

THE ROLE OF HYPOXIA-INDUCED RRM2B IN DNA REPLICATION

Iosifina-Petrina Foskolou

Jesus College

Thesis submitted to the University of Oxford in accordance with the
requirements for the degree of Doctor of Philosophy

Trinity Term, 2016
University of Oxford

Supervisor

Prof Ester M. Hammond (University of Oxford)

Assessment Committee

Prof Adrian L. Harris (University of Oxford)

Dr Kienan Savage (Queen's University Belfast)



The study presented in this thesis was performed at the Cancer Research UK and Medical Research Council Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford. This work was supported by Cancer Research UK Oxford Centre Prize DPhil Studentship/C38302/A12981.

ABSTRACT

The role of hypoxia-induced RRM2B in DNA replication

Submitted for Degree of Doctor of Philosophy

Iosifina- Petrina Foskolou, Jesus College, Trinity Term, 2016

Regions of low oxygen (hypoxia) occur in most solid tumours and correlate with poor patient prognosis due to their resistance to chemo- and radiotherapy and to their increased metastatic potential. Severe levels of hypoxia induce DNA replication stress characterised by an increased number of stalled replication forks and significantly reduced replication rates, which occurs in the absence of DNA damage. Ribonucleotide Reductase (RNR) is the only enzyme capable of *de novo* synthesis of dNTPs – the building blocks of DNA synthesis and repair. However, oxygen is essential for mammalian RNRs (RRM1/RRM2 and RRM1/RRM2B), leading us to question the source of dNTPs in hypoxia. Here we show that the RRM2B subunit of RNR is significantly induced in response to hypoxia in a universal manner. Interestingly, the hypoxic induction of RRM2B occurs both at transcriptional and translational levels and likely through two distinct mechanisms, one of which is p53-dependent. Most importantly, we demonstrate that RRM1/RRM2B enzyme is capable of retaining activity in hypoxia and therefore is favoured over RRM1/RRM2 in order to preserve on-going replication. We found two distinct mechanisms by which RRM2B maintains hypoxic activity and we identified specific RRM2B-residues (Y164 and K37/K151) responsible for this function. The importance of RRM2B in the response to tumour hypoxia is further illustrated by increased expression in the hypoxic regions of glioblastoma biopsies, roles in tumour growth and radioresistance, as well as prevention of DNA damage and apoptosis. In this study we present multi-disciplinary evidence, demonstrating the molecular rationale for the ability of RRM1/RRM2B to function in hypoxia. We propose that RRM2B has been evolutionary conserved so as to act as the hypoxic specific RNR subunit in order to be able to react promptly when this physiologically relevant stress occurs. Our data provide new insight into RNR biology, highlighting RRM2B as an important, hypoxic-specific, anti-cancer therapeutic target.

To my amazing parents and wonderful teachers

“Freedom of thought is best promoted by the gradual illumination of (wo)men’s minds which follows from the advance of science.” Charles Darwin (1809-1882)

ACKNOWLEDGMENTS

How could someone summarise four full years of work, fun, tension, laughter, experimenting and partying but most of all loving every single day in just one page... There are no doubts whom I shall first acknowledge and thank. Ester, as everybody knows, is not a typical supervisor. Knowledgeable, caring, always there for you whatever you need-seriously whatever; from finding the best collaborators to buying you beer(s) when nothing works. Ester, this single page is not enough to express my gratitude and thankfulness for all the opportunities you have given me, the help and support that made this thesis happen. You are a marvellous teacher and, as far as I am concerned, a real saviour. Thank you honestly for everything.

I would also like to thank my unofficial second supervisor Dr. Emily Flashman. Emily thank you for your patience and willingness to take me literally by the hand and teach me almost everything I know about biochemistry. I never expected that my DPhil would be so diverse - from molecular and cellular biology, and I could not have asked for a better supervisor to train and guide me in this “new” world.

A special thankyou to Prof Adrian Harris and Dr Kienan Savage for their time and effort to go through and examine my thesis.

A big thank you to everyone in Ester Hammond’s lab, past and present members that made research so much fun. A special thank to the Queen of the lab, Kasia, for all the support and expert advice; to Liam for going through this ‘marathon’ together with a rather pessimistic view that would always lighten up after a beer or two; to Greg for the critical thinking and unforgettable parties; to Shaliny for the thorough work and understanding or rather sharing the same OCDs; to Selva for being literally always there and to Cindy for being so encouraging and introducing me to yoga.

Monica and Dafni... Where shall I begin? Monica I still do not believe that you have abandoned us, such a traitor, going to Stanford... Just because it is sunny. Monica you are one of the most charismatic people I have ever met. I miss so much both our eternal scientific discussions and the great time we had almost every single day, COME BACK. Dafni τα λόγια είναι περιττά. I have said it numerous times; if it was not for you, my Oxonian times would be so much different. Thank you for listening and for being such a good friend. Πραγματικά δεν ξέρω τι θα έκανα χωρίς εσένα...

Many thanks to Christian Jorgensen, Carmen Domene and William Myers for the excellent collaboration and many hours enlightening me in computational and biophysical

studies. I wish all my future collaborations would be as helpful and interesting as these were. To many people from the Oncology and Chemistry department that have been extremely helpful throughout the years; Michael Stratford, Adam Hardy, Hanna Tarhonskaya, Lisa Folkes, Mick Woodcock and Graham Brown for the great collaboration and to Aswin, Delia, Leanne, Maria-Laura, Sofia and Nikola for your friendship. Special thanks to Kleopatra for going through the same phases and supporting each other.

Thanks to Hlias and Panos - the reason why I am in Oxofrd. Hlia thank you for the timeless support. Pano you have been a great teacher and friend all these years, thank you for still being here for me.

A massive thank you to my family. To my parents, Giorgos and Anna, that have supported every step I have made, trying to understand whatever I am saying even if it is ‘all Greek’ to them, for believing in me and helping me by any means they can. To my sisters, Maria and Fragkiska, as well as Petros, Giorgos and Philippos for making our family what it is.

Last but not least, a huge thank you to my (sur)real family. To Katerina (Mpalanika), Xristina, Katerina (Xatziz), Aggeliki, Giorgio and Niko. To all of our conversations, summers, days and nights that without, Athens would never have been the same. To Margaritoula mas, who left us so early but is always here with us.

Many Thanks! Σας ευχαριστώ πολύ όλους!

Αυτή η εργασία είναι αφιερωμένη στους γονείς μου, Γιώργο και Άννα. Πραγματικά δεν ξέρω πώς να σας ευχαριστήσω για ότι μου έχετε προσφέρει όλα αυτά τα χρόνια και προφανώς αυτή η μικρή παράγραφος δεν είναι αρκετή για να εκφράσω το πόσο σας αγαπάω. Ευχαριστώ που είστε πάντα δίπλα μου, που με στηρίζετε σε κάθε μου βήμα ακόμα και αν δεν συμφωνείτε με αυτό, που μου συμπαραστέκεστε σε κάθε αναποδιά και κυρίως που είστε τόσο τρελοί ώστε να σιγοντάρετε όποια φαινή ιδέα μου έρχεται στο μυαλό.

Σας ευχαριστώ πολύ για όλα!

TABLE OF CONTENTS

ABSTRACT.....	II
ACKNOWLEDGMENTS	IV
TABLE OF CONTENTS	VII
LIST OF TABLES	XIII
LIST OF FIGURES.....	XV
LIST OF ABBREVIATIONS	XIX
CHAPTER 1	1
INTRODUCTION	1
1.1 Cancer	1
1.2 Hypoxia and cancer.....	4
1.3 DNA replication stress.....	11
1.3.1 Causes of replication stress	13
1.3.2 The cellular response to replication stress	17
1.3.3 The hypoxia induced DNA damage response pathway	23
1.3.4 Consequences of replication stress	25
1.4 Nucleotide metabolism	27
1.4.1 Nucleotide biosynthesis	27
1.4.2 Cellular localisation of nucleotide biosynthesis	32
1.4.3 Regulation of nucleotide metabolism through nucleotide degradation	33
1.4.4 Oncogenes and tumour suppressor genes influence nucleotide metabolism	34
1.5 Ribonucleotide reductase (RNR)	35
1.5.1 Classes of RNR.....	35
1.5.2 The catalytic mechanism of RNR.....	39
1.5.3 The formation of the tyrosyl radical	41

1.5.4 RNR activity regulation (allosteric regulation).....	44
1.5.5 RNR activity regulation (cellular regulation)	47
1.5.6 The β subunit of RNR (RRM2 and RRM2B proteins)	49
1.5.7 RNR and cancer	52
1.5.8 RNR and hypoxia.....	58
AIM.....	60
CHAPTER 2.....	61
MATERIALS AND METHODS	61
2.1 Materials	61
2.1.1 General laboratory and cell culture reagents	61
2.1.2 Antibodies	65
2.1.3 Antibiotics.....	69
2.1.4 Primers	69
2.1.5 Constructs	73
2.2 Molecular Biology	74
2.2.1 Cloning.....	74
2.2.2 Transformation of bacteria.....	75
2.2.3 Extraction of plasmid DNA from bacterial cells	76
2.2.4 Agarose gel electrophoresis	76
2.3 Biochemistry	77
2.3.1 Cell lysis and SDS PAGE Electrophoresis	77
2.3.2 Western Blot	77
2.3.3 Immunoprecipitation (IP).....	78
2.3.4 Chromatin immunoprecipitation (ChIP)	79
2.3.5 Protein expression and purification	81

2.3.6 Site-directed mutagenesis	84
2.3.7 Enzymatic activity assay	84
2.3.8 Determination of CDP/dCDP	85
2.3.9 Circular dichroism (CD) spectrometry	85
2.3.10 Electron paramagnetic resonance (EPR)	85
2.4 Cellular biology	87
2.4.1 Drug treatments.....	87
2.4.2 Hypoxia treatment.....	87
2.4.3 Exposure to γ -irradiation.....	87
2.4.4 Cell lines	88
2.4.5 Construction of CRISPR-Cas9 RKO ^{RRM2B-/-} cell line.....	89
2.4.6 DNA transfection	94
2.4.7 Small interference RNA (siRNA).....	95
2.4.8 RNA extraction and purification.....	96
2.4.9 Quantitative PCR (qPCR).....	96
2.4.10 Immunofluorescence (IF) microscopy	97
2.4.11 Single-molecule DNA fiber analysis	98
2.4.12 Flow cytometry (FACS)	99
2.4.13 dNTP incorporation assay	100
2.4.14 Apoptosis (by cell morphology)	102
2.4.15 Colony survival.....	102
2.5 <i>In vivo</i> experiments	103
2.5.1 Immunohistochemistry (IHC).....	103
2.5.2 Xenograft study.....	103
2.5.3 Tissue IF.....	104

2.6 Statistical analysis	105
CHAPTER 3.....	106
HYPOXIA INDUCES THE RRM2B SUBUNIT OF RNR	106
3.1 Introduction.....	106
3.2 Results.....	108
3.2.1 Hypoxia induces DNA replication stress	108
3.2.2 Replication is not abrogated in hypoxia.....	111
3.2.3 The RRM2B subunit of RNR is induced in response to hypoxia	114
3.2.4 RRM2B is induced in hypoxia in a p53-dependent but HIF1 α -independent manner.....	119
3.3 Discussion	126
CHAPTER 4.....	127
BIOLOGICAL SIGNIFICANCE OF RRM2B INDUCTION IN HYPOXIA	127
4.1 Introduction.....	127
4.2 Results.....	129
4.2.1 RRM2B binds to RRM1 in hypoxia to form a functional RNR holoenzyme...	129
4.2.2 Depletion of RRM2B disrupts DNA replication in hypoxia	132
4.2.3 Depletion of RRM2B leads to DNA damage, loss of viability and delayed tumour growth.....	135
4.3 Discussion	141
CHAPTER 5.....	143
IN VITRO CHARACTERISATION OF THE TWO FORMS OF RNR ENZYME IN NORMOXIA AND HYPOXIA	143
5.1 Introduction.....	143
5.2 Results.....	145

5.2.1 The R1/R2B form of RNR enzyme retains activity in hypoxia.....	145
5.2.2 Adaptation of RRM2B in low oxygen environments	151
5.2.3 Identification of residues responsible for the adaptation of RRM2B to hypoxia	154
5.2.4 The role of Y164 and K37/K151 in the hypoxic adaptation of RRM2B.....	161
5.3 Discussion	168
CHAPTER 6.....	171
DISCUSSION	171
6.1 RRM2B induction in hypoxia.....	173
6.2 Molecular adaptations of RRM2B for hypoxic specific role.....	176
6.3 RRM2B and oxidative stress; could RRM2B generate oxygen to maintain hypoxic activity?.....	177
6.4 RRM2B as a clinical target.....	179
APPENDICES	181
APPENDIX I.....	181
Supporting material for construction of CRISPR-Cas9 RKO ^{RRM2B^{-/-}} cell line.....	181
A. Identification of the <i>RRM2B</i> transcript variants in RKO cells.....	181
B. FASTA of the <i>RRM2B</i> gene locus (NCBI Reference Sequence: NG_016617.1).183	
C. Guide sequences to target exon 1 of <i>RRM2B</i> variants	184
D. Western blot analysis of 24 clones picked targeting <i>RRM2B</i> exons 1.....	184
E. Guide sequence to target <i>RRM2B</i> exon 4.....	185
F. Western blot analysis of 20 clones picked targeting <i>RRM2B</i> exon 4.....	185
G. Sequencing validation of the RKO ^{RRM2B^{-/-}} cell line	186
APPENDIX II.....	187
Supporting material of the effects of RRM2B inhibition	187

A. Depletion of RRM2B disrupts DNA replication in hypoxia.....	187
B. Depletion of RRM2B leads to DNA damage.....	188
APPENDIX III	189
Supporting material for Molecular Dynamics (MD) simulations.....	189
A. Molecular Dynamics (MD) simulations	189
B. MD equilibration protocol.....	189
C. Code availability	190
D. MD simulation analysis	190
APPENDIX IV	192
Supporting material for structural differences between RRM2 and RRM2B.....	192
APPENDIX V.....	193
Published papers from this thesis.....	193
REFERENCES.....	194

LIST OF TABLES

Table 1.1 Human diseases correlated with defective RS and RS related signalling.....	26
Table 1.2 Examples of anticancer agents that target dNTP metabolism	31
Table 2.1 Chemicals and cell cultures reagents	62
Table 2.2 Antibodies used for western blotting	65
Table 2.3 Antibodies used for immunofluorescence (IF)	66
Table 2.4 Antibodies used for immunoprecipitation (IP)	66
Table 2.5 Antibodies used for chromatin immunoprecipitation (ChIP)	66
Table 2.6 Antibodies used for fluorescence-activated cell sorting (FACS)	66
Table 2.7 Antibodies used for Single-Molecule DNA Fiber Analysis	66
Table 2.8 Antibodies used for tissue immunofluorescence (Tissue IF).....	67
Table 2.9 Antibodies used for immunohistochemistry (IHC)	67
Table 2.10 Secondary antibodies used.....	67
Table 2.11 Primers used for quantitative PCR (qPCR)	70
Table 2.12 Primers used for ChIP-qPCR.....	70
Table 2.13 Primers used for Cloning	70
Table 2.14 Primers used for site-directed mutagenesis	71
Table 2.15 Primers used for verifying RRM2B isoforms.....	72
Table 2.16 Primers used for construction and verification of CRISPR-Cas9 RRM2B ^{-/-} cell line.....	72
Table 2.17 Primers used for dNTP incorporation assay	72
Table 2.18 Table of constructs.....	73
Table 5.1: Cluster distances for O ₂ -Fe sites and O ₂ entering frequencies for RRM2B and RRM2 proteins.....	152

Table 5.2: Quantitative analysis of the radical content of RRM2B and RRM2 proteins in normoxia and hypoxia.....	153
Table 5.3: Cluster distances for O ₂ -Fe sites and O ₂ entering frequencies for Y164C and K37E/K151E variants	162
Table 5.4: Quantitative analysis of the radical content of the RRM2B variants in normoxia and hypoxia.....	164

LIST OF FIGURES

Figure 1.1 Emerging hallmarks and enabling characteristics of cancer	3
Figure 1.2 Regulation HIF- α under normoxic and hypoxic conditions	7
Figure 1.3 Hallmarks of cancer regulated by hypoxia and HIFs.	10
Figure 1.4 Sources of replication stress	13
Figure 1.5 The ATR-mediated replication stress response	18
Figure 1.6 Schematic representation of ATR and ATM signalling pathways	21
Figure 1.7 <i>De novo</i> pathway of nucleotide biosynthesis	28
Figure 1.8 Main reactions in the salvage pathways of nucleotide biosynthesis	30
Figure 1.9 The basic catalytic reaction of RNR enzyme for dNDP formation.....	36
Figure 1.10 Class Ia RNR enzyme.....	38
Figure 1.11 The reaction mechanism for reduction of NDP by RNR	40
Figure 1.12 Stoichiometry of the tyrosyl generating reaction	42
Figure 1.13 The two forms of mammalian RNR enzyme.....	43
Figure 1.14 Allosteric regulation of RNR.....	45
Figure 1.15 Similarities and differences between RRM2 and RRM2B proteins.....	50
Figure 1.16 Examples of small-molecules RNR inhibitors	56
Figure 2.1 The three transcript variants of the human <i>RRM2B</i> gene.....	90
Figure 2.2 Schematic representation of the DNA fiber protocol in normoxia and hypoxia....	99
Figure 3.1 The Hypoxia-induced DDR.....	108
Figure 3.2 Hypoxia induces DNA replication stress	109
Figure 3.3 The accumulation of ssDNA observed in hypoxia decreases after prolonged exposure to these conditions	110
Figure 3.4 DNA replication is slower but not abrogated in hypoxia	111
Figure 3.5 Imbalanced nucleotide levels are observed in hypoxia	113

Figure 3.6 The RRM2B subunit of RNR is overexpressed in hypoxia	114
Figure 3.7 The RRM2B subunit of RNR is overexpressed in hypoxia in different cancer cell lines	115
Figure 3.8 <i>RRM2B</i> mRNA is induced in response to hypoxia	116
Figure 3.9 Different cancer cell lines show increased <i>RRM2B</i> mRNA in response to hypoxia	117
Figure 3.10 Increased RRM2B expression in the hypoxic areas of glioblastoma patient samples.....	118
Figure 3.11 The Hypoxic-induction of RRM2B is HIF-1 α independent.....	120
Figure 3.12 RRM2B is induced in an oxygen-dependent manner.....	121
Figure 3.13 Hypoxic-induction of <i>RRM2B</i> mRNA is p53 dependent	122
Figure 3.14 p53 directly binds to the <i>RRM2B</i> locus	123
Figure 3.15 Increased RRM2B protein levels were observed both in presence and absence of p53 in hypoxia.....	125
Figure 4.1 RNR switches subunits in response to hypoxia.....	129
Figure 4.2 Increased RRM2B levels bind to RRM1 in hypoxia compared to normoxia.....	130
Figure 4.3 Effective depletion of RRM2B protein after use of siRRM2B	131
Figure 4.4 Loss of RRM2B reduces nucleotide levels specifically in hypoxia.....	131
Figure 4.5 Depletion of RRM2B leads to less BrdU incorporation in hypoxia.....	132
Figure 4.6 Construction of CRISPR-Cas9 RKO ^{RRM2B-/-} cell line	133
Figure 4.7 Cells with decreased RRM2B levels show a persist formation of RPA foci	134
Figure 4.8 Loss of RRM2B leads to DNA damage in hypoxia	136
Figure 4.9 Cells with depleted RRM2B levels show elevated DSBs specifically in hypoxia	136
Figure 4.10 Depletion of RRM2B leads to loss of viability in hypoxia	137

Figure 4.11 Apoptosis is increased in hypoxia in the absence of RRM2B.....	138
Figure 4.12 Depletion of RRM2B results in smaller and more radiosensitive tumours.....	139
Figure 4.13 Increased levels of apoptosis in the hypoxic areas of RKO ^{RRM2B-/-} tumours	140
Figure 5.1 Expression and purification of the three recombinant RNR proteins.....	145
Figure 5.2 <i>In vitro</i> enzymatic activity assay of the RNR enzyme	146
Figure 5.3 No product formation is observed when only one of the RNR proteins is present in the <i>in vitro</i> enzymatic activity assay	147
Figure 5.4 The full reconstitution of both forms of RNR enzyme leads to product formation	148
Figure 5.5 The R1/R2B form of RNR enzyme retains activity in hypoxia	149
Figure 5.6 The R1/R2 form of RNR enzyme does not retain activity in hypoxia	150
Figure 5.7 MD simulations revealed three O ₂ tunnels in each small subunit of the RNR enzyme	152
Figure 5.8 EPR spectra of the tyrosyl radical in normoxia and hypoxia	153
Figure 5.9 Representative schematic of the key differences between RRM2 and RRM2B proteins.....	154
Figure 5.10 Expression and purification of the seven RRM2B variants	157
Figure 5.11 Substrate over product formation for Q127K and Y331F variants	158
Figure 5.12 dCDP formation in hypoxia for Y164F, F183Y and Y241H variants	158
Figure 5.13 dCDP formation in hypoxia for Y164C and K37E/K151E variants	160
Figure 5.14 The Y164C and K37E/K151E variants do not sustain activity in hypoxia.....	160
Figure 5.15 The phenylalanine network surrounding Y164 in RRM2B	161
Figure 5.16 Helix B of monomer-2 shows either an open or a closed conformation.....	163
Figure 5.17 EPR spectra of the tyrosyl radical for the Y164C, K37E/K151E and Q127K variants in normoxia and hypoxia.....	163

Figure 5.18 Introduction of pRRM2B ^{WT} in RKO ^{RRM2B-/-} cells increases the intracellular nucleotide levels in hypoxia.....	166
Figure 5.19 Introduction of pRRM2B ^{WT} rescued the null phenotype from apoptosis	167
Figure 6.1 Hypoxia induced RRM2B leads to continued DNA replication	172
Figure 6.2 Importance of RRM2B in hypoxic exposed cells.....	175
Figure I.A.1 RKO cells have all of the three transcript variants of <i>RRM2B</i> gene both in normoxia and in hypoxia	183
Figure I.A.2 Partial loss of RRM2B expression after targeting <i>RRM2B</i> exons 1.....	184
Figure I.A.3 Clones testing for RRM2B depletion after targeting <i>RRM2B</i> exon 4.....	185
Figure II.A.1 Inhibition of RRM2B results in persist formation of RPA foci.....	187
Figure II.B.1 Inhibition of RRM2B results in double strand breaks (DSBs)	188

LIST OF ABBREVIATIONS

53BP1	p53-binding protein 1
3AP	triapine
9-1-1	RAD9-RAD1-HUS1 complex
aa	amino acid
A	adenosine
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANGPT2	angiopoietin 2
ANOVA	analysis of variance
APC	Cdh1-anaphase-promoting complex
AraC	Arabinosylcytosine
ARNT	aryl hydrocarbon receptor nuclear translocator
Asn, N	asparagine
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATP	adenosine triphosphate
ATR	ataxia-telangiectasia and RAD3-related
ATRIP	ATR-interacting protein
BCL-2	B-cell lymphoma 2
BRCA1	breast cancer susceptibility gene 1
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
C	cytidine
CAIX	carbonic anhydrase IX
ccRRM2Bii	crucian carp RRM2B variant ii
CD	circular dichroism
Cdc6	cell division control protein 6
CDP	cytidine diphosphate
Cdt1	chromatin licensing and DNA replication factor 1
CFS	common fragile sites
ChIP	chromatin immunoprecipitation
CHK	checkpoint protein

CIF	clofarabine
CIFTP	clofarabine analogue triphosphate
CldU	5-chloro-2'-deoxyuridine
CMP	cytidine monophosphate
c-MYC	myc proto oncogene
CRISPR	clustered regularly interspaced short palindromic repeats
CT-L	cytotoxic T-lymphocyte
CTL	control
CTP	cytidine triphosphate
Cys, C	cysteine
dADP	deoxyadenosine diphosphate
dAMP	deoxyadenosine monophosphate
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
DC	dendritic cell
dCDP	deoxycytidine diphosphate
dCMP	deoxycytidine monophosphate
dCTP	deoxycytidine triphosphate
DDR	DNA damage response
DFO	deferoxamine mesylate
dGDP	deoxyguanosine diphosphate
dGMP	deoxyguanosine monophosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dNDP	deoxyribonucleoside diphosphate
dNTP	deoxyribonucleotide triphosphate
DSB	double-strand break
dTDP	deoxythymidine diphosphate
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate
dUDP	deoxyuridine diphosphate
dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate

<i>E.coli</i>	<i>Escherichia coli</i>
E2F	E2F transcription factor
EPR	electron paramagnetic resonance
ESCC	oesophageal squamous cell carcinoma
F₂C	gemcitabine
F₂CDP	gemcitabine analogue diphosphate
FACS	fluorescence activity sorting assay
FBS	foetal bovine serum
Fe	iron
FHIT	fragile histidine triad
FIH-1	factor-inhibiting HIF-1
G	guanosine
GDP	guanosine diphosphate
GFP	green fluorescent protein
Gln, Q	glutamine
Glu, E	glutamate
GLUT1	glucose transporter 1
GMP	guanosine monophosphate
GTP	guanosine triphosphate
H₂O₂	hydrogen peroxide
H3	histone H3
H3K9me3	trimethylation of H3 at lysine 9
HAF	hypoxia-associated factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	hypoxia-inducible factor
HPLC	high-performance liquid chromatography
HR	homologous recombination
HRE	hypoxia responsive element
HU	hydroxyurea
IdU	5-Iodo-2'-deoxyuridine
IF	immunofluorescence
IHC	immunohistochemistry
IP	immunoprecipitation
IR	ionising radiation

KAP-1	krüppel-associated box domain associated protein 1
LUC	luciferase
Lys, K	lysine
MAPK	mitogen-activated protein kinase
MCM	minichromosome maintenance
MD	molecular dynamics
MDM2	mouse double minute 2 homolog
MDR	multi-drug resistance
MDSC	myeloid derived suppressor cell
Me	methylation
MEF	mouse embryonic fibroblasts
MRE11	meiotic recombination 11 homolog A
MRN	MRE11/RAD50/NBS1
mtDNA	mitochondrial DNA
MYC	myelocytomatosis
NADPH	nicotinamide adenine dinucleotide phosphate
NDP	ribonucleoside diphosphate
NDPK	nucleoside diphosphate kinase
NHEJ	non-homologous end-joining
NO	nitric oxide
NSCLC	non-small cell lung cancer
NT5C2	5'-nucleotidase, cytosolic 2
NTP	ribonucleotide
O/N	overnight
Oct4	octamer-binding transcription factor 4
OD	optical density
ORC	origin recognition complex
OXPHOS	oxidative phosphorylation
PARP1	poly (ADP-ribose) polymerase 1
PCET	proton-coupled electron transfer
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF-β	platelet-derived growth factor-β
PHD	propyl-4-hydroxylases

Phe, F	phenylalanine
PI	propidium iodide
Pi	inorganic phosphate
PI3K	phosphatidylinositol-3-kinase
PI3KK	phosphatidylinositol-3-kinase like kinase
PIMO	pimonidazole
PLK	polo-like kinase
<i>P</i>_{O2}	partial pressure
pre-RC	pre-replicative complexes
PRPP	5-phospho-ribo-1-pyrophosphate
PTEN	phosphatase and tensin homolog
qPCR	quantitative real-time PCR
R1/R2	RRM1/RRM2 holoenzyme
R1/R2B	RRM1/RRM2B holoenzyme
RB	retinoblastoma
R-loops	RNA/DNA hybrids
RNA	ribonucleic acid
RNR	ribonucleotide reductase
ROS	reactive oxygen species
RPA	replication protein A
RPC	replisome progression complex
RPM	revolutions per minute
RRM1	ribonucleotide reductase regulatory subunit M1
RRM2	ribonucleotide reductase regulatory subunit M2
RRM2B	ribonucleotide reductase regulatory TP53 inducible subunit M2B
RS	replication stress
RT	room temperature
s.e.m.	standard error of the mean
SAMHD1	sterile α -motif and histidineaspartate domain-containing protein 1
SCF	Skip1/Cullin/F-box complex
SCR	scramble
SD	standard deviation
Ser, S	serine
sgRNA	single-guide RNA

siRNA	small interfering RNA
SMARCA1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A-like 1
SSB	single-strand break
ssDNA	single-stranded DNA
TAM	tumour associated macrophage
TDP	thymidine diphosphate
Tip60	tat-interactive protein 60
TLK1	tousled-like kinase
TMP	thymidine monophosphate
TMPK	thymidylate phosphate kinase
TOPBP1	DNA topoisomerase 2-binding protein 1
Treg	regulatory T-cell
TTP	thymidine triphosphate
Tyr, Y	tyrosine
U	uracil
UDP	uridine diphosphate
UMP	uridine monophosphate
UTP	uridine triphosphate
UV	ultraviolet
VEGF	vascular endothelial growth factor
VHL	Von-Hippel-Lindau
WT	wild type

CHAPTER 1

Introduction

1.1 Cancer

Cancer was first described by Hippocrates (Hippocratic Corpus, c. 410 - c. 360 BCE), who used the words *carcinos* (crab) and *carcinoma* to describe a range of tumours and swellings by their characteristic crab-like appearance (David and Zimmerman, 2010). Cancer is a leading cause of disease and death worldwide, with 14.1 million new cancer cases and 8.2 million deaths in 2012 (CRUK, 2014). Despite the large research effort, spanning many decades, cancer is still considered a complex disease that involves numerous changes in cell biology and physiology, which ultimately lead to uncontrolled growth (neoplasia) and the potential invasion of surrounding tissues and distant organs (Seyfried and Shelton, 2010). The origin of cancer is poorly understood, with a large number of known and unknown influences (such as chemicals, high-energy irradiation, viruses *etc.*) able to initiate malignant transformation. This characteristic of cancer, that a very specific process (malignant transformation) can be elicited by many different agents (carcinogens) led Szent-Gyorgyi to coin the term the “oncogenic paradox”, which is still considered unresolved (Szent-Gyorgyi, 1977).

Much more recently, Hanahan and Weinberg proposed the six hallmarks of cancer (Hanahan and Weinberg, 2000). Specifically, the authors proposed that as normal cells progress to a neoplastic state, they acquire six characteristics; resistance to cell death, sustained proliferative signalling, evasion of growth suppressors, activated invasion and metastasis, replicative immortality and induced angiogenesis. Together these hallmarks alter cellular physiology enabling cells to become tumourigenic and ultimately malignant (Hanahan and Weinberg, 2000). Hanahan and Weinberg updated the six hallmarks in 2011 to include two further emerging hallmarks and two enabling characteristics (Hanahan and Weinberg, 2011). Specifically, as illustrated in Figure 1.1, the emerging hallmarks include the deregulation of cellular energy metabolism and the ability of cancer to actively evade detection by the immune system, while the enabling characteristics include genome instability and tumour-promoting inflammation (Hanahan and Weinberg, 2011). Interestingly, the authors also highlighted the importance of the tumour microenvironment and specifically the hypoxic (low oxygen) regions that occur within many tumours, as they largely influence the mechanism of metabolic reprogramming and increase genomic instability (Bristow and Hill, 2008; Haider et al., 2016; Hanahan and Weinberg, 2011). Hypoxia, a feature central to the study presented here, plays a pivotal role in tumourigenesis and has been associated with the early stages of cancer development (Gorgoulis et al., 2005). A detailed study on how hypoxia contributes to tumour biology follows in Chapter 1.2.

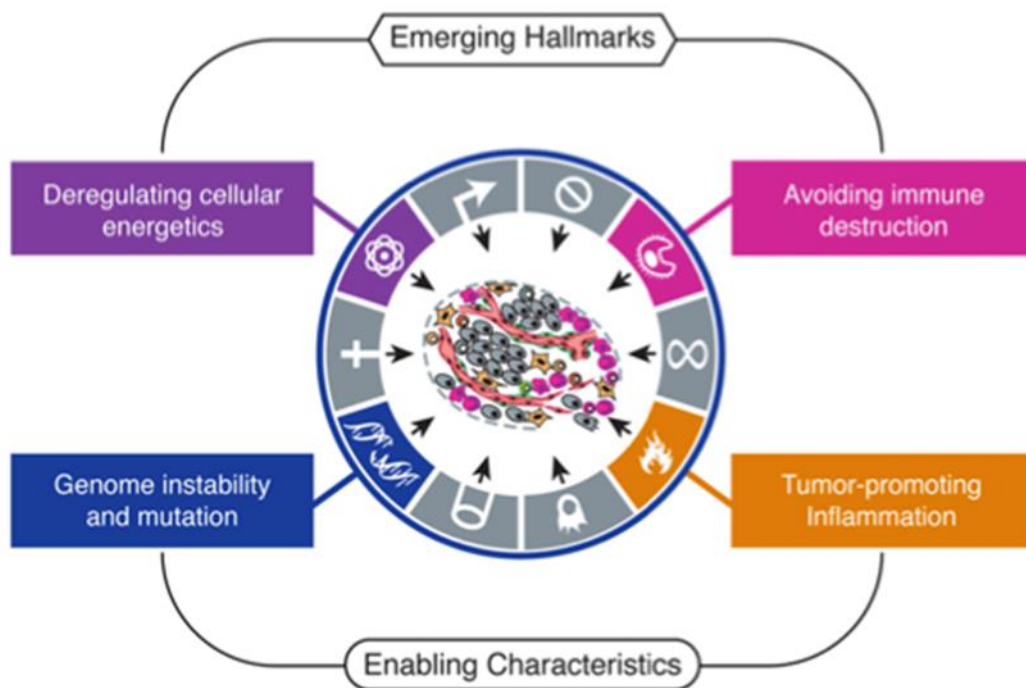


Figure 1.1 Emerging hallmarks and enabling characteristics of cancer

The diagram illustrates the two additional hallmarks and enabling characteristics of cancer. The “Deregulating cellular energetics” involves the capacity of a cancer cell to modify, or reprogram cellular metabolism in order to effectively support neoplastic proliferation. The “Avoiding immune destruction” allows cancer cells to evade immunological destruction, especially by T- and B-lymphocytes, macrophages and natural killer cells. The “Genome instability and mutation” endows cancer cells with genetic alterations that drive tumour progression. The “Tumour-promoting inflammation” shows the widely appreciated tumour-promoting consequences of the inflammatory response. Adapted and reprinted from (Hanahan and Weinberg, 2011) with permission from Elsevier, License Number: 3942961456507.

1.2 Hypoxia and cancer

Oxygen is an essential factor that controls numerous developmental processes and normal tissue homeostasis. At a cellular level, oxygen is required for oxidative metabolism, energy supply through the generation of ATP (adenosine triphosphate), and cell survival (Rankin and Giaccia, 2016). Therefore, an adequate supply of oxygen is essential for the proper function and survival of all aerobic organisms, while deregulation of oxygen homeostasis can trigger several pathological settings. Hypoxia, or decreased oxygen concentrations, occur when oxygen supply fails to meet demand (Wigerup et al., 2016). The exact definition of hypoxia is considered a challenging task since no clear-cut threshold exists between normoxia and hypoxia, as the normal oxygen partial pressure (P_{O_2}) varies between different tissues. Examples of “physiologically normal” tissue oxygen levels are 4.6% for brain, 5.6% for lung, 1.05-4.61% in skin, 13% in arterial blood and 5% in the liver (Dings et al., 1998; Hammond et al., 2014; Koh and Powis, 2012; Le et al., 2006; Wang et al., 2003). Hence, hypoxia as well as gradients of it (mild, moderate or severe) should be considered in context of what is normal for each particular tissue.

Clinically, hypoxia is defined as the state of reduced oxygen availability or decreased P_{O_2} below a critical threshold, thus restricting or even abrogating the function of organs, tissues or cells (Harris, 2002; Hockel and Vaupel, 2001; Zander and Vaupel, 1985). In solid tumours, hypoxia occurs as a result of a high metabolic demand and an abnormally formed/inefficient tumour vasculature, which leads to an imbalance between oxygen delivery and oxygen consumption (Patiar and Harris, 2006; Vaupel and Mayer, 2007). Specifically, within the tumour microenvironment, oxygen consumption rates are increased due to the metabolic demands of the proliferative tumour cells (Rankin and Giaccia, 2016). At the same time, oxygen delivery is limited due to a chaotic tumour vasculature, which is characterised

by distended or blocked capillaries and a leaky, sluggish and transient blood flow (Brown and Giaccia, 1998).

The malformed blood vessels are also considered responsible for the two types of hypoxia observed in tumours: namely chronic and cycling hypoxia. Chronic hypoxia was first described by Thomlinson and Gray in 1955, when they observed the presence of necrotic areas at the centres of large tumours that were surrounded by intact tumour cells (Hall, 2012; Thomlinson and Gray, 1955). The authors suggested the existence of a decreasing gradient of oxygen tensions within the tumour mass, which would eventually lead to complete anoxia at the centre of any tumour with a radius larger than 150 μm , if no functional blood vessels are present (Thomlinson and Gray, 1955). In 1979, Brown observed that in addition to regions of chronic hypoxia, rodent tumours contained acutely hypoxic cells that were produced by intermittent opening and closing of tumour blood vessels, leading to variations in the tumour tissue oxygen perfusion (Brown, 1979). Fluctuations in perfusion result in periods of poor or better oxygenation depending on vascular stability, and therefore cells may be exposed to acute hypoxia or anoxia followed by reoxygenation in a cyclic manner (cycling hypoxia) (Bristow and Hill, 2008; Michiels et al., 2016). Chronic and cycling hypoxia are heterogeneously distributed within the tumour mass and have both been shown to drive malignant progression.

The cellular response to hypoxia is critically regulated by the HIF (hypoxia-inducible factor) family of transcription factors. Each HIF transcription factor is composed of an oxygen sensitive α subunit (HIF-1 α , HIF-2 α , HIF-3 α) and a shared β subunit which is constitutively expressed (HIF-1 β /ARNT) (Brahimi-Horn and Pouyssegur, 2009). Under normoxic conditions, the HIF- α subunit is hydroxylated at conserved proline residues, which are recognised by the VHL (Von Hippel–Lindau) E3 ubiquitin ligase complex, leading to its ubiquitination and degradation (Ivan et al., 2001; Jaakkola et al., 2001) (Figure 1.2). HIF

propyl hydroxylation is mediated by the PHDs (propyl-4-hydroxylases), a group of enzymes which belong to the 2-oxoglutarate-dependent oxygenase superfamily, and their activity is dependent on oxygen and iron availability (Schofield and Ratcliffe, 2004). When oxygen is limited, PHDs cannot hydroxylate the HIF- α subunit, resulting in protein stabilisation, nuclear translocation and dimerisation with the HIF-1 β subunit in order to assemble the HIF transcription factor (Wang et al., 1995; Wigerup et al., 2016). In the nucleus, HIFs recognise HREs (hypoxia-responsive elements) in target genes and activate transcription by recruiting transcriptional coactivators such as p300/CBP (Freedman et al., 2002; Semenza, 2003; Wang and Semenza, 1993).

The HIF- α subunit is also regulated by a second oxygen-sensitive hydroxylation at a key asparagine residue, mediated by FIH-1 (factor-inhibiting HIF-1), leading to disruption of the HIF - p300/CBP interaction, thereby inhibiting the transcriptional activation potential of HIFs (Lando et al., 2002; Mahon et al., 2001). In addition, it has been demonstrated that HIF signalling can also be regulated by non-hypoxia-driven stimuli, including growth factor signalling (LaGory and Giaccia, 2016) (Figure 1.2). There are two main differences between oxygen-dependent and growth factor stimulation of HIF. Firstly, growth factor stimulation is cell type specific, whereas the stimulation of HIF through hypoxia is independent of cell type. Secondly, hypoxia stabilises the HIF- α subunit, whereas growth factors and other signalling genes increase HIF- α protein expression by activation of the PI3K (phosphatidylinositol-3 kinase) or MAPK (mitogen-activated protein kinase) pathways (Patlar and Harris, 2006; Richard et al., 1999; Semenza, 2003; Zhong et al., 2000).

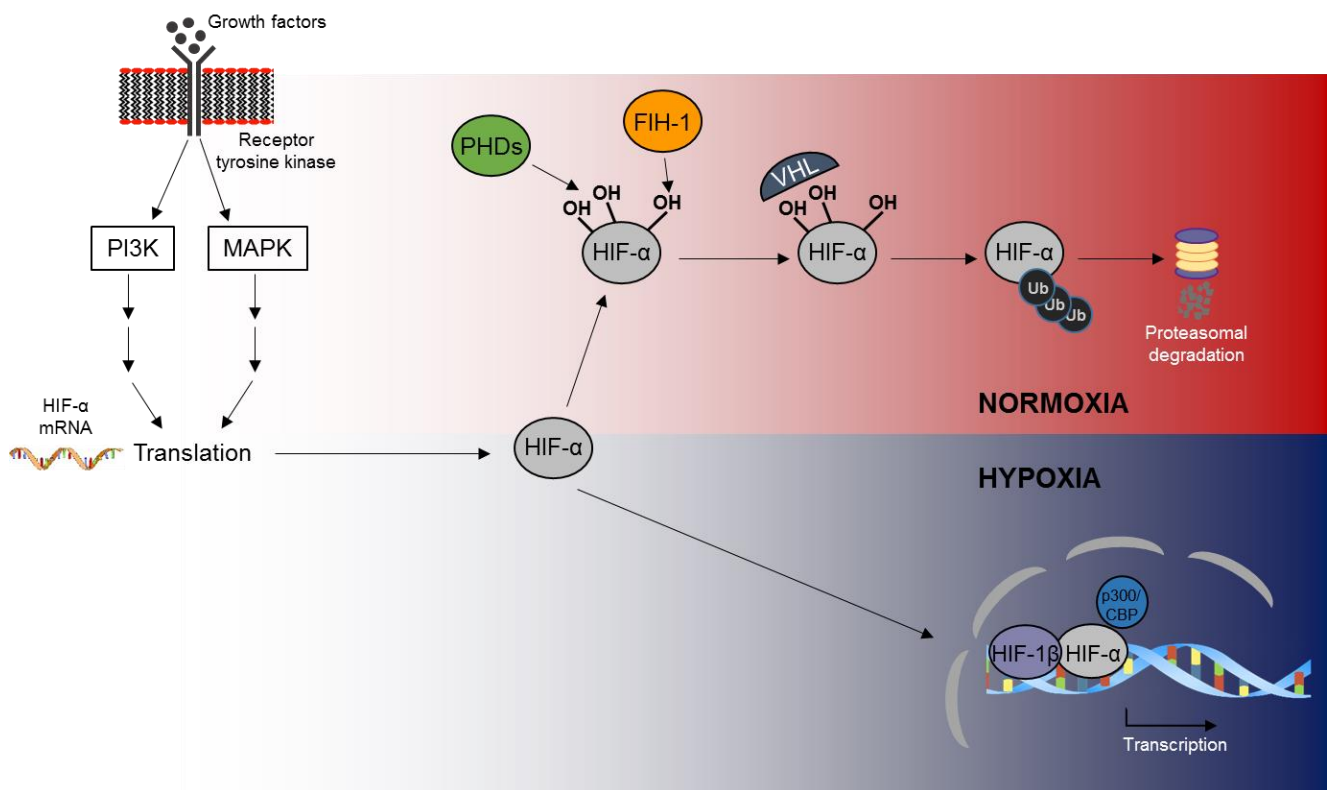


Figure 1.2 Regulation HIF- α under normoxic and hypoxic conditions

In normoxia, HIF- α is hydroxylated at conserved residues by PHDs and FIH-1. Hydroxylation by PHDs promotes degradation of HIF- α via an ubiquitin-dependent process executed by the VHL complex. Hydroxylation by FIH-1 inhibits HIF transcriptional activity by preventing interaction with p300/CBP. Under hypoxia, PHDs and FIH-1 are inactivated, which results in HIF- α stabilisation followed by translocation into the nucleus and dimerisation with HIF-1 β to form the HIF transcription factor. HIF, together with its co-activator p300/CBP, induces the transcription of numerous targets. HIF signalling is also regulated by non-hypoxia-driven stimuli, including growth factor signalling and activation of PI3K and MAPK pathways. HIF: hypoxia inducible factor; PHD: prolyl-4- hydroxylases; FIH-1: factor-inhibiting HIF-1; VHL: Von Hippel–Lindau; PI3K: phosphatidylinositol-3 kinase; MAPK: mitogen-activated protein kinase. Adapted from (Wigerup et al., 2016).

Once activated, HIF regulates the transcription of numerous genes which encode proteins involved in many different biological processes such as angiogenesis, glucose metabolism, cell proliferation and survival, pH regulation, invasion and metastasis as well as apoptosis (Harris, 2002; Semenza, 2003). These processes drive the adaptation and progression of cancer cells and contribute to the malignant phenotype and aggressive tumour behaviour (Harris, 2002; Wigerup et al., 2016). Thus, tumour hypoxia and HIFs are considered to affect most of the cancer hallmarks (Figure 1.3) (Wigerup et al., 2016). Interestingly, although the alternative HIF isoforms (HIF-1 and HIF-2) have many overlapping functions, they also show independent activities through the regulation of unique target genes and by differential interaction with important oncoproteins and tumour suppressors; hence they have distinct roles in regulating the tumour phenotype (Keith et al., 2012). Specifically, studies have shown that HIF-1 induces expression of glycolysis in various cell types, whereas unique HIF-2 targets seem to be cell type specific (Hu et al., 2003; Raval et al., 2005). For example, glucose metabolism is thought to be primarily regulated by HIF-1, while HIF-2 is specifically involved in Oct4-dependent stem cell pluripotency (Keith et al., 2012). Moreover, HIF-1 is thought to mediate the acute response to hypoxia, whereas HIF-2 is stabilised over longer time periods (Holmquist-Mengelbier et al., 2006). It has also been reported that the E3 ubiquitin ligase HAF (hypoxia-associated factor) switches cells from HIF-1 to HIF-2 dependent signalling by targeting HIF-1 α for degradation and increasing transactivation of HIF-2 α (Koh et al., 2011). Importantly, both HIF-1 α and HIF-2 α overexpression have been associated with poor clinical outcome in patients, with HIF-1 α expression having been observed in cervical cancer (Birner et al., 2000), endometrial carcinoma (Sivridis et al., 2002), oligodendroglioma (Birner et al., 2001a), ovarian cancer (Birner et al., 2001b) and different types of breast cancer (Bos et al., 2003; Kronblad et al., 2006; Schindl et al., 2002). HIF-2 α acts as an independent prognostic factor for breast cancer

(Helczynska et al., 2008) and has been correlated with poor survival in glioblastoma (Li et al., 2009), neuroblastoma (Holmquist-Mengelbier et al., 2006), head and neck squamous carcinoma (Koukourakis et al., 2002) and non-small lung cancer patients (Giatromanolaki et al., 2001).

The occurrence of hypoxic regions have been implicated in resistance to radio- and chemo-therapy as well as to increased genomic instability, invasion and metastatic potential of tumours (Bristow and Hill, 2008; Gray et al., 1953). With regards to chemotherapy, resistance can be mediated through different mechanisms, such as hypoxia-induced inhibition of proliferation, metabolic reprogramming, hypoxia-driven clonal selection of p53-mutated cells with low apoptotic potential, inhibition of DNA damage as well as poor drug delivery due to abnormally formed tumour vasculature (Graeber et al., 1996; Haider et al., 2016; Hockel and Vaupel, 2001; Sullivan and Graham, 2009; Tannock, 1968). In addition, tumour hypoxia diminishes response to radiation therapy (x- and γ - radiation) since radiosensitivity is progressively limited when P_{O_2} are lower than 25-30 mmHg (Hockel and Vaupel, 2001). This occurs because molecular oxygen is essential for the fixation of the DNA (deoxyribonucleic acid) damage formed by the free radicals produced after the interaction of radiation with intracellular water (Hall, 2012). As a result, 2-3 times the dose of radiation in the absence of oxygen is required to achieve the same biological effect (for example cell survival or amount of DNA breaks) as in conditions where oxygen is abundant (Bristow and Hill, 2008). This phenomenon is known as the “Oxygen Enhancement Ratio” and is associated with severe levels of hypoxia also known as radiobiologic hypoxia (Brown and Giaccia, 1998; Gray et al., 1953).

These extremely low oxygen tensions have also been associated with the induction of DNA replication stress (RS) and the subsequent activation of the DNA damage response pathway (DDR) (Hammond et al., 2002; Hammond et al., 2004; Olcina et al., 2013; Pires et

al., 2010b). Interestingly, it has been recently proposed that DNA RS should be considered as a new hallmark of cancer, since it is a dominant tumour characteristic and likely to drive tumour development through the induction of genomic instability and selection for evading apoptosis (Macheret and Halazonetis, 2015). The role and consequences of RS in cancer and specifically in the hypoxic microenvironment will be discussed in detail in Chapter 1.3.

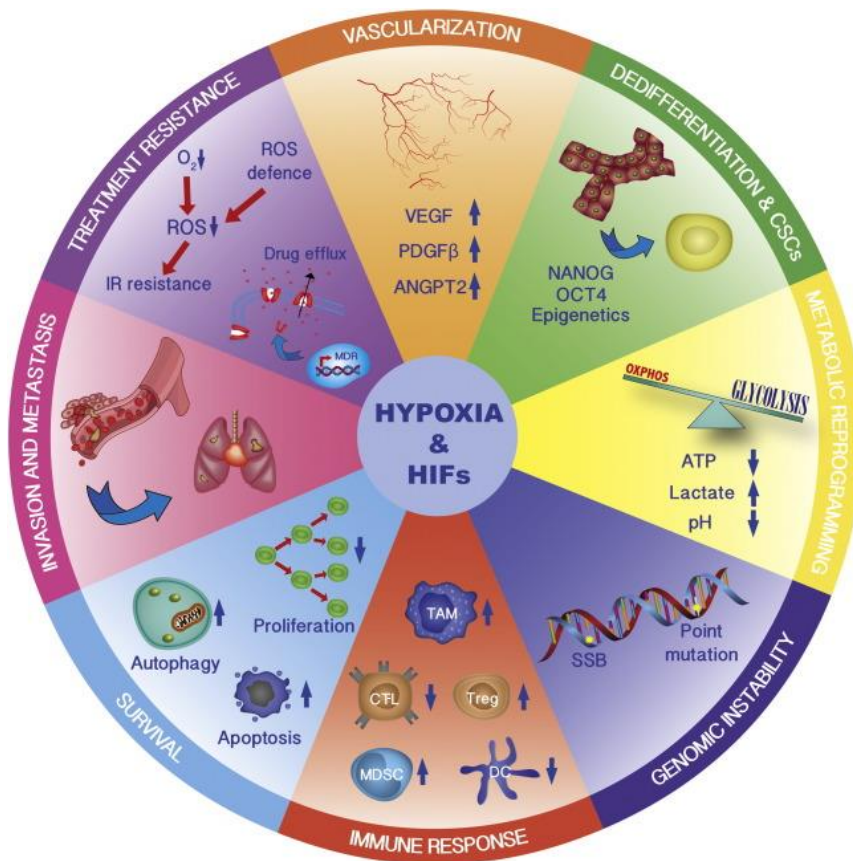


Figure 1.3 Hallmarks of cancer regulated by hypoxia and HIFs.

Hypoxia and HIFs regulate multiple cancer phenotypes. Targeting hypoxia/HIFs will thus inhibit several aspects of tumour progression, metastasis, and treatment resistance. VEGF: vascular endothelial growth factor; PDGF- β : platelet-derived growth factor- β ; ANGPT2: angiopoietin 2; OXPHOS: oxidative phosphorylation; SSB: single strand break; TAM: tumour associated macrophage; CTL: cytotoxic T-lymphocyte; Treg: regulatory T-cell; MDSC: myeloid derived suppressor cell; DC: dendritic cell; MDR: multi-drug resistance; ROS: reactive oxygen species; IR: ionising radiation. Adapted from and reprinted from (Wigerup et al., 2016) with creative commons attribution non-commercial no-derivatives license (CC BY-NC-ND).

1.3 DNA replication stress

Eukaryotic cells effectively regulate the replication of their genomes through a complex and tightly controlled process, which ensures the accurate duplication of the genetic information and the preservation of genome stability. DNA replication is one of the most important and well-regulated biological processes, that ensures that the genome will be duplicated just once per cell cycle, and that replication is complete before the initiation of mitosis. The importance of the DNA replication program is highlighted by the fact that not only the sequence of the events, but also the majority of proteins involved in this process are highly conserved in eukaryotes (Branzei and Foiani, 2010).

The initiation of DNA synthesis occurs at multiple regions along chromosomes known as replication origins, where the replication machinery is loaded and from which two replication forks progress in a bidirectional manner (Leonard and Mechali, 2013). The number of replication origins differs between organisms and depends on the genome size. For example, *Saccharomyces cerevisiae* contain around 12,000, whereas in humans the number of replication origins ranges from 30,000 to 50,000, as large numbers are required to establish replication forks at multiple sites for the timely duplication of large linear chromosomes (Cvetič and Walter, 2005; Mechali, 2010). At replication origin sites, initiator proteins interact with the DNA in order to assemble the pre-replicative complexes (pre-RCs), which in turn load and activate the DNA helicases to unwind the duplex DNA (Leonard and Mechali, 2013). Specifically, the origin recognition complex (ORC) first binds to DNA and then recruits additional replication factors, such as Cdc6 (cell division control protein 6) and Cdt1 (chromatin licensing and DNA replication factor 1), which subsequently recruit the MCM (minichromosome maintenance) helicase complex (Diffley, 2011).

The formation of pre-RCs occurs in the late mitosis/early G₁ phase of the cell cycle, and the process is known as “DNA replication licensing” (Blow and Gillespie, 2008). It is

important to note that each replication origin fires only once per cell cycle, and that only a subset of these preassembled origins will be activated in S phase (Masai et al., 2010; Yoshida et al., 2013). Since cells have to balance speed, accuracy and the distribution/consumption of resources (such as nucleotides and other replication factors), the firing of replication origins occurs sequentially in a regulated manner throughout S phase (Masai et al., 2010; Zeman and Cimprich, 2014). Interestingly, most replication origins will not fire at all, and instead remain “silenced” during DNA replication (dormant origins) (Blow et al., 2011; Woodward et al., 2006). If a replication fork is blocked or stalled, dormant or nearby origins will fire to ensure that replication is effectively completed (Branzei and Foiani, 2010).

DNA replication occurs concurrently with many other metabolic functions, such as transcription, mRNA processing and chromatin remodelling, thus a tight coordination between these processes is essential for the proper inheritance of genetic and epigenetic information (Magdalou et al., 2014). However, the DNA replication machinery is often challenged by many endogenous and exogenous obstacles, which can cause RS. Although RS is a widely recognised and significant cellular problem, which leads to genomic instability and loss of cancer cell survival, the exact definition of RS is still evolving (Zeman and Cimprich, 2014). In this study we have defined RS, as the incomplete or slow progression of DNA synthesis leading to the accumulation of single-stranded DNA (ssDNA), as a result of either slow, stalled or collapsed replication forks (Puigvert et al., 2016). The accumulation of ssDNA at replication forks is a principal characteristic of RS and usually forms when the helicases continue to unwind the parental DNA although the polymerases have stalled (Pacek and Walter, 2004). The events and factors responsible for initiation and accumulation of RS will be described.

1.3.1 Causes of replication stress

Impaired replication fork progression can be caused by numerous intracellular and extracellular sources (Figure 1.4). In a recent review, Magdalou *et al.*, classified known RS sources into four broad categories: (1) sources that alter origin firing; (2) obstacles to replication fork progression; (3) conflicts/collision between DNA replication and transcription and (4) inappropriate metabolic conditions that affect DNA replication (Magdalou *et al.*, 2014). Many of the factors that contribute to RS could however fall into more than one of the above categories.

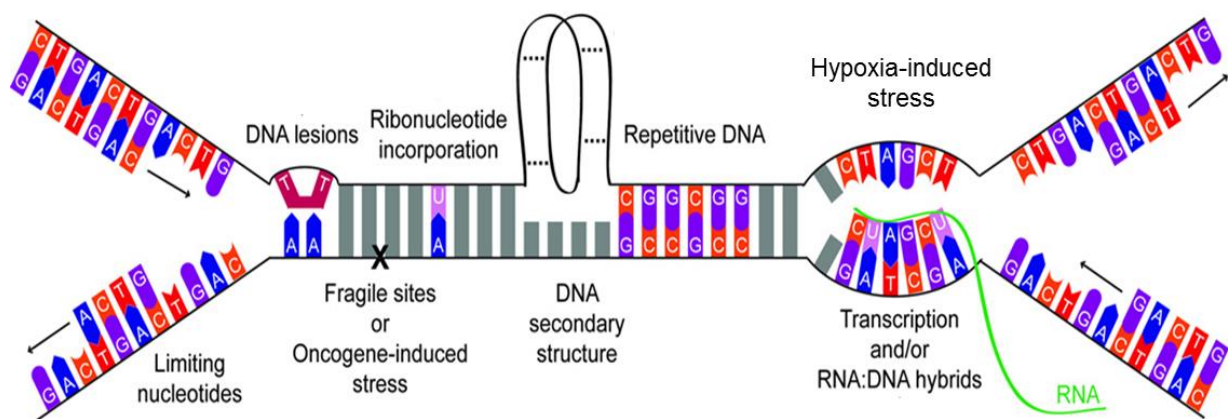


Figure 1.4 Sources of replication stress

Numerous impediments could interfere with DNA replication and result in RS. These include imbalanced nucleotide pools, DNA lesions and DSBs, misincorporation of ribonucleotides into DNA, secondary DNA structures, RNA/DNA hybrids (R-loops), oncogene overexpression and hypoxia. Adapted and reprinted from (Zeman and Cimprich, 2014), with permission from Nature Publishing Group, License Number: 3945900174132.

As previously discussed, during DNA replication the cell has to ensure that a sufficient number of origins fire sequentially during S phase in an orchestrated manner. Inappropriate firing of replication origins and, as a result, improper control of replication initiation, could be a major source of RS. Specifically, firing of more origins than required, could lead to nucleotide depletion and slow replication fork progression (Beck et al., 2012; Sorensen and Syljuasen, 2012). In contrast, insufficient origin firing could result in under-replication and subsequent loss of genetic information (Debatisse et al., 2012; Zeman and Cimprich, 2014).

Lack of the essential replication factors (nucleotides, replication machinery components, histones and histone chaperones) slow and destabilise replication forks and induce RS (Aguilera and Garcia-Muse, 2013; Bester et al., 2011; Sorensen and Syljuasen, 2012). The availability of deoxynucleotides (dNTPs) is considered as one of the most important factors, which when limiting, can impair the replication process (Bester et al., 2011; Poli et al., 2012). Replication rates during initiation and elongation are highly dependent on dNTP levels, which if low, could lead to slower replication fork progression and firing of more replication origins (Anglana et al., 2003). In addition, increased levels of dNTPs have been correlated with the accumulation of mutations and, as a consequence, genomic instability and increased cell survival (Chabes et al., 2003a). Thus, maintenance of optimal concentrations of dNTPs is critically important for the faithful replication of the genome, as imbalanced nucleotide pools induce RS and promote tumourigenesis (Bester et al., 2011; Chabosseau et al., 2011).

Unrepaired DNA lesions and double strand breaks (DSBs) are the most commonly recognised sources of RS because they form physical barriers to replication fork progression (Mailand et al., 2013; Zeman and Cimprich, 2014). Many agents (endogenous and exogenous) result in DNA damage including genotoxic agents and chemical mutagens, by-

products of cellular metabolism such as reactive oxygen species (ROS) as well as ionising radiation (IR) and ultraviolet light (UV) (Goldstein and Kastan, 2015). Misincorporation of ribonucleotides (rNTPs) instead of dNTPs into DNA, interferes with replication fork progression. Although misincorporated rNTPs are usually recognised and removed by activation of the RNase H2 enzyme, deficiencies in their removal from DNA have been shown to result in short deletion mutations, DSBs as well as RS *via* DNA strand misalignment (Nick McElhinny et al., 2010; Sparks et al., 2012; Williams et al., 2016). Additional obstacles to the replication machinery that lead to RS, include DNA-protein complexes, DNA sequences prone to form secondary DNA structures (such as hairpins and G-quadruplexes) and highly transcribed genes (Lambert and Carr, 2013; Magdalou et al., 2014; Zeman and Cimprich, 2014).

Interference between transcription and DNA replication is another source of RS. Since both transcription and replication share the same DNA template, inevitable collisions between the two complexes pose a significant problem for the replication machinery (Bermejo et al., 2012; Helmrich et al., 2013). Interestingly, it has been recently reported that highly transcribed regions which are replicated early during S phase, also known as ‘early replicating fragile sites’, are prone to DSB formation most likely due to collapsed replication forks after collision with the transcription complex (Barlow et al., 2013). In addition to the early replicating fragile sites, there are also some genomic regions (usually very large genes that are replicated late during S phase), which are particularly prone to accumulating DSBs during even moderate levels of RS (Debatisse et al., 2012; Smith et al., 2006). These loci, also known as ‘common fragile sites’ (CFS), are usually origin deficient regions and their sensitivity to breakage could be explained by the inability to activate dormant origins in order to rescue the stalled forks formed by DNA secondary structures and/or collisions with the transcriptional machinery (Helmrich et al., 2013; Zhang and Freudenreich, 2007).

Another transcription-related source of RS is the formation of RNA/DNA hybrids (R-loops). R-loops are three-stranded nucleic acid structures that are formed when nascent RNA hybridises with the template DNA strand, whilst the non-template ssDNA remains unpaired (Aguilera and Garcia-Muse, 2012). Depletion of factors that displace R-loops or prevent their resolution (such as the RNase H enzyme or the helicase Senataxin) can impede DNA replication, cause DNA damage and induce genomic instability (Aguilera and Garcia-Muse, 2012; Alzu et al., 2012).

Oncogene overexpression or constitutive activation is also considered an important source of RS, although the exact mechanism remains elusive. It has been proposed that oncogenes, such as MYC and Cyclin E, increase replication initiation and origin firing, a condition that would eventually lead to a shortage of nucleotides, limited replication factors and/or collisions with the transcriptional complex (Macheret and Halazonetis, 2015). Interestingly, it has been shown that elevated cellular nucleotide pools can rescue oncogene-induced RS, reduce DNA damage and decrease tumorigenicity of oncogene-overexpressing cells (Bester et al., 2011).

Severe hypoxia (<0.1% O₂) also leads to RS, although how remains somewhat unclear (Bencokova et al., 2009; Pires et al., 2010b). Importantly, mild hypoxia (for example 2% O₂) does not result in impaired DNA replication. It has been suggested, that the reason why only severe levels of hypoxia lead to RS, is that in the absence of oxygen the activity of the ribonucleotide reductase (RNR) enzyme, which provides the cell with dNTPs, is compromised (Pires et al., 2010b). The detailed understanding of hypoxia-induced RS, as well as the underlying mechanism/s and consequences of this defect, are questions pivotal to this study.

1.3.2 The cellular response to replication stress

The first and most crucial effect of RS is the activation of the DDR, following accumulation of long stretches of ssDNA. This ssDNA is usually formed either because the replicative helicases continue to unwind the DNA while the replicative polymerases are stalled, or because the newly synthesised DNA is degraded by the combined actions of nucleases and DNA helicases (Mimitou and Symington, 2011; Pacek and Walter, 2004). RPA (replication protein A) is the first responder to RS and acts by binding and stabilising the long ssDNA tracks (Glanzer et al., 2014). The primed ssDNA once coated with RPA, adjacent to the stalled newly synthesised double-stranded DNA, creates a primer-template structure that serves as a signalling platform for the activation of the RS response (Cimprich and Cortez, 2008). Specifically, after binding to ssDNA, RPA recruits and interacts with several factors involved in the ATR (ataxia-telangiectasia and RAD3-related) dependent DDR pathway. In brief, RPA first interacts with ATRIP (ATR-interacting protein), thereby recruiting and activating the ATR kinase, with additional cooperation from various stress response proteins (such as Rad9-Rad1-Hus1 (9-1-1) complex and the ATR-activator TOBP1) (Cimprich and Cortez, 2008; Zou and Elledge, 2003).

The ATR kinase, which is a member of PI3KK (phosphatidylinositol-3-kinase (PI3K) like kinase) family, plays a critical role in the cellular response to RS, and triggers a complex network of signalling pathways that act both locally and globally, in order to help the cell to survive and effectively complete DNA replication. Specifically, the local effect of ATR activation has been reported to be stabilisation and restart of stalled forks as well as suppression of recombination, whereas global effects include suppression of late origins and cell-cycle arrest (Figure 1.5) (Zeman and Cimprich, 2014).

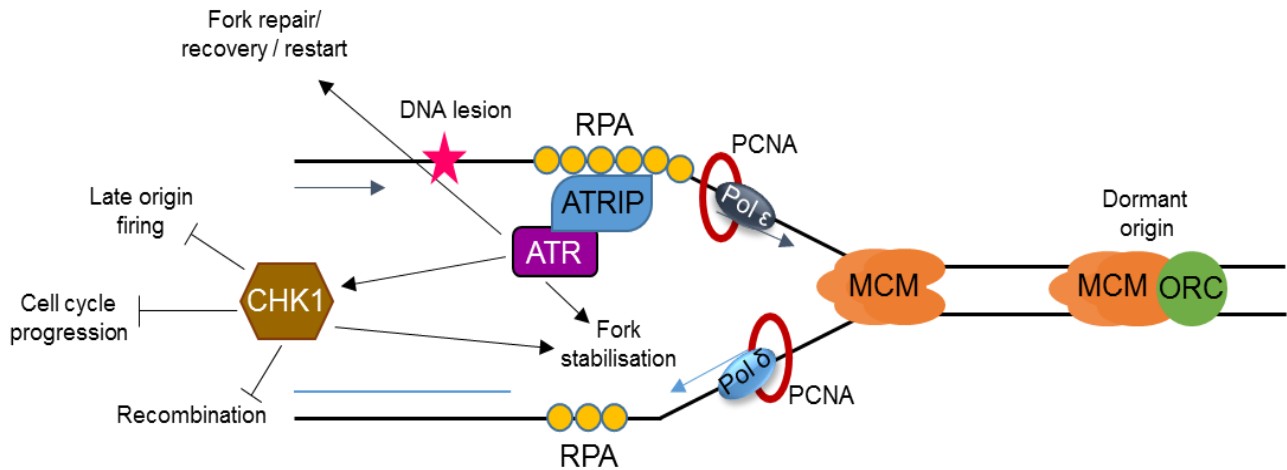


Figure 1.5 The ATR-mediated replication stress response

Parental DNA is unwound by the MCM complex and is replicated by the DNA polymerases that are bound to the replication cell nuclear antigen (PCNA), forming the leading and the lagging strands. In the event of a DNA lesion (pink star), DNA synthesis slows down and a primer-template structure is formed by RPA bound to the ssDNA, which serves as a signalling platform. This is recognised by ATRIP/ATR recruitment and ATR activation follows. ATR then initiates a local and global response by orchestrating a signalling cascade primarily mediated by the downstream target CHK1. This response promotes fork stabilisation and restart, and prevents cell cycle progression until replication is completed. Adapted from (Zeman and Cimprich, 2014).

The global actions of ATR provide the required time to resolve the stress, and allow the preservation of the resources needed (such as dNTPs, histones and RPA) around the stalled forks, in order to complete replication when the stress is resolved (Gunjan and Verreault, 2003; Toledo et al., 2013). When resources run low, ATR prevents the activation of late origins and entry into mitosis, primarily by phosphorylating one of its key downstream targets, CHK1 (checkpoint kinase 1) at Serine 345 (Ser, S345) (Dai and Grant, 2010; Maya-Mendoza et al., 2007). ATR has also been reported to regulate recombination at stalled and collapsed replication forks (Cimprich and Cortez, 2008). ATR-dependent substrates that maintain fork integrity are still under intensive investigation. It has been shown, that ATR by phosphorylating some of the key components of the replisome, can maintain the replisome in a competent state for replication (De Piccoli et al., 2012; Lopes et al., 2001). Additionally,

ATR recruits PLK1 (Polo-like kinase 1) through phosphorylation of MCM2, which in turn promotes dormant origin firing near to stalled replication forks (Trenz et al., 2008). Moreover, ATR phosphorylates the SMARCAL1 helicase preventing fork remodelling and fork collapse (Couch et al., 2013). ATR-dependent phosphorylation events that have been used as RS markers, include the histone variant pH2AX-S139 (γ H2AX) and pRPA (S33) (Marechal and Zou, 2013). However, it should be noted that not all cases of RS lead to activation of the ATR cascade, as in some cases not enough ssDNA is generated as only a few replication forks are involved (Magdalou et al., 2014).

In general, ATR-mediated fork stabilisation ensures that the fork will restart and continue faithful replication once the stress has been removed. However, some lesions can be bypassed as the replication machinery may continue replicating by re-priming after the lesion (Elvers et al., 2011). This generates ssDNA gaps that are ‘filled’ by lesion-specific polymerases or by fork remodelling (Mailand et al., 2013). Moreover, sometimes despite the RS response, stalled forks fail to restart and eventually collapse, resulting in DSB formation. These DSBs, which occur during RS, can be formed either by passive fork breakage or after the activation of endonucleases (such as Mus81), which cleave exposed structures (such as retained ssDNA or reversed forks) (Zeman and Cimprich, 2014). In the event of a DSB, cells respond by activating the DDR and the DNA repair pathways. Two main pathways are responsible for the repair of the DSBs, non-homologous end joining (NHEJ) which is error-prone and can occur throughout the cell cycle, and homologous recombination (HR) which is error-free and occurs in the S and G₂ phases of the cell cycle (Savage and Harkin, 2015). Although the NHEJ pathway predominates in mammalian cells, the HR pathway is usually the one required for the repair of the RS dependent DSBs (Goldstein and Kastan, 2015).

Generally, the DDR coordinates, in an orchestrated manner, cell cycle progression, DNA replication, DNA repair and apoptosis (Ciccia and Elledge, 2010). The DDR, in

addition to ATR signalling, includes activation of another two members of the PI3KK family: the ATM (ataxia-telangiectasia mutated) and DNA-PK (DNA-dependent protein) kinases (Goldstein and Kastan, 2015). It is generally considered, that the DDR activates the ATR pathway in response to RS and/or at single-stranded DNA breaks (SSBs), whereas in the event of DSBs, ATM and DNA-PK are the primary responders (Cimprich and Cortez, 2008; Jackson and Bartek, 2009). Importantly, ATM acts as the main kinase responder to DSBs, whereas DNA-PK is activated when DSBs are repaired through the NHEJ pathway (Collis et al., 2005). Of note, ATM activation occurs in the absence of DNA damage, in response to stresses such as hypoxia, heat, hypotonic and oxidative stress and agents that modify chromatin (Olcina et al., 2014). Regardless of the unique actions of the above kinases in the event of DNA damage, there is considerable crosstalk and collaboration between them. In particular, the ATR and ATM kinases share many common downstream targets and functions, and it has been shown that in some cases they can also activate each other (Cimprich and Cortez, 2008). A subset of the ATR and ATM downstream targets (common or not), and their usual cellular responses are shown in Figure 1.6.

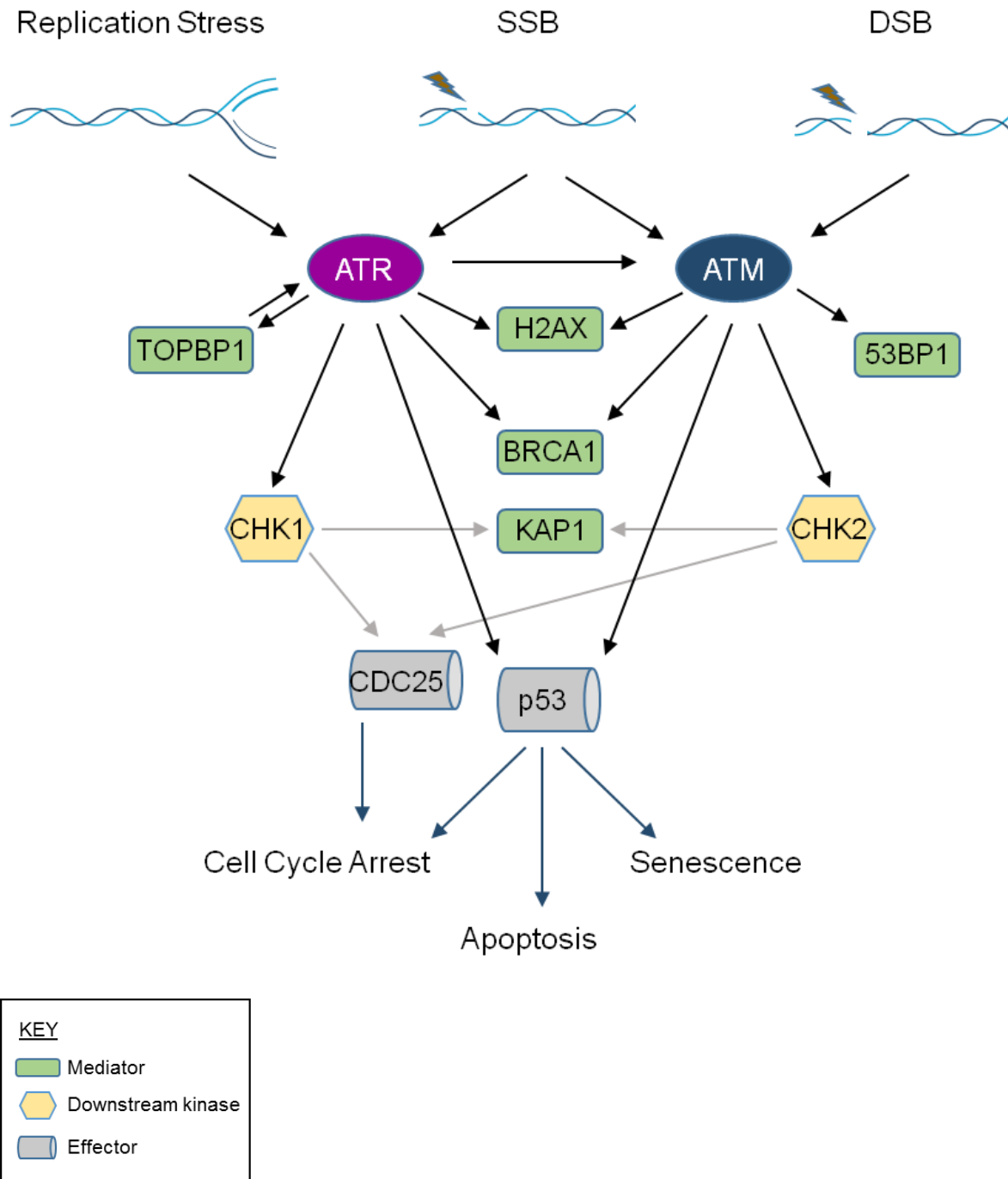


Figure 1.6 Schematic representation of ATR and ATM signalling pathways

Stalled forks due to replication stress, single strand breaks (SSB) and/or double strand break (DSB) can result in activation of the DNA damage response pathway, which will lead to activation of ATR and ATM kinases. Although the two pathways are usually activated by different lesions and each one has some unique targets, there is also considerable crosstalk and collaboration between them. Some important consequences following their activation are cell cycle arrest, senescence and apoptosis. For simplicity, only some of the ATR and ATM downstream targets are illustrated. The colour/shape coding of each protein involved in the cascade is given in the key. Adapted from (Olcina, 2014).

Two examples of downstream targets of the DDR which are activated by both ATR and ATM, are the repair factor BRCA1 (breast cancer susceptibility gene 1) and the tumour suppressor protein p53 (Cimprich and Cortez, 2008). BRCA1 is one of the key components of HR mediated DSB repair. It has many interacting partners and acts as a scaffold protein that forms multiple complexes in order to facilitate many different functions, such as DNA repair, cell cycle regulation, transcriptional regulation, protein ubiquitination, chromatin modification, mRNA splicing and apoptosis (Savage and Harkin, 2015). BRCA1 is crucial for the maintenance of genomic stability, as it can facilitate binding of ATM with the MRN (MRE11/RAD50/NBS1) complex at the DSB sites, it is required for efficient phosphorylation of p53 (S15) by ATM/ATR after exposure of cells to IR and UV and at the same time is involved in pre-mRNA splicing of genes involved in DNA damage signalling and repair (Fabbro et al., 2004; Foray et al., 2003; Lee et al., 2010; Savage et al., 2014). The tumour suppressor, p53 is also a key responder of the DDR, that has many cellular responses such as cell cycle arrest, senescence and apoptosis (Green and Kroemer, 2009). It was generally considered, that the tumour suppressive role of p53 was through transcriptional activation of DDR related genes, but recently this hypothesis has been questioned as the p53 targets involved in metabolism, ROS production and autophagy appear to be more important for this role (Brady et al., 2011).

1.3.3 The hypoxia induced DNA damage response pathway

As previously mentioned, severe levels of hypoxia (<0.1% O₂) lead to RS, which results in the activation of the DDR, but importantly this occurs without the concomitant generation of DNA damage (Bencokova et al., 2009). Strikingly, even prolonged exposure to these levels of hypoxia does not lead to detectable SSB or DSB formation (Hammond and Giaccia, 2004; Olcina et al., 2013). In contrast, significant levels of DNA damage can be readily detected during reoxygenation after periods of hypoxia (Hammond et al., 2003). The induction of RS in hypoxia is evidenced by both detection of specific RS markers (such as RPA-S33, γ H2AX and CHK1-S345) and by using the DNA fiber assay (Foskolou et al., 2016; Pires et al., 2010b). Specifically, Pires *et al.*, demonstrated that in response to severe hypoxia, replication slows down and consequently there is a decrease in origin firing and an increase in the number of stalled replication forks (Pires et al., 2010b). Additionally, Pires *et al.*, showed that RPA foci quickly accumulate when cells are exposed to severe hypoxia, indicating the presence of ssDNA (Pires et al., 2010b). Hypoxia induced RS leads to a robust activation of the DDR, which although generally shows the same characteristics as the ‘canonical’ DDR discussed above (activation of ATR/ATM signalling pathways and most of their downstream targets such as CHK1, CHK2, p53, KAP-1 *etc.*), it also exhibits some unique aspects, as discussed below.

Firstly, unlike activation of ATM in response to DSBs, the activation of ATM in hypoxia is independent of the MRN complex, further confirming the lack of DNA damage in these conditions (Bencokova et al., 2009). Olcina *et al.*, demonstrated that specific chromatin modifications are required in order for hypoxia induced ATM activation to take place (Olcina et al., 2013). Specifically, hypoxia increases trimethylation of H3 at lysine 9 (Lys, K9) (H3K9me3), which in combination with RS leads to activation of ATM signalling. Hypoxia-induced ATM activation promotes replication by protecting replication fork stability, since

ATM inhibition leads to slower replication rates under these conditions and accumulation of DNA damage (Olcina et al., 2013).

Both moderate and severe levels of hypoxia lead to repression of DNA repair pathways. Long hypoxic exposure leads to transcriptional and/or translational repression of many DNA repair proteins including BRCA1 (Bindra et al., 2007; Scanlon and Glazer, 2015). This repression of DNA repair pathways contributes to genomic instability, further highlighting the role of hypoxia in tumour initiation and progression. Moreover, p53 also shows an alternative response to hypoxia. For many years, it was thought that p53 predominantly acts as a transrepressor in hypoxia as opposed to a transactivator, as common targets (such as p21 and Bax) are not induced in these conditions (Hammond et al., 2006; Koumenis et al., 2001). However, Leszczynska *et al.*, recently reported a set of hypoxia-inducible p53 proapoptotic targets, which are associated with poor patient prognosis in numerous human cancers including breast, brain, colorectal, kidney, bladder, and melanoma (Leszczynska et al., 2015). Of note, these hypoxia-induced p53 targets are distinct from those induced in response to DNA damage and the primary role of p53 in hypoxia seems to be induction of apoptosis as opposed to cell cycle arrest. Graeber *et al.*, elegantly demonstrated that hypoxia drives the clonal selection of p53-mutated cells with low apoptotic potential. Specifically, the hypoxic regions of tumours with wild type (WT) p53 also had high levels of apoptosis, whereas regions that had lost p53 function had low levels of apoptosis (Graeber et al., 1996).

It is important to note that the hypoxia-induced DDR occurs independently of the HIF transcription factors and consequently moderate levels of hypoxia do not lead to ATR/ATM activation (Hammond and Giaccia, 2004; Papandreou et al., 2005). However, recent studies have highlighted crosstalk between those two pathways. Specifically, it has been reported that ATR activation contributes to HIF-1 α accumulation at the beginning of hypoxic exposure,

hypoxic DNA-PK activation protects HIF-1 α from degradation, while ATM phosphorylates HIF-1 α leading to its stabilisation in these conditions (Bouquet et al., 2011; Cam et al., 2010; Pires et al., 2012). Finally, although the DDR induction in response to severe hypoxia occurs very rapidly, most of the DDR targets show diminished phosphorylation following prolonged exposure to these conditions. A notable example is the phosphorylation of CHK1, which shows a rapid and robust induction after hypoxic exposure, which then declines after longer exposure (Pires et al., 2010a; Pires et al., 2010b).

1.3.4 Consequences of replication stress

Although the sources of RS vary, the consequences on cell fate are broadly similar. A defective DDR leads to persistent RS, which in turn results in deregulation of DNA replication and genomic instability followed by senescence or apoptosis (Magdalou et al., 2014). Cells that manage to evade apoptosis undergo clonal selection while they continue to accumulate mutations that help them adapt to their environment, including hypoxia. In addition, incomplete DNA replication or unrepaired DNA lesions result in chromosomal breakages, usually at prone regions such as at CFS (Macheret and Halazonetis, 2015). It has been reported that CFSs are also prone to atypical chromosome segregation events, like forming ultra-fine anaphase bridges (Chan et al., 2009). These delicate structures usually break during anaphase and lead to missegregation and improper translocation of chromosomal matter. Burrell *et al.*, found an increase in structural and numerical chromosomal instability, due to RS related DNA damage and abnormally formed chromosomes, as a result of chromosomal missegregation during mitosis (Burrell et al., 2013). These RS related inheritable genomic modifications are the driving forces of many human diseases. It is thought that the most common human disease which correlates with RS is cancer (Macheret and Halazonetis, 2015). A summary of selected human diseases known

to be associated with defects in RS or RS related signalling (DDR and DNA repair) is shown in Table 1.1.

Table 1.1 Human diseases correlated with defective RS and RS related signalling
Adapted from (Zeman and Cimprich, 2014).

Human Disease	Affected Pathway	Characteristics	Genes Involved
Aicardi-Goutieres syndrome	Removal of ribonucleotides; R-loops	Neurological dysfunction	<i>TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1</i>
Ataxia-ocular apraxia 2	Resolution of R-loops and transcription termination	Adolescent-onset cerebellar ataxia	<i>APTX, SETX</i>
Ataxia-telangiectasia	ATM signalling	Ataxia, weak immune system, cancer predisposition	<i>ATM</i>
Ataxia-telangiectasia-like disease	MRN complex; ATM/ATR activation	Neurodegeneration, ataxia	<i>MRE11</i>
Bloom syndrome	DNA remodelling; replication fork structure resolution	Premature ageing, growth retardation, cancer predisposition	<i>BLM</i>
Cancer	Many	Uncontrolled cell growth	Many
Ciliopathies	Centrosome; primary cilia formation	Dysfunction in degeneration of organs (kidney, retina, brain)	Many
Fanconi anemia	DNA inter-strand crosslink repair; replication fork protection	Heterogeneous: bone marrow failure, skeletal defects, cancer predisposition, hypopigmentation	More than 15 genes; mostly <i>FANCA, FANCC, FANCG</i>
Laminopathies	Nuclear envelope structure	Premature ageing	<i>LMNB1</i>
Meier-Gorlin syndrome	Origin licensing; centrosome maintenance	Growth retardation, microcephaly	<i>ORC1, ORC4, ORC6, CDT1, CDC6</i>
Nijmegen breakage syndrome	MRN complex; ATR/ATM activation	Growth retardation, microcephaly, cancer predisposition	<i>NBN</i>
Seckel syndrome	ATR signalling	Growth retardation, dwarfism, microcephaly, mental retardation	<i>ATR, CENPJ, DNA2</i>
Werner syndrome	DNA remodelling; replication fork structure resolution	Premature ageing, growth retardation, cancer predisposition	<i>WRN</i>
Xeroderma pigmentosum	Nucleotide excision repair	Cancer predisposition	<i>XPC, ERCC2, POLH</i>

1.4 Nucleotide metabolism

As already discussed, aberrations in dNTP pools, both in terms of dNTP pool levels and in terms of asymmetry of nucleotide ratios, could result in impairments of DNA replication fidelity and to increased genomic instability and disease. Many anticancer agents target nucleotide metabolism and have been used clinically for more than sixty years, therefore highlighting its importance for tumour initiation and progression.

1.4.1 Nucleotide biosynthesis

The biosynthesis of dNTPs involves both *de novo* and salvage pathways. In both cases, the first step of nucleotide biosynthesis starts with the action of RNR. RNR is the enzyme responsible for the rate-limiting step of *de novo* synthesis of dNTPs, which involves the stepwise assembly of purine and pyrimidine rings (Mathews, 2015). Specifically, RNR reduces ribonucleoside diphosphates (NDPs) to deoxyribonucleoside diphosphates (dNDPs), through a chemically “difficult” reaction, which requires the involvement of organic free radicals (discussed in detail in Chapter 1.5) (Reichard and Ehrenberg, 1983). After the reduction of NDPs to dNDPs, nucleoside diphosphate kinase (NDPK) is responsible for their conversion to dNTPs by phosphorylation (Kunz and Kohalmi, 1991). NDPK is an enzyme of low specificity, and phosphorylates all four dNDPs to dNTPs, without showing a preference for phosphate donor or acceptor. NDPK is also ubiquitously expressed in cells and has not been shown to have a role in regulating dNTP pool concentrations (Mathews, 2015). Interestingly, it has been reported that NDPK has an additional tumour-suppressive role that is independent of its kinase activity, and thus from its main role in nucleotide synthesis. The mechanism of NDPK mediated tumour suppression involves one of its human isoforms

(*NDPK-B* gene, encoding the PuF protein), which is a transcription factor and a suppressor of metastasis (Postel, 1999; Postel et al., 1993).

The pathway leading to deoxythymidine triphosphate (dTTP) *de novo* biosynthesis is more complicated than the other dNTPs, as additional steps are required. Specifically, first conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) is required, a reaction that is catalysed by thymidylate synthase. Then, dTMP is phosphorylated to dTDP by thymidylate phosphate kinase (TMPK), and finally NDPK phosphorylates dTDP to yield dTTP. dUMP is generated either from deoxyuridine triphosphate (dUTP), or deoxycytidine monophosphate (dCMP), both of which depend on RNR for their biosynthesis (Figure 1.7) (Reichard, 1988).

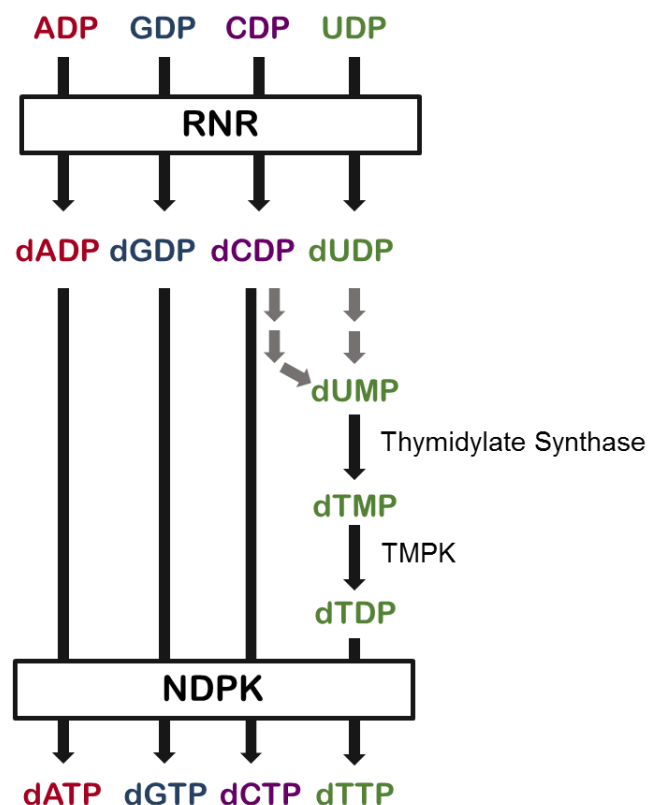


Figure 1.7 *De novo* pathway of nucleotide biosynthesis

RNR is the enzyme responsible for the rate-limiting step of *de novo* synthesis of dNTPs, as it reduces NDPs to dNDPs. Then, NDPK converts dNDPs to dNTPs by phosphorylation. dTTP *de novo* biosynthesis requires additional steps and more enzymes are needed, such as thymidylate synthase and TMPK.

In addition to *de novo* nucleotide synthesis, salvage pathways exist for reutilisation of nucleosides, as well as for purine and pyrimidine bases (collectively called nucleobases), which accumulate during nucleic acid degradation. Nucleobases are reutilised by reaction with the PRPP (5-phospho-ribo-1-pyrophosphate), in a reaction that is catalysed by phosphoribosyltransferases, whereas nucleosides are salvaged through the action of nucleoside kinases (such as thymidine kinase 1, thymidine kinase 2, deoxycytidine kinase and deoxyguanosine kinase) (Mathews, 2015). Interestingly, the pyrimidine (cytosine, thymine and uracil) salvage pathway has been reported to be more efficient than the purine (adenine and guanine) pathway (Reichard, 1988). The main salvage pathways of dNTP synthesis are illustrated in Figure 1.8. Importantly, many anticancer agents rely on these salvage pathways to be converted to active nucleotide analogues (Table 1.2). These drugs (e.g. 5-Fluorouracil, Arabinosylcytosine (AraC) *etc.*) are designed as analogues of nucleosides or nucleobases because nucleotides cannot be transported through the plasma membrane (Mathews, 2015).

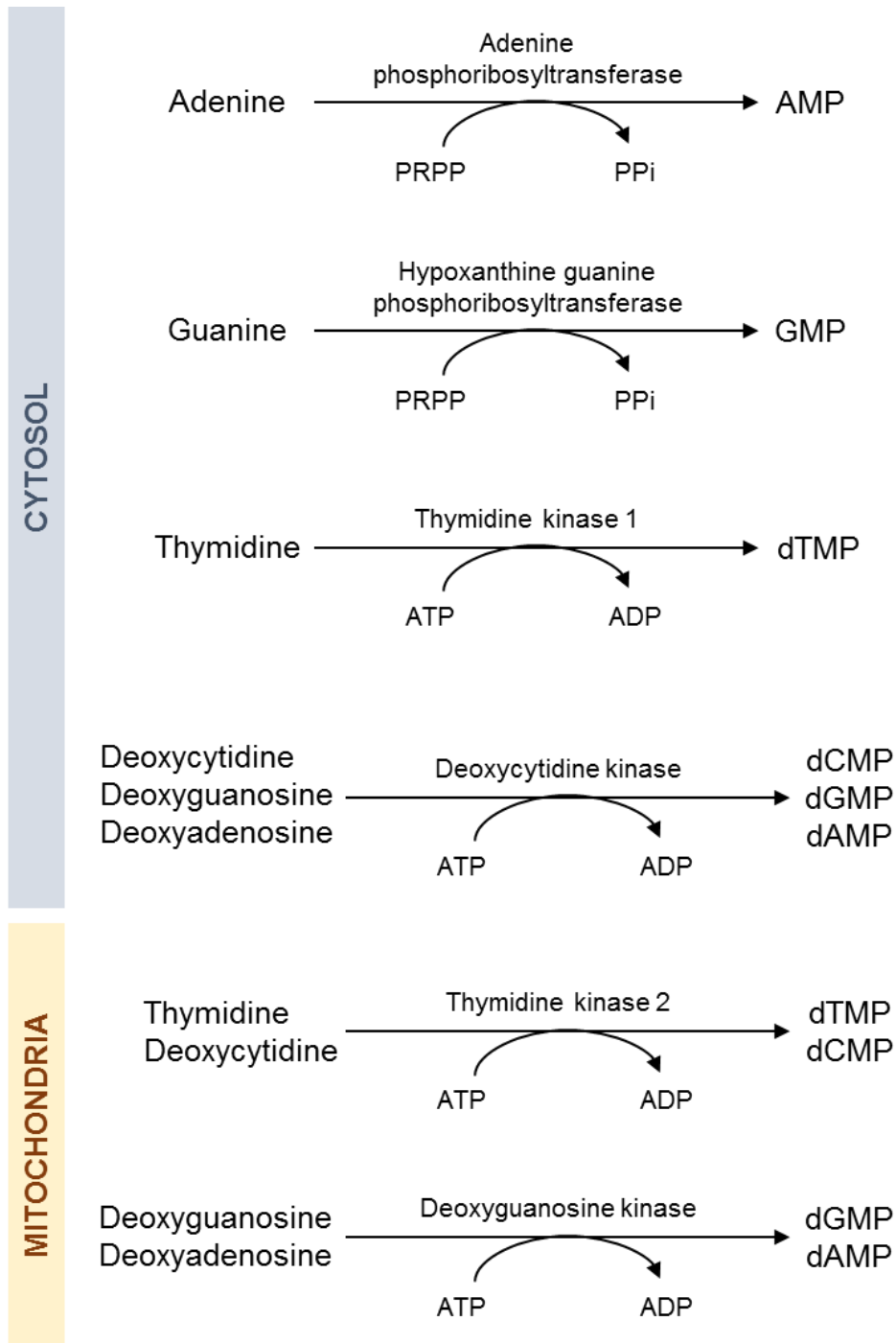


Figure 1.8 Main reactions in the salvage pathways of nucleotide biosynthesis

Thymidine kinase 2 and deoxyguanosine kinase are localised in mitochondria, whereas the other enzymes are primarily located in the cytosol. Nucleobases can be converted to ribonucleosides through the action of nucleoside phosphorylases, and in order then to be converted to deoxyribonucleosides, the action of RNR is required (*de novo* nucleotide biosynthesis). PRPP: 5-phosphoribosyl-1-pyrophosphate; P_i: inorganic phosphate. Adapted from (Mathews, 2015).

Table 1.2 Examples of anticancer agents that target dNTP metabolism

Adapted from (Mathews, 2015).

Agent	Targeted enzymes	Clinical use
<i>Folate analogues</i>		
Methotrexate	Dihydrofolate reductase	Acute lymphocytic leukaemia, osteosarcoma, choriocarcinoma, breast, head and neck, lung and bladder cancer
Pemetrexed	Thymidylate synthase and glycinamide ribonucleotide transformylase	Mesothelioma and lung cancer
<i>Pyrimidine analogues</i>		
5-Fluorouracil	Thymidylate synthase	Breast, colon, oesophageal, stomach, pancreas, head and neck cancers and premalignant skin
Arabinosylcytosine (AraC)	DNA polymerase	Acute myelogenous and acute lymphocytic leukaemia and non-Hodgkin lymphoma
Gemcitabine	RNR inhibition	Pancreatic, ovarian and lung cancers
5-Azacytidine	DNA cytosine methyltransferase	Myelodysplasia
<i>Purine analogues</i>		
6-Mercaptopurine	<i>De novo</i> purine nucleotide synthesis	Acute myelogenous and acute lymphocytic leukaemia and small-cell non-Hodgkin lymphoma
Fludarabine	DNA polymerase and RNR inhibition	Chronic lymphocytic leukaemia and low-grade lymphomas
Cladribine	Causes strand breaks after incorporation into DNA and RNR inhibition	Hairy cell leukaemia and low-grade lymphomas
Clofarabine	RNR inhibition	Paediatric acute leukaemias

Nucleotide biosynthesis is under strict regulation, since cells have to maintain two well-organised sets of dNTP pools that are functionally distinct, depending on the phase of the cell cycle. One pool is required for DNA replication and DNA repair of the genomic DNA during S phase, whereas the second is a smaller pool which has to be available throughout the cell cycle, for mitochondrial DNA (mtDNA) replication and the repair of genomic DNA outside of S phase (Mathews, 2015). The dNTP levels fluctuate throughout the cell cycle, reaching maximum levels in S phase. This is mostly due to differential expression of one of the RNR subunits (discussed below). Interestingly, thymidine kinase also shows differential expression depending on the phase of the cell cycle, whereas the expression of deoxycytidine kinase is constitutive throughout the cell cycle (Bianchi et al., 1997). The finding that the expression of some enzymes involved in pyrimidine biosynthesis fluctuates throughout the cell cycle, whereas others are ubiquitously expressed, should be taken into consideration before pyrimidine analogues are given to patients as anticancer agents (Mathews, 2015).

1.4.2 Cellular localisation of nucleotide biosynthesis

The location of dNTP biosynthesis is debateable, but the majority of studies agree that dNTPs are primarily synthesised in the cytosol (Leeds et al., 1985; Pontarin et al., 2008). Cytosolic dNTPs are then translocated either to the nucleus by passive diffusion through the nuclear pores, or to the mitochondria through dNTP transporters (Di Noia et al., 2014; Leeds et al., 1985). Although many studies have shown that RNR is cytosolic, Niida *et al.*, reported that in response to IR-dependent DNA damage, RNR translocates to the nucleus and associates with Tip60 (histone acetyltransferase 60 kDa Tat-interactive) at the sites of DNA damage (Engstrom and Rozell, 1988; Niida et al., 2010; Pontarin et al., 2008). Importantly, TMPK which converts dTMPs to dTDPs, seems to behave in a similar manner (Hu et al.,

2012). Inhibition of TMPK leads to accumulation of dUTP, which can be incorporated into DNA and result in DNA breaks (Hu et al., 2012). In general, the enzymes that take part in thymidylate metabolism (serine hydroxymethyltransferase, thymidylate synthase and dihydrofolate reductase) are essential for the integrity of DNA synthesis, as they prevent incorporation of uracil into DNA, and are therefore attractive targets for anticancer therapy (Anderson et al., 2012; Field et al., 2014; MacFarlane et al., 2011) (Table 1.2). It is an interesting question if the physical relocation of enzymes, especially in events of stress such as DNA damage, occurs to place the metabolic intermediates (e.g. dNTPs) at the sites of their utilisation. Further study is still needed to gain a better understanding into these cellular mechanisms and to be able to address these questions.

1.4.3 Regulation of nucleotide metabolism through nucleotide degradation

It is important to note that in addition to nucleotide biosynthesis, nucleotide degradation also has a critical impact on dNTP levels. Specifically, nucleotide pools are partly controlled by nucleotide kinases that perform ‘substrate cycle’ reactions, where interconversion between nucleosides and nucleotides takes place (Rampazzo et al., 2010). Interestingly, recent studies have shown that both nucleoside kinase and 5-nucleotidases represent sites of control of dNTP pools, as evidenced by the discovery that a number of lymphocytic leukaemia patients became resistant to purine analogues agents, due to overexpression of a mutant version of a 5'-nucleotidase enzyme (encoded by *NT5C2*) (Tzoneva et al., 2013). The degradation of dNTPs is also controlled by SAMHD1 (sterile α -motif and histidineaspartate domain-containing protein 1), which indiscriminately cleaves all dNTPs to deoxyribonucleosides and triphosphate products (Goldstone et al., 2011; Powell et al., 2011). Activation of SAMHD1 leads to depleted nucleotide pools, whereas inhibition causes accumulation of dNTPs and cell cycle arrest (Franzolin et al., 2013;

Mathews, 2015). SAMHD1 restricts lentivirus infection probably by depleting the infected cells of the essential dNTPs required for reverse transcription. Deficiency in *SAMHD1* has also been associated with the Aicardi-Goutieres syndrome in humans (Laguette and Benkirane, 2012; Powell et al., 2011). Recently, it was reported that SAMHD1, in addition to its dNTPase activity, also has ribonuclease activity (Ryoo et al., 2014). This could possibly explain why patients with Aicardi-Goutieres syndrome have problems in resolving R-loops (Table 1.1).

1.4.4 Oncogenes and tumour suppressor genes influence nucleotide metabolism

Deoxyribonucleotide metabolism can also be influenced by activation and expression of oncogenes as well as by tumour suppressor genes. For example, once activated, the RB (retinoblastoma) tumour suppressor depletes dNTP pools by decreasing the transcriptional expression of RNR, thymidylate synthase and dihydrofolate reductase (Angus et al., 2002). Moreover, downregulation of the MYC (myelocytomatosis) oncogene has been correlated with dNTP pool depletion by repression of thymidylate synthase, inosinate dehydrogenase and PRPPS2 (PRPP synthetase 2) (Mannava et al., 2008). Additionally, loss of the *FHIT* (fragile histidine triad) oncogene results in decreased dTTP pools through attenuated expression of the cytosolic form of thymidine kinase 1 (Saldivar et al., 2012). Finally, overexpression of the BCL-2 (B-cell lymphoma 2) oncoprotein has been associated with RS through inhibition of RNR and subsequent depletion of dNTPs (Xie et al., 2014).

1.5 Ribonucleotide reductase (RNR)

RNR is the only known enzyme responsible for the *de novo* conversion of the four standard ribonucleotides (5'-diphospho -adenosine, -cytidine, -guanosine and -uridine), to their corresponding 2'-deoxyribonucleotide counterparts. Ribonucleotides are synthesised from amino acids, tetrahydrofolate derivatives, NH_4^+ , CO_2 and 5-ribosyl-l-pyrophosphate. Such a synthetic pathway does not exist for deoxyribonucleotides, highlighting the major role of RNR in all living organisms (Stryer, 1995). The importance of RNR is also emphasised by the many different levels at which it is regulated, including allosteric and transcriptional control, cell cycle specific proteolysis in mammalian cells and inhibition through small proteins in yeast (Nordlund and Reichard, 2006). Although RNR has been extensively studied for more than 50 years, research into this enzyme continues to unearth new and fascinating attributes and regulatory mechanisms.

1.5.1 Classes of RNR

A common feature of all RNR enzymes, is the requirement of organic free radicals for substitution of the 2-OH group of a ribonucleotide with a hydrogen, which is the basic catalytic mechanism for deoxyribonucleotide formation (Figure 1.9) (Nordlund and Reichard, 2006). Different classes of RNRs employ different mechanisms for the organic radical generation (Kolberg et al., 2004). Specifically, RNRs have been divided into three classes depending on their metallofactor requirements, oxygen dependency and means by which this radical is formed (Eklund et al., 2001). Class I RNRs are characterised by a tyrosyl radical which is generated through an oxygen dependent mechanism and requires a diiron centre. Class I RNRs are found in almost all eukaryotic organisms as well as some bacteria, bacteriophages and viruses with a very limited distribution in Archea. Class II RNRs are

found in Archea and bacteria, which can live under both aerobic and anaerobic conditions, and are characterised by the formation of a thiyl radical that requires a cobaltous cofactor called adenosylcobalamin, which is a vitamin B₁₂ derivative. Of note, class II RNRs are unique amongst the three classes in that they prefer NTPs as a substrate instead of NDPs. Class III RNRs, are anaerobic and form a glycyl radical that requires an iron–sulfur centre in the presence of S-adenosylmethionine and reduced flavodoxin. Class III RNRs are found in strict, or facultative, anaerobic bacteria and some bacteriophages (such as bacteriophage T4) (Kolberg et al., 2004; Munro et al., 2013). Generally, class I and II RNRs are structurally more similar to each other, than either of them with class III. Many bacteria, such as *Escherichia coli* (*E. coli*), have been proposed to encode two or even three different classes of RNRs in their genome, which are expressed depending on the environment they are exposed to, and in some cases concomitant expression of different RNR classes has been observed, for example in some *Pseudomonas* species (Jordan et al., 1999).

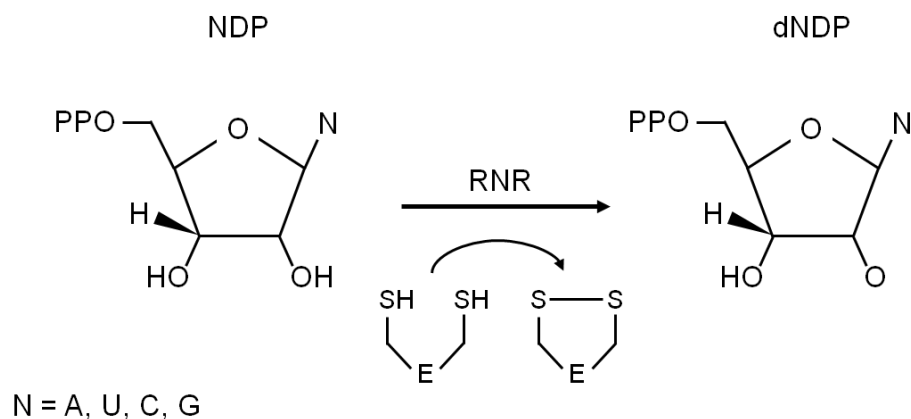


Figure 1.9 The basic catalytic reaction of RNR enzyme for dNDP formation

RNR catalyses *de novo* biosynthesis of dNDPs. An example of NDP reduction to dNDP by a Class I RNR enzyme is illustrated.

A: adenosine; U: uracil; C: cytidine and G: guanosine. Adapted from (Huang et al., 2014).

Higher organisms contain only class I RNRs, which are further subdivided based on their metal composition into three subclasses (Ia, Ib and Ic). Class Ia enzymes utilise the characteristic diiron centre, which reacts with oxygen and forms the diferric-tyrosyl radical ($\text{Fe}^{\text{III}}_2\text{-Y}^{\bullet}$). Class Ib enzymes utilise a diferric or dimanganic-tyrosyl radical ($\text{Mn}^{\text{III}}_2\text{-Y}^{\bullet}$). Class Ic enzymes, which have been characterised only *in vitro*, lack a tyrosyl radical and diiron centre, and instead utilise a manganese/iron metal centre (Cotruvo and Stubbe, 2011). Class Ia RNRs are found in eukaryotes, such as mammals and *Saccharomyces cerevisiae* and in a few prokaryotes, such as *E. coli*. Class Ib is found mostly in prokaryotes, such as *E. coli*, *Bacillus subtilis* and *Streptococcus sanguinis*. Few prokaryotes, like *E. coli*, contain both Ia and Ib RNRs (Huang et al., 2014).

All RNRs contain two subunits: the radical generator (hereafter called the β subunit) and the reductase (hereafter called the α subunit) (Figure 1.10). The β subunit produces and stores the aforementioned radical that is essential for initiation of NDP reduction, and as already discussed could differ between the different classes of RNR (Eklund et al., 2001). In contrast, all RNRs identified so far, share a structurally homologous α subunit, which binds NDPs and through a mechanism dependent on two cysteines (Cys, C), reduces them to dNDPs (described in detail below). In addition, the α subunit contains a third conserved Cys residue, at the active site that is located at the tip of a protein loop, in the centre of an α/β -barrel structural motif. This third Cys must be oxidised transiently (from β subunit) to a thiyl radical in order to initiate the NDP turnover (Kolberg et al., 2004; Stubbe and van Der Donk, 1998). Interestingly, despite the fact that α subunits in the different RNR classes share less than 10% of sequence homology, the overall folding of the active sites is extremely similar (Kolberg et al., 2004). The functional and structural similarities of the α subunit of RNRs, suggest that they have all evolved from a common ancestor, which would probably be more similar to today's class III RNR (Nordlund and Reichard, 2006; Stubbe et al., 2001).

In the remainder of Chapter 1.5, the mechanism, regulation and actions of Class Ia RNR (hereafter called RNR) which is the main focus of this work will be discussed.

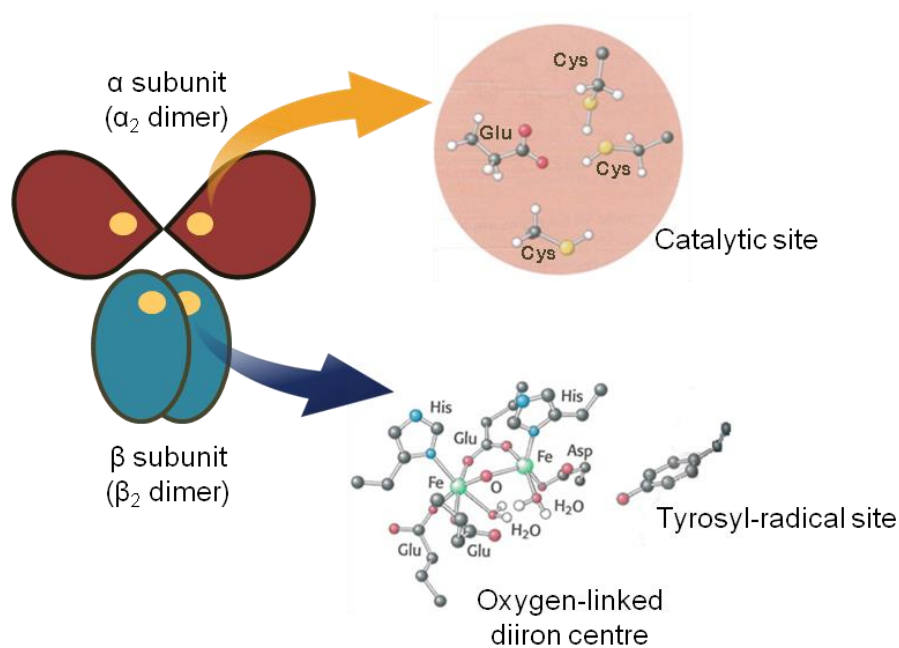


Figure 1.10 Class Ia RNR enzyme

The two subunits of RNR enzyme. The α subunit (illustrated as α_2 dimer) is the reductase, and contains the catalytic site. The β subunit (illustrated as β_2 dimer) is the radical generator, as it contains the characteristic (for Class Ia RNRs) oxygen-linked diiron centre which generates the tyrosyl radical.

1.5.2 The catalytic mechanism of RNR

The most well studied RNR is from *E. coli*, and so the following description of the catalytic mechanism for NDP reduction follows the *E. coli* numbering of the amino acid residues. The signal that triggers NDP turnover in RNR is not yet fully understood, however it is known that the α subunit must form a complex with the β subunit and binding of NDP is required (Kolberg et al., 2004). Firstly, a tyrosyl radical is generated in the β subunit, which is transferred to the α subunit in order to initiate catalysis (Stubbe et al., 2003). The tyrosyl radical formation and transfer will be described in detail in Chapter 1.5.3.

RNR dependent catalysis begins with the formation of a transient thiyl radical at a highly conserved Cys (for *E. coli*, C439) in the α subunit (Figure 1.11, state 1), which is tightly situated to the bound NDP (Kolberg et al., 2004). This thiyl radical will remove a hydrogen atom from the 3'-carbon of the ribose ring and thereby generate a ribose radical at the NDP (Figure 1.11, state 2). The ribose radical will make the 2'-OH group of the NDP more accessible to acid catalysis, and is therefore protonated by C225 and leaves as H₂O. This results in conversion of NDP to a 2'-ketyl radical (Figure 1.11, state 3). The C225 and C462 are then oxidised to a disulfide anion radical, and then transfer a hydrogen atom to NDP (Figure 1.11, state 4). The excess radical electron is then transferred to the 2'-position of the NDP, *via* a chain of hydrogen-bonded active site residues (asparagine 437 (Asn, N437) and glutamate 441 (Glu, E441)), and this results in a substrate radical (Figure 1.11, state 5). This radical then removes the hydrogen from C439, leading to completion of dNDP formation and to a thiyl radical in C439 (Figure 1.11, state 6). The radical state is then transferred back to the β subunit, which reforms the tyrosyl radical. For complete turnover, the disulphide bridge between C225 and C462 must be reduced, and this becomes possible through an electron transfer provided by a redox network that involves C754 and C759 in the α subunit, thioredoxin (or glutaredoxin) and the NADPH (nicotinamide adenine dinucleotide

phosphate) binding flavoprotein thioredoxin reductase. Thus, NADPH provides the reducing power for NDP reduction in RNRs (Holmgren and Sengupta, 2010; Kolberg et al., 2004).

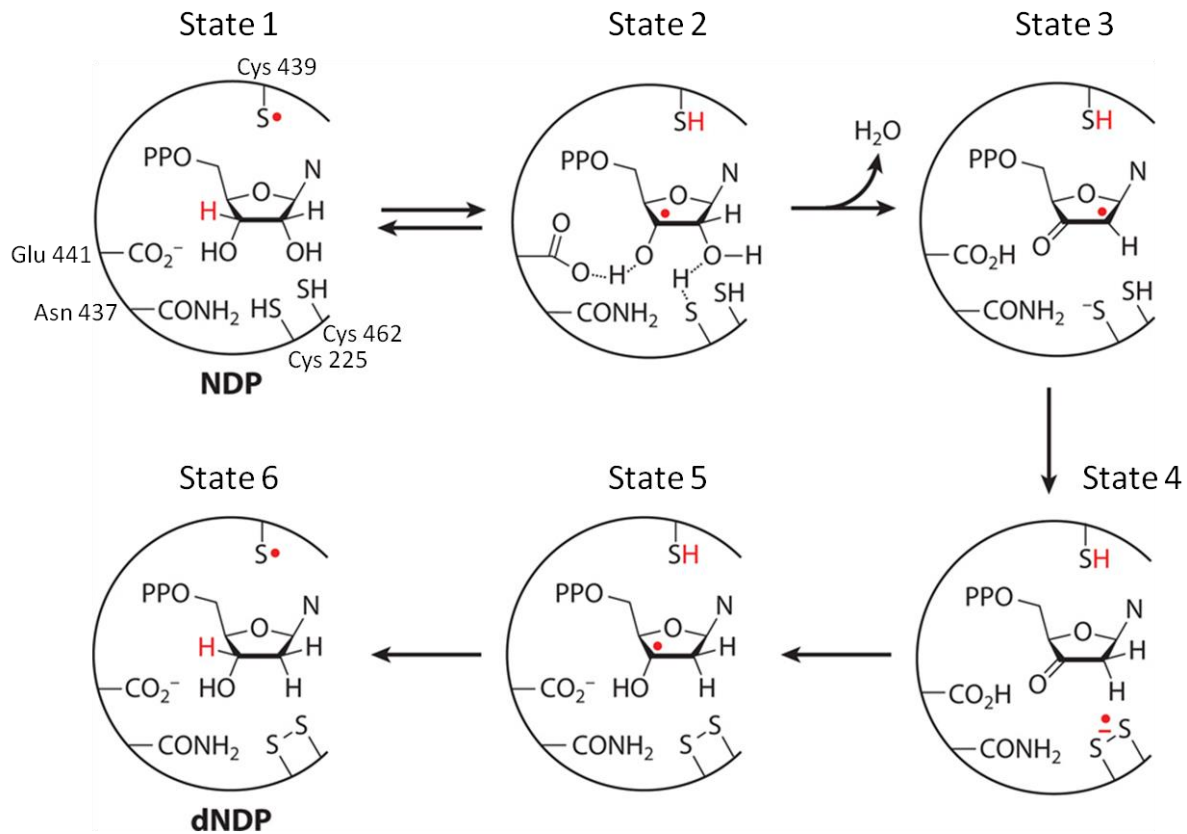


Figure 1.11 The reaction mechanism for reduction of NDP by RNR

RNR catalysis begins by formation of a transient thiyl radical at C439 in the α subunit. The thiyl radical will remove a hydrogen atom from the NDP, generating a 2'-OH group that will leave as H₂O. C225 and C462 are then oxidised and transfer a hydrogen atom to NDP. N437 and E441 are then involved and help for the NDP radical to be formed (state 5), which ultimately removes a hydrogen atom (red) from C439, completing as such the dNDP formation. This generates a thiyl radical in C439. The amino acid residues involved are given in *E. coli* numbering. The red circle symbolises the radical. Adapted and reprinted from (Cotruvo and Stubbe, 2011), no license permission is needed for non commercial use.

1.5.3 The formation of the tyrosyl radical

The initial radical needed for NDP reduction to dNDP is generated and stored in the β subunit, far from the active site in the α subunit. This radical is generated on a tyrosine (Tyr, Y) residue close to the diiron centre, and is protected from solvent as well as radical scavengers, as it is located in a hydrophobic pocket deep within the protein (Kolberg et al., 2004; Ormo et al., 1995).

Tyrosyl radical formation occurs when ions from the diiron centre react with molecular oxygen in a multistep process. The way that iron is incorporated into β , and how oxygen activates the diiron centre for radical initiation, is still poorly understood *in vivo*. However, detailed studies have been performed *in vitro*, especially on the purified β subunit of *E. coli*. Nordlund *et al.*, were first to reveal, by x-ray crystallography, that Y122 of the *E. coli* β subunit is in close proximity to the diiron centre, providing the first evidence that the diiron centre generates the tyrosyl radical (Figure 1.12) (Nordlund et al., 1990).

In vitro, the β subunit can exist in one of three states: apo- β (apo, without the metal) occurs when it lacks both the diiron centre and the tyrosyl radical, holo- β is when the diiron centre has been reconstituted and this results in formation of the tyrosyl radical, and meta- β is when it contains the diiron centre but the tyrosyl radical is in a reduced state. Holo- β is the only state that, when complexed with α subunit, RNR is active (Aye et al., 2015). In order for the reaction shown schematically in Figure 1.12 to take place, the apo- β requires the addition of iron (II) in the presence of oxygen. Then, the molecular oxygen has to be reduced to water, and this requires the involvement of four electrons. Two of those electrons come from the iron (II) which is oxidised to iron (III), one electron comes from the Y122 (generating the tyrosyl radical) and the fourth electron is thought to come from an external source, e.g. exogenous iron (II) (Kolberg et al., 2004).

Once formed the tyrosyl radical is then transferred through a proton-coupled electron transfer (PCET) to the α subunit *via* a pathway of hydrogen bonded amino acid chains (Ekberg et al., 1998; Minnihan et al., 2013). This generates the transient thiyl radical at the conserved Cys residue (C439 in *E. coli*) in the α subunit and allows catalysis to begin. The tyrosyl radical is not reduced after a single NDP reduction, but instead once formed, is stable enough to allow multiple turnovers until regeneration is necessary. Interestingly, the half-life of the tyrosyl radical varies between organisms, in *E. coli* RNR, it can last four days, whereas in human RNR it is only stable for ~25 min (Cotruvo and Stubbe, 2011; Thelander et al., 1983). The finding that the half-life of the tyrosyl radical in human RNR is less than half an hour raises the question of how RNR supplies dNTPs for DNA replication throughout S phase which lasts significantly longer. This is still a point of debate, and has led to the suggestion that either the radical is further stabilised *in vivo* or maintenance pathways exist in order to regenerate an active tyrosyl radical (Cotruvo and Stubbe, 2011).

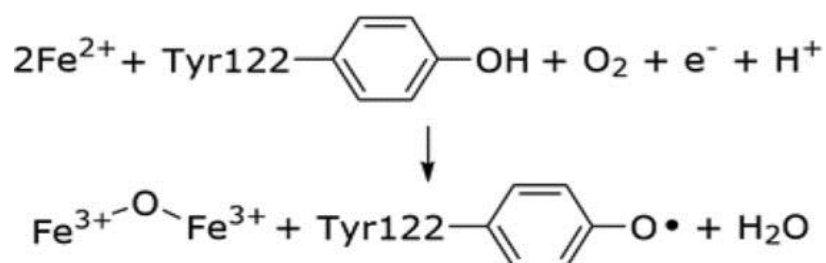


Figure 1.12 Stoichiometry of the tyrosyl generating reaction

The tyrosyl radical formation in the β subunit occurs when ions from the diiron centre react with molecular oxygen. The four electron reduction of oxygen requires an extra electron, in addition to the three electrons resulting from iron (II) oxidation to iron (III), and to the electron resulting from the tyrosyl radical formation (Y122' in *E. coli*). Adapted and reprinted from (Cotruvo and Stubbe, 2011), no license permission is needed for non commercial use.

It is important to note that the catalytically active holoenzyme of RNR has been suggested to be a $\alpha_2\beta_2$ tetramer at minimum (Minnihan et al., 2013; Uhlin and Eklund, 1994). In mammals, the α subunit is encoded by the *RRM1* (ribonucleotide reductase regulatory subunit M1) gene, whereas two different genes (*RRM2* and *RRM2B*), encode distinct β proteins: *RRM2* (ribonucleotide reductase regulatory subunit M2) and *RRM2B* (ribonucleotide reductase regulatory TP53 inducible subunit M2B), also known as p53R2 protein (Figure 1.13) (Aye et al., 2015). Although, it is generally believed that one tyrosyl radical is formed per β_2 subunit, it has been recently established that the tyrosyl radical is equally distributed between each β protein, which suggests the possibility of generation of two tyrosyl radicals per β_2 (Cotruvo and Stubbe, 2011).

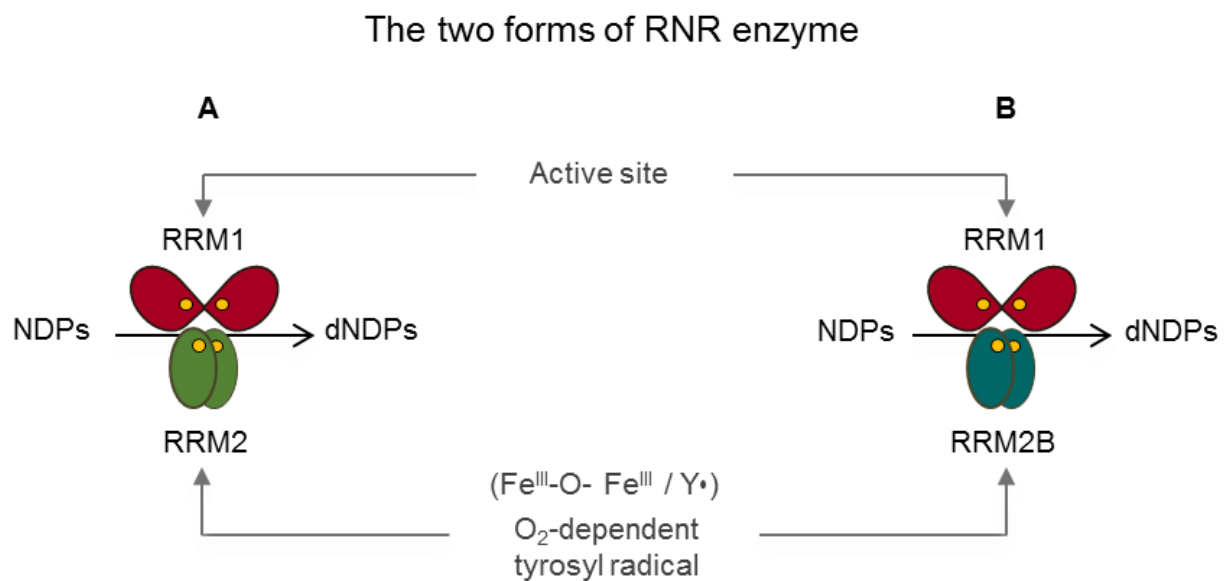


Figure 1.13 The two forms of mammalian RNR enzyme

Mammalian RNRs are a $\alpha_2\beta_2$ tetramer. α subunit is a RRM1 dimer, whereas β subunit can be an homodimer of either RRM2 or RRM2B (also known as p53R2) proteins. RRM1 contains the active catalytic site of NDP reduction, whereas RRM2 and RRM2B contain the oxygen dependent tyrosyl radical site that is essential for initiation of catalysis. **(A)** RRM1/RRM2 holoenzyme and **(B)** RRM1/RRM2B holoenzyme.

1.5.4 RNR activity regulation (allosteric regulation)

Since RNR is a principal component of dNTP homeostasis, its activity is under strict control through numerous distinct mechanisms, such as allosteric regulation, alterations in expression levels and localisation of its subunits as well as inhibitory factors (Aye et al., 2015). The allosteric regulation of RNR involves the α subunit (RRM1 protein), which in addition to the catalytic site (c-site; where the NDP reduction takes place), contains two different allosteric sites: the specificity site (s-site) and the overall activity site (a-site) (Figure 1.14 A) (Stryer, 1995). Hofer *et al.*, described the RNR allosteric regulatory system, as one of the most sophisticated known today, as it involves four substrates, four allosteric effectors and two effector binding sites (Hofer et al., 2012). Importantly, the allosteric regulation of RNR maintains both the relative ratios of the four different dNTPs as well as their absolute concentrations (Jonna et al., 2015). Specifically, the s-site determines substrate choice for catalysis in the c-site, whereas the a-site acts as a switch that can turn the enzyme on and off (Brown and Reichard, 1969). Importantly, all of the classes of RNRs (Class I, II and III) have the same s-site regulatory system, whereas the a-site predominates in Class I (with the exception of Class Ib) and Class III RNRs (Hofer et al., 2012).

Allosteric modulation through the s-sites, which are located in the interface of the α_2 dimer, prevents each concentration of individual dNTPs increasing in relation to the others (Hofer et al., 2012). This is possible as the s-site can bind ATP, dATP, dGTP and dTTP. $ATP_{(high)}/dATP_{(low)}$ stimulates reduction of CDP and UDP; dGTP stimulates reduction of ADP and dTTP stimulates reduction of GDP (Figure 1.14 B) (Nordlund and Reichard, 2006). Many studies of different species have shown that the s-site modulates substrate specificity *via* conformational protein changes. Specifically, the s-site is in contact with the c-site by a flexible loop. When one of the allosteric effectors binds to the s-site, the conformation of this loop changes in such a way that it makes the c-site more amenable to binding one substrate

over the others (Eriksson et al., 1997; Fairman et al., 2011; Larsson et al., 2004). Importantly, the s-site does not bind dCTP efficiently and therefore, additional regulation is needed for the dCTP/dTTP ratio through dCMP deaminase, which converts dCMP to dUMP (Hofer et al., 2012; Mathews, 2015). Interestingly, although the s-site controls the fine ratio between the four dNTPs, this does not mean that all dNTPs are represented equally in the dNTP pools. In all the systems examined to date, dGTPs are under-represented, constituting 5% or even less of the total dNTP levels (Martomo and Mathews, 2002). For example, in mammalian cells the following concentrations have been reported: dGTP (5 μM); dATP (24 μM); dCTP (29 μM) and dTTP (37 μM) (Traut, 1994). These concentrations are of course examples, as dNTP pool sizes vary between both different cell types and different organisms.

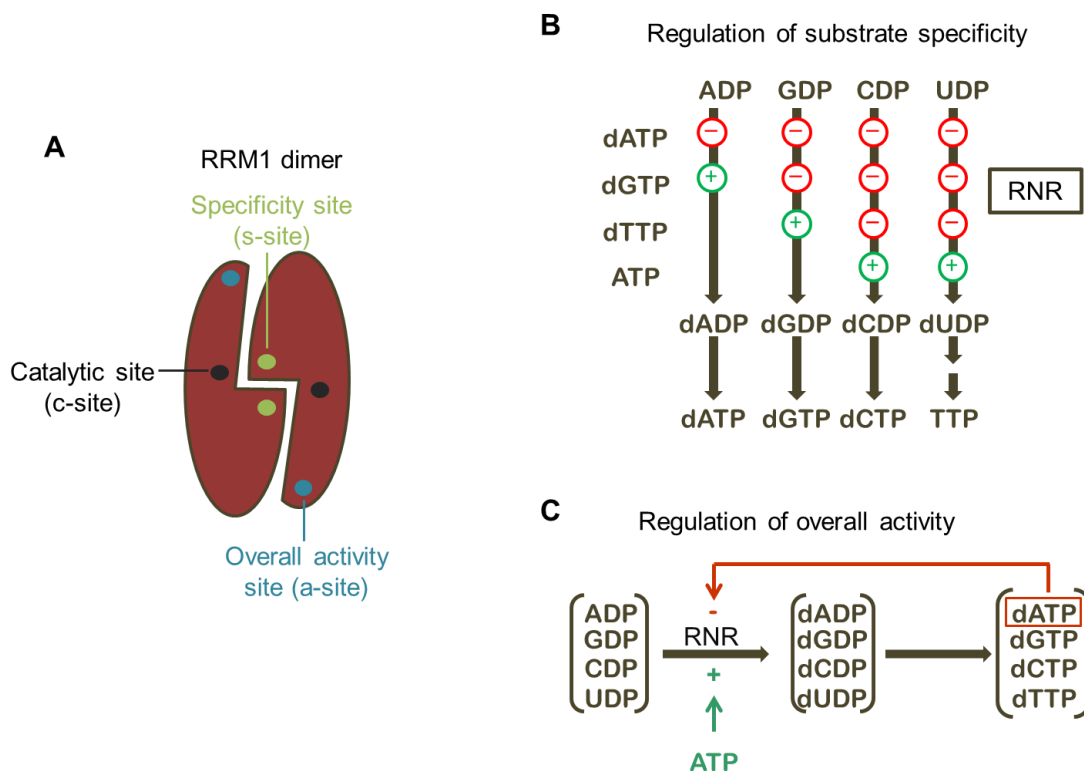


Figure 1.14 Allosteric regulation of RNR

(A) The RRM1 dimer of RNR contains the catalytic site (c-site), where the reduction of NDPs takes place, and two allosteric sites: the specificity site (s-site) and the overall activity site (a-site). **(B)** Regulation of substrate specificity in s-site maintains the relative ratios between the four dNTPs, by determining substrate choice for catalysis. **(C)** Regulation of overall activity in a-site switches the enzyme on/off, keeping the absolute concentrations of dNTPs in the appropriate levels. Adapted from (Stryer, 1995).

In addition to the substrate specificity control through the s-site, which is universal in all types of RNR, the control of the absolute concentrations of the four dNTPs does exist through the a-site. The a-site, is more unevenly distributed and is basically an ATP cone that is found in the N-terminus of the α subunit of some RNRs (not in Class Ib and scarcely in Class II) (Aravind et al., 2000; Hofer et al., 2012). The a-site can bind either ATP, which acts as an activator when the dNTP cellular levels are low, or dATP that acts as an inhibitor and is needed to turn off the enzyme when the dNTP levels are too high (Figure 1.14 C) (Kashlan et al., 2002). ATP has the same affinity for both a- and s- sites, whereas dATP shows 10-20 fold lower affinity for the a-site compared to the s-site (Brown and Reichard, 1969; Reichard et al., 2000). When dATP is low, it acts only as an s-site regulator, whereas at higher concentrations it binds both the s- and a-sites and turns the RNR enzyme off. In contrast, ATP has a lower affinity for both sites, but is still able to compete with the other dNTPs for substrate specificity in the s-site and with dATP in the a-site when the overall dNTP levels are low (Buckstein et al., 2008; Traut, 1994).

Binding of the allosteric effectors to the s-site, stimulates the α_2 dimerisation and promotes the binding with the β_2 dimer in order to form an active RNR $\alpha_2\beta_2$ complex. High dNTP levels mediate oligomerisation into tight complexes larger than the active $\alpha_2\beta_2$, and the two RNR subunits can no longer interact in a productive way. Specifically, in eukaryotic RNRs, a single dATP molecule binds to the a-site and mediates inhibition by promoting formation of an α hexamer ring (α_6). This α hexamer, even though it can bind β_2 to form a $\alpha_6\beta_2$ complex, disrupts the PCET between the two subunits leading to inhibition of the RNR enzyme (Fairman et al., 2011; Kashlan et al., 2002). Different organisms may show different inhibition mechanisms, such as *E. coli* which uses $\alpha_4\beta_4$ inhibitory complexes (Ando et al., 2011; Rofougaran et al., 2008). In a recent study, Jonna *et al.*, described a Class I RNR (from *Pseudomonas aeruginosa*), which binds two dATP molecules to its a-site, resulting in an

inactive α_4 complex. The authors suggest that the a-site (ATP cone) of RNR has been used as an evolutionary tool in order that the modern RNRs can be effectively regulated depending on specific organism requirements. This ensures control over the absolute concentrations of the dNTPs without the need for transcriptional or other non-allosteric related mechanisms (Jonna et al., 2015).

1.5.5 RNR activity regulation (cellular regulation)

In addition to the allosteric regulation of RNR, additional cellular mechanisms exist to keep control of dNTP biosynthesis. A major regulatory mechanism is the cell cycle phase-specific expression of the RNR subunits. In mammalian cells, the *RRM1* gene (α subunit of RNR) is mainly regulated transcriptionally and is overexpressed during S phase (Johansson et al., 1998). However, the levels of RRM1 protein remain stable throughout the cell cycle due to its long half-life (Engstrom et al., 1985). The RRM2 protein (one of the two forms of β subunit) is regulated at both the mRNA and protein levels. RRM2 is specifically overexpressed during S phase. During the G₂ phase Cyclin F (a Skip1/Cullin/F-box (SCF) complex ubiquitin ligase) recognises a KEN box motif in the N-terminus of RRM2, leading to degradation (D'Angiolella et al., 2012). In addition to Cyclin F-dependent degradation, RRM2 is also recognised by the Cdh1-anaphase-promoting complex (APC), which results in mitosis/G₁ phase-specific RRM2 proteolysis, again through recognition of the KEN box in RRM2 (Figure 1.13) (Chabes et al., 2003b). In contrast, RRM2B (the second form of β subunit) is ubiquitously expressed throughout the cell cycle (Nordlund and Reichard, 2006). The differences and/or similarities of the two forms of the β RNR subunit will be discussed in Chapter 1.5.6.

The subcellular localisation of RNR has been described as an additional regulatory mechanism, although in mammalian cells the aspect of re-localisation of RNR is still

controversial. As discussed in Chapter 1.4.2, recent studies propose RNR accumulation at the sites of DNA damage in the nucleus after IR (Niida et al., 2010). Generally, it has been shown that the DDR regulates RNR in multiple ways. After DNA damage due to genotoxic agents, *RRM1* and *RRM2* expression is upregulated (Filatov et al., 1996; Hurta and Wright, 1992). Most importantly, *RRM2B* is a direct target of p53, which induces its transcription after DNA damage (caused by several agents including UV, IR and adriamycin), in order to provide dNTPs for DNA repair (Tanaka et al., 2000; Tsai et al., 2006). In addition, in response to DNA damage, ATM stabilises *RRM2B* by direct phosphorylation at S72 (Chang et al., 2008). ATR is also involved in RNR regulation, by indirectly stabilising *RRM2*, through ATR-dependent downregulation of Cyclin F (D'Angiolella et al., 2012). Finally, another mechanism to regulate RNR is through RNR inhibitory proteins, which have been identified in both yeast and higher eukaryotes. The small protein inhibitors Sml1p and Spd1p have been reported to inhibit RNR in budding and fission yeast respectively, and the metazoan protein IRBIT, stabilises dATP in the allosteric a-site thereby inhibiting the enzyme (Arnaoutov and Dasso, 2014; Danielsson et al., 2008; Nestoras et al., 2010).

1.5.6 The β subunit of RNR (RRM2 and RRM2B proteins)

As briefly discussed earlier, the β subunit of mammalian RNR can be a homodimer of either RRM2 or RRM2B proteins (Figure 1.13). The two proteins share 83% sequence homology and can both bind RRM1 in order to form an active RNR holoenzyme (Figure 1.15) (Wang et al., 2009). RRM2 and RRM2B share the same mechanism of tyrosyl radical formation, which has been suggested to differ slightly from the canonical *E. coli* model (described in Chapter 1.5.2). Specifically, it has been proposed that human RNRs require two tyrosyls for the initiation of the tyrosyl radical instead of one, as in *E. coli* (Zhou et al., 2010). Zhou *et al.*, suggested that human RNRs employ a dityrosyl-diiron system for the initiation of the tyrosyl radical, and three-dimensional structural analysis revealed that these two residues are located on opposite sides of the diiron centre. In general, RRM2 and RRM2B proteins share several functional domains, including the iron ligands, the tyrosyl radical site, the hydrophobic pocket that surrounds the tyrosyl radical, the PCET pathway as well as the C-terminal sequence responsible for binding to the RRM1 protein (Shao et al., 2004).

Although RRM2 and RRM2B share many similarities, they also have key differences (Figure 1.15). Firstly, a major difference is that RRM2B lacks the KEN box that is found in the N-terminus of the RRM2 protein, which is recognised by SCF and APC complexes, leading to RRM2 cell cycle specific degradation (Chabes et al., 2003b; D'Angiolella et al., 2012). This means that the RRM1/RRM2 holoenzyme, is mostly responsible for providing dNTPs for S phase DNA replication, and repair in proliferating cells (Nordlund and Reichard, 2006). In contrast, the RRM1/RRM2B holoenzyme is believed to generate dNTPs for DNA repair, in quiescent cells, as well as for mtDNA replication (Bourdon et al., 2007; Guittet et al., 2001; Pontarin et al., 2012; Pontarin et al., 2007). Importantly, humans with RRM2B mutations have mitochondrial diseases, including mtDNA depletion syndrome, mitochondrial neurogastrointestinal encephalopathy and autosomal-dominant progressive external

ophthalmoplegia (Kollberg et al., 2009; Shaibani et al., 2009; Tyynismaa et al., 2009). Interestingly, mice lacking *RRM2B* are not embryonic lethal, but die early after weaning due to renal failure after attenuation of the dNTP pools and increased apoptosis (Kimura et al., 2003).

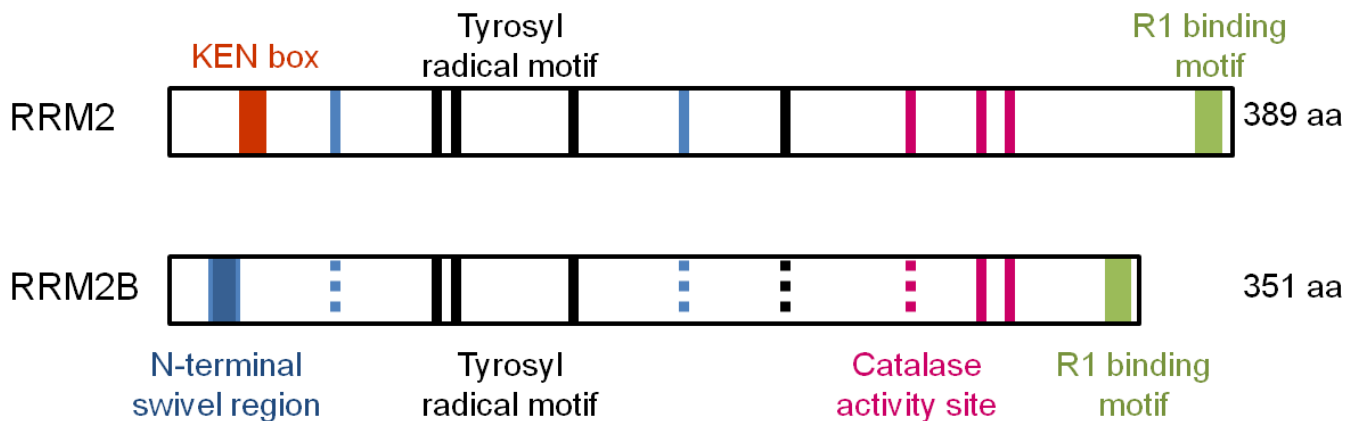


Figure 1.15 Similarities and differences between RRM2 and RRM2B proteins

RRM2 and RRM2B share 83% sequence homology but they also have key differences. Both proteins contain a C-terminal sequence responsible for binding with the RRM1 protein (green box) and share the same mechanism for tyrosyl radical formation (black lines). However, RRM2B lacks the KEN box (red) which is responsible for RRM2 cell cycle specific degradation. In blue are differences between the two proteins that lead to differences in iron-uptake capacities. Pink shows the responsible residues that lead to RRM2B specific catalase activity. Straight and dashed lines indicate homologous or not residues respectively.

Additionally, RRM2 and RRM2B show functional differences, including enzymatic activity, protection against ROS, iron-uptake capacity as well as different sensitivities to pharmacological RNR inhibitors. It has been shown that the kinetic activity of RRM2B-containing RNR is 20-50% lower than the RRM2-containing RNR, a feature that can be attributed to the lower binding affinity of RRM2B to RRM1 in comparison to RRM1/RRM2 (Shao et al., 2004; Yen et al., 2006). Moreover, RRM2B but not RRM2 has the unique ability of protecting cells against ROS. It has been shown that RRM2B exhibits catalase activity (through Y331, Y285, Y49 and Y241), and as a result breaks down H_2O_2 , thus offering cellular antioxidant protection (Xue et al., 2006). Finally, RRM2 and RRM2B show different

sensitivity to iron chelating agents and RNR inhibitors. Specifically, RRM2B shows a much higher sensitivity to deferoxamine mesylate (DFO: iron chelating agent) compared to RRM2 (Shao et al., 2004). This suggests that either RRM2B binds iron less efficiently than RRM2 or its diiron-radical centre is more accessible to the environment (Shao et al., 2004; Smith et al., 2009). On the other hand, RRM2B showed less sensitivity (2.5 fold) to the radical scavenger hydroxyurea (HU) than RRM2, but both proteins were equally sensitive to triapine (3AP), another RNR inhibitor (Shao et al., 2004). The sequence and structural differences between the two proteins, which may result in the functional differences observed in catalase activity, iron assimilation and radical accessibility, will be discussed in detail in Chapter 5.

1.5.7 RNR and cancer

RNR activity is highly correlated with cancer growth rates, and overexpression of the RNR subunits has in turn been correlated with many cancer types (Aye et al., 2015; Elford et al., 1970). Aye *et al.*, assessed the correlation of the expression of RNR genes in a large collection of human cancers using the ONCOMINE database. The authors found that *RRM2* was among the top 10% most overexpressed genes in 73 out of the 168 cancers analysed, *RRM1* was among the top 10% most overexpressed gene in 30 out of 170 studies and *RRM2B* was among the top 10% most overexpressed in 5 out of 90 studies (Aye et al., 2015). The authors continued with a TCGA analysis, in order to analyse changes in the RNR gene copy number, because the elevated RNR expression observed within the ONCOMINE study could be a result of cell cycle alterations, since many neoplasms have an increased S phase fraction. Interestingly, among the three RNR genes, *RRM2B* was the most frequently affected by copy number changes, and typically showed gains in many different cancers including breast, colon, ovarian, prostate and uterine cancers as well as glioblastoma. The copy number changes correlated with increased *RRM2B* expression in breast and prostate cancers, which supports a potential role for *RRM2B* as a tumour promoter (Aye et al., 2015). In the same TCGA study, *RRM2* and *RRM1* showed low and rare copy number changes respectively, in the malignancies tested.

RRM2 overexpression has been observed in gastric, ovarian, bladder and colorectal cancers, and has been correlated with tumour grade for breast and epithelial ovarian cancers (Liu et al., 2013; Ma et al., 2003; Morikawa et al., 2010a; Morikawa et al., 2010b; Wang et al., 2012). In addition, although *RRM2* levels are low in non-tumourigenic skin lesions, they are significantly higher in malignant melanomas and correlated with poor overall survival (Aird et al., 2013). Furthermore, it has been suggested that *RRM2* has oncogenic activity, due to cooperation with oncoproteins, which increase focus formation and anchorage-independent

growth in mouse cells (Fan et al., 1998; Fan et al., 1996). RRM2 has also been correlated with increased invasiveness in human carcinoma cells, as well as promoting tumour angiogenesis by elevating VEGF production (Duxbury et al., 2004b; Zhang et al., 2009). Interestingly, the synergy between *RRM2* and activated oncogenes in induced transformation is independent of its role in NDP reduction (Fan et al., 1996).

Increased *RRM2B* expression has been reported in melanoma, oral carcinoma, oesophageal squamous cell carcinoma and non-small cell lung cancer (NSCLC) (Matsushita et al., 2012; Okumura et al., 2006; Uramoto et al., 2006; Yanamoto et al., 2009; Yanamoto et al., 2003). The role of RRM2B in tumourigenesis and mutagenesis is still controversial. Due to its p53-inducibility and role in DNA repair, it was initially suggested that RRM2B would act as a tumour suppressor (Tanaka et al., 2000). It has been reported that under genotoxic stress RRM2B promotes p21 accumulation and thus G₁ arrest, in order to facilitate DNA repair and prevent mutagenesis (Xue et al., 2007). Moreover, RRM2B expression has been negatively correlated with colon adenocarcinoma metastasis and cancer cell invasiveness (Liu et al., 2011; Liu et al., 2006). However, as already mentioned, RRM2B overexpression has been correlated with many human cancers and its experimental suppression leads to impaired proliferation in cancer cells *in vitro* (Matsushita et al., 2012). Both RRM2 and RRM2B overexpression causes increased mutation frequency and error-prone DNA synthesis in cultured cells, especially when the DNA repair mechanisms are also defective, suggesting a synergistic effect in increasing tumourigenesis (Xu et al., 2008).

In contrast to the tumour promoting effects of RRM2 and RRM2B, RRM1 seems to act primarily as a tumour suppressor. Specifically, several studies show that RRM1 suppresses tumour initiation, and its overexpression in cultured cells results in reduced transformation, while *in vivo*, RRM1 overexpression leads to suppression of tumourigenesis and metastasis (Fan et al., 1997). In human lung cancer cells, increased levels of RRM1

suppress migration and invasion, by inducing expression of the phosphatase and tensin homolog (PTEN) (Gautam et al., 2003). In patients with NSCLC that underwent surgical resection, it was found that high levels of tumour-associated RRM1 expression, correlated with longer lifespan and later disease recurrence (Bepler et al., 2004). However, additional studies are required for the understanding of the tumour suppressive role of RRM1, especially through effects such as PTEN induction, as those roles could be independent of its main role in dNTP biosynthesis.

RNR is a long recognised important target for anticancer therapy. The most common drugs targeting RNR are small-molecule inhibitors, with a broad variety already in clinical use. Recent approaches though have uncovered potential uses of gene therapy, as well as new subunit-specific activity modulators. The small-molecule inhibitors that target RNR can be classified into two categories depending on which subunit they target. Nucleoside analogues are used to target the α subunit (RRM1 protein), since it is the subunit responsible for nucleotide binding, whereas redox active metal chelators target the β subunit (RRM2 and RRM2B proteins), to inhibit tyrosyl radical formation (Aye et al., 2015).

Some examples of clinically approved RNR inhibitors are illustrated in Figure 1.16. Two well-characterised nucleoside analogues are gemcitabine (F_2C) and clofarabine (CIF). The active form of F_2C is the substrate analogue diphosphate (F_2CDP), which irreversibly inactivates the α subunit by forming a covalent complex between the sugar of F_2 and α subunit (Stubbe and van der Donk, 1995). Interestingly, F_2CDP cannot inhibit the α subunit if it is not already complexed with β , as the whole holoenzyme is required for the formation of the thiyl radical that F_2CDP targets (Stubbe and van der Donk, 1995). F_2C is used in frontline therapy against pancreatic, bladder and lung cancers (Manegold et al., 2000).

CIF has a different mechanism of action against the α subunit. CIF's most abundant metabolite is the triphosphate (CIFTP), which reversibly inhibits the α subunit by binding to

its allosteric a-site leading to its hexamerisation (Aye et al., 2012a; Aye and Stubbe, 2011). Importantly, the RNR enzyme can be completely inhibited by brief exposure to saturating quantities of CIFTP, but activity is regained after prolonged exposure to the drug (Aye and Stubbe, 2011). CIF is used for treatment of refractory paediatric leukaemias (Bonate et al., 2006). More examples of nucleoside prodrugs that inhibit RNR include cytarabine, nelarabine, azacitidine, decitabine, cladribine and fludarabine. These drugs are usually used to treat haematological malignancies, but the molecular mechanisms underlying their RNR inhibition is still poorly understood (Bonate et al., 2006).

Despite their success, nucleoside analogues have challenges in the clinic, either because they are administered as inactive prodrugs, and thus necessitate a successful phosphorylation mechanism inside the cell in order to become active, or because cancer cells show resistance to these analogues, especially when the RRM1 is overexpressed (e.g. gemcitabine-resistant cancer cells with elevated levels of RRM1 (Jordheim et al., 2005)).

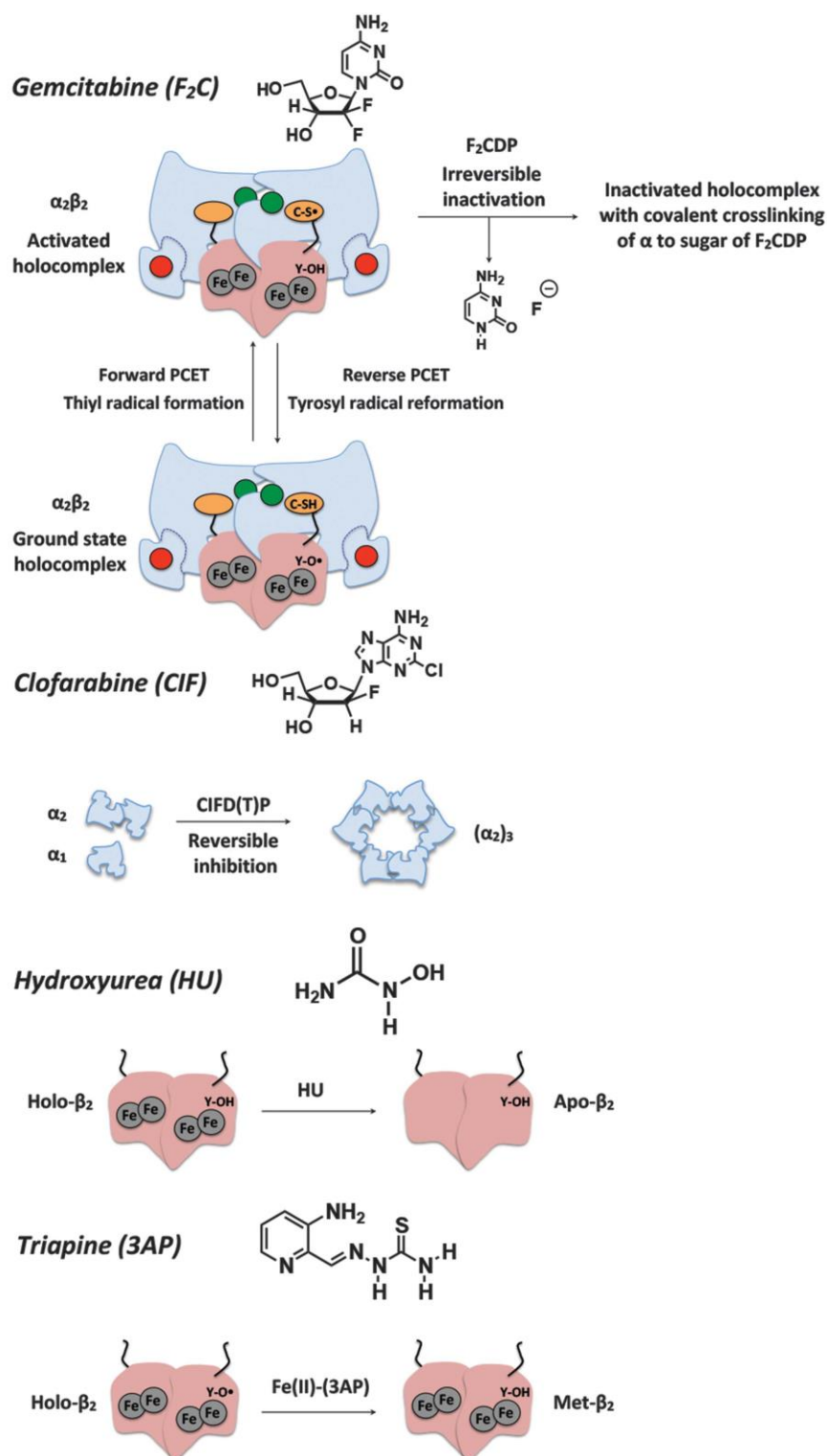


Figure 1.16 Examples of small-molecule RNR inhibitors

Gemcitabine and clofarabine are nucleoside analogs that target the α subunit of RNR, whereas hydroxyurea and triapine are small-molecule inhibitors that interact with and inhibit the β subunit of RNR. The figure illustrates the specific action of those drugs, which are discussed in detail in the text. Adapted and reprinted from (Aye et al., 2015), with permission from Nature Publishing Group, License Number: 3947720446999.

Two widely used drugs that target the β subunit are HU and 3AP (Figure 1.16). HU is used for the treatment of various cancers such as chronic myelogenous and acute myeloid leukaemias as well as glioblastoma (Hehlmann et al., 1993; Levin, 1992; Sterkers et al., 1998). HU acts as both an iron chelator and iron scavenger and treatment of the β subunit with HU leads to the formation of a catalytically incapable apo- β_2 form that lacks both the tyrosyl radical and the iron centre (Nyholm et al., 1993). Importantly, HU is not a ‘clean’ drug as it has been reported to target more metalloenzymes, such as carbonic anhydrase and matrix metalloproteinases (Temperini et al., 2006).

3AP is currently in clinical trials for various solid tumours (Nutting et al., 2009). Although it was initially thought that 3AP acts as an iron chelator, recent studies have shown that 3AP actually reacts with the iron (II) to form a Fe(II)-(3AP) complex, which is the active inhibitor *in vitro*. This complex reduces the tyrosyl radical in the β subunit, resulting in a met- β form that is catalytically incompetent (Aye et al., 2012b). Interestingly, 3AP is considered one of the most successful RNR inhibitors as it shows activity in HU-resistant tumours (Finch et al., 2000).

In addition to the small-molecule inhibitors of RNR, new studies highlight the importance of new and alternative approaches. For example, small interfering RNAs (siRNA) to knock-down RNR subunits could impair tumour cell proliferation and overcome resistance to DNA-damaging agents. Of note, a 20-mer phosphorothionate oligodeoxynucleotide (GTI-2040) that reduces *RRM2* mRNA has been developed, and delivery of this siRNA using nanoparticles or retroviruses has been shown to suppress pancreatic and head and neck cancers (Duxbury et al., 2004a; Lee et al., 2003; Rahman et al., 2012). Of course, application of these strategies in patients is still complicated with many unresolved challenges, but encouraging results have been observed.

1.5.8 RNR and hypoxia

One of the major characteristics of the Class I RNR enzymes, contained by almost all eukaryotic organisms, is that they rely on the presence of oxygen. As already discussed in detail, oxygen is essential to the Class I RNRs, as it reacts with the diiron centre to form a tyrosyl radical with the β subunit, and therefore initiate catalysis of nucleotide biosynthesis. This oxygen dependency of mammalian RNRs has led to the logical assumption that it is inactive in severely hypoxic conditions, something that has been verified for the RRM1/RRM2 form of mammalian RNRs (Brischwein et al., 1997; Chimpoy et al., 2000; Nordlund and Reichard, 2006; Probst et al., 1989; Thelander et al., 1983).

Specifically, early studies by Thelander *et al.*, using purified calf thymus RRM1/RRM2 holoenzyme, suggested that the tyrosyl radical has a continual requirement for oxygen and iron, so as not to be labile (Thelander et al., 1983). A few years later, Probst *et al.*, also highlighted the importance of oxygen for the RRM1/RRM2 form of RNR, derived from mammalian Ehrlich ascites tumour cells (of note, the RRM2B protein was only discovered by Tanaka *et al.*, in the year 2000). The authors used electron paramagnetic resonance (EPR) spectroscopy to monitor the tyrosyl radical of RRM2, and they observed that it declined when oxygen was limited, and reoxygenation was needed to restore it (Probst et al., 1989). However, previous work from the same lab using mammalian tumour cells in culture with different oxygen tensions, showed that although hypoxic cells failed to form new replication origins, the replication of the already active replisomes continued and was completed normally in these conditions (Probst et al., 1988). The authors suggested that the failure of the hypoxic cells to initiate replication, was a manifestation of the oxygen dependent regulation of DNA replication, but they did not comment on the ‘commitment’ of hypoxic cells to complete the already on-going replication (Probst et al., 1988). More importantly, when they used labelled NDPs ($[^3\text{H}]$ -ribo-Ctd) to monitor RNR activity in

culture, they observed that the rate of formed dNDPs ($[^3\text{H}]$ -deoxyribo-Ctd) was decreased upon deoxygenation, but it attained a steady state at a rate that was significantly different from zero (Probst et al., 1989). Pires *et al.*, reported similar results using human tumour cells, which when exposed to severe hypoxia showed compromised DNA replication, with minimal new replication origins and a decreased but detectable number of on-going replication forks; a finding that correlated with the cellular levels of dNTPs (Pires et al., 2010b). The paradox, that the main dNTP manufacturer (RNR) is oxygen dependent, and nevertheless there are sustained low amounts of dNTPs in severe hypoxic conditions, which results in commitment from the cell to complete on-going replication, is the main topic of investigation of this thesis.

It is worth noting that the oxygen dependency characterising eukaryotic RNRs, does not mean that life is restricted in hypoxic/anoxic environments. In fact, several species of lower vertebrates (fishes, amphibians and reptiles) survive in extremely hypoxic and anoxic habitats for days or even months. For example, the most anoxia-tolerant vertebrates, the Western painted turtle (*Chrysemys picta*) and the crucian carp (*Carassius carassius*), both survive without oxygen for several months (Bickler and Buck, 2007). Interestingly, the two species employ different mechanisms to meet the challenge of lack of oxygen. The painted turtle undergoes near-suspended animation, whereas crucian carp remains active and responsive in the absence of oxygen. Importantly, it was recently reported that crucian carp is capable of replicating its DNA in anoxia, and this was attributed to a RRM2B variant (ccRRM2Bii) which forms an active RNR enzyme, and supplies the cells with the required dNTPs (Sandvik et al., 2012). In addition, it has been reported that *RRM2B* is overexpressed in the hypoxia-tolerant rat *Spalax* (Shams et al., 2013). These evolutionary observations could shed light on the mechanisms that could be hijacked in human cancer cells experiencing and surviving under extreme hypoxic conditions.

AIM

The physiologically relevant stress of hypoxia leads to replication stress, but in contrast to chemical inducers of replication stress, exposure to hypoxia does not lead to the eventual accumulation of DNA damage, suggesting sustained DNA replication (Olcina et al., 2013). In support of this, it has been shown that hypoxic cells continue DNA replication once started, as well as maintaining low levels of dNTPs in hypoxia (Pires et al., 2010b; Probst et al., 1989). Since eukaryotic RNRs are oxygen dependent, we questioned the source of dNTPs in hypoxia. Our hypothesis was that the RRM1/RRM2B holoenzyme is capable of retaining activity in hypoxia, as it does in some lower vertebrates (crucian carp), and therefore is favoured over RRM1/RRM2, in order to preserve on-going replication. Further characterisation of the mechanisms by which RRM2B could maintain hypoxic activity was undertaken to test this hypothesis. In doing so, we furthered our understanding of RNR biology, as well as of the biological significance of hypoxia-induced RRM2B with respect to cancer.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 General laboratory and cell culture reagents

All cell culture media was supplied by Sigma-Aldrich UK and cell culture plastic ware obtained from Costar. In addition, the following reagents (Table 2.1) were used.

Table 2.1 Chemicals and cell cultures reagents

Name	Producer/UK distributor
(+/-) Dithiothreitol molecular biology grade (DTT)	Apollo Scientific
2-YT Broth	Thermo Fisher Scientific
Acetic acid	Sigma-Aldrich UK
Acrylamide/Bis Solution, 30%, 29:1	BioRad
Adenosine-5'-triphosphate (ATP)	Roche
Agarose	Sigma-Aldrich UK
Ammonium sulfate	Sigma-Aldrich UK
APS (ammonium persulfate)	Thermo Fisher Scientific
Blocking buffer LiCor	LiCor
Bromophenol blue	Thermo Fisher Scientific
BSA (bovine serum albumin)	Thermo Fisher Scientific
Calcium chloride	Thermo Fisher Scientific
Chloroform	Thermo Fisher Scientific
CldU (5-chloro-2'-deoxyuridine)	Sigma-Aldrich UK
Coomassie Brilliant Blue	Thermo Fisher Scientific
Copper Sulfate (CuSO ₄)	Sigma-Aldrich UK
Cytidine 5'-diphosphocholine sodium salt dehydrate (CDP)	Sigma-Aldrich UK
Crystal violet solution	Sigma-Aldrich UK
Deoxyadenosine 5'-Triphosphate, [8-3H(N)] (dATP-H3)	Perkin Elmer
Deoxyribonuclease I (DNase I)	Sigma-Aldrich UK
Deoxythymidine 5'-Triphosphate ((dTTP) Tetrasodium Salt) (dTTP-H3)	Perkin Elmer
DEPC (diethyl pyrocarbonatecarbonate)	Thermo Fisher Scientific
DharmaFECT 1	Thermo Fisher Scientific
DMEM (Dulbecco's modified Eagle's medium)	Sigma-Aldrich UK
DMSO (dimethylsulphoxide)	Thermo Fisher Scientific
EDTA (ethylenediaminetetraacetic acid)	Sigma-Aldrich UK
Ethanol	Thermo Fisher Scientific

Ethidium bromide	Sigma-Aldrich UK
FBS (foetal bovine serum)	Autogen Bioscience
Formaldehyde	Sigma-Aldrich UK
Glycerol	Thermo Fisher Scientific
Glycine	Sigma-Aldrich UK
H ₂ O ₂ (hydrogen peroxide)	Thermo Fisher Scientific
HCl (hydrochloric acid)	Sigma-Aldrich UK
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Thermo Fisher Scientific
IdU (5-iodo-2'-deoxyuridine)	Sigma-Aldrich UK
Igepal® CA-630	Sigma-Aldrich UK
Imidazol	Sigma-Aldrich UK
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich UK
LB (Luria broth)	Sigma-Aldrich UK
Lipofectamine LTX	Invitrogen/Life technologies
Lithium chloride (LiCl)	Sigma-Aldrich UK
Magnesium acetate (MgAcetate)	Sigma-Aldrich UK
Methanol	Thermo Fisher Scientific
Monosodium dihydrogen orthophosphate (NaH ₂ PO ₄)	Sigma-Aldrich UK
Nonidet™ P-40	Sigma-Aldrich UK
Paraformaldehyde	Thermo Fisher Scientific
PBS (phosphate buffered saline)	Thermo Fisher Scientific
Pepsin	Sigma-Aldrich UK
Phenol:chloroform:isoamyl-alcohol	Sigma-Aldrich UK
Phosphatase inhibitor cocktail tablets	Roche
PMSF (phenylmethylsulfonyl fluoride)	Sigma-Aldrich UK
Potassium dihydrogen phosphate (KH ₂ PO ₄)	Sigma-Aldrich UK
ProLong® Gold	Invitrogen/Life technologies
Propidium iodide (PI)	Sigma-Aldrich UK
Protease inhibitor cocktail tablets, EDTA-free	Roche

RNAse A (ribonuclease A)	Sigma-Aldrich UK
Sodium bicarbonate (NaHCO ₃)	Sigma-Aldrich UK
Sodium chloride (NaCl)	Sigma-Aldrich UK
Sodium deoxycholate	Sigma-Aldrich UK
Sodium dodecyl sulphate (SDS)	Thermo Fisher Scientific
Streptomycin sulfate	Sigma-Aldrich UK
Super optimal catabolite (SOC)	Sigma-Aldrich UK
SYBR® Green	Applied Biosystems
TEMED (tetramethylethylenediamine)	Thermo Fisher Scientific
Tetrabutylammonium hydroxide solution (TBAOH)	Sigma-Aldrich UK
Trichloroacetic acid (TCA)	Sigma-Aldrich UK
Tris-Glycine	Thermo Fisher Scientific
Tris-HCl (tris(hydroxymethyl)aminomethane hydrochloride)	Thermo Fisher Scientific
Triton-X-100	Thermo Fisher Scientific
TRIzol®	Invitrogen/Life technologies
Trypsin 0.05% EDTA	Sigma-Aldrich UK
Tween 20	Thermo Fisher Scientific
Urea	Thermo Fisher Scientific
β-mercaptoethanol	Thermo Fisher Scientific
β-Nicotinamide adenine dinucleotide phosphate (NADPH)	Sigma-Aldrich UK

2.1.2 Antibodies

Table 2.2 Antibodies used for western blotting

Name	Species	Dilution	Clone ID	Producer/Cat number
CHK1	Mouse	1:1000	-	Santa Cruz Biotechnology, sc-8408
HIF1- α	Mouse	1:500	54/HIF-1 α	Becton Dickinson Biosciences, # 610959
KAP-1	Rabbit	1:10000	-	Bethyl/Universal Biologicals Cambridge, # A300-274A
p53 (DO1)	Mouse	1:2000	-	Santa Cruz Biotechnology, sc-126
PARP1	Rabbit	1:1000	-	Cell Signaling/ <i>New England</i> BioLabs, #9542
Phospho-KAP-1 (S473)	Rabbit	1:1000	Poly6446	Biologend, # 644602
Phospho-KAP-1 (S824)	Rabbit	1:1000	-	Bethyl/Universal Biologicals Cambridge, # A300-767A
Phospho-53BP1 (S25)	Rabbit	1:1000	-	Bethyl/Universal Biologicals Cambridge, # A300-652A
Phospho-CHEK1 (S296)	Rabbit	1:1000	-	Cell Signaling/ <i>New England</i> BioLabs, # 2349
Phospho-CHEK1 (S317)	Rabbit	1:1000	-	Cell Signaling/ <i>New England</i> BioLabs, # 2344
Phospho-CHEK1 (S345)	Rabbit	1:1000	-	Cell Signaling/ <i>New England</i> BioLabs, # 2341
Phospho-p53 (S15)	Rabbit	1:500	-	Cell Signaling/ <i>New England</i> BioLabs, # 9284
RRM1	Goat	1:1000	-	Santa Cruz Biotechnology, sc-11733
RRM2	Goat	1:1000	-	Santa Cruz Biotechnology, sc-10844
RRM2	Mouse	1:1000	1E1	AbD Serotec, MCA3434Z
RRM2B	Goat	1:1000	-	Santa Cruz Biotechnology, sc-10840
β -Actin	Mouse	1:100000	AC-15	Santa Cruz Biotechnology, sc-69879
β -Tubulin	Rabbit	1:1000	-	Abcam, ab6046

Table 2.3 Antibodies used for immunofluorescence (IF)

Name	Species	Dilution	Clone ID	Producer/Cat number
53BP1	Rabbit	1:500	-	Novus Biologicals, NB100-904
RPA32	Rat	1:200	4E4	Cell Signaling/ New England BioLabs, # 2208
RRM2B	Goat	1:1000	-	Santa Cruz Biotechnology, sc-10840

Table 2.4 Antibodies used for immunoprecipitation (IP)

Name	Species	Dilution	Clone ID	Producer/Cat number
RRM1	Goat	3 mg/IP	-	Santa Cruz Biotechnology, sc-11733

Table 2.5 Antibodies used for chromatin immunoprecipitation (ChIP)

Name	Species	Dilution	Clone ID	Producer/Cat number
p53 (DO1)	Mouse	1 µg/IP	-	Santa Cruz Biotechnology, sc-126
p53 (FL-393)	Rabbit	1 µg/IP	-	Santa Cruz Biotechnology, sc-6243

Table 2.6 Antibodies used for fluorescence-activated cell sorting (FACS)

Name	Species	Dilution	Clone ID	Producer/Cat number
anti-BrdU	Mouse	1:500	B44	Becton Dickinson Biosciences (347580)

Table 2.7 Antibodies used for Single-Molecule DNA Fiber Analysis

Name	Species	Dilution	Clone ID	Producer/Cat number
Mouse anti-BrdU	Mouse	1:500	B44	Becton Dickinson Biosciences (347580)
Rat anti-BrdU	Rat	1:500	BU1/75 (ICR1)	Oxford Biotechnology, OBT0030

Table 2.8 Antibodies used for tissue immunofluorescence (Tissue IF)

Name	Species	Dilution	Clone ID	Producer/Cat number
Cleaved caspase 3	Rabbit	1:500	-	Cell Signaling/New England, Biolabs, # 9661
PIMO	Mouse	1:100	4.3.11.3	Hypoxyprome HP1-100

Table 2.9 Antibodies used for immunohistochemistry (IHC)

Name	Species	Dilution	Clone ID	Producer/Cat number
CAIX	Mouse	1:100	-	Prof. Adrian Harris, Oxford, UK
RRM2B	Goat	1:1000	-	Santa Cruz Biotechnology, sc-10840

Table 2.10 Secondary antibodies used

Name	Dilution	Application	Producer/Cat number
Alexa Fluor 680 goat anti-mouse IgG (H+L)	1:10000	WB	Invitrogen, #A21057
Alexa Fluor 680 goat anti-rabbit IgG (H+L)	1:10000	WB	Invitrogen, #A21076
Alexa Fluor 680 donkey anti-goat IgG (H+L)	1:10000	WB	Invitrogen, #A21084
IRDye 800CW donkey anti-mouse IgG	1:5000	WB	LiCor, #926-32212
IRDye 800CW donkey anti-rabbit IgG	1:5000	WB	LiCor, #926-32213
Alexa Fluor 488-conjugated goat anti-rabbit IgG	1:250	IF	Invitrogen, A11070
Alexa Fluor 488-conjugated donkey anti-rabbit IgG	1:250	IF	Invitrogen, A21206

Alexa Fluor 488-conjugated goat anti-mouse IgG	1:250	IF/FACS	Invitrogen, A11017
Alexa Fluor 488-conjugated donkey anti-goat IgG	1:500	IF	Invitrogen, A11055
Alexa Fluor 594-conjugated goat anti-rabbit IgG	1:250	IF	Invitrogen, A11072
Alexa Fluor 594-conjugated goat anti-rat IgG	1:250	IF	Invitrogen, A11007
Alexa Fluor 594-conjugated goat anti-mouse IgG	1:250	IF	Invitrogen, A11020
Alexa Fluor 594-conjugated donkey anti-goat IgG	1:500	IF	Invitrogen, A11058
Alexa Fluor 555-conjugated goat anti-rat IgG	1:500	DFA	Invitrogen, A21434
Alexa Fluor 488-conjugated goat anti-mouse F(ab') ₂ fragment	1:500	DFA	Invitrogen, A11017
Pierce™ Recombinant Protein A/G	1:5000	WB/IP	Thermo Fisher Scientific, 21186

2.1.3 Antibiotics

Ampicillin and kanamycin (Thermo Fisher Scientific) were made up as a stock solution of 50 mg/ml. The final concentrations of ampicillin and kanamycin were 100 µg/ml and 50 µg/ml respectively. All antibiotics were made up in sterile distilled water, aliquoted and stored at -20°C.

2.1.4 Primers

The primers used in this study are listed in Tables 2.11-2.16. Primers were either designed in this work (custom made) or adopted from previous published studies. For qPCR, primers were designed using NCBI Primer-Blast, which uses Primer3 software (Steve Rozen, Helen J. Skaletsky, 1998) and BLAST (Altschul et al., 1990); code available at (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The primers were designed so that amplification products would not exceed 250 base pairs (bp) in length, would span an exon-exon junction and would detect all gene variants of interest. For site-directed mutagenesis, primers were designed using the QuikChange Primer Design from Agilent Technologies (<http://www.genomics.agilent.com/primerDesignProgram.jsp>). For construction of a CRISPR-Cas9 RRM2B^{-/-} cell line, primers were designed with the CRISPR design tool (<http://crispr.mit.edu>) which scans the selected DNA sequence for possible CRISPR guides (20 nucleotides followed by a PAM sequence: NGG) and for possible off-target matches throughout the selected genome (Ran et al., 2013).

Table 2.11 Primers used for quantitative PCR (qPCR)

Oligonucleotide Name	Sequence	Reference
<i>18S</i> Forward	TAGAGGGACAAGTGGCGTTC	Custom made
<i>18S</i> Reverse	CGGACATCTAAGGGCATCAC	
<i>GLUT-1</i> Forward	ATACTCATGACCATCGCGCTAG	Custom made
<i>GLUT-1</i> Reverse	AAAGAAGGCCACAAAGCCAAAG	
<i>RRM1</i> Forward	ACCAACCGCCCACAACCTTT	Custom made
<i>RRM1</i> Reverse	TGCCATTAGTCCCAGCAATGT	
<i>RRM2</i> Forward	GTGGAGCGATTTAGCCAAGAA	(D'Angiolella et al., 2012)
<i>RRM2</i> Reverse	CACAAGGCATCGTTTCAATGG	
<i>RRM2B</i> Forward	TGGTGGAGCGCTTTAGTCAG	Custom made
<i>RRM2B</i> Reverse	CTATCCATCGCAAGGCCCAA	

Table 2.12 Primers used for ChIP-qPCR

Oligonucleotide Name	Sequence	Reference
<i>RRM2B</i> Forward-ChIP	CTTGCTGGGAAATCTTGACC	(Tanaka et al., 2007)
<i>RRM2B</i> Reverse-ChIP	CTGAGGCACTGGTCTAATGA	

Table 2.13 Primers used for Cloning

Oligonucleotide Name	Sequence	Reference
<i>Rrm2b</i> Forward-cloning <i>Bam</i> HI site	<u>TCGGATCC</u> ATGGGCGACCCGGAA AGG	(Shao et al., 2004)
<i>Rrm2b</i> Reverse-cloning <i>Not</i> I site	CTGAGGCACTGGTCTAATGATTG GATGCAGATTTTAAAG <u>CGGCCGC</u>	(Shao et al., 2004)
<i>Rrm2b</i> Forward-cloning <i>Xho</i> I site	<u>TCCTCGAG</u> ATGGGCGACCCGGAA AGG	Custom made

Table 2.14 Primers used for site-directed mutagenesis

Oligonucleotide Name	Sequence	Reference
RRM2B_Y164F_For	ATTTAATGCAATTGAAACCATGCC	Custom made
RRM2B_Y164F_Rev	CTTTGTTAAGAAAAAAGCAGATTG CAATCTGCTTTTTTCTTAACAAAGG GCATGGTTTCAATTGCATTAAAT	
RRM2B_Y164C_For	ATTTAATGCAATTGAAACCATGCC	Custom made
RRM2B_Y164C_Rev	CTGTGTTAAGAAAAAAGCAGATTG CAATCTGCTTTTTTCTTAACACAGG GCATGGTTTCAATTGCATTAAAT	
RRM2B_K37E_For	GAAGAGCCACTCCTAAGAGAGAGT	Custom made
RRM2B_K73E_Rev	TCTCGCCG CGGCGAGAACTCTCTCTTAGGAGT GGCTCTTC	
RRM2B_K151E_For	ACACTTACATCAGAGATCCCAAGG	Custom made
RRM2B_K151E_Rev	AAAGGGAATTTTTATTTAATGCA TGCATTAAATAAAAAATTCCCTTTCC TTGGGATCTCTGATGTAAGTGT	
RRM2B_F183Y_For	ATGGATAGCAGATAGAAAATCTAC	Custom made
RRM2B_F183Y_Rev	TTATGGGGAAAGAGTGG CCACTCTTTCCCCATAAGTAGATTT TCTATCTGCTATCCAT	
RRM2B_Y241H_For	ACTTTGCTTGCCTGATGTTCCAACA	Custom made
RRM2B_Y241H_Rev	CTTAGTAAATAAGCCTTC GAAGGCTTATTTACTAAGTGTTGG AACATCAGGCAAGCAAAGT	
RRM2B_Y331F_For	ATTTCTTTGAGAAACGAGTTTCAG	Custom made
RRM2B_Y331F_Rev	AGTTTCAGCGTTTTTGCAG CTGCAAAACGCTGAAACTCTGAAA CTCGTTTCTCAAAGAAAT	
RRM2B_Q127K_For	GCTCGCTGTTTCTATGGCTTTAAAA	Custom made
RRM2B_Q127K_Rev	TTCTCATCGAGAATGTTC GAACATTCTCGATGAGAATTTTAA AGCCATAGAAACAGCGAGC	

Table 2.15 Primers used for verifying RRM2B isoforms

Oligonucleotide Name	Sequence	Reference
RRM2B-var. 2-For	CCAGCCCGTTAGATTGCAAG	Custom made
RRM2B-var. 2-Rev	GAGTTCTCGCCGGTTTGTC	
RRM2B-var. 1-For	GCGGCCGGGCTGGATCAGG	Custom made
RRM2B-var. 1-Rev	GAGTTCTCGCCGGTTTGTC	
RRM2B-var. 1&3-Rev	TCATCTCTCACATCTTAGCCTTT	

Table 2.16 Primers used for construction and verification of CRISPR-Cas9 RRM2B^{-/-} cell line

Oligonucleotide Name	Sequence	Reference
<i>Rrm2b</i> -CRISPR 1/3-For	CACCGTTTCGGCGGAGTCTGCGCGAT	Custom made
<i>Rrm2b</i> -CRISPR 1/3-Rev	AAACATCGCGCAGACTCCGCCGAAC	
<i>Rrm2b</i> -CRISPR 2-For	CACCGAAATGTTATTCGCCGCGGTC	Custom made
<i>Rrm2b</i> -CRISPR 2-Rev	AAACGACCGCGGCGAATAACATTTTC	
<i>Rrm2b</i> -CRISPR 4-For	CACCGCCAGAGGCTCGCTGTTTCTA	Custom made
<i>Rrm2b</i> -CRISPR 4-Rev	AAACTAGAAACAGCGAGCCTCTGGC	
hU6 promoter-For	GAGGGCCTATTTCCCATGATTCC	(Ran et al., 2013)
<i>Rrm2b</i> -sequencing exon1-For	ACTCAATGGACAGGCGAGAA	Custom made
<i>Rrm2b</i> -sequencing exon1-Rev	GCCACAGTCCTCTTTCTTG	
<i>Rrm2b</i> -sequencing exon4-For	TGTTTGTTACATGACAGGCAGTTTT	Custom made
<i>Rrm2b</i> -sequencing exon4-Rev	CTTACGGCTTGGTTATGTTTCAGATC	

Table 2.17 Primers used for dNTP incorporation assay

Oligonucleotide Name	Sequence	Reference
dTTP-Forward	TTATTATTATTATTATTATTAGGCGGT GGAGGCGG	(Sherman and Fyfe, 1989)
dCTP-Forward	TTTGTTTGTTTGTTTGTTTGGGCGGTG GAGGCGG	
dGTP-Forward	TTTCTTTCTTTCTTTCTTTTCGGCGGTG GAGGCGG	
dATP-Forward	AAATAAATAAATAAATAAATGGCGG TGGAGGCGG	
dNTP-Reverse	CCGCCTCCACCGCC	

2.1.5 Constructs

Table 2.18 Table of constructs

Plasmid Name	Backbone	Reference
pET28b-6xHis RRM1	pET-28b DNA (Novagen #69865-3)	(Shao et al., 2004)
pET28a-6xHis RRM2	pET-28a DNA (Novagen #69864-3)	(Shao et al., 2004)
pET28a-6xHis RRM2B	pET-28a DNA (Novagen #69864-3)	Constructed in this work
HA-RRM2B	pcDNA3.1 (Invitrogen; V79520)	(D'Angiolella et al., 2012)
pET28a-6xHis RRM2B ^{Y164F}	pET-28a DNA (Novagen #69864-3)	Constructed in this work
pET28a-6xHis RRM2B ^{Y164C}	pET-28a DNA (Novagen #69864-3)	Constructed in this work
pET28a-6xHis RRM2B ^{K37E/K151E}	pET-28a DNA (Novagen #69864-3)	Constructed in this work
pET28a-6xHis RRM2B ^{F183Y}	pET-28a DNA (Novagen #69864-3)	Constructed in this work
pET28a-6xHis RRM2B ^{Y241H}	pET-28a DNA (Novagen #69864-3)	Constructed in this work
pET28a-6xHis RRM2B ^{Y331F}	pET-28a DNA (Novagen #69864-3)	Constructed in this work
pET28a-6xHis RRM2B ^{Q127K}	pET-28a DNA (Novagen #69864-3)	Constructed in this work
pRRM2B	pcDNA3.1 (Invitrogen; V79520)	Constructed in this work
pRRM2B ^{Y164C}	pcDNA3.1 (Invitrogen; V79520)	Constructed in this work
pRRM2B ^{K37E/K151E}	pcDNA3.1 (Invitrogen; V79520)	Constructed in this work
pRRM2B ^{Q127K}	pcDNA3.1 (Invitrogen; V79520)	Constructed in this work
pHRE-p53	pGL3-basic 5xHRE (Promega; E1751)	(Hammond et al., 2006)
pHRE-LUC	pGL3-basic 5xHRE (Promega; E1751)	(Hammond et al., 2006)
SpCas9-GFP-RRM2B_1/3	pSpCas9(BB)-2A-GFP (Addgene; 48138)	Constructed in this work
SpCas9-GFP-RRM2B_2	pSpCas9(BB)-2A-GFP (Addgene; 48138)	Constructed in this work
SpCas9-GFP-RRM2B_4	pSpCas9(BB)-2A-GFP (Addgene; 48138)	Constructed in this work

2.2 Molecular Biology

2.2.1 Cloning

Generation of the appropriate constructs was performed by isolation of the gene of interest by a donor plasmid following subcloning into a recipient plasmid by restriction digest and ligation. Specifically, appropriate primers were designed containing the restriction sites of interest followed by either the 5' or the 3' end of the gene of interest (Table 2.13). The restriction sites used were selected in order to flank but do not cut within the insert and they were in the desired location of the recipient plasmid (the insert was in the correct orientation).

Then, the gene of interest (insert) was isolated by PCR reaction as follows:

PCR Mix

10 x <i>Pfx</i> Amplification Buffer	10 µl
5 mM dNTP mixture	6 µl
50 mM MgSO ₄	2 µl
Primer mix (10 µM each)	3 µl
Donor DNA	10 - 200 ng
Platinum [®] <i>Pfx</i> DNA Polymerase (2.5 units)	1 µl
dd H ₂ O	up to 100 µl

PCR Conditions

1. 98°C 30 sec
2. 98°C 10 sec
3. 55°C 30 sec
4. 72°C 1 min
- Repeat steps 2 to 4 x 28 times
5. 72°C 10 min

The insert was then cleaned up by PCR impurities and primers using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Digestion of the insert and the recipient plasmid followed using the appropriate restriction enzymes according to the manufacturer's instructions (*New England BioLabs*). After digestion, the reactions were run on a freshly made 1% agarose gel and gel extraction of the insert and the recipient plasmid was performed using the QIAquick Gel Extraction Kit (Qiagen) according to

manufacturer's instructions. Finally, DNA Ligation was conducted to fuse the insert into the recipient plasmid as followed:

	A	B
Recipient plasmid	3 μ l	3 μ l
Insert (gene of interest)	-	12 μ l
H ₂ O	13 μ l	1 μ l

The mix was incubated at 45°C for 5 min followed by the addition of 2 μ l of T4 DNA Ligase Reaction Buffer and 2 μ l of T4 DNA Ligase (*New England BioLabs*). Overnight (O/N) incubation followed at 4°C. The ligation reaction was transformed into the bacterial strain of choice (Chapter 2.2.2). A diagnostic restriction digest with the enzymes used for the cloning followed, for at least 3 -5 bacterial colonies. The purified plasmids containing the insert were then sent for Sanger sequencing validation (Source Biosciences).

2.2.2 Transformation of bacteria

Bacterial cultures were grown in LB (Sigma) or 2-YT broth (Thermo Fisher Scientific) that were dissolved in Milli Q H₂O according to the manufacturer's instructions and sterilised by autoclaving at 121°C, 15 psi for 30 min. LB-agar plates were prepared by dissolving LB-agar (Sigma) in Milli Q H₂O according to the manufacturer's instructions and sterilised by autoclaving. Following boiling the LB-agar was allowed to cool to approximately 50°C before being supplemented with antibiotics. 25 ml of liquid LB agar was added to each sterile 9 cm diameter Petri dish around a Bunsen Burner flame. Plates were then allowed to set at RT and stored at 4°C until needed for bacterial transformations.

Bacterial transformations for plasmid production were carried out in One Shot[®] TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific). Specifically, 30 μ l of competent cells were thawed on ice prior to addition of 100 - 200 ng DNA for each transformation. Bacterial cells were incubated on ice for 30 min followed by a heat shock at 42°C for 30 sec. A further 2 min incubation of the cells on ice was then followed by addition

of 800 µl of SOC medium (Sigma-Aldrich UK). Transformation reactions were placed in an incubator at 37°C for 1 h while shaking at 225 rpm (Innova44 Incubator). Transformation reactions were spread onto LB-agar plates supplemented with the appropriate antibiotics (depending on the resistance of the transfected vector) and incubated O/N at 37°C.

2.2.3 Extraction of plasmid DNA from bacterial cells

A sterile tip was used to pick a single bacterial colony and was placed in 3 ml of LB medium in a round-bottomed 14 ml tube and grown at 37°C O/N. This culture was then expanded into a 2-litre culture flask in 100 ml of LB (with the appropriate antibiotic) at 37°C O/N. The following day, the O/N bacterial culture was harvested by centrifugation at 6000 x g for 15 min at 4°C and purification of plasmid DNA was performed by using Maxi-preparation kit (Qiagen) according to the manufacturer's instructions. DNA concentration was determined by measuring optical density (OD) at $A_{260\text{nm}}$ with 1 $A_{260\text{nm}}$ representing an equivalent of 50 µg of DNA in 1 ml distilled water. A ratio of absorbance of ≥ 1.8 at 260 nm and 280 nm indicated that DNA was pure and free from protein contamination.

2.2.4 Agarose gel electrophoresis

Agarose gels for DNA analysis were prepared by dissolving agarose in TBE Buffer (89 mM Tris-HCl, 89 mM Boric acid, 2 mM EDTA) to a final concentration of 1 - 2% (w/v) by heating the mixture to boiling point until agarose was dissolved and after the addition of 5 µl of ethidium bromide per 100 µl of agarose mixture. The samples were diluted with 6X Loading Buffer (0.25% Bromophenol blue, 30% glycerol in a 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 solution) before being loaded onto the gel. A DNA molecular weight marker (SmartLadder, Eurogentech) was run alongside the samples to assess DNA fragment size. Gel electrophoresis was carried out in TBE Buffer at 120 V for 1 h. DNA was visualised using an UV-transilluminator.

2.3 Biochemistry

2.3.1 Cell lysis and SDS PAGE Electrophoresis

Before SDS PAGE electrophoresis cells were scraped with 1 ml of PBS, pelleted and lysed in the appropriate amount (60-100 μ l) of UTB buffer (9 M urea, 75 mM Tris HCl, pH 7.5, 0.1 M β -mercaptoethanol). The samples were then sonicated for 20 seconds before being centrifuged for 15 min at 16.2 x g at 4°C to remove insoluble debris. The supernatant was then transferred to a fresh tube and 2 μ l of each sample was used for protein quantification using a Nanodrop meter. Absorbance of the protein sample was read at 280 nm. PROTEAN® TGX™ Precast Gels (BioRad) were used for the separation of the polypeptides. The appropriate volume of sample (25 μ g of total protein) was mixed with sample buffer (3.3% SDS, 6 M urea, 17 mM Tris-HCl (pH 7.5), 0.07 M β -mercaptoethanol, 0.01% Bromophenol blue), and heated at 100°C for 10 min. Protein ladder (Precision plus protein standards, BioRad) (5 μ l) was used for the analysis of the molecular weight of the bands. Gels were electrophoresed at 180 V for 45 min in Tris-glycine running buffer (25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS).

2.3.2 Western Blot

Proteins in the electrophoresed gels were electro-transferred onto nitrocellulose membranes (Trans-Blot Turbo Transfer Pack, BioRad) using the Trans-Blot® Turbo™ - Transfer System (BioRad). The membranes were then placed in LiCor buffer diluted 1:2 in Tris-Buffered Saline (TBS) (50 mM Tris-HCl, pH 7.6, 150 mM NaCl) for 1 h for blocking. Membranes were probed with primary antibody (diluted in 1:2 LiCor buffer: TBS-Tween) O/N at 4°C while rocking. All the primary antibodies used for western blotting are listed in Table 2.2. The following day the membranes were washed three times with TBS-Tween for 10 min each time and then probed with the secondary antibodies listed in Table 2.10 (diluted

1:2 in LiCor buffer: TBS-Tween) for 90 min at RT while rocking. After secondary incubation, the membranes were again washed three times in TBS-Tween and once with TBS. Membranes were developed using the Odyssey infrared imaging system.

2.3.3 Immunoprecipitation (IP)

Cells were seeded in 15 cm plates (at least two plates per condition, 5×10^6 cells per dish) and were used at about 60-70% confluence. At the end of each treatment, cells were washed three times with PBS and then scrapped into 1 ml PBS. After collection, cells were centrifuged $233 \times g$ for 5 min at 4°C . Cells were then lysed on ice with 1 ml IP Lysis Buffer (100 mM NaCl, 20 mM Tris-HCl pH 7.4, 5 mM MgCl_2 , 0.5% Nonidet P-40 in in Milli Q H_2O). Protease (complete EDTA free; Roche) and phosphatase (Phospho-Stop; Roche) inhibitors were added fresh. Samples were allowed to rotate end-over-end at 4°C for 10 min. A short sonication followed and the samples were then centrifuged at $16200 \times g$ for 10 min at 4°C . The supernatant was then transferred to a fresh tube and 2 μl of each sample was used for protein quantification using a Nanodrop meter. Absorbance of the protein sample was read at 280 nm. The sample with the lowest concentration was kept and the rest of the samples were diluted accordingly (all of the samples had the same final concentration). At this point, 50 μl of each sample was kept as an input control. Protein G agarose beads (Merck Millipore) were washed 3 times with IP Lysis Buffer and resuspended in one bed volume IP Lysis Buffer. 50 μl of bead slurry were added to each sample to pre-clear the samples prior to immunoprecipitation and the mix was placed at 4°C , rotating end-over-end for 30 min. The mix was then centrifuged at $233 \times g$ for 5 min at 4°C and the supernatant was collected. 100 μl of bead slurry and 3 mg of the antibody of interest (Table 2.4) or the IgG control (Cell Signaling) was added to the samples and placed at 4°C , rotating end-over-end O/N. The following day, beads were collected by centrifugation at $233 \times g$ at 4°C for 5 min and washed three times with IP Lysis Buffer. Between washes, samples were rotated end-over-end for 15

min at 4°C. Finally, the beads were resuspended in 30-50 µl of UTB buffer, boiled for 30 min and western blot analysis followed.

2.3.4 Chromatin immunoprecipitation (ChIP)

For ChIP analysis, cells were seeded in 15 cm plates (at least two plates per condition) and were used at 60-70% confluence. At the end of each treatment, cells were incubated with 1% formaldehyde in culture medium for 10 min at RT while rocking. The formaldehyde was then quenched with the addition of 1 M glycine in PBS for 5 min at RT while rocking. Cells were then washed with PBS and scraped into 5 ml PBS supplemented with protease inhibitor cocktail (Roche) per plate. After collection, cells were centrifuged 233 x g for 5 min at 4°C. The cell pellets were washed with PBS supplemented with protease inhibitor cocktail (Roche) and centrifuged at 233 x g for 5 min at 4°C. At this stage cells could be placed at -80°C in order to complete sample acquisition. When ready to proceed the cells were resuspended in cold SDS-cell lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1 and 25 x complete Pi in Milli Q H₂O) and incubated on ice for 10 min. Afterwards, samples were transferred to 1.5 ml Eppendorf tubes and cells were sonicated for 15 cycles at 4°C (10 seconds pulse on, 50 seconds pulse off) using a Diagenode Bioruptor plus sonicator. After sonication, the samples were centrifuged at 16200 x g for 10 min at 4°C and the supernatant was transferred to a 15 ml falcon. In order to verify that sonication had been optimum, a 50 µl aliquot of chromatin was taken before and after sonication for each sample (shearing control). Chromatin was then diluted 1:10 using ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl, 25 x complete Pi in Milli Q H₂O). Protein A agarose beads (Merck Millipore) were washed 3 times in ChIP dilution buffer and resuspended at 50% ChIP dilution buffer volume. 100 µl of 50% bead slurry were added to each sample to pre-clear the chromatin prior to immunoprecipitation and the mix was placed at 4°C, rotating end-over-end for 1 h. The mix was then centrifuged at 233 x g for 5 min at

4°C and the pre-cleared chromatin was divided into 3 ml aliquots. Samples were divided into: antibody of interest (Table 2.5), IgG control (combined non-specific mouse and rabbit control IgGs; Cell Signaling) or no antibody control (Input) and placed at 4°C, rotating end-over-end O/N. 90 µl bead slurry was added to each sample following this period for 1 h end-over-end at 4°C. Beads were then collected by centrifugation at 233 x g at 4°C for 5 min. At this point 500 µl of chromatin from the no antibody control was also collected as pre-cleared input. Beads were then washed with 1 ml low salt wash solution (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl in Milli Q H₂O) transferred to an Eppendorf tube and rotated end-over-end for 5 min at 4°C. Beads were collected by centrifugation at 400 x g for 3 min at 4°C and resuspended in 1 ml high salt wash solution (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl in Milli Q H₂O). Samples were placed rotating end-over-end for 5 min at 4°C before being centrifuged again at 400 x g for 3 min at 4°C. Samples were washed in 1 ml LiCl wash (0.25 M LiCl, 1 % Igepal, 1% Sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1 in Milli Q H₂O) and again allowed to rotate end-over-end at 4°C for 5 min, before centrifugation as before. Finally, samples were washed twice in 1 ml TE buffer (10 mM Tris-HCl pH 8.1, 1 mM EDTA in Milli Q H₂O) before elution in freshly made 250 µl elution buffer (0.1 M NaHCO₃, 1% SDS in Milli Q H₂O). Beads were eluted for 15 min end-over-end at RT. Following this time, beads were collected by centrifugation at 400 x g for 5 min at 4°C, before the elution process was repeated once again with a further 250 µl elution buffer. At this point, 400 µl elution buffer was added also to 100 µl of input sample. 20 µl of 5 M NaCl was added to each 500 µl volume of sample. Samples were inverted and centrifuged briefly before incubation at 65°C O/N. The 50 µl sonication control samples were also processed at this time by addition of 5 µl 5 M NaCl and incubation at 65°C O/N. The following day, 10 µl 0.5 M EDTA, 20 µl 1 M Tris-HCl pH 6.5 and 4 µl 10 mg/ml proteinase K mix were added to

each sample prior to incubation at 45°C for 1 h. DNA was then extracted by phenol-chloroform purification by addition of 500 µl phenol:chloroform: isoamyl-alcohol to each sample (50 µl to the shearing controls). Samples were vortexed to an emulsion and then centrifuged at 16200 x g 4°C for 10 min. The aqueous layer was then transferred to a new 1.5 ml Eppendorf. 50 µl of 3 M NaOAc was added to each sample followed by a short vortex and addition of 1 µl of 20 µg/µl glycogen and 1 ml 100% ice-cold ethanol. Samples were then placed at -20°C O/N. Samples were centrifuged the following day at 16200 x g 4°C for 45 min and the pellet was washed with 75% ethanol followed by 30 min centrifugation at 16200 x g 4°C. The supernatant was then removed and the pellet was air-dried. The pellet was then resuspended in 25 µl DEPC-treated H₂O.

For ChIP analysis, immunoprecipitation samples were diluted 1:6 in DEPC-treated H₂O prior to performing standard qPCR reaction for the gene of interest (primers listed in Table 2.12). The input samples were generally diluted 1:100. Fold enrichment was calculated relative to total p53 and as a % of input.

2.3.5 Protein expression and purification

The 6×His-tagged hRRM1 (pET28b-RRM1) and the 6×His-tagged hRRM2 (pET28a-RRM2) were kindly provided by Prof. JoAnne Stubbe (Massachusetts Institute of Technology, Cambridge, USA) and Dr. Yun Yen (Beckman Research Institute at City of Hope, Duarte, USA) respectively (Table 2.18). The 6×His-tagged hRRM2B (pET28a-RRM2B) was constructed in this work by cloning as described in Chapter 2.2.1, using as a donor plasmid the HA-RRM2B construct kindly provided by Dr. Vincenzo D'Angiolella (Table 2.18) and as a recipient plasmid the pET28a (Novagen). The *Rrm2b* Forward-cloning *Bam* *HI* site and *Rrm2b* Reverse-cloning *Not* *I* site primers (Table 2.13) were used for the extraction of the gene of interest. All constructs were sent for Sanger sequencing validation (Source Biosciences) before being used for protein overexpression. The constructs were then

transformed in *E.coli* BL21-Gold (DE3) competent cells (Agilent Technologies) as described in Chapter 2.2.2 and an O/N culture of the transformed bacteria was diluted 80-fold in 600 ml of 2×YT medium containing 50 µg/ml kanamycin. Each culture was grown at 37°C until OD_{600nm} = 0.7 - 0.9 and then 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added and the culture incubated O/N at 18°C. The growth conditions were experimentally optimised (different temperatures and times of incubation were tested) in order to have optimal soluble recombinant protein expression. The following day, cells were harvested by centrifugation at 7740 × g for 10 min at 4°C and the pellets were lysed with appropriate amount (5 ml/gr of pellet) of Lysis Buffer (50 mM Hepes pH 7.6; 0.1% Triton X-100; 10 mM β-mercaptoethanol; freshly added EDTA-free Protease Inhibitors and DNase I) at 4°C with vigorous agitation until the lysate was homogenous. The lysate was then sonicated (60% Amplitude 30 sec ON/30 sec OFF) and clarified by centrifugation at 20,000 × g for 20 min at 4°C. For purification of the RRM2 and RRM2B proteins, the soluble lysate was passed through a 40 µm filter (Merck Millipore) into a measuring cylinder and kept at 4°C. Meanwhile, a 5 ml His-Trap HP purification column (GE Healthcare) was directly coupled to an ÄKTA FPLC system (GE Healthcare) and was equilibrated with 40 ml His-Binding Buffer (50 mM Hepes pH 7.6; 500 mM NaCl; 5 mM imidazole). The lysate was then passed through the 5 ml His-Trap HP purification column, washed with at least 30-fold bed volumes of Wash Buffer (50 mM Hepes pH 7.6; 800 mM NaCl; 50 mM imidazole; 0.1% Triton X-100; 10 mM β-mercaptoethanol) and finally eluted with Elution Buffer (50 mM Hepes pH 7.6; 500 mM NaCl; 500 mM imidazole). Next, the proteins underwent buffer exchange with a PD-10 desalting column (GE Healthcare) into 25 mM Tris-HCl, pH 7.5 before finally being concentrated using an Amicon Ultra-15 ml Centrifugal Filter Unit (Merck Millipore) and stored at -80°C. For purification of the RRM1 protein, 2% streptomycin sulfate (from 10% stock) was added to the soluble lysate over the course of 10 min at 4°C while stirring. After

the addition of the streptomycin sulfate another 10 min of stirring followed. The lysate was then centrifuged at $20,000 \times g$ for 20 min at 4°C , the supernatant was collected and ammonium sulfate was then added to 60% saturation over the course of 10 min at 4°C while stirring. A 30 min incubation at 4°C while stirring followed and then a centrifugation at $20,000 \times g$ for 20 min at 4°C . The pellet was kept and was resuspended in Buffer A (50 mM NaH_2PO_4 , pH 7.0; 300 mM NaCl; 10 mM imidazole; 5 mM β -mercaptoethanol). A buffer exchange with Buffer A using a PD-10 desalting column (GE Healthcare) followed in order to take out the excess of the ammonium sulfate and the samples were incubated O/N at 4°C , rotating with TALON[®] Metal Affinity Resins (Clontech) that were already pre-equilibrated with Buffer A. The following day, the samples were centrifuged at $700 \times g$ for 5 min at 4°C , washed with 20 bed volumes of Buffer A while shaking for 10 min at 4°C . The washing step with Buffer A was repeated twice. After the last centrifugation, resins were diluted in one bed volume of Buffer A and were vigorously vortexed before they were transferred to a gravity flow column (BioRad) with an end-cap in place. The resins were allowed to settle out of suspension and then the end-cap was removed which allowed the buffer to drain. The column was then washed once with 5 bed volumes of Wash Buffer (50 mM NaH_2PO_4 , pH 7.0; 300 mM NaCl; 30 mM imidazole; 5 mM β -mercaptoethanol) and elute of the his-tagged protein (RRM1) followed by the addition of 5 bed volumes of Elution Buffer (50 mM NaH_2PO_4 , pH 7.0; 300 mM NaCl; 125 mM imidazole; 5 mM β -mercaptoethanol) to the column. Addition of ammonium sulfate to 60% saturation followed to the eluted protein over the course of 10 min at 4°C while stirring. An additional 30 min agitation at 4°C followed and then the sample was centrifuged at $20,000 \times g$ for 30 min at 4°C . The pellet was kept and was resuspended in 25 mM Tris-HCl, pH 7.5 and a buffer exchange with 25 mM Tris-HCl, pH 7.5 using a PD-10 desalting column (GE Healthcare) followed in order to take out the excess of the ammonium

sulfate. Finally, the protein was concentrated using an Amicon Ultra-15 ml Centrifugal Filter Unit (Merck Millipore) and stored at -80°C.

2.3.6 Site-directed mutagenesis

The specific mutations in RRM2B (Y164C; Y164F; F183Y; K31E/K151E; Y241H; Y331F and Q127K) were generated by PCR, using the pET28a-RRM2B vector as a template and the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions. The primers used are listed in Table 2.14. All constructs were verified by DNA sequencing. Expression and purification of the hRRM2B mutated proteins were performed in the same way as for hRRM2B described in Chapter 2.3.5.

2.3.7 Enzymatic activity assay

The activity of recombinant RNR was measured using the following reduction method. The enzyme concentration (R1/R2B or R1/R2) was the same in all cases and the limited factor was the presence or not of oxygen (normoxia versus hypoxia) (Chimploy et al., 2000). Specifically, a final concentration of 1.25 μ M of purified RRM1 was incubated with 2.5 μ M of either RRM2 or RRM2B protein. The reaction mixture contained 0.1 mM CDP, 50 mM HEPES (pH 7.5), 6 mM DTT, 8 mM magnesium acetate, 2 mM ATP and 1 mM NADPH in a final volume of 400 μ l. The RNR proteins purified were active without reassembling the iron centre (Shao et al., 2004; Zhou et al., 2010). In hypoxic samples (<0.1% O₂) the reaction mixture was assembled and the assay was carried out within an anaerobic chamber (Bactron, Shel Labs). Reactions were initiated by the addition of CDP, incubated at 37°C and aliquots of 50 μ l were quenched by the addition of 10 μ l of 3% tricarboxylic acid over a time course of the reaction. Subsequent dCDP and CDP levels were analysed by HPLC as described in Chapter 2.3.8. Graphs present either percentage of dCDP formation when normoxia and hypoxia were compared (data were normalised against the normoxic samples at 37°C at 30 min where the dCDP formation was considered 100%) or in

μM when the accumulation of dCDP formed in $<0.1\%$ O_2 at a 0-120 min time course was examined. The amount of product formed (dCDP) was determined.

2.3.8 Determination of CDP/dCDP

CDP and dCDP were determined by ion-pairing high performance liquid chromatography (HPLC) with UV detection (270 nm). HPLC was performed using a Shimadzu system with a photodiode array detector. The column used was an ACE C18, 3 μm , 125 x 3 mm (Hichrom) maintained at 35°C . A gradient separation was achieved using 10% methanol, 10 mM KH_2PO_4 , 10 mM TBAOH, pH6.9 (A) and 30% methanol, 6 mM TBAOH, 50 mM KH_2PO_4 , pH 7.0 (B), with a linear gradient of 25 – 47% B over 8 min, 47 – 80% B over 1 min, then returning to the start conditions for 3 min. The flow rate was 0.6 ml/min and the run time was 16.5 min.

2.3.9 Circular dichroism (CD) spectrometry

Circular dichroism (CD) spectra (Chirascan CD/Fluorimeter spectrometer (Applied Photophysics, UK) of hRRM2B and the mutants (0.2 mg/mL) were recorded in the wavelength range 260 to 185 nm in 100 mmol/L potassium phosphate buffer (pH 7.5). All measurements were carried out at 37°C using a 0.1-cm path length quartz cuvette. The data pitch was 0.1 nm with a 1-nm bandwidth at a scan speed of 1.0 nm/s. Each spectrum shown represents the average of three. All CD data were expressed as the mean residue ellipticity, $[\theta]$, $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$.

2.3.10 Electron paramagnetic resonance (EPR)

CW-EPR spectra were collected in the Center for Advanced ESR (CAESR) in the Inorganic Chemistry Laboratory at the University of Oxford. X-band measurements utilised a Bruker-Biospin Micro EMXplus spectrometer equipped with a PremiumX microwave bridge, a cylindrical TE011-mode resonator (SHQE-W), an ESR-900 liquid helium cryostat, and an

Oxford Instruments ITC-503s temperature controller. Measurements were performed within the limit of resolution by temperature, 40K, and under non-saturating conditions.

The protein concentration for all EPR samples was 200 μ M except for the hRRM2 protein which was 70 μ M. The protein samples (RRM2B, RRM2 or RRM2B variants) were diluted in 50 mM HEPES, pH 7.5 and were incubated either at 37°C for 5 min with oxygen present (normoxia) or at 37°C for 60 min in a Bactron II anaerobic chamber (<0.1% O₂). At the end of each treatment 20% (v/v) glycerol was added for vitrification during the low-temperature recordings. Samples were then transferred to EPR tubes (Sigma) and quick-frozen by immersion in liquid nitrogen. Spin quantitation was performed using the Bruker spectrometer hardware spin calibration, given the input dimensions of the precision Wilmad PQ-706 tubes. These results were verified with a 1 mM Cu^{II}-EDTA in 50 mM HEPES, pH 7.5, 20% (v/v) glycerol that was measured under non-saturating conditions at 100 K with 50 μ W microwave power. Results show concentration of tyrosyl radical (electron spins) per β subunit.

2.4 Cellular biology

2.4.1 Drug treatments

Hydroxyurea (HU) was used at a concentration of 2 mM for 6 h. Adriamycin was used at a concentration of 2 μ M for 6 h. 5-Bromo-2'-deoxyuridine (BrdU) was used at a concentration of 20 μ M for 30 min. All of the above compounds were purchased from Sigma-Aldrich UK.

2.4.2 Hypoxia treatment

A Bactron II anaerobic chamber (Shell Labs) was used for hypoxia treatment. Although no oxygen is supplied to this chamber, a complete absence of oxygen cannot be guaranteed at all times due to potential leaks. The oxygen concentration is estimated to be less than 0.1% at all times (<0.1% O₂). All experiments carried out in the anaerobic chamber, cells were plated on glass dishes to minimise the amount of potential oxygen introduced into the chamber. An *in vivo* 400 Ruskinn hypoxic chamber was used for oxygen concentration of 2%. The oxygen tension in the chambers was routinely tested either by the use of OxyLite probe (Oxford Optronix) or using the anaerobic paper indicator (BR0055B, OXOID). Cells were harvested inside the chambers with equilibrated solutions to avoid reoxygenation.

2.4.3 Exposure to γ -irradiation

Irradiations were carried out using a Gamma Service® GSR D1 irradiator which contains a Cs¹³⁷ source. The dose rates were 1.938 Gy/min and 1.233 Gy/min (depending on the distance from the source) as determined by the manufacturer. Samples were placed at the appropriate position and exposed to the required dose. The exposure time was calculated according to the dose rates of the system. After exposure to irradiation, cells were placed back in a humidified incubator (37°C and 5% CO₂) for 30 min and then cells were manipulated according to each experiment.

2.4.4 Cell lines

In this study, the following human cell lines were used: RKO colorectal cancer cells, HCT116 colorectal cancer cells, H1299 non-small cell lung carcinoma, U2OS osteosarcoma and U87-MG glioblastoma (purchased from ATCC); OE21 oesophageal squamous carcinoma cells (purchased from ECACC); RKO^{HIF-1 α +/+} and RKO^{HIF-1 α -/-} were generated by (Dang et al., 2006); HCT116^{p53+/+} and HCT116^{p53-/-} cell lines were generated by (Bunz et al., 1998); RKO^{RRM2B-/-} was constructed in this work as described in Chapter 2.4.5. All cells were cultured in DMEM supplemented with 10% FBS, in a standard humidified incubator at 37°C and 5% CO₂. Cells were passaged three to four times a week (depending on the cell line) and were not maintained in culture for more than 12-15 passages. Cells were trypsinised with Trypsin-EDTA after washing with PBS. Trypsin was inactivated by the addition of fresh media and cell density was determined using a Neuenbauer haemocytometer chamber. All cell lines were routinely tested for mycoplasma using the Plasmotest™ kit (InvivoGen) (carried out by Dr. Paul Newman) and found to be negative.

For cryopreservation of the above cell lines 1.5 ml cryovial (Thermo Fisher Scientific) were used. In brief, cells at 70-80% confluence were trypsinised, resuspended in fresh media and centrifuged at 400 x g for 6 min at room temperature. The supernatant was removed and the pellet of cells was resuspended in 1 ml of 90% FBS and 10% DMSO. The vial was placed in -80°C for 24 h before being stored in liquid nitrogen for long-term storage. To recover cells from liquid nitrogen, the cryovial was placed quickly in a 37°C water bath for cells to thaw and transferred to a 15 ml falcon containing pre-warmed medium. Cells were centrifuged at 400 x g for 6 min at room temperature. The supernatant was removed and the pelleted cells were resuspended in fresh medium and placed in a 75 cm² flask.

2.4.5 Construction of CRISPR-Cas9 RKO^{RRM2B-/-} cell line

In order to create a full *RRM2B* knock out RKO cell line the CRISPR-Cas9 system derived from *Streptococcus pyogenes* (SpCas9) was used. The CRISPR-Cas9 system stimulates a DSB at a target genomic locus by using RNA-guided nucleases (Garneau et al., 2010; Hsu and Zhang, 2012). Specifically, the Cas9 nuclease is targeted to genomic DNA by a single-guide RNA (sgRNA) that consists of a 20-nucleotide guide sequence and a partial direct repeat (PAM sequence - 5'-NGG for *S.pyogenes*). The guide sequence pairs with the DNA target directly upstream of the PAM repeat and mediates a double strand break (DSB) ~3 bp upstream of the PAM (Ran et al., 2013).

In order to create the CRISPR-Cas9 RKO^{RRM2B-/-} cell line, the canSAR database (Tym et al., 2016) was used to identify the copy number of the *RRM2B* gene in RKO cells. RKO cells contain four copies of the gene of interest. Since it has been reported that there are three different transcript variants of the *RRM2B* gene (each one encoding a different isoform; Figure 2.1), it was tested which isoform/s RKO cells express. Specific primers that recognise the three different transcript variants were designed (Table 2.15 and Appendix I-A.1) and qPCR (as described in Chapter 2.4.9) was carried out using cDNA from RKO cells, which were exposed either to normoxia or to hypoxia (6 h). It was determined that RKO cells express all three transcript variants of *RRM2B* both in normoxia and in hypoxia (Appendix I-A.2).

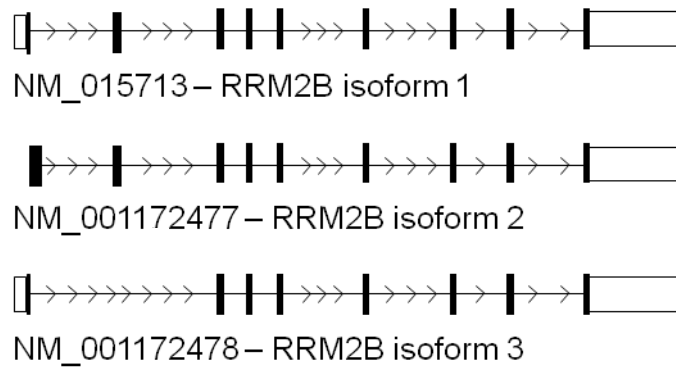


Figure 2.1 The three transcript variants of the human *RRM2B* gene

Transcript variants 1 and 3 (encoding for isoforms 1 and 3 respectively) share the same exon 1 whereas the transcript variant 2 (encoding for isoform 2) differs in the 5' UTR. Transcript variant 2 lacks a portion of the 5' coding region, and initiates translation at an alternate start codon, compared to variants 1 and 3. The encoded isoform 2 has a distinct N-terminus and is longer than isoforms 1 and 3. Transcript variant 3 lacks the alternate in-frame exon 2 in the 5' coding region, compared to variants 1 and 2, which results in a shorter protein (isoform 3), compared to isoforms 1 and 2.

Initially the beginning of the 5' UTR of the *RRM2B* gene was targeted and specifically the locus around the two exon 1 (variants 1&3 and variant 2). The guide sequences were designed by using the online CRISPR Design Tool (<http://tools.genome-engineering.org>). Specifically, the input sequence of 1-kb genomic fragment of the *RRM2B* exon locus 1 (Appendix I-B) was submitted to the online tool, which identified and ranked different suitable target sites and computationally predicted the off-target sites for each proposed sequence. Then, two proposed target sites (one for exon 1 of variants 1&3 and one for exon 1 of variant 2) which had the least off-target effects were chosen and the 20-bp identified sequences were ordered as custom made primers (Sigma) (Table 2.16 and Appendix I-C). Next, the sgRNA expression constructs (Table 2.18) were generated as follows:

I. Digestion of the pSpCas9(BB)-2A-GFP vector

DNA	5 µg
NEB Buffer 2.1	5 µl
Bbs I enzyme (NEB)	10 units
H ₂ O	top up to 50 µl

Digestion at 37°C for 2 h and then heat inactivation at 65°C for 20 min.

Digested DNA was run on a freshly made 1% agarose gel and gel extraction of the cut plasmid was performed using the QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions.

II. Annealing of guide sequence primers (Table 2.16)

Primer Forward	45 µl
Primer Reverse	45 µl
10 x annealing buffer	10 µl

The mix was incubated at 98°C for 10 min and then slowly cooled down at RT for at least 1

h. Annealing buffer (100 mM Tris-HCl pH 7.5, 1 M NaCl, 10 mM EDTA in Milli Q H₂O).

III. Ligation

	A	B	C
Digested vector (from I)	3 µl	3 µl	3 µl
Annealed primers (from II)	-	9 µl	13 µl
H ₂ O	13 µl	4 µl	-

The mix was incubated at 45°C for 5 min.

Next, 2 µl of T4 DNA Ligase Reaction Buffer and 2 µl of T4 DNA Ligase (*New England BioLabs*) was added to each mixture. O/N incubation followed at 4°C. The day after transformation into One Shot® TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific) was performed as described in Chapter 2.2.2 and the transformed bacterial cells were plated in LB ampicillin plates and allowed to grow O/N at 37°C. A sterile tip was used to pick a single bacterial colony the following day, which was grown in a small culture of 3 ml LB medium containing ampicillin at 37°C, O/N. At least five to six colonies were picked for each construct of interest (SpCas9-GFP-RRM2B_1/3 and SpCas9-GFP-RRM2B_2). The LB cultures were then used to isolate and purify the plasmid DNA by using the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. The purified plasmids were then sent for Sanger sequencing validation (Source Biosciences) using the hU6 promoter-For

primer (Table 2.16). One sample of each construct (SpCas9-GFP-RRM2B_1/3 and SpCas9-GFP-RRM2B_2) was kept and placed at -20°C.

Once the CRISPR-Cas9 plasmids were ready and validated, early passage RKO cells were thawed and kept in culture for one-two passages before they were transfected with both the SpCas9-GFP-RRM2B_1/3 and SpCas9-GFP-RRM2B_2 constructs. From each construct 1.25 µg of DNA was used (total 2.5 µg) and the transfection was carried out as described in Chapter 2.4.6. The day after transfection, cells were collected by trypsinisation, washed with PBS, centrifuged at 200 x g for 6 min at RT, resuspended in 1 ml PBS and finally filtered into a cell strainer cap Falcon™ Tube (Thermo Fisher Scientific). The concentration of the cell suspension was approximately 2×10^6 cells/ml. Isolation of clonal cell populations from the transfected cells was then performed using the FACS Aria™ III cell sorter (BD Biosciences) with the following parameters: GFP intensity per size of cell. Since not all sorted cells survived the FACS process, three 96-well plates were used to ensure an adequate number of clonal lines were derived. In each 96-well plate, 100 µl of DMEM supplemented with 10% FBS and 1% penicillin-streptomycin medium was added per well. Single cells were sorted into the 96-well plates and the plates were examined under the microscope to determine the presence of a single cell in most of the wells. The plates were then returned to the incubator and allowed to expand for 2-3 weeks. Ten days after sorting, the wells that contained single colonies were identified and marked. When the cells were more than 60% confluent, replica plates for passaging were prepared (one well for each clone in two different 96-well plates). Functional testing by IF was then performed on one of the replica plates for approximately 150 clones in total. Specifically, the media was aspirated from each well, cells were washed with PBS and fixed with 4% fixation buffer (4% (w/v) paraformaldehyde in PBS) for 15 min at RT. Following fixation, the cells were treated as described in Chapter 2.4.10. The RRM2B antibody (Table 2.3) was used to determine negative clones. After imaging the selected 150

clones, 24 promising clones were expanded and western blot analysis was carried out (Chapter 2.3.2). At this stage only a partial loss of the RRM2B expression was observed in some of the clones (Appendix I-D) meaning that not all of the copies and/or variants of the *RRM2B* were targeted. Clones No. 4, 5, 6 and 9 were checked to identify the one with the lowest RRM2B expression. Clone No. 4 was selected, expanded and used as a template cell line for the second attempt at complete knock out.

Since exon 1 varies between the different isoforms of the RRM2B, exon 4 was selected as the next best suitable region to stimulate a DSB in order to construct a full knock out cell line. A new sgRNA expression construct was made as described above. A 20-bp sequence targeting exon 4 was selected using the online CRISPR Design Tool (Table 2.16 and Appendix I-E). The SpCas9-GFP-RRM2B_4 construct (Table 2.18) was made and 2.5 µg of DNA was used for transfection. Isolation of clonal cell populations from the transfected cells was performed with the FACS Aria™ III cell sorter (BD Biosciences) and 500 clones in total were selected for functional testing by IF (Chapter 2.4.10). Expansion of 20 clones followed and western blot analysis (Chapter 2.3.2) was performed. One clone of the 20 tested (clone No. B2) showed no expression of RRM2B (Appendix I-F), and was selected as the CRISPR-Cas9 RKO^{RRM2B-/-} cell line.

The knock out cell line was then verified by sequencing in order to confirm the DSBs stimulated by Cas9 endonuclease. Genomic DNA of the RKO^{RRM2B-/-} cell line and RKO^{RRM2B+/+} cells (used as a negative control) were extracted using the Qiagen Blood and Cell Culture DNA mini kit (Qiagen) according to manufacturer's instructions. The DNA locus of interest was amplified by polymerase chain reaction (PCR) using either the *Rrm2b*-sequencing_exon1 or *Rrm2b*-sequencing_exon4 primers (Table 2.16). The Platinum® *Pfx* DNA Polymerase (Thermo Fisher Scientific) was used according to manufacturer's instructions. The PCR mix and conditions are described below:

PCR Mix

10 x <i>Pfx</i> Amplification Buffer	10 µl
10 x <i>Pfx</i> Enhancer	10 µl
5 mM dNTP mixture	6 µl
50 mM MgSO ₄	2 µl
Primer mix (10 µM each)	3 µl
Template genomic DNA (~ 200 ng/ µl)	20 µl
Platinum [®] <i>Pfx</i> DNA Polymerase (2.5 units)	1 µl
dd H ₂ O	48 µl
Total	100 µl

PCR Conditions

1.	98°C	30 sec
2.	98°C	10 sec
3.	55°C	30 sec
4.	72°C	1 min
Repeat steps 2 to 4 x 40 times		
5.	72°C	10 min

The PCR mix was then run on a freshly made 1% agarose gel and gel extraction was performed using the QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions. The extracted DNA was sent for Sanger sequencing validation (Source Biosciences) using the *Rrm2b*-sequencing_exon1 or *Rrm2b*-sequencing_exon4 primers. The sequencing results with the identified mutations are shown in Appendix I-G.

2.4.6 DNA transfection

Cells were transfected according to the manufacturer's instructions with Lipofectamine LTX (Invitrogen). The plasmids used for transfection are listed in Table 2.17. A final concentration of 2.5 µg of DNA was used. In brief, on the day before transfection, the appropriate number of cells that would give 70% confluency at the time of transfection were seeded in a 6 cm plate in antibiotic-free complete DMEM media. DNA and Reagent Plus were added in a 1:1 ratio in 200 µl Opti-Mem[®] media (Thermo Fisher Scientific). In a different tube 4 µl of Lipofectamine LTX was added to 200 µl Opti-Mem[®] media. Following a 5 min incubation, the mix containing the Lipofectamine LTX was added to the mix containing the DNA. After a further 20 min incubation, the media on the cells to be

transfected was replaced with 1.6 ml Opti-Mem® media plus 400 µl for each transfection. After 6 h of incubation at 37°C, the media of the cells was replaced with antibiotic-free complete DMEM media and cells were placed at 37°C O/N. The following day, cells were replated into glass dishes at a concentration that would allow the cells to be at 80% confluency at the time of the experiment.

2.4.7 Small interference RNA (siRNA)

Dharma-FECT 1 reagent (Thermo Fisher Scientific) was used for siRNA knockdown. RRM2B siRNA (sequence: UGAGUUUGUAGCUGACAGAUU) (Piao et al., 2009) (Sigma) was used in most of the experiments and second RRM2B siRNA#2 (sequence: GGAACAAGCUUAAAGCAGA) (Ambion/Life Technologies; s224156) was used where indicated; p53 siRNA (sequence: GUAAUCUACUGGGACGGAA) (Leszczynska et al., 2015) (Ambion /Life Technologies) or AllStars Negative Control siRNA (Qiagen) were transfected into cells at a final concentration of 50 nM according to the manufacturer's instructions. In brief, the appropriate number of cells that would allow 70% confluency at the time of transfection, were seeded in a 3.5 mm plate on the day before transfection in antibiotic-free complete DMEM media. The following day, 100 µM siRNA was mixed with 20 µl RNase free water and 25 µl antibiotic-free and serum-free DMEM media. In a different tube 5 µl Dharma-FECT 1 reagent was mixed with 345 µl antibiotic-free and serum-free DMEM media. The contents of each tube were mixed and 5 min incubation followed. The contents of both tubes were then mixed and the mixture left to incubate for 20 min. The media on the cells to be transfected was replaced with 1.6 ml of antibiotic-free complete DMEM media and after the 20 min incubation, 400 µl of each transfection was added to the cells. Cells were then left to incubate for 24 h at 37°C. The following day, cells were replated into glass dishes at a concentration that would allow the cells to be at 80% confluency at the time of the experiment. The transfection efficiency was optimised when the siRNAs were

first acquired. Observation and testing of the transfected cells was carried out in order to have the best possible depletion of the target of interest with the least cellular toxicity.

2.4.8 RNA extraction and purification

RNA was extracted using 1 ml of TRIzol[®] (Invitrogen/Life technologies) per 5 cm dish. Following addition of TRIzol[®], samples were incubated for 5 min at RT before the addition of chloroform (200 μ l). Samples were then vortexed (15 sec) before incubation for 3 min at RT. this was followed by centrifugation at 12000 x g at 4°C for 15 min. After centrifugation, the upper aqueous phase containing the RNA was carefully collected and transferred to a fresh tube. 500 μ l of isopropanol was then added per 1 ml TRIzol[®] and samples were incubated for 10 min at RT. Samples were then centrifuged at 12000 x g at 4°C for 15 min and the supernatant removed. The remaining pellet was washed with 75 % ethanol and the samples were vortexed briefly before another centrifugation at 7500 x g at 4°C for 5 min. The pellet was then allowed to air-dry and the RNA was resuspended in 50-100 μ l of DEPC-treated H₂O. RNA purity and concentration was determined using the Nanodrop meter by measuring the absorbance at 260 nm. An A260nm/A280nm ratio of 1.9-2.1 indicated that RNA was pure and protein-free (a reading below 1.6 indicated protein contamination).

2.4.9 Quantitative PCR (qPCR)

Verso (Thermo Fisher Scientific) cDNA (complementary DNA) synthesis kit was used to reverse transcribe cDNA from total RNA according to manufacturer's instructions. The cDNA was diluted in DEPC-treated water to 1:50 before it was used for qPCR experiments. Quantitative real time PCR (qPCR) was performed using the following reaction mix in a total volume of 20 μ l plus 5 μ l of diluted cDNA.

Reaction Mix

SYBR® Green PCR Master Mix (Applied Biosystems)	12.5 µl per reaction
Primer Forward	2.5 µl per reaction
Primer Reverse	2.5 µl per reaction
DEPC-treated H ₂ O	2.5 µl per reaction

The final concentration of primers was 100 nM except Glut 1 (300 nM) and 18S (500 nM). Three technical replicates from each sample were loaded into a 96-well plate. Reactions were carried out in a 7500 Fast real time PCR detection system (Applied Biosystems) with the following conditions: 20 sec at 50°C, 15 min at 95°C then 40 cycles of 15 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C, and finally a melt curve stage of 15 sec at 95°C, 1 min at 60°C, 30 sec at 95°C and 15 sec at 60°C.

For mRNA expression analysis, mRNA fold change levels were normalised to 18S ribosomal RNA and calculated using a $2^{-\Delta\Delta C_t}$ method. Error bars indicate the error between three biological experiments.

2.4.10 Immunofluorescence (IF) microscopy

For IF assays, cells were seeded on autoclaved cover glasses (Menzel-Glaser), left O/N in a standard humidified incubator at 37°C and 5% CO₂ and treated as described for each experiment. At the end of the experiment, the coverslips with the seeded cells were washed twice with PBS and fixed with 4% fixation buffer (4% (w/v) paraformaldehyde in PBS) for 15 min at RT. Following fixation, cells were washed three times with PBS and were then incubated with lysis buffer (1% PBS-Triton X-100) for 10 min at RT. After lysis, cells were washed once with PBS and were then incubated for 1 h at RT with blocking buffer (2% (w/v) BSA in 0.1% PBS-Triton X-100). Incubation with the appropriate primary antibody followed (diluted in 2% (w/v) BSA - 0.1% PBS-Triton X-100) for 90 min at 37°C in a humidified chamber. All primary antibodies used for IF are listed in Table 2.3. Three washes with PBS followed and then the cells were treated with the appropriate secondary antibodies (diluted in

2% (w/v) BSA - 0.1% PBS-Triton X-100) for 60 min at 37°C in a humidified chamber in the dark. Details of the secondary antibodies used are given in Table 2.10. Three further washes with PBS and a final wash with H₂O were carried out before mounting the slides in ProLong[®] Gold mounting medium with DAPI (Invitrogen/Life technologies). The slides were kept in the dark at 4°C before being analysed using a LSM780 (Carl Zeiss Microscopy Ltd) confocal microscope.

2.4.11 Single-molecule DNA fiber analysis

DNA fibers were performed as previously described with a few modifications (Foskolou et al., 2016). In brief, subconfluent cells (50-60% confluency) were sequentially pulse labelled with 25 mM CldU and 250 mM IdU for 20 min each and left at 21% O₂ in a 37°C incubator (normoxic samples). Cells were washed once with fresh warm media before the addition of the second (IdU) label. For hypoxic treated samples, cells were first placed for 2 h in the hypoxic chamber (<0.1% O₂) and then treated with 25 mM CldU for the indicated times (Figure 2.2). Cells were then washed once with fresh warm media and 250 mM IdU was added as it is indicated in Figure 2.2. All hypoxic samples treated in hypoxia for 6 h in total. At the end of the treatment, labelled cells were washed once with PBS and diluted to a concentration of 5×10^5 cells per ml. 2 µl of cell suspension was placed at the top of a slide and 5 min after cells were mixed with 7 µl of Spreading Buffer (200 mM Tris-HCl, pH 7.4, 50 mM EDTA, 0.5% SDS in Milli Q H₂O) and mixed gently. Cells were incubated for 2 min and the DNA fibers were spread by tilting the slide gently to an approximate angle of 30° - 40° angle. DNA fibers were then fixed with methanol/acetic acid (3:1 ratio). For staining, fibers were treated with 2.5 M HCl and incubated with rat anti-BrdU monoclonal antibody (for detection of CldU labelled tracts) and mouse anti-BrdU monoclonal antibody (for detection of IdU labelled tracts) for 1 h at RT (details of the antibodies used are shown in Table 2.7). Slides were then fixed with 4% paraformaldehyde and incubated with Alexa Fluor

555-conjugated goat anti-rat IgG and Alexa Fluor 488-conjugated goat anti-mouse for 1 h in a humidified chamber at 37°C (Table 2.10). Fibers were imaged using a Bio-Rad Radiance or LSM780 (Carl Zeiss Microscopy Ltd) confocal microscope and analysed using ImageJ software (NIH). The length of the fibre tracts that had incorporated both labels (CldU and IdU) was measured and replication rates were determined by the following formula: $(V(\text{CldU kb/min}) = [(x * 0.132 \mu\text{m}) * 2.59 \text{ kb}/\mu\text{m}] / t (\text{min})$, where x = length of CldU. At least 100 replication tracks were measured per experiment.

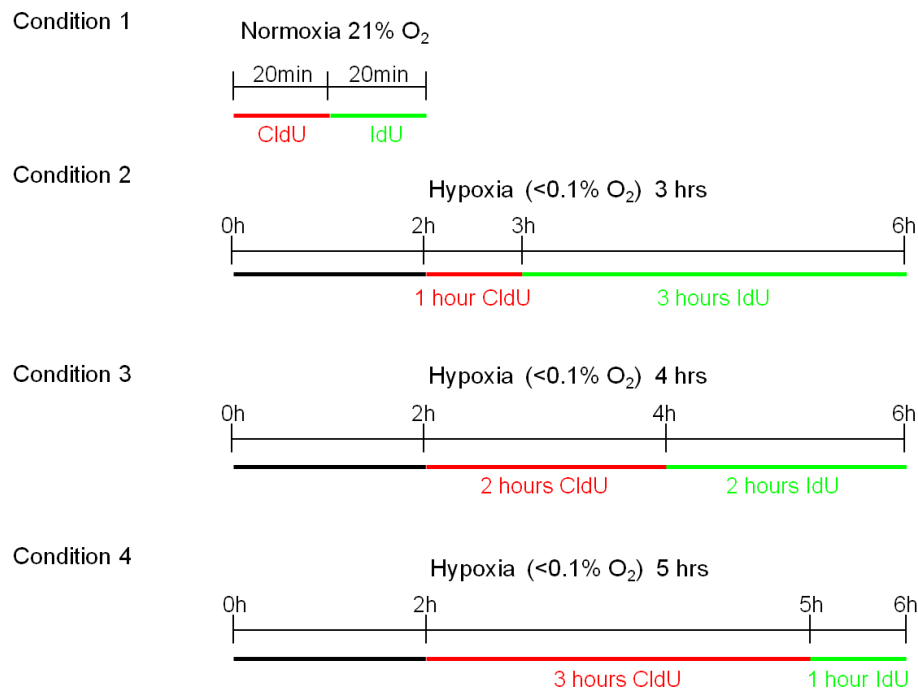


Figure 2.2 Schematic representation of the DNA fiber protocol in normoxia and hypoxia Condition 1: labelling in normoxia. Conditions 2-4: labelling times in hypoxia (<0.1% O₂).

2.4.12 Flow cytometry (FACS)

Cells (1×10^6 per condition) were seeded O/N and allowed to adhere. Cells were placed either in normoxia (21% O₂) or in hypoxia (<0.1% O₂ for 3 or 6 h) and 40 min prior the end of the time course cells were pulsed with 20 μM BrdU. Following treatment, cells were trypsinised and collected with 1 ml PBS. After collection, cells were washed with PBS and placed in 15 ml falcons. Centrifugation followed at 300 x g for 5 min and cells were then

fixed with 1 ml 70% ethanol while vortexing. Samples were placed at -20°C for at least 30 min. Samples were then centrifuged at 250 x g for 5 min and resuspended in 1 ml of 2 M HCl containing 0.1 mg/ml pepsin (Sigma) and incubated at RT for 20 min. Samples were then washed once in 2 ml PBS and centrifuged at 350 x g for 5 min and then washed once in 1ml 2% BSA in PBS and centrifuged at 350 x g for 5 min. Cells were then resuspended in 100 µl of 2% BSA in PBS and were then incubated with the primary antibody diluted 1:100 in 1% BSA in PBS for 90 min at RT followed by the secondary antibody diluted 1:200 in 1% BSA in PBS for 60 min (details of primary and secondary antibodies used are shown in Table 2.6 and 2.10 respectively). Finally, the pellets were resuspended in PBS containing PI (10 µg/ml) and incubated at 37°C for 30 min. Analysis was carried out for 10000 cells per experimental condition using Becton Dickinson FACSort. Samples were later analysed using CellQuest Pro and ModFit LT software.

2.4.13 dNTP incorporation assay

dNTP pool determination in whole-cell extracts was carried out based on the Sherman and Fyfe method (Sherman and Fyfe, 1989) with a few modifications as follows. For the preparation of cell extracts, cells (1×10^6 per condition) were seeded O/N in glass dishes (5 cm³) and allowed to adhere. The following day, cells were treated either in hypoxia (<0.1% O₂ for the indicated times) or with 2 mM HU (6 h). The normoxic samples (0 h of <0.1% O₂) were processed immediately after the beginning of the hypoxic and/or HU treatment. Samples were then carefully washed with PBS, trypsinised and collected with 1 ml PBS. 100 µl of cell suspension was kept for counting and the rest was centrifuged at 350 x g for 5 min. The cell pellet was then resuspended in 1 ml 60% iced cold methanol and kept at -20°C O/N. The following day, the samples were placed in boiling water for 3 min and then left at RT to cool down. Centrifugation at 16200 x g for 10 min at RT followed and the supernatant was then collected in a fresh Eppendorf. The supernatant was then dried by centrifugal

evaporation and finally dissolved in 50 µl of Milli Q H₂O. The samples were kept at -20°C until analysed. For the preparation of each primer mix a different set of primers was used supplemented with either [³H]dATP or [³H]dTTP (PerkinElmer). Specifically, 2.5 µM of each template (dCTP, dGTP, dTTP or dATP) forward primer and 2.5 µM of dNTP reverse primer (Table 2.17) was diluted in 1 ml Hepes Buffer (100 mM Hepes pH 7.5, 10 mM MgCl₂ diluted in Milli Q H₂O). Primers were next annealed at 90°C for 4 min and then let to cool down at RT. After annealing, each 1 ml of primer mix was supplemented with either 1 µl of [³H]dATP (1 µCi, for dCTP, dGTP and dTTP determination) or 1 µl of [³H]dTTP (2.5 µCi, for dATP determination). A stock of 20-25 ml of each primer mix was prepared which was subsequently aliquoted in 1.5 ml Eppendorfs and stored at -20°C in order to minimise pipetting errors. Reaction mixtures for dGTP and dCTP determination contained 2 µl cell extract, 48 µl of the appropriate (dGTP or dCTP) primer mix and 0.03 µl (1 U) Thermo Sequenase DNA Polymerase (GE Healthcare); for dTTP determination the reaction mixture contained 2 µl cell extract, 48 µl dTTP primer mix and 0.12 µl (1.25 U) Klenow Fragment (Thermo Fisher Scientific); for dATP determination the reaction mixture contained 1 µl cell extract, 49 µl dATP primer mix and 0.06 µl (0.625 U) Klenow Fragment (Thermo Fisher Scientific). The total assay volume was 50 µl and the reactions were started by addition of the enzyme. Reactions were incubated for 1 h either at 48°C (for dGTP and dCTP mixtures) or at RT (for dTTP and dATP mixtures). Following incubation, 48 µl of the reaction mixture were spotted onto circles (23 mm) Whatman DE81 paper (GE Healthcare) and let dry for 5 min at 65°C. The papers were then washed three times with 5% Na₂HPO₄ for 10 min each wash followed by rinsing once with distilled H₂O and once with 100% ethanol. After being dried, the radioactivity on the papers was measured in a LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter™) using 3.5 ml Optiphase HiSafe 3 (PerkinElmer) counting fluid. Data present percentage of dNTP incorporation compared to the positive control of each

experiment. In experiments where both siRNA and hypoxia treatment were used, a normalisation to each normoxic control was preceded the final analysis.

2.4.14 Apoptosis (by cell morphology)

To assess apoptosis by nuclear morphology both adherent and floating cells were collected in 15 ml falcon tubes and fixed for 15 min at RT with 4% paraformaldehyde. Samples were then washed with PBS and the nuclei were stained with ProLong[®] Gold mounting medium with DAPI (Thermo Fisher Scientific). Apoptosis was plotted as the percentage of cells with fragmented DNA per field of view, with at least ten fields of view quantified per experiment. Each apoptosis experiment was performed at least 3 times.

2.4.15 Colony survival

For clonogenic survival studies, exponentially growing cells were trypsinised and counted using a haemocytometer. 250 cells were seeded in each well of a 6-well plate in triplicate and were then allowed to adhere for 3 h under normal tissue culture conditions. Cells were then placed either in normoxia (21% O₂) or in hypoxia (<0.1% O₂). Post treatment the plates were transferred to a standard humidified incubator at 37°C and 5% CO₂ and colonies were allowed to form for approximately 10 days. Cells were then stained with 0.5% (w/v) crystal violet in 50% methanol and 20% ethanol. Colonies containing at least 50 cells were counted using a colony counter. The survival fraction was calculated using the following formula:

$$\text{Survival Fraction} = [(\text{number of colonies formed after treatment}) / (\text{number of cells seeded} \times \text{plating efficiency})] \times 100$$

$$\text{Plating efficiency} = \text{number of colonies obtained in the untreated control} / \text{number of cells seeded in the untreated control}$$

2.5 *In vivo* experiments

2.5.1 Immunohistochemistry (IHC)

For IHC staining, sections from two different patients with glioblastoma cancer were used. The brain tumour samples were provided by the Oxford Brain Bank (15/SC/0639), in part supported by the Oxford NIHR Biomedical Research Centre, consent was obtained for patient biopsies. The sections were dewaxed in Citoclear and rehydrated through a gradient of ethanol (100% - 50%). Endogenous horseradish peroxidase activity was quenched with 3% H₂O₂ for 30 min at RT. Blocking was performed with 2.5% horse serum for 2 h followed by incubation with Mouse on Mouse (M.O.M.TM) Blocking Reagent (Vector Laboratories) for 45 min at RT. Next, incubation with primary antibodies (Table 2.9) followed at 4°C O/N. The following day, secondary antibodies were used from the ImpressTM kit (Vector Laboratories) according to the manufacturer's instructions. Following this incubation, slides were stained with diaminobenzidine (DAB) solution (DAB+, Dako). Samples were counterstained with hematoxylin for 30 sec and mounted with DPX mounting media (Surgipath). Images were obtained using the Aperio ScanScope slide scanner.

2.5.2 Xenograft study

All animal procedures were performed in accordance with current UK legislation and were approved by the University of Oxford Biomedical Services Ethical Review Committee, Oxford, UK. For the tumour growth curves, female Balb/c nude mice or female athymic nude mice (Charles River, UK) were randomized and injected subcutaneously with 3×10^6 RKO^{RRM2B+/+} or RKO^{RRM2B-/-} cells in 25% (v/v) matrigel and serum-free DMEM. Tumour growth was monitored until they reached approximately 300-400 mm³ (volume = height x depth x width x π / 6). For the irradiation experiment, the tumours in half of the animals were irradiated with 10 Gy when tumour volumes reached 100 mm³. Tumours were measured

regularly, and tumour growth was plotted as a mean of tumour volumes $\pm$ SEM. Mice were injected with 50 mg/kg of pimonidazole (PIMO) 2 hr before the end of the experiment. Tumours were split in half and either preserved in formalin for wax embedding or were snap frozen and embedded in optimal cutting temperature compound (OCT).

2.5.3 Tissue IF

Tumours embedded in OCT were sectioned (5 μ m) and froze down at -20°C. Once removed from freezer, the sections were immediately fixed with 4% paraformaldehyde for 15 min at RT. Sections were then quenched with 50 mM NH₄Cl for 20 min and washed twice with 0.025% TBS-Triton X-100 for 5 min each. Blocking was performed with 1% BSA in TBS for 90 min at RT followed by incubation with Mouse on Mouse (M.O.M.TM) Blocking Reagent (Vector Laboratories) for 45 min at RT. The sections were stained with PIMO and cleaved caspase-3 primary antibodies (Table 2.8) at 4°C O/N. The following day, the sections were incubated with secondary antibodies (Molecular Probes) for 2 h RT and mounted with ProLong[®] Gold mounting medium with DAPI (Invitrogen/Life technologies). The apoptotic cells were counted within and outside of hypoxic regions. LSM780 software (Carl Zeiss Ltd) was used to determine the average of a single cell's surface area, and area of apoptotic cells (expressed as percentage) in whole hypoxic or normoxic regions was calculated from the following formula: number of apoptotic cells $\times$ average area of a single cell $\times$ 100 per area of total region measured. Images were taken from at least 3 RKO^{RRM2B+/+} and 3 RKO^{RRM2B-/-} tumours. Graphs show the percentage of apoptotic cells from all normoxic or hypoxic regions.

2.6 Statistical analysis

The statistical significance of differences between data sets was determined assessed by using the following tests. Student's *t* test (two-tailed paired, except for dNTP incorporation assays, where a two-tailed unpaired test was applied) was used in all experiments where two means were compared. One-way analysis of variance (ANOVA) was used to compare several means and two-way ANOVA was used for determination in a response that is affected by two factors (one- or two-way ANOVA analysis is indicated at the figure legends). Statistical significance was assumed if $P < 0.05$ or lower and is noted in the figures. Error bars represent mean $\pm$ standard error of the mean (s.e.m.).

CHAPTER 3

Hypoxia induces the RRM2B subunit of RNR

3.1 Introduction

Hypoxia induces RS, which triggers the activation of the DDR pathway, including ATR and ATM signalling. Notably, this occurs in the absence of detectable DNA damage (Bencokova et al., 2009; Hammond et al., 2002; Olcina et al., 2013). The activation of the DDR is associated with the early stages of cancer development, with hypoxia being one of the factors proposed to contribute to this pathway, as hypoxic regions are observed in preneoplastic lesions (Bartkova et al., 2005; Gorgoulis et al., 2005; Hammond et al., 2007). It has been suggested, that hypoxia-induced RS is in part due to imbalanced dNTP pools, resulting from compromised RNR activity in these oxygen-limited environments (Pires et al., 2010b).

As previously discussed, RNR is the only enzyme that catalyses the *de novo* conversion of NDPs to dNDPs, and as such plays a pivotal role in maintaining dNTP pools at optimum levels (Kolberg et al., 2004; Stubbe, 1998). Mammalian RNRs consist of two homodimeric subunits, which associate to form the holoenzyme. The α subunit (RRM1 dimer) contains the catalytic site, whereas the β subunit (RRM2 or RRM2B dimer) contains

the essential tyrosyl radical, which is formed during the reaction between oxygen and the diiron centre (Kolberg et al., 2004; Stubbe et al., 2003; Uhlin and Eklund, 1994).

RRM2B was first characterised by Tanaka *et al.*, as a p53-regulated RNR subunit (p53R2) and has been primarily associated with DNA repair and mitochondrial DNA synthesis (Pontarin et al., 2012; Tanaka et al., 2000; Wang et al., 2009). It has been shown that the activation of the DDR in response to hypoxia results in ATR- and ATM-dependent phosphorylation of p53 (S15) (Bencokova et al., 2009; Hammond et al., 2002; Hammond et al., 2007). For many years, hypoxia-induced p53 was thought to act primarily as a transcriptional repressor, as many well-known p53 targets are not induced in hypoxia (Hammond et al., 2006; Koumenis et al., 2001). However, it is now clear that in hypoxic conditions p53 also acts as a transactivator, which induces a specific subset of genes distinct from those induced in response to DNA damage (Leszczynska et al., 2015).

Intrigued by the absence of detectable DNA damage in hypoxic conditions, which implies that the integrity of the replication forks is preserved in hypoxia despite the induction of RS, we investigated the relationship between hypoxia-induced RS and nucleotide availability in these conditions.

3.2 Results

3.2.1 Hypoxia induces DNA replication stress

Previous studies from our lab have shown that in the physiological relevant stress of hypoxia (<0.1% O₂) there is induction of a robust DDR in the absence of DNA damage (Bencokova et al., 2009; Hammond et al., 2002; Olcina et al., 2013; Pires et al., 2010a). In order to investigate the hypoxia-induced DDR, we performed western blot analysis with colorectal RKO cancer cells that were exposed to hypoxia for different time points (Figure 3.1). We confirmed that, as previously reported, both ATR and ATM dependent targets (phosphorylated forms of CHK1 and KAP-1 respectively) are significantly induced after 3 hours of hypoxic exposure and that this induction then decreases over time.

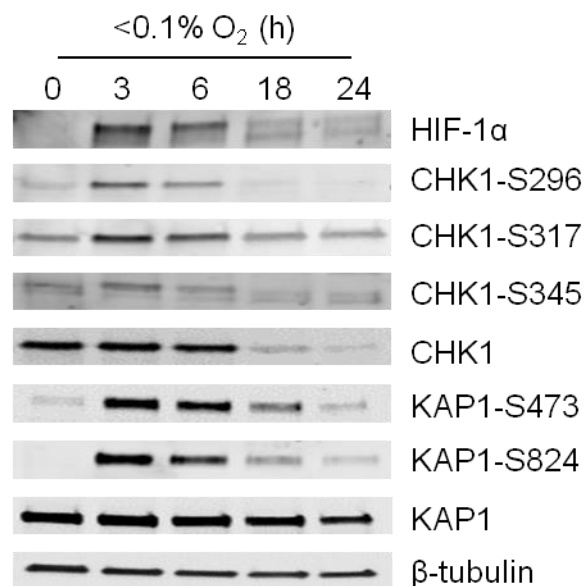


Figure 3.1 The Hypoxia-induced DDR

RKO cells were exposed to <0.1% O₂ for the times indicated. Western blot analysis was carried out with the antibodies indicated; β-tubulin was used as a loading control.

Next, we investigated hypoxia-induced RS, which has been proposed to be responsible for the activation of the observed DDR (Olcina et al., 2013; Pires et al., 2010b). Specifically, we used osteosarcoma U2OS cancer cells and exposed them to either normoxia or hypoxia (3 and 6 hours). Cells were pulse labelled with BrdU for 30 minutes before collection and FACS analysis (Figure 3.2). As expected, cells exposed to hypoxia showed a disruption of normal DNA replication. We observed a decrease in the BrdU positive population in $<0.1\%$ O_2 (Figure 3.2 A), which indicates slower and decreased DNA replication in these conditions. Specifically, the FACS analysis showed that hypoxic cells do incorporate some BrdU but to a lesser extent than in normoxic conditions, confirming the induction of RS.

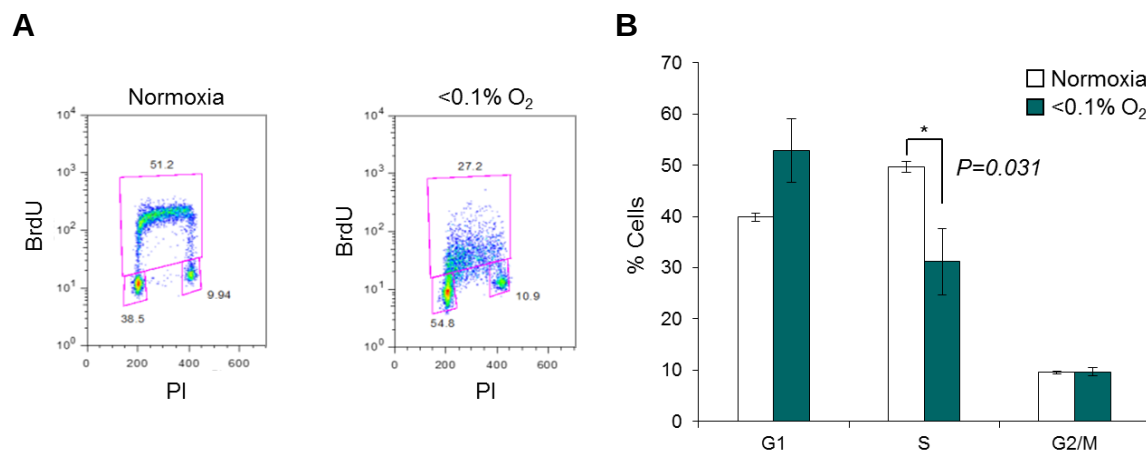


Figure 3.2 Hypoxia induces DNA replication stress

(A) U2OS cells exposed to normoxia or $<0.1\%$ O_2 (3 h). Cells were pulsed with BrdU (20 μM) for 30 min before collection. Cells were then stained for BrdU and for DNA content using propidium iodide (PI) and analysed by FACS. **(B)** Quantification of the percentage of U2OS cells in G₁, S or G₂/M phase of the cell cycle after exposure to normoxia or $<0.1\%$ O_2 (6 h). Cells were treated and analysed as in (A). Data show mean $\pm$ s.e.m. from $n=3$ biological experiments. Two-tailed, unpaired Student's t-test was applied

An alternative method to investigate RS, is through the detection of RPA foci which indicates ssDNA (Branzei and Foiani, 2010; Glanzer et al., 2014). As predicted, hypoxia leads to rapid accumulation of RPA foci, however, the percentage of cells in hypoxia with ssDNA decreases after prolonged exposure to these conditions (>6 hours) (Figure 3.3). These data suggest that the replication machinery continues on-going DNA synthesis in hypoxia. Thus, even though replication rates are slowed down in hypoxia (Pires et al., 2010b), it seems likely that it is not completely abrogated.

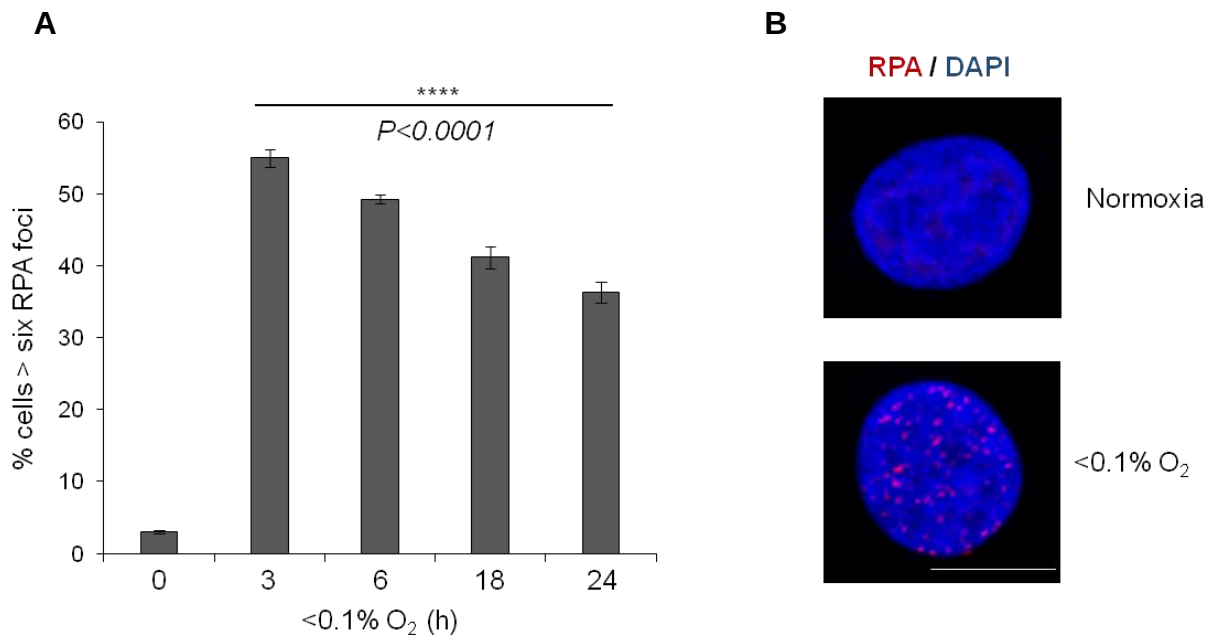


Figure 3.3 The accumulation of ssDNA observed in hypoxia decreases after prolonged exposure to these conditions

(A) Quantification of RPA foci in RKO cells exposed to <0.1% O₂ for the times indicated and stained for RPA32 foci. Data show mean $\pm$ s.e.m. from $n=3$ biological experiments. One-way ANOVA analysis was applied. **(B)** Representative images of RPA32 negative (normoxia) and positive (<0.1% O₂ – 3 h) cells. Cells were stained with RPA32 (red) indicating ssDNA and with DAPI (blue) to label nuclear DNA. Scale bar 10 μ m.

3.2.2 Replication is not abrogated in hypoxia

We hypothesised that, although uncoupling of the helicases and polymerases occurs in hypoxia, replication is not abrogated in these conditions. In order to investigate in detail how replication rates are affected by hypoxic exposure, we performed DNA fiber analysis. This technique is the most sensitive and specific assay available to date, for the direct measurement of DNA synthesis, as it allows the monitoring of DNA replication at the single molecule level (Foskolou et al., 2016). DNA fibers were generated following the exposure of RKO cells to normoxia and $<0.1\%$ O_2 for different time points, and DNA replication rates were quantified for the on-going replication structures. As shown in Figure 3.4, although DNA replication is significantly slower in hypoxia (25% of normal rate), it is not abolished in these conditions.

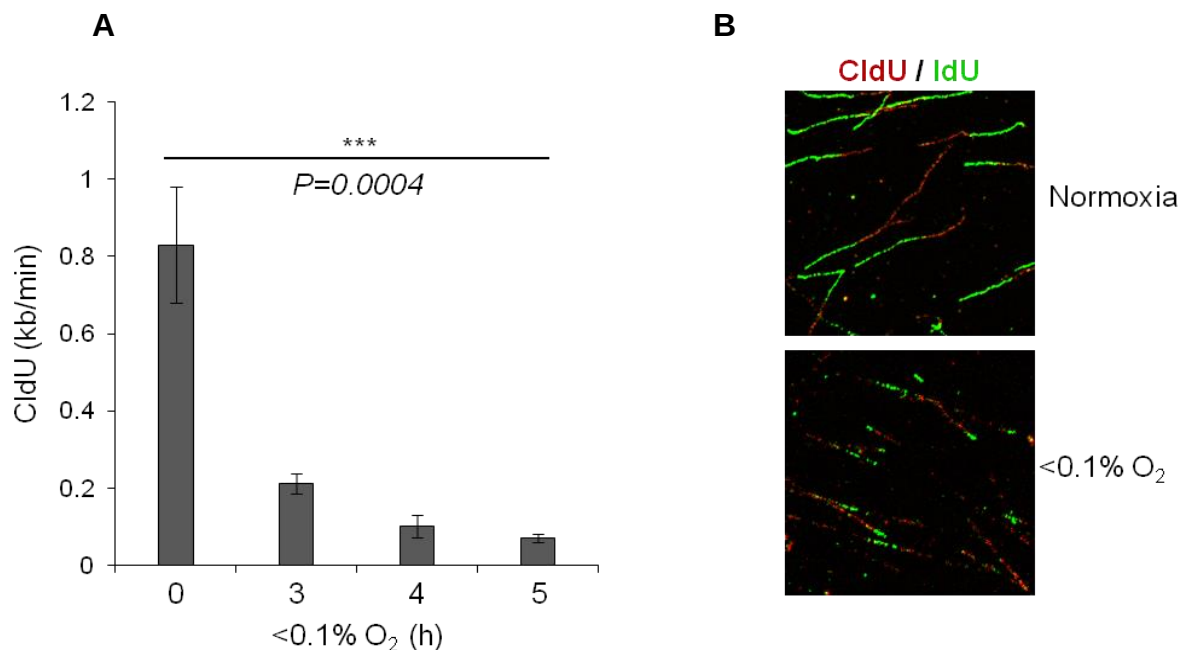


Figure 3.4 DNA replication is slower but not abrogated in hypoxia

(A) Quantification of replication rates by DNA fiber assay in RKO cells exposed to $<0.1\%$ O_2 for the times indicated. Cells were sequentially pulsed with CldU and IdU and stained as described in the Materials and Methods. Both labels were used in order to determine the on-going replication forks while only the first label (CldU) was used for determination of the replication rates. Data show mean $\pm$ s.e.m. from $n=3$ biological experiments. One-way ANOVA analysis was applied. **(B)** Representative images of replication structures from cells treated as in (A) and exposed to normoxia or $<0.1\%$ O_2 (5 h). Cells were stained with CldU (red) and IdU (green).

The finding that DNA replication proceeds in hypoxia, albeit at a compromised rate, made us question the status of dNTP levels in these conditions, as they are essential to maintain even the slow replication rates observed. dNTP incorporation assays were carried out in RKO cells exposed to either normoxia, hypoxia or HU. HU is an inhibitor of the RNR enzyme (Figure 1.13), which is used as a chemical inducer of RS, and results in lower intracellular dNTP levels (Krakoff et al., 1968). As shown in Figure 3.5, we found that although the dNTP pools were disrupted in hypoxia (with the purines [dGTP, dATP] being more affected than the pyrimidines [dCTP, dTTP]) there was nucleotide availability in these conditions.

The reason behind the difference observed between purine and pyrimidine levels due to both hypoxia and HU is generally poorly understood. As far as HU is concerned, it is well documented that HU results in partial depletion of the purine dNTP pools, whereas there are conflicting reports about its modulation on the pyrimidine levels with most studies showing an increase similar to our data (Bianchi et al., 1986b; Koc et al., 2004; Snyder, 1984). It has been suggested that the pyrimidine levels persist due to compensatory activities of the pyrimidine salvage pathways (Eriksson et al., 1987) or due to high amounts of deoxyuridine in the conditioned medium which cells could uptake and phosphorylate, contributing to the pyrimidine levels (Bianchi et al., 1986a). There are also reports suggesting that in conditions of RS, cells quickly scavenge the levels of the dATP and dGTP nucleosides in order to prevent cellular toxicity (Ullman et al., 1976). Importantly, hypoxia showed a similar dNTP levels as 6 hours of HU treatment (with the exception of the dCTP levels) confirming that one of the reasons why we observe RS in these conditions likely due to imbalanced nucleotide pools. The basal levels of dNTPs, which remain in hypoxia, may explain the slow replication rates observed.

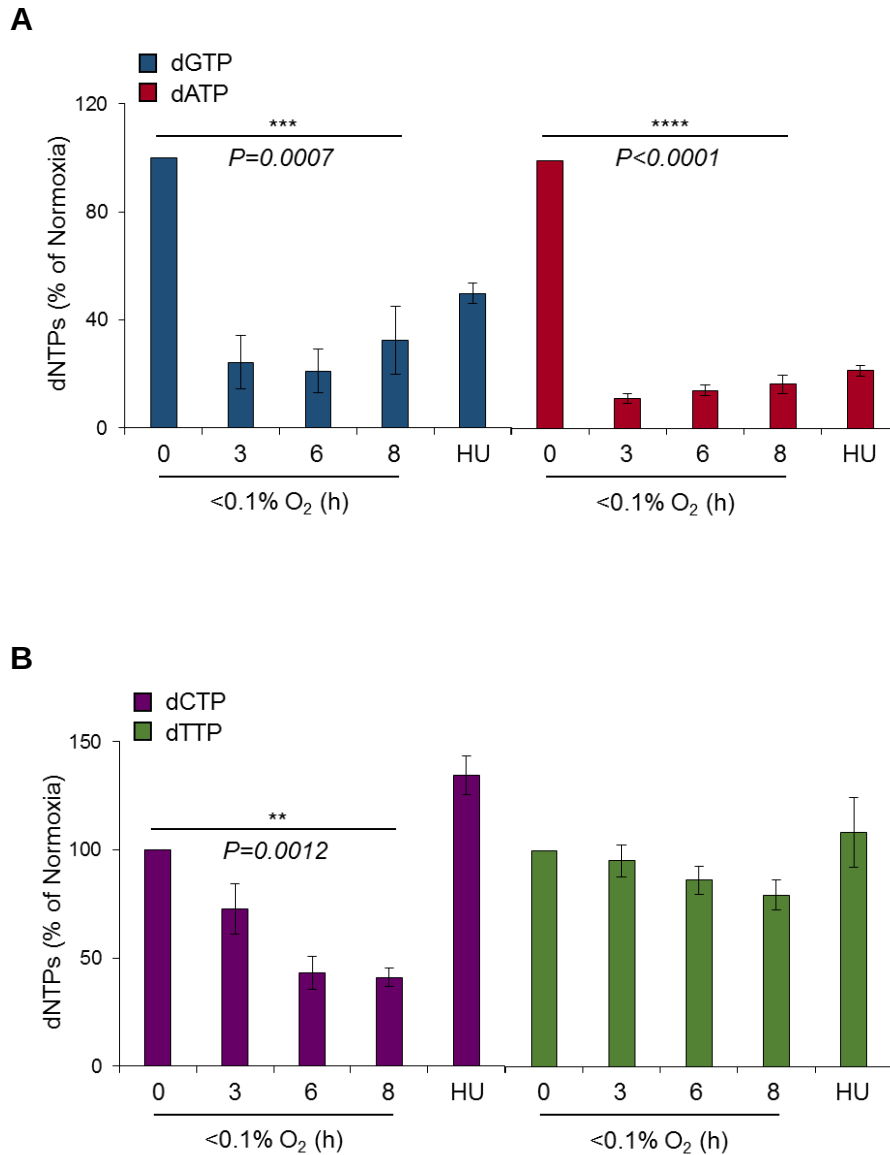


Figure 3.5 Imbalanced nucleotide levels are observed in hypoxia

Quantification of the dNTP levels in RKO cells exposed to $<0.1\% \text{ O}_2$ for the times indicated or to HU (2 mM, 6 h). **(A)** Purines; **(B)** Pyrimidines. Data show mean $\pm$ s.e.m. from $n=3$ biological experiments. One-way ANOVA analysis was applied.

3.2.3 The RRM2B subunit of RNR is induced in response to hypoxia

The nucleotide availability in hypoxia raised the possibility that RNR is able to retain some activity in hypoxia despite its oxygen dependency (Huang et al., 2014). Thus, we examined the levels of the three RNR subunits both in normoxic and hypoxic conditions. Expression analysis of the RNR complex in RKO cells demonstrated a 2.5-fold induction of the RRM2B protein in hypoxia both by western blot analysis and by immunofluorescence (Figure 3.6). In contrast, the RRM1 and RRM2 subunits of RNR showed a slight decrease after 24 hours of hypoxic exposure.

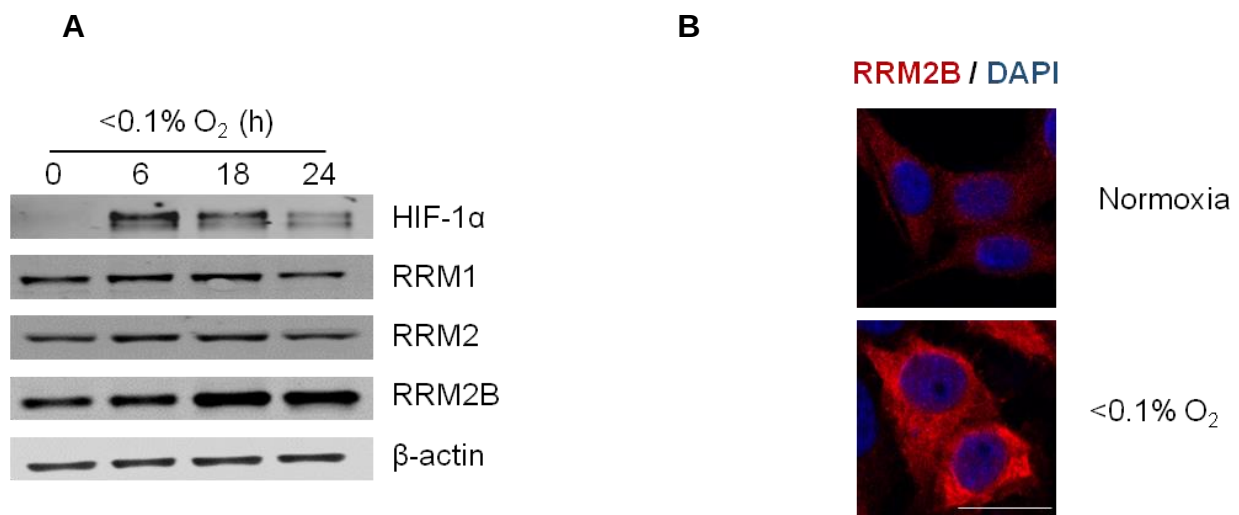


Figure 3.6 The RRM2B subunit of RNR is overexpressed in hypoxia

(A) RKO cells were exposed to <0.1% O₂ for the times indicated. Western blot analysis was carried out with the antibodies indicated; β-actin was used as a loading control. **(B)** RKO cells exposed to normoxia or <0.1% O₂ (18 h). Cells were stained for RRM2B (red) and DAPI (blue) and analysed by immunofluorescence. Scale bar 20 μm.

In order to verify that the induction of RRM2B in response to hypoxia was not cell line dependent, we tested numerous different cell lines including colorectal, osteosarcoma, glioblastoma, oesophageal squamous carcinoma and lung cancer cells. A significant induction of RRM2B protein (2 to 3.6-fold, depending on cell line) in response to hypoxia was observed. In the same hypoxic conditions the RRM2 small subunit decreased over time and the RRM1 remained either unchanged or showed a slight decrease (Figure 3.7).

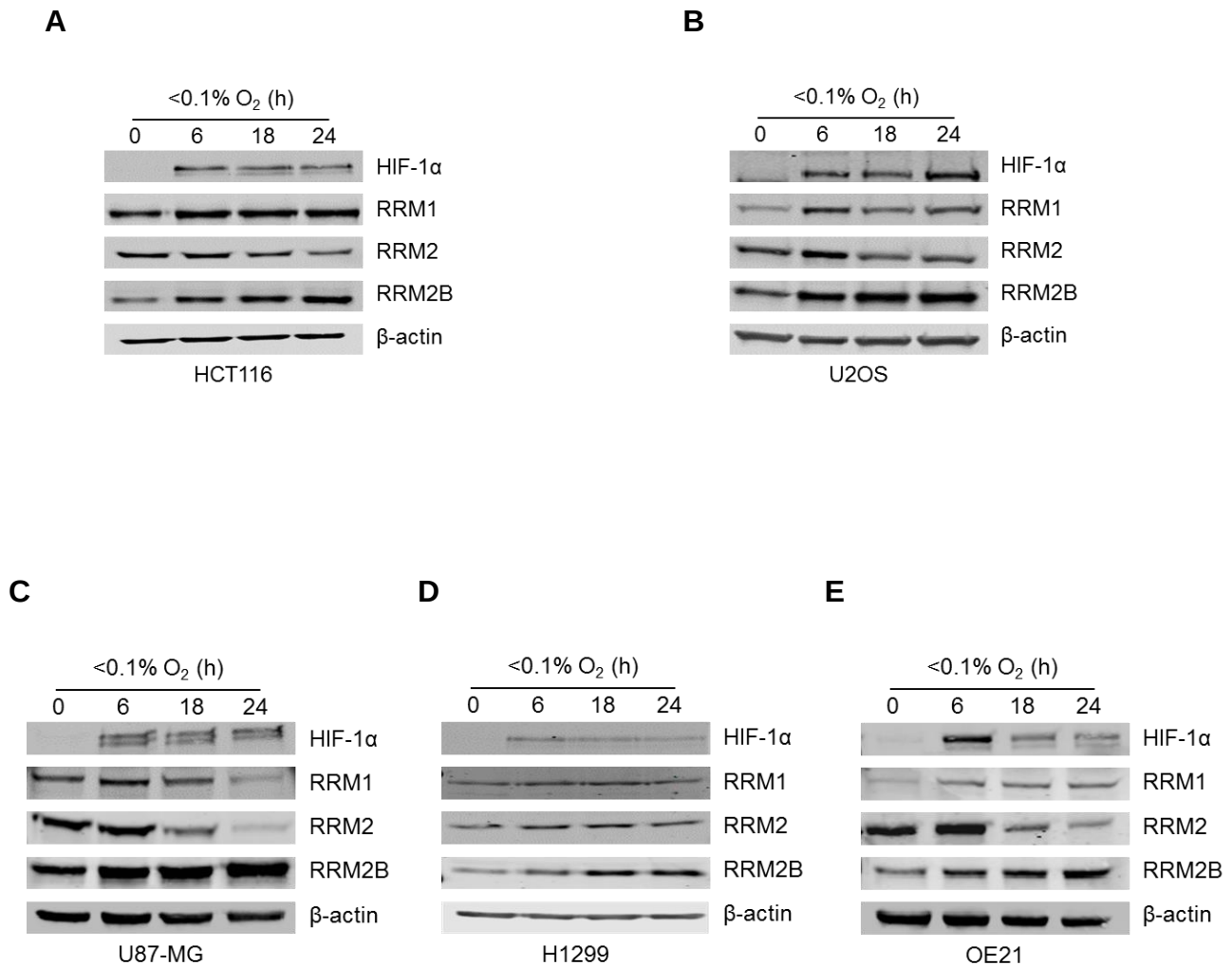


Figure 3.7 The RRM2B subunit of RNR is overexpressed in hypoxia in different cancer cell lines

Cells were exposed to <0.1% O₂ for the times indicated and western blot analysis was carried out with the antibodies indicated; β-actin was used as a loading control. **(A)** HCT116 colorectal cancer cells; **(B)** U2OS osteosarcoma cancer cells; **(C)** U87-MG glioblastoma cancer cells; **(D)** H1299 lung cancer cells and **(E)** OE21 oesophageal squamous carcinoma cells.

Analogous results were obtained when the mRNA levels of the RNR complex were assayed, with the *RRM2B* mRNA being increased by 4.6-fold in RKO cells (2-times more than the HIF-1 target, *GLUT1*) after 24 hours in hypoxic conditions. In contrast, the *RRM2* and *RRM1* mRNA levels decreased by 12.3 and 2.5-fold respectively (Figure 3.8). We then verified that the mRNA results obtained from RKO cells were not cell line specific as shown in Figure 3.9.

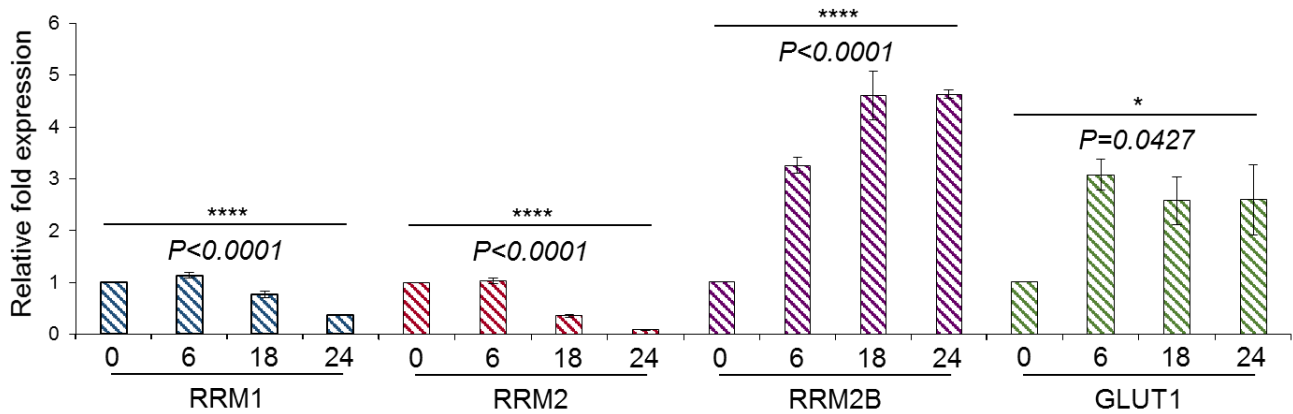


Figure 3.8 *RRM2B* mRNA is induced in response to hypoxia

RKO cells were exposed to $<0.1\%$ O_2 for the times indicated and the mRNA levels of the RNR subunits (*RRM1*, *RRM2*, *RRM2B*) were measured. *GLUT1* mRNA was used as a positive control for hypoxia (Semenza, 2002). All mRNA levels were normalised to *18S* and expressed as fold change. Data show the mean $\pm$ s.e.m. from $n=3$ biological experiments. One-way ANOVA analysis was applied.

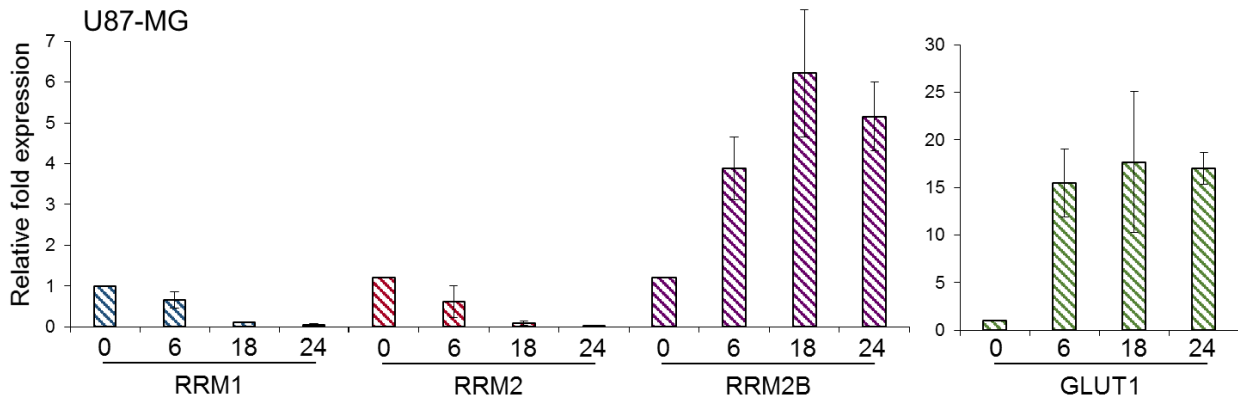
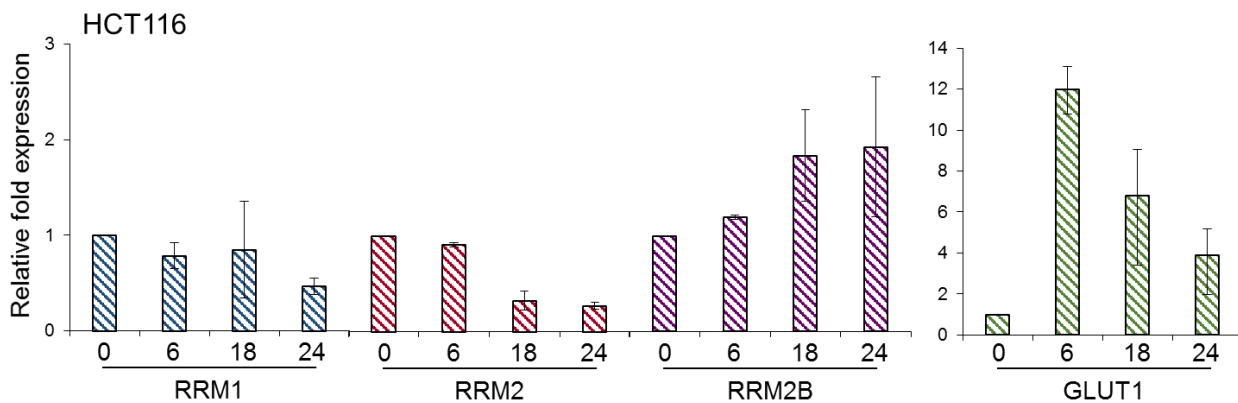
A**B**

Figure 3.9 Different cancer cell lines show increased *RRM2B* mRNA in response to hypoxia

Cells were exposed to $<0.1\%$ O_2 for the times indicated and the mRNA levels of the RNR subunits (*RRM1*, *RRM2*, *RRM2B*) were measured. *GLUT1* mRNA was used as a positive control. All mRNA levels were normalised to *18S* and expressed as fold change. **(A)** U87-MG glioblastoma cancer cells and **(B)** HCT116 colorectal cancer cells. Data show mean $\pm$ s.e.m. from $n=2$ biological experiments.

We then asked if the increased RRM2B expression that we observed *in vitro*, also occurred *in vivo*. In order to investigate this, we used biopsies from glioblastoma patient samples (kindly provided by the Oxford Brain Bank (15/SC/0639)) and performed immunohistochemistry (IHC) on serial sections stained for either RRM2B or carbonic anhydrase IX (CAIX) which defines the hypoxic areas (Semenza, 2002). As shown in Figure 3.10, we observed high RRM2B expression in the hypoxic regions of the glioblastoma patient samples, suggesting that the oxygen-dependent regulation of RRM2B also occurs *in vivo*. Of course, this is preliminary data and more patient samples should be stained, in order to be able to correlate elevated RRM2B levels with the most treatment-resistant tumour regions.

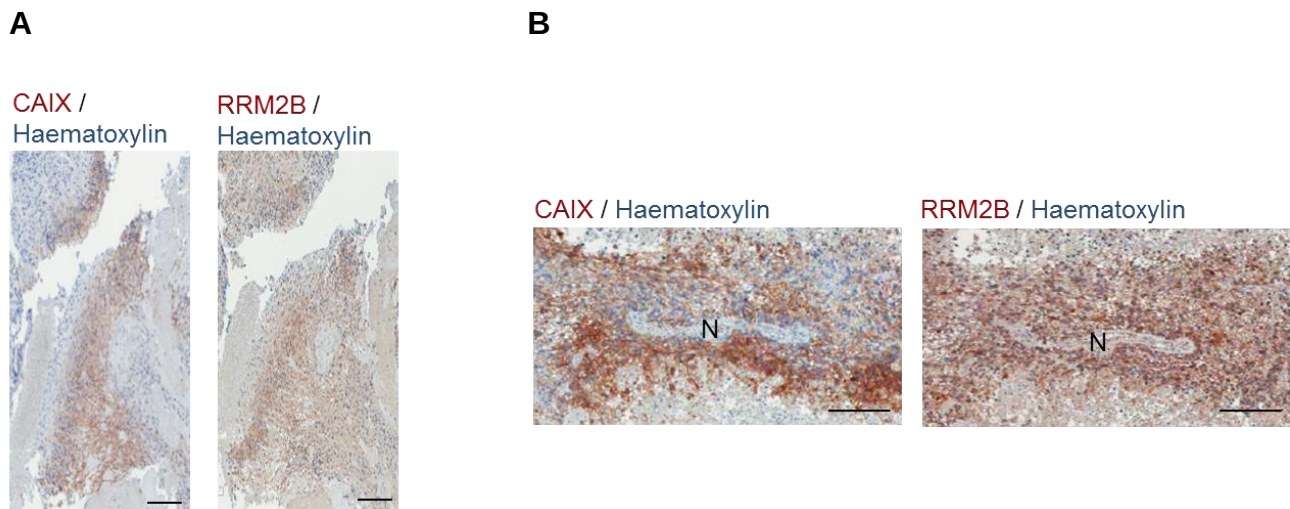


Figure 3.10 Increased RRM2B expression in the hypoxic areas of glioblastoma patient samples

Serial sections of two patient glioblastoma biopsies were analysed by IHC. Biopsies were dewaxed and stained with either CAIX (hypoxic areas) or RRM2B antibodies. **(A)** Patient #1 and **(B)** Patient #2. Scale bar 150 μ m. IHC staining was performed by Dr Katarzyna Leszczynska.

3.2.4 RRM2B is induced in hypoxia in a p53-dependent but HIF1 α -independent manner

Next, we employed different genetic tools in order to determine the molecular mechanism/s leading to RRM2B induction in hypoxia. Since HIF-1 is one of the most important hypoxic-specific transcription factors (Patlar and Harris, 2006), we started by investigating if there was any HIF-1 α dependency to the observed RRM2B induction in hypoxia. RKO^{HIF-1 α +/+} and RKO^{HIF-1 α -/-} cells were exposed to <0.1 % O₂ for the times indicated in Figure 3.11 and both the mRNA (Figure 3.11 A) and protein (Figure 3.11 B) levels were assayed. Interestingly, both the mRNA and the protein levels of RRM2B were induced in response to hypoxia and this was irrespective of the HIF-1 α status (Figure 3.11). Thus, we concluded from this experiment that RRM2B overexpression in hypoxia is HIF-1 α independent.

We then examined the oxygen dependency of RRM2B protein induction. RKO cells were exposed at 2% O₂ for 6, 18, 24 and 48 hours and at <0.1% O₂ for 6, 18 and 24 hours (Figure 3.12). As expected, HIF-1 α stabilisation was observed following the exposure of RKO cells to both of these oxygen tensions, whereas the RRM2B protein was induced only at <0.1% O₂, correlating as such with the p53 stabilisation pattern.

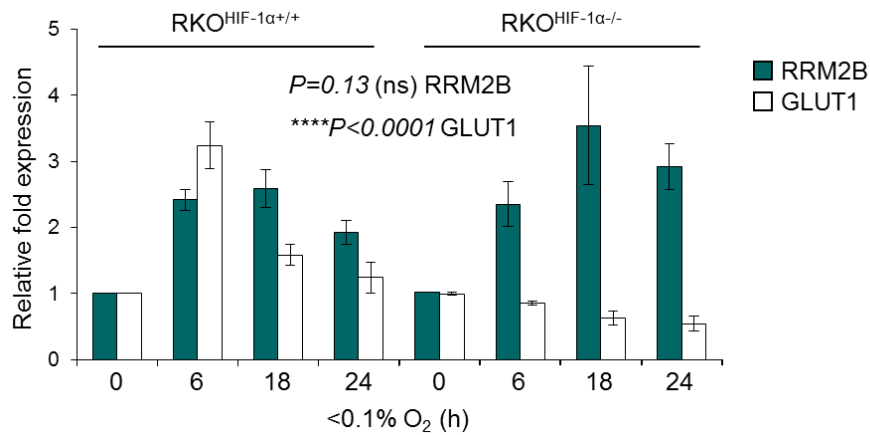
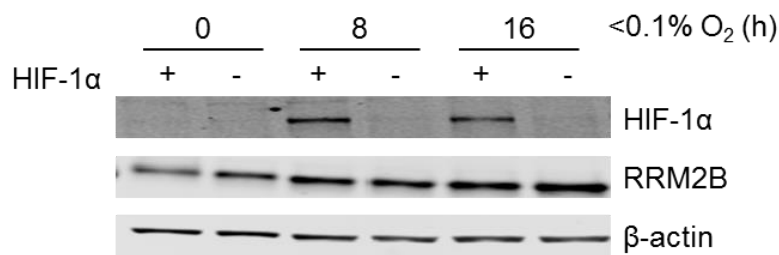
A**B**

Figure 3.11 The Hypoxic-induction of RRM2B is HIF-1α independent

RKO^{HIF-1α+/+} and RKO^{HIF-1α-/-} cells were exposed to <0.1% O₂ for the times indicated. **(A)** *RRM2B* and *GLUT1* mRNA levels were measured. *GLUT1* mRNA was used as a positive control for hypoxia. All mRNA levels were normalised to *18S* and expressed as fold change. Data show mean ± s.e.m. from n=3 biological experiments. Two-way ANOVA analysis was applied to determine HIF-1α dependency. **(B)** Western blot analysis was carried out with the antibodies indicated; β-actin was used as a loading control.

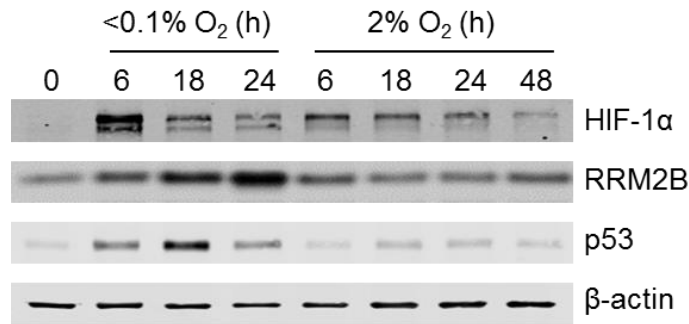


Figure 3.12 RRM2B is induced in an oxygen-dependent manner

RKO cells exposed to <0.1% O₂ and 2% O₂ for the times indicated. Western blot analysis was carried out with the antibodies indicated; β-actin was used as a loading control.

Given that RRM2B is a known p53 target under normoxic conditions (Tanaka et al., 2000), we questioned if p53 leads to RRM2B upregulation also in hypoxia. In order to answer this question, we used three alternative systems. Firstly, we used genetically modified HCT116 colorectal cancer cells that were either p53 deficient or WT p53. HCT116^{p53+/+} or HCT116^{p53-/-} cancer cells were exposed to normoxia and hypoxia and the *RRM2B* mRNA levels were tested. As shown in Figure 3.13 A, a clear p53-dependency for the induction of *RRM2B* mRNA in hypoxia was observed. We then used the H1299 lung cancer cell line, which is p53 deficient, and we overexpressed either the WT *p53* gene (pHRE-p53) or the control gene luciferase (pHRE-LUC). Both of the genes were under the hypoxia-specific HRE promoter that led to the overexpression of each protein only in hypoxia. As seen in Figure 3.13 B, the induction of *RRM2B* mRNA occurred only in the hypoxia-exposed H1299 cells in which p53 was expressed. Finally, we performed siRNA-mediated depletion of p53 in RKO colorectal cancer cells, which have WT p53 status. Again, a clear and statistically significant *RRM2B* mRNA induction was observed in a p53-dependent manner (Figure 3.13 C).

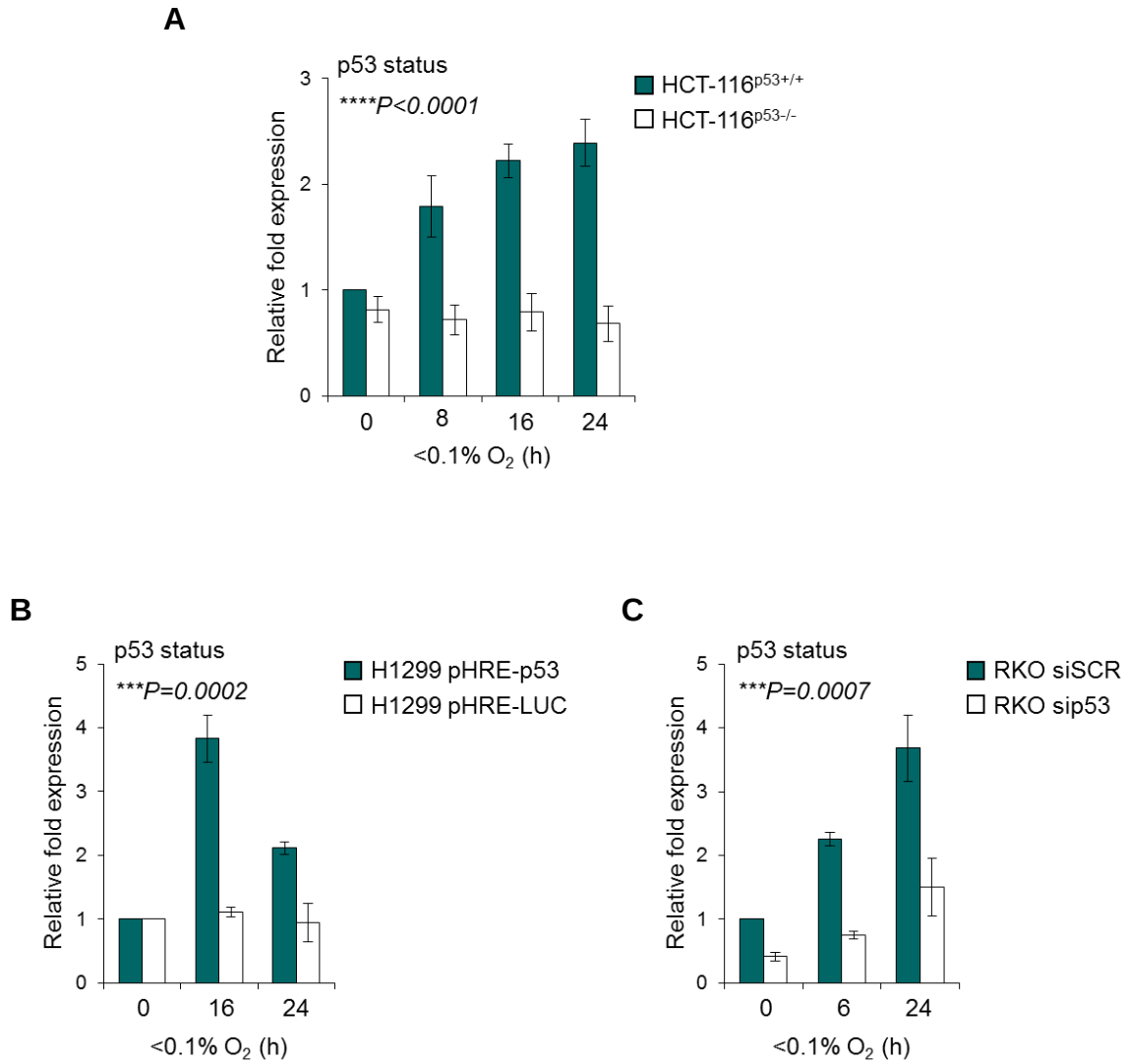


Figure 3.13 Hypoxic-induction of *RRM2B* mRNA is p53 dependent

(A) HCT116^{p53+/+} and HCT116^{p53-/-} cells; (B) H1299 cells transfected with either pHRE-p53 or pHRE-LUC constructs and (C) RKO cells treated with either sip53 or siRNA scramble (siSCR). All cells were exposed to <0.1% O₂ for the times indicated and *RRM2B* mRNA levels were measured. All mRNA levels were normalised to *18S* and expressed as fold change. Data show mean $\pm$ s.e.m. from n=3 biological experiments. Two-way ANOVA analysis was applied to determine p53 dependency.

To rule out the possibility of an indirect induction of *RRM2B* by p53, chromatin immunoprecipitation (ChIP) analysis was carried out. As shown in Figure 3.14 A, a significant enrichment of p53 was observed in response to hypoxia at the *RRM2B* locus whereas this was not the case following adriamycin (ADR) treatment. ADR is a chemical inducer of p53 through the induction of DNA damage (Hyun and Jang, 2015). Interestingly, p53 protein levels were highly induced in response to ADR but this did not lead to increased *RRM2B* protein or accumulation of p53 at the *RRM2B* locus. In contrast, the hypoxic-specific p53 induction led to accumulation of p53 on the *RRM2B* promoter and sequentially to a significant induction of *RRM2B* levels (Figure 3.14 B).

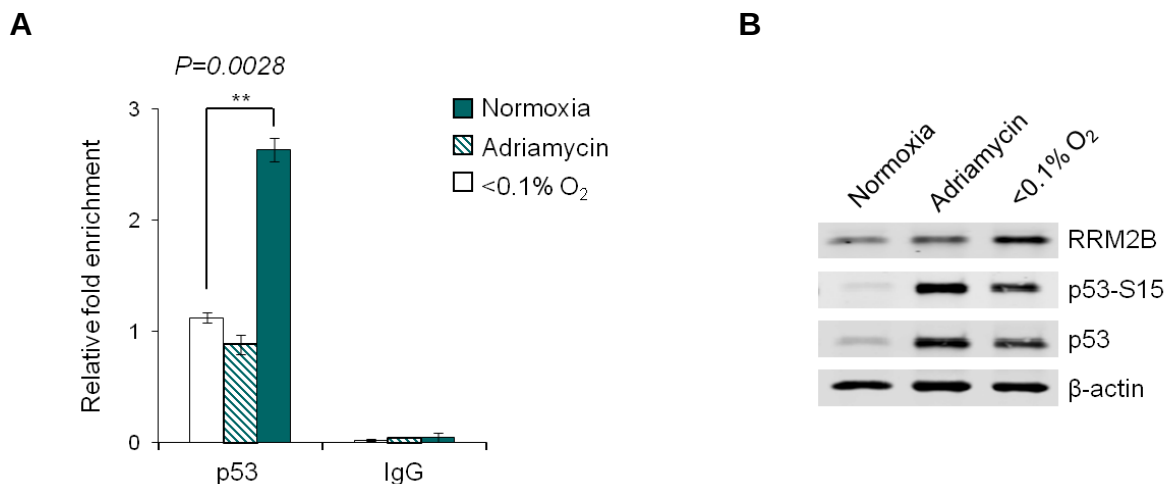


Figure 3.14 p53 directly binds to the *RRM2B* locus

(A) qPCR for p53 ChIP in RKO cells exposed to normoxia or <0.1% O₂ (6 h) or treated with adriamycin (2 μM, 6 h). p53 binding at *RRM2B* locus is expressed as fold change. Data are representative from n=3 biological experiments (graphs show mean of technical triplicates ± RQ_{max}/RQ_{min}). Two-tailed Student's t-test was applied. **(B)** RKO cells were treated as in (A) and western blot analysis was carried out with the antibodies indicated; β-actin was used as a loading control.

Finally, we also tested the protein levels of RRM2B in response to hypoxia in the presence or absence of p53. Specifically, by using the three different systems used previously to test the p53-dependent mRNA induction of *RRM2B* (Figure 3.13), we monitored the RRM2B protein levels following 6, 18 and 24 hours of hypoxic exposure. Interestingly, we observed a mild (1.3 to 1.7-fold depending on cell line) increase in RRM2B levels in <0.1% O₂ also in the absence of p53 (Figure 3.15), suggesting the presence of p53-independent mechanisms of RRM2B stabilisation in these conditions. This result highlights the importance of RRM2B protein in hypoxic conditions.

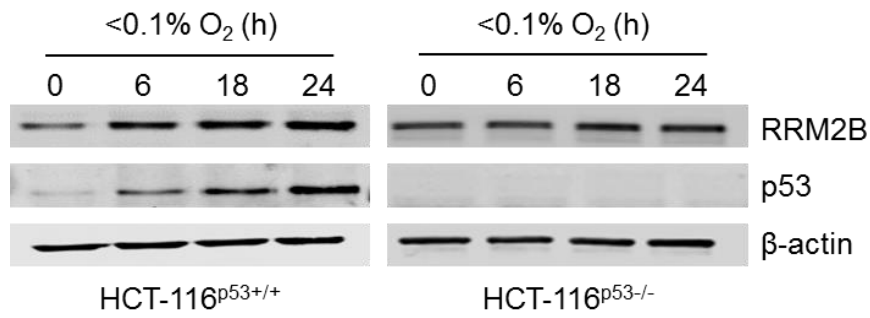
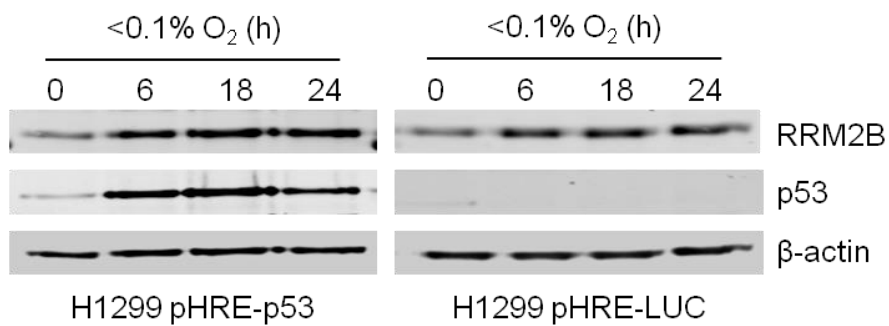
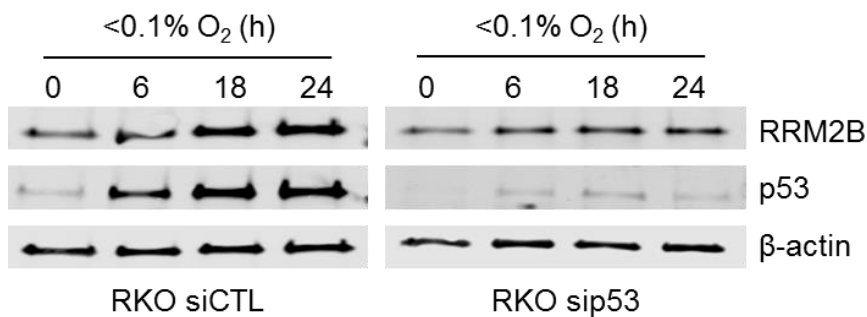
A**B****C**

Figure 3.15 Increased RRM2B protein levels were observed both in presence and absence of p53 in hypoxia

(A) HCT116^{p53+/+} and HCT116^{p53-/-} cells; (B) H1299 cells treated with either pHRE-p53 or pHRE-LUC constructs and (C) RKO cells treated with either sip53 or siSCR. All cells were exposed to <0.1 % O₂ for the times indicated and western blot analysis was carried out with the antibodies indicated; β-actin was used as a loading control.

3.3 Discussion

In this chapter we verified that in response to hypoxia there is RS, which is most likely the initiating signal for the activation of the DDR pathway in these conditions. We demonstrated that although replication rates are compromised in hypoxia, DNA replication proceeds under these conditions. We then measured the dNTP levels in hypoxia and observed clear imbalances in the dNTP pools, which could explain the RS and slow replication rates observed. Notably, dNTP pools are not completely depleted in hypoxia, as had been previously reported by Probst *et al.*, (Probst et al., 1989), leading us to question the source of the nucleotides in these conditions.

Expression analysis of the RNR complex demonstrated that both RRM2B protein and mRNA levels were significantly induced in response to hypoxia in a number of cancer cell lines, whereas the levels of the other β subunit of RNR (RRM2) were decreased over time in hypoxia. Importantly, preliminary IHC experiments showed increased RRM2B expression in the hypoxic regions of biopsies from glioblastoma patient samples, suggesting that the oxygen-dependent regulation of RRM2B occurs also *in vivo*. This result suggest that RRM2B could be a potential marker of the most aggressive and treatment-resistant tumour regions.

Finally, we investigated the molecular pathways that mediate RRM2B induction in hypoxia and verified that RRM2B is induced in a p53-dependent/HIF-1 α -independent manner. In addition, we showed that p53 binds directly to the response element at the *RRM2B* locus leading to transcriptional overexpression. Interestingly, a mild increase in RRM2B protein levels in p53-deficient cell lines was also observed, suggesting the presence of p53-independent mechanisms of RRM2B stabilisation and therefore highlighting the importance of RRM2B protein in hypoxia.

CHAPTER 4

Biological significance of RRM2B induction in hypoxia

4.1 Introduction

In contrast to RRM2, which is specifically expressed during the S phase of the cell cycle and is rapidly degraded after S phase completion, RRM2B is constitutively expressed throughout the cell cycle and has been reported to reach its highest levels at G₁/S transition (Chabes et al., 2003b; D'Angiolella et al., 2012; Liu et al., 2005). One of the first roles attributed to RRM2B was to provide cells with dNTPs during DNA repair in response to DNA damage, caused by agents such as UV, IR and adriamycin (Tanaka et al., 2000). In the absence of DNA damage, basal levels of RRM2B are observed in quiescent and non-proliferating cells, highlighting its central role in mtDNA synthesis (Hakansson et al., 2006; Pontarin et al., 2007). Inhibition of endogenous RRM2B with antisense oligonucleotides, attenuates DNA repair and inhibits cell survival after exposure to genotoxic agents, thus it has been suggested that RRM2B inhibition could improve response to chemotherapy (Devlin et al., 2008). In addition, siRNA-dependent RRM2B depletion has been shown to radiosensitise oesophageal squamous cell carcinoma (ESCC) cell lines, hence potentially improving radiotherapy (Yokomakura et al., 2007).

The induction of RRM2B in response to the physiological stress of hypoxia, shown in the Chapter 3, is a novel observation. The finding that the hypoxic induction of RRM2B was observed in numerous different cell lines, led us to address the role of RRM2B in hypoxia. Therefore, we continued our study by investigating the cellular responses of RRM2B depleted cells in both normoxia and hypoxia.

4.2 Results

4.2.1 RRM2B binds to RRM1 in hypoxia to form a functional RNR holoenzyme

In order to investigate the biological significance of hypoxia-induced RRM2B, we first verified that it forms a complex with the RRM1 subunit to reconstitute the RRM1/RRM2B (R1/R2B) holoenzyme in these conditions. RKO cells were exposed to either normoxia or hypoxia and immunoprecipitation assays were performed as described in Chapter 2.3.3. Specifically, we used the RRM1 antibody to immunoprecipitate the RRM1 subunit of RNR from whole cell extract, and by using western blot analysis we determined if the RRM2 and/or RRM2B protein were bound to RRM1 in the conditions examined. As shown in Figure 4.1, we observed increased RRM2B and decreased RRM2 levels bound to RRM1 in response to hypoxia. We then performed a time course in hypoxia to monitor the RRM2B levels bound to RRM1 (Figure 4.2), and observed a 4.7-fold increase in RRM2B protein bound to RRM1 after 18 hours of hypoxia compared to normoxia.

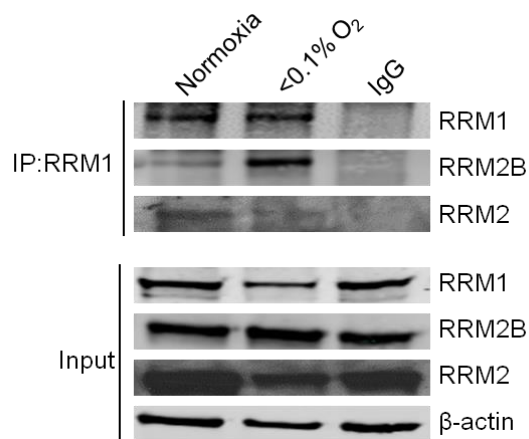


Figure 4.1 RNR switches subunits in response to hypoxia

RKO cells exposed to normoxia or <0.1% O₂ (18 h) and immunoprecipitation of the RRM1 protein was carried out. Western blot analysis using the antibodies indicated; β -actin was used as a loading control.

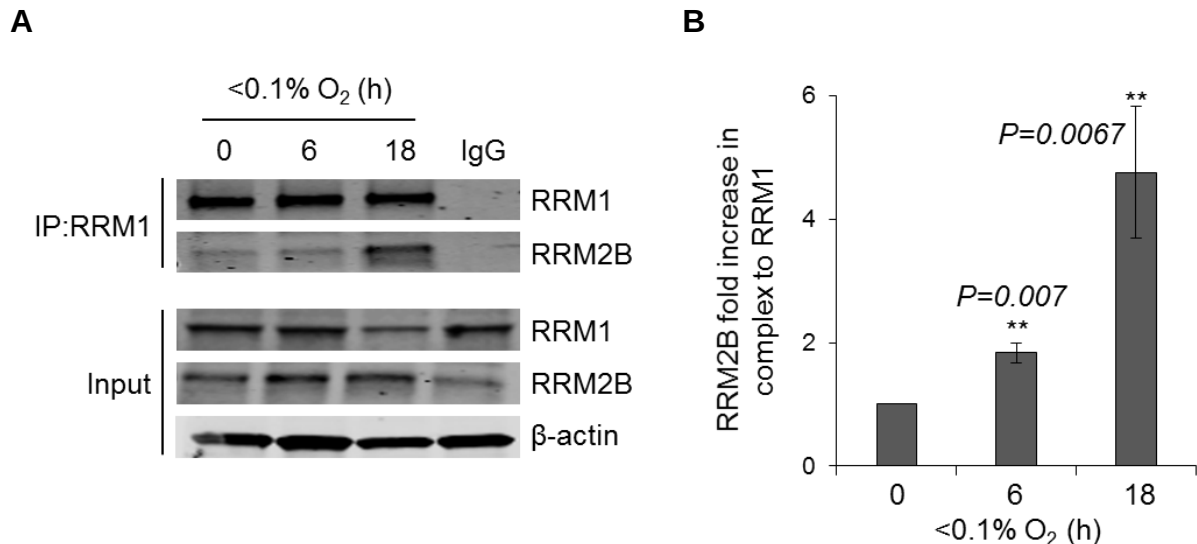


Figure 4.2 Increased RRM2B levels bind to RRM1 in hypoxia compared to normoxia
(A) RKO cells exposed to normoxia or <0.1% O₂ for the times indicated and immunoprecipitation of the RRM1 protein was carried out. Western blot analysis using the antibodies indicated; β-actin was used as a loading control. **(B)** Quantification of (A). Data show mean ± s.e.m. from n=3 biological experiments. Two-tailed, unpaired Student's t-test was applied comparing normoxic to hypoxic samples.

Since RRM1 and RRM2B interact to reconstitute the RNR holoenzyme in hypoxia, we then examined if the hypoxia-formed R1/R2B enzyme was also functional. In order to investigate this, we employed a specific siRNA to effectively deplete RRM2B expression. RKO cells were treated either with the siRRM2B or with the siRNA-scrambled sequence (siSCR), which was used to monitor possible non-specific off target effects. An efficient RRM2B depletion was observed (Figure 4.3). Then, we exposed the treated RKO cells to either normoxia or hypoxia (16 hours) and dNTP incorporation assays were carried out to investigate if disruption of the R1/R2B enzyme affects intracellular nucleotide levels. Interestingly, siRNA-mediated loss of RRM2B led to lower dNTP levels only in hypoxia and not in normoxia (Figure 4.4). Specifically, loss of RRM2B did not show a major effect on the dNTP pools in normoxic conditions compared to the negative control (Figure 4.4 A), whereas when cells were exposed to hypoxia, we observed 50-55% less pyrimidines (dCTPs and dTTPs) and 25-30% less purines (dATPs and dGTPs) compared to the control (Figure 4.4 B).

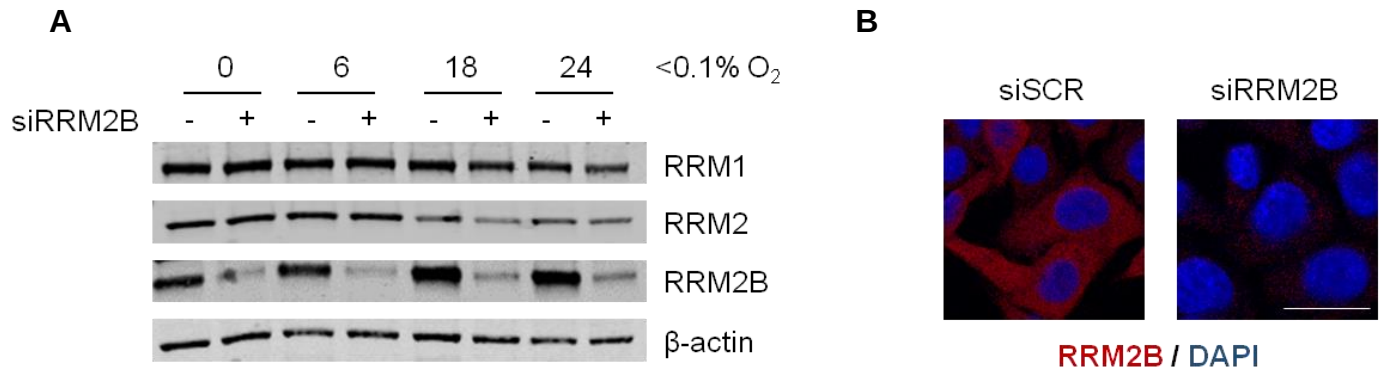


Figure 4.3 Effective depletion of RRM2B protein after use of siRRM2B

(A) RKO cells were treated with siRRM2B or siSCR and exposed to normoxia or <0.1% O₂ for the times indicated. Western blot analysis followed with the antibodies indicated; β-actin was used as a loading control. **(B)** RKO cells treated as in (A) and exposed to <0.1% O₂ (18 h). Cells were stained for RRM2B (red) and DAPI (blue) and analysed by fluorescence microscopy. Scale bar 20 μm.

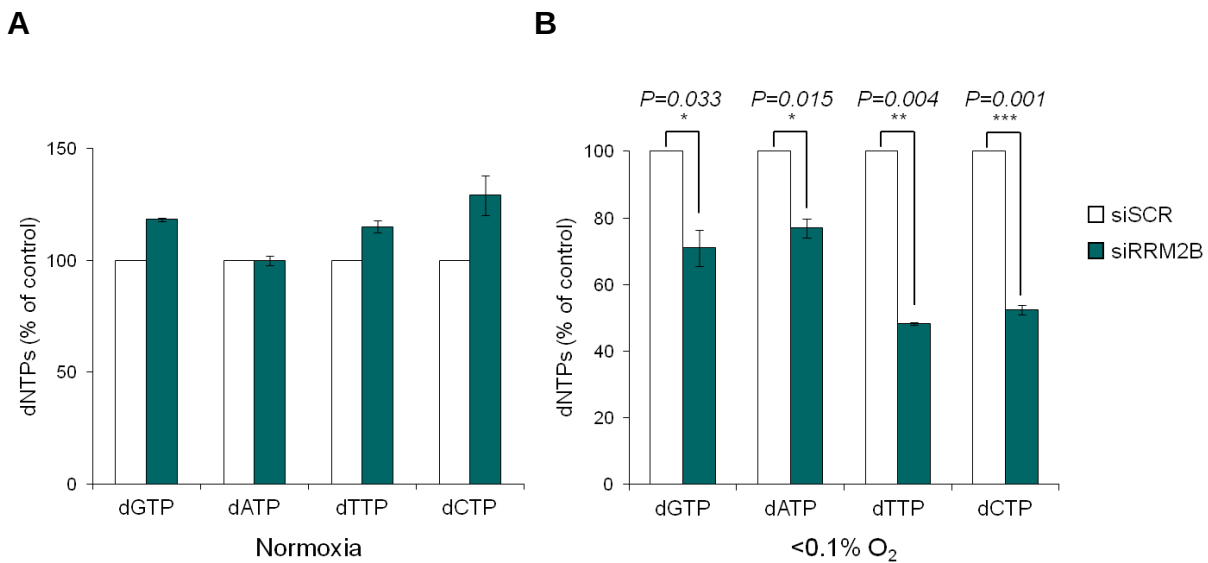


Figure 4.4 Loss of RRM2B reduces nucleotide levels specifically in hypoxia

(A) Quantification of dNTP levels in RKO cells treated with siRRM2B or siSCR and exposed to normoxia. Data are presented as percentage of the control (siSCR). **(B)** Quantification of dNTP levels in RKO cells treated as in (A) and exposed to <0.1% O₂ (16 h). Data were firstly normalised to the levels of dNTPs in normoxia of each group (siRRM2B or siSCR) and then compared and presented as percentage of the control (siSCR) in <0.1% O₂. Data show mean ± s.e.m. from n=3 biological experiments. Two-tailed, unpaired Student's t-test was applied.

4.2.2 Depletion of RRM2B disrupts DNA replication in hypoxia

Intrigued by the disruption of the dNTP pools after depletion of RRM2B in hypoxia (Figure 4.4), we asked if the loss of RRM2B would also affect DNA replication in these conditions. To test this, we firstly employed U2OS cells and treated them with either siRRM2B or siSCR followed by exposure to normoxia or hypoxia (3 hours). The cells were pulsed with BrdU for 30 minutes before collection, and FACS analysis was performed to examine if depletion of RRM2B will affect the amount of BrdU incorporated. As shown in Figure 4.5, S phase cells lacking RRM2B incorporated 37.5% less BrdU than the control treated cells in hypoxia, whereas loss of RRM2B in normoxia did not alter the BrdU incorporation levels.

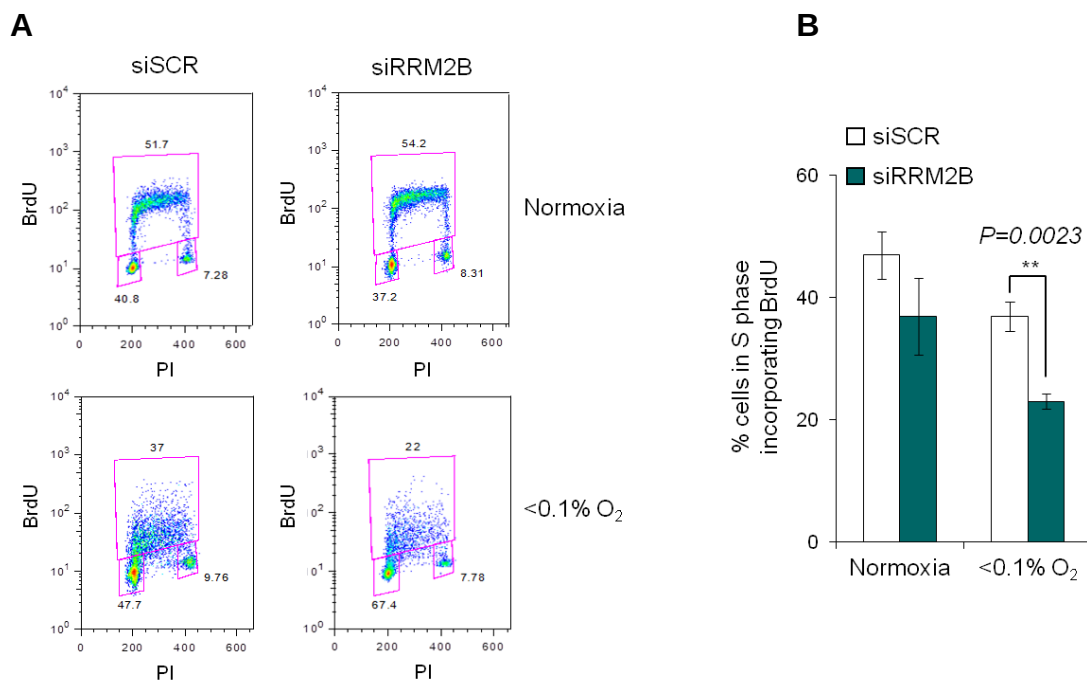


Figure 4.5 Depletion of RRM2B leads to less BrdU incorporation in hypoxia

(A) U2OS cells treated with either siRRM2B or siSCR, were exposed to normoxia or <0.1% O₂ (3 h). Cells were pulsed with BrdU (20 μ M, 30 min) before collection, stained for BrdU and PI and analysed by FACS. **(B)** Quantification of percentage of U2OS cells that incorporate BrdU while in S phase, after exposure to normoxia or <0.1% O₂ (3 h). Cells were treated and analysed as in (A). Data show mean $\pm$ s.e.m. from n=3 biological experiments. Two-tailed, paired Student's t-test was applied.

To further investigate the hypoxic role of RRM2B, we constructed a RRM2B knockout cell line (RKO^{RRM2B-/-}) using the CRISPR-Cas9 technology (Chapter 2.4.5 and Appendix I). As shown in Figure 4.6, targeting all three of the RRM2B isoforms in RKO cells created a complete knock out cell line. Importantly, the integrity of the other RNR subunits was not affected by the gene editing at *RRM2B*.

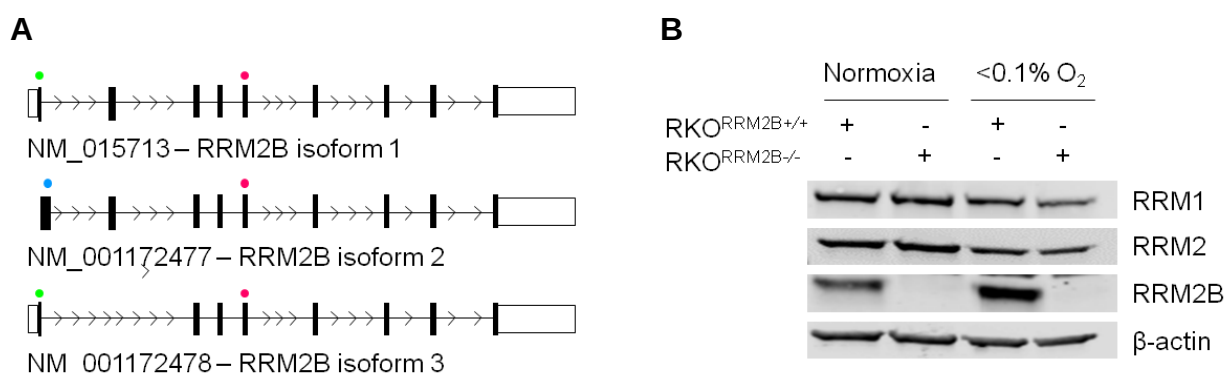


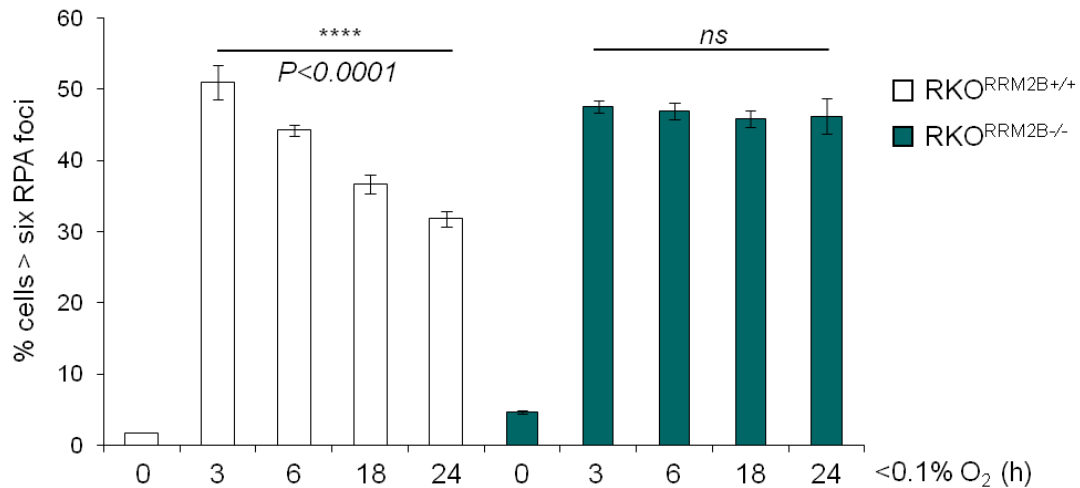
Figure 4.6 Construction of CRISPR-Cas9 RKO^{RRM2B-/-} cell line

(A) Schematic representation of the three isoforms of the *RRM2B* gene. Black rectangles represent exons. The coloured circles represent the positions targeted by the guided RNAs (sgRNA) in order to construct the RKO^{RRM2B-/-} cell line (CRISPR-Cas9 system). **(B)** Western blot analysis of RKO^{RRM2B+/+} and RKO^{RRM2B-/-} cell lines exposed to normoxia or <0.1% O₂ (18 h) with the antibodies indicated; β-actin was used as a loading control. The sgRNAs used to target the *RRM2B* did not affect the levels of either *RRM1* or *RRM2* genes.

Then, by using both knock-out and siRNA-treated RKO cells, we investigated the effect of RRM2B depletion on DNA replication. As previously discussed in Chapter 3.2.1, hypoxia leads to a rapid accumulation of ssDNA detectable by the presence of RPA foci, which decrease after prolonged exposure to <0.1% O₂ (Figure 3.3). Immunofluorescence assays, using the RPA32 antibody, were performed in order to monitor the levels of ssDNA over time in hypoxia in cells lacking RRM2B (Figure 4.7 and Appendix II-A.1). Importantly, while the control cells showed that in hypoxia there was a decrease in ssDNA after prolonged exposure to these conditions as expected, loss of RRM2B resulted in persistent RPA foci

even after 24 hours of hypoxic exposure. This finding suggests that the lack of RRM2B in hypoxia results in an accumulation of ssDNA and failure to complete DNA replication in these conditions.

A



B

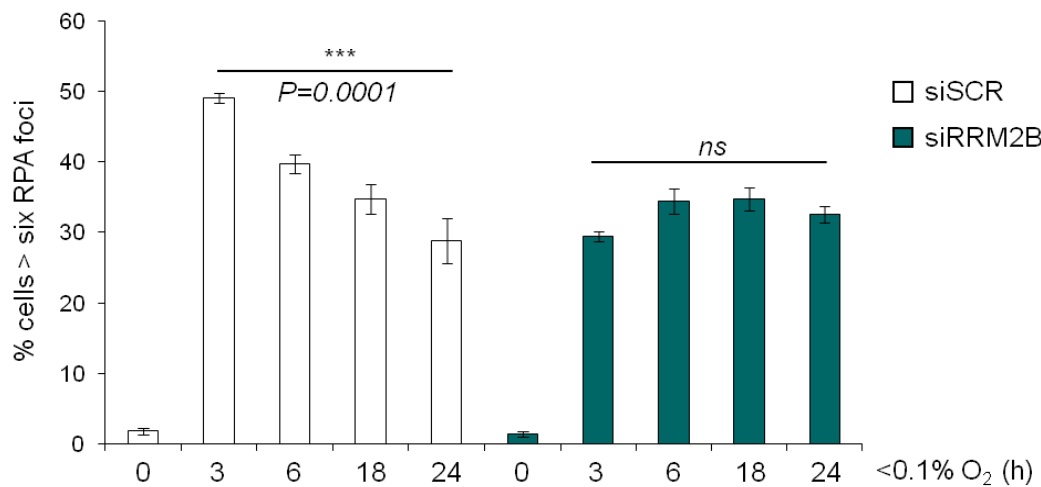


Figure 4.7 Cells with decreased RRM2B levels show persistent formation of RPA foci
(A) RKO^{RRM2B+/+} and RKO^{RRM2B-/-} cells were exposed to <0.1% O₂ for the times indicated, followed by IF staining for RPA32 foci, indicating ssDNA. **(B)** RKO cells were treated with either siRRM2B or siSCR and exposed to <0.1% O₂ for the times indicated. IF staining and quantification of RPA32 foci followed. For both (A) and (B), induction of ssDNA was quantified by counting cells with more than six RPA32 foci. Data show mean $\pm$ s.e.m. from $n=3$ biological experiments. One-way ANOVA analysis was applied.

4.2.3 Depletion of RRM2B leads to DNA damage, loss of viability and delayed tumour growth

Since DNA replication seems to be impaired in hypoxia in the absence of RRM2B, we continued by asking whether the loss of RRM2B would also affect DNA integrity and as a result overall survival. Firstly, we examined the consequences of RRM2B depletion on the accumulation of DNA damage. RKO cells with reduced levels of RRM2B (treated with either siRRM2B or siSCR), which were exposed to normoxia or $<0.1\%$ O_2 (6 hours), showed increased levels of phosphorylated 53BP1 indicating DNA damage specifically in hypoxia (Figure 4.8 A). In addition, when we performed IF to detect DNA damage, we determined that cells where the levels of RRM2B had not been effectively depleted did not accumulate 53BP1 foci after 6 hours of $<0.1\%$ O_2 , whereas cells with decreased RRM2B expression showed accumulation of 53BP1 foci indicating the presence of DSBs (Figure 4.8 B). Quantification of IF studies performed in both RKO siRRM2B treated and in our RRM2B knock out cell line demonstrated that the accumulation of DSBs was specific for the RRM2B depleted cells, which were exposed to hypoxia (Figure 4.9 and Appendix II-B.1). In contrast, no evidence of elevated DNA damage was observed in control cells and in cells lacking RRM2B in normoxia, something that was attributed to the RRM2-dependent supply of dNTPs when oxygen is abundant.

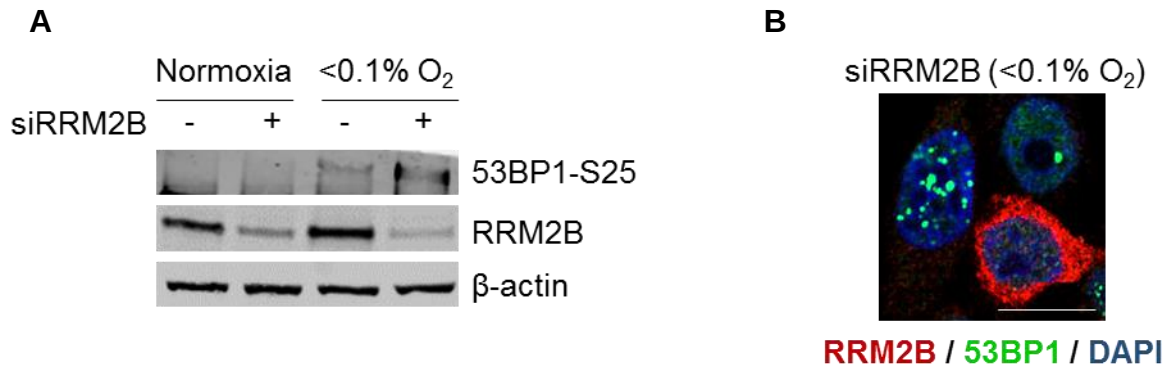


Figure 4.8 Loss of RRM2B leads to DNA damage in hypoxia

(A) RKO cells were treated with either siRRM2B or siSCR and exposed to normoxia or <0.1% O₂ (6 h). Western blot analysis followed with the antibodies indicated; β-actin was used as a loading control. (B) RKO cells treated as in (A) and IF staining followed with the antibodies indicated. Scale bar 20 μm.

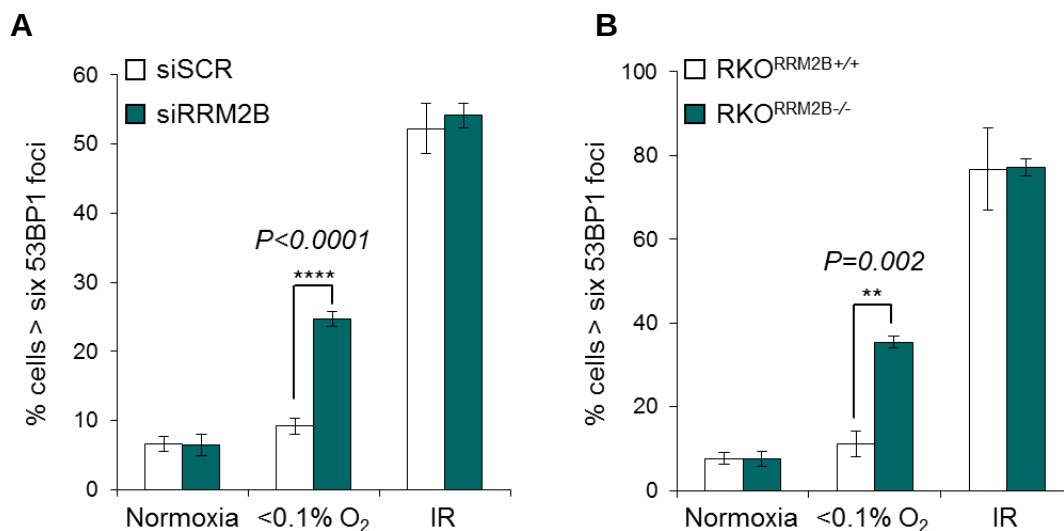


Figure 4.9 Cells with depleted RRM2B levels show elevated DSBs specifically in hypoxia

(A) RKO cells were treated with either siRRM2B or siSCR and exposed to normoxia, <0.1% O₂ (6 h) or to IR (5 Gy). IF staining was carried out and quantification of the percentage of cells with 53BP1 foci followed. IR was used as a positive control. (B) RKO^{RRM2B+/+} and RKO^{RRM2B-/-} cells were exposed to normoxia <0.1% O₂ (6 h) or to IR (5 Gy). IF staining was carried out and quantification of the percentage of cells with 53BP1 foci followed. For both (A) and (B), induction of DSBs was quantified by counting cells with more than six 53BP1 foci. Data show mean ± s.e.m. from n=3 biological experiments. Two-tailed, paired Student's t-test was applied.

The increased levels of DSBs, which were observed in hypoxic cells with depleted RRM2B, also correlated with decreased cell survival in these conditions. In detail, we used RKO cells treated with either siRRM2B or siSCR, and exposed them to different time points in hypoxia in order to perform a colony survival assay. As shown in Figure 4.10, we observed a significant loss of viability, with 77% less cells able to survive after 24 hours in hypoxia when RRM2B was silenced in comparison to siSCR. We also investigated if the loss of viability observed, correlated with the induction of apoptosis in RRM2B defective hypoxic cells. Indeed, siRRM2B treated RKO cells showed induced levels of apoptosis as determined both morphologically and by induction of PARP cleavage (Figure 4.11).

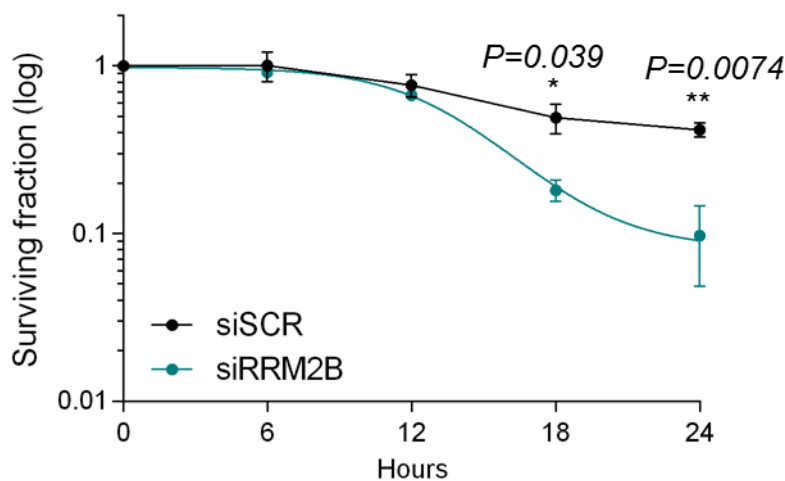


Figure 4.10 Depletion of RRM2B leads to loss of viability in hypoxia

RKO cells treated with siRRM2B or siSCR and exposed to $<0.1\%$ O_2 for the times indicated. After the appropriate time in the hypoxic chamber, all cells were placed in normoxic incubator and left for nine days to form colonies. Colonies were then visualised by crystal violet staining. Graphs show mean $\pm$ s.e.m. from $n=3$ biological experiments. Two-tailed, paired Student's t-test was applied.

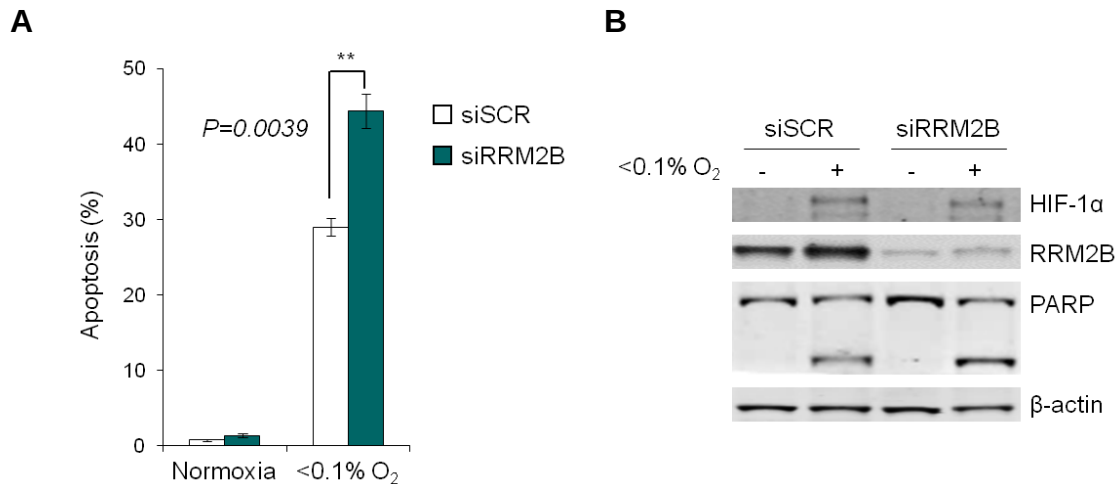


Figure 4.11 Apoptosis is increased in hypoxia in the absence of RRM2B

(A) Quantification of apoptotic RKO cells treated with siRRM2B or siSCR and exposed to normoxia or <0.1% O₂ (19 h). Apoptosis was detected morphologically with DAPI staining. Data show mean $\pm$ s.e.m. from n=3 biological experiments. Two-tailed, paired Student's t-test was applied. **(B)** Cells were treated as in (A) and western blot analysis was carried out with the antibodies indicated; β -actin was used as a loading control. PARP cleavage indicates induction of apoptosis.

Finally, we used RKO^{RRM2B+/+} and RKO^{RRM2B-/-} cells to perform *in vivo* studies. Specifically, nude mice were injected subcutaneously with RKO^{RRM2B-/-} and RKO^{RRM2B+/+} cells as described in Chapter 2.5.2 and tumour growth was monitored regularly. The study was repeated twice (Figure 4.12), and during the second experiment irradiation treatment was added (10 Gy) when tumour volumes reached 100 mm³. The animals were sacrificed when tumours reached approximately 300-400 mm³. As shown in Figure 4.12, the tumour xenografts showed delayed tumour growth when RRM2B was lacking, as well as an increase in radiosensitivity. Additionally, we performed IF staining on sections from the xenograft tumours, collected at the end of the first experiment (Figure 4.12 A). As illustrated in Figure 4.13, we observed a significant increase in apoptosis (monitored by cleaved Caspase 3) specifically in the hypoxic regions of the RRM2B^{-/-} tumours (hypoxic regions were determined by PIMO staining). Notably, we did not observe a difference in apoptosis in the non-hypoxic areas between the RRM2B^{+/+} and RRM2B^{-/-} tumours (Figure 4.13 B,C).

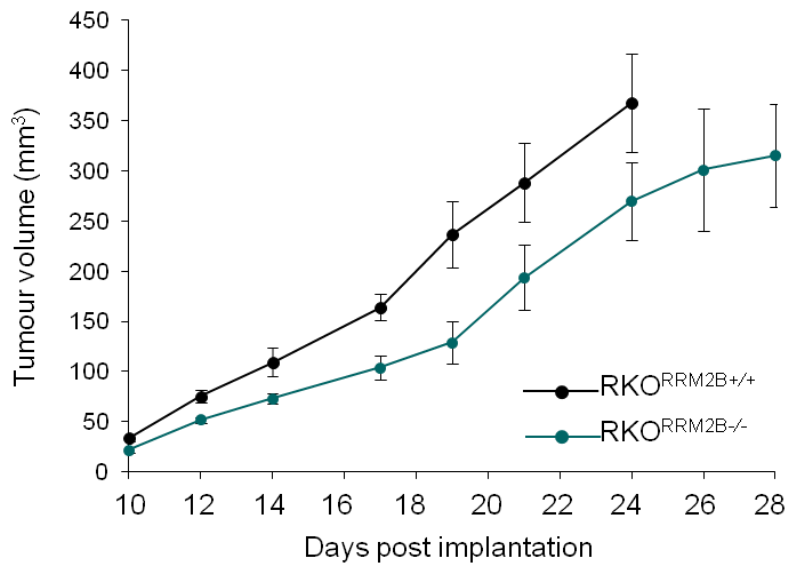
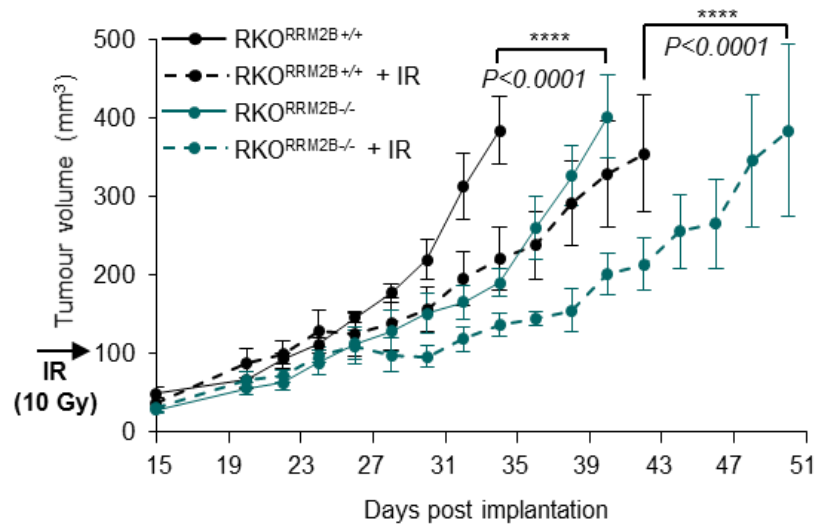
A**B**

Figure 4.12 Depletion of RRM2B results in smaller and more radiosensitive tumours

(A) RKO^{RRM2B+/+} and RKO^{RRM2B-/-} xenografts were grown in mice (n=5 for RKO^{RRM2B+/+} and n=6 for RKO^{RRM2B-/-}) and tumour volumes were measured on the days indicated. (B) RKO^{RRM2B+/+} and RKO^{RRM2B-/-} xenografts were grown in mice (n=4 per group) and tumour volumes were measured on the days indicated. Where indicated, irradiation (10 Gy) was given when tumours reached approximately 100 mm³. For both experiments, the tumour growth was monitored until they reached approximately 300-400 mm³. Data show mean $\pm$ s.e.m. For (A) two-tailed Student's t-test was applied and for (B) two-way ANOVA analysis was applied. Both experiments were performed by Dr Katarzyna Leszczynska.

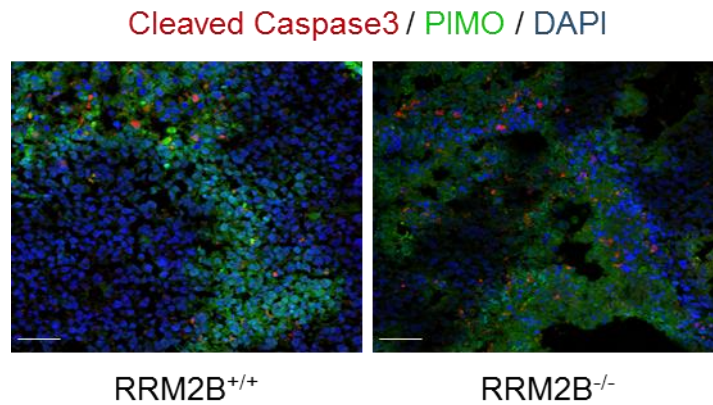
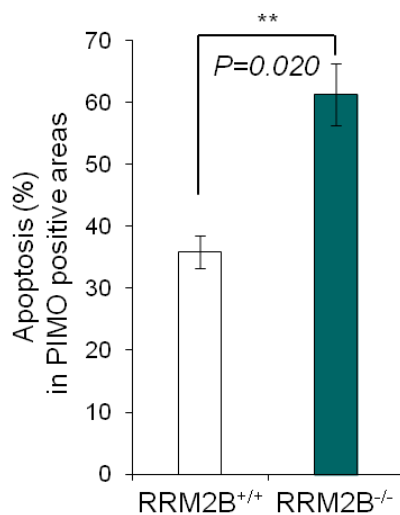
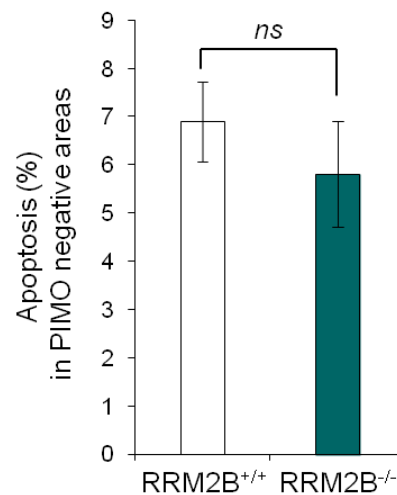
A**B****C**

Figure 4.13 Increased levels of apoptosis in the hypoxic areas of RKO^{RRM2B-/-} tumours
 RKO^{RRM2B+/+} and RKO^{RRM2B-/-} tumours were collected at the end of the xenograft experiment (Figure 4.12 A) and IF performed with the antibodies indicated. **(A)** Representative images of co-localisation of cleaved Caspase 3 (indicating apoptosis) with PIMO staining (indicating hypoxic areas) in RKO^{RRM2B+/+} or RKO^{RRM2B-/-} xenografts. Scale bars 50 μ m. **(B)** Quantification of the percentage of apoptosis within the hypoxic areas of the tumours (PIMO positive). **(C)** Quantification of the percentage of apoptosis within the non-hypoxic areas of the tumours (PIMO negative). Images from three different tumours (n=3) per group were counted. Data show mean $\pm$ s.e.m. Two-tailed, paired Student's t-test was applied.

4.3 Discussion

In this chapter, we studied the biological significance of RRM2B induction in hypoxia. Firstly, we verified that RRM2B is bound to the large subunit of RNR (RRM1) in order to reconstitute the full holoenzyme in hypoxic conditions. Importantly, we observed increased levels (4.7 fold) of RRM2B attached to RRM1 in response to hypoxia. We then investigated the cellular consequences of depleted levels of RRM2B in both normoxia and hypoxia. To address this, we employed two different tools to efficiently decrease or deplete RRM2B expression: siRNA treatment and CRISPR gene editing. Deletion of RRM2B led to reduced levels of dNTPs specifically in hypoxia with the pyrimidines (dCTPs and dTTPs) reduced to 50-55% and the purines (dATPs and dGTPs) to 25-30%. Our hypothesis is that purines were less affected (25-30% *versus* 50-55% for pyrimidines) because in hypoxia we generally saw low levels of purines (Figure 3.5), thus detection of a further decrease might be difficult due to technical limitations.

The disruption of dNTP pools in hypoxia had detrimental consequences for the cancer cells tested. Specifically, we observed that cells lacking RRM2B were unable to continue and complete DNA replication, as shown by both less BrdU incorporation and by maintained RPA foci after long exposures to hypoxia (indicating persistence of ssDNA). Most importantly, we found that the absence of RRM2B resulted in DNA damage and loss of viability specifically in hypoxic conditions. Cells lacking RRM2B in hypoxia accumulated 53BP1 foci, indicating DSBs, most probably due to the collapse of replication forks, due to the abrogation of DNA replication. It is likely, that this induction of DNA damage affected the survival of cells experiencing hypoxia, as we observed increased cell death by both colony survival assays and by induction of apoptosis. Taken together, our results suggest that the basal levels of dNTPs provided by R1/R2B (as opposed to R1/R2) in hypoxia are sufficient for on-going replication, preventing collapse of replication forks, which ultimately

would lead to DSBs, and loss of viability. Another experiment that could strengthened our point would be generation of DNA fibers in hypoxia in cells lacking RRM2B, in order to see if, and how much, DNA replication rates would be affected. Unfortunately, when we tried these experiments, the generated fibers were very malformed, which made the analysis of these data impossible.

Finally, we investigated if deletion of RRM2B had an effect on tumour growth *in vivo*. Notably, by performing xenograft studies using our RRM2B knock out cell line, we observed delayed tumour growth and increased radiosensitivity when RRM2B was missing. In addition, RRM2B deficient tumours showed increased apoptosis in comparison to tumours that were expressing physiological levels of RRM2B and this occurred specifically in the hypoxic areas. Taken together, our results suggest that RRM2B contributes to the increased resistance of the most aggressive fraction of tumours. Therefore, we continued our studies by investigating the hypoxic adaptation of RRM2B, by determining the enzymatic properties of both forms of RNR enzyme (R1/R2 *versus* R1/R2B) in normoxia and hypoxia (Chapter 5).

CHAPTER 5

***In vitro* characterisation of the two forms of RNR enzyme in normoxia and hypoxia**

5.1 Introduction

RRM2 and RRM2B share 83% sequence homology but show distinct expression patterns and function. The formation of the tyrosyl radical occurs *via* the same mechanism in both proteins and two tyrosine residues were shown to be involved in generation of the stable radical (Zhou et al., 2005). Specifically, in human RNRs, the stable radical is initiated at Tyr 176 (Y176) for RRM2 and Y138 in RRM2B (the corresponding residues for Y122 in *E. coli*) with the assistance of the nearby diiron centre (Nordlund and Reichard, 2006). In contrast to *E. coli*, human RNRs require an additional Tyr (Y162 in RRM2; Y124 in RRM2B) for the initiation of the stable radical, and it has been proposed that this mechanism differs from the ‘canonical’ *E. coli* mechanism, since a dityrosyl-diiron system is implicated (Zhou et al., 2005).

Extensive studies on the conserved diiron cluster active site have shown that a highly conserved glutamine (Gln, Q) residue (Q165 in RRM2 and Q127 in RRM2B), found between the diiron centre and Y176-RRM2 or Y138-RRM2B, is essential for the radical formation

probably by stabilising the radical-iron cluster (Zhou et al., 2010). More structural analysis of the diiron centre, revealed that there is a distinct phenylalanine (Phe, F) residue in RRM2B (F183), adjacent to Y124-RRM2B, which in the RRM2 protein is replaced by Y221. This tyrosine-to-phenylalanine switch in RRM2B alters its enzymatic activity and could be responsible for the lower activity of the R1/R2B holoenzyme (Shao et al., 2004; Zhou et al., 2010). Moreover, RRM2B and RRM2 show different physical and biochemical differences in the mobility and accessibility of the diiron centre. It has been proposed that this is due to pronounced structural differences between the two proteins, which mainly involve a N-terminal swivel region found specifically in RRM2B (Smith et al., 2009). Finally, an additional critical function of RRM2B is the antioxidant activity that manifests through scavenging ROS. This is a RRM2B unique function among the rest of the RNR subunits, and mainly involves Y331, Y258, Y49 and Y241 residues (Xue et al., 2006).

In Chapter 4, we verified that loss of RRM2B had detrimental consequences for cell fate in response to hypoxia. These results led us to hypothesise that RRM2B may have a distinct role from RRM2, and specifically that this activity might be prolonged under hypoxic conditions. Therefore, we continued our studies by investigating the potential hypoxic adaptation of RRM2B, through determination of the enzymatic properties of both forms of RNR enzyme (R1/R2 *versus* R1/R2B) in normoxia and hypoxia.

5.2 Results

5.2.1 The R1/R2B form of RNR enzyme retains activity in hypoxia

Firstly, we overexpressed and purified the three recombinant proteins of RNR: RRM1, RRM2 and RRM2B. Specifically, we used pET vectors carrying His-Tag sequences in order to overexpress each protein in *E.coli* (DE3) host cells followed by purification using His-Trap columns (Chapter 2.3.5). After expression and purification, we tested by SDS-PAGE, followed by coomassie blue staining that the purity of each protein was approximately 90% (Figure 5.1 A) and we confirmed each protein preparation by immunoblotting (Figure 5.1 B).

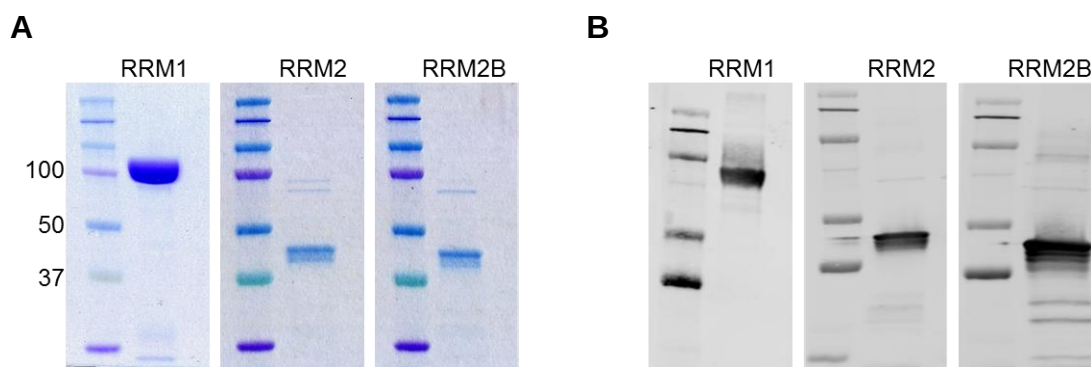


Figure 5.1 Expression and purification of the three recombinant RNR proteins
(A) SDS-PAGE electrophoresis followed by coomassie blue staining of the purified recombinant RNR proteins (RRM1, RRM2 and RRM2B). **(B)** Western blot analysis using the antibodies indicated for confirmation of the protein preparations.

In vitro enzymatic activity assays in normoxia and hypoxia were then carried out as described in Chapter 2.3.7, adapted from (Shao et al., 2004). Reconstitution of the RNR enzyme was achieved by mixing the purified RRM1 subunit with either the RRM2B or the RRM2 subunit in the appropriate buffer. The reaction was started by the addition of the substrate (CDP) and was carried out at 37°C (Figure 5.2). The product formation (dCDP) was quantified at multiple time points as described in Chapter 2.3.8.

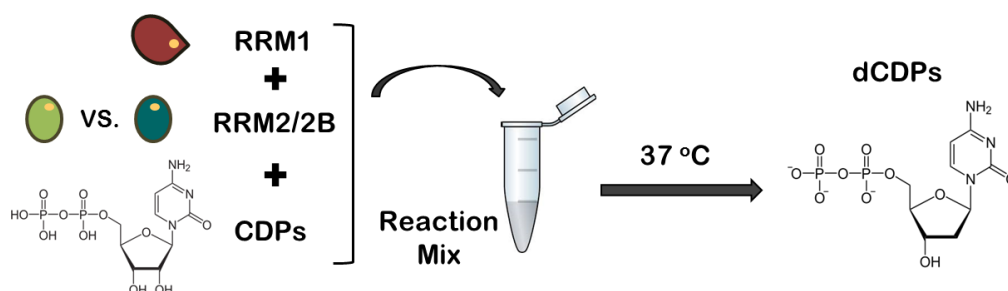


Figure 5.2 *In vitro* enzymatic activity assay of the RNR enzyme

Schematic representation of the *in vitro* RNR activity assay. The purified RRM1 subunit (red) was mixed either with the purified RRM2 (green) or with the RRM2B (blue) in the appropriate buffer, and the reaction started by the addition of the substrate (CDPs). After incubation at 37°C for the desired times, the samples were analysed to determine product formation (dCDPs).

Before examining the effect of oxygen on the activity of the two forms of the RNR, we confirmed that each of the proteins alone (either RRM1, RRM2 or RRM2B) was unable to convert any substrate to product (Figure 5.3). This verified that full reconstitution of the enzyme was needed in order for CDP reduction to take place. We then confirmed that in normoxic conditions the activity of the RRM2B-containing RNR was approximately 20-55% lower than that of RRM2-containing RNR (Figure 5.4), as has been previously described (Shao et al., 2004).

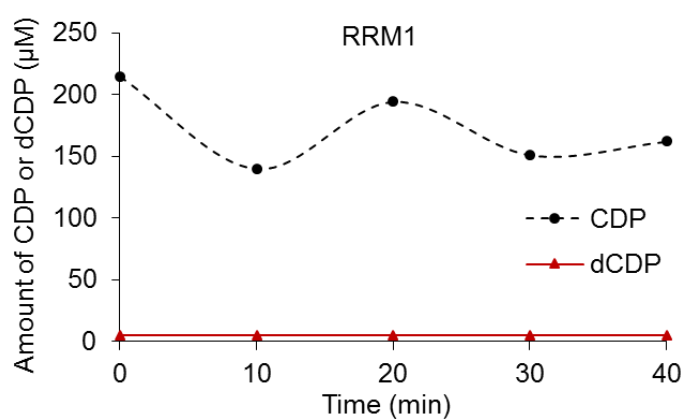
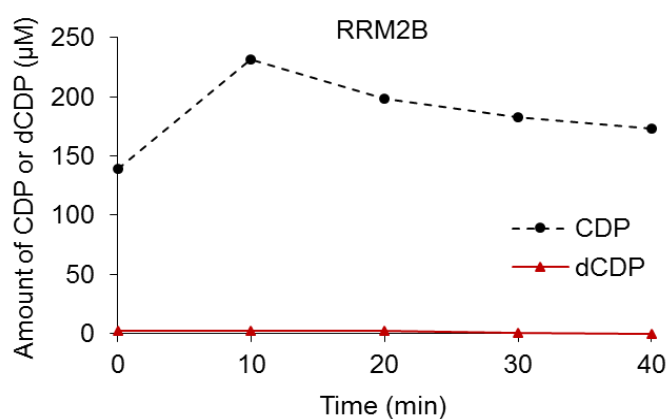
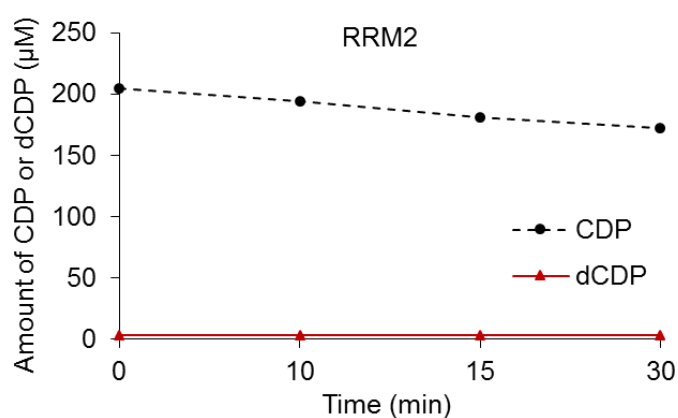
A**B****C**

Figure 5.3 No product formation is observed when only one of the RNR proteins is present in the *in vitro* enzymatic activity assay

5 μ M of the purified recombinant proteins **(A)** RRM1, **(B)** RRM2B and **(C)** RRM2 was mixed with the appropriate buffer and 250 μ M of CDP. The reaction mixture was incubated at 37°C in normoxia for the times indicated and the amount of the remaining substrate (CDP) or the product (dCDP) formed was examined (n=1).

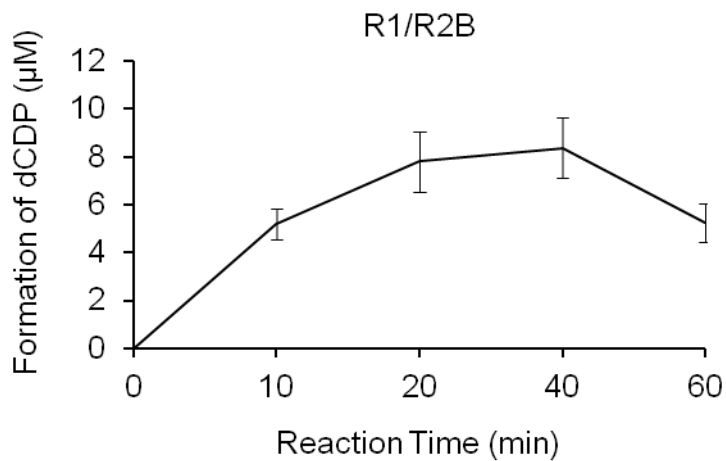
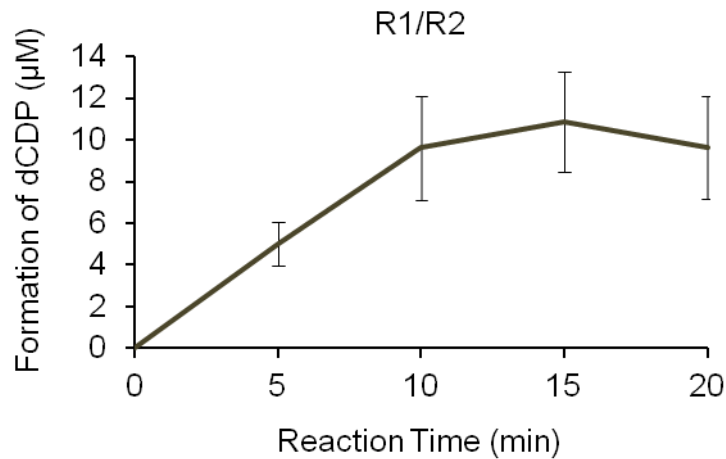
A**B**

Figure 5.4 The full reconstitution of both forms of RNR enzyme leads to product formation

5 μM of **(A)** RRM2B or **(B)** RRM2 was mixed with 9 μM of RRM1 in the appropriate buffer and 250 μM of CDP. The reaction mixture was incubated at 37°C in normoxia. At the times indicated, the amount of the dCDP formation was examined. Data show mean ± s.e.m. from n=2 biological experiments. The activity of the R1/R2B form of the RNR enzyme was lower than that of R1/R2 form. The specific activity at 37°C in normoxia at 20 min was 36.62 pmol/min/mg for R1/R2B and 41.9 pmol/min/mg for R1/R2.

We then continued by examining the effect of hypoxia on dCDP formation for the two forms of the RNR enzyme (R1/R2B and R1/R2). Specifically, 1.25 μM of RRM1 recombinant protein was mixed with an excess of RRM2B or RRM2 (2.5 μM) protein in the appropriate buffer either in normoxia or at $<0.1\%$ O_2 . Both reactions were incubated at 37°C , started by the addition of 100 μM CDP and, at the desired time points, the amount of dCDP formation was determined. In support of our hypothesis, the R1/R2B enzyme was able to sustain activity in both $<0.1\%$ O_2 and normoxia, and no statistically significant difference in the total amount of dCDP formed under either conditions after 30 minutes was observed (Figure 5.5 A). The R1/R2B enzyme continued converting CDP to dCDP for over 2 hours in hypoxia (Figure 5.5 B).

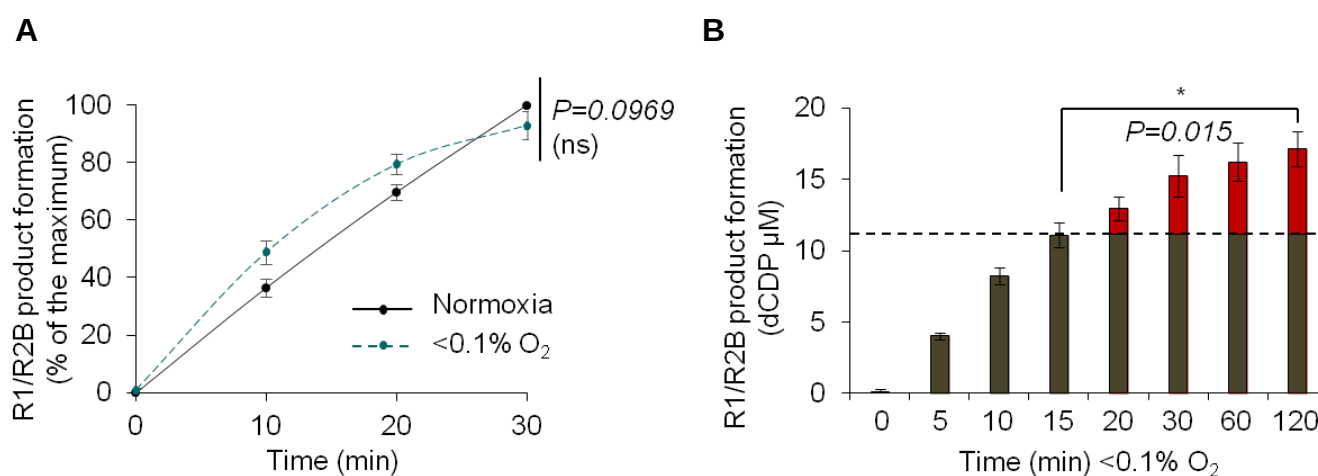


Figure 5.5 The R1/R2B form of RNR enzyme retains activity in hypoxia

(A) Product formation (% of the maximum, where maximum is the dCDP levels at 30 min in normoxia) for R1/R2B enzyme in normoxia and $<0.1\%$ O_2 . **(B)** dCDP (μM) in $<0.1\%$ O_2 for R1/R2B for the times indicated. Grey columns indicate the amount of dCDP formed up to 15 min in $<0.1\%$ O_2 ; red columns indicated the amount of dCDP formed after 15 min in $<0.1\%$ O_2 . Data show mean $\pm$ s.e.m. from $n=3$ biological experiments. In (A) two-way ANOVA was applied; in (B) two-tailed Student's t -test was applied.

In contrast, when the R1/R2 enzyme was tested, we observed a statistically significant decrease in dCDP formation in hypoxia compared to normoxia, meaning that this form of RNR does not sustain activity in hypoxic conditions (Figure 5.6 A). Moreover, the R1/R2 enzyme stopped dCDP formation after 15 minutes in $<0.1\%$ O_2 (Figure 5.6 B). Importantly, we verified that the inability of R1/R2 to convert CDP to dCDP after 15 minutes in hypoxia was not due to lack of available substrate (CDP) (Figure 5.6 C). Collectively, these data suggest that the compromised R1/R2 activity observed at $<0.1\%$ O_2 was a result of limited oxygen availability.

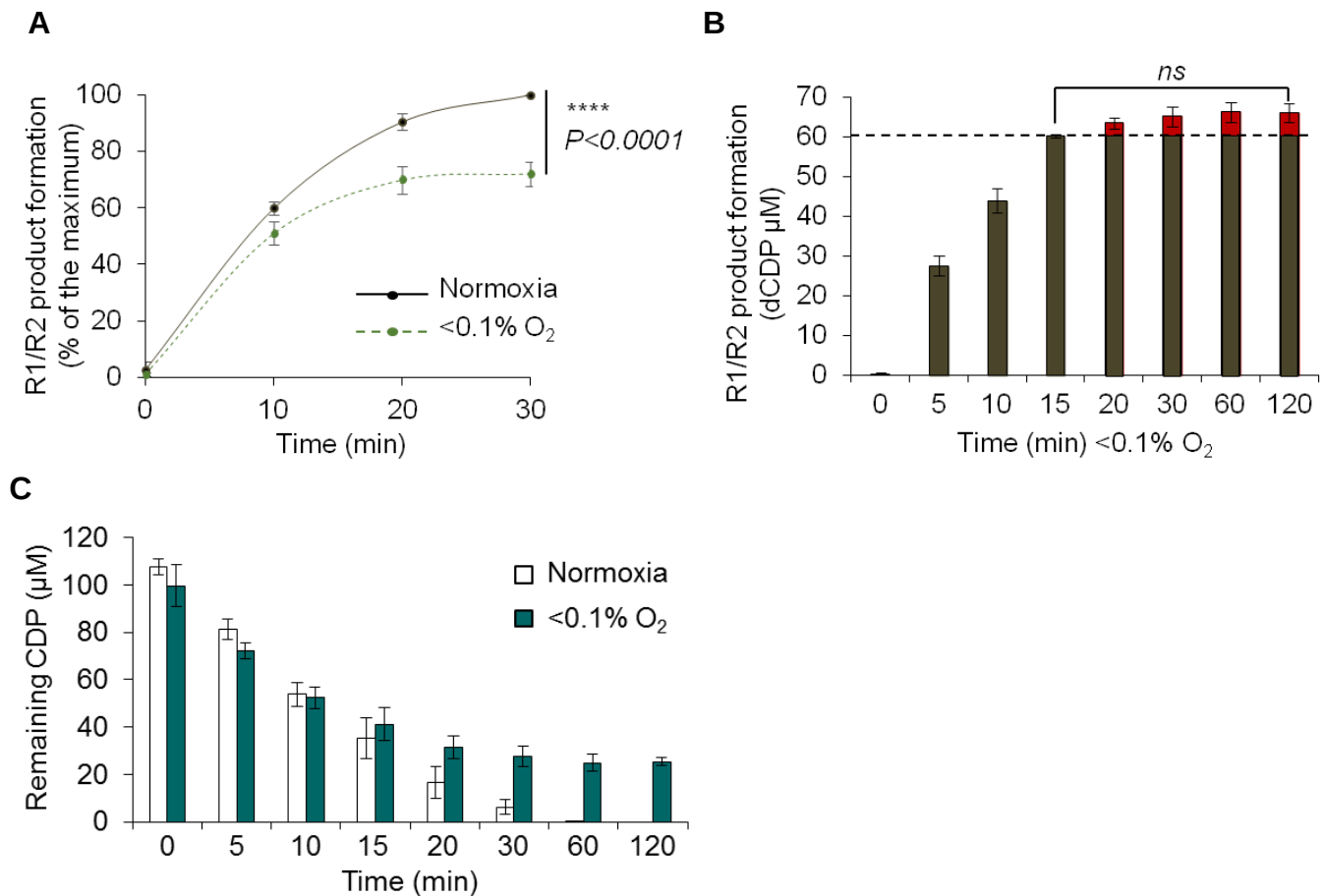


Figure 5.6 The R1/R2 form of RNR enzyme does not retain activity in hypoxia

(A) Product formation (% of the maximum, where maximum is the dCDP levels at 30 min in normoxia) for R1/R2 enzyme in normoxia and $<0.1\%$ O_2 . **(B)** dCDP (μM) in $<0.1\%$ O_2 for R1/R2 for the times indicated. Grey columns indicate the amount of dCDP formed up to 15 min in $<0.1\%$ O_2 ; red columns indicated the amount of dCDP formed after 15 min in $<0.1\%$ O_2 . **(C)** Substrate consumption (CDP) in normoxia and hypoxia for R1/R2. Data show mean $\pm$ s.e.m. from $n=3$ biological experiments. In (A) two-way ANOVA was applied; in (B) two-tailed Student's t-test was applied.

5.2.2 Adaptation of RRM2B in low oxygen environments

In order to understand why RRM2B retains activity in hypoxia whereas RRM2 does not, we initially investigated the theoretical differences and/or similarities in the dynamics of oxygen diffusion between the two proteins. Specifically, we used the published structures of RRM2B (PDB ID 3HF1) (Smith et al., 2009) and RRM2 (PDB ID 3VPN) (Smith et al., 2009) and carried out Molecular Dynamics (MD) simulations (Appendix III; simulations performed by Christian Jorgensen; Department of Chemistry, King's College). MD are computer simulations that permit the study of complex, dynamic processes that occur in biological systems by providing an estimation of how microscopic interactions occur between molecules (Allen, 2004). This analysis revealed that both RRM2B and RRM2 display three principal O₂ cavity tunnels (T1, T2 and T3) that could be employed as access points through which O₂ could travel from the bulk of the protein to the iron sites (Figure 5.7). Tunnel 1 (T1) showed connectivity between the iron site of monomer-1 and the outside protein surface, it is the longest tunnel and is similar in both proteins (RRM2B and RRM2). Tunnel 2 (T2) revealed connectivity between the two monomers of each protein and as shown in Figure 5.7, RRM2B showed a greater monomer connectivity through T2 compared to RRM2. Finally, tunnel 3 (T3) is connected with a pocket of Phe, which is known to create an open channel specifically in RRM2B (Smith et al., 2009) (Appendix IV).

Further analysis of those tunnels revealed two lines of evidence supporting the hypothesis that RRM2B could be a better oxygen-sequestering agent than RRM2. Specifically, we found that the predicted oxygen entering frequencies are higher for RRM2B (68% at monomer-1 and 22% at monomer-2) than for RRM2 (14% at monomer-1 and 0% at monomer-2). In addition, the distances that the oxygen needs to travel to reach the iron centres favour RRM2B (4.5-9.0 Å at monomer-1) over RRM2 (7.0-10.0 Å at monomer-1),

which indicate differential oxygen turnover susceptibilities between the two proteins (Table 5.1).

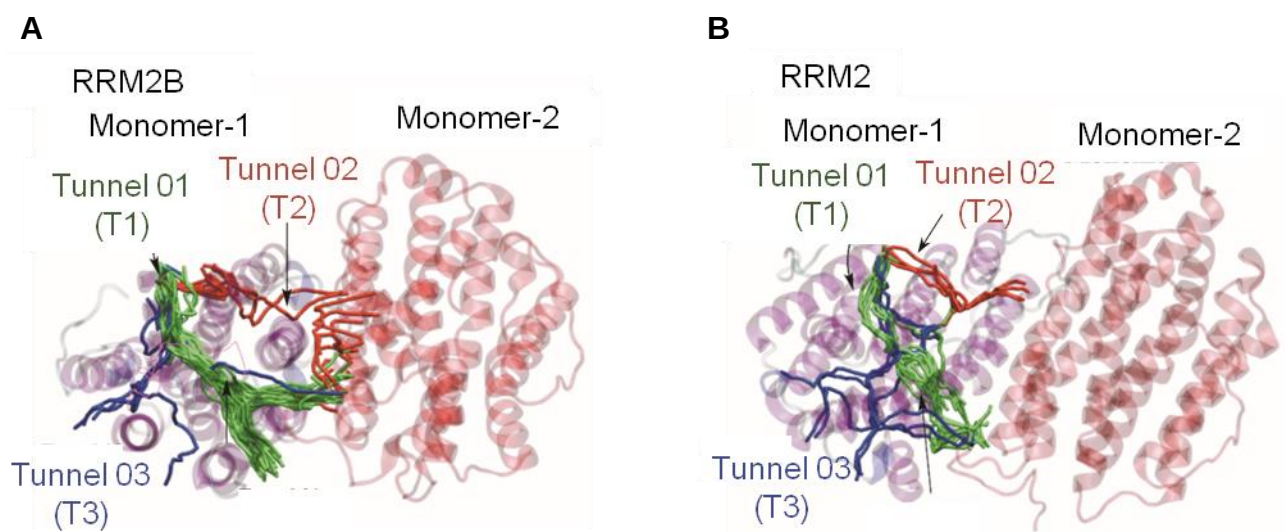


Figure 5.7 MD simulations revealed three O₂ tunnels in each small subunit of the RNR enzyme

The three O₂ cavity tunnels (T1, T2 and T3) in (A) RRM2B and (B) RRM2 proteins. The three tunnels could be employed by oxygen to travel from the bulk of the protein into the iron sites for the tyrosyl-radical to be formed. T1 (green) shows connectivity between the iron site of monomer-1 and the outside protein surface; T2 (orange) shows connectivity between the two monomers and T3 (blue) is connected with a pocket of Phes. (Simulations were performed by Christian Jorgensen).

Table 5.1: Cluster distances for O₂-Fe sites and O₂ entering frequencies for RRM2B and RRM2 proteins

System	$\langle d_{\text{O}_2\text{-Fe}} \rangle$ (Å)	O ₂ entering events	Entering frequency %
RRM2B Monomer 1	4.0 – 8.0	25	68
RRM2B Monomer 2	8.0	8	22
RRM2 Monomer 1	7.0 – 10.0	5	14
RRM2 Monomer 2	8.0– 11.0	0	0

(Performed by Christian Jorgensen)

We then investigated the tyrosyl radical content of the two proteins. To achieve this, we carried out electron paramagnetic resonance (EPR) spectroscopy to monitor the stability of the tyrosyl radical in RRM2B and RRM2 in normoxia and after 1 hour of hypoxia (Figure 5.8). Strikingly, we observed that 66% of the tyrosyl radical in RRM2B remained stable for 1 hour at <0.1% O₂, whilst in the same conditions only 43% remained stable in RRM2 (Table 5.2).

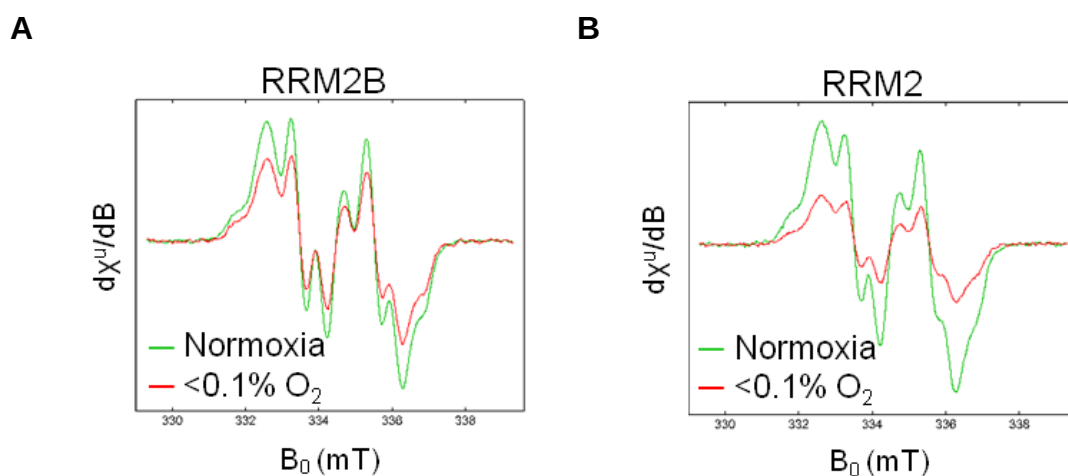


Figure 5.8 EPR spectra of the tyrosyl radical in normoxia and hypoxia

Spectra of the tyrosyl radical of RRM2B (A) and RRM2 (B) proteins as monitored by EPR spectroscopy in normoxia (green) and hypoxia (red). The amplitude of the EPR signals reflect the spin concentration (Table 5.2) and the radical content was quantified against a standard solution of 1 mM Cu^{II}-EDTA in 50 mM HEPES, pH 7.5. (Data analysis was performed with assistance from Dr. William Myers).

Table 5.2: Quantitative analysis of the radical content of RRM2B and RRM2 proteins in normoxia and hypoxia

EPR Sample	Spins / β subunit ($\times 10^{-3}$)	Hypoxia / Normoxia (%)
RRM2B Normoxia	9.84 ± 0.34	66 ± 3.35
RRM2B <0.1% O ₂	6.45 ± 0.15	
RRM2 Normoxia	30 ± 1.4	43 ± 4
RRM2 <0.1% O ₂	12.85 ± 0.75	

5.2.3 Identification of residues responsible for the adaptation of RRM2B to hypoxia

The results presented so far provide evidence that RRM2B is able to retain activity in hypoxia while the activity of RRM2 is compromised in the same conditions. To clarify whether there are structural requirements responsible for the hypoxic adaptation of RRM2B to hypoxia, we took a site-directed mutagenesis based approach. As RRM2B and RRM2 share 83% sequence homology (Zhou et al., 2010), we focused on the non-homologous residues, which have been suggested to confer structural and functional differences (Figure 5.9). Based on these key differences we constructed the following mutants: Y164C; Y164F; F183Y; K37E/K151E; Y241H; Y331F and Q127K.

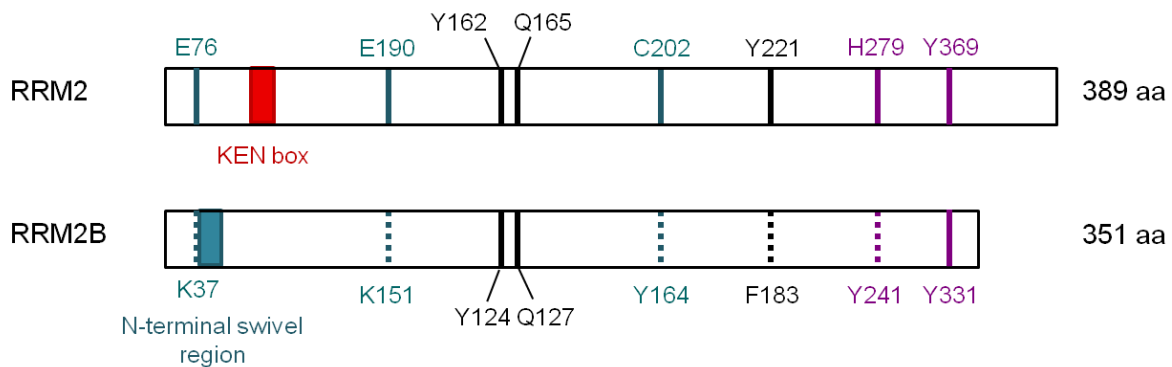


Figure 5.9 Representative schematic of the key differences between RRM2 and RRM2B proteins

Black indicates the crucial residues for tyrosyl radical formation. Blue indicates the RRM2B-specific N-terminal swivel region and the residues that are connected/affected by this region. Purple indicates some of the responsible residues for the RRM2B-specific catalase activity. Red indicates the KEN box that leads to RRM2-specific G₂ phase degradation. Straight and dashed lines indicate homologous or not residues respectively.

Firstly, we substituted the Y164 of RRM2B to Cys as it has been shown that the switch of C202 in RRM2 to Y164 in RRM2B results in the open Phe channel specifically in RRM2B (Appendix IV) (Smith et al., 2009). Importantly, our MD analysis showed that this residue is connected with the oxygen tunnel T3 (Figure 5.7). In addition, we substituted this residue with a Phe to mimic the crucian carp variant of RRM2B (ccRRM2Bii), which is known to maintain the stability of the tyrosyl radical under anoxic conditions (Sandvik et al., 2012).

Next, we mutated the F183 of RRM2B to Tyr (the analogous amino acid in RRM2), as this residue is adjacent to the essential residue (Y124-RRM2B/Y162-RRM2) and has been proposed to be responsible for the overall lower reductase activity of RRM2B compared to RRM2 (Zhou et al., 2010).

In addition, it has been reported that the RRM2B protein contains a N-terminal swivel region (residues 37-42), which communicates directly with three different regions in the opposite monomer and therefore exhibits a greater crosstalk between its monomers than the RRM2 protein (Smith et al., 2009). Specifically, through K37 and K151 (E76 and E190 in RRM2) the N-terminal swivel region of monomer-1 dictates conformational changes that stabilise helix B of monomer-2 in an open conformation. Also, this movement stabilises the full binuclear iron environment in monomer-2 specifically in RRM2B (Smith et al., 2009). Therefore, the double mutant K37E/K151E was created.

An additional important difference between RRM2B and RRM2, is the unique catalase activity that RRM2B exhibits. Specifically, there is a geometric structure in RRM2B composed of Y49, Y285, Y331 and Y241 that is responsible for its ability to break down H_2O_2 (Xue et al., 2006). The Y241 is the most important among these residues as replacement of this Tyr with a histidine (His, H) (the analogous residue in RRM2 is H279) led to inability of the protein to scavenge H_2O_2 (Xue et al., 2006). Replacement of Y331 of

RRM2B to Phe also lowers the catalase activity, but most importantly Y331F variant depletes the reductase activity of the protein as Y331 is part of the PCET pathway in RRM2B (Xue et al., 2006).

Finally, we constructed the Q127K mutant as a negative control as replacement of Q127 with Lys abolishes the reductase activity of RRM2B. It is known that both RRM2B and RRM2 proteins contain this conserved Gln residue (Q127-RRM2B/Q165-RRM2) which is essential for the radical formation through the formation of a hydrogen-bonding network and stabilisation of the radical-iron cluster (Zhou et al., 2010).

For the construction of the seven recombinant RRM2B variants the primers listed in Table 2.14 were used and site-directed mutagenesis was carried out as described in Chapter 2.3.6. The seven proteins were then overexpressed and purified to approximately 90% purity (Figure 5.10 A). Next, Circular Dichroism (CD) measurements were conducted as described in Chapter 2.3.9, to ensure that the proteins were properly folded. All of the RRM2B variants exhibited similar signatures to the WT protein confirming that all the mutants have the same secondary structure as WT RRM2B (Figure 5.10 B).

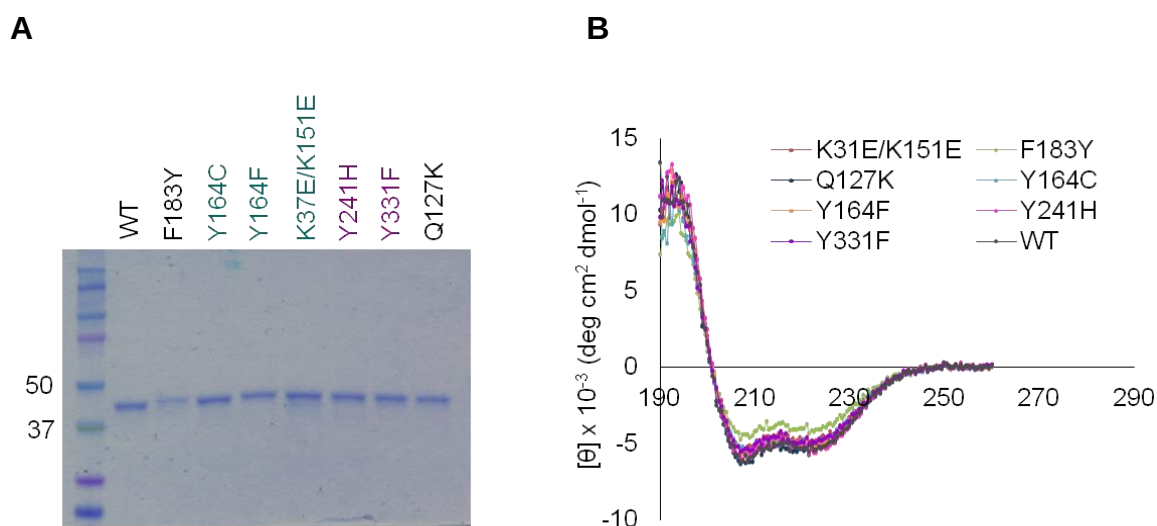


Figure 5.10 Expression and purification of the seven RRM2B variants

(A) SDS-PAGE electrophoresis followed by coomassie blue staining of the purified recombinant RRM2B variants (F183Y, Y164C, Y164F, K37E/K151E, Y241H, Y331F and Q127K). Protein ladder is shown and sizes 50 and 37 kD indicated. The colour code follows the one in Figure 5.9. **(B)** CD spectra of RRM2B WT and variant proteins to ensure the secondary structure of all variants tested; n=3 technical replicates.

We then conducted *in vitro* enzymatic activity assays for all variants in <0.1% O₂. As expected, the variants Q127K and Y331F did not show any dCDP formation, as substitution of these residues is known to abrogate the reductase activity of RRM2B (Figure 5.11). The variants Y164F, F183Y and Y241H showed sustained dCDP formation for over 2 hours in hypoxia (Figure 5.12) in a manner reminiscent of the WT RRM2B protein (Figure 5.5 B). Interestingly, the Y164F and F183Y variants showed increased product formation compared to the WT protein (Figure 5.12 A, B). That was an expected finding for the F183Y variant since the F183Y substitution has been shown to increase reductase activity of RRM2B (Zhou et al., 2010). With regards the Y164F variant, this finding could suggest an explanation for the efficiency of the RRM2B variant of crucian carp (ccRRM2Bii), which enables this fish to maintain cell division after seven days of complete anoxia (Sandvik et al., 2012).

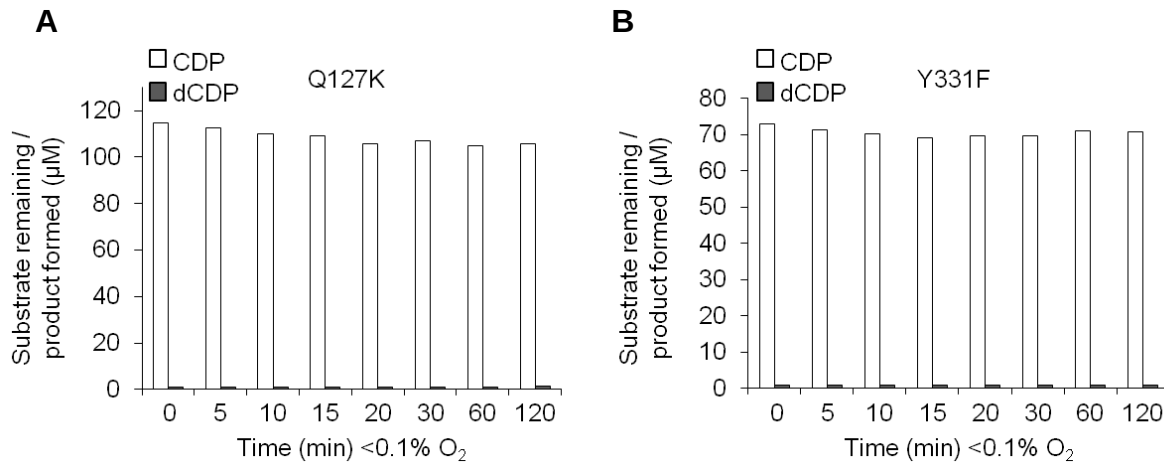


Figure 5.11 Substrate over product formation for Q127K and Y331F variants

(A) Q127K and **(B)** Y331F variants of RRM2B show zero turnover ratio of CDP to dCDP as disruption of those residues leads to diminished reductase activity in RRM2B (n=1).

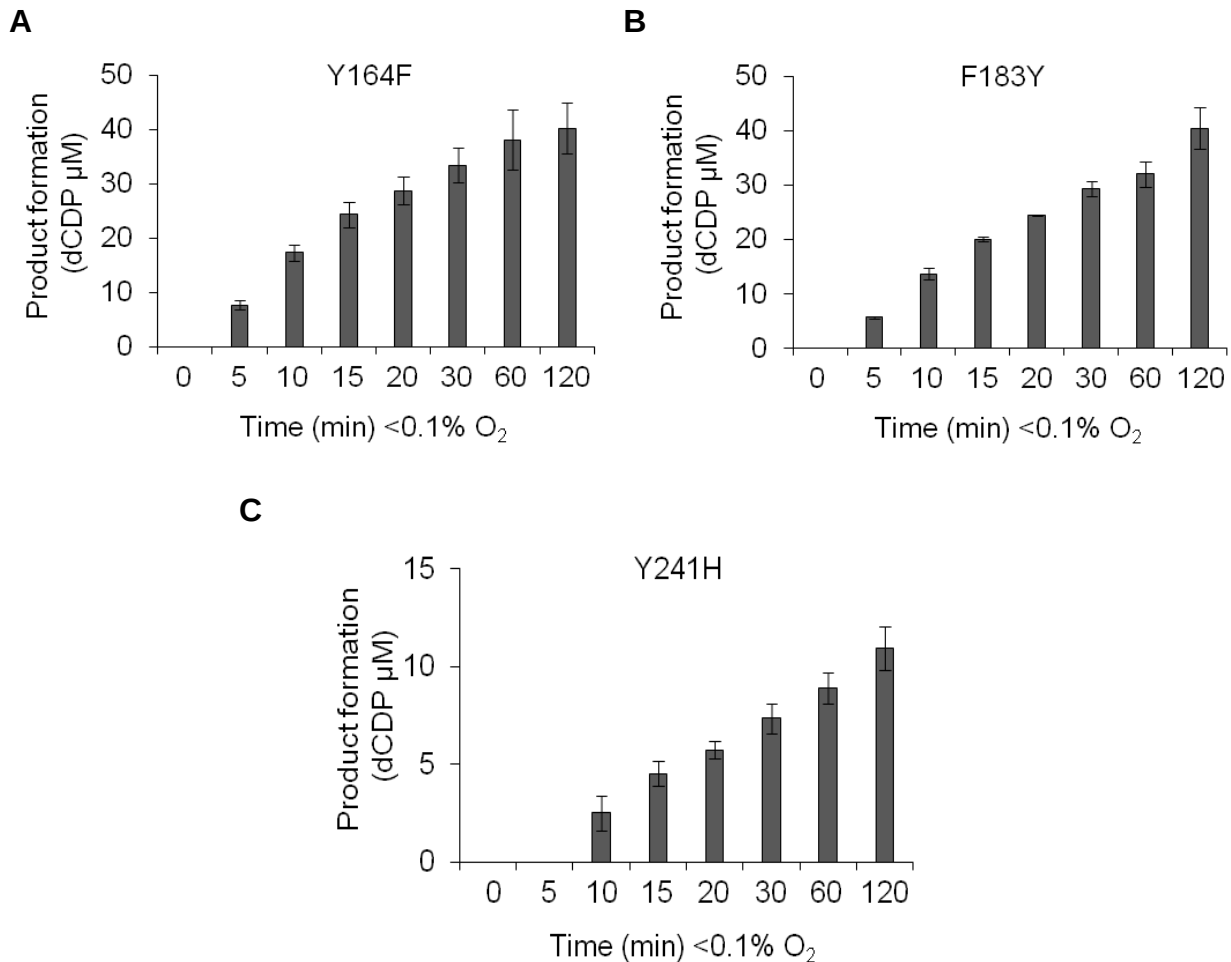


Figure 5.12 dCDP formation in hypoxia for Y164F, F183Y and Y241H variants

The variants **(A)** Y164F, **(B)** F183Y and **(C)** Y241H showed a sustained dCDP formation for over 2 h in <0.1% O₂ similar to WT RRM2B. Data show mean ± s.e.m. from n=2 biological experiments.

However, when the Y164C and K37E/K151E variants were tested, we observed that they stopped converting CDP to dCDP after ~30 minutes in <0.1% O₂ (Figure 5.13) in a manner reminiscent of the RRM2 protein (Figure 5.6 B). We then compared dCDP formation in normoxia and hypoxia for these two variants. Interestingly, we found that the ability of Y164C and K37E/K151E to reduce CDP to dCDP was compromised in hypoxia (Figure 5.14). These results indicate that these specific residues are critical for the role of RRM2B in hypoxia and that without them RRM2B activity is not sustained, similar to RRM2.

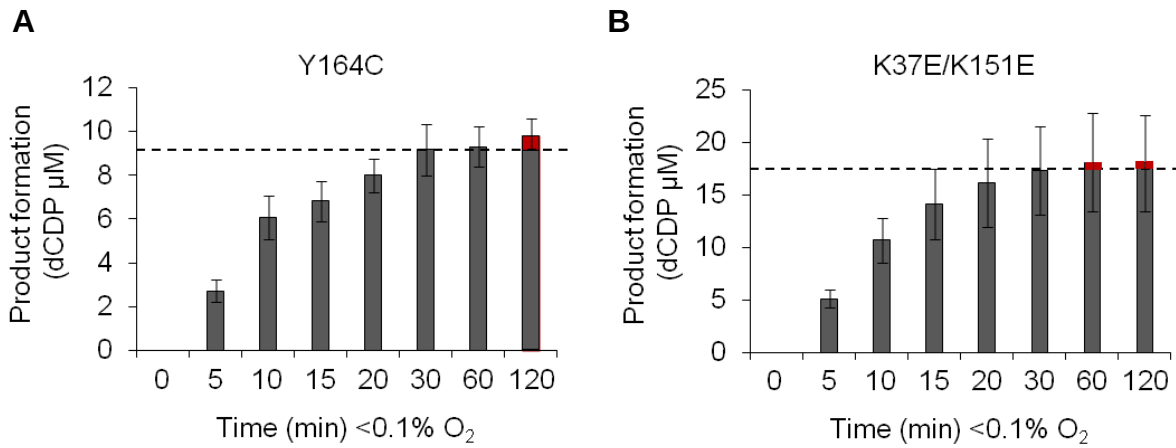


Figure 5.13 dCDP formation in hypoxia for Y164C and K37E/K151E variants

The variants **(A)** Y164C and **(B)** K37E/K151E stopped dCDP formation at 30 min <0.1% O_2 showing a distinct feature from WT RRM2B. Data show mean $\pm$ s.e.m. from $n=2$ biological experiments.

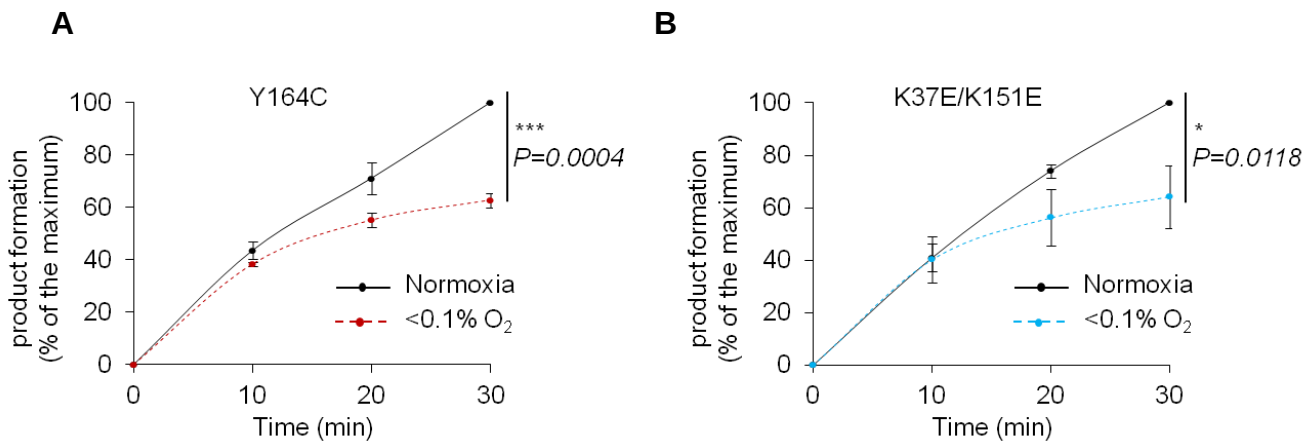


Figure 5.14 The Y164C and K37E/K151E variants do not sustain activity in hypoxia

Product formation (% of the maximum, where maximum is the dCDP levels at 30 min in normoxia) for **(A)** Y164C and **(B)** K37E/K151E variants in normoxia and <0.1% O_2 . Data show mean $\pm$ s.e.m. from $n=3$ (Y164C) and $n=4$ (K37E/K151E) biological experiments; two-way ANOVA was applied in both panels.

5.2.4 The role of Y164 and K37/K151 in the hypoxic adaptation of RRM2B

In order to further investigate the role of residues Y164 and K37/K151 of RRM2B, we conducted additional MD simulations and EPR analyses. Interestingly, our MD simulations showed major differences in the protein structure when the Y164C and K37E/K151E variants were compared with WT RRM2B (Figure 5.15 and Figure 5.16). Regarding Y164C, we found that two contacts in the WT RRM2B crystal structure (Y164-F197 and Y164-F198) were disrupted in monomer-1, which caused a side-chain rearrangement at F95. This rearrangement resulted in F95 moving closer to F197 in the Y164C variant, leading to closure of the Phe channel (Figure 5.15 and Appendix IV-A). Further analysis of the MD simulations showed that Y164C variant had a 4.8-fold lower oxygen entering frequency in monomer-1 compared to WT RRM2B. No changes in the oxygen entering frequency for the K37E/K151E variant were observed (Table 5.3).

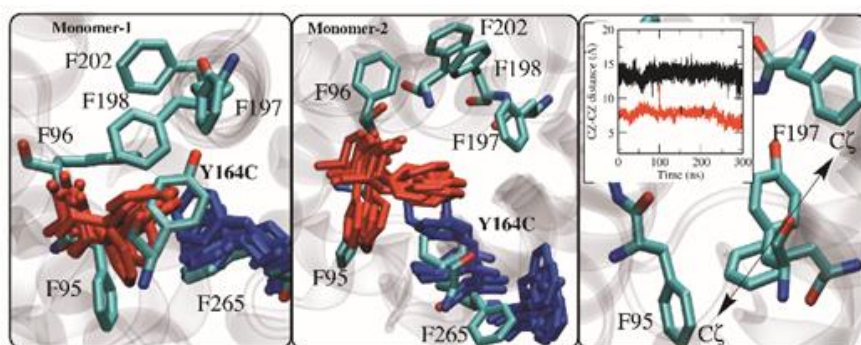


Figure 5.15 The phenylalanine network surrounding Y164 in RRM2B

MD simulations show a Phe conformation in the Y164C variant leading to closure of the Phe channel. The distance plot in monomer-1 reveals the effect of Y164C on the distance between F95-F197. Colour code: WT (black), Y164C (red). (Simulations were performed by Christian Jorgensen).

Table 5.3: Cluster distances for O₂-Fe sites and O₂ entering frequencies for Y164C and K37E/K151E variants

System	$\langle d_{\text{O}_2\text{-Fe}} \rangle$ (Å)	O ₂ entering events	Entering frequency %
RRM2B-WT Monomer 1	4.0 – 8.0	25	68
RRM2B-WT Monomer 2	8.0	8	22
Y164C Monomer 1	4.0 - 8.0	8	14
Y164C Monomer 2	8.0 - 12.0	17	29
K37E/K151E Monomer 1	4.0 - 8.0	37	64
K37E/K151E Monomer 2	8.0 - 12.0	6	16

(Performed by Christian Jorgensen)

The MD simulations also identified differences in the protein structure of the K37E/K151E variant. Specifically, we found a distortion in the bending angle of the helices in monomer-2, which leads to closure of the helix B open conformation in this variant (Figure 5.16). It has been previously reported that K37 and K151 in RRM2B (E76 and E190 in RRM2) stabilises the helix B of monomer-2 in an open conformation, which results in enhanced crosstalk between the two monomers of RRM2B (Smith et al., 2009). In contrast, the RRM2 protein showed a closed conformation of helix B in monomer-2, similar to the K37E/K151E variant of RRM2B (Figure 5.16).

EPR spectroscopy was then performed for the RRM2B variants. Interestingly, we observed that while most of the RRM2B variants tested behaved as the WT RRM2B, retaining 60-79% of their tyrosyl radical in hypoxia, the K37E/K151E variant retained only 28% of its tyrosyl radical in these conditions. The Q127K variant did not form a tyrosyl radical in either condition as expected (Figure 5.17 and Table 5.4). Since the K37/K151 residues affect the stability of the open conformation of helix B in monomer-2, the loss of the

sustained tyrosyl radical in hypoxia could be explained by the closure of helix B and the loss of essential crosstalk between the two monomers of the protein.

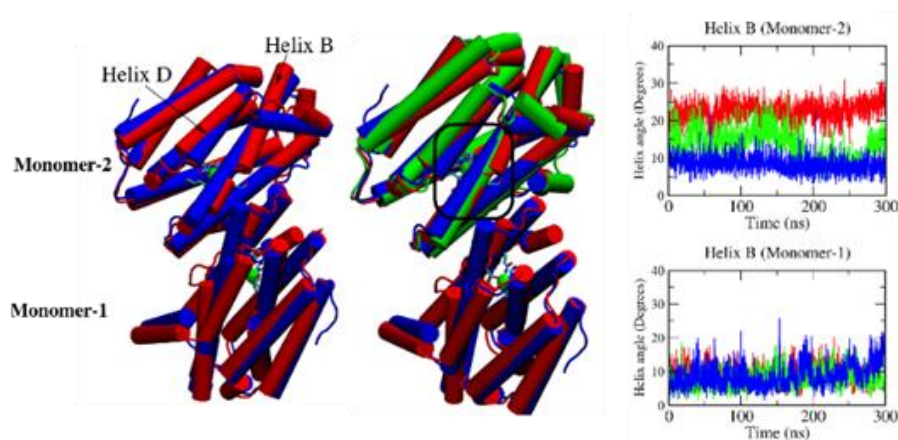


Figure 5.16 Helix B of monomer-2 shows either an open or a closed conformation

Comparison of the RRM2B (red) with RRM2 (blue) and K37E/K151E (green) with MD simulations revealed an open helix B in RRM2B and a closed helix B in RRM2 and K37E/K151E. The disruption in the bending angle of the helices in monomer-2 of the K37E/K151E variant led to closure of the helix B open conformation. Plots reveal the helix B bending angle. Black box shows the closure of helix B in K37E/K151E. (Simulations were performed by Christian Jorgensen).

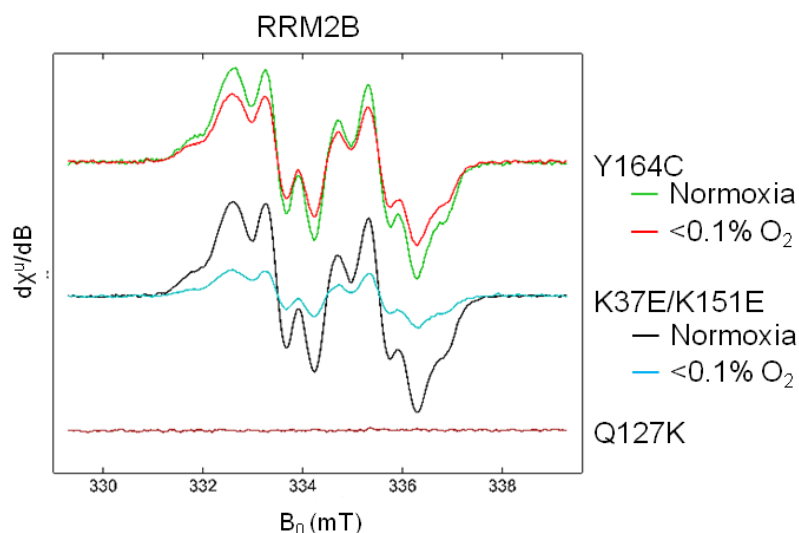


Figure 5.17 EPR spectra of the tyrosyl radical for the Y164C, K37E/K151E and Q127K variants in normoxia and hypoxia

Spectra of the tyrosyl radical of Y164C, K37E/K151E and Q127K variants as monitored by EPR spectroscopy in normoxia and hypoxia. The amplitude of the EPR signals reflect the spin concentration (Table 5.4) and the radical content was quantified against a standard solution of 1 mM Cu^{II}-EDTA in 50 mM HEPES, pH 7.5. Colour code: Y164C normoxia (green); Y164C hypoxia (red); K37E/K151E normoxia (black); K37E/K151E hypoxia (blue); Q127K normoxia and hypoxia (crimson). (Data analysis was performed with assistance from Dr. William Myers).

Table 5.4: Quantitative analysis of the radical content of the RRM2B variants in normoxia and hypoxia

EPR Sample	Spins / β subunit ($\times 10^{-3}$)	Hypoxia / Normoxia (%)
RRM2B-WT Normoxia	9.84 ± 0.34	66 ± 3.35
RRM2B-WT <0.1% O ₂	6.45 ± 0.15	
Y164C Normoxia	8.85 ± 0.95	70 ± 3.35
Y164C <0.1% O ₂	6.2 ± 0.9	
K37E/K151E Normoxia	8.9 ± 1.0	28 ± 0.25
K37E/K151E <0.1% O ₂	2.46 ± 0.29	
Y164F Normoxia	35.5 ± 0.5	60 ± 4
Y164F <0.1% O ₂	21.25 ± 1.25	
Y241H Normoxia	6.85 ± 0.05	72.5 ± 0.5
Y241H <0.1% O ₂	5 ± 0	
F183Y Normoxia	11.7 ± 1.1	79.5 ± 4.5
F183Y <0.1% O ₂	9.25 ± 0.35	
Q127K Normoxia	ND	ND*
Q127K <0.1% O ₂	ND	

*ND refers to non-detected

Finally, we investigated whether our findings are relevant to cells experiencing hypoxia and asked if the hypoxic adaptation of RRM2B depends on the identified residues (Y164 and K37/K151). To achieve this, rescue experiments were carried out using the RRM2B knockout cell line (RKO^{RRM2B^{-/-}}) transfected with the following vectors: pRRM2B^{WT}, pRRM2B^{Y164C}, pRRM2B^{K37E/K151E} and pcDNA3.1 (CTL).

We first checked the levels of the nucleotides in hypoxia in the transfected cells. As expected, when pRRM2B^{WT} was used, a significant increase in the intracellular nucleotide levels was observed in hypoxia compared to CTL, whereas transfection of pRRM2B^{Y164C} and pRRM2B^{K37E/K151E} showed significantly lower dNTP levels compared to pRRM2B^{WT} (Figure 5.18).

Next, the levels of apoptosis were monitored by both PARP cleavage and morphology. Importantly, we observed that pRRM2B^{WT} rescued the null phenotype from apoptosis since transfection of this plasmid led to less cleaved PARP and less cells with fragmented DNA compared to CTL (Figure 5.19). Additionally, when we transfected pRRM2B^{Y164C} and pRRM2B^{K37E/K151E} we observed that substitution of the key residues (Y164 and K37/K151) abrogated the rescue from apoptosis in both assays (Figure 5.19).

These results validate our *in vitro* enzymatic activity experiments and theoretical studies. Overall, we demonstrate the critical importance of the hypoxic induction of RRM2B and determine the mechanistic adaptation of RRM2B to hypoxia.

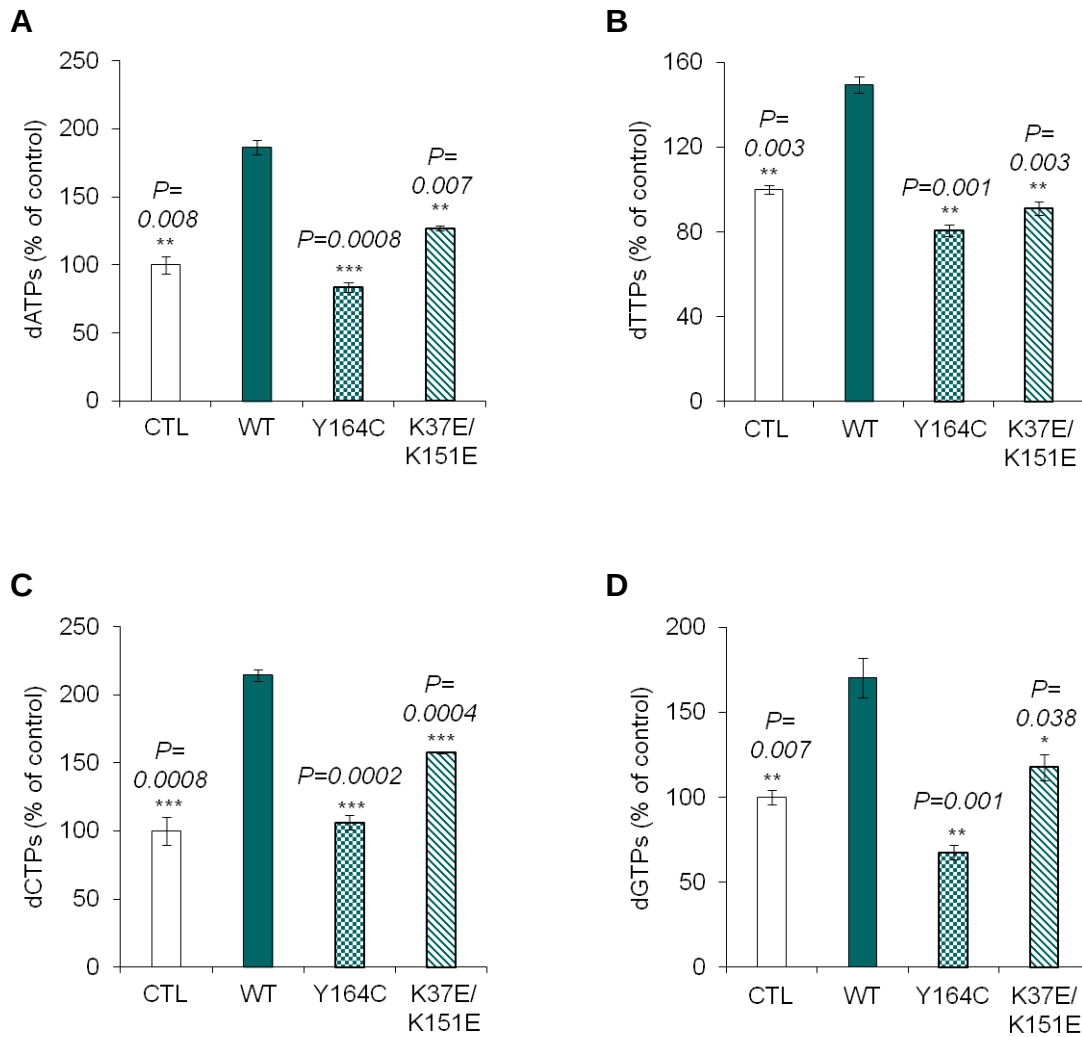


Figure 5.18 Introduction of pRRM2B^{WT} in RKO^{RRM2B-/-} cells increases the intracellular nucleotide levels in hypoxia

RKO^{RRM2B-/-} cells were transfected with either CTL, pRRM2B^{WT}, pRRM2B^{Y164C} or pRRM2B^{K37E/K151E} and exposed to <0.1% O₂ (16 h). Quantification of the dNTP levels in treated cells in hypoxia followed (A) dATP; (B) dTTP; (C) dCTP and (D) dGTP. Data were firstly normalised to the levels of the dNTPs in normoxia for each condition and then compared and presented as % of the CTL in <0.1% O₂. Data show mean ± s.e.m. from n=3 biological experiments. Two-tailed, unpaired Student's t-test was applied.

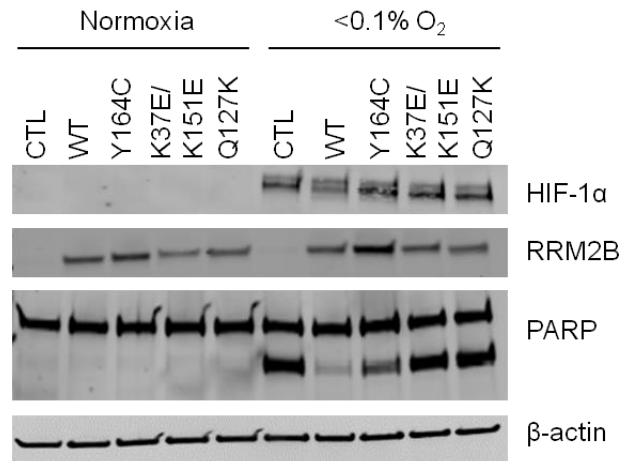
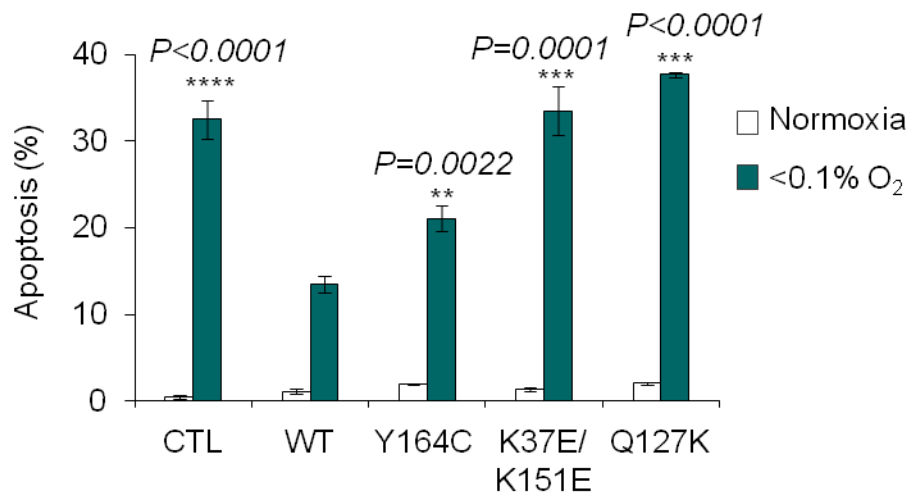
A**B**

Figure 5.19 Introduction of pRRM2B^{WT} rescued the null phenotype from apoptosis
(A) RKO^{RRM2B^{-/-}} cells were transfected with either CTL, pRRM2B^{WT}, pRRM2B^{Y164C} or pRRM2B^{K37E/K151E} and exposed to <0.1% O₂ (19 h). Western blot analysis was carried out with the antibodies indicated; β-actin was used as a loading control. PARP cleavage indicates induction of apoptosis. **(B)** Quantification of apoptotic cells treated as in (A) and exposed to normoxia or <0.1% O₂ (19 h). Apoptosis was detected morphologically with DAPI staining. Data show mean ± s.e.m. from n=3 biological experiments. Two-tailed, paired Student's t-test was applied.

5.3 Discussion

In this chapter, we studied the enzymatic properties of the two forms of RNR enzyme in both hypoxia and normoxia. By overexpressing and purifying the RNR proteins, we conducted *in vitro* experiments examining the function of both RNR forms (R1/R2B and R1/R2) in normoxia and hypoxia. As expected, in contrast to R1/R2 that does not retain activity in hypoxia, R1/R2B was able to maintain activity under hypoxic treatment. This is the first study verifying a preserved hypoxic activity for a mammalian RNR enzyme.

We then characterised the molecular attributes of the RRM2B protein, which promote continued hypoxic-activity. Our data indicate that RRM2B is capable of retaining hypoxic activity through two distinct mechanisms: (i) increased oxygen entering frequency and (ii) retained stability of the tyrosyl radical. Both of these mechanisms were compromised in RRM2, which neither behaved as a good oxygen-sequestering agent, nor was capable of retaining a stable tyrosyl radical in prolonged hypoxic exposure.

We followed our studies with mutagenesis assays that highlighted the importance of two regions (Y164 and K37/K151) of RRM2B. Substitution of either Y164 and K37/K151 caused the RNR to lose activity in hypoxia. Specifically, we found that Y164 is a key residue within RRM2B as it increases oxygen entering frequency within the protein, probably through oxygen T3 in monomer-1. Our MD analysis suggests that Y164, by keeping F95 at an optimum distance from F197, keeps the Phe channel in T3 open, an action that could allow oxygen to enter more easily into the protein. Substitution of this Tyr to Cys (the homologous amino acid in RRM2), leads to disruption of the Phe channel and a lower oxygen entering frequency in Y164C variant due to closure of this channel. Interestingly, when we substituted the same residue with Phe (the homologous amino acid in ccRRM2Bii) we observed that the recombinant protein had a higher activity both in normoxia and in hypoxia, and that activity was also preserved in hypoxia. Interestingly, in contrast to Cys, both Tyr and Phe amino acids

contain an aromatic ring facing the interior of the protein, which could result in keeping the Phe channel open. This finding could explain how the crucian carp maintains DNA replication in complete anoxia for several weeks (Sandvik et al., 2012).

We also found that through the K37/K151 residues, RRM2B retains 70% of its tyrosyl radical in hypoxia. Our MD analysis suggests that the two monomers of the small RNR subunits react differently in the overall production of the tyrosyl radical. It is possible that monomer-2 of RRM2B contributes more to the tyrosyl radical than in RRM2, specifically in hypoxia. In support of this hypothesis, our data showed a higher oxygen entering frequency in monomer-2 for RRM2B compared to RRM2 (22% and 0% respectively), most likely due to helix B open conformation that is found only in RRM2B. This hypothesis is also supported by the increased connectivity observed between the two monomers in RRM2B through oxygen T2. It is known that the stabilisation of helix B through K37/K151 affects the integrity of the diiron centre in monomer-2 (Smith et al., 2009). Our EPR analysis showed that in the K37E/K151E variant, the tyrosyl radical is affected specifically in hypoxia and not in normoxia. We propose that the tyrosyl radical of monomer-2 is better protected in RRM2B, and therefore contributes to the activity of the enzyme in hypoxic environments. This could also explain why RRM2B is less susceptible than RRM2 to the radical scavenger HU (Shao et al., 2004). Our proposed model, where both monomers contribute to tyrosyl radical formation, is in agreement with the finding that the tyrosyl radical is equally distributed between each subunit, which suggests the generation of two tyrosyl radicals per β_2 dimer (Cotruvo and Stubbe, 2011).

Most importantly, when we performed rescue experiments using our RRM2B knock-out cell line, we were able to verify our *in vitro* and theoretical findings, highlighting the importance of these residues for RRM2B activity in hypoxia. It is probable that RRM2B has been evolutionarily maintained in order to be used as the hypoxic-specific RNR small

subunit, especially as variants of RRM2B (ccRRM2Bii) have been found to be responsible for continued cell division in anoxia tolerant vertebrates (Sandvik et al., 2012). Here, we propose that one of the principal functions of RRM2B is actually to act as the hypoxic specific RNR subunit in order to be able to react promptly when this physiologically relevant stress occurs.

CHAPTER 6

Discussion

In this work, we demonstrated that in response to the physiological stress of hypoxia, RNR responds to limited oxygen availability by favouring the RRM2B subunit over RRM2. This property of RNR preserves replication fork integrity in hypoxia despite the persistence of RS, and allows continued DNA replication (Figure 6.1). Depletion of RRM2B in cells experiencing hypoxia, results in detrimental consequences for cell fate (failure to complete DNA replication, DNA damage and loss of viability). Loss of RRM2B in a xenograft model resulted in delayed tumour growth and increased radiosensitivity, further highlighting the biological importance of the hypoxic induction of RRM2B. Additionally, we characterised the molecular attributes of RRM2B, which promote continued activity in hypoxic environments. Also successfully identified the key residues for this unique hypoxic role. Importantly, we were able to correlate data using theoretical simulations, biochemical, cellular/molecular biology, xenograft models and immunohistochemistry on patient biopsies, all of which strongly support our proposed model. In this study we propose that RRM2B has been evolutionarily maintained in order to function as the hypoxia-specific RNR subunit and we demonstrate that this is essential for hypoxic cell viability. Our work explains the paradox of how hypoxic cells fulfil their commitment to continue on-going replication (an observation

first reported by Probst *et al.*, in 1988), despite the fact that RNR activity is oxygen dependent (Probst *et al.*, 1988).

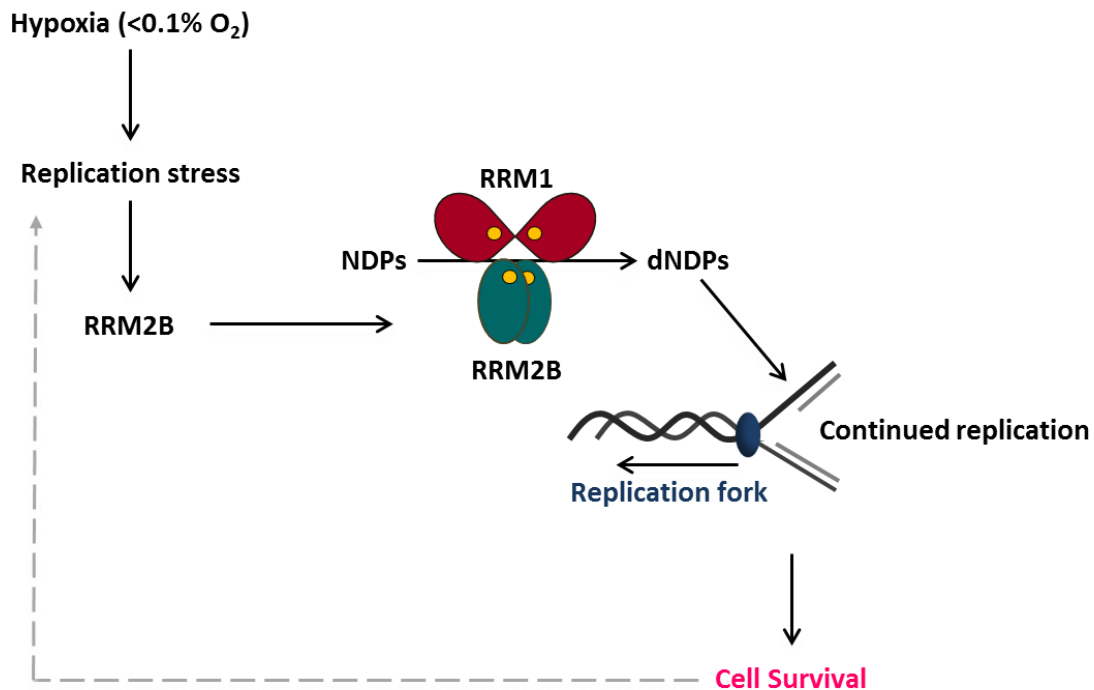


Figure 6.1 Hypoxia induced RRM2B leads to continued DNA replication

Severe levels of hypoxia lead to RS, which through the activation of the DDR, leads to induction of RRM2B. RNR favours the RRM2B subunit in hypoxia, which retains activity in these conditions providing the cell with a basal level of dNTPs. As a result, replication fork integrity is preserved, DNA replication continues albeit at a compromised rate and the cell avoids apoptosis and eventually loss of viability. The fact that RRM2B is unable to provide the appropriate amount of dNTPs for replication to continue at the same rates as in normoxia, results in the hypoxic-specific RS observed in cells that survive in these conditions.

6.1 RRM2B induction in hypoxia

Hypoxia induces RS whilst preserving the integrity of replication forks. Hypoxia-induced RS leads to activation of the DDR in the absence of detectable DNA damage. Given that the activity of RNR is oxygen dependent, we were intrigued by the source of dNTPs needed to maintain the slow replication rates observed in hypoxia. Interestingly, we found a universal induction of RRM2B in hypoxic cells, which is mainly p53-dependent and HIF-1 α -independent. However, in p53-deficient cells, a mild increase of RRM2B protein levels was also observed, suggesting the additional presence of p53-independent mechanisms for RRM2B stabilisation in hypoxia. This is not the first time that an induction of RRM2B has been observed in the absence of p53 (Cho and Yen, 2016). Specifically, a number of transcription factors have been recently described to upregulate RRM2B such as p73 (a structural homolog of p53 and a member of the same family), FOXO3 and E2F1 (Cho et al., 2014; Nakano et al., 2000; Qi et al., 2015). However, our results showed that the transcriptional induction of *RRM2B* is minimal in the absence of p53 in hypoxia, suggesting that the p53-independent mechanism/s of hypoxic RRM2B induction, most likely occur at the post-translational level.

Interestingly, it has been shown that ATM is capable of stabilising RRM2B through direct phosphorylation at S72, which inhibits hyper-ubiquitination and degradation by the E3 ubiquitin-protein ligase, MDM2 (mouse double minute 2 homolog) (Chang et al., 2008). It has been described previously that MDM2 expression is decreased in hypoxia, which could also contribute to stabilisation of RRM2B under these conditions (Alarcon et al., 1999). Moreover, nitric oxide (NO), which is reported to be elevated in hypoxia, has been shown to increase RRM2B in p53-deficient cells (Dweik, 2005; Guittet et al., 2008). Since all of these mechanisms are relevant to hypoxic stress, further investigation is needed to verify if they could be responsible for the p53-independent RRM2B induction in hypoxia. The existence of

different mechanisms that lead to RRM2B overexpression and stabilisation in hypoxia, highlights the importance of the RRM2B protein in these conditions. Of note, the basal levels of RRM2B expression show no significant association with p53 status (Cho and Yen, 2016). Since p53 is often mutated or deleted in human tumours, the involvement of mechanisms that lead to p53-independent basal expression of RRM2B, as well as protein stabilisation under hypoxia, suggest that RRM2B can perform its hypoxic role even in the absence of functional p53 (Wasylishen and Lozano, 2016).

The biological significance of induced RRM2B in hypoxia is highlighted by the detrimental consequences that the cells face after its depletion, specifically in hypoxic conditions (Figure 6.2). Loss of RRM2B leads to lower dNTP levels in hypoxia, which results in DNA damage, probably due to fork collapse and loss of viability. It would be interesting to measure the actual rates of DNA replication after depletion of RRM2B in hypoxia using the DNA fibre assay. Unfortunately, we were unable to generate good quality fibers from RRM2B-depleted cells that had been exposed to hypoxia. It is tempting to speculate that this was due to the fragility of the fibers as a result of destabilised replication forks after depletion of RRM2B. The DNA combing technique is now used routinely to monitor DNA replication in several organisms. Combing allows a high density of single DNA molecules to be uniformly stretched onto silane coated slides with a constant stretching factor, resulting in combed DNA molecules (Bianco et al., 2012; Foskolou et al., 2016; Michalet et al., 1997). It could be possible that using DNA combing, enough intact DNA fibers could be isolated to allow the measurement of replication rates in the absence of RRM2B in hypoxia. To date, we have been unable to perform these experiments due to the requirement of specific equipment (Combing System) for performing this technique.

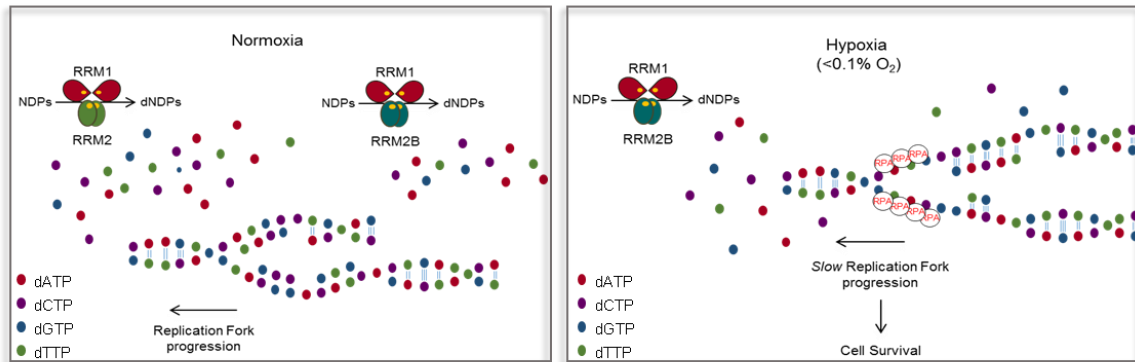
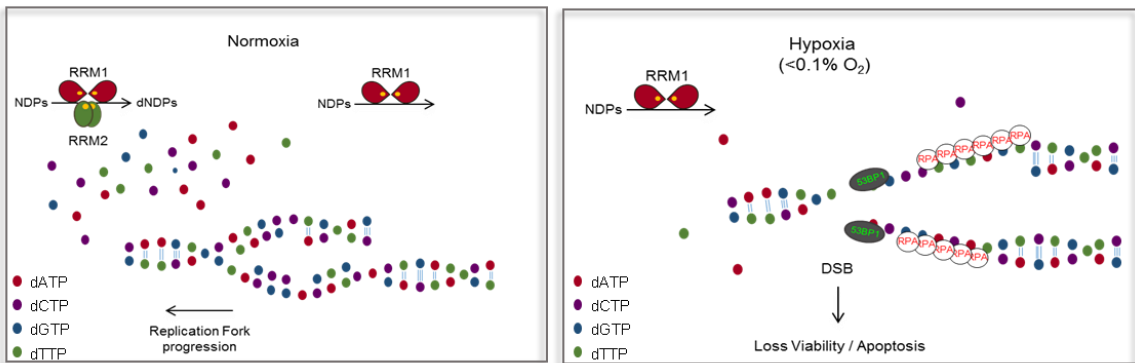
A**B**

Figure 6.2 Importance of RRM2B in hypoxic exposed cells

(A) During normoxia, both forms (R1/R2 and R1/R2B) of RNR enzyme are present, with the R1/R2 contributing more to the amount of dNTPs formed during S phase. In hypoxia, RNR is mostly reconstituted by the RRM2B subunit, which provides a basal level of dNTPs. Although the imbalanced dNTP pools and the subsequent induction of RS leads to accumulation of ssDNA (detected by RPA foci) in hypoxia, the basal levels of dNTPs provided are sufficient for DNA replication to continue at a compromised rate. This results in cellular adaptation and survival in hypoxia. **(B)** Depletion of RRM2B does not significantly affect dNTP pools in normoxia and replication proceeds normally. In hypoxia, RRM2B deletion leads to even lower levels of dNTPs and DSBs are observed (detected by 53BP1 foci) probably due to replication fork collapse. This would ultimately lead to loss of viability and induced apoptosis.

6.2 Molecular adaptations of RRM2B for hypoxic specific role

In order to investigate the molecular attributes of the RRM2B protein that promote continued hypoxic-activity, we performed biophysical experiments (EPR spectroscopy) and computational studies (MD simulations). By employing MD simulations, we proposed the existence of three oxygen tunnels that oxygen can use to enter the protein, in order to react with the diiron centre. MD simulations are a valuable tool in many areas of physics and chemistry which have been widely used to study the structure and dynamics of macromolecules, such as proteins and nucleic acids (Petrenko and Meller, 2001). However, experimental techniques are still preferred for determination of the structure and function of molecules of biological interest. Therefore, we tried to measure formation/decay of absorption attributable to the tyrosyl radical upon addition of oxygen, as has been reported for mouse RNR, by performing stopped-flow experiments (Yun et al., 2007). While tyrosyl radical formation is not necessarily reflective of oxygen addition rates, we nevertheless attempted to investigate whether radical formation rates upon the addition of oxygen differed between R2 and R2B to support our *in silico* findings. Unfortunately, both RRM2 and RRM2B recombinants repeatedly precipitated under these conditions and at the high concentrations necessary for the stopped-flow experiments, despite attempts to optimise the procedure. While it would have been of interest to compare tyrosyl radical formation rates, we do not believe that the experiment is necessary to support the conclusions we make, given the correlations we observe between *in silico*, *in vitro* and cellular results throughout this thesis.

6.3 RRM2B and oxidative stress; could RRM2B generate oxygen to maintain hypoxic activity?

A unique function of RRM2B amongst the other RNR proteins, is that it responds to oxidative stress by exhibiting anti-ROS potential (Cho et al., 2015; Kuo et al., 2012). *In vitro* studies have shown that RRM2B has catalase activity in addition to the reductase activity. Specifically, it was demonstrated that through residues Y331, Y258, Y49 and Y241 recombinant RRM2B uses intrinsic catalase activity to break down H_2O_2 (Xue et al., 2006). Recent studies have shown that silencing RRM2B expression, leads to increased intracellular ROS levels, mitochondria destabilisation and as a result induction of senescence. In response to oxidative stress RRM2B regulates mitochondrial and inflammatory pathways in a p53-independent manner (Cho et al., 2015; Kuo et al., 2012). Kimura *et al.*, have also demonstrated that *RRM2B*^{-/-} mouse embryonic fibroblasts (MEFs) derived from *RRM2B*-null mice, were significantly more sensitive to oxidative stress and had severely attenuated dNTP pools, compared to *RRM2B*^{+/+} MEFs (Kimura et al., 2003).

It has been reported that hypoxia could result in elevated ROS levels, thus the hypoxic induction of RRM2B could also be biologically beneficial due to anti-ROS properties. In agreement with this hypothesis, preliminary experiments we performed in cells with silenced RRM2B, showed increased ROS levels in cells experiencing hypoxia. It is tempting to speculate that through this catalase activity, RRM2B increases immediate oxygen availability by breaking down H_2O_2 to H_2O and O_2 . The locally elevated oxygen levels, could then be sequestered by RRM2B, and used for its conventional reductase function. Importantly, it has been suggested that among the identified catalase-responsible residues, Y241 is the most important, since replacement of this Tyr to His (the analogous residue in RRM2) led to inability of the protein to scavenge H_2O_2 (Xue et al., 2006). Interestingly, this Tyr is conserved between RRM2B variants including ccRRM2Bii and mouse RRM2B. When we

used recombinant RRM2B-Y241H, we observed that the enzymatic activity of the protein was lower both in normoxia and in hypoxia. This could be supportive of our hypothesis that Y241 is important for the overall reductase activity of RRM2B.

In order to test if H_2O_2 influences RRM2B activity in hypoxia, we attempted to perform *in vitro* experiments in the presence or absence of H_2O_2 . We observed that reductase activity of R1/R2B was not particularly affected by the presence of H_2O_2 in normoxia, in contrast to R1/R2, which lost activity when H_2O_2 was added. This verifies that only RRM2B is capable of resisting oxidative stress by breaking down H_2O_2 . Unfortunately, when we tried to perform the same experiments in hypoxia, we could not draw any conclusions, primarily due to technical and experimental limitations (very short half life of H_2O_2 and difficulties in conducting the H_2O_2 experiments in the hypoxic chamber).

Nevertheless, it would be interesting to investigate the hypothesis that some oxygen-requiring proteins have specific adaptations to facilitate prolonged activity in hypoxic conditions and the possibility that they may even create their own oxygen in such conditions. For example, another oxygen-requiring protein induced in hypoxia, which when silenced results in detrimental consequences for cell fate, specifically in hypoxic cells, is the squalene epoxidase enzyme (Haider et al., 2016). This enzyme is involved in cholesterol biosynthesis and requires oxygen as a co-factor for its enzymatic reaction. It has not been reported how this enzyme maintains its function in hypoxia or indeed if it has catalase activity. Our study of RRM2B may well shed light on the general mechanisms employed by enzymes, which require oxygen but are needed to function in hypoxia.

6.4 RRM2B as a clinical target

Studies using RRM2B knock-out mice have shown that *RRM2B* deficiency causes higher rates of spontaneous mutations, chromosomal instability and chronic inflammation-related tumour formation, and almost all the mice die early due to renal failure (Chang et al., 2013; Kimura et al., 2003). In addition, depletion of RRM2B has been correlated with growth inhibition and enhanced sensitivity to chemo- and radio-therapy in different cancer cell lines (Cho and Yen, 2016). Moreover, TCGA analysis has shown that copy number changes correlated with increased *RRM2B* expression in many different cancers including breast, colon, ovarian, prostate and uterine cancers as well as glioblastoma, therefore suggesting a potential role for RRM2B as a tumour promoter. Although the role of RRM2B in tumourigenesis and mutagenesis is still controversial, increased *RRM2B* expression has been reported in many different cancers (Aye et al., 2015). These data collectively support the hypothesis that RRM2B could be an attractive anticancer target.

In agreement, our data suggest that targeting R1/R2B activity specifically in hypoxic tumour cells might be an effective therapeutic strategy. The RNR holoenzyme is a well-established therapeutic target, with a number of RNR inhibitors (such as gemcitabine, clofarabine, hydroxyurea and triapine) already used in the clinic (Chapter 1.5.7). Designing specific RRM2B inhibitors has long been proposed, due to its pivotal role in supplying dNTPs during DNA repair (Kimura et al., 2003). However, the high sequence homology and the similarities in the mechanism of action with RRM2, are challenging obstacles for the design of specific inhibitors. Interestingly, new approaches such as genetic strategies represent a promising avenue for the future drug development. For example, delivery of siRNA against RRM2 by phosphorothionate oligodeoxynucleotides (GTI-2040) is in clinical trials for various solid tumours (Juhász et al., 2006; Lee et al., 2003; Leighl et al., 2009; Oh and Park, 2009). A similar approach could be modified to target RRM2B. It is tempting to

speculate that an agent specifically targeting RRM2B could be employed alongside patient stratification to identify those with significant tumour hypoxia and be used in combination with chemo- and radiotherapy. In addition, a specific anti-RRM2B drug could be used in combination with anti-angiogenic therapy, which is known to increase the hypoxic fraction in tumours (McIntyre and Harris, 2015). In general, many different studies including this one support the idea that inhibiting RRM2B may be an effective way to target the most aggressive and treatment resistant fraction of tumours.

APPENDICES

Appendix I

Supporting material for construction of CRISPR-Cas9 RKO^{RRM2B-/-} cell line

A. Identification of the *RRM2B* transcript variants in RKO cells

A.1 Primers for identifying the different variants (Table 2.15)

Homo sapiens ribonucleotide reductase M2 B (TP53 inducible) (RRM2B), transcript variant 1, mRNA

NCBI Reference Sequence: NM_015713.4

ATGGGCGACCCGAAAGGCCGGAAGCGGGCCGGCTGGATCAGGATGAGAGATCATCTTCAGACACCAACG
 AAAGTGAAATAAAGTCAAATGAAGAGCCACTCCTAAGAAAAGATTCTCGCCGGTTTGTATCTTTCCAAT
 CCAGTACCCTGATATTTGGAAAATGTATAAACAGGCACAGGCTTCCTTCTGGACAGCAGAAGAGGTCGAC
 TTATCAAAGGATCTCCCTCACTGGAACAAGCTTAAAGCAGATGAGAAGTACTTCATCTCTCACATCTTAG
 CCTTTTTCAGCCAGTGATGGAATTGTAAATGAAAATTGGTGGAGCGCTTTAGTCAGGAGGTGCAGGT
 TCCAGAGGCTCGCTGTTTCTATGGCTTTCAAATTCTCATCGAGAATGTTCACTCAGAGATGTACAGTTTG
 CTGATAGACACTTACATCAGAGATCCCAAGAAAAGGGAATTTTATTTAATGCAATTGAAACCATGCCCT
 ATGTTAAGAAAAAAGCAGATTTGGGCCTTGGGATGGATAGCAGATAGAAAATCTACTTTTGGGAAAGAGT
 GGTGGCCTTTGCTGCTGTAGAAGGAGTTTTCTTCTCAGGATCTTTTGTGCTATATTCTGGCTAAAGAAG
 AGAGGTCTTATGCCAGGACTCACTTTTTCCAATGAAGTCACTCAGCAGAGATGAAGGACTTCACTGTGACT
 TTGCTTGCCTGATGTTCCAATACTTAGTAAATAAGCCTTCAGAAGAAAGGGTCAGGGAGATCATTGTTGA
 TGCTGTCAAAATTGAGCAGGAGTTTTTAACAGAAGCCTTGCCAGTTGGCCTCATTGGAATGAATTGCATT
 TTGATGAAACAGTACATTGAGTTTGTAGCTGACAGATTACTTGTGGAACCTGGATTTCTCAAAGGTTTTTC
 AGGCAGAAAATCCTTTTGATTTTATGGAACATTTCTTTAGAAGGAAAAACAAATTTCTTTGAGAAACG
 AGTTTCAGAGTATCAGCGTTTTGCAGTTATGGCAGAAACACAGATAACGTCTTCACCTTGGATGCAGAT
 TTTTAA

Homo sapiens ribonucleotide reductase M2 B (TP53 inducible) (RRM2B), transcript variant 2, mRNA

NCBI Reference Sequence: NM_001172477.1

ATGTTGCTGTTGCGTCTTCCCCCTCACCGCAGTCAGGCCAGCCGTTAGATTGCAAGTTGCAGGACCGCT
 GTAGGAAATGTTATTCGCCGCGGTCAGGACAGGCTGTCCGCCCGCCCTCGCCGCAGCCTGGCTTCGTG
 TTGCGAGCGCCGGGAGGCCGTCCCCGGGGAGGGCGGAGGAAGGAGCTGACTTTGGGTTTTCGTCCCGCT

CGCTGCTCTGCCCCGGGGCCAGCCAAGGACGACGCTTGGAGGCCTCAGGCCGGGAGATCATCTTCAGACA
 CCAACGAAAGTGAAATAAAGTCAAAATGAAGAGCCACTCCTAAGAAAAGAGTTCTCGCCGGTTTGTATCTT
 TCCAATCCAGTACCCTGATATTTGGAAAATGTATAAACAGGCACAGGCTTCCTTCTGGACAGCAGAAGAG
 GTCGACTTATCAAAGGATCTCCCTCACTGGAACAAGCTTAAAGCAGATGAGAAGTACTTCATCTCTCACA
 TCTTAGCCTTTTTTGCAGCCAGTGATGGAATTGTAAATGAAAATTGGTGGAGCGCTTTAGTCAGGAGGT
 GCAGGTTCCAGAGGCTCGCTGTTTCTATGGCTTTCAAATTCTCATCGAGAATGTTCACTCAGAGATGTAC
 AGTTTGCTGATAGACACTTACATCAGAGATCCCAAGAAAAGGGAATTTTTATTTAATGCAATTGAAACCA
 TGCCCTATGTTAAGAAAAAAGCAGATTGGGCCCTTGGCATGGATAGCAGATAGAAAATCTACTTTTGGGGA
 AAGAGTGGTGGCCTTTGCTGCTGTAGAAGGAGTTTTCTTCTCAGGATCTTTTGCTGCTATATTCTGGCTA
 AAGAAGAGAGGTCTTATGCCAGGACTCACTTTTTCCAATGAACTCATCAGCAGAGATGAAGGACTTCACT
 GTGACTTTGCTTGCCTGATGTTCCAATACTTAGTAAATAAGCCTTCAGAAGAAAGGGTCAGGGAGATCAT
 TGTGATGCTGTCAAAATTGAGCAGGAGTTTTTAACAGAAGCCTTGCCAGTTGGCCTCATTGGAATGAAT
 TGCATTTTGATGAAACAGTACATTGAGTTTGTAGCTGACAGATTACTTGTGGAACCTTGATTCTCAAAGG
 TTTTTCAGGCAGAAAATCCTTTTGATTTTATGAAAACATTTCTTTAGAAGGAAAAACAAATTTCTTTGA
 GAAACGAGTTTCAGAGTATCAGCGTTTTGCAGTTATGGCAGAAACCACAGATAACGTCTTCACCTTGGAT
 GCAGATTTTTAA

Homo sapiens ribonucleotide reductase M2 B (TP53 inducible) (RRM2B), transcript variant 3, mRNA

NCBI Reference Sequence: NM_001172478.1

ATGGGCGACCCGGAAGGCCGGAAGCGGCCGGGCTGGATCAGGATGAGGTCGACTTATCAAAGGATCTCC
 CTCACTGGAACAAGCTTAAAGCAGATGAGAAGTACTTCATCTCTCACATCTTAGCCTTTTTCAGCCAG
 TGATGGAATTGTAAATGAAAATTGGTGGAGCGCTTTAGTCAGGAGGTGCAGGTTCCAGAGGCTCGCTGT
 TTCTATGGCTTTCAAATTCTCATCGAGAATGTTCACTCAGAGATGTACAGTTTGCTGATAGACACTTACA
 TCAGAGATCCCAAGAAAAGGGAATTTTTATTTAATGCAATTGAAACCATGCCCTATGTTAAGAAAAAAGC
 AGATTGGGCCCTTGGCATGGATAGCAGATAGAAAATCACTTTTGGGGAAGAGTGGTGGCCTTTGCTGCT
 GTAGAAGGAGTTTTCTTCTCAGGATCTTTTGCTGCTATATTCTGGCTAAAGAAGAGAGGTCTTATGCCAG
 GACTCACTTTTTCCAATGAACTCATCAGCAGAGATGAAGGACTTCACTGTGACTTTGCTTGCCTGATGTT
 CCAATACTTAGTAAATAAGCCTTCAGAAGAAAGGGTCAGGGAGATCATTGTTGATGCTGTCAAAATTGAG
 CAGGAGTTTTTAACAGAAGCCTTGCCAGTTGGCCTCATTGGAATGAATTGCATTTTGATGAAACAGTACA
 TTGAGTTTGTAGCTGACAGATTACTTGTGGAACCTTGATTCTCAAAGGTTTTTCAGGCAGAAAATCCTTT
 TGATTTTATGAAAACATTTCTTTAGAAGGAAAAACAAATTTCTTTGAGAAACGAGTTTCAGAGTATCAG
 CGTTTTGCAGTTATGGCAGAAACCACAGATAACGTCTTCACCTTGGATGCAGATTTTTAA

*Blue indicates primers RRM2B-var. 1-For & RRM2B-var. 1-Rev

Green indicates primers RRM2B-var. 2-For & RRM2B-var. 2-Rev

Yellow indicates primer RRM2B-var. 1&3-Rev

**letters red and blue indicate RRM2B primers that recognise all variants

A.2 Validation of all three *RRM2B* transcript variants in RKO cells

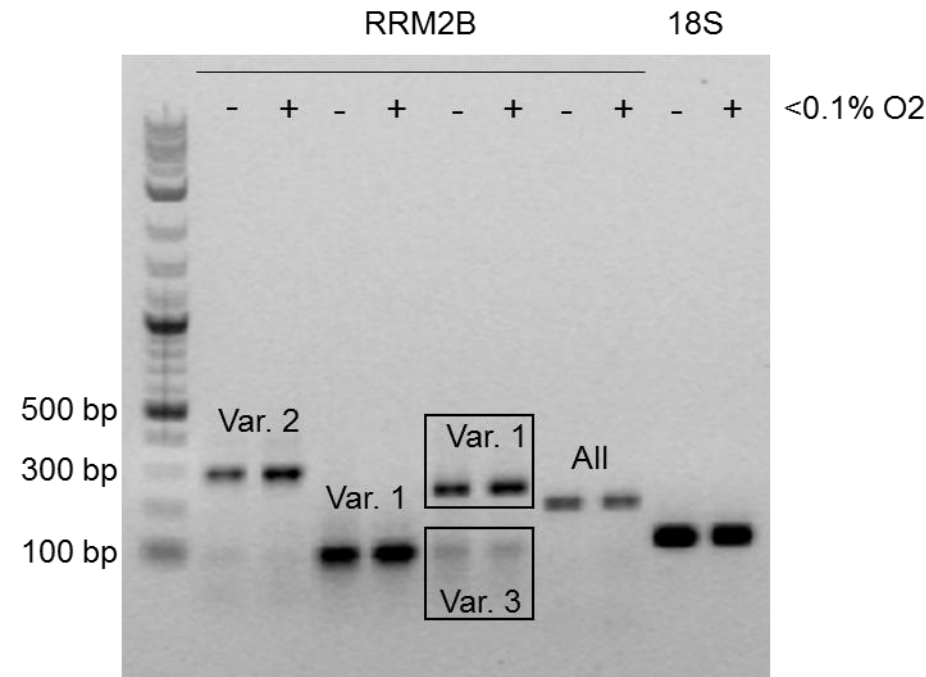
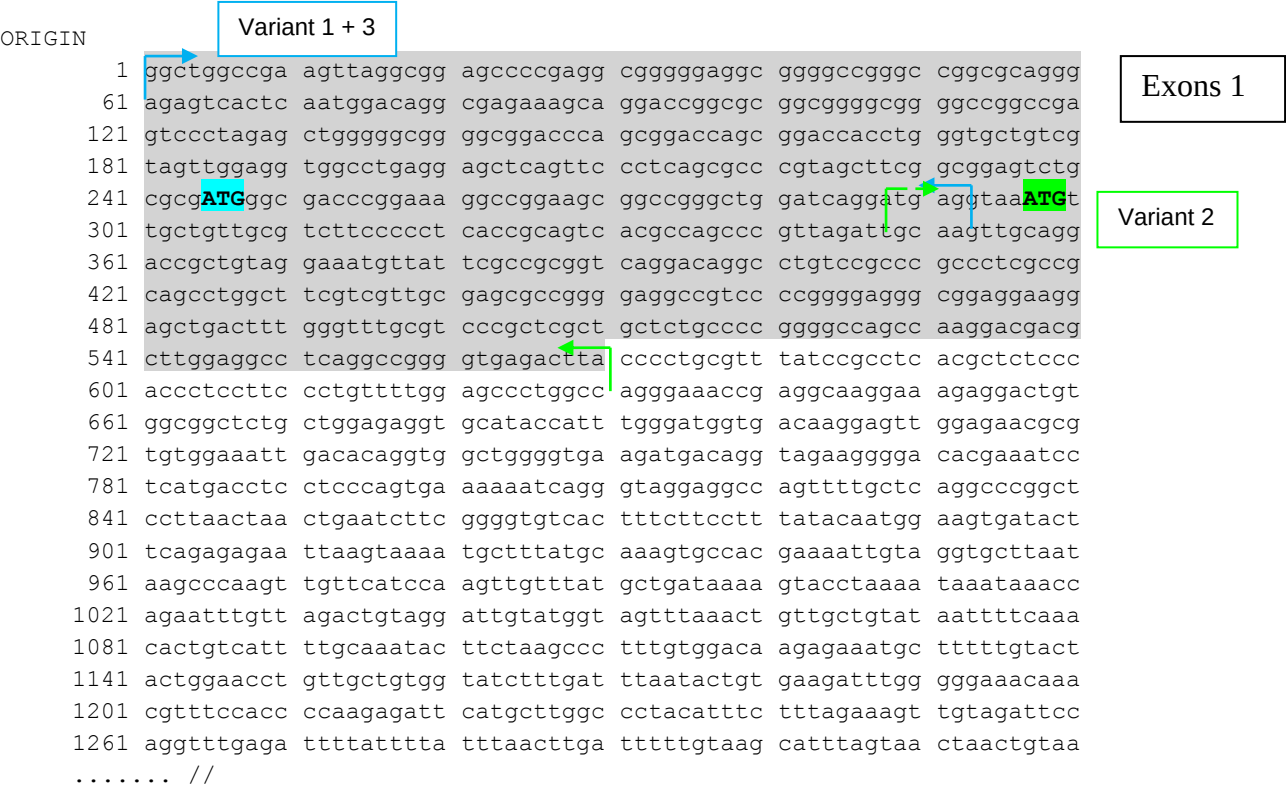


Figure I.A.1 RKO cells have all of the three transcript variants of *RRM2B* gene both in normoxia and in hypoxia

B. FASTA of the *RRM2B* gene locus (NCBI Reference Sequence: NG_016617.1)



C. Guide sequences to target exon 1 of *RRM2B* variants

Exon1:

```
gtccctagagctgggggcggggcgaccagcggaccagcggaccacctgggtgctgctgtagttggaggtggcctgaggagc
tcagtccctcagcgcccgtagcttccggcggagtgctgcgc^gATGggcgaccggaaggccggaagcgggcggtggatca
ggatgaggttaaATGttgctgttgctgtcttccccctcaccgcagtcacgccagcccgttagattgcaagttgcaggaccgctgt
aggaaatgttatttcgcccgcg^gtcaggacaggcctgtccgcccgccctcgccgcagcctggcttcgtcgttgcgagcgccggg
```

(^) indicates the position of the DSB

Primers:

Exon 1 (Variants 1 & 3)

<http://crispr.mit.edu/guides/1602824144488308>

Rrm2b-CRISPR 1/3-For 5'-CACCGTTCGGCGGAGTCTGCGCGAT
Rrm2b-CRISPR 1/3-Rev 5'-AAACATCGCGCAGACTCCGCCGAAC

Exon 1 (Variant 2)

<http://crispr.mit.edu/guides/1206707132539070>

Rrm2b-CRISPR 2-For 5'-CACCGAAATGTTATTTCGCCGCGGTC
Rrm2b-CRISPR 2-Rev 5'-AAACGACCGCGGCGGAATAACATTTC

D. Western blot analysis of 24 clones picked targeting *RRM2B* exons 1

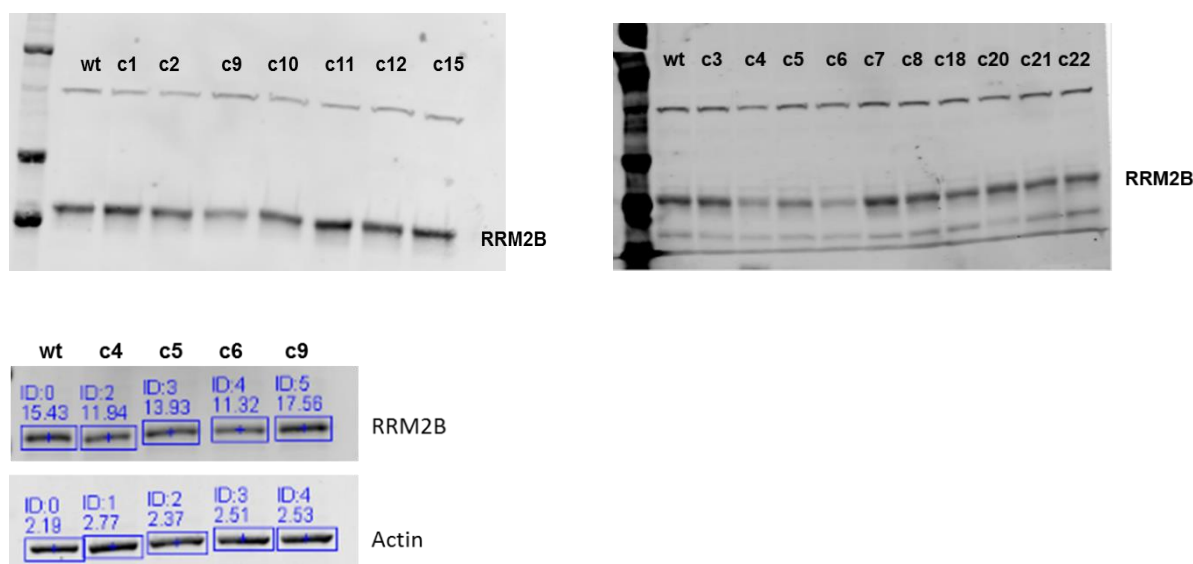


Figure I.A.2 Partial loss of RRM2B expression after targeting *RRM2B* exons 1

The most promising RKO clones were picked and the amount of the expressed RRM2B was calculated compared to β -actin loading control.

E. Guide sequence to target *RRM2B* exon 4

Exon4:

gtggagcgccttttagtcaggaggtgcaggttccagaggctcgcgtgttt[^]ctatggcctttcaaattctcatcgagaatgttcact
cagagatgtacagtttgctgatagacacttacatcagagatcccaagaaaag

([^]) indicates the position of the DSB

Primers:

Exon 4 (All variants)

<http://crispr.mit.edu/nick/2963088543231492>

Rrm2b-CRISPR 4-For 5'-CACCGCCAGAGGCTCGCTGTTTCTA

Rrm2b-CRISPR 4-Rev 5'-AAACTAGAAACAGCGAGCCTCTGGC

F. Western blot analysis of 20 clones picked targeting *RRM2B* exon 4

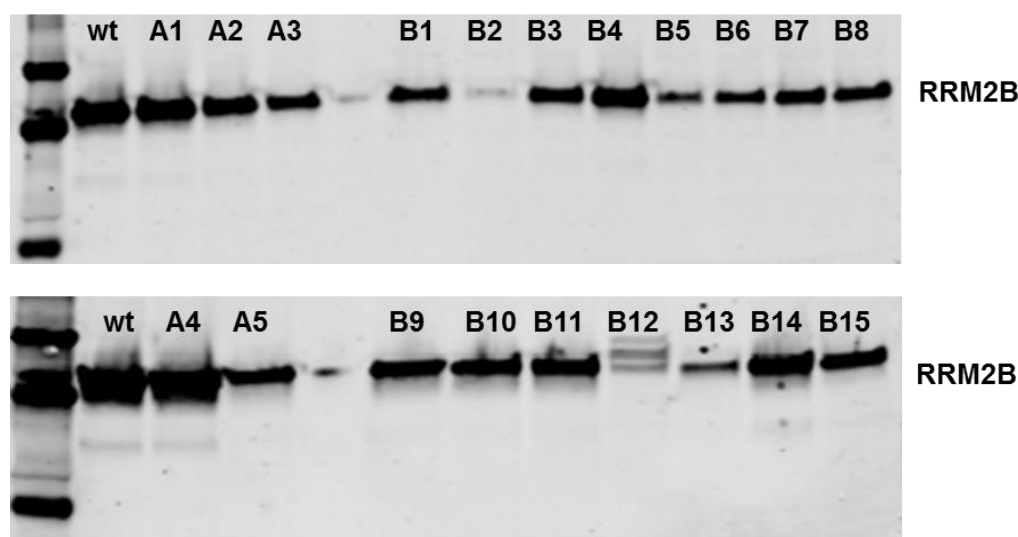


Figure I.A.3 Clones testing for *RRM2B* depletion after targeting *RRM2B* exon 4

One RKO-*RRM2B* knock out cell line (B2) was found from the ones tested. Further characterisation followed by sequencing analysis and comparison with WT RKO cells.

G. Sequencing validation of the RKO^{RRM2B-/-} cell line

actcaatggacagggcgagaaagcaggaccggcgcgggcgggcgggcgccgagtcctagagctggggcgggcgggcgacc
agcggaccagcggaccacctgggtgctgtcgtagttggaggtggcctgaggagctcagttccctcagcggcctagcttcggc
ggagtctgcgc

(Deletion)

gATGggcgacccggaaaggccggaagcggcggggctggatcaggatgaggtaaATGctgctgttgcttcttccccctcaccg
cagtcagccagcccgtagattgcaagttgcaggacgg_ctgta_ggaaatgttattcgccgcggtcaggacaggcctgtcc
(Mutations)

gcccgcctcgccgagcctggcttcgtcgttgcgagcgccggggaggccgtccccggggagggcgagggaaggagctgactt
tgggtttgctcccgctcgtgctctgccccggggccagccaaggacgacgcttgaggcctcaggccgggggtgagacttacc
cctgcgtttatccgcctcacgctctcccaccctccttcctgttttggagccctggccagggaaccgaggcaaggaaagagg
actgtggc

Appendix II

Supporting material of the effects of RRM2B inhibition

In order to validate that our siRRM2B results are not falsely positive due to siRNA off-target effects, a second siRNA sequence targeting RRM2B (siRRM2B#2) was used and indicative experiments were performed.

A. Depletion of RRM2B disrupts DNA replication in hypoxia

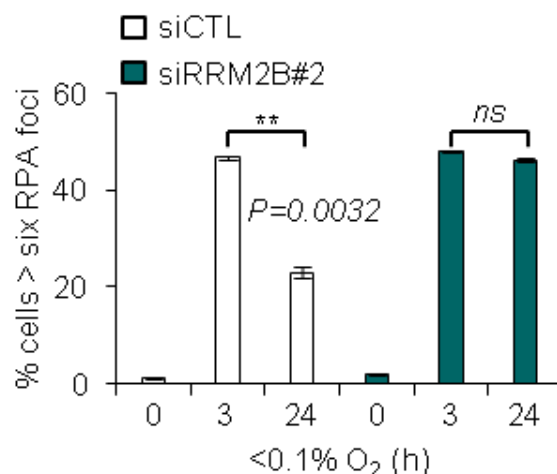


Figure II.A.1 Inhibition of RRM2B results in persist formation of RPA foci

RKO cells were treated with either siRRM2B#2 or siSCR and exposed to <0.1% O₂ for the times indicated. IF staining and quantification of RPA32 foci followed. Induction of ssDNA was quantified by counting cells with more than six RPA32 foci. Data show mean $\pm$ s.e.m. from n=3 biological experiments. Two-tailed, paired Student's t-test was applied.

B. Depletion of RRM2B leads to DNA damage

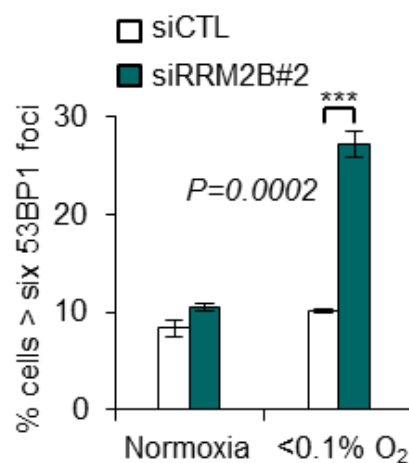


Figure II.B.1 Inhibition of RRM2B results in double strand breaks (DSBs)

RKO cells were treated with either siRRM2B#2 or siSCR and exposed to <0.1% O₂ for the times indicated. IF staining was carried out and quantification of the percentage of cells with 53BP1 foci followed. Induction of DSBs was quantified by counting cells with more than six 53BP1 foci. Data show mean $\pm$ s.e.m. from $n=3$ biological experiments. Two-tailed, paired Student's *t*-test was applied.

Appendix III

Supporting material for Molecular Dynamics (MD) simulations

A. Molecular Dynamics (MD) simulations

System setup: MD simulations of O₂ transport on hRRM2B (PDB id 3HF1; resolution 2.60 Å) (Smith et al., 2009), and hRRM2 (PDB id 3VPN; resolution 2.25 Å) were performed. The RRM2B structure comprises residues S29 to F311. The 3VPN X-ray structure is that of the E106D variant of native RRM2, and comprises residues M65 to M350. 200 randomly chosen water molecules outside the solvated protein were replaced by 200 O₂ molecules (0.5 M). The system was solvated in a box of dimensions (80, 80, 92) Å using the Solvate plug-in of VMD (Humphrey et al., 1996), with the total system size comprising ~61,000 atoms. In accordance with the Uniprot sequence alignment two variants of native RRM2B were prepared with the Mutate plugin in VMD (Humphrey et al., 1996), namely Y164C and K37E/K151E.

B. MD equilibration protocol

Calculations were done with NAMD 2.9 (Phillips et al., 2005), using the CHARMM 27 protein force-field (Brooks et al., 2009) with the CMAP correction (Buck et al., 2006), together with the TIP3P water model (Jorgensen et al., 1983). Oxygen LJ parameters were taken from CHARMM (Cohen et al., 2006; Cohen et al., 2005). After 1,000 steps of Conjugate-Gradient minimization, four classical molecular dynamics (MD) simulations of the O₂-containing solvated protein were carried out for 300 ns in the NPT ensemble. The high oxygen concentration was necessary to ensure sufficient sampling of protein cavities by O₂

on the accessible time scale of current simulations. It was verified that no gas clustering occurred in the solvent at this concentrations. The particle mesh Ewald (PME) algorithm was used for evaluation of electrostatics interactions beyond 12 Å, with a PME grid spacing of 1 Å, and NAMD defaults for spline and κ values (Darden et al., 1993). A cut-off at 12 Å was applied to non-bonded forces, both electrostatics and Van der Waals forces were smoothly switched off between the cut-off distance of 12 Å, and the switching distance of 10 Å, using the default NAMD switching function. A Verlet neighbour list with pairlist distance of 14 Å was used to only evaluate non-bonded neighbouring forces within the pairlist distance (Verlet, 1967). The lengths of covalent bonds involving hydrogen atoms were constrained by the SETTLE algorithm (Miyamoto and Kollman, 1992) in order to be able to use a 2-fs time-step. The multi time step algorithm Verlet-I/r-RESPA (Tuckerman et al., 1992; Verlet, 1967) was used to integrate the equations of motion. Non-bonded short-range forces were computed for each time step, while long-range electrostatic forces were updated every 2 time steps. The pressure was kept at 1 atm by the Nosé-Hoover Langevin piston (Langevin, 1908; Nosé, 1984a; Nosé, 1984b), with a damping time constant of 50 fs and a period of 100 fs. The temperature was maintained at 300 K by coupling the system to a Langevin thermostat, with a damping coefficient of 1 ps⁻¹. A total of 1.2 μ s of MD simulations were accrued.

C. Code availability

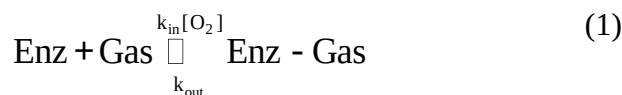
The standard MPI version 2.9 of the MD simulation code NAMD was employed (Phillips et al., 2005). A free license can be obtained at <http://www.ks.uiuc.edu/Research/namd/>. Version 1.9 of Visual Molecular Dynamics (VMD) was employed for analysis, with license obtained at <http://www.ks.uiuc.edu/Research/vmd/>.

D. MD simulation analysis

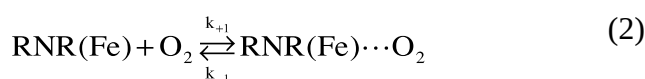
The Solvent-Accessible Surface Area (SASA) of both proteins RRM2B and RRM2 was calculated using the POPS (Parameter Optimised Surfaces) algorithm (Cavallo, 2003;

Fraternali and Van Gunsteren, 1996). The bending angle of helices was estimated using Bendix in VMD (Dahl et al., 2012).

The Diffusion-Reaction Model (Wang et al., 2013) presented in equation (1) was adopted to describe the diffusion of O₂ into the RNR active site:



This is a kinetic two-step model consisting of an initial diffusion step of the gas molecule, followed by metal-gas molecule chemical reaction step (Wang et al., 2011; Wang and Blumberger, 2012). Here, a classical force-field (FF) was employed, and only the diffusion and binding of O₂ gas molecules to the RNR iron centre are modelled:



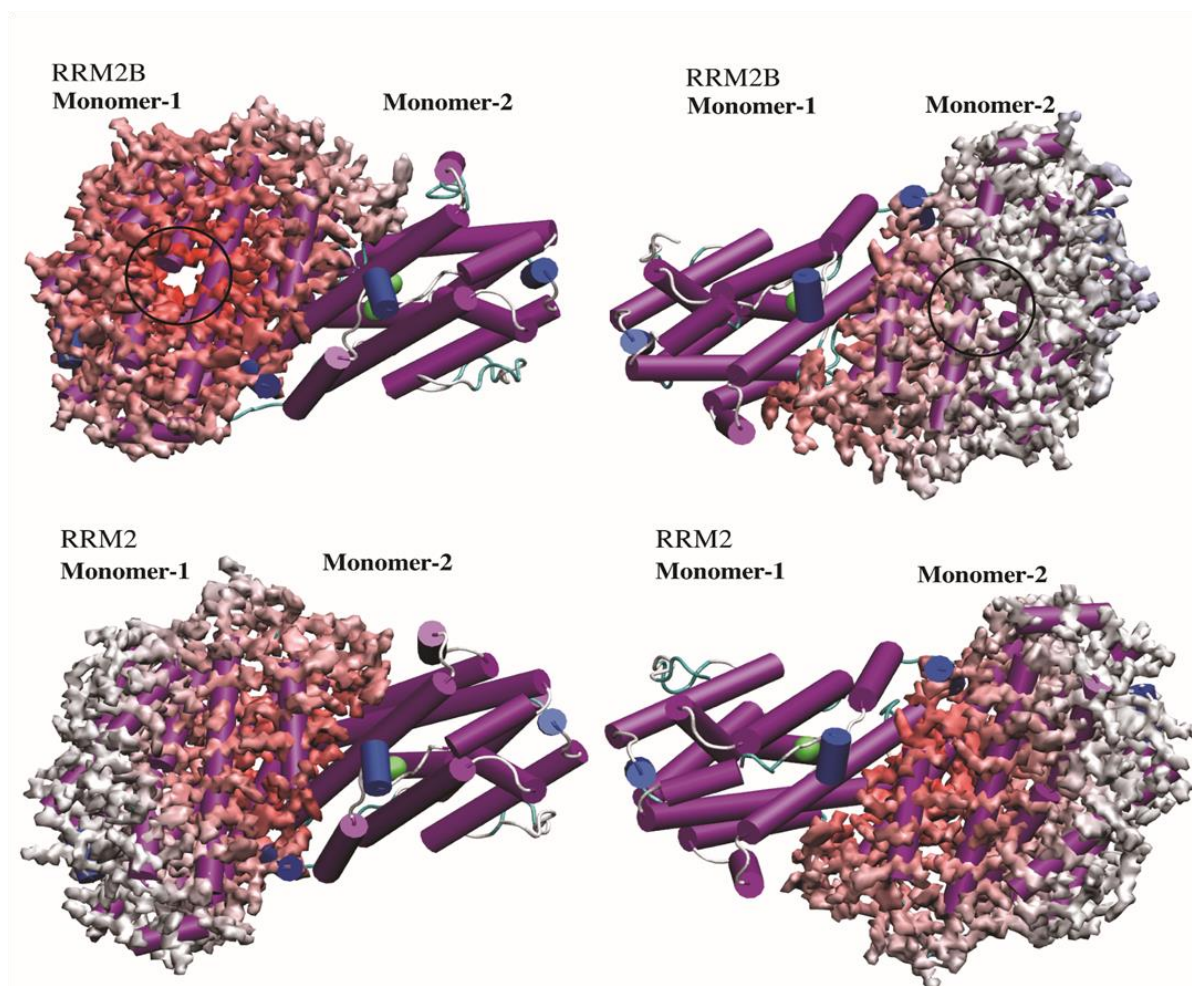
The simulation of O₂ diffusing in the solvated RNR enzymes yields diffusion statistics that enable the construction of a probability density map of the O₂-distribution within RNR (RRM2B or RRM2). This distribution is then divided into a set of M discrete protein cluster states that correspond to the probability maxima. Two parameters are used to characterise the diffusion of O₂. Firstly, a distance criterion is established to determine when an oxygen molecule has entered into the active site of hRRM2B and hRRM2, defined as the difference between the centre-of-mass (COM) of the iron ion and the O₂ molecule. Secondly, cut-off criteria of 6 Å, 8 Å, 9 Å and 10 Å were employed to generate statistics of the O₂ entering. An O₂ molecule is counted as having entered the cluster if $d_{\text{COM}}(\text{O}_2\text{-Fe}) < c$ where c is 6, 8, 9 or 10 Å.

Appendix IV

Supporting material for structural differences between RRM2 and RRM2B

The open phenylalanine channel in RRM2B protein

The switch of cysteine (C202) in RRM2 to tyrosine (Y164) in RRM2B, results in an open phenylalanine channel specifically in RRM2B.



Appendix V

Published papers from this thesis

- 1) **Foskolou IP**, Jorgensen C*, Leszczynska KB, Olcina MM, Tarhonskaya H, Haisma B, D'Angiolella V, Myers WK, Domene C, Flashman E and Hammond EM., Ribonucleotide reductase requires subunit switching in hypoxia to maintain DNA replication. *Mol Cell* (2017) Apr 11;(17) 30172-7. Reprinted from *Molecular Cell* with Creative Commons Attribution License (CC BY).
- 2) **Foskolou IP**, Biasoli D, Olcina MM, Hammond EM., Measuring DNA Replication in Hypoxic Conditions. *Adv Exp Med Biol.* 2016; 899:11-25. Reprinted from Springer with license number: 3957120004997.
- 3) Leszczynska KB, **Foskolou IP**, Abraham AG, Anbalagan S, Tellier C, Haider S, Span PN, O'Neill EE, Buffa FM, Hammond EM., Hypoxia-induced p53 modulates both apoptosis and radiosensitivity via AKT. *J Clin Invest.* 2015 Jun;125(6):2385-98. Reprinted from *Journal of Clinical Investigation* with license number: 3957120998100.
- 4) Olcina MM, **Foskolou IP**, Anbalagan S, Senra JM, Pires IM, Jiang Y, Ryan AJ, Hammond EM., Replication stress and chromatin context link ATM activation to a role in DNA replication. *Mol Cell.* 2013 Dec 12;52(5):758-66. Reprinted from *Molecular Cell* with Creative Commons Attribution License (CC BY).

References

- Aguilera, A., and T. Garcia-Muse. 2012. R loops: from transcription byproducts to threats to genome stability. *Mol Cell*. 46:115-124.
- Aguilera, A., and T. Garcia-Muse. 2013. Causes of genome instability. *Annu Rev Genet*. 47:1-32.
- Aird, K.M., G. Zhang, H. Li, Z. Tu, B.G. Bitler, A. Garipov, H. Wu, Z. Wei, S.N. Wagner, M. Herlyn, and R. Zhang. 2013. Suppression of nucleotide metabolism underlies the establishment and maintenance of oncogene-induced senescence. *Cell Rep*. 3:1252-1265.
- Alarcon, R., C. Koumenis, R.K. Geyer, C.G. Maki, and A.J. Giaccia. 1999. Hypoxia induces p53 accumulation through MDM2 down-regulation and inhibition of E6-mediated degradation. *Cancer Res*. 59:6046-6051.
- Allen, M.P. 2004. Introduction to Molecular Dynamics Simulation. NIC.
- Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *Journal of molecular biology*. 215:403-410.
- Alzu, A., R. Bermejo, M. Begnis, C. Lucca, D. Piccini, W. Carotenuto, M. Saponaro, A. Brambati, A. Cocito, M. Foiani, and G. Liberi. 2012. Senataxin associates with replication forks to protect fork integrity across RNA-polymerase-II-transcribed genes. *Cell*. 151:835-846.
- Anderson, D.D., C.F. Woeller, E.P. Chiang, B. Shane, and P.J. Stover. 2012. Serine hydroxymethyltransferase anchors de novo thymidylate synthesis pathway to nuclear lamina for DNA synthesis. *J Biol Chem*. 287:7051-7062.
- Ando, N., E.J. Brignole, C.M. Zimanyi, M.A. Funk, K. Yokoyama, F.J. Asturias, J. Stubbe, and C.L. Drennan. 2011. Structural interconversions modulate activity of Escherichia coli ribonucleotide reductase. *Proc Natl Acad Sci U S A*. 108:21046-21051.
- Anglana, M., F. Apiou, A. Bensimon, and M. Debatisse. 2003. Dynamics of DNA replication in mammalian somatic cells: nucleotide pool modulates origin choice and interorigin spacing. *Cell*. 114:385-394.
- Angus, S.P., L.J. Wheeler, S.A. Ranmal, X. Zhang, M.P. Markey, C.K. Mathews, and E.S. Knudsen. 2002. Retinoblastoma tumor suppressor targets dNTP metabolism to regulate DNA replication. *J Biol Chem*. 277:44376-44384.
- Aravind, L., Y.I. Wolf, and E.V. Koonin. 2000. The ATP-cone: an evolutionarily mobile, ATP-binding regulatory domain. *J Mol Microbiol Biotechnol*. 2:191-194.
- Arnautov, A., and M. Dasso. 2014. Enzyme regulation. IRBIT is a novel regulator of ribonucleotide reductase in higher eukaryotes. *Science*. 345:1512-1515.
- Aye, Y., E.J. Brignole, M.J. Long, J. Chittuluru, C.L. Drennan, F.J. Asturias, and J. Stubbe. 2012a. Clofarabine targets the large subunit (alpha) of human ribonucleotide reductase in live cells by assembly into persistent hexamers. *Chem Biol*. 19:799-805.
- Aye, Y., M. Li, M.J. Long, and R.S. Weiss. 2015. Ribonucleotide reductase and cancer: biological mechanisms and targeted therapies. *Oncogene*. 34:2011-2021.
- Aye, Y., M.J. Long, and J. Stubbe. 2012b. Mechanistic studies of semicarbazone triapine targeting human ribonucleotide reductase in vitro and in mammalian cells: tyrosyl radical quenching not involving reactive oxygen species. *J Biol Chem*. 287:35768-35778.
- Aye, Y., and J. Stubbe. 2011. Clofarabine 5'-di and -triphosphates inhibit human ribonucleotide reductase by altering the quaternary structure of its large subunit. *Proc Natl Acad Sci U S A*. 108:9815-9820.

- Barlow, J.H., R.B. Faryabi, E. Callen, N. Wong, A. Malhowski, H.T. Chen, G. Gutierrez-Cruz, H.W. Sun, P. McKinnon, G. Wright, R. Casellas, D.F. Robbiani, L. Staudt, O. Fernandez-Capetillo, and A. Nussenzweig. 2013. Identification of early replicating fragile sites that contribute to genome instability. *Cell*. 152:620-632.
- Bartkova, J., Z. Horejsi, K. Koed, A. Kramer, F. Tort, K. Zieger, P. Guldberg, M. Sehested, J.M. Nesland, C. Lukas, T. Orntoft, J. Lukas, and J. Bartek. 2005. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature*. 434:864-870.
- Beck, H., V. Nahse-Kumpf, M.S. Larsen, K.A. O'Hanlon, S. Patzke, C. Holmberg, J. Mejlvang, A. Groth, O. Nielsen, R.G. Syljuasen, and C.S. Sorensen. 2012. Cyclin-dependent kinase suppression by WEE1 kinase protects the genome through control of replication initiation and nucleotide consumption. *Mol Cell Biol*. 32:4226-4236.
- Bencokova, Z., M.R. Kaufmann, I.M. Pires, P.S. Lecane, A.J. Giaccia, and E.M. Hammond. 2009. ATM activation and signaling under hypoxic conditions. *Mol Cell Biol*. 29:526-537.
- Bepler, G., S. Sharma, A. Cantor, A. Gautam, E. Haura, G. Simon, A. Sharma, E. Sommers, and L. Robinson. 2004. RRM1 and PTEN as prognostic parameters for overall and disease-free survival in patients with non-small-cell lung cancer. *J Clin Oncol*. 22:1878-1885.
- Bermejo, R., M.S. Lai, and M. Foiani. 2012. Preventing replication stress to maintain genome stability: resolving conflicts between replication and transcription. *Mol Cell*. 45:710-718.
- Bester, A.C., M. Roniger, Y.S. Oren, M.M. Im, D. Sarni, M. Chaoat, A. Bensimon, G. Zamir, D.S. Shewach, and B. Kerem. 2011. Nucleotide deficiency promotes genomic instability in early stages of cancer development. *Cell*. 145:435-446.
- Bianchi, V., S. Borella, C. Rampazzo, P. Ferraro, F. Calderazzo, L.C. Bianchi, S. Skog, and P. Reichard. 1997. Cell cycle-dependent metabolism of pyrimidine deoxynucleoside triphosphates in CEM cells. *J Biol Chem*. 272:16118-16124.
- Bianchi, V., E. Pontis, and P. Reichard. 1986a. Changes of deoxyribonucleoside triphosphate pools induced by hydroxyurea and their relation to DNA synthesis. *J Biol Chem*. 261:16037-16042.
- Bianchi, V., E. Pontis, and P. Reichard. 1986b. Interrelations between substrate cycles and de novo synthesis of pyrimidine deoxyribonucleoside triphosphates in 3T6 cells. *Proc Natl Acad Sci U S A*. 83:986-990.
- Bianco, J.N., J. Poli, J. Saksouk, J. Bacal, M.J. Silva, K. Yoshida, Y.L. Lin, H. Tourriere, A. Lengronne, and P. Pasero. 2012. Analysis of DNA replication profiles in budding yeast and mammalian cells using DNA combing. *Methods*. 57:149-157.
- Bickler, P.E., and L.T. Buck. 2007. Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. *Annu Rev Physiol*. 69:145-170.
- Bindra, R.S., M.E. Crosby, and P.M. Glazer. 2007. Regulation of DNA repair in hypoxic cancer cells. *Cancer Metastasis Rev*. 26:249-260.
- Birner, P., B. Gatterbauer, G. Oberhuber, M. Schindl, K. Rossler, A. Proding, H. Budka, and J.A. Hainfellner. 2001a. Expression of hypoxia-inducible factor-1 alpha in oligodendrogliomas: its impact on prognosis and on neoangiogenesis. *Cancer*. 92:165-171.
- Birner, P., M. Schindl, A. Obermair, G. Breitenacker, and G. Oberhuber. 2001b. Expression of hypoxia-inducible factor 1alpha in epithelial ovarian tumors: its impact on prognosis and on response to chemotherapy. *Clin Cancer Res*. 7:1661-1668.

- Birner, P., M. Schindl, A. Obermair, C. Plank, G. Breitenecker, and G. Oberhuber. 2000. Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. *Cancer Res.* 60:4693-4696.
- Blow, J.J., X.Q. Ge, and D.A. Jackson. 2011. How dormant origins promote complete genome replication. *Trends Biochem Sci.* 36:405-414.
- Blow, J.J., and P.J. Gillespie. 2008. Replication licensing and cancer--a fatal entanglement? *Nat Rev Cancer.* 8:799-806.
- Bonate, P.L., L. Arthaud, W.R. Cantrell, Jr., K. Stephenson, J.A. Secrist, 3rd, and S. Weitman. 2006. Discovery and development of clofarabine: a nucleoside analogue for treating cancer. *Nat Rev Drug Discov.* 5:855-863.
- Bos, R., P. van der Groep, A.E. Greijer, A. Shvarts, S. Meijer, H.M. Pinedo, G.L. Semenza, P.J. van Diest, and E. van der Wall. 2003. Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer.* 97:1573-1581.
- Bouquet, F., M. Ousset, D. Biard, F. Fallone, S. Dauvillier, P. Frit, B. Salles, and C. Muller. 2011. A DNA-dependent stress response involving DNA-PK occurs in hypoxic cells and contributes to cellular adaptation to hypoxia. *J Cell Sci.* 124:1943-1951.
- Bourdon, A., L. Minai, V. Serre, J.P. Jais, E. Sarzi, S. Aubert, D. Chretien, P. de Lonlay, V. Paquis-Flucklinger, H. Arakawa, Y. Nakamura, A. Munnich, and A. Rotig. 2007. Mutation of RRM2B, encoding p53-controlled ribonucleotide reductase (p53R2), causes severe mitochondrial DNA depletion. *Nat Genet.* 39:776-780.
- Brady, C.A., D. Jiang, S.S. Mello, T.M. Johnson, L.A. Jarvis, M.M. Kozak, D. Kenzelmann Broz, S. Basak, E.J. Park, M.E. McLaughlin, A.N. Karnezis, and L.D. Attardi. 2011. Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. *Cell.* 145:571-583.
- Brahimi-Horn, M.C., and J. Pouyssegur. 2009. HIF at a glance. *J Cell Sci.* 122:1055-1057.
- Branzei, D., and M. Foiani. 2010. Maintaining genome stability at the replication fork. *Nat Rev Mol Cell Biol.* 11:208-219.
- Brischwein, K., M. Engelcke, H.J. Riedinger, and H. Probst. 1997. Role of ribonucleotide reductase and deoxynucleotide pools in the oxygen-dependent control of DNA replication in Ehrlich ascites cells. *Eur J Biochem.* 244:286-293.
- Bristow, R.G., and R.P. Hill. 2008. Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability. *Nat Rev Cancer.* 8:180-192.
- Brooks, B., C. Brooks, A. MacKerell, L. Nilsson, R. Petrella, B. Roux, Y. Won, G. Archontis, C. Bartels, and S. Boresch. 2009. CHARMM: the biomolecular simulation program. *Journal of computational chemistry.* 30:1545-1614.
- Brown, J.M. 1979. Evidence for acutely hypoxic cells in mouse tumours, and a possible mechanism of reoxygenation. *Br J Radiol.* 52:650-656.
- Brown, J.M., and A.J. Giaccia. 1998. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res.* 58:1408-1416.
- Brown, N.C., and P. Reichard. 1969. Role of effector binding in allosteric control of ribonucleoside diphosphate reductase. *J Mol Biol.* 46:39-55.
- Buck, M., S. Bouguet-Bonnet, R. Pastor, and A. MacKerell Jr. 2006. Importance of the CMAP correction to the CHARMM22 protein force field: dynamics of hen lysozyme. *Biophysical Journal.* 90:L36-L38.
- Buckstein, M.H., J. He, and H. Rubin. 2008. Characterization of nucleotide pools as a function of physiological state in Escherichia coli. *J Bacteriol.* 190:718-726.
- Bunz, F., A. Dutriaux, C. Lengauer, T. Waldman, S. Zhou, J.P. Brown, J.M. Sedivy, K.W. Kinzler, and B. Vogelstein. 1998. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science.* 282:1497-1501.

- Burrell, R.A., S.E. McClelland, D. Endesfelder, P. Groth, M.C. Weller, N. Shaikh, E. Domingo, N. Kanu, S.M. Dewhurst, E. Gronroos, S.K. Chew, A.J. Rowan, A. Schenk, M. Sheffer, M. Howell, M. Kschischo, A. Behrens, T. Helleday, J. Bartek, I.P. Tomlinson, and C. Swanton. 2013. Replication stress links structural and numerical cancer chromosomal instability. *Nature*. 494:492-496.
- Cam, H., J.B. Easton, A. High, and P.J. Houghton. 2010. mTORC1 signaling under hypoxic conditions is controlled by ATM-dependent phosphorylation of HIF-1 α . *Mol Cell*. 40:509-520.
- Cavallo, L. 2003. POPS: a fast algorithm for solvent accessible surface areas at atomic and residue level. *Nucleic Acids Research*. 31:3364-3366.
- Chabes, A., B. Georgieva, V. Domkin, X. Zhao, R. Rothstein, and L. Thelander. 2003a. Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. *Cell*. 112:391-401.
- Chabes, A.L., C.M. Pfleger, M.W. Kirschner, and L. Thelander. 2003b. Mouse ribonucleotide reductase R2 protein: a new target for anaphase-promoting complex-Cdh1-mediated proteolysis. *Proc Natl Acad Sci U S A*. 100:3925-3929.
- Chabosseau, P., G. Buhagiar-Labarchede, R. Onclercq-Delic, S. Lambert, M. Debatisse, O. Brison, and M. Amor-Gueret. 2011. Pyrimidine pool imbalance induced by BLM helicase deficiency contributes to genetic instability in Bloom syndrome. *Nat Commun*. 2:368.
- Chan, K.L., T. Palmai-Pallag, S. Ying, and I.D. Hickson. 2009. Replication stress induces sister-chromatid bridging at fragile site loci in mitosis. *Nat Cell Biol*. 11:753-760.
- Chang, L., R. Guo, Q. Huang, and Y. Yen. 2013. Chromosomal instability triggered by Rrm2b loss leads to IL-6 secretion and plasmacytic neoplasms. *Cell Rep*. 3:1389-1397.
- Chang, L., B. Zhou, S. Hu, R. Guo, X. Liu, S.N. Jones, and Y. Yen. 2008. ATM-mediated serine 72 phosphorylation stabilizes ribonucleotide reductase small subunit p53R2 protein against MDM2 to DNA damage. *Proc Natl Acad Sci U S A*. 105:18519-18524.
- Chimploy, K., M.L. Tassotto, and C.K. Mathews. 2000. Ribonucleotide reductase, a possible agent in deoxyribonucleotide pool asymmetries induced by hypoxia. *J Biol Chem*. 275:39267-39271.
- Cho, E.C., M.L. Kuo, J.H. Cheng, Y.C. Cheng, Y.C. Hsieh, Y.R. Liu, R.H. Hsieh, and Y. Yen. 2015. RRM2B-Mediated Regulation of Mitochondrial Activity and Inflammation under Oxidative Stress. *Mediators Inflamm*. 2015:287345.
- Cho, E.C., M.L. Kuo, X. Liu, L. Yang, Y.C. Hsieh, J. Wang, Y. Cheng, and Y. Yen. 2014. Tumor suppressor FOXO3 regulates ribonucleotide reductase subunit RRM2B and impacts on survival of cancer patients. *Oncotarget*. 5:4834-4844.
- Cho, E.C., and Y. Yen. 2016. Novel regulators and molecular mechanisms of p53R2 and its disease relevance. *Biochimie*. 123:81-84.
- Ciccio, A., and S.J. Elledge. 2010. The DNA damage response: making it safe to play with knives. *Mol Cell*. 40:179-204.
- Cimprich, K.A., and D. Cortez. 2008. ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol*. 9:616-627.
- Cohen, J., A. Arkhipov, R. Braun, and K. Schulten. 2006. Imaging the Migration Pathways for O₂, CO, NO, and Xe Inside Myoglobin. *Biophysical journal*. 91:1844-1857.
- Cohen, J., K. Kim, M. Posewitz, M.L. Ghirardi, K. Schulten, M. Seibert, and P. King. 2005. Molecular dynamics and experimental investigation of H₂ and O₂ diffusion in [Fe]-hydrogenase. *Biochemical Society Transactions*. 33:80-82.
- Collis, S.J., T.L. DeWeese, P.A. Jeggo, and A.R. Parker. 2005. The life and death of DNA-PK. *Oncogene*. 24:949-961.

- Cotruvo, J.A., and J. Stubbe. 2011. Class I ribonucleotide reductases: metallocofactor assembly and repair in vitro and in vivo. *Annu Rev Biochem.* 80:733-767.
- Couch, F.B., C.E. Bansbach, R. Driscoll, J.W. Luzwick, G.G. Glick, R. Betous, C.M. Carroll, S.Y. Jung, J. Qin, K.A. Cimprich, and D. Cortez. 2013. ATR phosphorylates SMARCA1 to prevent replication fork collapse. *Genes Dev.* 27:1610-1623.
- CRUK. 2014. International Agency for Research on Cancer and Cancer Research UK. World Cancer Factsheet. Cancer Research UK, London, 2014. Cancer Research UK, London, 2014.
- Cvetic, C., and J.C. Walter. 2005. Eukaryotic origins of DNA replication: could you please be more specific? *Semin Cell Dev Biol.* 16:343-353.
- D'Angiolella, V., V. Donato, F.M. Forrester, Y.T. Jeong, C. Pellacani, Y. Kudo, A. Saraf, L. Florens, M.P. Washburn, and M. Pagano. 2012. Cyclin F-mediated degradation of ribonucleotide reductase M2 controls genome integrity and DNA repair. *Cell.* 149:1023-1034.
- Dahl, A.C.E., M. Chavent, and M.S.P. Sansom. 2012. Bendix: intuitive helix geometry analysis and abstraction. *Bioinformatics.* 28:2193-2194.
- Dai, Y., and S. Grant. 2010. New insights into checkpoint kinase 1 in the DNA damage response signaling network. *Clin Cancer Res.* 16:376-383.
- Dang, D.T., F. Chen, L.B. Gardner, J.M. Cummins, C. Rago, F. Bunz, S.V. Kantsevov, and L.H. Dang. 2006. Hypoxia-inducible factor-1 α promotes nonhypoxia-mediated proliferation in colon cancer cells and xenografts. *Cancer Res.* 66:1684-1936.
- Danielsson, J., L. Liljedahl, E. Barany-Wallje, P. Sonderby, L.H. Kristensen, M.A. Martinez-Yamout, H.J. Dyson, P.E. Wright, F.M. Poulsen, L. Maler, A. Graslund, and B.B. Kragelund. 2008. The intrinsically disordered RNR inhibitor Sml1 is a dynamic dimer. *Biochemistry.* 47:13428-13437.
- Darden, T., D. York, and L. Pedersen. 1993. Particle mesh Ewald: An N $\cdot$ log(N) method for Ewald sums in large systems. *The Journal of Chemical Physics.* 98.
- David, A.R., and M.R. Zimmerman. 2010. Cancer: an old disease, a new disease or something in between? *Nat Rev Cancer.* 10:728-733.
- De Piccoli, G., Y. Katou, T. Itoh, R. Nakato, K. Shirahige, and K. Labib. 2012. Replisome stability at defective DNA replication forks is independent of S phase checkpoint kinases. *Mol Cell.* 45:696-704.
- Debatisse, M., B. Le Tallec, A. Letessier, B. Dutrillaux, and O. Brison. 2012. Common fragile sites: mechanisms of instability revisited. *Trends Genet.* 28:22-32.
- Devlin, H.L., P.C. Mack, R.A. Burich, P.H. Gumerlock, H.J. Kung, M. Mudryj, and R.W. deVere White. 2008. Impairment of the DNA repair and growth arrest pathways by p53R2 silencing enhances DNA damage-induced apoptosis in a p53-dependent manner in prostate cancer cells. *Mol Cancer Res.* 6:808-818.
- Di Noia, M.A., S. Todisco, A. Cirigliano, T. Rinaldi, G. Agrimi, V. Iacobazzi, and F. Palmieri. 2014. The human SLC25A33 and SLC25A36 genes of solute carrier family 25 encode two mitochondrial pyrimidine nucleotide transporters. *J Biol Chem.* 289:33137-33148.
- Diffley, J.F. 2011. Quality control in the initiation of eukaryotic DNA replication. *Philos Trans R Soc Lond B Biol Sci.* 366:3545-3553.
- Dings, J., J. Meixensberger, A. Jager, and K. Roosen. 1998. Clinical experience with 118 brain tissue oxygen partial pressure catheter probes. *Neurosurgery.* 43:1082-1095.
- Duxbury, M.S., H. Ito, E. Benoit, M.J. Zinner, S.W. Ashley, and E.E. Whang. 2004a. Retrovirally mediated RNA interference targeting the M2 subunit of ribonucleotide reductase: A novel therapeutic strategy in pancreatic cancer. *Surgery.* 136:261-269.

- Duxbury, M.S., H. Ito, M.J. Zinner, S.W. Ashley, and E.E. Whang. 2004b. RNA interference targeting the M2 subunit of ribonucleotide reductase enhances pancreatic adenocarcinoma chemosensitivity to gemcitabine. *Oncogene*. 23:1539-1548.
- Dweik, R.A. 2005. Nitric oxide, hypoxia, and superoxide: the good, the bad, and the ugly! *Thorax*. 60:265-267.
- Ekberg, M., S. Potsch, E. Sandin, M. Thunnissen, P. Nordlund, M. Sahlin, and B.M. Sjöberg. 1998. Preserved catalytic activity in an engineered ribonucleotide reductase R2 protein with a nonphysiological radical transfer pathway. The importance of hydrogen bond connections between the participating residues. *J Biol Chem*. 273:21003-21008.
- Eklund, H., U. Uhlin, M. Farnegårdh, D.T. Logan, and P. Nordlund. 2001. Structure and function of the radical enzyme ribonucleotide reductase. *Prog Biophys Mol Biol*. 77:177-268.
- Elford, H.L., M. Freese, E. Passamani, and H.P. Morris. 1970. Ribonucleotide reductase and cell proliferation. I. Variations of ribonucleotide reductase activity with tumor growth rate in a series of rat hepatomas. *J Biol Chem*. 245:5228-5233.
- Elvers, I., F. Johansson, P. Groth, K. Erixon, and T. Helleday. 2011. UV stalled replication forks restart by re-priming in human fibroblasts. *Nucleic Acids Res*. 39:7049-7057.
- Engström, Y., S. Eriksson, I. Jildevik, S. Skog, L. Thelander, and B. Tribukait. 1985. Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits. *J Biol Chem*. 260:9114-9116.
- Engström, Y., and B. Rozell. 1988. Immunocytochemical evidence for the cytoplasmic localization and differential expression during the cell cycle of the M1 and M2 subunits of mammalian ribonucleotide reductase. *EMBO J*. 7:1615-1620.
- Eriksson, M., U. Uhlin, S. Ramaswamy, M. Ekberg, K. Regnström, B.M. Sjöberg, and H. Eklund. 1997. Binding of allosteric effectors to ribonucleotide reductase protein R1: reduction of active-site cysteines promotes substrate binding. *Structure*. 5:1077-1092.
- Eriksson, S., S. Skog, B. Tribukait, and B. Wallström. 1987. Deoxyribonucleoside triphosphate metabolism and the mammalian cell cycle. Effects of hydroxyurea on mutant and wild-type mouse S49 T-lymphoma cells. *Exp Cell Res*. 168:79-88.
- Fabbro, M., K. Savage, K. Hobson, A.J. Deans, S.N. Powell, G.A. McArthur, and K.K. Khanna. 2004. BRCA1-BARD1 complexes are required for p53Ser-15 phosphorylation and a G1/S arrest following ionizing radiation-induced DNA damage. *J Biol Chem*. 279:31251-31258.
- Fairman, J.W., S.R. Wijerathna, M.F. Ahmad, H. Xu, R. Nakano, S. Jha, J. Prendergast, R.M. Welin, S. Flodin, A. Roos, P. Nordlund, Z. Li, T. Walz, and C.G. Dealwis. 2011. Structural basis for allosteric regulation of human ribonucleotide reductase by nucleotide-induced oligomerization. *Nat Struct Mol Biol*. 18:316-322.
- Fan, H., A. Huang, C. Villegas, and J.A. Wright. 1997. The R1 component of mammalian ribonucleotide reductase has malignancy-suppressing activity as demonstrated by gene transfer experiments. *Proc Natl Acad Sci U S A*. 94:13181-13186.
- Fan, H., C. Villegas, A. Huang, and J.A. Wright. 1998. The mammalian ribonucleotide reductase R2 component cooperates with a variety of oncogenes in mechanisms of cellular transformation. *Cancer Res*. 58:1650-1653.
- Fan, H., C. Villegas, and J.A. Wright. 1996. Ribonucleotide reductase R2 component is a novel malignancy determinant that cooperates with activated oncogenes to determine transformation and malignant potential. *Proc Natl Acad Sci U S A*. 93:14036-14040.
- Field, M.S., E. Kamynina, O.C. Agunloye, R.P. Liebenthal, S.G. Lamarre, M.E. Brosnan, J.T. Brosnan, and P.J. Stover. 2014. Nuclear enrichment of folate cofactors and methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) protect de novo thymidylate biosynthesis during folate deficiency. *J Biol Chem*. 289:29642-29650.

- Filatov, D., S. Bjorklund, E. Johansson, and L. Thelander. 1996. Induction of the mouse ribonucleotide reductase R1 and R2 genes in response to DNA damage by UV light. *J Biol Chem.* 271:23698-23704.
- Finch, R.A., M. Liu, S.P. Grill, W.C. Rose, R. Loomis, K.M. Vasquez, Y. Cheng, and A.C. Sartorelli. 2000. Triapine (3-aminopyridine-2-carboxaldehyde- thiosemicarbazone): A potent inhibitor of ribonucleotide reductase activity with broad spectrum antitumor activity. *Biochem Pharmacol.* 59:983-991.
- Foray, N., D. Marot, A. Gabriel, V. Randrianarison, A.M. Carr, M. Perricaudet, A. Ashworth, and P. Jeggo. 2003. A subset of ATM- and ATR-dependent phosphorylation events requires the BRCA1 protein. *EMBO J.* 22:2860-2871.
- Foskolou, I.P., D. Biasoli, M.M. Olcina, and E.M. Hammond. 2016. Measuring DNA Replication in Hypoxic Conditions. *Adv Exp Med Biol.* 899:11-25.
- Franzolin, E., G. Pontarin, C. Rampazzo, C. Miazzi, P. Ferraro, E. Palumbo, P. Reichard, and V. Bianchi. 2013. The deoxynucleotide triphosphohydrolase SAMHD1 is a major regulator of DNA precursor pools in mammalian cells. *Proc Natl Acad Sci U S A.* 110:14272-14277.
- Fraternali, F., and W.F. Van Gunsteren. 1996. An efficient mean solvation force model for use in molecular dynamics simulations of proteins in aqueous solution. *Journal of Molecular Biology.* 256:939-948.
- Freedman, S.J., Z.Y. Sun, F. Poy, A.L. Kung, D.M. Livingston, G. Wagner, and M.J. Eck. 2002. Structural basis for recruitment of CBP/p300 by hypoxia-inducible factor-1 alpha. *Proc Natl Acad Sci U S A.* 99:5367-5372.
- Garneau, J.E., M.E. Dupuis, M. Villion, D.A. Romero, R. Barrangou, P. Boyaval, C. Fremaux, P. Horvath, A.H. Magadan, and S. Moineau. 2010. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature.* 468:67-71.
- Gautam, A., Z.R. Li, and G. Bepler. 2003. RRM1-induced metastasis suppression through PTEN-regulated pathways. *Oncogene.* 22:2135-2142.
- Giatromanolaki, A., M.I. Koukourakis, E. Sivridis, H. Turley, K. Talks, F. Pezzella, K.C. Gatter, and A.L. Harris. 2001. Relation of hypoxia inducible factor 1 alpha and 2 alpha in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival. *Br J Cancer.* 85:881-890.
- Glanzer, J.G., S. Liu, L. Wang, A. Mosel, A. Peng, and G.G. Oakley. 2014. RPA inhibition increases replication stress and suppresses tumor growth. *Cancer Res.* 74:5165-5172.
- Goldstein, M., and M.B. Kastan. 2015. The DNA damage response: implications for tumor responses to radiation and chemotherapy. *Annu Rev Med.* 66:129-143.
- Goldstone, D.C., V. Ennis-Adeniran, J.J. Hedden, H.C. Groom, G.I. Rice, E. Christodoulou, P.A. Walker, G. Kelly, L.F. Haire, M.W. Yap, L.P. de Carvalho, J.P. Stoye, Y.J. Crow, I.A. Taylor, and M. Webb. 2011. HIV-1 restriction factor SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase. *Nature.* 480:379-382.
- Gorgoulis, V.G., L.V. Vassiliou, P. Karakaidos, P. Zacharatos, A. Kotsinas, T. Liloglou, M. Venere, R.A. Dittullo, Jr., N.G. Kastrinakis, B. Levy, D. Kletsas, A. Yoneta, M. Herlyn, C. Kittas, and T.D. Halazonetis. 2005. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature.* 434:907-913.
- Graeber, T.G., C. Osmanian, T. Jacks, D.E. Housman, C.J. Koch, S.W. Lowe, and A.J. Giaccia. 1996. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature.* 379:88-91.

- Gray, L.H., A.D. Conger, M. Ebert, S. Hornsey, and O.C. Scott. 1953. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br J Radiol.* 26:638-648.
- Green, D.R., and G. Kroemer. 2009. Cytoplasmic functions of the tumour suppressor p53. *Nature.* 458:1127-1130.
- Guittet, O., P. Hakansson, N. Voevodskaya, S. Fridd, A. Graslund, H. Arakawa, Y. Nakamura, and L. Thelander. 2001. Mammalian p53R2 protein forms an active ribonucleotide reductase in vitro with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells. *J Biol Chem.* 276:40647-40651.
- Guittet, O., A. Tebbi, M.H. Cottet, F. Vesin, and M. Lepoivre. 2008. Upregulation of the p53R2 ribonucleotide reductase subunit by nitric oxide. *Nitric Oxide.* 19:84-94.
- Gunjan, A., and A. Verreault. 2003. A Rad53 kinase-dependent surveillance mechanism that regulates histone protein levels in *S. cerevisiae*. *Cell.* 115:537-549.
- Haider, S., A. McIntyre, R.G. van Stiphout, L.M. Winchester, S. Wigfield, A.L. Harris, and F.M. Buffa. 2016. Genomic alterations underlie a pan-cancer metabolic shift associated with tumour hypoxia. *Genome Biol.* 17:140.
- Hakansson, P., A. Hofer, and L. Thelander. 2006. Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. *J Biol Chem.* 281:7834-7841.
- Hall, E.J.a.G., A.J. 2012. Radiobiology for the Radiologist. Lippincott Williams & Wilkins: Philadelphia, Lippincott Williams & Wilkins: Philadelphia, PA, USA, 2012.
- Hammond, E.M., M.C. Asselin, D. Forster, J.P. O'Connor, J.M. Senra, and K.J. Williams. 2014. The meaning, measurement and modification of hypoxia in the laboratory and the clinic. *Clin Oncol (R Coll Radiol).* 26:277-288.
- Hammond, E.M., N.C. Denko, M.J. Dorie, R.T. Abraham, and A.J. Giaccia. 2002. Hypoxia links ATR and p53 through replication arrest. *Mol Cell Biol.* 22:1834-1843.
- Hammond, E.M., M.J. Dorie, and A.J. Giaccia. 2003. ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. *J Biol Chem.* 278:12207-12213.
- Hammond, E.M., M.J. Dorie, and A.J. Giaccia. 2004. Inhibition of ATR leads to increased sensitivity to hypoxia/reoxygenation. *Cancer Res.* 64:6556-6562.
- Hammond, E.M., and A.J. Giaccia. 2004. The role of ATM and ATR in the cellular response to hypoxia and re-oxygenation. *DNA Repair (Amst).* 3:1117-1122.
- Hammond, E.M., M.R. Kaufmann, and A.J. Giaccia. 2007. Oxygen sensing and the DNA-damage response. *Curr Opin Cell Biol.* 19:680-684.
- Hammond, E.M., D.J. Mandell, A. Salim, A.J. Krieg, T.M. Johnson, H.A. Shirazi, L.D. Attardi, and A.J. Giaccia. 2006. Genome-wide analysis of p53 under hypoxic conditions. *Mol Cell Biol.* 26:3492-3504.
- Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell.* 100:57-70.
- Hanahan, D., and R.A. Weinberg. 2011. Hallmarks of cancer: the next generation. *Cell.* 144:646-674.
- Harris, A.L. 2002. Hypoxia--a key regulatory factor in tumour growth. *Nat Rev Cancer.* 2:38-47.
- Hehlmann, R., H. Heimpel, J. Hasford, H.J. Kolb, H. Pralle, D.K. Hossfeld, W. Queisser, H. Loffler, B. Heinze, A. Georgii, and et al. 1993. Randomized comparison of busulfan and hydroxyurea in chronic myelogenous leukemia: prolongation of survival by hydroxyurea. The German CML Study Group. *Blood.* 82:398-407.
- Helczynska, K., A.M. Larsson, L. Holmquist Mengelbier, E. Bridges, E. Fredlund, S. Borgquist, G. Landberg, S. Pahlman, and K. Jirstrom. 2008. Hypoxia-inducible

- factor-2alpha correlates to distant recurrence and poor outcome in invasive breast cancer. *Cancer Res.* 68:9212-9220.
- Helmrich, A., M. Ballarino, E. Nudler, and L. Tora. 2013. Transcription-replication encounters, consequences and genomic instability. *Nat Struct Mol Biol.* 20:412-418.
- Hockel, M., and P. Vaupel. 2001. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst.* 93:266-276.
- Hofer, A., M. Crona, D.T. Logan, and B.M. Sjöberg. 2012. DNA building blocks: keeping control of manufacture. *Crit Rev Biochem Mol Biol.* 47:50-63.
- Holmgren, A., and R. Sengupta. 2010. The use of thiols by ribonucleotide reductase. *Free Radic Biol Med.* 49:1617-1628.
- Holmquist-Mengelbier, L., E. Fredlund, T. Lofstedt, R. Noguera, S. Navarro, H. Nilsson, A. Pietras, J. Vallon-Christersson, A. Borg, K. Gradin, L. Poellinger, and S. Pahlman. 2006. Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. *Cancer Cell.* 10:413-423.
- Hsu, P.D., and F. Zhang. 2012. Dissecting neural function using targeted genome engineering technologies. *ACS Chem Neurosci.* 3:603-610.
- Hu, C.J., L.Y. Wang, L.A. Chodosh, B. Keith, and M.C. Simon. 2003. Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol Cell Biol.* 23:9361-9374.
- Hu, C.M., M.T. Yeh, N. Tsao, C.W. Chen, Q.Z. Gao, C.Y. Chang, M.H. Lee, J.M. Fang, S.Y. Sheu, C.J. Lin, M.C. Tseng, Y.J. Chen, and Z.F. Chang. 2012. Tumor cells require thymidylate kinase to prevent dUTP incorporation during DNA repair. *Cancer Cell.* 22:36-50.
- Huang, M., M.J. Parker, and J. Stubbe. 2014. Choosing the right metal: case studies of class I ribonucleotide reductases. *J Biol Chem.* 289:28104-28111.
- Humphrey, W., A. Dalke, and K. Schulten. 1996. VMD: Visual molecular dynamics. *Journal of Molecular Graphics.* 14:33-38.
- Hurta, R.A., and J.A. Wright. 1992. Alterations in the activity and regulation of mammalian ribonucleotide reductase by chlorambucil, a DNA damaging agent. *J Biol Chem.* 267:7066-7071.
- Hyun, S.Y., and Y.J. Jang. 2015. p53 activates G(1) checkpoint following DNA damage by doxorubicin during transient mitotic arrest. *Oncotarget.* 6:4804-4815.
- Ivan, M., K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J.M. Asara, W.S. Lane, and W.G. Kaelin, Jr. 2001. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science.* 292:464-468.
- Jaakkola, P., D.R. Mole, Y.M. Tian, M.I. Wilson, J. Gielbert, S.J. Gaskell, A. von Kriegsheim, H.F. Hebestreit, M. Mukherji, C.J. Schofield, P.H. Maxwell, C.W. Pugh, and P.J. Ratcliffe. 2001. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science.* 292:468-472.
- Jackson, S.P., and J. Bartek. 2009. The DNA-damage response in human biology and disease. *Nature.* 461:1071-1078.
- Johansson, E., K. Hjortsberg, and L. Thelander. 1998. Two YY-1-binding proximal elements regulate the promoter strength of the TATA-less mouse ribonucleotide reductase R1 gene. *J Biol Chem.* 273:29816-29821.
- Jonna, V.R., M. Crona, R. Rofougaran, D. Lundin, S. Johansson, K. Brannstrom, B.M. Sjöberg, and A. Hofer. 2015. Diversity in Overall Activity Regulation of Ribonucleotide Reductase. *J Biol Chem.* 290:17339-17348.

- Jordan, A., E. Torrents, I. Sala, U. Hellman, I. Gibert, and P. Reichard. 1999. Ribonucleotide reduction in *Pseudomonas* species: simultaneous presence of active enzymes from different classes. *J Bacteriol.* 181:3974-3980.
- Jordheim, L.P., O. Guittet, M. Lepoivre, C.M. Galmarini, and C. Dumontet. 2005. Increased expression of the large subunit of ribonucleotide reductase is involved in resistance to gemcitabine in human mammary adenocarcinoma cells. *Mol Cancer Ther.* 4:1268-1276.
- Jorgensen, W.L., J. Chandrasekhar, J.D. Madura, R.W. Impey, and M.L. Klein. 1983. Comparison of simple potential functions for simulating liquid water. *The Journal of Chemical Physics.* 79.
- Juhasz, A., A. Vassilakos, H.K. Chew, D. Gandara, and Y. Yen. 2006. Analysis of ribonucleotide reductase M2 mRNA levels in patient samples after GTI-2040 antisense drug treatment. *Oncol Rep.* 15:1299-1304.
- Kashlan, O.B., C.P. Scott, J.D. Lear, and B.S. Cooperman. 2002. A comprehensive model for the allosteric regulation of mammalian ribonucleotide reductase. Functional consequences of ATP- and dATP-induced oligomerization of the large subunit. *Biochemistry.* 41:462-474.
- Keith, B., R.S. Johnson, and M.C. Simon. 2012. HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. *Nat Rev Cancer.* 12:9-22.
- Kimura, T., S. Takeda, Y. Sagiya, M. Gotoh, Y. Nakamura, and H. Arakawa. 2003. Impaired function of p53R2 in Rrm2b-null mice causes severe renal failure through attenuation of dNTP pools. *Nat Genet.* 34:440-445.
- Koc, A., L.J. Wheeler, C.K. Mathews, and G.F. Merrill. 2004. Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools. *J Biol Chem.* 279:223-230.
- Koh, M.Y., R. Lemos, Jr., X. Liu, and G. Powis. 2011. The hypoxia-associated factor switches cells from HIF-1alpha- to HIF-2alpha-dependent signaling promoting stem cell characteristics, aggressive tumor growth and invasion. *Cancer Res.* 71:4015-4027.
- Koh, M.Y., and G. Powis. 2012. Passing the baton: the HIF switch. *Trends Biochem Sci.* 37:364-372.
- Kolberg, M., K.R. Strand, P. Graff, and K.K. Andersson. 2004. Structure, function, and mechanism of ribonucleotide reductases. *Biochim Biophys Acta.* 1699:1-34.
- Kollberg, G., N. Darin, K. Benan, A.R. Moslemi, S. Lindal, M. Tulinius, A. Oldfors, and E. Holme. 2009. A novel homozygous RRM2B missense mutation in association with severe mtDNA depletion. *Neuromuscul Disord.* 19:147-150.
- Koukourakis, M.I., A. Giatromanolaki, V. Liberis, and E. Sivridis. 2002. Hypoxia inducible factor 1 alpha and 2 alpha expression is independent of anemia in patients with stage I endometrial cancer. *Anticancer Res.* 22:4137-4140.
- Koumenis, C., R. Alarcon, E. Hammond, P. Sutphin, W. Hoffman, M. Murphy, J. Derr, Y. Taya, S.W. Lowe, M. Kastan, and A. Giaccia. 2001. Regulation of p53 by hypoxia: dissociation of transcriptional repression and apoptosis from p53-dependent transactivation. *Mol Cell Biol.* 21:1297-1310.
- Krakoff, I.H., N.C. Brown, and P. Reichard. 1968. Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. *Cancer Res.* 28:1559-1565.
- Kronblad, A., K. Jirstrom, L. Ryden, B. Nordenskjold, and G. Landberg. 2006. Hypoxia inducible factor-1alpha is a prognostic marker in premenopausal patients with intermediate to highly differentiated breast cancer but not a predictive marker for tamoxifen response. *Int J Cancer.* 118:2609-2616.

- Kunz, B.A., and S.E. Kohalmi. 1991. Modulation of mutagenesis by deoxyribonucleotide levels. *Annu Rev Genet.* 25:339-359.
- Kuo, M.L., A.J. Sy, L. Xue, M. Chi, M.T. Lee, T. Yen, M.I. Chiang, L. Chang, P. Chu, and Y. Yen. 2012. RRM2B suppresses activation of the oxidative stress pathway and is up-regulated by p53 during senescence. *Sci Rep.* 2:822.
- LaGory, E.L., and A.J. Giaccia. 2016. The ever-expanding role of HIF in tumour and stromal biology. *Nat Cell Biol.* 18:356-365.
- Laguette, N., and M. Benkirane. 2012. How SAMHD1 changes our view of viral restriction. *Trends Immunol.* 33:26-33.
- Lambert, S., and A.M. Carr. 2013. Impediments to replication fork movement: stabilisation, reactivation and genome instability. *Chromosoma.* 122:33-45.
- Lando, D., D.J. Peet, J.J. Gorman, D.A. Whelan, M.L. Whitelaw, and R.K. Bruick. 2002. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev.* 16:1466-1471.
- Langevin, P. 1908. On the theory of Brownian motion.
- Larsson, K.M., A. Jordan, R. Eliasson, P. Reichard, D.T. Logan, and P. Nordlund. 2004. Structural mechanism of allosteric substrate specificity regulation in a ribonucleotide reductase. *Nat Struct Mol Biol.* 11:1142-1149.
- Le, Q.T., E. Chen, A. Salim, H. Cao, C.S. Kong, R. Whyte, J. Donington, W. Cannon, H. Wakelee, R. Tibshirani, J.D. Mitchell, D. Richardson, K.J. O'Byrne, A.C. Koong, and A.J. Giaccia. 2006. An evaluation of tumor oxygenation and gene expression in patients with early stage non-small cell lung cancers. *Clin Cancer Res.* 12:1507-1514.
- Lee, J.H., A.A. Goodarzi, P.A. Jeggo, and T.T. Paull. 2010. 53BP1 promotes ATM activity through direct interactions with the MRN complex. *EMBO J.* 29:574-585.
- Lee, Y., A. Vassilakos, N. Feng, V. Lam, H. Xie, M. Wang, H. Jin, K. Xiong, C. Liu, J. Wright, and A. Young. 2003. GTI-2040, an antisense agent targeting the small subunit component (R2) of human ribonucleotide reductase, shows potent antitumor activity against a variety of tumors. *Cancer Res.* 63:2802-2811.
- Leeds, J.M., M.B. Slabaugh, and C.K. Mathews. 1985. DNA precursor pools and ribonucleotide reductase activity: distribution between the nucleus and cytoplasm of mammalian cells. *Mol Cell Biol.* 5:3443-3450.
- Leighl, N.B., S.A. Laurie, X.E. Chen, P. Ellis, F.A. Shepherd, J.J. Knox, G. Goss, R.L. Burkes, G.R. Pond, C. Dick, Y. Yen, J.A. Zwiebel, and M.J. Moore. 2009. A phase I/II study of GTI-2040 plus docetaxel as second-line treatment in advanced non-small cell lung cancer: a study of the PMH phase II consortium. *J Thorac Oncol.* 4:1163-1169.
- Leonard, A.C., and M. Mechali. 2013. DNA replication origins. *Cold Spring Harb Perspect Biol.* 5:a010116.
- Leszczynska, K.B., I.P. Foskolou, A.G. Abraham, S. Anbalagan, C. Tellier, S. Haider, P.N. Span, E.E. O'Neill, F.M. Buffa, and E.M. Hammond. 2015. Hypoxia-induced p53 modulates both apoptosis and radiosensitivity via AKT. *J Clin Invest.* 125:2385-2398.
- Levin, V.A. 1992. The place of hydroxyurea in the treatment of primary brain tumors. *Semin Oncol.* 19:34-39.
- Li, Z., S. Bao, Q. Wu, H. Wang, C. Eyler, S. Sathornsumetee, Q. Shi, Y. Cao, J. Lathia, R.E. McLendon, A.B. Hjelmeland, and J.N. Rich. 2009. Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells. *Cancer Cell.* 15:501-513.
- Liu, X., L. Lai, X. Wang, L. Xue, S. Leora, J. Wu, S. Hu, K. Zhang, M.L. Kuo, L. Zhou, H. Zhang, Y. Wang, Y. Wang, B. Zhou, R.A. Nelson, S. Zheng, S. Zhang, P. Chu, and Y. Yen. 2011. Ribonucleotide reductase small subunit M2B prognoses better survival in colorectal cancer. *Cancer Res.* 71:3202-3213.

- Liu, X., H. Zhang, L. Lai, X. Wang, S. Loera, L. Xue, H. He, K. Zhang, S. Hu, Y. Huang, R.A. Nelson, B. Zhou, L. Zhou, P. Chu, S. Zhang, S. Zheng, and Y. Yen. 2013. Ribonucleotide reductase small subunit M2 serves as a prognostic biomarker and predicts poor survival of colorectal cancers. *Clin Sci (Lond)*. 124:567-578.
- Liu, X., B. Zhou, L. Xue, J. Shih, K. Tye, W. Lin, C. Qi, P. Chu, F. Un, W. Wen, and Y. Yen. 2006. Metastasis-suppressing potential of ribonucleotide reductase small subunit p53R2 in human cancer cells. *Clin Cancer Res*. 12:6337-6344.
- Liu, X., B. Zhou, L. Xue, J. Shih, K. Tye, C. Qi, and Y. Yen. 2005. The ribonucleotide reductase subunit M2B subcellular localization and functional importance for DNA replication in physiological growth of KB cells. *Biochem Pharmacol*. 70:1288-1297.
- Lopes, M., C. Cotta-Ramusino, A. Pellicoli, G. Liberi, P. Plevani, M. Muzi-Falconi, C.S. Newlon, and M. Foiani. 2001. The DNA replication checkpoint response stabilizes stalled replication forks. *Nature*. 412:557-561.
- Ma, X.J., R. Salunga, J.T. Tuggle, J. Gaudet, E. Enright, P. McQuary, T. Payette, M. Pistone, K. Stecker, B.M. Zhang, Y.X. Zhou, H. Varnholt, B. Smith, M. Gadd, E. Chatfield, J. Kessler, T.M. Baer, M.G. Erlander, and D.C. Sgroi. 2003. Gene expression profiles of human breast cancer progression. *Proc Natl Acad Sci U S A*. 100:5974-5979.
- MacFarlane, A.J., D.D. Anderson, P. Flodby, C.A. Perry, R.H. Allen, S.P. Stabler, and P.J. Stover. 2011. Nuclear localization of de novo thymidylate biosynthesis pathway is required to prevent uracil accumulation in DNA. *J Biol Chem*. 286:44015-44022.
- Macheret, M., and T.D. Halazonetis. 2015. DNA replication stress as a hallmark of cancer. *Annu Rev Pathol*. 10:425-448.
- Magdalou, I., B.S. Lopez, P. Pasero, and S.A. Lambert. 2014. The causes of replication stress and their consequences on genome stability and cell fate. *Semin Cell Dev Biol*. 30:154-164.
- Mahon, P.C., K. Hirota, and G.L. Semenza. 2001. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev*. 15:2675-2686.
- Mailand, N., I. Gibbs-Seymour, and S. Bekker-Jensen. 2013. Regulation of PCNA-protein interactions for genome stability. *Nat Rev Mol Cell Biol*. 14:269-282.
- Manegold, C., P. Zatloukal, K. Krejcy, and J. Blatter. 2000. Gemcitabine in non-small cell lung cancer (NSCLC). *Invest New Drugs*. 18:29-42.
- Mannava, S., V. Grachtchouk, L.J. Wheeler, M. Im, D. Zhuang, E.G. Slavina, C.K. Mathews, D.S. Shewach, and M.A. Nikiforov. 2008. Direct role of nucleotide metabolism in C-MYC-dependent proliferation of melanoma cells. *Cell Cycle*. 7:2392-2400.
- Marechal, A., and L. Zou. 2013. DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harb Perspect Biol*. 5.
- Martomo, S.A., and C.K. Mathews. 2002. Effects of biological DNA precursor pool asymmetry upon accuracy of DNA replication in vitro. *Mutat Res*. 499:197-211.
- Masai, H., S. Matsumoto, Z. You, N. Yoshizawa-Sugata, and M. Oda. 2010. Eukaryotic chromosome DNA replication: where, when, and how? *Annu Rev Biochem*. 79:89-130.
- Mathews, C.K. 2015. Deoxyribonucleotide metabolism, mutagenesis and cancer. *Nat Rev Cancer*. 15:528-539.
- Matsushita, S., R. Ikeda, T. Fukushige, Y. Tajitsu, K. Gunshin, H. Okumura, M. Ushiyama, S. Akiyama, K. Kawai, Y. Takeda, K. Yamada, and T. Kanekura. 2012. p53R2 is a prognostic factor of melanoma and regulates proliferation and chemosensitivity of melanoma cells. *J Dermatol Sci*. 68:19-24.

- Maya-Mendoza, A., E. Petermann, D.A. Gillespie, K.W. Caldecott, and D.A. Jackson. 2007. Chk1 regulates the density of active replication origins during the vertebrate S phase. *EMBO J.* 26:2719-2731.
- McIntyre, A., and A.L. Harris. 2015. Metabolic and hypoxic adaptation to anti-angiogenic therapy: a target for induced essentiality. *EMBO Mol Med.* 7:368-379.
- Mechali, M. 2010. Eukaryotic DNA replication origins: many choices for appropriate answers. *Nat Rev Mol Cell Biol.* 11:728-738.
- Michalet, X., R. Ekong, F. Fougerousse, S. Rousseaux, C. Schurra, N. Hornigold, M. van Slegtenhorst, J. Wolfe, S. Povey, J.S. Beckmann, and A. Bensimon. 1997. Dynamic molecular combing: stretching the whole human genome for high-resolution studies. *Science.* 277:1518-1523.
- Michiels, C., C. Tellier, and O. Feron. 2016. Cycling hypoxia: A key feature of the tumor microenvironment. *Biochim Biophys Acta.* 1866:76-86.
- Mimitou, E.P., and L.S. Symington. 2011. DNA end resection--unraveling the tail. *DNA Repair (Amst).* 10:344-348.
- Minnihan, E.C., D.G. Nocera, and J. Stubbe. 2013. Reversible, long-range radical transfer in *E. coli* class Ia ribonucleotide reductase. *Acc Chem Res.* 46:2524-2535.
- Miyamoto, S., and P.A. Kollman. 1992. SETTLE: An analytical version of the SHAKE and RATTLE algorithm for rigid water models. *Journal of Computational Chemistry.* 13:952-962.
- Morikawa, T., R. Hino, H. Uozaki, D. Maeda, T. Ushiku, A. Shinozaki, T. Sakatani, and M. Fukayama. 2010a. Expression of ribonucleotide reductase M2 subunit in gastric cancer and effects of RRM2 inhibition in vitro. *Hum Pathol.* 41:1742-1748.
- Morikawa, T., D. Maeda, H. Kume, Y. Homma, and M. Fukayama. 2010b. Ribonucleotide reductase M2 subunit is a novel diagnostic marker and a potential therapeutic target in bladder cancer. *Histopathology.* 57:885-892.
- Munro, J.B., C.G. Jacob, and J.C. Silva. 2013. A novel clade of unique eukaryotic ribonucleotide reductase R2 subunits is exclusive to apicomplexan parasites. *J Mol Evol.* 77:92-106.
- Nakano, K., E. Balint, M. Ashcroft, and K.H. Vousden. 2000. A ribonucleotide reductase gene is a transcriptional target of p53 and p73. *Oncogene.* 19:4283-4289.
- Nestoras, K., A.H. Mohammed, A.S. Schreurs, O. Fleck, A.T. Watson, M. Poitelea, C. O'Shea, C. Chahwan, C. Holmberg, B.B. Kragelund, O. Nielsen, M. Osborne, A.M. Carr, and C. Liu. 2010. Regulation of ribonucleotide reductase by Spd1 involves multiple mechanisms. *Genes Dev.* 24:1145-1159.
- Nick McElhinny, S.A., D. Kumar, A.B. Clark, D.L. Watt, B.E. Watts, E.B. Lundstrom, E. Johansson, A. Chabes, and T.A. Kunkel. 2010. Genome instability due to ribonucleotide incorporation into DNA. *Nat Chem Biol.* 6:774-781.
- Niida, H., Y. Katsuno, M. Sengoku, M. Shimada, M. Yukawa, M. Ikura, T. Ikura, K. Kohno, H. Shima, H. Suzuki, S. Tashiro, and M. Nakanishi. 2010. Essential role of Tip60-dependent recruitment of ribonucleotide reductase at DNA damage sites in DNA repair during G1 phase. *Genes Dev.* 24:333-338.
- Nordlund, N., and P. Reichard. 2006. Ribonucleotide reductases. *Annual Review of Biochemistry.* 75:681-706.
- Nordlund, P., B.M. Sjöberg, and H. Eklund. 1990. Three-dimensional structure of the free radical protein of ribonucleotide reductase. *Nature.* 345:593-598.
- Nosé, S. 1984a. A molecular dynamics method for simulations in the canonical ensemble. *Molecular physics.* 52:255-268.
- Nosé, S. 1984b. A unified formulation of the constant temperature molecular dynamics methods. *The Journal of Chemical Physics.* 81:511-519.

- Nutting, C.M., C.M. van Herpen, A.B. Miah, S.A. Bhide, J.P. Machiels, J. Buter, C. Kelly, D. de Raucourt, and K.J. Harrington. 2009. Phase II study of 3-AP Triapine in patients with recurrent or metastatic head and neck squamous cell carcinoma. *Ann Oncol.* 20:1275-1279.
- Nyholm, S., L. Thelander, and A. Graslund. 1993. Reduction and loss of the iron center in the reaction of the small subunit of mouse ribonucleotide reductase with hydroxyurea. *Biochemistry.* 32:11569-11574.
- Oh, Y.K., and T.G. Park. 2009. siRNA delivery systems for cancer treatment. *Adv Drug Deliv Rev.* 61:850-862.
- Okumura, H., S. Natsugoe, N. Yokomakura, Y. Kita, M. Matsumoto, Y. Uchikado, T. Setoyama, T. Owaki, S. Ishigami, and T. Aikou. 2006. Expression of p53R2 is related to prognosis in patients with esophageal squamous cell carcinoma. *Clin Cancer Res.* 12:3740-3745.
- Olcina, M.M., and Hammond, E.M. 2014. Hypoxia and the DNA Damage Response. In *Hypoxia and Cancer: Biological Implications and Therapeutic Opportunities*. G. Melillo, editor. Springer New York © Springer Science+Business Media New York 2014. 21-30.
- Olcina, M.M., I.P. Foskolou, S. Anbalagan, J.M. Senra, I.M. Pires, Y. Jiang, A.J. Ryan, and E.M. Hammond. 2013. Replication stress and chromatin context link ATM activation to a role in DNA replication. *Mol Cell.* 52:758-766.
- Olcina, M.M., R.J. Grand, and E.M. Hammond. 2014. ATM activation in hypoxia - causes and consequences. *Mol Cell Oncol.* 1:e29903.
- Ormo, M., K. Regnstrom, Z. Wang, L. Que, Jr., M. Sahlin, and B.M. Sjoberg. 1995. Residues important for radical stability in ribonucleotide reductase from Escherichia coli. *J Biol Chem.* 270:6570-6576.
- Pacek, M., and J.C. Walter. 2004. A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication. *EMBO J.* 23:3667-3676.
- Papandreou, I., C. Krishna, F. Kaper, D. Cai, A.J. Giaccia, and N.C. Denko. 2005. Anoxia is necessary for tumor cell toxicity caused by a low-oxygen environment. *Cancer Res.* 65:3171-3178.
- Patiar, S., and A.L. Harris. 2006. Role of hypoxia-inducible factor-1alpha as a cancer therapy target. *Endocr Relat Cancer.* 13 Suppl 1:S61-75.
- Petrenko, R., and J. Meller. 2001. Molecular Dynamics. In eLS. John Wiley & Sons, Ltd.
- Phillips, J.C., R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, R.D. Skeel, L. Kale, and K. Schulten. 2005. Scalable molecular dynamics with NAMD. *J Comput Chem.* 26:1781-1802.
- Piao, C., M. Jin, H.B. Kim, S.M. Lee, P.N. Amatya, J.W. Hyun, I.Y. Chang, and H.J. You. 2009. Ribonucleotide reductase small subunit p53R2 suppresses MEK-ERK activity by binding to ERK kinase 2. *Oncogene.* 28:2173-2184.
- Pires, I.M., Z. Bencokova, C. McGurk, and E.M. Hammond. 2010a. Exposure to acute hypoxia induces a transient DNA damage response which includes Chk1 and TLK1. *Cell Cycle.* 9:2502-2507.
- Pires, I.M., Z. Bencokova, M. Milani, L.K. Folkes, J.L. Li, M.R. Stratford, A.L. Harris, and E.M. Hammond. 2010b. Effects of acute versus chronic hypoxia on DNA damage responses and genomic instability. *Cancer Res.* 70:925-935.
- Pires, I.M., M.M. Olcina, S. Anbalagan, J.R. Pollard, P.M. Reaper, P.A. Charlton, W.G. McKenna, and E.M. Hammond. 2012. Targeting radiation-resistant hypoxic tumour cells through ATR inhibition. *Br J Cancer.* 107:291-299.

- Poli, J., O. Tsaponina, L. Crabbe, A. Keszthelyi, V. Pantesco, A. Chabes, A. Lengronne, and P. Pasero. 2012. dNTP pools determine fork progression and origin usage under replication stress. *EMBO J.* 31:883-894.
- Pontarin, G., P. Ferraro, L. Bee, P. Reichard, and V. Bianchi. 2012. Mammalian ribonucleotide reductase subunit p53R2 is required for mitochondrial DNA replication and DNA repair in quiescent cells. *Proc Natl Acad Sci U S A.* 109:13302-13307.
- Pontarin, G., P. Ferraro, P. Hakansson, L. Thelander, P. Reichard, and V. Bianchi. 2007. p53R2-dependent ribonucleotide reduction provides deoxyribonucleotides in quiescent human fibroblasts in the absence of induced DNA damage. *J Biol Chem.* 282:16820-16828.
- Pontarin, G., A. Fijolek, P. Pizzo, P. Ferraro, C. Rampazzo, T. Pozzan, L. Thelander, P.A. Reichard, and V. Bianchi. 2008. Ribonucleotide reduction is a cytosolic process in mammalian cells independently of DNA damage. *Proc Natl Acad Sci U S A.* 105:17801-17806.
- Postel, E.H. 1999. Cleavage of DNA by human NM23-H2/nucleoside diphosphate kinase involves formation of a covalent protein-DNA complex. *J Biol Chem.* 274:22821-22829.
- Postel, E.H., S.J. Berberich, S.J. Flint, and C.A. Ferrone. 1993. Human c-myc transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis. *Science.* 261:478-480.
- Powell, R.D., P.J. Holland, T. Hollis, and F.W. Perrino. 2011. Aicardi-Goutieres syndrome gene and HIV-1 restriction factor SAMHD1 is a dGTP-regulated deoxynucleotide triphosphohydrolase. *J Biol Chem.* 286:43596-43600.
- Probst, H., H. Schiffer, V. Gekeler, H. Kienzle-Pfeilsticker, U. Stropp, K.E. Stotzer, and I. Frenzel-Stotzer. 1988. Oxygen dependent regulation of DNA synthesis and growth of Ehrlich ascites tumor cells in vitro and in vivo. *Cancer Res.* 48:2053-2060.
- Probst, H., H. Schiffer, V. Gekeler, and K. Scheffler. 1989. Oxygen Dependent Regulation of Mammalian Ribonucleotide Reductase In vivo and Possible Significance for Replicon Initiation. *Biochemical and Biophysical Research Communications.* 163:334-340.
- Puigvert, J.C., K. Sanjiv, and T. Helleday. 2016. Targeting DNA repair, DNA metabolism and replication stress as anti-cancer strategies. *FEBS J.* 283:232-245.
- Qi, J.J., L. Liu, J.X. Cao, G.S. An, S.Y. Li, G. Li, H.T. Jia, and J.H. Ni. 2015. E2F1 regulates p53R2 gene expression in p53-deficient cells. *Mol Cell Biochem.* 399:179-188.
- Rahman, M.A., A.R. Amin, X. Wang, J.E. Zuckerman, C.H. Choi, B. Zhou, D. Wang, S. Nannapaneni, L. Koenig, Z. Chen, Z.G. Chen, Y. Yen, M.E. Davis, and D.M. Shin. 2012. Systemic delivery of siRNA nanoparticles targeting RRM2 suppresses head and neck tumor growth. *J Control Release.* 159:384-392.
- Rampazzo, C., C. Miazzi, E. Franzolin, G. Pontarin, P. Ferraro, M. Frangini, P. Reichard, and V. Bianchi. 2010. Regulation by degradation, a cellular defense against deoxyribonucleotide pool imbalances. *Mutat Res.* 703:2-10.
- Ran, F.A., P.D. Hsu, J. Wright, V. Agarwala, D.A. Scott, and F. Zhang. 2013. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc.* 8:2281-2308.
- Rankin, E.B., and A.J. Giaccia. 2016. Hypoxic control of metastasis. *Science.* 352:175-180.
- Raval, R.R., K.W. Lau, M.G. Tran, H.M. Sowter, S.J. Mandriota, J.L. Li, C.W. Pugh, P.H. Maxwell, A.L. Harris, and P.J. Ratcliffe. 2005. Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. *Mol Cell Biol.* 25:5675-5686.
- Reichard, P. 1988. Interactions between deoxyribonucleotide and DNA synthesis. *Annu Rev Biochem.* 57:349-374.

- Reichard, P., and A. Ehrenberg. 1983. Ribonucleotide reductase--a radical enzyme. *Science*. 221:514-519.
- Reichard, P., R. Eliasson, R. Ingemarson, and L. Thelander. 2000. Cross-talk between the allosteric effector-binding sites in mouse ribonucleotide reductase. *J Biol Chem*. 275:33021-33026.
- Richard, D.E., E. Berra, E. Gothie, D. Roux, and J. Pouyssegur. 1999. p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. *J Biol Chem*. 274:32631-32637.
- Rofougaran, R., M. Crona, M. Vodnala, B.M. Sjöberg, and A. Hofer. 2008. Oligomerization status directs overall activity regulation of the Escherichia coli class Ia ribonucleotide reductase. *J Biol Chem*. 283:35310-35318.
- Ryoo, J., J. Choi, C. Oh, S. Kim, M. Seo, S.Y. Kim, D. Seo, J. Kim, T.E. White, A. Brandariz-Nunez, F. Diaz-Griffero, C.H. Yun, J.A. Hollenbaugh, B. Kim, D. Baek, and K. Ahn. 2014. The ribonuclease activity of SAMHD1 is required for HIV-1 restriction. *Nat Med*. 20:936-941.
- Saldivar, J.C., S. Miuma, J. Bene, S.A. Hosseini, H. Shibata, J. Sun, L.J. Wheeler, C.K. Mathews, and K. Huebner. 2012. Initiation of genome instability and preneoplastic processes through loss of Fhit expression. *PLoS Genet*. 8:e1003077.
- Sandvik, G.K., A.B. Tomter, J. Bergan, G. Zoppellaro, A.L. Barra, A.K. Rohr, M. Kolberg, S. Ellefsen, K.K. Andersson, and G.E. Nilsson. 2012. Studies of ribonucleotide reductase in crucian carp-an oxygen dependent enzyme in an anoxia tolerant vertebrate. *PLoS One*. 7:e42784.
- Savage, K.I., J.J. Gorski, E.M. Barros, G.W. Irwin, L. Manti, A.J. Powell, A. Pellagatti, N. Lukashchuk, D.J. McCance, W.G. McCluggage, G. Schettino, M. Salto-Tellez, J. Boulton, D.J. Richard, S.S. McDade, and D.P. Harkin. 2014. Identification of a BRCA1-mRNA splicing complex required for efficient DNA repair and maintenance of genomic stability. *Mol Cell*. 54:445-459.
- Savage, K.I., and D.P. Harkin. 2015. BRCA1, a 'complex' protein involved in the maintenance of genomic stability. *FEBS J*. 282:630-646.
- Scanlon, S.E., and P.M. Glazer. 2015. Multifaceted control of DNA repair pathways by the hypoxic tumor microenvironment. *DNA Repair (Amst)*. 32:180-189.
- Schindl, M., S.F. Schoppmann, H. Samonigg, H. Hausmaninger, W. Kwasny, M. Gnant, R. Jakesz, E. Kubista, P. Birner, G. Oberhuber, B. Austrian, and G. Colorectal Cancer Study. 2002. Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer. *Clin Cancer Res*. 8:1831-1837.
- Schofield, C.J., and P.J. Ratcliffe. 2004. Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol*. 5:343-354.
- Semenza, G. 2002. Signal transduction to hypoxia-inducible factor 1. *Biochem Pharmacol*. 64:993-998.
- Semenza, G.L. 2003. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer*. 3:721-732.
- Seyfried, T.N., and L.M. Shelton. 2010. Cancer as a metabolic disease. *Nutr Metab (Lond)*. 7:7.
- Shaibani, A., O.A. Shchelochkov, S. Zhang, P. Katsonis, O. Lichtarge, L.J. Wong, and M. Shinawi. 2009. Mitochondrial neurogastrointestinal encephalopathy due to mutations in RRM2B. *Arch Neurol*. 66:1028-1032.
- Shams, I., A. Malik, I. Manov, A. Joel, M. Band, and A. Avivi. 2013. Transcription pattern of p53-targeted DNA repair genes in the hypoxia-tolerant subterranean mole rat Spalax. *J Mol Biol*. 425:1111-1118.

- Shao, J., B. Zhou, L. Zhu, W. Qiu, Y.C. Yuan, B. Xi, and Y. Yen. 2004. In vitro characterization of enzymatic properties and inhibition of the p53R2 subunit of human ribonucleotide reductase. *Cancer Res.* 64:1-6.
- Sherman, P.A., and J.A. Fyfe. 1989. Enzymatic assay for deoxyribonucleoside triphosphates using synthetic oligonucleotides as template primers. *Anal Biochem.* 180:222-226.
- Sivridis, E., A. Giatromanolaki, K.C. Gatter, A.L. Harris, M.I. Koukourakis, Tumor, and G. Angiogenesis Research. 2002. Association of hypoxia-inducible factors 1 α and 2 α with activated angiogenic pathways and prognosis in patients with endometrial carcinoma. *Cancer.* 95:1055-1063.
- Smith, D.I., Y. Zhu, S. McAvoy, and R. Kuhn. 2006. Common fragile sites, extremely large genes, neural development and cancer. *Cancer Lett.* 232:48-57.
- Smith, P., B. Zhou, N. Ho, Y.C. Yuan, L. Su, S.C. Tsai, and Y. Yen. 2009. 2.6 Å X-ray crystal structure of human p53R2, a p53-inducible ribonucleotide reductase. *Biochemistry.* 48:11134-11141.
- Snyder, R.D. 1984. The role of deoxynucleoside triphosphate pools in the inhibition of DNA-excision repair and replication in human cells by hydroxyurea. *Mutat Res.* 131:163-172.
- Sorensen, C.S., and R.G. Syljuasen. 2012. Safeguarding genome integrity: the checkpoint kinases ATR, CHK1 and WEE1 restrain CDK activity during normal DNA replication. *Nucleic Acids Res.* 40:477-486.
- Sparks, J.L., H. Chon, S.M. Cerritelli, T.A. Kunkel, E. Johansson, R.J. Crouch, and P.M. Burgers. 2012. RNase H2-initiated ribonucleotide excision repair. *Mol Cell.* 47:980-986.
- Sterkers, Y., C. Preudhomme, J.L. Lai, J.L. Demory, M.T. Caulier, E. Wattel, D. Bordessoule, F. Bauters, and P. Fenaux. 1998. Acute myeloid leukemia and myelodysplastic syndromes following essential thrombocythemia treated with hydroxyurea: high proportion of cases with 17p deletion. *Blood.* 91:616-622.
- Stryer, L. 1995. Biochemistry. W.H. Freeman, New York. xxxiv, 1064 p. pp.
- Stubbe, J. 1998. Ribonucleotide reductases in the twenty-first century. *Proc Natl Acad Sci U S A.* 95:2723-2724.
- Stubbe, J., J. Ge, and C.S. Yee. 2001. The evolution of ribonucleotide reduction revisited. *Trends Biochem Sci.* 26:93-99.
- Stubbe, J., D.G. Nocera, C.S. Yee, and M.C. Chang. 2003. Radical initiation in the class I ribonucleotide reductase: long-range proton-coupled electron transfer? *Chem Rev.* 103:2167-2201.
- Stubbe, J., and W.A. van der Donk. 1995. Ribonucleotide reductases: radical enzymes with suicidal tendencies. *Chem Biol.* 2:793-801.
- Stubbe, J., and W.A. van Der Donk. 1998. Protein Radicals in Enzyme Catalysis. [Chem. Rev. 1998, 98, 705minus sign762. *Chem Rev.* 98:2661-2662.
- Sullivan, R., and C.H. Graham. 2009. Hypoxia prevents etoposide-induced DNA damage in cancer cells through a mechanism involving hypoxia-inducible factor 1. *Mol Cancer Ther.* 8:1702-1713.
- Szent-Gyorgyi, A. 1977. The living state and cancer. *Proc Natl Acad Sci U S A.* 74:2844-2847.
- Tanaka, H., H. Arakawa, T. Yamaguchi, K. Shiraishi, S. Fukuda, K. Matsui, Y. Takei, and Y. Nakamura. 2000. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature.* 404:42-49.
- Tanaka, T., S. Ohkubo, I. Tatsuno, and C. Prives. 2007. hCAS/CSE1L associates with chromatin and regulates expression of select p53 target genes. *Cell.* 130:638-650.

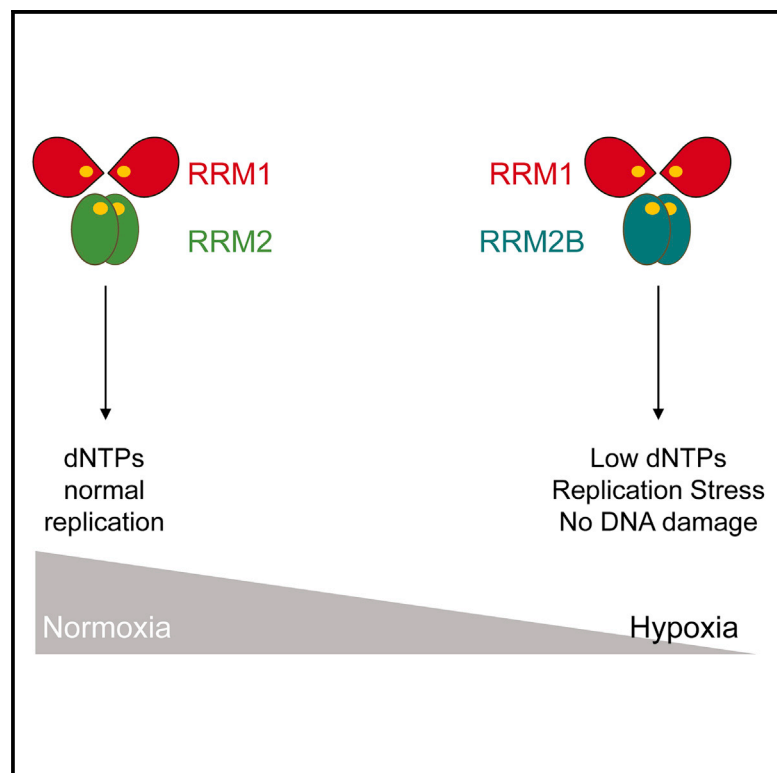
- Tannock, I.F. 1968. The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. *Br J Cancer*. 22:258-273.
- Temperini, C., A. Innocenti, A. Scozzafava, and C.T. Supuran. 2006. N-hydroxyurea--a versatile zinc binding function in the design of metalloenzyme inhibitors. *Bioorg Med Chem Lett*. 16:4316-4320.
- Thelander, L., A. Graslund, and M. Thelander. 1983. Continual presence of oxygen and iron required for mammalian ribonucleotide reduction: possible regulation mechanism. *Biochem Biophys Res Commun*. 110:859-865.
- Thomlinson, R.H., and L.H. Gray. 1955. The histological structure of some human lung cancers and the possible implications for radiotherapy. *Br J Cancer*. 9:539-549.
- Toledo, L.I., M. Altmeyer, M.B. Rask, C. Lukas, D.H. Larsen, L.K. Povlsen, S. Bekker-Jensen, N. Mailand, J. Bartek, and J. Lukas. 2013. ATR prohibits replication catastrophe by preventing global exhaustion of RPA. *Cell*. 155:1088-1103.
- Traut, T.W. 1994. Physiological concentrations of purines and pyrimidines. *Mol Cell Biochem*. 140:1-22.
- Trenz, K., A. Errico, and V. Costanzo. 2008. Plx1 is required for chromosomal DNA replication under stressful conditions. *EMBO J*. 27:876-885.
- Tsai, M.H., X. Chen, G.V. Chandramouli, Y. Chen, H. Yan, S. Zhao, P. Keng, H.L. Liber, C.N. Coleman, J.B. Mitchell, and E.Y. Chuang. 2006. Transcriptional responses to ionizing radiation reveal that p53R2 protects against radiation-induced mutagenesis in human lymphoblastoid cells. *Oncogene*. 25:622-632.
- Tuckerman, M., B.J. Berne, and G.J. Martyna. 1992. Reversible multiple time scale molecular dynamics. *The Journal of chemical physics*. 97:1990-2001.
- Tym, J.E., C. Mitsopoulos, E.A. Coker, P. Razaz, A.C. Schierz, A.A. Antolin, and B. Al-Lazikani. 2016. canSAR: an updated cancer research and drug discovery knowledgebase. *Nucleic Acids Res*. 44:D938-943.
- Tyynismaa, H., E. Ylikallio, M. Patel, M.J. Molnar, R.G. Haller, and A. Suomalainen. 2009. A heterozygous truncating mutation in RRM2B causes autosomal-dominant progressive external ophthalmoplegia with multiple mtDNA deletions. *Am J Hum Genet*. 85:290-295.
- Tzoneva, G., A. Perez-Garcia, Z. Carpenter, H. Khiabani, V. Tosello, M. Allegretta, E. Paietta, J. Racevskis, J.M. Rowe, M.S. Tallman, M. Paganin, G. Basso, J. Hof, R. Kirschner-Schwabe, T. Palomero, R. Rabadan, and A. Ferrando. 2013. Activating mutations in the NT5C2 nucleotidase gene drive chemotherapy resistance in relapsed ALL. *Nat Med*. 19:368-371.
- Uhlin, U., and H. Eklund. 1994. Structure of ribonucleotide reductase protein R1. *Nature*. 370:533-539.
- Ullman, B., A. Cohen, and D.W. Martin. 1976. Characterization of a cell culture model for the study of adenosine deaminase- and purine nucleoside phosphorylase-deficient immunologic disease. *Cell*. 9:205-211.
- Uramoto, H., K. Sugio, T. Oyama, T. Hanagiri, and K. Yasumoto. 2006. P53R2, p53 inducible ribonucleotide reductase gene, correlated with tumor progression of non-small cell lung cancer. *Anticancer Res*. 26:983-988.
- Vaupel, P., and A. Mayer. 2007. Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev*. 26:225-239.
- Verlet, L. 1967. Computer "Experiments" on Classical Fluids. I. Thermodynamical Properties of Lennard-Jones Molecules. *Physical Review*. 159:98-103.
- Wang, G.L., B.H. Jiang, E.A. Rue, and G.L. Semenza. 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci U S A*. 92:5510-5514.

- Wang, G.L., and G.L. Semenza. 1993. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc Natl Acad Sci U S A*. 90:4304-4308.
- Wang, L.M., F.F. Lu, S.Y. Zhang, R.Y. Yao, X.M. Xing, and Z.M. Wei. 2012. Overexpression of catalytic subunit M2 in patients with ovarian cancer. *Chin Med J (Engl)*. 125:2151-2156.
- Wang, P.-h., R.B. Best, and J. Blumberger. 2011. A microscopic model for gas diffusion dynamics in a [NiFe]-hydrogenase. *Physical Chemistry Chemical Physics*. 13.
- Wang, P.-h., M. Bruschi, L. De Gioia, and J. Blumberger. 2013. Uncovering a Dynamically Formed Substrate Access Tunnel in Carbon Monoxide Dehydrogenase/Acetyl-CoA Synthase. *Journal of the American Chemical Society*. 135:9493-9502.
- Wang, P.h., and J. Blumberger. 2012. Mechanistic insight into the blocking of CO diffusion in [NiFe]-hydrogenase mutants through multiscale simulation. *Proceedings of the National Academy of Sciences*. 109:6399-6404.
- Wang, W., C.P. Winlove, and C.C. Michel. 2003. Oxygen partial pressure in outer layers of skin of human finger nail folds. *J Physiol*. 549:855-863.
- Wang, X., A. Zhenchuk, K.G. Wiman, and F. Albertioni. 2009. Regulation of p53R2 and its role as potential target for cancer therapy. *Cancer Lett*. 276:1-7.
- Wasylishen, A.R., and G. Lozano. 2016. Attenuating the p53 Pathway in Human Cancers: Many Means to the Same End. *Cold Spring Harb Perspect Med*. 6.
- Wigerup, C., S. Pahlman, and D. Bexell. 2016. Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. *Pharmacol Ther*. 164:152-169.
- Williams, J.S., S.A. Lujan, and T.A. Kunkel. 2016. Processing ribonucleotides incorporated during eukaryotic DNA replication. *Nat Rev Mol Cell Biol*. 17:350-363.
- Woodward, A.M., T. Gohler, M.G. Luciani, M. Oehlmann, X. Ge, A. Gartner, D.A. Jackson, and J.J. Blow. 2006. Excess Mcm2-7 license dormant origins of replication that can be used under conditions of replicative stress. *J Cell Biol*. 173:673-683.
- Xie, M., Y. Yen, T.K. Owonikoko, S.S. Ramalingam, F.R. Khuri, W.J. Curran, P.W. Doetsch, and X. Deng. 2014. Bcl2 induces DNA replication stress by inhibiting ribonucleotide reductase. *Cancer Res*. 74:212-223.
- Xu, X., J.L. Page, J.A. Surtees, H. Liu, S. Lagedrost, Y. Lu, R. Bronson, E. Alani, A.Y. Nikitin, and R.S. Weiss. 2008. Broad overexpression of ribonucleotide reductase genes in mice specifically induces lung neoplasms. *Cancer Res*. 68:2652-2660.
- Xue, L., B. Zhou, X. Liu, Y. Heung, J. Chau, E. Chu, S. Li, C. Jiang, F. Un, and Y. Yen. 2007. Ribonucleotide reductase small subunit p53R2 facilitates p21 induction of G1 arrest under UV irradiation. *Cancer Res*. 67:16-21.
- Xue, L., B. Zhou, X. Liu, T. Wang, J. Shih, C. Qi, Y. Heung, and Y. Yen. 2006. Structurally dependent redox property of ribonucleotide reductase subunit p53R2. *Cancer Res*. 66:1900-1905.
- Yanamoto, S., G. Kawasaki, S. Yamada, I. Yoshitomi, H. Yoshida, and A. Mizuno. 2009. Ribonucleotide reductase small subunit p53R2 promotes oral cancer invasion via the E-cadherin/beta-catenin pathway. *Oral Oncol*. 45:521-525.
- Yanamoto, S., G. Kawasaki, I. Yoshitomi, and A. Mizuno. 2003. Expression of p53R2, newly p53 target in oral normal epithelium, epithelial dysplasia and squamous cell carcinoma. *Cancer Lett*. 190:233-243.
- Yen, Y., B. Chu, C. Yen, J. Shih, and B. Zhou. 2006. Enzymatic property analysis of p53R2 subunit of human ribonucleotide reductase. *Adv Enzyme Regul*. 46:235-247.
- Yokomakura, N., S. Natsugoe, H. Okumura, R. Ikeda, Y. Uchikado, Y. Mataka, H. Takatori, M. Matsumoto, T. Owaki, S. Ishigami, and T. Aikou. 2007. Improvement in radiosensitivity using small interfering RNA targeting p53R2 in esophageal squamous cell carcinoma. *Oncol Rep*. 18:561-567.

- Yoshida, K., A. Poveda, and P. Pasero. 2013. Time to be versatile: regulation of the replication timing program in budding yeast. *J Mol Biol.* 425:4696-4705.
- Yun, D., L. Saleh, R. Garcia-Serres, B.M. Chicaese, Y.H. An, B.H. Huynh, and J.M. Bollinger, Jr. 2007. Addition of oxygen to the diiron(II/II) cluster is the slowest step in formation of the tyrosyl radical in the W103Y variant of ribonucleotide reductase protein R2 from mouse. *Biochemistry.* 46:13067-13073.
- Zander, R., and P. Vaupel. 1985. Proposal for using a standardized terminology on oxygen transport to tissue. *Adv Exp Med Biol.* 191:965-970.
- Zeman, M.K., and K.A. Cimprich. 2014. Causes and consequences of replication stress. *Nat Cell Biol.* 16:2-9.
- Zhang, H., and C.H. Freudenreich. 2007. An AT-rich sequence in human common fragile site FRA16D causes fork stalling and chromosome breakage in *S. cerevisiae*. *Mol Cell.* 27:367-379.
- Zhang, K., S. Hu, J. Wu, L. Chen, J. Lu, X. Wang, X. Liu, B. Zhou, and Y. Yen. 2009. Overexpression of RRM2 decreases thrombospondin-1 and increases VEGF production in human cancer cells in vitro and in vivo: implication of RRM2 in angiogenesis. *Mol Cancer.* 8:11.
- Zhong, H., K. Chiles, D. Feldser, E. Laughner, C. Hanrahan, M.M. Georgescu, J.W. Simons, and G.L. Semenza. 2000. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res.* 60:1541-1545.
- Zhou, B., J. Shao, L. Su, Y.C. Yuan, C. Qi, J. Shih, B. Xi, B. Chu, and Y. Yen. 2005. A dityrosyl-diiron radical cofactor center is essential for human ribonucleotide reductases. *Mol Cancer Ther.* 4:1830-1836.
- Zhou, B., L. Su, Y.C. Yuan, F. Un, N. Wang, M. Patel, B. Xi, S. Hu, and Y. Yen. 2010. Structural basis on the dityrosyl-diiron radical cluster and the functional differences of human ribonucleotide reductase small subunits hp53R2 and hRRM2. *Mol Cancer Ther.* 9:1669-1679.
- Zou, L., and S.J. Elledge. 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science.* 300:1542-1548.

Ribonucleotide Reductase Requires Subunit Switching in Hypoxia to Maintain DNA Replication

Graphical Abstract



Authors

Iosifina P. Foskolou,
Christian Jorgensen,
Katarzyna B. Leszczynska, ...,
Carmen Domene, Emily Flashman,
Ester M. Hammond

Correspondence

ester.hammond@oncology.ox.ac.uk

In Brief

Foskolou et al. demonstrate that in hypoxic conditions the small subunit of the RNR enzyme is switched from RRM2 to RRM2B in order to facilitate nucleotide production and ongoing replication. They identify specific residues within RRM2B that are responsible for maintaining activity in hypoxia.

Highlights

- RRM2B is induced in response to hypoxia in both cell models and patient datasets
- RRM2B retains activity in hypoxic conditions and is the favored RNR subunit in hypoxia
- Loss of RRM2B has detrimental consequences for cell fate, specifically in hypoxia
- RRM2B depletion enhanced hypoxic-specific apoptosis and increased radiosensitivity

Ribonucleotide Reductase Requires Subunit Switching in Hypoxia to Maintain DNA Replication

Iosifina P. Foskolou,¹ Christian Jorgensen,² Katarzyna B. Leszczynska,¹ Monica M. Olcina,¹ Hanna Tarhonskaya,⁴ Bauke Haisma,¹ Vincenzo D'Angiolella,¹ William K. Myers,³ Carmen Domene,^{2,4} Emily Flashman,⁴ and Ester M. Hammond^{1,5,*}

¹Cancer Research UK and Medical Research Council Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford, Oxford OX3 7DQ, UK

²Department of Chemistry, King's College London, Britannia House, 7 Trinity Street, London SE1 1DB, UK

³Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR, UK

⁴Department of Chemistry, Chemistry Research Laboratory, University of Oxford, Mansfield Road, Oxford OX1 3TA, UK

⁵Lead Contact

*Correspondence: ester.hammond@oncology.ox.ac.uk

<http://dx.doi.org/10.1016/j.molcel.2017.03.005>

SUMMARY

Cells exposed to hypoxia experience replication stress but do not accumulate DNA damage, suggesting sustained DNA replication. Ribonucleotide reductase (RNR) is the only enzyme capable of de novo synthesis of deoxyribonucleotide triphosphates (dNTPs). However, oxygen is an essential cofactor for mammalian RNR (RRM1/RRM2 and RRM1/RRM2B), leading us to question the source of dNTPs in hypoxia. Here, we show that the RRM1/RRM2B enzyme is capable of retaining activity in hypoxia and therefore is favored over RRM1/RRM2 in order to preserve ongoing replication and avoid the accumulation of DNA damage. We found two distinct mechanisms by which RRM2B maintains hypoxic activity and identified responsible residues in RRM2B. The importance of RRM2B in the response to tumor hypoxia is further illustrated by correlation of its expression with a hypoxic signature in patient samples and its roles in tumor growth and radioresistance. Our data provide mechanistic insight into RNR biology, highlighting RRM2B as a hypoxic-specific, anti-cancer therapeutic target.

INTRODUCTION

Replication stress is a well-characterized tumor characteristic and has recently been considered as a potential new hallmark of cancer (Macheret and Halazonetis, 2015). Replication stress leads to the activation of the DNA damage response (DDR), which is associated with the early stages of cancer development (Bartkova et al., 2005; Gorgoulis et al., 2005). Hypoxia (low oxygen levels) is one of the most physiologically relevant driving forces of replication stress and is characterized by an increased number of stalled replication forks and significantly reduced replication rates. Importantly, hypoxia-induced replication stress

occurs in the absence of DNA damage (Olcina et al., 2013). Regions of hypoxia are observed in preneoplastic lesions in the majority of tumors. Hypoxia-induced replication stress is one of the factors proposed to contribute to the selection pressure to lose key components of the DDR, including p53 (Gorgoulis et al., 2005; Hammond et al., 2007; Graeber et al., 1996). The degree of tumor hypoxia is an indicator of poor patient prognosis due to resistance to most current therapies and increased metastatic spread (Begg et al., 2011; Höckel and Vaupel, 2001).

The importance of ribonucleotide reductase (RNR) lies in its unique ability to catalyze the rate-limiting step of the reduction of ribonucleotides (NDPs) to the corresponding deoxyribonucleotides (dNDPs), the precursors of DNA (Kolberg et al., 2004; Stubbe, 1998). Mammalian RNRs consist of two homodimeric subunits, which associate to form the holoenzyme. The large RRM1 dimer contains the catalytic site, whereas the smaller dimer (RRM2 or RRM2B) contains an oxygen-requiring di-iron tyrosyl-radical site, which is essential for catalysis (Kolberg et al., 2004; Uhlin and Eklund, 1994; Stubbe et al., 2003). RNR is highly regulated, as nucleotide imbalances can be detrimental to cell fate (Aye et al., 2015). Elevated deoxyribonucleotide triphosphate (dNTP) pools have been correlated with increased mutagenesis, while insufficient dNTPs cause replication stress and promote genomic instability (Bester et al., 2011; Burrell et al., 2013; Halazonetis et al., 2008; D'Angiolella et al., 2012).

Oxygen is essential for mammalian RNRs to oxidize the di-iron center found in the smaller subunit (RRM2 or RRM2B) (Huang et al., 2014). This generates the tyrosyl radical, which via a long-range pathway, transfers an electron to the catalytic site of RRM1, enabling the reduction of NDPs to dNDPs (Lundin et al., 2015; Kolberg et al., 2004; Stubbe et al., 2003). The mechanism by which iron is incorporated into the small subunit, and how oxygen activates the di-iron center for radical initiation, is still poorly understood and under intense investigation (Huang et al., 2014; Zhang et al., 2014). The oxygen dependency of the small RNR subunit has led to the logical assumption that RNR is inactive in severely hypoxic conditions, something that has been verified for the RRM1/RRM2 (R1/R2) version of the RNR enzyme (Brischwein et al., 1997; Chimpoy et al., 2000; Thelander et al., 1983; Reichard, 1993; Probst et al., 1989; Nordlund

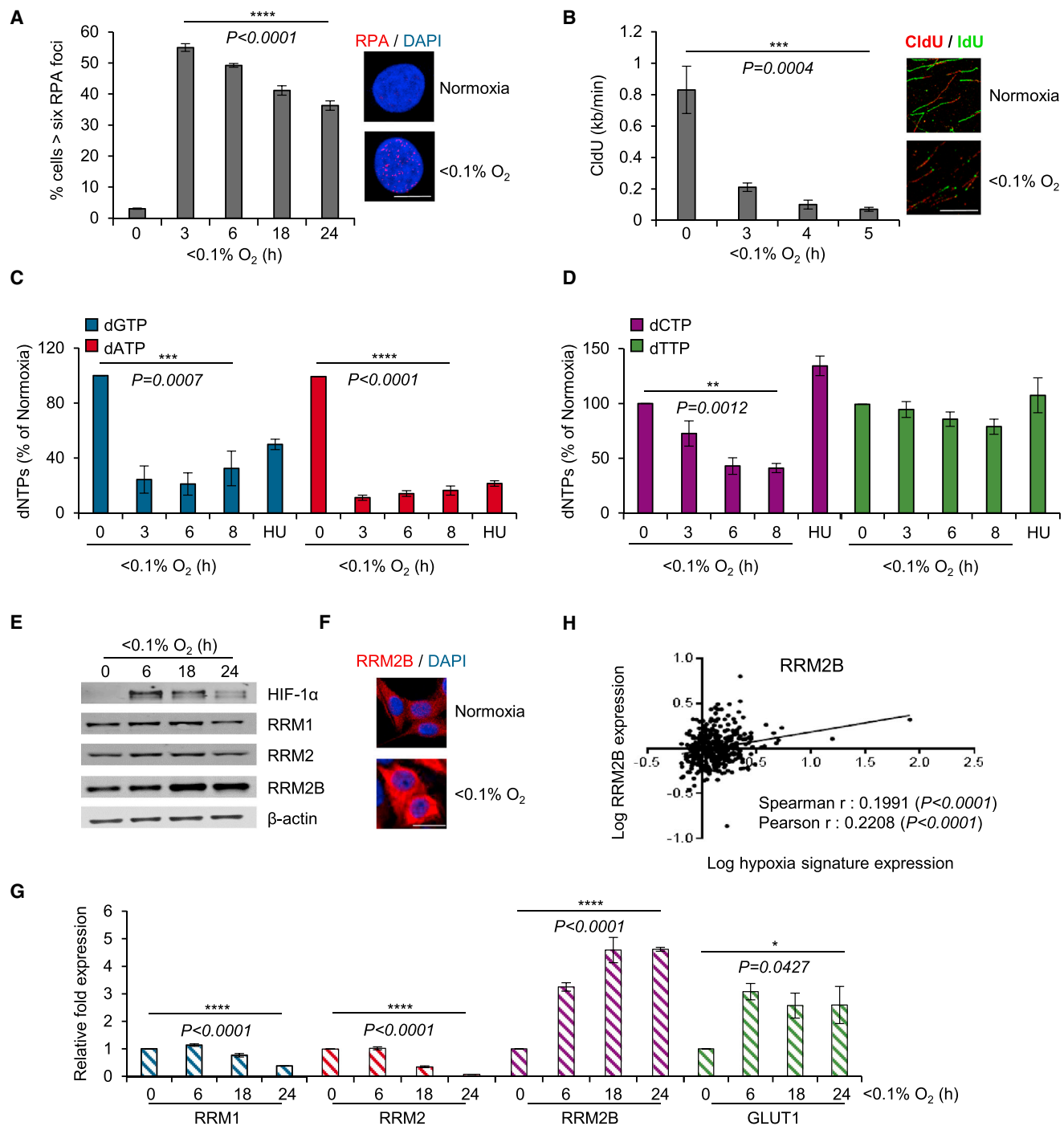


Figure 1. Replication Is Compromised, but Not Abrogated, in Oxygen Levels below 0.1%

(A) RPA32 foci in RKO cells exposed to $<0.1\%$ O_2 and representative images of RPA-negative (normoxia) and positive ($<0.1\%$ O_2 , 3 hr) cells. Scale bar, 10 μm .
 (B) Replication rates by DNA fiber analysis in RKO cells exposed to $<0.1\%$ O_2 and representative images of DNA fibers in normoxia and $<0.1\%$ O_2 (5 hr). Scale bar, 10 μm .
 (C and D) dNTP levels of purines (C) and pyrimidines (D) in RKO cells exposed to $<0.1\%$ O_2 or the radical-scavenger hydroxyurea (HU) (2 mM, 6 hr). Data represent percentage of the control (normoxia).
 (E) Immunoblots of RNR subunits in RKO cells exposed to $<0.1\%$ O_2 for the times indicated.
 (F) Representative images of RRM2B immunostaining in normoxia and $<0.1\%$ O_2 (18 hr) in RKO cells. Scale bar, 20 μm .

(legend continued on next page)

and Reichard, 2006). In contrast, we demonstrate that RNR is able to maintain dNDP formation by switching subunits in hypoxic conditions ($<0.1\%$ O_2). This switch is critical to preserve sufficient nucleotide levels for ongoing replication and avoid DNA damage accumulation and apoptosis in hypoxia. Interestingly, we found that the RRM1/RRM2B (R1/R2B) version of the RNR enzyme retains activity in hypoxia due to adaptation of RRM2B to these conditions through two distinct mechanisms. Specifically, we determined that most of the tyrosyl radical in RRM2B remained stable when exposed to $<0.1\%$ O_2 , a property that was diminished after disruption of the helix B open conformation (K37/K151) and the subsequent loss of the crosstalk between the two monomers. Furthermore, computational simulations suggest that Y164 plays a role in the fine-tuning of the oxygen-entering frequency of RRM2B, meaning it is likely to be a better oxygen-sequestering agent than RRM2. Collectively, our results reveal a critical role for the RNR (R1/R2B) enzyme in hypoxic conditions in maintaining sufficient dNTPs for ongoing replication and therefore preventing DNA damage, genomic instability, and subsequent loss of viability.

RESULTS

The RRM2B Subunit of RNR Is Induced in Response to Hypoxia

Cells in hypoxic conditions ($<0.1\%$ O_2) do not accumulate DNA damage, although they are characterized by the presence of replication stress, which leads to the activation of the DDR (Figures S1A and S1B) (Olcina et al., 2013). As predicted, hypoxia-induced replication stress leads to the accumulation of single-stranded DNA (ssDNA), which can be detected through the visualization of replication protein A (RPA) foci in S-phase cells (Pires et al., 2010). However, the percentage of cells in hypoxia with RPA foci decreases after prolonged exposure to these conditions (>6 hr) (Figure 1A). Therefore, our hypothesis is that although uncoupling of the helicases and polymerases occurs in hypoxia, replication can proceed. Using DNA fiber analysis, we confirmed that DNA replication is significantly slower in hypoxia (25% of normal rate) but is not completely abrogated (Figure 1B). dNTP incorporation assays were carried out in RKO cells exposed to hypoxia and demonstrated that, although the dNTP pools were disrupted (with the purines [dGTP, dATP] being more affected than the pyrimidines [dCTP, dTTP]), there was nucleotide availability in hypoxic conditions (Figures 1C and 1D). The differences observed between purine and pyrimidine pools are not fully understood. It has been suggested that pyrimidine levels persist due to compensatory activities of the pyrimidine salvage pathways or as a result of the deoxyuridine present in culture medium, which cells could uptake and phosphorylate, therefore contributing to pyrimidine levels, particularly dTTP (Eriksson et al., 1987; Bianchi et al., 1986). Interestingly, the pyrimidine salvage pathway has been

reported to be more efficient than the purine pathway, suggesting an explanation for why purine levels are more sensitive to hydroxyurea (HU) and potentially hypoxia (Reichard, 1988). Our data demonstrate sufficient nucleotide availability in hypoxia to allow replication to proceed, albeit at a compromised rate; thus raising the possibility that RNR is able to retain some activity in hypoxia.

Expression analysis of the RNR complex showed that the RRM2B protein is significantly induced (2- to 3.5-fold, depending on cell line) in response to hypoxia in cell lines, including colorectal, osteosarcoma, glioblastoma, and esophageal cancer cells, whereas the RRM2 small subunit decreased over time and the RRM1 remained unchanged or showed a slight decrease (Figures 1E, 1F, and S1C–S1F). As RKO cells showed the highest level of RRM2B expression, we chose it as a model line for the majority of our experiments (Figure S1G). Similar results were obtained when mRNA levels were assayed. *RRM2B* increased 4.6-fold after 24 hr in hypoxic conditions, whereas *RRM2* and *RRM1* mRNA levels decreased 12.3- and 2.5-fold, respectively (Figures 1G and S1H). Importantly, in silico TCGA (The Cancer Genome Atlas) analysis of colorectal adenocarcinoma patient cohorts demonstrated that *RRM2B* expression correlates significantly with the expression of a verified hypoxia signature (Figure 1H), suggesting that the oxygen-dependent overexpression of RRM2B also occurs in vivo (Li et al., 2014). In contrast, *RRM1* and *RRM2* expression did not correlate with the same hypoxic signature (Figures S1I and S1J). Interestingly, overexpression and genetic alterations in *RRM2B* correlated with worse overall and disease-free survival in colorectal cancer patients (Figures S1K–S1N).

The transcription factor HIF-1 (hypoxia-inducible factor 1) is known to mediate significant gene expression changes in response to hypoxia and has roles in DNA replication, DNA repair, and respiration (Hubbi et al., 2013; Fukuda et al., 2007; Crosby et al., 2009). Therefore, we investigated if the induction of RRM2B in hypoxia was dependent on HIF-1 α by utilizing RKO^{HIF-1 α +/+} and RKO^{HIF-1 α -/-} cells exposed to hypoxia (Figures 2A, S2A, and S2B). Interestingly, both the mRNA and the protein levels of RRM2B were induced in hypoxia irrespective of HIF-1 α status, in contrast to the well-documented HIF-1 α target GLUT1. Next, using RKO^{HIF-1 α +/+} cells exposed to either 2% or $<0.1\%$ O_2 , we investigated the oxygen dependency of the induction of RRM2B protein. RRM2B was induced in response to the lower level of hypoxia ($<0.1\%$ O_2), where a robust p53 induction was also observed but did not increase in response to 2% O_2 despite HIF-1 α stabilization (Figure 2B). This finding is in agreement with our previous studies demonstrating that the lower level of hypoxia ($<0.1\%$ O_2) induces replication stress and that this is the signal that initiates the DDR (including p53 stabilization) (Hammond et al., 2002; Olcina et al., 2013).

RRM2B was first characterized as a p53-regulated RNR subunit (p53R2) (Tanaka et al., 2000). Here, further analysis of

(G) mRNA levels of RNR subunits in RKO cells in $<0.1\%$ O_2 normalized to 18S.

(H) Expression of RRM2B (log10 conversion) in a colorectal adenocarcinoma TCGA dataset is shown against the hypoxia-inducible signature (log10 conversion). For all panels (except H), $n = 3$ (biological replicates); for (A)–(D) and (G), data show means $\pm$ SEM and one-way ANOVA analysis was applied; (ns) indicates a non-significant change. See also Figure S1.

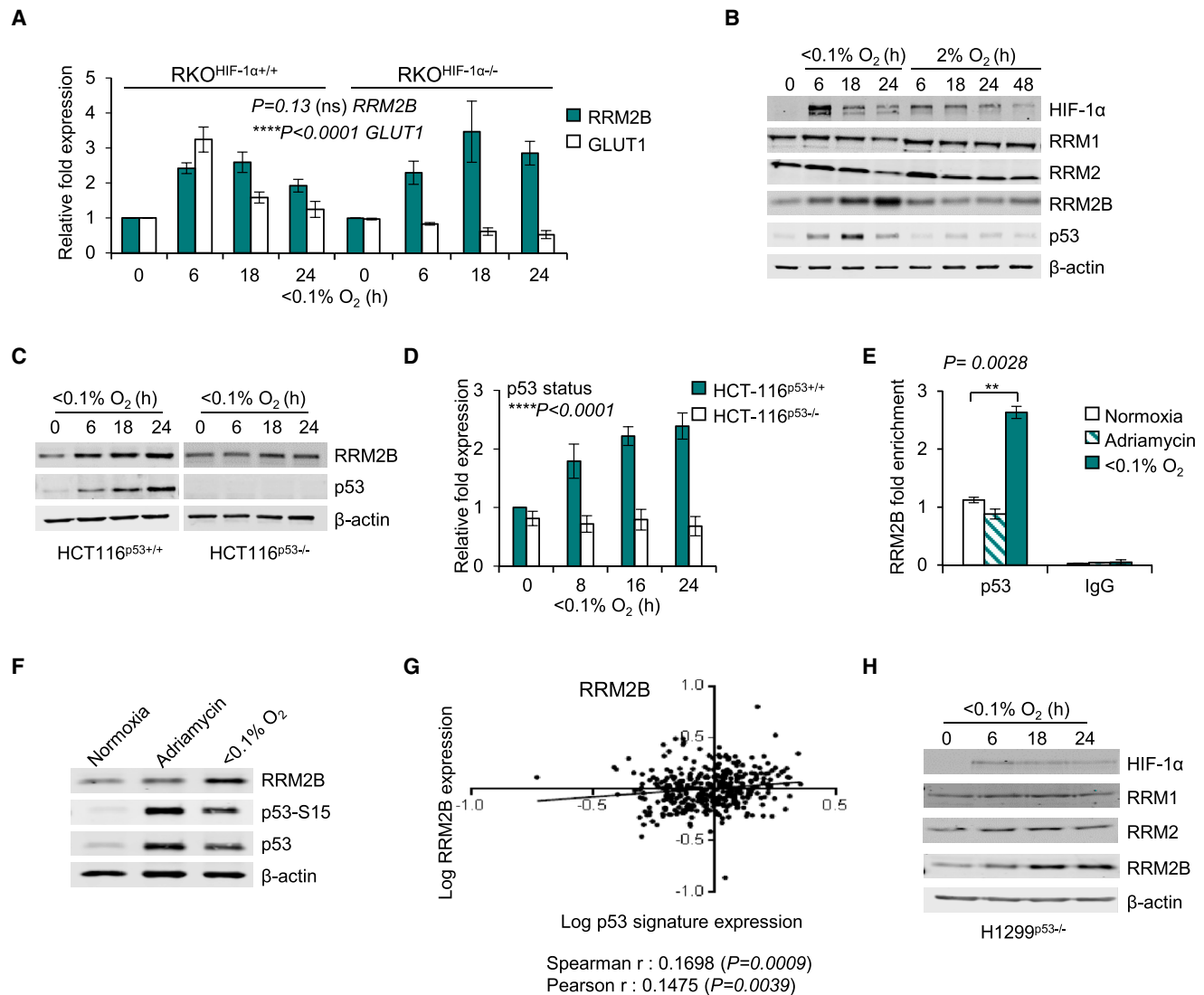


Figure 2. RRM2B Is Induced in Hypoxia

(A) mRNA levels of *RRM2B* and *GLUT1* in RKO^{HIF-1 α +/+} and RKO^{HIF-1 α -/-} cells in $<0.1\%$ O_2 assayed by qPCR and normalized to 18S. (B) Immunoblots of RKO cells exposed to 2% and $<0.1\%$ O_2 for the times indicated. p53 and RRM2B protein induction are observed only in $<0.1\%$ O_2 . (C and D) Immunoblots (C) and *RRM2B* mRNA levels (D) of HCT116^{p53+/+} and HCT116^{p53-/-} exposed to $<0.1\%$ O_2 for the times indicated. (E) qPCR for p53 ChIP in RKO cells treated with Adriamycin (2 μ M, 6 hr) or exposed to either normoxia or $<0.1\%$ O_2 (6 hr). (F) Immunoblots of RKO cells treated as in (E). (G) Expression of RRM2B (log10 conversion) in the colorectal adenocarcinoma TCGA datasets is shown against hypoxia dependent p53-inducible group of genes (log10 conversion). (H) Immunoblots of RNR subunits in H1299^{p53-/-} (non-small cell lung carcinoma) cells exposed to $<0.1\%$ O_2 for the times indicated. For all panels (except G), $n = 3$ (biological replicates); data in (A) and (D) represent mean $\pm$ SEM; and (E) shows representative mean of technical triplicates $\pm$ RQmax/RQmin. HIF-1 α status and p53 status was examined by two-way ANOVA analysis; two-tailed Student's t test was applied in (E); (ns) indicates a non-significant change. See also Figure S2.

the molecular pathways mediating RRM2B induction in hypoxia demonstrated that the hypoxic overexpression of RRM2B occurs in a p53-dependent manner (Figures 2C, 2D, and S2C–S2H). To rule out the possibility of an indirect mechanism of induction of RRM2B by p53, chromatin immunoprecipitation (ChIP) assays were carried out and demonstrated that p53 binds directly to the p53-response element at the *RRM2B* locus lead-

ing to transcriptional overexpression (Figures 2E, 2F, and S2I–S2K). Interestingly, although p53 expression was increased in response to the DNA damaging agent Adriamycin, this did not correlate with increased p53 binding to the p53-response element in *RRM2B* (Figures 2E and 2F). Most importantly, analysis of the TCGA colorectal adenocarcinoma patient cohorts showed that *RRM2B* expression significantly correlated with a

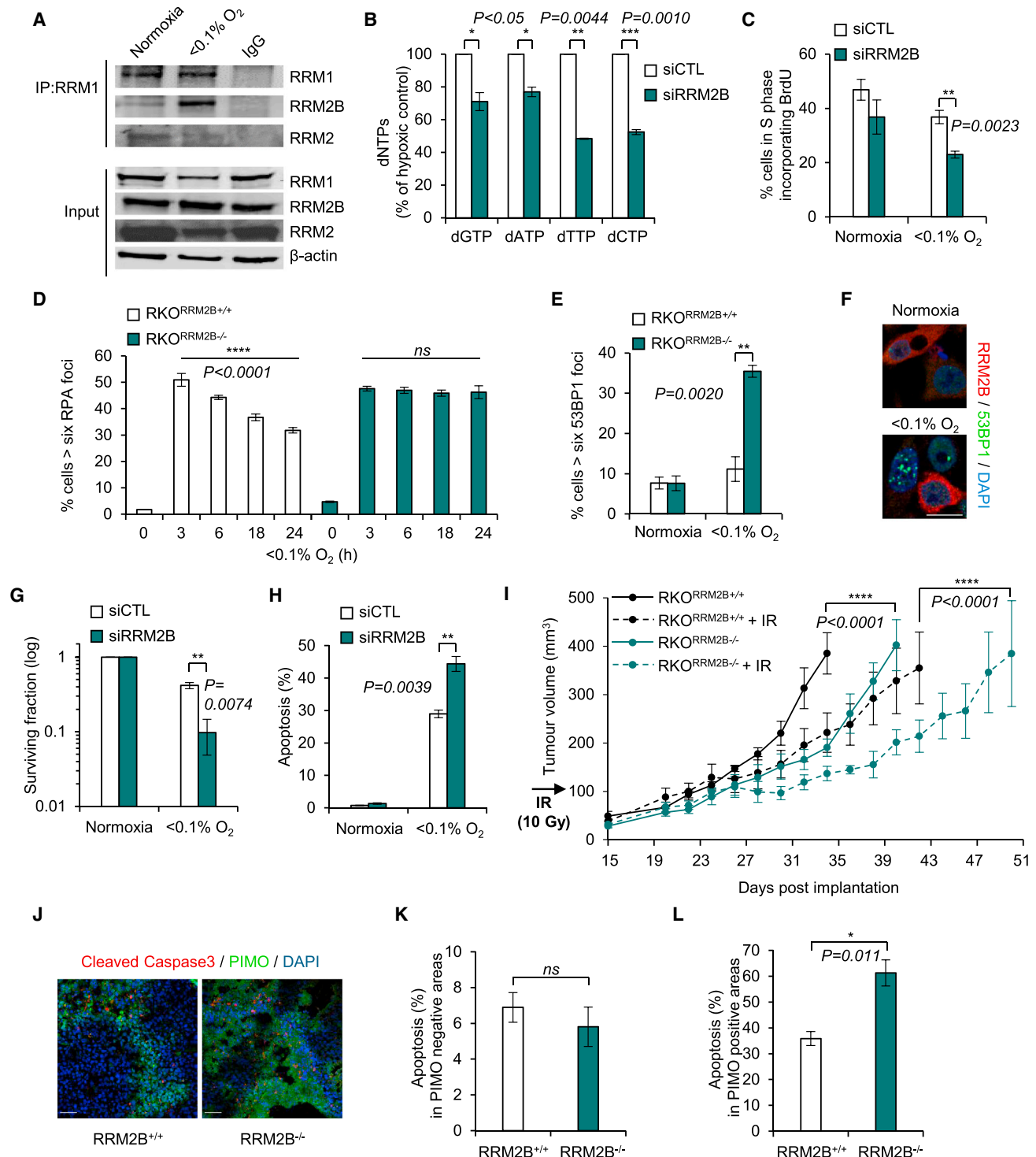


Figure 3. Effects of RRM2B Depletion in Hypoxia

(A) Immunoprecipitation of RRM1 followed by immunoblotting for RRM2B and RRM2 in normoxia and $<0.1\%$ O_2 (18 hr). (B) dNTP levels in RKO cells treated with non-specific (siCTL) or siRRM2B and exposed to $<0.1\%$ O_2 (16 hr). (C) FACS analysis of U2OS cells treated with siCTL or siRRM2B and exposed to normoxia or $<0.1\%$ O_2 (3 hr). Cells were pulsed with bromodeoxyuridine (BrdU) (20 μ M) 30 min before collection. (D) RPA32 foci in RKO^{RRM2B+/+} and RKO^{RRM2B-/-} cells after exposure to $<0.1\%$ O_2 . (E) 53BP1 foci in RKO^{RRM2B+/+} and RKO^{RRM2B-/-} cells exposed to normoxia or $<0.1\%$ O_2 (6 hr).

(legend continued on next page)

recently identified group of hypoxia-inducible p53-dependent genes (Leszczynska et al., 2015), suggesting that hypoxia- and p53-dependent expression of *RRM2B* occurs in human cancers (Figure 2G). Interestingly, in p53 null cell lines (H1299^{p53-/-}, HCT116^{p53-/-}) a mild (1.3- to 1.7-fold) increase in RRM2B protein levels was also observed in hypoxia (Figures 2C and 2H). These findings suggest that additional post-translational p53-independent mechanisms exist for RRM2B stabilization and therefore the importance of RRM2B in hypoxic conditions.

RRM2B Replaces RRM2 in Hypoxia

In order to investigate the biological significance of hypoxia-induced RRM2B, we first verified that it forms a complex with the RRM1 subunit to reconstitute the R1/R2B holoenzyme in <0.1% O₂. Immunoprecipitation assays demonstrated that increased levels (5.3-fold) of RRM2B protein were bound to the RRM1 subunit in hypoxia whereas the levels of RRM2 bound to RRM1 decreased by 1.8-fold (Figures 3A, S3A, and S3B). Next, we asked if the hypoxia-formed R1/R2B enzyme was functional. Small interfering RNA (siRNA)-mediated loss of RRM2B led to significantly lower intracellular dNTP levels in hypoxia (50%–55% less pyrimidines and 25%–30% less purines compared to the control [siCTL]) (Figures 3B and S3C). In contrast, the loss of RRM2B did not significantly affect the dNTP pools in normoxic conditions (Figure S3D). In addition, fluorescence-activated cell sorting (FACS) analysis demonstrated that S-phase U2OS cells lacking RRM2B incorporate 37.5% less BrdU than the control-treated cells in hypoxia (Figures 3C and S3E). These findings demonstrate that depletion of RRM2B in hypoxia leads to further disruption of the dNTP pools and indicate that ongoing replication is disrupted.

To further investigate the hypoxic role of RRM2B, we used CRISPR/Cas9 technology to construct a RRM2B knockout cell line (RKO^{RRM2B-/-}) (Figures S3F–S3I). RRM2B-depleted RKO cells (both knockout and siRNA treated) showed a persistent formation of RPA foci during long (24 hr) exposures to hypoxia, suggesting an accumulation of ssDNA and failure to complete DNA replication (Figures 3D and S4A–S4C). Furthermore, cells with reduced levels of RRM2B formed 53BP1 foci indicating the presence of double-strand breaks (DSBs) specifically in hypoxia (Figures 3E, 3F, and S4D–S4F). No evidence of elevated DNA damage was observed in control cells or in cells lacking RRM2B in normoxia; this was attributed to the RRM2-dependent supply of dNTPs when oxygen is abundant. The increased DSBs observed in hypoxic cells with depleted RRM2B correlated with (1) a significant loss of viability measured by colony survival assay (77% fewer cells survived after 24 hr of hypoxia when

RRM2B was silenced in comparison to siCTL) (Figure 3G) and (2) the induction of apoptosis as determined both morphologically and by induction of PARP cleavage (Figures 3H and S4G). Notably, xenograft tumors of RKO^{RRM2B+/+} and RKO^{RRM2B-/-} cells showed delayed tumor growth and increased radiosensitivity when RRM2B was lacking (Figures 3I and S4H). Additionally, a significant increase in apoptosis specifically in the hypoxic regions of the RRM2B^{-/-} tumors was observed (Figures 3J–3L) as determined by the presence of cleaved caspase-3 (apoptosis) in the pimonidazole (PIMO)-positive (hypoxic) regions. Increased apoptosis in the hypoxic fraction of the tumors offers a likely explanation for the increased radiosensitivity observed (Figure 3I).

The data presented so far suggest that the basal levels of dNTPs provided by R1/R2B (as opposed to R1/R2) are sufficient for ongoing replication in hypoxic cells, preventing the collapse of replication forks that would ultimately lead to DSBs and loss of viability. These data indicate that RRM2B contributes to the increased resistance of the most aggressive fraction of tumors; therefore, we investigated the hypoxic adaptation of RRM2B by determining the enzymatic properties of both forms of RNR enzyme (R1/R2 and R1/R2B) in normoxia and hypoxia.

RRM2B Retains Enzymatic Activity in Hypoxia

RRM1, RRM2, and RRM2B recombinant proteins were over-expressed and purified from *E. coli* (Figures S5A and S5B), without additional iron or reductants, to prevent the variability associated with in vitro assembly of the active cluster of the tyrosyl radical (Cotruvo and Stubbe, 2011). We quantified the iron bound to RRM2 and RRM2B using inductively coupled plasma (ICP) mass spectrometry and found comparable iron incorporation between the two recombinant proteins (Figure S5C). The level of iron incorporation was ~10% of the level that has been reported in a fully loaded RRM2, which would in turn predict lower enzymatic activity (Aye et al., 2012b; Ochiai et al., 1990).

The abilities of the two forms of the RNR enzyme (R1/R2B and R1/R2) to reduce cytidine diphosphate (CDP) (substrate) to deoxycytidine diphosphate (dCDP) (product) were assayed in normoxic and hypoxic conditions (<0.1% O₂). As hypothesized, the R1/R2B was able to sustain activity in both <0.1% O₂ and normoxia with no statistically significant difference in the total amount of dCDP formed under either condition after 30 min (Figure 4A). In fact, the R1/R2B enzyme continued converting CDP to dCDP for over 2 hr in hypoxia (Figures 4B and S5D). In contrast, the R1/R2 enzyme did not sustain activity in hypoxia (Figure 4C) and stopped producing dCDPs after 15 min in <0.1% O₂ (Figures 4D and S5D). Importantly, we verified that the R1/R2 enzyme did

(F) Representative images of 53BP1 foci in RRM2B-negative RKO cells treated with siRRM2B and exposed to normoxia or <0.1% O₂ (6 hr). Scale bar, 20 μm.

(G) Colony survival assay in RKO cells treated with siCTL or siRRM2B and exposed to normoxia or <0.1% O₂ (24 hr).

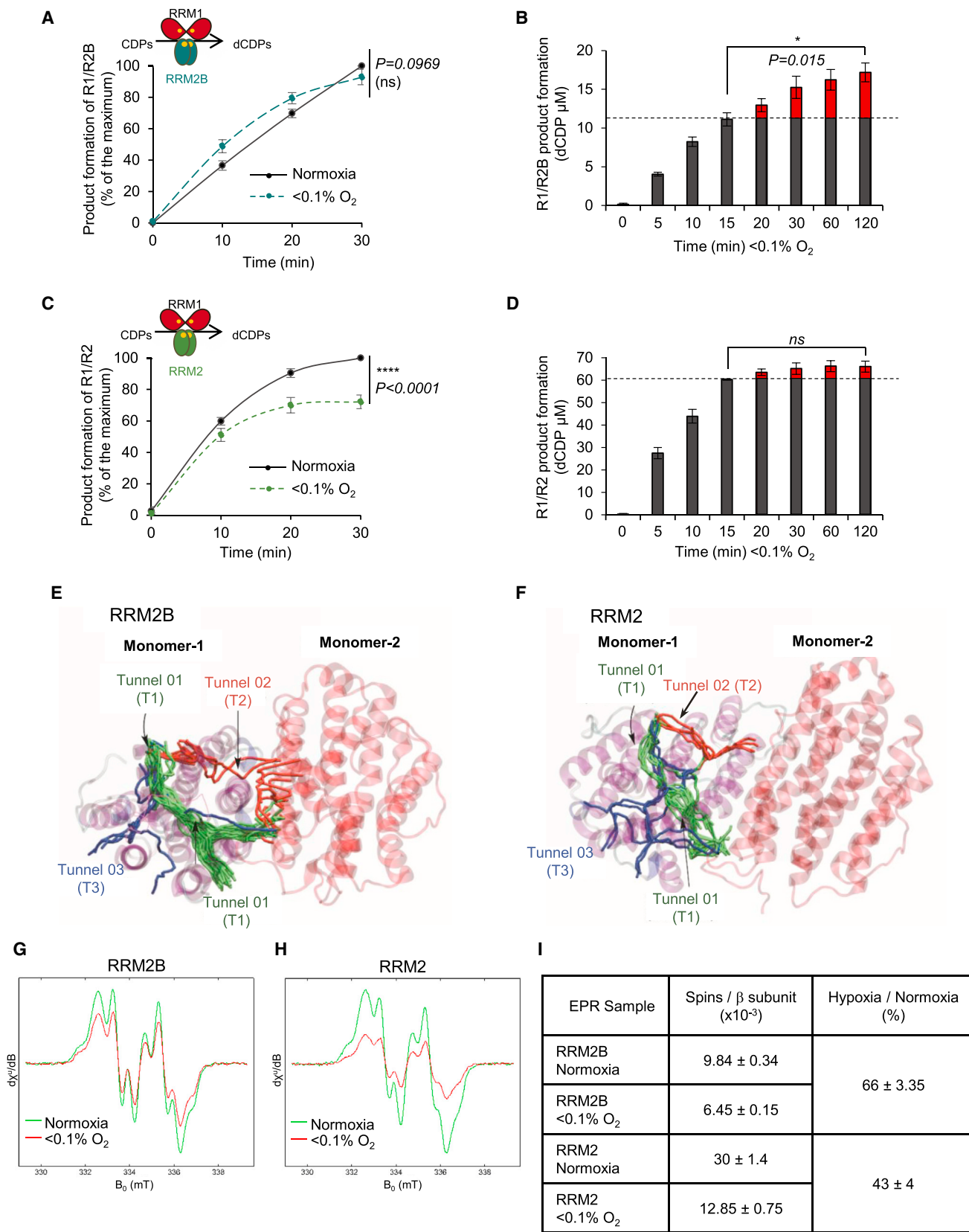
(H) Apoptosis detected morphologically in RKO cells treated with siCTL or siRRM2B and exposed to normoxia or <0.1% O₂ (19 hr).

(I) RKO^{RRM2B+/+} and RKO^{RRM2B-/-} cells were grown as xenografts in mice (n = 4 mice per each group). Where indicated, irradiation (10 Gy) was given when tumors reached ~100 mm³.

(J) Representative images of co-localization of cleaved caspase-3 (apoptosis) with PIMO (hypoxic areas) in RKO^{RRM2B+/+} or RKO^{RRM2B-/-} xenografts. Scale bars, 50 μm.

(K and L) Tumors were removed on day 28 post-implantation (from Figure S5H), and the level of apoptosis was quantified in normoxic areas (PIMO negative) (K) and hypoxic areas (PIMO positive) (L). Images from three different tumors (n = 3) per group were counted.

For all panels, n = 3 (biological replicates) unless otherwise stated. Data show mean ± SEM and two-tailed Student's t test was applied, except in (D), where one-way ANOVA analysis was applied, and (I), where two-way ANOVA analysis was applied. (ns) indicates a non-significant change. See also Figures S3 and S4.



(legend on next page)

Table 1. O₂ Residence Times around the Fe Metallocenter for RRM2B and RRM2 Proteins

System	Time (ns)	O ₂ , Fe Residence Time (ns)	O ₂ Entering Events	Entering Frequency (%)
RRM2B monomer 1	300	O ₂ , 79	25	68
		25		
		O ₂ , 80		
		19		
RRM2B monomer 2	300	O ₂ , 120	8	22
		36		
		O ₂ , 125		
		25		
RRM2 monomer 1	300	O ₂ , 93	5	14
		49		
		O ₂ , 107		
		9		
RRM2 monomer 2	300	O ₂ , 126	0	0
		38		
		O ₂ , 175		
		103		
RRM2 monomer 1	300	O ₂ , 19	3	14
		3		
		O ₂ , 22		
		11		
RRM2 monomer 2	300	O ₂ , 37	2	0
		2		
		O ₂ , 142		
		5		
RRM2 monomer 1	300	O ₂ , 8	19	0
		19		
		O ₂ , 65		
		20		
RRM2 monomer 2	300	O ₂ , 88	14	0
		14		
		O ₂ , 122		
		9		

not stop dCDP formation in hypoxia due to lack of available CDP (Figure S5E). Collectively, these data suggest that the compromised R1/R2 activity observed at <0.1% O₂ was a result of limited oxygen availability. It is important here to state that the oxygen does not affect the stability of the tyrosyl radical, and it is only required for tyrosyl radical formation (Stubbe, 2003). Once assembled, the tyrosyl radical can catalyze multiple turnovers until regeneration is necessary, with mammalian R1/R2 being stable for 15–25 min in vitro (Cotruvo and Stubbe, 2011). The key difference observed between R1/R2B and R1/R2 in hypoxia offers a mechanistic explanation for why the R1/R2B form of RNR is preferred in hypoxic conditions. It should be noted that, as previously described, the overall activity of the RRM2B-containing RNR enzyme is significantly lower than that of the RRM2-containing RNR (Shao et al., 2004) (Figures 4B and 4D). Therefore, although our data suggest that R1/R2B is the preferred RNR enzyme in hypoxia, the levels of available nucleotides are still reduced compared to normoxia, thus explaining the continued replication stress in these conditions (Figure 1A).

Adaption of RRM2B to Hypoxic Environments

Given the differences in normoxic and hypoxic activity between RRM2B and RRM2, we compared the potential oxygen accessibility for the proteins using a modeling approach. Using the only available published structures of RRM2B (PDB: 3HF1) and RRM2 (PDB: 3VPN) (Smith et al., 2009), we investigated the theoretical differences and/or similarities in the dynamics of oxygen diffusion between the two proteins using classical molecular dynamics (MD) simulations. The intention of this study was to understand how molecular oxygen accesses the core of the protein and to probe whether any differences could be related to the activities of RRM2/RRM2B in normoxia versus hypoxia. Our analysis revealed three principal oxygen cavity tunnels (T1–T3) in both proteins, which could be employed as access points (Figures 4E, 4F, and S5F). Analysis of T1–T3 suggested that RRM2B could act as a better oxygen-sequestering agent than RRM2. Specifically, we found that the predicted oxygen-entering frequencies are greater for RRM2B (68% at monomer 1 and 22% at monomer 2) than for RRM2 (14% at monomer 1 and 0% at monomer 2) (Table 1), which indicate differential oxygen-turnover susceptibilities between RRM2B and RRM2. In addition, free-energy calculations of oxygen entry through tunnels T1, T2, and T3 (Figure S5G) suggest that the origin of the selectivity between RRM2B and RRM2 for oxygen turnover resided primarily in tunnels T2 and T3, meaning that the energy barriers for oxygen to cross T2 and T3 are higher for RRM2 than for RRM2B.

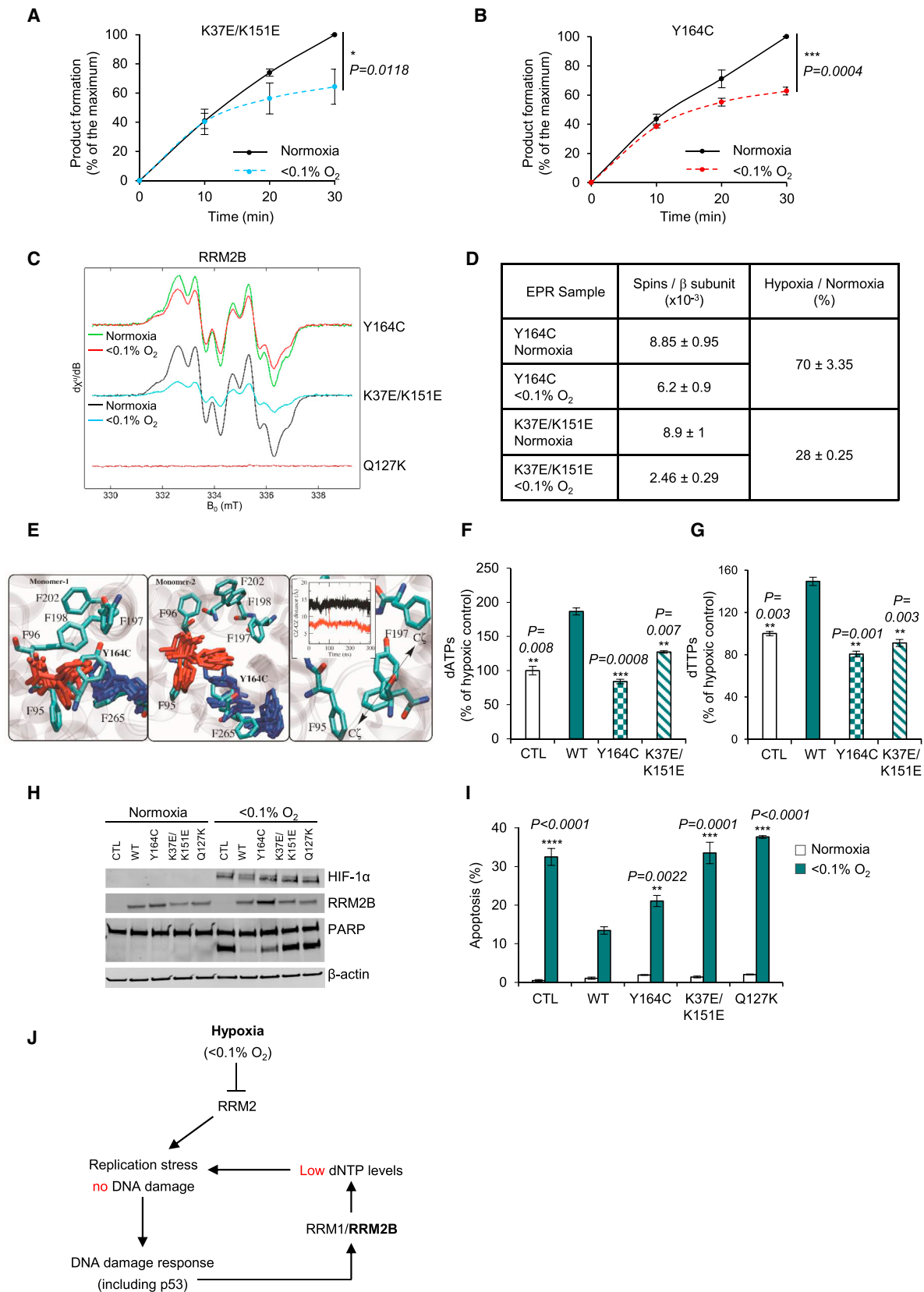
We then performed electron paramagnetic resonance (EPR) spectroscopy to monitor the stability of the tyrosyl radical of the two proteins in hypoxic conditions (Figures 4G and 4H). Strikingly, we observed that 66% ± 3.35% of the tyrosyl radical in RRM2B remained stable for 1 hr at 37°C in <0.1% O₂, while in the same conditions, only 43% ± 4% remained stable in RRM2 (Figure 4I). Collectively, our results provide compelling evidence that RRM2B is able to retain activity in hypoxia while the activity of RRM2 is compromised and therefore highlight a specific role for RRM2B in the hypoxic stress response.

Hypoxic Activity of RRM2B Is Dependent on K37/K151 and Y164

Despite the fact that RRM2 and RRM2B share 83% sequence homology, there are distinct functional and structural differences (Shao et al., 2004; Smith et al., 2009; Zhou et al., 2010; Xue et al., 2006; Wang et al., 2009b). The switch of C202 in RRM2 to Y164

Figure 4. RRM2B Retains Activity in Hypoxia

(A) Product formation (percentage of the maximum, where maximum is the dCDP levels at 30 min in normoxia) for R1/R2B enzyme in normoxia and <0.1% O₂. (B) dCDP (μM) in <0.1% O₂ for R1/R2B for the times indicated. Activity of R1/R2B enzyme at 37°C at 5 min in <0.1% O₂ was 19.57 nmol/min/mg RRM2B protein. Gray columns indicate the amount of dCDP formed up to 15 min in <0.1% O₂, and red columns indicate the amount of dCDP formed after 15 min in <0.1% O₂. (C) Product formation (percentage of the maximum, where maximum is the dCDP levels at 30 min in normoxia) for R1/R2 enzyme in normoxia and <0.1% O₂. (D) dCDP (μM) in <0.1% O₂ for R1/R2 for the times indicated. Activity of R1/R2 enzyme at 37°C at 5 min in <0.1% O₂ was 97.74 nmol/min/mg RRM2 protein. Gray columns indicate the amount of dCDP formed up to 15 min in <0.1% O₂, and red columns indicate the amount of dCDP formed after 15 min in <0.1% O₂. (E and F) Characterization of the oxygen tunnels (T1–T3) of RRM2B (E) and RRM2 (F). (G and H) EPR spectra of the tyrosyl radical of RRM2B (G) and RRM2 (H) in normoxia and <0.1% O₂, respectively. (I) Quantification of (G) and (H). Data present electron spins per β subunit. For all panels, n = 3 (biological replicates); for (A) and (C), data represent mean ± SEM and two-way ANOVA was applied; for (B) and (D), data represent mean ± SEM and two-tailed Student's t test was applied; (ns) indicates non significant change. See also Figure S5.



(legend on next page)

Table 2. O₂ Residence Times around the Fe Metallocenter for RRM2B Mutants

System	Time (ns)	O ₂ , Fe Residence Time (ns)	O ₂ Entering Events	Entering Frequency (%)
Y164C monomer 1	300	O ₂ , 57	8	14
		30		
		O ₂ , 79		
		6		
Y164C monomer 2	300	O ₂ , 124	17	29
		9		
		O ₂ , 139		
		60		
K37E/K151E monomer 1	300	O ₂ , 1	37	64
		9		
		O ₂ , 105		
		14		
K37E/K151E monomer 2	300	O ₂ , 167	6	16
		7		
		O ₂ , 191		
		16		
K37E/K151E monomer 1	300	O ₂ , 37	37	64
		30		
		O ₂ , 40		
		32		
K37E/K151E monomer 2	300	O ₂ , 47	6	16
		47		
		O ₂ , 27		
		27		
K37E/K151E monomer 1	300	O ₂ , 9	6	16
		9		
		O ₂ , 188		
		4		
K37E/K151E monomer 2	300	O ₂ , 190	6	16
		5		
		O ₂ , 198		
		3		

in RRM2B results in an open phenylalanine channel specifically in RRM2B (Smith et al., 2009), which is connected with oxygen T3 (Figures S5F and S5H). It has been proposed that F183 in RRM2B versus Y221 in RRM2 contributes to the differences in the enzymatic activity between the two proteins (Zhou et al., 2010). In addition, RRM2B has unique antioxidant capabilities, which are partially due to the Y241 and Y331 residues (H279 and Y369 in RRM2) (Xue et al., 2006). Finally, RRM2B through K37 and K151 (E76 and E190 in RRM2) exhibits greater crosstalk between its secondary structures, which stabilizes helix B in an open conformation (Smith et al., 2009). Based on these key differences between RRM2 and RRM2B (Figure S6A) and in order to determine the regions of RRM2B responsible for hypoxic adaptation, we constructed, overexpressed, and purified seven recombinant RRM2B mutants: Y164C, Y164F, F183Y, K37E/K151E, Y241H, Y331F, and Q127K (Figure S6B). The Q127K mutant was generated as a negative control, as it diminishes

enzymatic activity due to disruption of the stability of the radical cluster (Zhou et al., 2010).

The seven purified mutants showed the same secondary structure, determined by circular dichroism, as the wild-type RRM2B recombinant protein (Figure S6C). The levels of iron incorporation were also similar for the mutant with the exception of Q127K, as expected (Figure S6D). Reduction of CDP to dCDP was assayed in hypoxia for these mutants. Sustained reduction of CDP to dCDP was observed for 2 hr for most mutants, except Y164C and K37E/K151E, which both appeared to stop reducing substrate after ~30 min in hypoxia, reminiscent of R1/R2 (Figures S6E–S6K). For these two mutants, we then compared dCDP formation in normoxia and hypoxia and found that the ability to reduce CDP to dCDP in hypoxia was compromised in both the K37E/K151E and Y164C mutants (Figures 5A and 5B). These results indicate that these specific residues are critical to the role of RRM2B in hypoxia and that without them, RRM2B activity is not sustained, similar to RRM2.

To further probe the role of K37/K151 and Y164, we performed additional EPR analyses and MD simulations. Using EPR spectroscopy, we observed that while most of the RRM2B mutants behaved as the WT-RRM2B, which retains 60%–79% of its tyrosyl radical in hypoxia (Figure S7A), the K37E/K151E mutant retained only 28% of its tyrosyl radical in these conditions (Figures 5C and 5D). Since K37/K151 residues affect helix B of RRM2B monomer 2, the bending angle of the helices was estimated and showed that helix B in RRM2B is in an open conformation (20°–25° bending angle), whereas in RRM2, it is closed (8°–10°) (Figure S7B). Interestingly, MD simulations for the K37E/K151E variant showed a closure of helix B after 300 ns from 20° to 8° bending angle, therefore resembling the closed conformation of RRM2 (Figures S7B and S7C). Although these are theoretical studies that provide only indications of the protein conformation, the distortion of helix B in monomer 2 of the K37E/K151E mutant could suggest loss of the essential crosstalk between the two monomers, which may explain the loss of tyrosyl radical stability in hypoxia as demonstrated by our EPR analysis.

Additionally, MD simulations showed a 4.8-fold lower oxygen entering frequency for the Y164C mutant in comparison to WT-RRM2B, while we did not observe any changes in the oxygen entering frequency for the K37E/K151E mutant (Table 2).

Figure 5. Critical Roles of K37/K151 and Y164 in RRM2B

(A and B) Product formation (percentage of the maximum, where maximum is the dCDP levels at 30 min in normoxia) for K37E/K151E (A) and Y164C (B) in normoxia and <0.1% O₂.
 (C) EPR spectra of the tyrosyl radical of Y164C, K37E/K151E, and Q127K (as a negative control) in normoxia and <0.1% O₂.
 (D) Quantification of (C). Data present electron spins per β subunit.
 (E) The RRM2B phenylalanine network around Y164 and phenylalanine conformation in Y164C mutation. Distance plot reveals the effect of Y164C in F95–F197 distance. Color code: WT (black), Y164C (red).
 (F and G) dATP (F) and dTTP (G) levels in RKO^{RRM2B-/-} cells transfected with CTL, WT, Y164C, or K37E/K151E and exposed to <0.1% O₂ (16 hr).
 (H) Immunoblot for PARP cleavage in RKO^{RRM2B-/-} cells treated as in (F) and (G) plus Q127K and exposed to <0.1% O₂ (19 hr).
 (I) Apoptosis detected morphologically in RKO^{RRM2B-/-} cells treated as in (H).
 (J) Schematic representation of our proposed model. Hypoxia leads to severely compromised activity of RRM2, leading to replication stress. RRM2B is then induced through the DDR pathway to maintain ongoing replication. However, insufficient dNTPs are generated by R1/R2B, and replication stress is unresolved. The importance of RRM2B activity is that while it does not resolve replication stress, it does maintain replication fork integrity and prevents the accumulation of DNA damage and loss of genome stability.
 For (A), n = 3; for (B), n = 4 (biological replicates) and two-way ANOVA was applied; for (C), n = 2 (biological replicates); for (F)–(I), n = 3 (biological replicates); data represent means $\pm$ SEM and two-tailed Student's t test was applied. See also Figures S6 and S7.

This could be explained by disruption of the Y164-F197 and Y164-F198 contacts in monomer 1 of RRM2B, which could cause side-chain rearrangement of F95 in the Y164C mutant. This rearrangement may result in F95 moving closer to F197, leading to closure of the RRM2B specific phenylalanine channel (Figures 5E and S5H), therefore explaining the lower oxygen-entering frequency in Y164C mutant.

Finally, in order to demonstrate that our findings are relevant in cells experiencing hypoxia and that the hypoxic adaptation of RRM2B depends on the identified residues (Y164 and K37/K151), we performed rescue experiments by transfecting our RKO^{RRM2B^{-/-}} cells with the following constructs: pRRM2B^{WT}, pRRM2B^{Y164C}, pRRM2B^{K37E/K151E}, and pcDNA3.1 (CTL). As expected, when WT-RRM2B was reintroduced, a significant increase in the intracellular nucleotide levels was observed in hypoxia, whereas reintroduction of Y164C and K37E/K151E showed significantly lower dNTP levels compared to WT-RRM2B (Figures 5F, 5G, S7D, and S7E). Additionally, by monitoring apoptosis, we observed that WT-RRM2B rescued the null phenotype from apoptosis, whereas loss of the key residues (Y164 and K37/K151) abrogated this rescue (Figures 5H and 5I), therefore validating our in vitro experiments and theoretical studies. Overall, we demonstrate the critical importance of the hypoxic induction of RRM2B to mitigate replication stress and determine the molecular adaptations of RRM2B to hypoxia to support this function (Figure 5J).

DISCUSSION

We identified that in response to the physiological stress of hypoxia, RNR responds by isoform switching favoring RRM2B over RRM2. In hypoxia the activity of RRM2 is severely compromised due to the lack of available oxygen, leading to replication stress. RRM2B is able to retain its activity in hypoxia and is therefore induced to compensate and facilitate ongoing replication. This property of RRM1/RRM2B, although not sufficient to resolve replication stress, does preserve replication fork integrity and prevent the accumulation of DNA damage in hypoxia. We verified that depletion of RRM2B results in lower dNTP levels in hypoxic cells and that this has detrimental consequences for cell fate (failure to complete DNA replication, DNA damage, and loss of viability). Importantly, loss of RRM2B in a xenograft model showed delayed tumor growth, increased radiosensitivity, and increased apoptosis specifically in hypoxic areas, further highlighting the biological importance of the hypoxic induction of RRM2B.

Our data suggest that RRM2B is capable of retaining activity in hypoxia through two mechanisms: (1) increased oxygen-entering frequency and (2) enhanced stability of the tyrosyl radical. Our MD analyses suggested that Y164 could increase the oxygen-entering frequency through oxygen tunnel T3 in monomer 1 by keeping F95 an optimum distance from F197. We also found that through the K37/K151 residues, the RRM2B protein retains 66%–70% of its tyrosyl radical in hypoxia. Interestingly, monomer 2 of RRM2B showed a higher oxygen-entering frequency than RRM2 (22% and 0%, respectively), suggesting that the two monomers of the small RNR subunits could react differently in the overall production of the tyrosyl

radical. It is possible that monomer 2 contributes more to the tyrosyl radical in RRM2B than in RRM2, possibly due to its helix B open conformation, and that this could be more relevant in hypoxic conditions. In support of this hypothesis, EPR spectra of the double mutant, K37E/K151E, showed a marked reduction in the tyrosyl radical content in hypoxia, but not in normoxia. We propose that the tyrosyl radical of monomer 2 is better protected in RRM2B, therefore contributing to the prolonged activity of the enzyme in hypoxic environments. Our proposed model, where both monomers contribute to tyrosyl radical formation, is in agreement with the established finding that the tyrosyl radical is equally distributed between each small subunit, suggesting the possibility of generation of two tyrosyl radicals per dimer (Cotruvo and Stubbe, 2011). It is important to note that our MD simulations were carried out with the only currently available crystal structure of human RRM2B protein (PDB: 3HF1), which has limitations in its active site configuration and specifically in the iron center. However, we considered it unlikely that as a result of this, there would be large conformational changes in the global structure of the protein compared to its native state, at least to a degree that would influence our MD analysis. Indeed, this is supported by the results of our recombinant mutant analysis and, most importantly, our rescue experiments using our RRM2B knockout cell line. Collectively, this work highlights the importance of Y164, K37, and K151 for RRM2B activity in hypoxia. It is important to note that the recombinant proteins used in our biochemical assays were assembled without additional iron. The biochemistry of RNR, especially the reconstitution of the tyrosyl radical, is extremely complex and not fully understood. Additional studies that also consider the relevant factors for accurate assembly are required and will likely further illuminate the role of RRM2B in both normoxia and hypoxia.

To date, mammalian RRM2B has been primarily associated with DNA repair and mitochondrial DNA synthesis (Wang et al., 2009b; Pontarin et al., 2012). However, it is probable that RRM2B has been evolutionarily maintained in order to be used as the hypoxic-specific RNR small subunit, especially as variants of RRM2B protein have been found to be responsible for continued cell division in anoxia tolerant vertebrates (Sandvik et al., 2012). Here, we propose that one of the principal functions of RRM2B is to act as the hypoxic-specific RNR subunit in order to be able to react promptly when this physiologically relevant stress occurs. It is tempting to speculate that through antioxidant and catalase-like properties (Xue et al., 2006), RRM2B might even play a role in increasing immediate oxygen availability.

Interestingly, a number of previous reports have demonstrated that *RRM2B* is frequently affected by copy-number changes, typically showing gains, in a broad range of cancers (Chae et al., 2016; Jørgensen et al., 2013). This has led to the suggestion that *RRM2B* is a tumor promoter (Aye et al., 2015). Our study demonstrates that increased RRM2B expression in hypoxia maintains replication and prevents DNA damage, therefore providing a plausible explanation for why RRM2B is so often amplified in cancers.

Our data suggest that targeting R1/R2B enzyme specifically in hypoxic tumor cells might be an effective therapeutic strategy. RNR is a well-established therapeutic target, and a number of RNR inhibitors (such as gemcitabine, clofarabine, hydroxyurea,

and triapine) are being used clinically (Manegold et al., 2000; Aye and Stubbe, 2011; Aye et al., 2012a; Levin, 1992; Sterkers et al., 1998; Hehlmann et al., 1993; Nutting et al., 2009). Interestingly, delivery of siRNA against RRM2 by phosphorothionate oligodeoxynucleotides (GT1-2040) (Lee et al., 2003) is in clinical trials for various solid tumors (Juhász et al., 2006; Leigh et al., 2009; Oh and Park, 2009). It is tempting to speculate that if this approach was modified to target RRM2B and was employed alongside patient stratification to identify those with significant tumor hypoxia, it may be an effective way to target the most aggressive and treatment-resistant fraction of tumors.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
 - Cell culture and treatments
 - Hypoxia treatment
 - Immunoblotting and Immunofluorescence
 - RT-qPCR
 - Flow cytometry (FACS)
 - DNA Fiber analysis and dNTP pool determination
 - Immunoprecipitation (IP) and Chromatin IP (ChIP)
 - Colony survival and apoptosis
 - xCELLigence proliferation assay
 - Xenografts and tissue IF staining
 - TCGA RNA-sequencing (RNA-seq) analysis and analysis of colorectal cancer datasets
 - Protein overexpression and purification
 - dCDP formation assays
 - Determination of CDP/dCDP
 - Circular dichroism spectrometry
 - Inductively coupled plasma mass spectrometry (ICP-MS)
 - Electron paramagnetic resonance (EPR)
 - Molecular Dynamics (MD) simulations
 - MD equilibration protocol
 - Code availability
 - MD simulation analysis
 - Free-energy simulations of tunnel entry
 - Analysis of fenestrations
 - Statistical analysis
- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2017.03.005>.

AUTHOR CONTRIBUTIONS

I.P.F. conceived, designed, performed, and interpreted the majority of the experiments and wrote the manuscript. C.J. performed and interpreted the

MD simulations. K.B.L. performed the xenografts, immunohistochemistry (IHC), some experiments, and TCGA analysis. M.M.O. advised on some experiments and carried out TCGA analysis. B.H. assisted in developing the CRISPR clone. V.D. advised on the dNTP assay. H.T. assisted with enzyme assays. W.K.M. performed and analyzed the EPR studies. C.D. supervised, designed, and interpreted the MD simulations. E.F. supervised, designed, and interpreted in vitro enzymatic assays. E.M.H. conceived the project; conceived, designed, and interpreted the majority of the experiments; and wrote the manuscript. All authors commented on the manuscript.

ACKNOWLEDGMENTS

We thank Michael Stratford, Adam Hardy, Lisa Folkes, Mick Woodcock, Graham Brown, Philip Holdship, Ludovico Buti, Haoran Li, JoAnne Stubbe, and Yun Yen. CRUK Oxford Cancer Centre, CRUK Hartree Centre resources and the National Service were used for the Computational Chemistry Software. This work was supported by a Cancer Research UK (CR-UK) (H3RWGJ00.H307.1) grant (to E.M.H.), CR-UK Oxford Centre Prize DPhil Studentship C38302/A12981 (to I.P.F.), and a King's College London GTA studentship (to C.J.).

Received: October 11, 2016

Revised: February 13, 2017

Accepted: March 7, 2017

Published: April 13, 2017

SUPPORTING CITATIONS

The following references appear in the Supplemental Information: Hammond et al. (2006); Sherman and Fyfe (1989); Wang et al. (2007).

REFERENCES

- Ando, N., Li, H., Brignole, E.J., Thompson, S., McLaughlin, M.I., Page, J.E., Asturias, F.J., Stubbe, J., and Drennan, C.L. (2016). Allosteric inhibition of human ribonucleotide reductase by dATP entails the stabilization of a hexamer. *Biochemistry* 55, 373–381.
- Aye, Y., and Stubbe, J. (2011). Clofarabine 5'-di and -triphosphates inhibit human ribonucleotide reductase by altering the quaternary structure of its large subunit. *Proc. Natl. Acad. Sci. USA* 108, 9815–9820.
- Aye, Y., Brignole, E.J., Long, M.J., Chittuluru, J., Drennan, C.L., Asturias, F.J., and Stubbe, J. (2012a). Clofarabine targets the large subunit (α) of human ribonucleotide reductase in live cells by assembly into persistent hexamers. *Chem. Biol.* 19, 799–805.
- Aye, Y., Long, M.J., and Stubbe, J. (2012b). Mechanistic studies of semi-carbazone triapine targeting human ribonucleotide reductase in vitro and in mammalian cells: tyrosyl radical quenching not involving reactive oxygen species. *J. Biol. Chem.* 287, 35768–35778.
- Aye, Y., Li, M., Long, M.J., and Weiss, R.S. (2015). Ribonucleotide reductase and cancer: biological mechanisms and targeted therapies. *Oncogene* 34, 2011–2021.
- Bartkova, J., Horejsi, Z., Koed, K., Krämer, A., Tort, F., Zieger, K., Guldberg, P., Sehested, M., Nesland, J.M., Lukas, C., et al. (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 434, 864–870.
- Begg, A.C., Stewart, F.A., and Vens, C. (2011). Strategies to improve radiotherapy with targeted drugs. *Nat. Rev. Cancer* 11, 239–253.
- Bester, A.C., Roniger, M., Oren, Y.S., Im, M.M., Sarni, D., Chaoat, M., Bensimon, A., Zamir, G., Shewach, D.S., and Kerem, B. (2011). Nucleotide deficiency promotes genomic instability in early stages of cancer development. *Cell* 145, 435–446.
- Bianchi, V., Pontis, E., and Reichard, P. (1986). Changes of deoxyribonucleoside triphosphate pools induced by hydroxyurea and their relation to DNA synthesis. *J. Biol. Chem.* 261, 16037–16042.

- Brischwein, K., Engelcke, M., Riedinger, H.J., and Probst, H. (1997). Role of ribonucleotide reductase and deoxynucleotide pools in the oxygen-dependent control of DNA replication in Ehrlich ascites cells. *Eur. J. Biochem.* **244**, 286–293.
- Brooks, B.R., Brooks, C.L., 3rd, Mackerell, A.D., Jr., Nilsson, L., Petrella, R.J., Roux, B., Won, Y., Archontis, G., Bartels, C., Boresch, S., et al. (2009). CHARMM: the biomolecular simulation program. *J. Comput. Chem.* **30**, 1545–1614.
- Buck, M., Bouguet-Bonnet, S., Pastor, R.W., and MacKerell, A.D., Jr. (2006). Importance of the CMAP correction to the CHARMM22 protein force field: dynamics of hen lysozyme. *Biophys. J.* **90**, L36–L38.
- Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J.P., Sedivy, J.M., Kinzler, K.W., and Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* **282**, 1497–1501.
- Burrell, R.A., McClelland, S.E., Endesfelder, D., Groth, P., Weller, M.C., Shaikh, N., Domingo, E., Kanu, N., Dewhurst, S.M., Gronroos, E., et al. (2013). Replication stress links structural and numerical cancer chromosomal instability. *Nature* **494**, 492–496.
- Cavallo, L., Kleinjung, J., and Fraternali, F. (2003). POPS: a fast algorithm for solvent accessible surface areas at atomic and residue level. *Nucleic Acids Res.* **31**, 3364–3366.
- Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., Jacobsen, A., Byrne, C.J., Heuer, M.L., Larsson, E., et al. (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov.* **2**, 401–404.
- Chae, Y.K., Anker, J.F., Carneiro, B.A., Chandra, S., Kaplan, J., Kalyan, A., Santa-Maria, C.A., Platanius, L.C., and Giles, F.J. (2016). Genomic landscape of DNA repair genes in cancer. *Oncotarget* **7**, 23312–23321.
- Chimpoy, K., Tassotto, M.L., and Mathews, C.K. (2000). Ribonucleotide reductase, a possible agent in deoxyribonucleotide pool asymmetries induced by hypoxia. *J. Biol. Chem.* **275**, 39267–39271.
- Chipot, C., and Pohorille, A. (2007). *Free Energy Calculations* (Springer).
- Cohen, J., Kim, K., Posewitz, M., Ghirardi, M.L., Schulten, K., Seibert, M., and King, P. (2005). Molecular dynamics and experimental investigation of H(2) and O(2) diffusion in [Fe]-hydrogenase. *Biochem. Soc. Trans.* **33**, 80–82.
- Cohen, J., Arkhipov, A., Braun, R., and Schulten, K. (2006). Imaging the migration pathways for O₂, CO, NO, and Xe inside myoglobin. *Biophys. J.* **91**, 1844–1857.
- Cotruvo, J.A., and Stubbe, J. (2011). Class I ribonucleotide reductases: metal-ligand assembly and repair in vitro and in vivo. *Annu. Rev. Biochem.* **80**, 733–767.
- Crosby, M.E., Kulshreshtha, R., Ivan, M., and Glazer, P.M. (2009). MicroRNA regulation of DNA repair gene expression in hypoxic stress. *Cancer Res.* **69**, 1221–1229.
- D'Angiolella, V., Donato, V., Forrester, F.M., Jeong, Y.T., Pellacani, C., Kudo, Y., Saraf, A., Florens, L., Washburn, M.P., and Pagano, M. (2012). Cyclin F-mediated degradation of ribonucleotide reductase M2 controls genome integrity and DNA repair. *Cell* **149**, 1023–1034.
- Dahl, A.C.E., Chavent, M., and Sansom, M.S.P. (2012). Bendix: intuitive helix geometry analysis and abstraction. *Bioinformatics* **28**, 2193–2194.
- Dang, D.T., Chen, F., Gardner, L.B., Cummins, J.M., Rago, C., Bunz, F., Kantsevov, S.V., and Dang, L.H. (2006). Hypoxia-inducible factor-1 α promotes nonhypoxia-mediated proliferation in colon cancer cells and xenografts. *Cancer Res.* **66**, 1684–1936.
- Darden, T., York, D., and Pedersen, L. (1993). Particle mesh Ewald: an N $\cdot$ log(N) method for Ewald sums in large systems. *J. Chem. Phys.* **98**, 10089.
- Darve, E., and Pohorille, A. (2001). Calculating free energies using average force. *J. Chem. Phys.* **115**, 9169.
- Darve, E., Rodríguez-Gómez, D., and Pohorille, A. (2008). Adaptive biasing force method for scalar and vector free energy calculations. *J. Chem. Phys.* **128**, 144120.
- Den Otter, W.K. (2000). Thermodynamic integration of the free energy along a reaction coordinate in Cartesian coordinates. *J. Chem. Phys.* **112**, 7283.
- Den Otter, W.K., and Briels, W.J. (1998). The calculation of free-energy differences by constrained molecular-dynamics simulations. *J. Chem. Phys.* **109**, 4139.
- Eriksson, S., Skog, S., Tribukait, B., and Wallström, B. (1987). Deoxyribonucleoside triphosphate metabolism and the mammalian cell cycle. Effects of hydroxyurea on mutant and wild-type mouse S49 T-lymphoma cells. *Exp. Cell Res.* **168**, 79–88.
- Foskolou, I.P., Biasoli, D., Olcina, M.M., and Hammond, E.M. (2016). Measuring DNA Replication in Hypoxic Conditions. *Adv. Exp. Med. Biol.* **899**, 11–25.
- Fraternali, F., and Van Gunsteren, W.F. (1996). An efficient mean solvation force model for use in molecular dynamics simulations of proteins in aqueous solution. *J. Mol. Biol.* **256**, 939–948.
- Fukuda, R., Zhang, H., Kim, J.W., Shimoda, L., Dang, C.V., and Semenza, G.L. (2007). HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. *Cell* **129**, 111–122.
- Gao, J., Aksoy, B.A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S.O., Sun, Y., Jacobsen, A., Sinha, R., Larsson, E., et al. (2013). Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci. Signal.* **6**, p11.
- Gorgoulis, V.G., Vassiliou, L.V., Karakaidos, P., Zacharatos, P., Kotsinas, A., Liloglou, T., Venere, M., Dittullo, R.A., Jr., Kastrinakis, N.G., Levy, B., et al. (2005). Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* **434**, 907–913.
- Graeber, T.G., Osmanian, C., Jacks, T., Housman, D.E., Koch, C.J., Lowe, S.W., and Giaccia, A.J. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* **379**, 88–91.
- Halazonetis, T.D., Gorgoulis, V.G., and Bartek, J. (2008). An oncogene-induced DNA damage model for cancer development. *Science* **319**, 1352–1355.
- Hammond, E.M., Denko, N.C., Dorie, M.J., Abraham, R.T., and Giaccia, A.J. (2002). Hypoxia links ATR and p53 through replication arrest. *Mol. Cell. Biol.* **22**, 1834–1843.
- Hammond, E.M., Mandell, D.J., Salim, A., Krieg, A.J., Johnson, T.M., Shirazi, H.A., Attardi, L.D., and Giaccia, A.J. (2006). Genome-wide analysis of p53 under hypoxic conditions. *Mol. Cell. Biol.* **26**, 3492–3504.
- Hammond, E.M., Kaufmann, M.R., and Giaccia, A.J. (2007). Oxygen sensing and the DNA-damage response. *Curr. Opin. Cell Biol.* **19**, 680–684.
- Hehlmann, R., Heimpel, H., Hasford, J., Kolb, H.J., Pralle, H., Hossfeld, D.K., Queisser, W., Löffler, H., Heinze, B., Georgii, A., et al.; The German CML Study Group (1993). Randomized comparison of busulfan and hydroxyurea in chronic myelogenous leukemia: prolongation of survival by hydroxyurea. *Blood* **82**, 398–407.
- Höckel, M., and Vaupel, P. (2001). Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J. Natl. Cancer Inst.* **93**, 266–276.
- Huang, M., Parker, M.J., and Stubbe, J. (2014). Choosing the right metal: case studies of class I ribonucleotide reductases. *J. Biol. Chem.* **289**, 28104–28111.
- Hubbi, M.E., Kshitiz, Gilkes, D.M., Rey, S., Wong, C.C., Luo, W., Kim, D.H., Dang, C.V., Levchenko, A., and Semenza, G.L. (2013). A nontranscriptional role for HIF-1 α as a direct inhibitor of DNA replication. *Sci. Signal.* **6**, ra10.
- Humphrey, W., Dalke, A., and Schulten, K. (1996). VMD: visual molecular dynamics. *J. Mol. Graph.* **14**, 33–38, 27–28.
- Jorgensen, W.L., Chandrasekhar, J., Madura, J.D., Impey, R.W., and Klein, M.L. (1983). Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **79**, 926.
- Jørgensen, C.L., Ejlersen, B., Bjerre, K.D., Balslev, E., Nielsen, D.L., and Nielsen, K.V. (2013). Gene aberrations of RRM1 and RRM2B and outcome of advanced breast cancer after treatment with docetaxel with or without gemcitabine. *BMC Cancer* **13**, 541.

- Juhasz, A., Vassilakos, A., Chew, H.K., Gandara, D., and Yen, Y. (2006). Analysis of ribonucleotide reductase M2 mRNA levels in patient samples after GTI-2040 antisense drug treatment. *Oncol. Rep.* **15**, 1299–1304.
- Kolberg, M., Strand, K.R., Graff, P., and Andersson, K.K. (2004). Structure, function, and mechanism of ribonucleotide reductases. *Biochim. Biophys. Acta* **1699**, 1–34.
- Langevin, P. (1908). On the theory of Brownian motion. *C.R. Acad. Sci.* **146**, 530–533.
- Lee, Y., Vassilakos, A., Feng, N., Lam, V., Xie, H., Wang, M., Jin, H., Xiong, K., Liu, C., Wright, J., and Young, A. (2003). GTI-2040, an antisense agent targeting the small subunit component (R2) of human ribonucleotide reductase, shows potent antitumor activity against a variety of tumors. *Cancer Res.* **63**, 2802–2811.
- Leigh, N.B., Laurie, S.A., Chen, X.E., Ellis, P., Shepherd, F.A., Knox, J.J., Goss, G., Burkes, R.L., Pond, G.R., Dick, C., et al. (2009). A phase I/II study of GTI-2040 plus docetaxel as second-line treatment in advanced non-small cell lung cancer: a study of the PMH phase II consortium. *J. Thorac. Oncol.* **4**, 1163–1169.
- Leszczynska, K.B., Foskolou, I.P., Abraham, A.G., Anbalagan, S., Tellier, C., Haider, S., Span, P.N., O'Neill, E.E., Buffa, F.M., and Hammond, E.M. (2015). Hypoxia-induced p53 modulates both apoptosis and radiosensitivity via AKT. *J. Clin. Invest.* **125**, 2385–2398.
- Leszczynska, K.B., Göttgens, E.L., Biasoli, D., Olcina, M.M., Ient, J., Anbalagan, S., Bernhardt, S., Giaccia, A.J., and Hammond, E.M. (2016). Mechanisms and consequences of ATM1N repression in hypoxic conditions: roles for p53 and HIF-1. *Sci. Rep.* **6**, 21698.
- Levin, V.A. (1992). The place of hydroxyurea in the treatment of primary brain tumors. *Semin. Oncol.* **19** (3 Suppl 9), 34–39.
- Li, B., Qiu, B., Lee, D.S., Walton, Z.E., Ochocki, J.D., Mathew, L.K., Mancuso, A., Gade, T.P., Keith, B., Nissim, I., and Simon, M.C. (2014). Fructose-1,6-bisphosphatase opposes renal carcinoma progression. *Nature* **513**, 251–255.
- Lundin, D., Berggren, G., Logan, D.T., and Sjöberg, B.M. (2015). The origin and evolution of ribonucleotide reduction. *Life (Basel)* **5**, 604–636.
- Macheret, M., and Halazonetis, T.D. (2015). DNA replication stress as a hallmark of cancer. *Annu. Rev. Pathol.* **10**, 425–448.
- Manegold, C., Zatloukal, P., Krejcy, K., and Blatter, J. (2000). Gemcitabine in non-small cell lung cancer (NSCLC). *Invest. New Drugs* **18**, 29–42.
- Miyamoto, S., and Kollman, P.A. (1992). SETTLE: An analytical version of the SHAKE and RATTLE algorithm for rigid water models. *J. Comput. Chem.* **13**, 952–962.
- Mizuno, H., Kitada, K., Nakai, K., and Sarai, A. (2009). PrognosScan: a new database for meta-analysis of the prognostic value of genes. *BMC Med. Genomics* **2**, 18.
- Nordlund, P., and Reichard, P. (2006). Ribonucleotide reductases. *Annu. Rev. Biochem.* **75**, 681–706.
- Nosé, S. (1984a). A molecular dynamics method for simulations in the canonical ensemble. *Mol. Phys.* **52**, 255–268.
- Nosé, S. (1984b). A unified formulation of the constant temperature molecular dynamics methods. *J. Chem. Phys.* **81**, 511–519.
- Nutting, C.M., van Herpen, C.M., Miah, A.B., Bhide, S.A., Machiels, J.P., Buter, J., Kelly, C., de Raucourt, D., and Harrington, K.J. (2009). Phase II study of 3-AP Triapine in patients with recurrent or metastatic head and neck squamous cell carcinoma. *Ann. Oncol.* **20**, 1275–1279.
- Ochiai, E., Mann, G.J., Gräslund, A., and Thelander, L. (1990). Tyrosyl free radical formation in the small subunit of mouse ribonucleotide reductase. *J. Biol. Chem.* **265**, 15758–15761.
- Oh, Y.K., and Park, T.G. (2009). siRNA delivery systems for cancer treatment. *Adv. Drug Deliv. Rev.* **61**, 850–862.
- Olcina, M.M., Foskolou, I.P., Anbalagan, S., Senra, J.M., Pires, I.M., Jiang, Y., Ryan, A.J., and Hammond, E.M. (2013). Replication stress and chromatin context link ATM activation to a role in DNA replication. *Mol. Cell* **52**, 758–766.
- Phillips, J.C., Braun, R., Wang, W., Gumbart, J., Tajkhorshid, E., Villa, E., Chipot, C., Skeel, R.D., Kalé, L., and Schulten, K. (2005). Scalable molecular dynamics with NAMD. *J. Comput. Chem.* **26**, 1781–1802.
- Piao, C., Jin, M., Kim, H.B., Lee, S.M., Amatya, P.N., Hyun, J.W., Chang, I.Y., and You, H.J. (2009). Ribonucleotide reductase small subunit p53R2 suppresses MEK-ERK activity by binding to ERK kinase 2. *Oncogene* **28**, 2173–2184.
- Pires, I.M., Bencokova, Z., Milani, M., Folkes, L.K., Li, J.L., Stratford, M.R., Harris, A.L., and Hammond, E.M. (2010). Effects of acute versus chronic hypoxia on DNA damage responses and genomic instability. *Cancer Res.* **70**, 925–935.
- Pontarin, G., Ferraro, P., Bee, L., Reichard, P., and Bianchi, V. (2012). Mammalian ribonucleotide reductase subunit p53R2 is required for mitochondrial DNA replication and DNA repair in quiescent cells. *Proc. Natl. Acad. Sci. USA* **109**, 13302–13307.
- Probst, H., Schiffer, H., Gekeler, V., and Scheffler, K. (1989). Oxygen dependent regulation of mammalian ribonucleotide reductase in vivo and possible significance for replicon initiation. *Biochem. Biophys. Res. Commun.* **163**, 334–340.
- Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308.
- Reichard, P. (1988). Interactions between deoxyribonucleotide and DNA synthesis. *Annu. Rev. Biochem.* **57**, 349–374.
- Reichard, P. (1993). From RNA to DNA, why so many ribonucleotide reductases? *Science* **260**, 1773–1777.
- Sandvik, G.K., Tomter, A.B., Bergan, J., Zoppellaro, G., Barra, A.L., Röhr, A.K., Kolberg, M., Ellefsen, S., Andersson, K.K., and Nilsson, G.E. (2012). Studies of ribonucleotide reductase in crucian carp—an oxygen dependent enzyme in an anoxia tolerant vertebrate. *PLoS ONE* **7**, e42784.
- Shao, J., Zhou, B., Zhu, L., Qiu, W., Yuan, Y.C., Xi, B., and Yen, Y. (2004). In vitro characterization of enzymatic properties and inhibition of the p53R2 subunit of human ribonucleotide reductase. *Cancer Res.* **64**, 1–6.
- Sherman, P.A., and Fyfe, J.A. (1989). Enzymatic assay for deoxyribonucleoside triphosphates using synthetic oligonucleotides as template primers. *Anal. Biochem.* **180**, 222–226.
- Smith, P., Zhou, B., Ho, N., Yuan, Y.C., Su, L., Tsai, S.C., and Yen, Y. (2009). 2.6 Å X-ray crystal structure of human p53R2, a p53-inducible ribonucleotide reductase. *Biochemistry* **48**, 11134–11141.
- Smith, J.J., Deane, N.G., Wu, F., Merchant, N.B., Zhang, B., Jiang, A., Lu, P., Johnson, J.C., Schmidt, C., Bailey, C.E., et al. (2010). Experimentally derived metastasis gene expression profile predicts recurrence and death in patients with colon cancer. *Gastroenterology* **138**, 958–968.
- Sterkers, Y., Preudhomme, C., Lai, J.L., Demory, J.L., Caulier, M.T., Wattel, E., Bordessoule, D., Bauters, F., and Fenaux, P. (1998). Acute myeloid leukemia and myelodysplastic syndromes following essential thrombocythemia treated with hydroxyurea: high proportion of cases with 17p deletion. *Blood* **91**, 616–622.
- Stubbe, J. (1998). Ribonucleotide reductases in the twenty-first century. *Proc. Natl. Acad. Sci. USA* **95**, 2723–2724.
- Stubbe, J. (2003). Di-iron-tyrosyl radical ribonucleotide reductases. *Curr. Opin. Chem. Biol.* **7**, 183–188.
- Stubbe, J., Nocera, D.G., Yee, C.S., and Chang, M.C. (2003). Radical initiation in the class I ribonucleotide reductase: long-range proton-coupled electron transfer? *Chem. Rev.* **103**, 2167–2201.
- Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y., and Nakamura, Y. (2000). A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature* **404**, 42–49.
- Tanaka, T., Ohkubo, S., Tatsuno, I., and Prives, C. (2007). hCAS/CSE1L associates with chromatin and regulates expression of select p53 target genes. *Cell* **130**, 638–650.

- Thelander, L., Gräslund, A., and Thelander, M. (1983). Continual presence of oxygen and iron required for mammalian ribonucleotide reduction: possible regulation mechanism. *Biochem. Biophys. Res. Commun.* **110**, 859–865.
- Tuckerman, M., Berne, B.J., and Martyna, G.J. (1992). Reversible multiple time scale molecular dynamics. *J. Chem. Phys.* **97**, 1990–2001.
- Uhlen, U., and Eklund, H. (1994). Structure of ribonucleotide reductase protein R1. *Nature* **370**, 533–539.
- Van Gunsteren, W.F. (1989). Methods for calculation of free energies and binding constants: successes and problems. In *Computer Simulation of Biomolecular Systems: Theoretical and Experimental Applications*, W.F. van Gunsteren and P.K. Weiner, eds. (Escom), pp. 27–59.
- Verlet, L. (1967). Computer “experiments” on classical fluids. I. Thermodynamical properties of Lennard-Jones molecules. *Phys. Rev.* **159**, 98–103.
- Wang, P.-H., and Blumberger, J. (2012). Mechanistic insight into the blocking of CO diffusion in [NiFe]-hydrogenase mutants through multiscale simulation. *Proc. Natl. Acad. Sci. USA* **109**, 6399–6404.
- Wang, J., Lohman, G.J., and Stubbe, J. (2007). Enhanced subunit interactions with gemcitabine-5'-diphosphate inhibit ribonucleotide reductases. *Proc. Natl. Acad. Sci. USA* **104**, 14324–14329.
- Wang, J., Lohman, G.J., and Stubbe, J. (2009a). Mechanism of inactivation of human ribonucleotide reductase with p53R2 by gemcitabine 5'-diphosphate. *Biochemistry* **48**, 11612–11621.
- Wang, X., Zhenchuk, A., Wiman, K.G., and Albertioni, F. (2009b). Regulation of p53R2 and its role as potential target for cancer therapy. *Cancer Lett.* **276**, 1–7.
- Wang, P.-H., Best, R.B., and Blumberger, J. (2011). A microscopic model for gas diffusion dynamics in a [NiFe]-hydrogenase. *Phys. Chem. Chem. Phys.* **13**, 7708–7719.
- Wang, P.-H., Bruschi, M., De Gioia, L., and Blumberger, J. (2013). Uncovering a dynamically formed substrate access tunnel in carbon monoxide dehydrogenase/acetyl-CoA synthase. *J. Am. Chem. Soc.* **135**, 9493–9502.
- Xue, L., Zhou, B., Liu, X., Wang, T., Shih, J., Qi, C., Heung, Y., and Yen, Y. (2006). Structurally dependent redox property of ribonucleotide reductase subunit p53R2. *Cancer Res.* **66**, 1900–1905.
- Zhang, Y., Li, H., Zhang, C., An, X., Liu, L., Stubbe, J., and Huang, M. (2014). Conserved electron donor complex Dre2-Tah18 is required for ribonucleotide reductase metallofactor assembly and DNA synthesis. *Proc. Natl. Acad. Sci. USA* **111**, E1695–E1704.
- Zhou, B., Liu, X., Mo, X., Xue, L., Darwish, D., Qiu, W., Shih, J., Hwu, E.B., Luh, F., and Yen, Y. (2003). The human ribonucleotide reductase subunit hRRM2 complements p53R2 in response to UV-induced DNA repair in cells with mutant p53. *Cancer Res.* **63**, 6583–6594.
- Zhou, B., Su, L., Yuan, Y.C., Un, F., Wang, N., Patel, M., Xi, B., Hu, S., and Yen, Y. (2010). Structural basis on the dityrosyl-diiron radical cluster and the functional differences of human ribonucleotide reductase small subunits hp53R2 and hRRM2. *Mol. Cancer Ther.* **9**, 1669–1679.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat polyclonal anti-RRM1 (T-16)	Santa Cruz	Cat# sc-11733
Goat polyclonal anti-RRM2 (N-18)	Santa Cruz	Cat# sc-10844
Goat polyclonal anti-p53R2 (N-16) (RRM2B)	Santa Cruz	Cat# sc-10840
Mouse monoclonal anti-Chk1 (G-4)	Santa Cruz	Cat# sc-8408
Mouse monoclonal anti-p53 (DO-1)	Santa Cruz	Cat# sc-126
Rabbit polyclonal anti-p53 (FL-393)	Santa Cruz	Cat# sc-6243
Mouse monoclonal anti- β -Actin Antibody (AC-15)	Santa Cruz	Cat# sc-69879
Mouse anti-Human HIF-1 α Clone 54	BD Transduction Laboratories	Cat# 610958
Mouse anti-BrdU Clone B44	BD Transduction Laboratories	Cat# 347580
Rat monoclonal anti BrdU Clone BU1/75 (ICR1)	Bio-Rad	Cat# OB0030CX
Mouse monoclonal anti-RRM2 Clone 1E1	Bio-Rad	Cat# MCA3434Z
Rabbit polyclonal anti-KAP-1	Bethyl / Universal Biologicals	Cat# A300-274
Rabbit polyclonal anti-Phospho KAP-1 (S824)	Bethyl / Universal Biologicals	Cat# A300-767
Rabbit polyclonal anti-Phospho KAP-1 (S473)	BioLegend	Cat# 644602 RRID:AB_2241094
Rabbit polyclonal anti-53BP1	Novus Biologicals	Cat# NB100-904
Rabbit polyclonal anti-Phospho-p53 (Ser15)	Cell Signaling	Cat# 9284
Rat monoclonal anti-RPA32/RPA2 (4E4)	Cell Signaling	Cat# 2208
Rabbit polyclonal anti-Phospho-Chk1 (Ser296)	Cell Signaling	Cat# 2349
Rabbit polyclonal anti-Phospho-Chk1 (Ser317)	Cell Signaling	Cat# 2344
Rabbit polyclonal anti-Phospho-Chk1 (Ser345)	Cell Signaling	Cat# 2341
Rabbit polyclonal anti-PARP	Cell Signaling	Cat# 9542
Rabbit polyclonal anti-Cleaved Caspase-3 (Asp175)	Cell Signaling	Cat# 9661
Anti-mouse IgG, HRP-linked	Cell Signaling	Cat# 7076
Anti-rabbit IgG, HRP-linked	Cell Signaling	Cat# 7074
Rabbit polyclonal anti-beta Tubulin antibody	Abcam	Cat# ab6046
Mouse monoclonal anti-pimonidazole Clone 4.3.11.3)	Chemicon International	Cat# HP1-100
Alexa Fluor 680 goat anti-mouse IgG (H+L)	Invitrogen	Cat# A21057
Alexa Fluor 680 goat anti-rabbit IgG (H+L)	Invitrogen	Cat# A21076
Alexa Fluor 680 donkey anti-goat IgG (H+L)	Invitrogen	Cat# A21084
IRDye 800CW donkey anti-rabbit IgG	Li-Cor	Cat# 926-32213
IRDye 800CW donkey anti-mouse IgG	Li-Cor	Cat# 926-32212
Alexa Fluor 488-conjugated goat anti-rabbit IgG	Invitrogen	Cat# A11070
Alexa Fluor 488-conjugated donkey anti-rabbit IgG	Invitrogen	Cat# A21206
Alexa Fluor 488-conjugated goat anti-mouse IgG	Invitrogen	Cat# A11017
Alexa Fluor 488-conjugated donkey anti-goat IgG	Invitrogen	Cat# A11055
Alexa Fluor 594-conjugated goat anti-rabbit IgG	Invitrogen	Cat# A11072
Alexa Fluor 594-conjugated goat anti-rat IgG	Invitrogen	Cat# A11007
Alexa Fluor 594-conjugated goat anti-mouse IgG	Invitrogen	Cat# A11020
Alexa Fluor 594-conjugated donkey anti-goat IgG	Invitrogen	Cat# A11058
Alexa Fluor 555-conjugated goat anti-rat IgG	Invitrogen	Cat# A21434
Alexa Fluor 488-conjugated goat anti-mouse F(ab') ₂ fragment	Invitrogen	Cat# A11017
Pierce Recombinant Protein A/G	Thermo Fisher Scientific	Cat# 21186

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
BL21-Gold(DE3) Competent Cells	Agilent Technologies	Cat# 230132
One Shot TOP10 Chemically Competent <i>E. coli</i>	Thermo Fisher Scientific	Cat# C404010
Chemicals, Peptides, and Recombinant Proteins		
Hydroxyurea (HU)	Sigma	Cat# H8627
Doxorubicin hydrochloride (Adriamycin)	Sigma	Cat# D1515
5-Bromo-2'-deoxyuridine (BrdU)	Sigma	Cat# B5002
Propidium iodide (PI)	Sigma	Cat# S7109
Protease Inhibitor Cocktail	Roche	Cat# 04693159001
CldU (5-chloro-2'-deoxyuridine)	Sigma	Cat# C6891
IdU (5-iodo-2'-deoxyuridine)	Sigma	Cat# I7125
Cytidine 5'-diphosphocholine sodium salt dehydrate (CDP)	Sigma	Cat# C9755
Protein G Sepharose, Fast Flow	Sigma	Cat# P3296
Isopropyl β -D-1-thiogalactopyranoside (IPTG)	Sigma	Cat# I6758
Deoxyadenosine 5'-Triphosphate, [8-3H(N)] (dATP-H3)	Perkin Elmer	Cat# NET268250UC
Deoxythymidine 5'-Triphosphate ((dTTP) Tetrasodium Salt) (dTTP-H3)	Perkin Elmer	Cat# NET520A250UC
TRIzol Reagent	Thermo Fisher Scientific	Cat# 15596018
DharmaFECT 1	Dharmacon	Cat# T-2001
Lipofectamine Ltx	Thermo Fisher Scientific	Cat# 15338100
ProLong Gold	Thermo Fisher Scientific	Cat# P36931
Critical Commercial Assays		
Hypoxyprobe-1 Kit for the Detection of Tissue Hypoxia	Chemicon International	Cat# HP1-100
Real-Time and Dynamic Monitoring of Cell Proliferation and Viability for Adherent Cells	ACEA Biosciences Inc/ Cambridge Biosc	Cat# 00380601050
SYBR Green PCR Master Mix	Applied Biosystems	Cat# 4309155
Verso cDNA Synthesis Kit	Thermo Fisher Scientific	Cat# AB1453B
HisTrap HP columns	GE Healthcare	Cat# 17-5248-02
QIAprep Spin Miniprep Kit	QIAGEN	Cat# 27106
QuickChange II XL Site-Directed Mutagenesis Kit	Agilent Technologies	Cat# 200521
QIAquick PCR Purification Kit	QIAGEN	Cat# 28106
QIAquick Gel Extraction Kit	QIAGEN	Cat# 28704
Deposited Data		
Original imaging data	this study	http://dx.doi.org/10.17632/bp95v48kgm.1
Experimental Models: Cell Lines		
RKO	ATCC	CRL-2577
HCT116	ATCC	CCL-247
H1299	ATCC	CRL-5803
U2OS	ATCC	HTB-96
U87-MG	ATCC	HTB-14
OE21	ECACC	96062201
RKOHIF-1 α +/+ and RKOHIF-1 α -/-	Dang L.H. Laboratory	Dang et al., 2006
HCT116p53+/+ and HCT116p53-/-	Vogelstein B. Laboratory	Bunz et al., 1998
RKORRM2B-/-	This paper	N/A
Experimental Models: Organisms/Strains		
MICE: Female BALB/c nude were used for xenograft experiments	Charles River, UK	

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
siRNA-RRM2B (sequence: UGAGUUUGUAGCUGACAGAUU)	Sigma	Piao et al., 2009
siRNA#2 - RRM2B (sequence: GGAACAAGCUUAAAGCAGA)	Ambion / Life Technologies	s224156
siRNA-p53 (sequence: GUAAUCUACUGGGACGGAA)	Ambion / Life Technologies	Leszczynska et al., 2015
AllStars Negative Control siRNA	QIAGEN	SI03650318
Primers for RRM2B Forward-ChIP (sequence: CTTGCTGGGAAATCTTGACC)	Sigma	Tanaka et al., 2007
Primers used for site-directed mutagenesis Table S1	N/A	N/A
Primers used for dNTP incorporation assay Table S1	N/A	N/A
Primers used for quantitative PCR (qPCR) Table S1	N/A	N/A
Recombinant DNA		
Plasmids used in this work are listed in Table S2	N/A	N/A
Software and Algorithms		
7500 FAST Real-Time PCR thermocycler was used with v2.0.5 software	Applied Biosystems	http://www6.appliedbiosystems.com/support/software/7500/
The software CAVER 3.0 was used for the analysis of the evolution of lateral fenestrations during the MD simulations	N/A	N/A
The particle mesh Ewald (PME) algorithm was used for evaluation of electrostatics interactions	Darden et al., 1993	N/A
The multi time step algorithm Verlet-I/r-RESPA was used to integrate the equations of motion	Tuckerman et al., 1992 ; Verlet, 1967	N/A
The POPS (Parameter OPTimised Surfaces) algorithm was used for calculation of the Solvent-Accessible Surface Area (SASA) of both proteins RRM2B and RRM2	Cavallo et al., 2003 ; Fraternali and Van Gunsteren, 1996	N/A
The SETTLE algorithm was used for constrained the lengths of covalent bonds involving hydrogen atoms	Miyamoto and Kollman, 1992	N/A
Other		
For the analysis of RRM2B expression and genetic alterations in colorectal cancer datasets the cBioPortal and Prognoscan	http://www.cbioportal.org/ ; http://www.abren.net/Prognoscan/	Gao et al., 2013 ; Cerami et al., 2012 ; Mizuno et al., 2009
Gene Expression Omnibus; Smith et al., 2010	https://www.ncbi.nlm.nih.gov/geo	accession number GSE17536
ICP-MS was performed with ICP-MS Trace Element Analysis	https://www.earth.ox.ac.uk/research/services/geochemical-analysis/icpms/	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to, and will be fulfilled by, the lead contact, Dr. Ester M. Hammond (ester.hammond@oncology.ox.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Source of cell lines used in the study is reported in the reagent and resource table.

METHOD DETAILS

Cell culture and treatments

Source of cell lines used in the study is reported in the reagent and resource table. Cells were grown in DMEM with 10% FBS, in a standard humidified incubator at 37°C and 5% CO₂. All cell lines were routinely mycoplasma tested and found to be negative. RKO^{RRM2B-/-} was constructed in this work using CRISPR-Cas9 technology as previously described (Ran et al., 2013). *RRM2B* gene has three isoforms and in order to construct a full knock-out cell line two 20-bp target sgRNA sequences were used targeting exon 1: TTCGGCGGAGTCTGCGCGAT (isoforms 1 and 3) and AAATGTTATTGCGCGGTC (isoform 2). Lipofectamine Ltx (Invitrogen) was used for plasmid transfections according to manufacturer's recommendations (the plasmids used are listed in Table S2). Dharma-FECT 1 reagent (Thermo Fisher Scientific) was used for siRNA knockdown according to manufacturer's instructions. RKO or U2OS cells were transfected with the siRNA sequences reported in the reagent and resource table in a final concentration of 50 nM. Drugs were purchased from Sigma unless otherwise stated. Hydroxyurea (HU) was used at a concentration of 2 mM for 6 hr. Adriamycin was used at a concentration of 2 μM for 6 hr. BrdU (5-Bromo-2'-deoxyuridine) was used at a concentration of 20 μM.

Hypoxia treatment

Hypoxia treatments were carried out in a Bactron II anaerobic chamber (Shell labs) or an In vivo₂ 400 (Baker Ruskinn) (for oxygen concentrations at 2%). For experiments at < 0.1% O₂ cells were plated on glass dishes. Cells were harvested inside the chamber with equilibrated solutions. Oxygen concentrations were periodically verified using an Oxylite probe (Oxford Optronix, UK).

Immunoblotting and Immunofluorescence

For immunoblots, cells were lysed in UTB (9 M urea, 75 mM Tris-HCl pH 7.5 and 0.15 M β-mercaptoethanol) and sonicated briefly. The Odyssey infrared imaging technology was used (LI-COR Biosciences) and the Odyssey analysis system was used for quantification of the immunoblots. In each case, experiments were carried out in triplicate and a representative blot is shown unless otherwise stated. The antibodies used in this study are listed in the reagent and resource table.

For immunofluorescence, cells were seeded on autoclaved cover glasses (Menzel-Glaser). At the end of each experiment cells were fixed with 4% fixation buffer (4% (w/v) paraformaldehyde in PBS), lysed with 1% PBS-Triton X-100 and then incubated with blocking buffer (2% (w/v) BSA in 0.1% PBS-Triton X-100). Incubation with the appropriate primary and then secondary antibody followed (diluted in 2% (w/v) BSA - 0.1% PBS-Triton X-100) (antibodies used are listed in the reagent and resource table). Cells were visualized using a LSM780 (Carl Zeiss Microscopy Ltd) confocal microscope. At least 100 cells were counted per condition. Due to the presence of 53BP1 foci in the nuclei of unstressed cells, induction of DNA damage was quantified by counting cells with more than six foci. Induction of ssDNA was quantified by counting cells with more than six RPA32 foci.

RT-qPCR

RNA was prepared using Trizol (Invitrogen/Life Technologies). For qPCR expression analysis cDNA was reverse transcribed from total RNA using Verso kit (Thermo Scientific). qPCR was performed using SYBR Green PCR Master Mix kit (Applied Biosystems) in a 7500 FAST Real-Time PCR thermocycler with v2.0.5 software (Applied Biosystems). mRNA fold change was calculated using a 2^{-ΔΔCt} method in relation to the 18S reference gene. The qPCR graphs show the mean of three biological replicates ± s.e.m. The primers used for qPCR are listed in Table S1.

Flow cytometry (FACS)

FACS analysis was performed as previously described (Olcina et al., 2013). U2OS cells were treated with either RRM2B siRNA or negative control siRNA and were placed in normoxia or < 0.1% O₂ (3 hr). Samples were pulsed with BrdU (20 μM) for 30 min before collection. FACS analysis was carried out using a Becton Dickinson FACS Sort. Samples were later analyzed using CellQuest Pro and ModFit LT software. siRNAs and antibodies used are listed in the reagent and resource table.

DNA Fiber analysis and dNTP pool determination

Fiber analysis was carried out as previously described (Foskolou et al., 2016). In brief, sub confluent cells (50%–60% confluency) were sequentially pulse labeled with 25 mM CldU and 250 mM IdU for 20 min each and left at 21% O₂ in a 37°C incubator (normoxic samples). Cells were washed once with fresh warm media before the addition of the second (IdU) label. For hypoxic treated samples, cells were first placed for 2 hr in the hypoxic chamber (< 0.1% O₂) and then treated with 25 mM CldU for the indicated times, followed by the addition of 250 mM IdU for as long as needed for all samples to be in hypoxia for 6 hr in total. At the end of the treatment, labeled cells were lysed and spread with spreading buffer (200 mM Tris-HCl, pH 7.4, 50 mM EDTA, 0.5% SDS) in a tilting the slide. DNA fibers were then fixed with methanol/acetic acid (3:1 ratio) and stained with rat anti-BrdU monoclonal antibody (for detection of CldU labeled tracts) and mouse anti-BrdU monoclonal antibody (for detection of IdU labeled tracts). Source of antibodies is reported in the reagent and resource table. Fibers were imaged using a Bio-Rad Radiance or LSM780 (Carl Zeiss Microscopy Ltd) confocal microscope and analyzed using ImageJ software (NIH). The length of the fiber tracts that had incorporated both labels (CldU and IdU) was measured and replication rates were calculated with the following formula (V_{CldU} (kb/min) = $[(x \times 0.132 \mu\text{m}) \times 2.59 \text{ kb} / \mu\text{m}] / t$ (min), where x = length of CldU).

dNTP pool determination in whole-cell extracts was carried out as previously described (D'Angiolella et al., 2012). In brief, cells were seeded in glass dishes and treated either in $< 0.1\%$ O_2 (for the indicated times) or with 2 mM HU (6 hr). The normoxic samples were processed immediately after the beginning of the hypoxic and/or HU treatment. Cells were collected and resuspended in 60% iced cold methanol. Samples were then boiled, centrifuged and the supernatant was dried by centrifugal evaporation and finally dissolved in H_2O . For the preparation of each primer mix a different set of primers was used (listed in Table S1) supplemented with either $[^3H]dATP$ or $[^3H]dTTP$ (PerkinElmer). For dGTP and dCTP determination 1 U of Thermo Sequenase DNA Polymerase (GE Healthcare) was used; for dTTP determination 1.25 U Klenow Fragment (Thermo Fisher Scientific) and for dATP determination 0.625 U Klenow Fragment (Thermo Fisher Scientific) was used. The reactions were started by addition of the enzyme. Reactions were incubated for 1 hr either at $48^\circ C$ (for dGTP and dCTP mixtures) or at room temperature (for dTTP and dATP mixtures). Following incubation, the reaction mixture were spotted onto Whatman DE81 paper (GE Healthcare) and let dry. The papers were then washed with 5% Na_2HPO_4 , followed by rinsing with distilled H_2O and 100% EtOH. After being dried, the radioactivity on the papers was measured in a LS 6500 Multi-Purpose Scintillation Counter (Beckman CoulterTM) using 3.5 mL Optiphase HiSafe 3 (PerkinElmer) counting fluid. Data present percentage of dNTP incorporation compared to the positive control of each experiment. In experiments where both siRNA and hypoxia treatment were used, a normalization to each normoxic control was preceded the final analysis.

Immunoprecipitation (IP) and Chromatin IP (ChIP)

IP was carried out as previously described (Zhou et al., 2003). Briefly, RKO cells were lysed in lysis buffer (100 mM NaCl, 20 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.5% NP-40) with freshly added phosphatase/protease cocktail inhibitors (Roche). The lysates were incubated with Protein G Sepharose beads (Sigma) and the antibody of interest RRM1 (Santa Cruz Biotechnology: sc-11733) or a control IgG antibody (Invitrogen: A10535) with agitation, at $4^\circ C$ overnight. Lysates were washed with lysis buffer and solubilized in SDS-PAGE sample buffer. Antibodies used were: RRM1 (Santa Cruz Biotechnology: sc-11733), RRM2B (Santa Cruz Biotechnology: sc-10840), RRM2 (AbD Serotec, MCA3434Z) and for the detection of RRM2 the Pierce Recombinant Protein A/G (Thermo Fisher Scientific, 21186) secondary antibody was used.

ChIP in RKO cells treated as indicated in figure legend was carried out as previously described (Leszczynska et al., 2015). For each sample 2 μg of combined mouse p53 (DO-1; sc-126) and rabbit p53 (FL-393; sc-6243) (Santa Cruz) antibodies were used for immunoprecipitation. Combined non-specific mouse (7076) and rabbit (7074) (Cell Signaling) were used as control IgGs. A no antibody control sample was also included in each experiment. Bound fraction and input were analyzed by qPCR using specific primer set for the *RRM2B* locus: CTTGCTGGGAAATCTTGACC (Tanaka et al., 2007). Fold enrichment is expressed as a % of input and is normalized to total p53 in each sample. Graphs show the mean of technical triplicates $\pm RQ_{max}$ and RQ_{min} ; $n = 3$.

Colony survival and apoptosis

RKO cells were treated either with RRM2B siRNA or negative control siRNA and exposed to normoxia or hypoxia ($< 0.1\%$ O_2 for 24 hr). Subsequently all cells were placed in normoxic incubator and left for 9 days to form colonies, which were visualized by crystal violet staining. Graphs show the mean of three biological replicates $\pm$ s.e.m.

Apoptosis assay were performed as previously described (Leszczynska et al., 2015). In brief, both adherent and floating cells were collected and fixed with 4% paraformaldehyde. Samples were then washed and the nuclei were stained with ProLong Gold mounting medium with DAPI (Thermo Fisher Scientific). Apoptosis was plotted as the percentage of cells with fragmented DNA per field of view, with at least ten fields of view quantified per experiment. Each apoptosis experiment was performed at least 3 times.

xCELLigence proliferation assay

The xCELLigence Real-Time Cell Analyzer (ACEA Biosciences) DP Instrument equipped with an E-Plate was used for proliferation assays. Cells were seeded into 100 μL of media in 96X microplates. The attachment, spreading and proliferation of the cells were monitored every 15 min for 60 hr. For quantification, the cell index at indicated time points was averaged from three independent experiments. Values are presented as mean $\pm$ SEM of three independent experiments.

Xenografts and tissue IF staining

All animal procedures were performed in accordance with current UK legislation and were approved by the University of Oxford Biomedical Services Ethical Review Committee, Oxford, UK. For the tumor growth curves (xenografts), female BALB/c nude mice or female athymic nude mice (Charles River, UK) were randomized and injected subcutaneously with 3×10^6 RKO^{RRM2B+/+} or RKO^{RRM2B-/-} cells in 25% (v/v) matrigel and serum-free DMEM. Tumor growth was monitored until they reached approximately 300-400 mm³ (volume = height x depth x width x π / 6). For the irradiation experiment, the tumors in half of the animals were irradiated with 10 Gy when tumor volumes reached 100 mm³. Tumors were measured regularly, and tumor growth was plotted as a mean of tumor volumes $\pm$ SEM. Mice were injected with 50 mg/kg of pimonidazole (PIMO) 2 hr before the end of the experiment. Tumors were split in half and either preserved in formalin for wax embedding or were snap frozen and embedded in optimal cutting temperature compound (OCT).

Tissue IF staining were performed as previously described (Leszczynska et al., 2015). In brief, tumors embedded in OCT were sectioned (5 μm) and froze down at $-20^\circ C$. Sections were immediately fixed with 4% paraformaldehyde and then quenched with 50 mM NH_4Cl . Blocking was performed with 1% BSA in TBS, followed by incubation with Mouse on Mouse (M.O.M.) Blocking

Reagent (Vector Laboratories). The sections were stained with PIMO and cleaved caspase-3 primary antibodies, followed by secondary antibodies (listed in the reagent and resource table) and mounted with ProLong Gold mounting medium with DAPI (Invitrogen/Life technologies). The apoptotic cells were counted within and outside of hypoxic regions. Images were taken from at least 3 RKO^{RRM2B+/+} and 3 RKO^{RRM2B-/-} tumors. Graphs show the percentage of apoptotic cells from all normoxic or hypoxic regions.

TCGA RNA-sequencing (RNA-seq) analysis and analysis of colorectal cancer datasets

Raw RNA-seq data for 382 colorectal adenocarcinoma tumors were downloaded from the TCGA project (accessed through cBioportal: <http://www.cbioportal.org/>). To examine tumor-associated HIF-activity (referred to as hypoxia signature), raw data for each sequenced gene were rescaled to set the median equal to 1, and HIF-activity was quantified by averaging the normalized expression of 44 target genes, associated with HIF activity (encoding ADM, GFBP3, EDN2, PFKFB4, FLT1, TFR2, BNIP3L, TGFA, BNIP3, PGK1, EGLN1, LDHA, EGLN3, CP, TGFB3, PFKFB3, HK1, TFRC, EDN1, CDKN1A, CA9, HMOX1, SERPINE1, LOX, NDRG1, CA12, PDK1, VEGFA, ERO1L, RORA, P4HA1, MXI1, SLC2A1(GLUT1), STC2, MIF, DDIT4, ENO1, CXCR4, PLOD1, P4HA2, GAPDH, PGAM1, TMEM45A and PIM1) (Li et al., 2014). Log10 conversion of the hypoxia signature was plotted against Log10 conversion of raw data for RRM1, RRM2 and RRM2B. Two-tailed p value shown on each graph for each Pearson and Spearman r (correlation coefficient).

To examine tumor-associated p53-activity (referred to as p53 group), raw data for each sequenced gene were rescaled to set the median equal to 1, and p53-activity was quantified by averaging the normalized expression of 6 p53 target genes, associated with hypoxia-induced p53 activity (encoding BTG2, CYFIP2, INPP5D, KANK3, PHLA3 and SULF2) (Leszczynska et al., 2015, 2016). Log10 conversion of the p53 signature was plotted against Log10 conversion of raw data for RRM2B (also rescaled to set the median equal to 1). Two-tailed p value shown on each graph for each Pearson and Spearman r (correlation coefficient).

For the analysis of RRM2B expression and genetic alterations in colorectal cancer datasets the cBioPortal (<http://www.cbioportal.org/>) and Prognoscan (<http://www.abren.net/Prognoscan/>) tools were used on the 5th Dec 2016 (Gao et al., 2013; Cerami et al., 2012; Mizuno et al., 2009). Using cBioPortal the TCGA (provisional) dataset was analyzed (RNA Seq V2 RSEM) for alterations, including mutations, putative copy-number alterations, mRNA expression, mutations and survival probability (629 cancer patients). For the Prognoscan analysis, RRM2B expression was checked in all available colorectal cancer datasets and Kaplan-Meier graphs were extracted only for the statistically significant ($p < 0.05$) dependence between RRM2B expression (probe 223342_at) and survival probability. This analysis used the publicly available dataset at Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) with the accession number GSE17536 (Smith et al., 2010).

Protein overexpression and purification

The 6 × His-tagged hRRM1 (pET28a-RRM1) was kindly provided by Prof JoAnne Stubbe (Massachusetts Institute of Technology, Cambridge, USA), expressed in *E.coli* BL21-Gold (DE3) competent cells (Agilent Technologies) and as previously described (Ando et al., 2016). The 6 × His-tagged hRRM2 and hRRM2B (pET28a-RRM2, pET28a-RRM2B) were kindly provided by Dr. Yun Yen (Beckman Research Institute at City of Hope, Duarte, USA). The proteins were expressed in *E.coli* BL21-Gold (DE3) competent cells (Agilent Technologies) and purified as previously described (Shao et al., 2004) with minor modifications. Specifically, an overnight culture of the transformed bacteria was diluted 80-fold in 600 mL of 2 × YT medium containing 50 µg/mL kanamycin. Each culture was grown at 37°C until OD_{600nm} = 0.7 - 0.9 and then induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and incubated overnight at 18°C. Cells were harvested by centrifugation and the pellets were lysed with appropriate amount (5 mL/gr of pellet) Lysis Buffer (50 mM NaH₂PO₄ pH 7.0; 0.1% Triton X-100; 10 mM β-mercaptoethanol; freshly added EDTA-free Protease Inhibitors and DNase I) at 4°C with vigorous agitation until the lysate was homogeneous. The lysate was then sonicated (60% Amplitude 30 s ON / 30 s OFF) and clarified by centrifugation at 48,000 × *g* for 20 min at 4°C. Soluble lysate was passed through a His-Trap HP purification column (GE Healthcare), washed with at least 30-fold bed volume of Wash Buffer (50 mM NaH₂PO₄ pH 7.0; 800 mM NaCl; 50 mM imidazole; 0.1% Triton X-100; 10 mM β-mercaptoethanol) and finally eluted with Elution Buffer (50 mM NaH₂PO₄ pH 7.0; 300 mM NaCl; 125 mM imidazole). The protein then underwent buffer exchange into 25 mM Tris-HCl, pH 7.5 before being concentrated and stored at -80°C. The purified proteins showed approximately 90% purity. The apo-forms of RRM2 and RRM2B recombinant proteins used in ICP-MS experiments were overexpressed and purified as previously described (Wang et al., 2009a). Site-specific mutations in RRM2B (Y164C; Y164F; F183Y; K31E/K151E; Y241H; Y331F and Q127K) were generated by PCR, using the pET28a-RRM2B as a template and the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). All constructs were verified by DNA sequencing. Expression and purification of the hRRM2B mutated proteins were performed in the same way as for hRRM2B.

dCDP formation assays

The activity of recombinant RNR was measured using the following reduction method. The enzyme concentration (R1/R2B or R1/R2) was the same in all cases and the limited factor was the presence or not of oxygen (normoxia versus hypoxia). Specifically, a final concentration of 1.25 µM of purified RRM1 was incubated with 2.5 µM of either RRM2 or RRM2B protein. The reaction mixture contained 0.1 mM CDP, 50 mM HEPES (pH 7.5), 6 mM DTT, 8 mM magnesium acetate, 2 mM ATP and 1 mM NADPH in a final volume of 400 µL. The RNR proteins purified were active without reassembling the iron center (Shao et al., 2004; Zhou et al., 2010). In hypoxic

samples (< 0.1% O₂) the reaction mixture was assembled and the assay was carried out within an anaerobic chamber (Bactron, Shel Labs). Reactions were initiated by the addition of CDP, incubated at 37°C and aliquots of 50 μl were quenched by the addition of 10 μl of 3% tricarboxylic acid over a time course of the reaction. Subsequent dCDP and CDP levels were analyzed by HPLC. Graphs present either percentage of dCDP formation when normoxia and hypoxia were compared (data were normalized against the normoxic samples at 37°C at 30 min where the dCDP formation was considered 100%) or in μM when the accumulation of dCDP formed in < 0.1% O₂ at a 0–120 min time course was examined.

Determination of CDP/dCDP

CDP and dCDP levels following RNR activity assays were determined by ion-pairing HPLC with UV detection (270 nm). HPLC was performed using a Shimadzu system with a photodiode array detector. The column was an ACE C 18, 3 μm, 125 × 3 mm (Hichrom) maintained at 35°C. A gradient separation was achieved using 10% methanol, 10 mM KH₂PO₄, 10 mM TBAOH, pH 6.9 (A) and 30% methanol, 50 mM KH₂PO₄, 6 mM TBAOH, pH 7.0 (B), with a linear gradient of 25 – 47% B over 8 min, 47 – 80% B over 1 min, then returning to the started conditions 3 min. The flow rate was 0.6 ml/min and the running time was 16.5 min.

Circular dichroism spectrometry

Circular dichroism (CD) spectra (Chirascan CD/Fluorimeter spectrometer (Applied Photophysics, UK) of hRRM2B and the mutants (0.2 mg/mL) were recorded in the wavelength range 260 to 185 nm in 100 mmol/L potassium phosphate buffer (pH 7.5). All measurements were carried out at 37°C using a 0.1-cm path length quartz cuvette. The data pitch was 0.1 nm with a 1-nm bandwidth at a scan speed of 1.0 nm/s. Each spectrum shown represents the average of three. All CD data were expressed as the mean residue ellipticity, [θ], deg·cm²·dmol^{−1}.

Inductively coupled plasma mass spectrometry (ICP-MS)

The Thermo Finnigan Element 2 ICP-MS was used for quantitation of iron content in the recombinant purified proteins (40 μg of protein was used per 50 μl of reaction). Calibrations were obtained using external standards (a series of standards of known Fe concentrations were prepared and analyzed to gain a calibration linear, prior to the measurement of the samples). An external standard was diluted and measured from a dilution of High Purity Standards 10 ppm ICP-MS-68 A standard. All blanks, standards and samples were also spiked with 1 ng/g Rh, so that any general instrument drift could be normalized. Dilutions were made using a 2% HNO₃ solution, prepared using in-house distilled nitric acid and 18.2 Mohm DI water. All data results were first reported as elemental concentrations and then calculated in nM of Fe per μM of protein. Additional information can be found at (<https://www.earth.ox.ac.uk/research/services/geochemical-analysis/icpms/>).

Electron paramagnetic resonance (EPR)

CW-EPR spectra were collected in the Center for Advanced ESR (CAESR) in the Inorganic Chemistry Laboratory at the University of Oxford. X-band measurements utilized a Bruker-Biospin Micro EMX^{plus} spectrometer equipped with a PremiumX microwave bridge, a cylindrical TE₀₁₁-mode resonator (SHQE-W), an ESR-900 liquid helium cryostat, and an Oxford Instruments ITC-503 s temperature controller. Measurements were performed within the limit of resolution by temperature, 40K, and under non-saturating conditions.

The protein concentration for all EPR samples was 200 μM except for RRM2 protein, which was 70 μM. The protein samples (RRM2B, RRM2 or RRM2B mutants) were diluted in 50 mM HEPES, pH 7.5 and were incubated either at 37°C for 5 min with oxygen present (normoxia) or at 37°C for 60 min in a Bactron II anaerobic chamber (< 0.1% O₂). At the end of each treatment 20% (v/v) glycerol was added for vitrification during the low-temperature recordings. Samples were then transferred to EPR tubes and quick-frozen by immersion in liquid nitrogen. Spin quantitation was performed using the Bruker spectrometer hardware spin calibration, given the input dimensions of the precision Wilmad PQ-706 tubes. The results were verified with a 1 mM Cu^{II} EDTA in 50 mM HEPES pH 7.5, 20% (v/v) glycerol that was measured under non-saturating conditions at 100 K with 50 μW microwave power. The results show spin equivalents per β-subunit.

Molecular Dynamics (MD) simulations

System setup: MD simulations of O₂ transport on hRRM2B (PDB: 3HF1; resolution 2.60 Å) and hRRM2 (PDB: 3VPN; resolution 2.25 Å) were performed (Smith et al., 2009). The RRM2B structure comprises residues S29 to F311. The 3VPN X-ray structure is that of the E106D variant of native RRM2, and comprises residues M65 to M350. Four simulations were performed in which 200 randomly chosen water molecules outside the solvated protein were replaced by 200 O₂ molecules (0.5 M) termed the 100% oxygen level simulation. The random distribution of oxygen molecules precludes any potential artifacts arising from initial distribution. An additional two sets of simulations were performed to control for concentration effects; the first set up has a tenfold reduction in oxygen concentration, termed 10% oxygen level simulation with effective concentration of 50 mM, for RRM2B and RRM2. The second set does not include any oxygen molecules, termed 0% oxygen level simulation. Each system was solvated in a box of dimensions (80, 80, 92) Å using the Solvate plug-in of VMD (Humphrey et al., 1996), with the total system size comprising ~61,000 atoms. In accordance with the Uniprot sequence alignment two mutants of native RRM2B were prepared with the Mutate plugin in VMD (Humphrey et al., 1996), namely Y164C and K37E/K151E.

MD equilibration protocol

Calculations were done with NAMD 2.9 (Phillips et al., 2005), using the CHARMM 27 protein force-field (Brooks et al., 2009) with the CMAP correction (Buck et al., 2006), together with the TIP3P water model (Jorgensen et al., 1983). Oxygen Lennard-Jones parameters were taken from CHARMM (Cohen et al., 2005, 2006). For O₂ gas access in RRM2B and RRM2, the iron centers were modeled using CHARMM 27 force-field, with iron Lennard-Jones parameters $\epsilon_{\text{O}} = 0.00$ kcal/mol and $R_{\text{min}}/2 = 0.65$ Å. Oxygen (O₂) was modeled using CHARMM 27 Lennard-Jones parameters from heme oxygen, $\epsilon_{\text{O}} = -0.12$ kcal/mol and $R_{\text{min}}/2 = 1.7$ Å, as well as bond spring constants and bond spring lengths for O₂ as 1.23 Å and 600 kcal/mol/Å⁻¹, respectively. RRM2B contains the same diiron/dityrosyl cofactor that RRM2 does, and both RRM2B and RRM2 are biologically active as homodimers. In RRM2B, the active site iron coordination environment in Monomer-1 is a mono-iron site, and Monomer-2 has a di-iron site. In RRM2B, the active site iron coordination environment consists in both Monomer-1 and 2 of mono-iron sites.

After 1,000 steps of Conjugate-Gradient minimization, four classical molecular dynamics (MD) simulations of the O₂-containing solvated protein were carried out for 300 ns in the NPT ensemble. The high oxygen concentration was necessary to ensure sufficient sampling of protein cavities by O₂ on the accessible timescale of current simulations. It was verified that no gas clustering occurred in the solvent at this concentrations. The particle mesh Ewald (PME) algorithm was used for evaluation of electrostatics interactions beyond 12 Å, with a PME grid spacing of 1 Å, and NAMD defaults for spline and κ values (Darden et al., 1993). A cut-off at 12 Å was applied to non-bonded forces, both electrostatics and Van der Waals forces were smoothly switched off between the cut-off distance of 12 Å, and the switching distance of 10 Å, using the default NAMD switching function. A Verlet neighbor list with pairlist distance of 14 Å was used to only evaluate non-bonded neighboring forces within the pairlist distance (Verlet, 1967). The lengths of covalent bonds involving hydrogen atoms were constrained by the SETTLE algorithm (Miyamoto and Kollman, 1992) in order to be able to use a 2-fs time-step. The multi time step algorithm Verlet-l/r-RESPA (Tuckerman et al., 1992; Verlet, 1967) was used to integrate the equations of motion. Non-bonded short-range forces were computed for each time step, while long-range electrostatic forces were updated every 2 time steps. The pressure was kept at 1 atm by the Nosé-Hoover Langevin piston (Langevin, 1908; Nosé, 1984a, 1984b), with a damping time constant of 50 fs and a period of 100 fs. The temperature was maintained at 300 K by coupling the system to a Langevin thermostat, with a damping coefficient of 1 ps⁻¹. A total of 3.0 μ s of MD simulations were accrued.

Code availability

The standard MPI version 2.9 of the MD simulation code NAMD was employed (Phillips et al., 2005). A free license can be obtained at <http://www.ks.uiuc.edu/Research/namd/>. Version 1.9 of Visual Molecular Dynamics (VMD) was employed for analysis, with license obtained at <http://www.ks.uiuc.edu/Research/vmd/>.

MD simulation analysis

The Solvent-Accessible Surface Area (SASA) of both proteins RRM2B and RRM2 was calculated using the POPS (Parameter OPTimised Surfaces) algorithm (Cavallo et al., 2003; Fraternali and Van Gunsteren, 1996). The bending angle of helices was estimated using Bendix in VMD (Dahl et al., 2012).

The Diffusion-Reaction Model (Wang et al., 2013) presented in Equation (1) was adopted to describe the diffusion of O₂ into the RNR active site:



This is a kinetic two-step model consisting of an initial diffusion step of the gas molecule, followed by a metal-gas molecule chemical reaction step (Wang et al., 2011; Wang and Blumberger, 2012). Here, a classical force-field (FF) was employed, and only the diffusion and binding of O₂ gas molecules to the RNR iron center were modeled:



The simulation of O₂ diffusing in the solvated RNR enzymes yields diffusion statistics that enable the construction of a probability density map of the O₂-distribution within RNR (RRM2B or RRM2). This distribution is then divided into a set of M discrete protein cluster states that correspond to the probability maxima. Two parameters are used to characterize the diffusion of O₂. First, a distance criterion is established to determine when an oxygen molecule has entered into the active site of hRRM2B and hRRM2, defined as the difference between the center-of-mass (COM) of the iron ion and the O₂ molecule. Second, cut-off criteria of 8 Å, 9 Å and 10 Å were employed to generate statistics of the O₂ entering. An O₂ molecule is counted as having entered the cluster if $d_{\text{COM}}(\text{O}_2\text{-Fe}) < c$ where c is 8 or 9 Å.

Free-energy simulations of tunnel entry

Oxygen entry tunnels were assigned and characterized from classical MD simulations. As previously described, O₂ entry into WT RRM2B and RRM2 was identified to occur primarily through Tunnels T1, T2 and T3. Based on this assignment, equilibrium Adaptive-Biasing Force (ABF) MD simulations (Chipot and Pohorille, 2007; Darve and Pohorille, 2001; Darve et al., 2008; Van Gunsteren, 1989) were performed on oxygen entry into tunnels T1, T2 and T3 in Monomer-1 to determine relative differences in

the thermodynamics of oxygen entry into RRM2B and RRM2. In total, six ABF simulations per RNR were performed, totalling $\approx 0.5 \mu\text{s}$ of MD simulation dynamics. Each ABF simulation was performed with a force bias on a single oxygen molecule meaning these simulations constitute single-molecule biased MD simulations, using the collective variable ξ center-of-mass (COM) distance between Fe(III) and O_2 , $\xi(\mathbf{r}^N) = d(\text{O}_2 - \text{Fe(III)})$, with ξ ranging from 3 Å to 15 Å, in bins of width 0.25 Å. Each ABF simulation obtains ergodic sampling by applying every 500 timesteps a biasing force $\tilde{F}^{\text{ABF}}$ (3) onto the Newtonian equations of motion, whose value is updated on-the-fly (Den Otter, 2000; Den Otter and Briels, 1998) From the result, free-energy gradient along ξ , the Potential of Mean Force (PMF) along ξ is obtained from numerical integration of the gradient.

$$\tilde{F}^{\text{ABF}} = \nabla_r \tilde{A} = - \langle E(\xi) \rangle_{\xi} \nabla_r \xi \quad (3)$$

$$\left(\frac{dG(\xi)}{d\xi} \right) = \left\langle \frac{\partial U(\mathbf{r}^N)}{\partial \xi} \right\rangle - \beta^{-1} \left\langle \frac{\partial \ln |\underline{J}|}{\partial \xi} \right\rangle = - \langle E(\xi) \rangle_{\xi}. \quad (4)$$

Tunnel T1 in Monomer-1 extends for ~ 30 Å and connects the two faces of the protein. It was simulated as two separate tunnels of length ~ 15 Å in Monomer 1, denoted Tunnel T1 and T1', and was compared to a single simulation of Tunnel T1 in Monomer 2. Two simulations of Tunnels T2 and T3 were performed in RRM2B and RRM2.

Analysis of fenestrations

The software CAVER 3.0 was used for the analysis of the evolution of lateral fenestrations during the MD simulations, using 100 evenly chosen snapshots from the MD simulations, which is every 2 ns. The outer surface of the protein is calculated by rolling a large spherical probe around the surface of the protein and then internal cavities are identified using a smaller spherical probe (a radius of 0.8 Å was used here, and 12 probe spheres used). All identified tunnels are grouped into clusters based on relative proximities and with an 8 Å cut off for each cluster node, meaning that tunnels differing from the node by more than 8 Å are excluded. The values for shell radius and depth (15 Å) influence the definition of the protein molecular surface.

Statistical analysis

The statistical significance of differences between datasets was determined assessed by using the following test. Student's t test (two-tailed paired, except for dNTP incorporation assays, where a two-tailed unpaired test was applied) was used in all experiments where two means were compared. One-way analysis of variance (ANOVA) was used to compare several means and two-way ANOVA was used for determination in a response that is affected by two factors (one- or two-way ANOVA analysis is indicated at the figure legends). Statistical significance was assumed if $p < 0.05$ or lower and is noted in the figures. Error bars represent mean $\pm$ standard error of the mean (s.e.m.).

DATA AND SOFTWARE AVAILABILITY

Original imaging data have been deposited to Mendeley Data and are available at <http://dx.doi.org/10.17632/bp95v48kgm.1>

Supplemental Information

Ribonucleotide Reductase Requires Subunit

Switching in Hypoxia to Maintain DNA Replication

Iosifina P. Foskolou, Christian Jorgensen, Katarzyna B. Leszczynska, Monica M. Olcina, Hanna Tarhonskaya, Bauke Haisma, Vincenzo D'Angiolella, William K. Myers, Carmen Domene, Emily Flashman, and Ester M. Hammond

Figure S1

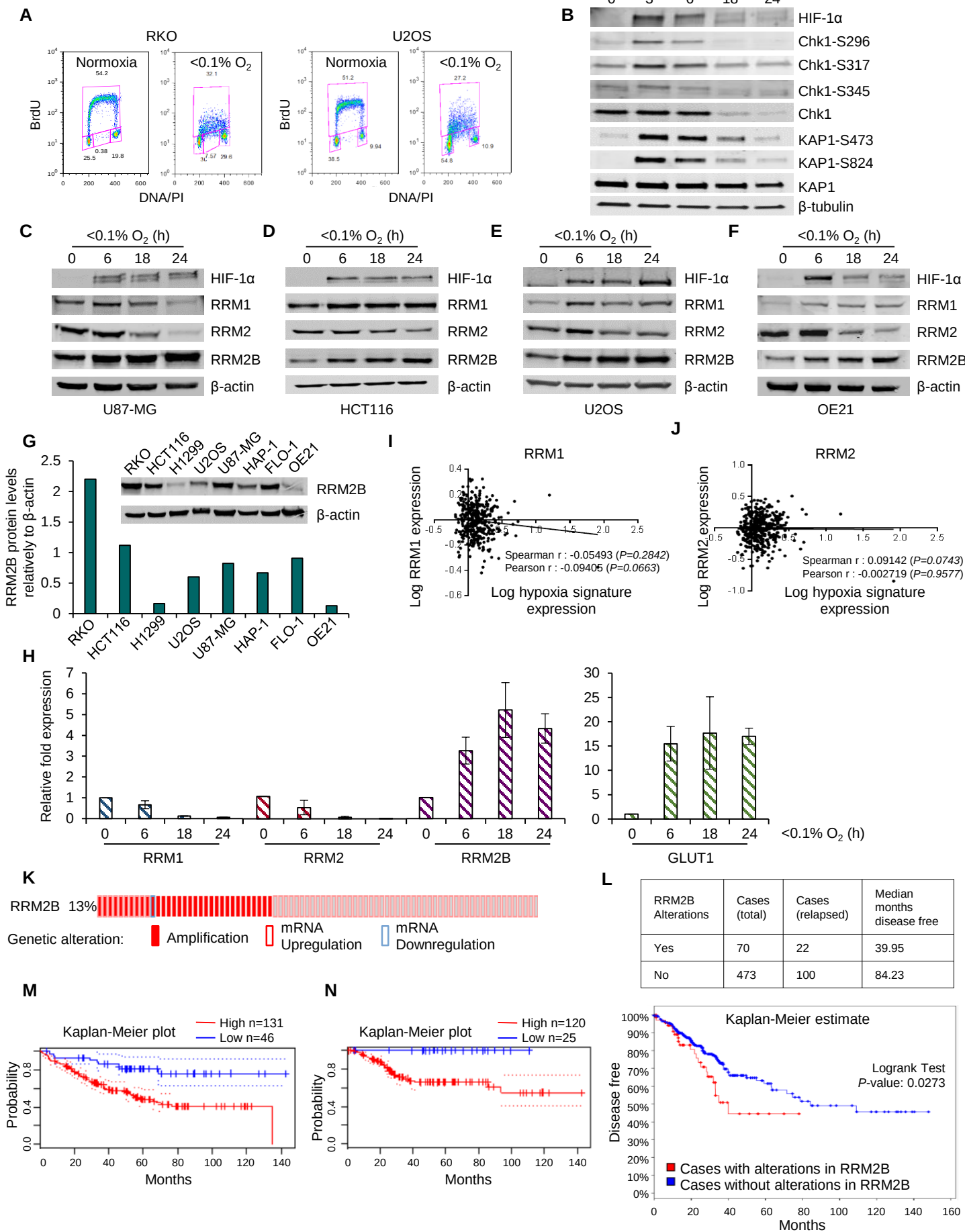


Figure S2

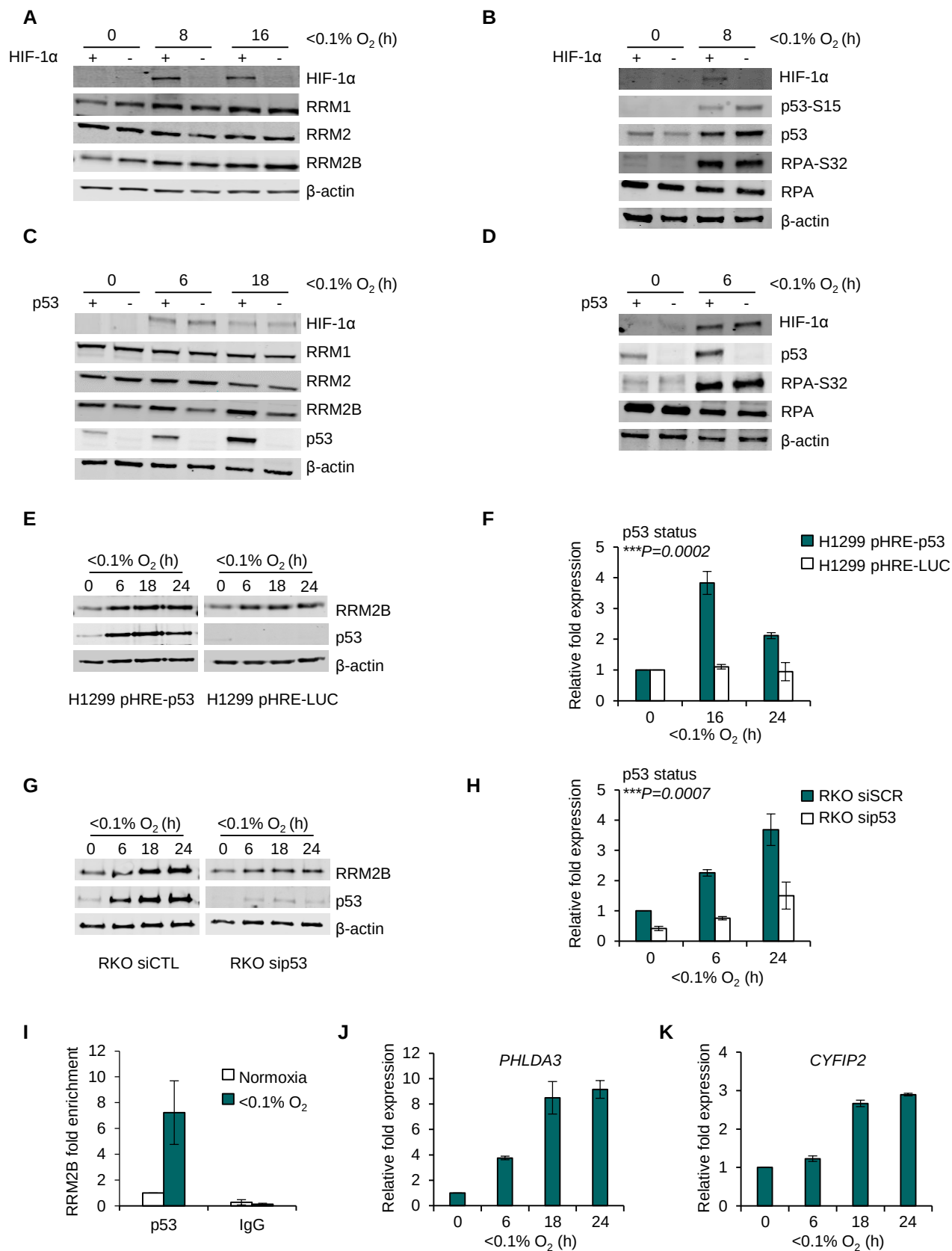


Figure S3

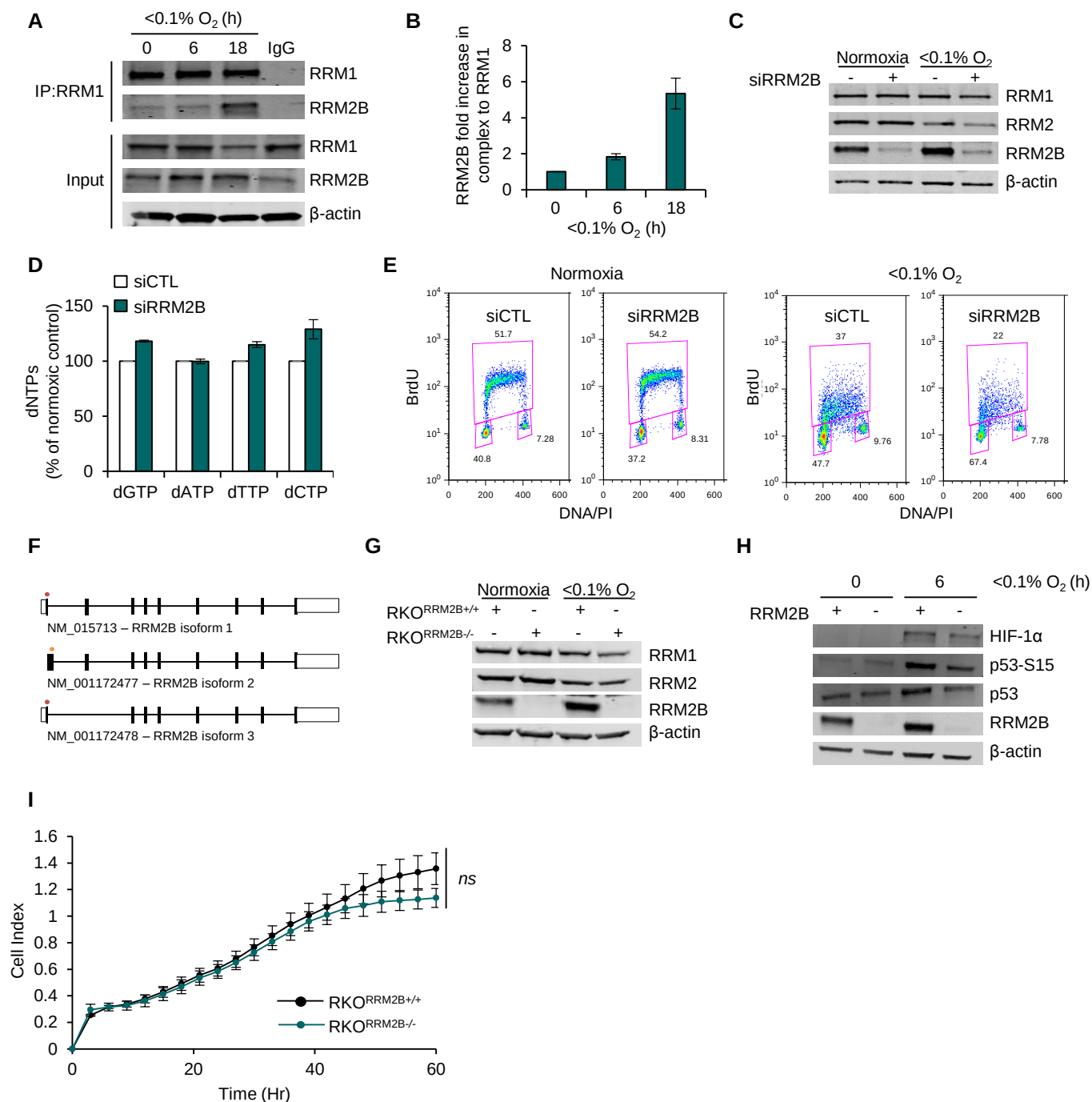


Figure S4

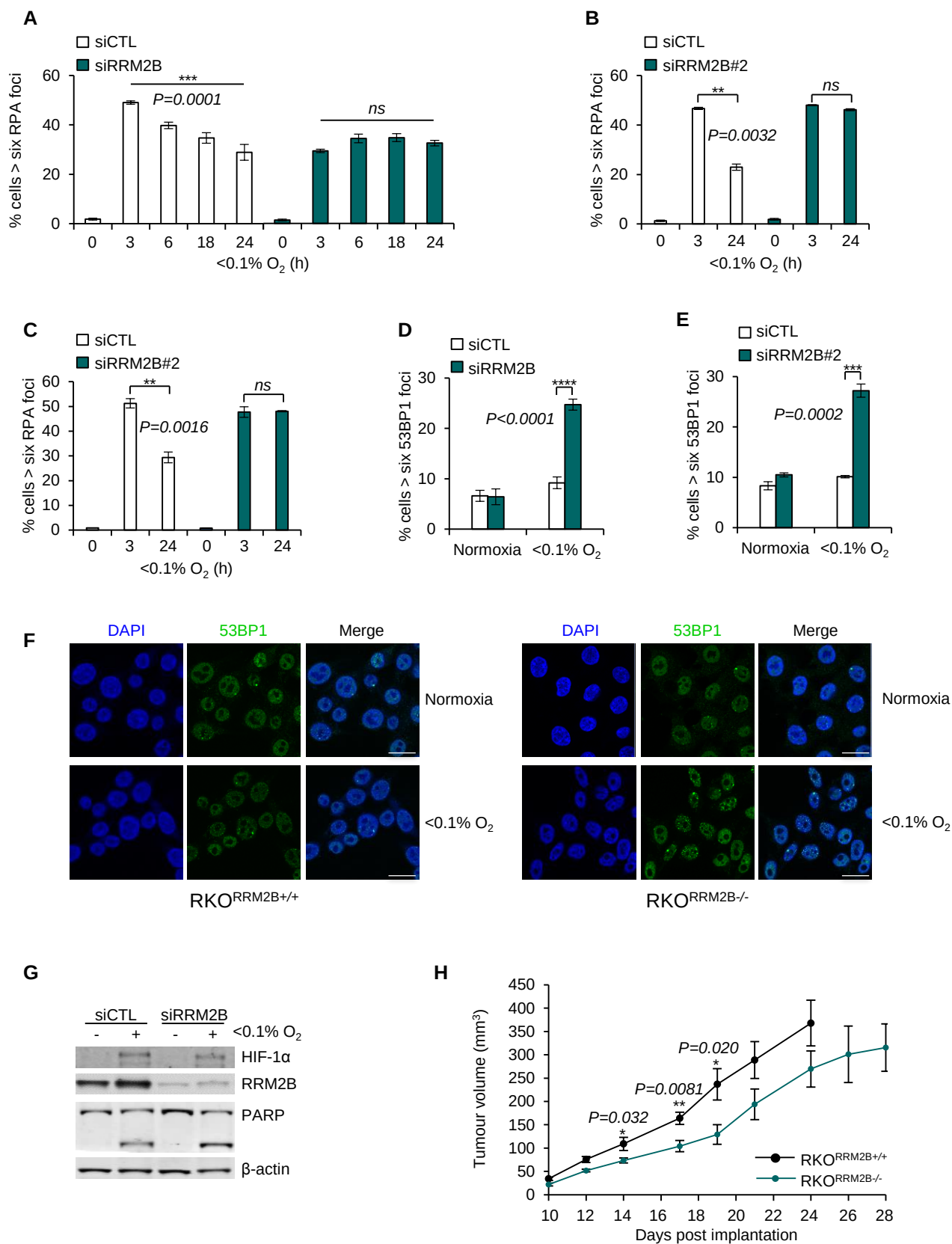


Figure S5

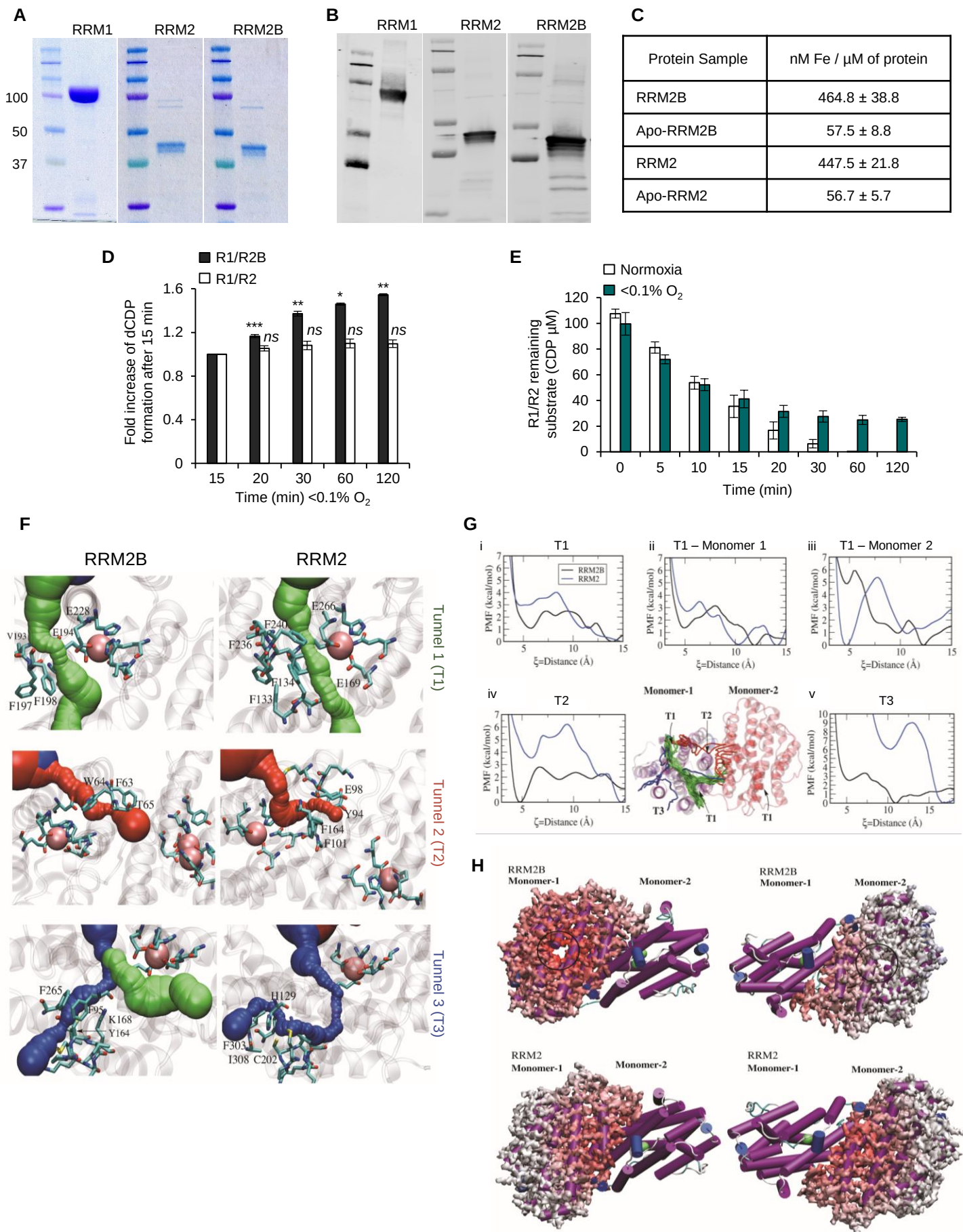


Figure S6

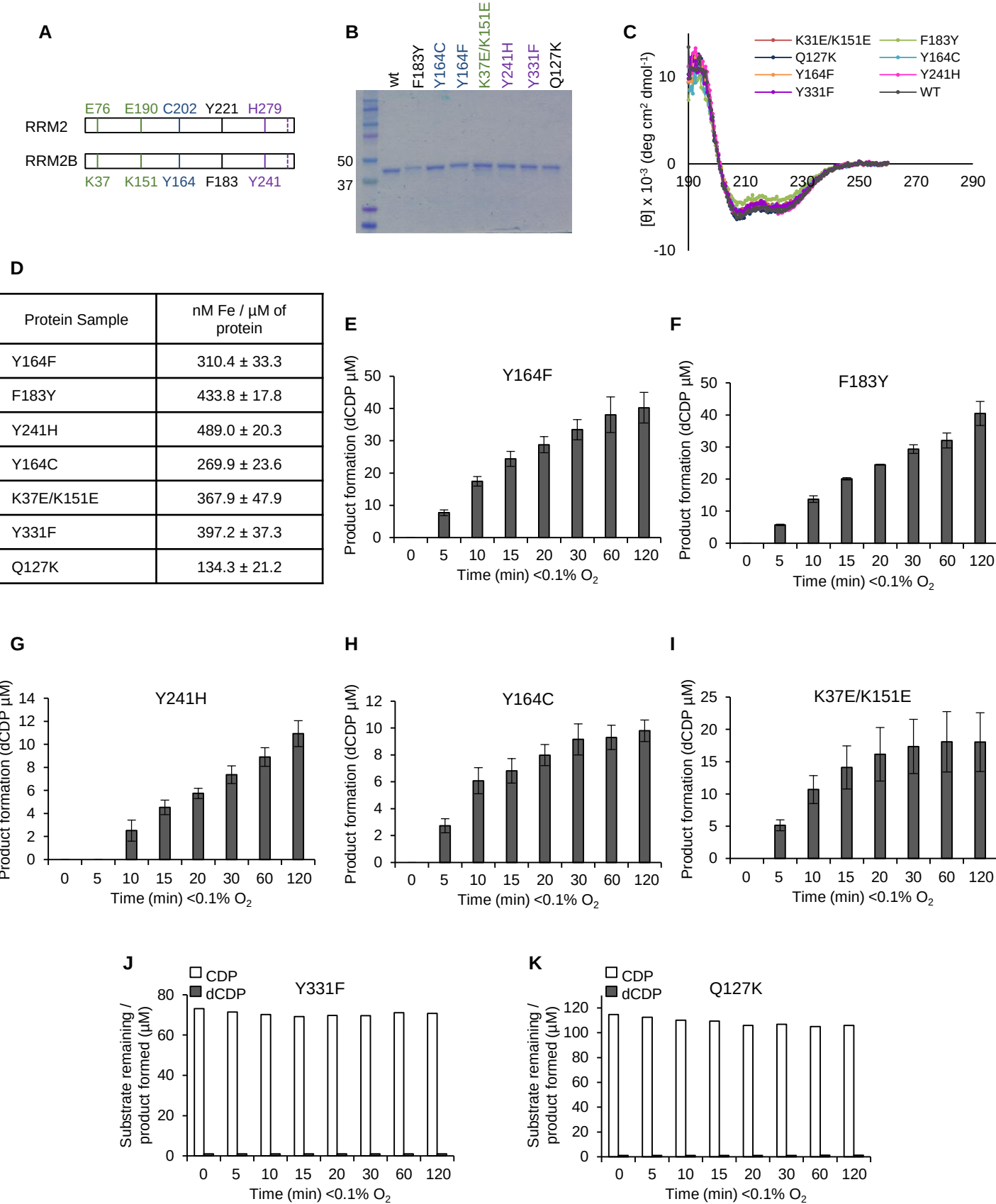
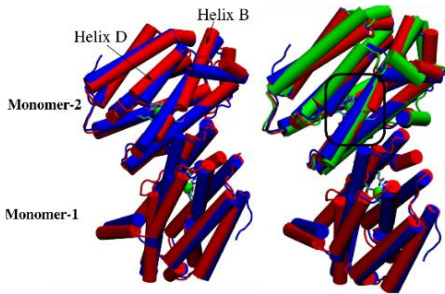


Figure S7

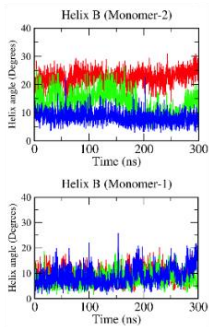
A

RRM2B EPR Sample	Spins / β subunit ($\times 10^{-3}$)	Hypoxia / Normoxia (%)
Y241H Normoxia	6.85 ± 0.05	72.5 ± 0.5
Y241H $<0.1\% \text{ O}_2$	5 ± 0	
F183Y Normoxia	11.7 ± 1.1	79.5 ± 4.5
F183Y $<0.1\% \text{ O}_2$	9.25 ± 0.35	
Y164F Normoxia	35.5 ± 0.5	60 ± 4
Y164F $<0.1\% \text{ O}_2$	21.25 ± 1.25	
Q127K Normoxia	ND	ND
Q127K $<0.1\% \text{ O}_2$	ND	

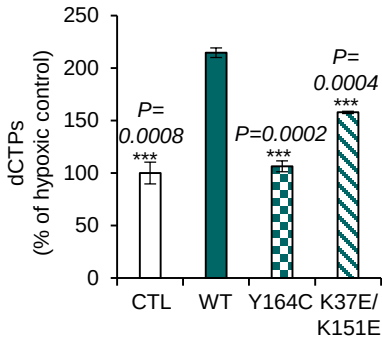
B



C



D



E

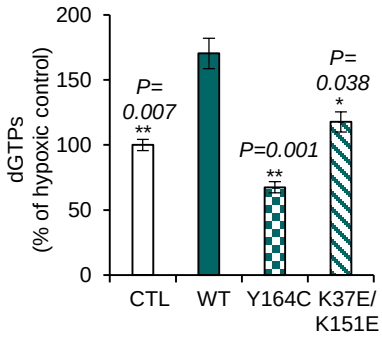


Figure S1. Related to Figure 1.

(A) Representative images of FACS analysis of RKO and U2OS cells exposed to normoxia or $<0.1\%$ O_2 (3 hr) indicating replication stress in hypoxic conditions. Cells were pulsed with BrdU (20 μ M) 30 min before collection. **(B)** Immunoblots for common DDR targets in RKO cells exposed to $<0.1\%$ O_2 for the times indicated. **(C-F)** Immunoblots of RNR subunits in different cancer cell lines exposed to $<0.1\%$ O_2 for the times indicated: (C) U87-MG (human glioblastoma); (D) HCT116 (colorectal carcinoma); (E) U2OS (human osteosarcoma) and (F) OE21 (human esophageal). **(G)** RRM2B levels relatively to β -actin in a panel of different cell lines in normoxia. RKO cells showed the highest expression of RRM2B protein. **(H)** mRNA levels of RNR subunits in U87-MG cells in $<0.1\%$ O_2 normalized to 18S. For all panels $n=3$ (biological replicates); for panel (H) data show means $\pm$ s.e.m. **(I)** Expression of RRM1 (Log10 conversion) in the colorectal adenocarcinoma TCGA datasets is shown against hypoxia-inducible signature (Log10 conversion). **(J)** Expression of RRM2 (Log10 conversion) in the colorectal adenocarcinoma TCGA datasets is shown against hypoxia-inducible signature (Log10 conversion). **(K)** The Cancer Genome Atlas (TCGA) colorectal adenocarcinoma dataset was analyzed for genetic alterations (including mutations, putative copy-number alterations and mRNA expression data (RNA Seq V2 RSEM)) in *RRM2B* gene using cBioPortal. The scheme shows only the 13% cases (83 out of 629), where the genetic alterations in *RRM2B* was found. No mutations were reported. **(L)** Kaplan-Meier curve showing disease free survival in cancer cases with or without alterations in *RRM2B* from K. **(M-N)** The expression of *RRM2B* and the associated survival probability in colorectal cancers using PrognoScan. The colorectal cancer patient dataset GSE17536 was analyzed using PrognoScan tool and overall survival (M), as well as disease-free survival (N) associated with high or low expression of *RRM2B* (probe 223342_at) are shown in Kaplan-Meier plots.

Figure S2. Related to Figure 2.

(A) Immunoblots of the RNR subunits in RKO^{HIF-1 α +/+} and RKO^{HIF-1 α -/-} cells exposed to <0.1% O₂ for the times indicated. RRM2B protein is induced in hypoxia irrespective of HIF-1 α status. **(B)** Immunoblots of common replication stress markers in RKO^{HIF-1 α +/+} and RKO^{HIF-1 α -/-} cells exposed to <0.1% O₂ (8 hr). **(C)** Immunoblots of the RNR subunits in HCT116^{p53+/+} and HCT116^{p53-/-} cells exposed to <0.1% O₂ for the times indicated. RRM2B protein is decreased in the absence of p53. **(D)** Immunoblots of the replication stress marker RPA32 in HCT116^{p53+/+} and HCT116^{p53-/-} cells exposed to <0.1% O₂ (6 hr). **(E-F)** Immunoblots (E) and *RRM2B* mRNA levels (F) of H1299 cells treated with either pHRE-p53 or pHRE-LUC and exposed to <0.1% O₂ for the times indicated. **(G-H)** Immunoblots (G) and *RRM2B* mRNA levels (H) of RKO cells treated with either sip53 or siCTL and exposed to <0.1% O₂ for the times indicated. **(I)** qPCR for p53 ChIP in HCT116 cells exposed to either normoxia or <0.1% O₂ (6 hr). **(J-K)** mRNA levels of hypoxia inducible p53 targets *PHLDA3* (J) and *CYFIP2* (K) in RKO cells exposed to <0.1% O₂ normalized to 18S. For all panels n=3 (biological replicates); panels (F, H, J and K) data show mean $\pm$ s.e.m. (I) shows representative mean of technical triplicates $\pm$ RQmax/RQmin. p53 status was examined by two-way ANOVA analysis; (ns) indicates non significant change.

Figure S3. Related to Figure 3.

(A) Immunoprecipitation of RRM1 for the times indicated, followed by immunoblotting for RRM2B in normoxia and <0.1% O₂. **(B)** Quantification of (A) from n=3 biological replicates. **(C)** Immunoblots of RNR subunits in RKO cells treated with either siCTL or siRRM2B and exposed to normoxia or <0.1% O₂ (18 hr). Effective depletion of RRM2B protein with the specific siRNA is observed in both conditions. **(D)** dNTP incorporation assay in RKO cells treated with either siCTL or siRRM2B in normoxia. **(E)** Representative images of FACS

analysis of U2OS cells treated with either siCTL or siRRM2B and exposed to normoxia or $<0.1\%$ O₂ (3 hr). **(F)** Representative figure of the three isoforms of *RRM2B* gene. Black rectangles represent exons. The colored ovals represent the positions targeted by the guided RNAs in order the RKO^{RRM2B^{-/-}} cell line to be constructed (CRISPR-Cas9 system). **(G)** Immunoblots of RNR subunits in RKO^{RRM2B^{+/+}} and RKO^{RRM2B^{-/-}} cell lines exposed to normoxia or $<0.1\%$ O₂ (18 hr). **(H)** Immunoblots of p53 status in RKO^{RRM2B^{+/+}} and RKO^{RRM2B^{-/-}} cell lines exposed to normoxia or $<0.1\%$ O₂ (6 hr). **(I)** Cell proliferation assay in normoxia using the xCELLigence system using RKO^{RRM2B^{+/+}} and RKO^{RRM2B^{-/-}} cells. For all panels n=3 (biological replicates); for panels (B, D, and I) data show mean $\pm$ s.e.m. and in panel (I) one-way ANOVA analysis was applied; (ns) indicates non significant change.

Figure S4. Related to Figure 3.

(A) Quantification of RPA32 foci in RKO cells treated with either siCTL or siRRM2B after exposure to $<0.1\%$ O₂ for the times indicated. **(B-C)** Quantification of RPA32 foci in RKO (B) or HCT116 (C) cells treated with either siCTL or siRRM2B#2 after exposure to $<0.1\%$ O₂ for the times indicated. **(D-E)** Quantification of 53BP1 foci in RKO cells treated with either siCTL or siRRM2B (D) or siRRM2B#2 (E) after exposure to normoxia or $<0.1\%$ O₂ (6 hr). **(F)** Representative images of 53BP1 foci in RKO^{RRM2B^{+/+}} and RKO^{RRM2B^{-/-}} cells exposed to normoxia or $<0.1\%$ O₂ (6 hr). Scale bar 20 μ m. **(G)** Immunoblot for PARP cleavage in RKO cells treated with either siCTL or siRRM2B and exposed to normoxia or $<0.1\%$ O₂ (19 hr). **(H)** RKO^{RRM2B^{+/+}} and RKO^{RRM2B^{-/-}} xenografts were grown in mice. Tumor volumes were measured for 5 (RKO^{RRM2B^{+/+}}) and 6 (RKO^{RRM2B^{-/-}}) mice per each group and a mean $\pm$ s.e.m. is shown. For all panels n=3 (biological replicates) otherwise stated; for panels (A-E and H) data show mean $\pm$ s.e.m. and two-tailed Student's t-test was applied; in panel (A) one-way ANOVA analysis was applied; (ns) indicates non significant change.

Figure S5. Related to Figure 4.

(A-B) Recombinant RRM1, RRM2 and RRM2B proteins were overexpressed in *E.coli* cells and purified to ~90% purity as shown with Coomassie blue staining (A) and Immunoblotting (B). **(C)** Quantitation of iron bound in the RRM2B and RRM2 proteins as overexpressed in *E.coli* cells using ICP-MS. As negative control the apo-forms of the proteins were used. **(D)** Fold increase of dCDP formation in <0.1% O₂ for the indicated times normalized to 15 min for either R1/R2B or R1/R2 enzymes. Graphs show the amount of dCDP formed after 15 min in <0.1% O₂ and each time point was compared with the previous to determine statistically significant dCDP formation. **(E)** Substrate consumption (CDP) in normoxia and hypoxia for R1/R2. For panel (C) n=3 (technical replicates); for panels (D-E) n=3 (biological replicates); in all panels data show mean $\pm$ s.e.m.; in panel (D) two-tailed Student's t-test was applied; (ns) indicates non significant change. **(F)** Identification and analysis of the RRM2B and RRM2 oxygen tunnels. MD simulations revealed three principle oxygen cavity tunnels (T1-3) in Monomer-1 in both RRM2B and RRM2 proteins. T1: reveals connectivity between Monomer-1 active site and the outside protein surface, with an average length 29.0 ± 16 Å in RRM2B to 15.0 ± 15 Å in RRM2. T2: reveals connectivity between Monomer-1 and Monomer-2. T3: connects either the Tyr164 (RRM2B) or the Cys202 (RRM2) with the pocket of phenylalanines. **(G)** Potential of Mean Force for oxygen entry shows differential functioning of RRM2B to RRM2. Comparison between RRM2B (black) and RRM2 (blue) PMF for diffusion (i) T1 and (ii) T1 in Monomer-1, compared to (iii) T1 in Monomer-2; (iv) T2 and (v) T3. The Potential of Mean Force (PMF) for diffusion in the individual tunnels between RRM2B (black) and RRM2 (blue) reveal differences in the tunnels of RRM2B and RRM2. T1 is similar in RRM2B and RRM2, precluding selectivity differences and with oxygen entry characterized by low barriers of 2-3 kcal mol⁻¹, as reported for extended tunnels in [Ni-Fe] hydrogenases (Wang et al., 2011, Wang et al., 2013). T1 in Monomer-2, showed

barriers of 5-6 kcal mol⁻¹, which might explain the lower O₂ turnover of Monomer-2 compared to Monomer-1 in the unbiased MD simulations. T2 appears to show a pronounced difference between RRM2B and RRM2, with a 3 kcal mol⁻¹ higher barrier for O₂ entry into RRM2 compared to RRM2B, as was rationalized in the crystal structure in which the T2 was proposed to be more open in RRM2B compared to RRM2 (Smith et al., 2009). T3 shows barrier height differences between RRM2B and RRM2 and suggest that RRM2B is a superior oxygen-sequestering agent. **(H)** RRM2B has an open phenylalanine channel. The switch of C202 in RRM2 to Y164 in RRM2B results in an open phenylalanine channel specifically in RRM2B.

Figure S6. Related to Figure 5.

(A) Representative figure of the key differences between RRM2 and RRM2B proteins. **(B)** Seven RRM2B variants were constructed with site-directed mutagenesis, overexpressed in *E.coli* cells and purified as shown with Coomassie blue staining to ~90% purity. **(C)** CD spectra of RRM2B WT and variant proteins ensuring that all proteins were properly folded; n=3 (technical replicates). **(D)** Quantitation of iron bound in the RRM2B variant proteins as overexpressed in *E.coli* cells using ICP-MS. **(E-I)** Product formation (dCDP) in <0.1% O₂ for (E) Y164F; (F) F183Y; (G) Y241H; (H) Y164C; (I) K37E/K151E. n=2 (biological replicates); data show mean ± s.e.m. **(J-K)** The substrate (CDP) over product formation for variants (J) Y331F and (K) Q127K showed zero turnover as expected. This is because in Y331F variant the pathway that transfers an electron to the catalytic site of RRM1 is disrupted (Xue et al., 2006), while in Q127K variant the stability of the tyrosyl radical cluster is disrupted (Zhou et al., 2010).

Figure S7. Related to Figure 5.

(A) Quantification of EPR spectra of the tyrosyl radical of mutant recombinant RRM2B proteins (Y241H, F183Y, Y164F and Q127K) in normoxia and <0.1% O₂. Data presented show electron spins per β subunit; n=2 (biological replicates); data show means $\pm$ s.e.m. **(B-C)** Comparison of RRM2B (red) with RRM2 (blue), revealing an open B-helix in RRM2B and a closed B-helix in RRM2. K37E/K151E (green) after 300 ns MD simulation has a closed B-helix. Plots (C) reveal Helix-B bending angle. **(D-E)** dCTP (D) and dGTP (E) in RKO^{RRM2B-/-} cells transfected with either CTL, WT, Y164C or K37E/K151E and exposed to <0.1% O₂ (16 hr); n=3 (biological replicates); data show means $\pm$ s.e.m. and two-tailed Student's t-test was applied.

Table S1. Primer Table. Related to the STAR Methods.

Oligonucleotide Name	Sequence	Reference
Primers used for quantitative PCR (qPCR)		
18S Forward	TAGAGGGACAAGTGGCGTTC	This paper
18S Reverse	CGGACATCTAAGGGCATCAC	
GLUT-1 Forward	ATACTCATGACCATCGCGCTAG	This paper
GLUT-1 Reverse	AAAGAAGGCCACAAAGCCAAAG	
RRM1 Forward	ACCAACCGCCCACAACCTTT	This paper
RRM1 Reverse	TGCCATTAGTCCCAGCAATGT	
RRM2 Forward	GTGGAGCGATTTAGCCAAGAA	(D'Angiolella et al., 2012)
RRM2 Reverse	CACAAGGCATCGTTTCAATGG	
RRM2B Forward	TGGTGGAGCGCTTTAGTCAG	This paper
RRM2B Reverse	CTATCCATCGCAAGGCCCAA	
INPP5D Forward	CGCCCACTAATCCTTGATGT	(Leszczynska et al., 2015)
INPP5D Reverse	GCTTGGACACCATGTTGATG	
CYFIP2 Forward	GGTCATGGAGGAACTGCTAA	(Leszczynska et al., 2015)
CYFIP2 Reverse	TCTTGGGCATCACCTCTATC	
Primers used for site-directed mutagenesis		
RRM2B_Y164F_For	ATTTAATGCAATTGAAACCATGCCCTT	This paper
RRM2B_Y164F_Rev	TGTTAAGAAAAAAGCAGATTG CAATCTGCTTTTTTCTTAACAAAGGGC ATGGTTTCAATTGCATTAAAT	
RRM2B_Y164C_For	ATTTAATGCAATTGAAACCATGCCCTT	This paper
RRM2B_Y164C_Rev	GTGTTAAGAAAAAAGCAGATTG CAATCTGCTTTTTTCTTAACACAGGGC ATGGTTTCAATTGCATTAAAT	
RRM2B_K37E_For	GAAGAGCCACTCCTAAGAGAGAGTTC	This paper
RRM2B_K73E_Rev	TCGCCG CGGCGAGAACTCTCTCTTAGGAGTGG CTCTTC	
RRM2B_K151E_For	ACACTTACATCAGAGATCCCAAGGAA	This paper
RRM2B_K151E_Rev	AGGGAATTTTTATTTAATGCA TGCATTAAATAAAAAATTCCCTTTCCTT GGGATCTCTGATGTAAGTGT	
RRM2B_F183Y_For	ATGGATAGCAGATAGAAAATCTACTT	This paper
RRM2B_F183Y_Rev	ATGGGGAAAGAGTGG CCACTCTTTCCCCATAAGTAGATTTTC TATCTGCTATCCAT	
RRM2B_Y241H_For	ACTTTGCTTGCCTGATGTTCCAACACT	This paper
RRM2B_Y241H_Rev	TAGTAAATAAGCCTTC GAAGGCTTATTTACTAAGTGTGGAA CATCAGGCAAGCAAAGT	

RRM2B_Y331F_For	ATTTCTTTGAGAAACGAGTTTCAGAG	This paper
RRM2B_Y331F_Rev	TTTCAGCGTTTTGCAG CTGCAAAACGCTGAAACTCTGAAACT CGTTTCTCAAAGAAAT	
RRM2B_Q127K_For	GCTCGCTGTTTCTATGGCTTTAAAATT	This paper
RRM2B_Q127K_Rev	CTCATCGAGAATGTTC GAACATTCTCGATGAGAATTTTAAAG CCATAGAAACAGCGAGC	
Primers used for dNTP incorporation assay		
dTTP-Forward	TTATTATTATTATTATTATTAGGCGGT GGAGGCGG	(Sherman and Fyfe, 1989)
dCTP-Forward	TTTGTGTTGTTTGTGTTTGGGCGGTG GAGGCGG	
dGTP-Forward	TTTCTTTCTTTCTTTCTTTCGGCGGTGG AGGCGG	
dATP-Forward	AAATAAATAAATAAATAAATGGCGGT GGAGGCGG	
dNTP-Reverse	CCGCCTCCACCGCC	

Table S2. Recombinant DNA Table. Related to the STAR Methods.

Plasmid Name	Backbone	Reference
pET28b-6xHis RRM1	pET-28a DNA (Novagen #69865-3)	(Wang et al., 2007)
pET28a-6xHis RRM2	pET-28a DNA (Novagen #69864-3)	(Shao et al., 2004)
pET28a-6xHis RRM2B	pET-28a DNA (Novagen #69864-3)	(Shao et al., 2004)
HA-RRM2B	pcDNA3.1 (Invitrogen; V79520)	(D'Angiolella et al., 2012)
pET28a-6xHis RRM2B ^{Y164F}	pET-28a DNA (Novagen #69864-3)	This paper
pET28a-6xHis RRM2B ^{Y164C}	pET-28a DNA (Novagen #69864-3)	This paper
pET28a-6xHis RRM2B ^{K37E/K151E}	pET-28a DNA (Novagen #69864-3)	This paper
pET28a-6xHis RRM2B ^{F183Y}	pET-28a DNA (Novagen #69864-3)	This paper
pET28a-6xHis RRM2B ^{Y241H}	pET-28a DNA (Novagen #69864-3)	This paper
pET28a-6xHis RRM2B ^{Y331F}	pET-28a DNA (Novagen #69864-3)	This paper
pET28a-6xHis RRM2B ^{Q127K}	pET-28a DNA (Novagen #69864-3)	This paper
pRRM2B ^{WT}	pcDNA3.1 (Invitrogen; V79520)	This paper
pRRM2B ^{Y164C}	pcDNA3.1 (Invitrogen; V79520)	This paper
pRRM2B ^{K37E/K151E}	pcDNA3.1 (Invitrogen; V79520)	This paper
pRRM2B ^{Q127K}	pcDNA3.1 (Invitrogen; V79520)	This paper
pHRE-p53	pGL3-basic 5xHRE (Promega; E1751)	(Hammond et al., 2006)
pHRE-LUC	pGL3-basic 5xHRE (Promega; E1751)	(Hammond et al., 2006)
SpCas9-GFP-RRM2B_1/3	pSpCas9(BB)-2A-GFP (Addgene; 48138)	This paper
SpCas9-GFP-RRM2B_2	pSpCas9(BB)-2A-GFP (Addgene; 48138)	This paper

Chapter 2

Measuring DNA Replication in Hypoxic Conditions

Iosifina P. Foskolou, Deborah Biasoli, Monica M. Olcina,
and Ester M. Hammond

Abstract It is imperative that dividing cells maintain replication fork integrity in order to prevent DNA damage and cell death. The investigation of DNA replication is of high importance as alterations in this process can lead to genomic instability, a known causative factor of tumor development. A simple, sensitive, and informative technique which enables the study of DNA replication, is the DNA fiber assay, an adaptation of which is described in this chapter. The DNA fiber method is a powerful tool, which allows the quantitative and qualitative analysis of DNA replication at the single molecule level. The sequential pulse labeling of live cells with two thymidine analogues and the subsequent detection with specific antibodies and fluorescence imaging allows direct examination of sites of DNA synthesis. In this chapter, we describe how this assay can be performed in conditions of low oxygen levels (hypoxia)—a physiologically relevant stress that occurs in most solid tumors. Moreover, we suggest ways on how to overcome the technical problems that arise while using the hypoxic chambers.

Keywords Hypoxia • DNA replication • DNA fiber assay

2.1 Introduction

DNA replication is one of the most important and well-regulated biological processes and enables the equal distribution of genetic information during cellular division. Eukaryotic cells start DNA replication in numerous regions (replication origins)

The authors disclose no potential conflicts of interest.

I.P. Foskolou • D. Biasoli • M.M. Olcina • E.M. Hammond (✉)
Cancer Research UK and Medical Research Council Oxford Institute
for Radiation Oncology, Department of Oncology, University of Oxford,
Old Road Campus Research Building, Oxford OX3 7DQ, UK
e-mail: iosifina.foskolou@oncology.ox.ac.uk; deborah.biasoli@oncology.ox.ac.uk;
molcina@stanford.edu; ester.hammond@oncology.ox.ac.uk

which are distributed along the chromosomes [1]. During the S-phase of the cell cycle, replication initiation proteins prepare the chromatin for the upcoming DNA replication and license the controlled firing of each replication origin [2]. Interestingly, not all replication origins fire during S-phase but instead most of them remain “silenced” during this process (dormant origins) and are usually activated when the major ones do not, for example, due to the presence of replication stress [3–8]. DNA replication is tightly monitored and cells need to ensure that the genome is duplicated efficiently and accurately in order to preserve genome stability [1, 9]. This fine control of DNA replication is of high importance since any defect in this biological process can result in DNA strand breaks and genomic instability, two well-known causative factors during tumor development [10].

Regions of low oxygen (hypoxia) occur in most solid tumors as a result of a high metabolic demand and an abnormally formed/inefficient tumor vasculature. The occurrence of hypoxic regions is an indicator of poor patient prognosis due to resistance to chemo- and radiotherapy as well as increased genomic instability, invasion, and metastatic potential [11–14]. Under severely hypoxic conditions (radiobiologic hypoxia) replication stress occurs, which is characterized by slow replication rates and the accumulation of stalled replication forks [15–18]. Moreover, hypoxia triggers the activation of DNA damage response (DDR) pathway including the ATR/ATM signaling without detectable DNA damage [19, 20]. Replication stress and the subsequent activation of the DDR pathway have been linked to the early stages of cancer development and have been proposed to act as a barrier to tumorigenesis [21, 22]. Hypoxia contributes to this pathway as hypoxic regions can be formed in preneoplastic lesions [21–23]. In order to study replication stress, accumulation of RPA foci and BrdU immunofluorescence can be used for the detection of single stranded DNA as well as the phosphorylation of common markers that are activated by the ATR pathway including γ H2AX, Chk1 (Ser 345), and RPA (Ser 33) [24, 25]. Although these techniques are commonly used, they are limited and provide only indications of DNA replication stress. The most specific replication stress assay available to date is the direct measurement of DNA synthesis using the DNA fiber assay [26]. Here, we describe the adaptation of this sensitive microscopy-based method, to visualize DNA replication both in conditions of normal oxygen levels and in hypoxia.

The DNA fiber assay enables the study of DNA replication at a single molecule level [27–29]. In this technique, two different thymidine analogues are used sequentially in live cells and are incorporated into the nascent DNA. Subsequent staining of those analogues allows the visualization of the replication structures through the use of specific antibodies and fluorescence imaging (Fig. 2.1). A quantitative analysis can then be carried out by measuring the rate of movement of active replication forks as well as a qualitative analysis by classifying the status of the replication structures (stalled, ongoing, or new origins). The sensitivity and the wide range of information that can be extracted from this technique combined with its low cost makes it a powerful tool for the investigation of DNA replication in physiologically relevant conditions.

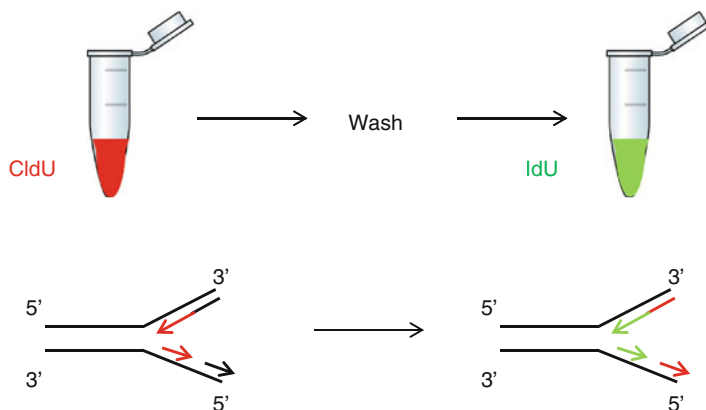


Fig. 2.1 Subsequent labeling of thymidine analogues (CldU and IdU) into the nascent DNA of live cells allows the visualization of the replication structures through the use of specific antibodies and fluorescence imaging

2.2 Solutions and Reagents

- Spreading Buffer (200 mM Tris-HCl, pH 7.4; 50 mM EDTA; 0.5 % SDS), once made stable for up to 6 months at room temperature.
- Fixation Buffer (Methanol/Acetic Acid 3:1), once made generally stable, keep at room temperature.
- Blocking Buffer (1 % BSA (Bovine Serum Albumin); 0.1 % Tween-20 diluted in 1× PBS), make fresh each time and keep at 4 °C.
- CldU (5-chloro-2'-deoxyuridine): Sigma-Aldrich UK (C6891-100MG). Stock solution 2.5 mM CldU diluted in culture media, once made stored at -20 °C for up to 2 months.
- IdU (5-iodo-2'-deoxyuridine): Sigma-Aldrich UK (I7125-5G). Stock solution 2.5 mM IdU diluted in culture media, once made stored at -20 °C for up to 2 months.
- Rat anti-BrdU: Oxford Biotechnology (OBT0030). Dilute 1:500 in blocking buffer and keep on ice. Make fresh each time.
- Mouse anti-BrdU: Becton Dickinson Biosciences (347580; 7580). Dilute 1:500 in blocking buffer and keep on ice. Make fresh each time.
- Alexa Fluor 555-conjugated goat anti-rat IgG: Invitrogen (A21434). Dilute 1:500 in blocking buffer and keep on ice in the dark. Make fresh each time.
- Alexa Fluor 488-conjugated goat anti-mouse F(ab')₂ fragment: Invitrogen (A11017). Dilute 1:500 in blocking buffer and keep on ice in the dark. Make fresh each time.

Coverslip—Cover Glasses: Menzel Gläser 22×50 mm, Fisher Scientific UK Ltd (MNJ-350-070P)

Slides—Adhesion slides: SuperFrost® Plus 76×26 mm, Thermo Scientific (631-0447)

2.3 Protocol

The following protocol is optimized for the RKO human colorectal cancer cell line (available from ATCC). Cell density and time of incubation may vary in different cell lines and may need to be adjusted. Cells are cultured in DMEM culture media supplemented with 10 % FBS, in a standard humidified incubator at 37 °C and 5 % CO₂. For hypoxia treatment, a Bactron II anaerobic chamber (Shell Labs) was used. No oxygen is supplied to this chamber; however, a complete absence of oxygen cannot be guaranteed at all times due to potential leaks. The oxygen concentration at any time is estimated to be no higher than 0.1 %.

2.3.1 Day 1

2.3.1.1 Seeding

- Seed in each 5 cm³ glass dish the number of cells needed to reach 50–60 % confluency on the following day. For RKO cells, 7×10^5 cells per glass dish are seeded. One dish per experimental condition is required
- Place the spreading buffer at 37 °C overnight
- Prepare CIdU (2.5 mM) and IdU (2.5 mM) stock solutions in the appropriate amount of culture media and store at –20 °C until use

Note: For experiments to be carried out in the anaerobic chamber, cells are plated on glass dishes to minimize the amount of potential oxygen being introduced into the chamber (since plastic absorbs and retains oxygen).

2.3.2 Day 2

2.3.2.1 Labeling

- Place CIdU stock solution at 37 °C and IdU stock solution at 50 °C to thaw
- Prepare fresh working solutions (25 µM CIdU and 250 µM IdU) by diluting the stock solutions in the appropriate amount of culture media. The CIdU stock solution is diluted 1:100 to prepare the working solution, whereas the IdU stock solution is diluted 1:10

2.3.2.2 Normoxic Samples

- Aspirate media from cells
- Add 5 ml of the working solution containing CIdU (25 µM) and incubate for 20 min at 21 % O₂ in a 37 °C incubator

- Wash once with media that does not contain any thymidine analogues
- Add 5 ml of the working solution containing IdU (250 μ M) and incubate for 20 min at 21 % O_2 in a 37 °C incubator
- Wash once with 1 $\times$ PBS and trypsinize cells
- Collect cells with 1 ml 1 $\times$ PBS by pipetting (do not scrape—cells should remain intact)
- Centrifuge to spin cells down (950 $\times g$ for 6 min)
- Dilute cells to a concentration of 5 $\times 10^5$ cells per ml and place them on ice
- Proceed straight to the spreading step

2.3.2.3 Hypoxic Samples

- Place the hypoxic samples in the chamber at the required oxygen tension
- *Note:* When labeling the hypoxic samples put the following in the pass box of the hypoxic chamber and let equilibrate for sufficient oxygen removal (at least 3 h)
 1. CldU and IdU working solutions
 2. Media that does not contain any thymidine analogues
 3. Spreading buffer
 4. Trypsin
 5. 1 $\times$ PBS
 6. Tips for P-20 pipet
 7. 5 and 10 ml pipets
 8. Appropriate number of slides (at least three per condition)
 9. Apparatus that has the appropriate height (2 cm) to tilt the slides (a small carton/box is recommended)
- 3 h After passing the cells into the chamber replace the media with 5 ml of the working solution containing CldU (25 μ M) and incubate for 2 h
- Wash once with media that does not contain any thymidine analogues
- Add 5 ml of the working solution containing IdU (250 μ M) and incubate for 1 h
- Wash once with 1 $\times$ PBS and trypsinize cells
- Collect cells with 1 ml 1 $\times$ PBS by pipetting (do not scrape)
- Centrifuge to spin cells down (950 $\times g$ for 6 min)
- Dilute cells in half of the volume of 1 $\times$ PBS used for normoxic samples
- Proceed straight to the spreading step

Note: The aforementioned labeling time for each thymidine analogue was optimized for our conditions. However, these times may need adjusting depending on the experimental procedure and the question to be addressed. The labeling time of each analogue is crucial and is taken into account under the analysis of the data so it is important to adjust appropriately, especially if different O_2 tensions are being used and/or drug treatments. For example, in the protocol described here, we have used shorter pulse labeling with the thymidine analogues in cells under normoxic conditions (20 min each) compared to the cells that are under hypoxic conditions

(2 h for CldU and 1 h IdU). This adjustment accounts for the fact that replication rates are much faster in the presence of O₂ due to the occurrence of replication stress under severely hypoxic conditions.

2.3.2.4 Spreading

- Spread each sample separately onto different slides (for each sample, use at least three slides and do not proceed with more than one sample at once since there will be insufficient time for the next steps)
- Pipet 2 μ l of cell suspension at the top of each slide on each of the three slides (around 2000 cells) (Fig. 2.2)
- Wait 3–5 min for the drop of cell suspension to flatten (Fig. 2.3)
- Add 7 μ l of spreading buffer and mix gently by stirring with a pipet tip (P-20)
- *Note:* While spreading use the P-20 pipet for pipetting both 2 μ l of cells and 7 μ l of spreading buffer and only the proposed slides. We have found that slides lacking a frosted section do not perform as well as those suggested here. While waiting for the drop to flatten, be careful not to let it dry. If the drop has flattened earlier than 3–5 min proceed straight to the next step.
- Incubate the slide at room temperature for 2 min
- Tilt the slides gently to an approximate angle of 30°–40° (Fig. 2.4)

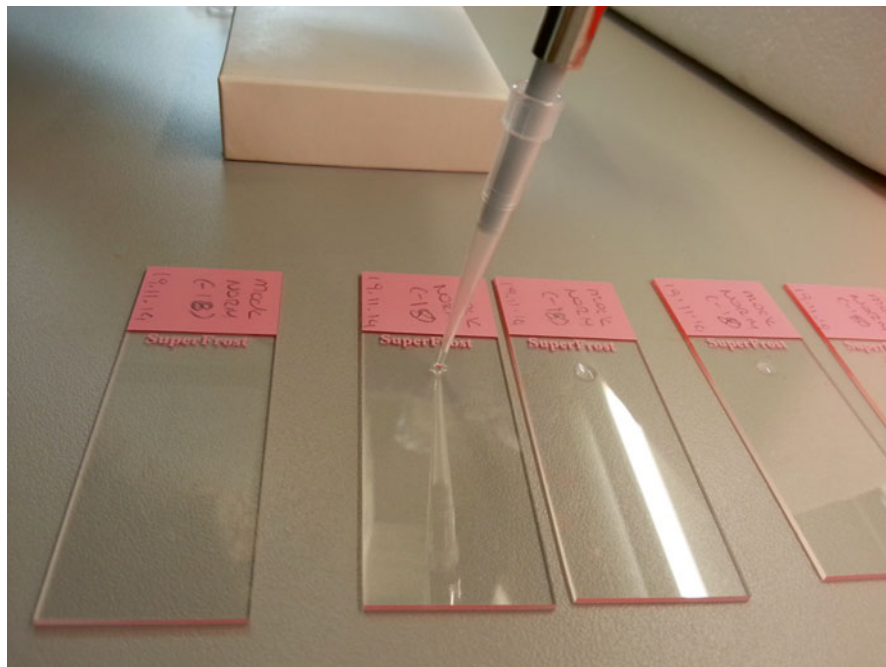


Fig. 2.2 Spreading procedure. Pipet 2 μ l of the cell suspension at the top of each slide with a P-20 pipet

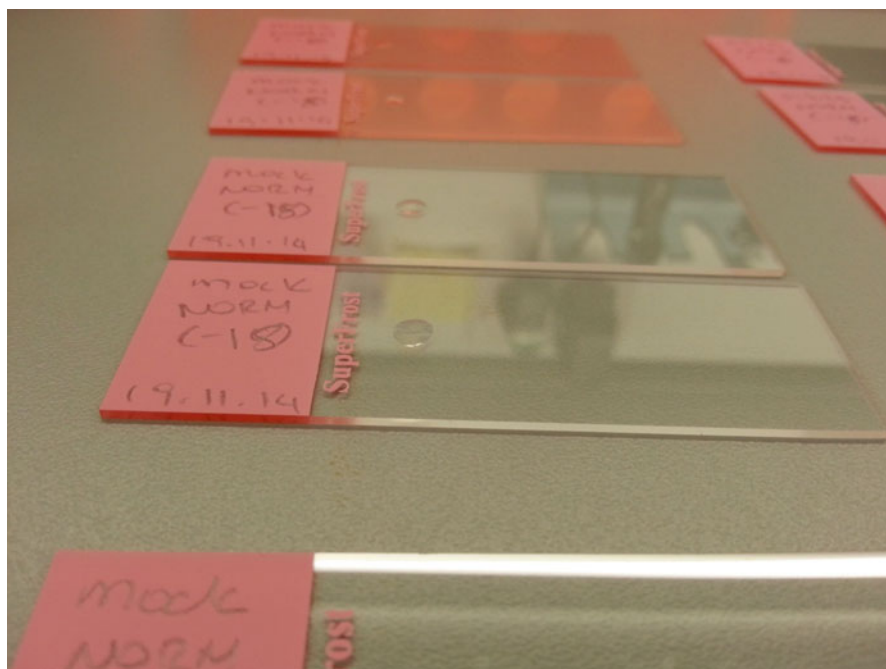


Fig. 2.3 Spreading procedure. Allow the drop of cell suspension for 3–5 min to flatten

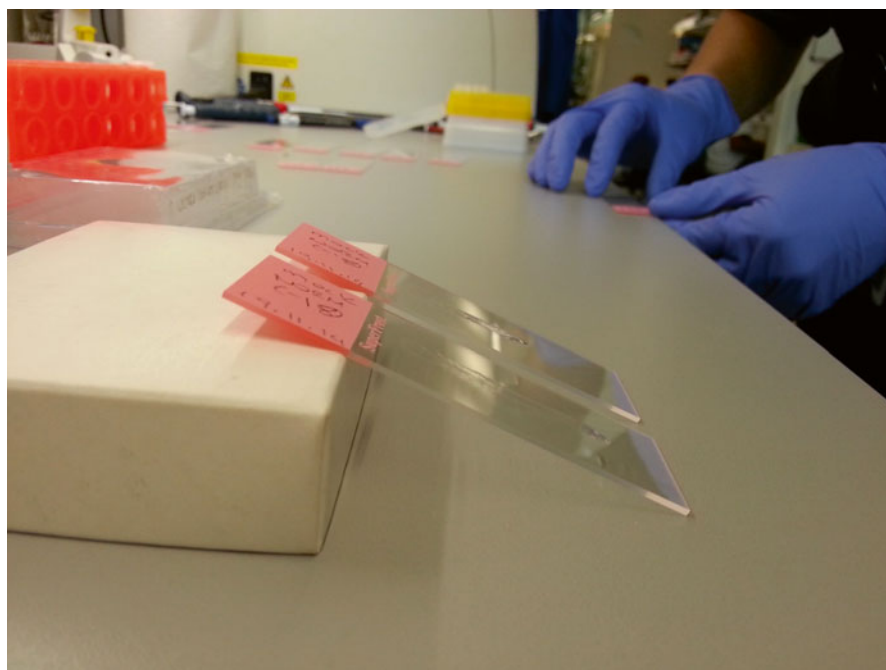


Fig. 2.4 Spreading procedure. Tilt the slides gently and let drops run down slowly to reach the bottom of the slide (approximately 20 s)

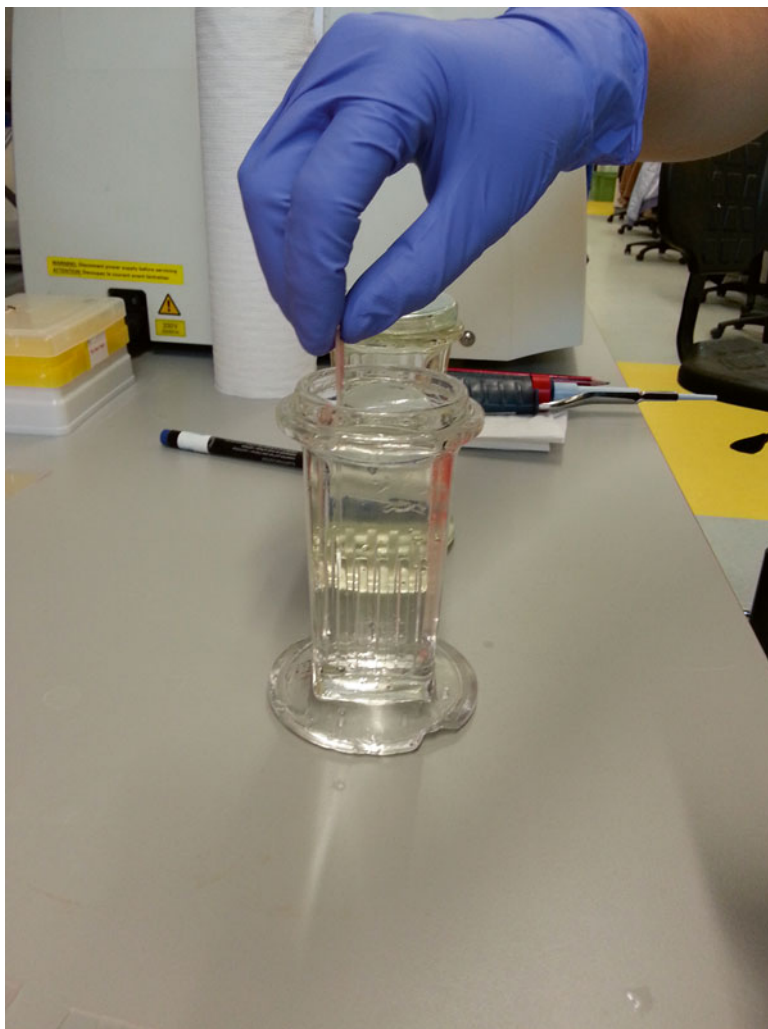


Fig. 2.5 Spreading procedure. Fix slides in fixation buffer for 10 min and air-dry

- Let the drops run down the slide slowly until they reach the bottom and air-dry
- While waiting for the drop to run down the slide proceed with the next sample
- Dip and fix in fixation buffer for 10 min (Fig. 2.5)
- Air-dry and store at 4 °C until staining

2.3.3 Day 3

2.3.3.1 Staining

- Wash the slides twice with deionized water for 5 min at RT
- Wash once with 2.5 M HCl for 5 min RT
- Discard the HCl
- Incubate with 2.5 M HCl for 80 min RT
- Discard the HCl and rinse twice with 1× PBS
- Wash twice with blocking buffer for 5 min each
- Discard the blocking buffer
- Incubate with blocking buffer for 1 h
- Add to each slide 150 µl of the primary antibodies diluted in blocking buffer (1:500 α-BrdU rat and 1:500 α-BrdU mouse) and cover with a coverslip
- Incubate at 37 °C for 1 h
- Wash three times with 1× PBS for 5 min each
- Incubate with 4 % PFA for 10 min (caution: do not incubate with PFA for more than 15 min)
- Wash three times with 1× PBS for 5 min each
- Incubate with blocking buffer for 1 min
- Discard the blocking buffer
- Incubate with blocking buffer for 5 min
- Discard the blocking buffer
- Incubate with blocking buffer for 25 min
- Discard the blocking buffer
- Add to each slide 150 µl of the secondary antibodies diluted in blocking buffer (1:500 α-rat 555 and 1:500 α-mouse 488) and cover with a coverslip
- Incubate at 37 °C for 1 h
- Rinse twice with 1× PBS
- Incubate with blocking buffer for 1 min
- Discard blocking buffer
- Incubate with blocking buffer for 5 min
- Discard blocking buffer
- Incubate with blocking buffer for 25 min
- Discard blocking buffer and rinse twice with 1× PBS
- Mount with mounting media (make sure the mounting media does not contain a nuclear dye such as DAPI, Hoechst, or Propidium Iodide) and cover with appropriate coverslip
- Store at 4 °C in the dark until imaging

2.3.4 Day 4

2.3.4.1 Imaging

Fibers are imaged using a LSM780 (Carl Zeiss Microscopy Ltd) confocal microscope and each image is converted in a TIF file for data analysis. While imaging try to focus on the green label, which is usually brighter when visualized with a $\times 60$ lens. When the fibers are focused go to a field with single and well separated fibers and image with both labels. Avoid fields where you have too many fibers since this will complicate the subsequent quantification.

Note: Try to image the samples as soon as possible as fluorescence intensity decreases with time.

2.4 Analysis of the Data

Import the acquired images to a picture analysis program (e.g., ImageJ). For the qualitative analysis, count the percentage of fibers that have incorporated only the first label (stalled forks), both labels (ongoing forks) and only the second label (new origins) (Fig. 2.6). For the quantitative analysis, measure the length of the fiber tracts only of the ongoing forks and use the following formula to determine the replication rates ($V_{(\text{IdU kb/min})} = [(x \times 0.132 \text{ } \mu\text{m}) \times 2.59 \text{ kb}/\mu\text{m}]/t \text{ (min)}$, where x = length of IdU in the program used). In general, the length of at least 100 fibers should be measured per condition and 100–200 replication structures should be counted. Each individual experiment should be repeated at least 3 times.

Note: For ImageJ software (NIH) the following settings are used

Qualitative analysis

1. Open TIF images with the ImageJ program
2. Go to Plugins from the toolbar and select Analyze
3. From Analyze select Cell Counter (Fig. 2.7)

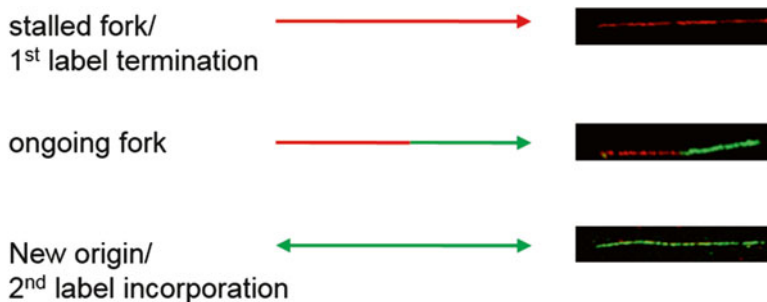


Fig. 2.6 Qualitative analysis of the data. Count the percentage of fibers that had incorporated only the first label (stalled forks), both labels (ongoing forks), or only the second label (new origins)

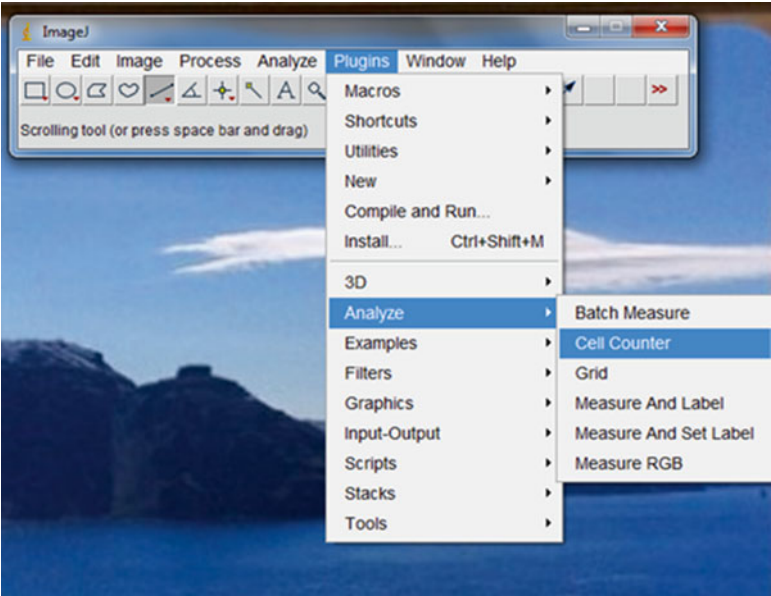
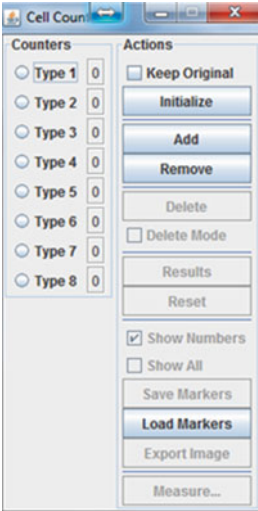


Fig. 2.7 Select the Cell Counter option from the ImageJ software (NIH) for the qualitative analysis of the data

Fig. 2.8 Select from the Cell Counter window a different type to mark stalled forks, ongoing forks, or new origins and press Initialize to start counting



4. From Cell Counter use a different type (left column at the Cell Counter window) for stalled forks, ongoing forks, or new origins and press Initialize to start counting (Fig. 2.8)

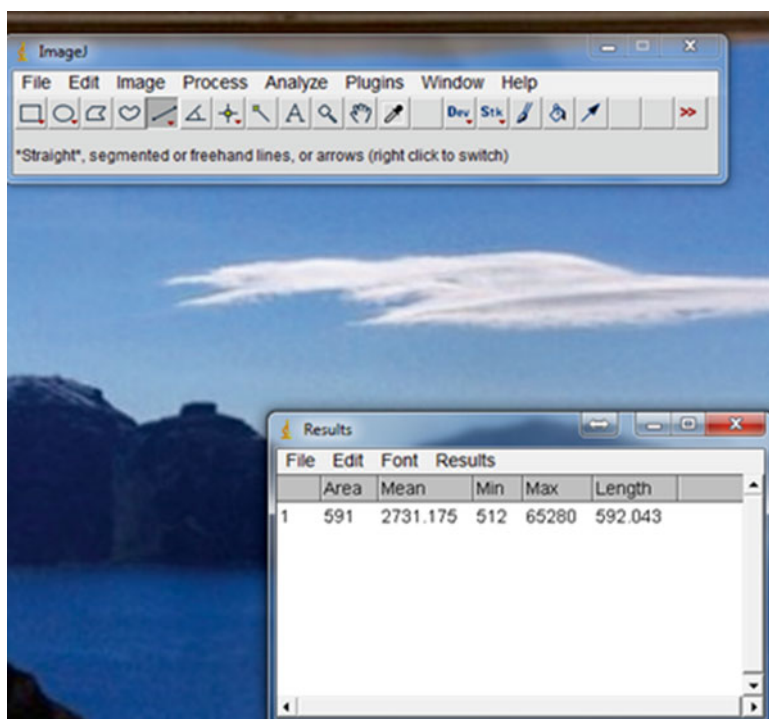


Fig. 2.9 For the quantitative analysis of the data, select the segmented line from the toolbar to measure the length of each fiber. When all measurements are completed use from the Results window the column named as Length (*right column*) to complete the formula that determines the replication rates (see data analysis)

Quantitative analysis

1. Open TIF images with the ImageJ program
2. Right click to select segmented line from toolbar
3. Go to your image and draw a line following each fiber
4. Go to Analyze from toolbar and select Measure
5. When all measurements are completed use from the Results table the length of each fiber (Fig. 2.9)

2.5 Troubleshooting

2.5.1 Unintentional Reoxygenation of Hypoxic Samples

Make sure that the media containing the thymidine analogues is well equilibrated before use in hypoxic chamber. Allow at least 2 h equilibration of the media in the pass box with the appropriate catalysts if required by the hypoxia chamber type.

2.5.2 *Less DNA Fibers in Hypoxic Conditions*

Usually it is difficult to count the amount of cells per sample in hypoxic conditions and this could result in less starting material in these conditions. Make sure that you spread two or three different concentrations per sample (usually 2× and/or 4× the amount you used for the normoxic samples).

2.6 Conclusion

The DNA fiber assay is a unique technique that allows most parameters that determine DNA synthesis during S-phase to be assessed individually, on the level of single replication forks as opposed to whole cell populations [30]. Previous genomic approaches that provide an overall replication profile of a cell population (such as FACS analysis with BrdU staining) are not sensitive enough for the investigation of DNA replication dynamics. In contrast, the DNA fiber technique presented here gives both quantitative and qualitative read-outs. Moreover, this technique enables the study of the changes to replication dynamics which may occur in response to different stresses and/or damaging agents. The investigation of the DNA replication process is indispensable not only for the further characterization of replication itself, but also to understand the alterations that occur in pathological conditions such as cancer. Hypoxic induction of DNA replication stress is characterized by an increased number of stalled replication forks and significantly reduced replication rates. The DNA fiber-labeling strategy is a great tool for the investigation of both the immediate and longer term changes to replication dynamics in these environments. A limitation of this technique though is the possibility of breakage of the DNA fibers while spreading them on a glass surface, which can lead to misinterpretation of the data. The need for controlling the orientation, density, and stretching of DNA fibers led to the development of the DNA combing technique, which has been successfully used to monitor DNA replication in a variety of organisms [26]. In this technique, DNA is stretched onto salinized slides with a constant stretching factor resulting in thousands of combed DNA molecules per slide, allowing detailed analysis of origin firing efficiency. The requirement for specific equipment (Combing System) for performing the aforementioned technique, however, limits its use in hypoxia due to technical problems (there is a possibility of reoxygenation of the samples if they have to be transferred outside of the chamber for the use of the equipment).

Acknowledgments We thank all members of the Hammond lab, past and present, for their assistance in the development of this protocol. DB and EMH are supported by Cancer Research UK (grant awarded to EMH). This work was supported by Cancer Research UK (CR-UK) grant number C38302/A12981, through a Cancer Research UK Oxford Centre Prize DPhil Studentship (awarded to IPF).

References

1. Branzei D, Foiani M. Maintaining genome stability at the replication fork. *Nat Rev Mol Cell Biol.* 2010;11:208–19.
2. Masai H, Matsumoto S, You Z, Yoshizawa-Sugata N, Oda M. Eukaryotic chromosome DNA replication: where, when, and how? *Annu Rev Biochem.* 2010;79:89–130.
3. Dershowitz A, Newlon CS. The effect on chromosome stability of deleting replication origins. *Mol Cell Biol.* 1993;13:391–8.
4. Ge XQ, Jackson DA, Blow JJ. Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress. *Genes Dev.* 2007;21:3331–41.
5. McIntosh D, Blow JJ. Dormant origins, the licensing checkpoint, and the response to replicative stresses. *Cold Spring Harb Perspect Biol.* 2012;4.
6. Schwob E. Flexibility and governance in eukaryotic DNA replication. *Curr Opin Microbiol.* 2004;7:680–90.
7. Woodward AM, Gohler T, Luciani MG, Oehlmann M, Ge X, Gartner A, Jackson DA, Blow JJ. Excess Mcm2-7 license dormant origins of replication that can be used under conditions of replicative stress. *J Cell Biol.* 2006;173:673–83.
8. Wyrick JJ, Aparicio JG, Chen T, Barnett JD, Jennings EG, Young RA, Bell SP, Aparicio OM. Genome-wide distribution of ORC and MCM proteins in *S. cerevisiae*: high-resolution mapping of replication origins. *Science.* 2001;294:2357–60.
9. Zeman MK, Cimprich KA. Causes and consequences of replication stress. *Nat Cell Biol.* 2014;16:2–9.
10. Halazonetis TD, Gorgoulis VG, Bartek J. An oncogene-induced DNA damage model for cancer development. *Science.* 2008;319:1352–5.
11. Brown JM, Giaccia AJ. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res.* 1998;58:1408–16.
12. Erler JT, Bennewith KL, Nicolau M, Dornhofer N, Kong C, Le QT, Chi JT, Jeffrey SS, Giaccia AJ. Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature.* 2006;440:1222–6.
13. Gray LH, Conger AD, Ebert M, Hornsey S, Scott OC. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br J Radiol.* 1953;26:638–48.
14. Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst.* 2001;93:266–76.
15. Hammond EM, Dorie MJ, Giaccia AJ. Inhibition of ATR leads to increased sensitivity to hypoxia/reoxygenation. *Cancer Res.* 2004;64:6556–62.
16. Olcina MM, Foskolou IP, Anbalagan S, Senra JM, Pires IM, Jiang Y, Ryan AJ, Hammond EM. Replication stress and chromatin context link ATM activation to a role in DNA replication. *Mol Cell.* 2013;52:758–66.
17. Pires IM, Bencokova Z, Milani M, Folkes LK, Li JL, Stratford MR, Harris AL, Hammond EM. Effects of acute versus chronic hypoxia on DNA damage responses and genomic instability. *Cancer Res.* 2010;70:925–35.
18. Welford SM, Giaccia AJ. Hypoxia and senescence: the impact of oxygenation on tumor suppression. *Mol Cancer Res.* 2011;9:538–44.
19. Bencokova Z, Kaufmann MR, Pires IM, Lecane PS, Giaccia AJ, Hammond EM. ATM activation and signaling under hypoxic conditions. *Mol Cell Biol.* 2009;29:526–37.
20. Hammond EM, Denko NC, Dorie MJ, Abraham RT, Giaccia AJ. Hypoxia links ATR and p53 through replication arrest. *Mol Cell Biol.* 2002;22:1834–43.
21. Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldborg P, Sehested M, Nesland JM, Lukas C, Orntoft T, Lukas J, Bartek J. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature.* 2005;434:864–70.
22. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio Jr RA, Kastrinakis NG, Levy B, Kleitas D, Yoneta A, Herlyn M, Kittas C, Halazonetis TD. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature.* 2005;434:907–13.

23. Hammond EM, Kaufmann MR, Giaccia AJ. Oxygen sensing and the DNA-damage response. *Curr Opin Cell Biol.* 2007;19:680–4.
24. Marechal A, Zou L. DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harb Perspect Biol.* 2013;5.
25. Nam EA, Cortez D. ATR signalling: more than meeting at the fork. *Biochem J.* 2011;436:527–36.
26. Bianco JN, Poli J, Saksouk J, Bacal J, Silva MJ, Yoshida K, Lin YL, Tourriere H, Lengronne A, Pasero P. Analysis of DNA replication profiles in budding yeast and mammalian cells using DNA combing. *Methods.* 2012;57:149–57.
27. Jackson DA, Pombo A. Replicon clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S phase in human cells. *J Cell Biol.* 1998;140:1285–95.
28. Petes TD, Williamson DH. Fiber autoradiography of replicating yeast DNA. *Exp Cell Res.* 1975;95:103–10.
29. Takeuchi F, Hanaoka F, Goto M, Akaoka I, Hori T, Yamada M, Miyamoto T. Altered frequency of initiation sites of DNA replication in Werner's syndrome cells. *Hum Genet.* 1982;60:365–8.
30. Merrick CJ, Jackson D, Diffley JF. Visualization of altered replication dynamics after DNA damage in human cells. *J Biol Chem.* 2004;279:20067–75.

Hypoxia-induced p53 modulates both apoptosis and radiosensitivity via AKT

Katarzyna B. Leszczynska,¹ Iosifina P. Foskolou,¹ Aswin G. Abraham,¹ Selvakumar Anbalagan,¹ Céline Tellier,¹ Syed Haider,¹ Paul N. Span,² Eric E. O'Neill,¹ Francesca M. Buffa,¹ and Ester M. Hammond¹

¹Cancer Research UK and Medical Research Council Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford, Oxford, United Kingdom. ²Radboud University Medical Centre, Department of Radiation Oncology 874, Nijmegen, Netherlands.

Restoration of hypoxia-induced apoptosis in tumors harboring p53 mutations has been proposed as a potential therapeutic strategy; however, the transcriptional targets that mediate hypoxia-induced p53-dependent apoptosis remain elusive. Here, we demonstrated that hypoxia-induced p53-dependent apoptosis is reliant on the DNA-binding and transactivation domains of p53 but not on the acetylation sites K120 and K164, which, in contrast, are essential for DNA damage-induced, p53-dependent apoptosis. Evaluation of hypoxia-induced transcripts in multiple cell lines identified a group of genes that are hypoxia-inducible proapoptotic targets of p53, including inositol polyphosphate-5-phosphatase (*INPP5D*), pleckstrin domain-containing A3 (*PHLDA3*), sulfatase 2 (*SULF2*), B cell translocation gene 2 (*BTG2*), cytoplasmic FMR1-interacting protein 2 (*CYFIP2*), and KN motif and ankyrin repeat domains 3 (*KANK3*). These targets were also regulated by p53 in human cancers, including breast, brain, colorectal, kidney, bladder, and melanoma cancers. Downregulation of these hypoxia-inducible targets associated with poor prognosis, suggesting that hypoxia-induced apoptosis contributes to p53-mediated tumor suppression and treatment response. Induction of p53 targets, *PHLDA3*, and a specific *INPP5D* transcript mediated apoptosis in response to hypoxia through AKT inhibition. Moreover, pharmacological inhibition of AKT led to apoptosis in the hypoxic regions of p53-deficient tumors and consequently increased radiosensitivity. Together, these results identify mediators of hypoxia-induced p53-dependent apoptosis and suggest AKT inhibition may improve radiotherapy response in p53-deficient tumors.

Introduction

Regions of low/insufficient oxygen (hypoxia) are a pathophysiological feature of most solid tumors (1). It has been demonstrated conclusively that patients with significant levels of tumor hypoxia have a worse prognosis irrespective of treatment modality (2–4). Within a tumor, gradients of oxygen concentrations exist, which are likely to reach complete anoxia in avascular areas (5, 6). Cells exposed to severe hypoxia (<0.1% O₂) are particularly radioreistant and induce a replication stress-mediated DNA damage response (DDR), which includes stabilization and activation of the p53 tumor suppressor (7). Replication stress refers to the slowing or stalling of replication forks (8). Importantly, hypoxia induces a DDR in the absence of detectable DNA damage (9). p53 is rapidly stabilized and activated in response to a wide variety of cellular stresses, including DNA damage, oncogene expression, nutrient deprivation, ribosomal dysfunction, and hypoxia (<0.1% O₂) (10). Tumor hypoxia is potentially one of the earliest driving forces to mutate p53 function during tumorigenesis (11). The majority of human cancers (over 50%) have lost WT p53 function due to mutations in the DNA-binding domain (DBD) of the protein (10, 12). We recently demonstrated that even mild levels of hypoxia (2% O₂) combined with oncogene-induced replication stress can

lead to DDR signaling and that this can occur before the accumulation of DNA damage (7).

Under conditions of hypoxia-induced replication stress, p53 is phosphorylated and stabilized by the ATR and, to a lesser extent, ATM kinases (9, 13). In addition, p53 stabilization is facilitated by decreased expression of MDM2 and/or by phosphorylation of MDMX E3 ubiquitin ligases (14, 15). Once activated by hypoxia, p53 induces apoptosis through the intrinsic pathway (16, 17) and requires the transcriptional activity of p53 (18, 19). However, the exact p53 target genes responsible for apoptosis in hypoxia remain poorly characterized. Isolated reports exist, which describe the involvement of known proapoptotic targets, such as *PUMA* or *BAD*, in hypoxia-induced and p53-dependent apoptosis, although these tend to be restricted to specific cell lines/types, for example, cardiomyocytes (20). In contrast, a number of reports demonstrate that genes such as *PUMA*, *BAX*, or *DR5* are not induced by p53 in various cancer cell lines during hypoxia-induced apoptosis (18, 21, 22). Previous studies have suggested that p53 acts predominantly as a transrepressor rather than a transactivator during hypoxia (18–21). However, few targets of p53-mediated transrepression have been reported (18–21). Interestingly, recent studies investigating the tumor-suppressive function of p53 demonstrated that the most characterized p53 targets crucial for DNA damage-induced cell cycle arrest, senescence, or apoptosis (*p21*, *PUMA*, *NOXA*) appear to be dispensable for suppressing tumorigenesis (23–25). Instead, p53 targets involved in regulation of energy metabolism, ROS production, and autophagy

► Related Commentary: p. 2264

Conflict of interest: The authors have declared that no conflict of interest exists.

Submitted: December 8, 2014; **Accepted:** March 19, 2015.

Reference information: *J Clin Invest*. 2015;125(6):2385–2398. doi:10.1172/JCI80402.

(e.g., *TIGAR*, *GLS2*, or *ULK1*) appear to be crucial in tumor suppression (24–26). Recently, using genetically engineered mouse knockin models in the transactivation domains of p53, Brady et al. demonstrated that the genes critical for the acute DDR of p53 and for tumor suppression differ. Brady et al. identified 14, mostly novel, p53 targets required for p53-mediated tumor suppression (23). The induction of these genes was found to be dependent on both of the transactivation domains of p53 and an intact DBD. More recently, additional mouse models showed that acetylation of p53 at specific lysine residues K117/K161/K162 (corresponding to K120/K164 in humans) in the DBD are essential for DNA damage-induced apoptosis but are dispensable for p53-dependent tumor suppression (24). These studies have led to the conclusion that p53-induced apoptosis is not required for tumor suppression.

In this study, we investigated the p53-mediated transcriptional response to hypoxia and specifically the mechanism of p53-induced apoptosis in these conditions. Hypoxia-induced p53-dependent apoptosis was reliant on DNA binding (R175) but not acetylation in the DBD (K120, K164). We show that a group of 6 validated hypoxia-inducible p53 target genes was found to predict p53 status in samples from patients with cancer; in addition, we show that when these genes were underexpressed they correlated with poor clinical outcome in two independent analyses of large human breast cancer cohorts, with a total of more than 3,000 patients, and other human cancers. Two of the p53 targets induced in hypoxia, pleckstrin domain-containing A3 (*PHLDA3*) and inositol polyphosphate-5-phosphatase (*INPP5D*, which encodes SHIP-1), were found to induce apoptosis through inhibition of AKT signaling. We show that by mimicking the function of WT p53 in hypoxia using a pharmacological inhibitor of AKT we can increase apoptosis specifically in hypoxic areas of tumors with nonfunctional p53 but not in tumors with WT p53. Furthermore, increased apoptosis in the hypoxic fraction induced through AKT inhibition correlated with significant radiosensitization in an esophageal tumor model.

Results

Hypoxia-induced p53 transactivates specific target genes. Using hypoxia-inducible p53 constructs in p53-null H1299 cells, we investigated the role of DNA binding and acetylation of p53 in hypoxia-induced apoptosis (18). Mutation of the acetylation sites in the p53 DBD (K120R, K164R, K120R/K164R) had no significant effect on the level of hypoxia-induced apoptosis, in contrast to a structural mutation of the DBD (R175H), which prevented DNA binding and abrogated apoptosis entirely (Figure 1, A and B). However, in agreement with previous reports, acetylation at K120 and/or K164 was critical for p53-dependent apoptosis in response to camptothecin-induced DNA damage (Supplemental Figure 1, A and B) (27). These data immediately suggested that the mechanism of hypoxia-induced p53-dependent apoptosis differs from the response to DNA-damaging agents (24, 27). Using the same inducible system, we carried out an expression array to compare the transcriptional response to WT p53 and p53^{R175H} in hypoxia. As expected, the well-characterized p53 proapoptotic targets, including *BAX*, *PUMA*, *NOXA*, or *BID*, were not upregulated by p53 in response to

hypoxia (18). Instead, a number of other p53 targets were identified, of which the most significant are shown in Figure 1C (<http://www.ncbi.nlm.nih.gov/geo/>; accession number GSE53893). Notably and in contrast to our previous study in *myc/ras*-transformed mouse embryonic fibroblasts, we did not observe significant levels of p53-mediated gene repression (18). *PHLDA3*, one of the most significantly induced genes in hypoxia, was previously identified as 1 of 14 genes critical for p53-mediated tumor suppression (23). This led us to ask whether any of the other p53 targets identified by Brady et al. were also hypoxia regulated. *PHLDA3* stood out as the most hypoxia inducible from this group of 14 genes; however, KN motif and ankyrin repeat domains 3 (*KANK3*), *ABHD4*, and *DEF6* were also potential p53 targets in hypoxia, with some cell line dependence (Figure 1D and Supplemental Figure 1, C–E). *GLUT1* served as hypoxia-inducible control and *BAX* as a typical DNA damage-inducible p53 target. Together, these data support our hypothesis that p53 induces a stress-specific transcriptional response in hypoxia. Most importantly, these data suggest that p53-dependent apoptosis in response to hypoxia could still play a role in tumor suppression.

We focused on genes that have been linked with apoptosis (*INPP5D*, *PHLDA3*, sulfatase 2 [*SULF2*], Bcell translocation gene 2 [*BTG2*], cytoplasmic FMR1-interacting protein 2 [*CYFIP2*]) or tumor suppression (*KANK3*) (23, 28–31). Using quantitative PCR (qPCR), we validated these genes as being hypoxia inducible in a p53-dependent manner in the HCT116 isogenic cell lines (*p53*^{+/+} and *p53*^{-/-}) (Figure 2A). We also demonstrated that, in addition to the DBD, the transactivation domains of p53 (*p53*²², *p53*²³, *p53*⁵³, *p53*⁵⁴) were required to induce these targets in hypoxia (Supplemental Figure 2, A and B). p53 response elements (REs) have been identified in *PHLDA3*, *SULF2*, *CYFIP2*, and *KANK3*, so we used ChIP to determine whether p53 was enriched at these sites in hypoxia (Figure 2B and refs. 32–34). In each case, there was a clear accumulation of p53 at the REs in hypoxia, demonstrating direct transactivation of these targets in response to hypoxia as well as the DNA-damaging stresses previously reported. In addition, we confirmed that *PHLDA3* and *SULF2* proteins were induced in hypoxia in a p53-dependent manner, again using *p53*^{+/+} and *p53*^{-/-} HCT116 cells or RKO cells treated with siRNA to p53 (Supplemental Figure 2, C–E). We also observed a hypoxic induction of both *PHLDA3* mRNA and *PHLDA3* protein in a human nontumor lung fibroblast cell line, WI38, suggesting that this regulation is not restricted to tumor cells (Supplemental Figure 2, F and G).

Previous reports suggest an interaction between p53 and HIF-1 α (35). We have determined that *PHLDA3* and *INPP5D* were induced in response to <0.1% O₂ when both p53 and HIF-1 were induced but not in response to 2% O₂ when only HIF-1 was stabilized (Supplemental Figure 3, A–C). This was further verified in *HIF1A*^{+/+} and *HIF1A*^{-/-} RKO cells, in which *PHLDA3* and *INPP5D* were highly induced by hypoxia irrespective of HIF-1 α status, as opposed to the HIF-1 α target, *GLUT1*, which was not induced in the *HIF1A*^{-/-} cell line (Supplemental Figure 3, D–G). This demonstrates that HIF-1 α does not contribute to the induction of the hypoxia-induced p53-dependent genes described.

Hypoxia-induced p53 specifically transactivates the *INPP5D* transcript associated with stem cells. *INPP5D* has been recently

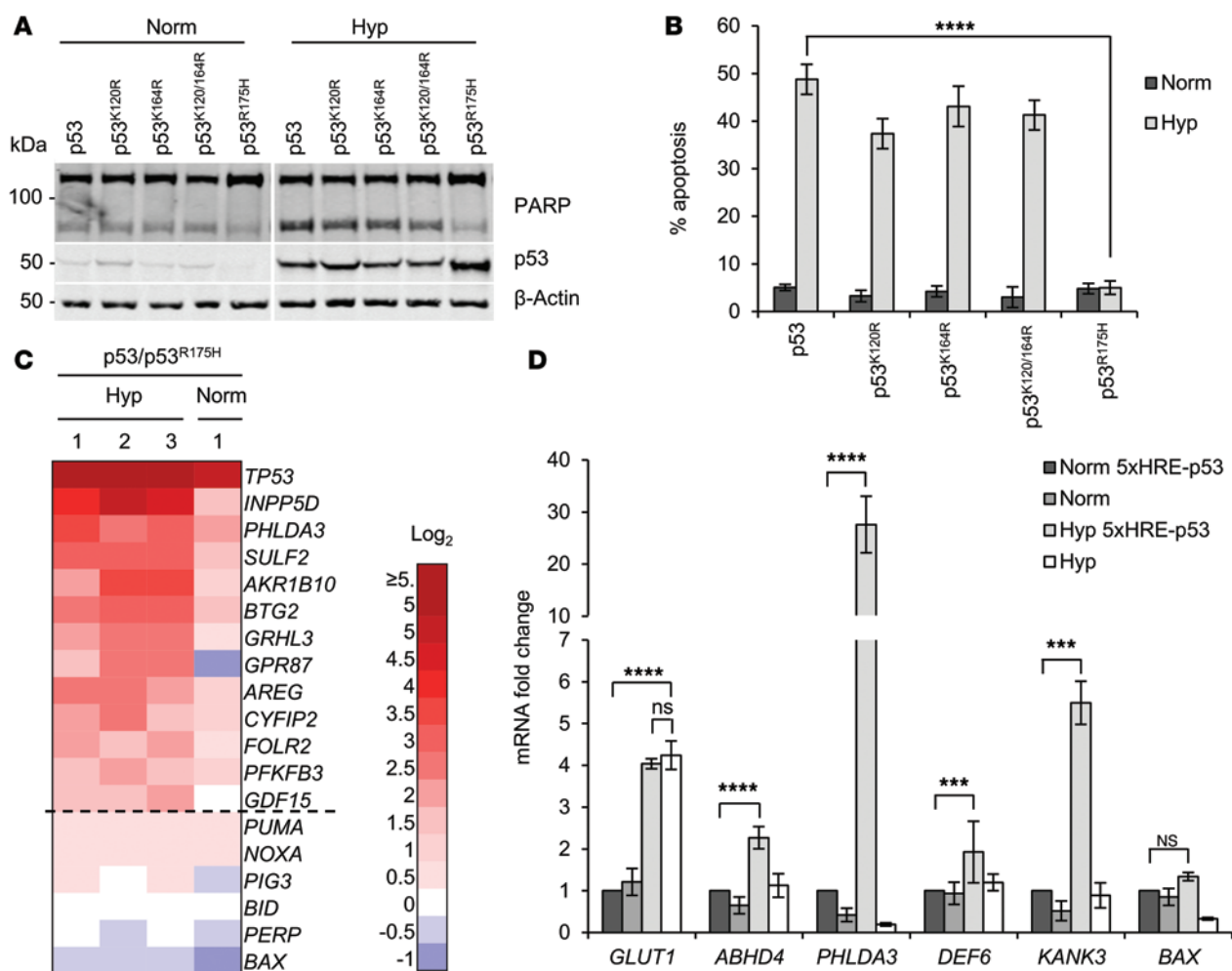


Figure 1. Hypoxia-induced p53-dependent apoptosis is associated with the expression of specific genes. (A and B) Apoptosis in H1299 cells expressing either WT p53 (p53) or the mutants (K120R, K164R, K120/164R, R175H) from the 5xHRE promoter and exposed to 24 hours of hypoxia (Hyp) or normoxia (Norm) detected by PARP cleavage or morphologically, respectively. For Western blotting, the lanes were run on the same gel but were noncontiguous. The bar graph shows mean $\pm$ SEM (combined $n = 3$, 2-way ANOVA test [**** $P < 0.0001$]). (C) Microarray gene expression analysis in H1299 cells transfected with either 5xHRE-p53 or 5xHRE-p53^{R175H} and exposed to 16 hours of hypoxia. Heat map showing the top candidate genes induced by hypoxia in a p53-dependent manner. Well-characterized proapoptotic p53 targets are shown in the bottom 6 rows. Fold change ratios of gene expression for 5xHRE-p53/5xHRE-p53^{R175H} samples are shown in red for upregulated and blue for downregulated genes. Columns numbered 1 to 3 represent technical replicates of hypoxic samples. (D) qPCR testing of the hypoxic regulation of p53 target genes identified as being essential to its tumor-suppressive role by Brady et al. (22) in H1299 cells transfected with 5xHRE-p53 and exposed to 24 hours of hypoxia. The bar graph shows mean $\pm$ SEM (combined $n = 3$, 2-way ANOVA test [$P < 0.0001$] followed by 2-tailed Student's t test [*** $P < 0.001$; **** $P < 0.0001$]).

described as a p53 target gene with 2 p53 REs in intron 9 (36). We carried out ChIP analysis using both these REs and found that one of them was moderately enriched for p53 binding in hypoxia (Supplemental Figure 4A). To investigate further and as multiple *INPP5D* transcript variants exist, we asked which transcript was induced in hypoxia (37). By carrying out PCR with specific primers, we determined that RKO and HCT116 cells expressed only the short *INPP5D* transcript (U50040), which was further induced by hypoxia (Figure 2C and Supplemental Figure 4B). This particular transcript is transcribed from an internal promoter in intron 5 of the full-length *INPP5D* gene (37). We aligned this region with the corresponding mouse and rat loci of the *INPP5D* gene and found a region of conserved similarity in the sequence directly preceding the cDNA of the short *INPP5D*. Within this region, we identified a novel putative p53 RE, which

was conserved between species (chr2:233,139,693–233,139,712 of hg38 human genome assembly) (Figure 2D). Using ChIP, we verified that this sequence is a bona fide p53 RE and that occupancy of this region by p53 was enhanced in hypoxia to a similar level as that seen for *PHLDA3*, *SULF2*, *CYFIP2*, or *KANK3* (Figure 2E). The closely related member of the *INPP5D* gene family, *INPL1*, did not increase in hypoxia (Supplemental Figure 4C). The expression of short *INPP5D* has been previously characterized mainly in mouse embryonic stem cells, although the mechanism of induction is unclear (37). We found that expression of this particular transcript was increased in response to hypoxia, not only in cancer cells but also in embryonic stem cells (Supplemental Figure 4D). This correlated with an accumulation of p53 protein and induction of apoptosis in the embryonic stem cells in hypoxia (Supplemental Figure 4E). These data together demon-

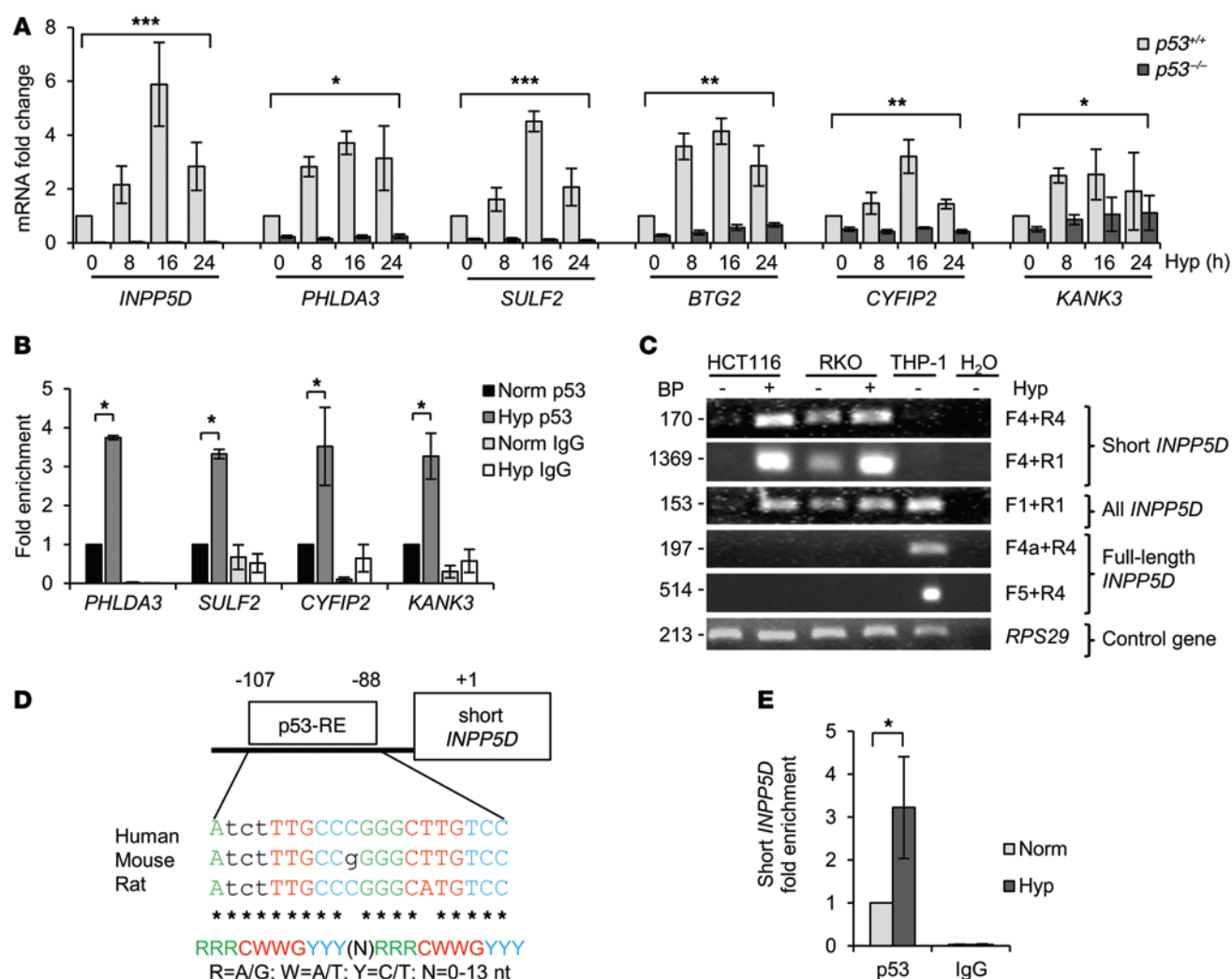


Figure 2. Transcriptional activity of p53 is required to induce expression of a specific group of genes in hypoxia. (A) Validation of p53 targets by qPCR in *p53*^{+/+} and *p53*^{-/-} HCT116 cells exposed to hypoxia. The bar graph shows mean $\pm$ SEM (combined $n = 3$, 2-way ANOVA test [$*P < 0.05$; $**P < 0.01$; $***P < 0.001$]). (B) qPCR for p53 ChIP in HCT116 cells exposed to 8 hours of normoxia or hypoxia for the genes indicated. The bar graph shows mean $\pm$ SEM (combined $n = 3$, 2-way ANOVA test [$P < 0.0001$] followed by 2-tailed Student's t test [$*P < 0.05$]). (C) PCR for the *INPP5D* transcripts using a combination of primers (marked as arrows in Supplemental Figure 2A) specifically detecting the short *INPP5D* transcript (F4+R4 or F4+R1), all *INPP5D* transcripts (F1+R1), or the full-length *INPP5D* transcript (F4a+R4 or F5+R4) on the cDNA from HCT116 or RKO cells exposed to 16 hours of hypoxia. THP-1 cDNA was used as a control for the expression of the full-length *INPP5D*. *RPS29* is shown as a control gene. (D) Alignment of the putative p53 RE of the short *INPP5D* in human, mouse, and rat. A consensus p53 RE is shown below the alignment. (E) qPCR for p53 ChIP at the putative p53 RE in the short *INPP5D* promoter in HCT116 cells exposed to 8 hours of normoxia or hypoxia. The bar graph shows mean $\pm$ SEM (combined $n = 3$, 2-tailed Student's t test [$*P < 0.05$]).

strate that in response to hypoxia p53 induces expression of a specific short *INPP5D* transcript, the expression of which was previously associated predominantly with embryonic stem cells.

The collective expression of hypoxic p53 targets is regulated in cancers and correlates with patient prognosis. To investigate the relevance of the p53 target genes induced in response to hypoxia in cancer patients, we analyzed all available Oncomine data sets using our validated p53 hypoxic targets: *PHLDA3*, *INPP5D*, *SULF2*, *BTG2*, *CYFIP2*, and *KANK3*. The underexpression of this group of genes correlated significantly with *TP53* mutation in 9 studies (Supplemental Figure 5A); this represents a statistically significant enrichment with respect to association with any other mutation (hypergeometric test, $P = 0.0279$). To further demon-

strate the significance of this result, we defined an equally sized control group of known p53 target genes that are not induced by hypoxia (Figure 1C) but have well-characterized roles in DNA damage-induced apoptosis: *PUMA*, *BID*, *PERP*, *BAX*, *PIG3*, and *NOXA* (10). Although the analysis of this control group did identify 3 data sets where the underexpression of these genes was associated with *TP53* mutation (Supplemental Figure 5B), this was not a statistically significant enrichment (hypergeometric test, $P = 0.599$). This led us to conclude that the p53-dependent genes identified here in vitro as hypoxia inducible are likely to be regulated by p53 in vivo. Moreover, the hypoxia-inducible group of genes (*PHLDA3*, *INPP5D*, *SULF2*, *BTG2*, *CYFIP2*, and *KANK3*) was a better predictor of p53 status than a control group of

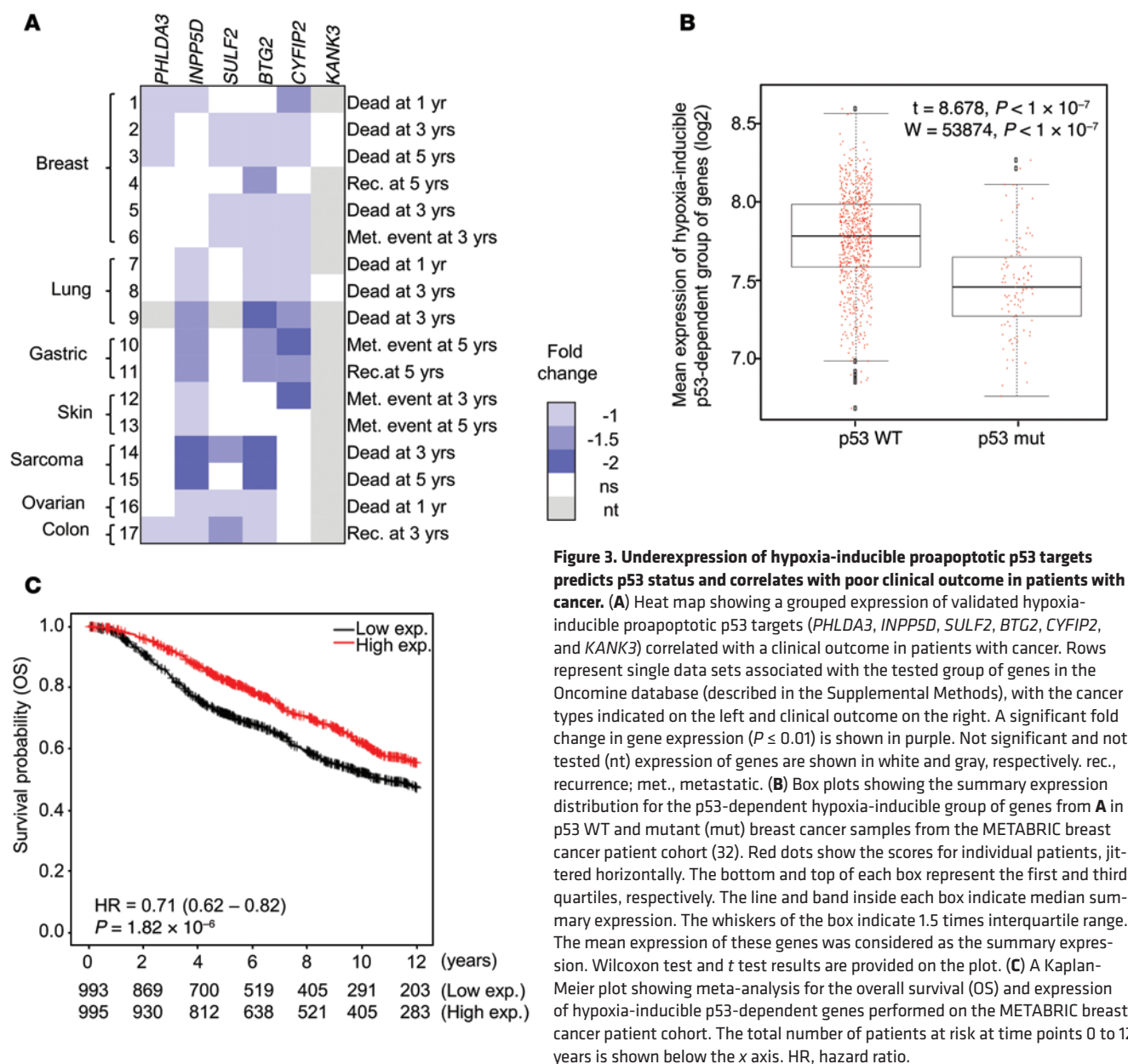


Figure 3. Underexpression of hypoxia-inducible proapoptotic p53 targets predicts p53 status and correlates with poor clinical outcome in patients with cancer. (A) Heat map showing a grouped expression of validated hypoxia-inducible proapoptotic p53 targets (*PHLDA3*, *INPP5D*, *SULF2*, *BTG2*, *CYFIP2*, and *KANK3*) correlated with a clinical outcome in patients with cancer. Rows represent single data sets associated with the tested group of genes in the Oncomine database (described in the Supplemental Methods), with the cancer types indicated on the left and clinical outcome on the right. A significant fold change in gene expression ($P \leq 0.01$) is shown in purple. Not significant and not tested (nt) expression of genes are shown in white and gray, respectively. rec., recurrence; met., metastatic. (B) Box plots showing the summary expression distribution for the p53-dependent hypoxia-inducible group of genes from A in p53 WT and mutant (mut) breast cancer samples from the METABRIC breast cancer patient cohort (32). Red dots show the scores for individual patients, jittered horizontally. The bottom and top of each box represent the first and third quartiles, respectively. The line and band inside each box indicate median summary expression. The whiskers of the box indicate 1.5 times interquartile range. The mean expression of these genes was considered as the summary expression. Wilcoxon test and *t* test results are provided on the plot. (C) A Kaplan-Meier plot showing meta-analysis for the overall survival (OS) and expression of hypoxia-inducible p53-dependent genes performed on the METABRIC breast cancer patient cohort. The total number of patients at risk at time points 0 to 12 years is shown below the x axis. HR, hazard ratio.

well-known p53 proapoptotic targets (*PUMA*, *BID*, *PERP*, *BAX*, *PIG3*, and *NOXA*) induced in response to DNA damage.

We then tested whether collective expression of the group of genes induced by p53 under hypoxia (*PHLDA3*, *INPP5D*, *SULF2*, *BTG2*, *CYFIP2*, and *KANK3*) was associated with any consistent clinical outcome in patients with cancer. An Oncomine search of all the available data for patients with cancer identified 17 data sets in which underexpression of this group of genes was significantly associated with a poor clinical outcome in patients with breast, lung, gastric, melanoma, sarcoma, ovarian, and colorectal cancers (Figure 3A). Conversely, no studies were found in which overexpression of this group of genes correlated with poor clinical outcome. The expression of these genes tested individually in Oncomine did not show any consistent pattern of their expression correlating with patient prognosis, suggesting that their concomitant regulation is the relevant factor for

clinical outcome. When we again compared the control group of well-characterized p53 proapoptotic genes (*PUMA*, *BID*, *PERP*, *BAX*, *PIG3*, and *NOXA*), we found that only 4 data sets were significantly associated with their collective expression; notably, in all 4 cases, it was the overexpression, rather than underexpression, of these genes that correlated with poor clinical outcome (Supplemental Figure 5C).

Next, we carried out independent meta-analyses on publicly available, large, and well-characterized breast cancer patient cohorts. The first analysis was performed on 6 curated retrospective breast cancer data sets deposited in the Gene Expression Omnibus repository, in which clinical information was published for a total of 1,050 cases (GSE6532KI, GSE6532GUY, GSE6532OXF, GSE9195, GSE1456, and GSE2034). Tests for heterogeneity showed that the 6 data sets could be merged safely ($P = 0.785$). The p53 status of these samples is

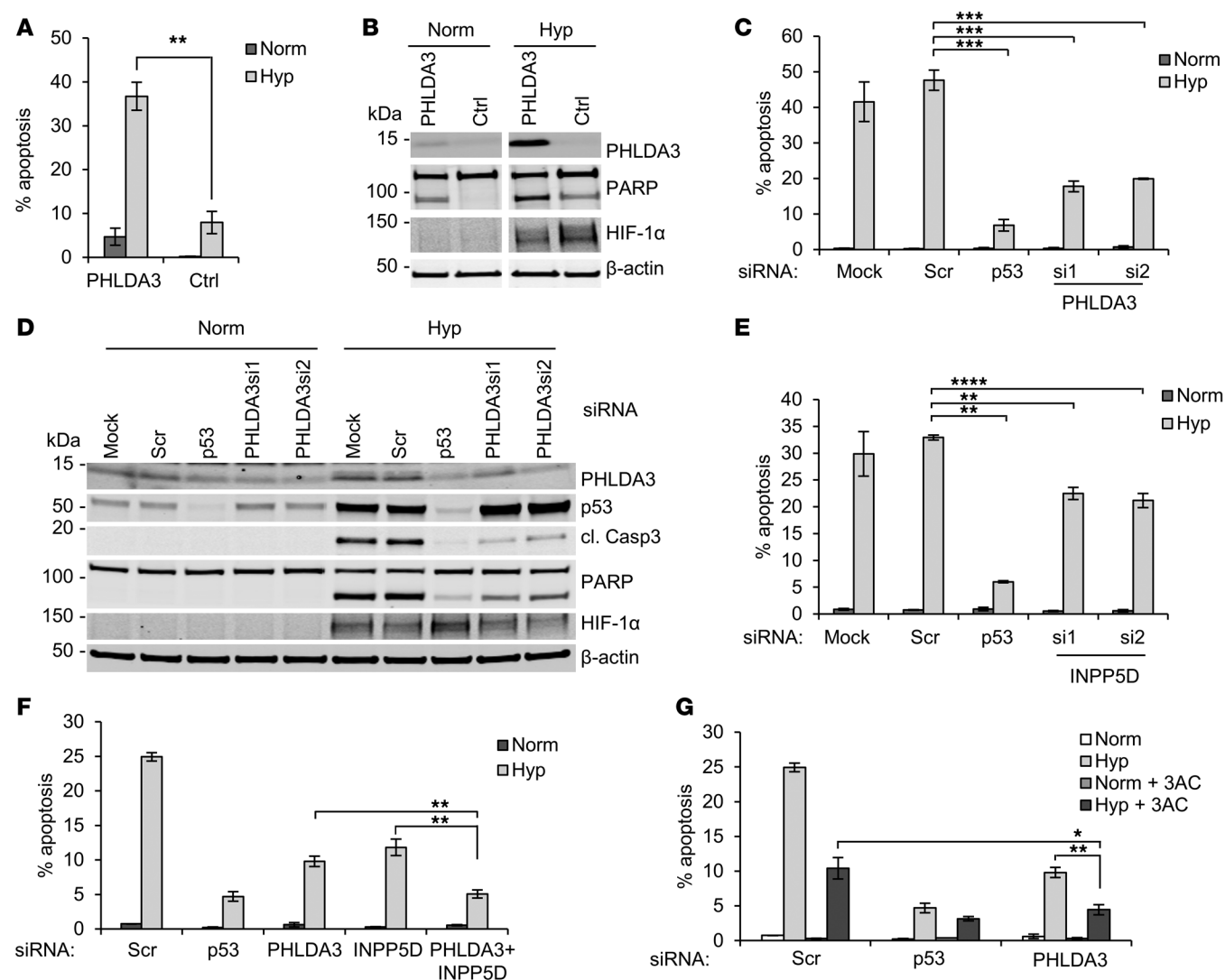


Figure 4. p53 targets *PHLDA3* and *INPP5D* mediate apoptosis in hypoxia. (A and B) Apoptosis detected morphologically or by Western blotting for PARP cleavage, respectively, in H1299 cells transfected with 5xHRE-PHLDA3 and exposed to 24 hours of hypoxia. The bar graph shows mean $\pm$ SEM ($n = 3$; 2-tailed Student's t test [$^{**}P < 0.01$]). For Western blotting, the lanes were run on the same gel but were noncontiguous. (C and D) Apoptosis detected morphologically or by Western blotting for cleaved PARP or caspase-3 (cl. Casp3) in RKO cells treated with nonspecific (Scr), PHLDA3, or p53 siRNA and exposed to 18 hours of hypoxia or normoxia. The bar graph shows mean $\pm$ SEM ($n = 3$; 2-way ANOVA test [$P < 0.0001$] followed by 2-tailed Student's t test [$^{***}P < 0.01$]). (E and F) Apoptosis detected in RKO cells treated with nonspecific, PHLDA3, INPP5D, or p53 siRNA as indicated and exposed to 14 hours of hypoxia or normoxia. The bar graphs show mean $\pm$ SEM ($n = 3$; 2-way ANOVA tests [$P < 0.0001$ for both graphs] followed by 2-tailed Student's t test [$^{**}P < 0.01$; $^{****}P < 0.0001$]). (G) Apoptosis detected morphologically in RKO cells treated with nonspecific, p53, or PHLDA3 siRNA and exposed to 14 hours of hypoxia or normoxia in the presence or absence of INPP5D/SHIP-1 inhibitor 3AC. The bar graph shows mean $\pm$ SEM ($n = 3$; 2-way ANOVA test [$P < 0.0001$] followed by 2-tailed Student's t test [$^{*}P < 0.05$; $^{**}P < 0.01$]).

unknown, and so we used a recently described p53 gene signature to determine the likely p53 status and then asked whether our group of genes was expressed in a p53-dependent manner (38). Using this approach, we found that our p53-dependent hypoxia-inducible group of genes was expressed at a significantly lower level in p53-mutated samples ($P < 1 \times 10^{-7}$; Supplemental Figure 6A). This provided independent validation for the conclusion reached through earlier Oncomine analysis, showing that the group of hypoxic p53 targets is significantly and consistently associated with p53 mutation status in vivo. Next, we found that underexpression of hypoxic p53 targets correlated with a lower recurrence-free survival, with a summary effect hazard ratio

of 0.63 (95% confidence limits of 0.42 and 0.96) in the breast cancer data sets (Supplemental Figure 6B). We then carried out a similar meta-analysis on the data from the recently published METABRIC study, which consists of a large cohort of patients with breast cancer, for a total of 2,000 human cancer samples (39). The p53 status in a subset of these samples was known, and we confirmed once more in an independent data set that lower expression of our hypoxia-inducible p53-dependent genes significantly correlated with p53 mutation status (Figure 3B). As expected, breast cancer subtypes associated with high rates of p53 mutations, such as basal-like cancers (40), showed the lowest expression of the group of genes (Supplemental Figure 7).

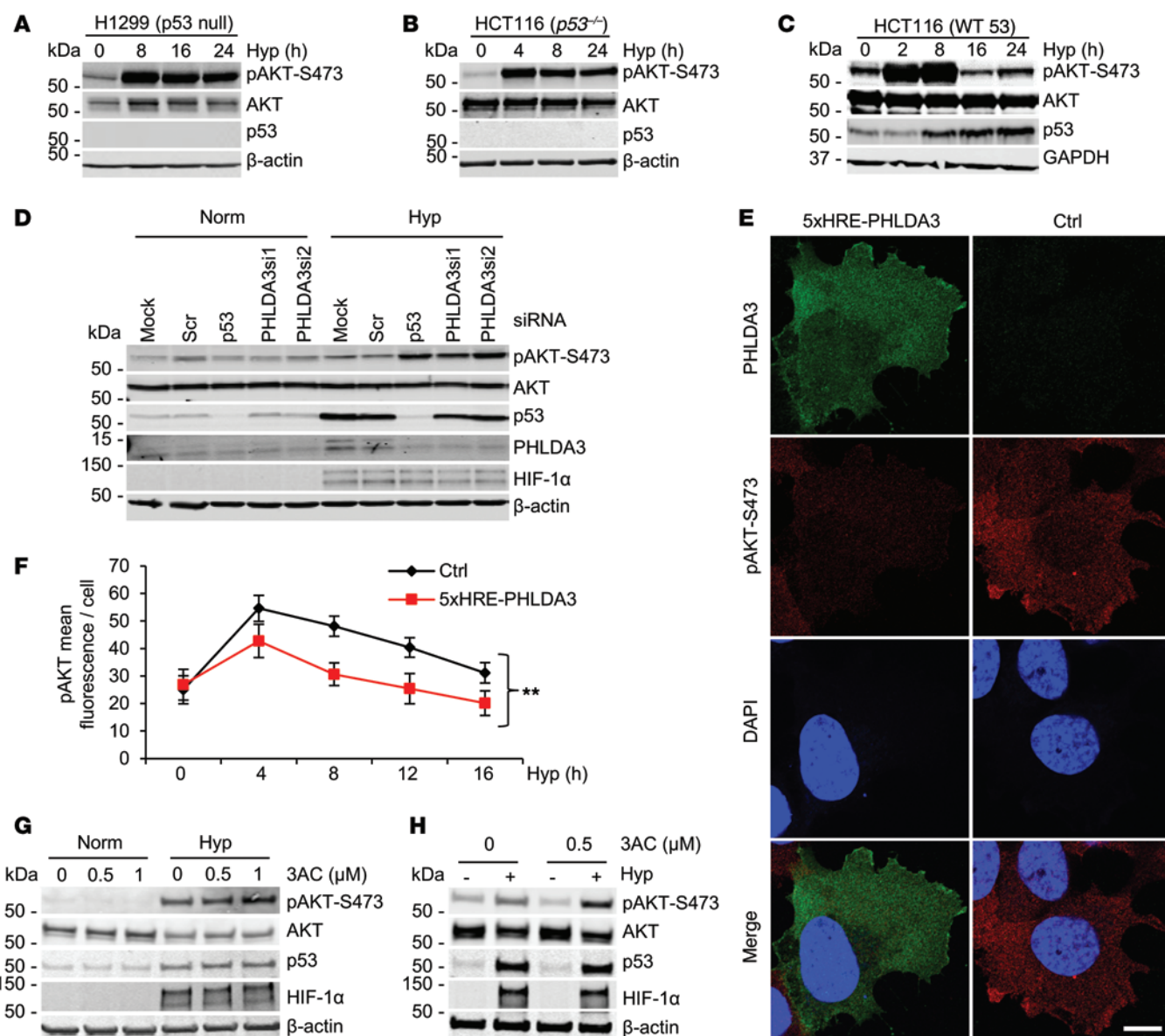


Figure 5. Hypoxia-induced activation of AKT is attenuated by p53/PHLDA3/SHIP-1 signaling. (A–C) Western blotting comparing AKT-S473 phosphorylation in H1299, HCT116 (*p53*^{-/-}), and WT HCT116 cells, respectively, exposed to hypoxia for the times indicated. (D) Western blotting for antibodies indicated in RKO cells transfected with either p53 or PHLDA3 siRNA and exposed to 8 hours of hypoxia. (E) Representative images of immunofluorescent containing with anti-PHLDA3 (green) and anti-pAKT-S473 (red) antibodies in H1299 cells transfected with 5xHRE-PHLDA3 or control and exposed to hypoxia for 12 hours. Scale bar: 10 μm. (F) Hypoxic time course for the mean intensity of pAKT-S473 fluorescence per cell in control or PHLDA3-expressing cells from E. The plot shows mean ± SEM (*n* = 3, 2-way ANOVA test [***P* < 0.01]). (G) Western blotting in HCT116 cells exposed to 18 hours of normoxia or hypoxia in the presence or absence of SHIP-1 inhibitor, 3AC. (H) Western blotting in RKO cells exposed to 14 hours of normoxia or hypoxia in the presence or absence of 3AC.

This analysis also highlighted that these genes are expressed at higher levels in tumor tissue compared with that in normal tissue. Again, the lower expression of this group of genes correlated with poor patient outcome, as measured by overall survival over 12 years in the entire METABRIC cohort (Figure 3C).

PHLDA3 and *INPP5D* mediate *p53*-dependent apoptosis in hypoxia. Since the collective expression of the *p53* target genes inducible in response to hypoxia significantly correlated with the clinical outcome of patients with cancer, we went on to investigate the function of these genes in hypoxia. Interestingly, two of the most highly induced *p53* targets in hypoxia, *PHLDA3*

and *INPP5D*, were both previously shown to induce apoptosis through negative regulation of AKT signaling (32, 37, 41). This led us to hypothesize that *p53* can induce apoptosis in hypoxia via inhibition of AKT and that this is reliant on the coordinated functions of more than one target gene. We expressed a 5xHRE-PHLDA3 construct in *p53*-null H1299 cells and exposed them to hypoxia. As expected, PHLDA3 localized to the plasma membrane and significantly increased the percentage of apoptosis (Figure 4, A and B, and Supplemental Figure 8A). siRNA-mediated knockdown of *PHLDA3* significantly compromised hypoxia-induced apoptosis in RKO cells, although not to the same

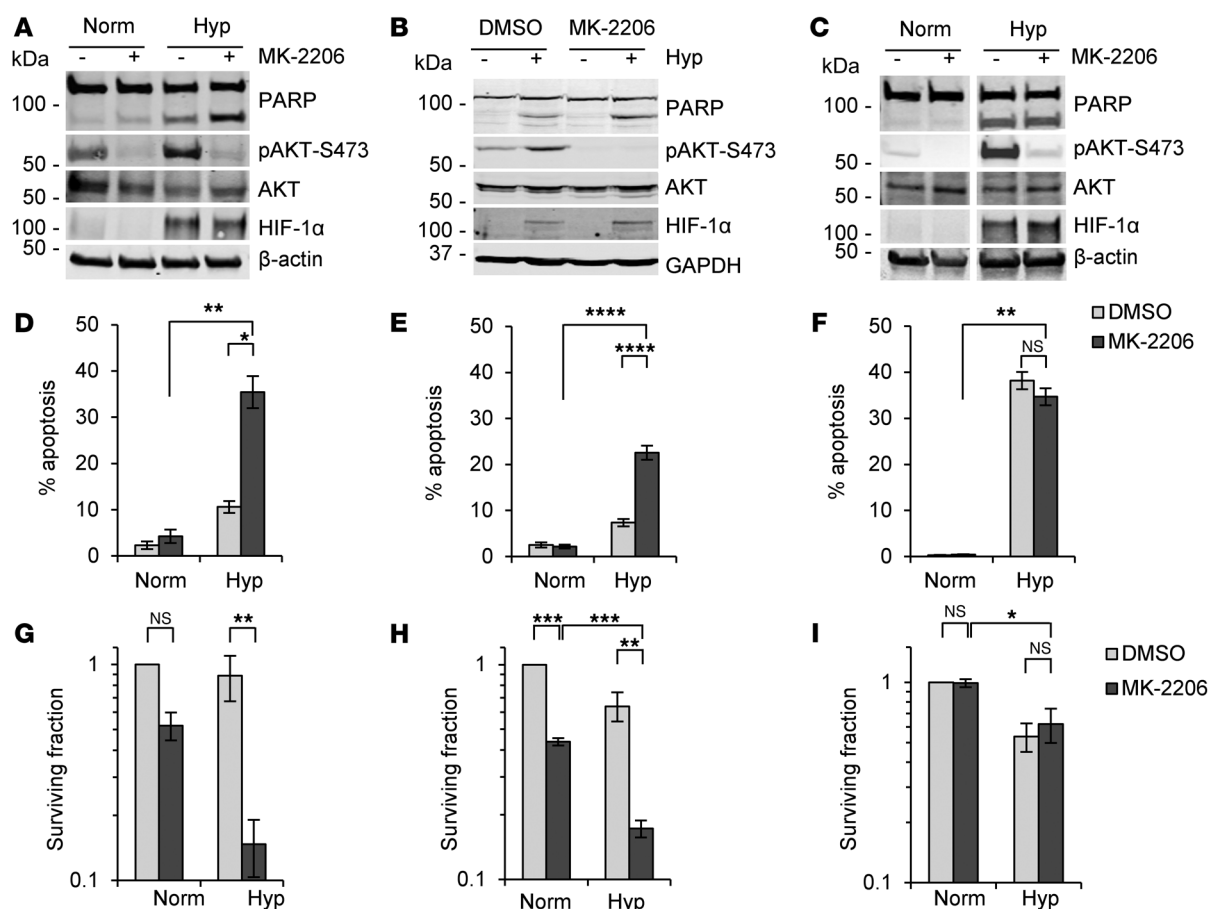


Figure 6. Pharmacological inhibition of AKT sensitizes p53-deficient hypoxic tumor cells. (A–C) Apoptosis detected by PARP cleavage in OE21, H1299, and HCT116 cells, respectively, exposed to 24 hours of hypoxia or normoxia in the presence of MK-2206 or DMSO. For Western blotting in C, the lanes were run on the same gel but were noncontiguous. (D–F) Apoptosis detected morphologically in OE21, H1299, and HCT116 cells, respectively, exposed to 24 hours of hypoxia or normoxia in the presence of MK-2206 or DMSO. (G–I) Colony survival assay in OE21, H1299, and HCT116 cells, respectively, exposed to 24 hours of hypoxia or normoxia in the presence of MK-2206 or DMSO. All the bar graphs show mean $\pm$ SEM ($n = 3$; 2-tailed Student's t test [$*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$]).

extent as p53 siRNA knockdown (Figure 4, C and D). Likewise, siRNA knockdown of short *INPP5D* also significantly impaired hypoxia-induced apoptosis in these cells (Figure 4E). However, when both p53 targets, *PHLDA3* and short *INPP5D*, were knocked down, the decrease in hypoxia-induced apoptosis was more profound than the levels of apoptosis observed with single knockdown of *PHLDA3* or *INPP5D* alone and similar to the levels of apoptosis induced by p53-targeted siRNA, suggesting that these targets synergize in order to mediate apoptosis (Figure 4F). The efficiency of *PHLDA3*, p53, and *INPP5D* knockdown is quantified in Supplemental Figure 8, B–D. The proapoptotic function of the SHIP-1 protein (encoded by *INPP5D*) is reliant on its phosphatase domain, which is included in the short *INPP5D* transcript (37). Given that the phosphatase activity of SHIP-1 is essential for the induction of apoptosis, we investigated the SHIP-1-specific phosphatase domain inhibitor, 3AC (42). The use of 3AC decreased hypoxia-induced apoptosis in a dose-dependent manner in a number of p53 WT cell lines (Supplemental Figure 8, E–G). In addition, when the SHIP-1 inhibitor, 3AC, was combined with *PHLDA3* siRNA knockdown in RKO cells, we found that the combination reduced apoptosis further than 3AC

combined with a nontargeting siRNA (Figure 4G). In summary, these data confirm that both p53 targets, *PHLDA3* and short *INPP5D*, contribute to p53-dependent apoptosis in hypoxia.

Hypoxia-induced activation of AKT is sustained in the absence of p53 function. In contrast to our hypothesis that p53 induces apoptosis in hypoxia by inhibiting AKT signaling, previous studies have demonstrated that hypoxia activates AKT (43, 44). We also found that hypoxia potentially induced phosphorylation of AKT at S473 (pAKT-S473). However, we noted that this activation of AKT was only sustained in p53-null or p53 mutant cells (HCT116 *p53*^{−/−}, H1299, OE21, or PSN1) compared with p53 WT cells (RKO or HCT116) (Figure 5, A–C, and Supplemental Figure 8, H–J). These data support our hypothesis that p53 may be negatively regulating AKT activation in hypoxia. In further support of this, siRNA-mediated depletion of endogenous *PHLDA3* or p53 in RKO cells increased pAKT-S473 in hypoxia (Figure 5D). We also found that overexpression of *PHLDA3* in H1299 cells significantly reduced hypoxic activation of AKT, as judged by the fluorescent intensity of pAKT-S473 staining in *PHLDA3*-expressing cells compared with that in *PHLDA3*-negative cells (Figure 5, E and F). In addition, inhibition of

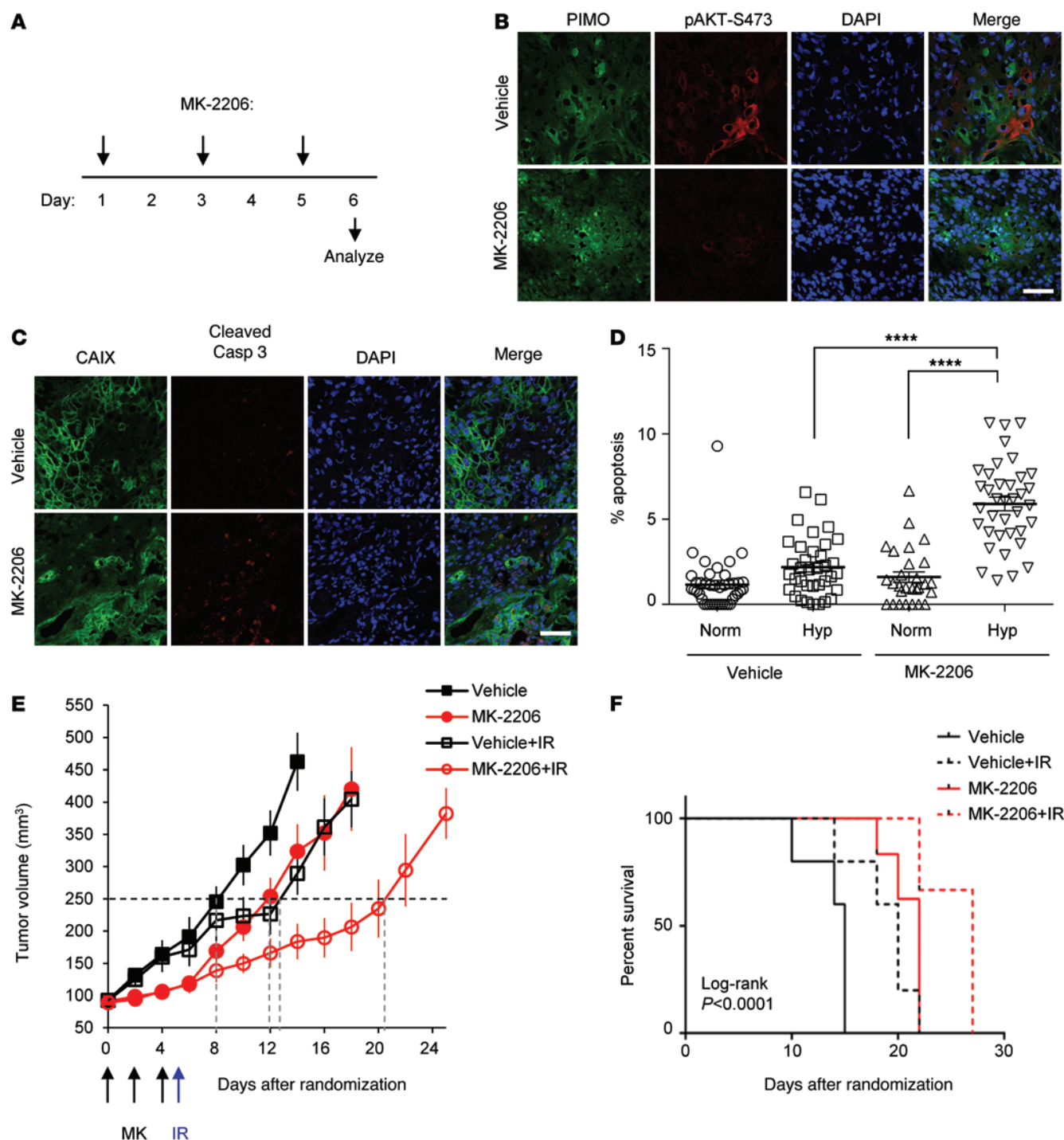


Figure 7. Pharmacological inhibition of AKT induces apoptosis in hypoxic regions of p53-deficient tumors and increases radiosensitivity. (A) MK-2206 treatment in OE21 xenografts. Mice were given 60 mg/kg MK-2206 or vehicle alone on 3 alternate days, and, 24 hours after the last dose, tumors were collected for analysis. (B) Representative images of colocalization of pAKT-S473 with hypoxic areas of OE21 xenografts stained with pimonidazole antibody (PIMO). Scale bar: 50 μ m. (C) Representative images of colocalization of cleaved caspase-3 with hypoxic areas of OE21 xenografts stained with CAIX antibody. Scale bar: 50 μ m. (D) Plots showing the percentage of apoptosis in either normoxic or hypoxic regions from all the regions analyzed. The bar represents the mean $\pm$ SEM from 3 animals per each group (2-tailed Student's *t* test [**** $P < 0.0001$]). (E) OE21 xenografts were grown in mice as in A, and a day after the last dose of MK-2206 or vehicle, tumors were treated with a single dose of IR (10 Gy). Tumor volumes were measured for 5 (vehicle), 5 (vehicle+IR), 6 (MK-2206), and 6 (MK-2206+IR) mice per each group, and mean $\pm$ SEM is shown. (F) A Kaplan-Meier plot showing the survival probability of mice in each treatment group (log-rank [Mantel-Cox] test with $P < 0.0001$ shown for all the curves compared together; comparisons of 2 single curves at the time were significant [$P < 0.05$] for all the combinations except the Vehicle+IR group versus MK-2206 group).

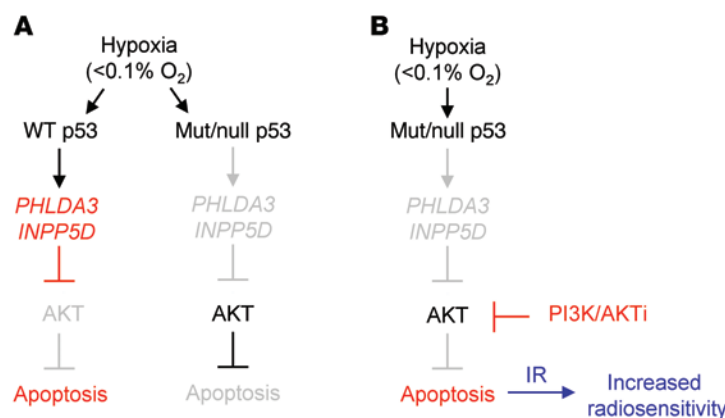


Figure 8. AKT inhibition radiosensitizes p53-deficient hypoxic tumors. (A) In response to hypoxia, p53 induces specific target genes, the role of which includes the induction of apoptosis. One of the mechanisms of apoptosis induction by p53 in hypoxia is through the inhibition of AKT signaling via *PHLDA3* and *INPP5D*. In the p53-deficient tumors, the expression of *PHLDA3* and *INPP5D* is abrogated, which facilitates activation of AKT and consequently inhibition of apoptosis. (B) Targeting p53-deficient tumors with PI3K/AKT inhibitors (PI3K/AKTi) is a promising strategy to restore the signaling of p53 in the hypoxic cells and eliminate therapy-resistant tumor fractions.

SHIP-1 with 3AC increased pAKT-S473 in hypoxia in HCT116 and RKO cells (Figure 5, G and H).

Inhibition of AKT increases apoptosis in p53-deficient hypoxic cells. Our data suggest that one of the functions of hypoxia-induced p53 is to restrict AKT signaling and drive cells into apoptosis. We asked whether the use of pharmacological inhibitors of AKT could be an effective approach to target hypoxic tumor cells. A panel of cell lines, expressing mutant p53 or not, were exposed to the allosteric AKT inhibitor MK-2206, and apoptosis assays were carried out. We observed a significant induction of apoptosis in cells (OE21, H1299, PSN1) treated with MK-2206 in combination with hypoxia but not with MK-2206 alone (Figure 6, A, B, D, and E, and Supplemental Figure 9, A–D). As expected, low levels of apoptosis were observed in these cells (p53 mutant/null) in response to hypoxia alone. This was also reflected in a difference in overall survival, as measured by clonogenic assay (Figure 6, G and H). In contrast, treatment with MK-2206 did not further increase hypoxia-induced apoptosis or affect overall survival in HCT116 cells expressing WT p53 (Figure 6, C, F, and I) or H1299 cells transfected with the HRE-p53 construct (Supplemental Figure 9, C and D). These data demonstrate that p53 inhibits AKT signaling in hypoxia and hypoxic cells that have lost p53 function are more sensitive to inhibition of AKT.

Inhibition of AKT increases apoptosis in hypoxic regions of p53-deficient tumors and increases radiosensitivity. To validate these findings *in vivo*, we grew OE21 (p53^{p.S90fs31X, c.269C>T, c.270delC}), PSN1 (p53^{K132Q}), and HCT116 (p53^{WT}) cells as tumor xenografts and treated them with either MK-2206 or vehicle alone (Figure 7A; Supplemental Figure 10, A and B; Supplemental Figure 11A; and Supplemental Figure 12A). As expected, pAKT-S473 was induced in the pimonidazole-positive hypoxic regions of these tumors, and this was efficiently inhibited by treatment with MK-2206, as judged by immunofluorescent staining or Western blotting of harvested tumors (Figure 7B, Supplemental Figure 10, and Supplemental Figure 11B). Most importantly, we found that AKT inhibition significantly increased apoptosis in the hypoxic areas of the p53-deficient tumors (Figure 7, C and D, and Supplemental Figure 11, C and D) but not in those of the p53 WT tumors (Supplemental Figure 12, B and C), as determined by costaining of cleaved caspase-3 and CAIX. These data support our hypothesis that the mechanism of p53-dependent hypoxia-induced apoptosis includes AKT inhibition and that this can be exploited *in vivo* through pharmacological inhibitors.

A decrease in the hypoxic fraction of solid tumors through increased apoptosis would be predicted to increase radiosensitivity. To test this, we chose to use an esophageal cancer model, due to the high rates of p53 mutation and the significant correlation between p53 status and patient prognosis in this cancer (45). OE21 xenografts were grown, and the mice were treated with either 60 mg/kg MK-2206 or vehicle on 3 alternate days, followed by a single dose of 10 Gy irradiation (IR) (Figure 7E). MK-2206 or IR alone delayed tumor growth from 100 to 250 mm³ for approximately 4 days in comparison to that in the vehicle-treated control group. However, the combined MK-2206 and IR treatment had a profound effect in delaying tumor growth from 100 to 250 mm³ for 12 days when compared with vehicle treatment or for 8 days when compared with either treatment with IR or MK-2206 alone (Figure 7E). This was reflected by the significantly prolonged median survival of mice treated with the combined therapy in comparison to either treatment alone (Figure 7F). These data confirmed our hypothesis that the radiosensitizing potential of AKT inhibitors could in part be mediated through a decrease in the hypoxic fraction of tumors that have lost functional p53 (Figure 8).

Discussion

Our study demonstrates that p53 is transactivation competent in response to replication stress induced by hypoxia (<0.1% O₂). A validated subset of the genes identified was significantly and consistently associated with p53 mutation status and patient prognosis in several clinical cohorts of patients with cancer, which both confirms the p53 dependency and highlights the importance of the hypoxia-induced p53 response. Two of the genes identified (*PHLDA3* and *INPP5D*) both inhibit AKT signaling and promote apoptosis in hypoxia. This suggests that tumors that have lost p53 function may be more sensitive to AKT inhibition, due to restoration of proapoptotic signaling in hypoxia (Figure 8). In support of this, we demonstrate increased apoptosis in hypoxia in response to an AKT inhibitor (MK-2206) in the absence of functional p53 both *in vitro* and in OE21 and PSN1 tumors *in vivo*. Most significantly, we found that esophageal p53-deficient tumors that had increased apoptosis specifically in hypoxic areas also showed an increased response to radiotherapy.

Recently a number of mouse models have been used to determine the mechanism of p53-mediated tumor suppression. These include the mutation of key residues such as K117/K161/K162

(corresponding to K120/K164 in humans) or coordinated knock-out of genes, such as *PUMA*, *NOXA*, and *p21* (24, 25). These studies demonstrate that DNA damage-induced p53-dependent apoptosis mediated by the classic target genes is not required for tumor suppression. This is supported by our finding that the levels of *PUMA*, *BID*, *PERP*, *BAX*, *PIG3*, and *NOXA* as a group do not predict patient outcome in clinical samples. Our study demonstrates that the hypoxia-induced transcriptional response of p53 uses alternative proapoptotic pathways, which substantially differ from those induced by genotoxic stress. Hypoxia-induced p53-mediated apoptosis could therefore be a crucial component of tumor suppression. In support of this hypothesis, some of the hypoxia-inducible p53 targets, such as *PHLDA3* and *KANK3*, have already been linked with p53-mediated tumor suppression (23, 46). By carrying out thorough and independent analyses of large clinical cohorts of breast and other cancers, we found that poor patient prognosis and progression of disease are significantly and consistently associated with diminished expression of the hypoxia-inducible p53-dependent group of genes, suggesting that their function is lost in aggressive cancers.

Previous studies have demonstrated that hypoxia-induced p53 is proapoptotic and suggest that this is the primary role for p53 in hypoxia (11, 18, 21). The relevance of apoptosis was further supported by our finding that a number of the hypoxia-induced targets identified here had previously been described as proapoptotic, as opposed to having roles in alternative p53-mediated responses. However, it is probable that hypoxia-induced p53 has additional functions, i.e., other than driving cells into apoptosis. For example, it is well known that cancer cells have increased glycolytic flux and suppressed oxidative phosphorylation, even in the presence of oxygen, and recently, the tumor-suppressive function of p53 was attributed to inhibition of glycolytic flux through induction of TIGAR (24). Interestingly, in hypoxia, TIGAR has been showed to protect from cell death via regulation of mitochondrial reactive oxygen species (47). Therefore, it would be interesting to investigate whether any of the targets identified in this study have roles in regulating cell metabolism under hypoxic conditions and whether this could in turn play a role in tumor suppression.

INPP5D, which encodes SHIP-1, specifically converts phosphatidylinositol-(3,4,5)-trisphosphate [PI(3,4,5)P₃] to PI(3,4)P₂ and therefore depletes the pool of PI3K-generated PI(3,4,5)P₃ at the plasma membrane, resulting in impaired AKT activation (41). However, AKT can be also recruited to the plasma membrane by PI(3,4)P₂ as well as PI(3,4,5)P₃. Therefore, it is significant that hypoxia-induced p53 also transactivates *PHLDA3*, which, through its pleckstrin homology domain, can bind both PI(3,4,5)P₃ and PI(3,4)P₂, leading to efficient inactivation of AKT (32, 48). This confirms that one of the principle roles of hypoxia-induced p53 is to restrict protumorigenic PI3K/AKT signaling.

AKT activation in hypoxic regions of breast tumors has been linked with poor patient prognosis (49). One of the known functions of AKT is to block apoptosis through phosphorylation of multiple targets, for example, caspase-3/9 or BAD (50). Therefore, inhibition of AKT is a promising strategy to eliminate cancer cells via reactivation of apoptosis. Many PI3K/AKT inhibitors are currently undergoing clinical trials, yet surprisingly, the available clinical data show little evidence for single-agent activity (51, 52). However, several studies describe promising antitumor activity of

PI3K/AKT inhibitors when combined with radiotherapy (53–57). AKT inhibition has also been demonstrated to improve radiosensitivity through normalization of the tumor vasculature (58). Our study, for the first time to our knowledge, demonstrates that inhibition of AKT in hypoxia may have a different effect with respect to p53 status and suggests that patients with cancer stratified for high tumor hypoxia and loss of p53 may get the most benefit from combined radiotherapy and AKT inhibition. The group of 6 genes identified and validated in this study offers a promising candidate biomarker to identify patients who could benefit from this approach. In summary, by fully characterizing the p53 transcriptional response to hypoxia, we have identified a stress-specific proapoptotic response, which could be exploited therapeutically and is relevant to patient prognosis.

Methods

Cell lines and plasmid transfection. RKO and HCT116 (colorectal carcinoma), H1299 (non-small-cell lung carcinoma), OE21 (esophageal squamous carcinoma), PSN1 (pancreatic adenocarcinoma), WI38 (lung fibroblasts), CCE (mouse embryonic stem cells), and THP-1 (acute monocytic leukemia) cell lines and plasmid transfection procedures are described in the Supplemental Methods.

Hypoxia/drug treatment. A Bactron chamber (Shel Lab) was used for studies at <0.1% O₂ and an in vivo 400 chamber (Baker Ruskinn) was used for 2% O₂. All hypoxic treatments were performed at <0.1% O₂ unless indicated otherwise. The following drug concentrations were used: 25 μM etoposide (Sigma-Aldrich); 1 μM MK-2206 (Selleck Chemicals); and 0.5–1 μM 3AC (Calbiochem).

Immunoblotting. Both adherent and detached cells were collected and lysed in SDS lysis buffer (10 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% SDS, 0.1 mM β-mercaptoethanol, protease/phosphatase inhibitors). The following antibodies were used: p53 (DO-I, Santa Cruz), p53 (C-19, Santa Cruz), β-actin (AC-15, Santa Cruz), p53 (1C12, Cell Signaling Technology), p53-S15 (9284, Cell Signaling Technology), PARP (9542, Cell Signaling Technology), cleaved caspase-3 (9661, Cell Signaling Technology), pAKT-S473 (4060, Cell Signaling Technology), AKT (4691, Cell Signaling Technology), *PHLDA3* (ab22822, Abcam), γH2AX (JBW301, Upstate-Millipore), H2AX (DR1016, Calbiochem), HIF-1α (610958, BD Biosciences), and GAPDH (6C5, Novus Biologicals). The Odyssey infrared system (LI-COR) was used to image Western blots.

siRNA knockdown. RKO cells were transfected using DharmaFECT 1 reagent (Thermo Fisher Scientific). siRNA duplexes were used at 50 nM, and 24 hours after knockdown, cells were replated to be used in assays up to 72 hours after transfection. siRNA sequences are listed in the Supplemental Methods.

Apoptosis detection. Apoptosis by nuclear morphology was assessed in combined adherent and detached cells fixed in 4% paraformaldehyde. Apoptosis was measured as the percentage of cells with fragmented DNA per 10 fields in every treatment. Graphs show mean values ± SEM from 3 independent experiments.

Colony survival assay. H1299, OE21, and HCT116 cells were treated either with DMSO or 1 μM MK-2206 and exposed to 24 hours of hypoxia or normoxia. Subsequently, all cells were placed in a normoxic incubator and left for 9 days to form colonies, which were visualized by crystal violet staining. Graphs show mean values ± SEM from 3 independent experiments.

Immunofluorescence. Cells were grown on glass coverslips and stained as previously described (59). The following antibodies were used: PHLDA3 (ab22822, Abcam) and pAKT-S473 (4060, Cell Signaling Technology). A LSM780 confocal microscope (Carl Zeiss Microscopy Ltd) was used. The fluorescent intensity of pAKT-S473 per cell was measured from ≥ 15 randomly selected cells imaged for each condition and was plotted as a mean of intensities $\pm$ SEM from 3 independent experiments.

RNA extraction, microarray analysis, and qPCR. RNA was prepared using TRIzol (Invitrogen/Life Technologies). Gene expression array analysis was carried out using Agilent Sure Print HD arrays by Oxford Gene Technology. Differential gene expression regulated by p53 in hypoxia ($<0.1\%$ O₂) or in normoxia (21% O₂) was calculated as a $\log_2(p53/p53^{175})$ of intensities, and *t* test with Benjamini and Hochberg *P* value adjustment was applied (60). For qPCR expression analysis, cDNA was reverse transcribed from total RNA using the Verso Kit (Thermo Scientific). qPCR was performed using the SYBR Green PCR Master Mix Kit (Applied Biosystems) in a 7500 FAST Real-Time PCR thermocycler with v2.0.5 software (Applied Biosystems). Sequences of qPCR primers are shown in the Supplemental Methods. mRNA fold change was calculated using a $2^{-\Delta\Delta Ct}$ method in relation to the 18S reference gene. The qPCR graphs show the mean $\pm$ SEM of 3 biological replicates.

ChIP. ChIP was carried out on HCT116 cells as described previously (7). Combined p53 antibodies (DO-1 and FL-393, Santa Cruz) or combined nonspecific mouse and rabbit control IgGs (Cell Signaling Technology) were used for immunoprecipitation. For the qPCR, input samples were used at 100-fold dilution and ChIP samples were used at 6-fold dilution. Fold enrichment at specific promoter sites was calculated in relation to the input samples. Primers used for qPCR are listed in the Supplemental Methods.

Analysis of human tumor samples. The methods, selection, and curation of data sets for the meta-analysis based on Gene Expression Omnibus cohorts have been described previously (4). Briefly, the prognostic meta-analysis was carried out using the *rmeta* R package (<http://cran.r-project.org/>). A fixed-effect model was used. The log hazard ratios and standard errors were obtained for each study from Cox proportional hazards regression. Study results were combined using the generic inverse-variance method. The variance was estimated as the square of the standard errors; this follows a χ^2 distribution, with approximately $k - 1$ degrees of freedom (k = number of studies). When groups of genes were considered, summary expression was estimated both as mean and median expression. In data sets in which p53 mutation status was not known, the p53 signature and related model were used to predict p53 mutation status as described previously (38). METABRIC data (39) were stratified by median expression of the gene signature to achieve balanced groups, and a Cox proportional hazards model was used to test equality of the survival distributions.

Oncomine analysis. Oncomine (<https://www.oncomine.org/>, research edition 4.4.3) results were generated using an odds ratio 2.0, *P* value of 0.01, and mRNA as a data type. No other filtering on data sets was applied, so the whole Oncomine cohort was used.

In vivo experiments. All animal procedures were performed in accordance with current United Kingdom legislation. Female athymic nude mice (BALB/c nude) (Harlan) were injected s.c. in the flank with 5×10^6 OE21 cells, 5×10^6 HCT116 cells, or 1×10^6 PSN-1 human pancreatic ductal carcinoma cells, the latter ones with 4×10^6 LTC-14 stellate cells.

Animals were randomized, and treatments were started when tumor volumes reached 100 mm³ (volume = height $\times$ depth $\times$ width $\times \pi/6$). Animal groups received either vehicle (25% cyclodextrin) or 60 mg/kg MK-2206 s.c. (PSN1 and HCT116) or orally (OE21) on 3 alternate days. Twenty-four hours after the last dose, tumors were harvested and snap frozen. Tumors embedded in OCT were sectioned (5 μ m) and immunofluorescence stained for hypoxia with CAIX antibody (M75 mouse monoclonal; ref. 61) or hypoxyprom-1 pimonidazole antibody (clone 4.3.11.3, Hypoxyprom) and with cleaved caspase-3 antibody (9661, Cell Signaling Technology). Apoptotic cells were counted within and outside of hypoxic regions. LSM780 software (Carl Zeiss) was used to determine the average of a single cell's surface area, and area of apoptotic cells (expressed as percentage) in whole hypoxic or normoxic regions was calculated from the following formula: number of apoptotic cells $\times$ average area of a single cell $\times 100$ /area of total region measured. Images were taken from at least 2 tumors treated with vehicle and 3 tumors treated with MK-2206. Graphs show the percentage of apoptotic cells from all normoxic or hypoxic regions and treatments analyzed in every tumor. The mean $\pm$ SEM is indicated by the bar. For the tumor growth curves, animals were s.c. injected with OE21 cells and randomized when average tumor volumes reached 100 mm³. Animals received vehicle or 60 mg/kg of MK-2206 on 3 alternate days, as above, and 24 hours after the last dose, the tumors in half of the animals in each treatment group were irradiated with 10 Gy. Tumors were measured regularly, and tumor growth was plotted as a mean of tumor volumes $\pm$ SEM. The number of mice for each treatment is as follows: 5 for vehicle, 5 for vehicle plus IR, 6 for MK-2206, and 6 for MK-2206 plus IR.

Statistics. Statistical tests other than the ones on clinical samples were performed using GraphPad Prism 6 software (GraphPad Software Inc.) and are indicated in figure legends for particular experiments. These included 2-way ANOVA tests, 2-tailed Student's *t* test, and log-rank (Mantel-Cox) tests. *P* values of less than 0.05 were considered significant.

Study approval. All animal experiments were approved by the University of Oxford Biomedical Services Ethical Review Committee. Ethical approval for the analysis of human breast cancer samples was obtained from the Molecular Taxonomy of Breast Cancer International Consortium.

Acknowledgments

We thank Adrian Harris, Amato Giaccia, Anderson Ryan, and Emmanouil Fokas for critical reading of the manuscript; Rieko Ohki for reagents; and Sally Hill, Karla Watson, and Mike Stratford for technical assistance. K.B. Leszczynska and E.M. Hammond are supported by Cancer Research UK (grant awarded to E.M. Hammond). Additional funds were provided by the Royal Society (awarded to E.M. Hammond) and European Union FP7 program (awarded to F.M. Buffa). This study makes use of data generated by the Molecular Taxonomy of Breast Cancer International Consortium, which was funded by Cancer Research UK and the British Columbia Cancer Agency Branch.

Address correspondence to: Ester M. Hammond, Cancer Research UK and Medical Research Council Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford, Old Road Campus Research Building, Oxford, OX3 7DQ. Phone: 01865.617320; E-mail: Ester.Hammond@oncology.ox.ac.uk.

1. McKeown SR. Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. *Br J Radiol.* 2014;87(1035):20130676.
2. Begg AC, Stewart FA, Vens C. Strategies to improve radiotherapy with targeted drugs. *Nat Rev Cancer.* 2011;11(4):239–253.
3. Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst.* 2001;93(4):266–276.
4. Buffa FM, Harris AL, West CM, Miller CJ. Large meta-analysis of multiple cancers reveals a common, compact and highly prognostic hypoxia metagene. *Br J Cancer.* 2010;102(2):428–435.
5. Chitneni SK, Palmer GM, Zalutsky MR, Dewhirst MW. Molecular imaging of hypoxia. *J Nucl Med.* 2011;52(2):165–168.
6. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res.* 1989;49(23):6449–6465.
7. Olcina MM, et al. Replication stress and chromatin context link ATM activation to a role in DNA replication. *Mol Cell.* 2013;52(5):758–766.
8. Mazouzi A, Velimezi G, Loizou JI. DNA replication stress: Causes, resolution and disease. *Exp Cell Res.* 2014;329(1):85–93.
9. Hammond EM, Denko NC, Dorie MJ, Abraham RT, Giaccia AJ. Hypoxia links ATR and p53 through replication arrest. *Mol Cell Biol.* 2002;22(6):1834–1843.
10. Brady CA, Attardi LD. p53 at a glance. *J Cell Sci.* 2010;123(pt 15):2527–2532.
11. Graeber TG, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature.* 1996;379(6560):88–91.
12. Brosh R, Rotter V. When mutants gain new powers: news from the mutant p53 field. *Nat Rev Cancer.* 2009;9(10):701–713.
13. Hammond EM, Dorie MJ, Giaccia AJ. ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. *J Biol Chem.* 2003;278(14):12207–12213.
14. Alarcon R, Koumenis C, Geyer RK, Maki CG, Giaccia AJ. Hypoxia induces p53 accumulation through MDM2 down-regulation and inhibition of E6-mediated degradation. *Cancer Res.* 1999;59(24):6046–6051.
15. Lee JH, et al. Hypoxia activates tumor suppressor p53 by inducing ATR-Chk1 kinase cascade-mediated phosphorylation and consequent 14-3-3 γ inactivation of MDMX protein. *J Biol Chem.* 2012;287(25):20898–20903.
16. Hammond EM, Giaccia AJ. The role of p53 in hypoxia-induced apoptosis. *Biochem Biophys Res Commun.* 2005;331(3):718–725.
17. Soengas MS, et al. Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science.* 1999;284(5411):156–159.
18. Hammond EM, et al. Genome-wide analysis of p53 under hypoxic conditions. *Mol Cell Biol.* 2006;26(9):3492–3504.
19. Johnson TM, Hammond EM, Giaccia A, Attardi LD. The p53QS transactivation-deficient mutant shows stress-specific apoptotic activity and induces embryonic lethality. *Nat Genet.* 2005;37(2):145–152.
20. Li YZ, Lu DY, Tan WQ, Wang JX, Li PF. p53 initiates apoptosis by transcriptionally targeting the antiapoptotic protein ARC. *Mol Cell Biol.* 2008;28(2):564–574.
21. Koumenis C, et al. Regulation of p53 by hypoxia: dissociation of transcriptional repression and apoptosis from p53-dependent transactivation. *Mol Cell Biol.* 2001;21(4):1297–1310.
22. Fei P, et al. Bnip3L is induced by p53 under hypoxia, and its knockdown promotes tumor growth. *Cancer Cell.* 2004;6(6):597–609.
23. Brady CA, et al. Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. *Cell.* 2011;145(4):571–583.
24. Li T, et al. Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. *Cell.* 2012;149(6):1269–1283.
25. Valente LJ, et al. p53 efficiently suppresses tumor development in the complete absence of its cell-cycle inhibitory and proapoptotic effectors p21, Puma, and Noxa. *Cell Rep.* 2013;3(5):1339–1345.
26. Kenzelmann Broz D, et al. Global genomic profiling reveals an extensive p53-regulated autophagy program contributing to key p53 responses. *Genes Dev.* 2013;27(9):1016–1031.
27. Mellert HS, et al. Deacetylation of the DNA-binding domain regulates p53-mediated apoptosis. *J Biol Chem.* 2011;286(6):4264–4270.
28. Adamsen BL, Kravik KL, Clausen OP, De Angelis PM. Apoptosis, cell cycle progression and gene expression in TP53-depleted HCT116 colon cancer cells in response to short-term 5-fluorouracil treatment. *Int J Oncol.* 2007;31(6):1491–1500.
29. Hong JW, Ryu MS, Lim IK. Phosphorylation of serine 147 of tis21/BTG2/pc3 by p-Erk1/2 induces Pin-1 binding in cytoplasm and cell death. *J Biol Chem.* 2005;280(22):21256–21263.
30. Mongroo PS, et al. IMP-1 displays cross-talk with K-Ras and modulates colon cancer cell survival through the novel proapoptotic protein CYFIP2. *Cancer Res.* 2011;71(6):2172–2182.
31. Kerley-Hamilton JS, Pike AM, Li N, DiRenzo J, Spinella MJ. A p53-dominant transcriptional response to cisplatin in testicular germ cell tumor-derived human embryonal carcinoma. *Oncogene.* 2005;24(40):6090–6100.
32. Kawase T, et al. PH domain-only protein PHLDA3 is a p53-regulated repressor of Akt. *Cell.* 2009;136(3):535–550.
33. Jackson RS 2nd, Cho YJ, Stein S, Liang P. CYFIP2, a direct p53 target, is leptomycin-B sensitive. *Cell Cycle.* 2007;6(1):95–103.
34. Chau BN, et al. Identification of SULF2 as a novel transcriptional target of p53 by use of integrated genomic analyses. *Cancer Res.* 2009;69(4):1368–1374.
35. Sermeus A, Michiels C. Reciprocal influence of the p53 and the hypoxic pathways. *Cell Death Dis.* 2011;2:e164.
36. Lion M, et al. Interaction between p53 and estradiol pathways in transcriptional responses to chemotherapeutics. *Cell Cycle.* 2013;12(8):1211–1224.
37. Tu Z, et al. Embryonic and hematopoietic stem cells express a novel SH2-containing inositol 5'-phosphatase isoform that partners with the Grb2 adapter protein. *Blood.* 2001;98(7):2028–2038.
38. Miller LD, et al. An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. *Proc Natl Acad Sci U S A.* 2005;102(38):13550–13555.
39. Curtis C, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature.* 2012;486(7403):346–352.
40. Silwal-Pandit L, et al. TP53 mutation spectrum in breast cancer is subtype specific and has distinct prognostic relevance. *Clin Cancer Res.* 2014;20(13):3569–3580.
41. Liu Q, et al. SHIP is a negative regulator of growth factor receptor-mediated PKB/Akt activation and myeloid cell survival. *Genes Dev.* 1999;13(7):786–791.
42. Brooks R, et al. SHIP1 inhibition increases immunoregulatory capacity and triggers apoptosis of hematopoietic cancer cells. *J Immunol.* 2010;184(7):3582–3589.
43. Chen EY, Mazure NM, Cooper JA, Giaccia AJ. Hypoxia activates a platelet-derived growth factor receptor/phosphatidylinositol 3-kinase/Akt pathway that results in glycogen synthase kinase-3 inactivation. *Cancer Res.* 2001;61(6):2429–2433.
44. Stegeman H, et al. Activation of AKT by hypoxia: a potential target for hypoxic tumors of the head and neck. *BMC Cancer.* 2012;12(1):463.
45. Yao W, et al. Association of p53 expression with prognosis in patients with esophageal squamous cell carcinoma. *Int J Clin Exp Pathol.* 2014;7(10):7158–7163.
46. Ohki R, et al. PHLDA3 is a novel tumor suppressor of pancreatic neuroendocrine tumors. *Proc Natl Acad Sci U S A.* 2014;111(23):E2404–E2413.
47. Cheung EC, Ludwig RL, Vousden KH. Mitochondrial localization of TIGAR under hypoxia stimulates HK2 and lowers ROS and cell death. *Proc Natl Acad Sci U S A.* 2012;109(50):20491–20496.
48. Viernes DR, Choi LB, Kerr WG, Chisholm JD. Discovery and development of small molecule SHIP phosphatase modulators. *Med Res Rev.* 2014;34(4):795–824.
49. Wennemers M, et al. Hypoxia regulation of phosphokinases and the prognostic value of pAKT in breast cancer. *Int J Biol Markers.* 2013;28(2):151–160.
50. Stiles BL. PI-3-K and AKT: Onto the mitochondria. *Adv Drug Deliv Rev.* 2009;61(14):1276–1282.
51. Pal SK, Reckamp K, Yu H, Figlin RA. Akt inhibitors in clinical development for the treatment of cancer. *Expert Opin Investig Drugs.* 2010;19(11):1355–1366.
52. Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and opportunities. *Nat Rev Drug Discov.* 2014;13(2):140–156.
53. Diaz R, et al. The novel Akt inhibitor Palomid 529 (P529) enhances the effect of radiotherapy in prostate cancer. *Br J Cancer.* 2009;100(6):932–940.
54. Chen W, Wu S, Zhang G, Wang W, Shi Y. Effect of AKT inhibition on epithelial-mesenchymal transition and ZEB1-potentiated radiotherapy in nasopharyngeal carcinoma. *Oncol Lett.* 2013;6(5):1234–1240.
55. Zhang T, et al. Inhibition of PI3 kinases

- enhances the sensitivity of non-small cell lung cancer cells to ionizing radiation. *Oncol Rep.* 2010;24(6):1683–1689.
56. Prevo R, et al. Class I PI3 kinase inhibition by the pyridinylfuranopyrimidine inhibitor PI-103 enhances tumor radiosensitivity. *Cancer Res.* 2008;68(14):5915–5923.
57. Hamilton G, et al. AKT regulates NPM dependent ARF localization p53mut stability in tumors. *Oncotarget.* 2014;5(15):6142–6167.
58. Fokas E, et al. Dual inhibition of the PI3K/mTOR pathway increases tumor radiosensitivity by normalizing tumor vasculature. *Cancer Res.* 2012;72(1):239–248.
59. Bencokova Z, Kaufmann MR, Pires IM, Lecane PS, Giaccia AJ, Hammond EM. ATM activation and signaling under hypoxic conditions. *Mol Cell Biol.* 2009;29(2):526–537.
60. Benjamini Y, Hochberg Y. Controlling the false discovery rate — a practical and powerful approach to multiple testing. *J Roy Stat Soc B Met.* 1995;57(1):289–300.
61. Pastorek J, et al. Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment. *Oncogene.* 1994;9(10):2877–2888.

Replication Stress and Chromatin Context Link ATM Activation to a Role in DNA Replication

Monica M. Olcina,¹ Iosifina P. Foskolou,¹ Selvakumar Anbalagan,¹ Joana M. Senra,¹ Isabel M. Pires,¹ Yanyan Jiang,¹ Anderson J. Ryan,¹ and Ester M. Hammond^{1,*}

¹The Gray Institute for Radiation Oncology and Biology, Department of Oncology, University of Oxford, Oxford, OX3 7DQ, UK

*Correspondence: ester.hammond@oncology.ox.ac.uk

<http://dx.doi.org/10.1016/j.molcel.2013.10.019>

This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Open access under CC BY license.

SUMMARY

ATM-mediated signaling in response to DNA damage is a barrier to tumorigenesis. Here we asked whether replication stress could also contribute to ATM signaling. We demonstrate that, in the absence of DNA damage, ATM responds to replication stress in a hypoxia-induced heterochromatin-like context. In certain hypoxic conditions, replication stress occurs in the absence of detectable DNA damage. Hypoxia also induces H3K9me3, a histone modification associated with gene repression and heterochromatin. Hypoxia-induced replication stress together with increased H3K9me3 leads to ATM activation. Importantly, ATM prevents the accumulation of DNA damage in hypoxia. Most significantly, we describe a stress-specific role for ATM in maintaining DNA replication rates in a background of increased H3K9me3. Furthermore, the ATM-mediated response to oncogene-induced replication stress is enhanced in hypoxic conditions. Together, these data indicate that hypoxia plays a critical role in the activation of the DNA damage response, therefore contributing to this barrier to tumorigenesis.

INTRODUCTION

Replication stress (RS) leads to fork stalling as a result of nucleotide pool depletion and/or the generation of DNA lesions (Murga et al., 2011). RS triggers a DNA damage response (DDR) in an attempt to resolve the insult, ensure fork integrity, and restart DNA synthesis (Harper and Elledge, 2007). The DDR is a complicated signaling cascade composed of sensors, transducers, and effectors that collectively orchestrate a response to the initial stress: RS or DNA single- or double-strand breaks (DSBs). The ataxia-telangiectasia mutated (ATM) serine/threonine kinase is a phosphatidylinositol-3-kinase-like kinase that has been characterized as an important component of the DDR involved in sensing and responding to DSBs (Jackson and Bartek, 2009). ATM is present as an inactive dimer that becomes activated upon dissociation into monomers and intermolecular autophosphorylation on serine 1981 (Bakkenist and Kastan, 2003). Phos-

phatases such as PP2A and WIP-1 are also important for the regulation of overall ATM activity following DNA damage (Goodarzi et al., 2004; Shreeram et al., 2006).

Low oxygen levels (hypoxia) occur in the majority of solid tumors and are associated with tumor development and progression. Importantly, severe hypoxia (<0.1% O₂) can induce RS and activation of the DDR (Bencokova et al., 2009; Bristow and Hill, 2008). In hypoxia, areas of single-stranded DNA accumulate, leading to ataxia-telangiectasia and Rad3-related (ATR) activation. Surprisingly, the hypoxia-induced DDR also includes rapid activation of ATM, despite the lack of associated DNA damage. It is important to note that the activity of the MRN (Mre11-Rad50-Nbs1) complex is not necessary for the activation of ATM under hypoxic conditions. Interestingly, oxidative stresses such as those attributable to H₂O₂ also induce ATM activity independent of the MRN complex in the absence of DNA breaks (Guo et al., 2010a). However, ATM signaling under oxidative stress differs from hypoxia-induced ATM, because the former does not include KAP-1 (S824) or H2AX (S139) phosphorylation (Bencokova et al., 2009; Guo et al., 2010b). KAP-1 phosphorylation at S824 occurs in an ATM-dependent manner in response to a variety of stresses including ionizing radiation (IR) and hypoxia (Ziv et al., 2006).

Here we have verified that ATM can respond to RS in the absence of DNA damage, providing this occurs in the presence of increased levels of H3K9me3. This observation led us to question the role of ATM in these conditions. Most significantly, loss of ATM during exposure to hypoxia-induced RS led to an accumulation of DNA damage and a decrease in DNA replication rates. This study highlights a stress- and context-specific role for ATM in DNA replication in conditions of RS.

RESULTS

Replication Stress Can Induce ATM Activity in the Absence of DNA Damage

Exposure to hypoxia (<0.1% O₂) does not induce detectable levels of DNA damage using available assays (Bencokova et al., 2009) (Figures S1A–S1C available online). Despite this, ATM is rapidly phosphorylated and downstream targets are phosphorylated in an ATM-dependent manner. Hypoxia-induced ATM targets include both CHK2 (T68) and KAP-1 (S824) (Figure 1A). An increase in phosphorylation of KAP-1 at residue 473 was also observed in response to hypoxia, but

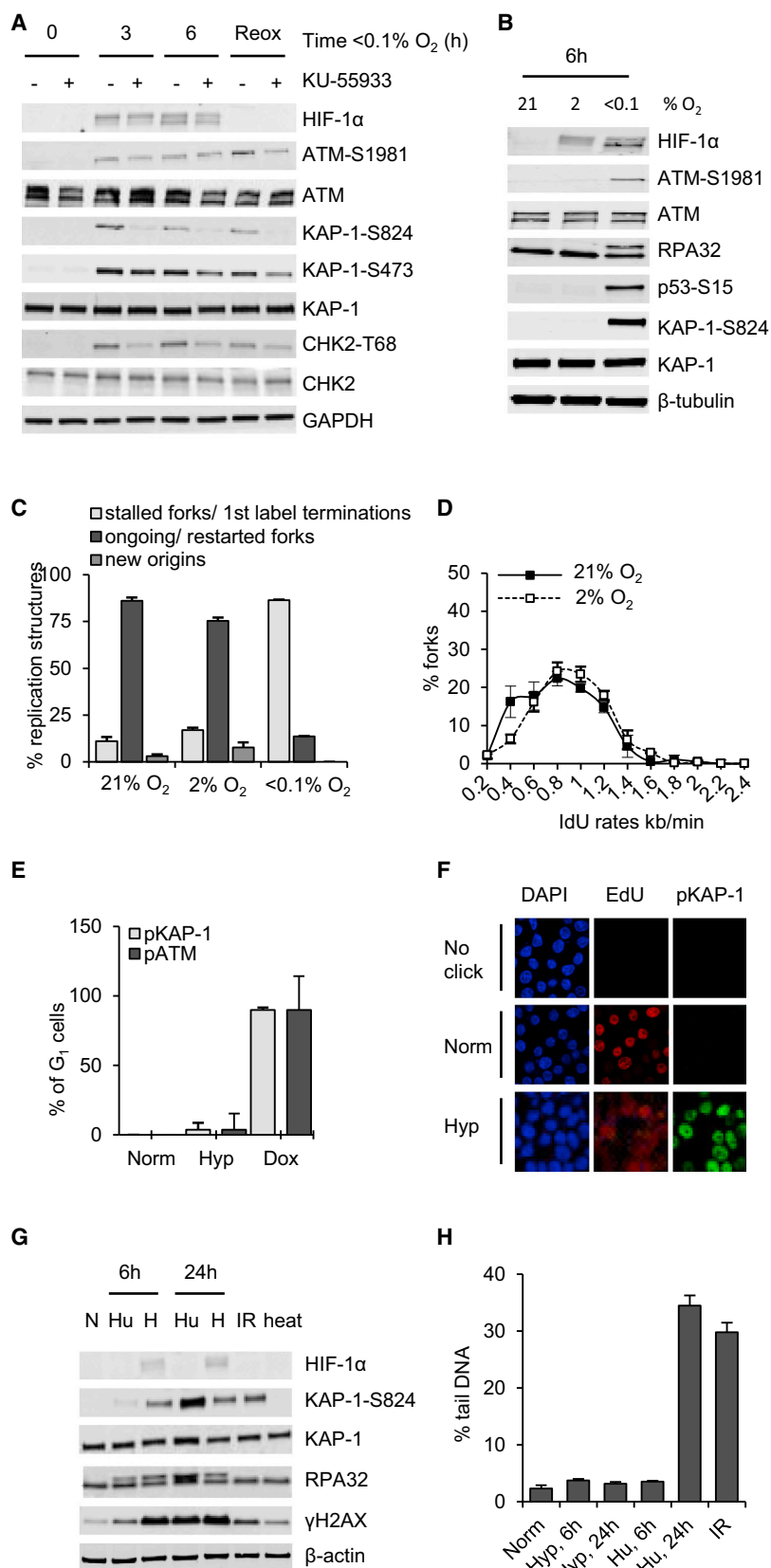


Figure 1. Hypoxia-Induced Replication Stress and ATM Activity

(A) RKO cells were exposed to <0.1% O₂ for the times indicated in the presence of DMSO or ATM inhibitor (KU-55933). Reox (6 hr, <0.1% O₂ followed by 1 hr of 21% O₂). Western blotting (WB) was carried out with the antibodies indicated; GAPDH was the loading control.

(B) RKO cells were exposed to 21%, 2%, or <0.1% O₂. WB was carried out as indicated; β -tubulin was the loading control.

(C) RKO cells were exposed to 21%, 2%, or <0.1% O₂ and DNA fibers were scored. Shown is a graph of the replication structures.

(D) The graph indicates the distribution of fork rates for the second label for the forks scored in (C) for cells exposed to normoxia (21% O₂) or hypoxia (2% O₂, 6 hr). IdU, 5-iodo-2'-deoxyuridine.

(E) The quantification of the number of stained G₁ cells is shown. pKAP-1, KAP-1-S824; pATM, ATM-S1981. Norm (21% O₂); Hyp (<0.1% O₂, 6 hr); Doxorubicin (Dox; 4 hr).

(F) RKO cells were labeled with EdU and costained with KAP-1-S824. Hyp (<0.1% O₂, 6 hr). A representative image of the colocalization of KAP-1-S824 and EdU staining is shown. DAPI, 4',6-diamidino-2-phenylindole.

(G) RKO cells were exposed to N (21% O₂), Hu (6 or 24 hr), H (<0.1% O₂, 6 or 24 hr), IR (5 Gy, harvested 30 min post IR), or heat (42.5°C, 1 hr). WB was carried out with the antibodies indicated; β -actin was the loading control.

(H) RKO cells were treated as shown and an alkaline comet assay was carried out. Norm (21% O₂), Hyp (<0.1% O₂, 6 or 24 hr), Hu (6 or 24 hr), IR (5 Gy, lysed immediately after IR). Shown is the % comet tail DNA.

Data are represented as mean $\pm$ SEM. See also Figure S1.

was not significantly affected by ATM inhibition, in agreement with the finding that it is dependent on CHK1 (Blasius et al., 2011). To study a possible link between the hypoxia-induced ATM response and RS, we investigated the activation of ATM in response to 2% and <0.1% O₂. Both of these levels of oxygen induce a hypoxic response, including HIF-1 α stabilization. However, only in conditions of <0.1% O₂ is ATM phosphorylated at serine 1981 and downstream ATM/ATR targets activated (Figure 1B). Importantly, exposure to <0.1% O₂ leads to phosphorylation of RPA32, which is again indicative of RS in these conditions. Analysis of replication structures in 21%, 2%, and <0.1% O₂ demonstrated that hypoxia (<0.1% O₂) induces a significant increase in the number of stalled forks (Pires et al., 2010). In contrast, the numbers of stalled or ongoing forks exposed to 21% or 2% O₂ are similar (Figure 1C). In addition, replication rates are unaffected by exposure to 2% O₂ when compared to the replication rates of cells exposed to 21% O₂ (Figure 1D). These data suggest that hypoxia-induced RS signals to ATM and, therefore, that in hypoxic conditions, <0.1% O₂, ATM-mediated signaling should be restricted to S phase. We determined that KAP-1 is phosphorylated at S824 in approximately 50% of RKO cells exposed to <0.1% O₂ (Figure S1D). In contrast, a population enriched for G₁ cells and exposed to hypoxia showed little or no induction of KAP-1-S824 or ATM-S1981 (Figure 1E). By labeling cells with 5-ethynyl-2'-deoxyuridine (EdU), we confirmed that, in hypoxia, cells in S phase accumulated KAP-1-S824 (Figure 1F; Figure S1E). This was further supported by fluorescence-activated cell sorting analysis (Figure S1F).

The ATM-dependent signaling to KAP-1 in response to RS induced by hypoxia was surprising given that previous studies have shown that KAP-1 is only phosphorylated in the presence of DNA damage (Goodarzi et al., 2009). RKO cells were exposed to hydroxyurea (Hu) or hypoxia (<0.1% O₂) for either 6 or 24 hr. Irradiation was used as a DNA-damaging control, and heat (42°C) as a non-DNA-damaging control (Figure S1G) (Hunt et al., 2007). Acute exposure (6 hr) to both Hu and hypoxia led to phosphorylation of RPA32, confirming RS (Figure 1G). Importantly, although the short acute exposure to both Hu and hypoxia induced RS, neither induced DNA damage detectable by comet assay (Figure 1H). In contrast to the response to hypoxia-induced RS, acute exposure to Hu did not induce KAP-1 phosphorylation. After a longer exposure to Hu (24 hr) significant KAP-1 phosphorylation was observed, although this correlated with an induction of DNA damage detected by comet assay (Figure 1H). These data suggest that ATM does respond to RS but that this is context specific. Interestingly, loss of ATM does not sensitize cells to replication stresses such as Hu (Bolderson et al., 2004). However, inhibition of ATM, using KU-55933, during exposure to hypoxia (<0.1% O₂) led to decreased colony survival, suggesting an important role for ATM in these conditions (Figure S1H).

Hypoxia-Induced H3K9me3 Correlates with ATM Activity In Vitro and In Vivo

Hypoxia-induced RS occurs within a specific chromatin context. Many hypoxia-induced histone modifications have been reported (Johnson et al., 2008). However, the consequences of

hypoxia-induced chromatin changes as well as the regulation of many of the enzymes that lead to such changes are poorly understood (Melvin and Rocha, 2012). We investigated the induction of a number of histone marks in hypoxia and after reoxygenation. As expected, hypoxia (<0.1% O₂) led to the induction of pan-nuclear γ H2AX indicative of RS, whereas milder hypoxic conditions (2% O₂) did not (Figures 2A and 2B; Figure S1A) (Toledo et al., 2011). An increase in H3K9me3 under both hypoxic conditions was observed, which returned to baseline levels after reoxygenation. In contrast, we only saw a mild induction in H3K9me2 in response to <0.1% O₂, and did not observe marked differences in H3K27me3 levels in response to either <0.1% or 2% O₂. Interestingly, the increase in H3K9me3 was inversely related to the level of oxygen. H3K9me3 is enriched in chromocenters corresponding to centromeric and pericentric heterochromatin (Guenatri et al., 2004). In response to hypoxia (<0.1% O₂), H3K9me3 enrichment occurred throughout the nucleus and in all cell-cycle phases (Figure 2C; Figure S2A). H3K9me3 recruits chromodomain-containing proteins such as HP1 β , which can regulate heterochromatin formation (Nielsen et al., 2002). HP1 β -H3K9me3 binding is proposed to allow H3K9 methylation on neighboring nucleosomes through the recruitment of Suv39h1 and other methyltransferases by HP1 β (Loyola et al., 2009). The expression of HP1 β in hypoxia, however, has not been studied to date. Here we observed a transient increase in expression of chromatin-bound HP1 β after 6 hr of hypoxia (Figure 2D; Figure S2B).

We observed that two methyltransferases responsible for H3K9me3, SETDB1 and Suv39h2, were induced at the protein but not mRNA level in response to hypoxia (<0.1% O₂) (Figures S2C–S2E). Furthermore, the protein levels of SETDB1 were decreased in response to reoxygenation, reflecting the drop in H3K9me3 observed in response to reoxygenation. Levels of Suv39h2 were notably increased in response to reoxygenation and IR, suggesting that this methyltransferase is DNA damage responsive.

We hypothesized that RS in the presence of increased H3K9me3 could be linked to ATM activation in the absence of damage. In order to verify that H3K9me3 occurs in the hypoxic areas of tumors and correlates with ATM activation, subcutaneous Calu6 xenograft tumors were stained for H3K9me3, the hypoxia marker CAIX, and ATM-S1981. Importantly, in CAIX-positive regions of the tumor, there was a clear association with both high expression of H3K9me3 and ATM-S1981 (Figures 2E and 2F; Figure S2F).

Hypoxia-Induced H3K9me3 Is Required for ATM Activity

To test whether increased H3K9me3 was required for ATM signaling in hypoxia, we investigated the effects of loss of H3K9me3 on ATM activation. One of the roles of KAP-1 is to act as a transcriptional corepressor by facilitating H3K9me3 spreading (Schultz et al., 2002). Upon treatment with KAP-1 siRNA a modest reduction in H3K9me3 was observed. Interestingly, this correlated with a similar reduction in phosphorylated ATM (Figures S2G and S2H). We then used Suv39h1/2^{+/+} and Suv39h1/2^{-/-} mouse embryonic fibroblasts (MEFs) to investigate the direct role of these two methyltransferases in ATM phosphorylation in hypoxia. As expected, the Suv39h1/2^{-/-}

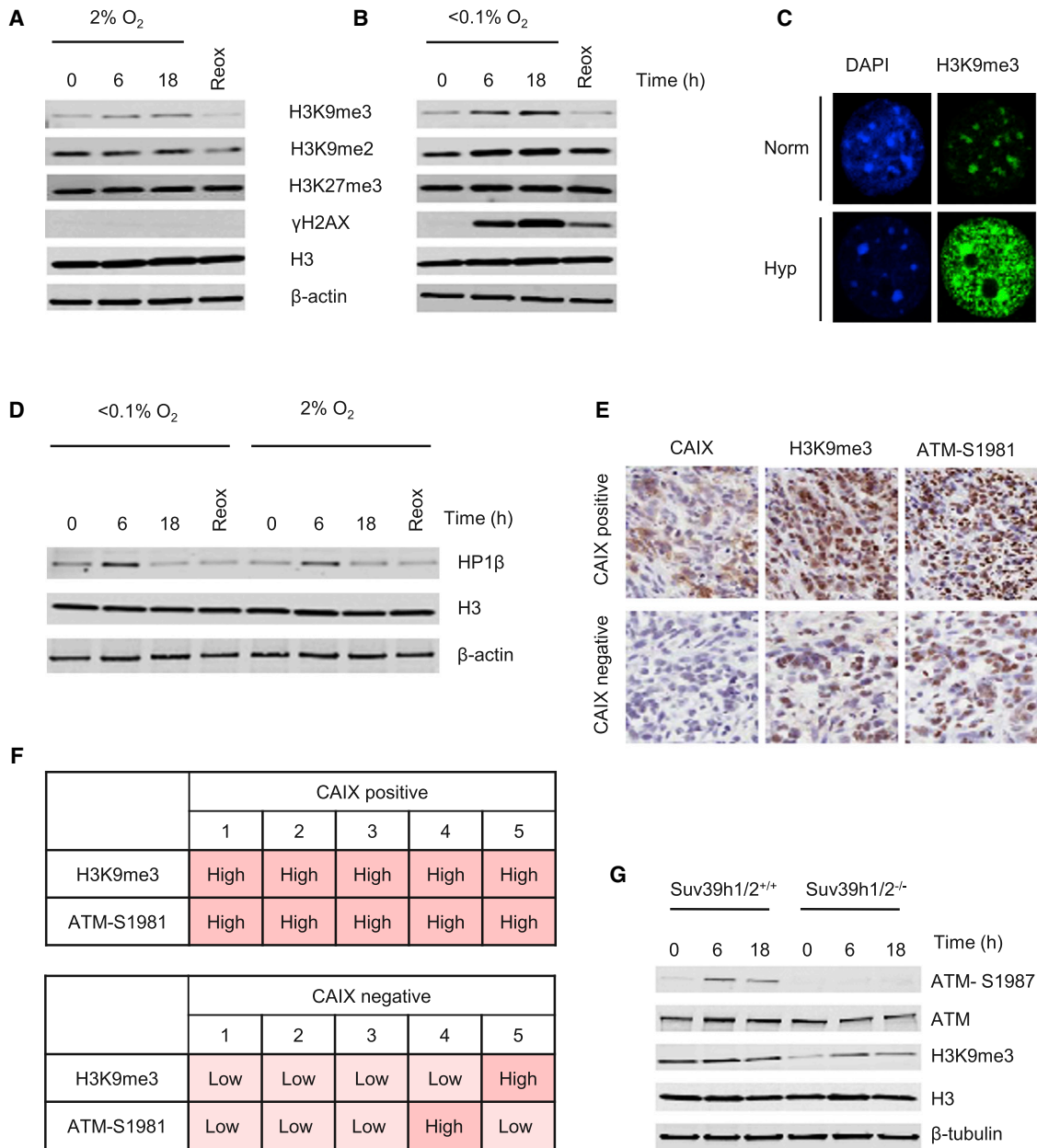


Figure 2. Hypoxia-Induced H3K9me3 Correlates with ATM Activity In Vitro and In Vivo

(A and B) RKO cells were treated with either 2% (A) or <0.1% O₂ (B). Reox (6 hr, <0.1% O₂ followed by 2 hr of 21% O₂). The chromatin modifications investigated are shown between the Western blots.

(C) Mouse NIH 3T3 cells were fixed and stained for H3K9me3 following exposure to Norm (21% O₂) or Hyp (<0.1% O₂, 20 hr).

(D) RKO cells were treated as in (A) and (B). WB was carried out.

(E) Serial sections from Calu6 xenografts were stained for H3K9me3, CAIX, or ATM-S1981. Shown are representative images of regions positive or negative for CAIX staining and the corresponding H3K9me3 and ATM-S1981 staining in brown.

(F) Five CAIX-positive and -negative regions were scored for both H3K9me3 and ATM-S1981.

(G) Suv39h1/2^{+/+} and Suv39h1/2^{-/-} MEFs were treated as indicated (Hyp, <0.1% O₂). WB was carried out; β-tubulin was the loading control.

See also Figure S2.

MEFs showed a decreased level of H3K9me3 in both normoxia and hypoxia. Most importantly, ATM phosphorylation was markedly decreased in these cells following exposure to hypoxia (<0.1% O₂) (Figure 2G; Figure S2I). As previously re-

ported, we also observed reduced ATM phosphorylation in response to IR in the Suv39h1/2^{-/-} cell line (Figure S2J) (Sun et al., 2009). Lentiviral knockdown of Suv39h1 and Suv39h2 also showed a reduction in H3K9me3 levels and impaired

hypoxia-induced ATM activity (Figure S2K). ATM activation in response to RS, therefore, occurs in a specific hypoxia-induced chromatin context, which includes increased levels of H3K9me3.

Because H3K9me3 is associated with gene repression, we hypothesized that this chromatin mark may be contributing to hypoxia-induced ATM activation through repression of a factor that normally inhibits ATM phosphorylation, such as an ATM-specific phosphatase. In support of this, both WIP-1 and PP2A-C appear genetically regulated by Suv39h1/2 (Figures S2L and S2M). We measured the enrichment of H3K9me3 on PP2A-C by chromatin immunoprecipitation and observed a moderate increase in H3K9me3 enrichment in response to hypoxia (Figures S2N–S2P). In addition, we verified that the chromatin accessibility of the PP2A-C gene was reduced and that mRNA expression decreased in response to hypoxia (Figures S2Q and S2R). Finally, we assessed the functional activity of PP2A-C and found a significant reduction in hypoxic conditions (<0.1% O₂) (Figure S2S). Collectively, these data suggest that H3K9me3 can facilitate ATM activation in response to hypoxia (<0.1% O₂) through repression of ATM-specific phosphatases, including PP2A.

Hypoxia-Induced ATM Has a Role to Play in DNA Replication

The dependence on H3K9me3 for ATM activation in response to RS in hypoxia led us to hypothesize that ATM could be involved in facilitating replication through H3K9me3-rich regions. To address this possibility, we first investigated the specific localization of H3K9me3 using the recently described iPOND (isolation of proteins on nascent DNA) technique (Sirbu et al., 2012). This technique allowed us to determine whether the hypoxia-induced increase in H3K9me3 was also associated with or around replication forks (Figure S3A). The exposure to EdU was optimized to ensure equal amounts of DNA were pulled down in each condition (Figure S3B). Thymidine-chase experiments were also carried out to ensure specific enrichment of H3K9me3 in areas associated with the replication fork (Figure S3C). iPOND analysis demonstrated that there was an increase in H3K9me3 in the vicinity of the replication fork in hypoxia (<0.1% O₂). PCNA was used as a replication fork-associated control (Figure 3A). The presence of H3K9me3 and associated heterochromatic factors around the replication fork could contribute to hypoxia-induced RS and potentially lead to a requirement for ATM activity in order to preserve fork integrity. To test this hypothesis, RKO cells were exposed to hypoxia in the presence of the ATM inhibitor and the accumulation of DNA damage was measured by increased 53BP1 focus formation. Whereas no significant increase in damage was seen in KU-55933-treated cells in normoxia, increased DNA damage was observed in hypoxic cells treated with KU-55933 (Figure 3B). Similar results were obtained upon siRNA-mediated depletion of ATM (Figure S3D). Furthermore, ATM inhibition or knockdown following exposure to 2% O₂ (where no RS occurs) did not lead to a significant increase in DNA damage, suggesting that the role of ATM in preserving fork integrity in hypoxia is restricted to conditions in which RS is observed (Figures S3E and S3F). To investigate this further,

H1299 cells expressing Fucci plasmids (Sakaue-Sawano et al., 2008) were used in order to assess the specific cell-cycle phase in which increased 53BP1 foci were observed following exposure to <0.1% O₂. Notably, the majority of the 53BP1 foci observed in hypoxia following treatment with the ATM inhibitor were present in the S phase population (Figure S3G). To validate this finding, RKO cells were labeled with EdU and exposed to hypoxia in the presence or absence of the ATM inhibitor. The majority of the DNA damage detected was present in the EdU-positive/S phase population (Figure 3C). Interestingly, in cells with EdU staining representative of heterochromatin replication in mid/late S phase, the 53BP1 foci clustered in areas thought to be associated with replicating heterochromatin (Nakayasu and Berezney, 1989). These data support the model that loss of ATM activity leads to the accumulation of DNA damage in hypoxic conditions associated with RS and an H3K9me3-rich environment.

It is important to note that the iPOND analysis also showed a dramatic reduction in H3K9me3 in response to reoxygenation, indicating a dynamic oxygen-dependent regulation of this chromatin modification. Therefore, we predicted that loss of ATM during reoxygenation-induced replication restart would have no effect on the DNA damage levels. In support of this, we saw no increase in reoxygenation-induced DNA damage following ATM inhibition (Figure 3B). These findings suggest that replication should be unaffected by ATM inhibition when H3K9me3 is reduced during reoxygenation-induced replication restart. Notably, DNA fiber analysis showed that in conditions of reoxygenation, replication restart was unaffected by ATM inhibition. Surprisingly, however, following reoxygenation the number of new origins increased in the presence of the ATM inhibitor (Figure 3D; Figure S3H). Importantly, inhibition of ATM under normoxic conditions had a nonsignificant effect on origin firing, pointing to a stress-specific role for ATM in regulating appropriate replication restart in hypoxic conditions (Figure 3E).

The data so far pointed to a role for ATM in facilitating replication in hypoxia where there is an increase in H3K9me3. We measured replication rates in cells in either normoxia or during hypoxia (<0.1% O₂) following ATM inhibition/knockdown (Figure 3F; Figures S3I and S3J). Loss of ATM did not have a significant effect on replication rates in normoxia. In contrast, replication rates were significantly slower in hypoxic cells treated with the ATM inhibitor compared to the hypoxic controls. These data demonstrate a stress-specific role for ATM in DNA replication.

Replication Stress in Hypoxic Conditions Leads to ATM Activation

Our data demonstrate that ATM responds to RS when it occurs in hypoxic cells enriched for H3K9me3. This model suggests that exposure to alternative sources of RS in an environment including increased H3K9me3 would also induce ATM activity. To address this, we used a combination of 2% O₂ (because it would lead to H3K9me3 in the absence of RS) and Hu. Importantly, acute Hu treatment in normoxia does not increase H3K9me3 levels either globally (Figure 4A) or at the replication fork (Figure S4A). As predicted, neither 2% O₂ alone nor a 6 hr exposure to Hu in normoxia induced ATM-S1981 or

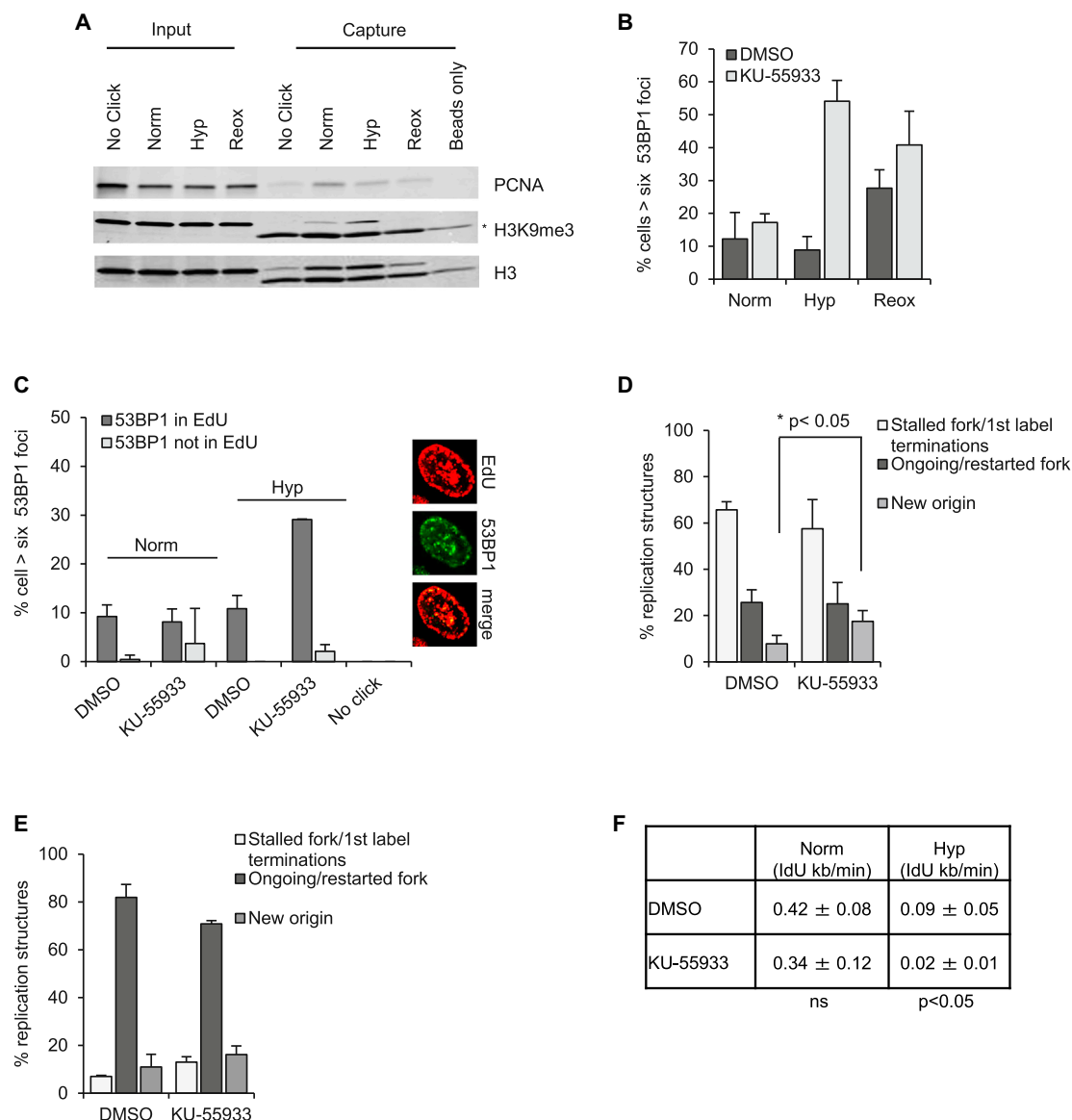


Figure 3. Hypoxia-Induced ATM Has a Role in Replication

(A) iPOND was carried out following exposure of cells to either Norm (21% O₂), Hyp (6 hr, <0.1% O₂), or Reox (6 hr, <0.1% O₂ followed by 2 hr of 21% O₂). The asterisk denotes a nonspecific band observed in all lanes at the end of the gel.

(B) RKO cells were treated with DMSO or KU-55933 and exposed to Norm (21% O₂), Hyp (<0.1% O₂, 6 hr), or Reox (6 hr, <0.1% O₂ followed by 1 hr of 21% O₂). The quantification of 53BP1 foci counted in three independent experiments is shown.

(C) RKO cells were labeled with EdU, treated with DMSO or KU-55933, and exposed to Norm (21% O₂) or Hyp (<0.1% O₂, 3 hr). EdU labeling times were 1 hr for Norm and 3 hr for Hyp conditions.

(D) RKO cells were treated with DMSO or KU-55933 and exposed to hypoxia (<0.1% O₂, 5 hr) followed by 1 hr of reox (21% O₂). DNA fibers were scored.

(E) RKO cells were treated as above but in normoxia (21% O₂).

(F) The replication rates of cells treated with DMSO or KU-55933 and exposed to Norm (21% O₂) or Hyp (<0.1% O₂, 6 hr) were scored. An average of the average IdU incorporation rates for each of three independent experiments is shown. ns, nonsignificance.

Data are represented as mean ± SEM. See also Figure S3.

KAP-1-S824 phosphorylation. In contrast, a notable induction of both ATM-S1981 and KAP-1-S824 was seen when the Hu-treated cells were incubated at 2% O₂ (Figure 4A). As expected, the combination of Hu and 2% O₂ had led to RS, as shown by increased numbers of RPA32 foci (Figures S4B

and S4C), but without resulting in the accumulation of DNA damage (Figure 4B). In order to further investigate the finding that RS can induce ATM activity in a hypoxic chromatin context, we made use of an alternative method to induce RS. We asked whether oncogene-induced RS in hypoxic

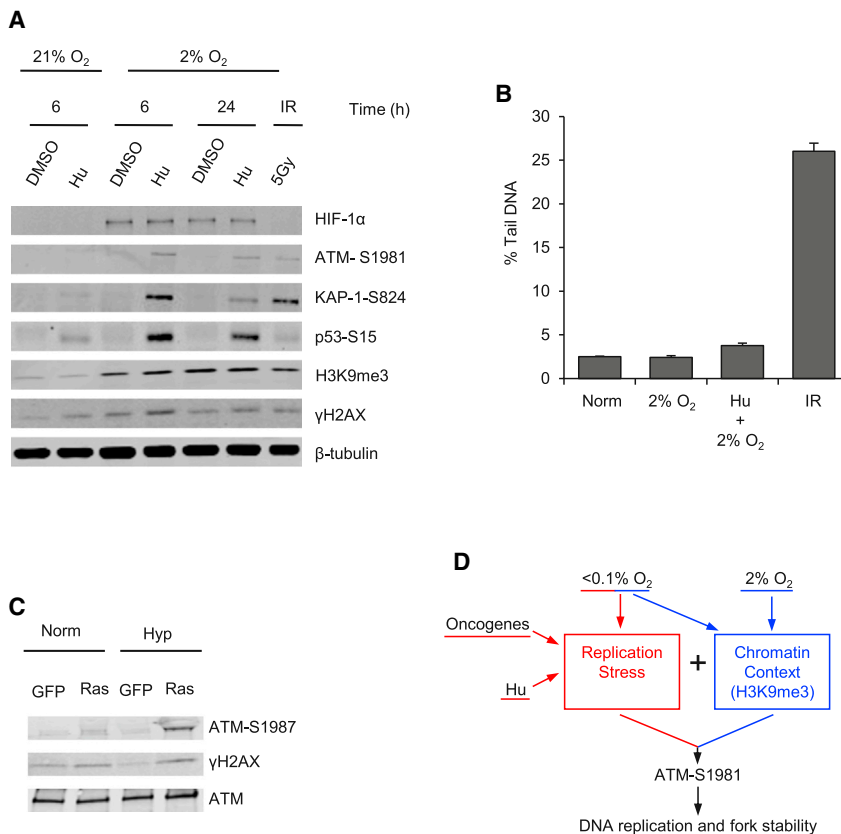


Figure 4. Both Hu- and Oncogene-Induced Replication Stress Lead to ATM Activation in Hypoxic Conditions

(A) RKO cells were treated as indicated. Hu (6 hr) was used in either normoxia (21% O₂) or hypoxia (2% O₂). WB was carried out; β-tubulin was the loading control.

(B) RKO cells were exposed to the O₂ concentrations indicated for a period of 6 hr. Hu was added where indicated for 6 hr. Cells exposed to IR (5 Gy) were lysed immediately after IR. Shown is the % comet tail DNA. Data are represented as mean ± SEM.

(C) p53^{-/-} MEFs with overexpressed mutant K-Ras or GFP were exposed to Norm (21% O₂) or Hyp (2% O₂, 6 hr). WB was carried out as indicated.

(D) RS in the absence of DNA damage induced by either hypoxia (<0.1% O₂), oncogene expression, or Hu treatment leads to ATM activation and downstream signaling only in the hypoxia-induced chromatin context. As demonstrated, one of the critical components of this chromatin context is H3K9me3. Once active, ATM is involved in maintaining DNA replication. See also Figure S4.

conditions, in the absence of DNA damage, might lead to an enhanced DDR activation. Mutant K-Ras was overexpressed in cells exposed to normoxia or hypoxia (2% O₂) for 6 hr (Figures S4D and S4E). Consistent with our hypothesis, we only observed significant ATM phosphorylation when mutant K-Ras was overexpressed in p53^{-/-} primary MEFs in hypoxic (2% O₂) conditions (Figure 4C). The p53^{-/-} background was chosen to avoid the entry of cells into senescence upon overexpression of the oncogene. Collectively, these data support a model whereby ATM only responds to RS in the absence of DNA damage if it occurs in a chromatin context associated with hypoxia and specifically increased H3K9me3 (Figure 4D). These data suggest that hypoxia and RS may work together in mediating an early barrier to tumorigenesis, and that such a barrier may be initiated by ATM activation even in the absence of detectable damage.

DISCUSSION

Hypoxia (<0.1% O₂) induces ATM activity in response to RS in a context that includes elevated H3K9me3. The induction of ATM in these specific situations pointed to a potential role for ATM in resolving RS. In response to the absence or inhibition of ATM activity, hypoxic S phase cells accumulated significant levels of DNA damage. This could suggest a kinase-dependent role for ATM in protecting replication structures in areas rich in factors associated with heterochromatin. A possible mechanism

for this could be through hypoxia-induced ATM-dependent phosphorylation of KAP-1, which has a characterized role in chromatin relaxation (Goodarzi et al., 2011; Ziv et al., 2006). The pan-nuclear localization of ATM and KAP-1 phosphorylation in hypoxia could reflect the need to globally facilitate chromatin relaxation given the widespread localization of H3K9me3 in hypoxia (Figure 2C). Alternatively, ATM-mediated signaling to additional targets may also be relevant, such as CHK1/CHK2. We have shown previously that in hypoxic conditions, CHK1 phosphorylates and inactivates TLK1, which has a role in repressing the histone chaperone ASF1 (Groth et al., 2003; Pires et al., 2010). Interestingly, in conditions of RS, ASF1 buffers evicted H3K9me3 and, therefore, loss of ASF1 during hypoxia-induced RS could contribute to the increased levels of H3K9me3 that we observed around replication forks (Jasencakova et al., 2010). Most interestingly, we determined that inhibition of ATM activity in hypoxic conditions led to decreased DNA replication rates. Together, these data demonstrate a stress-specific role for ATM in facilitating DNA replication during RS.

In addition, we have identified a stress-responsive role for ATM in replication restart. Inhibition of ATM increased origin firing during reoxygenation-induced replication restart whereas having no effect on normal replication. Given that no new origins are licensed once cells have entered S phase, the increase in origin firing following ATM inhibition likely reflects an increase in the firing of dormant origins (Ge and Blow, 2010). This would suggest a role for ATM in regulating dormant origin firing in response to replication restart following RS in the absence of DNA damage.

We observed a global increase in H3K9me3 occurring in response to both 2% as well as <0.1% O₂. This increase, however, was more prominent in response to <0.1% O₂, and coincided with increased protein levels of some of the principal H3K9 methyltransferases. MDM2 has been shown to posttranslationally downregulate methyltransferases such as Suv39h1 (Mungamuri et al., 2012). It is possible that decreased MDM2 expression in hypoxia contributes to the stabilization of some of the histone methyltransferases under these conditions (Alarcón et al., 1999). Furthermore, the decreased activity of the 2-oxoglutarate-dependent family of histone demethylases under hypoxic conditions undoubtedly contributes to the hypoxia-induced H3K9me3, although this was not investigated in this study (Sánchez-Fernández et al., 2013). We also observed that hypoxia-induced H3K9me3 is enriched on PP2A-C. These data indicate that H3K9me3 could serve to negatively regulate PP2A following hypoxia-induced RS, thereby contributing to ATM phosphorylation and activation. It is also possible that ATR-mediated signaling contributes to hypoxia-induced ATM activation (Stiff et al., 2006).

Recent reports have demonstrated a role for TIP60 in facilitating ATM activation particularly in regions of heterochromatin in response to IR (Kaidi and Jackson, 2013; Sun et al., 2009). However, Price and colleagues showed a dependency on MRN to recruit the ATM-TIP60 complex to sites of irradiation-induced DSBs. Given the absence of DSBs and the lack of requirement for the MRN complex in ATM activation in hypoxia, it seems unlikely this mechanism is involved in hypoxia-induced ATM activation (Bencokova et al., 2009; Cam et al., 2010).

Our model supports the theory that the chromatin context in which RS occurs can strongly influence the DDR. A role for Suv39h1 in preventing Ras-induced lymphoma development in vivo has already been demonstrated, supporting the concept that Suv39h1/2-mediated regulation of ATM activity in a hypoxic context could halt tumorigenesis in response to oncogenic stress (Braig et al., 2005). We propose that the hypoxia-induced chromatin context could have a role to play in facilitating the activation of the DDR (and in particular ATM) in response to RS before DNA damage accumulates. Therefore, cells proliferating at an increased rate due to oncogenic activation in a hypoxic environment would activate ATM independent of the levels of DNA damage. This implies that the barrier to tumorigenesis induced by the DDR in hypoxic preneoplastic lesions could occur earlier than predicted and, most importantly, before the accumulation of damage in response to oncogene activation (Bartkova et al., 2005; Gorgoulis et al., 2005). The hypoxia-dependent induction of the DDR early in tumorigenesis will significantly contribute to the selective pressure to lose/mutate genes such as ATM or p53 and therefore drive development of therapy-resistant disease (Graeber et al., 1996; Squatrito et al., 2010).

EXPERIMENTAL PROCEDURES

Cell lines, treatments, and details of siRNA and oligonucleotide sequences used are available in the [Supplemental Experimental Procedures](#). Immunoblotting and immunofluorescence were carried out as previously described

(Bencokova et al., 2009). Comet assays, DNA fiber analysis, and iPOND analysis were carried out as described previously in [Parsons et al. \(2012\)](#), [Pires et al. \(2010\)](#), and [Sirbu et al. \(2012\)](#), respectively, with modifications to allow hypoxic/reoxygenation treatments. Detailed experimental procedures are available in the [Supplemental Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2013.10.019>.

ACKNOWLEDGMENTS

We are grateful to Thomas Jenuwein for the Suv39h1/2 MEFs, Bianca Sirbu and David Cortez for advice on the iPOND procedure, and Amato Giaccia, Vincenzo D'Angiolella, Roger Grand, Wojciech Niedzwiedz, Denise Chan, and Colin Goding for helpful discussions. This work was supported by a Cancer Research UK grant (awarded to E.M.H.) and an MRC Centenary award (awarded to M.M.O.).

Received: June 17, 2013

Revised: August 28, 2013

Accepted: October 7, 2013

Published: November 21, 2013

REFERENCES

- Alarcón, R., Koumenis, C., Geyer, R.K., Maki, C.G., and Giaccia, A.J. (1999). Hypoxia induces p53 accumulation through MDM2 down-regulation and inhibition of E6-mediated degradation. *Cancer Res.* 59, 6046–6051.
- Bakkenist, C.J., and Kastan, M.B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 421, 499–506.
- Bartkova, J., Horejsí, Z., Koed, K., Krämer, A., Tort, F., Zieger, K., Guldberg, P., Sehested, M., Nesland, J.M., Lukas, C., et al. (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 434, 864–870.
- Bencokova, Z., Kaufmann, M.R., Pires, I.M., Lecane, P.S., Giaccia, A.J., and Hammond, E.M. (2009). ATM activation and signaling under hypoxic conditions. *Mol. Cell. Biol.* 29, 526–537.
- Blasius, M., Forment, J.V., Thakkar, N., Wagner, S.A., Choudhary, C., and Jackson, S.P. (2011). A phospho-proteomic screen identifies substrates of the checkpoint kinase Chk1. *Genome Biol.* 12, R78.
- Bolderson, E., Scorch, J., Helleday, T., Smythe, C., and Meuth, M. (2004). ATM is required for the cellular response to thymidine induced replication fork stress. *Hum. Mol. Genet.* 13, 2937–2945.
- Braig, M., Lee, S., Lodenkemper, C., Rudolph, C., Peters, A.H.F.M., Schlegelberger, B., Stein, H., Dörken, B., Jenuwein, T., and Schmitt, C.A. (2005). Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* 436, 660–665.
- Bristow, R.G., and Hill, R.P. (2008). Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability. *Nat. Rev. Cancer* 8, 180–192.
- Cam, H., Easton, J.B., High, A., and Houghton, P.J. (2010). mTORC1 signaling under hypoxic conditions is controlled by ATM-dependent phosphorylation of HIF-1 α . *Mol. Cell* 40, 509–520.
- Ge, X.Q., and Blow, J.J. (2010). Chk1 inhibits replication factory activation but allows dormant origin firing in existing factories. *J. Cell Biol.* 191, 1285–1297.
- Goodarzi, A.A., Jonnalagadda, J.C., Douglas, P., Young, D., Ye, R., Moorhead, G.B., Lees-Miller, S.P., and Khanna, K.K. (2004). Autophosphorylation of ataxia-telangiectasia mutated is regulated by protein phosphatase 2A. *EMBO J.* 23, 4451–4461.
- Goodarzi, A.A., Noon, A.T., and Jeggo, P.A. (2009). The impact of heterochromatin on DSB repair. *Biochem. Soc. Trans.* 37, 569–576.

- Goodarzi, A.A., Kurka, T., and Jeggo, P.A. (2011). KAP-1 phosphorylation regulates CHD3 nucleosome remodeling during the DNA double-strand break response. *Nat. Struct. Mol. Biol.* **18**, 831–839.
- Gorgoulis, V.G., Vassiliou, L.V., Karakaidos, P., Zacharatos, P., Kotsinas, A., Liloglou, T., Venere, M., Ditullio, R.A., Jr., Kastrinakis, N.G., Levy, B., et al. (2005). Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* **434**, 907–913.
- Graeber, T.G., Osmanian, C., Jacks, T., Housman, D.E., Koch, C.J., Lowe, S.W., and Giaccia, A.J. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* **379**, 88–91.
- Groth, A., Lukas, J., Nigg, E.A., Silljé, H.H., Wernstedt, C., Bartek, J., and Hansen, K. (2003). Human Toslled like kinases are targeted by an ATM- and Chk1-dependent DNA damage checkpoint. *EMBO J.* **22**, 1676–1687.
- Guenatri, M., Bailly, D., Maison, C., and Almouzni, G. (2004). Mouse centric and pericentric satellite repeats form distinct functional heterochromatin. *J. Cell Biol.* **166**, 493–505.
- Guo, Z., Deshpande, R., and Paull, T.T. (2010a). ATM activation in the presence of oxidative stress. *Cell Cycle* **9**, 4805–4811.
- Guo, Z., Kozlov, S., Lavin, M.F., Person, M.D., and Paull, T.T. (2010b). ATM activation by oxidative stress. *Science* **330**, 517–521.
- Harper, J.W., and Elledge, S.J. (2007). The DNA damage response: ten years after. *Mol. Cell* **28**, 739–745.
- Hunt, C.R., Pandita, R.K., Laszlo, A., Higashikubo, R., Agarwal, M., Kitamura, T., Gupta, A., Rief, N., Horikoshi, N., Baskaran, R., et al. (2007). Hyperthermia activates a subset of ataxia-telangiectasia mutated effectors independent of DNA strand breaks and heat shock protein 70 status. *Cancer Res.* **67**, 3010–3017.
- Jackson, S.P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. *Nature* **461**, 1071–1078.
- Jasencakova, Z., Scharf, A.N., Ask, K., Corpet, A., Imhof, A., Almouzni, G., and Groth, A. (2010). Replication stress interferes with histone recycling and pre-deposition marking of new histones. *Mol. Cell* **37**, 736–743.
- Johnson, A.B., Denko, N., and Barton, M.C. (2008). Hypoxia induces a novel signature of chromatin modifications and global repression of transcription. *Mutat. Res.* **640**, 174–179.
- Kaidi, A., and Jackson, S.P. (2013). KAT5 tyrosine phosphorylation couples chromatin sensing to ATM signalling. *Nature* **498**, 70–74.
- Loyola, A., Tagami, H., Bonaldi, T., Roche, D., Quivy, J.P., Imhof, A., Nakatani, Y., Dent, S.Y., and Almouzni, G. (2009). The HP1 α -CAF1-SetDB1-containing complex provides H3K9me1 for Suv39-mediated K9me3 in pericentric heterochromatin. *EMBO Rep.* **10**, 769–775.
- Melvin, A., and Rocha, S. (2012). Chromatin as an oxygen sensor and active player in the hypoxia response. *Cell. Signal.* **24**, 35–43.
- Mungamuri, S.K., Benson, E.K., Wang, S., Gu, W., Lee, S.W., and Aaronson, S.A. (2012). p53-mediated heterochromatin reorganization regulates its cell fate decisions. *Nat. Struct. Mol. Biol.* **19**, 478–484.
- Murga, M., Campaner, S., Lopez-Contreras, A.J., Toledo, L.I., Soria, R., Montaña, M.F., D'Artista, L., Schleker, T., Guerra, C., Garcia, E., et al. (2011). Exploiting oncogene-induced replicative stress for the selective killing of Myc-driven tumors. *Nat. Struct. Mol. Biol.* **18**, 1331–1335.
- Nakayasu, H., and Berezney, R. (1989). Mapping replicational sites in the eucaryotic cell nucleus. *J. Cell Biol.* **108**, 1–11.
- Nielsen, P.R., Nietlispach, D., Mott, H.R., Callaghan, J., Bannister, A., Kouzarides, T., Murzin, A.G., Murzina, N.V., and Laue, E.D. (2002). Structure of the HP1 chromodomain bound to histone H3 methylated at lysine 9. *Nature* **416**, 103–107.
- Parsons, J.L., Khoronenkova, S.V., Dianova, I.I., Ternette, N., Kessler, B.M., Datta, P.K., and Dianov, G.L. (2012). Phosphorylation of PNKP by ATM prevents its proteasomal degradation and enhances resistance to oxidative stress. *Nucleic Acids Res.* **40**, 11404–11415.
- Pires, I.M., Bencokova, Z., Milani, M., Folkes, L.K., Li, J.L., Stratford, M.R., Harris, A.L., and Hammond, E.M. (2010). Effects of acute versus chronic hypoxia on DNA damage responses and genomic instability. *Cancer Res.* **70**, 925–935.
- Sakaue-Sawano, A., Kurokawa, H., Morimura, T., Hanyu, A., Hama, H., Osawa, H., Kashiwagi, S., Fukami, K., Miyata, T., Miyoshi, H., et al. (2008). Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* **132**, 487–498.
- Sánchez-Fernández, E.M., Tarhonskaya, H., Al-Qahtani, K., Hopkinson, R.J., McCullagh, J.S., Schofield, C.J., and Flashman, E. (2013). Investigations on the oxygen dependence of a 2-oxoglutarate histone demethylase. *Biochem. J.* **449**, 491–496.
- Schultz, D.C., Ayyanathan, K., Negorev, D., Maul, G.G., and Rauscher, F.J., III. (2002). SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes Dev.* **16**, 919–932.
- Shreeram, S., Demidov, O.N., Hee, W.K., Yamaguchi, H., Onishi, N., Kek, C., Timofeev, O.N., Dudgeon, C., Fornace, A.J., Anderson, C.W., et al. (2006). Wip1 phosphatase modulates ATM-dependent signaling pathways. *Mol. Cell* **23**, 757–764.
- Sirbu, B.M., Couch, F.B., and Cortez, D. (2012). Monitoring the spatiotemporal dynamics of proteins at replication forks and in assembled chromatin using isolation of proteins on nascent DNA. *Nat. Protoc.* **7**, 594–605.
- Squatrito, M., Brennan, C.W., Helmy, K., Huse, J.T., Petri, J.H., and Holland, E.C. (2010). Loss of ATM/Chk2/p53 pathway components accelerates tumor development and contributes to radiation resistance in gliomas. *Cancer Cell* **18**, 619–629.
- Stiff, T., Walker, S.A., Cerosaletti, K., Goodarzi, A.A., Petermann, E., Concannon, P., O'Driscoll, M., and Jeggo, P.A. (2006). ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. *EMBO J.* **25**, 5775–5782.
- Sun, Y., Jiang, X., Xu, Y., Ayrapetov, M.K., Moreau, L.A., Whetstone, J.R., and Price, B.D. (2009). Histone H3 methylation links DNA damage detection to activation of the tumour suppressor Tip60. *Nat. Cell Biol.* **11**, 1376–1382.
- Toledo, L.I., Murga, M., Zur, R., Soria, R., Rodriguez, A., Martinez, S., Oyarzabal, J., Pastor, J., Bischoff, J.R., and Fernandez-Capetillo, O. (2011). A cell-based screen identifies ATR inhibitors with synthetic lethal properties for cancer-associated mutations. *Nat. Struct. Mol. Biol.* **18**, 721–727.
- Ziv, Y., Bielopolski, D., Galanty, Y., Lukas, C., Taya, Y., Schultz, D.C., Lukas, J., Bekker-Jensen, S., Bartek, J., and Shiloh, Y. (2006). Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. *Nat. Cell Biol.* **8**, 870–876.