

The Role of the Transcription Factor BRN2 in Melanoma

Thesis submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy in Biomedical and Clinical Sciences

Dr. Katharine Herbert MBChB (hons)
Green Templeton College, University of Oxford
Hilary Term 2018

Primary supervisor: Professor Colin Goding
Secondary supervisor: Professor Mark Middleton

Ludwig Institute for Cancer Research
Nuffield Department of Medicine
University of Oxford

Funding: Wellcome Trust Clinician Research Training Fellowship

Disclaimer

I declare that this submission is my own written work. To the best of my knowledge, it contains no material that has been written by another, with the exception of the methods relating to our collaborators' work as detailed below. Nor has it been previously submitted in the same or a different form as academic work, to this or any other university.

The results presented derive from my own experiments, with the following exceptions: Figures 3.3.1a, 3.3.3 and 3.4.2 (data derived from experiments by Jean-Philippe Lambert, Lunenfeld-Tanenbaum Research Institute, Toronto); Figure 3.6.1 (RNA-Seq analysis and associated methods by Pakavarin Louphrasitthiphol and Jagat Chuahan, Goding Lab); Figure 3.8.2 (*in silico* modelling done in conjunction with Guy Williamson); Figures 3.9.1 and 3.9.2 (work and associated methods done by Carla Daniela Robles-Espinoza and Nicola Roberts; Sanger Institute, Cambridge)

Katharine Herbert

Hilary Term 2018

Abstract

The Role of the Transcription Factor BRN2 in Melanoma

Katharine Herbert, Green Templeton College
DPhil Biomedical and Clinical Sciences, Hilary Term 2018

Expression of the POU-domain transcription factor BRN2 is crucial for the developing brain and drives proliferation and invasiveness in cancers of neural crest origin including melanoma. However, our understanding of BRN2 function and how its behaviour is modulated remains incomplete. We therefore aimed to investigate how protein:protein interaction and post-translational modification might influence the activity of BRN2 and how this impacts on melanoma cell phenotype.

We show that, surprisingly, BRN2 binds DNA-damage response proteins rather than transcriptional co-factors and that following UVB irradiation, BRN2 rescues cells from apoptosis. Expression of apoptosis-induction genes in response to UVB is enhanced following knockdown of BRN2. Significantly BRN2 expression in tumours also correlates with an increased mutational burden. Mechanistically, BRN2 is rapidly recruited to DNA-damage sites in a PARP-dependent manner and requires DNA binding for efficient recruitment and rescue from apoptosis. Furthermore, post-translational modification of the transactivating domain of BRN2 by the UV-induced p38 stress kinase modifies the recruitment kinetics of BRN2 and its DNA binding ability, and p38 signalling disrupts a key BRN2 protein:protein interaction.

Our findings identify a novel role for BRN2 in promotion of survival of melanoma cells in the face of genomic insult. These findings help us to understand the mechanisms that melanoma cells employ to tolerate high levels of genome instability with potential implications for neuronal development and tumours such as small cell lung cancer and neuroendocrine prostate cancer.

Acknowledgements

I would like to thank my supervisor Professor Colin Goding for giving me the opportunity to work in his lab and for his guidance, ideas and feedback over the course of my DPhil. I am indebted to my lab colleagues for teaching me so many different experimental techniques. I owe a special debt of gratitude to my friend, colleague and mentor Dr. Paola Falletta who has been instrumental in guiding my scientific development. She has taught me most of the methods used in my thesis and provided unending support in planning, executing and analysing experiments whilst teaching me Italian and keeping me motivated. Thanks also to Dr. Romuald Binet for helping me approach the DNA damage questions; Dr. Rasmus Freter for teaching me about protein purification and kinase assays and Dr. Paka Louphrasittiphol for his help with RNA-Seq. I am grateful to have had such a fun and supportive environment to work in and thank all the Goding lab members, past and present, for making it so enjoyable. With particular thanks to Dr. Paola Falletta, Dr. Hans Friedrichsen, Dr. Yurena Vivas Garcia, Dr. Luis Sanchez del Campo Ferrer, Dr. Kao Chin Ngeow, Dr. Jenny Thomas, Helen Middleton, Dr. Ioanna Ledaki, Sizhu Lu, Laura Mosteo Lopez, Richard Lisle and Diogo Dias for their advice, support and friendship.

I am grateful to the Ludwig Institute for Cancer Research for the wonderful facilities and support, particularly Mark Shipman and the microscope facilities. I am grateful to our collaborators for bringing their expertise to address my experimental questions: JP Lambert from the Lunenfield-Tanenbaum institute in Toronto and Daniela Robles-Espinoza at the Sanger Institute in Cambridge. I would also like to thank my

second supervisor Professor Mark Middleton for funding support and his ongoing mentorship.

I am grateful to the Wellcome Trust for funding my Clinician Research Training Fellowship which has provided me with precious time and resources to enable me to focus fully on lab research whilst out of programme from my clinical training. I am extremely grateful to Professor Paul Klenerman and his colleagues for giving me this opportunity by awarding me the fellowship. Thanks also to Green Templeton College and my College Advisor Professor Ken Reid.

I am fortunate to have such a good support network away from the lab, particularly my parents Alicia and Martin, who have boldly proofread my entire thesis. I would like to thank Patrick, Rachel and Jen for their friendship and help with our chaotic menagerie: Donny, Rebus and Sheldon. Finally, to my husband Guy, I could not have done this without your unwavering support, inspiration, love and advice. Thank you for everything, I'm looking forward to moving on to the next adventure with you and our boys.

Table of Contents

Disclaimer	i
Abstract	ii
Acknowledgements	iii
Table of Contents	v
List of Figures	ix
List of Tables	xii
List of Abbreviations	xiii
Chapter 1 Introduction	1
1.1 Phenotypic Heterogeneity in Development and Cancer	1
1.1.1 Introduction	1
1.1.2 Neural-Crest Migration and Cancer Metastasis	1
1.1.3 Tumour Heterogeneity	3
1.1.4 Mechanisms of Phenotype Control	6
1.2 Transcription Factors and Their Control Mechanisms	7
1.2.1 Introduction	7
1.2.2 Protein-Protein Interaction and Chromatin Remodelling	7
1.2.3 Post-Translational Modification	9
1.3 POU Domain Transcription Factors	13
1.3.1 Introduction	13
1.3.2 The POU-Domain Protein Family	13
1.3.3 DNA Binding by POU Domain Proteins	16
1.3.4 POU Domain and BRN2 Post-Translational Modification	18
1.3.5 POU-Domain Protein Interactors	21
1.4 BRN2 In Development and Disease	24
1.4.1 Introduction	24
1.4.2 BRN2 in Cancer	24
1.4.3 BRN2 in Development	28
1.5 UV Radiation and the Melanocyte-Lineage Response	33
1.5.1 Introduction	33
1.5.2 UV Radiation	33

1.5.3 UVB-induced DNA Damage	34
1.5.4 UVA-induced DNA Damage	36
1.5.5 The DNA Damage Response	39
1.5.6 UV Activation of Stress Kinases	43
1.5.7 UV Response in Melanocytes and Melanoma	48
1.5.8 UV-Induced DNA Damage and Genomic Instability in Melanoma	50
1.6 Summary	52
1.7 Aims	53
Chapter 2 Materials and Methods	54
2.1 DNA methods	54
2.1.1 Restriction Enzyme Sub-Cloning	54
2.1.2 Site-Directed Mutagenesis	56
2.1.3 Dot-Blot Assay	57
2.2 RNA methods	63
2.2.1 RNA Seq Sample Preparation	63
2.3 Cell methods	63
2.3.1 Cell Culture	63
2.3.2 Transient Transfection	64
2.3.3 Stable Transfection - Piggybac	64
2.3.4 Stable Overexpression – Lentiviral Cell Lines	65
2.3.5 RNA interference	65
2.3.6 UV Irradiation	66
2.3.7 λ Phosphatase Treatment	66
2.4 Cell-based assays	67
2.4.1 Luciferase Assay	67
2.4.2 Flow Cytometry	67
2.4.3 Mitotic Shake	68
2.4.4 Laser Microirradiation (LMI), Fixed and Live Cells	69
2.4.5 Immunofluorescence	70
2.5 Protein methods	74
2.5.1 Whole Cell Extracts	74
2.5.2 FLAG Immunoprecipitation	74
2.5.3 Bacterially-Purified Protein Production	75
2.5.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)	76
2.5.5 Coomassie Protein Staining	76
2.5.6 Western Blotting	77

2.5.7 Phos-Tag™ Gel Electrophoresis	77
2.5.8 <i>in vitro</i> Kinase Assay (IVKA)	78
2.5.9 Electrophoretic Mobility Shift Assay (EMSA)	78
2.6 Proteomics	80
2.6.1 Affinity Purification–Mass Spectrometry (AP-MS)	80
2.6.2 Phosphoproteomics	80
2.7 <i>in silico</i> methods	82
2.7.1 Sequence Alignment	82
2.7.2 3D Structure Analysis	82
2.7.3 BRN2 Interactome Analysis	83
2.7.4 Gene Ontology	83
2.7.5 RNA-Seq Analysis	84
2.7.6 GSEA and GSVA analyses	84
2.7.7 TCGA Bioinformatics Analysis	84
Chapter 3 Results	87
3.1 Introduction	87
3.2 Generating A Stable-Inducible BRN2_FLAG Cell Line	88
3.3 Identifying BRN2 Binding Partners by AP-MS	92
3.4 BRN2 Binds to DDR Proteins Via its DNA Binding Domain	98
3.5 BRN2 Protects Melanoma Cells from UVB-Induced Apoptosis	103
3.6 BRN2 Expression Correlates with Genes Involved in Apoptosis	123
3.7 BRN2 Is Recruited to DNA Damage in a PARP Dependent Manner	127
3.8 Motif-Specific DNA Binding Is Required for BRN2 Recruitment and Apoptosis Rescue	134
3.9 BRN2 Expression <i>in vivo</i> Correlates with Genomic Instability	145
Chapter 4 Results	149
4.1 Introduction	149
4.2 BRN2 Can Be Phosphorylated by Stress-Activated Protein Kinases	150
4.3 p38 Activation Results in BRN2 Phosphorylation <i>in cellulo</i>	163
4.4 p38 Phosphorylation Disrupts BRN2 Association with DDR Proteins	170

4.5 BRN2 N-Terminal Region Inhibits the Function of the POU Domain and Is Modulated by Phosphorylation	177
Chapter 5 Discussion	183
5.1 Introduction	183
5.2 BRN2 Suppresses UVB-Induced Apoptosis in Melanoma Cells	185
5.3 BRN2 Associates with DNA Damage Response Pathways	191
5.4 p38-Mediated Phosphorylation Modulates BRN2 Function	199
5.5 A Proposed Model for Dynamic Regulation of BRN2 Activity	204
5.6 BRN2 and Genome Instability	208
5.7 Conclusions	210
References	213

List of Figures

Figure 1.1.1 Tumour heterogeneity drives therapy resistance and metastasis	5
Figure 1.3.1 BRN2 (POU3F2)	15
Figure 1.3.2 BRN2 phosphorylation sites	20
Figure 1.5.1 UV-induced DNA damage and the DNA damage response	38
Figure 1.5.2 Initiation of the DDR - recruitment of DDR proteins to sites of damage	42
Figure 3.2.1 Development of stable, inducible BRN2 WT _FLAG cell lines	89
Figure 3.2.2 BRN2 subcellular localisation is dependent on expression levels	91
Figure 3.3.1 BRN2 binding partners identified by AP-MS	93
Figure 3.3.2 BRN2 binds to proteins involved in the DNA damage response	95
Figure 3.3.3 Summary figure of BRN2 binding partners	97
Figure 3.4.1 Generation of BRN2 truncation mutant cell lines	99
Figure 3.4.2 BRN2 truncation mutant binding partners identified by AP-MS	100
Figure 3.4.3 DNA repair pathway remains overrepresented in truncation mutant binding partners	101
Figure 3.5.1 Validation of siRNA against BRN2	104
Figure 3.5.2 CPD dynamics are not affected by BRN2 expression levels	106
Figure 3.5.3 BRN2 KD increases persistence of γ H2AX after UVB irradiation	107
Figure 3.5.4 BRN2 KD does not affect cell cycle distribution	109
Figure 3.5.5 BRN2 KD increases persistence of γ H2AX after UVB irradiation	110
Figure 3.5.6 H2AX is phosphorylated in all cell cycle phases, independently of BRN2 levels	112
Figure 3.5.7 BRN2 KD increases susceptibility to cell death after UVB irradiation	114
Figure 3.5.8 BRN2 overexpression delays G1 progression	115
Figure 3.5.9 Knockdown of BRN2 does not significantly affect MITF levels	117
Figure 3.5.10 Overexpression of BRN2 represses MITF	118

Figure 3.5.11 BRN2 overexpression reduces persistence of γ H2AX after UVB irradiation	120
Figure 3.5.12 Overexpression of BRN2 rescues melanoma cells from UVB-induced cell death	121
Figure 3.6.1 DNA damage-induced apoptotic pathway upregulation is increased following BRN2 knockdown	124
Figure 3.6.2 Knockdown of BRN2 alters expression of apoptosis-associated genes in response to UVB	125
Figure 3.7.1 Optimisation of laser micro-irradiation (LMI)	128
Figure 3.7.2 Endogenous BRN2 is recruited to sites of DNA Damage in a PARP-dependent manner	129
Figure 3.7.3 PARP inhibition reduces interaction between BRN2 and Ku80 following damage	130
Figure 3.7.4 BRN2 recruitment to sites of damage is abolished by 20 minutes	131
Figure 3.7.5 BRN2 recruitment is PARP dependent and transient	133
Figure 3.8.1 BRN2 DNA binding domain and contact residues are conserved across paralogues	134
Figure 3.8.2 N442A mutation in Oct-1 is predicted to break H bonds with DNA	135
Figure 3.8.3 N401A mutation in BRN2 inhibits DNA binding in vitro	136
Figure 3.8.4 N401A mutation in BRN2 fails to repress MITF promoter	137
Figure 3.8.5 Intensity of BRN2 recruitment is tag-dependent	139
Figure 3.8.6 N401A mutation in BRN2 inhibits recruitment to sites of damage	141
Figure 3.8.7 Generation of a stable BRN2N401A cell line	142
Figure 3.8.8 BRN2N401A fails to rescue melanoma cells from UVB-induced apoptosis	144
Figure 3.9.1 BRN2 expression in tumours correlates positively with increased mutational burden	146
Figure 3.9.2 Correlation of SNV burden to BRN2 is specific	148
Figure 4.2.1 UVB irradiation activates DNA damage and stress response pathways	150
Figure 4.2.2 Sequence homology between BRN2 orthologues	152
Figure 4.2.3 Schemata of BRN2 mutants to be used in IVKAs	154

Figure 4.2.4 p38 can phosphorylate BRN2	156
Figure 4.2.5 p38 can phosphorylate residues 91S, 96S and 442T	157
Figure 4.2.6 JNK can phosphorylate BRN2	159
Figure 4.2.7 JNK can phosphorylate residue 442T	160
Figure 4.2.8 ERK can phosphorylate BRN2	161
Figure 4.2.9 Non-specific band in ERK IVKA impairs interpretation	162
Figure 4.3.1 Optimisation of H ₂ O ₂ as an activator of the p38 pathway	164
Figure 4.3.2 BRN2 is phosphorylated following p38 pathway activation	166
Figure 4.3.3 Phosphoproteomics of BRN2	168
Figure 4.4.1 BRN2 association with Ku80 is not modulated by p38 activation	170
Figure 4.4.2 p38 inhibitor validation	171
Figure 4.4.3 p38 inhibition interrupts BRN2:Ku80 interaction	173
Figure 4.4.4 p38 inhibition blocks stabilisation of BRN2 binding to Ku80	174
Figure 4.4.5 p38 inhibition alters the dynamics of BRN2 recruitment post-LMI	176
Figure 4.5.1 BRN2 N-terminal impedes DNA binding	178
Figure 4.5.2 Proposed model for BRN2 activation by stress response kinases	179
Figure 4.5.3 Non-phosphorylatable BRN2 binds less well to Ku80 than WT	181
Figure 4.5.4 Phosphorylated BRN2 binds DNA with higher abundance	182

List of Tables

Table 1 Restriction enzyme sub-cloning for <i>in cellulo</i> assays	59
Table 2 Restriction enzyme sub-cloning for <i>in vitro</i> assays	60
Table 3 Primers for Site Directed Mutagenesis	62
Table 4 Drugs used in cell culture	66
Table 5 Primary Antibodies	72
Table 6 Secondary Antibodies	73
Table 7 BRN2 orthologues	82
Table 8 Human BRN2 paralogues	82

List of Abbreviations

6-4pp	6-4 photoproducts
7-DHC	7-dehydrocholesterol
AADC	Aromatic Lammino Acid Decarboxylase (DDC gene)
Ab	Antibody
ALC1	Amplified in Liver Cancer 1 (CHD1L gene)
AP1	Activator Protein 1 transcription factor (JUN)
AP-MS	Affinity Purification Mass Spectrometry
Ascl1/Mash1	Achaete-Scute Family bHLH transcription factor 1
ATF2	Activating Transcription Factor 2
ATM	Ataxia Telangiectasia mutated kinase
ATP	Adenosine Triphosphate
ATR	Ataxia Telangiectasia and Rad3 related kinase
ATRIP	ATR interacting protein
<i>B. taurus</i>	<i>Bos taurus</i>
Bax	Bcl-2 associated X protein
BCL2	Apoptosis regulateor Bcl-2
BER	Base Excision Repair
bHLH	basic-helix-loop-helix
BMP	Bone Morphogenetic Protein
bp	base pair
BRCA1/2	Breast Cancer Susceptibility Protein 1/2
BRN1-5	Brain transcription factors
BRN1	POU3F3
BRN2	POU3F2
BRN3a	POU4F1
BRN3b	POU4F2
BRN4	POU3F4
BRN5	POU6F1
C terminal	Carboxy-terminus
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>

C>T	Cytosine to Thymine
CAK	CDK activating kinase
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CDK	Cyclin Dependent Kinase
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
CFC	Chlorofluorocarbon
ChIP	Chromatin immunoprecipitation
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
co-IP	co-Immunoprecipitation
CPD	Cyclobutane pyrimidine dimers
CREB	cAMP Responsive Element Binding Protein
CRH	Corticotrophin releasing hormone
CSA/B	Cockayne Syndrome proteins A/B
CST	Cell Signalling Technology
CTD	Carboxy-terminal domain
CTP	Cytidine Triphosphate
<i>D. melanogaster</i>	<i>Drosophila melanogaster</i>
<i>D. rerio</i>	<i>Danio rerio</i>
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DCT	Dopachrome Tautomerase
DDR	DNA Damage Response
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DNAPK	DNA protein kinase
Dox	Doxycycline
DSB	Double-strand break
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ECL	Enhanced Chemiluminescence

EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial to mesenchymal transition
ER	Estrogen Receptor
ES cells	Embryonic Stem Cells
FACS	Fluorescence-activated cell sorting
FAS	Fas Cell Surface Death Receptor
FDR	False Discovery Rate
FGF	Fibroblast Growth Factor
FITC	Fluorescein isothiocyanate
FoxD3	Forkhead Box D3
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
GADD45 α	Growth Arrest and DNA Damage Inducible alpha
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green-fluorescent protein
GG-NER	Global genomic nucleotide excision repair
GO	Gene ontology
Grb2	Growth factor receptor-bound protein 2
GSEA	Gene Set Enrichment Analysis
GST	Glutathione S-transferase
GSVA	Gene Set Variance Analysis
<i>H. sapiens</i>	<i>Homo sapiens</i>
H2AX	Histone H2AX
H ₂ O ₂	Hydrogen Peroxide
H-bond	Hydrogen Bond
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	hepatocyte growth factor
HLTF	helicase-like transcription factor
HMGN1	High Mobility Group Nucleosome Binding Domain 1
Hox	Homeobox
HPRT	Hypoxanthine Phosphoribosyltransferase 1
HR	Homologous Recombination

IF	Immunofluorescence
IPO5	Importin 5
IPTG	Isopropyl- β D-thiogalactoside
IR	Ionising Radiation
IVKA	<i>in vitro</i> Kinase Assay
JNK	c-Jun Kinase
kb	kilobase
KD	knockdown
kDa	kilo-Dalton
KIT	Stem cell growth factor receptor Kit
Klf4	Kruppel Like Factor 4
KRAS	GTPase Kras
Ku70	Ku Antigen 70 (XRCC6)
Ku80	Ku Antigen 80 (XRCC5)
LB	Lysogeny Broth
Lef1/Tcf	Lymphoid Enhancer Binding Factor 1/T Cell-specific Factor
LMI	Laser micro-irradiation
<i>M. musculus</i>	<i>Mus musculus</i>
MAPK	Mitogen activated protein kinase
MAPKAPK2 or MK2	MAPK Activated protein kinase 2
MAPKKK	MAPK kinase kinase
MC1R	Melanocortin 1 Receptor
MCS	Multi-cloning site
MDM	MDM2 (proto-oncogene)
MEF2	Myocyte Enhancer Factor 2
MEK	MAK/ERK kinase (mitogen-activated protein kinase kinase)
MET	Mesenchymal to epithelial transition
miRNA	microRNA
MITF	Microphthalmia-Associated transcription factor
ML-IAP	Melanoma Inhibitor of Apoptosis Protein (BIRC7)
MMR	Mismatch Repair
MORE	More palindromic Oct1 Responsive Element
MRN	Mre11/Rad50/Nbs1 Complex

mRNA	messengerRNA
MSH	MutS homologue
mTORC1	mTOR complex 1
Myt1l	Myelin Transcription Factor 1 Like
N terminal	Amino-terminus
NER	Nucleotide excision repair
NF-Y	Nuclear Transcription Factor Y
NFκB	Nuclear Factor Kappa B
NHEJ	Non-Homologous End Joining
NLS	Nuclear localisation signal
NORE	N-Oct-3 Responsive Element
NRAS	GTPase Nras
NSCLC	Non-Small Cell Lung Cancer
OH·	hydroxyl radical
Oct	Octamer proteins
O-GlcNAc	O-linked β-N-Acetylglucosamine
p38 SAPK	p38 Stress Associated Protein Kinase
p53	Tumour Protein P53
PANTHER	Protein Analysis Through Evolutionary Relationships
PAR	poly(ADP) ribose
PARP	poly(ADP) ribose polymerase
PARPi	poly(ADP) ribose polymerase inhibitor
PAX	Paired box transcription factor
PB	PiggyBac
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline - Tween 0.1%
PCR	Polymerase chain reaction
PD	Parkinson's disease
PI3K	Phosphoinositide 3-kinase
PIKK	PI3K-related kinase
Pit1	Pituitary-Specific Positive Transcription Factor 1 (POU1F1)
PKA	Protein Kinase A
PKM2	Pyruvate kinase M2

PMSF	Phenylmethane Sulfonyl Fluoride
PNKP	Polynucleotide kinase phosphatase
polyQ	Poly Glutamine
POMC	Pro-Opio Melanocortin
PORE	Palindromic Oct1 Responsive Element
POU	Pit-Oct-Unc
POU _H	POU homeodomain
POU _S	POU specific domain
PQBP1	Poly Glutamine Binding Protein 1
PTEN	Phosphatase and Tensin Homolog
PTM	Post-translational modification
RAF	RAF proto-oncogene serine/threonine-protein kinase
RAS	GTPase Ras
Rb	Retinoblastoma
RFC	Replication family C
rmANOVA	repeated measures Analysis of Variance
RNA	Ribonucleic acid
RNA Pol II	RNA polymerase II
ROS	Reactive Oxygen Species
RPA	Replication Protein A
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SD	Standard Deviation
SEM	Standard Error of the Mean
S.O.C.	Super Optimal broth with Catabolite repression
SAINT	Significance Analysis of Interactome
SCLC	Small Cell Lung Cancer
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SILAC	Stable Isotope Labelling with Amino acids in Cell Culture
siRNA	small interfering RNA
SNP	Single-nucleotide polymorphism
SNV	Single-nucleotide variants
Sos	Son of sevenless homolog

SOX	Sry box
SSB	Single Strand Break
SSC	Saline Sodium Citrate
SUMO	Small Ubiquitin-like Modifier
TB	Terrific Broth
TBP	TATA-box binding protein
TCGA	The Cancer Genome Atlas
TC-NER	Transcription coupled nucleotide excision repair
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylene-diamine
TERT	Telomerase Reverse Transcriptase
TF	Transcription factor
TNF α	Tumour Necrosis Factor alpha
TRP1	Tyrosinase Related Protein 1 (TYRP1)
TTF1	Thyroid Transcription Factor 1
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Unc86	<i>C. elegans</i> POU5F1 homologue
USF-1	Upstream Transcription Factor 1
UTR	Untranslated region
UV	Ultraviolet Radiation
UVA	Ultraviolet A 315-400 nm
UVB	Ultraviolet B 280-315 nm
UVC	Ultraviolet C 100-280 nm
UV-DDB	UV-Damaged DNA Binding Protein (DDB1 gene)
WB	Western Blot
WT	Wild Type
<i>X. laevis</i>	<i>Xenopus laevis</i>
XP	Xeroderma Pigmentosum
XPC	Xeroderma Pigmentosum Group C
α MSH	alpha-Melanocyte Stimulating Hormone
γ H2AX	phosphorylated histone H2AX

Chapter 1 | Introduction

1.1 | Phenotypic Heterogeneity in Development and Cancer

1.1.1 | Introduction

Development from a single-cell to a multicellular organism requires cells to acquire and utilise a range of phenotypic properties including self-renewal, proliferation, differentiation, senescence, migration and apoptosis. The phenotype of a cell is dependent on its transcriptional programme activating a precise combination of genes in a cell type-specific manner (Niwa 2018). The switching on and off of gene expression in an appropriate spatiotemporal pattern is the key to the successful development of organisms (Davidson 2010). The complex gene-regulatory networks which make this possible are co-opted by cancer cells and confer the ability to recapitulate developmental behaviours, which when inappropriately regulated allow the pathological growth and metastasis of tumours (Hanahan and Weinberg 2000, 2011). If we consider cancer to be the consequence of mis-regulated developmental mechanisms, then, understanding how cancer cells hijack developmental pathways can help us to better target cancer therapy.

1.1.2 | Neural-Crest Migration and Cancer Metastasis

Whilst phenotypic plasticity is relevant to many aspects of development and tumour biology, one of the hallmarks of cancer which has proven almost impossible to treat and results in the death of patients, is the ability to metastasise (Steeg 2016). In both

development and cancer, cells need to alter their transcriptional programme to acquire the requisite set of phenotypic attributes to successfully move from one site in an organism and set up a colony in a new site. This process, frequently termed epithelial-to-mesenchymal transition (EMT), and the reverse process, mesenchymal-to-epithelial transition (MET) are achieved through modification of gene expression (Thiery et al. 2009). Neural crest cells use this process when they migrate distally from the neural crest to their peripheral locations during development (Theveneau and Mayor 2012), whilst *in situ* tumour cells can employ these processes to metastasise and create a secondary tumour at a new site (Hanahan and Weinberg 2011).

Melanoma is a particularly good model for studying the molecular events underpinning metastasis as it is a highly metastatic cancer arising from a neural crest cell lineage with a highly migratory developmental programme (Gupta et al. 2005). Melanoma arises from melanocyte-lineage cells that reside in the basal layer of the epidermis at the epidermal/dermal junction. The melanocyte lineage originates in the neural crest cells of the ectoderm, from where melanoblasts migrate, predominantly via the dorsolateral pathway (Erickson and Goins 1995), in response to chemoattractants from the developed dermis (Thomas and Erickson 2008). Melanoblasts invade and come to rest in the basal layer of the epidermis where they give rise to stem cells but also differentiate and mature into pigment-producing melanocytes (Bosserhoff et al. 2011).

Owing to their developmental origins, melanocyte lineage cells are ontogenically programmed to have high invasive and metastatic capacity (Mort et al. 2015). Although melanocytes are not epithelial, the process of melanoma metastasis still involves the activation of the EMT programme (Vandamme and Berx 2014; Alonso et al. 2007). Many of the transcriptional programmes involved in neuronal and melanocyte development are conserved in melanomas and other tumours arising from cells of neural crest origin, such

as small cell lung cancer. Therefore developmental biology can inform melanoma biology and vice versa (Liu et al. 2014).

1.1.3 | Tumour Heterogeneity

Nutrients and oxygen are rate-limiting for normal cell growth and proliferation. Tumours tend to have regions of high hypoxia and low nutrient availability, in part due to aberrant tumour vasculature (Tatum 2006). The dysregulation of cancer cell growth entails the loss of the usual homeostatic mechanisms that link cell proliferation to microenvironmental cues, thus tumours can thrive despite physiologically unfavourable conditions (Gupta and Massagué 2006). In addition to a competition for resources, tumour cells also have to contend with exogenous and endogenous stress such as chemotherapy or reactive oxygen stress. As a result of these evolutionary pressures, tumours tend to comprise a mix of cell phenotypes, with selection driven by environmental stressors such as hypoxia (Fluegen et al. 2017). This tumour heterogeneity, which can derive from the genetic or transcriptional level, is particularly evident in tumours with high mutational burden such as melanoma (Lawrence et al. 2013; Krepler et al. 2017). (see Fig 1.1.1a)

The treatment of metastatic melanoma has been transformed over the last decade by the use of novel targeted therapies including mitogen activated protein kinase (MAPK) pathway and immune checkpoint inhibition, increasing median survival from 8-12 months to more than 24 months (Hodi et al. 2016; Ugurel et al. 2017). However not all patients respond to these therapies and many develop resistance and disease progression. One of the key mechanisms of therapy resistance is tumour heterogeneity (Roesch 2014), as the susceptibilities being targeted by a particular therapy will not be shared by all the cells in the tumour. The resistant cells can therefore survive the treatment and fuel tumour expansion. This has profound consequences for the way in which we approach cancer

therapy. Understanding the basis of the ability of cells to change their phenotype is critical in designing treatment strategies.

Two models describe how phenotypic heterogeneity may arise. The genetic model proposes that an acquired genetic mutation may result in phenotypic change by altering the function of a gene product from the mutated locus (Hoek and Goding 2010). If the protein deriving from the irreversibly mutated gene is phenotypically relevant, then there is likely to be a unidirectional alteration in the cell phenotype. In contrast, the phenotype-switching model posits that when a cell phenotype changes, the transcriptional output of the cell is changed, but not the underlying genome; therefore, it is a dynamic and reversible condition (Hoek and Goding 2010). Phenotype-switching allows cells to be dynamic in their relationship with the microenvironment and react to exogenous threats to their survival such as chemotherapy, nutrient limitation and immune surveillance. (Hoek et al. 2008; Hoek and Goding 2010) (see Fig. 1.1.1a). The phenotypic-switch in response to environmental cues is exemplified by the glutamine starvation response. This is an example of how an exogenous signal, nutrient limitation, can affect the behaviour of a cell through alteration of the transcriptional and post-transcriptional programme to lead to acquisition of metastatic capacity and therapy resistance (Falletta et al. 2017).

Critical components of gene expression programmes are transcription factors which can modulate gene transcription in response to specific signals (Niwa 2018; Morris 2016). There is evidence from *in vivo* live cell imaging using a melanoma model to support the phenotype-switching model at a transcription-factor level during metastasis. The expression of two transcription factors, microphthalmia-associated transcription factor (MITF), which controls pigmentation in melanocytes, and BRN2 which can modulate MITF, were evaluated during melanoma metastasis. Loss of MITF expression was inferred by loss of pigment; GFP expression was driven by the BRN2 promoter.

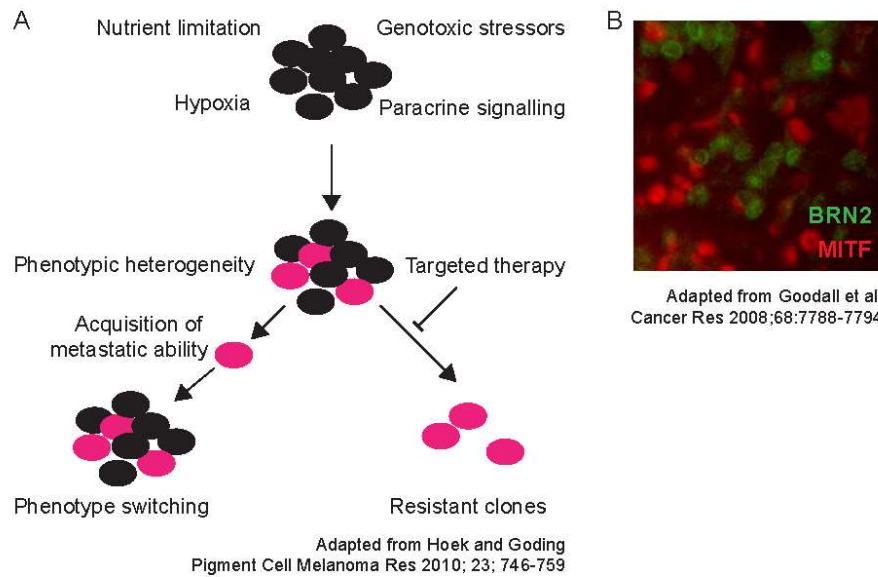


Figure 1.1.1 | Tumour heterogeneity drives therapy resistance and metastasis

(A) Schematic illustrating the principles of genetic and phenotypic tumour heterogeneity driven by evolutionary pressures deriving from the tumour microenvironment. Circles represent individual melanoma cells. Pink cells represent cells with phenotypic differences to Black cells which could derive from genomic mutation or transcriptional programme modification. Tumour heterogeneity results in variable response to therapy. Phenotype switching through transcriptional control allows reversible acquisition of hallmark features of tumour cells. Adapted from Hoek and Goding 2010 (B) Immunofluorescence of a human melanoma metastasis MITF (Red); BRN2 (Green). Adapted from Goodall et al. 2008

Migrating metastatic melanoma cells were predominantly unpigmented with high BRN2 expression. In contrast the resulting metastases were heterogeneous, comprising cells of either BRN2 high or low expression (Pinner et al. 2009). This heterogeneity was also demonstrated in tumour samples with mutually exclusive patterns of MITF and BRN2 expression (Goodall et al. 2008). The implication is that the expression of these two transcription factors can be switched on and off dependent on cellular context (see Fig. 1.1.1b)

1.1.4 | Mechanisms of Phenotype Control

To understand how a cell acquires a different phenotype, we need to understand how cell phenotypes are modulated mechanistically. Alterations in gene and protein function can occur at the transcriptional, or post-transcriptional level, which includes translational and post-translational control. The transcriptional programme is dependent on the integration of upstream signalling pathways with versatile complexes in the nucleus, in particular transcription factors, transcriptional co-factors and chromatin remodellers (Mitchell and Tjian 1989). In this way exogenous signals can be transduced to the nucleus, via intra-cellular signalling cascades, affect transcriptional output from specific gene loci and ultimately protein expression and therefore cell phenotype.

Transcribed mRNA is translated to create the proteins which manifest the cellular phenotype. However not all mRNA is translated, it can be maintained in a pool to be translated at a point when the protein is required quickly in response to an external cue, with ribosomal specificity as a control mechanism (Shi et al. 2017), or it can be degraded prior to translation (Houseley and Tollervey 2009). Translational control can be global, so that upregulation or downregulation of translation is co-ordinated according to the availability of nutrients or other stressors. However, individual mRNA translation to protein can also be controlled, particularly at the initiation phase of translation (Sonenberg and Hinnebusch 2009). Aberrancy in translational control resulting in acquisition of new phenotypic traits has emerged as a key driver of oncogenesis (Vaklavas et al. 2017). Finally, post-translational modification is a mechanism by which proteins are modified enzymatically or chemically, for example addition of a phosphate group by a kinase, or oxidation by free radicals. These modifications can change the properties of the protein and depending on its role in the cell, this can alter the cell phenotype.

1.2 | Transcription Factors and Their Control Mechanisms

1.2.1 | Introduction

Transcription factors (TF) are a class of proteins that determine cell phenotype by regulating the onset, rate and amount of transcription of a single gene, or a set of genes, modulating a plethora of biological processes. TFs bind to specific DNA motifs through their DNA-binding domains to act as transcription activators or repressors, altering the output from a specific gene locus (Mitchell and Tjian 1989). The specificity and activity of transcription factors for target sites can be modified by conformational changes induced by binding co-factors or DNA and by post-translational modification, resulting in sequestration or alteration of DNA binding affinity (Reiter et al. 2017). In addition to direct promoter binding, TFs can also modulate transcription through chromatin remodelling, either directly or by recruitment of remodelling complexes (Lai and Pugh 2017; Andrey and Mundlos 2017). Here we discuss the three key mechanisms of TF activity modulation: protein-protein interaction, chromatin remodelling and post-translational modification, with emphasis on the latter.

1.2.2 | Protein-Protein Interaction and Chromatin Remodelling

Transcription factor activity and specificity are frequently modified by protein-protein interactions, for example with other transcription factors (Jolma et al. 2015), transcriptional co-regulators (Naar et al. 2001; Tjian and Maniatis 1994), and chromatin remodelling complexes (Luo and Dean 1999). Control of access to DNA is a way of modulating transcriptional output. DNA is tightly packaged in nucleosomes, consisting of histone proteins wrapped in DNA. This ‘closed’ conformation is referred to as

heterochromatin and there is little transcriptional activity from heterochromatic DNA. In order to transcribe a gene, transcription factors need to bind DNA to help recruit the transcriptional machinery and initiate transcription. This open DNA is referred to as euchromatin (Venkatesh and Workman 2015).

Modulation of nucleosomal architecture to facilitate switching between transcriptionally-active euchromatin and more silent heterochromatin is achieved in part through modification of histone tails by post-translational modifications. Some modifications such as phosphorylation and acetylation directly change the physical properties of the histones and therefore the interaction with DNA. However, a complex and dynamic system of covalent modifications of the lysine residues in the histone tails, referred to as the histone code (Jenuwein and Allis 2001), is responsible for indirect chromatin architectural restructuring in part through the recruitment of chromatin remodelling complexes. Canonically this is achieved via bromodomain and chromodomain containing proteins. Lysine methylation, recognised by Royal family chromodomain-containing proteins such as HP1 tend to promote heterochromatin conformation. In contrast, lysine acetylation, recognised by bromodomain containing proteins such as p300/CBP, is associated with transcriptionally-active chromatin (Taverna et al. 2007). Combinatorial modifications and paired recruitment of readers with different “reader” domains allows a complex and flexible readout of histone modifications to facilitate specific and dynamic remodelling of chromatin and thus control of gene transcription (Andrews et al. 2016).

The balance of transcriptional activators or repressors at a gene promotor affects its transcription. The components of transcriptional and chromatin remodelling complexes have overlapping functions. For example, the histone acetyltransferase p300/CBP has roles in histone modification, transcription factor binding and in

recruitment of RNA Polymerase II (RNA Pol II) to transcription sites (Tie et al. 2009; Boijja et al. 2017; Bedford et al. 2010).

1.2.3 | Post-Translational Modification

Amino acids can be subject to a wide range of post-translational modifications (PTM) including small chemical modifications such as phosphorylation or methylation (Clarke 1993); addition of carbohydrates by glycosylation to form glycoproteins (Xu and Ng 2015); attachment of chains of protein such as ubiquitin (Yau and Rape 2016) or SUMO (Matunis et al. 1996); or modification by nucleotide-derived additions such as poly(ADP)ribose (PAR) (Jungmichel et al. 2013). PTMs can alter the stability, localisation or function of a protein.

Phosphorylation, the dynamic addition of a 75 Da phosphate group by a protein kinase, is one of the most abundant post-translational modifications (Olsen et al. 2006) and can affect the structure and charge of the modified amino acid and consequently the overall 3D structure and function of the modified protein (Nishi et al. 2011). An induced conformational change in a transcription factor can alter its affinity and specificity for both DNA target binding sites and interaction with co-factors (Garza et al. 2010). The polar amino acids serine, threonine and tyrosine are the most well-described and commonly phosphorylated sites in eukaryotes. The abundance of phosphorylated serine, threonine and tyrosine sites based on phospho-mass spectrometry of 2000 phosphoproteins are 86.4%, 11.8% and 1.8% respectively (Olsen et al. 2006). There is also emerging evidence for the importance in eukaryotes of phosphorylation of side chains of other amino-acids such as histidine, which is critical in prokaryotes (Fuhs and Hunter 2017). The importance of serine phosphorylation sites is illustrated by the finding

that whilst serine is one of the most frequently mutated amino acids, serines at phospho-sites are evolutionarily conserved (Creixell et al. 2012).

Kinase signalling cascades transduce a message from outside the cell to the nucleus by a series of phosphorylation events, with one of the main endpoints being transcription factors (Karin and Hunter 1995). Time-course phosphoproteomics has demonstrated that in response to growth factor signals, tyrosine phosphorylation events occur early, accruing immediately following stimulation. Serine/threonine phosphorylation events however occur with a delay, peaking 5 minutes after EGFR stimulation (Olsen et al. 2006). This reflects the fact that tyrosine phosphorylation activity predominates at transmembrane receptor or membrane-associated kinases such as KIT and EGFR (Schlessinger 2000), whilst serine/threonine kinases tend to act in the cytosol and nucleus. Although transcription factors can be phosphorylated by tyrosine kinases, the serine/threonine kinases are particularly critical for transcription factor modification. Serine/threonine protein kinases comprise a diverse group of kinases, operating in multiple different pathways and are the main signal transducers of the cell within the cytosol and nucleus. This large group includes the kinase families, MAPKs (Roux and Blenis 2004) and cyclin dependent kinases (CDKs) (Malumbres and Barbacid 2005).

MAPKs modulate tissue homeostasis through modulation of growth and death pathways whilst CDKs control progression through the cell cycle. The MAPK and CDK pathways are frequently deregulated in cancer cells (Malumbres and Barbacid 2009; Imperial et al. 2017). Physiologically and in development, tissues need to respond to stress signals to modulate their access to nutrients, metabolism and growth. The MAPK pathways are composed of a series of protein kinases which create signalling cascades to amplify and transduce an external signal into a transcriptional and translational response.

The EGFR>RAS>RAF>MEK>ERK>RSK pathway is the best described and is also frequently dysregulated in cancer (Imperial et al. 2017). The small GTPase RAS is activated by the co-factors Grb2 and Sos, after activation of the tyrosine kinase growth receptor EGFR by growth factors and other mitogenic signals. RAS activates RAF which phosphorylates MEK which in turn phosphorylates the MAPK ERK that then activates RSK. Mutant NRAS and BRAF are two of the key driver genes in melanoma (Colombino et al. 2012; Reddy et al. 2017), providing constitutively active pro-growth signalling uncoupled from microenvironmental signals. Similarly, mutant KRAS is frequently overexpressed in colon and lung cancers (Bos et al. 1987; Dogan et al. 2012).

Regions of kinases critical to their phosphorylation activity are the ATP binding pockets, regulatory domains and catalytic domains (Ubersax and Ferrell 2007). To catalyse the phosphorylation event, the kinase needs to dock on the substrate protein and the catalytic domain must access the hydroxyl group of the side chain being targeted for phosphorylation. The specificity of a kinase for its target site is critical to ensure fine control of protein function. This is partly achieved through context of the target amino acid. The amino acids flanking the phospho-target therefore can dictate kinase specificity through creating the correct physicochemical environment for kinase activity (Ubersax and Ferrell 2007). The conserved sequences around the phosphorylated residue are known as phosphorylation motifs or consensus sequences. The MAPKs and CDKs preferentially phosphorylate serine/threonine followed by a proline (S/TP) (Roux and Blenis 2004; Malumbres and Barbacid 2005). Another class of serine/threonine kinases, the PI3K-related kinases (PIKKs), favour a serine/threonine-glutamine (S/TQ) motif and phosphorylate kinase and transcription factor targets in response to DNA damage and will be discussed later (Jackson and Bartek 2009).

For MAPKs the target motif of S/TP is generic, yet the kinase protein targets are very specific. Kinase specificity can be further determined by sequences which promote association of substrate and kinase, allowing the catalytic domain access to the phosphorylation site. These docking sites increase kinase affinity for a target by bringing the catalytic domain into close proximity with the consensus motif (Biondi and Nebreda 2003). Docking sites on substrates and the reciprocal docking groove on the kinase have evolved to confer remarkable specificity for MAPKs to their targets (Tanoue et al. 2000, 2001; Zeke et al. 2015).

1.3 | POU Domain Transcription Factors

1.3.1 | Introduction

POU domain proteins are key regulators of development, particularly stem cell identity and cell fate determination (Veenstra et al. 1997; Takahashi and Yamanaka 2006), and are also implicated in the pathogenesis of a range of cancers. Therefore, they are a fascinating group of proteins to study in order to better understand the role of transcription factors in cell-fate specification in both development and cancer.

1.3.2 | The POU-Domain Protein Family

The POU-domain protein transcription factor family was originally described three decades ago (Herr et al. 1988), it comprises a group of DNA-binding proteins with a highly conserved DNA binding region which recognise and bind the octamer motif (ATGCAAAT) or variants thereof. The family was codified by Herr et al. combining the data that had been gathered up to that point on four octamer-binding proteins. These were termed Pit1, Oct1, Oct2 and *C. elegans* Unc86 (the vertebrate homologue is BRN3), therefore the proteins were referred to as POU domain proteins. Whilst the N-terminal region of these POU domain proteins is highly variable, they all have in common a DNA binding domain, which consists of two highly conserved regions separated by a flexible and non-conserved linker region. The DNA binding domain is comprised of the 75 amino acid POU specific domain (POU_S) and the 60 amino acid POU homeodomain (POU_H) which is highly similar to the embryonic patterning homeobox (Hox) genes, described originally in *D. melanogaster* (Herr et al. 1988; Gehring 1993). However, the linker region is variable between POU domain proteins and dramatically affects the way in

which the proteins can bind the DNA. Therefore, the family has been divided into six classes according to characteristics of the linker region and the tissue-specific expression patterns of the POU proteins (Malik et al. 2018).

1.3.2.1 | Tissue-specific expression of POU-domain proteins

Whilst one of the canonical octamer-binding proteins, Oct1, is ubiquitously expressed (Fletcher et al. 1987), the other three have tissue-specific expression. Pit1, also known as GHF-1, is expressed in the developing pituitary and in prolactin and growth hormone-releasing pituitary neurons in the developed brain (Bodner et al. 1988). Unc86 knockout in *C. elegans* resulted in disruption of neuronal development only (Finney and Ruvkun 1990). Oct2 is specific to the B-cells of the lymphoid system (Staudt et al. 1986). Subsequently many tissue-specific POU domain factors have been identified. In addition to Pit1, there are a notable number of neuronal-specific POU domain proteins. Originally termed N-Oct proteins, class III and IV POU domain proteins, now referred to as BRN proteins, are widely expressed in the central nervous system and derived tissues (Schreiber et al. 1990). They are required for correct neuronal cell fate decisions in the developing brain and so their expression patterns are tightly modulated, to ensure development of the appropriate neuron in the correct spatiotemporal pattern.

1.3.2.2 | The neural POU-domain factor BRN2 (POU3F2)

BRN2 (POU3F2) is a class III POU domain protein with a 15 amino acid linker between the two POU domains. Brn2 is an intronless gene located on human chromosome 6q16 (Atanasoski et al. 1995). It has a short C-terminal domain 3' of the POU_H domain. The N-terminal domain is intrinsically disordered and has no clear protein motifs, but includes polyglycine and polyglutamine stretches as well as a proline-rich region (see Fig. 1.3.1). However, the function of these regions has not been established. The polyglutamine stretch is translated from a CAG trinucleotide repeat expansion which, in

some proteins, are known to be pathogenic and demonstrate anticipation in neurological disorders such as Huntington's disease (Paulson 2018; Blum et al. 2013). BRN2 was originally identified in brain tissue and neuronal cell lines where it was named N-Oct-3 (Schreiber et al. 1990; He et al. 1989). Initially a second species termed N-Oct-5 was identified and shown to arise as a consequence of an *in vitro* protein cleavage event (Atanasoski et al. 1997). Simultaneously, melanoma-specific octamer-binding protein was described, originally referred to as Oct-M1 and Oct-M2. It was then shown that N-Oct-3 and Oct-M1 were indeed the same protein, whilst Oct-M2 corresponded to the *in vitro* cleavage product N-Oct-5 (Eisen et al. 1995; Thomson et al. 1993; Cox et al. 1988). This same protein had previously been identified in neuronal tumours (Schreiber et al. 1990) and multiple small cell lung cancer cell lines, but no non-small cell lung cancers (Schreiber et al. 1992). Thus, the same octamer-binding protein has been described in neuronal development and several cancers in cell types with neural crest origins.

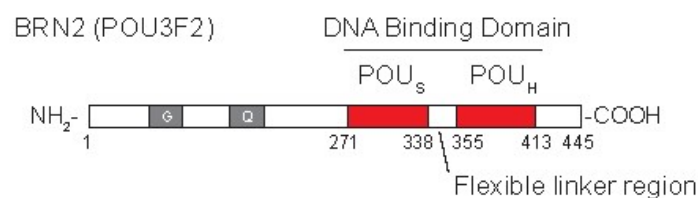


Figure 1.3.1 | BRN2 (POU3F2)

Schematic illustrating the domains of BRN2. G= polyglutamine tract; Q=polyglycine tract; POU_s = POU specific domain; POU_H = POU homeodomain. Numbers refer to amino acids (*mus musculus*)

1.3.3 | DNA Binding by POU Domain Proteins

POU domain proteins characteristically have a wide range of transcriptional targets and act as both activators and repressors. This is achieved by the flexibility in binding site recognition afforded by the fact that the binding to the octamer motif is bipartite. Each half of the POU domain, POU_S and POU_H, is composed of 4 and 3 α helices respectively with helix-turn-helix motifs between the second and third helices in both domains, noted to have similarity to the bacteriophage λ -repressor (Assa-Munt et al. 1993; Klemm et al. 1994). Oct1 POU_S domain binds the first five bases (ATGCA), whilst the POU_H domain binds AAAT. The two binding domains make contact with the DNA on opposite sides of the DNA in the major groove. The variable linker region which separates the POU_S and POU_H regions wraps around the minor groove, both assisting in binding through tethering the second binding domain close to the octamer motif, and by helping to alter the DNA conformation to promote binding (Klemm et al. 1994). The linker region's flexibility contributes to the ability of each POU domain protein to bind a wide range of targets (van Leeuwen et al. 1997; Jacobson et al. 1997; Li et al. 1993). The BRN2 linker, which is highly conserved throughout class III POU proteins, is particularly dynamic, promoting conformational changes that alter the target range for BRN2 (Blaud et al. 2004; Alazard et al. 2007).

Unlike homeodomain proteins, POU domain proteins do not need to dimerise in order to bind DNA. For example, the DNA-Oct1 complex was originally described as monomeric (Klemm et al. 1994). However, depending on the DNA binding site, POU domain proteins can bind DNA as heterodimers (Jerabek et al. 2017), homodimers or as monomers, where the two separate DNA binding domains act in concert to behave like a dimer (Phillips and Luisi 2000; Malik et al. 2018). BRN2 is particularly versatile in its dimerisation requirements, but it exists as a monomer when not bound to DNA (Alazard

et al. 2007). But it should be noted that many of these studies were undertaken with the BRN2-POU domain only, without the potentially trans-activating N-terminal, potentially complicating our understanding of BRN2 dimerisation. In binding to DNA, BRN2 can homodimerise (Alazard et al. 2005) or heterodimerise with non-POU (Blaud et al. 2004; Millevoi et al. 2001) and POU domain proteins such as BRN3a (Rhee et al. 1998).

The canonical octamer site binding described in Oct1 is just one of several related sequences that POU domain proteins can recognise. Multiple variations of the octamer site have been identified, initially through random oligonucleotide selection in electrophoretic mobility shift assays (EMSA) (Gruber et al. 1997; Rhee et al. 1998). Oct1 is able to bind variations on the octamer motif which have been termed PORE (Palindromic Oct1 Responsive Element), or MORE (More palindromic Oct1 Responsive Element). Whilst the DNA binding site motif is adaptable, the contact amino acids are conserved; three in the third helix of the helix-turn-helix region of the POU_S domain (QTxxxR), two in the putative nuclear-localisation (NLS) at the start of the POU_H domain (Sock et al. 1996) (RxxR) and finally an Asparagine at the 3' end of the POU_H domain (Klemm et al. 1994; Phillips and Luisi 2000). In mouse BRN2 these correspond to Q307, T308, R312, R357, R360, N401. Mutation of the POU_S contact residues in Oct-1 (Q44, T45, R49) interrupts DNA binding and transcriptional function of Oct-1 (van Leeuwen et al. 1995)

BRN2 is highly flexible in its binding site specificity and in addition to octamer, MORE and PORE sites, it can bind a further modification of the octamer site, referred to as the NORE (N-Oct-3 Responsive Element) (Alazard et al. 2005). The repetition of octamer-like sequences in these palindromic-style recognition motifs facilitates the non-cooperative binding of two POU domain proteins on opposite sides of the DNA (Alazard et al. 2007). The 3D structure of BRN2 has not been fully solved, although broad features

were identified by circular dichroism (Cabos-Siguier et al. 2009). Full-length BRN2 *in vitro* has similar binding affinity to the DNA binding domain alone (Cabos-Siguier et al. 2009). An *in silico* model of BRN2 simulated binding to MORE DNA has recently been generated (do Vale Coelho et al. 2016), which confirms the likely importance of the six amino acids described above in forming hydrogen bonds with the MORE DNA binding site. The flexibility and versatility of the POU domain proteins results in a huge diversity of target sites (Phillips and Luisi 2000). Genome-wide ChIP-chip of BRN2 revealed 2108 binding sites with 1700 candidate target genes representing genes with a diverse range of functions (Kobi et al. 2010). This study also indicated that BRN2 has binding site recognition capabilities beyond octamer, MORE, PORE and NORE motifs, with binding sites characterised by AT-rich sequences (Kobi et al. 2010).

1.3.4 | POU Domain and BRN2 Post-Translational Modification

As described above, a common way to alter the specificity of transcription factors for their target sites, and thus regulate gene expression, is through post-translational modification. The activity of transcription factors can also be affected by post-translational modification of other proteins which interact with them, affecting parameters such as their subcellular localisation or stability. For example, Oct-1 is released from the nuclear envelope in response to c-Jun Kinase (JNK)-mediated phosphorylation of LaminB1 in response to stress, which results in higher availability of Oct1 to bind its target sites (Boubriak et al. 2017). Whilst relatively little is known about the post-translational modifications which affect BRN2, several classes of PTM have been described to alter the function of Oct4 and Oct1, including ubiquitination, sumoylation, O-GlcNACylation and phosphorylation (Malik et al. 2018). Here we focus on phosphorylation as a modifier of transcription factor activity.

Phosphorylation of a conserved threonine-serine motif located at the start of the POU_H domain (adjacent to the DNA-contacting arginine), modulates Oct1 and Pit1 DNA binding affinity (Segil et al. 1991; Kapiloff et al. 1991; Caelles et al. 1995), whilst mutation of the equivalent site on murine Oct4 (serine 228) reduces its reprogramming ability (Jin et al. 2016). Pit1 is phosphorylated in response to growth and stress signals with a consequent alteration in its binding affinity for target octamer DNA binding sites, which was achieved through mitotic phase (M-phase) specific phosphorylation on threonine 220 (Kapiloff et al. 1991; Caelles et al. 1995). Oct1 transcriptional activity is modulated physiologically in a cell cycle dependent manner by phosphorylation on serine 385, which is in the same position as Pit1 residue 220 (Roberts et al. 1991; Segil et al. 1991). These phosphorylation events have been ascribed *in vitro* and *in vivo* to be Protein Kinase A (PKA)-dependent. This conserved mechanism has also been demonstrated in BRN2. Phosphorylation of murine BRN2 by PKA at serine 362 (analogous to Oct1 serine 385 and Pit1 220) has been shown to alter BRN2 DNA binding affinity *in vitro* particularly for PORE and NORE sites (Nieto et al. 2007). Simultaneous mutation of serine 362 and the preceding threonine 361 to non-phosphorylatable alanine was shown to impair DNA binding and activation at the Paired Box 3 (Pax3) promoter (Berlin et al. 2012). However there is also evidence that cell cycle regulated kinases may also phosphorylate this site (Caelles et al. 1995).

Phosphorylation at other sites has also been shown to affect POU domain behaviour. Another conserved serine site, upstream of the linker region in the POU_S domain (analogous to S319 in *m. musculus* BRN2) is phosphorylated in response to stress in Oct1 and Oct4 and reduces their DNA binding affinity for MORE sites (Kang et al. 2009). Prior to this data, Oct-1 had already been described as a stress sensor (Tantin et al. 2005) and can be stabilised by DNA Protein Kinase (DNAPK) phosphorylation of

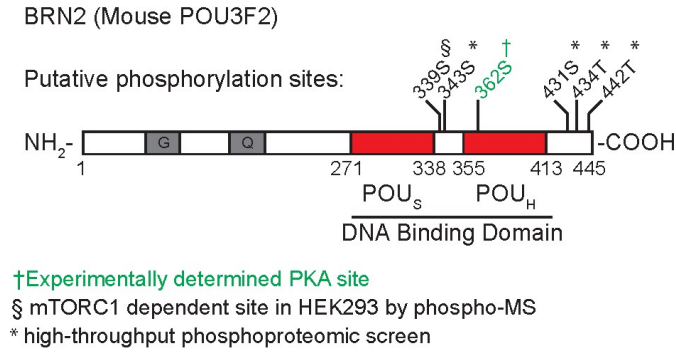


Figure 1.3.2 | BRN2 phosphorylation sites

Schematic illustrating BRN2 putative phosphorylation sites, identified experimentally or by phosphoproteomic screen, as indicated. Numbers refer to amino acid residues in *m. musculus*. PKA = Protein Kinase A.

non-POU domain regions following ionising radiation (Wang et al. 2009; Schild-Poulter et al. 2003). The class VI POU domain TF BRN5 (POU6f1, TCFβ), predominantly expressed in the thymus and brain, binds more strongly to its octamer recognition sequences following stress-induced phosphorylation by JNK (Kasibhatla et al. 1999). The target sites are close to the PKA site described above but are non-conserved.

Although no other BRN2 phosphorylation sites have been described experimentally, several have been identified in high-throughput phosphoproteomic screening. Serine 341 of human BRN2, which corresponds to serine 343 in mouse, has been identified as phosphorylated in HEK293 (Christensen et al. 2010; Franz-Wachtel et al. 2012; Gauci et al. 2009; Stuart et al. 2015), Jurkat (Mertins et al. 2013; Mayya et al. 2009) and also two melanoma cell lines A375 and WM239A (Stuart et al. 2015). Hsu et al. also identified serine 339 in HEK293 cells as phosphorylated in an mTOR complex 1

(mTORC1) specific manner (Hsu et al. 2011). In addition to these sites, Cell Signalling Technology (CST) curation sets on PhosphoSitePlus have identified serine 431 and threonines 434, 442 (mouse) as phosphorylation sites (Hornbeck et al. 2015). It is notable that there was only 10-20% sequence coverage of BRN2 in SILAC experiments (Mertins et al. 2013). This means that high-throughput data are potentially missing many BRN2 phosphorylation sites. Exploring these potential phosphorylation sites is a key aim of this thesis (see Fig. 1.3.2).

1.3.5 | POU-Domain Protein Interactors

POU-domain proteins interact with other classes of transcription factor to modulate gene expression, either directly or by co-operative binding. They also bind to the transcription machinery including the transcriptional co-activators p300 and TATA-box binding protein (TBP) (Smit et al. 2000). Other classes of POU domain protein interactors include kinases, with CDK activating kinase (CAK) interacting with multiple POU domain proteins (Inamoto et al. 1997). The pyruvate kinase (PKM2) has been shown to interact with Oct4 and can enhance or inhibit Oct4 driven transcription its target genes, depending on the cellular context (Lee et al. 2008; Morfouace et al. 2014)

1.3.5.1 POU-domain proteins interaction with non-POU transcription factors

Transcription factor families implicated in POU-domain factor interaction include Lim-homeodomain, bHLH, Sry box (SOX), and Paired box (PAX) families. Lim-homeodomain and POU proteins form complexes on DNA binding sites and co-regulate expression from target genes (Certel 2004). This has been demonstrated for several Lim:POU pairs in a number of different neuronal lines. Many of the reported interactions involve class IV POU domain proteins including Unc86 with Mec3 during *C. elegans*

touch receptor neuron development (Xue et al. 1993) and BRN3a and BRN3b with Isl1 in sensory neurons and retinal ganglion cells respectively (Li et al. 2014; Dykes et al. 2011). An interaction has been demonstrated in *D. melanogaster* between the class III POU domain protein Drifter and two Lim-domain proteins that drives cell fate specification in *Drosophila* motor neurons (Certel 2004). The bHLH transcription factor Ascl1/Mash1 modulates neurogenesis and binds to the promoters of genes involved in neuronal development co-operatively and at adjacent binding sites with class III POU domain factors BRN1 and 2 to positively regulate gene transcription (Castro et al. 2006).

The SOX transcription factor family is critical for early-fate decisions in developing tissues and are also widely expressed in adult tissues (Pevny and Lovell-Badge 1997). SOX 9 and 10 are key melanocyte transcription factors implicated in both development of the melanocyte lineage in addition to adult melanocyte lineage specification (Harris et al. 2013). SOX and POU proteins are often co-expressed and frequently interact and co-operate in gene expression, particularly in neuronal development, embryonic stem cell and neuronal progenitor cell maintenance (Lodato et al. 2013; Kuhlbrodt et al. 1998; Miyagi et al. 2006).

The pairing of different SOX/POU combinations has led to the suggestion that there might be a code which dictates spatiotemporal differences in gene expression and consequently regulates cell fate determination (Kuhlbrodt et al. 1998). SOX and POU factor interaction is implicated in driving the expression of Nestin during neuronal development, with SOX2 and BRN2 co-operating on the Nestin enhancer to drive its transcription. Several other combinations of SOX and POU factors were demonstrated to recapitulate this activity (Tanaka et al. 2004). Control of the SOX/POU complex is in part modulated by BRN2 subcellular localisation and cell cycle dependent phosphorylation (Tanaka et al. 2004; Sunabori et al. 2008). However, in melanoma cells

it was subsequently demonstrated that SOX9/10 can drive the expression of Nestin in a BRN2-independent manner (Flammiger et al. 2009). Smit et al. investigated candidate BRN2 binding partners using a mammalian two-hybrid system, complemented by *in vitro* GST-pull down assays to confirm direct protein-protein interaction (Smit et al. 2000). This study confirmed BRN2 binding to the key melanocyte transcription factors Sox10 and Pax3. Finally, binding of BRN2 to the transcription factor NF-Y is required for promoter activation of the neuronal gene AADC, which encodes an enzyme required for catalysing the synthesis of monoamine neurotransmitters (Dugast and Weber 2001).

1.3.5.2 | *BRN2 interaction domains*

Whilst many protein-protein interactions are mediated by the POU domain, this region alone is not sufficient for full binding efficiency. Rather the N-terminal domain appears to play an important role in modulating BRN2 protein interactions, including with transcriptional co-activator p300 (Smit et al. 2000). Other interacting proteins do not require the POU domain for binding. The polyglutamine (polyQ) tract located in the N-terminal region of BRN2 is bound by the polyglutamine binding protein PQBP1, resulting in impairment of BRN2 gene transcription and resultant increased susceptibility of embryonic carcinoma cells to UV irradiation and other stressors (Waragai et al. 1999). In hypothalamic cells, the mutant huntingtin protein binds to the polyQ tract and suppresses BRN2-driven transcription by promoting its sequestration from DNA, thus reducing the production of BRN2-driven neuropeptides (Yamanaka et al. 2010).

1.4 | BRN2 In Development and Disease

1.4.1 | Introduction

BRN2 is critical for correct cortical neuronal spatiotemporal organisation, hypothalamic development and neuronal precursor survival (Dominguez et al. 2013; Tanaka et al. 2004; Sunabori et al. 2008; Nakai et al. 1995). In addition to the central nervous system, BRN2 is expressed in cells of neural crest origin such as cultured melanoblasts and peripheral neuroendocrine cells. Although absent or at low levels in fully differentiated cells (Bossertoff et al. 2011; Cook et al. 2003; Goodall et al. 2004a), BRN2 re-emerges as a critical driver of proliferation and invasiveness during oncogenesis in melanoma, small cell lung cancer and neuroendocrine prostate cancer (Sakaeda et al. 2013; Bishop et al. 2016; Ishii et al. 2013; Goodall et al. 2004a).

1.4.2 | BRN2 in Cancer

1.4.2.1 | *Melanoma*

Although BRN2 is not expressed in melanocytes, it is expressed in melanoma cells (Sturm et al. 1994). In melanoma, the bHLH-LZ transcription factor MITF is termed the master regulator of transcription because the phenotype of the melanocyte lineage cell has been shown to correlate with the activity of MITF. The rheostat model suggests that as the activity of MITF increases, melanocyte-lineage cells are more differentiated (Hoek and Goding 2010; Carreira et al. 2006). When MITF is knocked down, DNA damage is induced and cells undergo senescence (Strub et al. 2011; Giuliano et al. 2010). However, at low levels of MITF, either directly measured (Cheli et al. 2011) or inferred through loss of pigmentation (Pinner et al. 2009), cells are stem-cell like with low proliferative

rate but high invasive capacity and frequently have high expression of BRN2 in addition to stem-cell markers Oct4 and Nanog (Pinner et al. 2009; Cheli et al. 2011).

MITF and BRN2 are expressed in a mutually exclusive pattern in melanoma *in vivo* (See Fig 1.1b) (Goodall et al. 2008). The question of how MITF and BRN2 co-regulate each other remains controversial. BRN2 represses MITF and MITF can represses BRN2 indirectly via miRNA-211 (Boyle et al. 2011; Goodall et al. 2008). The resulting positive feedback loop between the two transcription factors is proposed to promote segregation of their expression to generate a heterogeneous population of cells, reflecting tumour phenotypic heterogeneity in melanoma. This has been recapitulated in melanoma sphere culture *in vitro* (Thurber et al. 2011). However, in some *in vitro* cellular contexts BRN2 can activate MITF (Wellbrock et al. 2008).

Given the importance of MITF activity levels in driving phenotypic variation in melanocyte-lineage cells and melanoma, establishing how MITF is controlled is of great importance in understanding melanoma biology. Therefore, investigating how one of its modulators, BRN2, is regulated is key to understanding the balance of transcription factor dominance in melanoma cells and the associated phenotypes. The diversity of BRN2 targets and flexibility of its binding would suggest that BRN2 is modulated dynamically by post-translational modifications and co-factor binding to alter its DNA binding properties. Very little is known about this aspect of BRN2 biology. However, there are indications that the transcriptional behaviour of BRN2 can be affected by exogenous signals. For example, GADD45a transcription is upregulated by BRN2 binding to its promoter following UVB irradiation, in a p53 independent manner (Lefort et al. 2001).

The expression of BRN2 is promoted by the key melanoma-driving signalling pathways BRAF, PI3K and Wnt (Bonvin et al. 2012; Goodall et al. 2004b, 2004a). BRN2

expression is promoted by MAPK signalling, demonstrated by upregulation of BRN2 expression in Ras-transformed melanocytes. Furthermore, mutant BRAF signalling in melanoma cells increased expression from the BRN2 promoter by 20-fold (Goodall et al. 2004b). Pax3 binds to the BRN2 promoter and activates its transcription in response to PI3K signalling, promoting invasiveness in melanoma cells (Bonvin et al. 2012). The modulation between Pax3 and BRN2 is reciprocal, with BRN2 able to bind and modulate transcription from the Pax3 promoter both positively and negatively, dependent on BRN2 phosphorylation status (Berlin et al. 2012).

Wnt/ β -catenin is a developmentally important pathway which can promote asymmetric stem-cell division (Habib et al. 2013) and proliferation in melanocytes through upregulation of MITF expression (Takeda et al. 2000). The BRN2 promoter has a Lef1/Tcf binding site which is bound by TFs downstream of β -catenin with consequent upregulation of BRN2 expression and promotion of proliferation (Goodall et al. 2004a). Wnt signalling has been implicated in cancer progression in many tumour types, including lung cancer and melanoma (Murakami et al. 2001; Tammela et al. 2017). BRN2 induction by Wnt/ β -catenin promotes proliferation; in other melanoma contexts, the Wnt pathway promotes EMT, invasion and metastasis (Murakami et al. 2001; Dissanayake et al. 2007; Weeraratna et al. 2002). Variations in the effect on phenotypic trait induced by Wnt signalling may be a result of differences between the often antagonistic Wnt1/3a and Wnt5a signalling which mediate the canonical and non-canonical pathways respectively (Webster et al. 2015).

1.4.2.2 | *BRN2 in other tumours of neural-crest origin*

Although BRN2 was observed to be highly expressed in small cell lung cancer (SCLC) 25 years ago (Schreiber et al. 1992), its role in controlling gene expression in this setting has only recently been described (Sakaeda et al. 2013; Ishii et al. 2013). SCLC

accounts for 15-20% of lung cancer and is clinically and molecularly distinct from non-small cell lung cancers (Calbo et al. 2005). SCLC is noted for being particularly aggressive and metastatic, and although initially it is highly chemosensitive, there is a high frequency of disease recurrence and thus five year survival is only 7% (Gazdar et al. 2017). Although there is debate over the cell of origin in the development of SCLC, the tumour cells have many features of neuroendocrine cells and may originate from neuroendocrine cells in the lining of the bronchi (Sutherland and Berns 2010; Park et al. 2011). SCLC overexpresses the proneural TF bHLH *Ascl1* (George et al. 2015), which as discussed above, co-operates with *BRN1/2* to modulate neurogenesis (Castro et al. 2006). *Ascl1* and another bHLH TF, *NeuroD1*, contribute to the expression of neuroendocrine specific gene expression and have been implicated in maintenance of SCLC growth and survival (Ishii et al. 2013). Their expression in SCLC is dependent on *BRN2*, although it is not known if *BRN2* directly binds their promoters (Ishii et al. 2013). Furthermore, expression of the TF thyroid transcription factor 1 (*TTF1*), which is expressed in >90% of SCLC is directly driven by *BRN2* binding to its promoter (Sakaeda et al. 2013).

A role for *BRN2* has also been recently identified in the driving of proliferation in neuroendocrine prostate cancer. A variety of neuroendocrine cells are found in the prostate which contribute towards the secretory functions of the adult prostate and have been demonstrated to derive from the neural crest (Szczyrba et al. 2017). Although neuroendocrine prostate cancer deriving directly from these cells is a rare tumour, the highly prevalent prostate adenocarcinoma contains neuroendocrine prostate cells which contribute towards metastasis and therapy resistance and are associated with poor prognosis (Cui et al. 2016; Terry and Beltran 2014). There is also evidence that prostate

adenocarcinoma cells can transform into neuroendocrine cells, and BRN2 has recently been implicated in this process (Bishop et al. 2016).

Prostate cancer growth is predominantly driven by the androgen receptor present on normal prostate cells and therapy is targeted against this receptor by androgen blockade through deprivation or interruption of the androgen receptor pathway. The growth of neuroendocrine cells is androgen-independent and thus they are resistant to therapy (Terry and Beltran 2014). Bishop et al. demonstrated that BRN2 drives differentiation of prostate cells into neuroendocrine cells by activating neuroendocrine transcriptional programmes, similar to those described in SCLC. BRN2 and androgen receptor expression inversely correlate in prostate cancer, with an increase in BRN2 expression in advanced neuroendocrine prostate cancer. The androgen receptor represses BRN2 expression in normal prostate cancer cells, thus the loss of the androgen receptor-bearing cells through treatment targeting them, promotes the growth of BRN2 expressing, aggressive, resistant neuroendocrine cells (Bishop et al. 2016).

1.4.3 | BRN2 in Development

1.4.3.1 | *BRN2 in neuronal development*

In the adult brain, BRN1 and BRN2 are usually co-expressed and are present, as assessed by *in situ* hybridisation, in subsets of neurons in most regions of the central nervous system (He et al. 1989). BRN2 expression in the developing brain displays spatiotemporal dynamism with variation in anatomical location of expression throughout neural ontogeny (He et al. 1989). BRN2 knockout mice develop a normal cortex as there is redundancy between the class III POU factors BRN1 and 2, thus BRN1 can protect cortical development in *brn2^{-/-}* mice (Sugitani et al. 2002). However, homozygous double

knockout mice for *Brn1/2* are not viable (McEvilly et al. 2002). BRN2 is instrumental in organisation of the upper layers of the mammalian neocortex and is expressed in the adult brain in layers II-V which develop during mid-neurogenesis between E13-16 in the mouse brain. Expression of BRN2 is switched on in neural progenitors as they begin migration from deeper to upper layers of the cortex at E13.5 in the mouse embryo (Dominguez et al. 2013). Experimental overexpression of BRN1/2 earlier in development results in precocious migration of upper layer cortical neurons, whilst electroporation of dominant-negative BRN1/2 results in complete failure of neuron migration to the cortical plate; rather they remain in the ventricular zone and continue to express the neural progenitor marker Pax6. This recapitulates the finding that the upper cortical layers fail to develop in *brn1/2^{-/-}* mice (Sugitani et al. 2002; McEvilly et al. 2002). Specification of neural primordial cells in the spinal cord also depends on the spatiotemporal regulation of BRN2 expression, which in concert with SOX transcription factors, ensures the appropriate expression of the intermediate filament protein Nestin. This is achieved through cytoplasmic sequestration of BRN2 in the intermediate zone whilst it remains nuclear in the ventricular and subventricular zones (Tanaka et al. 2004).

BRN2 expression is required for hypothalamic development (Schonemann et al. 1995). The hypothalamus is a neuroendocrine structure with well-defined discrete functional regions which develop from neural progenitors whose fate specificity is largely dependent on POU domain factors. BRN1, BRN2 and BRN4 are expressed in the developing hypothalamus in partially overlapping regions (Schonemann et al. 1995). BRN2 knockout mice fail to form the neurons of the supraventricular and supraoptic nuclei, which are responsible for producing corticotrophin releasing hormone (CRH), vasopressin and oxytocin (Schonemann et al. 1995). *brn2^{-/-}* mice fail to survive beyond postnatal day 10, probably as a consequence of the failure of the hypothalamic-pituitary-

adrenal axis which results from lack of CRH (Nakai et al. 1995; Schonemann et al. 1995). Neural POU-domain proteins are critical for cell-fate determination in the developing brain, as described above. In addition to this, BRN2 and BRN3a/b have been shown to be critical for post-mitotic neuronal survival and inhibition of apoptosis in the developing nervous system (Gascoyne et al. 2004; Schonemann et al. 1995; Sugitani et al. 2002).

1.4.3.2 | *BRN2 in neural crest lineage development*

The neural crest is a transient structure in development, derived from the ectoderm, which is located on the dorsal surface of the neural tube. The neural crest gives rise to a diverse range of cells which migrate during development, invade mesodermal tissues and differentiate into specialised cells, including the pigmented melanocyte lineage (Theveneau and Mayor 2012). Cell-fate specification in neural crest cells is induced by morphogenetic signalling pathways including BMP, Wnt and FGF (Mayanil 2013). The response to these signals is dependent on Pax3 and Sox9/10, which induce the expression of various lineage specifiers (Mayanil 2013). In the case of the melanocyte-lineage, they induce the master regulator transcription factor MITF (Thomas and Erickson 2008). The precursor cells to the fully differentiated melanocytes are neural crest melanoblasts, these specified cells express lineage specific markers such as Dct, MITF and Kit from E10.5-11, after they have delaminated and begun migration along the dorsolateral route from the neural crest (Opdecamp et al. 1997). The transcription factor networks which control the maintenance of melanoblasts and their terminal differentiation to melanocytes in culture involve the interplay of BRN2, PAX3, SOX10 and MITF (Cook et al. 2005).

However, there is conflicting data surrounding the role of BRN2 in melanoblasts, as it has not been detected in melanoblasts *in vivo*. On the other hand, *in vitro* studies have demonstrated that BRN2 is expressed in unpigmented melanoblasts but is

downregulated on differentiation into melanocytes (Cook et al. 2003; Bosserhoff et al. 2011). Specified melanoblasts are capable of reversion to a bipotent more stem-like state rather than commitment to melanocyte differentiation (Dupin et al. 2000). Endothelin 3 which induces this fate reversal is required to maintain BRN2 expression in cultured melanoblasts (Cook et al. 2003). These observations implicate BRN2 in the early phase of melanoblast development, potentially preceding MITF-driven lineage specific gene expression. Neural crest delamination and ventral migration of other cell types occurs from E9-9.5, thus specification of cell populations within the neural crest must precede the appearance of identifiable melanoblasts (E10.5-11) (Krispin et al. 2010a). Lineage tracing has established that melanocyte precursor lineage restriction does indeed occur prior to migration from the neural crest and therefore prior to expression of MITF (Krispin et al. 2010b). This could explain the failure to detect BRN2 expression in melanoblasts *in vivo*, which are identified by lineage specific marker expression.

The divergence from a common neuronal/melanocyte precursor is signalled by the loss of FoxD3 expression in a subset of cells which are still within the neural crest (Nitzan et al. 2013; Krispin et al. 2010b). FoxD3 inhibits melanocytic specification through repression of MITF expression. Both direct promoter repression, and indirect via inhibition of Pax3, have been proposed as mechanisms by which FoxD3 impairs MITF expression, although the full mechanisms are not completely understood (Thomas and Erickson 2009; Curran et al. 2010, 2009). It is possible that the negative feedback loop between BRN2 and MITF, established in melanoma, may be of relevance in the early-melanoblast setting. There is a precedent for POU-domain-FoxD3 co-regulation. In embryonic stem (ES) cells, FoxD3 is required for both maintenance of expression of the POU-domain factor Oct4 and therefore the stem cell pool, but is also required for lineage specification (Hanna et al. 2002). Oct4 directly binds to the DNA-binding domain of

FOXD3, restricting its ability to activate transcription of lineage specific genes and therefore maintains pluripotency of the ES cell (Guo et al. 2002). BRN2 and FOXD3 could co-operate to prevent the lineage specific expression of MITF that drives forward melanocyte specification.

1.4.3.3 | POU domain proteins and cellular reprogramming

POU domain proteins are implicated in cellular reprogramming and stem-cell maintenance. Oct4 is one of the canonical Yamanaka factors, capable of inducing and maintaining stem-cell pluripotency (Takahashi and Yamanaka 2006). Addition of BRN4 and TCF3 to the stem-cell factors Sox2, Klf4 and c-Myc can reprogram fibroblasts into neuronal stem cells (Han et al. 2012). BRN2 in combination with the bHLH TF Ascl1/Mash1 and the zinc finger TF Myt1l have been demonstrated to drive cellular reprogramming to generate neurons from other cell types including fibroblasts and cardiac myocytes (Vierbuchen et al. 2012; Chuang et al. 2017). Conversely, BRN2 and the related class III POU-domain protein BRN4 can drive differentiation of neural stem cells to dopaminergic neurons (Tan et al. 2014; Tian et al. 2015), with potential therapeutic benefit for neurodegenerative diseases such as Parkinson's disease (PD).

1.5 | UV Radiation and the Melanocyte-Lineage Response

1.5.1 | Introduction

As described in section 1.1.4, phenotype modification mediated by transcriptional change is often triggered as a response to exogenous stimuli. One of the most important exogenous signals in melanocyte lineage biology is UV irradiation, therefore here we describe the impact of UV on pigmented cell phenotypes and the mechanisms involved in modulation of this response. To mitigate the harmful effects of UV irradiation, cells have a complex UV response which is principally composed of two parts: firstly, the response to DNA damage, and secondly, the activation of stress response kinases.

1.5.2 | UV Radiation

Sunlight is composed of a spectrum of electromagnetic waves ranging from ultraviolet (UV) through visible light to infrared. The shortest and therefore highest energy wavelength is UVC at 100-280 nm and is highly damaging to DNA, causing double strand breaks as well as base damage. However, the majority of UVC is filtered out by the atmosphere. The main physiologically relevant solar irradiation spectra are therefore UVB and UVA, 280-315 nm and 315-400 nm respectively. Only about 5% of UVB reaches the earth's surface, as it is absorbed by the atmosphere and ozone. However, the thinning of the ozone layer, partly as a consequence of CFC pollution, means that more UVB can travel unimpeded to earth. UVA is not absorbed by the atmosphere and therefore comprises the majority of UV irradiation which reaches the earth's surface (Hockberger 2002).

Although UV irradiation is a powerful mutagen, organisms have evolved to benefit from it in diverse ways, from photosynthesis in the plant kingdom, to UV vision and metabolic processes in the animal kingdom (Hockberger 2002). From a human perspective, UVB and UVA irradiation is successfully used in the treatment of hyperproliferative skin disorders such as psoriasis and eczema (Lim et al. 2015). UVB irradiation is physiologically extremely important to animals, including humans, for the production of Vitamin D from 7-dehydrocholesterol (7-DHC) in the skin (Holick 1981). Vitamin D is then activated by hydroxylation events in the liver and then kidney (Holick 1981). Vitamin D is critical for proper bone development and maintenance, deficiency in childhood results in Ricket's disease, whilst in adults it presents as osteomalacia. Vitamin D deficiency has also been implicated in the pathogenesis of multiple diseases in diverse organ systems including multiple sclerosis, type I diabetes, depression and poor foetal health outcomes (Hossein-Nezhad and Holick 2013; Sintzel et al. 2017).

1.5.3 | UVB-induced DNA Damage

UVB is readily absorbed by DNA resulting in direct DNA base damage which can also be converted to strand breaks. Absorption of UVB by DNA results in direct DNA damage in the form of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4pps). The formation of these dimers occurs instantaneously with the absorption of UVB, but also persists after exposure to UV via free-radical-driven chemiexcitation of melanin, producing DNA damaging free radicals (Premi et al. 2015). CPDs consist of covalent bond formation between adjacent pyrimidine bases which results in a four-carbon ring. The resultant intra-strand crosslinking between thymines and/or cytosines, disrupts the structure of the DNA leading to replicative and transcriptional errors if not repaired. In most organisms, CPDs can be repaired by exposure to visible light in the blue spectrum,

which activates CPD photolyase enzymes. This mechanism is not preserved in placental mammals, therefore the damaged bases have to be actively repaired by DNA damage repair pathways (Zhang et al. 2017; Selby and Sancar 2006). In the case of nucleotide disruption, such as CPDs, this is achieved by the nucleotide excision repair pathway (NER) (Wood 1997). The failure to fully repair CPDs results in a UV signature, characterised by C>T mutation. The importance of an intact NER pathway in response to UV exposure is highlighted clinically by the autosomal recessive syndrome Xeroderma Pigmentosum (XP) which is characterised by XP gene mutations resulting in failure of the global genomic nucleotide excision repair (GG-NER) pathway and a high susceptibility to melanoma and other cancers (Cleaver 2005; Marteijn et al. 2014).

The NER pathways comprise the overlapping transcription coupled and global genome nucleotide excision pathways (TC-NER and GG-NER). Both pathways remove structurally deforming DNA lesions with diverse origins including UV-induced CPDs. The difference between the two pathways is in the initial damage recognition and repair factor recruitment. In non-transcribed regions GG-NER is responsible for identification and repair of distorting base damage. The CPD is recognised by the UV-DDB complex which surveys non-transcribed DNA for damage. It binds to and exaggerates the distortion to create a kink in the helix which can then be recognised by the XPC-containing complex, leading to recruitment of further effector complexes of the NER pathway (Marteijn et al. 2014).

The TC-NER, however, is a more active and immediate process with damage to actively transcribed DNA recognised and repaired on the template strand (Hu et al. 2015). The distortion caused by CPD lesions stalls the RNA pol II transcriptional machinery, displacing it from the DNA and triggers DNA repair by TC-NER (Chiou et al. 2017). Cockayne Syndrome proteins A and B (CSA, ERCC8; CSB, ERCC6) are critical for

recruitment of NER components in TC-NER (Fousteri et al. 2006). Clinically, Cockayne syndrome (CS) is a consequence of mutations in ERCC6/8 and resultant impairment of TC-NER and is characterised by marked neurological dysfunction, premature aging and sunlight sensitivity. Interestingly there is no increased cancer risk, in contrast to XP gene mutations as described above (Hoeijmakers 1994). (See Fig 1.5.1a)

However, the CS and XP gene driven syndromes are thought to be a combination of both defective DNA damage repair but also inefficient transcriptional activity. This overlapping phenotype is likely the result of the multifunctional proteins involved in damage repair and transcription. For example, the multi-subunit containing transcription initiation complex TFIIH is recruited by XPC to sites of UV-induced damage and assists in the opening of DNA for excision of damaged bases during NER. However, it is also a critical component of the pre-initiation complex required for Pol II-driven basal transcription, in which its kinase subunits phosphorylate the Pol II C terminal domain and other transcription factors, and again helps to open DNA to promote access of the transcription machinery to the DNA. Furthermore, TFIIH helps to recruit NER factors such as the XP and CS proteins described here, potentially enhancing the efficiency of transcription (Compe and Egly 2012).

1.5.4 | UVA-induced DNA Damage

UVA is lower energy than UVB and therefore is not absorbed by DNA and so does not directly damage it. UVA, however, is highly mutagenic, principally invoking indirect DNA damage by generation of reactive oxygen species (ROS) (Masaki et al. 1999). The interaction of reactive oxygen species with DNA results in oxidative base damage, single-strand and double-strand breaks, which can result in mutations due to

errors in repair (Jackson and Bartek 2009). Therefore, although the NER pathway is the most important in ensuring appropriate repair of direct UVB damage, SSB and DSB repair pathway competence are critical for ensuring the appropriate repair of cells with more extensive damage which can be caused by UVA-activated ROS. Additionally, in replicating cells, stalling of DNA polymerase leads to collapse of the replication fork at unrepaired CPDs and results in double-strand breaks and secondary activation of DSB repair pathways (Limoli et al. 2002) (See Fig. 1.5.1a).

DSBs are repaired principally by one of two pathways, Non-homologous end-joining (NHEJ) or Homologous recombination (HR). The latter requires a sister chromatid and can therefore only occur in late S/G2. HR is less error-prone than NHEJ and is favoured in transcriptionally active chromatin (Aymard et al. 2014). NHEJ is initiated following the recognition of DSBs by the DNAPK complex, comprised of Ku70, Ku80 (XRCC5/6) and DNA-PKcs (PRKDC), which form a trimeric complex at double-strand breaks (DSBs). This complex is critical to NHEJ pathway function through scaffolding, recruitment and phosphorylation of other DDR proteins (Smith and Jackson 1999). In addition to cell cycle phase during which DNA damage occurs, the choice of repair pathway is influenced by chromatin state, DDR kinase signalling and PARP activity (Ceccaldi et al. 2015).

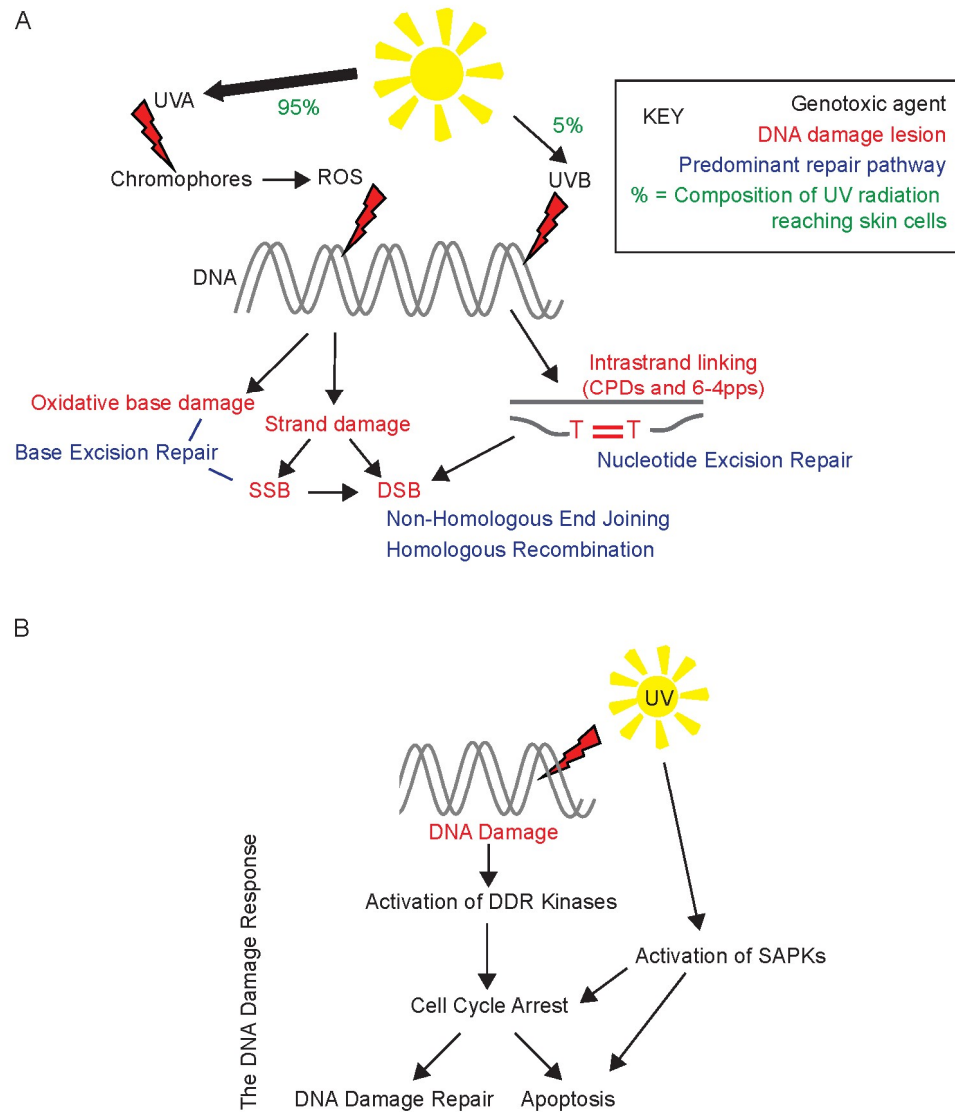


Figure 1.5.1 | UV-induced DNA damage and the DNA damage response

(A) Schematic illustrating the DNA damaging effects of UV radiation. Black text- Genotoxic agent; Red text – DNA damage lesion; Blue text – DNA repair pathway; Green text- Composition of UV spectrum of physiological relevance, following absorption of short wavelengths by the earth's atmosphere. ROS = Reactive Oxygen Species; CPDs = cyclobutene pyrimidine dimers; 6-4pps = 6-4 photoproducts; SSB = Single Strand Breaks; DSB = Double Strand Breaks. (B) Schematic representing the DNA Damage Response (DDR) and parallel activation of SAPKs (stress activated protein kinases) in response to UV radiation.

1.5.5 | The DNA Damage Response

The three key features of the DNA damage response (DDR) are DNA damage repair, cell cycle arrest and apoptosis. In order to maintain the integrity of the genome, there are a plethora of DNA damage repair pathways, which are specific to different forms of DNA damage. These aim to repair mutations and breaks in DNA to restore the original DNA sequence and prevent genomic mutation. To facilitate this, DNA damage leads to recruitment of proteins which induce DDR signalling cascades with the downstream consequences of cell cycle arrest whilst damage is repaired; or triggering of apoptosis if damage cannot be appropriately repaired (see Figs 1.5.1b and 1.5.2) (Jackson and Bartek 2009). In this way cells are prevented from continued proliferation with mutated genomic DNA.

The tumour suppressor TF p53 is a core component of the protective effector mechanisms of the DDR (Kastan et al. 1991). p53 transcriptional targets principally comprise three groups; pro-apoptotic genes, DNA damage repair genes and cell cycle arrest genes (Riley et al. 2008). p53 controls the G1/S checkpoint (Kuerbitz et al. 1992), upregulating the transcription of genes which enact a G1/S arrest, following activation by ATM signalling, the most critical of which is p21. p53 has also been implicated in the G2/M checkpoint (Agarwal et al. 1995). Failure of the p53 induction of G1 arrest and apoptosis allows proliferation of cells with damaged DNA and consequent accumulation of mutations leading to genomic instability. p53 is frequently mutated or deleted in cancer (Lane 1992). However, in melanoma, p53 is often wildtype (WT); instead, components of p53 pathways are frequently mutated (Hodis et al. 2012; Kaufmann et al. 2008). For example, CDKN2A, a key effector of the p53 cell cycle checkpoint pathway, is often mutated or deleted in melanoma and is implicated in familial melanoma syndromes (Hodis et al. 2012; Shain et al. 2015; Laud et al. 2006). The CDKN2A gene locus codes

for p16^{INK4A} and p14^{ARF}. Inhibition of CDK4 and CDK6 by p16^{INK4A} prevents progression through the G1/S checkpoint by activation of the tumour suppressor Retinoblastoma (Rb) (Serrano et al. 1993). p14^{ARF} meanwhile inhibits MDM, therefore activating p53 and consequently triggering cell cycle arrest (Stott et al. 1998).

Excessive DNA damage can result in apoptosis, which is a programmed cell death mediated by caspase degradation of organelles (Taylor et al. 2008; Kerr et al. 1972). This final common pathway can be activated by either the intrinsic or extrinsic pathways. The intrinsic pathway is also known as mitochondrial pathway as the activation of caspase 9 leading to apoptosis is triggered by cytochrome c release from mitochondria following activation by intracellular stress pathways (Taylor et al. 2008). The extrinsic pathway is driven by the death receptor FasR, which can be activated by death signals such as TNF α and Fas ligand. The intrinsic pathway is modulated by the activity of Bcl-2 family of proteins, some of which have pro-apoptotic functions whilst others inhibit apoptosis. Activated p53 induces the transcription of pro-apoptotic Bcl-2 family members Puma Noxa and Bax and inhibits the transcription of the anti-apoptotic protein Bcl2 (Taylor et al. 2008). The pro-apoptotic Bcl-2 proteins then activate the mitochondrial pathway resulting in induction of the execution phase of apoptosis characterised by DNA fragmentation, membrane blebbing and the generation of apoptotic bodies (Kerr et al. 1972).

The interlinked pathways that control damage-induced cell cycle arrest and apoptosis are activated by DNA damage signalling. The PI3K-related kinases (PIKKs) ATM, ATR and DNAPK are principally responsible for initiating the signalling cascades which modulate the cell cycle and death pathways (Jackson and Bartek 2009; Blackford and Jackson 2017). They are serine/threonine kinases with a propensity for phosphorylating serine/threonine-glutamine motifs (S/TQ). DNAPK and ATM are

activated in response to DSB detection. The Ku proteins Ku70 and Ku80 (XRCC5/6) recruit DNAPK to DSBs and initiate the NHEJ pathway, whilst ATM is recruited to DSBs by the MRN complex (Mre11, Rad50, Nbs1), aided by PARP. ATR is recruited by ATRIP to RPA-coated single strand DNA, which can be generated by resection of DSBs, ROS and stalled replication forks and is the main signal initiator following UV-induced damage (Sancar et al. 2004).

PIKK signalling in promoting cell cycle arrest was first recognised in cells bearing ATM mutations. ATM loss results in failure to activate p53 with resultant failure to initiate the G1/S checkpoints following exposure to ionising radiation (Kastan et al. 1992; Turenne et al. 2001). A-T mutated (ATM) kinase is mutated in the autosomal-recessive disease Ataxia-Telangiectasia (Savitsky et al. 1995). In addition to telangiectasia and progressive neurological decline with cerebellar predominance, manifesting as ataxia, patients were noted to have immunodeficiency (van Os et al. 2017), cancer predisposition and marked radiosensitivity (Blackford and Jackson 2017). Radiosensitivity has also been associated with a mutating SNP in the ATM gene (Andreassen et al. 2016). This is a consequence of both failed checkpoint-activation and impairment of DSB repair in cells with deficient ATM activity (Kühne et al. 2004).

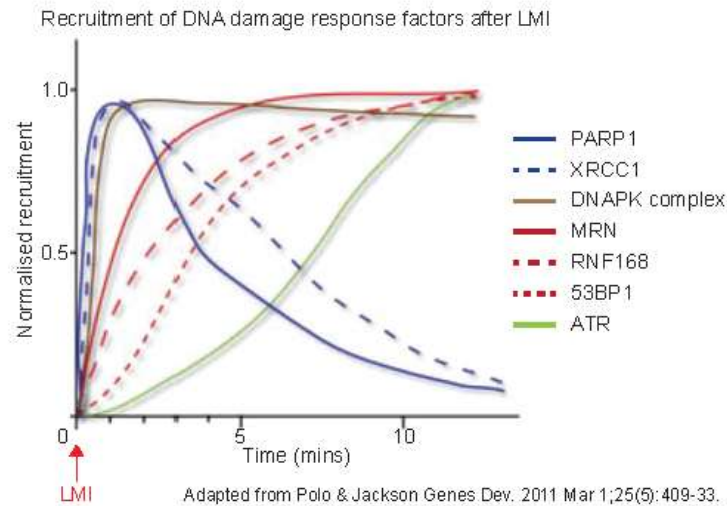


Figure 1.5.2 | Initiation of the DDR - recruitment of DDR proteins to sites of damage

Graph adapted from Polo & Jackson 2011; timing of DDR proteins recruitment to sites of DNA damage induced by laser micro-irradiation (LMI).

As with most kinases, ATM and ATR, and to a lesser extent DNAPK are prolific phosphorylators with an expanding list of targets which includes other kinases (Matsuoka et al. 2007). The histone variant H2AX is phosphorylated on serine 139 (γ H2AX) by both ATM and ATR. At DSBs, γ H2AX is involved in maintenance and stabilisation of the repair complexes and is visible as punctate foci by immunofluorescence (Tanaka et al. 2009; Rogakou et al. 1998). H2AX is also phosphorylated in response to UV damage during NER, but manifests as a pan-nuclear staining, serving as a biomarker for damage and subsequent apoptosis (de Feraudy et al. 2010; Marti et al. 2006; Halicka et al. 2005). The main effector kinases of the ATM/ATR pathways are CHK2 and CHK1 respectively.

These phosphorylate a range of targets which include p53. They phosphorylate both serine 15 and serine 20: the former activates p53's transcriptional activity, whilst the latter blocks p53's ubiquitination and subsequent proteasomal degradation, thus stabilising it. (Roos and Kaina 2013).

1.5.6 | UV Activation of Stress Kinases

UV irradiation triggers the activation of the stress-activated protein kinases (SAPKs) p38 and Jun kinase (JNK) (Adler et al. 1995; Chen and Bowden 1999). The SAPKs are part of the Mitogen-activated protein kinases (MAPK) family. They transduce external “stress” signals to the nucleus to affect cell phenotype. These kinases have been ascribed both tumour suppressor and oncogenic roles, depending on the cell type and microenvironmental conditions, and can either work in concert or play opposing roles (Wagner and Nebreda 2009) (see Fig. 1.5.1b).

1.5.6.1 | *c-Jun N-terminal kinases (JNK)*

c-Jun N-terminal kinases (JNK) have been described to have both oncogenic and tumour suppressive functions (Wagner and Nebreda 2009). Multiple isoforms exist as a result of alternative splicing (Gupta et al. 1996); whether JNK has pro or anti-tumorigenic activity depends on cross-talk between JNK isoforms and other MAPK pathways, notably p38 pathways, in addition to the cellular environment (Staples et al. 2010). Loss-of-function mutations in JNK3 are prevalent in brain tumours (Yoshida et al. 2001), whilst in liver and prostate cancer JNK1 expression is upregulated (Sakurai et al. 2006; Vivanco et al. 2007). JNK activity differs in different tissues but also in response to different types and levels of exogenous stress. Here we will focus on the JNK response to UV irradiation.

JNK has been implicated in melanoma cell survival by regulating both proliferative and apoptotic pathways (Alexaki et al. 2008). In B-cell lymphoma JNK is critical for the transduction of the constitutively active BCR-ABL growth receptor signals, specifically the upregulation of anti-apoptotic Bcl2 family members in order to promote survival and contribute towards oncogenic transformation (Hess et al. 2002). Ras-induced transformation of fibroblasts and lung epithelial cells in a p53^{-/-} background is dependent on JNK. In this context, JNK signalling prevents contact inhibition of growth, promoting survival through anti-apoptotic pathways (Cellurale et al. 2011). Its oncogenic functions are in part realised through phosphorylation of the AP1 transcription factor complex which is a variable heterodimer composed of c-Jun and c-Fos family members. The DNA binding sites of AP1 are situated in a large and varied number of gene promoters and it is capable of driving multiple aspects of oncogenic behaviour. JNK also targets and impairs the p53 pathway, thus promoting anti-apoptotic activity in response to stress, which is another oncogenic mechanism.

However, JNK also possesses tumour suppressive activity through regulation of the expression and activity of Bcl2 family members, both by direct phosphorylation and by affecting their sub-cellular localisation through 14-3-3 family phosphorylation. JNK activation positively regulates apoptosis in endometrial cancer, thus acting as a tumour suppressor (Reno et al. 2009). JNK is required for activation of mitochondrial apoptotic pathways with release of cytochrome c following UV treatment of fibroblasts (Tournier et al. 2000).

1.5.6.2 | p38 SAPK

There are four p38 isoforms, encoded by four genes, *MAPK11-14*. The majority of data relate to the α isoform (MAPK14) which is the most ubiquitously and abundantly expressed. p38 β (MAPK11) is also ubiquitous but expressed at extremely low levels.

The γ (MAPK12) and δ (MAPK13) isoforms have tissue-restricted expression patterns, predominating in neural tissue (Wagner and Nebreda 2009). How p38 is activated remains incompletely understood. The receptor tyrosine kinase at the head of the p38 pathway is unknown, however p38 lies downstream of MKK3 and MKK6 (mitogen-activated protein kinase kinases) which phosphorylate it at T180 and Y182, further autophosphorylation maintains p38 activity. p38 subsequently phosphorylates a range of transcription factors such as ATF2 and MEF2 and protein kinases such as its key effector MK2 (Ono and Han 2000). Reinhardt et al. demonstrated that p38MAPK/MK2 pathway is not only activated by UV but also by drugs which induce DNA damage. This might indicate that it is the DNA damaging effect of UVB which activates p38 (Reinhardt et al. 2007). However, whilst p38 activation following DNA damaging chemotherapeutics is dependent on ATR, ATM or DNAPK function, UVB induced p38 activation is independent of these kinases (Reinhardt et al. 2007). The UV-dependent induction of p38 SAPK can be mediated by XPC, the bulky DNA lesion sensor (see section 1.5.3), which is a key DNA damage recognition component of the NER pathway (Schreck et al. 2016; Marteiijn et al. 2014).

In addition to UV, p38 can be activated by multiple different forms of stress, most notably inflammatory cytokines (Ono and Han 2000). What is interesting, is that the consequences of p38 activity are variable depending on both cellular context and source of stress. The role of p38 in response to UV is controversial; it has been implicated in both induction of apoptosis and cell cycle checkpoint activation (Bulavin et al. 2001; Bulavin 1999), but in other cellular contexts is critical for survival and differentiation (Reinhardt et al. 2007; Phong et al. 2010). Some of the controversies arise from finer dissection of mechanisms as specificity and application of small molecule inhibitors has improved over the past two decades. However, the root cause of these controversies is

that the activity and impact of MAPK cascades is highly dependent on cell context. There is complex and dynamic crosstalk between signalling cascades which affects the outcome of the kinase activity on cell phenotype (Wagner and Nebreda 2009). Unfortunately, much of the literature surrounding the role of p38 in checkpoint induction and the DNA damage response involves the use of DNA damaging drugs or ionising radiation (IR), rather than UV. It is evident from studies where UV has been compared to drugs or IR, that the response is not the same, therefore generalising conclusions based on the literature is counterproductive (Pandey et al. 1996). The contradictory reports about the function of p38 which lead to it being described both as a tumour suppressor and an oncogene could be explained by the fact that p38 has multiple activators and targets. Uncoupling its outputs could allow for selection of one function over the other, dependent on cellular context (Dolado et al. 2007).

p38 is involved in the activation of the G2/M checkpoint following UV irradiation, however, the relative importance of p38 and the specific role it plays again appears to be context specific (Wagner and Nebreda 2009). The regulation of CDC25B phosphorylation by p38-activated MK2 in response to UV modulates the initiation of the G2/M checkpoint by promoting the sequestration of CDC25B in the cytoplasm through 14-3-3 binding and therefore preventing progression into mitosis (Bulavin et al. 2001; Manke et al. 2005). This p38-dependent G2 checkpoint activation was suggested to be specific to the UV irradiation response and is not required following ionising radiation (IR) (Bulavin et al. 2001). Furthermore, in a chemotherapy setting, the CDC25B-dependent mechanism of G2/M checkpoint activation described above has been ascribed to the DDR kinase Chk1, and the role of p38/MK2 in this setting is at best redundant, but possibly antagonistic (Xiao et al. 2006). The p38/MK2 driven G2/M checkpoint activation in response to chemotherapy-induced DNA damage was confirmed to be

redundant in p53 WT cells, however it becomes critical in p53-deficient cells, suggesting that the checkpoint activation function of p53 could be substituted by p38 activation (Reinhardt et al. 2007).

Reports vary as to the importance of p38 vs ATR/Chk1 in the onset of the G2/M checkpoint in response to UV. It may be that the relative importance of the interlinked kinase pathways depends on the timing of the damage in relation to the cell cycle. Bulky DNA adducts, such as CPDs, generated in G1/S are likely to result in replication-fork stalling and transcriptional stress, with consequent strong induction of the DDR, including ATR/Chk1 activation. However, in G2 the DDR to UV lesions is muted. In contrast p38 is activated through all stages of the cell cycle (Warmerdam et al. 2013).

p38 has been implicated in both the promotion and inhibition of apoptosis. Again, this is likely to be highly dependent on cellular context and crosstalk with other pathways. There is evidence across a range of cancers to indicate that the role of p38 at the G2 DNA damage checkpoint is not in triggering of the checkpoint itself but rather in survival promotion. In melanoma, p38 activation upregulates TNF α via ATF2, in concert with downregulation of the death receptor Fas via NF κ B, thus promoting cell survival (Ivanov and Ronai 2000, 1999). In multiple cancer cell lines, p38 activation is required for upregulation of anti-apoptotic members of the Bcl2 family (Phong et al. 2010). p38 also protects cells from apoptosis by suppressing ROS and therefore avoiding activation of death pathways and thus promoting tumour cell survival in breast cancer cells following DNA crosslinking by Cisplatin treatment and in mouse embryonic fibroblasts following treatment by H₂O₂ (Pereira et al. 2013; Gutiérrez-Uzquiza et al. 2012).

However, in contrast, p38 sensing of oncogene-induced ROS in epithelial cells, followed by activation of apoptosis has suggested that p38 acts as a tumour suppressor to

prevent malignant transformation by oncogenes (Dolado et al. 2007). Indeed, in transformed cells with high levels of ROS, p38 activity is impaired (Dolado et al. 2007). In keratinocytes, UVB-induced apoptosis via caspase 3 cleavage is mediated through p38 activation (Shimizu et al. 1999). Further tumour suppressor activity has been ascribed to p38 by data showing its contribution towards p53-driven apoptosis in response to DNA-damaging agents such as UV both by directly phosphorylating p53 and by stabilising transcriptional co-activators such as p18^{HAMLET} (Cuadrado et al. 2007; Bulavin 1999).

1.5.7 | UV Response in Melanocytes and Melanoma

In the skin, fully differentiated melanocytes respond to paracrine signalling from keratinocytes in response to UV irradiation and generate eumelanin pigment (melanogenesis) to be taken up by keratinocytes to act as a photoprotectant. The resultant pigmentation can shield hypodermal cells from the high levels of genomic insult which result from UV irradiation, by absorbing mutagenic energy directly or from ROS (Nasti and Timares 2015). Melanogenesis is initiated by paracrine signalling by POMC-derived melanocortin ligands α -MSH and ACTH, secreted from keratinocytes, signalling via the melanocortin 1 receptor (MC1R) which activates cAMP and promotes CREB-driven MITF transcription, which in turn activates transcription of pigmentation genes including Dct, TRP1 and Tyrosinase (Bertolotto et al. 1998; Nasti and Timares 2015). UV can activate pigmentation enzymes directly or through inducing a transcriptional response. Following activation by UV, p38 phosphorylates the MITF-related TF USF-1, activating the transcription of both POMC and MC1R, thus promoting pigmentation and initiating the tanning response (Galibert et al. 2001; Corre et al. 2004).

As described above in section 1.5.5, activation of the DNA damage response is a vital component of the UV response. MITF is required for the suppression of DNA-damage induced senescence (Giuliano et al. 2010). It has also been shown to upregulate APE-1, required for DNA damage in response to ROS, thus promoting survival (Liu et al. 2009). Furthermore MITF promotes melanocyte survival through upregulation of genes involved in inhibition of apoptosis including ML-IAP and Bcl2 (Dynek et al. 2008; McGill et al. 2002).

In addition to melanogenesis, UV stimulates proliferation in melanocytes promoting an increase in pigment-producing cells, and therefore increasing photoprotection. UV irradiation of melanocytes induces a p38 and JNK dependent phosphorylation and activation of the transcription CREB which is a key proliferation activator in melanocyte lineage cells (Tada et al. 2002; Bertolotto et al. 1998). In human melanoma cells, promotion of proliferation by hepatocyte growth factor (HGF) by Cyclin D1 upregulation is mediated at the transcriptional level by ATF2. This pathway is at least partly dependent on both p38 and JNK (Recio and Merlino 2002). However, p38 has also been implicated in differentiation of melanocyte-lineage cells; α -MSH can induce differentiation of murine melanoma cells via p38 (Smalley and Eisen 2000). This was recapitulated in human cells with a notably greater and more sustained induction of p38 following α -MSH treatment or cAMP activation with forskolin. The cell cycle arrest and differentiation was associated with a p38-dependent increase in hypophosphorylated, active Rb protein (Smalley and Eisen 2002).

1.5.8 | UV-Induced DNA Damage and Genomic Instability in Melanoma

Genomic instability comprises changes to the structure and function of DNA, through base changes, chromosomal translocations and failures in chromosomal segregation which result in aneuploidy (Carter et al. 2006). Impaired DNA damage response (DDR) is a hallmark of cancer, resulting in increased genomic instability and oncogenesis (Hanahan and Weinberg 2011). Tissues which are chronically exposed to mutagens have the highest frequency of somatic mutations. For example, lung cancer and melanoma have notoriously high mutational burdens and heterogeneity of mutations as a result of exposure to tobacco and UV respectively (Lawrence et al. 2013). This is reflected in the high genomic instability seen in melanomas (Alexandrov et al. 2013). In the past decade, a series of so-called mutational signatures have been described. Comparison of somatic mutations from whole genome or exome sequencing in over 7000 tumour samples compared to normal tissue identified 21 signatures (Alexandrov et al. 2013).

The signatures are combinations of somatic mutations which have been linked to specific DNA damaging processes such as ageing. For signatures where the mutagen is identifiable with high probability, they have provided insight into the impact of specific DNA damaging agents on somatic mutation at base level and subsequent cancer development (Alexandrov et al. 2013). One of the signatures comprises high C>T mutation rate with untranscribed strand bias and is highly overrepresented in melanoma and squamous head and neck tumours and probably corresponds to UV exposure (Alexandrov et al. 2013). The C>T mutation resulting from UVB irradiation is due to the propensity of the NER pathway to replace bases with adenine, therefore, although T-T dimers result in correct repair of bases, dimers involving cytosine are mutated to thymine (Choi et al. 2006; Brash 2015; Pfeifer et al. 2005). The mechanisms which contribute to

the accumulation of C>T transition mutations in melanoma are not fully understood. However, mutations in the MC1R gene, implicated in melanoma susceptibility, have been correlated to increased burden of single-nucleotide variants (SNVs), particularly C>T mutations (Robles-Espinoza et al. 2016). This suggests that MC1R signalling is involved in protecting melanoma cells from genome instability.

The C>T UV mutational signature has been implicated in both the onset and evolution of melanoma, as it increases during melanoma lesions progression (Shain et al. 2015; Lawrence et al. 2013; Alexandrov et al. 2013; Hodis et al. 2012). Melanoma has a very high burden of somatic mutations compared to other tumours (Alexandrov et al. 2013; Pleasance et al. 2010; Hodis et al. 2012). However, only some of these will have a material effect on the biology of the melanoma, and much work has been done to separate the signal from noise to identify the so-called ‘driver mutations’ from the inert ‘passenger mutations’ (Pleasance et al. 2010). Driver mutations in melanoma include the BRAF^{V600E} mutation and NRAS mutations which result in constitutively activated growth signalling and deregulated proliferation, driving the development of melanoma (Pleasance et al. 2010; Hodis et al. 2012). Such point mutations have been successfully targeted by small molecule inhibitors such as vemurafenib which directly targets mutant BRAF or MEK inhibitors like trametinib which target the kinase downstream of mutant NRAS (Flaherty et al. 2010, 2012; Ugurel et al. 2017). However, these inhibitors have had limited efficacy, both in duration of response, and in proportion of patients with disease response (Trunzer et al. 2013; Ugurel et al. 2017; Rughani et al. 2013).

1.6 | Summary

Tumour phenotypic heterogeneity, either genetic or transcriptionally derived, contributes to therapy resistance in cancer treatment. Mixed populations of cells with different phenotypic traits render both conventional and targeted therapies unable to completely eradicate a tumour. Understanding the mechanisms of generation and control of phenotypic heterogeneity is critical in moving forward the treatment of cancer. The melanoma phenotype is known to be dependent on the expression and activity of the transcription factor MITF. The POU domain transcription factor BRN2 can modulate MITF transcription bi-directionally, dependent on context. How this dynamic control is achieved is unknown. Here we have introduced the generalised molecular mechanisms by which transcription factor activity can be modified with particular focus on post-translational modification by phosphorylation. Subsequently we discussed the specifics of POU domain family function and control. Although here we use melanoma as a model system, we have described how BRN2 has key roles in development and a range of cancers, which means that our results may be applicable beyond melanoma. Finally, the transcriptional programme of a cell is strongly influenced by exogenous stimuli which are transmitted to the nucleus via intracellular signalling pathways. UV is the most important exogenous agent in melanoma development, exerting its influence both through direct DNA damage and activation of signalling cascades. The resultant genome instability in UV damaged cells is a key contributor to oncogenesis and progression in melanoma.

1.7 | Aims

The aims of this thesis are to understand how BRN2 function might be modified to affect melanoma cell phenotype. We chose to approach this question by investigating two of the key mechanisms by which transcriptional factor behaviour is controlled: protein-protein interactions and post-translational modifications. In the first results chapter we look at the BRN2 interactome, in the second, we investigate BRN2 phosphorylation in the context of UV-activated stress response kinases.

Chapter 2 | Materials and Methods

2.1 | DNA methods

2.1.1 | Restriction Enzyme Sub-Cloning

2.1.1.1 | *PCR of template DNA*

Primers for PCR were designed using Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) with addition of unique restriction enzyme site and are listed in Tables 1 and 2. Inserts were amplified with AccuPrime Taq DNA polymerase High Fidelity kit (Invitrogen; 12346094) from pre-existing vectors in the Goding lab database or newly generated point mutants. PCR reactions were set up with 10 ng template DNA, 1 pmol each of forward and reverse primers, in 10X AccuPrime Buffer I with 0.2 µl AccuPrime High Fidelity Taq DNA Polymerase in a final volume 50 µl made up with ultrapure water (18Ω). The PCR program was run on a T100™ Thermal Cycler (BioRad) or Veriti Thermal Cycler (ThermoFisher) as per manufacturer's instructions, optimised as follows: Initial denaturing step 94 °C 100 s; 30 cycles [Denature 94 °C 20 s; Anneal 57 °C 20 s; Extend 68 °C 1 min per kb]; final extension 68 °C 5mins.

2.1.1.2 | *Agarose gel electrophoresis*

PCR product size was verified by agarose gel electrophoresis. 0.8 % agarose gels were prepared in 1X Tris-borate-EDTA (TBE) with the DNA dye PeqGREEN (VWR; 732-3196) to visualise DNA. 50 bp, 100 bp or 1 kb HyperLadders™ (Bioline; BIO-33054, BIO-33056 or BIO-33053) were run alongside a 4 µl aliquot of PCR product which was

prepared with 5X loading buffer (Bioline, BIO-37041). Gels were then visualised under UV light in a UVIdoc (UVItec, Cambridge, UK)

2.1.1.3 | *Restriction enzyme digestion*

The remaining PCR product was purified with QIAquick PCR Purification Kit (Qiagen; 28106) according to the manufacturer's instructions and eluted in 30 µl ultrapure water (18Ω). Amplified, purified inserts were then cloned into a target backbone vector. Simultaneous digestion of 2 µg backbone and PCR insert was carried out using High Fidelity (HF®) restriction endonucleases (New England Biolabs: EcoR1 HF #R3101L; Not1 HF #R3189L; BamH1 HF #R3136L; Kpn1 #R3142S) in 10X CutSmart® buffer (New England Biolabs; B7204S) in final volume 50 µl made up with nuclease-free water. The digested vector and insert were then purified with the QIAquick PCR Purification Kit (Qiagen; 28106) according to the manufacturer's instructions. The digestion products were eluted in 30 µl ultrapure water (18Ω).

2.1.1.4 | *Ligation*

Ligations were carried out at RT for 1 hour with T4 DNA ligase (New England Biolabs; M0202S) with 10X T4 DNA Ligase Buffer (New England Biolabs; B0202S) in final volume 40 µl made up with ultrapure water (18Ω). A 2.5 µl aliquot was transformed into *E. coli* (see below) whilst the remainder of the ligation mix was incubated at 16 °C overnight. Where transformation of the initial ligation mix failed to produce bacterial colonies, the remaining mix was precipitated in 10 volumes of butan-2-ol, dried for 5 minutes at RT and re-suspended in minimum volume ultrapure water (18Ω) possible prior to re-transformation.

2.1.1.5 | Transformation

25 µl *E. coli* Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen; 18265017) was thawed on ice. A 2.5 µl aliquot of ligation product was then incubated with the bacteria on ice for 5 minutes in a 1.5 ml Eppendorf tube. The bacteria were then transformed by heatshock in a waterbath at 42 °C for 35 seconds. The tube was then incubated on ice for 2 minutes and then 150 µl S.O.C. media added (ThermoFisher; 15544034). The transformation mix was then incubated on a shaker at 37 °C at 220 rpm for 45 minutes. Bacteria were then spread on pre-warmed ampicillin or kanamycin pre-poured LB-Agar plates and incubated overnight at 37 °C.

2.1.1.6 | Colony PCR

Colonies grown on antibiotic selection plates were picked and screened by colony PCR with MyTaq Red mix (Bioline; BIO-25044) using amplifying primers located in the plasmid backbone that flanked the insert. PCR products were then run on Agarose gels as described above and colonies with correct size plasmid insert were grown as minicultures in LB media.

2.1.1.7 | Plasmid purification

DNA plasmid was purified from the bacterial culture using QIAprep Spin Miniprep kits (Qiagen; 27106). Plasmids were eluted in 35 µl ultrapure water (18Ω). The concentration of purified DNA was measured by spectrophotometry on a NanoDrop 2000 (ThermoFisher). DNA was sent for Sanger sequencing using sequencing primers which flanked the insert.

2.1.2 | Site-Directed Mutagenesis

QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies; #210519-3) was used to according to the manufacturer's instructions to create point

mutants. Primers were designed using the QuikChange Primer Design Program. Available at: <https://www.genomics.agilent.com/primerDesignProgram.jsp>. See Table 3. PCR reactions were set-up in 20 µl final volume with 10 ng template DNA, 0.6 pmol each of forward and reverse primers, 0.4 µl dNTPs, 0.6 µl QuikSolution, 2 µl 10X QuikChange Lightning buffer and 0.4 µl QuikChange Lightning Enzyme. PCR program was run on T100™ Thermal Cycler (BioRad) or Veriti Thermal Cycler (ThermoFisher) as per manufacturer's instructions, optimised as follows: Initial denaturing step 95 °C 120 s; 18 cycles [Denature 95 °C 20 s; Anneal 55-60 °C 20 s; Extend 68 °C 30 s per kb]; final extension 68 °C 5 mins. Template DNA was then digested with addition of 0.8 µl Dpn1 restriction enzyme at 37°C for 1 hour. DNA was incubated with XL10 gold ultracompetent *E. coli* (Agilent; 200315) on ice for 30 minutes in 14 ml Falcon tubes and then transformed by heatshock at 42 °C for 30s. The tube was then incubated on ice for 2 minutes prior to the addition of 150 µl S.O.C. media (ThermoFisher; 15544034). The transformation mix was then incubated at 37 °C on a shaker at 220 rpm for 45 minutes. Bacteria were then spread on pre-warmed ampicillin or kanamycin pre-poured LB-Agar plates and incubated overnight at 37 °C.

Colonies were picked and grown into cultures in 3 ml LB overnight at 37 °C at 220 rpm. DNA plasmid was purified from the bacterial culture using QIAprep Spin Miniprep kits (Qiagen; 27106). Plasmid was eluted in 35 µl ultrapure water (18Ω). DNA was sent for Sanger sequencing to confirm the point mutation.

2.1.3 | Dot-Blot Assay

DNA was purified from cell pellets with PureLink® Genomic DNA kit (Invitrogen; K182002). The concentration of purified DNA was measured by spectrophotometry on a NanoDrop 2000 (ThermoFisher). For immunodetection of CPDs,

100 ng DNA was diluted into a final volume of 75 μ l 0.4M NaOH, 10 mM EDTA. Samples were then denatured by boiling at 95 °C for 10 mins and then neutralised with cold 2 M ammonium acetate pH 7.0 on ice. A 96 well dot-blot apparatus (Jencons; 286-437 DHM-96) was assembled with 3 layers of 3 mm Whatman blotting paper and nitrocellulose membrane (GE Healthcare Amersham™ Protran™ NC; 45-004-000) pre-soaked in SSC 6X (SSC 20X: 3 M NaCl, 0.3 M $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, pH 7.0). Wells were rinsed with 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA) under vacuum; samples were then pipetted into wells and run through under vacuum. Following one rinse under vacuum with 2X SSC, DNA was fixed to the membrane by heating under vacuum at 80 °C for 2 hours on a gel dryer. The membrane was washed in PBS-Tween 0.1% (PBS-T), blocked in 5% milk in PBS-T at RT for 30 mins and then incubated with primary antibody against CPD at 4 °C overnight in PBS-T. Following 4 x 15 min washes in PBS-T, the membrane was incubated with secondary HRP-conjugated antibody for 1 hour. The membrane was then incubated with Amersham™ ECL™ (GE Healthcare, RPN2106) following 4 x 15 min washes in PBS-T, and then exposed to radiographic film. The membrane was then rinsed before incubation with SYBR®-Gold (Invitrogen; S11494. 1:10,000 in PBS-T) for 20 minutes. The membrane was then imaged in a GelDoc (Biorad) using trans UV illumination. Immunoblot and DNA staining were then analysed using ImageJ software.

Table 1 | Restriction enzyme sub-cloning for *in cellulo* assays

Construct	Template	Backbone	Primer name	Primer sequence
Transient mammalian expression: pCMV				
pCMV mCh BRN2 WT 3xF	pCMV mCh P2A MCS 3xF	pCMV BRN2 WT 3xF	Kpn1_mCherry	tttggtaccATGGTGAGCAAGGGCGAGG
			mCherry_BamH1	aaaggatccCTTGTACAGCTCGTCCATGCCG
pCMV BRN2 442A 3xF	pCMV BRN2 WT 3xF	p3xF CMV 14	Not1_BRN2	tttgcggccgcATGGCGACCGCAGCGTCTAA
			BRN2_442A_BamH1	aaaggatccCTGGACGGGCGCC
pCMV BRN2 7A 3xF	pCMV BRN2 6A 442T 3xF	p3xF CMV 14	Not1_BRN2	tttgcggccgcATGGCGACCGCAGCGTCTAA
			BRN2_442A_BamH1	aaaggatccCTGGACGGGCGCC
Stable, inducible mammalian expression: PiggyBac				
pPB-hCMV1 Brn2 WT 3XF	pCMV Brn2 WT 3XF	pPB-hCMV1-cHApA-MCS puro	EcoR1-BRN2 N term	tttGAATTCGCCATGGCGACCGCAGCGTCT
			3xF_Not1	aaaGCGGCCGCTCACTACTTGTTCATCGTCATCCTTGT
pPB-hCMV1 Brn2 N term3XF	pCMV Brn2 N term3XF	pPB-hCMV1-cHApA-MCS puro	EcoR1-BRN2 N term	tttGAATTCGCCATGGCGACCGCAGCGTCT
			3xF_Not1	aaaGCGGCCGCTCACTACTTGTTCATCGTCATCCTTGT
pPB-hCMV1 Brn2 ΔN 3XF	pCMV Brn2 ΔN 3XF	pPB-hCMV1-cHApA-MCS puro	EcoR1-BRN2 ΔN	tttGAATTCGCCATGGACACGCCGACCTCAGAC
			3xF_Not1	aaaGCGGCCGCTCACTACTTGTTCATCGTCATCCTTGT
pPB-hCMV1 Brn2 ΔNC 3XF	pCMV Brn2 ΔNC 3XF	pPB-hCMV1-cHApA-MCS puro	EcoR1-BRN2 ΔN	tttGAATTCGCCATGGACACGCCGACCTCAGAC
			3xF_Not1	aaaGCGGCCGCTCACTACTTGTTCATCGTCATCCTTGT
pPB-hCMV1 Brn2 ΔC 3XF	pCMV Brn2 ΔC 3XF	pPB-hCMV1-cHApA-MCS puro	EcoR1-BRN2 N term	tttGAATTCGCCATGGCGACCGCAGCGTCT
			3xF_Not1	aaaGCGGCCGCTCACTACTTGTTCATCGTCATCCTTGT
pPB-hCMV1 Brn2 ΔNLS 3XF	pCMV Brn2 ΔNLS 3XF	pPB-hCMV1-cHApA-MCS puro	EcoR1-BRN2 N term	tttGAATTCGCCATGGCGACCGCAGCGTCT
			3xF_Not1	aaaGCGGCCGCTCACTACTTGTTCATCGTCATCCTTGT
Lentiviral expression				
CSII-EF mCh-P2A-3xF BRN2 WT	pCMV BRN2 WT 3xF	CSII-EF mCh-P2A-3xF MCS	BamH1_BRN2	tttgatccATGGCGACCGCAGCGTCTAAC
			BRN2_442T_Not1	aaagcgggcgcTCACTGGACGGGCGTCTGCACC
CSII-EF mCh-P2A-3xF BRN2 N401A	pCMV BRN2 N401A 3xF	CSII-EF mCh-P2A-3xF MCS	BamH1_BRN2	tttgatccATGGCGACCGCAGCGTCTAAC
			BRN2_442T_Not1	aaagcgggcgcTCACTGGACGGGCGTCTGCACC

Table 2 | Restriction enzyme sub-cloning for *in vitro* assays

Construct	Template	Backbone	Primer name	Primer sequence
Bacterial expression: pGEX				
pGEX 4T1 GST BRN2 WT	pCMV Brn2 WT 3XF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442T_NotI	aaagcggccgcTCACTGGACGGGCGTCTGCACC
pGEX 4T1 GST BRN2 442E	pCMV BRN2 442E 3xF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442E_NotI	aaagcggccgcTCACTGGACTGGCTCTTGTA TACTCCGT
pGEX 4T1 GST BRN2 6A 91S	pCMV Brn2 6A 91S 3XF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442A_NotI	aaagcggccgcTCACTGGACGGGCGCCTGCAC
pGEX 4T1 GST BRN2 6A 96S	pCMV Brn2 6A 96S 3XF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442A_NotI	aaagcggccgcTCACTGGACGGGCGCCTGCAC
pGEX 4T1 GST BRN2 6A 266T	pCMV Brn2 6A 266T 3XF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442A_NotI	aaagcggccgcTCACTGGACGGGCGCCTGCAC
pGEX 4T1 GST BRN2 6A 343S	pCMV Brn2 6A 343S 3XF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442A_NotI	aaagcggccgcTCACTGGACGGGCGCCTGCAC
pGEX 4T1 GST BRN2 6A 415T	pCMV Brn2 6A 415T 3XF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442A_NotI	aaagcggccgcTCACTGGACGGGCGCCTGCAC
pGEX 4T1 GST BRN2 6A 434T	pCMV Brn2 6A 434T 3XF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442A_NotI	aaagcggccgcTCACTGGACGGGCGCCTGCAC
pGEX 4T1 GST BRN2 6A 442T	pCMV Brn2 6A 442T 3XF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442T_NotI	aaagcggccgcTCACTGGACGGGCGTCTGCACC
pGEX 4T1 GST BRN2 91A	pCMV BRN2 91A 3xF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442T_NotI	aaagcggccgcTCACTGGACGGGCGTCTGCACC
pGEX 4T1 GST BRN2 96A	pCMV BRN2 96A 3xF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442T_NotI	aaagcggccgcTCACTGGACGGGCGTCTGCACC
pGEX 4T1 GST BRN2 266A	pCMV BRN2 266A 3xF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442T_NotI	aaagcggccgcTCACTGGACGGGCGTCTGCACC

Construct	Template	Backbone	Primer name	Primer sequence
pGEX 4T1 GST BRN2 343A	pCMV BRN2 343A 3xF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442T_Not1	aaagcggccgcTCACTGGACGGGCGTCTGCACC
pGEX 4T1 GST BRN2 434A	pCMV BRN2 434A 3xF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442T_Not1	aaagcggccgcTCACTGGACGGGCGTCTGCACC
pGEX 4T1 GST BRN2 442A	pCMV BRN2 442A 3xF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442A_Not1	aaagcggccgcTCACTGGACGGGCGCCTGCAC
pGEX 4T1 GST BRN2 7A	pCMV BRN2 7A 3xF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442A_Not1	aaagcggccgcTCACTGGACGGGCGCCTGCAC
pGEX 4T1 GST BRN2 N term	pCMV Brn2 N term3XF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2 N term_Not1	aaaGCGGCCGCTCATGAGGTCTGGCGTGTCTCCTCGTC
pGEX 4T1 GST BRN2 ΔN	pCMV Brn2 ΔN 3XF	pGEX 4T1 GST S2A	BamH1_ΔN BRN2	tttggatccATGGACACGCCGACCTCAGAC
			BRN2_442T_Not1	aaagcggccgcTCACTGGACGGGCGTCTGCACC
pGEX 4T1 GST BRN2 ΔNC	pCMV Brn2 ΔNC 3XF	pGEX 4T1 GST S2A	BamH1_ΔN BRN2	tttggatccATGGACACGCCGACCTCAGAC
			BRN2_ΔC_Not1	aaaGCGGCCGCTCACCTTTTCTCTTTCTGTCTCCTGTTAC
pGEX 4T1 GST BRN2 ΔC	pCMV Brn2 ΔC 3XF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_ΔC_Not1	aaaGCGGCCGCTCACCTTTTCTCTTTCTGTCTCCTGTTAC
pGEX 4T1 GST BRN2 ΔNLS--	pCMV Brn2 ΔNLS 3XF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442T_Not1	aaagcggccgcTCACTGGACGGGCGTCTGCACC
pGEX 4T1 GST BRN2 91/96EE	pCMV BRN2 91/96EE 3xF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442T_Not1	aaagcggccgcTCACTGGACGGGCGTCTGCACC
pGEX 4T1 GST BRN2 442E	pCMV BRN2 442E 3xF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442E_Not1	aaagcggccgcTCACTGGACTGGCTCTTGTACTCCGT
pGEX 4T1 GST S2A Empty	Adaptors	pGEX 4T1 GST S2A	BamH1_stop	GATCCTGAACGTGTC
			stop_Not1	GACTTGACACGCCGG
pGEX 4T1 GST BRN2 N401A	pCMV BRN2 N401A 3xF	pGEX 4T1 GST S2A	BamH1_BRN2	tttggatccATGGCGACCGCAGCGTCTAAC
			BRN2_442T_Not1	aaagcggccgcTCACTGGACGGGCGTCTGCACC

(Table 2 continued | Restriction enzyme sub-cloning for *in vitro* assays)

Table 3 | Primers for Site Directed Mutagenesis

Product	Template	Point mutation	Forward primer	Reverse primer
pCMV Brn2 4A + 266A 3XF	pCMV Brn2 4A* 3XF	BRN2 T266A	tcggacgaggacgcgccgacctcag	ctgaggtcggcgcgccctcgtccga
pCMV Brn2 4A + 415A 3XF	pCMV Brn2 4A* 3XF	BRN2 T415A	agaaagagaaaaggatggccctcccggaggg	ccctccgggaggggccatcctttctctttct
pCMV Brn2 6A 442T 3XF	pCMV Brn2 4A + 266A 3XF	BRN2 T415A	agaaagagaaaaggatggccctcccggaggg	ccctccgggaggggccatcctttctctttct
pCMV Brn2 6A 91S 3XF	pCMV BRN2 7A 3xF	BRN2 A91S	gtggaccacgggctgccatgccgcc	ggcggcgatggcagcccggtgtccac
pCMV Brn2 6A 96S 3XF	pCMV BRN2 7A 3xF	BRN2 A96S	ctggcctagggggctggtggaccacggg	cccgtggtccaccagccccctaggccag
pCMV Brn2 6A 266T 3XF	pCMV BRN2 7A 3xF	BRN2 A266	ctgaggtcggcgtgtcctcgtccga	tcggacgaggacacgccgacctcag
pCMV Brn2 6A 343S 3XF	pCMV BRN2 7A 3xF	BRN2 A343S	ctatgctggtgggctgcccaggatgagt	actcatcctcgggcagccccaccagcatag
pCMV Brn2 6A 415T 3XF	pCMV BRN2 7A 3xF	BRN2 A415T	ccctccgggaggggtcatcctttctctttct	agaaagagaaaaggatgaccctcccggaggg
pCMV Brn2 6A 434T 3XF	pCMV BRN2 7A 3xF	BRN2 A434T	tggtgtggtggcgtgtccctactacc	gggtagtagggacacgccaccacacca
pCMV mCh BRN2 91A 3xF	pCMV mCh BRN2 WT 3xF	BRN2 S91A	ggaccacggggcgccgtgccgc	gcggcgacggcgccccgtgtcc
pCMV mCh BRN2 91/96AA 3xF	pCMV mCh BRN2 91A 3xF	BRN2 S96A	ctggcctagggggcggtggaccacggg	cccgtggtccaccgccccctaggccag
pCMV BRN2 N401A 3xF	pCMV BRN2 WT 3xF	BRN2 N401A	tttcttttctgtctcctggcacaaaacaaactctcaccacc	ggtggtgagagtgtgtttgtgccaggagacagaaagagaaa
pGEX 4T1 GST S2A	pGEX 4T1 GST	GST S2A	cacaggaacagtattcatggcccctat	ccaataacctagtataggggcatgaat

*BRN2 4A = (91,96,343,434)

2.2 | RNA methods

2.2.1 | RNA Seq Sample Preparation

RNA was extracted using the RNeasy kit (QIAGEN #74106), and QC was performed on a Bioanalyzer (for RIN ≥ 9.5). ERCC ExFold RNA Spike-In Mixes (Ambion) were added prior to Library prep using QuantSeq Forward kit (LEXOGEN #015.96), using 500 ng starting material to minimise the PCR amplification step. Samples were sequenced on HiSeq 2500 (Illumina) carried out using the Wellcome Trust genomic service, Oxford.

2.3 | Cell methods

2.3.1 | Cell Culture

Cells were cultured as monolayers in 10% CO₂ at 37 °C and regularly tested for mycoplasma. All media contained GlutaMAXTM and Phenol Red and were supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin (10,000 Units/ml, Gibco, ThermoFisher) except where indicated. The following human melanoma cell lines and any modified versions were cultured in RPMI-1640 (Gibco, ThermoFisher): 501mel, IGR37, IGR39, SKMel28, NA8-Mel, SKMel2, WM852 and SKMel30. The following cell lines and any modified versions were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, ThermoFisher): U-2-OS, Phoenix and B16 melanoma. Where necessary, drugs were diluted in water or DMSO as indicated and then diluted in culture media prior to cell treatment (see Table 4).

2.3.2 | Transient Transfection

Mammalian cells were transfected using the FuGENE® 6 Transfection Reagent lipofection system (Promega; E2691) as per manufacturer's instructions. Cells were cultured to 50-60% confluence and then media changed to antibiotic-free media (RPMI or DMEM according to cell line, as above). FuGENE® 6 Transfection Reagent was incubated with OptiMEM (Gibco, ThermoFisher) for 5 minutes, DNA was then added at a Fugene:DNA ratio of 3:1 and incubated for 30 minutes prior to transfection. For one well of a 6 well-plate, 2 µg DNA was incubated with 6 µl of FuGENE®6 Transfection Reagent in 100 µl Optimem. Cells were then incubated for 24 hours prior to assays being performed as indicated.

2.3.3 | Stable Transfection - Piggybac

The FuGENE® 6 lipofection system was used as above to generate polyclonal stable, doxycycline-inducible BRN2 FLAG cell lines in 501mel cells. pPB-hCMV1 Brn2 WT 3XF and truncation mutant vectors were co-transfected with the PiggyBac (PB) transposase vector pPyCAG-PBase and the Tet-On System vector with a Neomycin resistance cassette pPB-CAG-rtTA-IRES-Neo in a ratio of 9:0.5:0.5. 501mel cells were cultured to 50% confluency in 10 cm dish and transfected with total of 12 µg DNA. Transfection reagents were prepared in OptiMEM (Gibco, ThermoFisher) at a FuGENE® to DNA plasmid ratio of 3:1 as above.

Following transfection, cell lines were subjected to double selection with Puromycin 3 µg/ml (p8833, Sigma-Aldrich) and Genetecin® 750 µg/ml (G418, Gibco, ThermoFisher) for 48-72h until death of all control 501mel cells treated in parallel. Monoclonal cell lines from BRN2 WT Flag were established by colony picking and were screened by immunofluorescence. BRN2 FLAG expression was induced with Doxycycline as indicated (D9891, Sigma-Aldrich).

2.3.4 | Stable Overexpression – Lentiviral Cell Lines

A lentiviral vector (pCSII EF1alpha) containing the sequence encoding BRN2 WT or N401A and mCherry fluorescent protein separated by a P2A self-cleaving peptide was used to establish stable cell lines in 501mel cells. Virus was produced by transient transfection into Phoenix cells with Lipofectamine 2000 (Invitrogen; 11668019) according to the manufacturer's instructions together with vectors encoding Gag/Pol, Rev, and the vesicular stomatitis virus (VSV) envelope. Virus-containing supernatant was harvested at 48 and 72 h post-transfection, passed through a 0.45- μ m filter and target cells were incubated with limiting dilutions of virus from 1:1 to 1:125 for 72 hours. Infection efficiency was measured by mCherry expression using flow cytometry with a FACSCantoII (BD Biosciences). Target cells with equivalent infection efficiency were expanded cell sorted by FACS according to mCherry expression to achieve a 100% positive cell population with the same levels of the exogenous protein expression.

2.3.5 | RNA interference

siRNA oligomers targeting BRN2 were purchased from Dharmacon (siBRN2#1; custom siRNA, target sequence: AAGCGCAGAGCCTGGTGCAGG) and Sigma-Aldrich (siBRN2#2; SASI_Hs01-00196791). Adequate knockdown efficiency was confirmed for two siRNA by WB and IF. 501mel cells cultured in antibiotic free media were cultured to 40% confluence and transfected with control siRNA or BRN2 siRNA at stock concentrations of 20 μ M using Lipofectamine® RNAiMAX as per manufacturer's instructions with RNAiMAX:siRNA ratio 1:0.6. Cells were then cultured for up to 72 hours. If media change was required for assays, media was changed for all cells at first treatment time point.

2.3.6 | UV Irradiation

Cells were cultured in individual 35 mm or 60 mm plates for UVB, or 10 cm plates for UVC. Prior to UV irradiation, media was exchanged for PBS. For UVB irradiation, the plate, with lid removed, was placed in a custom-made chamber and subjected to sham treatment or irradiated with 150 J/m² UVB using a 302 nm bulb (UVM-24 EL series UV 4W; UVP, LLC) calibrated using a light detector at the appropriate wavelength. For UVC, the plate, with lid removed was placed in a UVC crosslinker and treated with doses of 25 or 50 J/m² at 254nm (CL-1000; UVP, LLC). PBS was removed and fresh media was replaced and cells were incubated for remainder of time course.

2.3.7 | λ Phosphatase Treatment

Cells were collected by scraping from 6 well plates in 150 μ l ice-cold λ phosphatase buffer (1X λ phosphatase buffer, 1X MnCl₂ in PBS). Samples were sonicated at 4°C for 30 seconds in a water bath sonicator (Diagenode Bioruptor). The sample was then divided in two. Phosphatase inhibitors (PhosSTOP™ Roche, Sigma-Aldrich #04906845001; 1 tablet/10 ml buffer) were added to the first aliquot. 0.5 μ l λ phosphatase was added to the second. Both samples were then incubated at 30°C for one hour. 2X Laemmli sample buffer was then added, samples were denatured at 95°C for 5 minutes prior to running on Phos-tag™ gel (See Phos-tag™ gel electrophoresis).

Table 4 | Drugs used in cell culture

Drug name	Target	Solvent	Supplier	Product #
Olaparib	PARP inhibitor	DMSO	Cayman Chemicals	10621
SB203580	p38 inhibitor	DMSO	Sigma-Aldrich	S8307
SB202190	p38 inhibitor	DMSO	Sigma-Aldrich	S7067
SB239063	p38 inhibitor	DMSO	Sigma-Aldrich	S0569
Doxycycline	Tetracycline response element	Water	Sigma-Aldrich	D9891
Hydrogen Peroxide	ROS generator	Water	Sigma-Aldrich	H1009

2.4 | Cell-based assays

2.4.1 | Luciferase Assay

501mel cells were plated in 24 well plates and transfected by FuGENE® 6 lipofection as above at FuGENE:DNA ratio 3:1, in triplicate with 200 ng MITF promoter-luciferase reporter vector and 200 ng pCMV BRN2 FLAG expression vectors or pCMV FLAG Empty control per well. After 48 hours, cells were analysed using a Luciferase Assay System (Promega; E1500) as per manufacturer's instructions. Passive lysis was carried out with 50 µl Lysis buffer per well, at RT, shielded from light on a rotating shaker for 15 minutes. 25 µl aliquots of the samples, without debris, were pipetted into a white 96 well plate. 50 µl of Luciferase Assay Substrate was then added to each well and the plate immediately loaded into a plate-reader and luminescence detected by photo-multiplier tube (PMT) (Promega; Glomax-Multi). Triplicate experimental luminescence readings were normalised to the MITF promoter_luciferase reporter + pCMV FLAG empty sample.

2.4.2 | Flow Cytometry

2.4.2.1 | Fixed cells: γ H2AX and Cell cycle

Cells which had been subjected to UV irradiation, mitotic shake or drug treatments as indicated were grown to 70-80% confluency. They were washed in PBS, trypsinised and resuspended in staining solution SS5 (FBS 5%, EDTA 2 mM, NaNH₃ 0.01% in PBS) and centrifuged at 125 g for 5 minutes to pellet. Cells were fixed by resuspending the cell pellet in 70% EtOH in PBS and incubated on ice for 1 hour. The fixed cells were then centrifuged and the pellet resuspended in 250 µl of staining mix (PBS with RNase A 0.1 mg/ml, 0.05% Triton X100) with DNA dye/antibodies as follows. Either 1: TO-PRO™-3 Iodide (642/661) 1:1000, Phospho-H2A.X(Ser139)-

FITC Antibody 1:250; Or 2: Propidium Iodide 50 µg/ml and incubated at 37 °C for 45 minutes. Cells were washed in PBS, pelleted by centrifugation and resuspended in 250 µl SS5. 10-50,000 cells were measured by FACS on BD FACSCanto II (BD Biosciences) and data were analysed using FlowJo software (7.6.1).

2.4.2.2 | *Live cells: Apoptosis*

Apoptosis was analysed by FACS using CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit (Thermofisher; C10427) as per manufacturer's instructions. Prior to application of the kit, cells were collected as follows: 24 hours after irradiation, media was collected from cells at 70-80% confluency, the adherent cells were then washed in PBS, trypsinised and resuspended in collected media. Samples were centrifuged at 125 g for 5 minutes and cell pellets resuspended in 250 µl SS5 (FBS 5%, EDTA 2 mM, NaNH₃ 0.01% in PBS) with 1:1000 CellEvent™ Caspase 3/7 Green Detection Reagent: 511/533. Samples were incubated, shielded from light, at 37 °C for 30 minutes. SYTOX® AADvanced™: 546/ 647 live/dead stain 1:1000 was added 5 minutes prior to FACS analysis. Samples were then analysed on BD LSRFortessa Cell Analyzer (BD Biosciences) and data were analysed using FlowJo software (7.6.1).

2.4.3 | **Mitotic Shake**

Three 175 cm² flasks per cell line were cultured up to 70-80% confluency. Cells were synchronised in mitosis by shaking two of three source flasks to detach low adherent, mitotic cells, these were then collected and reseeded on new plates. This was repeated 2 hourly for 24 hours, alternating through the three source flasks. After 24 hours samples were collected for FACS analysis of Cell Cycle as above.

2.4.4 | Laser Microirradiation (LMI), Fixed and Live Cells

2.4.4.1 | *Live LMI*

For live imaging U-2-OS cells were cultured in glass-bottomed dishes. GFP-BRN2 or mCherry-BRN2 constructs or empty fluorescent controls were transfected by transient transfection with FuGENE® 6, as above, 24 h prior to irradiation. Cells were incubated with Hoechst 33258 10 µg/ml (Invitrogen, H3569) at 37 °C for 1 hour prior to irradiation. For live imaging, DMEM was replaced with FluoroBrite DMEM (Gibco, ThermoFisher, A18967), supplemented with 10% FBS, 1 % Penicillin-Streptomycin and 4 mM glutamine. Cells were irradiated using a multiphoton laser at 750 nm with 100 mW power via a Plan-Apochromat 63x/1.40 Oil DIC M27 objective with fully open pinhole on LSM 710 confocal microscope (Carl Zeiss AG) with Zen software. Live images were obtained every 10s from 30s pre-irradiation to 420s after irradiation. Where indicated, cells were incubated with DMSO, PARP inhibitor (Olaparib 10 µM; Cayman Chemicals, 10621) or p38 inhibitor (SB203580 10 µM; Sigma-Aldrich, S8307) for 3 hours prior to and during irradiation. Images were analysed using ImageJ and Matlab (2015b) software. Background to Stripe intensity ratio was calculated for each time point and expressed as percentage change in intensity compared to background. Statistical significance of differences in stripe intensity was assessed using repeated measures ANOVA.

2.4.4.2 | *Fixed LMI*

For fixed imaging, 501mel cell lines were cultured on 18 mm diameter, #1 thickness, round glass coverslips (NeuVtro; 1001/18) in 12 well plates to 60 % confluency. One hour prior to LMI, Hoechst 33258 10 µg/ml (Invitrogen, H3569) was added to the well and incubated at 37 °C. Fresh media was then added prior to transferring plates to the microscopy room. Coverslips were then extracted from the culture dish with forceps, the bottom surface was dried, and the coverslip was transferred to a dry, custom-

adapted culture dish with an aperture cut out for access by the microscope objective. 3 drops of media were then added to the coverslip with a Pasteur pipette. The dish was transferred to the microscope stage. The Z-plane was fixed near to the centre of the nuclei using Hoescht and Brightfield/DIC (T-PMT detector) as markers. Cells were then irradiated using a multiphoton laser at 750 nm with 100 mW power in a 5x5 tile via a Plan-Apochromat 20x/0.8 M27 objective with fully open pinhole on LSM 710 confocal microscope (Carl Zeiss AG) with Zen software.

After the specified time course incubation, prior to fixation, soluble proteins were pre-extracted. All steps were carried out at RT. Coverslips were gently washed for a few seconds in Cytoskeletal buffer (CSK) (10 mM PIPES pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂), soluble proteins were then extracted in CSK-T buffer (CSK buffer, Triton X-100 0.5%) for 45-75 seconds, depending on the cell line RNaseA 0.3 mg/ml was added to CSK-T for pre-extraction of RNA-bound proteins when staining for Ku80. After a brief wash in CSK, coverslips were fixed in 4% PFA for 12 minutes and then washed in PBS twice prior to continuing immunofluorescence protocol as below.

2.4.5 | Immunofluorescence

2.4.5.1 | Fixation and permeabilisation of non-LMI immunofluorescence samples

Cells to be assayed by immunofluorescence were cultured on 10x10mm, #1.5 thickness, square coverslips to 60-80% confluency. Coverslips were transferred directly from culture dish, to 24 well plates with 4 % PFA and fixed for 12 minutes. Following two 3-5 minute washes in PBS, cells were permeabilised in PBS-Triton 0.2 % for 10 minutes.

2.4.5.2 | *Immunofluorescence: staining and imaging*

All coverslips were blocked in 5 % Bovine Serum Albumin (BSA; Sigma-Aldrich) for 20 minutes at RT. Coverslips were incubated with primary antibodies in 5% BSA for 20 mins at RT or for LMI samples, overnight at 4 °C (Table 5). After three 3-5 minute PBS washes, DAPI and secondary Alexa-conjugated antibodies (Table 6) were incubated in 5% BSA for 20 mins, or 1 hour for LMI samples, at RT protected from light. After four 3-5 minute PBS washes, coverslips were mounted on glass slides using Mowiol® 4-88 mounting medium (Mowiol® 4-88 12 % w/v, Glycerol 30 %w/v, Tris pH 8.5 120 mM). Slides were imaged with LSM 710 confocal microscopes (Carl Zeiss AG) using Zen software. Images were processed and analysed using Image J.

Table 5 | Primary Antibodies

Protein target	Species	Isotype	Supplier	Product #	Dilution	
					WB	IF
ATM	mouse	IgG1(κ)	Abcam	ab2618	1:1,000	
BRN2	rabbit	IgG	Cell Signaling	12137	1:2,000	1:200
CPDs	mouse	IgG2a,κ	Cosmobio	CAC-NM-DND-001	Immunoblot: 1:2,000	
ERK2	mouse	IgG2bκ	Santa Cruz Biotechnologies	sc-1647	1:5,000	
ERK2	rabbit	polyclonal	Santa Cruz Biotechnologies	sc-154	1:10,000	
FLAG® M2	mouse	IgG1	Sigma-Aldrich	F1804	1:10,000	1:500
GADD45a	rabbit	polyclonal	Santa Cruz Biotechnologies	sc-797	1:500	
GAPDH	rabbit	polyclonal	Sigma-Aldrich	G9545	1:5,000	
GST	rabbit	polyclonal	Santa Cruz Biotechnologies	sc-459	EMSA: 1 µl /reaction	
Ku80	rabbit	IgG	Abcam	ab79391	1:1,000	1:200
MAPKAPK-2	rabbit	IgG	Cell Signaling	12155	1:1,000	
MITF	rabbit	polyclonal	Santa Cruz Biotechnologies	HPA003259	1:2,000	1:200
MITF	mouse	IgG	Homemade	n/a	1:10,000	1:500
p38 MAPK	rabbit	polyclonal	Cell Signaling	9212	1:1,000	
Phospho ATM (S1981)	rabbit	polyclonal	Abcam	ab81292	1:500	
Phospho MAPKAPK-2 (T334)	rabbit	IgG	Cell Signaling	3007	1:500	
Phospho p53 (S15)	rabbit	polyclonal	Cell Signaling	9284L	1:500	
Phospho Erk1/2 (T202/Y204)	rabbit	IgG	Cell Signaling	4377	1:500	
Phospho H2AX (S139)(γH2AX)	mouse	IgG1	Millipore	05-636	1:500	1:200
Phospho H2AX (S139)(γH2AX)	rabbit	IgG	Cell Signaling	9718	1:500	1:200
Phospho H2AX (S139)(γH2AX)-FITC	mouse	IgG1	BioLegend	613403	FACS: 1:250	
Phospho -JNK	mouse	IgG1	Santa Cruz Biotechnologies	sc-6254	1:500	
Phospho -p38 MAPK (T180/Y182)	rabbit	polyclonal	Cell Signaling	9211	1:500	
α-Tubulin	rabbit	polyclonal	Abcam	ab18251	1:5,000	

Table 6 | Secondary Antibodies

Species	Target species	Isotype	Conjugate	Supplier	Product #	Use (dilution)
goat	mouse	IgG (H + L)	HRP	Biorad	170-6516	WB (1:10,000)
goat	rabbit	IgG (H + L)	HRP	Biorad	170-6515	WB (1:10,000)
donkey	mouse	IgG (H + L)	Alexa Fluor™ 488	Life technologies	A-21202	IF (1:500)
donkey	rabbit	IgG (H + L)	Alexa Fluor™ 488	Life technologies	A-21206	IF (1:500)
donkey	mouse	IgG (H + L)	Alexa Fluor™ 546	Life technologies	A10036	IF (1:500)
donkey	rabbit	IgG (H + L)	Alexa Fluor™ 546	Life technologies	A10040	IF (1:500)

2.5 | Protein methods

2.5.1 | Whole Cell Extracts

Whole cell extracts were collected by addition of 1X Laemmli sample buffer (Tris-HCl pH 6.8 62.5 mM, SDS 2%, Glycerol 12.5%, Bromophenol Blue 0.1%, β -mercaptoethanol 1%) to adherent cells in a culture dish following one wash in PBS. Cells were scraped, collected into 1.5 ml Eppendorf tubes, sonicated using a probe sonicator (US Sonics Vibracell, 100 w, 20 KHz) at 50 Amps for 5s and then denatured at 95 °C for 5 minutes. Samples were stored at -20 °C.

2.5.2 | FLAG Immunoprecipitation

Immunoprecipitation of FLAG-tagged proteins for co-immunoprecipitation Western Blots, AP-MS and phosphoproteomics were prepared as follows (Lambert et al. 2014). Adherent cells were trypsinised, collected in complete media and centrifuged at 125 g for 5 minutes. Cell pellets were snap frozen on dry ice and lysed by freeze-thaw in 250 μ l lysis buffer (50 mM Hepes-NaOH pH 8.0, 100 mM KCl, 2 mM EDTA, 0.1% NP40, 10% glycerol, 1 mM PMSF, 1 mM DTT and Sigma-Aldrich protease inhibitor cocktail, P8340, 1:500). Phosphatase inhibitors were also added for phosphoproteomics analysis (PhosSTOP™ Roche, Sigma-Aldrich #04906845001; 1 tablet/10 ml buffer). Samples were sonicated at 4°C for 30 seconds in a water bath sonicator (Diagenode Bioruptor). Samples were incubated with 1 μ L of benzonase (Sigma-Aldrich #E1014; 250 units/ μ L) at 4 °C for 1 hour. Samples were centrifuged at 16,873 g for 20 mins at 4 °C, 10% of the supernatant was retained as Input, the remainder was incubated for 2 hours at 4 °C on a rotator with anti-FLAG M2 magnetic beads (Sigma-Aldrich, #M8823), pre-equilibrated in lysis buffer. Beads were then washed five times in lysis buffer. For co-immunoprecipitation studies, the beads were resuspended in equal volume of 2X Laemmli sample buffer, boiled at 95 °C for 5 minutes and then stored at -20 °C.

2.5.3 | Bacterially-Purified Protein Production

Purified N-terminal GST-tagged BRN2 WT and mutant proteins were produced by restriction enzyme sub-cloning into a bacterial expression system as described above (pGEX-4T-1 vector, GE-Healthcare). 10 ng of DNA were transformed by heatshock at 42 °C into BL21 competent *E. coli* (Made in lab, R. Siddaway and R. Freter) and grown on ampicillin-LB Agar plates overnight at 37 °C. Colonies were expanded at 37 °C in Terrific Broth (TB) Culture Medium (Tryptone 1.2% w/v, Yeast Extract 2.4% w/v, Glycerol 0.4% v/v, 17 mM KH₂PO₄, 72 mM K₂HPO₄ in water). Protein production was induced by adding Isopropyl-β D-thiogalactoside (IPTG) (0.5 mM) to cultures with OD₆₀₀ 0.6-0.8 and incubating overnight at RT. Proteins were harvested by sonicating bacterial pellets resuspended in Lysis Buffer (NaCl 300 mM, Tris pH 8.0 20 mM, DTT 5 mM, 1x protease inhibitor) with six cycles of 12s on, 18s off at 40 amps using a probe sonicator (US Sonics Vibracell, 100w, 20 KHz). Supernatant was collected following 20 mins centrifugation at 20,000 g at 4 °C. Proteins were then purified by overnight GST pull-down with Glutathione Sepharose 4B beads (17-0756-01, GE Healthcare Life Sciences) in Lysis Buffer. Purified proteins were left on beads for IVKA or eluted from beads with glutathione (see below). Proteins were stored at 4 °C in an excess of lysis or elution buffer. To prepare proteins for SDS-PAGE and Coomassie staining, beads were diluted in equivalent volume of 2X Laemmli sample buffer and boiled at 95 °C for five minutes.

2.5.3.1 | Glutathione elution

GST-tagged proteins were eluted from Glutathione Sepharose 4B beads by incubation with reduced glutathione. Two volumes of beads were incubated with one volume of elution buffer (50 mM Tris HCl pH 8.0, 10 mM reduced glutathione) for 10 minutes with end-over-end rotation in a 1.5 ml Eppendorf on a rotor at room temperature.

Beads were then pelleted by centrifugation at 500 g for 5 mins. The supernatant was collected and the process repeated for two further elutions. The three eluates were combined. For EMSA, eluted protein was stored at 4 °C; for SDS-PAGE and Coomassie staining an equal volume of 2X Laemmli Sample buffer was added and samples were boiled at 95 °C for five minutes.

2.5.4 | SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Whole cell lysates, immunoprecipitated samples or purified proteins prepared in Laemmli sample buffer were resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-PROTEAN® Tetra electrophoresis system (Biorad). 1 mm, 10 % 37.5:1 Bis:Acrylamide Tris-Glycine-SDS resolving gels were prepared unless otherwise specified (10 % w/v Bis:Acrylamide 37.5:1 (Severn Biotech, 20-2100-10), 0.4 M Tris HCl pH 8.9, 0.1 % w/v SDS, 0.05 % v/v N,N,N',N'-tetramethylethylenediamine (TEMED), 0.1 % w/v Ammonium persulfate). Isopropanol was layered over the gel to form a level surface, until polymerised. Following removal of isopropanol, 4 % stacking gel (4 % w/v Bis:Acrylamide 37.5:1 (Severn Biotech, 20-2100-10), 0.12 M Tris HCl pH 6.8, 0.1 % w/v SDS, 0.4 % v/v N,N,N',N'-tetramethylethylenediamine (TEMED), 0.04 % w/v Ammonium persulfate) was added with 10 or 15 well combs used. Once polymerised, gels were assembled in a miniProtean tank system (Biorad). Samples were loaded alongside a protein standard ladder (Biorad, Precision Plus Protein™ All Blue Prestained Protein Standards #1610373 or Precision Plus Protein™ Dual Color Standards, 1610374). Gels were then run in 1x running buffer (52.2 mM Tris Base, 53.3 mM Glycine, 0.1 % w/v SDS) at 70 to 130 V until the dye front had left the gel.

2.5.5 | Coomassie Protein Staining

To visualise proteins, gels were stained with Coomassie blue (50 % Methanol v/v, 5 % Glacial Acetic Acid v/v, spatula-tip Coomassie Brilliant Blue R, Sigma, B7920).

Gels were microwaved in Coomassie blue stain for 45 seconds. Gels were then incubated at RT on a rocker in Coomassie destain solution (20 % Methanol v/v, 10 % Glacial Acetic Acid v/v) for at least one hour. Alternatively, commercially available Coomassie stain was used (InstantBlue Protein Stain, Expedeon, ISB1L). This required 15 minutes incubation at RT on a rocker and then gels were rinsed in ultrapure water (18Ω). Destained gels were imaged in a GelDoc (BioRad). ImageJ software was used to compare band intensity.

2.5.6 | Western Blotting

Proteins were transferred from gels to nitrocellulose membrane (GE Healthcare Amersham™ Protran™ NC; 45-004-000) using wet transfer in Tris-Glycine transfer buffer (31.4 mM Tris Base, 192 mM Glycine) at 400 mA for 75 minutes with ice blocks using the Mini Trans-Blot® Cell system (Biorad). Membranes were stained with Ponceau S (0.1 % w/v Ponceau S, 5 % v/v acetic acid) to visualise transfer and facilitate membrane cutting to allow blotting with multiple antibodies. Following washes in PBS-Tween 0.1 % (PBS-T), membranes were blocked in 5 % non-fat milk in PBS-T at RT for 45 minutes. Membranes were then incubated with primary antibodies in 5 % non-fat milk at 4 °C overnight. Dilutions as indicated in Table 5. Following three PBS-T washes, membranes were incubated in secondary HRP-conjugated antibodies (see Table 6) 1:10,000 for 45 mins at RT. Membranes were again washed three times in PBS-T and then processed with Amersham™ ECL™ (GE Healthcare, RPN2106) prior to radiographic film exposure.

2.5.7 | Phos-Tag™ Gel Electrophoresis

To increase separation between phosphorylated and non-phosphorylated BRN2 species, SuperSep™ Phos-tag™ 50 µmol/l gels were used (Wako 198-17981). The gels were run as above in Tris-Glycine-SDS running buffer using the Mini-PROTEAN®

Tetra electrophoresis system (Biorad) (see SDS-PAGE). Prior to transfer, gels were incubated in 1X transfer buffer with 10mM EDTA for 20 minutes, three times, on a rocker at RT. Following one further wash in 1X transfer buffer with no EDTA, proteins were transferred to nitrocellulose membranes and immunoblotted as described above (Western Blotting).

2.5.8 | *in vitro* Kinase Assay (IVKA)

A master mix was prepared of 50 ng per reaction of purified kinase with cold ATP (30 nM) in kinase buffer (HEPES 20 mM, MgCl₂ 10 mM, DTT 1 mM) and this was pre-incubated for 1 hour at 30 °C. 2.5 nCi per reaction ³²P-ATP was added (ATP, [γ -³²P]-10 mCi/ml EasyTide Lead, Perkin Elmer) to the master mix. 2 μ l of the Glutathione-sepharose beads bound to purified GST-tagged proteins, described above, were mixed with 8 μ l of kinase buffer in radiation-safe 2 ml Eppendorf tubes. 10 μ l of hot master mix was aliquoted into each tube. The kinase reactions were incubated for 3 hours at 30 °C. 7 μ l 4X Laemmli sample buffer was added to each sample, they were then denatured at 95°C for 5 mins and resolved on a 12% bis-acrylamide 200:1 Tris-Glycine-SDS gel with 26 lanes. Following Coomassie Blue staining, gels were dried on a gel dryer and then films exposed to the radioactive gel overnight. Coomassie gel was imaged with a camera. Kinases: p38 α -SAPK2a (Millipore, 14-251); JNK2 2 α 2/SAPK 1a (Millipore, 14-329); p42 MAP Kinase (NEB, P6080).

2.5.9 | Electrophoretic Mobility Shift Assay (EMSA)

DNA probes were designed to flank the BRN2 binding sites on two BRN2 target genes *MITF* and *KITL*. 30bp complimentary oligos with CTAG overhangs were hybridized and then labelled with CTP, [α -³²P]-10 mCi/ml EasyTide Lead (Perkin Elmer) in NEB buffer 2, BSA 0.2 μ g/ μ l, DTT 2 nM, dATP, dTTP, dGTP, Klenow at RT for 30 mins. DNA probes were then purified using a Nucleotide Removal Kit (Qiagen

28304). Probes were eluted in 100ul water and stored at -20 °C. EMSA reaction was carried out in final volume 20 µl bandshift buffer (25 mM HEPES (pH7.4); KCl 150 mM, Glycerol 10%, DTT 5 nM, BSA 0.5 µg/µl, dIdC 50 ng/µl).

Proteins were run on Coomassie, quantified by ImageJ and diluted accordingly. 6 µl of 2X Laemmli sample buffer was added to 6 µl of the diluted protein and run on a Coomassie to confirm dilution ratios. 2 µl of the diluted protein was added to duplicate reactions and 1 µl of BRN2 or GST antibody added to one reaction and incubated on ice for 20 mins. 1 µl of purified, labelled probe was then added and the mixture was incubated on ice for 20 mins. 8 % 55:1 Bis:Acrylamide (Severn Biotech) gels in 0.5X Tris-Borate-EDTA buffer (TBE) (UltraPure™ TBE Buffer, 10X, Invitrogen, 15581044) were made with a 20 lane comb. Samples were loaded with a GelPilot loading dye (Qiagen, 239901) in the first lane to visualise the progress of electrophoresis. The gel was then run in 0.5X TBE at 200V for 105 minutes. Gels were then dried on a gel dryer under vacuum at 80 °C for 50-60 minutes. Photographic films were exposed to the radioactive gel for between 30 minutes and three days.

2.6 | Proteomics

2.6.1 | Affinity Purification–Mass Spectrometry (AP-MS)

Stable, doxycycline-inducible 501mel BRN2-FLAG-expressing cell lines were sent to our collaborators for AP-MS (J.P. Lambert, Samuel Lunenfeld Research Institute, Toronto). AP-MS for interaction partners was carried out as previously described (Lambert et al. 2013). Briefly, after doxycycline induction (24 h, 10 ng/ ml), BRN2 FLAG was immunoprecipitated from 501mel BRN2-FLAG expressing cell lines (see section 2.5.2). Following wash steps, samples were digested on-beads in 7.5 µl of 20mM Tris-HCl (pH 8.0) containing 500ng Trypsin overnight at 37°C. Following centrifugation, the supernatant underwent repeat Trypsin digestion for 3-4 h at 37°C. Samples were stored in 2% formic acid at -80°C prior to MS. The samples were then analysed on the AB SCIEX 5600 Triple TOF in data dependent acquisition mode (DDA). The DDA parameters for acquisition on the TripleTOF 5600 were 1 MS scan (250 ms; mass range 400-1250) followed by up to 50 MS/MS scans (50 ms each). Candidate ions between charge states 2-5 and above a minimum threshold of 200 counts per second were isolated using a window of 0.7 amu. Previous candidate ions were dynamically excluded for 20 sec with a 50 mDa window.

2.6.2 | Phosphoproteomics

501mel_BRN2 WT_FLAG cells were transiently transfected with either pCMV_Empty_FLAG or pCMV_p38_FLAG vectors. After 24 hours, they were subjected to control or H₂O₂ treatment (2.5 mM 30 minutes). Cell pellets were then collected and FLAG-tagged BRN2 and p38 were immunoprecipitated as described above (FLAG co-immunoprecipitation). All solutions contained phosphatase inhibitors (PhosSTOP™ Roche, Sigma-Aldrich #04906845001; 1 tablet/10 ml buffer). Following

the final wash post-IP, the beads were resuspended in 30 μ l of 50 mM Ammonium Bicarbonate and submitted to proteomics facility for phospho-proteomics. Briefly, samples were digested on beads overnight with 200 ng Elastase per sample at 37°C prior to preparation for phospho-Mass Spectrometry. Data searched using the PEAKS search engine was prepared by the proteomics facility.

2.7 | *in silico* methods

2.7.1 | Sequence Alignment

To create sequence alignments, protein sequences were downloaded from Uniprot (Bateman et al. 2017) (See Tables 7 and 8). Sequences were aligned using T-Coffee (Notredame et al. 2000) and formatted using the BOXSHADE server (available at: https://embnet.vital-it.ch/software/BOX_form.html).

Table 7 | BRN2 orthologues

Species	Uniprot entry number	Uniprot entry name
<i>Homo sapiens</i>	P20205	PO3F2_HUMAN
<i>Mus musculus</i>	P31360	PO3F2_MOUSE
<i>Bos taurus</i>	Q2KIB8	Q2KIB8_BOVIN
<i>Danio rerio</i>	P79746	PO3F2_DANRE
<i>Xenopus laevis</i>	P70030	P3F2B_XENLA

Table 8 | Human BRN2 paralogues

Protein	Uniprot entry number	Uniprot entry name
BRN2	P20205	PO3F2_HUMAN
BRN3A	Q01851	PO4F1_HUMAN
OCT1	P14859	P0251_HUMAN
OCT4	Q01860	PO5F1_HUMAN
PIT1	P28069	PIT1_HUMAN

2.7.2 | 3D Structure Analysis

To create Oct1 crystal structure images, the PDB ID:1Oct (DOI: <http://dx.doi.org/10.2210/pdb1oct/pdb>) (Klemm et al. 1994) was downloaded from the Protein Database (PDB) (Berman et al. 2000). The code was altered to create an *in silico* mutation of residue N422 to alanine. Images were created using the UCSF Chimera 1.11.2 package (Pettersen et al. 2004).

2.7.3 | BRN2 Interactome Analysis

Raw data generated by AP-MS was analysed by collaborators (J.P. Lambert) as previously described (Lambert et al. 2013). Peptides were identified through searching the files containing candidate ions using Mascot (version 2.3.02) and Comet (2012.01 rev. 3) applying the following parameters: one missed cleavage site, methionine oxidation and asparagine/glutamine deamidation as variable modifications. Search results were combined and further analysed using the iProphet tool from the Trans-Proteomics Pipeline (Shteynberg et al. 2011; Keller and Shteynberg 2011). Peptides which did not meet the minimum PeptideProphet significance p value of 0.05 were filtered out. Significant interaction partners were identified by subjecting data to Significance Analysis of Interactome (SAINT) analysis in which the BRN2_FLAG data were compared directly to 3xFLAG empty control samples. A False Discovery Rate <1% was applied to reduce false positives (Knight et al. 2015; Choi et al. 2011; Liu et al. 2016). Cytoscape software (v.3.5.1) was then used to produce an interactome scheme from the received data (Shannon et al. 2003).

2.7.4 | Gene Ontology

Protein lists generated from AP-MS BRN2 interactome analysis were analysed by Gene Ontology, using online tools available at <http://pantherdb.org/> (Mi et al. 2013). Gene ontology overrepresentation analysis was carried out using PANTHER GO-slim Biological Process gene sets to compare frequency of proteins identified as BRN2 binding partners with expected frequency compared to reference genome. Binomial test for significance with p-value <0.05 cut-off was applied. Biological Processes with >4-fold overrepresentation were considered.

2.7.5 | RNA-Seq Analysis

RNA-Seq data were analysed by P. Louphrasitthiphol and J. Chuahan (Goding lab). Raw fastq files were trimmed of poly-A using cutadapt (Martin 2011) and mapped using STAR (Dobin et al. 2013) against hg38 (GRCh38, 2015). Counts per gene from STAR were used as input for differential gene expression analysis using EdgeR (Robinson et al. 2009). Reads for each sample set were first filtered for genes whose expression is <1 count-per-million prior to glmQLFTest. Genes with a p-value ≤ 0.05 and meet the specific fold-changes were taken for further analysis. Heatmaps of RNA-seq samples were generated from the edgeR-library normalised reads of genes whose differential gene expression has p-value ≤ 0.05 before further filtering for genes with read counts ≥ 2 counts in all the replicates of either treated or control samples. Raw reads were log2 transformed, centered normalised around mean and hierarchical clustering performed using complete linkage using Gene Cluster 3.0 (de Hoon et al. 2004). The output matrix was used to generate the heat-map for visualisation using TreeView (Saldanha 2004).

2.7.6 | GSEA and GSVA analyses

GSEA analyses were carried out using javaGSEA2-3.0 (Subramanian et al. 2005). 10,000 permutations were carried out for each probe geneset. GSVA analyses were performed using the Bioconductor package GSVA (Hanzelmann et al. 2013). The genesets used were obtained from the Molecular Signatures Database (Subramanian et al. 2005). The GSVA matrix was then clustered and displayed as heatmap using Pheatmap (<https://cran.r-project.org/web/packages/pheatmap/index.html>).

2.7.7 | TCGA Bioinformatics Analysis

TCGA analysis was performed by the D. Robles-Espinoza and N. Roberts at The Wellcome Trust Sanger Institute, Cambridge. Files with FPKM gene expression

measurements were downloaded on 2017/11/20 from TCGA (<https://portal.gdc.cancer.gov/projects/TCGA-SKCM>) with the following filters: Project ID: TCGA-SKCM; Sample type: Metastatic, primary tumour, additional metastatic; Workflow type: HTSeq-FPKM; Data category: Transcriptome profiling. We added $\log_2(\text{FPKM}+1)$ values for genes POU3F2 (BRN2, ENSG00000184486), POU4F1 (BRN3a, ENSG00000152192) and POU4F2 (BRN3b, ENSG00000151615) as variables to the negative binomial model previously published (Robles-Espinoza et al. 2016). This left 271 samples in the model as two did not have available FPKM measurements (TCGA-EE-A3AE and TCGA-GN-A269). We also added $\log_2(\text{mean FPKM}(\text{MITF target genes}))$ as a variable, where MITF target genes = MITF (ENSG00000187098), BCL2 (ENSG00000171791), CDK2 (ENSG00000123374), CDK4 (ENSG00000135446), DCT (ENSG00000080166), HIF1A (ENSG00000100644), MLANA (ENSG00000120215), PMEL (ENSG00000185664), PPARGC1A (ENSG00000109819), PTEN (ENSG00000171862), RAB27A (ENSG00000069974), SHC4 (ENSG00000185634), TRPM1 (ENSG00000134160), TYR (ENSG00000077498), TBX2 (ENSG00000121068), MCOLN1 (ENSG00000090674), DIAPH1 (ENSG00000131504), and MET (ENSG00000105976). For each of the six basic SNV classes (C>A, C>G, C>T, T>A, T>C and T>G), we modelled the relationship between expected somatic SNV burden and these variables using negative binomial regression with a log link, controlling for all available clinical variables in the TCGA cohort, as described previously (Robles-Espinoza et al. 2016). The most common constellation of clinical variables, for which the prediction depicted with the blue dashed line was performed, consists of a male patient from the University of Sydney with a non-ulcerated tumour in the extremities and an age of diagnosis of 57 years old, Breslow thickness of 2.5mm, Clark level IV, and metastasis to a regional lymph node.

$\text{Log}_2(\text{FPKM}+1)$ values for BRN3a and BRN3b were 0.09167 and 0 respectively, and $\log_2$ of the median of FPKM values of MITF targets was 7.032.

Chapter 3 | Results

BRN2 Protects Melanoma Cells from UVB-Induced Apoptosis

3.1 | Introduction

BRN2 plays a critical role in development and a range of cancers, yet remarkably little is known about how BRN2 exerts its effects, or how its activity is modulated. There is evidence that the activity of BRN2 on the MITF promoter is context dependent (Goodall et al. 2008; Wellbrock et al. 2008). A key mechanism by which transcription factor activity is modified is by the binding of co-factors. To date, there is little data identifying BRN2 binding partners. Therefore, in this chapter we investigate the hypothesis that BRN2 activity is modulated by co-factor binding.

3.2 | Generating A Stable-Inducible BRN2_FLAG Cell Line

To establish what co-factors might be mediating BRN2 function, we used a tandem-affinity mass-spectrometry approach to perform an unbiased search for BRN2 interactors. As there is no anti-BRN2 antibody available that is sufficiently efficient for use in immunoprecipitation, an exogenous tagged expression system was used. The Piggybac transposon (PB) system allows genomic integration of a doxycycline inducible cassette under control of the pCMV promoter. BRN2 expression: is highly variable across melanoma cell lines (Fig. 3.2.1a), therefore we assessed a panel of cell lines for expression of BRN2 protein and we decided to use 501mel melanoma cells as a model system since they express endogenous BRN2 and are easily transfectable, allowing both BRN2 overexpression and knock-down assays.

The BRN2 mouse cDNA sequence, which only differs from the human sequence by the presence of 2 additional glycines in the N terminal polyglycine tract, was cloned, with a triple FLAG (3xF) C-terminal tag, into a modified PB vector (K. Ngeow) with an additional puromycin resistant cassette (Fig. 3.2.1b). Following transfection of the PB-BRN2-3xFLAG vector with a transposase expression vector and neomycin resistance plasmid, cells with successfully integrated PB vector were selected by double selection with geneticin and puromycin. Initial screening of the 501mel_BRN2WT_FLAG cell line by Western Blot (WB), demonstrated good induction of BRN2_FLAG using doxycycline, but also some leakiness, with a low level of BRN2_FLAG expression evident without doxycycline treatment (Fig. 3.2.1c). Screening by immunofluorescence (IF) revealed that not only was BRN2_FLAG expression in the polyclonal cell line leaky, but that although close to 100% of cells were positive for FLAG expression, the expression level was very heterogenous (Fig. 3.2.1d). BRN2_FLAG was primarily expressed in the nucleus, but with doxycycline induction, some BRN2_FLAG was also

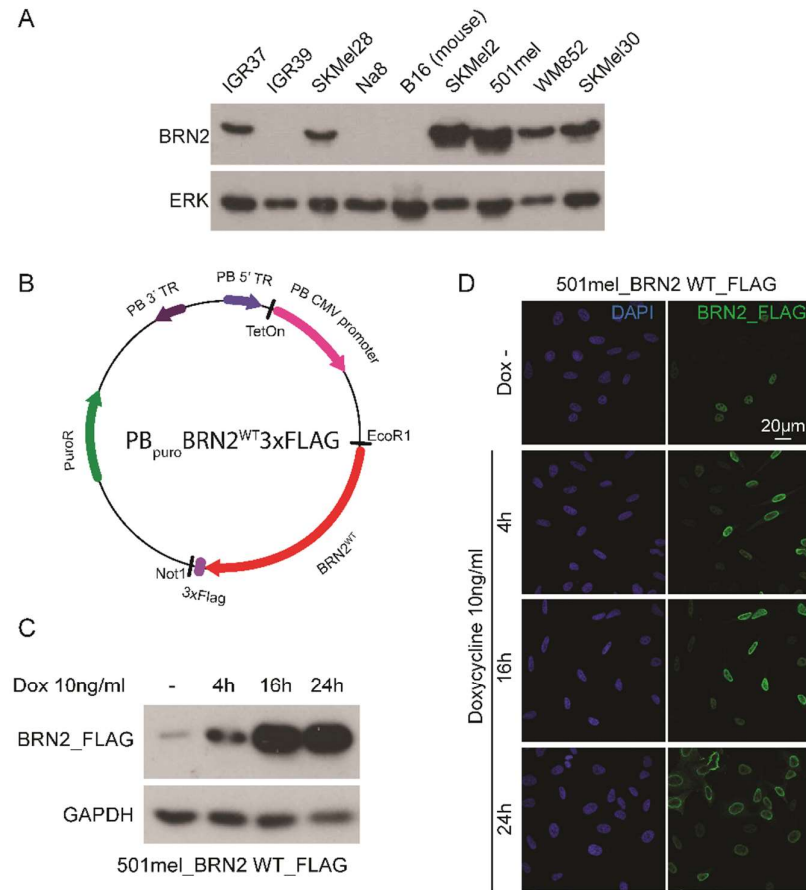


Figure 3.2.1 | Development of stable, inducible BRN2 WT_FLAG cell lines

(A) Western blot of BRN2 levels in a panel of melanoma cell lines. ERK is used as loading control. (B) Schematic of PiggyBac transposon vector (pPBhCMV1cHApA-MCS) modified to contain a puromycin resistance cassette and with BRN2 WT_FLAG cloned into the MCS with EcoR1-Not1 cloning strategy. (C) Western blot and (D) IF of FLAG expression in 501mel_BRN2 WT_FLAG with a time course induction of BRN2_FLAG expression with 10ng/ml doxycycline. GAPDH is used as loading control. IF stained with anti-FLAG antibody and DAPI nuclear counterstain.

present in the cytoplasm. In order to create a model in which all cells had similar expression levels of exogenous BRN2, colonies derived from single cells were picked and screened by WB and IF. It became evident that the subcellular localisation of BRN2_FLAG was dependent on the absolute amount of BRN2_FLAG being expressed in the cell (Fig. 3.2.2 a,b). Clone #2 has very high levels of BRN2_FLAG expression following doxycycline induction with a nucleocytoplasmic distribution. In contrast, clone #1, with and without doxycycline treatment, and uninduced clone #2 all have low levels of expression and BRN2_FLAG remains in the nucleus (Fig. 3.2.2b). It is possible that there is a low level in the cytoplasm too, but it is undetectable by immunofluorescence.

The leakiness of the system is an advantage because it allows the use of a cell line with stable expression of BRN2_FLAG without the need for doxycycline induction, which could be compared to 501mel cells as a control. Clone #1 (Fig. 3.2.2a,b) has low levels of stable expression, whilst clone #2 has high levels of expression (Fig. 3.2.2a,b). This meant that for immunoprecipitation studies clone #2 could be used, whilst phenotype assays could be undertaken with clone #1, to have an exogenous expression level as close to endogenous as (Fig. 3.2.2c).

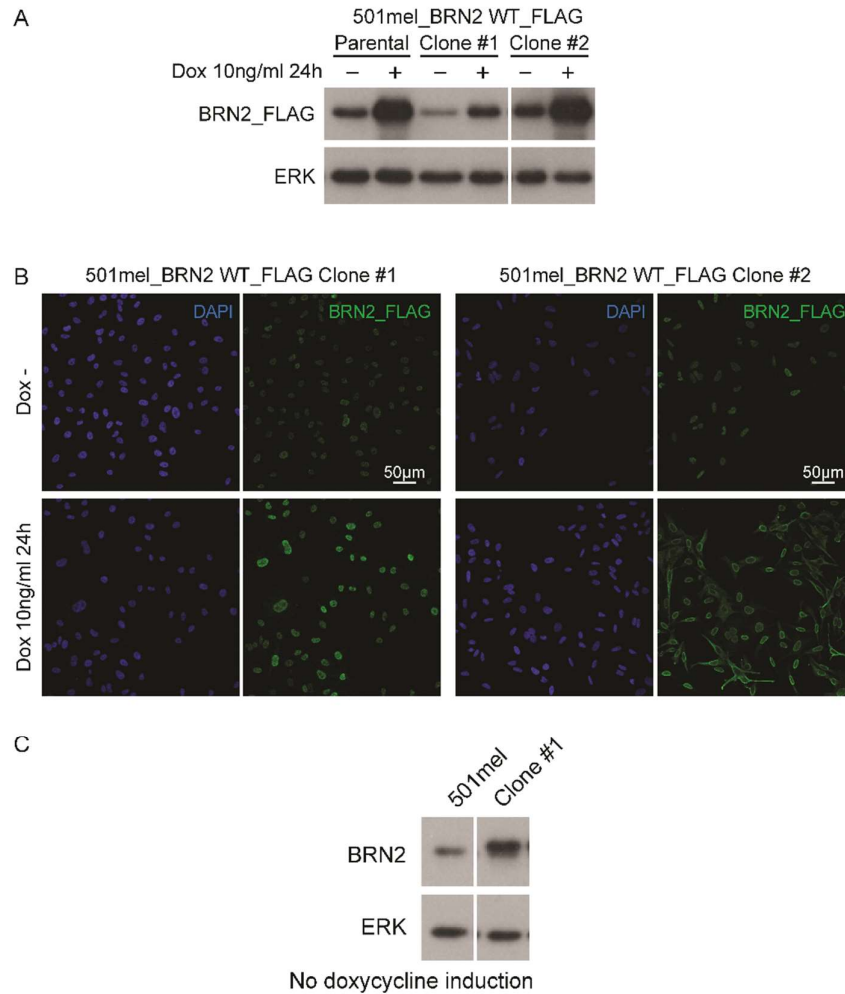


Figure 3.2.2 | BRN2 subcellular localisation is dependent on expression levels

(A) Western blot showing BRN2_FLAG expression in 501mel_BRN2 WT_FLAG parental cell line and clonal lines #1 and #2 (established by colony picking), with and without 24hours 10ng/ml doxycycline induction. ERK as loading control. (B) IF of 501mel_BRN2 WT_FLAG clonal lines #1 and #2, with and without doxycycline induction. Anti-FLAG antibody with DAPI nuclear counterstain. (C) Western blot of 501mel cells compared to 501mel_BRN2 WT_FLAG clone #1, with no doxycycline induction, showing BRN2 expression levels. ERK is used as a loading control.

3.3 | Identifying BRN2 Binding Partners by AP-MS

The 501mel doxycycline-inducible parental BRN2_FLAG cell line was used to identify BRN2 binding partners by affinity purification mass spectrometry (AP-MS). Following 24 h 10 ng/ml doxycycline induction, immunoprecipitation of the FLAG-tagged BRN2 WT protein was performed by our collaborators (J.P. Lambert, University of Toronto) using magnetic anti-FLAG beads. After a Significance Analysis of INTeractome (SAINT) was performed and a threshold false discovery rate of < 1% was applied (Choi et al. 2011; Liu et al. 2016) to the raw AP-MS data, 66 proteins were identified with significant binding affinity to BRN2_FLAG. The spectral count, as represented by the heatmap (Fig. 3.3.1a), indicates the abundance of the binding partners. We validated the data by co-immunoprecipitation of one of the highest abundance binding partners, Ku80 (XRCC5), with BRN2_FLAG. Magnetic anti-FLAG beads were again used to immunoprecipitate BRN2_FLAG from whole cell lysates, following a modified version of the protocol used in sample preparation for AP-MS. The Input (10%) and IP samples were separated by SDS-PAGE and WB against FLAG and Ku80 was performed. This confirms that Ku80 interacts with BRN2_FLAG (Fig. 3.3.1b).

Gene ontology overrepresentation analysis using PANTHER (Mi et al. 2013) was then carried out and we were surprised to find that the most overrepresented PANTHER GO-slim biological process was DNA repair with 18.15-fold enrichment compared to reference genome (Binomial test $p=5.73 \times 10^{-8}$) (Fig. 3.3.2a). DNA lesions resulting from DNA damage vary from the level of individual base damage through to double strand breaks and each type of lesion has a dedicated repair pathway with distinct mechanisms. However, the many DDR proteins involved in these pathways are very promiscuous. Analysis of BRN2 binding partners demonstrates that the identified proteins are involved across many DDR pathways (Fig. 3.3.2b) with no pathway predominant (Fig. 3.3.2c).

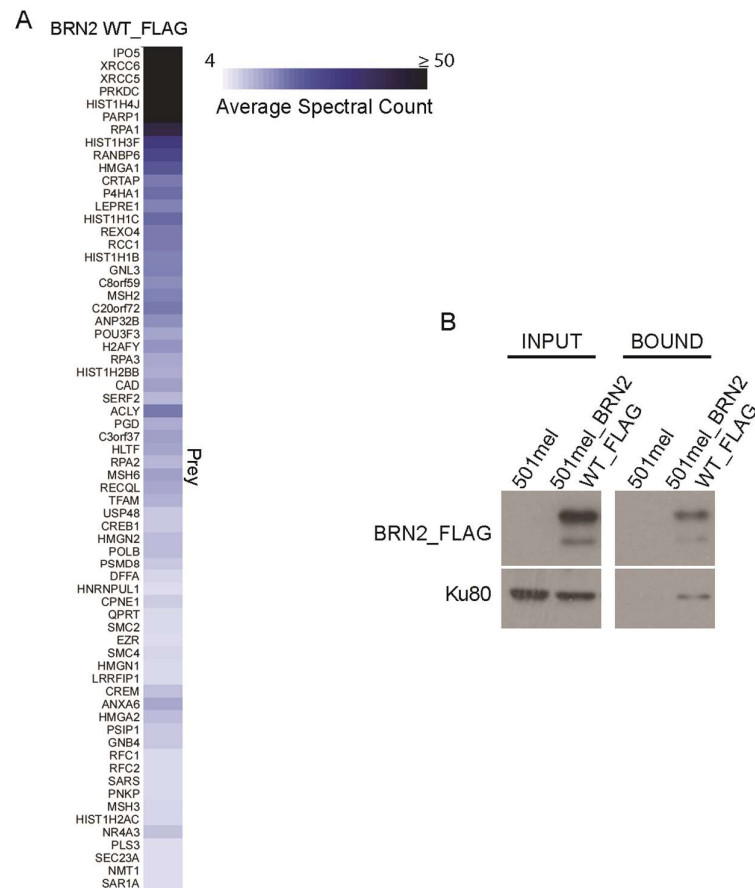


Figure 3.3.1 | BRN2 binding partners identified by AP-MS

(A) Heatmap representing average spectral count of proteins identified to bind to BRN2 by AP-MS after SAINT analysis. False discovery rate cut off =1%. Data and Figure produced by JP Lambert, Lunenfeld-Tanenbaum Institute, Toronto. (B) Western blot showing co-immunoprecipitation of Ku80 with BRN2 following FLAG-pull down; anti-FLAG and anti-Ku80 antibodies.

Three of the top four binding partners were Ku70, Ku80 (XRCC5/6) and DNA-PK_{cs} (PRKDC) which form a trimeric complex (DNAPK) at double strand breaks (DSBs). The DNAPK complex is critical in the initiation of repair via the non-homologous end-joining (NHEJ) pathway through scaffolding, recruitment and phosphorylation of other DDR proteins (Smith and Jackson 1999). Poly(ADP-ribose) polymerase 1 (PARP1) catalyses the PARylation of histones and proteins at sites of DNA damage (Jungmichel et al. 2013) and assists in chromatin remodelling, recruitment of repair factors and stabilisation of repair complexes at sites of DNA damage in several pathways. A role for PARP1 has been well described in DSB repair by homologous recombination (HR) (Hu et al. 2014) and both the classical and alternative non-homologous end joining pathways (cNHEJ; altNHEJ) (Mansour et al. 2010; Ruscetti et al. 1998). The critical interaction between PARP1 and BRCA1/2 in HR has been successfully exploited in the treatment of BRCA mutated cancers with PARP inhibitors (Bryant et al. 2005). In addition, PARP1 function is also crucial in excision pathways such as NER (Pines et al. 2012).

Replication protein A family members (RPA1 and RPA2) were identified in the AP-MS data; these proteins coat single stranded DNA and their canonical role is in facilitating recruitment of repair factors to double strand breaks and stalled replication forks (Zou and Elledge 2003). However, RPA is also one of the group of minimal essential proteins for effective nucleotide excision repair (NER) (Araújo et al. 2000). MutS homologue (MSH) family members are canonically mismatch repair (MMR) proteins. MSH 2, 4 and 6 were identified as BRN2 binding partners. MSH2 in particular has been implicated in interactions with both HR and BER pathways in addition to MMR (Edelbrock et al. 2013; Zhang et al. 2007; Gu et al. 2002). Another family with multiple members identified as prey to the BRN2-flag bait was replication family C (RFC) 1 and 2. RFC proteins are predominantly involved in the NER pathways,

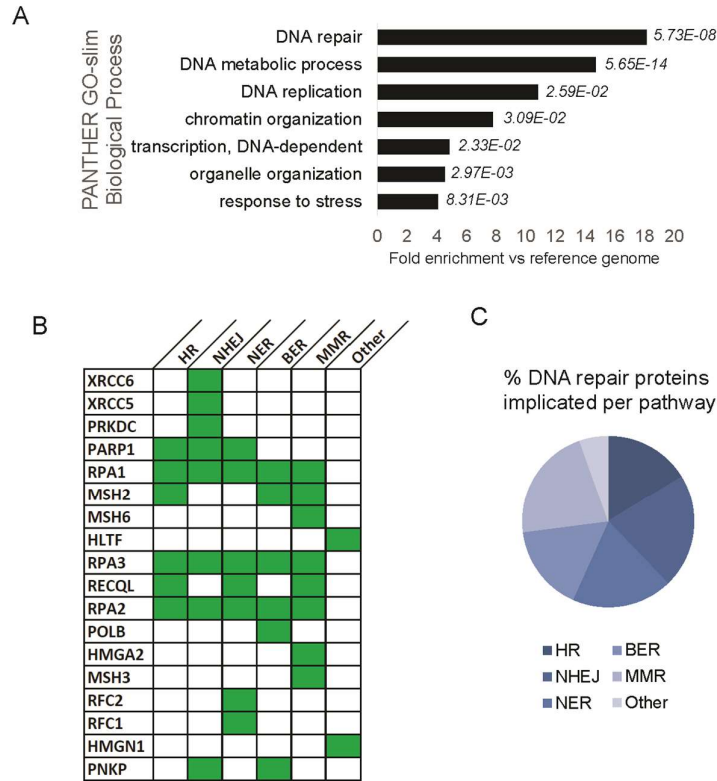


Figure 3.3.2 | BRN2 binds to proteins involved in the DNA damage response

(A) Gene ontology analysis of BRN2 binding partners. PANTHER GO-slim Biological Processes with ≥ 4 fold enrichment on over-representation analysis. Binomial test p-values in italics. (B) Table of DNA damage repair pathways in which BRN2 binding partners are involved. HR=homologous recombination; NHEJ=non-homologous end joining; NER=nucleotide excision repair; BER=base excision repair; MMR=mismatch repair. (C) Distribution of DNA binding partners across DNA damage pathways.

crucial for recruiting and stabilising repair complexes to allow post-incisional DNA repair (Overmeer et al. 2010).

Helicase-like transcription factor (HLTF) is involved in promoting post-replication DNA damage repair, preventing stalled replication forks from contributing to genomic instability (Blastyak et al. 2010). Intriguingly, loss of HLTF in the mouse brain results in failure to repair DNA damage and increased apoptosis (Helmer et al. 2013). HMGN1 is a key component of transcription-coupled-NER (TC-NER), where it assists in remodelling of chromatin around UV-induced DNA lesions (Fousteri et al. 2006). Polynucleotide kinase phosphatase (PNKP) is a DNA-end processing enzyme with kinase activity at 5' and phosphatase activity at 3' DNA ends of DSBs in NHEJ (Koch et al. 2004) and single-strand break (SSB) repair by BER (Whitehouse et al. 2001; Della-Maria et al. 2012).

Fig. 3.3.3 summarises the AP-MS data showing the 18 proteins implicated in DNA repair and a further 7 histone proteins that bind to BRN2^{WT}, weighted by average spectral count. The distance from BRN2 is inversely proportional to the spectral count. This demonstrates that, with the exception of Importin 5 (IPO5), implicated in nuclear import, the highest abundance binders are all DNA repair proteins or histones. When considering the functions of the DNA damage response-associated proteins which were identified as BRN2 binding partners, it is notable that these principally comprise factors implicated in chromatin remodelling, marking of the DNA damage and recruitment of DDR pathway components during the initiation of the DDR, suggesting that BRN2 may be involved in the early phases of DNA damage recognition and response rather than a specific pathway.

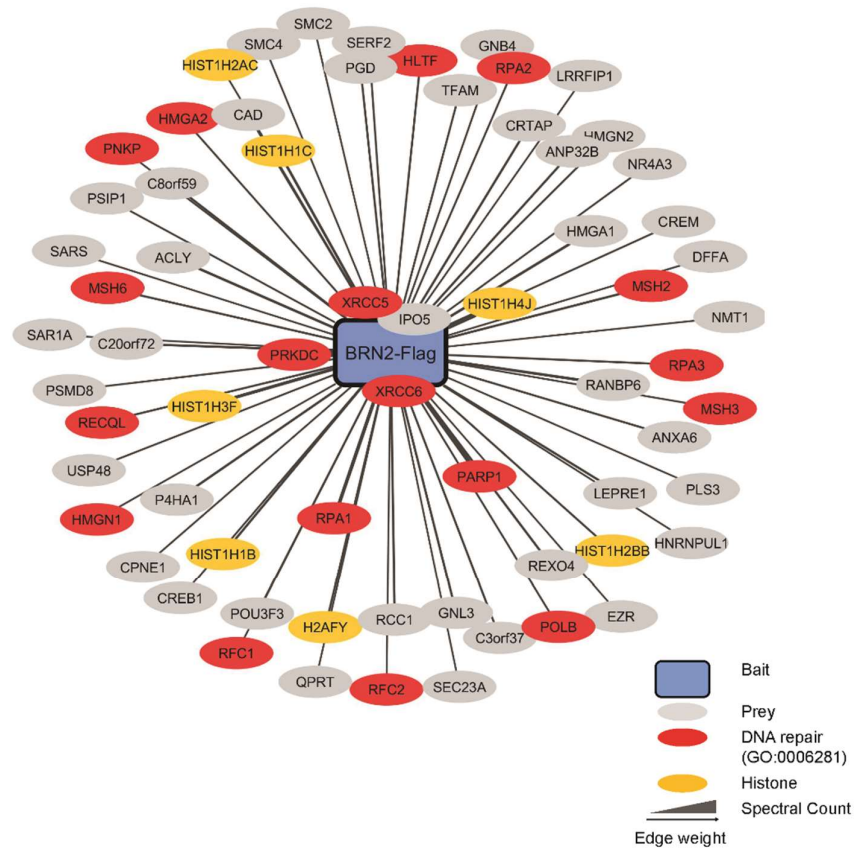


Figure 3.3.3 | Summary figure of BRN2 binding partners

BRN2 binding partners arranged by Cytoscape Edge-weighted Spring-embedded Layout where the summed spectral count is inversely proportional to prey distance from BRN2 and edge weight is proportional to prey abundance. GO annotation: Red, DNA repair; Yellow, Histones; Grey, other. Data generated by JP Lambert, Lunenfeld-Tanenbaum Institute, Toronto.

3.4 | BRN2 Binds to DDR Proteins Via its DNA Binding Domain

To gain insight into the mechanism of binding and potential role of the BRN2:DDR protein interactions we determined which region of BRN2 binds to this group of proteins. BRN2 is composed of a highly disordered, unique N-terminal, notable for polyglutamine and polyglycine tracts but with no established protein domains; a highly conserved POU DNA-binding domain; and a short, partially conserved C-terminal domain (Fig. 3.4.1a, top panel). The POU domain is highly conserved both in orthologues and paralogues, with specificity of POU domain DNA binding conferred in part by the non-conserved and flexible linker region. To establish where DDR proteins were binding, truncation mutants were created consisting of the N-terminal region alone (N-term), the highly conserved POU domain, without the N-terminal (Δ N) or without the short C-terminal domain (Δ C) (Fig. 3.4.1a, bottom panel). 501mel_BRN2_FLAG cell lines were created with these truncated mutants as for BRN2 WT, using the PB vector. Expression, size and localization of these mutants were validated by WB (Fig. 3.4.1b) and IF (Fig. 3.4.1c). As expected, localisation of WT, Δ N and Δ C was predominantly nuclear since the POU_H domain has a recognized nuclear localization signal (NLS) (Sock et al. 1996; Kalderon et al. 1984). Expression levels and induction efficiency were almost identical for WT and Δ C. Δ N had higher expression levels and consequently can be seen to a greater degree in the cytoplasm than WT. The N-terminal construct was only seen in the cytoplasm as the NLS is in the POU_H domain.

AP-MS was performed on these cell lines as described for BRN2 WT. In addition to the 66 proteins identified with BRN2 WT as bait, a further 79 proteins were identified with significant binding to the truncation mutants of BRN2 (Fig. 3.4.2). Here the absolute abundance of the prey is indicated by the shade of blue, whilst relative abundance between the different BRN2_FLAG constructs is denoted by the area of the circle.

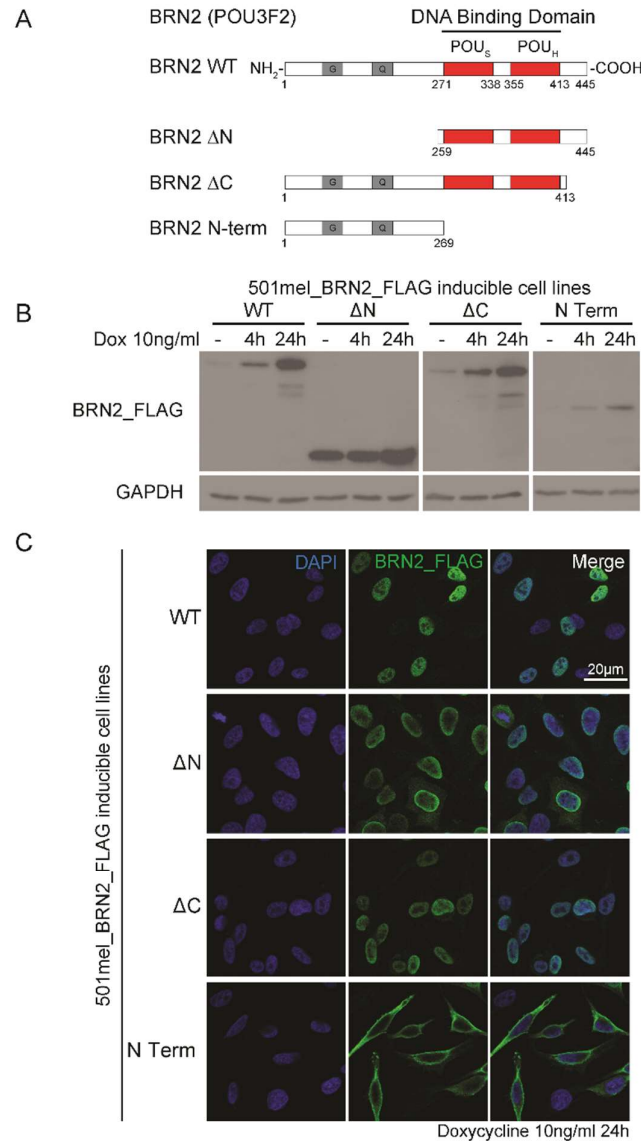


Figure 3.4.1 | Generation of BRN2 truncation mutant cell lines

(A) Upper schematic represents full length BRN2 (POU3F2). G=polyglycine tract; Q=polyglutamine tract; POU_S = POU specific domain; POU_H = POU homeodomain. Lower schematics represent truncation mutants (ΔN 259-445; ΔC 1-413; N-term 1-269). (B) Western blot showing expression levels and differential size of BRN2 WT and truncation mutant cell lines. All cell lines were created in 501mel with PB vector. Time-course of induction of FLAG expression with 10ng/ml doxycycline. GAPDH is used as a loading control. (C) Immunofluorescence of cell lines in (B) after 24 hours induction with 10ng/ml doxycycline. Cells stained with antibody against FLAG, with DAPI nuclear counterstain.

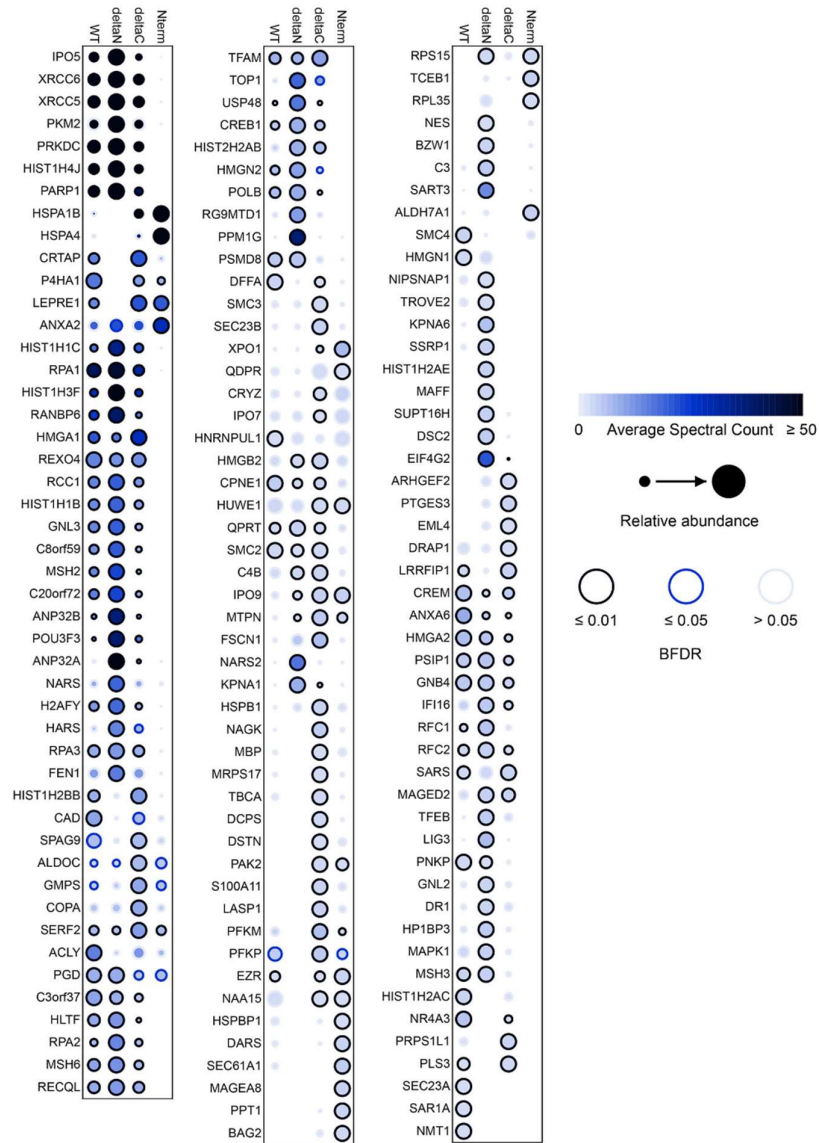


Figure 3.4.2 | BRN2 truncation mutant binding partners identified by AP-MS

Dot-plot of AP-MS data showing results of all BRN2 constructs. Size of dots represents relative abundance, colour of dots represents average spectral count, colour of circle encompassing dot represents False Discovery Rate (FDR) cut off. Data and Figure produced by JP Lambert, Lunenfield-Tanenbaum Institute, Toronto.

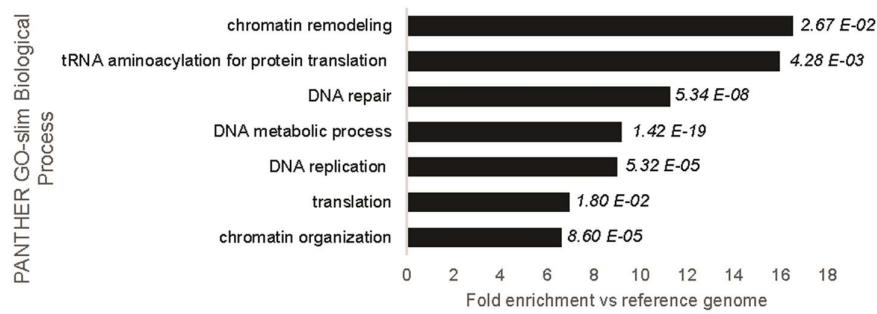


Figure 3.4.3 | DNA repair pathway remains overrepresented in truncation mutant binding partners

Gene ontology analysis of BRN2 binding partners. PANTHER GO-slim Biological Processes with ≥ 4 fold enrichment on over-representation analysis. Binomial test *p*-values in italics.

Gene ontology analysis of all 145 proteins again identified DNA repair as one of the most overrepresented biological processes (11.26 fold; binomial test $p=5.34 E -08$). Chromatin remodelling GO term also became significantly overrepresented (6.60 fold; binomial test $p=8.60 E -05$) (Fig. 3.4.3). The “tRNA aminoacylation for protein translation” GO term BRN2 binding proteins are HARS, DARS, SARS, NARS and NARS2 which are all cytoplasmic proteins. Although this may be an artefact of overexpression, it is consistent with endogenous BRN2 being observed in the cytoplasm in some regions of the brain (Tanaka et al. 2004).

The N-term mutant does not bind any DDR proteins, however, as it is not expressed in the nucleus this negative result alone does not rule out its binding potential. The ΔC mutant bound the proteins with similar affinity to WT, suggesting that the C-terminal is not critical for binding. Finally, the ΔN mutant bound the DDR proteins with much higher affinity than WT. Taken together, these data indicate that the interactions with DDR proteins are mediated via the highly conserved POU DNA binding domain. The higher spectral counts with the ΔN mutant suggest that the N-terminal region might inhibit BRN2 binding with DDR proteins. However, it is also possible that the higher expression of BRN2 ΔN_FLAG compared to WT is responsible for the increased affinity. The hypothesis that the BRN2 N-terminal may modulate the functions of the BRN2 POU domain will be explored further in Chapter 4.

3.5 | BRN2 Protects Melanoma Cells from UVB-Induced Apoptosis

Having established that BRN2 associates with various DDR pathway proteins, we wanted to investigate if BRN2 influences the DDR. A knockdown strategy (KD) was used to establish if lack of BRN2 affected the extent and dynamics of UVB-induced DNA damage repair. siRNA against BRN2 mRNA was transfected into 501mel cells. Four different commercial siRNAs purporting to target BRN2 were trialled. KD efficiency was validated by WB (Fig. 3.5.1a) and IF (Fig. 3.5.1b). Two of the four BRN2-targeting siRNA were found to have effective knock down of BRN2 at the protein level (siBRN2 #1 and siBRN2 #2). Figure 3.5.1c is a scaled schema of BRN2 mRNA with the location of the target sequences for the two successful siRNAs #1 and #2. The advantage of having one siRNA that targets the CDS and another in the 3' UTR is that potentially they can be used in stably overexpressing cells to knockdown only endogenous (siBRN2 #2) or both ectopic and endogenous BRN2 (siBRN2 #1).

In melanoma, mutations are predominately caused by UVB irradiation (Alexandrov et al. 2013), associated with a progressive increase in the C>T UV mutational signature during melanoma lesion progression (Hodis et al. 2012; Shain et al. 2015). Moreover, in response to UVB radiation BRN2 activity is modified, resulting in activation of *GADD45* in a p53-independent fashion (Lefort et al. 2001; Pedoux et al. 2002). We therefore chose to use UVB as a physiologically relevant DNA damaging agent to investigate the role of BRN2 in the DDR. Following siRNA-mediated depletion of BRN2, 501mel cells were subjected to UVB irradiation with a physiologically relevant dose (150J/m²) (Pedoux et al. 2002; Lisby et al. 2005). Endogenous levels of BRN2 are not notably affected by UVB irradiation (Fig. 3.5.1d).

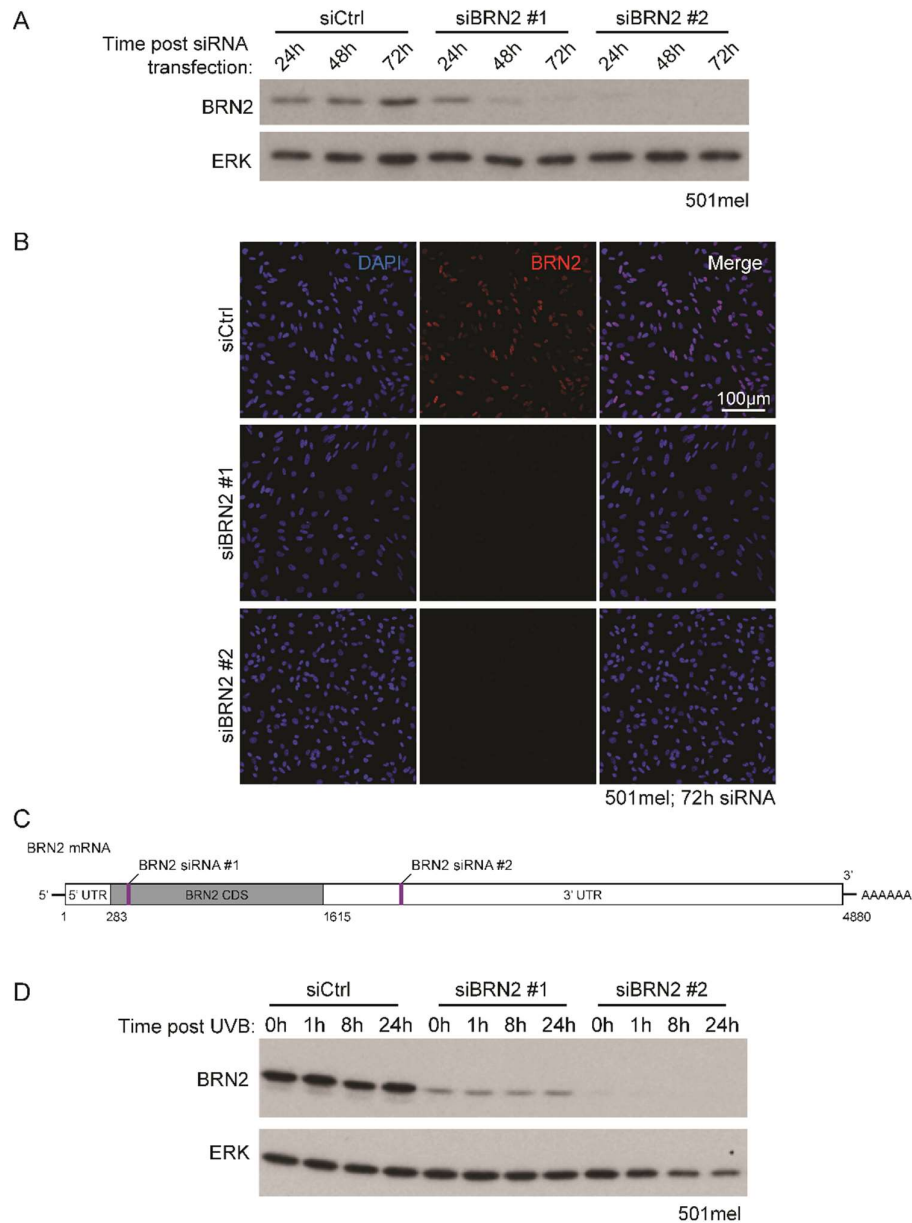


Figure 3.5.1 | Validation of siRNA against BRN2

(A) Representative Western blot showing validation of siBRN2#1 and #2 in 501mel cells with siCtrl treated cells as control. Time corresponds to hours post transfection of siRNA. Antibodies against BRN2, and ERK is used as a loading control. (B) Immunofluorescence of 501mel cells 72 hours after siRNA transfection with siBRN2#1 and #2. Antibodies against BRN2 with DAPI nuclear counterstain. (C) Schematic of BRN2 mRNA to scale, location of siRNA target sequences denoted by purple band. UTR= untranslated region; CDS = coding sequence. Numbers represent base number of mRNA sequence. (D) Representative Western blot showing BRN2 expression levels in siCtrl and siBRN2 treated 501mel cells following UVB treatment timecourse (150J/m² UVB), begun 48 hours after siRNA transfection. ERK is used as a loading control.

The main DNA damage lesions induced by UVB are thymine dimers in the form of cyclobutane pyrimidine dimers (CPD) and 6-4 pyrimidine-pyrimidone photoproducts (6-4PP) which are repaired by the nucleotide excision repair (NER) pathway. We investigated the dynamics of CPD induction and resolution by Dot-blot immunoassay (Fig. 3.5.2). In response to UVB irradiation, CPDs are formed within an hour and begin to be repaired by 12 hours, with almost complete resolution by 48 hours. Although the dot-blot itself seems to show a difference in antibody staining intensity following BRN2 KD (Fig. 3.5.2a, left panel), once the CPD dot has been normalised to the DNA loading, as measured by SYBR-Gold staining, (Fig. 3.5.2a, right panel), there was no significant difference in the dynamics of thymine dimer formation and resolution following BRN2 siRNA (Fig. 3.5.2a, quantified in b).

Although UVB principally induces thymine dimers, it also leads to double-strand breaks (DSB) either directly, through ROS induction, or conversion of unrepaired dimers. The histone protein H2AX is phosphorylated at Serine 139 (γ H2AX) by DDR kinases ATM, ATR and DNA-PK_{cs} in response to DSBs (Rogakou et al. 1998). H2AX phosphorylation is propagated along the chromatin producing large, extensive foci that can be assayed by immunofluorescence using confocal microscopy or FACS (Huang and Darzynkiewicz 2006; Toledo et al. 2014; Tanaka et al. 2009).

BRN2 was again knocked down by siRNA in 501mel cells. 48 hours after siRNA treatment, the cells were subjected to UVB irradiation, cells were then fixed and stained for immunofluorescence with antibodies against BRN2 and γ H2AX. 1 hour after damage γ H2AX staining was not significantly different by IF in siCtrl and siBRN2 cells, suggesting that damage induction is very similar in the presence or absence of BRN2 (Fig. 3.5.3a, quantified in 3.5.3b). However, in the siBRN2 treated cells, γ H2AX staining was higher at 8 hours than siCtrl and persisted at elevated levels to 24 hours compared to

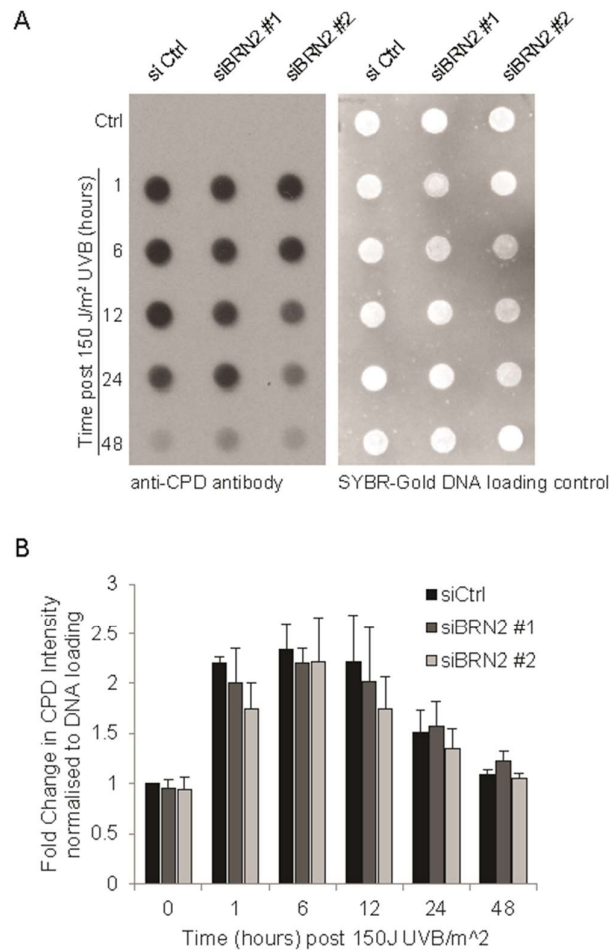


Figure 3.5.2 | CPD dynamics are not affected by BRN2 expression levels

(A) Dot-blot assay of DNA extracted from 501mel cells pre-treated with siCtrl or siBRN2 #1 or #2 and then subjected to a timecourse of UVB irradiation. 100 ng DNA loaded per dot. Left panel: immunoblot with primary anti-CPD antibody, secondary HRP conjugated antibody; Right panel: DNA loading control, membrane incubated with SYBR-Gold and imaged on transilluminator. (B) Quantification of CPD dot-blot assay: immunoblot intensity measured using FIJI and normalised to DNA staining intensity. Data shown are Mean $\pm$ SD of four biological replicates.

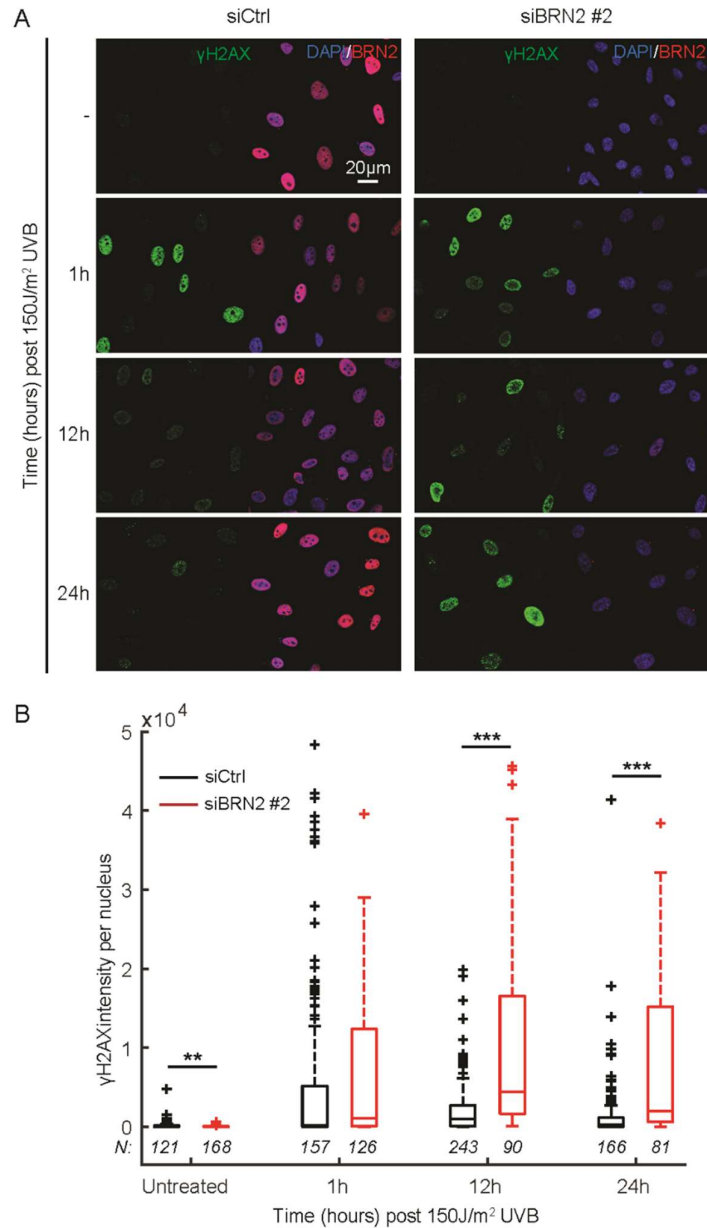


Figure 3.5.3 | BRN2 KD increases persistence of γ H2AX after UVB irradiation

(A) Representative IF of 501mel cells treated with siCtrl or siBRN2 #2 for 48 hours prior to UVB irradiation time course, as indicated, stained with antibodies against γ H2AX and BRN2 with DAPI nuclear counterstain. (B) Box-plot of quantification of IF (Matlab) in (A) using FIJI to analyse the intensity of γ H2AX per nucleus. *N*= number of nuclei analysed. Asterisks represent *p*-values of unpaired Student's *t*-test between siCtrl and siBRN2 #2 at each time point. ** *p*<0.01; *** *p*<0.001.

siCtrl where the levels were progressively decreasing. Since in IF γ H2AX staining was variable between cells within the population, we hypothesized it could depend on the cell cycle phase of each single cell. Therefore, we examined DNA content and γ H2AX staining concurrently in a large population of cells using flow cytometry (Fig. 3.5.4) which allows the analysis the γ H2AX levels in the different cell cycle phases (Huang et al. 2005).

Cells were treated as for IF, with a UVB time course following siRNA-mediated KD of BRN2. Fixed, permeabilised cells were then double stained with γ H2AX-FITC and Topro3. Since γ H2AX is physiologically elevated during S-phase, in order to be able to compare γ H2AX levels between conditions, we needed to understand if KD of BRN2 affects the cell cycle distribution of the cell population. We first confirmed that siBRN2 transfection alone does not affect the distribution of cells through the cell cycle by analysing the DNA content of cells using Topro3 (Fig. 3.5.4a, quantified in Fig. 3.5.4b - untreated). UVB treatment induced first a slight G1 accumulation by 8 hours and a marked G2/M accumulation by 24 hours in all conditions. Although there were some statistical differences between siCtrl and siBRN2 cell cycle distribution, the percentage changes are marginal, indicating that any differences in γ H2AX abundance between siCtrl and siBRN2 following UVB irradiation is unlikely to be a result of BRN2 effect on the cell cycle (Fig. 3.5.4a, quantified in Fig. 3.5.4b).

γ H2AX staining, as measured by fold change in median γ H2AX-FITC, was slightly elevated at baseline, but not significantly, suggesting that BRN2 KD may result in a degree of background DNA damage (Fig. 3.5.5a, quantified in 3.5.5b). We confirmed the observations from IF that initial damage induction is the same or only slightly increased at 1 hour following DNA damage with or without BRN2 KD. However, the persistence of γ H2AX becomes evident at 8h in the more robust BRN2 KD (siBRN2 #2;

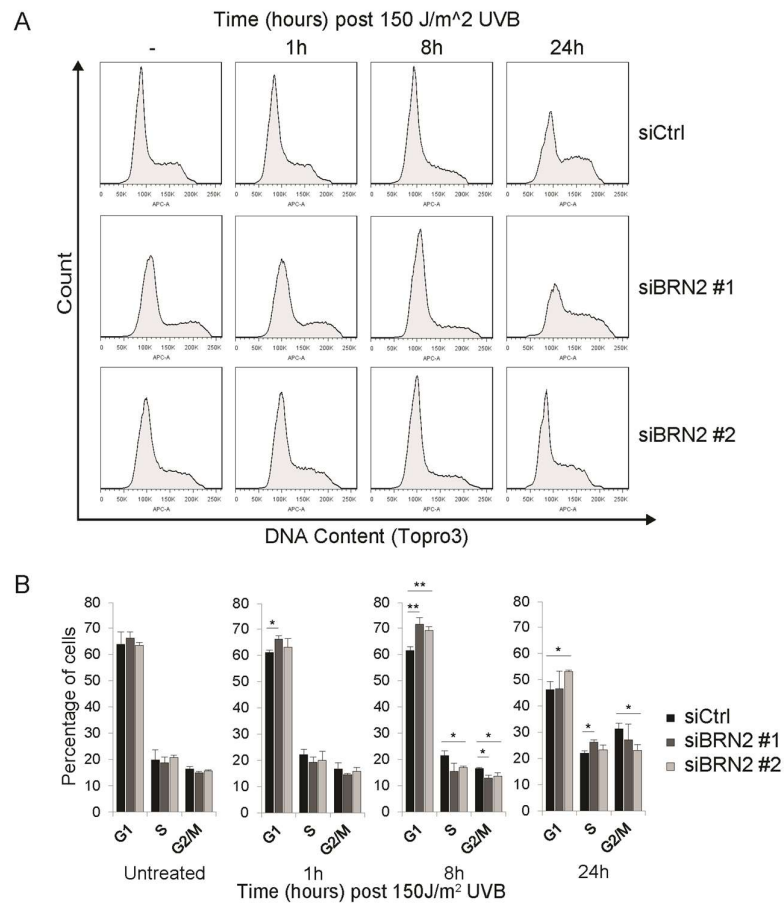


Figure 3.5.4 | BRN2 KD does not affect cell cycle distribution

(A-B) FACS analysis of 501mel cells treated with siCtrl and two siBRN2 (#1 and #2) for 48 hours prior to UVB irradiation, time course as indicated. (A) Representative histograms showing Topro3 staining, quantified in (B) Cell cycle distribution analysis for each siRNA treatment shown in (A) at each timepoint. Data presented are mean $\pm$ SD of at least three biological replicates. Inter-sample comparison by unpaired Student's t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

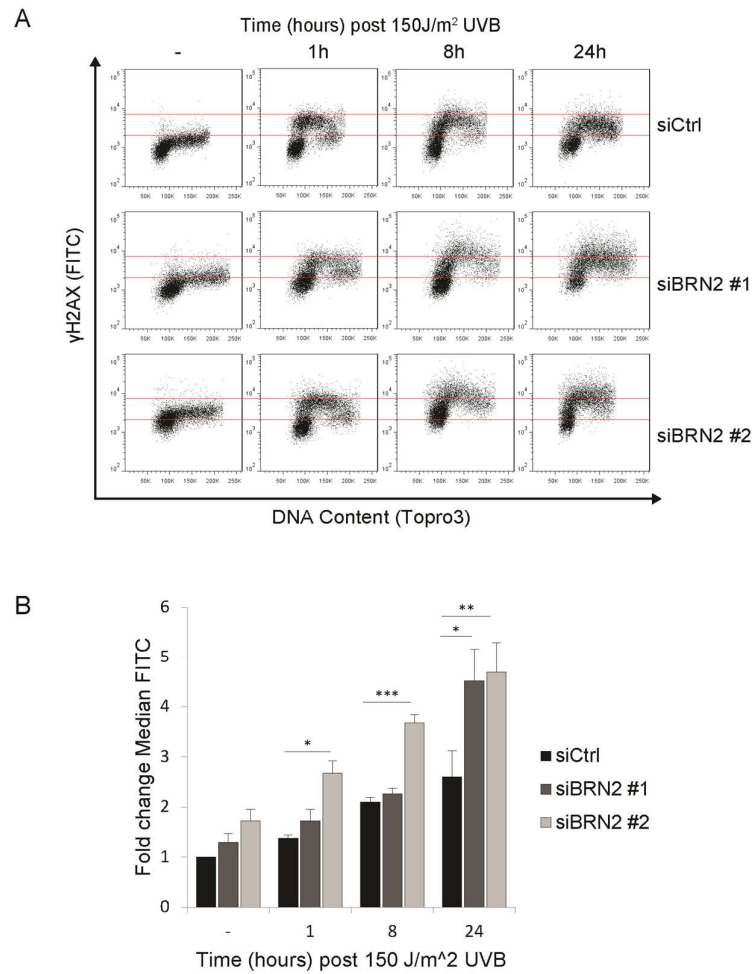


Figure 3.5.5 | BRN2 KD increases persistence of γH2AX after UVB irradiation

(A-B) Flow cytometry analysis of 501mel cells treated with siCtrl and two siBRN2 (#1 and #2) for 48 hours prior to UVB irradiation, time course as indicated. Cells were stained with FITC-conjugated γH2AX antibody and DNA was stained with Topro-3 and analysed by flow cytometry. (A) Representative flow cytometry plot. Lower red line delineates negative vs positive γH2AX staining, upper red line delineates highest positive staining in siCtrl. (B) Quantification of γH2AX positive cells in (A). Data represent fold change in median FITC-γH2AX intensity compared to untreated siCtrl: mean ±SD of at least three biological replicates. Inter-sample comparison by unpaired Student's t-test (* p<0.05; ** p<0.01).

see Fig. 3.5.1) and is clear at 24 hours for both siBRN2 #1 and #2 (Fig. 3.5.5a, quantified in 3.5.5b). There are a number of ways of analysing the change in γ H2AX staining intensity. A large proportion of the cells remain in the γ H2AX negative G1 cloud in the FACS plot and therefore lower the median staining intensity which limits the fold change. However, when the subjective nature of gating positive and negative regions in a dynamic FACS plot is considered, median staining intensity is the most objective measure. As demonstrated above (see Fig. 3.5.4), there was not a dramatic difference in cell cycle phase distribution between siCtrl and siBRN2 conditions, therefore the importance of the G1 cloud should be consistent between siCtrl and siBRN2 treated cells. To confirm this, the proportion of γ H2AX positive cells as a percentage of the total number of cells in each cell cycle phase was analysed at 1 hour. This shows that there was no relationship between BRN2 expression and the cell cycle phase in which H2AX was being phosphorylated (Fig. 3.5.6).

Having shown that γ H2AX levels are elevated and persistent following BRN2 KD, to establish if this corresponds to elevated DSB presence, Comet assays were performed in which the length and intensity of the comet tail reflects the prevalence of DNA strand breaks. There was no significant difference in DSB presence as measured by Comet tail frequency (data not shown). This may reflect that it is difficult to detect differences due to a low frequency of DSB induction by UVB in all conditions. However, more interestingly it reflects that γ H2AX is not purely a DSB marker, particularly when present through all phases of the cell cycle (Marti et al. 2006): persistence of γ H2AX can indicate increased apoptosis, and does not necessarily represent persistence of the DSBs themselves (Halicka et al. 2005). Importantly, the analysis of cell cycle phase distribution of γ H2AX staining described above, showed that H2AX is being phosphorylated throughout the cell cycle, not just in S-phase (Fig. 3.5.6). In addition, the nuclear

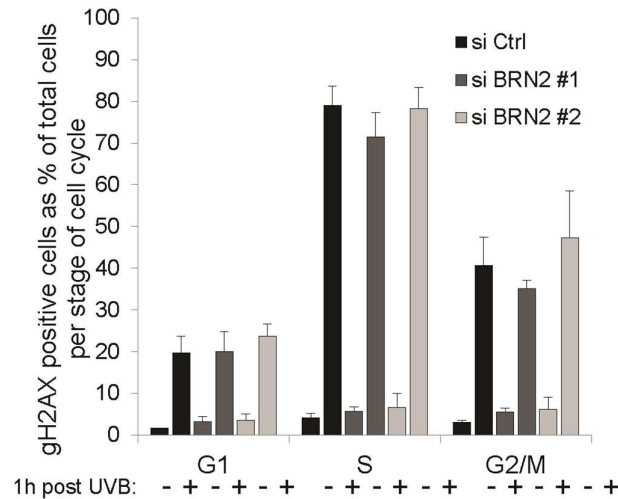


Figure 3.5.6 | H2AX is phosphorylated in all cell cycle phases, independently of BRN2 levels

Analysis of γ H2AX positivity per cell cycle phase 1 hour after UVB treatment compared to untreated cells. Data represents percentage of cells positive for γ H2AX staining as a percentage of total number of cells in each cell cycle phase. Data shown is mean $\pm$ SD of three biological replicates. Inter-sample comparison by un-paired Student's t-test comparing either - UVB or + UVB at each cell cycle phase. All p values >0.5 .

distribution of γ H2AX staining in IF is predominantly pan-nuclear rather than punctate, which is associated with apoptosis induction (de Feraudy et al. 2010). Furthermore, some cells show a ring of γ H2AX staining within the nucleus around the periphery which has been termed an apoptotic ring. It is an epigenetic marker of the early stages of apoptosis in which heterochromatic regions of DNA begin to undergo fragmentation. This fragmentation triggers DDR kinase activation and hence phosphorylation of H2AX. However, the caspase-mediated cleavage also targets proteins involved in recruitment of downstream repair factors, thus curtailing the DDR and avoiding unwanted repair of the newly cleaved DNA (Solier and Pommier 2014).

Therefore, the elevated levels of γ H2AX in UVB-treated siBRN2 cells may indicate that cells lacking BRN2 are more sensitive to UVB-induced apoptosis. To investigate if the persistence of γ H2AX represented increased apoptosis, we performed FACS on cells stained for the activated apoptotic protein cleaved caspase 3/7. There was a significant increase in cleaved caspase 3/7 positive cells 24 h after UVB treatment following knockdown of BRN2 with both siRNAs, whereas control cells with maintained levels of BRN2 were resistant to induction of apoptosis (Fig. 3.5.7a, quantified in b).

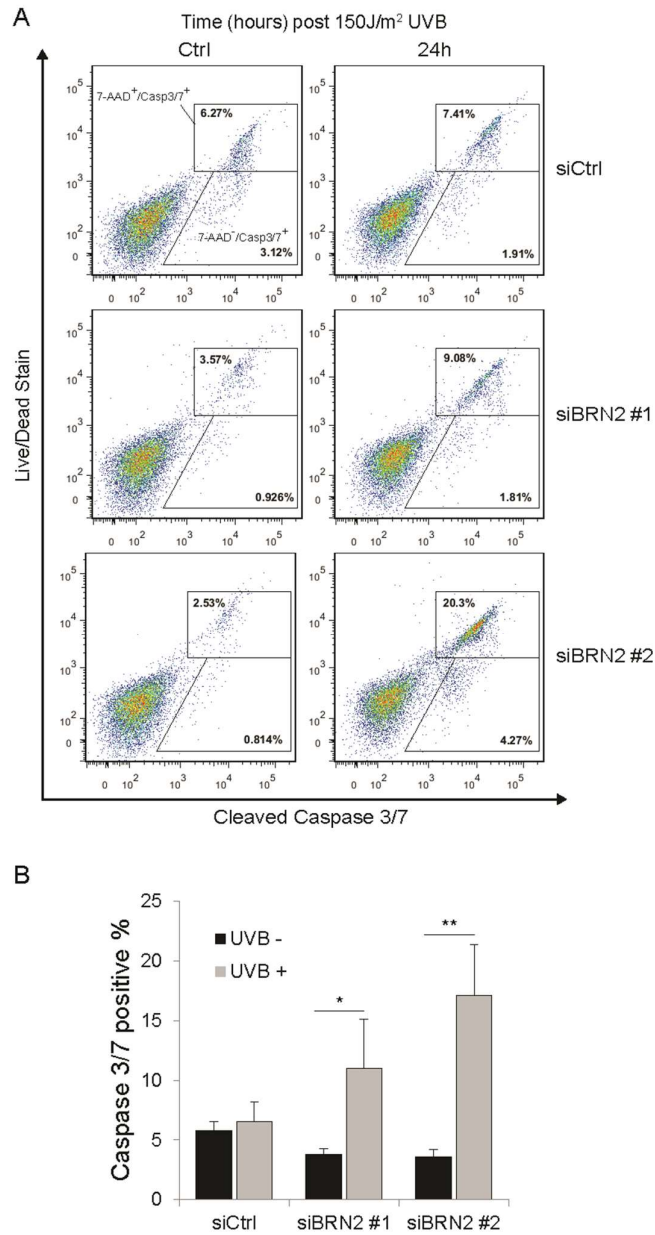


Figure 3.5.7 | BRN2 KD increases susceptibility to cell death after UVB irradiation

(A-B) Apoptosis assay using cleaved caspase 3/7 in conjunction with 7-AAD live/dead stain to identify apoptotic population by Flow cytometry 24 hours after UVB treatment in siCtrl or siBRN2 treated 501mel cells. (A) Representative Flow cytometry plots, apoptotic and dead cells are isolated in the two gated regions which are both Caspase 3/7 positive, upper is double positive for 7-AAD and Caspase 3/7. Combined populations quantified in (B). Data presented are mean $\pm$ SD of at least three biological replicates. Intrasample comparison by paired Student's t-test (* $p < 0.05$; ** $p < 0.01$).

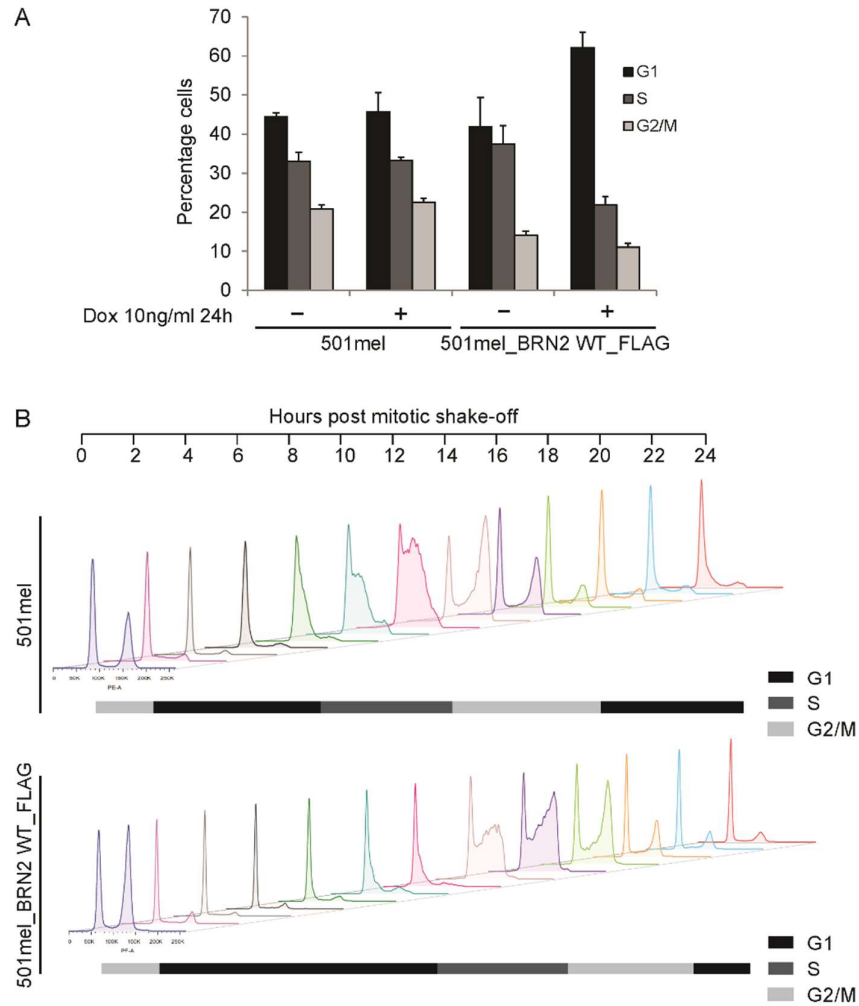


Figure 3.5.8 | BRN2 overexpression delays G1 progression

(A) Cell cycle distribution of 501mel cells compared to 501mel_BRN WT_FLAG (clone#2) with and without doxycycline induction (10 ng/ml, 24hours). Cells were collected, fixed, permeabilised and stained with propidium iodide and then analysed by FACS. Data presented are mean $\pm$ SD of three replicates. (B) Sequential cell cycle profiles of propidium iodide stained 501mel or 501mel_BRN WT_FLAG (clone#2) cells without doxycycline induction which were subjected to mitotic shake-off every 2 hours for 24 hours. Shaded bar represents progression through cell cycle. Black – G1, Dark grey – S, light grey – G2/M.

To confirm the BRN2-specific role in DDR and apoptosis, reciprocal experiments were carried out in BRN2-stably overexpressing 501mel_BRN2 WT_FLAG cells. Since cell cycle distribution can affect the interpretation of γ H2AX assays we initially assessed the effects of BRN2_FLAG overexpression on the cell cycle. Strong induction of BRN2_FLAG overexpression with doxycycline resulted in a marked G1 accumulation, that was not apparent without doxycycline treatment (Fig. 3.5.8a). However, as discussed above, the PB system was demonstrated to be leaky and there is a low level of BRN2_FLAG overexpression without doxycycline treatment (see Figs 3.2.1-3.3.2). To investigate any subtle cell cycle phenotype, a ‘mitotic shake-off assay’ was used to track the progression through the cell cycle. This is a method of synchronising cells without the use of drugs, by exploiting the loose attachment of mitotic cells to the culture dish. Gentle shaking of the flask dislodges mitotic cells, which can then be collected, replated and assayed at a specified time after collection. The doubling time of 501mel cells is approximately 16 hours, therefore cells were collected every 2 hours over a 24-hour period and analysed by FACS for cell cycle distribution. 501mel and 501mel_BRN2WT_FLAG cells were compared. BRN2_FLAG expressing cells were shown to have a delayed G1 progression of 2-4 hours (Fig. 3.5.8b).

It is known that MITF knockdown results in a G1 arrest (Carreira et al. 2006), it is therefore possible that the cell cycle effect we see in BRN2 overexpression is a consequence of BRN2-mediated MITF repression. However, the relationship between BRN2 and MITF remains controversial. Whilst *in vivo* expression of BRN2 and MITF has been shown to be mutually exclusive (Goodall et al. 2008), it has been demonstrated that *in vitro*, BRN2 can upregulate MITF expression (Wellbrock et al. 2008). We therefore investigated the effect of BRN2 on MITF expression in our experimental systems. Following BRN2 depletion using siRNA in 501mel cells, MITF levels are

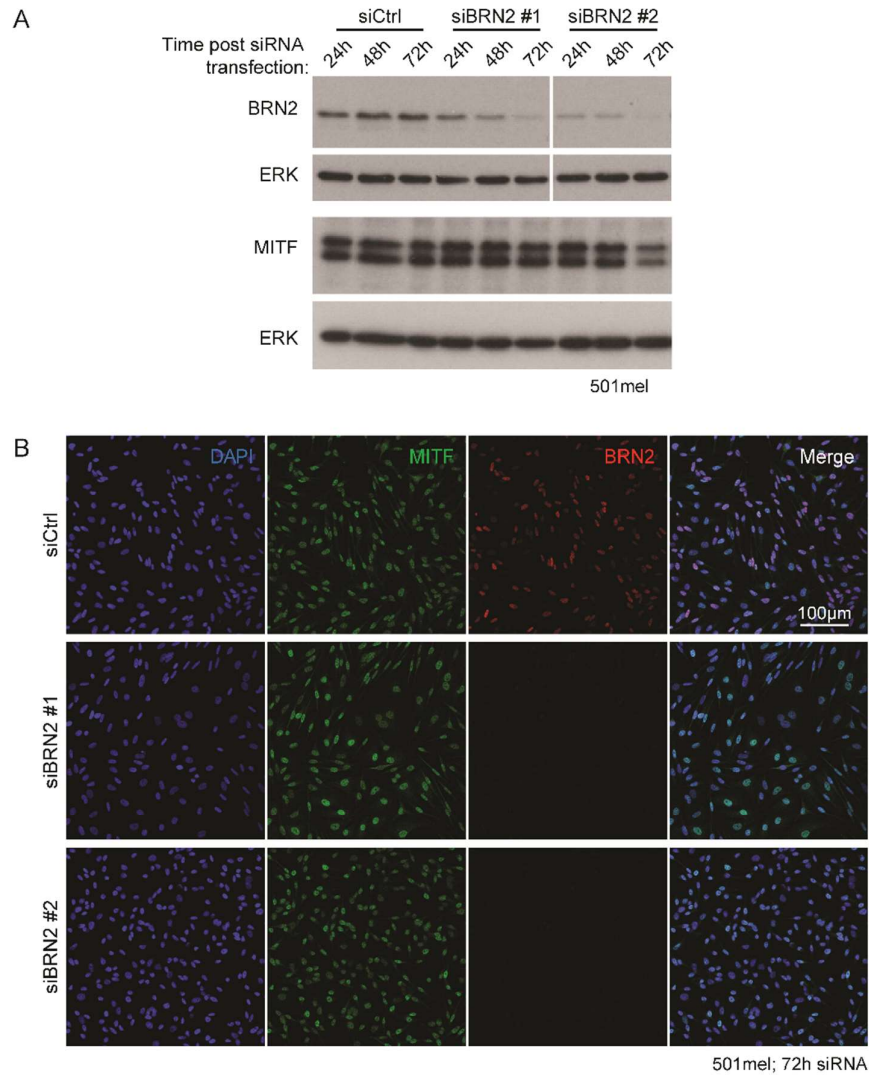


Figure 3.5.9 | Knockdown of BRN2 does not significantly affect MITF levels

(A) Western blot of 501mel cells treated with siCtrl or siBRN2 (#1 or #2) for time course as indicated. Same samples run on two gels in parallel. Antibodies against BRN2 and MITF with ERK used as a loading control. (B) IF of 501mel cells treated with siCtrl or siBRN2 (#1 or #2) at 72 hours. Antibodies against MITF and BRN2 with DAPI counterstain.

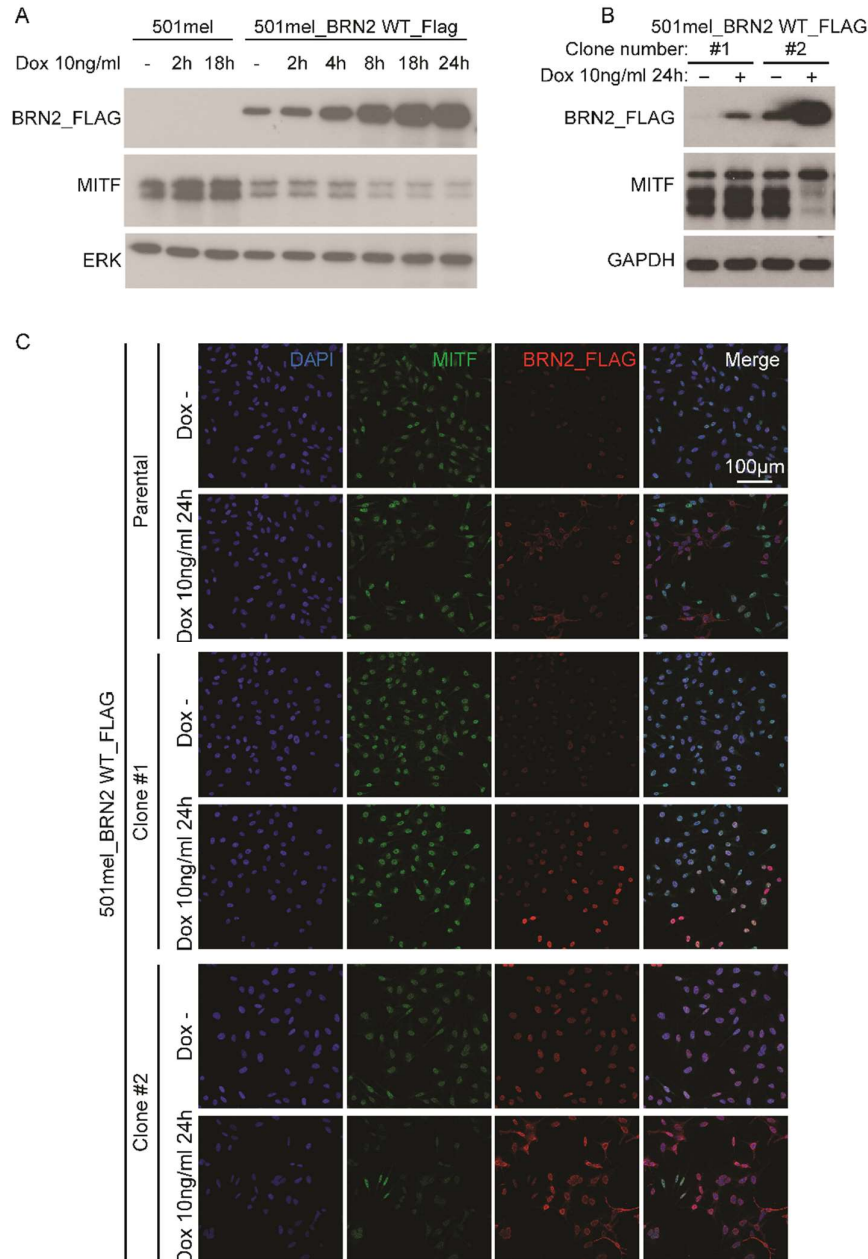


Figure 3.5.10 | Overexpression of BRN2 represses MITF

(A) Western blot of 501mel and 501mel_BRN2 WT_FLAG (parental) cell lines, with a time-course induction of doxycycline 10 ng/ml. Antibodies against BRN2_FLAG, MITF and ERK as loading control. (B) Western blot showing FLAG and MITF expression levels in 501mel_BRN2 WT_FLAG clone#1 and clone#2 with and without doxycycline induction (10ng/ml, 24 hours). (C) IF of 501mel_BRN2 WT_FLAG parental, clone#1 or clone#2 following 24 hours doxycycline 10 ng/ml induction. Antibodies against MITF, FLAG (BRN2), with DAPI nuclear counterstain.

maintained, or slightly reduced by 72 hours with the more robust siRNA (#2) (Fig. 3.5.9 a,b). By contrast, in 501mel_BRN2 WT_FLAG cell lines, there is clearly a loss of MITF protein expression when BRN2_FLAG is highly expressed, as in the parental line or with doxycycline induction in parental and clone #2 (Fig. 3.5.10 a-c). However, when low levels of BRN2 are overexpressed, either in the uninduced clone #2, or in clone #1 with or without doxycycline induction, the reduction in MITF is absent (Fig. 3.5.10b,c). Therefore for the overexpression DNA damage assays, the low overexpression BRN2 cell line (Clone#1) was used to avoid any issues surrounding any effect of BRN2 on the expression of MITF which is known to regulate DNA damage repair genes (Strub et al. 2011).

As with endogenous BRN2, the amount of BRN2_FLAG expressed is unchanged by UVB treatment (Fig. 3.5.11a). By FACS analysis, the BRN2_FLAG cells seem to have nuclei with higher DNA content as assessed by Topro3 staining and represented by a rightwards shift and expansion of the FACS plot along the X axis. This could potentially falsely elevate the median γ H2AX, thus diminishing differences between the two cell lines. Despite this, γ H2AX levels are reduced compared to 501mel control cells at 8 hours after UVB treatment, although there is no difference at 24 hours (Fig. 3.5.11b, quantified in c). Significantly, we demonstrated that we could rescue the phenotype of UVB induced apoptosis by overexpression of BRN2 (Fig. 3.5.12a, quantified in b) as assayed by Caspase 3/7 staining.

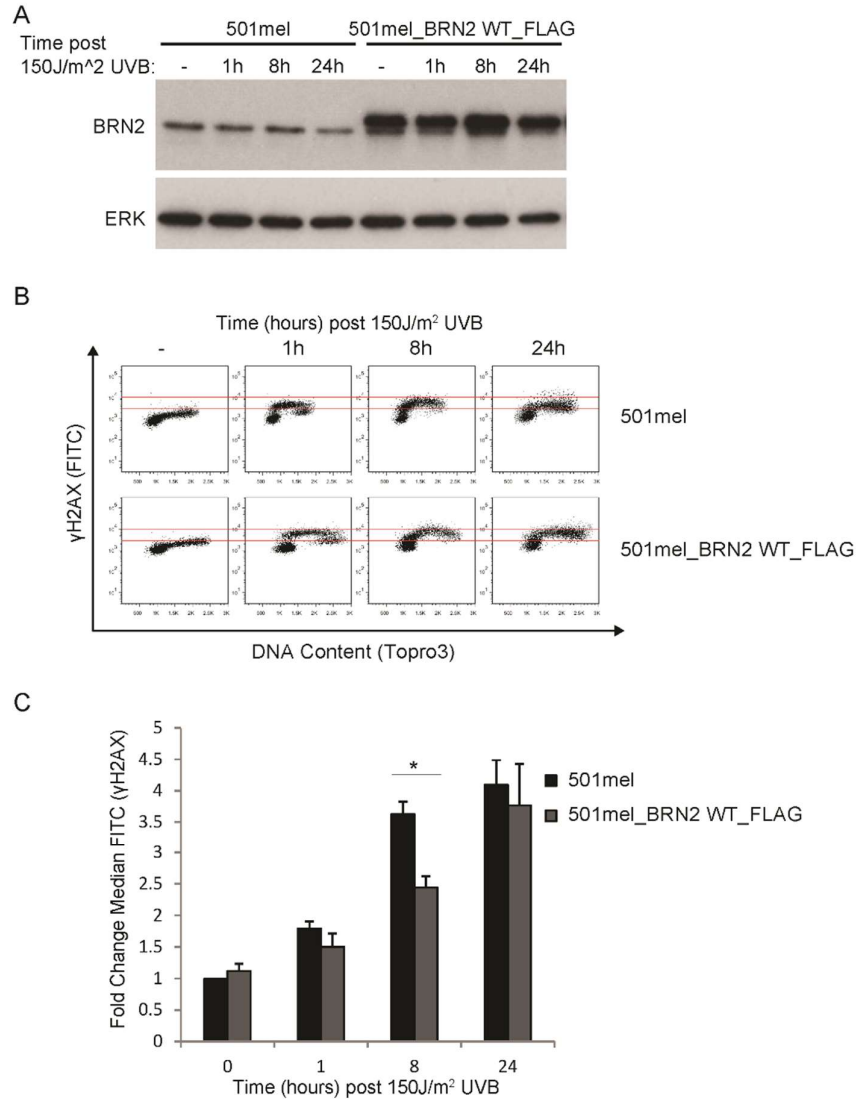


Figure 3.5.11 | BRN2 overexpression reduces persistence of γ H2AX after UVB irradiation

(A) Representative Western blot showing BRN2 expression levels in 501mel and stable 501mel_BRN2_WT_FLAG cells following UVB treatment. ERK as loading control. (B) Representative Flow cytometry plot of FITC-conjugated γ H2AX and Topro3 staining in 501mel and stable 501mel_BRN2_WT_FLAG cells following UVB treatment. Lower red line delineates negative vs positive γ H2AX staining, upper red line delineates highest positive staining in siCtrl. (C) Quantification of γ H2AX positive cells in (B) Data shown represents fold change in median FITC relative to 501mel untreated and is shown as mean $\pm$ SD of three biological replicates. Inter-sample comparison by un-paired Student's t-test (* $p < 0.05$).

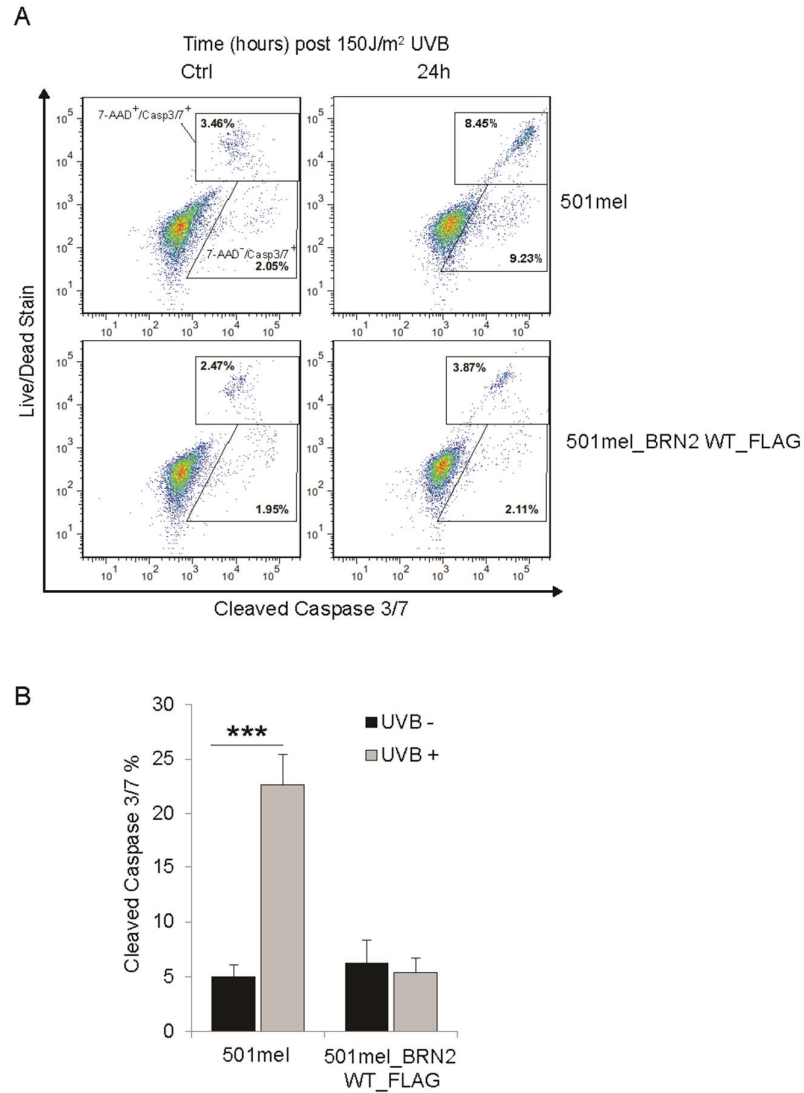


Figure 3.5.12 | Overexpression of BRN2 rescues melanoma cells from UVB-induced cell death

(A-B) Apoptosis assay using cleaved caspase 3/7 in conjunction with 7-AAD live/dead stain to identify apoptotic population by Flow cytometry 24 hours after UVB treatment 501mel and stable 501mel_BRN2 WT_FLAG cell lines. (A) Representative Flow cytometry plots, apoptotic and dead cells are isolated in the two gated regions which are both Caspase 3/7 positive, upper is double positive for 7-AAD and Caspase 3/7. Combined populations quantified in (B). Data presented are mean $\pm$ SD of at least three biological replicates. Intrasample comparison by paired Student's t-test (***) $p < 0.001$.

It is noteworthy that in this experiment 501mel cells showed a four-fold elevation in γ H2AX levels at 24 hours which translated to an increase from 5% to around 25% caspase 3/7 positive cells (Fig. 3.5.12a, b). This is in contrast to the 501mel siCtrl cells which only demonstrated a doubling of γ H2AX levels at 24 hours and consequently had little change in percentage of apoptotic cells (see Fig. 3.5.5). The difference between the 501mel and 501mel siCtrl treated cells means that the two experiments, KD and overexpression, must be treated separately.

3.6 | BRN2 Expression Correlates with Genes Involved in Apoptosis

We have clearly shown that BRN2 KD results in increased apoptosis following DNA damage and that BRN2 overexpression can rescue cells from apoptosis. Given the canonical role of BRN2 as a transcription factor, we therefore sought to understand the transcriptional landscape in siBRN2 treated cells following UVB damage. RNA-Seq was carried out on 501mel cells treated with siCtrl or siBRN2. Triplicate RNA samples were collected at time 0, 6, 12 and 24 hours after UVB damage. Knockdown of BRN2 was confirmed by RNA-Seq. Gene set variance analysis (GSVA) showed that expression of genes involved in apoptosis pathways (Fig. 3.6.1a) is significantly upregulated following BRN2 KD and that this upregulation persists to 24 hours after damage (Fig. 3.6.1b) (Wu et al. 2010; Liberzon et al. 2015). This contrasts with siCtrl-treated cells which show a smaller increase in apoptosis pathway gene expression at 6 -12 hours which is absent at 24 hours (Fig. 3.6.1b).

Gene set enrichment analysis (GSEA) was then carried out using the RNA-Seq data correlated against the identified differentially expressed gene sets. Pairwise comparisons between BRN2 KD and siCtrl-treated cells at each time point, normalised to time 0, were performed. This allowed us to see if there was a significant overrepresentation of the apoptosis gene sets identified in GSVA in the siBRN2 cells compared to the siCtrl cells. At 12 and 24 hours after UVB irradiation, up to four of the studied apoptosis gene sets (3.6.1a) were significantly positively correlated with genes which were overexpressed in the siBRN2 treated cells compared to siCtrl treated cells. Here we show the GSEA analysis at 24 hours (Fig. 3.6.1c). The correlation of the Hallmark Apoptosis gene set with genes upregulated in siBRN2 treated cells compared to control at 24 hours is shown as an example (Fig. 3.6.1d).

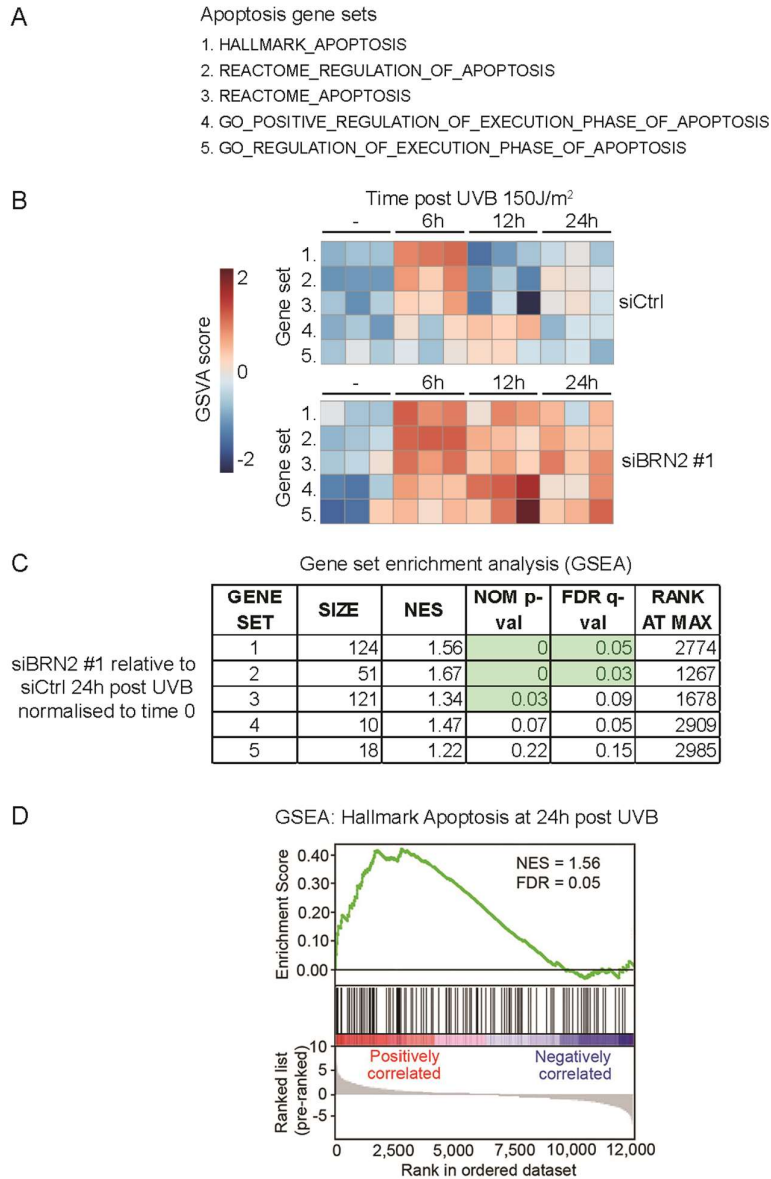


Figure 3.6.1 | DNA damage-induced apoptotic pathway upregulation is increased following BRN2 knockdown

(A) Legend for apoptosis gene sets referenced in (B) and (C). (B) Heatmap showing gene set variance analysis (GSVA) for gene sets relating to apoptosis. Data from RNA-Seq of 501mel siCtrl or siBRN2 #1 treated cells, subjected to a timecourse of UVB irradiation as indicated. (C) Table showing Gene set enrichment analysis (GSEA) for the apoptosis gene sets in siBRN2 cells compared to siCtrl cells at 24h after UVB irradiation. NES - normalised enrichment score; NOM p-val – nominal *p*-value; FDR q-val- False discovery rate *q*-value (D) Enrichment plot of GSEA for Hallmark Apoptosis gene set plotted by enrichment of gene expression in siBRN2 compared to siCtrl treated cells 24 hours after UVB irradiation. Analysis by P. Louphrasittiphol

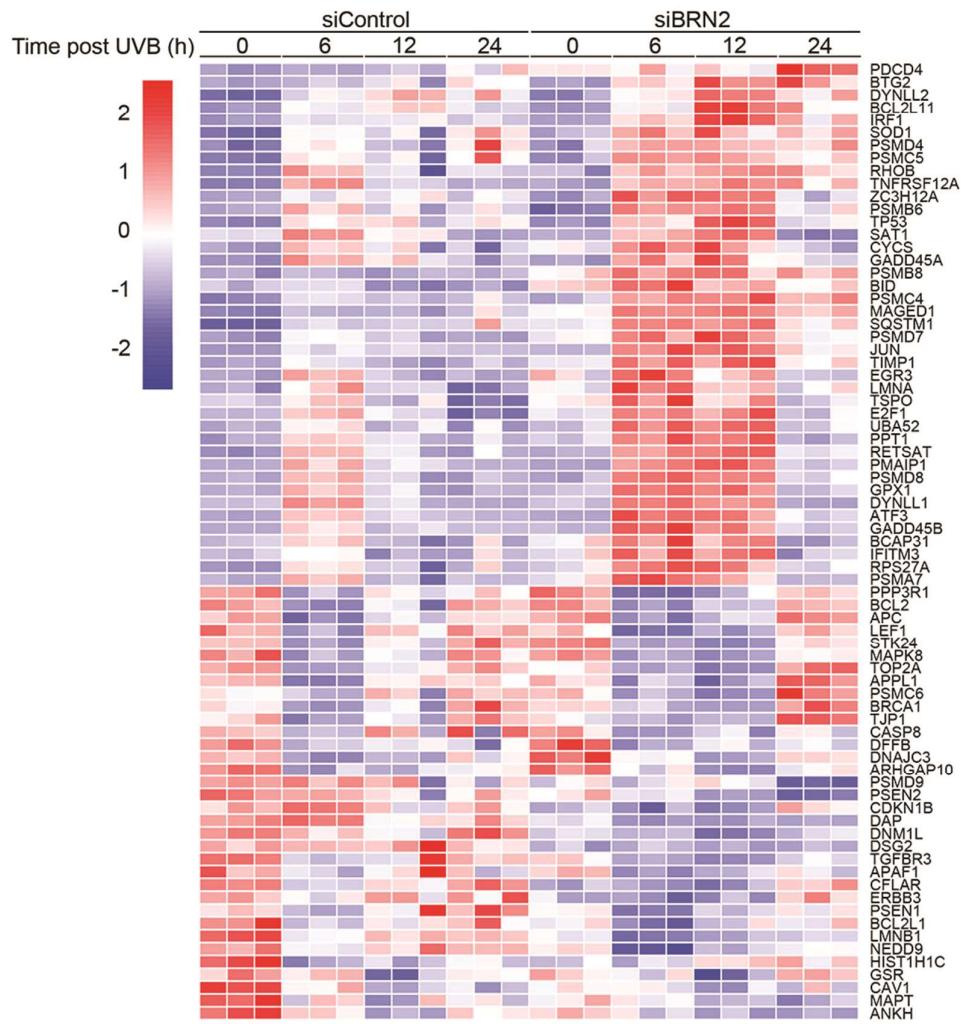


Figure 3.6.2 | Knockdown of BRN2 alters expression of apoptosis-associated genes in response to UVB

Heatmap represents normalised read counts of mRNA levels for individual genes contained within the identified apoptotic gene sets. Data derived from triplicate 3' RNA-Seq of cells transfected with control or siBRN2 for 24 h followed by UVB irradiation. Cells were harvested at indicated times after UVB treatment. Heatmap scale = Z-score. Analysis by J. Chuahan.

Examination of the overlap between each apoptotic signature gene set revealed that a core set of genes associated with apoptosis were either robustly up- or down-regulated following UVB irradiation in the BRN2 depleted cells (Fig. 3.6.2). This is particularly marked at 6-12 hours which would fit with the apoptosis phenotype being evident by 24 hours. Some genes identified in this analysis are known to be bound or regulated directly by BRN2 or related factors. For example, *BCL2* and *BRCA1* are known to be directly regulated by BRN3a (Budhram-Mahadeo et al. 1999). GADD45a is a known, UVB-dependent, direct target of BRN2 (Lefort et al. 2001). Whilst APC, APPL1 and PMAIP1 were identified in BRN2 ChIP-chip experiments as direct targets (Kobi et al. 2010).

3.7 | BRN2 Is Recruited to DNA Damage in a PARP Dependent Manner

Having established that BRN2 binds to DDR proteins, particularly those involved in recognition and onset of the DDR, and that its loss results in increased apoptosis following DNA damage, we used laser micro-irradiation (LMI) to establish if BRN2 can be recruited to sites of DNA damage. Fig. 3.7.1a shows a schema for live LMI in which fluorescently-tagged proteins can be visualised recruiting to sites of damage. The U-2-OS osteosarcoma cell line is a canonical cell model for live cell imaging LMI as they are very robust and optimal for imaging. Using U-2-OS also has the advantage of looking beyond the melanoma environment as neither BRN2 or MITF or other melanoma specific proteins are expressed in U-2-OS. The assay can also be performed with fixed cells and immunofluorescence of endogenous or exogenous proteins. The cells are pre-incubated with Hoechst which both sensitises the DNA to LMI and acts as a nuclear stain to facilitate imaging and LMI. There is a recognised phenomenon of decreased Hoechst intensity in the path of the LMI which represents chromatin decondensation (Strickfaden et al. 2016); these Hoechst negative stripes can be used as a marker of the LMI irradiation (Fig. 3.7.1a). The LMI protocol was optimised to establish the parameters of laser power that resulted in appropriate Hoechst negative stripes but without causing catastrophic damage to the integrity of the nuclei; this corresponds to 100 mW from the multiphoton laser at 750 nm (Fig. 3.7.1b). For live imaging, U-2-OS cells stably expressing GFP-tagged Ku80 were used as a positive control (Fig. 3.7.1c) with Empty-GFP used as a negative control (Fig. 3.7.1d). In fixed imaging slides were co-stained against BRN2 and the DNA damage markers Ku80 and γ H2AX.

We used fixed LMI followed by immunofluorescence in 501mel cells to demonstrate that endogenous BRN2 co-localises with sites of DNA damage. At 90 seconds after LMI, BRN2 accumulates at the damaged area and co-localises with the two

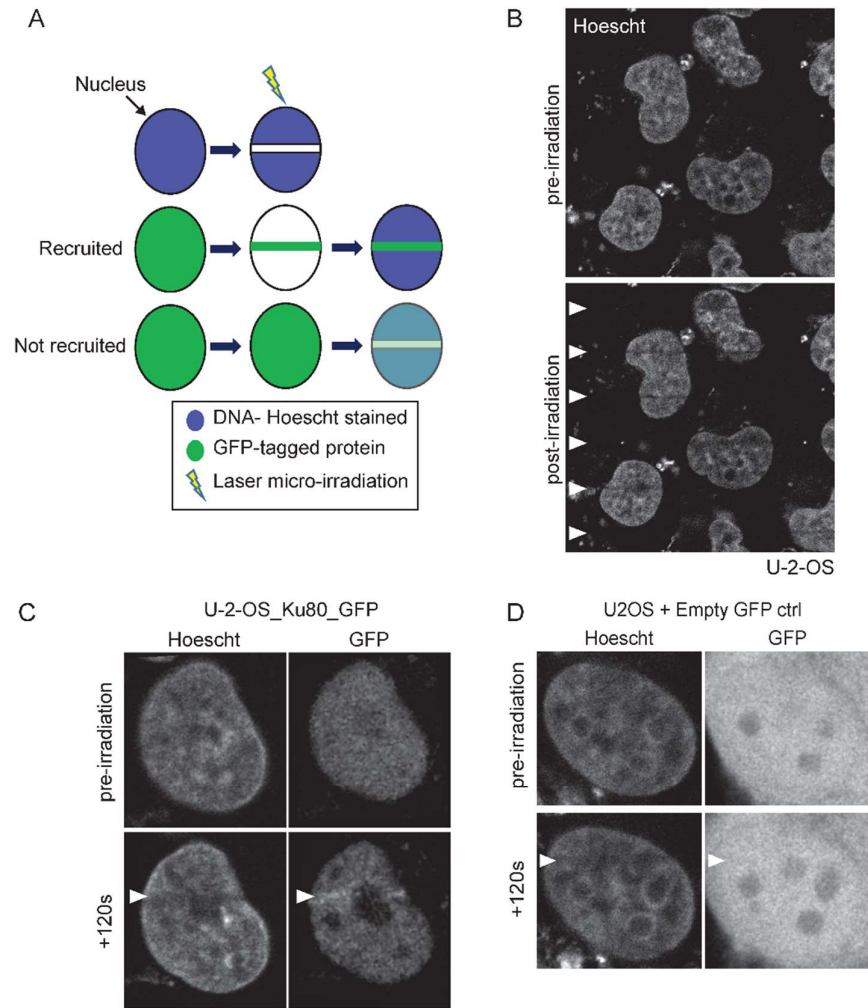


Figure 3.7.1 | Optimisation of laser micro-irradiation (LMI)

(A) Scheme describing LMI protocol. Blue – Hoescht 33258 staining marks nucleus; Green – fluorescent-tagged protein; lightning bolt – multiphoton laser micro-irradiation. (B) Live image of Hoescht 33258 stained U2OS cells before and after LMI, showing Hoescht “negative” stripes at sites of irradiation (represented by white arrows). (C) stable U2-OS_Ku80_GFP cells before and at 120 s post irradiation - positive control for LMI. (D) U-2-OS cells transiently transfected with Empty GFP; before and at 120 s post-irradiation - negative control for GFP.

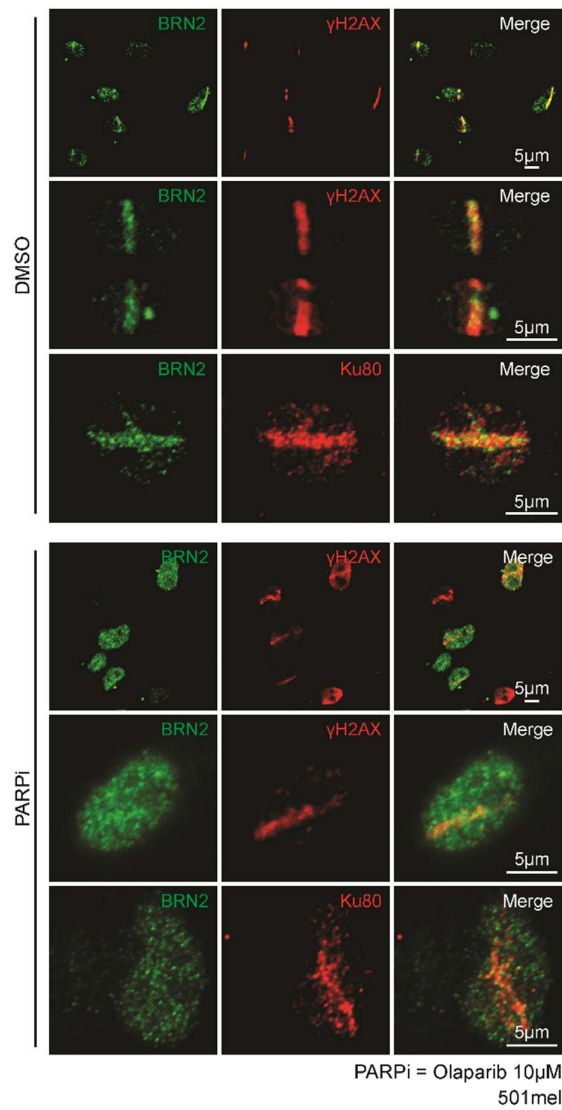


Figure 3.7.2 | Endogenous BRN2 is recruited to sites of DNA Damage in a PARP-dependent manner

Representative IF of 501mel cells 90 s post LMI. Cells were pre-treated with DMSO (top panel) or 10 μ M PARP inhibitor Olaparib (bottom panel) for minimum 3 hours prior to irradiation. Cells were stained with antibodies against γ H2AX or Ku80 and BRN2.

well established DNA damage markers γ H2AX and Ku80 (Fig. 3.7.2, top panel). PARP1 is one of the early DNA damage response proteins, it is also one of the most highly enriched binding partners of BRN2 (see Figs 3.3.1, 3.4.2). Following treatment with the PARP inhibitor Olaparib (10 μ M) (PARPi), BRN2 no longer co-localises with sites of damage. Importantly we demonstrated that both γ H2AX and Ku80 are still recruited (Fig. 3.7.2, bottom panel). To confirm that PARP inhibition disrupts the ability of BRN2 to bind to its DDR response protein partners, BRN2_FLAG was immunoprecipitated from 501mel_BRN2 WT_FLAG overexpressing cells which had been pre-treated with PARPi and then subjected to 50J/m² UVC irradiation (Fig. 3.7.3). In DMSO treated cells, Ku80 co-immunoprecipitated with BRN2_FLAG as expected, and there was no disruption to the association triggered by UV treatment. Following PARPi pre-treatment alone Ku80 and BRN2 continued to co-IP but following UV treatment Ku80 interaction with BRN2 was substantially diminished (Fig. 3.7.3). This suggests that PARP may be required to maintain the BRN2:Ku80 interaction following UV irradiation and DNA damage.

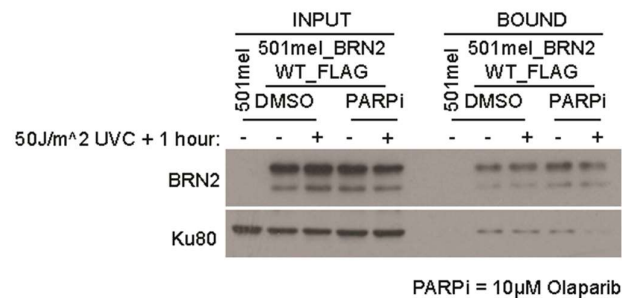


Figure 3.7.3 | PARP inhibition reduces interaction between BRN2 and Ku80 following damage

Western blot showing co-immunoprecipitation of Ku80 with BRN2 following Flag-pull down. 501mel (Ctrl) cells or stable 501mel_BRN2 WT_FLAG were treated with DMSO or PARPi for at least 3 hours prior to UVC irradiation. Cells were collected 1 hour post UV treatment.

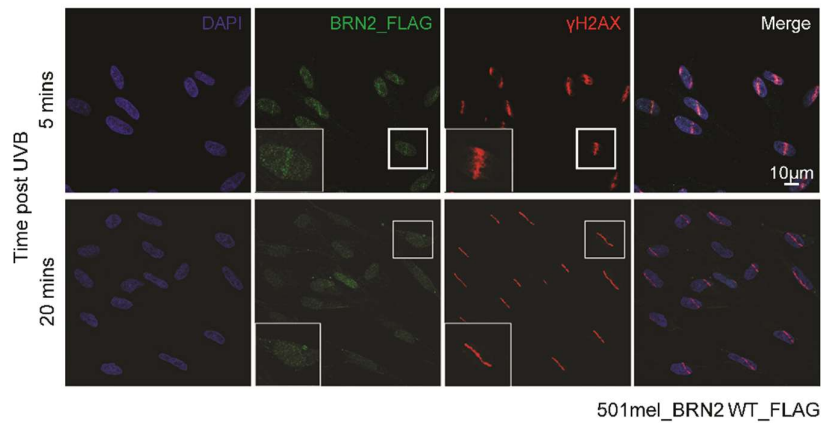


Figure 3.7.4 | BRN2 recruitment to sites of damage is abolished by 20 minutes

Representative immunofluorescence of 501mel_BRN2 WT_FLAG cells following laser micro-irradiation treatment at (A) 5 mins and (B) 20, 30 and 50 mins post-irradiation. Cells were stained with antibodies against γ H2AX and BRN2, with DAPI nuclear counterstain.

The recruitment of BRN2 was confirmed by fixed imaging following LMI in 501mel_BRN2 WT_FLAG cells, which further revealed that BRN2 recruitment was indeed present at 5 minutes but was abolished by 20 mins post irradiation, whilst γ H2AX continues to be localised to irradiated stripes throughout (Fig. 3.7.4). This indicated that the time window in which BRN2 associates with DNA damage sites is very early following damage induction, and potentially quite short.

To further investigate the dynamics of BRN2 recruitment, live cell LMI was used. GFP-BRN2 was transfected into U-2-OS osteosarcoma cells. Cells with equivalent levels of GFP expression were identified to minimise the variability in recruitment that might be caused by expression levels alone. In DMSO treated controls, BRN2 recruitment is evident immediately after irradiation and peaks at 4.5 minutes, before tailing off again

(Fig. 3.7.5a quantified in b). Live imaging was only done for 7 minutes as bleaching of GFP much beyond this complicates the analysis and interpretation of accumulation at the stripe.

Taken together with the fixed imaging time-course, the data show that BRN2 recruitment to laser irradiated sites occurs almost immediately, has peaked by 5 minutes and is lost completely by 20 minutes. This indicates an early and dynamic association of BRN2 with sites of DNA damage, as suggested by the range of DNA damage proteins identified by mass spec. The dependence on PARP activity for BRN2 recruitment was then confirmed in the live system, with no GFP-BRN2 detectable at sites of LMI damage in cells pre-treated with PARP inhibitor (Fig. 3.7.5a quantified in b). Thus, BRN2 is transiently recruited to sites of DNA damage and co-localises with Ku80 immediately following damage in a PARP dependent manner.

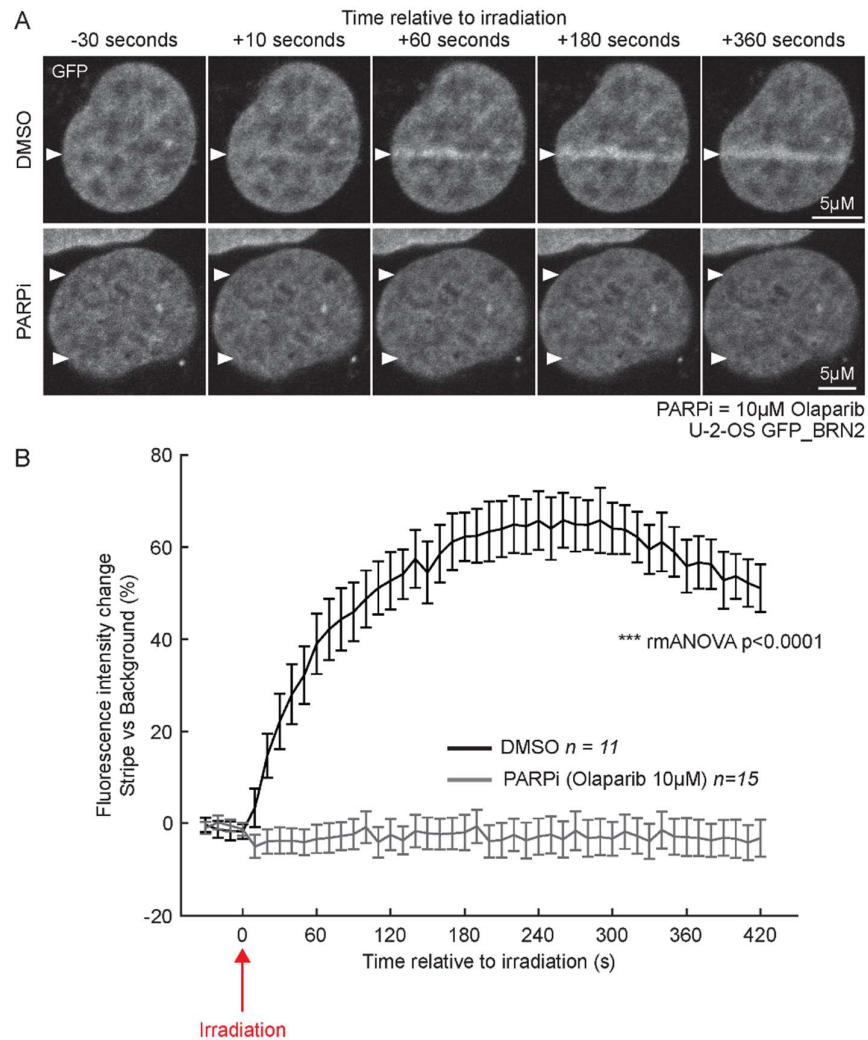


Figure 3.7.5 | BRN2 recruitment is PARP dependent and transient

(A) Representative still images from live imaging of U-2-OS cells transiently transfected with GFP_BRN2 WT expression vector with or without PARP inhibitor treatment (Olaparib 10µM). Time (s) relative to irradiation. (B) Quantification of live imaging shown in (A). Data shown are the mean fluorescence intensity change of irradiated stripe vs background per cell expressed as mean $\pm$ SEM (N : DMSO=11; PARPi=15), curves compared by repeated measures ANOVA. *** $p < 0.0001$.

3.8 | Motif-Specific DNA Binding Is Required for BRN2 Recruitment and Apoptosis Rescue

To establish if DNA binding is required for BRN2 recruitment to sites of DNA damage, a non-DNA binding mutant had to be created as there is no validated BRN2 non-DNA binding mutant. There is no crystal structure of BRN2, however, the POU domain of multiple paralogues has been crystallised on DNA and six contact residues have been identified (Phillips and Luisi 2000). Since the POU domain is highly conserved, these residues were mapped onto the BRN2 DNA binding domain and the corresponding residues in homologues identified (Fig. 3.8.1). Two of the six residues are in the nuclear localisation signal; therefore they were not considered as suitable mutation targets. The 3D structure of Oct1 crystallised on DNA predicts two H bonds between Asparagine 422

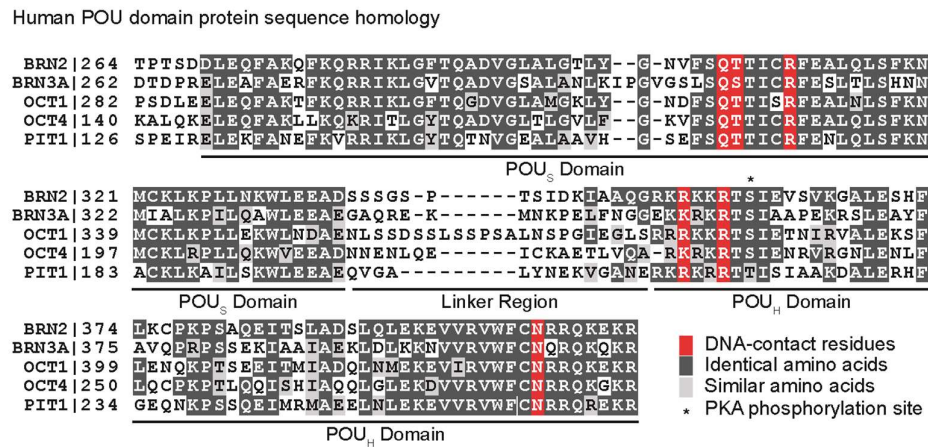


Figure 3.8.1 | BRN2 DNA binding domain and contact residues are conserved across paralogues

Schematic showing POU domain sequence homology between BRN2, BRN3A, OCT1, OCT4, PIT1. Red=DNA-contact residues; Dark Grey=Identical amino acids; Light Grey = similar amino acids.

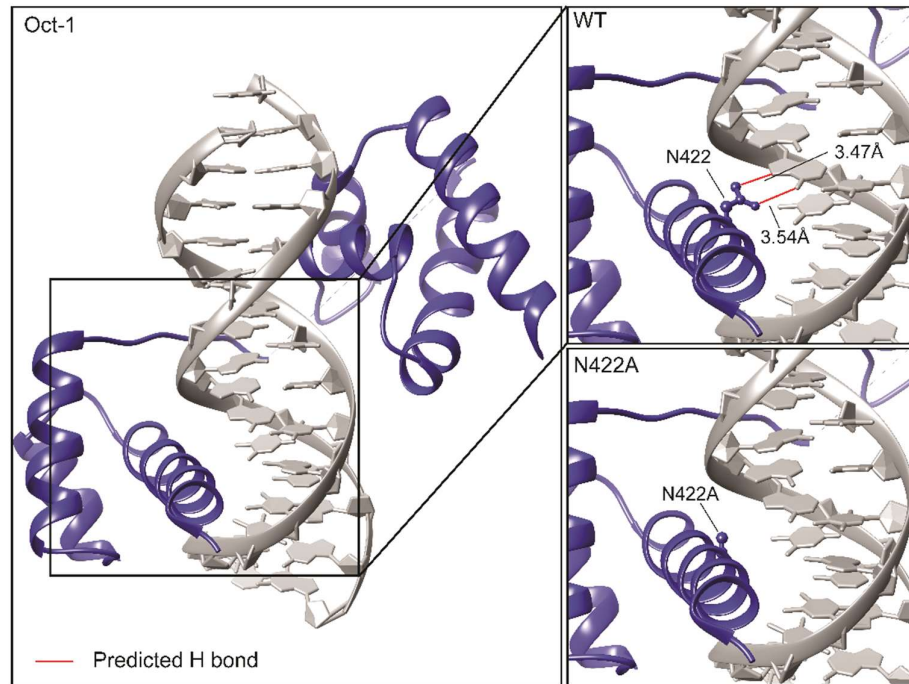


Figure 3.8.2 | N442A mutation in Oct-1 is predicted to break H bonds with DNA

Images showing crystal structure of Oct-1 on DNA. Upper inset: Residue N442 on Oct-1 (corresponding to N401 in BRN2) depicted as molecular structure, with two predicted H bonds (red lines). Lower inset: Residue N442 mutated to Alanine (N442A) demonstrating predicted loss of H bonds.

and an adenine in the DNA major groove (Klemm et al. 1994), which was predicted to be disrupted following *in silico* mutation to Alanine (Fig. 3.8.2) An Alanine mutant on the corresponding Asparagine in BRN2 (N401A) was generated using site-directed mutagenesis.

To validate that BRN2^{N401A} fails to bind DNA, Electrophoretic Mobility Shift Assays (EMSA) with bacterially-expressed, purified GST-tagged BRN2^{WT} and BRN2^{N401A} were performed. Two known BRN2 binding sites on the MITF and Kit ligand

promoters were used as DNA probes, which were hybridised and radiolabelled with $\alpha^{32}\text{P}$ -CTP (Fig. 3.8.3a) (Kobi et al. 2010). GST-BRN2^{WT} bound both probes (Fig. 3.8.3b). Pre-incubation of the GST-BRN2 protein with anti-BRN2 antibody resulted in a supershift of the radio-opaque band, confirming that BRN2 was in complex with the radiolabelled probe. GST-BRN2^{N401A} however, was unable to bind either DNA probe, despite being present in excess, as demonstrated by Coomassie staining of a gel run in parallel with the pre-incubation mix (Fig. 3.8.3b).

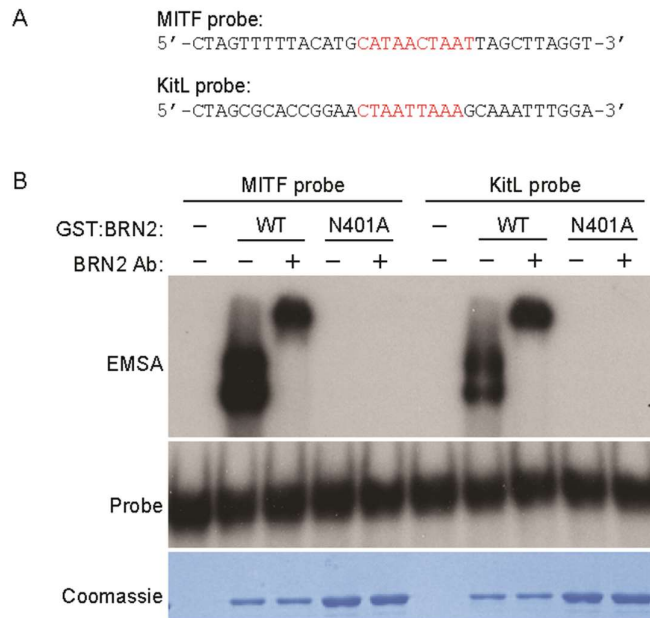


Figure 3.8.3 | N401A mutation in BRN2 inhibits DNA binding in vitro

(A) Full sequence of DNA probes used in EMSA. (B) Representative image of electrophoretic mobility shift assay (EMSA) of purified GST-BRN2^{WT} or GST-BRN2^{N401A} incubated for 30 mins with radiolabelled DNA probes of validated BRN2 binding motifs. Addition of BRN2 Antibody shows super-shift demonstrating specificity. Coomassie staining of SDS-Page gel run in parallel to show protein loading.

Non-DNA binding by the BRN2^{N401A} mutant was confirmed in a mammalian system by transient co-overexpression in 501mel cells of BRN2^{WT}_FLAG or BRN2^{N401A}_FLAG with a well-characterised BRN2-binding site_Luciferase reporter derived from the MITF promoter (Goodall et al. 2008). BRN2^{WT}_FLAG represses Luciferase expression by more than 90%. However, in the presence of BRN2^{N401A}_FLAG, Luciferase expression is only reduced by around 20% (Fig. 3.8.4 a,b). Together these show that mutation of Asparagine 401 to Alanine (N401A) in the BRN2 POU homeodomain leads to impairment of sequence-specific DNA binding.

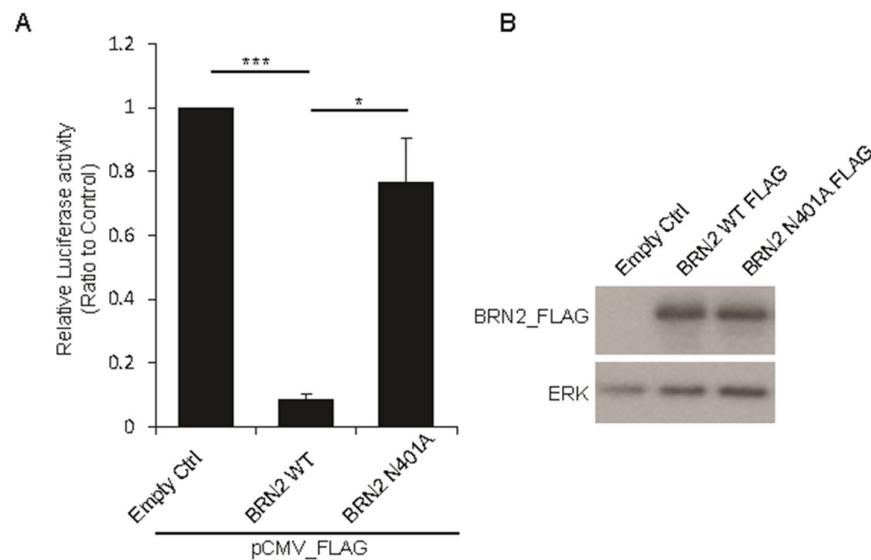


Figure 3.8.4 | N401A mutation in BRN2 fails to repress MITF promoter

(A) Luciferase activity of an MITF promoter luciferase reporter in 501mel cells co-transfected with Empty_FLAG, BRN2^{WT}_FLAG or BRN2^{N401A}_FLAG expression vectors. Results are expressed as mean $\pm$ SD of three biological replicates. Inter-sample comparison by unpaired Student's t-test (* $p < 0.05$; *** $p < 0.001$). (B) Western blot shows FLAG expression levels, ERK is used as a loading control.

Having created a non-DNA binding mutant, the question of whether BRN2 requires sequence-specific DNA binding for recruitment to sites of DNA damage could now be addressed. BRN2^{WT} and BRN2^{N401A} were cloned into an mCherry vector under the pCMV promoter so that live LMI could be carried out. Initially the aim was to co-express GFP-tagged BRN2^{WT} and mCherry-tagged BRN2^{N401A} so that the experiment could be carried out with both BRN2 constructs at the same time, allowing for direct comparison of stripe dynamics. However, in order to validate that the fluorescent tag didn't affect recruitment, the assay was carried out with GFP-BRN2^{WT} and mCherry-BRN2^{WT}. It quickly became apparent that there is a technical issue with the way in which GFP-tagged BRN2^{WT} was recruited compared to mCherry-BRN2^{WT}. Although mCherry BRN2^{WT} was recruited with very similar temporal dynamics to GFP-BRN2^{WT}, the intensity of the stripes was much less with the mCherry tag (Fig. 3.8.5a, quantified in b). It is not clear if this is an artefact of the microscope detection of mCherry vs GFP or the way in which the structure of the GFP and mCherry differ.

GFP-derived proteins originate from the jellyfish *Aequora victoria* (Morise et al. 1974). They have a β -barrel structure surrounding the chromophore. mCherry is a red fluorescent protein which is a monomeric derivative of DsRed which was extracted from the sea anemone *Dicosoma striata* (Shaner et al. 2004). As with all fluorescent proteins, mCherry also has a β -barrel structure surrounding the chromophore. However the structure of the β -barrel is distinct to that of GFP in that it is more elliptical in cross-section (Yarbrough et al. 2001). The structure of the β -barrel determines the maximum intensity of emission by the chromophore (Day and Davidson 2009). This could explain why the range of intensity of recruitment of GFP-BRN2 was four-fold greater than that of the mCherry-tagged BRN2 in the live LMI experiments as the intrinsic brightness of mCherry is only 50% that of EGFP (Day and Davidson 2009).

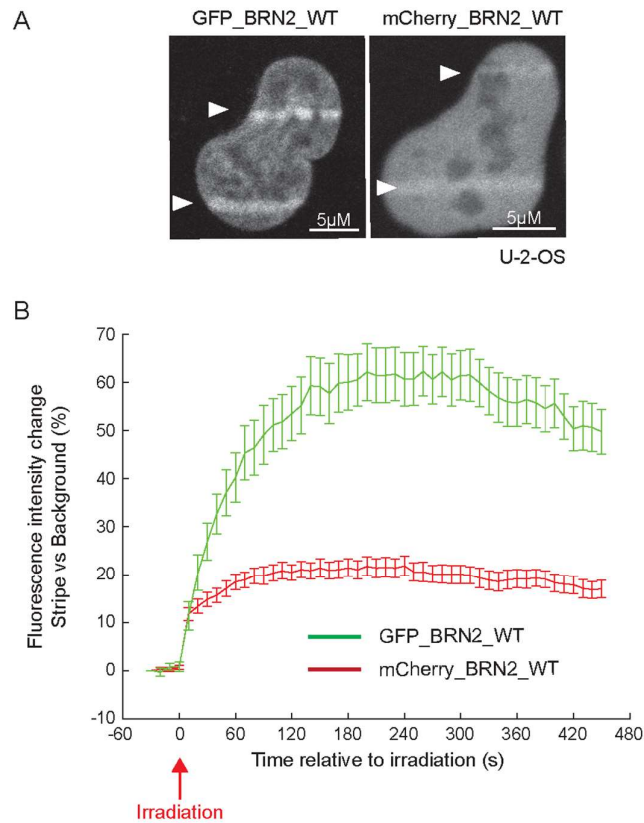


Figure 3.8.5 | Intensity of BRN2 recruitment is tag-dependent

(A) Still images of U-2-OS cells transiently transfected with either GFP BRN2^{WT} or mCherry_BRN2^{WT} and subjected to live LMI. White arrows indicate site of LMI. (B) Quantification of live imaging shown in (A). Data shown are the mean fluorescence intensity change of irradiated stripe vs background per cell expressed as mean $\pm$ SEM. Data are shown as mean percentage change in stripe intensity compared to the background expression. (N : GFP=17; mCherry= 36), curves compared by repeated measures ANOVA. *** $p < 0.0001$.

Whatever the underlying cause of this discrepancy, to compare WT and mutant we used the same fluorescent tag. mCherry-BRN2^{N401A} was transfected into U-2-OS cells and LMI with live imaging was carried out. Whilst the mCherry-BRN2^{WT} was again recruited to sites of DNA damage, at the peak of recruitment, 150s after damage, mCherry-BRN2^{N401A} was recruited four-fold less than mCherry-BRN2^{WT} (rmANOVA $p < 0.0001$; Fig. 3.8.6a, quantified in b). Although it should be noted that recruitment was not completely abolished, which may reflect that the mutant still retains some DNA binding ability or that DNA binding ability significantly enhances recruitment, rather than being a critical component of the mechanism. This demonstrates that BRN2 recruitment to sites of DNA damage, in addition to PARP, is at least in part dependent on its DNA binding ability. It may be that PARP indirectly affects recruitment through altering accessibility of the chromatin to DNA binding by BRN2.

To examine the impact of the non-DNA binding mutant on apoptosis, we generated FLAG-tagged BRN2^{WT} or BRN2^{N401A} stable expressing 501mel cell lines using a lentiviral system. The plasmid expresses mCherry fluorescent protein separated from the FLAG_BRN2 constructs by a P2A self-cleaving peptide, allowing a stoichiometric expression of the fluorescent protein and BRN2, and avoiding tag-induced localisation artefacts (Fig. 3.8.7a). Cell sorting by FACS based on mCherry expression was performed prior to analysis of other markers to ensure that we had equivalent levels of BRN2 constructs or the empty vector (Fig. 3.8.7b). FLAG_BRN2 expression levels were confirmed by WB in the sorted cell populations (Fig. 3.8.7c). To remove endogenous BRN2 and leave the FLAG-tagged exogenous proteins, the cell lines were treated with siBRN2 #2 which targets the 3' UTR (see Fig. 3.5.1). Endogenous BRN2 was at least partially knocked down, leaving exogenous BRN2-FLAG constructs;

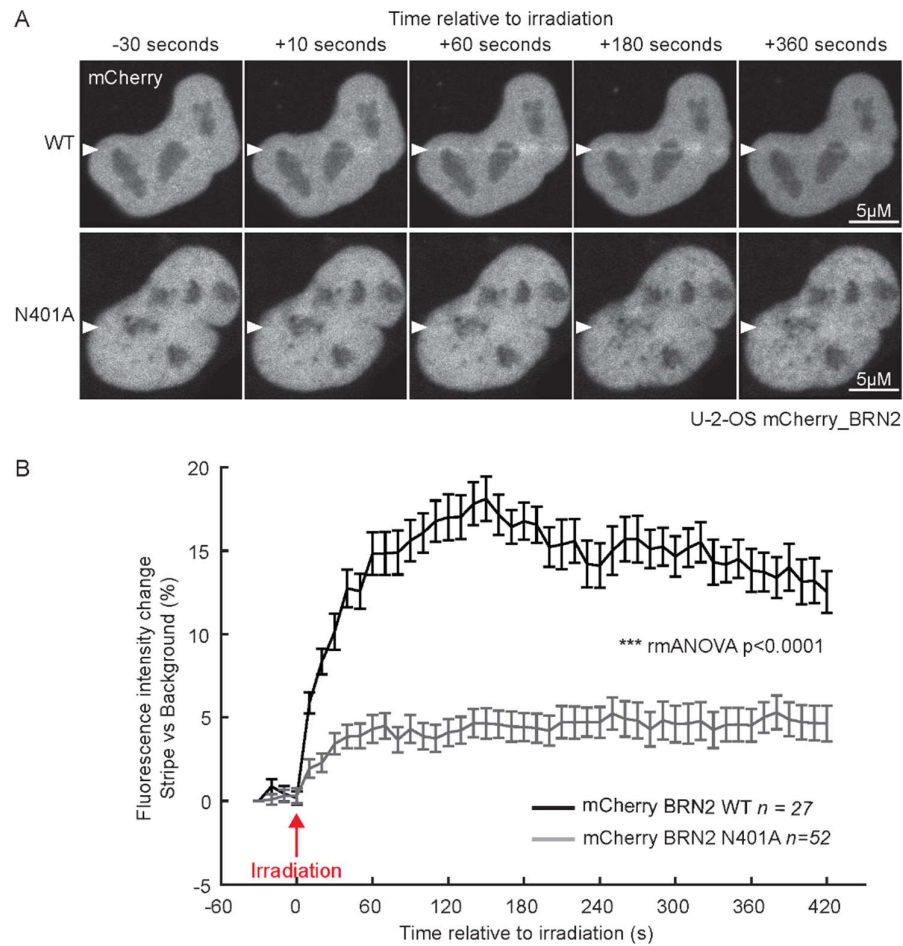


Figure 3.8.6 | N401A mutation in BRN2 inhibits recruitment to sites of damage

(A) Representative still images from live imaging of U-2-OS cells transiently transfected with mCherry_BRN2^{WT} or mCherry_BRN2^{N401A} expression vectors. Time (s) relative to irradiation. (B) Quantification of live imaging shown in (A). Data shown are the mean fluorescence intensity change of irradiated stripe vs background per cell expressed as mean $\pm$ SEM (N : BRN2^{WT}=27; BRN2^{N401A}=52), curves compared by repeated measures ANOVA. *** $p < 0.0001$.

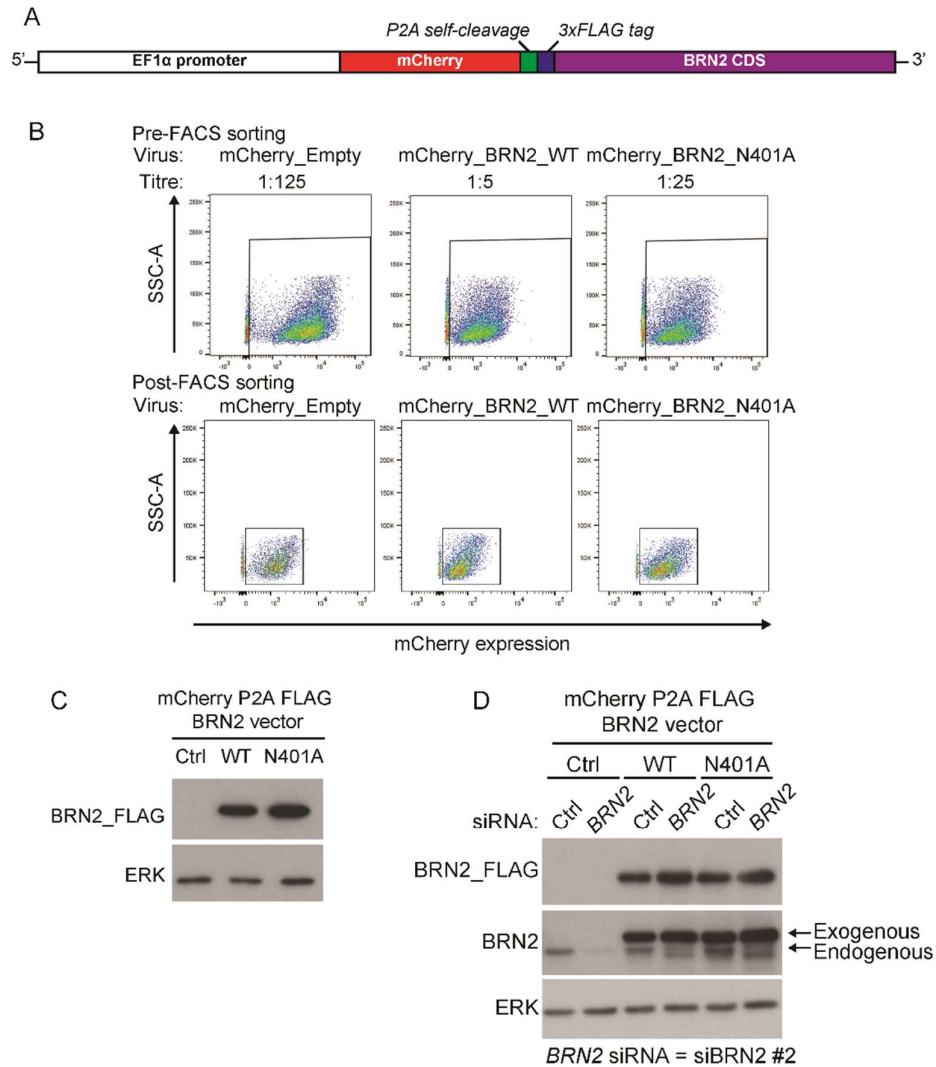


Figure 3.8.7 | Generation of a stable BRN2N401A cell line

(A) Scheme of lentiviral vector transfected into Phoenix cells to produce lentivirus (B) FACS plots showing 501mel cells stably infected with mCherry_P2A_FLAG-BRN2^{WT}, mCherry_P2A_FLAG-BRN2^{N401A} or mCherry_P2A_FLAG-Control virus. (Upper panel) Box = mCherry pos cells; Titre = dilution of lentivirus infected into 501mel cells. (Lower panel) mCherry expression levels post-FACS sorting showing narrower mCherry expression levels and 90-95% mCherry positivity. (C-D) Western blots of cell extracts from 501mel cells infected with mCherry_P2A_FLAG-Control, mCherry_P2A_FLAG-BRN2^{WT} and mCherry_P2A_FLAG-BRN2^{N401A} 501mel cells post-sorting; (C) BRN2_FLAG, ERK as loading control. (D) after endogenous BRN2 KD by siBRN2#2. BRN2_FLAG, endogenous BRN2, ERK loading control.

however, the fold change in endogenous BRN2 between siCtrl and siBRN2 was insignificant compared to the exogenous levels (Fig. 3.8.7d). Therefore, downstream assays were carried out without KD of endogenous BRN2.

The question to answer, was whether the non-DNA binding mutant, BRN2^{N401A}, could rescue the siBRN2 apoptosis phenotype as BRN2^{WT} overexpression had. It was not possible to assay for γ H2AX by FACS as previously because the permeabilisation step results in loss of the cytoplasmic mCherry and therefore we could not reliably sort a FLAG_BRN2 positive population. However, given that the γ H2AX staining levels are a proxy for apoptosis in the previous experiments, the more interesting downstream phenotype assay is apoptosis. The Cleaved caspase 3/7 assay is performed in live cells, therefore we could sort on mCherry expression first (Fig. 3.8.8a) It was confirmed that 24h after UVB treatment, BRN2^{WT} is able to decrease the percentage of apoptotic cells (Fig. 3.8.8b, quantified in c). BRN2^{N401A} then failed to rescue the cells from the apoptosis caused by UVB dependent DNA damage (Fig. 3.8.8b, quantified in c).

Together these data using the newly generated DNA non-binding BRN2 mutant (BRN2^{N401A}) indicate that BRN2 DNA-binding ability is necessary for both recruitment to sites of DNA damage and for protection from apoptosis following UVB-induced DNA damage.

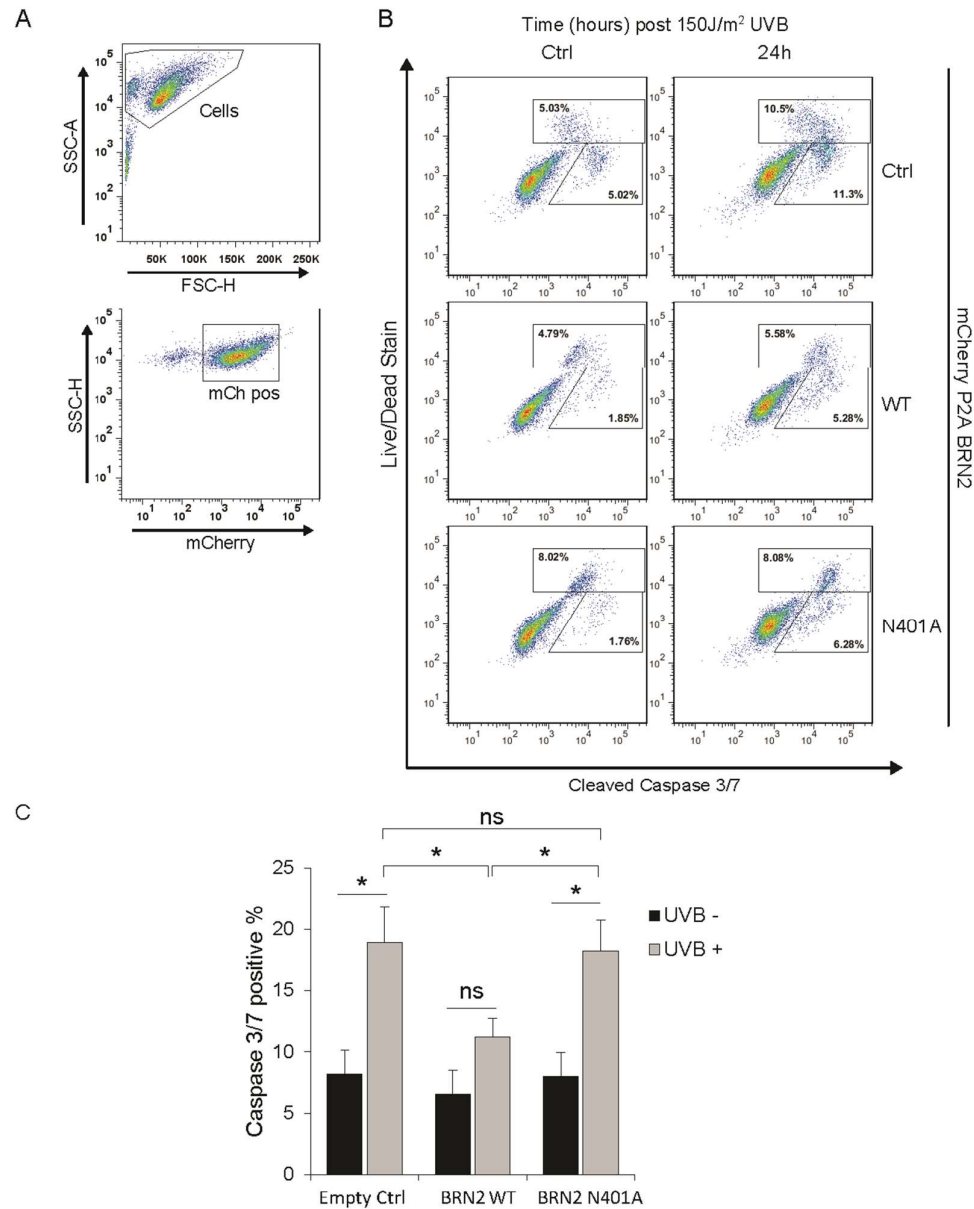


Figure 3.8.8 | BRN2N401A fails to rescue melanoma cells from UVB-induced apoptosis

(A-C) Apoptosis assay using cleaved caspase 3/7 in conjunction with 7-AAD live/dead stain to identify apoptotic population by FACS 24 h after UVB treatment in mCherry_P2A_FLAG-Control, mCherry_P2A_Flag-BRN2^{WT} and mCherry_P2A_Flag-BRN2^{N401A} 501mel cells. (A) Representative FACS plots of gating strategy to select mCherry positive population (B) Representative FACS plots of apoptosis assay; Upper box: double positive for Caspase3/7 and 7AAD; Lower box: Caspase 3/7 positive. Combined populations quantified in (C). Data presented are mean $\pm$ SD of at least three biological replicates. Intrasample comparison by paired Student's t-test ($*p < 0.05$).

3.9 | BRN2 Expression *in vivo* Correlates with Genomic Instability

It is clear from the data so far that BRN2 expression is positively correlated with protection of melanoma cells in culture from DNA damage-induced apoptosis. However, although it cannot be conclusively stated that BRN2 does not increase DNA damage repair, all experiments to establish differences in DNA lesion repair gave negative results. Indeed, there were no striking differences in DNA repair pathway gene expression analysed by RNA-Seq (not shown). This leads to the intriguing hypothesis that BRN2 assists cells to survive despite the presence of mutations; that BRN2 may be assisting cells to bypass DNA damage checkpoints therefore avoiding apoptosis despite the persistence of DNA lesions. This would, over time lead to populations of cells with an increased number of mutations and therefore increased likelihood of genomic instability. If this were the case it would contribute to our understanding of how melanoma cells accrue genetic mutations yet continue to survive, and indeed why they are so resistant to traditional DNA damaging chemotherapeutic agents.

To address this hypothesis our collaborator (Daniela Robles Espinoza, Sanger Institute) modelled the relationship between expected somatic SNV burden and BRN2 expression levels using data from the TCGA melanoma cohort (Fig. 3.9.1). As expected in melanoma the C>T transition was the highest frequency SNV, denoting both UV and ageing signatures (Hodis et al. 2012; Alexandrov et al. 2013). We observed evidence for a positive association between BRN2 expression and somatic SNV burden for all six mutation classes (C>A, C>G, C>T, T>A, T>C, T>G). Negative binomial regression models adjusted for all available clinical variables showed that BRN2 expression level is a statistically significant predictor of SNV burden for all mutation classes ($P < 0.05$ after Benjamini-Hochberg correction for six mutation classes), with the most dramatic correlation being with C>T transition. This means that there is a positive correlation

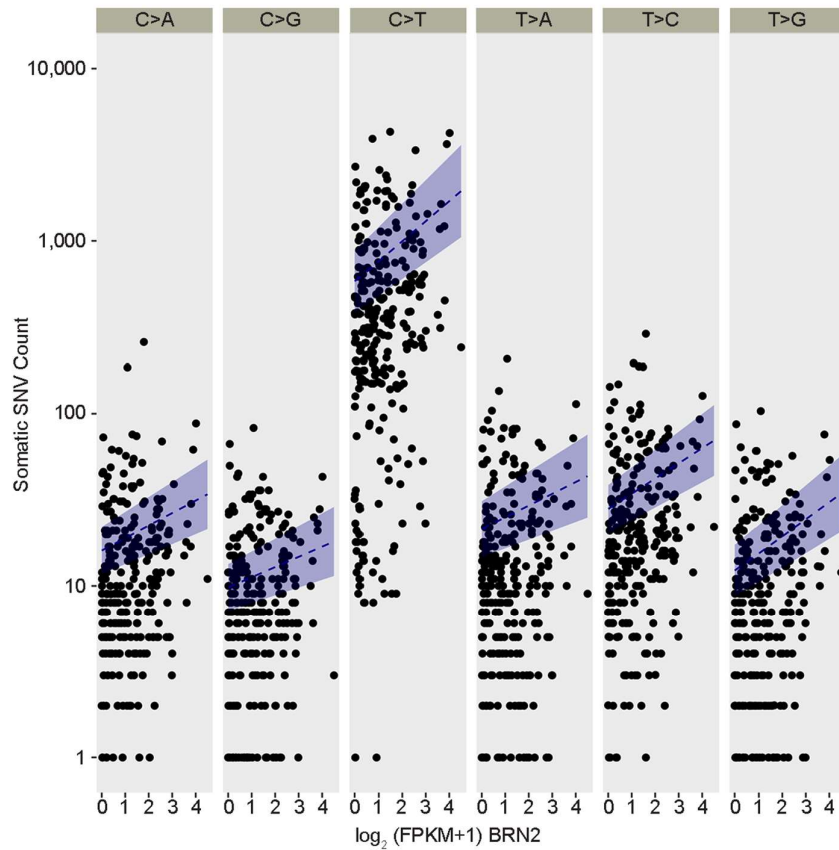


Figure 3.9.1 | BRN2 expression in tumours correlates positively with increased mutational burden

SNV counts are plotted against $\log_2(\text{FPKM}+1)$ BRN2 values. For each SNV class, the blue dashed line (and ribbon) charts the predicted mean mutation burden (and 95% confidence interval) of a patient with the most common constellation of values for clinical variables (see Methods) as the $\log_2(\text{FPKM}+1)$ BRN2 level increases from the minimum to the maximum observed in the TCGA dataset, with all other clinical variables held fixed. Data by Daniela Robles-Espinoza, Sanger Institute, Cambridge.

between BRN2 expression and mutational burden, particularly UVB-associated mutations. This supports the hypothesis that BRN2 does not help to repair UVB-induced damage, but rather helps melanoma cells to survive despite damage. The specificity of BRN2 in this mechanism was supported by the lack of a similar correlation when SNV frequency was analysed in conjunction other POU domain factors, BRN3a (POU4f1) (Fig. 3.9.2a), BRN3b (POU4f2) (Fig. 3.9.2b), which was poorly expressed in most melanomas. Decoupling of BRN2 function from MITF pathways was indicated by the lack of correlation with a gene set comprising well-characterised MITF-target genes as a surrogate marker for MITF activity (Fig. 3.9.2c). Although direct comparisons are difficult, the magnitude of the correlation with somatic SNV burden appears to be similar between BRN2 and that recently reported for MC1R RHC variants (Robles-Espinoza et al. 2016) that are important risk factors for melanoma.

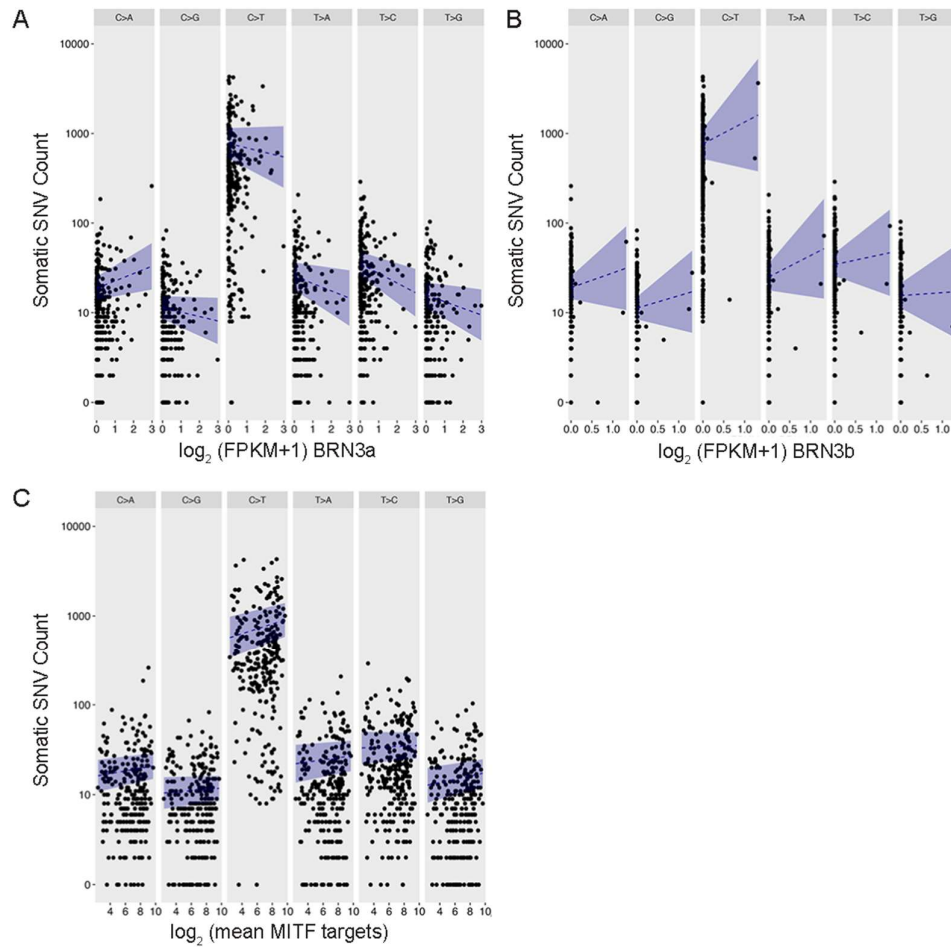


Figure 3.9.2 | Correlation of SNV burden to BRN2 is specific

SNV counts were plotted against $\log_2(\text{FPKM}+1)$ values for BRN3a (A), BRN3b (B) or the average expression of a well-characterised MITF target gene set (mean FPKM counts) (C). For each SNV class, the blue dashed line (and ribbon) charts the predicted mean mutation burden (and 95% confidence interval) of a patient with the most common constellation of values for clinical variables as the $\log_2(\text{FPKM}+1)$ BRN3a, BRN3b, or MITF-target mean gene set expression value increases from the minimum to the maximum observed in the TCGA dataset, with all other clinical variables held fixed. In these models and for this prediction, the value for the BRN2 expression variable was set to 1.023459, the median $\log_2(\text{FPKM}+1)$. Data by Daniela Robles-Espinoza, Sanger Institute, Cambridge.

Chapter 4 | Results

PTM by p38 SAPK modifies BRN2 activity

4.1 | Introduction

The data to this point demonstrate a role for BRN2 in the DNA damage response. BRN2 binds to DNA damage response proteins and is recruited to sites of DNA damage in a PARP-dependent manner, facilitated by its ability to bind DNA. The presence of BRN2 appears to protect melanoma cells from apoptosis following DNA damage, with little evidence of an increased rate of efficiency of DNA damage repair itself. These observations led to the hypothesis that BRN2 plays a role in suppressing apoptosis in the face of persistent DNA damage. The *in vivo* corollary of this is realised in the strikingly positive correlation of BRN2 expression with mutational burden in melanomas.

However, so far the evidence for BRN2 DDR-related activity is circumstantial. There remain significant questions regarding the molecular mechanism by which BRN2 effects the protection from apoptosis, and what its role at the site of DNA damage is. Although there is an upregulation of apoptosis-related genes following DNA damage in BRN2 KD cells, suggesting a repressive role of BRN2 on the expression of these genes, there is no evidence that this modulation of gene expression is directly controlled by BRN2. One way in which specificity of TF binding to target genes is modulated, is by post-translational modification (PTM) of the TF to alter its conformation, subcellular localisation, or ability to bind either co-factors or DNA binding sites. As described in the introduction, phosphorylation is a well-established PTM for altering the function of POU domain proteins. In this chapter we investigate the hypothesis that UVB-induced phosphorylation of BRN2 modifies its behaviour.

4.2 | BRN2 Can Be Phosphorylated by Stress-Activated Protein Kinases

The apoptosis-rescue phenotype was identified in response to UVB irradiation that activates stress pathways which include the stress-associated protein kinases such as JNK and p38 SAPK, in addition to MAPKs such as ERK. Fig. 4.2.1 shows the early and late effects of UVB on p38 SAPK phosphorylation in the 501mel cell line. As shown previously, BRN2 expression is not altered by UVB irradiation. p38 phosphorylation in response to UVB peaks at 4 hours, and diminishes by 24 hours. Induction of the DDR is demonstrated by phosphorylation of the kinase ATM, and its downstream target Histone H2AX (γ H2AX) (Fig. 4.2.1).

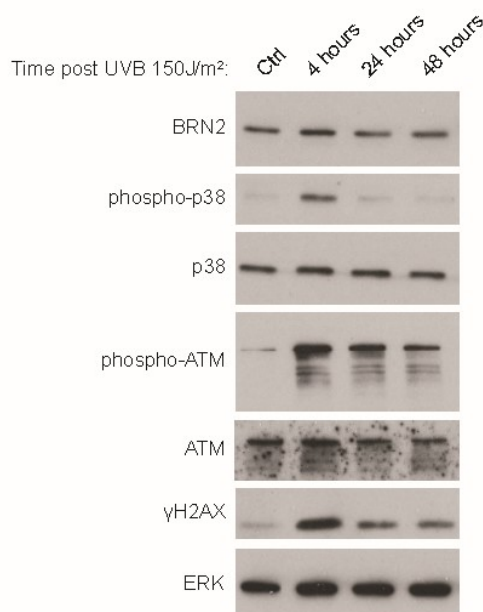


Figure 4.2.1 | UVB irradiation activates DNA damage and stress response pathways

Western blot of 501mel cells treated with 150 J/m² UVB over a time-course as indicated. Antibodies against BRN2, phospho-p38, p38, phospho-ATM, ATM, γ H2AX. Erk is used as a loading control

To understand the effect that any potential phosphorylation might have on BRN2 function, it was important to establish if and where phosphorylation occurs. MAPK/SAPK families phosphorylate serine/threonine proline (S/TP) residues. The more conserved the residue across BRN2 orthologues, the more critical the role of the phosphorylation residue is likely to be. For ease of understanding, all residues will be referred to by their position in the *M. musculus* sequence as mouse cDNA was used experimentally for all mutational analyses. Sequence alignment of BRN2 from *H. sapiens*, *M. musculus*, *B. taurus*, *D. rerio* and *X. laevis* shows almost complete conservation of the POU domain and short C-terminal (*M. musculus* 271-445) (Fig. 4.2.2). Additionally, the N terminal region (*M. musculus* 1-270) in the three mammalian species is almost completely conserved, with the exception of the shortening of the polyglutamine tract (*M. musculus* 129-151) by two and seven glutamine residues between *M. musculus* and *H. sapiens* and *B. taurus* respectively. This is the only deviation from full homology between *M. musculus* and *H. sapiens*, which means that there is not likely to be any significant consequence of using the mouse cDNA rather than the human sequence experimentally. The *D. rerio* and *X. laevis* N-terminal is very similar to the mammalian sequence but is lacking both the polyglycine and polyglutamine tracts and has a reduced proline rich region (Fig. 4.2.2).

In mammalian BRN2 there are seven S/TP residues, providing potential target phosphorylation motifs for cyclin dependent kinases as well as ERK, JNK and p38 stress-activated kinases: serines 91 and 96 (S91S, S96) in the N-terminal between the polyglycine and polyglutamine stretches; threonine 266 (T266) in the conserved region immediately preceding the POU Specific domain (POU_S); serine 343 (S343) in the linker domain; and threonines 415, 434 and 442 in the C-terminal domain (T415, T434, T442). Six of these are conserved beyond the mammalian class, with S91 not conserved in

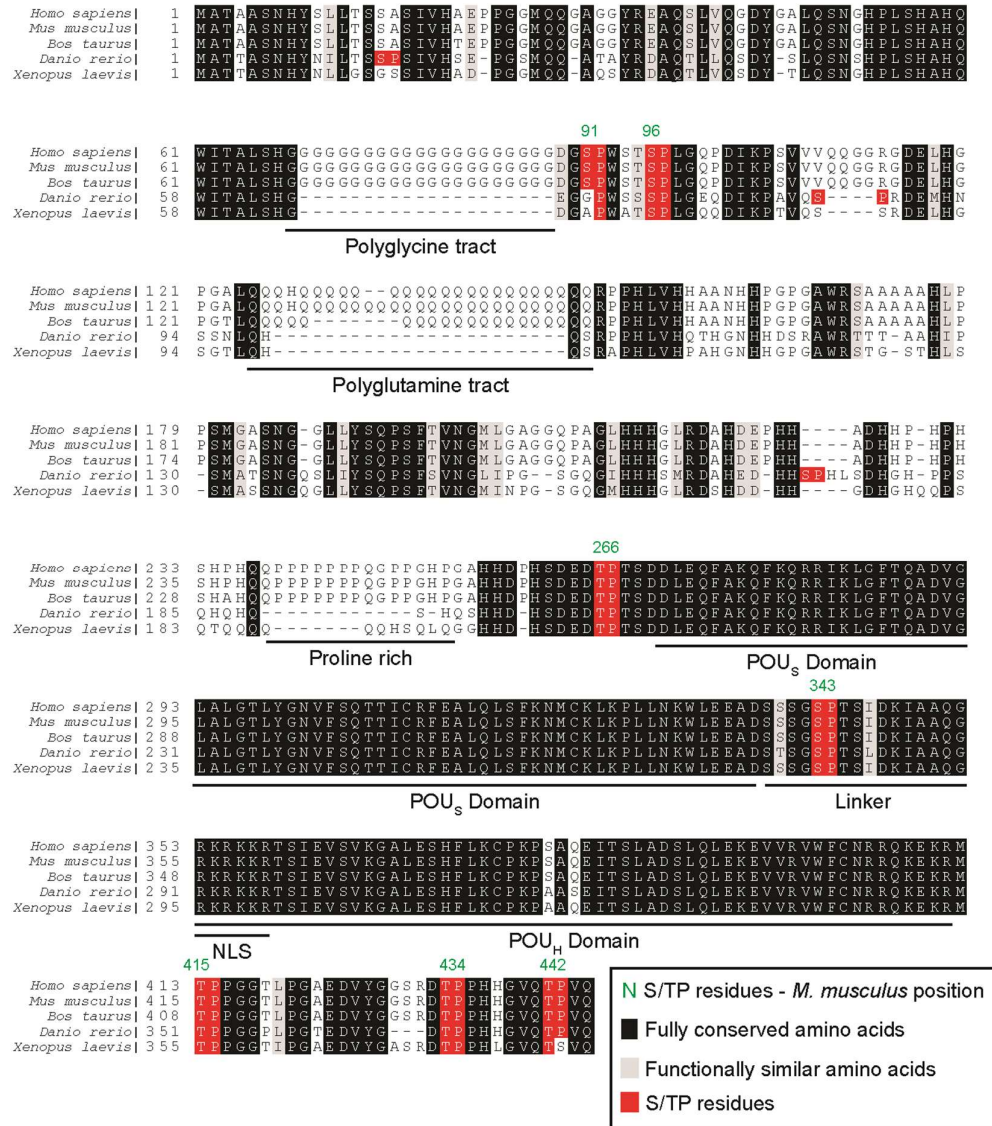


Figure 4.2.2 | Sequence homology between BRN2 orthologues

Schematic showing sequence alignment of BRN2 orthologues in *H. sapiens*, *M. musculus*, *B. taurus*, *D. rerio* and *X. laevis*. Numbering of S/TP sites refers to the position of the S/T in the *M. musculus* sequence. Shading: Red=S/TP sites; Black =Identical amino acids; Light Grey = similar amino acids; POU_S= POU specific domain; POU_H= POU homeodomain; NLS = nuclear localisation signal.

D. rerio and *X. laevis*. The threonine corresponding to T442 in *X. laevis* is conserved, however it is followed by a serine, rather than a proline, meaning that it is still a potential phospho-site. In addition, there are three extra, non-conserved SP sites in the N-terminal region of *D. rerio* only (Fig. 4.2.2).

Alanine is a non-polar amino acid with a small side-chain which consists of a single methyl group. It is similar to serine and threonine but lacks the polar hydroxyl group which is hydrolysed by ATP to add a phosphate group and is therefore not phosphorylatable. Although it is one carbon smaller than threonine it has been used effectively as a non-phosphorylatable replacement for both amino acids. To establish which of the phosphorylation S/TP sites can be phosphorylated by candidate kinases, *in vitro* kinase assays (IVKAs) were used. BRN2 proteins were expressed in bacteria and purified for use in IVKA using a GST-tag to allow purification of the expressed proteins. The GST sequence has an SP site at S2, which if phosphorylated by the candidate kinases would make interpretation of the assay difficult, therefore prior to cloning of BRN2 cDNA into the vector, GST S2 was mutated to alanine by site-directed mutagenesis (data not shown). To narrow down which residues might be phosphorylated, BRN2 WT and truncation mutants were cloned into the pGEX_GST_MCS vector (Fig. 4.2.3a). In addition to the N-term, Δ N and Δ C proteins used in the AP-MS (see Fig. 3.4.1), an additional two mutants were used. Δ NC comprises the POU domain alone with slight overhangs at either end (259-413); Δ NLS which has a stretch of five amino acids that are implicated in nuclear localisation of BRN2 deleted by SDM at the start of the POU homeodomain (Δ 355-360) (Sock et al. 1996). In the IVKA, Δ NLS should behave as full length BRN2 as it is a cell-free system (Fig. 4.2.3a).

As the truncation mutants would not provide sufficient resolution to identify any individual phospho sites, and also because the lack of a full-length sequence would affect

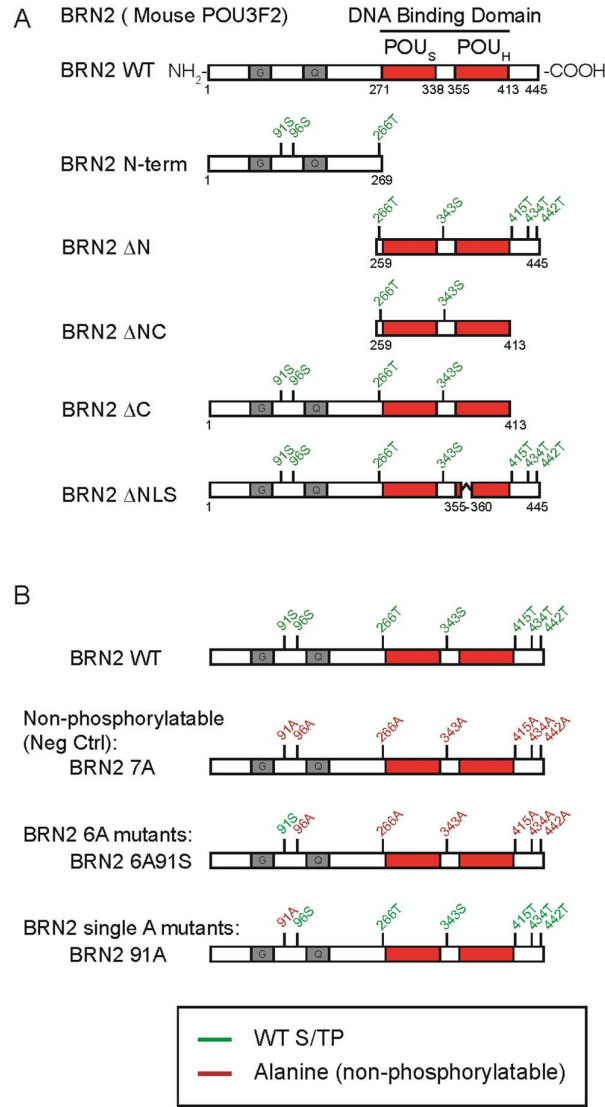


Figure 4.2.3 | Schemata of BRN2 mutants to be used in IVKAs

(A) Full length mouse BRN2 (POU3F2) (1-445). G=polyglycine tract; Q=polyglutamine tract; POU_S= POU specific domain; POU_H= POU homeodomain. Truncation mutants (N-term 1-269; ΔN 259-445; ΔNC 259-413; ΔC 1-413; ΔNLS 1-445Δ355-360). (B) Phospho-site mutant schemata. BRN2 WT with serine (S) and threonine (T) residues shown; BRN2 7A; BRN2 6A mutants – 6A 91S as example; BRN2 single A mutants – BRN2 91A as example. Green = WT residue; Red = mutated residue.

the protein's tertiary structure, each potential phosphoacceptor site was mutated from serine or threonine to alanine using site-directed mutagenesis. A negative control was generated by mutagenesis of all seven S/TP sites (7A), then seven proteins which were only phosphorylatable on one site were generated (6A xS) and finally seven proteins where only one residue had been mutated to alanine were used (xA) (Fig. 4.2.3b).

Purified GST or the C terminal domain of Pol II (Pol II CTD) were prepared as negative and positive controls respectively. The Pol II CTD consists of a series of tandem repeats of the amino acid sequence YSPTSPS. *in vivo* it is phosphorylated in a spatiotemporally regulated manner by a range of CDKs which results in modification of transcription through the cell cycle (Hsin et al. 2014; Heidemann et al. 2013). *in vitro* it can be used as a positive control for phosphorylation by most S/TP-specific kinases. IVKAs were performed by incubating the 21 purified GST_BRN2 proteins or controls, with $\gamma^{32}\text{P}$ -ATP and candidate kinases before analysis by PAGE. The first IVKA was carried out with p38 SAPK, a kinase activated by UV irradiation. The Coomassie stained gel shows protein expression (Fig. 4.2.4a, bottom panel) whilst the upper panel shows the radiograph with $\gamma^{32}\text{P}$ -ATP generated opacities (Fig. 4.2.4a, upper panel). The control proteins behaved as expected, GST was not phosphorylated, whilst the Pol II CTD was heavily phosphorylated. p38 SAPK phosphorylated BRN2 WT and every mutant except ΔNC , which indicated that p38 can phosphorylate both the N and C terminal regions of BRN2 (Fig. 4.2.4b). This means that p38 could phosphorylate anywhere within the N and C terminal domains and therefore only the S/TP motif within the POU domain (S343) could be excluded.

Therefore, the point mutants were analysed (Fig. 4.2.5a). Importantly the 7A mutant was clearly not phosphorylated, validating its use as a negative control. The 6A

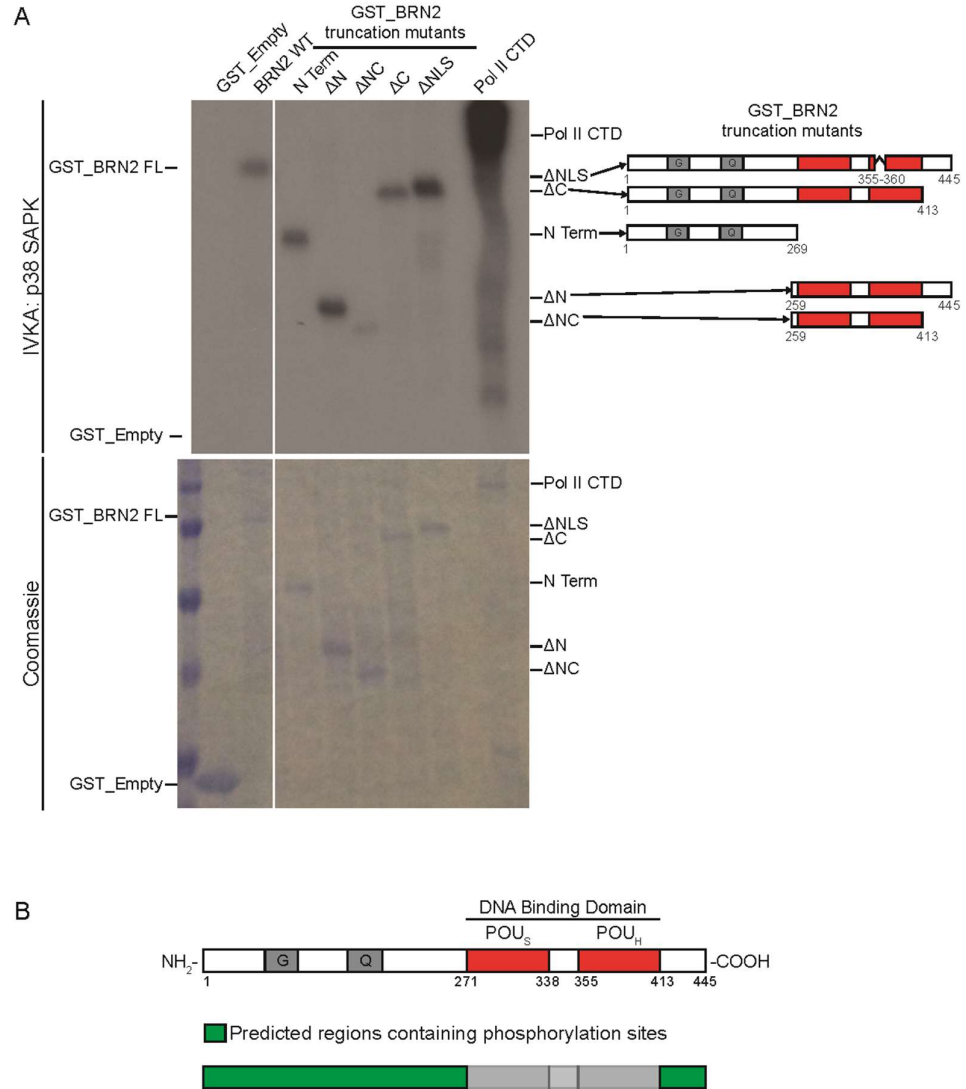


Figure 4.2.4 | p38 can phosphorylate BRN2

(A) (Upper panel) IVKA of purified GST_BRN2 WT or truncation mutants incubated with $\gamma^{32}\text{P}$ -ATP and p38 α /SAPK. Negative control - Empty_GST; Positive control - Pol II CTD = polymerase II C-terminal domain. Schemata of truncation mutants shown to side for clarity of interpretation. (Lower panel) Coomassie staining of the IVKA gel. (B) Scheme showing interpretation of the IVKA with region of BRN2 where p38 can phosphorylate highlighted in green. BRN2 WT shown for reference.

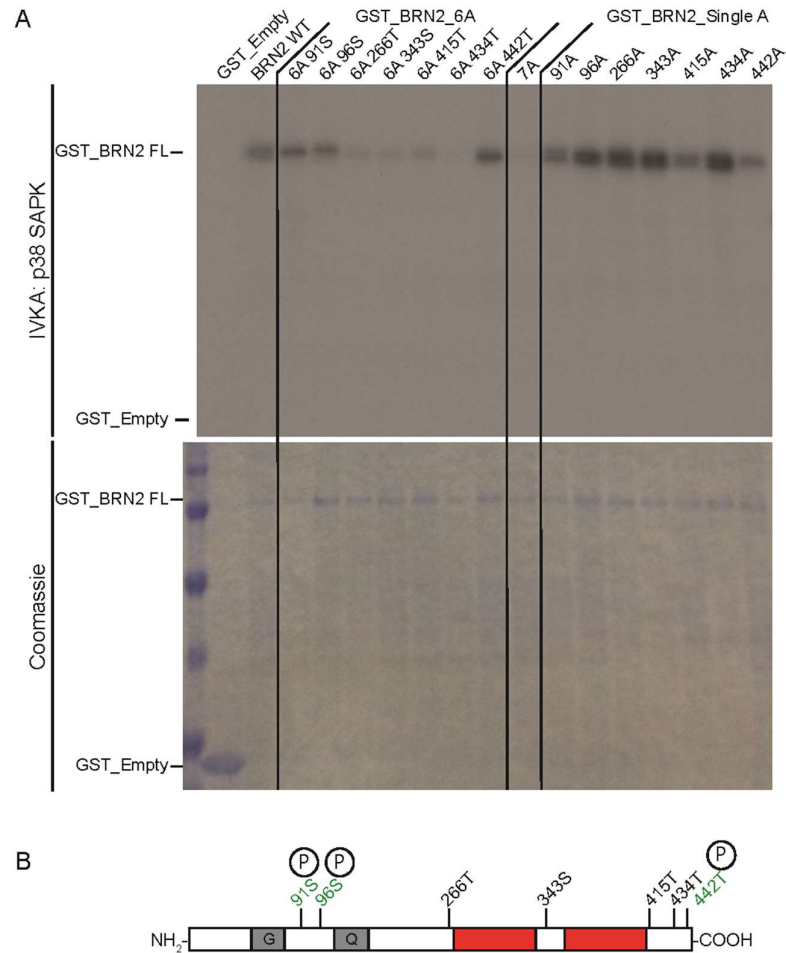


Figure 4.2.5 | p38 can phosphorylate residues 91S, 96S and 442T

(A) (Upper panel) IVKA of purified GST_BRN2 WT, 6A or single A mutants incubated with $\gamma^{32}\text{P}$ -ATP and p38 α SAPK. Negative control - Empty_GST; Positive control - Pol II CTD = polymerase II C-terminal domain. (Lower panel) Coomassie staining of the IVKA gel. (B) Scheme showing interpretation of the IVKA with S/TP residues where p38 can phosphorylate highlighted in green.

mutants clearly show that p38SAPK can phosphorylate BRN2 residues 91S, 96S and 442T (Fig. 4.2.5b). The single alanine mutants show a corresponding reduction in phosphorylation when 91A and 442A mutations are present, although the 96A mutant is still phosphorylated efficiently. Mutation of T415 to A (415A) appears to reduce phosphorylation of BRN2, although T415 itself does not appear to be phosphorylated in the 6A 415T mutant (Fig. 4.2.5a).

The IVKA was repeated with JNK (Figs. 4.2.6, 4.2.7) and ERK (Fig. 4.2.8). The phosphorylation pattern of BRN2 following incubation with JNK2 α is even clearer than the p38 experiment. Again, the GST control was not phosphorylated whilst the Pol II CTD positive control was phosphorylated (Fig. 4.2.6a). Full length BRN2 WT was phosphorylated and Δ NLS had similar phosphorylation signal to WT. Strikingly BRN2 Δ N was very strongly phosphorylated whilst there was complete absence of signal from N Term and Δ C. There was a faint background signal from Δ NC, although on Coomassie staining, there was a heavier protein load.

When taken together, these phosphorylation patterns indicate that JNK is phosphorylating a residue in the C-terminal region (T415, T434 or T442) (Fig. 4.2.6b). To pinpoint the residue(s) involved, the point mutants were then analysed (Fig. 4.2.7a). Here it was clear that the only 6A mutant with a phosphorylation signal was 6A 442T. Similarly, the only single A mutant with no phosphorylation signal was 442A. This indicates that JNK can only phosphorylate the T442 (Fig. 4.2.7b).

Finally, the IVKA was performed using ERK (Fig. 4.2.8a). As previously, GST was not phosphorylated and Pol II CTD was very strongly phosphorylated. However, it is very difficult to interpret the truncation mutants; there is some signal from all five truncation mutants, suggesting they are all phosphorylated and therefore there are

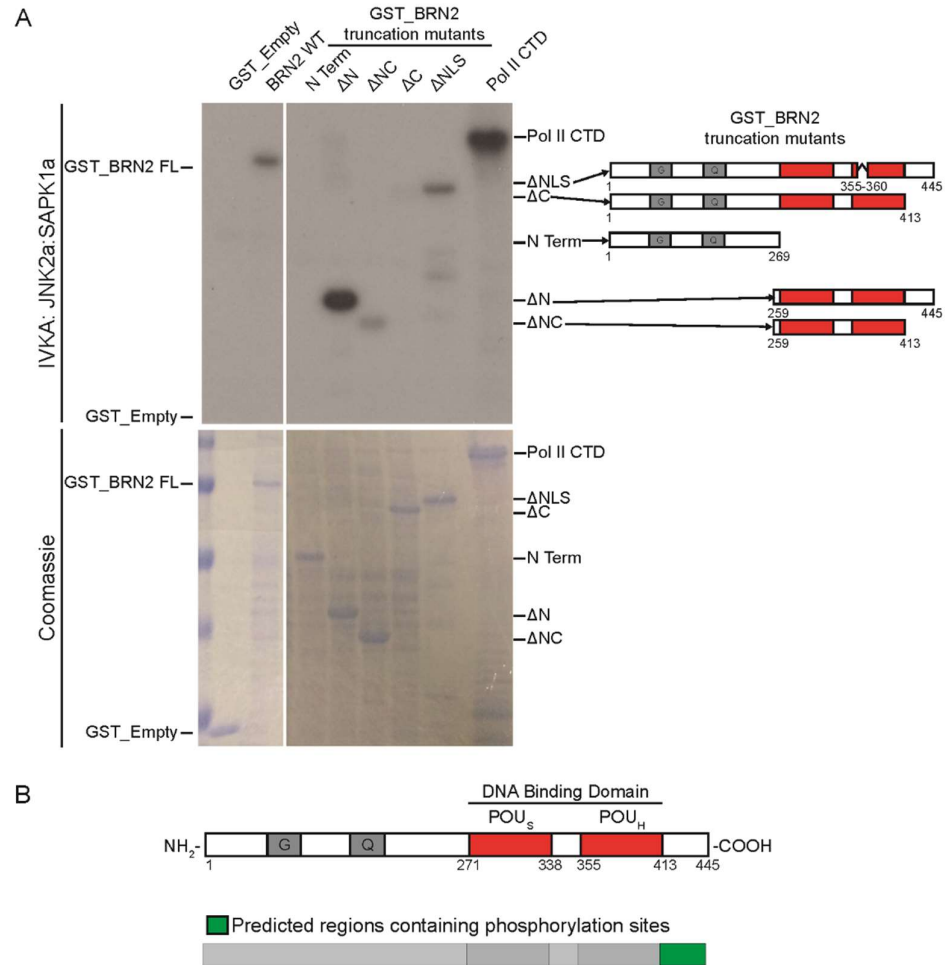


Figure 4.2.6 | JNK can phosphorylate BRN2

(A) (Upper panel) IVKA of purified GST_BRN2 WT or truncation mutants incubated with $\gamma^{32}\text{P}$ -ATP and JNK2 α :SAPK1 α . Negative control - Empty_GST; Positive control - Pol II CTD = polymerase II C-terminal domain. Schemata of truncation mutants shown to side for clarity of interpretation. (Lower panel) Coomassie staining of the IVKA gel. (B) Scheme showing interpretation of the IVKA with the region of BRN2 where JNK can phosphorylate highlighted in green. BRN2 WT shown for reference.

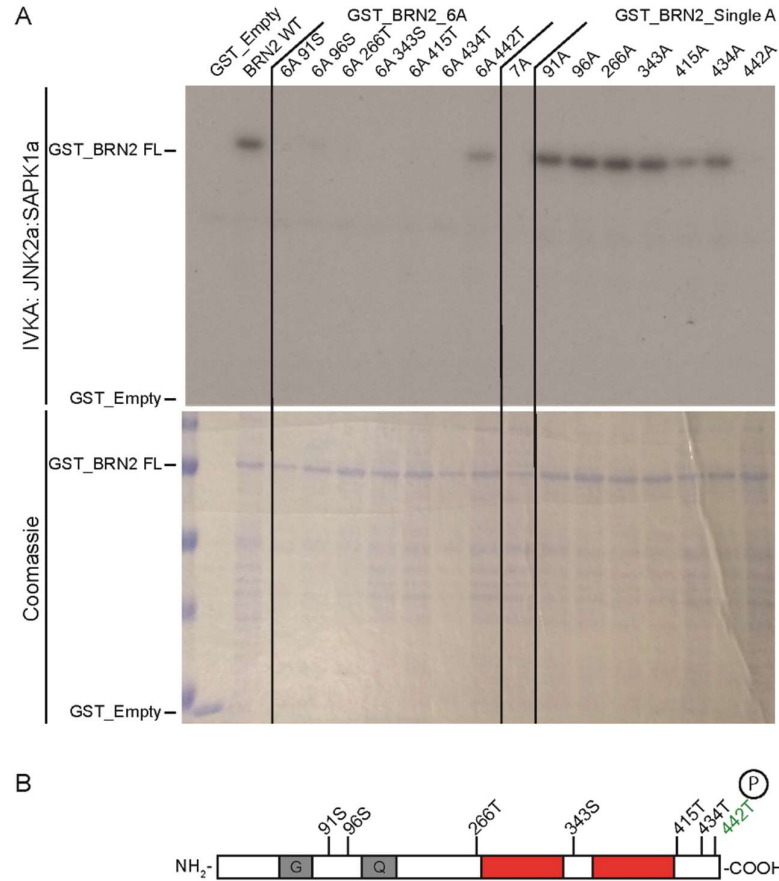


Figure 4.2.7 | JNK can phosphorylate residue 442T

(A) (Upper panel) IVKA of purified GST_BRN2 WT, 6A or single A mutants incubated with $\gamma^{32}\text{P}$ -ATP and JNK2 α :SAPK1 α . Negative control - Empty_GST; Positive control - Pol II CTD = polymerase II C-terminal domain. (Lower panel) Coomassie staining of IVKA gel. (B) Scheme showing interpretation of the IVKA with the S/TP residue where JNK can phosphorylate highlighted in green.

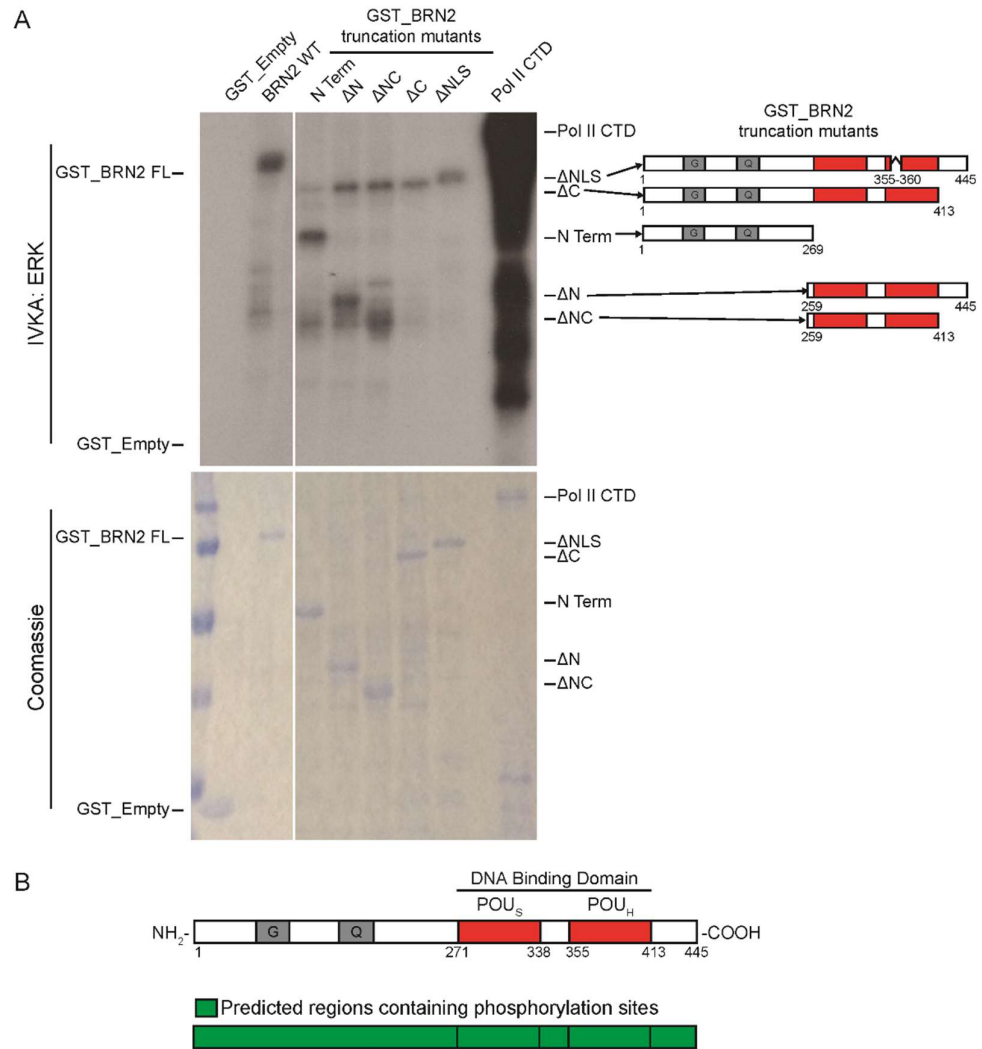


Figure 4.2.8 | ERK can phosphorylate BRN2

(A) (Upper panel) IVKA of purified GST_BRN2^{WT} or truncation mutants incubated with $\gamma^{32}\text{P}$ -ATP and ERK. Negative control - Empty_GST; Positive control - Pol II CTD = polymerase II C-terminal domain. Schemata of truncation mutants shown to side for clarity of interpretation. (Lower panel) Coomassie staining of the IVKA gel. (B) Scheme showing interpretation of the IVKA with the region of BRN2 where ERK can phosphorylate highlighted in green. BRN2 WT shown for reference.

multiple ERK phosphorylation sites (Fig. 4.2.8b). However, there is also a non-specific band which runs at a very similar size to BRN2 WT (Fig. 4.2.9). Therefore, it is not possible to interpret the point mutant IVKA as the non-specific band cannot be distinguished reliably from the GST_BRN2_WT band.

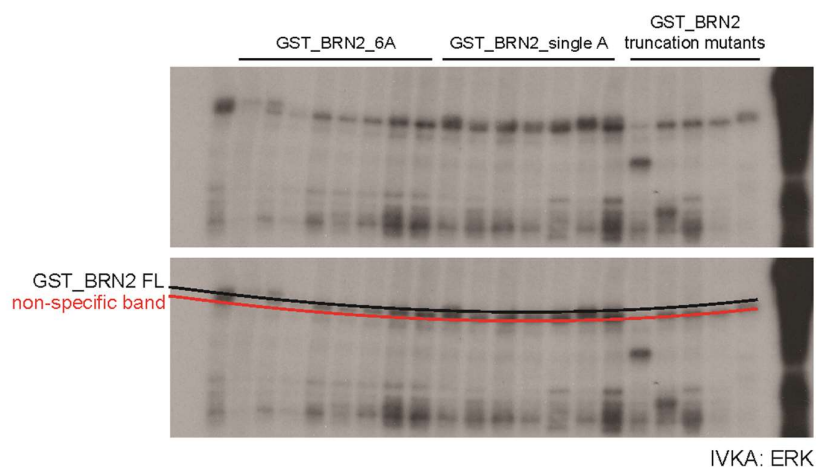


Figure 4.2.9 | Non-specific band in ERK IVKA impairs interpretation

(Upper panel) Full image of an ERK IVKA demonstrating non-specific band (lower panel) in (red) and BRN2 full length (black)

4.3 | p38 Activation Results in BRN2 Phosphorylation *in cellulo*

The IVKA data presented above in section 4.2 shows that BRN2 can be phosphorylated by p38, JNK and ERK *in vitro*. However, it is important to show that this phosphorylation event occurs in cells and that there is a mechanistic or phenotypic effect. We focussed on p38 since we did not have a conclusive answer from the IVKA data of where ERK phosphorylates BRN2, and the tools available for investigating the role of JNK were sub-optimal. The phospho-JNK antibody produces many non-specific bands and the inhibitor has a strong yellow colour in solution which disrupted the live cell imaging experiments. The question of JNK's role remains open and an interesting avenue for future work but is beyond the scope of this thesis.

The first question to answer was whether p38 phosphorylates BRN2 *in cellulo*. This could be addressed initially by WB analysis of the electrophoresis pattern of BRN2 species, however the gold standard for establishing the presence of a phosphorylation event would be phospho-mass spectrometry. The UVB assays had previously been conducted in a chamber with a UVB bulb, optimised for 60 mm dishes. Although it was possible to use a bigger culture dish in the chamber, it was less reliable in terms of ensuring uniform application of UVB. Therefore, other modalities of triggering p38 activation were trialled. p38 SAPK is activated in response to multiple stressors, including UVB. However, p38 is also a key component of the cellular response to reactive oxygen species (ROS), either endogenously derived, or as the product of exogenous stress, including UV irradiation. The use of H₂O₂ as a ROS generator reliably activated p38. A dose titration in 501mel cells using a 30-minute treatment time established an appropriate dose of 2.5 mM, this induced p38 phosphorylation without significant cell death (Fig. 4.3.1a). Using 2.5 mM H₂O₂, a time course was then conducted. This shows phosphorylation of both p38 and ERK after 15 minutes of treatment with no notable

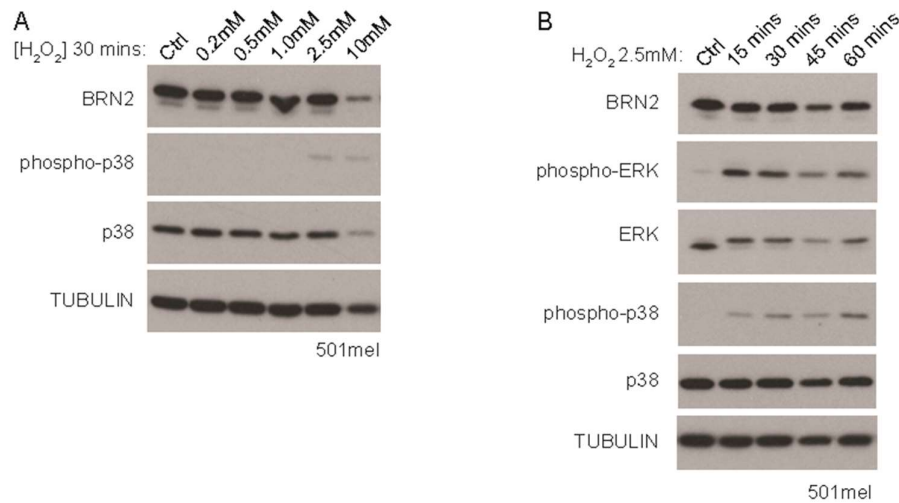


Figure 4.3.1 | Optimisation of H_2O_2 as an activator of the p38 pathway

(A) Western blot of 501mel cells treated with increasing concentrations of H_2O_2 from 0.2mM to 10mM for 30 minutes. Tubulin is used as a loading control. (B) Western blot of 501mel cells treated with 2.5mM H_2O_2 over a timecourse from 15 to 60 minutes. Tubulin is used as a loading control.

increase in kinase phosphorylation with extended activation time (Fig. 4.3.1b). For subsequent experiments, cells were treated with 2.5 mM H_2O_2 for 30 minutes, cells were washed with PBS and then incubated in normal media for the time courses indicated.

Phosphorylation events, particularly transient events, are often present at very low stoichiometry. In a proteome-wide analysis of phosphorylation stoichiometry in NSCLC cells, <15% of MAPK targets had high phosphorylation stoichiometry (Tsai et al. 2015). Therefore, it can be difficult to identify the phosphorylated population of a protein from a cellular extract. Experimental options include the use of Phos-tag™ gels which preferentially retain phosphorylated species by manganese chelation, delaying the

migration of the phosphorylated form thus increasing the migration difference between non-phosphorylated and phosphorylated species in an SDS-PAGE system (Kinoshita et al. 2006, 2015). Alternatively, phospho-enrichment of the samples by affinity chromatography using a PhosphoProtein Purification Kit (Qiagen 37101) can enrich the phosphorylated species from a whole cell extract (Dunn et al. 2010). Although BRN2 was retained in the phospho-enriched sample, it was also present in unstimulated cells, which demonstrates that BRN2 is phosphorylated at baseline, but does not help to establish if p38 is responsible for this (data not shown).

Next, enhancement of p38 activation in the cell model was developed. To do this, a p38_FLAG plasmid under the pCMV promoter was transiently overexpressed in stable 501mel_BRN2 WT_FLAG cell lines. With the addition of H₂O₂, in the control cells, p38 was immediately activated by phosphorylation, as was the DDR kinase ATM. Transient transfection with the p38_FLAG vector increased the amount of phospho-p38 generated by H₂O₂ treatment. It is notable that p38 expression alone appears to cause basal ATM activation. (Fig. 4.3.2a). It was difficult to see any change in BRN2 on this WB in response to p38 activation, therefore a Phos-tagTM gel was used. In addition, to try to retain any phosphorylation events, an aliquot of the collected cells was treated with phosphatase inhibitors, whilst another aliquot was treated with λ phosphatase, to remove any phosphorylation and act as a control for any observed band shifts on the gel. The Phos-tagTM gel, probed with an anti-BRN2 antibody, revealed a slower migrating band above the main BRN2 band in all phosphatase inhibitor treated samples following H₂O₂ treatment (Fig. 4.3.2b). The band disappeared following λ phosphatase treatment, confirming that the slower migration is due to phosphorylation. The clearest phosphorylation band is in the 501mel cells with co-transfection of p38_FLAG. However, these data do not confirm that p38 is responsible for the phosphorylation as

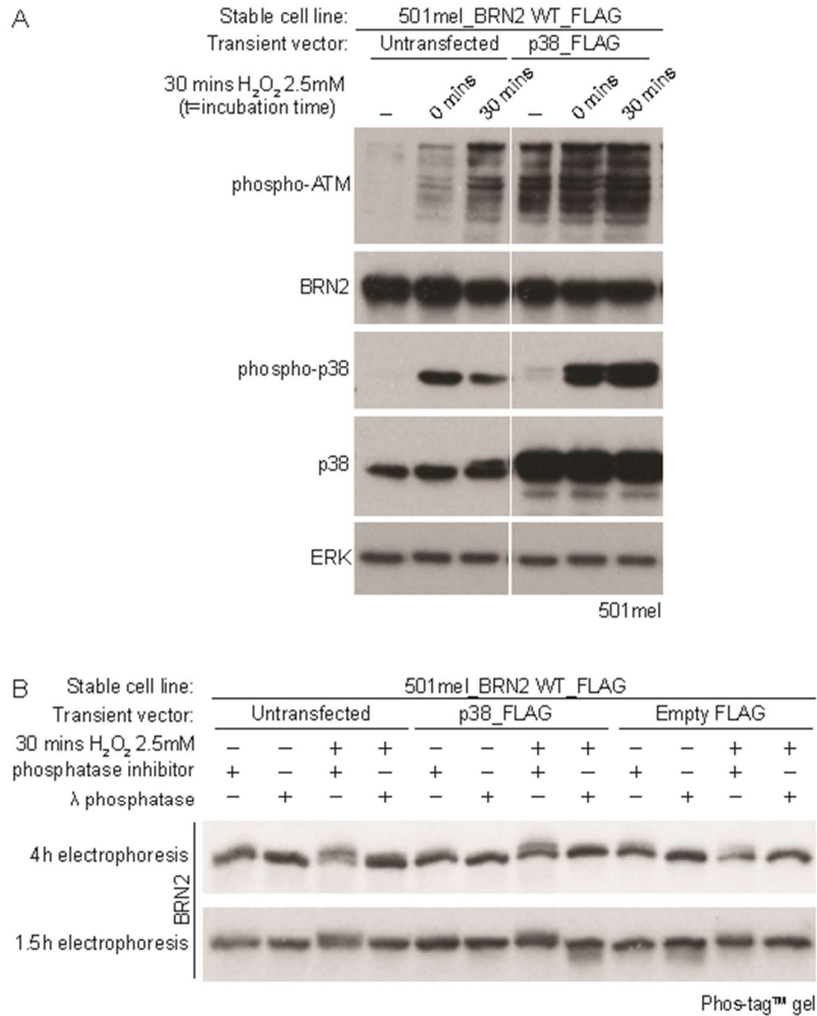


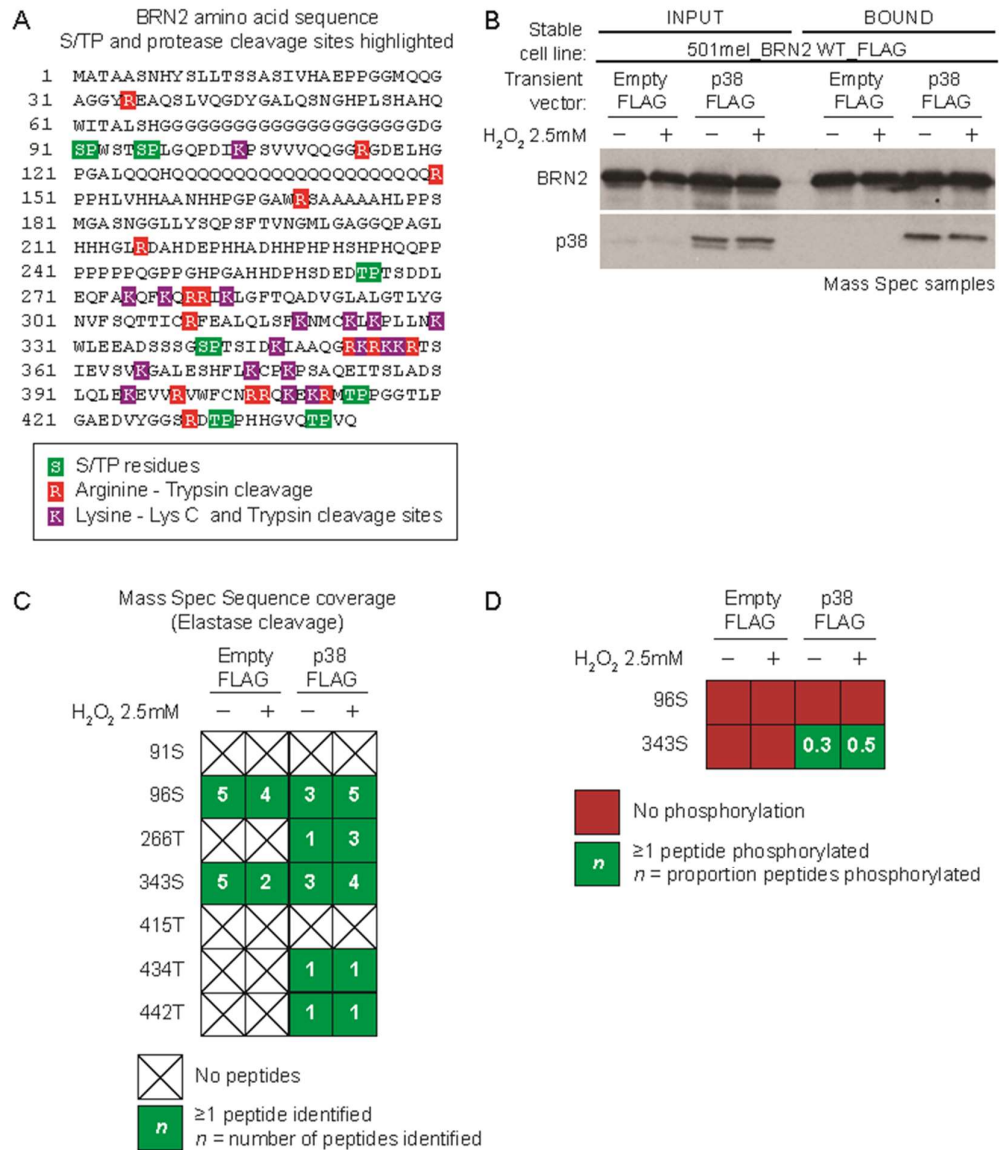
Figure 4.3.2 | BRN2 is phosphorylated following p38 pathway activation

(A) Western blot of 501mel_BRN2 WT_FLAG stable cell line, untransfected or transiently transfected with pCMV_p38_FLAG. The cells were then treated with 2.5 mM H₂O₂ for 30 minutes and then either collected immediately or after 30 minutes incubation in fresh media. ERK as loading control. (B) Western blot of Phos-tag™ gel run with 501mel_BRN2 WT_FLAG stable cell line either untransfected, or transiently transfected with pCMV_p38_FLAG or Empty_FLAG vector. Cells were treated with 2.5 mM H₂O₂ for 30 minutes prior to collection in PBS. Aliquots were then treated with phosphatase inhibitor or incubated with λ phosphatase before running on the gel. Figure shows two electrophoresis times, as indicated. Membrane probed with anti-BRN2 antibody.

other kinases including ERK are activated under the experimental conditions used (see Fig. 4.3.1b). However, it does show that BRN2 is phosphorylated in response to stress pathway activation.

Following optimisation of this stress induction model and confirmation that it results in phosphorylation of BRN2, the next step was to identify the phosphorylation events by mass spectrometry to establish which of the three residues established as p38 targets *in vitro*, S91, S96 and T442, were phosphorylated *in cellulo*. The standard proteases used to cleave proteins to produce peptides of 10-20 amino acids suitable for flight in mass spectrometry are Trypsin and LysC or LysN which cleave at arginine and lysine residues (Gauci et al. 2009). Examining the BRN2 amino acid sequence, it is evident that, particularly in the N-terminal region, there is an unusual dearth of arginine and lysine residues (Fig. 4.3.3a). This may explain why, in the phosphoproteomic screens discussed in section 1.3.4, there is very poor coverage of BRN2, stated as 10-20% in one SILAC experiment (Mertins et al. 2013). We therefore used elastase as the cleavage enzyme. Elastase has unpredictable and more non-specific cleavage sites. The result is that more useable peptides might be generated, and therefore more phospho-sites identified, but with the disadvantage that it is not possible to reliably establish stoichiometry due to an increase in the sample complexity (Wang et al. 2008; Tsiatsiani and Heck 2015)

Stable 501mel_BRN2 WT_FLAG cell lines were transiently co-transfected with p38_FLAG, p38 activation was then induced with H₂O₂ as above. Samples were purified by FLAG IP on anti-FLAG magnetic beads (Fig. 4.3.3b) and the bound fractions were submitted for phospho-mass spectrometry, following elastase treatment. As expected, there was an increase in coverage compared to what would have been predicted with Trypsin or LysC, varying from 37% to 67%. However, only residues S96 and S343 were

**Figure 4.3.3 | Phosphoproteomics of BRN2**

(A) BRN2 amino acid sequence; highlighted residues: Red=Arginine; Purple = Lysine; Green = S/TP. (B) WB of FLAG IP of BRN2_FLAG and p38_FLAG from 501mel_BRN2 WT_FLAG stable cell line, transiently transfected with pCMV_p38_FLAG or Empty_FLAG vector; anti-BRN2 and anti-p38 antibodies. (C) Table summarising peptides identified by phosphoMS following protease cleavage with elastase. Rows indicate S/TP residue present on peptide. Green box= peptide identified; N = number of peptides; Cross = no peptide identified. (D) Table summarising the phosphorylation status of the S/TP residues where all four experimental conditions had a peptide identified. Red box= no phosphorylation identified; Green box = ≥1 phosphorylated peptide. N= proportion of identified peptides with phosphorylation identified.

covered in every experimental condition (Fig. 4.3.3c). The presence of phosphorylation was assessed where all controls and experimental conditions could be analysed. This showed that peptides containing S343 were phosphorylated in the presence of exogenous p38 with or without H₂O₂ treatment. However, the proportion of identified peptides with the residue phosphorylated was only 0.3 without ROS induction and 0.5 following ROS induction (Fig. 4.3.3d). As a consequence, the S343 site could not be confidently called as a positively identified phosphorylation site in any of the samples. Unfortunately, the phospho-mass spectrometry therefore did not take us any further forward in confirming phosphorylation of the predicted residues. However, the data do show that activation of stress pathways leads to an increase in BRN2 phosphorylated species.

4.4 | p38 Phosphorylation Disrupts BRN2 Association with DDR Proteins

Having established that BRN2 can be phosphorylated in the presence of p38, the next question was whether the p38 pathway has any bearing on BRN2 involvement in the DDR pathway. To determine if p38 activation affects BRN2 binding to DDR proteins, a co-IP was performed in 501mel_BRN2 WT_FLAG stable cells following activation of the p38 pathway as above with H₂O₂ (Fig. 4.4.1). Triplicate samples were analysed by WB and probed for BRN2, Ku80 and phospho-p38, which was a positive control for pathway activation. When looking at the bound fraction, initially it looks as though p38 activation leads to an increase in Ku80 binding to BRN2_FLAG. However, in the H₂O₂ treated samples, the input levels of Ku80 are higher, and the FLAG IP has been more efficient, therefore it is not possible to conclude if p38 activation promotes association of BRN2 to Ku80 (Fig. 4.4.1).

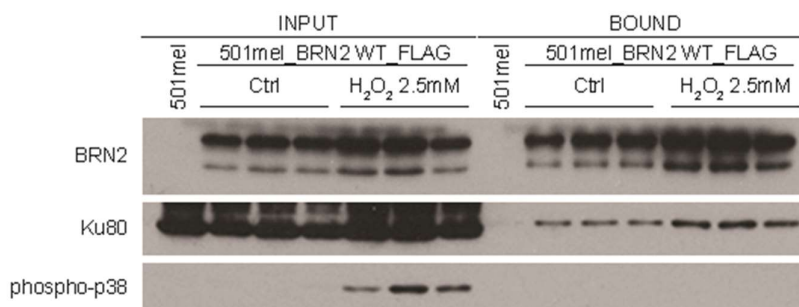


Figure 4.4.1 | BRN2 association with Ku80 is not modulated by p38 activation

Western blot of FLAG immunoprecipitation of BRN2_FLAG from 501mel_BRN2 WT_FLAG stable cell line treated with or without 2.5mM H₂O₂ for 30 minutes prior to collection. Samples run in triplicate, one untreated 501mel cell sample run as IP control.

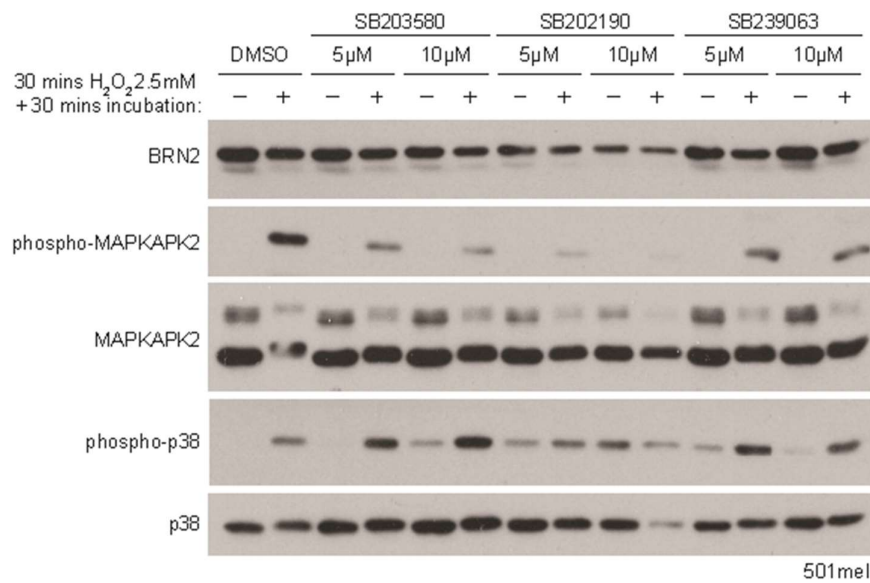


Figure 4.4.2 | p38 inhibitor validation

Western blot showing 501mel cells pre-treated with DMSO or p38 inhibitor for at least three hours, as indicated, followed by 2.5mM H₂O₂ for 30 minutes followed by 30 minutes incubation. p38 inhibitor present throughout. Antibodies as shown, total p38 as loading control.

The reciprocal experiment provides a clearer answer. Established p38 inhibitors were validated using MAPKAPK2 phosphorylation as a marker of downstream p38 kinase activity (Fig. 4.4.2). Despite lower loading of the SB202190 10 μM treated sample in particular, it is clear that compared to DMSO treated cells, all three p38 inhibitors worked, with SB202190 and SB203580 the more convincing of the three. Experiments were then conducted using either SB203580 which is the most established p38i, but SB202190 was also used as it is a more specific inhibitor (Bain et al. 2007). However it should be noted that despite improved specificity with successive generations of kinase inhibitors, particularly at higher concentrations, as used here, there is some off-target inhibition of other S/TP kinases such as JNK, AKT and ERK.

As discussed above, the H₂O₂ experimental system was set up to allow analysis of large cell numbers and with reliably high levels of p38 activation when optimising for mass spectrometry. To investigate the effect of p38 pathway modulation on BRN2 recruitment and behaviour in the DDR *in cellulo*, it was important to move back to the UV irradiation system in which the original observations discussed in the previous chapter were made. Initially UVC was used for practical reasons, as it is easier to treat bigger and multiple dishes in the UVC crosslinker than the purpose-made UVB chamber. Validation of p38 activation by UVC is shown in Fig. 4.4.3a in SKMel28 melanoma cell line. Cells were pre-treated with p38 inhibitor (p38i; SB203580, 10 µM) prior to irradiation with UVC (Fig. 4.4.3b). The phospho-p38 bands in the UVC treated lanes confirm that UVC treatment leads to p38 phosphorylation in the 501mel_BRN2 WT_FLAG cell line too. The inhibitor does not inhibit the phosphorylation of p38 but rather p38 kinase activity towards downstream targets such as p53, hence the levels of phospho-p38 are equivalent in DMSO and p38i conditions (Fig. 4.4.3b). As expected, in DMSO treated cells, Ku80 binds to BRN2 with and without p38 activation. In undamaged cells treated with p38i this association is maintained. However, this blot shows a loss of association between Ku80 and BRN2 when cells pre-treated with p38i are subjected to p38 induction via UVC (Fig. 4.4.3b).

It was then established that enough material for Co-IP could be gathered from 60 mm dishes, therefore the experiment was repeated with UVB irradiation in a time course to establish dynamics of association of BRN2 with DDR proteins. Ku80 input levels and BRN2_FLAG IP efficiency was comparable in all 501mel_BRN2 WT_FLAG lanes. As before, p38 inhibition alone did not affect the association of Ku80 with BRN2. Interestingly, UVB treatment led to a reduction in BRN2 binding to Ku80 at 5 minutes

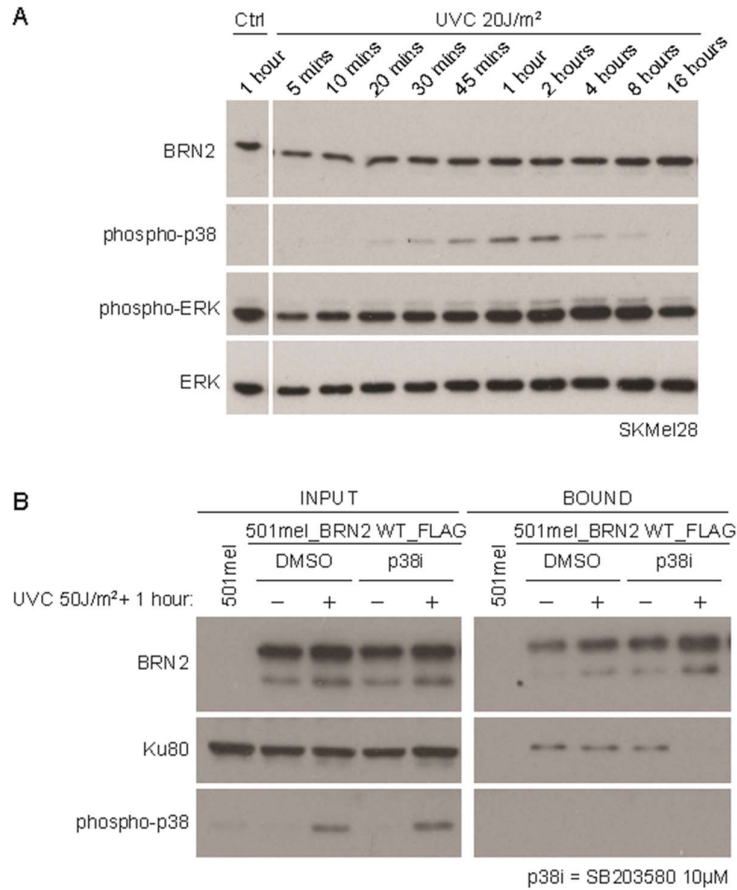


Figure 4.4.3 | p38 inhibition interrupts BRN2:Ku80 interaction

(A) Western blot showing p38 and ERK phosphorylation in SKMel28 cells treated with 20J/m² UVC in a time course. Ctrl is mock treated SKMel28. (B) Western blot of FLAG immunoprecipitation of BRN2_FLAG from 501mel_BRN2^{WT}_FLAG stable cell line either treated with DMSO control or p38 inhibition with SB 203580 10μM for 3 hours prior to irradiation with UVC 50J/m² and incubated for 1 hour prior to collection. One untreated 501mel cell sample run as IP control.

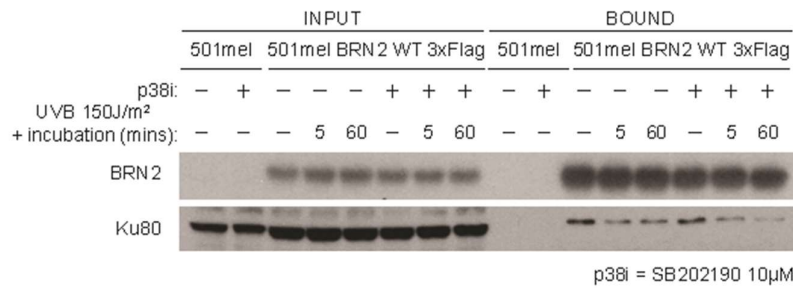


Figure 4.4.4 | p38 inhibition blocks stabilisation of BRN2 binding to Ku80

Western blot of FLAG immunoprecipitation of BRN2_FLAG from 501mel_BRN2_WT_FLAG stable cell line either treated with DMSO control or p38 inhibition with SB 202190 10 μM for 3 hours prior to irradiation with UVB 150 J/m² for either 5 minutes or 1 hour prior to collection. 501mel cells with DMSO or p38i treatment run as IP controls.

which was sustained at 60 minutes in DMSO treated cells. Reduction of BRN2 binding to Ku80 was similar at 5 minutes in p38i treated cells but much more pronounced after 60 minutes. This indicates that p38 activity helps in the maintenance of BRN2 binding to Ku80, rather than affecting the initial binding itself (Fig. 4.4.4).

To further investigate how the dynamics of BRN2 recruitment are affected by p38 activity, live LMI was used. U-2-OS cells were transiently transfected with GFP_BRN2. Cells were then pre-treated with p38i (SB203580 10μM) or DMSO as control. In the DMSO treated cells, as previously (see Fig. 3.7.5), GFP_BRN2 was rapidly recruited to the laser irradiated stripes after irradiation and peaking at 4.5 minutes, before beginning to tail off (Fig. 4.4.5a, quantified in b). However, when cells were pre-treated with p38i prior to irradiation, BRN2 recruited to the laser stripes, with a much quicker kinetic of onset and higher peak; Fluorescence was around 70% above baseline and peaked by 2

minutes, compared to a peak of around 60% at 4.5 minutes in the control cells. The off-kinetic was also much faster, immediately tailing off following the peak of fluorescence. By the end of the experiment at 7 minutes, the amount of GFP_BRN2 detectable at stripes in p38i treated cells was around 20% above baseline compared to around 50% in the DMSO-treated cells (Fig. 4.4.5a, quantified in b).

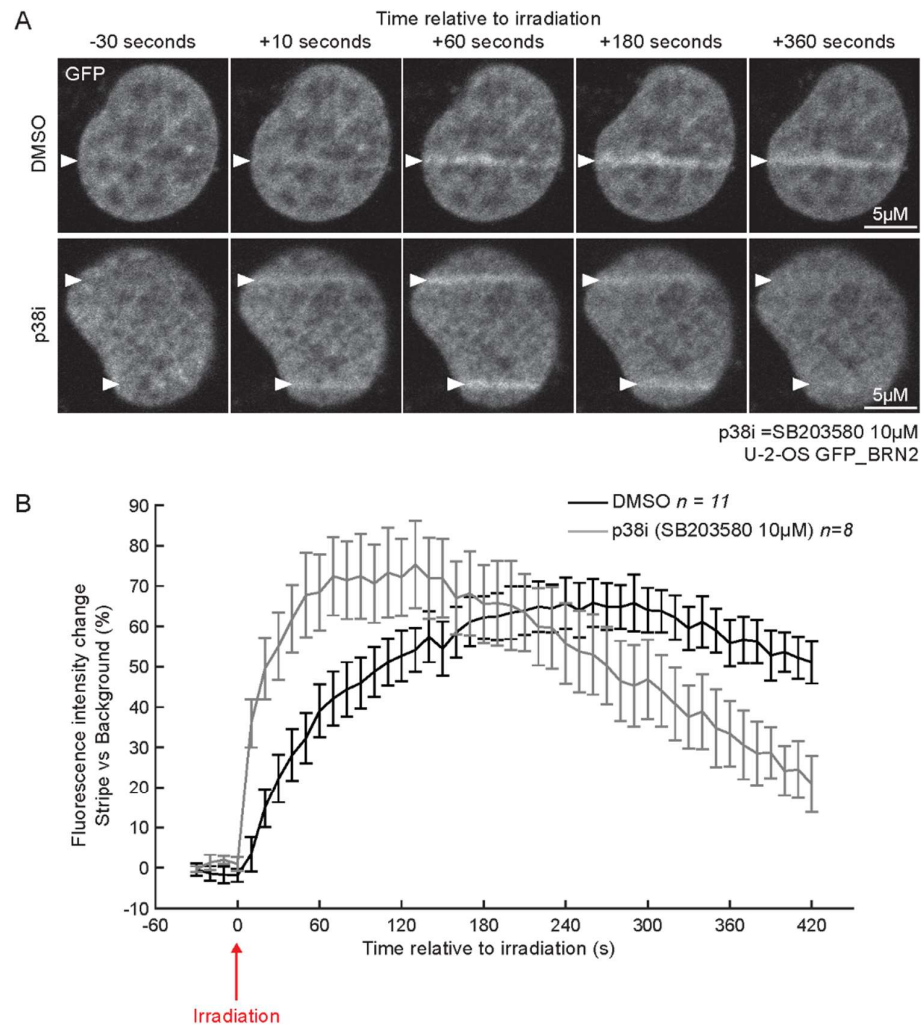


Figure 4.4.5 | p38 inhibition alters the dynamics of BRN2 recruitment post-LMI

(A) Representative still images from live imaging of U2OS cells transiently transfected with GFP_BRN2^{WT} expression vector with or without p38 inhibitor treatment (SB203580 10 μ M). Time (s) relative to irradiation. (B) Quantification of live imaging shown in (A). Data shown are the mean fluorescence intensity change of irradiated stripe vs background per cell expressed as mean $\pm$ SEM (N : DMSO=11; p38i=8).

4.5 | BRN2 N-Terminal Region Inhibits the Function of the POU Domain and Is Modulated by Phosphorylation

The live LMI data with p38i treatment support the hypothesis driven by the co-IP experiments, that p38 pathway activation stabilises the interaction of BRN2 with DDR proteins and its retention at sites of DNA damage. To address the question as to whether direct p38 phosphorylation of BRN2 affects its interaction with Ku80, we needed to further investigate the effect of the p38 phosphorylation sites on BRN2 function. The data from the p38 IVKA demonstrated that the target residues for p38 phosphorylation are S91, S96 in the N-terminal region and T442 at the end of the C-terminal domain (see Figs. 4.2.4, 4.2.5). Therefore, we began by looking more closely at the function of the non-POU domain regions of BRN2.

The AP-MS data indicated that the BRN2 Δ N had higher binding frequencies than BRN2 WT to the BRN2-binding partners (see Fig. 3.4.2). However, as discussed previously, this could be due to the level of BRN2_FLAG expression differing in the cell lines. Even so, this observation begged the question: does the loss of the N-terminal region enhance DNA binding? Put another way – Does the N-terminal inhibit the functions of the POU domain. To answer this, GST-tagged BRN2 WT, BRN2 Δ N, and BRN2 NTerm were purified from bacteria and an EMSA run using a radiolabelled probe containing the sequence of the BRN2 binding site in the MITF promoter (Fig. 4.5.1). Run in duplicate, this EMSA demonstrates that GST_BRN2 WT binds to the DNA probe, whilst GST_BRN2 NTerm does not bind at all, despite being present in excess, as shown by the Coomassie gel run in parallel. This confirms that the POU domain is required for DNA binding. Strikingly GST_BRN2 Δ N binds to the probe with much higher abundance than GST_BRN2 WT (Fig. 4.5.1). This supports the hypothesis that the N-terminal region somehow obstructs the function of the POU domain.

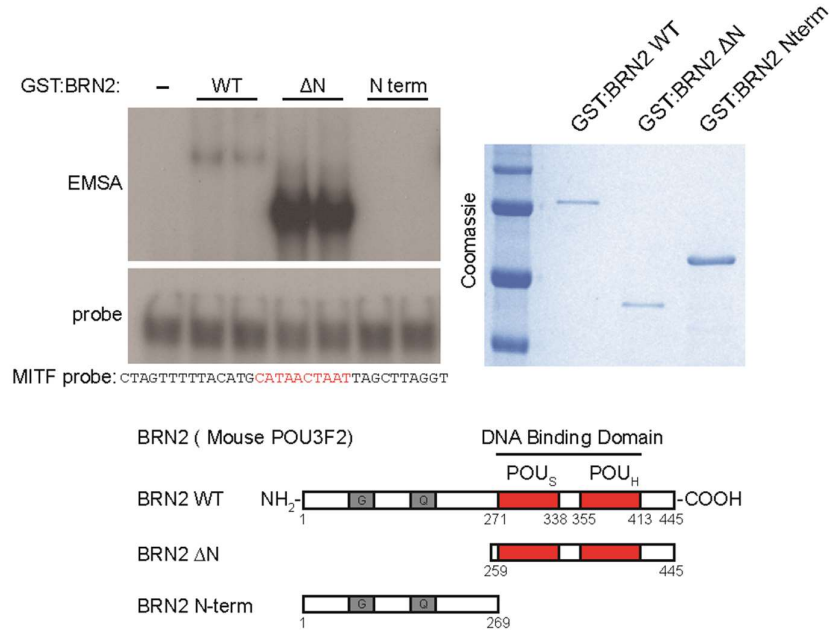


Figure 4.5.1 | BRN2 N-terminal impedes DNA binding

Representative image of electrophoretic mobility shift assay (EMSA) of purified GST-BRN2^{WT}, GST-BRN2^{ΔN} or GST-BRN2^{N term}, incubated for 30 mins with radiolabelled DNA probe of a validated BRN2 binding motif. Run in duplicate. Coomassie staining of SDS-Page gel run in parallel to show protein loading. Schema of mutants to aid interpretation.

Taken together, the data so far lead to a model in which BRN2, with unphosphorylated S91, S96 and T442 residues, adopts a “closed conformation” in which the N-terminal region limits the function of the POU domain. Clearly it does not completely block it as BRN2 WT can still bind the DNA probe. Following activation of stress-response kinases such as p38 and JNK, the N- and C-terminal S/TP residues are phosphorylated, altering the interaction of the N-terminal with the POU domain leading to an “open conformation” which allows better access of the POU domain to its binding

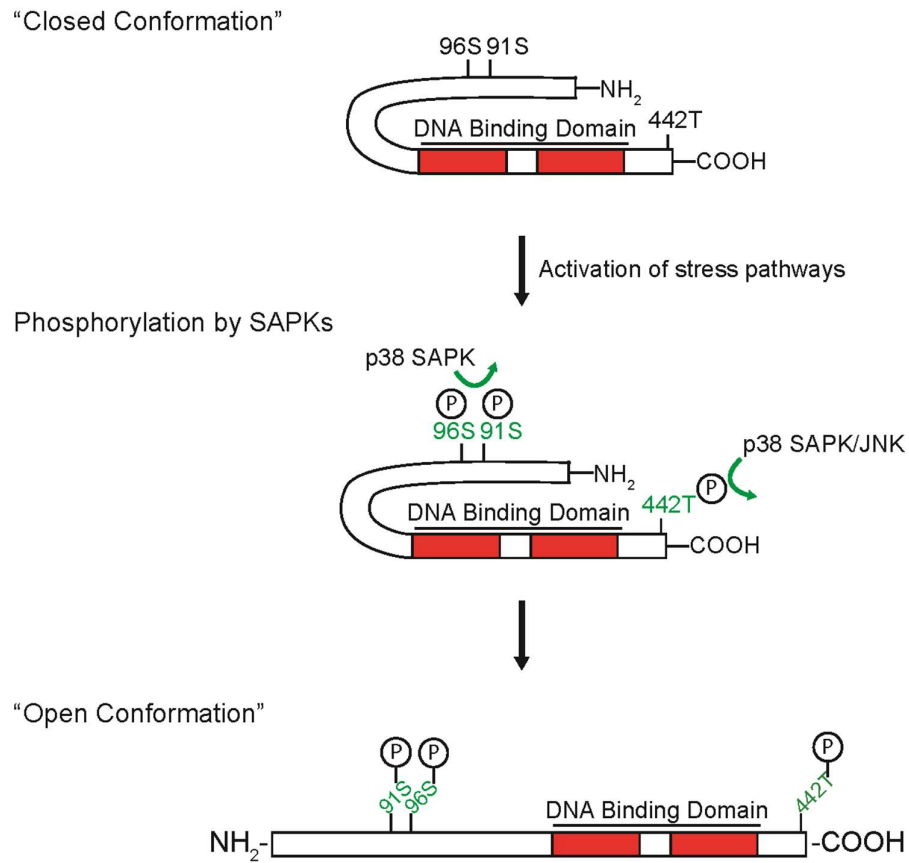


Figure 4.5.2 | Proposed model for BRN2 activation by stress response kinases

(Top panel) "closed conformation" shows BRN2 N-terminal inhibiting accessibility of POU domain (DNA-Binding Domain). (Middle panel) Phosphorylation of target residues by p38 and/or JNK indicated by green arrows. (Bottom panel) "open conformation" showing increased accessibility of POU domain following phosphorylation of 91S, 96S and 442T.

partners and DNA target sequences (Fig. 4.5.2). In this predicted model, non-phosphorylatable full-length BRN2 would bind less well to Ku80 and other binding partners, whilst phosphorylated BRN2 would bind with higher affinity.

Given that the N-terminal region seems to be the most important for affecting interactions, we concentrated on the two N-terminal residues 91S and 96S. These were both mutated to alanine by site directed mutagenesis, referred to as BRN2^{AA} (Fig. 4.5.3a). BRN2^{WT}_FLAG and BRN2^{AA}_FLAG expression vectors were both transiently transfected into 501mel cells and a FLAG-IP was carried out (Fig. 4.5.3b). There is an equal amount of BRN2_FLAG expression in the bound fraction for both constructs. However, there is a noticeable reduction in the amount of Ku80 immunoprecipitated with BRN2^{AA}_FLAG compared to BRN2^{WT}_FLAG. This suggests that the unphosphorylatable residues result in reduced BRN2:Ku80 interaction, but may also suggest that the residues are phosphorylated at baseline in the 501mel cells. This could be due to steady state activity of p38, or there could be an additional kinase responsible for the phosphorylation of these residues, opening up the intriguing idea that BRN2 transcriptional specificity could be modulated by a number of S/TP kinases.

Next, to establish if phosphorylation of the residues enhances POU domain availability, phosphomimetic mutants were produced in the GST-tagged bacterial expression system to test in EMSAs. S91 and S96 were mutated by site-directed mutagenesis to glutamate residues, producing BRN2^{91/96EE}, and in addition the T442 residue was mutated to glutamate, producing BRN2^{442E} (Fig. 4.5.4a). The purified GST-tagged proteins were run in an EMSA, as before, using a radiolabelled probe with the sequence of the BRN2 binding site on the MITF promoter (Fig. 4.5.4b). Here an anti-GST antibody produces a supershift in the second lane of each protein, confirming that the radiopaque complex seen contains the GST-tagged proteins. The Coomassie gel

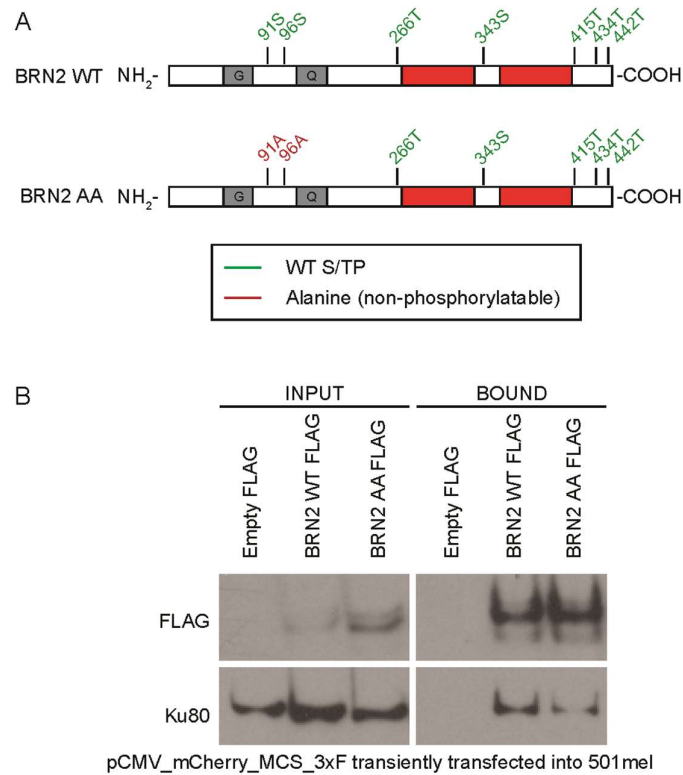


Figure 4.5.3 | Non-phosphorylatable BRN2 binds less well to Ku80 than WT

(A) BRN2 schema showing generation of BRN2^{AA} mutant by site-directed mutagenesis of 91S and 96S to alanine. (B) Western blot of FLAG immunoprecipitation of BRN2_{FLAG} from 501mel cells transiently transfected with Empty_{FLAG}, BRN2^{WT}_{FLAG} or BRN2^{AA}_{FLAG} vectors. Antibodies against FLAG and Ku80.



182

Chapter 5 | Discussion

5.1 | Introduction

The first aim of this thesis was to determine how modulation of BRN2 activity might contribute towards phenoplasticity in melanoma cells. To this end, in Chapter 3, we began by investigating BRN2 binding partners by AP-MS. However, this data generated the surprising result that BRN2 binds to multiple DNA damage response factors. The main aim then became to understand how BRN2 might be involved in the DNA damage response. We showed that BRN2 expression is inversely correlated with UVB-induced apoptosis and expression of apoptosis gene sets. We then established that it is recruited to sites of DNA damage in a PARP dependent manner and that effective recruitment and rescue from apoptosis require intact DNA binding ability. We correlated BRN2 expression in tumours to increased mutational burden and therefore highlight a potential role for BRN2 in contributing towards development of genomic instability in melanoma. We also demonstrated that although BRN2 represses MITF in a dose-dependent manner in cultured 501mel cells, the levels of overexpression used in our DNA damage assays did not affect MITF level. Therefore, BRN2 probably protects cells from UVB-induced apoptosis through an MITF-independent pathway.

However, there still remained questions regarding the molecular mechanisms governing the dynamic regulation of BRN2 function. Therefore, as described in Chapter 4, we aimed to investigate how post-translational modification of BRN2 by UV-induced kinases might facilitate the modulation of BRN2 function to effect protection of melanoma cells from UVB-induced apoptosis. Our results revealed that BRN2 can be phosphorylated on multiple S/TP motifs by the MAPK/SAPK family kinases p38, JNK

and ERK. We then demonstrated that BRN2 is phosphorylated in response to p38 activation in cells and that p38 activity is required for maintenance of BRN2 association with sites of DNA damage. Finally, we showed that p38 phosphorylation sites modulate the DNA binding affinity of BRN2. Together this leads us to a mechanistic model in which phosphorylation of the non-POU regions of BRN2 in response to UVB, triggers a conformational change which alters the accessibility of the DNA-binding domain, enhancing BRN2 DNA binding and protein:protein interactions, ultimately leading to altered transcriptional repertoire and therefore contributing to the melanoma progression.

5.2 | BRN2 Suppresses UVB-Induced Apoptosis in Melanoma Cells

Here we demonstrated that BRN2 deficient melanoma cells have persistent elevation of γ H2AX following UVB irradiation, in a pan-nuclear distribution known to be associated with apoptosis (de Feraudy et al. 2010). We demonstrated by cleaved caspase 3/7 staining that there is an increase in apoptosis following UVB irradiation of BRN2-depleted cells and that this can be rescued by overexpression of BRN2 in a DNA-binding-dependent manner. We hypothesised that BRN2 assists bypassing of the cell cycle checkpoints and thus enables cells to avoid apoptosis despite the persistence of damaged DNA. An alternative hypothesis is that the increase in apoptosis in the absence of BRN2 was a consequence of failure to repair damaged DNA with a consequent upregulation of apoptosis pathway activation.

There are a number of ways to assay for apoptosis in a cell culture system, all of which have technical and interpretation limitations. Apoptosis is a programmed cell death which is initiated by the extrinsic pathway of death receptor induced signalling or intrinsic pathways mediated by BCL-2 family members. They both end in a common mitochondrial pathway of caspase-mediated cell death (Taylor et al. 2008). The available assays make use of the range of morphological features of apoptotic cells including nuclear condensation, membrane blebbing and formation of apoptotic bodies (Kerr et al. 1972) and molecular features such as exposure of the membrane-binding protein Annexin V (Vermes et al. 1995) and cleavage of the caspases which signifies activation of the apoptosis effector proteins (Nicholson 1999). We chose to use a FACS-based method for analysing the expression of cleaved caspase 3/7 which is a critical step in the executor or mitochondrial pathway. Co-staining with a DNA stain such as 7-AAD allows distinction between early apoptotic and necrotic cell populations as membrane integrity is retained in the early phases of apoptosis whilst necrosis is characterised by immediate membrane

permeability and hence DNA staining without experimental membrane permeabilisation (Krysko et al. 2008). *In vivo*, fragmented, apoptotic cells, known as apoptotic bodies, are phagocytosed and therefore do not become necrotic (Kerr et al. 1972). In tissue culture, there is an absence of phagocytosis and therefore cells undergoing apoptosis become secondarily necrotic (Krysko et al. 2008; Fink et al. 2005). Therefore, in the late phases of apoptosis, dual staining by caspase 3/7 and 7-AAD cannot reliably distinguish necrotic from late phase apoptotic cells in tissue culture (Krysko et al. 2008). For interpretation here, we therefore counted all caspase 3/7 positive cells as apoptotic, as caspase 3/7 cleavage is not a feature of primary necrosis (Kroemer et al. 2009). We analysed one time point after UVB irradiation (24h) for apoptosis prevalence. However, to obtain a full picture of the kinetics and modes of death induced by UVB in this setting, a time-course analysis of apoptosis could be performed. It is also possible to analyse different features of apoptosis to support the data we have already gathered. For example, analysis of the sub-G1 population in DNA-dyed cell cycle analysis is a proxy measure for fragmented and condensed DNA and is a useful screening tool for the presence of apoptosis. TUNEL analysis to assay for DNA fragmentation, and Annexin V for loss of membrane structure are other possibilities (Vermes et al. 1995; Gorczyca et al. 1993). However, given the complementary data from RNA-Seq and γ H2AX staining which support the apoptosis phenotype, we did not venture further with confirmatory apoptosis assays.

One confounding feature of our apoptosis results was that we found that the rates of apoptosis were not equivalent in the 501mel cells treated with control siRNA compared to those in untreated 501mel cells. Although these two conditions were never examined at the same time, all apoptosis experiments were conducted a minimum of three times, and results were highly reproducible. The cells treated with siRNA prior to the apoptosis assay required an extra wash step to remove the lipofection reagent, which is a stressful

event per se, prior to commencing the UVB irradiation. It is possible that cells that might be more susceptible to UVB-induced apoptosis were washed away prior to irradiation and thus the baseline apoptosis induction was reduced in the siCtrl cells. Whatever the cause of this discrepancy in the baseline apoptosis rates for the siRNA and overexpression experiments, the data are reproducible, and the experiments are internally well controlled. Therefore, we are unable to compare apoptosis rates across the experiments, only within the experiments.

The gene set variance analysis (GSVA) analysis of the RNA-Seq data demonstrated that, as expected, apoptosis pathways are transiently upregulated in melanoma cells following UVB irradiation. However, following siRNA-mediated BRN2 knockdown, the upregulation of these apoptosis gene sets was of greater magnitude, and persisted at 24 hours after UV irradiation. GSEA analysis confirmed an inverse correlation of BRN2 expression with genes contained in the apoptosis gene sets at 12 and 24 hours after UVB irradiation. The upregulation of apoptosis gene sets in siBRN2 treated cells is supported by the finding that increased apoptosis is observed in BRN2 KD cells at 24 hours after UVB irradiation. DNA binding ability was demonstrated to be essential for BRN2 rescue of the apoptosis phenotype by use of the newly described non-DNA binding mutant BRN2^{N401A}.

BRN2 can act as a repressor as well as an activator of gene transcription. It may be that loss of BRN2 releases the genes implicated in apoptosis induction from the repressive activity of BRN2, allowing transcription and therefore overexpression. However, despite the demonstrated requirement for DNA binding to prevent apoptosis, it is still not possible to conclude that BRN2 is directly regulating the genes whose expression is altered upstream of the apoptosis phenotype. A genome wide ChIP-chip analysis in the same 501mel cell line identified more than 2000 target genes with BRN2-

bound promoters (Kobi et al. 2010). There is a small overlap between these identified targets and those genes found to be upregulated in the RNA-Seq data set. However, it should be noted that BRN2 gene specificity has previously been shown to be modulated by UVB, with BRN2 transcriptional regulation at the GADD45 α promoter specifically dependent on UVB irradiation (Lefort et al. 2001). It is of note that GADD45 α is not amongst the significantly modulated or occupied sites identified in the ChIP-chip analysis (Kobi et al. 2010) suggesting that BRN2 may bind a larger repertoire of genes than identified in this study. In the RNA-Seq data here, GADD45 α is one of the apoptosis genes upregulated following BRN2 KD in response to UVB. This contradicts previous reports that GADD45 α upregulation in response to UVB is BRN2-dependent (Lefort et al. 2001). It is likely that promoter occupancy by BRN2 is dynamic and modulated by exogenous signals such as UVB. Therefore, we cannot rule out that BRN2 directly affects transcription of apoptosis-related genes following UVB irradiation.

MITF activity is critical for melanoma cell phenotype determination, as summarised by the rheostat model (Carreira et al. 2006; Hoek and Goding 2010). Of particular relevance to the data described in this thesis, knockdown of MITF expression invokes the DNA damage response and drives cells towards a senescence phenotype, which is a permanent exit from the cell cycle and therefore an escape from usual cell cycle checkpoints which would lead to apoptosis (Strub et al. 2011; Giuliano et al. 2010). It is worth noting that in these experiments, siRNA mediated KD of MITF in 501mel cells was associated with a >2 fold increase in BRN2 mRNA expression (Strub et al. 2011). In addition, MITF has been shown to be critical for melanocyte and melanoma cell survival and it can drive the expression of the anti-apoptotic BCL-2 protein (McGill et al. 2002). As BRN2 and MITF expression can be linked through a feedback loop (Boyle et al. 2011; Goodall et al. 2008), it is pertinent to consider whether the phenotypic

effects which follow BRN2 KD are merely a consequence of altered BRN2-dependent MITF expression. We have shown that siRNA-mediated KD of BRN2 does not significantly affect the protein levels of MITF. We also showed that overexpression of BRN2_FLAG represses MITF in a dose-dependent manner, however the expression level used in the apoptosis rescue assays was not sufficient to repress MITF. Therefore, it is likely that the effects of BRN2 described here are independent of MITF.

BRN2 and other neuronal POU domain proteins are critical for neuronal development and function of adult neurons. Neurons are post-mitotic and therefore as a population are very susceptible to depletion through cell death. Indeed, many neurodegenerative disorders are characterised by loss of specific cell populations. Parkinson's disease (PD), for example, results from excessive cell death in dopaminergic neurons in the substantia nigra of the basal ganglia in response to mitochondrial oxidative stress (Franco-Iborra et al. 2016). BRN2 is critical for survival of neuronal precursors in the developing hypothalamus (Schonemann et al. 1995). Similarly, the class IV POU-domain protein BRN3a has been shown to be critical for neuronal survival and inhibition of apoptosis in sensory neurons of the developing nervous system (Gascoyne et al. 2004; McEvilly et al. 1996). BRN3a upregulates the anti-apoptotic protein Bcl-2 and suppresses p53 activation of apoptosis (Hudson et al. 2005; Smith et al. 1998)

BRN3a, like BRN2 is not expressed in melanocytes but is expressed in melanoma and contributes to melanoma cell proliferation and survival. siRNA mediated knockdown of BRN3a in melanoma cells resulted in cell cycle arrest and apoptosis (Hohenauer et al. 2013). This may be due to an increase in DSBs in the absence of BRN3a and thus upregulation of p53-driven arrest and apoptosis. In BRN3a-driven suppression of neuronal apoptosis, there is direct association with p53 (Hudson et al. 2005), however in melanoma cells there was no evidence of direct p53 interaction with BRN3a (Hohenauer

et al. 2013). Our data do not show a cell cycle arrest or increase in DSBs on knockdown of BRN2, therefore the mechanisms involved in BRN2 suppression of apoptosis are likely to be at least partially distinct to those invoked by BRN3a. Nevertheless, promotion of survival through suppression of apoptosis appears to be a conserved feature of POU domain factors in neuronal development, stem cell maintenance and cancer.

5.3 | BRN2 Associates with DNA Damage Response Pathways

It is possible that the protection of cells from apoptosis by BRN2 may be achieved through a non-transcriptional mechanism, but for which direct association with sites of damage and/or DNA damage response proteins is necessary. The data presented in this thesis demonstrate that BRN2 is recruited to sites of DNA damage in a PARP and DNA-binding dependent manner within 5 minutes of damage induction by LMI. We also demonstrated by AP-MS that BRN2-FLAG binds to proteins involved in diverse DNA damage pathways. Notably most of the identified prey are involved in the early recognition, signalling and recruitment phases of DNA damage. The kinetics of BRN2 recruitment are similar to PARP, that is, a quick or immediate peak in localisation to sites of LMI-induced damage (Polo and Jackson 2011) (See Fig 1.5.2). However, PARP quickly dissociates from LMI tracts within a few minutes. BRN2 recruitment, however, peaks at 5 minutes and gradually declines thereafter. This pattern of recruitment and retention is much closer to that of the DNAPK complex components (Ku70, Ku80 and DNAPK_{cs}) (Polo and Jackson 2011) (See Fig 1.5.2).

It should be noted that the cells prepared for AP-MS were not subjected to exogenous genotoxic stress prior to sample preparation. This indicates that BRN2 associates with DNA damage repair machinery before damage occurs. BRN2 is therefore primed for involvement in the DNA damage repair process. Due to its pre-existing interaction with damage recognition proteins such as PARP and DNAPK complex, BRN2 could then be recruited immediately following damage. This early and close association with damaged DNA would provide the opportunity for PTM by DNA damage response kinases, potentially altering its DNA and co-factor binding properties.

There is prior evidence for POU domain protein interaction with DDR proteins and their involvement in the DNA damage response. Direct association of the ubiquitously-

expressed POU domain factor Oct1 with DDR pathways has been described through its phosphorylation by the DDR kinase DNAPK at multiple sites on its non-conserved trans-activator domain with resultant protein stabilisation and enhanced Ku80 association (Schild-Poulter et al. 2001, 2003, 2007). Oct1 deficient cells do not appropriately upregulate cellular stress response genes and as a consequence are hypersensitive to genotoxic stressors (Tantin et al. 2005). Oct1 DNA binding affinity is enhanced following DNA damage and it upregulates target gene expression in a p53-independent manner (Zhao et al. 2000). It is possible that tissue-specific POU-domain proteins such as BRN2 recapitulate the function and mechanisms of their more ubiquitous family members. Although the Oct1 DNAPK phosphorylation does not involve conserved sites, it is possible that this mechanism has converged.

The PIKKs ATM, ATR and DNAPK target SQ motifs. BRN2 has two evolutionarily conserved SQ sites, one is proximal to the POU_S domain in the N-terminal, the second is in the POU_S domain. There is therefore potential for DNAPK or other PIKK phosphorylation of BRN2. Phosphorylation is a key mechanism for altering transcription factor DNA binding affinity and specificity, as demonstrated in BRN2 with PKA phosphorylation of 361/362 TS (Nieto et al. 2007; Berlin et al. 2012). The glutamine residue of the SQ motif within the POU_S domain is one of the six DNA contact residues identified in the Oct-1 crystal structure. Therefore it is plausible that PIKK phosphorylation of this residue could alter DNA binding specificity and therefore affect retention at sites of damage or the BRN2 transcriptional programme.

We demonstrated that the recruitment of BRN2 to sites of DNA damage following LMI is PARP dependent. PARP enzymes catalyse the PARylation of a vast array of substrates (Jungmichel et al. 2013) and are involved in modulating a number of nuclear processes, most notably the DNA damage response (Lindahl et al. 1995; Satoh and

Lindahl 1992). PARP activity is essential for the immediate response to DNA damage, particularly the recruitment of complexes involved in the DNA damage response (Cruz et al. 2015; Ahel et al. 2009). Poly(ADP-ribose) (PAR) is a bulky modification composed of branch chains which acts primarily as a signal for recruitment of both chromatin remodelling complexes such as ALC1 (Ahel et al. 2009) and DNA damage repair proteins such as XRCC1 (El-Khamisy et al. 2003). PARP is known to assist in chromatin remodelling at sites of DNA damage both directly through PARylation of histone tails (Messner et al. 2010), but also by recruitment of chromatin remodellers (Ahel et al. 2009). With PARP inhibition the chromatin retains a more closed formation following LMI (Strickfaden et al. 2016). In addition to components of the DDR, PARP activity is critical for the recruitment of a range of transcription factors, including class II lymphoid-specific POU-domain protein Oct-2, to sites of DNA damage (Izhar et al. 2015). In this screen for proteins recruited to sites of DNA damage, of 35 transcription factors (TFs) tested, 25 were recruited. Furthermore of 31 proteins recruited to LMI sites, 23 were PARP dependent. For three proteins tested, the DNA-binding domain was found to be necessary for recruitment (Izhar et al. 2015). This observation led the authors to suggest that transcription factor recruitment to sites of DNA damage is merely a non-specific function of proteins with DNA-binding domains responding to the presence of decondensed chromatin, facilitated by PARP activity (Izhar et al. 2015).

This interpretation would suggest that the PARP-dependent recruitment of BRN2 we have observed is non-specific. The LMI experiments conducted using the BRN2^{N401A} DNA non-binding mutant do indicate that DNA binding is required for recruitment of BRN2, however this does not demonstrate that it is sufficient. Indeed, there is still some residual recruitment of BRN2^{N401A}, which suggests that another mechanism might be involved in BRN2 association with the site of DNA damage. It is possible that the low-

level recruitment reflects the fact that BRN2^{N401A} may have some residual DNA binding ability; although *in vitro* no binding of the non-DNA binding mutant to the DNA probes was detected. However, there was some repression of the luciferase reporter, suggesting that *in cellulo*, there is some DNA binding activity. Alternatively, the luciferase-reporter repression could occur via interaction with other DNA-binding proteins.

With the exception of small non-coding RNAs (D'Adda di Fagagna 2014), transcription is inhibited at sites of DNA damage, therefore it is likely that TFs with DNA binding ability have a non-canonical function when binding to DNA in the vicinity of damage (Moné et al. 2001). It is known that many proteins which are not formally recognised as DDR proteins are recruited to sites of DNA damage. The observed phenomenon of TF recruitment to sites of DNA damage and the potential existence of a role for TFs in DNA damage response has been gathering traction recently, but is not yet fully understood (Izhar et al. 2015). Work on ATF2 and E2F suggests that the ability of TFs to remodel chromatin may be harnessed in the context of DNA damage to enhance recruitment of DDR proteins and act as a scaffold for efficient DNA damage repair (Chen et al. 2011; Bhoumik et al. 2005). Whilst neither E2F or ATF2 require an intact DNA-binding domain for their activity at DNA damage sites, whether this is the case for other recruited TFs is unclear (Izhar et al. 2015). Conversely, DDR proteins have been implicated in transcriptional control outside of the context of DNA damage, utilising their specialised roles of chromatin remodelling and recruitment and complex formation to assist in the fine tuning of transcriptional behaviour (Fong et al. 2013).

We have demonstrated both phenotypic and transcriptional evidence of increased apoptosis activation in the absence of BRN2. γ H2AX pan-nuclear staining does not correlate to the amount or persistence of DSBs (Halicka et al. 2005; Marti et al. 2006), rather it is associated with apoptosis and is a feature of UVB irradiation (de Feraudy et

al. 2010). However, γ H2AX staining is a well-established DNA damage marker, and we could not exclude that its persistence in BRN2 KD cells was not associated with failed DNA damage repair. Therefore, to address our alternative, but not necessarily mutually exclusive, hypothesis that BRN2 enhances damage repair, thus reducing apoptosis and to elucidate the mechanisms by which apoptosis is upregulated, we wanted to establish if the dynamics of DNA damage lesion induction and repair were affected by BRN2.

UVB predominantly introduces intra-strand linking in the form of cyclobutane pyrimidine dimers (CPDs). The capacity for NER repair of UV-induced CPDs is maintained from melanocytes to melanoma cells (Gaddameedhi et al. 2010). Although the process of repairing CPDs can result in strand breakage, this is not the predominant feature of UVB induced DNA damage (de Feraudy et al. 2010). To determine if there was a BRN2-dependent change in the temporal dynamics of CPD and strand break repair, DNA damage repair assays were performed. First we investigated CPD repair, carried out by the nucleotide excision repair pathway, the disruption of which is a feature of the cancer-predisposition syndrome Xeroderma Pigmentosa (Cleaver 2005). In the AP-MS, BRN2-FLAG bound to multiple components of the NER pathway including PARP1, RPA and RFC proteins, although it should be noted that many of these proteins are involved in several DNA damage pathways (see Fig. 3.3.2). A comparison of the dynamics of CPD formation and repair in siCtrl treated cells vs siBRN2 treated cells failed to detect any significant difference, indicating that in our hands, the NER pathway in response to UVB-induced damage is not dependent on BRN2.

Given that the predominant exogenous genotoxic insult for melanocytes and melanoma is UVB irradiation, we did not assay other forms of damage. However, the AP-MS, as discussed above, does show BRN2 interacting with proteins involved in pathways which would not usually be associated with UVB-induced DNA damage.

Although not predominantly caused by UVB irradiation, DSBs were of interest for two main reasons. Firstly, the top three BRN2-FLAG preys were the three members of the DNAPK complex (Ku70, Ku80 and DNAPK_{cs}) which is critical for recognition of DSBs and initiation of the NHEJ repair pathway (Smith and Jackson 1999; Blackford and Jackson 2017). Secondly, increased amount of H2AX phosphorylation can be associated with DSBs (Tanaka et al. 2009). However, as described above, the nuclear distribution and cell cycle profile of γ H2AX in our experiments was not consistent with DSB-associated staining.

There exists a huge number of DNA damage repair assays to examine discrete pathways. For example, the *HPRT*-mutation repair assay can be used to assay mismatch repair (MMR) deficiency (Glaab and Tindall 1997). To investigate strand break repair further, comet assays were used. As we would predict that the majority of strand breaks, if present, in a UVB context would be SSB resulting from replication fork stalling at bulky adducts during S phase and from secondary ROS mediated strand breakage (Ciccia and Elledge 2010), an alkaline comet assay was used. Alkaline comet assays favour the detection of SSBs over DSBs and can also be used to detect repair efficiency of UVB lesions that are repaired by NER (Olive and Ban  th 2006; Alapetite et al. 1996). Alkaline comet assays, using an H₂O₂-treated positive control, were used to screen for differences in strand break formation and resolution but there were very few cells which developed comet tails following UVB irradiation which rendered statistical comparison of different experimental conditions impossible.

In addition to γ H2AX staining, a number of other markers have been used for assessing strand breaks by immunofluorescence (Huang et al. 2005). Immunofluorescence for Ku80, as used in the LMI experiments, can be used to detect DSB recognition by the NHEJ pathway (Britton et al. 2013). Likewise, ATM activation

downstream of MRN recognition of DSBs can be indicated by phospho-ATM and 53BP1 foci, whilst RPA and Rad51 foci are markers of single-strand ends generated by the processing of DSBs by the HR pathway (Polo and Jackson 2011). Pilot experiments were conducted to investigate DSB-associated foci in UVB irradiated siRNA-mediated BRN2 depleted cells, however initial results demonstrated no apparent BRN2-dependent difference in foci.

In our hands therefore, we concluded that there was no evidence to support a BRN2-dependent enhancement of damage or impairment of repair, at least under the culture conditions used here. However, given the importance of BRN2 in multiple other tissues which are not exposed to UV, such as the developing brain, prostate and lung cancer, it is entirely plausible that BRN2 may play a role in the DDR to other genotoxic agents which cause different DNA damage lesions such as DSBs.

As we had established that there was a positive association between elevated γ H2AX and apoptosis induction in the absence of BRN2, we considered γ H2AX to be a proxy measure of apoptosis induction rather than a marker of DNA damage, as previously described in the literature and discussed above. Therefore, further DNA damage repair assays were not pursued, in order to focus on the observed apoptotic phenotype. However, it is clearly difficult to prove a negative, and further work is required to investigate fully whether the temporal dynamics and prevalence of DNA damage lesions resulting from UV irradiation is BRN2-dependent. To this end, the piloted IF and Comet assays described above could be extended to confirm or refute the negative results from the pilot experiments. One issue with DNA damage assays is that the induction of damage by an exogenous agent such as UV is not uniform across a population of cells. It would be informative to employ novel methods to investigate molecular events surrounding DSBs and repair pathway choice in a much more precise manner than the more

established techniques described here. The use of the restriction-enzyme *Asi*SI under the control of an ER promoter enables the induction of a quantifiable number of DSBs (Iacovoni et al. 2010; Chailleux et al. 2014); when combined with immunofluorescence and ChIP, this enables the assessment of the behaviour of DDR-associated factors at sites of damage (Iacovoni et al. 2010; Caron et al. 2012) and repair pathway choice in a known chromatin context (Aymard et al. 2014).

5.4 | p38-Mediated Phosphorylation Modulates BRN2 Function

Having demonstrated that UVB-induced apoptosis is modulated in a BRN2-dependent manner, we investigated the role of PTM by UV-associated kinases in BRN2 regulation. We have shown that p38 can phosphorylate BRN2 *in vitro* on three sites (S91, S96 and T442) and we then showed *in cellulo* that p38 activation is associated with an increased prevalence of phosphorylated BRN2 species. However, these results do not definitively show that BRN2 is directly phosphorylated by p38 physiologically. Unfortunately, we were unable to successfully use phosphoproteomics to confirm which sites are phosphorylated and the stoichiometry of any phosphorylation events. In an age when high-throughput proteomic technology is becoming increasingly accessible, phosphoproteomics has emerged as the gold-standard for characterising protein phosphorylation *in vivo* (Walther and Mann 2010). Mass spectrometry has enabled identification of phosphorylation sites, and is very sensitive when combined with methods to increase the concentration of phosphorylated species such as strong-ion exchange chromatography (Beausoleil et al. 2004). Combination of mass spectrometry with SILAC and time-course studies has allowed the dynamic investigation of phosphorylation in cells (Olsen et al. 2006; Ong et al. 2002). Due to the unusual amino-acid sequence of BRN2, it is not amenable to appropriate proteolytic cleavage to produce peptides with the required characteristics for mass spectrometry and therefore we had very limited sequence coverage. If we had been able to use this tool further then we could have used p38-specific inhibitor treated samples to increase our confidence that any phosphorylation events identified in the presence of activated p38, were p38-specific.

Although elucidating the molecular dynamics of p38-dependent BRN2 phosphorylation would have contributed to further understanding the mechanisms of BRN2 function, the downstream phenotype assays have more bearing on establishing

clinical significance of any such molecular event. We used p38 inhibitors to determine if association of BRN2 with DNA damage response proteins is p38-dependent. Live-imaging LMI in conjunction with p38 inhibition demonstrated that p38 affects both the on and off kinetics of BRN2 recruitment to the laser-irradiated damage tract. The faster disappearance of BRN2 from LMI stripe suggests that p38 activity is required for the stabilisation or retention of BRN2 at sites of damage. This would support the fact that Ku80 binding to BRN2 is reduced following p38 inhibition as observed by co-immunoprecipitation.

Why the on-kinetic, or recruitment, of BRN2 is accelerated following p38 inhibition is not clear. The dynamics of PARP recruitment to DNA damage and the associated modulation of chromatin compaction are similar to the dynamics of BRN2 recruitment that we observed in the presence of p38 inhibition (Polo and Jackson 2011) (see Fig. 1.5.2). The recruitment kinetics of BRN2 in the presence of p38 inhibition would therefore fit with the hypothesis generated by the Izhar et al. screen of multiple TFs being recruited to sites of damage, that TF presence at sites of damage is merely a consequence of PARP-mediated chromatin decondensation (Izhar et al. 2015). The very fact that BRN2 association with the DNA damage stripe in control conditions is different to this and is affected by the function of p38 suggests a more active mechanism of promotion and maintenance of recruitment to sites of damage in a p38-dependent manner. Of course, this does not prove that p38 activity is required specifically for BRN2 recruitment. It may be that p38 activity alters the dynamics of PARP or indeed Ku80 recruitment which has a knock-on effect on BRN2 recruitment. Here we did not examine the recruitment of Ku80 and PARP with p38 inhibition. It would be possible to use stable Ku80-GFP U-2-OS cells to establish the dynamics of Ku80 recruitment with and without p38 inhibition. We demonstrated in the previous results chapter that PARP inhibition does not affect the

recruitment of Ku80 to the LMI stripe. Therefore, even if p38 affects PARP recruitment, the implication would be that this would not then have an impact on Ku80 recruitment. Given the fact that p38 inhibition interrupts the association of BRN2 and Ku80, the prediction would be that Ku80 recruitment is unaffected by p38 inhibition, rather it is the disruption of BRN2:Ku80 binding which alters BRN2 recruitment dynamics.

To investigate further whether the p38-dependence of BRN2 recruitment was due to a direct effect on BRN2, we used amino-acid substitution experiments to replicate the predicted-effect of p38 phosphorylation on BRN2. The S/TP motifs identified as p38 targets in the *in vitro* kinase assays were mutated to alanine to mimic loss of p38-mediated phosphorylation or glutamate to mimic p38 phosphorylation. Phosphorylated serine or threonine have a very similar chemical structure to the negatively charged amino acids aspartate and glutamate, so these two amino acids are often used as substitutes to mimic phosphorylation (Thorsness and Koshland 1987). Experimentally, there are limitations to the preciseness of the mimicry as both the size and charge of glutamate and aspartate are not fully comparable to phospho-serine/threonine which means that the biological functions mediated by the phosphorylated S/T residue may not be fully re-capitulated by the phosphomimetic residue. Intriguingly, it has been suggested that the structural and chemical similarities of phospho-S/T to D and E is a driver of evolutionary development of phosphorylation sites resulting from a mutation of D/E to S/T. This would introduce the possibility of dynamic protein function by switching between the non-phosphorylated and phosphorylated S/T, rather than the static D/E, equivalent to the phosphorylated S/T (Pearlman et al. 2011; Creixell et al. 2012). Complementary experiments with the non-phosphorylatable and phosphomimetic BRN2 mutants demonstrated that the POU domain functions of protein:protein interaction and DNA binding are positively regulated by phosphorylation.

To establish if this phospho-site dependent alteration in BRN2 DNA and protein binding affected the recruitment of BRN2 to sites of DNA damage, we aimed to use the phospho-site mutants in live LMI experiments. However, pilot LMI studies showed no difference in recruitment kinetics between BRN2 WT and either the S91/S96 mutants (non-phosphorylatable AA or phosphomimetic EE) (data not shown). This could indicate that these phosphorylation sites have no impact on BRN2 conformation. However, it is also possible that artefactual consequences of fluorescent-tagging render impossible the inference of structural change through point mutation. Fluorescent tags can affect the function and structure of the protein of interest to which they are tagged (Crivat and Taraska 2012). This can be caused by oligomerisation of the fluorescent protein, although this has been substantially reduced by use of structural GFP variants such as enhanced GFP (EGFP) (Cinelli et al. 2000), as we used here. Fluorescent proteins are 27 kDa with a size of 3x4x4nm; they can therefore cause steric hindrance of protein function or they can interact with the protein and alter the conformation of the protein, particularly when there is no flexible linker between tag and protein (Crivat and Taraska 2012).

We wanted to address whether p38 phosphorylation is involved in effecting the BRN2-dependent apoptotic phenotype that results from UVB-irradiation. To do this, we needed to assay for apoptosis with and without p38 inhibition. Unfortunately, p38 inhibition alone following UVB irradiation resulted in high levels of baseline γ H2AX and cell death, therefore we were unable to fruitfully use these assays to demonstrate a downstream phenotype of p38-dependent BRN2 recruitment or phosphorylation. To take this question forward, rather than inhibiting p38 and thus interfering with the cellular response to stress resulting from UVB irradiation, we would want to employ a more BRN2-specific assay. Generation of stable cell lines expressing the phosphomimetic or non-phosphorylatable BRN2 proteins could be used to perform the apoptosis assays. The

prediction would be that the non-phosphorylatable BRN2 fails to rescue the apoptotic phenotype, as was the case with the N401A mutant. Conversely, we would expect the phosphomimetic version to rescue cells from UVB-induced apoptosis as well as, or more so, than the WT protein.

Although we established that phosphomimetic BRN2 increases DNA binding *in vitro* to known BRN2-binding sites, we did not investigate whether this results in altered transcription *in cellulo*. Further work to investigate this would require transcriptomics such as RNA-Seq in a cell line stably expressing the phosphomimetic BRN2 in which endogenous BRN2 has been stably knocked-down. Therefore, we can only speculate as to the effect on transcription of p38-mediated BRN2 phosphorylation.

5.5 | A Proposed Model for Dynamic Regulation of BRN2 Activity

We demonstrated here that post-translational modification of the transactivation N-terminal region of BRN2 by the UV-activated kinase p38, affects BRN2 DNA binding ability and recruitment to sites of DNA damage. The mechanistic model arising from the data described in this thesis (see Fig. 4.5.2) predicts that the N-terminal domain obstructs both DNA binding and protein:protein interactions mediated through the POU domain. Phosphorylation of S/TP sites in the N- and C-terminal domains by p38, and potentially other kinases like JNK and ERK, results in a conformational change which increases availability of the POU domain and consequently alters BRN2 function. This could be achieved through both modulation of DNA binding and therefore transcriptional activity, and protein:protein interaction which affects its recruitment to sites of DNA damage.

Although the successful application of an *in cellulo* assay would have enabled us to infer the functional effect of a proposed conformational change in BRN2 resulting from mutation of phosphorylation sites, it would not prove that there was a conformational change. In order to test the physical predictions of this model, that phosphorylation alters the conformation of BRN2, there are a number of structural assays we could try. Firstly, a simple biochemical assay like proteolytic clipping would give an indication as to whether the phosphosite mutants trigger a change in the 3D structure of BRN2. More robust methods to assess the conformation of BRN2 would require structural biology techniques, the gold standard of which is X-ray crystallography (Holcomb et al. 2017). The phosphomimetic and non-phosphorylatable proteins could be used alongside BRN2 WT to compare conformational change. However, to date no crystal structure of BRN2 exists and such a project would be likely fraught with technical difficulties, not least because the N-terminal domain of BRN2 is intrinsically disordered and therefore crystallisation is likely to be problematic (Dale et al. 2003).

The alternative is the rapidly advancing field of molecular dynamic simulation which uses computer modelling to analyse the structure of proteins and can be used to predict conformational changes of proteins when bound to ligands or in complexes (Forster 2002; Karplus and McCammon 2002; Hospital et al. 2015). It would be interesting to use *in silico* methods to predict whether there are structurally-important intramolecular bonds which are dependent on the identified p38 phosphorylation sites. The structure of isolated POU domains crystallised on DNA has been determined, for example Oct1 (Klemm et al. 1994), which we used here to design a non-DNA binding mutant. The transactivation domains of POU domain proteins are not conserved and therefore extrapolation of other protein crystal structures to BRN2 is not possible.

There is no solved crystal structure of BRN2, however, Evangelista do Vale Coelho et al. built an *in silico* model of full length *homo sapiens* BRN2 and undertook a molecular dynamic (MD) simulation of its association with MORE DNA (do Vale Coelho et al. 2016). This demonstrates that the two regions of BRN2 which underwent the most conformational change during dynamic modelling were the flexible linker region between the POU_S and POU_H domains, and the transactivation domain which we have termed the N-terminal region. Interestingly in the N-terminal region, the highest peak of root-mean square fluctuation (RMSF), which indicates high potential for conformational change (Martínez 2015), during the MD stimulation was at about position 100 in the BRN2 sequence,. This is immediately after the two predicted p38 phosphorylation sites (S91 and S96). This *in silico* data support the concept embodied in our predicted model that conformational change of BRN2 by PTM of the N-terminal or transactivation domain could alter accessibility of the DNA binding site.

The DNA probe shown in the EMSAs using phospho-site mutant BRN2 (see Figs. 4.5.1 and 4.5.4) was the MITF promoter BRN2 binding site. The modulation of the MITF

promoter by BRN2 is well described, with both activation and repression observed dependent on cellular context (Goodall et al. 2008; Wellbrock et al. 2008). However, the mechanisms which facilitate this dynamic regulation have not yet been described. There has been speculation that the multiple BRN2 binding sites on the MITF promoter may have distinct roles in the regulation of MITF expression (Kobi et al. 2010). It is possible that a conformational change induced by p38 phosphorylation, as that hypothesised in our model, could influence how BRN2 binds to different regions of the MITF promoter. Whether these different promoter sites are PORE, MORE or NORE or another degenerate form may dictate the ability of BRN2 to bind to them, dependent on its conformation. A genome-wide ChIP-Seq experiment might reveal binding to more of the BRN2 regulated genes than is currently apparent.

A second DNA probe was used to successfully validate the EMSA result (data not shown). This was the BRN2 binding site identified by ChIP chip on the Kit ligand (*KITL*) promoter, also known as stem cell factor (SCF or steel) (Kobi et al. 2010). Overexpression of BRN2 led to increased endogenous KitL expression, whilst siRNA-mediated knockdown of BRN2 resulted in decreased KitL expression demonstrating that BRN2 positively regulates the KitL promoter (Kobi et al. 2010). The Kit ligand receptor c-Kit is a tyrosine kinase transmembrane growth factor receptor which activates multiple signalling pathways including MAPK. c-Kit signalling is required for development and survival of melanocyte precursors in the neural crest and migration of melanoblasts to the epidermis (Wehrle-Haller and Weston 1995; Guo et al. 1997; Giebel and Spritz 1991). c-Kit mutation or copy number variation is present in about one third of acral, mucosal and chronic sun damage exposed melanomas, but is mutually exclusive to the BRAF and NRAS mutations found in intermittently sun-exposed skin (Curtin et al. 2006). The phenotypic effect of c-Kit signalling in melanoma appears to be context dependent, with

tumour-suppressor activity in BRAF/NRAS-mutated sun-exposed melanoma and oncogene function in chronic or non-exposed melanomas (Neiswender et al. 2017). Constitutive activation of mutant c-Kit results in increased activation of PI3K/MTOR and MAPK pathways, with p38 being the most highly-activated kinase (Sanlorenzo et al. 2016). Increased BRN2 phosphorylation and therefore DNA binding resulting from c-Kit-driven activation of p38 could promote KitL transcription, leading to a positive feedback loop of autocrine potentiation of c-Kit signalling. The effect of this on melanoma phenotype would likely depend on the wider context of the melanoma cell, including BRAF and NRAS mutational status.

5.6 | BRN2 and Genome Instability

The potential corollary of continued viability of cells with damaged DNA would be an increase in mutational burden and consequent promotion of genomic instability. Genomic instability is a hallmark of cancer and is correlated with poor clinical outcomes (Carter et al. 2006; Hanahan and Weinberg 2000). By studying the SNV rate in whole exome sequencing of TCGA melanoma samples in comparison to BRN2 expression, we identified that there was a significant positive correlation with the C>T transition SNV. The C>T mutation denotes a UV signature and results from base substitution during NER following UVB induction of CPDs (Alexandrov et al. 2013; Brash 2015; Pfeifer et al. 2005). Melanoma has the highest burden of SNVs of all tumour types, which reflects its pathophysiology as a consequence of UV exposure (Alexandrov et al. 2013). Interestingly, the mutational signatures of melanoma are distinct depending on their anatomical location and associated sun exposure. Acral, mucosal or chronically exposed regions are a distinct genomic entity from the more aggressive, invasive lesions which arise from intermittently exposed skin and have a higher C>T mutational rate, representing a UV signature (Curtin et al. 2006; Akbani et al. 2015; Curtin et al. 2005).

Progression of melanoma from benign naevus or precursor cell to metastatic melanoma is marked by the accumulation of mutations in genes which contribute towards the acquisition of proliferative, invasive and metastatic characteristics which facilitate disease progression (Shain et al. 2015). The key genes which are mutated, deleted or amplified and as a consequence drive melanoma progression have been well characterised (Hodis et al. 2012; Akbani et al. 2015). These include activating point mutations in driver genes such as BRAF and NRAS (Carr and Mackie 1994; Pollock et al. 2003) or regulatory regions such as the TERT promoter (Huang et al. 2013; Horn et al. 2013); gene deletion of tumour suppressors such as PTEN and CDKN2A (Curtin et

al. 2005). However, along with these specific genes, there is a generalised increase in the C>T mutational signature and SNV burden through melanoma lesion progression (Shain et al. 2015). Genomic instability characterised by copy number variation becomes a feature in advanced, invasive melanoma (Shain et al. 2015; Alexandrov et al. 2013). High levels of BRN2 expression have been shown to correlate with a slow-cycling, invasive population with metastatic ability (Pinner et al. 2009). This would support the data that we have generated here, that BRN2 promotes survival of cells despite the presence of DNA damage and its expression is correlated with high SNV burden. Loss of genomic integrity, broadly speaking, can result from instability at the nucleotide or chromosomal level (Lengauer et al. 1998). Here we have investigated nucleotide instability through SNV frequency. It would be interesting to correlate chromosomal instability (CIN) with BRN2 expression through karyotyping of melanoma cells. We would predict that the increased SNV burden identified, is associated with greater structural genomic instability, in the form of chromosomal translocations and amplifications (Shain et al. 2015; Carter et al. 2006).

Interestingly, we found that the increased mutational burden was specific to BRN2 expression with no significant correlation found between SNV frequency and MITF, BRN3a and BRN3b expression. As discussed above, BRN3a and 3b have been implicated in apoptosis protection, survival and DNA damage repair (Hudson et al. 2005; McEvilly et al. 2002; Hohenauer et al. 2013; Gascoyne et al. 2004). Our failure to demonstrate a BRN2-dependence for DNA damage repair rates is supported by the increased SNV frequency in tumours overexpressing BRN2. The contrast with BRN3a suggests an uncoupling of the mechanisms by which POU-domain factors are involved in apoptosis and DNA damage repair, rather than both being enacted through a common pathway such as p53 modulation.

5.7 | Conclusions

We have identified a role for BRN2 in rescuing melanoma cells following UVB irradiation and promoting the survival of cells without any evidence of enhanced DNA damage repair. Indeed, we showed that BRN2 expression is positively correlated with mutational burden in melanoma, suggesting that BRN2 supports cell survival in the face of DNA damage which would otherwise lead to senescence or apoptosis. This novel function of BRN2 could also be relevant in other BRN2-associated cancers such as small cell lung cancer and neuroendocrine prostate cancer, subject to other forms of DNA damaging agents (Bishop et al. 2016; Ishii et al. 2013). Association of BRN2 with DNA damage response factors, and recruitment to sites of damage may facilitate an as yet undescribed non-transcriptional mechanism for BRN2 function in the UV response. Induction of apoptosis pathway gene expression in the absence of BRN2 may reflect the removal of BRN2 direct promoter repression. However it could also result from the removal of a BRN2-critical inhibition of the usual DNA damage signalling pathways through the protein:protein interactions established here.

BRN2 recruitment and retention at sites of DNA damage is PARP and p38 dependent respectively. The requirement for p38 to maintain the association of BRN2 with damaged DNA links UV-activated signalling to BRN2 function. BRN2 DNA binding is also necessary for efficient recruitment and retention. This indicates that the requirement for PARP function may be indirect through the PARP-driven decondensation of DNA allowing DNA-binding by BRN2. Although we have not identified the function of BRN2 at sites of DNA damage, given the downstream phenotype of apoptotic suppression following DNA damage, there are a number of possible events that might occur. Firstly, BRN2 may be further modified at the site of

DNA damage response by PIKK kinases such as ATM, ATR or DNAPK. We showed by AP-MS that BRN2 binds to all three subunits of the DNAPK complex, this is therefore a promising candidate kinase. Secondly, BRN2 recruitment may promote its binding to other components of DNA damage signalling pathways which affect apoptosis gene activation. The pivotal protein in this context would be p53, but interruption of any of the components of p53-driven apoptotic gene transcription would contribute towards apoptosis suppression.

How melanoma cells escape sensitivity to UV irradiation and remain viable is a fundamental question in melanoma biology. The full range of molecular mechanisms that facilitate melanoma cells' avoidance of apoptosis in response to UV damage and allow tumour progression, despite, or indeed because of a stepwise accrual of mutations are not yet fully understood. High levels of genomic instability however may be a double-edged sword for melanoma and other tumours. Genome instability is associated with advanced melanoma and poor clinical outcomes (Shain et al. 2015; Carter et al. 2006). However, the high rate of neoepitope generation through successive acquisition of new mutations may in part explain the susceptibility of melanoma to immunotherapy and provide a basis for creating personalised neoantigen vaccines (Palmieri et al. 2017; Turajlic et al. 2017; Ott et al. 2017; Snyder et al. 2014). Whilst BRN2 expression is associated with a more invasive melanoma cell phenotype, this newly described role in protection from DNA-damage dependent apoptosis may result in higher neoantigen formation, increasing susceptibility of BRN2^{high} melanoma cells to immunotherapy.

BRN2 has not previously been demonstrated to be essential for melanoma cell survival, however, it is critical for hypothalamic neuron survival during neurogenesis (Schonemann et al. 1995). The class IV POU domain factors BRN3a/b have been shown to inhibit apoptosis in the developing nervous system (McEvilly et al. 1996; Hudson et

al. 2005). In melanoma cells loss of BRN3a is associated with increased DNA damage, cell cycle arrest and apoptosis in the absence of any exogenous stress (Hohenauer et al. 2013). This is not the same phenotype as we have observed with BRN2, as siRNA of BRN2 alone did not result in a damage or apoptosis phenotype. Rather BRN2 role is in response to UV-induced DNA damage. Promotion of cell survival appears to be a conserved POU-domain protein function; however, the mechanisms and pathways involved are likely to be context and tissue specific. The non-POU regions of the POU-domain families are poorly conserved and therefore mechanisms of post-translational control outwith the POU domain are not conserved.

Given their importance in stem cell maintenance, embryological development and cancer, conservation across POU domain proteins of this novel function of BRN2 in promoting cell survival in the face of DNA damaging agents could have important consequences for understanding the pathophysiology and influencing the treatment of a range of cancers and neurodegenerative disease.

References

- Adler V, Kim J, Dolan L, Ronai Z. 1995. UV Irradiation and Heat Shock Mediate JNK Activation via Alternate Pathways. *J Biol Chem* **270**: 26071–26077.
- Agarwal ML, Agarwal A, Taylor WR, Stark GR. 1995. p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proc Natl Acad Sci USA* **92**: 8493–8497.
- Ahel D, Ho Z, Wiechens N, Polo SE, Garcia-wilson E, Ahel I, Flynn H, Skehel M, West SC, Jackson SP, et al. 2009. Poly(ADP-ribose)-Dependent Regulation of DNA Repair by the Chromatin Remodeling Enzyme ALC1. *Science* **325**: 1240–1244.
- Akbani R, Akdemir KC, Aksoy BA, Albert M, Ally A, Amin SB, Arachchi H, Arora A, Auman JT, Ayala B, et al. 2015. Genomic Classification of Cutaneous Melanoma. *Cell* **161**: 1681–1696.
- Alapetite C, Wachter T, Sage E, Moustacchi E. 1996. Use of the alkaline comet assay to detect DNA repair deficiencies in human fibroblasts exposed to UVC, UVB, UVA and γ -rays. *Int J Radiat Biol* **69**: 359–369.
- Alazard R, Blaud M, Elbaz S, Vossen C, Icre G, Joseph G, Nieto L, Erard M. 2005. Identification of the “NORE” (N-Oct-3 responsive element), a novel structural motif and composite element. *Nucleic Acids Res* **33**: 1513–1523.
- Alazard R, Mourey L, Ebel C, Konarev PV., Petoukhov MV., Svergun DI, Erard M. 2007. Fine-tuning of intrinsic N-Oct-3 POU domain allostery by regulatory DNA targets. *Nucleic Acids Res* **35**: 4420–4432.
- Alexaki V, Javelaud D, Mauviel A. 2008. JNK supports survival in melanoma cells by controlling cell cycle arrest and apoptosis. *Pigment Cell Melanoma Res* **21**: 429–438.
- Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SAJR, Behjati S, Biankin AV., Bignell GR, Bolli N, Borg A, Børresen-Dale A-L, et al. 2013. Signatures of mutational processes in human cancer. *Nature* **500**: 415–421.
- Alonso SR, Tracey L, Ortiz P, Pérez-Gómez B, Palacios J, Pollán M, Linares J, Serrano S, Sáez-Castillo AI, Sánchez L, et al. 2007. A high-throughput study in melanoma identifies epithelial-mesenchymal transition as a major determinant of metastasis. *Cancer Res* **67**: 3450–3460.
- Andreassen CN, Rosenstein BS, Kerns SL, Ostrer H, De Ruyscher D, Cesaretti JA, Barnett GC, Dunning AM, Dorling L, West CML, et al. 2016. Individual patient data meta-analysis shows a significant association between the ATM rs1801516 SNP and toxicity after radiotherapy in 5456 breast and prostate cancer patients. *Radiother Oncol* **121**: 431–439.
- Andrews FH, Strahl BD, Kutateladze TG. 2016. Insights into newly discovered marks and readers of epigenetic information. *Nat Chem Biol* **12**: 662–668.
- Andrey G, Mundlos S. 2017. The three-dimensional genome: regulating gene expression during pluripotency and development. *Development* **144**: 3646–3658.

- Araújo S, Tirode F, Coin F, Pospiech H. 2000. Nucleotide excision repair of DNA with recombinant human proteins: definition of the minimal set of factors, active forms of TFIIH, and modulation by CAK. *Genes Dev* **14**: 349–359.
- Assa-Munt N, Mortishire-Smith RJ, Aurora R, Herr W, Wright PE. 1993. The solution structure of the Oct-1 POU-specific domain reveals a striking similarity to the bacteriophage λ repressor DNA-binding domain. *Cell* **73**: 193–205.
- Atanasoski S, Schreiber E, Fontana A, Herr W. 1997. N-Oct 5 is generated by in vitro proteolysis of the neural POU-domain protein N-Oct 3. *Oncogene* **14**: 1287–94.
- Atanasoski S, Toldo S, Malpiero U, Schreiber E, Fries R, Fontana A. 1995. Isolation of the Human Genomic Brain-2/N-Oct-3 Gene (Pouf3) and Assignment To Chromosome-6Q16. *Genomics* **26**: 272–280.
- Aymard F, Bugler B, Schmidt CK, Guillou E, Caron P, Briois S, Iacovoni JS, Daburon V, Miller KM, Jackson SP, et al. 2014. Transcriptionally active chromatin recruits homologous recombination at DNA double-strand breaks. *Nat Struct Mol Biol* **21**: 366–74.
- Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, Melauchlan H, Klevernic I, Arthur JSC, Alessi DR, Cohen P. 2007. The selectivity of protein kinase inhibitors: a further update. *Biochem J* **408**: 297–315.
- Bateman A, Martin MJ, O'Donovan C, Magrane M, Alpi E, Antunes R, Bely B, Bingley M, Bonilla C, Britto R, et al. 2017. UniProt: The universal protein knowledgebase. *Nucleic Acids Res* **45**: D158–D169.
- Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, Cohn MA, Cantley LC, Gygi SP. 2004. Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc Natl Acad Sci USA* **101**: 12130–12135.
- Bedford DC, Kasper LH, Fukuyama T, Brindle PK. 2010. Target gene context influences the transcriptional requirement for the KAT3 family of CBP and p300 histone acetyltransferases. *Epigenetics* **5**: 9–15.
- Berlin I, Denat L, Steunou A-L, Puig I, Champeval D, Colombo S, Roberts K, Bonvin E, Bourgeois Y, Davidson I, et al. 2012. Phosphorylation of BRN2 Modulates Its Interaction with the Pax3 Promoter To Control Melanocyte Migration and Proliferation. *Mol Cell Biol* **32**: 1237–1247.
- Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. 2000. The protein data bank. *Nucleic Acids Res* **28**: 235–242.
- Bertolotto C, Abbe P, Hemesath TJ, Bille K, Fisher DE, Ortonne JP, Ballotti R. 1998. Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes. *J Cell Biol* **142**: 827–835.
- Bhoomik A, Takahashi S, Breitweiser W, Shiloh Y, Jones N, Ronai Z. 2005. ATM-dependent phosphorylation of ATF2 is required for the DNA damage response. *Mol Cell* **18**: 577–587.
- Biondi RM, Nebreda AR. 2003. Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. *Biochem J* **372**: 1–13.
- Bishop JL, Thaper D, Vahid S, Davies A, Ketola K, Kuruma H, Jama R, Nip KM, Angeles A,

- Johnson F, et al. 2016. The Master Neural Transcription Factor BRN2 is an Androgen Receptor Suppressed Driver of Neuroendocrine Differentiation in Prostate Cancer. *Cancer Discov.*
- Blackford A, Jackson SP. 2017. ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response. *Mol Cell* 801–817.
- Blastyak A, Hajdu I, Unk I, Haracska L. 2010. Role of Double-Stranded DNA Translocase Activity of Human HLTf in Replication of Damaged DNA. *Mol Cell Biol* 30: 684–693.
- Blaud M, Vossen C, Joseph G, Alazard R, Erard M, Nieto L. 2004. Characteristic patterns of N Oct-3 binding to a set of neuronal promoters. *J Mol Biol* 339: 1049–1058.
- Blum ES, Schwendeman AR, Shaham S. 2013. PolyQ disease: Misfiring of a developmental cell death program? *Trends Cell Biol* 23: 168–174.
- Bodner M, Castrillo JL, Theill LE, Deerinck T, Ellisman M, Karin M. 1988. The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell* 55: 505–518.
- Boija A, Mahat DB, Zare A, Holmqvist PH, Philip P, Meyers DJ, Cole PA, Lis JT, Stenberg P, Mannervik M. 2017. CBP Regulates Recruitment and Release of Promoter-Proximal RNA Polymerase II. *Mol Cell* 68: 491–503.e5.
- Bonvin E, Falletta P, Shaw H, Delmas V, Goding CR. 2012. A Phosphatidylinositol 3-Kinase-Pax3 Axis Regulates Brn-2 Expression in Melanoma. *Mol Cell Biol* 32: 4674–4683.
- Bos JL, Fearon ER, Hamilton SR, Verlaan-de Vries M, van Boom JH, van der Eb AJ, Vogelstein B. 1987. Prevalence of ras gene mutations in human colorectal cancers. *Nature* 327: 293–7.
- Bosserhoff AK, Ellmann L, Kuphal S. 2011. Melanoblasts in culture as an in vitro system to determine molecular changes in melanoma. *Exp Dermatol* 20: 435–440.
- Boubriak II, Malhas AN, Drozd MM, Pytowski L, Vaux DJ. 2017. Stress-induced release of Oct-1 from the nuclear envelope is mediated by JNK phosphorylation of lamin B1. *PLoS One* 12: 1–22.
- Boyle GM, Woods SL, Bonazzi VF, Stark MS, Hacker E, Aoude LG, Dutton-Regester K, Cook AL, Sturm R a., Hayward NK. 2011. Melanoma cell invasiveness is regulated by miR-211 suppression of the BRN2 transcription factor. *Pigment Cell Melanoma Res* 24: 525–537.
- Brash DE. 2015. UV signature mutations. *Photochem Photobiol* 91: 15–26.
- Britton S, Coates J, Jackson SP. 2013. A new method for high-resolution imaging of Ku foci to decipher mechanisms of DNA double-strand break repair. *J Cell Biol* 202.
- Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, Kyle S, Meuth M, Curtin NJ, Helleday T. 2005. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 434: 913–917.
- Bulavin D V. 1999. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. *EMBO J* 18: 6845–6854.
- Bulavin D V., Higashimoto Y, Popoff IJ, Gaarde WA, Basrur V, Potapova O, Appella E, Fornace AJ. 2001. Initiation of a G2/M checkpoint after ultraviolet radiation requires p38

- kinase. *Nature* **411**: 102–107.
- Cabos-Siguier B, Steunou AL, Joseph G, Alazard R, Ducoux-Petit M, Nieto L, Monsarrat B, Erard M, Clottes E. 2009. Expression and purification of human full-length N Oct-3, a transcription factor involved in melanoma growth. *Protein Expr Purif* **64**: 39–46.
- Caelles C, Hennemann H, Karin M. 1995. M-phase-specific phosphorylation of the POU transcription factor GHF-1 by a cell cycle-regulated protein kinase inhibits DNA binding. *Mol Cell Biol* **15**: 6694–6701.
- Calbo J, Meuwissen R, Montfort E V., Tellingén O V., Berns A. 2005. Genotype-Phenotype Relationships in a Mouse Model for Human Small-Cell Lung Cancer. *Cold Spring Harb Symp Quant Biol* **70**: 225–232.
- Caron P, Aymard F, Iacovoni JS, Briois S, Canitrot Y, Bugler B, Massip L, Losada A, Legube G. 2012. Cohesin protects genes against γ H2AX induced by DNA double-strand breaks. *PLoS Genet* **8**.
- Carr J, Mackie RM. 1994. Point mutations in the Nras oncogene in malignant melanoma and congenital naevi. *Br J Dermatol* **131**: 72–77.
- Carreira S, Goodall J, Denat L, Rodriguez M, Nuciforo P, Hoek KS, Testori A, Larue L, Goding CR. 2006. Mitf regulation of Dia1 controls melanoma proliferation and invasiveness. *Genes Dev* **20**: 3426–3439.
- Carter SL, Eklund AC, Kohane IS, Harris LN, Szallasi Z. 2006. A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. *Nat Genet* **38**: 1043–1048.
- Castro DS, Skowronska-Krawczyk D, Armant O, Donaldson IJ, Parras C, Hunt C, Critchley J a., Nguyen L, Gossler A, Göttgens B, et al. 2006. Proneural bHLH and Brn Proteins Coregulate a Neurogenic Program through Cooperative Binding to a Conserved DNA Motif. *Dev Cell* **11**: 831–844.
- Cellurale C, Sabio G, Kennedy NJ, Das M, Barlow M, Sandy P, Jacks T, Davis RJ. 2011. Requirement of c-Jun NH 2 -Terminal Kinase for Ras-Initiated Tumor Formation. *Mol Cell Biol* **31**: 1565–1576.
- Certel SJ. 2004. Specification of Drosophila motoneuron identity by the combinatorial action of POU and LIM-HD factors. *Development* **131**: 5429–5439.
- Chailleux C, Aymard F, Caron P, Daburon V, Courilleau C, Canitrot Y, Legube G, Trouche D. 2014. Quantifying DNA double-strand breaks induced by site-specific endonucleases in living cells by ligation-mediated purification. *Nat Protoc* **9**: 517–528.
- Cheli Y, Guiliano S, Botton T, Rocchi S, Hofman V, Hofman P, Bahadoran P, Bertolotto C, Ballotti R. 2011. Mitf is the key molecular switch between mouse or human melanoma initiating cells and their differentiated progeny. *Oncogene* **30**: 2307–2318.
- Chen J, Zhu F, Weeks RL, Biswas AK, Guo R, Li Y, Johnson DG. 2011. E2F1 promotes the recruitment of DNA repair factors to sites of DNA double-strand breaks. *Cell Cycle* **10**: 1287–1294.
- Chen W, Bowden GT. 1999. Activation of p38 MAP kinase and ERK are required for ultraviolet-B induced c-fos gene expression in human keratinocytes. *Oncogene* **18**: 7469–

7476.

- Chiou Y-Y, Hu J, Sancar A, Selby CP. 2017. RNA polymerase II is released from the DNA template during transcription-coupled repair in mammalian cells. *J Biol Chem* **293**: 2476–2486.
- Choi H, Larsen B, Lin Z-Y, Breitzkreutz A, Mellacheruvu D, Fermin D, Qin ZS, Tyers M, Gingras A-C, Nesvizhskii AI. 2011. SAINT: probabilistic scoring of affinity purification-mass spectrometry data. *Nat Methods* **8**: 70–73.
- Choi JH, Besaratinia A, Lee DH, Lee CS, Pfeifer GP. 2006. The role of DNA polymerase ϵ in UV mutational spectra. *Mutat Res* **599**: 58–65.
- Christensen GL, Kelstrup CD, Lyngsø C, Sarwar U, Bøgebo R, Sheikh SP, Gammeltoft S, Olsen J V., Hansen JL. 2010. Quantitative Phosphoproteomics Dissection of Seven-transmembrane Receptor Signaling Using Full and Biased Agonists. *Mol Cell Proteomics* **9**: 1540–1553.
- Chuang W, Sharma A, Shukla P, Li G, Mall M, Rajarajan K, Abilez OJ, Hamaguchi R, Wu JC, Wernig M, et al. 2017. Partial Reprogramming of Pluripotent Stem Cell-Derived Cardiomyocytes into Neurons. *Sci Rep* **7**: 44840.
- Ciccia A, Elledge SJ. 2010. The DNA Damage Response: Making It Safe to Play with Knives. *Mol Cell* **40**: 179–204.
- Cinelli R a, Ferrari A, Pellegrini V, Tyagi M, Giacca M, Beltram F. 2000. The Enhanced Green Fluorescent Protein as a Tool for the Analysis of Protein Dynamics and Localization: Local Fluorescence Study at the Single-molecule Level. *Photochem Photobiol* **71**: 771–776.
- Clarke S. 1993. Protein Methylation. *Curr Opin Cell Biol* **5**: 977–983.
- Cleaver JE. 2005. Opinion: Cancer in xeroderma pigmentosum and related disorders of DNA repair. *Nat Rev Cancer* **5**: 564–573.
- Colombino M, Capone M, Lissia A, Cossu A, Rubino C, De Giorgi V, Massi D, Fonsatti E, Staibano S, Nappi O, et al. 2012. BRAF/NRAS mutation frequencies among primary tumors and metastases in patients with melanoma. *J Clin Oncol* **30**: 2522–2529.
- Compe E, Egly JM. 2012. TFIIH: When transcription met DNA repair. *Nat Rev Mol Cell Biol* **13**: 343–354.
- Cook AL, Donatien PD, Smith AG, Murphy M, Jones MK, Herlyn M, Bennett DC, Leonard JH, Sturm R a. 2003. Human melanoblasts in culture: expression of BRN2 and synergistic regulation by fibroblast growth factor-2, stem cell factor, and endothelin-3. *J Invest Dermatol* **121**: 1150–9.
- Cook AL, Smith AG, Smit DJ, Leonard JH, Sturm RA. 2005. Co-expression of SOX9 and SOX10 during melanocytic differentiation in vitro. *Exp Cell Res* **308**: 222–235.
- Corre S, Primot A, Sviderskaya E, Bennett DC, Vaultont S, Goding CR, Galibert MD. 2004. UV-induced expression of key component of the tanning process, the POMC and MC1R genes, is dependent on the p-38-activated upstream stimulating factor-1 (USF-1). *J Biol Chem* **279**: 51226–51233.

- Cox PM, Temperley SM, Kumar H, Goding CR. 1988. A distinct octamer-binding protein present in malignant melanoma cells. *Nucleic Acids Res* **16**: 11047–11056.
- Creixell P, Schoof EM, Tan CSH, Linding R. 2012. Research article: Mutational properties of amino acid residues: implications for evolvability of phosphorylatable residues. *Philos Trans R Soc B Biol Sci* **367**: 3058–3058.
- Crivat G, Taraska JW. 2012. Imaging proteins inside cells with fluorescent tags. *Trends Biotechnol* **30**: 8–16.
- Cruz GMS, Kong X, Silva BA, Khatibzadeh N, Thai R, Berns MW, Yokomori K. 2015. Femtosecond near-infrared laser microirradiation reveals a crucial role for PARP signaling on factor assemblies at DNA damage sites. *Nucleic Acids Res* **44**.
- Cuadrado A, Lafarga V, Cheung PCF, Dolado I, Llanos S, Cohen P, Nebreda AR. 2007. A new p38 MAP kinase-regulated transcriptional coactivator that stimulates p53-dependent apoptosis. *EMBO J* **26**: 2115–2126.
- Cui Y, Sun Y, Hu S, Luo J, Li L, Li X, Yeh S, Jin J, Chang C. 2016. Neuroendocrine prostate cancer (NEPCa) increased the neighboring PCa chemoresistance via altering the PTHrP / p38 / Hsp27 / androgen receptor (AR)/ p21 signals. *Oncogene* **35**: 6065–6076.
- Curran K, Lister JA, Kunkel GR, Prendergast A, Parichy DM, Raible DW. 2010. Interplay between Foxd3 and Mitf regulates cell fate plasticity in the zebrafish neural crest. *Dev Biol* **344**: 107–118.
- Curran K, Raible DW, Lister JA. 2009. Foxd3 controls melanophore specification in the zebrafish neural crest by regulation of Mitf. *Dev Biol* **332**: 408–417.
- Curtin JA, Busam K, Pinkel D, Bastian BC. 2006. Somatic activation of KIT in distinct subtypes of melanoma. *J Clin Oncol* **24**: 4340–4346.
- Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, Cho K-H, Aiba S, Bröcker E-B, LeBoit PE, et al. 2005. Distinct Sets of Genetic Alterations in Melanoma. *N Engl J Med* **353**: 2135–2147.
- D’Adda di Fagagna F. 2014. A direct role for small non-coding RNAs in DNA damage response. *Trends Cell Biol* **24**: 171–178.
- Dale GE, Oefner C, D’Arcy A. 2003. The protein as a variable in protein crystallization. *J Struct Biol* **142**: 88–97.
- Davidson EH. 2010. Emerging properties of animal gene regulatory networks. *Nature* **468**: 911–920.
- Day RN, Davidson MW. 2009. The fluorescent protein palette: tools for cellular imaging. *Chem Soc Rev* **38**: 2887.
- de Feraudy S, Revet I, Bezrookove V, Feeney L, Cleaver JE. 2010. A minority of foci or pan-nuclear apoptotic staining of γ H2AX in the S phase after UV damage contain DNA double-strand breaks. *Proc Natl Acad Sci* **107**: 6870–6875.
- de Hoon MJL, Imoto S, Nolan J, Miyano S. 2004. Open source clustering software. *Bioinformatics* **20**: 1453–1454.

- Della-Maria J, Hegde ML, McNeill DR, Matsumoto Y, Tsai MS, Ellenberger T, Wilson DM, Mitra S, Tomkinson AE. 2012. The interaction between polynucleotide kinase phosphatase and the DNA repair protein XRCC1 is critical for repair of DNA alkylation damage and stable association at DNA damage sites. *J Biol Chem* **287**: 39233–39244.
- Dissanayake SK, Wade M, Johnson CE, O’Connell MP, Leotlela PD, French AD, Shah K V., Hewitt KJ, Rosenthal DT, Indig FE, et al. 2007. The Wnt5A/protein kinase C pathway mediates motility in melanoma cells via the inhibition of metastasis suppressors and initiation of an epithelial to mesenchymal transition. *J Biol Chem* **282**: 17259–17271.
- do Vale Coelho IE, Arruda DC, Taranto AG. 2016. In silico studies of the interaction between BRN2 protein and MORE DNA. *J Mol Model* **22**: 1–10.
- Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29**: 15–21.
- Dogan S, Shen R, Ang DC, Johnson ML, D’Angelo SP, Paik PK, Brzostowski EB, Riely GJ, Kris MG, Zakowski MF, et al. 2012. Molecular epidemiology of EGFR and KRAS mutations in 3,026 lung adenocarcinomas: Higher susceptibility of women to smoking-related KRAS-mutant cancers. *Clin Cancer Res* **18**: 6169–6177.
- Dolado I, Swat A, Ajenjo N, De Vita G, Cuadrado A, Nebreda AR. 2007. p38 α MAP Kinase as a Sensor of Reactive Oxygen Species in Tumorigenesis. *Cancer Cell* **11**: 191–205.
- Dominguez MH, Ayoub AE, Rakic P. 2013. POU-III transcription factors (Brn1, Brn2, and Oct6) influence neurogenesis, molecular identity, and migratory destination of upper-layer cells of the cerebral cortex. *Cereb Cortex* **23**: 2632–2643.
- Dugast C, Weber MJ. 2001. NF-Y binding is required for transactivation of neuronal aromatic L-amino acid decarboxylase gene promoter by the POU-domain protein Brn-2. *Mol Brain Res* **89**: 58–70.
- Dunn JD, Reid GE, Bruening ML. 2010. Techniques for phosphopeptide enrichment prior to analysis by mass spectrometry. *Mass Spectrom Rev* **29**: 29–54.
- Dupin E, Glavieux C, Vaigot P, Le Douarin NM. 2000. Endothelin 3 induces the reversion of melanocytes to glia through a neural crest-derived glial-melanocytic progenitor. *Proc Natl Acad Sci USA* **97**: 7882–7887.
- Dykes IM, Tempest L, Lee S-I, Turner EE. 2011. Brn3a and Islet1 Act Epistatically to Regulate the Gene Expression Program of Sensory Differentiation. *J Neurosci* **31**: 9789–9799.
- Dynek JN, Chan SM, Liu J, Zha J, Fairbrother WJ, Vucic D. 2008. Microphthalmia-associated transcription factor is a critical transcriptional regulator of melanoma inhibitor of apoptosis in melanomas. *Cancer Res* **68**: 3124–3132.
- Edelbrock MA, Kaliyaperumal S, Williams KJ. 2013. Structural, molecular and cellular functions of MSH2 and MSH6 during DNA mismatch repair, damage signaling and other noncanonical activities. *Mutat Res Mol Mech Mutagen* **743–744**: 53–66.
- Eisen T, Easty DJ, Bennett DC, Goding CR. 1995. The POU domain transcription factor Brn-2: elevated expression in malignant melanoma and regulation of melanocyte-specific gene expression. *Oncogene* **11**: 2157–2164.

- El-Khamisy SF, Masutani M, Suzuki H, Caldecott KW. 2003. A requirement for PARP-1 for the assembly or stability of XRCC1 nuclear foci at sites of oxidative DNA damage. *Nucleic Acids Res* **31**: 5526–5533.
- Erickson CA, Goins TL. 1995. Avian neural crest cells can migrate in the dorsolateral path only if they are specified as melanocytes. *Development* **121**: 915–24.
- Falletta P, Sanchez-del-Campo L, Chauhan J, Effern M, Kenyon A, Kershaw CJ, Siddaway R, Lisle R, Freter R, Daniels MJ, et al. 2017. Translation reprogramming is an evolutionarily conserved driver of phenotypic plasticity and therapeutic resistance in melanoma. *Genes Dev* **31**: 1–16.
- Fink SL, Cookson BT, Fink SL, Cookson BT. 2005. Apoptosis , Pyroptosis , and Necrosis : Mechanistic Description of Dead and Dying Eukaryotic Cells. *Infect Immun* **73**: 1907–1916.
- Finney M, Ruvkun G. 1990. The unc-86 gene product couples cell lineage and cell identity in *C. elegans*. *Cell* **63**: 895–905.
- Flaherty K, Puzanov I, Kim K, Ribas A, McArthur G, Sosman J, O'Dwyer P, Lee R, Grippo J, Nolop K, et al. 2010. Inhibition of Mutated, Activated BRAF in Metastatic Melanoma. *N Engl J Med* **363**: 809–819.
- Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, Hamid O, Schuchter L, Cebon J, Ibrahim N, et al. 2012. Combined BRAF and MEK Inhibition in Melanoma with BRAF V600 Mutations. *N Engl J Med* **367**: 1694–1703.
- Flammiger A, Besch R, Cook AL, Maier T, Sturm R a, Berking C. 2009. SOX9 and SOX10 but not BRN2 are required for nestin expression in human melanoma cells. *J Invest Dermatol* **129**: 945–953.
- Fletcher C, Heintz N, Roeder RG. 1987. Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. *Cell* **51**: 773–81.
- Fluegen G, Avivar-Valderas A, Wang Y, Padgen MR, Williams JK, Nobre AR, Calvo V, Cheung JF, Bravo-Cordero JJ, Entenberg D, et al. 2017. Phenotypic heterogeneity of disseminated tumour cells is preset by primary tumour hypoxic microenvironments. *Nat Cell Biol* **19**.
- Fong YW, Cattoglio C, Tjian R. 2013. The Intertwined Roles of Transcription and Repair Proteins. *Mol Cell* **52**: 291–302.
- Forster MJ. 2002. Molecular modelling in structural biology. *Micron* **33**: 365–384.
- Fousteri M, Vermeulen W, van Zeeland AA, Mullenders LHF. 2006. Cockayne Syndrome A and B Proteins Differentially Regulate Recruitment of Chromatin Remodeling and Repair Factors to Stalled RNA Polymerase II In Vivo. *Mol Cell* **23**: 471–482.
- Franco-Iborra S, Vila M, Perier C. 2016. The Parkinson Disease Mitochondrial Hypothesis. *Neurosci* **22**: 266–277.
- Franz-Wachtel M, Eisler SA, Krug K, Wahl S, Carpy A, Nordheim A, Pfizenmaier K, Haussler A, Macek B. 2012. Global Detection of Protein Kinase D-dependent Phosphorylation Events in Nocodazole-treated Human Cells. *Mol Cell Proteomics* **11**: 160–170.

- Fuhs SR, Hunter T. 2017. pHisphorylation: the emergence of histidine phosphorylation as a reversible regulatory modification. *Curr Opin Cell Biol* **45**: 8–16.
- Gaddameedhi S, Kemp MG, Reardon JT, Shields JM, Smith-Roe SL, Kaufmann WK, Sancar A. 2010. Similar nucleotide excision repair capacity in melanocytes and melanoma cells. *Cancer Res* **70**: 4922–4930.
- Galibert M-D, Carreira S, Goding C. 2001. The Usf-1 transcription factor is a novel target for the stress- responsive p38 kinase and mediates UV-induced Tyrosinase expression. *EMBO J* **20**: 5022–5031.
- Garza AMS, Khan SH, Kumar R. 2010. Site-Specific Phosphorylation Induces Functionally Active Conformation in the Intrinsically Disordered N-Terminal Activation Function (AF1) Domain of the Glucocorticoid Receptor. *Mol Cell Biol* **30**: 220–230.
- Gascoyne DM, Thomas GR, Latchman DS. 2004. The effects of Brn-3a on neuronal differentiation and apoptosis are differentially modulated by EWS and its oncogenic derivative EWS/Fli-1. *Oncogene* **23**: 3830–3840.
- Gauci S, Helbig AO, Slijper M, Krijgsveld J, Heck AJR, Mohammed S. 2009. Lys-N and trypsin cover complementary parts of the phosphoproteome in a refined SCX-based approach. *Anal Chem* **81**: 4493–4501.
- Gazdar AF, Bunn PA, Minna JD. 2017. Small-cell lung cancer: What we know, what we need to know and the path forward. *Nat Rev Cancer* **17**: 725–737.
- Gehring WJ. 1993. Exploring the homeobox. *Gene* **135**: 215–221.
- George J, Lim JS, Jang SJ, Cun Y, Ozretić L, Kong G, Leenders F, Lu X, Fernández-Cuesta L, Bosco G, et al. 2015. Comprehensive genomic profiles of small cell lung cancer. *Nature* **524**: 47–53.
- Giebel LB, Spritz RA. 1991. Mutation of the KIT (mast/stem cell growth factor receptor) protooncogene in human piebaldism. *Genetics* **88**: 8696–8699.
- Giuliano S, Cheli Y, Ohanna M, Bonet C, Beuret L, Bille K, Loubat A, Hofman V, Hofman P, Ponzio G, et al. 2010. Microphthalmia-associated transcription factor controls the DNA damage response and a lineage-specific senescence program in melanomas. *Cancer Res* **70**: 3813–3822.
- Glaab WE, Tindall KR. 1997. Mutation rate at the hprt locus in human cancer cell lines with specific mismatch repair-gene defects. *Carcinogenesis* **18**: 1–8.
- Goodall J, Carreira S, Denat L, Kobi D, Davidson I, Nuciforo P, Sturm R a., Larue L, Goding CR. 2008. Brn-2 represses microphthalmia-associated transcription factor expression and marks a distinct subpopulation of microphthalmia-associated transcription factor-negative melanoma cells. *Cancer Res* **68**: 7788–7794.
- Goodall J, Martinuzzi S, Dexter TJ, Champeval D, Carreira S, Larue L, Goding CR. 2004a. Brn-2 expression controls melanoma proliferation and is directly regulated by beta-catenin. *Mol Cell Biol* **24**: 2915–2922.
- Goodall J, Wellbrock C, Dexter TJ, Roberts K, Marais R, Goding CR. 2004b. The Brn-2 transcription factor links activated BRAF to melanoma proliferation. *Mol Cell Biol* **24**: 2923–31.

- Gorczyca W, Gong J, Darzynkiewicz Z. 1993. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyltransferase and nick translation assays. *Cancer Res* **53**: 1945–1951.
- Gruber CA, Rhee JM, Gleiberman A, Turner EE. 1997. POU domain factors of the Brn-3 class recognize functional DNA elements which are distinctive, symmetrical, and highly conserved in evolution. *Mol Cell Biol* **17**: 2391–2400.
- Gu Y, Parker A, Wilson TM, Bai H, Chang DY, Lu AL. 2002. Human MutY homolog, a DNA glycosylase involved in base excision repair, physically and functionally interacts with mismatch repair proteins human MutS homolog 2/human MutS homolog 6. *J Biol Chem* **277**: 11135–11142.
- Guo CS, Wehrle-Haller B, Rossi J, Ciment G. 1997. Autocrine regulation of neural crest cell development by steel factor. *Dev Biol* **184**: 61–9.
- Guo Y, Costa R, Ramsey H, Starnes T, Vance G, Robertson K, Kelley M, Reinbold R, Scholer H, Hromas R. 2002. The embryonic stem cell transcription factors Oct-4 and FoxD3 interact to regulate endodermal-specific promoter expression. *Proc Natl Acad Sci USA* **99**: 3663–3667.
- Gupta GP, Massagué J. 2006. Cancer Metastasis: Building a Framework. *Cell* **127**: 679–695.
- Gupta PB, Kuperwasser C, Brunet JP, Ramaswamy S, Kuo WL, Gray JW, Naber SP, Weinberg RA. 2005. The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. *Nat Genet* **37**: 1047–1054.
- Gupta S, Barrett T, Whitmarsh a J, Cavanagh J, Sluss HK, Dériard B, Davis RJ. 1996. Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO J* **15**: 2760–70.
- Gutiérrez-Uzquiza Á, Arechederra M, Bragado P, Aguirre-Ghiso JA, Porras A. 2012. p38 α mediates cell survival in response to oxidative stress via induction of antioxidant genes: Effect on the p70S6K pathway. *J Biol Chem* **287**: 2632–2642.
- Habib SJ, Chen B, Tsai F, Anastassiadis K, Meyer T, Betzig E, Nusse R. 2013. A Localized Wnt Signal Orients Asymmetric Stem Cell Division in Vitro. *Science* **339**: 1445–1448.
- Halicka HD, Huang X, Traganos F, King MA, Dai W, Darzynkiewicz Z. 2005. Histone H2AX phosphorylation after cell irradiation with UV-B: Relationship to cell cycle phase and induction of apoptosis. *Cell Cycle* **4**: 339–345.
- Han DW, Tapia N, Hermann A, Hemmer K, Höing S, Araújo-Bravo MJ, Zaehres H, Wu G, Frank S, Moritz S, et al. 2012. Direct reprogramming of fibroblasts into neural stem cells by defined factors. *Cell Stem Cell* **10**: 465–472.
- Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: The next generation. *Cell* **144**: 646–674.
- Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. *Cell* **100**: 57–70.
- Hanna LA, Foreman RK, Tarasenko IA, Kessler DS, Labosky PA. 2002. Requirement for Foxd3 in maintaining pluripotent cells of the early mouse embryo. *Genes Dev* **16**: 2650–2661.
- Hanzelmann S, Castelo R, Guinney J. 2013. GSEA: gene set variation analysis for microarray

- and RNA-Seq data. *BMC Bioinformatics* **14**: 7.
- Harris ML, Buac K, Shakhova O, Hakami RM, Wegner M, Sommer L, Pavan WJ. 2013. A Dual Role for SOX10 in the Maintenance of the Postnatal Melanocyte Lineage and the Differentiation of Melanocyte Stem Cell Progenitors. *PLoS Genet* **9**: 1–14.
- He X, Treacy MN, Simmons DM, Ingraham HA, Swanson LW, Rosenfeld MG. 1989. Expression of a large family of POU-domain regulatory genes in mammalian brain development. *Nature* **340**: 35–41.
- Heidemann M, Hintermair C, Voß K, Eick D. 2013. Dynamic phosphorylation patterns of RNA polymerase II CTD during transcription. *Biochim Biophys Acta* **1829**: 55–62.
- Helmer RA, Foreman O, Dertien JS, Panchoo M, Bhakta SM, Chilton BS. 2013. Role of Helicase-Like Transcription Factor (Hltf) in the G2 / M Transition and Apoptosis in Brain. *PLoS One* **8**: 1–13.
- Herr W, Sturm R a, Clerc RG, Corcoran LM, Baltimore D, Sharp P a, Ingraham H a, Rosenfeld MG, Ruvkun G. 1988. The POU domain : a large conserved region in the mammalian The POU domain : a large conserved region in the mammalian pit-1 , oct-1 , oct-2 , and Caenorhabditis elegans unc-86 gene products. *Genes Dev* **86**: 1513–1516.
- Hess P, Pihan G, Sawyers CL, Flavell RA, Davis RJ. 2002. Survival signaling mediated by c-Jun NH 2 -terminal kinase in transformed B lymphoblasts. **32**: 201–205.
- Hockberger PE. 2002. A History of Ultraviolet Photobiology for Humans, Animals and Microorganisms¶. *Photochem Photobiol* **76**: 561.
- Hodi FS, Chesney J, Pavlick AC, Robert C, Grossmann KF, McDermott DF, Linette GP, Meyer N, Giguere JK, Agarwala SS, et al. 2016. Combined nivolumab and ipilimumab versus ipilimumab alone in patients with advanced melanoma: 2-year overall survival outcomes in a multicentre, randomised, controlled, phase 2 trial. *Lancet Oncol* **17**: 1558–1568.
- Hodis E, Watson IR, Kryukov G V., Arold ST, Imielinski M, Theurillat JP, Nickerson E, Auclair D, Li L, Place C, et al. 2012. A landscape of driver mutations in melanoma. *Cell* **150**: 251–263.
- Hoeijmakers JHJ. 1994. Human nucleotide excision repair syndromes: Molecular clues to unexpected intricacies. *Eur J Cancer* **30**: 1912–1921.
- Hoek KS, Eichhoff OM, Schlegel NC, Döbbeling U, Kobert N, Schaerer L, Hemmi S, Dummer R. 2008. In vivo switching of human melanoma cells between proliferative and invasive states. *Cancer Res* **68**: 650–656.
- Hoek KS, Goding CR. 2010. Cancer stem cells versus phenotype-switching in melanoma. *Pigment Cell Melanoma Res* **23**: 746–759.
- Hohenauer T, Berking C, Schmidt A, Haferkamp S, Senft D, Kammerbauer C, Fraschka S, Graf SA, Irmeler M, Beckers J, et al. 2013. The neural crest transcription factor Brn3a is expressed in melanoma and required for cell cycle progression and survival. *EMBO Mol Med* **5**: 919–934.
- Holcomb J, Spellmon N, Zhang Y, Doughan M, Li C, Yang Z. 2017. Protein crystallization: Eluding the bottleneck of X-ray crystallography. *AIMS Biophys* **4**: 557–575.

- Holick MF. 1981. The cutaneous photosynthesis of previtamin D3: A unique photoendocrine system. *J Invest Dermatol* **77**: 51–58.
- Horn S, Figl A, Rachakonda PS, Fischer C, Sucker A, Gast A, Kadel S, Moll I, Nagore E, Hemminki K, et al. 2013. TERT promoter mutations in familial and sporadic melanoma. *Science* **339**: 959–961.
- Hornbeck P V., Zhang B, Murray B, Kornhauser JM, Latham V, Skrzypek E. 2015. PhosphoSitePlus, 2014: Mutations, PTMs and recalibrations. *Nucleic Acids Res* **43**: D512–D520.
- Hospital A, Goñi R, Orozco M, Gelpi J. 2015. Molecular dynamics simulations: advances and applications. *Adv Appl Bioinforma Chem* **8**: 37–47.
- Hosseini-Nezhad A, Holick MF. 2013. Vitamin D for health: A global perspective. *Mayo Clin Proc* **88**: 720–755.
- Houseley J, Tollervey D. 2009. The Many Pathways of RNA Degradation. *Cell* **136**: 763–776.
- Hsin J-P, Xiang K, Manley JL. 2014. Function and Control of RNA Polymerase II C-Terminal Domain Phosphorylation in Vertebrate Transcription and RNA Processing. *Mol Cell Biol* **34**: 2488–2498.
- Hsu PP, Kang SA, Rameseder J, Zhang Y, Ottina KA, Lim D, Peterson TR, Choi Y, Gray NS, Yaffe MB, et al. 2011. The mTOR-Regulated Phosphoproteome Reveals a Mechanism of mTORC1-Mediated Inhibition of Growth Factor Signaling. *Science* **332**: 1317–1322.
- Hu J, Adar S, Selby CP, Lieb JD, Sancar A. 2015. Genome-wide analysis of human global and transcription-coupled excision repair of UV damage at single-nucleotide resolution. *Genes Dev* **29**: 948–960.
- Hu Y, Petit SA, Ficarro SB, Toomire KJ, Xie A, Lim E, Cao SA, Park E, Eck MJ, Scully R, et al. 2014. PARP1-driven poly-ADP-ribosylation regulates BRCA1 function in homologous recombination-mediated DNA repair. *Cancer Discov* **4**: 1430–1447.
- Huang FW, Hodis E, Xu MJ, Kryukov G V., Chin L, Garraway LA. 2013. Highly recurrent TERT promoter mutations in human melanoma. *Science* **339**: 957–959.
- Huang X, Darzynkiewicz Z. 2006. Cytometric assessment of histone H2AX phosphorylation: a reporter of DNA damage. *Methods Mol Biol* **314**: 73–80.
- Huang X, Halicka HD, Traganos F, Tanaka T, Kurose A, Darzynkiewicz Z. 2005. Cytometric assessment of DNA damage in relation to cell cycle phase and apoptosis. *Cell Prolif* **38**: 223–243.
- Hudson CD, Morris PJ, Latchman DS, Budhram-mahadeo VS. 2005. Brn-3a Transcription Factor Blocks p53-mediated Activation of Proapoptotic Target Genes Noxa and Bax in Vitro and in Vivo to Determine Cell Fate. *J Biol Chem* **280**: 11851–11858.
- Iacovoni JS, Caron P, Lassadi I, Nicolas E, Massip L, Trouche D, Legube G. 2010. High-resolution profiling of γ H2AX around DNA double strand breaks in the mammalian genome. *EMBO J* **29**: 1446–1457.
- Imperial R, Toor OM, Hussain A, Subramanian J, Masood A. 2017. Comprehensive pancancer genomic analysis reveals (RTK)-RAS-RAF-MEK as a key dysregulated pathway in

- cancer: Its clinical implications. *Semin Cancer Biol* In Press.
- Inamotc S, Segil N, Pan ZQ, Kimura M, Roeder RG. 1997. The cyclin-dependent kinase-activating kinase (CAK) assembly factor, MAT1, targets and enhances CAK activity on the POU domains of Octamer transcription factors. *J Biol Chem* **272**: 29852–29858.
- Ishii J, Sato H, Sakaeda M, Shishido-Hara Y, Hiramatsu C, Kamma H, Shimoyamada H, Fujiwara M, Endo T, Aoki I, et al. 2013. POU domain transcription factor BRN2 is crucial for expression of ASCL1, ND1 and neuroendocrine marker molecules and cell growth in small cell lung cancer. *Pathol Int* **63**: 158–168.
- Ivanov VN, Ronai Z. 1999. Down-regulation of tumor necrosis factor alpha expression by activating transcription factor 2 increases UVC-induced apoptosis of late-stage melanoma cells. *J Biol Chem* **274**: 14079–14089.
- Ivanov VN, Ronai Z. 2000. p38 protects human melanoma cells from UV-induced apoptosis through down-regulation of NF- κ B activity and Fas expression. *Oncogene* **19**: 3003–3012.
- Izhar L, Adamson B, Ciccia A, Lewis J, Pontano-Vaites L, Leng Y, Liang AC, Westbrook TF, Harper JW, Elledge SJ. 2015. A Systematic Analysis of Factors Localized to Damaged Chromatin Reveals PARP-Dependent Recruitment of Transcription Factors. *Cell Rep* **11**: 1486–1500.
- Jackson SP, Bartek J. 2009. The DNA-damage response in human biology and disease. *Nature* **461**: 1071–8.
- Jacobson EM, Li P, Leon-Del-Rio A, Rosenfeld MG, Aggarwal AK. 1997. Structure of Pit-1 POU domain bound to DNA as a dimer: Unexpected arrangement and flexibility. *Genes Dev* **11**: 198–212.
- Jenuwein T, Allis CD. 2001. Translating the histone code. *Science (80)* **293**: 1074–1080.
- Jerabek S, Ng CK, Wu G, Arauzo-Bravo MJ, Kim K, Esch D, Malik V, Chen Y, Velychko S, MacCarthy CM, et al. 2017. Changing POU dimerization preferences converts Oct6 into a pluripotency inducer. *EMBO Rep* **18**: 319–333.
- Jin W, Wang L, Zhu F, Tan W, Lin W, Chen D, Sun Q, Xia Z. 2016. Critical POU domain residues confer Oct4 uniqueness in somatic cell reprogramming. *Sci Rep* **6**: 1–12.
- Jolma A, Yin Y, Nitta KR, Dave K, Popov A, Taipale M, Enge M, Kivioja T, Morgunova E, Taipale J. 2015. DNA-dependent formation of transcription factor pairs alters their binding specificity. *Nature* **527**: 384–388.
- Jungmichel S, Rosenthal F, Altmeyer M, Lukas J, Hottiger MO, Nielsen ML. 2013. Proteome-wide identification of poly(ADP-Ribosyl)ation targets in different genotoxic stress responses. *Mol Cell* **52**: 272–285.
- Kalderon D, Roberts BL, Richardson WD, Smith AE. 1984. A short amino acid sequence able to specify nuclear location. *Cell* **39**: 499–509.
- Kang J, Gemberling M, Nakamura M, Whitby FG, Handa H, Fairbrother WG, Tantin D. 2009. A general mechanism for transcription regulation by Oct1 and Oct4 in response to genotoxic and oxidative stress. *Genes Dev* **23**: 208–222.

- Kapiloff MS, Farkash Y, Wegner M, Rosenfeld MG. 1991. Variable effects of phosphorylation of Pit-1 dictated by the DNA response elements. *Science* **253**: 786–789.
- Karin M, Hunter T. 1995. Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr Biol* **5**: 747–757.
- Karplus M, McCammon JA. 2002. Molecular dynamics simulations of biomolecules. *Nat Struct Biol* **9**: 646–652.
- Kasibhatla S, Tailor P, Bonefoy-Berard N, Mustelin T, Altman A, Fotadar A. 1999. Jun kinase phosphorylates and regulates the DNA binding activity of an octamer binding protein, T-cell factor beta1. *Mol Cell Biol* **19**: 2021–2031.
- Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. 1991. Participation of p53 Protein in the Cellular Response to DNA Damage. *Cancer Res* **5**: 6304–6311.
- Kastan MB, Zhan Q, El-Deiry WS, Carrier F, Jacks T, Walsh W V., Plunkett BS, Vogelstein B, Fornace AJ. 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* **71**: 587–597.
- Kaufmann WK, Nevis KR, Qu P, Ibrahim JG, Zhou T, Zhou Y, Simpson DA, Helms-Deaton J, Cordeiro-Stone M, Moore DT, et al. 2008. Defective cell cycle checkpoint functions in melanoma are associated with altered patterns of gene expression. *J Invest Dermatol* **128**: 175–187.
- Keller A, Shteynberg D. 2011. Software Pipeline and Data Analysis for MS/MS Proteomics: The Trans-Proteomic Pipeline. In *Bioinformatics for Comparative Proteomics* (eds. C.H. Wu and C. Chen), pp. 169–189, Humana Press, Totowa, NJ.
- Kerr JFR, Wyllie AH, Currie AR. 1972. Apoptosis: a Basic Biological Phenomenon With Wide- Ranging Implications in Tissue Kinetics. *Br J Cancer* **26**: 239–257.
- Kinoshita E, Kinoshita-Kikuta E, Koike T. 2015. Advances in Phos-tag-based methodologies for separation and detection of the phosphoproteome. *Biochim Biophys Acta* **1854**: 601–8.
- Kinoshita E, Kinoshita-Kikuta E, Takiyama K, Koike T. 2006. Phosphate-binding Tag, a New Tool to Visualize Phosphorylated Proteins. *Mol Cell Proteomics* **5**: 749–757.
- Klemm JD, Rould MA, Aurora R, Herr W, Pabo CO. 1994. Crystal structure of the Oct-1 POU domain bound to an octamer site: DNA recognition with tethered DNA-binding modules. *Cell* **77**: 21–32.
- Knight JDR, Liu G, Zhang JP, Pasculescu A, Choi H, Gingras A-C. 2015. A web-tool for visualizing quantitative protein-protein interaction data. *Proteomics* **15**: 1432–1436.
- Kobi D, Steunou A-L, Dembélé D, Legras S, Larue L, Nieto L, Davidson I. 2010. Genome-wide analysis of POU3F2/BRN2 promoter occupancy in human melanoma cells reveals Kitl as a novel regulated target gene. *Pigment Cell Melanoma Res* **23**: 404–418.
- Koch CA, Agyei R, Galicia S, Metalnikov P, O'Donnell P, Starostine A, Weinfeld M, Durocher D. 2004. Xrcc4 physically links DNA end processing by polynucleotide kinase to DNA ligation by DNA ligase IV. *EMBO J* **23**: 3874–3885.
- Krepler C, Sproesser K, Brafford P, Beqiri M, Garman B, Xiao M, Shannan B, Watters A, Perego M, Zhang G, et al. 2017. A Comprehensive Patient-Derived Xenograft Collection

- Representing the Heterogeneity of Melanoma. *Cell Rep* **21**: 1953–1967.
- Krispin S, Nitzan E, Kalcheim C. 2010a. The dorsal neural tube: A dynamic setting for cell fate decisions. *Dev Neurobiol* **70**: 796–812.
- Krispin S, Nitzan E, Kassem Y, Kalcheim C. 2010b. Evidence for a dynamic spatiotemporal fate map and early fate restrictions of premigratory avian neural crest. *Development* **137**: 585–595.
- Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny M V., El-Deiry WS, Golstein P, Green DR, et al. 2009. Classification of cell death: Recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ* **16**: 3–11.
- Krysko D V., Vanden Berghe T, Parthoens E, D’Herde K, Vandenabeele P. 2008. Methods for distinguishing apoptotic from necrotic cells and measuring their clearance. In *Methods in Enzymology*, pp. 307–341.
- Kuerbitz SJ, Plunkett BS, Walsh W V., Kastan MB. 1992. Wild-Type p53 is a Cell Cycle Checkpoint Determinant Following Irradiation. *Proc Natl Acad Sci USA* **89**: 7491–7495.
- Kuhlbrodt K, Herbarth B, Sock E, Enderich J, Hermans-Borgmeyer I, Wegner M. 1998. Cooperative function of POU proteins and SOX proteins in glial cells. *J Biol Chem* **273**: 16050–16057.
- Kühne M, Riballo E, Rief N, Ku M, Rothkamm K, Jeggo PA, Lo M. 2004. A Double-Strand Break Repair Defect in ATM-Deficient Cells Contributes to Radiosensitivity. *Cancer Res* **64**: 500–508.
- Lai WKM, Pugh BF. 2017. Understanding nucleosome dynamics and their links to gene expression and DNA replication. *Nat Rev Mol Cell Biol* **18**: 548–562.
- Lambert J-P, Iovesev G, Couzens AL, Larsen B, Taipale M, Lin Z-Y, Zhong Q, Lindquist S, Vidal M, Aebersold R, et al. 2013. Mapping differential interactomes by affinity purification coupled with data-independent mass spectrometry acquisition. *Nat Methods* **10**: 1239–1245.
- Lambert JP, Tucholska M, Pawson T, Gingras AC. 2014. Incorporating DNA shearing in standard affinity purification allows simultaneous identification of both soluble and chromatin-bound interaction partners. *J Proteomics* **100**: 55–59.
- Lane DP. 1992. Cancer. p53, guardian of the genome. *Nature* **358**: 15–16.
- Laud K, Marian C, Avril MF, Barrois M, Chompret A, Goldstein AM, Tucker MA, Clark PA, Peters G, Chaudru V, et al. 2006. Comprehensive analysis of CDKN2A (p16INK4A/p14ARF) and CDKN2B genes in 53 melanoma index cases considered to be at heightened risk of melanoma. *J Med Genet* **43**: 39–47.
- Lawrence MS, Stojanov P, Polak P, Kryukov G V., Cibulskis K, Sivachenko A, Carter SL, Stewart C, Mermel CH, Roberts SA, et al. 2013. Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* **499**: 214–218.
- Lee J, Kim HK, Han YM, Kim J. 2008. Pyruvate kinase isozyme type M2 (PKM2) interacts and cooperates with Oct-4 in regulating transcription. *Int J Biochem Cell Biol* **40**: 1043–1054.

- Lefort K, Rouault JP, Tondereau L, Magaud JP, Doré JF. 2001. The specific activation of gadd45 following UVB radiation requires the POU family gene product N-Oct3 in human melanoma cells. *Oncogene* **20**: 7375–7385.
- Lengauer C, Kinzler KW, Vogelstein B. 1998. Genetic instabilities in human cancers. *Nature* **396**: 643–649.
- Li P, He X, Renee Gerrero M, Mok M, Aggarwal A, Rosenfeld MG. 1993. Spacing and orientation of bipartite DNA-binding motifs as potential functional determinants for POU domain factors. *Genes Dev* **7**: 2483–2496.
- Li R, Wu F, Ruonala R, Sapkota D, Hu Z, Mu X. 2014. Isl1 and Pou4f2 form a complex to regulate target genes in developing retinal ganglion cells. *PLoS One* **9**: 1–13.
- Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. 2015. The Molecular Signatures Database Hallmark Gene Set Collection. *Cell Syst* **1**: 417–425.
- Lim HW, Silpa-Archa N, Amadi U, Menter A, Van Voorhees AS, Lebwohl M. 2015. Phototherapy in dermatology: A call for action. *J Am Acad Dermatol* **72**: 1078–1080.
- Limoli CL, Giedzinski E, Bonner WM, Cleaver JE. 2002. UV-induced replication arrest in the xeroderma pigmentosum variant leads to DNA double-strand breaks, gamma -H2AX formation, and Mre11 relocalization. *Proc Natl Acad Sci USA* **99**: 233–238.
- Lindahl T, Satoh MS, Poirier GG, Klungland A. 1995. Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. *Trends Biochem Sci* **20**: 405–411.
- Lisby S, Gniadecki R, Wulf HC. 2005. UV-induced DNA damage in human keratinocytes: Quantitation and correlation with long-term survival. *Exp Dermatol* **14**: 349–355.
- Liu F, Fu Y, Meyskens FL. 2009. MiTF regulates cellular response to reactive oxygen species through transcriptional regulation of APE-1/Ref-1. *J Invest Dermatol* **129**: 422–31.
- Liu G, Knight JDR, Zhang JP, Tsou CC, Wang J, Lambert JP, Larsen B, Tyers M, Raught B, Bandeira N, et al. 2016. Data Independent Acquisition analysis in ProHits 4.0. *J Proteomics* **149**: 64–68.
- Liu J, Fukunaga-Kalabis M, Li L, Herlyn M. 2014. Developmental pathways activated in melanocytes and melanoma. *Arch Biochem Biophys* **563**: 13–21.
- Lodato MA, Ng CW, Wamstad JA, Cheng AW, Thai KK, Fraenkel E, Jaenisch R, Boyer LA. 2013. SOX2 Co-Occupies Distal Enhancer Elements with Distinct POU Factors in ESCs and NPCs to Specify Cell State. *PLoS Genet* **9**: 1–19.
- Luo RX, Dean DC. 1999. Chromatin Remodeling and Transcriptional Regulation. *JNCI J Natl Cancer Inst* **91**: 1288–1294.
- Malik V, Zimmer D, Jauch R. 2018. Diversity among POU transcription factors in chromatin recognition and cell fate reprogramming. *Cell Mol Life Sci*. 1–26.
- Malumbres M, Barbacid M. 2009. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer* **9**: 153–166.
- Malumbres M, Barbacid M. 2005. Mammalian cyclin-dependent kinases. *Trends Biochem Sci*

30: 630–641.

- Manke IA, Nguyen A, Lim D, Stewart MQ, Elia AEH, Yaffe MB. 2005. MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G 2/M transition and S phase progression in response to UV irradiation. *Mol Cell* **17**: 37–48.
- Mansour WY, Rhein T, Dahm-Daphi J. 2010. The alternative end-joining pathway for repair of DNA double-strand breaks requires PARP1 but is not dependent upon microhomologies. *Nucleic Acids Res* **38**: 6065–6077.
- Marteijn JA, Lans H, Vermeulen W, Hoeijmakers JHJ. 2014. Understanding nucleotide excision repair and its roles in cancer and ageing. *Nat Rev Mol Cell Biol* **15**: 465–481.
- Marti TM, Hefner E, Feeney L, Natale V, Cleaver JE. 2006. H2AX phosphorylation within the G1 phase after UV irradiation depends on nucleotide excision repair and not DNA double-strand breaks. *Proc Natl Acad Sci USA* **103**: 9891–9896.
- Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* **17**: 10.
- Martínez L. 2015. Automatic identification of mobile and rigid substructures in molecular dynamics simulations and fractional structural fluctuation analysis. *PLoS One* **10**: 1–10.
- Masaki H, Okano Y, Sakurai H. 1999. Generation of active oxygen species from advanced glycation end-products (AGEs) during ultraviolet light A (UVA) irradiation and a possible mechanism for cell damaging. *Biochim Biophys Acta* **1428**: 45–56.
- Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y, et al. 2007. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* **316**: 1160–1166.
- Matunis MJ, Coutavas E, Blobel G. 1996. A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol* **135**: 1457–1470.
- Mayanil CS. 2013. Transcriptional and epigenetic regulation of neural crest induction during neurulation. *Dev Neurosci* **35**: 361–372.
- Mayya V, Lundgren DH, Hwang S-I, Rezaul K, Wu L, Eng JK, Rodionov V, Han DK. 2009. Quantitative Phosphoproteomic Analysis of T Cell Receptor Signaling Reveals System-Wide Modulation of Protein-Protein Interactions. *Sci Signal* **2**: 1–16.
- McEvelly RJ, Erkman L, Luo L, Sawchenko PE, Ryan AF, Rosenfeld MG. 1996. Requirement for Brn-3.0 in differentiation and survival of sensory and motor neurons. *Nature* **384**: 574–577.
- McEvelly RJ, Ortiz de Diaz M, Schonemann MD, Hooshmand F, Rosenfeld MG. 2002. Transcriptional regulation of cortical neuron migration by POU domain factors. *Science* **295**: 1528–1532.
- McGill GG, Horstmann M, Widlund HR, Du J, Motyckova G, Nishimura EK, Lin YL, Ramaswamy S, Avery W, Ding HF, et al. 2002. Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. *Cell* **109**: 707–718.
- Mertins P, Qiao JW, Patel J, Udeshi ND, Clauser KR, Mani DR, Burgess MW, Gillette MA,

- Jaffe JD, Carr SA. 2013. Integrated proteomic analysis of post-translational modifications by serial enrichment. *Nat Methods* **10**: 634–637.
- Messner S, Altmeyer M, Zhao H, Pozivil A, Roschitzki B, Gehrig P, Rutishauser D, Huang D, Caflisch A, Hottiger MO. 2010. PARP1 ADP-ribosylates lysine residues of the core histone tails. *Nucleic Acids Res* **38**: 6350–6362.
- Mi H, Muruganujan A, Casagrande JT, Thomas PD. 2013. Large-scale gene function analysis with the PANTHER classification system. *Nat Protoc* **8**: 1551–66.
- Millevoi S, Thion L, Joseph G, Vossen C, Ghisolfi-Nieto L, Erard M. 2001. Atypical binding of the neuronal POU protein N-Oct3 to noncanonical DNA targets: Implications for heterodimerization with HNF-3 β . *Eur J Biochem* **268**: 781–791.
- Mitchell PJ, Tjian R. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**: 371–378.
- Miyagi S, Nishimoto M, Saito T, Ninomiya M, Sawamoto K, Okano H, Muramatsu M, Oguro H, Iwama A, Okuda A. 2006. The Sox2 regulatory region 2 functions as a neural stem cell-specific enhancer in the telencephalon. *J Biol Chem* **281**: 13374–13381.
- Moné MJ, Volker M, Nikaido O, Mullenders LHF, Van Zeeland AA, Verschure PJ, Manders EMM, Van Driel R. 2001. Local UV-induced DNA damage in cell nuclei results in local transcription inhibition. *EMBO Rep* **2**: 1013–1017.
- Morfouace M, Lalier L, Oliver L, Cheray M, Pecqueur C, Cartron P-F, Vallette FM. 2014. Control of glioma cell death and differentiation by PKM2-Oct4 interaction. *Cell Death Dis* **5**: 1–8.
- Morise H, Shimomura O, Johnson FH, Winant J. 1974. Intermolecular energy transfer in the bioluminescent system of *aequorea*. *Biochemistry* **13**: 2656–2662.
- Morris SA. 2016. Direct lineage reprogramming via pioneer factors; a detour through developmental gene regulatory networks. *Development* **143**: 2696–2705.
- Mort RL, Jackson IJ, Patton EE. 2015. The melanocyte lineage in development and disease. *Development* **142**: 1387–1387.
- Murakami T, Toda S, Fujimoto M, Ohtsuki M, Byers HR, Etoh T, Nakagawa H. 2001. Constitutive activation of Wnt/ β -catenin signaling pathway in migration-active melanoma cells: Role of LEF-1 in melanoma with increased metastatic potential. *Biochem Biophys Res Commun* **288**: 8–15.
- Naar AM, Lemon BD, Tjian R. 2001. Transcriptional Coactivator Complexes. *Annu Rev Biochem* **70**: 475–501.
- Nakai S, Kawano H, Yudate T, Nishi M, Kuno J, Nagata A, Jishage K, Hamada H, Fujii H, Kawamura K, et al. 1995. The POU domain transcription factor Brn-2 is required for the determination of specific neuronal lineages in the hypothalamus of the mouse. *Genes Dev* **9**: 3109–3121.
- Nasti TH, Timares L. 2015. MC1R, eumelanin and pheomelanin: Their role in determining the susceptibility to skin cancer. *Photochem Photobiol* **91**: 188–200.
- Neiswender J V., Kortum RL, Bourque C, Kasheta M, Zon LI, Morrison DK, Ceol CJ. 2017.

- KIT suppresses BRAFV600E-mutant melanoma by attenuating oncogenic RAS/MAPK signaling. *Cancer Res* **77**: 5820–5830.
- Nicholson DW. 1999. Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ* **6**: 1028–1042.
- Nieto L, Joseph G, Stella A, Henri P, Burlet-Schiltz O, Monsarrat B, Clottes E, Erard M. 2007. Differential Effects of Phosphorylation on DNA Binding Properties of N Oct-3 Are Dictated by Protein/DNA Complex Structures. *J Mol Biol* **370**: 687–700.
- Nishi H, Hashimoto K, Panchenko AR. 2011. Phosphorylation in protein-protein binding: Effect on stability and function. *Structure* **19**: 1807–1815.
- Nitzan E, Krispin S, Pfaltzgraff ER, Klar A, Labosky PA, Kalcheim C. 2013. A dynamic code of dorsal neural tube genes regulates the segregation between neurogenic and melanogenic neural crest cells. *Development* **140**: 2269–2279.
- Niwa H. 2018. The principles that govern transcription factor network functions in stem cells. *Development* **145**: 1–13.
- Notredame C, Higgins DG, Heringa J. 2000. T-coffee: a novel method for fast and accurate multiple sequence alignment 1 Edited by J. Thornton. *J Mol Biol* **302**: 205–217.
- Olive PL, Banáth JP. 2006. The comet assay: a method to measure DNA damage in individual cells. *Nat Protoc* **1**: 23–9.
- Olsen J V., Blagoev B, Gnäd F, Macek B, Kumar C, Mortensen P, Mann M. 2006. Global, In Vivo, and Site-Specific Phosphorylation Dynamics in Signaling Networks. *Cell* **127**: 635–648.
- Ong S-E, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. 2002. Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC, as a Simple and Accurate Approach to Expression Proteomics. *Mol Cell Proteomics* **1**: 376–386.
- Ono K, Han J. 2000. The p38 signal transduction pathway: activation and function. *Cell Signal* **12**: 1–13.
- Opdecamp K, Nakayama A, Nguyen MT, Hodgkinson C A, Pavan WJ, Arnheiter H. 1997. Melanocyte development in vivo and in neural crest cell cultures: crucial dependence on the Mitf basic-helix-loop-helix-zipper transcription factor. *Development* **124**: 2377–2386.
- Ott PA, Hu Z, Keskin DB, Shukla SA, Sun J, Bozym DJ, Zhang W, Luoma A, Giobbie-Hurder A, Peter L, et al. 2017. An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature* **547**: 217–221.
- Overmeer RM, Gourdin AM, Giglia-Mari A, Kool H, Houtsmuller AB, Siegal G, Fousteri MI, Mullenders LHF, Vermeulen W. 2010. Replication Factor C Recruits DNA Polymerase δ to Sites of Nucleotide Excision Repair but Is Not Required for PCNA Recruitment. *Mol Cell Biol* **30**: 4828–4839.
- Palmieri G, Colombino M, Cossu A, Marchetti A, Botti G, Ascierto PA. 2017. Genetic instability and increased mutational load: which diagnostic tool best direct patients with cancer to immunotherapy? *J Transl Med* **15**: 17.
- Pandey P, Raingeaud J, Kaneki M, Weichselbaum R, Davis RJ, Kufe D, Kharbanda S. 1996.

- Activation of p38 Mitogen-activated Protein Kinase by c-Abl-dependent and -independent Mechanisms. *J Biol Chem* **271**: 23775–23779.
- Park K-S, Liang M-C, Raiser DM, Zamponi R, Roach RR, Curtis SJ, Walton Z, Schaffer BE, Roake CM, Zmoos A-F, et al. 2011. Characterization of the cell of origin for small cell lung cancer. *Cell Cycle* **10**: 2806–2815.
- Paulson H. 2018. Repeat expansion diseases. *Handb Clin Neurol* **147**: 105–123.
- Pearlman SM, Serber Z, Ferrell JE. 2011. A mechanism for the evolution of phosphorylation sites. *Cell* **147**: 934–946.
- Pedoux R, Lefort K, Cuenin C, Cortes U, Kellner K, Doré J-F, Nakazawa H. 2002. Specific induction of gadd45 in human melanocytes and melanoma cells after UVB irradiation. *Int J Cancer* **98**: 811–6.
- Pereira L, Igea A, Canovas B, Dolado I, Nebreda AR. 2013. Inhibition of p38 MAPK sensitizes tumour cells to cisplatin-induced apoptosis mediated by reactive oxygen species and JNK. *EMBO Mol Med* **5**: 1759–1774.
- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. 2004. UCSF Chimera - A visualization system for exploratory research and analysis. *J Comput Chem* **25**: 1605–1612.
- Pevny LH, Lovell-Badge R. 1997. Sox genes find their feet. *Curr Opin Genet Dev* **7**: 338–344.
- Pfeifer GP, You Y-H, Besaratinia A. 2005. Mutations induced by ultraviolet light. *Mutat Res* **571**: 19–31.
- Phillips K, Luisi B. 2000. The virtuoso of versatility: POU proteins that flex to fit. *J Mol Biol* **302**: 1023–1039.
- Phong MS, Van Horn RD, Li S, Tucker-Kellogg G, Surana U, Ye XS. 2010. p38 mitogen-activated protein kinase promotes cell survival in response to DNA damage but is not required for the G(2) DNA damage checkpoint in human cancer cells. *Mol Cell Biol* **30**: 3816–26.
- Pines A, Vrouwe MG, Marteiijn JA, Typas D, Luijsterburg MS, Cansoy M, Hensbergen P, Deelder A, de Groot A, Matsumoto S, et al. 2012. PARP1 promotes nucleotide excision repair through DDB2 stabilization and recruitment of ALC1. *J Cell Biol* **199**: 235–249.
- Pinner S, Jordan P, Sharrock K, Bazley L, Collinson L, Marais R, Bonvin E, Goding C, Sahai E. 2009. Intravital imaging reveals transient changes in pigment production and Brn2 expression during metastatic melanoma dissemination. *Cancer Res* **69**: 7969–7977.
- Pleasance ED, Cheetham RK, Stephens PJ, McBride DJ, Humphray SJ, Greenman CD, Varela I, Lin ML, Ordóñez GR, Bignell GR, et al. 2010. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature* **463**: 191–196.
- Pollock PM, Harper UL, Hansen KS, Yudt LM, Stark M, Robbins CM, Moses TY, Hostetter G, Wagner U, Kakareka J, et al. 2003. High frequency of BRAF mutations in nevi. *Nat Genet* **33**: 19–20.
- Polo S, Jackson S. 2011. Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. *Genes Dev* **25**: 409–33.

- Premi S, Wallisch S, Manu CM, Weiner AB, Bacchiocchi A, Wakamatsu K, Bechara EJ, Halaban R, Douki T, Brash DE. 2015. Chemiexcitation of melanin derivatives induces DNA photoproducts long after UV exposure. *Science* **347**: 842–47.
- Recio JA, Merlino G. 2002. Hepatocyte growth factor/scatter factor activates proliferation in melanoma cells through p38 MAPK, ATF-2 and cyclin D1. *Oncogene* **21**: 1000–1008.
- Reddy BY, Miller DM, Tsao H. 2017. Somatic driver mutations in melanoma. *Cancer* **123**: 2104–2117.
- Reinhardt HC, Aslanian AS, Lees JA, Yaffe MB. 2007. p53-Deficient Cells Rely on ATM- and ATR-Mediated Checkpoint Signaling through the p38MAPK/MK2 Pathway for Survival after DNA Damage. *Cancer Cell* **11**: 175–189.
- Reiter F, Wienerroither S, Stark A. 2017. Combinatorial function of transcription factors and cofactors. *Curr Opin Genet Dev* **43**: 73–81.
- Reno EM, Haughian JM, Jackson TA, Thorne AM, Bradford AP. 2009. c-Jun N-terminal kinase regulates apoptosis in endometrial cancer cells. *Apoptosis* **14**: 809–820.
- Rhee JM, Gruber CA, Brodie TB, Trieu M, Turner EE. 1998. Highly cooperative homodimerization is a conserved property of neural POU proteins. *J Biol Chem* **273**: 34196–34205.
- Riley T, Sontag E, Chen P, Levine A. 2008. Transcriptional control of human p53-regulated genes. *Nat Rev Mol Cell Biol* **9**: 402–412.
- Roberts SB, Segil N, Heintz N. 1991. Differential Phosphorylation of the Transcription Factor Oct1 During the Cell Cycle. *Science* **253**: 1022–1026.
- Robinson MD, McCarthy DJ, Smyth GK. 2009. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**: 139–140.
- Robles-Espinoza CD, Roberts ND, Chen S, Leacy FP, Alexandrov LB, Pornputtapong N, Halaban R, Krauthammer M, Cui R, Timothy Bishop D, et al. 2016. Germline MC1R status influences somatic mutation burden in melanoma. *Nat Commun* **7**: 1–7.
- Roesch A. 2014. Tumor heterogeneity and plasticity as elusive drivers for resistance to MAPK pathway inhibition in melanoma. *Oncogene* **34**: 1–7.
- Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. 1998. Double-stranded Breaks Induce Histone H2AX phosphorylation on Serine 139. *J Biol Chem* **273**: 5858–5868.
- Roos WP, Kaina B. 2013. DNA damage-induced cell death : From specific DNA lesions to the DNA damage response and apoptosis. *Cancer Lett* **332**: 237–248.
- Roux PP, Blenis J. 2004. ERK and p38 MAPK-Activated Protein Kinases : a Family of Protein Kinases with Diverse Biological Functions. *Microbiol Mol Biol Rev* **68**: 320–344.
- Rughani MG, Gupta A, Middleton MR. 2013. New treatment approaches in melanoma: current research and clinical prospects. *Ther Adv Med Oncol* **5**: 73–80.
- Ruscetti T, Lehnert BE, Halbrook J, Trong H Le, Hoekstra MF, Chen DJ, Peterson SR. 1998. Stimulation of the DNA-dependent Protein Kinase by Poly (ADP-Ribose) Polymerase. *J Biol Chem* **273**: 14461–14467.

- Sakaeda M, Sato H, Ishii J, Miyata C, Kamma H, Shishido-Hara Y, Shimoyamada H, Fujiwara M, Endo T, Tanaka R, et al. 2013. Neural lineage-specific homeoprotein BRN2 is directly involved in TTF1 expression in small-cell lung cancer. *Lab Invest* **93**: 408–21.
- Sakurai T, Maeda S, Chang L, Karin M. 2006. Loss of hepatic NF-kappa B activity enhances chemical hepatocarcinogenesis through sustained c-Jun N-terminal kinase 1 activation. *Proc Natl Acad Sci U S A* **103**: 10544–51.
- Saldanha AJ. 2004. Java Treeview - Extensible visualization of microarray data. *Bioinformatics* **20**: 3246–3248.
- Sancar A, Lindsey-Boltz LA, Ünsal-Kaçmaz K, Linn S. 2004. Molecular Mechanisms of Mammalian DNA Repair and the DNA Damage Checkpoints. *Annu Rev Biochem* **73**: 39–85.
- Sanlorenzo M, Vujic I, Posch C, Ma J, Lin K, Lai K, Lee D, Vujic M, Oses-Prieto JA, Chand S, et al. 2016. Oncogenic KIT mutations in different exons lead to specific changes in melanocyte phospho-proteome. *J Proteomics* **144**: 140–147.
- Satoh MS, Lindahl T. 1992. Role of poly(ADP-ribose) formation in DNA repair. *Nature* **356**: 356–358.
- Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle D, Smith S, Uziel T, Sfez S, et al. 1995. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* **268**: 1749–1753.
- Schild-Poulter C, Pope L, Giffin W, Kochan JC, Ngsee JK, Traykova-Andonova M, Haché RJG. 2001. The Binding of Ku Antigen to Homeodomain Proteins Promotes Their Phosphorylation by DNA-dependent Protein Kinase. *J Biol Chem* **276**: 16848–16856.
- Schild-Poulter C, Shih A, Tantin D, Yarymowich NC, Soubeyrand S, Sharp PA, Haché RJG. 2007. DNA-PK phosphorylation sites on Oct-1 promote cell survival following DNA damage. *Oncogene* **26**: 3980–3988.
- Schild-Poulter C, Shih A, Yarymowich NC, Haché RJG. 2003. Down-regulation of histone H2B by DNA-dependent protein kinase in response to DNA damage through modulation of octamer transcription factor 1. *Cancer Res* **63**: 7197–205.
- Schlessinger J. 2000. Cell Signaling by Receptor Tyrosine Kinases A large group of genes in all eukaryotes encode for. *Cell* **103**: 211–225.
- Schonemann MD, Ryan AK, McEvilly RJ, O'Connell SM, Arias CA, Kalla KA, Li P, Sawchenko PE, Rosenfeld MG. 1995. Development and survival of the endocrine hypothalamus and posterior pituitary gland requires the neuronal POU domain factor Brn-2. *Genes Dev* **9**: 3122–3135.
- Schreck I, Grico N, Hansjosten I, Marquardt C, Bormann S, Seidel A, Kvietkova DL, Pieniazek D, Segerbäck D, Diabaté S, et al. 2016. The nucleotide excision repair protein XPC is essential for bulky DNA adducts to promote interleukin-6 expression via the activation of p38-SAPK. *Oncogene* **35**: 908–918.
- Schreiber E, Harshman K, Kemler I, Malipiero U, Schaffner W, Fontana A. 1990. Astrocytes and glioblastoma cells express novel octamer-DNA binding proteins distinct from the ubiquitous Oct-1 and B cell type Oct-2 proteins. *Nucleic Acids Res* **18**: 5495–5503.

- Schreiber E, Himmelmann A, Malipiero U, Tobler A, Stahel R, Fontana A. 1992. Human Small Cell Lung Cancer Expresses the Octamer DNA-binding and Nervous System-specific Transcription Factor N-Oct 3 (Brain-2). *Cancer Res* **52**: 6121–6124.
- Segil N, Roberts SB, Heintz N. 1991. Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. *Science* **254**: 1814–1816.
- Selby CP, Sancar A. 2006. A cryptochrome/photolyase class of enzymes with single-stranded DNA-specific photolyase activity. *Proc Natl Acad Sci USA* **103**: 17696–17700.
- Serrano M, Hannon GJ, Beach D. 1993. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366**: 704–707.
- Shain AH, Yeh I, Kovalyshyn I, Sriharan A, Talevich E, Gagnon A, Dummer R, North J, Pincus L, Ruben B, et al. 2015. The Genetic Evolution of Melanoma from Precursor Lesions. *N Engl J Med* **373**: 1926–1936.
- Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE, Tsien RY. 2004. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat Biotechnol* **22**: 1567–1572.
- Shannon P, Markiel A, Owen Ozier 2, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2498–2504.
- Shi Z, Fujii K, Kovary KM, Genuth NR, Röst HL, Teruel MN, Barna M. 2017. Heterogeneous Ribosomes Preferentially Translate Distinct Subpools of mRNAs Genome-wide. *Mol Cell* **67**: 71–83.e7.
- Shimizu H, Banno Y, Sumi N, Naganawa T, Kitajima Y, Nozawa Y. 1999. Activation of p38 mitogen-activated protein kinase and caspases in UVB-induced apoptosis of human keratinocyte HaCaT cells. *J Invest Dermatol* **112**: 769–74.
- Shteynberg D, Deutsch EW, Lam H, Eng JK, Sun Z, Tasman N, Mendoza L, Moritz RL, Aebersold R, Nesvizhskii AI. 2011. iProphet: Multi-level Integrative Analysis of Shotgun Proteomic Data Improves Peptide and Protein Identification Rates and Error Estimates. *Mol Cell Proteomics* **10**:12
- Sintzel MB, Rametta M, Reder AT. 2017. Vitamin D and Multiple Sclerosis: A Comprehensive Review. *Neurol Ther* 1-27
- Smalley K, Eisen T. 2000. The involvement of p38 mitogen-activated protein kinase in the alpha-melanocyte stimulating hormone (alpha-MSH)-induced melanogenic and anti-proliferative effects in B16 murine melanoma cells. *FEBS Lett* **476**: 198–202.
- Smalley KSM, Eisen TG. 2002. Differentiation of human melanoma cells through p38 MAP kinase is associated with decreased retinoblastoma protein phosphorylation and cell cycle arrest. *Melanoma Res* **12**: 187–192.
- Smit DJ, Smith AG, Parsons PG, Muscat GEO, Sturm RA. 2000. Domains of Brn-2 that mediate homodimerization and interaction with general and melanocytic transcription factors. *Eur J Biochem* **267**: 6413–6422.
- Smith GCM, Jackson SP. 1999. The DNA-dependent protein kinase. *Genes Dev* **13**: 916–934.

- Smith MD, Ensor EA, Coffin RS, Boxer LM, Latchman DS. 1998. Bcl-2 transcription from the proximal P2 promoter is activated in neuronal cells by the Brn-3a POU family transcription factor. *J Biol Chem* **273**: 16715–16722.
- Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, Walsh LA, Postow MA, Wong P, Ho TS, et al. 2014. Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N Engl J Med* **371**: 2189–2199.
- Sock E, Enderich J, Rosenfeld MG, Wegner M. 1996. Identification of the Nuclear Localization Signal of the POU Domain Protein Tst-1/Oct6. *J Biol Chem* **271**: 17512–17518.
- Solier S, Pommier Y. 2014. The nuclear γ -H2AX apoptotic ring: Implications for cancers and autoimmune diseases. *Cell Mol Life Sci* **71**: 2289–2297.
- Sonenberg N, Hinnebusch AG. 2009. Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets. *Cell* **136**: 731–745.
- Staples CJ, Owens DM, Maier J V, Cato ACB, Keyse SM. 2010. Cross-talk between the p38a and JNK MAPK Pathways Mediated by MAP Kinase Phosphatase-1 Determines Cellular Sensitivity to UV Radiation. *J Biol Chem* **285**: 25928–25940.
- Staudt LM, Singh H, Sen R, Wirth T, Sharp PA, Baltimore D. 1986. A lymphoid-specific protein binding to the octamer motif of immunoglobulin genes. *Nature* **323**: 640–643.
- Steeg PS. 2016. Targeting metastasis. *Nat Rev Cancer* **16**: 201–218.
- Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S, Palmero I, Ryan K, Hara E, Vousden KH, et al. 1998. The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *EMBO J* **17**: 5001–5014.
- Strickfaden H, McDonald D, Kruhlak MJ, Haince JF, Th'Ng JPH, Rouleau M, Ishibashi T, Corry GN, Ausio J, Underhill DA, et al. 2016. Poly(ADP-ribosyl)ation-dependent transient chromatin decondensation and histone displacement following laser microirradiation. *J Biol Chem* **291**: 1789–1802.
- Strub T, Giuliano S, Ye T, Bonet C, Keime C, Kobi D, Le Gras S, Cormont M, Ballotti R, Bertolotto C, et al. 2011. Essential role of microphthalmia transcription factor for DNA replication, mitosis and genomic stability in melanoma. *Oncogene* **30**: 2319–2332.
- Stuart SA, Houel S, Lee T, Wang N, Old WM, Ahn NG. 2015. A Phosphoproteomic Comparison of B-RAF^{V600E} and MKK1/2 Inhibitors in Melanoma Cells. *Mol Cell Proteomics* **14**: 1599–1615.
- Sturm RA, O'Sullivan BJ, Thomson JA, Jamshidi N, Pedley J, Parsons PG. 1994. Expression studies of pigmentation and POU-domain genes in human melanoma cells. *Pigment Cell Res* **7**: 235–240.
- Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, et al. 2005. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci* **102**: 15545–15550.
- Sugitani Y, Nakai S, Minowa O, Nishi M, Jishage KI, Kawano H, Mori K, Ogawa M, Noda T. 2002. Brn-1 and Brn-2 share crucial roles in the production and positioning of mouse

- neocortical neurons. *Genes Dev* **16**: 1760–1765.
- Sunabori T, Tokunaga A, Nagai T, Sawamoto K, Okabe M, Miyawaki A, Matsuzaki Y, Miyata T, Okano H. 2008. Cell-cycle-specific nestin expression coordinates with morphological changes in embryonic cortical neural progenitors. *J Cell Sci* **121**: 1204–1212.
- Sutherland KD, Berns A. 2010. Cell of origin of lung cancer. *Mol Oncol* **4**: 397–403.
- Szczyrba J, Niesen A, Wagner M, Wandernoth PM, Aumüller G, Wennemuth G. 2017. Neuroendocrine cells of the prostate derive from the neural crest. *J Biol Chem* **292**: 2021–2031.
- Tada A, Pereira E, Beitner-johnson D, Kavanagh R, Abdel-malek ZA. 2002. Mitogen- and Ultraviolet-B-Induced Signaling Pathways in Normal Human Melanocytes. *J Invest Dermatol* **118**: 316–322.
- Takahashi K, Yamanaka S. 2006. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell* **126**: 663–676.
- Takeda K, Yasumoto KI, Takada R, Takada S, Watanabe KI, Udono T, Saito H, Takahashi K, Shibahara S. 2000. Induction of melanocyte-specific microphthalmia-associated transcription factor by Wnt-3a. *J Biol Chem* **275**: 14013–14016.
- Tammela T, Sanchez-Rivera FJ, Cetinbas NM, Wu K, Joshi NS, Helenius K, Park Y, Azimi R, Kerper NR, Wesselhoeft RA, et al. 2017. A Wnt-producing niche drives proliferative potential and progression in lung adenocarcinoma. *Nature* **545**: 355–359.
- Tan X, Zhang L, Zhu H, Qin J, Tian M, Dong C, Li H, Jin G. 2014. Brn4 and TH synergistically promote the differentiation of neural stem cells into dopaminergic neurons. *Neurosci Lett* **571**: 23–28.
- Tanaka S, Kamachi Y, Tanouchi A, Hamada H, Jing N, Kondoh H. 2004. Interplay of SOX and POU factors in regulation of the Nestin gene in neural primordial cells. *Mol Cell Biol* **24**: 8834–8846.
- Tanaka T, Halicka HD, Traganos F, Darzynkiewicz Z. 2009. Cytometric Analysis of DNA Damage: Phosphorylation of Histone H2AX as a Marker of DNA Double-Strand Breaks (DSBs). *Methods Mol Biol* **523**: 161–168.
- Tanoue T, Adachi M, Moriguchi T, Nishida E. 2000. A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nat Cell Biol* **2**: 110–116.
- Tanoue T, Maeda R, Adachi M, Nishida E. 2001. Identification of a docking groove on ERK and p38 MAP kinases that regulates the specificity of docking interactions. *EMBO J* **20**.
- Tantin D, Schild-Poulter C, Wang V, Haché RJG, Sharp PA. 2005. The octamer binding transcription factor Oct-1 is a stress sensor. *Cancer Res* **65**: 10750–10758.
- Tatum JL. 2006. Hypoxia: Importance in tumor biology, noninvasive measurement by imaging, and value of its measurement in the management of cancer therapy. *Int J Radiat Biol* **82**: 699–757.
- Taverna SD, Li H, Ruthenburg AJ, Allis CD, Patel DJ. 2007. How chromatin-binding modules interpret histone modifications: Lessons from professional pocket pickers. *Nat Struct Mol Biol* **14**: 1025–1040.

- Taylor RC, Cullen SP, Martin SJ. 2008. Apoptosis: Controlled demolition at the cellular level. *Nat Rev Mol Cell Biol* **9**: 231–241.
- Terry S, Beltran H. 2014. The Many Faces of Neuroendocrine Differentiation in Prostate Cancer Progression. *Front Oncol* **4**: 1–9.
- Theveneau E, Mayor R. 2012. Neural crest delamination and migration: From epithelium-to-mesenchyme transition to collective cell migration. *Dev Biol* **366**: 34–54.
- Thiery JP, Acloque H, Huang RYJ, Nieto MA. 2009. Epithelial-Mesenchymal Transitions in Development and Disease. *Cell* **139**: 871–890.
- Thomas AJ, Erickson CA. 2009. FOXD3 regulates the lineage switch between neural crest-derived glial cells and pigment cells by repressing MITF through a non-canonical mechanism. *Development* **136**: 1849–1858.
- Thomas AJ, Erickson CA. 2008. The making of a melanocyte: The specification of melanoblasts from the neural crest. *Pigment Cell Melanoma Res* **21**: 598–610.
- Thomson JAF, Parsons PG, Sturm RA. 1993. In vivo and in vitro expression of octamer binding proteins in human melanoma metastases, brain tissue, and fibroblasts. *Pigment Cell Res* **6**: 13–22.
- Thorsness PE, Koshland DE. 1987. Inactivation of isocitrate dehydrogenase by phosphorylation is mediated by the negative charge of the phosphate. *J Biol Chem* **262**: 10422–10425.
- Thurber AE, Douglas G, Sturm EC, Zabierowski SE, Smit DJ, Ramakrishnan SN, Hacker E, Leonard JH, Herlyn M, Sturm RA. 2011. Inverse expression states of the BRN2 and MITF transcription factors in melanoma spheres and tumour xenografts regulate the NOTCH pathway. *Oncogene* **30**: 3036–3048.
- Tian C, Li Y, Huang Y, Wang Y, Chen D, Liu J, Deng X, Sun L, Anderson K, Qi X, et al. 2015. Selective Generation of Dopaminergic Precursors from Mouse Fibroblasts by Direct Lineage Conversion. *Sci Rep* **5**: 12622.
- Tie F, Banerjee R, Stratton CA, Prasad-Sinha J, Stepanik V, Zlobin A, Diaz MO, Scacheri PC, Harte PJ. 2009. CBP-mediated acetylation of histone H3 lysine 27 antagonizes Drosophila Polycomb silencing. *Development* **136**: 3131–3141.
- Tjian R, Maniatis T. 1994. Transcriptional activation: A complex puzzle with few easy pieces. *Cell* **77**: 5–8.
- Toledo LI, Altmeyer M, Rask MB, Lukas C, Larsen DH, Povlsen LK, Bekker-Jensen S, Mailand N, Bartek J, Lukas J. 2014. ATR prohibits replication catastrophe by preventing global exhaustion of RPA. *Cell* **155**: 1088–1103.
- Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-sagi D, Jones SN, Flavell RA, Davis RJ. 2000. Requirement of JNK for Stress- Induced Activation of the Cytochrome c – Mediated Death Pathway. *Science* **288**: 870–875.
- Trunzer K, Pavlick a. C, Schuchter L, Gonzalez R, McArthur G a., Hutson TE, Moschos SJ, Flaherty KT, Kim KB, Weber JS, et al. 2013. Pharmacodynamic Effects and Mechanisms of Resistance to Vemurafenib in Patients With Metastatic Melanoma. *J Clin Oncol* **31**: 1767–1774.

- Tsai CF, Wang YT, Yen HY, Tsou CC, Ku WC, Lin PY, Chen HY, Nesvizhskii AI, Ishihama Y, Chen YJ. 2015. Large-scale determination of absolute phosphorylation stoichiometries in human cells by motif-targeting quantitative proteomics. *Nat Commun* **6**: 1–8.
- Tsiatsiani L, Heck AJR. 2015. Proteomics beyond trypsin. *FEBS J* **282**: 2612–2626.
- Turajlic S, Litchfield K, Xu H, Rosenthal R, McGranahan N, Reading JL, Wong YNS, Rowan A, Kanu N, Al Bakir M, et al. 2017. Insertion-and-deletion-derived tumour-specific neoantigens and the immunogenic phenotype: a pan-cancer analysis. *Lancet Oncol* **18**: 1009–1021.
- Turrenne GA, Paul P, Laflair L, Price BD. 2001. Activation of p53 transcriptional activity requires ATM's kinase domain and multiple N-terminal serine residues of p53. *Oncogene* **20**: 5100–5110.
- Ubersax JA, Ferrell JE. 2007. Mechanisms of specificity in protein phosphorylation. *Nat Rev Mol Cell Biol* **8**: 530–541.
- Ugurel S, Röhm J, Ascierto PA, Flaherty KT, Grob JJ, Hauschild A, Larkin J, Long GV, Lorigan P, McArthur GA, et al. 2017. Survival of patients with advanced metastatic melanoma: the impact of novel therapies—update 2017. *Eur J Cancer* **83**: 247–257.
- Vaklavas C, Blume SW, Grizzle WE. 2017. Translational Dysregulation in Cancer: Molecular Insights and Potential Clinical Applications in Biomarker Development. *Front Oncol* **7**: 1–16.
- van Leeuwen HC, Strating MJ, Cox M, Kaptein R, van der Vliet PC. 1995. Mutation of the Oct-1 POU-specific recognition helix leads to altered DNA binding and influences enhancement of adenovirus DNA replication. *Nucleic Acids Res* **23**: 3189–3197.
- van Leeuwen HC, Strating MJ, Rensen M, De Laat W, van Der Vliet PC. 1997. Linker length and composition influence the flexibility of Oct-1 DNA binding. *EMBO J* **16**: 2043–2053.
- van Os NJH, Jansen AFM, van Deuren M, Haraldsson A, van Driel NTM, Etzioni A, van der Flier M, Haaxma CA, Morio T, Rawat A, et al. 2017. Ataxia-telangiectasia: Immunodeficiency and survival. *Clin Immunol* **178**: 45–55.
- Vandamme N, Berx G. 2014. Melanoma Cells Revive an Embryonic Transcriptional Network to Dictate Phenotypic Heterogeneity. *Front Oncol* **4**: 1–6.
- Veenstra GJ, van der Vliet PC, Destree OH. 1997. POU domain transcription factors in embryonic development. *Mol Biol Rep* **24**: 139–55.
- Venkatesh S, Workman JL. 2015. Histone exchange, chromatin structure and the regulation of transcription. *Nat Rev Mol Cell Biol* **16**: 178–189.
- Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. 1995. A novel assay for apoptosis Flow cytometric detection of phosphatidylserine early apoptotic cells using fluorescein labelled expression on Annexin V. *J Immunol Methods* **184**: 39–51.
- Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M. 2012. Direct conversion of fibroblasts to functional neurons. *Nature* **77**: 7–8.
- Vivanco I, Palaskas N, Tran C, Finn SP, Getz G, Kennedy NJ, Jiao J, Rose J, Xie W, Loda M, et al. 2007. Identification of the JNK Signaling Pathway as a Functional Target of the

- Tumor Suppressor PTEN. *Cancer Cell* **11**: 555–569.
- Wagner EF, Nebreda AR. 2009. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer* **9**: 537–549.
- Walther TC, Mann M. 2010. Mass spectrometry-based proteomics in cell biology. *J Cell Biol* **190**: 491–500.
- Wang B, Malik R, Nigg EA, Körner R. 2008. Evaluation of the low-specificity protease elastase for large-scale phosphoproteome analysis. *Anal Chem* **80**: 9526–9533.
- Wang P, Wang Q, Sun J, Wu J, Li H, Zhang N, Huang Y, Su B, Li R, Liu L, et al. 2009. POU homeodomain protein Oct-1 functions as a sensor for cyclic AMP. *J Biol Chem* **284**: 26456–65.
- Waragai M, Lammers CH, Takeuchi S, Imafuku I, Udagawa Y, Kanazawa I, Kawabata M, Mouradian MM, Okazawa H. 1999. PQBP-1, a novel polyglutamine tract-binding protein, inhibits transcription activation by Brn-2 and affects cell survival. *Hum Mol Genet* **8**: 977–987.
- Warmerdam DO, Brinkman EK, Marteijn J a, Medema RH, Kanaar R, Smits VAJ. 2013. UV-induced G2 checkpoint depends on p38 MAPK and minimal activation of ATR-Chk1 pathway. *J Cell Sci* **126**: 1923–30.
- Webster MR, Kugel CH, Weeraratna AT. 2015. The Wnts of change: How Wnts regulate phenotype switching in melanoma. *Biochim Biophys Acta* **1856**: 244–251.
- Weeraratna AT, Jiang Y, Hostetter G, Rosenblatt K, Duray P, Bittner M, Trent JM. 2002. Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. *Cancer Cell* **1**: 279–288.
- Wehrle-Haller B, Weston JA. 1995. Soluble and cell-bound forms of steel factor activity play distinct roles in melanocyte precursor dispersal and survival on the lateral neural crest migration pathway. *Development* **121**: 731–742.
- Wellbrock C, Rana S, Paterson H, Pickersgill H, Brummelkamp T, Marais R. 2008. Oncogenic BRAF regulates melanoma proliferation through the lineage specific factor MITF. *PLoS One* **3**: 1–9.
- Whitehouse CJ, Taylor RM, Thistlethwaite A, Zhang H, Karimi-Busheri F, Lasko DD, Weinfeld M, Caldecott KW. 2001. XRCC1 stimulates human polynucleotide kinase activity at damaged DNA termini and accelerates DNA single-strand break repair. *Cell* **104**: 107–117.
- Wood RD. 1997. Nucleotide excision repair in mammalian cells. *J Biol Chem* **272**: 23465–23468.
- Wu G, Feng X, Stein L, Hanahan D, Weinberg R, Vogelstein B, Kinzler K, Itoh S, Itoh F, Goumans M, et al. 2010. A human functional protein interaction network and its application to cancer data analysis. *Genome Biol* **11**: R53.
- Xiao Z, Xue J, Sowin TJ, Zhang H. 2006. Differential roles of checkpoint kinase 1, checkpoint kinase 2, and mitogen-activated protein kinase-activated protein kinase 2 in mediating DNA damage-induced cell cycle arrest: implications for cancer therapy. *Mol Cancer Ther* **5**: 1935–1943.

- Xu C, Ng DTW. 2015. Glycosylation-directed quality control of protein folding. *Nat Rev Mol Cell Biol* **16**: 742–752.
- Xue D, Tu Y, Chalfie M. 1993. Cooperative interactions between the *Caenorhabditis elegans* homeoproteins UNC-86 and MEC-3. *Science* **261**: 1324–1328.
- Yamanaka T, Tosaki A, Miyazaki H, Kurosawa M, Furukawa Y, Yamada M, Nukina N. 2010. Mutant huntingtin fragment selectively suppresses Brn-2 POU domain transcription factor to mediate hypothalamic cell dysfunction. *Hum Mol Genet* **19**: 2099–2112.
- Yarbrough D, Wachter RM, Kallio K, Matz M V., Remington SJ. 2001. Refined crystal structure of DsRed, a red fluorescent protein from coral, at 2.0-Å resolution. *Proc Natl Acad Sci USA* **98**: 462–467.
- Yau R, Rape M. 2016. The increasing complexity of the ubiquitin code. *Nat Cell Biol* **18**: 579–586.
- Yoshida S, Fukino K, Harada H, Nagai H, Imoto I, Inazawa J, Takahashi H, Teramoto A, Emi M. 2001. The c-Jun NH2-terminal kinase3 (JNK3) gene: Genomic structure chromosomal assignment, and loss of expression in brain tumors. *J Hum Genet* **46**: 182–187.
- Zeke A, Bastys T, Alexa A, Garai Á, Mészáros B, Kirsch K, Dosztányi Z, Kalinina O V, Reményi A, Agnarsson I, et al. 2015. Systematic discovery of linear binding motifs targeting an ancient protein interaction surface on MAP kinases. *Mol Syst Biol* **11**: 837.
- Zhang M, Wang L, Zhong D. 2017. Photolyase: Dynamics and electron-transfer mechanisms of DNA repair. *Arch Biochem Biophys* **632**: 158–174.
- Zhang N, Liu X, Li L, Legerski R. 2007. Double-strand breaks induce homologous recombinational repair of interstrand cross-links via cooperation of MSH2, ERCC1-XPF, REV3, and the Fanconi anemia pathway. *DNA Repair (Amst)* **6**: 1670–1678.
- Zhao H, Jin S, Fan F, Fan W, Tong T, Zhan Q. 2000. Activation of the Transcription Factor Oct-1 in Response to DNA Damage. *Cancer Res* **60**: 6276–6280.
- Zou L, Elledge SJ. 2003. Sensing DNA Damage Through ATRIP Recognition of RPA-ssDNA Complexes. *Science* **300**: 1542–1548.