

DNA damage responses to loss of telomere integrity

Ana Rita Cabral Martins Carlos

A thesis presented for the degree of Doctor of Philosophy in Radiobiology

Telomeres and Genome Stability Group

The Gray Institute for Radiation Oncology & Biology

Department of Oncology

University of Oxford

Wolfson College

Michaelmas 2012

Index

Index..... i

Acknowledgements..... iv

Declaration..... vi

Abstract..... vii

List of figures..... viii

List of tables..... xi

Abbreviations..... xii

Chapter 1 - Introduction 1

1.1 Telomere structure and function..... 2

1.2 Key aspects of telomere maintenance: lengthening, replication and capping 5

1.2.1 Telomere length homeostasis 5

1.2.2 Telomere replication 10

1.2.3 Telomere protection..... 15

1.3 Telomere dysfunction and the DNA damage response 18

1.3.1 DNA damage response triggered by dysfunctional telomeres 20

1.3.2 DNA end-processing reactions at dysfunctional telomeres..... 24

1.3.3 Dysfunctional telomeres and cell cycle checkpoints 29

1.4 Telomere dysfunction and cancer 33

1.5 Aim of the work..... 37

Chapter 2 Materials and Methods 39

2.1 Cell lines and treatments..... 39

2.2 Establishment of doxycycline (DOX) inducible TRF2 shRNA cell line 39

2.3 siRNA transfection..... 40

2.4 siRNA transfection of siRNA libraries..... 40

2.5 MEF retroviral transduction 43

2.6 Immunoblotting 44

2.7 Immunofluorescence 45

2.8 Antibodies 46
2.9 Combined FISH and immunofluorescence (IF-FISH) 47
2.10 Telomeric FISH 48
2.11 Chromosome orientation FISH (CO-FISH) 48
2.12 Real-Time quantitative PCR (qPCR) 50
2.13 Fluorescence-activated cell sorting (FACS) 51
2.14 Pulsed field gel electrophoresis of single-strand telomeric DNA 52

Chapter 3 Results – Part 1: High-throughput screening for factors involved in telomere biology 53

3.1 Introduction 53
3.2 Results and discussion 55
3.2.1 A screen for factors required for RAP1 and TRF2 foci formation using a human Phosphatase siRNA library 55
3.2.2 A screen for factors required for RAP1 and TRF2 foci formation using a human Ubiquitinome siRNA library 61
3.2.3 Validation of hits from Phosphatase and Ubiquitinome siRNA library screens 63
3.2.4 A screen for factors required for TIF formation using human DNA repair library 70
3.2.5 DNA repair library screen and validation 74

Chapter 4 Results – Part 2: CtIP/BRCA1 and EXO1 play distinct roles in the formation of telomeric end-to-end fusions 82

4.1 Introduction 82
4.2 Results and discussion 84
4.2.1 BRCA1 and CtIP are not required for DNA damage signalling after TRF2 depletion 84
4.2.2 BRCA1 and CtIP are required for the telomeric fusions in TRF2-depleted cells 87
4.2.3 EXO1 is also required for end-joining of uncapped telomeres 91
4.2.4 EXO1, but not CtIP, is required to remove the 3’ telomeric overhang at uncapped telomeres 93

Chapter 5 Discussion 98

5.1 siRNA screens as useful tools to unravel new components involved in telomere biology 98
5.2 siRNA screens as tools to compare the general DNA damage response to the one triggered from uncapped telomeres 101

5.3 DDR factors BRCA1 and CtIP are required for telomeric end-to-end fusions 103

5.4 EXO1 involvement in telomeric fusions and possible connection with A-NHEJ mechanisms 105

5.5 CtIP and EXO1 play different roles in telomeric end-joining 108

5.6 Multiple pathways act at telomeres uncapped through TRF2 depletion 113

Acknowledgements

This thesis would not have been possible without the guidance and support of several individuals and institutions to who I am truly grateful.

I would like to start by thanking my supervisor, Dr. Madalena Tarsounas, to give me the chance to join her laboratory. It has been a valuable experience, both professionally and personally, which greatly contributed to development of my career. She has been a precious help in improving my knowledge and skills throughout my 4 years of the DPhil. She has also been very important integrating me in the scientific community. Thanks to her I had the chance to be in contact with several well-known researches from various countries and establish fruitful collaborations.

Secondly I have to thank the Portuguese Foundation for Science and Technology who has supported my living costs and partially my work (scholarship number SFRH / BD / 47326 / 2008; QREN - POPH program co-funded by the European Social Fund and the Portuguese Ministry for Education and Science). Without this grant my thesis would not have been possible.

I also would like to thank everybody in the laboratory, both past and current members for always being there for me and making the lab into a 'home'. In particular I have to thank Sophie, our precious lab manager, who also had a valuable role in optimizing some of the techniques used in my work and also for all the time she spent in the microscope taking pictures of my slides. A special thanks to Jose for all the useful discussions and suggestions and for his help setting up some of the cell lines used in this work. Thanks also to Tiago who also helped with testing some reagents. To Maria, Chunyan, Natsuko, Panagiotis, Kirsten, Nikola, Eliana, thank you!

A general thanks to everybody in The Gray Institute for Radiation Oncology and Biology who in one way or another helped me during these four years. Especially I would like to thank Cecilia Lundin, Daniel Ebner and Bastian Evers who helped me to set up

the conditions for the siRNA screens performed in the Department and also for the troubleshooting.

Outside the department I have to thank our collaborators. Starting by Claudia and Jiri Lukas who gave me the chance to go to their laboratory and use one of their siRNA libraries. It was a rewarding experience that allowed me to grow even more as a scientist. Thanks to Eros Denchi who kindly accepted to run my samples in their pulsed field electrophoresis system. This greatly improved my work.

Last but not least I have to thank family and friends (both the long term ones and the ones that I was really fortunate to meet here in Oxford) who have supported me every step of the way. Without them everything would me much harder.

Thank you all!!!!

Declaration

I declare that:

The work here presented is wholly my own work.

No part of my thesis has been accepted or is currently being submitted for any degree, diploma or certificate or other qualification in this University or elsewhere.

Abstract

Linear genomes end in characteristic structures consisting of repetitive DNA and proteins: the telomeres. These play two critical roles: on one hand they avoid the of loss of genetic information due to the incomplete replication of the chromosome ends and on the other, they provide capping structures for chromosome termini, differentiating them from double strand breaks. Telomeres contain specialized proteins (the shelterin complex), as well as proteins present elsewhere on the chromosomes (chromatin remodelling, DNA damage repair and response factors). Interestingly, several DNA damage factors are required for proper telomere maintenance, drawing a thin line between telomere protection and their recognition as broken DNA ends.

Loss of telomere integrity has severe consequences for the cell, namely it can induce replicative senescence and cellular aging, or it can contribute to tumorigenesis. How telomeres are capped and how they are perceived by the cell when they become dysfunctional is essential for our understanding of the contribution of loss of telomere integrity to aging and disease. In order to unravel new factors involved in telomere maintenance, siRNA screens were performed. The optimization process has confirmed both telomeric foci and telomere dysfunction-induced foci (TIFs) as suitable readouts and the screens performed generated a list of potential candidate genes involved in telomere biology. Although some of the candidate genes tested in this work failed the validation process, other genes deserve further analysis.

In addition this work also studied the role of several DNA damage factors at uncapped telomeres. Furthermore, BRCA1, CtIP and EXO1 were found to be critical for the formation of end-to-end fusions generated after TRF2 inactivation. The requirement of this proteins in this process, suggests that not only that not only the classical non-homologous end joining (C-NHEJ) pathway is active at TRF2-depelted telomeres, but emphasises the multiplicity of mechanisms that act to repair dysfunctional telomeres.

List of figures

Figure 1.1 – Telomere structure. 2

Figure 1.2 – Telomeric complexes in mammals, *S.pombe* and *S. cerevisae*. 3

Figure 1.3 – Telomerase allows telomere lengthening. 7

Figure 1.4 – Alternative lenghthening of telomeres. 10

Figure 1.5 – Telomeric replication in mammalian cells. 15

Figure 1.6 – Shelterin complex and acessory proteins protect telomeres. 18

Figure 1.7 – Comparison between the cannonical and telomeric DDR. 29

Figure 1.8 – DNA damage checkpoint activation after telomere dysfunction. 33

Figure 1.9 – Telomere crisis. 34

Figure 2.1 – Schematic representation of the CO-FISH protocol. 50

Figure 3.1 - Selection of the cell line and cell density for the Phosphatase library screen. 55

Figure 3.2 - Selection of the siRNA transfection conditions for the Phosphatase Library. 56

Figure 3.3 - TRF2 and RAP1 siRNA-mediated depletion in U2OS cells using optimized conditions. 59

Figure 3.4 – Z-score analysis of the number TRF2/RAP1 foci. 61

Figure 3.5 - Z-score analysis of the number TRF2/RAP1 foci. 62

Figure 3.6 - Hit validation by immunofluorescence for TRF2 and RAP1. 65

Figure 3.7 - TIF analysis in U2OS cells treated with siRNA hits isolated from Phosphatase and Ubiquitinome library screens. 67

Figure 3.8 – RNF41 depletion reduces both TRF2 and RAP1 protein levels. 68

Figure 3.9 – siRNA number 1 targets TRF2 mRNA. 70

Figure 3.10 – Inducible system for the expression of TRF2 shRNA in U2OS cells. 70

Figure 3.11 – TRF2 is efficiently depleted in U2OS cells using DOX-inducible TRF2 shRNA. 71

Figure 3.12 – Time course after DOX addition. 72
Figure 3.13 – Diagram of reverse transfection protocol for the siRNA library. 73
Figure 3.14 – RNF8 siRNA reduces number of 53BP1 foci formed after TRF2 depletion. 73
Figure 3.15 – The siRNA screen for factors that triggered 53BP1 foci induced by TRF2 abroagation using a DNA repair library. 75
Figure 3.16 – Secondary screen using selected hits from the DNA damage repair library. 77
Figure 3.17 – siRNAs for CHD3, SUV39H2 and TSC1 do not target 53BP1. 78
Figure 3.18 – The decrease in the number of 53BP1 foci is not consistent with the level of depletion triggered by individual siRNAs. 80
Figure 3.19 – Formation of 53BP1 foci is not impaired in *Tsc1*$^{-/-}$, *Tsc2*$^{-/-}$ or *Suv39h1/2DN* MEFs treated with TRF2 shRNA or IR. 81
Figure 4.1 - BRCA1/CtIP are not required for ATM-mediated signalling triggered by TRF2 depletion. 86
Figure 4.2 - CtIP inhibition alone does not cause CHK2 activation, but only in combination with TRF2 depletion. 88
Figure 4.3 - BRCA1 and CtIP are required for the telomeric fusions induced by TRF2 inactivation. 89
Figure 4.4 – The decrease in chromosome fusions in BRCA1- and CtIP-deficient MEFs lacking TRF2, is not due to changes in proliferation. 89
Figure 4.5 - Co-depletion of BRCA1 and CtIP does not have any additive effect in the fusions of TRF2 deplered cells. 90
Figure 4.6 - BRCA1 or CtIP inactivation together with TRF2 inhibition changes the chromatid fusions phenotype. 91
Figure 4.7 - EXO1, BLM, DNA2 and WRN were efficiently depleted from TRF2-depleted cells using shRNAs. 92

Figure 4.8 - EXO1 but not DNA2, WRN or BLM, is required to process uncaped telomeres. 93
Figure 4.9 - Absence of both BRCA1 and 53BP1 does not restore fusions in TRF2-depleted cells. 94
Figure 4.10 – 53BP1 contribution to the end-joining of uncapped telomeres is dominant over CtIP or EXO1..... 95
Figure 4.11 – CtIP, but not EXO1, is required to process the 3’ telomeric overhang. 97
Figure 5.1 – Multiple pathways act to repair telomeres uncapped through TRF2 depletion. 115

List of tables

Table 2.1 - siRNA sequences..... 42

Table 2.2 - shRNA sequences 44

Table 2.3 - RT qPCR primers..... 51

Table 3.1 - Z-factor of the siRNA assay..... 60

Table 3.2 – Selected hits from the Phosphatase and Ubiquitinome siRNA libraries 64

Abbreviations

9-1-1	Complex formed by Rad9-Hus1-Rad1
53BP1	Tumour protein p53 binding protein 1
ALT	Alternative lengthening of telomeres
APB	ALT-associated promyelocytic leukaemia bodies
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
BFB	Breakage-fusion-bridge cycle
BLM	Bloom's syndrome protein
BRCA1	Breast cancer susceptibility protein 1
BRCA2	Breast cancer susceptibility protein 2
Ccq1	Coiled-coil protein quantitatively enriched
CDC_	Cell division cycle; example Cdc13, CDC25A, CDC25C
CDK	Cyclin-dependent kinase
CHD3	Chromodomain helicase DNA binding protein 3
CHK1	Checkpoint kinase-1
CHK2	Checkpoint kinase-2
Crb2	Crumbs homolog 2; 53BP1 homolog in *Schizosaccharomyces pombe*
CSR	Class switch recombination
CST	Complex formed by Cdc13-Stn1-Ten1 in *Saccharomyces cerevisiae* and by CTC1-STN1-TEN1 in mammals
CTC1	Conserved telomere maintenance component 1; Cdc13 homolog in mammals; CST component
CtIP	CTBP-interacting protein also known as retinoblastoma binding protein 8 (RBBP8)

DC	Dyskeratosis congenita
DDR	DNA damage response
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
ssDNA	Single stranded deoxyribonucleic acid
DNA2	DNA replication helicase 2
DNA-PK	DNA-activated protein kinase, catalytic polypeptide
DOX	Doxycycline
DSB	Double strand break
ERCC1	Excision repair cross complementing group 1
EXO1	Exonuclease 1
FACS	Fluorescence-activated cell sorting
FANCA	Fanconi anemia, complementation group A
FANCD2	Fanconi anemia group D2 protein
FBS	Foetal bovine serum
FISH	Fluorescence in situ hybridization
CO-FISH	Chromosome orientation fluorescence in situ hybridization
IF-FISH	Combined immunofluorescence and fluorescent in situ hybridization
γH2AX	Phosphorylated histone 2AX
H3K9m3	Histone 3 lysine 9 trimethylated
H4K20me2	Histone 4 dimethylation at lysine 20
HBS	Hepes-buffered saline
HDAC_	Histone deacetylase; example HDAC8
HP1	Heterochromatin protein 1
HR	Homologous recombination
IR	Ionizing radiation

KAP-1	KRAB domain–associated protein 1
KO	Knock out
LIG3	Ligase III
LIG4	Ligase IV
MDC1	Mediator of DNA-damage checkpoint 1
Mec1	ATR homolog in *Saccharomyces cerevisiae*
MEFs	Mouse embryonic fibroblasts
MMEJ	Microhomology-mediated alternative end joining
MRE11	Meiotic recombination 11; MRN component
MRN	Complex formed by MRE11, RAD50 and NBS1 in mammals and in *Schizosaccharomyces pombe*
MRX	*Saccharomyces cerevisiae* homolog for MRN complex, composed by Mre11, Nbs1 and Xrs2
MTS	Multitelomeric signal
NBS1	Nijmegen breakage syndrome protein; MRN component
NHEJ	Non-homologous end-joining
A-NHEJ	Alternative non-homologous end-joining
C-NHEJ	Classic non-homologous end-joining
NuRD	Nucleosome remodelling and deacetylase complex
OB-fold	Oligonucleotide/oligosaccharide binding - fold
p53	Tumour protein p53
PARP1	Poly (ADP-ribose) polymerase 1
PARP2	Poly (ADP-ribose) polymerase 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
PML	Promyelocytic leukaemia

POT1	Protection of telomeres 1
Poz1	Pot1-associated protein in *Schizosaccharomyces pombe*
RAD_	Radiation resistant protein; example RAD51, RAD54
Rad3	Radiation resistant protein 3; ATR homolog in *Schizosaccharomyces pombe*
RAP1	Repressor/Activator protein 1, also known as TERF2IP
scRap1	*Saccharomyces cerevisiae* Repressor/Activator protein 1
RB	Retinoblastoma protein
Rif1	Rap1-interacting factor 1
Rif2	Rap1-interacting factor 2
RNA	Ribonucleic acid
RNase A	Ribonuclease A
snoRNA	Small nucleolar RNA
shRNA	Short interference ribonucleic acid
siRNA	Small interference ribonucleic acid
SSC	Saline-sodium citrate
RNF8	Ring-finger protein 8, ubiquitin protein ligase
RNP	Ribonucleoprotein
hnRNPA1	Heterogeneous nuclear ribonucleoprotein A1
RPA	Replication protein A
RTEL1	Regulator of telomere elongation helicase 1
RT qPCR	Real time quantitative polymerase chain reaction
Sae2	SUMO activating enzyme 2; CtIP homolog in *Saccharomyces cerevisiae*
Sgs1	Salivary gland secretion 1; RecQ homolog in *Saccharomyces cerevisiae*
SMC_	Structural maintenance of chromosomes; example SMC5
STN1	Suppressor of Cdc13; CST component
SUV39H2	Suppressor of variegation 3-9 homolog 2

Taz1	Telomere-associated protein in *Schizosaccharomyces pombe*; TRF1/2 homolog
Tel1	ATM homolog in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*
TEN1	Telomeric pathways in association with Stn1, number 1; CST component
TERC	Telomerase RNA component
TERF2IP	Telomeric repeat binding factor 2 interacting protein; also known as RAP1
TERRA	Telomeric repeat-containing RNA
TERT	Telomerase reverse transcriptase
TIF	Telomere dysfunction-induced foci
TIN2	TRF2- and TRF1-interacting nuclear protein 2
T-loop	Telomeric displacement loop
TOPBP1	DNA topoisomerase II binding protein 1
TPP1	POT1- and TIN2-interacting protein
Tpz1	TPP1 homolog in *Schizosaccharomyces pombe*
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor 2
TSC1	Tuberous sclerosis 1
UV	Ultraviolet
WB	Western blotting
WRN	Werner's syndrome protein
XPF	Xeroderma pigmentosum group F
XRCC_	X-ray repair complementing defective repair in Chinese hamster cells; example XRCC3

Chapter 1 - Introduction

Telomeres play key roles maintaining genome stability through protection of chromosome termini, thus preventing their recognition as broken DNA ends and activation of deleterious DNA damage responses. In mammals, telomeres consist of arrays of tandem TTAGGG repeats, with length varying on average between 10 kb in humans and 50 kb in mouse, and associated telomere-binding proteins. The telomere concept was suggested as early as the first half of the 20th century by studies of Barbara McClintock and Hermann J. Muller. Barbara McClintock noticed that 'if chromosomes are broken by various means, the broken ends appear to be adhesive and tend to fuse with one another' (McClintock, 1941). This initiates what is now known as the "bridge-breakage-fusion" cycle, which can continue during successive nuclear divisions. Herman Muller reported that 'the terminal gene must have a special function, that of sealing the end of the chromosome' (Muller, 1938). At this point the identity of 'the terminal gene' and the mechanisms it mediates to cap the chromosome end were unknown. Studies conducted since then unravelled not only the telomere structure, but more importantly, the reactions required for its integrity, essential to avoid joining of chromosome ends, which could potentially limit cell proliferation and lead to cell death.

Three distinct mechanisms act to maintain telomere integrity in most types of cells. Firstly, telomeric repeats must be constantly replenished in order to counteract the end replication problem, i.e. the incomplete replication of the lagging strand, and/or accidental loss of telomeric sequences, due for example to failed DNA replication. Secondly, mammalian telomeres, due to their G-rich repetitive DNA sequence and protective structures, represent a natural obstacle for passing replication forks. Thus specialized mechanisms have evolved to facilitate telomere

replication and the restart of stalled or broken replication forks. Thirdly, chromosome termini must be protected against nucleolytic degradation and end-to-end fusions. This is primarily accomplished by shelterin, a capping protein complex specifically associated with chromosome termini. If the uncapping occurs, then it is critical to eliminate such a cell. For that, cells have evolved a very tight control system, the cell cycle checkpoints.

1.1 Telomere structure and function

Telomeres of different eukaryotes share certain structural features. The 5' strand of the telomeric DNA is usually rich in guanosine and devoid of cytosine (being commonly referred to as the G-rich strand) and conversely the 3' strand is rich in cytosine and devoid of guanosine (C-rich strand). Additionally the telomeric repeats are double stranded (ds) DNA for most of their length, but they end in a single stranded overhang on the G-rich strand (Figure 1.1A). This overhang is typically not exposed, but thought to invade the double stranded telomeric DNA, displacing the G-rich strand and annealing with the C-rich one (Griffith et al., 1999). This leads to the formation of a displacement loop (D loop) that at the telomeres is referred to as T-loop (Figure 1.1B).

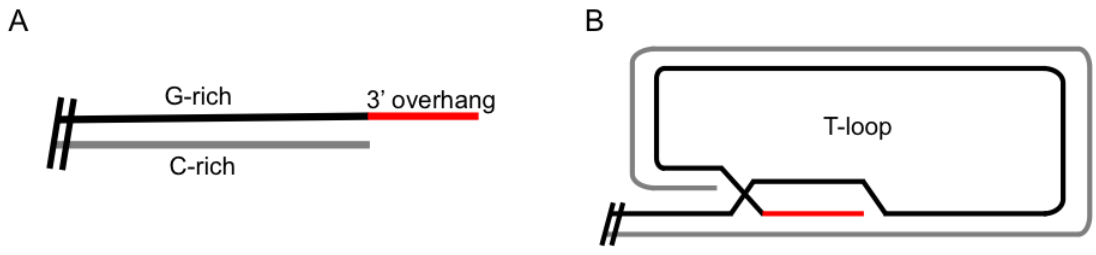


Figure 1.1 – Telomere structure.

A) Representation of a telomere showing the G-rich and C-rich strands. The G-rich strand ends in a single strand 3' overhang. B) Telomeric D-loop also called T-loop.

In mammalian cells, telomeres are composed of TTAGGG repeats associated with the protein complex called shelterin. This complex consists of six proteins: Telomeric Repeat binding Factor 1 and 2 (TRF1 and TRF2), Protection Of Telomeres 1 (POT1), TRF2- and TRF1-Interacting Nuclear protein 2 (TIN2), POT1- and TIN2-interacting protein (TPP1) and transcriptional Repressor/Activator Protein 1 (RAP1, also known as Telomeric Repeat binding Factor 2, Interacting Protein, TERF2IP) (Figure 1.2A). From the six proteins above mentioned only TRF1 and TRF2 are able to bind directly to the ds telomeric DNA through their Myb domain (Court et al., 2005; Hanaoka et al., 2005). On the other hand, POT1 is the only factor able to bind to the single stranded (ss) DNA through its OB-folds (Baumann and Cech, 2001; Lei et al., 2004; Loayza et al., 2004). TRF1/TRF2 and POT1 are bridged together by TIN2 and TPP1, providing the connection between telomeric ssDNA and dsDNA (Kim et al., 2004; Liu et al., 2004; Ye et al., 2004a; Ye et al., 2004b). RAP1 localizes to the telomeres through its binding to TRF2 (Li et al., 2000). Together these proteins form a stable complex that is involved in telomere protection and maintenance, being responsible for repression of DNA repair reactions, telomere length regulation and T-loop assembly.

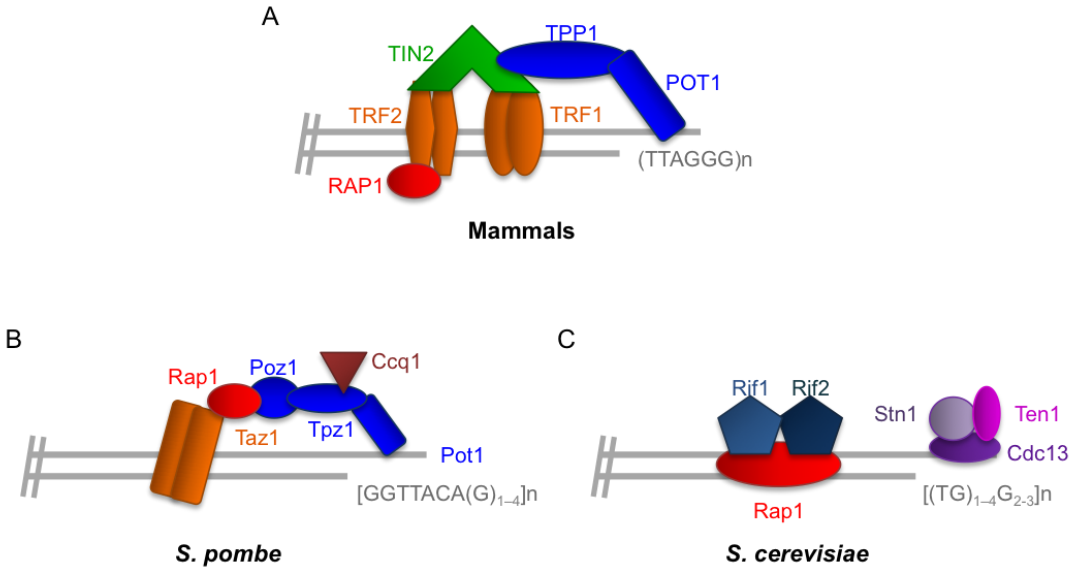


Figure 1.2 – Telomeric complexes in mammals, *S.pombe* and *S. cerevisae*.
Comparison between the telomeric complexes of mammals (A), *S. pombe* (B) and *S. cerevisiae* (C).

As telomere protection by the shelterin complex is so critical, similar systems to protect chromosome ends are found in other organisms. Interestingly, the structure of *Schizosaccharomyces pombe* telomeres resembles the one of mammalian cells (Figure 1.2B). Similarly to the mammalian shelterin complex, also *S. pombe* has one protein binding to telomeric dsDNA (Taz1) and the other to ssDNA overhang (Pot1) (Baumann and Cech, 2001; Cooper et al., 1997). More recently, TPP1 homolog (Tpz1) was also identified, as well as the other telomere-associated proteins, Ccq1 and Poz1 (Miyoshi et al., 2008). *S. pombe* Rap1 also binds to the TRF homolog Taz1, as observed in mammalian cells (Chikashige and Hiraoka, 2001; Kanoh and Ishikawa, 2001). In contrast, *Saccharomyces cerevisiae* telomeres harbour a distinct set of telomeric proteins. In this organism, Rap1 plays a key role in telomere maintenance and protection (Conrad et al., 1990; Lustig et al., 1990). Contrary to the mammalian RAP1, scRap1 is able to bind directly to dsDNA not only at the telomeres but also at other loci where it acts as a gene repressor/activator (reviewed in Shore, 1994) (Figure 1.2C). Although the conformation of its Myb domain prevents the mammalian RAP1 from binding directly to the DNA (Hanaoka et al., 2001), recent studies have shown that it might also have an important extra-telomeric role (Martinez et al., 2010; Teo et al., 2010). *S. cerevisiae* Rap1 is also known to interact with Rif1 and Rif2 (Hardy et al., 1992; Wotton and Shore, 1997), two factors required for telomere length regulation. Interestingly, mammalian RIF1 is not a telomeric protein, but is part of the DNA damage response (DDR) machinery and can only be found at telomeres that have lost their integrity (Silverman et al., 2004; Xu and Blackburn, 2004). Additionally, the CST (Cdc13/Stn1/Ten1) complex, which binds to the single stranded telomeric overhang, is also a critical player in telomere protection in yeast (Gao et al., 2007), Cdc13 being the functional homolog of POT1. Although no discernible mammalian CST homologs have initially been found, recent studies were able to identify the mammalian CST (CTC1/STN1/TEN1) complex (Miyake et al., 2009; Surovtseva et al., 2009).

Apart from the core telomeric proteins, several accessory factors have been described. The vast majority of these factors are known to be involved in the DDR, e.g. the MRN complex (MRE11-RAD50-NBS1) (Zhu et al., 2000), Ku70/80 (Hsu et al., 1999; Jaco et al., 2004), ERCC1/XPF (Zhu et al., 2003), PARP1 and PARP2 (Dantzer et al., 2004; Gomez et al., 2006), 9-1-1 (Francia et al., 2006), RAD51D (Tarsounas et al., 2004), BRCA2/RAD51 (Badie et al., 2010; Min et al., 2012), RAD54 (Jaco et al., 2003), RecQ helicases (Opresko et al., 2002), RTEL1 (Ding et al., 2004) and Apollo (Freibaum and Counter, 2006; Wu et al., 2010; Ye et al., 2010). Studies further exploring the telomeric proteome have suggested that additional factors with potential telomeric roles are still to be characterized (Dejardin and Kingston, 2009; Giannone et al., 2010; Kim et al., 2009).

Altogether these proteins contribute to maintain the telomeric integrity that is critical for cell survival. Moreover, they ensure the two critical functions of telomeres: to prevent the loss of genetic information due to the incomplete replication of the chromosome termini and to provide a capping structure for chromosome ends allowing them to be distinguished from a double strand break (DSB) and protecting them from degradation and fusion.

1.2 Key aspects of telomere maintenance: lengthening, replication and capping

1.2.1 Telomere length homeostasis

As telomeres are of such critical importance for cell viability, their maintenance is a crucial process. Due to the end replication problem that arises in linear chromosomes, telomere erosion occurs in every round of replication. In mammals, the majority of cell types do not have an active mechanism to maintain their telomere

length and when telomeres become too short they impact on cell homeostasis triggering senescence or apoptosis. In contrast, some organisms like yeast and some mammalian cell types like embryonic stem cells, have coupled replication with a mechanism that allows the addition of new telomeric sequences.

The increment of telomeric repeats at chromosome termini is a highly regulated process carried out by a specialized ribonucleoprotein complex, the telomerase. The telomerase holoenzyme comprises an enzyme with reverse transcriptase activity (TERT) and a RNA template (TERC) (reviewed in Palm and de Lange, 2008) (Figure 1.3). Telomerase RNA harbours different secondary structures and includes a region that is complementary to the telomeric repeats, allowing for both initial annealing and the subsequent elongation process. The 3' end of TERC contains an H/ACA box, which can also be found in small nucleolar RNAs (snoRNAs) and that is involved in their maturation (Mitchell et al., 1999a). The H/ACA box is a critical domain for the association of the ribonucleoprotein (RNP) complex involved in the snoRNA processing. Furthermore, some of the RNP components have been ascribed a critical function in telomerase biogenesis, including GAR1, NHP2, NOP10 and dyskerin (Mitchell et al., 1999b; Pogacic et al., 2000; Wang and Meier, 2004). Mutations in these components have been associated with dyskeratosis congenital (DC), a premature ageing syndrome, where cells display very short telomeres (Mitchell et al., 1999b; Vulliamy et al., 2008).

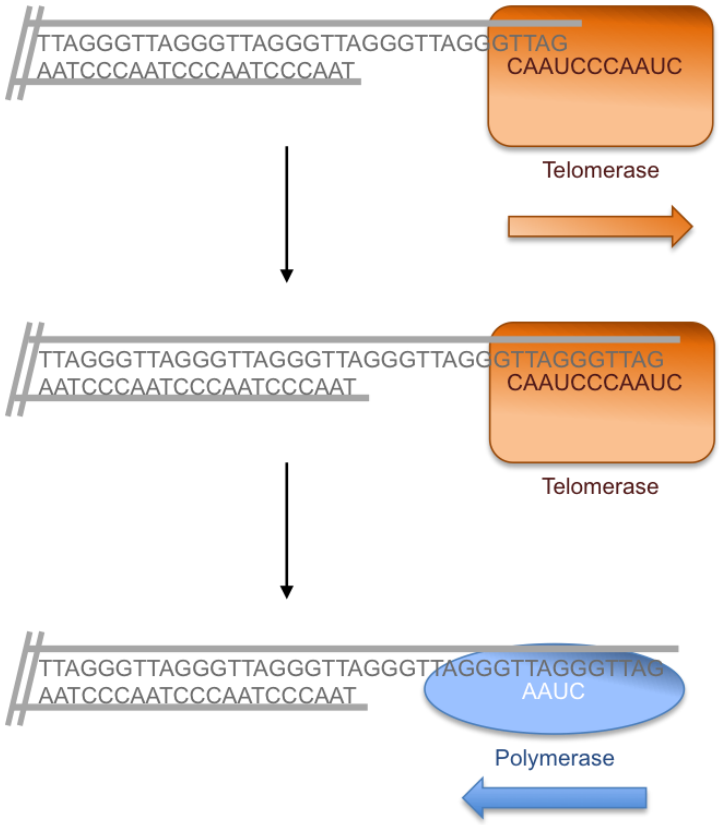


Figure 1.3 – Telomerase allows telomere lengthening.

Telomerase comprises the reverse transcriptase holoenzyme TERT and the RNA template TERC, which serves as a template for the addition of new telomeric repeats to the G-rich strand. Complementation of the C-rich strand is performed by DNA polymerase. This enzyme uses a RNA primer which is removed at the end of the polymerization creating the 3' overhang.

Apart from telomerase biogenesis, regulation of telomerase activity is a key factor in the maintenance of telomere length homeostasis. Maintaining telomeres at a fairly constant length is a critical process to maintain genome stability. If telomeres become critically short, the DNA damage machinery is activated leading to cell cycle arrest. If, in contrast, they are too long, this could represent an additional replication effort, due to the repetitive nature of the telomeres.

Several telomeric proteins act in different organisms in *cis* to ensure this tight control. In yeast, Rap1, Rif1 and Rif2 form a critical complex involved in a negative feedback loop that allows the telomeres to be kept under a certain range of lengths (reviewed in Smogorzewska and de Lange, 2004). Additionally, Cdc13, a ssDNA-

binding telomeric protein, has been shown to have dual role controlling telomere elongation. On one hand it is critical for the recruitment of yeast telomerase to the telomeres (Nugent et al., 1996), on the other, it also seems to act as a negative regulator of telomere elongation, preventing the continuous telomere lengthening (Chandra et al., 2001; Grandin et al., 1997). In mammalian cells, all the components of the shelterin complex act as negative regulators of telomere length (reviewed in Palm and de Lange, 2008; and Smogorzewska and de Lange, 2004). TRF1 role in modulating telomerase activity is probably the best characterized. TRF1 has three distinct binding partners: Tankyrase 1 and 2, two poly(ADP-ribose) polymerases (PARP) that interact with TRF1 inhibiting its binding to the telomeres and promoting telomere elongation; and TIN2, which avoids TRF1 poly(ADP-ribosyl)ation by tankyrases, allowing it to bind to the telomeric DNA and preventing telomerase access (Cook et al., 2002; Smith and de Lange, 2000; Smith et al., 1998; Ye and de Lange, 2004). Similarly to *S. cerevisae*, telomeric ssDNA binding factor POT1 plays an important role in telomere length homeostasis. POT1 displacement from the telomeres leads to telomerase-dependent telomere elongation (Loayza and De Lange, 2003). Moreover, POT1 and telomerase compete for the binding to the 3'-end of the telomeric overhang (Kelleher et al., 2005), suggesting that POT1 acts as a sensor for the overhang length. Interestingly, the POT1-TPP1 complex acts as a telomerase processivity factor during telomere extension (Wang et al., 2007), implying that POT1, like Cdc13 in yeast, has a dual role. If POT1 is associated with the 3' overhang, it is likely to inhibit telomerase binding and subsequent telomere elongation. In contrast, when telomeres become too short, the POT1-TPP1 complex can act as a positive regulator of telomere length.

Although for many years it was believed that telomeres were transcriptionally silent, recent work led to the identification of a telomeric repeat-containing RNA (TERRA), a large non-coding RNA that is an integral component of telomeric heterochromatin (Luke and Lingner, 2009). TERRA localizes to telomeres, being a

novel structural component that is required for several aspects of telomere maintenance, including telomere replication and lengthening (Azzalin et al., 2007; Flynn et al., 2011; Schoeftner and Blasco, 2008). Furthermore, TERRA was proposed as a new negative regulator of telomerase, according to *in vitro* results showing that a TERRA-mimicking RNA oligonucleotide is able to inhibit telomerase activity (Schoeftner and Blasco, 2008). Additionally, a negative correlation between telomere length and TERRA abundance has also been described (Azzalin et al., 2007; Schoeftner and Blasco, 2008).

Apart from the structural telomeric components, other factors including the ubiquitin ligase MKRN1 (Kim et al., 2005), the Akt protein kinase (Kang et al., 1999) or the histone deacetylase 8, HDAC8 (Lee et al., 2006a), have also been reported to act as modulators of telomerase, stressing the importance of telomere length control.

Although telomerase-dependent telomere elongation is the most widely used mechanism for telomere extension, some cells are able to retain their telomeric length using an alternative mechanism called Alternative Lengthening of Telomeres (ALT) (reviewed in Cesare and Reddel, 2010). This process is dependent on homologous recombination (HR), although the exact mechanism by which it occurs is still uncertain. It was proposed that one telomeric overhang could strand invade the dsDNA of another telomere promoting inter-telomeric recombination and generating telomeres of very heterogeneous sizes (Dunham et al., 2000) (Figure 1.4A). Apart from intra-telomeric recombination, intra-chromosomal recombination has also been described (Wang et al., 2004). This process involves resolution and consequent loss of the T-Loop structure, with formation of telomeric circles (T-circles) and chromosomes with very short telomeres (Figure 1.4B). Telomere length in ALT cells involves different mechanisms. The first mechanism to be described was MRN complex-dependent (Jiang et al., 2005; Zhong et al., 2007). Later on, other proteins such as the SMC5-SMC6 complex (Potts and Yu, 2007) or the Fanconi anemia proteins FANCD2 and FANCA (Fan et al., 2009) have also been shown to be

required for telomere elongation in ALT cells. In addition, HR proteins such as RAD51, BRCA2, RAD51D have also been found to colocalize to ALT-associated promyelocytic leukaemia bodies (APBs), sub-nuclear compartments characterised by the presence of telomeric sequences and telomere-associated proteins, which are considered hallmarks of ALT cells (Tarsounas and West, 2005).

The diversity of mechanisms involved in telomere lengthening in ALT cells stresses once more the importance of telomere length homeostasis.

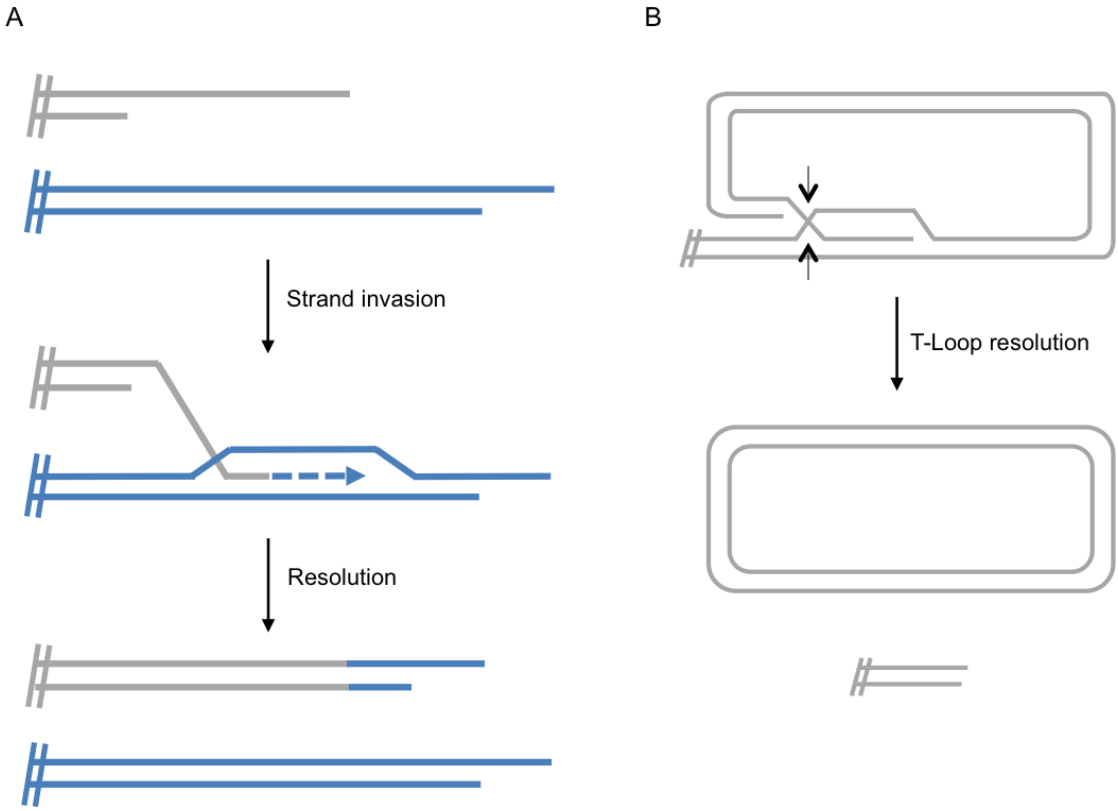


Figure 1.4 – Alternative lenghthening of telomeres.
ALT can occur through inter-chromosomal invasion, where one telomere uses another one as a template to mediate telomere elongation (A) or it can be mediated by intra-strand recombination, which results in T-Loop resolution with consequent loss of T-circle and generation of a very short telomere.

1.2.2 Telomere replication

Due to their repetitive DNA sequence, telomeres face a major problem: DNA replication. In addition, telomeric chromatin shows characteristics that might render

replication even more challenging. In vertebrates, telomeric chromatin is enriched for H3K9m3 mark, providing a binding site for Heterochromatin Protein 1 (HP1) and turning the telomeres into a heterochromatic region (reviewed in Schoeftner and Blasco, 2009). Likewise, other structures such as T-loops and G-quadruplexes are also obstacles to overcome. These properties renders the telomeres similar to fragile sites, i.e. specific loci that exhibit gaps and breaks on metaphase chromosomes, as a consequence of chromosome instability and partial inhibition of DNA synthesis. Moreover, yeast telomeres, like fragile sites, are replicated only in late S-phase (Raghuraman et al., 2001). In contrast, in mammalian cells, telomere replication seems to occur throughout S-phase (Hultdin et al., 2001; Wright et al., 1999).

So far no origin of replication has been identified inside the telomeric sequence, the closest origin being in the subtelomeric region. Therefore, it is not surprising that several studies reported that replication forks can naturally pause or stall at telomeres (Ivessa et al., 2002; Makovets et al., 2004; Verdun and Karlseder, 2006). Another challenge for telomere replication is the presence of telomere capping complexes bound to the telomeric DNA. In human cells, the shelterin components are bound to the telomeres throughout the cell cycle, even though levels of TRF1 and POT1 seem to be reduced in S or G2 phases of the cell cycle, respectively (Verdun et al., 2005). Interestingly, DDR factors such as ATM, ATR, RPA, MRE11 and BRCA2/RAD51 were shown to localize at functional telomeres during S/G2, in response to two different DNA damage signals (Badie et al., 2010; Verdun et al., 2005; Verdun and Karlseder, 2006). The first event is thought to occur when the replication fork stalls at the telomeres and the DNA damage machinery is required for the restart of the stalled fork (Figure 1.5A). The second signal might be prompted by the generation of short 3' overhangs at the end of the lagging strand. After replication, telomeres restore their T-loop conformation in a process that was shown *in vitro* to be dependent on homologous recombination factors RAD51 and XRCC3 (Verdun and Karlseder, 2006). Altogether this suggests that DNA damage

machinery plays an important role in allowing telomere replication and further processing to be completed.

In addition to DDR factors and contrary to what was initially assumed, some telomeric proteins can have an important role themselves in promoting replication fork progression. The first insight came from work in fission yeast, where replication of telomeric DNA requires the dsDNA binding protein, Taz1 (Miller et al., 2006). Recent work has suggested that the mammalian telomeric dsDNA binding proteins, TRF1 and TRF2, also play a role in telomere replication (Martinez et al., 2009; Sfeir et al., 2009; Ye et al., 2010). Furthermore, these proteins recruit helicases (*e.g.* WRN, BLM and RTEL1) and topoisomerases (Topoisomerase 2α) to telomeres in order to facilitate replication fork progression (Crabbe et al., 2004; Sfeir et al., 2009; Ye et al., 2010). Recently, attention has been brought in particular to RTEL1, a helicase critical to remove T-loop structures and prevent formation of G-quadruplexes, therefore promoting telomere replication (Uringa et al., 2012; Vannier et al., 2012).

The fragile telomere phenotype is characterized by the occurrence of multitelomeric signals (MTS), a hallmark of telomere replication defects (Martinez et al., 2009; Okamoto et al., 2008; Sfeir et al., 2009). These were first reported in embryonic stem cells after conditional deletion of TRF1 (Okamoto et al., 2008). Since then, these defects have been reported in the absence of other proteins, including DNA repair factors such as ATR (Martinez et al., 2009) and BRCA2/RAD51 (Badie et al., 2010) or the CST components CTC1 and STN1 (Price et al., 2010). Moreover, CTC1 and STN1 have been identified as co-factors of DNA polymerase α (Pol α) - primase and showed to act by increasing the affinity of Pol α to the primase template, promoting lagging strand synthesis (Casteel et al., 2009). Even though only recently the role of mammalian CST complex in telomeric replication started to be uncovered, it is highly likely that this complex would play a role similar to its yeast counterparts

(Figure 1.5A). Moreover, a recent study using conditional CTC1 mouse embryonic fibroblasts (MEFs), revealed that CTC1 is required for efficient telomere replication (Gu et al., 2012). Similarly, knockdown of CST components in human cells was also reported to lead to replication defects both in telomeric and non-telomeric regions (Huang et al., 2012; Stewart et al., 2012).

Replication protein A (RPA) is a ssDNA-binding heterotrimeric complex with diverse cellular roles including DNA replication and DDR (reviewed in Sakaguchi et al., 2009). RPA binds to the single stranded telomeric DNA during S phase (Verdun and Karlseder, 2006), most likely by competing with the telomeric protein POT1, for binding to the ssDNA. Another interesting fact is that, after binding to the ssDNA, RPA promotes the recruitment of ATR, triggering a DNA damage checkpoint (Zou and Elledge, 2003), while POT1 binding to the telomeres suppresses ATR activation (Denchi and de Lange, 2007; Guo et al., 2007). This raises the question of how the RPA-POT1 switch is regulated and how this affects telomere replication. Recent work by Flynn and co-workers showed that this switch is orchestrated by heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and the telomeric repeat-containing RNA (TERRA) in a sequential manner throughout S phase (Flynn et al., 2011), thus explaining how telomeres lose the DNA response factor RPA and reassociate with the capping protein POT1.

An interesting question is how telomeres are processed after replication and what is the mechanism underlying the generation of the 3' overhang. Recently it has been suggested that Apollo, a TRF2-interacting protein, might play a critical role in this process (Wu et al., 2010; Ye et al., 2010). During telomere replication, TRF2 regulates the exonuclease activity of Apollo allowing the generation of 3' overhangs. Additionally, TRF2 and Apollo cooperate with topoisomerase 2, allowing efficient replication by relieving the topological stress of the telomeric region (Ye et al., 2010). Whilst the role of Apollo has been suggested to be critical for the generation of the leading strand of the telomeres, EXO1 has been demonstrated to be the

exonuclease required for both leading and lagging strand processing (Wu et al., 2012) (Figure 1.5B). Extensive resection mediated by this exonuclease is counteracted by the CST complex, which promotes the fill-in reaction, generating the final overhangs.

In *S. cerevisiae* formation of the 3' overhang after replication requires the same machinery as the one involved in DSB repair, being mediated by proteins such as MRX (MRN homolog) complex, Sae2 (CtIP homolog), Exo1, Sgs1 (WRN/BLM homologs) and Dna2 (Bonetti et al., 2009; Larrivee et al., 2004). In mammalian cells, the roles of MNR complex and BLM are dispensable for the post-replication processing (Wu et al., 2012); but the roles of DNA2, WRN and CtIP still need to be determined.

Although several recent studies have shed new light on telomere replication, the exact replication process is still to be unravelled.

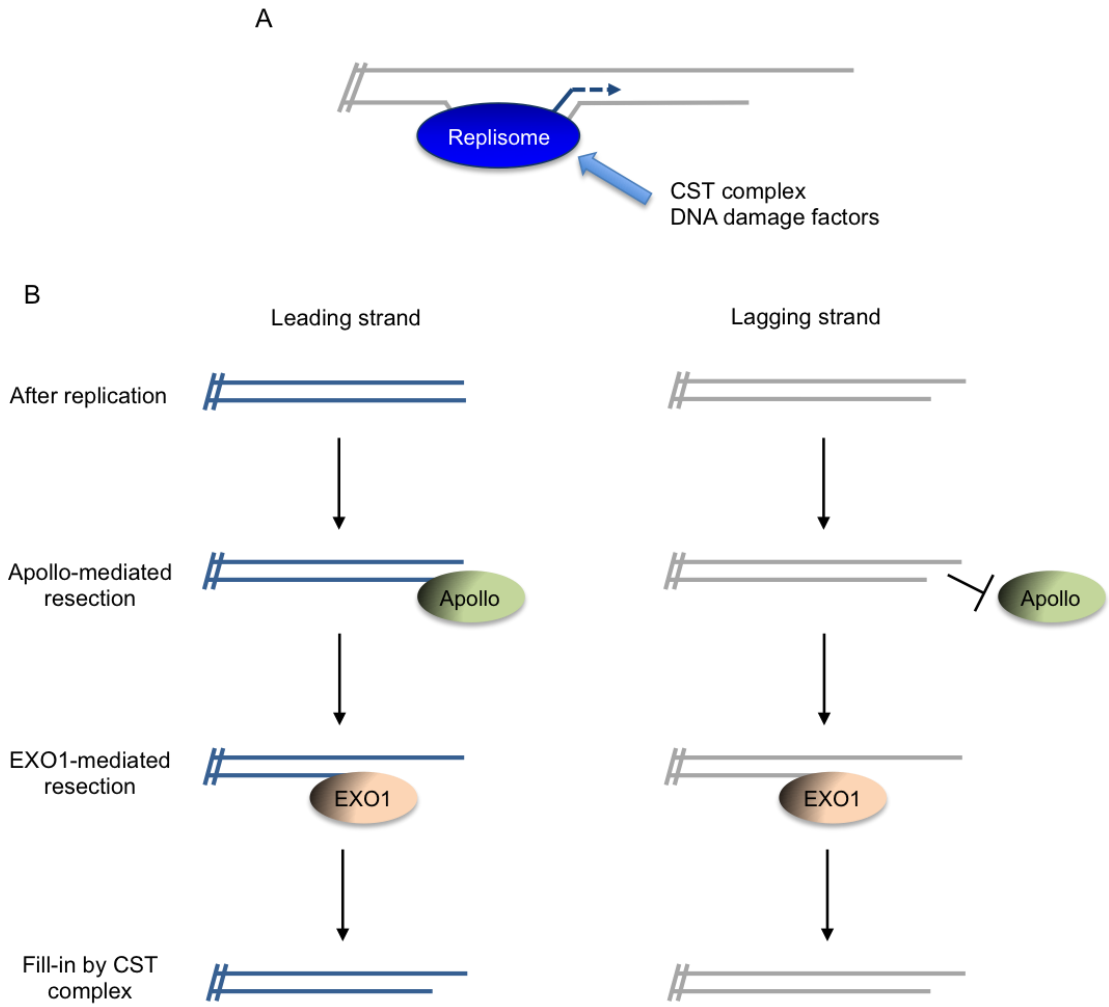


Figure 1.5 – Telomeric replication in mammalian cells.

A) The replisome ensures telomere replication. Factors such as the CST complex or DNA repair proteins help to facilitate this process. B) After replication, the 3' overhang is processed. In leading telomeres, processing is mediated first by Apollo and then by EXO1. Lagging strand telomeres are processed by EXO1 alone. In order to generate the final 3' overhang, the CST complex promotes fill-in synthesis.

1.2.3 Telomere protection

The discovery of telomerase solved the end-replication, but not the end-capping problem. In addition to telomere length maintenance, the assembly of capping structures is vital for telomere integrity.

When devoid of protective structures, the 3' single strand telomeric overhang is exposed and can trigger a DNA damage response. The shelterin complex in mammalian cells and the telomeric proteins in other organisms play crucial roles in

safeguarding the chromosome ends (Palm and de Lange, 2008). TRF2 and POT1 are two key players that prevent the DNA damage response by repressing activation of the two main DNA damage kinases, ATM and ATR, respectively (Denchi and de Lange, 2007; Guo et al., 2007) (Figure 1.6A). POT1 has an additional role in protecting the telomeres from 5'-end resection, thus avoiding the formation of extremely long overhangs (Kibe et al., 2010; Palm et al., 2009). Interestingly, the genome of rodents contains two *POT1* isoforms, *POT1a* and *POT1b*, which bear distinct telomeric functions. While POT1a prevents ATR activation, POT1b represses resection of the 5' telomeric end (Hockemeyer et al., 2006; Kibe et al., 2010), which emphasizes the importance of both roles of POT1 in telomere capping.

Apart from a role in telomere replication, TRF1 is also required for telomere capping through inhibition of both ATM and ATR (Martinez et al., 2009; Sfeir et al., 2009). In fact TRF1 seems to be critical to avoid ATR activation during S-phase (Sfeir et al., 2009), consistent with its importance in regulating telomere replication.

Telomeric proteins are constituents of capping structures in other organisms such as fission yeast, budding yeast or the fruit fly (reviewed in Ciapponi and Cenci, 2008; Jain and Cooper, 2010). Similarly to mammals, also in *S. pombe* Pot1 and its interacting partner Ccq1 repress the checkpoint cascade triggered by Rad3, the ATR homolog (Carneiro et al., 2010; Pitt and Cooper, 2010; Tomita and Cooper, 2008). In *S. cerevisae*, lack of the ssDNA binding protein Cdc13, leads to Mec1 (ATR homolog) -dependent Rad9 activation and to cell cycle arrest (Garvik et al., 1995; Jia et al., 2004).

While the role of the budding yeast CST component, Cdc13, in telomere capping was long established, only recently the function of the mammalian CST complex in telomere maintenance has been characterized (Gu et al., 2012; Huang et al., 2012; Miyake et al., 2009; Surovtseva et al., 2009; Wu et al., 2012) (Figure 1.6B). Similar to the Cdc13 mutants in yeast, depletion of mammalian CTC1 or STN1 also leads to increase in the overhang size, suggesting that in addition to POT1, the CST

complex also plays an important role in protecting the 5' end from resection (Garvik et al., 1995; Miyake et al., 2009; Surovtseva et al., 2009).

Intriguingly, several DDR factors also play a critical role in telomere protection (reviewed in Ueno, 2010) (Figure 1.6B). In fact, inactivation of ATM in human cells (Rad3 in *S. pombe* and Tel1 in *S. cerevisiae*) causes telomere shortening (Dahlen et al., 1998; Lustig and Petes, 1986; Metcalfe et al., 1996).

In addition to checkpoint activation, another hallmark of telomere uncapping is the formation of chromosome end-to-end fusions. Dysfunctional telomeres are sensed by the cells as DSBs and usually repaired through an error-prone pathway the nonhomologous end-joining (NHEJ).

Apart from ATM whose abrogation leads to chromosome fusions in *S. cerevisiae* (Chan and Blackburn, 2003; Naito et al., 1998), the other important double strand break sensors, the kinase DNA-PK and the Ku70/80 heterodimer, also plays roles in telomere capping, as their inactivation causes telomere fusions (Bailey et al., 1999; Gilley et al., 2001; Hsu et al., 2000; Jaco et al., 2004). Furthermore, ATM and Ku70/80 are recruited to telomeres under physiological conditions through their interaction with TRF2 (Karlseder et al., 2004; Song et al., 2000). TRF2 also interacts with other DNA repair proteins including the MRN complex (Zhu et al., 2000) and PARP1, a polymerase that catalyses the poly(ADP-ribosyl)ation of several substrates involved in DNA repair (Gomez et al., 2006). This not only emphasizes the close relation between DDR proteins and telomeric proteins, but also reinforces the role of TRF2 as a central factor in telomere maintenance.

Additional DNA repair proteins such as the histone acetyltransferase GCN5 (Atanassov et al., 2009), the mismatch repair protein MSH2 (Campbell et al., 2006) or the RecQ helicases WRN and BLM (Callen and Surralles, 2004; Crabbe et al., 2007; Crabbe et al., 2004; Du et al., 2004), were also reported to be directly or indirectly required for proper telomere capping.

Overall, the involvement of such a complex protein network in telomere

capping clearly shows how important is telomere integrity for cell viability.

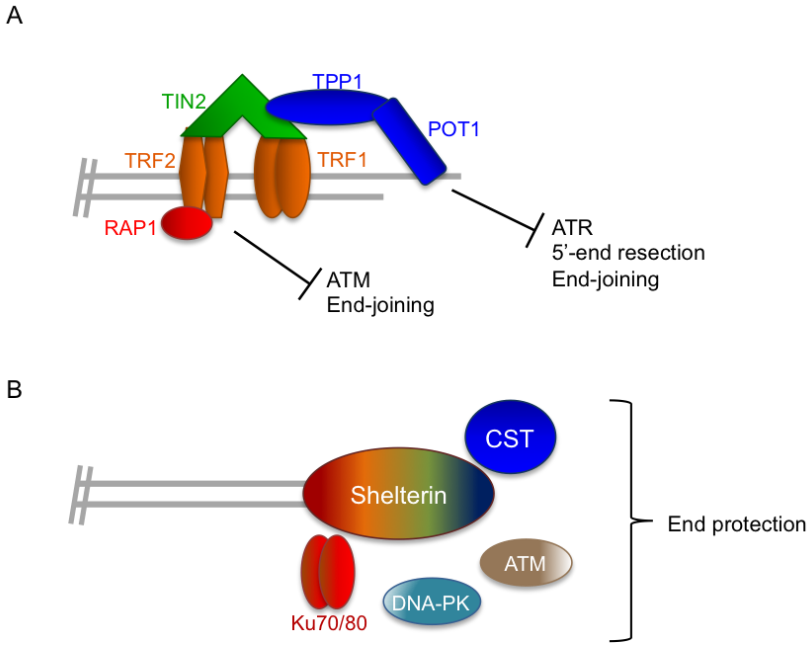


Figure 1.6 – Shelterin complex and acessory proteins protect telomeres.

A) Shelterin complex acts to protect telomeres from checkpoint activation mediated by ATM and ATR, 5'-end resection and end-joining reactions. B) Apart from the shelterin complex also other proteins including the CST complex and the DNA damage factors contribute for chromosome end protection.

1.3 Telomere dysfunction and the DNA damage response

The genome can be exposed to various types of endogenous (e.g. reactive oxygen species, as recombination and replication intermediates) and exogenous damages (e.g. ultraviolet radiation and chemotherapeutic drugs) (reviewed in Sancar et al., 2004). In order to rapidly repair these damages, cells evolved several mechanisms of DNA repair.

When a DSB is produced in a cell, for example by reactive oxygen species or as a consequence of replication fork arrest and collapse, two main pathways act to repair such damage: HR and NHEJ. The choice between HR and NHEJ is thought to be primarily dependent on the cell cycle. HR is a three-step reaction (strand invasion, branch migration and Holliday junction formation) dependent on key factors such as BRCA2 and RAD51 (reviewed in Sancar et al., 2004). HR requires an intact DNA

template, usually a sister chromatid and thus it takes place in the S/G2 phases of the cell cycle. NHEJ, on the other hand, is based on the re-joining of two broken ends through the sequential action of Ku heterodimer, DNA-PK catalytic subunit and DNA Ligase IV and has been shown to occur primarily in G1 (reviewed in Sancar et al., 2004). Interestingly, apart from the classical NHEJ pathway (C-NHEJ), the other pathways of end-joining that do not require some of the canonical components have gained more impact in the last decade. These pathways are generally referred to as alternative NHEJ (A-NHEJ) or backup NHEJ (B-NHEJ) (reviewed in Iliakis, 2009; McVey and Lee, 2008; Nussenzweig and Nussenzweig, 2007).

For the cell to be able to repair a break, it is essential that DNA damage checkpoints are activated. The three main checkpoint kinases involved in DNA repair are the phophoinositide 3-kinase related kinases ATM, ATR and DNA-PK. Once activated, these kinases lead to signal transduction primarily through a series of phosphorylation and ubiquitylation events that promote activation of DNA repair factors and cell cycle arrest.

The recruitment of checkpoint kinases to DNA lesions requires generation of DNA repair intermediates. For instance, a DSB is first processed by the MRN complex, generating a ssDNA region that provides the substrate for the activation of checkpoint responses mediated through ATM (reviewed in Branzei and Foiani, 2008). Additionally, the presence of a ssDNA leads to the recruitment of RPA and then the ssDNA-RPA complex confers the activating signal for ATR recruitment. DNA-PK can also be recruited to a DSB when Ku70/80 heterodimer is bound at the sites of break.

ATM activation triggers a series of events that ultimately promote repair. ATM phosphorylates the histone H2AX (γH2AX) (Burma et al., 2001), allowing the damage signal to be spread to the chromatin surrounding the break. This signal is not only important for the recruitment of more ATM molecules, but also of other proteins that

mediate the damage response (e.g. γH2AX, RNF8, MDC1, MRN, 53BP1, BRCA1). Additionally, ATM directly phosphorylates the checkpoint kinase-2 (CHK2) promoting cell cycle arrest (Matsuoka et al., 1998).

The RPA-bound ssDNA is the platform for recruitment of ATR and ATR-interacting partners such as ATRIP, TOPBP1 and the 9-1-1 complex (reviewed in Branzei and Foiani, 2008). Like ATM, ATR is responsible for the phosphorylation of several targets including γH2AX, the Fanconi anemia proteins FANCD2 and FANCI and most importantly the checkpoint kinase-1 (CHK1).

CHK1, CHK2 and cyclin-dependent kinases (CDK) are essential for the crosstalk between DDR factors and the cell cycle control machinery. This ensures activation of the correct repair pathway (NHEJ or HR) at the appropriate stage of the cell cycle (reviewed in Branzei and Foiani, 2008).

1.3.1 DNA damage response triggered by dysfunctional telomeres

In addition to DNA lesions described above, DDR can be triggered by dysfunctional telomeres. In the recent years, several studies have helped our understanding of how dysfunctional telomeres are perceived by the DDR machinery. However, the involvement of DDR proteins both in the DNA damage response emanating from uncapped telomeres and in telomere maintenance remains a conundrum in telomere biology.

Spontaneous telomere uncapping occurs in senescent cells due to erosion of telomeric repeats and in normally growing cells due to loss of protective structures (d'Adda di Fagagna et al., 2003; Takai et al., 2003). In the latest case, this physiological uncapping is required for telomeres to be replicated and elongated during S/G2. When the telomeres become dysfunctional they resemble DSBs and lead to checkpoint activation and recruitment of DDR factors to the telomeres (e.g. ATM, γH2AX and 53BP1). These can be visualized as microscopically defined

telomere-dysfunction induced foci or TIFs (reviewed in d'Adda di Fagagna et al., 2004; and Palm and de Lange, 2008). Due to the similarities between an uncapped telomere and a DSB, the recruitment of DDR factors to dysfunctional telomeres leads to repair by end-joining. Chromosomes with uncapped termini will generate NHEJ-mediated end-to-end fusions. Depending on the extent of the uncapping this can lead to several concatenated chromosomes.

Artificial removal of TRF2 or POT1 from telomeres has revealed distinct pathways for telomeres to avoid the DDR. Whilst loss of TRF2 leads to ATM activation, loss of POT1 triggers an ATR-dependent response (Celli and de Lange, 2005; Denchi and de Lange, 2007; Guo et al., 2007), suggesting specific roles for telomeric proteins in the prevention of the DNA damage activation at the telomeres. It remains unclear the exact mechanism by which TRF2 prevents ATM activation, but might do so by helping to maintain the T-loop structure, thus avoiding the exposure of the 3' overhang. POT1 repression of the ATR pathway is associated with the fact that POT1 is able to displace RPA from telomeres (Gong and de Lange, 2010). Although less abundant, POT1 has a greater affinity for telomeric DNA than RPA. In *S. cerevisiae*, deletion of *cdc13*, the POT1 functional homolog, leads to checkpoint activation dependent on Mec1 (ATR homolog) and Rad9 (Garvik et al., 1995; Lydall and Weinert, 1995). While in *S. pombe* Pot1 deletion elicits a Rad3 (ATR homolog) – mediated checkpoint response (Pitt and Cooper, 2010). Surprisingly, taz1 mutants (the *S. pombe* TRF1/2 homolog) do not activate checkpoint and their vegetative growth is normal (Cooper et al., 1997). Even though telomeres lacking Taz1 are able to recruit checkpoint sensors, such as RPA and Rad9, they fail to recruit Crb2 (53BP1 homolog), which plays a critical role in signal transduction and Chk1 activation (Carneiro et al., 2010). Moreover, in addition to H2A phosphorylation, also histone 4 dimethylation at lysine 20 (H4K20me2) is required for efficient Crb2 recruitment to sites of damage. H4K20me2 is absent in functional *S. pombe* telomeres, which prevents checkpoint transduction. So far, a similar link between

specific telomeric histone modifications and DNA damage at telomeres has not been described in vertebrates, but this study is an example of how chromatin structure can impact on telomere biology.

Apart from preventing the DNA checkpoint activation, telomeres also evade repair by the DDR machinery. When telomeres become dysfunctional by TRF2 removal, factors such as MRN or Ku70/80 bind to the telomeres and promote LIG4-dependent NHEJ (Celli et al., 2006; Deng et al., 2009; Dimitrova and de Lange, 2009; Smogorzewska et al., 2002). Interestingly, Ku70 also plays an important role in functional telomeres repressing HR, which can be visualized by the increased number of sister chromatid exchanges at telomeres that lack Ku70 (Celli et al., 2006). This demonstrates the importance of preventing HR at telomeres, thus avoiding unequal exchanges of telomeric DNA, which can give rise to critically short telomeres. In addition, and due to the interaction between TRF2 and the Ku heterodimer, this further stresses how essential is the presence of TRF2 in avoiding NHEJ at the telomeres.

In *S. pombe, taz1* mutants undergo NHEJ in a Ku70-dependent manner (Ferreira and Cooper, 2001), showing that this process is transversal across eukaryotes. The formation of end-to-end fusions also occurs in cells lacking the Taz1-interacting protein Rap1 (Miller et al., 2005). Similarly, inactivation of Rap1 in *S. cerevisiae* also leads to telomere fusions, which requires core components of NHEJ, including Ku70/80, Lig4 and the MRX complex (Pardo and Marcand, 2005). In human cells, the use of a RAP1 construct that binds to telomeric DNA in the absence of TRF2 also showed that RAP1 can play a role in NHEJ, reducing the number of fusions observed after TRF2 removal from the telomeres (Sarthy et al., 2009). However, in mouse cells, RAP1 cannot prevent NHEJ (Martinez et al., 2010; Sfeir et al., 2010). Instead it is critical for preventing telomeric sister chromatid exchanges (Sfeir et al., 2010). Moreover, recent work showed that the role previously attributed to TRF2 in preventing HR (Celli et al., 2006), is not due to TRF2 itself but to its

function of recruiting RAP1 to the telomeres (Sfeir et al., 2010). The differences between human and mouse RAP1 might be due to the different experimental approaches used and are not mutually exclusive. RAP1 can act primarily by preventing HR at telomeres, which is supported by its ability to interact with Ku70/80 (O'Connor et al., 2004). Additionally, in the absence of TRF2, a version of RAP1 protein that binds to the telomeres could potentially repress NHEJ, like its yeast counterparts.

In mammalian telomeres that lack TRF2, the connection between the checkpoint activation and NHEJ seems to rely on 53BP1, an important ATM target that mediates checkpoint responses (Dimitrova et al., 2008). Supporting this, in 53BP1-deficient cells, depletion of TRF2 did not result in the formation of fusions. The exact mechanism by which 53BP1 promotes NHEJ at uncapped telomeres is not clear. Data from time-lapse microscopy showed that changes in chromatin dynamics after TRF2 depletion were dependent on 53BP1 (Dimitrova et al., 2008), suggesting that 53BP1 might help to bring together two uncapped telomeres allowing repair by NHEJ. The role of 53BP1 in joining two broken ends was also suggested to be required during immunoglobulin class-switch recombination, a process that involves DNA cleavage and subsequent long-range joining of DNA breaks (reviewed in Stavnezer et al., 2008).

Other telomeric proteins such as TPP1, POT1 and TRF1 are also required to prevent chromosome end-to-end fusions, strengthening the importance of telomere capping in NHEJ prevention (Deng et al., 2009; Hockemeyer et al., 2006; Hockemeyer et al., 2005; Kibe et al., 2010; Martinez et al., 2009). Moreover, telomere uncapping by removal of a particular telomeric component has revealed specific ways in which end joining can occur. While absence of TRF2 leads to chromosome fusions occurring primarily in G1 (Konishi and de Lange, 2008), POT1 deletion leads primarily to chromatid fusions thought to occur after replication in S phase (Hockemeyer et al., 2006). The presence of sister chromatid fusions also

suggests that POT1 might play a role preventing HR at telomeres (Hockemeyer et al., 2006). Furthermore, while TRF2 abrogation leads to chromosome fusions that are LIG4-dependent and mediated by C-NHEJ reaction, the fusions formed after removal of TPP1/POT1 complex are LIG4-independent and enhanced after Ku70 depletion, suggesting that they are the result of A-NHEJ (Rai et al., 2010). Interestingly, absence of C-NHEJ components such as Ku or DNA-PKcs also lead to spontaneous telomere fusions (Bailey et al., 1999; Gilley et al., 2001; Hsu et al., 2000), suggesting that these factors, which are present at functional telomeres, may act together with telomeric proteins to prevent HR and A-NHEJ.

1.3.2 DNA end-processing reactions at dysfunctional telomeres

As DNA break repair is vital to cell survival, the break has to be rapidly recognized, processed and healed. Similarly to DSBs, uncapped telomeres are also recognized and processed by the DNA repair machinery, but exactly how this occurs is still to be determined. The absence of a specific telomeric component elicits different DNA repair pathways (e.g. TRF2 inactivation triggers C-NHEJ, while POT1 abrogation triggers A-NHEJ) suggesting that telomere processing is specific to removal of each shelterin component.

As with most DNA repair pathways, C-NHEJ requires nucleases to resect the sites of break before they can be properly ligated. The Ku heterodimer is thought to be the first factor to arrive to the damage site and enable the recruitment of the Artemis:DNA-PKcs nuclease complex (reviewed in Lieber, 2010). Ku:DNA complexes also promote binding of DNA polymerases (Pols μ and λ) to fill-in newly-synthesized DNA. Subsequently, the two broken ends are ligated by a very versatile ligase, the complex XLF:XRCC4:LIG4.

The roles of Ku heterodimer and Artemis:DNA-PKcs nuclease complex are thought to be critical for end-to-end fusions of dysfunctional telomeres. Paradoxically,

these components are also required for the proper telomere capping, as cells lacking these proteins harbour telomeric fusions (Bailey et al., 1999; Gilley et al., 2001; Hsu et al., 2000; Jaco et al., 2004; Rooney et al., 2003). This further suggests alternative ways to process uncapped telomeres.

Similarly to a DSB, TRF2-depleted telomeres have to be processed before being engaged in LIG4-dependent repair. The 3' telomeric overhang is removed to generate blunt ends that can be efficiently ligated. The precise mechanism behind this processing is still uncertain, but some players have been identified. Interestingly, the first protein reported to be required for removal of 3' telomeric overhangs was ERCC1/XPF (Zhu et al., 2003), a 3' flap endonuclease involved in nucleotide excision repair and Ku80-independent microhomology endjoining (Ahmad et al., 2008). Additionally, the MRN complex is also critical for the end-to-end fusions that arise after TRF2 abrogation (Deng et al., 2009; Dimitrova and de Lange, 2009). The MRN/X complex is composed by MRE11, RAD50 and NBS1 (or Xrs2 in *S. cerevisae*) and can be found in most eukaryotes. The MRN/X complex is a critical sensor of DSBs, but it also functions in tethering the DNA end and promoting NHEJ (reviewed in Stracker and Petrini, 2011). After binding to a DSB, MRN/X complex together with CtIP (or Sae2 in *S. cerevisae*), initiates resection reaction through removal of a short ssDNA sequence. In *S. cerevisae*, it is well established that, following this initial resection, MRX further recruits Dna2, Exo1 and Sgs1 (a member of the RecQ helicases family), which promote extensive resection, critical for DSB repair by HR (reviewed in Stracker and Petrini, 2011). In yeast, the roles of MRX complex, Sae2, Dna2, Exo1 and Sgs1 in telomere resection have been characterized (Longhese et al., 2010). These proteins are required after telomere replication for generation of the 3' ssDNA overhang, but their function in processing uncapped telomeres is not yet fully understood. In mammalian cells, the MRN complex is required for processing TRF2-depleted telomeres (Deng et al., 2009; Dimitrova and de Lange, 2009). MRE11 acts by promoting resection of the 3' overhang, since mouse cells expressing

a nuclease-deficient MRE11 allele retain the 3' overhang and consequently are unable to form end-to-end fusions after TRF2 depletion (Deng et al., 2009). The MRN complex is not only critical for NHEJ, but also for sustaining the ATM signalling that emanates from uncapped telomeres after TRF2 depletion (Deng et al., 2009; Dimitrova and de Lange, 2009). Furthermore, ATM itself is required not only for the fusions that occur after TRF2 depletion, but also for processing the 3' overhang (Denchi and de Lange, 2007), pointing once more to the importance of these upstream DNA damage factors in the processing of uncapped telomeres.

Although the role of MRN complex in telomere biology has been elucidated, very little is known about the function of CtIP, DNA2, EXO1 or the RecQ helicases in telomere processing. CtIP is a key factor in DSB repair, promoting the initial resection that is required for the repair by HR, but it has also been implicated in microhomology-mediated alternative end joining (MMEJ) (reviewed in You and Bailis, 2010) (Figure 1.7), an end joining pathway that requires small homologous DNA sequences. MMEJ is a Ku-independent pathway and for this reason has been considered as part of the A-NHEJ mechanisms (McVey and Lee, 2008). Due to the repetitiveness of the telomere sequence, it is very likely that pathways that involve homology-mediated repair might also act at telomeres.

Apart from MMEJ, CtIP is also involved in another repair pathway that requires the presence of long homology regions, the single strand annealing (SSA) (Mimitou and Symington, 2008; Yun and Hiom, 2009). SSA is considered a backup pathway of HR, involving a strand invasion reaction similarly to canonical HR. In vertebrates, SSA has been shown not only to require CtIP, but also the MRN complex, ERCC1/XPF, BRCA1 and RAD52 (Adair et al., 2000; Sargent et al., 2000; Stark et al., 2004; Yun and Hiom, 2009). The contribution of ERCC1/XPF and MRN (Deng et al., 2009; Dimitrova and de Lange, 2009; Zhu et al., 2003), raises the interesting possibility that this pathway may also play a role in end-joining at the chromosome termini. Moreover, in fission yeast SSA plays a critical role in the fusions of short

dysfunctional telomeres (Wang and Baumann, 2008).

RecQ helicases play an important role in HR and in DNA replication due to their ability to unwind complex DNA structures and to promote resection (reviewed in Chu and Hickson, 2009). In *S. cerevisae*, the RecQ helicase Sgs1 is required for processing telomeres after replication, leading to the formation of 3' overhang (Bonetti et al., 2009). The RecQ homologs in mammals, WRN and BLM, have also been implicated in telomere replication and are believed to facilitate fork progression through complex chromatin structures present at telomeres (Crabbe et al., 2004; Sfeir et al., 2009), but have not yet been described to play a role in formation of the 3' overhang.

Furthermore, DNA2 and EXO1, are also required for resection of DSBs in yeast and in humans (Nimonkar et al., 2011; Peng et al., 2012; Zhu et al., 2008) (Figure 1.7) and for telomere processing after replication in yeast (Bonetti et al., 2009). Recently, mouse EXO1 was also ascribed a role in post-replication processing of the telomeres (Wu et al., 2012), being involved in the generation of the 3' overhang of both telomeric leading and lagging strands. Nevertheless, their role in processing uncapped telomeres in mammalian cells has not been addressed yet. So far the only link between EXO1 and telomere dysfunction in mammals comes from a study which shows that mice simultaneously harbouring dysfunctional telomeres and EXO1 deletion, accumulated less DNA damage and live longer (Schaetzlein et al., 2007). Interestingly, in *S. pombe* Exo1 has been shown to be required for processing DSB, but not dysfunctional telomeres in *taz1* mutants (Tomita et al., 2003). On the other hand, Dna2 is important for the generation of a G-rich overhang in both wt and *taz1* mutants (Tomita et al., 2004).

Analysis of telomeres uncapped by POT1 further substantiates the diversity of pathways involved in processing uncapped telomeres. POT1 abrogation leads to end-to-end fusions mediated by A-NHEJ, which has been shown to require MRN, CtIP and RNF8 (Deng et al., 2009; Rai et al., 2011; Rai et al., 2010). RNF8 is also

critical for C-NHEJ to occur at dysfunctional telomeres after TRF2 inactivation (Peuscher and Jacobs, 2011; Rai et al., 2011). In this context, RNF8 is required not only for the ubiquitylation events that regulate the recruitment of several DNA damage factors (e.g. 53BP1) to dysfunctional telomeres, but also for the processing the 3' overhang (Peuscher and Jacobs, 2011). Moreover, 53BP1 role in promoting telomeric fusions has also been described (Dimitrova et al., 2008), together with a critical function in preventing over-resection at telomeres (Sfeir and de Lange, 2012). This emphasizes the importance of 53BP1 not only for the end-joining reactions, but also in telomere protection.

Although in the recent years several studies have contributed to our knowledge on how uncapped telomeres are processed, future studies are needed to elucidate how distinct DNA repair pathways act to process and ligate uncapped telomeres.

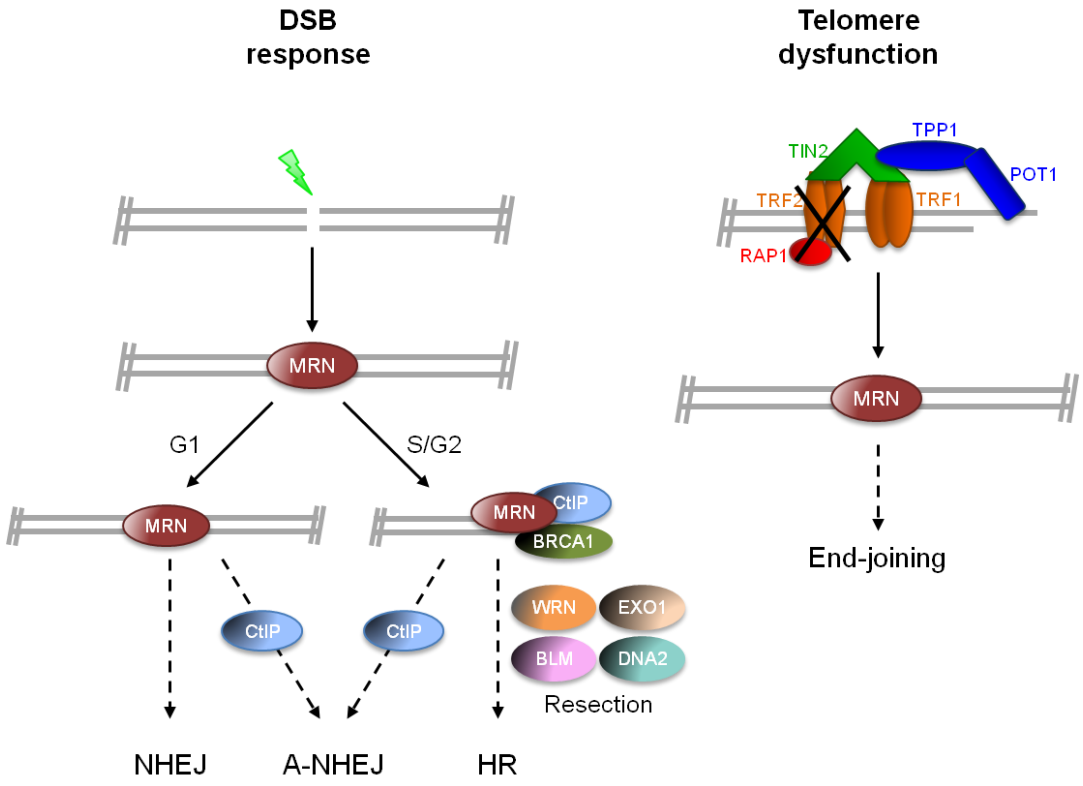


Figure 1.7 – Comparison between the cannonical and telomeric DDR.

When a double strand break is introduced in the genome, the MRN complex is one of the first factors to be recruited to the sites of break (left). In G1, MRN promotes repair by NHEJ, while in S/G2, its interaction with CtIP and BRCA1 promotes resection. These reaction are further facilitated by several helicases and nucleases including WRN, BLM, DNA2 and EXO1, ultimately allowing repair by HR. In addition, alternative mechanisms of end-joining (A-NHEJ) that require factors such as CtIP also act in DSB repair. Similarly, telomere dysfunction triggered by TRF2 abrogation requires the MRN complex to allow efficient repair (right). The requirement of other factors involved in resection and A-NHEJ is still unclear.

1.3.3 Dysfunctional telomeres and cell cycle checkpoints

Similarly to other types of DNA damage, dysfunctional telomeres elicit checkpoint activation. This leads to cell cycle arrest allowing the cell enough time to either repair the damage or trigger apoptosis.

So far, three main DNA damage checkpoints have been identified: G1/S, intra-S and G2/M. The first checkpoint prevents cells from entering S-phase with damages that might compromise initiation of replication; the second checkpoint not only allows repair of the damage that escaped the G1/S checkpoint, but also of any damage

encountered during S-phase that might lead to a replication block. Finally, the G2/M checkpoint prevents cells from entering mitosis in the presence of DNA damage (reviewed in Sancar et al., 2004).

Because they resemble DSBs, dysfunctional telomeres are likely to trigger G1/S arrest, primarily through activation of the checkpoint kinase ATM, which in turn activates target molecules, including p53 and CHK2 (Denchi and de Lange, 2007; Karlseder et al., 1999) (Figure 1.8). After its phosphorylation in response to DNA damage, CHK2 phosphorylates in turn CDC25A targeting it for proteosome-dependent degradation. CDC25A is a phosphatase that dephosphorylates CDKs, which drive the cells through the cell cycle (reviewed in Sancar et al., 2004). CDC25A degradation disables the progression through the cell cycle and consequentially prevents initiation of replication.

Lack of shelterin components such as TRF2 triggers G1/S arrest and leads to senescence or apoptosis, in a process that is p53-dependent (Karlseder et al., 1999; Smogorzewska and de Lange, 2002; van Steensel et al., 1998). Moreover, cells without functional TRF2 and that simultaneously lack p53 not only fail to arrest in G1/S (Smogorzewska and de Lange, 2002), but are also able to form end-to-end fusions through NHEJ reactions, largely confined to G1 (Konishi and de Lange, 2008).

Apart from the above-mentioned artificial telomere uncapping, it is well established that transient telomere uncapping naturally occurs in every S-phase of the cell cycle. This allows telomere replication and elongation. It is believed that, following telomere replication, spontaneously uncapped telomeres elicit an ATM-dependent response, which directs the recruitment of factors needed to restore telomere protection in G2/M (Verdun et al., 2005; Verdun and Karlseder, 2006). For this reason, in normally cycling cells, the G2/M checkpoint might represent a critical barrier ensuring proper telomere capping after replication.

If telomere re-capping process following replication is not successful, telomeres

can be recognised as DNA damage in G2/M and trigger a DDR that will induce TIF formation in mitotic cells (Thanasoula et al., 2010). Furthermore, removal of telomeric proteins such as TPP1 and POT1, in yeast or vertebrates, also triggers G2/M arrest, most likely through exposure of the telomeric 3' ssDNA (Churikov et al., 2006; Pang et al., 2003; Pitt and Cooper, 2010; Tejera et al., 2010).

Most of the work performed on p53 in the past decades has focused on its role in regulating cell cycle arrest at the G1/S transition in response to DNA damage and other types of cellular stress. Nevertheless, other studies also support a role for the p53 pathway during the G2/M transition in response to DNA damage (reviewed in Taylor and Stark, 2001). Recently, p53 has been shown to participate in the G2/M checkpoint responsive to uncapped telomeres (Thanasoula et al., 2010) (Figure 1.8). Similarly to its role in the general DDR, p53 is likely to act as sensor of uncapped telomeres, delaying mitotic entry and allowing capping processes to be completed.

The absence of a functional p53 leads to increase not only in the number of mitotic TIFs that label naturally uncapped telomeres, but also in mitotic TIFs labelling short telomeres or telomeres lacking TRF2, TRF1 or POT1 (Thanasoula et al., 2010; Thanasoula et al., 2012).

Although p53 plays a crucial role as a keeper of telomere integrity ensuring that cells enter mitosis with intact telomeres, an additional CHK1 and CHK2-dependent pathway, also seems to act (Thanasoula et al., 2012). Intriguingly, phosphorylation of p53 at the Ser20 of p53 by CHK1/2 in response to DNA damage (reviewed in Sancar et al., 2004), is not important for checkpoint activation after telomere uncapping, while phosphorylation of p53 at Ser15 by ATM/ATR is indispensable for the G2/M response (Thanasoula et al., 2012). This provides evidence for the presence of two distinct pathways that contribute to the protection of telomere integrity (Figure 1.8).

Interestingly, both CHK1 and CHK2 seem to act redundantly in monitoring telomere uncapping at the G2/M transition (Thanasoula et al., 2012). Consistent with previous reports in mouse (Celli and de Lange, 2005; Denchi and de Lange, 2007;

Guo et al., 2007), POT1 depletion induces CHK1 activation, whilst TRF2 depletion leads to CHK2 activation during G2/M in human cells (Thanasoula et al., 2012) (Figure 1.8).

The CHK1/2-dependent response to general DNA damage leads to inactivation of the CDC25 phosphatases, resulting in cell cycle arrest (reviewed in Sancar et al., 2004). While G1/S progression seems to be controlled primarily by CDC25A stability, the G2/M transition depends on all three mammalian CDC25 phosphatases, CDC25A/B/C, which are phosphorylated by both CHK1 and CHK2 kinases, and consequently inactivated through different mechanisms (reviewed in Kiyokawa and Ray, 2008). In particular, CDC25A phosphorylation at Ser76 by CHK1 targets it for proteosome-mediated degradation (Busino et al., 2003; Donzelli et al., 2004; Jin et al., 2008; Jin et al., 2003), while CDC25C is inactivated mainly through phosphorylation on Ser216 by both CHK1 and CHK2 (Matsuoka et al., 1998; Peng et al., 1997; Sanchez et al., 1997; Zhou et al., 2000).

In accordance with differential activation of CDC25 phosphatases, recent work has shown that CDC25A and C are differentially regulated after POT1 or TRF2 abrogation (Thanasoula et al., 2012). CDC25A is only down-regulated after POT1 depletion, which triggers a CHK1-dependent G2/M arrest. In contrast, CDC25C is involved in the G2/M response triggered by either TRF2 or POT1 depletion, which leads to CHK2 and CHK1 phosphorylation, respectively.

Although CDC25A degradation via proteosome in response to DNA damage is well established, very little is known about the DNA damage-induced degradation of CDC25C (reviewed in Kiyokawa and Ray, 2008). CDC25C degradation via ubiquitin-proteasome pathways has only been described after particular conditions such as the G2/M arrest induced by arsenite, a known human carcinogen (Chen et al 2002 PNAS). Interestingly, and in contrast to general DNA damage, telomeric damage, through TRF2 or POT1 abrogation also leads to CDC25C degradation (Thanasoula et al., 2012) (Figure 1.8). This marks not only a DNA damage checkpoint activation

distinct from the cannonical DNA damage, but most importantly constitutes a novel mechanism for maintenance of the telomere dysfunction-induced G2/M arrest.

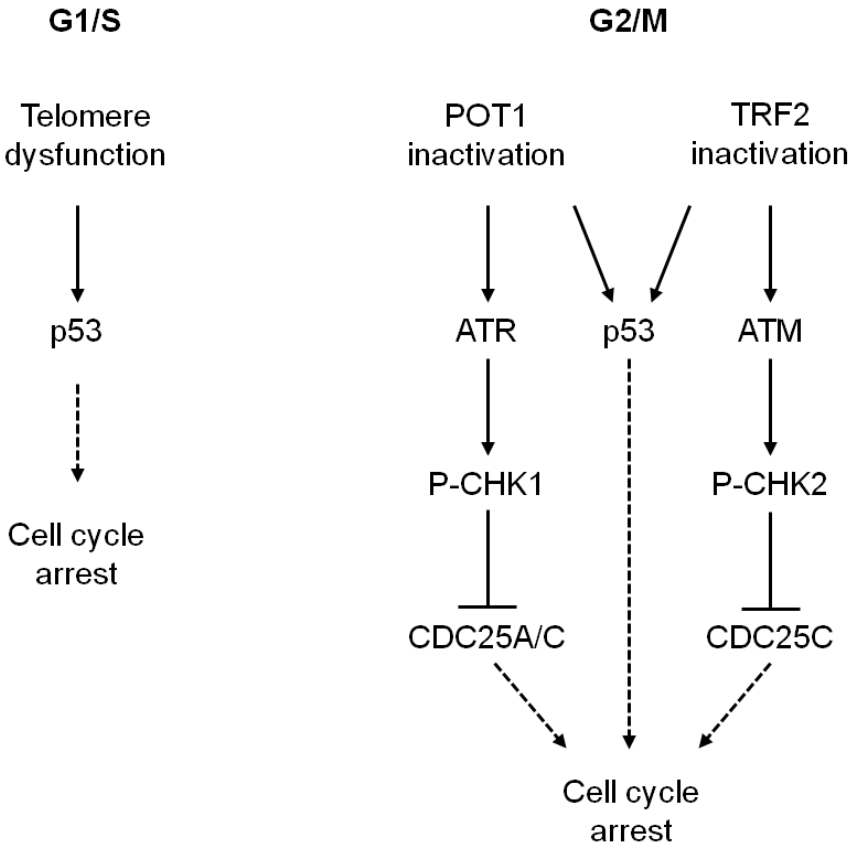


Figure 1.8 – DNA damage checkpoint activation after telomere dysfunction.

Two main DNA damage checkpoints are induced after telomere dysfunction: the G1/S (left) and the G2/M (right) checkpoints. In the G2/M checkpoint, inactivation of POT1 and TRF2 differentially trigger CHK1 and CHK2 activation, respectively. This either promotes destruction of both CDC25A and CDC25C, in the case of POT1 abrogation, or of CDC25C alone, eventually leading to G2/M arrest.

1.4 Telomere dysfunction and cancer

In the recent decades it became obvious that telomere maintenance is fundamental in preventing cancer onset and development. In fact, loss of telomere integrity has been linked with two main hallmarks of cancer: enabling replicative immortality and promoting genome instability (Hanahan and Weinberg, 2011).

In every round of replication, and due to the end replication problem, telomeres become progressively shorter. Thus, telomere shortening has been linked to the

number of cell division that a cell can undergo (Allsopp et al., 1995; Hastie et al., 1990). If the number of divisions reaches the point of crisis, i.e. surpasses the limited number of cell divisions, also called the Hayflick limit, cells enter replicative senescence and cellular ageing (Hornsby, 2011). Cancer cells have the ability to overcome this problem through replicative immortality. In approximately 90% of the cancers reactivation of telomerase, the enzyme responsible for the addition of telomeric repeats, is the mechanism of replicative immortality. In some other cases, telomere elongation is achieved by activation of the ALT pathway. This illustrates how important telomere maintenance is for replicative immortality, one of the cancer hallmarks, in order to achieve tumorigenesis. In fact, telomere shortening has been observed in primary tumours as a consequence of the high replicative potential of cancer cells (de Lange et al., 1990; Hastie et al., 1990) and precedes reactivation of telomerase (Chin et al., 2004). Additionally, telomere shortening has been linked with potential cancer predisposition (Wu et al., 2003), which might be explained by the fact that individuals with shorter telomeres will reach telomere crisis earlier and more cells can become tumorigenic.

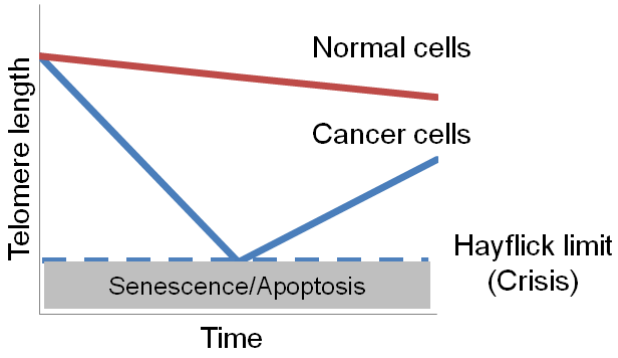


Figure 1.9 – Telomere crisis.

Normal cells shorten their telomeres in every round of replication. Pre-cancerous cells have a higher proliferative rate and for this reason the telomeres rapidly get short until they reach a point of crisis, the so-called Hayflick limit. At this point, cells can either undergo senescence/apoptosis or can reactivate mechanisms that will allow telomere elongation, becoming this way virtually immortal, as cancer cells mostly are.

Apart from cancer, telomere shortening is also accelerated in several chronic diseases, such as hypertension, coronary heart diseases or chronic human immunodeficiency, or in premature ageing (reviewed in Blasco, 2005; von Figura et al., 2009). Recent studies have also suggested that some cells with short telomeres might overexpress specific markers, such as stathmin, EF-1α, and chitinase, which can be used as biomarkers of telomere dysfunction (Jiang et al., 2008). This may allow for a more prompt detection of telomere dysfunction-related diseases.

Although the decrease in the number of telomeric repeats contributes to replicative senescence, it is in fact the ensuing of telomere deprotection that elicits telomere crisis (Karlseder et al., 2002). This might be linked to the fact that lower numbers of telomeric repeats bind less components of the shelterin complex. Furthermore, telomeric proteins such as POT1 and TRF2 have been directly implicated in preventing checkpoint activation that leads to activation of p53 or RB pathways, two mechanisms that can trigger senescence (Karlseder et al., 1999; Smogorzewska and de Lange, 2002; Thanasoula et al., 2010; Thanasoula et al., 2012).

Dysfunctional telomeres can lead to the formation of dicentric chromosomes as a consequence of end-to-end fusions. If the checkpoint machinery is not fully functional, these fused chromosomes will progress through the cell cycle. During mitosis the centromeres of the same dicentric chromosome might be pulled apart, causing the chromosome to break. Broken chromosomes are likely to be repaired through end-joining, giving rise to a new dicentric chromosome. This process of repeated fusions and breaks is called breakage-fusion-bridge (BFB) cycle and is known to greatly contribute to the genomic instability observed in cancer. In fact, the presence of telomere fusions has been identified in human breast carcinomas or in chronic lymphocytic leukaemia (Lin et al., 2010; Tanaka et al., 2012). This further highlights the importance of telomere dysfunction as a cancer hallmark (Feldser et al., 2003; Maser and DePinho, 2002; Murnane, 2012; Wanat and Johnson, 2012).

When checkpoints are disrupted, the presence of dysfunctional telomeres can have an additional consequence: mitotic catastrophe (Hornsby, 2011). Mitotic catastrophe is the failure in cytokinesis, which can also be promoted by the presence of chromosome fusions and can result in tretraploidization. Recently, a direct link between tetraploidization and permanent telomere damage has been established (Davoli et al., 2010), further stressing the importance of maintaining telomere integrity in order to avoid cancer development.

Although the link between dysfunctional telomeres and cancer is certain, the connection between mutations of specific telomeric subunits and cancer remains elusive. The study of mouse models lacking shelterin components shed some light to this subject (reviewed in Martinez and Blasco, 2010). The knockout (ko) mice of most telomeric subunits are embryonic lethal (Celli and de Lange, 2005; Chiang et al., 2004; Karlseder et al., 2003; Kibe et al., 2010; Wu et al., 2006). Only RAP1 ko and POT1b ko are viable (Hockemeyer et al., 2006; Sfeir et al., 2010). Mice that harbour p53 deletion together with TPP1 or TRF1 deletion are not only viable, but also display increase incidence of carcinomas (Else et al., 2009; Martinez et al., 2009), suggesting a scenario in which p53 mutations are followed by defects on telomere proteins might promote cancer. Furthermore, overexpression of TRF2, TRF1, TIN2 and POT1 is detectable in some human tumours (Bellon et al., 2006; Hu et al., 2010; Kondo et al., 2004; Munoz et al., 2005; Oh et al., 2005) . In other tumours seems to prevail a balance between overexpression and reduced expression of telomere-associated proteins (Salhab et al., 2008). In this study while telomerase and tankyrase 1 were found to be overexpressed other factors such as POT1 and tankyrase 2 were found downregulated, possibly acting as tumour suppressors by negatively regulating telomere elongation.

Mutations in telomeric components have great impact in premature ageing syndromes. In particular, dyskeratosis congenita (DC) is strongly linked to mutations in components of telomerase or in TIN2 (reviewed in Walne and Dokal, 2009).

Recently, mutations in CTC1 have also been connected to DC (Keller et al., 2012). Moreover, not only core telomeric components, but also telomeric accessory factors have been linked to premature ageing syndromes characterized by the presence of short telomeres (Blasco, 2005). These include mutations in ATM, causing ataxia telangiectasia; MRE11, causing ataxia telangiectasia-like disorder; NBS1, linked to Nijmegen breakage syndrome; WRN and BLM helicases, responsible for Werner and Bloom syndrome, respectively; and FANC genes, involved in Fanconi anaemia.

Altogether, this shows how telomere dysfunction can have an impact not only at a cellular level, promoting for example telomere shortening, but also at the organismal level, leading to aging and disease.

1.5 Aim of the work

Telomere integrity is crucial to prevent cells from entering senescence, apoptosis or tumorigenesis. When telomeres become dysfunctional end-to-end fusions occur. These fusions promote genomic instability either through repetitive BFB cycles or through tetraploidization and consequent aneuploidy. Understanding the mechanisms by which telomeres become uncapped and how uncapped telomeres are processed is important to comprehend the physiological consequences of telomere dysfunction. In addition, is also important to understand what mechanisms allow dysfunctional telomeres to escape senescence or apoptosis and to trigger tumorigenesis. In the recent decades, an incredible effort has been made to obtain answers to these questions, but our understanding of telomere biology is still far from being complete.

The first aim of the work presented in my thesis was to characterize a higher-order complex with end-capping function through identification of new proteins required to maintain the integrity of the shelterin complex and to participate in the

DNA damage signalling at uncapped telomeres. Secondly, this work intended to provide further knowledge on how telomeres are processed and repaired through end-joining reaction. There is a thin line between telomere capping and dysfunctional telomeres, known to cause disease. Thus, this work will further our understanding of the mechanism by which a telomere is recognized as damage and how the cell acts to repair it, avoiding to trigger several know pathologies.

Chapter 2 Materials and Methods

2.1 Cell lines and treatments

The human osteosarcoma cell line U2OS, the human cervical cancer cell line HeLa 1.2.11 and MEFs were cultivated in monolayers in DMEM medium (Life Technologies or Sigma Aldrich) supplemented with antibiotics (penicillin and streptomycin, Sigma Aldrich) and 10% foetal bovine serum (FBS, Life Technologies). U2OS cells expressing tetracycline regulated expression vector (CR-UK) were cultivated in DMEM medium (Life Technologies or Sigma Aldrich) supplemented with antibiotics (penicillin and streptomycin, Sigma Aldrich) and 10% tetracycline-free FBS (Lonza). Primary *Brca1*$^{F/-}$ MEFs were a gift from Jos Jonkers. In these cells the *Brca1* exons 5 and 6 are flanked by loxP recombination sites and a split puromycin resistance marker, allowing reconstitution of the puromycin resistance gene after Cre-recombinase treatment (Bouwman et al., 2010). Cells were originally referred to as *Brca1*^{SCo}, here they are referred as *Brca1*$^{F/-}$ for simplification. MEFs were treated with 0.2 μg/mL KaryoMAX® Colcemid (Life Technologies) for 4-6 h or overnight before harvesting.

2.2 Establishment of doxycycline (DOX) inducible TRF2 shRNA cell line

U2OS expressing tetracycline (Tet) regulated expression vector were transfected using Lipofectamine 2000 (Life Technologies) with TRF2 short hairpin RNA (shRNA) (target sequence: CAGAAGTGGACTGTAGAAG) cloned onto pSuperior vector. After transfection cells were selected with 1 μg/mL puromycin for 2 weeks. Single cell clones were established by serial dilution in 96 well plates. Wells with one colony were selected and the cells amplified. After addition of DOX (2

μg/mL; Sigma Aldrich), different clones were tested by Western blotting and immunofluorescence for efficient depletion of TRF2.

2.3 siRNA transfection

For initial optimization cells were reverse-transfected using 0.1-0.5% (v/v) of Dharmafect 1 (Dharmacon) or RNAiMax (Life Technologies) transfection reagents and 15-150 nM of small interference RNA (siRNA) duplexes targeting RAP1 (Life Technologies), TRF2 (Dharmacon), RAD51 (Life Technologies) and for RNF8 (Mailand et al., 2007) (Table 2.1). A scrambled siRNA (Ambion) was used as negative control. Cells were analysed by immunoblotting 24 h, 48 h and 72 h after transfection.

2.4 siRNA transfection of siRNA libraries

For the Phosphatase library screen (96-well plates containing a pool of 3 siRNAs targeting ~270 genes; Stealth™ RNAi Human Phosphatase Collection, Life Technologies), U2OS cells were reverse transfected using 0.1% (v/v) Dharmafect 1 (Dharmacon) transfection reagent per well and 30 nM of siRNAs. siRNAs targeting RAP1 and TRF2 and a scrambled siRNA were used as positive and negative controls, respectively. AllStars Hs Cell Death control (Catalogue number 1027298; Qiagen) was used as a transfection control. The cells were analysed by immunofluorescence 48 h after transfection.

For the Ubiquitinome library screen (Lab-Tek chambers containing two individual siRNAs targeting ~1200 genes; Ambion; siRNA arrays were prepared as previously described (Erfle et al., 2007)), U2OS cells were transfected as previously described (Doil et al., 2009). As a control for the transfection siRNA targeting the

INCENP gene were used. Cells were analysed by immunofluorescence 72 h after transfection. This siRNA screen was a collaboration with the laboratory of Dr. Jiri Lukas, who provided the library and the facilities.

For hit validation, U2OS cells were transfected using the conditions described for the Phosphatase library.

For the in-house siRNA library, containing a subset of the whole genome library including siRNAs targeting genes involved in different pathways, such as DNA repair or telomere biology (96-well plates containing a pool of 4 siRNAs targeting ~600 genes; Qiagen). U2OS cells expressing DOX inducible TRF2 shRNA cells were reverse transfected using 0.1% (v/v) Dharmafect 1 (Dharmacon) transfection reagent per well and 10 nM of siRNAs. siRNAs targeting RNF8 and a scrambled siRNA were used as positive and negative controls, respectively (Table 2.1). AllStars Hs Cell Death control (Qiagen) was used as a transfection control. The cells were analysed by immunofluorescence 72 h after transfection. For the secondary screen, all the selected hits were tested using the four deconvoluted siRNAs present in the library and reverse transfection was carried on as described above.

For hit validation, U2OS and HeLa 1.2.11 were transfected using 0.16% or 0.22%, respectively, Dharmafect 1 (Dharmacon) per well and a 30 nM concentration of the of siRNA (Ambion). The cells were analysed 72 h after transfection by immunofluorescence, combined FISH and immunofluorescence, immunoblotting or real-time PCR.

Table 2.1 - siRNA sequences

Gene	Sequence	Code	Reference/ Supplier
RAP1	GGCGGAGGCGATGGATTTGGGCAAA	HSS147631	Life Technologies
	CCACCCAUUCCUCGACUCUGUUCGU	HSS147632	
	AUUAACUGCCGAAUGAUCUUAAUGG	HSS182475	
	GAGCCCUCCUGAUUUUGAAtt	s28940	Applied Biosystems
	GGCAGUUAAUGGAGAAGUUtt	s28941	
	AGAAAUCCAGGAGAAUGAAtt	s224298	
	TTGGGATAGATTCCAAATAAA	SI03247027	Qiagen
	CCGGATGTCTTTCTTTACCTA	SI03246691	
	CTCGGAGGATTGAATTTCGAA	SI04226908	
	ATGGTAGGTGAGGTGGTTAAA	SI04179763	
TRF2	CAGAAGUGGACUGUAGAAGUU		(Takai et al., 2003)
	GUACAACCAAUAUAACAAAtt	s13992	Applied Biosystems
	GGUCGAAUCCAGUAGAAAAtt	s13991	
	GAACAAGCGCAUGACAAUAtt	s13993	
	TGGGCTATGTAGGCAGGTTAA	SI04310719	Qiagen
	AAGGAACTTAGTCTGTTATGA	SI04261159	
	TACCCATTTGTTAACCGAACA	SI04238507	
	CAGGAGCATGGTTCCTAATAA	SI00742637	
RAD51	UCAAGUGGAUGGAGCAGCGAUGUUU	HSS109012	Life Technologies
	GAAGCUGAAGCUAUGUUCGCCAUUA	HSS109013	
	GCGCCAAAGAAGGAGCUAAUAAAUA	HSS109014	
KIAA1333	GAAGGGUCCUUGUCAAAGAtt	s31129	Applied Biosystems
DTL	CUCUGAGUCUAGAAAUAGAtt	s226738	Applied Biosystems
UFM1	CAGACUGCUGGAAAUGUUUtt	s28345	Applied Biosystems
ABTB2	CACACUUUUUGAACAAUAAtt	s24599	Applied Biosystems
CALCOCO1	GGAGAGGACCCAAAACCAAtt	s33535	Applied Biosystems
COPS6	AGGAAUAUUAUUACACCAAtt	s21616	Applied Biosystems
WDR53	CGACCACUCUGGAUUACAAtt	s51360	Applied Biosystems
VPRBP	GGCAGGACAUGGUACCUAUtt	s18761	Applied Biosystems

ATE1	GGAUCUCAUAGACCGAGGAtt	s21886	Applied Biosystems
MLL2	CCACUCUCAUCAAAUCCGAtt	s15604	Applied Biosystems
RNF41	GGAUGAUCUCGACUCCUGAtt	s19860	Applied Biosystems
PPP5C	CAGAUGUACGAGCUCUUUAtt	s11012	Applied Biosystems
PPP2R3A	CGAUCUGUCUCGAUACAAUtt	s10977	Applied Biosystems
RNF8	AAUGCGGAGUAUGAAUAUGAA		(Mailand et al., 2007)
Scrambled	-	1027280	Qiagen

2.5 MEF retroviral transduction

Retroviral transduction of MEFs was performed using HEK293T as a viral packaging cell line. HEK293T were grown to 80% confluency and transfected using the calcium phosphate method (Graham and van der Eb, 1973). Briefly, pCL-Eco helper vector was mixed with either pBabe plus retroviral vector encoding 'Hit-and-run' Cre recombinase (Silver and Livingston, 2001) or pBabe alone in the presence of 300 mM calcium chloride. In order to produce calcium phosphate-DNA complexes, 2X Hepes saline buffer (HBS) buffer (280 mM NaCl, 50 mM HEPES and 1.48 mM Na_2HPO_4 in water; pH adjusted between 7 and 7.2) was added to the mixture while bubbling. When the solution turned opalescent it was added to the HEK293T. For shRNA transfection, retroviral constructs in pRetroSuper were transfected along with pCL-Eco helper vector (Table 2.2). CtIP shRNA was cloned in pMX-pie-IRES-GFP. For MRE11, EXO1, DNA2, BLM and WRN, a mix of both shRNA was used. Media was changed 24 h after transfection and then 24 h after the cell supernatant was harvested and used to infect the recipient MEFs. Approximately $1x10^5$ MEFs were plated 24 h before the first infection. Two additional infections were performed with 10-12 h intervals. Twenty-four hours after the last infection, cells were washed and

incubated in fresh medium containing 3 μg/mL puromycin for 3 days. Two days later, exponentially growing MEFs were either collected and processed for immunoblotting, RT qPCR, FACS or arrested in mitosis by addition of colcemid and processed for metaphase spreads preparation as described below.

Table 2.2 - shRNA sequences

Gene	Target sequence	Reference
GFP	GCTGACCCTGAAGTTCATCTT	(Tarsounas et al., 2004)
TRF2	GAACAGCTGTGATGATTAA	(Deng et al., 2009)
CtIP	GCTCTCTATGTACAAATGAATTA	(Bunting et al., 2010) A gift from André Nussenzweig
MRE11	No.1 - GCTTGTAAGAACTTGGCTAAA	TRC library database
	No. 2 - GCGCATTAAAGGGAGAAAGAT	
NBS1	TTTGACTCAAACTGGTTAC	A gift from Jacqueline Jacobs
53BP1	GCTATTGTGGAGATTGTGTTT	(Bouwman et al., 2010)
EXO1	No.1 - GGGTCAAGCCGATTCTCATATTT	TRC library database
	No.2 - GTCAAGCCGATTCTCATATTT	
DNA2	No.1 - GGGAGATCAGAGGACTATTAC	TRC library database
	No.2 - ATGGCAGGTGACAGGATTATT	
BLM	No.1 - GCCAGGTTATCTGTGCGACAAT	TRC library database
	No.2 - CGAAGGAAACTCACGTCAATA	
WRN	No.1 - GCAAGGCAGAAACACGCTAAT	TRC library database
	No.2 - GTCGAGCCATAGAGTCTTTAAT	

2.6 Immunoblotting

Preparation of fractionated samples was performed essentially as described (Takei et al., 2001). Briefly, cells were harvested by trypsinization, washed with cold phosphate buffered saline (PBS) and re-suspended in cold buffer A (50 mM Tris pH 8.0, 0.05% NP-40, 10% Glycerol, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 1 mM DTT, Protease and Phosphatase inhibitors). Following extraction on ice for 15 min, the cell suspension was cleared by centrifugation at 16, 000 × *g*. The supernatant was kept (cytoplasmic fraction) and the pellet (nuclei) was ressuspended in buffer B (50 mM

Tris pH 8.0, 0.4% NP-40, 10% Glycerol, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.42 M NaCl, 1 mM DTT, Protease and Phosphatase inhibitors) and kept on ice for 15 min. The supernatant was cleared by centrifugation at 16, 000 × *g* and stored (nuclear soluble fraction). The pellet (chromatin bound fraction) was resuspended in SDS-PAGE loading buffer and sonicated. For the preparation of whole cell extracts, trypsinized cells were directly ressuspended in SDS-PAGE loading buffer and sonicated afterwards. Volumes corresponding to an equal protein concentration (quantified using Nanodrop, Thermo Scientific) were analysed by gel electrophoresis followed by Western blotting. NuPAGE-Novex 3-8% Tris-Acetate and 10% Bis-Tris gels (Life Technologies) or ClearPage 10% SDS TEO-Cl gels (C.B.S. Scientific) were run in SDS-running buffers and transferred according to manufacturers‘ instructions. Gels were stained using SimplyBlue™ SafeStain (Life Technologies). Quantification of western blots was performed with ImageJ 1.43k (National Institutes of Health, USA).

2.7 Immunofluorescence

Cells transfected with siRNA were stained by immunofluorescence 48 h or 72 h after transfection. Cells were washed in PBS, and swollen in hypotonic solution (85.5 mM NaCl and 5 mM MgCl2) for 5 min. Cells were then fixed with 4% paraformaldehyde or 3% buffered formaldehyde for 10 min at room temperature and permeabilized by adding 0.03% SDS to the fixative. After washing and blocking with antibody dilution buffer (1% goat -serum, 0.3% BSA, 0.005% Triton X-100 in PBS), cells were incubated 1.5 h, 3 h or overnight with primary antibody. Secondary antibody was added for 30 min to 1 h. After washing, coverslips were dried and mounted on microscope slides using the ProLong Antifade kit (Life Technologies) supplemented with 1 µg/mL DAPI. For 96-well plates and Lab-Tek chambers, cells

were incubated with PBS supplemented with 1 μg/mL DAPI for 5 min. Finally, an equal volume of PBS was added to all the wells or Lab-Tek chambers.

Images of 96-well plates were acquired using the IN Cell Analyzer 1000 (GE Healthcare) with a 20x air objective and the subsequent analysis was accomplished using IN Cell Analyzer 1000 Workstation software (GE Healthcare). Image acquisition of Lab-Tek chambers was performed on a wide-field fluorescence microscope (Zeiss Axiovert 200) equipped with motorized stage, 20x air objective (PlanApochromat 20x/0.8) and CCD camera (Coolsnap HQ, Roper Scientific) operated by Metamorph software (version 6.2r6; Molecular Devices). Further analysis was performed with ImageJ 1.43k (National Institutes of Health, USA). For coverslips analysis, the specimens were viewed with an inverted microscope (DMI6000B; Leica) and fluorescence imaging workstation equipped with an HCX Plan-Apochromat 100°— NA 1.4-0.7 oil objective. Images were acquired using a digital camera (DFC350 FX R2; Leica) using LAS-AF software (Leica) and analysed with ImageJ 1.43k (National Institutes of Health, USA). All z-factor and z-score calculations were performed using Microsoft Excel (Microsoft Office).

2.8 Antibodies

The following antibodies were used for immunofluorescence staining: mouse monoclonal anti-RAP1 (5G7, Madalena Tarsounas‘ Laboratory); rabbit polyclonal antibodies raised against TRF2 (SWE38, (Tarsounas et al., 2004)) and 53BP1 (NB100-304, Novus Biologicals and sc-22760, Santa Cruz).

The following antibodies were used for immunoblotting: rabbit polyclonal antibodies raised against RAP1 (SWE39, (Tarsounas et al., 2004)), TRF2 (SWE53,(Thanasoula et al., 2010)), mouse TRF2 (NB100-57130, Novus Biologicals), RAD51 (sc-8349, Santa Cruz), 53BP1 (NB100-304, Novus Biologicals), BRCA1

(Bouwman et al., 2010), MRE11 (NB100-142, Novus Biologicals); mouse monoclonal antibodies raised against CHK2/Cds1 (Clone 7, Upstate), CtIP (a gift from Richard Baer), NBS1 (ab49958, Abcam); mouse polyclonal anti-TRF2 (MTA18, Madalena Tarsounas' Laboratory). As loading controls for immunoblotting we used the following antibodies: mouse monoclonal antibody raised against α-tubulin (TAT-1; Cancer Research UK Monoclonal Antibody Services), WRN (a gift from Thomas Helleday) and rabbit polyclonal antibody raised against histone H3 (a gift from Dr. Alain Verreault, University of Montreal), and SMC1 (BL308, Bethyl Laboratories).

2.9 Combined FISH and immunofluorescence (IF-FISH)

Immunofluorescence was performed as described above. FISH was performed immediately after incubation with the secondary antibody. Coverslips were washed, fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, washed again three times in 1xPBS for 5 min each time and left to dry. Telomeric probe mix [10 mM Tris pH 7.5, 2.175 mM $MgCl_2$, 0.08 mM citric acid, 7.2 mM Na_2HPO_4 pH7.0, 70% deionized formamide (Chemicon Int.), 0.5 μg/mL Cy3-conjugated PNA $[CCCTAA]_3$ telomeric probe (Applied Biosystems) and 0.25% blocking reagent 100 mM maleic acid and 50 mM NaCl pH7.5 (Roche) in dH_2O] was dropped onto the slides and coverslip added. Following denaturation on a hot plate at 80 °C for 3 min, the coverslips were incubated at room temperature for 1.5 h in a dark humidified chamber. After washing twice in formamide wash [70% formamide (Fluka), 10 mM Tris, 0.1% BSA (Fluka)] and three times in PBS, followed by a single wash in destiled water, the slides were left to dry before mounting, as described for the immunofluorescence protocol. The specimens were viewed with an inverted microscope (DMI6000B; Leica) and fluorescence imaging workstation equipped with an HCX Plan-Apochromat 100°— NA 1.4-0.7 oil objective, images were acquired

using a digital camera (DFC350 FX R2; Leica) using LAS-AF software (Leica) and analysed with ImageJ 1.43k (National Institutes of Health, USA).

2.10 Telomeric FISH

Mitotic cells were collected by mitotic shake-off following 4 h or up to overnight incubation with 0.2 μg/mL colcemid (Life Technologies) and swollen in hypotonic buffer (0.03 M Sodium Acetate) at 37°C for 25 minutes. Few drops of freshly prepared fixative (3:1 mix of methanol:glacial acetic acid (Fisher Scientific)) were added to the cells, which were then centrifuged. Next, the supernatant was aspirated leaving 1 mL for resuspending the cells, to which 11 mL of fixative were added drop wise. Cells were washed with fixative once more, dropped onto slides pre-soaked in 45% acetic acid and left to dry overnight. Slides were then washed in PBS for 10 min and dehydrated with sequential washes in 70, 90 and 100% ethanol. FISH staining was performed with a telomeric FISH probe as previously described for IF-FISH. After air drying, the slides were mounted with Vectashield (Vector Laboratories). The images were acquired using the same system as described for IF-FISH.

2.11 Chromosome orientation FISH (CO-FISH)

Cells were plated subconfluently and Bromodeoxyuridine (BrdU) alone or together with Bromodeoxycytidine (BrdC) (3:1 ratio) were added to a final concentration of 10 μM approximately 20 h before harvesting the cells. To obtain metaphases, 0.2 μg/mL colcemid was added to the cells 4-6 h before collecting. Mitotic cells were harvested, fixed and spread onto slides as described for Telomeric FISH. Cells were then processed and stained for CO-FISH (Figure 2.1). After washing in PBS for 10 min, the slides were treated with 0.5 mg/ mL RNase A (Sigma

Aldrich) in PBS for 10 min at 37 °C. Following washes in PBS and 2 x saline-sodium citrate (SSC), the DNA was stained with 0.5 μg/mL Hoechst 33258 (Sigma Aldrich) in 2xSSC for 15 min. In order to introduce DNA breaks, slides were abundantly washed with water and then exposed to ultraviolet (UV; 365 nm) light for 25 min. Following PBS washes, the DNA was digested with 3 U/μL of Exonuclease III (Promega). The slides were washed again with PBS and then dehydrated with sequential washes of 70, 90 and 100% ethanol. When dried, the first telomeric probe mix (same as described for IF-FISH) containing 0.5 μg/mL Cy3-conjugated PNA $[TTAGGG]_3$ telomeric probe (Applied Biosystems) was added to the slides. DNA was denatured on a hot plate at 80 °C for 3 min, then slides were incubated for 1.5 h at room temperature washed twice with formamide wash, as described for IF-FISH. Next the slides were washed with PBS, dehydrated with ethanol and after air drying, were incubated with the second telomeric probe mix containing 0.5 μg/mL FITC-conjugated PNA $[CCCTAA]_3$ telomeric probe (Applied Biosystems) for 1.5 h at room temperature. Following washes with formamide wash and PBS and dehydration with ethanol, slides were dried and mounted using Vectashield (Vector Laboratories). The images were acquired using the same system as described for IF-FISH.

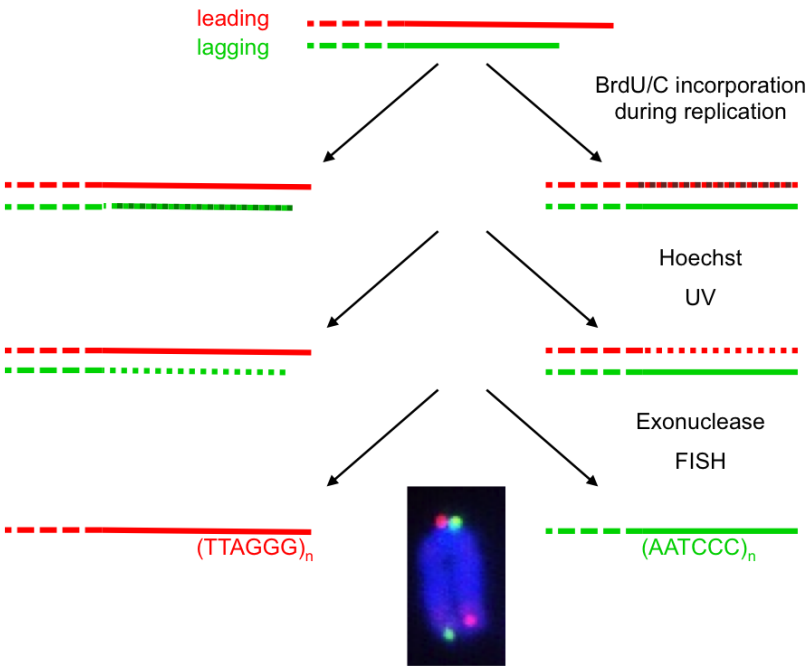


Figure 2.1 – Schematic representation of the CO-FISH protocol.

2.12 Real-Time quantitative PCR (qPCR)

siRNA-transfected U2OS cells were processed for real-time PCR using Power SYBR Green Cells-to-CT Kit (Ambion) according to manufacture's instructions. Reverse transcriptase reactions were performed in a Veriti® 96-Well Thermal Cycler (Applied Biosystems, Life Technologies Corporation). For each PCR reaction 0.2-0.4 μM of primers were used in a final volume of 10 μL (Table 2.3). The reaction was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies Corporation). To avoid amplification of contaminating genomic DNA, for each pair of primers design in this work (PerlPrimer 1.1.18, Primer-BLAST), as well as the ones previously described (Lin et al., 2006), at least one primer was placed at the junction between two exons. All real-time PCR reactions were performed in triplicate. 18S and actin primers were used as endogenous controls.

Table 2.3 - RT qPCR primers

Gene	Target sequence	Reference
RAP1	F: GCCACCCGGGAGTTTGA R: GGGTGGATCATCATCACACATAGT	(Lin et al., 2006)
TRF2	F: ACCAGGGCCTGTGGAAAAG R: GGTGGTTGGAGGATTCCGTA	(Lin et al., 2006)
RNF41	F: AGACATCCAGCTGCTAAAGG R: GTTCACCCACTCTAGGATCTC	This work
SUV39H2	F: GAATCACAGCTGTGACCC R: GGCTGTGGTCAATAGAATCTG	This work
TSC1	F: GCTAGAACAGTTTGGTAGTGG R: CGACATTTGCTTGTTGGG	This work
CHD3	F: AAGATGAAGAGGGAGGCAC R: TGGACTGGTTGTTCTTGAG	This work
EXO1	F: ACCAGCTCGTGTTCGACCCCA R: CACCGACGTACTGCCCAGCG	This work
DNA2	F: ACCACCATCTGTGCCCTGGTGA R: AACGGCGGAGTGCGTGTAGC	This work
BLM	F: TGTCGGCGCGCGGAGTTT R: ACAGCAGCCATGATCCTCACTCA	This work
18S	F: AGTCCCTGCCCTTTGTACACA R: GATCCGAGGGCCTCACTAAAC	(Lin et al., 2006)
Actin	F: GCTCTGGCTCCTAGCACCAT R: CCACCGATCCACACAGAGTAC	Tarsounas' laboratory

F – forward primer; R – reverse primer

2.13 Fluorescence-activated cell sorting (FACS)

Cells were harvested by trypsinization, washed once in cold PBS fixed in ice-cold 70% ethanol overnight at -20 °C. Following washes with PBS, cells were treated with 10 µg/mL RNAse A (Sigma Aldrich) for 10 min at 37 °C. Cells were finally stained with 5 µg/mL propidium iodide (Sigma Aldrich). At least 10,000 cells were analysed by flow cytometry (Becton Dickison).

2.14 Pulsed field gel electrophoresis of single-strand telomeric DNA

Approximately, $1x10^6$ cells were harvested by trypsinization and the pellets were kept frozen until further analysis. Cells were then processed for overhang analysis essentially as previously described (Sfeir and de Lange, 2012). Briefly, cells were ressuspended in PBS, mixed with 2% agarose (1:1 ratio) in order to obtain cell plugs. The plugs were then digested overnight at 55 °C in proteinase K digestion buffer (10 mM Tris-HCl pH 8.0, 250 mM EDTA, 0.2% sodium deoxycholate, and 1% sodium lauryl sarcosine). After washes with TE, plugs were digested with 60 U MboI and 60U AluI overnight at 37 °C. The digested DNA was resolved for 24 h on a 1% agarose/0.5X TBE gel. The gel was then dried at room temperature and hybridized overnight at 50°C with γ-32P-ATP end-labeled $[AACCCT]_4$ probe in Church mix (0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 0.7% SDS, 0.1% BSA). The gel was washed at 50°C three times in 4XSSC (30 min each), once in 4XSSC/0.1% SDS (30 min), and exposed to a PhosphoImager screen. After capturing the single-stranded telomere signal, the gel was denatured with 0.5 M NaOH/1.5 M NaCl for 30 min, neutralized with two 30-min washes in 0.5 M Tris-HCl pH 7.5/3 M NaCl, prehybridized in Church mix for 30 min at 55°C, and hybridized overnight with the same probe at 55°C. The next day, the denatured gel was washed in the same way as the native gel and exposed to capture the total telomere signal. ImageQuant software was used to quantify the single-stranded telomere overhang signal and the signal from total telomeric DNA in the denatured gel. The cell pellets used for this assay were processed and analysed in Dr. Eros Denchi laboratory.

Chapter 3 Results – Part 1: High-throughput screening for factors involved in telomere biology

3.1 Introduction

High-throughput screens are extensively used to unravel new mechanisms important for cell physiology and to give new insights into existing pathways, for example those linked to cell survival. With this technique one can run biochemical, genetic or pharmacological assays using libraries containing millions of compounds, in a time effective manner. In mammalian cells, due to the difficulties obtaining complete knockouts of a certain gene, siRNA and shRNA provide rapid tools for the study of gene function. In mammalian cells, siRNA libraries are commonly used to explore existing cellular pathways and find new factors. In order to successfully run high-throughput screens, careful optimization of several parameters is required. These parameters include cell type, which should be suitable for the read-out, concentration of both transfection reagent and siRNA, and duration of the assay. Apart from experimental optimization, the adequate statistical tools (e.g. normalization formulae, hit identification thresholds) should also be chosen according to the type of screen and the read-out (Malo et al., 2006). For example, the use of siRNA screens allowed very important discoveries in the DNA repair field, which opened new lines of investigation, including the identification of a role for RNF8 and RNF168 E3 ligases in the DDR (Doil et al., 2009; Kolas et al., 2007; Stewart et al., 2009). The exact mechanism involving these proteins is still under investigation.

Phosphorylation and ubiquitylation are post-translational modifications essential for several cellular processes. Phosphorylation is often associated with protein regulation and is particularly important in signal transduction (Ubersax and Ferrell, 2007), while ubiquitylation is commonly involved in protein degradation, even though it can also be a regulatory mechanism required in several different pathways

such as endocytosis and DNA repair (Pickart, 2001). In telomere biology, examples of protein phosphorylation include DNA damage-induced phosphorylation of TRF2 by ATM (Tanaka et al., 2005) and TRF1 phosphorylation by Plk1 (Wu et al., 2008) or by Akt (Chen et al., 2009), which control TRF1 ability to bind to telomeres and its roles in telomere length maintenance, respectively. Although a potential SQ/TQ target site for ATM/ATR kinases has been identified in RAP1 (Matsuoka et al., 2007), its functionality has not yet been addressed. In addition, RAP1 contains a BRCT domain, which could be involved in binding phosphorylated proteins, as shown for other proteins harbouring BRCT domains, such as BRCA1 (Yu et al., 2003). More recently, several groups studied the role of ubiquitylation and SUMOylation at telomeres (Atanassov et al., 2009; Kim et al., 2005; Lee et al., 2006b; Potts and Yu, 2007). These processes are crucial for telomere maintenance and telomeric protein turnover. Both RAP1 and TRF2 are SUMOylated in ALT cells (Potts and Yu, 2007), as a pre-requisite for the PML (promyelocytic leukaemia) bodies formation. In contrast, ubiquitylation reactions involving these two telomeric proteins have not yet been described.

A critical aspect of telomere biology is understanding how dysfunctional telomeres are recognized and processed. When telomeres become dysfunctional several DNA damage factors are recruited to the chromosome termini (d'Adda di Fagagna et al., 2003; Takai et al., 2003). Some of these factors including the MRN complex (Deng et al., 2009; Dimitrova and de Lange, 2009), 53BP1 (Dimitrova et al., 2008) and RNF8 (Peuscher and Jacobs, 2011; Rai et al., 2011) are also required to process uncapped telomeres allowing end-joining to occur. Although the studies performed so far clearly suggest a strong resemblance between uncapped telomeres and DSBs, the network of proteins involved in the repair of damaged telomeres is far from being completely understood.

Unravelling the cellular roles of RAP1 and TRF2 and understanding how absence of these proteins affects the DNA repair pathways at the telomeres, can

bring new insights into telomere biology and into the mechanisms that prevent recognition of chromosome termini as broken DNA ends.

3.2 Results and discussion

3.2.1 A screen for factors required for RAP1 and TRF2 foci formation using a human Phosphatase siRNA library

Three critical parameters require extensive optimization before embarking on a high-throughput siRNA screen: cell line selection, optimal cell density in each well and siRNA transfection conditions (Figure 3.1, Figure 3.2).

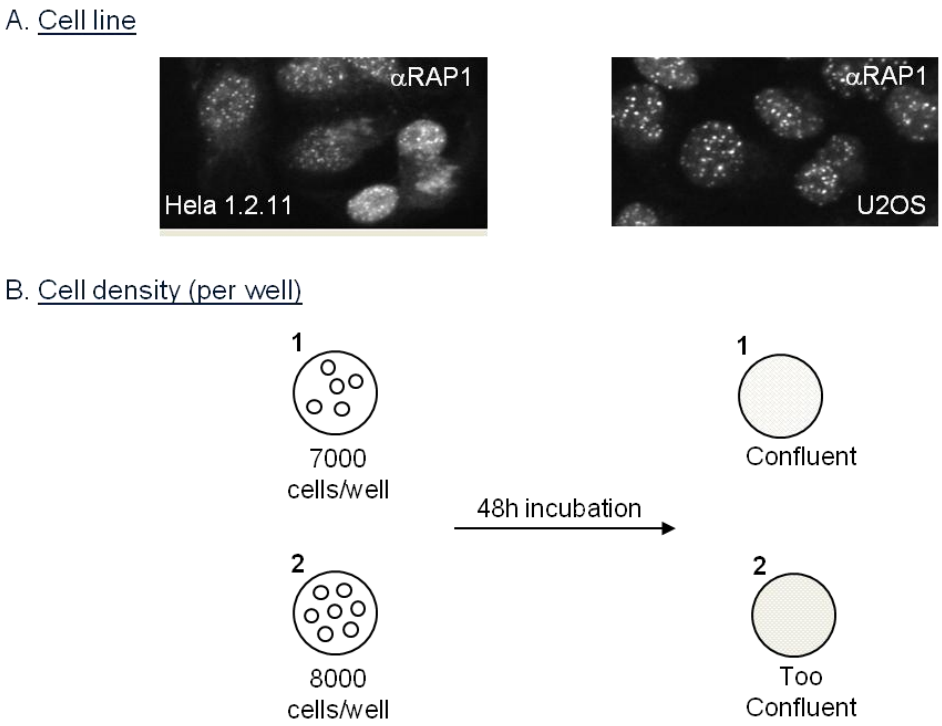


Figure 3.1 - Selection of the cell line and cell density for the Phosphatase library screen.

A) HeLa 1.2.11 and U2OS cells were stained with anti-RAP1 antibody and analysed by immunofluorescence in 96-well plates. Images were acquired using IN Cell Analyzer 1000 (GE Healthcare) and analysed with IN Cell Analyzer 1000 Workstation software. B) Quantification of cell confluency 48h after plating. Red squares indicate the final conditions chosen.

To select the best cell line for the screen, both HeLa 1.211 and U2OS cell lines were immunostained with anti-TRF2 and anti-RAP1 antibodies to visualize telomeric foci.

Due to their bigger cell size, growth in a perfect monolayer and even distribution throughout the well, U2OS were found to be more suitable for this analysis (Figure 3.1A, right panel). The optimal cell density after 48h growth was achieved by plating 7000 cells per well (Figure 3.1B).

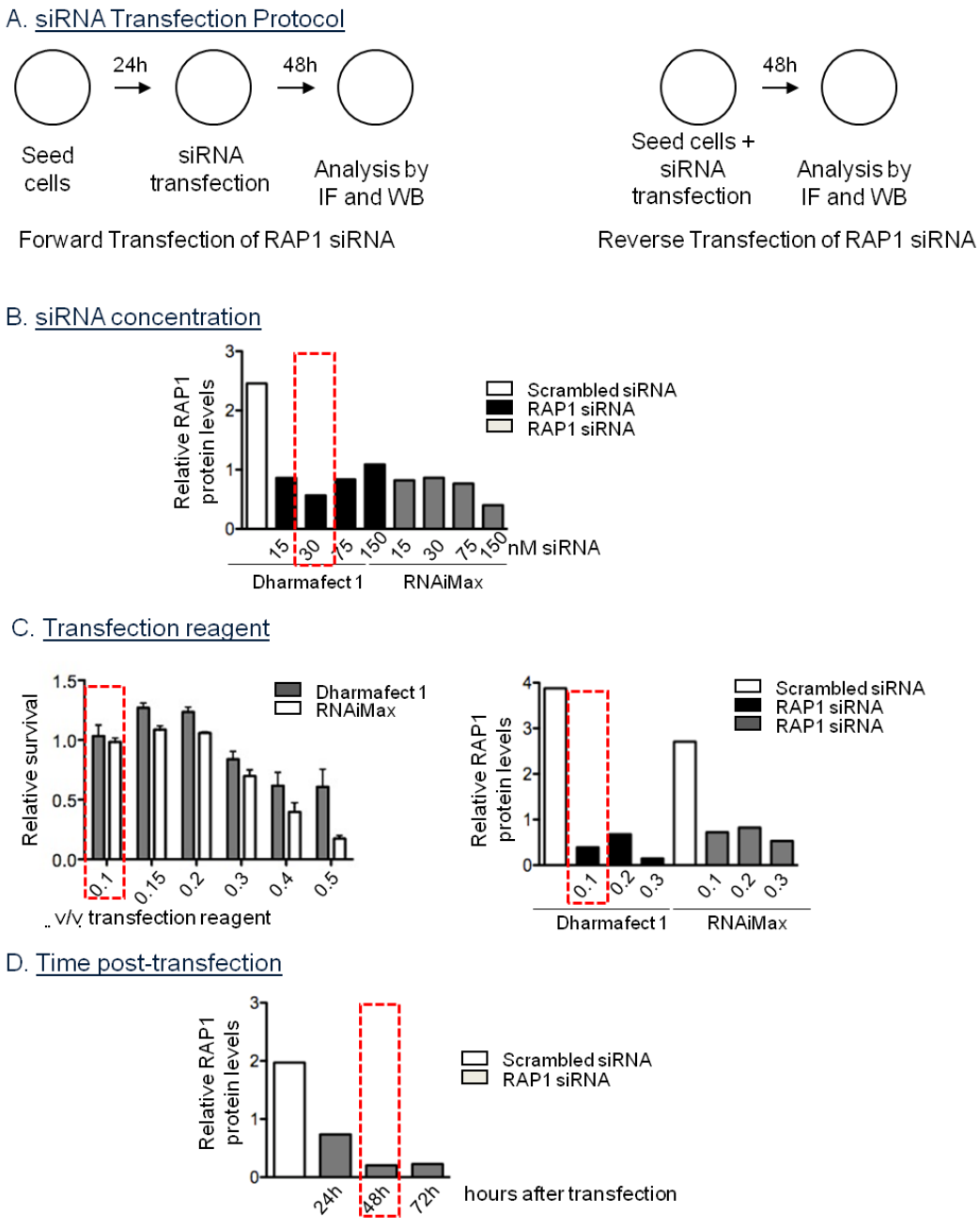


Figure 3.2 - Selection of the siRNA transfection conditions for the Phosphatase Library.

A) Diagram of forward and reverse siRNA transfection methods tested in U2OS cells. B) U2OS cells were reverse transfected with 15, 30, 75 and 150 nM siRNA. Western blot analysis was performed with anti-RAP1 and anti-Tubulin antibodies. Relative protein levels were quantified using ImageJ software. Tubulin was used as endogenous control. C)

Evaluation of toxicity and depletion efficiency of Dharmafect 1 and Lipofectamine RNAiMax transfection reagents. Relative protein levels were determined as in B). D) U2OS cells were reverse-transfected using 30 nM of siRNA and 1% Dharmafect 1. Time points were collected at 24, 48 and 72 h post-transfection. Relative protein levels were determined as in B). Red squares indicate the final conditions chosen.

To determine the best transfection protocol, forward versus reverse transfection procedures, as well as various siRNA concentrations, transfection reagents and post-transfection incubation times were tested (Figure 3.2). Reverse transfection protocol revealed several advantages comparing to the forward-transfection, in particular the reduction of total time of the assay without affecting transfection efficiency. Combination of maximum transfection efficiency and minimum toxicity was found when using U2OS cells reverse-transfected with 30 nM of siRNA and 0.1% (v/v) of Dharmafect 1 transfection reagent (Figure 3.2B and C). The analyses were performed 48 h after transfection, since increase of the assay up to 72h did not improve the level of RAP1 depletion.

siRNAs targeting three different genes were selected: RAP1, TRF2, both of which were included as positive controls for screening the library, and RAD51. Protein levels were determined by Western Blot and immunofluorescence (Figure 3.3A and B). In Western blot, all siRNAs tested efficiently abolished the expression of the corresponding proteins (Figure 3.3A). Similarly, in immunostained cells, the RAP1 and TRF2 siRNAs abolished the accumulation of these proteins into telomeric foci (Figure 3.3B). Importantly, TRF2 siRNA also inhibited RAP1 focal accumulation, consistent with RAP1 being recruited to telomeres by TRF2 (Li et al., 2000).

Antibody staining conditions were optimized using high-throughput image acquisition and analysis for TRF2/RAP1 foci quantification. The main goal was to obtain a read-out where the difference between negative (scrambled siRNA) and positive controls (siRAP1 and siTRF2) was large enough to make the screen feasible. Feasibility of the assay can be determined using z-factor, which compares

the standard deviation of positive and negative controls. An assay where z-factor equals 1 is an ideal assay, if z-factor values are between 0.5 and 1 the assay is considered excellent and values below 0.5 renders the assay not suitable (Zhang et al., 1999). The number and intensity of both RAP1 and TRF2 foci were quantified after depletion by siRNA transfection (Figure 3.3C). As above, TRF2 depletion led to a decrease in both TRF2 and RAP1 foci, whilst RAP1 depletion affected only the RAP1 foci. Based on the values obtained for number and intensity of foci for both RAP1 and TRF2, the z-factor was calculated (Table 3.1). A z-factor above 0.5 was obtained for the number of RAP1 or TRF2 foci predicting an excellent performance for the assay. In contrast, analysis of the intensity of either RAP1 or TRF2 foci gave z-factor values lower than 0.5. Thus, a screen was performed for factors that would lead to a decrease in numbers of TRF2 and/or RAP1 foci.

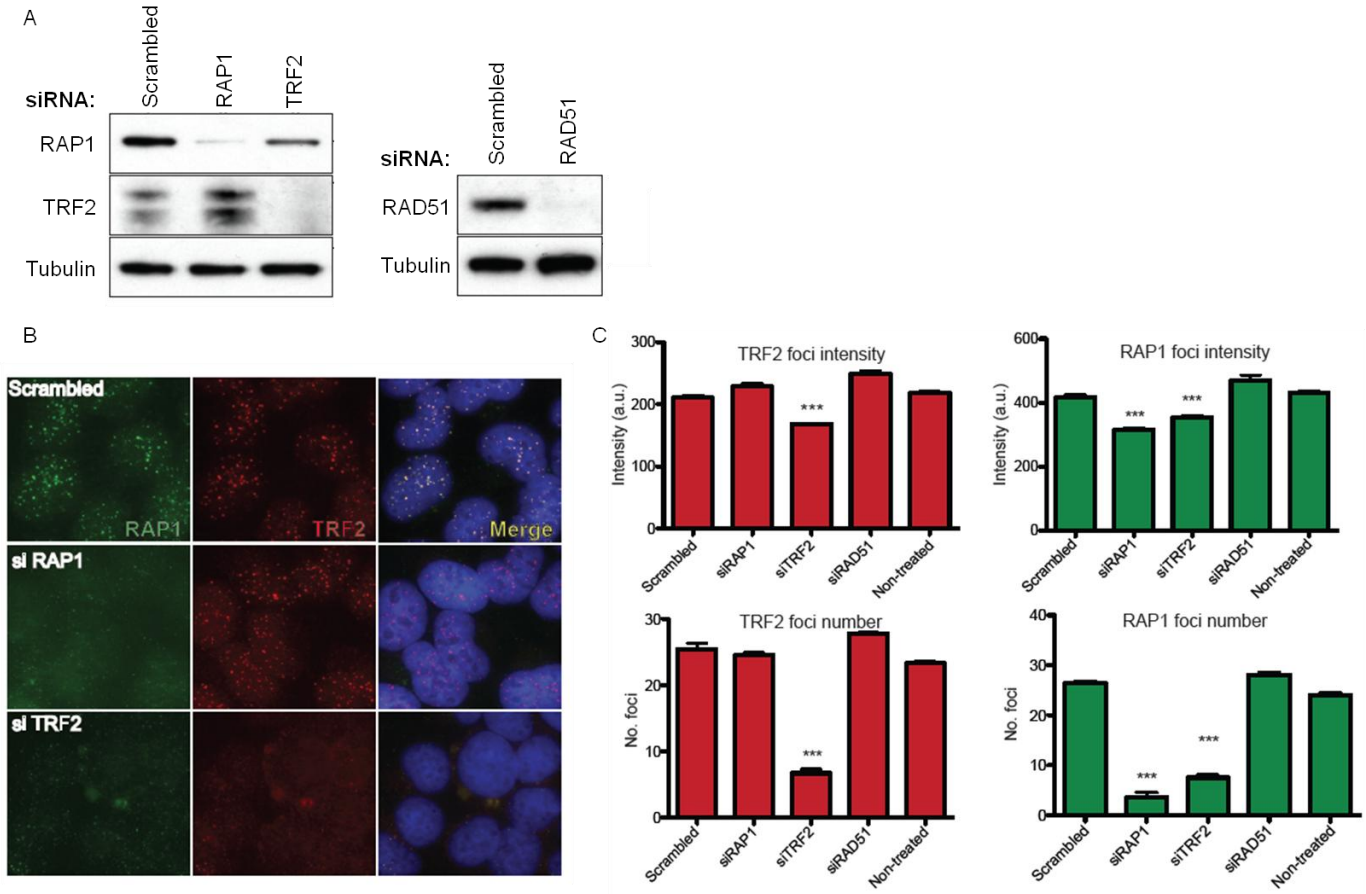


Figure 3.3 - TRF2 and RAP1 siRNA-mediated depletion in U2OS cells using optimized conditions.

A) U2OS cells were reverse-transfected with siRNAs targeting RAP1, TRF2, RAD51 and control scrambled siRNA. Cells were collected 48 hours post-transfection and the efficiency of the depletion assessed by Western blotting. B) Cells transfected as in A) were analysed by immunofluorescence using antibodies against TRF2 (red) and RAP1 (green). C) The intensity and the number of TRF2 and RAP1 foci were quantified in cells stained as in B). Bars represent standard deviation. Statistical significance of reduced TRF2/RAP1 staining relative to scrambled siRNA was calculated with a student's t-test. ***, $P < 0.0001$. a.u. – arbitrary units.

Table 3.1 - Z-factor of the siRNA assay

	RAP1 foci number	RAP1 foci intensity	TRF2 foci number	TRF2 foci intensity
Z-Factor*	0.62	-0.15	0.54	0.35

* Z-factor $= 1 - \frac{3' \left(s_p + s_n\right)}{\left|m_p - m_n\right|}$, where σ_p is the standard deviation of the positive control (RAP1 or TRF2 siRNA-treated samples) and σ_n is the standard deviation of the negative control (scrambled siRNA-treated samples); μ_p is the mean of the positive control and μ_n of the negative control.

Using conditions optimized as above, the screen of a human Phosphatase siRNA library was run in triplicate in 96-well plates. The data was analysed and normalised using the z-score, a formulae that allows the comparison between a particular sample and the average of all the samples (Malo et al., 2006). The z-score values for each siRNA target were plotted in descending order (Figure 3.4). As a threshold level for selecting the hits, a value equal to two times the standard deviation of the scrambled siRNA control was chosen.

The depletion of 13 of the phosphatases from the library was found to decrease the number of TRF2 foci. Of these 2 showed a significant reduction in RAP1 foci number, PPP1R2 and PPP2R3A. The first gene scored higher for RAP1 and the second for TRF2 (Figure 3.4). PPP1R2 acts both as an inhibitory and activating signaling molecule, with an important role controlling cell division. PPP2R3A encodes one of the four major Ser/Thr phosphatases and is implicated in the negative control of cell growth and division. Being the top hits for RAP1 and TRF2, PPP1R2 and PPP2R3A were selected for further analysis (Table 3.2). In addition, PPP5C, a protein previously described to play a role in the DNA damage repair pathway (Ali et al., 2004; Kang et al., 2009), and which inhibition using the siRNA phosphatase library also produced a considerable reduction in the number of TRF2 foci, was also selected for validation.

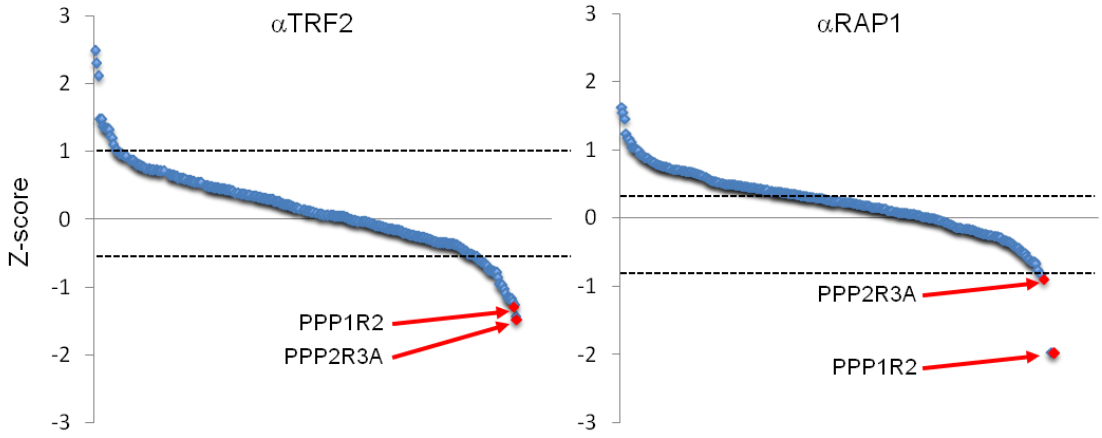


Figure 3.4 – Z-score analysis of the number TRF2/RAP1 foci.

Z-score values based on the TRF2 and RAP1 foci number per nucleus, determined in cells transfected with siRNAs of the Phosphatase library. The z-score was calculated using the formula: $z\text{-}score = \frac{n - m}{s}$; where **n** is the number of foci in the well x, **μ** is the average number of foci in the plate k and **s** is the standard deviation in the plate k. Arrows indicate examples of selected hits. Dashed lines indicate the threshold used for hit selection, which corresponds to the average of the scrambled siRNA plus/minus two times its standard deviation.

3.2.2 A screen for factors required for RAP1 and TRF2 foci formation using a human Ubiquitinome siRNA library

Due to the importance of the ubiquitylation activities in the regulation of DNA damage response pathways (Doil et al., 2009; Kolas et al., 2007; Mailand et al., 2007; Stewart et al., 2009), the potential requirement for ubiquitylation-related factors for the formation of TRF2 and RAP1 foci was also investigated. This was accomplished by using an Ubiquitinome siRNA library, available in Dr. Jiri Lukas laboratory (Danish Cancer Society, Copenhagen, Denmark) a cell array format (384 samples per plate), with mixtures of siRNA duplexes and transfection reagent spotted on Lab-Tek chambers and preserved in a gelatin and fibronectin matrix (Erfle et al., 2007). In comparison to the 96-well plate format, the advantages of this system are: the ability to screen high-content libraries, longer shelf-life and more homogeneous staining throughout the chamber. Since the optimal transfection

conditions and cell line for this system have been previously determined (Doil et al., 2009; Erfle et al., 2007), limited optimisation was focused on the staining conditions and the settings for the image acquisition.

The z-score for each sample was calculated as above and the same threshold was used as criteria to select the hits (Figure 3.5). In contrast to the Phosphatase library, which contained a pool of 3 different siRNAs for each gene, the Ubiquitinome library contained two individually spotted siRNAs that targeted the same gene. The selection of the hits took into consideration the fact that both individual siRNA targeting one gene had to score above the defined threshold. RNF41, a RING-domain E3 ligase required for the degradation of molecules involved in different pathways, such as tumor cell growth and apoptosis, and COPS6, a component of the COP9 signalosome involved in cell cycle regulation; scored for both TRF2 and RAP1 and were selected for further analysis. In addition ABTB2, (unknown function), UFM1 (an ubiquitin-like protein that is conjugated to target proteins by E1-like protein in a manner analogous to ubiquitylation) and MLL2 (histone methyltransferase with functions as transcriptional regulator) were also chosen for validation (Table 3.2).

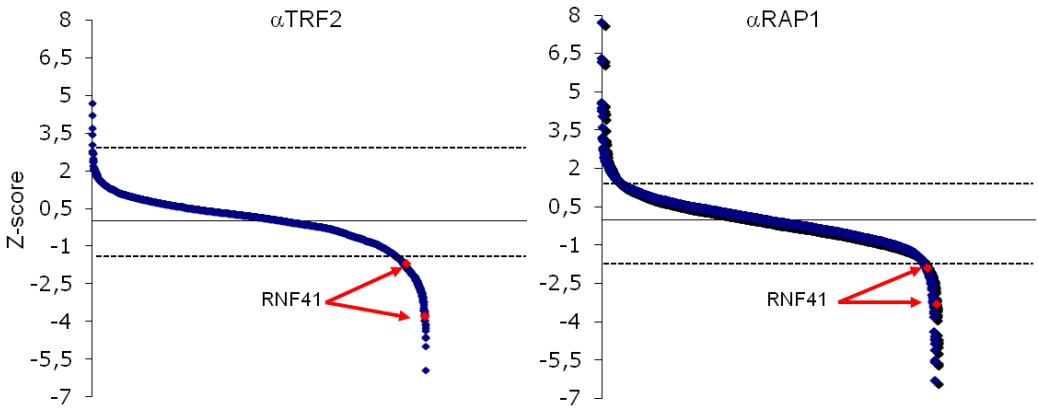


Figure 3.5 - Z-score analysis of the number TRF2/RAP1 foci.

Z-score values based on the TRF2 and RAP1 foci number per nucleus, determined in cells transfected with siRNAs of the Ubiquitinome library. The z-score was calculated using the formula: $z\text{-}score = \frac{n - m}{s}$; where **n** is the number of foci in the well x, **μ** is the average number of foci in the plate k and **s** is the standard deviation in the plate k. Arrows indicate examples of selected hits. Dashed lines indicate the threshold used for hit selection, which

corresponds to the average of the scrambled siRNA plus/minus two times its standard deviation.

In addition to the screen described above, a separate screen of the Ubiquitinome library using the frequency of dysfunctional telomeres as readout was performed. The aim of this approach was to identify factors implicated in the DNA damage response pathway at the telomeres. Telomere dysfunction triggered by TRF2 (Denchi and de Lange, 2007; Takai et al., 2003), POT1 (Denchi and de Lange, 2007; Hockemeyer et al., 2005), TPP1 (Kibe et al., 2010) or TRF1 (Martinez et al., 2009; Sfeir et al., 2009) deletion causes accumulation of DNA damage factors (e.g. γH2AX, 53BP1) at telomeres into complexes known as TIFs (d'Adda di Fagagna et al., 2003; Takai et al., 2003). In this analysis, an anti-53BP1 antibody was used to mark DNA damage sites and a FISH telomeric PNA probe to label telomeres. The co-localization of both signals indicated activation of DNA damage pathways at the telomeres. The z-score values determined for 53BP1 foci were ranked and the ones with the highest score were further analysed for 53BP1 co-localization with the telomeric probe. From this analysis DTL (E3 ligase that promotes genomic stability either through the prevention of re-replication or by its involvement in the early G2/M checkpoint), KIAA1333 (G2/M-phase specific E3 ubiquitin ligase, which delocalizes rapidly from the nucleolus after DNA damage) and CALCOCO1 (putative role as co-activator of transcription), UFM1 and ABTB2 were selected as factors whose depletion produced a considerable number of TIFs (Table 3.2). Both UFM1 and ABTB2 were also selected based on their ability to reduce the number of RAP1 and TRF2 foci, respectively.

3.2.3 Validation of hits from Phosphatase and Ubiquitinome siRNA library screens

In an attempt to reproduce the results obtained in the screens, each one of the fourteen hits, selected from both the Phosphatase and Ubiquitinome Libraries

Table 3.2), was siRNA-depleted in U2OS cells in 96 well plates, followed by immunofluorescence analysis with anti-RAP1 and anti-TRF2 antibodies (Figure 3.6). As expected siRNAs targeting TRF2 or RAP1 greatly reduced the number of foci, confirming the suitability of the transfection and staining conditions for this analysis. RNF41 also showed a clear reduction in the number of TRF2 and RAP1 foci, confirming this protein as a good candidate. Depletion of UFM1 also showed reduction in the number of RAP1 foci, although to a lesser extent.

Table 3.2 – Selected hits from the Phosphatase and Ubiquitinome siRNA libraries

Gene	Library	RAP1 foci	TRF2 foci
PPP1R2	Phosphatase	X	X
PPP2R3A	Phosphatase	X	X
PPP5C	Phosphatase		X
COPS6	Ubiquitinome	X	X
RNF41	Ubiquitinome	X	X
ABTB2	Ubiquitinome	*	X
UFM1	Ubiquitinome	X	
MLL2	Ubiquitinome	X	
ATE1	Ubiquitinome		X
CALCOCO1	Ubiquitinome		X
VPRBP	Ubiquitinome		
WDR53	Ubiquitinome		X
DTL	Ubiquitinome	TIFs	
KIAA1333	Ubiquitinome	TIFs	

X - siRNA that scored above the threshold

* - Only one of the siRNA against ABTB2 scored for RAP1

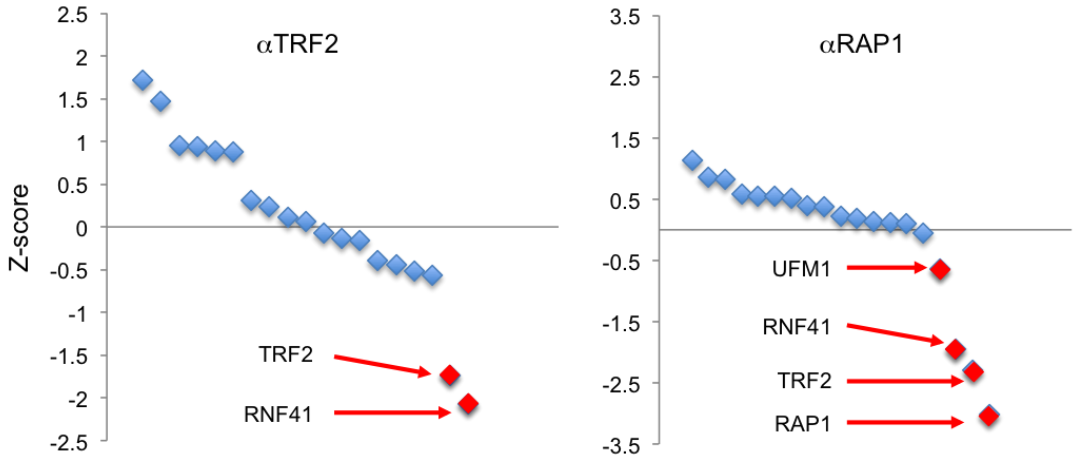


Figure 3.6 - Hit validation by immunofluorescence for TRF2 and RAP1.

Z-score values based on the TRF2 and RAP1 foci number per nucleus were determined in cells transfected with siRNAs of the selected hits. The z-score was calculated using the formula: $z\text{-}\ score = \frac{n\text{-}\ m}{s}$; where **n** is the number of foci in the spot x, **μ** is the average number of foci in the plate k and **s** is the standard deviation in the plate k. Factors with lower Z-score are shown in red.

Next TIF formation upon depletion of these fourteen proteins in U2OS cells was also analysed. As controls, we used RAP1, TRF2 and scrambled siRNA duplexes (Figure 3.7A and B). As expected, TRF2 siRNA-mediated depletion resulted in robust TIF induction (Takai et al., 2003). No TIFs were detected when RAP1 was depleted from these cells (Figure 3.7A). This is in accordance with recent mouse data which demonstrates that RAP1 knockout MEFs do not form TIFs and do not phosphorylate ATM/ATR targets CHK2 and CHK1 (Sfeir et al., 2010). In control cells transfected with scrambled siRNA it was possible to detect between zero and three 53BP1 foci per nucleus (Figure 3.7A-C), which did not co-localize preferentially with telomeres. DTL, KIAA1333, UFM1 and ABTB2, which scored highly for TIFs in the Ubiquitinome library screen, also scored in both validation experiments performed afterwards (Figure 3.7C and D). Moreover, RNF41-depleted cells showed an increase in the number of TIFs, once more suggesting it as a good candidate for further analysis. In addition, depletion of DTL led to a robust increase in the number of TIFs (Figure 3.7C and D). Importantly, DTL depletion also increased the number of non-telomeric

53BP1 foci and decreased viability in U2OS cells. This essential function of DTL might be explained by its role in G2/M checkpoint activation, which promotes cell cycle arrest upon DNA damage (Sansam et al., 2006). Thus, the increase in the number of 53BP1 foci can be explained by its requirement in maintaining genomic stability.

Taken together the results shown in Figure 3.6 and Figure 3.7, suggested that RNF41 was the strongest candidate for a factor required for telomere integrity, since its depletion efficiently reduced the number of TRF2 and RAP1 foci and triggered TIF formation. RNF41 is an E3 ligase involved in the degradation of proteins such as ErbB3 (Qiu and Goldberg, 2002) and BRUCE (Qiu et al., 2004), or activation of factors, such as TBK1 (Wang et al., 2009). To evaluate whether depletion of RNF41 could impact on TRF2 and RAP1 protein stability, analysis of the protein levels by Western blotting, was performed. RNF41 was depleted using siRNA in HeLa and U2OS cells. Interestingly, both cells lines showed reduction in the levels of TRF2 and RAP1, in the chromatin-bound protein fraction and in the whole-cell extract analysed (Figure 3.8), suggesting that RNF41 could stabilize TRF2 alone, since it is known that loss of TRF2 triggers RAP1 degradation (Celli and de Lange, 2005; Denchi and de Lange, 2007)(Figure 3.3), or both TRF2 and RAP1.

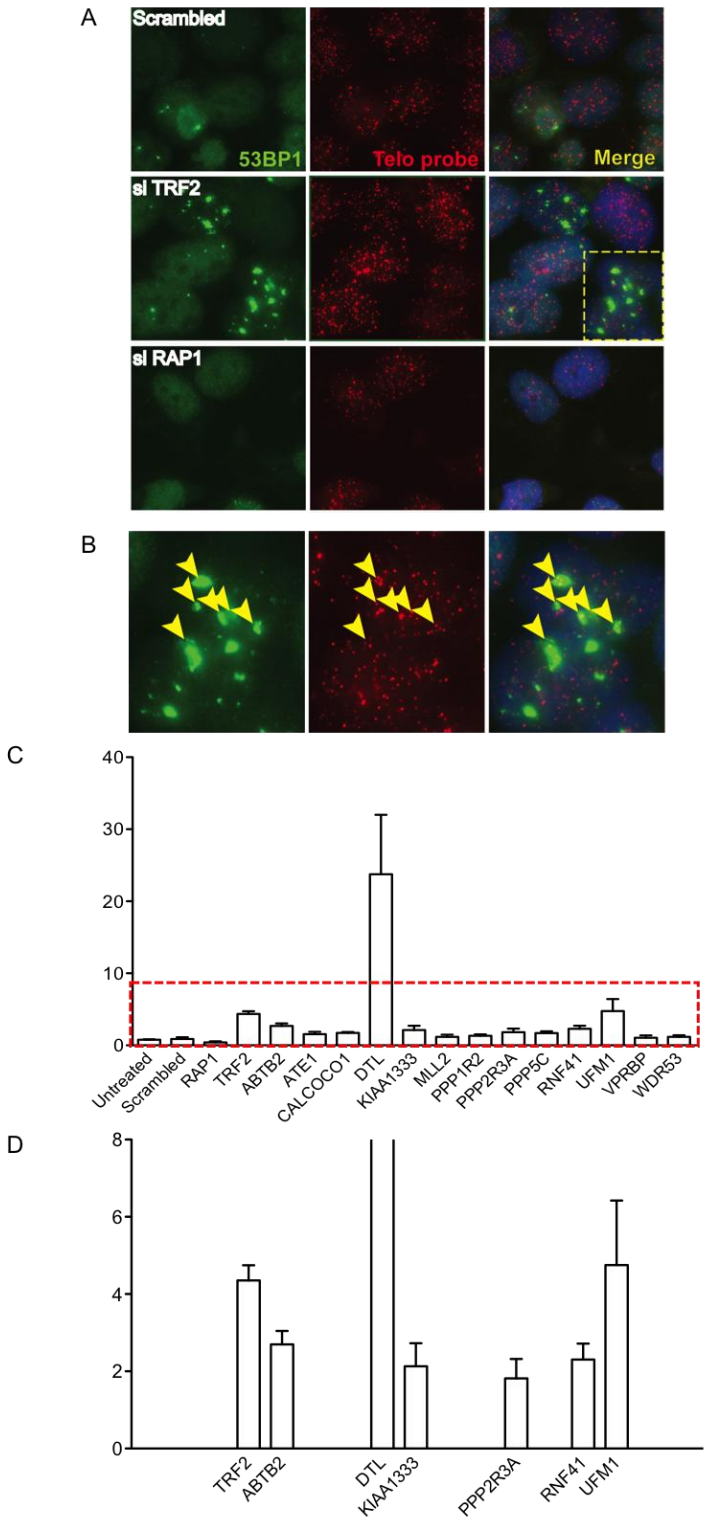


Figure 3.7 - TIF analysis in U2OS cells treated with siRNA hits isolated from Phosphatase and Ubiquitinome library screens.

A) U2OS cells were transfected with RAP1, TRF2 or scrambled siRNA duplexes, then stained with an antibody against 53BP1 (green) and FISH telomeric probe (red). B) Magnification of the inset in A). Arrowheads indicate TIFs. C) U2OS cells were transfected with RAP1, TRF2 or scrambled siRNA duplexes, as well as siRNAs for the hits isolated from library screens. The number of TIFs per nucleus was quantified using a co-localization function of ImageJ

software and plotted as shown. D) Subset of samples analysed in C) showing only the hits that induced more than two TIFs per cell.

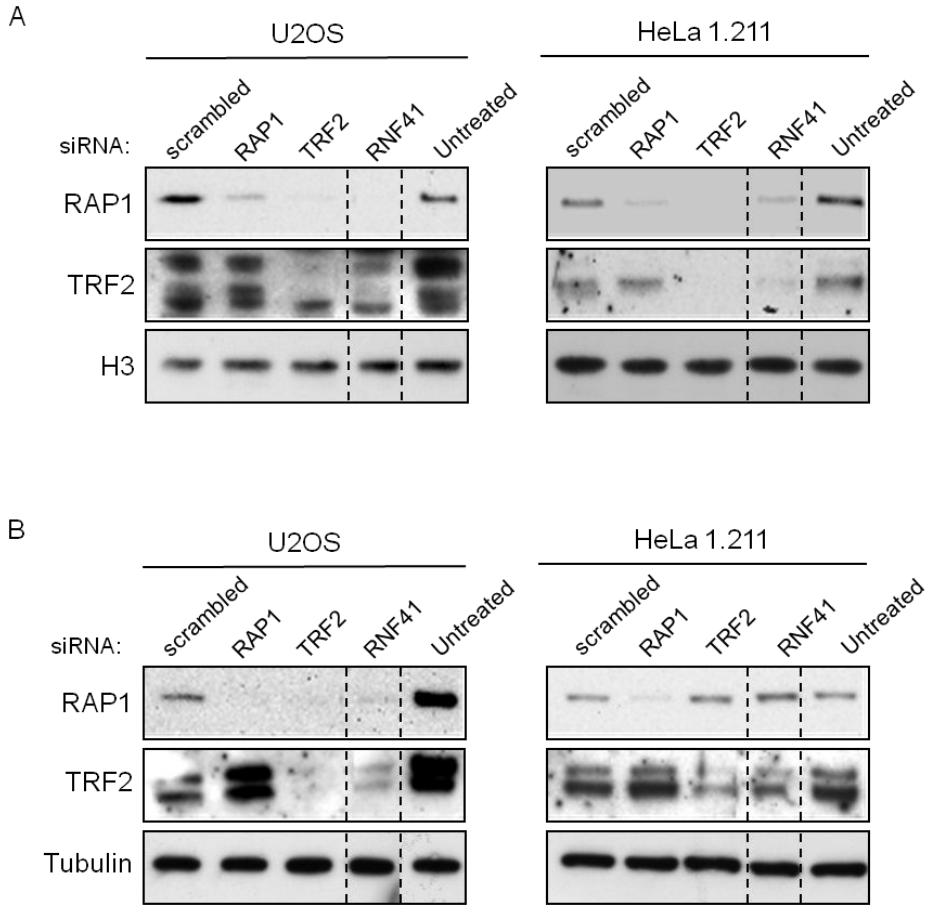


Figure 3.8 – RNF41 depletion reduces both TRF2 and RAP1 protein levels.

A) U2OS and HeLa 1.211 cells were reverse-transfected with RAP1, TRF2, RNF41 or scrambled siRNA duplexes. Seventy two hours after were harvested and subjected to cell fractionation. Western blots show the chromatin-bound fraction. H3 was used as loading control. B) Cells transfected as in A) were harvested and the whole cell extract was used for Western blotting. Tubulin was used as loading control. Dashed lines represent areas of the same gel that were cropped.

To further confirm these results an additional siRNA targeting RNF41 was tested. Surprisingly, the second siRNA did not reduced TRF2 protein levels (Figure 3.9A), in spite of the fact that both siRNAs targeting RNF41 led to its efficient depletion, as access by qPCR (Figure 3.9B).

The observation of these differences between the two siRNAs suggested that RNF41 could be a false positive hit. Frequently false positive hits arise from off-target effects. One of the most common off-target effects of siRNA duplexes is caused by

annealing of the seed region of the siRNA antisense strand with the 3' untranslated region of unrelated mRNAs, thus causing non-specific downregulation through a mechanism similar to the one triggered by microRNAs (Jackson and Linsley, 2010). To investigate this, the levels of TRF2 mRNA were determined by qPCR. RNF41 siRNA number 1 reduced the levels of TRF2 mRNA, in contrast to the siRNA number 2 (Figure 3.9C). This strongly suggests the presence of an off-target effect mediated by the siRNA number 1. In fact, the alignment of the RNF41 siRNA number 1 with the 3' UTR region of TRF2 mRNA revealed a homology sequence of 12 nucleotides with one mismatch in the middle, highly similar to a microRNA-like structure. This further suggested that RNF41 siRNA target non-specifically TRF2 mRNA.

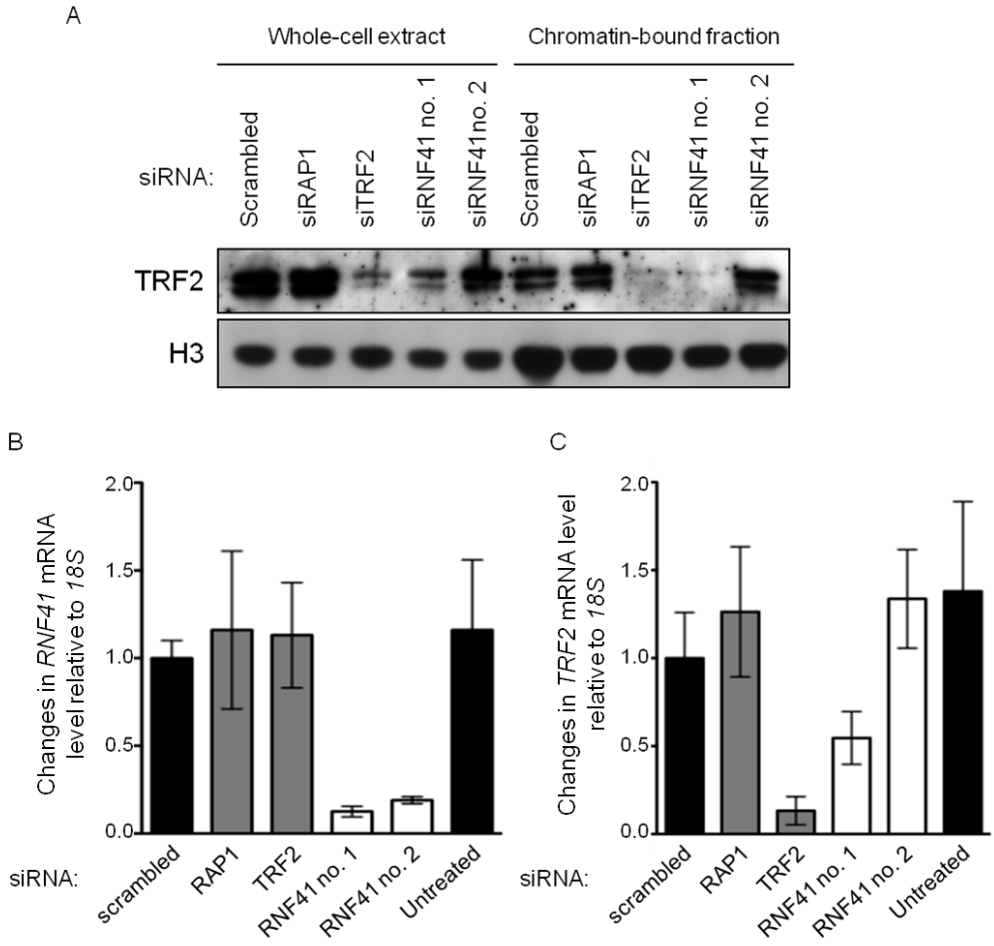


Figure 3.9 – siRNA number 1 targets TRF2 mRNA.

A) U2OS were reverse transfected with RAP1, TRF2, RNF41 no1 and no2 or scrambled siRNA duplexes and 72 h after were harvested and subjected to cell fractionation or the whole cell extract was used directly for Western blotting. H3 was used as loading control. B) Cells transfected as in A) were harvested for qPCR 24 h after transfection. qPCR was performed using RNF41 primers. 18S was used as endogenous control. C) Same as in B) but using TRF2 primers.

3.2.4 A screen for factors required for TIF formation using human DNA repair library

Telomere dysfunction constitutes a clear threat to genomic stability. For this reason it is important to understand the DDR triggered from uncapped telomeres and to compare it to the canonical DNA damage cascade. From the work presented above, the use of TIFs as a readout for telomere dysfunction emerged as a promising approach. The siRNA library selected to perform another study targeted a collection of genes linked to DNA repair, allowing to study their effect on TIF formation. To perform this screen, a cell line expressing a DOX inducible TRF2 shRNA was established (Figure 3.10). This cell line allows the stable expression of shRNA for TRF2 after DOX addition. Moreover, single-cell clones were selected in order to ensure the homogeneous expression of the TRF2 shRNA the population used for the analysis.

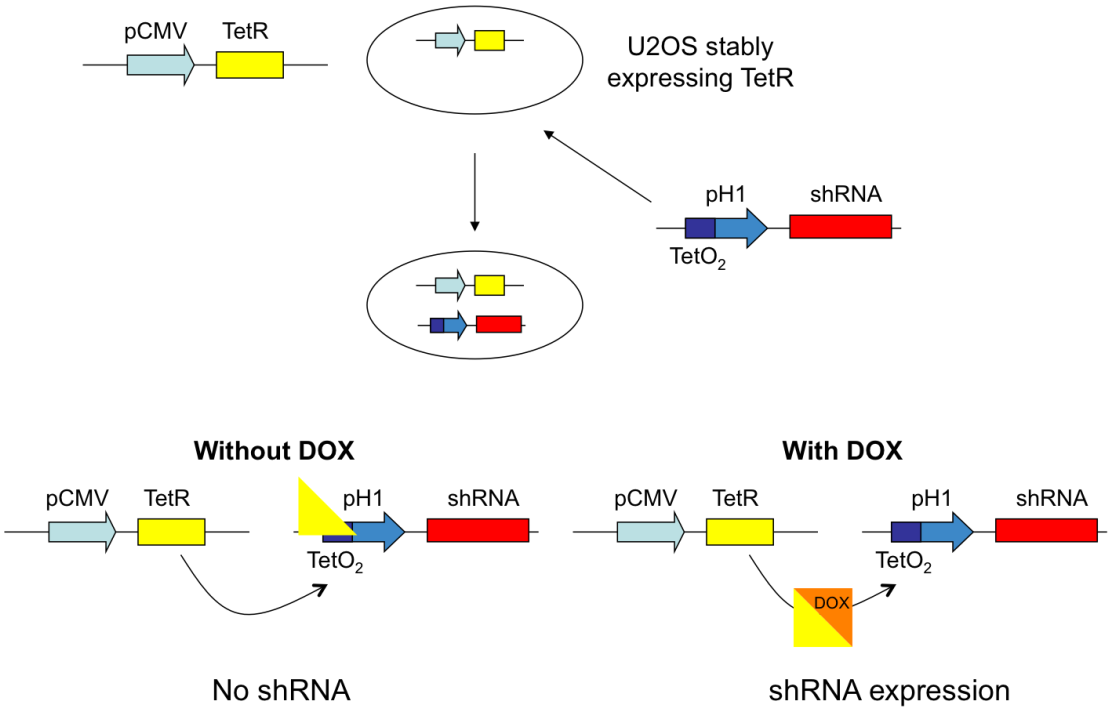


Figure 3.10 – Inducible system for the expression of TRF2 shRNA in U2OS cells.
U2OS cells stably expressing tetracycline repressor (TetR) where transfected with a pSuperior construct harboring a tetracycline operator ($TetO_2$) and an shRNA targeting TRF2 (top). Without DOX TetR is bound to $TetO_2$, preventing expression of the shRNA. When DOX is added to the media it sequesters TetR preventing its binding to $TetO_2$ and allowing expression of the shRNA.

Efficient TRF2 depletion after DOX addition was assessed by Western blot and immunofluorescence analysis (Figure 3.11A and B). Additionally, TIF formation after DOX-inducible TRF2 depletion was also tested (Figure 3.11B and C). As expected, expression of TRF2 shRNA by DOX addition, led to a decrease in TRF2 levels and an increase in the number of 53BP1 foci, used as a marker for DNA damage. In addition, the co-localisation of 53BP1 foci with the telomeres was also studied using IF-FISH and showed a robust overlap between 53BP1 foci and the telomeric FISH signal (Figure 3.11C).

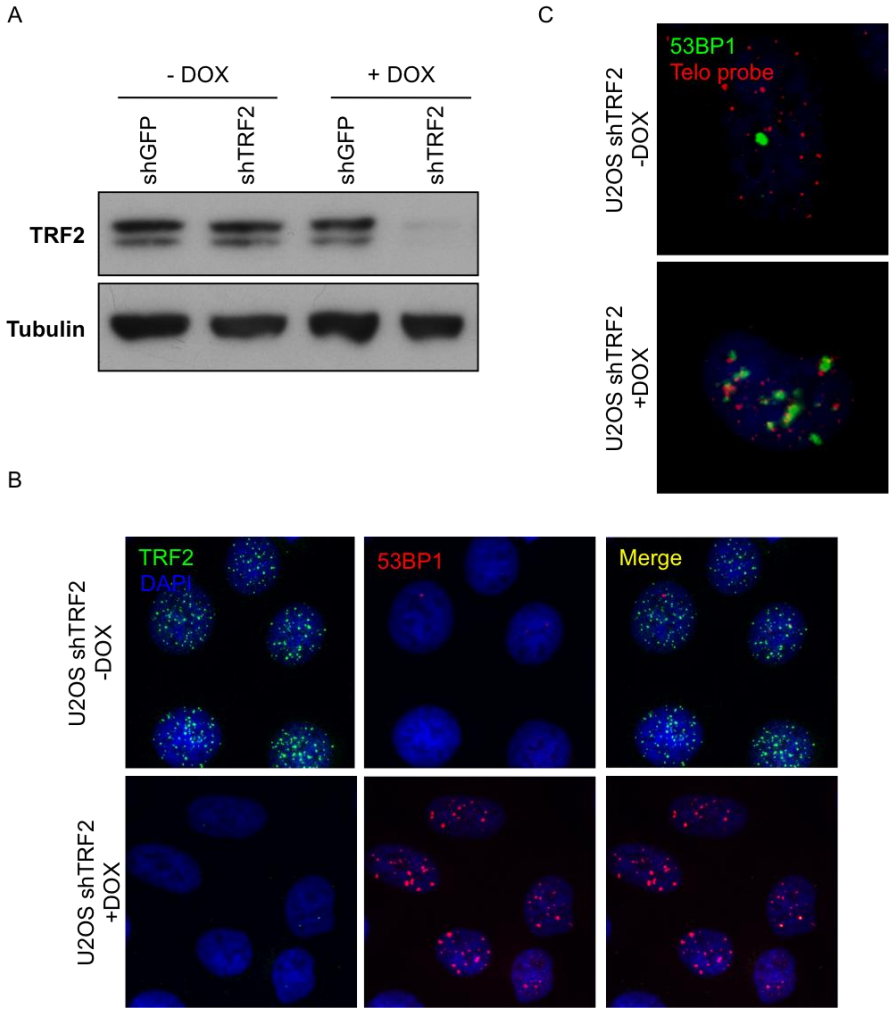


Figure 3.11 – TRF2 is efficiently depleted in U2OS cells using DOX-inducible TRF2 shRNA.

A) U2OS cells expressing inducible TRF2 shRNA or GFP control shRNA were analysed by Western blotting after DOX addition. Tubulin was used as loading control. B) U2OS cells expressing inducible TRF2 shRNA were analysed by immunofluorescence after DOX addition. C) Cells as in B) were stained by immunofluorescence with 53BP1 antibody and the telomeres were labeled using telomeric PNA FISH probe.

Having selected single-cell clones based on their ability to efficiently deplete TRF2 and produce TIFs upon DOX treatment, the conditions for the siRNA screen were further optimized. To determine the time point post-DOX addition after which the maximum number of TIFs was reached, a time course was performed. Analysis of the number of TIFs 2, 3 and 6 days after DOX revealed that the highest difference between treated and untreated cells was found 6 days after DOX treatment, when DOX addition triggered an average of 6 TIFs per cell (Figure 3.12). Therefore, this was the time point selected for the siRNA screen analysis.

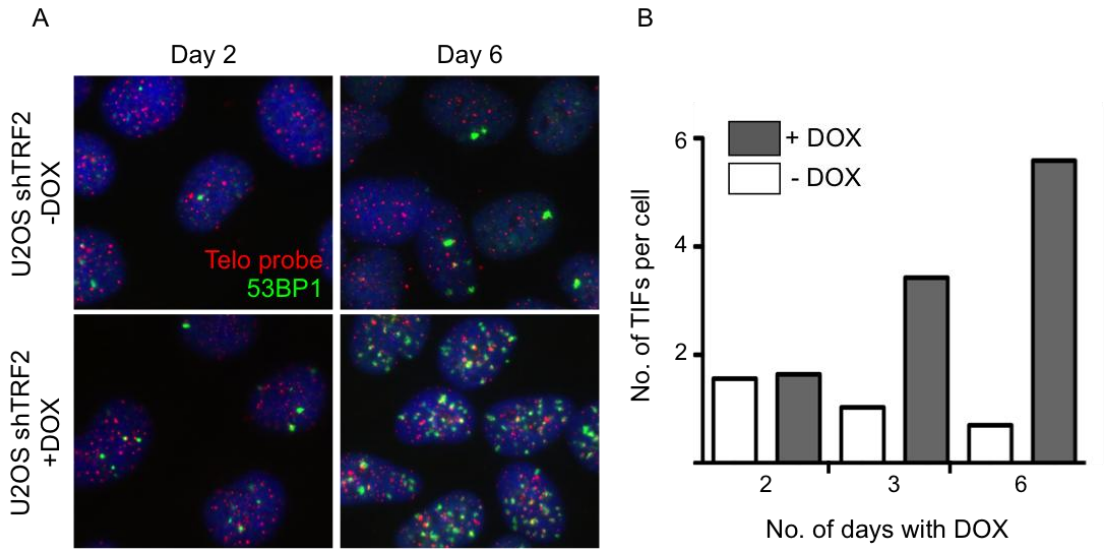


Figure 3.12 – Time course after DOX addition.

A) Cells seeded in 10 cm dishes were treated with DOX or left untreated; coverslips were then fixed and stained using the IF-FISH protocol. B) Analysis of the staining done in A).

Additional optimization involved determining the best plating density (11 000 cells per well of a 96-well plate) and transfection conditions, including siRNA concentration (final of 10 nM) and transfection procedure. The final transfection protocol is depicted in Figure 3.13. As a negative control for this screen, siRNA targeting RNF8 was selected and its efficiency was tested under the selected conditions (Figure 3.14). RNF8 has been previously described to be required for 53BP1 foci formation after DNA damage (Kolas et al., 2007; Mailand et al., 2007).

More recently it was also shown to be required for the DNA damage signal elicited by uncapped telomeres (Peuscher and Jacobs, 2011; Rai et al., 2011). Consistent with these results, the 53BP1 foci present after DOX-induced depletion of TRF2 were efficiently abolished by RNF8 depletion (Figure 3.14).

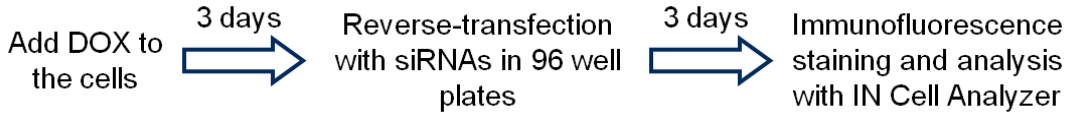


Figure 3.13 – Diagram of reverse transfection protocol for the siRNA library.

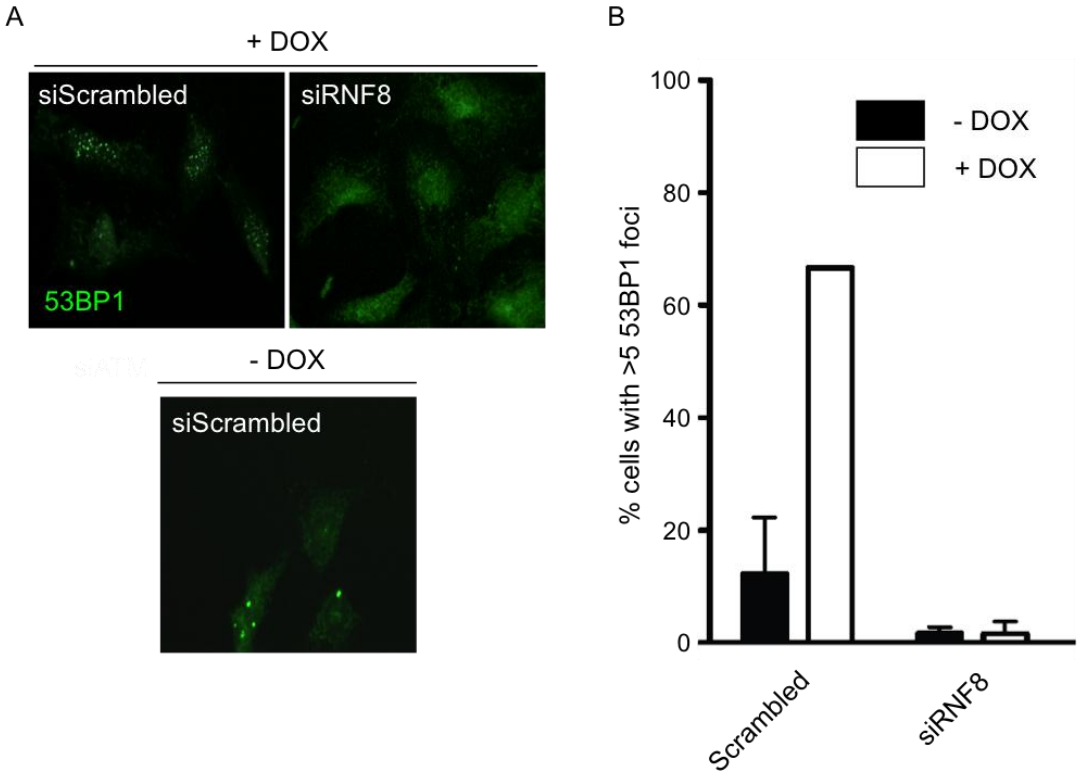


Figure 3.14 – RNF8 siRNA reduces number of 53BP1 foci formed after TRF2 depletion.

A) Cells were transfected as described in Figure 3.13, treated with DOX or left untreated and stained using the immunofluorescence protocol. B) Analysis of the staining in A).

3.2.5 DNA repair library screen and validation

Using the conditions optimized above, cells expressing TRF2 shRNA were reverse-transfected with the DNA repair library and stained with 53BP1 antibody as a marker for telomeric damage. The screen was run in triplicate and RNF8 siRNA was included in every plate as a control, since RNF8 depletion reduces the number of 53BP1 foci (Kolas et al., 2007; Mailand et al., 2007). Data was analysed and the z-score for the percentage of cells with less than 5 53BP1 foci was calculated for every sample (Figure 3.15). The RNF8 siRNA control clearly showed a reduction of the number of 53BP1 foci, validating the feasibility of the screen. Amongst the siRNAs that reduced the number of 53BP1 foci were the siRNA targeting 53BP1 itself and siRNAs for H2AX and ATM. This is consistent with the idea that phosphorylated H2AX is important for the stabilization of 53BP1 foci (Celeste et al., 2003) and that the ATM pathway is activated after TRF2 depletion (Denchi and de Lange, 2007). In addition, also MDC1 depletion reduced the number of TIFs, although it did not score above the threshold. This is in accordance with the previously proposed idea that MDC1 abrogation impairs 53BP1 binding to the telomeres (Dimitrova et al., 2008).

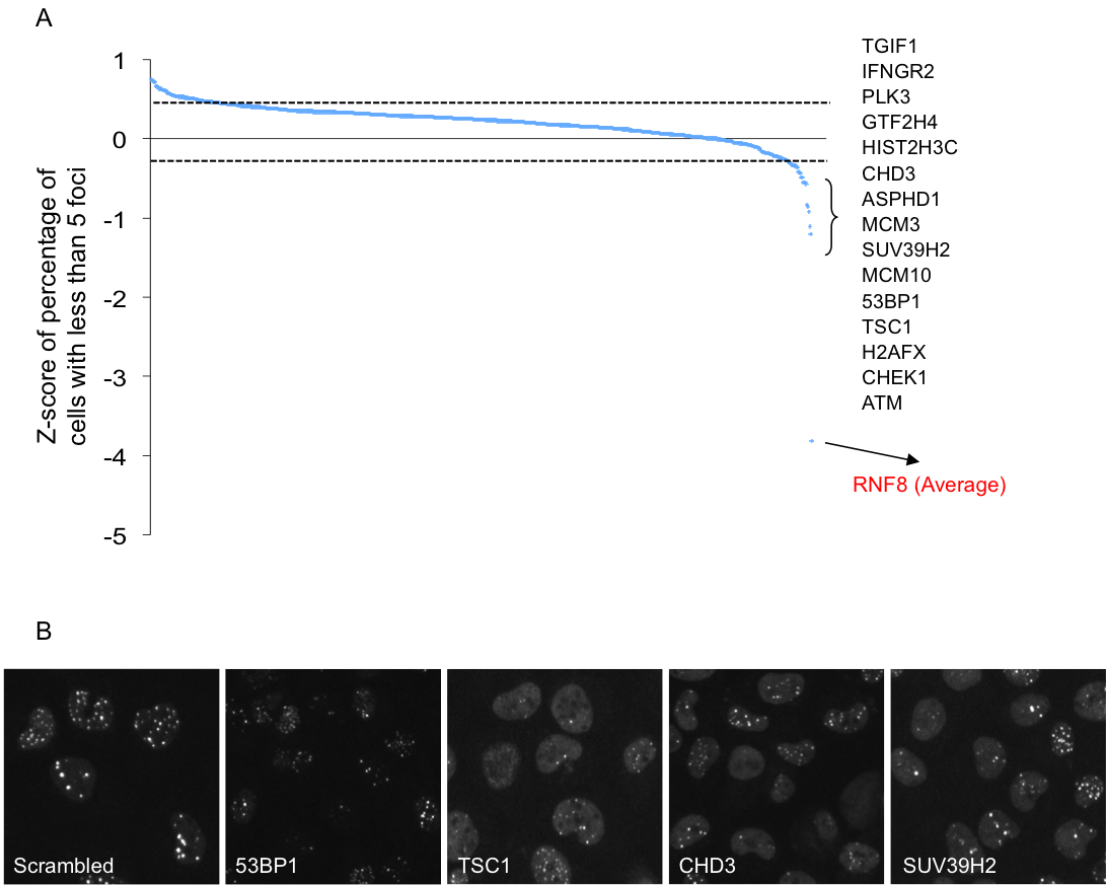


Figure 3.15 – The siRNA screen for factors that triggered 53BP1 foci induced by TRF2 abrogation using a DNA repair library.

A) Cells were reverse-transfected with the DNA repair library as described in Figure 3.13 and stained with immunofluorescence protocol. The z-score for every sample was calculated and plotted. Dashed lines indicate the threshold used for hit selection, which corresponds to the average of the scrambled siRNA plus/minus two times its standard deviation. Hits that scored below the threshold (defined as 2 times the standard deviation of the scrambled) are shown. The average z-score for RNF8 siRNA, negative control, is also shown. B) Pictures showing examples of factors that scored as hits in the siRNA screen described in A).

Additional potential hits included factors involved in chromatin structure/remodelling (CHD3, SUV39H2, HIST2H3C, HMGN1), replication (MCM10, MCM3, ORC2L), transcription (GTF2H4, TGIF1) and other cellular processes (IFNGR2, TSC1, ASPHD1, PLK3). Some of the hits (SUV39H2, TSC1, MCM10, PLK3, HMGN1, CHD3, ORC2 and TGIFII) were selected for a secondary screen. Each of the selected hits was targeted by four different siRNAs and the secondary screen was performed using the same conditions as the original siRNA screen. In order to compare the effect of these hits on the general DDR pathway, cells without

DOX were transfected with siRNAs and then irradiated with 10 Gy, one hour before being stained. Furthermore, during this analysis the effect of the depletion of these genes on the number of spontaneous 53BP1 foci was also addressed. Interestingly, it was possible to observe a clear overlap between the hits that scored higher in the cells depleted of TRF2 or cells treated with IR, suggesting a similar requirement of these factors in both types of DNA damage (Figure 3.16A and B). When analysing the effect of these factors on the spontaneous 53BP1 foci, none of them seemed to have a clear effect, except for RNF8 siRNA (Figure 3.16C). Comparing the results obtained for individual siRNA targeting the same gene, it was possible to observe that for CHD3, TSC1 and SUV39H2 two different siRNA scored above the defined threshold. Since off-target effects constitute an important source of false positives, the protein levels of 53BP1 were analysed in cells transfected with the siRNAs targeting CHD3, TSC1 and SUV39H2. No differences in 53BP1 levels were found between the scrambled and the siRNAs (Figure 3.17), suggesting that the reduction in the number of 53BP1 foci after TRF2 depletion or IR is not a consequence of these siRNA targeting non-specifically the 53BP1 mRNA.

CHD3 (chromodomain helicase DNA binding protein 3) is a factor involved in chromatin remodelling and a component of the Nucleosome Remodeling and Deacetylase (NuRD) complex. Chromatin remodelling is required for many cellular processes, including transcription and DNA repair. Moreover, a link between the ATM kinase and chromatin remodelling has been previously established (Goodarzi et al., 2008). After DNA damage, ATM phosphorylates KAP-1, a transcriptional co-repressor that promotes heterochromatinization by concentrating several silencing activities, including the NuRD complex, in the chromatin (Goodarzi et al., 2010). KAP1-1 phosphorylation is thought to interfere with the interaction between CHD3 and SUMO1, which displaces CHD3 from the damaged heterochromatin allowing repair (Goodarzi et al., 2011).

.

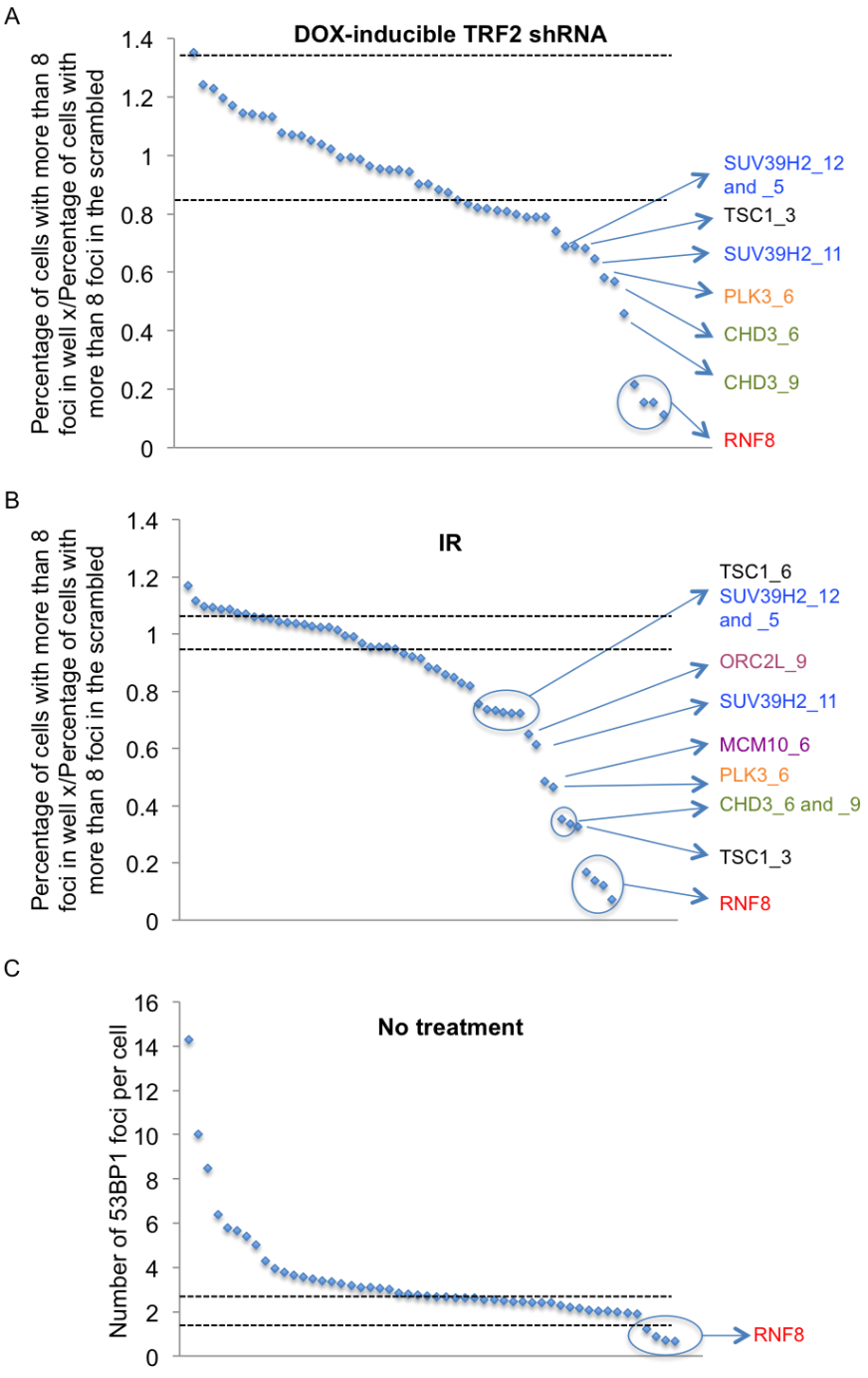


Figure 3.16 – Secondary screen using selected hits from the DNA damage repair library.

A) Cells were reverse-transfected with the selected siRNA subset of the DNA repair library, as described in Figure 3.13. Cells were then stained with immunofluorescence protocol and the z-score for every sample was calculated and plotted. Dashed lines indicate the threshold used for hit selection, which corresponds to the average of the scrambled siRNA plus/minus two times its standard deviation. Hits that scored below the threshold (defined as 2 times the standard deviation of the scrambled) are shown. The RNF8 siRNA used as negative control, is also shown. Cells were also reverse-transfected with the subset of the DNA repair library and either irradiated with 10 Gy 1 h before analysis by immunofluorescence (B) or left untreated (C). As in A), selected hits and RNF8 siRNA control are indicated.

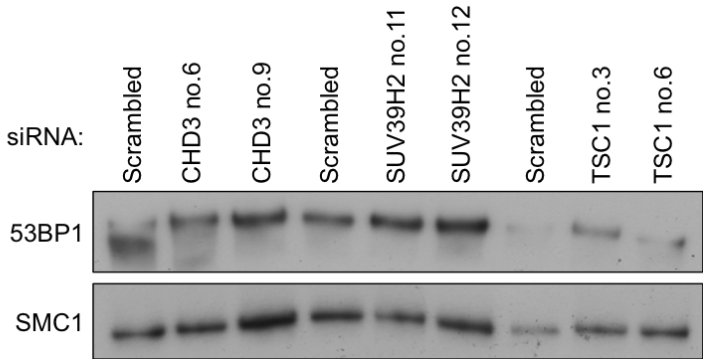


Figure 3.17 – siRNAs for CHD3, SUV39H2 and TSC1 do not target 53BP1.

U2OS cells were reverse-transfected with the indicated siRNAs. Seventy two hours after transfection cells were harvested for Western blot. SMC1 was used as loading control.

SUV39H2 is a methyltransferase that promotes heterochromatinization through dimethylation and trimethylation of the histone H3 on lysine 9. Like KAP-1, the absence of SUV39H1/2 from the chromatin also facilitates DNA repair (Goodarzi et al., 2008), once more implying that loss of compact chromatin structure is required for DNA repair. In addition, SUV39H1 and 2 have been implicated in telomere length regulation (Garcia-Cao et al., 2004). Their absence leads to abnormal telomere elongation, which established a link between telomere elongation and epigenetic regulation.

TSC1 (Tuberous sclerosis 1) is a tumour suppressor gene, which forms a complex with TSC2 that functions as a GTPase-activating protein (GAP). This complex is an important negative regulator of the mTOR pathway, which responds to a wide range of stimuli and plays important roles in several cellular processes including protein synthesis and survival (reviewed in Laplante and Sabatini, 2012). Moreover, mTOR is regulated in response to reactive oxygen species in a process that relies on ATM-dependent TSC2 activation (Alexander et al., 2010), suggesting a connection between DNA repair pathways and TSC1/2 signalling. Furthermore, TSC1 has been identified as a putative substrate for the ATM/ATR kinases

(Matsuoka et al., 2007), although this connection has not been previously characterized.

To further validate the results of the siRNA screen, the number of 53BP1 foci was compared between cells transfected with scrambled siRNA and the ones transfected with siRNAs for SUV39H2, TSC1 and CHD3. For each target gene, six different siRNAs were used, including the four siRNAs present in the library. Unfortunately, for all three genes, the reduction in the number of 53BP1 foci did not correlate with the level of depletion obtained with each siRNA (Figure 3.18). For SUV39H2, siRNAs numbers 11 and 12 reduced the number of 53BP1 foci by about 40% of the scrambled siRNA (Figure 3.18A), yet the highest depletion efficiency was achieved with siRNAs numbers 5 and S1, as determined by qPCR (Figure 3.18B). For TSC1, only siRNA number 3 reduced the number of 53BP1 foci (approximately 50% reduction compared to the scrambled siRNA), even though other siRNAs also led to a high level of knockdown (Figure 3.18). Similarly, siRNAs 6 and 9 targeting CHD3 reduced considerably the number of 53BP1 foci (to about 40 and 50%, of the scrambled siRNA respectively), despite the fact that these siRNA were not the most efficient, according to qPCR results. Together, these results point to a lack of correlation between depletion of these genes and the reduction in the number of 53BP1 foci.

An additional study was performed to address the potential role of TSC1/2 in DDR using *Tsc1*$^{-/-}$, *Tsc2*$^{-/-}$ and WT MEFs. Surprisingly, both TRF2 depletion and IR triggered 53BP1 foci formation in *Tsc1*$^{-/-}$ and *Tsc2*$^{-/-}$ (Figure 3.19). This was in contrast to what was previously observed in U2OS cells transfected with siRNA for TSC1 (Figure 3.15, Figure 3.16). Similarly, analysis of MEFs null for both *Suv39h1* and *Suv39h2* (Suv39h1/2DN) did not show any reduction in the number of 53BP1 foci, either after TRF2 abrogation or IR (Figure 3.19), further suggesting that these factors are not involved in the DDR.

.

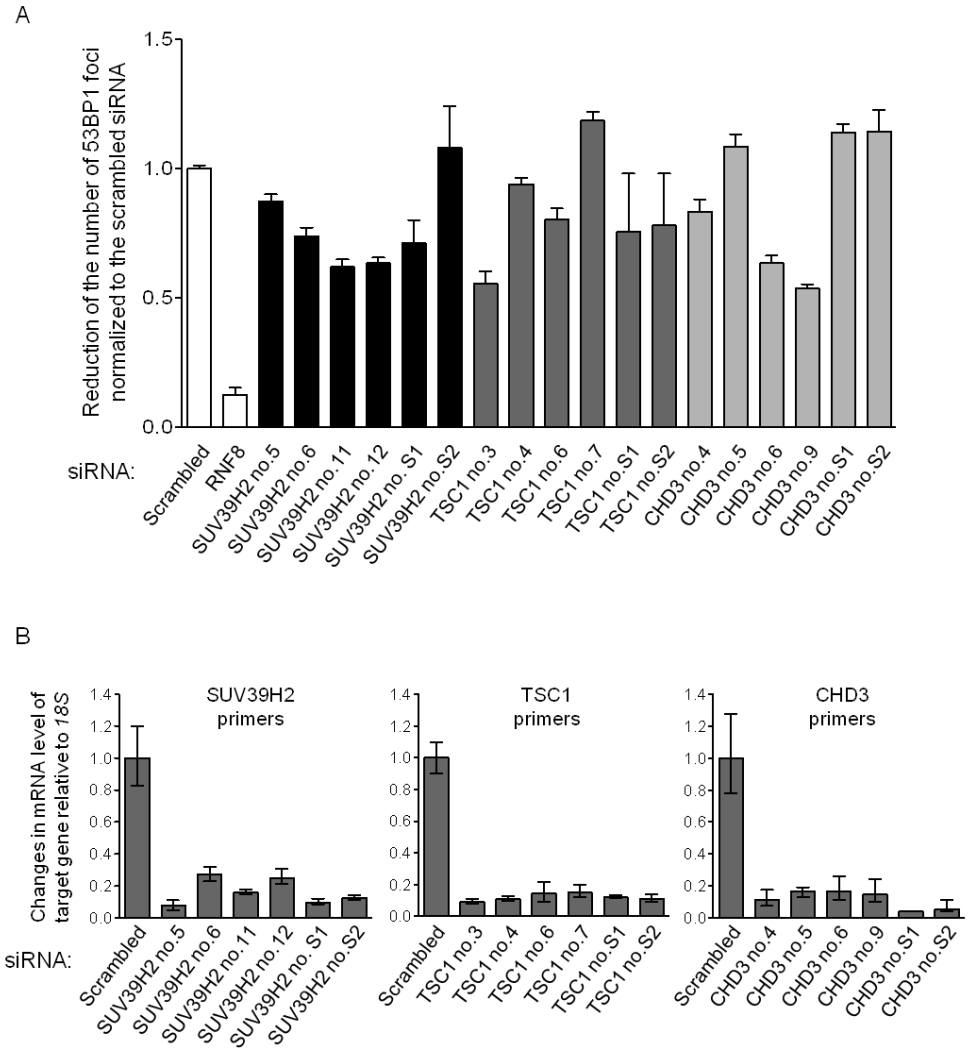


Figure 3.18 – The decrease in the number of 53BP1 foci is not consistent with the level of depletion triggered by individual siRNAs.

A) U2OS cells expressing TRF2 shRNA were reverse-transfected with the indicated siRNAs. Seventy two hours after transfection cells were fixed and stained for immunofluorescence using anti-53BP1 antibody. Images were analysed and the number of 53BP1 foci was quantified and normalized to the number of 53BP1 foci in cells transfected with the scrambled siRNA. B) Depletion in the cells transfected as in A) was analysed by qPCR with the indicated primers. 18S was used as endogenous control.

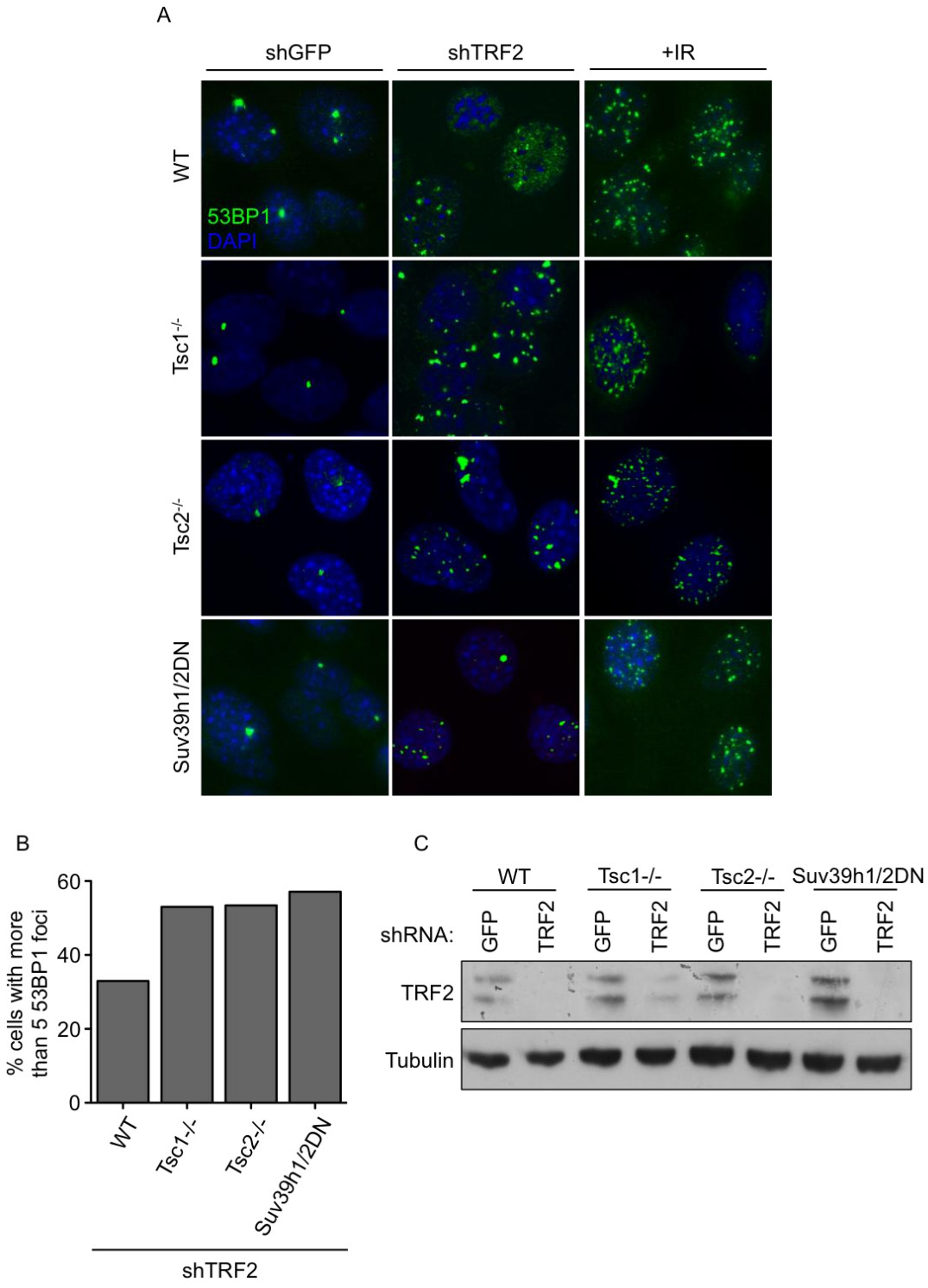


Figure 3.19 – Formation of 53BP1 foci is not impaired in *Tsc1*$^{-/-}$, *Tsc2*$^{-/-}$ or *Suv39h1/2DN* MEFs treated with TRF2 shRNA or IR.

A) WT, *Tsc1*$^{-/-}$, *Tsc2*$^{-/-}$ or *Suv39H1/2DN* MEFs were treated with shRNA for GFP or TRF2, or irradiated with 10 Gy, before immunofluorescence staining with 53BP1 antibody. B) Quantification of the number of 53BP1 foci in cells transfected with TRF2 shRNA. C) MEFs treated as in A) were harvested for Western blot. Tubulin was used as a loading control.

Chapter 4 Results – Part 2: CtIP/BRCA1 and EXO1 play distinct roles in the formation of telomeric end-to-end fusions

4.1 Introduction

DNA DSBs represent the most deleterious type of DNA damage. In order to repair such damage, two major pathways have emerged: HR and NHEJ (Branzei and Foiani, 2008). While HR requires an intact homologous template in order to repair, NHEJ promotes re-ligation of two broken ends without the need of a template.

One of the first factors to be recruited to the sites of damage is the MRN complex. This complex plays a role both in HR and in NHEJ, contributing to the decision of the repair pathway that will be engaged (Lamarche et al., 2010). CtIP, another factor involved in repair, undergoes CDK-dependent phosphorylation during S/G2, promoting its interaction with the MRN complex and binding to the tumour suppressor protein BRCA1. This allows formation of a complex that plays a critical role in DSB resection (You and Bailis, 2010). Extensive resection is a key aspect for the choice of HR as the pathway of repair, since it allows generation of ssDNA tails required for the homology search and strand invasion.

Both the MRN complex and CtIP are directly required to stimulate resection (Sartori et al., 2007; You et al., 2009). On the other hand, the role of BRCA1 in resection is thought to be through CtIP activation. After CDK-dependent phosphorylation of CtIP on S327, BRCA1 interacts with CtIP through this phosphorylation site and promotes CtIP ubiquitylation (Yu et al., 2006). Both phosphorylation and ubiquitylation of CtIP are required for its localization to the sites of DNA damage. Linked to this essential role of BRCA1 in CtIP activation, it has been recently shown that BRCA1 and 53BP1 play a critical role in the decision between

HR and NHEJ (Bunting et al., 2010). While BRCA1 promotes resection and therefore HR reactions, 53BP1 blocks resection and channels repair through NHEJ.

In addition to MRE11 and CtIP, other factors such as EXO1, DNA2 and the RecQ homologs WRN and BLM, also contribute to DNA resection. Moreover, it has been proposed based primarily on yeast data that these factors act to accomplish resection in a “two-step” model (reviewed in Huertas, 2010; Mimitou and Symington, 2009). MRN and CtIP may carry out an initial step of limited resection, which is then followed by a second step of extensive resection. This second step can be either mediated mainly by EXO1 or by a complex involving BLM/WRN and DNA2 (Gravel et al., 2008; Mimitou and Symington, 2008; Nimonkar et al., 2011; Nimonkar et al., 2008; Zhu et al., 2008). The ssDNA tails generated through resection are then bound by RPA, allowing the recruitment of the RAD51 recombinase (reviewed in Suwaki et al., 2011). This allows strand invasion of a homolog template and initiates HR repair.

Whilst resection and HR are mainly active in S/G2, NHEJ appears as the prevailing repair mechanism of DSBs during G1. As mentioned above, this mechanism does not required an homologous template, in contrast it relies only on the end joining of the two broken DNA ends in a three-step process: 1) capture of the two broken ends; 2) formation of a molecular bridge between the two DNA ends (both steps mediated by Ku70/80 and DNA-PK that act as a platform for the binding of other factors); and 3) limited processing and re-ligation of the broken ends (involves a series of enzymes, including nucleases and polymerases; the final ligation requires the XLF:XRCC4:LIG4 complex) (Sancar et al., 2004).

Recently, another DSB repair pathway has been the subject of several studies. This is the so-called MMEJ (reviewed in McVey and Lee, 2008), which can also be classified as an alternative NHEJ mechanism. In contrast to the classical NHEJ, MMEJ is Ku70/80-independent and requires an initial step of limited end resection. In yeast, this step requires the MRN/X complex, Exo1 and Sae2 (the CtIP homolog in yeast) (Decottignies, 2007; Lee and Lee, 2007; Ma et al., 2003). In vertebrates, the

requirement for MRN and CtIP in the resection stage of MMEJ has also been described (Quennet et al., 2011; Xie et al., 2009; Yun and Hiom, 2009), however the role of EXO1 has not yet been defined. Nevertheless, EXO1 is required for class switch recombination (Bardwell et al., 2004), a process that requires an alternative NHEJ mechanism (Nussenzweig and Nussenzweig, 2007; Stavnezer et al., 2008), suggesting that it might also be implicated in MMEJ.

Recent data has shown that the end-joining of dysfunctional telomeres is not only dependent on the classic NHEJ, but also involves alternative NHEJ pathways (Rai et al., 2010; Sfeir and de Lange, 2012), suggesting that a more complex network is required for telomere processing. Moreover, examining the processing of uncapped telomeres is a powerful model to study the repair pathways in a defined chromosomal context.

4.2 Results and discussion

4.2.1 BRCA1 and CtIP are not required for DNA damage signalling after TRF2 depletion

TRF2 depletion triggers an ATM-dependent response (Palm and de Lange, 2008), which is mediated by the MRN complex (Deng et al., 2009; Dimitrova and de Lange, 2009). The MRN complex is a critical component of the repair pathway essential for the initial processing of the break (reviewed in Branzei and Foiani, 2008). This processing is then further stimulated by CtIP, which in turn requires BRCA1 to be activated.

To address whether BRCA1 or CtIP have an impact on the signalling that emanates from telomeres uncapped by TRF2 depletion, *Brca1*$^{F/-}$ MEFs were treated with Cre-recombinase, to allow deletion of BRCA1, or with CtIP shRNA in combination with TRF2 shRNA (Figure 4.1A). MRE11 and NBS1 shRNAs were used as controls for factors known to decrease the ATM response after TRF2 depletion. Efficient depletion of all these factors was confirmed by Western blotting. To

determine the effect of CtIP on the ATM cascade, CHK2 phosphorylation was used as readout. In contrast to the MRN complex, BRCA1 and CtIP are not required for the ATM-dependent signalling cascade, since CHK2 phosphorylation was not affected by their inactivation (Figure 4.1A). In order to verify that CHK2 phosphorylation was due to TRF2 depletion and was not a consequence of BRCA1 or CtIP inactivation, P-CHK2 levels were compared in cells deleted of BRCA1 or depleted of CtIP, alone or in combination with TRF2 abrogation (Figure 4.2). As previously reported, BRCA1 deletion by itself triggered CHK2 activation (Bouwman et al., 2010), yet similar results were not observed when CtIP was inactivated. Consistent with this, CtIP is not required for ATM signalling in response to damaged chromatin in *Xenopus* extracts (You et al., 2009). This further demonstrates that BRCA1 and CtIP are not required for CHK2 phosphorylation after TRF2 inactivation.

TRF2 abrogation leads to the recruitment of DNA damage factors to the telomeres, which can be visualized as TIFs. TIF analysis revealed that BRCA1 and CtIP are not required for TIF-formation after TRF2 depletion, in contrast to MRE11 (Figure 4.1B and C). While in the case of MRE11 co-depletion with TRF2 the number of cells with more than 5 TIFs dropped from approximately 80% to 10% compared to TRF2 shRNA, when TRF2 is co-depleted with BRCA1 or CtIP this percentage is not significantly affected. Taken together, these results suggest that CtIP and BRCA1 are not required for the DNA damage signalling cascade triggered by TRF2 abrogation.

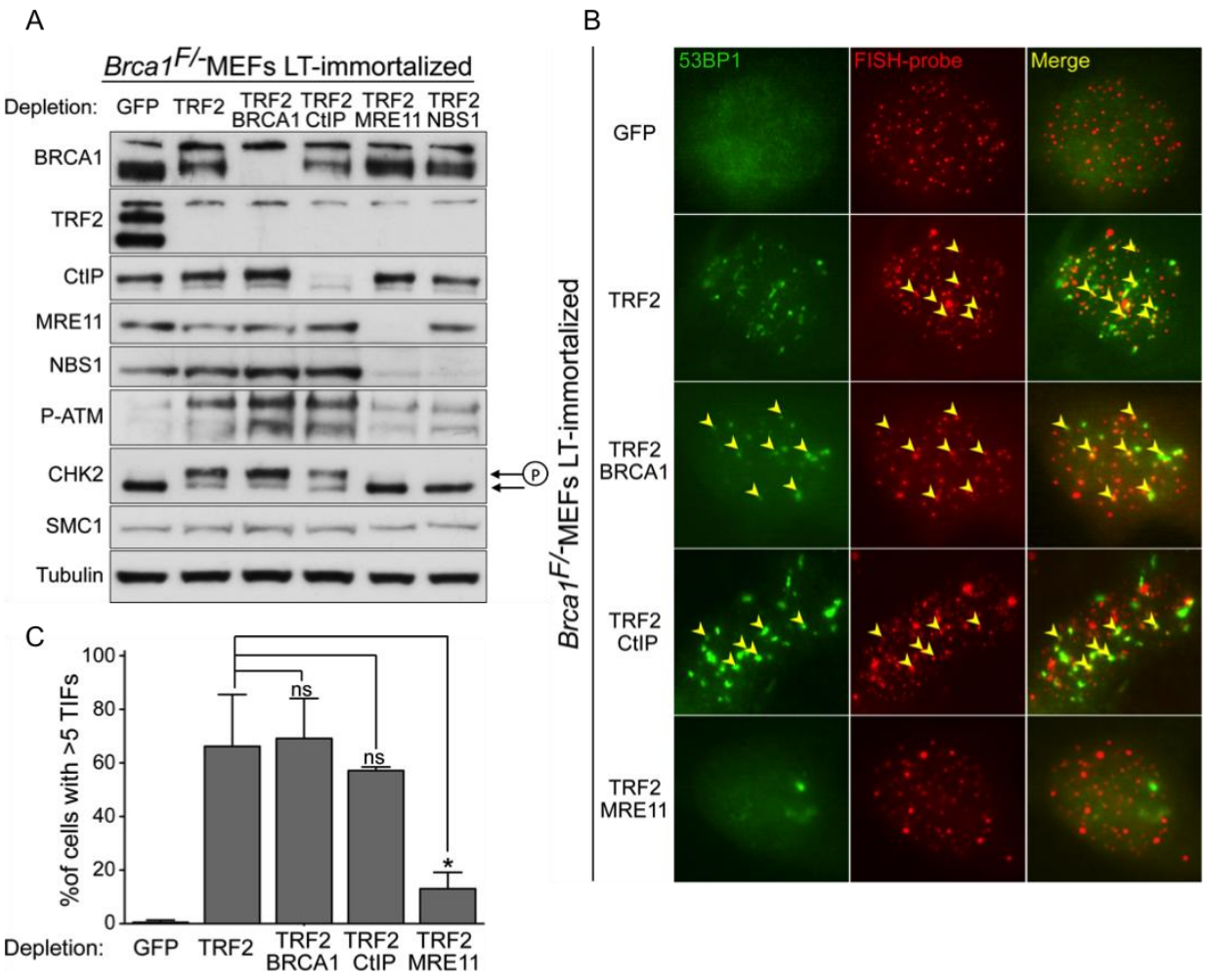


Figure 4.1 - BRCA1/CtIP are not required for ATM-mediated signalling triggered by TRF2 depletion.

A) *Brca1*$^{F/-}$ LT-immortalized MEFs were infected three times with retroviruses expressing the indicated shRNAs or Cre-recombinase, followed by selection with puromycin for 72h. Cell extracts were prepared 5 days after selection and analysed by Western blotting. SMC1 and tubulin were used as loading controls. B) Cells treated as in A) were stained with IF-FISH protocol and the number of TIFs was analysed. 53BP1 antibody was used to label DNA damage foci and telomeric probed to label telomeres. C) Quantification of the IF-FISH staining shown in B). Error bars represent SD of two independent experiments. The statistical significance of the number of TIFs was evaluated using an unpaired two-tailed t-test. ns - not significant; * - *P-value* < 0.05.

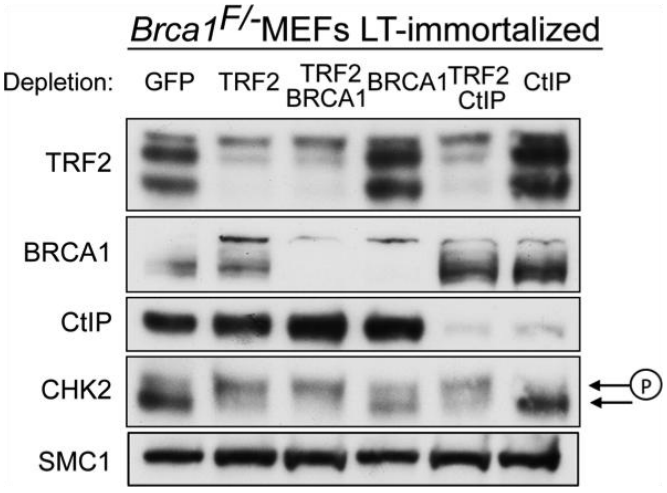


Figure 4.2 - CtIP inhibition alone does not cause CHK2 activation, but only in combination with TRF2 depletion.

$BRCA1^{F/-}$ LT-immortalized MEFs were infected three times with retroviruses expressing the indicated shRNAs or Cre-recombinase, followed by selection with puromycin for 72h. Cell extracts were prepared 5 days after selection and analysed by Western blotting. SMC1 was used as loading control.

4.2.2 BRCA1 and CtIP are required for the telomeric fusions in TRF2-depleted cells

In addition to DDR activation, chromosome end-joining is also a consequence of TRF2 depletion. To address if BRCA1 and CtIP were required for the end-joining of telomeres uncapped by TRF2 inactivation, BRCA1 or CtIP were abrogated together with TRF2. Surprisingly, BRCA1 deletion caused a significant reduction in the number of fusions (Figure 4.3). Whilst in the TRF2-depleted cells the percentage of total fusions is around 20%, in BRCA1-deleted cells there is a reduction of approximately half to two-thirds in the number of fusions (Figure 4.3). A similar phenotype was observed after CtIP depletion. The effect on end-joining was not due to changes in cell proliferation or defects in cell cycle progression (Figure 4.4), since abrogation of BRCA1 or CtIP did not have any additional impact on the number of population doublings or BrdU-positive cells. Moreover, co-inactivation of BRCA1 and CtIP in TRF2-depleted cells did not lead to an additional reduction in the number of fusions (Figure 4.5), suggesting that BRCA1 and CtIP act in the same pathway.

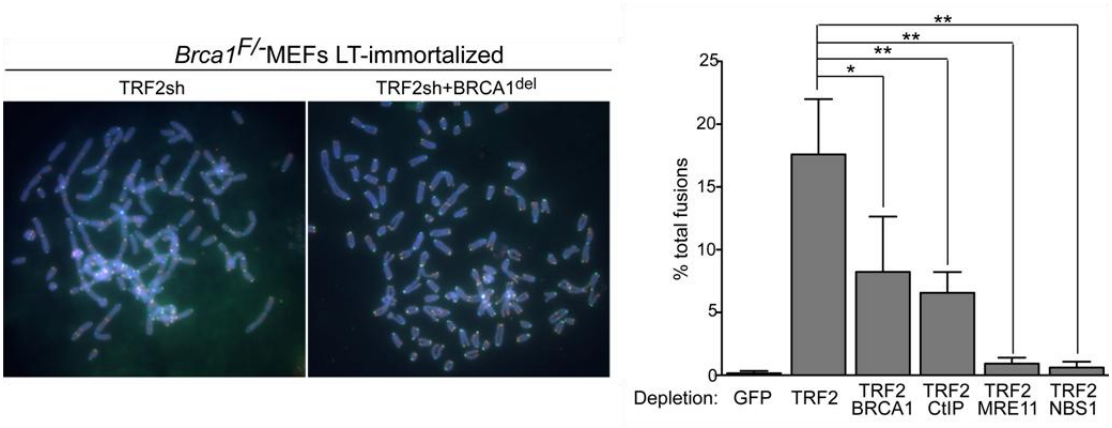


Figure 4.3 - BRCA1 and CtIP are required for the telomeric fusions induced by TRF2 inactivation.

$BRCA1^{F/-}$ LT-immortalized MEFs were infected three times with retroviruses expressing the indicated shRNAs or Cre-recombinase, followed by selection with puromycin for 72 h. Five days after selection cells were treated with colcemid for 4-6 h. Metaphases were harvested and stained with CO-FISH protocol. Quantification of the number of total telomeric fusions in each cell type is shown.

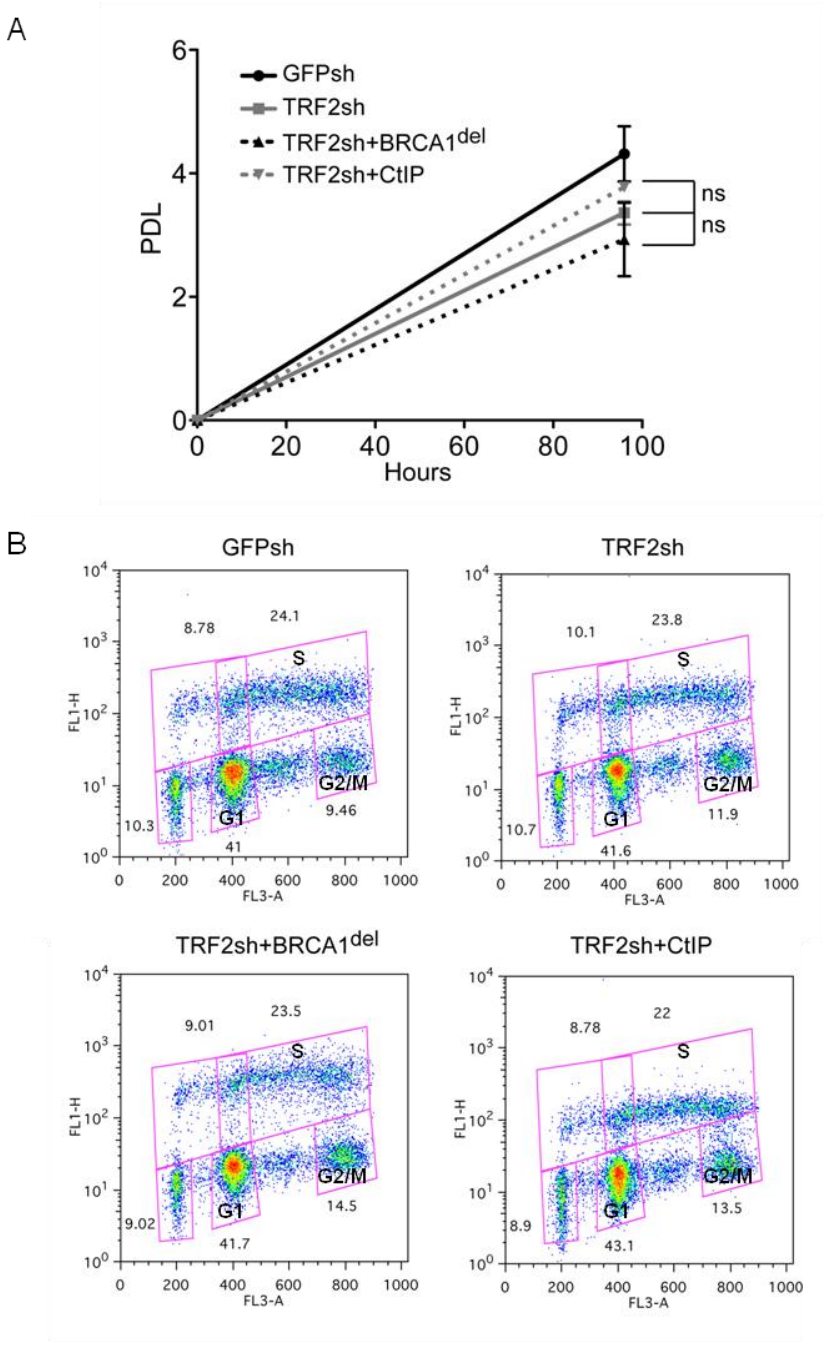


Figure 4.4 – The decrease in chromosome fusions in BRCA1- and CtIP-deficient MEFs lacking TRF2, is not due to changes in proliferation.

A) *Brca1*$^{F/-}$ LT-immortalized MEFs were infected three times with retroviruses expressing the indicated shRNAs or Cre, followed by selection with puromycin for 72 h. After selection, cells were re-plated and proliferation was assessed by using a resazurin proliferation assay. PDL – population doublings. Error bars represent SD of three independent experiments. The statistical significance was evaluated using an unpaired two-tailed t-test. ns - not significant. B) Cell cycle profile of cells treated as in A) at the time of analysis. Cells were pulsed with BrdU 30 min before harvesting. Fixed cells were stained with anti-BrdU antibody and propidium iodide, and then analysed by flow cytometry. The percentage of cells in each stage of the cell cycle is indicated. The cell cycle stage for the predominant polyploidy population is shown.

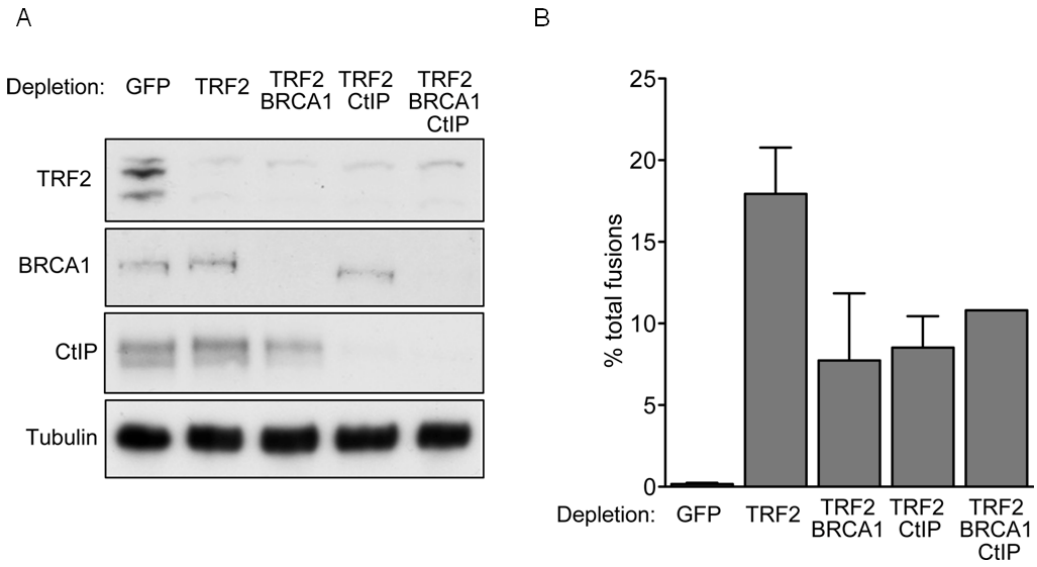


Figure 4.5 - Co-depletion of BRCA1 and CtIP does not have any additive effect in the fusions of TRF2 depleted cells.

A) *Brca1*$^{F/-}$ LT-immortalized MEFs were infected three times with retroviruses expressing the indicated shRNAs or Cre, followed by selection with puromycin for 72 h. 5 days after selection cells were analysed by Western blotting. Tubulin was used as loading control. B) Cells infected as in A) were treated with colcemid overnight and then metaphases were harvested and stained according to Q-FISH protocol. Quantification of the number of total fusions of cells is shown. Error bars represent SD of two independent experiments.

Upon inhibition of the MRN complex, formation of telomeric end-to-end fusions is highly impaired (Deng et al., 2009; Dimitrova and de Lange, 2009). In this context the remaining fusions displayed an increased percentage of chromatid fusions, which involved the leading strand of the telomere (Deng et al., 2009; Dimitrova and de Lange, 2009) (Figure 4.6). Interestingly, BRCA1 and CtIP abrogation also led to an increase in the number of chromatid fusions, from less than 5% in the TRF2-depleted cells to approximately 15-20% after co-inactivation of either BRCA1 or CtIP (Figure 4.6A). This increase in the number of chromatid fusions was even higher when TRF2 was co-depleted with either MRE11 or NBS1 (approximately 50% chromatid fusions). Further analysis of the chromatid fusions revealed that, and contrary to what has been described for MRN complex, the chromatid fusions remaining after BRCA1 or CtIP inactivation involved both the leading and the lagging strand of the telomeres, the lagging strand fusions being slightly more abundant (Figure 4.6B).

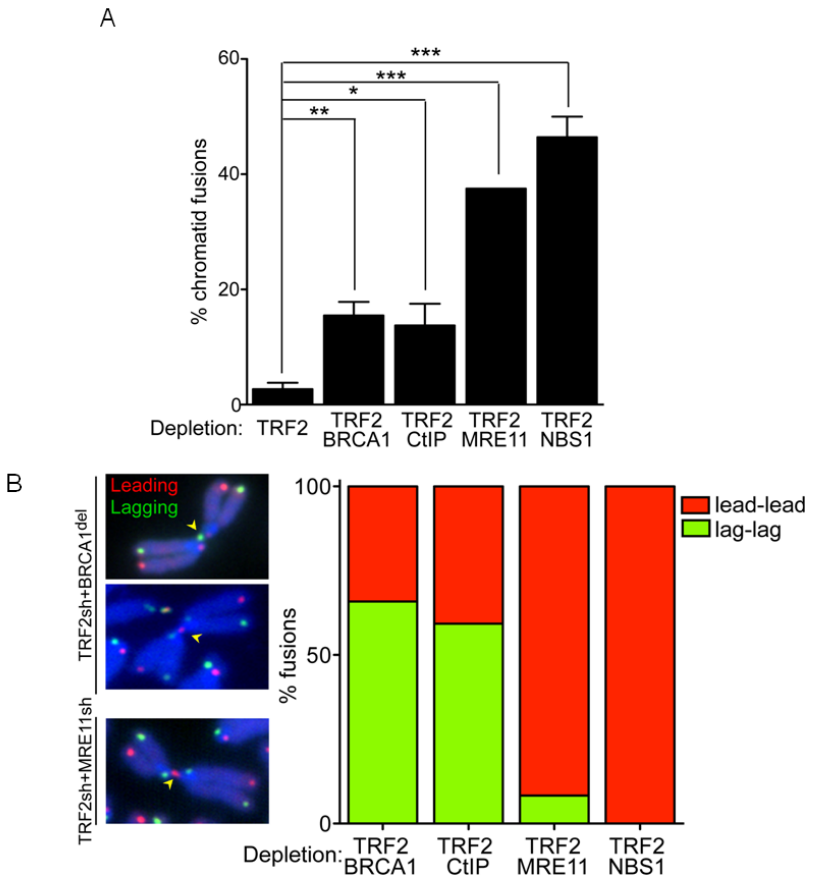


Figure 4.6 - BRCA1 or CtIP inactivation together with TRF2 inhibition changes the chromatid fusions phenotype.

A) *Brca1*$^{F/-}$ LT-immortalized MEFs were infected three times with retroviruses expressing the indicated shRNAs or Cre-recombinase, followed by selection with puromycin for 72 h. Five days after selection cells were treated with colcemid for 4-6h. Metaphases were harvested and stained with CO-FISH protocol. Quantification percentage of chromatid fusions is shown. B) Analysis of the type of chromatid fusions. Lead indicates leading strand fusions and lag, lagging strand fusions. Error bars represent SD of at least three independent experiments. The statistical significance was evaluated using an unpaired two-tailed t-test. ns - not significant; * - *P-value* < 0.05; ** - *P-value* < 0.01; *** - *P-value* < 0.001.

4.2.3 EXO1 is also required for end-joining of uncapped telomeres

After the initial DSB processing, which is mediated by the MRN complex and CtIP, additional proteins including EXO1, DNA2, WRN and BLM, act to promote further resection (Huertas, 2010; Mimitou and Symington, 2009). In order to understand if EXO1, DNA2, WRN and BLM are also required to process telomeres uncapped by TRF2 depletion, TRF2 was depleted in MEFs together with the these proteins. Depletion was assessed by qPCR or Western blotting (Figure 4.7).

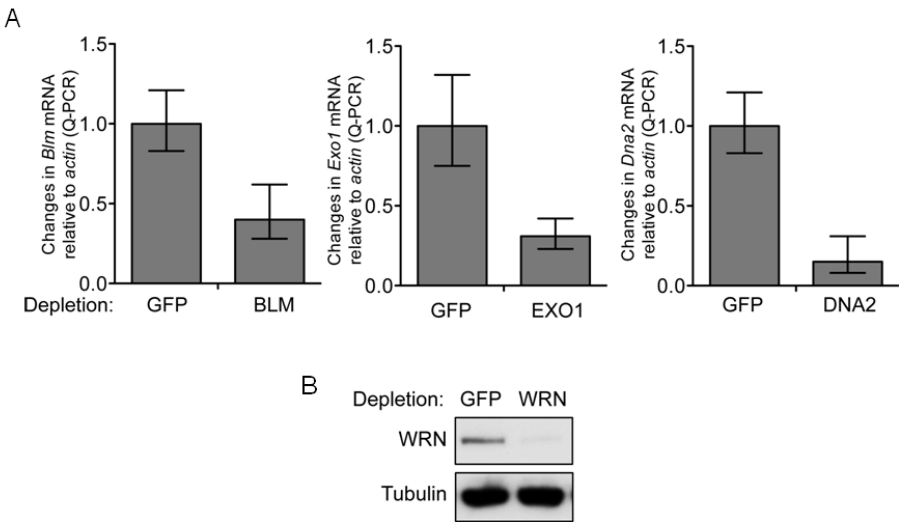


Figure 4.7 - EXO1, BLM, DNA2 and WRN were efficiently depleted from TRF2-depleted cells using shRNAs.

A) WT LT-immortalized MEFs were infected three times with retroviruses expressing the indicated shRNAs, followed by selection with puromycin for 72 h. Five days after selection cells were analysed by Real time qPCR (A) or Western blotting (B). For Real time qPCR, actin was used as endogenous control. Samples were normalized to shGFP control. For Western blotting, tubulin was used as loading control.

Interestingly, only EXO1 inhibition together with TRF2 shRNA caused a significant reduction in the number of telomere fusions when compared to TRF2 depletion alone (Figure 4.8A). The reduction in the number of fusions in the EXO1-depleted cells was not due to changes in the proliferation rate (Figure 4.8B). In contrast, BLM, WRN and DNA2 co-inactivation with TRF2 shRNA led to an increased number of fusions (Figure 4.8A). For BLM and WRN this is consistent with their possible function in telomere capping, because cells deficient for BLM and WRN alone show telomere dysfunction phenotypes including the presence of chromosome fusions (Callen and Surralles, 2004; Crabbe et al., 2007; Crabbe et al., 2004; Du et al., 2004). For DNA2 this suggests that this protein might also play a role in capping telomeres, similarly to WRN and BLM.

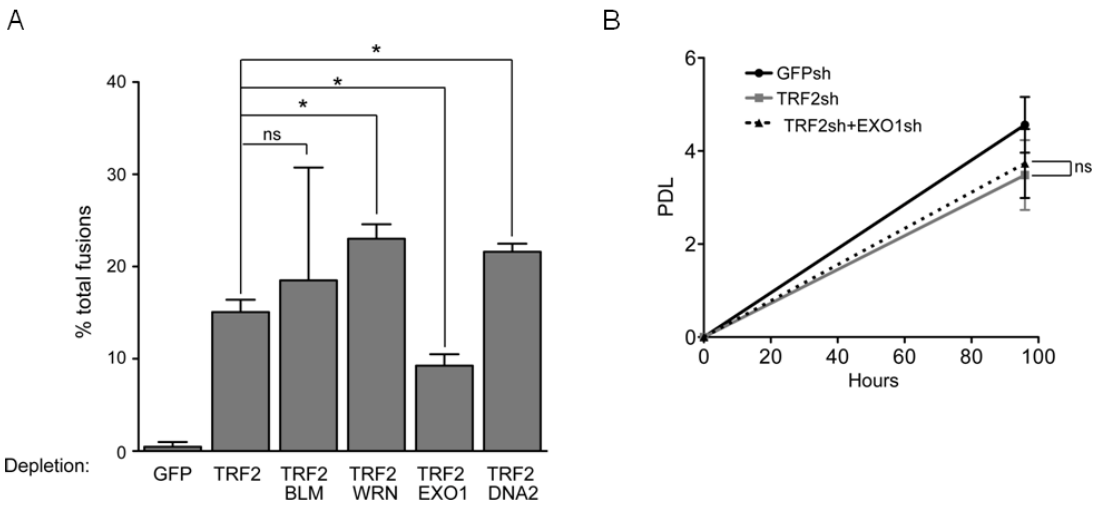


Figure 4.8 - EXO1 but not DNA2, WRN or BLM, is required to process uncapped telomeres.

A) WT LT-immortalized MEFs were infected three times with retroviruses expressing the indicated shRNAs, followed by selection with puromycin for 72 h. Five days after selection cells were treated with colcemid overnight. Metaphases were harvested and stained with Q-FISH protocol. Quantification of the number of total fusions of cells is shown. B) Cells infected as in (A) were plated and the proliferation was assessed using a resazurin-based proliferation assay. PDL represents the number of population doublings. Error bars represent SD of three independent experiments. The statistical significance was evaluated using an unpaired two-tailed t-test. ns - not significant; * - *P-value* < 0.05.

4.2.4 EXO1, but not CtIP, is required to remove the 3' telomeric overhang at uncapped telomeres

BRCA1 and 53BP1 play opposite roles in DNA end resection. While 53BP1 binding to the sites of break prevents resection, BRCA1 is thought to displaced 53BP1 from the break and promote resection, for example by activating CtIP (Bunting et al., 2010). In the absence of both BRCA1 and 53BP1, resection can take place. 53BP1 was also found to prevent the extensive resection mediated by CtIP and BLM at shelterin-free telomeres (Sfeir and de Lange, 2012). In addition, in TRF2-depleted telomeres 53BP1 seems to promote telomere mobility facilitating end-joining (Dimitrova et al., 2008), in a similar way to what has been described for V(D)J recombination, a process involved in the assembly of antigen receptor genes in the developing B and T cells (Difilippantonio et al., 2008). To address how BRCA1 and 53BP1 affect the fusions in the context of TRF2 depletion, BRCA1 and 53BP1 were

co-inactivated in TRF2-depleted cells (Figure 4.9A). The percentage of telomeric fusions obtained when BRCA1 and 53BP1 were abrogated at the same time was similar to the one observed when 53BP1 was depleted alone (Figure 4.9B). This suggested that 53BP1 role in promoting telomere mobility, rather then preventing resection, is more critical for the overall end-joining process.

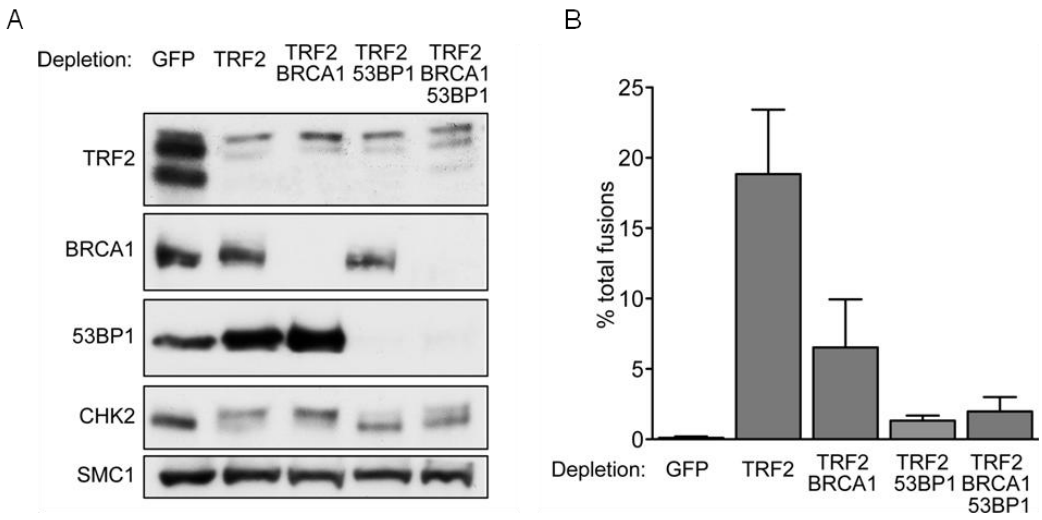


Figure 4.9 - Absence of both BRCA1 and 53BP1 does not restore fusions in TRF2-depleted cells.

A) *Brca1*$^{F/-}$ LT-immortalized MEFs were infected three times with retroviruses expressing the indicated shRNAs or Cre, followed by selection with puromycin for 72 h. Five days after selection cells were analysed by Western blotting. SMC1 was used as loading control. B) Cells infected as in A) were treated with colcemid overnight. Metaphases were harvested and stained using the Q-FISH protocol. Quantification of the number of total fusions of cells is shown. Error bars represent SD of two independent experiments.

To further address the role of 53BP1 in regulating resection, the effect of 53BP1 co-depletion with either CtIP or EXO1 was analysed. As expected, a similar percentage of fusions was detected when 53BP1 was depleted alone or in combination with either EXO1 or CtIP (Figure 4.10B). This suggested that 53BP1 is critical for all fusions to occur and that the remaining fusions in the EXO1- or CtIP-depleted cells may follow the classical NHEJ pathway. When analysing the checkpoint responses, EXO1 inactivation did not abrogate the CHK2 phosphorylation that occurs after TRF2 depletion, similarly to CtIP inactivation (Figure 4.10A). Interestingly, depletion of 53BP1 alone or in combination with CtIP or EXO1 reduced

CHK2 phosphorylation. This is consistent with previous data showing that 53BP1 is required for ATM signalling after IR (Mochan et al., 2003; Peng and Chen, 2003; Wang et al., 2002). Studies from other groups failed to observe this phenotype after telomere uncapping (Dimitrova et al., 2008; Rai et al., 2010), this is most likely due to the model of study used, since both studies were conducted using 53BP1 knockout MEFs.

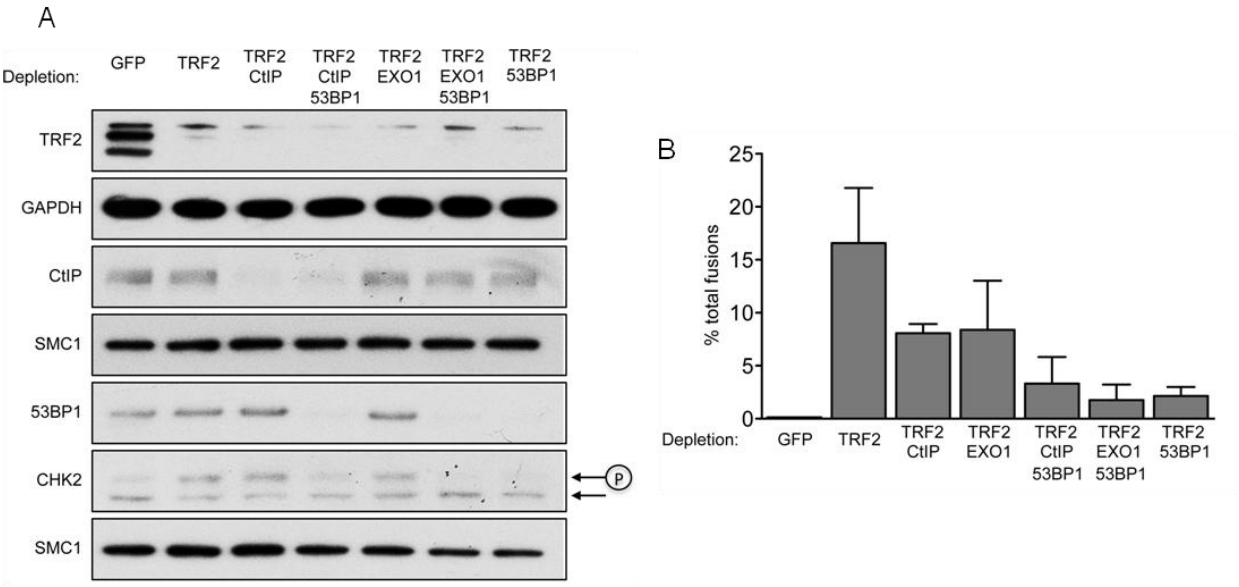


Figure 4.10 – 53BP1 contribution to the end-joining of uncapped telomeres is dominant over CtIP or EXO1.

A) WT LT-immortalized MEFs were infected three times with retroviruses expressing the indicated shRNAs or Cre, followed by selection with puromycin for 72 h. Five days after selection cells were analysed by Western blotting. SMC1 and GAPDH were used as loading control. B) Cells infected as in A) were treated with colcemid overnight. Metaphases were harvested and stained according to the Q-FISH protocol. Quantification of the percentage of total fusions is shown. Error bars represent SD of two independent experiments.

One possible role for CtIP and EXO1 is to remove the 3' overhang after TRF2 abrogation by endonucleolytic incision or exonuclease digestion. The resulting blunt end allows the fusions to occur. It has been recently established that both CtIP and EXO1 play a role in the resection of 'shelterin-free' telomeres (Sfeir and de Lange, 2012). To address this possibility in TRF2-depleted telomeres, the overhang signal was analysed by pulsed field gel electrophoresis (Figure 4.11). Interestingly, EXO1 but not CtIP was required for resection of the 3' telomeric overhang, thus suggesting

that these proteins play different roles in telomeres uncapped by TRF2 abrogation and shelterin-free telomeres. Furthermore, depletion of 53BP1 also prevented the resection of the 3' overhang, as previously described (Dimitrova et al., 2008). Although at telomeres lacking the entire shelterin complex, 53BP1 abrogation leads to extensive resection (Sfeir and de Lange, 2012), this is not the case for TRF2-depleted telomeres (Figure 4.11). Here, when TRF2 was depleted together with 53BP1, no extensive resection was observed, and no additional role for CtIP and EXO1 was detected, as seen by the similar intensity of the overhang signal in GFP-depleted and 53BP1-depleted cells. Together, these results show that the activities of CtIP and EXO1 are different when telomeres are totally uncapped and more similar to DSBs, than when telomeres only lack TRF2. In the first case, both CtIP and EXO1 are required for end-processing (Sfeir and de Lange, 2012), whilst in the second only EXO1 plays a role. CtIP functions during end-joining need to be further investigated. One possibility is that CtIP mediates bridging of telomeric ends, a scenario that has been proposed in yeast during DSBs repair (Clerici et al., 2005). Another possibility is that, like in humans, CtIP only acts to stimulate EXO1 resection activity (Tomimatsu et al., 2012) and what is observed in the double depleted TRF2 and CtIP is only a delayed processing compared to TRF2-depleted cells.

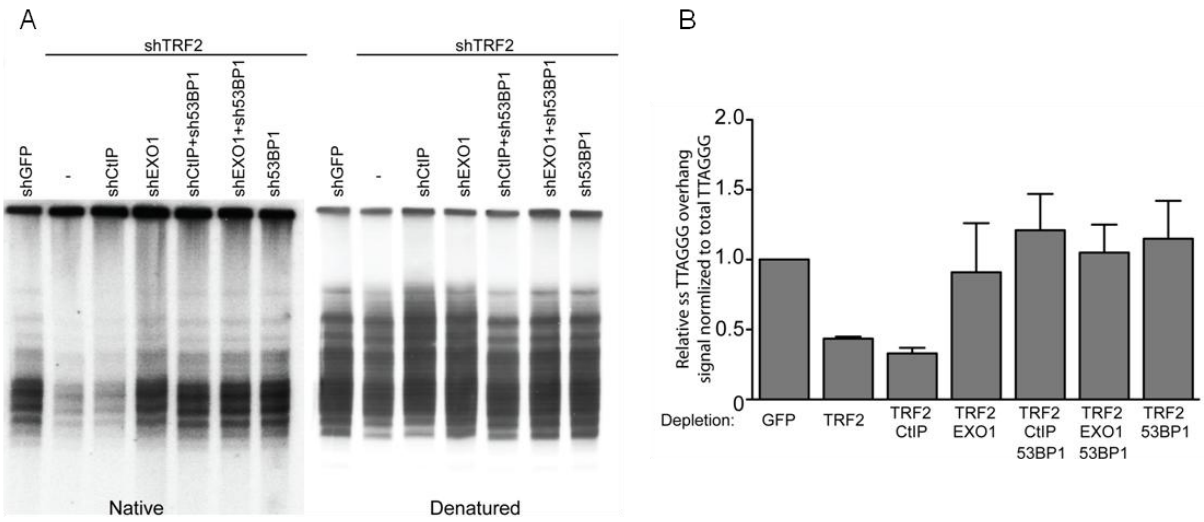


Figure 4.11 – CtIP, but not EXO1, is required to process the 3' telomeric overhang.

A) WT LT-immortalized MEFs were infected three times with retroviruses expressing the indicated shRNAs or Cre, followed by selection with puromycin for 72 h. Five days after selection cells were harvested and were processed for Pulsed field gel electrophoresis and probed with $(AACCCT)_4$ telomeric probe. Representative Pulsed field gel with samples run under native and denatured conditions is shown. B) Quantification of the overhang intensity of cell treated as in A). All samples run under native condition were normalized to the respective sample run under denatured conditions. Cells with various treatments were compared to GFP shRNA-treated sample (defined as 1). Error bars represent SD of two independent experiments.

Chapter 5 Discussion

5.1 siRNA screens as useful tools to unravel new components involved in telomere biology

Although, in the last decades the core telomeric complex, the shelterin, has been defined in great detail (reviewed in Palm and de Lange, 2008), full understanding of telomere biology is still on-going. Apart from shelterin, many other proteins play a role at the chromosome termini, including DNA damage factors such as MRN (Zhu et al., 2000), Ku70/80 (Hsu et al., 1999; Jaco et al., 2004) or BRCA2/RAD51 (Badie et al., 2010; Min et al., 2012). Additional work has also suggested that many other factors are still to be uncovered (Dejardin and Kingston, 2009; Giannone et al., 2010; Kim et al., 2009).

As telomeres are critical for maintenance of genomic stability, a full understanding of their structure and function is vital. For this reason, in the present work siRNA screens were used in order to identify novel factors required for telomere function. Two main aspects were taken into account in this analysis. On one hand, post-translational modifications such as phosphorylation or ubiquitylation, which may impact on the assembly of TRF2 and RAP1, two of the shelterin components, and on the other hand, additional factors that may be required for the DNA damage response triggered after removal of TRF2 from the telomeres.

Both phosphorylation and ubiquitylation events play important roles at the telomeres. For TRF1, several studies have highlighted the impact of these modifications for the stability and activity of this protein (Walker and Zhu, 2012). Phosphorylation of TRF1 by PLK1 is essential for its binding to the telomeres (Wu et al., 2008), while phosphorylation of TRF1 by CDK1 is decisive in the regulation between telomere-bound and free TRF1 (McKerlie and Zhu, 2011). In turn, this regulation impacts on the resolution of the sister chromatids. Furthermore, other

factors including FBX4 and RLIM, contribute to TRF1 ubiquitylation and are involved in its proteasome-dependent degradation (Her and Chung, 2009; Lee et al., 2006b).

Previous studies have underlined the importance of TRF2 in the recruitment of additional factors to the telomeres (Giannone et al., 2010; Kim et al., 2009). However the mechanistic details of these interactions and whether they regulate TRF2 binding to the telomeres still remains elusive. In particular, the interaction between TRF2 and RAP1, which has been long established (Li et al., 2000), has not been fully characterized yet. Moreover, little is known about the impact of TRF2 post-translational modifications on its function or its interaction with binding partners. It is also worth noting that, even though there is only one known isoform of human TRF2, this protein is detected as a doublet by Western blotting (Figure 3.3). Yet, no clear post-translation modification has been ascribed to this feature. So far, TRF2 phosphorylation has only been characterized after DNA damage and both ATM and CHK2 seem to be required for this event (Buscemi et al., 2009; Huda et al., 2009; Tanaka et al., 2005). In addition only ubiquitylation by SIAH1, a p53-inducible E3 ubiquitin ligase, has been linked to the regulation of TRF2 protein stability (Fujita et al., 2010). Thus, this work aimed to further characterize factors required for TRF2 and RAP1 foci formation and also potential mediators of the interaction between TRF2 and RAP1.

To address this, siRNA screens using libraries containing siRNAs targeting both phosphatases and ubiquitylation-related genes were used in this study. The screens generated several potential candidates, which were evaluated in subsequent studies (Figure 3.4, Figure 3.5, Table 3.2). These candidates included factors that only affected RAP1 foci or only TRF2 foci or both (Table 3.2). Factors that affect only RAP1 foci, would help to understand how the interaction between TRF2 and RAP1 is regulated, while factors that affect both TRF2 and RAP1 may potentially be involved in TRF2 regulation. Moreover, TRF2 deletion (Celli and de Lange, 2005; Denchi et al., 2006), depletion (Figure 3.3) or over-expression of a dominant negative form of

TRF2 (Sarthy et al., 2009) not only prevents RAP1 localization to telomeres, but also impacts on its stability. Factors that would only affect TRF2 foci formation will be then less likely to occur. They would potentially be involved in the control of RAP1 stability after TRF2 removal from the telomeres. How RAP1 could be binding to the telomeres in the absence of TRF2 is not intuitive and maybe be explained by its ability to interact with other telomeric accessory proteins (Karlseder et al., 2002; O'Connor et al., 2004; Zhu et al., 2000; Zhu et al., 2003). Nevertheless, in the present work it was possible to identify some potential candidates that only affected TRF2, but not RAP1, localization to the telomeres and the ones with higher z-score values were selected for further analysis.

Although siRNA screens are powerful tools since they allow us to study the impact of the depletion of a vast variety of target genes on a given cellular process, they also have disadvantages including the occurrence of false positive hits. False positive candidates are mainly due to off-target effects of individual siRNAs (Jackson and Linsley, 2010). The off-target effects can arise primarily from either a partial or complete annealing of the siRNA to a non-target mRNA or can be a consequence of the delivery vesicles used. Although initial studies have suggested that siRNAs were highly specific for a given mRNA, when more information became available it became obvious that siRNAs can indeed have suboptimal specificity (Jackson and Linsley, 2010). Moreover, these potential off-target effects can lead to measurable undesirable phenotypes, such a cell death (Fedorov et al., 2006). Indeed, validation of the hits obtained from the screens performed in this work (Figure 3.4, Figure 3.5), clearly demonstrated that the highest-scoring gene, RNF41 (Figure 3.6, Figure 3.8), had an off-target effect (Figure 3.9). One of the siRNAs targeting RNF41 was found to also reduce the levels of TRF2 mRNA (Figure 3.9C), suggesting that this siRNA could possible anneal with the 3' UTR region of TRF2 mRNA reducing its expression, which is known as a common source of off-target effects. Despite this negative result, the screen design proved to have the potential to characterise novel proteins

involved in telomere biology. Other hits obtained from these screens probably deserve a second analysis with siRNAs from different sources. The focus should be on hits that either reduce both RAP1 and TRF2 foci, or RAP1 foci alone. In fact, the secondary screen performed with the candidate hits suggests that UFM1, an ubiquitin-like modifier (Figure 3.6), should be present in further studies. Interestingly, UFM1 depletion impacts on the levels of RAP1 foci alone. In spite of the fact that initial studies have not succeeded in the validation of this hit, designing additional siRNAs can potentially reveal whether it is a bona fide player in RAP1 foci formation or stability of RAP1 protein.

5.2 siRNA screens as tools to compare the general DNA damage response to the one triggered from uncapped telomeres

A key aspect of genomic stability is the maintenance of telomere integrity. Telomere dysfunction can arise primarily from either telomere uncapping (i.e. removal of shelterin components or other accessory telomeric factors) or telomere shortening (reviewed in Palm and de Lange, 2008). TRF2 is a key component of the shelterin complex and its removal from telomeres triggers activation and recruitment of DNA damage factors to the telomeres (d'Adda di Fagagna et al., 2003; Takai et al., 2003). Curiously, several of these factors play important roles in the maintenance of telomere integrity, including the MRN complex (Zhu et al., 2000) and Ku70/80 (Hsu et al., 1999; Jaco et al., 2004). This raises the question of how is the balance between telomere protection and repair managed by the DNA repair components. To address whether there are different components of the DDR activated after telomere dysfunction or after general DNA damage, a cell line harbouring an inducible TRF2 shRNA was established in this study (Figure 3.10). This cell line provides a useful tool to identify new proteins involved in the DDR triggered after TRF2 depletion by

quantification of TIFs. The suitability of this cell line was verified by the fact that siRNAs targeting 53BP1, ATM and γH2AX and which were present in the DNA repair library, reduced the number of TIFs (Figure 3.15). All these factors are key DNA damage proteins known to be recruited to the telomeres when they become dysfunctional (d'Adda di Fagagna et al., 2003; Takai et al., 2003).

At the same time as this study, Lackner and co-workers have also shown the feasibility of siRNA screens using TIFs as a readout (Lackner et al., 2011). In this work, the authors focused on factors that increased the number of TIFs, using TRF2 depletion by siRNA as a control. This screen allowed them to identify a new factor, the splicing factor and transcriptional co-activator SKIIP, which produced a weak TIF phenotype when depleted. Furthermore, the depletion of this gene also produced non-telomeric DNA damage, similarly to what was found in the work presented here through the depletion of DTL (Figure 3.7). Once more, this points to the similarities between the general DNA damage response and the one triggered by dysfunctional telomeres. The screen also allowed the identification of new candidates required for the DDR triggered by deprotected telomeres, including the chromatin-remodelling factor CHD3, histone methyltransferase SUV39H2 and tumour suppressor TSC1 (Figure 3.15, Figure 3.16). However, further validation with siRNAs distinct from the ones in the library failed to demonstrate the requirement of these factors on the DDR (Figure 3.18). This reiterates the importance of the validation with several different siRNAs, in order to confirm the requirement of a candidate gene for the pathway under study.

DDRs triggered by introducing DNA damage anywhere in the chromosomes or specifically at telomeres are expected to share some similarities (reviewed in d'Adda di Fagagna et al., 2004; Palm and de Lange, 2008). However, some studies have shown that differences between these two responses can occur, either due to distinct chromatin modifications (Carneiro et al., 2010), or different organization of the DDR cascade (Peuscher and Jacobs, 2011) or distinct signals for checkpoint activation

(Thanasoula et al., 2012). Altogether, this points to the necessity of further studying the loss of telomere integrity, in order to increase our understanding of the mechanisms that trigger genomic instability.

5.3 DDR factors BRCA1 and CtIP are required for telomeric end-to-end fusions

Telomere dysfunction not only triggers activation of the DNA damage signalling pathway mediated by ATM and ATR, but also leads to repair through mechanisms including classical-NHEJ, alternative-NHEJ and homologous recombination (de Lange, 2009). Due to the resemblance between uncapped telomeres and DSBs, it is likely that the processing might occur similarly in both cases. Moreover, specific shelterin components can act to suppress a given DNA damage pathway, although some components have been shown to be involved in the protection against more than one type of DNA repair (reviewed in de Lange, 2009). For example, POT1 was first characterized as a telomeric factor required for protection against HR (Palm et al., 2009; Wu et al., 2006). Nonetheless, recent studies have implicated POT1/TPP1 complex also in suppression of A-NHEJ (Rai et al., 2010). Similarly, TRF2 has been shown to prevent HR (Celli et al., 2006; Wang et al., 2004) and also C-NHEJ (Celli and de Lange, 2005; Konishi and de Lange, 2008; Rai et al., 2010) at the telomeres. Interestingly, recent studies demonstrated that suppression of HR by TRF2 is in fact a consequence of RAP1 removal from telomeres (Sfeir et al., 2010), thus suggesting that one telomeric factor can inhibit multiple DNA damage pathways at the telomeres.

One critical aspect of telomere end-joining is DNA processing of chromosome ends. Processing of DSBs requires different proteins depending on which pathway acts to repair the break. HR involves extensive resection of DNA ends mediated by

several proteins including MRN, CtIP, EXO1, DNA2 and the RecQ helicases, WRN and BLM and leading to formation of ssDNA tails. In contrast, during C-NHEJ prevails the repair without processing (reviewed in Bernstein and Rothstein, 2009; Hiom, 2010; Mimitou and Symington, 2009). If processing occurs during C-NHEJ, it is thought to be mediated by Artemis, which creates blunt-ends at the break site (Ma et al., 2004; Pawelczak and Turchi, 2010). A-NHEJ, on the other hand, requires processing in order to repair the break (McVey and Lee, 2008). In particular MMEJ requires limited resection in order to expose the microhomology sequences, which will then allow the repair of the break. This resection requires DNA damage factors including MRN and CtIP (McVey and Lee, 2008).

Analysis of telomeres uncapped through TRF2 removal has revealed that several DNA repair proteins including ATM (Denchi and de Lange, 2007), MRN complex (Deng et al., 2009; Dimitrova and de Lange, 2009) and XPF (Zhu et al., 2003) are required for processing of the 3' overhang in order to allow end-joining to occur. Although other factors, such as CtIP, BRCA1 or EXO1, have also been implicated in DSB resection and subsequent repair (Bernstein and Rothstein, 2009; Mimitou and Symington, 2009), their role in the end-joining after TRF2 abrogation has not been addressed yet.

In the present study, shRNA-mediated depletion of TRF2 revealed that both BRCA1 and CtIP are required for telomeric end-joining (Figure 4.3). Classically both BRCA1 and CtIP have been associated to repair mediated by HR (You and Bailis, 2010). CtIP is phosphorylated in CDK-dependent manner during the S and G2 phases of the cell cycle, promoting its binding to BRCA1 and MRN complex and allowing resection to occur (Chen et al., 2008; Yu and Chen, 2004). Resection is active in S phase and stimulates HR, also through the availability of a DNA template molecule for strand invasion reactions (You and Bailis, 2010). More recently, both CtIP and BRCA1 have been linked to repair by MMEJ (Yun and Hiom, 2009) and previous studies have already suggested that BRCA1 also played a role in end-

joining mechanisms (Zhong et al., 2002a; Zhong et al., 2002b). The fact that BRCA1 and CtIP are required for telomere end-joining (Figure 4.3), suggests that, in addition to C-NHEJ, other repair pathways such as HR or A-NHEJ, are acting at the chromosome termini after TRF2 abrogation to generate fusions. Interestingly, these proteins are not required for the end-joining of all the telomeres but only between 50-70% of them, once more suggesting that multiple pathways must act to repair uncapped telomeres. Moreover, the inactivation of CtIP and BRCA1 also led to an increase in the proportion of chromatid fusions (Figure 4.6). These fusions have been shown to be characteristic of S phase since they require the presence of the sister chromatid or homologous chromosome to occur (Dimitrova and de Lange, 2009; Konishi and de Lange, 2008). An increase in the number of these fusions may suggest that CtIP and BRCA1 are required to process uncapped telomeres, perhaps following their replication, in the S/G2 phases of the cell cycle.

5.4 EXO1 involvement in telomeric fusions and possible connection with A-NHEJ mechanisms

To address if CtIP and BRCA1 mediate repair of dysfunctional telomeres through an HR-related mechanism, the role of EXO1, DNA2, WRN and BLM in telomeric end-joining was also assessed. Interestingly, only EXO1 abrogation decreased the number of fusions after TRF2 depletion, similarly to the effects of BRCA1 and CtIP inactivation (Figure 4.8). This implies that a very specific pathway acts to process telomeric DNA ends.

Whilst in yeast the role of EXO1 has been extensively characterized (Tran et al., 2004), in mammalian cells EXO1 role in processing DSBs has only recently been addressed. Similar to what has been described in yeast, in human cells two pathways act to allow extensive resection: one that is dependent on EXO1 and the

other that involves primarily BLM/WRN and DNA2 (Nimonkar et al., 2011). In the EXO1-dependent pathway, this nuclease can either act alone to resect the break or its activity can be further stimulated by BLM (Nimonkar et al., 2011; Nimonkar et al., 2008; Zhu et al., 2008). In addition, human EXO1 resects DNA breaks, although less efficiently, even in the absence of MRN-CtIP complex, suggesting a more central role for this protein in the DNA repair in humans (Tomimatsu et al., 2012). Furthermore, the nuclease activity of EXO1 seems to be limited by CtIP, which can be an important mechanism to control over-resection (Eid et al., 2010).

Data from the present study suggests a mechanism of telomere resection by EXO1 that is independent of BLM (Figure 4.8), pointing to a pathway of resection that does not follow the canonical HR mechanism. Furthermore, yeast EXO1 has also been implicated in repair by MMEJ (Decottignies, 2007; Lee and Lee, 2007), but its role in mammalian MMEJ has not yet been addressed. The data presented in this thesis strongly suggests an alternative mechanism of end-joining acting at telomeres uncapped by TRF2 depletion. With this work, it was shown that not only MRN complex but also BRCA1, CtIP and EXO1 are required for telomeric fusions after TRF2 depletion (Figure 4.8, Figure 4.3). Apart from EXO1, all the other proteins have been linked to A-NHEJ mechanisms (Quennet et al., 2011; Xie et al., 2009; Yun and Hiom, 2009). Interestingly, several of the proteins implicated in telomere fusions have also been linked to class switch recombination (CSR), a process essential to create the diversity of existing antibodies and which has been associated with A-NHEJ repair mechanisms (Yan et al., 2007). MRN complex facilitates A-NHEJ during generation of CSR intermediates (Dinkelmann et al., 2009), although it still remains unclear whether it does so by first tethering the DNA ends and then promoting processing of DNA termini. Similarly, 53BP1, which is essential for telomeric fusions (Dimitrova et al., 2008), has also been described to facilitate class switch recombination (Difilippantonio et al., 2008; Manis et al., 2004). More recently, CtIP has been shown to promote MMEJ during CSR, although its precise role remains

unclear (Lee-Theilen et al., 2011). Interestingly, EXO1 deletion also leads to reduced levels of CSR (Bardwell et al., 2004), which is the closest involvement of EXO1 in A-NHEJ mechanisms in mammalian cells. The data presented here together with the similarities between CSR and telomeric end-joining (i.e. the requirement in both processes of end-joining of distant chromosomal regions), strongly suggest that EXO1 is also required for A-NHEJ at the telomeres.

Addressing the requirement of other factors associated with A-NHEJ, primarily Ligase III (Audebert et al., 2004; Sfeir and de Lange, 2012; Wang et al., 2006), will to establish the involvement of A-NHEJ after TRF2 depletion. In addition, although the role of Ligase IV in C-NHEJ has long been demonstrated (reviewed in Lieber, 2010), this ligase has also been associated to CSR (Han and Yu, 2008; Roddam et al., 2002), a process that requires the A-NHEJ pathway (Yan et al., 2007). Furthermore, studies have revealed that not all the C-NHEJ components are required for CSR; while Ku70/80, Ligase IV and also its interacting partner XRCC4 play a role in CSR (Casellas et al., 1998; Han and Yu, 2008; Manis et al., 1998; Roddam et al., 2002; Soulas-Sprauel et al., 2007), Artemis and DNA-PK do not seem to be required for this type of recombination (Bosma et al., 2002; Kiefer et al., 2007; Rooney et al., 2005). This further highligths the multiplicity of pathways involved in DNA repair or recombination processes. A recent study using MEFs where telomeres are totally devoid of a capping structure, has shown that there are distinct requirements for Ku70/80 and Ligase IV in the formation of end-to-end fusions (Sfeir and de Lange, 2012). While Ligase IV deletion leads to reduction in the frequency of telomeric fusions from approximately 80% to around 10%, MEFs where Ku80 has been deleted still show 65% fusions. The remaining fusions in this background are dependent on Ligase III and PARP-1, both implicated in A-NHEJ mechanisms (reviewed in McVey and Lee, 2008). Altogether, these results suggest that, when completely uncapped, the telomeres are processed and ligated through both C-NHEJ and A-NHEJ. Surprisingly, however resembling what was described for CSR,

telomeric fusions can rely on Ligase IV as the main factor involved in the ligation step regardless of C- or A-NHEJ being the pathway engaged in repair.

Consistent with this diversity of end-joining pathways at telomeres the present study establishes that apart from C-NHEJ, an alternative end-joining mechanism acts to repair TRF2-devoided telomeres. This is indicated by the requirement of factors such as CtIP, BRCA1 and EXO1 in the end-joining of the chromosome termini (Figure 4.8, Figure 4.3). Altogether, this points out not only the complexity of repair pathways present in a cell, but also the importance of having multiple mechanisms that allow the rapid re-establishment of normal function of vital processes such as CSR or telomere maintenance.

5.5 CtIP and EXO1 play different roles in telomeric end-joining

A key step in DSB repair is resection of the DNA break (reviewed in Bernstein and Rothstein, 2009; Hiom, 2010; Huertas, 2010; Mimitou and Symington, 2009). This is a 5'-3' process that allows the generation of 3' overhangs, which are required for the recombinogenic reactions during HR-mediated repair. This mechanism is known to be dependent on several nucleases and helicases including EXO1 and CtIP, both acting to promote 5'-3' resection. Although both EXO1 and CtIP have been implicated in DSB resection, their interdependence and their requirement in limiting the extent of resection is not fully understood. In *S. cerevisiae*, Sae2 (CtIP homolog) mutants show slower DSB repair, this mechanism is not completely abrogated (Clerici et al., 2005). Interestingly, and even though Sae2 role as nuclease has been established, in Sae2 mutants, resection is delayed, but not impaired (Clerici et al., 2005). In addition, the resection products in these mutants are stable for longer than in WT cells, suggesting additional roles for Sae2 in the DSB repair. Interestingly, Sae2 have also been implicated in bridging of the DNA broken ends

(Clerici et al., 2005), although whether it does so directly or by affecting the MRX function is still not clear.

Furthermore, analysis of *exo1* mutants in yeast also showed that repair is only slightly affected (Mimitou and Symington, 2008), while in *sgs1/exo1* double mutants the DSB processing is substantially compromised (Mimitou and Symington, 2008; Zhu et al., 2008). Moreover, both Sae2 and MRX complex are required only to accelerate the rate of initiation of resection, but this process can still occur in their absence (Mimitou and Symington, 2008; Zhu et al., 2008). This further supports the presence of a two-step resection mechanism, where resection is initiated by MRX-Sae2 and continued by Exo1, Sgs1 and Dna2 (reviewed in Mimitou and Symington, 2009).

In vertebrates, CtIP inactivation impairs 5'-3' resection, thus preventing ssDNA formation and subsequent RPA recruitment (Huertas and Jackson, 2009; Sartori et al., 2007; You et al., 2009). Even though CtIP is essential for resection during HR, pathways involving EXO1 and RecQ homologs, WRN and BLM, are also required for repair (Gravel et al., 2008; Nimonkar et al., 2011; Nimonkar et al., 2008). This suggests that CtIP by itself cannot efficiently promote resection of DSBs.

Interestingly, while in *S. cerevisiae* the recruitment of Exo1 to the sites of break is independent of Sae2 (Shim et al., 2010), in mammalian cells EXO1 has recently been shown to be recruited to DSBs in a CtIP- and MRN-dependent manner (Eid et al., 2010). The same study also suggests that CtIP may act to restrain EXO1 nucleolytic activity, thus preventing extensive resection. In addition, even though Sae2 has been shown to act directly as a nuclease (Lengsfeld et al., 2007), the data currently available for CtIP has failed to show unequivocally this connection. Additionally, CtIP stimulates MRE11 endonucleolytic activity (Sartori et al., 2007), which suggests that CtIP role might be to regulate resection rather than acting directly as a nuclease.

While during DSB repair the generation of 3' ssDNA tails is a critical step that allows the subsequent healing of the break, the presence of such 3' tails is a characteristic of the chromosome termini. Furthermore, one critical step in the formation of telomeric end-to-end fusions is the processing of the 3' telomeric overhang. So, in contrast to DSB repair, the processing of an uncapped telomere does not rely primarily on 5'-3' resection, but is linked to the removal of the 3' overhang.

Even though, the MRN complex has been previously implicated in the processing of the telomeric overhang (Deng et al., 2009; Dimitrova and de Lange, 2009), the roles of CtIP and EXO1 have not been addressed yet. Intriguingly, the present work shows that in TRF2-depleted telomeres CtIP and EXO1 play distinct roles in processing of the 3' overhang (Figure 4.11). While EXO1 depletion prevents excision of the overhang, CtIP abrogation allows this processing to occur, despite the fact that both EXO1 and CtIP are required for end-joining. One possibility is that CtIP plays an additional role during fusion establishment, other than resection. Furthermore, one can speculate that similarly to Sae2 (Clerici et al., 2005), the CtIP role in telomere fusions mediated by A-NHEJ, might occur through bridging of the two dysfunctional telomeres. Another possible explanation is that CtIP only acts to stimulate resection mediated by EXO1, a mechanism that has been previously suggested to occur in human cells (Tomimatsu et al., 2012). In this context and comparing the same time point, TRF2-depleted cells are expected to harbour more fusions than doubly-depleted TRF2 and CtIP cells, as a consequence of faster processing. In the later, the overhang would still be resected, but with slower kinetics and this resection product in the absence of CtIP could be more stable, similarly to yeast (Clerici et al., 2005). This can further explain the simultaneous absence of overhang and fusions at the analysed time point. In addition, the pulsed field electrophoresis used in this study does not allow the visualisation of short resection tails and thus it is not possible to exclude that CtIP might mediate resection of short

stretches of DNA. Analyses of microhomologies at the fusion sites in the presence and absence of CtIP will also allow to establish whether TRF2-depleted MEFs show the characteristic microhomology sequences found after MMEJ (McVey and Lee, 2008) and whether these sequences are CtIP-dependent. In late generation telomerase deficient mice, microhomology sequences were found at the telomere junction sites (Hemann et al., 2001). These mice harbour very short telomeres and no telomeric DNA was found at the sites of fusion, suggesting a mechanism of end-joining that requires erosion of telomeric DNA. In human cells, very short telomeres tend to fuse and the fusion sites are characterised by the deletion of one or both telomeres and by the presence of microhomologies at the fusion point (Capper et al., 2007). The same study revealed that after siRNA-mediated depletion of TRF2, the fusion sites are flanked by telomeric sequences, suggesting the absence of extensive telomere DNA deletions, although the presence of microhomologies cannot be excluded. Furthermore, analysis of telomeric fusions using fusion PCR and allele-specific single-telomere length analysis (STELA), was carried out only for two chromosomes, XpYp and 17p, thus raising the possibility that fusions occurring between other chromosomes might involve additional processes. Moreover, EXO1 and CtIP are not required for all the fusions observed after TRF2-abrogation in MEFs (Figure 4.3, Figure 4.8, Figure 4.10), which can suggest that different chromosome ends might be processed through different mechanisms. Whether this is related with distinct processing at different cell cycles stages or with specific chromosome sequences still remains to be determined.

CtIP and EXO1 are only partially required for the extensive resection in telomeres simultaneously lacking the shelterin complex and 53BP1 (Sfeir and de Lange, 2012), thus suggesting that both factors can process uncapped telomeric DNA. In cells where TRF2 and 53BP1 are simultaneously depleted, neither CtIP nor EXO1 have any additional effect (Figure 4.10) and the absence of telomeric fusions might be explained by the inability to process the 3’ overhang. In this scenario,

53BP1 could be required for the initiation of the processing and not only to prevent extensive resection from occurring.

In conclusion, while EXO1 is a new factor required for the 3’ overhang processing that allows telomeric end-joining, CtIP role does not seem to be directly linked to resection. Analysis, of EXO1 and CtIP doubly-depleted cells will certainly provide more information about the interplay of these two proteins at dysfunctional telomeres.

5.6 Multiple pathways act at telomeres uncapped through TRF2 depletion

In addition to the classical pathways of DSB repair, HR and C-NHEJ, the so-called alternative repair pathways have received great attention in the recent years (Iliakis, 2009; McVey and Lee, 2008; Nussenzweig and Nussenzweig, 2007). In fact, A-NHEJ is required for essential processes such as CSR or V(D)J recombination. Several mechanisms that lead to telomeric end-to-end fusions have been described (de Lange, 2009). The presence of telomeric fusions as a consequence of telomere dysfunction, presents a clear threat to genomic integrity. Understanding better how uncapped telomeres are processed and fused will unravel new mechanisms that normally sustain genomic stability and will provide a model to dissect distinct DSB repair pathways. It is established that, following TRF2 depletion, several C-NHEJ components are critical for telomeric fusions to occur (Celli et al., 2006; Smogorzewska et al., 2002). In addition, factors such as XPF (Zhu et al., 2003), MRN (Deng et al., 2009; Dimitrova and de Lange, 2009), RNF8 (Peuscher and Jacobs, 2011; Rai et al., 2011), 53BP1 (Dimitrova et al., 2008), BRCA1, CtIP and EXO1 (this study) also participate in the formation of telomeric fusions.

Thus, it is proposed here that re-joining of TRF2-depleted telomeres may occur through two main pathways: one that follows the C-NHEJ mechanism (Figure 5.1, Left) and another that requires additional proteins including BRCA1, CtIP and EXO1 (Figure 5.1, Right) and is most likely an A-NHEJ reaction. Both pathways can occur simultaneously at telomeres uncapped through TRF2 depletion. Importantly, Ku70 interacts with TRF2 (Song et al., 2000) and inhibits A-NHEJ at telomeres (Sfeir and de Lange, 2012). Therefore, in the absence of TRF2, Ku70/80 may be removed from telomeres, rendering them more prone to A-NHEJ mechanisms. TRF2 removal has also been suggested to lead to a partial removal of POT1 from telomeres (Denchi and de Lange, 2007; Yang et al., 2005). Since POT1 is another known repressor of A-NHEJ at telomeres (Rai et al., 2010), this further suggests that TRF2-deficient

telomeres are prone to A-NHEJ mechanisms. The precise role of CtIP and EXO1 in this pathway requires further studies. One possibility is that CtIP acts to stimulate EXO1-mediated resection and bridging of DNA ends. In addition CtIP can act after the overhang removal in order to generate short overhangs characteristic of MMEJ (McVey and Lee, 2008). EXO1 is a 5'→3' exonuclease, as well as a flap endonuclease (Lee and Wilson, 1999). Thus, its role in uncapped telomeres processing by A-NHEJ is either to further resect the 5' end of the C-rich telomeric strand, allowing microhomology search, or creating a 3' overhang that could become the substrate for other flap nucleases, such as ERCC1/XPF, previously implicated in uncapped telomeres processing (Zhu et al., 2003). In addition, in yeast Exo1 has been shown to act in a bidirectional resection process together with MRX complex (Garcia et al., 2011). In this case Mre11 first nicks the strand to be resected and second promotes resection in a bidirectional manner; while Exo1 resects away from the break in a 5'-3' direction, Mre11 resects in a 3' to 5' direction towards the break. This scenario can also occur at uncapped telomeres after TRF2 removal. EXO1 and MRE11 can act together to promote overhang removal in a process that involves a first step that generates a nick in the DNA and then further resection that promotes overhang removal and repair by alternative end-joining mechanisms. Alternatively, EXO1 could act itself primarily as a flap endonuclease to promote excision of the 3' overhang. The exact mechanism of A-NHEJ remains unclear, an example currently being addressed is the interplay between LIG3 and LIG4 in the final stage of repair. Unravelling this will allow to further understand how cells solve the loss of telomere integrity problem and uncover a key step in the progression to tumorigenesis.

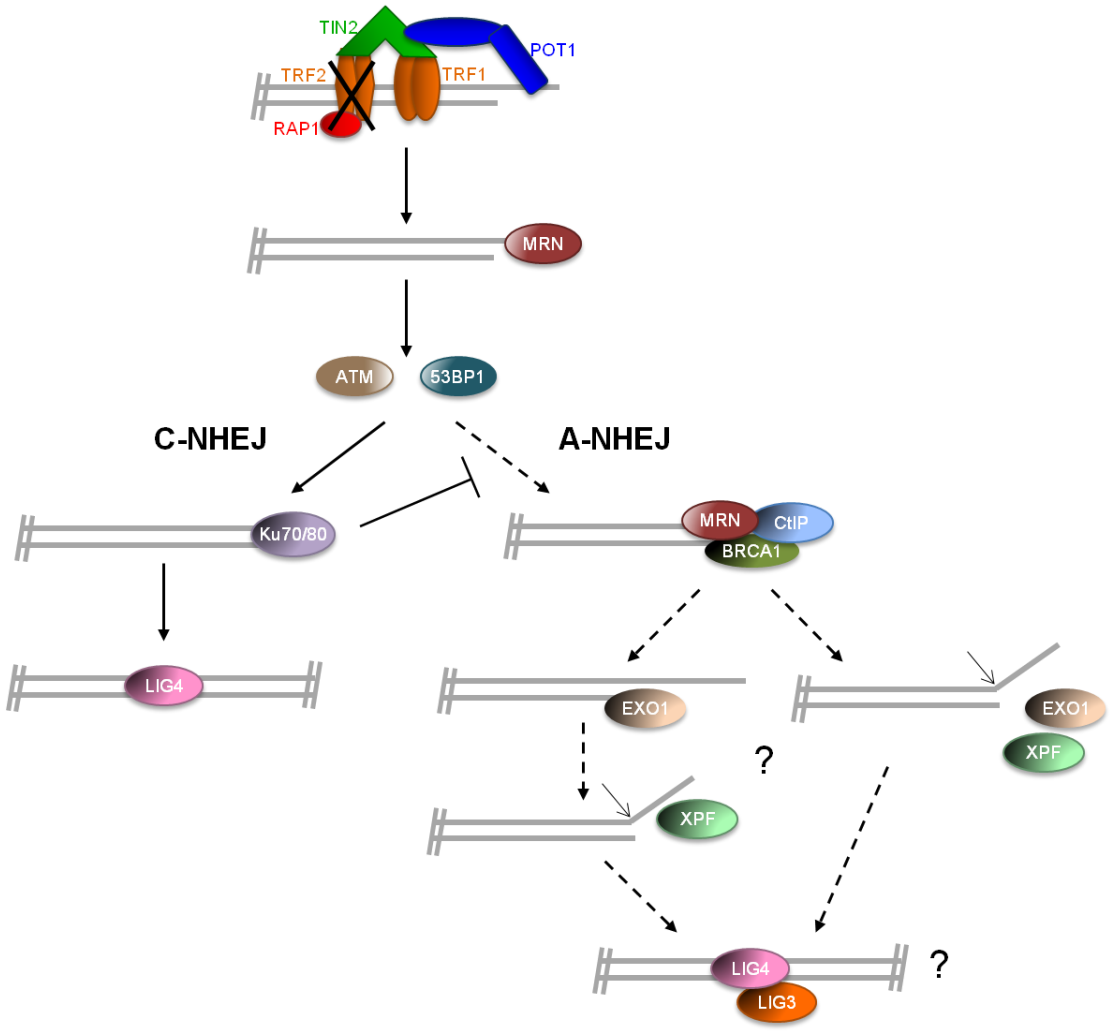


Figure 5.1 – Multiple pathways act to repair telomeres uncapped through TRF2 depletion.

TRF2 depletion leads to recruitment of MRN complex, activation of ATM and 53BP1 localization to telomeres, which facilitate repair either through C-NHEJ (Left) or A-NHEJ (Right). C-NHEJ is a well-established repair pathway, known to require factors such as the heterodimer Ku70/80 and LIG4. A-NHEJ is a less defined mechanism, known to be dependent on several proteins. The MRN-BRCA1-CtIP complex may be required for bridging the ends and initiation of resection. Resection could be extended by EXO1 until microhomology sequences are found. Before ligation, the ssDNA flaps must be removed, possibly by XPF/ERCC1. In addition, EXO1 can act as a flap nuclease itself, promoting excision of the 3' overhang. Finally, ligation could be mediated by LIG4 or LIG3 or both.

References

Adair, G. M., Rolig, R. L., Moore-Faver, D., Zabelshansky, M., Wilson, J. H., and Nairn, R. S. (2000). Role of ERCC1 in removal of long non-homologous tails during targeted homologous recombination. Embo J *19*, 5552-5561.

Ahmad, A., Robinson, A. R., Duensing, A., van Drunen, E., Beverloo, H. B., Weisberg, D. B., Hasty, P., Hoeijmakers, J. H., and Niedernhofer, L. J. (2008). ERCC1-XPF endonuclease facilitates DNA double-strand break repair. Mol Cell Biol *28*, 5082-5092.

Alexander, A., Cai, S. L., Kim, J., Nanez, A., Sahin, M., MacLean, K. H., Inoki, K., Guan, K. L., Shen, J., Person, M. D*., et al.* (2010). ATM signals to TSC2 in the cytoplasm to regulate mTORC1 in response to ROS. Proc Natl Acad Sci U S A *107*, 4153-4158.

Ali, A., Zhang, J., Bao, S., Liu, I., Otterness, D., Dean, N. M., Abraham, R. T., and Wang, X. F. (2004). Requirement of protein phosphatase 5 in DNA-damage-induced ATM activation. Genes Dev *18*, 249-254.

Allsopp, R. C., Chang, E., Kashefi-Aazam, M., Rogaev, E. I., Piatyszek, M. A., Shay, J. W., and Harley, C. B. (1995). Telomere shortening is associated with cell division in vitro and in vivo. Exp Cell Res *220*, 194-200.

Atanassov, B. S., Evrard, Y. A., Multani, A. S., Zhang, Z., Tora, L., Devys, D., Chang, S., and Dent, S. Y. (2009). Gcn5 and SAGA regulate shelterin protein turnover and telomere maintenance. Mol Cell *35*, 352-364.

Audebert, M., Salles, B., and Calsou, P. (2004). Involvement of poly(ADP-ribose) polymerase-1 and XRCC1/DNA ligase III in an alternative route for DNA double-strand breaks rejoining. J Biol Chem *279*, 55117-55126.

Azzalin, C. M., Reichenbach, P., Khoriauli, L., Giulotto, E., and Lingner, J. (2007). Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. Science *318*, 798-801.

Badie, S., Escandell, J. M., Bouwman, P., Carlos, A. R., Thanasoula, M., Gallardo, M. M., Suram, A., Jaco, I., Benitez, J., Herbig, U*., et al.* (2010). BRCA2 acts as a RAD51 loader to facilitate telomere replication and capping. Nat Struct Mol Biol *17*, 1461-1469.

Bailey, S. M., Meyne, J., Chen, D. J., Kurimasa, A., Li, G. C., Lehnert, B. E., and Goodwin, E. H. (1999). DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes. Proc Natl Acad Sci U S A *96*, 14899-14904.

Bardwell, P. D., Woo, C. J., Wei, K., Li, Z., Martin, A., Sack, S. Z., Parris, T., Edelmann, W., and Scharff, M. D. (2004). Altered somatic hypermutation and reduced class-switch recombination in exonuclease 1-mutant mice. Nat Immunol *5*, 224-229.

Baumann, P., and Cech, T. R. (2001). Pot1, the putative telomere end-binding protein in fission yeast and humans. Science *292*, 1171-1175.

Bellon, M., Datta, A., Brown, M., Pouliquen, J. F., Couppie, P., Kazanji, M., and Nicot, C. (2006). Increased expression of telomere length regulating factors TRF1, TRF2 and TIN2 in patients with adult T-cell leukemia. Int J Cancer *119*, 2090-2097.

Bernstein, K. A., and Rothstein, R. (2009). At loose ends: resecting a double-strand break. Cell *137*, 807-810.

Blasco, M. A. (2005). Telomeres and human disease: ageing, cancer and beyond. Nat Rev Genet *6*, 611-622.

Bonetti, D., Martina, M., Clerici, M., Lucchini, G., and Longhese, M. P. (2009). Multiple pathways regulate 3' overhang generation at S. cerevisiae telomeres. Mol Cell *35*, 70-81.

Bosma, G. C., Kim, J., Urich, T., Fath, D. M., Cotticelli, M. G., Ruetsch, N. R., Radic, M. Z., and Bosma, M. J. (2002). DNA-dependent protein kinase activity is not required for immunoglobulin class switching. J Exp Med *196*, 1483-1495.

Bouwman, P., Aly, A., Escandell, J. M., Pieterse, M., Bartkova, J., van der Gulden, H., Hiddingh, S., Thanasoula, M., Kulkarni, A., Yang, Q.*, et al.* (2010). 53BP1 loss rescues BRCA1 deficiency and is associated with triple-negative and BRCA-mutated breast cancers. Nat Struct Mol Biol *17*, 688-695.

Branzei, D., and Foiani, M. (2008). Regulation of DNA repair throughout the cell cycle. Nat Rev Mol Cell Biol *9*, 297-308.

Bunting, S. F., Callen, E., Wong, N., Chen, H. T., Polato, F., Gunn, A., Bothmer, A., Feldhahn, N., Fernandez-Capetillo, O., Cao, L.*, et al.* (2010). 53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks. Cell *141*, 243-254.

Burma, S., Chen, B. P., Murphy, M., Kurimasa, A., and Chen, D. J. (2001). ATM phosphorylates histone H2AX in response to DNA double-strand breaks. J Biol Chem *276*, 42462-42467.

Buscemi, G., Zannini, L., Fontanella, E., Lecis, D., Lisanti, S., and Delia, D. (2009). The shelterin protein TRF2 inhibits Chk2 activity at telomeres in the absence of DNA damage. Curr Biol *19*, 874-879.

Busino, L., Donzelli, M., Chiesa, M., Guardavaccaro, D., Ganoth, D., Dorrello, N. V., Hershko, A., Pagano, M., and Draetta, G. F. (2003). Degradation of Cdc25A by beta-TrCP during S phase and in response to DNA damage. Nature *426*, 87-91.

Callen, E., and Surralles, J. (2004). Telomere dysfunction in genome instability syndromes. Mutat Res *567*, 85-104.

Campbell, M. R., Wang, Y., Andrew, S. E., and Liu, Y. (2006). Msh2 deficiency leads to chromosomal abnormalities, centrosome amplification, and telomere capping defect. Oncogene *25*, 2531-2536.

Capper, R., Britt-Compton, B., Tankimanova, M., Rowson, J., Letsolo, B., Man, S., Haughton, M., and Baird, D. M. (2007). The nature of telomere fusion and a definition of the critical telomere length in human cells. Genes Dev *21*, 2495-2508.

Carneiro, T., Khair, L., Reis, C. C., Borges, V., Moser, B. A., Nakamura, T. M., and Ferreira, M. G. (2010). Telomeres avoid end detection by severing the checkpoint signal transduction pathway. Nature *467*, 228-232.

Casellas, R., Nussenzweig, A., Wuerffel, R., Pelanda, R., Reichlin, A., Suh, H., Qin, X. F., Besmer, E., Kenter, A., Rajewsky, K., and Nussenzweig, M. C. (1998). Ku80 is required for immunoglobulin isotype switching. Embo J *17*, 2404-2411.

Casteel, D. E., Zhuang, S., Zeng, Y., Perrino, F. W., Boss, G. R., Goulian, M., and Pilz, R. B. (2009). A DNA polymerase-{alpha}{middle dot}primase cofactor with homology to replication protein A-32 regulates DNA replication in mammalian cells. J Biol Chem *284*, 5807-5818.

Celeste, A., Fernandez-Capetillo, O., Kruhlak, M. J., Pilch, D. R., Staudt, D. W., Lee, A., Bonner, R. F., Bonner, W. M., and Nussenzweig, A. (2003). Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. Nat Cell Biol *5*, 675-679.

Celli, G. B., and de Lange, T. (2005). DNA processing is not required for ATM-mediated telomere damage response after TRF2 deletion. Nat Cell Biol *7*, 712-718.

Celli, G. B., Denchi, E. L., and de Lange, T. (2006). Ku70 stimulates fusion of dysfunctional telomeres yet protects chromosome ends from homologous recombination. Nat Cell Biol *8*, 885-890.

Cesare, A. J., and Reddel, R. R. (2010). Alternative lengthening of telomeres: models, mechanisms and implications. Nat Rev Genet *11*, 319-330.

Chan, S. W., and Blackburn, E. H. (2003). Telomerase and ATM/Tel1p protect telomeres from nonhomologous end joining. Mol Cell *11*, 1379-1387.

Chandra, A., Hughes, T. R., Nugent, C. I., and Lundblad, V. (2001). Cdc13 both positively and negatively regulates telomere replication. Genes Dev *15*, 404-414.

Chen, L., Nievera, C. J., Lee, A. Y., and Wu, X. (2008). Cell cycle-dependent complex formation of BRCA1.CtIP.MRN is important for DNA double-strand break repair. J Biol Chem *283*, 7713-7720.

Chen, Y. C., Teng, S. C., and Wu, K. J. (2009). Phosphorylation of telomeric repeat binding factor 1 (TRF1) by Akt causes telomere shortening. Cancer Invest *27*, 24-28.

Chiang, Y. J., Kim, S. H., Tessarollo, L., Campisi, J., and Hodes, R. J. (2004). Telomere-associated protein TIN2 is essential for early embryonic development through a telomerase-independent pathway. Mol Cell Biol *24*, 6631-6634.

Chikashige, Y., and Hiraoka, Y. (2001). Telomere binding of the Rap1 protein is required for meiosis in fission yeast. Curr Biol *11*, 1618-1623.

Chin, K., de Solorzano, C. O., Knowles, D., Jones, A., Chou, W., Rodriguez, E. G., Kuo, W. L., Ljung, B. M., Chew, K., Myambo, K.*, et al.* (2004). In situ analyses of genome instability in breast cancer. Nat Genet *36*, 984-988.

Chu, W. K., and Hickson, I. D. (2009). RecQ helicases: multifunctional genome caretakers. Nat Rev Cancer *9*, 644-654.

Churikov, D., Wei, C., and Price, C. M. (2006). Vertebrate POT1 restricts G-overhang length and prevents activation of a telomeric DNA damage checkpoint but is dispensable for overhang protection. Mol Cell Biol *26*, 6971-6982.

Ciapponi, L., and Cenci, G. (2008). Telomere capping and cellular checkpoints: clues from fruit flies. Cytogenet Genome Res *122*, 365-373.

Clerici, M., Mantiero, D., Lucchini, G., and Longhese, M. P. (2005). The Saccharomyces cerevisiae Sae2 protein promotes resection and bridging of double strand break ends. J Biol Chem *280*, 38631-38638.

Conrad, M. N., Wright, J. H., Wolf, A. J., and Zakian, V. A. (1990). RAP1 protein interacts with yeast telomeres in vivo: overproduction alters telomere structure and decreases chromosome stability. Cell *63*, 739-750.

Cook, B. D., Dynek, J. N., Chang, W., Shostak, G., and Smith, S. (2002). Role for the related poly(ADP-Ribose) polymerases tankyrase 1 and 2 at human telomeres. Mol Cell Biol *22*, 332-342.

Cooper, J. P., Nimmo, E. R., Allshire, R. C., and Cech, T. R. (1997). Regulation of telomere length and function by a Myb-domain protein in fission yeast. Nature *385*, 744-747.

Court, R., Chapman, L., Fairall, L., and Rhodes, D. (2005). How the human telomeric proteins TRF1 and TRF2 recognize telomeric DNA: a view from high-resolution crystal structures. EMBO Rep *6*, 39-45.

Crabbe, L., Jauch, A., Naeger, C. M., Holtgreve-Grez, H., and Karlseder, J. (2007). Telomere dysfunction as a cause of genomic instability in Werner syndrome. Proc Natl Acad Sci U S A *104*, 2205-2210.

Crabbe, L., Verdun, R. E., Haggblom, C. I., and Karlseder, J. (2004). Defective telomere lagging strand synthesis in cells lacking WRN helicase activity. Science *306*, 1951-1953.

d'Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N. P., and Jackson, S. P. (2003). A DNA damage checkpoint response in telomere-initiated senescence. Nature *426*, 194-198.

d'Adda di Fagagna, F., Teo, S. H., and Jackson, S. P. (2004). Functional links between telomeres and proteins of the DNA-damage response. Genes Dev *18*, 1781-1799.

Dahlen, M., Olsson, T., Kanter-Smoler, G., Ramne, A., and Sunnerhagen, P. (1998). Regulation of telomere length by checkpoint genes in Schizosaccharomyces pombe. Mol Biol Cell *9*, 611-621.

Dantzer, F., Giraud-Panis, M. J., Jaco, I., Ame, J. C., Schultz, I., Blasco, M., Koering, C. E., Gilson, E., Menissier-de Murcia, J., de Murcia, G., and Schreiber, V. (2004). Functional interaction between poly(ADP-Ribose) polymerase 2 (PARP-2) and TRF2: PARP activity negatively regulates TRF2. Mol Cell Biol *24*, 1595-1607.

Davoli, T., Denchi, E. L., and de Lange, T. (2010). Persistent telomere damage induces bypass of mitosis and tetraploidy. Cell *141*, 81-93.

de Lange, T. (2009). How telomeres solve the end-protection problem. Science *326*, 948-952.

de Lange, T., Shiue, L., Myers, R. M., Cox, D. R., Naylor, S. L., Killery, A. M., and Varmus, H. E. (1990). Structure and variability of human chromosome ends. Mol Cell Biol *10*, 518-527.

Decottignies, A. (2007). Microhomology-mediated end joining in fission yeast is repressed by pku70 and relies on genes involved in homologous recombination. Genetics *176*, 1403-1415.

Dejardin, J., and Kingston, R. E. (2009). Purification of proteins associated with specific genomic Loci. Cell *136*, 175-186.

Denchi, E. L., Celli, G., and de Lange, T. (2006). Hepatocytes with extensive telomere deprotection and fusion remain viable and regenerate liver mass through endoreduplication. Genes Dev *20*, 2648-2653.

Denchi, E. L., and de Lange, T. (2007). Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. Nature *448*, 1068-1071.

Deng, Y., Guo, X., Ferguson, D. O., and Chang, S. (2009). Multiple roles for MRE11 at uncapped telomeres. Nature *460*, 914-918.

Difilippantonio, S., Gapud, E., Wong, N., Huang, C. Y., Mahowald, G., Chen, H. T., Kruhlak, M. J., Callen, E., Livak, F., Nussenzweig, M. C.*, et al.* (2008). 53BP1 facilitates long-range DNA end-joining during V(D)J recombination. Nature *456*, 529-533.

Dimitrova, N., Chen, Y. C., Spector, D. L., and de Lange, T. (2008). 53BP1 promotes non-homologous end joining of telomeres by increasing chromatin mobility. Nature *456*, 524-528.

Dimitrova, N., and de Lange, T. (2009). Cell cycle-dependent role of MRN at dysfunctional telomeres: ATM signaling-dependent induction of nonhomologous end joining (NHEJ) in G1 and resection-mediated inhibition of NHEJ in G2. Mol Cell Biol *29*, 5552-5563.

Ding, H., Schertzer, M., Wu, X., Gertsenstein, M., Selig, S., Kammori, M., Pourvali, R., Poon, S., Vulto, I., Chavez, E.*, et al.* (2004). Regulation of murine telomere length by Rtel: an essential gene encoding a helicase-like protein. Cell *117*, 873-886.

Dinkelmann, M., Spehalski, E., Stoneham, T., Buis, J., Wu, Y., Sekiguchi, J. M., and Ferguson, D. O. (2009). Multiple functions of MRN in end-joining pathways during isotype class switching. Nat Struct Mol Biol *16*, 808-813.

Doil, C., Mailand, N., Bekker-Jensen, S., Menard, P., Larsen, D. H., Pepperkok, R., Ellenberg, J., Panier, S., Durocher, D., Bartek, J.*, et al.* (2009). RNF168 binds and amplifies ubiquitin conjugates on damaged chromosomes to allow accumulation of repair proteins. Cell *136*, 435-446.

Donzelli, M., Busino, L., Chiesa, M., Ganoth, D., Hershko, A., and Draetta, G. F. (2004). Hierarchical order of phosphorylation events commits Cdc25A to betaTrCP-dependent degradation. Cell Cycle *3*, 469-471.

Du, X., Shen, J., Kugan, N., Furth, E. E., Lombard, D. B., Cheung, C., Pak, S., Luo, G., Pignolo, R. J., DePinho, R. A.*, et al.* (2004). Telomere shortening exposes functions for the mouse Werner and Bloom syndrome genes. Mol Cell Biol *24*, 8437-8446.

Dunham, M. A., Neumann, A. A., Fasching, C. L., and Reddel, R. R. (2000). Telomere maintenance by recombination in human cells. Nat Genet *26*, 447-450.

Eid, W., Steger, M., El-Shemerly, M., Ferretti, L. P., Pena-Diaz, J., Konig, C., Valtorta, E., Sartori, A. A., and Ferrari, S. (2010). DNA end resection by CtIP and exonuclease 1 prevents genomic instability. EMBO Rep *11*, 962-968.

Else, T., Trovato, A., Kim, A. C., Wu, Y., Ferguson, D. O., Kuick, R. D., Lucas, P. C., and Hammer, G. D. (2009). Genetic p53 deficiency partially rescues the adrenocortical dysplasia phenotype at the expense of increased tumorigenesis. Cancer Cell *15*, 465-476.

Erfle, H., Neumann, B., Liebel, U., Rogers, P., Held, M., Walter, T., Ellenberg, J., and Pepperkok, R. (2007). Reverse transfection on cell arrays for high content screening microscopy. Nat Protoc *2*, 392-399.

Fan, Q., Zhang, F., Barrett, B., Ren, K., and Andreassen, P. R. (2009). A role for monoubiquitinated FANCD2 at telomeres in ALT cells. Nucleic Acids Res *37*, 1740-1754.

Fedorov, Y., Anderson, E. M., Birmingham, A., Reynolds, A., Karpilow, J., Robinson, K., Leake, D., Marshall, W. S., and Khvorova, A. (2006). Off-target effects by siRNA can induce toxic phenotype. Rna *12*, 1188-1196.

Feldser, D. M., Hackett, J. A., and Greider, C. W. (2003). Telomere dysfunction and the initiation of genome instability. Nat Rev Cancer *3*, 623-627.

Ferreira, M. G., and Cooper, J. P. (2001). The fission yeast Taz1 protein protects chromosomes from Ku-dependent end-to-end fusions. Mol Cell *7*, 55-63.

Flynn, R. L., Centore, R. C., O'Sullivan, R. J., Rai, R., Tse, A., Songyang, Z., Chang, S., Karlseder, J., and Zou, L. (2011). TERRA and hnRNPA1 orchestrate an RPA-to-POT1 switch on telomeric single-stranded DNA. Nature *471*, 532-536.

Francia, S., Weiss, R. S., Hande, M. P., Freire, R., and d'Adda di Fagagna, F. (2006). Telomere and telomerase modulation by the mammalian Rad9/Rad1/Hus1 DNA-damage-checkpoint complex. Curr Biol *16*, 1551-1558.

Freibaum, B. D., and Counter, C. M. (2006). hSnm1B is a novel telomere-associated protein. J Biol Chem *281*, 15033-15036.

Fujita, K., Horikawa, I., Mondal, A. M., Jenkins, L. M., Appella, E., Vojtesek, B., Bourdon, J. C., Lane, D. P., and Harris, C. C. (2010). Positive feedback between p53 and TRF2 during telomere-damage signalling and cellular senescence. Nat Cell Biol *12*, 1205-1212.

Gao, H., Cervantes, R. B., Mandell, E. K., Otero, J. H., and Lundblad, V. (2007). RPA-like proteins mediate yeast telomere function. Nat Struct Mol Biol *14*, 208-214.

Garcia-Cao, M., O'Sullivan, R., Peters, A. H., Jenuwein, T., and Blasco, M. A. (2004). Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases. Nat Genet *36*, 94-99.

Garcia, V., Phelps, S. E., Gray, S., and Neale, M. J. (2011). Bidirectional resection of DNA double-strand breaks by Mre11 and Exo1. Nature *479*, 241-244.

Garvik, B., Carson, M., and Hartwell, L. (1995). Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol Cell Biol *15*, 6128-6138.

Giannone, R. J., McDonald, H. W., Hurst, G. B., Shen, R. F., Wang, Y., and Liu, Y. (2010). The protein network surrounding the human telomere repeat binding factors TRF1, TRF2, and POT1. PLoS One *5*, e12407.

Gilley, D., Tanaka, H., Hande, M. P., Kurimasa, A., Li, G. C., Oshimura, M., and Chen, D. J. (2001). DNA-PKcs is critical for telomere capping. Proc Natl Acad Sci U S A *98*, 15084-15088.

Gomez, M., Wu, J., Schreiber, V., Dunlap, J., Dantzer, F., Wang, Y., and Liu, Y. (2006). PARP1 Is a TRF2-associated poly(ADP-ribose)polymerase and protects eroded telomeres. Mol Biol Cell *17*, 1686-1696.

Gong, Y., and de Lange, T. (2010). A Shld1-controlled POT1a provides support for repression of ATR signaling at telomeres through RPA exclusion. Mol Cell *40*, 377-387.

Goodarzi, A. A., Jeggo, P., and Lobrich, M. (2010). The influence of heterochromatin on DNA double strand break repair: Getting the strong, silent type to relax. DNA Repair (Amst) *9*, 1273-1282.

Goodarzi, A. A., Kurka, T., and Jeggo, P. A. (2011). KAP-1 phosphorylation regulates CHD3 nucleosome remodeling during the DNA double-strand break response. Nat Struct Mol Biol *18*, 831-839.

Goodarzi, A. A., Noon, A. T., Deckbar, D., Ziv, Y., Shiloh, Y., Lobrich, M., and Jeggo, P. A. (2008). ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin. Mol Cell *31*, 167-177.

Graham, F. L., and van der Eb, A. J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology *52*, 456-467.

Grandin, N., Reed, S. I., and Charbonneau, M. (1997). Stn1, a new Saccharomyces cerevisiae protein, is implicated in telomere size regulation in association with Cdc13. Genes Dev *11*, 512-527.

Gravel, S., Chapman, J. R., Magill, C., and Jackson, S. P. (2008). DNA helicases Sgs1 and BLM promote DNA double-strand break resection. Genes Dev *22*, 2767-2772.

Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A., Moss, H., and de Lange, T. (1999). Mammalian telomeres end in a large duplex loop. Cell *97*, 503-514.

Gu, P., Min, J. N., Wang, Y., Huang, C., Peng, T., Chai, W., and Chang, S. (2012). CTC1 deletion results in defective telomere replication, leading to catastrophic telomere loss and stem cell exhaustion. Embo J.

Guo, X., Deng, Y., Lin, Y., Cosme-Blanco, W., Chan, S., He, H., Yuan, G., Brown, E. J., and Chang, S. (2007). Dysfunctional telomeres activate an ATM-ATR-dependent DNA damage response to suppress tumorigenesis. Embo J *26*, 4709-4719.

Han, L., and Yu, K. (2008). Altered kinetics of nonhomologous end joining and class switch recombination in ligase IV-deficient B cells. J Exp Med *205*, 2745-2753.

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Hanaoka, S., Nagadoi, A., and Nishimura, Y. (2005). Comparison between TRF2 and TRF1 of their telomeric DNA-bound structures and DNA-binding activities. Protein Sci *14*, 119-130.

Hanaoka, S., Nagadoi, A., Yoshimura, S., Aimoto, S., Li, B., de Lange, T., and Nishimura, Y. (2001). NMR structure of the hRap1 Myb motif reveals a canonical three-helix bundle lacking the positive surface charge typical of Myb DNA-binding domains. J Mol Biol *312*, 167-175.

Hardy, C. F., Sussel, L., and Shore, D. (1992). A RAP1-interacting protein involved in transcriptional silencing and telomere length regulation. Genes Dev *6*, 801-814.

Hastie, N. D., Dempster, M., Dunlop, M. G., Thompson, A. M., Green, D. K., and Allshire, R. C. (1990). Telomere reduction in human colorectal carcinoma and with ageing. Nature *346*, 866-868.

Hemann, M. T., Strong, M. A., Hao, L. Y., and Greider, C. W. (2001). The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. Cell *107*, 67-77.

Her, Y. R., and Chung, I. K. (2009). Ubiquitin Ligase RLIM Modulates Telomere Length Homeostasis through a Proteolysis of TRF1. J Biol Chem *284*, 8557-8566.

Hiom, K. (2010). Coping with DNA double strand breaks. DNA Repair (Amst) *9*, 1256-1263.

Hockemeyer, D., Daniels, J. P., Takai, H., and de Lange, T. (2006). Recent expansion of the telomeric complex in rodents: Two distinct POT1 proteins protect mouse telomeres. Cell *126*, 63-77.

Hockemeyer, D., Sfeir, A. J., Shay, J. W., Wright, W. E., and de Lange, T. (2005). POT1 protects telomeres from a transient DNA damage response and determines how human chromosomes end. Embo J *24*, 2667-2678.

Hornsby, P. J. (2011). Cellular aging and cancer. Crit Rev Oncol Hematol *79*, 189-195.

Hsu, H. L., Gilley, D., Blackburn, E. H., and Chen, D. J. (1999). Ku is associated with the telomere in mammals. Proc Natl Acad Sci U S A *96*, 12454-12458.

Hsu, H. L., Gilley, D., Galande, S. A., Hande, M. P., Allen, B., Kim, S. H., Li, G. C., Campisi, J., Kohwi-Shigematsu, T., and Chen, D. J. (2000). Ku acts in a unique way at the mammalian telomere to prevent end joining. Genes Dev *14*, 2807-2812.

Hu, H., Zhang, Y., Zou, M., Yang, S., and Liang, X. Q. (2010). Expression of TRF1, TRF2, TIN2, TERT, KU70, and BRCA1 proteins is associated with telomere shortening and may contribute to multistage carcinogenesis of gastric cancer. J Cancer Res Clin Oncol *136*, 1407-1414.

Huang, C., Dai, X., and Chai, W. (2012). Human Stn1 protects telomere integrity by promoting efficient lagging-strand synthesis at telomeres and mediating C-strand fill-in. Cell Res.

Huda, N., Tanaka, H., Mendonca, M. S., and Gilley, D. (2009). DNA damage-induced phosphorylation of TRF2 is required for the fast pathway of DNA double-strand break repair. Mol Cell Biol *29*, 3597-3604.

Huertas, P. (2010). DNA resection in eukaryotes: deciding how to fix the break. Nat Struct Mol Biol *17*, 11-16.

Huertas, P., and Jackson, S. P. (2009). Human CtIP mediates cell cycle control of DNA end resection and double strand break repair. J Biol Chem *284*, 9558-9565.

Hultdin, M., Gronlund, E., Norrback, K. F., Just, T., Taneja, K., and Roos, G. (2001). Replication timing of human telomeric DNA and other repetitive sequences analyzed by fluorescence in situ hybridization and flow cytometry. Exp Cell Res *271*, 223-229.

Iliakis, G. (2009). Backup pathways of NHEJ in cells of higher eukaryotes: cell cycle dependence. Radiother Oncol *92*, 310-315.

Ivessa, A. S., Zhou, J. Q., Schulz, V. P., Monson, E. K., and Zakian, V. A. (2002). Saccharomyces Rrm3p, a 5' to 3' DNA helicase that promotes replication fork progression through telomeric and subtelomeric DNA. Genes Dev *16*, 1383-1396.

Jackson, A. L., and Linsley, P. S. (2010). Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. Nat Rev Drug Discov *9*, 57-67.

Jaco, I., Munoz, P., and Blasco, M. A. (2004). Role of human Ku86 in telomere length maintenance and telomere capping. Cancer Res *64*, 7271-7278.

Jaco, I., Munoz, P., Goytisolo, F., Wesoly, J., Bailey, S., Taccioli, G., and Blasco, M. A. (2003). Role of mammalian Rad54 in telomere length maintenance. Mol Cell Biol *23*, 5572-5580.

Jain, D., and Cooper, J. P. (2010). Telomeric strategies: means to an end. Annu Rev Genet *44*, 243-269.

Jia, X., Weinert, T., and Lydall, D. (2004). Mec1 and Rad53 inhibit formation of single-stranded DNA at telomeres of Saccharomyces cerevisiae cdc13-1 mutants. Genetics *166*, 753-764.

Jiang, H., Schiffer, E., Song, Z., Wang, J., Zurbig, P., Thedieck, K., Moes, S., Bantel, H., Saal, N., Jantos, J.*, et al.* (2008). Proteins induced by telomere dysfunction and DNA damage represent biomarkers of human aging and disease. Proc Natl Acad Sci U S A *105*, 11299-11304.

Jiang, W. Q., Zhong, Z. H., Henson, J. D., Neumann, A. A., Chang, A. C., and Reddel, R. R. (2005). Suppression of alternative lengthening of telomeres by Sp100-mediated sequestration of the MRE11/RAD50/NBS1 complex. Mol Cell Biol *25*, 2708-2721.

Jin, J., Ang, X. L., Ye, X., Livingstone, M., and Harper, J. W. (2008). Differential roles for checkpoint kinases in DNA damage-dependent degradation of the Cdc25A protein phosphatase. J Biol Chem *283*, 19322-19328.

Jin, J., Shirogane, T., Xu, L., Nalepa, G., Qin, J., Elledge, S. J., and Harper, J. W. (2003). SCFbeta-TRCP links Chk1 signaling to degradation of the Cdc25A protein phosphatase. Genes Dev *17*, 3062-3074.

Kang, S. S., Kwon, T., Kwon, D. Y., and Do, S. I. (1999). Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. J Biol Chem *274*, 13085-13090.

Kang, Y., Lee, J. H., Hoan, N. N., Sohn, H. M., Chang, I. Y., and You, H. J. (2009). Protein phosphatase 5 regulates the function of 53BP1 after neocarzinostatin-induced DNA damage. J Biol Chem *284*, 9845-9853.

Kanoh, J., and Ishikawa, F. (2001). spRap1 and spRif1, recruited to telomeres by Taz1, are essential for telomere function in fission yeast. Curr Biol *11*, 1624-1630.

Karlseder, J., Broccoli, D., Dai, Y., Hardy, S., and de Lange, T. (1999). p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science *283*, 1321-1325.

Karlseder, J., Hoke, K., Mirzoeva, O. K., Bakkenist, C., Kastan, M. B., Petrini, J. H., and de Lange, T. (2004). The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response. PLoS Biol *2*, E240.

Karlseder, J., Kachatrian, L., Takai, H., Mercer, K., Hingorani, S., Jacks, T., and de Lange, T. (2003). Targeted deletion reveals an essential function for the telomere length regulator Trf1. Mol Cell Biol *23*, 6533-6541.

Karlseder, J., Smogorzewska, A., and de Lange, T. (2002). Senescence induced by altered telomere state, not telomere loss. Science *295*, 2446-2449.

Kelleher, C., Kurth, I., and Lingner, J. (2005). Human protection of telomeres 1 (POT1) is a negative regulator of telomerase activity in vitro. Mol Cell Biol *25*, 808-818.

Keller, R. B., Gagne, K. E., Usmani, G. N., Asdourian, G. K., Williams, D. A., Hofmann, I., and Agarwal, S. (2012). CTC1 Mutations in a patient with dyskeratosis congenita. Pediatr Blood Cancer.

Kibe, T., Osawa, G. A., Keegan, C. E., and de Lange, T. (2010). Telomere protection by TPP1 is mediated by POT1a and POT1b. Mol Cell Biol *30*, 1059-1066.

Kiefer, K., Oshinsky, J., Kim, J., Nakajima, P. B., Bosma, G. C., and Bosma, M. J. (2007). The catalytic subunit of DNA-protein kinase (DNA-PKcs) is not required for Ig class-switch recombination. Proc Natl Acad Sci U S A *104*, 2843-2848.

Kim, H., Lee, O. H., Xin, H., Chen, L. Y., Qin, J., Chae, H. K., Lin, S. Y., Safari, A., Liu, D., and Songyang, Z. (2009). TRF2 functions as a protein hub and regulates telomere maintenance by recognizing specific peptide motifs. Nat Struct Mol Biol *16*, 372-379.

Kim, J. H., Park, S. M., Kang, M. R., Oh, S. Y., Lee, T. H., Muller, M. T., and Chung, I. K. (2005). Ubiquitin ligase MKRN1 modulates telomere length homeostasis through a proteolysis of hTERT. Genes Dev *19*, 776-781.

Kim, S. H., Beausejour, C., Davalos, A. R., Kaminker, P., Heo, S. J., and Campisi, J. (2004). TIN2 mediates functions of TRF2 at human telomeres. J Biol Chem *279*, 43799-43804.

Kiyokawa, H., and Ray, D. (2008). In vivo roles of CDC25 phosphatases: biological insight into the anti-cancer therapeutic targets. Anticancer Agents Med Chem *8*, 832-836.

Kolas, N. K., Chapman, J. R., Nakada, S., Ylanko, J., Chahwan, R., Sweeney, F. D., Panier, S., Mendez, M., Wildenhain, J., Thomson, T. M.*, et al.* (2007). Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase. Science *318*, 1637-1640.

Kondo, T., Oue, N., Yoshida, K., Mitani, Y., Naka, K., Nakayama, H., and Yasui, W. (2004). Expression of POT1 is associated with tumor stage and telomere length in gastric carcinoma. Cancer Res *64*, 523-529.

Konishi, A., and de Lange, T. (2008). Cell cycle control of telomere protection and NHEJ revealed by a ts mutation in the DNA-binding domain of TRF2. Genes Dev *22*, 1221-1230.

Lackner, D. H., Durocher, D., and Karlseder, J. (2011). A siRNA-based screen for genes involved in chromosome end protection. PLoS One *6*, e21407.

Lamarche, B. J., Orazio, N. I., and Weitzman, M. D. (2010). The MRN complex in double-strand break repair and telomere maintenance. FEBS Lett *584*, 3682-3695.

Laplante, M., and Sabatini, D. M. (2012). mTOR signaling in growth control and disease. Cell *149*, 274-293.

Larrivee, M., LeBel, C., and Wellinger, R. J. (2004). The generation of proper constitutive G-tails on yeast telomeres is dependent on the MRX complex. Genes Dev *18*, 1391-1396.

Lee-Theilen, M., Matthews, A. J., Kelly, D., Zheng, S., and Chaudhuri, J. (2011). CtIP promotes microhomology-mediated alternative end joining during class-switch recombination. Nat Struct Mol Biol *18*, 75-79.

Lee, B. I., and Wilson, D. M., 3rd (1999). The RAD2 domain of human exonuclease 1 exhibits 5' to 3' exonuclease and flap structure-specific endonuclease activities. J Biol Chem *274*, 37763-37769.

Lee, H., Sengupta, N., Villagra, A., Rezai-Zadeh, N., and Seto, E. (2006a). Histone deacetylase 8 safeguards the human ever-shorter telomeres 1B (hEST1B) protein from ubiquitin-mediated degradation. Mol Cell Biol *26*, 5259-5269.

Lee, K., and Lee, S. E. (2007). Saccharomyces cerevisiae Sae2- and Tel1-dependent single-strand DNA formation at DNA break promotes microhomology-mediated end joining. Genetics *176*, 2003-2014.

Lee, T. H., Perrem, K., Harper, J. W., Lu, K. P., and Zhou, X. Z. (2006b). The F-box protein FBX4 targets PIN2/TRF1 for ubiquitin-mediated degradation and regulates telomere maintenance. J Biol Chem *281*, 759-768.

Lei, M., Podell, E. R., and Cech, T. R. (2004). Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection. Nat Struct Mol Biol *11*, 1223-1229.

Lengsfeld, B. M., Rattray, A. J., Bhaskara, V., Ghirlando, R., and Paull, T. T. (2007). Sae2 is an endonuclease that processes hairpin DNA cooperatively with the Mre11/Rad50/Xrs2 complex. Mol Cell *28*, 638-651.

Li, B., Oestreich, S., and de Lange, T. (2000). Identification of human Rap1: implications for telomere evolution. Cell *101*, 471-483.

Lieber, M. R. (2010). The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem *79*, 181-211.

Lin, T. T., Letsolo, B. T., Jones, R. E., Rowson, J., Pratt, G., Hewamana, S., Fegan, C., Pepper, C., and Baird, D. M. (2010). Telomere dysfunction and fusion during the progression of chronic lymphocytic leukemia: evidence for a telomere crisis. Blood *116*, 1899-1907.

Lin, X., Gu, J., Lu, C., Spitz, M. R., and Wu, X. (2006). Expression of telomere-associated genes as prognostic markers for overall survival in patients with non-small cell lung cancer. Clin Cancer Res *12*, 5720-5725.

Liu, D., Safari, A., O'Connor, M. S., Chan, D. W., Laegeler, A., Qin, J., and Songyang, Z. (2004). PTOP interacts with POT1 and regulates its localization to telomeres. Nat Cell Biol *6*, 673-680.

Loayza, D., and De Lange, T. (2003). POT1 as a terminal transducer of TRF1 telomere length control. Nature *423*, 1013-1018.

Loayza, D., Parsons, H., Donigian, J., Hoke, K., and de Lange, T. (2004). DNA binding features of human POT1: a nonamer 5'-TAGGGTTAG-3' minimal binding site, sequence specificity, and internal binding to multimeric sites. J Biol Chem *279*, 13241-13248.

Longhese, M. P., Bonetti, D., Manfrini, N., and Clerici, M. (2010). Mechanisms and regulation of DNA end resection. Embo J *29*, 2864-2874.

Luke, B., and Lingner, J. (2009). TERRA: telomeric repeat-containing RNA. Embo J *28*, 2503-2510.

Lustig, A. J., Kurtz, S., and Shore, D. (1990). Involvement of the silencer and UAS binding protein RAP1 in regulation of telomere length. Science *250*, 549-553.

Lustig, A. J., and Petes, T. D. (1986). Identification of yeast mutants with altered telomere structure. Proc Natl Acad Sci U S A *83*, 1398-1402.

Lydall, D., and Weinert, T. (1995). Yeast checkpoint genes in DNA damage processing: implications for repair and arrest. Science *270*, 1488-1491.

Ma, J. L., Kim, E. M., Haber, J. E., and Lee, S. E. (2003). Yeast Mre11 and Rad1 proteins define a Ku-independent mechanism to repair double-strand breaks lacking overlapping end sequences. Mol Cell Biol *23*, 8820-8828.

Ma, Y., Lu, H., Tippin, B., Goodman, M. F., Shimazaki, N., Koiwai, O., Hsieh, C. L., Schwarz, K., and Lieber, M. R. (2004). A biochemically defined system for mammalian nonhomologous DNA end joining. Mol Cell *16*, 701-713.

Mailand, N., Bekker-Jensen, S., Faustrup, H., Melander, F., Bartek, J., Lukas, C., and Lukas, J. (2007). RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins. Cell *131*, 887-900.

Makovets, S., Herskowitz, I., and Blackburn, E. H. (2004). Anatomy and dynamics of DNA replication fork movement in yeast telomeric regions. Mol Cell Biol *24*, 4019-4031.

Malo, N., Hanley, J. A., Cerquozzi, S., Pelletier, J., and Nadon, R. (2006). Statistical practice in high-throughput screening data analysis. Nat Biotechnol *24*, 167-175.

Manis, J. P., Gu, Y., Lansford, R., Sonoda, E., Ferrini, R., Davidson, L., Rajewsky, K., and Alt, F. W. (1998). Ku70 is required for late B cell development and immunoglobulin heavy chain class switching. J Exp Med *187*, 2081-2089.

Manis, J. P., Morales, J. C., Xia, Z., Kutok, J. L., Alt, F. W., and Carpenter, P. B. (2004). 53BP1 links DNA damage-response pathways to immunoglobulin heavy chain class-switch recombination. Nat Immunol *5*, 481-487.

Martinez, P., and Blasco, M. A. (2010). Role of shelterin in cancer and aging. Aging Cell *9*, 653-666.

Martinez, P., Thanasoula, M., Carlos, A. R., Gomez-Lopez, G., Tejera, A. M., Schoeftner, S., Dominguez, O., Pisano, D. G., Tarsounas, M., and Blasco, M. A. (2010). Mammalian Rap1 controls telomere function and gene expression through binding to telomeric and extratelomeric sites. Nat Cell Biol *12*, 768-780.

Martinez, P., Thanasoula, M., Munoz, P., Liao, C., Tejera, A., McNees, C., Flores, J. M., Fernandez-Capetillo, O., Tarsounas, M., and Blasco, M. A. (2009). Increased telomere fragility and fusions resulting from TRF1 deficiency lead to degenerative pathologies and increased cancer in mice. Genes Dev *23*, 2060-2075.

Maser, R. S., and DePinho, R. A. (2002). Connecting chromosomes, crisis, and cancer. Science *297*, 565-569.

Matsuoka, S., Ballif, B. A., Smogorzewska, A., McDonald, E. R., 3rd, Hurov, K. E., Luo, J., Bakalarski, C. E., 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.

Matsuoka, S., Huang, M., and Elledge, S. J. (1998). Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science *282*, 1893-1897.

McClintock, B. (1941). The Stability of Broken Ends of Chromosomes in Zea Mays. Genetics *26*, 234-282.

McKerlie, M., and Zhu, X. D. (2011). Cyclin B-dependent kinase 1 regulates human TRF1 to modulate the resolution of sister telomeres. Nat Commun *2*, 371.

McVey, M., and Lee, S. E. (2008). MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. Trends Genet *24*, 529-538.

Metcalfe, J. A., Parkhill, J., Campbell, L., Stacey, M., Biggs, P., Byrd, P. J., and Taylor, A. M. (1996). Accelerated telomere shortening in ataxia telangiectasia. Nat Genet *13*, 350-353.

Miller, K. M., Ferreira, M. G., and Cooper, J. P. (2005). Taz1, Rap1 and Rif1 act both interdependently and independently to maintain telomeres. Embo J *24*, 3128-3135.

Miller, K. M., Rog, O., and Cooper, J. P. (2006). Semi-conservative DNA replication through telomeres requires Taz1. Nature *440*, 824-828.

Mimitou, E. P., and Symington, L. S. (2008). Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. Nature *455*, 770-774.

Mimitou, E. P., and Symington, L. S. (2009). DNA end resection: many nucleases make light work. DNA Repair (Amst) *8*, 983-995.

Min, J., Choi, E. S., Hwang, K., Kim, J., Sampath, S., Venkitaraman, A. R., and Lee, H. (2012). The breast cancer susceptibility gene BRCA2 is required for the maintenance of telomere homeostasis. J Biol Chem *287*, 5091-5101.

Mitchell, J. R., Cheng, J., and Collins, K. (1999a). A box H/ACA small nucleolar RNA-like domain at the human telomerase RNA 3' end. Mol Cell Biol *19*, 567-576.

Mitchell, J. R., Wood, E., and Collins, K. (1999b). A telomerase component is defective in the human disease dyskeratosis congenita. Nature *402*, 551-555.

Miyake, Y., Nakamura, M., Nabetani, A., Shimamura, S., Tamura, M., Yonehara, S., Saito, M., and Ishikawa, F. (2009). RPA-like mammalian Ctc1-Stn1-Ten1 complex binds to single-stranded DNA and protects telomeres independently of the Pot1 pathway. Mol Cell *36*, 193-206.

Miyoshi, T., Kanoh, J., Saito, M., and Ishikawa, F. (2008). Fission yeast Pot1-Tpp1 protects telomeres and regulates telomere length. Science *320*, 1341-1344.

Mochan, T. A., Venere, M., DiTullio, R. A., Jr., and Halazonetis, T. D. (2003). 53BP1 and NFBD1/MDC1-Nbs1 function in parallel interacting pathways activating ataxia-telangiectasia mutated (ATM) in response to DNA damage. Cancer Res *63*, 8586-8591.

Muller, H. J. (1938). The remaking of chromosomes. Collecting Net *8*, 182–198.

Munoz, P., Blanco, R., Flores, J. M., and Blasco, M. A. (2005). XPF nuclease-dependent telomere loss and increased DNA damage in mice overexpressing TRF2 result in premature aging and cancer. Nat Genet *37*, 1063-1071.

Murnane, J. P. (2012). Telomere dysfunction and chromosome instability. Mutat Res *730*, 28-36.

Naito, T., Matsuura, A., and Ishikawa, F. (1998). Circular chromosome formation in a fission yeast mutant defective in two ATM homologues. Nat Genet *20*, 203-206.

Nimonkar, A. V., Genschel, J., Kinoshita, E., Polaczek, P., Campbell, J. L., Wyman, C., Modrich, P., and Kowalczykowski, S. C. (2011). BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN constitute two DNA end resection machineries for human DNA break repair. Genes Dev *25*, 350-362.

Nimonkar, A. V., Ozsoy, A. Z., Genschel, J., Modrich, P., and Kowalczykowski, S. C. (2008). Human exonuclease 1 and BLM helicase interact to resect DNA and initiate DNA repair. Proc Natl Acad Sci U S A *105*, 16906-16911.

Nugent, C. I., Hughes, T. R., Lue, N. F., and Lundblad, V. (1996). Cdc13p: a single-strand telomeric DNA-binding protein with a dual role in yeast telomere maintenance. Science *274*, 249-252.

Nussenzweig, A., and Nussenzweig, M. C. (2007). A backup DNA repair pathway moves to the forefront. Cell *131*, 223-225.

O'Connor, M. S., Safari, A., Liu, D., Qin, J., and Songyang, Z. (2004). The human Rap1 protein complex and modulation of telomere length. J Biol Chem *279*, 28585-28591.

Oh, B. K., Kim, Y. J., Park, C., and Park, Y. N. (2005). Up-regulation of telomere-binding proteins, TRF1, TRF2, and TIN2 is related to telomere shortening during human multistep hepatocarcinogenesis. Am J Pathol *166*, 73-80.

Okamoto, K., Iwano, T., Tachibana, M., and Shinkai, Y. (2008). Distinct roles of TRF1 in the regulation of telomere structure and lengthening. J Biol Chem *283*, 23981-23988.

Opresko, P. L., von Kobbe, C., Laine, J. P., Harrigan, J., Hickson, I. D., and Bohr, V. A. (2002). Telomere-binding protein TRF2 binds to and stimulates the Werner and Bloom syndrome helicases. J Biol Chem *277*, 41110-41119.

Palm, W., and de Lange, T. (2008). How shelterin protects mammalian telomeres. Annu Rev Genet *42*, 301-334.

Palm, W., Hockemeyer, D., Kibe, T., and de Lange, T. (2009). Functional dissection of human and mouse POT1 proteins. Mol Cell Biol *29*, 471-482.

Pang, T. L., Wang, C. Y., Hsu, C. L., Chen, M. Y., and Lin, J. J. (2003). Exposure of single-stranded telomeric DNA causes G2/M cell cycle arrest in Saccharomyces cerevisiae. J Biol Chem *278*, 9318-9321.

Pardo, B., and Marcand, S. (2005). Rap1 prevents telomere fusions by nonhomologous end joining. Embo J *24*, 3117-3127.

Pawelczak, K. S., and Turchi, J. J. (2010). Purification and characterization of exonuclease-free Artemis: Implications for DNA-PK-dependent processing of DNA termini in NHEJ-catalyzed DSB repair. DNA Repair (Amst) *9*, 670-677.

Peng, A., and Chen, P. L. (2003). NFBD1, like 53BP1, is an early and redundant transducer mediating Chk2 phosphorylation in response to DNA damage. J Biol Chem *278*, 8873-8876.

Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S., and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science *277*, 1501-1505.

Peng, G., Dai, H., Zhang, W., Hsieh, H. J., Pan, M. R., Park, Y. Y., Tsai, R. Y., Bedrosian, I., Lee, J. S., Ira, G., and Lin, S. Y. (2012). Human nuclease/helicase DNA2 alleviates replication stress by promoting DNA end resection. Cancer Res *72*, 2802-2813.

Peuscher, M. H., and Jacobs, J. J. (2011). DNA-damage response and repair activities at uncapped telomeres depend on RNF8. Nat Cell Biol *13*, 1139-1145.

Pickart, C. M. (2001). Mechanisms underlying ubiquitination. Annu Rev Biochem *70*, 503-533.

Pitt, C. W., and Cooper, J. P. (2010). Pot1 inactivation leads to rampant telomere resection and loss in one cell cycle. Nucleic Acids Res *38*, 6968-6975.

Pogacic, V., Dragon, F., and Filipowicz, W. (2000). Human H/ACA small nucleolar RNPs and telomerase share evolutionarily conserved proteins NHP2 and NOP10. Mol Cell Biol *20*, 9028-9040.

Potts, P. R., and Yu, H. (2007). The SMC5/6 complex maintains telomere length in ALT cancer cells through SUMOylation of telomere-binding proteins. Nat Struct Mol Biol *14*, 581-590.

Price, C. M., Boltz, K. A., Chaiken, M. F., Stewart, J. A., Beilstein, M. A., and Shippen, D. E. (2010). Evolution of CST function in telomere maintenance. Cell Cycle *9*, 3157-3165.

Qiu, X. B., and Goldberg, A. L. (2002). Nrdp1/FLRF is a ubiquitin ligase promoting ubiquitination and degradation of the epidermal growth factor receptor family member, ErbB3. Proc Natl Acad Sci U S A *99*, 14843-14848.

Qiu, X. B., Markant, S. L., Yuan, J., and Goldberg, A. L. (2004). Nrdp1-mediated degradation of the gigantic IAP, BRUCE, is a novel pathway for triggering apoptosis. Embo J *23*, 800-810.

Quennet, V., Beucher, A., Barton, O., Takeda, S., and Lobrich, M. (2011). CtIP and MRN promote non-homologous end-joining of etoposide-induced DNA double-strand breaks in G1. Nucleic Acids Res *39*, 2144-2152.

Raghuraman, M. K., Winzeler, E. A., Collingwood, D., Hunt, S., Wodicka, L., Conway, A., Lockhart, D. J., Davis, R. W., Brewer, B. J., and Fangman, W. L. (2001). Replication dynamics of the yeast genome. Science *294*, 115-121.

Rai, R., Li, J. M., Zheng, H., Lok, G. T., Deng, Y., Huen, M. S., Chen, J., Jin, J., and Chang, S. (2011). The E3 ubiquitin ligase Rnf8 stabilizes Tpp1 to promote telomere end protection. Nat Struct Mol Biol *18*, 1400-1407.

Rai, R., Zheng, H., He, H., Luo, Y., Multani, A., Carpenter, P. B., and Chang, S. (2010). The function of classical and alternative non-homologous end-joining pathways in the fusion of dysfunctional telomeres. Embo J *29*, 2598-2610.

Roddam, P. L., Rollinson, S., O'Driscoll, M., Jeggo, P. A., Jack, A., and Morgan, G. J. (2002). Genetic variants of NHEJ DNA ligase IV can affect the risk of developing multiple myeloma, a tumour characterised by aberrant class switch recombination. J Med Genet *39*, 900-905.

Rooney, S., Alt, F. W., Lombard, D., Whitlow, S., Eckersdorff, M., Fleming, J., Fugmann, S., Ferguson, D. O., Schatz, D. G., and Sekiguchi, J. (2003). Defective DNA repair and increased genomic instability in Artemis-deficient murine cells. J Exp Med *197*, 553-565.

Rooney, S., Alt, F. W., Sekiguchi, J., and Manis, J. P. (2005). Artemis-independent functions of DNA-dependent protein kinase in Ig heavy chain class switch recombination and development. Proc Natl Acad Sci U S A *102*, 2471-2475.

Sakaguchi, K., Ishibashi, T., Uchiyama, Y., and Iwabata, K. (2009). The multi-replication protein A (RPA) system--a new perspective. Febs J *276*, 943-963.

Salhab, M., Jiang, W. G., Newbold, R. F., and Mokbel, K. (2008). The expression of gene transcripts of telomere-associated genes in human breast cancer: correlation with clinico-pathological parameters and clinical outcome. Breast Cancer Res Treat *109*, 35-46.

Sancar, A., Lindsey-Boltz, L. A., Unsal-Kacmaz, K., and Linn, S. (2004). Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem *73*, 39-85.

Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S. J. (1997). Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science *277*, 1497-1501.

Sansam, C. L., Shepard, J. L., Lai, K., Ianari, A., Danielian, P. S., Amsterdam, A., Hopkins, N., and Lees, J. A. (2006). DTL/CDT2 is essential for both CDT1 regulation and the early G2/M checkpoint. Genes Dev *20*, 3117-3129.

Sargent, R. G., Meservy, J. L., Perkins, B. D., Kilburn, A. E., Intody, Z., Adair, G. M., Nairn, R. S., and Wilson, J. H. (2000). Role of the nucleotide excision repair gene ERCC1 in formation of recombination-dependent rearrangements in mammalian cells. Nucleic Acids Res *28*, 3771-3778.

Sarthy, J., Bae, N. S., Scrafford, J., and Baumann, P. (2009). Human RAP1 inhibits non-homologous end joining at telomeres. Embo J *28*, 3390-3399.

Sartori, A. A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., Baer, R., Lukas, J., and Jackson, S. P. (2007). Human CtIP promotes DNA end resection. Nature *450*, 509-514.

Schaetzlein, S., Kodandaramireddy, N. R., Ju, Z., Lechel, A., Stepczynska, A., Lilli, D. R., Clark, A. B., Rudolph, C., Kuhnel, F., Wei, K.*, et al.* (2007). Exonuclease-1 deletion impairs DNA damage signaling and prolongs lifespan of telomere-dysfunctional mice. Cell *130*, 863-877.

Schoeftner, S., and Blasco, M. A. (2008). Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. Nat Cell Biol *10*, 228-236.

Schoeftner, S., and Blasco, M. A. (2009). A 'higher order' of telomere regulation: telomere heterochromatin and telomeric RNAs. Embo J *28*, 2323-2336.

Sfeir, A., and de Lange, T. (2012). Removal of shelterin reveals the telomere end-protection problem. Science *336*, 593-597.

Sfeir, A., Kabir, S., van Overbeek, M., Celli, G. B., and de Lange, T. (2010). Loss of Rap1 induces telomere recombination in the absence of NHEJ or a DNA damage signal. Science *327*, 1657-1661.

Sfeir, A., Kosiyatrakul, S. T., Hockemeyer, D., MacRae, S. L., Karlseder, J., Schildkraut, C. L., and de Lange, T. (2009). Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication. Cell *138*, 90-103.

Shim, E. Y., Chung, W. H., Nicolette, M. L., Zhang, Y., Davis, M., Zhu, Z., Paull, T. T., Ira, G., and Lee, S. E. (2010). Saccharomyces cerevisiae Mre11/Rad50/Xrs2 and Ku proteins regulate association of Exo1 and Dna2 with DNA breaks. Embo J *29*, 3370-3380.

Shore, D. (1994). RAP1: a protean regulator in yeast. Trends Genet *10*, 408-412.

Silver, D. P., and Livingston, D. M. (2001). Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. Mol Cell *8*, 233-243.

Silverman, J., Takai, H., Buonomo, S. B., Eisenhaber, F., and de Lange, T. (2004). Human Rif1, ortholog of a yeast telomeric protein, is regulated by ATM and 53BP1 and functions in the S-phase checkpoint. Genes Dev *18*, 2108-2119.

Smith, S., and de Lange, T. (2000). Tankyrase promotes telomere elongation in human cells. Curr Biol *10*, 1299-1302.

Smith, S., Giriat, I., Schmitt, A., and de Lange, T. (1998). Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. Science *282*, 1484-1487.

Smogorzewska, A., and de Lange, T. (2002). Different telomere damage signaling pathways in human and mouse cells. Embo J *21*, 4338-4348.

Smogorzewska, A., and de Lange, T. (2004). Regulation of telomerase by telomeric proteins. Annu Rev Biochem *73*, 177-208.

Smogorzewska, A., Karlseder, J., Holtgreve-Grez, H., Jauch, A., and de Lange, T. (2002). DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2. Curr Biol *12*, 1635-1644.

Song, K., Jung, D., Jung, Y., Lee, S. G., and Lee, I. (2000). Interaction of human Ku70 with TRF2. FEBS Lett *481*, 81-85.

Soulas-Sprauel, P., Le Guyader, G., Rivera-Munoz, P., Abramowski, V., Olivier-Martin, C., Goujet-Zalc, C., Charneau, P., and de Villartay, J. P. (2007). Role for DNA repair factor XRCC4 in immunoglobulin class switch recombination. J Exp Med *204*, 1717-1727.

Stark, J. M., Pierce, A. J., Oh, J., Pastink, A., and Jasin, M. (2004). Genetic steps of mammalian homologous repair with distinct mutagenic consequences. Mol Cell Biol *24*, 9305-9316.

Stavnezer, J., Guikema, J. E., and Schrader, C. E. (2008). Mechanism and regulation of class switch recombination. Annu Rev Immunol *26*, 261-292.

Stewart, G. S., Panier, S., Townsend, K., Al-Hakim, A. K., Kolas, N. K., Miller, E. S., Nakada, S., Ylanko, J., Olivarius, S., Mendez, M.*, et al.* (2009). The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage. Cell *136*, 420-434.

Stewart, J. A., Wang, F., Chaiken, M. F., Kasbek, C., Chastain, P. D., 2nd, Wright, W. E., and Price, C. M. (2012). Human CST promotes telomere duplex replication and general replication restart after fork stalling. Embo J *31*, 3537-3549.

Stracker, T. H., and Petrini, J. H. (2011). The MRE11 complex: starting from the ends. Nat Rev Mol Cell Biol *12*, 90-103.

Surovtseva, Y. V., Churikov, D., Boltz, K. A., Song, X., Lamb, J. C., Warrington, R., Leehy, K., Heacock, M., Price, C. M., and Shippen, D. E. (2009). Conserved telomere maintenance component 1 interacts with STN1 and maintains chromosome ends in higher eukaryotes. Mol Cell *36*, 207-218.

Suwaki, N., Klare, K., and Tarsounas, M. (2011). RAD51 paralogs: roles in DNA damage signalling, recombinational repair and tumorigenesis. Semin Cell Dev Biol *22*, 898-905.

Takai, H., Smogorzewska, A., and de Lange, T. (2003). DNA damage foci at dysfunctional telomeres. Curr Biol *13*, 1549-1556.

Takei, Y., Swietlik, M., Tanoue, A., Tsujimoto, G., Kouzarides, T., and Laskey, R. (2001). MCM3AP, a novel acetyltransferase that acetylates replication protein MCM3. EMBO Rep *2*, 119-123.

Tanaka, H., Abe, S., Huda, N., Tu, L., Beam, M. J., Grimes, B., and Gilley, D. (2012). Telomere fusions in early human breast carcinoma. Proc Natl Acad Sci U S A *109*, 14098-14103.

Tanaka, H., Mendonca, M. S., Bradshaw, P. S., Hoelz, D. J., Malkas, L. H., Meyn, M. S., and Gilley, D. (2005). DNA damage-induced phosphorylation of the human telomere-associated protein TRF2. Proc Natl Acad Sci U S A *102*, 15539-15544.

Tarsounas, M., Munoz, P., Claas, A., Smiraldo, P. G., Pittman, D. L., Blasco, M. A., and West, S. C. (2004). Telomere maintenance requires the RAD51D recombination/repair protein. Cell *117*, 337-347.

Tarsounas, M., and West, S. C. (2005). Recombination at mammalian telomeres: an alternative mechanism for telomere protection and elongation. Cell Cycle *4*, 672-674.

Taylor, W. R., and Stark, G. R. (2001). Regulation of the G2/M transition by p53. Oncogene *20*, 1803-1815.

Tejera, A. M., Stagno d'Alcontres, M., Thanasoula, M., Marion, R. M., Martinez, P., Liao, C., Flores, J. M., Tarsounas, M., and Blasco, M. A. (2010). TPP1 is required for TERT recruitment, telomere elongation during nuclear reprogramming, and normal skin development in mice. Dev Cell *18*, 775-789.

Teo, H., Ghosh, S., Luesch, H., Ghosh, A., Wong, E. T., Malik, N., Orth, A., de Jesus, P., Perry, A. S., Oliver, J. D.*, et al.* (2010). Telomere-independent Rap1 is an IKK adaptor and regulates NF-kappaB-dependent gene expression. Nat Cell Biol *12*, 758-767.

Thanasoula, M., Escandell, J. M., Martinez, P., Badie, S., Munoz, P., Blasco, M. A., and Tarsounas, M. (2010). p53 prevents entry into mitosis with uncapped telomeres. Curr Biol *20*, 521-526.

Thanasoula, M., Escandell, J. M., Suwaki, N., and Tarsounas, M. (2012). ATM/ATR checkpoint activation downregulates CDC25C to prevent mitotic entry with uncapped telomeres. Embo J.

Tomimatsu, N., Mukherjee, B., Deland, K., Kurimasa, A., Bolderson, E., Khanna, K. K., and Burma, S. (2012). Exo1 plays a major role in DNA end resection in humans and influences double-strand break repair and damage signaling decisions. DNA Repair (Amst) *11*, 441-448.

Tomita, K., and Cooper, J. P. (2008). Fission yeast Ccq1 is telomerase recruiter and local checkpoint controller. Genes Dev *22*, 3461-3474.

Tomita, K., Kibe, T., Kang, H. Y., Seo, Y. S., Uritani, M., Ushimaru, T., and Ueno, M. (2004). Fission yeast Dna2 is required for generation of the telomeric single-strand overhang. Mol Cell Biol *24*, 9557-9567.

Tomita, K., Matsuura, A., Caspari, T., Carr, A. M., Akamatsu, Y., Iwasaki, H., Mizuno, K., Ohta, K., Uritani, M., Ushimaru, T.*, et al.* (2003). Competition between the Rad50 complex and the Ku heterodimer reveals a role for Exo1 in processing double-strand breaks but not telomeres. Mol Cell Biol *23*, 5186-5197.

Tran, P. T., Erdeniz, N., Symington, L. S., and Liskay, R. M. (2004). EXO1-A multi-tasking eukaryotic nuclease. DNA Repair (Amst) *3*, 1549-1559.

Ubersax, J. A., and Ferrell, J. E., Jr. (2007). Mechanisms of specificity in protein phosphorylation. Nat Rev Mol Cell Biol *8*, 530-541.

Ueno, M. (2010). Roles of DNA repair proteins in telomere maintenance. Biosci Biotechnol Biochem *74*, 1-6.

Uringa, E. J., Lisaingo, K., Pickett, H. A., Brind'Amour, J., Rohde, J. H., Zelensky, A., Essers, J., and Lansdorp, P. M. (2012). RTEL1 contributes to DNA replication and repair and telomere maintenance. Mol Biol Cell *23*, 2782-2792.

van Steensel, B., Smogorzewska, A., and de Lange, T. (1998). TRF2 protects human telomeres from end-to-end fusions. Cell *92*, 401-413.

Vannier, J. B., Pavicic-Kaltenbrunner, V., Petalcorin, M. I., Ding, H., and Boulton, S. J. (2012). RTEL1 dismantles T loops and counteracts telomeric G4-DNA to maintain telomere integrity. Cell *149*, 795-806.

Verdun, R. E., Crabbe, L., Haggblom, C., and Karlseder, J. (2005). Functional human telomeres are recognized as DNA damage in G2 of the cell cycle. Mol Cell *20*, 551-561.

Verdun, R. E., and Karlseder, J. (2006). The DNA damage machinery and homologous recombination pathway act consecutively to protect human telomeres. Cell *127*, 709-720.

von Figura, G., Hartmann, D., Song, Z., and Rudolph, K. L. (2009). Role of telomere dysfunction in aging and its detection by biomarkers. J Mol Med (Berl) *87*, 1165-1171.

Vulliamy, T., Beswick, R., Kirwan, M., Marrone, A., Digweed, M., Walne, A., and Dokal, I. (2008). Mutations in the telomerase component NHP2 cause the premature ageing syndrome dyskeratosis congenita. Proc Natl Acad Sci U S A *105*, 8073-8078.

Walker, J. R., and Zhu, X. D. (2012). Post-translational modifications of TRF1 and TRF2 and their roles in telomere maintenance. Mech Ageing Dev *133*, 421-434.

Walne, A. J., and Dokal, I. (2009). Advances in the understanding of dyskeratosis congenita. Br J Haematol *145*, 164-172.

Wanat, J. J., and Johnson, F. B. (2012). Telomere stability and carcinogenesis: an off-again, on-again relationship. J Clin Invest *122*, 1962-1965.

Wang, B., Matsuoka, S., Carpenter, P. B., and Elledge, S. J. (2002). 53BP1, a mediator of the DNA damage checkpoint. Science *298*, 1435-1438.

Wang, C., Chen, T., Zhang, J., Yang, M., Li, N., Xu, X., and Cao, X. (2009). The E3 ubiquitin ligase Nrdp1 'preferentially' promotes TLR-mediated production of type I interferon. Nat Immunol *10*, 744-752.

Wang, C., and Meier, U. T. (2004). Architecture and assembly of mammalian H/ACA small nucleolar and telomerase ribonucleoproteins. Embo J *23*, 1857-1867.

Wang, F., Podell, E. R., Zaug, A. J., Yang, Y., Baciu, P., Cech, T. R., and Lei, M. (2007). The POT1-TPP1 telomere complex is a telomerase processivity factor. Nature *445*, 506-510.

Wang, M., Wu, W., Wu, W., Rosidi, B., Zhang, L., Wang, H., and Iliakis, G. (2006). PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. Nucleic Acids Res *34*, 6170-6182.

Wang, R. C., Smogorzewska, A., and de Lange, T. (2004). Homologous recombination generates T-loop-sized deletions at human telomeres. Cell *119*, 355-368.

Wang, X., and Baumann, P. (2008). Chromosome fusions following telomere loss are mediated by single-strand annealing. Mol Cell *31*, 463-473.

Wotton, D., and Shore, D. (1997). A novel Rap1p-interacting factor, Rif2p, cooperates with Rif1p to regulate telomere length in Saccharomyces cerevisiae. Genes Dev *11*, 748-760.

Wright, W. E., Tesmer, V. M., Liao, M. L., and Shay, J. W. (1999). Normal human telomeres are not late replicating. Exp Cell Res *251*, 492-499.

Wu, L., Multani, A. S., He, H., Cosme-Blanco, W., Deng, Y., Deng, J. M., Bachilo, O., Pathak, S., Tahara, H., Bailey, S. M.*, et al.* (2006). Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. Cell *126*, 49-62.

Wu, P., Takai, H., and de Lange, T. (2012). Telomeric 3' overhangs derive from resection by Exo1 and Apollo and fill-in by POT1b-associated CST. Cell *150*, 39-52.

Wu, P., van Overbeek, M., Rooney, S., and de Lange, T. (2010). Apollo contributes to G overhang maintenance and protects leading-end telomeres. Mol Cell *39*, 606-617.

Wu, X., Amos, C. I., Zhu, Y., Zhao, H., Grossman, B. H., Shay, J. W., Luo, S., Hong, W. K., and Spitz, M. R. (2003). Telomere dysfunction: a potential cancer predisposition factor. J Natl Cancer Inst *95*, 1211-1218.

Wu, Z. Q., Yang, X., Weber, G., and Liu, X. (2008). Plk1 phosphorylation of TRF1 is essential for its binding to telomeres. J Biol Chem *283*, 25503-25513.

Xie, A., Kwok, A., and Scully, R. (2009). Role of mammalian Mre11 in classical and alternative nonhomologous end joining. Nat Struct Mol Biol *16*, 814-818.

Xu, L., and Blackburn, E. H. (2004). Human Rif1 protein binds aberrant telomeres and aligns along anaphase midzone microtubules. J Cell Biol *167*, 819-830.

Yan, C. T., Boboila, C., Souza, E. K., Franco, S., Hickernell, T. R., Murphy, M., Gumaste, S., Geyer, M., Zarrin, A. A., Manis, J. P.*, et al.* (2007). IgH class switching and translocations use a robust non-classical end-joining pathway. Nature *449*, 478-482.

Yang, Q., Zheng, Y. L., and Harris, C. C. (2005). POT1 and TRF2 cooperate to maintain telomeric integrity. Mol Cell Biol *25*, 1070-1080.

Ye, J., Lenain, C., Bauwens, S., Rizzo, A., Saint-Leger, A., Poulet, A., Benarroch, D., Magdinier, F., Morere, J., Amiard, S.*, et al.* (2010). TRF2 and apollo cooperate with topoisomerase 2alpha to protect human telomeres from replicative damage. Cell *142*, 230-242.

Ye, J. Z., and de Lange, T. (2004). TIN2 is a tankyrase 1 PARP modulator in the TRF1 telomere length control complex. Nat Genet *36*, 618-623.

Ye, J. Z., Donigian, J. R., van Overbeek, M., Loayza, D., Luo, Y., Krutchinsky, A. N., Chait, B. T., and de Lange, T. (2004a). TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres. J Biol Chem *279*, 47264-47271.

Ye, J. Z., Hockemeyer, D., Krutchinsky, A. N., Loayza, D., Hooper, S. M., Chait, B. T., and de Lange, T. (2004b). POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex. Genes Dev *18*, 1649-1654.

You, Z., and Bailis, J. M. (2010). DNA damage and decisions: CtIP coordinates DNA repair and cell cycle checkpoints. Trends Cell Biol *20*, 402-409.

You, Z., Shi, L. Z., Zhu, Q., Wu, P., Zhang, Y. W., Basilio, A., Tonnu, N., Verma, I. M., Berns, M. W., and Hunter, T. (2009). CtIP links DNA double-strand break sensing to resection. Mol Cell *36*, 954-969.

Yu, X., and Chen, J. (2004). DNA damage-induced cell cycle checkpoint control requires CtIP, a phosphorylation-dependent binding partner of BRCA1 C-terminal domains. Mol Cell Biol *24*, 9478-9486.

Yu, X., Chini, C. C., He, M., Mer, G., and Chen, J. (2003). The BRCT domain is a phospho-protein binding domain. Science *302*, 639-642.

Yu, X., Fu, S., Lai, M., Baer, R., and Chen, J. (2006). BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP. Genes Dev *20*, 1721-1726.

Yun, M. H., and Hiom, K. (2009). CtIP-BRCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle. Nature *459*, 460-463.

Zhang, J. H., Chung, T. D., and Oldenburg, K. R. (1999). A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen *4*, 67-73.

Zhong, Q., Boyer, T. G., Chen, P. L., and Lee, W. H. (2002a). Deficient nonhomologous end-joining activity in cell-free extracts from Brca1-null fibroblasts. Cancer Res *62*, 3966-3970.

Zhong, Q., Chen, C. F., Chen, P. L., and Lee, W. H. (2002b). BRCA1 facilitates microhomology-mediated end joining of DNA double strand breaks. J Biol Chem *277*, 28641-28647.

Zhong, Z. H., Jiang, W. Q., Cesare, A. J., Neumann, A. A., Wadhwa, R., and Reddel, R. R. (2007). Disruption of telomere maintenance by depletion of the MRE11/RAD50/NBS1 complex in cells that use alternative lengthening of telomeres. J Biol Chem *282*, 29314-29322.

Zhou, B. B., Chaturvedi, P., Spring, K., Scott, S. P., Johanson, R. A., Mishra, R., Mattern, M. R., Winkler, J. D., and Khanna, K. K. (2000). Caffeine abolishes the mammalian G(2)/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity. J Biol Chem *275*, 10342-10348.

Zhu, X. D., Kuster, B., Mann, M., Petrini, J. H., and de Lange, T. (2000). Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. Nat Genet *25*, 347-352.

Zhu, X. D., Niedernhofer, L., Kuster, B., Mann, M., Hoeijmakers, J. H., and de Lange, T. (2003). ERCC1/XPF removes the 3' overhang from uncapped telomeres and represses formation of telomeric DNA-containing double minute chromosomes. Mol Cell *12*, 1489-1498.

Zhu, Z., Chung, W. H., Shim, E. Y., Lee, S. E., and Ira, G. (2008). Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. Cell *134*, 981-994.

Zou, L., and Elledge, S. J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science *300*, 1542-1548.