



ATM/ATR-Dependent Responses to Dysfunctional Telomeres during the G2/M Transition

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DECLARATION

I declare:

The work presented in this thesis is my own.

No portion of this work has been previously submitted for another qualification at this, or any other university.

Signed

Maria Thanasoula

ABSTRACT

Mammalian telomeres are nucleoprotein complexes at the end of chromosomes containing a specific protein complex, called shelterin. Shelterin protects chromosome ends from the DNA damage response (DDR), by facilitating the formation of a telomeric capping structure, called the T-loop. During their elongation in S phase, telomeres become transiently uncapped and can be sensed as DNA damage in G2 phase. This leads to the recruitment of DDR factors, such as phosphorylated histone H2AX (γ H2AX), to the telomeres forming the so-called, telomere dysfunction-induced foci (TIFs). My PhD work described here, indicates that DNA damage occurring during interphase can persist after entry into mitosis, indicated by the detection of γ H2AX at a subset of mitotic telomeres in human and mouse cells. This accumulation of γ H2AX to mitotic telomeres is ATM-dependent and the γ H2AX-labelled uncapped telomeres that persist, are shorter than the average telomere length for the entire cell population.

Most importantly, my work suggests that telomere uncapping, naturally occurring or artificially induced, is detected by two parallel ATM/ATR-dependent pathways at the G2/M transition: a p53/p21-dependent pathway through the ATM/ATR-mediated phosphorylation of p53 at Ser15 and a CHK1/CHK2-dependent pathway that acts through negative regulation of CDC25 phosphatases. In particular, telomere uncapping triggered by TRF2 depletion leads to CHK2-dependent CDC25A degradation, while POT1 depletion results in CHK1-mediated CDC25A and CDC25C degradation. Both pathways act as sensors of unprotected telomeres at the G2/M transition and block cell cycle progression

through inhibition of CDK1/Cyclin B complex, allowing telomere re-capping before entry into mitosis. This mechanism protects telomere integrity by the maintenance of a cell cycle stage conducive for capping reactions and thereby prevents genomic instability induced by telomere dysfunction.

Finally, I studied the cellular functions of 3 poorly characterised shelterin components, TRF1, RAP1 and TPP1, in telomere protection. TRF1 and to a lesser extent RAP1 were shown to be important for telomere protection by suppressing DDR at the telomeres, while TPP1 was shown to be mainly responsible for the recruitment of the catalytic subunit of telomerase, TERT, to the chromatin, contributing to telomere maintenance.

In conclusion, my work on both human and mouse models, reveals an important part of the DDR pathways activated by dysfunctional telomeres, as well as the molecular mechanisms underlying the cell cycle specific regulation of telomere capping, which ensures that only cells with intact telomeres enter mitosis.

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ABBREVIATIONS

°C	degree Celsius
53BP1	p53-binding protein 1
ADB	antibody dilution buffer
Ala	alanine
ALT	alternative lengthening of telomeres
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related protein
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CaCl ₂	calcium chloride
CDC25	cell division cycle 25/dual specificity phosphatase 25
CDK	cyclin dependent kinase
CDK1/cdc2	cyclin dependent kinase 1/cell division control protein 2 homolog
CENP-F	centromere protein F
ChIP	chromatin immunoprecipitation
CHK1	checkpoint kinase 1
CHK2	checkpoint kinase 2
cm	centimetres
Cre	Cre recombinase

DAPI	4,6-diamidino-2-phenylindole
DDR	DNA damage response
D-loop	displacement loop
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA-protein kinase
DNA-PKcs	DNA-PK catalytic subunit
Dox	doxycycline
ds	double - stranded
DSBs	double strand breaks
FACS	fluorescence - activated cell sorting analysis
FBS	foetal bovine serum
FCS	fetal calf serum
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
<i>g</i>	relative centrifugal force
G4	4 th generation
GADD45	growth arrest and DNA damage protein 45
GFP	green fluorescent protein

Gy	Gray
h	hour/hours
H3	histone 3
HCl	hydrochloric acid
His	histidine
HR	homologous recombination
IF	immunofluorescence
IgG	immunoglobulin G
IR	ionising radiation
kb	kilobases
KCl	potassium chloride
LT	SV40 large T-antigen
MDC1	mediator of DNA damage checkpoint 1
MDM2	transformed 3T3 cell double minute 2, p53 binding protein (mouse)
MEFs	mouse embryonic fibroblasts
mg	milligrams
MgCl ₂	magnesium chloride
min	minutes
ml	millilitres
mm	millimetres

mM	millimolar
Mre11	meiotic recombination element 11 homolog
MRN complex	MRE11/RAD50/NBS1
mRNA	messenger RNA
mTERT	mouse TERT
Myb	myeloblastosis oncogene
Na ₂ HPO ₄	sodium monohydrogen phosphate
NaCl	sodium chloride
NBS1	Nijmegen breakage syndrome protein 1
NFκB	nuclear factor kappa B
NHEJ	non-homologous end joining
O/N	overnight
p21	protein 21
p53	protein 53
PBS	phosphate buffered saline
PLK1	polo-like kinase 1
PNA	peptide nucleic acid
POT1	protection of telomeres
Q-FISH	quantitative FISH
QRT-PCR	quantitative reverse transcription-polymerase chain reaction

RAP1	the human ortholog of the yeast repressor/activator protein 1
RNA	ribonucleic acid
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	serine
shRNA	short hairpin RNA
siRNA	small interfering RNA
ss	single - stranded
TERC	telomerase RNA component
TERT	telomerase reverse transcriptase
Thr	threonine
TIF	telomere-dysfunction induced foci
TIN2	TRF2 and TRF1-interacting nuclear protein 2
T-loop	telomeric D-loop
TPP1/TINT1	TIN2-interacting protein 1
TRF1	telomeric repeat binding factor 1
TRF2	telomeric repeat binding factor 2
Tris	N,N,N',N'-tetramethyl-ethylene-diamine

Tyr	tyrosine
UV	ultraviolet light
Ve	vector
Wb	Western blotting
wt	wild-type
γ H2AX	gamma-Histone 2AX
μ g	micrograms
μ l	microlitres
μ M	micromolar

CHAPTER 1

Introduction

1.1 Telomeres and shelterin complex

Telomeres are nucleoprotein complexes consisting of long stretches of double-stranded (ds) deoxyribonucleic acid (DNA) tandem repeats at the ends of linear chromosomes, which terminate in a 3' single-stranded (ss) DNA overhang (Figure 1A). The DNA strand of the telomeres that constitutes the 3' -end is generally rich in guanosine and devoid of cytosine and is called the G-rich strand. The majority of mammalian cells use the sequence TTAGGG at their chromosome ends. The length of telomeric repeats varies between different mammals, from 10-15 kilobases (kb) in human at birth, to 20-50 kb, in the laboratory mice and rats (de Lange et al., 1990; Hastie et al., 1990; Kipling and Cooke, 1990; Lejnine et al., 1995). The 3' ss DNA overhang is conserved throughout the eukaryotic kingdom and its length varies between 50-500 nucleotides.

Human somatic cells lack detectable telomere length maintenance mechanisms resulting in a progressive loss of telomeric length in each cell division, the so-called end replication problem (Watson, 1972). Many cells use telomerase, a specialised reverse transcriptase (Greider and Blackburn, 1985), in order to maintain their telomeres to a certain length, in a process called telomere homeostasis. Telomerase is a ribonucleoprotein complex comprised of a reverse transcriptase (telomerase reverse transcriptase, TERT) and a ribonucleic acid (RNA) moiety (telomerase RNA component, TERC) (Feng et al., 1995; Greider and Blackburn, 1985, 1987; Lingner et al., 1997; Nakamura et al., 1997). Telomeres are thought to be replicated at the end of S phase by telomerase (Zhao et al., 2009) and in order to be replicated they have to be in their linear form.

However, the telomeres are not usually present in the cells in their linear form as they resemble double strand breaks (DSBs) and therefore can be recognised as DNA

damage and trigger a DDR, described below. Instead, as revealed by electron microscopy, mouse and human telomeres are organised in a large duplex lariat structure, called the T-loop [telomeric Displacement (D)-loop; (Griffith et al., 1999a); Figure 1A]. T-loops are presumably formed through strand invasion of the duplex telomeric repeat by the 3' overhang. The overhang then anneals with the C-rich strand, displacing the G-strand at this site into a D-loop. T-loops are thought to protect the telomeres by hiding the telomere terminus from the DNA damage repair machinery and they can be further stabilised by the binding of several telomeric factors in a process also known as telomere capping.

A number of proteins have been characterised, that either bind directly and specifically to the telomeric DNA sequences, or are recruited to the telomeres through interactions with other DNA binding factors (de Lange, 2005; Palm and de Lange, 2008). Shelterin, a complex of six proteins in mammalian cells, binds to the telomeres and protects chromosome ends against degradation and fusion (Figure 1B). It is composed of: TRF1 and TRF2 [telomeric repeat binding factor 1 and 2; (Bilaud et al., 1997; Broccoli et al., 1997; Chong et al., 1995; Zhong et al., 1992)], POT1 [protection of telomeres 1; (Baumann and Cech, 2001)], TIN2 [TRF2 and TRF1-interacting nuclear protein 2; (Kim et al., 1999b)], RAP1 [(the human ortholog of the yeast repressor/activator protein 1; (Li et al., 2000)] and TPP1 [formerly known as TINT1 for TIN2-interacting protein 1; (Houghtaling et al., 2004; Liu et al., 2004; Ye et al., 2004)].

Figure 1

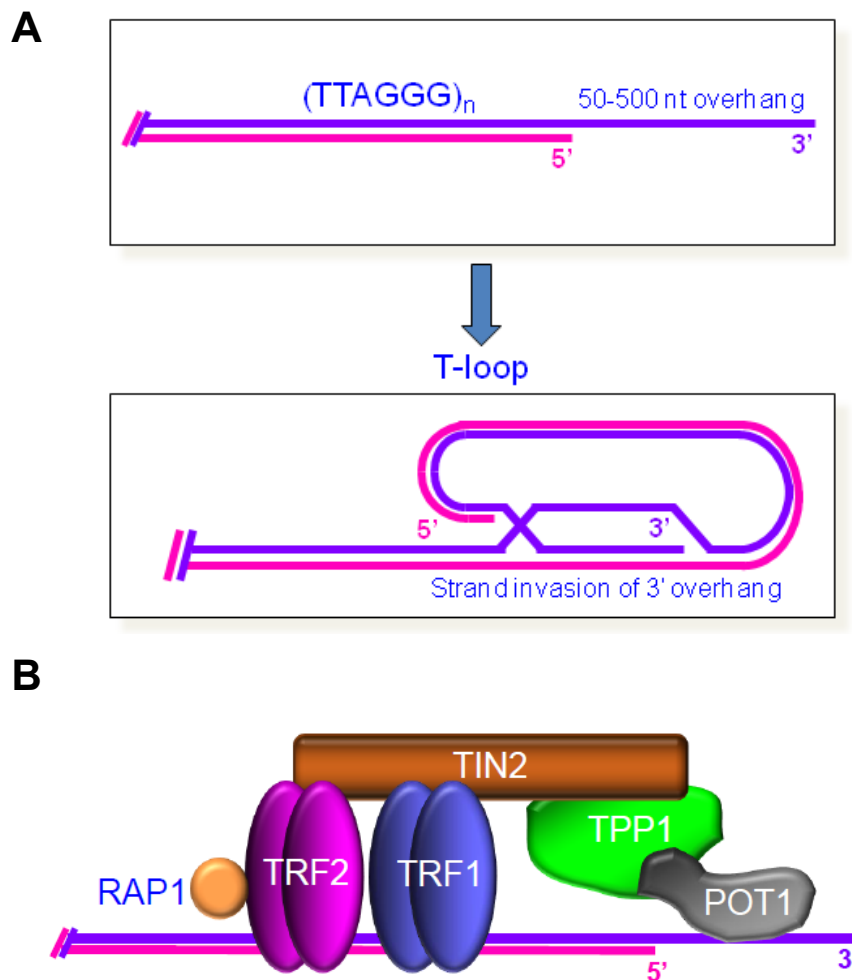


Figure 1: Structures of the mammalian telomeric DNA, the T-loop and the shelterin complex

(A) Human chromosomes end in an array of TTAGGG repeats that varies in length. The telomere terminus contains a long G-strand ss 3' overhang, which acts as a substrate for telomerase. After their replication telomeres form the T-loop configuration by strand invasion of the 3' overhang. (B) Schematic representation of how shelterin might be positioned on telomeric DNA. TRF1 and TRF2 are bound to the ds telomeric DNA, whereas POT1 binds to the ss TTAGGG repeats. TIN2 is directly bound to TRF1, TRF2 and TPP1 and is acting as a bridge between the ds and ss DNA-binding proteins of the shelterin complex. RAP1 is recruited to the shelterin complex via interaction with TRF2.

TRF1 and TRF2 are acting together to anchor the shelterin complex onto the telomeric ds DNA and play a key role in telomere protection and telomere length regulation (Palm and de Lange, 2008). They do not interact directly (Broccoli et al., 1997; Fairall et al., 2001), but they share a common domain structure consisting of the TRF homology domain and a C-terminal SANT/ myeloblastosis oncogene (Myb) DNA-binding domain (Bianchi et al., 1997; Billaud et al., 1997; Broccoli et al., 1997; Chen et al., 2008; Chong et al., 1995; Court et al., 2005; Hanaoka et al., 2005). Both of them are responsible for the recruitment of the other shelterin components, RAP1, TIN2, TPP1 and POT1, to the telomeres.

TRF1 and TRF2 are also well characterised in the context of telomere protection and elongation. TRF1 and TRF2 inhibition by the overexpression in human cells of truncated forms of these proteins, which act as dominant negative and inhibit the binding of the endogenous TRF1 and TRF2 to the telomeres, leads to telomere elongation, telomere uncapping and DDR activation at the telomeres (van Steensel and de Lange, 1997; Karlseder et al., 1999). TRF2 was also studied using mouse embryonic fibroblasts (MEFs) established from mouse models that allow the conditional *Trf2* gene deletion. According to this study TRF2 contributes to telomere maintenance by suppressing the ataxia telangiectasia mutated (ATM)-dependent DDR at the telomeres and the formation of end-to-end fusions between the uncapped telomeres through inhibition of non-homologous end joining (NHEJ) (Denchi and de Lange, 2007).

RAP1 is a poorly characterised binding partner of TRF2 [(Li et al., 2000); Figure 1B]. Mammalian RAP1 lacks DNA-binding activity and therefore, is dependent on TRF2 for its telomeric localisation and stability (Li and de Lange, 2003; Li et al., 2000). Most of RAP1 is lost from telomeres upon *Trf2* gene deletion (Celli and de Lange, 2005).

TIN2 has a central position in the shelterin complex and is able to bind TRF1, TRF2 and TPP1. TIN2 recruits TPP1 to the complex and it provides a bridge between the shelterin components which bind to ds telomeric DNA, including TRF1 and TRF2 and

POT1 which binds to the ss telomeric DNA [(Houghtaling et al., 2004; Kim et al., 2004; Kim et al., 1999b; Ye and de Lange, 2004; Ye et al., 2004); Figure 1B].

TPP1 is the connector of TIN2 with POT1 and is mainly responsible for the recruitment of POT1 to telomeres (Liu et al., 2004; Ye et al., 2004). It was found that human TPP1 recruits POT1 to telomeres through its binding to the carboxyl terminus of POT1 and that small interference RNA (siRNA)-mediated inhibition of TPP1 or over-expression of TPP1 mutants deficient for POT1 binding leads to removal of all detectable POT1 from telomeres in human cells (Liu et al., 2004). Moreover, impaired TPP1 function, both in mouse and human cells, can lead to telomere deprotection and long 3' telomeric overhang phenotype, similar to POT1 loss (Denchi and de Lange, 2007; Hockemeyer et al., 2007; Liu et al., 2004; Xin et al., 2007; Ye et al., 2004). However, the role of TPP1 in telomere protection apart from regulation of POT1 binding to telomeric DNA was not yet fully elucidated due to the lack of a mouse model for the complete knock-out of the TPP1 protein.

Finally, POT1 is involved in telomere protection by binding to the 3' telomeric ss overhang and the ss DNA in the D-loop of the T-loop configuration [(Hughes et al., 2000; Lin and Zakian, 1996; Mitton-Fry and Wuttke, 2002; Nugent et al., 1996; Theobald and Schultz, 2003); Figure 1B]. As already mentioned, human POT1 is recruited to telomeric DNA through its interaction with TPP1 and can bind directly to the ss G-rich strand substrates *in vitro* (Liu et al., 2004). In mouse cells there are two POT1 proteins found, POT1a and POT1b (He et al., 2006; Hockemeyer et al., 2006; Wu et al., 2006), that are both shown to require interaction with TPP1 for their telomeric localisation (Hockemeyer et al., 2007). POT1a and POT1b were shown to have distinct functions in telomere protection. In particular, it was reported that in MEFs, POT1a inhibits activation of the ATM and Rad3-related (ATR) kinase and contributes to the repression of the NHEJ at newly replicated telomeres. On the other hand, conditional *Pot1b* gene deletion leads to

unscheduled resection of the 5'-ended telomeric DNA strand, resulting in long 3' overhangs (Denchi and de Lange, 2007).

In addition to promoting and stabilising the T-loop structure, shelterin components contribute to telomere length regulation as part of their protective role at the telomeres. For instance, TRF1, as well as, TIN2, TPP1 and POT1 act as negative regulators of telomerase-mediated telomere elongation (Houghtaling et al., 2004; Kim et al., 1999b; Liu et al., 2004; Smogorzewska et al., 2000; Ye et al., 2004). The amount of TRF1 and other shelterin components increases with the number of TTAGGG repeats and therefore can be representative of the telomeric length (van Steensel and de Lange, 1997). For example, increasing the amount of TRF1 bound to the telomeres through over-expression leads to progressive telomere shortening, whereas a dominant negative form of TRF1 that removes the endogenous TRF1 from the telomeres leads to telomere elongation (van Steensel and de Lange, 1997). Moreover, POT1 also participates in telomere length regulation by acting as a *cis*-inhibitor of telomerase through binding to the 3' overhang, the substrate of telomerase (Loayza and de Lange, 2003; Ye et al., 2004).

The components of the shelterin complex recruit to chromosome ends several other proteins, a subset of which are DDR factors. Together with these “accessory” factors, the shelterin complex performs the essential function of protecting chromosome ends against attrition and illegitimate recognition as DNA damage (Blasco, 2005; Palm and de Lange, 2008).

1.2 DDR and cell cycle checkpoints

The maintenance of genome integrity is essential for the proper function and survival of all organisms. This task is particularly critical due to constant assault on the DNA by genotoxic agents (both endogenous and exogenous), nucleotide misincorporation during DNA replication and the intrinsic biochemical instability of the DNA itself (Lindahl, 1993).

Failure to repair DNA lesions may result in blockages of transcription and replication, mutagenesis and cellular cytotoxicity (Friedberg et al., 1995). In humans, DNA damage is involved in a variety of genetically inherited disorders, aging (Finkel and Holbrook, 2000) and carcinogenesis (Hoeijmakers, 2001). All eukaryotic cells have evolved a multifaceted response to deal with the potentially deleterious effects of DNA damage, the so-called DDR. Upon detection of DNA damage or replication fork stalling, cell cycle checkpoints are activated to arrest cell cycle progression to allow time for repair before the damage is passed on to daughter cells. Thus, DDR leads to activation of DNA repair pathways, or induction of senescence or initiation of apoptosis, when the level of damage incurred is too high (Zhou and Elledge, 2000).

The main checkpoints of the mammalian cell cycle are: the G1/S checkpoint which prevents damaged DNA from being replicated, the intra-S phase checkpoint that monitors replication progression and decreases the rate of DNA synthesis following DNA damage, and the G2/M cell cycle checkpoint, that allows suspension of the cell cycle progression prior to chromosome segregation (Figure 2).

Some of the early events of the DDR in mammalian cells include phosphorylation of histone H2AX by several different kinases depending on the type of the DNA damage introduced and activation of early DDR mediators responsible for the signal amplification (Stucki and Jackson, 2006). Such factors are the mediator of DNA checkpoint 1 (MDC1), which is recruited to the sites of DNA damage through direct interaction with γ H2AX (Stewart et al., 2003; Ward et al., 2003a), p53 binding protein 1 [53BP1;(Ward et al., 2003a; Ward et al., 2003b)] and Nijmegen breakage syndrome protein 1 (NBS1) which is a component of the meiotic recombination element 11 homolog (MRE11)/RAD50/NBS1 (MRN) complex and one of the primary sensors of DNA damage in most cells [(Bartek and Lukas, 2007; Petrini and Stracker, 2003); Figure 2].

The key regulators of the checkpoint response in mammalian cells are the ATM and ATR protein kinases. Both of these proteins belong to a structurally unique family of serine-threonine kinases characterised by a C-terminal catalytic motif containing a phosphatidylinositol 3-kinase domain (Abraham, 2001; Shiloh, 2001). Although ATM and ATR appear to phosphorylate many of the same cellular substrates (Kim et al., 1999a), they generally respond to distinct types of DNA damage. ATM is the primary mediator of the response to DSBs that can arise by exposure to ionizing radiation (IR) and various others DNA damaging agents, such as bleomycin, camptothecin and cisplatin. ATR, on the other hand, plays only a back-up role in the DSB response, but coordinates the response to ultraviolet (UV) radiation-induced damage and DNA replication fork stalling. One of the main roles of ATM and ATR kinases in both G1/S and G2/M cell cycle checkpoints is to induce the accumulation and activation of the tumour suppressor p53 protein.

In normally growing cells, p53 levels are low due to interaction with the inhibitor protein MDM2 (HDM2 in humans), which targets p53 for nuclear export and proteasome-mediated degradation in the cytoplasm [(Alarcon-Vargas and Ronai, 2002); Figure 2]. p53 stabilisation and enhancement of its transcriptional activity occurs through phosphorylation at Serine 15 (Ser15) (Dumaz and Meek, 1999), directly by ATM or ATR in response to IR, or only by ATR in response to UV irradiation and DNA replication fork stalling. Activated p53 then regulates a number of target genes, several of which are also involved in the DDR, e.g. MDM2, growth arrest and DNA damage protein 45 (GADD45) and p21 (Figure 2).

In addition to p53, other downstream targets of ATM and ATR are activated following DNA damage. In response to IR, ATM activates checkpoint kinase 2 (CHK2) through phosphorylation at Threonine 68 (Thr68) (Buscemi et al., 2001; Matsuoka et al., 2000), which in turn phosphorylates Ser20 of p53 (Figure 2). The Ser20 phosphorylation of p53 blocks p53/MDM2 interaction, resulting in p53 accumulation (Chehab et al, 1999,

2000; Shieh et al., 2000; Hirao et al., 2000). On the other hand, ATR is required for checkpoint kinase 1 (CHK1) activation in response to IR or UV (Abraham, 2001; Cuadrado et al., 2006; Jazayeri et al., 2006; Kastan and Lim, 2000; Shiloh, 2003), via its phosphorylation at Ser317 and Ser345 (Jiang et al., 2003; Liu et al., 2000; Zhao and Piwnica-Worms, 2001). There is also evidence for an ATM- and NBS1-dependent phosphorylation of CHK1 at Ser317 (Gatei et al., 2003). The role of ATR in p53-Ser20 phosphorylation and subsequent stabilisation is indirect, compared with that of the ATM/CHK2 pathway, demonstrated by *in vitro* CHK1-dependent Ser20 phosphorylation (Shieh et al., 2000).

There are also p53-independent pathways leading to cell cycle arrest activated by CHK1 and CHK2 kinases during the DDR, described more analytically in part 1.4. One of these pathways involves the phosphorylation of the dual specificity phosphatase CDC25 family members (Bartek and Lukas 2003; Kastan and Bartek 2004), which prevents them from activating cyclin-dependent kinases (CDKs) through removal of inhibitory phosphorylations. This, in turn, prevents activation of the various CDK/Cyclin complexes, a critical step that leads to exit from the blockage triggered by checkpoint activation and allows cell cycle progression (Figure 2).

Figure 2

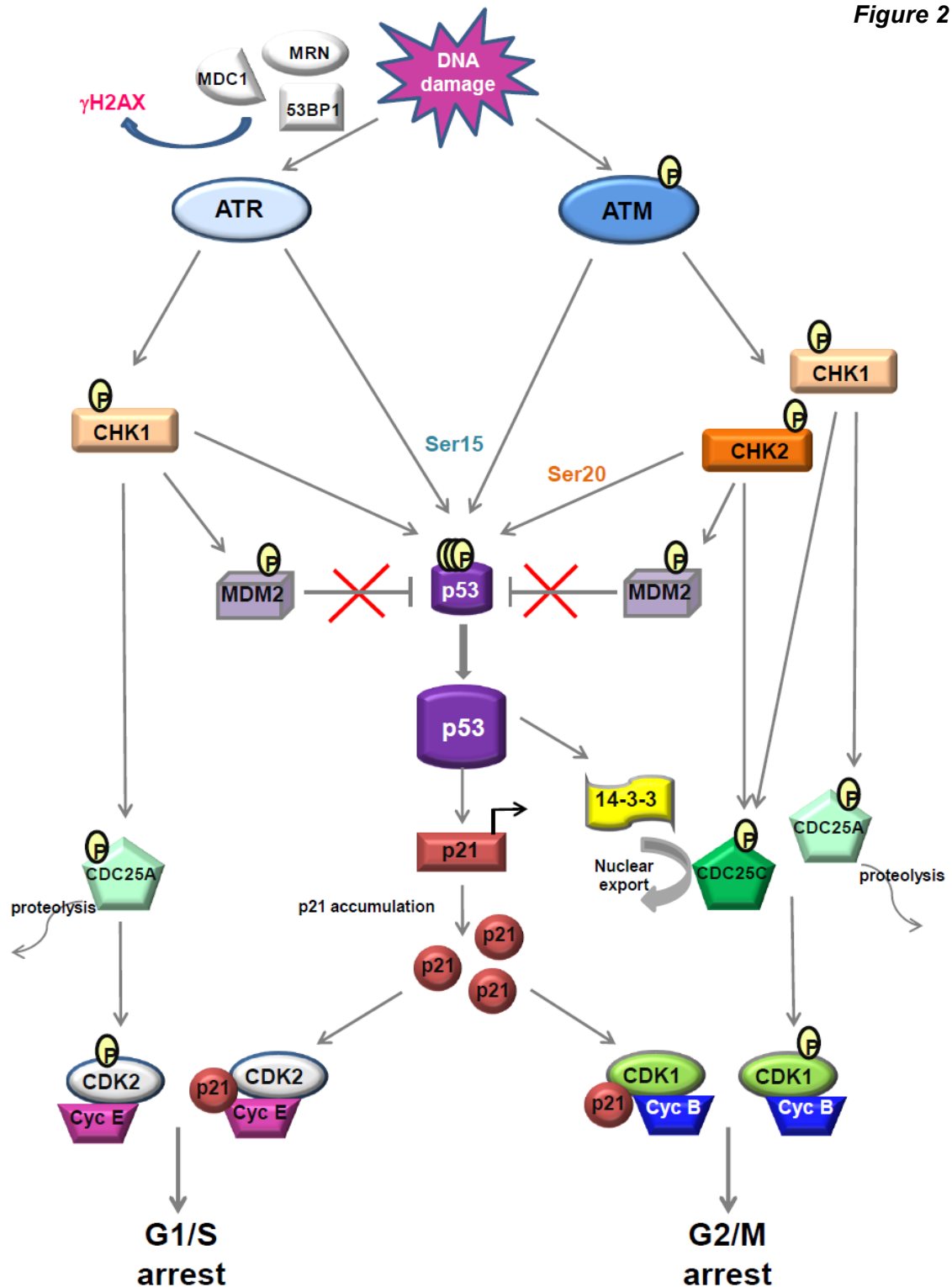


Figure 2: The DDR leading to G1/S and G2/M cell cycle arrest

In both G1/S and G2/M checkpoint arrest, ATM and ATR kinases are activated by DNA damage and then phosphorylate CHK1 and CHK2 kinases which in turn either activate p53, or inhibit CDC25 phosphatases. Alternatively, ATM and ATR directly activate p53 through Ser15 phosphorylation. After activated, p53 leads to p21 accumulation, which induces cell cycle arrest via transcriptional downregulation of CDK2/Cyclin E complex at G1/S, or CDK1/Cyclin B complex at G2/M. CDC25A is inhibited only by CHK1 at both G1/S and G2/M, whilst CDC25C is inhibited by both CHK1 and CHK2, but specifically at G2/M.

1.3 Telomere dysfunction and DDR

Telomere dysfunction can be the result of either critical shortening of the telomeres or loss of telomere protective factors in normally growing cells (d'Adda di Fagagna et al., 2003; Takai et al., 2003). It has been shown in both cases that dysfunctional telomeres can be sensed as DNA damage triggering the DDR (d'Adda di Fagagna et al., 2003; Takai et al., 2003). DDR activation can cause cell cycle arrest leading to either senescence or apoptosis. Alternatively, DDR can engage DNA repair machinery to join unprotected telomeres into deleterious end-to-end fusions, which can result in genomic instability, cell cycle arrest or cell death (Palm and de Lange, 2008). Thus, the signal emanating from dysfunctional telomeres resembles that triggered by unrepaired DSBs signalling.

Telomeres which have lost their protective structures become uncapped and co-localise with several DDR factors including γ H2AX, 53BP1, MRN complex, phosphorylated ATM and RAD17 (Takai et al., 2003). In senescent cells with short or uncapped telomeres, γ H2AX co-localises with checkpoint proteins such as ATM, 53BP1, MDC1 and NBS1 at telomeres (d'Adda di Fagagna et al., 2003). Telomeric accumulation of all these DNA damage factors leads to the formation of microscopically defined TIFs (d'Adda di Fagagna et al., 2003; Takai et al., 2003). It has been also shown by chromatin immunoprecipitation (ChIP) analysis that in cells undergoing senescence triggered by telomere shortening or uncapping, chromosome ends are directly associated with γ H2AX (d'Adda di Fagagna et al., 2003).

γ H2AX is a robust marker for DSBs, facilitating the assembly of DNA repair factors at the break site (Stucki and Jackson, 2006). H2AX can be phosphorylated by a number of different kinases in response to various DNA damaging agents. ATR is considered to be the primary kinase responsible for H2AX phosphorylation in response to replicative stress and UV radiation, while ATM targets H2AX primarily in response to IR

(Ward and Chen, 2001; Ward et al., 2004). Both of them phosphorylate H2AX at Ser139. DNA-protein kinase catalytic subunit (DNA-PKcs) contributes minimally to H2AX phosphorylation when ATM is present. However, in the absence of ATM or NBS1, which ensures rapid recruitment of ATM to the site of the DSB (Falck et al., 2005; You et al., 2005), DNA-PKcs seems to be the main kinase phosphorylating H2AX in response to IR (Hickson et al., 2004; Stiff et al., 2004).

ATM and ATR can also phosphorylate CHK1 and CHK2 kinases in response to DNA damage, leading to cell cycle arrest. More specifically, ATM, activated mainly by the presence of DSBs leads to CHK2 phosphorylation, while ATR mainly activated by the formation of ss DNA, phosphorylates CHK1. There are also data supporting an ATM-dependent activation of CHK1 in response to IR (Gatei et al., 2003; Sorensen et al., 2003). Activation of ATM and ATR can also happen in response to artificial telomere uncapping caused by the inhibition or deletion of individual shelterin components. In particular, in MEFs, telomeres artificially uncapped by conditional *Trf2* gene deletion elicit an ATM-dependent DDR leading to CHK2 phosphorylation and NHEJ of dysfunctional telomeres (Denchi and de Lange, 2007). In similar experiments, *Pot1a* gene deletion leads to activation of ATR kinase and induction of NHEJ at newly replicated telomeres, while POT1b represses unscheduled resection of the 5'-ended telomeric DNA strand, resulting in long 3' overhangs in *Pot1b*-deleted cells (Denchi and de Lange, 2007). Apart from the fact that NHEJ occurs in G1, there is very little known about cell-cycle specific DDR to uncapped telomeres in mouse and human cells, as well as about the roles of the rest of the shelterin components in the context of telomere protection and the responses triggered by their loss.

1.4 G2/M checkpoint regulation by checkpoint kinases CHK1 and CHK2

The G2/M checkpoint response to DNA damage such as DSBs or dysfunctional telomeres, that resemble unrepaired DSBs, leads to cell cycle arrest through inhibition of the main mitosis-promoting complex cyclin-dependent kinase 1 (CDK1)/Cyclin B. Progression into mitosis requires activation of CDK1 by its binding to Cyclin B and phosphorylation of Thr161 by CDK-activating kinase (Pines, 1995a, b). During G2-phase, the CDK1/Cyclin B complex is kept inactive by phosphorylation at tyrosine 15 (Tyr15) and Thr14 of CDK1 (Booher et al., 1997; Liu et al., 1997; Parker and Piwnica-Worms, 1992). The DDR in G2/M involves two main pathways activated by ATM and ATR kinases, both leading to CDK1/Cyclin B complex inhibition. One pathway includes p53 activation and stabilisation, either directly by ATM or ATR kinases via phosphorylation at Ser15, or indirectly by CHK1 and CHK2 kinases through phosphorylation at Ser20. Subsequently, p53 activates several factors such as p21, GADD45 and 14-3-3 σ , all leading to inhibition of CDK1/Cyclin B complex either via transcriptional regulation or regulation of its subcellular localisation.. A second pathway of the G2/M response to DNA damage involves activation of CHK1 and CHK2 by ATM/ATR, leading either to activation of p53 or, most commonly, to negative regulation of CDC25 phosphatases, also leading to CDK1/Cyclin B complex inhibition and cell cycle arrest before entry into mitosis.

CHK1 and CHK2 are key signal transducers in the network of genome integrity checkpoints (Bartek and Lukas, 2003) activated by ATM and ATR. CHK2 is a kinase stably expressed throughout the cell cycle and its activation requires its dimerisation and autophosphorylation, whereas CHK1 is largely expressed specifically during S and G2 phases and its activation does not require dimerisation or autophosphorylation (Bartek and Lukas, 2003). It is also known that CHK1 and CHK2 have mitotic roles controlling mitotic entry, mitotic exit and progression through mitosis in human cells (Bartek and Lukas, 2003).

After their activation, CHK1 and CHK2 initiate additional waves of phosphorylation to amplify signalling. In both G1/S and G2/M checkpoints, CHK1 and CHK2 are involved in the phosphorylation and regulation of CDC25 dual-specificity phosphatases that belong to the tyrosine phosphatase family, with roles in driving cells through the cell cycle (Bartek and Lukas, 2003; Kastan and Bartek, 2004). These enzymes remove crucial inhibitory phosphorylations on CDK/Cyclin complexes, the most important being dephosphorylation of Tyr15 in the adenosine triphosphate -binding loop of CDK1 and cyclin-dependent kinase 2 (CDK2) (Reinhardt and Yaffe, 2009). This allows the activation of CDK1/Cyclin B complex and progression in mitosis. In human cells, CDC25 proteins are encoded by a multigene family, consisting of *CDC25A*, *CDC25B*, and *CDC25C* (Galaktionov and Beach, 1991; Nagata et al., 1991; Sadhu et al., 1990). All three display more than 60% identity to each other within the carboxyl terminal, which encodes the catalytic domain.

During G2/M transition, one of the roles of CHK1 and CHK2 is to phosphorylate and inactivate CDC25A and CDC25C. The general mechanism for CDC25 inhibition after DNA damage via their CHK1- and CHK2-dependent phosphorylation, involves the ubiquitin-mediated degradation of CDC25A (Mailand et al., 2002; Sorensen et al., 2003), or the binding of CDC25A and CDC25C to 14-3-3 proteins leading to their subsequent cytoplasmic sequestration (Chen et al., 2003; Matsuoka et al., 1998). Altogether, this chain of events prevents activation of CDK1/Cyclin B complexes and progression in mitosis. In addition to CHK1/2-dependent negative regulation of CDC25 phosphatases at G2/M, CDC25C is also positively regulated by CDK1/Cyclin B complex itself and a positive feedback loop has been proposed (Hoffmann et al., 1993; Izumi and Maller, 1993; Strausfeld et al., 1994). Indeed, CDC25C phosphorylated by CDK1/Cyclin B is capable of inducing partial mitotic activation (Hoffmann et al., 1993; Strausfeld et al., 1994), while phosphorylation of CDC25C at Ser214 in mitosis inhibits its phosphorylation at Ser216 ensuring that CDK1 remains active once mitosis is initiated (Bulavin et al., 2003). This is a control mechanism that maintains the proper order of cell cycle

transitions.

In both human and mouse cells, CHK1 is important for the activation of the G2/M checkpoint in response to IR (Siljuasen et al., 2004; Sorensen et al., 2003; Zhao et al., 2002) and several other DNA damaging agents, such as camptothecin and doxorubicin (Xiao et al., 2003). Inhibition of CHK1 by chemical inhibitors (UCN-01 and CEP-3891) impairs the intra-S and G2/M checkpoints induced by IR and leads to mitotic nuclear fragmentation and cell death (Siljuasen et al., 2004). In response to IR (Sorensen et al., 2003; Zhao et al., 2002) or UV light (Mailand et al., 2000), CHK1 phosphorylates CDC25A at Ser123, which leads to its ubiquitin-mediated destruction or loss of phosphatase activity and G2/M arrest (Mailand et al., 2002; Sorensen et al., 2003). Phosphorylation of CDC25A by CHK1 also creates a 14-3-3 binding site that specifically inhibits its interaction with CDK1/Cyclin B complex (Chen et al., 2003). IR-induced proteolytic degradation of CDC25A is CHK1-dependent (Jin et al., 2008; Sorensen et al., 2003) and ATM is also required for this process (Sorensen et al., 2003). Additionally, CHK1 is also involved in CDC25C regulation during G2/M. Similarly to CDC25A, phosphorylation of CDC25C at Ser216 by CHK1 following IR creates a binding site for 14-3-3, which sequesters CDC25C to the cytoplasm and blocks its interaction with CDK1/Cyclin B complex (Peng et al., 1997; Sanchez et al., 1997).

CHK1 and Polo-like kinase 1 (PLK1), functionally interact to activate CDK1/Cyclin B complex (Kastan and Bartek, 2004), in the regulation of G2/M checkpoint. G2/M arrest triggered by DNA damage requires ATM and CHK1-dependent inhibition of PLK1 (Smits et al., 2000). However, during checkpoint adaptation, a process in which cell division can happen despite the presence of DNA damage, CHK1 and PLK1 control exit from the IR-induced G2/M checkpoint via independent pathways (Siljuasen et al., 2006).

Unlike CHK1, the importance of CHK2 for the DNA damage-induced G2/M checkpoint is highly controversial. Human CHK2 is unable to phosphorylate CDC25A or

support its ubiquitination *in vitro*. Studies in *CHK2*^{-/-} HCT116 cells showed that CHK2 is dispensable for CDC25A degradation and G2/M checkpoint activation after IR (Jin et al., 2008). Although CHK2 is required for p53 stabilisation in response to IR and other DNA damaging agents (Chehab et al., 1999, 2000; Shieh et al., 2000; Falck et al., 2001; Hirao et al., 2000), e.g. via its phosphorylation at Ser20 (Chehab et al., 2000; Hirao et al., 2000), there is also data supporting the opposite. Deletion of *CHK2* in human cells by homologous recombination (HR) showed that even in the absence of CHK2 the G2/M checkpoint in response to IR is completely functional resulting in p53 stabilisation and p21 activation (Jallepalli et al., 2003). Moreover, studies on *Chk1*^{+/-} and *Chk2*^{-/-} mice have shown that unlike IR-induced G1/S and intra-S phase checkpoints that require both CHK1 and CHK2, IR-induced G2/M checkpoint activation requires only CHK1 and not CHK2 (Niida et al., 2005). Consistent with this, the G2/M checkpoint is not impaired in *Chk2*^{-/-} MEFs, while p53 stabilisation and accumulation after IR is intact in the absence of CHK2 (Takai et al., 2000). Finally, *Chk2*^{-/-} mouse embryonic stem cells are defective for p53 stabilization and activation of p21 after IR, but can have a normal IR-induced G2/M arrest (Hirao et al., 2000).

On the other hand, recent evidence supports CHK2 activation by ATM during the G2/M transition in response to doxorubicin-induced DNA damage (Yang et al., 2010). Moreover, IR-induced phosphorylation of CDC25C at Ser216 by CHK2 results in its sequestration to the cytoplasm through binding to 14-3-3, and inhibits its interaction with CDK1/Cyclin B complex leading to G2/M arrest (Matsuoka et al., 1998). Regulation of cell cycle G2/M arrest by CHK2 seems to be exclusively dependent on CDC25C inhibition, as there is no data supporting the inhibition of CDC25A by CHK2 during G2/M. In conclusion, although the field of G2/M responses to general DNA damage is quite well elucidated there is very little known about the ATM/ATR- or CHK1/CHK2/CDC25-dependent signalling pathways involved in the detection of telomere dysfunction and especially in human cells.

Importantly, CHK1 and CHK2 have been identified as tumour suppressors. The first evidence that genetic alteration in *CHK2* may predispose to cancer was the finding of rare germline mutations in the *CHK2* gene in patients with Li-Fraumeni syndrome [LFS; (Bell et al., 1999)]. LFS is a familial cancer syndrome often linked to germline mutations in the *p53* gene. Nevertheless, in all the cases with *CHK2* mutations *p53* was wild type (wt) suggesting that germline mutations of *CHK2* may represent an alternative genetic defect predisposing to LFS. More studies (Ingvarsson et al., 2002; Sodha et al., 2002) support the involvement of *CHK2* defects in breast cancer and colon cancer susceptibility, and indicate distinct modes of action for different genetic variants of this tumour suppressor.

Somatic mutations of *CHK2* have been found in small subsets of diverse types of sporadic human malignancies, including carcinomas of the breast (Sullivan et al., 2002), lung (Haruki et al., 2000), vulva (Reddy et al., 2002), urinary bladder, colon (Bell et al., 1999), ovary (Miller et al., 2002), osteosarcomas (Miller et al., 2002), and lymphomas (Hangaishi et al., 2002; Tavor et al., 2001; Tort et al., 2002). Some of these mutants showed decreased or lost kinase activity (Matsuoka et al., 2001; Wu et al., 2001), while others are defective in recognizing their substrates such as CDC25A and *p53* (Falck et al., 2001a; Falck et al., 2001b; Li et al., 2002). Moreover, there is a significant subset of human tumours with low or undetectable CHK2 protein levels in the apparent absence of any *CHK2* mutations (Bartkova et al., 2001; Sullivan et al., 2002; Tort et al., 2002; Vahteristo et al., 2002), suggesting that these low CHK2 protein levels can be the result of post-transcriptional or post-translational regulation.

In contrast to the functionally overlapping CHK2 kinase, which qualifies as a tumour suppressor, cancer-associated defects of *CHK1* are rare and limited to carcinomas of the colon, stomach, and endometrium (Bertoni et al., 1999; Menoyo et al., 2001; Vassileva et al., 2002). Furthermore, a shorter isoform of CHK1 mRNA, has been detected in a subset of small cell lung carcinomas (Haruki et al., 2000). Despite the fact that CHK1 function and its analogy with CHK2 fit a candidate tumour suppressor gene,

the complete deficiency of *Chk1* in mice results in early embryonic lethality. However, deletion of *CHK1* in a *p53*-deficient chicken cell line is tolerable (Zachos et al., 2003), similarly with the heterozygous truncation mutations of *CHK1* in some human tumours (Bertoni et al., 1999). Therefore, the functional impact of *CHK1* mutations in cancer cells (usually resulting in truncated forms of CHK1) given their heterozygous state, remain unclear. It is conceivable that hypomorphic mutations of *CHK1* or *CHK1* defects that occur during a stage of cancer that the cells are less prone to apoptosis (such as those with mutant p53), may contribute to enhanced genetic instability in some tumours.

1.5 G2/M checkpoint regulation by p53

A major component of the cellular response to chromosomal DSBs is p53. p53 is a tumour suppressor protein mutated in about half of all human tumours. It protects mammals from genomic instability and neoplasia by inducing apoptosis, DNA repair and cell cycle arrest in response to a variety of cellular stress. One of the most critical components of the response triggered by DNA damage is p53-dependent cell cycle arrest at the G1 phase. At G1/S, p53 is activated in response to DNA damage by ATM/ATR or CHK1/CHK2 kinases, which inhibit p53 interaction with MDM2, resulting in its stabilisation. Thereafter, p53 activates mainly p21 leading to cell cycle arrest through transcriptional regulation of several proteins (Bartek and Lukas, 2001; Ekholm and Reed, 2000; Hartwell and Kastan, 1994; Ryan et al., 2001; Sherr and Roberts, 1999; Vogelstein et al., 2000).

p53 is also implicated in the regulation of entry into mitosis when cells enter G2 with damaged DNA. Although it has been shown that *p53*-null cell types can still arrest in G2 in response to DNA damage (Kastan et al., 1991), the importance of p53 in G2/M arrest has been reported under specific conditions. For example, in human fibroblasts, the inactivation of p53 by human papilloma virus E6 protein resulted in increased mitotic entry

after IR (Filatov et al., 1998; Kaufmann et al., 1997; Thompson et al., 1997). A clearer role of p53 in the G2/M checkpoint was revealed by using a derivative of the human colorectal tumor cell line HCT116 in which p53 was inactivated by HR (Bunz et al., 1998). These cells were able to arrest at G2/M in response to IR, but this arrest was not stable, compared with isogenic cells expressing wild-type p53, which exhibited a prolonged G2/M arrest. Therefore, p53 is not required for the initial G2/M arrest in HCT116 cells but is essential for the maintenance of the arrest.

As already mentioned, p53 can be directly activated by ATM and ATR via its phosphorylation at Ser15 in response to DNA damage and genotoxic stress (Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999). Phosphorylation of Ser15 represents an early cellular response to a variety of genotoxic stress (Shieh et al., 1997; Siliciano et al., 1997). p53 that has been phosphorylated at Ser15 *in vitro* displays reduced binding to MDM2 (Shieh et al., 1997). Because the association with MDM2 targets p53 for a proteasome-mediated degradation and inhibits its transactivating function (Haupt et al., 1997; Kubbutat et al., 1997), Ser15 phosphorylation promotes both the accumulation and functional activation of p53 in response to DNA damage. In addition, p53 can be activated and stabilised via its phosphorylation at Ser20 (Chehab et al., 1999). Substitution of Ser20 was sufficient to abrogate p53 stabilisation in response to both IR and UV light. Both IR and UV light-induced phosphorylation of p53 at Ser20, weakened the interaction of p53 with MDM2 *in vitro* (Chehab et al., 1999). CHK2, rather than CHK1, is believed to be mainly responsible for this phosphorylation event at both G1/S and G2/M cell cycle checkpoints (Chehab et al., 2000; Hirao et al., 2000; Shieh et al., 2000).

Activated p53 blocks cells at the G2/M checkpoint through downregulation of CDK1, required to enter mitosis (Nurse, 1990). CDK1 is mainly inhibited by three transcriptional targets of p53, GADD45, p21, and 14-3-3 σ . p53 can also directly transcriptionally repress *Cyclin B* and *CDK1* genes, in order to ensure the maintenance of the G2/M arrest (Taylor and Stark, 2001).

p21, the best characterized p53 target, participates in both G1/S and G2/M checkpoints. HCT116 colorectal tumor cells lacking p21 did not arrest stably in G2 after exposure to IR, similarly to HCT116 cells lacking p53 (Bunz et al., 1998). Failure to arrest was associated with levels of CDK1 kinase activity higher than that observed in p21-proficient cells. Some of the mechanisms suggested to mediate regulation of the G2 checkpoint by p21 include the inhibition of CDK activities either by direct binding of p21 to CDK1/Cyclin B complex (Boulaire et al., 2000), or through inhibition of proteins involved in the generation of active CDK1, such as CDK2 (Guadagno and Newport, 1996).

An additional mechanism by which p53 contributes to the G2/M arrest involves regulation of the subcellular localisation of CDK1. In order to progress through mitosis, CDK1/Cyclin B complex has to be translocated to the nucleus by importin α and β . In the presence of DNA damage at the G2/M, 14-3-3 σ , another downstream target of p53, can bind to CDK1/Cyclin B complex and sequester it in the cytoplasm inhibiting its activation and cell cycle progression (Hermeking et al., 1997).

p53 is also activated by dysfunctional telomeres. It has been shown that TRF2 inhibition can lead to p53- and ATM-dependent apoptosis in a subset of mammalian cell types (Karlseder et al., 1999). Moreover, when primary human fibroblasts were cultured for many population doublings, the progressive telomere shortening eventually resulted in structural changes at the telomeres and the induction of p53- and p16/retinoblastoma protein-dependent replicative senescence (Harley et al., 1990; Karlseder et al., 2002; Shay et al., 1991). In addition, production of G-rich ss fragments during telomere shortening under oxidative stress conditions in human cells is sufficient to trigger a p53/p21-dependent cell cycle arrest (Saretzki et al., 1999).

Hepatocellular carcinoma is a disease commonly associated with functional inactivation of p53. In mouse models telomere dysfunction produced by mouse *mTERT* gene knockout, led to advanced hepatocellular carcinoma disease, under chronic liver

damage conditions (Farazi et al., 2006). Therefore, attenuated p53 function in combination with telomere-induced chromosomal instability may play critical and cooperative roles in the progression of tumourigenesis. Consistent with this, telomerase-deficient MEFs which also lack p53 have a lower ability to arrest at the G2/M transition in response to dysfunctional telomeres than their p53-wild type counterparts (Chin et al., 1999). More studies in TERC-deficient MEFs, support a key role of p53 in mediating proliferative arrest in response to telomere damage *in vivo* (Feldser and Greider, 2007; Stout and Blasco, 2009). Despite these data connecting p53 with telomere dysfunction, very little is known about the cell cycle specific role of p53 in response to telomere dysfunction, such as telomere uncapping naturally occurring during S/G2 phase or induced disruption of telomere protective structures.

1.6 Telomeres and human disease

Emerging evidence demonstrates that telomere biology is involved in the pathophysiology of aging and human diseases, including cardiovascular disease, Type 2 diabetes, neurological diseases and cancer [reviewed in (Zhu et al., 2011)]. In particular, telomeres have been metaphorically proposed to be cancer's Achilles heel, since they are essential to stabilise and protect linear chromosomes (Zumstein and Lundblad, 1999). Since telomere dysfunction, induced either by loss of telomere-binding proteins or erosion of telomeric sequences is associated with loss of cell viability through induction of apoptosis (Zumstein and Lundblad, 1999), many anti-cancer treatments are focused on telomerase inhibition. Indeed, studies in mice that lack TERC showed that telomerase inhibition in mammalian cells leads to telomere shortening, increased chromosomal instability and loss of viability (Blasco et al., 1997). Furthermore, inhibition of telomerase in various cancer cell lines, also led to telomere shortening and cell death or differentiation (Hahn et

al., 1999; Kondo et al., 1998). These data suggested that telomerase inhibition might suppress tumour growth by leading to an accelerated telomere shortening and cell death.

Importantly, 85% of human cancers are telomerase-positive (Shay and Bacchetti, 1997) and many of the remaining 15% of cancers maintain their telomeres by a telomerase-independent mechanism termed alternative lengthening of telomeres (ALT) (Bryan et al., 1995; Bryan et al., 1997). ALT appears to involve DNA repair proteins and HR-dependent replication of telomeric DNA (Dunham et al., 2000; Lundblad and Blackburn, 1993; McEachern and Blackburn, 1996), while the T-loop structure could also account for telomerase-independent telomere elongation (Griffith et al., 1999b; Palm and de Lange, 2008). Therefore, it is conceivable that anticancer treatments based only on telomerase inhibition may be inefficient. This notion is supported by studies in mouse models lacking telomerase showing that a fraction of telomerase-deficient mice appear to develop lymphomas at a higher frequency than the wild type counterparts (Rudolph et al., 1999). These tumours are possibly the consequence of loss of checkpoints associated with telomere loss (Chin et al., 1999). However, different tissues have different sensitivities to telomere loss, as for example the skin of telomerase-deficient mice is resistant to chemical tumorigenesis (Gonzalez-Suarez et al., 2000), indicating that telomerase inhibition in skin tumours might be able to inhibit their growth. Our knowledge so far indicates that telomeres are a potentially good target for new anticancer therapies. In the past most of the efforts to compromise telomere function in cancer cells have involved telomerase inhibition. However, future studies should focus not only on development of telomerase inhibitors but also on inhibitors of telomeric proteins, disruption of telomere structure, or disruption of alternative telomere maintenance pathways. There is also a great necessity to generate new mouse models, similar to the ones already generated that support the conditional deletion of genes encoding a number of shelterin components (Denchi and de Lange, 2007), that will allow a thorough study of how telomere protection can be regulated and what are the *in vivo* consequences of the

loss of checkpoint pathways that monitor telomere uncapping and protect telomere integrity.

CHAPTER 2

Materials and methods

2.1 Cell lines, culture conditions and treatments

HeLa 1.2.11, HeLa OHIO, U2OS, HCT116 wild type and $p53^{-/-}$ cells (Bunz et al., 1998), MO59J, MO59K (Allalunis-Turner et al., 1997; Anderson et al., 2001), SAOS-2 and WI38-VA13 (all obtained from Cancer Research UK Cell Services) were cultivated in monolayers in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with antibiotics (penicillin and streptomycin, Sigma Aldrich) and 10% fetal bovine serum (FBS, Invitrogen). Human T4 lymphocytes carrying an inducible TRF2^{ΔMΔB} expression construct (van Steensel et al., 1998) were cultivated in DMEM supplemented with antibiotics (penicillin and streptomycin), 10% FBS and 100 ng/ml doxycycline (Dox; Sigma Aldrich). Cells were collected at the time of Dox removal (day 0), as well as 3 and 7 days later. Primary MEFs were isolated from day 13.5 embryos as previously described (Blasco et al., 1997; Herrera et al., 1999; Jacks et al., 1994; Martinez et al., 2009) and cultivated in DMEM supplemented with antibiotics (penicillin and streptomycin) and 10% FBS (serum from United States, Invitrogen). $p21^{-/-}$ MEFs with $p21$ exon 3 deleted (Brugarolas et al., 1995) were a gift from M. Serrano (CNIO). Littermate-derived isogenic wild-type and $p53^{-/-}$ null MEFs, isogenic $Trf1^{F/F} p53^{-/-}$ and $Trf1^{F/F} p53^{+/+}$ floxed for the $Trf1$ exon 1, $Tpp1^{F/F}$ MEFs, isogenic $Rap^{+/+}$ and $Rap1^{F/F}$ MEFs immortalized by SV40 Large T antigen (LT) expression, as well as MEFs deleted for the germline copy of the mouse telomerase RNA gene (mTR) established from C57BL6 embryos of 4th generation (G4) $Terc^{-/-}$ immortalized by p53 depletion [short hairpin RNA (shRNA) for p53; (Dirac and Bernards, 2003)] have been previously described (Herrera et al., 1999; Jacks et al., 1994; Martinez et al., 2009), all gifts from Maria Blasco.

γ -irradiation was carried out using a ¹³⁷Cs source at the doses indicated. Human cells were arrested in mitosis by adding 0.1 mg/ml of KARYOMAX colcemid (GIBCO) to

the media overnight (O/N) or 0.1 mg/ml 4 hours (h) prior to collection in the case of SAOS-2 and WI38-VA13 cells. Primary MEFs were treated with 0.1 mg/ml colcemid O/N and another 0.1 mg/ml for 4 h prior to collection or irradiation. ATM inhibitor Ku-55933 (Hickson et al., 2004; R&D Chemicals) was added to the media at 20 μ M final concentration in dimethyl sulfoxide (DMSO) 1 h prior to collection or irradiation. In the case of the synchronised U2OS cells, Ku-55933 was added to the media at 20 μ M final concentration, 6 h after double thymidine block release (see part 2.2) and 4 h prior to collection. Caffeine (SIGMA) was added to the media in a final concentration of 5 mM, 6 h after double thymidine block release and 4 h prior to collection. ATR inhibitor ETP-46464 [a gift from Oscar Fernandez Capetillo;(Toledo et al., 2011)] was added again 6 h after double thymidine block release and 4 h prior to collection at a final concentration of 5 μ M. Similarly, the inhibitors UCN-01 (Merck-Chemicals) and Gö6976 (Tocris Bioscience) were added to the media in a final concentration of 300 nM each, 6 h after double thymidine block release and 4 h prior to collection.

2.2 Synchronisation of human cells

Synchronisation of HeLa 1.2.11, HCT116 and U2OS cells was performed using a double thymidine block. Exponentially growing cells were arrested by addition of thymidine (SIGMA) (2 mM) to the growth media for 16 h, followed by two washes in 1 X phosphate buffered saline {PBS; [sodium chloride (NaCl) 137 mM, potassium chloride (KCl) 2.7 mM, sodium monohydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$) 8.1 mM, potassium dihydrogen phosphate (KH_2PO_4) 1.76 mM, pH 7.4]} and released into fresh media for 10 h. Cells were then arrested a second time by addition of thymidine (2 mM) and 16 h incubation, then washed as above and released into fresh media. Samples were collected for Western blotting (Wb), phospho-histone-H3 (Ser10) or bromodeoxyuridine (BrdU) staining and fluorescence - activated cell sorting (FACS) analysis for the indicated timepoints.

2.3 FACS analysis

Cells were collected at the indicated timepoints, washed once in 1 X PBS and centrifuged at 300xg for 5 min. Each pellet was resuspended in 300 µl 1 X PBS and 700 µl cold absolute ethanol was added dropwise, while vortexing gently. The samples were fixed O/N at -20°C. The samples were centrifuged for 5 min at 300xg, the pellets were washed with 1 X PBS and resuspended in a final volume of 500 µl PBS. Ribonuclease A (SIGMA) was added at a final concentration of 20 mg/ml and an incubation of 15 min at 37°C followed. Finally, propidium iodide was added at a final concentration of 50 mg/ml and the samples were stored at 4°C O/N until analysed by FACS.

2.4 Mitotic index and phospho-histone H3 (Ser10) staining

For determination of the mitotic index of the *Trf1*-floxed MEFs, cells were treated O/N with colcemid, 4 and 6 days after puromycin selection. Then all cells were collected by trypsinisation, fixed in methanol and spread on slides. Following that, the slides were mounted using ProLong Antifade kit (Invitrogen) supplemented with 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI). Cell nuclei stained by DAPI were visualised under the microscope and mitotic cells were determined by their characteristic structure.

For determination of the mitotic index of the HCT116 cells, cells were synchronized with double thymidine block, released and collected for the indicated timepoints. Then all cells were fixed and stained by IF as described below using an antibody against phospho-H3 (Ser10) in order to determine which cells were in mitosis. For each individual experiment 200 nuclei were scored.

For determination of the mitotic fraction in human cells, cells and media were collected at the indicated timepoints. They were washed once with PBS and centrifuged

at 300xg for 5 min. The pellets were directly resuspended first in 300 µl of PBS and then 700 µl of ethanol was added. O/N incubation at -20°C followed. The fixed cells were washed with PBS twice and blocked with FACS incubation buffer [0.5% bovine serum albumin (BSA) in PBS] for 45 min at room temperature (RT) rotating. The cells were centrifuged at 300xg for 5 min and incubated with anti-phospho-histone H3 (Ser10) antibody at 1:50 dilution in FACS incubation buffer for 2 h at RT. The cells were again centrifuged at 300xg for 5 min and the pellets were washed once with FACS incubation buffer. Incubation for 1 h at RT with the secondary antibody fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (immunoglobulin G) antibody (Jackson ImmunoResearch), diluted 1:100 followed. The cells were washed in 1 X PBS and resuspended in 500 µl of PBS. Finally, Ribonuclease A at a final concentration of 20 mg/ml and propidium iodide at a final concentration of 50 mg/ml was added. The samples were stored at 4°C until analysed by FACS.

2.5 Brdu/DNA staining of nuclei extracted from fixed cells

Prior to collection U2OS cells were stained with 20 µM BrdU (SIGMA) for 20-30 min at 37°C. After collection they were resuspended in 300 µl of PBS and 700 µl of ethanol and stored at -20°C for at least 30 min up to O/N. They were then centrifuged at 250xg for 5 min and resuspended in 1 ml of 2 M hydrochloric acid (HCl) containing 0.1 mg/ml pepsin. Incubation at RT for 20 min followed, one wash in PBS and one in 0.5% FCS (Fetal Calf Serum)/0.5% TWEEN in PBS. The cells were centrifuged at 700xg and resuspended in PBS containing 2% FCS and anti-BrdU mouse monoclonal antibody in a dilution of 1:100 (BD, clone 44) for 90 min at RT. The cells were then a centrifuged at 700xg for 5 min, resuspended in 100 µl PBS containing FCS and FITC-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch) (1:200 dilution) and incubated for 60 min at RT in the dark. Finally, the cells were washed in PBS and resuspended in 0.5 ml of PBS with

propidium iodide at a final concentration of 50 mg/ml. The samples were stored at 4°C until analysed by FACS.

2.6 siRNA-mediated depletion in human cells

U2OS and HCT116 cells were transfected using DharmaFect transfection reagent (Thermo Scientific). $1.5\text{--}2 \times 10^6$ cells were reverse-transfected in 10 cm plates. Cells were transfected with 40 nM siRNA/plate, the media was changed 24 h later and the cells were collected and processed 48 h post transfection. All the siRNAs used were obtained from Dharmacon Research and their sequences are given in part 2.15. Two days after siRNA transfection, U2OS cells were either treated with 0.1 mg/ml colcemid (Gibco), then harvested by trypsinisation and processed for combined immunofluorescence (IF) - fluorescent *in situ* hybridization (FISH) or harvested by trypsinisation and processed for Wb.

For cell synchronisation, the first dose of 2 mM thymidine was added to U2OS cells 48 h after transfection. FACS analysis of DNA content and phospho-histone H3- and BrdU- stained cells was performed as previously described. GFPsi, TRF2si and POT1si – treated SAOS-2 and WI38-VA13 cells were transfected with empty vector, wild type and mutants p53 Serine15Alanine (Ser15Ala) and Serine20Alanine (Ser20Ala) DNA constructs [gift from Dr. Xuan Liu; (Shouse et al., 2008)] using Lipofectamine (Invitrogen) 24 h post siRNA treatment. 3×10^6 cells were transfected in 10 cm plates with 24 µg of DNA/plate, the media was changed 5 h later and the cells were collected 24 h later for FACS analysis of phospho-histone H3 stained cells and Wb.

2.7 Quantitative reverse transcription polymerase chain reaction (QRT-PCR)

For determination of POT1 depletion, the levels of messenger RNA (mRNA) were measured 24 h after siRNA transfection by QRT-PCR using the Fast SYBR® Green Cells-to-CT™ kit from Applied Biosystems. The primers used for POT1 amplification were Forward 5'- TCAGATGTTATCTGTCAATCAGAACCT and Reverse 5'- TGTTGACATCTTTCTACCTCGTATAATGA (SIGMA). We also used as an internal control for the QRT-PCR the levels of ribosomal RNA 18S with the following primers: Forward 5'- AGTCCCTGCCCTTTGTACACA and Reverse 5'- GATCCGAGGGCCTCACTAAAC (Lin et al., 2006).

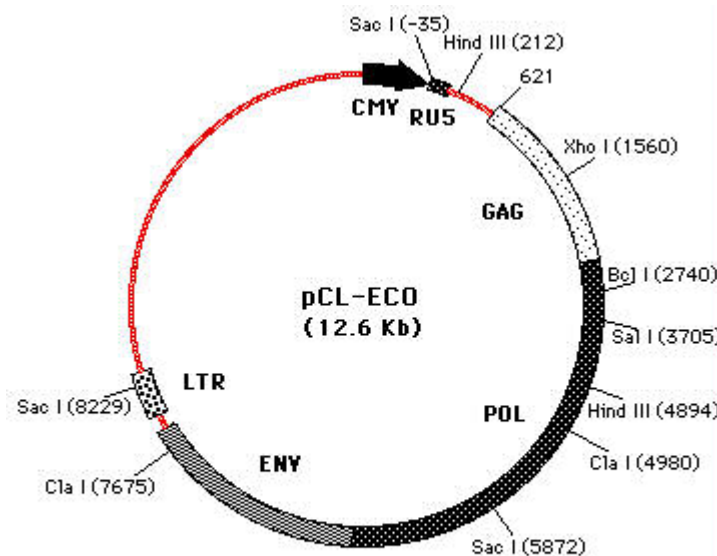
2.8 Retroviral infection of MEFs

293T cells seeded into 10 cm dishes (2×10^6 per dish) were transfected the next day with 10 µg of the pBabeGFP empty vector, or the Hit and Run-MMPCreGFP expression vector and 10 µg of the packaging plasmid (pCL-Eco) in 100 µl 2.5M calcium chloride (CaCl_2) and water up to 1 ml. One ml of 2 X HBS buffer (16.4 g NaCl, 11.9 g HEPES, 0.21 g Na_2HPO_4 ; add dH_2O up to 1L, pH 7.00-7.15) was added to the DNA/ CaCl_2 mix while bubbling with air and the final mix was added to each plate of 293T cells dropwise. In this step the 293T cells receive all the plasmids necessary to produce the retroviral particles that will be afterwards released to the growth media and will be collected and used for the infection of the recipient cells. The pBabeGFP vector is an empty vector serving as a control, while the Hit and Run-MMPCreGFP vector contains the gene expressing the Cre recombinase enzyme responsible for the excision of the genes that are flanked with loxP sites in the genome of the recipient MEFs. This Cre recombinase is called Hit and Run as it can be self-excised after its expression, therefore limiting any unspecific excision events that can be a result of the Cre recombinase long-term expression in the recipient cells. Next day, the growth media was removed and replaced with 10 ml of fresh media. The recipient MEFs were seeded ($2-3 \times 10^5$ per 10 cm dish) and incubated at 37°C O/N. Next

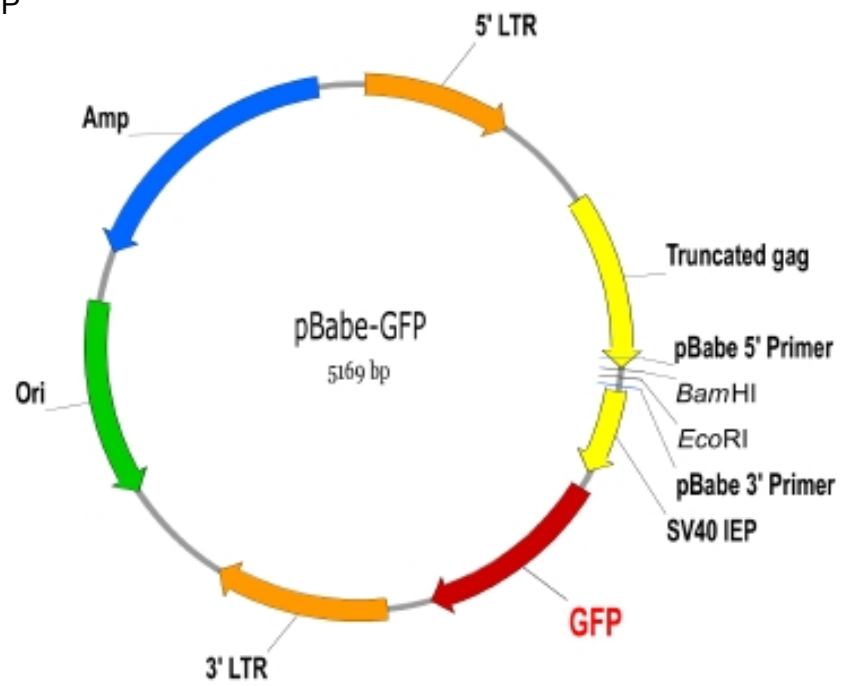
day the retrovirus-containing media was collected from the 293T cells and replaced with fresh growth media in order to produce and release more retroviral particles for the next round of infections. The retrovirus-containing media was centrifuged at 300xg for 10 min at RT, filtered through 0.45 mm filter, transferred into a clean tube and diluted with growth media 1:2 in order to remove any remaining 293T cells that can potentially contaminate the recipient cell population. Polybrene (hexadimethrine bromide, Sigma H9268) was added at a final concentration of 8 µg/ml for increased infection efficiency. The media was removed from the recipient cells and they were covered with the diluted viral supernatant (10 ml/10 cm dish). Next day the infection was repeated once in the morning and once again 7 h later as described above. Finally, the MEF media was replaced with a fresh one containing puromycin as selecting agent (3 µg/ml final concentration) and the cells were kept in selection for 4 to 6 days.

Vectors used for retroviral infection:

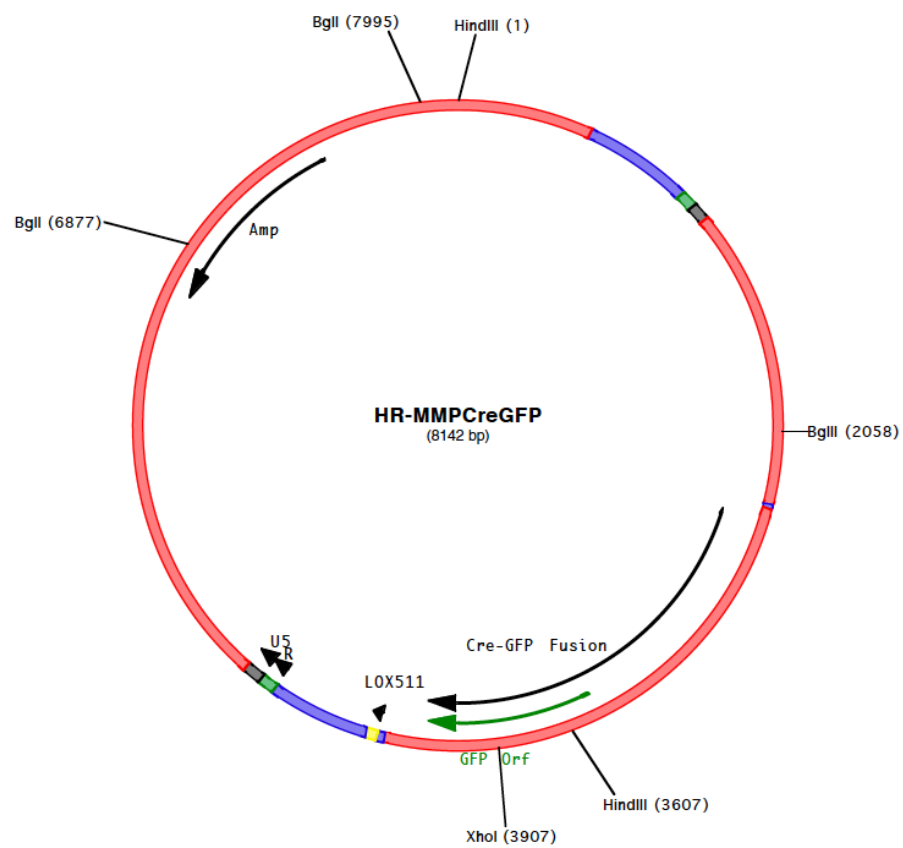
A) pCL-ECO



B) pBabe-GFP



C) HR-MMPCreGFP



2.9 Metaphase collection and immunofluorescence (IF)

For IF staining, cells were seeded in 10 cm plates 24 h prior to colcemid treatment (1.5×10^6 cells/10 cm dish). When IR was used, cells were allowed to recover for 2 h before collection. To prepare mitotic chromosome spreads, mitotic shake off was performed when a large number of round cells, possibly metaphases, were obtained. Alternatively, all the cells were collected by trypsinisation. The cells were centrifuged at 300xg for 5 min and the pellet was incubated in hypotonic solution (30 mM N,N,N',N'-tetramethylethylene-diamine (Tris) pH 8.2, 50 mM sucrose, 17 mM sodium citrate) for 5 min. Cells were resuspended in 100 mM sucrose, pH 8.2 and spread on microscope slides previously dipped in fixative [1% paraformaldehyde, 5 mM sodium borate (pH 9.2), 0.15% Triton X-100 (Barry and Alberts, 1994)]. The slides were left to dry at RT for 2.5 h in a wet chamber. They were washed twice in 1 X PBS with 0.4% Photoflo (SIGMA) and fixed in 4% paraformaldehyde in dH₂O (pH 8.5-9) for 10 min at RT. Cells were then permeabilised in 1 X PBS with 0.2% Triton X-100 at RT for 5 min and blocked in 1 x antibody dilution buffer (ADB; 1% goat -serum, 0.3% BSA, 0.005% Triton X-100 in PBS). Antibody staining was performed as previously described (Tarsounas et al., 2003). Briefly, the slides were incubated with primary antibody diluted in 1 X ADB O/N at RT in the dark. Then 3 washes followed on a rotating platform for 5-10 min each: 2 washes in 1 X ADB with 0.4% Photoflo and one wash in 1 X ADB with 0.005% Triton. The slides were then incubated with secondary antibody diluted 1/400 in 1 X ADB for 1h at RT in the dark and a 5-10 min wash in 1 X PBS with Photoflo on a rotating platform followed. The slides were then mounted using ProLong Antifade kit (Invitrogen) supplemented with 1 µg/ml of DAPI. Specimens were viewed with a Leica DMI6000B inverted microscope and fluorescence imaging workstation equipped with a HCX PL APO 100x/1.4-0.7 oil objective, and images acquired using a Leica DFC350 FX R2 digital camera using LAS-AF software (Leica). Brightness levels and contrast adjustments were applied to the whole image using Photoshop CS3 (Adobe).

2.10 Metaphase collection with cytospin

Mitotic cells were resuspended at a concentration of 10^6 cells/ml in pre-warmed hypotonic solution [(10 mM Tris pH7.5, 10 mM NaCl, 5 mM magnesium chloride (MgCl_2))] at 37°C for 5 min. Each cell suspension was diluted 1:4 with hypotonic solution and the cells were spread on slides using Cytospin 3 apparatus. 250-500 μl of cell suspension (~100,000 cells) was loaded per spot on slide and the slides were spun 300xg for 5 min. The slides were left to dry fast at RT (at least 90% dry) and then were immediately immersed into ice-cold fixative solution (4% paraformaldehyde, 250mM HEPES, 1 X PBS, 0.1-0.5% Triton X-100) at 4°C for 20 min. Then they were washed with ice-cold 1 X PBS for 4-5 times. The cells were permeabilised with 0.5% Triton X-100 (concentration of Triton X-100 was optimised for different proteins) in 1 X PBS at 4°C for 20 min. The slides were then washed again with ice-cold 1 X PBS for 4-5 times and blocked with 1 X ADB in for 15-20 min at RT.

2.11 IF – FISH

Mitotic cell collection and IF protocol were performed as described above. FISH was performed immediately after incubation with the secondary antibody. Slides were washed, fixed in 2-4% paraformaldehyde in PBS (pH 8.5) for 10 min at room temperature, then washed again 3 times in 1 X PBS for 5 min each time and left to dry for 5-20 min. 15 μl of telomeric probe mix [10 mM Tris pH7.5, 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 $\mu\text{g/ml}$ Cy3-TEL- peptide nucleic acid (PNA) probe [(CCCTAA)₆; 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 was added. Following denaturation on a hot plate at 80°C for 3 min,

the slides were incubated at RT 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 1 X PBS and dH₂O, the slides were left to dry for about 20 min before mounting them as described above. Quantitative analysis of telomeric signals was performed as previously described (Blasco et al., 1997; Tarsounas et al., 2004).

2.12 Cell extracts fractionation

The cells were collected by trypsinisation, centrifuged at 300xg and washed with cold PBS. The pellets were resuspended in a concentration of 10⁷ cells/0.5ml in ice-cold buffer A+ (HEPES pH7.9 10 mM, KCl 10 mM, MgCl₂ 1.5 mM, sucrose 0.34 M, glycerol 10%, in water). They were then incubated on ice for 5 min and centrifuged for 5 min at 1300xg at 4°C. The supernatant (S1) contained cytoplasmic proteins, while the pellet contained the nuclei (P1). S1 was further cleaned by centrifugation for 20 min at 20,000xg at 4°C. The pellet P1 was washed once more in buffer A+ and centrifuged again for 5 min at 1300xg at 4°C. Each pellet was resuspended in a concentration of 10⁷ cells/0.25 ml in ice-cold buffer B (ethylene-diamine-tetraacetic acid 3 mM and ethylene-glycol-tetraacetic acid 0.2 mM in water). The cell suspensions were incubated on ice for 30 min and then centrifuged for 5 min at 1700xg at 4°C. The supernatants were transferred (S3 = soluble nuclear protein) into fresh tubes. The pellets (P3 = insoluble chromatin) were washed once more with buffer B and centrifuged for 5 min at 1700xg at 4°C. The pellets (P3) were resuspended in sodium dodecyl sulfate (SDS) gel loading buffer (10⁷ cells/150 µl) and sonicated on ice to break down the DNA with the following settings: power dial ~50%, time dial at ~20"; 10-15 pulses. The samples were analysed by Wb.

2.13 Western blotting (Wb)

Cells were harvested by trypsinisation, washed with cold PBS and re-suspended in SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer (0.16 M Tris-HCl pH6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue, 100mM final concentration of dithiothreitol) at a concentration of 2×10^7 cells/ml. The samples were sonicated, boiled at 100°C for 5 min and centrifuged for 10 min at 20,000xg. Volumes corresponding to equal numbers of cells were analysed by gel electrophoresis followed by Wb. NuPAGE-Novex 10% Bis-Tris and NuPAGE-Novex 3-8% Tris-Acetate gels (Invitrogen) were run in SDS-running buffers according to manufacturer's instructions using the XCell SureLock Mini-Cell system (Invitrogen). The proteins were transferred onto a nitrocellulose membrane using 1 X Transfer Buffer (Invitrogen) with 10% methanol by semi-dry transfer.

For Wb the membranes were blocked by blocking buffer [5% milk in PBS-Tween (0.05%)] for 1 h and the primary antibody incubation was done at 4°C O/N in blocking buffer. Secondary antibody incubation was done in blocking buffer similarly to primary antibody incubation for 1 h at RT and then the membranes were washed in PBS-Tween before antibody detection. For antibody detection 3 different sets of detection reagents [Horse Radish Peroxidase (HRP) Luminol Enhancer Reagent and HRP Peroxide Reagent] were used depending on the intensity of the signal: Immobilon Western detection reagent 1 and 2 (Millipore) is medium intensity; the other two detection reagent 1 and 2 (Thermo Scientific) and Lumigen solution A and B (GE Healthcare) are either less or far more sensitive. An automatic X-ray film processor was used for the exposure of special X-ray films (Fujifilm).

2.14 Antibodies

The following antibodies were used for Wb: rabbit polyclonal antisera raised against human TRF2 (Tarsounas et al., 2004), mouse TRF2 [2390; (Munoz et al., 2005)], mouse

TERT (Martin-Rivera et al., 1998), mouse TIN2 (ab13791-100, abcam), human RAP1 (Tarsounas et al., 2004), human POT1 (MTA40), human histone H3 (a gift from A. Verreault), Ser15-p53 (Cell Signalling) and human p21 (C-19, Santa Cruz), human CHK1 (Cell Signalling), human CDC25C (C-20, Santa Cruz) human phospho-CHK1 Ser137 (Cell Signalling) and human phospho-CHK2 Thr68 (Cell Signalling); mouse monoclonal antibodies raised against phosphorylated ATM-Ser1981 (Cell Signalling), mouse TRF1 (TRF-78, Abcam), p53 (DO-1, Santa Cruz Biotechnology), 14-3-3 σ (Upstate), CHK2 (Clone 7, Millipore), CDC25A (F-6, Santa Cruz Biotechnology), Cdc2/CDK1 p34 (B322, Santa Cruz Biotechnology) and α -tubulin [Cancer Research UK Monoclonal Antibody Service; (Tarsounas et al., 2004)]. Additional antibodies used for IF detection were: mouse monoclonal antibodies raised against MDC1 (Abcam), phosphorylated histone H2AX-Ser139 (JBW301; Upstate), phosphorylated ATM-Ser1981 (Cell Signalling) and rabbit polyclonal antiserum raised against 53BP1 (Novus), centromere protein F (CENP-F; ab5, Abcam) and mouse TRF1 (Munoz et al., 2005).

2.15 siRNAs sequences

All the siRNA sequences were purchased from Dharmacon Research:

- a) green fluorescent protein [GFP;(Tarsounas et al., 2004)]: CGA CUG GGA CUU
CAA GUA G
- b) hp53: CGU AGA AUA GGC UCA CCU UAA
- c) hp21 (CDKN1A ON-TARGETplus set of 4 siRNAs): CGA CUG UGA UGC GCU
AAU G, CCU AAU CCG CCC ACA GGA A, CGU CAG AAC CCA UGC GGC A,
AGA CCA GCA UGA CAG AUU U
- d) hTRF2 (Takai et al., 2003): AGG AGC ACA CCG UGU CCC GAU UU, CAG AAG
UGG ACU GUA GAA GUU

- e) hTRF1 (TERF1 ON-TARGETplus set of 4 siRNAs): CAA AUU CUC AUA UGC
CUU U, CAG UAG UAG UCC UUU GAU A, AGA GUA ACC UAU AAG CAU G,
UAC CAG AGU UAA AGC AUA U
- f) hPOT1 (SiGenome no18): CAG GAG UAC UAG AAG CCU A
- g) hChk1 (CHEK1, ON-TARGETplus no10 and no12): CAA GAU GUG UGG UAC
UUU A, CCA CAU GUC CUG AUC AUA U
- h) hChk2 (CHEK2 siGenome SMARTpool): CUC AGG AAC UCU AUU CUA U, AAA
CGC CGU CCU UUG AAU A, GCU AAA UCA UCC UUG CAU C, GAA AUU GCA
CUG UCA CUA A

CHAPTER 3

Results

Part 1

3.1 DDR factors at human telomeres during mitosis

Spontaneous DNA damage is present in most cells and is believed to occur primarily during DNA replication, in the S phase of the cell cycle. Telomere uncapping as a form of spontaneous damage also leads to checkpoint activation and recruitment of DDR factors (e.g. 53BP1, MDC1, γ H2AX and ATM) to the telomere, visualised as TIFs (Takai et al., 2003; d'Adda di Fagagna et al., 2003). As previously mentioned in the introduction, telomere capping is a process regulated by cell cycle progression. Telomeres become transiently uncapped during every cell cycle following their replication in S phase and the ensuing ATM-dependent response is thought to promote the re-assembly of protective telomeric structures in G2 phase (Verdun et al., 2005).

To monitor telomere re-capping at the G2/M transition under physiological conditions, we synchronised human HeLa 1.2.11 cells at late G1/S phase by double thymidine block and release. The cells in late S and G2 phase were identified by IF staining against CENP-F. CENP-F shows a characteristic diffused staining during these phases of the cell cycle (Figure 3A) (Fletcher et al., 2003). Several γ H2AX foci were detectable in G2 cells by IF and a subset of them localised to telomeres, indicative of TIFs (Figure 3A). Telomeres were detected by FISH using a telomeric (CCCTAA)₆-PNA probe. Quantification of the TIFs showed that the majority of the G2 cells (around 50%) have 2-5 TIFs/cell (Figure 3B).

Figure 3

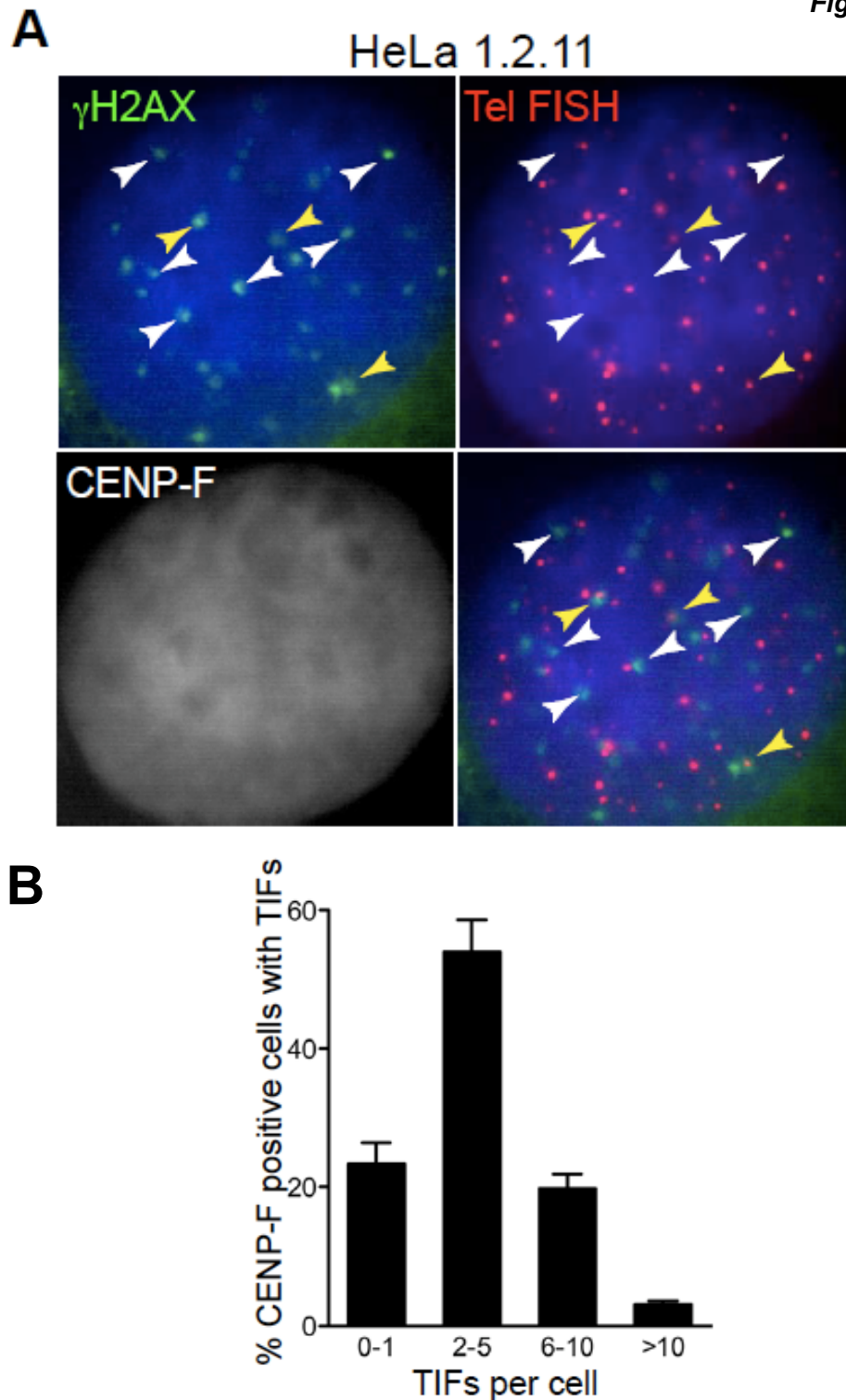
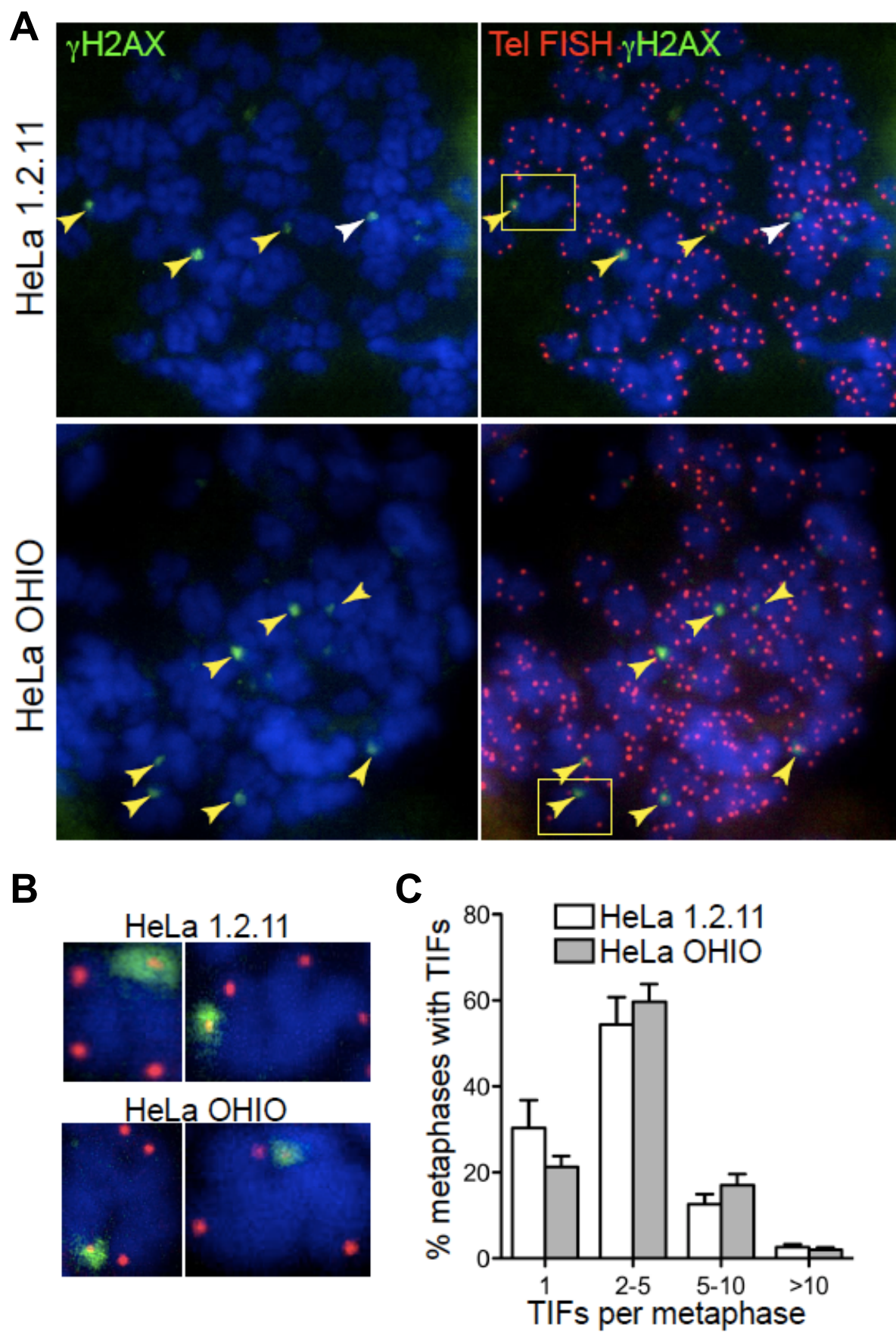


Figure 3: TIF detection in HeLa cells during late S/G2

(A) HeLa 1.2.11 cells grown on coverslips were stained with anti- γ H2AX (green) and anti-CENP-F antibodies (white). Telomeres were visualised with a Cy3-conjugated (CCCTAA)₆-PNA probe (Tel FISH; red). DNA was counter stained with DAPI (blue). White arrows indicate γ H2AX foci that do not co-localise with telomeres. Yellow arrows indicate TIFs. (B) TIF frequency was quantified in 100 CENP-F positive cells per experiment. Error bars represent standard deviation (SD) of three independent experiments.

Upon entry into mitosis, a fraction of γ H2AX-labelling persisted and it exclusively co-localised with telomeres in cells arrested in mitosis after colcemid treatment. To confirm γ H2AX localisation at telomeres, we visualised TIFs on mitotic chromosomes by co-localisation of γ H2AX and telomeric FISH signals (Figures 4A, B). The frequency of mitotic TIFs was comparable with that of interphase TIFs (2-5 TIFs/cell) (Figures 3B), suggesting a persistence of G2-uncapped telomeres into mitosis. γ H2AX labelling of mitotic telomeres was found not only in the HeLa 1.2.11 cells, which carry relatively long telomeres [approx. 17 kb; (Canela et al., 2007)], but also in the HeLa OHIO cells with shorter telomeres [approx. 3.4 kb; (Canela et al., 2007)] (Figures 4A, B). The number of γ H2AX-labelled telomeres in mitotic HeLa OHIO cells was even higher than in HeLa 1.2.11 cells (Figure 4C). We further analysed the association of γ H2AX foci with mitotic telomeres in two other human cell lines, MO59K and MO59J (Figure 6B). Mitotic TIFs were observed in these cell lines as well, indicating that the phenomenon observed is not cell type-specific. In addition to γ H2AX, we also detected MDC1 at mitotic telomeres (Figure 4D), a damage response mediator at the G2/M transition which is recruited to the sites of DNA damage through a direct interaction with γ H2AX (Stewart et al., 2003; Ward et al., 2003a). In contrast, 53BP1, another damage response protein (Ward et al., 2003a; Ward et al., 2003b), was not detected at mitotic TIFs (Figure 4D). The same was true for NBS1, one of the primary sensors of DNA damage in most cells (Bartek and Lukas, 2007; Petrini and Stracker, 2003) (data not shown).

Figure 4



D

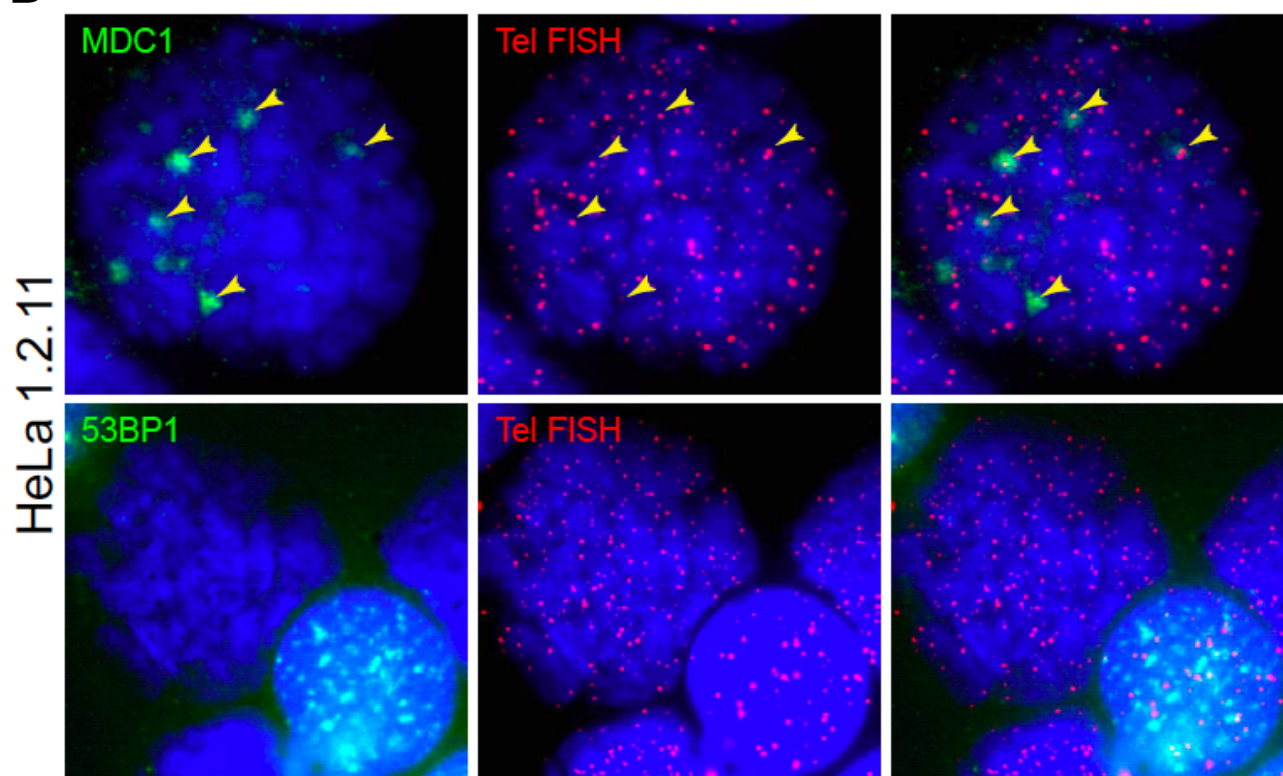


Figure 4: γ H2AX persists at telomeres during mitosis

(A) Mitotic chromosomes isolated from HeLa 1.2.11 and HeLa OHIO cells arrested in mitosis with colcemid were spread onto glass slides via the cytopspin method. Preparations were fixed and stained with anti- γ H2AX monoclonal antibody (green). Telomeres were visualised with a Cy3-conjugated (CCCTAA)₆-PNA probe (red). Yellow arrows indicate TIFs. White arrows point at γ H2AX foci that do not co-localise with telomeres. (B) Enlarged image of the areas marked with yellow rectangles in (A) illustrating TIFs. (C) Quantification of TIFs in HeLa 1.2.11 and HeLa OHIO cells. One hundred metaphases were scored for each cell line. Error bars represent standard deviation (SD) of three independent experiments. (D) HeLa 1.2.11 cells were arrested in mitosis with colcemid and mitotic chromosomes were spread onto glass slides using the cytopspin method. Preparations were immunostained with anti-MDC1 or anti-53BP1 antibodies (green). Telomeres were visualised with a Cy3-conjugated (CCCTAA)₆-PNA probe (red). DNA was counter stained with DAPI (blue). Yellow arrows indicate TIFs.

3.2 Mitotic TIFs mark short, uncapped telomeres

Short human telomeres can become uncapped more easily and thus be recognised as DNA damage (Meier et al., 2007). Therefore, we sought to address whether a correlation existed between telomere length and the occurrence of mitotic TIFs. Our initial results suggested that HeLa OHIO cells, which have shorter telomeres, show a slightly higher incidence of telomeric γ H2AX staining during mitosis compared to HeLa 1.2.11 cells (Figure 4C). This observation was confirmed by IF-FISH performed on mitotic chromosome preparations of HeLa 1.2.11 (Figure 5A). As the intensity of the FISH signals is proportional to the telomere length we were able to observe that nuclei with stronger telomeric FISH signals, indicative of long telomeres (Figure 5A, long) typically lack TIFs. In contrast, TIFs were abundant in metaphases with shorter telomeres, based on the lower FISH signal intensity (Figure 5A, short). To quantitatively assess whether γ H2AX-labelling was linked to short telomere length, we measured the intensities of individual telomeric FISH signals in mitotic HeLa 1.2.11 cells using the Q-FISH technique and recorded their γ H2AX-status in the same preparation. This technique allowed us to estimate the average telomere length of the total population at around 17 kb, as well as the average length of the telomeres labelled by γ H2AX in that population (Figure 5B). This analysis revealed that shorter telomeres were indeed more likely to be labelled by γ H2AX. 20% of γ H2AX-positive telomeres, based on analysis of 530 telomeres, but only 7% of all telomeres from approximately 200 metaphases analysed, were shorter than 10 kb in length (Figure 5B). Thus, γ H2AX-associated telomeres are shorter than the average for all telomeres (p value <0.0001, rank sum Wilcoxon test), consistent with the idea that shorter telomeres could become uncapped more easily and labelled by γ H2AX.

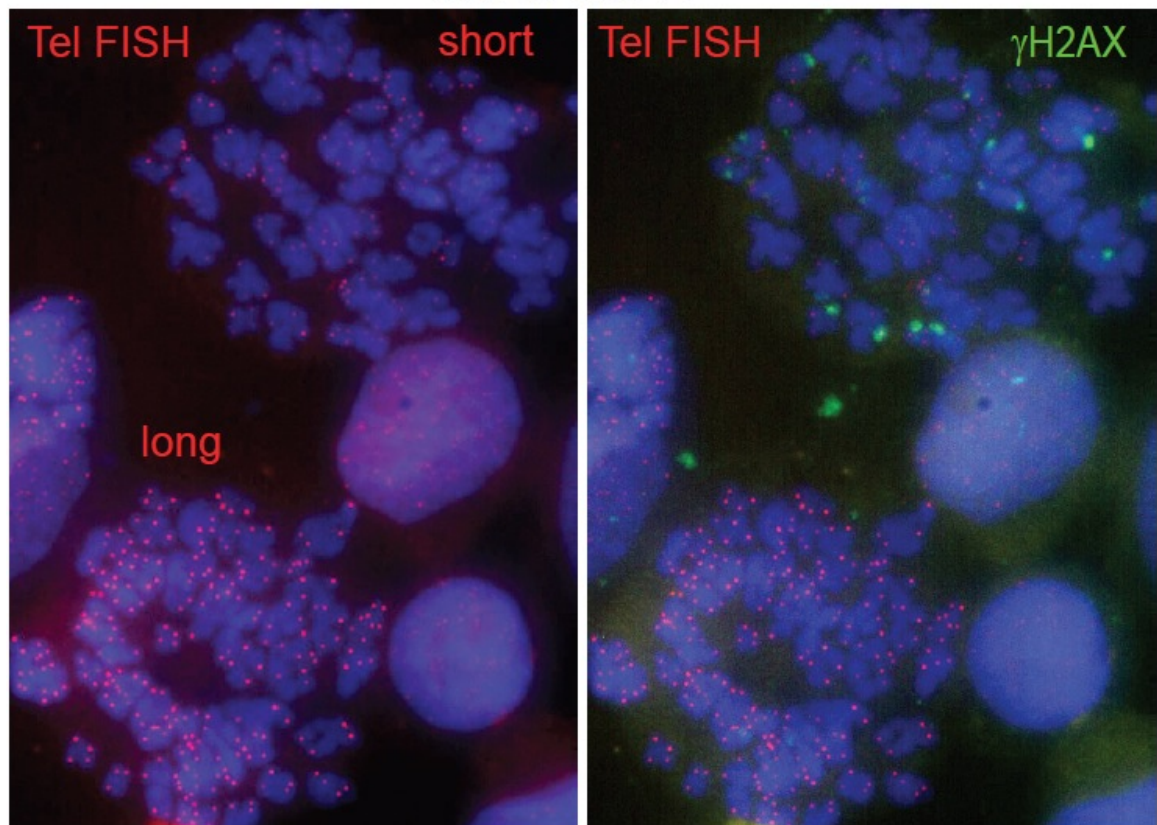
To determine whether mitotic γ H2AX-labelling is indicative of uncapped telomeres that could be sensed as DNA damage and trigger DDR we artificially created telomere uncapping by overexpression of a truncated form of TRF2 lacking both Myb and N-

terminal basic domain [TRF2^{ΔMΔB}; (van Steensel et al., 1998)] in human T4 cells. TRF2 protein, similarly with TRF1, binds to telomeric DNA as a homodimer requiring two Myb DNA-binding domains for stable association with its target *in vivo* and *in vitro* (Bianchi et al., 1997; van Steensel and de Lange, 1997). TRF2^{ΔMΔB} still has the dimerisation domain but not the DNA-binding domain therefore when overexpressed it acts as a dominant-negative, by inhibiting the binding of the endogenous TRF2 to the telomeres resulting in an artificial telomere uncapping. In the cell line employed here the TRF2^{ΔMΔB} expression is under the control of a Dox-repressible promoter. 3 days after a removal of Dox from the growth medium, the expression TRF2^{ΔMΔB} could be detected by Wb, whilst maximum expression was observed at day 7 post-induction (Figure 5D). Before induction of TRF2^{ΔMΔB}, only few TIFs were detected on spread metaphase chromosomes, similar to what we observed in HeLa cells. In contrast, 7 days after the induction of TRF2^{ΔMΔB}, the majority of mitotic telomeres were labelled by γH2AX (Figure 5C). These data suggest that telomeres can display mitotic γH2AX staining. Therefore we can conclude that TIFs persisting into mitosis in cells with intact telomere structure may reflect telomeres that have remained uncapped after their replication in S phase.

Figure 5

A

HeLa 1.2.11



B

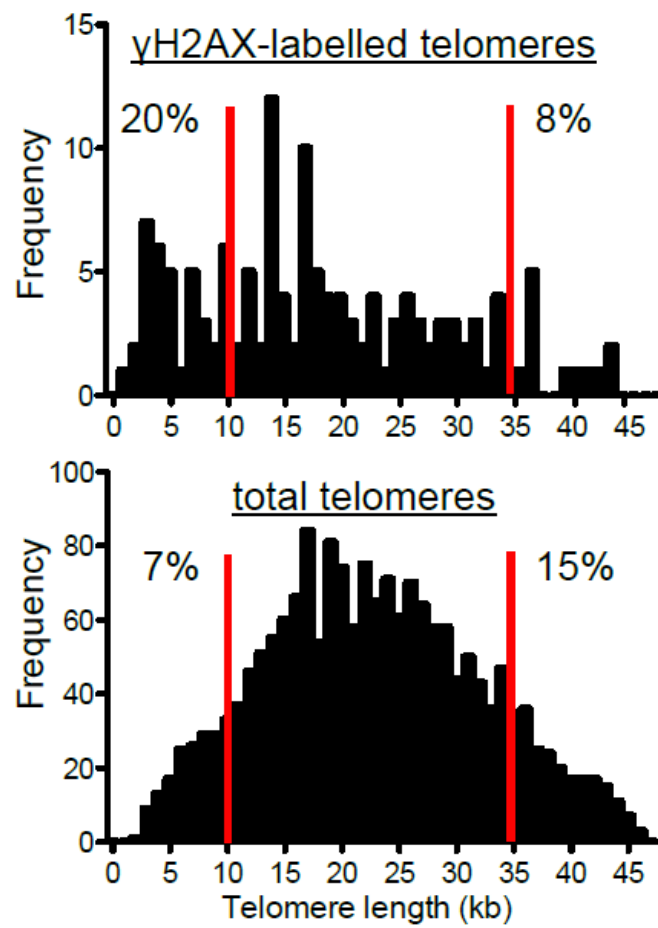


Figure 5

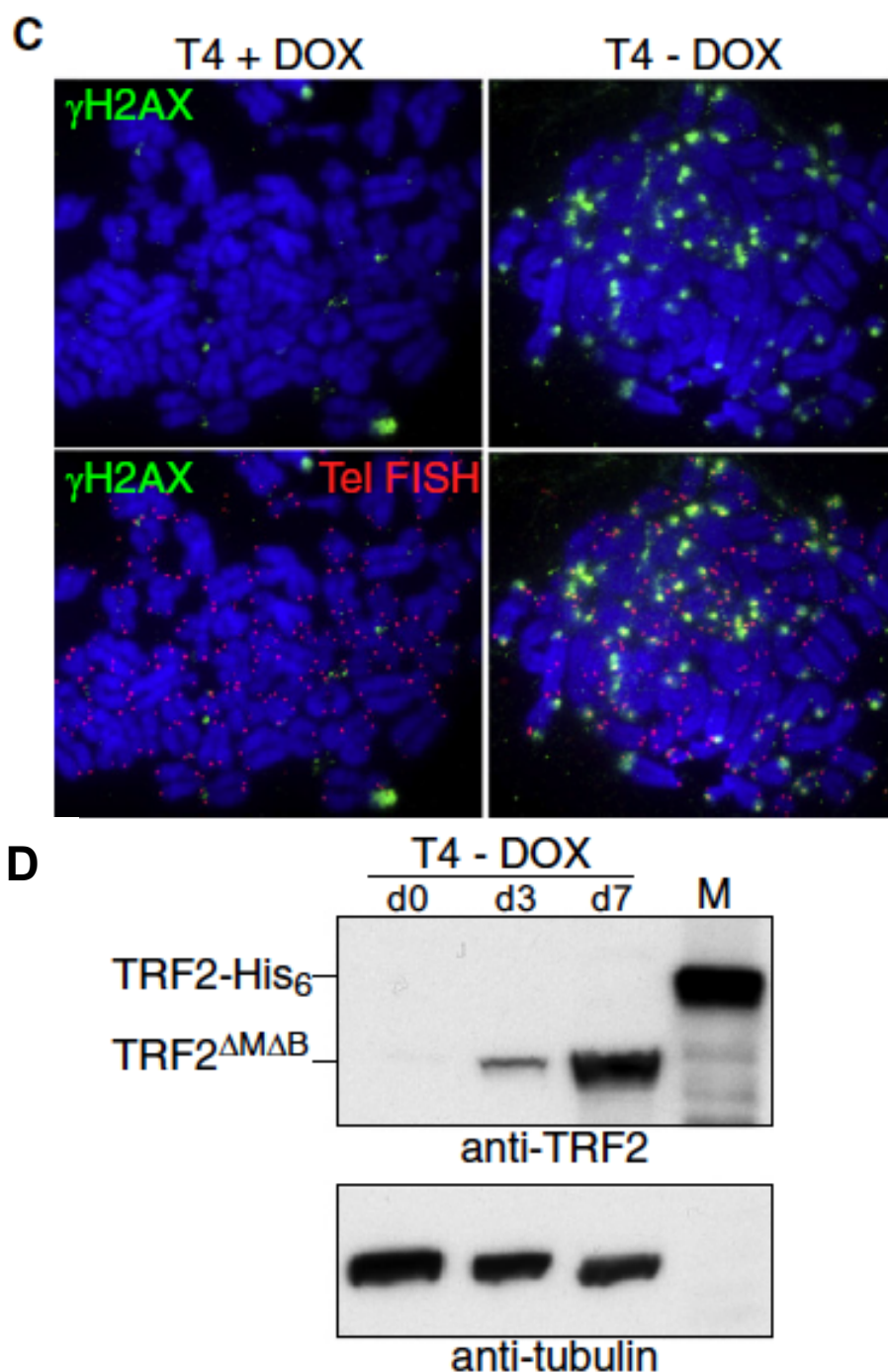


Figure 5: γ H2AX associates preferentially with short, uncapped telomeres

(A) HeLa 1.2.11 cells were arrested in mitosis with colcemid, and mitotic chromosomes were spread onto glass slides via the cytospin method. Preparations were stained with anti- γ H2AX antibodies (green) and a Cy3-conjugated (CCCTAA)₆-PNA probe (red). DNA was counterstained with DAPI (blue). (B) For Q-FISH analysis of telomere length distribution, $n = 4814$ telomeres were analysed, of which 315 stained positive for γ H2AX. (C) Control cells (T4 + Dox) or cells expressing the human TRF2^{ΔMΔB} variant (T4 – Dox) were arrested in mitosis with colcemid, and mitotic chromosomes were spread onto glass slides. Preparations were stained with anti- γ H2AX antibodies (green) and a Cy3-conjugated (CCCTAA)₆-PNA probe (red). (D) TRF2^{ΔMΔB} expression in T4 cells was monitored by Wb at day 0, 3, 7 post-induction. Recombinant full-length human TRF2-His₆, migrating more slowly because of its epitope tag, served as a specificity control. Tubulin was used as a loading control.

3.3 γ H2AX detected at mitotic telomeres requires ATM but not DNA-PKcs

We next tried to identify the kinase required for the H2AX phosphorylation event detected at uncapped mitotic telomeres. ATM is one of the major kinases phosphorylating H2AX in response to DNA damage in interphase (Ward and Chen, 2001; Ward et al., 2004) and it has been previously shown that TIF formation at uncapped telomeres during S phase is ATM-dependent (Takai et al., 2003). Consistent with previous reports, we found that mitotic TIFs were strongly reduced when HeLa 1.2.11 cells were treated with the ATM inhibitor Ku55933 (Hickson et al., 2004) (Figure 6A, B). Quantification of the percentage of cells with more than 5 γ H2AX telomeric foci showed a 50% decrease in the level of this phosphorylation event in ATM-inhibited HeLa cells (Figure 6C). In comparison, MO59J cells, deficient in DNA-PK activity (Kuhne et al., 2003), showed similar levels of mitotic TIFs to those of HeLa and MO59K, DNA-PK-proficient control cells. Instead, ATM inhibition prevented mitotic TIF formation in MO59J and MO59K cells to the same degree as in HeLa cells (Figure 6C). This demonstrates that γ H2AX-labelling of mitotic telomeres depends on ATM, but not DNA-PK, characteristic for the response to uncapped telomeres and consistent with the critical requirement for ATM activity in chromosomal regions of high heterochromatinization (Goodarzi et al., 2008), such as telomeres (Gonzalo et al., 2006).

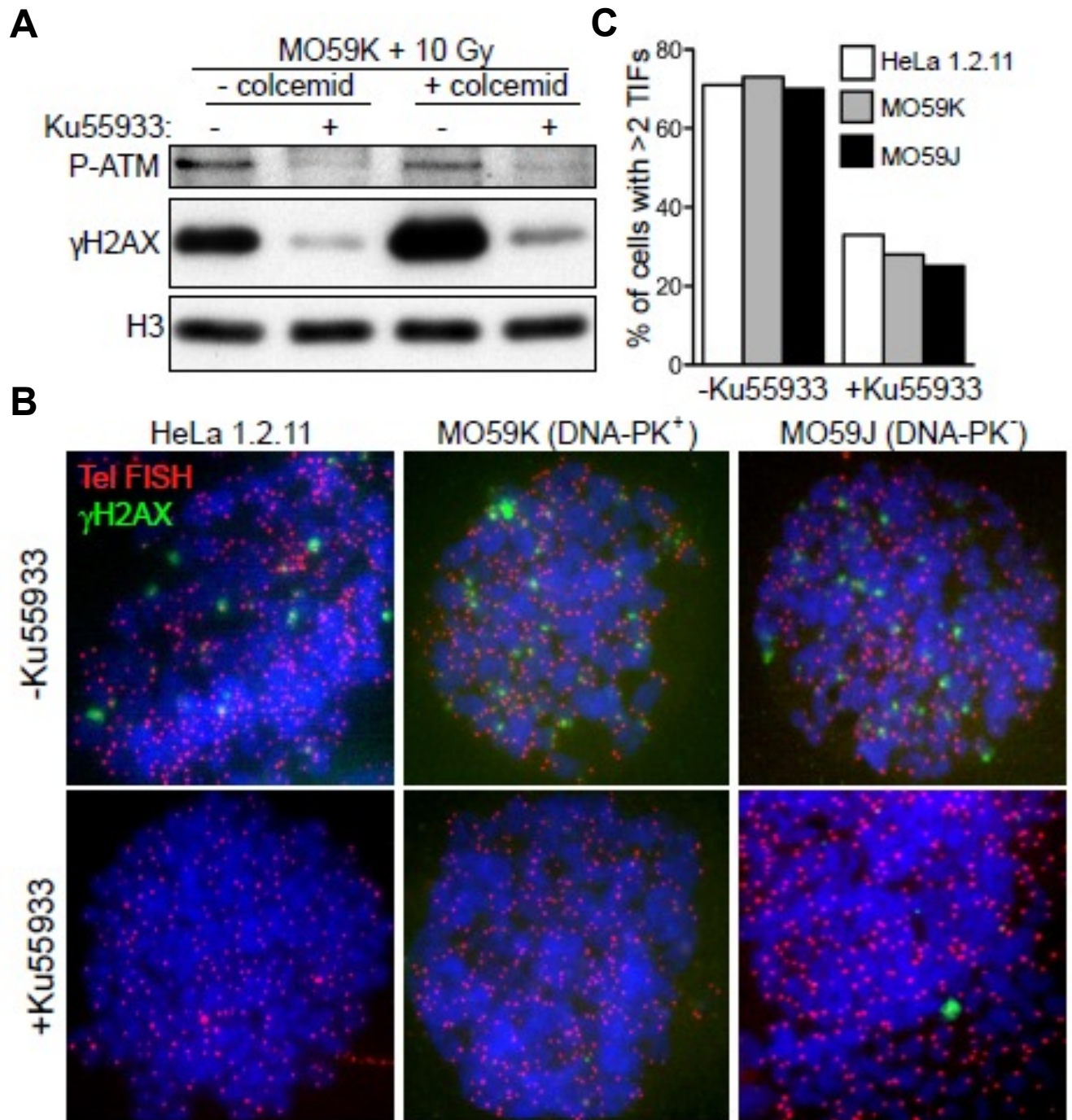


Figure 6: ATM-dependency of mitotic TIF formation

(A) Inhibition of ATM activity by Ku55933 was analysed by Wb of irradiated MO59K cells. Ku55933 (10 μ M) was added to cells arrested in mitosis with colcemid or untreated cells 1 h prior to exposure to 10 Gy of IR. Cells were collected and immunoblotted as indicated. (B) For TIF detection, HeLa 1.2.11, MO59J and MO59K cells were arrested in mitosis with colcemid. Ku55933 (ATM inhibitor) or DMSO control was added 1 h before collection. Mitotic chromosomes were spread onto glass slides, fixed and stained with anti- γ H2AX (green) and a Cy3-conjugated (CCCTAA)₆-PNA probe (red). (C) Quantification of TIFs of mitotic chromosomes shown in (B).

3.4 The p53/p21 pathway prevents accumulation of γ H2AX at mitotic uncapped telomeres

Occurrence of mitotic TIFs in cells that are not exposed to any exogenous DNA damage, was a very interesting observation that raised several questions concerning the mechanism underlying that event. Telomere capping after DNA replication is thought to be completed during the G2 phase of the cell cycle (Verdun et al., 2005), therefore the widespread presence of mitotic TIFs in a range of human cell lines came as a surprise. However, the cell lines used in our studies so far lacked functional p53, which is a major cell cycle regulator known to be activated in response to DNA damage and block cell cycle progression (Chin et al., 1999; Sharpless and DePinto, 2002) or induce apoptosis (Karlseder et al., 1999). To investigate whether p53 plays a role in detecting or preventing mitotic uncapped telomeres, we first analysed the human U2OS cell line that is proficient for p53 function (Stott et al., 1998). After siRNA-mediated depletion of p53 in these cells (Figure 7A), we used them for mitotic TIF detection. γ H2AX-labelling on mitotic chromosomes treated with control siRNA was rarely seen (0-1 TIFs/cell), but increased to frequencies similar to HeLa cells after siRNA-mediated depletion of p53 from these cells (2-5 TIFs/cell) (Figure 7B, C). We similarly analysed the consequences of p21 depletion, a downstream target of p53, showing a similar increase of mitotic TIFs to 2-5 TIFs/cell (Figure 7D).

Figure 7

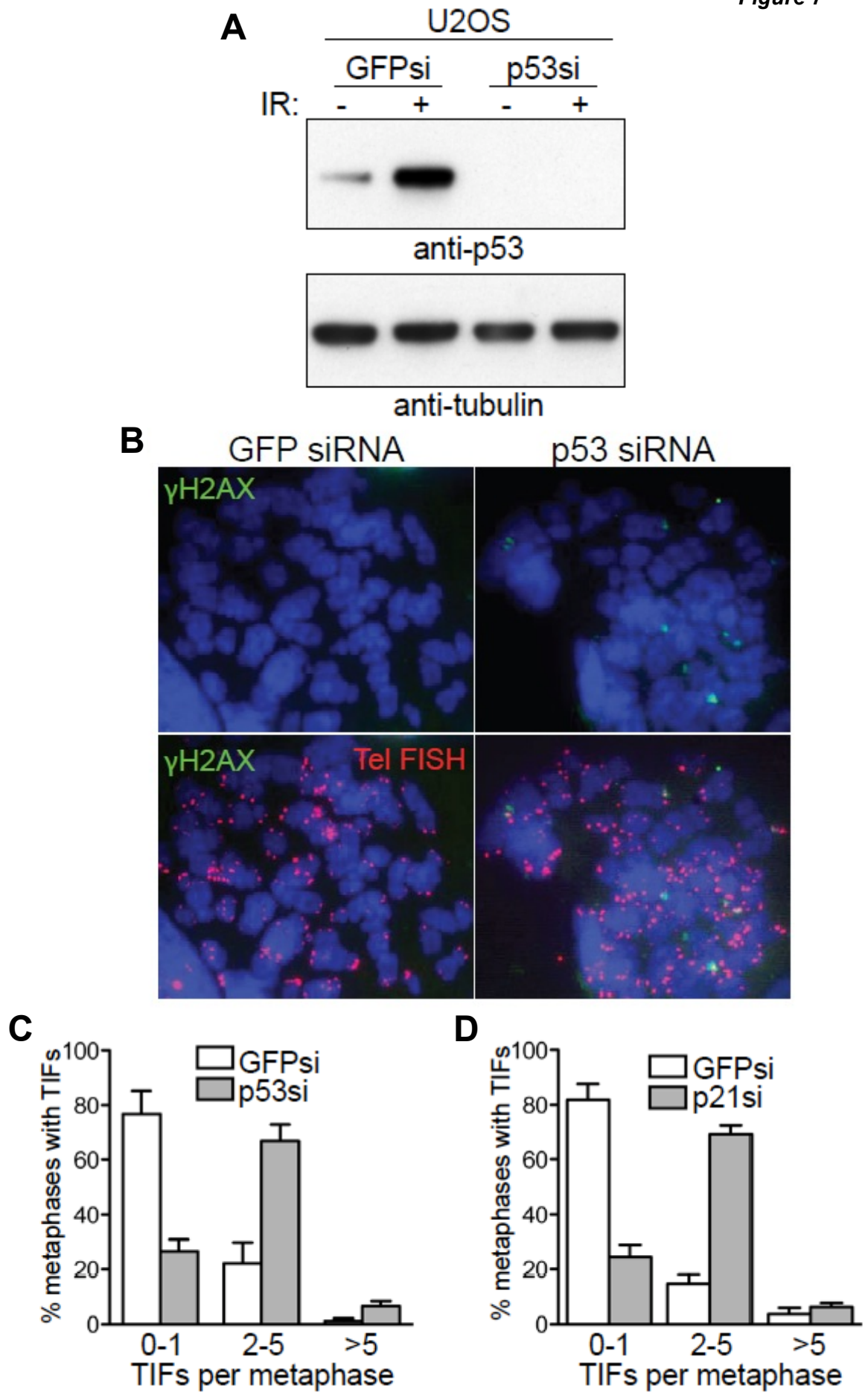
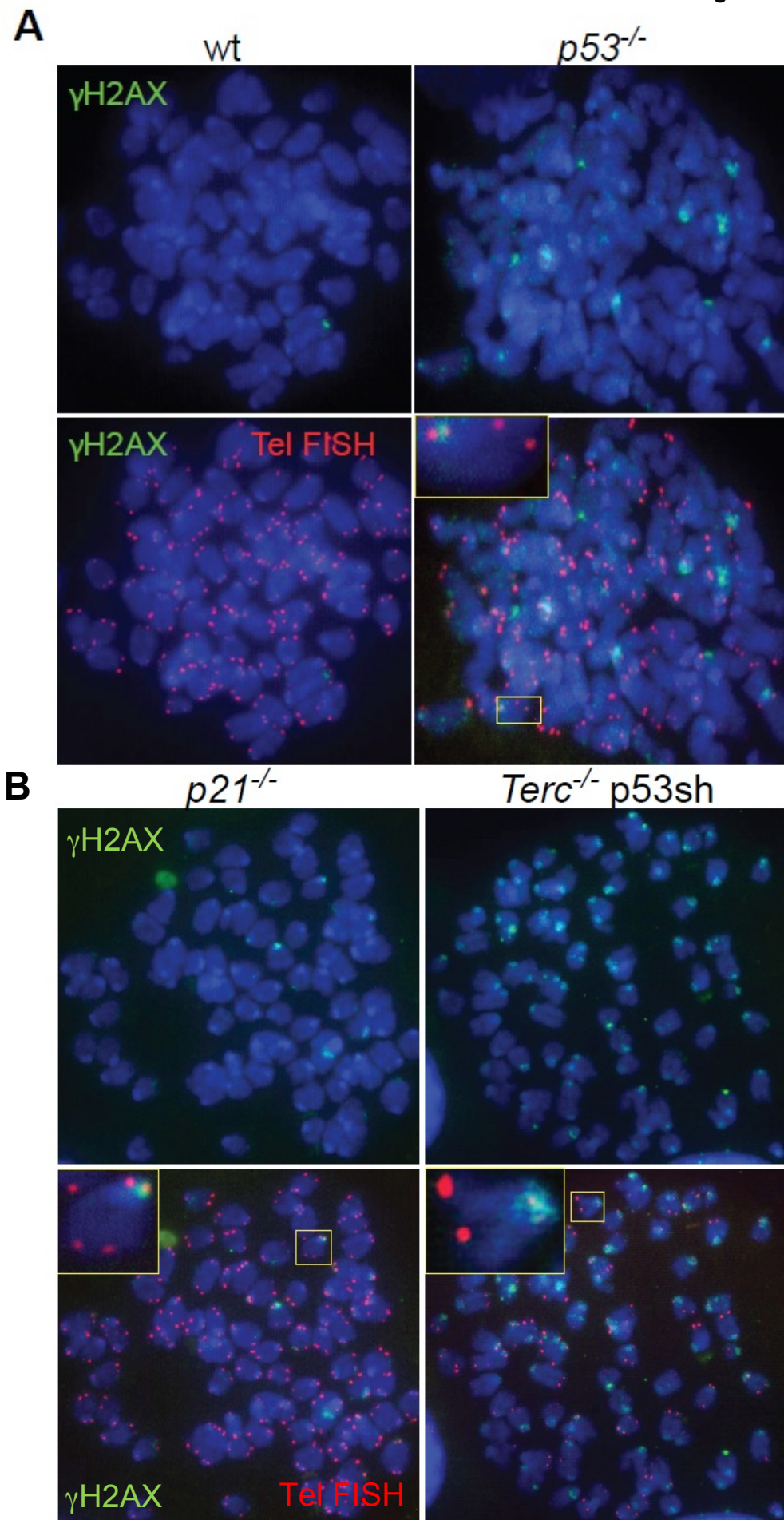


Figure 7: Occurrence of uncapped, γ H2AX-labelled mitotic telomeres is prevented by p53 and p21

(A) Wb analysis of siRNA-mediated p53 depletion. U2OS cells were transfected with control or p53 siRNAs and irradiated (10 Gy) 48 h post-transfection. After 2 h, extracts were prepared and immunoblotted as indicated. (B) U2OS cells were transfected with control or p53 siRNAs and incubated with colcemid 48 h post transfection. Mitotic chromosomes were spread onto glass slides using the cytopsin method and stained with anti- γ H2AX antibodies (green) and a Cy3-conjugated (CCCTAA)₆-PNA probe (red). DNA was counter stained with DAPI (blue). (C) Quantification of TIF frequency in cells prepared as in (B). (D) U2OS cells were transfected with control or p21 siRNAs and incubated with colcemid 48 h post transfection. Mitotic TIFs were quantified as in (C). For each quantification 100-150 metaphases were scored.

To confirm the dependency of mitotic TIFs occurrence on the *p53* status of mouse cells as well, we analysed isogenic MEFs carrying wild type *p53* or a targeted deletion in the *p53* gene (Jacks et al., 1994). γ H2AX-labelling of mitotic chromosomes was again rarely seen in wild type MEFs (0-1 TIFs/cell), but occurred frequently in *p53*^{-/-} MEFs (2-5 TIFs/cell) (Figure 8A). As was the case in human HeLa cells, Q-FISH analysis revealed that γ H2AX-staining was preferentially observed at the shorter telomeres of *p53*^{-/-} MEFs (data not shown). As a control of γ H2AX association with dysfunctional telomeres we used late generation *Terc*^{-/-} T cells, in which γ H2AX association with sites close to the telomeres of mitotic chromosomes has been previously reported (Hao et al., 2004) (Figure 8B). These cells lack the RNA component of telomerase, called TERC, and therefore they have short and dysfunctional telomeres. When we quantified mitotic TIFs in *Terc*^{-/-} shp53 MEFs (Herrera et al., 1999) with short telomeres (G4; approx. 20.9 kb average) we found a substantial increase compared to wild-type MEFs (approx. 36.4 kb average) (Figures 8B, C). This confirms our observation that shorter telomeres are more prone to persisting in an uncapped state. These results suggest that p53 is required either for efficient capping of telomeres in G2 before the time of mitotic entry, or to prevent progression into mitosis while telomere capping reactions are in progress. p53 has already been shown to delay mitotic entry in response to DNA damage by upregulation of its downstream target p21 (Bunz et al., 1998). To test whether this hypothesis is correct in the case of telomere uncapping, which is also a type of DNA damage, we used MEFs carrying a *p21* deletion (Brugarolas et al., 1995). In *p21*-deficient MEFs we observed mitotic TIFs as frequently as in MEFs lacking *p53* (Figure 8B, C). These data suggest that a possible role of p53 is to delay cell cycle progression into mitosis by p21 induction when uncapped telomeres persist.

Figure 8



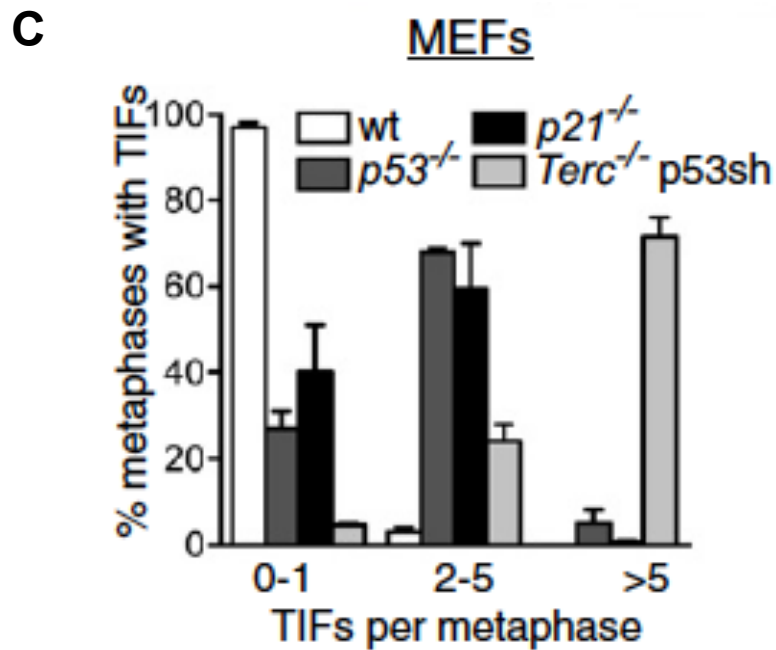


Figure 8: p53 and p21 prevent progression into mitosis in the presence of uncapped telomeres in MEFs

(A, B) MEFs established from wild-type, *p53*^{-/-}, *p21*^{-/-}, or *Terc*^{-/-} *p53*sh embryos were arrested in mitosis with colcemid, and mitotic chromosomes were spread via the cytopsin method. Preparations were fixed and stained with anti- γ H2AX monoclonal antibody (green). Telomeres were visualised with a Cy3-conjugated (CCCTAA)₆-PNA probe (red). DNA was counterstained with DAPI (blue). Insets show enlarged images of the areas marked with yellow rectangles. (C) TIF frequency was determined by counting the number of foci in 100–150 metaphases. Error bars represent SD of three independent experiments.

3.5 The p53/p21 pathway protects telomere integrity by affecting the telomere capping reaction at the G2/M transition

As p53 clearly affected the presence of mitotic TIFs in both human and mouse cells, we next tried to address whether p53 itself affects the telomere capping reaction, directly or indirectly. Therefore, we synchronised cells from a pair of isogenic human colorectal cancer cell lines, proficient or deleted for p53 [(HCT116 $p53^{+/+}$ or $p53^{-/-}$); (Bunz et al., 1998)], by double thymidine block and release. We monitored the occurrence of TIFs at the G2/M transition, 7 – 11 h after the second release. At 7 h, both $p53^{+/+}$ and $p53^{-/-}$ cells had completed DNA replication and stained positive for the G2 specific marker CENP-F [(Badie et al., 2009); data not shown]. Most cells exhibited more than 2 TIFs at this time regardless of their p53 status. By the time cells entered mitosis at 8 h, as seen by the increase in phospho-histone H3 (Ser10) staining, characteristic for mitotic cells (Figure 9B), TIFs were no longer observed in $p53^{+/+}$ cells. In contrast, TIFs remained detectable in most $p53^{-/-}$ cells throughout G2/M transition until the majority of cells have entered mitosis at 10 h (Figure 9A). This suggests that p53 is required for efficient completion of telomere capping and that the p53 response pathway may impact on the capping reaction. Alternatively, HR reactions that are part of telomere capping may become disfavoured as $p53^{-/-}$ cells enter mitosis prematurely (Esashi et al., 2005; Verdun and Karlseder, 2006). As reported previously (Bunz et al., 1998) and as shown here by staining with phospho-histone H3 (Ser10) (Figure 9B), a marker for the G2/M transition, mitotic entry of $p53^{-/-}$ cells was somewhat advanced compared to $p53^{+/+}$ cells. This means that a potential role of p53 in the protection of telomere integrity is to cause a G2/M delay in order to allow telomeres to become protected before the cells enter mitosis.

Figure 9

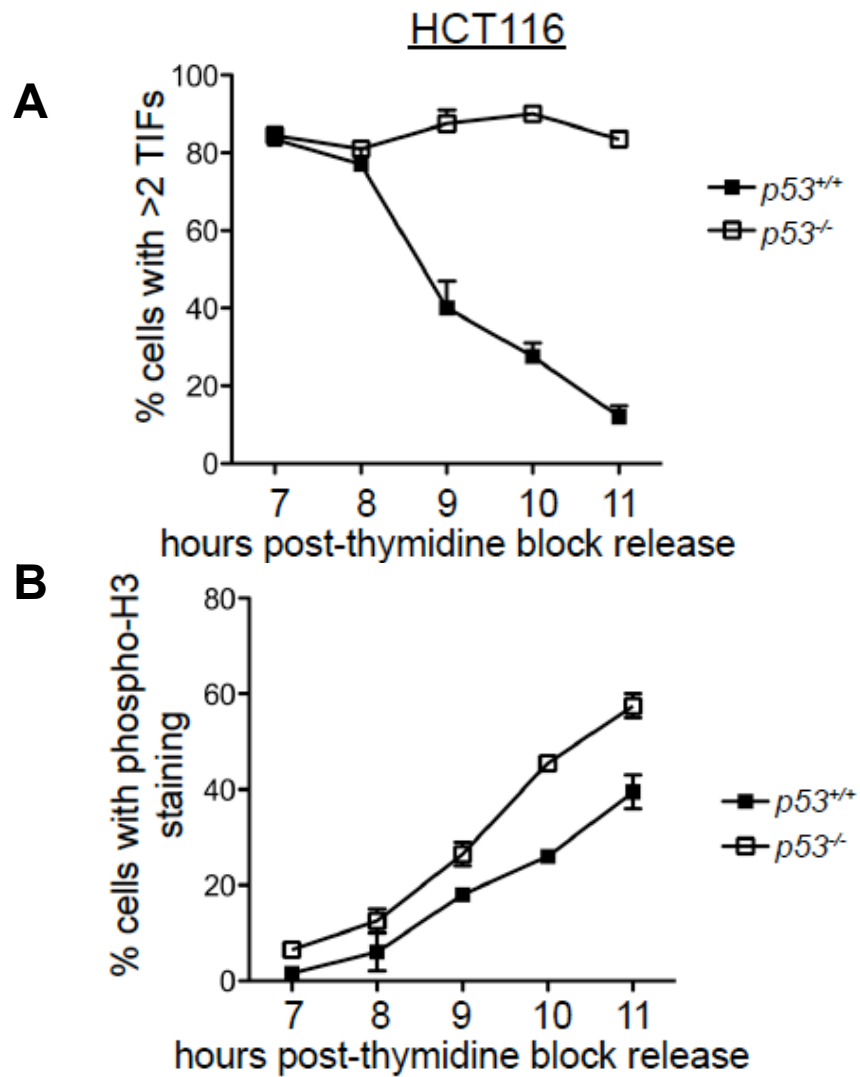


Figure 9: p53 is required for efficient telomere capping

(A) HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells synchronised at the G1/S transition using double thymidine block were collected at the indicated times after release in fresh media. Cells were then stained with anti- γ H2AX monoclonal antibody and telomeres visualised with a Cy3-conjugated (CCCTAA)₆-PNA probe. For TIF quantification 30-50 cells were scored for each timepoint. (B) IF staining with phosphorylated histone H3 antibody identified cells in mitosis. For quantification of ph-H3 positive cells 200 cells were scored. Error bars represent SD of two independent experiments.

3.6 Artificially uncapped telomeres trigger ATM/ATR and p53/p21-dependent G2/M checkpoint activation

To gain mechanistic insight into the control of p53 activation at the G2/M transition and taking into account the ATM-dependent TIFs detected during mitosis (Figure 6), we monitored in synchronised human cells the ATM/ATR-dependent p53 phosphorylation at Ser15 by Wb, an event known to occur in response to DNA damage (Girard et al., 2002; Siliciano et al., 1997). Ser15 phosphorylation occurred in S phase and also at the G2/M transition of the cell cycle, as monitored by FACS analyses of DNA content and phospho-H3 staining (Figure 10A). Concomitant activation of p21 was observed only in G2/M, emphasising its importance at this stage of the cell cycle. Another target of p53 at the G2/M, 14-3-3 σ (Taylor and Stark, 2001), showed a similar induction pattern to that of p21, raising the possibility that 14-3-3 σ is also involved in the response to telomere damage.

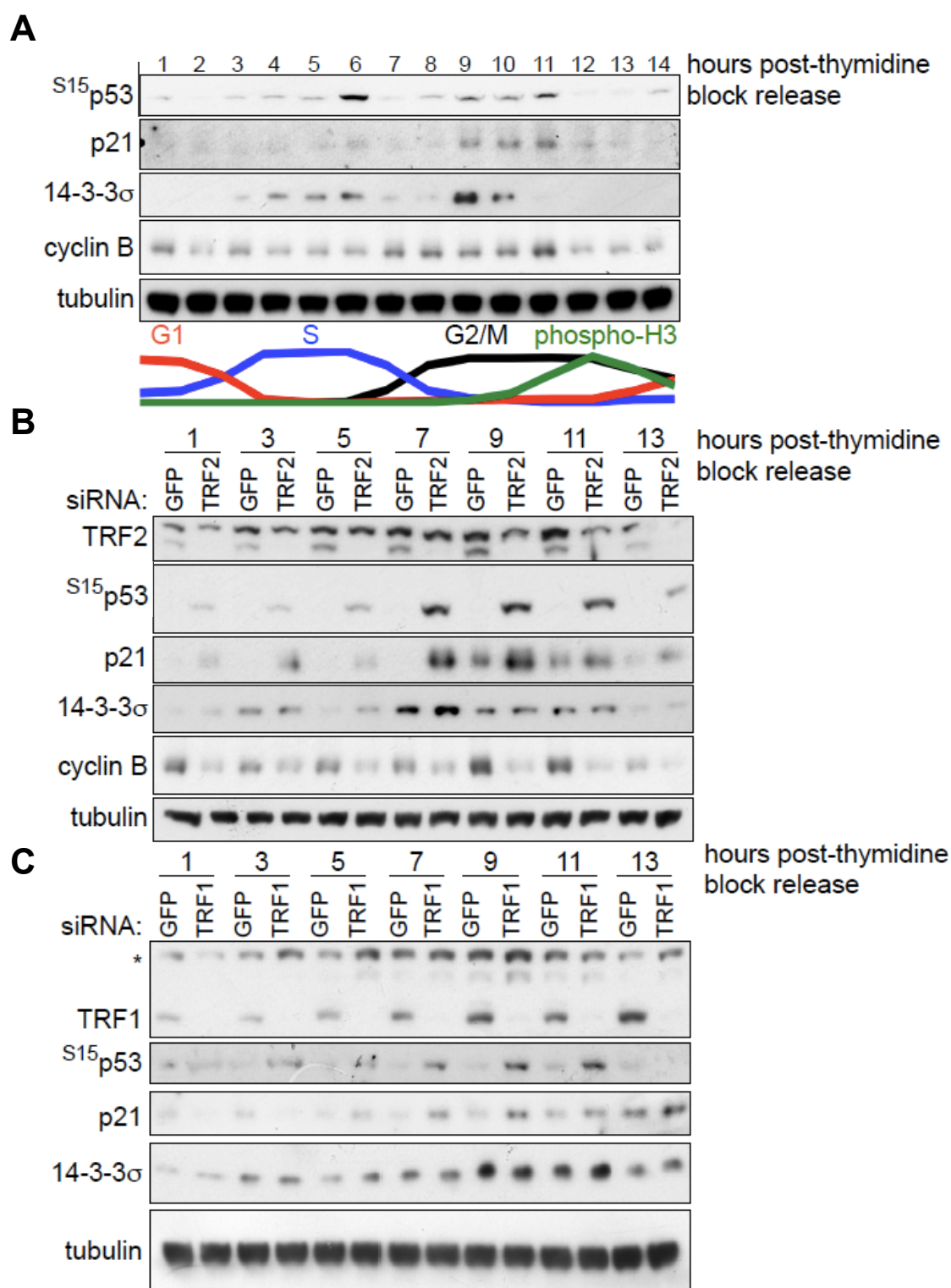
To test this hypothesis, we decided to produce artificial telomere uncapping by disrupting telomere protective structures through depletion of components of the shelterin complex in human cells and monitor the p53 pathway activation. First, we synchronised by double thymidine block and release U2OS cells that have been depleted of TRF2 using siRNA (Figure 10B). Whilst increased ATM/ATR-dependent p53 phosphorylation at Ser15, as well as p21 induction occurred specifically in response to TRF2 depletion and only during the G2/M transition (7-11 h), 14-3-3 σ expression was not significantly augmented. This suggests that p21, but to a lesser degree 14-3-3 σ , acts downstream of p53 in response to telomere dysfunction at the G2/M transition.

Following that observation we were interested in whether the depletion of other components of the shelterin complex have the same effect or if the G2/M response to artificially uncapped telomeres varies depending on the shelterin component inhibited. Therefore, we repeated the same experiment but this time depleting TRF1 by siRNA (Figure 10C), a telomere binding factor required for telomere protection and stability,

which shares biochemical and cellular functions with TRF2 (Martinez et al., 2009). We saw exactly the same pattern of p53/p21 activation by ATM/ATR specifically at the G2/M transition and only after depletion of TRF1. Similarly 14-3-3 σ did not seem to be important for G2/M response to TRF1 depletion since it was activated regardless of the presence or absence of TRF1.

Furthermore, POT1, a TPP1-interacting protein, is unique among shelterin components in its ability to bind specifically to ss telomeric DNA, thus suppressing ATR-dependent checkpoint responses (Palm and de Lange, 2008). Importantly, we observed p53/p21 activation at the G2/M transition in cells lacking POT1 (Figure 10D, E), a response similar to that triggered by TRF1 and TRF2 inhibition. This suggested that ATR and ATM signalling converge to activate p53 in response to uncapped telomeres.

Figure 10



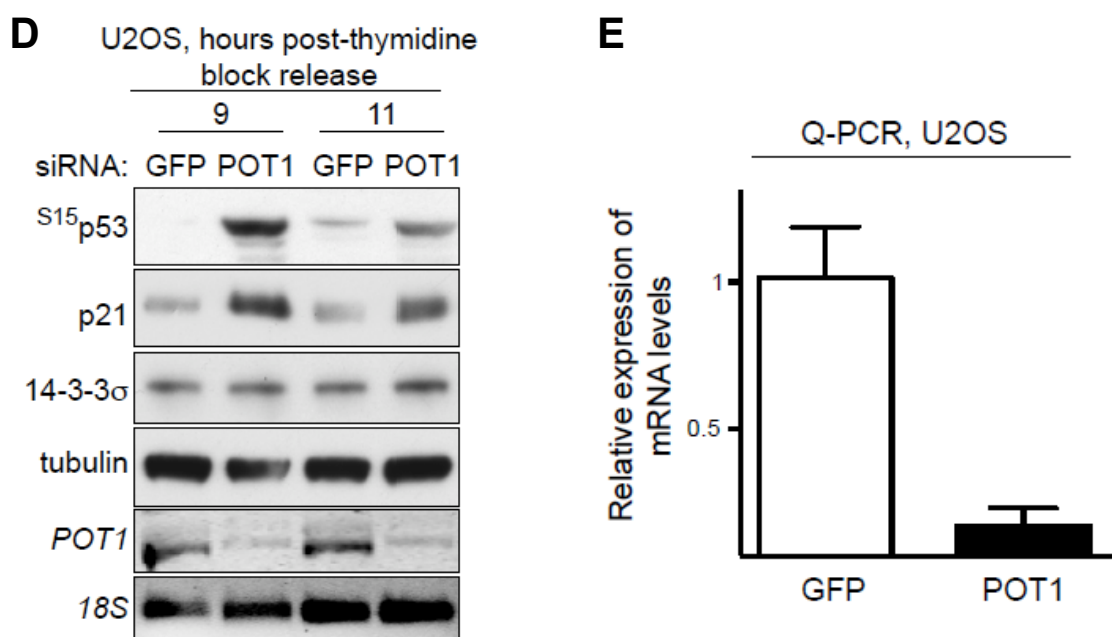


Figure 10: p53 undergoes ATM/ATR-dependent phosphorylation at the G2/M transition in response to uncapped telomeres in human cells

(A) U2OS cells were synchronised at the G1/S transition via a double thymidine block. Extracts prepared from cells at the indicated times after release were immunoblotted as indicated. (B) U2OS cells transfected with control GFP or TRF2 siRNAs were grown for 48 h before the initiation of synchronisation by double thymidine block and release. Extracts prepared at the indicated times after release were immunoblotted as shown. (C) U2OS cells transfected with control GFP or TRF1 siRNAs were grown for 48 h before the initiation of synchronisation by double thymidine block and release. Extracts prepared at the indicated times after release were immunoblotted as shown.* indicates non-specific band. (D) U2OS cells transfected with control GFP or POT1 siRNAs were grown for 48 h before synchronisation by double thymidine block and release. Cells collected at the indicated times after release were processed for Wb or QRT-PCR analysis as indicated. Tubulin and 18S were used as controls for Wb and QRT-PCR, respectively. (E) Q-PCR analysis of POT1 mRNA levels of POT1si-treated cells vs GFPsi-treated cells collected 24 h after transfection.

3.7 Artificially uncapped telomeres trigger a p53/p21-dependent delay of mitotic entry

Since our results clearly suggested the activation of the p53/p21 pathway at the G2/M phase of the cell cycle in response to telomere damage triggered by the depletion of at least 3 different shelterin components, it was important to discover the specific role of that pathway in protecting telomere integrity. As mentioned above, our data (Figure 9) along with previous findings supporting the role of p53/p21 in preventing progression of cells to mitosis in the presence of DNA damage (Bunz et al., 1998; Taylor and Stark, 2001), suggested that the role of p53 is to delay the G2/M transition in the presence of uncapped telomeres. In order to directly address the ability of p53 to delay mitotic entry we produced artificial telomere uncapping through depletion of components of the telomeric complex in mouse and human cells and compared mitotic indexes between $p53^{+/+}$ and $p53^{-/-}$ cells.

First, $p53^{+/+}$ and $p53^{-/-}$ primary MEFs carrying homozygous floxed *Trf1* alleles (*Trf1^{F/F}*) (Martinez et al., 2009) were infected with retroviruses encoding Cre recombinase or vector control. Cre recombinase was responsible for the excision of *Trf1* gene which was flanked by loxP sites. Deletion of *Trf1*, as monitored by Wb, resulted in marked telomere uncapping visualised by γ H2AX-labelled telomeres (Figure 11A). To analyse mitotic entry of these cells following telomere uncapping, we determined the fraction of cells in mitosis at the indicated times after infection by DAPI staining of cells spread onto microscopy slides after colcemid treatment (Figure 11B). $p53^{+/+}$ cells displayed a very low mitotic index after uncapping, compared to *Trf1* positive control cells. $p53^{-/-}$ cells showed a higher mitotic index in the TRF1-proficient background, compared to $p53^{+/+}$ cells, and this was not affected after loss of telomere protection by *Trf1* deletion. FACS analysis of DNA content (Figure 11C) confirmed that the low mitotic index in $p53^{-/-}$ cells with uncapped telomeres was due to an arrest of cells in G2/M rather than G1/S. Collectively, these data suggest that in MEFs p53 is activated in the presence of telomeres that become uncapped due to TRF1 loss and prevents mitotic entry.

Figure 11

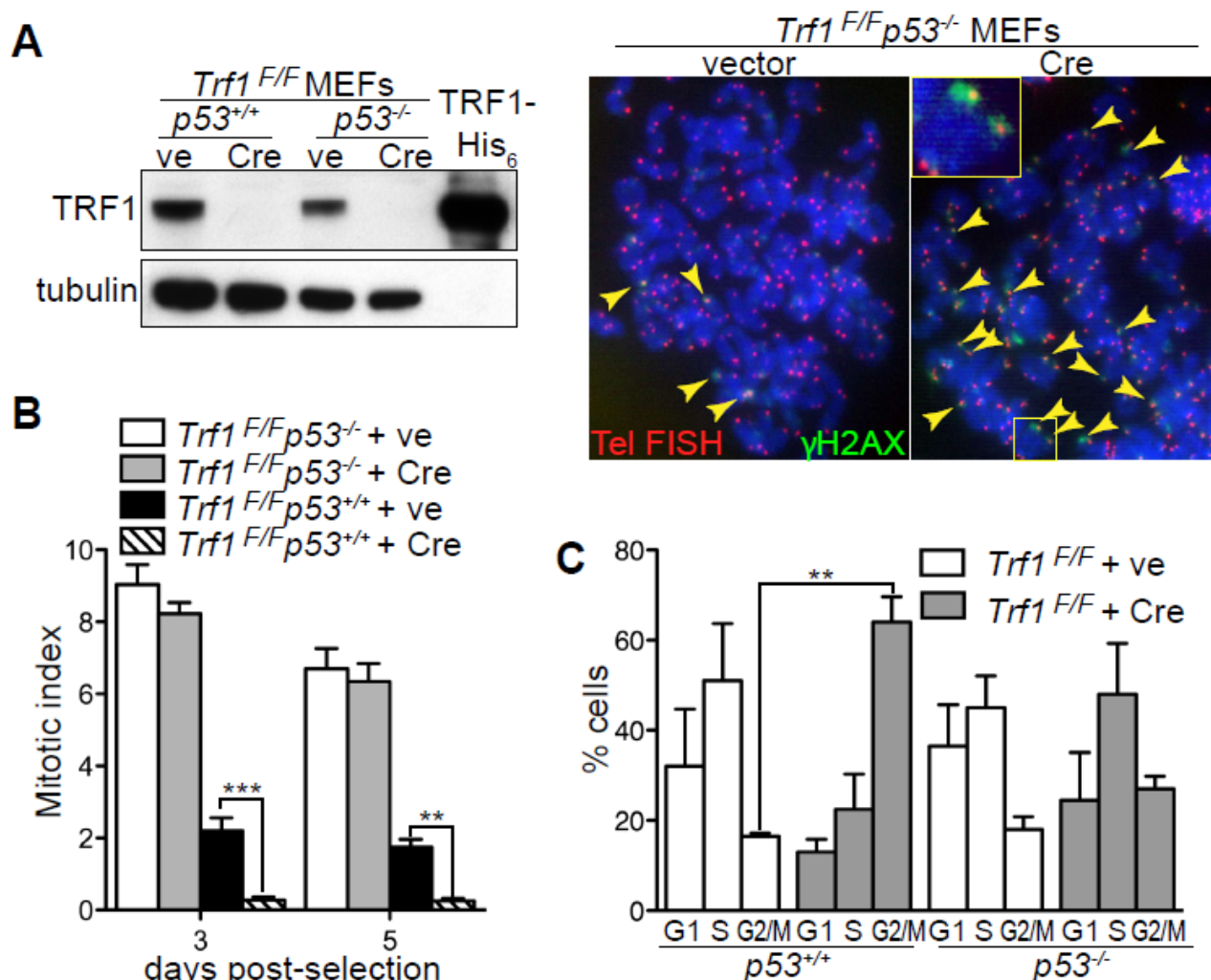


Figure 11: p53 controls the G2/M transition in response to uncapped telomeres in MEFs

(A) Wb detection of mouse TRF1 in primary *p53*^{+/+}*Trf1*^{F/F} and *p53*^{-/-}*Trf1*^{F/F} MEFs of the indicated genotypes treated with empty pBabe vector (ve) or Cre recombinase (Cre) 3 days after selection. Recombinant human TRF1-His₆ served as a specificity control. Tubulin was used as loading control. *p53*^{-/-}*Trf1*^{F/F} MEFs were arrested in mitosis with colcemid and then mitotic chromosomes were spread via the cytospin method and stained with anti-γH2AX monoclonal antibody (green). Telomeres were visualised with a Cy3-conjugated (CCCTAA)₆-PNA probe (red). DNA was counterstained with DAPI (blue). Yellow arrows indicate TIFs. (B) The mitotic index was determined in n = 350–500 cells for each genotype, treatment, and time point. (C) FACS analysis of DNA content was performed on propidium iodide-stained cells. Error bars represent SD of two independent experiments. p values were calculated with an unpaired two-tailed t test. **p < 0.01 and ***p < 0.001.

Similarly with the *Trf1*^{-/-} MEFs, p53 was required to prevent mitotic entry of human cells with uncapped telomeres (Figure 12). We first depleted TRF2 using siRNA in *p53*^{+/+} or *p53*^{-/-} HCT116 cells in order to induce artificial telomere uncapping shown by the significant increase in the number of γ H2AX-labelled telomeres by IF-FISH (Figure 12A). We then examined the ability of cells to enter mitosis by phospho-H3 (Ser10) staining of fixed cells. Their ability to enter mitosis was significantly impaired only in the presence of functional p53 indicated by the lower levels of phospho-H3 positive cells (Figure 12B). Additionally, human U2OS cells depleted of p21 and TRF2 entered mitosis at a higher rate compared to p21-proficient cells (Figure 12C). This suggests that a p53-dependent pathway recognises uncapped telomeres and delays mitotic entry via activation of p21. Consistent with this idea, we observed p21 induction following siRNA-mediated depletion of TRF2 by Wb (Figure 12C).

Figure 12

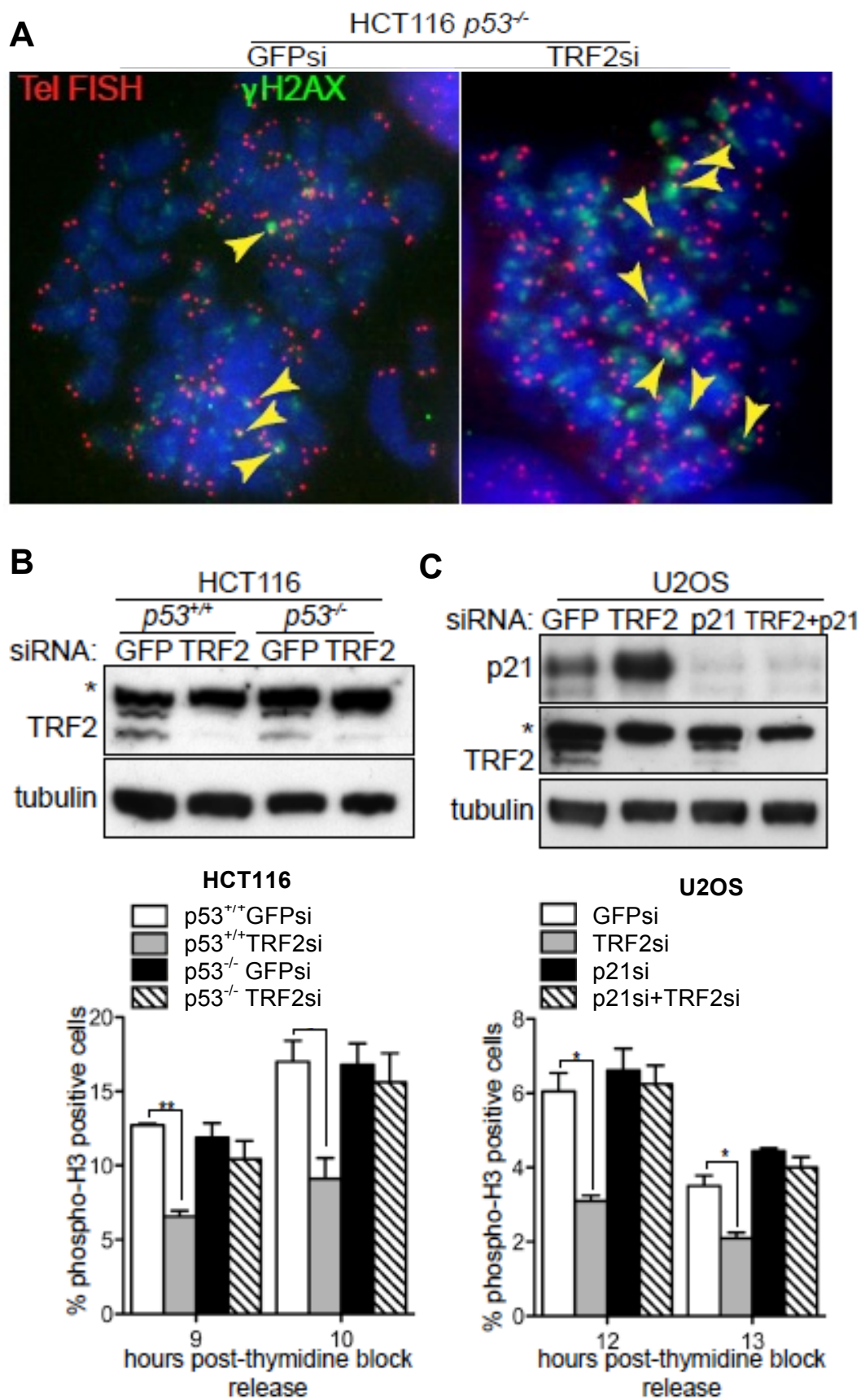


Figure 12: p53 and p21 prevent mitotic entry with uncapped telomeres in human cells

(A) HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells were transfected with control or TRF2 siRNAs. 48 h post transfection, cells were treated with colcemid, spread on glass slides and stained with anti- γ H2AX monoclonal antibody (green) while telomeres were visualised with a Cy3-conjugated (CCCTAA)₆-PNA probe (red), as shown for HCT116 $p53^{-/-}$ (yellow arrows indicate TIFs), or (B) were collected and immunoblotted as indicated. *, non-specific band. Cells were synchronised at the G1/S transition using a double thymidine block initiated 48 h after transfection, and collected at the indicated times after release. Cells were stained with propidium iodide and an antibody against phosphorylated histone H3 (Ser10) and analysed by flow cytometry. n=10,000 cells were analysed for each sample. p values were calculated using an unpaired two-tailed t test. *, $p<0.05$ and **, $p<0.01$. (C) U2OS cells were transfected with control, p21 or TRF2 siRNAs and analysed as in (B).

3.8 p53 prevents mitotic entry with telomeres uncapped through loss of TRF2 or POT1

Next we set out to address whether p53 acts as the effector of the two different types of telomere damage, caused by the disruption of a ds or ss DNA-binding telomeric protein. For that we used human U2OS cells in which we abrogated telomere capping by depleting either TRF2, which is a ds telomeric DNA-binding factor or POT1, which is a ss telomeric DNA-binding factor, alone or in conjunction with p53 (Figure 13A). We then monitored mitotic entry of these cells by measuring phosphorylation of histone H3 at Ser10 in (Figure 13B). We performed this analysis in cells released from a double thymidine block for 10 h, when a significant proportion of cells were entering mitosis, as evaluated by FACS analyses of DNA content (Figure 13E). When either TRF2 or POT1 were depleted by siRNA, the ability of cells to enter mitosis was impaired only in the presence of functional p53 (Figure 13B). Upon concomitant inhibition of p53, both TRF2- and POT1-depleted cells entered mitosis at a slightly higher rate than GFP siRNA-treated control cells. This indicates that both types of telomere damage known to activate either an ATM- or ATR-dependent response also triggered p53-dependent G2/M arrest. In the same cells we monitored TIF formation at uncapped mitotic telomeres by γ H2AX co-localisation with the telomeric FISH signal (Figure 13C). The level of mitotic TIFs increased significantly upon co-depletion of p53 with either TRF2 or POT1 (Figure 13D), consistent with p53 playing a central role in monitoring capping reactions at the G2/M transition.

Figure 13

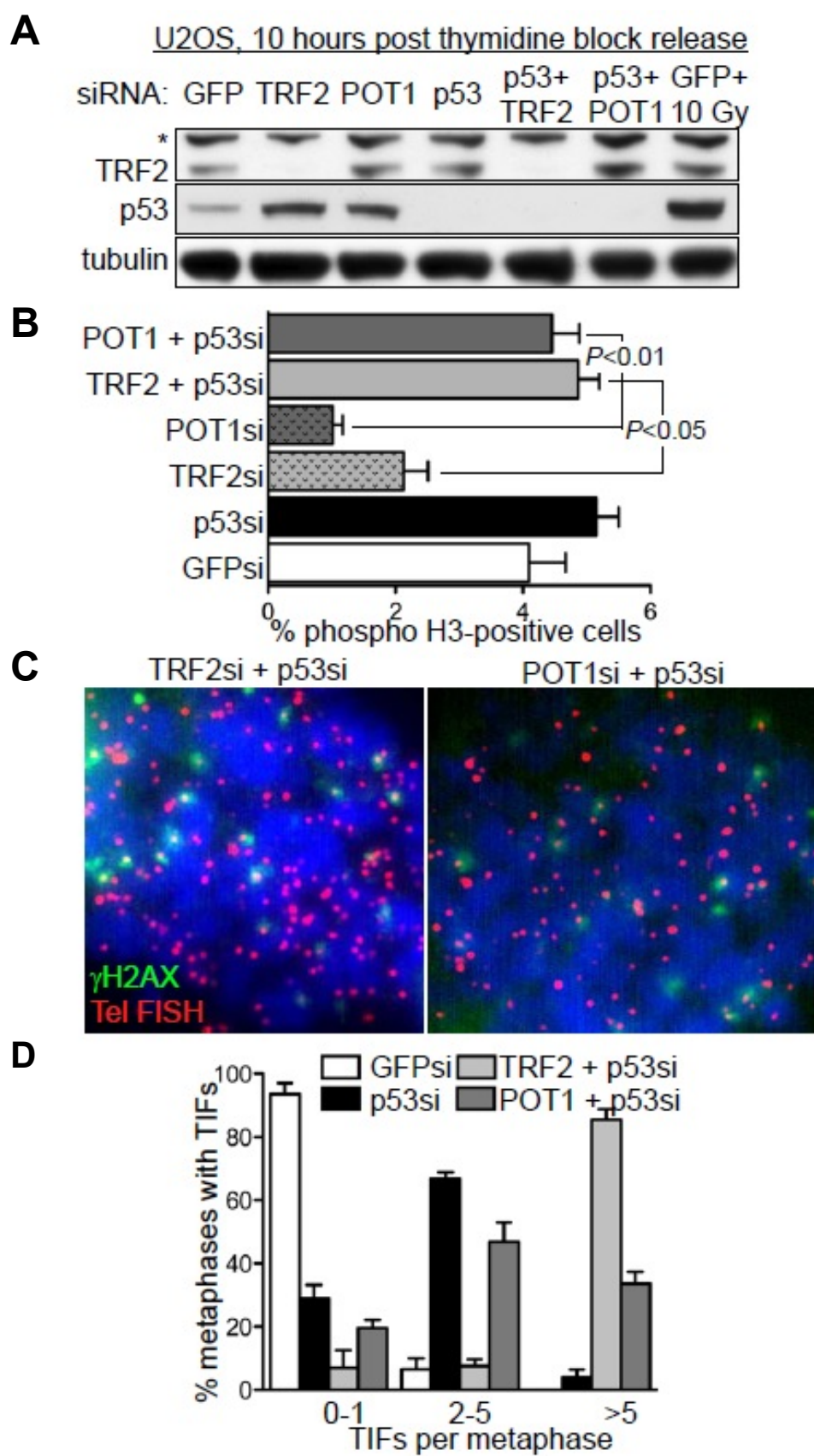


Figure 13

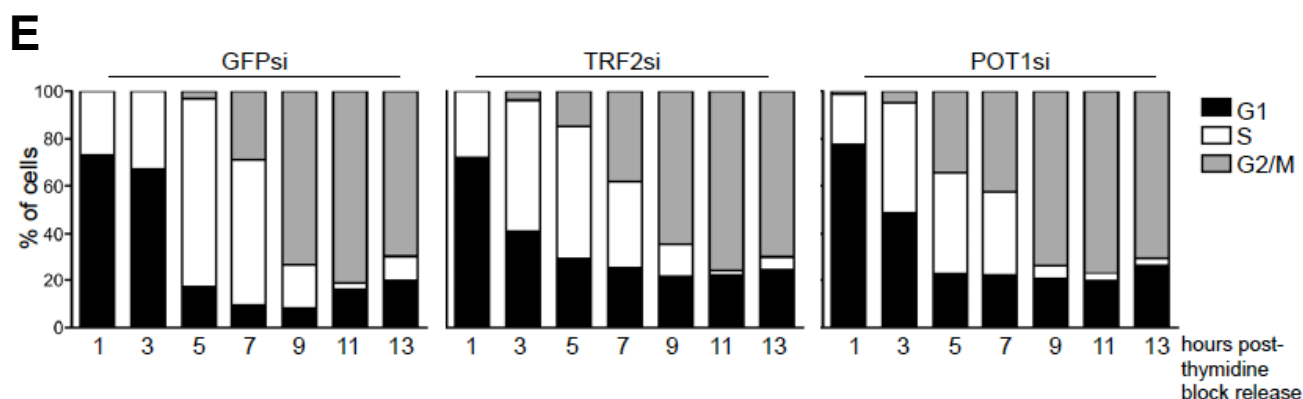


Figure 13: POT1 and TRF2 depletion from telomeres triggers a p53-dependent G2/M checkpoint response

(A) U2OS cells transfected with siRNA as shown were synchronised by double thymidine block and release. After 10 h, extracts were prepared and immunoblotted as shown. Extracts from cells exposed to 10 Gy of IR were used as a control for p53 induction. (B) Cells treated as in (A) were stained with propidium iodide and an antibody against phosphorylated histone H3-Ser 10 and analysed by flow cytometry. $n=10,000$ cells were analysed for each sample. Error bars represent SD of two independent experiments. P values were calculated using an unpaired two-tailed t test. (C) Mitotic chromosomes isolated from U2OS cells treated with siRNA and arrested in mitosis with colcemid were spread onto glass slides using the cytospin method. Preparations were fixed and stained with anti- γ H2AX monoclonal antibody (green). Telomeres were visualised with a Cy3-conjugated (CCCTAA)₆-PNA probe (red). (D) Quantification of TIFs in U2OS cells treated as in (C). 100 metaphases were scored for each cell line. Error bars represent SD of two independent experiments. (E) FACS analysis of DNA content of U2OS cells following POT1, TRF1 and TRF2 siRNA-mediated inhibition. Cells were synchronised at the G1/S transition 48 h after siRNA transfection using a double thymidine block, and collected at the indicated times after release. Cells were stained with propidium iodide and analysed by flow cytometry. $n=10,000$ cells were analysed for each sample.

3.9 Uncapped telomeres trigger G2/M arrest through ATM/ATR-dependent p53 phosphorylation at Ser15

ATM/ATR-dependent p53 phosphorylation occurs in response to telomere damage at the G2/M transition preventing cell cycle progression, whilst p53 inactivation enables cells with dysfunctional telomeres to enter mitosis. Thus, we tested whether inhibition of ATM and ATR kinases could rescue the mitotic entry block imposed by loss of TRF2 or POT1, similarly to p53 abrogation. We treated U2OS cells synchronised in G2/M with the ATM inhibitor Ku55933 or ATR inhibitor ETP-46464, following TRF2 or POT1 siRNA-mediated depletion (Figure 14A). Although both inhibitors reduced the phosphorylation of p53, H2AX and CHK1 in response to telomere damage, only the ATM inhibitor abrogated CHK2 phosphorylation induced by TRF2 depletion. This is consistent with the notion that CHK2 is only phosphorylated and activated by ATM in response to damage (Matsuoka et al., 2000). The low rate of mitotic entry following telomere uncapping by either POT1 or TRF2 depletion was rescued by both ATM and ATR inhibition (Figure 14B), suggesting that both ATM and ATR are required for the G2/M response to damaged telomeres. A similar effect was observed when depleted cells were treated with caffeine, known to compromise both ATM and ATR activities [(Sarkaria et al., 1999); Figure 14C].

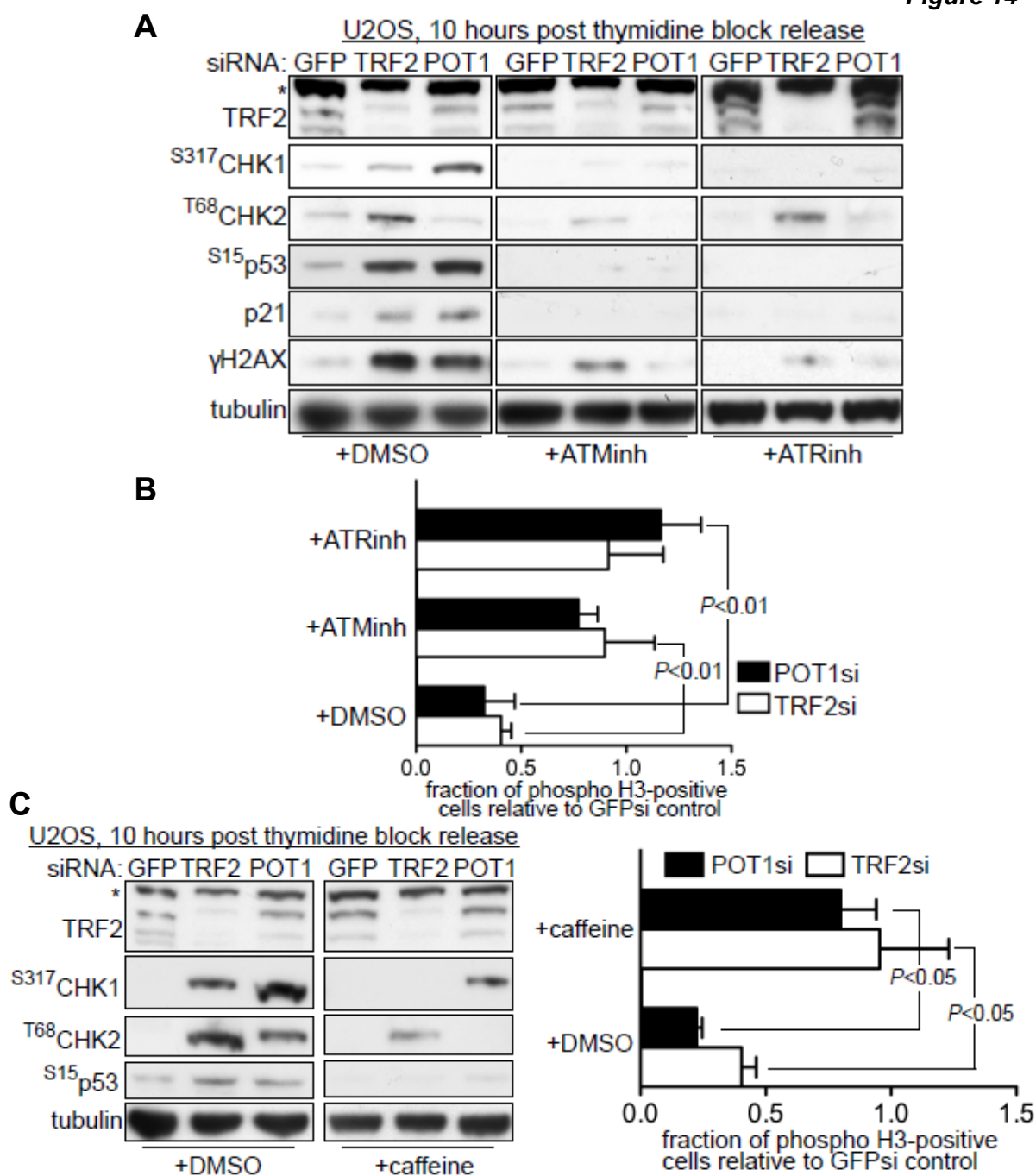


Figure 14: ATM/ATR-dependent G2/M checkpoint activation prevents mitotic entry in response to telomeres uncapped by TRF2 or POT1 inhibition

(A) U2OS cells transfected with TRF2, POT1 or control GFP siRNAs were grown for 48 h before synchronisation by double thymidine block and release in fresh media for 10 h. ATM inhibitor (Ku55933), ATR inhibitor (ETP-46464) or solvent (DMSO) were added to the media 4 h prior to collection. Cell extracts were prepared and immunoblotted as shown. (B) U2OS cells treated as in (A) were stained with propidium iodide and an antibody against phosphorylated histone H3-Ser 10 and analysed by flow cytometry. $n=10,000$ cells were analysed for each sample. Error bars represent SD of three independent experiments. (C) U2OS cells were transfected with siRNAs and synchronised as in (A). Caffeine or solvent (DMSO) were added to media 4 h prior to collection. Cells were collected and immunoblotted as shown or stained with propidium iodide and an antibody against phospho-H3 (Ser 10) and analysed by flow cytometry as in (B).

Both ATR and ATM kinases have the ability to phosphorylate p53 at Ser15 following exposure to DNA damaging agents (Canman et al., 1998; Tibbetts et al., 1999) and we have detected the same phosphorylation event in response to telomere dysfunction triggered by loss of TRF1, TRF2 or POT1 during the G2/M transition (Figure 10). In addition, p53 can be phosphorylated at Ser20 by CHK1 and CHK2 checkpoint kinases in response to DNA damage (Chehab et al., 2000; Shieh et al., 2000; Hirao et al., 2000). Thus, we tested whether mutation of either of these two residues could alter the ability of p53 to mediate the G2/M arrest in response to telomeres rendered dysfunctional through TRF2 or POT1 inhibition. Human SAOS-2 cells, in which p53 function is compromised (Stott et al., 1998), were transfected with constructs expressing p53 variants carrying Ser15Ala and Ser20Ala substitutions, or wild type p53, following TRF2 or POT1 siRNA-mediated depletion. Exogenously expressed wild type or mutant p53 could be detected in all cells except for those transfected with vector only (Figure 15A). As expected, cells expressing the Ser15Ala mutant p53 lacked Ser15 phosphorylation in response to TRF2 depletion, as well as detectable p21 induction. We next examined mitotic entry following telomere uncapping through TRF2 and POT1 siRNA-mediated depletion in these cells. As expected, progression into mitosis was impaired upon TRF2 or POT1 inhibition in cells expressing wild-type p53 (Figure 15B), suggestive of p53-dependent checkpoint activation. The Ser20Ala mutant p53 showed a rate of mitotic entry similar to wild type. In contrast, cells in which ATM/ATR-dependent phosphorylation of p53 was abrogated by mutation of Ser15, entered mitosis at a higher rate comparable to the control vector-transfected, p53-deficient cells (Figure 15B). Similarly, Ser15Ala mutation rescued mitotic entry in TRF2- and POT1-depleted WI38-VA13 human cells (Figure 15C), also known to lack a functional p53 pathway (Stott et al., 1998). These results demonstrate that ATM/ATR-dependent phosphorylation of p53 at Ser15 is specifically required for the G2/M checkpoint activation in response to telomeres uncapped through TRF2 or POT1 inhibition.

Figure 15

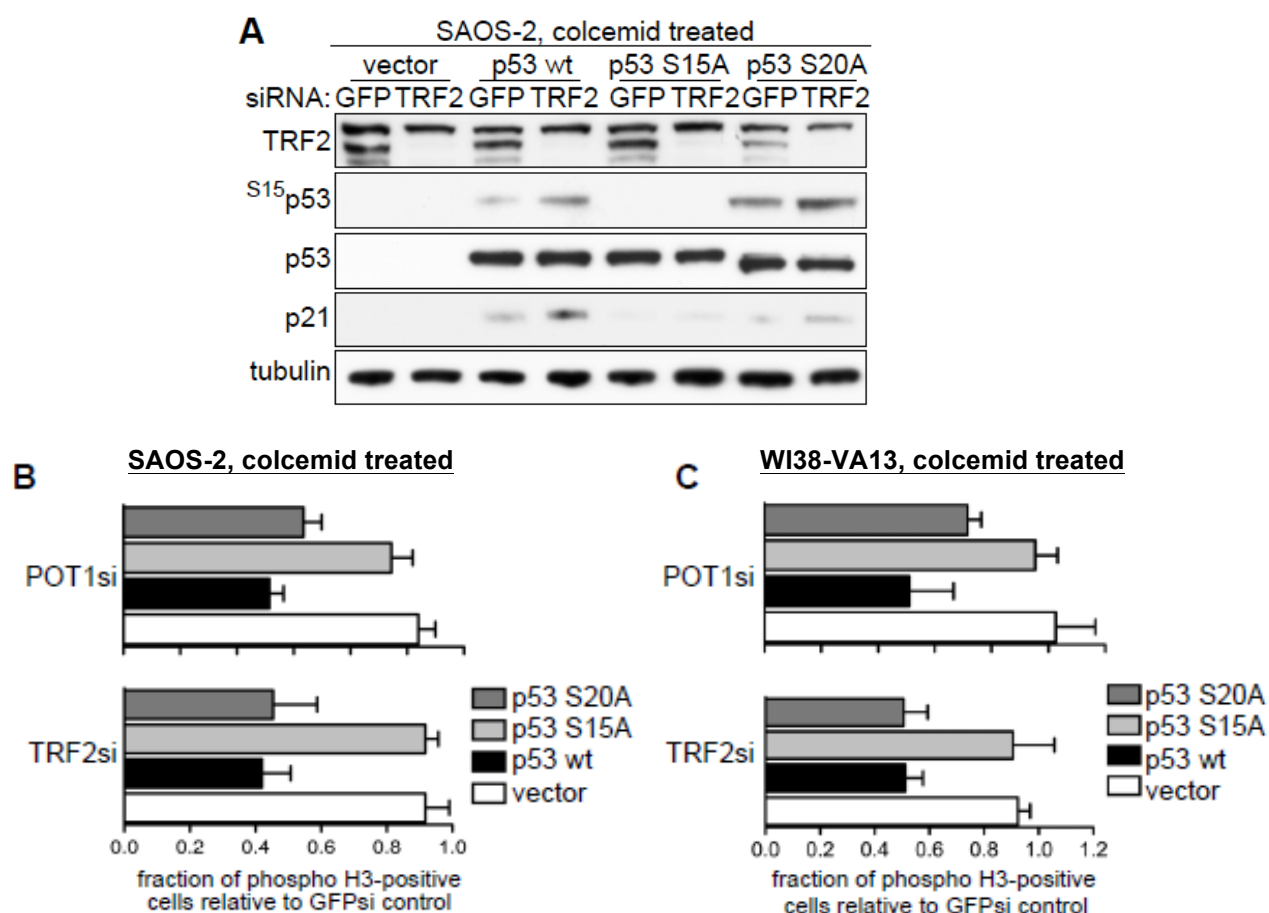


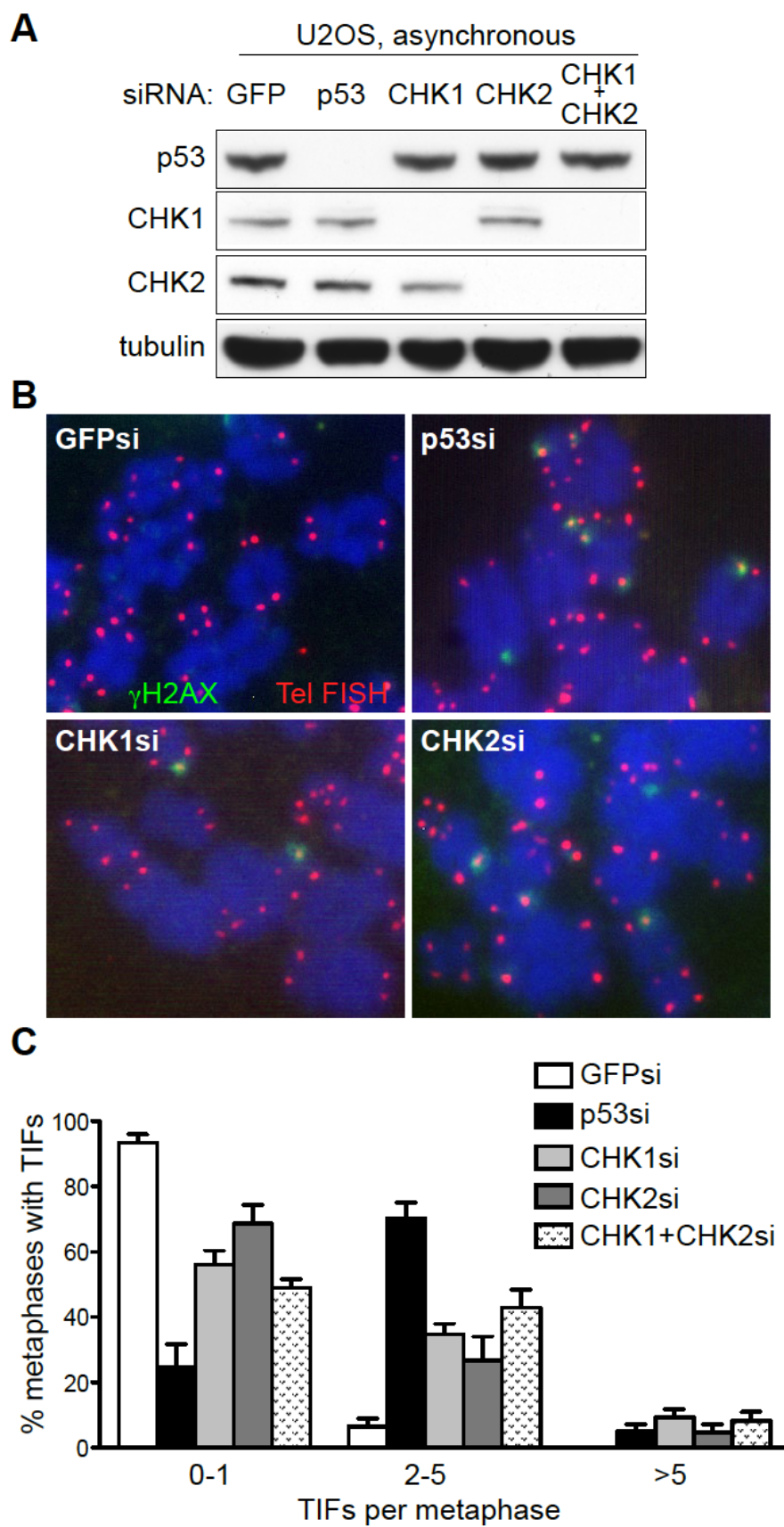
Figure 15: ATM/ATR-dependent activation of p53 at Ser15 prevents mitotic entry in response to telomeres uncapped by TRF2 or POT1 inhibition

(A) SAOS-2 cells were transfected with p53-encoding constructs or vector alone, 24 h after treatment with TRF2 or control GFP siRNAs. Cell extracts prepared 24 h later were immunoblotted as shown. Tubulin was used as a loading control. (B) SAOS-2 cells and (C) WI38-VA13 cells treated as in (A) were stained with propidium iodide and an antibody against phosphorylated histone H3 (Ser10) and analysed by flow cytometry. $n=10,000$ cells were analysed for each sample. Error bars represent SD of at least two independent experiments. The p value was calculated using an unpaired two-tailed t test.

3.10 p53/p21 and CHK1/CHK2 provide distinct pathways to monitor telomere capping at G2/M during physiological cell cycle progression

After establishing the role of p53 in the G2/M response to dysfunctional telomeres we were interested in the roles of other key regulators of the G2/M DDR. Therefore, we addressed whether CHK1 and CHK2 checkpoint kinases, known to play a role in the G2/M response to unrepaired DSBs (Bartek and Lukas, 2003), could also monitor telomere capping prior to entry into mitosis similarly to p53. We thus, inhibited CHK1 and CHK2 expression with siRNA in asynchronous U2OS cells (Figure 16A) and monitored the frequency of uncapped telomeres in mitosis using combined IF-FISH staining of mitotic chromosomes (Figure 16B). γ H2AX labelling of mitotic chromosomes is rarely seen in U2OS cells, but it increases after p53 or p21 siRNA-mediated depletion (Figure 16). Similarly, we found that the number of γ H2AX-labelled telomeres in mitotic U2OS cells depleted of CHK1 or CHK2 is higher than in control cells (Figure 16C). This effect however is milder than that caused by p53 abrogation, even when CHK1 and CHK2 are depleted concomitantly. Moreover, there was a robust p53 phosphorylation at Ser15 and p21 induction particularly in CHK1-deficient cells, while CHK1 and CHK2 were both phosphorylated in the absence of p53 (Figure 16D). Moreover, CHK1 phosphorylation was enhanced in cells lacking CHK2 and conversely, CHK2 phosphorylation was detectable in cells lacking CHK1 at G2/M, suggesting a compensatory effect and crosstalk between the ATM and ATR-dependent checkpoint response pathways (Bartek and Lukas, 2003). We conclude that, although p53 provides the major pathway for uncapped telomere detection at the G2/M transition during unchallenged cell cycle progression, the checkpoint kinases CHK1 and CHK2 are also required for successful completion of telomere capping reactions before entry into mitosis. These 3 pathways possibly act in parallel to efficiently protect telomere integrity.

Figure 16



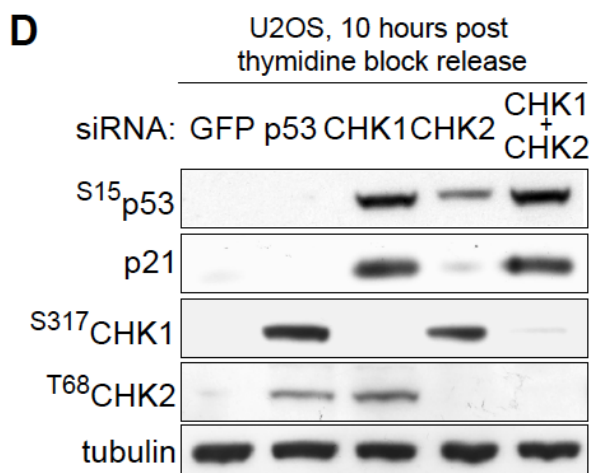


Figure 16: CHK1 and CHK2 are required for the G2/M transition during physiological cell cycle progression

(A) U2OS cells were transfected with siRNA and grown for 48 h. Cell extracts were prepared and immunoblotted as indicated. (B) U2OS cells treated with p53, CHK1, CHK2 or GFP control siRNAs for 48 h were arrested in mitosis with colcemid and mitotic chromosomes were spread onto glass slides using the cytopsin method. Preparations were stained with an anti- γ H2AX antibody (green) and a Cy3-conjugated (CCCTAA)₆-PNA probe (red). DNA was counter stained with DAPI (blue). (C) TIF frequency was determined by counting the number of foci in 100-150 metaphases. Error bars represent SD of at least two independent experiments. (D) U2OS cells transfected with the indicated siRNAs were grown for 48 h before the initiation of synchronisation by double thymidine block and release in fresh media for 10 h. Cell extracts were immunoblotted as shown.

3.11 CHK1 and CHK2 mediate the G2/M responses to POT1 and TRF2 depletion respectively

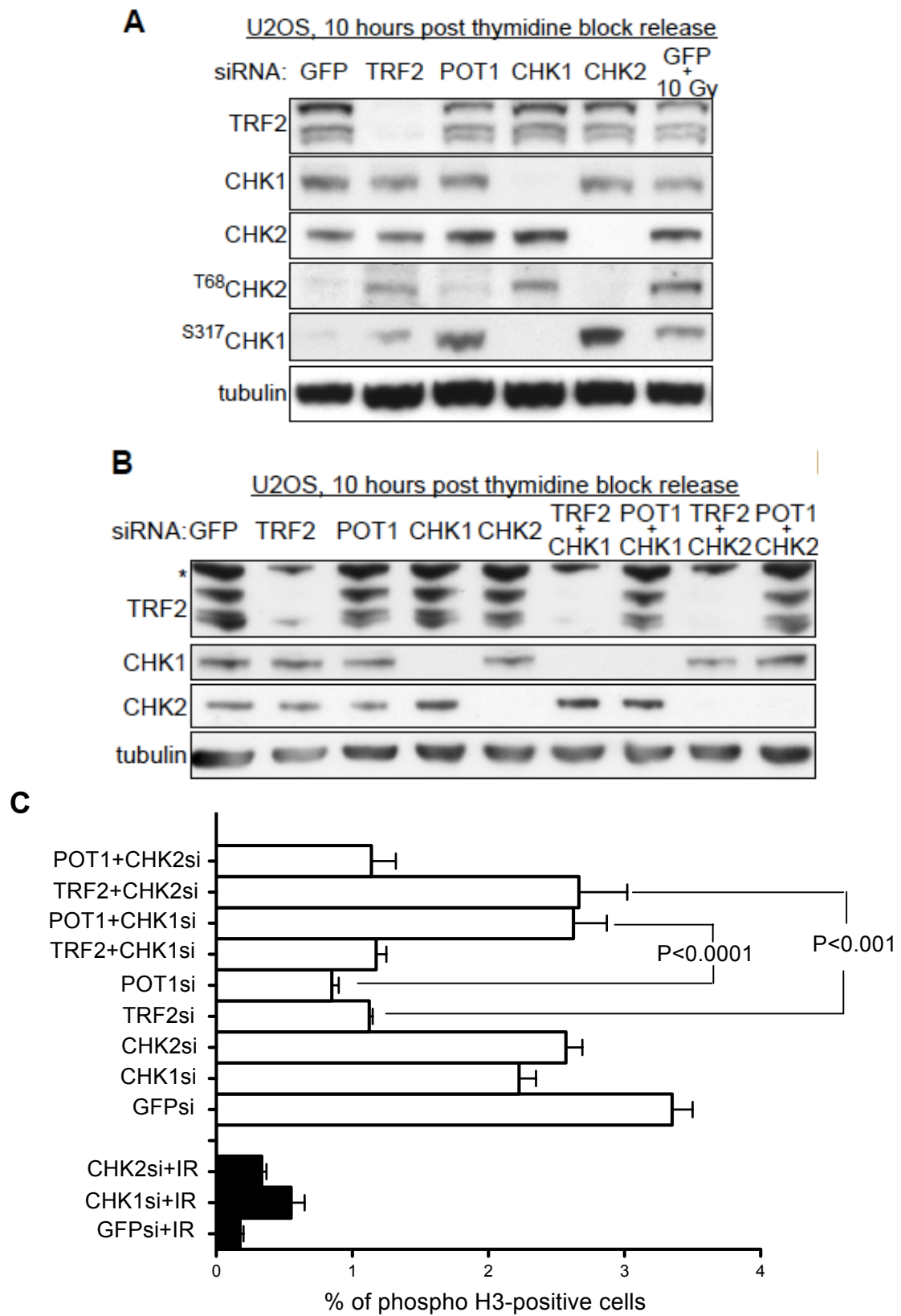
After confirming a role of CHK1 and CHK2 in the monitoring of telomere uncapping during the physiological cell cycle progression, we next set out to see whether they can also regulate the response to artificial telomere uncapping, similarly with p53. As previously shown, conditional deletion of TRF2 in MEFs triggers ATM-dependent CHK2 phosphorylation, whilst inactivation of POT1 induces ATR-dependent CHK1 phosphorylation (Denchi and de Lange, 2007). This led to the conclusion that TRF2 inhibits ATM signalling at mouse telomeres. Similarly, POT1 inhibits ATR-mediated checkpoint responses in mouse cells. The assumption that human and mouse telomeres are structurally and functionally identical has been recently challenged by the discovery that mouse telomeres have two functionally distinct POT1 proteins, POT1a and POT1b (He et al., 2006; Hockemeyer et al., 2006; Wu et al., 2006). Whilst combining features of both, human POT1 cannot compensate for the concomitant loss of *Pot1a* and *Pot1b* genes in mouse (Palm et al., 2009) suggesting a clear evolutionary divergence in shelterin composition and function from rodent to human telomeres. Thus, we addressed whether the G2/M DDR emanating from human telomeres uncapped by TRF2 or POT1 siRNA-mediated depletion is similar to the one in mouse.

In human cells synchronised at the G2/M transition, we found that TRF2 depletion led to phosphorylation of CHK2 at Thr68, whilst POT1 depletion induced CHK1 phosphorylation at Ser317 (Figure 17A). This observation suggests that the response to human uncapped telomeres is similar to the mouse models, in which loss of TRF2 triggers an ATM-dependent DDR and POT1 deficiency activates ATR. Importantly, we detected CHK1 phosphorylation indicative of ATR activation in cells lacking TRF2. This is consistent with the reported ATM-dependent activation of ATR in response to DNA damage (Adams et al., 2006; Cuadrado et al., 2006; Garcia-Muse and Boulton, 2005; Jazayeri et al., 2006; Myers and Cortez, 2006; Zou and Elledge, 2003). We also noticed

an enhanced CHK1 phosphorylation in cells lacking CHK2 and CHK2 phosphorylation in cells lacking CHK1, similarly with figure 16D (Figure 17A).

To directly address the ability of CHK1 and CHK2 to signal telomere damage and delay mitotic entry in the presence of artificially uncapped telomeres, we abrogated telomere protective structures through inhibition of TRF2 and POT1, alone, or in conjunction with CHK1 and CHK2 (Figure 17B). We then synchronised depleted cells by double thymidine block and release, and measured their ability to enter mitosis by staining with phospho-H3 (Ser10). As a control, cells exposed to 10 Grays (Gy) of IR were analysed in the same way. Irradiated GFP siRNA-transfected U2OS cells arrest at the G2/M transition and this arrest is mildly alleviated by CHK1 inhibition and to a lesser extent by CHK2 inhibition (Figure 17C), confirming the role of CHK1 but not CHK2 in the induction of G2/M arrest after IR (Syljuasen et al., 2006). As previously shown, loss of POT1 or TRF2 expression impairs entry into mitosis. We were also able to detect a minor, not statistically significant decrease in the mitotic index of the cells depleted only for CHK1 and CHK2 (Figure 17C), in contrast with p53 depletion which leads to complete abrogation of the G2/M checkpoint as previously shown (Figure 13B). Mitotic progression is restored by co-depletion of TRF2 in conjunction with CHK2 and of POT1 in conjunction with CHK1 (Figure 17C). Similar abrogation of the G2/M arrest was observed in POT1-depleted cells treated with CHK1 inhibitors (Figure 18). To further test the specificity of the G2/M response to TRF2- or POT1-mediated uncapping, we measured TIF levels in the depleted cells. TIFs occurred at high frequency in TRF2/POT1- and CHK2/CHK1-double-depleted metaphases (Figure 17D), most likely due to more cells progressing into mitosis.

Figure 17



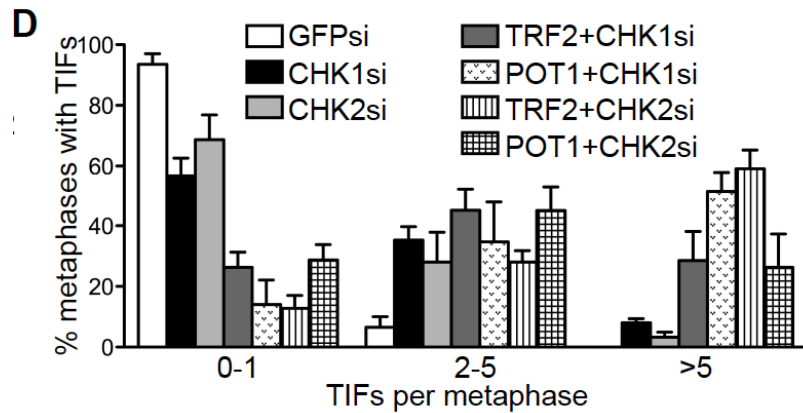


Figure 17: CHK1 and CHK2 mediate the G2/M responses to POT1 and TRF2 depletion respectively

(A) U2OS cells transfected with siRNA were grown for 48 h before the initiation of synchronisation by double thymidine block and release. Cells collected 10 h after release were processed for Wb as indicated. Extracts from cells exposed to 10 Gy of IR were used as a control for CHK1 and CHK2 phosphorylation. Tubulin was used as a loading control. (B) U2OS cells transfected with siRNA were grown for 48 h before the initiation of synchronisation by double thymidine block and release. Cells collected 10 h after release were processed for Wb as indicated. (C) U2OS cells treated as in (B) were stained with propidium iodide and an antibody against phosphorylated histone H3 (Ser10) and analysed by flow cytometry. $n=10,000$ cells were analysed for each sample. Error bars represent SD of at least three independent experiments. P values were calculated using an unpaired two-tailed t test. Only the analysis of statistically significant samples is shown. (D) U2OS cells treated as in (B) were arrested in mitosis with colcemid and mitotic chromosomes were spread onto glass slides using the cytopsin method. TIF frequency was determined by counting the number of γ H2AX foci co-localising with telomeres identified with a $(CCCTAA)_6$ -PNA probe, in 100-150

Figure 18

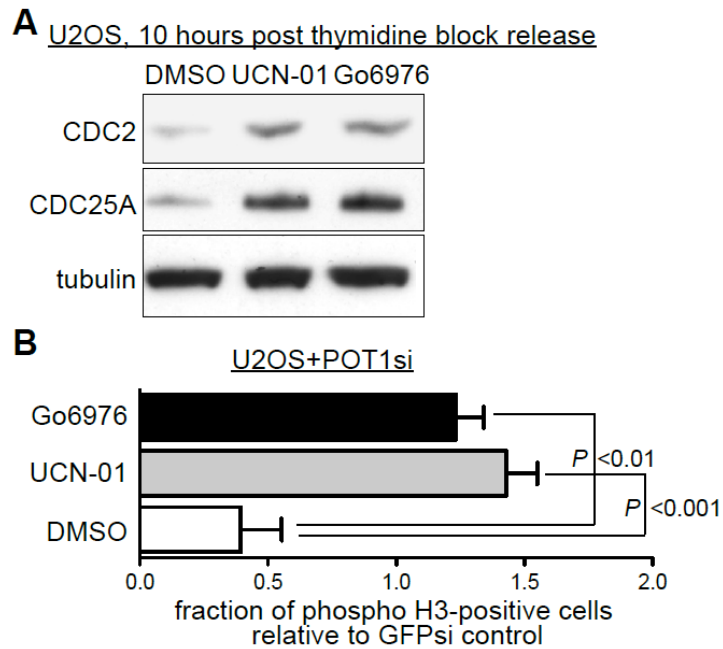


Figure 18: CHK1 chemical inhibition rescues the G2/M arrest in POT1-depleted cells

(A) U2OS cells were synchronised by double thymidine block and release in fresh media for 10 h. UCN-01 and Go6976 inhibitors, or DMSO control were added to the media 4 h before collection. Cell extracts were immunoblotted as shown. (B) U2OS cells transfected with POT1 or control GFP siRNAs were grown for 48 h before the initiation of synchronisation by double thymidine block and release in fresh media for 10 h. UCN-01 and Go6976 inhibitors, or DMSO control were added to the media 4 h before collection. Cells were stained with propidium iodide and an antibody against phospho-histone H3 (Ser 10) and analysed by flow cytometry. $n=10,000$ cells were analysed for each sample. Error bars represent SD of two independent experiments.

3.12 CHK1 and CHK2 mediate the G2/M response to uncapped telomeres by regulating the stability of CDC25 family members

Next, we were interested in addressing the downstream targets of CHK1 and CHK2 that are responsible for the cell cycle arrest in the presence of uncapped telomeres. It is known that during unchallenged cell proliferation, CDC25 phosphatases promote G2/M transition by removing phosphorylated groups from the CDK1/Cyclin B complex. Upon exposure to DNA damaging agents, activated CHK1 and CHK2 destabilise CDC25A and CDC25C to induce cell cycle arrest (Bartek and Lukas, 2003; Kastan and Bartek, 2004). To address whether CHK1 and CHK2 could act to prevent mitotic entry in response to uncapped telomeres through a similar mechanism, we monitored CDC25A and CDC25C levels in cells synchronised at the G2/M transition. As expected, we detected both phosphatases, as well as the mitosis-promoting CDK1 expression in control cells transfected with GFP siRNA (Figure 19A, B). Inhibition of TRF2 expression significantly reduced CDC25C levels, but not CDC25A (Figure 19A). Consistent with the proliferation arrest in these cells being rescued by CHK2 inactivation (Figure 17C), we found that CDC25C levels were restored when CHK2, and to a lesser extent CHK1, expression was abrogated in TRF2-depleted cells. Interestingly, POT1 depletion led to a decrease in both CDC25A and CDC25C expression levels (Figure 19B). These were restored efficiently by CHK1 concomitant inhibition. CDK1 levels were decreased in both cases, consistent with impaired progression into mitosis in cells lacking either TRF2 or POT1 (Figure 17C). These results support the concept that telomere uncapping through TRF2 loss triggers primarily ATM/CHK2-dependent signalling, whilst POT1-depleted telomeres elicit an ATR/CHK1 response.

Figure 19

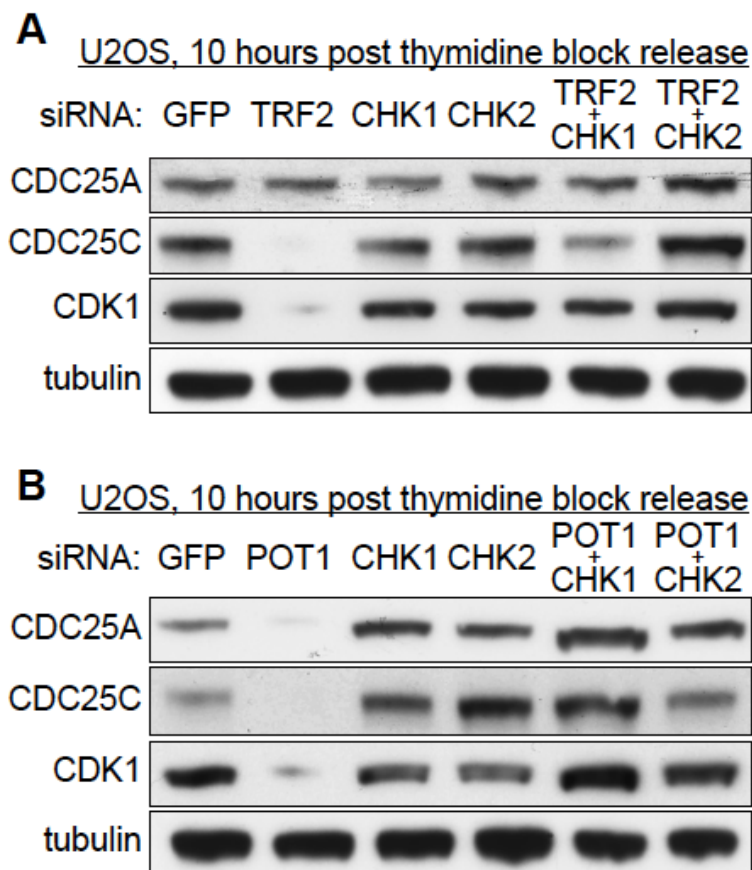


Figure 19: CHK1 and CHK2 control the stability of CDC25 phosphatases during G2/M arrest elicited by TRF2 or POT1 inhibition

(A, B) U2OS cells transfected with siRNA were grown for 48 h before the initiation of synchronisation by double thymidine block and release. Cells collected 10 h after release were processed for Wb as indicated. Tubulin was used as a loading control.

All these data allow us to propose a model for the G2/M response to dysfunctional telomeres (Figure 20). According to that model 3 pathways are regulating the telomere capping reaction at the G2/M during the physiological cell cycle progression: p53/p21 pathway, CHK1 pathway and CHK2 pathway. Based on our data, all the 3 pathways are acting in parallel in order to ensure efficient telomere capping before mitotic entry. The effect of all these pathways is more substantial after artificial telomere uncapping by TRF2 or POT1 depletion in human cells. In that case, ATM/ATR are activated in order to further activate both p53/p21 and CHK1/2 pathway leading to G2/M arrest by CDK1/CyclinB inhibition in order to allow telomere re-capping before mitotic entry (Figure 20A). Furthermore, this model can become more accurate, defining that CHK1 is responsible for the G2/M arrest after POT1 depletion through degradation of CDC25A and CDC25C, while CHK2 results in G2/M arrest after TRF2 depletion via inhibition of CDC25C (Figure 20B).

Figure 20

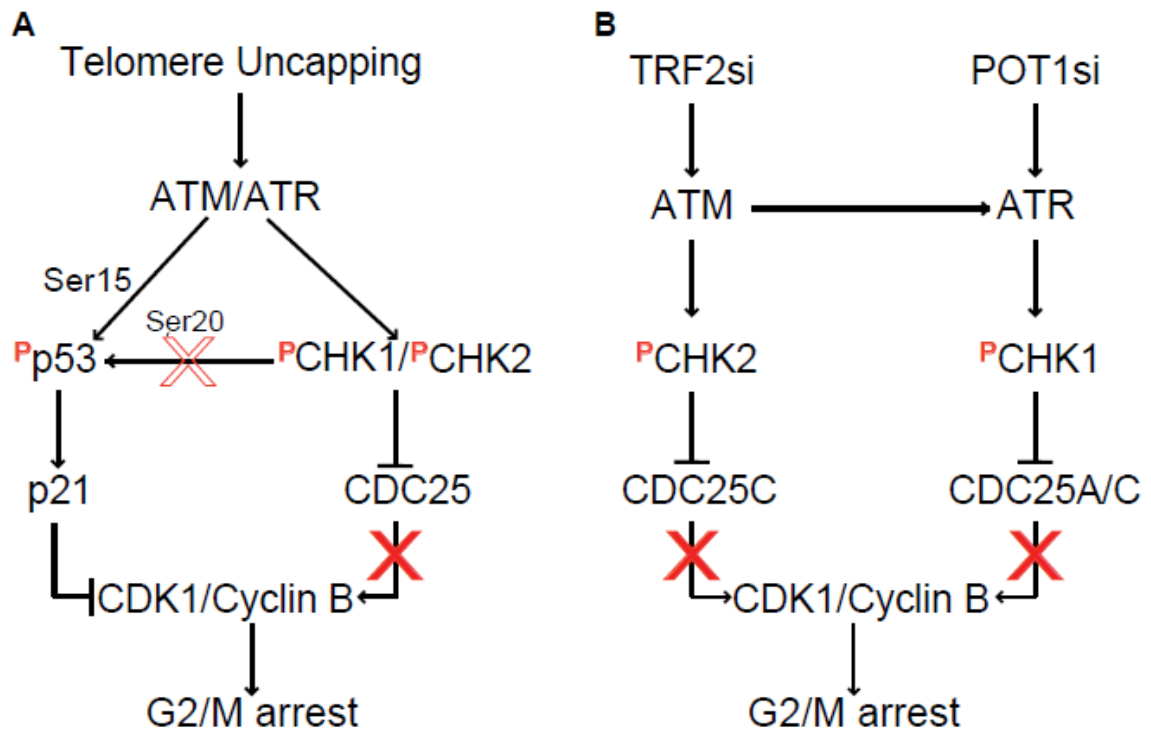


Figure 20: Model for G2/M signalling in response to artificially uncapped telomeres

(A) Uncapped telomeres trigger ATM/ATR responses leading to phosphorylation of p53 at Ser15 or activation of CHK1/CHK2 checkpoint kinases. Suppression of CDK1 activity through p21 induction or CDC25 degradation blocks cell cycle progression into mitosis. (B) Telomere uncapping through TRF2 inhibition activates ATM signalling during G2/M transition, which leads to CDC25C degradation and cell cycle arrest. POT1 depletion elicits ATR responses leading to CHK1-dependent CDC25A and CDC25C destruction. ATM activation can promote ATR signalling, similarly to the G2/M response to IR (Hastie et al., 1990; Jazayeri et al., 2006).

CHAPTER 4

Results

Part 2

4.1 TRF1 deficiency results in DDR activation at telomeres

Disruption of telomere protective structures through the inhibition of components of the shelterin complex can lead to DDR and subsequently be the source of great genomic instability through the formation of fusions between the unprotected telomere ends. However, in the context of telomere protection only the role of TRF2 and POT1 was well established in mouse cells, with TRF2 protecting the telomeres by inhibiting the ATM-dependent DNA damage signalling and POT1 by inhibiting the ATR-dependent DNA damage signalling (Denchi and de Lange, 2007). Considering that all the 6 proteins composing the shelterin complex are encoded by essential genes (Palm and de Lange, 2008), we were interested to address the cellular functions of other components of the shelterin complex and their role in telomere maintenance.

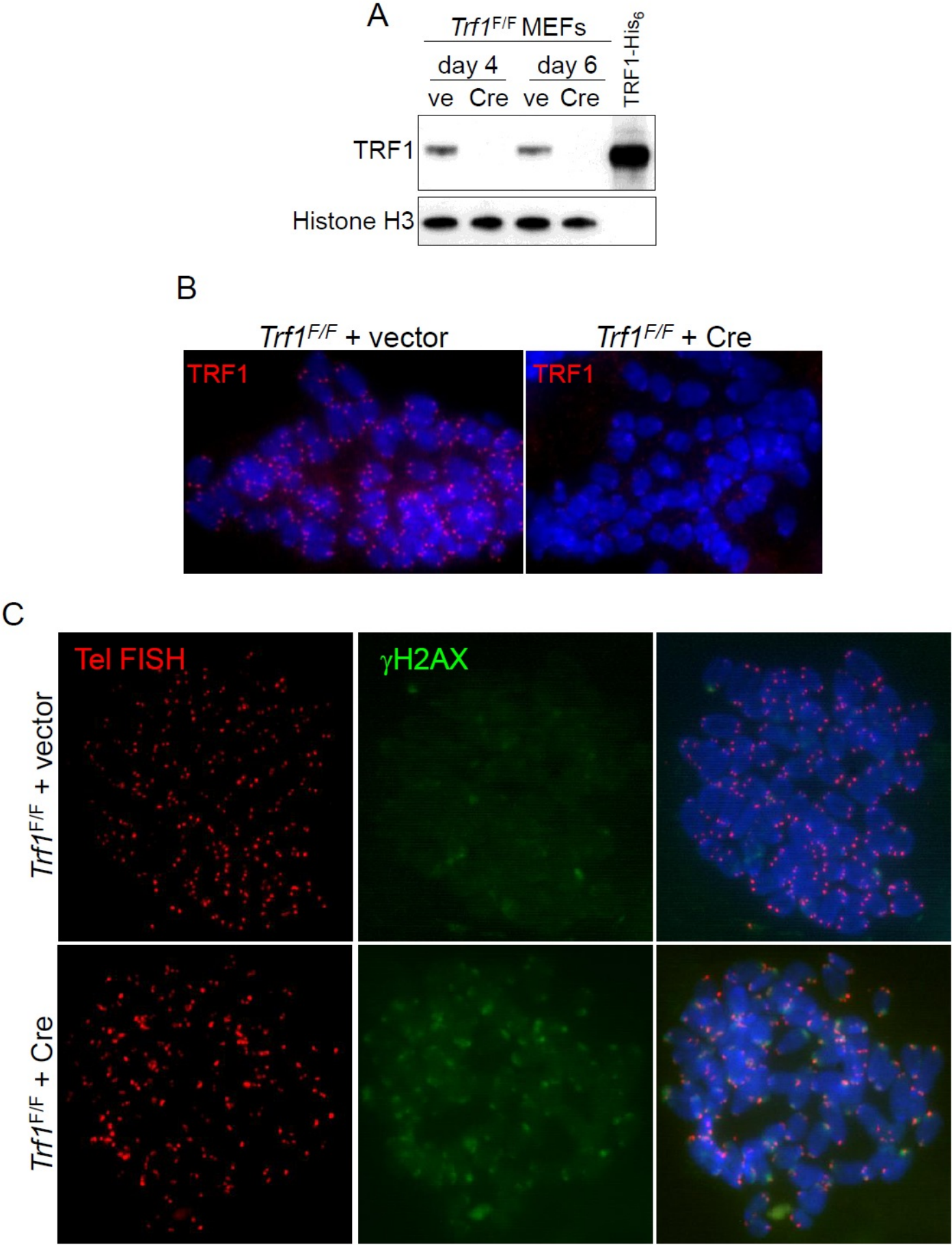
First, we studied in collaboration with Maria Blasco's group, the role of TRF1 in telomere protection. TRF1 shares similar structural features with TRF2 and its inhibition by the overexpression of a dominant negative has similar consequences with TRF2 inhibition for telomere protection (van Steensel and de Lange, 1997). Maria Blasco's laboratory generated a mouse model for the conditional deletion of the *Trf1* gene, where *Trf1* exon 1 was flanked by loxP sites (Martinez et al., 2009).

We addressed whether TRF1 abrogation resulted in increased telomeric damage in LT-immortalised MEFs. To this end, transient expression of Cre recombinase from the self-inactivating Hit-and-Run retrovirus [H&R; (Silver and Livingston, 2001)], resulted in efficient loss of TRF1 gene expression as monitored by Wb at 4 and 6 days after selection (Figure 21A). Concomitantly, the telomere-associated TRF1 signal on metaphase

chromosome spreads became undetectable by IF (Figure 21B). Most of these cells showed γ H2AX foci at telomeres of spread mitotic chromosomes, with at least one telomere per pair of sister chromatids being labelled by γ H2AX (Figure 21C). As mentioned above γ H2AX foci associated with critically short/dysfunctional telomeres are also known as TIFs (d'Adda di Fagagna et al., 2003; Takai et al., 2003). *Trf1*-deleted MEFs showed a very robust TIF induction, with 40% of the metaphases carrying 10–20 TIFs and 50% having >20 foci (Figure 21D).

As expected, TIF induction following *Trf1* deletion was accompanied by DDR activation. Phosphorylation of ATM at Ser1981 and its downstream checkpoint kinases CHK1 at Ser345 and CHK2 (as detected by mobility shift) was detected by Wb (Figure 21E). Also an indication that the checkpoint was activated was the high phosphorylation of H2AX itself (Figure 21E). As a positive control, we used wild type MEFs exposed to 10 Gy of IR, which show a complete shift of the CHK2 lower band and high levels of ATM and CHK1 phosphorylation. Our data suggest that TRF1 has a similar function with TRF2 in telomere protection by inhibiting the ATM-dependent DDR at the telomeres.

Figure 21



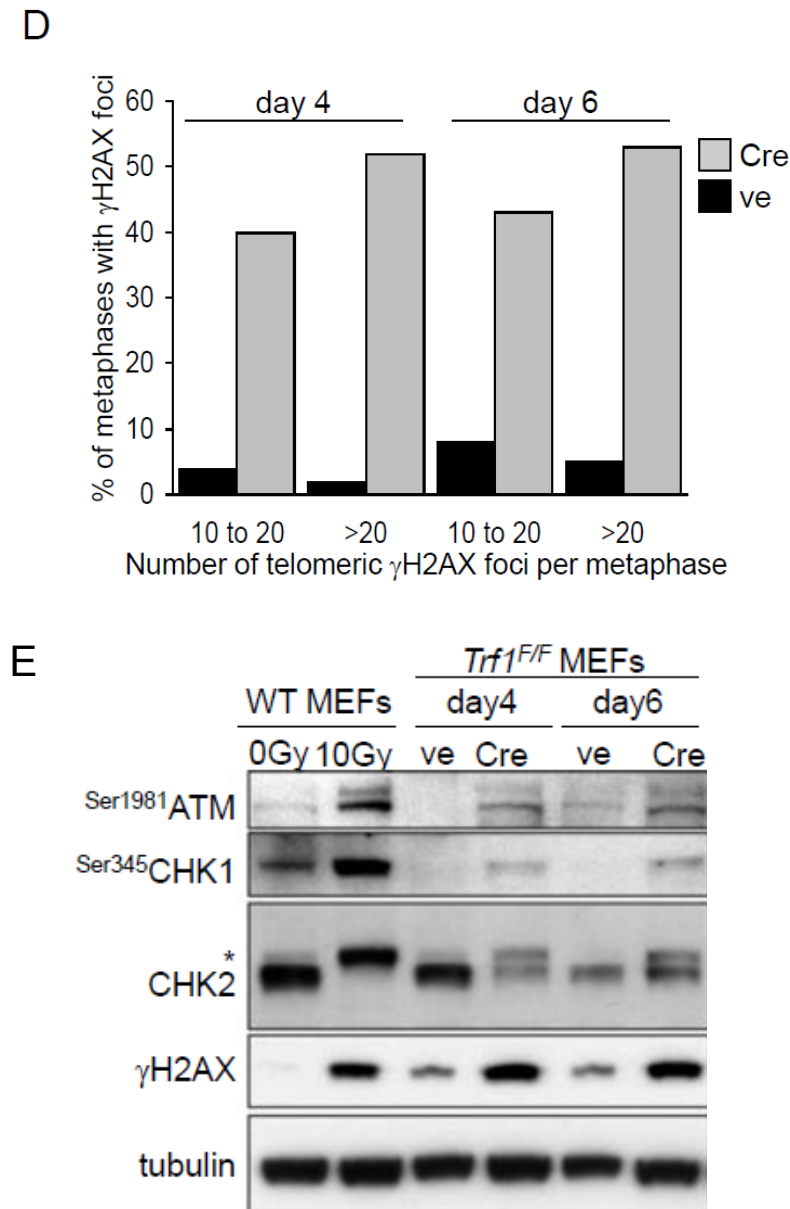


Figure 21: Activation of DDR in TRF1-deficient MEFs

(A) Wb detection of mouse TRF1 in *Trf1^{F/F}* MEFs treated with empty pBabe vector (ve) and H&R Cre (Cre). Extracts from cells collected 4 and 6 days post-selection were immunoblotted as indicated. Recombinant human TRF1-His₆, migrating more quickly due to the smaller size of the human TRF1 protein, served as a control for antibody specificity. Histone H3 was used as loading control. (B) IF detection of mouse TRF1 in *Trf1^{F/F}* MEFs treated with empty vector and Cre. Metaphase chromosome spreads were stained with anti-TRF1 rabbit polyclonal antibody (red). DNA was counterstained with DAPI (blue). (C) IF detection of γ H2AX (green) combined with FISH staining of the telomeres (red) in *Trf1^{F/F}* MEFs treated with empty vector and Cre. (D) The percentage of metaphase nuclei exhibiting 10–20 or >20 γ H2AX foci was determined for at least 50 metaphases prepared as in (C) and collected 4 and 6 days post-selection. (E) Wb detection of DDR factors in *Trf1^{F/F}* MEFs treated with empty vector and Cre. Extracts were collected 4 and 6 days post selection as indicated. MEFs treated with 0 or 10 Gy of IR served as a control for checkpoint activation. *, corresponds to phosphorylated CHK2. Tubulin was used as a loading control.

4.2 RAP1 abrogation does not disrupt binding of other shelterin components to telomeres

Next we were interested in addressing the cellular functions of another shelterin component, RAP1. As mentioned in the introduction, RAP1 is a small protein that cannot bind directly to the telomeric DNA but only via its interaction with TRF2. In order to study its role in telomere capping and protection, we used LT-immortalised MEFs derived from *Rap1^{F/F}* conditional mice generated in Maria Blasco's laboratory (Martinez et al., 2010). For the generation of the *Rap1^{F/F}* allele, *Rap1* exon 3 was flanked by loxP sites and its deletion eliminated the *Rap1* telomere localization domain and the nuclear localization signal (Martinez et al., 2010).

We treated the *Rap1*-floxed MEFs with Cre recombinase in order to efficiently delete *Rap1* gene shown by Wb (Figure 22A). We further confirmed efficient *Rap1* deletion by IF using an anti-RAP1 antibody on metaphase spreads (Figure 22B). As RAP1 was a very poorly characterised protein, we tested whether deletion of *Rap1* gene affects the binding of other shelterin components to the chromatin. We, thus, performed chromatin fractionation assays, which showed that binding of TRF1, TRF2, POT1, TPP1 and TIN2 to chromatin was normal in *Rap1*-null cells, while no binding of RAP1 was observed (Figure 22C, D). As control, histone H3 showed normal binding to chromatin in both genotypes (Figure 22C). These data indicate that RAP1 is not affecting the binding to the chromatin of most of the other shelterin components including TRF1, TRF2 and POT1 and is not required for their recruitment to the telomeres. However, we were able to observe a minor increase in the amount of TIN2 bound to the chromatin after the deletion of *Rap1* (Figure 22C), which could mean that the loss of RAP1 could slightly affect the recruitment of TIN2 to the chromatin possibly through interaction with TRF2 since this is the protein connecting TIN2 with RAP1 in the shelterin complex.

4.3 *Rap1* deletion results in increased telomere damage and checkpoint activation

To measure DNA damage at telomeres in the absence of RAP1, we determined their co-localization with γ H2AX foci, similar to the analysis of the *Trf1*-deleted MEFs. *Rap1* deletion resulted in more than 70% of *Rap1*-deleted cells showing more than three TIFs per metaphase, whereas these events were rare in control cells (Figure 23A, B). Moreover, CHK1 and CHK2 checkpoint kinases were phosphorylated in *Rap1*-deleted cells but not in wild type cells (Figure 23C). Nevertheless, as shown in Figure 21D *Trf1*-null MEFs showed a much higher number of TIFs and consistent with that, a far more robust activation of CHK1 and CHK2 kinases (Figure 21E) than the *Rap1*-null MEFs. Therefore, the comparison between the DNA damage checkpoint activation after *Rap1* and *Trf1* deletion (Figure 23D) suggests that TRF1 is much more potent regulator of telomere protection than RAP1.

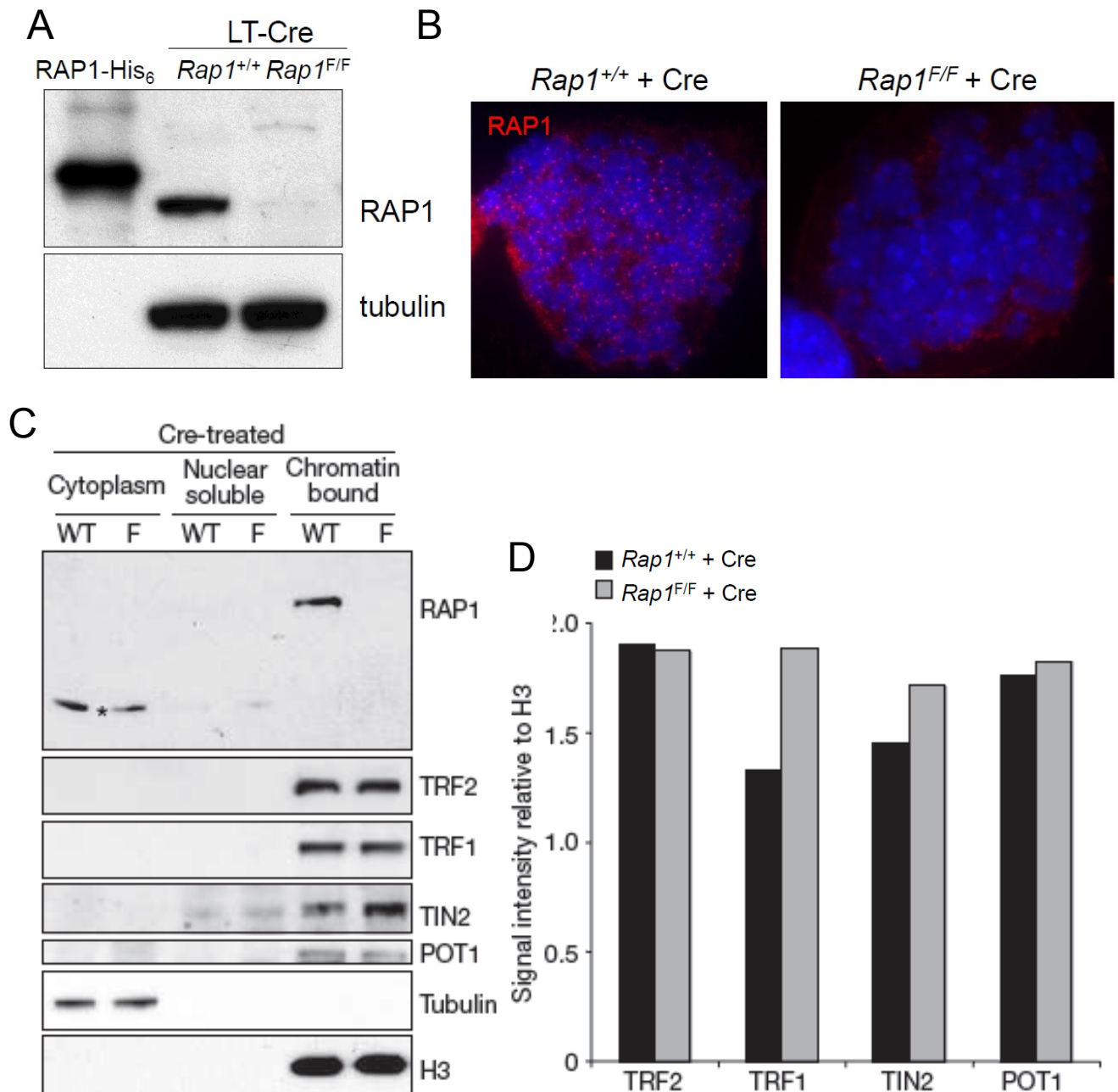
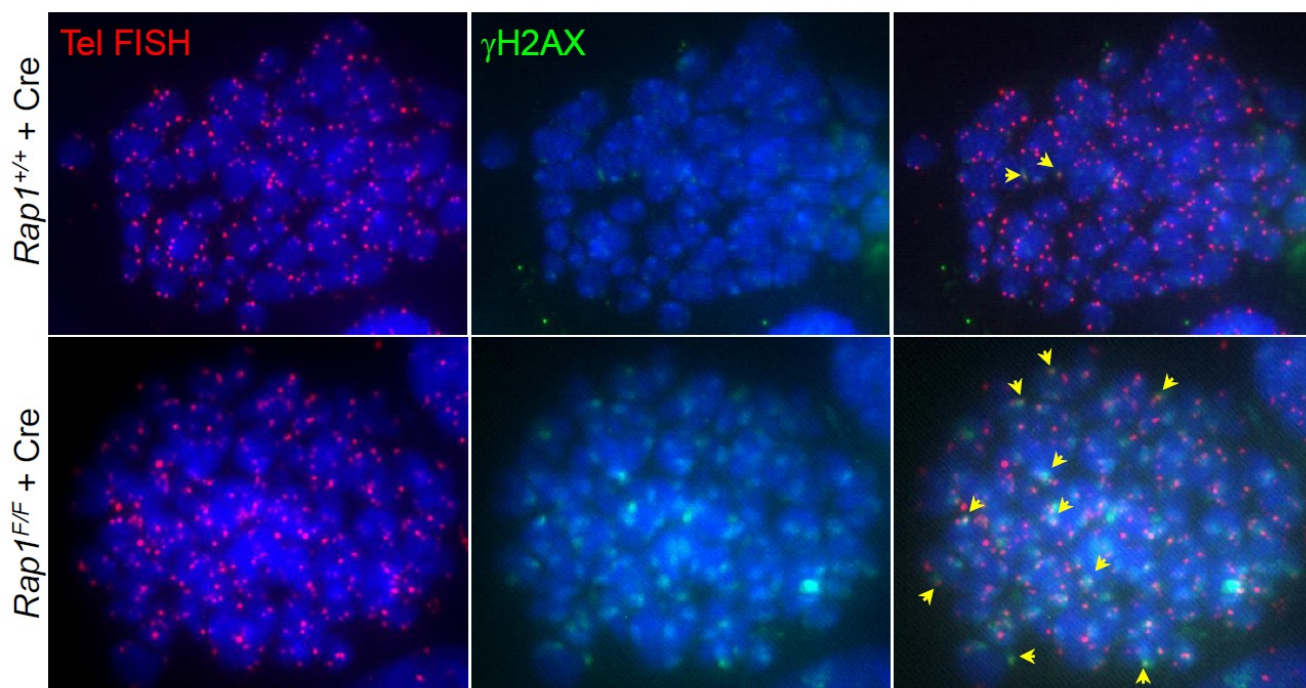


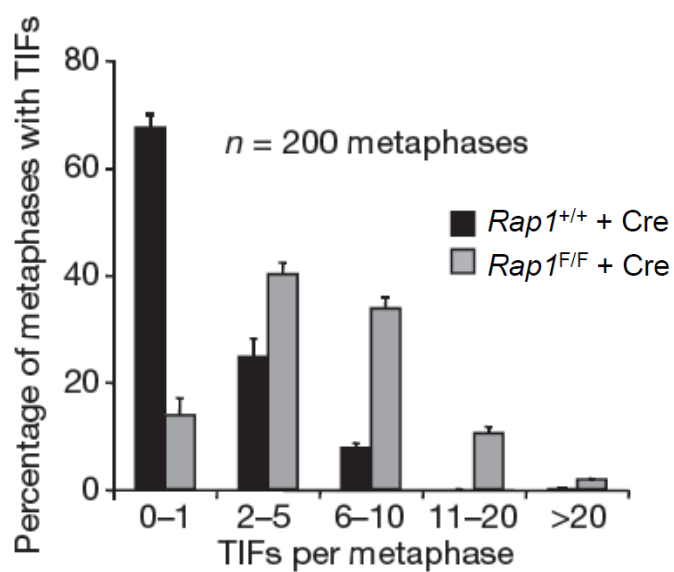
Figure 22: *Rap1* deletion does not affect the binding of other shelterin components to telomeres

(A) Wb detection of mouse RAP1 in *Rap1^{+/+}* or *Rap1^{F/F}* MEFs treated with Cre. Recombinant human RAP1-His₆, served as a control. Tubulin was used as a loading control. (B) IF detection of mouse RAP1 in *Rap1^{+/+}* and *Rap1^{F/F}* MEFs treated with Cre. Metaphase chromosome spreads were stained with anti-Rap1 rabbit polyclonal antibody (red). DNA was counterstained with DAPI (blue). (C) Subcellular fractionation of the indicated MEFs. Note the absence of full-length RAP1 in *Rap1*-null MEFs. A putative truncated form of RAP1 (*) is restricted to cytoplasmic extracts. Tubulin was used as a loading control for the cytoplasmic fraction, and histone H3 for the chromatin-bound fraction. (D) Quantification of protein levels relative to H3 shown in (C).

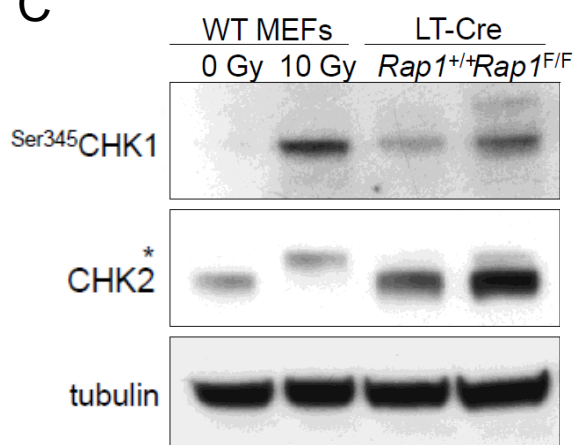
A



B



C



D

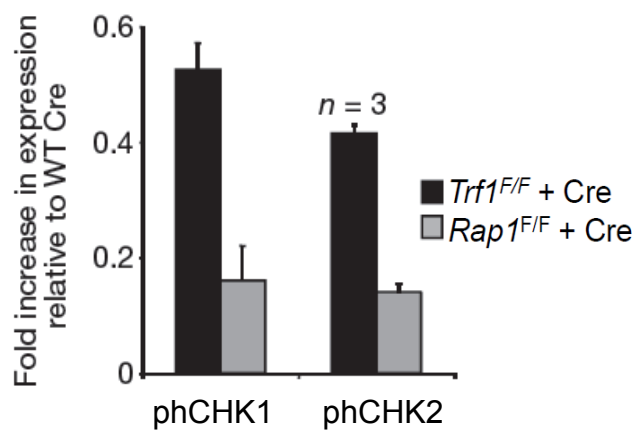


Figure 23: *Rap1* deletion results in increased telomere damage and checkpoint activation

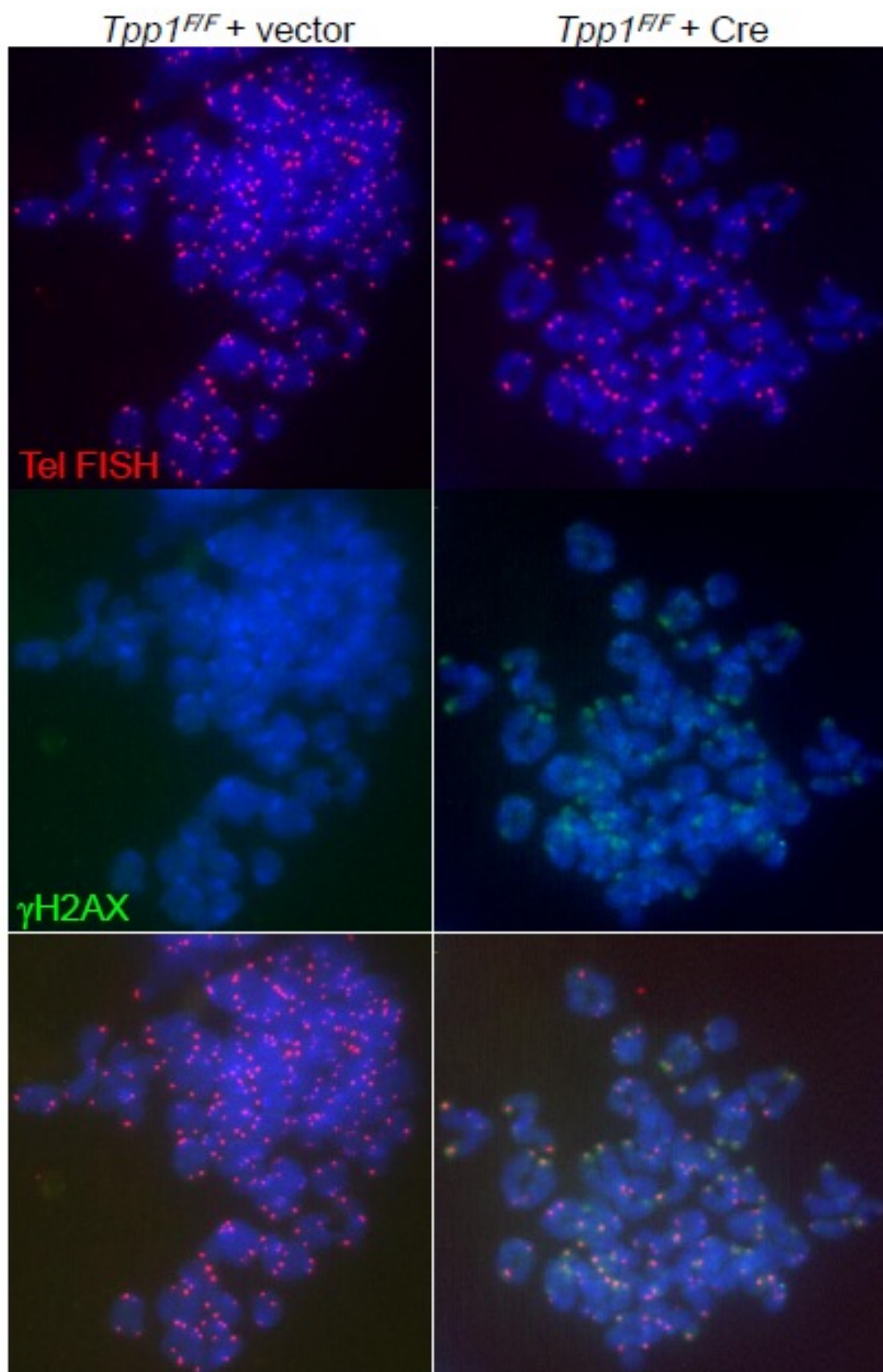
(A) Metaphase spreads of *Rap1* wild-type and *Rap1*-deleted MEFs treated with colcemid were stained with anti- γ H2AX monoclonal antibody (green). Telomeres were visualised with a Cy3-conjugated (CCCTAA)₆-PNA probe (red). Yellow arrows indicate TIFs. (B) Percentage of metaphases with the indicated number of γ H2AX foci from a total of 200 metaphases per genotype prepared as in (A). (C) Wb of phospho-CHK1 (Ser345) and phospho-CHK2 in the indicated MEFs. Wild type MEFs treated with 0 or 10 Gy of IR were controls for checkpoint activation. Tubulin was used as a loading control. (D) Fold increases in phospho-CHK1 (Ser345) and phospho-CHK2 levels detected by Wb in (C) in *Rap1*-deleted cells compared with controls. * indicates the band corresponding to the phosphorylated form of CHK2.

4.4 TPP1 abrogation results in increased telomere uncapping and checkpoint activation

Finally, we studied the consequences of the loss of another poorly characterised shelterin component, TPP1. Several evidence indicate that TPP1 forms a heterodimer with POT1 and mediates the recruitment of POT1 to telomeres (Hockemeyer et al., 2007; Houghtaling et al., 2004; Liu et al., 2004; Palm et al., 2009; Xin et al., 2007; Ye et al., 2004). To study TPP1 role in telomere protection we used LT-immortalised MEFs derived from *Tpp1*^{F/F} conditional mice generated in Maria Blasco's laboratory, in which *Tpp1* exons 1, 2, 3 and 4 were flanked by loxP sites (Tejera et al., 2010). We inhibited TPP1 expression after transient expression of the Cre recombinase H&R-encoding retrovirus. Efficient deletion of *Tpp1* gene was confirmed by QRT-PCR (Tejera et al., 2010). To determine whether *Tpp1* deletion results in the activation of checkpoint responses induced by telomere dysfunction, we addressed whether TPP1 abrogation leads to telomere damage and TIF formation. Most of the TPP1-deficient cells showed increased number of γ H2AX foci at telomeres of spread mitotic chromosomes (Figure 24A), reaching 6–20 TIFs per metaphase (Figure 24B). TIF induction following *Tpp1* deletion was accompanied by phosphorylation of the CHK1 and CHK2 checkpoint kinases (Figure 24C), whereas this was not observed in TPP1 wild type cells served as controls. As a positive control, wild type MEFs exposed to 10 Gy of IR showed a complete shift of the CHK2 lower band and a robust CHK1 phosphorylation at Ser345. We therefore concluded that TPP1 is required for efficient telomere protection by suppressing the ATM- and ATR-dependent DNA damage signalling.

Figure 24

A



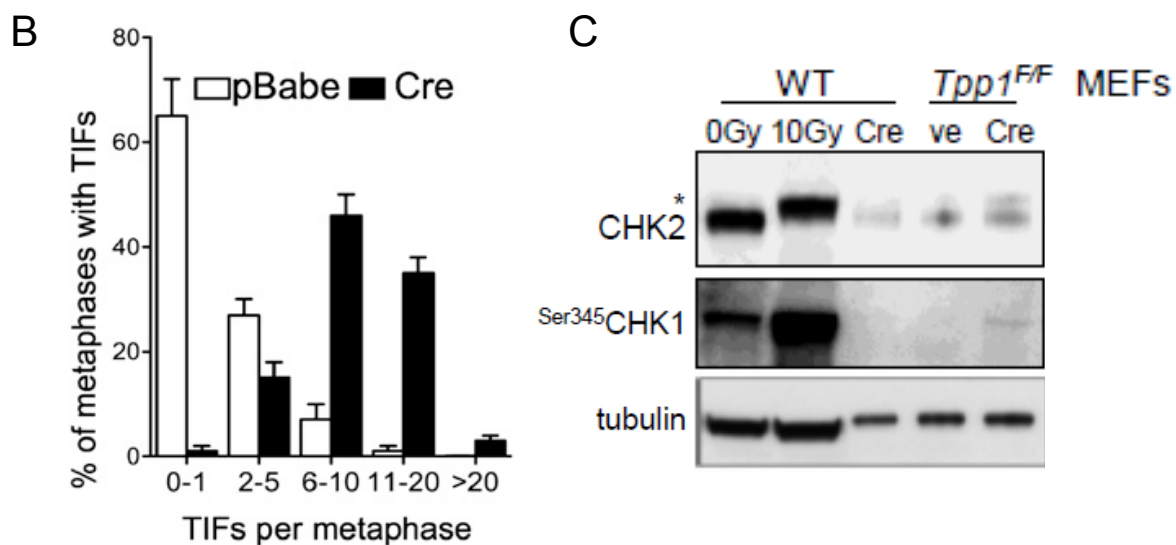


Figure 24: DDR activation in *Tpp1*-deleted MEFs

(A) Metaphase spreads of *Tpp1^{F/F}* MEFs treated with pBabe empty vector or Cre vector were stained with anti- γ H2AX monoclonal antibody (green). Telomeres were visualised with a Cy3-conjugated (CCCTAA)₆-PNA probe (red). (B) The percentage of metaphase nuclei exhibiting the indicated number of TIFs per metaphase was determined for at least 50 metaphases per genotype prepared as in (A). (C) Wb detection of phospho-CHK1 (Ser345) and phospho-CHK2 in the indicated MEFs treated with Cre vector. Wild type MEFs treated with 0 or 10 Gy of IR served as a control for checkpoint activation. Tubulin was used as a loading control. * indicates the band corresponding to the phosphorylated form of CHK2.

4.5 TPP1 is responsible for the recruitment of TERT to the chromatin and its deletion does not affect the chromatin binding of the other shelterin components

TPP1 in addition to recruiting POT1 to the telomeres, was also reported to promote the interaction between TIN2, TRF1 and TRF2 (Chen et al., 2007; O'Connor et al., 2006). This suggests that TPP1 could play an essential role in organizing function in shelterin and that its deletion could affect TRF1 and TRF2. Therefore, we set out to address whether binding of TRF1, TRF2, and RAP1 to the chromatin was affected by *Tpp1* deletion. In chromatin fractionation assays, binding of TRF1, TRF2, and RAP1 to chromatin was similar in wild type and *Tpp1*-deficient MEFs (Figure 25A, B).

Based on its direct interaction with telomerase (Xin et al., 2007), TPP1 has been proposed to regulate telomerase at chromosome ends. In addition, as already mentioned shelterin components such as TRF1, TPP1, TIN2 and POT1 have been proposed to participate in telomere length regulation (Houghtaling et al., 2004; Kim et al., 1999b; Liu et al., 2004; Smogorzewska et al., 2000; Ye et al., 2004). Therefore, we were also interested in addressing whether TPP1 is required for the binding of the catalytic subunit of telomerase, called TERT, to telomeres *in vivo*. As shown in figure 25A and B, *Tpp1*-deleted MEFs showed decreased TERT binding to chromatin in the presence of normal binding of H3 and the other shelterin components. This is an indication that TPP1 may play an important role in recruiting telomerase to the chromatin and that in the absence of TPP1, TERT may be destabilised or degraded since it is not detectable in any of the soluble nuclear and cytoplasmic fractions (Figure 25A).

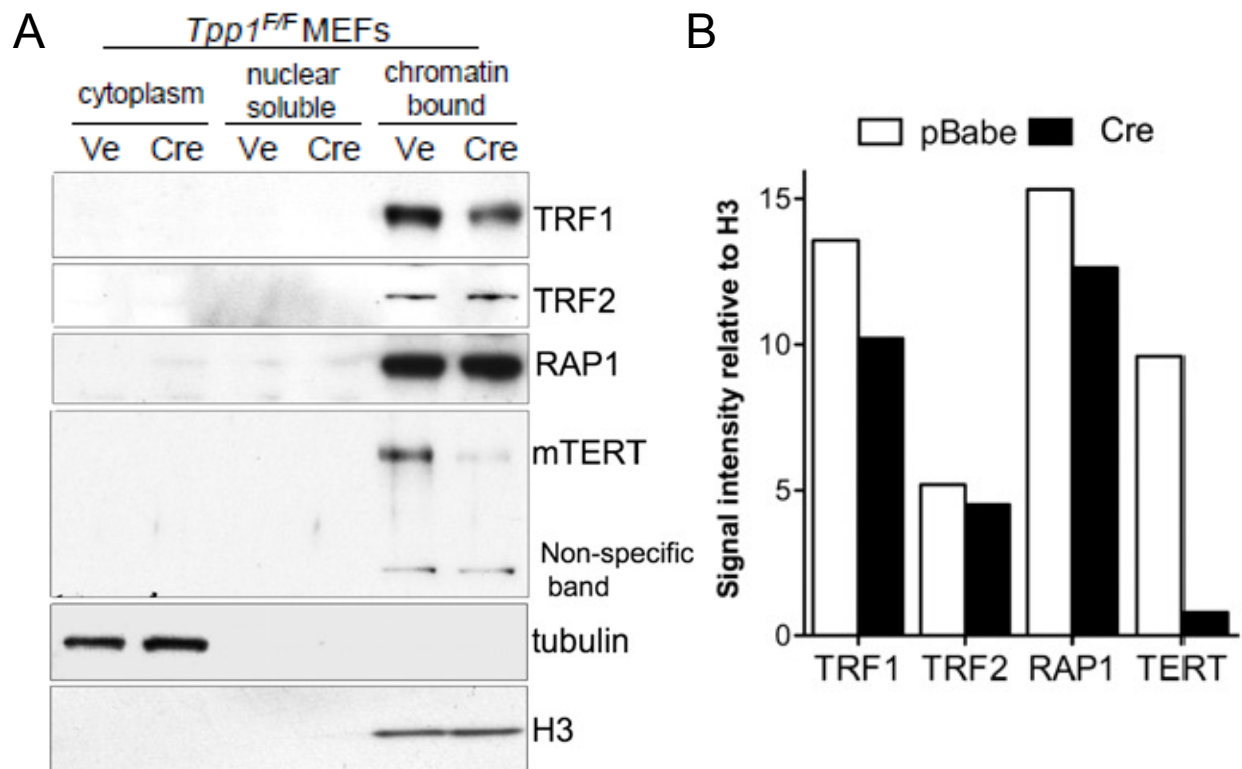


Figure 25: TPP1 recruits TERT to telomeres in the absence of changes in TRF1 or other shelterin components levels

(A) Subcellular fractionation of *Tpp1^{F/F}*-Cre treated or control MEFs treated with pBabe vector. The different subcellular fractions were immunoblotted with the indicated antibodies. Note a decrease in mTERT bound to chromatin in *Tpp1*-deleted MEFs compared to control MEFs, whereas a nonspecific band produced by the TERT antibody did not change. Tubulin was used as a loading control for the cytoplasmic fraction and histone H3 for the chromatin-bound fraction. (B) Quantification of different proteins shown in (A) relative to H3 levels.

CHAPTER 5

Discussion

Part 1

My PhD thesis work was mostly focused on the study of the DDR triggered by telomere dysfunction, as well as the molecular mechanisms underlying the cell cycle specific regulation of telomere capping. This ensures that only cells with intact telomeres progress into mitosis. We used human and mouse cells, in which we monitored the response to both natural telomere uncapping during the physiological cell cycle progression and also to telomere uncapping induced by disruption of protective structures through siRNA-mediated depletion or conditional gene deletion of various shelterin components.

5.1 Checkpoint responses to telomere dysfunction in interphase and mitosis

The response to spontaneous or intrinsic DNA damage is an important aspect of the cellular defence and protection mechanism. Spontaneous damage can arise from natural chemical and physical lesions introduced into cellular DNA by thermal, as well as oxidative insults. These natural DNA changes include depurination, depyrimidination, deamination, ss breaks, DSBs, base modifications and DNA–protein crosslinks (Billen, 1990). Another source of endogenous DNA damage is replication fork stalling that can greatly contribute to genomic instability (Branzei and Foiani, 2005). There is previous evidence of DDR activation in response to spontaneous DNA damage without the introduction of any exogenous damage, including the detection of spontaneous 53BP1 foci in un-irradiated interphase cells [(DiTullio et al., 2002); Figure 4D].

It is well established that telomere uncapping naturally occurring during the physiological cell cycle progression is a form of spontaneous DNA damage. In every S phase of the cell cycle telomeres become transiently uncapped in order to be replicated

and elongated by telomerase. Moreover, telomere dysfunction due to telomere shortening or uncapping in senescent cells can also trigger the DDR during interphase leading to ATM-dependent γ H2AX and 53BP1 foci formation (d'Adda di Fagagna et al., 2003; Meier et al., 2007; Takai et al., 2003). Together these data suggest that during interphase telomere dysfunction can signal the accumulation of DDR factors to the site of the dysfunctional telomere. However, little is known about the molecular mechanisms of telomere-triggered checkpoint activation and whether this process is controlled by the cell cycle surveillance machinery. It is believed that following telomere replication the ensuing ATM-dependent response is responsible for the recruitment of all the factors needed to restore telomere protection in G2/M (Verdun et al., 2005; Verdun and Karlseder, 2006). According to the data presented here, if the capping process following replication is not successful, telomeres can be recognised as DNA damage at G2/M and trigger a DDR, illustrated by the γ H2AX-labelling of the uncapped telomeres (Figure 3).

In contrast with the DDR induced by telomere dysfunction during interphase, mitotic checkpoints are poorly understood. Recent data suggest that treatment with DSB-inducing agents during mitosis activates a "primary" DDR comprised of early signalling events, including ATM and DNA-PK activation, H2AX phosphorylation and recruitment of MDC1 to damaged sites (Giunta et al., 2010). However, mitotic cells do not show recruitment of early DDR factors (e.g. E3 ubiquitin ligases RNF8 and RNF168 and 53BP1) at DSB sites. Consistent with these observations, we found that only a few components of the DDR machinery, namely γ H2AX and MDC1, are able to be recruited to dysfunctional telomeres during mitosis (Figure 4). This suggests that mitotic DDR to dysfunctional telomeres may engage a distinct set of factors than interphase signalling. Alternatively, it can explain why TIFs formed in interphase can be persistent and more difficult to repair in mitosis. It is conceivable that the remaining checkpoint proteins, apart from γ H2AX and MDC1 (Figure 4), cannot access the site of the damaged telomere probably due to the highly compacted telomeric heterochromatin during mitosis.

Moreover, we observed that most of the endogenous DNA damage occurring during interphase is repaired before entry into mitosis. In contrast, spontaneous telomeric damage, that resembles DSB and is visualised as TIFs, persists even after entry into mitosis (Figure 3, 4), suggestive of dysfunctional mitotic entry checkpoints.

My work demonstrated that shorter mitotic telomeres can be preferentially labelled by γ H2AX (Figure 5). These could be telomeres becoming naturally uncapped during the physiological cell cycle progression, unable to reform T-loop protective structures and therefore, targeted by cellular nucleases leading to telomere shortening. These properties make them more prone to trigger a DDR, as already shown for shortened telomeres in cells undergoing replicative senescence (d'Adda di Fagagna et al., 2003), or cells deficient for telomerase activity (Jacks et al., 1994; Herrera et al., 1999). Alternatively, the short length of certain telomeres may be the reason for their failing to acquire a protected status, through strand invasion and formation of the T-loop. Also, the amount of protective factors that can bind to the shortened telomeric DNA is not enough to fulfil all the necessary capping functions. Consistent with this hypothesis, it was shown that the amount of the shelterin components, such as TRF1, bound to the telomeres increases with the number of TTAGGG repeats and can be representative of the telomeric length [(van Steensel and de Lange, 1997); reviewed in (Palm and de Lange, 2008)].

5.2 Roles of p53, CHK1 and CHK2 in the regulating responses to uncapped telomeres during the G2/M transition

My work suggests that telomere capping is a process regulated mainly by the cell cycle G2/M checkpoint and requires a functional p53-dependent pathway. Traditionally, much research on p53 has focused on its role in regulating cell cycle arrest at the G1/S transition in response to general DNA damage and other types of cellular stress. An ATM/p53-dependent checkpoint that detects altered telomeric state at the G1/S transition was proposed to act as a regulator of the cellular response to telomere dysfunction in human cells over-expressing TRF2^{AMAB} (Karlseder et al., 1999). However, several reports also support a role for the p53 pathway at the G2/M transition [reviewed in (Taylor and Stark, 2003; Kastan and Bartek; 2004)]. Here we report that telomere uncapping during normal cell cycle progression triggers a p53-dependent checkpoint at the G2/M transition (Figure 10). The nature of the signal can be the linear telomere structure transiently generated during DNA replication in S phase, which resembles an unrepaired DSB. This linear telomere can initiate the reactions required for elongation, processing and capping following DNA replication, at S/G2 (Verdun et al., 2005), while reactions related to those of DNA repair by HR are required to form a protective T-loop structure (Verdun and Karlseder, 2006). Our results suggest that p53, similarly to its role in the general DDR, acts as sensor of uncapped telomeres and delays mitotic entry in order to allow capping processes to be completed. Consistent with this, Chin et al. (1999) reported that telomerase-deficient MEFs, which also lack p53 have a lower ability to arrest at the G2/M transition in response to dysfunctional telomeres than their wild type counterparts.

We report the existence of a second pathway additional to p53 which acts as a keeper of telomere integrity to ensure that the cells enter mitosis with intact and protected telomeres. This pathway requires CHK1 and CHK2 kinases, well characterised players of the general DNA damage response in both human and mouse cells. At the G1/S

checkpoint, CHK1 and CHK2 are acting cooperatively to prevent tumourigenesis by regulating partly redundant, but mainly non-redundant responses to DNA damage or genotoxic stress (Niida et al., 2010). On the other hand, the G2/M checkpoint response to general DNA damage is thought to be regulated in a slightly different way. CHK1 is highly expressed in G2 phase and is thought to be indispensable and mainly responsible for the G2/M checkpoint activation, while CHK2 is ubiquitously expressed throughout the cell cycle and is considered to be an amplifier of the signal produced by CHK1 at the G2/M (Bartek and Lukas, 2003).

My results clearly indicate that CHK1 and CHK2 act redundantly in monitoring telomere uncapping at the G2/M (Figure 16, 17). Consistent with the established mechanisms in MEFs, my results showed that POT1 depletion induced CHK1 activation, whilst TRF2 depletion led to CHK2 activation during G2/M (Figure 17). An indication that CHK1 can be more potent activator of the G2/M checkpoint in response to telomere uncapping is the stronger G2/M arrest and the lower mitotic entry accomplished after CHK1 activation compared with CHK2 activation in response to POT1 and TRF2 depletion, respectively (Figure 17C).

The CHK1/2-dependent G2/M responses to general DNA damage and telomere deprotection, require inactivation of the CDC25 phosphatases, which leads to G2/M arrest. In particular, CDC25A has been only shown to be targeted for degradation by CHK1, but not CHK2 (Jin et al., 2008; Xiao et al., 2003; Zhao et al., 2002; Sorensen et al., 2003; Niida et al., 2005). This is consistent with our data showing that CDC25A is only down-regulated after POT1 depletion which clearly triggers a CHK1-dependent G2/M arrest. On the other hand, CDC25C is negatively regulated by both CHK1 and CHK2 (Sanchez et al., 1997; Peng et al., 1997; Matsuoka et al., 1998). Our findings demonstrated that CDC25C is involved in the G2/M response triggered by either TRF2, or POT1 depletion, leading to CHK2 and CHK1 phosphorylation respectively (Figure 19A, B). This means that CDC25C may be a general regulator of telomere damage response

at G2/M. CDC25A is induced only by POT1 depletion and seems dispensable for the response to POT1 depletion. This is in agreement with the information coming from previous studies on the DDR suggesting that CDC25C is important only for the intra-S and G2/M checkpoints and is highly expressed specifically at S and G2 phases, in contrast with CDC25A which is thought to be involved in all cellular checkpoints and expressed at the same levels throughout the cell cycle (Bartek and Lukas, 2003).

Importantly, our data suggest that CDC25C regulation in response to telomere uncapping is mechanistically different than that in response to DNA damaging agents. CDC25C is mainly phosphorylated at Ser216 after DNA damage creating a binding site for 14-3-3, which sequesters CDC25C outside the nucleus and inhibits cell cycle progression (Peng et al., 1997; Sanchez et al., 1997; Matsuoka et al., 1998). CDC25C can also be phosphorylated and thus, positively regulated by Cyclin B creating a positive feedback loop which ensures cell cycle progression and completion of mitosis (Hoffman et al., 1993; Izumi and Maller, 1993; Strausfield et al., 1994). How this equilibrium is maintained and ensures cell cycle progression only after completion of DNA repair, remains unknown.

Most importantly, there is very little evidence for DNA damage-induced degradation of CDC25C. Under certain conditions, for example the G2/M arrest induced by arsenite, a known human carcinogen, is stabilised by CDC25C degradation via ubiquitin-proteasome pathways (Chen et al., 2002). Consistent with this, our data also suggest that CDC25C is downregulated after TRF2 or POT1 depletion during G2/M (Figure 19). This is, therefore, a novel mechanism for maintenance of telomere dysfunction-induced G2/M arrest.

5.3 Functional comparison of p53- and CHK1/2-dependent pathways in telomere capping control at G2/M

In the context of checkpoint activation in response to spontaneous telomere dysfunction my work provides evidence that p53, CHK1, and CHK2 are all activated at G2/M during the physiological cell cycle progression in the presence of naturally uncapped telomeres. Our results indicate that they protect telomere integrity possibly by facilitating telomere capping reaction before entry into mitosis (Figure 16). This is evident by the increase of mitotic TIFs after depletion of p53, CHK1 and CHK2 in U2OS cells (Figure 16B, C). Importantly, p53 seems to contribute more than CHK1 and CHK2 to telomere capping before mitotic entry, since its depletion leads to a higher increase in the number of mitotic TIFs than CHK1 or CHK2 depletion (Figure 16B, C). Moreover, it is notable that at G2/M during physiological cell cycle progression there is a higher activation of Ser15-p53 after CHK1 and CHK2 inhibition, as well as enhanced activation of CHK1 after p53 or CHK2 depletion, and of CHK2 after p53 or CHK1 depletion (Figure 16D). This observation suggests that p53, CHK1 and CHK2 possibly regulate 3 parallel pathways at G2/M during the physiological cell cycle progression all converging to the protection of the cells against endogenous DNA damage, such as naturally uncapped telomeres.

The p53- and CHK1/2-dependent pathways seem to be distinct in their telomere capping function during G2/M transition. Although both pathways lead to G2/M arrest and inhibition of mitotic entry in the presence of artificially uncapped telomeres produced by the disruption of various shelterin components, our results indicate that p53 and CHK1/2-dependent pathways act in parallel to mediate the response to artificial telomere uncapping. According to Figure 15, Ser20 p53 which is phosphorylated by CHK1/2 (Hirao et al., 2000; Shieh et al., 2000, Chehab et al., 2000) in response to DNA damage, is not important for the checkpoint activation after telomere uncapping, whilst phosphorylation of p53 at Ser15 by ATM/ATR is indispensable for the G2/M response (Figure 15). Therefore, we suggest that there is no cross talk between the p53- and CHK1/2 -dependent

pathways, activated by telomere uncapping at G2/M. Both pathways are activated by ATM/ATR in the presence of dysfunctional telomeres and lead to CDK1/Cyclin B inhibition and G2/M arrest, p53 through p21 induction and CHK1/2 through inhibition of CDC25 phosphatases.

Although both p53 and CHK1/2 are part of the ATM/ATR-dependent G2/M response to uncapped telomeres and they both lead to cell cycle delay allowing telomere re-capping to occur before mitotic entry, our data suggest that p53 is a more sensitive sensor of telomere damage than the two checkpoint kinases. p53 efficiently detects low levels of endogenous damage arising from naturally uncapped telomeres arising during the physiological cell cycle progression and acts to inhibit mitotic entry (Figure 13B). On the other hand, CHK1 and CHK2 need a higher threshold of telomere damage in order to be activated (e.g. caused by TRF2 and POT1 depletion) and they promote G2/M arrest only after artificial uncapping of telomeres through POT1 or TRF2 depletion (Figure 17C).

Another feature of p53, that distinguishes it from CHK1/2 in the context of telomere capping regulation, is that p53 is a more potent regulator of the G2/M response not only to naturally uncapped telomeres, but also after artificial telomere uncapping. Its depletion in cells lacking TRF2 or POT1 fully restores the mitotic entry of the cells to wild type levels (Figure 13B), whilst the effect of CHK1 and CHK2 is milder (Figure 17C). Indeed, CHK1 or CHK2 inhibition is not sufficient to abolish the G2/M checkpoint response, in contrast with p53 (Figure 13B).

The importance of p53 in telomere capping control may be due to its role in sustaining a prolonged G2/M cell cycle arrest, rather than initiating it (Bunz et al., 1998; Taylor and Stark, 2001). In contrast, CHK1 and CHK2 are required for G2/M checkpoint initiation in response to various genotoxic insults (Bartek et al., 2001; Bartek and Lukas, 2003; McGowan, 2002). As telomere capping is thought to be a lengthy process, p53 and to a lesser extent CHK1 and CHK2, are required to sustain the G2/M arrest until all

telomeres become capped.

5.4 Human and mouse models for the study of DDR to telomere dysfunction

Our results on human cells combined with previous findings based on the study of mouse models, suggest that there is a strong similarity between the DDR to uncapped telomeres in human and mouse cells. In mouse cells *Pot1* deletion results in ATR-dependent DNA damage signalling, whereas TRF2 deletion leads to ATM-dependent DDR (Denchi and de Lange, 2007). Consistent with this, we found that in human cells siRNA –mediated TRF2 depletion leads to CHK2 activation and G2/M arrest, whilst POT1 depletion induces CHK1-dependent inhibition of mitotic progression (Figure 17). Importantly, according to our model (Figure 20), p53 pathway is also downstream of ATM/ATR and is strongly activated by artificial uncapping caused by both TRF2 and POT1 depletion (Figure 10, 13). Our data showed release of the G2/M arrest in both TRF2si- and POT1si-treated cells after inhibition of either ATM or ATR (Figure 14B). Moreover, abrogation of p53-dependent pathway is accomplished by either ATM or ATR inhibition (Figure 13A), which means that both kinases are necessary for efficient p53-dependent checkpoint activation to telomere uncapping. Thus, both ATM and ATR inhibition resulted in increased mitotic entry compared to control cells regardless of the protein depleted (Figure 13B). It is worth mentioning that mitotic entry of POT1-depleted cells was rescued to a higher extent than TRF2-depleted cells after treatment with the ATR inhibitor (Figure 14B). This may be due to efficient phospho-CHK1 abrogation in the POT1-depleted cells, in contrast with the TRF2-depleted cells, where phospho-CHK2 was still detectable after treatment with the ATR inhibitor (Figure 14B). This observation is consistent with the notion that ATM can be an activator of ATR and that CHK1 can also be phosphorylated by ATM (Adams et al., 2006; Cuadrado et al., 2006; Garcia-Muse and Boulton, 2005; Jazayeri et al., 2006; Myers and Cortez, 2006; Zou and Elledge, 2003). Overall our studies on human cells

together with previous studies on mouse cells reveal a great level of similarity between the human and mouse cellular responses to uncapped telomeres, thus making mouse models reliable systems for the study of genomic instability caused by dysfunctional telomeres.

5.5 p53-, CHK1- and CHK2-dependent pathways for telomere capping regulation in tumour suppression

The study of ATM/ATR-dependent pathways involved in protection of telomere integrity during the cell cycle can also contribute to our understanding of how loss of these checkpoints can promote onset of tumourigenesis. The majority of cancers are telomerase-positive and inhibition of telomerase in cancer cell lines led to severe telomere shortening and loss of viability in many cases (Shay and Bacchetti, 1997). Therefore, most of the anticancer therapies in the past were focused on telomerase inhibition. However, some cancers maintain their telomeres by telomerase-independent ALT mechanisms (Bryan et al., 1995; Bryan et al., 1997). A connection between telomere dysfunction and p53-dependent tumour suppression is supported by the observation that the majority of the cancer cell lines that use ALT mechanisms for telomere maintenance lack functional p53, while a study on glioblastomas found that 78% of ALT-positive tumours were p53-deficient, while 79% of telomerase-positive tumours retained wild-type p53 (Chen et al., 2006a; Chen et al., 2006b). Moreover, in late generation telomerase-null mice with dysfunctional telomeres, p53 loss was required for tumours to form. Furthermore, in the tumours that were positive for the ALT phenotypic markers derived from these animals, p53 function was lost (Laud et al., 2005; Chin et al., 1999).

In addition to p53, CHK1 and CHK2 can also act as tumour suppressors either alone, or together with p53. In particular, *CHK1* is mutated in a limited number of human tumours and in most cases the effects of CHK1 deficiency in tumourigenesis is apparent

only in the absence of p53 (Zachos et al., 2003; Bertoni et al., 1999). This means that CHK1 alone is not a potent tumour suppressor, but has to act cooperatively with p53. Consistent with this, we report a parallel function of CHK1, CHK2 and p53 at the G2/M checkpoint in order to protect telomere integrity before mitotic entry, with p53 being the main regulator (Figure 13, 16).

CHK2 is mutated or downregulated in a number of tumours and its deficiency is able to promote tumourigenesis, even in the presence of intact p53 (Bell et al., 1999). However, there is data supporting cooperative function of CHK2 with p53 in the DDR and the promotion of tumourigenesis. It was reported that after IR, 53BP1 is required for ATM-dependent CHK2 phosphorylation and efficient G2/M arrest in human cancer cell lines with mutant p53 (DiTullio et al., 2002). Most importantly, in these cells and in a subset of human primary breast and colon carcinomas, constitutive phosphorylation of CHK2 at Thr68 by ATM was observed, even in the absence of IR or any other exogenous DNA damage (DiTullio et al., 2002). Such checkpoint activation in cells without any external DNA damage raises the question about the origin of the stimulus that triggered this ATM-CHK2 pathway activation. Such an endogenous stimulus could be genomic instability caused by defective telomere protection during physiological cell cycle progression, in the absence of functional p53-dependent G2/M pathway. Alternatively, persistent spontaneous telomere uncapping and subsequent CHK2 activation may increase the selective pressure in human cancers to mutate p53, which is an important aspect of cancer biology.

All these data indicate that the loss of checkpoints that monitor telomere dysfunction and block cell cycle progression until recapping is completed, can promote tumourigenesis. This telomere damage does not include only telomere shortening due to loss of telomerase or ALT pathways, but also loss or alteration of telomeric protective structures, or most importantly, telomere uncapping naturally occurring in every round of cell division after DNA replication. Here we report a physiologic p53- or CHK1/CHK2-

dependent response to naturally uncapped telomeres, a discovery that further extends our understanding of these important tumour suppressors.

CHAPTER 6

Discussion

Part 2

An additional focus of my PhD was to define DDR to dysfunctional telomeres using mouse models for the conditional deletion of the genes encoding 3 shelterin components, TRF1, RAP1 and TPP1. These mouse models were generated in Maria Blasco's laboratory and allowed us to study the roles of these poorly characterised proteins in DDR regulation and telomere capping and elongation, based on the consequences of their complete abrogation. This approach was superior to the shRNA-mediated depletion of these proteins used in previous studies.

6.1 TRF1 promotes telomere protection and replication and acts as tumour suppressor

TRF1 has been attributed roles in telomere length regulation, telomere cohesion, and telomere silencing (Dynek and Smith, 2004; Koering et al., 2002; Munoz et al., 2009; Schoeftner and Blasco, 2008; van Steensel and de Lange, 1997). Most of these studies were based on TRF1 over-expression experiments in both human and mouse cells. However, the impact of TRF1 abrogation in the context of the adult organism has not been studied so far. In the PhD work presented here, we used MEFs generated from *Trf1*-floxed mice, as a system to study the cellular functions of TRF1 and our data indicate that TRF1 acts as a suppressor of the DDR at mammalian telomeres (Figure 21). We further showed that TRF1 has an essential role in telomere integrity by preventing generation of telomere fusions (particularly, sister telomere fusions; Martinez et al., 2009). In particular, conditional deletion of *Trf1* in MEFs leads to dramatic TIF induction, which we visualised at mitotic telomeres (Figure 21C, D). This occurs concomitantly with a robust activation of

ATM/ATR and the downstream CHK1 and CHK2 checkpoint kinases (Figure 21E). Altogether, our findings indicate that *Trf1* deletion induces telomere defects at the molecular level, including telomere uncapping leading to end-to-end fusions, cellular functions similar to that performed by TRF2 (Celli and de Lange, 2005; Denchi and de Lange, 2007; Karlseder et al., 2004).

In addition to TRF1 performing a similar function with TRF2 in telomere protection as indicated by our results, a more specific role for TRF1 than TRF2 in telomere maintenance was recently suggested. TRF1 was shown to be required for efficient duplication of telomeres (Sfeir et al., 2009). In particular, *Trf1* conditional deletion in MEFs leads to activation of ATR in S phase and the *Trf1*-deleted cells show a fragile-site phenotype in metaphase. These data suggest that TRF1, in contrast with TRF2 and POT1 whose functions in suppressing DDR at the telomeres are needed throughout the cell cycle (Celli and de Lange, 2005; Hockemeyer et al., 2006; Konishi and de Lange 2008), has a more specific role restricted mainly in S phase.

Apart from TRF1 role in protecting the telomeres by suppressing DDR and the formation end-to-end fusions, it also acts as a tumour suppressor according to the results of our collaborators in Maria Blasco's laboratory. In particular, *Trf1* deletion had a profound impact on cancer and degenerative pathologies in mice (Martinez et al., 2009). The abnormalities caused to the mice due to TRF1 abrogation were characteristic of human diseases produced by mutations in telomerase-related genes and the presence of short telomeres, such as dyskeratosis congenita, aplastic anemia, and idiopathic pulmonary fibrosis (Armanios et al., 2007; Mitchell et al., 1999; Tsakiri et al., 2007; Vulliamy et al., 2001). These data suggest that TRF1 normally acts as a tumour suppressor in the context of the organism by preventing telomere-induced genomic instability (Martinez et al., 2009). In conclusion, the *Trf1* conditional knockout mice described in this work represent a mouse model in which full abrogation of a telomere-binding protein, without having to simultaneously abrogate telomerase expression, leads

to induction of severe telomere damage and higher incidence of neoplastic lesions (Martinez et al., 2009).

6.2 RAP1 has telomeric and non-telomeric roles, contributing in both telomere protection and transcriptional control

We next addressed the DDR to telomere dysfunction triggered by the conditional deletion of *Rap1* in MEFs. Our main observation was that RAP1 also takes part in telomere capping and protection but its effect is much milder than TRF1. We were able to see telomere uncapping represented by TIF formation in mitotic chromosomes after *Rap1* deletion, however both the number of TIFs produced and the checkpoint activation was much lower than in the case of *Trf1* deletion (Figure 23). Moreover, according to the data produced by our collaborators chromatin-bound RAP1 did not significantly inhibit end-to-end fusions in MEFs, in contrast with previous studies that support a RAP1-dependent pathway for protection against telomere fusions (Bae and Baumann, 2007; Sarthy et al., 2009). Therefore, we conclude that the contribution of RAP1 in protecting the telomeres from DDR activation or end-to-end fusions is minor compared with TRF1 and TRF2. Consistent with our findings, similar recent studies on MEFs conditionally deleted for *Rap1* or MEFs expressing a mutant TRF2 that cannot bind RAP1, showed that RAP1 was dispensable for the essential functions of TRF2 (Kipling and Kearsey, 1990). These functions include repression of ATM-dependent signalling and NHEJ. In addition to that, the *Rap1*-deleted mice are viable and fertile (Kipling and Kearsey, 1990). These observations together with our results indicate that the contribution of RAP1 in telomere protection by inhibiting both DDR and end-to-end fusions is not important compared with other shelterin components, namely TRF1 and TRF2.

The fact that the role of RAP1 in telomere protection is minor compared with TRF1 and TRF2 proteins, suggests that RAP1 may have other primary functions. Indeed, it was

shown by ChIP coupled with ultrahigh-throughput sequencing performed by our collaborators, that RAP1 binds to both telomeres and extratelomeric sites through the (TTAGGG)₂ consensus motif which were enriched at subtelomeric regions (Martinez et al., 2010). *Rap1* deletion led to several transcriptional changes including genes that contain RAP1-binding sites, suggesting a role of RAP1 in transcriptional control. The RAP1-dependent transcriptional changes included genes involved in cancer, cell adhesion, and metabolism (Martinez et al., 2010) and were different from those associated with severe telomere dysfunction (Schoeftner et al., 2009). It is therefore unlikely that this effect in transcriptional regulation is due to the mild telomere dysfunction induced by *Rap1* deletion, shown by our data (Figure 23). In conclusion, mammalian RAP1 has both telomeric and non-telomeric roles, as was previously shown for TERT and other shelterin components (Bradshaw et al., 2005; Deng et al., 2003; Mignon-Ravix et al., 2002; Park et al., 2009; Smogorzewska et al., 2000; Zhang et al., 2008). Furthermore, our data are supported by recent findings demonstrating another non-telomeric role of RAP1 in NFκB (Nuclear Factor kappa B) signalling (Teo et al., 2010). More specifically, RAP1 was identified in a gain-of-function screen for positive regulators of the NFκB signalling pathway. In this screen RAP1 was associated with IκB kinases, which are responsible for the phosphorylation and subsequent degradation of NFκB inhibitors. Moreover, consistent with a role for RAP1 outside the nucleus, human cells (but not mouse fibroblasts) contain a considerable fraction of RAP1 in the cytoplasm which is not associated with TRF2 (Takai et al., 2010).

6.3 TPP1 promotes telomere maintenance by suppressing DDR and by recruiting TERT to the chromatin

We also describe a mouse model for complete TPP1 abrogation that allowed us to study the DDR triggered by *Tpp1* gene deletion. TPP1 binds to both POT1a and POT1b

proteins found in mouse cells (Hockemeyer et al., 2008; Wang et al., 2007) and previous studies based on shRNA-mediated depletion of TPP1 demonstrated that TPP1 contributes to the normal function of both POT1a and POT1b (Hockemeyer et al., 2007; Liu et al., 2004; Palm et al., 2009; Xin et al., 2007; Ye et al., 2004). Mouse POT1a prevents activation of the ATR kinase and contributes to the repression of the NHEJ at newly replicated telomeres, whereas POT1b represses unscheduled resection of the 5'-ended telomeric DNA strand, resulting in long 3' overhangs in *Pot1b*-deleted cells (He et al., 2009; Hockemeyer et al., 2006; Hockemeyer et al., 2008). However, it was not known whether TPP1 has additional roles in the shelterin complex. Our results indicate that TPP1 abrogation leads to DDR activation at chromosome ends, including induced CHK1 and CHK2 phosphorylation (Figure 24) and increased telomere fusions and fragility (Tejera et al., 2010). This suggests a role of TPP1 in telomere protection. Recent similar studies on *Tpp1*-conditionally deleted MEFs also indicate that TPP1 contributes to telomere protection and its function is mediated by POT1a and POT1b (Kibe et al., 2010). In addition to that, *Tpp1* deletion resulted in the release of POT1a and POT1b from chromatin and loss of these proteins from telomeres. This suggests that TPP1 is required for the association of POT1a and POT1b with the telomeres, but not for their stability (Kibe et al., 2010).

Finally, we demonstrated that TPP1 is required for TERT recruitment to the chromatin (Figure 25), as well as efficient telomere elongation (Tejera et al., 2010). This finding is consistent with previous data supporting that TPP1 is essential for telomere elongation during nuclear reprogramming of MEFs into induced pluripotent stem cells, a process that involves telomere elongation by telomerase (Marion et al., 2009a; Marion et al., 2009b). Interestingly, TERT and TPP1 have similar patterns of mRNA expression during embryonic development (Martin-Rivera et al., 1998; Vlangos et al., 2009), further suggesting their co-regulation. Moreover, the epithelial pathologies as a result of *Tpp1* deletion in mice (Tejera et al., 2010) are similar to those produced in human diseases

associated with mutations in telomerase-related genes and the presence of dysfunctional telomeres (Armanios et al., 2007; Mitchell et al., 1999; Tsakiri et al., 2007; Vulliamy et al., 2001). These observations make *Tpp1*-deleted mice a useful model for understanding human disease. In conclusion, we suggest that TPP1 has a dual role in telomere protection and elongation, thus maintaining telomere function and preventing the early onset of degenerative pathologies in mice.

Overall, our work showed that all the 3 shelterin components, TRF1, RAP1 and TPP1, contribute to telomere maintenance in mouse cells. In particular, TRF1 and RAP1 protect the telomeres by suppressing DDR, while TPP1 is required for both telomere protection and elongation by suppressing DDR and by recruiting TERT to the chromatin.

Our work revealed several aspects of the cell cycle-specific regulation of telomere capping and the effects of the loss of essential checkpoint pathways which detect telomere dysfunction in human cells. The study of a certain stage of the cell cycle and the DDR triggered at this specific stage by cell synchronisation is an approach that was poorly used in the previous studies on either human or mouse cells. However, there are still questions that need to be answered in the future. An example is the effect of telomere uncapping triggered by the disruption of other shelterin components in human cells such as TPP1 or TIN2 which have also been related to human pathologies (Savage et al., 2011; Augereau et al., 2011; Sasa et al., 2011). Moreover, a cell cycle-specific analysis of the *Trf1*-, *Rap1*- or *Tpp1*-deleted MEFs would allow us to see the effects of telomere uncapping in mouse cells at certain stages of the cell cycle similarly with the experiments done on human cells. This could contribute more to our knowledge on checkpoint responses to dysfunctional telomeres and how they are regulated in mouse cells including the pathways downstream of CHK1 and CHK2 kinases which have not yet been studied in the context of telomere uncapping.

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