The function of classical and alternative non-homologous end-joining pathways in the fusion of dysfunctional telomeres

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Repair of DNA double-stranded breaks (DSBs) is crucial for the maintenance of genome stability. DSBs are repaired by either error prone non-homologous end-joining (NHEJ) or error-free homologous recombination. NHEJ precedes either by a classic, Lig4-dependent process (C-NHEJ) or an alternative, Lig4-independent one (A-NHEJ). Dysfunctional telomeres arising either through natural attrition due to telomerase deficiency or by removal of telomere-binding proteins are recognized as DSBs. In this report, we studied which end-joining pathways are required to join dysfunctional telomeres. In agreement with earlier studies, depletion of Trf2 resulted in end-to-end chromosome fusions mediated by the C-NHEJ pathway. In contrast, removal of Tpp1-Pot1a/b initiated robust chromosome fusions that are mediated by A-NHEJ. C-NHEJ is also dispensable for the fusion of naturally shortened telomeres. Our results reveal that telomeres engage distinct DNA repair pathways depending on how they are rendered dysfunctional, and that A-NHEJ is a major pathway to process dysfunctional telomeres.

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Introduction

Telomeres are nucleoprotein structures that provide both end protection and a mechanism for the maintenance of chromosomal ends. In mammals, telomeres consist of TTAGGG repetitive sequences that associate with and are protected by shelterin, a core complex of telomere-binding proteins that includes the double-stranded DNA-binding proteins TRF1 and TRF2 and protection of telomeres 1 (POT1) that interacts with its binding partner TPP1 to protect single-stranded (ss) G-rich

overhangs (Palm *et al*, 2009). Telomeres are maintained by the enzyme telomerase, which is limiting in human somatic cells, resulting in progressive telomere shortening. Dysfunctional telomeres that can no longer exert end-protective functions are recognized as DNA double-stranded breaks (DSBs) by the DNA damage repair (DDR) pathway. This leads to the activation of checkpoint sensors including the Mre11–Rad50–Nbs1 (MRN) complex and 53BP1, the ATM and ATR signal transducing kinases and downstream effectors including Chk1, Chk2 and p53. The cellular consequences of activating the DDR by dysfunctional telomeres include induction of cellular growth arrest, cellular senescence or apoptosis (Deng *et al*, 2008).

Mammalian DSBs are repaired primarily by homologous recombination (HR) or non-homologous end joining (NHEJ). HR initiates error-free repair between homologous repeat sequences, requires the MRN complex to tether DNA ends (Williams *et al*, 2008), and the associated protein CtIP (Sae2 in budding yeast) to help generate the 3' ss DNA substrate necessary for HR (Sartori *et al*, 2007; Mimitou and Symington, 2008). NHEJ-mediated error-prone repair also requires MRN and is composed of two pathways: classic NHEJ (C-NHEJ) that requires Ku70/86 and the DNA ligase IV (Lig4)-Xrcc4 complex, and alternative NHEJ (A-NHEJ). A-NHEJ is a robust, evolutionarily conserved repair pathway in which DSB repair products display short tracks of microhomology at the repair junctions and the joining reaction uses DNA Ligase III (Ma *et al*, 2003; Audebert *et al*, 2004; Guirouilh-Barbat *et al*, 2004; Wang *et al*, 2005, 2007; Haber, 2008). Mechanistically, A-NHEJ is associated with the generation of 3' ss overhang at the sites of DSBs, and this process involves MRN and CtIP (Corneo *et al*, 2007; Yan *et al*, 2007; Bennardo *et al*, 2008; Deriano *et al*, 2009; Dinkelmann *et al*, 2009; Rass *et al*, 2009; Xie *et al*, 2009).

Recent data suggest that telomeres use different telomere-binding proteins to prevent uncapped telomeres from engaging in distinct signalling pathways. TRF2 specifically represses ATM signalling (Denchi and de Lange, 2007; Guo *et al*, 2007; Deng *et al*, 2009), and removal of TRF2 elicits C-NHEJ that requires ATM, MRN and 53BP1 (Dimitrova *et al*, 2008; Attwooll *et al*, 2009; Deng *et al*, 2009; Dimitrova and de Lange, 2009). In contrast, POT1 specifically represses the ATR pathway (Denchi and de Lange, 2007; Guo *et al*, 2007; Deng *et al*, 2009). The observation that different signalling pathways are repressed by specific shelterin components raises the interesting possibility that distinct DNA repair pathways at telomeres might become activated on removal of specific shelterin protein complexes.

The central function for C-NHEJ of DSBs in a large variety of organisms, coupled with data indicating that removal of TRF2 initiates C-NHEJ, has fueled the notion that naturally shortened telomeres are also processed through this pathway. Indeed, fusion of dysfunctional telomeres in late generation

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telomerase null (*mTerc*^{-/-}) mice bearing critically shortened telomeres has been shown to require the C-NHEJ core factors Ku86 and DNA-PK_{cs} (Espejel *et al*, 2002). However, a recent report indicating that chromosome fusions in *mTerc*^{-/-} mice with critically shortened telomeres take place independent of Lig4 and DNA-PK_{cs} challenges this assumption (Maser *et al*, 2007).

In this study, we used mouse embryonic fibroblasts (MEFs) in which specific components of the C-NHEJ pathway are deleted genetically to understand how dysfunctional telomeres are joined together. Removal of Trf2 led to the activation of Lig4 and 53BP1-dependent C-NHEJ-mediated fusions. Surprisingly, removal of Tpp1–Pot1a/b from *53BP1*^{-/-} or *Lig4*^{-/-} MEFs resulted in robust end-to-end chromosome fusions. These chromosome fusions are suppressed by Ku70 and are dependent upon CtIP, indicating that they are mediated by the A-NHEJ pathway. Finally, we show that chromosome fusions arising from naturally shortened telomeres do not use C-NHEJ. Our results show that depending upon how telomeres are rendered dysfunctional profoundly influences the choice of DNA repair pathways used, and that telomeres devoid of Tpp1–Pot1a/b engage the A-NHEJ pathway to mediate chromosome end-to-end fusions.

Results

Multiple domains of 53BP1 are required for fusion of telomeres lacking Trf2

53BP1 is a critical DDR protein that has functions in both sensing and repair of all DSBs. It is rapidly recruited to sites of IR-induced DNA breaks (Schultz *et al*, 2000; Rappold *et al*, 2001; Xia *et al*, 2001) as well as to dysfunctional telomeres (Takai *et al*, 2003; d'Adda di Fagagna *et al*, 2003). Stable association of 53BP1 with DSBs requires its interaction with phosphorylated H2AX and the mediator of DNA damage checkpoint 1 (Lou *et al*, 2003; Stewart *et al*, 2003). 53BP1 also binds to DNA and participates directly in Lig4-mediated repair (Iwabuchi *et al*, 2003; Charier *et al*, 2004), and is epistatic to Ku70 (Nakamura *et al*, 2006). These data suggest that 53BP1 is an important accessory C-NHEJ factor. In accord with its function in the maintenance of genomic stability, deletion of 53BP1 from the mouse genome results in increased sensitivity to IR and chromosomal abnormalities consistent with a defect in DNA repair (Morales *et al*, 2003; Ward *et al*, 2003). However, repair of IR-induced DNA damage is not markedly affected by the absence of 53BP1, suggesting that 53BP1 might be involved in the repair of a subset of IR-induced DSBs (Ward *et al*, 2004). To ascertain the impact of 53BP1 deficiency on the DDR mediated by dysfunctional telomeres, we uncapped telomeres in both 53BP1 competent and *53BP1*^{-/-} SV40LT immortalized MEFs by removing Trf2 with retrovirus-mediated short hairpin RNA against Trf2 (shTrf2) (Deng *et al*, 2009). Removal of Trf2 initiated a robust DNA damage response at telomeres, resulting in the formation of dysfunctional telomere-induced DNA damage foci (TIFs) (Takai *et al*, 2003; d'Adda di Fagagna *et al*, 2003) (Figure 1A; Supplementary Figure S1A) and phosphorylation of 53BP1 (Supplementary Figure S1B, S1C). In the absence of 53BP1, removal of Trf2 did not adversely impact upon γ -H2AX TIF formation at telomeres (Figure 1A; Supplementary Figure S1A), indicating that the initial response to DNA damage is unaffected upon 53BP1 loss.

In support of this observation, 53BP1 deficiency did not affect dysfunctional telomere-induced phosphorylation of ATM or its downstream substrate Chk2 (Supplementary Figure S1B and C). In sharp contrast, fusion of Trf2-uncapped telomeres in the form of end-to-end chromosome fusions was reduced ~40-fold in the setting of 53BP1 deficiency (Figure 1B; Supplementary Figure S1D). These results indicate that although 53BP1 is dispensable for the initial DNA damage signalling response induced by telomere uncapping, it is required for C-NHEJ-mediated fusion of Trf2-depleted telomeres.

We next asked which regions of 53BP1 are required for C-NHEJ of Trf2-depleted telomeres. 53BP1 is a large nuclear protein composed of multiple functional domains (Figure 1C). The N-terminal portion of 53BP1 contains 15(S/T)Q motifs that are phosphorylated by members of the phosphoinositide 3-kinase-related protein kinase family, including ATM and ATR, following IR-induced DNA damage (Schultz *et al*, 2000; Rappold *et al*, 2001; Xia *et al*, 2001; Ward *et al*, 2006; Matsuoka *et al*, 2007). The 53BP1 homo-oligomerization domain is required for 53BP1 to oligomerize with itself and is essential for efficient repair of DSBs (Adams *et al*, 2005; Ward *et al*, 2006), as a D1256A mutation compromised IR-induced DNA damage focus formation (Zgheib *et al*, 2009). The glycine-arginine-rich (GAR) domain is required for PRMT1-dependent methylation, although how this relates to repair of damaged DNA is unclear (Adams *et al*, 2005). The Tudor domain directly interact with chromatin on H4-K20me2, and a point mutation in this domain (D1521R) completely abolishes recruitment of 53BP1 to sites of DNA damage (Botuyan *et al*, 2006). Finally, the C-terminal BRCT motif seems not to have a significant function in IR-induced repair of DSBs (Ward *et al*, 2006). To ascertain which domain of 53BP1 is required for fusion of uncapped telomeres, we stably expressed wild-type HA-tagged 53BP1 as well as HA-tagged 53BP1 mutants (Figure 1C; Supplementary Figure S2) in *53BP1*^{-/-} MEFs, treated these cells with shTrf2 and analysed the ability of these reconstituted cell lines to both sense (using the TIF assay) and join (by monitoring chromosome fusions) Trf2-depleted telomeres. Full-length (FL) wild-type 53BP1, the 53BP1 mutants 15AQ (in which all 15 serines/threonines were replaced with alanines), Δ NH3 (N-terminal residues 1–1052 deleted), Δ GAR and the BRCT domain mutant BRCT^{S1853A} all efficiently localized to telomeres to restore TIF formation after removal of Trf2 (Figure 1D and E). However, localization to dysfunctional telomeres was reduced four-fold in the oligomerization domain point mutant Oligo^{D1256A}, despite the ability of this mutant to respond to ATM signalling (Figure 1D; Supplementary Figure S2). In contrast to a recent report (Dimitrova *et al*, 2008), the Tudor domain point mutant Tudor^{D1521R} was unable to localize to Trf2-deficient telomeres when examined 2, 4 and 8 days after infection, even though the expression of this mutant remained robust in *53BP1*^{-/-} MEFs (Figure 1D and E; Supplementary Figure S2 and data not shown). This result is in agreement with an earlier report documenting the inability of 53BP1 Tudor^{D1521R} to localize to IR-induced DNA breaks (Botuyan *et al*, 2006). Control experiments confirmed that unlike the other 53BP1 mutants examined, both Oligo^{D1256A} and Tudor^{D1521R} mutants failed to localize to sites of IR-induced DNA damage (Supplementary Figure S3). Examination of metaphase spreads revealed that only the

Figure 1 53BP1 domains required for telomere localization and repair. (A) γ -H2AX-positive TIFs in $53BP1^{+/+}$ and $53BP1^{-/-}$ MEFs after Trf2 depletion. (B) SV40LT immortalized $53BP1^{+/+}$ and $53BP1^{-/-}$ MEFs were treated with control vector or Trf2 shRNA for 120 h, metaphases were prepared and telomere fusions were visualized by Tam-OO-(CCCTAA)₄ telomere peptide nucleic acid (PNA; red) and 4,6-diamidino-2-phenylindole (DAPI; blue). (C) Schematic of 53BP1 domains and its deletion and point mutants used in this study. (D) 53BP1 TIFs and chromosome fusions in $53BP1^{-/-}$ MEFs reconstituted with full-length 53BP1 and the indicated 53BP1 deletion or point mutants. For TIFs, cells stably infected with Trf2 shRNA were fixed after 72 h and stained with anti-53BP1 antibody (green), telomere-PNA FISH (red) and DAPI (blue). For telomere fusions, metaphases prepared from reconstituted cell lines infected with Trf2 shRNA after 120 h were visualized by telomere PNA-FISH (red) and DAPI (blue). Arrows point to representative fused chromosomes. (E) Quantification of 53BP1 TIFs. A minimum of 100 cells were examined and cells with >4 TIFs were scored as TIF positive. Mean values were derived from at least three experiments. Error bars: s.d. (F) Quantification of telomere fusion frequencies. A minimum of 1600 chromosomes were analysed and mean values derived from at least three experiments presented. Error bars: s.d.

53BP1 FL construct, the Δ GAR and BRCT^{S1853A} mutants were able to restore shTrf2-mediated chromosome fusions in $53BP1^{-/-}$ MEFs to near wild-type levels (Figure 1D and F; Supplementary Figure S4). The 15AQ, Δ NH3 and Tudor^{D1521R} mutants were all unable to promote any chromosome fusions, and a significant reduction in the number of long chain chromosome fusions was observed in the Oligo^{D1256A} mutant (Figure 1D and F; Supplementary Figure S4). These results reveal that efficient accumulation of 53BP1 to Trf2-depleted telomeres requires intact Tudor and the oligomerization domains of 53BP1. Subsequent fusion of Trf2-depleted telomeres requires the oligomerization domain and N-terminal phosphorylation. As the Tudor domain mutant was not able to localize to uncapped telomeres, we were not able to ascertain its possible telomere repair functions. These results also indicate that GAR methylation and the BRCT motifs do not function in maintaining genomic stability in the context of telomere dysfunction.

Removal of Tpp1-Pot1a/b from telomeres elicits robust chromosome fusions in the absence of 53BP1

We have recently shown that under certain conditions, the Tpp1-Pot1a/b complex is able to protect the ss G-overhang from engaging in end joining (Deng *et al*, 2009). To determine whether the lack of chromosome fusions observed in shTrf2-treated $53BP1^{-/-}$ MEFs is due to the protective effects of the Tpp1-Pot1a/b complex at telomeres, we expressed the dominant-negative mutant Tpp1^{ARD} in $53BP1^{-/-}$ MEFs to efficiently remove endogenous Tpp1-Pot1a/b complex from telomeres (Guo *et al*, 2007; Xin *et al*, 2007; Deng *et al*, 2009). Expression of Tpp1^{ARD} in $53BP1^{-/-}$ MEFs elicited robust γ -H2AX TIF formation (Supplementary Figure S5). In contrast to shTrf2-treated $53BP1^{-/-}$ MEFs, in which chromosome fusions occurred at a frequency of 0.8%, expression of Tpp1^{ARD} in $53BP1^{-/-}$ MEFs increased end-to-end chromosome fusions 23-fold (to ~18%, $P < 0.001$) (Figure 2A–C). This result was surprising, and pointed to the likelihood that

Figure 2 Chromosome fusions in the absence of Tpp1-Pot1a/b are mediated by alternative NHEJ. **(A)** SV40LT immortalized 53BP1^{+/+} or 53BP1^{-/-} MEFs were treated with the indicated DNA constructs for 120 h, metaphases prepared and telomeres visualized by telomere FISH (FITC-OO-(TTAGGG)₄ (green, leading strand) and Tam-OO-(CCCTAA)₄ (red, lagging strand)). Arrows point to fused chromosomes. **(B)** Quantification of the chromosome fusion frequencies in 53BP1^{+/+} MEFs observed in **(A)**. A minimum of 2000 chromosome ends were analysed per cell line. Error bars: s.d. ***P* < 0.001 calculated using a two-tailed Student's *t*-test. **(C)** Quantification of the chromosome fusion frequencies in 53BP1^{-/-} MEFs observed in **(A)**. A minimum of 2500 chromosome ends were analysed per cell line. Error bars: s.d. **P* < 0.05, ***P* < 0.001 calculated using a two-tailed Student's *t*-test. **(D)** Quantification of T-SCEs in 53BP1^{+/+} MEFs. A minimum of 1500 chromosome ends were scored per cell line. Error bars: s.d. **(E)** Quantification of T-SCEs in 53BP1^{-/-} MEFs. A minimum of 2100 chromosome ends were scored per cell line. Error bars: s.d. **(F)** SV40LT immortalized Lig4^{-/-} MEFs were treated with the indicated DNA constructs for 120 h, metaphases prepared and telomeres visualized by CO-FISH (FITC-OO-(TTAGGG)₄ (green) and Tam-OO-(CCCTAA)₄ (red)). Arrows point to fused chromosomes. **(G)** Quantification of the chromosome fusion frequencies observed **(F)**. A minimum of 1500 chromosome ends were analysed per cell line and the mean value derived from three independent experiments are given. Error bars: s.d.

telomeres devoid of Trf2 were joined through a different mechanism from telomeres devoid of Tpp1-Pot1a/b. Importantly, as 53BP1 was required to promote efficient C-NHEJ-mediated fusion of Trf2-depleted telomeres, the chromosome fusions observed when the Tpp1-Pot1a/b complex was removed from telomeres in the absence of 53BP1 could not be mediated the C-NHEJ pathway. Indeed, the number of fused chromosomes observed did not differ significantly whether the Tpp1-Pot1a/b complex was removed in the presence or absence of 53BP1 (Figure 2B and C). Depletion of both Trf2 and the Tpp1-Pot1a/b complex from 53BP1^{-/-} MEFs increased the number of fused chromosomes 48-fold (to

involve ~38% of ends, *P* < 0.001) (Figure 2A–C), suggesting that both Trf2 and Tpp1-Pot1a/b are required to completely repress C-NHEJ-independent fusions. Although robust, the fusion efficiency of this C-NHEJ-independent fusion mechanism is still ~2-fold less than C-NHEJ-mediated fusions.

Chromosome-orientation FISH (CO-FISH) analysis revealed that compared with 53BP1^{+/+} MEFs, telomere sister chromatid exchanges (T-SCEs), a marker for homology-directed repair (HDR), were elevated 12-fold in 53BP1^{-/-} MEFs devoid of Trf2 (Figure 2D and E). This result suggests that 53BP1 cooperates with Trf2 to repress HDR at telomeres, an observation reminiscent of the cooperative effects between

Ku70 and Trf2 in preventing HDR at dysfunctional telomeres (Celli *et al*, 2006). In contrast, the elevated T-SCE levels observed when the Tpp1-Pot1a/b complex was removed from telomeres were not significantly altered in the setting of 53BP1 deficiency (Figure 2D and E). Taken together, these results suggest that 53BP1 only represses HDR at telomeres initiated by removal of Trf2.

Chromosome fusions in the absence of Tpp1-Pot1a/b are mediated by alternative NHEJ

Although telomere HDR is increased in the absence of 53BP1 and Trf2, end-to-end chromosome fusions could only be generated through some form of NHEJ-mediated process and not through HDR, as all telomeric sequences are oriented in a 5'-3' direction and are not amenable to HDR. To test the hypothesis that the Lig4-independent A-NHEJ pathway might be involved in joining telomeres devoid of Tpp1-Pot1a/b, we removed either Trf2 and/or Tpp1-Pot1a/b from telomeres of SV40LT immortalized Lig4 null MEFs. Lig4 is the critical core component essential for C-NHEJ, and in agreement with our data regarding 53BP1 null MEFs, chromosome fusions elicited by shTrf2 were strictly dependent on C-NHEJ. In agreement with earlier observations in the absence of Lig4, removal of Trf2 resulted in the fusion of only 0.7% of chromosome ends (Figure 2F and G) (Celli and de Lange, 2005). In contrast, removal of Tpp1-Pot1a/b from telomeres of *Lig4*^{-/-} MEFs increased end-to-end chromosome fusions 11-fold (to ~8% of chromosome ends, $P < 0.002$). Depletion of both Trf2 and the Tpp1-Pot1a/b complex further elevated this fusion percentage 26-fold (to involve ~18% of all chromosome ends, $P < 0.001$) (Figure 2F and G). Interestingly, the ~2-fold increase in the number of chromosome fusions observed in 53BP1 deficient, *Lig4*^{-/-} MEFs devoid of both Trf2 and the Tpp1-Pot1a/b complex suggests that 53BP1 may have a function in suppressing A-NHEJ at telomeres (Figure 2G).

Removal of Ku70 enhances A-NHEJ-mediated fusion of telomeres devoid of Tpp1-Pot1a/b

The Ku70-80 heterodimer is required for C-NHEJ-mediated repair of DNA breaks, including telomeres devoid of Trf2 (Celli *et al*, 2006). Recent reports suggest that this complex also has a major function in repressing A-NHEJ at endonuclease-induced DNA breaks (Guirouilh-Barbat *et al*, 2007; Bennardo *et al*, 2008). To test the hypothesis that the Ku70-80 heterodimer has a function repressing A-NHEJ-mediated fusion of dysfunctional telomeres, we removed Trf2 and/or the Tpp1-Pot1a/b complex from SV40LT immortalized Ku70 null MEFs. In agreement with published results, chromosome fusions were dramatically reduced when Trf2 was depleted from *Ku70*^{-/-} MEFs, again supporting the observation that telomeres devoid of Trf2 were joined primarily by C-NHEJ (Figure 3A and C). In sharp contrast, a 55-fold increase in the number of chromosome fusions (involving ~21% of all chromosome ends, $P < 0.001$) were observed when Tpp1-Pot1a/b was removed from *Ku70*^{-/-} MEFs, increasing to 130-fold (51% of ends, $P < 0.001$) when both Trf2 and Tpp1-Pot1a/b were depleted from telomeres (Figure 3A and C). To ensure that C-NHEJ was not inadvertently activated at telomeres in the absence of Ku70, we repeated this experiment in Ku70 null MEFs in which Lig4 was efficiently depleted with shRNA (Deng *et al*, 2009) (Supplementary

Figure S6A). The number of chromosome fusions observed when Tpp1-Pot1a/b was removed from telomeres in the setting of both Lig4 and Ku70 deficiency remained elevated and not significantly different from those observed in *Ku70*^{-/-} MEFs (53-fold increase over vector control when Tpp1-Pot1a/b is removed, 101-fold when both Trf2 and Tpp1-Pot1a/b are removed) (Figure 3B and C). These results support the notion that removal of Tpp1-Pot1a/b results in the generation of dysfunctional telomeric ends that are joined primarily through the A-NHEJ pathway that is normally repressed by Ku70.

Rad51 is dispensable for A-NHEJ-mediated telomere fusions

A-NHEJ-mediated DNA repair is characterized by the presence of short microhomologies at the repair junction that seems to be mechanistically distinct from HDR, which uses extensive sequence homology during the repair process and is critically dependent on Rad51 (Bennardo *et al*, 2008; Haber, 2008). However, A-NHEJ in yeast relies on genes involved in HDR (Decottignies, 2007), and an HDR pathway has recently been observed to repair yeast telomeres devoid of Pot1 (Wang and Baumann, 2008). To ascertain whether efficient A-NHEJ-mediated telomere fusions observed after depletion of Tpp1-Pot1a/b is dependent on the HDR pathway, Rad51 was efficiently depleted from *53BP1*^{-/-} or *Lig4*^{-/-} MEFs (Supplementary Figure S6B and C). Depletion of Rad51 did not significantly alter the number of fused chromosomes observed when both Trf2 and Tpp1-Pot1a/b were subsequently removed from telomeres (Figure 3D and E), suggesting that HDR was not used to ligate chromosome ends when Tpp1-Pot1a/b is removed from mammalian telomeres.

A-NHEJ-mediated telomere fusions requires ATR and CtIP

Removal of Trf2 from telomeres induces a DDR that preferentially activates ATM, whereas removal of Tpp1-Pot1a/b preferentially activates ATR (Denchi and de Lange, 2007; Guo *et al*, 2007; Deng *et al*, 2009). These results suggest that the respective losses of Trf2 and Tpp1-Pot1a/b might initiate different signalling events, resulting in the activation of distinct repair processes at telomeres. To determine whether A-NHEJ-mediated chromosome fusions requires an intact ATM or ATR pathway, Trf2 and/or Tpp1-Pot1a/b was depleted from ATM null MEFs. Although shTrf2-induced chromosome fusions were abolished in the absence of ATM, the number of end-to-end chromosome fusions observed when Tpp1-Pot1a/b was removed from ATM null MEFs was unaffected (Figure 4A and B). In contrast, removal of Trf2, but not Tpp1-Pot1a/b, from 53BP1 proficient, ATR-deficient MEFs resulted in chromosome fusions, suggesting that ATM, but not ATR, is required to fuse telomeres devoid of Trf2 (Figure 4C). Depletion of ATR from *53BP1*^{-/-} MEFs expressing Tpp1^{ARD} completely abolished end-to-end chromosome fusions (Figure 4A and C; Supplementary Figure S7A). A similar phenotype was observed when both Trf2 and Tpp1-Pot1a/b were depleted from shATR-treated *53BP1*^{-/-} MEFs. Taken together, these results suggest that activation of ATR signalling is crucial for A-NHEJ-mediated fusion of telomeres devoid of Tpp1-Pot1a/b.

The MRN complex has recently been shown to be crucial for the generation of the 3' ss telomeric overhang of

Figure 3 Ku70 represses A-NHEJ of telomeres devoid of Tpp1-Pot1a/b. (A) *Ku70*^{-/-} cells were treated with DNA constructs as indicated, metaphases prepared and telomeres visualized by telomere FISH (FITC-OO-(TTAGGG)₄ (green) and Tam-OO-(CCCTAA)₄ (red)). Arrows point to representative fused chromosomes. (B) *Ku70*^{-/-} cells stably expressing shLig4 were treated with DNA constructs as indicated, and metaphases prepared as in (A). Arrows point to representative fused chromosomes. (C) Quantification of the chromosome fusion frequency observed in (A, B). A minimum of 2000 chromosome ends were analysed per cell line and the mean value derived from two independent experiments are given. Error bars: s.d. **P* < 0.4, ***P* < 0.001 calculated using a two-tailed Student's *t*-test. (D) *53BP1*^{-/-} or *Lig4*^{-/-} cells stably expressing shRad51 were treated with shTrf2 and Tpp1^{ΔRD}, and metaphases prepared as in (A). Almost all the chromosomes are fused. (E) Quantification of the chromosome fusion frequency observed in (D). A minimum of 2000 chromosome ends were analysed and the mean value derived from three independent experiments are given. Error bars: s.d.

leading-strand telomeres by promoting 5' end resection (Attwooll *et al*, 2009; Deng *et al*, 2009; Dimitrova and de Lange, 2009). It is also required to produce the 3' ends of I-SceI-induced DNA breaks to initiate A-NHEJ (Rass *et al*, 2009; Xie *et al*, 2009). The dramatic increase in chromosome fusions observed in *53BP1*^{-/-} MEFs after depletion of Tpp1-Pot1a/b suggest the possibility that nucleolytic processing of telomeric ends to generate ss DNA might be required for A-NHEJ-mediated chromosome fusions. To test this hypothesis, we examined the status of the 3' ss overhang using an in-gel hybridization assay. In contrast to shTRF2-treated *53BP1*^{-/-} MEFs, which showed rapid reduction of the 3' overhang due to C-NHEJ-mediated processing, the 3' overhang increased ~1.5-fold in *53BP1*^{-/-} MEFs when Tpp1-Pot1a/b is removed from telomeres (Supplementary Figure S8). Treatment with the 3' end specific exonuclease ExoI revealed that these overhangs were indeed ss telomeric repeats (data not shown). As CtIP interacts directly with Mre11 and likely contributes to DNA resection to generate ss DNA intermediates (Sartori *et al*, 2007), we examined the function of CtIP in the end joining of chromosomes. End-to-end chromosome fusions in *53BP1*^{-/-} MEFs treated with

both shTrf2 and Tpp1^{ΔRD} were reduced to near background levels when CtIP was also depleted (Figure 4D and E; Supplementary Figure S7B). In addition, depletion of CtIP resulted in a 3.4-fold reduction in the number of T-SCEs observed after Trf2 and Tpp1-Pot1a/b were removed from telomeres (Figure 4F). Taken together, these results suggest that CtIP is critically important for A-NHEJ-mediated fusion of telomeric ends after removal of Tpp1-Pot1a/b, and that both A-NHEJ and HDR at telomeres require functional CtIP.

Chromosome fusions are present in *53BP1*^{-/-} mice bearing naturally shortened telomeres

Our results provide mechanistic insights into how removal of different components of the mammalian telomere complex (Trf2 versus Tpp1-Pot1a/b) results in the activation of independent DNA repair pathways (C-NHEJ versus A-NHEJ). We next asked whether dysfunctional telomeres arising from natural telomere attrition in the absence of telomerase are fused by either the C-NHEJ or the A-NHEJ pathways, as there is currently controversy as to how naturally shortened telomeres are joined. There are data suggesting that fusion of dysfunctional telomeres in telomerase null (*mTerc*^{-/-}) mice

Figure 4 ATR and CtIP are required for A-NHEJ of telomeres lacking Tpp1-Pot1a/b. **(A)** Metaphase spreads prepared from *ATM*^{-/-} MEFs or *53BP1*^{-/-} MEFs stably expressing shATR were treated with the indicated DNA constructs, metaphases prepared and analysed by telomere FISH (FITC-OO-(TTAGGG)₄ (green) and Tam-OO-(CCCTAA)₄ (red)). Arrows point to representative fused chromosomes. **(B)** Quantification of the chromosome fusion frequencies observed in *ATM*^{-/-} MEFs. A minimum of 2100 chromosomes were analysed and the mean value derived from two independent experiments are given. Error bars: s.d. **(C)** Quantification of the chromosome fusion frequencies observed in shATR treated *53BP1*^{-/-} and *ATR*^{Δ/-} *53BP1*^{+/+} MEFs (Guo *et al*, 2007). A minimum of 1200 chromosomes were analysed per cell line and the mean value derived from two independent experiments are given. Error bars: s.d. **(D)** Metaphase spreads prepared from *53BP1*^{-/-} MEFs expressing the indicated DNA constructs were analysed by CO-FISH [FITC-OO-(TTAGGG)₄ (green) and Tam-OO-(CCCTAA)₄ (red)]. Arrows point to representative fused chromosomes. **(E)** Quantification of the chromosome fusion frequencies observed in **(D)**. A minimum of 2500 chromosomes were analysed per cell line and the mean value derived from two independent experiments are given. Error bars: s.d. **(F)** Quantification of T-SCE frequencies observed in shATR treated *53BP1*^{-/-} MEFs. A minimum of 1500 chromosome ends were scored per cell line. Error bars: s.d.

requires the C-NHEJ core factors Ku86 and DNA-PK_{cs} (Espejel *et al*, 2002). However, a recent report indicates that chromosome fusions in *mTerc*^{-/-} mice with critically shortened telomeres take place independent of Lig4 and DNA-PK_{cs} (Maser *et al*, 2007). To help resolve these discrepancies, we engineered mice with short dysfunctional telomeres in the presence or absence of 53BP1. We reasoned that as 53BP1 is absolutely required for C-NHEJ-mediated fusion of dysfunctional telomeres, any chromosome fusions we observe in *mTerc*^{-/-} *53BP1*^{-/-} mice is not likely to be mediated by C-NHEJ. We crossed *mTerc*^{+/+} *53BP1*^{-/-} mice with generation 1 (G1) *mTerc*^{-/-} *53BP1*^{+/+} mice to generate the following cohorts: *mTerc*^{+/+} *53BP1*^{-/-} controls, G1-2 *mTerc*^{-/-} *53BP1*^{+/+}, G1-2 *mTerc*^{-/-} *53BP1*^{-/-} mice with long telomeres and G4 *mTerc*^{-/-} *53BP1*^{+/+}, G4 *mTerc*^{-/-} *53BP1*^{+/+}, G4 *mTerc*^{-/-} *53BP1*^{-/-} mice with short dysfunctional telomeres. *53BP1*^{-/-} mice do not readily develop spontaneous

cancer (Figure 5A). However, when exposed to 4 Gy IR almost 100% of *53BP1*^{-/-} mice die from lymphomas by 30 weeks of age (Figure 5A) (Morales *et al*, 2003; Ward *et al*, 2003). Compared with G2 *mTerc*^{-/-} *53BP1*^{+/+} controls, G2 *mTerc*^{-/-} *53BP1*^{-/-} mice succumb to lymphomas with a median age of ~55 weeks (*P* < 0.01) (Figure 5A). This increased tumor incidence is suppressed in G4 *mTerc*^{-/-} *53BP1*^{-/-} mice with critically shortened telomeres, highlighting the tumor suppressive effects of short telomeres in the setting of a competent p53 pathway (Cosme-Blanco *et al*, 2007) (Figure 5B).

The biological consequences stemming from telomere dysfunction in the setting of 53BP1 deficiency was examined *in vivo* in the intestinal epithelium, a highly proliferative tissue compartment used to quantitatively assess *in situ* telomere dynamics (Wong *et al*, 2003; Cosme-Blanco *et al*, 2007). We monitored the well-characterized *in vivo* hallmarks

Figure 5 C-NHEJ is dispensable for chromosome fusions of naturally shortened telomeres. **(A)** Kaplan–Meier (KM) tumor-free survival curve of *53BP1*^{+/+} (wt), *53BP1*^{-/-} + 5 Gy IR, *G1-2 mTerc*^{-/-} *53BP1*^{+/+} and *G1-2 mTerc*^{-/-} *53BP1*^{-/-} mice. **(B)** KM tumor-free survival curve of *G4 mTerc*^{-/-} *53BP1*^{+/+} and *G4 mTerc*^{-/-} *53BP1*^{-/-} mice. **(C)** Quantification of anaphase bridges and **(D)** apoptotic bodies observed in the intestinal crypts of mice of the indicated genotypes. Error bars: s.d. **(E, F)** Representative images of metaphase spreads derived from bone marrow (BM) of *G4 mTerc*^{-/-} *53BP1*^{+/+} and *G4 mTerc*^{-/-} *53BP1*^{-/-} mice. Arrows point to fused chromosomes. A minimum of 30 metaphases were analysed per genotype. **(G)** Quantification of number of chromosome fusions per metaphase in bone marrow and **(H)** thymic lymphomas of mice of the indicated genotypes. A minimum of 30 metaphases were analysed per genotype. Error bars: s.d.

of telomere dysfunction: elevated levels of anaphase bridge formation and TUNEL-positive apoptosis in mouse intestinal crypt (Wong *et al*, 2003; Cosme-Blanco *et al*, 2007). Compared with wild-type intestines, a modest increase in anaphase bridges and apoptotic bodies were observed in intestines from *G2 mTerc*^{-/-} *53BP1*^{+/+} mice. Both markers of telomere dysfunction increased dramatically (~30-fold) in *G4 mTerc*^{-/-} *53BP1*^{+/+} intestines (Figure 5C and D). This response to dysfunctional telomeres was unaltered in the setting of *53BP1* deficiency (Figure 5C and D), suggesting that *53BP1* does not modulate the DNA damage responses in mouse intestines stemming from critically shortened telomeres. Metaphase spreads derived from MEFs, bone marrow (BM) and lymphomas from our mouse cohorts revealed that chromosome fusions were minimal in early generation telomerase null MEF and BM. In contrast, all *G4 mTerc*^{-/-} MEFs and BMs yielded ~0.8 end-to-end chromosome fusions irrespective of *53BP1* status (Figure 5E–G and data not shown). The number of fused chromosomes rose to ~5 per metaphase in *G4 mTerc*^{-/-} *53BP1*^{-/-} lymphomas (Figure 5H). Taken together, these results suggest that chromosome fusions as a result of natural telomeres attrition do not require functional *53BP1*, and thus take place through mechanisms independent of C-NHEJ. Instead, we speculate that A-NHEJ is

likely the mechanism responsible for fusion of critically shortened telomeres. This notion is supported by data demonstrating that fusion sites of critically shortened human telomeres possess regions of microhomologies as well as extensive telomere deletions, both critical features of A-NHEJ-mediated end joining (Capper *et al*, 2007).

To ascertain whether these critically shortened telomeres are joined through A-NHEJ, we monitored the telomere status at chromosome fusion sites using telomere FISH, as primers to sequence telomere fusion junctions in mice are not yet available. We hypothesized that telomere signal intensities at chromosome fusion sites mediated by A-NHEJ would be distinct from fusion sites generated by C-NHEJ, as extensive end processing is characteristic of A-NHEJ-mediated fusions. Fusion sites from chromosomes derived from five cell types were examined: (1) *53BP1*^{+/+} MEFs devoid of Trf2 (C-NHEJ-mediated fusions), (2) *Lig4* null MEFs expressing shTrf2 and *Tpp1*^{ARD} (A-NHEJ-mediated fusions), (3) *53BP1* null MEFs expressing *Tpp1*^{ARD} (A-NHEJ-mediated fusions), (4) *G4mTerc*^{-/-} *53BP1*^{-/-} MEFs (critically shortened telomeres, *53BP1* deficiency) and (5) *G4mTerc*^{-/-} *53BP1*^{+/+} MEFs (critically shortened telomeres, *53BP1* heterozygous). CO-FISH revealed that 97% of chromosome fusion sites observed in wild-type MEFs in the absence of Trf2 possess robust

Figure 6 Telomere-FISH analysis of chromosome fusion sites. (A) Metaphases prepared from 53BP1^{+/+} MEFs depleted of Trf2, Lig4^{-/-} MEFs depleted of both Tpp1 and Trf2, and 53BP1^{-/-} MEFs depleted of Tpp1 were analysed by CO-FISH (FITC-OO-(TTAGGG)₄ (green, to detect the leading strand) and Tam-OO-(CCCTAA)₄ (red, to detect the lagging strand)). Metaphases from G4mTerc^{-/-} 53BP1^{-/-} and G4mTerc^{-/-} 53BP1^{+/-} MEFs were analysed by Tam-OO-(CCCTAA)₄ telomere FISH (red). Telomere intensities at chromosome fusion sites were scored. White arrows: robust telomeric signals at fusion sites, with approximately equal intensity lagging- and leading-strand telomeres; yellow arrow: attenuated telomeric signals (either leading- or lagging-strand telomere) at fusion sites; red arrows: fusion sites devoid of telomeric signals. (B) Quantification of telomere signals at chromosome fusion sites. A minimum of 18 metaphases were analysed per cell type. Error bars: s.d.

telomeric signals at the sites of fusion, with equal intensity of leading- and lagging-strand telomeric DNA present (Figure 6A and B). In contrast, ~43 of Lig4^{-/-} MEFs devoid of Trf2 and Tpp1 display fusion sites with unequal intensity of lagging- or leading-strand telomeres, suggesting that processing of telomeric DNA has taken place before chromosome fusions. In 53BP1^{-/-} MEFs expressing Tpp1^{ΔRD}, ~60% of chromosome fusion sites possess attenuated telomeric signals or completely lacked telomeric signals. When we examined chromosome fusion sites in G4mTerc^{-/-} 53BP1^{+/-} or 53BP1^{-/-} MEFs, almost all fusion sites completely lacked telomeric signals (Figure 6A and B). These results support the hypothesis that the chromosome fusions observed in G4mTerc^{-/-} 53BP1^{-/-} MEFs are likely due to engagement of the A-NHEJ pathway.

Discussion

In this report, we show that mammalian telomeres protect chromosome ends from engaging in both C-NHEJ and A-NHEJ-mediated end-to-end chromosome fusions (Figure 7). How telomeres are rendered dysfunctional profoundly influences the choice of DNA end-joining pathways used to initiate chromosome fusions. In agreement with earlier results, removal of Trf2 from telomeres initiates C-NHEJ-mediated fusions that is dependent on functional ATM, Lig4 and 53BP1 (Smogorzewska *et al*, 2002; Celli and de Lange,

Figure 7 Model of how telomeres are protected from engaging in inappropriate end-joining reactions. Telomeres are normally protected by the TRF2-RAP1 and TPP1-POT1 from engaging in inappropriate fusion reactions. Removal of TRF2-RAP1 initiates downstream DDR events to activate ATM/53BP1 for Lig4-mediated C-NHEJ. Removal of TPP1-POT1 stimulates ATR and CtIP to activate A-NHEJ. Natural telomere attrition results in chromosome fusions that do not require factors involved in C-NHEJ. We speculate that A-NHEJ is used to fuse naturally shortened telomeres.

2005). In contrast, Tpp1-Pot1a/b represses A-NHEJ-mediated fusions at telomeres. A-NHEJ of telomeres devoid of Tpp1-Pot1a/b also requires ATR and CtIP. We found that Trf2, Ku70 and 53BP1 all cooperate with Tpp1-Pot1a/b to repress

A-NHEJ at telomeres. In addition, naturally shortened telomeres are fused by mechanisms independent of C-NHEJ. Finally, HDR at telomeres is repressed by Tpp1-Pot1a/b and by Trf2 in cooperation with 53BP1.

Removal of TRF2 from telomeres initiates Lig4-dependent C-NHEJ-mediated end-to-end chromosome fusions that seem to be evolutionarily conserved, as deletion of the TRF2 homolog Taz1 from fission yeast also result in C-NHEJ-mediated chromosome fusions (Miller *et al*, 2005). TRF2 inhibits C-NHEJ through several distinct mechanisms: TRF2 can directly bind to telomeric DNA to repress C-NHEJ (Bae and Baumann, 2007) and also can bind to ATM to block its activation, thereby repressing C-NHEJ-mediated telomere fusions (Karlseder *et al*, 2004; Denchi and de Lange, 2007; Guo *et al*, 2007). In addition, TRF2 cooperates with Mre11 to promote the generation of 3' overhangs in newly replicated leading-strand telomeres, which are poor substrates for C-NHEJ (Attwooll *et al*, 2009; Deng *et al*, 2009; Dimitrova and de Lange, 2009). Finally, TRF2 is required to protect against the 3'-5' nucleolytic activity of Mre11 at telomeres, as depletion of TRF2 results in rapid nucleolytic degradation of the 3' ss overhang, generating telomere substrates amenable to C-NHEJ (Deng *et al*, 2009).

Recent evidence suggests that 53BP1 is recruited to chromatin flanking DSBs through histone ubiquitylation (Huen *et al*, 2007) and interaction of its Tudor domain with dimethylated histone residues (Huyen *et al*, 2004; Botuyan *et al*, 2006). This interaction is thought to increase the mobility of deprotected telomeres and is essential for C-NHEJ-mediated fusions (Dimitrova *et al*, 2008). Consistent with a function for 53BP1 in joining all distant DNA ends, 53BP1 is also required for variable, diversity and joining (VDJ) recombination (Difilippantonio *et al*, 2008) and class-switch recombination in lymphoid cells (Manis *et al*, 2004; Ward *et al*, 2004). Although we were unable to show that a Tudor domain mutant of 53BP1 localizes to uncapped telomeres, our data support a function for 53BP1 participation in C-NHEJ-mediated fusion of Trf2-depleted telomeres through the use of both the phosphorylation and homo-oligomerization domains. Although the 15AQ phosphorylation and Δ N-terminus 53BP1 mutants efficiently localized to dysfunctional telomeres, they were unable to support C-NHEJ, indicating that ATM and ATR-mediated phosphorylation of 53BP1 is critical for this joining reaction. In agreement with this notion, C-NHEJ of Trf2-depleted telomeres is completely compromised in the setting of ATM deficiency (Figure 4A). Our data are consistent with the requirement for ATM/ATR-mediated phosphorylation of the SQ/TQ sites in the N-terminus of 53BP1 to promote IR-induced DNA repair (Ward *et al*, 2006). The 53BP1 oligomerization mutant displayed reduced affinity for localizing to Trf2-depleted telomeres, consistent with earlier results demonstrating that 53BP1 oligomerization is essential for its localization to damaged DNA and formation of IR-induced DNA damage foci (Ward *et al*, 2006; Zgheib *et al*, 2009). This observation raises the possibility that 53BP1 oligomerization might be important for efficient fusion of dysfunctional telomeres similar to that observed at DSB repair, a notion reinforced by our observation that a reduction in the number of long chain end-to-end chromosome fusions was found in Trf2-depleted 53BP1^{-/-} MEFs reconstituted with the 53BP1 oligomerization mutant. We speculate that oligomerization of 53BP1 might contribute to fusion of

telomeres, perhaps by promoting direct synapsis between the two Trf2-uncapped telomeric ends to facilitate C-NHEJ-mediated DNA ligation. Consistent with a possible function in binding to and sequestering the ss DNA substrate, we found that 53BP1 potently repressed HDR of telomeres devoid of Trf2. Our results are in agreement with a recently published report demonstrating that 53BP1 prevents exonuclease-mediated resection of DSBs, preventing HR-mediated repair (Bunting *et al*, 2010).

As C-NHEJ of telomeres devoid of Trf2 was efficiently abolished in 53BP1^{-/-} MEFs, we used these cells to address the contribution of other components of the shelterin complex in mediating telomere end protection. Surprisingly, removal of Tpp1-Pot1a/b from telomeres resulted in robust end-to-end chromosome fusions in the absence of 53BP1, suggesting that these fusions are not the result of C-NHEJ. We confirmed this observation using Lig4^{-/-} MEFs. Since Lig4 (along with its partner XRCC4) has the most specific function in C-NHEJ, our observation indicates that end-to-end chromosome fusions observed in the absence of Tpp1-Pot1a/b occurs independent of C-NHEJ. The observation of robust chromosome fusions in Ku70^{-/-} and ATM^{-/-} MEFs after Tpp1-Pot1a/b depletion further strengthen the notion that A-NHEJ is the likely mechanism responsible for fusion of telomeres devoid of Tpp1-Pot1a/b. A-NHEJ requires ss DNA substrates that results in extensive deletions and possess regions of microhomology at fusion junctions (Ma *et al*, 2003; Guirouilh-Barbat *et al*, 2007), and we speculate that Tpp1-Pot1a/b repress nucleolytic processing of telomeric ends required to engage A-NHEJ. Indeed, chromosome fusion sites ligated through A-NHEJ display diminished telomeric sequences. Our data also suggest that the putative nuclease CtIP is essential for A-NHEJ of telomeres devoid of Tpp1-Pot1a/b. In mammalian cells, both the Mre11 nuclease and CtIP are implicated in the generation of DNA substrates necessary for A-NHEJ (Rass *et al*, 2009), as recent data suggest that its yeast homolog Sae2 cooperates with Mre11 to initiate resection at DSBs in a 5'-3' manner before extensive resection is performed by other nucleases including ExoI and Dna2 (Sartori *et al*, 2007; Mimitou and Symington, 2008). A-NHEJ of uncapped telomeres also seems to be repressed by both Ku70 and 53BP1. Although a function for Ku70 in repressing A-NHEJ has been described earlier (Bennardo *et al*, 2008), a function for 53BP1 in repressing A-NHEJ has only recently documented (Bothmer *et al*, 2010). We speculate that 53BP1 (Bothmer *et al*, 2010; Bunting *et al*, 2010), like Ku70 (Soutoglou *et al*, 2007), binds to and protects DNA ends from resection to prevent the generation of DNA substrates amenable to A-NHEJ-mediated repair.

Using the telomerase knockout mouse, we examined how naturally shortened telomeres are fused in the absence of C-NHEJ by genetically deleting 53BP1. Late generation *mTerc*^{-/-} primary cells (MEFs, BMs and lymphomas) all exhibit end-to-end chromosome fusions with fusion sites devoid of telomeric sequences, in the presence or absence of 53BP1. These results suggest that C-NHEJ is not required for fusion of naturally eroded telomeres. In support of this observation, 53BP1 also does not seem to have a significant function in checkpoint responses initiated by critically shortened telomeres, as it is dispensable for the activation of p53-dependent apoptosis. These results reinforce earlier observations documenting C-NHEJ-independent chromo-

some fusions in *mTerc*^{-/-}*Lig4*^{-/-} cells (Maser *et al*, 2007). We speculate that end-to-end chromosome fusions arising from critically shortened telomeres require nucleolytic processing and regions of microhomologies that are substrates for A-NHEJ. This notion is supported by the observation that critically shortened human telomeres fuse with extensive processing at fusion sites, with microhomologies observed at fusion points (Capper *et al*, 2007).

In conclusion, our results challenge the current model of telomere end protection in which uncapped telomeres, as well as telomeres arising from natural shortening, are all thought to be joined via the C-NHEJ pathway. Rather, engagement of distinct DNA repair pathways seems to depend upon how telomeres are rendered dysfunctional. Tpp1-Pot1a/b is required to repress A-NHEJ at telomeres, and this mechanism is likely responsible for fusing naturally shortened telomeres as well. As C-NHEJ and A-NHEJ repair pathways both operate in the G1/S phase of the cell cycle, the choice of how a DSB is joined by a particular repair pathway has a large bearing on the types of DNA products generated. The formation of chromosomal rearrangements as a result of incorrectly repaired breaks could result in increased chromosome instability and progression to cancer. A-NHEJ mediated repair results in genomic instability and this pathway has been implicated in promoting chromosomal aberrations, including translocations and deletions (Guirouilh-Barbat *et al*, 2007; Weinstock *et al*, 2007). Indeed, both spontaneous and radiation-induced leukaemias possess chromosomal rearrangements consistent with A-NHEJ, suggesting that utilization of this repair pathway contributes to tumorigenesis (Greaves and Wiemels, 2003). Along this line, mouse models with critically shortened telomeres or telomeres lacking Tpp1 drive genomic instability in the setting of p53 deficiency, resulting in increased tumorigenesis (Artandi *et al*, 2000; Rappold *et al*, 2001; Maser *et al*, 2007; Else *et al*, 2009). Chromosomal alterations generated by A-NHEJ is therefore likely to account for much of the genomic instability that occurs in human carcinomas, and targeting this pathway might represent a novel therapeutic approach in the future.

Materials and methods

Antibodies

A polyclonal antibody against 53BP1 has been described earlier (Morales *et al*, 2003). Anti-phospho 53BP1 Ser29 was a gift from Dr Junjie Chen (MD Anderson Cancer Center). Anti- γ -H2AX, anti-p-Chk2, anti-p-ATM Ser1981 and ATR were obtained from Upstate, BD Transduction laboratories, Rockland Immunochemical and Cell Signaling, respectively. Anti-CtIP, anti-ATM and anti-Rad51 was obtained from Santa Cruz. Anti-HA, anti- γ -tubulin (clone GTU-488), anti-Ligase 4 were purchased from Sigma. Anti-mouse Trf2 was obtained from Dr Jan Karlseder, Salk Institute.

shRNA sequences

The murine Trf2 and ATR shRNA-targeting sequences, GAA-CAGCTGTGATGATTAA and GGAGATGCAACTCGTTTAA, respectively were each cloned into the pRetro-Super vector (Stratagene) and used as described in the text. The lentivirus-based murine CtIP, 53BP1 and Lig4 shRNA were purchased from Sigma. shRNA against Rad51 was a generous gift from Magdalena Tarsounas (University of Oxford).

Generation of early and late generation *mTerc*^{-/-}/*53BP1*^{-/-} mouse and MEFs

53BP1 knockout mice have been described earlier (Morales *et al*, 2003; Manis *et al*, 2004). We crossed *mTerc*^{+/+}*53BP1*^{-/-} mice with

generation 1 (G1) *mTerc*^{-/-}*53BP1*^{+/+} mice to generate the following cohorts: *mTerc*^{+/+}*53BP1*^{+/+}, *mTerc*^{+/+}*53BP1*^{-/-} controls, G1-2 *mTerc*^{-/-}*53BP1*^{+/+}, G1-2 *mTerc*^{-/-}*53BP1*^{-/-} mice with long telomeres, and G4 *mTerc*^{-/-}*53BP1*^{+/+}, G4 *mTerc*^{-/-}*53BP1*^{-/-} mice with short dysfunctional telomeres. *mTerc*^{+/+}*53BP1*^{+/+} and *mTerc*^{+/+}*53BP1*^{-/-} MEFs from E13.5 days embryos were immortalized at passage 2 with pBabeSV40LT. Primers and conditions used to genotype *mTerc* and *53BP1* as described earlier (Morales *et al*, 2003).

Apoptotic and anaphase bridge analyses

Intestine harvested from mice were fixed in 10% neutral-buffered formalin and embedded in paraffin. Apoptotic and anaphase bridging indices were determined from 5- μ m-thick sections of fixed tissues stained with haematoxylin and eosin (H&E).

Construction of FL, deletion and point mutants of 53BP1

FL human 53BP1 cloned in pLPCX1-puro retroviral expression vector (Adams *et al*, 2005) was used for making various deletion and point mutations using site-directed mutagenesis (Stratagene) following the manufacturer's protocol. All the mutations were confirmed by sequencing. 53BP1 FL and the various deletion/point mutants were introduced into *mTerc*^{+/+}*53BP1*^{+/+} and *mTerc*^{+/+}*53BP1*^{-/-} cells by two consecutive retroviral infections at 12-h interval. Stable pools of infected MEFs selected in the presence of 2 μ g/ml puromycin were used for further studies.

Culture of MEFs and retroviral infection

53BP1 wild-type and null MEFs were cultured in DMEM supplemented with 10% FCS and maintained in 5% CO₂ at 37°C. For viral particle packaging, 293T cells were transiently transfected with pCL Eco using Lipofectamine Plus (Invitrogen) following the manufacturer's protocol. Viral supernatant were collected 48–72 h post-transfection, filtered through 0.45 μ m membranes and directly used to infect the *53BP1*^{+/+} *mTerc*^{+/+} and *53BP1*^{-/-} *mTerc*^{+/+} MEFs by two consecutive retroviral infections at 12-h interval. After 120 h of second infection, cells grown in puromycin were harvested for peptide-nucleic acid (PNA)-telomere FISH and CO-FISH analysis.

Telomere-induced foci

After 72 h of retroviral infection with Trf2 shRNA and 53BP1 mutants, cells grown on cover slips were fixed for 10 min in 2% sucrose/2% paraformaldehyde at RT followed by PBS washes. Cover slips were blocked for 1 h in blocking solution (0.2% fish gelatin, 0.5% BSA in 1XPBS). The cells were incubated with a primary, anti-rabbit 53BP1 antibody or anti-mouse γ -H2AX antibody for 2 h at RT. After PBS washes, cover slips were incubated with the appropriate Alexa fluor secondary antibody for 1 h followed by washes in PBS. Next, the cover slips were fixed with 4% paraformaldehyde for 10 min at RT, washed extensively in PBS. Hybridizing mix (70% formamide, 2% BSA, 100 μ g/ml tRNA) containing PNA 5'-Tam-OO-(CCCTAA)₄-3' probe (Applied Biosystem) was added to each cover slip and the cells were denatured by heating for 3 min at 80°C on a heat block. After 2 h incubation at RT in the dark, cells were washed twice with 70% formamide/0.1% Tween 20/0.1% BSA/10 mM Tris-HCl, pH 7.5 followed by three washes in 50 mM Tris-HCl, pH 7.5/150 mM NaCl/0.1% BSA/0.1% Tween-20. DNA was counterstained with DAPI. A minimum of 100 cells with greater than four 53BP1 or γ -H2AX signals colocalized with telomere signals were imaged on a Nikon Eclipse 800 microscope.

PNA FISH and CO-FISH

Metaphase chromosomes from MEFs were prepared 4–7 h after colcemid treatment. Chromosomes were fixed and telomere FISH with PNA Tam-OO-(CCCTAA)₄- probe (Applied Biosystem) was performed as described earlier (Wu *et al*, 2006; Guo *et al*, 2007; Deng *et al*, 2009). For CO-FISH, metaphase spreads were incubated sequentially with Tam-OO-(CCCTAA)₄- 3' and 5'-FITC-OO-(TTAGGG)₄ probes as described earlier (Wu *et al*, 2006; Guo *et al*, 2007; Deng *et al*, 2009). Images were captured with SBIG and Photometrics CCD cameras on a Nikon Eclipse 800 microscope as described earlier and processed with MetaMorph Premier (Molecular Devices). A minimum of 30 metaphases from each sample were analysed in detail.

Telomere length and G-strand overhang assays

For in-gel detection of telomeric length and G-strand overhang, a total of 2×10^6 cells were subjected to pulse-field electrophoresis. In-gel hybridization of the native gel with p-32P-C3TA2 oligonucleotides and subsequent denaturation and hybridization with p-32P-T2AG3 oligonucleotides were performed as described earlier (Deng *et al*, 2009).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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