Identification of novel factors controlling DNA damage responses at uncapped telomeres
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DNA-damage response and repair activities at uncapped telomeres depend on RNF8

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Loss of telomere protection causes natural chromosome ends to become recognized by DNA-damage response and repair proteins. These events result in ligation of chromosome ends with dysfunctional telomeres, thereby causing chromosomal aberrations on cell division. The control of these potentially dangerous events at deprotected chromosome ends with their unique telomeric chromatin configuration is poorly understood. In particular, it is unknown to what extend bulky modification of telomeric chromatin is involved. Here we show that uncapped telomeres accumulate ubiquitylated histone H2A in a manner dependent on the E3-ligase RNF8. The ability of RNF8 to ubiquitylate telomeric chromatin is associated with its capacity to facilitate accumulation of both 53BP1 and phospho-ATM at uncapped telomeres and to promote non-homologous end-joining of deprotected chromosome ends. In line with the detrimental effect of RNF8 on uncapped telomeres, depletion of RNF8, as well as of the E3-ligase RNF168, reduces telomere-induced genome instability. This indicates that, besides suppressing tumourigenesis by mediating repair of DNA double-strand breaks, RNF8 and RNF168 might enhance cancer development by aggravating telomere-induced genome instability.

Natural chromosome ends present a particularly challenging problem to cells. They should not be treated as free ends of a DNA double-strand break (DSB), as this would lead to loss of proliferation by checkpoint activation and to end-joining activities that can cause genomic instability. Genome instability is an important driving factor in the development of cancer as it increases the probability that cells acquire genetic changes required for tumour development. Telomeres are highly specialized complex structures of DNA, RNA and proteins that have evolved to protect chromosome ends. Telomeres consist of long stretches of TTAGGG-repeats that provide binding sites for the telomere-specific protein complex known as shelterin, which is composed of TRF1, TRF2, RAP1, TIN2, TPP1 and POT1. Shelterin controls telomere length and structure, protects chromosome ends from activating ATM and ATR DNA-damage checkpoint responses and prevents inappropriate activities by the homologous recombination and non-homologous end-joining (NHEJ) repair machineries.

The recognition and repair of DNA DSBs occurs within the context of chromatin and is facilitated by chromatin modification and remodelling. Importantly, telomeres represent unique chromatin structures. Besides consisting of hexameric DNA repeats and high concentrations of unique proteins, telomeric chromatin resembles constitutive heterochromatin by containing
trimethyl-H3K9, trimethyl-H4K20 and HP1. Telomeric nucleosomes show a shorter repeat length, hypersensitivity to micrococcal nuclease and increased mobility. In addition, telomeres contain unique structural arrangements in the form of G-quadruplexes and T-loops and are difficult for replication forks to pass through. Together, these special chromatin features not only affect the maintenance of telomeres by telomerase and telomere replication, but probably also impact on other processing activities such as by DNA-repair proteins. Indeed, DNA-damage responses at DNA DSBs or uncapped telomeres have in part different requirements. Therefore, it is important to investigate how (modification of) telomeric chromatin is involved in the control of DNA-damage response and repair activities at uncapped telomeres.

While the assembly of DNA-repair and signalling complexes at DNA DSBs has been shown to be under the control of a regulatory ubiquitylation pathway mediated by the RNF8 and RNF168 ubiquitin ligases, it is unknown whether post-translational modification by ubiquitylation occurs at mammalian telomeres on deprotection. To address this we first investigated whether the E3 ligase RNF8 would contribute to DNA-damage response and processing activities at uncapped telomeres. We made use of a well-characterized experimental system, which allows for fast and reversible uncapping of telomeres and relies on the temperature-sensitive inactivation of TRF2. In this system, deletion of Trf2 (also known as Terf2) in p53-/- mouse embryonic fibroblasts (MEFs) is compensated by a Trf2 allele that harbours the I468A mutation in the MYB/SANT DNA-binding domain (Trf2ts). At the permissive temperature of 32°C the TRF2ts protein is functional; it localizes to telomeres and protects these from eliciting a DNA-damage response. At non-permissive temperatures (37 to 40°C), TRF2ts becomes inactive and detaches.

**Figure 1. RNF8 contributes to telomere-induced genome instability.** (a) Schematic timeline for analysis of different aspects of the telomere-damage response using TRF2ts. At the permissive temperature of 32°C TRF2ts protects telomeres from activating DNA-damage responses. At the non-permissive temperature of 39°C, TRF2-mediated telomere protection is lost. When placing cells back at 32°C, TRF2ts becomes functional again and telomere-protection is restored. (b) Long-term inactivation of TRF2 by growing TRF2ts cells for 12 days at 39°C leads to loss of cell viability, but not when TRF2ts cells are generated in a DNA ligase IV-deficient background. (c) Survival assay of TRF2ts cells infected with pRS control retrovirus or pRS-Lig4 shRNA3 retrovirus, showing that DNA ligase IV knockdown by RNAi can rescue a portion of TRF2ts cells from lethal genome instability induced by growing TRF2ts cells for 12 days at 39°C. Plates were stained 4 weeks after placing cells back at 32°C. (d) Quantitative PCR with reverse transcription (qRT-PCR) analysis of DNA ligase IV expression levels of cells shown in c. (e) Survival assay of TRF2ts cells (C15 clone) infected with control or Rnf8 shRNA lentiviruses (Plko) or retroviruses (pRS), stained 4 weeks after cells were grown for 12 days at 39°C. (f) Photograph of TRF2ts cells infected with pLKO control or pLKO-Rnf8 shRNA1 lentivirus, grown for 12 days at 39°C. (g) qRT-PCR analysis of mouse Rnf8 expression levels of cells shown in e and f. Scale bars, 100µm.
DNA-damage response and repair activities at uncapped telomeres depend on RNF8

**Diagram a:**
- 39 °C for 12 days
- 32 °C for 12 days
- TIF, Metaphases, DNA-PFGE
- Survival

**Diagram b:**
- Ligase IV+/+
- Ligase IV−/

**Diagram c:**
- Control
- Lig4 shRNA3

**Diagram d:**
- Relative expression level

**Diagram e:**
- Control
- Rnf8 shRNA1

**Diagram f:**
- Control
- Rnf8 shRNA1

**Diagram g:**
- Relative expression level
from telomeres. This causes activation of the ATM kinase signalling pathway, accumulation of DNA-damage response proteins at telomeres into TIFs (telomere-dysfunction-induced foci), and NHEJ-dependent chromosome end fusions17.

To determine whether the TRF2ts system can be used for the swift and potentially genome-wide identification of factors that contribute to telomere-induced genome instability, we monitored TRF2ts cells kept at the non-permissive temperature for prolonged periods of time (Fig. 1a). After 12 days at the non-permissive temperature, all cells had either arrested or died as a consequence of severe chromosome end fusion, which drives cells into crisis. This condition was not reversed after placing cells back at the permissive temperature (Fig. 1b,c,e controls panels). We reasoned that interference with the ability of cells to form chromosome fusions between uncapped telomeres should read out as enhanced survival from prolonged telomere uncapping. Indeed, deletion or RNA interference (RNAi)-mediated inhibition of DNA ligase IV, the ligase responsible for joining most TRF2-deprotected telomeres18,19, enabled survival from severe telomere uncapping following prolonged culturing of TRF2ts cells at the non-permissive temperature (Fig. 1b-d).

Interestingly, when we reduced RNF8 levels with independent short hairpin RNA (shRNA) vectors in multiple TRF2ts clones and kept these cells for 12 days at the non-permissive temperature, we observed a markedly increased survival both during growth at the non-permissive temperature and after restoration of telomere protection (Fig. 1e-g). Furthermore, another Rnf8 shRNA recently emerged as a top hit in a genome-wide RNAi screen that we carried out using TRF2ts to identify factors that contribute to telomere-induced genome instability (our unpublished results). The enhanced survival of Rnf8 knockdown cells under conditions of long-term TRF2 inactivation was indeed due to a decrease in telomere fusions. After 24 h of TRF2 inactivation, analysis of telomere fluorescence in situ hybridization (FISH)-stained metaphase spreads indicated that approximately 35% of chromosomes were fused. However, knockdown of Rnf8 lowered the degree of telomere fusions with 75%, to 9% of the chromosomes fused (Fig. 2a,b). Growth rates were not significantly different between wild-type or Rnf8-knockdown TRF2ts cells, indicating that the NHEJ defect observed with Rnf8 knockdown was not due to changes in cell-cycle progression.

Telomeres end in single-stranded G-rich overhangs that undergo Lig4/NHEJ-dependent degradation upon loss of TRF2-mediated protection18-20. Whereas telomeres in control cells had lost around 60% of their G-overhangs after 48 h of TRF2 inactivation, RNF8-depleted cells showed no significant G-overhang loss (Fig. 2c,d). This indicates that RNF8 function is required for G-overhang degradation. As single-strand overhangs are not suitable substrates for NHEJ, this observation explains why Rnf8-knockdown cells show fewer fusions on telomere uncapping. Maintenance of the telomeric G-overhang and compromised fusion of uncapped telomeres has also been observed in the absence of ATM, NBS1, MDC1, 53BP1, DNA-ligase IV and KU7018-25. As all these proteins were present in RNF8-depleted cells, the failure of RNF8-depleted cells to efficiently degrade
G-overhangs and form telomere fusions in a Lig4/NHEJ-dependent manner was not due to insufficient levels of these DNA-damage response proteins (Fig. 3d and data not shown).

Since in the absence of RNF8 uncapped telomeres were not processed properly, we examined the recognition of uncapped telomeres by DNA-damage response proteins. Without TRF2ts inactivation we observed barely any accumulation of phosphorylated ATM (p-ATM), phosphorylated histone H2AX (γ-H2AX) or 53BP1 at telomeres into TIFs (Fig. 3b and Supplementary Fig. S1), nor did a temperature-shift from 32°C to 39°C alone induce TIFs (Supplementary Fig. S2). In contrast, after culturing TRF2ts cells for 3 h at the non-permissive temperature of 39°C to inactivate TRF2, γ-H2AX was readily detected at telomeres. Its accumulation seemed not to be influenced by RNF8 (Fig. 3a,b). In line with this, induction of γ-H2AX on telomere uncapping was also visible in total cell lysates and its levels did not significantly differ between control and Rnf8-knockdown cells (Fig. 3d). However, under the same conditions we observed a very strong reduction in the accumulation of 53BP1 at telomeres in RNF8-depleted cells, whereas overall 53BP1 protein levels remained unaffected (Fig. 3a,b,d). The effect of RNF8 on 53BP1 TIFs was independent of the method of TRF2 inactivation and not restricted to mouse cells or TRF2 inhibition, as RNF8 knockdown also prevented 53BP1 TIFs following TRF2 knockdown in human BJ fibroblasts (Supplementary Fig. S3) and after TPP1 inhibition (Fig. 3c).

Interestingly, Rnf8 knockdown also inhibited accumulation of p-ATM at telomeres shortly after uncapping (Fig. 3a,b). This was unexpected, as ATM acts upstream of γ-H2AX and 53BP1 and is not known to be affected by RNF8 at early time points after DNA or telomere-damage. As was the case for 53BP1, total p-ATM levels in whole cell extracts appeared largely normal (Fig. 3d). However, a reduction both in 53BP1 and in p-ATM that was strongly associated with chromatin was also observed by immunoblotting of differential KCL-extracted cells (Supplementary Fig. S4). After telomere uncapping 53BP1 and p-ATM exist in two chromatin-bound forms that are either sensitive or refractory to extraction with 420 mM KCl. After 3 h of telomere uncapping, p-ATM increased in both fractions, but most pronouncedly in the 420 mM KCl-resistant fraction, suggesting that p-ATM becomes more tightly associated with chromatin. This was diminished in cells with Rnf8 knockdown, suggesting loss of more tightly bound p-ATM. Similarly, Rnf8 knockdown prohibited accumulation of 53BP1 in the 420 mM KCl-resistant fraction.

Despite the reduced accumulation of p-ATM at uncapped telomeres, ATM-mediated DNA-damage signalling seemed largely normal, as judged by extensive phosphorylation of H2AX and checkpoint kinase 2 (CHK2), although CHK2 phosphorylation seemed partially compromised in Rnf8-knockdown cells (Fig. 3d). The CHK2 and H2AX phosphorylation observed despite severely reduced telomeric association of p-ATM could be explained by remaining p-ATM activity or contribution of ATR signalling activated by single-stranded telomeric DNA. Consistent with previous characterization of TRF2ts17, we observed phosphorylation of the ATR target CHK1 on TRF2ts inactivation (Fig. 3d). This
Chapter 3

**a**

Control

![Control](image1)

Rnf8 shRNA

![Rnf8 shRNA](image2)

**b**

Percentage of chromosomes fused

<table>
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<th>39°C</th>
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<tbody>
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<td>Control</td>
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<td>0</td>
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<tr>
<td>Rnf8 shRNA</td>
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**c**

Native ss TTAGGG

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Denatured Total TTAGGG

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<td>+</td>
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**d**

Relative ss TTAGGG

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<td>Rnf8 shRNA</td>
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* P = 0.000045
is explained by partial release of POT1 from telomeres when TRF2 is removed, resulting in reduced POT1-mediated repression of ATR at single-stranded telomeric DNA^{17, 22}. The accumulation of 53BP1 at DNA DSBs is thought to depend on both methylation of Lys 20 on histone H4 and ubiquitylation of histones H2A and H2AX. The latter is controlled by RNF8 and RNF168^{26-31}. As in the absence of RNF8 53BP1 failed to accumulate at uncapped telomeres, we asked whether telomere uncapping would result in H2A ubiquitylation at telomeres. Clear distinct foci of ubiquitylated H2A (Ub-H2A) were readily detected at telomeres after TRF2 inactivation in TRF2ts MEFs (Fig. 4a,b). These foci were not observed either at the permissive temperature, or in wild-type MEFs when shifted from 32°C to 39°C (Fig. 4a,b and Supplementary Fig. S2). Ub-H2A TIFs were almost completely absent in RNF8-depleted cells (Fig. 4a,b). Furthermore, 3 h after telomere uncapping we observed mono- and di-ubiquitylated species of γ-H2AX (Fig. 4c). In particular, di-ubiquitylated γ-H2AX was reduced in RNF8-depleted cells experiencing telomere deprotection (Fig. 4c). This indicates that H2A ubiquitylation at uncapped telomeres and ubiquitylation of γ-H2AX in cells with uncapped telomeres are dependent on the activity of RNF8. Consistent with this, RNF8 accumulated at telomeres after TRF2 inactivation (Fig. 4d). In addition, we observed a strong increase in Ub-H2A foci in E6E7-expressing human BJ fibroblasts with critically short telomeres due to passaging towards crisis (data not shown). Thus, not only shelterin inhibition, but also telomere deprotection due to loss of telomere repeats, as a consequence of replication, is accompanied by H2A ubiquitylation.

The RING-domain of RNF8 is required for protein ubiquitylation and 53BP1 accumulation at DNA DSBs^{26-29}. To examine whether the RING-domain is also important for the ability of RNF8 to control protein ubiquitylation and 53BP1 accumulation at uncapped telomeres we complemented Rnf8 knockdown with RNAi-resistant wild-type RNF8 or the RNF8^{C406S} RING-domain.
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Figure 3. Impaired 53BP1 and p-ATM accumulation at uncapped telomeres on knockdown of Rnf8. (a) Representative images of immunofluorescence FISH (IF-FISH) detection of p-ATM Ser 1987, γ-H2AX, 53BP1 and telomere repeats in TRF2ts MEFs grown at 39 °C for 3 h. Scale bar, 10µm. (b) Quantification of p-ATM, γ-H2AX and 53BP1 foci at telomeres in TRF2ts MEFs, with or without Rnf8 knockdown, after 3 h at 39 °C to deprotect telomeres. (c) Quantification of 53BP1 foci in p53 shRNA-immortalized MEFs infected with control or Rnf8 shRNA2 retroviruses and subsequently with control retrovirus or Tpp1 shRNA2 retrovirus to inhibit the shelterin component TPP1. Error bars represent s.d., n=5 replicates (d) Western blot analysis of DNA-damage response and repair proteins after 0, 3, 24, 48 h of telomere uncapping in whole-cell lysates of TRF2ts MEFs infected with control retrovirus or Rnf8 shRNA2 retrovirus. The asterisk in the 53BP1 lane marks a non-specific band. The asterisk in the CHK2 lane indicates phosphorylated CHK2. Immunoblotting for γ-tubulin serves as a loading control. As a positive control for CHK1 phosphorylation we included a lysate of cells treated for 3 h with 2 mM hydroxyurea (HU). Uncropped images of blots are shown in Supplementary Fig. S6.
dependent histone ubiquitylation and thereby orchestrate the accumulation of 53BP1 and BRCA1 at DNA-lesions\textsuperscript{30, 31}. As for RNF8, depletion of RNF168 rescued cells from lethal telomere-induced genome instability induced by inactivation of TRF2 (Supplementary Fig. S5). This suggests that RNF168, like RNF8, is involved in controlling the processing of deprotected telomeres by NHEJ.

Collectively, our data show that post-translational modification of telomeric chromatin by ubiquitylation through the E3 ligase RNF8 plays a critical role in the recognition and repair of deprotected telomeres and in telomere-induced genome instability in mammalian cells. Our results indicate that RNF8 affects the processing and end-joining of deprotected telomeres in at least two ways. RNF8 facilitates

Figure 4. Telomere uncapping induces ubiquitylation of H2A and H2AX in an RNF8-dependent manner. (a) Representative images of IF-FISH detection of Ub-H2A at telomeres in TRF2ts MEFs grown at 32 °C or for 3 h at the non-permissive temperature of 39 °C in the absence or presence of Rnf8-shRNA2-mediated knockdown. (b) Quantification of Ub-H2A foci at telomeres in TRF2ts MEFs grown at 32 °C or for 3 h at 39 °C with or without Rnf8 knockdown. Error bars represent s.d., n=4 independent experiments. (c) Western blots of whole-cell lysates showing RNF8-dependent induction of γ-H2AX ubiquitylation after 0, 3, 24 and 48 h of telomere uncapping in TRF2ts cells. (d) IF-FISH detection of transfected haemagglutinin (HA)-RNF8 at telomeres in TRF2ts MEFs grown for 6 h at 39 °C to inactivate TRF2 and uncap telomeres. Scale bars, 10µm.

Chapter 3

\[ TTAGGG \quad Ub-H2A \quad Merge (+Topro) \]

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Telomere uncapping induces ubiquitylation of H2A and H2AX in an RNF8-dependent manner. (a) Representative images of IF-FISH detection of Ub-H2A at telomeres in TRF2ts MEFs grown at 32 °C or for 3 h at the non-permissive temperature of 39 °C in the absence or presence of Rnf8-shRNA2-mediated knockdown. (b) Quantification of Ub-H2A foci at telomeres in TRF2ts MEFs grown at 32 °C or for 3 h at 39 °C with or without Rnf8 knockdown. Error bars represent s.d., n=4 independent experiments. (c) Western blots of whole-cell lysates showing RNF8-dependent induction of γ-H2AX ubiquitylation after 0, 3, 24 and 48 h of telomere uncapping in TRF2ts cells. (d) IF-FISH detection of transfected haemagglutinin (HA)-RNF8 at telomeres in TRF2ts MEFs grown for 6 h at 39 °C to inactivate TRF2 and uncap telomeres. Scale bars, 10µm.\]
DNA repair at telomeres by inducing ubiquitylation of histone H2A, H2AX and possibly other substrates, thereby promoting accumulation of 53BP1, which is required for efficient long-range end-joining of uncapped telomeres by increasing chromatin mobility²³. Intriguingly, RNF8 also promotes p-ATM accumulation or retention at deprotected telomeres shortly (3 h) after telomere uncapping. As ATM contributes to DNA repair by increasing chromatin elasticity in a manner dependent on the transcriptional co-repressor KAP1 and by promoting chromatin

![Figure 5. 53BP1, p-ATM and Ub-H2A accumulation at uncapped telomeres and telomere-induced genome instability depend on the RNF8 FHA and E3-ligase domains.](image)

(a) Expression of Flag-tagged murine RNF8 RR, RNF8 C406S RR and RNF8 R42A RR proteins after retroviral infection in TRF2ts MEFs. RR refers to silent mutations introduced to confer resistance to RNAi by Rnf8 shRNA1. (b) Examples of Ub-H2A foci in wild-type TRF2ts cells or cells with knockdown of Rnf8 and complemented with wild-type murine RNF8 or the C406S or R42A RNF8 mutants. Scale bar, 10 µm. (c) Quantification of IF-FISH detection of 53BP1, p-ATM and Ub-H2A foci at telomeres (TIFs) in TRF2ts MEFs infected with control viruses or infected with Rnf8 shRNA1 lentivirus together with either control retrovirus, RNAi-resistant Flag-tagged RNF8, RNF8 C406S or RNF8 R42A retrovirus. TIFs were scored after 3 h at the non-permissive temperature to inactivate TRF2ts. (d) Survival assay of TRF2ts cells infected with control or Rnf8 shRNA1 lentiviruses and control or RNAi-resistant Flag-tagged RNF8, RNF8 C406S or RNF8 R42A retroviruses. The effect of Rnf8 shRNA1, wildtype RNF8 and mutant RNF8 on cell growth in the absence of telomere damage was monitored after 12 days at 32 °C. Their effect on survival from 12 days of telomere deprotection was monitored at 4 weeks of growth at 32 °C.
decondensation via H2B ubiquitylation, RNF8-mediated control of p-ATM might enhance processing and repair at uncapped telomeres via increasing chromatin accessibility to DNA-repair proteins.

RNF8 has been shown to act as a tumour suppressor in mice by mediating repair of DNA DSBs, thereby preventing genomic instability. Our work raises the interesting possibility that RNF8 might also enhance cancer development by facilitating telomere fusion, which leads to genome instability in DNA-damage checkpoint-deficient cells. Interestingly, high-level amplification of Rnf8 in lung cancer and leukaemia cells revealed by the Cancer Genome Project (Sanger Institute) and reports in the Oncomine database on elevated Rnf8 copy numbers and Rnf8 messenger RNA overexpression in multiple cancers indicate that here Rnf8 might act as an oncogene rather than a tumour suppressor. Although RNF8 overexpression might interfere with normal DNA-repair functions, it is tempting to speculate that RNF8 overexpression potentially enhances cancer development by specifically promoting telomere-driven genomic instability. Further insights in the contribution of RNF8-mediated control of telomere-induced genomic instability to tumourigenesis could come from studying the impact of RNF8 on cancer development in mouse models for telomere uncapping.

Methods

Cell culture and growth assays

TRF2<sup>−/−</sup>; p53<sup>−/−</sup>; TRF2ts MEFs or TRF2<sup>−/−</sup>; p53<sup>−/−</sup>; Lig4<sup>−/−</sup> TRF2ts MEFs were generated as described previously. TRF2<sup>box/−</sup>; p53<sup>−/−</sup>; MEFS or TRF2<sup>box/−</sup>; p53<sup>−/−</sup>; Lig4<sup>−/−</sup> MEFS were infected with a pWLZH-TRF2I468A retrovirus, infected with a Hit-and-Run Cre retrovirus to delete the remaining Trf2 allele, and clones were generated that were genetically and functionally tested for absence of endogenous Trf2 and temperature-sensitive complementation by the exogenous Trf2ts allele. All results were confirmed in 2 independent clones (C15 and B17). MEFs, Phoenix, 293 and human diploid BJ fibroblasts (ATCC) expressing the ecotropic receptor for retroviral infection were grown in DMEM with 50 U of penicillin, 0.05 mg ml<sup>−1</sup> streptomycin, 2 mM L-glutamine and 10% FBS. For colony survival assays, TRF2ts cells were plated at 150,000 cells per 15 cm dish, allowed to adhere at 32 °C and then placed for 12 days at 39 °C, after which they were returned to 32 °C. At 0, 2 or 4 weeks after returning the plates to 32 °C, plates where fixed with 10% formalin and stained with 0.1% crystal violet.

Retroviral and lentiviral transductions

For retroviral infection ecotropic phoenix producer cells (ATCC) were seeded at 2.5 x 10<sup>6</sup> cells per 10 cm dish and transfected using CaPO4 precipitation of 20 µg of retroviral vector DNA. Medium was refreshed at 16 and 24 h after adding the DNA/CaPO4 precipitate and viral supernatants were harvested, filtered through a 0.22 µm syringe filter at approximately 48, 62 and 72 h post-transfection. Viral supernatants were either frozen on dry ice and stored at −80°C until use, or used immediately. For infection cells were overlaid with viral supernatant supplemented with 4 µg ml<sup>−1</sup> polybrene. MEFs were infected once and human cells, engineered to express the ecotropic viral receptor, were infected twice to achieve 100% infection
efficiency. Cells were transduced with pRetrosuper-puro retroviruses encoding shRNAs targeting mouse Rnf8 (shRNA2: 5'-GCATCAGGCTCTAATGGAA-3'), mouse Lig4 (shRNA3: 5'-GGATCAGA-GACGAGTTACT-3'), mouse and human Trf2 (shRNA2: 5'-GAACAGCTGTA-GATTAA-3'), mouse Tpp1 (shRNA2: 5'-GCTGTGTTCACGTGTCTG-3') and human RNF8 (shRNA3: 5'-ACATGAAAGCCGTTATGAAT-3'). The use of Trf2 shRNA2, Tpp1 shRNA2 and RNF8 shRNA3 was described before 25, 28, 38. Mouse Rnf8 complementary DNA was obtained from Open Biosystems. For complementation experiments we cloned Flag-tagged mouse Rnf8 cDNA, as well as the C406S and R42A mutant cDNAs, in pBabeblas retrovirus. The target sequence of Plko-Rnf8 shRNA1 was changed to 5'-CGGAAGGTGGAATGTCCCATT-3' to create shRNA-resistant constructs RNF8RR, RNF8C406S RR and RNF8R42A RR. The C406S, R42A and RNAi-resistant mutations were made by QuickChange Site-Directed mutagenesis (Stratagene). For lentiviral transduction with Plko-puro shRNA lentiviruses obtained from Mission library clones (Sigma) cells were incubated for 6 h in diluted virus that was produced in 293 cells. Lentiviruses used in the experiments shown were: mouse Rnf8 shRNA1 (TRC 39429: 5'-CGGAAAGGTGGAATGTCCCATT-3'), mouse Rnf168 shRNA77 (TRC#40877: 5'-CTGGACAAGAATCAAAGGAAA-3') and mouse Rnf168 shRNA74 (TRC#40874: 5'-CCGGAAGAAATCTCTGGTCAA-3').

Pulsed-field gel electrophoresis and In-gel detection of telomeric DNA

Pulsed-field gel electrophoresis of mouse telomere repeats was carried out as described 17. ExoI digestion and hybridization with a 32P-labeled (TTAGGG)4 probe specific for the C-rich strand was done to verify that the hybridization signal obtained with a 32P-labeled (CCCTAA)4 probe for the G-rich telomere strand was indeed derived from 3' single-stranded telomeric TTAGGG repeat strands.

Immunoblotting

Whole-cell lysates were prepared in SDS sample buffer, protein concentration was determined by standard BCA protein assay (Pierce) and equal amounts of protein were separated on precast 4-12% Bis-Tris or 3-8% Tris-acetate gels (Invitrogen). Immunoblotting was done according to standard methods using IRDye800CW- and IRDye680-labeled secondary antibodies for detection on the Odyssey Infrared imager (LI-COR). Primary antibodies used were against phospho-ATM (p-Ser 1987 of mATM, p-Ser 1981 of hATM, no 4526, Cell Signaling, 1:1,000), 53BP1 (NB100-305,
Novus, 1:500), phospho-H2AX Ser 139 (clone JBW301, Upstate, 1:1,000), CHK2 (611570, BD, 1:500), phospho-CHK1 Ser 345 (no 2348 Cell Signaling, 1:1,000), MDC1 (ab11169, abcam, 1:1,000), UBC13 (ab25885, abcam, 1:2,000), Ligase IV (H300, scbt, 1:300), γ-tubulin (GTU-88, Sigma, 1:5,000), lamin A/C (636, scbt, 1:1,000).

Immunofluorescence, IF-FISH, metaphase chromosome analysis

For Immunofluorescence or IF-FISH cells were grown on eight-well chamber slides (Lab-Tek), pre-extracted with 0.5% triton/PBS on ice and fixed with 4% paraformaldehyde in PBS. The IF-FISH procedure was carried out essentially as described17. Primary Antibodies used were against phospho-ATM (p-Ser 1987 of mATM, p-Ser 1981 of hATM, no 4526, Cell Signaling, 1:1,000), 53BP1 (NB100-305, Novus, 1:500), ubiquitylated histone H2A (clone E6C5, Millipore, 1:1,000), HA tag (3F10, Roche, 1:1,000). Telomere repeats were detected by FISH using a FITC-OO-(CCCTAA) peptide nucleic acid custom probe (Biosynthesis). DNA was stained with Topro-3 and slides were mounted in Vectashield (Vector Laboratories). Cell harvesting, preparation of metaphase spreads and telomere FISH for metaphase chromosome analysis was done as described17. Confocal fluorescence images were obtained on a Leica TCS SP2 confocal system equipped with an Ar, Kr and HeNe laser system. Images were acquired using a 63x NA 1.32 oil objective and standard LCS software. Possible crosstalk between different fluorochromes, which could cause false-positive colocalization of signals, was completely avoided by careful selection of imaging conditions. Standard filter combinations and Kalman averaging was used. Digital images of metaphases were captured using a Zeiss AxioObserver Z1 microscope with a Hamamatsu C4742-80-12AG ORCA-ER CCD camera and Axiovision software.

Differential KCl extraction of chromatin

Differential KCl extraction of chromatin was carried out as described39, with minor modifications. Cells were washed twice in ice-cold PBS and collected by scraping in PBS. After centrifugation, cell pellets were resuspended in 10 times pellet volume of buffer C-150 (20 mM HEPES pH 7.9, 25% glycerol, 5 mM MgCl, 0.2% NP40, 1 mM dithiothreitol, 1mM phenylmethyl sulphonyl fluoride, protease inhibitor cocktail (Roche), 1 mM Na3VO4, 5 mM β-glycerophosphate, 1 mM NaF and 150 mM KCl). After 15 min on ice, supernatants containing soluble cytoplasmic and nucleoplasmic proteins were collected by centrifugation at 3,000 r.p.m. for 5 min at 4°C. Pellets were resuspended in buffer C-420 (as above, but with 420 mM KCl) and incubated for 15 min on ice. Supernatants containing chromatin-associated proteins were collected by centrifugation at 14,000 r.p.m. for 10 minutes at 4°C. Final pellets were resuspended in SDS sample buffer and sheared by passing through a 25G needle.

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References

19. Celli, G.B. & de Lange, T. DNA processing is not required for ATM-mediated telomere
Chapter 3

DNA-damage response and repair activities at uncapped telomeres depend on RNF8

Supplementary Figures

Figure S1. p-ATM, γ-H2AX and 53BP1 do not form foci at telomeres in TRF2ts MEFs at the permissive temperature in the presence of wild-type or reduced levels of RNF8. Representative images of IF-FISH detection of p-ATM, γ-H2AX, 53BP1 and telomere repeats in TRF2ts MEFs grown at 32°C to illustrate that at the permissive temperature of 32°C, when TRF2ts can participate in telomere protection, there is no accumulation of DNA damage foci at telomeres. See Fig. 3b for quantifications. Dashed white lines indicate the positions of the cells. Scale bar, 10 µm.
Figure S2. No p-ATM, γ-H2AX, 53BP1 and Ub-H2A foci at 39°C in the absence of telomere deprotection. Representative images of IF-FISH detection of p-ATM, γ-H2AX, 53BP1 and Ub-H2A and telomere repeats in SV40 immortalized wild-type MEFs to illustrate that a temperature shift from 32°C to 39°C for 3 h by itself does not induce DNA damage foci at telomeres or elsewhere. Scale bar, 10 μm.
DNA-damage response and repair activities at uncapped telomeres depend on RNF8.

Figure S3. RNF8 controls efficient 53BP1 TIF formation upon TRF2 knockdown in human BJ fibroblasts. Quantification of 53BP1 foci in human diploid BJ fibroblasts infected with control or RNF8 shRNA2 retroviruses and control or TRF2 shRNA2 retroviruses.

Figure S4. RNF8 depletion prevents strong association of p-ATM and 53BP1 with chromatin upon telomere uncapping. Analysis of 53BP1 and p-ATM levels upon differential KCl isolation of chromatin from TRF2ts cells infected with control or Rnf8 shRNA2 retrovirus and grown at 32 °C s or for 3 h at 39 °C s to inactivate TRF2ts. Ponceau S staining and immunostaining for Lamin A/C serve a loading controls. Inactivation of TRF2 in wild type TRF2ts MEFs, but not in RNF8-depleted TRF2ts MEFs increases the level of 53BP1 that is tightly bound to chromatin and refractory to 420 mM KCL extraction. Similarly, TRF2 inactivation increases the levels of ATM phosphorylated at Ser 1987 (corresponding to Ser 1981 in human). The fraction of p-ATM that is tightly bound to chromatin and resistant to 420mM KCl extraction is reduced in RNF8-depleted cells. However the fraction of p-ATM that can be extracted with 420mM KCl is not different between wild-type or RNF8-depleted TRF2ts cells.
Figure S5. RNF168 depletion protects against lethal genome instability resulting from severe telomere deprotection. (a) Survival assay of TRF2ts cells (B17 clone) infected with Plko control lentivirus or Plko-Rnf168 shRNA lentiviruses. For comparison, the effect is shown of retroviral PRS-Rnf8 shRNA2, addressed in parallel in the same experiment. (b) qRT-PCR analysis of mouse Rnf168 expression levels of cells shown in a.
DNA-damage response and repair activities at uncapped telomeres depend on RNF8

Figure S6. Full scans of the immunoblots shown in Fig. 3d.