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Loss of Rap1 induces telomere recombination in absence of NHEJ or a DNA damage signal

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Abstract

Shelterin is an essential telomeric protein complex that prevents DNA damage signaling and DNA repair at mammalian chromosome ends. Here we report on the role of the TRF2-interacting factor Rap1, a conserved shelterin subunit of unknown function. We removed Rap1 from mouse telomeres either through gene deletion or by replacing TRF2 with a mutant that does not bind Rap1. Rap1 was dispensable for the essential functions of TRF2 – repression of ATM kinase signaling and non-homologous end-joining (NHEJ) – and mice lacking telomeric Rap1 were viable and fertile. However, Rap1 was critical for the repression of homology-directed repair (HDR), which can alter telomere length. The data reveal that HDR at telomeres can take place in absence of DNA damage foci and underscore the functional compartmentalization within shelterin.

Keywords

shelterin; Rap1; NHEJ; HDR; ATM; ATR

The shelterin subunit TRF2 is a main player in the repression of the telomeric DNA damage response (1). Deletion of TRF2 results in activation of the ATM kinase and rampant telomere fusions mediated by NHEJ. TRF2 also contributes to the repression of HDR, which can create undesirable telomeric sister chromatid exchanges (T-SCEs). HDR at telomeres is unleashed in Ku70/80 deficient cells upon deletion of either TRF2 or the two POT1 proteins (2,3). The repression of ATM signaling, NHEJ, and HDR by TRF2 could potentially involve its interacting partner Rap1, which depends on TRF2 for its stable expression and recruitment to telomeres (4,5). Telomere protection is one of the functions of the distantly-related Rap1 orthologs in yeast. In Saccharomyces cerevisiae and Schizosccharomyces pombe, Rap1 contributes to the repression of NHEJ at chromosome ends, whereas Kluyveromyces lactis Rap1 represses HDR (6-9). Human Rap1 affects telomere length homeostasis and a tethering experiment has suggested that Rap1 can repress telomere fusions (10-12). Here we use two complementary approaches to determine how Rap1 loss affects telomere function. Gene targeting was used to generate mouse cells lacking a functional Rap1 gene. In addition, we generated mouse cells devoid of the endogenous TRF2 that were complemented with a TRF2 mutant incapable of binding Rap1. The two approaches yielded the same results.

Because the first exon of the mouse *Rap1* gene immediately abuts the essential KARS lysyl-tRNA-synthetase gene, we developed a conditional knockout strategy to delete exon 2 (Fig. 1A-C). The $Rap1^{\Delta ex2}$ allele generated by Cre recombinase treatment of $Rap1^{F/F}$ cells can

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potentially encode a Rap1 fragment that lacks the TRF2-binding domain (Fig. 1A). We verified that this truncated form of Rap1, if it were produced, would not bind chromatin or localize to telomeres (fig. S1A-C). IF and immunoblotting showed that Cre-treated SV40LT-immortalized *Rap1*$^{F/F}$ MEFs indeed lacked any detectable Rap1 protein and ChIP showed the loss of Rap1 from telomeres (Fig. 1D-F, fig. S1D). As the expression and localization of other shelterin components were not significantly affected (Fig. 1D-F, fig. S1E), the phenotypes of *Rap1*$^{\Delta ex2/\Delta ex2}$ MEFs should inform specifically on the telomeric function of Rap1.

The growth rate of the *Rap1*$^{\Delta ex2/\Delta ex2}$ MEFs was similar to control cells, regardless of whether the cells were immortalized with SV40LT, and primary MEFs lacking wild type Rap1 did not show a growth arrest or p53 activation (Fig. 1G, fig. S1F, G). Furthermore, *Rap1*$^{\Delta ex2/\Delta ex2}$ mice were born at the expected frequencies and were fertile (Fig. 1H). The survival of *Rap1*$^{\Delta ex2/\Delta ex2}$ cells and mice argues that *Rap1* deletion does not result in major telomere dysfunction, which is known to be lethal. This conclusion was further corroborated by infecting *Rap1*$^{\Delta ex2/\Delta ex2}$ MEFs with an shRNA targeting exon 1 (Fig. 1A, fig. S1H), which did not induce a growth arrest or other phenotypes typical of telomere dysfunction (see Fig. 3, data not shown).

In the second approach to remove Rap1 from telomeres, we used previously characterized *TRF2*$^{F/-}$*p53*$^{-/-}$ MEFs (4) to replace the endogenous TRF2 with a mutant that does not bind to Rap1. In order to identify amino acids critical for Rap1 interaction, we searched for highly conserved, TRF2-specific motifs in the previously mapped Rap1 binding region (aa 260-360; (5)). A short predicted helix at position 290 was conserved in TRF2 orthologs but not in TRF1 (fig. S2A,B). Two mutations in this region (A289S and F290S) reduced the interaction between Rap1 and TRF2 in co-IP experiments (fig. S2C). To generate TRF2$^{\Delta Rap1}$, amino acids 284-297 were deleted (Fig. 2A). TRF2$^{\Delta Rap1}$ failed to bind to Rap1 in co-IP experiments whereas it retained its interaction with the TRF2-interacting protein Apollo (Fig. 2B). Wild type TRF2 and TRF2$^{\Delta Rap1}$ were expressed in *TRF2*$^{F/-}$*p53*$^{-/-}$ MEFs and the endogenous TRF2 was removed with Cre (Fig. 2C). Although TRF2$^{\Delta Rap1}$ localized to telomeres efficiently, IF and ChIP indicated that the telomeres lacked Rap1 and the overall level of Rap1 in the cells was reduced (Fig. 2C-E, fig. S3A). Other shelterin components were affected to an extent (<2-fold; Fig. 2D,E) that is not expected to be functionally significant as heterozygous MEFs and mice lacking one copy of *TRF1*, *TPP1*, *TRF2*, or *POT1a/b* display no telomere defect. Consistent with the viability of *Rap1*$^{\Delta ex2/\Delta ex2}$ cells, cells expressing TRF2$^{\Delta Rap1}$ proliferated at the same rate as cells expressing wild type TRF2 (fig. S3B).

Telomeres lacking Rap1 were examined for the hallmarks of telomere dysfunction. *Rap1*$^{\Delta ex2/\Delta ex2}$ cells did not show Telomere Dysfunction-Induced Foci (TIFs; (13)), which are telomeric DNA damage foci that report on ATM and/or ATR signaling at chromosome ends, and phosphorylation of Chk1 and Chk2 was not evident (Fig. 3A-C). Further depletion of Rap1 mRNA with an shRNA also failed to elicit a DNA damage signal in *Rap1*$^{\Delta ex2/\Delta ex2}$ cells (Fig. 3B). Consistent with these results, TRF2$^{\Delta Rap1}$ was equal to wild type TRF2 in its ability to repress TIFs in cells lacking the endogenous TRF2 (Fig. 3D). The mutant form of TRF2 also repressed the induction of Chk-2 phosphorylation to the same extent as wild type TRF2 (Fig. 3E). The low level of Chk2-P observed in Cre-treated TRF2- and TRF2$^{\Delta Rap1}$-expressing cells is likely due to Cre-induced DNA damage, since the phosphorylation of Chk2 was diminished when using a version of Cre that eventually disappears from the cells due to self-deletion (fig. S4).

Furthermore, telomere fusions were not induced by deletion of *Rap1* and TRF2$^{\Delta Rap1}$ had the same ability as wild type TRF2 to repress NHEJ at telomeres (Fig. 3F-H). However, as NHEJ of telomeres lacking TRF2 requires active DNA damage signaling (14), the lack of telomere fusions could be due to the lack of ATM signaling. We therefore used a TPP1 shRNA to activate ATR kinase signaling at telomeres. This approach previously resulted in the reactivation of

NHEJ at telomeres of TRF2/ATM deficient cells (14). Despite the telomeric ATR kinase signal elicited by the TPP1 shRNA, (Fig. 3B,D), Rap1 removal from telomeres did not induce their fusion (Fig. 3G,H).

These data argue against a requirement for Rap1 in either the repression of NHEJ or ATM kinase signaling and explain why the deletion of Rap1 did not curb cellular or organismal viability. In addition, Rap1 was not required for the maintenance of several other features of mouse telomeres, including the maintenance of telomere length over three generations of mouse breeding and in cultured cells, the amount of single-stranded telomeric DNA, the telomeric nucleosomal organization, the methylation status of H3K9 in telomeric chromatin, and the level of telomeric UUAGGG RNAs (TERRA, (15)) (fig. S5).

HDR threatens telomere integrity because unequal T-SCEs can change telomere lengths. T-SCEs are most frequent when either *TRF2* or *POT1a/b* are deleted from Ku-deficient cells (2,3), although low levels of T-SCEs have been reported for *POT1a* deficiency alone (16). To determine whether Rap1 was required for TRF2-mediated repression of T-SCEs, we introduced TRF2$^{\Delta Rap1}$ into SV40LT-immortalized *TRF2$^{F/-}$Ku70$^{-/-}$* MEFs which display frequent T-SCEs upon deletion of *TRF2* with Cre ((2) and Fig. 4). Whereas the telomeric exchanges were repressed by wild type TRF2, TRF2$^{\Delta Rap1}$ failed to block the telomeric HDR (Fig. 4A-C). The frequency of T-SCEs was the same whether the cells expressed TRF2$^{\Delta Rap1}$ or no TRF2. Furthermore, T-SCEs were induced by Cre-mediated deletion of Rap1 from *Rap1$^{F/F}$Ku70$^{-/-}$* cells (Fig. 4E). The T-SCEs occurred despite absence of TIFs in cells lacking both Ku70 and telomeric Rap1 (fig. S6).

These data indicate that Rap1 functions at mouse telomeres to repress HDR. Repression of recombination is important since unequal exchanges can curb the viability of daughter cells that inherit shortened telomeres and uncontrolled HDR promotes telomerase-independent telomere maintenance. The mechanism by which Rap1 affects HDR remains to be elucidated. Rap1 has the domain structure of an adaptor protein, combining three protein-protein interaction modules in one polypeptide. Its C-terminus serves to anchor the protein in shelterin and the N-terminal BRCT domain, when dimerized in the shelterin complex, could bind a phosphorylated target protein. The third potential protein interaction module in Rap1 is its Myb-type motif. Myb motifs often bind DNA, but the surface charge of the Myb domain in mammalian Rap1 makes it more suitable to bind to another protein (17). This view of Rap1 as an adaptor explains how the Rap1 orthologs can fulfill diverse functions in different organisms as alterations in one of the protein-interaction domains could endow Rap1 with a new interacting partner and thus instigate a new function.

These results further underscore the remarkable compartmentalization within shelterin (Fig. 4F). Shelterin contains at least four proteins dedicated to distinct functions. The replication of telomeric DNA is facilitated by TRF1 (18) and TPP1/POT1 are required for the repression of ATR signaling (1,19). TRF2 is the predominant repressor of ATM signaling and NHEJ and the current data show that these functions of TRF2 do not require Rap1. Finally, our results identify a fifth component of shelterin, Rap1, as an important repressor of HDR. Repression of HDR also requires TPP1/POT1 since removal of either Rap1 or POT1a/b result in telomere recombination. In a parallel pathway, Ku70/80 inhibits HDR but it has not been established whether this function is telomere specific (2). The extensive separation of function within shelterin permitted the observation that telomeres can undergo HDR without being detected by the ATM and ATR kinase pathways. When HDR takes place at telomeres lacking TRF2 or POT1a/b, DNA damage signaling results in the formation of TIFs. In the case of Rap1 removal, however, the telomeres lack detectable TIFs, yet are susceptible to HDR. Thus, consistent with the telomere recombination events in yeast lacking both Mec1 and Tel1 (20), the formation of DNA damage foci at telomeres is not a prerequisite for HDR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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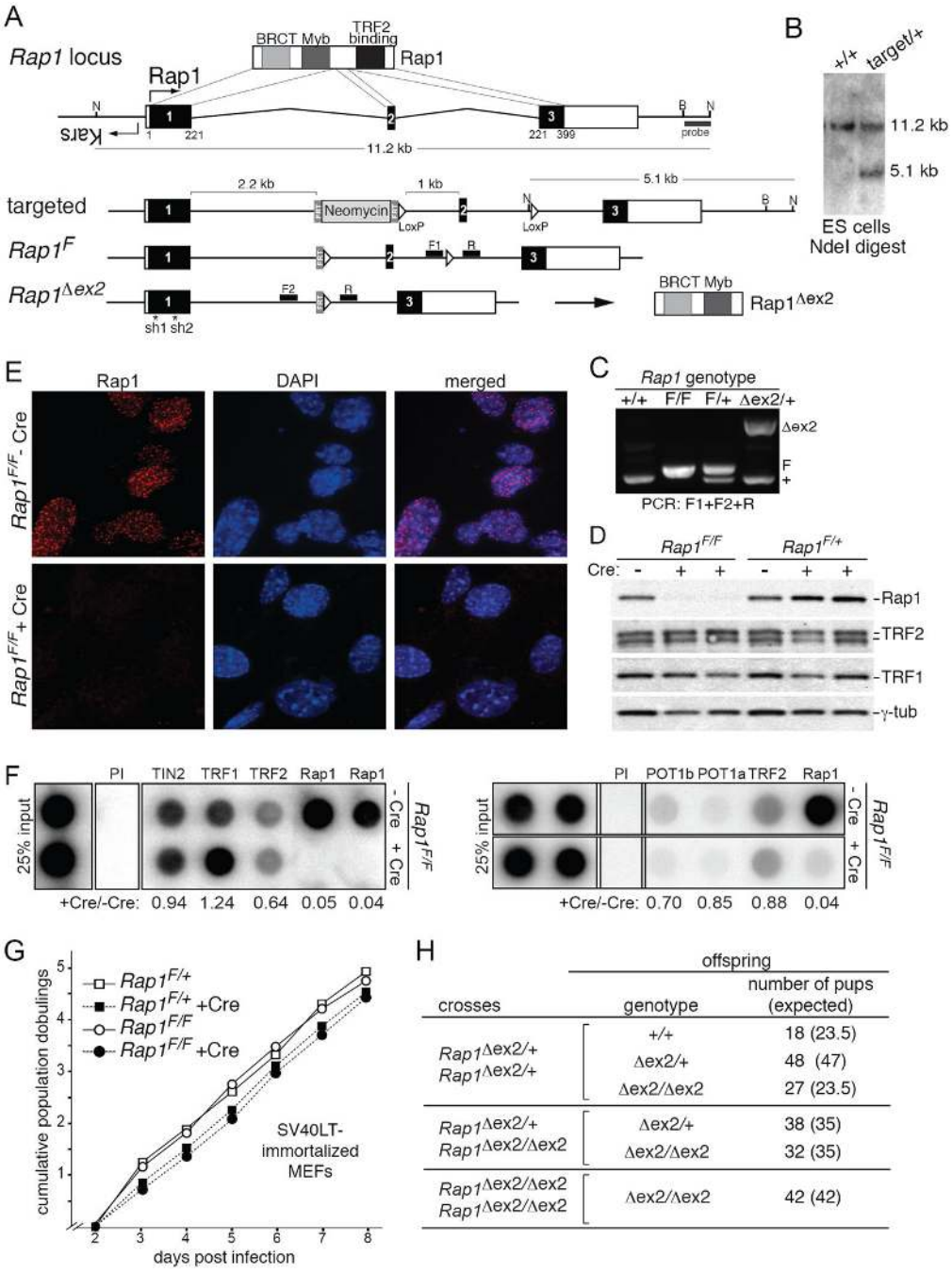


Fig 1. Deletion of Rap1 does not affect cell and organismal viability

(A) Schematic of Rap1 (top), and maps of the mouse *Rap1 (Terf2ip)* locus, the targeting construct, the floxed allele, and the Δex2 allele. N, *Nde*I; B, *BamH*I; F1, F2, and R, PCR primers. Rap1 shRNAs are indicated at the bottom. At right, the $Rap1^{\Delta ex2}$ encoded protein. (B) Genomic blot of *Nde*I-digested DNA from wild type and targeted ES cells. Probe as in (A). (C) PCR genotyping of tail DNAs. Primers in (A). (D) Immunoblots for Rap1 (Ab1252), TRF2 (Ab1254), and TRF1 (Ab1449) from $Rap1^{F/F}$ and $Rap1^{F/+}$ MEFs five days after Cre, First and second +Cre lanes, H&R-Cre and pWZL-Cre, respectively. (E) Loss of Rap1 IF signal from $Rap1^{F/F}$ MEFs at day 5 after Cre. Red, Rap1; green, telomeric FISH; blue, DNA (DAPI). (F) Telomeric ChIPs on $Rap1^{F/F}$ MEFs at day 5 after Cre. Numbers represent ratios of % telomeric

DNA in the ChIPs (corrected for pre-immune (PI) signal) on cells + and -Cre. (G) Proliferation of SV40LT-immortalized *Rap1*$^{F/F}$ and *Rap1*$^{F/+}$ MEFs infected with pWZL-Cre or vector. (H) Offspring from *Rap1*$^{\Delta ex2/+}$ and *Rap1* $^{\Delta ex2/\Delta ex2}$ intercrosses.

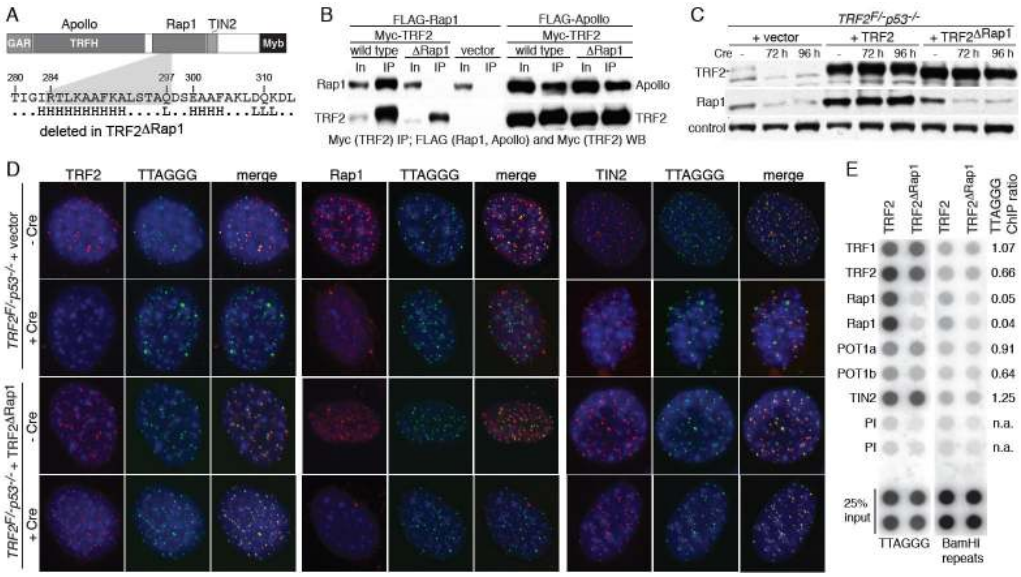
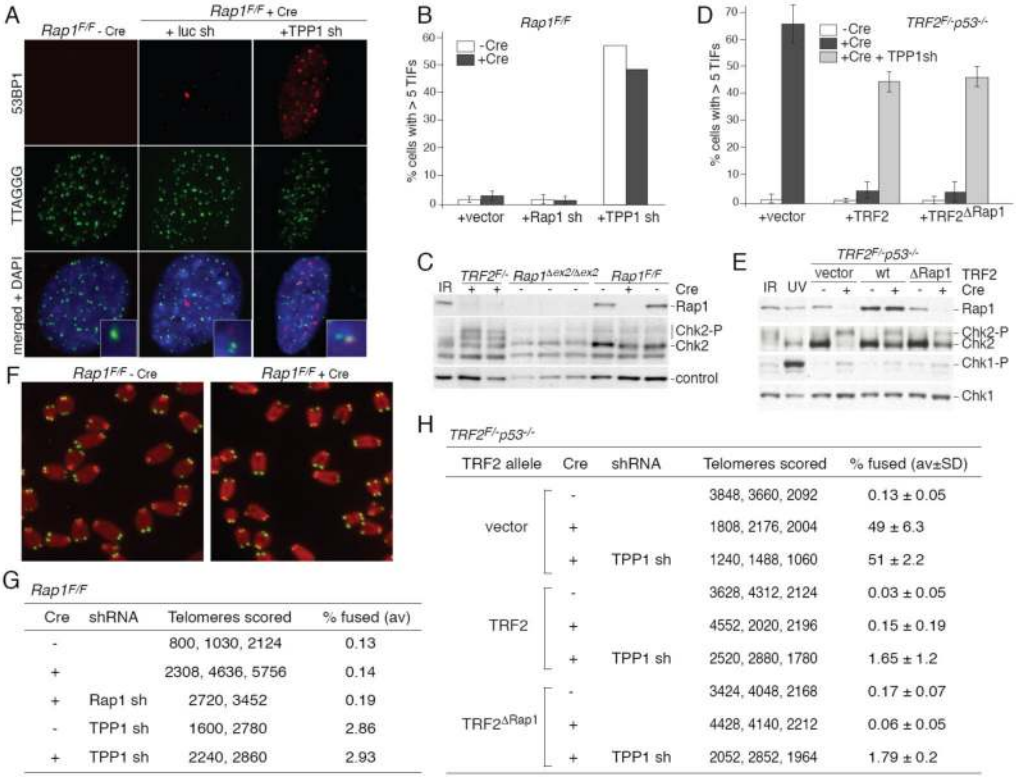


Fig. 2. A TRF2 mutant deficient for Rap1 binding

(A) Schematic of the TRF2$^{\Delta Rap1}$ mutant. H, predicted helix. (B) Co-IP of Myc-TRF2 or Myc-TRF2$^{\Delta Rap1}$ with FLAG-tagged Rap1 or Apollo from co-transfected 293T cells. In, 2.5% of input. (C) Immunoblots for TRF2 and Rap1 from *TRF2$^{F/-}$p53$^{-/-}$* MEFs expressing TRF2, TRF2$^{\Delta Rap1}$, or vector control, 72 and 96 hours after H&R-Cre. (D) IF-FISH to monitor TRF2, Rap1, and TIN2 at telomeres in *TRF2$^{F/-}$p53$^{-/-}$* MEFs expressing TRF2$^{\Delta Rap1}$ or vector control at day 4 after Cre. (E) Telomeric ChIP of *TRF2$^{F/-}$p53$^{-/-}$* MEFs expressing TRF2 or TRF2$^{\Delta Rap1}$ at day 7 post Cre. Duplicate dot blots were probed for telomeric DNA or the dispersed BamHI repeats. ChIP ratios represent the % telomeric DNA recovered in TRF2$^{\Delta Rap1}$ vs. TRF2 expressing cells calculated as in Fig. 1.



G *Rap1F/F*

Cre	shRNA	Telomeres scored	% fused (av)
-		800, 1030, 2124	0.13
+		2308, 4636, 5756	0.14
+	Rap1 sh	2720, 3452	0.19
-	TPP1 sh	1600, 2780	2.86
+	TPP1 sh	2240, 2860	2.93

H *TRF2F/-p53-/-*

TRF2 allele	Cre	shRNA	Telomeres scored	% fused (av±SD)
vector	-		3848, 3660, 2092	0.13 ± 0.05
	+		1808, 2176, 2004	49 ± 6.3
	+	TPP1 sh	1240, 1488, 1060	51 ± 2.2
TRF2	-		3628, 4312, 2124	0.03 ± 0.05
	+		4552, 2020, 2196	0.15 ± 0.19
	+	TPP1 sh	2520, 2880, 1780	1.65 ± 1.2
TRF2ΔRap1	-		3424, 4048, 2168	0.17 ± 0.07
	+		4428, 4140, 2212	0.06 ± 0.05
	+	TPP1 sh	2052, 2852, 1964	1.79 ± 0.2

Fig. 3. No DNA damage signal or NHEJ at the telomeres lacking Rap1

(A) TIF assay on *Rap1*$^{F/F}$ MEFs treated with Cre and the indicated shRNA. Red, IF for 53BP1; green, telomeric FISH; blue, DNA (DAPI). (B) TIF assay quantification. Averages of two independent experiments (n≥100 nuclei each) and SEMs. (C) Chk2-P in Rap1-deficient MEFs. TRF2 null cells and IR-treated cells (1 hr post 2 Gy,) serve as positive controls. (D) Quantification of TIF assays on *TRF2*$^{F/-}$*p53*$^{-/-}$ cells expressing TRF2, TRF2$^{\Delta Rap1}$ or vector control at day 4 post Cre. Mean of three independent experiments (n≥100 nuclei each) and SDs. (E) Chk1 and Chk2 phosphorylation in *TRF2*$^{F/-}$*p53*$^{-/-}$ MEFs expressing TRF2, TRF2$^{\Delta Rap1}$ or vector control. UV (1 hr post 25 J/m2) and IR (1 hr post 2 Gy) treated cells serve as positive controls. (F) Metaphase chromosomes from *Rap1*$^{F/F}$ cells 5 days post Cre. Red, DNA (DAPI); green, telomeric FISH. (G) Quantification of telomere fusions, detected as in (F) in Rap1$^{F/F}$ MEFs with the indicated Cre and shRNA treatments. Average (av) % of telomeres fused is given. (H) Quantification of telomere fusions in *TRF2*$^{F/-}$*p53*$^{-/-}$ MEFs (+ or − Cre, day 4) complemented with TRF2 or TRF2$^{\Delta Rap1}$ or vector control and treated with TPP1 shRNA as indicated.

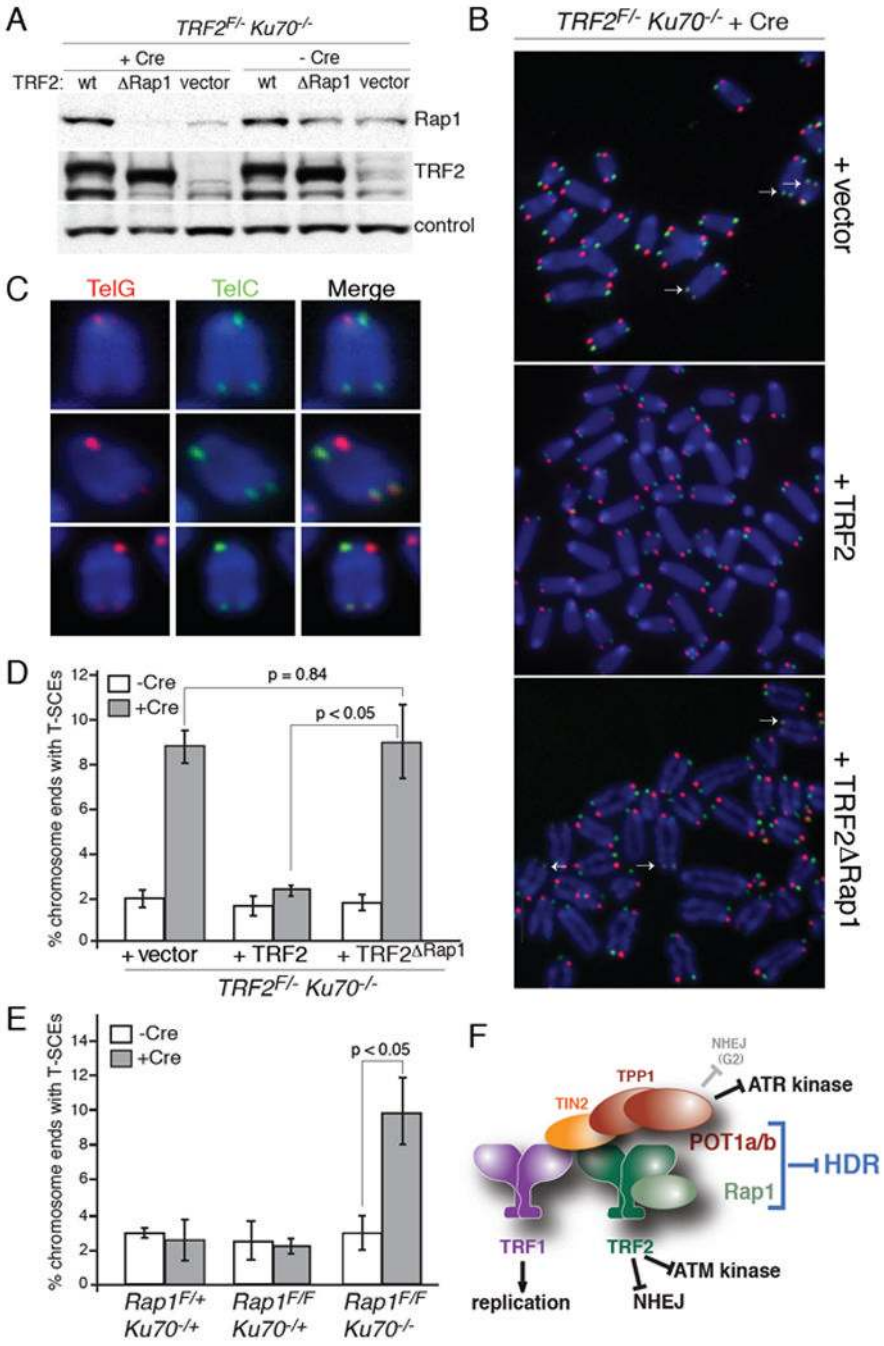


Fig. 4. Rap1 is a repressor of telomere recombination

(A) Rap1 and TRF2 from *TRF2*$^{F/-}$*Ku70*$^{-/-}$ MEFs expressing TRF2, TRF2$^{\Delta Rap1}$, or vector control analyzed 4 days after Cre. (B) CO-FISH analysis on cells as in (A). Arrows: T-SCEs. (C) Enlarged T-SCE events in Cre-treated TRF2$^{F/-}$Ku70$^{-/-}$ MEFs expressing TRF2$^{\Delta Rap1}$. (D) Quantification of T-SCEs as assessed in (B). Bars represent averages from three independent experiments (n>1100 chromosome ends each) and SDs. P values based on Student's two-tailed t-test. (E) Quantification of T-SCEs as assessed in (B) in cells of the indicated Rap1 and Ku70 status. Method as in (D). Errors bars: SEMs except for SDs for *Rap1*$^{F/F}$*Ku70*$^{-/-}$. (F) The functions of shelterin components. See text for details.