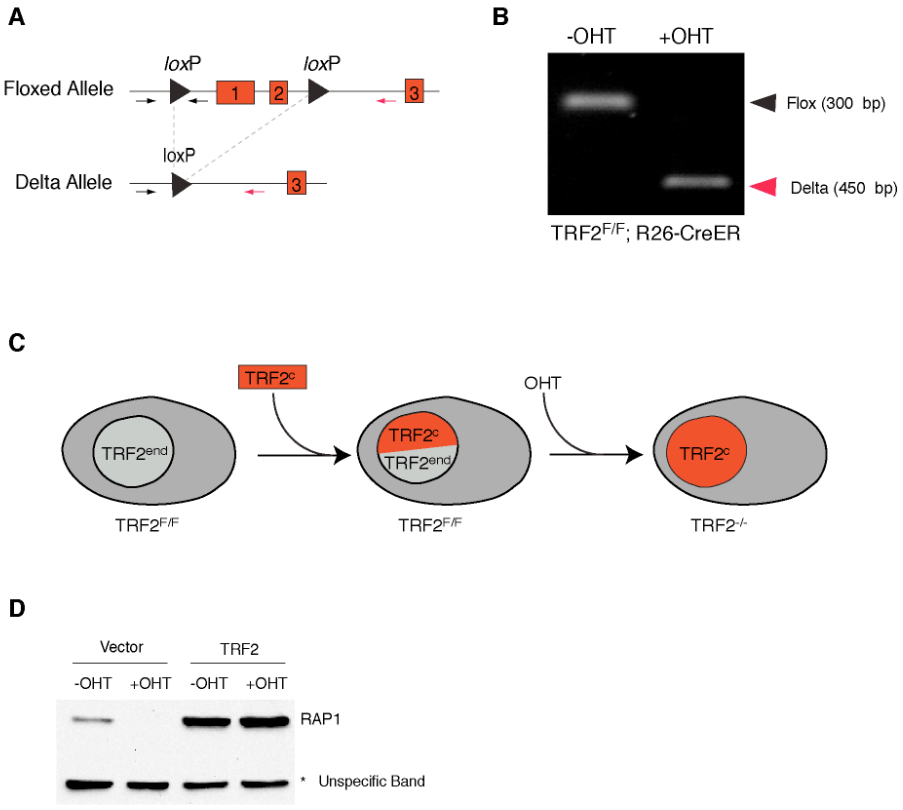
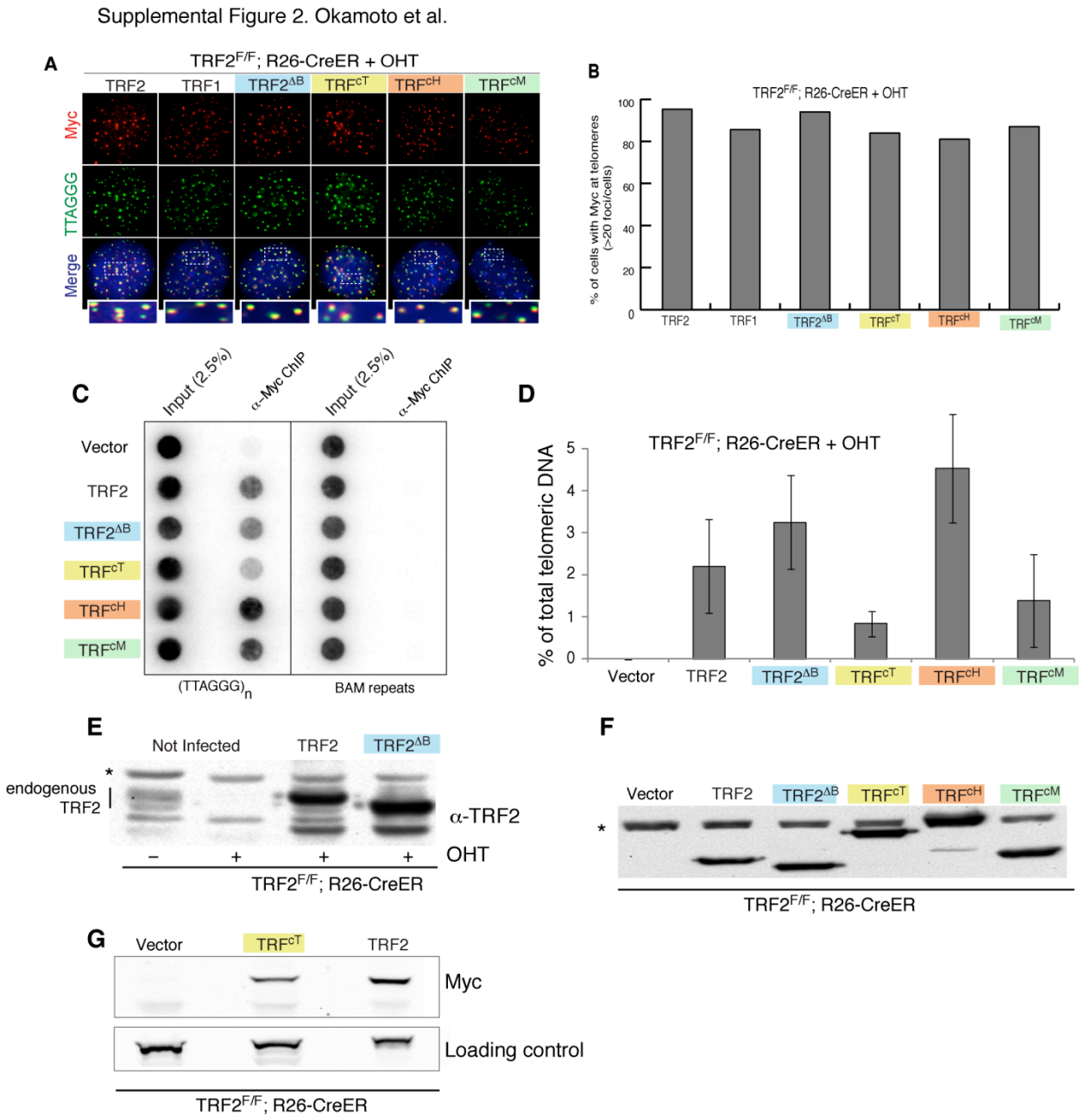
Supplemental Figure 1. Okamoto et al.



Supplemental Figure 1 Experimental system.

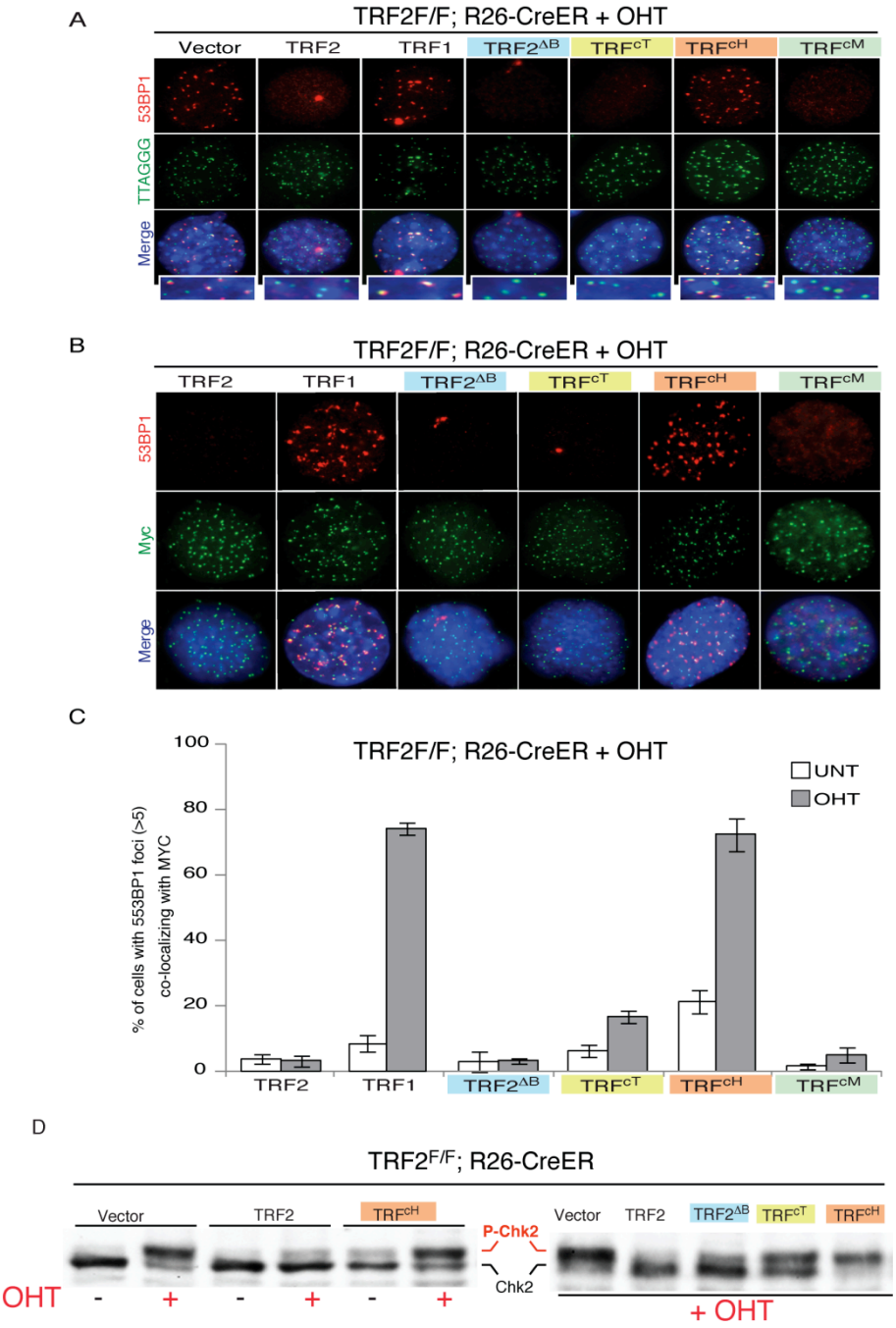
A Schematic representation of the TRF2 floxed allele and the deleted allele resulting from CRE-mediated recombination. **B** Tamoxifen induced CRE recombination in $TRF2^{F/F}$; R26-CreER cells was detected by PCR. **C** Schematic representation of the experimental approach used to determine whether TRF chimeras (TRF^{c}) can complement for the loss of endogenous TRF2 ($TRF2^{end}$). $TRF2^{F/F}$; R26-CreER cells were infected with TRF^{c} constructs, treated with OHT to induce CRE mediated deletion of TRF2 and analyzed 96 hours later. **D** Functional validation of the approach used. Cells were infected with an empty vector (vector) or TRF2 and treated with OHT. As expected depletion of endogenous TRF2 results in RAP1 destabilization in control cells. Ectopic expression of TRF2 complements loss of endogenous TRF2.



Supplemental Figure 2. TRF chimeras localize to telomeres.

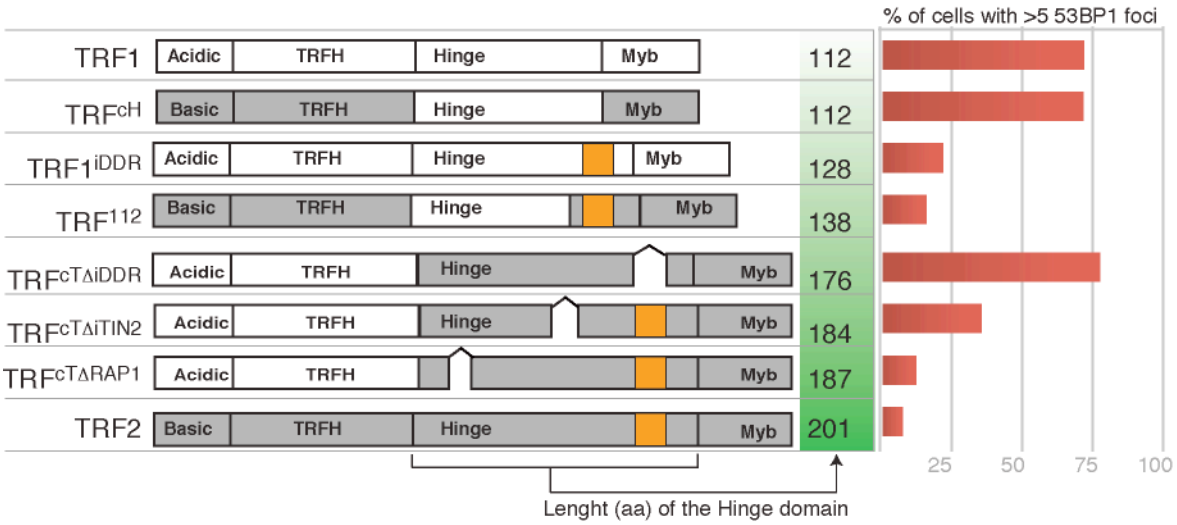
A Cells infected with the indicated constructs were stained with myc (to detect ectopically expressed proteins) and for telomere DNA (TTAGGG). **B** Quantification of data shown A. Cells with more than 20 Myc foci at telomeres were scored as positive. **C** Anti-Myc chromatin IP (ChIP) was stained for telomere DNA (TTAGGG) or control BAM repeats. **D** Quantification of the experiment described in C. **E** Whole cell lysate of cells treated as indicated was assayed for TRF2 expression. **F** Whole cell lysate of cells treated as described were assayed for expression of chimera alleles (Myc Staining). **G** Whole cell lysate of cells treated as described were assayed for expression of the indicated construct (Myc Staining) and loading control (Vinculin).

Supplemental Figure 3. Okamoto et al.



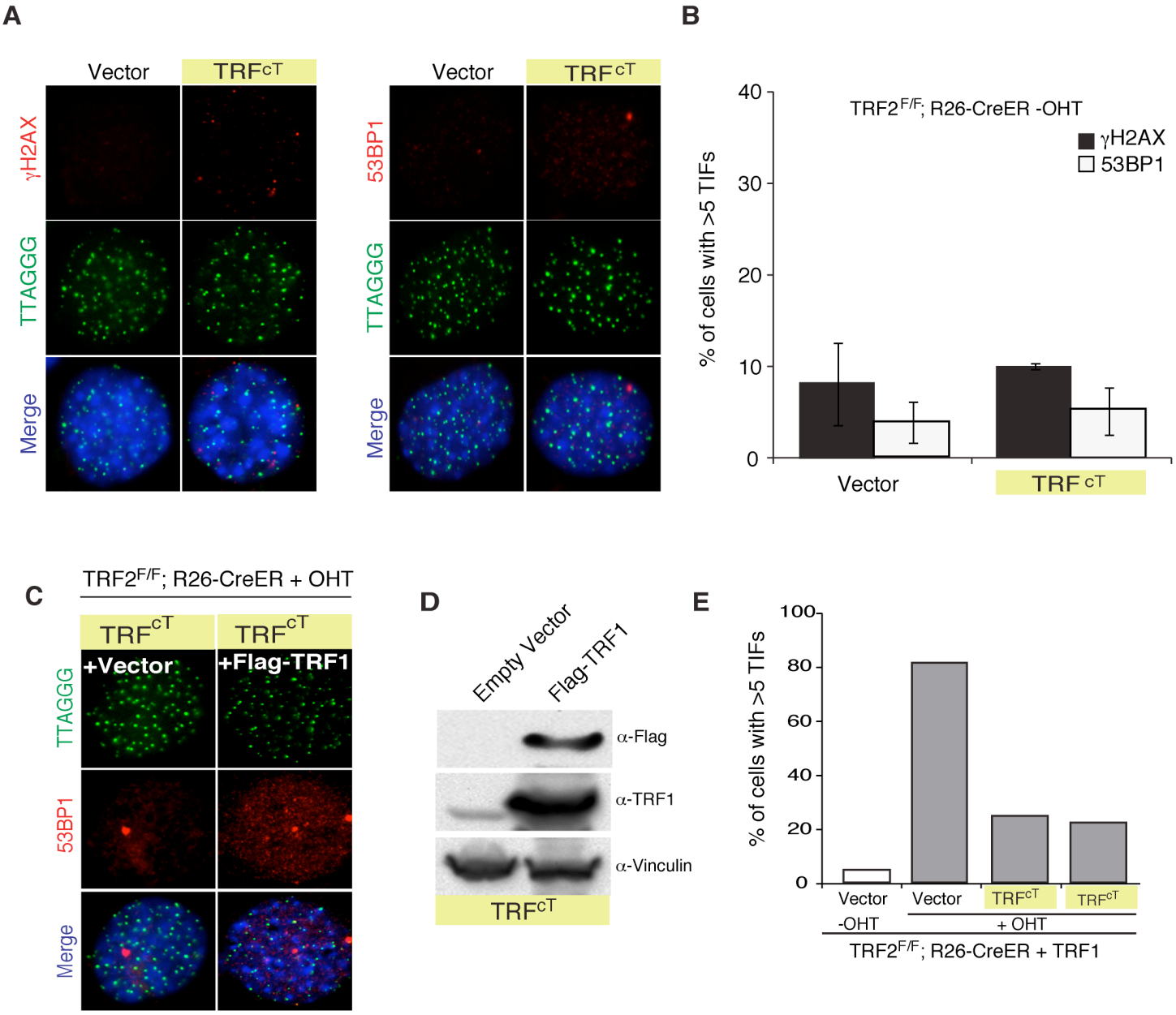
Supplemental Figure 3. A critical role for TRF2 Hinge domain to prevent 53BP1 localization at telomeres. A Cells were infected with the indicated constructs were harvested 4 days post OHT treatment and stained for 53BP1 (red) and telomere DNA (green). **B** Cells treated as in A were stained for 53BP1 (red) and Myc (green). Note colocalization of 53BP1 to TRF^{cH}. **C** Quantification of the data shown in panel A. TIFs were analyzed in cells either treated with OHT (gray bars) or untreated (white bars). **D** CHK2 activation (p-CHK2) was assessed by western blot using whole cell lysates of cells with the indicated genotype.

Supplemental Figure 4. Okamoto et al.



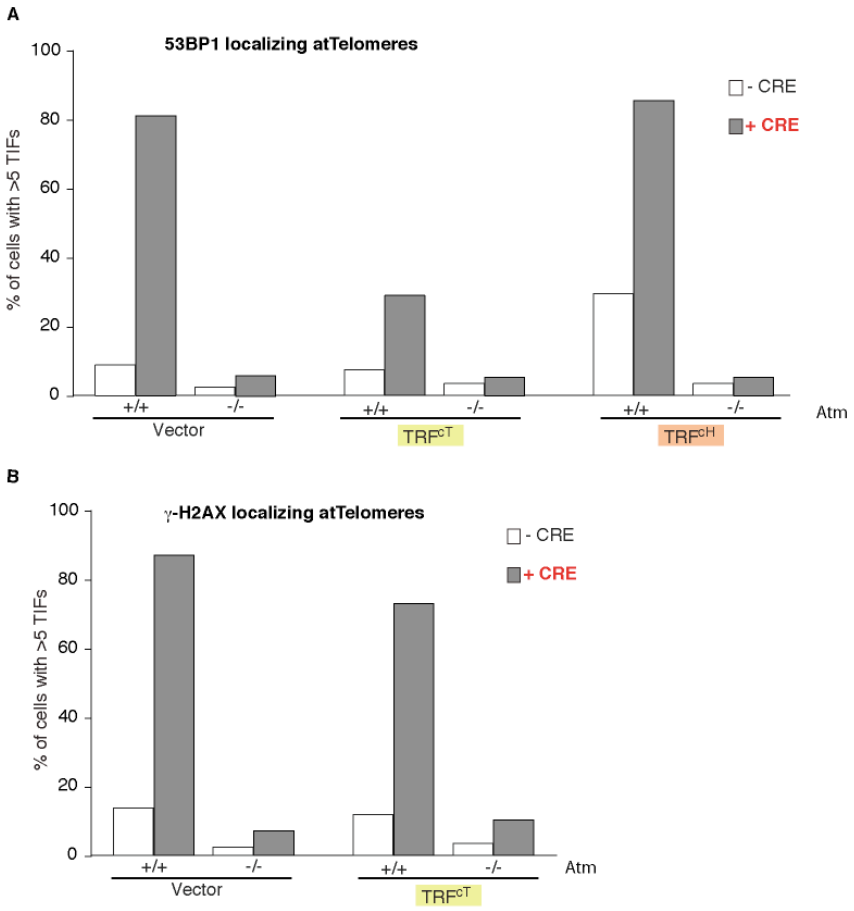
Supplemental Figure 4. Size of the HINGE domain does not matter for TRF2-mediated end protection. Alleles used in this study are sorted based on the size of their HINGE domain. Graph indicates the % of cells expressing the indicated allele (in the absence of endogenous TRF2) with 53BP1 TIFs.

Supplemental Figure 5. Okamoto et al.



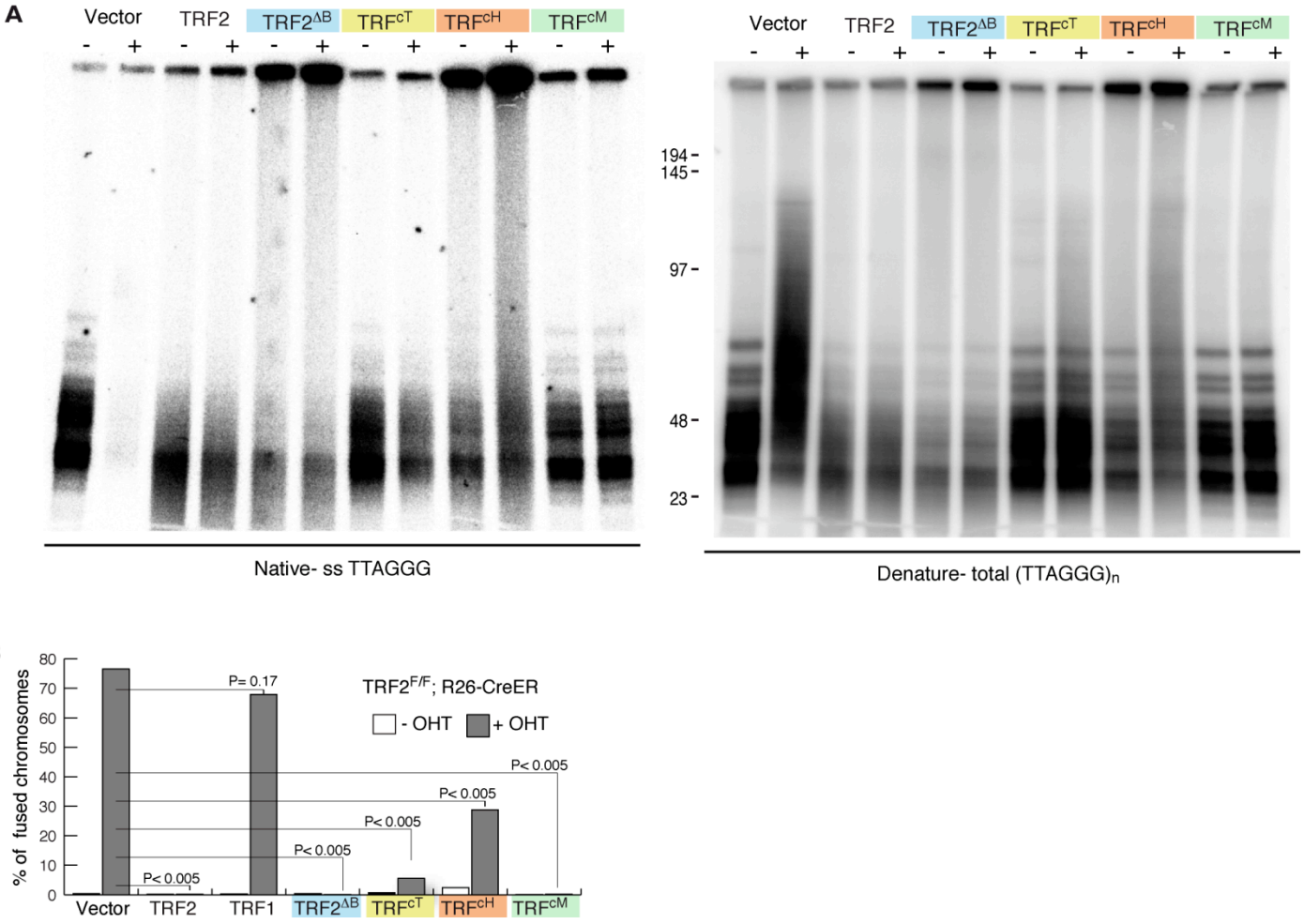
Supplemental Figure 5. The TRF^{cT} is not a dominant negative allele. A TRF2 proficient cells infected with the indicated constructs were stained for telomeric DNA (TTAGGG, green), γH2AX (green) or 53BP1 (green). **B** Quantification of data shown in A. Note that expression of the TRF^{CT} allele does not induce DNA damage foci at telomeres. **C** TRF2 deficient cells (+OHT) expressing the TRF^{cT} allele were transduced with wild type FLAG tagged TRF1 or a vector control and stained for 53BP1 (red) and telomeric DNA (TTAGGG, green). **D** Whole cell lysate of cells treated as described in C were assayed for expression of the exogenous TRF1 (FLAG), endogenous TRF1 and a loading control (Vinculin). **E** Quantification of data shown in C. Note that TRF1 overexpression does not rescue TIF formation in cells expressing the TRF^{cT} allele.

Supplemental Figure 6. Okamoto et al.

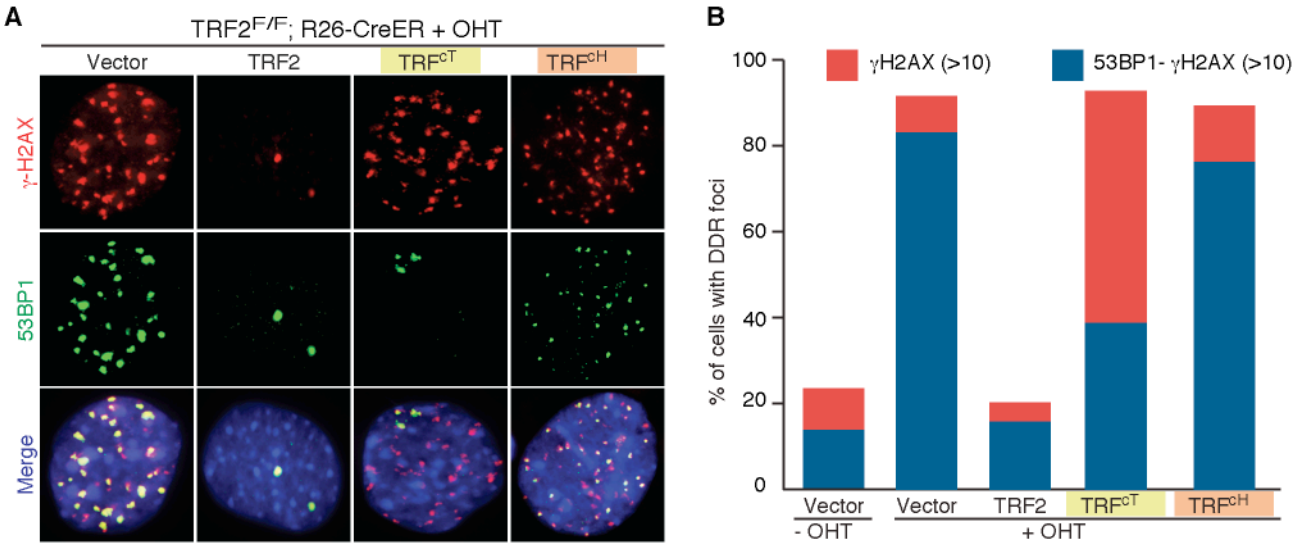


Supplemental Figure 6. ATM-dependent DNA damage at telomeres A TRF2$^{F/F}$ cells either ATM proficient (+/+) or ATM deficient (-/-) were infected with the indicated constructs. 4 Days post CRE induction (+Cre) 53BP1 localization at telomeres was assessed by FISH-IF analysis. Cells with more than 5 53BP1 foci at telomere were scored as positive. As expected TRF2 depletion induces an ATM-dependent DDR. Note that in a similar manner the DNA damage detected in cells expressing the TRF^{cH} allele is ATM-dependent. **B** Cells of the same genotype and treatment as in A were analyzed for the localization of γH2AX at telomeres. Note that the DNA damage signal detected in cells expressing the TRF^{cT} allele is ATM-dependent.

Supplemental Figure 7. Okamoto et al.



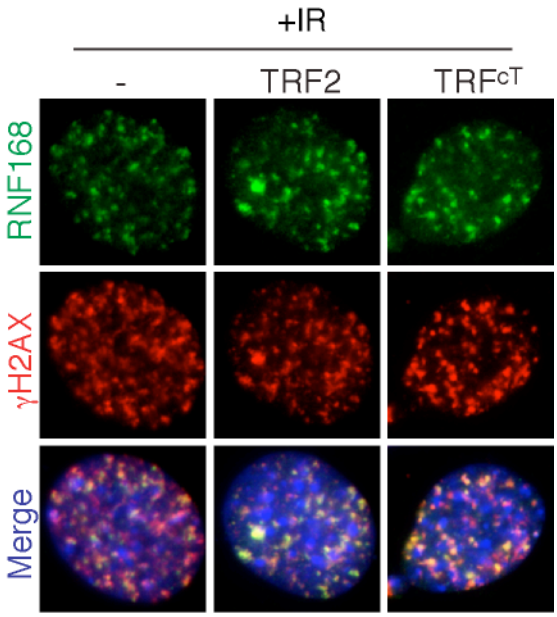
Supplemental Figure 7. Analysis of chromosome fusions in cells expressing TRF chimeras. **A** Genomic DNA from TRF2$^{F/F}$ MEFs infected with the indicated constructs and either treated with OHT (+) or untreated (-) was analyzed by in-gel telomere blotting. The left image shows hybridization signal using the TelC probe ([CCCTAA]$_4$) under native conditions detecting the telomeric 3' overhang. The right image shows the total telomeric hybridization signal obtained with the same probe after in-gel denaturation of the DNA. Note the frequent appearance of slow migrating telomeric fragments (indicative of end-to-end fusions) in cells infected with vector controls (second lane on the left). **B** MEFs were retroviral infected with the indicated constructs, treated with 4-hydroxytamoxifen (+OHT) and harvested 4 days later. Metaphase spreads were stained for telomere DNA (see Figure 1 d for representative images) and analyzed for chromosome with telomere fusions. The graph indicates the % of chromosome with telomere fusions observed in MEFs expressing the indicated alleles. Supplemental Table 1 lists the number of chromosome analyzed/ condition.



Supplemental Figure 8. Uncoupling of γH2AX localization from 53BP1 recruitment.

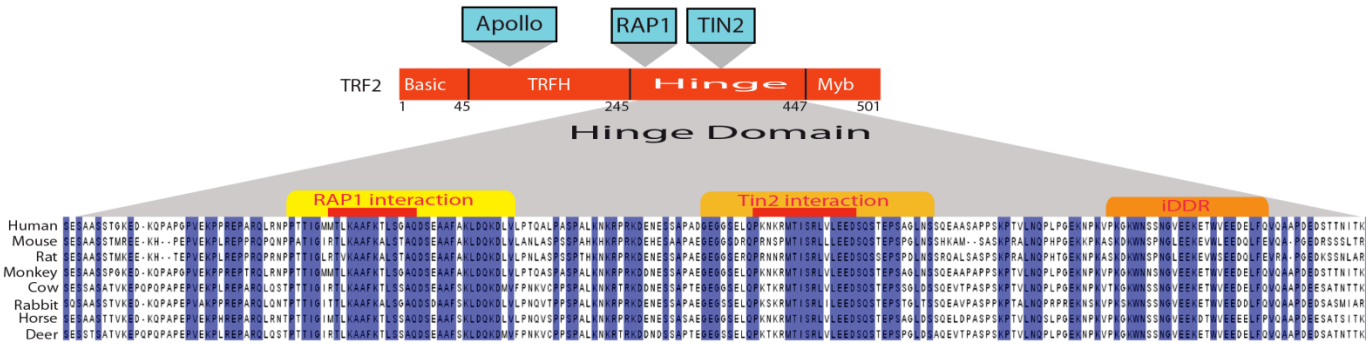
A MEFs of the indicated genotype and infected with the indicated construct were stained for γH2AX (red), 53BP1 (green) and DAPI (blue). Note the colocalization of 53BP1 with γH2AX in vectors control cells as well as in cells expressing the TRF^{cH} allele. In contrast, cells expressing the TRF^{cT} allele show γH2AX without 53BP1 localization. **B** Quantification of data shown in panel A. The Blue line represents the % of cells in which 53BP1 foci (>10) colocalized with γH2AX, the red bar the % of cells in which γH2AX foci were detected in the absence of 53BP1.

Supplemental Figure 9. Okamoto et al.



Supplemental Figure 9. Efficient RNF168 IRIFs in cells expressing the TRF^{cT} allele. TRF2$^{F/F}$ MEFs infected with the indicated constructs were irradiated **(2Gy)** and harvested 1 hour later. Cells were stained with γH2AX (red) and RNF168 (green).

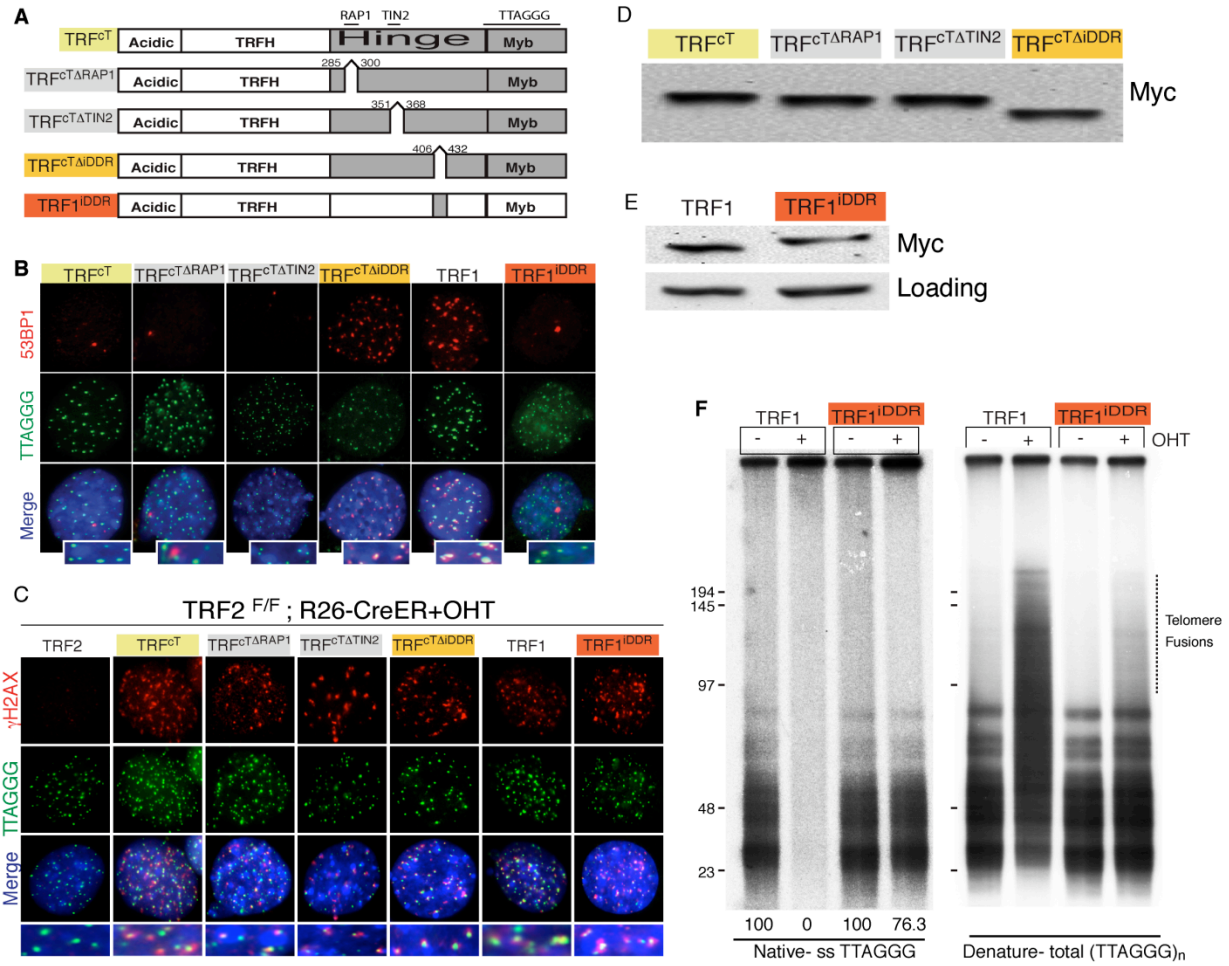
Supplemental Figure 10. Okamoto et al.



Supplemental Figure 10. TRF2 Hinge domain.

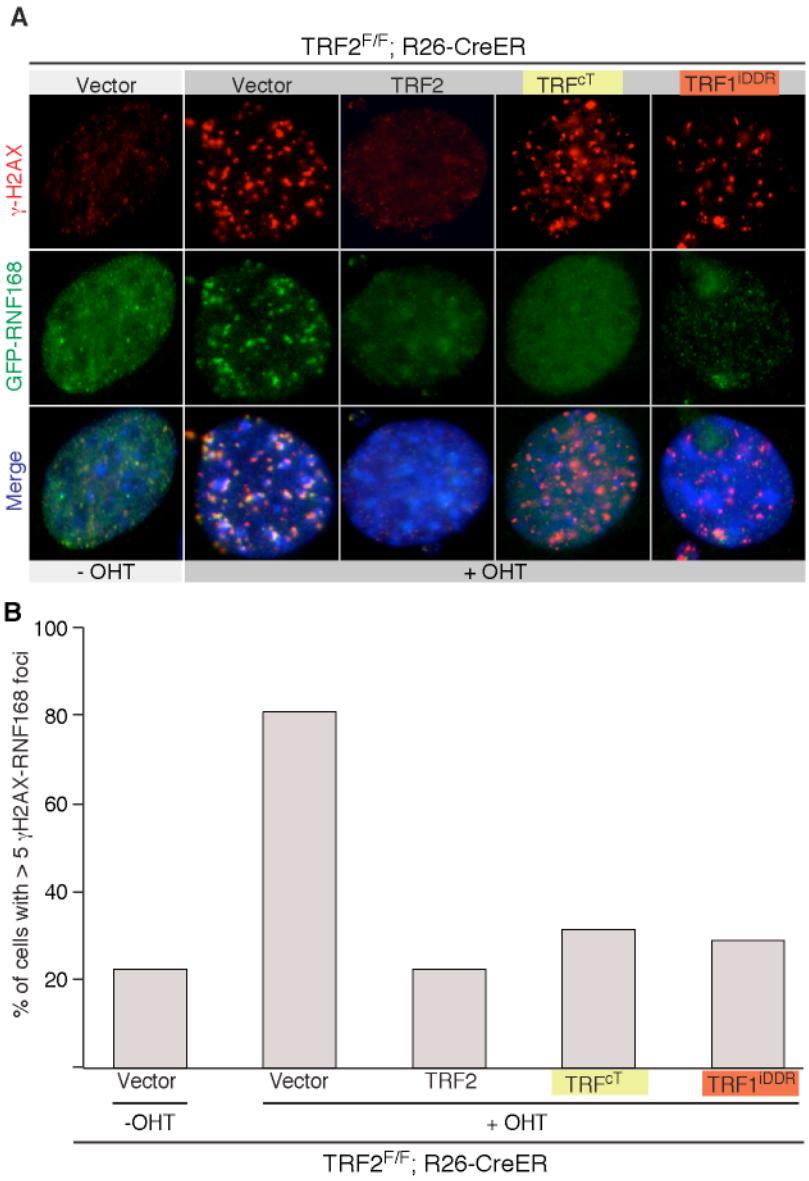
Alignment of the Hinge domain of mammalian TRF2 molecules.

Supplemental Figure 11. Okamoto et al



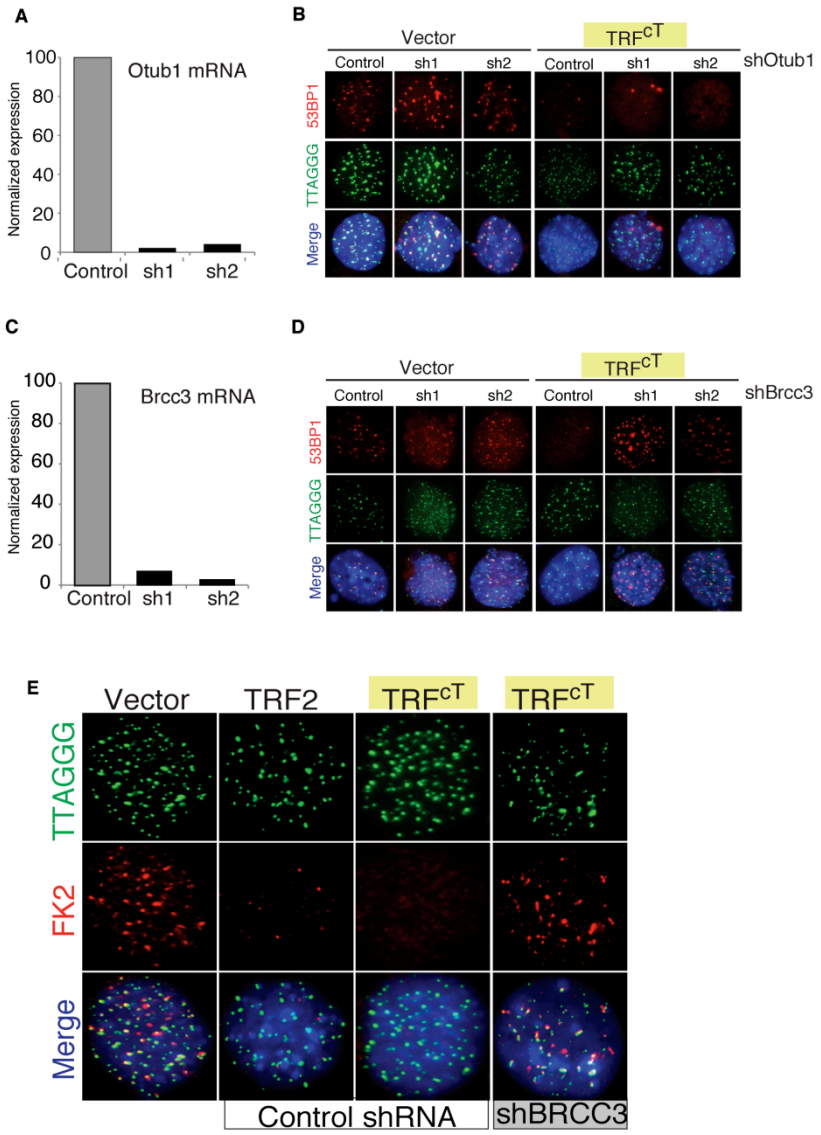
Supplemental Figure 11. The C-terminal portion of TRF2's Hinge domain is necessary and sufficient to prevent 53BP1 localization at telomeres. **A** Schematic representation of chimeric alleles used to probe the role of TRF2 Hinge domain in end protection. **B** MEFs were retroviral infected with the indicated constructs, treated with 4-hydroxytamoxifen (+OHT) and harvested 4 days later. Cells were stained for telomere DNA (green), 53BP1 IF (red) and DAPI (blue). **C** MEFs treated as described in B were stained for telomeric DNA (TTAGGG, green), γH2AX (red) and DAPI (blue). **D** Whole cell lysate of cells treated as described in A was assayed for expression of the indicated alleles (Myc staining). **E** Whole cell lysate of cells treated as described were assayed for expression of the indicated construct (Myc staining) and loading control (Vinculin). **F** Genomic DNA from TRF2$^{F/F}$ MEFs infected with the indicated constructs and either treated with OHT (+) or untreated (-) was analyzed by in-gel telomere blotting. The left image shows hybridization signal using the TelC probe ([CCCTAA]$_4$) under native conditions detecting the telomeric 3' overhang. The right image shows the total telomeric hybridization signal obtained with the same probe after in-gel denaturation of the DNA. Note the frequent appearance of slow migrating telomeric fragments (indicative of end-to-end fusions) in cells infected with TRF1 (second lane on the left) compared to cells infected with the TRF1^{iDDR} allele. TRF2$^{-/-}$ MEFs were complemented with the indicated alleles and stained for telomeric DNA (TTAGGG, green), γH2AX (red) and DAPI (blue).

Supplemental Figure 12. Okamoto et al.



Supplemental Figure 12. The iDDR region of TRF2 mediates the inhibition of RNF168 recruitment at telomeres. **A** TRF2$^{F/F}$ MEFs infected with the indicated constructs were infected with a GFP-RNF168 construct and treated with OHT to induce Cre- mediated TRF2 depletion. Cells were stained for γH2AX (red) GFP (green) and DAPI (blue). **B** Quantification of the data shown in A.

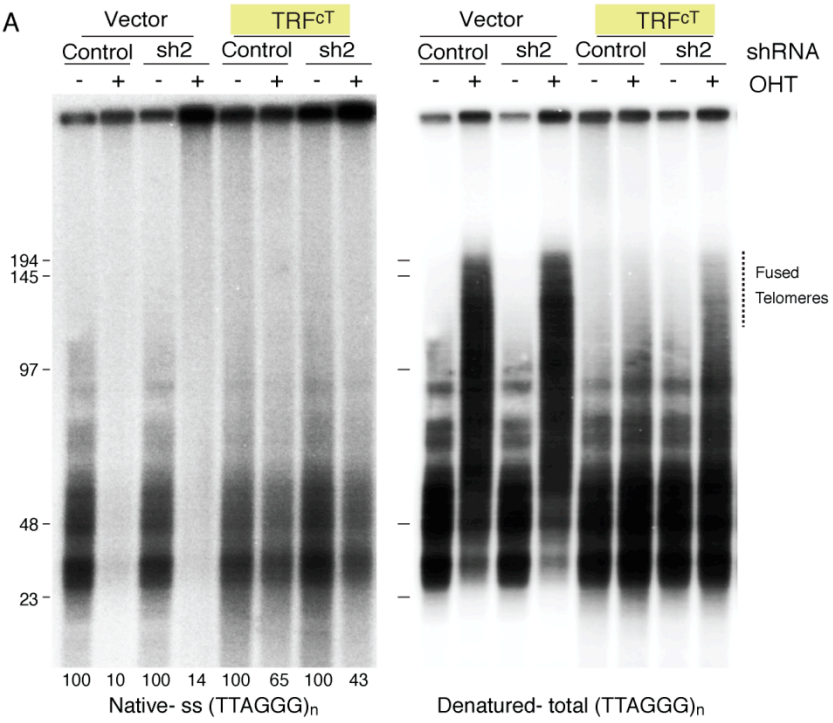
Supplemental Figure 13. Okamoto et al.

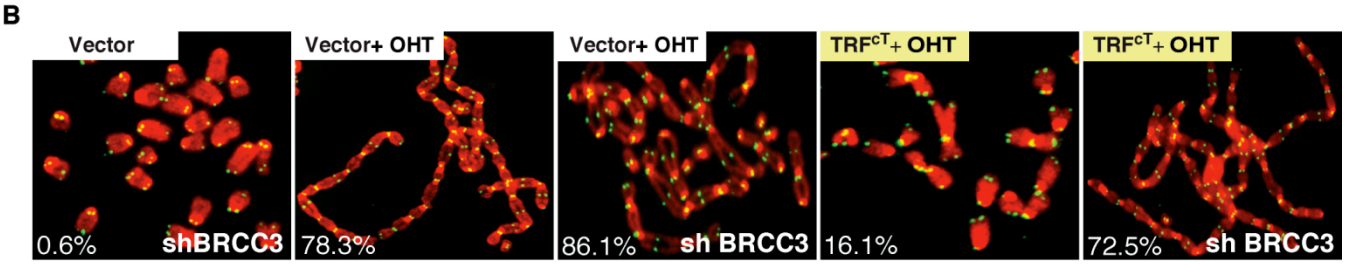


Supplemental Figure 13. Inhibition of the DUB enzymes OTUB1 and BRCC3.

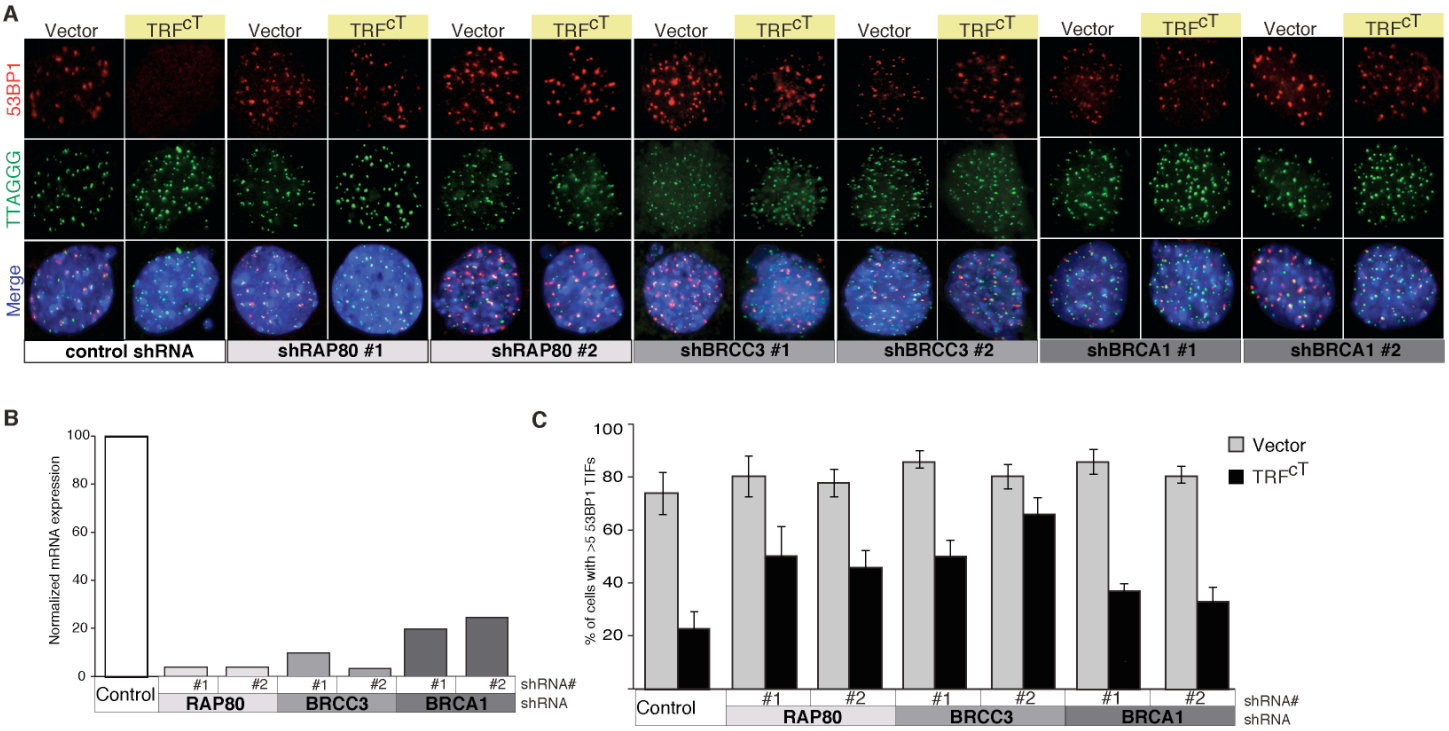
A MEFs were infected with a control vector or with two independent shRNAs specific for Otub1. Relative expression of Otub1 was assessed by qPCR and was normalized to the expression in the vector control (set to 100%). **B** TRF2$^{F/F}$ MEFs infected with the indicated constructs were treated with OHT to induce Cre-mediated TRF2 depletion. Cells were stained for telomeric DNA (TTAGGG, green), 53BP1 (red) and DAPI (blue). **C** MEFs were infected with a control vector or with two independent shRNA specific for BRCC3. Relative expression of BRCC3 was assessed by qPCR and was normalized to the expression in the vector control (set to 100%). **D** TRF2$^{F/F}$ MEFs infected with the indicated constructs were treated with OHT to induce Cre mediated TRF2 depletion. Cells were stained for telomeric DNA (TTAGGG, green), 53BP1 (red) and DAPI (blue). **C** MEFs treated as described in A were stained for polyubiquitin chains (FK, red) and telomeres (TTAGGG, green).

Supplemental Figure 14. Okamoto et al.





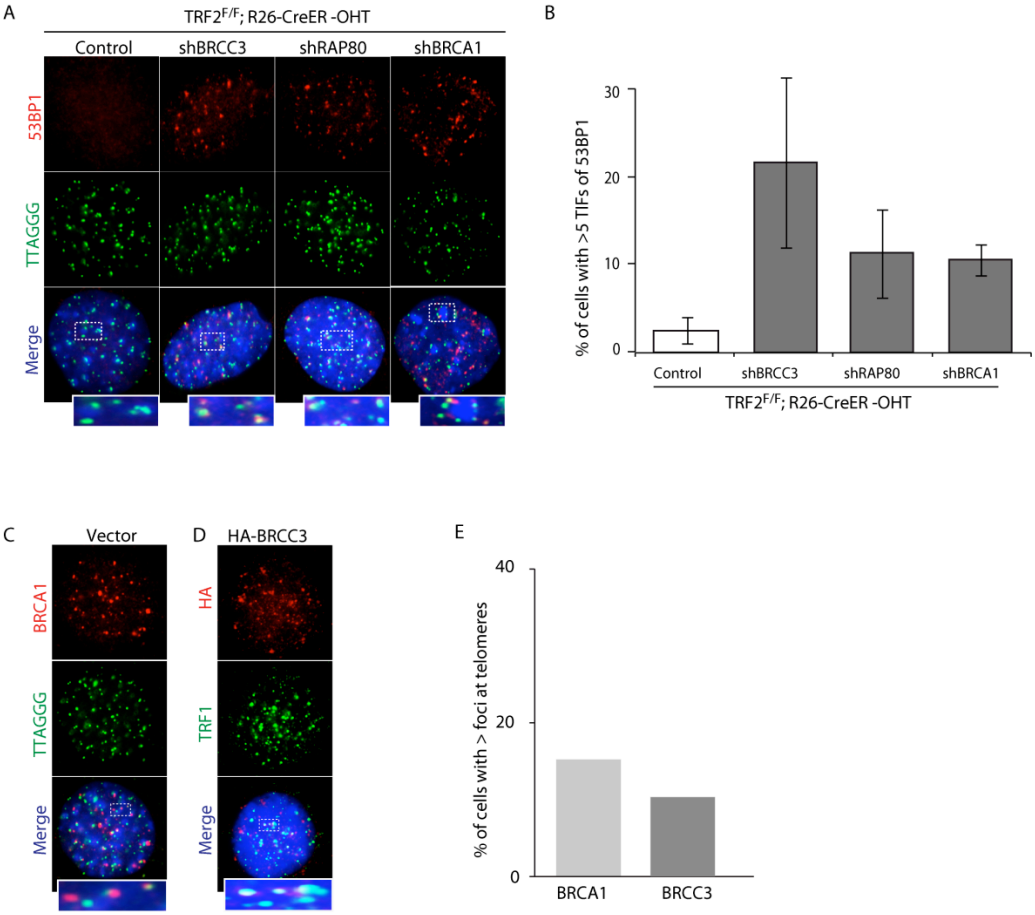
Supplemental Figure 14. BRCC3 inhibition results in telomere fusions in cells expressing the TRF2^{cT} allele. A Genomic DNA from TRF2$^{F/F}$ MEFs infected with the indicated constructs and either treated with OHT (+) or untreated (-) was analyzed by in-gel telomere blotting. The left image shows hybridization signal using the TelC probe ([CCCTAA]$_4$) under native conditions detecting the telomeric 3' overhang. The right image shows the total telomeric hybridization signal obtained with the same probe after in-gel denaturation of the DNA. Note the frequent appearance of slow migrating telomeric fragments (indicative of end-to-end fusions). **B** Metaphase spreads of OHT-treated TRF2$^{F/F}$ MEFs infected with either a vector control (Vector) or TRF^{cT}, were infected with either a control shRNA construct (control) or BRCC3 shRNA#2. Metaphase spreads were stained for telomere DNA (green) and DAPI (red). Percentages of chromosomes with fusions are indicated.



Supplemental Figure 15. The BRCA1 complex mediates end-protection.

A TRF2$^{F/F}$ MEFs were infected with a control vector or with two independent shRNAs specific for RAP80, BRCC3 or BRCA1. Cells were treated with OHT to induce Cre-mediated TRF2 depletion. Cells were stained for telomeric DNA (TTAGGG, green), 53BP1 (red) and DAPI (blue). **B** MEFs were infected with the indicated shRNA constructs. Relative expression of Rap80, BRCC3 or BRCA1 was assessed by qPCR and was normalized to the expression in the vector control (set to 100%). **C** Quantification of the data shown in A.

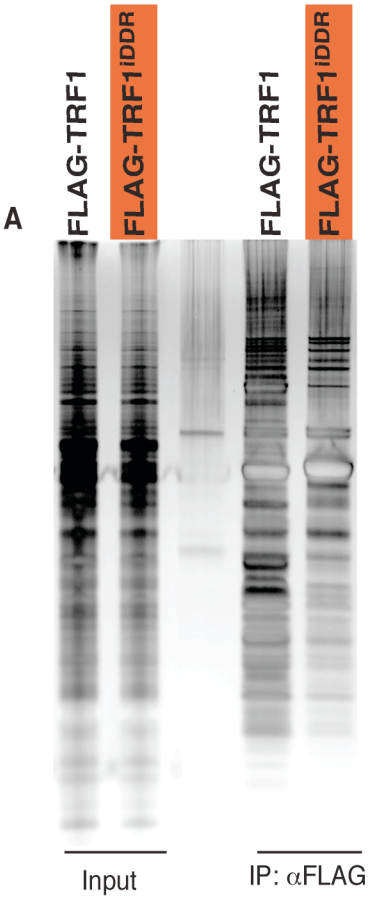
Supplemental Figure 16. Okamoto et al.



Supplemental Figure 16. The BRCA1 complex mediates end-protection.

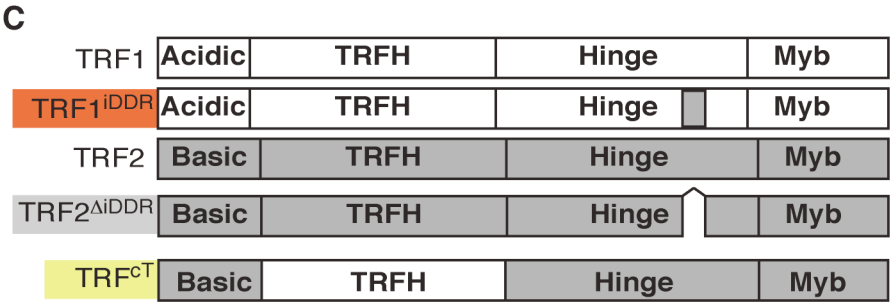
A TRF2$^{F/F}$ MEFs were infected with a control vector or with two independent shRNAs specific for RAP80, BRCC3 or BRCA1. Cells were stained for telomeric DNA (TTAGGG, green), 53BP1 (red) and DAPI (blue). **B** Quantification of the data shown in A. **C** Untreated MEFs were stained for telomeric DNA (TTAGGG, green) BRCA1 (red) and DAPI (blue). **D** MEFs infected with HA-BRCC3 were stained for HA (red), TRF1 (green) and DAPI (blue). **E** Quantification of the data shown in C and D.

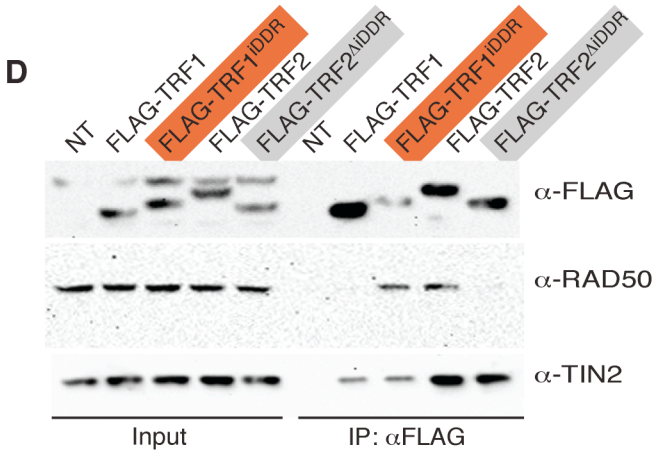
Supplemental Figure 17. Okamoto et al.

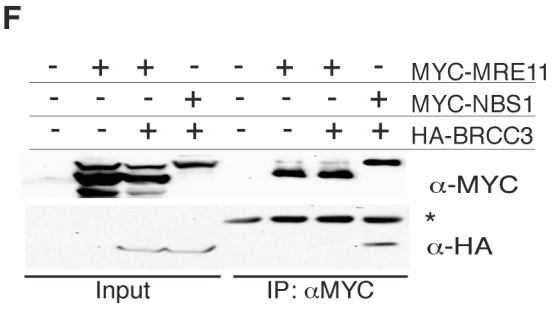


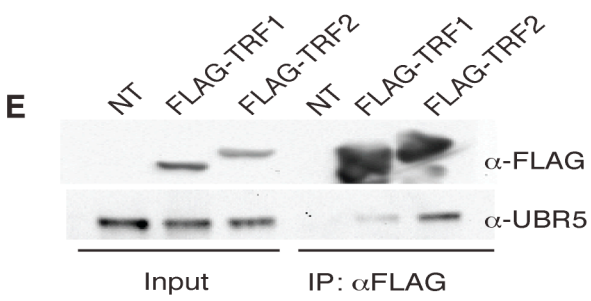
B

Swissprot	Protein	TRF1 IP	TRF1^{iDDR} IP	Delta spectra
A6H8Y5	NBN	0	3.7	1
Q92878	RAD50	0.3	10.3	1
P49959	MRE11	0.3	6.0	0.9
O95071	UBR5	1.3	10.7	0.9
P54274	TRF1	79.3	117	0.6
Q9BSI4	TIN2	6.7	8.3	0.6





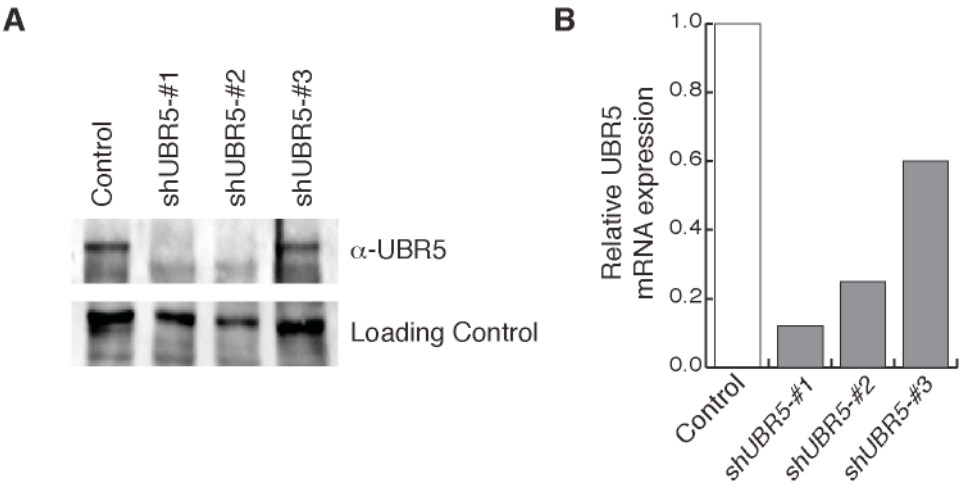




Supplemental Figure. 17 Identification of proteins associated with TRF2 iDDR region.

A HEK293 cells were transfected with FLAG–tagged TRF1 or FLAG–tagged TRF1^{iDDR} allele. TRF1 was immunopurified with anti-FLAG agarose resin, protein eluates were separated by Nu-PAGE and proteins were stained with silver stain. **B** Protein eluates isolated as described in A were analyzed by nanoflow liquid chromatography mass spectrometry. Numbers of spectra identified were normalized by protein abundance (*http://pax-db.org/#!home*). Relative enrichment of proteins in the TRF1^{iDDR} relative to the TRF1 control sample was evaluated based on the "Delta spectra value" [(spectra TRF1^{iDDR}- spectra TRF1)/ spectra TRF1^{iDDR}]. Isolated proteins resulting from three independent experiments were ranked based on the combined Delta spectra values. **C** Schematic representation of the constructs used for IP shown in D, E and F. **D** and **E** HEK293 cells were transfected with cDNAs encoding the indicated FLAG-tagged proteins. FLAG-tagged immunoprecipitates (IP) from cell extracts with anti-FLAG resin were immunoblotted as indicated. NT represents the non-transfected controls. **F** HEK293 cells were co-transfected with cDNAs encoding the indicated FLAG and MYC-tagged proteins. MYC-tagged immunoprecipitates (IP) from cell extracts with anti-FLAG resin were immunoblotted as indicated.

Supplemental Figure 18. Okamoto et al.



Supplemental Figure. 18 shRNA-mediated knockdown of UBR5.

A MEFs were infected with control vector or three independent shRNAs for UBR5. Cells were harvested three days post infection. Whole cell lysates were immunobloted with anti-UBR5 antibody and a loading control. **B** Cells were treated as described in A. Relative expression of Ubr5 was assessed by qPCR and was normalized to the expression in the vector control (set to 100%).

Supplemental Table 1. Okamoto et al.

Complementation construct	OHT	Brcc3 shRNA	Chromosomes	Chromosomes with fusions	% of chromosome fusions	p values	relative to:
Vector	-	-	1314	20	1.5		
	+	-	1562	1223	78.3	1	vector + OHT
TRF2	-	-	1516	3	0.2		
	+	-	1831	3	0.2	2.963E-50	vector + OHT
TRF1	-	-	2791	34	1.2		
	+	-	4065	2699	66.4	0.1742	vector + OHT
TRF $^{\Delta B}$	-	-	1840	16	0.9		
	+	-	1509	22	1.5	1.469E-49	vector + OHT
TRF ^{cT}	-	-	3222	40	1.2		
	+	-	2358	100	4.2	4.301E-31	vector + OHT
TRF ^{cH}	-	-	1306	31	2.4		
	+	-	2834	817	28.8	5.374E-19	vector + OHT
TRF ^{cM}	-	-	1084	1	0.1		
	+	-	1094	2	0.2	1.620E-33	vector + OHT
TRF1 ^{iDDR}	-	-	2541	39	1.5		
	+	-	3156	605	19.2	5.093E-23	TRF1 + OHT
Vector	-	+	945	6	0.6		
	+	+	1211	1043	86.1	0.2700147	vector + OHT
TRF ^{cT}	-	+	1441	34	2.4		
	+	+	1171	849	72.5	1.57E-23	TRF^{cT} + OHT
TRF1 ^{iDDR}	-	+	937	21	2.2		
	+	+	1016	665	65.5	0.4733229	TRF1 + OHT

Supplemental Table 1. Metaphase spreads analysis. Summary of chromosome analysis performed on metaphase spreads derived from TRF2$^{F/F}$; R26-CreER cells infected with the indicated complementation constructs and either treated with tamoxifen (OHT) to induce Cre-mediated recombination or left untreated. When indicated cells were infected with a lentivirus encoding an shRNA against Brcc3. The number of chromosomes analyzed and chromosomes with fusions is indicated. P values (chi square) are indicated to test significance relative to the indicated condition. Non significant values ($p>0.05$) are highlighted.

	template	forward primer	reverse primer
TRF^{cT}	TRF1	pBabe-F	GAGGCAGCGGACTCAGATTTTAAAGCTTTTGCCGCTGCCT
	TRF2	AGGCAGCGGCAAAAGCTTTAAAATCTGAGTCCGCTGCCTC	pBabe-R
TRF^{cH}	TRF2	pBabe-F	TTTTGCTTTCCAAAGCCTTTTTGGCCATCG
	TRF1	CGATGGCCAAAAAGGCTTTGGAAAGCAAAAGGACAAGAAC	CACTTCTGCTTTTTTGTTATTCGATGTTTTTCAGGAGTTA
	TRF2	TAACTCCTGAAAAACATCGAATAACAAAAAAGCAGAAGTG	pBabe-R
TRF^{cM}	TRF2	pBabe-F	CATGCCTGTCTTTTTCTAGCATTGGTTGTACTGTCTTCAT
	TRF1	ATGAAGACAGTACAACCAATGCTAGAAAAAGACAGGCATG	pBabe-R
TRF^{cTDRAP1}	cmT	pBabe-F	GCAAAGGCTGCCTCAGAATCCATTCCAATGGTGGTTGGAG
	TRF2	CTCCAACCACCATTGGAATGGATTCTGAGGCAGCCTTTGC	Babe-R
TRF^{cTDTIN2}	cmT	pBabe-F	CTGGGCTCAGTACTCTGGCTCTGCAGTTCCGAGCCACCCT
	TRF2	AGGGTGGCTCGGAACTGCAGAGCCAGAGTACTGAGCCCAG	Babe-R
TRF^{cTDiDDR}	cmT	pBabe-F	TCATCTGGTGCTGCCTGAACTACTTTGGGATTCTTCTCTC
	TRF2	GAGAGAAGAATCCCAAAGTAGTTCAGGCAGCACCAGATGA	Babe-R
TRF1^{iDDR}	TRF1	pBabe-F	CTGTTCCACTTGCCTTTGGGAGGAGTTCCTACTCTTCTTT
	TRF2	AAAGAAGAGTAGGAACTCCTCCCAAAGGCAAGTGGAACAG	GTTACCGGCTGACTCTTTGATTGAAACAGTTCATCCTCTT
	TRF1	AAGAGGATGAACTGTTTCAATCAAAGAGTCAGCCGGTAAC	pBabe-R

Supplemental Table 2. Primers used to generate the TRF chimeras used in this study.

TRF chimera constructs were generated by PCR amplification of each TRF region using as templates myc-TRF1 and myc-TRF2 constructs. Amplified PCR products were mixed and amplified with external primers (pBabe-F and pBabe-R) to obtain the final alleles.

Supplemental Table 3. List of proteins identified by Mass Spectrometry. Three independent experiments were performed in HEK293 cells transfected with FLAG–tagged TRF1 or FLAG–tagged TRF1^{iDDR}. Cells were lysed (50 mM Tris–HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% TritonX-100) and immunopurified with anti-FLAG agarose resin (Sigma). After washing, proteins were eluted by competition with FLAG peptide (Sigma). For mass spectrometry analysis samples were denatured, reduced and alkylated prior to an overnight digestion with trypsin. Peptide mixtures were analyzed by nanoflow liquid chromatography mass spectrometry using an Eksigent nanopump (Dublin, CA) and LTQ-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) using a 7 step MudPIT separation. In this table proteins that were identified in three independent experiments in the TRF1^{iDDR} sample are shown. Average numbers of spectra identified in the TRF1 sample (average TRF1) and in the TRF1iDDR sample (average TRF1 iDDR) are shown. Proteins are ranked based on the relative enrichment in the TRF1^{iDDR} samples calculated based on the "Delta spectra value" (Spectra TRF1^{iDDR} / Spectra TRF1+ spectra TRF1^{iDDR}). Proteins with a "delta spectra value" greater or equal to 0.9 were considered as enriched in the TRF1^{iDDR} sample. In yellow are highlighted the proteins that were further characterized in this study.