

Supplemental Figure Legends

Supplemental Figure 1. Cell proliferation is not inhibited by expression of shTpp1. A. Early passage (P2) primary p53^{-/-} MEFs were infected with control vectors, shRNA-1, and -2, and subjected to puromycin selection. Subsequently, cells numbers were measured at the indicated time points, with day 0 representing the first day after puromycin selection. Error bars represent s.e.m. B. TPP1 knockdown induce ATM-dependent 53BP1 foci at telomeres. ATM^{+/+} or ATM^{-/-} MEFs infected with shTPP1-1 were analyzed by telomere PNA-FISH (red) and antibody to 53BP1 (green). Merged image is shown on the right.

Supplemental Figure 2. TPP1 RD domain is required for interaction with POT1a or POT1b. A. Full length mTpp1, showing indicated domains, and Tpp1 deletion mutants Tpp1^{ΔRD} and Tpp1^{ΔC}. RD: Pot1a/Pot1b-recruitment domain. S/T: Serin-rich region. TID: TIN2-interacting domain. B. IP-Western analysis indicating that HA-Tpp1 pulls down Myc-Pot1a or Flag-Pot1b in 293HEK cells, while HA-tagged Tpp1^{ΔRD} mutant do not interact with either Pot1 proteins. C. IP-Western analysis indicating that Myc-Pot1a pulls down HA-Tpp1 but not HA-Tpp1^{ΔRD}. D. IP-Western analysis indicating that Flag-Pot1b pulls down HA-Tpp1 but not HA-Tpp1^{ΔRD}.

Supplemental Figure 3. Tpp1 knockdown extends telomere G-rich overhang. A. In-gel overhang assay of telomeres derived from MEFs of the indicated ATM genotypes expressing either TPP1 shRNA (sh) or control vector (v). Native: detection of single-stranded (ss) (TTAGGG)_n repeats; Denatured: detection of both single stranded and double-stranded telomeric repeat segments after denaturation of the gel. B. Quantification of overhang signals shown in (A). Signals were quantitated and the single-stranded telomeric signal (from native gel) was normalized to total telomeric DNA signal (from denatured gel) in the same lane. C. Quantitation of total telomere signals from denatured gel.

Supplemental Figure 4. Quantitation of TIFs. A. Quantitation of number of γ-H2AX positive TIFs per cell in ATM^{+/+} or ATM^{-/-} MEFs in which Pot1a and Pot1b have been

depleted. B: quantitation of percentage of the cells with ≥ 5 of γ -H₂AX positive TIFs in ATM^{+/+} or ATM^{-/-} Pot1a and Pot1b knockdown MEFs. Error bars represents s.e.m. C, D: Quantitation of γ -H₂AX positive TIFs with telomeres in ATR^{F/-} or ATR ^{Δ -} MEFs in which Pot1a and Pot1b have been depleted. C: percentage of cells containing ≥ 5 γ -H₂AX positive TIFs. D: total number of γ -H₂AX positive TIFs per cell. Error bars represents s.e.m. E, F: Quantitation of γ -H₂AX positive TIFs with telomeres in ATR^{F/-} or ATR ^{Δ -} MEFs transiently depleted of Tpp1. E: total number of γ -H₂AX positive TIFs per cell. F: percentage of cells containing ≥ 5 γ -H₂AX positive TIFs. Error bars represents s.e.m.

Supplemental Figure 5. Generation of Pot1a^{F/-}ATR^{F/-} and Pot1^{F/-}ATR^{F/+} MEFs. A. RT-PCR of untreated (UT) or AdCre treated Pot1a^{F/-}ATR^{F/-} MEFs, showing that Cre-mediated deletion results in the elimination of the floxed alleles and the emergence of the deleted (Δ) alleles for both Pot1a and ATR. B. Pot1a depletion activates an ATR-dependent DDR at telomeres. Pot1a^{F/-}ATR^{F/-} and Pot1^{F/-}ATR^{F/+} MEFs were either untreated or treated with AdCre to remove the floxed alleles. Pot1 ^{Δ -}ATR^{F/+} cells displayed robust TIF formation that is abrogated upon removal of ATR.

Supplemental Figure 6. Tpp1 Knockdown in ATM null cells induces anaphase bridge formation, chromosomal instability and tumorigenesis *in vivo*. A. Elevated anaphase bridges (arrowheads) observed in ATM^{-/-} cells expressing Tpp1-shRNA-1 (a, b). Two nuclei connected with a chromatin bridge contains telomeric signals (c, red signals denote telomeres, arrowheads point to telomeric signal within the bridge). B. Quantitation of anaphase bridges. Error bars represent s.e.m. C. Tpp1 knockdown in ATM^{-/-} cells formed tumors *in vivo*. ATM^{-/-} cells expressing control vector, Tpp1-shRNA-1 or-2 were injected into the flanks of SCID mice. Tumors are indicated with arrows. D. Tumors isolated from SCID mice induced by knockdown of Tpp1 as shown in (C). E. Telomere-FISH on metaphase spreads derived from Tpp1 knockdown, ATM and p53 null tumor cells. The telomeric hybridization signal is shown in red and DAPI counterstained chromosomes are blue. Arrows indicate chromosomal aberrations. F. Quantitation of the frequency of cytogenetic aberrations as indicated in tumor cells generated from Tpp1-knockdown, ATM null MEFs.

Supplemental Table 1. SCID tumor injections. Cell lines of the indicated genotypes were injected into SCID mice and then tumors were harvested at $\sim 1 \text{ cm}^3$. Number of tumor cell lines injected per site are indicated.

Supplemental Materials and Methods

Immunofluorescence staining and TIF (Telomere dysfunction Induced Foci)

analysis. Cells with different genotypes were infected and selected for 7-10 days for stable expression of various retroviral constructs. The cells were grown on 8-well glass chambers, fixed with 2% paraformaldehyde and permeabilized in 0.5% NP-40. Cells were blocked in PBG (0.2% [w/v] cold water fish gelatin [Sigma G-17765]; 0.5% BSA in PBS) and incubated with the antibodies in PBG: TRF1, 53BP1, or γ -H2AX. Secondary antibodies against mouse or rabbit IgG were labeled with Alexa 488 (Molecular Probes) and Rhodamine Red-X (RRX, Jackson) respectively. TIF assay was performed as described (Wu et al. 2006) using the same primary and secondary antibodies as above and a Tamra-(TTAGGG)₃ PNA telomere probe (Applied Biosystems). DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI) and slides were mounted in 90% glycerol/10% PBS containing 1 $\mu\text{g}/\text{mL}$ p-phenylene diamine (Sigma). Digital images were acquired on a Nikon Eclipse 800 with x63 and x100 plan-apo objectives and photographed with a cooled CCD camera. CCD chip nonlinearities were removed by taking bias and dark current frames and the optical train flat fielded to eliminate vignetting. Individual raw images were taken through DAPI, Rhodamine and FITC narrowband filters and stacked in MetaMorph and PhotoShop CS were utilized to compose the final images. The same amount of linear histogram stretch was applied to all raw images before stacking to maintain the same degree of contrast enhancement in all images. Only cells with ≥ 5 γ -H2AX signals co-localized with telomere signal (TTAGGG)₄ were scored.

Telomere-TdT assay. Labeling unprotected telomeres with FITC conjugated deoxy-Uridine was performed essentially as described (Verdun et al., 2005; Wu et al., 2006) except that the TdT incubation time was 30 minutes at 37 °C to minimize background. Co-localization of the TdT signal with telomeres was performed by fixing TdT-labeled

cells with 2% paraformaldehyde, washing 3 times in PBS, denaturation at 80°C for 3 minutes, followed by incubation in PNA-FISH hybridization solution containing 0.25 ug/ml TRITC-OO-(TTAGGG)₄. Images were analyzed as described above.

Cell proliferation, senescence-associated β -gal assay. For growth curves, 4 x 10⁴ infected MEFs were plated in triplicate into six-well plates. At 0, 2, 4, 6 and 8 days post plating, cells were fixed and stained with crystal violet. BrdU incorporation was performed essentially as described (Smogorzewska et al, 2002). SA β -gal assay was performed as described (Dimri et al, 1995).

In-gel Telomeric G-overhang assay. In-gel G-overhang assay was performed essentially as described (Hemann and Greider 1999). Following electrophoresis, gels were dried down at 40°C and prehybridized at 50°C for 1 h in Church mix (0.5 M Na₂HPO₄ (pH 7.2), 1 mM EDTA, 7% SDS, and 1% BSA), followed by hybridization at 50°C overnight with an end-labeled (CCCTAA)₄ oligonucleotide. After hybridization, gels were washed three times with 4 × SSC for 30 min and once with 4 × SSC/0.1% SDS. Gels were exposed to PhosphoImager screens. Following G-overhang assay, gels were alkali denatured (0.5 M NaOH and 1.5 M NaCl), neutralized (3 M NaCl and 0.5 M Tris-HCl (pH 7.0)), rinsed with H₂O, and reprobbed with the (CCCTAA)₄ oligonucleotide at 55°C and then processed as previously. To determine the relative overhang signal, the signal intensity for each lane was determined before and after denaturation using Imagequant software. The G-overhang signal was normalized to the total telomeric DNA and this normalized value was compared between samples.

Immunoprecipitation and Western blot. To determine the interaction between mouse POT1a, POT1b and TPP1, HA-tagged TPP1 constructs and full-length Myc-POT1a or Flag-POT1b were co-transfected into 293T cells. Extracts were isolated and IP-Western analysis was performed as described (He et al, 2006; Wu et al., 2006).

Telomere-FISH and anaphase bridge determination. For chromosomes analysis, cells were arrested in metaphase with colcemid (Invitrogen). Cells were incubated in hypotonic solution (75 mM KCl), fixed in methanol:acetic acid (3:1), and spread on cold, wet, ethanol-cleaned slides. Air-dried slides were rehydrated in 1 X PBS, post-fixed in 4% formaldehyde, rinsed in 1X PBS, digested in pepsin (0.5 mg/ml, 0.01 N HCl), rinsed, post-fixed in 4% formaldehyde and dehydrated in a graded ethanol series, and air-dried.

Then TRITC-OO-(TTAGGG)₄ peptide nuclei acid probes (Applied Biosystems) was applied under a sealed coverslip denatured at 80°C and hybridization was performed as described (Wu et al, 2006). Slides were counterstained in DAPI (1 mg/ml in 1 X PBS) and mounted in VECTASHIELD (Vector Laboratories). Metaphase chromosomes from mouse tumors were prepared as described (Rudolph et al., 1999) and subjected to Giemsa staining or telomere-FISH with TRITC-OO-(TTAGGG)₄ probe. Depending on the quality of metaphase spreads, 15-20 metaphases from each tumor sample were analyzed in detail. Digital images were captured with Metamorph Premiere (Molecular Devices) and processed with Metamorph and Adobe Photoshop CS. Anaphase bridges were quantitated from a minimum of 200 metaphase plates undergoing anaphase and determining percentage possessing a distinct chromatin bridge after DAPI staining.

Soft-agar growth and subcutaneous tumorigenicity assays. At the time of plating in soft agar, cultures were trypsinized and counted, and 2 times 10⁵ total cells were mixed with 1.5 ml of 0.45% low melting point (LMP) agarose-DMEM (top layer) and then poured on top of 1.5 ml of solidified 0.75% LMP agarose-DMEM (bottom layer) in six-well plate. Colonies were counted and photographed after 6 weeks. To analyze the tumorigenicity in vivo, Cells (5 times 10⁶) were resuspended in 400 μl of phosphate-buffered saline solution and injected into SCID mice as previously described (Chang et al., 2003). Tumors were monitored daily and mice sacrificed when tumors reach 2 cm in greatest dimension. Animals were treated in a human manner in accordance with the U.T. M.D. Anderson Cancer Center guidelines for the animal laboratory care and use.

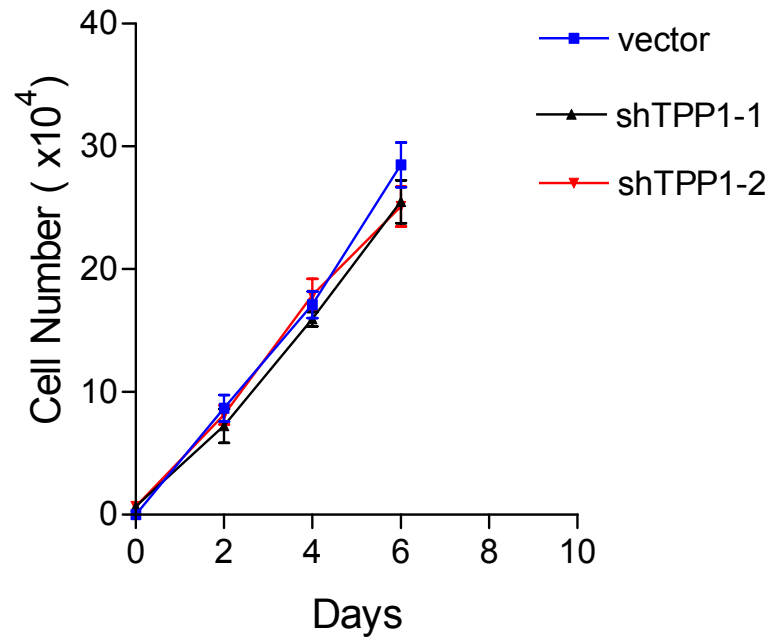
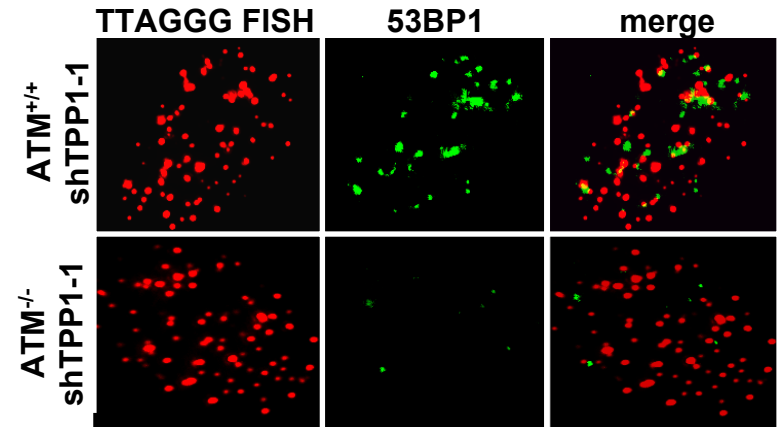
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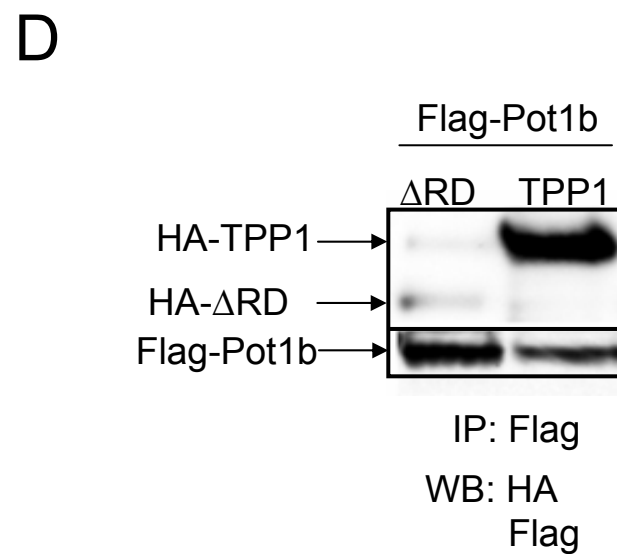
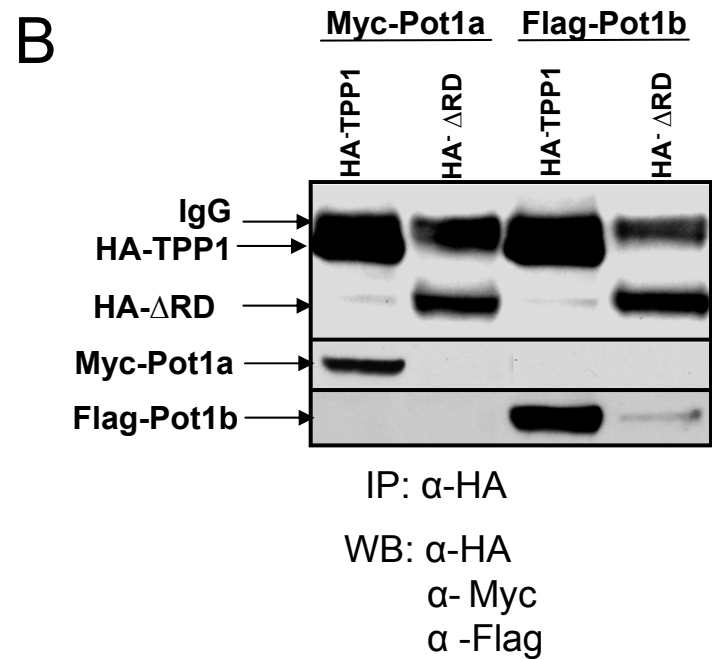
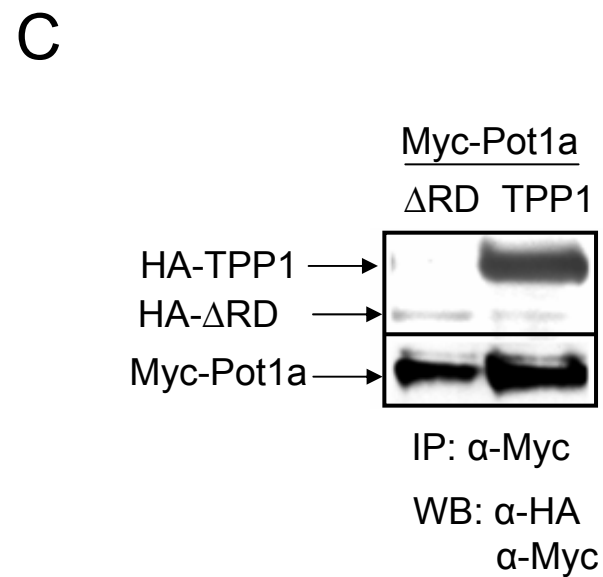
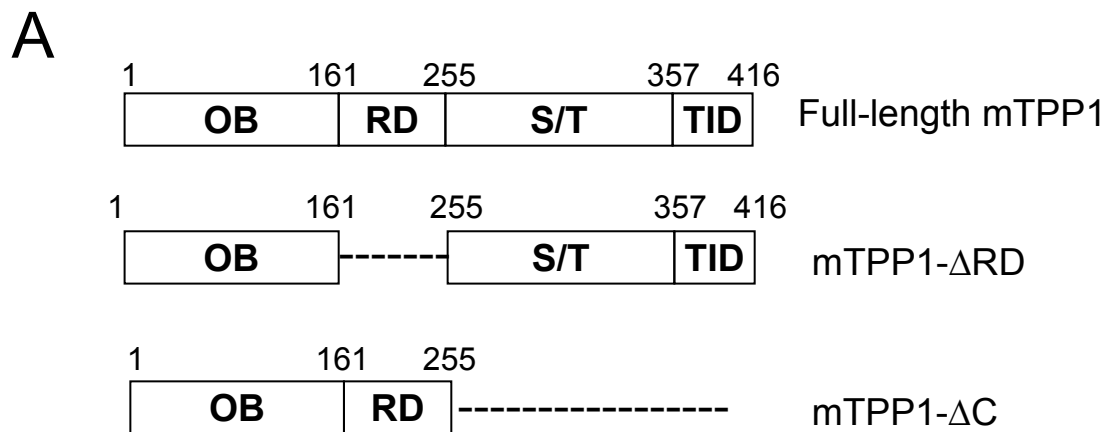
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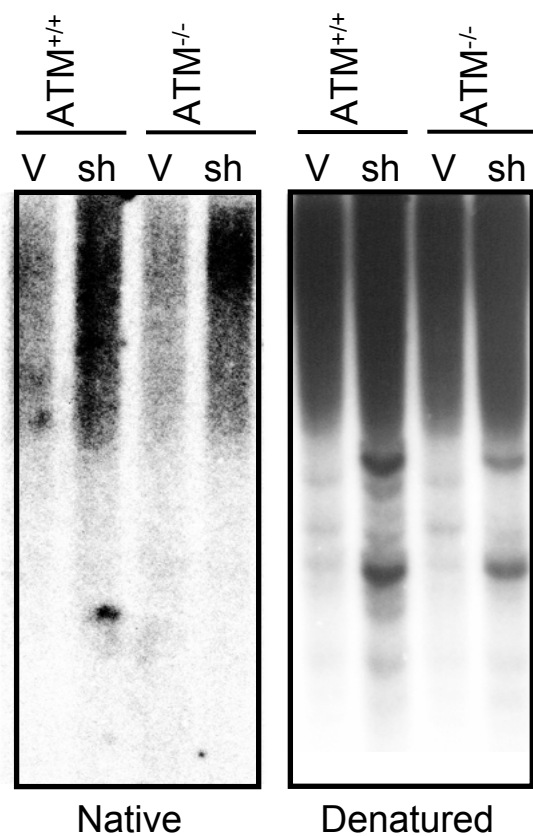
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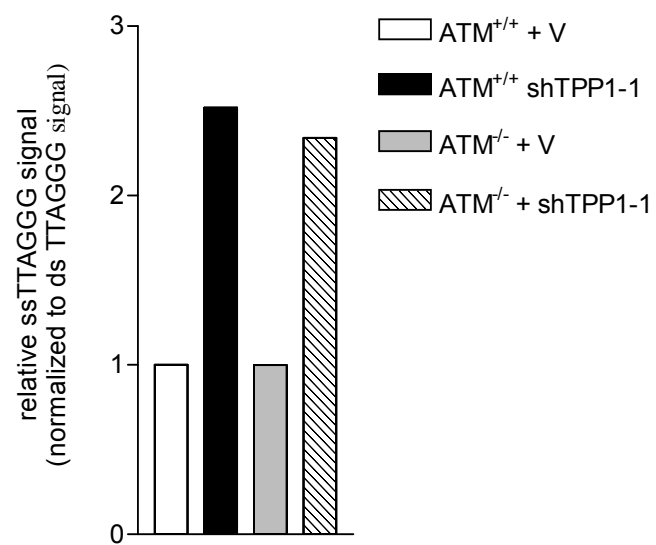
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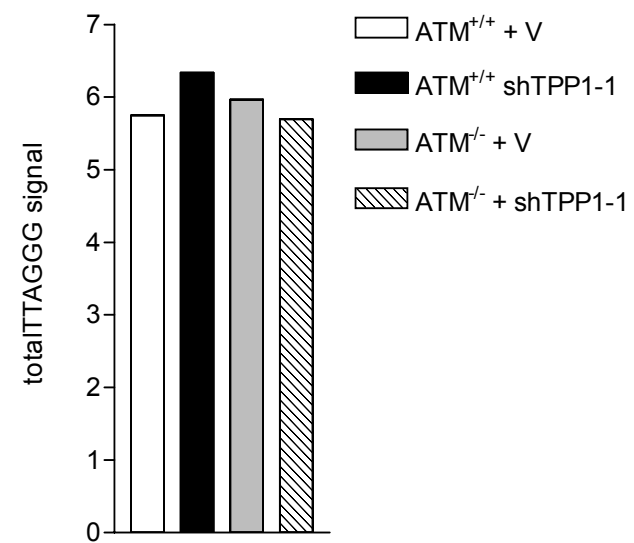
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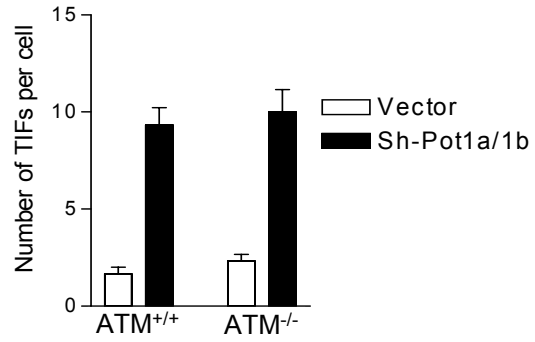
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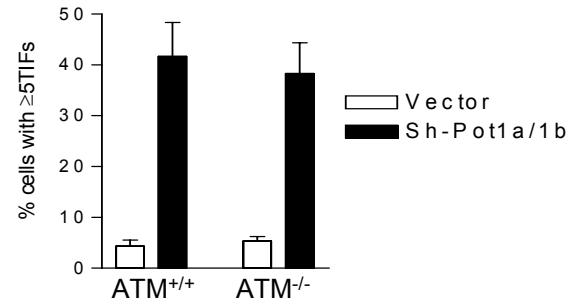
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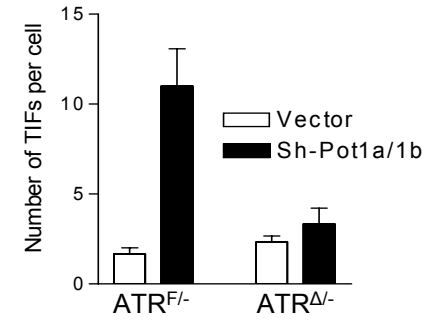
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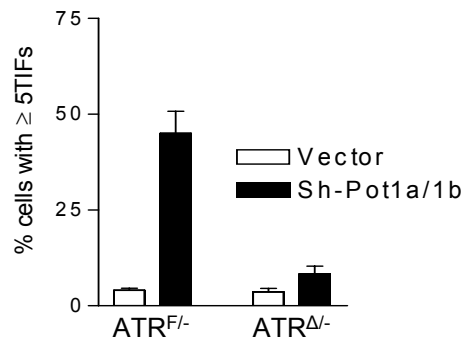
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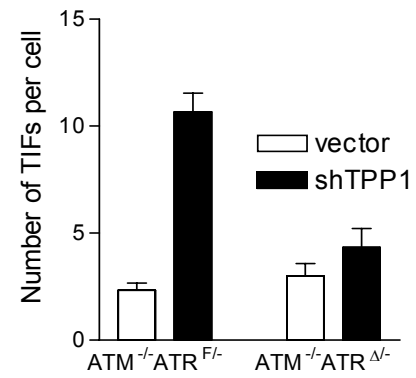
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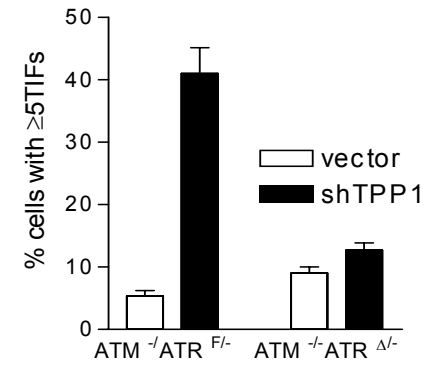
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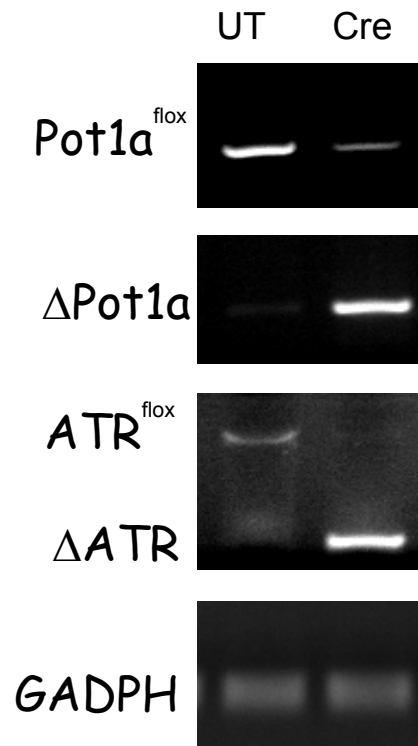
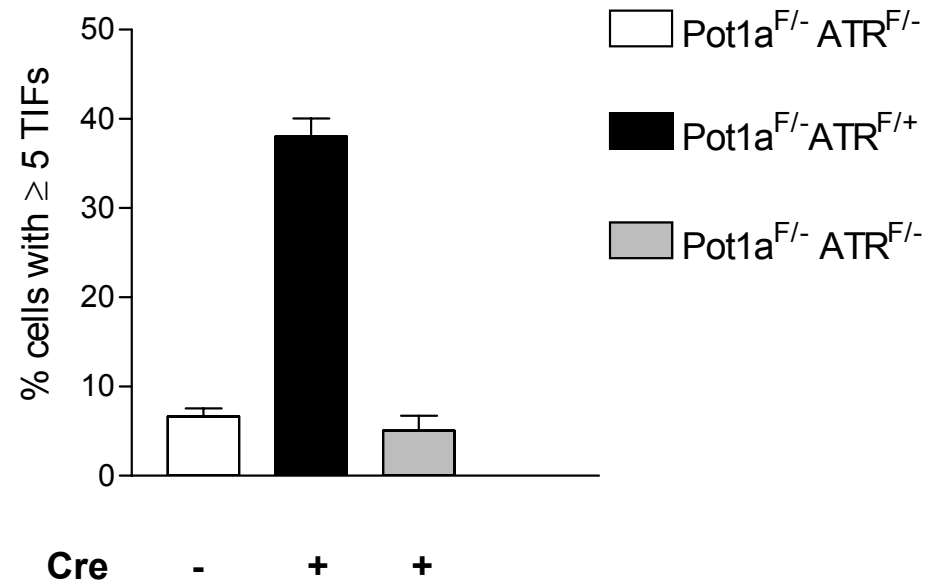


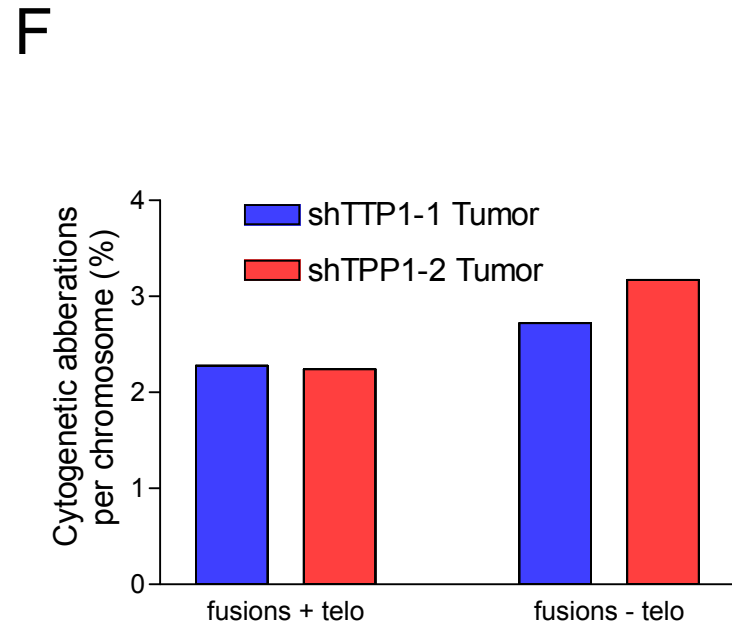
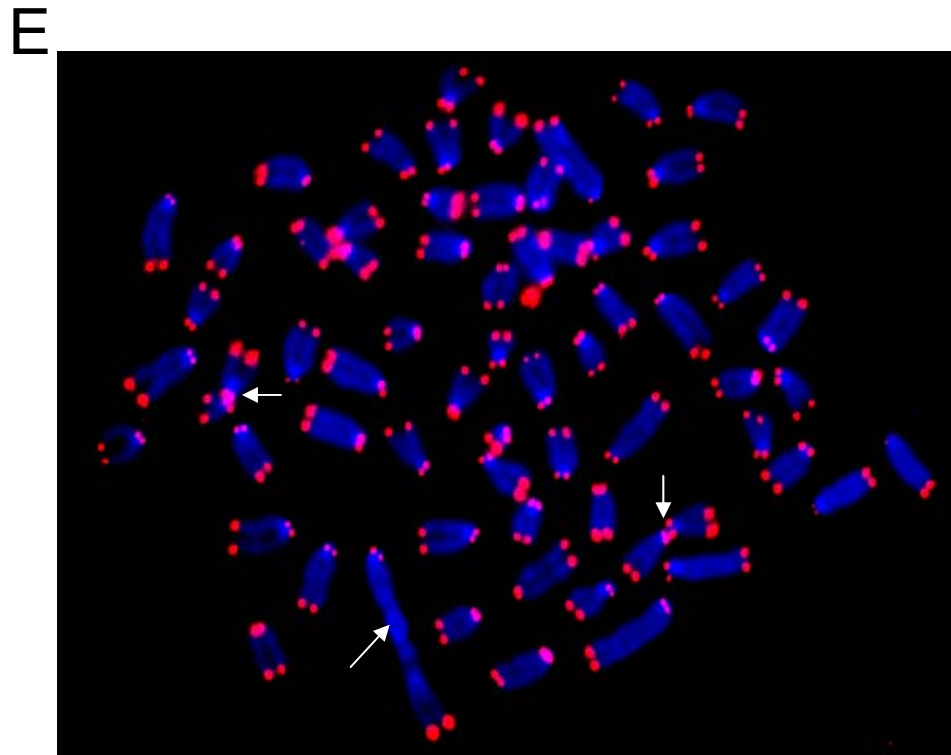
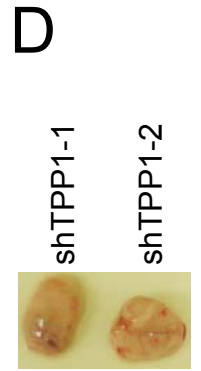
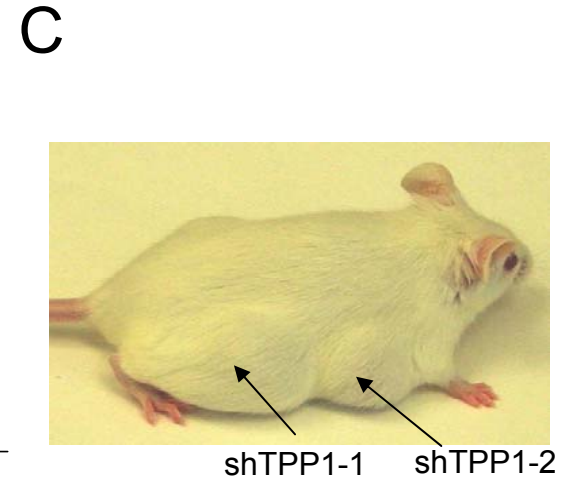
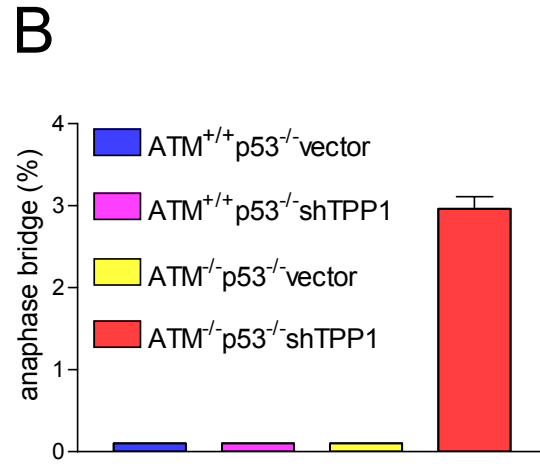
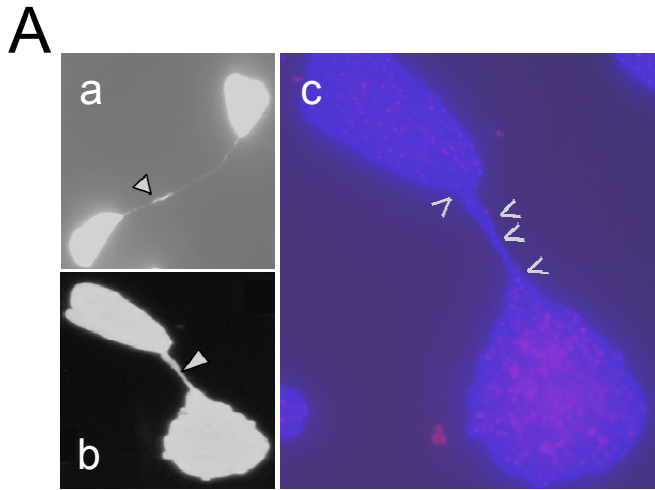
E



F



A**B**



Genotype	Tumors/sites injected
ATM ^{+/+} p53 ^{-/-} vector	0/3
ATM ^{+/+} p53 ^{-/-} shTPP1-1	0/3
ATM ^{+/+} p53 ^{-/-} shTPP1-2	0/3
ATM ^{-/-} p53 ^{-/-} vector	0/4
ATM ^{-/-} p53 ^{-/-} shTPP1-1	4/4
ATM ^{-/-} p53 ^{-/-} shTPP1-2	4/4

5 x 10⁵ cells injected per site