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14. ABSTRACT
Telomeres, the protective elements at the ends of chromosomes, must be maintained for cells to proliferate indefinitely. In many human cancers, the telomeric DNA is replenished by the enzyme telomerase. However, a second pathway for telomere maintenance, referred to as the ALT pathway, has increasingly been recognized in human cancers. The genetic basis for activation of ALT is not known, but recent data have identified mutations and loss of ATRX protein as being hallmarks of ALT-immortalized cell lines and tumors. Our efforts to understand the mechanism by which loss of ATRX facilitates telomere recombination have uncovered a novel role for this protein in promoting telomere cohesion. Furthermore, disruption of telomere cohesion, in combination with telomere dysfunction, is capable of promoting the inappropriate non-sister telomere interactions that would facilitate the recombination-mediated mechanism of telomere maintenance in ALT cells. I have determined that ALT cells show evidence of telomere dysfunction, specifically telomere replication problems, and may also have disruptions to the Rif1/BRCA1 pathways controlling homologous recombination repair. These defects, in combination with a telomere cohesion defect resulting from loss of ATRX, could facilitate the repair with non-sister telomeres that abolishes both template use and register, allowing the telomere recombination necessary for maintenance of ALT cells.

15. SUBJECT TERMS
telomere, ALT, ATRX, cohesion, Rif1, BRCA1

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Introduction

Human cancer cells require a telomere maintenance mechanism for their unlimited proliferative potential. Most achieve this by up-regulating telomerase, an enzyme that catalyzes the de novo addition of telomeric repeats to the end of chromosomes[1, 2, 3]. A significant minority of tumors activates a telomerase-independent mechanism referred to as alternative lengthening of telomeres (ALT)[4, 5]. The characterization of ALT has remained largely descriptive since this mechanism was identified, and the recurring features of long and heterogeneous telomeres, extrachromosomal telomeric circles, and ALT-associated PML bodies (APBs) have been the basis for attempts to identify tumors reliant on ALT[4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14]. However, it is unclear whether these characteristics are relevant for the ALT mechanism of telomere maintenance or sufficient for properly identifying tumors reliant on ALT.

Several important observations have provided insight into the mechanism of telomere maintenance in ALT cells. Cell fusion experiments that rescued the ALT phenotype suggested ALT might result from recessive mutations and loss of a normal function[15]. The requirement for mutational alterations is also consistent with the low frequency of ALT induction in vitro. Additionally, multiple lines of evidence suggest that despite the repression of homology-directed repair (HDR) at functional telomeres, ALT cells maintain their telomeres by a telomere-specific recombination mechanism[16, 17, 18, 19].

The genetic requirements for the activation of telomere recombination and ALT are largely unknown, but work by us and others have identified mutations and loss of the ATRX protein as being hallmarks of ALT-immortalized cell lines and tumors[20, 21]. ATRX is a member of the SNF2 family of chromatin remodeling proteins, and can use ATP hydrolysis to translocate nucleosomes in cis along the DNA[22]. It also cooperates with DAXX to deposit the histone variant H3.3 at telomeres and other G-rich repetitive sequences in a replication-independent chromatin assembly pathway[23, 24, 25, 26]. The function of ATRX/DAXX and H3.3 at telomeres is unclear, but loss of ATRX is associated with reduced telomeric loading of HP1α, upregulation of TERRA, and telomere dysfunction in mouse ES

cells[27, 25]. ATRX patient cells also show changes in the patterns of DNA methylation at highly repetitive sequences[28]. Finally, loss of ATRX has been associated with defects in mitotic progression and sister chromatid cohesion, resulting in the formation of lobulated nuclei, micronuclei, and chromatin bridges [29, 30, 31].

Our efforts are currently focused on understanding the mechanism by which loss of ATRX facilitates telomere-specific recombination, and determining whether ALT-immortalized cells display specific sensitivities that can lead to more effective treatments for ALT positive cancers.

Key Words

Telomere, ALT, ATRX, cohesion, Rif1, BRCA1

Overall Project Summary

As described in my previous report, loss of ATRX alone is not sufficient to unleash telomere recombination and it does not function in the shelterin or Ku-mediated pathways of HDR repression at telomeres. Our data also definitively exclude the ATRX-DAXX-H3.3 pathway as the relevant mechanism through which telomere recombination is unleashed, given that H3.3 remains associated with telomeric DNA in both ATRX-deficient ALT cells and ATRX-null MEFs. I have also excluded changes to DNA methylation patterns or nucleosomal organization as contributing to the suppression of HDR, since subtelomeric DNA methylation and telomeric nucleosomal organization were unchanged by loss of ATRX.

I became particularly interested in the reported cohesion function of ATRX upon recognizing the high degree of overlap between the phenotypes of defective chromosome cohesion and those of ATRX loss. Both show inaccurate chromosome segregation, an inability to maintain the processivity and stability of replication forks, an increased sensitivity to replication inhibitors, as well as changes in gene transcription[32, 33, 34, 31, 35, 36, 24]. Thus, many of the phenotypes associated with ATRX deficiency could potentially result from chromosome

cohesion defects. And importantly, there is a telomere-specific cohesin ring complex (containing SA1 instead of SA2), potentially allowing for differential regulation of telomere cohesion versus arm cohesion[37].

To assess telomere and arm cohesion, I performed FISH using probes for the subtelomeric and arm regions of mouse chromosomes 8 and 10. All probes were confirmed to localize correctly on metaphase chromosome spreads, and FISH signals in interphase cells were then scored as either a single focus or a doublet (indicative of premature separation and thus a cohesion defect, as previously described[37]). CRE-mediated deletion of ATRX resulted in a significant increase in telomere doublets for both chromosome 8 and chromosome 10 in two independent cell lines (Figure 1A-D). ATRX loss had no significant effect on the percentage of arm signals observed as doublets, suggesting ATRX is important specifically for the establishment of telomere cohesion.

I also examined telomere and arm cohesion in a subset of ALT cell lines and telomerase-positive controls. FISH probes for the subtelomeric and arm regions of human chromosome 4 were confirmed to localize properly on metaphase chromosome spreads, and signals in interphase cells were again scored as either a single focus or a doublet (Figure 1E). The percentage of arm signals observed as doublets varied significantly in this panel of cells, making direct comparisons of the percentage of telomere doublets inappropriate and likely inaccurate. To normalize for this varying amount of separation observed, I plotted the ratio of telomere doublets to arm doublets within each of the three independent experiments for each cell line (Figure 1F). The telomerase-positive cells consistently show ratios of less than one, indicating they have less separation of the telomere signals than the arm signals. The ALT cells generally have ratios greater than one, indicating telomere separation is more frequent than separation of the arm signals. And interestingly, the ALT cell lines segregated based on ATRX status. Those with mutations in ATRX showed the highest ratios and about a two-fold increase compared to the telomerase-positive cells, consistent with the two-fold increase in telomere doublets observed after ATRX deletion in MEFs. The ALT cell lines with no alterations to ATRX

expression or localization showed more modest increases, if any, relative to the telomerase-positive cells. Although the initial results in the ALT cells appear consistent with those observed in ATRX-deficient MEFs, the variability necessitates that additional ALT cell lines be examined to confirm the presence and extent of a telomere-specific cohesion defect.

To examine telomere cohesion in a different context, I generated MEFs that would allow the conditional deletion of both ATRX and TPP1. Deletion of the shelterin protein TPP1 leads to telomere deprotection and increases in sister-telomere fusion events, both due to loss of Pot1 from single-stranded telomere overhangs[38]. If loss of ATRX does indeed cause a telomere-specific cohesion defect, we hypothesized that the combined loss of ATRX and TPP1 could increase fusion events with non-sister telomeres. Using the previously described CO-FISH assay to differentially label sister chromatids, metaphase chromosome spreads from cells lacking TPP1, ATRX, or both TPP1 and ATRX were analyzed in at least four independent experiments for both sister and non-sister fusion events. As shown in Figure 2, deletion of TPP1 causes a significant increase in sister chromatid fusions, as expected, while ATRX deletion causes no increase. The combined deletion of ATRX and TPP1 also causes a significant increase in sister chromatid fusions. The median percentage and range of fusions do not differ from those observed after deletion of TPP1 alone (Figure 2B), suggesting that loss of ATRX does not impact these fusion events. Deletion of TPP1 also causes a small but significant increase in non-sister fusions, with an average of 1.1% of chromatids fused. While deletion of ATRX alone has no impact on non-sister fusions, the combined deletion of ATRX and TPP1 elevates non-sister fusions to an average of 2.4%, significantly greater than that observed with TPP1 deletion alone (Figure 2C). These results are consistent with ATRX loss causing a telomere-specific cohesion defect, and highlight the opportunity for ATRX-deficient and deprotected telomeres to attempt repair using non-sister telomeres.

Deletion of ATRX can increase fusions between non-sister telomeres under conditions of telomere dysfunction (TPP1 deletion). However, loss of ATRX alone is not sufficient to promote these events, suggesting there may not

be sufficient telomere damage in this context to elicit HDR repair. Consistent with this idea, ATRX deficient MEFs do not display telomere-dysfunction induced foci (TIFs; data not shown). Most markers used to identify TIFs are associated with the presence of DNA double-stranded breaks, but I also wanted to assess potential telomere replication problems since stalled forks could also require resolution via HDR repair. The repetitive telomeric DNA can present a challenge to the replication machinery, and gives rise to defects that resemble those of common fragile sites[39]. Instead of the normal telomere FISH signal of a single dot, multiple signals are observed at a chromatid as if the telomeric DNA is decondensed or broken. This abnormal FISH pattern is referred to as a fragile telomere.

I analyzed two independent MEF lines after deletion of ATRX and found no increase in fragile telomeres (Figure 3A-B). The DNA polymerase alpha inhibitor aphidicolin accentuates replication problems at both telomeres and common fragile sites[40, 39]. Although aphidicolin generally elevated the percentage of fragile telomeres in both cell lines, no further increases were observed with deletion of ATRX (Figure 3A-B). This suggests that loss of ATRX is not sufficient to cause telomere replication problems in MEFs. However, deletion of ATRX from a human cell line was recently reported to cause replication defects, as evidenced by DNA fiber labeling experiments[35]. A more sensitive technique may be necessary to visualize replication problems present in the ATRX-deficient MEFs, or this could highlight a difference in the severity of ATRX loss in mouse versus human cells. The analysis of ALT and telomerase-positive cells does reveal a consistent and significant increase in fragile telomeres in several ALT cell lines (Figure 3C-D). Fragile telomeres were observed in ALT cells regardless of the ATRX status. Although loss of ATRX could potentially contribute to this fragile phenotype, there are likely other pervasive ATRX-independent replication problems in these cells that promote telomere dysfunction.

The telomere cohesin complex uniquely contains the protein SA1, while chromosome arm cohesion is maintained by a ring complex containing SA2[37].

To determine whether ATRX promotes telomere cohesion through the same pathway as the SA1-containing cohesin ring complex, I scored telomere and arm doublets in interphase cells after silencing of SA1, deletion of ATRX, or the combined loss of SA1 and ATRX (Figure 4A-C). Silencing SA1 caused a significant increase in telomere doublets while having no effect on the percentage of arm doublets observed, consistent with previously published results[37]. Deletion of ATRX again caused a significant increase specifically in telomere doublets, and to a similar extent as that observed with loss of SA1. Importantly, the combined loss of SA1 with ATRX caused no further defect in telomere cohesion. This suggests ATRX and SA1 not only function in the same pathway to promote telomere cohesion, but that loss of ATRX may be as detrimental to telomere cohesion as loss of the cohesin protein (Figure 4D).

Similarly, I also examined chromatid fusion events in the ATRX, TPP1, and ATRX-TPP1 MEFs after silencing of SA1 (Figure 4D-F). Knockdown of SA1 had no significant effect on the percentage of sister fusions observed after loss of ATRX and TPP1, alone or in combination (Figure 4E). Although silencing of SA1 had no effect on the percentage of non-sister fusions observed in ATRX-deficient cells, the percentage of non-sister fusions after TPP1 deletion was significantly increased by loss of SA1 (Figure 4F). This suggests that defects in telomere cohesion, combined with telomere dysfunction, can promote fusion events with non-sister telomeres. Deletion of both ATRX and TPP1 again resulted in a significant increase in non-sister fusions, and loss of SA1 did not further exacerbate this phenotype (Figure 4F). The data shows that loss of ATRX is again as detrimental as loss of the cohesin subunit, confirms that ATRX and SA1 function in the same pathway to promote telomere cohesion, and highlights the importance of the cohesion pathway in preventing inappropriate telomere associations.

After establishing that ATRX and SA1 promote telomere cohesion through the same mechanism, I performed telomere ChIP to determine whether loss of ATRX affected the loading of SA1 at telomeres. Deletion of ATRX transiently (Hit and Run Cre) or stably (pWZL-Cre) did not affect the amount of telomeric DNA

associated with SA1 (Figure 5). This suggests loading of SA1 (and perhaps the cohesin ring complex) still occurs at telomeres, but cohesion establishment may be disrupted in other ways, possibly via influences on important cohesin accessory proteins. ATRX deletion also had no effect on the association of TRF1, TRF2, TIN2, or TPP1 with telomeric DNA (Figure 5), indicating shelterin binding to telomeres is unaltered by the absence of ATRX and does not contribute to the observed cohesion defect.

Despite the telomere cohesion defect observed in ATRX-deficient MEFs, loss of ATRX alone is not sufficient to unleash the telomere recombination characteristic of ALT cells. Although ALT and telomerase are not mutually exclusive, the ALT cell lines do not exhibit telomerase activity while the ATRX MEFs do. To ensure that the presence of active telomerase is not masking any effect of ATRX loss, I generated ATRX MEFs deficient in telomerase activity and assessed telomere recombination again using the previously described CO-FISH assay. Although the background level of exchanges was elevated in these cells, there was again no difference in the percentage of telomere exchanges observed after deletion of ATRX (data not shown). We also completed the whole genome sequencing of several ALT cell lines hoping to uncover additional genetic mutations that may be relevant for the ALT mechanism of telomere maintenance. Nearly 2000 mutations were identified and then narrowed down to 46 genes of interest based on the presence of mutations in two distinct panels of ALT cell lines, thereby excluding likely SNPs. Preliminary immunoblots for some genes did not reveal any pervasive changes in protein expression in our panel of ALT cell lines, although some mutations may warrant further investigation. For example, several mutations were identified in a component of the Anaphase Promoting Complex (APC), Cdc27. These mutations cluster in the major interacting surface by which Cdc27 links to other proteins. The functional relevance of the Cdc27 mutations could be examined by depleting the endogenous protein in the ALT cell lines while expressing the wild type allele and assessing cell survival or changes to ALT phenotypes.

We have not pursued potential genes of interest from the sequencing effort because no gene mutation was as pervasive as ATRX, but also because a new protein of interest has attracted our attention. Data presented at a scientific meeting earlier this year showed ALT telomeres undergoing long-range movements that are dependent on HDR proteins, suggesting the presence of enhanced levels of single-stranded DNA. The de Lange laboratory has recently discovered a role for the protein Rif1 in blocking resection and the generation of single-stranded DNA at telomeres and other sites of DNA damage[41], and we therefore thought it was necessary to examine Rif1 in our panel of ALT cell lines. As shown in Figure 6, Rif1 protein levels vary widely in the ALT cells, particularly within the JFCF6 jejunal cell lines and the IIICF breast cell lines, each panel derived from a single patient. Even with long exposure times some ALT cell lines show little to no detectable protein by immunoblot analysis. Rif1 protein levels were also variable in the telomerase-positive cell lines though (Hela 1.3, BJ hTERT, and RPE hTERT), highlighting the concern about identifying the appropriate control cell line to use for comparison. The dramatic fluctuations in Rif1 expression from different cell lines originating from a single patient does suggest this pathway is targeted, to some extent, in ALT cells and warrants further investigation.

To determine whether these changes in protein expression had any functional effect, I examined Rif1 foci formation after treating the cells with IR. Rif1 is recruited to sites of damage and has a critical role in determining the choice of DSB repair pathway employed. In G1, recruitment of Rif1 prevents the accumulation of BRCA1 at DSBs and blocks resection, thereby promoting NHEJ. In S/G2, Rif1 recruitment to sites of damage is blocked, thereby allowing recruitment of BRCA1, resection, and promoting repair via HDR[42]. Sites of IR-induced DNA damage were identified by the presence of γH2AX foci, a marker of DNA damage signaling that is visible immediately after irradiation and provides the platform for the recruitment of many downstream signaling factors. The telomerase-positive and ALT cell lines examined all showed ~70-90% of cells with γH2AX foci and, surprisingly, all showed similar percentages of cells with

Rif1 at these sites of damage (Figure 7). The HeLa cells invariably had more intense Rif1 foci than all other cell lines examined, but the percentage of ALT cells with Rif1 foci was consistent with that observed in the BJ hTERT or RPE hTERT cell lines.

Given the mutual exclusion of Rif1 and BRCA1 at sites of damage, I also examined BRCA1 foci formation after irradiation to see if the reduced protein levels of Rif1 manifested as an increase in cells with BRCA1 foci. The telomerase-positive and ALT cell lines again showed similar percentages of cells with DNA damage, as indicated by γH2AX foci formation (Figure 8). The percentage of damaged cells containing BRCA1 foci was much more variable than that observed for Rif1. And although not apparent from scoring all cells containing BRCA1 foci, several ALT cell lines appeared to have more intense BRCA1 foci than those observed in the telomerase-positive cells. My initial experiments were performed with a single dose of irradiation and analyzed at a single time point. Although the dose of irradiation administered was relatively low, the two-hour time point examined may be late enough after irradiation to mask an early recruitment defect for Rif1. And while cell cycle analysis is necessary to confirm these populations did not contain dramatically different percentages of cells in S/G2, the highly variable level of BRCA1 foci formation together with the variation in foci intensity suggest further experiments are necessary and possibly very informative. The ALT cell lines could each individually adjust the Rif1 and BRCA1-mediated pathways in different ways, with the ultimate effect of enhancing single-stranded DNA to promote HDR. A better readout of these pathways might therefore be foci formation of the single-stranded DNA binding protein RPA, which I am currently investigating.

The potential alterations to the Rif1/BRCA1 pathways may be an important contributor to the ALT mechanism of telomere maintenance, particularly in cooperation with ATRX deficiencies. Telomere replication problems, both dependent and independent of ATRX loss, would elevate the occurrence of stalled and collapsed forks within telomeric DNA. In the absence of sufficient Rif1 protein, excessive resection of these forks may occur and

generate enhanced levels of single-stranded DNA that render the telomeres highly recombinogenic. Combined with the telomere-specific cohesion defect resulting from loss of ATRX, defective forks could be repaired using non-sister telomeres, thereby abolishing the restriction on both template use and register. This proposed mechanism could thus provide an explanation for the observed changes in telomere length associated with ALT, as well as the characteristic telomere recombination events.

Key Research Accomplishments

- Established ATRX as a key protein mediating telomere cohesion
- Confirmed ATRX functions in the SA1-mediated pathway of telomere cohesion
- Identified telomere cohesion defects as capable of promoting non-sister telomere interactions (relevant for the ALT mechanism of telomere maintenance)
- Identified a telomere-specific cohesion defect in ALT cell lines

Conclusion

I have clearly established a role for ATRX in promoting sister telomere cohesion, and demonstrated that disruption of this critical function can promote the inappropriate non-sister telomere interactions that would facilitate the recombination-mediated mechanism of telomere maintenance in ALT cells. I believe this accomplishes the goal outlined in *Task 1* of my SOW, and identifies the functional significance of the ATRX mutations in ALT cells. Despite the characteristic disruption of ATRX in ALT cell lines, I have demonstrated in MEFs and other laboratories have reported in human cells that deletion of ATRX alone is not sufficient to unleash telomere recombination or promote immortalization via the ALT pathway. Due to the fact that other laboratories have examined and reported their findings that cells deficient in ATRX alone do not preferentially induce ALT, it is not beneficial to invest a substantial amount of time repeating these experiments, as I had proposed in *Task 2* of my SOW. Instead, I am now

focused on identifying the additional pathway disruptions that, when combined with ATRX deficiencies, can promote the characteristics of ALT. My data in MEFs suggest ATRX deficiency alone may not be sufficient to cause telomere deprotection or telomere replication problems, an apparent requisite for initiation of the inappropriate HDR repair events. I have demonstrated that ALT telomeres do display elevated levels of telomere replication problems though (Figure 3C-D). Additionally, I have preliminary evidence suggesting the Rif1/BRCA1 pathways may be perturbed in ALT cell lines. The combination of telomere replication problems and Rif1 deficiencies could certainly increase the abundance of telomeric single-stranded DNA and promote HDR-mediated repair. However, in the context of efficient sister telomere cohesion these repair events would preferentially occur with the sister chromatid and produce no changes in telomere length or template. The disruption of telomere cohesion by loss of ATRX is critical because it allows repair to occur with non-sister telomeres, and it is this abolished restriction on both template use and register that is necessary to effect the mechanism responsible for telomere maintenance in ALT cells. Confirming the deficiencies in the Rif1 pathway and determining whether the combined loss of ATRX and Rif1 can facilitate telomere recombination is currently a high priority. *Task 3* of my SOW also remains a critical undertaking, and identifying any chemotherapeutic sensitivities of these cells will be immensely useful in employing better treatment options for patients with tumors reliant on ALT.

Publications, Abstracts and Presentations

Nothing to report.

Inventions, Patents and Licenses

Nothing to report.

Reportable Outcomes

Nothing to report.

Other Achievements

Nothing to report.

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Appendices

Figure 1. ATRX-deficient cells display a telomere-specific cohesion defect. **A.** Immunoblot for ATRX and γtubulin (loading control) in two independent $ATRX^{flox}$ MEF lines before and 96hr after CRE infection. **B.** FISH analysis of $ATRX^{flox}$ interphase cells before and 96hr after CRE infection, using probes for the subtelomeric and arm regions of chromosomes 8 and 10, as indicated. **C.** Quantitation of the FISH signals observed as doublets for the telomere and arm probes of chromosome 10 after deletion of ATRX from two independent cell lines. Approximately 100 FISH signals for each probe were analyzed, and bars represent the average and SD from four independent experiments. **D.** Quantitation of the FISH signals observed as doublets for the telomere and arm probes of chromosome 8 after deletion of ATRX from two independent cell lines. Approximately 100 FISH signals for each probe were analyzed, and bars represent the average and SD from three independent experiments. **E.** FISH analysis of telomerase-positive and ALT interphase cells using probes for the

subtelomeric and arm regions of chromosome 4. **F.** Ratio of the percent telomere doublets to percent arm doublets for the chromosome 4 FISH signals analyzed. Approximately 100 FISH signals for each probe were analyzed in triplicate for each cell line.

Figure 2. ATRX deletion enhances non-sister telomere associations. A. Immunoblot for ATRX and γtubulin (loading control) in the indicated MEF cells before and 96hr after CRE infection. Graphs display the quantitation of sister chromatid **(B)** and non-sister chromatid **(C)** fusion events observed after deletion of TPP1 and ATRX, individually and collectively, from four independent experiments. The boxes represent the minimum and maximum values observed with a bar at the median. **D.** Representative images showing CO-FISH staining of metaphase chromosomes from the indicated cell lines used to assess sister and non-sister chromatid fusions. Circles highlight the indicated fusion event.

Figure 3. ALT cell lines, but not ATRX-deficient MEFs, show evidence of telomere replication problems. A. Representative images showing CO-FISH staining of metaphase chromosomes from $ATRX^{flox}$ MEFs before and 96hr after CRE-mediated deletion. Cells were additionally treated with 0.3μM aphidicolin for 16hr prior to harvest where indicated. **B.** Quantitation of fragile telomeres in two independent $ATRX^{flox}$ MEF lines after CRE infection and after aphidicolin treatment. Bars represent average and SD from three independent experiments. **C.** Representative images of telomere FISH signals observed in telomerase-positive and ALT cell lines. **D.** Quantitation of fragile telomeres in telomerase-positive (HeLa and JFCF6/T.1F) and ALT cell lines from three independent experiments. Boxes represent the minimum and maximum values observed with a bar at the median.

Figure 4. ATRX and SA1 promote telomere cohesion through a common pathway. A. Immunoblot for ATRX, SA1, and γtubulin (loading control) in $ATRX^{flox}$ MEFs infected with an shRNA targeting SA1 (or empty vector) followed by CRE infections, where indicated. **B.** FISH analysis of $ATRX^{flox}$ interphase cells after knockdown of SA1, deletion of ATRX, or both. The FISH probe for the subtelomeric region of chromosome 10 is shown in green and that for the arm region of chromosome 10 is red. **C.** Quantitation of FISH signals observed as doublets for the telomere and arm probes of chromosome 10 after knockdown of SA1, deletion of ATRX, or both. Approximately 100 FISH signals for each probe were analyzed, and bars represent the mean and SEM from two independent experiments. **D.** Immunoblot for ATRX, SA1 and γtubulin in the indicated MEF cell lines infected with an empty vector (pLKO) or shRNA to SA1, followed by CRE infections, where indicated. Graphs display the quantitation of sister chromatid fusions **(E)** and non-sister chromatid fusions **(F)** observed in the indicated cell lines after knockdown of SA1, deletion of ATRX/TPP1, or both. Bars represent the mean and SEM from two independent experiments.

Figure 5. Deletion of ATRX does not affect shelterin or SA1 association with telomeric DNA. A. Telomeric ChIP for the shelterin proteins TRF1, TRF2, TIN2, TPP1, and Pot1, along with ATRX and SA1 in $ATRX^{flox}$ MEFs before and after CRE-mediated ATRX deletion (96hr after H&R; ~40 population doublings after pWZL). Pre-immune serum was used as a negative control. **B.** Quantitation of the ChIP data shown in A. Bars indicate the average and SD from three independent experiments.

Figure 6. Rif1 protein levels are highly variable in ALT cell lines. Immunoblot for Rif1 and actin in telomerase-positive (BJ hTERT, RPE hTERT, and HeLa) and ALT cell lines. Center panel shows a longer exposure of the same Rif1 immunoblot depicted directly above.

Figure 7. Irradiation-induced Rif1 foci formation appears normal in ALT cell lines. A. Immunofluorescence staining of Rif1 and γH2AX in telomerase-positive (HeLa, BJ hTERT, RPE hTERT) and ALT cell lines 2hr after receiving 3Gy of irradiation. **B.** Quantitation of the percentage of cells containing γH2AX foci (>10/cell), and γH2AX-positive cells containing Rif1 co-localizing foci (>5/cell).

Figure 8. Irradiation-induced BRCA1 foci formation is highly variable in ALT cell lines. A. Immunofluorescence staining of BRCA1 and γH2AX in telomerase-positive (HeLa, BJ hTERT, RPE hTERT) and ALT cell lines 2hr after receiving 3Gy of irradiation. **B.** Quantitation of the percentage of cells containing γH2AX foci (>10/cell), and γH2AX-positive cells containing BRCA1 co-localizing foci (>5/cell).

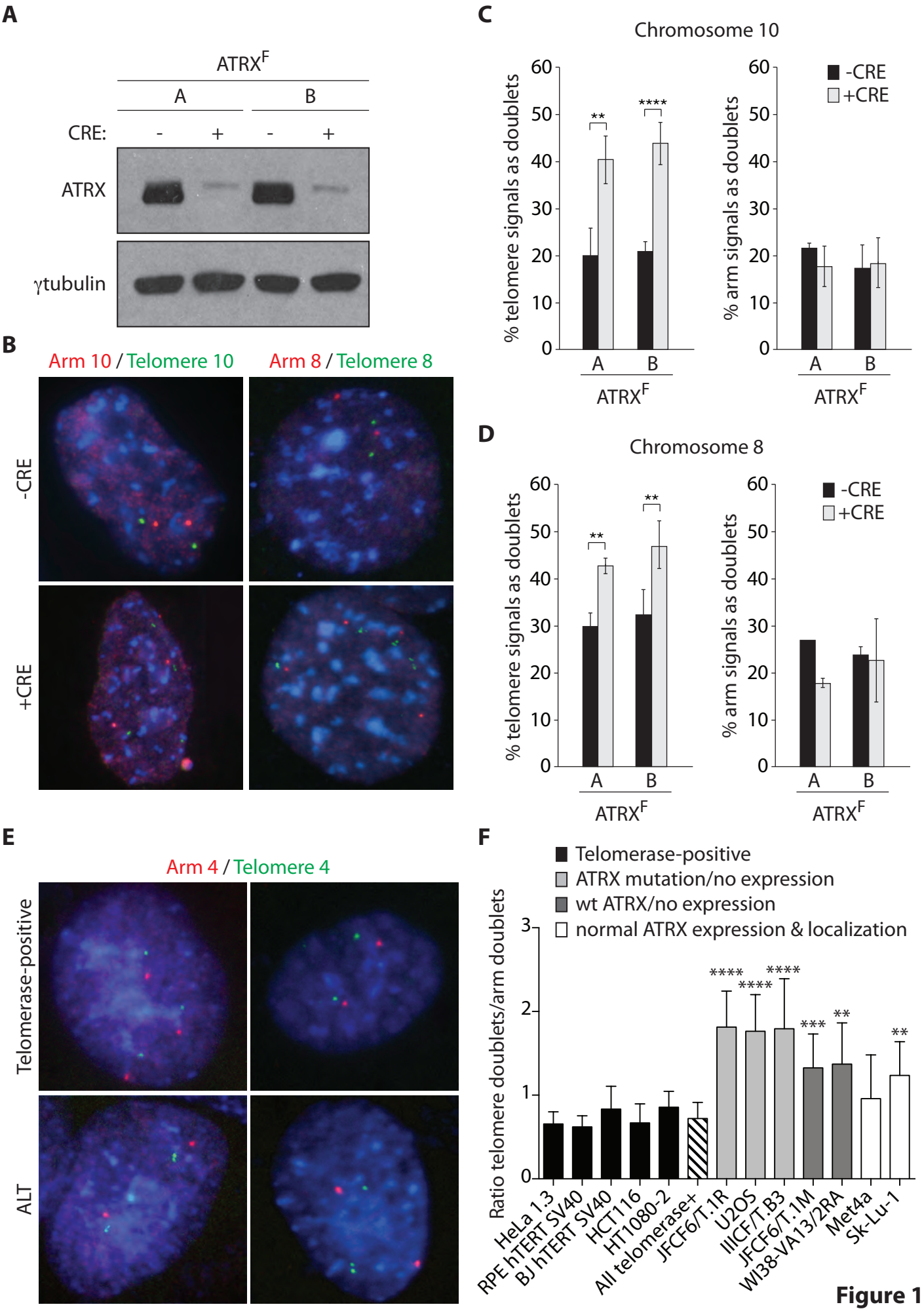
A
ATRX^F
A
B
CRE:
-
+
-
+
ATRX
γtubulin
B
Arm 10 / Telomere 10
Arm 8 / Telomere 8
-CRE
+CRE
C
Chromosome 10
% telomere signals as doublets
% arm signals as doublets
-CRE
+CRE
**
****
A
B
ATRX^F
D
Chromosome 8
% telomere signals as doublets
% arm signals as doublets
-CRE
+CRE
**
**
A
B
ATRX^F
E
Arm 4 / Telomere 4
Telomerase-positive
ALT
F
Telomerase-positive
ATRX mutation/no expression
wt ATRX/no expression
normal ATRX expression & localization
Ratio telomere doublets/arm doublets
****
****
****
***
**
**
HeLa 1.3
RPE hTERT SV40
BJ hTERT SV40
HCT116
HT1080-2
All telomerase+
JFCF6/T.1R
U2OS
IIICF/T.B3
JFCF6/T.1M
WI38-VA13/2RA
Met4a
Sk-Lu-1

Figure 1

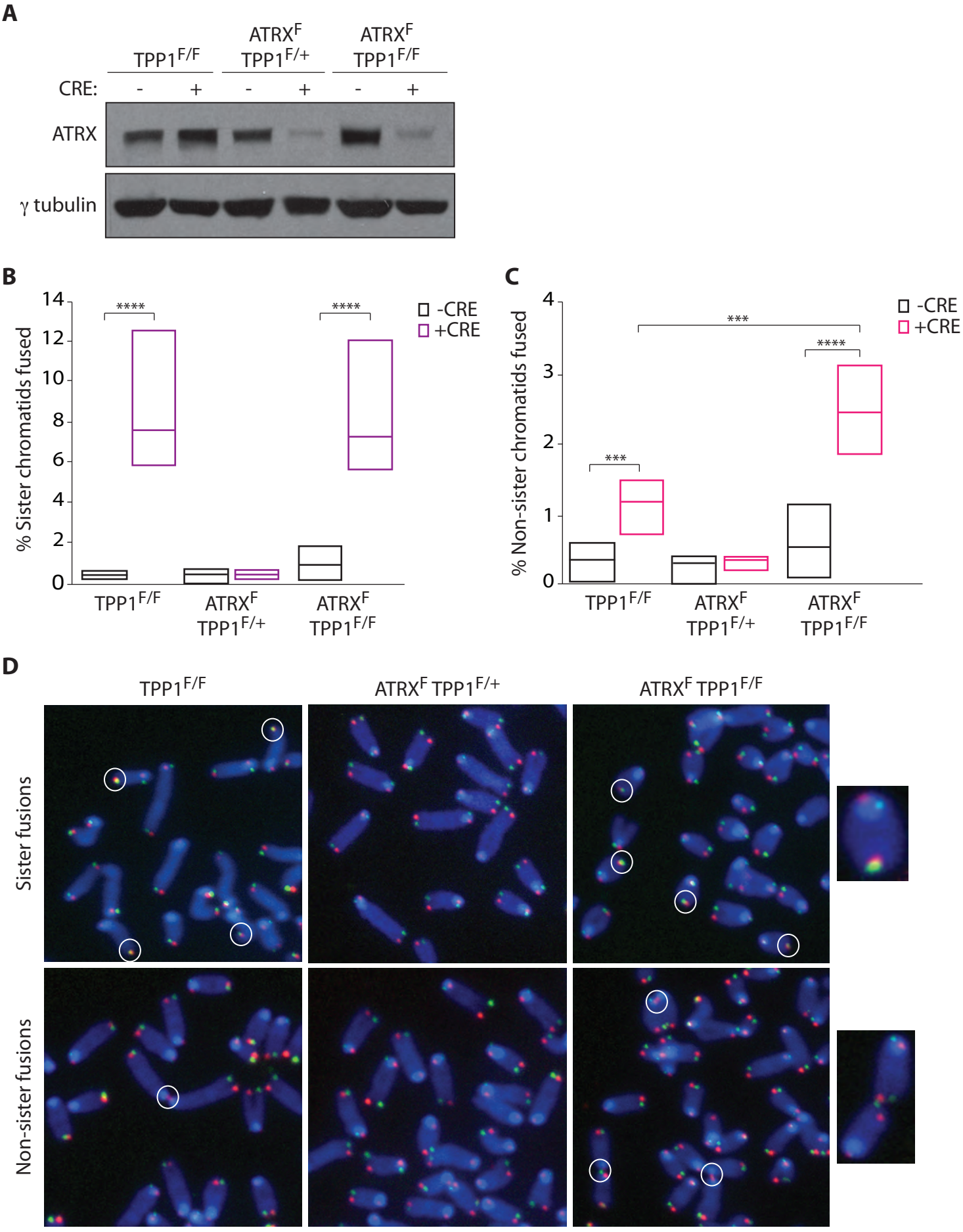
A
TPP1F/F
ATRXF TPP1F/+
ATRXF TPP1F/F
CRE: - + - + - +
ATRX
γ tubulin
B
% Sister chromatids fused
14
12
10
8
6
4
2
0
****
****
-CRE
+CRE
TPP1F/F
ATRXF TPP1F/+
ATRXF TPP1F/F
C
% Non-sister chromatids fused
4
3
2
1
0
***
***
****
-CRE
+CRE
TPP1F/F
ATRXF TPP1F/+
ATRXF TPP1F/F
D
TPP1F/F
ATRXF TPP1F/+
ATRXF TPP1F/F
Sister fusions
Non-sister fusions

Figure 2

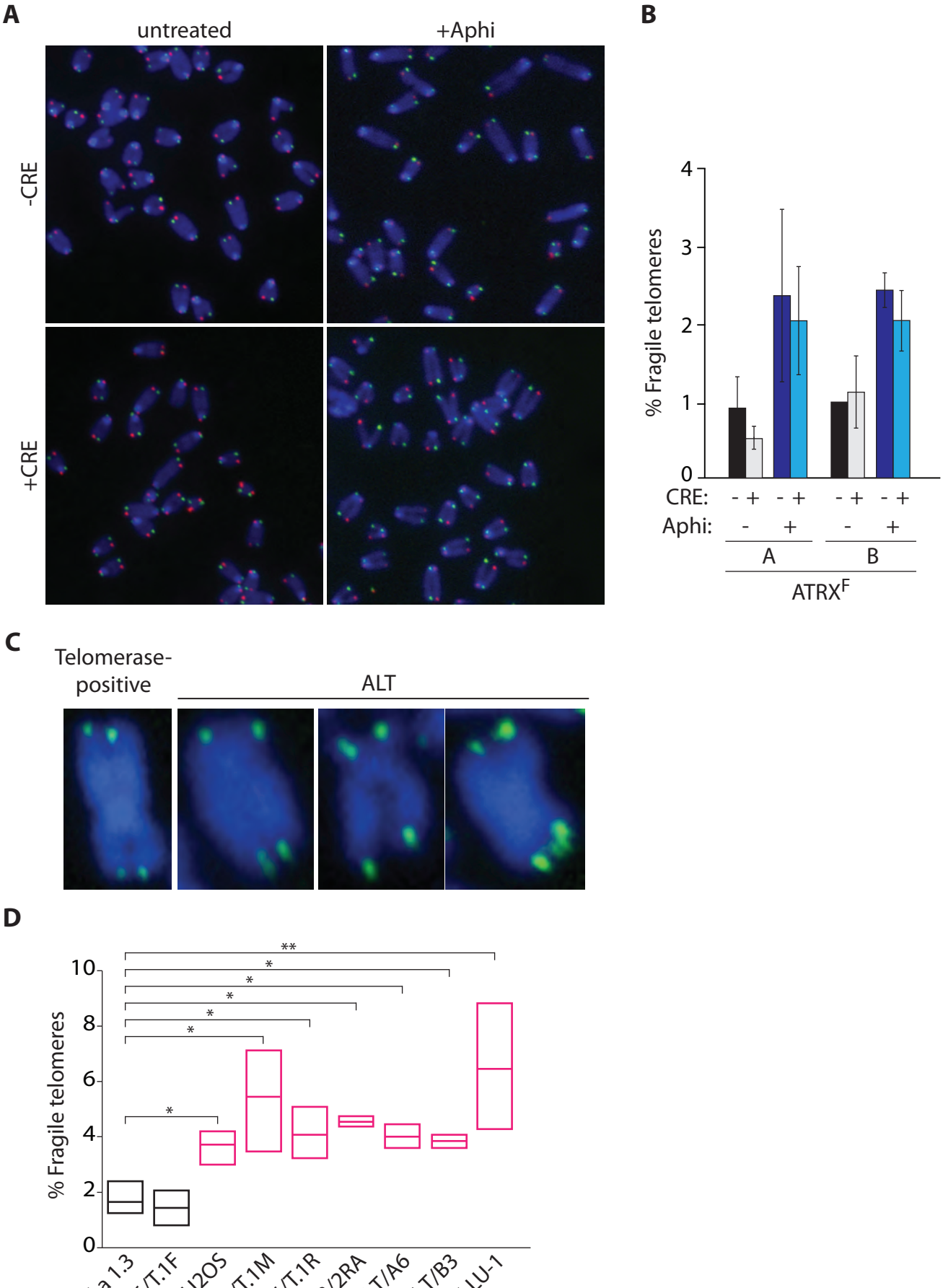
A
untreated
+Aphi
-CRE
+CRE
B
% Fragile telomeres
0
1
2
3
4
CRE: - + - + - + - +
Aphi: - + - +
A
B
ATRX^F
C
Telomerase-positive
ALT
D
% Fragile telomeres
0
2
4
6
8
10
**
*
*
*
*
*
*
HeLa 1.3
JFCF6/T.1F
U2OS
JFCF6/T.1M
JFCF6/T.1R
WI38.VA13/2RA
IIICF-T/A6
IIICF-T/B3
SK-LU-1

Figure 3

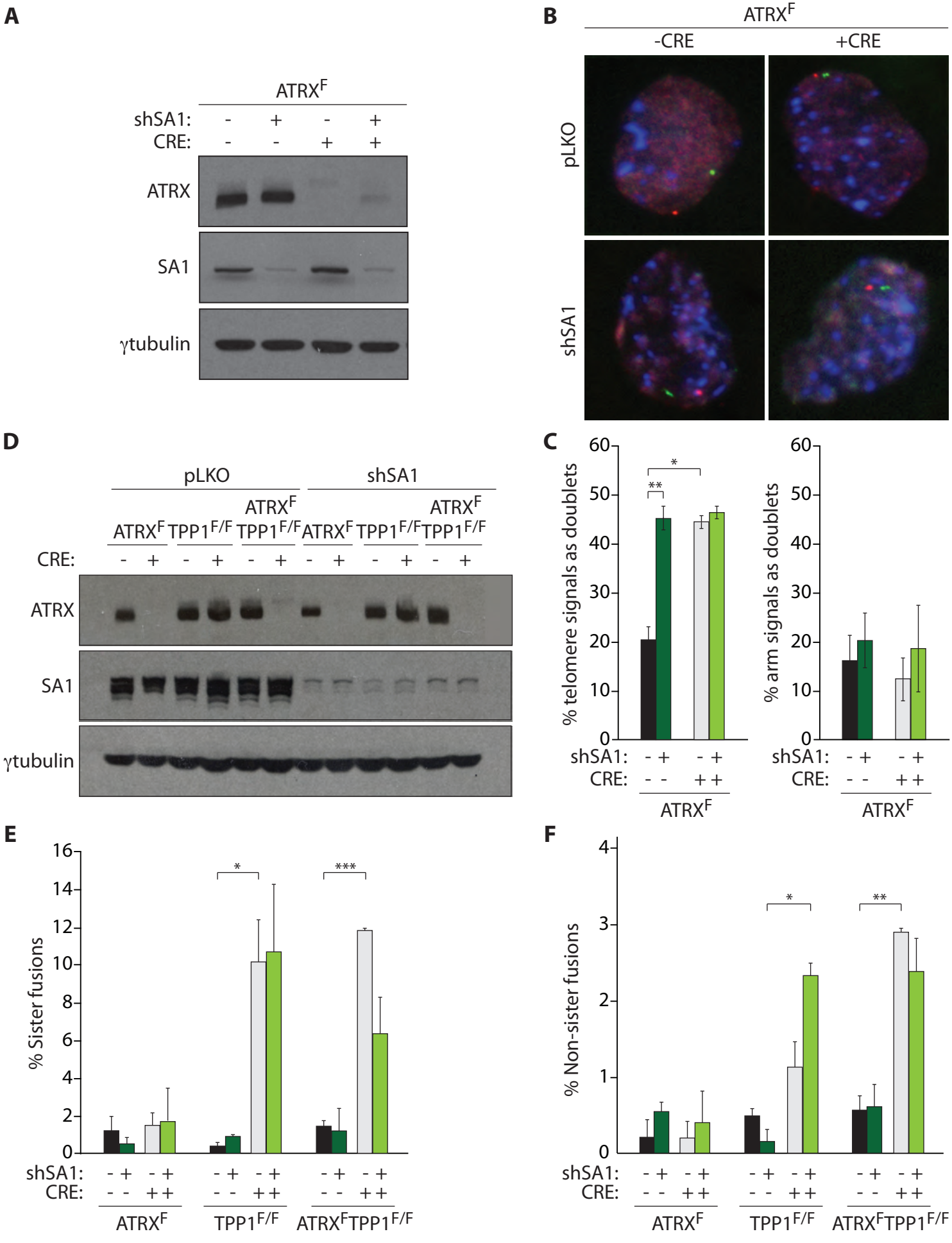
A
ATRX^F
shSA1: - + - +
CRE: - - + +
ATRX
SA1
γtubulin
B
ATRX^F
-CRE
+CRE
pLKO
shSA1
C
% telomere signals as doublets
% arm signals as doublets
shSA1: - + - +
CRE: - - + +
ATRX^F
D
pLKO
shSA1
ATRX^F
TPP1^F/F
ATRX^F TPP1^F/F
CRE: - + - + - + - + - + - +
ATRX
SA1
γtubulin
E
% Sister fusions
shSA1: - + - + - + - + - + - +
CRE: - - + + - - + + - - + +
ATRX^F
TPP1^F/F
ATRX^F TPP1^F/F
F
% Non-sister fusions
shSA1: - + - + - + - + - + - +
CRE: - - + + - - + + - - + +
ATRX^F
TPP1^F/F
ATRX^F TPP1^F/F

Figure 4

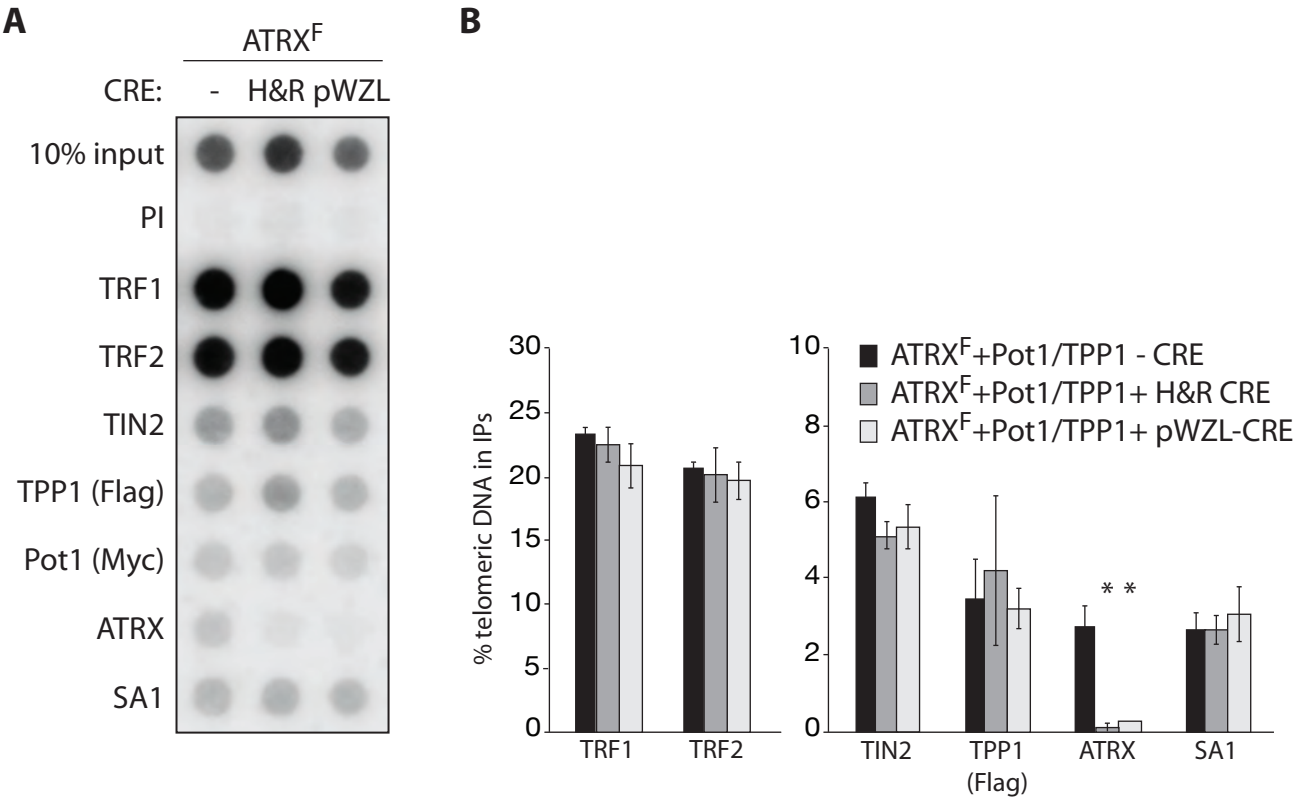
A
ATRX^F
CRE: - H&R pWZL
10% input
PI
TRF1
TRF2
TIN2
TPP1 (Flag)
Pot1 (Myc)
ATRX
SA1
B
% telomeric DNA in IPs
30
25
20
15
10
5
0
10
8
6
4
2
0
ATRX^F+Pot1/TPP1 - CRE
ATRX^F+Pot1/TPP1+ H&R CRE
ATRX^F+Pot1/TPP1+ pWZL-CRE
* *
TRF1
TRF2
TIN2
TPP1 (Flag)
ATRX
SA1

Figure 5

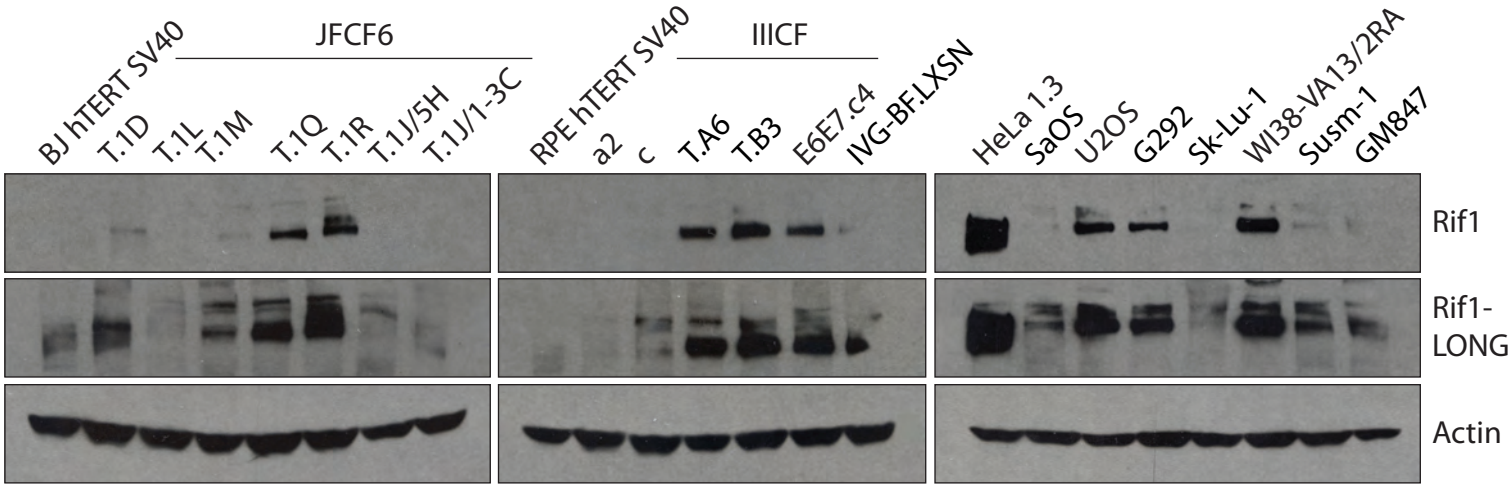
JFCF6
IIICF
BJ hTERT SV40
T.1D
T.1L
T.1M
T.1Q
T.1R
T.1J/5H
T.1J/1-3C
RPE hTERT SV40
a2
c
T.A6
T.B3
E6E7.c4
IVG-BF.LXSN
HeLa 1.3
SaOS
U2OS
G292
Sk-Lu-1
WI38-VA13/2RA
Susm-1
GM847
Rif1
Rif1-LONG
Actin

Figure 6

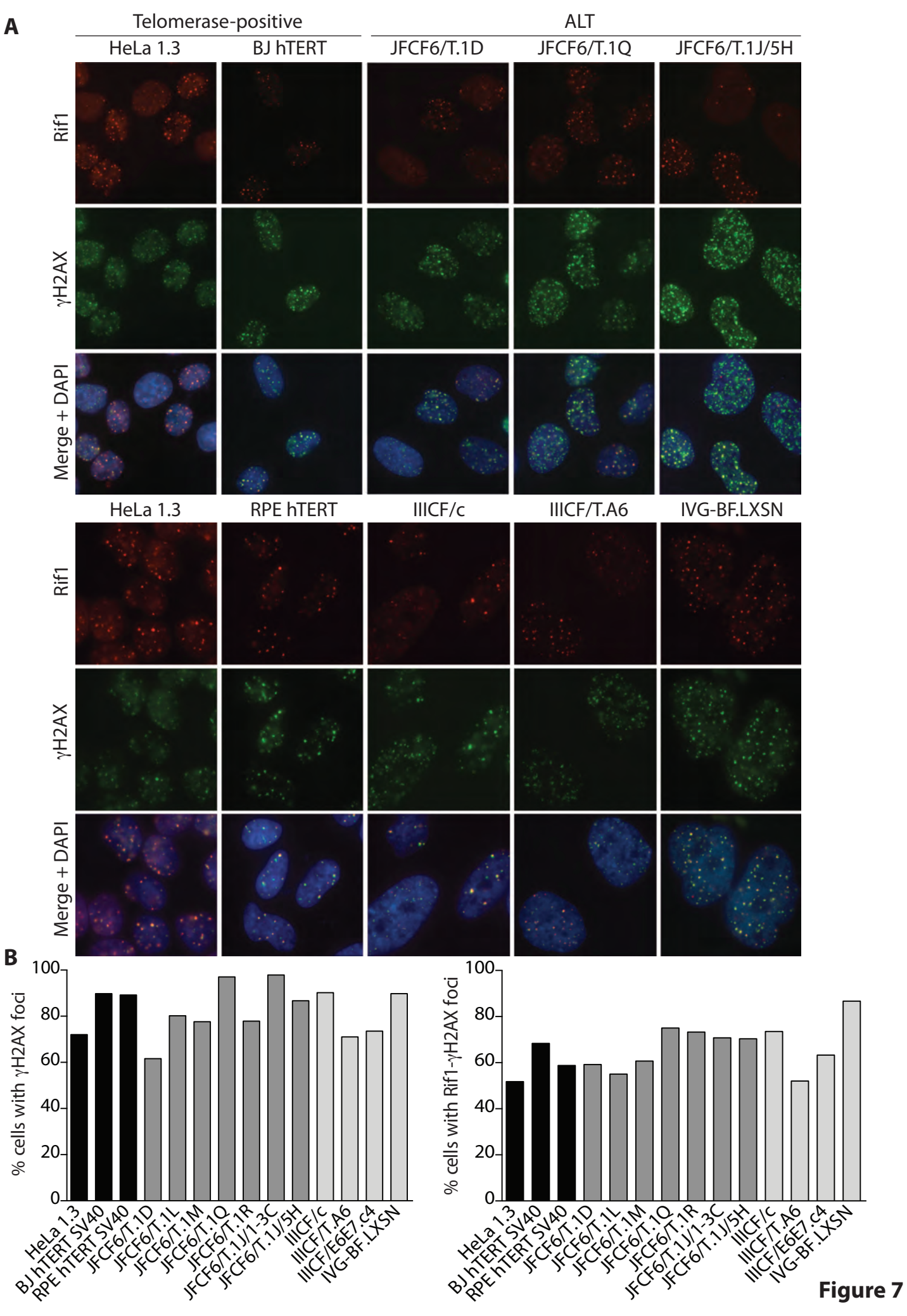
A
Telomerase-positive
ALT
HeLa 1.3
BJ hTERT
JFCF6/T.1D
JFCF6/T.1Q
JFCF6/T.1J/5H
Rif1
γH2AX
Merge + DAPI
HeLa 1.3
RPE hTERT
IIICF/c
IIICF/T.A6
IVG-BF.LXSN
Rif1
γH2AX
Merge + DAPI
B
% cells with γH2AX foci
100
80
60
40
20
0
HeLa 1.3
BJ hTERT SV40
RPE hTERT SV40
JFCF6/T.1D
JFCF6/T.1L
JFCF6/T.1M
JFCF6/T.1Q
JFCF6/T.1R
JFCF6/T.1J/1-3C
JFCF6/T.1J/5H
IIICF/c
IIICF/T.A6
IIICF/E6E7.c4
IVG-BF.LXSN
% cells with Rif1-γH2AX foci
100
80
60
40
20
0
HeLa 1.3
BJ hTERT SV40
RPE hTERT SV40
JFCF6/T.1D
JFCF6/T.1L
JFCF6/T.1M
JFCF6/T.1Q
JFCF6/T.1R
JFCF6/T.1J/1-3C
JFCF6/T.1J/5H
IIICF/c
IIICF/T.A6
IIICF/E6E7.c4
IVG-BF.LXSN

Figure 7

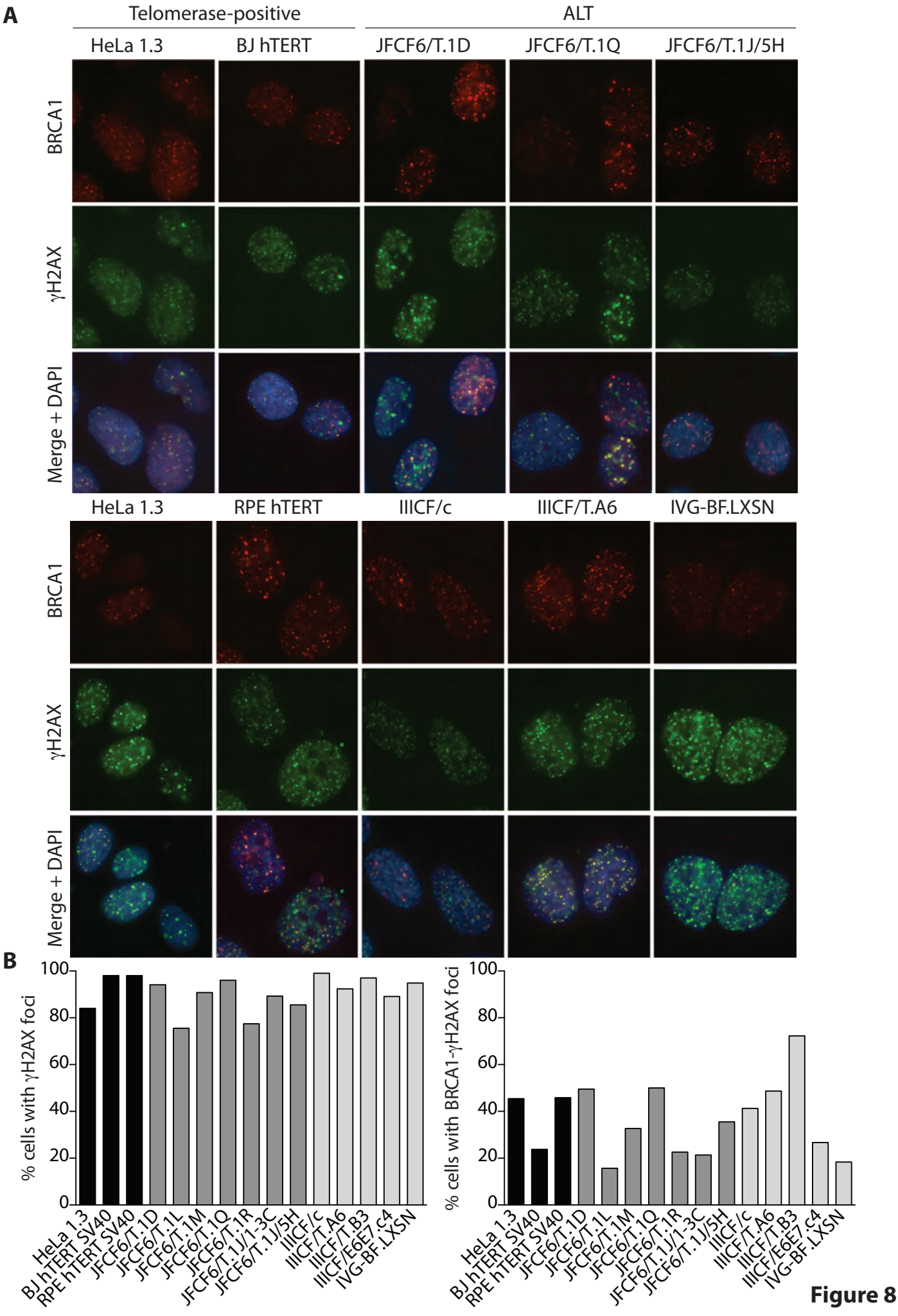
A
Telomerase-positive
ALT
HeLa 1.3
BJ hTERT
JFCF6/T.1D
JFCF6/T.1Q
JFCF6/T.1J/5H
BRCA1
γH2AX
Merge + DAPI
HeLa 1.3
RPE hTERT
IIICF/c
IIICF/T.A6
IVG-BF.LXSN
BRCA1
γH2AX
Merge + DAPI
B
% cells with γH2AX foci
100
80
60
40
20
0
HeLa 1.3
BJ hTERT SV40
RPE hTERT SV40
JFCF6/T.1D
JFCF6/T.1L
JFCF6/T.1M
JFCF6/T.1Q
JFCF6/T.1R
JFCF6/T.1J/1-3C
JFCF6/T.1J/5H
IIICF/c
IIICF/T.A6
IIICF/T.B3
IIICF/E6E7.c4
IVG-BF.LXSN
% cells with BRCA1-γH2AX foci
100
80
60
40
20
0
HeLa 1.3
BJ hTERT SV40
RPE hTERT SV40
JFCF6/T.1D
JFCF6/T.1L
JFCF6/T.1M
JFCF6/T.1Q
JFCF6/T.1R
JFCF6/T.1J/1-3C
JFCF6/T.1J/5H
IIICF/c
IIICF/T.A6
IIICF/T.B3
IIICF/E6E7.c4
IVG-BF.LXSN

Figure 8