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RESEARCH ARTICLE

# Cdk-dependent phosphorylation regulates TRF1 recruitment to PML bodies and promotes C-circle production in ALT cells

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## ABSTRACT

TRF1, a duplex telomeric DNA binding protein, is implicated in homologous-recombination-based alternative lengthening of telomeres, known as ALT. However, how TRF1 promotes ALT activity has yet to be fully characterized. Here we report that Cdk-dependent TRF1 phosphorylation on T371 acts as a switch to create a pool of TRF1, referred to as (pT371)TRF1, which is recruited to ALT-associated PML bodies (APBs) in S and G2 phases independently of its binding to telomeric DNA. We find that phosphorylation of T371 is essential for APB formation and C-circle production, both of which are hallmarks of ALT. We show that the interaction of (pT371)TRF1 with APBs is dependent upon ATM and homologous-recombination-promoting factors Mre11 and BRCA1. In addition, (pT371)TRF1 interaction with APBs is sensitive to transcription inhibition, which also reduces DNA damage at telomeres. Furthermore, overexpression of RNaseH1 impairs (pT371)TRF1 recruitment to APBs in the presence of campothecin, an inhibitor that prevents topoisomerase I from resolving RNA–DNA hybrids. These results suggest that transcription-associated DNA damage, perhaps arising from processing RNA–DNA hybrids at telomeres, triggers (pT371)TRF1 recruitment to APBs to facilitate ALT activity.

**KEY WORDS: Cdk, TRF1, PML bodies, ALT**

## INTRODUCTION

In most human somatic cells, telomeres shorten with each round of DNA replication, in part because of an inability of DNA polymerases to fill in the gap left from removal of the last RNA primer (Levy et al., 1992). When the length of telomeric DNA becomes critically short, the DNA damage response is activated, triggering the induction of replicative senescence (d'Adda di Fagagna et al., 2003). About 85-90% of human cancers avoid replicative senescence and gain unlimited growth potential by activating telomerase (Shay and Bacchetti, 1997). The remaining 10-15% of human cancers do not activate telomerase but instead maintain their telomere length through a homologous-recombination-based mechanism, referred to as alternative lengthening of telomeres (ALT) (Cesare and Reddel, 2010). ALT tends to be associated with aggressive cancers including osteosarcomas, soft tissue sarcomas, gastric carcinomas, astrocytomas and neuroblastomas, as well as a subset of *in vitro* transformed cell lines (Henson and Reddel, 2010).

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ALT cells carry several hallmarks (Cesare and Reddel, 2010), which include telomere length heterogeneity, a high level of extra-chromosome telomeric DNA such as C-circles, as well as PML bodies containing telomeric chromatin, referred to as ALT-associated PML bodies (APBs) (Henson and Reddel, 2010). APBs contain many proteins involved in DNA replication, recombination and repair (Chung et al., 2011), including the Mre11–Rad50–Nbs1 complex (Nbs1 is also known as NBN) (Wu et al., 2003, 2000), BRCA1, Rad51, Rad52 and RPA (Yeager et al., 1999). It has been reported that APB formation requires Nbs1, which mediates the recruitment of Mre11, Rad50 and BRCA1 to APBs (Wu et al., 2003). DNA synthesis has been shown to occur at APBs (Grobelny et al., 2000; Wu et al., 2000) and has been found to be dependent upon ATM and ATR (Nabetani et al., 2004). It has been suggested that APBs might be sites where homologous-recombination-mediated telomere maintenance takes place (Draskovic et al., 2009; Wu et al., 2000). Disruption in the formation of APBs leads to telomere shortening in ALT cells (Zhong et al., 2007), suggesting that APBs are involved in ALT activity.

TRF1, a subunit of the shelterin (also known as telosome) complex that also includes TRF2 (also known as TERF2), TIN2 (also known as TINF2), hRap1 (also known as TERF2IP), TPP1 and POT1 (de Lange, 2005; Liu et al., 2004), is a multifunctional protein that is implicated in telomere length maintenance (Ancelin et al., 2002; van Steensel and de Lange, 1997), cell cycle progression (Shen et al., 1997; Zhou et al., 2003), resolution of sister telomeres (Canudas et al., 2007; McKerlie and Zhu, 2011) as well as DNA double-strand break repair (Kishi et al., 2001; McKerlie et al., 2013). In telomerase-expressing cells, TRF1 acts as a negative regulator of telomere length maintenance (Ancelin et al., 2002; van Steensel and de Lange, 1997) whereas in ALT cells, TRF1 is implicated as a positive mediator of homologous-recombination-based telomere maintenance (Jiang et al., 2007). Depletion of TRF1 impairs the formation of APBs (Jiang et al., 2007). TRF1 is reported to be SUMOylated and SUMOylation has been suggested to promote APB formation (Potts and Yu, 2007). However, whether other types of post-translational modifications such as phosphorylation might regulate ALT activity has remained largely uncharacterized.

TRF1 undergoes extensive phosphorylation (Walker and Zhu, 2012). It has been reported that Cdk1 phosphorylates TRF1 on T371 and that this phosphorylation regulates sister telomere resolution and DNA double-strand break repair in telomerase-expressing cells (McKerlie et al., 2013; McKerlie and Zhu, 2011); however, the role of this phosphorylation in ALT cells has yet to be characterized. Here, we report that Cdk-dependent phosphorylation of TRF1 on T371 promotes TRF1 interaction with APBs in S and G2 phases independently of its binding to telomeric DNA. Loss of TRF1 phosphorylation on T371 impairs APB formation and C-circle production, indicative of its important role in promoting ALT

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activity. We demonstrate that (pT371)TRF1 is associated with a subset of ALT telomeres that are predominantly dysfunctional as evidenced by their accumulation of γH2AX. The interaction of (pT371)TRF1 with APBs is dependent upon ATM and homologous-recombination-promoting factors Mre11 and BRCA1. We show that transcription inhibition not only impairs (pT371)TRF1 interaction with APBs but also reduces telomeric accumulation of γH2AX. Furthermore, overexpression of RNaseH1 impairs (pT371)TRF1 interaction with APBs in the presence of camptothecin, an inhibitor of topoisomerase I. Taken together, these results suggest that Cdk activity in S and G2 phases controls TRF1 interaction with APBs and that this interaction is triggered by transcription-associated DNA damage, perhaps arising from processing RNA–DNA hybrids at telomeres.

## RESULTS

### Cdk-dependent punctate nuclear staining of phosphorylated (pT371)TRF1

Cdk1 phosphorylates TRF1 on T371 and this phosphorylation creates a stable pool of TRF1, referred to as (pT371)TRF1, which exists largely free of telomere chromatin (McKerlie and Zhu, 2011). To investigate if (pT371)TRF1 might play a role in the regulation of ALT activity, we first examined the nuclear staining of (pT371)TRF1 in several ALT cell lines (GM847, U2OS and WI38VA13/2RA) through analysis of indirect immunofluorescence with a phospho-specific anti-TRF1-pT371 antibody. In all ALT cell lines examined, (pT371)TRF1 was found to exhibit punctate nuclear staining in about 50% of interphase cells whereas its nuclear staining in the remaining population of interphase cells was diffuse and barely detectable (Fig. 1A; Fig. S1A). This staining was in sharp contrast to anti-TRF1 staining, which predominantly detects telomere-bound TRF1 and was observed in all interphase cells (Fig. S1B). These results suggest that the punctate nuclear staining of (pT371)TRF1 might be cell-cycle regulated.

To investigate the nature of cell-cycle-dependent staining of (pT371)TRF1, we performed dual indirect immunofluorescence with an anti-pT371 antibody in conjunction with an antibody against cyclin A, a marker for S and G2 cells. The punctate nuclear staining of (pT371)TRF1 was overwhelmingly seen in cells staining positive for cyclin A (Fig. 1B). Over 80% and 90% of GM847 and U2OS cells, respectively, exhibiting punctate nuclear staining of (pT371)TRF1 were positive for cyclin A (Fig. 1C), suggesting that the punctate nuclear staining of (pT371)TRF1 occurs predominantly in the S and G2 phases of the cell cycle.

T371 of TRF1 is reported to be a target of Cdk1 (McKerlie and Zhu, 2011). To investigate if Cdk activity might regulate the punctate nuclear staining of (pT371)TRF1 in S and G2 cells, we subjected GM847 cells to a double thymidine block. Western analysis revealed that TRF1 phosphorylation on T371 started to increase as cells progressed through S and G2 phases, peaking 10 h post-release and falling sharply 16 h post-release from a double thymidine block (Fig. 1D), in agreement with previous finding (McKerlie and Zhu, 2011). We observed that the majority of GM847 cells at 10 h post-release were in mitosis and therefore we did not include them for examination of interphase nuclear staining of (pT371)TRF1. The number of GM847 cells exhibiting punctate nuclear staining of (pT371)TRF1 increased as cells progressed through S and G2 phases and then dropped sharply as cells entered G1 phase at 16 h post-release from a double thymidine block (Fig. 1E). In addition, we found that treatment with Cdk inhibitor roscovitine for one hour led to a significant reduction in the number of GM847 cells exhibiting the punctate nuclear staining of (pT371)TRF1 (Fig. 1F). Taken together, these results suggest that Cdk activity regulates the punctate nuclear staining of (pT371)TRF1, and further imply that the lack of detectable nuclear staining of (pT371)TRF1 in cyclin-A-negative cells probably results from a lack of Cdk-dependent T371 phosphorylation in G1 cells.

### Phosphorylated (pT371)TRF1 is preferentially associated with dysfunctional telomere ends

Our finding of punctate nuclear staining of (pT371)TRF1 prompted us to investigate if (pT371)TRF1 might be associated with ALT telomeres. Immunofluorescence combined with fluorescence *in situ* hybridization (IF-FISH) analysis by using an anti-pT371 antibody (red) in conjunction with a FITC-conjugated PNA probe against telomeric DNA (green) revealed that the number of punctate anti-pT371 foci (red) in a given interphase cell was always less than that of punctate telomere foci (green) (Fig. 2A). Whereas all punctate anti-pT371 foci seemed to colocalize well with telomeric DNA, not all telomere foci contained detectable (pT371)TRF1 staining (Fig. 2A), suggesting that (pT371)TRF1 is associated with a subset of interphase ALT telomeres.

Telomeres in ALT cells are known to elicit a DNA damage response (Cesare et al., 2009; Cho et al., 2014; Dunham et al., 2000; Wu et al., 2000). In agreement with previous findings, we found that GM847 cells exhibited a high level of genomic instability as evidenced by their accumulation of γH2AX staining (Fig. 2B). We estimated that at least 80% of GM847 cells exhibited the formation of γH2AX foci (Fig. 2C). The number of γH2AX foci per GM847 cell ranged from less than 5 to greater than 15 foci (Fig. 2C). Although the total number of cells exhibiting γH2AX foci did not change significantly throughout the cell cycle (Fig. 2C), the number of cells exhibiting greater than 15 γH2AX foci dropped sharply 16 h post-release from a double thymidine block (Fig. 2C), suggesting that the amount of DNA damage per cell is higher in S/G2 cells than in G1 cells.

Analysis of dual indirect immunofluorescence with an anti-pT371 antibody in conjunction with an anti-γH2AX antibody revealed very good colocalization between punctate anti-pT371 foci and a subset of γH2AX foci in GM847 interphase cells (Fig. 2B), suggesting that (pT371)TRF1 is associated with dysfunctional telomeres. Further analysis of (pT371)TRF1 colocalization with γH2AX in synchronized GM847 cells revealed that the level of (pT371)TRF1 colocalization with γH2AX was cell-cycle regulated, dipping to the lowest level 16 h post-release from a double thymidine block (Fig. 2D). The colocalization of (pT371)TRF1 with γH2AX was predominantly seen in GM847 cells exhibiting greater than 15 γH2AX foci (Fig. 2D). Taken together, these results suggest that (pT371)TRF1 might be recruited to telomeres in response to DNA damage.

We also investigated the association of (pT371)TRF1 with telomeres on metaphase chromosome spreads from GM847 cells. Analysis of indirect immunofluorescence revealed that (pT371)TRF1 was also associated with only a subset of metaphase chromosome ends (Fig. 2E). In addition, (pT371)TRF1 was found to be almost always associated with telomere ends that were also enriched for γH2AX (Fig. 2E). These results suggest that (pT371)TRF1 is preferentially recruited to dysfunctional telomere ends.

### Phosphorylated (pT371)TRF1 is a component of ALT-associated PML bodies (APBs)

Analysis of dual indirect immunofluorescence with an anti-pT371 antibody in conjunction with an anti-PML antibody in

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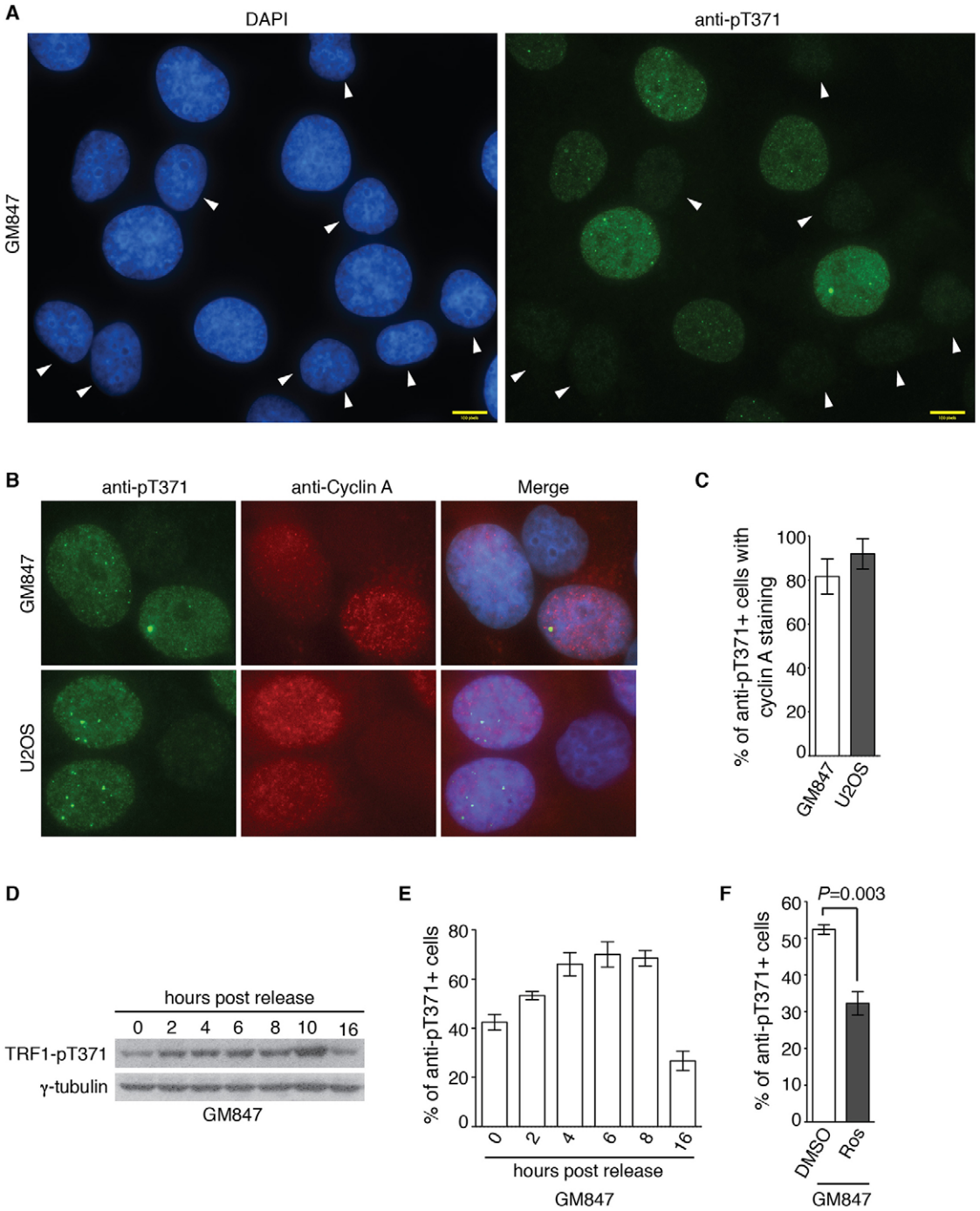


**Fig. 1. Cell cycle-dependent association of (pT371)TRF1 with ALT telomeres.** (A) Indirect immunofluorescence with rabbit anti-pT371 antibody. Cell nuclei were stained with DAPI in blue in this and subsequent figures. Arrowheads indicate nuclei with barely detectable anti-pT371 staining. (B) Immunofluorescence analysis with both anti-pT371 and anti-cyclin-A antibodies. (C) Quantification of the percentage of cells staining positive for both (pT371)TRF1 and cyclin A. A total of 500 cells from each independent experiment were scored in blind for each cell line. Standard deviations from three independent experiments are indicated. (D) Western analysis of GM847 cells post-release from a double thymidine block as indicated. Immunoblotting was carried out with an anti-pT371 antibody. The γ-tubulin blot was used as a loading control in this and subsequent figures. (E) Quantification of the percentage of synchronized GM847 cells staining positive for (pT371)TRF1. A total of 1000 cells from each independent experiment were scored in blind. Standard deviations from three independent experiments are indicated. (F) Quantification of the percentage of cells staining positive for (pT371)TRF1. GM847 cells were treated with either DMSO or roscovitine (20 μM), a Cdk inhibitor, for 1 h prior to IF analysis. Scoring was done as described in E. Standard deviations from three independent experiments are indicated.

ALT cell lines GM847 and U2OS revealed consistent and reproducible colocalization of labeled pT371 foci with PML foci (Fig. 3A), suggesting that (pT371)TRF1 is a component of APBs. This notion was further supported by IF-FISH analysis involving triple staining with an anti-pT371 antibody, an anti-PML antibody and a FITC-conjugated PNA probe against telomeric DNA, which demonstrated colocalization of (pT371)TRF1 foci with both telomeric DNA and PML (Fig. 3B).

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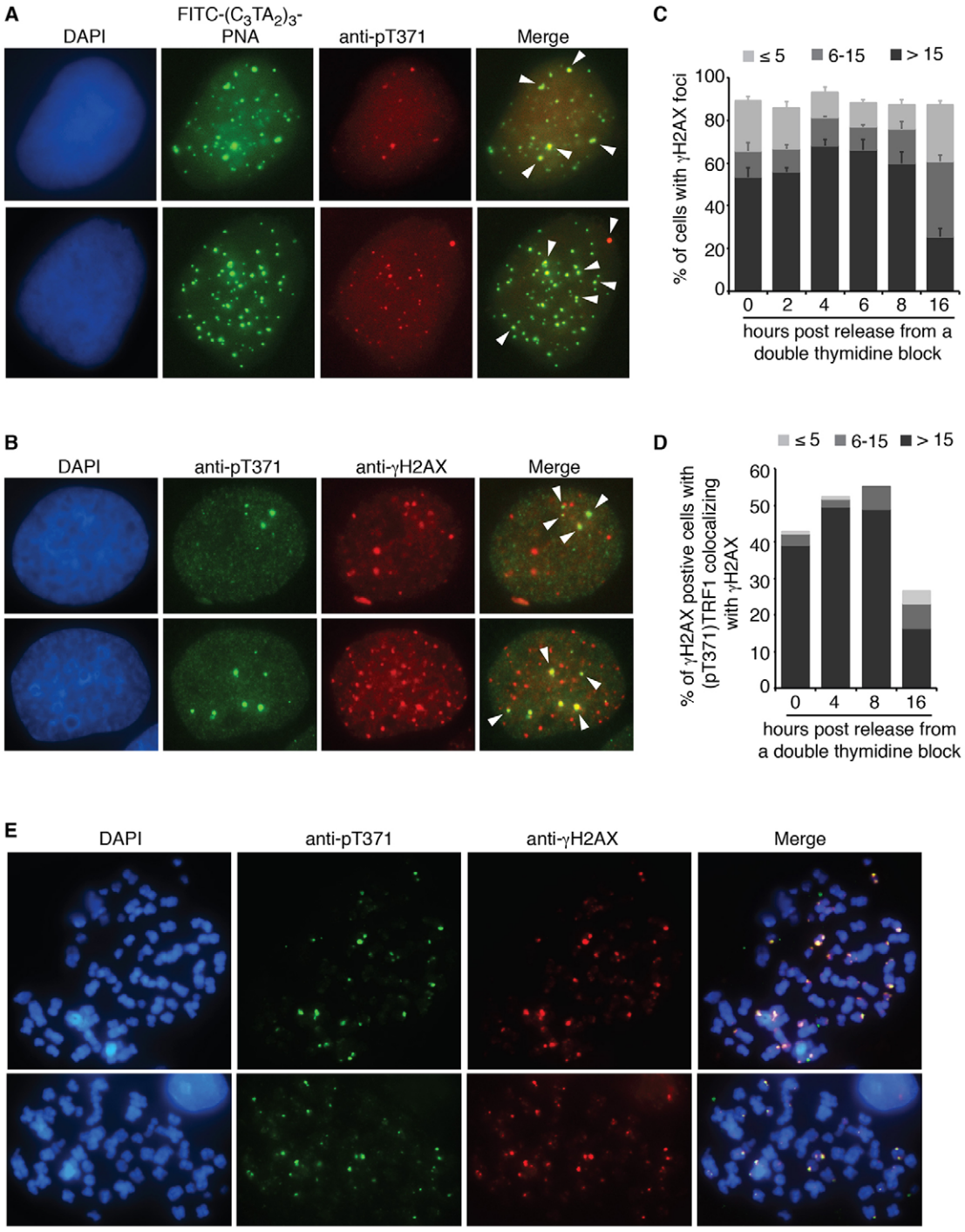


**Fig. 2. Phosphorylated (pT371)TRF1 is associated with a subset of ALT telomeres that are predominantly dysfunctional.** (A) IF-FISH analysis of interphase GM847 cells with a FITC-conjugated telomere-containing PNA probe (green) in conjunction with an anti-pT371 antibody (red). Arrowheads indicate colocalization of (pT371)TRF1 with telomeres. (B) Immunofluorescence analysis of interphase GM847 cells with both anti-pT371 and anti-γH2AX antibodies. Arrowheads indicate colocalization of (pT371)TRF1 with γH2AX foci. (C) Quantification of the percentage of synchronized GM847 cells exhibiting γH2AX foci as indicated. A total of 1000 cells from each independent experiment were scored in blind. Standard deviations from three independent experiments are indicated. Light grey bars, 1-5 γH2AX foci per cell; dark grey bars, 6-15 γH2AX foci per cell; black bars, >15 γH2AX foci per cell. (D) Quantification of the percentage of γH2AX-positive GM847 cells exhibiting (pT371)TRF1 colocalization with γH2AX foci as indicated. Scoring of colocalization was done in blind from captured cell images. The number of cells scored for each time point: 171 (0 h); 149 (4 h); 149 (8 h) and 208 (16 h). Light grey bars, 1-5 γH2AX foci per cell; dark grey bars, 6-15 γH2AX foci per cell; black bars, >15 γH2AX foci per cell. (E) Immunofluorescence analysis of metaphase GM847 cells with both anti-pT371 antibody and anti-γH2AX antibodies.

### TRF1 phosphorylation on T371 is needed to support APB formation and C-circle production

TRF1 is crucial for the formation of APBs (Jiang et al., 2007; Potts and Yu, 2007), which are hallmarks of ALT cells and are composed of shelterin proteins and repair factors such as Nbs1, a component of the Mre11 complex essential for APB formation (Jiang et al., 2007; Wu et al., 2003; Zhong et al., 2007). We observed the colocalization of TRF2, hRap1, TIN2 and Nbs1 with

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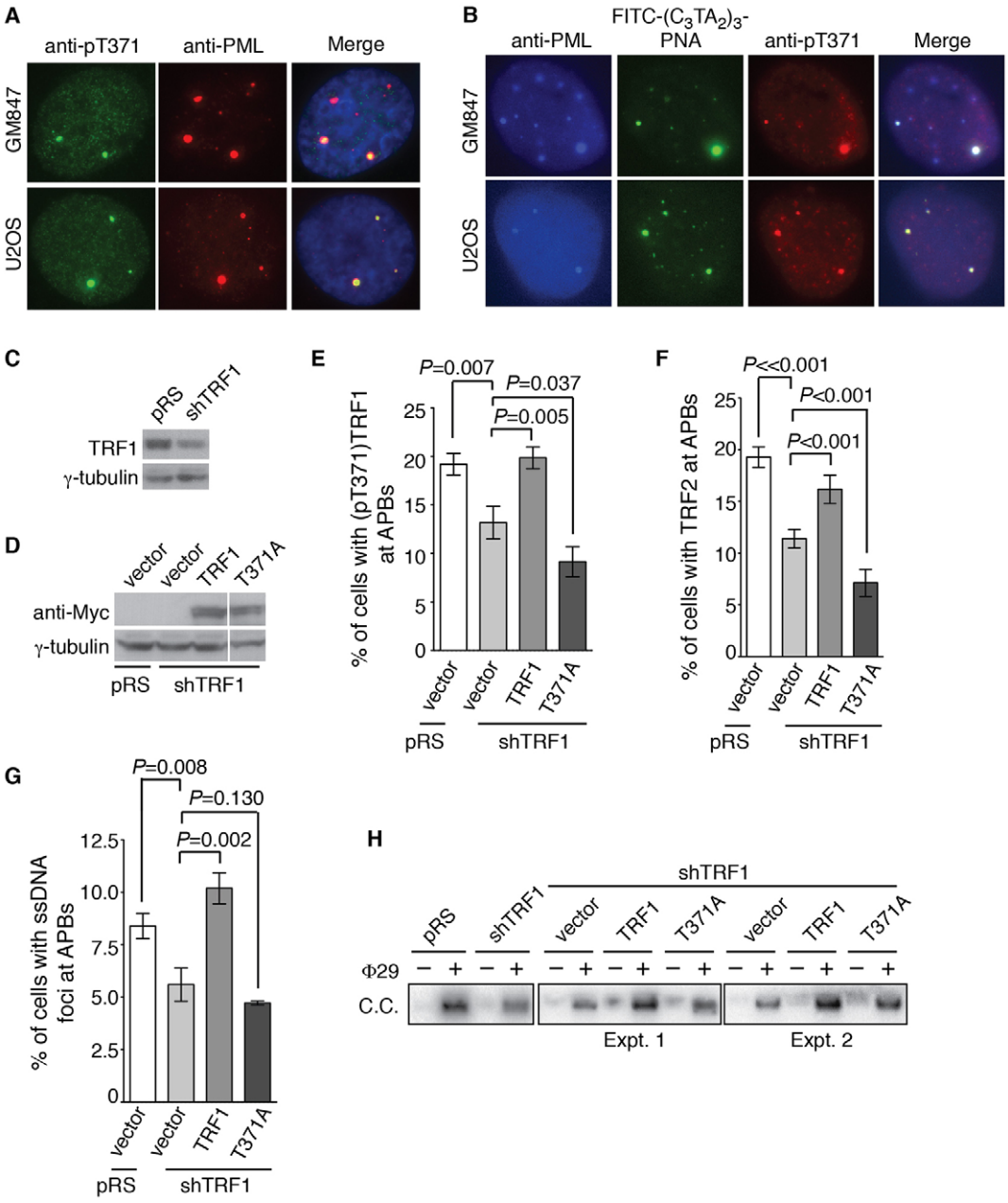


**Fig. 3. TRF1 phosphorylation at T371 is needed to support APB formation and C-circle production.** (A) Immunofluorescence analysis of GM847 and U2OS cells with both anti-pT371 and anti-PML antibodies. (B) IF-FISH analysis of GM847 and U2OS cells with a FITC-conjugated telomere-containing PNA probe in conjunction with anti-pT371 and anti-PML antibodies. (C) Western analysis of GM847 cells expressing the vector alone (pRS) or shRNA against TRF1 (shTRF1) as indicated. Immunoblotting was performed with anti-TRF1 and anti-γ-tubulin antibodies. (D) Western analysis of pRS- and shTRF1-expressing GM847 cells complemented with either the vector alone or various Myc-tagged TRF1 alleles as indicated. Immunoblotting was performed with anti-Myc and anti-γ-tubulin antibodies. (E) Quantification of the percentage of GM847 cells as indicated with (pT371)TRF1 at APBs. A total of 500 cells from each experiment were scored for each cell line as indicated in blind. Standard deviations from three independent experiments are indicated. (F) Quantification of the percentage of GM847 cells as indicated with TRF2 at APBs. Scoring was done as described in E. Standard deviations from six independent experiments are indicated. (G) Quantification of the percentage of cells with ssDNA foci at APBs. GM847 cells as indicated were incubated in the presence of 10 μM BrdU (Sigma) for 24 h prior to immunofluorescence analysis with anti-BrdU and anti-PML antibodies. A total of 1000 cells from each independent experiment were scored for each cell line in blind. Standard deviations from three independent experiments are indicated. (H) Analysis of C-circle formation. C.C., C-circles.

PML at APBs in GM847 cells (Fig. S2A), in agreement with previous findings (Jiang et al., 2007; Wu et al., 2003; Zhong et al., 2007). To investigate if phosphorylation of TRF1 on T371 might be involved in APB formation, we depleted endogenous TRF1 in GM847 cells (Fig. 3C) and then complemented TRF1-depleted GM847 cells with Myc-tagged shTRF1-resistant wild-type TRF1 or TRF1 carrying an amino acid substitution of T371A. The expression of Myc-tagged TRF1-T371A was comparable with that of Myc-tagged wild-type TRF1 (Fig. 3D). Depletion of TRF1 not only impaired the localization of (pT371)TRF1 to APBs (Fig. 3E) but also resulted in a significant decrease in the number of GM847 cells exhibiting colocalization of PML with TRF2 (Fig. 3F), hRap1 (Fig. S2B), TIN2 (Fig. S2C) and Nbs1 (Fig. S2D). Overexpression of Myc-tagged wild-type TRF1

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suppressed the impaired association of (pT371)TRF1, TRF2, hRap1, TIN2 and Nbs1 with PML bodies in TRF1-depleted GM8487 cells (Fig. 3E,F; Fig. S2B-D), whereas introduction of Myc-tagged TRF1 carrying a T371 mutation failed to do so (Fig. 3E,F; Fig. S2B-D). Taken together, these results suggest that phosphorylation of TRF1 on T371 is necessary for the assembly of APBs. Furthermore, our finding that Myc-tagged TRF1-T371A fails to rescue (pT371)TRF1 association with APBs confirms that the observed staining of pT371 at APBs is specific.

APBs are thought to be sites of homologous-recombination-mediated telomere synthesis and therefore, we asked if T371 phosphorylation might play a role in the generation of single-stranded DNA (ssDNA), a key intermediate of homologous recombination, within APBs. To visualize the presence of ssDNA within APBs, we performed dual indirect immunofluorescence with an anti-PML antibody in conjunction with an anti-BrdU antibody under non-denaturing conditions (Fig. S2E). We observed the presence of ssDNA within APBs in about 8-9% of GM847 cells expressing the vector alone (Fig. 3G). Depletion of TRF1 led to a significant decrease in the number of cells containing ssDNA within APBs (Fig. 3G). This decrease was suppressed by Myc-tagged wild-type TRF1 but not by Myc-tagged TRF1 carrying a T371A mutation (Fig. 3G), indicating that T371 phosphorylation is necessary for the generation of ssDNA within APBs. These results suggest that T371 phosphorylation is needed to facilitate homologous-recombination-mediated telomere synthesis.

We also examined the role of T371 phosphorylation in the production of C-circles, another key feature of ALT activity. Analysis of C-circle assays revealed that depletion of TRF1 resulted in a decrease in the level of C-circles in GM847 cells (Fig. 3H). Introduction of Myc-tagged wild-type TRF1 reproducibly rescued the level of C-circles in TRF1-depleted cells, whereas overexpression of Myc-tagged TRF1 carrying a T371A mutation failed to do so (Fig. 3H), indicating that T371 phosphorylation plays an important role in C-circle production in ALT cells.

### ATM and homologous-recombination-promoting factors Mre11 and BRCA1 mediate the association of (pT371)TRF1 with APBs

We have shown that (pT371)TRF1 is preferentially associated with dysfunctional telomere ends at APBs. To investigate how (pT371)TRF1 might be recruited to APBs, we asked if ATM, a master regulator of DNA damage response, might play a role in the regulation of (pT371)TRF1 association with APBs. To address this question, we performed indirect immunofluorescence with an anti-pT371 antibody in GM847 cells stably expressing the vector alone or shRNA against ATM. Depletion of ATM impaired the recruitment of (pT371)TRF1 to APBs (Fig. 4A,B). The impaired recruitment of (pT371)TRF1 to APBs was also observed in GM847 cells treated with KU55933, a specific inhibitor of ATM (Fig. 4C,D). Conversely, treatment with NU7026, a specific inhibitor of DNA-PKcs, had little impact on the association of (pT371)TRF1 with APBs (Fig. 4C,D). Knockdown or inhibition of ATM as well as inhibition of DNA-PKcs had little effect on the percentage of GM847 cells staining positive for cyclin A (Fig. 4E,F), indicating that the impaired recruitment of (pT371)TRF1 to APBs in ATM-depleted or -inhibited cells is unlikely to result from a change in the number of cyclin A-positive cells. Furthermore, knockdown of ATM did not alter the level of (pT371)TRF1 (Fig. 4G). Taken together, these results suggest that ATM mediates the association of (pT371)TRF1 with APBs.

APBs are thought to be sites of homologous-recombination-mediated telomere synthesis and therefore we examined if homologous-recombination-promoting factors such as Mre11 and BRCA1 might mediate the association of (pT371)TRF1 with APBs. Treatment with Mirin, a specific inhibitor of Mre11 (Dupre et al., 2008), led to a significant reduction in the number of GM847 cells with (pT371)TRF1 localized at APBs (Fig. 4C,D). Depletion of BRCA1 also impaired the association of (pT371)TRF1 with APBs (Fig. 4A,H). Conversely, depletion of 53BP1, a non-homologous-end-joining (NHEJ)-promoting factor, had little effect on (pT371)TRF1 association with APBs (Fig. 4A,H). Depletion of either BRCA1 or 53BP1 had little impact on the level of (pT371)TRF1 (Fig. 4I,J). Futhermore, no significant change in the number of cyclin-A-positive cells was detected as a result of Mirin treatment or depletion of either BRCA1 or 53BP1 (Fig. 4F,K). Collectively, these results suggest that the association of (pT371)TRF1 with APBs is dependent upon homologous-recombination-promoting factors but not NHEJ-promoting factors.

### Transcription-associated DNA damage at telomeres mediates the association of (pT371)TRF1 with APBs

It has been reported that transcription of telomeric DNA is upregulated in ALT cells (Arora et al., 2014; Azzalin et al., 2007). Collision between the transcription and DNA replication machineries is known to give rise to DNA double-strand breaks (DSBs) (Helmrich et al., 2013). Therefore we reasoned that enhanced transcription at ALT telomeres might be a source of DNA damage that triggers the recruitment of (pT371)TRF1 to APBs. To investigate this hypothesis, we treated GM847 cells with transcription inhibitor DRB. Analysis of indirect immunofluorescence with an anti-TRF2 antibody in conjunction with an antibody against γH2AX, a marker for DNA damage, revealed that treatment with DRB led to a significant reduction in the number of cells exhibiting γH2AX colocalization with TRF2 (Fig. 5A,B), suggesting that ALT telomeres are associated with transcription-induced DNA damage. Treatment with DRB also significantly reduced the number of cells exhibiting (pT371)TRF1 association with APBs (Fig. 5C,D). The impaired association of (pT371)TRF1 with APBs was also observed in GM847 cells treated with another transcription inhibitor, actinomycin D (Fig. 5C,E), as well as in U2OS cells treated with either DRB or actinomycin D (Fig. S3A,B). By contrast, treatment with DRB did not affect the number of cyclin-A-positive GM847 cells (Fig. S3C), nor did it affect the number of GM847 cells with PML bodies (Fig. S3D). DRB treatment also had little impact on the level of (pT371)TRF1 and PML proteins in GM847 cells (Fig. S3E). Taken together, these results suggest that (pT371)TRF1 association with APBs is dependent upon active transcription, and further imply that transcription-associated DNA damage at telomeres mediates (pT371)TRF1 association with APBs.

Earlier, we showed that ATM regulates (pT371)TRF1 association with APBs. To investigate the epistatic relationship between ATM and transcription, we treated GM847 cells with both ATM inhibitor KU55933 and transcription inhibitor DRB. The combined treatment with both KU55933 and DRB did not lead to any further reduction in the localization of (pT371)TRF1 to APBs compared with treatment with either KU55933 or DRB alone (Fig. 5F), suggesting that ATM and transcription act in the same epistatic pathway in regulating the recruitment of (pT371)TRF1 to APBs.



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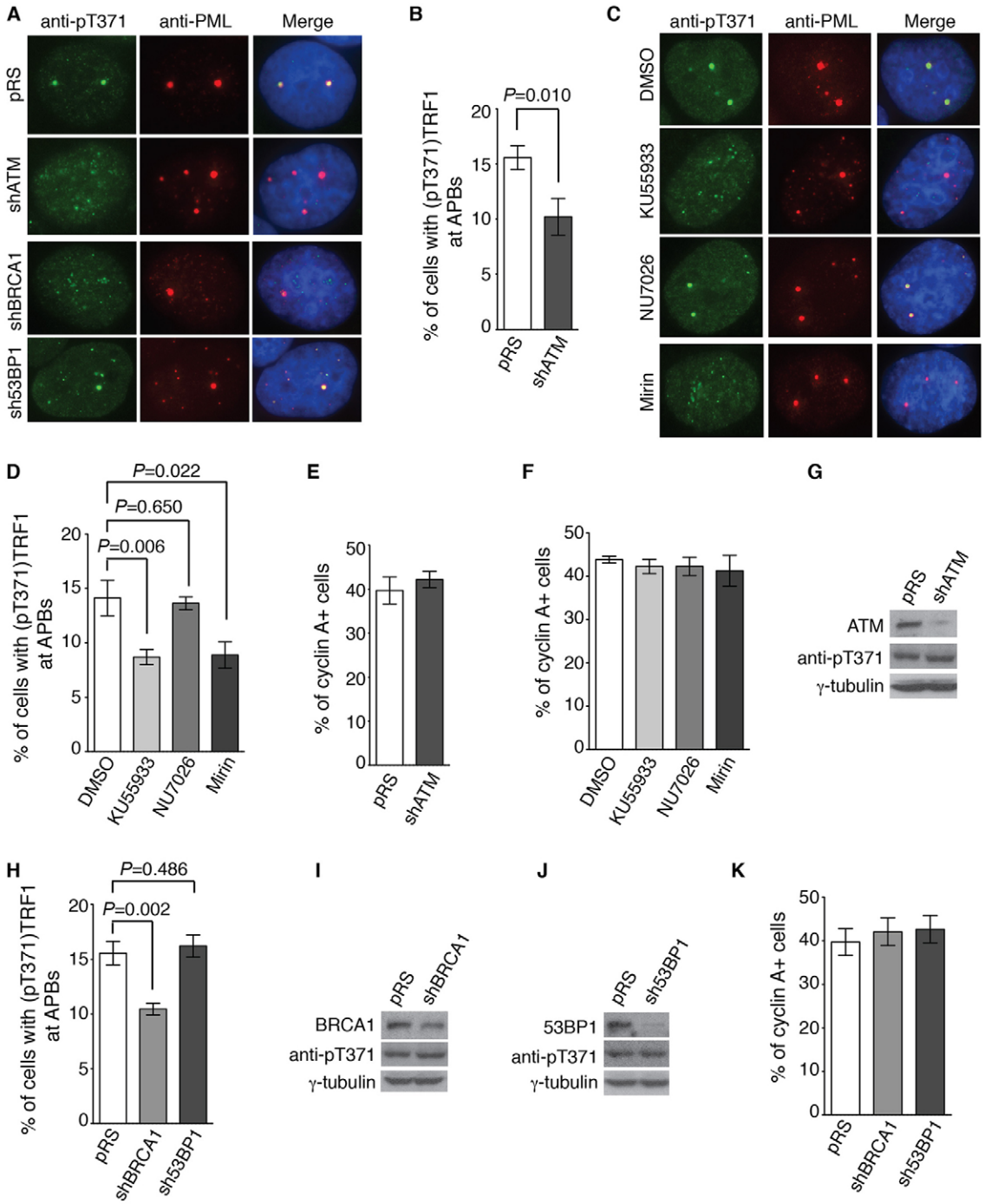


**Fig. 4. Association of (pT371)TRF1 with APBs is dependent upon ATM, Mre11 and BRCA1 but not DNA-PKcs and 53BP1.** (A) Immunofluorescence analysis of staining with anti-pT371 and anti-PML antibodies in GM847 cells expressing the vector alone or shRNA against ATM, BRCA1 or 53BP1 as indicated. (B) Quantification of the percentage of pRS- and shATM- expressing cells with (pT371)TRF1 at APBs. A total of 1000 cells from each independent experiment were scored for each cell line in blind. Standard deviations from three independent experiments are indicated. (C) Immunofluorescence analysis of staining with anti-pT371 and anti-PML antibodies in GM847 cells treated with either DMSO, KU55933 or NU7026 for 1 h prior to fixation. (D) Quantification of the percentage of GM847 cells with (pT371)TRF1 at APBs from C. Scoring was done as described in B. Standard deviations from three independent experiments are indicated. (E) Quantification of the percentage of pRS- and shATM-expressing GM847 cells staining positive for cyclin A. Scoring was done as described in B. Standard deviations from three independent experiments are indicated. (F) Quantification of the percentage of DMSO-, KU55933-, NU7026- and Mirin-treated GM847 cells staining positive for cyclin A. Scoring was done as described in B. Standard deviations from three independent experiments are indicated. (G) Western analysis of GM847 stably expressing the vector alone (pRS) or shRNA against ATM (shATM). Immunoblotting was performed with anti-ATM, anti-pT371 and anti-γ-tubulin antibodies. (H) Quantification of the percentage of pRS-, shBRCA1- and sh53BP1-expressing GM847 cells with (pT371)TRF1 at APBs. Scoring was done as described in B. Standard deviations from three independent experiments are indicated. (I) Western analysis of GM847 cells stably expressing the vector alone (pRS) or shRNA against BRCA1 (shBRCA1). Immunoblotting was performed with anti-BRCA1, anti-pT371 and anti-γ-tubulin antibodies. (J) Western analysis of GM847 cells stably expressing the vector alone (pRS) or shRNA against 53BP1 (sh53BP1). Immunoblotting was performed with anti-53BP1, anti-pT371 and anti-γ-tubulin antibodies. (K) Quantification of the percentage of pRS-, shBRCA1- and sh53BP1-expressing GM847 cells staining positive for cyclin A. Scoring was done as described in B. Standard deviations from three independent experiments are indicated.

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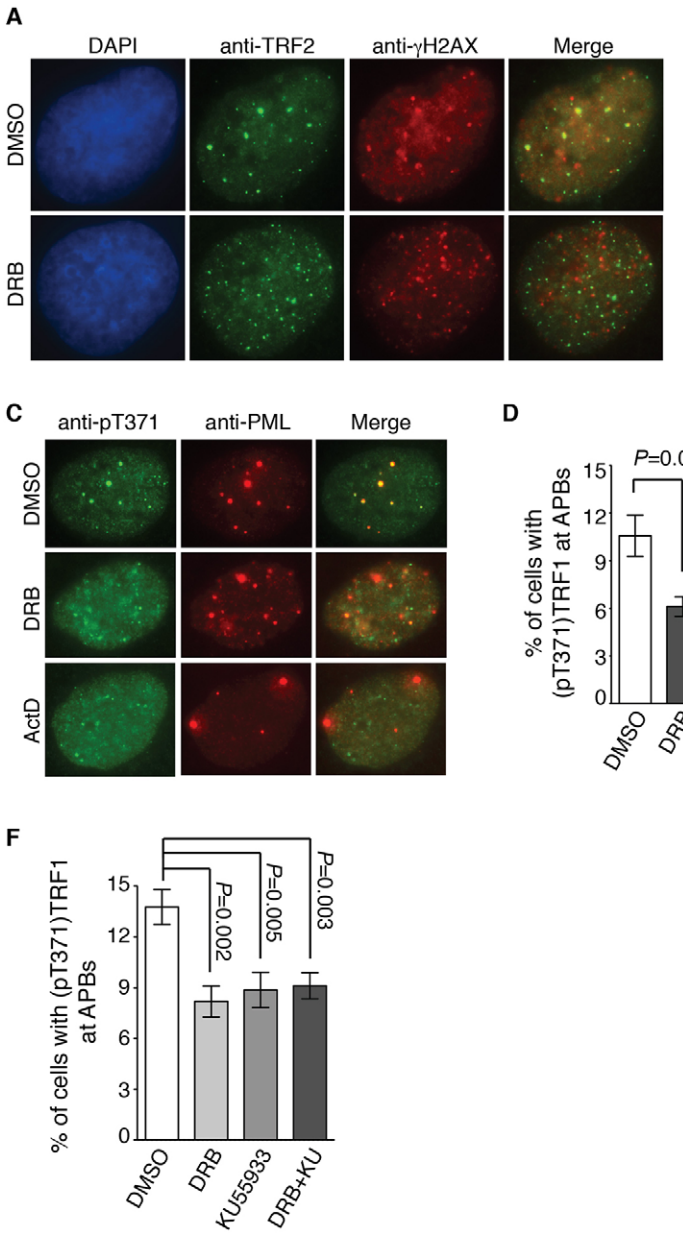


**Fig. 5. Transcription inhibition reduces telomeric accumulation of γH2AX and impairs (pT371)TRF1 interaction with APBs.** (A) Immunofluorescence analysis of staining with anti-TRF2 or anti-γH2AX antibodies in GM847 cells treated with either DMSO or DRB for 3 h prior to fixation. (B) Quantification of the percentage of DMSO- and DRB-treated GM847 cells exhibiting TRF2 colocalization with γH2AX. A total of 1000 cells from each independent experiment were scored in blind. Standard deviations from three independent experiments are indicated. (C) Immunofluorescence analysis of staining with anti-pT371 and anti-PML antibodies in GM847 cells treated with DMSO, DRB or actinomycin D (ActD). Treatment with ActD was done for 2 h whereas treatment with DRB was carried out for 3 h. (D) Quantification of the percentage of DMSO- and DRB-treated GM847 cells exhibiting (pT371)TRF1 at APBs. Scoring was done as described in B. Standard deviations from three independent experiments are indicated. (E) Quantification of the percentage of DMSO- and actinomycin-D (ActD)-treated GM847 cells exhibiting (pT371)TRF1 at APBs. Scoring was done as described in B. Standard deviations from three independent experiments are indicated. (F) Quantification of the percentage of cells with (pT371)TRF1 at APBs. GM847 cells were treated with DMSO, DRB, KU55933 or a combination of DRB and KU55933 prior to fixation. Scoring was done as described in B. Standard deviations from three independent experiments are indicated.

### RNA-DNA hybrids modulate the association of (pT371)TRF1 with APBs

Telomeric DNA is known to be transcribed into a large non-coding RNA (Azzalin et al., 2007), referred to as TERRA, which can form RNA–DNA hybrids with telomeric DNA. RNA–DNA hybrids, also known as R loops, can be processed to give rise to DNA double-strand breaks (Hamperl and Cimprich, 2014; Sollier et al., 2014). To investigate if RNA–DNA hybrids might regulate (pT371)TRF1 recruitment to APBs, we generated U2OS cell lines stably expressing the vector alone or Myc-tagged RNaseH1 (Fig. 6A). Overexpression of Myc-tagged RNaseH1 did not result in any significant change in (pT371)TRF1 recruitment to APBs (Fig. 6B and data not shown). At the same time, we also did not detect any change in the level of TERRA in our Myc-RNaseH1-overexpressing U2OS cells (Fig. 6C), which was inconsistent with a previous report that overexpression of RNaseH1 decreases the TERRA level (Arora et al., 2014). We reasoned that this discrepancy in the level of TERRA might result from a difference in the level of RNaseH1 expression between the two studies although RNaseH1 expression was readily detectable in our cell line (Fig. 6A). Alternatively, it was possible that RNA–DNA hybrids at telomeres might exist transiently and that they might be sensitive to experimental conditions. To further investigate the role of RNA–DNA hybrids in regulating (pT371)TRF1 association with APBs, we turned to campothecin (CPT), which has been reported to induce the formation of RNA–DNA hybrids (Sollier et al., 2014; Sordet et al., 2009). CPT is an inhibitor of topoisomerase I, which prevents the formation of RNA–DNA hybrids and resolves conflicts between transcription and replication (Hamperl and Cimprich, 2014; Tuduri et al., 2009). CPT can also block DNA re-ligation and promotes the induction of single-strand breaks, which can be converted into DNA double-strand breaks during S phase. We found that treatment with CPT led to a significant reduction in (pT371)TRF1 association with APBs in RNaseH1-expressing U2OS cells, whereas it had little effect on (pT371)TRF1 association with APBs in vector-expressing U2OS cells (Fig. 6B). The latter suggests that (pT371)TRF1

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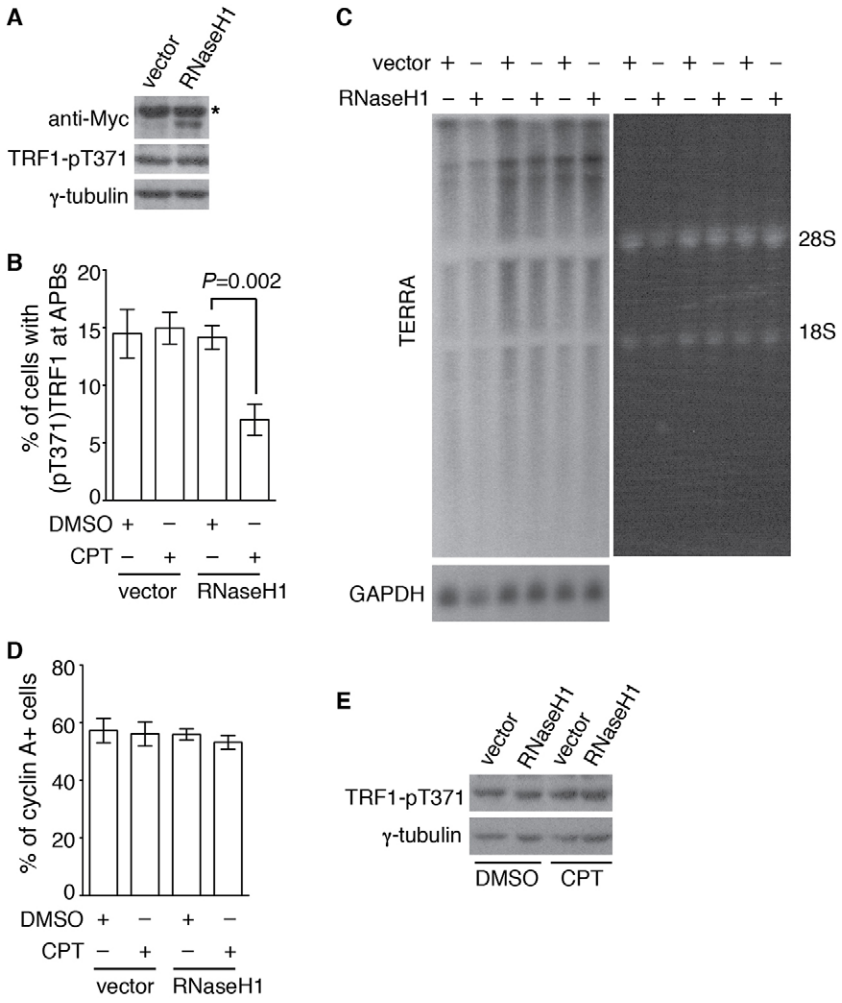


**Fig. 6. Overexpression of RNaseH1 impairs (pT371) TRF1 recruitment to APBs in the presence of camptothecin (CPT).** (A) Western analysis of U2OS cells stably expressing vector alone or Myc-tagged RNaseH1. Immunoblotting was performed with anti-Myc, anti-pT371 and anti-γ-tubulin antibodies. Asterisk represents a non-specific band. (B) Quantification of the percentage of cells exhibiting (pT371)TRF1 at APBs. U2OS cells stably expressing the vector alone or Myc-tagged RNaseH1 were treated with DMSO or 5 μM CPT for 2 h prior to immunofluorescence analysis. A total of 1000 cells from each independent experiment were scored in blind. Standard deviations from three independent experiments are indicated. (C) Analysis of TERRA expression from U2OS cells stably expressing the vector alone or Myc-tagged RNaseH1. Three pairs of total RNA isolated from three independent experiments were subjected to TERRA analysis. Northern blotting was performed with a $^{32}$P-labeled telomeric DNA-containing probe, as shown in the left main panel. The northern blot of GAPDH shown on the left bottom panel was used as a loading control. The right panel was taken from the ethidium-bromide-stained agarose gel. The position of 28S and 18S ribosomal RNA is indicated. (D) Quantification of cyclin-A-positive cells. U2OS cells stably expressing the vector alone or Myc-tagged RNaseH1 were treated with DMSO or 5 μM CPT for 2 h prior to immunofluorescence analysis. Immunofluorescence analysis was performed with an anti-cyclin-A antibody. Scoring was done as described in B. Standard deviations from three independent experiments are indicated. (E) Western analysis of DMSO- and CPT-treated U2OS cells stably expressing the vector alone or Myc-tagged RNaseH1. Immunoblotting was performed with anti-pT371 and anti-γ-tubulin antibodies.

association with APBs is unlikely promoted by CPT-induced DNA breaks alone. In addition, CPT treatment did not significantly alter the number of cyclin-A-positive cells (Fig. 6D), nor did it affect the level of (pT371)TRF1 (Fig. 6E). These results altogether suggest that (pT371)TRF1 association with APBs might be regulated by RNA–DNA hybrids.

### The telomeric DNA binding activity of TRF1 is dispensable for its interaction with PML bodies but is crucial for both the production of C-circles and the assembly of APBs

Our earlier finding that (pT371)TRF1 is preferentially associated with dysfunctional telomeres at APBs suggests that TRF1 might interact with APBs independently of its binding to telomeric DNA per se. To address this hypothesis, we generated a Myc-tagged TRF1 deletion allele lacking the C-terminal Myb-like DNA binding domain (TRF1-ΔM). Myc-tagged TRF1-ΔM co-migrated with a non-specific protein band observed in the lane containing Myc-tagged wild-type TRF1 (Fig. 7A). Extracting the density of the non-specific protein band, we estimated that the expression of Myc-tagged TRF1-ΔM in TRF1-depleted GM847 cells was similar to wild-type TRF1 (Fig. 7A). Analysis of dual indirect immunofluorescence with an anti-Myc antibody in conjunction with an anti-PML antibody revealed that Myc-tagged TRF1-ΔM was competent in interacting with PML bodies (Fig. S4A). The number of cells containing Myc-tagged TRF1-ΔM at PML bodies was indistinguishable from that of cells containing Myc-tagged wild-type TRF1 (Fig. 7B). By contrast, unlike Myc-tagged wild-type TRF1, Myc-tagged TRF1-ΔM was predominantly free of chromatin (Fig. 7C), indicating that the observed association of Myc-tagged TRF1-ΔM to PML bodies is unlikely to result from its interaction with telomeric DNA. These results suggest that the Myb-like DNA binding domain of TRF1 is dispensable for its association with PML bodies.

It has been reported that R425 in the Myb-like DNA binding domain of TRF1 makes direct base contacts with telomeric DNA (Court et al., 2005). To gain further evidence about the role of the telomeric DNA binding activity in TRF1 association with PML bodies, we also generated Myc-tagged TRF1 carrying a R425V mutation. Myc-tagged TRF1-R425V was expressed at a level similar to Myc-tagged wild-type TRF1 (Fig. S4B) and was found to be largely soluble (Fig. S4C), in agreement with previous findings that the R425V mutation abrogates TRF1 binding to telomeric DNA both *in vivo* and *in vitro* (Fairall et al., 2001; McKerlie and Zhu, 2011). Analysis of indirect immunofluorescence revealed that Myc-tagged TRF1-R425V was able to localize to PML bodies indistinguishably from Myc-tagged wild-type TRF1 (Fig. S4D,E), further supporting the notion that TRF1 might interact with PML bodies in ALT cells independently of its telomeric DNA activity.

We also examined the role of the telomeric DNA binding activity of TRF1 in supporting both C-circle production and APB assembly. Unlike Myc-tagged wild-type TRF1, overexpression of Myc-tagged

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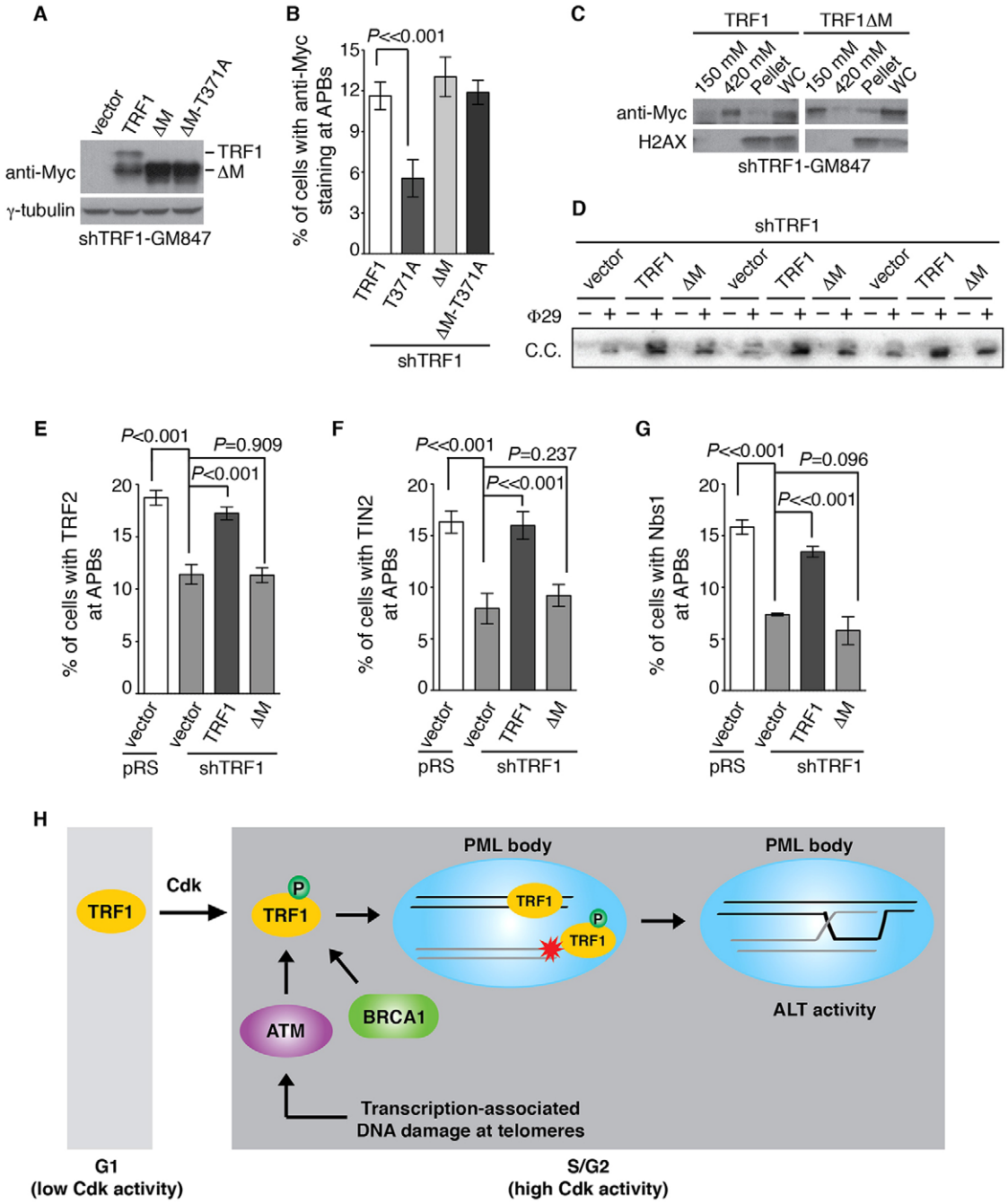


**Fig. 7. The Myb-like DNA binding domain of TRF1 is dispensable for its association with APBs but is required for the recruitment of other shelterin proteins and Nbs1 to APBs.** (A) Western analysis of TRF1-depleted GM847 cells overexpressing the vector alone and various Myc-tagged TRF1 alleles as indicated. Immunoblotting was performed with anti-Myc and anti-γ-tubulin antibodies. (B) Quantification of the percentage of cells with Myc staining at APBs. Immunofluorescence analysis was performed with anti-Myc and anti-PML antibodies. A total of 1000 cells from each independent experiment were scored for each cell line in blind. Standard deviations from three independent experiments are indicated. (C) Analysis of differential salt extraction of chromatin of TRF1-depleted GM847 cells overexpressing various Myc-tagged TRF1 alleles. Immunoblotting was performed with anti-Myc and anti-H2AX antibodies. The H2AX blot was used as a control for differential salt extraction of chromatin. WC, whole cell lysate. (D) Analysis of C-circle formation. The blot represents three pairs of C-circle assays from three independent experiments. C.C., C-circles. (E) Quantification of the percentage of cells with TRF2 at APBs. Immunofluorescence analysis was performed with anti-TRF2 and anti-PML antibodies. Scoring was done as described in B. Standard deviations from three independent experiments are indicated. (F) Quantification of the percentage of cells with TIN2 at APBs. Immunofluorescence analysis was performed with anti-TIN2 and anti-PML antibodies. Scoring was done as described in B. Standard deviations from three independent experiments are indicated. (G) Quantification of the percentage of cells with Nbs1 at APBs. Immunofluorescence analysis was performed with anti-Nbs1 and anti-PML antibodies. Scoring was done as described in B. Standard deviations from three independent experiments are indicated. (H) Model for Cdk-dependent control of TRF1 interaction with APBs. See the text for details.

TRF1-ΔM failed to rescue the level of C-circles in TRF1-depleted GM847 cells (Fig. 7D). Overexpression of Myc-tagged TRF1-ΔM also failed to rescue the association of TRF2, TIN2 and Nbs1 with PML bodies in TRF1-depleted GM847 cells (Fig. 7E-G). The failure to rescue the association of TRF2, TIN2 and Nbs1 with PML bodies in TRF1-depleted GM847 cells was also observed with

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Myc-tagged mutant TRF1-R425V (Fig. S4F-H). These results suggest that the telomeric DNA binding activity of TRF1 is crucial for both the production of C-circles and the assembly of other shelterin and repair proteins at PML bodies.

### Deletion of the Myb-like DNA binding domain voids the need for T371 phosphorylation in regulating TRF1 interaction with PML bodies

Earlier, we showed that Myc-tagged TRF1 carrying a T371A mutation fails to rescue (pT371)TRF1 association with APBs in TRF1-depleted GM847 cells (Fig. 3E), prompting us to investigate the ability of Myc-tagged TRF1-T371A to localize to PML bodies. Analysis of dual indirect immunofluorescence with an anti-Myc antibody in conjunction with an anti-PML antibody revealed that the T371A mutation significantly impaired the colocalization of Myc-tagged TRF1 with PML bodies (Fig. 7B; Fig. S4A), suggesting that TRF1 interaction with PML bodies requires its phosphorylation on T371.

To investigate if T371 phosphorylation might be needed for the association of Myc-tagged TRF1-ΔM with PML bodies, we introduced the T371A mutation into Myc-tagged TRF1-ΔM. The expression of Myc-tagged TRF1-ΔM-T371A was indistinguishable from that of Myc-tagged TRF1-ΔM (Fig. 7A). Analysis of indirect immunofluorescence with an anti-Myc antibody revealed that the mutation of T371A had little effect on the association of Myc-tagged TRF1-ΔM with PML bodies (Fig. 7B; Fig. S4A). These results suggest that T371 phosphorylation becomes dispensable for TRF1 association with PML bodies in the absence of the Myb-like DNA binding domain. These results further imply that T371 phosphorylation acts as a switch to create a pool of TRF1 to be recruited to PML bodies independently of its binding to telomeric DNA.

## DISCUSSION

In this report, we have uncovered that Cdk-dependent phosphorylation of TRF1 on threonine 371 promotes TRF1 to interact with APBs in S and G2 phases independently of its binding to telomeric DNA. We have shown that TRF1 phosphorylation on T371 is essential for the formation of APBs and the production of C-circles, both of which are hallmarks of ALT cells. We have demonstrated that the interaction of (pT371)TRF1 with APBs is dependent upon ATM and homologous-recombination-promoting factors such as Mre11 and BRCA1. Furthermore, we have shown that the interaction of (pT371)TRF1 with APBs is sensitive to transcription inhibition, which reduces the accumulation of γH2AX at telomeres. These results suggest that transcription-associated DNA damage might act as a trigger to recruit (pT371)TRF1 to dysfunctional telomeres to facilitate homologous-recombination-mediated ALT activity (Fig. 7H). It is possible that this process might occur both within and outside of PML bodies in ALT cells.

We have shown that (pT371)TRF1 is preferentially associated with dysfunctional telomere ends in response to DNA damage rather than binding to telomeric DNA per se, in agreement with previous finding that (pT371)TRF1 can be recruited to sites of DNA damage independently of telomeric DNA (McKerlie et al., 2013). It has been reported that (pT371)TRF1 facilitates DNA end resection to promote homologous-recombination-mediated repair of DNA double-strand breaks (McKerlie et al., 2013). Perhaps, (pT371)TRF1 might help process dysfunctional telomere ends for homologous-recombination-mediated events in ALT cells.

TRF1 binds to duplex telomeric DNA and it is also found to interact directly with PML (Hsu et al., 2012; Yu et al., 2010) and PML-associated proteins such as Nbs1 at APBs (Wu et al., 2000). In addition, TRF1 is reported to be SUMOylated and that its SUMOylation promotes APB formation (Potts and Yu, 2007). SUMOylated TRF1 is found to interact with PML-IV (Hsu et al., 2012), and it has been suggested that noncovalent binding of PML to SUMOylated TRF1 and other shelterin proteins might promote the recruitment of telomere heterochromatin to PML bodies (Potts and Yu, 2007). Alternatively, SUMOylation of TRF1 and other shelterin proteins might take place within PML bodies to facilitate the maintenance of APBs (Potts and Yu, 2007). However, whether TRF1 binding to telomeric DNA is important for its interaction with PML bodies has yet to be characterized. In this report, we have shown that deletion of the Myb-like DNA binding domain of TRF1 does not affect its interaction with PML bodies although it abrogates TRF1 binding to telomere chromatin. These results suggest that TRF1 interaction with PML bodies in ALT cells can occur independently of its binding to telomeric DNA. It would be of interest to investigate if deletion of the Myb-like DNA binding domain might affect SUMOylation of TRF1.

We have shown that deletion of the Myb-like DNA binding domain suppresses the deficiency in localizing to PML bodies demonstrated by TRF1 carrying a T371A mutation, suggesting that Cdk-dependent TRF1 phosphorylation on T371 acts as a switch to create a pool of TRF1 that can be recruited to PML bodies independently of its binding to telomeric DNA. Although the Myb-like DNA binding domain is dispensable for TRF1 localization to PML bodies, it is necessary to support the recruitment of other shelterin and repair factors to PML bodies as well as the production of C-circles. These results suggest that TRF1 interaction with PML bodies is a separable process from the recruitment of telomere heterochromatin to PML bodies, both of which are necessary for APB formation and C-circle production. These results further imply that Cdk activity in S and G2 cells controls the function of TRF1 in these two processes to facilitate ALT activity (Fig. 7H).

It has been reported that depletion of TRF1 impairs APB formation (Jiang et al., 2007), however little is known about the role of TRF1 in other features of ALT cells such as C-circle prodution, telomere length heterogeneity and telomere sister chromatin exchange (T-SCE). We have shown that depletion of TRF1 impairs not only APB formation but also C-circle production. However, we did not detect any significant change in telomere length heterogeneity and T-SCE in our TRF1-depleted ALT cells (F.R.W., A.H. and X.-D.Z., unpublished data). It is possible that an incomplete depletion of TRF1 in ALT cells might have contributed to this lack of change in telomere length heterogeneity and T-SCE. Future studies would be needed to investigate the role of TRF1 in telomere length heterogeneity and T-SCE in ALT cells.

We have shown that the formation of APBs is sensitive to transcription inhibition, which also reduces the accumulation of γH2AX at ALT telomeres, suggesting that transcription-associated DNA damage at telomeres might act as a trigger to induce ALT. Telomeric DNA is transcribed into a large non-coding telomeric DNA-containing RNA, referred to as TERRA (Azzalin et al., 2007). In telomerase-positive cancer cells, TERRA expression is cell cycle regulated, plunging to its lowest level as cells progress from S to G2 phase, which is thought to prevent deleterious interference with DNA replication of telomeric tracts (Flynn et al., 2015; Porro et al., 2010). Conversely, ALT cells are found to be defective in regulating TERRA expression (Flynn et al., 2015). Not only are TERRA levels increased in ALT cells (Arora et al., 2014; Azzalin et al., 2007) but

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also they remain highly elevated in S and G2 phases (Flynn et al., 2015). We have shown that association of (pT371)TRF1 with APBs is sensitive to RNaseH1 in the presence of camptothecin, an inhibitor of topoisomerase I, an enzyme known to prevent the formation of RNA–DNA hybrids and to resolve conflicts between transcription and replication (Hamperl and Cimprich, 2014; Tuduri et al., 2009). RNA–DNA hybrids can be processed into DNA double-strand breaks (DSBs) (Sollier et al., 2014), and recently, it has been reported that DNA DSBs at ALT telomeres induce telomere clustering, APB formation as well as other homologous-recombination-based ALT activities (Cho et al., 2014). Perhaps, telomeric RNA–DNA hybrids arising from transcription and replication collision might be a natural source of DNA damage that triggers ALT.

## MATERIALS AND METHODS

### DNA constructs and inhibitor treatment

Retroviral expression constructs for shTRF1, shATM, shBRCA1, sh53BP1 as well as various full-length TRF1 alleles (wild-type TRF1, and TRF1 mutant alleles T371A and R425V) have been previously described (McKerlie et al., 2013; McKerlie and Zhu, 2011). The retroviral construct for TRF1 lacking the entire Myb-like DNA binding domain (missing the last 60 amino acids from 379 to 439) (TRF1-ΔM) was generated through PCR using shTRF1-resistant wild-type TRF1 as a template. The sequence of primers for cloning TRF1-ΔM will be made available upon request. The QuickChange site-directed mutagenesis kit (Strategene) was used to create a T371A mutation in the TRF1-ΔM allele.

Inhibitors used include: KU55933 (Sigma), a specific inhibitor for ATM; NU7026 (Sigma), a specific inhibitor for DNA-PKcs; Mirin (Sigma), a specific inhibitor for Mre11 (Dupre et al., 2008); roscovitine (Sigma), a Cdk inhibitor; camptothecin (Sigma); DRB (Cayman Chemical) and actinomycin D (Sigma), transcription inhibitors. KU55933, NU7026 and roscovitine were each used at 20 μM. Mirin and DRB were each used at 100 μM. Actinomycin D was used at 2 μg/ml.

### Cell culture and synchronization

Cells were grown in DMEM medium with 5% fetal bovine serum (FBS) for GM847 (a gift from Titia de Lange, Rockefeller University), U2OS (ATCC), WI38VA13/2RA (Zhu et al., 2003) and Phoenix cells (Zhu et al., 2003), supplemented with non-essential amino acids, L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cell lines were tested to be free of mycoplasma contamination. Retroviral gene delivery was carried out as described (Mitchell et al., 2009) to generate stable cell lines. TRF1-depleted GM847 cells stably expressing various TRF1 alleles were maintained in the selection medium containing either puromycin (2 μg/ml) or hygromycin (90 μg/ml), alternating every 2 weeks for the entirety of the experiments. For analysis of APB formation and C-circle production, we used cell lines that were kept in culture for less than a month following their generation. For analysis of telomere length heterogeneity, cell lines were cultured continuously for up to 2 months.

Cell synchronization at the G1/S boundary was carried out essentially as described with some modifications (Zhu et al., 2000). GM847 and U2OS cells were first arrested with thymidine (2.5 mM) for 17 h, followed by washing in PBS for three times and then released into fresh media for 14 h. Subsequently, cells were arrested again with 2.5 mM thymidine for 17 h and washed in PBS for three times before their release into fresh media for 0-16 h.

### Protein extracts, differential salt extraction of chromatin and immunoblotting

Protein extracts, differential salt extraction of chromatin and immunoblotting were performed as described (McKerlie et al., 2013; McKerlie and Zhu, 2011). Phospho-specific anti-pT371-TRF1 antibody has been previously described (McKerlie and Zhu, 2011). Mouse anti-TRF1 antibody and rabbit antibodies against total TRF1 (van Steensel and de Lange, 1997), TRF2 (Zhu et al., 2000), hRap1 (Li et al., 2000) and TIN2 (Ye et al., 2004) were generous gifts from Titia de Lange, Rockefeller University. Other antibodies include: Nbs1 (a kind gift from John Petrini, Memorial Sloan-Kettering Cancer Center); PML (sc-966, Santa Cruz); PML (ab53773, Abcam); anti-BrdU (NB200-569, Novus Biologicals); BRCA1 (MS110, Abcam); BRCA1 (07-434, Millipore); Cyclin A (6E6, Abcam); γ-H2AX (05-636, Milipore); 53BP1 (612522, BD Biosciences) and γ-tubulin (GTU88, Sigma).

### IF-FISH

Immunofluorescence combined with fluorescence *in situ* hybridization (IF-FISH) was carried out as follows. Immunofluorescence was performed as described (Mitchell and Zhu, 2014; Zhu et al., 2003). FISH analysis was carried out essentially as described (McKerlie et al., 2012). For triple staining, blocked coverslips were incubated with both rabbit anti-pT371 (at 1:500) and mouse anti-PML (at 1:200) antibodies in blocking buffer (1 mg/ml BSA, 3% goat serum, 0.1% Triton X-100 and 1 mM EDTA in PBS) at room temperature for 2 h. After three washes in PBS, coverslips were incubated with both TRITC-conjugated donkey anti-rabbit (1:250; Cat. no. 715-025-152, Jackson Laboartories) and AMCA-conjugated donkey anti-mouse (1:250; Cat. no. 715-156-150, Jackson Laboratories) at room temperature for 30 min. Subsequently, coverslips were processed as described (McKerlie et al., 2012). Cell images were then recorded on a Zeiss Axioplan 2 microscope with a Hammamatsu C4742-95 camera and processed in Open Lab.

### Metaphase chromosome spreads

Metaphase chromosome spreads were essentially prepared as described (Batenburg et al., 2012; Zhu et al., 2003). Cells were arrested in nocodazole (0.1 μg/ml) for 120 min. Following arrest, cells were collected by shake-off, spun down and incubated at 37°C for 10 min in RSB buffer [10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 5 mM $MgCl_2$]. Cell droplets were spun onto glass coverslips, fixed in PBS-buffered 2% paraformaldehyde and followed by standard immunofluorescence as described above.

### Northern analysis of TERRA and C-circle amplification assays

Northern analysis of TERRA was carried out as described (Batenburg et al., 2012). C-circle amplification assays were performed essentially as described (Henson et al., 2009). Briefly, each DNA sample (10 μl or 50 ng) was incubated with or without 7.5 U ϕ29 DNA polymerase (NEB) in a 20 μl reaction containing 9.25 μl of premix (0.2 mg/ml BSA, 0.1% Tween 20, 1 mM ATP, 1 mM dTTP, 1 mM dGTP, 1× ϕ29 buffer) at 30°C for 8 h. Following heat inactivation of ϕ29 at 65°C for 20 min, samples were separated on a 0.6% agarose gel in 0.5× Tris-borate-EDTA (TBE) at 1.75 V/cm for 12-16 h. Gels were dried at 50°C and then hybridized with a $^{32}$P-end-labeled single-strand $(CCCTAA)_3$ probe under native conditions as described (Lackner et al., 2012). Gels were exposed to PhosphorImager screens and scanned using a Typhoon FLA9500 biomolecular imager (GE Healthcare).

### Statistical analysis

A Student's two-tailed *t*-test was used to derive all *P*-values.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

F.R.W., A.H., and J.R.W. performed the experiments. J.R.W. and X.-D.Z. conceived hypotheses and designed the project. All authors contributed to data analysis and manuscript writing.

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**Supplementary information**
Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.186098.supplemental

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