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A role for monoubiquitinated FANCD2 at telomeres in ALT cells

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ABSTRACT

Both Fanconi anemia (FA) and telomere dysfunction are associated with chromosome instability and an increased risk of cancer. Because of these similarities, we have investigated whether there is a relationship between the FA protein, FANCD2 and telomeres. We find that FANCD2 nuclear foci colocalize with telomeres and PML bodies in immortalized telomerase-negative cells. These cells maintain telomeres by alternative lengthening of telomeres (ALT). In contrast, FANCD2 does not colocalize with telomeres or PML bodies in cells which express telomerase. Using a siRNA approach we find that FANCA and FANCL, which are components of the FA nuclear core complex, regulate FANCD2 monoubiquitination and the telomeric localization of FANCD2 in ALT cells. Transient depletion of FANCD2, or FANCA, results in a dramatic loss of detectable telomeres in ALT cells but not in telomerase-expressing cells. Furthermore, telomere loss following depletion of these proteins in ALT cells is associated with decreased homologous recombination between telomeres (T-SCE). Thus, the FA pathway has a novel function in ALT telomere maintenance related to DNA repair. ALT telomere maintenance is therefore one mechanism by which monoubiquitinated FANCD2 may promote genetic stability.

INTRODUCTION

Fanconi anemia (FA) is a multigenic disease associated with bone marrow failure and cancer susceptibility, particularly acute myelogenous leukemia (AML) and squamous cell carcinoma of the head and neck (1,2). FA cells display chromosome fragility, characterized by chromosome breakage and the formation of radial chromosomes, in response to DNA interstrand crosslinkers such as mitomycin C (MMC). FA cells are also hypersensitive to MMC (2–4). Together, these results suggest that Fanconi anemia is linked to a defect in the cellular response to DNA damage. Importantly, the role of FA proteins in DNA damage responses is not well understood.

The genes for 13 FA subtypes (A, B, C, D1, D2, E, F, G, I, J, L and M and N) have been identified (2,4,5). The encoded FA proteins cooperate in the FA pathway (3). Eight of the FA proteins (A, B, C, E, F, G, L and M) assemble into a nuclear core complex that is required for the monoubiquitination of FANCD2 and FANCI (2–4). Monoubiquitination of FANCD2 is critical for the normal function of DNA damage responses. A non-ubiquitinable mutant of FANCD2, mutated at the site of ubiquitin conjugation, is completely incapable of restoring MMC resistance to FANCD2-deficient cells (3,6,7). In contrast, a non-ubiquitinable mutant of FANCI partially restores MMC resistance in FANCI-deficient cells (8).

Monoubiquitination of FANCD2 is required for the assembly of this protein into nuclear foci during an unperturbed S phase or in response to DNA damage (3,9). Consistent with a role for FANCD2 in DNA damage responses, FANCD2 foci colocalize with BRCA1 and RAD51 foci (9). The ATR checkpoint kinase, along with the FA nuclear core complex, is involved in the regulation of FANCD2 monoubiquitination and the assembly of FANCD2 foci in response to DNA damage (10).

Telomere dysfunction can occur either through progressive telomere shortening or other changes in telomere structure that leave the chromosome ends unprotected (11,12). Similar to FA, telomere dysfunction results in chromosome instability, in particular chromosome end

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fusions and complex rearrangements (11,12). The shared chromosome instability phenotype suggests that FA proteins could have some role in telomere function.

While expression of telomerase is the predominant mechanism of stabilizing telomeres in human tumor cells, the alternative lengthening of telomeres (ALT) pathway is utilized in about 10–15% of human tumors (13,14). The ALT pathway maintains telomeres through homologous recombination (15,16). ALT cells display a corresponding increase in sister chromatid exchanges at telomeres (T-SCE), as compared to non-ALT cells (17,18). Importantly, telomeres in ALT cells have a more heterogeneous length and display increased chromosome instability, including breaks, fusions and fragments, as compared to telomerase-expressing cells (19,20).

Another characteristic of human ALT cells is that promyelocytic leukemia (PML) nuclear bodies (APBs for ALT-associated PML bodies) colocalize with telomeres during late S phase/G2 (21–23). Other proteins involved in DNA damage responses, such as NBS1 and MRE11 (22), BRCA1 (24), BLM (23,25), RPA (26,27) and SMC5/6 (28), also localize to APBs. Relatively little is known, however, about how, or whether, these proteins function at ALT telomeres.

The function of FA proteins in DNA damage responses has been largely characterized in non-ALT cells (2). Here we demonstrate that FANCD2 localizes to telomeres in immortalized telomerase-negative ALT cells, but not in immortalized/transformed lines expressing telomerase. We find that the localization of FANCD2 to ALT telomeres requires FANCA and FANCL, either with or without exposure to DNA damaging agents. Furthermore, we demonstrate that the ATR checkpoint kinase is required for the localization of FANCD2 to telomeres after ALT cells are exposed to MMC. Our results also demonstrate that transient depletion of either FANCD2 or FANCA is associated with a decrease in recombination between the telomeres (T-SCE) and with a dramatic loss of detectable telomeres in ALT cells. In contrast, depletion of FANCD2 or FANCA does not result in decreased T-SCE or loss of detectable telomeres in telomerase-expressing cells. Our results suggest that monoubiquitinated FANCD2 has a role in the maintenance of telomeres at chromosome ends in ALT cells that may be related to its function in cellular responses to DNA damage. Importantly, ALT telomeres may serve as a model system for understanding the function of FA proteins in homologous recombination.

MATERIALS AND METHODS

Cell culture

Transformed ALT cell lines (U2OS, GM847 and WI38/VA13) and telomerase-expressing cell lines (HeLa, MCF7 and 293-EBNA) (26) were cultured in DMEM (Invitrogen) supplemented with 15% fetal bovine serum (Sigma), 100 U/ml Penicillin, 100 μg/ml Streptomycin and 2 mM L-glutamine at 37°C, 5% CO_2.

DNA replication was arrested by treatment with 2 mM hydroxyurea (HU), added from a 200 mM stock in growth medium which was kept at –20°C. Mitomycin C was added from a 3 mM stock in ethanol kept at –20°C.

Immunofluorescence microscopy

Adherent cells were grown on 12 mm diameter glass coverslips, coated with poly-D-lysine, for a minimum of 24 h prior to treatment or fixation. For detection of ATR, cells were fixed with 3% paraformaldehyde for 10 min. Cells were then permeabilized with PBS containing 0.5% Triton X-100 for 5 min at RT. For immunolocalization of all other antigens, cells were fixed with 2% paraformaldehyde for 20 min at RT, permeabilized and processed for immunofluorescence microscopy as we have described previously (29).

For immunolocalization of FANCD2 on mitotic chromosomes, chromosome spreads were prepared. Cells were incubated with 1 μg/ml nocodazole for 3 h and cells were swollen in 0.8% sodium citrate for 5 min at 37°C. Spreads were prepared by centrifugation at 1200 rpm for 2 min using a Thermo Shandon Cytospin 4 apparatus. Cells were then fixed with 2% paraformaldehyde for 20 min at RT.

Primary antibodies included anti-FANCD2 (E35, 1:200, rabbit, obtained from Dr Alan D'Andrea, Dana-Farber Cancer Institute), anti-ATR (Ab-2, Calbiochem, 1:100, rabbit), anti-PML (N-19 from Santa Cruz, 1:300, goat) and anti-TRF1 (TRF-78 from Abcam, 1:100, mouse). Secondary antibodies included FITC-conjugated donkey anti-goat IgG, FITC-conjugated donkey anti-mouse or anti-rabbit IgG and Rhodamine B-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch; 1:500).

Labeled cells were observed with a Zeiss Axiovert 200M microscope and images were collected with a Hamamatsu Camera using Openlab software (Improvision). Images were processed into figures using Photoshop (Adobe).

Combined immunofluorescence microscopy and fluorescence *in situ* hybridization (FISH)

To simultaneously detect FANCD2 or TRF1 with specific antibodies, and telomeric DNA with a fluorescent DNA probe, cells were grown on glass coverslips coated with poly-D-lysine. Cells were fixed for 10 min with 3% paraformaldehyde in PBS, and then extracted with 0.5% Triton X-100 in PBS for 5 min on ice. Following a PBS wash, cells were incubated with primary antibodies to FANCD2 (E35) or TRF1 (TRF78) as described previously (29). Secondary antibodies were diluted 200-fold. Cells were washed three times with PBS, post-fixed with 3% paraformaldehyde in PBS for 10 min at RT, and then exposed to two 10 min incubations with 70% ethanol at −20°C. Samples were air dried and hybridization mixture (50% formamide, 10% dextran sulfate, 5X Denhardt's solution, 2X SSC, 0.5 ng/ml yeast tRNA and 0.3 μg/ml Cy3-PNA telomeric probe [Cy3-$(CCCTAA)_3$]) was added. Cells were incubated at 80°C for 3 min and then overnight at RT with protection from ambient light. Cells were washed for 30 min at RT with 50% formamide/2X SSC, then twice for 5 min each with 2X SSC. Finally, cells were washed twice with PBS and were mounted in Vectashield containing DAPI.

SiRNA and transfection

Expression of targeted genes was knocked down by transient expression of siRNAs directed against ATR, FANCA and GFP, as we have described previously (10). SiRNAs directed against FANCD2 (30) and FANCL (31) were also utilized. Cells were either left untreated or were exposed to 2 mM HU or 0.5 μM mitomycin C for 24 h. Cells were harvested 96 h post-transfection.

ShRNA and transduction

FANCA was depleted in GM847 ALT cells using a lentiviral shRNA. The LeGO-G vector (32) was kindly provided by Kristoffer Weber and Dr Boris Fehse (University Hospital Eppendorf, Hamburg, Germany). Virus was produced by transfection of 293T cells with LeGO-G, along with the packaging plasmids pRRE and pRSV-Rev, and pVSV-G (32). Virus was concentrated by ultracentrifugation and then centrifuged through a 20% sucrose cushion for additional purification. Virus was aliquoted and stored at –80°C. Target cells were transduced 3X, at approximately 12 h intervals, with virus in growth medium containing 8 μg/ml polybrene. Cells were analyzed at 96 h after beginning transduction.

Immunoblotting

Cells were lysed, protein concentrations determined from supernatants, and sample buffer added to combined supernatants and pellets to prepare whole cell lysates as we have described previously (29). Equivalent amounts of proteins were loaded for each sample, followed by SDS–PAGE (6% polyacrylamide, *bis*-acrylamide). Proteins were transferred to nitrocellulose, blocked, and incubated with primary antibodies as previously described (9). Antibodies included anti-FANCA (1:1000) generously provided by Dr Maureen Hoatlin (Oregon Health & Sciences University, Portland, OR, USA), anti-FANCD2 (E35, 1:1000) (3), anti-FANCL (1:2000) (31) from Dr Ruhikanta Meetei (Cincinnati Children's Research Foundation), anti-ATR (1:500) and anti-actin (C4, 1:2000) from Dr James Lessard (Cincinnati Children's Research Foundation). Membranes were washed, incubated with HRP-linked secondary antibodies (Amersham), and signals detected by chemiluminescence (Amersham) as previously described (9).

Assay of signal-free ends by FISH

Following transfection with siRNAs, U2OS and HeLa cells were exposed to 1 μg/ml nocodazole for 5 h. All cells were collected and pooled following trypsinization. Cells were swollen, fixed, dried onto slides and prepared for hybridization, as described previously (33). Hybridization mix [70% formamide, 0.06X SSC, 0.2% bovine serum albumin, 0.5 ng/ml yeast tRNA (Sigma) and 0.5 ng/ml Cy3-labeled telomeric peptide nucleic acid (PNA) probe (Applied Biosystems)] was applied and covered with a plastic coverslip. Slides were placed on a heat block at 83°C for 3 min and hybridized for 3 h at RT. Coverslips were then removed, and slides were washed twice in 70% formamide and 0.06X SSC, pH 7.2, then twice in PBS containing 0.05% Triton X-100. Slides were then mounted with a coverslip using Vectashield that contained DAPI.

Images were collected with constant settings and no threshold signal, so that only the brightest metaphase telomeric signals approached saturating intensity. Due to the wide heterogeneity of telomere lengths in ALT cells (16,19), longer exposure times were utilized to image telomeres in HeLa cells in a linear range. Images of metaphases with spread chromosomes were collected and signal-free ends were counted from merged images.

Measurements of telomere sister chromatid exchange (T-SCE)

T-SCE was analyzed by chromosome orientation fluorescence *in situ* hybridization (CO-FISH) as previously described (34), with slight modification. Briefly, U2OS or HeLa cells were grown overnight and then incubated with 10 μM bromodeoxyuridine (BrdU) for 16 h, with 1 μg/ml nocodazole added for the last 5 h. Cells were collected following trypsinization and were resuspended in 75 mM KCl for 20 min prior to fixation and preparation of metaphase spreads. Following incubation with Hoechst 33258, treatment with UV light (using a Stratalinker 1800 UV irradiator), and digestion with Exonuclease III (Promega), cells were hybridized overnight with 0.5 ng of Cy3-$(TTAGGG)_3$ telomeric probe. Slides were then washed and mounted with a coverslip and Vectashield containing DAPI.

Images were collected and T-SCE counts were made from merged images. A telomere signal split between sister chromatids at a particular end of a chromosome was counted as a T-SCE event. In cases where both chromosome ends displayed a split signal, this was counted as two T-SCE events. To determine the number of T-SCE events relative to the number of chromosome ends with a detectable signal, the total number of T-SCE events was divided by the number of chromosome ends with a detectable telomere signal, whether single or split.

Flow FISH

Telomere lengths in interphase cells were quantitated by FISH with Cy_3-OO-$(CCCTAA)_3$ probe using flow cytometry, as previously described (35). Flow cytometry was performed on a FacsCalibur instrument (Becton–Dickinson). Aggregated cells were gated out.

RESULTS

FANCD2 colocalizes with telomeres in ALT cells

The FA protein FANCD2 forms nuclear foci during S phase in the absence of exogenous DNA damage (9). Whether S phase FANCD2 foci associate with specific substructures within the nucleus is unknown. Since another DNA damage response protein, NBS1, colocalizes with telomeres in non-ALT cells (36), we tested whether spontaneous FANCD2 foci colocalize with telomeres in cells which maintain telomere length by either of two different mechanisms: telomerase expression or ALT.

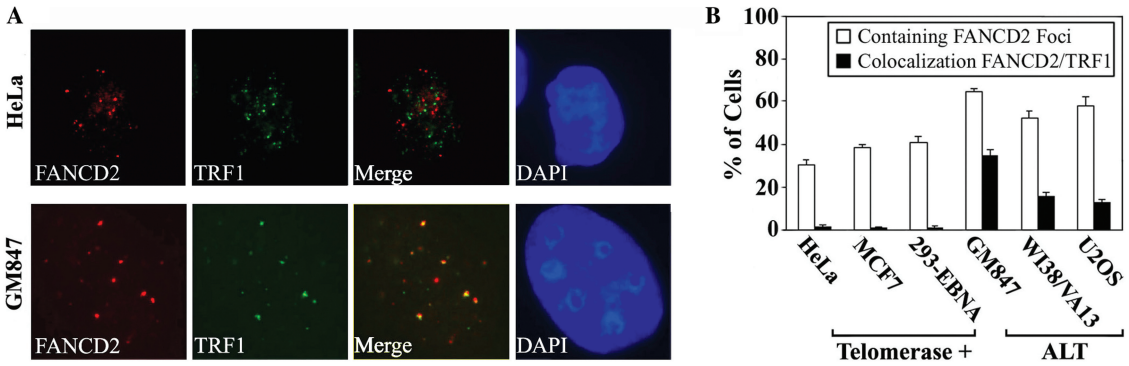

Figure 1. FANCD2 colocalizes with the telomere-binding protein TRF1 in ALT cells. (**A**) Images of FANCD2 foci (red) and TRF1 foci (green) in the telomerase-expressing line, HeLa, and in the ALT cell line, GM847. The position of the nucleus is shown by counterstaining with DAPI. (**B**) Quantification of the colocalization of FANCD2 foci with TRF1 foci. The percentage of three telomerase-expressing cell lines (HeLa, MCF7 and 293-EBNA) and three ALT cell lines (GM847, WI38/VA13 and U2OS) with five or more FANCD2 foci, or with two or more FANCD2 foci which colocalized with the telomeric protein TRF1, is shown. Two foci were used as the standard for colocalization, since telomeres can aggregate in ALT cells into a small number of bright telomeric foci (22,26,38). Each bar represents the average of three counts of at least 150 cells each ±SD. The levels of colocalization of FANCD2 with TRF1 in ALT cells were statistically different from those seen in telomerase-expressing cells ($P < 0.01$).

In initial experiments, we assayed colocalization of FANCD2 foci with the telomere-binding protein TRF1 (37) (Figure 1). FANCD2 did not colocalize with TRF1 in the telomerase-expressing cell line HeLa (Figure 1A) or in proliferating primary human fibroblasts (GM00038) (data not shown). We did find, however, that FANCD2 colocalized with TRF1 in the ALT cell line, GM847 (Figure 1A). Telomeres aggregate (cluster) in ALT cells (22,26,38). As a result, ALT cells frequently have a smaller number of TRF1 foci, which are larger than those detected in telomerase-expressing cells. We found that FANCD2 also colocalized with the telomeric protein TRF2 in 35.9 ± 2.1% of GM847 ALT cells, but not in HeLa cells (1.3 ± 0.6%).

A quantitative analysis of three telomerase-expressing cell lines (HeLa, MCF7 and 293-EBNA) and three ALT cell lines (GM847, WI38 VA13/R2 and U2OS) showed that spontaneous FANCD2 foci formed in each of the cell lines (Figure 1B). FANCD2 foci specifically colocalized with TRF1 at telomeres in each of the ALT cell lines (34.8 ± 2.6%, 15.9 ± 1.7% and 12.4 ± 1.3% of randomly cycling GM847, WI38 VA13/R2 and U2OS cells, respectively), but not in any of the telomerase-expressing cell lines which were tested (less than 2% of randomly cycling HeLa, MCF7 or 293-EBNA cells) (Figure 1B).

We examined whether colocalization of FANCD2 with TRF1 is cell-cycle regulated. For these experiments, ALT (U2OS) cells were synchronized using a double thymidine treatment. Just prior to release from synchronization (0 h), U2OS cells were at the G1-S boundary (Figure 2A). Cells were in mid-S phase at 6 h after release and in G2-mitosis by 12 h of release from synchronization. At 15 h after release from synchronization, an accumulation of cells with a 2N DNA content was evident, indicating passage through mitosis and return to G1.

Nearly all U2OS cells had FANCD2 foci prior to release from double-thymidine synchronization (0 h) (Figure 2B). The percentage of cells with FANCD2 foci was relatively constant until it began to decline at 12 h following release from synchronization. The percentage of cells with FANCD2 foci declined further as cells divided and returned to G1 at 15 h of release.

In contrast, the colocalization of FANCD2 foci with telomeres (TRF1) in ALT cells was low in early S phase (0 and 3 h following release from synchronization). Colocalization of FANCD2 foci with TRF1 foci peaked dramatically in late S/G2/mitosis at 9–12 h following release from synchronization, and decreased at 15 h following release from synchronization when many cells had re-entered G1 (Figure 2C). We assayed for the localization of FANCD2 to spread mitotic chromosomes to determine whether FANCD2 colocalizes with ALT bodies during mitosis (Figure 2D). FANCD2 foci did not associate with mitotic chromosomes. We conclude that colocalization of FANCD2 foci with TRF1 foci in ALT cells is restricted to late S phase/G2, thus explaining why colocalization is not observed in every ALT cell that displays spontaneous FANCD2 foci.

As an alternative measure of the colocalization of FANCD2 foci with telomeres in ALT cells, we labeled cells with FANCD2 antibodies and detected telomeric repeat DNA by simultaneous FISH (37,39). We found that FANCD2 foci colocalized with telomeric DNA in two different ALT cell lines, GM847 and U2OS (Figure 3A). In contrast, FANCD2 foci did not colocalize with telomeric DNA in telomerase-expressing HeLa cells (data not shown). Furthermore, TRF1 uniformly colocalized with telomeric DNA in interphase GM847 cells (Figure 3B), and in HeLa cells (data not shown), validating the use of TRF1 antibody in subsequent figures for the identification of telomeres by immunofluorescence microscopy. Consistent with a recent report (40), we also found that TRF1 strongly colocalized with telomeric DNA following the induction of DNA damage (data not shown).

The promyelocytic leukemia protein (PML) forms ALT-associated PML bodies (APBs) (21,22,26), which also contain telomeric DNA, telomere-binding proteins, and certain DNA repair proteins. To better understand the behavior of FANCD2 in ALT cells, we sought to

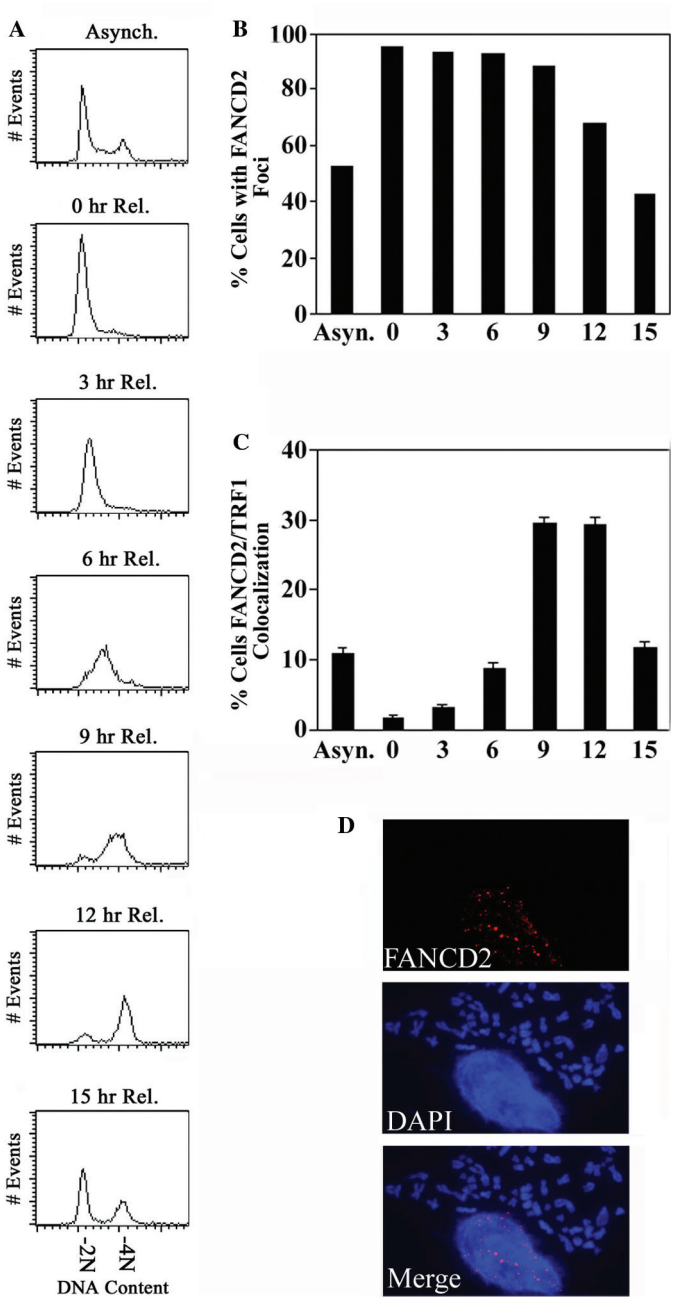

Figure 2. FANCD2 colocalizes with TRF1 foci in ALT cells in a cell-cycle-specific manner. (**A**) Cell-cycle distributions of asynchronous U2OS ALT cells, or at various timepoints following double-thymidine synchronization, were determined by flow cytometric measurements of DNA content. (**B** and **C**) The percentage of cells with five or more FANCD2 foci (B), or with two or more FANCD2 foci colocalized with TRF1 foci (C), in asynchronous populations or at timepoints following release from double-thymidine synchronization. Each bar represents the average of three counts of 150 or more cells ±SD. (**D**) A representative field of GM847 ALT cells, treated with 1 μg/ml nocodazole for 5 h to arrest mitotic progression, contains an interphase cell and a cell with mitotic chromosomes detected by counterstaining with DAPI (blue). FANCD2 foci (red) were detected with E35 antibody.

determine whether FANCD2 colocalizes with APBs. FANCD2 foci colocalized with PML in the ALT cell line GM847, but did not colocalize with PML bodies in HeLa cells (Figure 4A).

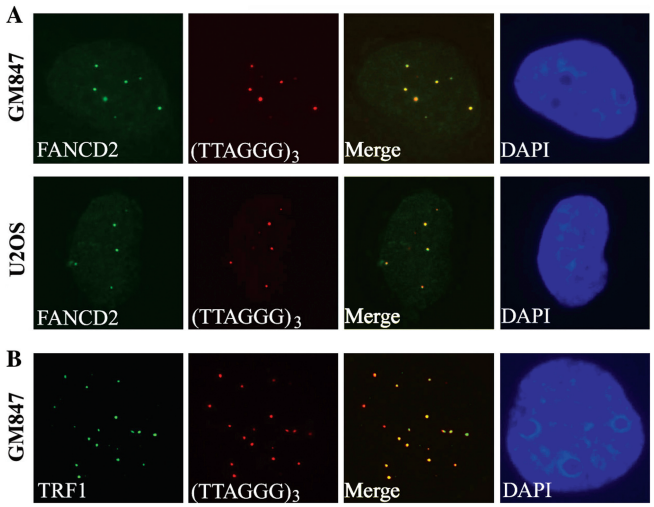

Figure 3. FANCD2 colocalizes with telomeric DNA repeats in ALT cells. (**A**) FANCD2 was detected with antibodies (green) and telomeric DNA was detected with a Cy3-PNA probe (red) in the ALT cell lines GM847 and U2OS. (**B**) TRF1 was detected with anti-TRF1 antibodies (green) and telomeric DNA (red) was detected with a Cy3-PNA probe in GM847 cells.

A comparison of three telomerase-expressing cell lines (HeLa, MCF7 and 293-EBNA) and three ALT cell lines (GM847, WI38 VA13/R2 and U2OS) shows that nearly all cells had PML foci, regardless of the mechanism of telomere maintenance (Figure 4B). FANCD2 colocalized with PML in each of the ALT cell lines (15.3 ± 1.1%, 10.9 ± 0.9% and 3.6 ± 0.6% of randomly cycling GM847, WI38 VA13/R2 and U2OS cells, respectively), but in none of the three telomerase-expressing cell lines tested (less than 1% of randomly cycling populations of HeLa, MCF7 or 293-EBNA cells) (Figure 4B).

Because FANCD2 preferentially colocalizes with TRF1 at late S phase/G2 (Figure 2), and because PML also colocalizes with telomeres late in the cell cycle (21–23), we sought to determine whether FANCD2 colocalizes with PML bodies in a cell-cycle-dependent manner in ALT cells. For this purpose, we synchronized GM847 cells using a double thymidine treatment (Supplementary Figure 1). GM847 cells were utilized for these experiments due to the relatively high level of colocalization of FANCD2 and PML bodies in asynchronous populations of this ALT cell line (Figure 4B). Synchronization of GM847 cells was similar to results obtained for U2OS cells in Figure 2A. GM847 cells had a 2N DNA content, consistent with synchronization at the G1/S boundary, prior to release from the second thymidine treatment (Supplementary Figure 1). In contrast, cells displayed a 4N DNA content, indicating the accumulation of GM847 ALT cells at G2-M, at 12 h following release from the second thymidine treatment.

While over 85% of GM847 cells displayed FANCD2 foci at both timepoints, only 10.80 ± 2.11% of cells showed colocalization of FANCD2 and PML foci prior to release from synchronization (Figure 4C). In comparison, over 50% of GM847 ALT cells displayed colocalization of FANCD2 and PML foci at G2-M 12 h following

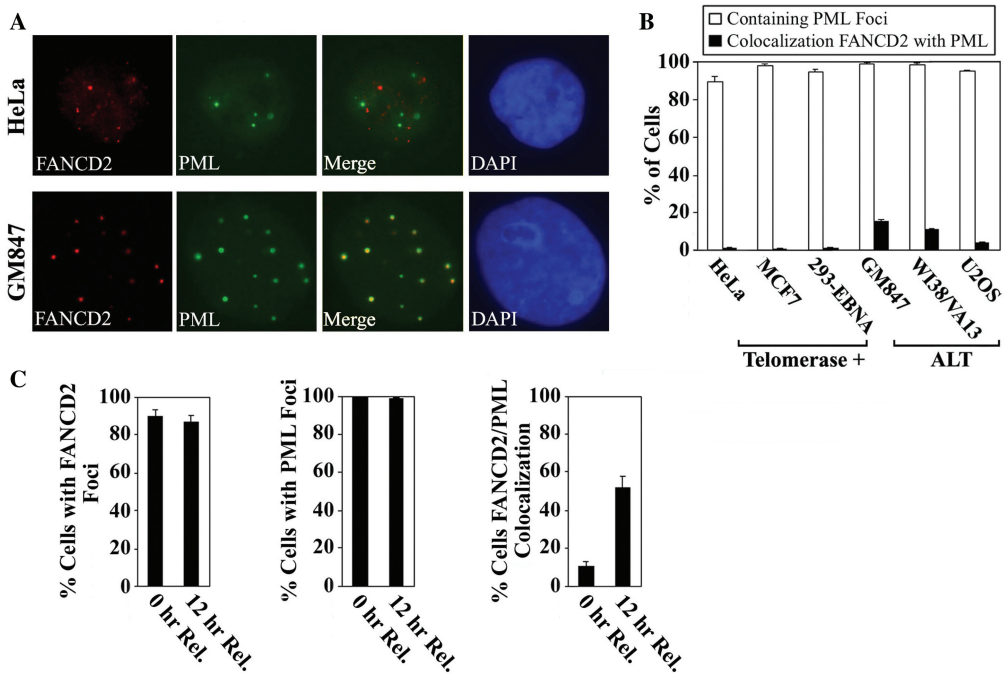

Figure 4. FANCD2 colocalizes with PML in ALT bodies. (**A**) FANCD2 foci (red) and PML bodies (green) were detected in GM847 (ALT cell line) and in HeLa cells (telomerase-expressing cell line). (**B**) Quantification of the assembly of PML bodies and their colocalization with FANCD2 foci in various telomerase-expressing cell lines and ALT cell lines. The percentage of cells with five or more PML foci, or in which two or more FANCD2 foci colocalized with PML, is shown for each cell line. The levels of colocalization of FANCD2 foci with PML foci in ALT cells were statistically different from those observed in telomerase-expressing cells ($P < 0.01$). (**C**) The percentage of GM847 ALT cells with five or more FANCD2 foci, five or more PML foci, or with two or more colocalized FANCD2 and PML foci at 0 or 12 h following release from double-thymidine synchronization is shown. The levels of FANCD2 colocalization with PML foci at 0 and 12 h of release were statistically different ($P < 0.01$). Each bar represents the average of three counts of at least 150 cells each ±SD (B and C).

release from the second thymidine treatment. Over 99% of cells had PML bodies at either timepoint. Thus FANCD2 colocalizes with PML bodies and with TRF1 foci at the same stages of the cell cycle.

FANCA, FANCL and ATR are required for the colocalization of FANCD2 foci with TRF1 foci in ALT cells

Since monoubiquitination is required for the assembly of FANCD2 foci in telomerase-expressing cells during S phase and in response to DNA damage (3,9), we sought to determine whether monoubiquitination is required for the colocalization of FANCD2 foci with TRF1 foci in ALT cells. For this purpose, we depleted FANCA, which is a component of the FA nuclear core complex that is required for FANCD2 monoubiquitination (3,10), using a siRNA that we have described previously (10). Depletion of FANCA in ALT cells (U2OS) inhibited FANCD2 monoubiquitination, but did not deplete FANCD2, both in randomly cycling cells and following exposure to HU (Figure 5A). SiRNAs directed against FANCA also strongly inhibited FANCD2 monoubiquitination in HeLa telomerase-expressing cells (data not shown).

The percentage of U2OS cells transfected with siGFP that contained five or more FANCD2 foci increased following treatment with MMC (Figure 5B). Thus, the assembly of FANCD2 foci is induced by DNA damage both in telomerase-expressing cells (10) and in ALT cells. A smaller number of U2OS ALT cells with FANCD2 foci was observed following depletion of FANCA, as compared to control cells transfected with GFP siRNA. This was true for cells grown either with or without exposure to MMC for 24 h (Figure 5B).

The colocalization of FANCD2 foci and TRF1 foci in U2OS ALT cells is quantified in Figure 5B. Importantly, treatment with MMC induced a strong increase in the colocalization of FANCD2 with TRF1 foci in cells transfected with a control siRNA directed against GFP (Figure 5B). Thus, the association of FANCD2 foci with telomeres in ALT cells may be related to a DNA damage response at this site.

While depletion of FANCA had no effect on TRF1 foci (data not shown), it inhibited colocalization of FANCD2 and TRF1 foci, both in untreated populations of U2OS ALT cells and following treatment with MMC for 24 h. In particular, the percentage of cells which displayed colocalization of FANCD2 and TRF1 foci following treatment with MMC was $44.3 \pm 1.3\%$ and $15.7 \pm 1.1\%$ in cells transfected with siGFP and siFANCA, respectively (Figure 5B). In contrast, while FANCA protein levels were suppressed in HeLa cells by the siRNA directed against FANCA, less than 2% of HeLa cells had FANCD2 foci colocalized with TRF1 foci, either with or without FANCA depletion (data not shown).

Next we sought to determine whether FANCA is required for the cell-cycle-dependent colocalization of

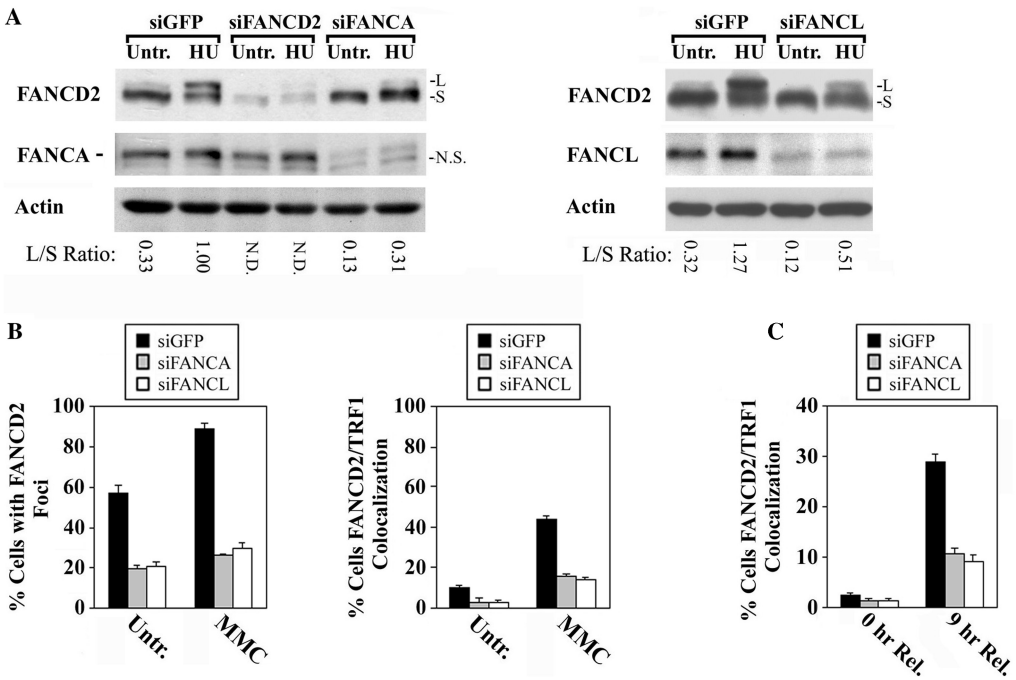

Figure 5. FANCA and FANCL are required for the colocalization of FANCD2 foci with TRF1 foci in ALT cells. (**A**) U2OS (ALT) cells were transfected with siRNAs directed against either FANCA or FANCD2, or with a control siRNA directed against GFP. In an independent experiment, U2OS (ALT) cells were transfected with siRNAs directed against FANCL or GFP. Depletion of FANCA or FANCD2 (left panel), or FANCL (right panel) was assayed on immunoblots. Cells were left untreated or were exposed to HU for 24 h. The ratio of monoubiquitinated (-L) to non-ubiquitinated (-S) FANCD2 is indicated (L/S ratio). Due to the low levels of FANCD2 protein present following transfection with the siRNA directed against FANCD2 (siFANCD2), it was not possible to accurately measure the L/S ratio for these cells and this value was not determined (N.D.). Antibodies directed against FANCA also recognized a non-specific band (N.S.). Immunoblots for actin are shown as a loading control. (**B**) Quantification of the percentage of U2OS cells, transfected with siRNAs directed against GFP, FANCA or FANCL, which had five or more FANCD2 foci in untreated populations or following exposure to 0.5 μM MMC for 24 h (left). Quantification of the percentage of U2OS cells, transfected with siRNAs directed against GFP, FANCA, or FANCA, which had two or more FANCD2 foci colocalized with TRF1 foci is also shown (right panel in figure part). Cells were left untreated or were exposed to 0.5 μM MMC for 24 h. (**C**) Quantification of the percentage of U2OS cells, transfected with siRNAs directed against GFP, FANCA or FANCL, which had two or more FANCD2 foci colocalized with TRF1 foci, at 0 or 9 h following release from double-thymidine synchronization. Each bar represents the average of three counts of 150 or more cells ±SD, except that three counts from two experiments were included for cells transfected with siGFP. The behavior of FANCD2 foci was statistically different in cells transfected with siFANCA or siFANCL, as compared to controls transfected with siGFP ($P < 0.01$) (B and C).

FANCD2 with TRF1 foci (Figure 5C). U2OS ALT cells were transfected with a control siRNA that targeted GFP or with a siRNA directed against FANCA. Cells were then synchronized in early G1 using a double thymidine treatment, as demonstrated in Figure 2A. The colocalization of FANCD2 foci with TRF1 foci was dramatically increased in cells at late S/G2 at 9 h following release from synchronization, as compared to cells in early G1 that had not been released from synchronization (Figure 5C). Critically, depletion of FANCA strongly inhibited the colocalization of FANCD2 with the telomeric marker, TRF1, in cells during late S phase/G2 of the cell cycle (Figure 5C). At 9 h release from synchronization, the percentage of cells that displayed colocalization of FANCD2 and TRF1 foci was 28.7 ± 1.7% and 10.7 ± 1.1% of cells transfected with siGFP and siFANCA, respectively.

To confirm the requirement for FANCD2 to be monoubiquitinated in order to colocalize with telomeres, we tested the role of another component of the FA nuclear core complex, FANCL (Figure 5). Importantly, FANCL is the apparent E3 ubiquitin ligase for FANCD2 (31). For this purpose, we depleted FANCL using a siRNA that has been described previously (31). Depletion of FANCL in ALT cells (U2OS) inhibited FANCD2 monoubiquitination, both in randomly cycling cells and following exposure to HU (Figure 5A). Furthermore, depletion of FANCL in U2OS ALT cells inhibited the assembly of FANCD2 foci either with or without exposure to MMC (Figure 5B). Depletion of FANCL also resulted in inhibition of the colocalization of FANCD2 foci with TRF1 foci in U2OS ALT cells, both in untreated populations and following treatment with MMC for 24 h (Figure 5B). The percentage of cells that displayed colocalization of FANCD2 foci with TRF1 foci in MMC-treated U2OS ALT cells was 44.3 ± 1.3% and 13.8 ± 1.4% following transfection with siRNAs directed against GFP and FANCL, respectively.

We also determined that FANCL, like FANCA, is similarly required for cell-cycle-dependent colocalization of FANCD2 with TRF1 foci (Figure 5C). Depletion of FANCL inhibited the colocalization of FANCD2 foci with TRF1 in cells synchronized in early S phase (0 h Rel.) and at late S phase/G2 (9 h Rel.). In particular, the percentage of cells that displayed colocalization of FANCD2 foci with TRF1 foci at 9 h after release from synchronization was 28.7 ± 1.7% and 9.2 ± 1.2% in cells

transfected with siGFP and siFANCL, respectively. Given that depletion of FANCA or FANCL similarly inhibits FANCD2 monoubiquitination and the colocalization of FANCD2 with TRF1 foci, we conclude that monoubiquitination of FANCD2 is required for its assembly into ALT-associated PML bodies.

The assembly of FANCD2 foci in response to DNA damage or replication stress is regulated by the ATR checkpoint kinase in telomerase-expressing cells (10). Depletion of ATR in U2OS ALT cells using a siRNA we have described previously (10), inhibited FANCD2 monoubiquitination in HU-treated cells (Figure 6A). But depletion of ATR did not inhibit FANCD2 monoubiquitination in untreated cells. Quantification shows that depletion of ATR resulted in a decrease in the percentage of cells treated with MMC that had FANCD2 foci (Figure 6B) and in the percentage of cells in which FANCD2 foci colocalized with TRF1 foci (Figure 6C). In cells treated with MMC, 45.5 ± 1.4 and $6.3 \pm 1.7\%$ of U2OS cells transfected with siGFP and siATR, respectively, displayed colocalization of FANCD2 and TRF1 foci. But depletion of ATR did not alter the number of TRF1 foci (data not shown). Thus, the DNA damage-induced assembly of FANCD2 foci at telomeres in ALT cells requires ATR.

To better understand how ATR regulates the colocalization of FANCD2 foci with TRF1 in ALT cells, we sought to determine whether ATR colocalizes with TRF1 foci in such cells (Figure 6D). As determined by immunofluorescence microscopy, ATR foci colocalized with TRF1 foci in untreated GM847 ALT cells (Figure 6D). ATR also colocalized with TRF1 foci in GM847 cells following treatment with MMC, but did not colocalize with TRF1 in telomerase-expressing HeLa cells (data not shown). Thus, ATR may regulate the monoubiquitination of FANCD2 at ALT telomeres by localizing to ALT-associated PML bodies.

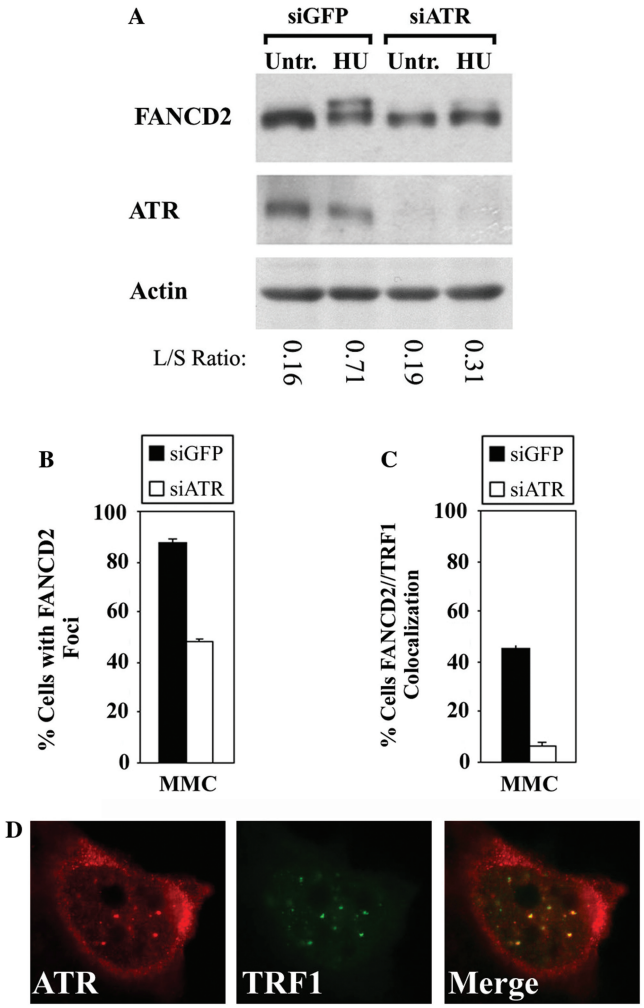

Figure 6. ATR is required for the colocalization of FANCD2 foci with TRF1 foci in ALT cells following induction of DNA damage. (**A**) U2OS ALT cells were transfected with a siRNA directed against ATR or with a control siRNA directed against GFP. Immunoblots for ATR and FANCD2 demonstrate ATR depletion and effects on FANCD2 monoubiquitination, respectively. Cells were either left untreated or were exposed to HU for 24 h. The ratio of monoubiquitinated (-L) to non-ubiquitinated (-S) FANCD2 is indicated (L/S ratio). An immunoblot for actin is shown as a loading control. (**B**) Quantification of the percentage of U2OS cells, transfected with siRNAs directed against either GFP or ATR, which had five or more FANCD2 foci following treatment with 0.5 mM MMC for 24 h. (**C**) Quantification of the percentage of U2OS cells, transfected with siRNAs directed against either GFP or ATR, which had two or more FANCD2 foci colocalized with telomeres detected with antibodies to TRF1 following treatment with 0.5 mM MMC for 24 h. Each bar represents the average of three counts of 150 or more cells ±SD, and differences in the behavior of FANCD2 foci were statistically different in cells transfected with siGFP and siATR ($P < 0.01$) (B and C). (**D**) ATR (red) and TRF1 (green) display a similar pattern of nuclear foci in untreated GM847 ALT cells. Colocalization is demonstrated by merged images (not shown). There is an apparent non-specific signal detected by anti-ATR antibodies outside of the nucleus.

Depletion of FANCA or FANCD2 results in loss of telomere signals at chromosome ends in ALT cells

To determine whether FANCD2 has a function at telomeres in ALT cells, we performed FISH of metaphase spreads using a Cy3-labeled telomeric peptide-nucleic acid (PNA) probe. As examples, representative images are shown for U2OS (ALT) and HeLa (telomerase-expressing) cells which were transfected with a control siRNA directed against GFP (Figure 7A). Importantly, telomeres were readily detectable in metaphase spreads from each cell line. As previously reported, however, telomere lengths were more heterogeneous in ALT cells (16,19). Thus, the longest telomeres in U2OS cells yielded signals of greater intensity, detected with Cy3-PNA, than in HeLa cells. Furthermore, while nearly all telomeres were detectable in HeLa cells, a striking number of telomeres in U2OS cells were not detectable with the Cy3-PNA probe (Figure 7A).

As a measure of altered telomere function, we quantified the percentage of signal-free telomeres in U2OS or HeLa cells transfected with siRNAs directed against GFP, FANCA or FANCD2 (Figure 7B). This assay has been described previously (19,41). Depletion of FANCA or FANCD2 with the appropriate siRNA was effective in both U2OS (ALT) (Figure 5A) and HeLa (telomerase-expressing) cells (10). While $17.6 \pm 2.3\%$ of the telomeres were signal-free in U2OS cells transfected with the control

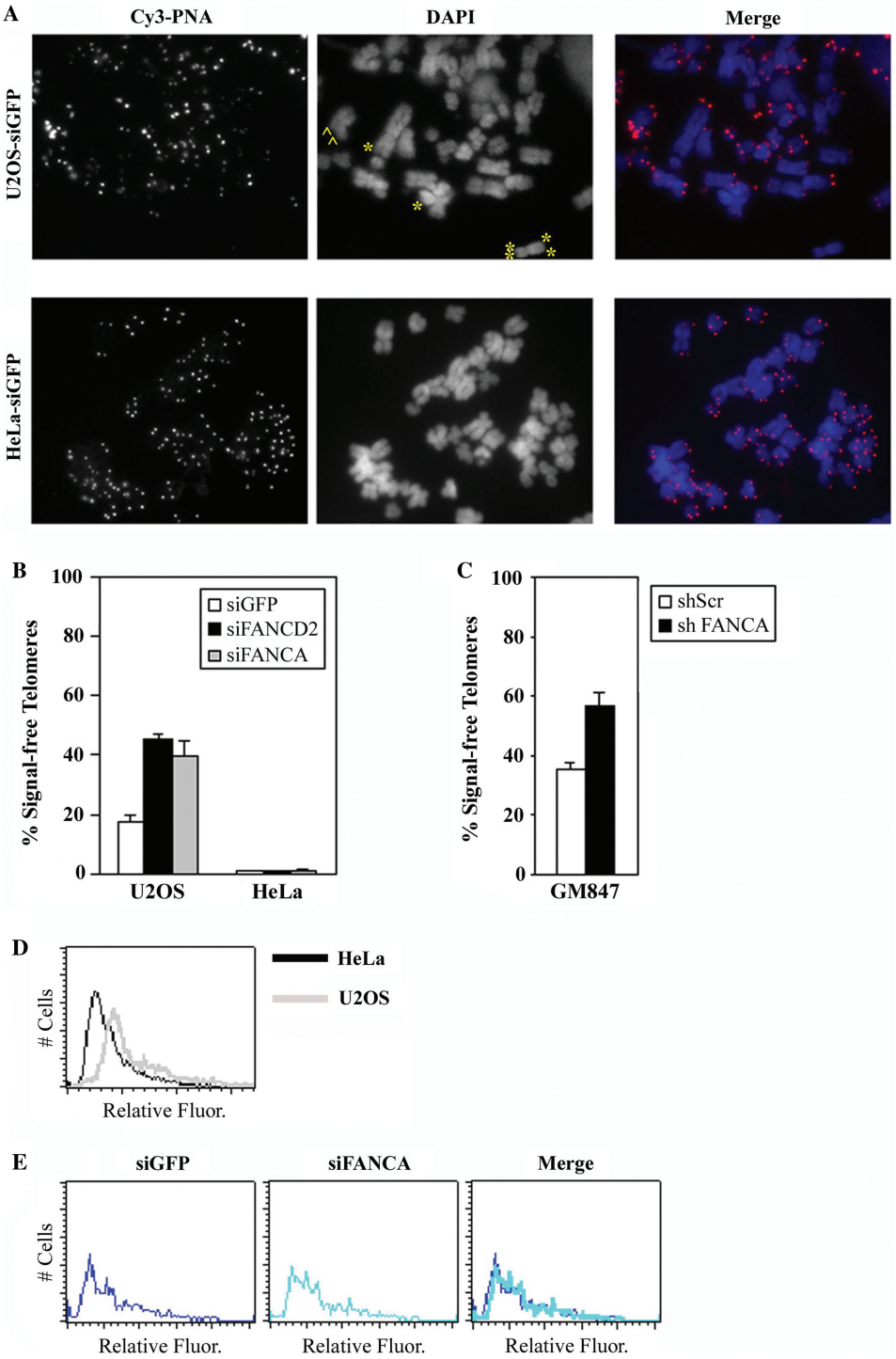

Figure 7. Depletion of FANCA or FANCD2 results in an increased frequency of chromosome ends in ALT cells which are undetectable by *in situ* hybridization with a probe for telomeric DNA. (**A**) Representative images for chromosomes assayed with a Cy3-labeled telomeric probe (PNA) (red) or stained with DAPI (blue) in U2OS (ALT) or HeLa (telomerase-expressing) cells transfected with siRNAs against GFP (control). The telomeric signal was more uniform in HeLa cells than in U2OS cells. Abnormally long telomeres and some telomere ends which were undetectable with Cy3-PNA probe are indicated in the images for U2OS cells by ^ and * symbols, respectively. (**B** and **C**) The percentage of signal-free chromosome ends, quantified from images obtained from metaphase spreads of HeLa or U2OS cells (B) or GM847 cells (C). U2OS and HeLa cells were transfected with siRNAs against GFP (control), FANCA or FANCD2. GM847 cells were transduced with shRNAs against FANCA or a scrambled control (shScr). Cells were examined at 4 days following transfection or transduction. In each case, over 4000 telomeres were examined. For each value, the average of four counts of five or more metaphases each is shown with the SD. Levels of signal-free telomeres in U2OS or GM847 ALT cells that contained si/shRNAs against FANCA or FANCD2 were statistically different ($P < 0.01$) from cells transfected/transduced with control si/shRNAs. (**D**) Histogram showing the distribution of telomere lengths in HeLa and U2OS cells, as determined by flow FISH using Cy_3-OO-$(CCCTAA)_3$. Results obtained in HeLa (black line) and U2OS (grey line) cells are overlayed for purposes of comparison. (**E**) Histograms of telomere lengths (relative fluorescence units) in U2OS ALT cells transfected with a control siRNA directed against GFP (left, dark blue line) or a siRNA directed against FANCA (center, light blue line). An overlay of these results is shown at right. Different settings for flow cytometry were utilized in figure parts D and E.

siRNA targeting GFP, the percentage of signal-free telomere ends was increased in U2OS cells in which either FANCD2 or FANCA was depleted over a period of only 4 days ($45.5 \pm 1.7\%$ and $39.7 \pm 5.3\%$, respectively) (Figure 7B). Only a small percentage of telomeres were signal-free in HeLa cells (less than 1.2% of total telomere ends). Importantly, depletion of FANCA or FANCD2 in HeLa cells had no effect on the quantity of signal-free ends, relative to cells transfected with the control siRNA targeting GFP (Figure 7B).

To determine whether FANCA has a role in telomere maintenance in multiple ALT cell lines, we also depleted FANCA in GM847 cells and assayed for signal-free telomere ends (Figure 7C). SiRNA oligonucleotides were not effective in GM847 cells (data not shown), so we instead developed a lentiviral shRNA that targets FANCA. Using this reagent, FANCA was effectively depleted and FANCD2 monoubiquitination was inhibited (Supplementary Figure 2). While $35.3 \pm 1.9\%$ of the telomeres were signal-free in GM847 cells transduced with the control shRNA, the percentage of signal-free telomere ends was increased in GM847 cells depleted of FANCA over a period of only 4 days ($56.5 \pm 4.9\%$) (Figure 7C).

We utilized flow FISH for quantification of telomere length (Figure 7D and E). This approach measures the combined length of all telomeres in the cell. Flow FISH was capable of distinguishing a greater overall telomere length in U2OS ALT cells [peak signal at 165 relative fluorescence units (RFU), mean signal of 231 RFU] as compared to HeLa telomerase-expressing cells [peak signal at 96 RFU, mean signal of 127 RFU] (Figure 7D). Importantly, depletion of FANCA had a negligible effect on overall telomere length in U2OS cells (Figure 7E). These results suggest that depletion of FANCA or FANCD2 results in destabilization of a subset of telomeres in ALT cells, perhaps the shortest telomeres, rather than a gradual attrition of all telomeres.

ALT cells maintain telomeres through homologous recombination (15,16). Homologous recombination at ALT telomeres is shown schematically in Figure 8A, and a more detailed consideration of the molecular steps that may be involved can be found elsewhere (42,43). Because depletion of FANCA or FANCD2 results in the loss of detectable telomeres in ALT cells (Figure 7), we sought to determine whether FANCA and FANCD2 are required for telomeric recombination in ALT cells. Sister chromatid exchange at telomeres (T-SCE) can be measured by CO-FISH (34). According to the protocol that we utilized (shown schematically in Figure 8B), newly synthesized strands were degraded and only the pre-existing strands were detected by hybridization with a strand-specific telomere probe [Cy3-$(CCCTAA)_3$]. In the absence of a T-SCE event, only a single signal at each end of the chromosome was observed. In contrast, a T-SCE event resulted in a signal that was split between the chromatids on a particular end of the chromosome (Figure 8B). Less frequently, both ends of a chromosome underwent T-SCE and each arm displayed a signal.

In the examples shown, T-SCE (indicated by a yellow dot) was seen with a higher frequency in ALT U2OS cells transfected with siGFP than in cells in which FANCD2 was depleted for 4 days (Figure 8C). It should be noted that, in the examples shown, some chromosome ends lacked telomeres which were detectable by CO-FISH.

The incidence of T-SCE per 100 chromosomes is quantified in Figure 8D and E. Critically, transient depletion of FANCA or FANCD2 in ALT U2OS cells resulted in an equivalent decrease in total T-SCE events (25.0 ± 3.8 and 23.7 ± 3.3 per 100 chromosomes, respectively) relative to controls transfected with siGFP (45.7 ± 2.5 per 100 chromosomes) (Figure 8D). Consistent with the previous reports (17,18), T-SCE was much less frequent in HeLa, which is not an ALT cell line. Furthermore, depletion of FANCA or FANCD2 had no effect on the frequency of T-SCE in HeLa, as compared to populations transfected with siGFP (Figure 8D). Transient depletion of FANCA in GM847 ALT cells also resulted in a decrease in total T-SCE events relative to controls transduced with a control shRNA (40.6 ± 5.2 and 18.9 ± 2.1 per 100 chromosomes in cells containing shScr or shFANCA, respectively) (Figure 8E).

Because depletion of FANCA or FANCD2 decreases the frequency of detectable telomeres in U2OS cells (Figure 7), we also determined the number of T-SCE events relative to the number of chromosome ends with detectable telomeres (Figure 8F). Depletion of either FANCA or FANCD2 in U2OS ALT cells resulted in a decrease in T-SCE at chromosome ends with telomeres detectable by CO-FISH, whether a single signal or split signal, as compared to U2OS cells transiently transfected with siGFP (33.2 ± 1.4, 20.2 ± 2.1 and 21.3 ± 2.1 per 100 signal positive ends in cells transfected with siGFP, siFANCA and siFANCD2, respectively). Depletion of FANCA in GM847 ALT cells also resulted in a decrease in T-SCE at chromosome ends with telomeres detectable by CO-FISH, as compared to GM847 cells transduced with a control shRNA (18.2 ± 2.3 and 11.8 ± 0.8 per 100 signal positive ends in cells transduced with shScr and shFANCA, respectively) (Figure 8G). Thus, we have demonstrated a requirement for FANCA for T-SCE in two different ALT cell lines. Together, our results demonstrate that FANCA and FANCD2 contribute to telomere maintenance in ALT cells, at least in part, through a role in homologous recombination.

DISCUSSION

The FA protein FANCD2 colocalizes with telomeres specifically in cell lines which maintain telomere length through ALT. The colocalization of FANCD2 with telomeres in ALT cells requires two different components of the FA nuclear core complex, FANCA and FANCL. Furthermore, depletion of FANCA, or FANCD2 itself, increases the number of chromosome ends that lack detectable telomere signals. Depletion of FANCA or FANCD2 also results in a decrease in homologous recombination (HR) between telomeres (T-SCE). Together, our results suggest that monoubiquitinated FANCD2 is involved in ALT telomere maintenance, at least in part, through a role in T-SCE.

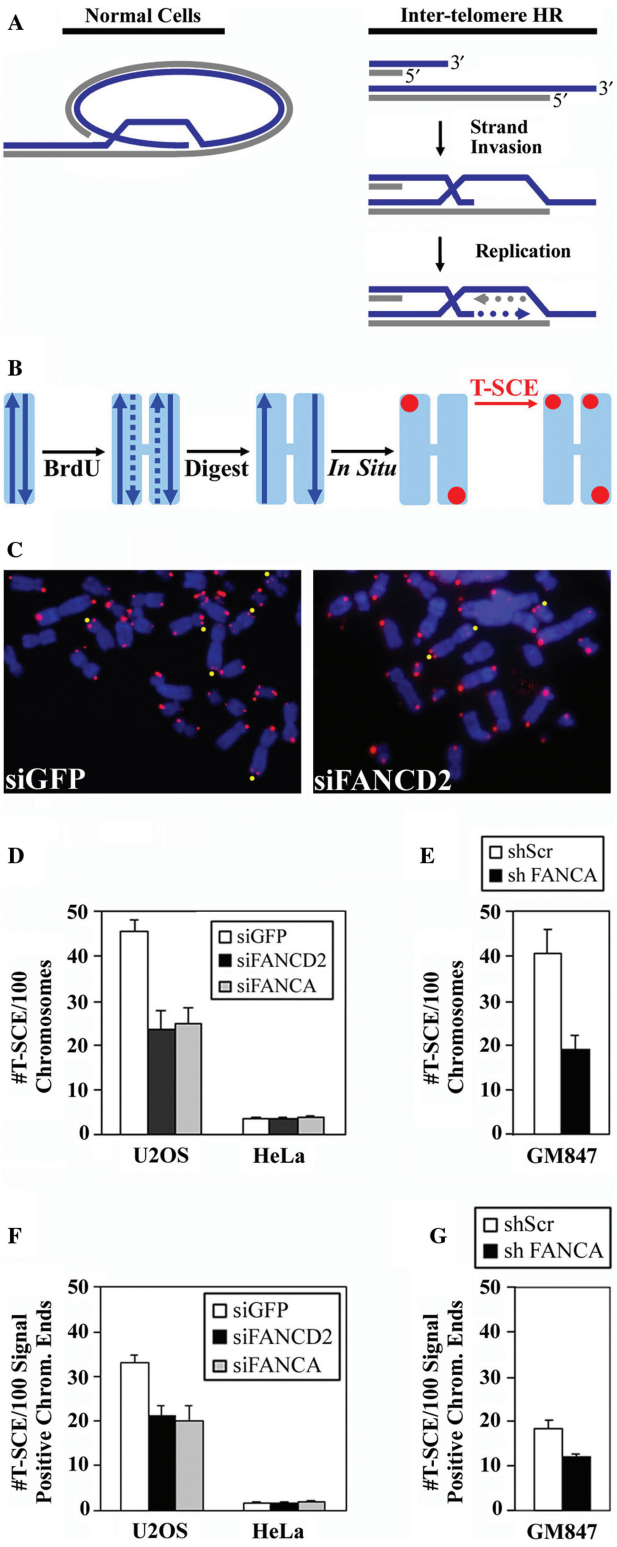

Figure 8. FANCA and FANCD2 are involved in telomere sister chromatid exchange (T-SCE) in ALT cells but not in telomerase-expressing cells. (**A**) Schematic of homologous recombination at telomeres. In normal cells, the 3′ telomeric overhang loops back and invades duplex DNA in the same telomere to form a T-loop that prevents the telomere from being recognized as a DNA double-strand break. In ALT cells, a 3′ overhang of telomeres that is not protected in a T-loop can initiate homologous recombination by invading another telomere. Because telomeres are composed of 5′-TTAGGG-3′ repeats, strand invasion can occur anywhere within the other telomere. Replication can lengthen both strands of the invading telomere. Recombination can be completed by resolution of the Holliday junction. (**B**) Schematic of the CO-FISH protocol utilized. Newly synthesized strands were labeled with BrdU (dashed lines), and the strands were nicked, following treatment with Hoecsht33258 and UV radiation, and nicked strands digested with Exonuclease III. This left only the parental DNA strands (solid lines). Telomeres were detected with a strand-specific Cy3-labeled probe (PNA) by FISH (indicated by red spot). This diagram also shows a signal split between sister chromatids, indicating T-SCE. (**C**) Representative images of CO-FISH for U2OS cells transfected with siRNAs against GFP (control) or FANCD2. Telomeres remaining after digestion of newly replicated strands were detected with a Cy3-labeled telomeric probe (PNA) (red) and the entire chromosome was stained with DAPI (blue), as shown in merged images. Representative T-SCE events are indicated by yellow dots. More T-SCE events were observed in cells treated with siGFP than in those treated with siFANCD2. Some chromosome ends lacked detectable telomeres, and not all T-SCE events are visible in the merged images shown. (D and E) The number of T-SCE events per 100 chromosomes is shown for U2OS (ALT) or HeLa (telomerase-expressing) cells (**D**), or GM847 cells (**E**). (**F** and **G**) The number of T-SCE events per 100 chromosome ends detectable with the telomere probe is shown for U2OS or HeLa cells (F), or GM847 cells (G). U2OS and HeLa cells were transiently transfected with siRNAs that targeted GFP, FANCA or FANCD2, and GM847 cells were transduced with shRNAs directed against FANCA or a scrambled control (shScr) (D–G). Cells were analyzed 4 days after transfection or transduction. Values represent the average of four groups of nine metaphases each ±SD (D–G). A minimum of 2000 chromosomes were examined for each sample. Levels of T-SCE in U2OS or GM847 ALT cells depleted of FANCA or FANCD2 were statistically different ($P < 0.01$) from levels in cells transfected or transduced with control shRNAs.

A role in maintaining or stabilizing telomeres in ALT cells represents a novel function for monoubiquitinated FANCD2 which is related to the maintenance of a stable genome. While many DNA damage response proteins localize to ALT telomeres (22–28,44–47), few, including NBS1 and SMC5/6, have been directly implicated in the maintenance of ALT telomeres (28,46). Thus, our results, which demonstrate a role for the FA pathway, are important for understanding ALT telomere maintenance. In turn, ALT telomeres may provide an important model for analysis of the function of FA proteins in HR.

FANCD2 foci colocalize with TRF1 foci in ALT cells

We have demonstrated the colocalization of FANCD2 with telomeres in ALT cells by independent means, including colocalization with the telomere-binding protein TRF1 and with telomeric DNA. We have not, however, observed co-immunoprecipitation of FANCD2 with TRF1 or TRF2, either in the chromatin fraction that contains monoubiquitinated FANCD2 (7) or under conditions of increased FANCD2 monoubiquitination following the induction of DNA damage (data not shown). FANCD2 may interact with other DNA damage response proteins present at the ALT telomere rather than interacting directly with TRF1 or TRF2. Alternatively, monoubiquitinated FANCD2 might interact with TRF1 or TRF2 only transiently in ALT cells, perhaps in the context of HR between telomeres.

Regulation of the colocalization of FANCD2 with TRF1 in ALT cells

Our results demonstrate that the FA nuclear core complex and ATR are required both for the distribution of FANCD2 to ALT telomeres and to sites of DNA damage induced by various genotoxic agents (3,7,10). In fact, FANCD2 may localize to ALT telomeres as part of a specialized DNA damage response. This is supported by our finding that treatment with MMC induces an increased colocalization of FANCD2 with ALT telomeres (Figure 5B). Furthermore, ALT cells contain telomeres with DNA termini recognized as DNA double-strand breaks (47).

ATR colocalizes with telomeres in ALT cells (Figure 6D), and thus may directly regulate FANCD2 monoubiquitination at this site. We have not, however, determined whether ATR is required for ALT telomere maintenance.

FANCA and FANCD2 are required for telomere maintenance in ALT cells

We have demonstrated, by an increased frequency of chromosome ends lacking detectable telomeres (Figure 7), that depletion of either FANCA or FANCD2 results in an alteration of telomeres in ALT cells. This has been examined by a widely utilized method of FISH with a probe that recognizes TTAGGG telomeric repeats (41,48,49). Importantly, transient depletion of FANCA or FANCD2 over a period of 4 days results in a similar increase in the frequency of signal-free ends in U2OS ALT cells. Together, our results suggest that FANCA and FANCD2 may function in a pathway, leading to FANCD2 monoubiquitination, that contributes to ALT telomere maintenance.

In addition to gradual telomere attrition, ALT cells display rapid decreases or increases of telomere length of multiple kilobases (50). We propose that a deficiency for FANCD2 or its monoubiquitination may lead to rapid telomere shortening in ALT cells due, at least in part, to a demonstrated defect in the T-SCE that is required for telomere lengthening. Changes in overall telomere length are not detected in ALT cells by flow FISH following transient depletion of FANCA (Figure 7E). This may indicate that monoubiquitinated FANCD2 has a role in maintaining only the shortest telomeres in ALT cells. Monoubiquitinated FANCD2 might also normally act to stabilize or cap linear DNA present at APBs (51). In this context, it is interesting that the telomeric protein, POT1b, is involved both in stabilizing telomeres and in regulating T-SCE (52).

While lymphoblasts from FA patients have shorter telomeres relative to age-matched controls (53–55), a previous study using mouse and human cells deficient for FA nuclear core complex proteins demonstrated no direct role for the FA pathway in telomere maintenance in non-ALT cells (56). This is in accord with our finding that FANCD2 does not colocalize with telomeres in non-ALT cells and therefore is not expected to have a role in telomere maintenance in such cells.

Functions of FA proteins in homologous recombination

We demonstrate that deficiency for FANCA or FANCD2 results in a decrease in T-SCE, which is a form of homologous recombination required for ALT telomere maintenance (15,16). It should be noted that not all proteins that have an integral role in homologous recombination are required for T-SCE or for ALT telomere maintenance. An example of this is RAD54 (17). Thus, FA proteins may be involved in a specific form of HR that is utilized to maintain ALT telomeres.

Deficiency for FA proteins, such as FANCA, FANCG and FANCD2, is associated with a modest defect in DNA double-strand break-induced HR (57–59). Based upon such results, it has been suggested that FA proteins, other than BRCA2/FANCD1 and its partner, PALB2/FANCN, have a regulatory role in HR, rather than a direct mechanistic function in this type of repair (60). The assays of DNA double-strand break-induced HR described earlier utilized a reporter introduced into DNA (57–59). In contrast, the function of FA proteins in T-SCE represents homologous recombination in a physiological context. As such, ALT telomeres may represent an important system for understanding the function of FA proteins in DNA repair.

Roles of DNA damage response proteins at APBs

Incorporation of bromodeoxyuridine at APBs during late S phase/G2 (21–23) could be related either to replication of telomeric DNA or homologous recombination at telomeres. We suggest that monoubiquitinated FANCD2 has a function in the maintenance of ALT telomeres that is related to DNA repair rather than DNA replication. First, we have demonstrated a function for FANCA and FANCD2 in T-SCE. Second, FA proteins have been implicated in DNA repair in non-ALT cells (57–59), and FANCD2 is not a constitutive component of the replication machinery (61). Third, neither FANCD2 nor its monoubiquitination significantly influences the rate of bulk DNA replication (9). Finally, similar to FANCD2, other DNA repair-related proteins, such as NBS1 and SMC5/6, have a role in T-SCE and colocalize with APBs during late S phase/G2 (22,28).

How T-SCE acts to maintain ALT telomeres is not well understood. The DNA repair proteins WRN and Ku70, and the telomeric proteins Pot1 a and b, suppress sister chromatid exchange at telomeres (T-SCE) (48,52,62,63). In contrast, the FA pathway, along with SMC5/6 (28), appears to be required for the T-SCE that drives telomere maintenance in ALT cells (15,16). The functional relationship of SMC5/6 to FA proteins in T-SCE, if any, is unknown at present.

Interestingly, our results suggest that two different ubiquitin-related or ubiquitin-like protein modifications are involved in ALT telomere maintenance. It has been reported that sumoylation of the telomere-binding proteins TRF1 and TRF2 is dependent upon SMC5/6. Further, SMC5/6 are required for the formation of APBs and for T-SCE in ALT cells (28). Our findings suggest that monoubiquitination of FANCD2 is also

required for colocalization of this protein with APBs and for T-SCE in ALT cells.

Consequences of the telomeric localization of FANCD2 in ALT cells for cancer formation and for therapy

Given our finding that FANCA and FANCD2 are specifically involved in the maintenance of telomeres in ALT cells, it is expected that tumors in FA patients likely express telomerase, rather than relying on the ALT pathway for telomere stabilization. Indeed, the most prevalent malignancies in FA patients are AML and squamous carcinoma of the head and neck (1). These cancers utilize ALT with a low frequency (14,64).

Because of a defect in the cellular response to DNA damage, FA patients are potentially hypersensitive to radiotherapy or chemotherapy (1,65). If tumors in FA patients uniquely develop through reactivation of telomerase, perhaps inhibition of telomerase (66) will be a more effective therapeutic strategy to which FA patients are not hypersensitive.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

REFERENCES

1. Alter,B.P. (2003) Cancer in Fanconi anemia, 1927–2001. *Cancer*, **97**, 425–440.
2. Taniguchi,T. and D'Andrea,A.D. (2006) Molecular pathogenesis of Fanconi anemia: recent progress. *Blood*, **107**, 4223–4233.
3. Garcia-Higuera,I., Taniguchi,T., Ganesan,S., Meyn,M.S., Timmers,C., Hejna,J., Grompe,M. and D'Andrea,A.D. (2001) Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol. Cell*, **7**, 249–262.
4. Thompson,L.H. (2005) Unraveling the Fanconi anemia-DNA repair connection. *Nat. Genet.*, **37**, 921–922.
5. Grompe,M. and van de Vrugt,H. (2007) The Fanconi family adds a fraternal twin. *Dev. Cell*, **12**, 661–662.
6. Taniguchi,T., Garcia-Higuera,I., Xu,B., Andreassen,P.R., Gregory,R.C., Kim,S.T., Lane,W.S., Kastan,M.B. and D'Andrea,A.D. (2002) Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. *Cell*, **109**, 459–472.
7. Montes de Oca,R., Andreassen,P.R., Margossian,S.P., Gregory,R.C., Taniguchi,T., Wang,X., Houghtaling,S., Grompe,M. and D'Andrea,A.D. (2005) Regulated interaction of the Fanconi anemia protein, FANCD2, with chromatin. *Blood*, **105**, 1003–1009.
8. Smogorzewska,A., Matsuoka,S., Vinciguerra,P., McDonald,E.R. 3rd, Hurov,K.E., Luo,J., Ballif,B.A., Gygi,S.P., Hofmann,K., D'Andrea,A.D. *et al*. (2007) Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. *Cell*, **129**, 289–301.
9. Taniguchi,T., Garcia-Higuera,I., Andreassen,P.R., Gregory,R.C., Grompe,M. and D'Andrea,A.D. (2002) S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. *Blood*, **100**, 2414–2420.
10. Andreassen,P.R., D'Andrea,A.D. and Taniguchi,T. (2004) ATR couples FANCD2 monoubiquitination to the DNA-damage response. *Genes Dev.*, **18**, 1958–1963.
11. Desmaze,C., Soria,J.C., Freulet-Marriere,M.A., Mathieu,N. and Sabatier,L. (2003) Telomere-driven genomic instability in cancer cells. *Cancer Lett.*, **194**, 173–182.
12. Maser,R.S. and DePinho,R.A. (2002) Connecting chromosomes, crisis, and cancer. *Science*, **297**, 565–569.
13. Reddel,R.R., Bryan,T.M., Colgin,L.M., Perrem,K.T. and Yeager,T.R. (2001) Alternative lengthening of telomeres in human cells. *Radiat. Res.*, **155**, 194–200.
14. Henson,J.D., Neumann,A.A., Yeager,T.R. and Reddel,R.R. (2002) Alternative lengthening of telomeres in mammalian cells. *Oncogene*, **21**, 598–610.
15. Dunham,M.A., Neumann,A.A., Fasching,C.L. and Reddel,R.R. (2000) Telomere maintenance by recombination in human cells. *Nat. Genet.*, **26**, 447–450.
16. Varley,H., Pickett,H.A., Foxon,J.L., Reddel,R.R. and Royle,N.J. (2002) Molecular characterization of inter-telomere and intra-telomere mutations in human ALT cells. *Nat. Genet.*, **30**, 301–305.
17. Akiyama,K., Yusa,K., Hashimoto,H., Poonepalli,A., Hande,M.P., Kakazu,N., Takeda,J., Tachibana,M. and Shinkai,Y. (2006) Rad54 is dispensable for the ALT pathway. *Genes Cells*, **11**, 1305–1315.
18. Londono-Vallejo,J.A., Der-Sarkissian,H., Cazes,L., Bacchetti,S. and Reddel,R.R. (2004) Alternative lengthening of telomeres is characterized by high rates of telomeric exchange. *Cancer Res.*, **64**, 2324–2327.
19. Scheel,C., Schaefer,K.L., Jauch,A., Keller,M., Wai,D., Brinkschmidt,C., van Valen,F., Boecker,W., Dockhorn-Dworniczak,B. and Poremba,C. (2001) Alternative lengthening of telomeres is associated with chromosomal instability in osteosarcomas. *Oncogene*, **20**, 3835–3844.
20. Chang,S., Khoo,C.M., Naylor,M.L., Maser,R.S. and DePinho,R.A. (2003) Telomere-based crisis: functional differences between telomerase activation and ALT in tumor progression. *Genes Dev.*, **17**, 88–100.
21. Grobelny,J.V., Godwin,A.K. and Broccoli,D. (2000) ALT-associated PML bodies are present in viable cells and are enriched in cells in the G(2)/M phase of the cell cycle. *J. Cell Sci.*, **113**, 4577–4585.
22. Wu,G., Lee,W.H. and Chen,P.L. (2000) NBS1 and TRF1 colocalize at promyelocytic leukemia bodies during late S/G2 phases in immortalized telomerase-negative cells. Implication of NBS1 in alternative lengthening of telomeres. *J. Biol. Chem.*, **275**, 30618–30622.
23. Lillard-Wetherell,K., Machwe,A., Langland,G.T., Combs,K.A., Behbehani,G.K., Schonberg,S.A., German,J., Turchi,J.J., Orren,D.K. and Groden,J. (2004) Association and regulation of the BLM helicase by the telomere proteins TRF1 and TRF2. *Hum. Mol. Genet.*, **13**, 1919–1932.
24. Wu,G., Jiang,X., Lee,W.H. and Chen,P.L. (2003) Assembly of functional ALT-associated promyelocytic leukemia bodies requires Nijmegen Breakage Syndrome 1. *Cancer Res.*, **63**, 2589–2595.

25. Stavropoulos,D.J., Bradshaw,P.S., Li,X., Pasic,I., Truong,K., Ikura,M., Ungrin,M. and Meyn,M.S. (2002) The Bloom syndrome helicase BLM interacts with TRF2 in ALT cells and promotes telomeric DNA synthesis. *Hum. Mol. Genet.*, **11**, 3135–3144.
26. Yeager,T.R., Neumann,A.A., Englezou,A., Huschtscha,L.I., Noble,J.R. and Reddel,R.R. (1999) Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. *Cancer Res.*, **59**, 4175–4179.
27. Grudic,A., Jul-Larsen,A., Haring,S.J., Wold,M.S., Lonning,P.E., Bjerkvig,R. and Boe,S.O. (2007) Replication protein A prevents accumulation of single-stranded telomeric DNA in cells that use alternative lengthening of telomeres. *Nucleic Acids Res.*, **35**, 7267–7278.
28. Potts,P.R. and Yu,H. (2007) The SMC5/6 complex maintains telomere length in ALT cancer cells through SUMOylation of telomere-binding proteins. *Nat. Struct. Mol. Biol.*, **14**, 581–590.
29. Andreassen,P.R. and Margolis,R.L. (1994) Microtubule dependency of p34cdc2 inactivation and mitotic exit in mammalian cells. *J. Cell Biol.*, **127**, 789–802.
30. Howlett,N.G., Taniguchi,T., Durkin,S.G., D'Andrea,A.D. and Glover,T.W. (2005) The Fanconi anemia pathway is required for the DNA replication stress response and for the regulation of common fragile site stability. *Hum. Mol. Genet.*, **14**, 693–701.
31. Meetei,A.R., de Winter,J.P., Medhurst,A.L., Wallisch,M., Waisfisz,Q., van de Vrugt,H.J., Oostra,A.B., Yan,Z., Ling,C., Bishop,C.E. *et al.* (2003) A novel ubiquitin ligase is deficient in Fanconi anemia. *Nat. Genet.*, **35**, 165–170.
32. Weber,K., Bartsch,U., Stocking,C. and Fehse,B. (2008) A multicolor panel of novel lentiviral "gene ontology" (LeGO) vectors for functional gene analysis. *Mol. Ther.*, **16**, 698–706.
33. Zijlmans,J.M., Martens,U.M., Poon,S.S., Raap,A.K., Tanke,H.J., Ward,R.K. and Lansdorp,P.M. (1997) Telomeres in the mouse have large inter-chromosomal variations in the number of T2AG3 repeats. *Proc. Natl Acad. Sci. USA*, **94**, 7423–7428.
34. Bailey,S.M., Brenneman,M.A. and Goodwin,E.H. (2004) Frequent recombination in telomeric DNA may extend the proliferative life of telomerase-negative cells. *Nucleic Acids Res.*, **32**, 3743–3751.
35. Rufer,N., Dragowska,W., Thornbury,G., Roosnek,E. and Lansdorp,P.M. (1998) Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. *Nature Biotech.*, **16**, 743–747.
36. Zhu,X.D., Kuster,B., Mann,M., Petrini,J.H. and de Lange,T. (2000) Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. *Nat. Genet.*, **25**, 347–352.
37. Chong,L., van Steensel,B., Broccoli,D., Erdjument-Bromage,H., Hanish,J., Tempst,P. and de Lange,T. (1995) A human telomeric protein. *Science*, **270**, 1663–1667.
38. Molenaar,C., Wiesmeijer,K., Verwoerd,N.P., Khazen,S., Eils,R., Tanke,H.J. and Dirks,R.W. (2003) Visualizing telomere dynamics in living mammalian cells using PNA probes. *EMBO J.*, **22**, 6631–6641.
39. Herbig,U., Jobling,W.A., Chen,B.P., Chen,D.J. and Sedivy,J.M. (2004) Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). *Mol. Cell*, **14**, 501–513.
40. Williams,E.S., Stap,J., Essers,J., Ponnaiya,B., Luijsterburg,M.S., Krawczyk,P.M., Ullrich,R.L., Aten,J.A. and Bailey,S.M. (2007) DNA double-strand breaks are not sufficient to initiate recruitment of TRF2. *Nat. Genet.*, **39**, 696–698.
41. Wang,Y., Erdmann,N., Giannone,R.J., Wu,J., Gomez,M. and Liu,Y. (2005) An increase in telomere sister chromatid exchange in murine embryonic stem cells possessing critically shortened telomeres. *Proc. Natl Acad. Sci. USA*, **102**, 10256–10260.
42. McEachern,M.J. and Haber,J.E. (2006) Break-induced replication and recombinational telomere elongation in yeast. *Annu. Rev. Biochem.*, **75**, 111–135.
43. Tarsounas,M. and West,S.C. (2005) Recombination at mammalian telomeres: an alternative mechanism for telomere protection and elongation. *Cell Cycle*, **4**, 672–674.
44. Tarsounas,M., Munoz,P., Claas,A., Smiraldo,P.G., Pittman,D.L., Blasco,M.A. and West,S.C. (2004) Telomere maintenance requires the RAD51D recombination/repair protein. *Cell*, **117**, 337–347.
45. Opresko,P.L., Otterlei,M., Graakjaer,J., Bruheim,P., Dawut,L., Kolvraa,S., May,A., Seidman,M.M. and Bohr,V.A. (2004) The Werner syndrome helicase and exonuclease cooperate to resolve telomeric D loops in a manner regulated by TRF1 and TRF2. *Mol. Cell*, **14**, 763–774.
46. Zhong,Z.H., Jiang,W.Q., Cesare,A.J., Neumann,A.A., Wadhwa,R. and Reddel,R.R. (2007) Disruption of telomere maintenance by depletion of the MRE11/RAD50/NBS1 complex in cells that use alternative lengthening of telomeres. *J. Biol. Chem.*, **282**, 29314–29322.
47. Nabetani,A., Yokoyama,O. and Ishikawa,F. (2004) Localization of hRad9, hHus1, hRad1, and hRad17 and caffeine-sensitive DNA replication at the alternative lengthening of telomeres-associated promyelocytic leukemia body. *J. Biol. Chem.*, **279**, 25849–25857.
48. Laud,P.R., Multani,A.S., Bailey,S.M., Wu,L., Ma,J., Kingsley,C., Lebel,M., Pathak,S., DePinho,R.A. and Chang,S. (2005) Elevated telomere-telomere recombination in WRN-deficient, telomere dysfunctional cells promotes escape from senescence and engagement of the ALT pathway. *Genes Dev.*, **19**, 2560–2570.
49. Erdmann,N., Liu,Y. and Harrington,L. (2004) Distinct dosage requirements for the maintenance of long and short telomeres in mTert heterozygous mice. *Proc. Natl Acad. Sci. USA*, **101**, 6080–6085.
50. Murnane,J.P., Sabatier,L., Marder,B.A. and Morgan,W.F. (1994) Telomere dynamics in an immortal human cell line. *EMBO J.*, **13**, 4953–4962.
51. Fasching,C.L., Neumann,A.A., Muntoni,A., Yeager,T.R. and Reddel,R.R. (2007) DNA damage induces alternative lengthening of telomeres (ALT) associated promyelocytic leukemia bodies that preferentially associate with linear telomeric DNA. *Cancer Res.*, **67**, 7072–7077.
52. He,H., Multani,A.S., Cosme-Blanco,W., Tahara,H., Ma,J., Pathak,S., Deng,Y. and Chang,S. (2006) POT1b protects telomeres from end-to-end chromosomal fusions and aberrant homologous recombination. *EMBO J.*, **25**, 5180–5190.
53. Adelfalk,C., Lorenz,M., Serra,V., von Zglinicki,T., Hirsch-Kauffmann,M. and Schweiger,M. (2001) Accelerated telomere shortening in Fanconi anemia fibroblasts–a longitudinal study. *FEBS Lett.*, **506**, 22–26.
54. Leteurtre,F., Li,X., Guardiola,P., Le Roux,G., Sergere,J.C., Richard,P., Carosella,E.D. and Gluckman,E. (1999) Accelerated telomere shortening and telomerase activation in Fanconi's anaemia. *Br. J. Haematol.*, **105**, 883–893.
55. Callen,E., Samper,E., Ramirez,M.J., Creus,A., Marcos,R., Ortega,J.J., Olive,T., Badell,I., Blasco,M.A. and Surralles,J. (2002) Breaks at telomeres and TRF2-independent end fusions in Fanconi anemia. *Hum. Mol. Genet.*, **11**, 439–444.
56. Franco,S., van de Vrugt,H.J., Fernandez,P., Aracil,M., Arwert,F. and Blasco,M.A. (2004) Telomere dynamics in FANCG-deficient mouse and human cells. *Blood*, **104**, 3927–3935.
57. Yamamoto,K., Ishiai,M., Matsushita,N., Arakawa,H., Lamerdin,J.E., Buerstedde,J.M., Tanimoto,M., Harada,M., Thompson,L.H. and Takata,M. (2003) Fanconi anemia FANCG protein in mitigating radiation- and enzyme-induced DNA double-strand breaks by homologous recombination in vertebrate cells. *Mol. Cell Biol.*, **23**, 5421–5430.
58. Nakanishi,K., Yang,Y.G., Pierce,A.J., Taniguchi,T., Digweed,M., D'Andrea,A.D., Wang,Z.Q. and Jasin,M. (2005) Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. *Proc. Natl Acad. Sci. USA*, **102**, 1110–1115.
59. Yang,Y.G., Herceg,Z., Nakanishi,K., Demuth,I., Piccoli,C., Michelon,J., Hildebrand,G., Jasin,M., Digweed,M. and Wang,Z.Q. (2005) The Fanconi anemia group A protein modulates homologous repair of DNA double-strand breaks in mammalian cells. *Carcinogenesis*, **26**, 1731–1740.
60. Mirchandani,K.D. and D'Andrea,A.D. (2006) The Fanconi anemia/BRCA pathway: a coordinator of cross-link repair. *Exp. Cell Res.*, **312**, 2647–2653.

61. Hussain,S., Wilson,J.B., Medhurst,A.L., Hejna,J., Witt,E., Ananth,S., Davies,A., Masson,J.Y., Moses,R., West,S.C. *et al.* (2004) Direct interaction of FANCD2 with BRCA2 in DNA damage response pathways. *Hum. Mol. Genet.*, **13**, 1241–1248.
62. Celli,G.B., Denchi,E.L. and de Lange,T. (2006) Ku70 stimulates fusion of dysfunctional telomeres yet protects chromosome ends from homologous recombination. *Nat. Cell Biol.*, **8**, 885–890.
63. Wu,L., Multani,A.S., He,H., Cosme-Blanco,W., Deng,Y., Deng,J.M., Bachilo,O., Pathak,S., Tahara,H., Bailey,S.M. *et al.* (2006) Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. *Cell*, **126**, 49–62.
64. Henson,J.D., Hannay,J.A., McCarthy,S.W., Royds,J.A., Yeager,T.R., Robinson,R.A., Wharton,S.B., Jellinek,D.A., Arbuckle,S.M., Yoo,J. *et al.* (2005) A robust assay for alternative lengthening of telomeres in tumors shows the significance of alternative lengthening of telomeres in sarcomas and astrocytomas. *Clin. Cancer Res.*, **11**, 217–225.
65. Alter,B.P. (2002) Radiosensitivity in Fanconi's anemia patients. *Radiother. Oncol.*, **62**, 345–347.
66. Mokbel,K. (2003) The evolving role of telomerase inhibitors in the treatment of cancer. *Curr. Med. Res. Opin.*, **19**, 470–472.