ZNF365 Promotes Stability of Fragile Sites and Telomeres

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ABSTRACT

Critically short telomeres activate cellular senescence or apoptosis, as mediated by the tumor suppressor p53, but in the absence of this checkpoint response, telomere dysfunction engenders chromosomal aberrations and cancer. Here, analysis of p53-regulated genes activated in the setting of telomere dysfunction identified Zfp365 (ZNF365 in humans) as a direct p53 target that promotes genome stability. Germline polymorphisms in the ZNF365 locus are associated with increased cancer risk, including those associated with telomere dysfunction. On the mechanistic level, ZNF365 suppresses expression of a subset of common fragile sites, including telomeres. In the absence of ZNF365, defective telomeres engage in aberrant recombination of telomere ends, leading to increased telomere sister chromatid exchange and formation of anaphase DNA bridges, including ultra-fine DNA bridges, and ultimately increased cytokinesis failure and aneuploidy. Thus, the p53-ZNF365 axis contributes to genomic stability in the setting of telomere dysfunction.

SIGNIFICANCE: The contribution of the p53-ZNF365-telomere axis in the suppression of genomic instability illuminates how alterations in this pathway may confer increased cancer risk for individuals harboring germline alterations in the ZNF365 locus. Cancer Discov; 3(7); 1–14. ©2013 AACR.

INTRODUCTION

Telomeres maintain chromosome structural integrity and thus promote genomic stability. Dysfunctional telomeres provoke inappropriate DNA repair followed by chromosome nondisjunction events and breakage-fusion-bridge cycles, and, therefore, are major instigators of the genomic instability in diverse human cancer types (reviewed in ref. 1). The multiprotein shelterin complex consisting of TRF1, TRF2, POT1, RAP1, TPP1, and TIN2 forms the telomere loop, or t-loop, that protects telomere ends by preventing their recognition as damaged DNA (2). To date, studies have shown a number of proteins that are recruited to replicate structurally constrained telomeres: TRF2 can recruit Topo2a and Apollo to resolve the positive superhelical strain and protect telomeres (3), and TRF1 plays an essential role in telomere replication (4). In addition, the RecQ helicases WRN and Bloom’s syndrome (BLM) play essential roles in DNA replication, recombination, and repair, and in the maintenance of functional telomeres (5). In particular, BLM is critical for the resolution of late-replicating structures as part of the BTR complex observed on ultra-fine DNA bridges (UFB; ref. 6). Telomeres are often incompletely replicated until late anaphase, and intermediate structures are associated with Fanconi anemia proteins in response to replication inhibition (7).

The genetic interactions between telomere dysfunction and the molecular circuitry underlying the telomere checkpoint response continue to be active areas of investigation. Previous studies have shown functional interactions between telomeres and DNA damage-sensing, transduction (ATM, ATR), and repair molecules (Ligase IV, DNA-PK, Ku70) at the organismal level, where combined deficiencies accelerated telomere attrition and dysfunction, culminating in genomic catastrophe and accelerated aging (8–11). The acute loss of telomere function by expression of dominant-negative TRF2 (telomere repeat-capping protein) leads to the activation of ATM and p53 in human cell culture models, consistent with their roles in sensing telomere dysfunction and eliciting cellular checkpoint responses (12).

In the in vivo setting, genetic analysis of the interplay between telomere dysfunction and ATM or p53 has revealed opposing phenotypic consequences upon loss of ATM versus p53 in the telomerase knockout mouse. In late generation mTerc−/− Atm+/− mice and derivative cells, there is increased genomic instability and preservation of p53-mediated senescence and apoptosis, leading to severe tissue atrophy and degeneration, premature aging, and suppression of cancer compared with mTerc+/− Atm−/− controls. In contrast, late generation mTerc−/− p53−/− mice and cells show restoration of cellular proliferation and survival, an increase in organ cellularity, and enhanced tumorigenesis with altered tumor spectrum, underscoring the critical role of p53 status in dictating genome integrity and cellular and organismal fates with regard to degeneration or cancer (10, 13, 14).

Many correlative studies in human cancer and numerous other studies have supported the view that persistent DNA damage signaling resulting from telomere dysfunction provides pressure to deactivate critical checkpoints and sets

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the stage for accumulation of chromosomal aberrations and aneuploidy (15). A recent study showed the molecular basis of tetraploidization by unprotected telomeres in the absence of POT1 and p53, further implicating dysfunctional telomeres as a cause of genomic instability in human cancer (16).

Genomic instability is a prominent feature of hereditary and sporadic cancers in which we observe the loss of DNA repair and checkpoint genes, increased replication stress associated with activated oncogenes, and erosion of telomeres (reviewed in ref. 17). Functional DNA repair and cellular checkpoint processes promote proper replication of the genome in part by resolving replication blockage. Hereditary defects in DNA repair pathway components such as WRN, BLM, and BRCA1/2 cause DNA breaks during replication, resulting in chromosomal rearrangement (18, 19). In particular, inherently unstable common fragile sites (CFS) tend to break or recombine following partial replication inhibition. Of relevance to our study, such genomic fragile sites include telomeres that are particularly sensitive to replication blockade (4, 6).

Despite the large body of well-described biologic outcomes, the molecular mechanisms underlying the role of p53 in suppressing the genomic instability associated with telomere dysfunction are not well understood. In this current study, we attempt to dissect the circuitry of the p53-mediated checkpoint response by analyzing transcriptional changes associated with telomere dysfunction. Our study identified ZNF365 as a necessary target whose activation by p53 in the presence of critically short telomeres contributes to genomic stability. We provide evidence that loss of ZNF365 leads to increased expression of CFS and dysfunctional telomeres, aberrant sister telomere recombination, and increased aneuploidy. Furthermore, ZNF365 expression is downregulated in triple-negative breast cancer (TNBC), in line with multiple genome-wide association studies defining ZNF365 as a major locus of breast cancer susceptibility in BRCA2-mutant patients (20, 21). Together, these results support the view that ZNF365 is a novel player contributing to genomic stability.

### RESULTS

**p53 Reactivation in Cells with Telomere Dysfunction Causes Robust Gene Expression Changes Resembling a Cellular Checkpoint Response**

To define the p53-mediated transcriptome associated with telomere dysfunction, we used a generation 4 (G4) telomerase-negative, ATM-negative (G4 mTerc<sup>−/−</sup> Atm<sup>−/−</sup>) skin fibroblast model that retains a robust p53-dependent telomere checkpoint. Consistent with previous work, G4 mTerc<sup>−/−</sup> Atm<sup>−/−</sup> p53<sup>−/−</sup> (triple knockout, TKO) skin fibroblasts show inactivation of cellular checkpoints that provide genomic stability. These cells exhibit frequent telomere signal-free ends and significantly shorter telomeres compared with p53 single knockout cells (10) (Fig. 1A–C).

In the TKO cells, we introduced an inducible p53 allele encoding a p53–estrogen receptor fusion protein (p53ER) that becomes functional upon the addition of 4-hydroxytamoxifen (4-OHT; ref. 22). We chose a time point of 4 hours after 4-OHT induction to catalog potential direct targets of p53 in the transcriptome, as known transcriptional targets of Cdkn1a and Mdm2 started to show robust induction (Supplementary Fig. S1A and S1B). Zfp365 (ZNF365 in humans) ranked high among other genes (Mdm2, Phlda3, Gdf15, Ckap2, Gte1, Sen2, Cdkn1a; 2-way ANOVA, P < 0.0005) that were previously linked to p53 biology (http://linkage.rocketfeller.edu/p53; Fig. 1D and Supplementary Table S1).

Consistent with the microarray data, Zfp365 expression peaks at 4 hours upon p53 reactivation followed by restoration to normal basal levels (Fig. 1E). A survey of the Zfp365 5 kb promoter region revealed a putative p53-binding element at position −84 bp relative to the transcriptional start site that is conserved across multiple species (Fig. 1F). A 120-bp fragment, which encompasses the proximal promoter region containing this putative p53-binding element, showed an approximate 6-fold increase in luciferase activity with enforced p53 expression. Collectively, these results identify Zfp365 as a novel transcriptional target of p53 in the context of cells experiencing telomere dysfunction.

**ZNF365 Plays a Role in Telomere Biology and Is Implicated in Breast Cancer Risk**

Next, we characterized ZFP365 in both telomere biology and in human cancers with dysfunctional telomeres. ZFP365 showed a partial colocalization with a fraction of telomeres by immunofluorescence (IF) coupled with telomere FISH. This is in contrast to TRF1 localization, which is mostly telomeric. Its colocalization with telomeres becomes detectable only after aphidicolin (Aph) treatment-mediated replication challenges, suggesting it may transiently localize to the stalled replication sites, including telomeric/subtelomeric regions (Fig. 1G). We further examined its expression and localization pattern in multiple murine and human cell types. Among the human orthologs of ZFP365, ZNF365-A isoform, which bears 98% sequence homology, showed identical subcellular localization with ZFP365, whereas ZNF365-B or -C isoforms, which have different C-termini, primarily localized to centrosomes (Supplementary Fig. S2A and S2B).

Hereafter, we refer to both ZFP365 and ZNF365-A collectively as ZNF365 based on their indistinguishable features. Together, our results suggest that ZNF365 is regulated at the levels of expression and localization in the setting of telomere dysfunction, suggesting a potential role in telomere biology.

**Critical differences and dysfunctional telomeres are universally present in early lesions of human breast cancer (23).** Recent reports alluding to ZNF365 as a major genetic locus for breast cancer risk in BRCA2 mutation carriers (20, 21), we surveyed its expression in TNBC versus non-TNBC in the Cancer Genome Atlas (TCGA) dataset. The former has been shown to exhibit “BRCAness” [i.e., defective double-strand break (DSB) repair capacity] that is shared with BRCA mutation carriers (24). Interestingly, expression analysis showed the lowest expression of ZNF365 in TNBC (n = 49), whereas average expression levels remained high in non-TNBC (n = 300), suggesting that loss of ZNF365 expression may correlate with BRCA status (Fig. 2A, t test P = 7.32e-06). Furthermore, the level of ZNF365 expression stratified the TCGA cohort (n = 2,978) into two groups that exhibited a significant
Figure 1. Significantly shortened telomeres in TKO in comparison with p53 knockout (p53KO) fibroblasts and induction of Zfp365 expression by p53.

A, representative photographs of TKO (left) and p53KO (right) nuclei stained for DNA (DAPI, bottom) and telomere PNA-FISH (FITC, top).

B, histograms that represent mean numbers of telomere-positive ends (B) and mean integrals of PNA signals (signal area × signal intensity) per nucleus (C; mean ± SD, n = 10) suggest the presence of shorter telomeres in the TKO model. PNA, peptide nucleic acid.

D, TKO cells were transfected with p53ER and p53 was reactivated by 4-OHT. Top-ranked genes induced by p53 are listed (FDR < 0.05, P < 0.0005 by 2-way ANOVA, n = 5).

E, enforced p53 activation induced time-dependent Zfp365 expression in TKO cells. TKO cells were transduced with retroviral particles for p53ER and treated with 100 nmol/L 4-OHT for multiple time points. None of the transduced cells and ethanol treatment were used as controls. For indicated time points, Zfp365 expression was analyzed by qRT-PCR.

F, top, a putative p53-binding element conserved in multiple species locates at 84 bp upstream of Zfp365 transcription start site. Bottom, luciferase activity assay using a 120 bp DNA fragment of the Zfp365 promoter region cloned into pGL4 vector. Either GFP- or p53-encoding plasmids were cotransfected and analyzed for the luciferase activity after 72 hours.

G, ZFP365 partially localizes at telomeres after replication challenge with Aph. On the left, IF-FISH staining for ZFP365-v5 (red), TRF1 (red), and telomeres (TelC-FITC) suggests greater colocalization of TRF1 with telomeres, as further illustrated by comparison of the percentage of telomeres positive for TRF1 or ZFP365-v5 plotted on the right (mean ± SD, n = 30).
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Figure 2. ZNF365 expression varies in different subtypes of breast cancer. A, boxed plot of ZNF365 expression levels among human normal breast tissues (n = 244), TNBC (n = 49), and non-TNBC (n = 300) in Oncomine TCGA datasets. B, Kaplan-Meier survival plot showed the prognostic effect of ZNF365 expression (206448_at) on the relapse-free survival over 10 years. HR and log-rank analysis were conducted. C, representative immunohistochemical staining of a tissue microarray with specific antibody against ZNF365. Higher magnification images of the boxed regions are shown on the left. Scale bar, 200 μm.

difference in 10-year, relapse-free survival by Kaplan–Meier analysis (Fig. 2B; HR = 0.74, log-rank $P = 1.5e-05$).

Next, we stained for ZNF365 in a set of tissue microarrays (TMA) comprising cohorts of normal breast tissue (n = 18) and TNBC (n = 141) as well as non-TNBC (luminal type A, n = 145). Consistently, ZNF365 exhibited nuclear expression in normal breast epithelium and non-TNBC, whereas its expression declined significantly in TNBC (Fig. 2C, Table 1). Together, ZNF365 expression varies in different subtypes of breast cancer where distinct levels of telomere dysfunction are present.

Loss of ZNF365 Contributes to Telomere Dysfunction

To further investigate ZNF365 function, we conducted loss of function studies with short hairpin–mediated knockdown in mouse and human cells (Supplementary Fig. S3A).

Table 1. Analysis of the expression level of ZNF365 by immunohistochemical staining of a tissue microarray

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<th>Case</th>
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<td>Mean</td>
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Upon knocking down of ZNF365, numerous 53BP1-positive foci appeared, with many localizing to telomeres, and cultures showed robust activation of p53 as well as an early-senescent phenotype (Fig. 3A–C). To measure the degree of telomere dysfunction, we determined the anaphase bridge index (ABI) out of total late anaphase. ZNF365-depleted TKO cells exhibited a high ABI, indicating the presence of unseparated sister chromatids or DNA bridges (Fig. 3D).

Disruption of a telomere structural component (i.e., TRF2, POT1a) activates DNA damage responses (12, 25, 26). Consequently, aberrant repair of telomeres by the mechanism of nonhomologous end joining mediates chromosomal instability induced by the breakage-fusion-bridge cycle (27, 28). Thus, we posited that p53 activation and cellular senescence responses in ZNF365-depleted cells might be due to disrupted telomeres and ensuing recombination events on unprotected telomeres that lead to DNA damage signaling. Our analysis of the metaphases showed increased p-p chromosomal and intrachromatid fusions in shZNF treated ZNF365-depleted HeLa cells compared with control Scr controls (Supplementary Fig. S3B).

Most strikingly, there was a significant increase in telomere sister chromatid exchange (T-SCE) and telomere defects in multiple cell types (U2OS, IMR90, and TKO) upon ZNF365 knockdown with 2 independent short hairpin RNAs (shRNA; Fig. 3E and Supplementary Fig. S4A–S4C). In early-passage IMR90 cells with sufficient telomere reserve, T-SCE occurred in less than 2 to 3 cell cycles after knocking down ZNF365 (Fig. 3E and Supplementary Fig. S4A). These results suggest that T-SCE is an outcome of an exacerbated dysfunctional state at telomeres, and acute loss of ZNF365 is sufficient to cause such changes in cells with longer telomeres (Supplementary Table S2). Furthermore, the average telomere length was not significantly affected in cells with T-SCE. Instead, we observed a high degree of variation in the distribution of telomere intensity (Fig. 3F, note larger SD in ZNF365 knockdown cells). Moreover, no telomere signals were followed by early cellular senescence (Fig. 4E). These changes were accompanied by large variations in telomere length (Scr, ranges from 440 to 7507 vs. siZNF, 4 to 9867), suggesting telomere phenotypes might occur at the acutely shortened telomeres. These results show that ZNF365 is essential to prevent defective telomeres, regardless of telomere length.

Our observation of increased 53BP1 foci in ZNF365-depleted cells, even in the absence of exogenous genotoxic stress, indicated the presence of widespread DNA damage (Fig. 4F). These findings pointed to a potential role for ZNF365 in genomic stability for nontelomeric DNA. Notably, chromosomal breakage in ZNF365-depleted culture increased significantly upon Aph treatment, indicating conversion of stalled replication forks into DNA DSBs (Fig. 5A). These increased DSBs point to the involvement of ZNF365 in the maintenance of nontelomeric and low-dose Aph-sensitive fragile sites.

To examine the effect of ZNF365 deficiency on CFS expression, we surveyed the well-characterized genomic region of 16q23.2 (FRA16D). Consistent with our prediction, fragile site expression at 16q23.2 increased significantly in Aph-treated ZNF365-depleted HeLa cells compared with control cells (Fig. 5B). However, expression at other fragile sites such as 3p14.2 (FRA3B) and 12p12.1 was marginal, suggesting a site-specific role of ZNF365 (Supplementary Fig. S5A). Together, our results indicate that ZNF365 is a necessary element in the maintenance of a subset of genomic regions that are sensitive to replication stress, including telomeres.

**Loss of ZNF365 Leads to Defective Telomere Structures and FRA16D Expression**

Telomeres are topologically strained genomic regions where cellular DNA replication forks are subject to stalling. Interestingly, ZNF365 knockdown increased the number of telomeres that highly resemble “fragile telomeres,” as previously described for abnormally fused or amplified sister telomeres in TRF1-deficient cells (Fig. 4A). In addition, telomere defects resulting from ZNF365 knockdown were classified into several structural classes: telomere heterogeneity, loss, fragility, and fusion (intra- and interchromosomal; Fig. 4A). ZNF365 depletion increased the incidence of telomere defects in TKO and U2OS cells compared with the control Scr cells (Fig. 4B and C).

The high incidence of these telomere defects in ZNF365-deficient cells prompted us to speculate that replication of telomeric regions was affected, as similarly observed in TRF1-deficient cells (4). Therefore, we examined the effect of replication challenge on the frequency of defective telomeres. Consistent with previous studies, HeLa cells treated with low-dose Aph showed fragile telomeres, albeit less prominently than ZNF365-deleted HeLa cultures (Fig. 4D). In ZNF365-deficient HeLa cells, we mainly observed fragility of telomeres with infrequent fusion events. Overall, the level of telomere defects was much less prominent than that in telomerase-negative cell types that we examined, suggesting telomerase may have resolved some of the telomere defects.

To determine whether fragility is due to shortened telomeres, knockdown of ZNF365 was conducted in early-passage (< 5) IMR90 primary fibroblasts with sufficiently long telomeres (29). In IMR90, acute knockdown of ZNF365 caused telomere defects, including heterogeneity and fragility, which were followed by early cellular senescence (Fig. 4E). These changes were accompanied by large variations in telomere length (Scr, ranges from 440 to 7507 vs. siZNF, 4 to 9867), suggesting telomere phenotypes might occur at the acutely shortened telomeres. These results show that ZNF365 is essential to prevent defective telomeres, regardless of telomere length.

**Lack of ZNF365 Increases Mitotic UBF and Aneuploidy**

Next, we determined the outcome of ZNF365 deficiency in mitotic cells. Both Scr and shZNF U2OS cells treated with Aph increased UBFs, as determined by BLM staining–positive structures (Fig. 5C). Fanconi anemia proteins cooperate in DNA replication and recombination and are activated at stalled replication forks (30, 31). In particular, persistent FANCD2 (FD2) foci localize to UBFs originating from CFS, including FRA16D (7). Because knockdown of ZNF365 increased expression of a subset of CFS under replication challenge, we scored and compared the frequency of both
Figure 3. Depletion of ZNF365 causes damaged telomeres and cellular senescence. A, depletion of ZNF365 increased 53BP1-positive telomeres. 53BP1 IF staining (red) combined with FISH for TelC (green) showed frequency of 53BP1-positive TelC telomeres out of total telomeres in HeLa Scr (nontargeting scrambled sequence) and shZNF cells. The quantitative results are shown in the bottom right of the panel. **, \( P = 0.005 \); n.d, not determined. B, activation of p53 by Zfp365 knockdown in p16 Ink4a-null mouse embryonic fibroblasts (MEF). Cultures were harvested 96 hours after shRNA knockdown and phospho-p53 (S15) immunoblotting was conducted with Zeocin-treated cell lysate as a positive control. C, knockdown of Zfp365 in early-passage MEFs (P = 2) led to senescence [bottom, bright field; top, senescence associated β-galactosidase (SA-β-gal) staining] phenotype. The percentage of the SA-β-gal–positive cells was plotted on the right side. **, \( P < 0.05 \). D, a representative DAPI staining (green) image shows anaphase bridges from TKO shZfp culture. The anaphase bridge index (number of nuclei with anaphase bridges)/total number of anaphase nuclei) is plotted on the left (mean ± SD; 3 independent experiments). The inset shows the higher magnification of an anaphase bridge. E, frequency of T-SCE per chromosome end (%). F, representative TEL-FISH (TelC, green) images in TKO Scr, TKO shZfp, U2OS Scr, and U2OS shZNF cells. Inserts show the distribution diagram of telomere length. Right, the average telomere length (mean ± SD) of each cell type is plotted (number of telomeres analyzed). ns, not significant.
Figure 4. Loss of ZNF365 causes defective telomeres. A, representative images of defective telomeres (heterogeneity, loss, fragility, and intra- and interchromosomal fusions) observed in ZNF365 knockdown TKO and U2OS cells as in B and C. B and C, Knockdown of ZNF365 increased defective telomeres in (B) TKO primary murine fibroblasts and (C) U2OS cells. Frequencies of defective telomeres are plotted in the bottom panel. D, pictures of fragile telomere structures shown in metaphase prepared from HeLa Scr and shZNF cells treated with 0.3 μmol/L Aph for 24 hours. Frequency of telomere defects (pointed by red arrowheads, higher magnification images are shown in the inset) with or without Aph treatment are plotted in the bottom panel (n = 10). E, ZNF365 siRNA-treated early-passage (P = 5) IMR90 cells exhibit increased telomere defects. The inset shows a representative fragile telomere. Top right, quantified telomere defects plot. Bottom right, SA-β-gal staining in IMR90 Scr and shZNF cells showing increased senescence in the latter. F, 53BP1 IF staining (red, nuclei marked) shows increased DSB in 2 independent shZNF-expressing U2OS cells compared with Scr control expressing ones. Number of 53BP1-positive foci per nuclei (mean ± SD) is plotted (n = 5) **, P < 0.01.
FD2-associated and total UFBs. In ZNF365-depleted cells, an increased number of UFBs originated from unresolved DNA replication sites under Aph-induced replicative stress, as evidenced by associated FD2 foci on the structure (Fig. 5D and E) (32). This finding is similar to what has been observed in cells with RAD51 or Fanconi anemia gene depletion that are defective for recombination and repair (7).

To confirm that telomeres are indeed among the unresolved replication intermediates in ZNF365-depleted cells, IF-FISH of FD2 and TelC was conducted. In interphase nuclei, FD2 colocalized with multiple TelC-positive foci, some of which formed sister foci in mitotic cells (Fig. 5F). The number of FD2- and TelC-positive sister foci in mitotic cells increased, consistent with increased telomere defects in ZNF365-depleted cells (Fig. 5G). Furthermore, coexisting with BLM or FD2 detected a fraction of ZNF365 on the UFBs and the mitotic FD2 sister foci (Fig. 5H and I) as well as colocalized with TelC and FRA16D (data not shown), suggesting its potential role in the resolution of replication intermediates at these sites. Together, our results show that ZNF365 deficiency suppresses timely resolution of replication intermediates, increasing FD2-associated UFBs.

Interestingly, numerous metaphases from ZNF365-silenced cells exhibited aneuploidy. In particular, cells with p53-deficient backgrounds became aneuploid upon ZNF365 depletion (Fig. 5J). There was a concomitant increase in centrosome number and appearance of multinucleated cells in culture, suggesting frequent cytokinesis failure in ZNF365-depleted cells (Supplementary Fig. S5B and S5C). Similarly, the increased number of anaphase DNA bridges in Fanconi anemia–mutant cells correlated with a higher rate of cytokinesis failure, resulting

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**Figure 5.** Loss of ZNF365 leads to common fragile site expression and aneuploidy. A, elevated chromosome breakage in shZNF HeLa cells treated with 0.3 μmol/L Aph for 24 hours (n = 25). The number of chromosome breakage per metaphase (mean ± SD) is plotted on the right. Arrows point to representative chromosome breakages. B, chromosomal aberrations in the FRA16D region. Left, FISH for 16q23.2 showing that the probe maps to the site of a chromosomal fragment originating from FRA16D in HeLa shZNF cells treated with Aph. Arrows point to genomic regions with 16q23.2 amplification. Inset shows a representative FRA16D duplication observed in HeLa shZNF cells. Right, frequency of aberrations (mean ± SD) at 16q23.2 (n = 25) in HeLa Scr and shZNF cells. C, BLM-coated UFB structure frequently seen in Aph-treated anaphase U2OS cells. U2OS cells were treated with 0.3 μmol/L Aph for 24 hours and stained for BLM and counterstained with DAPI. OL, overlay of BLM and DAPI staining. D, FD2-associated BLM-coated bridge structure (pointed by yellow arrow) from Aph-treated ZNF365-depleted U2OS cells. U2OS shZNF cells were treated with 0.3 μmol/L Aph for 24 hours and stained for BLM (red) and FD2 (green) and counterstained with DAPI. E, percentages of mitotic U2OS Scr or shZNF cells with BLM-positive UFB alone or FANCD2 (FD2) and BLM double-positive UFB are plotted. F, FD2 (red) and telomere (green) IF-FISH staining shows a fraction of telomeres are associated with FD2 foci in interphase nuclei (top left) or FD2 sister foci in mitotic U2OS shZNF cells (bottom left). Arrows point to double positive foci. G, percentage of FD2-positive telomere sister foci in mitotic U2OS Scr and shZNF cells (n = 17). (continued on following page)
Telomere Checkpoint Suppresses Genomic Instability

in binucleated cells and increased apoptosis observed in patients with Fanconi anemia (33). As mentioned earlier, the increased presence of anaphase DNA bridges including UFBs may contribute to chromosomal and genomic instability in ZNF365-depleted cells (Fig. 3D). It is conceivable that DNA bridges are likely the outcome of failed resolution of stalled and incomplete replication of telomeres and CFS in the absence of ZNF365. Collectively, our results suggest that ZNF365 is necessary for the timely completion of replication in a subset of fragile sites and thus suppresses cytokinesis failure and aneuploidy.

**DISCUSSION**

In this study, we explored the p53 transcriptome in the context of telomere dysfunction and identified ZNF365 as a key effector of genome maintenance. Our characterization of ZNF365 provides novel insights into feedback regulation of genomic stability by a telomere dysfunction-induced checkpoint. Our findings gain added significance in light of the observation that the dysfunctional telomere is one of the hallmarks of degenerative and cancer-prone conditions (17).

In cells with telomere dysfunction, one of the most robustly upregulated genes upon reconstitution of p53 activity was ZNF365. Our studies show that the biology of ZNF365 and p53 are intimately linked, as p53 regulates ZNF365 expression, and loss of ZNF365 function activates p53 by exacerbating telomere defects, forging a feedback regulatory loop. This feedback regulatory pattern further corroborates p53's fail-safe action to preserve genomic stability as well as ZNF365's effector action (Fig. 5K). Similarly, checkpoint activation in Pot1a-deficient cells promoted bypass of mitosis and endoreplication in p53-null cells, providing a common mechanism for the development of aneuploidy by dysfunctional telomeres (16).

A subset of telomerase-negative cancers use an ALT mechanism to maintain telomere length by adding telomeric repeats.
repeats to chromosome ends (34, 35). ALT features highly heterogeneous telomere lengths, rapid changes in telomere length, and a greatly elevated level of recombination at telomeres (36–38). Loss of ZNF365 leads to telomere phenotypes partially fulfilling these criteria in both ALT-positive U2OS cells and ALT-negative mouse embryonic fibroblasts and normal human fibroblasts. Furthermore, ZNF365-deficient cells did not exhibit upregulation of APBs, which are a specialized type of promyelocytic leukemia bodies that contain telomeric fragments, telomere-binding proteins, and factors involved in recombination, and, thus, provide a catalytic surface for telomere recombination (39, 40, 41). However, it is formally possible that APB is revealed only under conditions such as methionine starvation, or ALT does not necessarily involve APBs as previously described (41, 42). A lack of colocalization of telomeres and PML may suggest APBs are not the site for induced T-SCE in ZNF365 knockdown cells and loss of ZNF365 may upregulate ALT through alternative mechanisms. Furthermore, elevated T-SCE in ZNF365 knockdown cells could reflect defects in a repair of stalled replication forks or DNA breaks that maintain telomeres, resulting in recombination and rearrangement. Indeed, our characterization of ZNF365 suggests its potential role in DSB repair and replication fork recovery. We tested whether ZNF365 is involved in the recovery of stalled replication forks induced by hydroxyurea (HU) based on the possibility that increased DSB in knockdown cells is due to the conversion of arrested replication forks. Consistent with our hypothesis, ZNF365-depleted cells showed significantly decreased length of replication fork tract after HU-mediated replication arrest (Supplementary Fig. S6). Our results place ZNF365 at stalled forks where it promotes resolution and recovery of replication. It is noteworthy that defects in stalled fork recovery are not limited to telomeres, suggesting a general role of ZNF365 in maintenance of genomic stability.

Unlike premature senescence after many rounds of cell division in WRN- or BLM-defective cells, knocking down ZNF365 causes an early growth arrest, a phenotype seen in cells with an acute loss of TRF1 or TRF2 function (4, 43). In terms of telomere phenotype, ZNF365-deficient cells did not exhibit significant telomere end-to-end fusion unless there was preexisting telomere attrition (e.g., TKO, Supplementary Fig. S3B), contrasting with that of TRF2-deleted cells (43). On the basis of the similarity to the telomere phenotype of TRF1-deficient cells, defective telomeres seen in ZNF365-deficient cells arise most likely from replication problems. Indeed, telomeres pose a structural hindrance to the machinery of DNA replication and thus may constitute fragile sites where replication fork stalling may significantly increase the rate of recombination (4, 44), as evidenced by increased T-SCE in our study.

Our observation that ZNF365 maintains stability of a specific fragile site (i.e., FRA16D) aside from telomeres suggests that ZNF365 may have sequence-specific interactions with this CFS. A recent study showed that TRF1 specifically stabilized 4q14, a CFS bearing an interstitial telomeric sequence TTAGGG, whereas it did not affect non telomeric CFS, including FRA16D (45). One plausible mechanism would be the presence of a ZNF365 DNA-binding motif within FRA16D and (sub)telomeric genomic regions. As a protein containing a single C2H2 zinc finger domain, ZNF365 homodimerizes (data not shown) and possibly tethers to specific DNA sequences. The exact mechanism of how ZNF365 participates in resolution of late-replicating CFS and telomeres warrants further investigation.

A previous study reported that misexpression of ZNF365 led to cytokinesis failure, suggesting that its deregulation may contribute to the development of genomic instability during tumorigenesis (46). Of high relevance to our findings, recent genome-wide association studies have identified single-nucleotide polymorphisms (SNP) within ZNF365 that bear significant correlation with breast cancer susceptibility (21, 47). In particular, an independent study identified SNPs within the same intron as a significant modifier locus to BRCA2-mutant patient susceptibility to breast cancer (20). It is possible that these SNPs have other relevant functional links apart from affecting ZNF365 expression or function. Nevertheless, our study provides a potential molecular mechanism by which alteration of ZNF365 instigates genomic instability. Whether ZNF365 functionally contributes to breast or other cancer pathogenesis warrants future investigation. A better understanding of this telomere dysfunction–initiated cellular checkpoint response may illuminate novel therapeutic approaches for neoplastic and degenerative diseases linked to telomere biology.

METHODS

Antibodies and Cell Lines

We used antibodies against 53BP1 (Bethyl labs), TRF1 (gift from T. de Lange), FANCD2 (Novus), BLM (C-18; Santa Cruz), v5 (Invitrogen), ZNF365 (clone 3c23), lamin A/C, and pericentrin (Abcam). U2OS, Saos-2, HeLa, and IMR90 cells were obtained from the American Type Culture Collection (ATCC). Cells were tested for the absence of mycoplasma and authenticated by standard DNA microsatellite short tandem repeats (PowerPlex 1.2 System, Promega) and the resulting DNA fingerprints were matched to the reference published by ATCC.

Time-Dependent Transcriptome Analysis after p53ER Activation

Ear skin fibroblasts isolated from G4 mTerc−/− Atn−/− p53−/+ TKO mice were used to study the effects of p53 reactivation. Cells were transduced with retroviral particles for pBabe-p53ER12 (p53ER, provided by Dr. Gerard Evan at the University of California San Francisco, San Francisco, CA) and both uninfected cells and p53ER-expressing cells were treated with 100 mmol/L OHT for multiple time points to catalog the p53 transcriptome. We used the Affymetrix Mouse Genome 430 2.0 Array to analyze total RNA. To identify p53 targets, fold changes were calculated as a ratio of the mean expression values of the OHT-treated p53ER-infected or uninfected samples at 4 hours. For data analysis, the best-route combination of analysis methods was used. Briefly, background and perfect matched/mismatched corrections were conducted by Affymetrix Microarray Suite 5.0, and expression summary values for each probe set were calculated in Bioconductor (http://www.bioconductor.org/). In addition, hierarchical clustering was conducted in dCHIP (http://biosun1.harvard.edu/complab/dchip/)
for both microarray studies to assess similarities and differences in gene expression among samples. Two-way ANOVA was carried out on the expression profiles of all samples to assess the significance of interaction between p53-containing virus infection and OHT treatment, assigning each probe set a P-value. To exclude other factors potentially affecting p53 activity, we set the criteria for fold change by p53 + 4-OHT/mock-infected + 4-OHT of more than 1.5; and this fold change over p53 + vehicle/mock-infected + vehicle is also more than 1.5. In addition, fold change of p53 + 4-OHT/mock-infected + 4-OHT is at least 1.2 times over all other comparisons: p53-0 hour/mock infection-0 hour, p53 + vehicle/p53-0 hour, mock infected + vehicle/mock infected-0 hour. Microarray data have been deposited to GEO with accession number GSE44599.

**Assay for T-SCE**

Cells were labeled with 10 μg/mL bromodeoxyuridine (BrdUrd) for 12 to 16 hours, and metaphase spreads were used for chromosome orientation FISH assay to detect T-SCE. Slides were rehydrated in PBS and treated with 0.5 mg/mL RNase A for 10 minutes at 37°C, followed by staining with 0.5 μg/mL Hoechst 33258 in 2× saline-sodium citrate (SSC) for 15 minutes, and exposed to UV light. BrdUrd-substituted DNA strands were digested with 10 U/μL of Exonuclease III for 10 minutes. Slides were dehydrated in an ethanol series and hybridized with TelG-Cy3 (1:5,000, Panagene) for 1 hour, then further hybridized for 2 hours with TelC-FITC (1:500, Bio-Synthesis, Inc.). After hybridization, slides were dehydrated in ethanol series, air-dried, and mounted.

**Knockdown of ZNF365**

The RNAi Consortium clone IDs for the shRNAs used in this study are as follows: shZNF-1, TRCN0000128973; shZNF-2, TRCN0000128835; shZNF-7, TRCN0000175966; shZNF-8, TRCN0000176181. To deplete ZNF365 acutely in IMR90 cells, cells were transfected with ZNF siRNA sequences for quantitative real-time PCR analysis of ZNF365 expression are available upon request.

**Tel-FISH**

Cells were treated with 0.1 μg/mL colcemid and prepared for metaphase spread. Slides were treated with pepsin and RNase, washed and dehydrated through an ethanol series, hybridized with a TelG-Cy3 or Tel-C-FITC peptide nucleic acid probe (15,000 or 1:500, respectively) by denaturation at 80°C for 5 minutes, and then incubated for 1 hour. Slides were washed with 70% formamide and 0.06x SSC, followed by PBS containing 0.02% (v/v) Tween-20, and mounted. The length of telomeres was quantified with TFL-TELO software.

**Fragile Site-FISH**

Cells were treated with Aph (0.3 μmol/L) for 24 hours. The telomere, 3p14, 12p12, and 16q23 probes were labeled by nick translation using digoxigenin-11-DUTP (Roche) and were detected by rhodamine-conjugated antibody. For fragile site analysis, the chromaternal bands involved in aberrations were matched to the location of published fragile sites. The fused, duplicated, broken, and aberrant signals were scored.

**IF-FISH**

Cells grown on coverslips were fixed in 2% paraformaldehyde, permeabilized in 0.2% Triton X-100, and incubated with primary antibodies followed by Alexa-conjugated secondary antibodies. The cover slips were fixed in 2% paraformaldehyde and dehydrated in an ethanol series. Hybridizing solution containing TelC-FITC (1:500, Bio-Synthesis, Inc.) was dropped on the coverslip and denatured at 70–80°C, followed by hybridization for 2 hours. Slides were washed with 70% formamide and 0.06x SSC, followed by PBS containing 0.02% (v/v) Tween-20, and mounted.

**TMA Analysis**

In-house-constructed TMA sets for different subtypes of breast cancers were stained with monoclonal ZNF365 antibody. Slides were manually scored based on no signal (0), weak (1), moderate (2), and strong (3) staining criteria and calculated as H-score as previously described (48). The significance of the difference between H-scores was determined by a nonparametric Wilcoxon rank-sum test.

**Statistical Analysis**

All data represent the average from at least 3 independent experiments, with at least 100 cells counted per experiment. The unpaired 2-tailed Student t test or one-way ANOVA test with Tukey Honestly Significant Difference posttest was used to determine significance for all experiments, unless noted otherwise. HR and log-rank analysis were conducted as described (49).

**Disclosure of Potential Conflicts of Interest**

S.J. Shin is a consultant/advisory board member of SignalGenetics, LLC. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Zhang, S.J. Shin, F. Foerster, Y. Xiao, Z. Chen, J.-H. Paik
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