Subtelomeric p53 binding prevents accumulation of DNA damage at human telomeres

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Abstract

Telomeres and tumor suppressor protein TP53 (p53) function in genome protection, but a direct role of p53 at telomeres has not yet been described. Here, we have identified non-canonical p53-binding sites within the human subtelomeres that suppress the accumulation of DNA damage at telomeric repeat DNA. These non-canonical subtelomeric p53-binding sites conferred transcription enhancer-like functions that include an increase in local histone H3K9 and H3K27 acetylation and stimulation of subtelomeric transcripts, including telomere repeat-containing RNA (TERRA). p53 suppressed formation of telomere-associated γH2AX and prevented telomere DNA degradation in response to DNA damage stress. Our findings indicate that p53 provides a direct chromatin-associated protection to human telomeres, as well as other fragile genomic sites. We propose that p53-associated chromatin modifications enhance local DNA repair or protection to provide a previously unrecognized tumor suppressor function of p53.

Keywords: chromatin; DNA damage; telomere; TERRA; TP53; tumor suppressor
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Introduction

Telomeres are the repetitive DNA structures that protect the ends of linear chromosomes from nucleolytic degradation and attrition during DNA replication (reviewed in Blackburn, 2005, and Cech, 2004). The terminal repeats of telomeres are bound by a set of proteins, collectively termed Shelterin or the Telosome, which perform multiple functions in telomere maintenance that are essential for cellular division and genome stability (de Lange, 2005; Xin et al., 2008). Telomere repeat length is highly regulated, and a minimal repeat number is required to provide chromosome end protection (Suram & Herbig, 2014). Shelterin/Telosome proteins prevent linear DNA ends from being mistaken for damaged DNA and eliciting a DNA damage response that leads to cellular replicative arrest or apoptosis (Palm & de Lange, 2008).

The chromosomal elements that extend beyond the terminal repeats, referred to as the subtelomeres, are also known to contribute to genome integrity and cellular development (Riethman et al., 2005; Stong et al., 2014). For example, deletions of the subtelomeric D4Z4 array can give rise to facioscapulohumeral muscular dystrophy (FSHD; Sacconi et al., 2014). Subtelomeres have complex repetitive and duplicated DNA structures, as well as many coding and non-coding genes, and retrotransposon-like elements (Riethman et al., 2005). Transcription of subtelomeric genes can be regulated by complex epigenetic effects imposed by the telomere repeats (Baur et al., 2001; Stadler et al., 2013). Telomere position effect through the spreading of repressive heterochromatin has been described in many organisms, including human cells (Baur et al., 2001). More recent studies indicate that telomere position effect can be mediated by long-range DNA-looping interactions between telomere repeats and subtelomeric genes (Robin et al., 2014).

Transcripts generated within subtelomeres that include telomere repeat sequences have been identified and referred to as telomere repeat encoding RNA (TERRA; Azzalin et al., 2007; Azzalin & Lingner, 2014). TERRA has been implicated in several functions, including heterochromatin formation (Deng et al., 2009) and inhibition of telomerase loading and enzymatic activity (Schoeftner & Blasco, 2008; Redon et al., 2010). TERRA can be induced by DNA damage and a requirement for p53 has been observed (Caslini et al., 2009). The transcriptional start site for TERRA in human chromosomes appears to localize to a repetitive GC-rich element, referred to as a 29mer, that is located within many subtelomeres (Nergadze et al., 2009). We have shown that the chromosome-organizing factors CTCF and cohesin bind in close proximity to these elements and are typically enriched in RNA polymerase II (Deng et al., 2012a). Although these elements are likely to regulate TERRA transcription, TERRA transcripts can also initiate from more centromerically located sites in the subtelomere (Porro et al., 2014). In mouse, the bulk of TERRA transcripts appear to be generated from a single subtelomere, but they can associate in trans with the remaining...
telomeres (de Silanes et al., 2014). Telomeric and subtelomeric transcription can be regulated by developmental, environmental, and stress-related signals (Feuerhahn et al., 2010; Arora et al., 2011; Bah & Azzalin, 2012). TERRA transcripts can accumulate in some cancer cells and tissues (Deng et al., 2012b), and TERRA transcription is dampened in the absence of p53 (Caslini et al., 2009). The mechanism through which p53 may regulate TERRA has not been explored in molecular detail.

p53 is a master regulator of genome integrity (Lane, 1992). DNA damage-induced p53 DNA binding activates transcription of genes important for cell cycle control (e.g., p21) and apoptosis (e.g., Bax) (Bieging & Attardi, 2012). Genomewide studies have revealed that p53 can bind to many loci in the cellular genome, including sites not associated with transcription control (Kenzelmann Broz et al., 2013). These studies also reveal functionally distinct p53-binding sites. Here, we identify a new class of p53-binding sites that are located in human subtelomeres in close proximity to terminal telomere repeat tracts. We show that p53 binding to these sites can confer local chromatin changes associated with increased genome stability. We propose that p53 binding in the subtelomere provides direct protection to telomere DNA, in part, through alteration of histone modifications and activation of transcriptional enhancer-like elements.

Results

Genomewide ChIP-Seq studies have revealed unexpected distribution of chromosome binding for many DNA-binding proteins. In one p53 ChIP-Seq study, 542 high confidence p53-binding sites were identified (Wei et al., 2006). However, many of these could not be readily assigned to proximal promoters of known p53 target genes (Wei et al., 2006). To investigate the possibility that p53 may bind to sites proximal to telomeres, we generated p53 ChIP-Seq data to compare untreated and DNA damage-treated cells. We used these data, in combination with publically available published data, to map high confidence binding sites to the subtelomeres, using the most recent build of the human subtelomere genome (Deng et al., 2012a; Stong et al., 2014). Peak-calling algorithms specifically optimized for subtelomere analysis revealed that unique and statistically robust p53 peaks were present in a number of subtelomeres (Fig 1A and B, Appendix Fig S1 and Appendix Table S1). We also observed numerous p53-binding sites in mouse subtelomeres (Appendix Fig S2), including the mouse chromosome 18q that is implicated as the template for the majority of mouse TERRA transcripts (de Silanes et al., 2014).

In human, the major p53-binding peaks mapped to the subtelomeric repeat element (SRE) region and co-localized with a sequence element defined by RepeatMasker (Tempel, 2012) (http://www.repeatmasker.org) as a MER31-int/(TG)n/LTR10C string of nucleotides. This element is shared by the subterminal duplcicon families A, B, and D and is located in the intron of a transcribed subterminal gene/pseudogene family annotated as L23a-like (Ambrosini et al., 2007; Riethman, 2008). Eleven of the 12 subtelomeric sites are localized between 2 and 12 kb from the start of the terminal repeat tract; the remaining one is located 9 kb from an Internal telomere-like sequence (ITS), within an extensive SRE region, approximately 160 kb from the 8p telomere. LTR10C is highly enriched in subtelomeric DNA regions (defined here as the terminal 500 kb of each chromosome arm), whereas MER31-int is slightly enriched at subtelomeres. However, their co-localization is almost exclusive to subtelomeres, and all of these subtelomeric co-localization sites correspond to p53-binding peaks. Only 3 non-subtelomeric sites have the co-localized MER31-int and LTR10C (Appendix Fig S1). Two of these, which bind to p53, correspond to previously identified non-subtelomeric sites of subtelomere-derived duplcicons, one at an ancestral chromosome fusion junction of primate telomeres on 2q and the second at a 3p pericentromeric site carrying a copy of the subterminal D duplcicon. The third non-subtelomeric site does not bind p53 and lacks the (TG)n repeat sequence present at the junction of all of the other co-localized MER31-int and LTR10C repeats.

The subtelomeric DNA sequences corresponding to the twelve p53 peaks were analyzed further (Fig 1C). Eighty base pairs centered on the midpoint of each MACS2-called peak were selected and aligned with the others using CLUSTAL (Higgins et al., 1996). It was apparent from this initial alignment that the twelve sequences fell into two groups, one corresponding to eight peaks and a second corresponding to four. Peak heights for the first group of eight sites were approximately twice that of the second group of 4, so we analyzed the two groups separately using MEME-ChIP to locate motifs. In both cases, the top-scoring motif corresponded to a p53-binding site. Alignment of these MEME-generated motifs with consensus p53-binding site identified from genomewide ChIP-Seq data set (Wei et al., 2006) showed that the subtelomeric sequences with stronger p53 binding contained a motif corresponding to three consecutive p53 consensus half-sites, whereas the second group of sequences contained two consecutive consensus half-sites (Fig 1D). Each half-site in both sequences contained the non-variant central C (A/T)/T/A motif identified originally (el-Deiry et al., 1992).

To begin to assess the significance of these subtelomeric p53-binding sites, we first validated the p53 binding by ChIP–qPCR (Fig 2). We designed qPCR primers for the p53-binding site found in the prototypical subtelomere repeat element found most proximal to the terminal repeat TTAGGG in chromosomes 18q and 13q. We first compared p53 binding in HCT116 p53 null (p53−/−) and p53 wt (p53+/+) cells treated with 50 μM etoposide or control DMSO. Induction of total and phospho-S15 p53, as well as DNA damage-associated γH2AX, was monitored by Western blot (Fig 2A). We observed a strong activation of p53 in HCT116 p53+/+ cells as expected, and while γH2AX was induced in both cells, we observed a reduced level of γH2AX in p53−/− cells. ChIP–qPCR assays confirmed that p53 bound to both the 18q- and 13q-binding sites in p53+/+ cells, but not in p53−/− cells (Fig 2B). We also assayed p53 binding induced by nutrient deprivation stress associated with growth in serum-free Hank’s buffered saline solution (HBSS) (Fig 2C and D). We compared two different p53 antibodies (Ab6 and FL) in p53−/− or p53+/+ HCT116 cell lines. We found that growth in HBSS for 4 h induced high levels of p53 binding in HCT116 p53+/+ cells (Fig 2C). Growth in HBSS produced a strong (>10-fold) induction of p53 binding to the 18q and 13q subtelomeres, as well as to p21 promoter element. No binding was observed in p53−/− cells, or with control IgG. We also assayed p53 binding in the H1299 lung carcinoma cell lines which conditionally expresses a doxycycline-inducible p53 (Fig 2D). Doxycycline-induced p53 bound detectably to p53-binding sites at both the 18q subtelomere and p21 promoter. Growth in serum-free HBSS led to a further ~10-fold increase in p53 binding at
both 18q and p21. As a negative control we showed that p53 did not bind to a control set of primers located in a promoter “desert” IGX1A (Fig 2B, middle panel). These findings indicate that p53 binding can be induced by various stress conditions, or by simple p53 induction in an inducible system, at both 18q and 13q subtelomeres.

To explore the potential function of subtelomeric p53-binding sites, we tested the 18q subtelomere p53-binding site for its ability to function as a transcriptional response element (Fig 3). The subtelomeric p53-binding site from chromosome 18q was inserted upstream of the SV40 early promoter core element driving the
luciferase reporter gene. We found that a single copy of the 18q p53-binding site conferred transcriptional responsiveness to p53 (Fig 3A). Transient transfection of the p53 expression vector induced the 18q p53-Luc reporter by ~100-fold, similar to the levels reached with a canonical binding site from the mdm2 promoter. These findings indicate that the subtelomeric p53-binding site in 18q can function as a p53-dependent transcription response element.

To assess whether p53 contributes to the subtelomeric transcription, we assayed the RNA expression by RT–qPCR (Fig 3B–D). We first assayed HCT116 p53−/− and p53+/+ cells treated with etoposide for p53-dependent transcription. As expected, etoposide induced p21 transcription > 20-fold in a p53-dependent manner. We also assayed a subtelomeric transcript PARD6G located ~12 kb centromerically from the p53-binding site in chromosome 18q. We found that etoposide induced PARD6G ~4-fold in a p53-dependent manner and relative to untreated cells. We next asked whether etoposide induced TERRA. We failed to observe a general induction of TERRA by Northern blot (data not shown), but did observe significant activation of chromosome-specific TERRA, including those that contain p53-binding sites within their subtelomeres. We show that etoposide treatment induced TERRA transcripts for subtelomere 13q and 2q by 7-fold in a p53-dependent manner (Fig 3C).
found that HBSS treatment led to a p53-dependent induction of TERRA transcription from subtelomere 18q using two different chromosome-specific primer sets (Fig 3D). Additionally, we observed that weak, but consistent DNA damage-induced p53 ChIP-Seq peaks colocalized with the CTCF-cohesin-RNA polymerase II peaks site in TERRA transcription at many subtelomeres (Appendix Fig S3). Although these weaker binding sites did not correspond to sequence-specific p53 binding, they suggest that DNA damage-induced p53 may function at TERRA promoter elements. Taken together, these findings suggest that p53 contributes to the stress-induced activation of subtelomeric (e.g., PARD6G) and TERRA-like transcripts from multiple chromosomes.

The role of p53 in response to DNA damage is well established, but the possibility that this protein functions in a specific and direct role to protect subtelomeres from DNA damage has not been explored in detail. To establish a system to study p53 function at subtelomeres, we treated cells with a sublethal dose of etoposide (Fig 4). We found that 1 μM etoposide led to a partial induction of p53 and low levels of γH2AX by Western blot (Fig 4A). HCT p53+/− cells treated with 50 μM etoposide arrested predominantly

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**Figure 3. Transcriptional activity of subtelomeric p53-response elements.**

A Luciferase test constructs containing either empty test vector (top), p53-response elements from 18q subtelomere (middle) or MDM2 promoter (lower) were assayed in HCT p53−/− cells transfected with two independent preparations of empty vector or p53 WT expression vector. 

B RT–qPCR measure of p21 (top panel) or PARD6G (lower panel) in HCT116 p53−/− or p53+/+ cells after treatment with DMSO or 50 μM etoposide. 

C RT–qPCR analysis of TERRA transcripts from subtelomere 13q (upper) or 2q (lower). 

D RT–qPCR analysis of TERRA transcripts from 18q using two independent primer sets pr1 (left panel) or pr2 (right panel) from HCT116 p53+/+ cells cultured with serum (gray bars) or in HBSS lacking serum (black bars).

Data information: Error bars represent SD, and * indicates P-value < 0.05 using Student’s t-test comparing treated and untreated samples. n = 3.
in S phase, whereas p53−/− cells accumulated in G2/M (Fig 4B), suggesting that their DNA damage response is mechanistically distinct. To determine whether p53 provided a function important for cell viability in response to DNA damage, we performed a clonogenic survival assay (Fig 4C). HCT wt and p53−/− cells were exposed to sublethal concentrations of etoposide (ranging from 1–2.5 μM) for 24 h and then assayed by replating at low density. Colony quantification demonstrated that p53+/+ cells had a significantly greater survival advantage compared to p53−/− cells (Fig 4D). We next asked whether telomere DNA damage-induced foci (TIFs) accumulated at higher frequency in cells lacking p53, relative to wt controls (Fig 4E and F). TIFs were scored based on the colocalization of γH2AX DNA damage-associated foci with telomere DNA foci. We found that cells with >4 TIFs per cell were significantly enriched in p53−/− cells, relative to wt cells (Fig 4E and F).

**Figure 4.** p53-dependent cell viability and telomere protection in response to DNA damage.

- **A** Western blot of total p53, γH2AX, and GAPDH in HCT116 p53−/− or p53+/+ cells treated with 1 μM or 50 μM etoposide for 24 h.
- **B** Cell cycle analysis of HCT116 p53−/− or p53+/+ cells untreated or treated with 50 μM etoposide for 24 h. Cell cycle was determined by FACS analysis of propidium iodide-stained cells.
- **C** Cell clonal survival assay for HCT116 p53−/− or p53+/+ cells treated with DMSO or 1, 1.5, or 2 μM etoposide for 24 h prior to replating.
- **D** Quantification of clonal survival assay relative total cell numbers (top panel) or normalized to DMSO controls (lower panel).
- **E** TIF assay for HCT116 p53−/− or p53+/+ cells treated with 1 μM etoposide for 3 h. Telomere DNA FISH with TelC probe (red), γH2AX (green), and DAPI (blue).
- **F** Quantification of TIF assay for % cells with >4 TIFs per cell for p53+/+ (black) or p53−/− (red) cell.
- **G** Telomere length assay showing p53−/− or p53+/+ cells treated with either DMSO, 1 μM, or 50 μM etoposide. Ethidium bromide (EtBr)-stained agarose gels (left panel), Southern blot hybridized with Tel C probe (middle panel) or with a Satellite DNA (right panel). Quantification of telomere DNA signal relative to invariant bands in α Sat.
- **H** Two-dimensional agarose gel analysis of telomeric DNA from HCT p53−/− or p53+/+ treated with 50 μM etoposide for 24 h. Ethidium gel (top) and Southern blot probed with TelG probe (lower panel). Single-stranded telomere DNA (red arrow) and recombination/replication-associated structures (blue arrow) are indicated.

Data information: Error bars represent SD and * indicates P-values < 0.05 determined by Student’s t-test (TLA), or chi-square (TIF assay). n = 3.
The DNA integrity of the telomeres was examined by telomere length assays (TLA) using Au/MboI restriction digest followed by Southern blot hybridization. HCT116 p53\(^{-/-}\) or p53\(^{+/+}\) cells were treated with 0, 1, or 50 \(\mu\)M etoposide for 24 h and then assayed by TLA (Fig 4G). Ethidium bromide staining revealed that DNA isolated from all samples was similar in size distribution and relative abundance, although small molecular wt DNA increased in etoposide treatment, and was greater in p53\(^{+/+}\) cells (Fig 4F, left panel). Interestingly, Southern blotting with TelC probe revealed a striking loss of telomere DNA signal from high molecular wt (\(~5\) kb) telomere DNA in p53\(^{+/+}\) cells treated with etoposide. This loss of telomere DNA was not observed in p53\(^{-/-}\) cells (Fig 4G, middle panel). The same membrane was stripped and hybridized to a probe for alpha satellite DNA (Fig 4G, right panel). This analysis revealed the loss of a high molecular weight species (12 kb), but a constant amount of most other species. The relative loss of telomere DNA signal was quantified relative to the constant signal in the alpha satellite DNA and revealed a 50% loss of telomeric DNA in p53\(^{+/+}\) cells. To further examine the rapid loss of telomeric DNA, we assayed telomere DNA using 2D agarose gels (Fig 4H). In p53\(^{+/+}\) cells, etoposide induced a modest increase in complex DNA structures associated with recombination and replication intermediates (red arrow). Interestingly, p53\(^{+/+}\) cells showed an increased accumulation of these DNA structures in the absence of etoposide, and all telomeric DNA structures, including single-stranded telomeric DNA (blue arrows) diminished in response to etoposide treatment. While the precise structures formed in the absence of p53 and in response to etoposide are not completely clear, these findings suggest that p53 influences telomere DNA structure and promotes telomere repeat DNA stability in response to etoposide treatment.

To explore the mechanism of p53 protection of subtelomeric DNA, we assayed the p53-dependent histone modification changes that occur in response to DNA damage stress. We designed a series of primers spanning the 18q and 13q subtelomeres surrounding the p53-binding sites. This series of primers were used for analysis of ChIP DNA from p53, histones H3K9Ac, H3K27Ac, γH2AX, and IgG control (Fig 5). As before, we compared HCT116 p53\(^{-/-}\) and p53\(^{+/+}\) cell lines for their response to etoposide treatment. As expected, we found that p53 binding was enriched in p53\(^{+/+}\) cells after etoposide treatment at p53-binding site positions 1,974 bp and 1,958 on 18q, and 9,620 and 9,838 on 13q. p53 also bound to the positive control p21 promoter and did not bind substantially to other regions of the 18q subtelomere or in cells lacking p53, indicating that the ChIP assay is specific for p53.

p53 is known to associate with histone acetyltransferases important for transcriptional activation functions. We therefore tested whether histone promoter-like H3K9ac and H3K27ac were elevated in a p53-dependent manner. We found that H3K9ac was elevated at the p53-binding sites (1,974 in 18q and 9,620 in 13q) in p53\(^{+/+}\) cells in response to etoposide treatment (Fig 5A). H3K9ac was also elevated at proximal position telomeric to p53-binding sites (705 in 18q and 7,702 in 13q) in p53\(^{+/+}\) cells, but no significant increase was observed at other sites or in p53\(^{-/-}\) cells. H3K9ac also increased at the p21 promoter where p53 binds and activates transcription. H3K9ac did not increase significantly in p53\(^{-/-}\) cells at any of these sites. Histone H3K27ac was also found to be induced at subtelomeres in a p53-dependent manner, but the pattern was different than that of H3K9ac (Fig 5). H3K27ac was substantially increased at the p53-binding sites in 18q and 13q, as well as elevated at additional sites more distal to p53. Etoposide treatment led to an increase in H3K27ac at more telomeric positions in 18q, while H3K27ac expanded more centromeric orientation in 13q. H3K27ac did not accumulate in p53\(^{+/+}\) cells in response to etoposide. These findings indicate that H3K27ac and H3K9ac accumulate at the subtelomeric p53-binding sites and surrounding regions in a p53-dependent manner (Fig 5A). We also demonstrate subtelomeric histone acetylation increases in a p53-dependent manner in H1299 cell lines expressing dox-inducible p53 (Appendix Fig S4). These findings indicate that subtelomeric p53-binding sites induce local histone acetylation in response to genotoxic stress.

To investigate the potential relationship between histone acetylation and the DNA damage response, we assayed the pattern of γH2AX accumulation at the subtelomeres in response to DNA damage (Fig 5, lower panels). Similar to what was observed in TIF assays, we found that subtelomeric γH2AX was highly elevated in etoposide-treated p53\(^{-/-}\) cells. The distribution of γH2AX was extended throughout most of the 18q and 13q subtelomeres, with the highest enrichment at the regions proximal to the telomere repeats. In contrast, γH2AX did not accumulate to similar levels in p53\(^{+/+}\) cells, suggesting that p53 contributes to the suppression of γH2AX accumulation at the subtelomere (Fig 5A and B). We also tested whether p53, γH2AX, TRF2, or TRF1 binding was altered at the telomere repeats in response to etoposide treatment in p53\(^{-/-}\) compared to p53\(^{+/+}\) cells (Fig 5C). We observed that γH2AX levels increased at telomere repeats in response to etoposide, but this effect was independent of p53 status. On the other hand TRF1 and TRF2 binding increased modestly at telomere repeats in a p53-positive, but not in p53 null cells (Fig 5C). TRF2 was next examined for its binding to subtelomeric positions (Fig 5E). We found that etoposide stimulated TRF2 interactions with subtelomeric sequences most proximal to the terminal repeat junction, but only in p53-positive cells. These findings suggest that p53 can alter the interaction of TRFs with telomeric and subtelomeric DNA in response to DNA damage.

To better understand the p53-dependent changes in subtelomeric chromatin, we assayed the temporal regulation of histone modifications at subtelomeres (Fig 6). We first assayed the global changes in the levels of histone γH2AX, pan H2AX, H3K9ac, panH3, and GAPDH in HCT 116 p53\(^{-/-}\) and p53\(^{+/+}\) cells (Fig 6A). We found that p53 levels increased from 8–16 h after etoposide treatment in p53\(^{+/+}\) cells. γH2AX accumulated in p53\(^{+/+}\) cells by 8 h and continued to increase for up to 24 h. The relative formation of γH2AX was ~2–3 fold greater at all time points in p53\(^{-/-}\) relative to p53\(^{+/+}\) cells. Total H2AX (pan H2AX) also increased in response to etoposide treatment, but to a lesser extent than γH2AX. However, no differences were observed in H2AX levels in p53\(^{-/-}\) relative to p53\(^{+/+}\) cells. Etoposide treatment had no discernable effect on the global levels of H3K9ac or pan H3 in either p53\(^{-/-}\) or p53\(^{+/+}\) cells. These findings corroborate our previous observation that p53\(^{-/-}\) cells accumulate lower levels of γH2AX relative to p53\(^{+/+}\) cells (Fig 6B), and are consistent with γH2AX ChIP assays at 18q (Fig 5A), as well as with TIF assays (Fig 4) showing increase accumulation in p53 null cells.

The temporal regulation of γH2AX binding at a subtelomere was analyzed by ChIP (Fig 6B). γH2AX binding accumulated over time

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Figure 5. p53-dependent histone modification changes in response to DNA damage stress.

A, B HCT116 p53−/− (left panels) or p53+/+ (right panels) were treated with DMSO (black) or 50 μM etoposide (red) for 24 h and assayed by ChIP–qPCR at various locations relative to the TTAGGG repeats across the chromosome 18q subtelomere (A) or 13q subtelomere (B) or for control regions (p21 promoter, GKN1/2, PARKIN) as indicated below. ChIP–qPCR for p53, H3K9Ac, H3K27Ac, γH2AX, or IgG control were quantified as % input. * indicates P-value < 0.05 using Student’s t-test comparing ChIP values in p53+/+ relative to p53−/− cells for specific primer pairs.

C, D Dot blot analysis of telomere repeat or Alu repeat DNA from p53+/+ or p53−/− HCT116 cells treated with DMSO or etoposide (ETP) (as described in A), that were subjected to ChIP with antibodies to IgG, p53, γH2AX, TRF2, or TRF1. Quantitation of three independent ChIP assays for TRF1 and TRF2 is shown in (D).

E ChIP–qPCR for TRF2 is shown for cells treated as in (A) using primers across the subtelomeres of 18q (left) or 13q (right).

Data information: Error bars represent SD and * indicates P-values < 0.05 determined by Student’s t-test. n = 3.
Figure 6.
at 18q subtelomere in p53−/− cells. In contrast, γH2AX binding appeared to have much reduced binding in p53+/− cells, with a weak binding peak occurring at 16 h at some sites. In the absence of p53, γH2AX binding accumulated to much greater levels at positions close to the telomere repeats. These findings are consistent with a role of p53 in suppressing γH2AX accumulation at subtelomeres.

Recent studies have shown some p53-binding sites function as enhancer-like elements that can generate low abundance enhancer-RNAs (eRNAs; Melo et al., 2013; Allen et al., 2014). To determine whether subtelomeric p53-binding sites were associated with eRNA-like species, we assayed the 18q subtelomeric region for low abundance transcripts (Fig 6C and D). We first confirmed that etoposide treatment led to robust activation of the p21 transcript in a p53-dependent manner (Fig 6C). p21 mRNA levels increased more than ~10-fold by 8 h after etoposide treatment and continued to increase up to 24 h. Similarly, PARD6G RNA increased ~8-fold by 8 h after etoposide treatment in p53−/− cells, but not in p53−/− cells. We previously demonstrated that 18q TERRA transcripts increased in response to DNA damage in a p53-dependent manner (Fig 6B). TERRA transcripts could only be detected with telomere-specific reverse transcription to enrich for low abundance RNA. To determine if any additional RNA species, like eRNAs, are expressed in the 18q subtelomere region, we used random primed cDNA and the primer sets used for the ChIP assays to measure RT–PCR products from the 18q subtelomere (Fig 6D). We identified an RT–PCR signal at positions 1,974 and 2,354 in p53−/− and p53+/− cells. This RNA species increased ~5-fold in p53+/+ cells, but did not increase in p53−/− cells in response to etoposide treatment. No RNA species were identified in the absence of reverse transcription, arguing that the signal is RNA dependent (Fig 6D, lower panels, and Appendix Fig S5). Similar transcription patterns were observed when cells were treated with another DNA damaging agent, bleomycin (Appendix Fig S6), indicating that these effects are not specific to DNA damage induced by etoposide. Moreover, metadata analysis of published GRO-Seq data sets from Nutlin-3a-treated cells revealed a strong activation of subtelomeric transcription (Leville et al., 2015; Fig 6E and Appendix Fig S7). Activation of p53 by Nutlin-3a led to an increase in both sense and anti-sense transcripts initiating at the PARD6G gene. We also observed a lower level of bidirectional transcription initiating from the p53-binding sites in the 18q subtelomere. Integrating published ChIP-Seq data sets revealed that histone modifications associated with active transcription (H3K4me1, H3K27ac, and H3K4me3) were detected at the PARD6G transcript initiation sites (Taberlay et al., 2014). A similar pattern of p53-activated transcription and histone modifications could be observed at other subtelomeres with p53-binding sites (Appendix Fig S7), and the pattern is summarized in Fig 6F. Taken together, these observations suggest that short, non-annotated RNAs covering the regions encompassing the p53-binding sites can be induced by DNA damage stress in a p53-dependent manner. They also suggest that these p53-binding sites serve as enhancers for neighboring subtelomeric transcripts, like PARD6G.

To investigate the contribution of the p53 site in the 18q subtelomere, we used CRISPR genome editing combined with homologous recombination to delete the 18q p53-binding site in a stable HCT116 cell line (Fig 7A). We first determined that a pair of CRISPR-edited cell lines (18q WT and 18q Δp53) had a similar global response to etoposide with respect to p53 activation and γH2AX formation (Fig 7B), as well as p21 transcription activation (Fig 7C). Interestingly, we observed that p53-dependent activation of PARD6G transcription was compromised in 18q Δp53 cells relative to WT controls (Fig 7D and Appendix Fig S8). We also found that the subtelomeric transcripts surrounding the p53-binding site were not induced in response to etoposide in 18q Δp53 cell lines (Fig 7E and Appendix Fig S8). Finally, we examined the formation of γH2AX in response to etoposide treatment. We found that γH2AX accumulated at higher levels at most subtelomeric positions tested in the 18q Δp53 relative to 18q WT CRISPR cell line (Fig 7F and Appendix Fig S8). Additionally, we validated that human diploid fibroblasts depleted for p53 with shRNA (Kung et al., 2015) had similar changes in subtelomere regulation as did HCT116 cancer cell lines (Appendix Fig S9). We found that p53 depletion from human diploid fibroblasts increased global γH2AX in response to etoposide, along with the accumulation of γH2AX at subtelomeres, and reduced 18q subtelomeric transcription relative to normal diploid fibroblasts (Appendix Fig S9). Taken together, these findings support the model that non-canonical p53-binding sites in human subtelomeres provide enhancer-like functions that have direct impact on the local chromatin structure and DNA damage response at subtelomeric DNA (Fig 8).

Discussion

p53 is known as the “guardian of the genome” because of its essential role in preventing the accumulation of genetic aberrations associated with cancer cell evolution (Lane, 1992). p53 has...
well-characterized DNA-binding function that activates transcription of genes required for cell cycle arrest and apoptosis (Sigal & Rotter, 2000; Olivier et al., 2010; Shah et al., 2012). However, p53 tumor suppressor activity is thought to involve functions in addition to the activation of these target genes (Bieging et al., 2014). Recent genomewide studies reveal that p53-binding sites vastly exceed p53 responsive genes and occur at sites that cannot be assigned to transcriptional targets (Chang et al., 2014). Whether the many p53-binding sites have a tumor suppressor or genome maintenance function remains an important unanswered question.

We have shown here that p53 binds to multiple human and mouse subtelomeres. We have identified non-canonical p53-binding sites found within eleven human subtelomeres at positions close to the terminal repeat tracts. These p53-binding sites are also found within the acrocentric short-arm telomeres that contain the 4p- and 4q-like subterminal duplicon A sequence family (Youngman et al., 1992). The distinct class of subtelomeric p53-binding motifs lie at the edge of LTR10C elements that are positioned adjacent to a MER31-int sequence with a variable stretch of (TG)n between the two. This configuration of p53-binding sites appears to be unique to human subtelomeres. p53-binding sites have also been reported to occur throughout the human genome within a subclass of Alu repeat transposable elements (Cui et al., 2011) and throughout primate genomes within hERV elements (Wang et al., 2007). Thus, mobile element dispersion of p53-binding sites may provide a genomic adaptive advantage.

Genomewide studies have revealed that p53-binding sites can be functionally distinct. Integrated analyses of multiple ChIP-Seq and mRNA expression profiling studies indicate that p53-binding sites can be aggregated into two major classes, higher affinity sites responsible for cell cycle arrest, and weaker affinity, cooperative binding sites responsible for proapoptotic and autophagy functions (Kenzelmann Broz et al., 2013; Schlereth et al., 2013). A high-resolution ChIP-Seq analysis of stress-induced p53-binding sites revealed that p53 may regulate anti-sense non-coding RNAs near DNA damage response genes (Chang et al., 2014). This study revealed that p53 binds to > 2,000 response elements, of which only a small percentage could be assigned to the regulation of 151 annotated genes. Many of the p53 sites were found associated with LTRs, and to be enriched with TFIIB and RNAPII, and produce anti-sense transcripts. These transcripts may be equivalent to the eRNAs.
Materials and Methods

Cells and plasmid DNA

HCT116+/+ and −/− cells were generously contributed by Bert Vogelstein’s laboratory (Johns Hopkins School of Medicine). HCT116 cells were cultured in Dulbecco’s modification of Eagle’s medium (DMEM, Cellgro, 10-027) with 10% fetal bovine serum (FBS), 1% Pen/Strep, and 4.5 g/l glucose and L-glutamine. H1299 Tet-inducible p53 cell lines were cultured in DMEM, 1% Pen/Strep and with Tet system approved 10% FBS (Clontech, 631106). A concentration of 0.75 μg/ml doxycycline was used to induce p53. Etoposide (Sigma) 50 mM in DMSO was added to cells, at 1, 2, or 50 μM final concentration for 24 h or times indicated. For transient transfection assays, 5×10⁶ HCT116 p53+/− cells were plated per 24-well, transfected using Lipofectamine 2000 (Invitrogen) in duplicate with 0.01 μg renilla, 0.2 μg reporter template, and either 0.05 μg or 0.2 μg of the effector plasmid. Cells were collected and lysed 24 h post-transfection for analysis using the Promega dual-luciferase reporter kit on a Wallac Victor1420 multilabel counter. HCT116 18q WT and 18q Δp53 clones were generated using CRISPR Cas9 cleavage targeting a single site neighboring the 18q p53-binding site, combined with co-transfection of 2-kb DNA fragment for homologous recombination to produce clones lacking a 70-bp region spanning the non-canonical p53-binding site and the adjacent (GT)ₙ repeat element (Sanjana et al., 2014). Clones were identified by PCR and validated by sequencing.

ChIP-Seq

ChIP-Seq was performed essentially as described previously (Lee et al., 2006). HCT116 cells were treated with 5 μM camptothecin (Sigma) for 8 h, then crosslinked and harvested. Cells were sonicated using the Bioruptor sonicator (Diagenode) for 15 min, 30 s on, and 30 s off at high power. Antibody incubation was performed overnight at 4°C with 15 μg FL939 (Santa Cruz), as described (Sykes et al., 2006). ChIP-Seq analysis and mapping to human subtelomeres was described previously (Deng et al., 2012a; Stong et al., 2014). Deep sequencing was performed using the Applied Biosystems-SOLiD 5500xl. ChIP-Seq data sets were comprised of color space short reads that were mapped using SHRIMP 2.2.3 (Rumble et al., 2009), allowing for reads mapping up to 101 mapping positions (≈0 102). Once mapping positions were determined the pipeline followed was the same as Illumina-generated ChIP-Seq data sets.

ChIP–qPCR

ChIP–qPCR and telomere ChIP dot blots were performed essentially as described previously (Deng et al., 2012a). All qPCR primers are available upon request and included in the Appendix (Appendix Table S2).

Antibodies

The following antibodies were used: mouse anti-p53 (Ab6) (Calbiochem OP43); rabbit anti-phospho-p53 (Ser15) (Cell Signaling 9284); mouse anti-phospho-Histone H2A.X (Ser139) (Millipore 05-636); rabbit anti-Histone H2A.X (Millipore 07-627); rabbit anti-
acetyl-Histone H3 (Lys9) (Millipore 07-352); rabbit anti-Histone H3 (K4me1) (abcam ab8895); rabbit anti-Histone H3 (K27Ac) (abcam ab4729); rabbit anti-Histone H3, CT, pan (Millipore 07-690); rabbit anti-GAPDH (14C10) (Cell Signaling 2118); and rabbit TRF1 and TRF2 antibodies were produced at Pocono Rabbit Farms and described previously (Deng et al., 2012a).

Telomere length assay

TLA assay and two-dimensional agarose gel analyses were performed essentially as described previously (Deng et al., 2012a). Probes employed for the telomere assay are TelC (TAACCCTAACCTAAACCCCCT), TelG (GSP) designed to complement telomere repeats (ccctaaccctaacctacccct), and α Sat repeat probe mixtures (TTTCTTTGATAGTGCAAGTTTTGAAAC ATTCTTTTAAAATTATCAGCAG + TGGACATTCTCAGACCTCCTTTTAG GCTAATCGTTGAAGAAAGGAAATCTGTC + CATTAAACAAAGACAGA GAACATTCACAAAATCCTTTATGATGCTGCA).

Clonogenicity assay

Cells were treated with an appropriate concentration of etoposide, then 24 h later, they were trypsinized, counted, and replated at a constant density, typically 1,000 cells per 6 well. Colonies were permitted to grow out over approximately 2 weeks then were fixed and stained in 0.5% crystal violet/6% glutaraldehyde. Colonies were photographed and quantified for % coverage of total area.

RT–PCR

RNA was isolated from cells by suspending well in 1 ml TRIzol (Life Technologies) at RT. About 200 μl chloroform was added, and the sample was shaken by hand for 15 s. After 2–3 min at RT, samples were centrifuged at 12,000 × g for 15 min at 4°C. About 550 μl of the upper aqueous phase was transferred to a new eppendorf, mixed with 500 μl isopropanol, and incubated at RT for 10 min. RNA is precipitated at 12,000 × g for 10 min at 4°C, washed in 75% ethanol, dried briefly, and dissolved in DNase I reaction mix prepared in DEPC-treated water. DNase I treatment proceeds at 37°C for 15 min at 4°C, then 24 h later, they were trypsinized, counted, and replated at a constant density, typically 1,000 cells per 6 well. Colonies were permitted to grow out over approximately 2 weeks then were fixed and stained in 0.5% crystal violet/6% glutaraldehyde. Colonies were photographed and quantified for % coverage of total area.

Data availability

ChIP-Seq data have been submitted to NCBI GEO Database with accession number GSE75528.

References


Author contributions

ST, GAA, NS, OV, AW, JAM, KB, ZW, and ZD performed experiments, designed experiments, and interpreted data. ST, HR, SBM, MM, and PML designed experiments, interpreted data, and wrote the manuscript.