Telomere dysfunction and cell survival: Roles for distinct TIN2-containing complexes

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Abstract

Telomeres are maintained by three DNA binding proteins, TRF1, TRF2 and POT1, and several associated factors. One factor, TIN2, binds TRF1 and TRF2 directly and POT1 indirectly. These and two other proteins form a soluble complex that may be the core telomere-maintenance complex. It is not clear whether subcomplexes exist or function *in vivo*. Here, we provide evidence for two TIN2 subcomplexes with distinct functions in human cells. TIN2 ablation by RNA interference caused telomere uncapping and p53-independent cell death in all cells tested. However, we isolated two TIN2 complexes from cell lysates, each selectively sensitive to a TIN2 mutant (TIN2-13, TIN2-15C). In cells with wild-type p53 function, TIN2-15C was more potent than TIN2-13 in causing telomere uncapping and eventual growth arrest. In cells lacking p53 function, TIN215C more than TIN2-13 caused genomic instability and cell death. Thus, TIN2 subcomplexes likely have distinct functions in telomere maintenance, and may provide selective targets for eliminating cells with mutant p53.

Telomeres are the repetitive DNA sequence and specialized proteins that cap the ends of linear eukaryotic chromosomes and protect them from degradation or fusion by DNA repair processes. Telomere integrity and length maintenance are essential for prolonged cell proliferation and are thought to play important roles in suppressing aging and cancer[1,2].

Telomere length is generally maintained by telomerase, a reverse transcriptase that adds telomeric DNA repeats to chromosome ends. Telomere length homeostasis also depends on proteins that act in *cis* at the telomeres to control telomerase recruitment or access[3]. Most human cells do not express telomerase. Because DNA replication machineries cannot fully copy DNA 3' ends, such cells lose 50-200 bp of telomeric DNA with each S phase and enter a permanent growth arrested state termed senescence when telomeres become critically short[1]. Both telomerase-expressing and telomerase-negative cells utilize and require a host of proteins to ensure a proper protective telomeric structure.

The precise structure of mammalian telomeres is not known. However, a 't-loop' structure, in which the 3' overhang loops back on the double stranded telomeric DNA and invades the duplex, has been inferred by electron microscopy and biochemical experiments[4]. The t-loop model explains how the telomeric ends are protected from recognition by DNA repair machineries. This protection is sometimes termed capping. Telomeres can lose capping when they become critically short, presumably too short to form a t-loop, or when telomeric proteins that participate in telomere capping are defective.

Several telomere-associated proteins are known to be important for telomere length regulation and capping[1,3,5]. These include the direct telomeric DNA binding proteins TRF1, TRF2, and POT1, proteins that associate with these telomeric DNA binding actors (e.g., TIN2, RAP1, tankyrases), and a variety of proteins involved in other processes, such as DNA repair and recombination. Of

the direct DNA binding proteins, TRF1 binds double stranded telomeric DNA and is an important regulator of telomere length[6]. In contrast, TRF2, which also binds double stranded telomeric DNA, is more important for telomere capping[7-9] and t-loop formation[10]. POT1 binds the single-stranded 3' overhang and is likely a terminal regulator of telomere length and end protection[11].

TIN2 is an important telomere-associated protein because it binds both TRF1[12] and TRF2[13,14,15], and indirectly interacts with POT1 via the intermediary protein TPP1, also termed pTOP[16], PIP1[17], and TINT1[18]. TIN2 participates in the regulation of telomere length through its interactions with TRF1[12] and TPP1[16,17,18]. In addition, TIN2 appears to be a critical component in forming telomere complexes that function in end-protection[13].

The functions of the three telomeric DNA binding proteins (TRF1, TRF2 and POT1) are very likely coordinated. Perturbations to either TRF1 or TRF2, or their associated proteins POT1, RAP1 or TIN2, influence both telomere length and capping[6,11-13,19-22]. These observations suggest that TRF1, TRF2, POT1, and TIN2 may function in the same pathway. Consistent with this idea, six proteins co-purified in a large molecular weight complex[14,15,23]. This complex may be the core molecular machinery that regulates mammalian telomeres. On the other hand, gel filtration identified a TRF2-RAP1 complex that also contains TIN2 and POT1 but not TRF1[14]. Further, when TRF1 is removed from telomeres, TIN2 and TPP1 remain at telomeres via increased association with TRF2[18]. And, although POT1 was shown to be associated with TRF1[19], POT1 and TRF2 also form a complex with telomeric DNA, and POT1 overexpression protects against loss of telomeric single-stranded DNA caused by expression of a dominant negative TRF2 (DN-TRF2)[22]. Thus, there may be distinct telomeric complexes that participate in maintaining telomere length and capping.

It is not yet clear whether there is a single TIN2 complex, which always contains TRF1, TRF2 and TPP1/POT1 and their interacting proteins, or whether

TIN2 forms multiple complexes, some of which contain TRF1, while others contain TRF2[13,14,18]. Further, although it is hypothesized that telomeric complexes respond to telomere shortening, it is not known which telomere complexes are important for telomere capping, cellular senescence and cell death. Here, we report that at least two major TIN2-complexes can be identified by immunoprecipitation of cell lysates, and that one of these is crucial for cellular senescence and p53-independent cell survival.

Results

TIN2 depletion disrupts both TRF1 and TRF2 and causes p53-independent cell death

To understand the effects of TIN2 on cell fate and telomeric complex integrity, we used RNA interference (RNAi) to ablate TIN2 expression. Short hairpin (sh) RNAs[24] complementary to three regions of the TIN2 mRNA (T2i-1, T2i-2, T2i-3) were expressed in HT1080 human fibrosarcoma cells. Two of these shRNAs, T2i-2 and T2i-3, reduced TIN2 protein levels by 80-90% (Fig. 1a). T2i-2 and T2i-3 (not shown) also reduced TRF1 and TRF2 protein levels (Fig. 1b), consistent with their reported degradation upon removal from telomeres[25], and T2i-2 reduced focal immunostaining, indicative of telomeric localization, of all three proteins (TIN2, TRF1, and TRF2) (Fig. 1c). Thus, loss of TIN2 completely disrupted telomeres, as determined by loss of TRF1 and TRF2 protein levels and telomeric occupancy.

Following the loss of telomeric TIN2, TRF1 and TRF2, T2i-2 and T2i-3 induced caspase-dependent cell death, indicative of apoptosis (Fig. 1d). Cell death was not observed when cells expressed an insertless vector, a non-specific shRNA or T2i-1, which did not reduce TIN2 expression (Figs. 1a and d). T2i-2 also induced apoptosis (Fig. 1e) in primary human fibroblasts (strains HCA2 and WI-38). Strikingly, inactivation of p53 by expression of GSE-22, a short peptide that disrupts p53 tetramerization in these cells[26] failed to rescue

WI-38 cells from undergoing T2i-2-induced apoptosis (Fig 1e). The loss of p53 activity was confirmed by the absence of detectable p21 immunostaining in GSE-expressing, but not control, cells (Fig. 1f). Thus, TIN2 was essential for TRF1 and TRF2 stability and occupancy at telomeres, as well as the viability of normal and tumor-derived human cells, regardless of p53 status. This result suggests that loss of TIN2 promotes a seriously aberrant telomeric structure that induces p53-independent cell death, similar to the effects of complete telomere disruption caused by expression of a mutant telomerase template RNA[27].

Two major TIN2-complexes are formed in cells

TIN2-13 and TIN3-15C are TIN2 truncation mutants with different binding capabilities for TRF1 and TRF2 (Fig. 2a). TIN2-15C retains the TRF2[13] and TPP1/POT1 (Fig. 2b) binding domains, but lacks TRF1 binding[12,16]. TIN215C interacts less strongly than wild-type TIN2 with TRF2, as determined by immunoprecipitation and yeast two-hybrid analyses[13], suggesting additional TIN2 domains are needed for tight TRF2 binding. By contrast, TIN2-13 is a C-terminal TIN2 fragment that retains TRF1 binding[12], but lacks TRF2[13] and TPP1/POT1[17] binding.

We used these TIN2 mutants to identify TIN2 complexes in cells, and determine their role in the p53-independent cell death caused by complete loss of TIN2. We overexpressed epitope-tagged POT1, TIN2 or TRF1 (V5-tagged POT1, Flag-TIN2, HA-tagged TRF1) in HT1080 cells and used epitope-tag immunoprecipitation to isolate complexes from cell lysates. HA antibodies (Fig. 2c, lanes 10, 11) precipitated a major TRF1-TIN2 complex and a minor of TRF1-TIN2-POT1/TRF2 (visible when the gel is overexposed). These findings suggest that most TRF1 proteins form a complex with TIN2, at least under the physiological salt conditions used in the immunoprecipitation buffer. V5-antibodies precipitated a major POT1-TPP1-TIN2-TRF2 complex, regardless of whether TRF1 was overexpressed, and minor TRF1-TIN2-POT1-TRF2 and TRF1-TIN2-POT1 complexes (overexposed inset). Because POT1 does not

interact directly with TRF1[19], and interacts with TRF2[22] and TPP1[16,17,18], this result suggests that POT1 forms a more stable complex with TIN2-TRF2 than with TIN2-TRF1. FLAG antibodies (lanes 6, 7) precipitated a TIN2-TRF2-POT1 complex in the absence of overexpressed TRF1; TRF1 overexpression reduced formation of this complex and favored the formation of a TRF1-TIN2 complex. These results confirm that TRF1 and TRF2 compete with TIN2 to form separable complexes[13,14,18]. For simplicity, we term these complexes as follows: TRF1-TIN2, TIN2 complex A; TIN2-TRF2-POT1, TIN2 complex B; TRF1-TIN2-POT1-TRF2, combined complex C (Fig. 2d).

TIN2 mutants have distinct effect on TIN2-complexes.

We tested the effects of the TIN2 mutants on the formation of complexes A, B and C. Based on the complexes we identified (Fig. 2d), we predicted that TIN2-13, which lacks TPP1 and TRF2 binding[16,17] , would perturb complexes A and C, whereas TIN2-15C, which lacks TRF1 binding, would perturb complexes B and C.

We expressed V5-POT1 plus TIN2 (wild-type), TIN2-13 or TIN2-15C in HT1080 cells, and immunoprecipitated cell lysates using V5 antibodies (Fig. 3a, lanes 5-8). When TIN2-13 was expressed, V5 antibodies did not precipitate TIN2-13, and the presence of TIN2-13 did not alter the level of endogenous TIN2 in the precipitate (compare lanes 6 and 7). This result indicates that V5-POT1 mainly precipitated TIN2 B complexes, and that TIN2-13 does not disrupt B complexes, as predicted. This result also confirms the inability of TIN2-13 to interact with TRF2[13] and POT1[17].

By contrast, when TIN2-15C was expressed, V5 antibodies precipitated TIN2-15C, and the presence of TIN2-15C reduced the level of endogenous TIN2 in the precipitate (lane 8). This result suggests that TIN2-15 disrupted complex B formation, as predicted, and is consistent with the finding that TIN2-15C reduces TRF2 stability in cells and dissociates TRF2 from telomeres[13].

We also expressed HA-TRF1 plus wild-type TIN2, TIN2-13 or TIN2-15C, and immunoprecipitated cell lysates using HA antibodies (Fig. 3b, lanes 13-16). Expression of TIN2-13 reduced the binding of endogenous TIN2 to HA-TRF1 (compare lanes 13 and 15), but expression of TIN2-15C did not disrupt this interaction (compare lanes 13 and 16). Thus, as predicted, TIN2-13 disrupted TIN2-TRF1 complexes (complex A), but expression of TIN2-15C did not.

We conclude that the TIN2 mutants TIN2-13 and TIN2-15C disrupt different TIN2 complexes, at least by immunoprecipitation analysis, and thus favor the formation of different telomeric complexes when expressed in cells.

Effects of TIN2 mutants in cells

To determine the biological effects of the TIN2 complexes, we expressed GFP (control), TIN2-13 or TIN2-15C in primary human fibroblasts (strain HCA2). We first tested presenescent (P) or replicatively senescent (S) cells, with or without p53 inactivation by GSE-22, and measured apoptotic cell death using a sensitive cumulative assay[28] (Fig. 4a). In contrast to the robust cell death caused by complete loss of TIN2 (Fig. 1d,e), TIN-13 or TIN2-15C caused little or no cell death when expressed in presenescent or senescent cells with normal p53 function (Fig. 4a). However, when p53 was inactivated, the fraction of apoptotic cells increased in both cell populations (Fig. 4a). Notably, co-expression of TIN2-13 and GSE-22 elevated apoptosis only about 2-fold above control levels (caused by GFP and GSE-22), whereas co-expression of TIN2-15C and GSE-22 increased cell death 8-10 fold (Fig. 4a). These findings suggest that disruption of telomeric complexes B and C (by TIN-15C) had more severe consequences for cell survival than disruption of complex A (by TIN2-13), and that the cell death caused by disrupting these complexes is p53-independent.

Late generation mice lacking both telomerase and p53 progress very few additional generations relative to telomerase deficiency alone, suggesting the existence of a telomere-dependent, p53-independent cell death pathway[29]. To

further test the idea that disruption of different telomeric complexes differentially affects p53-independent cell survival, we used lenti-viral vectors to express TIN2 mutants and GSE-22 in senescent HCA2 fibroblasts, which have several short dysfunction telomeres[30], similar to those in late generation telomerase-deficient mice[31]. Because HCA2 cells express low levels of p16 (not shown), which inhibits the proliferation of cells that lack p53 function, p53 inactivation causes senescent HCA2 cells to resume proliferation, as reported for other low p16-expressing primary human fibroblasts[32]. We therefore measured the ability of senescent HCA2 cells to form colonies following p53 inactivation by GSE-22 and disruption of telomere complexes by TIN2 mutants or a dominant-negative TRF2 protein (DN-TRF2) that was shown to uncap telomeres and induce p53-dependent cell death[9] (Fig. 4b). Senescent cells readily formed colonies upon expression of GSE-22, as expected[32]. Subsequent expression of GFP (control), TIN2-13, or DN-TRF2 had no effect on colony formation over >30 days. By contrast, subsequent expression of TIN2-15C initially had no effect on cell proliferation (for 3-10 days; not shown), but then dramatically reduced colony formation. The differences between TIN2-15C and TIN2-13 or DN-TRF2 in supporting colony formation could not be explained by differences in protein expression levels because TIN2-13 and DN-TRF2 were expressed more robustly than TIN2-15C (Fig. 4c).

Together, these findings suggest that p53-dependent cell growth requires both TIN2 A and B complexes. By contrast, p53-independent cell growth does not require TIN2 A complexes, which are disrupted by TIN2-13 (Fig. 3), but does require TIN2 B complexes, which are disrupted by TIN2-15C (Fig. 3). DN-TRF2, which lacks the TRF2 DNA binding domain and causes p53-dependent cell death[9], is clearly not equivalent to TIN-15C in its biological activity, possibly because it does not affect POT1 function in B complexes (see Discussion).

We confirmed the selective sensitivity of p53-deficient cells to TIN2-15C using several human cancer cell lines. These included HT1080 fibrosarcoma

cells (which were reported to undergo no cell death upon expression of DN-TRF2[9]), MDA-MB-231 and MDA-MB-157 breast cancer cells, and PPC-1 prostate cancer cells. We used a transfected pIRES2-eGFP vector[13], and measured apoptotic cell death. TIN2-15C expression induced significant cell death within 48 h in all these p53-deficient cancer cells (Fig. 4d), whereas TIN2-13 was 2- to 8-fold less effective, depending on the cell line (Fig. 4d). Compared to normal cells in which p53 was inactivated by GSE-22, apoptosis was more robust in the cancer cells, being evident as early as 18 h after transfection, presumably because the cancer cells harbor multiple mutations that disrupt cell growth and survival pathways. Nonetheless, in all cases, TIN2-15C, but to a lesser extent TIN-13, caused substantial cell death in p53-deficient cells (Fig. 4d), but very little cell death in normal cells (Fig. 4a).

Normal cells responded to TIN2-15C by undergoing senescence. TIN2-15C, much more than TIN2-13, retarded cell proliferation (Fig. 5a) and induced the senescence-associated-β-galactosidase (Fig. 5b). Both mutants also induced 53BP1/γH2AX foci, most of which localized to telomeres[13] (and not shown), indicating uncapped dysfunctional telomeres[33]. The TIN2 mutants induced 53BP1 foci in presenescent cells, as well as replicatively senescent cells, which already have several telomeric 53BP1 foci. In all cases, TIN2-15C induced many more of these foci than TIN2-13 (Fig. 5c), suggesting that TIN2-15C is more potent than TIN3-13 at uncapping telomeres. These results also suggest that B complexes are more important than A complexes for protecting cells from telomere- and p53-independent cell death.

Chromosomal abnormalities induced by TIN2 mutants

Although p53-deficient cancer cell lines underwent rapid cell death in response to TIN-15C, p53 inactivation and TIN2-15C expression did not cause rapid cell death in presenescent or senescent human fibroblasts (in contrast to the effect of TIN2 depletion). Thus, additional events, such as cell cycle

progression and subsequent chromosome fusion, might be necessary for the death of cells with minimal lesions that inactivate p53 and complex B functions.

To test this possibility, we expressed GFP (control), TIN2-13 or TIN2-15C in HCA2 cells in which p53 was inactivated by GSE-22. Cells expressing TIN2-15C had a significantly higher number of chromosome fusions, compared to cells expressing TIN2-13 (Table 1). We observed no chromosome fusions in cells expressing GFP. The level of chromosome fusions in cells expressing TIN2-15C was similar to that reported for cells expressing DN-TRF2[7,22,34], consistent with the finding that expression of TIN2-15C reduces TRF2 at telomeres[13]. Thus, TIN2-15C may cause the death of p53-deficient cells with no other genetic lesions by indirectly driving chromosome fusions, breakage and mitotic catastrophe.

Telomerase does not rescue TIN2-15C lethality

TIN2-15C may cause chromosome fusions by accelerating telomere shortening. To test this idea, we co-expressed the telomerase catalytic subunit (hTERT) and TIN2-15C in senescent HCA2 cells in which p53 was inactivated by GSE-22. Expression of telomerase in p53-inactivated senescent cells prevented the crisis that limits the proliferative capacity of these cells to approximately 20 population doublings[32]. However, co-expression of telomerase and TIN2-15C did not rescue the cells from loss of proliferative potential (Fig. 6a,b). This result suggests that expression of TIN2-15C in p53-deficient senescent cells does not induce cell death by accelerating telomere shortening. Rather, the results suggest that TIN2-15C causes cell death by disrupting telomeric structure and capping.

Discussion

TIN2 telomeric complexes.

Six telomere-associated proteins (TRF1-TIN2-TPP1-POT1-TRF2-hRAP1) have been isolated as a single soluble complex[14,15]. TIN2 occupies a unique position in this complex because it interacts directly with TRF1 and TRF2, and indirectly (through TPP1) with POT1. In these studies, TRF2-complexes contained TIN2 and POT1, but not TRF1, suggesting that TRF1 is not required for the TRF2-TIN2-TPP1-POT1 interaction[18]. TRF1 and TRF2 bind non-cooperatively along arrays of telomere repeats and have high off-rates *in vitro*[35] and *in vivo*[36]. These results suggest that TIN2 may form dynamic TRF1 or TRF2-POT1 complexes at telomeres. We identified two major soluble complexes (TRF1-TIN2, complex A, and TIN2-TRF2-POT1, complex B), in addition to a single minor complex, supporting the idea that TRF1 and TRF2 complexes are separable.

We utilized two TIN2 mutants with distinct abilities to disrupt the major TIN2 complexes (A and B). The N-terminal deletion mutant TIN2-13 affected the TRF1-TIN2 complex A, whereas the C-terminal deletion mutant TIN2-15C affected TRF2-complexes. If the major telomeric complex were a single entity (complex C), TIN2-13 and TIN2-15C should disrupt this complex similarly. However, our results are more consistent with the existence of two separable complexes, which may have different functions in cells.

TIN2 complexes may function differently.

In normal cells, telomeres shorten with each division, resulting eventually in replicative senescence. TIN2-15C, which we show here disrupts B complexes, removes TRF2 from telomeres and induces DNA damage foci, but does not reduce TRF1 at telomeres[13]. Expression of DN-TRF2 also removes TRF2, with DNA damage foci co-localizing with TRF1[33]. Senescent cells with short telomeres and telomeric DNA damage foci frequently lack TRF2 but not TRF1[37].

Therefore, it is possible that short telomeres preferentially attract A complexes, and that senescent cells cannot form enough B (TIN2-TRF2-POT1) complexes because TIN2 is bound primarily in A (TRF1-TIN2) complexes. Alternatively, A and B complexes may have different preferred locations along the telomeric DNA and at the t-loop, and may cooperate in telomere capping. Short telomeres may preferentially lose B complexes owing to their location, for example enrichment at or near t-loops.

TRF1-TIN2 complexes stimulate interactions between telomeric DNA tracts *in vitro*, suggesting that this complex modulates a tertiary telomeric structure[38]. Thus, TIN2-13, which disrupts this complex (A complex), may decrease the complexity of the telomeric structure. On the other hand, deletion of TRF2 or POT1 causes telomere uncapping and chromosome end-to-end fusions, suggesting that the B complex is essential for telomere end protection[39,40]. Consistent with this view, we found that TIN2-15C, which disrupts B complexes, uncapped telomeres, evident as telomeric DNA damage foci, and caused telomeric fusions. Thus, we propose that TRF1-TIN2 (A) complexes and TIN2-TRF2-TPP1/POT1 (B) complexes have different locations at telomeres and cooperate to form the telomeric cap (Fig. 6c). B complexes are more important for ensuring a proper terminal or t-loop structure, whereas A complexes modulate the telomeric tertiary structure and enhance the stability and function of B complexes. Although TIN2-13 induces some telomere uncapping and telomeric fusions, the effects of TIN2-13 are less pronounced than those of TIN2-15C, suggesting that A complexes support the functions of B complexes.

As an alternative model, A and B complexes may cooperate to form the six protein complex (C complex), and this complex may be the essential entity for telomere capping. TIN2-13 may be less efficient than TIN2-15C at disrupting C complexes. TPP1 may be an important regulator of C complex formation because loss of TPP1 can reduce the TRF1-TRF2-Rap1 interaction [23].

Telomere-dependent, p53-independent cell survival.

Late generation mice lacking both telomerase and p53 have a higher incidence of cancer relative to animals lacking only telomerase[29,41] defining p53 as a key regulator of the response to telomere dysfunction. However, mice deficient in both p53 and telomerase lose sterility after only a few generations, defining a second p53-independent block to cell viability[29]. Expression of dominant-negative telomerase proteins[42] or mutant telomerase RNAs[43] induces death in cells with either wild type or mutant p53, supporting the idea that loss of viability caused by telomere dysfunction is not dependent on a functional p53 response. By contrast, cell death or growth inhibition due to expression of DN-TRF2 is p53-dependent[8,9]. Our results indicate that B complex disruption by TIN2-15C causes p53-independent cell death, suggesting that DN-TRF2 and TIN2-15C have different effects on the integrity of this complex.

In the absence of p53 activity, senescent cells that express little or no p16 can resume growth, although their proliferative potential is eventually limited by severe telomere shortening, crisis and massive cell death[32]. Expression of telomerase eliminated this growth limitation. Expression of TIN2-13 and DN-TRF2 only minimally affected this p53-independent growth resumption, whereas TIN2-15C expression accelerated this growth limitation. Recent findings[34,39,40] suggest an explanation for why the effects of TIN2-15C differ from those of DN-TRF2. Although, POT1deficiency causes a telomere uncapping phenotype similar to that caused by TRF2 loss, POT1 and TRF2 likely have distinct functions in protecting telomeres, regulating nucleolytic processing and controlling recombination at telomeres. Thus, TIN2-15C and DN-TRF2 likely inactivate TRF2 and POT1 differently in B complexes. Since DN-TRF2 induces p53-dependent apoptosis[9], whereas TIN2-15C caused p53-independent cell death, our findings offer an explanation for why ablation of TIN2 in cells (Fig. 1), and in the mouse germ line[44], causes cell lethality or senescence, regardless of p53 status.

It remains to be understood how telomere dysfunction causes p53-independent cell death, how dysfunctional telomeres are sensed as DNA damage, and nature of the p53-dependent and p53-independent sensing mechanisms. Despite these gaps in our knowledge, telomerase inactivation has been proposed as an anti-cancer strategy because most cancer cells maintain telomere length by expressing this enzyme. Likewise, disruption of TIN2-complexes may provide a strategy for killing cancer cells, most of which lack p53 function. TIN2-15C induced cell death in p53-inactivated cancer cells, but induced growth arrest in p53 positive normal cells. Therefore, TIN2-15C sensitive complexes are more critical for cell survival in p53-negative cancer cells, compared to normal cells.

Experimental Procedures:

Cell culture, senescence and apoptosis characterization: We cultured all cells, and measured senescence-associated β-galactosidase, as described[45] . We determined cell death by collapse of the mitochondrial membrane potential[46] or by a sensitive cumulative assay in which cells are incubated with a caspase inhibitor for 3 days and scored for cytosolic cytochrome c[28].

Purification of TIN2 complexes: We expressed FLAG-tagged TIN2 and HA-tagged TRF1 in HT1080 cells using the retroviral vector pLXSN, as described[12]. V5-tagged POT1[47] and myc-TIN2 mutants (TIN2-13 or TIN2-15C) were expressed using the pIRE2-EGFP vector (Bio Science) and transient transfection using Fugene 6 (Roche). Cells (6×10^6) were washed with phosphate buffered saline (PBS), and 1 ml RIPA buffer {50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10% glycerol, protease inhibitor cocktail (Roche)} was added to each plate. After incubation on ice for 30 min, cells were collected by scraping and centrifugation at 4° C, and the supernatant (cell lysate) was recovered.

Immunoprecipitation and western analyses: We incubated cell lysates (200-300 μg protein) with 2 μg HA antibody (Roche), 10 μg FLAG M2 antibody (Sigma) or 2 μg V5 antibody (Invitrogen) for 2 h at 4˚ C, and added 50 μl of a 50 % protein A-Sepharose slurry (Pharmacia) for 2 h at 4˚ C. We washed the immune complexes with RIPA buffer and analyzed proteins by western blotting as described[12,46]. Primary antibodies were anti-TRF2 mouse monoclonals (Imgenex), rabbit polyclonal anti-HA- or -TRF2 or monoclonal anti-HA (Santa Cruz), monoclonal anti-V5 (Invitrogen), and polyclonal anti-TIN2[12].

Immunostaining: We immunostained cells as described[12]. Briefly, we cultured cells in chamber slides, fixed with 4% formalin, permeabilized with 0.5% Triton-X100, and stained with mouse anti-TRF2 (Imgenex), polyclonal anti-TIN2[12], polyclonal anti-53BP1 (Abcam), mouse anti-p21 (Pharmigen) or control 10% goat

serum (Vector). After washing, we stained with secondary antibodies conjugated to Texas Red or FITC (Molecular probes), and counterstained the nuclei with DAPI.

Chromosome analyses: Telomeres were visualized by *in situ* hybridization (FISH) on metaphase spreads using a telomeric protein nucleic acid (PNA) probe, as described[48]. Cells treated with colcemid (0.1μg/ml) for 4 h were trypsinized and collected at 1000 X *g* (5 min). After hypotonic swelling in 30 mM Na citrate for 20 min at 37^{o} C, the cells were fixed in methanol:acetic acid (3:1). FISH using a Cy3-labeled $(CCCTAA)_3$ peptide nucleic acid (PNA) probe (Applied Biosystem) and scoring for telomeric fusions were performed as described[49].

shRNA and expression vectors: Where indicated, cells were infected with a retrovirus expressing GSE-22[26], selected and then transfected with expression vectors or infected with lenti-viruses, as described[13,32]. To ablate TIN2 expression, we synthesized double stranded DNAs to target the TIN2 mRNA (T2i-1:caggtgaagcagctgtcag; T2i-2: ggtcatatctaatcctgag, T2i-3: gtggtggtggagctgatc) or SATB1, a nuclear protein that is not expressed in fibroblasts or HT1080 cells (N/S (non-specific): aacagctactattgccact). We cloned the DNA into the pSuper vector[24], and transiently transfected packaging cells using FuGene6 (Roche). We cloned TIN2 mutants into the bicistronic pIRES2-EGFP (Clontech) or pPRL-Sin18-lenti vector[50]. Lentiviruses were used at equivalent titers, sufficient to infect approximately 80-90% of cells.

Colony formation assay: Senescent cells (less than 2% labeling index) were plated with 5 X 10^4 cells in 6 well plates and infected with lentiviruses expressing GFP, TIN2-15C, TIN2-13, hTERT or GSE-22 as described[32]. The cells were washed with PBS twice, fixed and stained with 0.1 % crystal violet in 10% ethanol for 5 min at room temperature, washed with PBS and dried.

Figure Legends

FIGURE 1. Effects of TIN2 ablation on TRF1, TRF2 and cell viability.

a. **RNAi reduces TIN2 expression.** HT1080 cells were transiently transfected with pSuper vectors expressing shRNAs corresponding to a non-expressed (non-specific, N/S) mRNA or one of three distinct regions in the TIN2 mRNA (T2i-1, T2i-2, T2i-3), as described in Methods. Transfection efficiency varied from 60-80%. Cell lysates were analyzed 48 h later by western blotting for TIN2 expression, with tubulin used as a loading control.

b. **TIN2 reduction decreases TRF1 and TRF2 protein expression.** Lysates from HT1080 cells transfected with the N/S (control) or T2i-2 pSuper vectors described above were analyzed for TIN2, TRF1, TRF2 and tubulin by western blotting.

c. **TIN2 reduction decreases TRF1 and TRF2 foci .** HT1080 cells transfected with the N/S (control) or T2i-2 pSuper vectors described above were immunostained for TIN2 and TRF1 (left panels) or TRF2 (right panels). Arrowheads indicate nuclei (visible by DAPI) that lack TIN2 and TRF1 (left panels) or TRF2 (right panels) immunostaining.

d. **TIN2 reduction induces apoptosis in human tumor cells.** HT1080 cells were transiently transfected with pSuper vectors containing no insert (Vector) or N/S (control), T2i-1, T2i-2, or T2i-3 shRNAs and analyzed 48 h later for apoptosis as described in Methods. Transfection efficiency was 60-80%. Where indicated, the caspase inhibitor ZVAD (100 μM) was added 8 h after transfection. 200 cells were analyzed for apoptosis in three independent experiments.

e. **TIN2 reduction induces apoptosis in normal human cells.** Normal human fibroblasts (HCA2 and WI-38) were transiently co-transfected with pSuper vectors containing no insert (Vector) or T2i-1 or T2i-2 shRNAs, and a lenti-GFP vector at a ratio of 10:1. 48 h later, GFP positive cells were analyzed for apoptosis as described in Methods. Where indicated, WI-38 cells were first

infected with a retrovirus expressing GSE-22, selected and then infected with the pSuper and GFP vectors. 200 cells were analyzed in three experiments.

f. GSE-22 inactivates p53 activity in normal cells.

WI38 cells were infected with a retrovirus expressing GSE-22 or insertless virus, selected, plated and then irradiated with 10 Gy X-ray. The cells were fixed 19 hrs later and immunostained for p21.

FIGURE 2. TIN2-mediated complexes.

a. TIN2 deletion mutants. Wild type TIN2 (aa 1-354) showing N-terminal (N-term), TRF1-interaction (TRF1-Int) and C-terminal (C-term) domains, and deletion mutants TIN2-13 (aa 180-354) and TIN2-15C (aa 1-257).

b. Interaction of TIN2-15C with POT1/TPP1. Lysates from HT1080 cells that transiently expressed Myc-TIN2-15C and V5-POT1 were precipitated using anti-Myc or V5 antibodies. Unprecipitated lysates (10%) and the immune precipitates were analyzed for POT1 and TIN2-15C by western blotting (WB).

c. TIN2-complexes. We prepared lysates from HT1080 cells that transiently expressed V5-tagged POT1 and stably expressed FLAG-TIN2 (lanes 2,4,6,8) or HA-TRF1 (1,10) or both Flag-TIN2 and HA-TRF1[12] (lanes 3,5,7,9,11). We isolated TIN2 complexes using FLAG, V5 and HA antibodies and analyzed the lysates (10%, Input) and immunoprecipitates by western blotting (Western) for the indicated proteins.

d. Proposed TIN2 complexes. A-complex, TIN2-TRF1; B-complex, TIN2-TRF2-TPP1-POT1; C-complex, TRF1-TIN2-TRF2-TPP1-POT1. hRAP interacts with TRF2[20], and is presumed to be present in complexes B and C.

FIGURE 3. TIN2 complexes disrupted by TIN2 mutants

a. TIN2-TRF2-POT1 complexes disrupted by TIN2-15C, but not TIN2-13. Lysates from HT1080 cells transiently expressing WT-TIN2, control vector, TIN2-13 or TIN2-15C with V5-POT1 were immunoprecipitated (IP) by anti-V5 antibody. The lysates (15%, Input) and immune precipitates were analyzed

for TIN2, V5-POT and tubulin by western blotting (WB) (lanes 1-8). The precipitating heavy chain is indicated (IgG).

b. TRF1-TIN2 complexes disrupted by TIN2-13, but not TIN2-15C. Lysates from HT1080 cells transiently expressing WT-TIN2, vector, TIN2-13 and TIN2-15C with HA-TRF1 were immunoprecipitated (IP) by anti-HA antibody. The lysates (15%, Input) and precipitates were analyzed for TIN2 and HA-TRF1 and tubulin by western blotting (WB) (lanes 9-18). The precipitating heavy chain is indicated (IgG).

FIGURE. 4. Effects ofTIN2 mutants in presenescent and senescent cells.

a. Cell death caused by TIN2-15C in presenescent cells. Where indicated, presenescent (P) HCA2 cells were first infected with a retrovirus expressing GSE-22 and selected for 2-3 days. Cells were then infected with lentiviruses expressing GFP, TIN2-13 or TIN2-15C. Where indicated, senescent (S) HCA2 cells were first infected with lentiviruses expressing GFP, TIN2-13 or TIN2-15C, and then infected with a lentivirus expressing GSE-22. Cells were scored for cell death by assessing mitochondrial release of cytochrome c as described [28]. 300 cells were scored in two or three independent experiments.

b. Effects of TIN2-15C on senescent cells reactivated by GSE-22. 5×10^4 senescent HCA2 cells were infected with lentiviruses expressing GFP (control), TIN2-13, TIN2-15C or DN-TRF2, and then infected with lenti-GSE-22. Colonies were stained at the indicated days after infection as described in Methods.

c. Expression levels of TIN2 mutants and DN-TRF2. Lysates from cells presenescent cells infected with the indicated lenti-viruses were analyzed for TIN2 and TRF2 by western blotting (WB).

d. Cell death induced by TIN2-15C in p53-negative cancer cells. Human HT1080 fibrosarcoma, MDA-MB-231 breast cancer, MDA-MB-157 breast cancer and PPC-1 prostate cancer cells were transiently transfected with pIRES2-eGFP vectors expressing no insert (vector), TIN2-13 or TIN2-15C. 48 h later, the cells were analyzed for GFP fluorescence, and GFP-positive

cells were scored for cell death by collapse of the mitochondrial membrane potential as described[46]. 200 GFP positive cells were scored in two or three independent experiments for each transfection.

FIGURE. 5. Effects of TIN2 mutants in normal cells.

a. TIN2-15C suppresses proliferation of normal cells. We infected presenescent HCA2 cells with lenti-viruses expressing GFP, wild type (wt) TIN2, TIN2-13 or TIN2-15C and determined cell number at the indicated number of days thereafter. Plotted are cumulative cell numbers vs. days in culture.

b. TIN2-15C induces cellular senescence. HCA2 cells were infected with lenti-GFP or lenti-TIN2-15C and assessed 10 d later for senescence-associated β-galactosidase, as described[45]. Replicatively senescent HCA2 cells were used as positive controls.

c. TIN2-13 and TIN2-15C induces damage foci in presenescent and senescent cells. Presenescent (P) and senescent (S) HCA2 cells were infected with lenti-TIN2-13 or lenti-TIN2-15C and stained 48 h later for expression of the mutant proteins and γH2AX or 53BP1 foci. Shown are the % cells with varying numbers of foci per nucleus.

FIGURE 6.

a/b. Telomerase does not rescue cell death by TIN2-15C. 5 X 10^4 senescent cells were infected with lentiviruses expressing GFP (control), the telomerase catalytic subunit hTERT, TIN2-15C or both TIN2-15C and hTERT. The cells were then infected with lenti-GSE-22. After 2 d, the cells were subcultured and plated for colony formation. Colonies were fixed and stained 61 days later.

b. Colonies were counted 5, 11 and 61 days after plating.

c. Proposed model for TIN2 complexes at telomeres.
Complex A and B cooperate, albeit at different positions on telomeres, to form t-loops or other terminal structures. Complex B may localize preferentially or

uniquely near t-loop junctions, whereas A complexes may modulate the tertiary structure of telomeres and promote B complex stability. TIN2-13 disrupts complex A, thereby reducing the tertiary telomeric structure and destabilizing B complexes, resulting in partial or mild disruption of t-loops and telomere uncapping. TIN2-15C directly disrupts B complexes, resulting in severe disruption of t-loops and telomere uncapping. Cells expressing wild type p53 and TIN2-13 or TIN2-15 undergo growth arrest, whereas cells lacking functional p53 undergo cell death. In both cases, the consequences of TIN2-15C expression are more severe than that of expressing TIN-13.

Table 1.

Presenescent HCA2 cells were infected with a retroviruses expressing GSE-22, selected and then infected with lentiviruses expressing GFP, TIN2-13 or TIN2-15C. The cells were treated with colcemid (0.1μg/ml) for 4 h and telomeres of metaphase chromosomes were identified by PNA-FISH, as described in Experimental procedures.

TABLE 1. Telomeric fusions caused by expression of TIN2 mutants.

HCA2 cells + GSE-22	Metaphases analyzed (No)	Cells with telomere fusions (%)	Fusions /Metaphase	Fusions /Chromosome
Vector	53	0	0	0
TIN2-13	53	18.8	0.18	0.0034
TIN2-15C	61	75.0	0.85	0.0175

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