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The human CTC1/STN1/TEN1 complex regulates telomere maintenance in ALT cancer cells

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ABSTRACT

Maintaining functional telomeres is important for long-term proliferation of cells. About 15% of cancer cells are telomerase-negative and activate the alternative-lengthening of telomeres (ALT) pathway to maintain their telomeres. Recent studies have shown that the human CTC1/STN1/TEN1 complex (CST) plays a multi-faceted role in telomere maintenance in telomerase-expressing cancer cells. However, the role of CST in telomere maintenance in ALT cells is unclear. Here, we report that human CST forms a functional complex localizing in the ALT-associated PML bodies (APBs) in ALT cells throughout the cell cycle. Suppression of CST induces telomere instabilities including telomere fragility and elevates telomeric DNA recombination, leading to telomere dysfunction. In addition, CST deficiency significantly diminishes the abundance of extrachromosomal circular telomere DNA known as C-circles and t-circles. Suppression of CST also results in multinucleation in ALT cells and impairs cell proliferation. Our findings imply that the CST complex plays an important role in regulating telomere maintenance in ALT cells.

1. Introduction

Telomeres, the physical termini at linear chromosomes, preserve genome stability by distinguishing natural chromosomal termini from broken DNA ends and protecting chromosomes from degradation and inappropriate repair activities [1–3]. Maintenance of functional telomeres is essential for long-term cell proliferation. In the majority of immortalized cell lines, including germline cells and ~85% of cancer cells, telomeres are maintained by telomerase-catalyzed *de novo* addition of telomeric repeats, allowing for indefinite cellular proliferation [4,5]. The remaining ~15% of human tumor cells lack telomerase activity, and maintain their telomeres using the alternative lengthening of telomeres (ALT) pathway [6–8].

Telomeric DNA, consisting of repetitive double-stranded $(TTAGGG/AATCCC)_n$ repeats and single-stranded G-rich 3′ overhangs, is bound by a group of proteins that play an important role in maintaining telomere stability. The shelterin complex, consisting of TRF1, TRF2, POT1, TPP1, TIN2, and RAP1, shields chromosome ends from being recognized as damaged DNA, as evidenced by numerous studies showing that shelterin components prevent the activation of ATM/ATR damage response pathways at telomeres (reviewed in [9]). Another important telomere maintenance complex is the CTC1-STN1-TEN1 (CST) complex, a trimeric protein complex that binds to single-stranded DNA with high affinity [10–16]. Several recent studies have shown that the CST complex is important for telomere maintenance in a multifaceted manner. First and foremost, it facilitates efficient replication of telomeric DNA, thereby preventing catastrophic telomere loss [12–14]. As a result, suppression of individual components of CST increases the frequencies of fragile telomeres and leads to telomere loss in human somatic cells and mammalian cells [12–14]. CST is also involved in the late S/G2-specific synthesis of telomeric C-strands referred to as C-strand fill-in, and depletion of CST results in excessively long G-overhangs [12–15]. Additionally, CST may compete with shelterin POT1-TPP1 for binding to telomeric DNA and restrict telomerase extension of telomeres [17]. The importance of CST in maintaining telomere stability is underscored by genetic studies showing that CTC1 and STN1 mutations cause the Coats Plus syndrome and dyskeratosis congenita [18–22], two diseases that are associated with telomere maintenance defects.

To date, the role of CST in telomere maintenance has mainly been investigated in non-ALT cells, and its role in ALT-mediated telomere maintenance is largely unknown. Although it appears that ALT cells

Abbreviations: ALT, alternative lengthening of telomeres; CST, the CTC1/STN1/TEN1 trimeric complex; APBs, ALT-associated promyelocytic leukemia bodies; PML, promyelocytic leukemia; ECTR, extrachromosomal circular telomere repeats; TC, t-circle; CC, C-circle; HDR, homology-directed recombination; T-SCE, telomere sister chromatid exchange; SFE, signal free ends; FT, fragile telomere; CO-FISH, chromosome-oriented FISH; FISH, fluorescent in situ hybridization; PFGE, pulse-field gel electrophoresis; RCA, rolling circle amplification; TRF, telomere restriction fragment analysis

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employ homology-directed synthesis for telomere maintenance [8,23–25], the mechanism for the ALT pathway remains largely elusive. In addition, ALT cells show several characteristics distinct from non-ALT cells. First, ALT cells contain ALT-associated promyelocytic leukemia (PML) bodies (APBs), which are special PML nuclear bodies containing telomeric DNA, shelterin proteins and repair factors [25–27]. Mounting evidence suggests a model that APBs may provide sites to accumulate telomeric repeats and relevant proteins to facilitate telomere synthesis events [26,28–30]. Second, ALT cells show high frequency of telomere sister chromatid exchange (T-SCE), which is presumably caused by elevated levels of homology-directed repair (HDR) events at telomere repeats [31,32]. Third, telomere lengths in ALT cells are extremely heterogeneous [6,7]. Moreover, abundant extrachromosomal telomere repeats (ECTRs) are detectable in ALT cells. These ECTR molecules are predominantly composed of double-stranded telomeric circles named t-circles (TCs) and partially single-stranded circles referred to as C-circles (CCs) or G-circles that possess intact continuous C- or G-rich strands [24,33]. CCs are much more abundant than G-circles, and are found to be more specific and quantifiable to ALT activity than G-circles and TCs [24]. Lastly, while only 3′ G-rich overhangs are detectable in non-ALT cells, abundant 5′ C-rich overhangs are present in ALT cells [34]. These 5′ C-overhangs have been implicated in the telomere recombination pathway [35].

In this study we set out to examine the effect of CST suppression on telomere maintenance in ALT cells. We report that CTC1 and STN1 display punctate nuclear staining that colocalizes with APBs in ALT cells. Suppression of CST significantly decreases CC and TC abundance, elevates telomere abnormalities including T-SCE and fragile telomeres, and induces telomere DNA damage. Moreover, CST suppression limits ALT cell proliferation and dramatically increases the formation of multinucleated polyploid cells. Our results demonstrate that CST plays an important role in telomere maintenance in ALT cells, and suggest that targeting CST may be a potential therapeutic approach for inhibiting the growth of ALT-positive cancer cells.

2. Materials and methods

2.1. Cell culture

U2OS cells stably expressing Flag-CTC1 were constructed by retroviral transduction of pBabe- Flag-CTC1 [36], followed by hygromycin selection. All cells were cultured at 37 °C under 5% CO_2 in DMEM supplemented with 10% fetal bovine serum or cosmic calf serum (HyClone). Double thymidine block was used to synchronize U2OS cells. Briefly, exponentially growing cells were treated with thymidine (2 mM) for 14 h, followed by a cell wash with prewarmed DMEM (three times) and then released into fresh media for 10 h. The second thymidine (2 mM) was then added to medium for 12–16 h, followed by a cell wash with pre-warmed DMEM (three times) before cells were released into fresh medium containing serum. Cells were collected at different time points for analyzing DNA contents using a Beckman Coulter EPICS® XL™ flow cytometer.

2.2. Antibodies

The following primary antibodies were used: rabbit α-OBFC1/STN1 (Santa Cruz), mouse α-OBFC1/STN1 (Sigma), rabbit α-CTC1 (ThermoFisher), rabbit α-TRF2 (Santa Cruz), rabbit α-PML (Santa Cruz), mouse α-PML (Santa Cruz), rabbit α-FLAG (Cell Signaling), mouse α-FLAG (Sigma), mouse α-actin (Millipore). Secondary antibodies were horseradish peroxidase conjugated anti-mouse IgG and anti-rabbit IgG (BD Biosciences) for western blotting, Dylight 488-anti-mouse IgG (ThermoFisher) and Dylight 549-anti-rabbit IgG (ThermoFisher) for immunofluorescence.

2.3. RNAi

STN1 siRNA sequences targeting GAUCCUGUGUUUCUAGCCU (siSTN1-1) and GCUUAACCUCACAACUUAA (siSTN1-2) were described previously [13]. STN1 shRNA sequences targeting GAUCCUGUGUUUCUAGCCU (shSTN1-1) GCUUAACCUCACAACUUAA (shSTN1-2) [13], GGACUGCCAGAAACCAAAT (shSTN1-4), CTC1 shRNA sequences targeting GTGTTTCCTTTGACCATCA (shCTC1-1), GAAAGTCTTGTCCGGTATT (shCTC1-2), and control siRNA targeting luciferase (shLUC) were described previously [36].

2.4. Immunofluorescence (IF), Immunofluorescence-FISH (IF-FISH)

For IF or IF-FISH, cells grown on chamber slides were fixed with 4% paraformaldehyde and permeabilized in 0.15% Triton X-100. In experiments with pre-permeabilization, cells were treated with 0.1% Triton X-100 for 5 min on ice prior to paraformaldehyde fixation [10]. Cells were then blocked for 1 h in 3% BSA at 37 °C, incubated with primary antibodies for overnight at 4 °C, washed 3 times with PBS and incubated with fluorescence-conjugated secondary antibodies for 1 h at 37 °C. Cells were washed again and counterstained with DAPI. Images were taken under Zeiss AxioImager M2 epifluorescence microscope. For IF-FISH, after staining for protein antigens, slides were re-fixed with 4% paraformaldehyde for 10 min at r.t. Slides were then denatured in hybridization buffer [10 mM Tris pH 7.5, 70% formamide, 0.5% blocking solution (Roche)] at 90 °C for 5 min and hybridized with peptide nucleic acid (PNA) probes Alexa488-OO-$(TTAGGG)_3$ (TelG) or Cy5-OO-$(CCCTAA)_3$ (TelC) at r.t. for 2 h as described previously [13]. Slides were then washed with 10 mM Tris pH7.5/70% formamide for 15 min, followed by washing two times in 0.1 M Tris pH7.5/0.15 M NaCl/0.08% Tween-20 for 5 min each. Slides were dried and DAPI containing mounting medium (Vector Labs) was applied for microscopy visualization.

2.5. Chromosome-oriented FISH (CO-FISH)

CO-FISH was performed as described previously [37]. Briefly, cells were cultured in the presence of 10 μM BrdU and BrdC (3:1) for one population doubling prior to colcemid treatment. Slides containing metaphase spreads were incubated with Hoechst 33258 for 15 min, exposed to UV light for 30 min, treated with Exonuclease III (Promega) for 10 min at room temperature, and then sequentially hybridized to PNA Alexa488-OO-(TTAGGG)3 and Cy3-OO-(CCCTAA)3 probes at the room temperature for 2 h. Slides were then washed and dehydrated in ethanol series. DNA was counterstained with DAPI, and images were taken under Zeiss AxioImager M2 epifluorescence microscope.

2.6. C-Circle assay (CC assay)

CC assay was performed as described previously [24]. Briefly, 40 ng of genomic DNA was digested with AluI and HaeIII, and then subjected to rolling circle amplification in the presence of 1 mM of each dATP, dGTP, dTTP, with or without 10 U Φ29 DNA polymerase (NEB). Reaction was incubated at 30 °C for 8 h, then at 65 °C for 20 min. Equal amount of reaction solution from each sample was subjected to agarose gel electrophoresis under non-denaturing condition to detect amplified long G-strand products that migrated minimally from the wells. For quantitative CC assay, an aliquot of reaction products was diluted with 2×SSC and slot-blotted onto Hybond-N^+ membrane (GE Healthcare). DNA was UV-crosslinked and hybridized with the ^{32}P-$(CCCTAA)_3$ probe. Meanwhile, an equal amount of reaction products was denatured in 0.1 M NaOH at 65 °C for 10 min and then hybridized with ^{32}P-Alu probe on slot blot. Membranes were exposed to Phosphor screens to obtain signals in the linear range, and were scanned on a STORM 860 (GE Healthcare) with the ImageQuant software. Signals from telomeric probe hybridization (native membrane) were normal-