TCAB1 prevents nucleolar accumulation of the telomerase RNA to promote telomerase assembly

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Abstract

Localization of a wide variety of RNAs to non-membrane bound cellular compartments such as nucleoli, Cajal bodies, and stress-granules is critical for their function and stability. The molecular mechanisms that underly the recruitment and exclusion of specific RNAs from these phase-separated organelles is poorly understood. Telomerase is a ribonucleoprotein (RNP), that is composed of the reverse transcriptase protein TERT, the telomerase RNA (TR), and several auxiliary proteins that associate with TR, including TCAB1. Here we show that, that in the absence of TCAB1, TR is trapped in the nucleolus, while TERT localizes to the nucleoplasm and is excluded from the nucleolus, which prevents telomerase assembly. Thus, nucleolar phase separation constitutes a barrier that counteracts telomerase assembly and TCAB1 is required to exclude the telomerase RNA from the nucleolus. Our work provides general insight into the mechanism and functional consequences of RNA recruitment to non-membrane bound organelles and proposes a new model explaining the critical role of TCAB1 in telomerase function.

Introduction

Human cells contain a number of non-membrane bound organelles that carry out critical cellular functions. For instance, nucleoli and Cajal bodies are phase-separated nuclear organelles that play important roles in the biogenesis and maturation of many cellular RNAs (Hyman et al., 2014; Mitrea and Kriwacki, 2016). Nucleoli and Cajal bodies contain a wide range of small nucleolar and small Cajal body-specific RNAs (snoRNAs and scaRNAs, respectively). A subset of these snoRNAs and scaRNAs are bound by the H/ACA complex, which contains NOP10, NHP2, GAR1, and the pseudouridylase dyskerin, which modifies ribosomal and spliceosomal RNA precursors and other RNAs (Angrisani et al., 2014). A key difference between snoRNAs and scaRNAs is the presence of the Cajal-body box (CAB-box) motif in scaRNAs that directly associates with the telomerase Cajal body protein 1 (TCAB1, also known as WRAP53) (Jády et al., 2004; Schmidt and Cech, 2015; Venteicher et al., 2009). TCAB1 is required for the recruitment of scaRNAs to Cajal bodies and in its absence scaRNAs localize to the nucleolus (Venteicher et al., 2009). Therefore, TCAB1 controls which phase-separated nuclear organelle scaRNAs associate with. Importantly, the molecular mechanism by which TCAB1 drives exclusion of scaRNAs from the nucleolus and facilitates their recruitment to Cajal bodies is unknown. In addition, it is unclear whether miss-localization of scaRNAs to the nucleolus has functional consequences.

The telomerase RNA (TR) is a scaRNA and like other scaRNAs its association with nucleoli and Cajal bodies is controlled by TCAB1 (Schmidt and Cech, 2015). Telomere maintenance by telomerase is essential for continuous proliferation of stem cell

populations in the human body and most cancers require telomerase activity for their survival (Stewart and Weinberg, 2006). To compensate for the incomplete replication of chromosome ends, telomerase appends TTAGGG repeats to the telomeric single-stranded overhang (Schmidt and Cech, 2015). Telomerase-mediated telomere maintenance requires three critical steps: Telomerase assembly, telomerase recruitment to telomeres, and telomeric repeat synthesis (Schmidt and Cech, 2015). Mutations in several genes have been identified that cause deficiencies in one of these critical steps and lead to a variety of diseases known as telomere syndromes, characterized by premature depletion of stem cell populations (Armanios and Blackburn, 2012). In addition, telomerase is inappropriately activated in >85% of cancers (Stewart and Weinberg, 2006). While telomerase recruitment to telomeres (Nandakumar and Cech, 2013) and telomerase catalysis (Wu et al., 2017) have been studied extensively, much less is known about telomerase assembly. Importantly, telomerase assembly could be targeted to reduce telomerase activity in cancer cells, or to increase telomerase function in patients affected by genetically defined telomerase deficiency syndromes (Nagpal et al., 2020; Shukla et al., 2020).

Telomerase is a complex ribonucleoprotein (RNP). The core components of telomerase are the telomerase reverse transcriptase (TERT) protein, TR, the H/ACA complex, and TCAB1 (Schmidt and Cech, 2015). The primary function of the H/ACA complex is to stabilize TR, by directly binding to its 3'-end, preventing the exonucleolytic degradation of TR (Stuart et al., 2015; Tummala et al., 2015). The 3'-end formation of TR is tightly regulated by the competing activities of the poly-(A) polymerase PAPD5 and the nuclease

PARN (Shukla et al., 2016; Tseng et al., 2015). Loss of TCAB1 function leads to telomere attrition in a variety of cell lines (Chen et al., 2018; Venteicher et al., 2009; Vogan et al., 2016; Zhong et al., 2011). In addition, multiple mutations in TCAB1 have been identified that cause misfolding of TCAB1 and lead to dyskeratosis congenita, a telomere syndrome (Freund et al., 2014; Zhong et al., 2011). While these observations highlight that TCAB1 is necessary for telomere maintenance, the underlying molecular mechanism is unclear. Initially, it was proposed that TCAB1 is required for telomerase recruitment to telomeres (Stern et al., 2012; Venteicher et al., 2009). A more recent study suggested that TCAB1 is required for the correct folding of TR, and that its absence causes a reduction in telomerase activity (Chen et al., 2018). Importantly, all previous studies have come to the conclusion that TCAB1 is not required for telomerase assembly.

Here we analyze telomerase assembly in intact cells and by purification of the telomerase RNP and demonstrate that, contrary to previous findings, TCAB1 promotes telomerase assembly *in vivo*. Using a combination of genetic perturbations, biochemical analysis of telomerase function, cell biological approaches, and single-molecule live cell imaging, we show that in the absence of TCAB1, TR is sequestered in the nucleolus while TERT is excluded from the nucleolus. The spatial separation of TERT and TR that we observe in our experiments is inconsistent with proper telomerase assembly. Furthermore, we show that the limited amount of telomerase that can assemble in the absence of TCAB1 is fully active and can localize to telomeres, suggesting that TCAB1 is not required for the enzymatic function of telomerase or its recruitment to telomeres. In addition, we demonstrate that the diffusion properties of TERT in cells that lack TCAB1 closely

resemble those of TERT that is not bound to TR. We conclude that the sequestration of TR in the nucleolus, when TCAB1 is absent, prevents its association with TERT, and that this lack of telomerase assembly is the molecular mechanism underlying the critical role of TCAB1 in telomere maintenance. Our results also demonstrate that the nucleolar phase separation constitutes a barrier for telomerase assembly and suggest that incompletely assembled RNPs are tightly associated with the nucleolus and do not readily enter the nucleoplasm.

Results

Loss of TCAB1 leads to nucleolar accumulation of TR

To confirm that TR is sequestered in the nucleolus in the absence of TCAB1, we attempted to knock-out TCAB1 in parental HeLa cells and HeLa cells expressing 3xFLAG-HaloTag-TERT and mEos3.2-TRF2 from the endogenous *TERT* and *TRF2* loci (referred to as Halo-TERT from here on) using Cas9 and a single guide RNA targeting the first exon of the *TCAB1* locus (Figure 1 – figure supplement 1). We isolated clonal cell lines that lacked a wild-type *TCAB1* allele (Figure 1 – figure supplement 1) and analyzed TCAB1 expression using Western blot. While a TCAB1 antibody targeting the N-terminus of TCAB1 failed to detect TCAB1 in our knock-out clones (Figure 1 – figure supplement 1), antibodies raised against more C-terminal TCAB1 fragments detected a truncated form of TCAB1 (Figure 1 – figure supplement 1). The size of the truncated TCAB1 is consistent with the use of methionine 129 as an alternative start codon. Importantly, the truncated form of TCAB1 localizes to Cajal bodies (Figure 1 – figure supplement 1) and scaRNA localization is unaffected in its presence (Figure 1 – figure supplement 1), indicating that it is at least partially functional. To avoid expression of the truncated TCAB1 variant, we knocked out TCAB1 using Cas9 with two guide RNAs to delete exons 2 and 3 from the *TCAB1* gene, which removes the coding sequence for residues 144-214 of TCAB1 and results in a frame shift (Figure 1 – figure supplement 2). TCAB1 knock-out was validated by Southern blot, PCR, Western blot, and immunofluorescence imaging (IF, Fig. 1A-C, Figure 1 – figure supplement 2). Fluorescence *in situ* hybridization (FISH) demonstrated that TR accumulates in the nucleolus in cells that lack TCAB1, as indicated by co-localization of TR and nucleolar dyskerin signals. Importantly, expression of GFP-

TCAB1 in TCAB1 knock-out cells rescued TR localization to Cajal bodies (Figure 1 – figure supplement 2), confirming that the miss-localization of TR to nucleoli is caused by absence of TCAB1. These observations confirm that TCAB1 is required to prevent TR accumulation in the nucleolus (Fig. 1C).

Figure 1. TR is localized to nucleoli in TCAB1 knock-out cells. (A-B) Western blot demonstrating the absence of TCAB1 protein in TCAB1 knock-out cell lines generated from (A) HeLa and (B) Halo-TERT parental cell lines (probed with Proteintech TCAB1 antibody). (C) Immunofluorescence with anti-dyskerin and anti-TCAB1 antibodies coupled to fluorescence in-situ hybridization with probes against TR, demonstrating the absence of TCAB1 and TR localization to nucleoli in TCAB1 knock-out cells (scale bar = 5 μ m).

TERT is excluded from nucleoli

Our previous observations demonstrated that TERT does not enter nucleoli in human cancer cells (Schmidt et al., 2016). To confirm these results, we performed single-

molecule imaging of 3xFLAG-HaloTag-TERT in living HeLa cells. Consistent with our previous results, 3xFLAG-HaloTag-TERT was not observed entering or overlapping with nucleoli, which are discernible as circular shapes in the nucleus under transmitted light

illumination (Fig. 2A,B, Figure 2 – figure supplement 1, Movie 1,2). TERT trajectories in close proximity to nucleoli revealed that their movement is biased away from the nucleolus, suggesting that TERT is repelled by the nucleolus (Fig. 2B). To exclude the possibility that nucleolar exclusion is a consequence of the 3xFLAG-HaloTag on the N-terminus of TERT used in our experiments, we transiently expressed the 3xFLAG-HaloTag fused to a nuclear localization sequence (NLS) in HeLa cells. Single-molecule imaging demonstrated that the nuclear 3xFLAG-HaloTag signals overlapped with the nucleolus (Figure 2 – figure supplement 1, Movie 3). Similar to the 3xFLAG-HaloTag alone, 3xFLAG-HaloTag-dyskerin also localized to the nucleolus (Figure 2 – figure supplement 1). These results demonstrate that 3xFLAG-HaloTag-TERT is excluded from the nucleolus and that this exclusion is not caused by the 3xFLAG-HaloTag but instead is an intrinsic property of the TERT protein.

TERT and TR localize to distinct nuclear compartments in cells lacking TCAB1

It is well established that telomerase assembly is incomplete in human cancer cells, which leads to substantial pools of TERT and TR that are not assembled into telomerase RNPs (Xi and Cech, 2014). Together with our observation that TERT molecules rarely enter the nucleolus (Schmidt et al., 2016) this suggests that both free TERT and assembled telomerase RNPs do not enter the nucleolus. To test whether, like TR, TERT accumulates in the nucleolus in cells lacking TCAB1, we carried out single-molecule imaging of 3xFLAG-HaloTag-TERT. Strikingly, TERT localization is unchanged in cells lacking TCAB1 (Fig. 2A,B). This suggests that unlike TR, TERT does not accumulate in nucleoli when TCAB1 is absent.

The experiments described so far demonstrate that TCAB1 is enriched in nucleoli and TERT is excluded from nucleoli in cells lacking TCAB1, but they do not simultaneously detect TERT and TR in the same cell. To overcome this limitation, we over-expressed mCherry-TERT and TR in TCAB1 knock-out cells. In controls, TERT and TR colocalized with dyskerin at telomeres marked by TRF2 (Fig. 3A). In addition, analysis of the TERT signal across the nucleus and nucleolus revealed that TERT was depleted from the nucleolus (Fig. 3B). Importantly, mCherry-dyskerin localized to the nucleolus, demonstrating that the mCherry-tag does not lead to nucleolar exclusion of its fusion partner (Figure 3 – figure supplement 1). In cells lacking TCAB1, mCherry-TERT was diffusely localized in the nucleoplasm, localized to a subset of telomeres, and was depleted from the nucleolus (Fig. 3A,C). Similar to endogenous TR, overexpressed TR

was enriched in the nucleolus in TCAB1 knock-out cells (Fig. 3A). In addition, TR was frequently co-localized

Figure 3. TERT and TR localize to distinct nuclear compartments in TCAB1 knock-out cells. (A) IF-FISH images of control and TCAB1 knock-out HeLa cells (3xFLAG-Halo-TERT, mEOS3.2-TRF2) overexpressing mCherry-TERT and TR (scale bar = 5 μ m). Cells were probed with antibodies against dyskerin and mCherry, and FISH probes specific to TR. The intrinsic fluorescence of mEOS3.2-TRF2 was used to detect telomeres. TR and TERT are co-localized at telomeres in control cells. In TCAB1 knock-out cells, TR is enriched in nucleoli, while TERT is depleted from nucleoli in both control and TCAB1 knock-out cells. Both TERT and TR also localize to telomeres in TCAB1 knock-out cells. **(B-C)** Line scans of **(B)** control and **(C)** TCAB1 knock-out cells along the dashed white lines in Fig. 3A, demonstrating the enrichment of TR (green) in nucleoli (blue) in TCAB1 knock-out cells and the depletion of TERT (magenta) from nucleoli in both control and TCAB1 knock-out cells. **(D)** Quantification of the fraction of cells showing TR localization exclusively to telomeres, to telomeres and nucleoli, or only to nucleoli in cells over-expressing mCherry-TERT and TR (3 independent experiments, >100 cells per experiments, mean $\pm$ standard deviation).

with telomeres when mCherry-TERT and TR were overexpressed in cells that lack TCAB1 (Fig. 3A,C-D). Similar TR localization patterns were found when overexpressing

untagged TERT and TR (Figure 3 – figure supplement 1) and untagged TERT was excluded from nucleoli, confirming that nucleolar exclusion is an intrinsic property of TERT (Figure 3 – figure supplement 1). Together these results demonstrate that, when overexpressed in TCAB1 knock-out cells, TR is enriched in nucleoli and TERT is depleted from nucleoli, consistent with a failure of nucleolar TR to assemble with TERT. In addition, our observations suggest that overexpression can partially overcome the sequestration of TR in the nucleolus when TCAB1 is absent, allowing a fraction of TR to bind to TERT and localize to telomeres.

TCAB1 promotes telomerase RNP assembly

Previous studies by other laboratories have concluded that telomerase assembly is unaffected by the absence of TCAB1 and whether TCAB1 is required for telomerase activity is controversial (Chen et al., 2018; Venteicher et al., 2009; Vogan et al., 2016). To assess the role of TCAB1 in telomerase assembly, we overexpressed TERT and TR in parental HeLa and TCAB1 knock-out cells and immuno-purified TERT using a well-established anti-TERT antibody (Cohen et al., 2007). To quantify telomerase assembly, we measured TERT levels by Western blot and determined the amount of TR co-purified using Northern blot (Fig. 4A,B). The amount of TR associated with TERT purified from TCAB1 knock-out cells was significantly reduced compared to parental cells, for both HeLa ($31 \pm 9\%$, mean $\pm$ SEM, $p = 0.001$) and Halo-TERT ($33 \pm 4\%$, mean $\pm$ SEM, $p = 0.001$) cell lines (Fig. 4D). As a second measure of telomerase RNP assembly we quantified the amount of dyskerin associated with TERT (Fig. 4C). Since TR bridges TERT and dyskerin, dyskerin co-purified with TERT directly reports on the presence of

TR. Consistent with the reduction in TR, the amount of dyskerin bound to TERT was also significantly reduced when purified from cells lacking TCAB1 compared to parental controls for both HeLa ($22 \pm 8\%$, mean $\pm$ SEM, $p = 0.002$) and Halo-TERT ($32 \pm 9\%$, mean $\pm$ SEM, $p = 0.004$) cell lines (Fig. 4C,E). Importantly, we also confirmed that TCAB1

Figure 4. Telomerase Assembly is reduced in the absence of TCAB1. (A) Western blots analyzing TERT immunoprecipitation (using a sheep anti-TERT antibody) probed with a rabbit anti-TERT antibody (Abcam) and a TCAB1 antibody. (B) Northern blot of RNA extracted from input and purified TERT samples probed with three radiolabeled DNA oligonucleotides complementary to TR. Standards are *in vitro* transcribed full-length TR and truncated TR₂₈₋₃₂₄. TR₂₈₋₃₂₄ was added to samples prior to RNA extraction as loading and recovery control. (C) Western blots to analyze immunopurified telomerase RNP composition. A single membrane was cut into two pieces that were probed with TERT and dyskerin antibodies, respectively. (D-F) Quantification of the amount of (D) TR, (E) dyskerin, and (F) the ratio of TR to TERT in TERT purifications from TCAB1 knock-out cells compared to parental controls (n = 4-5, mean). The dashed lines indicate the level in telomerase purified from wild-type TCAB1 control cells which was normalized to 1.0.

is absent from telomerase purified from TCAB1 knock-out cells (Fig. 4A). As a final measure of telomerase assembly, we determined the ratio between TR and TERT (Fig. 4A,B,F), which was also significantly reduced when TERT was purified from TCAB1 knock-out cells (HeLa: $18 \pm 3\%$, mean $\pm$ SEM, $p < 0.001$, Halo-TERT: $17 \pm 5\%$, mean $\pm$ SEM, $p < 0.001$). Altogether, these results demonstrate that telomerase assembly is

significantly reduced in the absence of TCAB1 and that overexpression of TERT and TR is not sufficient to overcome this defect in telomerase RNP formation.

TCAB1 is not required for telomerase catalytic activity

Previous work by others has suggested that the defects in telomere maintenance

Figure 5. The specific activity of telomerase is unchanged in the absence of TCAB1. (A-B) Direct telomerase extension assay of telomerase immuno-purified from parental (WT) and TCAB1 knock-out (TKO) (A) HeLa and (B) Halo-TERT cell lines. LC1 and LC2, radiolabeled DNA oligonucleotide loading controls. In -Ab samples the TERT antibody was omitted during the immuno-purification. (C) Quantification of telomerase activity in samples from TCAB1 knock-out cells relative to parental controls (n = 4, mean). (D) Specific activity of telomerase purified from TCAB1 knock-out cells relative to parental controls (n = 4, mean). Specific activity was calculated by dividing the relative activity (see Fig. 5C) by the relative amount of TR present in immuno-purified TERT samples (see Fig. 4D). The dashed lines indicate the activity level in telomerase purified from wild-type TCAB1 control cells which was normalized to 1.0.

observed in TCAB1 knock-out cells are the consequence of reduced telomerase activity caused by the miss-folding of the telomerase RNA rather than impaired telomerase assembly (Chen et al., 2018). This TR folding model predicts that the specific activity of telomerase is reduced. To determine the specific activity of telomerase purified from TCAB1 knock-out cells, we measured telomerase activity using the direct telomerase extension assays (Fig. 5A,B, Figure 5 – figure supplement 1), and divided the activity by the amount of TR in the respective telomerase sample (Fig. 4B,D), which is a direct measure of the quantity of telomerase RNP present. Total activity of telomerase purified from TCAB1 knock-out cells was significantly reduced relative to telomerase purified from parental controls telomerase (Fig. 5C, HeLa: $24 \pm 6\%$, mean $\pm$ SEM, $p < 0.001$, Halo-TERT: $34 \pm 9\%$, mean $\pm$ SEM, $p = 0.005$), consistent with observations by others (Chen et al., 2018). In contrast, the specific activity of telomerase derived from cells that lack TCAB1 was not significantly reduced compared to controls (Fig. 5D, HeLa: $90 \pm 9\%$, mean $\pm$ SEM, $p = 0.35$, Halo-TERT: $76 \pm 10\%$, mean $\pm$ SEM, $p = 0.1$). Together these observations suggest that TCAB1 is required for telomerase assembly and that the limited amount of telomerase RNP that can form in the absence of TCAB1 is fully active.

TCAB1 is required for telomerase assembly in living cells

The experiments presented thus far demonstrate that telomerase assembly is significantly reduced in the absence of TCAB1 but were carried out in fixed cells or cell lysates. To analyze telomerase assembly in intact cells, we carried out live cell single-molecule imaging of 3xFLAG-HaloTag-TERT and determined the diffusion coefficient of TERT particles (Fig. 6A, Movie 4, Figure 6 – figure supplement 1). The diffusion co-

efficient is a measure of the rate of movement of a molecule and depends on the size of the complex it is part of and reports on molecular interactions formed with other sub-cellular structures. Analysis of the diffusion coefficients of TERT trajectories in control cells revealed three distinct populations of TERT particles (Fig. 6B-D). A static population ($D_S = 0.03 \mu\text{m}^2/\text{s}$, 12%) which likely represents assembled telomerase RNPs bound to telomeres or Cajal bodies, a slowly diffusion population ($D_{F1} = 0.35 \mu\text{m}^2/\text{s}$, 28%) and a rapidly diffusing population ($D_{F2} = 1.54 \mu\text{m}^2/\text{s}$, 60%). The slowly diffusing population likely includes assembled telomerase RNPs, while the rapidly diffusing particles represents TERT molecules, which are not assembled with TR (Fig. 6B). Importantly, these diffusion coefficients are largely consistent with our previous results using a distinct method to determine their values (Schmidt et al., 2016). In the absence of TCAB1, the diffusion coefficient of the freely diffusing TERT population was increased ($D_{F2} = 1.78 \pm 0.04 \mu\text{m}^2/\text{s}$, mean $\pm$ SEM, $p = 0.04$, Fig. 6C) and the fraction of the TERT populations that includes assembled telomerase RNPs was significantly reduced ($F_{\text{Slow+Static}} = 29 \pm 1\%$, mean $\pm$ SEM, $p = 0.02$, Fig. 6D). This observation is consistent with our model that in the absence of TCAB1, telomerase assembly is defective. To determine the degree to which telomerase assembly is affected in cells lacking TCAB1, we knocked out TR, completely abolishing telomerase assembly. TR knock-out was confirmed by PCR and Sanger sequencing, FISH, and qPCR (Figure 6 – figure supplement 1). Similar to control cells and TCAB1 knock-out cells, TERT was also excluded from nucleoli in cells lacking TR (Fig. S6D). Strikingly, the diffusion coefficients and the fraction of slow and static TERT particles in cells lacking TR closely resembled those of TCAB1 knockout cells (Fig. 6A-D, Figure 6 – figure supplement 1, Movie 4-5). To analyze the

Figure 6. Telomerase assembly is reduced in TCAB1 knock-out cells. (A) TERT particle trajectories from control, TCAB1 knock-out, and TR knock-out cells expressing 3xFLAG-HaloTag TERT (JF646, scale bar = 2 μm). **(B)** Diagram of distinct populations of TERT particles detected in control cells. **(C)** Diffusion coefficient of the rapidly diffusing TERT population in control, TCAB1 knock-out, and TR knock-out cells (3 independent experiments, >15 cells per experiment per cell line, mean $\pm$ standard deviation, complete data in Fig. S6D). **(D)** Fraction of slow plus static TERT particles in control, TCAB1 knock-out, and TR knock-out cells expressing 3xFLAG-HaloTag TERT (3 independent experiments, >15 cells per experiment per cell line, mean $\pm$ standard deviation, complete data in Fig. S6D). **(E)** Model for the regulation of telomerase assembly by TCAB1. In the absence of TCAB1, TR is sequestered in the dense fibrillar component (DFC) of the nucleolus, which is separated from the nucleoplasm by the granular component (GC) of the nucleolus.

interaction of TERT with telomeres, we filtered out TERT trajectories that came into proximity with telomeres marked by mEOS3.2-TRF2, as previously described (Schmidt et al., 2016). To assess the interaction of TERT with telomeres, we plotted the step-size vs. the distance from the closest telomere for each step of these trajectories (Figure 6 – figure supplement 2). In control cells, we observed an enrichment of smaller step sizes and particles in close proximity to telomeres, consistent with TERT interactions with the telomere (Figure 6 – figure supplement 2). In contrast, TERT trajectories from TCAB1 knock-out cells lacked this enrichment, and the step size vs. distance from the closest telomere plots were identical to those from TR knock-out cells (Figure 6 – figure supplement 2). In addition, diffusion analysis using SpotOn revealed that the fraction of static TERT particles at telomeres was reduced from 12% in control cells to 4-5% in TCAB1 and TR knock-out cells (Figure 6 – figure supplement 2). These observations indicate that in the absence of either TCAB1 or TR, stable interactions of telomerase with telomeres occur at a lower frequency because they require base pairing of TR with the chromosome end (Schmidt et al., 2018). Together these results demonstrate that telomerase assembly is strongly reduced in the absence of TCAB1.

Discussion

The experiments described in this study demonstrate that TCAB1 promotes telomerase assembly. In the absence of TCAB1 the telomerase RNA is targeted to the nucleolus via its association with dyskerin and other components of the H/ACA complex. In contrast to TR, TERT cannot enter the nucleolus, preventing its association with TR in cells that lack TCAB1. This demonstrates that nucleolar phase separation counteracts telomerase assembly. In addition, we demonstrate that sequestration of TR in the phase-separated nucleolus in the absence of TCAB1 can be partially overcome by telomerase overexpression, leading to telomerase assembly and localization to telomeres. This suggests that the nucleolus has a limited capacity to accommodate cellular RNAs and that TCAB1 is not required for telomerase recruitment to telomeres. Finally, we demonstrate that while telomerase assembly is limited the specific activity of telomerase is unchanged in the absence of TCAB1, which excludes a role of TCAB1 in telomerase catalytic function. Altogether our work completely reshapes our understanding of the role of TCAB1 in telomerase function in human cells and provides insight into the role phase-separated organelles play in RNP assembly and function.

TCAB1 promotes telomerase assembly

The importance of TCAB1 for telomere maintenance is undisputed (Chen et al., 2018; Venteicher et al., 2009). Knock-out or depletion of TCAB1 results in telomere shortening (Chen et al., 2018; Venteicher et al., 2009; Vogan et al., 2016). All previous work also concluded that TR is enriched in the nucleolus in the absence of TCAB1 (Chen et al., 2018; Stern et al., 2012; Venteicher et al., 2009; Vogan et al., 2016; Zhong et al., 2011).

Finally, all prior studies propose that TCAB1 is not required for telomerase assembly but instead plays a role telomerase trafficking to Cajal bodies and telomeres or is required for telomerase catalysis (Chen et al., 2018; Stern et al., 2012; Venteicher et al., 2009; Vogan et al., 2016; Zhong et al., 2011). In contrast, our results demonstrate that in the absence of TCAB1, TERT and TR are localized to the distinct sub-cellular compartments, the nucleoplasm and the nucleolus, respectively. This spatial separation strongly reduces telomerase assembly, which leads to reduced number of telomerase RNPs per cell and in turn telomere shortening. Our conclusions are based on a variety of quantitative approaches analyzing telomerase assembly by purification of the telomerase RNP and in intact cells. Analysis of TERT localization in living and fixed cells clearly demonstrates that TERT is excluded from the nucleolus. Importantly, we confirmed that the nucleolar exclusion of TERT is not a consequence of the protein-tags used to visualize TERT in these experiments. Purification of telomerase from TCAB1 knock-out cells further confirmed that TCAB1 is required for telomerase assembly *in vivo*. The amount of TR and dyskerin associated with TERT is substantially reduced in the absence of TCAB1. Finally, we used live cell single-molecule imaging to demonstrate that the diffusion pattern of TERT molecules and their interactions with telomeres are significantly different in TCAB1 knock-out cells compared to control cells. More importantly, TERT dynamics in the absence of TCAB1 are comparable to those in TR knock-out cells where telomerase assembly is impossible. Altogether, these observations support our model that TCAB1 promotes telomerase assembly by extracting TR from the nucleolus to facilitate its assembly with TERT. Our model is further supported by work from Vogan *et al.*, which demonstrated that truncated TR (hTRmin) that lacks the H/ACA region and therefore

cannot bind dyskerin, accumulates in the nucleoplasm and is excluded from nucleoli (Vogan et al., 2016). Importantly, in cells that express hTRmin, TCAB1 is not required for telomere maintenance (Vogan et al., 2016), consistent with TCAB1 promoting telomerase assembly by extracting TR from the nucleolus.

TCAB1 is not required for telomerase catalysis

Previous work by others has reported conflicting results regarding the role of TCAB1 in telomerase catalysis, ranging from full enzymatic activity in initial reports to substantial activity defects in the most recent study (Chen et al., 2018; Venteicher et al., 2009; Vogan et al., 2016). Importantly, both our work and the only other study that analyzed the role of TCAB1 in telomerase activity using the “gold-standard” direct telomerase extension assay concluded that telomerase activity is reduced to approximately 20-30% in the absence of TCAB1. In both cases, telomerase was purified from TCAB1 knock-out cells using an antibody raised against TERT prior to measuring telomerase activity. While both studies concur on the degree to which telomerase activity is reduced in the absence of TCAB1, the proposed underlying molecular mechanisms differ. Chen *et al.* propose that TCAB1 is required for proper folding of the CR4/CR5 region of the telomerase RNA, which directly associates with TERT, without affecting telomerase assembly (Chen et al., 2018). Recent structural analysis of the telomerase RNP from human cells revealed that TCAB1 is located far away from the CR4/CR5 region of TR (Figure 6 – figure supplement 3)(Ghanim et al., 2021). TCAB1 resides within the H/ACA lobe of while CR4/CR5 is located within the catalytic lobe of the telomerase RNP. The two lobes are connected by the P1 and P4.2 helices which can adopt multiple conformations. Importantly, TCAB1 was not located

near the CR4/CR5 region of TR in any of the observed telomerase RNP conformations (Ghanim et al., 2021). Although it is possible that telomerase can adopt additional conformations, based on the currently available structural information it is difficult to rationalize a molecular mechanism by which TCAB1 could specifically promote CR4/CR5 folding. In addition, due to the miss-folding of TR telomerase was proposed to adopt a low activity state in the absence of TCAB1. Experimentally such a low activity state would manifest itself as a reduction in the specific activity of telomerase (telomerase activity per assembled telomerase RNP). Our experiments strongly suggest that, while telomerase assembly is reduced in the absence of TCAB1, the limited amount of telomerase that can assemble is fully active (i.e. does not have reduced specific activity). Both, our enzymatic analysis and the positioning of TCAB1 within the telomerase RNP do not support a role of TCAB1 in TR folding and telomerase catalysis but are fully consistent with TCAB1 promoting telomerase assembly.

TCAB1 is not necessary for telomerase recruitment to telomeres

TCAB1 is necessary for the localization of scaRNAs to Cajal bodies and previous work suggested that it is also required for telomerase recruitment to telomeres (Stern et al., 2012; Venteicher et al., 2009). Our observations demonstrate that when TERT and TR are overexpressed in TCAB1 knockout cells a fraction of TERT can assemble with TR and localize to telomeres. Telomerase recruitment to telomeres requires a direct interaction between TERT and TPP1 (Nandakumar et al., 2012; Schmidt et al., 2014; Zhong et al., 2012), therefore TR that localizes to telomeres must be assembled with TERT. These results are fully consistent with our model that the spatial separation of TR and TERT in

the absence of TCAB1 prevents telomerase assembly. When TR is overexpressed the capacity of the nucleolus to sequester TR may be saturated and excess TR can assemble with TERT and localize to telomeres. Importantly, our observations also suggests that TCAB1 is not required for telomerase localization to telomeres.

Molecular mechanism of telomerase RNP assembly

The results presented in this study demonstrate that, in the absence of TCAB1, telomerase assembly is reduced but not completely abolished. It is likely that TR bound by the H/ACA complex is in an equilibrium between localizing to nucleoplasm and the nucleolus. In the presence of TCAB1, this equilibrium is shifted towards the nucleoplasm allowing TR to associate with TERT (Fig. 6E). In contrast, in the absence of TCAB1, the majority of TR is trapped in the nucleolus effectively reducing the amount of TR that is available for assembly with TERT. In addition, the nucleolus disassembles during mitosis and does not reform until early G1-phase of the cell cycle (Hernandez-Verdun, 2014). In cells lacking TCAB1, nucleolar disassembly should lead to the release of TR, which could transiently allow telomerase assembly during cell division or prior to reformation of the nucleolus in G1-phase. Both, a reduction of TR available for assembly and transient assembly during cell division are plausible models to explain the limited amount of telomerase that can form in the absence of TCAB1.

The sub-cellular location and order in which telomerase RNP components associate with TR in human cells is largely unknown (Fig. 6E). Our results demonstrate that telomerase does not assemble in the nucleolus. In addition, it is unlikely that telomerase assembly requires intact Cajal bodies. Multiple studies have shown that

knock-out of coilin, which eliminates Cajal bodies, does not impact telomerase activity or telomere maintenance (Chen et al., 2018; Vogan et al., 2016). Our single-molecule live cell imaging of TERT has demonstrated that TERT is almost exclusively localized to the nucleus, it is therefore likely that human telomerase assembles in the nucleoplasm. But it cannot be ruled out that in human cells TR is transiently exported to the cytoplasm to assemble with nascent TERT protein, as is the case in *S. cerevisiae* (Gallardo et al., 2008).

The H/ACA complex has been shown to associate with scaRNAs at the site of their transcription (Darzacq et al., 2006). While co-transcriptional association of the H/ACA complex with TR has not been demonstrated, binding of the H/ACA complex proteins to TR is likely the first step in telomerase RNP assembly, because of their critical role in TR stability and 3'-end formation (Stuart et al., 2015; Tummala et al., 2015). Once TR is bound to dyskerin, TR will be targeted to the nucleolus, unless it associates with TCAB1 (Fig. 6E). Like TERT, TCAB1 is excluded from nucleoli (Fig. 1, Fig. S2D), and dyskerin and TR are localized to the dense fibrillar component (DFC, Fig. 6E) of the nucleolus (Yao et al., 2019), which is separated from the nucleoplasm by the granular component (GC, Fig. 6E) (Lafontaine et al., 2021). Thus, it is unlikely that TCAB1 can bind to TR localized to the DFC of the nucleolus. We therefore believe that TCAB1 association with TR prevents the entry of TR into the nucleolus rather than extracting TR which is already localized to the DFC of the nucleolus. The association of TR with either TERT or TCAB1 should prevent its sequestration in the nucleolus, but the order in which TERT and TCAB1 bind to TR cannot be inferred from our experiments, or observation by others. Since

TCAB1 is substantially more abundant than TERT it is likely that TCAB1 encounters TR before TERT does.

Regulation of RNP assembly by nucleolar phase-separation

In addition to the mechanistic insight into the role of TCAB1 in telomerase function, our results also demonstrate that nucleolar phase separation can effectively regulate telomerase RNP assembly in the nucleus of human cells. How RNA molecules are specifically recruited into, excluded from, or expelled from non-membrane bound organelles is a key unanswered question. One model suggests that gradual replacement of non-specific, multivalent interactions of pre-ribosomal RNAs with nucleolar proteins such as NPM1 and fibrillarin, with specific, high-affinity interactions with ribosomal proteins leads to the ejection of mature ribosomal subunits from the nucleolus (Riback et al., 2020). In this model a key driving force for the retention of RNA in the nucleolus is regions of RNA not yet bound by ribosomal proteins, that are available to interact with nucleolar proteins (Riback et al., 2020). By analogy, this model would explain why TR bound by the H/ACA complex but not TERT would be sequestered in the nucleolus. In addition to the interactions formed by the H/ACA complex with nucleolar proteins and RNA, the regions of TR that are bound by TERT in the context of telomerase (i.e. the pseudoknot, template, and CR4/CR5) would be available to form non-specific, multivalent interactions with nucleolar proteins to strengthen the association of TR with the nucleolus and prevent its release.

TR is a unique among the scaRNAs because it contains the additional domains that associate with TERT. Most other box H/ACA scaRNAs are substantially shorter (<150

nucleotides) than TR (451 nucleotides), and do not contain large regions that are not bound by proteins and could form non-specific interactions with nucleolar proteins. It is therefore possible, that in cells lacking TCAB1, TR is strongly retained in the nucleolus while other scaRNAs are less tightly bound, because they lack additional interaction sites with nucleolar proteins. This provides a potential explanation for the phenotypes observed in patients with TCAB1 mutations that suffer from dyskeratosis congenita. The patients have a clear deficiency in telomerase function but no defects in splicing have been reported (Zhong et al., 2011), which would be the consequence of complete loss of scaRNA function and their critical role in spliceosome maturation.

How TCAB1 binding leads to the exclusion of TR and other scaRNA from the nucleolus remains a key unanswered question. TCAB1 interacts with a very short sequence motif in TR, which is far removed from the TR regions that associate with TERT (Fig. S8) (Ghanim et al., 2021). It is therefore unlikely that TCAB1 binding leads to expulsion of TR from the nucleolus by reducing the number of non-specific, multivalent interactions TR can form with nucleolar proteins. As outlined above, we believe that TCAB1 prevents localization of scaRNAs to the nucleolus, rather than extracting scaRNAs that are already localized to the DFC. One potential explanation is that TCAB1 counteracts scaRNA recruitment to the nucleolus by inhibiting the nucleolar localization signals within dyskerin (Heiss et al., 1999). Dissecting the molecular mechanism by which TCAB1 leads to exclusion of TR from the nucleolus in future studies will undoubtedly shed light on the fundamental principles RNP recruitment to non-membrane bound organelles and its physiological role in cell biology.

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Author contributions

B.S.A.-M. carried out IF-FISH experiments, telomerase purifications, analyzed telomerase assembly, generated mCherry- and 3xFLAG-HaloTag-dyskerin plasmids, determined their sub-cellular localization, and edited the manuscript. G.I.P. maintained cell lines, established TCAB1 and TR knockout cell lines and carried out IF-FISH experiments. K.A.-B. assisted in establishing the TR knockout cell line and carried out characterization of the TR knockout cells. S.B.C. purified and characterized the anti-TERT sheep antibody. L.H. and K.Y. characterized TCAB1 knock-out clones using Southern blots. J.C.S. carried out all other experiments, designed the research, analyzed data, and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Materials and Methods

Cell Lines and Tissue Culture

All cell lines were based on HeLa-EM2-11ht (Weidenfeld et al., 2009) and were cultured in Dulbecco's Modified Eagle Medium including L-glutamine (Gibco) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO₂. Live cell imaging was carried out using CO₂ independent media supplemented with 2 mM GlutaMAX (Life Technologies), 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO₂. For single-molecule imaging of HaloTag-TERT cell were cultured in homemade imaging dishes made by gluing 22x22 mm Nexterion coverslips (170 ± 5 µm, Schott) onto the bottom of plastic 3.5 x 1.0 cm cell culture dishes with a hole in the middle using an epoxy adhesive. Prior to chamber assembly the coverslips were washed with 1 M KOH and 100% for 30 min each in a sonicating water bath. To enrich for cells in S-phase for live cell imaging experiments, cultures we treated with complete media including 2 mM thymidine for a minimum of 16 hours. Cells were released 2 hours prior to imaging by replacing the thymidine containing media with fresh media without thymidine. Puromycin selection was carried out at a concentration of 1 µg/ml.

Plasmid Construction and Genome Editing

All plasmids were generated by Gibson assembly (NEB) using standard protocols or by inverse PCR. All plasmids will be made available on Addgene. All Cas9 and sgRNA expression plasmids were based on pX330 (Cong et al., 2013). The homologous recombination donor for the TR knockout was generated by assembling the genomic

sequences immediately upstream and downstream (~500 bp each) of the TR sequence flanking a puromycin resistance cassette into HpaI linearized pFastBac. The 3xFLAG-HaloTag-NLS plasmids was generated by adding a 3xFLAG-tag to a previously described HaloTag-NLS plasmid (a kind gift from X. Darzacq and A. Hansen) (Hansen et al., 2018). The 3xFLAG-HaloTag-dyskerin plasmid was generated by replacing TERT in our previously described 3xFLAG-HaloTag-TERT expression plasmid with the dyskerin coding sequence (Schmidt et al., 2016). The mCherry-dyskerin plasmid was generated by replacing TERT in our previously described mCherry-TERT expression plasmid with the dyskerin coding sequence (Schmidt et al., 2014). All transfections were carried out using Lipfectamine 2000 (Invitrogen) using the manufacturer's instructions. TCAB1 was knocked-out using a single sgRNA or two separate sgRNA and Cas9 encoding plasmids that were transfected alongside a GFP-expression plasmid. 24 hours after transfection single-cell clones were sorted using the GFP signal. TCAB1 knock-out clones were screened by PCR and confirmed by western blot, Southern Blotting of the *TCAB1* locus and immunofluorescence imaging. TR was knocked out by transfecting two sgRNA plasmids and a homologous recombination donor plasmid. 48 hours after transfection puromycin selection was initiated and 1 week after the initiation of selection single-cell clones were generated by dilution into 96-well plates. TR knock-out was confirmed using PCR and Sanger sequencing, fluorescence *in situ* hybridization, and RT-qPCR.

Immunofluorescence and Fluorescence *In Situ* Hybridization Imaging

Fixed cell immunofluorescence imaging and fluorescence *in situ* hybridization was carried out as previously described (Schmidt et al., 2014). Briefly, cells grown on coverslips were

539 fixed in PBS supplemented with 4% formaldehyde. When using the HaloTag for
540 fluorescence detection cells were incubated with 100 nM of JF646 HaloTag-ligand for 30
541 min prior to fixation. Unincorporated ligand was removed by 3 washes with complete
542 media followed by placing the cells back in the incubator for 10 min to let additional dye
543 leak out of the cells. mEOS3.2-TRF2 was detected using the intrinsic fluorescence of
544 green form of mEOS3.2. After removing the fixation solution using 2 PBS washes,
545 coverslips were transferred into aluminum foil covered humidity chambers with a parafilm
546 layer and rinsed with 1 ml of PBS with 0.2% Triton X-100. Cells were then incubated in
547 blocking buffer (PBS, 0.2% Triton X-100, 3% BSA) for 30 minutes, followed by incubation
548 with primary antibodies diluted in blocking buffer for 1 hour. All primary antibodies (see
549 key resource table) were used at a concentration of 1 µg/ml. After three washes with PBS
550 + 0.2% Triton X-100, coverslips were incubated with secondary antibodies diluted in PBS
551 + 0.2% Triton X-100 for 1 hour. All secondary antibodies (see key resource table) were
552 used at a concentration of 4 µg/ml. Cells were washed three times PBS + 0.2% Triton X-
553 100 prior to a second fixation with PBS + 4% formaldehyde. In cases where nuclear
554 staining was used the first of the three washing steps also included 0.1 µg/ml HOECHST.
555 After the second fixation steps coverslips were dehydrates in three steps with ethanol
556 (70%, 95%, 100%), re-hydrated in 2xSSC + 50% formamide, blocked for 1 hour in
557 hybridization buffer (100 mg/ml dextran sulfate, 0.125 mg/ml *E. coli* tRNA, 1 mg/ml
558 nuclease free BSA, 0.5 mg/ml salmon sperm DNA, 1 mM vanadyl ribonucleoside
559 complexes, 50% formamide, 2xSSC) at 37°C, before incubating the coverslips in
560 hybridization buffer supplemented with three TR probes (30 ng per coverslip) over night
561 at 37°C. Probe sequences were previously described (Tomlinson et al., 2006). After

hybridization coverslips were washed twice for 30 minutes in 2xSSC + 50% formamide and then mounted on slides using ProLong Antifade Diamond mounting media (Life Technologies). Microscopy was carried out using a DeltaVision Elite microscope using a 60x PlanApo objective (1.42 NA) and a pco.edge sCMOS camera. We acquired 20 Z-sections spaced by 0.2 μ m, followed by image deconvolution and maximum intensity projection of the sections using the DeltaVision Softworx software.

Single-Molecule Live Cell Imaging

Live cell single-molecule imaging was carried out on a Olympus IX83 inverted microscope equipped with a 4-line cellTIRF illuminator (405 nm, 488 nm, 561 nm, 640 nm lasers), a Olympus UAPO 100x TIRF objective (1.49 NA), a CAIRN TwinCam beamsplitter, 2 Andor iXon 897 Ultra EMCCD cameras, and a blacked-out environmental control enclosure. The microscope was operated using the Olympus cellSense software. 3xFLAG-HaloTag-TERT was labeled for 2 min in complete media supplemented with 100 nM JF646-HaloTag ligand (Grimm et al., 2015). After removing the HaloTag-ligand with three washes in complete media, cells were placed back in the incubator for 10 min to allow unincorporated dye to leak out of the cells. Cells were then transferred into CO₂ independent media and put on the microscope which was heated to 37°C. Single-molecule imaging was carried out at 50 or 100 frames per second using highly inclined laminated optical sheet illumination (Tokunaga et al., 2008). Movies were typically 20 seconds in length (2000 frames) and were followed by a transmitted light acquisition to visualize overall cell morphology.

RT-qPCR

RNA samples for RT-qPCR analysis were generated by using RNA mini prep kits (Zymogen) using ~2 million cells as starting material. Reverse transcription was carried out using random hexamer primers and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. qPCR was carried out using the Maxima SYBR Green qPCR master mix (Thermo Scientific) using primers for GAPDH and TR according to the manufacturer's instructions. All qPCR reactions were carried out in triplicates and three independent biological replicates were analyzed.

Southern Blotting

Southern blotting was carried out using standard protocols (Southern, 2006). Briefly, genomic DNA generated by phenol-chloroform extraction after cell lysis using TE supplemented with 0.5% SDS and 0.1 mg/ml Proteinase K, was digested with BamHI (generating a 1394 bp fragment spanning exons 1-3 of the *TCAB1* locus) and separated on a 0.8% agarose gel. The DNA was then transferred on a Hybond-N+ nylon membrane using capillary transfer. The *TCAB1* locus was detected using radioactive probes (alpha-³²P-dCTP) generated by randomly primed DNA synthesis using an 800 bp PCR product overlapping with the 1394 bp restriction fragment as a template and Klenow polymerase (NEB).

Western Blotting

Mini-PROTEAN TGX stain-free gels (Bio-Rad) were used for SDS-PAGE. Total protein was detected using a ChemiDoc MP (Bio-Rad) after a 45 second UV activation. Western

transfer was carried out using the Trans-Blot Turbo transfer system (Bio-Rad) according to the manufacturer's instructions using the mixed molecular weight transfer setting. Immuno-blotting was carried out using standard protocols. The TCAB1 antibody (Proteintech) was used at a 1:2000 dilution, the TERT antibody (Abcam) was used at a 1:4000 dilution, and the dyskerin antibody (Santa Cruz Biotech) was used at a 1:200 dilution. Secondary antibodies were used at a 1:10000 dilution.

Northern Blotting

RNA was extracted from cell lysates and purified telomerase samples supplemented with 10 ng of a loading and recovery control (*in vitro* transcribed TR 34-328) using the RNeasy Mini kit (Qiagen) and eluted in 30 ul of RNase free water. 15 ul of eluted RNA was mixed with 15 ul of 2x formamide loading buffer (0.1XTBE, 25 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 93% formamide) and heated to 60 °C for 5 min. Samples were separated on a 6% TBE, 7M Urea, polyacrylamide gel (Life Technologies), and transferred to a Hybond N+ membrane (Cytiva) using a wet-blotting apparatus in 1x TBE for 2 hours at 0.5 A of constant current in the cold room. After transfer, membranes were UV-crosslinked, and pre-hybridized in Church buffer for 2 hours at 50 °C. Three DNA oligos complementary to TR were radioactively labeled using T4 PNK (NEB) and ~10x10⁶ cpm of each probe were added to the membrane. Hybridization was carried out at 50 °C overnight. Membranes were washed three times with 2xSSC, 0.1% SDS prior to exposure to a storage phosphorescence screen (Cytiva) which was then imaged on an Amersham Typhoon IP phosphoimager (Cytiva).

Telomerase Expression and Purification

Cell lines were transfected in 15 cm tissue culture plates at ~90% confluency (~25-30x10⁶ cells) using 7.5 µg of TERT plasmid, 30 µg of TR plasmid and 75 µl of Lipofectamine 2000 in 1875 µl of Opti-MEM (Cristofari and Lingner, 2006). Transfected cells were split to three 15 cm dishes 24 hours after transfection. 48 hours after transfection cells were counted, harvested, and snap frozen in liquid nitrogen. Cells were lysed in 1 ml of CHAPS lysis buffer supplemented with 5 µl of RiboLock RNase inhibitor (10 mM TRIS pH 7.5, 1 mM MgCl₂, 1 mM EGTA pH 8.0, 0.5% CHAPS, 10% glycerol) per 100x10⁶ cells and rotated at 4 °C for 30 min. Lysates were cleared in a table-top centrifuge at 21,000xg for 15 min at 4 °C. Identical cell equivalents were used for all samples. 45 µg of anti-TERT antibody was added per ml of cleared lysate and samples were rotated for 1 hour at 4 °C. Lysates were then added to 100 µl of protein G agarose and rotated for 1 hour at 4 °C. The resin was spun down at 1000xg and washed four times with 1 ml of Buffer W (20 mM HEPES pH 7.9, 300 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1% Triton X-100, 10% glycerol). TERT was eluted in 100 µl of Buffer W supplemented with 5 µl of 1 mM TERT peptide by rotating for 30 min at room temperature.

Telomerase Activity Assays

Telomerase assays were carried out in 20 µl of reaction buffer (50 mM TRIS pH 8.0, 150 mM KCl, 1 mM MgCl₂, 2 mM DTT, 100 nM TTAGGGTTAGGGTTAGG oligo, 10 µM dATP, 10 µM dGTP, 10 µM dTTP, 0.165 µM dGTP [α -³²P] 3000 Ci/mmol) including 2 µl of purified telomerase for 1 hour at 30 °C. Telomerase was incubated with the substrate oligo for 15 min at room temperature, prior to initiating the reaction by addition of dNTPs.

Reactions were stopped by adding 100 μ l of 3.6 M of ammonium acetate supplemented with 20 μ g of glycogen and 32P 5'-end labeled loading control oligos (TTAGGGTTAGGGTTAGGG, TTAGGGTTAGGGTTAG). Reaction products were precipitated using 500 μ l of ice-cold ethanol and stored at -20 °C over-night. Reaction products were spun down in a table-top centrifuge at max speed for 30 min at 4 °C, washed with 500 μ l of 70% ethanol, and spun down again speed for 30 min at 4 °C. The 70% ethanol was decanted, and the reaction products were dried in an Eppendorf vacuum concentrator at 45 °C. Reaction products were resuspended in 20 μ l of loading buffer (0.05XTBE, 25 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, 46.5% formamide) and incubated at 95 °C for 5 min. 10 μ l of each sample was separated on a 12% polyacrylamide, 7 M urea sequencing gel pre-run for 45 min at 90W. Gels were dried and exposed to a storage phosphorescence screen (Cytiva) and imaged on an Amersham Typhoon IP phosphoimager (Cytiva).

Single-Particle Tracking

Single-particle tracking was carried out in MATLAB 2019a using a batch parallel-processing version of SLIMfast modified to allow the input of TIFF files (kindly provided by Xavier Darzacq and Anders Hansen) (Hansen et al., 2018), an implementation of the Multiple-Target-Tracing algorithm (Sergé et al., 2008), with the following settings: Exposure Time = 10 ms, NA = 1.49, Pixel Size = 0.16 μ m, Emission Wavelength = 664 nm, $D_{\max}$ = 5 μ m²/s, Number of gaps allowed = 2, Localization Error = 10⁻⁵, Deflation Loops = 0. Diffusion coefficients and the fraction of molecules in each distinct particle population were determined using the MATLAB version of the Spot-On tool (kindly

provided by Xavier Darzacq and Anders Hansen) (Hansen et al., 2018) with the following settings: TimeGap = 10 ms or 20 ms, dZ = 0.700 μ m, GapsAllowed = 2, TimePoints = 8, JumpsToConsider = 4, BinWidth = 0.01 μ m, PDF-fitting, D_Free1_3State = [1 25], D_Free2_3State = [0.1 1], D_Bound_3State = [0.0001 0.1]. For all experiments we carried out 3 independent biological replicates with at least 15 cells for each cell line. The complete data set can be found in supplemental figure S6D. The statistical significance of differences in particle fractions and diffusion coefficients were assessed using a two-tailed T-Test.

Quantification of Fixed Cell Imaging

For the quantification of cellular TR distribution in control and TCAB1 knockout cells we assigned cells into one of three categories: Cells with TR only at telomeres, Cells with TR only in nucleoli, Cells with TR at telomeres and in nucleoli. We carried out 3 independent biological replicates and counted a minimum of 100 cells for control and TCAB1 knockout cells.

Quantification of RT-qPCR data

RT-qPCR experiments were carried out in triplicate and the TR Ct value was normalized to the GAPDH Ct value. The mean Δ Ct (Ct of TR – Ct of GAPDH) value from three independent experiments and the corresponding standard deviation is shown in figure S3B.

Quantification of Western Blots, Northern Blots, and Telomerase Activity Assays

Gel images from Western Blots, Northern Blots, and Telomerase Activity Assays were analyzed using ImageQuant TL 8.2. To quantify TR levels in Northern blots the TR band intensity was normalized to the loading and recovery control signal added to the RNA sample prior to RNA purification. To quantify telomerase activity the whole lane intensity starting at repeat 1 was determined and divided by the sum of the loading control signals. Telomerase processivity was calculated by dividing product intensity > 7 repeats by the total signal in the respective lane. The statistical significance of the observed differences was calculated using a two-tailed T-test using a minimum of three biological replicates. Each biological replicate (independent telomerase expression and purification) was analyzed in technical triplicate.

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848 Supplementary Information

Figure 1 – Figure Supplement 1. (A) Strategy to knock-out *TCAB1* with a single Cas9 and a single sgRNA targeting exon 1 of the *TCAB1* gene. **(B)** PCR of *TCAB1* knock-out clones using the primers indicated in **(A)** demonstrating the absence of a WT *TCAB1* allele. **(C)** Sanger sequencing of the PCR product derived from Halo-TERT TKO1 **(B)** demonstrating deletion causing a frame shift. **(D)** Model of *TCAB1* protein indicating the antigens used to raise the indicated *TCAB1* antibodies. **(E-G)** Western blots of parental cells and *TCAB1* knock-out clones using **(E)** the Novus Biologicals, **(F)** Abcam, and **(G)** Proteintech *TCAB1* antibodies, demonstrating the presence of a truncated *TCAB1* protein in the putative *TCAB1* knock-out cells. **(H)** IF using antibodies against coilin and *TCAB1* (Abcam) demonstrating that truncated *TCAB1* can be detected in Cajal bodies (white arrows) in putative *TCAB1* knock-out clones. **(I)** IF using coilin and *TCAB1* (Novus Biologicals) antibodies coupled to FISH with probes against the scaRNA ACA57, demonstrating that the Novus Biologicals antibody fails to detect the truncated *TCAB1* and scaRNA localization is unchanged in putative *TCAB1* knock-out cells.

Figure 1 – Figure Supplement 2. (A) Strategy to knock-out TCAB1 using Cas9 and two sgRNAs targeting introns 1 and 3. **(B)** Southern blot of genomic DNA digested with BamHI from parental cells and TCAB1 knock-out clones using a probes generated from a PCR product of the TCAB1 gene indicated in **(A)** demonstrating the expected truncation of the *TCAB1* gene in Halo-TERT TCAB1 KO 2. HeLa TCAB1

KO 2 carries larger deletions completely removing exons 1 and 2 from the *TCAB1* gene. (C) PCR using primers indicated in (A) of genomic DNA from parental cells and *TCAB1* knock-out clones confirming the deletion of critical regions of the *TCAB1* gene show in (B). (D) Immuno-fluorescence with anti-dyskerin and anti-TCAB1 antibodies coupled to fluorescence in-situ hybridization with probes against TR, demonstrating that expression of GFP-TCAB1 in *TCAB1* knockout cells rescues TR localization to Cajal bodies (scale bar = 5 μ m).

Figure 2 – Figure Supplement 1. (A) Images of HeLa cells transiently expressing GFP-nucleolin to mark nucleoli. The GFP-nucleolin signal overlaps with circular shapes visible under transmitted light illumination (scale bar = 2 μ m). (B) Images of HeLa cells transiently expressing GFP-nucleolin and 3xFLAG-HaloTag-NLS labeled with JF646. The 3xFLAG-HaloTag-NLS signal (maximum intensity projection of 1000 frames of a single-molecule imaging movie) clearly overlaps with the GFP-nucleolin signal (red dashed outline), demonstrating that 3xFLAG-HaloTag-NLS can enter the nucleolus (scale bar = 2 μ m). (C) Images of HeLa cells transiently expressing 3xFLAG-HaloTag-dyskerin labeled with JF646 and probed with an antibody against dyskerin, demonstrating that 3xFLAG-HaloTag-dyskerin localizes to the nucleolus (scale bar = 5 μ m).

Figure 3 – Figure Supplement 1. (A) Images of HeLa cells transiently expressing mCherry-dyskerin probed with antibodies against mCherry and dyskerin, demonstrating that mCherry dyskerin localizes to the nucleolus (scale bar = 5 μ m). **(B)** Images of control and TCAB1 knock-out HeLa cells (3xFLAG-Halo-TERT, mEOS3.2-TRF2) overexpressing untagged TERT and TR. TR was detected using FISH and TRF2 was visualized using the fluorescence signal from mEOS3.2-TRF2. In control cells TR co-localizes with telomeres, while it is enriched in nucleoli and localized to telomeres in TCAB1 KO cells (scale bar = 5 μ m). **(C)** Quantification of the fraction of cells showing TR localization exclusively to telomeres, to telomeres and nucleoli, or only to nucleoli (2 independent experiments, >100 cells per experiments, mean $\pm$ standard deviation). **(D)** Images of HeLa cells and TCAB1 knockout cells transiently expressing untagged TERT and TR, probed with TERT (Abcam) and dyskerin antibodies and FISH for TR demonstrating that untagged TERT is excluded from nucleoli marked by dyskerin in parental and TCAB1 knock-out cells.

Figure 5 – Figure Supplement 1. Direct telomerase extension assay of telomerase immuno-purified from parental (WT) and TCAB1 knock-out (TKO) HeLa and Halo-TERT cell lines. LC1 and LC2, radiolabeled DNA oligonucleotide loading controls.

Figure 6 – Figure Supplement 1. (A) PCR analysis of the TR locus in parental and TR knock-out clones. Both TR knock-out clones show PCR products with reduced length that were confirmed to be knock-outs by Sanger sequencing. **(B)** Images of control and TR knock-out cells probed with an antibody against coilin and FISH probes specific for TR, demonstrating the lack of TR signal in TR knock-out cells (scale bar = 5 μ m). **(C)** Determination of TR levels in control, TCAB1 knock-out, and TR knock-out cells, using RT-qPCR with primers specific to TR normalized to GAPDH (3 independent biological replicates, 3 technical replicates for each biological replicate, mean $\pm$ standard deviation). **(D)** Maximum intensity projection of 2000 frames of a 3xFLAG-HaloTag (JF646) TERT movie (bottom), demonstrating that the TERT signal does not overlap with the nucleolus detected as circular shape in the transmitted light image (top, red dashed line). **(E)** Fitting of single-particle tracking data of TERT from control, TCAB1 knock-out, and TR knock-out cells expressing 3xFLAG-HaloTag-TERT using the Spot-On tool.

Figure 6 – Figure Supplement 2. (A) Analysis of the step size of telomeric TERT particles relative to the distance of the particle to the closest telomere (pooled results from 3 independent biological replicates with 19-30 cells analyzed per replicate). TERT molecules bound to the telomere are expected to have small step sizes and a short distance to the closest telomere, which is apparent in the enrichment of events in the lower quadrants in the WT control. This enrichment is not observed in TCAB1 and TR knock-out cells. **(B)** Spot-On analysis of telomeric TERT particles (pooled results from 3 independent biological replicates with 19-30 cells analyzed per replicate). The fraction of bound TERT particles in TCAB1 and TR knock-out cells is 4-5%, compared to 12% in the WT control cells.

Figure 6 – Figure Supplement 3. (A) Structure of the telomerase RNP showing the distance between TCAB1 and CR4/CR5 (13.4 nm). TR in blue, CR4/CR5 in red, and TCAB1 in green (Ghanim et al., 2021).

Movie Legends

Movie 1. Single-particle tracking of 3xFLAG-HaloTag-TERT labeled with JF646 in a control cell acquired at 100 frames per second. Trajectories with a minimum of 5 localizations are displayed. 150x150 pixels with a pixel size of 0.16 μm .

Movie 2. Single-particle tracking of 3xFLAG-HaloTag-TERT labeled with JF646 in a TCAB1 knockout cell acquired at 100 frames per second. Trajectories with a minimum of 5 localizations are displayed. 150x150 pixels with a pixel size of 0.16 μm .

Movie 3. Movie of cell expressing GFP-nucleolin (red) and 3xFLAG-HaloTag-NLS (green) labeled with JF646 acquired at 100 frames per second, showing overlap of 3xFLAG-HaloTag-NLS with nucleoli. 140x140 pixels with a pixel size of 0.16 μm .

Movie 4. Movie of 3xFLAG-HaloTag-TERT labeled with JF646 in a control (left), TCAB1 knockout (middle), and TR knockout (right) cell acquired at 100 frames per second. Each panel is 150x150 pixels in size with a pixel size of 0.16 μm .

Movie 5. Single-particle tracking of 3xFLAG-HaloTag-TERT labeled with JF646 in a TR knockout cells acquired at 100 frames per second. Trajectories with a minimum of 5 localizations are displayed. 150x150 pixels with a pixel size of 0.16 μm .