



# TRF2 recruits nucleolar protein TCOF1 to coordinate telomere transcription and replication

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## Abstract

Telomeres are transcribed into telomeric RNA termed as TERRA. However, the transcription itself and excessive TERRA may interfere with telomere replication during S phase. The mechanism that coordinates telomere transcription and replication is unknown. Here, we report that TCOF1 leaves the nucleolus and is recruited to telomeres specifically during S phase by interacting with TRF2. Therein, TCOF1 acts to suppress telomere transcription by binding and inhibiting Pol II. Thus, TERRA is limited to low levels in S phase. Depletion of TCOF1 leads to abnormally elevated TERRA and formation of DNA/RNA hybrids (R-loops) at telomeres, which induces replication fork stalling and fragile telomeres. Importantly, telomere replication defect induced by TCOF1 deficiency can be rescued by either masking TERRA or expressing an R-loop eraser RNase H1, demonstrating a critical role of TCOF1 in coordinating telomere transcription and replication. These findings link nucleolus to telomeres and uncover a novel function of TCOF1 on ensuring telomere integrity.

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## Introduction

DNA replication and transcription compete for the same DNA template, and encounters naturally occur between conventional DNA replication machinery and RNA polymerase complex, leading to so called “transcription–replication conflict” [1]. In addition, transcription products, especially long noncoding RNAs (lncRNAs), may have a negative impact on DNA replication. Increased evidences support the idea that nascent RNA transcripts can impede replication fork by forming R-loops (three-strand structure with a RNA/DNA hybrid) or RNA-DNA triplex [2, 3]. Therefore, the coordinated mechanism, although not yet fully understood, is important to prevent the potential replication defects induced by unscheduled expression of lncRNAs.

Human telomeres are composed of tandem repeats of short DNA sequences (TTAGGG/CCCTAA)<sub>n</sub> and associated shelterin proteins [4, 5]. As a typical fragile site, telomeres bear high loads of replication stress that may induce genome instability if not properly resolved. Meanwhile, telomeres in eukaryotes are transcribed into long noncoding RNA known as TERRA (telomeric repeat-containing RNA) [6]. The study in *Saccharomyces cerevisiae* showed that abnormal expression of TERRA results in replication fork collapse and telomere shortening [7]. Because human telomeres replicate through all S phase [8],

it is speculated that expression of TERRA must be strictly regulated during S phase in order to prevent a potential “transcription–replication conflict” or replication stress induced by excessive TERRA forming R-loops.

TRF2 is a component of shelterin complex that plays a critical role in chromosome end protection by telomeres [9]. Notably, increasing evidences link TRF2 to telomere replication. For instance, TRF2 has been reported to work with Apollo to relieve topological stress during telomere replication [10]. Moreover, TRF2 may promote the formation of pre-replication complex by recruiting an origin recognition complex to telomeres [11]. Intriguingly, it has also been reported that depletion of TRF2 leads to increased level of TERRA [12]. However, the mechanism underlying regulation of TERRA expression by TRF2 remain elusive and how TRF2–TERRA axis affects telomere replication is unclear.

TCOF1, so called Treacle protein, is a nucleolar factor that regulates ribosomal DNA (rDNA) transcription in nucleolus through interacting with the upstream binding factor (UBF) [13]. Mutations in *TCOF1* gene lead to deficient ribosome biogenesis that is proposed to associate with the pathogenesis of Treacher Collins Syndrome (TCS), a severe genetic disorder that includes abnormal craniofacial development [14]. It has been recently reported that TCOF1 is a mobile protein that shuttles between the nucleoplasm and the nucleoli [15]. Moreover, TCOF1 has non-nucleolus function during DNA damage repair in cells with high reactive oxygen species [16]. Here, we discovered that TCOF1 is recruited by TRF2 to telomeres specifically during S phase of cell cycle, where it suppresses the transcription activity of RNA polymerase II (Pol II). The loss of TCOF1 leads to a significant increase of TERRA abundance. Excessive TERRA increases the amount of R-loops at telomeres, resulting in telomere replication defects, telomere fragility, and genome instability.

## Results

### Transportation of TCOF1 from nucleoli to telomeres during S phase

The *TCOF1* gene encoded protein, called treacle, is a typical nucleolar protein that is located in nucleolus, a small region inside the nucleus where rRNAs are produced. Interestingly, we observed that in human bone osteosarcoma U2OS and cervical carcinoma HeLa cells, TCOF1 proteins are fully colocalized with fibrillarin, a marker of nucleolus, during non-S phases of the cell cycle (EdU negative). However, many of TCOF1 are located outside of nucleoli during S phase (EdU positive) (Figs. 1a, b and S1a, b). When cells were synchronized at S phase, up to 70% U2OS and 90% HeLa cells displayed 30% and 40% on

average of TCOF1 being outside of nucleoli, respectively (Figs. 1c–e and S1c–e). Non-nucleolus localization of TCOF1 during S phase was also confirmed by over-expression of GFP-TCOF1 in U2OS (Fig. S1f–h).

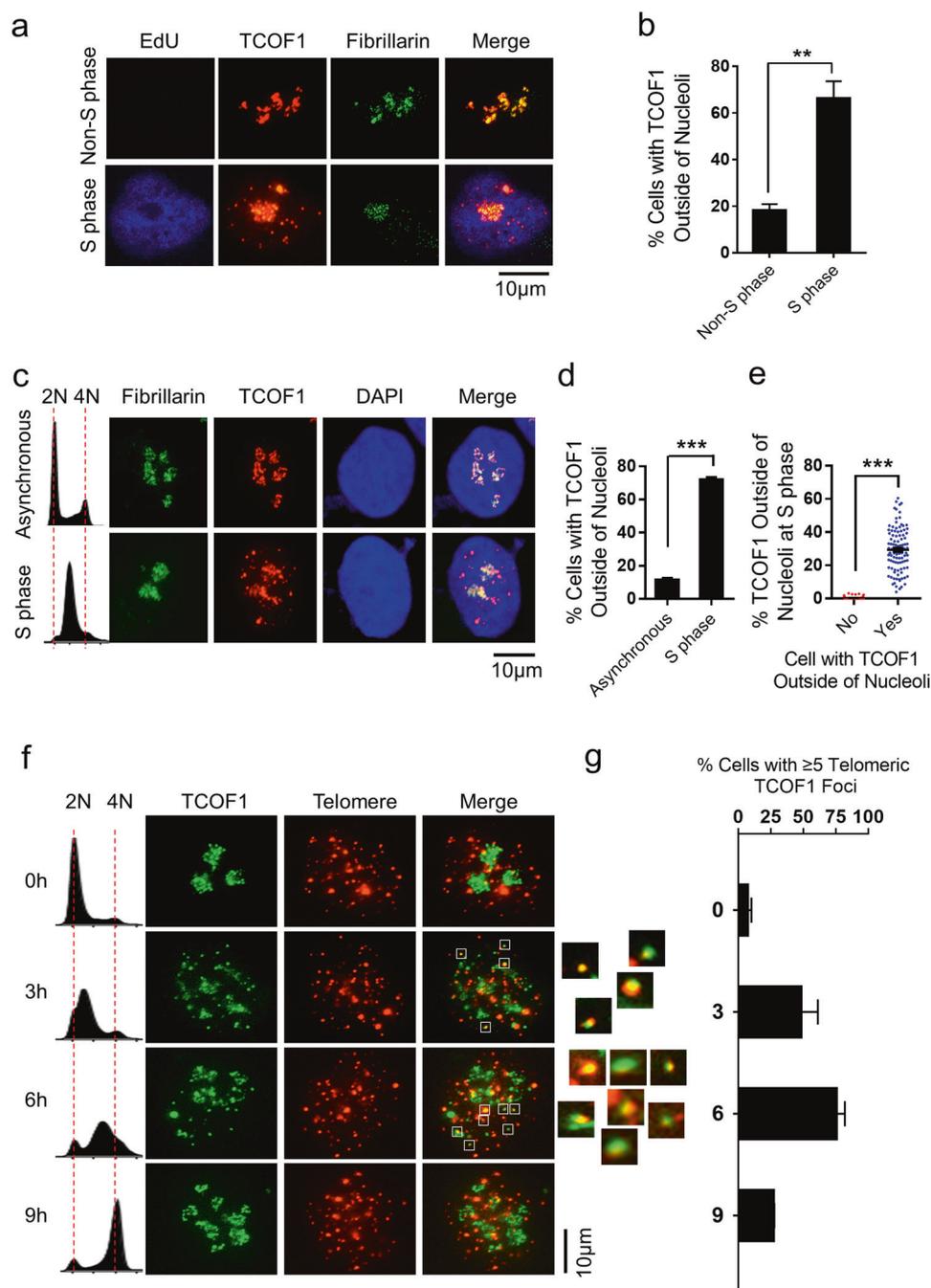
Co-staining TCOF1 and telomeres by immunofluorescence (IF) and fluorescence in situ hybridization (FISH) showed S phase-specific co-localization of TCOF1 with telomeres in telomerase-negative U2OS (Fig. 1f, g) and telomerase-positive HeLa cells (Fig. S1i–k). Notably, only a portion of TCOF1 localizes to telomeres in S phase, likely due to that TCOF1 has another functions at other locations during S phase. Cell-cycle dependent association of TCOF1 with telomeres was also examined by chromatin immunoprecipitation (ChIP) followed by hybridization with telomeric probe. The result confirmed a specific enrichment of TCOF1 at telomeres during S phase (Fig. S1l–n).

### TRF2 recruits TCOF1 to telomeres

TCOF1 may localize to telomeres through interacting with shelterin component. Previous IP-MS assay identified that TCOF1 is on the list of TRF2-associated proteins [17]. To test a potential interaction between TCOF1 and TRF2, we first performed IF experiment to determine the spatial localization of TCOF1 and TRF2. The result showed S phase-specific co-localization of GFP-tagged TCOF1 and TRF2 in U2OS cells (Fig. 2a, b). Furthermore, we performed co-immunoprecipitation (co-IP) experiments to examine a physical interaction between TCOF1 and TRF2. We observed that TRF2 is co-precipitated with endogenous TCOF1 (Fig. 2c) and ectopically expressed GFP-tagged TCOF1 in U2OS and 293T cells, respectively (Fig. 2d).

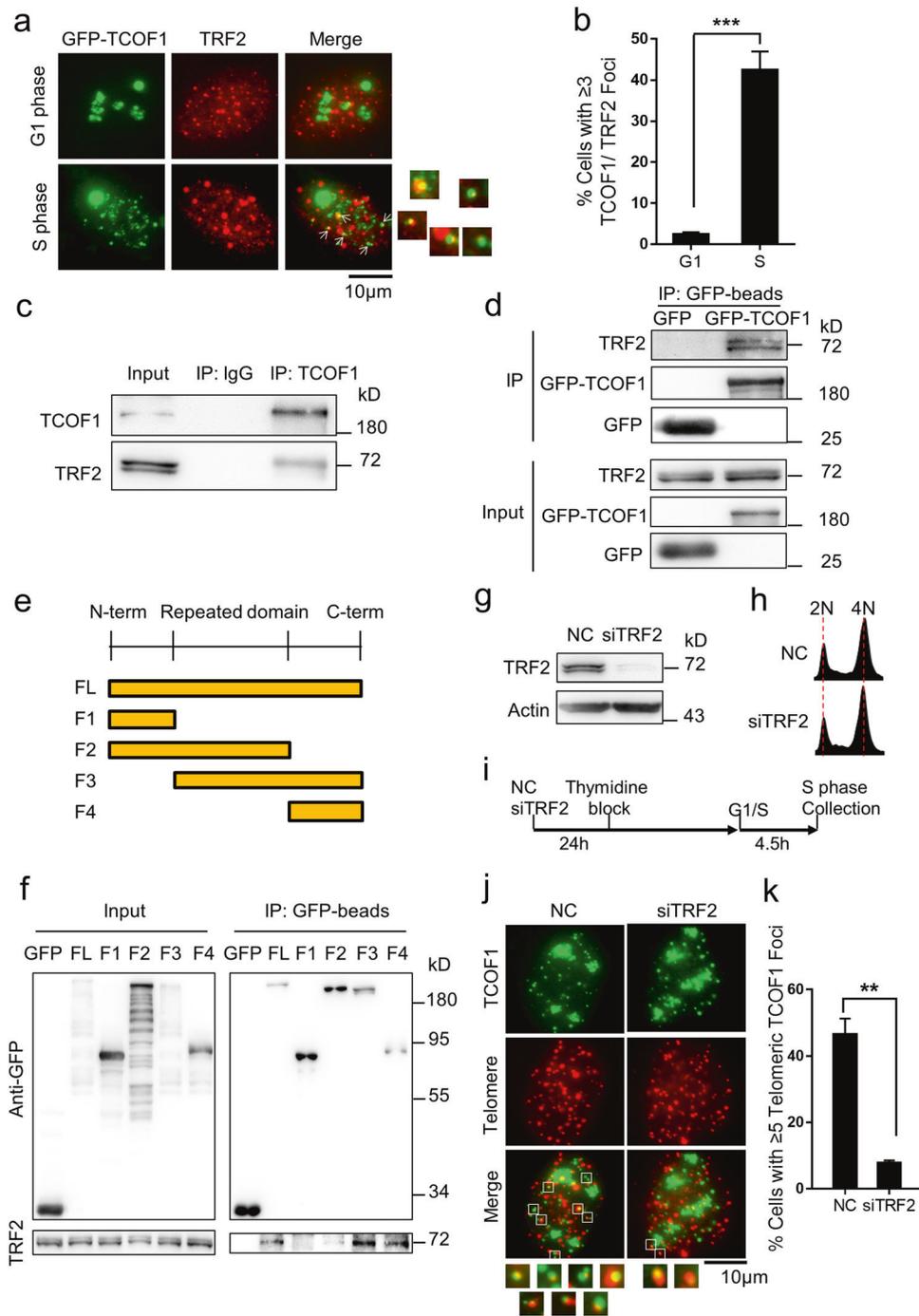
TCOF1 consists of three distinct domains: N-terminal domain, repeated domain and C-terminal domain containing nuclear and nucleolar localization signals [18]. To explore which domain is responsible for interacting with TRF2, full-length and truncated TCOF1 with one or two domains depleted were expressed in 293T cells and their interaction with TRF2 was tested by co-IP (Fig. 2e). The result showed that TCOF1 binds to TRF2 mainly through its C-terminal domain (Fig. 2f).

If TCOF1 is recruited to telomeres through interacting with TRF2, it is speculated that depletion of TRF2 would abolish the localization of TCOF1 at telomeres. To test it, TCOF1 and telomeres were co-stained in wild-type and TRF2-depleted U2OS cells. Indeed, TRF2 depletion significantly decreased the number of TCOF1 foci at telomeres (Fig. 2g–k). In addition, depletion of TRF2 did not change the cell cycle of U2OS, excluding the possibility that decrease in TCOF1 foci at telomeres is due to changed length of S phase (Fig. 2h).



**Fig. 1 S phase-specific localization of TCOF1 at telomeres.** **a** Localization of TCOF1 in nuclei of U2OS cells during S and non-S phase of cell cycle. EdU labeling was used to indicate S phase cells. Nucleoli were shown by staining the fibrillarin marker protein. **b** Quantification of **(a)**. The percentage of cells with TCOF1 localized outside of nucleoli was determined ( $n \geq 100$  cells). All values are the average  $\pm$ SEM of three independent experiments. The unpaired Student's two-tailed  $t$ -test was used to determine the statistical significance (\*\* $P < 0.01$ ). **c** U2OS cells were synchronized at G1/S and released into S phase for 4.5 h, corresponding to middle S phase. Asynchronous cells were used as a control. Left: flow cytometry analysis of the cell cycle. Right: co-staining of TCOF1 and fibrillarin in cells. **d** Quantification of **(c)**. The percentage

of cells with TCOF1 localized outside of nucleoli was determined. All values are the average  $\pm$ SEM of three independent experiments ( $n \geq 100$  cells). **e** Quantification of **(c)**. The percentage of TCOF1 outside of nucleoli in individual cells during S phase was quantified by determining the fluorescence intensity of TCOF1 after IF staining ( $n \geq 100$  cells). The unpaired Student's two-tailed  $t$ -test was used to determine the statistical significance (\*\* $P < 0.001$ ). **f** U2OS cells were synchronized at G1/S and released for 0, 3, 6, and 9 h, respectively. Left: flow cytometry analysis of the cell cycle. Right: co-staining TCOF1 and telomeres at indicated timing. **g** Quantification of **(f)**. The percentage of cells with  $\geq 5$  telomeric TCOF1 foci was determined ( $n \geq 100$  cells). All values are the average  $\pm$ SEM of three independent experiments.



**TERRA transcription is regulated by TCOF1**

TCOF1 regulates the transcription of ribosomal RNA (rRNA) in the nucleoli [13]. To study a potential function of TCOF1 on telomeres, we examined telomere transcription in wild-type and TCOF1-depleted U2OS cells. We observed that depletion of TCOF1 by siRNA leads to significant increase of TERRA (Figs. S2a and 3a, b), suggesting that

TCOF1 may act to suppress the transcription of TERRA. To test it, TERRA transcripts from individual subtelomere/telomeres were determined by RT-qPCR using subtelomere-specific PCR primers [19]. The level of TERRA transcribed from six representative chromosomes (6p, 7p, XpYp, 17p, 17q, and XqYq) were all increased upon depletion of TCOF1 in U2OS (Fig. 3c), HeLa and normal lung fibroblast MRC5 cells (Fig. S2b, c). Flow

◀ **Fig. 2 TRF2 recruits TCOF1 to telomeres.** **a** U2OS cells stably expressing GFP-tagged TCOF1 were synchronized at G1/S and released for 0, 4.5 h, corresponding to the G1, S phase, respectively. Cells were stained with antibody against TRF2. **b** Quantification of (a). The percentage of cells with  $\geq 3$  TCOF1/TRF2 foci was determined ( $n \geq 100$  cells). All values are the average  $\pm$ SEM of three independent experiments. The unpaired Student's two-tailed *t*-test was used to determine the statistical significance ( $***P < 0.001$ ). **c** Interaction between endogenous TCOF1 and endogenous TRF2 was detected by immunoprecipitation with IgG or anti-TCOF1 antibody in U2OS cells. **d** Co-immunoprecipitation of ectopically expressed GFP-tagged TCOF1 and endogenous TRF2 in 293T cells. **e** A diagram of various TCOF1 constructs is shown. **f** Four GFP-tagged deletion constructs of TCOF1, lacking both the repeated domain and the C-terminal domain (F1), the C-terminal domain (F2), the N-terminal domain (F3), or both the N-terminal domain and the repeated domain (F4) were used to co-immunoprecipitate with endogenous TRF2 after transfection of 293T cells. Full-length TCOF1 (FL) was used as a positive control. **g** Western blot was performed to determine the protein levels of endogenous TRF2 after siRNA treatment. **h** Flow cytometry analysis of the cell cycle in U2OS cells transfected with NC and siTRF2. **i** Time course of thymidine block and IF-FISH following siRNA treatment. **j** Immunofluorescence and FISH detection of TCOF1 foci at telomeres in U2OS cells transfected with NC or siTRF2. **k** Quantification of (j). The percentage of cells with  $\geq 5$  telomeric TCOF1 foci was determined ( $n \geq 100$  cells). All values are the average  $\pm$ SEM of three independent experiments. The unpaired Student's two-tailed *t*-test was used to determine the statistical significance ( $**P < 0.01$ ).

cytometry analysis revealed that TCOF1 depletion leads to cell cycle arrest at S phase (Fig. S2d). Notably, it has been previously reported [20] and further demonstrated by our experiment (Fig. S2e) that S phase cells bear a low level of TERRA. Therefore, it is unlikely that increase of TERRA upon TCOF1 depletion is caused by S phase arrest. Conversely, overexpression of TCOF1 decreased the expression of all six transcripts in U2OS cells (Fig. S2f). TERRA forms foci in nucleus that can be visualized by RNA-FISH [6]. We demonstrated that TERRA foci, which are sensitive to RNase A digestion, are visible in U2OS cells and that depletion of TCOF1 increases both number and the intensity of TERRA foci (Fig. S2g–i).

It is previously reported that TERRA abundance changes in a cell-cycle dependent manner with a specific decrease during S phase [20]. This observation was confirmed by our RNA-FISH experiment (Fig. 3d, e). However, when TCOF1 was depleted by siRNA, the overall intensity, which indicates the amount of TERRA within cell, was significantly increased, and we observed no difference in TERRA abundance between S/G2 (Cyclin A positive) and G1 (Cyclin A negative) cells (Fig. 3d, e). Altogether, these results suggested that TCOF1 is specifically required to reduce TERRA levels during S phase.

Since TCOF1 is recruited to telomeres by interacting with TRF2 (Fig. 2f), it is speculated that depletion of TRF2 would increase telomere transcription and TERRA levels. To test it, the transcripts from specific chromosomes were

determined by RT-qPCR in the absence or presence of TRF2. Indeed, we found that depletion of TRF2 significantly increases the levels of chromosome-specific TERRA transcripts (Fig. S2j).

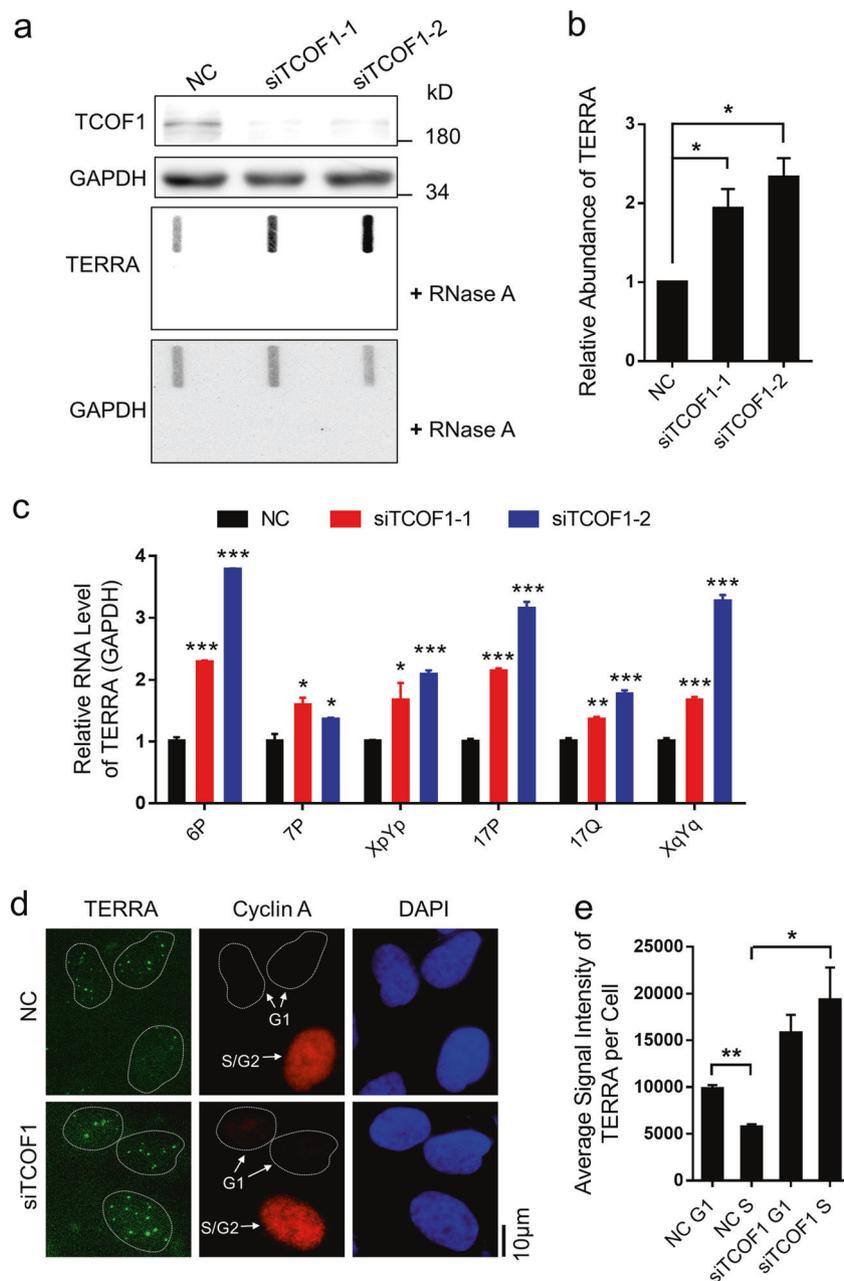
### TCOF1 suppresses telomere transcription by binding to Pol II

It has been showed that TERRA is transcribed by RNA Pol II [21]. We suspected that TCOF1 may suppress TERRA transcription by inactivating Pol II at telomeres. To test it, we first examined whether TCOF1 binds to Pol II in cells. Co-IP experiments using antibody recognizing endogenous Pol II showed that TCOF1 co-precipitates with Pol II (Fig. 4a), and vice versa (Fig. 4b). Moreover, Pol II was also co-precipitated with GFP-tagged TCOF1, demonstrating the interaction between TCOF1 and Pol II (Fig. 4c). Specifically, co-IP experiment using synchronized G1 or S phase cells demonstrated that the interaction between Pol II and TCOF1 primarily occurs at S phase (Fig. 4d).

Truncated TCOF1 were expressed in 293T cells to identify the domain that interacts with Pol II (Fig. 2e). Co-IP experiment showed that F2 and F3, both consisting of repeated domain of TCOF1, is sufficient to pull down Pol II (Fig. 4e). Therefore, TCOF1 adopts different domains, C-terminal and repeated domains, to interact with TRF2 and Pol II, respectively.

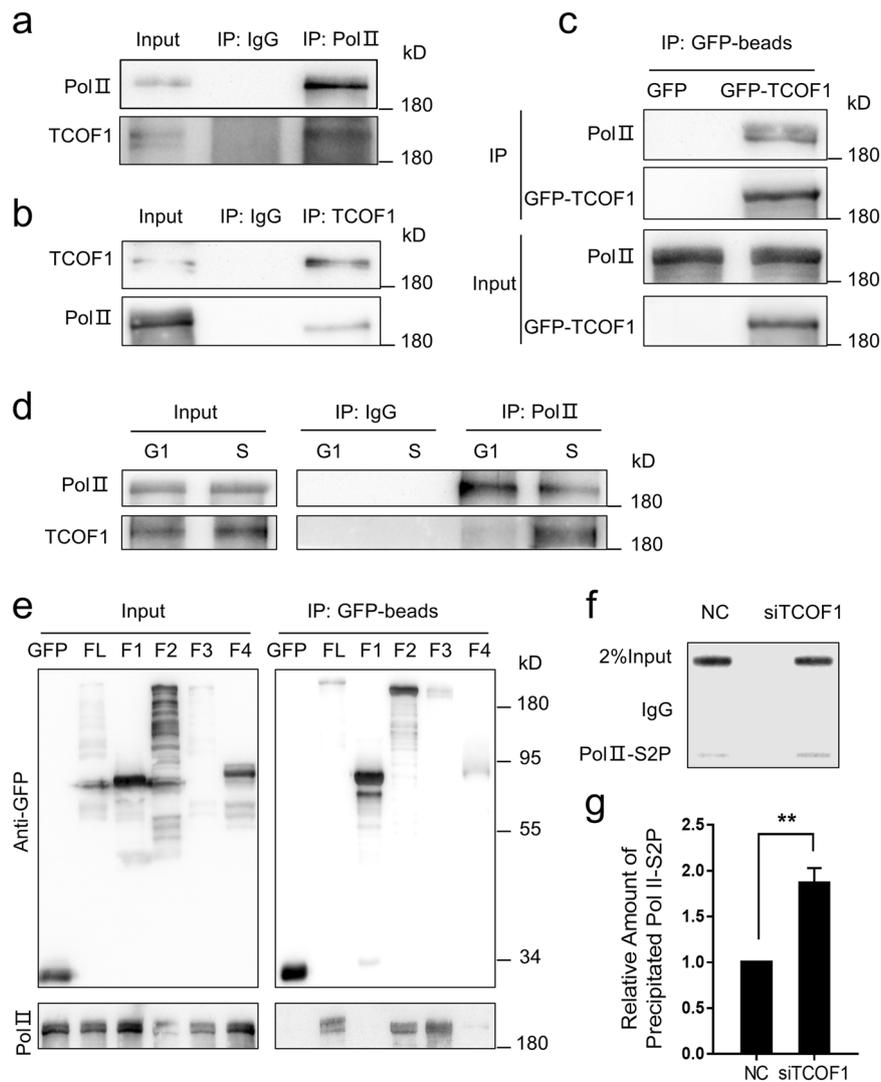
Next, we assessed how TCOF1 influences the activity of Pol II at telomeres. Pol II-S2P, a phosphorylated Pol II at ser-2 of its heptapeptide repeat, is considered as a marker for active or elongating Pol II [22]. Using ChIP followed by hybridization with telomeric probe, we determined the amount of Pol II-S2P at telomeres in the presence or absence of TCOF1. The result showed that Pol II-S2P at telomeres is significantly increased when TCOF1 was depleted in U2OS cells (Fig. 4f, g). Moreover, we demonstrated that increase of telomeric Pol II-S2P is not due to S phase arrest induced by TCOF1 depletion, because S phase cells display lower level of Pol II-S2P at telomeres compared to asynchronous cells (Fig. S3a, b).

To explore the mechanism by which TCOF1 suppresses Pol II elongation activity, truncated TCOF1 with depleted N-terminal, Repeated or C-terminal domain ( $\Delta$ N,  $\Delta$ R,  $\Delta$ C) was expressed in TCOF1-deficient U2OS cells (Fig. S3c, d). Interestingly, we found that only full-length, but not any form of truncated TCOF1, is able to reduce Pol II-S2P at telomeres (Fig. S3e, f). This result indicated that in addition to repeated domain that is responsible for interacting with Pol II (Fig. 4e) and C-terminal domain that is needed for positioning TCOF1 to telomeres by associating with TRF2 (Fig. 2f), N-terminal domain is



**Fig. 3 TCOF1 negatively regulates transcription of TERRA.** **a** Slot-blot analysis of TERRA levels in NC (negative control), siTCOF1-1 and siTCOF1-2 transfected U2OS cells. Western blot was performed to determine knockdown efficiency of TCOF1. The total RNA was purified from 0.5 M cells and used for detection of TERRA and GAPDH (Control) by slot blot. The membrane was first hybridized with GAPDH probe, stripped and then hybridized with telomeric C-rich probe. **b** Quantification of (a). The relative abundance of TERRA was determined by quantifying the hybridization signals. All values are the average  $\pm$ SEM of three independent experiments. The unpaired Student's two-tailed *t*-test was used to determine the statistical significance ( $*P < 0.05$ ). **c** RT-qPCR analysis of levels of chromosome-specific TERRA transcripts in U2OS cells transfected

with NC, siTCOF1-1 or siTCOF1-2. Subtelomere-specific primers were used. All values are the average  $\pm$ SEM of three independent experiments. The unpaired Student's two-tailed *t*-test was used to determine the statistical significance ( $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ). **d** RNA-FISH detection of TERRA in NC or siTCOF1 transfected U2OS cells stained with Cyclin A. Cyclin A staining was used to indicate S and G2 phase cells. **e** Quantification of (d). The average signal intensity of TERRA per cell was determined by quantifying the hybridization intensity ( $n \geq 100$  cells). All values are the average  $\pm$ SEM of three independent experiments. The unpaired Student's two-tailed *t*-test was used to determine the statistical significance ( $*P < 0.05$ ;  $**P < 0.01$ ).



**Fig. 4 TCOF1 suppresses telomere transcription by binding to RNA pol II.** **a** Interaction between endogenous TCOF1 and endogenous RNA pol II was detected by immunoprecipitation with IgG or anti-RNA pol II antibody in U2OS cells. **b** Interaction between endogenous TCOF1 and endogenous RNA pol II was detected by immunoprecipitation with IgG or anti-TCOF1 antibody in U2OS cells. **c** Co-immunoprecipitation of ectopically expressed GFP-tagged TCOF1 and endogenous RNA pol II in 293T cells. **d** Interaction between TCOF1 and RNA pol II during G1 or S phase was determined by immunoprecipitation using IgG or anti-RNA pol II antibody. U2OS cells stably expressing TCOF1 were synchronized at G1/S and released for 0, 4.5 h, corresponding to the G1, S phase, respectively.

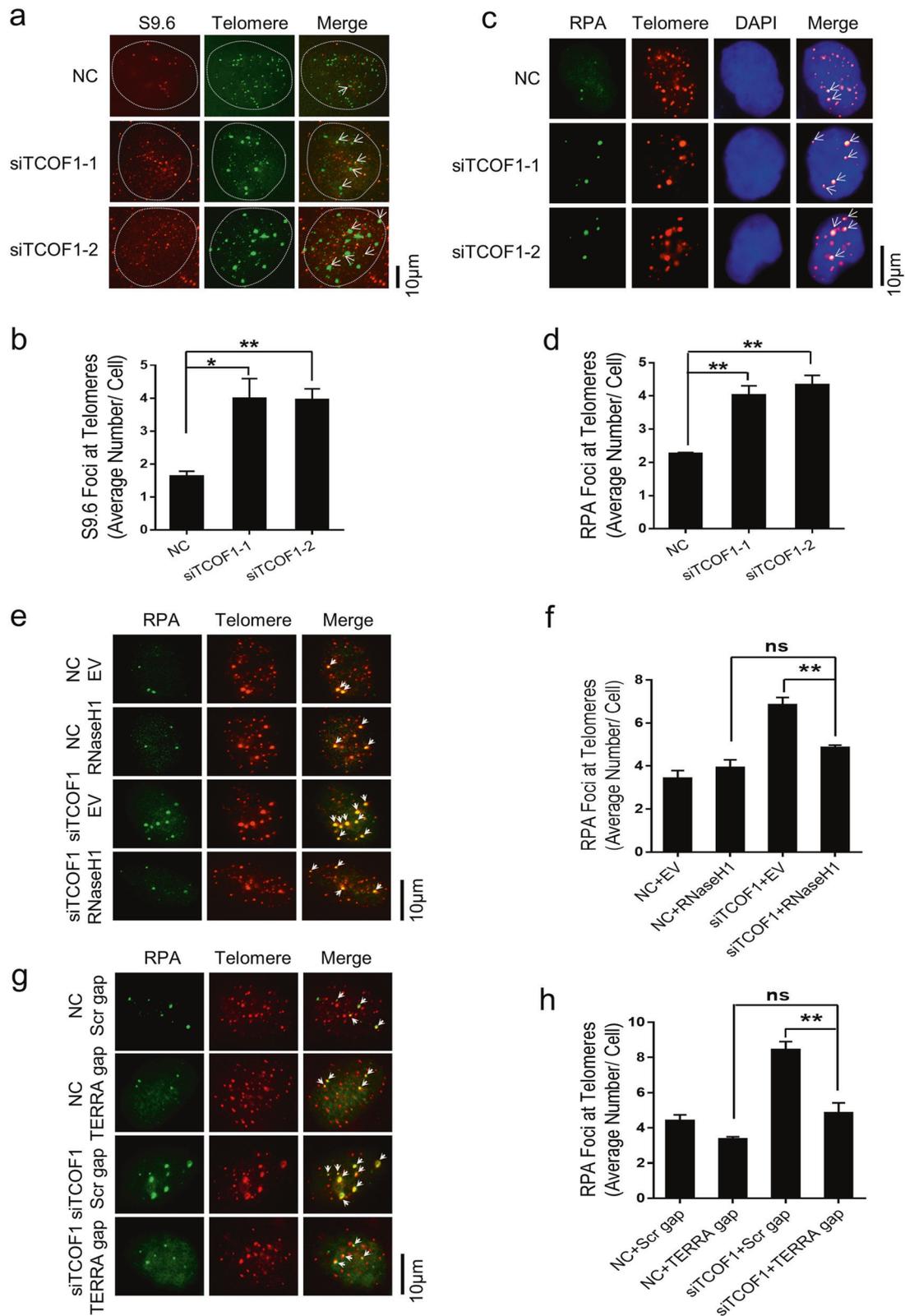
also required to suppress Pol II-mediated transcription at telomeres.

### TCOF1 deficiency stimulates R-loop formation at telomeres

Actively transcribed telomere may form the structure termed R-loops that can be detected by S9.6, a specific antibody that recognizes RNA-DNA hybrids. In addition,

To avoid the artificial interaction occurring during cell lysis, cells were first cross-linked with DSP and then lysed in RIPA buffer containing 0.1% SDS. **e** Four GFP-tagged deletion constructs of TCOF1 (F1, F2, F3, F4) were used to co-immunoprecipitate with endogenous RNA pol II after transfection of 293T cells. Full-length TCOF1 (FL) was used as a positive control. **f** ChIP analyses of RNA pol II-S2P at telomeres in normal (NC) and TCOF1-deficient U2OS cells (siTCOF1). **g** Quantification of (f). The relative amount of precipitated RNA pol II-S2P at telomeres was determined (Signal intensity of Pol II-S2P above IgG/2% input). All values are the average  $\pm$ SEM of three independent experiments. The unpaired Student's two-tailed *t*-test was used to determine the statistical significance (\*\* $P < 0.01$ ).

excessive TERRA may also form R-loop structure by invading into telomeric DNA [23]. In consistent with increased TERRA upon TCOF1 depletion, we observed a significant increase of S9.6 foci at telomeres, indicating more R-loops forming in telomeric DNA (Fig. 5a, b). Global and telomeric S9.6 foci were sensitive to RNase H digestion, confirming the nature of R-loops visualized by S9.6 (Fig. S4a–c). In addition, DNA-RNA immunoprecipitation using S9.6 (DRIP) followed by hybridization with



telomeric probe also demonstrated the increase of R-loops forming at telomeres in U2OS cells when TCOF1 was depleted (Fig. S4d, e).

R-loops may impede the progress of replication fork, leading to fork stalling [3], which is characterized by accumulation of PCNA or RPA protein on stalled

◀ **Fig. 5 R-loops accumulate at telomeres upon TCOF1 depletion.** **a** Detection of telomeric R-loops in U2OS cells transfected with NC (negative control), siTCOF1-1 or siTCOF1-2. R-loops and telomeres were visualized by immunofluorescence using S9.6 antibody and telomere FISH, respectively. **b** Quantification of (a). The mean number of S9.6 foci at telomeres per cell was determined ( $n \geq 100$  cells). All values are the average  $\pm$ SEM of three independent experiments. The unpaired Student's two-tailed *t*-test was used to determine the statistical significance ( $*P < 0.05$ ;  $**P < 0.01$ ). **c** Immunofluorescence and FISH detection of RPA foci at telomeres in U2OS cells transfected with NC, siTCOF1-1 or siTCOF1-2. **d** Quantification of (c). The average number of telomeric RPA foci per cell was determined ( $n \geq 100$  cells). All values are the average  $\pm$ SEM of three independent experiments. The unpaired Student's two-tailed *t*-test was used to determine the statistical significance ( $**P < 0.01$ ). **e** Immunofluorescence and FISH detection of RPA foci at telomeres in TCOF1-deficient U2OS cells with overexpressed RNase H1. **f** Quantification of (e). The average number of telomeric RPA foci per cell was determined ( $n \geq 100$  cells). All values are the average  $\pm$ SEM of three independent experiments. The unpaired Student's two-tailed *t*-test was used to determine the statistical significance ( $**P < 0.01$ ). **g** Immunofluorescence and FISH detection of RPA at telomeres in NC or siTCOF1 transfected U2OS cells treated with scrambled LNA gapmer or TERRA targeting LNA gapmer for 24 h. **h** Quantification of (g). The average number of telomeric RPA foci per cell was determined ( $n \geq 100$  cells). All values are the average  $\pm$ SEM of three independent experiments. The unpaired Student's two-tailed *t*-test was used to determine the statistical significance ( $**P < 0.01$ ).

replication forks [24, 25]. Consistent with this speculation, we observed significant increase of PCNA and RPA foci at telomeres in TCOF1-depleted U2OS cells (Figs. 5c, d and S4f–k).

To verify that stalled replication fork is caused by R-loops, human RNase H1, which specifically digests RNA in RNA/DNA hybrids and thus removes R-loops, was overexpressed in TCOF1-depleted U2OS cells (Fig. S5a). As expected, RNase H1 expression significantly reduced the number of S9.6 foci at telomeres (Fig. S5b, c). Accordingly, telomeric RPA foci decreased to a background level (Fig. 5e, f). In addition, we also used a locked nucleic acid (LNA) gapmer to mask TERRA [26]. Expectedly, LNA gapmer transfected control and TCOF1-depleted cells displayed much less TERRA FISH signals (Fig. S6a–c). As a result, RPA foci at telomeres decreased to a background level in TCOF1-deficient cells transfected with TERRA targeting LNA gapmer (Fig. 5g, h).

### TCOF1 depletion leads to fragile telomere phenomena

The above results supported that TCOF1 acts to suppress the telomere transcription and R-loop formation, thus preventing replication fork stalling at telomeres. Replication defects at telomeres would result in a fragile-site phenotype, termed fragile telomeres [27]. In consistent with these speculations, we observed that depletion of TCOF1 induces fragile telomere phenomena including increased telomere-free ends and

multiple telomere signals (MTS) at the metaphase chromosome in U2OS and telomerase-positive HeLa cells (Figs. 6a–c and S7a–c). However, transfection of LNA gapmer that masks TERRA reduced fragile telomeres to a background level (Fig. 6a–c).

As a result of telomere replication defects, we observed a significant increase of DNA damage response (DDR) at telomeres (termed as TIF, Telomere dysfunction Induced Foci) in TCOF1-depleted U2OS, HeLa and MRC5 cells (Figs. 6d, e and S7d–g). Moreover, overexpression of RNase H1 decreased the number of TIF in TCOF1-depleted U2OS cells, demonstrating that excessive TERRA and R-loops activate DDR at telomeres (Fig. S7h, i). Furthermore, when TCOF1 is depleted, significantly increased cells displayed micronuclei, an indicator of genome instability (Fig. S7j, k) [28].

## Discussion

Here, we demonstrated that unintended increase of TERRA during S phase results in formation of R-loops that impede telomere replication. In addition, we discovered a previously unknown function of TCOF1 in facilitating telomere replication by suppressing telomere transcription during S phase. These findings highlight the importance of nucleolus in maintaining telomere integrity.

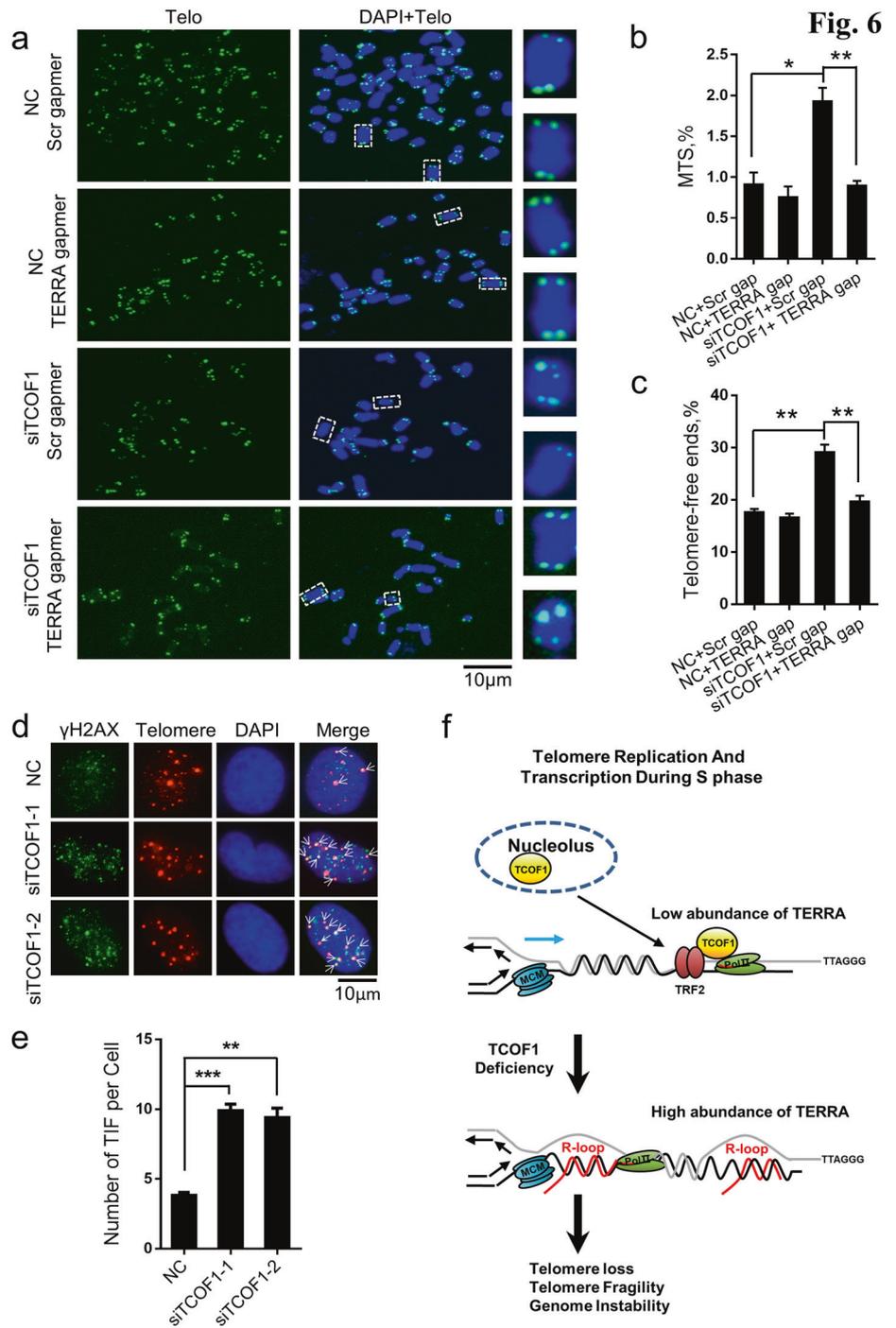
### Telomere transcription and excessive TERRA interfere with telomere replication

The frequent occurrence of transcription and DNA replication in cells results in many encounters between transcription and replication machineries. Unscheduled encounters often lead to transcription–replication conflicts and genome instability [1]. Telomeres are duplicated by conventional semi-conservative DNA replication, and replication forks could initiate within both telomere and subtelomere [4, 29]. However, telomere transcription is only initiated from the subtelomere and processes toward telomere [6]. Thus, the replication fork may encounter transcription machinery in both a head-on and co-directional orientation. Suppression of telomere transcription by TCOF1 during S phase is important for preventing potential transcription–replication conflict. Our result showed that in the absence of TCOF1, increased amount of active/elongating Pol II was observed at telomeres, which is consistent with increased amount of R-loops forming in telomeric DNA and accumulation of RPA and PCNA at telomeres indicating the stalling of replication fork (Figs. 4f, g, 5a–d, and S4f–k).

In addition, excessively expressed RNA transcripts may block replication fork movement by forming high-level structures [3]. In the context of telomere, TERRA may form R-loop structure with telomeric DNA that impedes the

**Fig. 6 TCOF1 depletion leads to fragile telomere phenomena.**

**a** Metaphase telomere FISH detection of telomere loss or multiple telomeres signals at ends of chromosomes. NC or siTCOF1 transfected U2OS cells were treated with scrambled LNA gapmer or TERRA targeting LNA gapmer for 24 h, then treated with nocodazole for 6 h, and subjected to FISH. **b** Quantification of **(a)**. The percentage of chromosomes with MTSs was calculated. For each group, 1000 or more chromosomes were examined. **c** Quantification of **(a)**. The percentage of chromosomes with one or more telomere-free ends was calculated. For each group, 1000 or more chromosomes were examined. All values are the average  $\pm$ SEM of three independent experiments. The unpaired Student's two-tailed *t*-test was used to determine the statistical significance ( $*P < 0.05$ ;  $**P < 0.01$ ). **d** Immunofluorescence and FISH detection of  $\gamma$ H2AX foci at telomeres in U2OS cells transfected with NC, siTCOF1-1 or siTCOF1-2. **e** Quantification of **(d)**. The average number of telomeric  $\gamma$ H2AX foci per cell was determined ( $n \geq 100$  cells). All values are the average  $\pm$ SEM of three independent experiments. The unpaired Student's two-tailed *t*-test was used to determine the statistical significance ( $**P < 0.01$ ;  $***P < 0.001$ ). **f** Proposed working model.



progress of replication fork. To prevent it, cells may need to decrease the amount of TERRA during S phase. Given that TERRA has a half-life of only 2 h [12], it is speculated that TERRA level would rapidly decreases if telomere transcription is suppressed. Indeed, TERRA level is much lower during S phase than G1 phase when TCOF1 is present that suppresses telomere transcription. However, in the absence of TCOF1, the levels of TERRA do not decrease from G1 phase to S phase (Fig. 3d, e).

**Duo functions of TERRA during telomere replication**

It has been previously reported that cells from human immunodeficiency, centromeric region instability, and facial anomalies syndrome have abnormally elevated TERRA levels that is associated with advanced telomere replication timing, telomere shortening and telomere aberrations such as chromosomes with telomere-free ends [30]. Here, we demonstrated that high level of TERRA

during S phase is associated with replication fork stalling and chromosome aberrations indicative of fragile telomeres. In contrast, it has also been previously proposed that TERRA may promote telomere replication through facilitating the initiation of the replication fork [31]. Seemed opposite role of TERRA in telomere replication could be interpreted as implying that while only limited number of TERRA is required for the initiation of replication, excessive TERRA has opposite effect by forming high structure such as R-loops, which instead impede the progression of telomere replication fork. In this scenario, TCOF1 plays an important role to limit TERRA at low levels during S phase.

### TCOF1 suppresses telomere transcription

The nucleolus is a nonmembranous nuclear organelle, which serves as a site for rDNA transcription, rRNA processing, and ribosome assembly [32]. Both rDNA and telomeric DNA are composed of repetitive sequences, which could be transcribed into RNA and have been considered as fragile sites for replication [27, 33]. In nucleolus, the central repeated domain of TCOF1 binds with RNA polymerase I, while C-terminus of TCOF1 is involved in rDNA promoter recognition and UBF recruitment [34]. With a striking similarity, we found that the central repeated domain of TCOF1 binds with RNA Pol II at telomeres and C-terminus of TCOF1 interacts with TRF2 (Figs. 2f and 4e).

In the nucleolus, TCOF1 enhances rDNA transcription by interacting with UBF [13]. However, in the context of DNA damage, TCOF1 plays an opposing role to suppress rDNA transcription through recruiting NBS1 [15]. Thereby, serving as a factor to regulate transcription, TCOF1 is able to both promote and suppress rDNA transcription depending on physiological situation and protein factors associating with it. Here, we proposed that TCOF1 is recruited to telomeres by interacting with TRF2, where it acts to suppress Pol II-mediated transcription.

It has been showed that TCOF1 depletion-induced replication defects on telomeres can be rescued by either masking TERRA (Fig. 5g, h) or expression of RNase H1 (Fig. 5e, f). These results excluded the possibility that TCOF1 affects telomere replication through altering ribosome biogenesis. In addition, we found that depletion of TCOF1 does not affect the amounts of TRF1 and TRF2 (data not shown).

### New function of TCOF1 and its relevance to TCS

TCS is a disorder of craniofacial development that is caused at the cellular level by a deficiency in the formation and survival of neural crest cells [14]. Although the pathogenesis of TCS is not fully understood, it has been proposed that mutations in *TCOF1* lead to deficient ribosome biogenesis that

subsequently causes apoptosis of neuroepithelial cell in embryos via a nucleolar stress-induced pathway [35]. Here, we found that TCOF1 deficiency results in telomere replication defects, causing fragile telomeres and genome instability. Undoubtedly, telomere stability is important factor for survival and proliferation of neuroepithelial cells [36]. Telomere replication defects and genome instability caused by mutations in *TCOF1* gene may contribute to pathogenesis of TCS. The underlying mechanism is worth further investigation.

## Materials and methods

### Cell culture and treatment

U2OS, HeLa, 293T, and MRC5 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured at 37 °C and 5% CO<sub>2</sub>. U2OS cells were grown in DMEM (Hyclone) supplemented with 10% newborn calf serum (PAA) and 1% penicillin/streptomycin (Hyclone). MRC5, 293T and HeLa cells were grown in DMEM (Hyclone) with 10% fetal bovine serum (GIBICO) and 1% penicillin/streptomycin (Hyclone). All the cell lines were identified by standardized short tandem repeat analysis. Mycoplasma was regularly examined during cell culturing, and no contamination occurred during this study. Sequences of the various siRNAs used in the study are: NC (negative control): 5'-UUCUCCGAACGUGUCACGUGdTdT-3'; siTCOF1-1: 5'-GGAAUCAGAUAGUGAGGAAdTdT-3'; siTCOF1-2: 5'-GAGGAUUCUUCAAGCAGUGAGGAAUdTdT-3'; siTRF2: 5'-ACAGAAGCAGUGGUCGA AUCdTdT-3'

### Cell-cycle synchronization

Cells were synchronized using the “double thymidine” approach, as previously described with a minor modification [37]. Briefly, U2OS cells were synchronized with thymidine (2 mM) for 20 h, washed with PBS (3×), then released into fresh medium for 12 h. Thymidine (2 mM) was then added for 17 h followed by washing with PBS (3×) before release into fresh medium.

### EdU labeling

Briefly, U2OS or HeLa cells grown on coverslips were cultured with medium supplemented with 10 μM EdU for 15 min. Cells were washed, fixed with 4% paraformaldehyde. Coverslips were stained at room temperature for 30 min with staining buffer (1 mM CuSO<sub>4</sub>, 10 μM FAM-azide, and 10 mM sodium ascorbate in PBS). After washing with PBST (3×), EdU-positive cells were visualized using a Zeiss microscope.

## Immunofluorescence (IF) and immunofluorescence in situ hybridization (IF-FISH)

IF and IF-FISH experiments were performed as previously described [38]. Briefly, cells grown on coverslips were fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.5% Triton X-100 for 30 min and blocked with 5% goat serum for 1 h. Fixed cells were incubated with primary antibody at 4 °C overnight, followed by incubation with secondary antibody for 1 h at room temperature. The coverslips were stained with DAPI and visualized using a Zeiss microscope.

For IF-FISH experiments, following secondary antibody incubation, coverslips were fixed with 4% paraformaldehyde for 30 min and sequentially dehydrated with 75, 95, and 100% ethanol. The coverslips were denatured at 85 °C for 5 min, then hybridized with PNA probe (TelC-Alexa488 or TelG-Cy3, Panagene) for 2 h at 37 °C. The coverslips were washed, stained with DAPI and visualized using a Zeiss microscope. Antibodies used are as follows: anti- $\gamma$ H2AX antibody (Cell Signaling Technology, 9718S), anti-PCNA (Abcam, ab92552), anti-RPA32 (Millipore, MABE285), anti-TCOF1 (Santa Cruz Biotechnology, sc-374536), anti-Fibrillarin (Abcam, ab166630), anti-Cyclin A (Abcam, ab181591), anti-TRF2 (Millipore, MABE1135-25UG).

## qPCR and RNA Slot blot

Total RNA was extracted with RNAiso Plus (Takara) and cDNA was prepared with PrimeScript II 1st Strand cDNA synthesis Kit (Takara) following the manufacturer's instruction. qPCR reactions were performed with 2 $\times$  Real-Star Power SYBR Mixture (GenStar, China). GAPDH was used for normalization. qPCR primers are listed in the Supplementary Table. RNA purified from the same amount of cells (0.5 M) was blotted onto membrane and RNA slot blot was performed as previously described [39].

## Transfection of TERRA antisense LNA gapmer

TERRA was masked by an LNA gapmer mediated approach as previously described [26]. The LNA gapmer contains phosphorothioate backbone modifications indicated by "\*" in the sequence below. The sequence of the LNA gapmer and the position of the LNA modifications are designed by Qiagen. Briefly, U2OS cells were transfected with NC or siTCOF1 siRNAs. 24 h post transfection, cells were then transfected with LNA gapmer by using Lipo3000 and incubated for 24 h. TERRA or control antisense LNA gapmer includes sequences 5'-T\*A\*A\*C\*C\*C\*T\*A\*A\*C\*C\*C\*T\*A\*A\*C-3' or 5'-C\*A\*C\*G\*T\*C\*T\*A\*T\*A\* C\*A\*C\*A\*C-3', respectively.

## Immunofluorescence in situ hybridization (IF-FISH) for detection of R-loop at telomeres

IF-FISH for R-loop detection was performed as previously described with a minor modification [40]. Briefly, cells were trypsinized and swelled in prewarmed 75 mM KCl solution and incubated for 15 min at 37 °C. Cells were pelleted and washed twice with methanol: acetic acid (3:1) at room temperature. Cells were transferred to slides. Slides were left to dry and blocked with 5% BSA in PBS for 1 h. Slides were incubated with S9.6 antibody (Kerafast) overnight at 4 °C, followed by incubation with secondary antibody for 1 h at room temperature. Slides were fixed with 4% paraformaldehyde, dehydrated and heat-denatured as described above. Then slides were hybridized with PNA probe (TelC-Alexa488 or TelG-Cy3, Panagene), stained with DAPI and observed using a Zeiss microscope.

## DRIP for detection of R-loop at telomeres

DRIP was performed as previously described with a minor modification [41]. Briefly, cells were collected, suspended in lysis buffer (0.5% SDS, 140 mM NaCl, 10 mM Tris-HCl [pH 7.4], 5 mM EDTA, PMSF) and subjected to sonication. Genomic DNA was extracted with phenol/chloroform procedure and treated with or without RNase H (Takara). Samples were purified, suspended in DRIP buffer (50 mM HEPES [pH 7.5], 140 mM NaCl, 5 mM EDTA, 0.05% Triton-X) and incubated with S9.6 antibody (Kerafast) overnight at 4 °C, followed by incubation with Protein A/G Plus-Agarose (Santa Cruz Biotechnology). DNA was washed, eluted, purified and analyzed by Slot-blot assay.

## Metaphase telomere FISH

Metaphase telomere FISH was performed as previously described with a minor modification [31]. Briefly, cells were incubated with nocodazole (0.5  $\mu$ g/ml) for 6 h. Cells were collected and swelled in prewarmed 75 mM KCl solution and incubated for 30 min at 37 °C. Cells were fixed three times with methanol: acetic acid (3:1, 10 ml) at room temperature. Cells were transferred to slides. Slides were left to dry, fixed with 3.7% formaldehyde in PBS for 2 min, dehydrated with ethanol series, and hybridized with PNA probe (TelC-Alexa488 or TelG-Cy3, Panagene). The slides were stained with DAPI and visualized using a Zeiss microscope.

## Western blot, immunoprecipitation (IP), and chromatin immunoprecipitation (ChIP)

Western blot, IP, and ChIP assays were carried out according to standard protocols. Antibodies used are as

follows: anti-TCOF1 (Santa Cruz Biotechnology, sc-374536), anti-GAPDH (Proteintech, 60004-1-Ig), anti-Flag (Sigma-Aldrich, F1804), anti-Tubulin (Proteintech, 66031-1-Ig), anti-TRF2 (Millipore, MABE1135-25UG), anti-RNA pol II CTD repeat YSRTSPS (phospho S2) antibody (Abcam, ab5095), anti-RNA pol II CTD repeat YSRTSPS antibody (Abcam, ab26721).

## Statistics

The results are shown as means  $\pm$  SEM and the Student's two-tailed unpaired *t*-test was used to determine the statistical significance (\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001). For every figure, statistical tests are justified as appropriate.

## Data availability

All relevant data are within the paper and its Supplemental files.

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**Author contributions** YZ, HH, and XN designed the experiments; XN, DX, YX, HZ, and XL performed the experiments; XN, YG, HL, and TZ analyzed the data; YZ, HL, and HH oversaw the project and wrote the paper.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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