CTC1 deletion results in defective telomere replication, leading to catastrophic telomere loss and stem cell exhaustion

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The proper maintenance of telomeres is essential for genome stability. Mammalian telomere maintenance is governed by a number of telomere binding proteins, including the newly identified CTC1–STN1–TEN1 (CST) complex. However, the *in vivo* functions of mammalian CST remain unclear. To address this question, we conditionally deleted *CTC1* from mice. We report here that *CTC1* null mice experience rapid onset of global cellular proliferative defects and die prematurely from complete bone marrow failure due to the activation of an ATR-dependent G2/M checkpoint. Acute deletion of *CTC1* does not result in telomere deprotection, suggesting that mammalian CST is not involved in capping telomeres. Rather, *CTC1* facilitates telomere replication by promoting efficient restart of stalled replication forks. *CTC1* deletion results in increased loss of leading C-strand telomeres, catastrophic telomere loss and accumulation of excessive ss telomere DNA. Our data demonstrate an essential role for *CTC1* in promoting efficient replication and length maintenance of telomeres. The EMBO Journal (2012) 31, 2309–2321. doi:10.1038/emboj.2012.96; Published online 24 April 2012
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Introduction

Mammalian telomeres consist of TTAGGG repeats, ending in single-stranded (ss) G-rich overhangs that play important roles in the protection and replication of chromosome ends. Telomeres are protected by the telomere binding proteins TRF2–RAP1 and TPP1–POT1. Telomere length independent removal of these proteins results in the activation of a p53-dependent DNA damage response (DDR) at telomeres, engaging either non-homologous end joining (NHEJ) or homologous recombination (HR) repair pathways, respectively (Wu *et al.*, 2006; Denchi and de Lange, 2007;

Guo *et al.*, 2007; Rai *et al.*, 2010). Chromosomal rearrangements as a result of incorrectly repaired DNA breaks could lead to increased cancer formation (McKinnon and Caldecott, 2007).

Semi-conservative DNA replication is used to replicate most telomere DNA, while telomerase is required to elongate the lagging G-strand. In budding yeast, the Cdc13, Stn1 and Ten1 (CST) protein complex plays important roles in telomere length regulation (Nugent *et al.*, 1996; Grandin *et al.*, 1997, 2001; Puglisi *et al.*, 2008). CST binds to the ss G-overhang, modulates telomerase activity and interacts with DNA polymerase α (Pol α) to couple leading- and lagging-strand DNA synthesis (Qi and Zakian, 2000; Chandra *et al.*, 2001; Bianchi *et al.*, 2004; Grossi *et al.*, 2004). The recent identification that the STN1 and TEN1 interacting protein CTC1 (conserved telomere maintenance component 1) binds ss DNA and stimulates Pol α -primase activity suggests that mammalian CST also functions in mediating lagging-strand telomere replication (Goulian and Heard, 1990; Casteel *et al.*, 2009; Miyake *et al.*, 2009; Surovtseva *et al.*, 2009; Dai *et al.*, 2010). However, how mammalian CST promotes telomere replication is not known.

In addition to its putative function in telomere replication, mammalian CST is also implicated in telomere end protection. shRNA-mediated deletion of STN1 and CTC1 results in telomere deprotection and telomere erosion (Miyake *et al.*, 2009; Surovtseva *et al.*, 2009). These results, together with recent observations that STN1 associates with TPP1 (Wan *et al.*, 2009), suggest that mammalian CST and shelterin might have overlapping functions in telomere end protection.

In this study, we report that formation of the CST complex is abrogated in the absence of CTC1. *CTC1* null mice are viable but die prematurely from complete bone marrow (BM) failure due to the activation of a G2/M checkpoint. While end-to-end chromosome fusions are prominent in *CTC1* null splenocytes and late-passage *CTC1*^{−/−} mouse embryo fibroblasts (MEFs), they are absent during early passages. These results indicate that unlike deletion of shelterin components, deletion of *CTC1* does not result in acute telomere deprotection. Rather, the DDR and chromosome fusions observed in the absence of *CTC1* are a consequence of rapid, catastrophic telomere shortening, coupled with the accumulation of ss G-overhangs. We demonstrate that CTC1 functions to promote efficient replication of telomeric DNA. Our results therefore provide mechanistic insights into the functions of CTC1 in telomere replication and telomere length maintenance.

Results

Complete BM failure and premature death in *CTC1* null mice

To ascertain the *in vivo* functions of CTC1, we generated conditional *CTC1* knockout mice. The *CTC1* gene has 24 exons and encodes a protein of 1121 amino acids (aa), with

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exon 2 containing the translation initiation start site. We engineered the *CTC1* locus and flanked exon 6 with loxP sites in the targeting vector (Figure 1A; Supplementary Figure S1A and B). Deletion of exon 6 is predicted to generate a frameshift mutation, resulting in premature termination of the *CTC1* open reading frame. Two independently targeted ES cell lines were generated and used to produce *CTC1*^{F/+} and *CTC1*^{F/F} mice and MEFs (Supplementary Figure S1C). Expression of Cre-recombinase in *CTC1*^{F/F} MEFs resulted in efficient deletion of exon 6 and the generation of a transcript encoding a severely truncated protein of only 234 aa, lacking all of the four predicted OB-folds required to interact with STN1 and TEN1 (Supplementary Figure S1B and D) (Theobald and Wuttke, 2004; Gao *et al*, 2007; Sun *et al*, 2009, 2011). Indeed, endogenous STN1 protein level was greatly reduced, and its localization to telomeres was undetectable in *CTC1*^{-/-} MEFs (Supplementary Figure S2A and B). These results suggest that the CST complex is likely unstable in the absence of CTC1.

We crossed *CTC1*^{F/F} mice with the zona pellucida 3 (ZP3)-Cre deleter mouse to generate *CTC1*^{-/-} animals. While *CTC1* null mice appeared grossly normal right after birth, they were consistently smaller than their wild-type (WT) littermates, developed sparse fur coverings and had a median lifespan of only 24 days (Figure 1B; Supplementary Figure S2C). Endogenous STN1 protein levels were markedly reduced or undetectable in all *CTC1*^{-/-} tissues examined (Supplementary Figure S2D). Gross inspections revealed that compared with WT controls, *CTC1* null mice possess significantly smaller thymi and spleens, although other organs appeared to be of normal size and weight in relationship to body weight (Figure 1C; Supplementary Figure S3). Histological examination of femurs derived from *CTC1*^{-/-} mice just before they died revealed complete BM failure, with a total absence of trilineage haematopoiesis and replacement of the BM by stromal adipose tissues that is likely the cause for premature death (Figure 1D). This BM failure phenotype is reminiscent of the haematopoietic defects observed in *Pot1b*^{-/-}; *mTR*^{+/-} mice, although in comparison BM defects in *CTC1*^{-/-} mice occurred much more rapidly (Hockemeyer *et al*, 2008; He *et al*, 2009). Since BM failure in *Pot1b*^{-/-}; *mTR*^{+/-} mice is due to compromised haematopoietic stem cell (HSC) function (Wang *et al*, 2011), we examined the HSC status of *CTC1* null mice. FACS analyses of BM derived from 4/5 *CTC1* null mice revealed severe depletion of both LK (Lin⁻, Sca-1⁻, c-kit⁺) and LSK (Lin⁻, Sca-1⁺, c-kit⁺) cells, populations enriched in HSCs and multipotent progenitors (0.022% in *CTC1*^{-/-} BM versus 2.54% for WT controls). These results suggest that HSCs were depleted in a majority of *CTC1* null mice, likely resulting in complete BM failure (Figure 1E). Interestingly, *in vivo* BrdU labelling of a younger *CTC1* null mouse revealed apparently normal numbers of LK cells, but with a significantly higher percentage of abnormal LSK cells in the G2/M-phase of the cell cycle compared with WT controls (42.3% for *CTC1*^{-/-} BM versus 2.51% for WT controls) (Figure 1E). We surmised that in the absence of *CTC1*, proliferating HSCs experience a G2/M cell-cycle arrest. Because the majority of *CTC1* null mice already display this phenotype at birth, we isolated fetal livers from 15.5-day-old WT and *CTC1*^{-/-} embryos. Compared with WT controls, a three-fold reduction in the total number of LSK cells was observed in 100% of *CTC1*^{-/-} fetal livers, with a two-fold increase in the number

of cells arrested in G2/M (Supplementary Figure S4A). Taken together, these data indicate that *CTC1* deletion resulted in a profound G2/M arrest in HSCs, leading to BM failure and premature death.

Proliferative defects in *CTC1* null cells

To further examine the impact of *CTC1* deletion on cellular proliferation, we performed *in vivo* BrdU incorporation experiments in WT and *CTC1* null mice. Deletion of *CTC1* resulted in an overall decrease in cellular proliferative capacity, with reduced BrdU incorporation in highly proliferative tissues including the skin, small intestine and testis (Figure 2A; Supplementary Figure 4B). Analysis of *CTC1*^{-/-} splenocytes revealed a cell-cycle profile indicative of a growth arrest phenotype, including the diminished expression of the proliferative markers phosphorylated pRB, p130 and PCNA and increased expression of p21, which promotes cell-cycle arrest in the setting of DNA damage (Figure 2B). Profound proliferative arrest was also observed in *CTC1* null MEFs, manifested as rapid growth arrest and a 20-fold increase in senescence-like phenotype by passage 2 (Figure 2C–E).

Chromosome fusions and progressive telomere loss in the absence of *CTC1*

The increased p21 expression in *CTC1*^{-/-} splenocytes suggested that the observed proliferative arrest could occur as a consequence of increased DNA damage. Since dysfunctional telomeres are recognized as damaged DNA, we examined the status of telomeres in *CTC1* null cells. A profound defect in telomere end protective function was observed in *CTC1* null cells. Compared with WT controls, 30% of chromosome ends in *CTC1* null splenocytes lacked telomeric signals, and end-to-end chromosome fusions were observed in 7% of all chromosomes examined (Figure 3A and B). None of these fusions possessed any telomere signals at fusion sites, suggesting that they arose as a consequence of critical telomere attrition. In support of this notion, TRF Southern analysis revealed extensive telomere loss in 5/5 of spleens and BMs isolated from *CTC1* null mice (Figure 3C; Supplementary Figure S5). An ~3-fold progressive increase in the number of fused chromosomes and telomere-free chromosome ends were observed in *CTC1* null MEFs from passages 10 to 19, along with a dramatic decline in total telomere length by passage 13, to 21% of total telomere signal observed in WT controls (Figure 3D–F). These results indicate that rapid and catastrophic telomere attrition occurred in the absence of *CTC1*, culminating in critical telomere shortening, chromosome fusions and compromised cellular functions.

***CTC1* deletion results in increased 3' G-overhang**

A consequence of *CTC1* deletion is increased formation of the 3' ss G-overhang. We therefore examined the status of the G-overhang in *CTC1* null tissues and MEFs. Non-denaturing in-gel hybridization revealed that *CTC1* null splenocytes and total BM exhibited a 23- to 34-fold increase in the signal intensity of the ss G-overhang (Figure 4A; Supplementary Figure S5). This dramatic increase in both overhang signal intensity and length heterogeneity correlated inversely with overall telomere length, with the greatest amount of the ss G-overhang signal observed in tissues with the shortest total telomere length. ss TTAGGG repeats were sensitive to Exonuclease I treatment, suggesting that they originated at

Figure 1 Conditional deletion of *CTC1*. (A) Schematic showing *CTC1* genomic locus, targeting construct and *CTC1* null allele. Black boxes, coding exons, white box, non-coding exon; red box, exon 6; arrowheads, loxP sites; green rectangle, PGK-neo gene; EV, EcoRV; blue rectangles, left and right arm probes for genomic Southern. (B) Kaplan-Meier survival curve of WT and *CTC1* null mice. Number of mice analysed is indicated. (C) Weight of spleens from WT and *CTC1* null mice. *P* = 0.0013. Two-tailed *t*-test was used to calculate statistical significance. (D) Histology of femurs derived from 25-day-old WT and *CTC1* null mice. Scale bar, 100 μm. (E) Top: Expression of Sca-1 and c-Kit in multilineage negative BM cells from WT and *CTC1*^{-/-} mice. Bottom: BrdU/7-AAD FACS profiles of BM LSK cells (Lin⁻ Sca-1⁺ c-kit⁺) derived from aged WT and *CTC1*^{-/-} mice. Results were expressed as percentage of total nucleated BM cells for each fraction. Number of mice analysed is indicated.

the 3' end (Figure 4B). In contrast to the increased ss TTAGGG repeats observed in *POT1b*^{-/-}; *mTR*^{+/-} mice (He *et al*, 2009), increased ss G-overhang was observed only in rapidly proliferating *CTC1* null splenocytes and BM and not in quiescent liver cells (Supplementary Figure S6). We next asked how quickly this increase in ss G-overhang signal intensity occurred after *CTC1* deletion. Tamoxifen treatment of *CAG-Cre^{ER}-CTC1^{F/F}* MEFs revealed that the increase in ss TTAGGG repeat intensity occurred rapidly,

within 9 days after activation of Cre-recombinase, prior to the onset of any observable telomere shortening or evidence of significant telomere loss at chromosome ends (Figure 4C; Supplementary Figure S7A-D). This result suggests that acute removal of *CTC1* induced ss G-strand formation that is not accompanied immediately by telomere dysfunction.

The marked ss TTAGGG repeat heterogeneity observed in *CTC1* null cells prompted us to use native and denaturing 2D

Figure 2 Deletion of *CTC1* results in attenuated proliferation. (A) Tissues isolated from *in vivo* BrdU-labelled mice were harvested and serial sections processed for H&E and immunostaining with an anti-BrdU antibody. (B) Immunoblots of pRB, p130, PCNA and p21 in splenocytes of the indicated genotypes. γ -tubulin served as loading control. (C) Proliferative defects in primary *CTC1* null MEFs. Two independent lines per genotype are shown. (D) SA- β -galactosidase staining and (E) quantification of WT and *CTC1* null MEFs at passage 2. Mean values were derived from at least three experiments. Two-tailed *t*-test was used to calculate statistical significance. Error bars: standard error of the mean (s.e.m.).

Figure 3 Chromosomal fusions and telomere deletion in *CTC1* null cells. (A) Telomere-FISH analysis on metaphase chromosome spreads of *CTC1*^{+/+} and *CTC1*^{-/-} splenocytes using Tam-OO-(CCCTAA)₄ telomere peptide nucleic acid (red) and 4,6-diamidino-2-phenylindole (DAPI, blue). A minimum of 180 metaphases were analysed per genotype. White arrows, chromosome fusion sites; green arrows, representative signal-free ends. (B) Quantification of chromosome aberrations in (A). Left, telomere signal-free chromosome ends; right, chromosome fusions. A minimum of 30 metaphases were examined. Mean values were derived from at least three experiments. Two-tailed *t*-test was used to calculate statistical significance. *P* values are indicated. Error bars: s.e.m. (C) *HinfI/RsaI* digested spleen and BM genomic DNA of the indicated genotypes were hybridized under denatured conditions with a ³²P-labelled [CCCTAA]₄-oligo to detect total telomere DNA. Telomere signal intensity (%) was quantified using EtBr staining intensity as the total DNA loading control. Error bars: s.e.m. (D) Telomere-FISH and DAPI (left) and DAPI only (right) analysis on metaphase chromosome spreads of immortalized *CTC1*^{+/+} and *CTC1*^{-/-} MEFs at passages 10 and 19 using Tam-OO-(CCCTAA)₄ telomere peptide nucleic acid (red) and DAPI (blue). White arrows, left panel, telomere signal-free ends, right panel, chromosome fusion sites. (E) Quantification of increased chromosome aberrations with passage in (D). Left, telomere signal-free chromosome ends; right, chromosome fusions. A minimum of 30 metaphases were examined. Mean values were derived from a minimum of 2 cell lines per genotype. Two-tailed *t*-test was used to calculate statistical significance. *P* values: **: <0.005, ***: <0.0005. Error bars: s.e.m. (F) left, *HinfI/RsaI* digested *CTC1*^{+/+} and *CTC1*^{-/-} MEF genomic DNA of the indicated passages were hybridized under denatured conditions with a ³²P-labelled [CCCTAA]₄-oligo to detect total telomere DNA. Telomere signal intensity (%) was quantified by setting WT total telomere DNA as 100%. Right, graph showing rapid telomere loss with passage observed in *CTC1*^{-/-} MEFs.

gel electrophoresis to determine whether other DNA conformations (linear or circular forms) are generated at telomeres following *CTC1* deletion. 2D gels performed on DNAs from WT MEFs and splenocytes under native conditions revealed an arc of ss G-rich telomeric DNA, corresponding to the 3' ss G-overhang (Figure 4D) (Wu *et al*, 2006; Nabetani and

Ishikawa, 2009; Oganessian and Karlseder, 2011). Under denaturing conditions, both ss and double-stranded (ds) telomeric DNAs were observed (Figure 4D). In *CTC1* null MEFs and splenocytes, the same DNA conformations were observed, with an increased amount of ss G-DNA present even when total ds telomeric signals were barely

Figure 4 Deletion of *CTC1* results in increased ss G-overhang. (A) In-gel hybridization analysis of genomic DNA isolated from BM and spleens of *CTC1*^{+/+} and *CTC1*^{-/-} mice under native (top panel) and after denaturation (bottom panel) with a ³²P-labelled [CCCTAA]₄-oligo probe to detect ss (top) and total (bottom) telomere DNA. Overhang signal intensity (%) was normalized to the total telomeric signals. (B) In-gel hybridization analysis of genomic DNA isolated from *CTC1*^{+/+} and *CTC1*^{-/-} MEFs treated with (+) or without (-) Exo I under native (top) and after denaturation (bottom). Gels were hybridized with a ³²P-labelled [CCCTAA]₄-oligo probe. (C) In-gel hybridization analysis of genomic DNA isolated from *Cre*^{ER}*CTC1*^{F/F} (two independent lines) and *CTC1*^{+/+} MEFs treated with 4-HT for the indicated times. Overhang signal intensity (%) was normalized to the total telomeric signals and compared with *CTC1*^{F/F} MEFs without 4-HT and WT MEFs plus 4-HT. (D) 2D gel electrophoresis analysis (first dimension: molecular weight, second dimension: DNA conformation) of genomic DNA isolated from *CTC1*^{+/+} and *CTC1*^{-/-} MEFs and splenocytes. Left panels, schematic of DNA conformations revealed under native (top) and denaturing (bottom) conditions. Right panels, 2D analysis of restriction enzyme digested genomic DNAs under native (top) and denaturing (bottom) conditions. White arrowheads, single-strand DNA; thick white arrowhead, double-strand linear DNA; black arrowheads, t-circles. DNAs were first fractionated under native conditions, hybridized with a ³²P-labelled [CCCTAA]₄-oligo probe to detect ss DNA species, then denatured and probed for all DNA conformations.

detectable (Figure 4D). Surprisingly, we detected the presence of prominent extrachromosomal telomeric repeat (ERCT) DNA in the form of circular ds telomere (t)-circles in *CTC1* null MEFs (Figure 4D). T-circles are a hallmark of cells that maintain their telomeres via the HR based alternative lengthening of telomeres (ALT) mechanism of telomere maintenance (Cesare *et al*, 2009; Nabatani and Ishikawa, 2009; Oganessian and Karlseder, 2011), suggesting that HR at telomeres is elevated in the absence of *CTC1*.

DDR in cells lacking *CTC1*

Protection of the ss telomeric overhang requires the coordinated activities of RPA and POT1 (Flynn *et al*, 2011). Since POT1 is typically limiting in mammalian cells (Kibe *et al*, 2010), we hypothesized that in the absence of *CTC1*, the POT1-TPP1 complex would be insufficient to fully protect the excessive ss telomeric DNA from interacting with RPA, resulting in subsequent activation of DNA damage checkpoint proteins ATR and its downstream kinase Chk1. In

support of this notion, shRNA-mediated depletion of *CTC1* results in the initiation of a DDR, manifested as increased phosphorylation of ATR and Chk1 (Figure 5A; Supplementary Figure S7E and F). In *CTC1*^{-/-} MEFs, activation of both ATR, ATM, Chk1 and Chk2 were observed, accompanied by rapid growth arrest and a senescence-like phenotype (Figure 2C–E and Figure 5A–C). Overexpression of *CTC1*, but not *STN1* alone, in *CTC1* null MEFs completely repressed checkpoint protein activation and rescued the proliferative defect, indicating that the DDR was due to *CTC1* deletion (Figure 5C and D). Deletion of *CTC1* did not affect the localization of shelterin complex at telomeres (Figure 5E; Supplementary Figure S8). Interestingly, telomere dysfunction induced DNA damage foci (TIFs) were not observed in *CTC1* null MEFs. Instead, γ -H2AX-positive foci were visible in the nucleus without obvious association with telomeric signals (Figure 5F). *CTC1* null MEFs were able to elicit robust TIF formation when endogenous POT1a and POT1b were removed from telomeres by overexpression of the dominant negative allele *TPP1*^{ARD} (Figure 5F). These results suggest that either the DDR observed was not due to telomere defects, or that they occurred on a subset of telomeres too short to be visualized by the TIF assay. Evidence of elevated global DNA damage (increased number of broken chromosomes, chromatids and fragments) was not detected in metaphase spreads from *CTC1* null MEFs (Figure 3A and D), suggesting that the observed DDR originated from chromosome ends devoid of telomere sequences. To test this hypothesis, we examined the co-localization of γ -H2AX and telomere-FISH signals on mitotic chromosomes (Cesare *et al*, 2009; Thanasoula *et al*, 2010). WT control MEFs infected with shRNA against *TRF2* or expressing *TPP1*^{ARD} displayed prominent γ -H2AX co-staining with telomeres, indicating that uncapped telomeres devoid of TRF2 or POT1–TPP1 activate a DDR (Figure 5G and H and data not shown). In contrast, in *CTC1* null MEFs γ -H2AX staining was observed only at chromosome ends missing telomere signals (Figure 5G and H). These results suggest that unlike cells missing shelterin components, the DDR in *CTC1* null MEFs occurs only at chromosome ends devoid of telomeres.

***CTC1* is required for efficient replication and restart of stalled replication forks at telomeres**

The repetitive nature of telomere DNA sequences poses a special challenge to the DNA replication machinery (Gilson and Geli, 2007; Sampathi and Chai, 2011). Telomere binding proteins are thought to be required to promote efficient telomere replication and prevent replication fork stalling at telomeres (Martinez *et al*, 2009; Sfeir *et al*, 2009; Badie *et al*, 2010). Given that the excessive ss G-overhang DNA induced by *CTC1* deficiency could arise as a defect in the synthesis of C-strand telomeres, we suspected that deletion of *CTC1* hindered efficient replication of telomeres. To test whether *CTC1* promotes efficient replication of telomere DNA, we examined WT and *CTC1* null MEFs for the presence of fragile telomeres, a hallmark of telomere replication defects (Sfeir *et al*, 2009). A significant increase in the number of fragile telomeres was observed in the absence of *CTC1*, with the number of fragile telomeres observed in *CTC1*^{-/-} MEFs equivalent to those observed in aphidicolin-treated WT controls (Figure 6A and B). *CTC1*^{-/-} MEFs treated with aphidicolin grew extremely poorly and generated few meta-

phases containing fragile telomeres. These results suggest that *CTC1* is required for proper telomere replication. To further test this hypothesis, we measured the efficiency of BrdU incorporation into telomeres during the S-phase, using BrdU labelling and CsCl separation of replicated leading/lagging telomeres (Dai *et al*, 2010). Cells were synchronized at the G1/S boundary and then released into S-phase in the presence of BrdU, and cells were collected after replication completed (Supplementary Figure S9A). Replicated and unreplicated DNA was separated on CsCl density gradients, and telomeres detected by slot-blot with a telomere probe (Supplementary Figure S9B). Acute deletion of *CTC1* in *CAG-Cre*^{ER}; *CTC1*^{F/F} MEFs was accomplished by treatment with 4-hydroxy tamoxifen (4-HT) during the synchronization process for 3 days. *CTC1* deletion resulted in decreased replication of both leading- and lagging-strand telomeres, accompanied by an increase in unreplicated telomeres (Figure 6C; Supplementary Figure S9C). To examine whether the long-term loss of *CTC1* preferentially affect C-strand synthesis at the lagging telomeres, we utilized chromosome-orientation FISH (CO-FISH) to monitor the status of leading- and lagging-strand telomeres in *CTC1* null MEFs. Compared with WT and early passage *CTC1* null MEFs, a significant decrease in the intensity of leading C-strand telomere signals was observed in late-passage *CTC1* null MEFs (Figure 6D and E). Taken together, these results suggest that replication of telomere DNA was less efficient in the absence of *CTC1*, with a preferential decrease in the amount of leading C-strand telomeres observed at late passages.

Since telomeres resemble difficult to replicate fragile sites, additional factors might be required to restart stalled telomere replication. To examine whether *CTC1* plays a role for efficient restart of stalled forks at telomeres, cells were synchronized and released into S-phase. At mid-S-phase, cells were treated with hydroxyurea (HU) for 1 h to arrest replication forks. After the removal of HU, BrdU was added to media for 1 h to pulse label daughter strands replicated from restarted forks (Supplementary Figure S9D). Cells were then collected immediately after BrdU labelling and the heavier BrdU-containing telomere DNA was then separated from non-BrdU-incorporated telomeres using the CsCl density separation technique (Figure 6F). The percentage of BrdU-labelled telomeres among the total telomere molecules represents the efficiency of the restart of stalled fork specifically at the telomeric region. Since the time for pulse labelling was short, the majority of telomeres was unlabelled and the population of BrdU-incorporated telomeres was low (Figure 6F). Comparison of BrdU-labelled telomeres revealed that *CTC1* deletion led to a statistically significant reduction in telomeric BrdU incorporation after the removal of HU (Figure 6G). Taken together, our data suggest that *CTC1* may play a role for the efficient restart of stalled replication forks at telomeres.

Discussion

In this study, we generated a conditional *CTC1* knockout mouse to demonstrate an essential role for *CTC1* in regulating telomere replication. Deletion of *CTC1* in mice resulted in G2/M cell-cycle arrest in haematopoietic stem cells, culminating in complete BM failure and premature death. This growth arrest phenotype is reminiscent of the Mec1-mediated G2/M cell-cycle arrest observed when the CST complex is disrupted in budding

yeast (Garvik *et al*, 1995; Grandin *et al*, 1997, 2001; Jia *et al*, 2004). Mechanistically, our data suggest that CTC1 facilitates telomere replication by promoting efficient restart of stalled replication forks. *CTC1* deletion results in increased loss of leading C-strand telomeres, catastrophic telomere loss and accumulation of excessive ss telomere DNA that activates an ATR-dependent DDR. Collectively, these findings suggest that

mammalian CTC1 is required to prevent rapid telomere loss by promoting its efficient and complete replication.

The CST complex appears to possess evolutionarily conserved functions in DNA replication (Giraud-Panis *et al*, 2010; Nakaoka *et al*, 2011). In yeast, Cdc13 and Stn1 interact with Pol α and couple telomeric C- and G-strand synthesis, preventing the formation of excessively long G-overhangs.

CST could either mediate C-strand fill in following G-strand extension by telomerase and/or prevent excessive C-strand resection by nucleases (Qi and Zakian, 2000; Grossi *et al*, 2004; Petreaca *et al*, 2006; Puglisi *et al*, 2008). In mouse cells, the shelterin component POT1b functions to prevent C-strand resection by unknown nucleases (Hockemeyer *et al*, 2008; He *et al*, 2009). Since the G-overhang appears to be of normal length in quiescent *CTC1*^{-/-} liver cells, CTC1 is unlikely to play a significant role in mediating C-strand resection in all cell types. We favour a role for CTC1 in facilitate priming and synthesis of telomere DNA chains on the lagging-strand template, and/or promoting efficient re-initiation of lagging telomere DNA synthesis by DNA Pol α at stalled replication forks. These functions might be especially important if a replication stall uncouples DNA Pol α and replication helicase activities the fork (Yao and O'Donnell, 2009). In the absence of CTC1, fork stalling at telomeres would give rise to ss G-strand DNA that are insensitive to Exonuclease I digestion. Replication fork stalling appears to be a particularly vexing problem at telomeres, since the aberrant G-rich secondary structures are postulated to cause steric hindrance, making them difficult substrates to replicate. Therefore, besides the CST complex, several other telomere binding proteins and associated factors have evolved to facilitate replication fork progression through telomeres (Crabbe *et al*, 2004; Miller *et al*, 2006; Martinez *et al*, 2009; Sfeir *et al*, 2009; Badie *et al*, 2010; Ye *et al*, 2010). While we postulate that the CST complex is required for efficient replication of telomeres, we cannot rule out the possibility that they also participate in general DNA replication and the restart of stalled forks at non-telomere regions.

Stalled replication forks at telomeres might also initiate aberrant recombination events that could account in part for the catastrophic loss of total telomeric DNA observed in *CTC1* null cells (Lustig, 2003; Miller *et al*, 2006). The loss of bulk telomeric DNA observed in the BM of *CTC1* null mice occurred much more rapidly than the progressive loss of telomere sequences observed in *POT1b*^{-/-}; *mTR*^{+/-} mice, another mouse model in which rapid telomere attrition results in haematopoietic defects (He *et al*, 2009; Wang *et al*, 2011). In contrast to the *POT1b*^{-/-}; *mTR*^{+/-} mouse, in which telomere shortening and increased G-overhang was observed in all organs examined, telomere defects in *CTC1* null animals were limited only to rapidly proliferative tissues (haematopoietic system, skin, small intestine) and were not observed in the quiescent liver. These results further support a defect in telomere replication as a mechanism for the phenotypes observed in *CTC1* null mice. Since HR is the

primary pathway utilized to restart stalled replication forks, we postulate that increased aberrant HR at telomere replication forks in the absence of CTC1 results in catastrophic telomere shortening. In support of this notion, T-circles, a product of cells that utilize HR for telomere maintenance (Wang *et al*, 2004; Nabetani and Ishikawa, 2009; Oganessian and Karlseder, 2011), are prominent in *CTC1* null MEFs, suggesting that CTC1 is required to repress aberrant HR at telomere replication forks. It is intriguing to note that *Arabidopsis* *CTC1* mutants also display elevated recombination and extensive telomere loss prior to end-to-end chromosome fusions (Surovtseva *et al*, 2009).

The mammalian CST complex is thought to play a role in telomere end protection (Miyake *et al*, 2009; Surovtseva *et al*, 2009). However, our studies suggest that telomere protection mediated by the CST complex is distinct from that mediated by the shelterin complex. Removal of mammalian TRF2-RAP1 or TPP1-POT1 complexes results in immediate telomere dysfunction, manifested as recruitment of ATM and ATR to chromosome ends, respectively, robust TIF formation, and initiation of NHEJ and HR-mediated repair reactions, while overall telomere length remains intact (Wu *et al*, 2006; Denchi and de Lange, 2007; Guo *et al*, 2007; Deng *et al*, 2008; Dimitrova *et al*, 2008; Rai *et al*, 2010). By contrast, increased TIF formation was not observed in *CTC1* null MEFs, even at late passages. We postulate that in the absence of CTC1, the increased ss G-overhang generated over time could not be completely protected by TPP1-POT1, leading to the recruitment of RPA and subsequent activation of ATR/Chk1 (Flynn *et al*, 2011, 2012). Increased telomere attrition ultimately results in the displacement of the shelterin complex from chromosome ends, leading to the recruitment of γ -H2AX and the activation of ATM/Chk2. Our results suggest that in contrast to the shelterin complex, which is involved in both telomere end protection and replication, the CST complex promotes telomere replication and does not play a direct role in repressing a DDR at mammalian telomeres. It would be interesting to understand whether shelterin cooperates with CST to regulate mammalian telomere replication.

Several inherited human BM failure syndromes are due to defects in telomere maintenance. For example, Dyskeratosis congenita (DC) is characterized by very short telomeres, BM failure, cutaneous phenotypes and increased cancer incidence (reviewed in Young, 2010). Recently, whole-exome sequencing revealed that missense mutations in *CTC1* results in the human disorder Coats plus (Anderson *et al*,

Figure 5 *CTC1* deletion activates a DDR. (A) Immunoblot of pATR, p53 and p21 in WT MEFs treated with the indicated shRNAs. γ -Tubulin served as loading control. (B) Immunoblot of pATM, pChk1 and pChk2 in two independent lines of MEFs of the indicated genotypes. γ -Tubulin served as loading control. (C) Immunoblot of pATM, pChk1, pChk2, Flag-CTC1, HA-STN1 and endogenous STN1 in *CTC1* null MEFs reconstituted with indicated cDNAs. γ -tubulin served as loading control. (D) Proliferation of SV40 immortalized *CTC1* null MEFs reconstituted with the indicated cDNAs. (E) Telomere-PNA FISH demonstrating the localization of shelterin components to telomeres in *CTC1*^{-/-} MEFs. Cells were stained with the indicated antibodies (green), telomere-PNA FISH (red) and DAPI (blue). Localization of the indicated shelterin proteins on telomeres (telo) in *CTC1* null MEFs. (F) γ -H2AX-positive TIFs in WT (top) and *CTC1*^{-/-} (bottom) MEFs, expressing either vector or *TPP1*^{ARD} cDNA. Cells were fixed 72 h after retroviral infection, stained with anti- γ -H2AX antibody (green), Tam-OO-(CCCTAA)₄ telomere peptide nucleic acid (red) and DAPI (blue). (G) WT MEFs expressing *TPP1*^{ARD} or *CTC1*^{-/-} MEFs were arrested in mitosis with colcemid, metaphase spreads prepared and stained with anti- γ -H2AX antibodies (green), Tam-OO-(CCCTAA)₄ telomere peptide nucleic acid (red) and DAPI (blue). Arrows point to sites of γ -H2AX localization. Insets show enlarged images. (H) *CTC1*^{-/-} MEFs (two independent lines), WT MEFs treated with shRNA against TRF2 or expressing *TPP1*^{ARD} were assayed for TIF formation. Quantification of the number of TIFs with or without visible telomere signals co-localizing with γ -H2AX staining are indicated.

Figure 6 *CTC1* is required for efficient replication and efficient restart of stalled replication fork at telomeres. (A) Left, examples of the fragile telomere phenotype observed in *CTC1* null MEFs and WT MEFs treated with 0.2 μ M aphidicolin. Arrows point to fragile telomeres. (B) Quantification of fragile telomeres observed in (A). (C) Summary of telomere replication in *CTC1*^{F/F} and *CAG-Cre*^{ER}; *CTC1*^{F/F} MEFs treated with or without 4-hydroxytamoxifen (4-HT) to delete *CTC1*. Two-tailed *t*-test was used to calculate statistical significance. $n \geq 4$, Error bars: s.e.m. (D) Examples of CO-FISH images of partial metaphase spreads from WT (PD 49), *CTC1*^{-/-} (PD14) and *CTC1*^{-/-} (PD58) MEFs. Red signals: G-strand telomeres, green signals: C-strand telomeres. Red arrowheads point to loss of lagging-strand telomeres and green arrowheads point to loss of leading-strand telomeres. (E) Quantification of signal intensity of leading- and lagging-strand telomeres in (D). Two independent cell lines were analysed, and a minimum of 35 metaphases and 1500 chromosomes were scored per genotype and passage. Mean values were derived from two cell lines. The two-tailed *t*-test was used to calculate statistical significance. Error bars: s.e.m. (F) Distribution of telomere signals on CsCl gradient after releasing from HU treatment. BrdU pulse-labelled telomere DNA following HU treatment was separated on CsCl gradient. Fractions were collected from the bottom of the gradient and slot-blot was used to quantitate the amount of telomeres in each fraction. Telomere signals from telomeres with density ≥ 1.755 g/ml are amplified in the insert. (G) Percentage of BrdU-containing replicated telomeres. One-tailed *t*-test was used to calculate statistical significance. $n = 3$. Error bars: s.e.m.

2012; Polvi *et al*, 2012; reviewed in Savage, 2012). Coats plus is an autosomal recessive disorder characterized by retinal telangiectasia, intracranial calcifications, osteopenia and gastrointestinal bleeding (Tolmie *et al*, 1988; Crow *et al*, 2004). Interestingly, white blood cells from Coats plus patients with compound heterozygous mutations have very short telomeres, suggesting that telomere maintenance function is compromised in these patients. Indeed, some Coats plus patients develop a normocytic anaemia that reflects a degree of BM failure (Anderson *et al*, 2012). These exciting clinical results suggest that similar to the phenotypes observed in our *CTC1* null mice, human *CTC1* missense mutations also confer haematopoietic defects. Understanding how these point mutations impact upon *CTC1* function should reveal additional insights into the function of the CST complex at telomeres. We postulate that patients with a family history of unexplained BM failure and without the characteristic DC mutations affecting telomere length maintenance might have mutations in *CTC1*, and perhaps in other members of the CST complex as well. *CTC1* mutations should therefore be examined in patients with inherited BM failure lacking mutations in genes encoding shelterin components and proteins involved in telomerase function.

Materials and methods

Generation of *CTC1* conditional knockout mice

A long-range PCR strategy was used to obtain three correct genomic fragments for generating a conditional targeting vector. A 3.3-kb *KpnI*-*XhoI* fragment (5' arm), a 5.1-kb *Sall*-*NotI* fragment (3' arm), and a 1.2-kb *HindIII*-*Sall* fragment containing exon 5 and the second loxP at 3' end were inserted into pKOII vector (Wu *et al*, 2006). The targeting vector was linearized with *NotI* and electroporated into AB2.1 ES cells. ES cells were screened by long-range PCR and two selected ES cell candidates (1A11, 3E3) with correct recombination were further confirmed by Southern analysis and injected into C57BL/6 blastocysts. Chimeric mice were mated with WT C57BL/6 to generate *CTC1*^{F/+} offspring. *CTC1*^{F/+} mice were intercrossed to obtain *CTC1*^{F/F} homozygous mice. Female *CTC1*^{F/F} mice were mated with ZP3-Cre or chicken actin (CAG)-Cre male mice to give rise to *CTC1*^{F/+};ZP3-Cre or *CTC1*^{F/+};CAG-Cre^{ER} mice. Female *CTC1*^{F/+};ZP3-Cre mice were crossed with WT mice to obtain *CTC1*^{+/-} heterozygous mice. The homozygous *CTC1*^{-/-} mice were generated by intercrossing *CTC1*^{+/-} mice. All mice were maintained according to Yale University IACUC-approved protocols.

Generation of *CTC1*^{-/-} and *CTC1*^{F/F}, CAG-Cre^{ER} MEF

CTC1^{-/-} MEF were isolated from E13.5 embryos by intercrossing *CTC1*^{+/-} mice. *CTC1*^{F/F}; CAG-Cre^{ER} or *CTC1*^{F/F} MEFs were obtained from E13.5 embryos by intercrossing *CTC1*^{F/+};CAG-Cre with *CTC1*^{F/+} mice. Primary MEFs at passages 1 or 2 were immortalized with SV40 large-T antigen retrovirus. To acutely delete *CTC1*, *CTC1*^{F/F}; CAG-Cre^{ER} MEF was treated with 1 μ M 4-HT for 2 days and maintained in the presence of 10 nM 4-HT for 1 week. All MEFs were cultured in DMEM with 10% serum.

Isolation of BM, splenocytes and fetal liver cells

Mice were injected with BrdU (i.p.) at the dosage of 150 mg/kg 2 h before euthanizing. BM cells were flushed from hind limb bones through a 21-gauge needle into HBSS+ (Hanks balanced salt solution (Invitrogen, Carlsbad, CA)), 2% FBS (Invitrogen) and 10 mM HEPES. Fetal liver from E15.5 embryo was dissociated by passing 21-gauge needle. Spleen was smashed between two pieces of glass slide. The cells were passed through 40 μ m cell strainer to make sure of single-cell suspensions. Red blood cells were removed by the lysis buffer (3% acetic acid in methylene blue (Stem Cell Technologies, Vancouver, BC, Canada)). Fetal liver cells were incubated in GMEM media with 10% serum in the presence of 10 μ M

BrdU for 2 h at 37°C. To obtain metaphase chromosome, splenocytes were suspended in DMEM with 10% serum at 37°C for 3 h and treated with Colcemid for 1 h.

Flow cytometry

In all, 1×10^7 total BM or fetal liver cells were centrifuged and resuspended in 200 μ l HBSS+. Cells were stained for 15 min with a cocktail of antibodies including nine lineage markers: Ter-119, CD3, CD4, CD8, B220, CD19, IL-7R α , GR-1 and Mac-1 (eBioscience, San Diego, CA) conjugated to APC-Cy-7 (47-4317 eBioscience). In addition, the cocktail also contained anti-Sca-1-PE (12-5981 eBioscience) and anti-c-Kit-APC (17-1171 eBioscience). After staining, cells were washed, resuspended in HBSS+ and analysed by flow cytometry (Calibur or LSRII, BD). LSK populations were selected based on low or negative expression of the mature lineage markers and dual-positive expression for Sca-1 and c-Kit. The analysis of BrdU incorporation was performed using the FITC BrdU flow kit (BD Pharmingen, Cat#552598). FlowJo software was used for all data analysis.

Generation of mouse *STN1* antibody, expression plasmids and commercial antibodies used

Full-length mouse *STN1* cDNA was obtained from RT-PCR and subcloned into pRSET-A (Invitrogen) vector. His-tagged *STN1* fusion protein was expressed in BL21DE3 bacteria and purified by Nickel-charged agarose beads according to Invitrogen's protocols. Rabbit antiserum were raised by injection of purified fusion protein (Ptlab) and affinity purified by cellulose membrane containing fusion protein. Full-length mouse *CTC1* and *STN1* expression vectors were generated by inserting full-length cDNAs into pQCXIP retrovirus vectors. Anti-mouse TRF1 and TRF2 were gifts from Jan Karlseder (Salk). Commercial antibodies used were Anti-Phospho-Chk1 (Ser345) (#2348), Anti-Phospho-ATR (Ser428) (#2853), Anti-Phospho-ATM (Ser1981) (#4526) from Cell Signaling; Anti-pRB (#554136) and Anti-Chk2 (#611570) from BD Pharmingen; Anti-p21 (sc-6246), Anti-p130 (sc-317) and Anti-PCNA (sc-7907) from Santa Cruz; Anti-Flag, HA, γ -tubulin from Sigma; Anti- γ -H2AX (#05-636) from Upstate; Anti-RAP1 (#A300-306) from Bethyl.

Knockdown of *STN1* or *CTC1* with shRNA

Lentivirus-based shRNAs against mouse *STN1* and *CTC1* were purchased from Sigma (sequences available on request). The knockdown efficiency of different shRNA was tested by either RT-PCR or immunoblotting. Total RNA was extracted with Trizole reagent (Invitrogen) and reverse transcription was performed with Super transcriptase III Kit (Invitrogen). PCR was performed with SybrGreen PCR Kit (Biolab) and reactions were run on an ABI PCR machine.

Histology and immunohistochemistry

Tissues were fixed in 10% formalin, paraffin embedded, sectioned and stained for haematoxylin and eosin. BrdU staining was done by Yale Pathology Histology Service Lab.

PNA-FISH, CO-FISH, TIF and immunofluorescence-FISH assays

Cells were treated with 0.5 μ g/ml of Colcemid for 4 h before harvest. Trypsinized cells were treated with 0.06 M KCl, fixed with methanol:acetic acid (3:1) and spread on glass slides. Metaphase spreads were hybridized with 5'-Tam-OO-(CCCTAA)₄-3' probe. For CO-FISH, cells were treated with BrdU for 14 h before addition of Colcemid. Metaphase spreads were sequentially hybridized with 5'-FITC-OO-(TTAGGG)₄-3' and 5'-Tam-OO-(CCCTAA)₄ probes. For the TIF assay, cells were seeded in eight-well chambers and immunostained with primary antibodies and FITC-secondary antibody, then hybridized with 5'-Tam-OO-(CCCTAA)₄-3' probe. IF-FISH on metaphase spreads were performed as described (Thanasoula *et al*, 2010).

TRF Southern and 2D-gel

In all, 5–10 μ g total genomic DNA were separated by pulse-field gel electrophoresis (Bio-Rad). The gels were dried at 50°C and prehybridized at 58°C in Church mix (0.5 M NaH₂PO₄, pH 7.2, 7% SDS) and hybridized with γ -³²P-(CCCTAAA)₄ oligonucleotide probes

at 58°C overnight. Gels were washed with 4 × SSC, 0.1% SDS buffer at 55°C and exposed to Phosphorimager screens. After in-gel hybridization for the G-overhang under native conditions, gels were denatured with 0.5 N NaOH, 1.5 M NaCl solution and neutralized with 3 M NaCl, 0.5 M Tris-Cl, pH 7.0, then reprobed with (TTAGGG)₄ oligonucleotide probes to detect total telomere DNA. To determine the relative G-overhang signals, the signal intensity for each lane was scanned with Typhoon (GE) and quantified by ImageQuant (GE) before and after denaturation. The G-overhang signal was normalized to the total telomeric DNA and compared between samples. For 2D-gel TRF Southern assay, 20 µg of total genomic DNA was resolved in the first dimension in a 0.4% agarose gel at 1.5 V/cm, EB stained and cut out. The gel slice was casted into a second 1% agarose gel at a 90° orientation from the first gel and resolved at 8 V/cm. The steps for hybridization were same as the described above.

Synchronization of cell-cycle and CsCl gradient grade separation of leading and lagging daughter telomeres from MEFs

For synchronizing MEFs, cells were serum starved in DMEM-0.1% FBS for 48 h and then released into fresh DMEM-10% FBS containing 1 µg/ml aphidicolin for 24 h. Aphidicolin was then removed by washing cells with pre-warmed PBS (three times) before released into fresh DMEM-10% FBS. MEFs synchronized in G1/S boundary were released into DMEM-10% FBS supplemented with 100 µM BrdU for 9 h. Cells were collected, genomic DNA was isolated with DNAeasy DNA isolation kit (Qiagen), digested with *AluI/RsaI/CfoI/MspI/HaeIII/HinfI* at 37°C overnight, mixed with CsCl solution, centrifuged in vertical rotor VTi65 (Beckman Coulter) for 16–22 h (45 000 r.p.m., 21°C). Fractions were collected, denatured in 1 M NaOH for 10 min, neutralized with 6 × SSC, and used for slot-blot. Blot was hybridized to telomere probe at 42°C overnight. All procedures were performed in dark.

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Measurement of the efficiency of restart of stalled replication fork

Cells were synchronized at G1/S boundary and then released to S-phase for 3 h. HU (4 mM) was added to stall replication fork for 1 h. Cells were then washed with pre-warmed media for three times and released into DMEM-10% FBS containing 100 µM BrdU for 1 h. Procedures following this step were performed in dark. Cells were collected and genomic DNA was isolated, digested with *AluI/RsaI/CfoI/MspI/HaeIII/HinfI* at 37°C for overnight, mixed with CsCl solution, and centrifuged at 39 000 r.p.m. 21°C for 16–22 h. Fractions were collected, denatured in 1 M NaOH, neutralized with 6 × SSC, and used for slot-blot. Blot was hybridized to telomere probe at 42°C overnight. Telomeres with the ≥ 1.755 g/ml were considered replicated DNA, since the complete BrdU-incorporated lagging telomeres were located at density ~ 1.760 g/ml (Supplementary Figure S7C and D).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: PG, WC and SC conceived the project and designed the experiments. PG, JM, YW, CH and WC performed the experiments and generated data for the figures. PG, WC and SC analysed and interpreted the data, composed the figures and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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