**CTC1-mediated C-strand fill-in is an essential step in telomere length maintenance**

Xuyang Feng, Shih-Jui Hsu, Christopher Kasbek, Mary Chaiken and Carolyn M. Price

## SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

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**Figure S1. CTC1 gene disruption in HCT116 cells.** Cells were grown with/without tamoxifen for the indicated times. (A) Growth curve showing effect of tamoxifen on *CTC1*$^{F/+}$ cells. (B) Representative Western blots showing STN1 and TEN1 protein levels in *CTC1*$^{F/F}$ and *CTC1*$^{-/-}$ cells. Actin served as a loading control. (C) FACS analysis of DNA content to show cell cycle profile. (D) β-galactosidase staining after 20 days growth with/without tamoxifen. Arrows indicate senescent cells.

**Figure S2. Quantification of telomere FISH signals and γH2AX localization to telomeres and signal free ends.** (A) Metaphase spreads from CTC1 conditional cells grown with tamoxifen for the indicated times were hybridized with telomere and centromere probe and scored for Signal Free Ends (SFE), sister chromatid associations or telomere fusions. Values represent % of chromosomes with one or more SFE, associated sisters, a SFE on only one sister chromatid (single SFE) or SFE on both sisters (double SFE). Chromosome fusions (chromosome or chromatid) were scored for number of fusions per 100 chromosomes. Values represent the mean from ≥3 independent experiments; ≥2,000 chromosomes were scored per time point. (B) Representative image of chromosome spreads from CTC1 conditional cells showing non-telomeric γH2AX staining on chromosome arms. (C) Quantification of γH2AX on chromosome spreads from CTC1 conditional cells treated with/without tamoxifen for 4 days. Telomeres were detected by FISH, γH2AX by immunostaining as described for Fig. 3. Chromosomes with γH2AX foci on chromosome arms (Non-Tel), at one or more telomeres with detectable telomeric DNA (Tel) or at signal free ends (SFE). N = 3 independent experiments, mean ± S.E.M.

**Figure S3. G-overhang, telomere length and telomerase activity analysis in CTC1 conditional cells with/without expression of rescuing FLAG-CTC1 allele.** (A) Non-denaturing in-gel hybridization with TAA($C_3TA_2$)$_3$ probe to the telomeric C-strand. CTC1 conditional cells were treated with tamoxifen for the indicated times. (B) Western blot showing expression of FLAG-CTC1 in *CTC1*$^{F/F}$ cells. Blot was probed with antibody to FLAG or to actin as a loading control. (C) Rescue of G-overhang elongation after expression of FLAG-CTC1 rescuing allele. CTC1 conditional cells were grown with tamoxifen for the indicated times and G-overhang abundance was examined by in-gel hybridization. Gels show hybridization of TAA($C_3TA_2$)$_3$ probe to genomic DNA under native (top) and denaturing (bottom) conditions. (D-E) Southern blots showing length of telomere restriction fragments. Brackets indicate telomeres that appear to have undergone elongation or shortening in *CTC1*$^{-/-}$ cells. Probe was TAA($C_3TA_2$)$_3$. Mean telomere length is indicated below each lane. (D) CTC1 conditional cells. (E) CTC1 conditional cells with or without FLAG-CTC1 rescuing allele. (F) TRAP assay showing telomerase activity in *CTC1*$^{F/F}$ and *CTC1*$^{-/-}$ cells after 7 days of tamoxifen treatment. (F) Cartoon illustrating how loss of C-strand fill-in leads to progressive telomere shortening at each round of telomere replication. First round of replication shows products generated from leading and lagging strand telomeres. The second round of

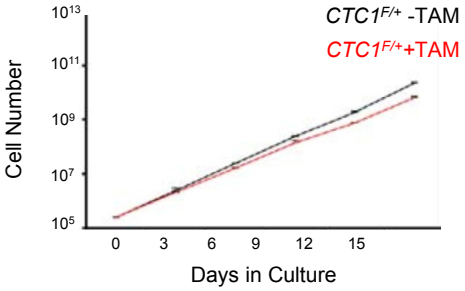
replication shows products generated from the lagging strand telomere only. Shortening will be further increased by exonuclease processing steps (not illustrated).

**Figure S4. Analysis of Pot1 overexpression.** (A) Western blots showing level of exogenous FLAG-POT1 expression relative to endogenous POT1. Cell extracts were prepared from *CTC1$^{F/F}$* cells or *CTC1$^{F/F}$* cells expressing POT1-FLAG. Duplicate lanes from the same gel were transferred to membrane and probed with FLAG (left) or POT1 (right) antibody. The top portion of each blot was probed with antibody to actinin as a loading control. (B) Raw data from Fig. 4B-C showing quantification of telomeric and non-telomeric localization of γH2AX staining. (C) Representative slot blots from ChIP experiments to quantify RPA, γH2AX or TRF2 localization to telomeres. Samples were from CTC1 conditional cells that did/did not express exogenous POT1-FLAG. Cells were grown with/without tamoxifen for 7 days. (D) Raw data from ChIP experiments shown in Fig. 4D, Fig S4C and Fig. S4E. (E) Quantification of TRF2 ChIP to showing changes in TRF2 localization to telomeres after CTC1 loss and POT1 overexpression. N = 3 experiments mean ± S.E.M. (F) Growth curve showing effect of POT1 expression on growth of *CTC1$^{F/F}$* and *CTC1$^{-/-}$* cells. Cells were grown with tamoxifen for 7 days then the tamoxifen was removed because the transient drug treatment reduced stress on the cells and maximized the effect of POT1 overexpression.

**Figure S5. Southern blot analysis of telomere length in CTC1 conditional cells with/without FLAG-POT1 overexpression.** Telomeric restriction fragments were detected by hybridization with $TAA(C_3TA_2)_3$ probe. Mean telomere length is indicated below each lane. Brackets indicate telomeres that show shortening after culture in tamoxifen.

Feng. et al. supplementary figure 1

A

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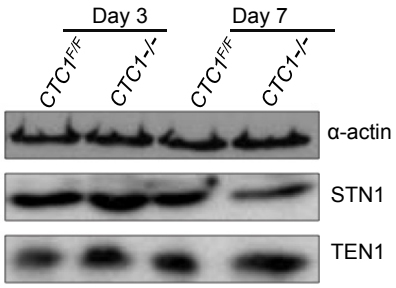


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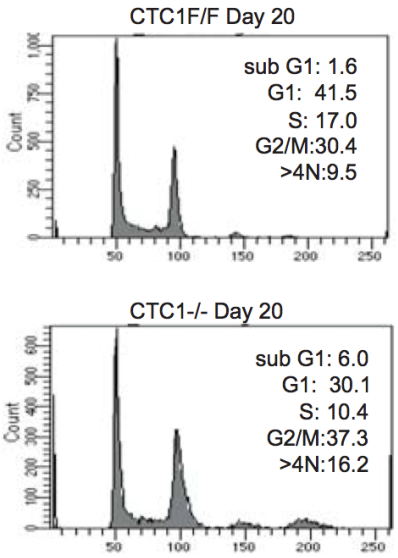


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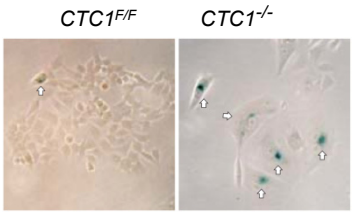
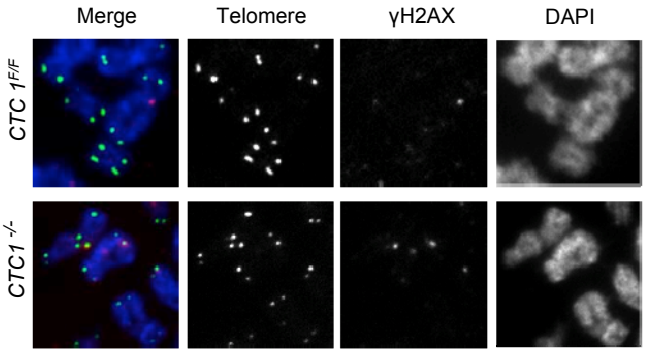


Feng. et al. supplementary figure 2

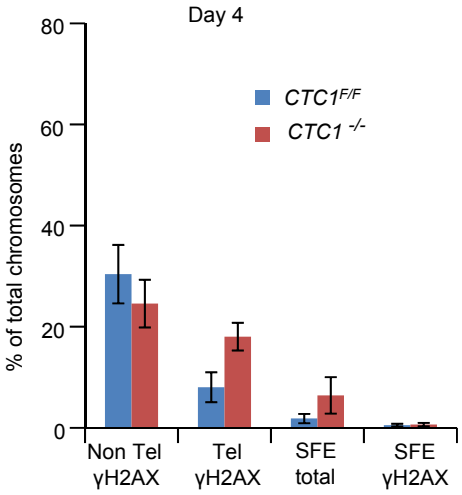
A

| Day of TAM treatment | SFE | Sister chromatid association | Chromosome fusion | Single SFE | Double SFE |
|---|---|---|---|---|---|
| day 0 | 2.26% | 0.87% | 0.00% | 1.15% | 1.12% |
| day 7 | 3.58% | 2.09% | 0.17% | 2.09% | 1.49% |
| day 10 | 4.10% | 2.16% | 0.08% | 2.33% | 1.78% |
| day 14 | 6.04% | 1.68% | 0.31% | 2.56% | 3.49% |

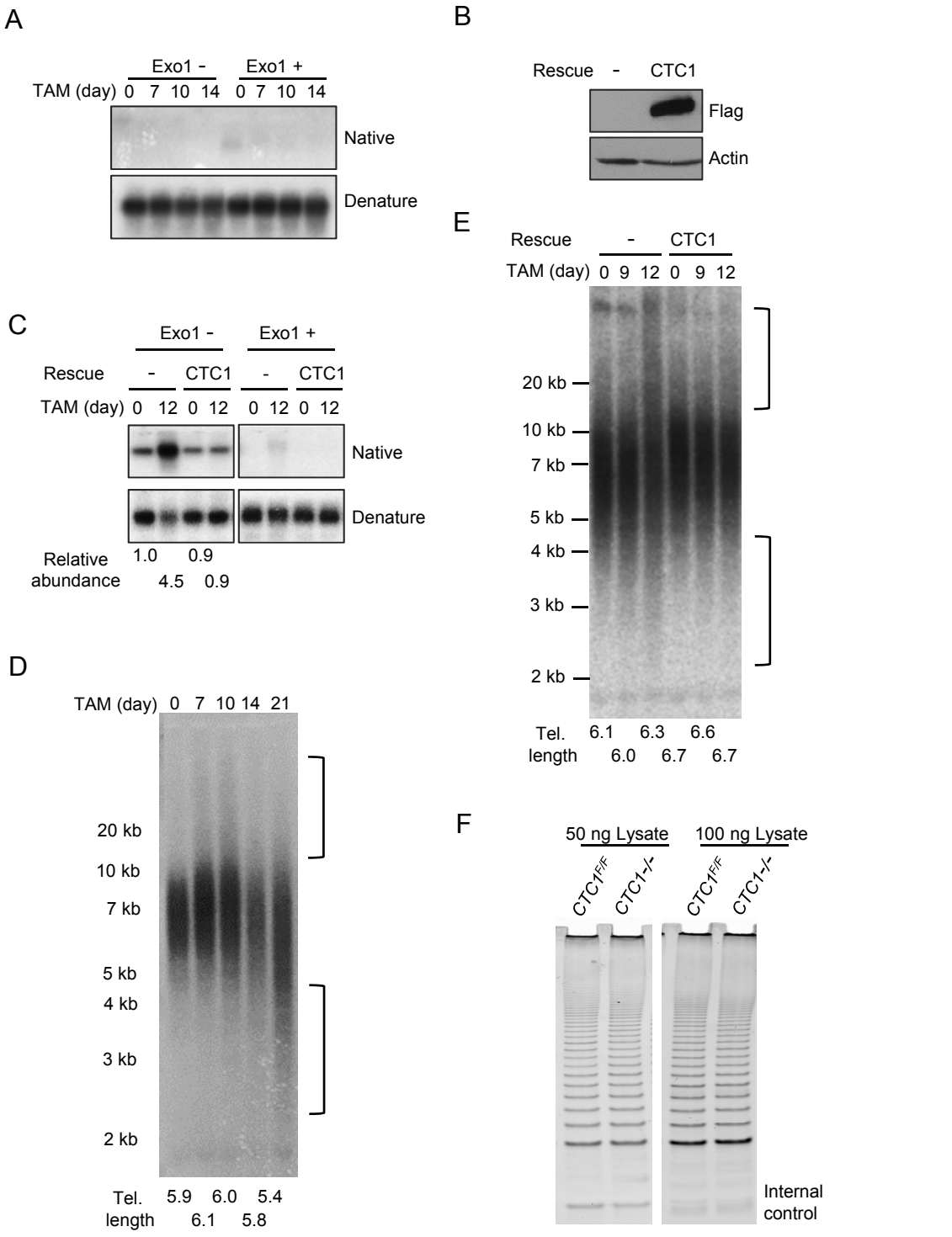
B




C

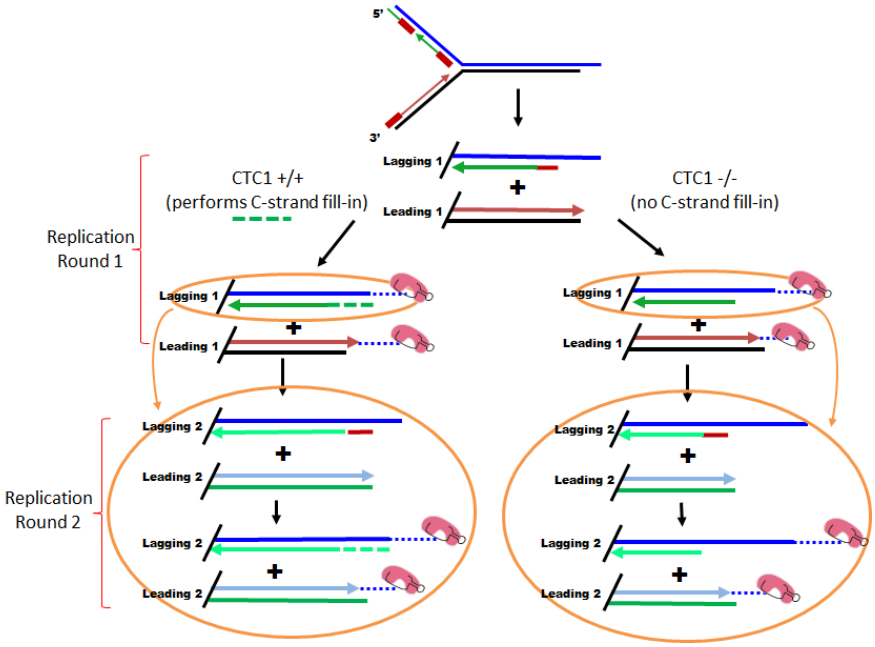


Feng. et al. supplementary figure 3A-F



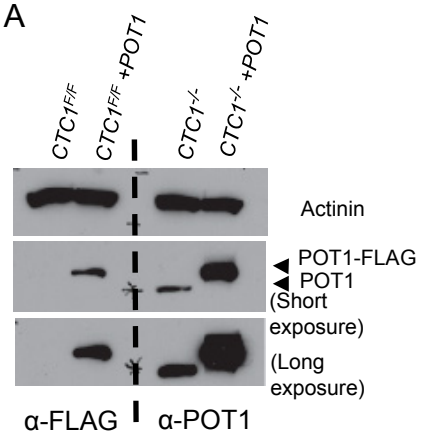
Feng. et al. supplementary figure 3G

G

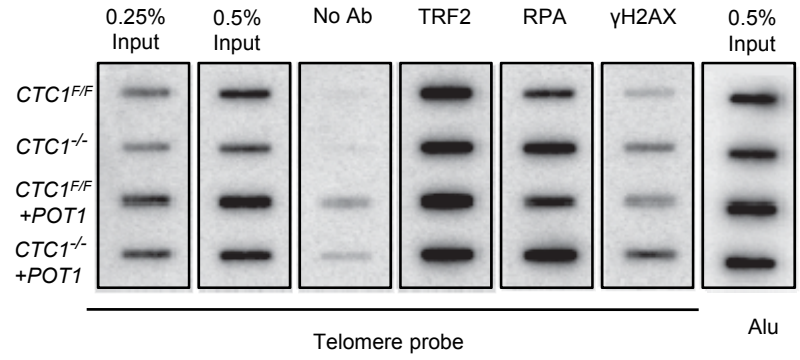


Feng. et al. supplementary figure 4

A




C



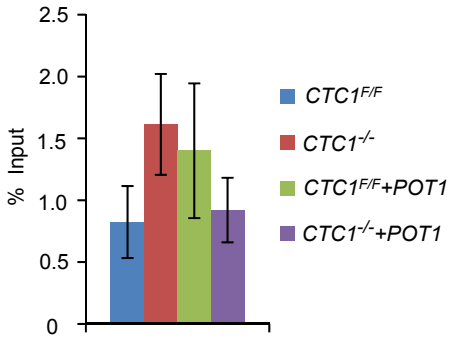

B

| Exp. 1 | $CTC1^{F/F}$ | $CTC1^{-/-}$ | $CTC1^{F/F}$+POT1 | $CTC1^{-/-}$+POT1 |
|---|---|---|---|---|
| NonTel γH2AX | 27.8% (83/229) | 28.9% (71/246) | 33.8% (109/323) | 24.9% (97/390) |
| Tel γH2AX | 11.4% (34/229) | 51.2% (126/246) | 20.4% (66/323) | 28.5% (111/390) |
| SFE γH2AX | 0% (0/229) | 0.4% (1/246) | 0.9% (3/323) | 1.5% (6/390) |

| Exp. 2 | $CTC1^{F/F}$ | $CTC1^{-/-}$ | $CTC1^{F/F}$+POT1 | $CTC1^{-/-}$+POT1 |
|---|---|---|---|---|
| NonTel γH2AX | 23% (17/74) | 23.2% (46/198) | 20.8% (16/77) | 27.6% (32/116) |
| Tel γH2AX | 5.4% (4/74) | 30.3% (60/198) | 13% (10/77) | 18.1% (21/116) |
| SFE γH2AX | 0% (0/74) | 1% (2/198) | 0% (0/77) | 0% (0/116) |

| Exp. 3 | $CTC1^{F/F}$ | $CTC1^{-/-}$ | $CTC1^{F/F}$+POT1 | $CTC1^{-/-}$+POT1 |
|---|---|---|---|---|
| NonTel γH2AX | 19.9% (40/201) | 11.1% (12/108) | 12% (27/226) | 10.6% (49/464) |
| Tel γH2AX | 4.5% (9/201) | 42.6% (46/108) | 6.6% (15/226) | 17.2% (80/464) |
| SFE γH2AX | 1.5% (3/201) | 0.9% (1/108) | 0.4% (1/226) | 0.2% (1/464) |

E



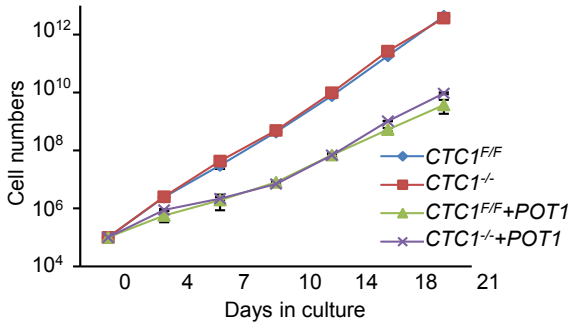

D

| yH2AX ChIP | Exp. 1 | Exp. 2 | Exp. 3 | Average |
|---|---|---|---|---|
| $CTC1^{F/F}$ | 0.10% | 0.05% | 0.05% | 0.07% |
| $CTC1^{-/-}$ | 0.40% | 0.29% | 0.29% | 0.33% |
| $CTC1^{F/F}$+POT1 | 0.10% | 0.04% | 0.08% | 0.07% |
| $CTC1^{-/-}$+POT1 | 0.20% | 0.21% | 0.19% | 0.20% |

| RPA ChIP | Exp. 1 | Exp. 2 | Exp. 3 | Average |
|---|---|---|---|---|
| $CTC1^{F/F}$ | 0.28% | 0.10% | 0.17% | 0.18% |
| $CTC1^{-/-}$ | 0.94% | 0.34% | 0.61% | 0.63% |
| $CTC1^{F/F}$+POT1 | 0.21% | 0.13% | 0.29% | 0.21% |
| $CTC1^{-/-}$+POT1 | 0.43% | 0.19% | 0.55% | 0.39% |

| TRF2 ChIP | Exp. 1 | Exp. 2 | Exp. 3 | Average |
|---|---|---|---|---|
| $CTC1^{F/F}$ | 0.95% | 1.03% | 0.49% | 0.82% |
| $CTC1^{-/-}$ | 1.15% | 1.77% | 1.92% | 1.61% |
| $CTC1^{F/F}$+POT1 | 0.78% | 1.62% | 1.80% | 1.40% |
| $CTC1^{-/-}$+POT1 | 0.90% | 0.67% | 1.19% | 0.92% |

F



Feng. et al. supplementary figure 5