PNAS

Supporting Information for

Extrachromosomal Telomere DNA Derived from Excessive Strand Displacements

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This PDF file includes:

Figure S1

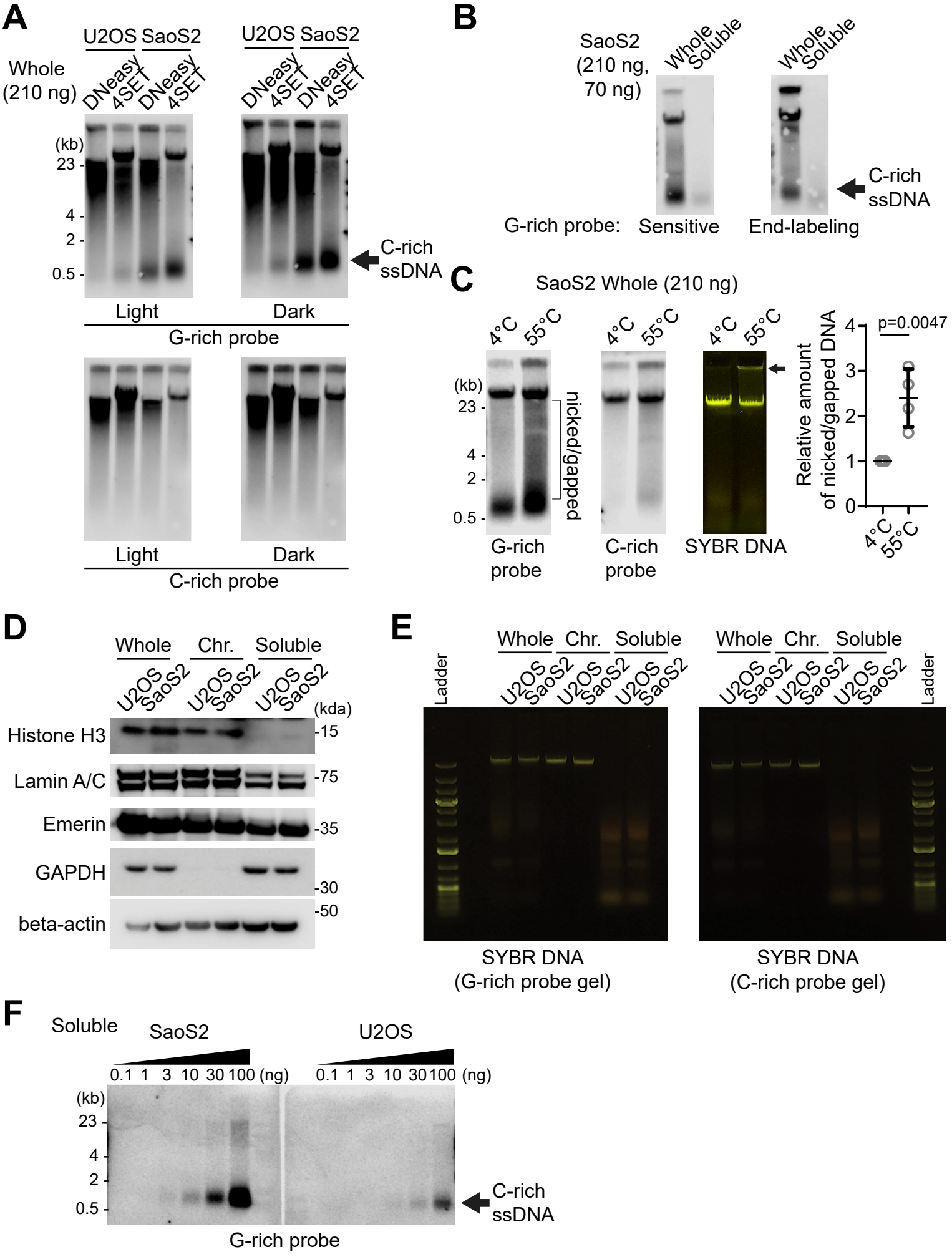

A
U2OS SaoS2
U2OS SaoS2
Whole (210 ng)
DNeasy 4SET DNeasy 4SET
DNeasy 4SET DNeasy 4SET
(kb)
23
4
2
0.5
C-rich ssDNA
Light
Dark
G-rich probe
Light
Dark
C-rich probe
B
SaoS2 (210 ng, 70 ng)
Whole
Soluble
Whole
Soluble
C-rich ssDNA
G-rich probe:
Sensitive
End-labeling
C
SaoS2 Whole (210 ng)
4°C 55°C
4°C 55°C
4°C 55°C
(kb)
23
4
2
0.5
nicked/gapped
G-rich probe
C-rich probe
SYBR DNA
Relative amount of nicked/gapped DNA
p=0.0047
4
3
2
1
0
4°C
55°C
D
Whole
Chr.
Soluble
U2OS SaoS2 U2OS SaoS2 U2OS SaoS2
(kda)
Histone H3
Lamin A/C
Emerin
GAPDH
beta-actin
-15
-75
-35
-30
-50
E
Ladder
Whole Chr. Soluble
Whole Chr. Soluble
U2OS SaoS2 U2OS SaoS2 U2OS SaoS2
U2OS SaoS2 U2OS SaoS2 U2OS SaoS2
Ladder
SYBR DNA
(G-rich probe gel)
SYBR DNA
(C-rich probe gel)
F
Soluble
SaoS2
U2OS
0.1 1 3 10 30 100 (ng)
0.1 1 3 10 30 100 (ng)
(kb)
23
4
2
0.5
C-rich ssDNA
G-rich probe

Figure S1

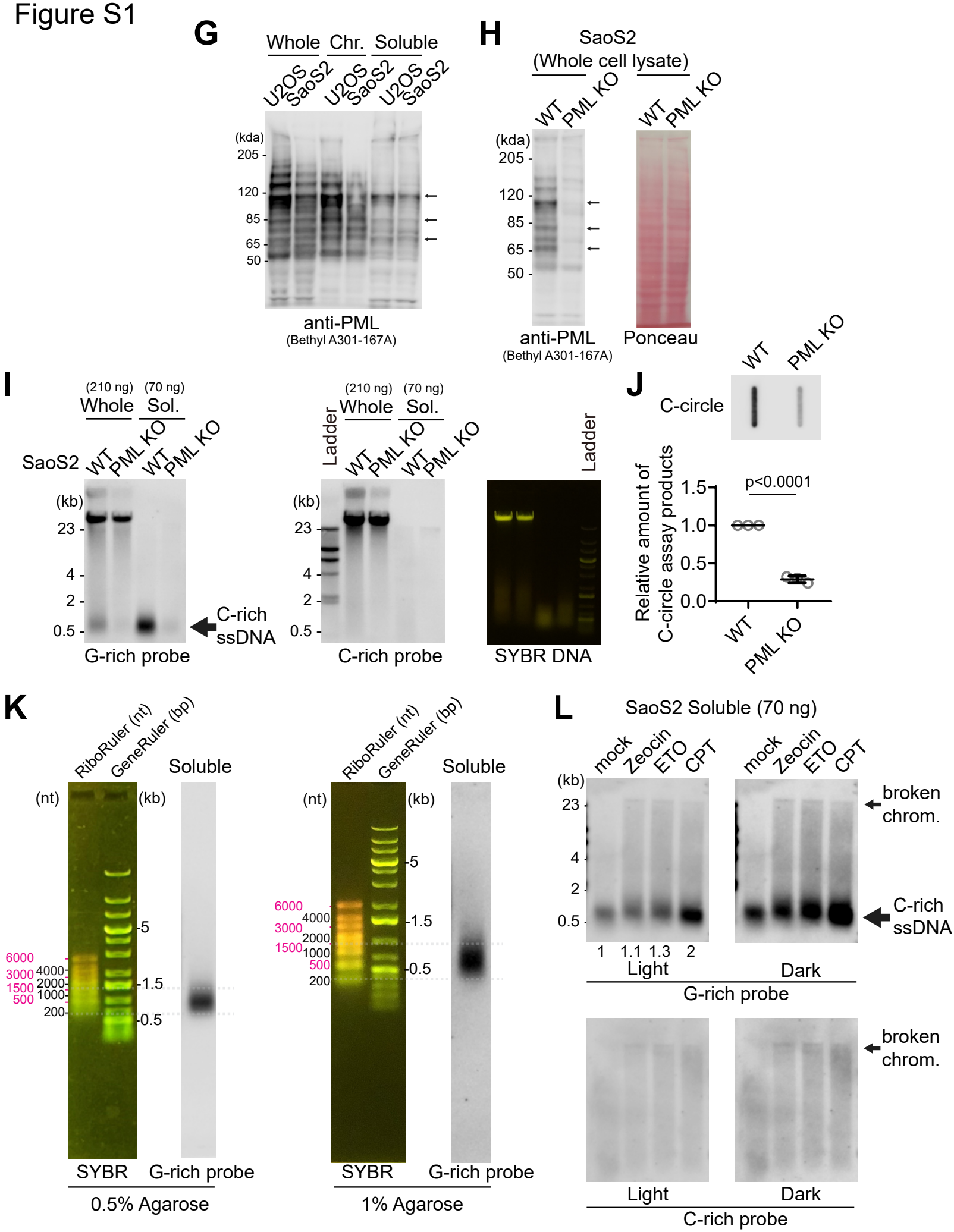
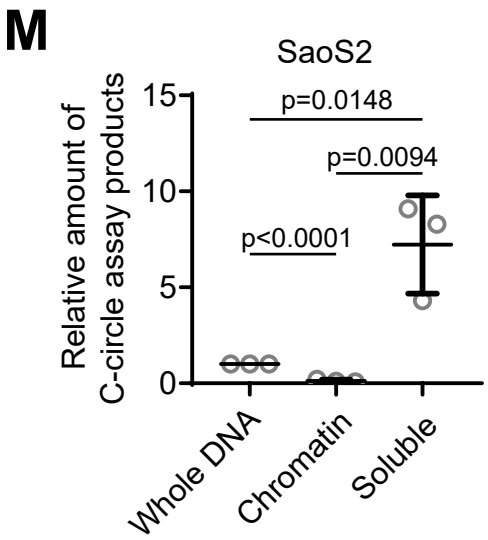
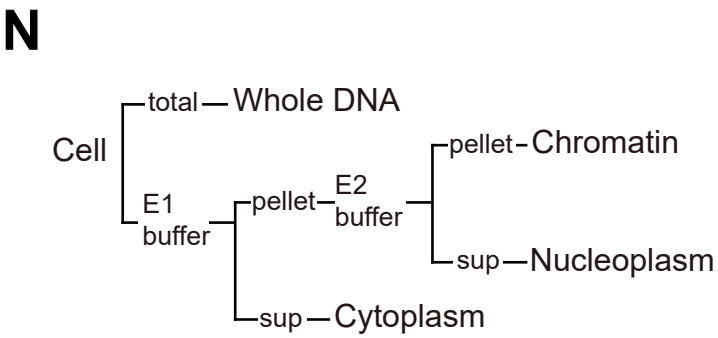
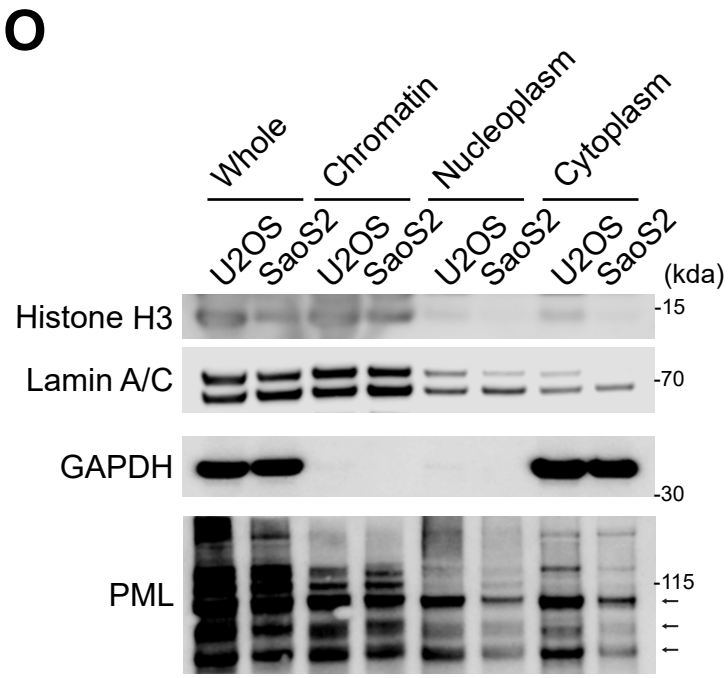
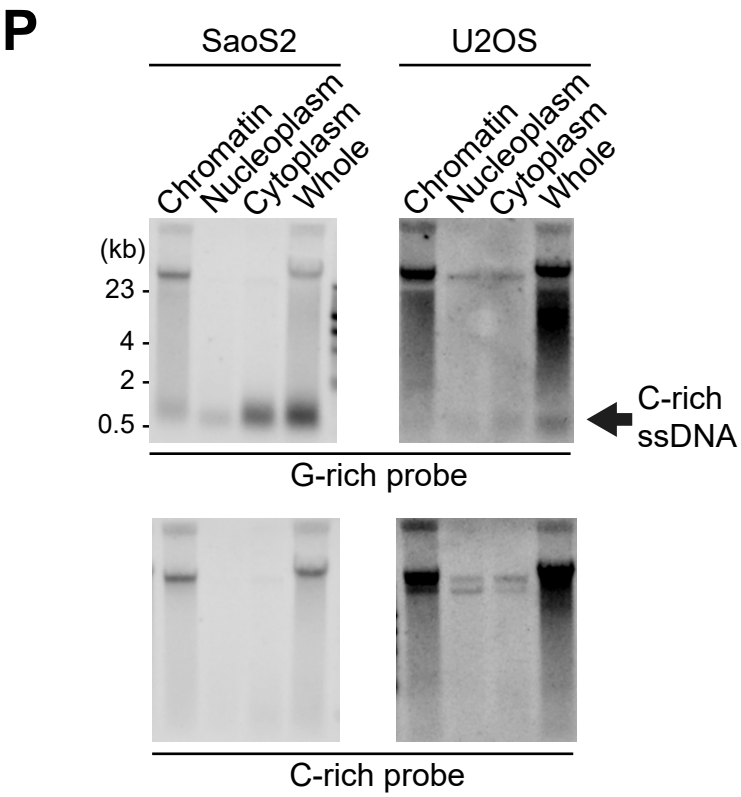

G
Whole
Chr.
Soluble
U2OS
SaoS2
(kda)
205
120
85
65
50
anti-PML
(Bethyl A301-167A)
H
SaoS2
(Whole cell lysate)
WT
PML KO
anti-PML
(Bethyl A301-167A)
Ponceau
I
(210 ng)
(70 ng)
Whole
Sol.
SaoS2
(kb)
23
4
2
0.5
C-rich
ssDNA
G-rich probe
Ladder
C-rich probe
SYBR DNA
J
C-circle
Relative amount of
C-circle assay products
p<0.0001
1.5
1.0
0.5
0.0
K
RiboRuler (nt)
GeneRuler (bp)
Soluble
(nt)
(kb)
6000
4000
3000
2000
1500
1000
500
200
5
1.5
0.5
SYBR
G-rich probe
0.5% Agarose
1% Agarose
L
SaoS2 Soluble (70 ng)
mock
Zeocin
ETO
CPT
broken
chrom.
C-rich
ssDNA
1
1.1
1.3
2
Light
Dark
G-rich probe
C-rich probe

Figure S1





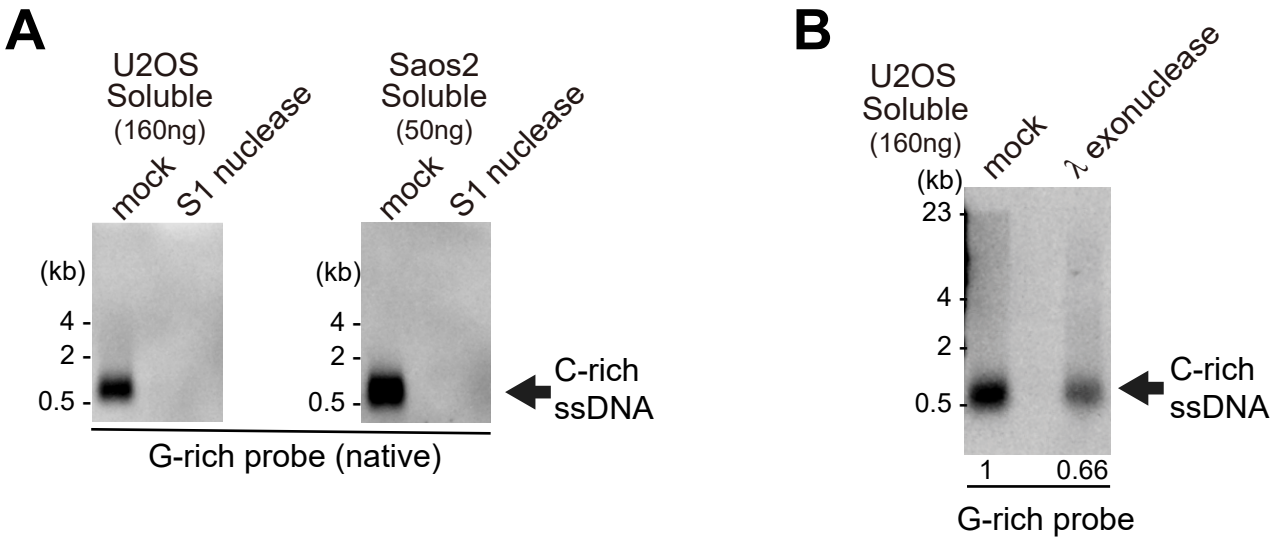




Whole - 140ng
Chromatin&Nucleoplasm&Cytoplasm - 70ng

Figure S1, related to Figure 1. 4SET assay, a simple and efficient method for detecting single-stranded extrachromosomal telomeric DNA. (A) Comparison of genomic DNA purification between DNeasy Blood & Tissue Kit (Qiagen, 69504) and our 4SET method in U2OS and SaoS2 cells. **(B)** Comparison between Dig-labeled strand-specific probes and standard end-labeling probes. **(C)** Comparison of 4SET results with DNA dissolved at 4°C overnight vs 55°C for 2 hours, followed by 4°C overnight. Observed smear signals potentially indicate the presence of nicked or gapped DNA. The arrow marked in the gel well is likely indicative of a DNA intermediate structure. This may be a result of annealing coupled with spontaneous branch migration, processes that are potentially facilitated by the high temperature conditions. The graph to the right displays the quantified signals potentially indicative of nicked or gapped DNA (mean ± SD; unpaired t-test). **(D)** Western blot analysis of the whole, chromatin (Chr.), and soluble using anti-Histone H3, Lamin A/C, Emerin, GAPDH, and beta-actin for the validation of cell fractions. **(E)** SYBR DNA images (loading control) for G-rich probe and C-rich probe gels in Fig. 1C. **(F)** 4SET assay for soluble fractions of U2OS and SaoS2 cells to determine the minimum sample requirement. **(G)** Western blot analysis of the whole, chromatin (Chr.), and soluble fractions using PML antibody. **(H)** Western blot analysis of Saos2 Wild-type (WT) and PML Knock-out cells using PML antibody, along with a Ponceau image as a loading control. **(I)** 4SET assay for SaoS2 WT and PML Knock-out cells after fractionation into Whole and soluble fractions. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA staining of gel image (loading control). **(J)** C-circle assay using DNA in (I) (top). Quantification of C-circle assay (mean ± SD; unpaired t-test) (bottom). **(K)** 4SET assay conducted using a nucleotide ladder (RiboRuler) to measure the size of C-rich single-stranded DNA in SaoS2 soluble fraction, analyzed on both 0.5% and 1% agarose gels. **(L)** 4SET assay for SaoS2 treated with Zeocin (100 μg/ml), Etoposide (ETO) (10 μM), or Camptothecin (CPT) (0.25 μM) for 24 hrs. **(M)** Quantification of the C-circle assay in Fig. 1E (SaoS2 cells). Relative amount of C-circle assay products (mean ± SD; unpaired t-test). **(N)** Cell fractionation using E1 and E2 buffers to separate chromatin, nucleoplasm, and cytoplasm. **(O)** Western blot analysis conducted using the fractionated DNAs in (N) using anti-Histone H3, Lamin A/C, GAPDH, and PML for the validation of cell fractions. **(P)** 4SET assay for fractionated DNAs in (N).

Figure S2


A
U2OS
Soluble
(160ng)
mock
S1 nuclease
Saos2
Soluble
(50ng)
mock
S1 nuclease
(kb)
4
2
0.5
(kb)
4
2
0.5
C-rich
ssDNA
G-rich probe (native)
B
U2OS
Soluble
(160ng)
mock
λ exonuclease
(kb)
23
4
2
0.5
C-rich
ssDNA
1
0.66
G-rich probe

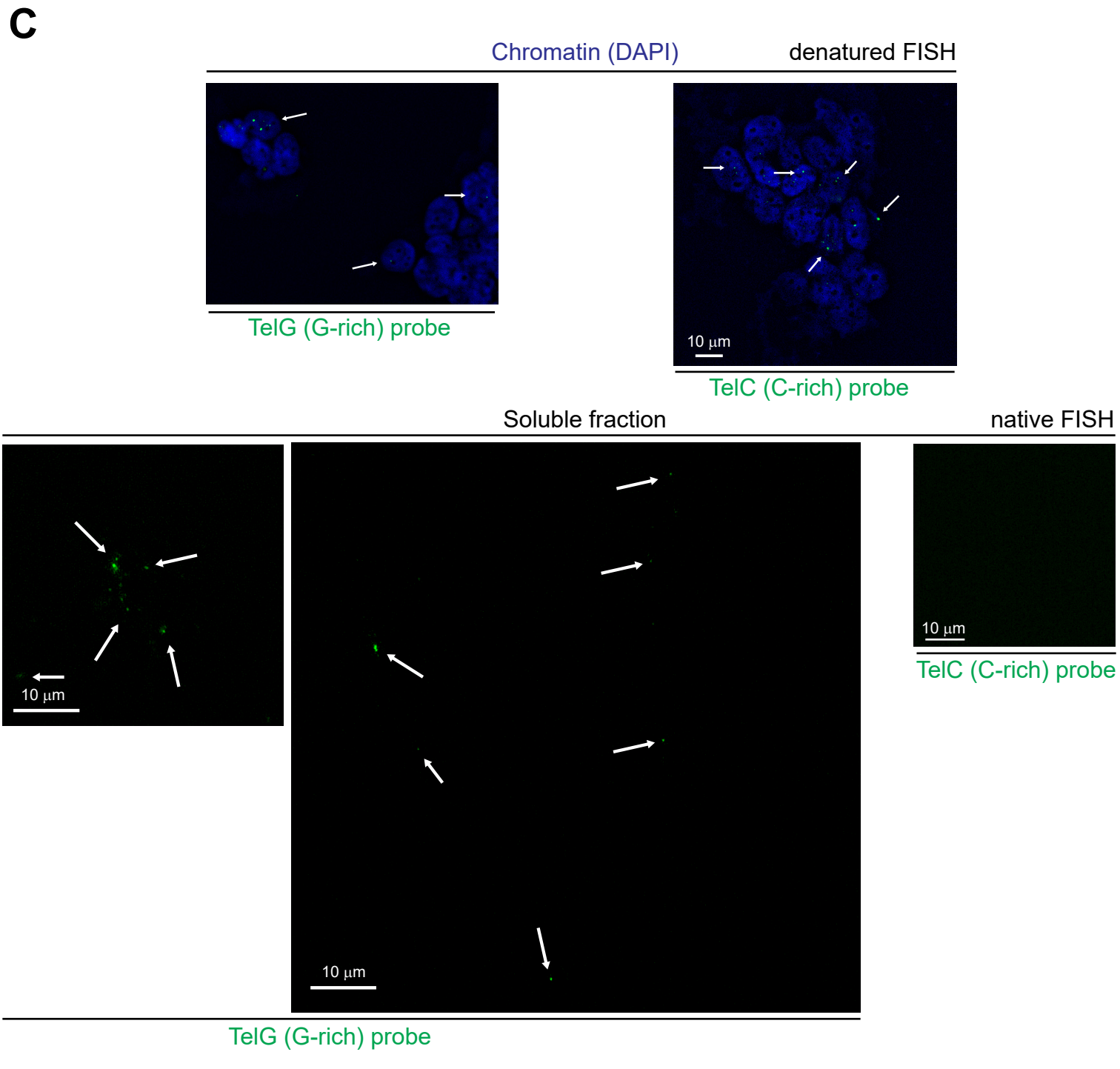

C
Chromatin (DAPI)
denatured FISH
TelG (G-rich) probe
10 μm
TelC (C-rich) probe
Soluble fraction
native FISH
10 μm
10 μm
TelC (C-rich) probe
10 μm
TelG (G-rich) probe

Figure S2, related to Figure 2. The presence of C-rich telomeric single-stranded DNA. (A) S1 nuclease assay on U2OS and SaoS2 soluble DNA fractions. **(B)** Lambda exonuclease assay on U2OS soluble DNA fraction. **(C)** Telomere-FISH with TelG (G-rich) and TelC (C-rich) PNA probes for chromatin (denatured FISH) and soluble fractions (native FISH).

Figure S3

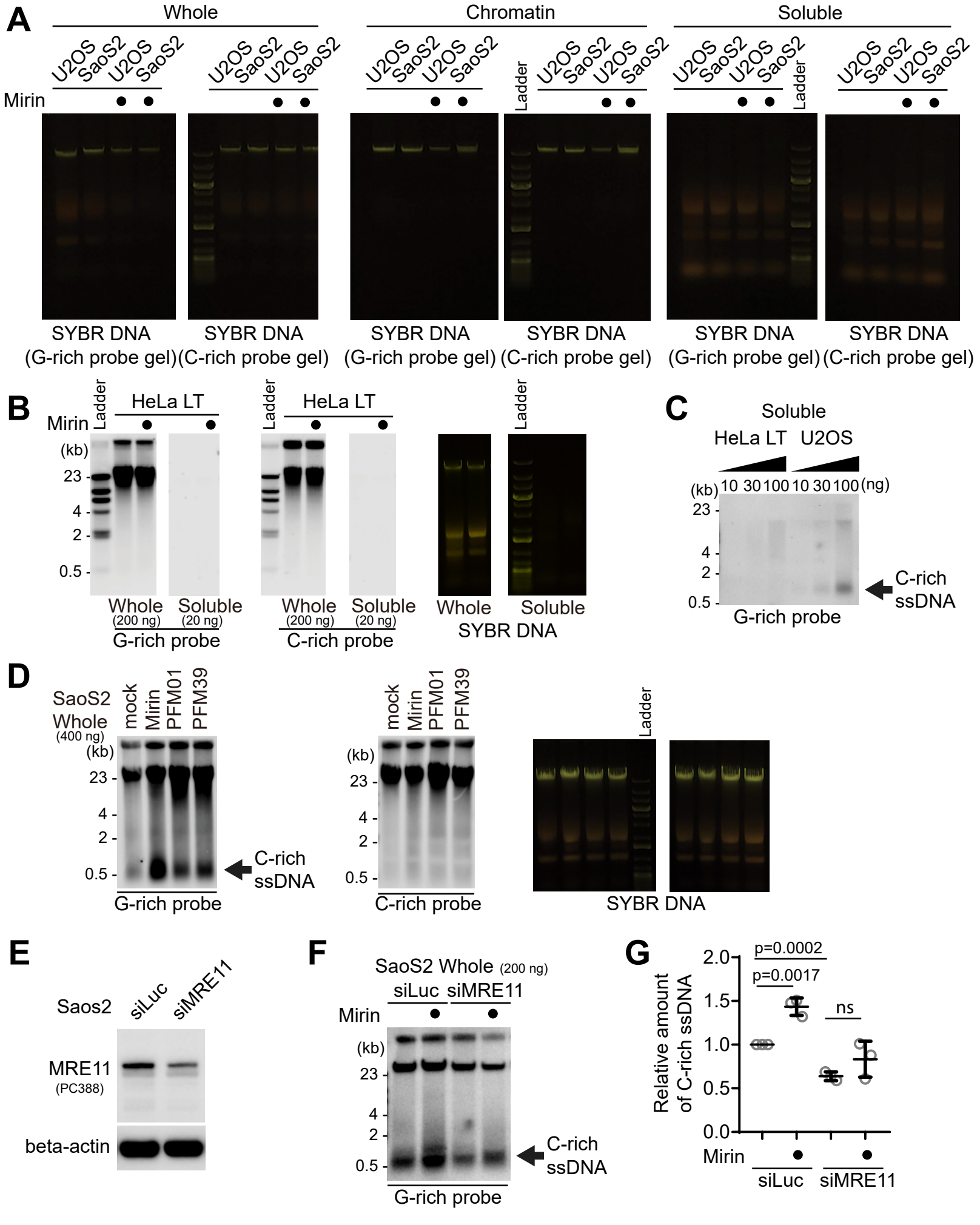

A
Whole
Chromatin
Soluble
U2OS SaoS2 U2OS SaoS2
Mirin
Ladder
SYBR DNA
(G-rich probe gel)
(C-rich probe gel)
B
HeLa LT
Mirin
Ladder
(kb)
23
4
2
0.5
Whole (200 ng)
Soluble (20 ng)
G-rich probe
C-rich probe
SYBR DNA
C
Soluble
HeLa LT U2OS
10 30 100 10 30 100 (ng)
C-rich ssDNA
G-rich probe
D
SaoS2 Whole (400 ng)
mock
Mirin
PFM01
PFM39
C-rich ssDNA
G-rich probe
C-rich probe
SYBR DNA
E
Saos2
siLuc
siMRE11
MRE11 (PC388)
beta-actin
F
SaoS2 Whole (200 ng)
siLuc siMRE11
Mirin
C-rich ssDNA
G-rich probe
G
Relative amount of C-rich ssDNA
p=0.0002
p=0.0017
ns
Mirin
siLuc
siMRE11

Figure S3

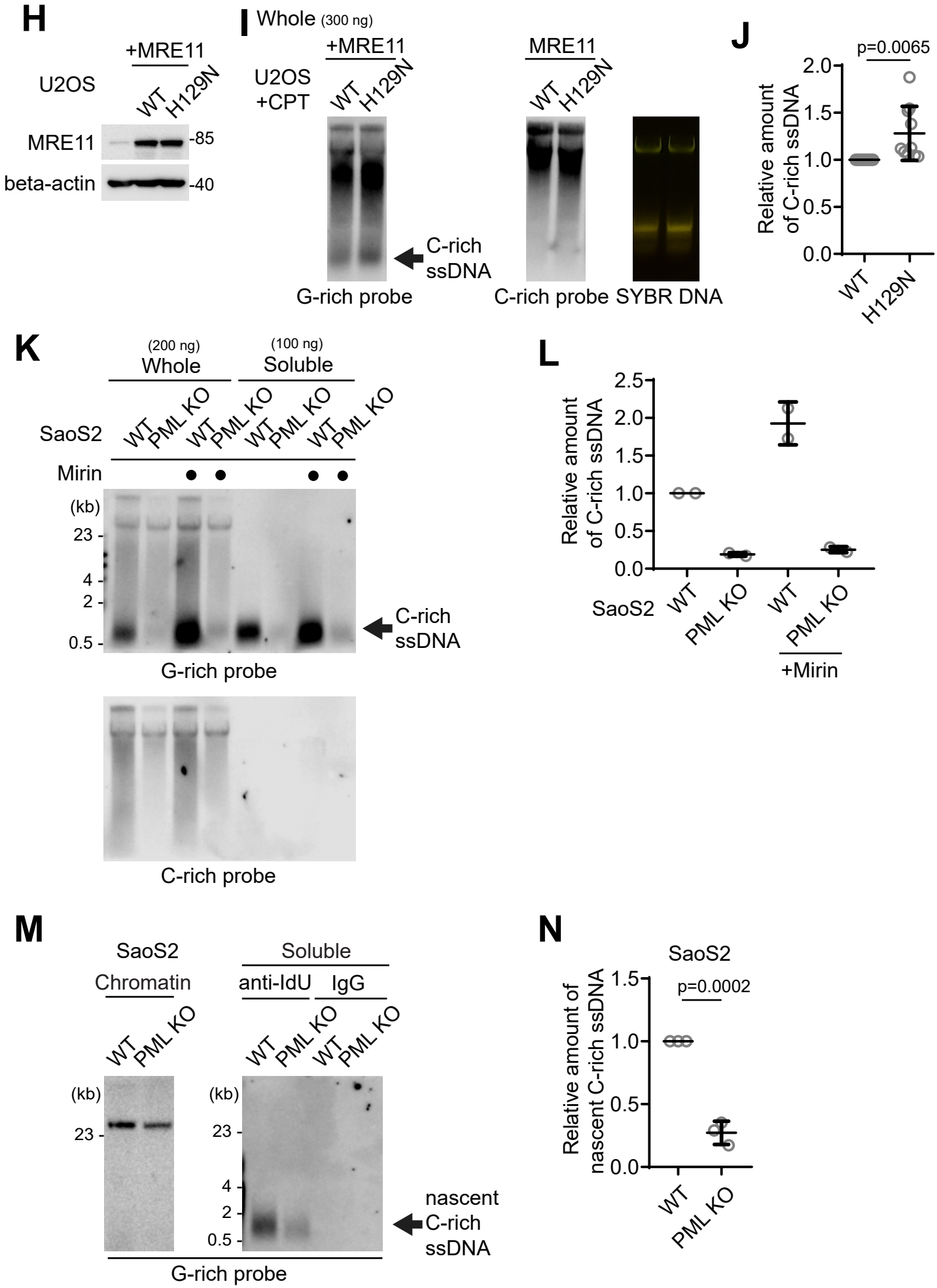

H
+MRE11
U2OS
WT
H129N
MRE11
-85
beta-actin
-40
I
Whole (300 ng)
+MRE11
U2OS
+CPT
WT
H129N
C-rich
ssDNA
G-rich probe
MRE11
WT
H129N
C-rich probe
SYBR DNA
J
p=0.0065
Relative amount
of C-rich ssDNA
2.0
1.5
1.0
0.5
0.0
WT
H129N
K
(200 ng)
Whole
(100 ng)
Soluble
SaoS2
WT
PML KO
Mirin
(kb)
23
4
2
0.5
C-rich
ssDNA
G-rich probe
C-rich probe
L
Relative amount
of C-rich ssDNA
2.5
2.0
1.5
1.0
0.5
0.0
SaoS2
WT
PML KO
+Mirin
M
SaoS2
Chromatin
Soluble
anti-IdU
IgG
WT
PML KO
(kb)
23
4
2
0.5
nascent
C-rich
ssDNA
G-rich probe
N
SaoS2
p=0.0002
Relative amount of
nascent C-rich ssDNA
1.5
1.0
0.5
0.0
WT
PML KO

Figure S3, related to Figure 3. MRE11 nuclease activity suppresses the generation of C-rich ssDNA. (A) SYBR DNA image (loading control) for G-rich probe and C-rich probe gels in Fig. 3D. **(B)** 4SET assay for HeLa LT cells with or without Mirin treatment. Samples were fractionated into soluble DNA fraction or whole DNA. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. SYBR DNA image (loading control) for G-probe gel. **(C)** 4SET assay for soluble DNA fractions of HeLa LT and U2OS cells**. (D)** 4SET assay for SaoS2 cells with Mirin, PFM01, PFM39 or mock (control) treatments. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA image (loading control) for G-rich probe and C-rich probe gels. **(E)** Western blot of SaoS2 post-transfection with siLuciferase (siLuc) or siMRE11. **(F)** 4SET for SaoS2, with and without Mre11 treatment post siLuc or siMRE11s transfection. **(G)** Quantification of 4SET from F, presenting the quantification of relative amount of C-rich ssDNAs (mean ± SD; unpaired t-test). **(H)** Western blot of U2OS cells expressing MRE11 WT or H129N. **(I)** 4SET assay for MRE11 WT or H129N expressing U2OS cells treated with CPT (0.25 μM) for 48 hrs **(J)** Quantification of 4SET from I, representing the quantification of relative amount of C-rich ssDNAs (mean ± SD; unpaired t-test). **(K)** 4SET for SaoS2 WT and SaoS2 PML KO with/without Mirin treatment. **(L)** Quantification of 4SET from K, showing relative C-rich ssDNA (whole) levels (mean ± SD). **(M)** Native IdU pulldown was conducted using IdU (3D4) antibody for Saos2 WT and Saos2 PML KO after IdU incorporation. IgG served as a negative control. Chromatin DNA was also included to control for DNA quantity. **(N)** Quantification of the native IdU-pulldown assay in M; Relative amount of nascent C-rich ssDNAs (mean ± SD; unpaired t-test).

Figure S4

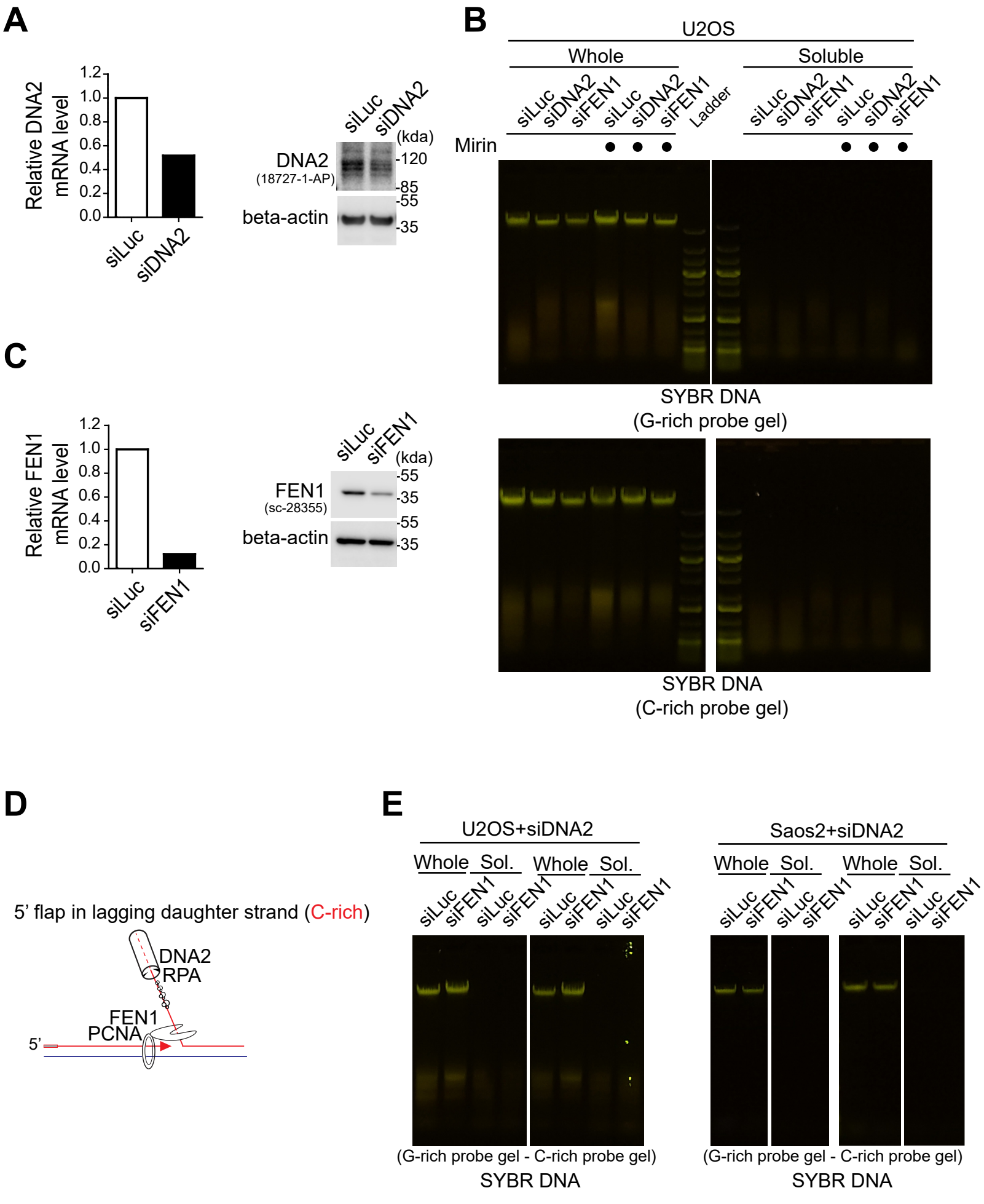
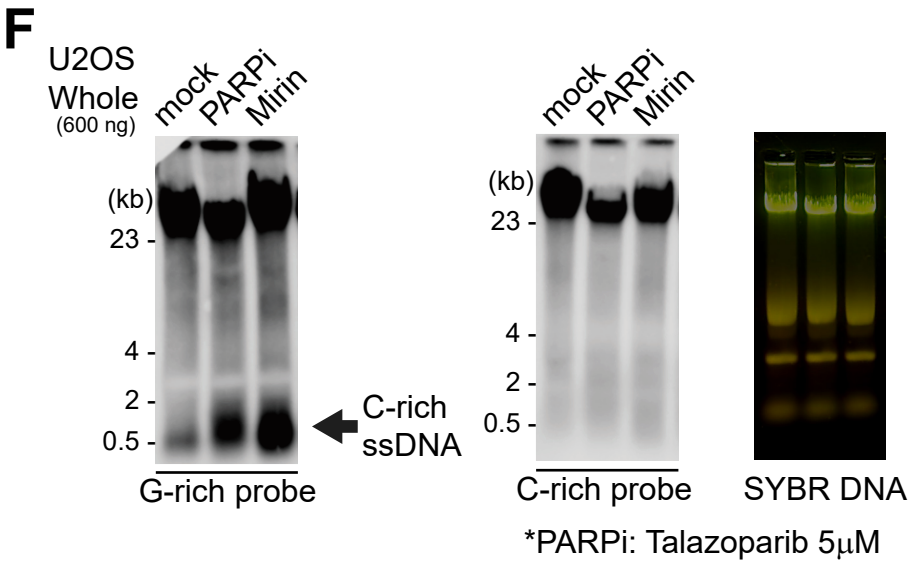
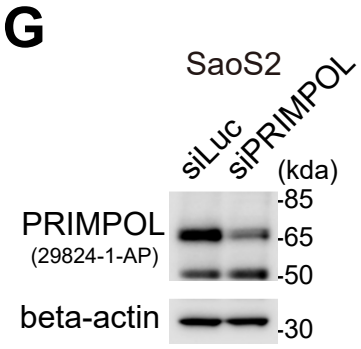
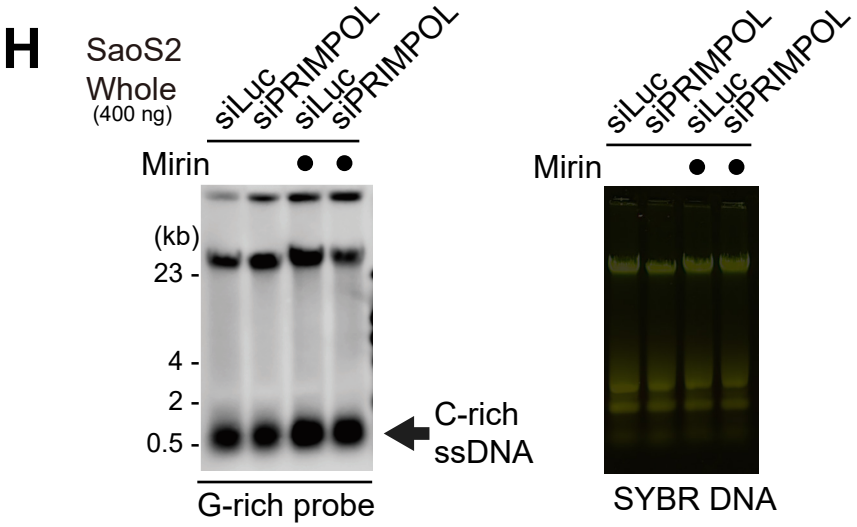
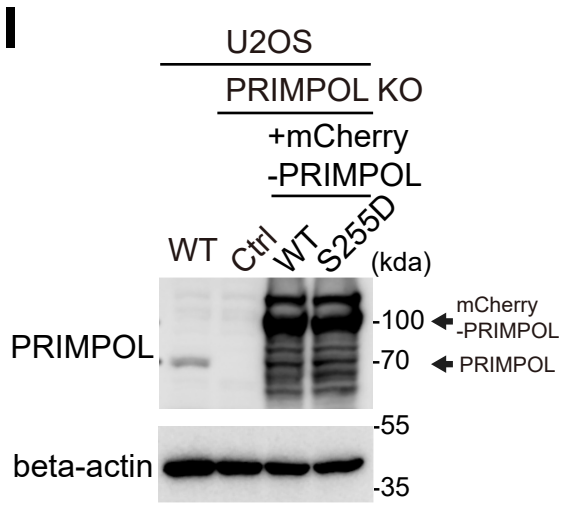

A
Relative DNA2 mRNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
siLuc
siDNA2
siLuc
siDNA2
(kda)
DNA2
(18727-1-AP)
-120
-85
beta-actin
-55
-35
B
U2OS
Whole
Soluble
siLuc
siDNA2
siFEN1
siLuc
siDNA2
siFEN1
Ladder
siLuc
siDNA2
siFEN1
siLuc
siDNA2
siFEN1
Mirin
SYBR DNA
(G-rich probe gel)
SYBR DNA
(C-rich probe gel)
C
Relative FEN1 mRNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
siLuc
siFEN1
siLuc
siFEN1
(kda)
FEN1
(sc-28355)
-55
-35
beta-actin
-55
-35
D
5' flap in lagging daughter strand (C-rich)
DNA2
RPA
FEN1
PCNA
5'
E
U2OS+siDNA2
Whole
Sol.
Whole
Sol.
siLuc
siFEN1
siLuc
siFEN1
siLuc
siFEN1
siLuc
siFEN1
(G-rich probe gel - C-rich probe gel)
SYBR DNA
Saos2+siDNA2
Whole
Sol.
Whole
Sol.
siLuc
siFEN1
siLuc
siFEN1
siLuc
siFEN1
siLuc
siFEN1
(G-rich probe gel - C-rich probe gel)
SYBR DNA

Figure S4


F
U2OS
Whole
(600 ng)
mock
PARPi
Mirin
(kb)
23
4
2
0.5
C-rich
ssDNA
G-rich probe
C-rich probe
SYBR DNA
*PARPi: Talazoparib 5μM


G
SaoS2
siLuc
siPRIMPOL
(kda)
PRIMPOL
(29824-1-AP)
beta-actin
85
65
50
30


H
SaoS2
Whole
(400 ng)
siLuc
siPRIMPOL
siLuc
siPRIMPOL
Mirin
(kb)
23
4
2
0.5
C-rich
ssDNA
G-rich probe
SYBR DNA


I
U2OS
PRIMPOL KO
+mCherry
-PRIMPOL
WT
Ctrl
WT
S255D
(kda)
PRIMPOL
beta-actin
100
70
55
35
mCherry
-PRIMPOL
PRIMPOL

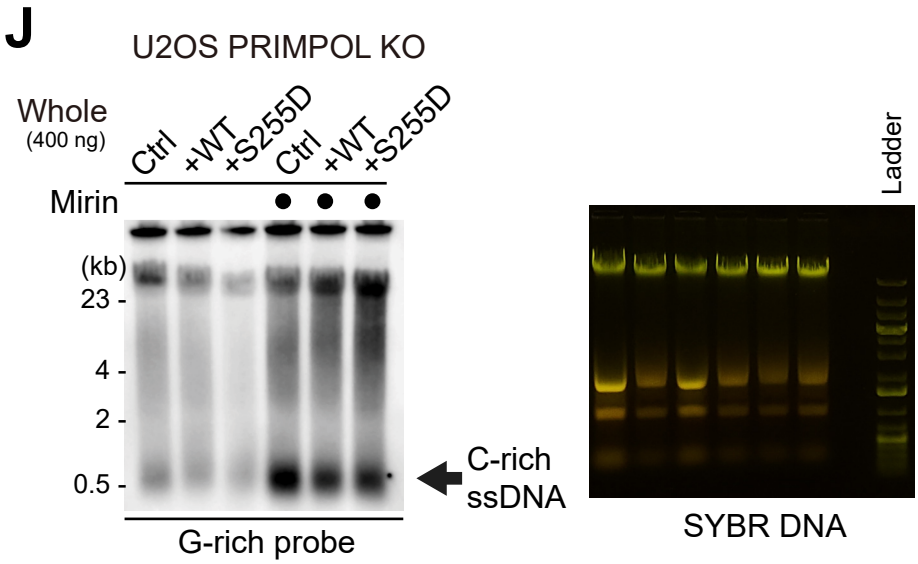

J
U2OS PRIMPOL KO
Whole
(400 ng)
Ctrl
+WT
+S255D
Ctrl
+WT
+S255D
Mirin
(kb)
23
4
2
0.5
C-rich
ssDNA
G-rich probe
Ladder
SYBR DNA

Figure S4, related to Figure 4. C-rich ssDNAs are derived from lagging strand during the Okazaki fragment processing. (A) Relative amount of DNA2 mRNA levels measured by quantitative-PCR (left) and DNA2 Western blot (right) of U2OS cells transfected with siRNAs targeting DNA2 or control (siLuc). **(B)** SYBR DNA images (loading control) for G-rich probe and C-rich probe gels in Fig. 4C. **(C)** Relative amount of FEN1 mRNA levels measured by quantitative-PCR (left) and FEN1 Western blot (right) of U2OS cells transfected with siRNAs targeting FEN1 or control (siLuc). **(D)** Illustration depicting 5' flap processing at lagging strand telomeres by DNA2-RPA or FEN1-PCNA. **(E)** SYBR DNA images (loading control) for G-rich probe and C-rich probe gels in Fig. 4E. **(F)** 4SET assay for U2OS cells with PARPi (Talazoparib), Mirin, or mock (control) treatments. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA images (loading control) for G-rich probe and C-rich probe gels. **(G)** Western blot of SaoS2 post-transfection with siLuc or siPRIMPOL. **(H)** 4SET assay for SaoS2 cells after transfection of siRNAs targeting PRIMPOL, or control (siLuc) with or without Mirin treatment. G-rich probe was used to detect C-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA image (loading control) for G-rich probe. **(I)** Western blot of U2OS WT and U2OS PRIMPOL KO cells post-transfection with mCherry-PRIMPOLs (WT or S255D) or control vector. **(J)** 4SET assay for U2OS PRIMPOL KO cells after transfection of PRIMPOL WT, S255D, or control cDNA plasmid with or without Mirin treatment. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA images (loading control) for G-rich probe and C-rich probe gels.

Figure S5

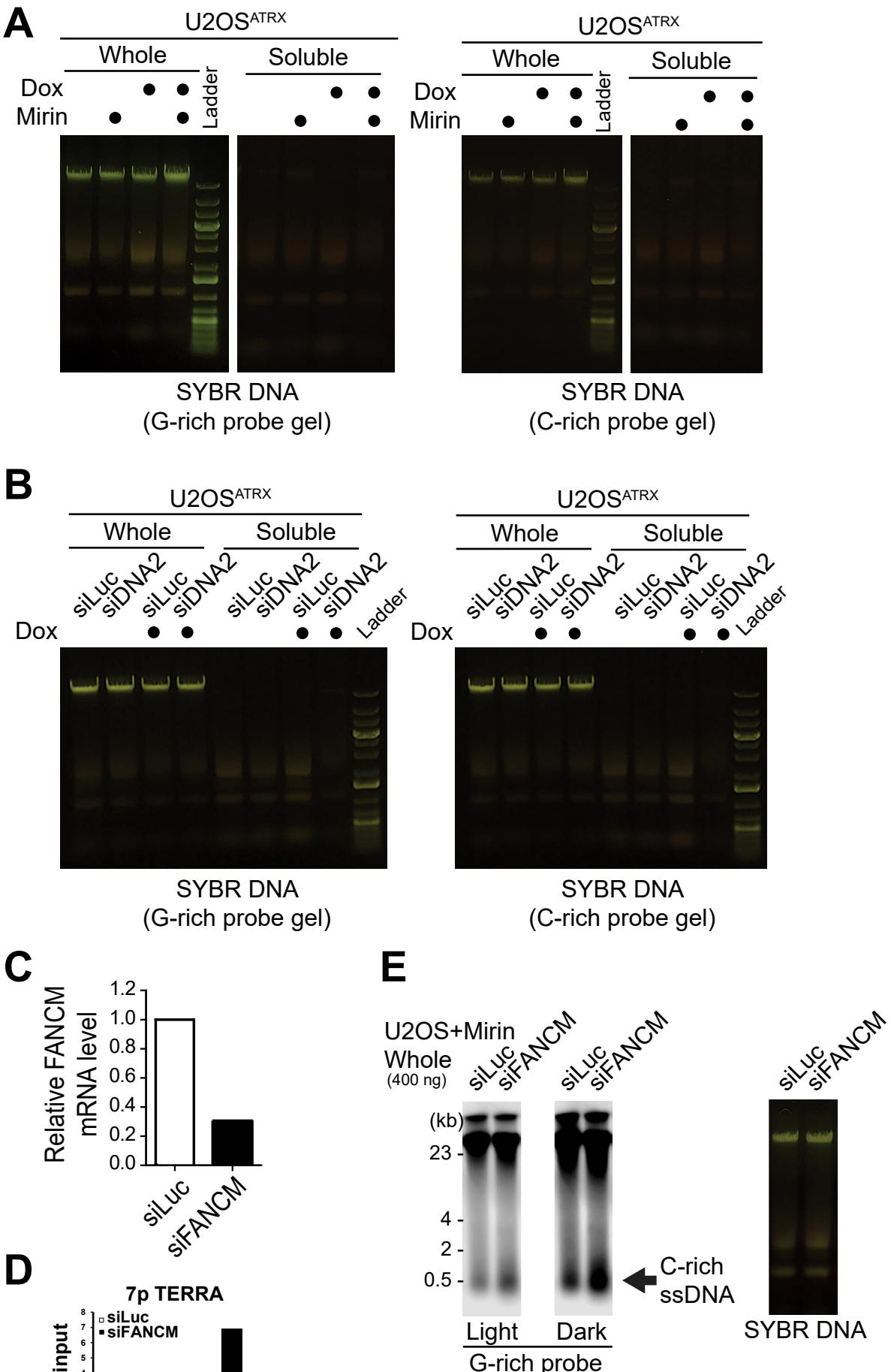

A
U2OS^ATRX
Whole
Soluble
Dox
Mirin
Ladder
SYBR DNA
(G-rich probe gel)
U2OS^ATRX
Whole
Soluble
Dox
Mirin
Ladder
SYBR DNA
(C-rich probe gel)
B
U2OS^ATRX
Whole
Soluble
siLuc
siDNA2
Dox
Ladder
SYBR DNA
(G-rich probe gel)
U2OS^ATRX
Whole
Soluble
siLuc
siDNA2
Dox
Ladder
SYBR DNA
(C-rich probe gel)
C
Relative FANCM mRNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
siLuc
siFANCM
D
7p TERRA
siLuc
siFANCM
% of input
IgG
S9.6
E
U2OS+Mirin
Whole
(400 ng)
siLuc
siFANCM
siLuc
siFANCM
(kb)
23
4
2
0.5
C-rich
ssDNA
Light
Dark
G-rich probe
siLuc
siFANCM
SYBR DNA

Figure S5

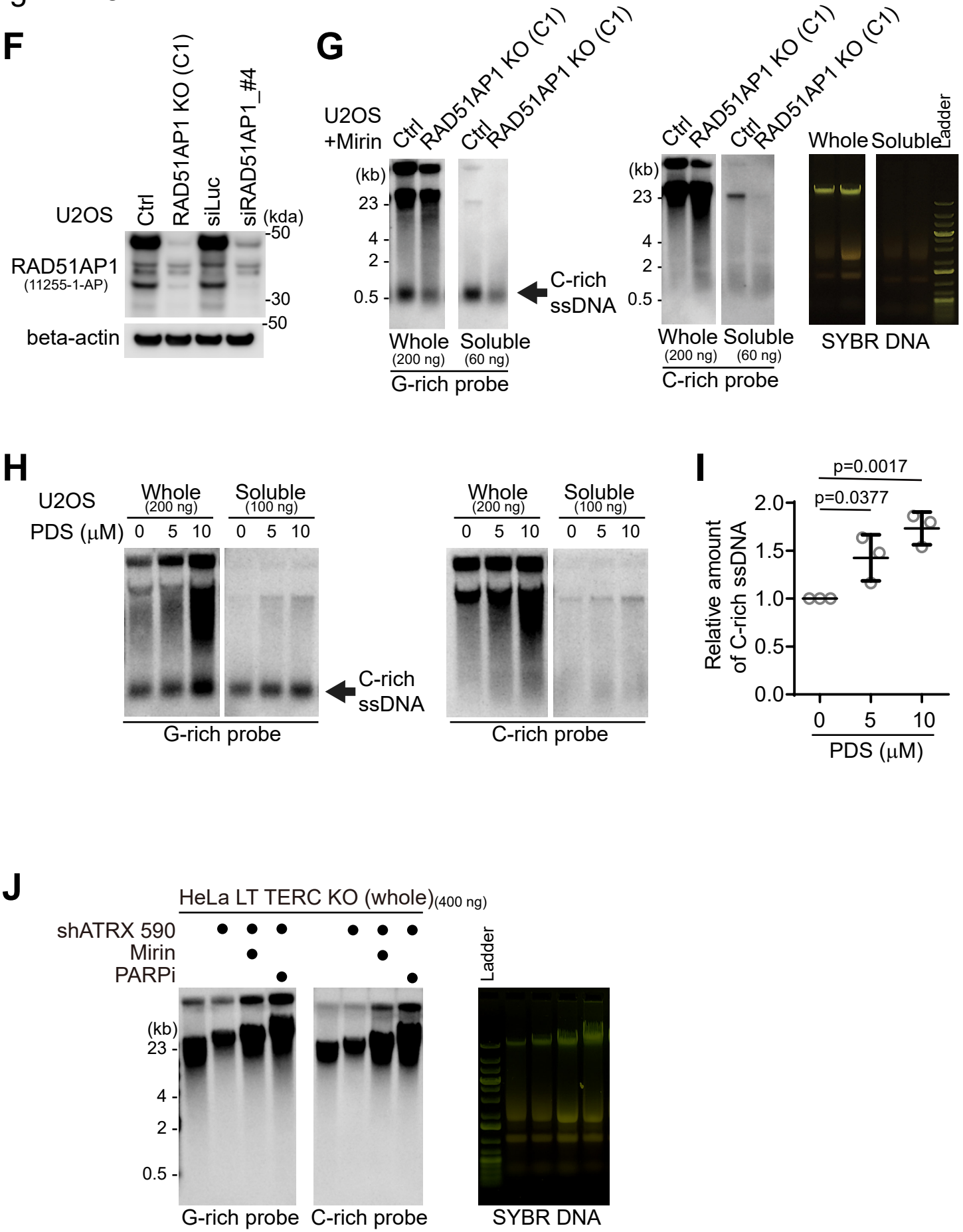
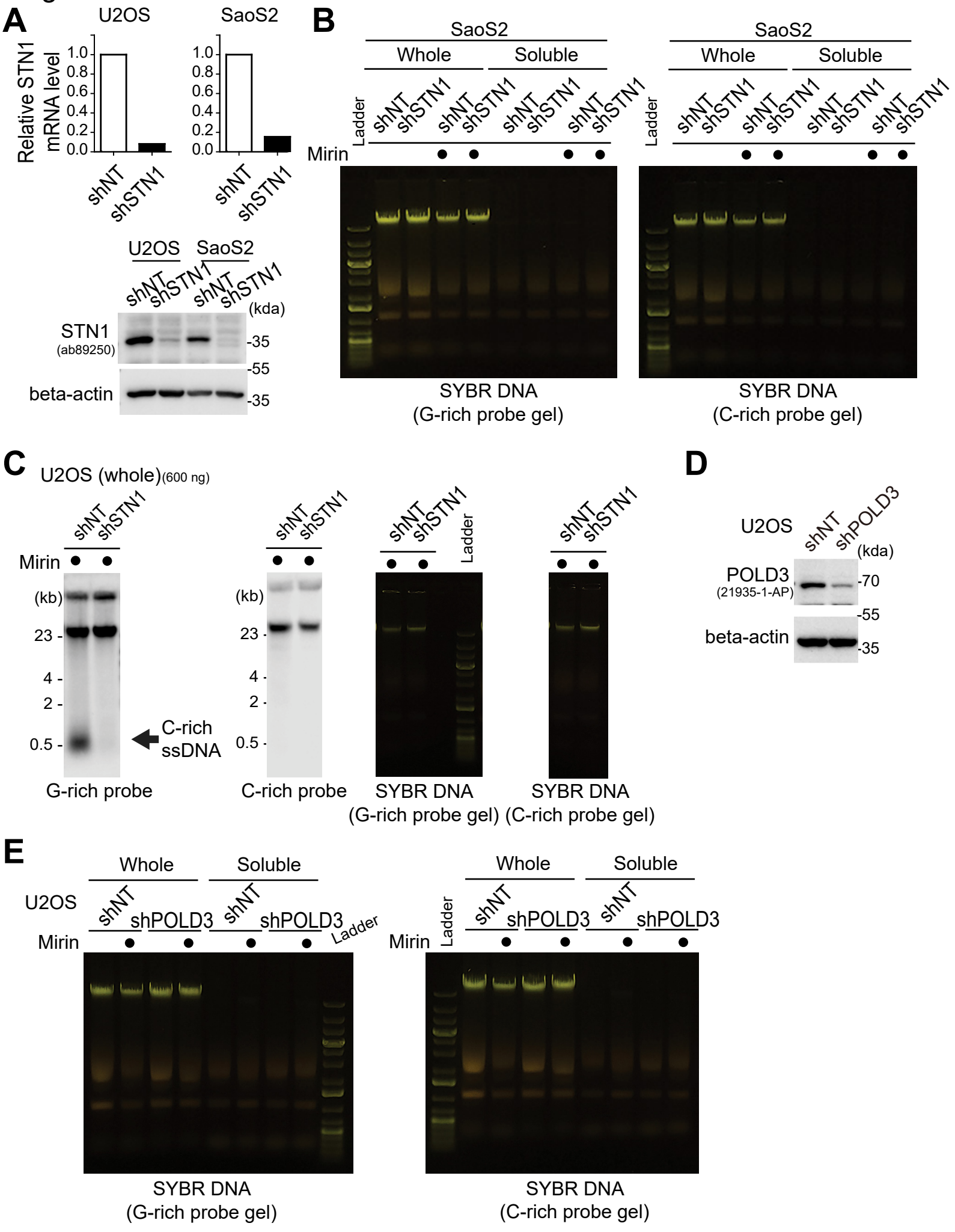

F
U2OS
Ctrl
RAD51AP1 KO (C1)
siLuc
siRAD51AP1_#4
(kda)
RAD51AP1
(11255-1-AP)
-50
-30
beta-actin
-50
G
U2OS
+Mirin
Ctrl
RAD51AP1 KO (C1)
Ctrl
RAD51AP1 KO (C1)
(kb)
23
4
2
0.5
C-rich
ssDNA
Whole
(200 ng)
Soluble
(60 ng)
G-rich probe
Ctrl
RAD51AP1 KO (C1)
Ctrl
RAD51AP1 KO (C1)
(kb)
23
4
2
0.5
Whole
(200 ng)
Soluble
(60 ng)
C-rich probe
Whole
Soluble
Ladder
SYBR DNA
H
U2OS
Whole
(200 ng)
Soluble
(100 ng)
PDS (μM)
0 5 10
0 5 10
C-rich
ssDNA
G-rich probe
Whole
(200 ng)
Soluble
(100 ng)
0 5 10
0 5 10
C-rich probe
I
p=0.0017
p=0.0377
Relative amount
of C-rich ssDNA
2.0
1.5
1.0
0.5
0.0
0
5
10
PDS (μM)
J
HeLa LT TERC KO (whole)(400 ng)
shATRX 590
Mirin
PARPi
(kb)
23
4
2
0.5
G-rich probe
C-rich probe
Ladder
SYBR DNA

Figure S5, related to Figure 5. ATRX suppresses the generation of C-rich ssDNAs. (A) SYBR DNA images (loading control) for G-rich probe and C-rich probe gels in Fig. 5D. **(B)** SYBR DNA images (loading control) for G-rich probe and C-rich probe gels in Fig. 5G. **(C)** Relative amount of FANCM mRNA levels measured by quantitative-PCR in U2OS cells transfected with siRNAs targeting FANCM or control (siLuc). **(D)** Relative quantification of R-loops at telomeres using the 7p TERRA primer following DNA/RNA hybrid immunoprecipitation with the S9.6 antibody or IgG in U2OS cells transfected with siRNAs targeting FANCM or control (siLuc). **(E)** 4SET assay for U2OS cells after transfection of siRNAs targeting FANCM or control (siLuc) with Mirin treatment. G-rich probe was used to detect C-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA image (loading control) for G-rich probe gel. **(F)** Western blot analysis of U2OS RAD51AP1 KO (C1) or control. **(G)** 4SET assay for U2OS RAD51AP1 KO (C1) or control with Mirin treatment. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA image (loading control) for G-rich probe gel. **(H)** 4SET assay for U2OS treated with Pyridostatin (PDS; 0, 5, 10 μM, 48 hrs). **(I)** Quantification of 4SET from H, representing the quantification of relative amount of C-rich ssDNAs (mean ± SD; unpaired t-test). **(J)** 4SET assay for HeLa LT TERC KO or HeLa LT TERC KO cells expressing shRNA targeting ATRX gene (ATRX 590) with or without Mirin or PARPi treatment. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. SYBR DNA image (loading control) for G-rich probe gel.

Figure S6


A
U2OS
SaoS2
Relative STN1 mRNA level
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0.8
0.6
0.4
0.2
0.0
shNT
shSTN1
U2OS
SaoS2
shNT
shSTN1
shNT
shSTN1
(kda)
STN1
(ab89250)
-35
-55
beta-actin
-35
B
SaoS2
Whole
Soluble
Ladder
shNT
shSTN1
Mirin
SYBR DNA
(G-rich probe gel)
C
U2OS (whole)(600 ng)
Mirin
(kb)
23
4
2
0.5
C-rich ssDNA
G-rich probe
C-rich probe
SYBR DNA
(G-rich probe gel)
SYBR DNA
(C-rich probe gel)
D
U2OS
shNT
shPOLD3
(kda)
POLD3
(21935-1-AP)
-70
-55
beta-actin
-35
E
Whole
Soluble
U2OS
shNT
shPOLD3
Mirin
Ladder
SYBR DNA
(G-rich probe gel)
SYBR DNA
(C-rich probe gel)

Figure S6

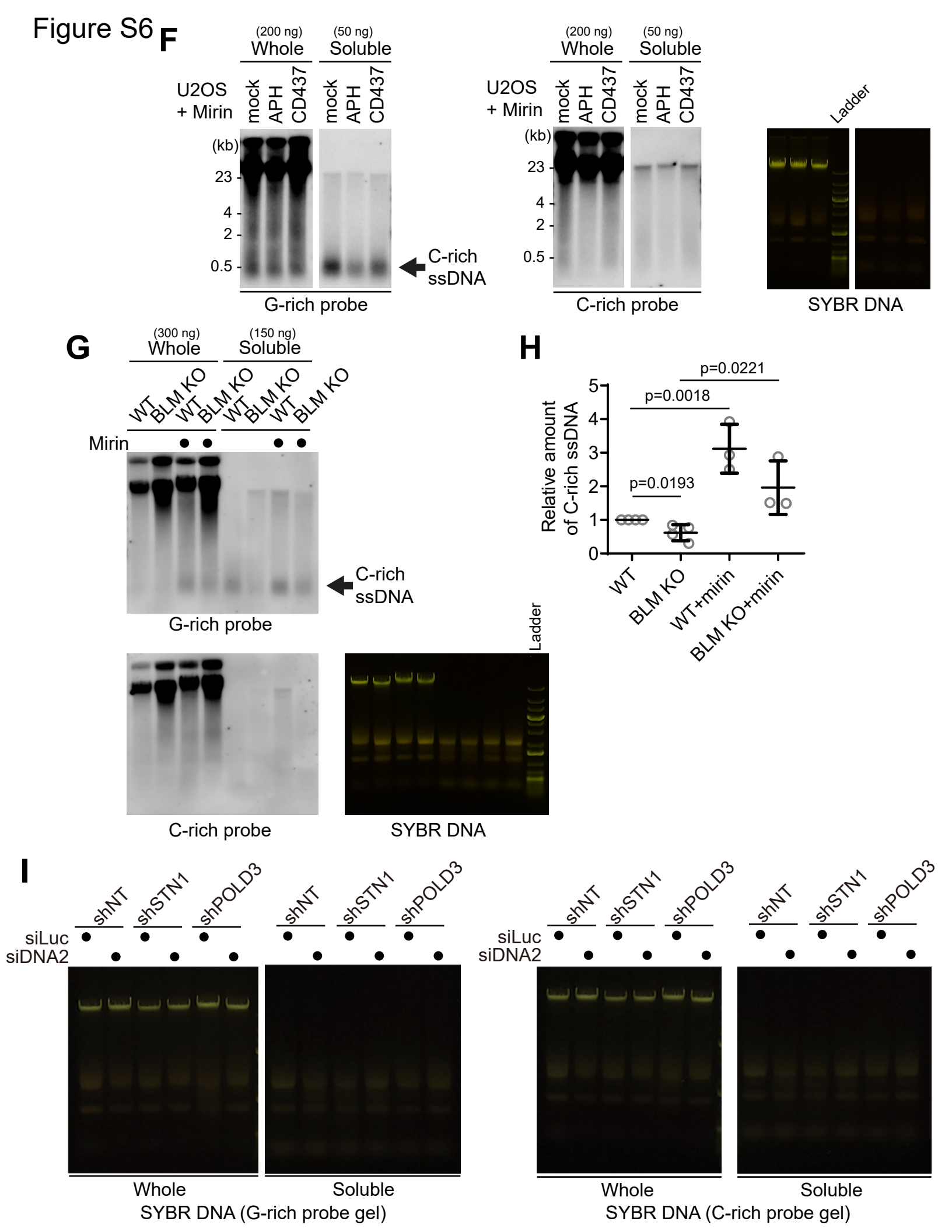
F
(200 ng)
Whole
(50 ng)
Soluble
U2OS
+ Mirin
mock
APH
CD437
(kb)
23
4
2
0.5
C-rich
ssDNA
G-rich probe
C-rich probe
Ladder
SYBR DNA
G
(300 ng)
(150 ng)
WT
BLM KO
Mirin
H
p=0.0221
p=0.0018
p=0.0193
Relative amount
of C-rich ssDNA
WT+mirin
BLM KO+mirin
I
shNT
shSTN1
shPOLD3
siLuc
siDNA2
Whole
Soluble
SYBR DNA (G-rich probe gel)
SYBR DNA (C-rich probe gel)

Figure S6, related to Figure 6. CST complex-mediated priming and subsequent strand displacements by DNA polymerase delta generates C-rich ssDNAs. (A) Relative amount of STN1 mRNA levels measured by quantitative-PCR (top) and STN1 Western blot (bottom) in U2OS and SaoS2 cells expressing shRNA targeting STN1 gene or control (shNT). **(B)** SYBR DNA images (loading control) for G-rich probe and C-rich probe gels in Fig. 6D. **(C)** 4SET assay for U2OS cells expressing shRNAs targeting STN1, or non-targeting (NT) control. Cells were treated with Mirin (50 μM, 48 hr). SYBR DNA images (loading control) for G-rich probe and C-rich probe gels. **(D)** Western blot of U2OS cells expressing shRNA targeting POLD3 gene or control (shNT). **(E)** SYBR DNA images (loading control) for G-rich probe and C-rich probe gels in Fig. 6F. **(F)** 4SET assay for Mirin treated U2OS cells along with aphidicolin, CD437 or control. Samples were fractionated into soluble DNA fraction or whole DNA. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA image (loading control) for G-rich probe gel. **(G)** 4SET assay for Mirin treated U2OS WT or BLM KO cells. G-rich probe was used to detect C-rich telomeric sequences, and C-rich probe was used to detect G-rich telomeric sequences. C-rich single stranded DNAs are indicated by an arrow. SYBR DNA image (loading control) for G-rich probe gel. **(H)** Quantification of the 4SET assay in G; as the relative amount of C-rich ssDNA (whole). (mean ± SD; unpaired t-test). **(I)** SYBR DNA images (loading control) for G-rich probe and C-rich probe gels in Fig. 6G.

Extended Methods

Cell culture

U2OS, SaoS2, and HeLa LT cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, Penicillin-Streptomycin (Gibco, 15070063). To prevent mycoplasma contaminations, we regularly used Plasmocin Prophylactic (InvivoGen) to maintain the cells according to the manufacturer's protocol.

Cells were subjected to the following treatments with specific concentrations and durations: Mirin (50 μM, 48 hr), Talazoparib (5 μM, 48 hr), Aphidicolin (0.2 μM, 48 hr), CD437 (0.2 μM, 48 hr), Pyridostatin (5 or 10 μM, 48 hr), Camptothecin (0.25 μM, 24 hr), Etoposide (10 μM, 48 hr), Zeocin (100 μg/ml, 48 hr), and doxycycline treatment (1 μg/ml, 24 or 48 hr for U2OS expressing pCW MRE11 WT or H129N, 1 week for U2OS^{ATRX}). Negative control (mock) samples were untreated.

siRNA-mediated knockdown

siRNA oligos were purchased from Sigma-Aldrich (10 nmol) and purified by desalting. The dTdT overhangs were added to ensure the stability of siRNA. The siRNAs were transfected into the cells at a final concentration of 20 nM, along with 2 μl of Lipofectamine 3000 (Thermo scientific, L3000001) according to the manufacturer's protocol.

The following siRNAs were used:
siLuc: UCGAAGUAUUCCGCGUACGUU,
siDNA2#1: CAGUAUCUCCUCUAGCUAGUU (1),
siDNA2#2: AUAGCCAGUAGUAUUCGAUUU (1),
siFEN1#1: UGACAUCAAGAGCUACUUU (2),
siFEN#2 : CCAUUCGCAUGAUGGAGAA (2),
siPRIMPOL: GAGGAAACCGUUGUCCUCAGUG (3),
siFANCM: AAGCUCAUAAAGCUCUCGGAA (4),
siMRE11: ACAGGAGAAGAGAUCAACU (5),

Lentiviral shRNA knockdown and sgRNA knockout.

Lentiviruses were generated by transfecting shRNA vectors targeting human POLD3 (6), STN1 (7), ATRX(8), or the control vector, or sgRNA vectors targeting human PML exon 3 (sense: CACCGGcggtaccagcgcgactacg, antisense: AAACcgtagtcgcgctggtaccgCC) inserted in pLentiV2 vector (Addgene, 52961) along with pMD2.G and psPAX into 293T cells. Transfection was carried out using TransIT (Mirus) following the manufacturer's protocol. After 24 hours, the medium was replaced with fresh medium, and cells were incubated for an additional 24 hours. Supernatants containing the lentiviruses were collected and passed through a 0.45 μm syringe filter. The collected supernatants were then concentrated using 4x virus concentrator solution. This solution consisted of phosphate-buffered saline (PBS) at pH 7.2 containing 1.2 M sodium chloride (NaCl) and 40% (v/w) PEG-8000. The concentrated virus pellets were used to infect target cells, and 0.5 μg/ml polybrene was added to enhance viral transduction. Cells were selected under either 5 μg/ml Blasticidin-S (Invivogen) for two weeks or 5 μg/ml Puromycin (Invivogen) for 5 days to establish stable cell lines expressing the desired shRNA or sgRNA. After selection, cells are cloned and confirmed by RT-qPCR or Western blot. Proper biosafety procedures were followed when working with lentiviruses.

Plasmid

cDNAs for MRE11 WT and H129N were obtained from Addgene (#177168, #177169) and inserted into pCW vector. The pCW-MRE11 WT or H129N vectors were employed to produce lentivirus for a

doxycycline-inducible expression system of either MRE11 WT or H129N, following above mentioned-protocol.
cDNA encoding PRIMPOL was synthesized from Integrated DNA Technologies (IDT) and cloned into pCS2(+) NLS-mCherry vector. PRIMPOL S255D mutant was generated using Q5 site-directed mutagenesis kit (NEB). The pCS2 NLS-mCherry-PRIMPOL WT or S255D vectors were used for transfection in U2OS PRIMPOL KO cells using TransIT (Mirus) according to the manufacturer's protocol. cDNA expression and transfection efficiency were assessed by mCherry signal and western blot.

Strand-Specific Southern for Single-stranded Extrachromosomal Telomeres (4SET)

- **DNA preparation procedure**

Cells were harvested using Trypsin-EDTA and then divided into separate tubes for fractionation. The cells were pelleted via centrifugation (500 g, 5 min) and washed with PBS. Cell pellets can be preserved at -80°C prior to 4SET analysis. For the whole DNA fraction, cells were lysed by adding lysis buffer (0.1 M Tris pH 8.0, 0.1 M EDTA, 1% SDS). To obtain the chromatin and soluble fractions, cells were treated with extraction buffer (PBS, 5 mM EDTA, 0.1% Triton X-100) and subjected to pipetting over ten times. The cell lysate was subsequently centrifuged at 1500 g for 5 min to separate the chromatin pellet from the soluble supernatant. The chromatin pellet was then lysed using lysis buffer. After obtaining the whole, chromatin, and soluble fractions, NaCl was added to each fraction (final concentration 1.6 M NaCl). The fractions were then centrifuged at 4°C, 15,000 g for 20 minutes to remove cell debris and obtain the DNA-containing supernatant. To precipitate the DNA, Glycogen (GlycoBlue Coprecipitant, 75 μg) was added to the supernatant, followed by the addition of 75% of the supernatant volume of isopropanol.
It has been observed that the isopropanol/ethanol precipitation procedure can result in unreliable nucleotides recovery efficiency, particularly for small nucleic acids when the concentration of nucleic acid is low (9). During isopropanol/ethanol DNA precipitation, the presence of bulk DNA/RNA can act as a carrier, which facilitates the precipitation of small-sized nucleic acids. To overcome this issue, we introduced excessive amounts of glycogen (75 μg) as a carrier during the isopropanol precipitation step. Adding glycogen led to a minimum 10% increase in DNA recovery compared to the procedure without glycogen. Additionally, we incubated the mixture at -20 degrees Celsius for a minimum of 3 hours to maximize the precipitation of single-stranded DNA fragments.
The mixture was subjected to additional centrifugation at 4°C, 15,000 g for 20 minutes to obtain the DNA pellet. Subsequently, the DNA pellet was washed twice with 70% ethanol by centrifuging at 15,000 g for 5 minutes each time. Finally, the DNA pellet was dissolved in DW (distilled water) and incubated overnight at 4 °C to ensure complete dissolution. The DNA concentration was measured using Qubit (Qubit dsDNA HS Assay Kit, Invitrogen) or a nanodrop spectrophotometer.

- **Southern blot procedure**

For electrophoresis, 100-400 ng of whole DNA, measured using Qubit, was loaded into 0.6% Agarose gel and run at 40 V (2.9V/cm) in TAE (Tris-acetate EDTA) buffer. Subsequently, the agarose gel underwent sequential incubations in the following solutions: Depurination solution (0.2 M HCl) for 5 minutes, Denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes, and Neutralization Buffer (0.5 M Tris pH 8.0, 1.5 M NaCl) for 20 minutes. The DNA was then transferred onto a Nylon membrane (Nylon Membranes, positively charged, Roche) using a Vacuum Blotting System (VacuGene XL) at 30 mbar for 1 hour in 4xSSC (Saline Sodium Citrate) buffer. Following this, the membrane was incubated with 1 nM DIG (Digoxigenin)-labeled Telomere probes in Hybridization solution (DIG Easy Hyb, Roche) at 42°C. Afterward, the membrane underwent two washes with Wash buffer 1 (2xSSC, 0.1% SDS) and two washes with Wash buffer 2 (0.5xSSC, 0.1% SDS) for 10 minutes each. The membrane was then

blocked with Blocking solution (1% Blocking reagent, Roche 11096176001, in Maleic acid buffer pH 7.4) for 30 minutes, followed by incubation with 0.2% Anti-Digoxigenin-AP Fab fragments (Roche) in Blocking solution for 1 hour. Subsequently, the membrane underwent two washes with Wash buffer 3 (Maleic acid, 0.3% tween-20) for 15 minutes each. Finally, the membrane was incubated with 1% CDP-star (Roche 11759051001) in AP buffer (50 mM Tris pH 9.5, 100 mM NaCl) for detection, which was performed using the Odyssey Imaging System (LI-COR 2800).

- **Strand specific probe generation procedure**

For the generation of strand-specific probes, we made minor modifications to the previous protocol (10). Annealed templates (TC, TG, and UP* in the list below) were generated at a concentration of 40 µM in NEBuffer#2.1 (NEB) buffer. The templates were then incubated with 50 units of exo-Klenow enzyme (NEB) and 1 mM dNTP with 0.35 mM DIG-dUTP (biorbyt) at 25°C overnight, followed by heat inactivation at 75°C for 20 minutes. Subsequently, we performed a 10-unit T4 DNA polymerase reaction to improve specificity. The double-stranded oligos were purified using NucleoSpin Gel and PCR clean-up (MN) with NTI buffer following the manufacturer's instructions. Next, a 20-unit lambda exonuclease reaction was carried out to obtain single-stranded oligos for 1 hour at 37°C. Finally, the single-stranded telomere probes were purified using NucleoSpin Gel and PCR clean-up (MN) with NTC buffer.
Following oligos were used:
Telomere C-rich probe template (TC):
5'[Phos]GGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGATAGTTGAGAGTC-3'
Telomere G-rich probe template (TG):
5'[Phos]CCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAGATAGTTGAGAGTC-3'
Universal priming oligo (UP*): 5'-[Phos]GACTCTCAAC*T*A*T*C*T*A-3'
(*) indicates phosphorothioate bond

CsCl separation (Cesium gradient analysis)

CsCl separation was performed as previously with minor modification (11). Briefly, cells were treated with 100 µM IdU for 20 hours, in conjugation with 10 nM FudR and 100 nM PARG inhibitor to promote IdU-incorporation and the recovery of C-rich ssDNAs. Post-incorporation, genomic DNA was extracted, yielding approximately 200 µg using the Puregene Core Kit A from Qiagen. The extracted DNA was then subjected to digestion using a combination of HinfI and RsaI enzymes. Following digestion, the DNA samples were mixed with a CsCl solution to achieve a target density of 1.770 g/ml. Centrifugation of these samples was performed at a speed of 55,000 rpm for 20 hours, utilizing a VTi-90 vertical rotor from Beckman. After centrifugation, the fractions were collected and a slot blot analysis for each fraction was executed.

Cell fractionation using E1 and E2 buffer.

Cells were lysed in E1 buffer (140 mM NaCl, 1 mM EDTA, 50 mM HEPES-KOH pH 7.5, 0.5% NP-40, 10% glycerol, and 0.25% Triton X-100). After mixing, the mixture was centrifuged at 2000 g for 2 minutes. The supernatant was prepared as the cytoplasm fraction. The pellet was then washed twice with E1 buffer, centrifuging at 1000 g for 2 minutes each time. On the second wash, the pellet was incubated on ice for 15 minutes. Subsequently, the pellet was resuspended in E2 buffer (200 mM NaCl, 1 mM EDTA, 10 mM Tris pH 8, and 0.5 mM EGTA pH 8). After a 20-minute incubation on ice, the supernatant was collected (nucleoplasm fraction). The remaining pellet was designated as the chromatin fraction.

Nascent C-rich ssDNA pull-down (native IdU-immunoprecipitation)

IdU-pulldown was conducted under native conditions (without heat or denaturation), as detailed described (37). Cells were exposed to 100 µM IdU for 20 hours (with Mirin concurrently in Fig.3).

Genomic DNAs from chromatin and soluble fractions were harvested from approximately 10 million cells. 1 µg of this genomic DNA from soluble fraction was mixed with 3 µg of anti-IdU/BrdU antibody (3D4 clone, BD Science) in BrdU-IP buffer composed of PBS and 0.0625% Triton X-100 overnight. This mixture was then subjected to a 1-hour incubation with Protein G magnetic beads. The immunoprecipitated DNA was subsequently eluted using an elution buffer (10 mM Tris pH 8 and 1% SDS). The elution was performed at room temperature and agitated at 1000 rpm for 1 hour. This was then purified using NucleoSpin Gel and PCR clean-up (MN) with NTC buffer. Eluted samples were analyzed using a native agarose gel electrophoresis.

C-circle assay

Genomic DNAs were subjected to incubation with 10 units of phi29 polymerase (NxGen phi29), 0.2 mM dNTP mix, and 1X phi29 buffer at 30°C for 12 hours, followed by a 20-minute incubation at 65°C. Subsequently, the samples were loaded onto a Nylon membrane using a slot-blot technique. The subsequent steps of hybridization and detection followed the same procedure as described in the 4SET method.

Western Blot

Samples were treated with Laemmli loading buffer (2% SDS, 5% beta-mercaptoethanol, 10% Glycerol, 0.002% Bromophenol Blue, 62.5 mM Tris-base) and subjected to 10 seconds of ultrasonication. The prepared samples were then separated on a Bis-Tris or Tris-HCl polyacrylamide gels and transferred to a PVDF membrane using the iBlot 2 Dry Blotting or Trans-Blot System. After blocking with 5% skim milk or 3% BSA in PBS-T (PBS-0.1% Tween-20) for 1 hour at room temperature, the membrane was incubated with the primary antibody overnight at 4°C. Following PBS-T wash, the membrane was incubated with the secondary antibody for 1 hour at room temperature. After an additional PBS-T wash, the ECL solution was applied, and detection was performed using the Odyssey Imaging System (LI-COR 2800).

Antibodies

The following antibodies were used for Western Blot: anti-Beta actin (Sigma-Aldrich, A5316), anti-Histone H3 (Cell signaling, 2650), anti-ATRX (Santa Cruz, sc-15408), anti-hMRE-11 (Calbiochem, PC388), anti-GAPDH (Cell signaling, 97166), anti-Lamin A/C (Santa Cruz, sc-376248), anti-Emerin (Santa Cruz, sc-25284), anti-RAD51AP1 (Proteintech, 11255), anti-DAXX (Santa Cruz, sc-8043), anti-DNA2 (Proteintech, 18727-1-AP), anti-FEN1 (Santa Cruz, sc-28355), anti-PRIMPOL (Proteintech, 29824-1-AP), anti-STN1 (Abcam, ab89250), and anti-POLD3 (Proteintech, 21935-1-AP).

RNA extraction and quantification

For RNA quantification, total RNA was extracted by using Direct-zol RNA Miniprep kits (ZYMO) according to the manufacturer’s protocol. cDNAs were synthesized using RevertAid First Strand cDNA synthesis kit (Thermo scientific) and qPCRs were performed using TB Green Premix Ex Taq II (Tli RNase H Plus) (Takara) according to the manufacturer's protocol.
The following DNA probes were used for qPCR:
GAPDH F: TGAGAACGGGAAGCTTGTCA,
GAPDH R: AGCATCGCCCCACTTGATT,
STN1 F: TGCACAGAAAGATCCACCGG,
STN1 R: AGGCCAAGATGTGCAGGAA,
DNA2 F: ACTGTGAAAAGCGCCTGGT,
DNA2 R: AGATGTGCAGTCTCCCTCCA,
FEN1 F: TTCTTCAAGGTGACCGGCTC,

FEN1 R: TTAAACTTCCCTGCTGCCCC,
FANCM F: AGACCTTTATTGCCGCCGT,
FANCM R: TTCGTTGGGGCCATGAAGA

Statistical Analyses

Statistical analyses for the experiments were conducted using GraphPad Prism 8. The graph displays all individual values. Detailed information regarding the tests and corresponding p-values is provided in the figures and their respective legends.

Extended Discussion

4SET, an efficient and simple method to detect extrachromosomal single stranded DNAs

We demonstrate an efficient and simple method to detect extrachromosomal telomeric ssDNAs called 4SET (Strand-Specific Southern-blot for Single-stranded Extrachromosomal Telomeres) assay. This technique can be performed within two days by molecular biologists at an intermediate level of expertise, without the need for specialized or expensive laboratory equipment, allowing us to investigate single-stranded extrachromosomal telomeres in a time-efficient and cost-effective manner. Additionally, this assay offers a strand-specific approach, allowing for the discrimination of specific telomere strands during analysis. For example, by employing the 4SET assay, here we proposed the model of underlying mechanisms of generation of single-stranded extrachromosomal telomeres which are derived from strand displacement of lagging strands. Furthermore, the simplicity and efficiency of the 4SET assay make it suitable for implementation in various research settings, such as assessing other repeat sequences of interest.

Comparison between 4SET and C-circle Assays and the Contribution of the 4SET Assay

In PML knockout Saos-2 cells, the 4SET assay failed to detect any C-rich ssDNAs (Fig. S1I), whereas the C-circle method identified decreased levels of C-circles (Fig. S1J). This observation raises concerns about the sensitivity of the 4SET assay compared to the C-circle assay, particularly in detecting circular forms of C-rich ssDNAs. Such a discrepancy indicates a differential sensitivity between the two assays in identifying circular forms of C-rich ssDNAs.

However, it is important to note that while the C-circle assay is adept at amplifying and detecting C-circles, it relies on Phi29-polymerase-mediated rolling circle amplification for 12-18 hrs, which can nonspecifically amplify other DNA replication/recombination intermediates (Fig. 1 in Henson et al. (2017) (12)). This characteristic necessitates a higher threshold for determining ALT activity in cancer cells, often set at a 5-fold difference compared with non-ALT cells (13). In contrast, our 4SET assay

exhibits a high specificity, consistently failing to detect C-rich ssDNA signals in non-ALT cells. This specificity brings into question the nature of residual C-circle levels in PML KO cells – whether they are true C-circle signals or artefacts of DNA replication/recombination intermediate amplification (12).

Moreover, the unique ability of the 4SET assay to isolate and detect C-rich ssDNAs originating from the daughter strand of lagging strands, as presented in Fig 2, is an advancement. This capability is crucial for hypothesizing that C-rich ssDNAs are derived from the Okazaki fragment processing, providing a foundational understanding that is not attainable with the C-circle assay alone.

In essence, while both assays have their merits, the 4SET assay contributes new insights, particularly regarding the origin and formation mechanism of C-rich ssDNAs, thereby complementing and extending the findings obtainable through the C-circle assay, as we demonstrate a new model here.

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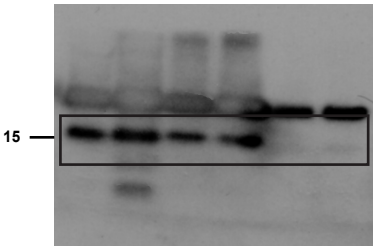
Uncropped WB

Figure S1D



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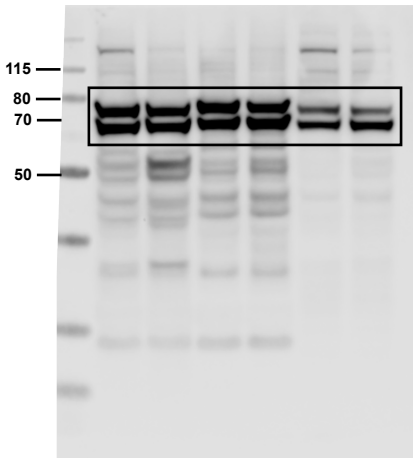

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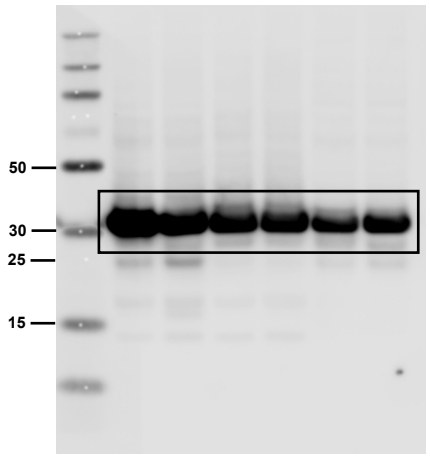

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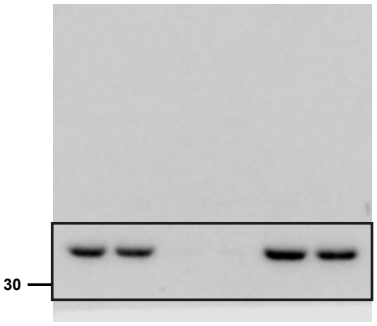

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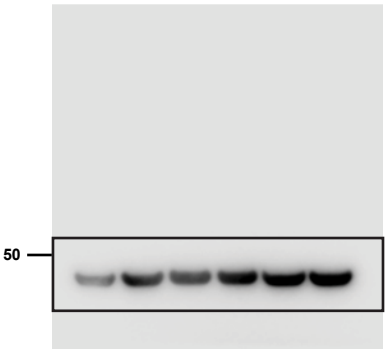

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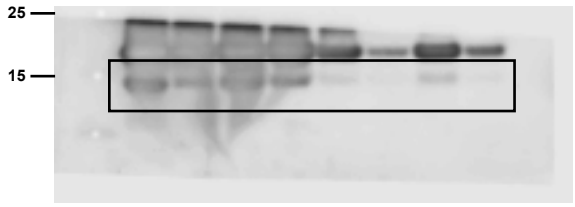
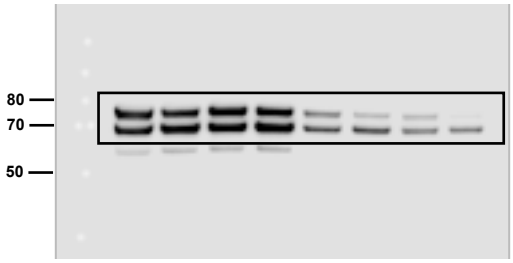
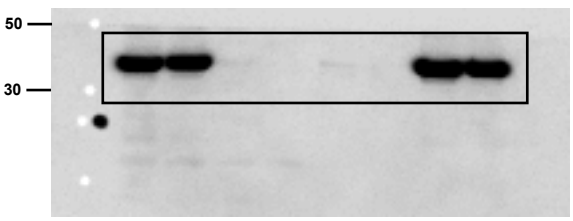
Figure S1O



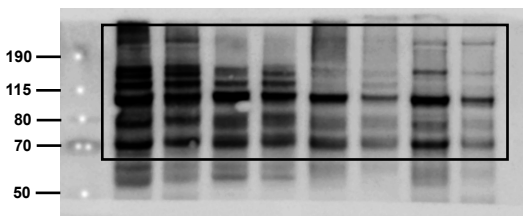
IB : H3



IB : Lamin A/C



IB : GAPDH



IB : PML

Figure S3E

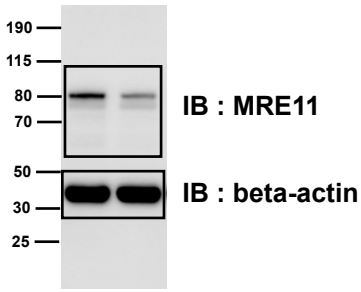


Figure S3H

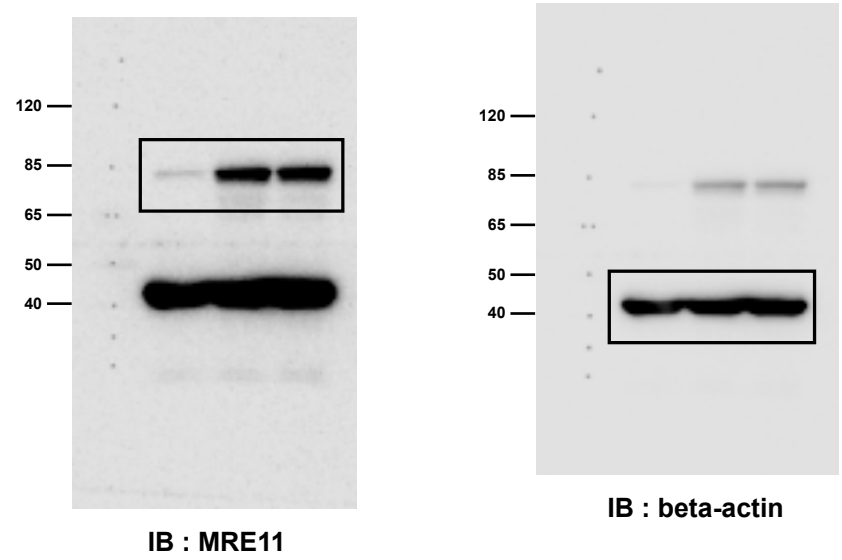


Figure S4A

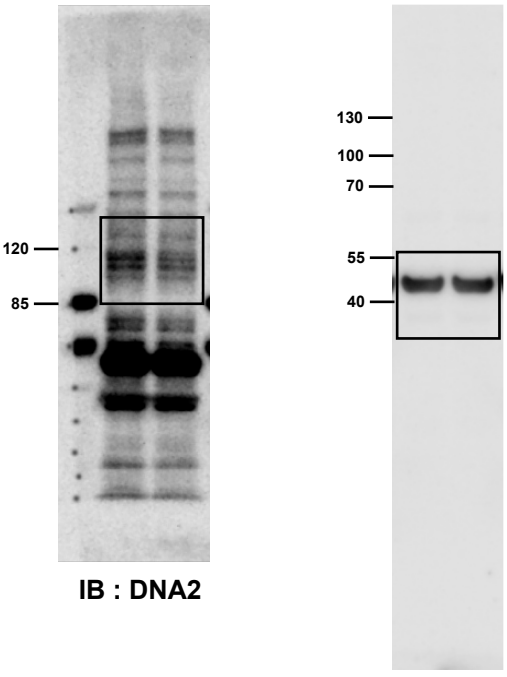


Figure S4C

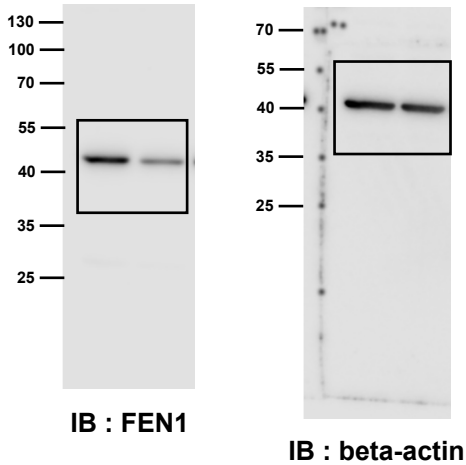


Figure S4G

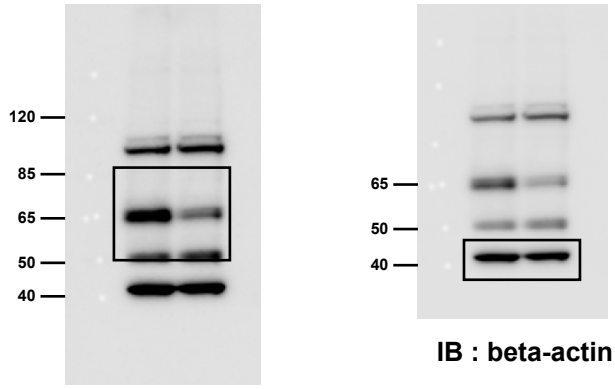


Figure S4I

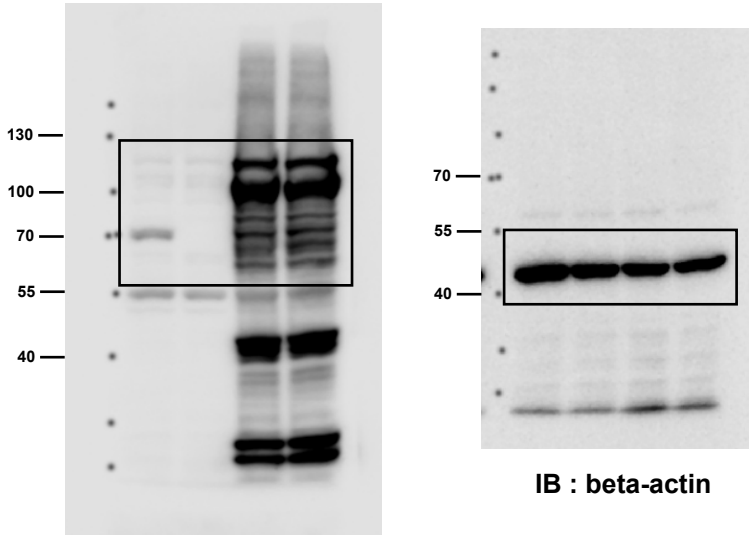


Figure 5A

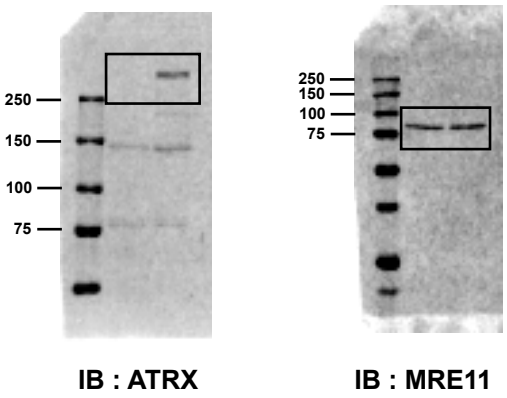


Figure S5F

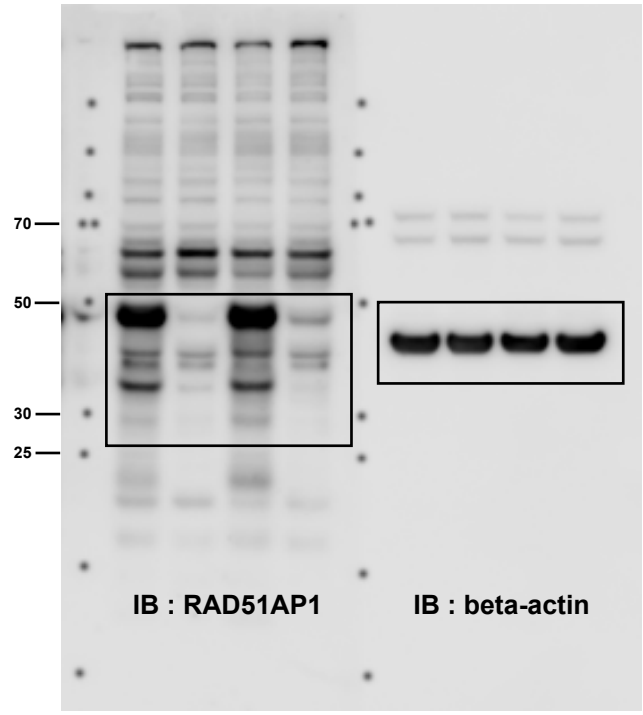
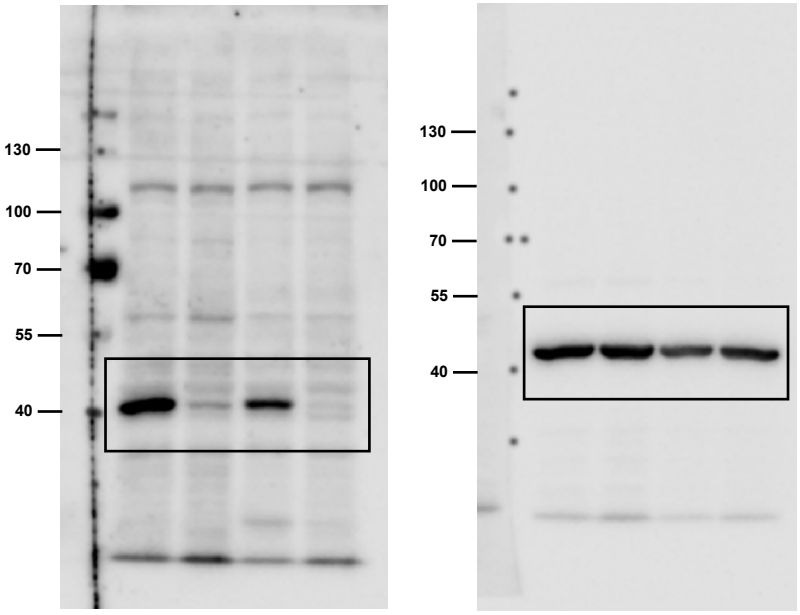
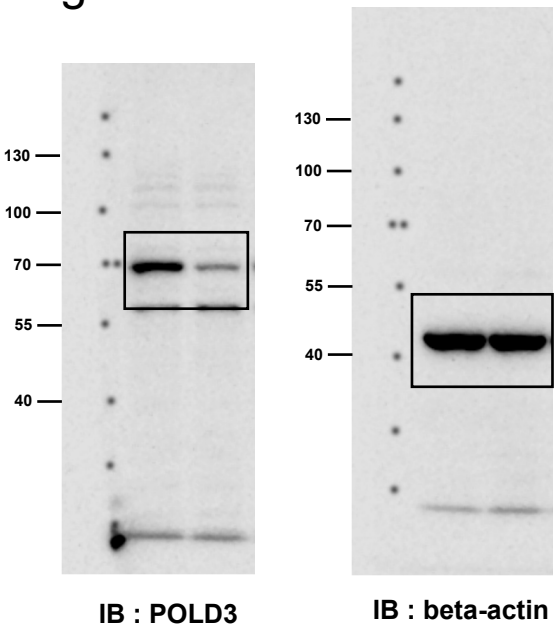


Figure S6A



IB : STN1 IB : beta-actin

Figure S6D



IB : POLD3 IB : beta-actin