SUPLEMENTAL INFORMATION

Supplemental figure legends

Fig. S1 (related to Fig. 1). Effects of TERRA on telomeric R-loops and ALT in RAD52 KO cells

(A) Representative images of TERRA localization at APBs in U2OS WT and RAD52 KO cells analyzed by RNA-ISH (TelC PNA probe) and immunostaining (PML and TRF2 antibodies). **(B)** APBs and TERRA foci were quantified in U2OS WT and RAD52 KO cells (from A; upper panels) (n>350). Red lines: means. ****: P-value <0.0001. The correlations between APBs and TERRA foci in U2OS WT and RAD52 KO cells were analyzed (lower panels). R values were calculated with Prism-9 software using the simple linear regression analysis. **(C)** Representative images of TERRA foci in U2OS WT cells treated with Control or TERRA LNA (100 nM) for 24 hr (upper panel). Quantification of TERRA foci in cells treated with Control or TERRA LNA (bottom left panel), and in cells treated with Control LNA and with or without RNaseA (125 ng/ml, 1hr) (right). Red lines: mean value. ****: P value <0.0001. **(D)** Indicated amounts of total RNA from U2OS cells transfected with Control or TERRA LNA (100 nM, 24 hr) was analyzed by dot blot using the TelC probe (18-nt). Input RNA was normalized using the GAPDH probe. The RNaseA (125 ng/ml, 1hr) treated sample was used as a negative control. TERRA levels were quantified over background in the lower panel (a representative of three experiments). **(E)** RNA was isolated from U2OS cells treated with Control or TERRA LNA, extensively digested with DNase I, and analyzed by RT-qPCR using primers specific to a sub-telomeric sequence in chromosome 10q. Error bars: SD (n=4, 4 technical duplicates). ***: P value 0.0006. **(F)** Cell cycle profiles of U2OS cells after Control or TERRA LNA treatments (100 nM, 30 hr). **(G)** DRIP samples of U2OS WT cells were treated with or without RNaseH (5 units for overnight at 37 ^{0}C) and then analyzed by telomere-specific or β-actin-specific qPCR. Error bars: SEM (n=2). *: P value 0.014, **: P value 0.002. **(H)** U2OS WT and RAD52 KO cells were analyzed by the C-circle assay. The RCA+/RCA- ratios reflect the relative levels of C-circle amplification. Error bars: SEM (n=2). **: P value 0.002. **(I)** Telomeric R-loops in U2OS RAD52 KO cells were analyzed by PLA using S9.6 and TRF2 antibodies. S9.6 and TRF2 antibodies alone were used as negative controls (n>140). Red lines: mean intensities. ****: P value <0.0001. **(J)** U2OS WT and RAD52 KO cells were quantified for telomeric DNA:RNA hybrids with the TRF2-S9.6 PLA assay. Red lines: mean intensities. ****:

P value <0.0001. **(K)** APBs were quantified in U2OS WT and RAD52 KO cells treated with Control or TERRA LNA (shown in Fig. 1B) (n>120). **: P value 0.001, NS: not significant. **(L)** Doxycycline-mediated induction of RNaseH1-GFP in U2OS cells was confirmed by western blot (left panel). U2OS cells expressing RNaseH1-GFP were transfected with Control or TERRA LNA, treated with 200 nM Doxycycline for 26 hr, and then analyzed for C-circle levels (right panel). Error bars: SEM (n=3). *: P value 0.013, ****: P value <0.0001, NS: not significant.

Fig. S2 (related to Fig. 2). The ability of RAD51AP1 to promote telomeric R-loop formation and its effects on telomeric R-loops and ALT in RAD52 KO cells

(A) Increasing concentrations of RAD51AP1 were incubated with TERRA-IR800 (60-nt), and reaction products were separated on a native acrylamide gel. **(B)** TERRA-IR800 was incubated with increasing concentrations of RAD51AP1 and dsDNA containing telomeric sequences. Reaction products were separated an agarose gel and telomeric R-loops were quantified (a representative image of three independent experiments). **(C)** Increasing concentrations of $RAD51AP1^{WT}$ or $RAD51AP1^{DBM}$ were incubated with TERRA-IR800, and reaction products were separated on a native acrylamide gel. Binding efficiency was quantified by measuring the reduction of free TERRA in the gel. Mean values of two experiments (n=2) are shown. **(D)** Increasing concentrations of $RAD51AP1^{WT}$ or $RAD51AP1^{DBM}$ were incubated with TERRA-IR800 (60-nt, 60 nM) and dsDNA containing telomeric sequences. **(E)** Telomeric R-loops were generated with RAD51AP1 (0.5 μM), biotin-labeled TERRA (48-nt, 30 nM), and dsDNA containing telomeric sequences. The R-loops were captured with streptavidin beads (10 μL) and quantified by qPCR using primers specific to the dsDNA. The effects of omitting various components of the reaction were analyzed. A sample of a complete reaction was treated with RNaseH (0.5 unit) to confirm the presence of R-loops. The level of R-loop in the sample with no protein (lane 4) was defined as 1, and other samples were normalized to it. **(F)** RAD51, RAD52, or RAD51AP1 (0.5 μM each) was incubated with biotin-labeled TERRA and dsDNA containing telomeric sequences. The resulting telomeric R-loops were quantified as in E. The level of R-loops in each sample was compared to a no protein control sample, and the relative levels of protein-dependent R-loops are shown. Error bars: SEM, n=2. **(G)** Western blots confirming the knockdown of RAD51AP1. **(H)** Western blots confirming RAD52 KO and unchanged RAD51AP1 levels in RAD52 KO cells. **(I)** The TERRA foci colocalized with APBs in U2OS cells

treated with siControl and siRAD51AP1 were quantified (left panel). Red lines: means (n>180). NS: not significant. The levels of TERRA in U2OS cells treated with siControl or siRAD51AP1 were analyzed by RT-qPCR after extensive DNase I digestion (right panel). Error bars: SEM (n=2). *: P value 0.039. **(J)** SAOS2 and SKLU1 cells treated with siControl or siRAD51AP1 were quantified for telomeric DNA:RNA hybrids with the TRF2-S9.6 PLA. Red lines: means (n>100). ****: P value <0.0001. **(K)** SAOS2 and SKLU1 cells treated with siControl or siRAD51AP1 were quantified for the EdU signals at APB. Red lines: means (n>75). P value 0.109 (left) and 0.043 (right).

Fig. S3 (related to Fig. 3). RAD51AP1 promotes telomere maintenance and cell proliferation, and prevents senescence in RAD52 KO cells

(A) Western blots confirming RAD52 KO and RAD52, 51AP1 DKO in U2OS cells. **(B)** Representative images of telomeric FISH signals in U2OS WT, RAD52 KO, and RAD52, 51AP1 DKO (clones C16 and C18) cells. Signal free ends (SFEs) at telomeres were quantified in Fig. 3B. **(C)** Quantification of the β- galactosidase positive cells in the samples of Fig 3E.

Fig. S4 (related to Fig. 4). Telomeric R-loops promote G4 formation

(A) A schematic to explain how TERRA-mediated DNA:RNA hybrids in telomeric R-loops prevent D-loop formation and how G4s may promote telomeric D-loop formation after removal of DNA:RNA hybrids. The gray hexagon represents RAD51AP1. **(B)** U2OS cells were treated with DMSO or TMPYP4 (4 μM) for 6 hr, and the G4s at telomeres were analyzed by PLA using G4 (1H6) and TRF2 antibodies (n>130). To specifically detect G4s in DNA, the samples were treated with RNaseA (10 μg/ml) at 37 ºC for 1 hr. **(C)** U2OS RAD52 KO cells treated with siControl or siRNaseH1 were quantified for telomeric G4s by PLA using G4 (1H6) and TRF2 antibodies. Red lines: means (n>300). ****: P value <0.0001. **(D)** U2OS RAD52 KO cells treated with siControl or siSenataxin were quantified for telomeric G4s as in C. Red lines: means (n>300). ****: P value <0.0001. **(E)** U2OS RAD52 KO cells were transfected with Control or TERRA LNA, treated with DMSO or 200 nM Doxycycline for 26 hr to induce RNaseH1-GFP, and quantified for telomeric G4s by PLA. Red lines: mean intensities (n>170). **: P value 0.001, ****: P value <0.0001. **(F)** SAOS2 and SKLU1 cells treated with siControl or siRAD51AP1 were quantified for telomeric G4s by PLA. Red lines: means (n>60). ****: P value <0.0001.

Fig. S5 (related to Fig. 5). Knockdown of RNaseH1

Western blot confirming the knockdown of RNAseH1.

Fig. S6 (related to Fig. 6). A biotin-ssDNA and qPCR based assay to quantify telomeric D-loop formation

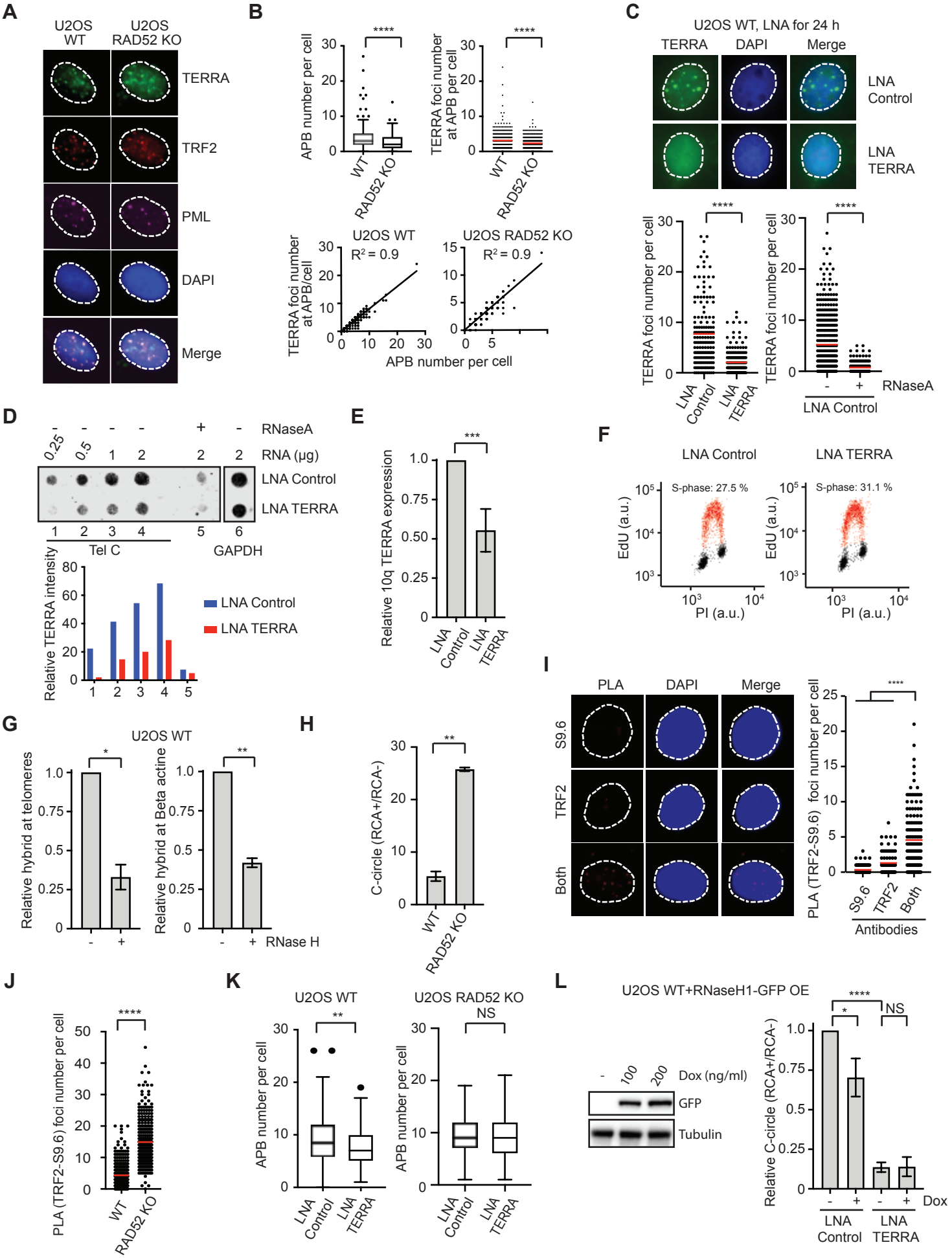
The experimental scheme to generate and quantify telomeric D-loops using biotin-labeled telomeric ssDNA and qPCR (Left). Biotin-TelG-ssDNA (60 nM) was incubated with RAD52 (0.3 μM) and dsDNA containing telomeric sequences. The resulting telomeric D-loops were captured with streptavidin beads (10 μL) and quantified by qPCR using primers specific to the dsDNA. Error bars: SEM (n=2).

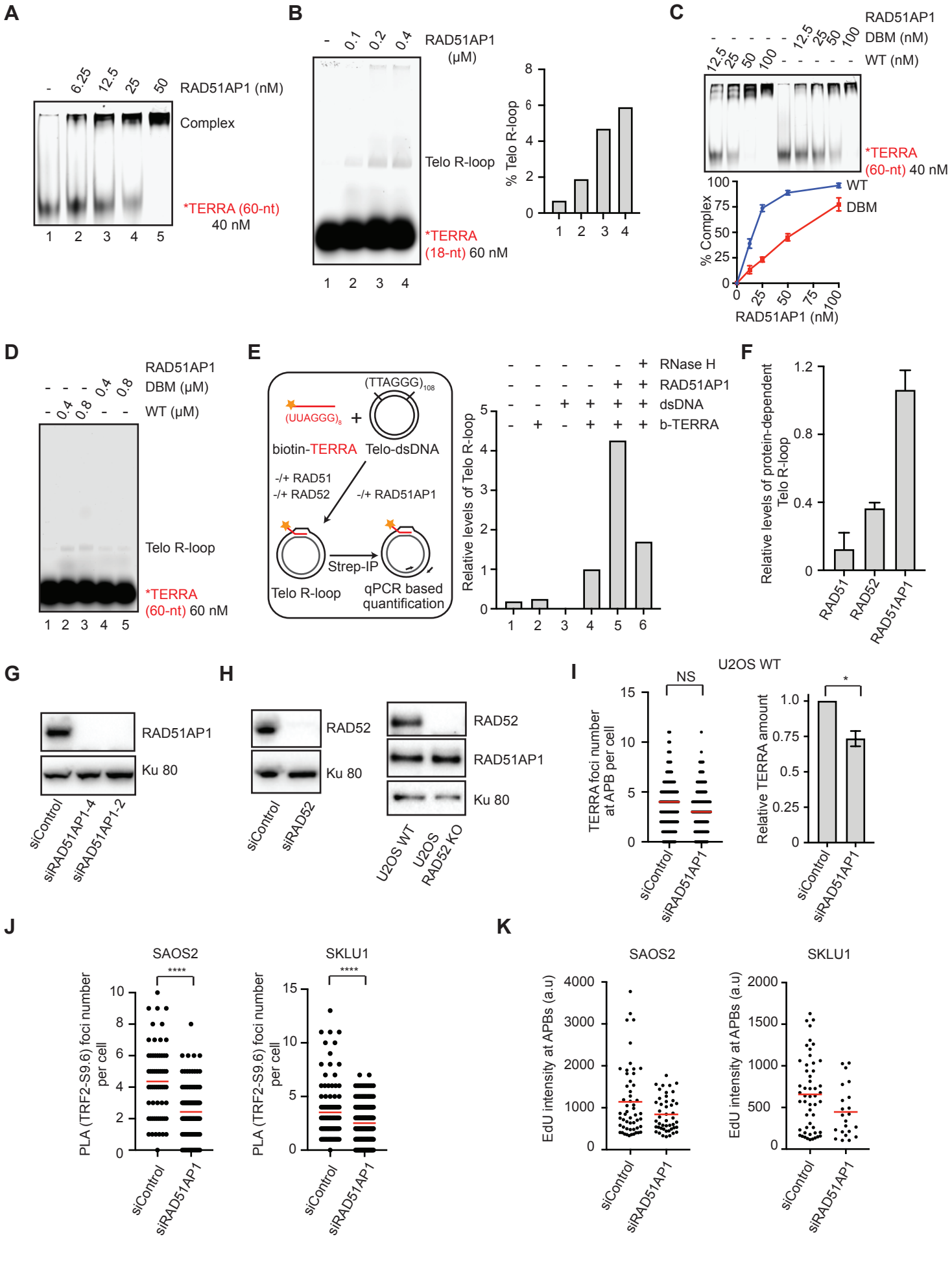
Table S1. List of Oligonucleotides used in this study (related to Figures 1, 2, 3, 4 and 5)

Sequence (5' to 3')	Oligo name
Silencer™ Select Negative Control No. 2	Control siRNA, 4390846
GCAGUGUAGCCAGUGAUUAtt	RAD51AP1 siRNA- 4
GAGUGAGGAUAAUGACGAAtt	RAD51AP1 siRNA- 2
AAAUCUAUUUGCUCGCUUUCAGAGGtt	BLM siRNA
GGGAAAGAGGUGAUCAACAtt	RNaseH1-2 siRNA
GCGCAGAGCCGUAUGCAAAtt	RNaseH1-3 siRNA
GCCAGAUCGUAUACAAUUA	Senataxin siRNA
+C*+A*+C*G*T*C*T*A*T*A*C*A*C*+C*+A*+C*	LNA Control
+T*+A*+A*C*C*C*T*A*A*C*C*C*T*+A*+A*+C*	LNA TERRA
TTAGGGTTAGGGTTAGGGTTAGGGTTAGGG TTAGGGTTAGGGTTAGGGTTAGGGTTAGGG	TelG-ssDNA (60-nt) and biotin-TelG-ssDNA
TTAGGGTTAGGGTTAGGG	TelG-ssDNA (18-nt)
UUAGGGUUAGGGUUAGGGUUAGGGUUAGG GUUAGGGUUAGGGUUAGGGUUAGGGUUAG GG	TERRA (60-nt)
UUAGGGUUAGGGUUAGGG	TERRA (18-nt)
UUAGGGUUAGGGUUAGGGUUAGGGUUAGG GUUAGGGUUAGGGUUAGGG	Biotin-TERRA (48-nt)
TTAGGGTTAGGGTTAGGGTTAGGGTTAGGG TTAGGG	Biotin-TelG-ssDNA (36-nt)
GGTTTTTGAGGGTGAGGGTGAGGGTGAGGG TGAGGGT	Telo-F (for telomere qPCR)
TCCCGACTAT CCCTATCCCTATCCCTATCCCTATCCCTA	Telo-R (for telomere qPCR)
CAGCAAGTGGGAAGGTGTAATCC	36B4-F (for genomic DNA qPCR)
CCATTCTATCATCAACGGGTACAA	36B4-R (for genomic DNA qPCR)
TAACCCTAACCCTAACCCTAACCCTAACCC	TelC-30 (for dot blot & RT)
GTAGACCCACGACATACTCAGCACCGGCCTC ACCCCATT	GAPDH (for dot blot)
AGGTCCACCACTGACAC	RT primer for GAPDH
AACCTGAACCCTAACCCTCC	qPCR FP for 10q
ATTGCAGGGTTCAAGTGCAG	qPCR RP for 10q
GATCATCAGCAATGCCTCC	qPCR FP for GAPDH
AGGTCCACCACTGACAC	qPCR RP for GAPDH

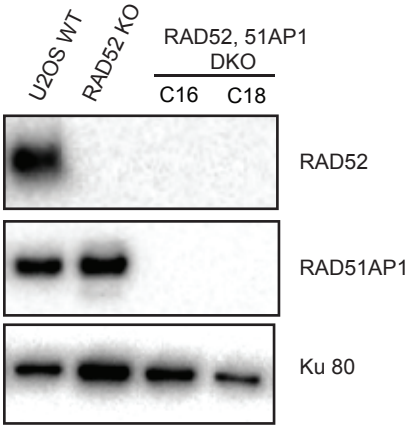
GGGACTATTTGGGGGTGTCT	Beta-actin FP (for qPCR)
TCCCATAGGTGAAGGCAAAG	Beta-actin RP (for qPCR)
AGATTTGGATAAGATTACTG	Guide #17 targeting RAD51AP1 Exon 5
TTCCGGAGATTGTTCAAGTT	Guide #21 targeting RAD51AP1 Exon 3

+modified bases; *modified phospho-backbone.

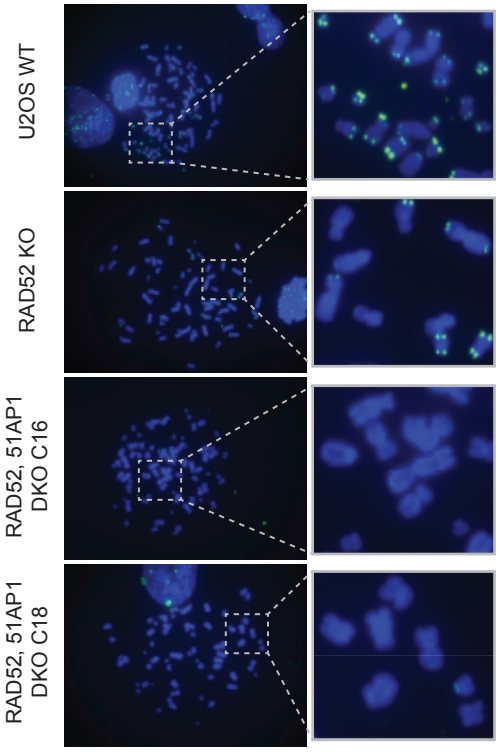

A
U2OS WT
U2OS RAD52 KO
TERRA
TRF2
PML
DAPI
Merge
B
APB number per cell
TERRA foci number at APB per cell
WT
RAD52 KO
U2OS WT
U2OS RAD52 KO
R² = 0.9
TERRA foci number at APB/cell
APB number per cell
C
U2OS WT, LNA for 24 h
TERRA
DAPI
Merge
LNA Control
LNA TERRA
TERRA foci number per cell
RNaseA
D
RNaseA
RNA (µg)
0.25
0.5
1
2
LNA Control
LNA TERRA
Tel C
GAPDH
Relative TERRA intensity
E
Relative 10q TERRA expression
F
LNA Control
LNA TERRA
S-phase: 27.5 %
S-phase: 31.1 %
EdU (a.u.)
PI (a.u.)
G
U2OS WT
Relative hybrid at telomeres
Relative hybrid at Beta actine
RNase H
H
C-circle (RCA+/RCA-)
I
PLA
DAPI
Merge
S9.6
TRF2
Both
PLA (TRF2-S9.6) foci number per cell
Antibodies
J
PLA (TRF2-S9.6) foci number per cell
K
U2OS WT
U2OS RAD52 KO
NS
APB number per cell
L
U2OS WT+RNaseH1-GFP OE
Dox (ng/ml)
GFP
Tubulin
Relative C-circle (RCA+/RCA-)
Dox


A
RAD51AP1 (nM)
Complex
*TERRA (60-nt) 40 nM
B
RAD51AP1 (μM)
Telo R-loop
*TERRA (18-nt) 60 nM
% Telo R-loop
C
RAD51AP1 DBM (nM)
WT (nM)
*TERRA (60-nt) 40 nM
% Complex
WT
DBM
RAD51AP1 (nM)
D
RAD51AP1 DBM (μM)
WT (μM)
Telo R-loop
*TERRA (60-nt) 60 nM
E
biotin-TERRA
Telo-dsDNA
-/+ RAD51
-/+ RAD52
-/+ RAD51AP1
Strep-IP
Telo R-loop
qPCR based quantification
RNase H
RAD51AP1
dsDNA
b-TERRA
Relative levels of Telo R-loop
F
Relative levels of protein-dependent Telo R-loop
RAD51
RAD52
RAD51AP1
G
RAD51AP1
Ku 80
siControl
siRAD51AP1-4
siRAD51AP1-2
H
RAD52
Ku 80
siControl
siRAD52
RAD51AP1
U2OS WT
U2OS RAD52 KO
I
U2OS WT
NS
*
TERRA foci number at APB per cell
Relative TERRA amount
siControl
siRAD51AP1
J
SAOS2
SKLU1
****
PLA (TRF2-S9.6) foci number per cell
K
EdU intensity at APBs (a.u)

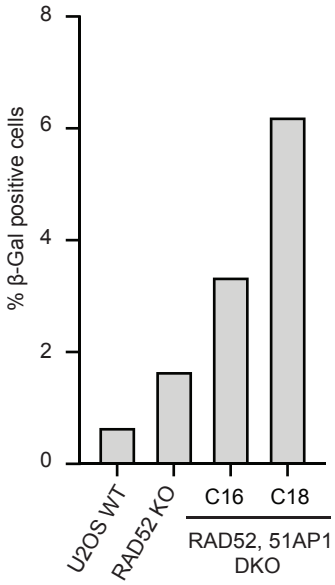
A


U2OS WT
RAD52 KO
RAD52, 51AP1 DKO
C16
C18
RAD52
RAD51AP1
Ku 80

B


U2OS WT
RAD52 KO
RAD52, 51AP1 DKO C16
RAD52, 51AP1 DKO C18

C


% β-Gal positive cells
0
2
4
6
8
U2OS WT
RAD52 KO
C16
C18
RAD52, 51AP1 DKO

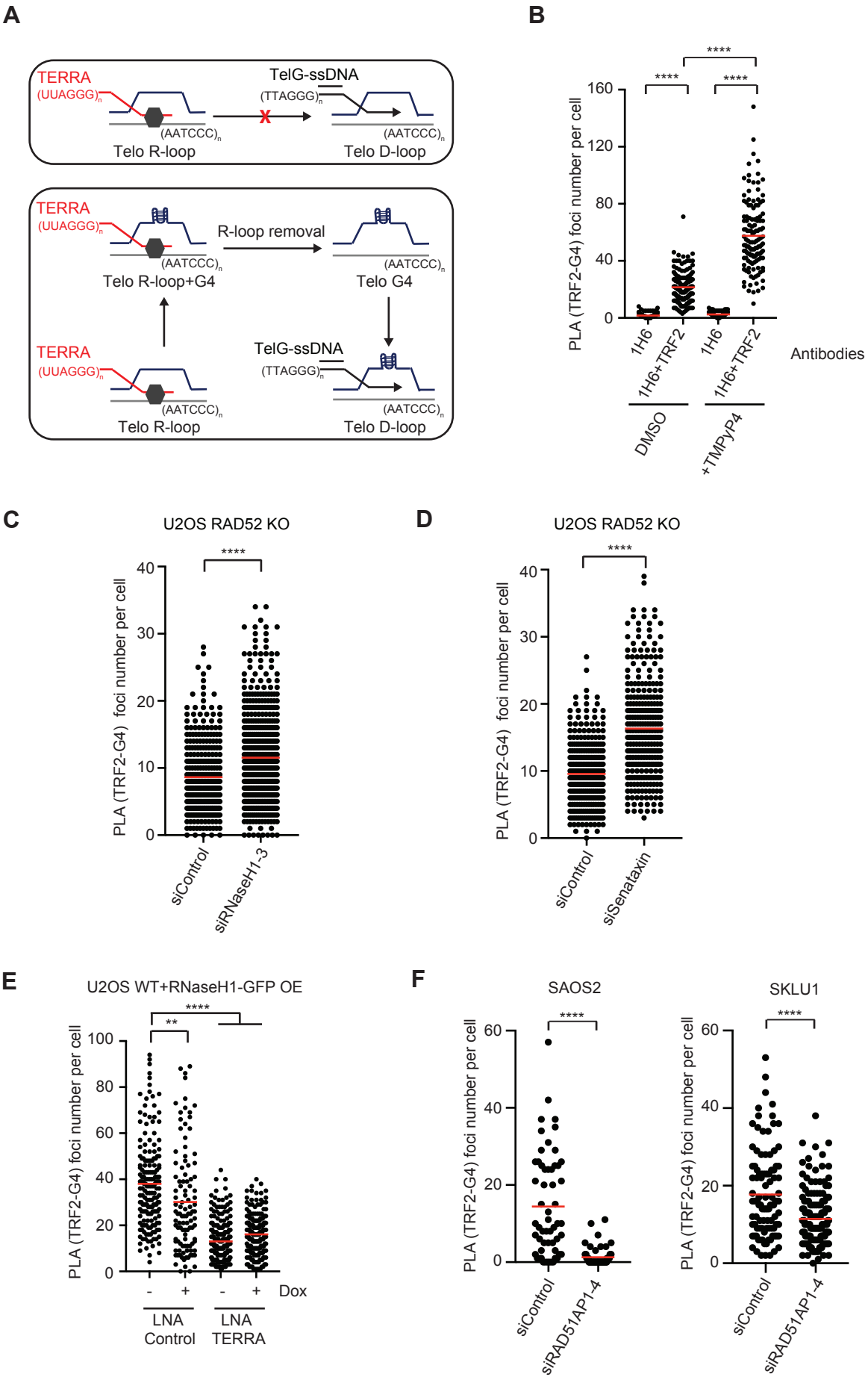

A
TERRA
(UUAGGG)n
TelG-ssDNA
(TTAGGG)n
(AATCCC)n
Telo R-loop
Telo D-loop
R-loop removal
Telo R-loop+G4
Telo G4
B
PLA (TRF2-G4) foci number per cell
1H6
1H6+TRF2
DMSO
+TMPyP4
Antibodies
C
U2OS RAD52 KO
siControl
siRNaseH1-3
D
siSenataxin
E
U2OS WT+RNaseH1-GFP OE
Dox
LNA Control
LNA TERRA
F
SAOS2
SKLU1
siRAD51AP1-4

Fig. S5

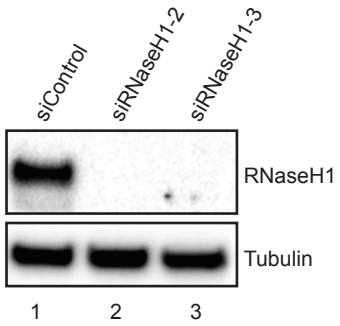
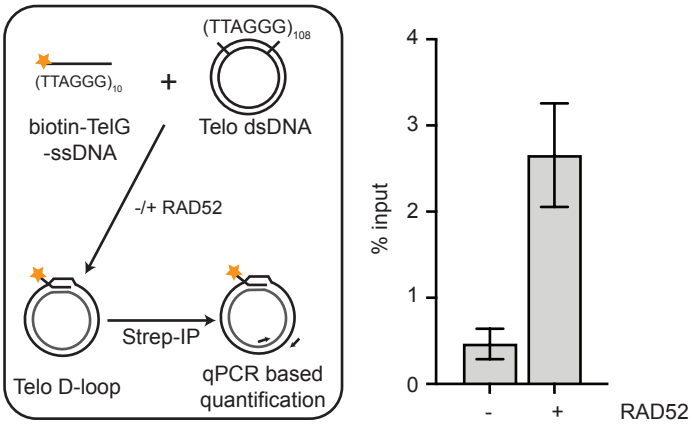


Fig. S6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(TTAGGG)10
+
biotin-TelG
-ssDNA
Telo dsDNA
-/+ RAD52
Strep-IP
Telo D-loop
qPCR based
quantification
% input
4
3
2
1
0
-
+
RAD52