FANCM suppresses DNA replication stress at ALT telomeres by disrupting TERRA R-loops

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SUPPLEMENTAL TABLE AND FIGURE LEGENDS:

Supplemental Table S1: siRNA used in this study

Fig. S1: Depletion of FANCM in Saos-2, but not HeLa and MG63 cells, induces pronounced C-circles formation. Saos-2, or HeLa, or MG63 cells were transfected with siRNA targeting either luciferase (siLuc) or FANCM (siFM and siFM-U). Genomic DNA was extracted and used for C-circle assay. "–Φ" indicates samples with no Phi(Φ)29 DNA polymerase added. The amount of C-circles in siLuc transfected cells is used to normalize C-circles in other siRNA transfected cells. All error bars are standard deviation of the mean obtained from two different experiments. Standard two-tailed Student's t-test: $**p<0.01$.

Fig. S2: Depletion of the FANCM complex has no pronounced effects on the telomere length. U-2 OS cells were transfected with siRNA targeting either luciferase (siLuc), FAAP24 (siFAPP24-1 and siFAAP24-2), FANCM (siFM and siFM-U), MHF1, MHF2, or both MHF1 and MHF2. Genomic DNA was extracted from the siRNA transfected cells and digested with Hindf I and Rsa I and then run on a 0.8% agarose gel.

Fig. S3: Knockdown efficiency of different siRNA in U2-OS cells.

Fig. S4: Depletion of FANCD2 does not affect checkpoint activation and ssDNA formation at telomeres in FANCM deficient cells. siRNA transfected U2-OS cells were co-stained with antibodies recognizing pChk1 and TRF1, or pRPA and TRF1. All error bars are standard deviation of the mean obtained from three different experiments. Standard two-tailed Student's t-test: n.s., not significant.

Fig. S5: Histogram graphs show the percentage of cells with the indicated number of ABP foci per nucleus (A) and the percentage of cells with the indicated number of TERRA associated APB foci (B, D). siRNA transfected cells were stained with TERRA probe, and antibodies recognizing PML or TRF2. (C) U2-OS cells stably express either wild-type (WT) RNase H1 or

mutant (Mut) RNase H1. The *p* values were calculated by two-tailed Student's t-test. n= number of cells.

Fig. S6: Histogram graphs show the percentage of cells with the indicated number of telomeric R-loops foci (S9.6 and TRF2 colocalization) per nucleus (A, C) and the percentage of cells with the indicated number of TERRA R-loops (TERRA, S9.6 and TRF2 colocalization) foci per nucleus (B, D). RNase III was added to siRNA transfected cells to remove double-stranded RNA signals prior to immune-RNA FISH for TERRA, S9.6 and TRF2 (C, D). The *p* values in A to D were calculated by two-tailed Student's t-test. n= number of cells. Pretreatment of RNase H prior to S9.6 staining reduces the overall S9.6 signals in FANCM knockdown (siFM) cells (E), indicating the specificity of the S9.6 signals for DNA-RNA hybrids. (F to G) siRNA transfected U2-OS cells were co-transfected with either empty vector or a plasmid expressing RNase H1. The expression level of RNase H1 is shown in (F). One set of cells were co-stained with antibodies recognizing pChk1 and TRF1 (G). Another set of cells were used for C-circle analysis (H). All error bars in F and G are standard deviation of the mean obtained from two different experiments. Standard two-tailed Student's t-test: $*p<0.05$, $**p<0.01$.

Fig. S7: A model of TERRA R-loops induced replication stress response at ALT telomeres. In FANCM deficient ALT cells, TERRA R-loops accumulate at telomeres, which then lead to pausing/stalling of replication forks. Repair/re-start of the stalled replication forks via BIR leads to the up-regulation of C-circles formation.

Supplemental Table S1: siRNA used in this study

	Name	Sequence	References
Control	Luc	UCGAAGUAUUCCGCGUACGUU	(Pan et al., 2017)
FANCM complex	FANCM	AAGCUCAUAAAGCUCUCGGAA	(Pan et al., 2017)
	FANCM-U	AAAGACCUCUCACAAUAUU	(Pan et al., 2017)
	FAAP24-1	CCGGAUGAGUGAACAAUACUU	(Pan et al., 2017)
	FAAP24-2	AUUUUCGAGGAUGGCUUGACA	(Pan et al., 2017)
	MHF1	DHARMACON, L-032895-01	(Pan et al., 2017)
	MHF2	DHARMACON, L-016829-01	(Pan et al., 2017)
Dissolvase complex	BLM	CCCACUACUUUGCAAGUAA	(Pan et al., 2017)
	TOP3A	ACAUCGGGUUUGAGAUUAU	(Yang et al., 2012)
	RMI1	AGCCUUCACGAAUGUUGAU	(Yang et al., 2012)
Checkpoint kinases	ATR	CCUCCGUGAUGUUGCUUGA	(Pan et al., 2017)
	Chk1	AAGCGUGCCGUAGACUGUCCA	(Pan et al., 2017)
BIR	Pol D3	TGGCATTATGTCTAGGACTAA	(Costantino et al., 2014)
DNA polymerase	Pol D1	GAGAGAGCAUGUUUGGGUA	(Dilley et al., 2016)
	Pol H	CTGGTTGTGAGCATTCGTGTA	(Dilley et al., 2016)
HR	BRCA1	GAAGGAGCUUUCAUCAUUC	(Pan et al., 2017)
	BRCA2	GGGAAACACUCAGAUUAAAUU	(Ahrabi et al., 2016)
	PALB2	DHARMACON, D-012928-04	(Orthwein et al., 2015)
	Rad51	UGUAGCAUAUGCUCGAGCG	(Dilley et al., 2016)

	Rad52	Qiagen, SI03035123	(Sotiriou et al., 2016)
DNA end resection nuclease	CtIP	GCUAAAACAGGAACGAAUC	(Pan et al., 2017)
	Mre11	GGAGGUACGUCGUUUCAGA	(Pan et al., 2017)
Cohesion-like complex	Smc5	GAAGCAAGAUGUUAUAGAA	(Min et al., 2017)
	Smc6	AGAGCGGCUUACUGAACUA	(Min et al., 2017)
Other FA	FANCD2-1	GGAGAUUGAUGGUCUACUA	(Kais et al., 2016)
	FANCD2-2	CAGAGUUUGCUUCACUCUCUA	(Murina et al., 2014)

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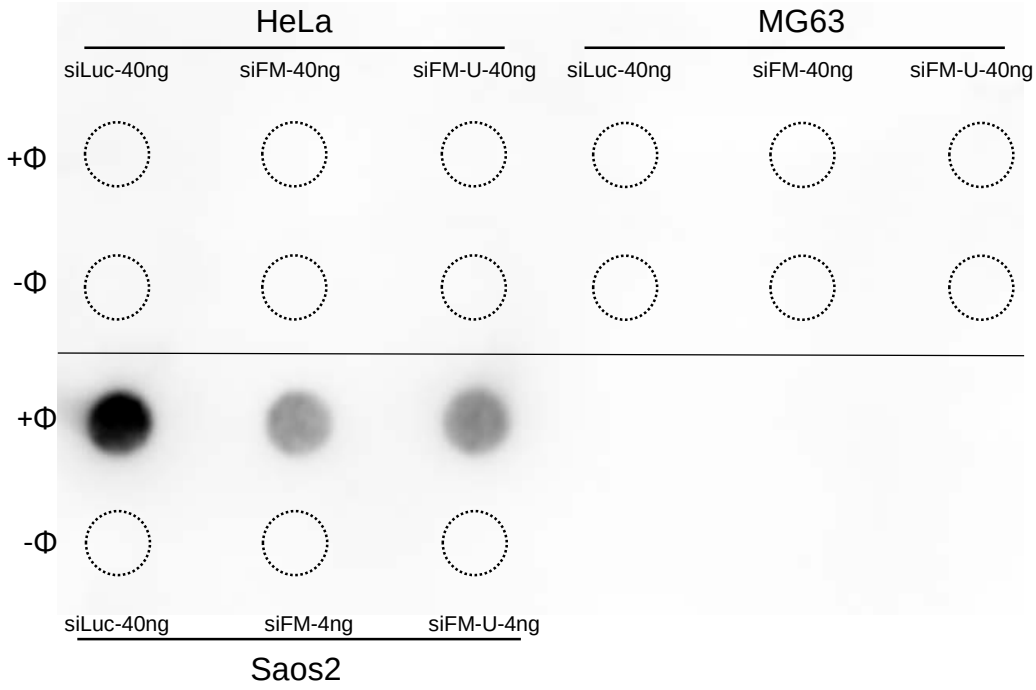
Fig S1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MG63
siLuc-40ng
siFM-40ng
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siLuc-40ng
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+Φ
-Φ
+Φ
-Φ
siLuc-40ng
siFM-4ng
siFM-U-4ng
Saos2

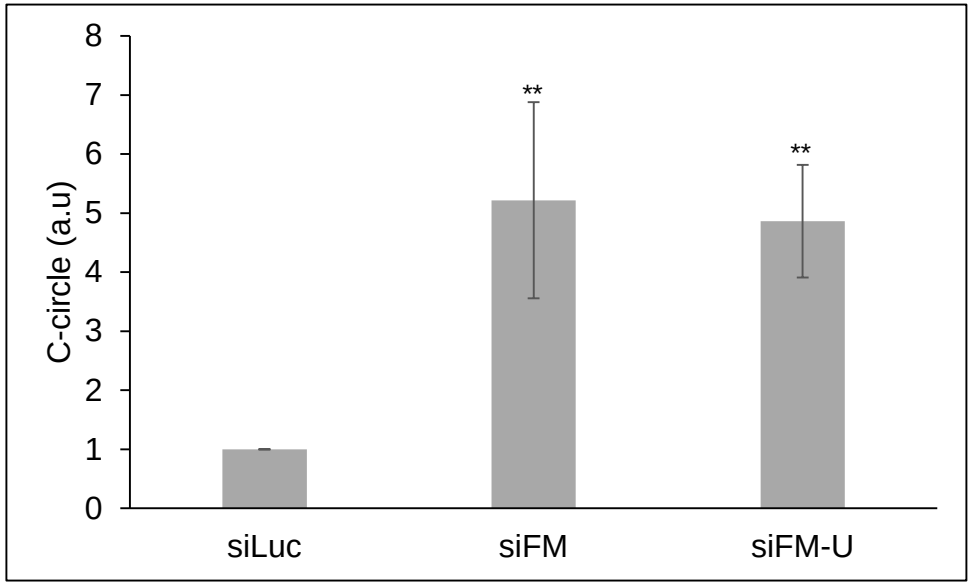
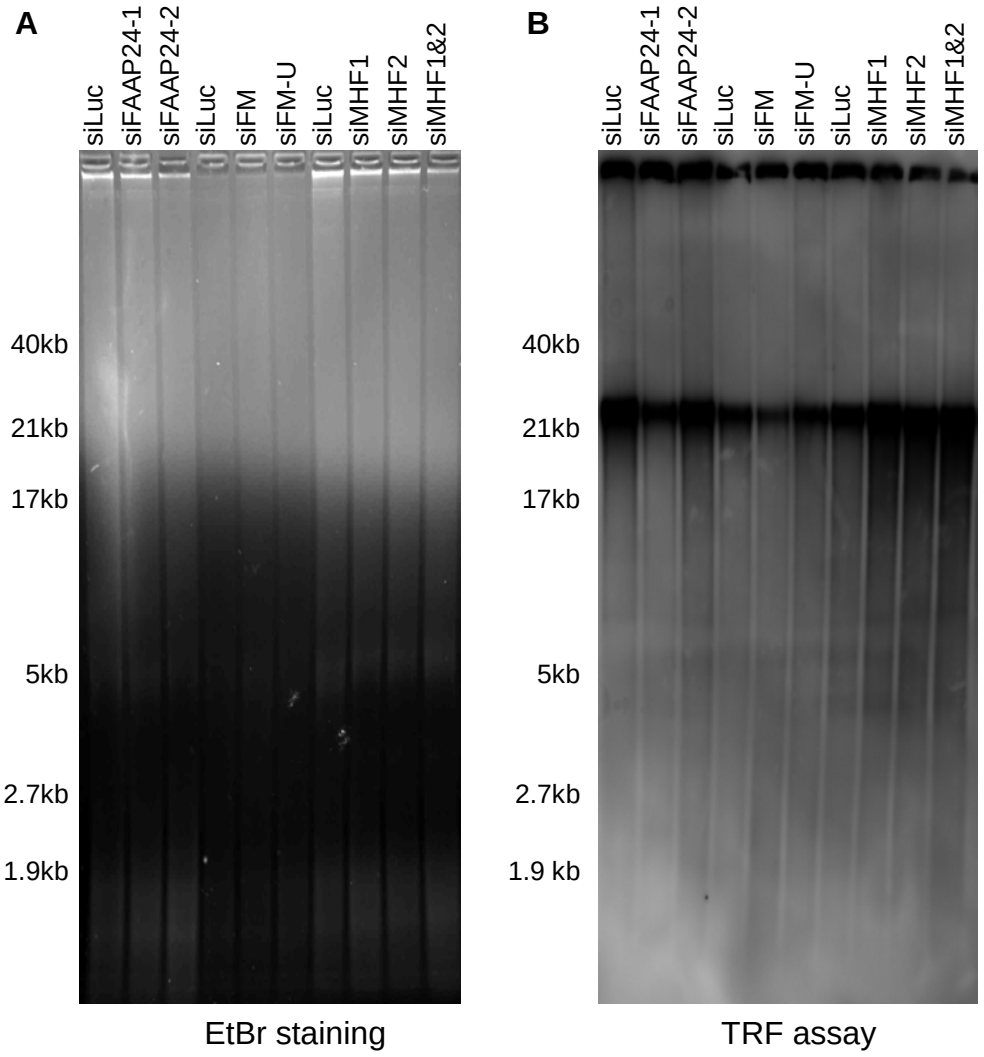

C-circle (a.u)
0
1
2
3
4
5
6
7
8
**
**
siLuc
siFM
siFM-U

Fig.S2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21kb
17kb
5kb
2.7kb
1.9kb
EtBr staining
B
siLuc
siFAAP24-1
siFAAP24-2
siLuc
siFM
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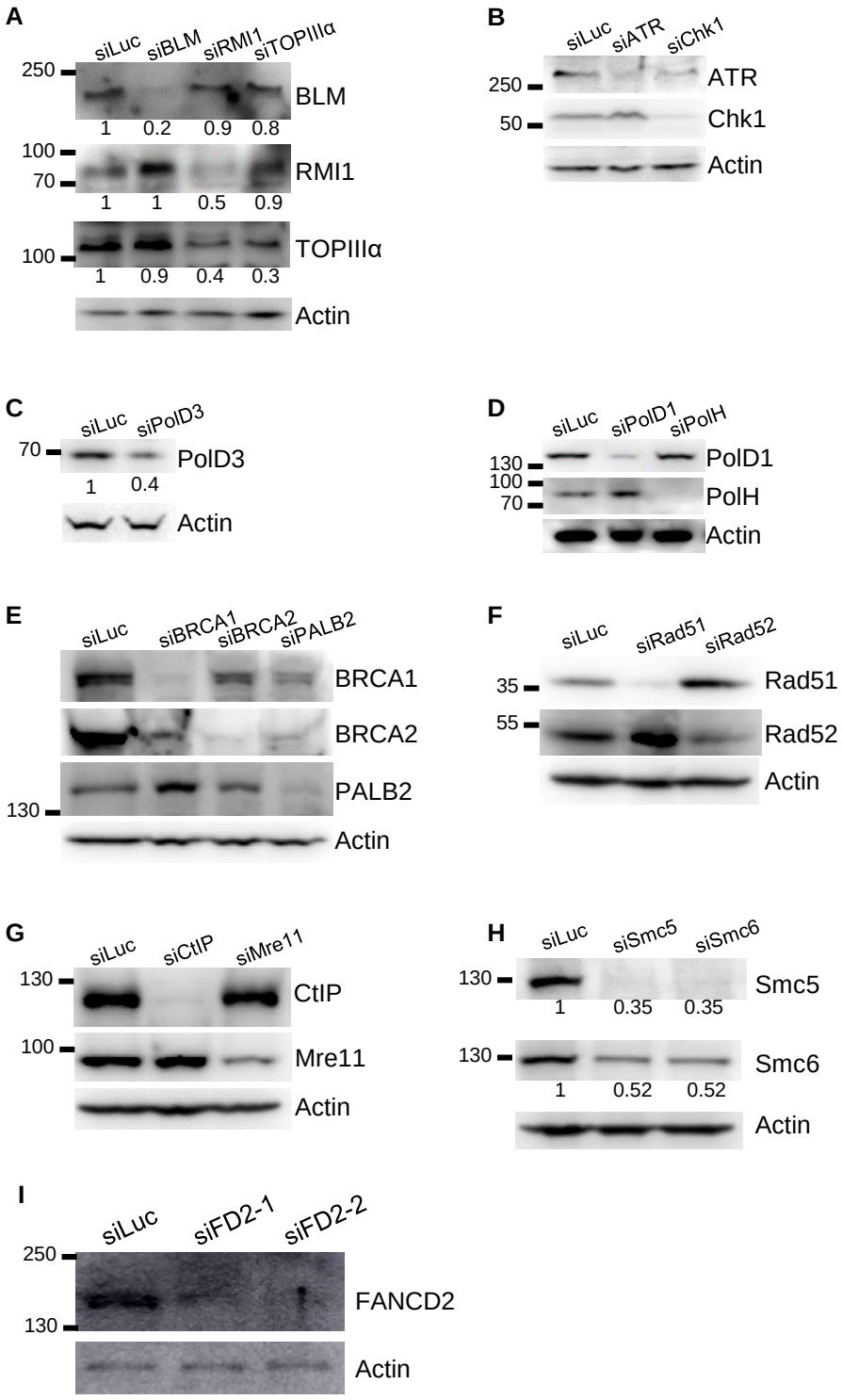
TRF assay


A
siLuc
siBLM
siRMI1
siTOPIIIα
250
BLM
1 0.2 0.9 0.8
100
70
RMI1
1 1 0.5 0.9
100
TOPIIIα
1 0.9 0.4 0.3
Actin
B
siLuc
siATR
siChk1
250
ATR
50
Chk1
Actin
C
siLuc
siPolD3
70
PolD3
1 0.4
Actin
D
siLuc
siPolD1
siPolH
130
100
70
PolD1
PolH
Actin
E
siLuc
siBRCA1
siBRCA2
siPALB2
BRCA1
BRCA2
130
PALB2
Actin
F
siLuc
siRad51
siRad52
35
Rad51
55
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Actin
G
siLuc
siCtIP
siMre11
130
CtIP
100
Mre11
Actin
H
siLuc
siSmc5
siSmc6
130
Smc5
1 0.35 0.35
130
Smc6
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Actin
I
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siFD2-1
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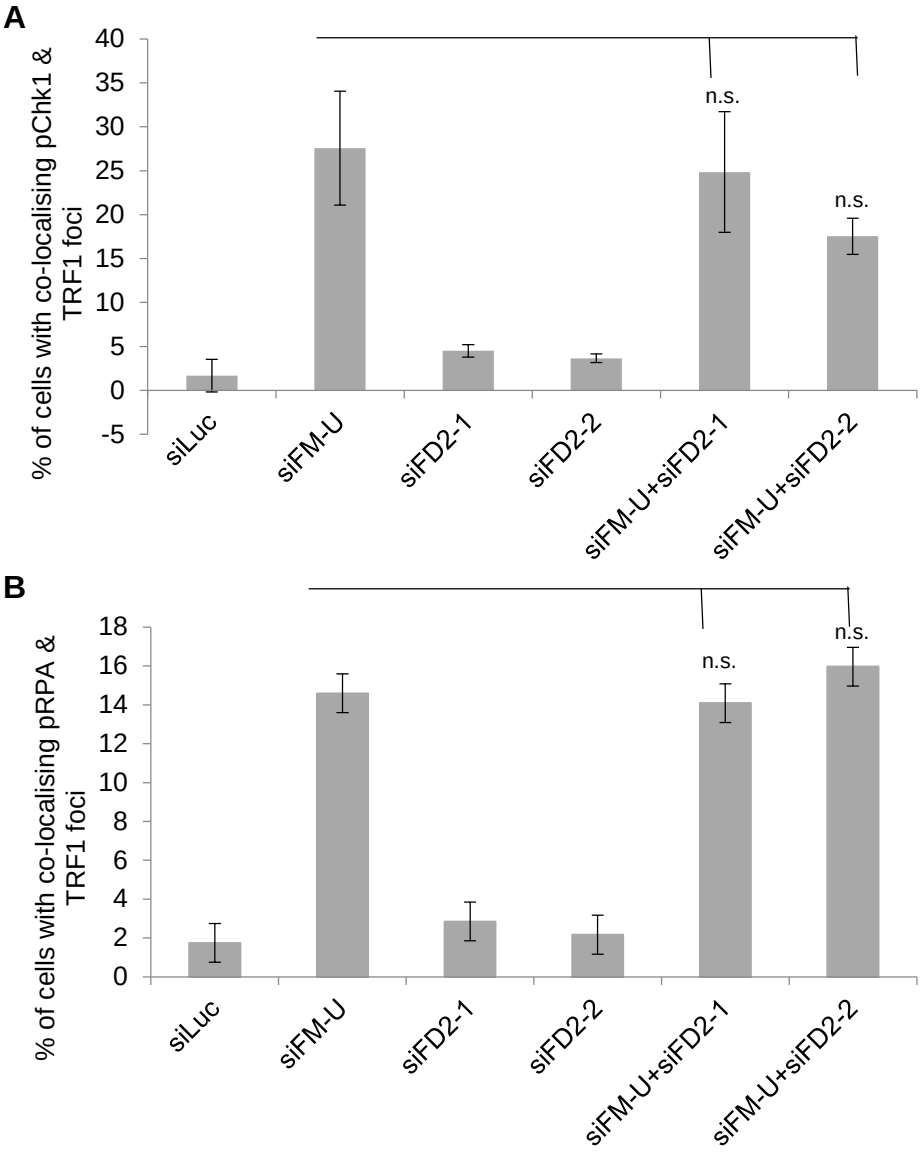
130
FANCD2
Actin


A
% of cells with co-localising pChk1 & TRF1 foci
40
35
30
25
20
15
10
5
0
-5
n.s.
n.s.
siLuc
siFM-U
siFD2-1
siFD2-2
siFM-U+siFD2-1
siFM-U+siFD2-2
B
% of cells with co-localising pRPA & TRF1 foci
18
16
14
12
10
8
6
4
2
0
n.s.
n.s.
siLuc
siFM-U
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siFM-U+siFD2-2

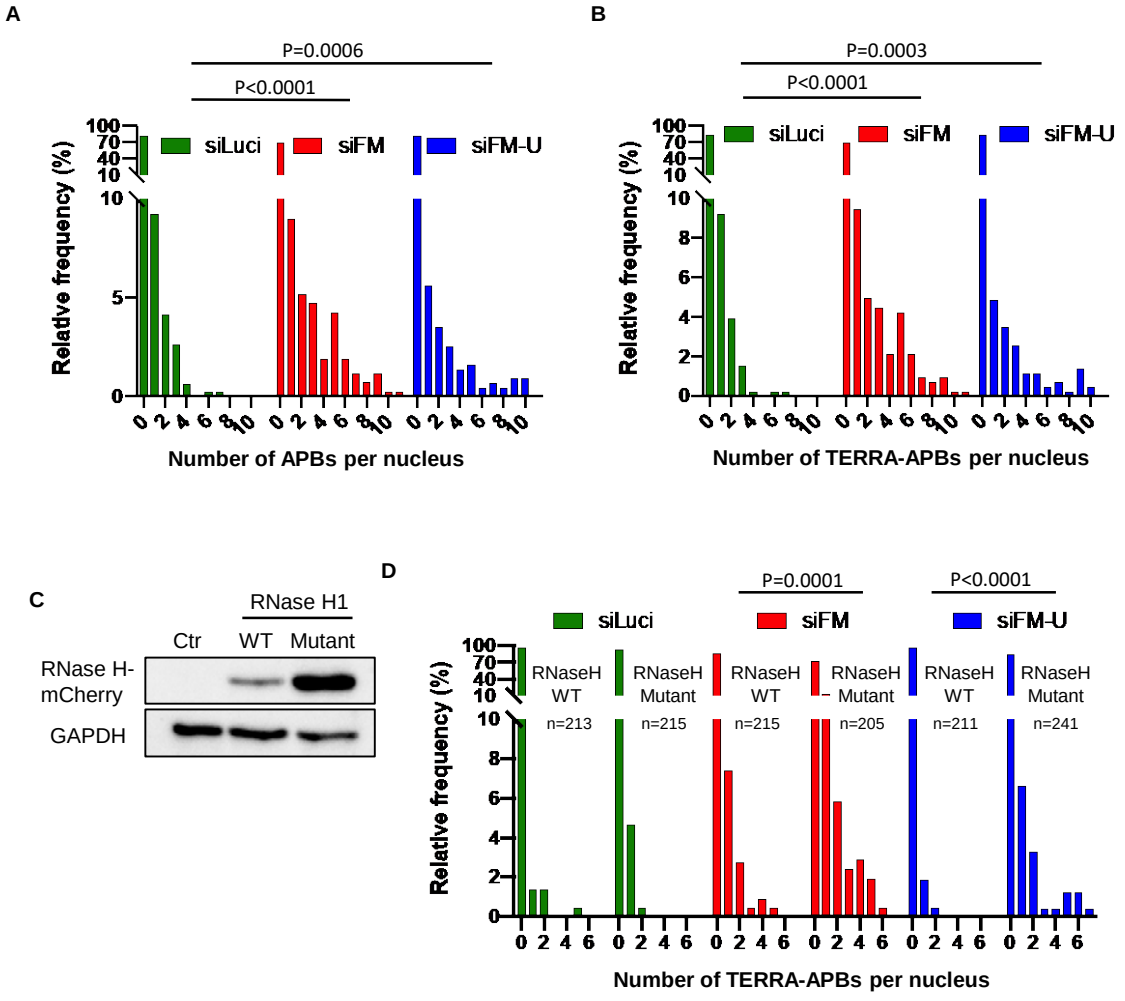

A
P=0.0006
P<0.0001
siLuci
siFM
siFM-U
Relative frequency (%)
Number of APBs per nucleus
B
P=0.0003
P<0.0001
siLuci
siFM
siFM-U
Relative frequency (%)
Number of TERRA-APBs per nucleus
C
RNase H1
Ctr
WT
Mutant
RNase H-mCherry
GAPDH
D
P=0.0001
P<0.0001
siLuci
siFM
siFM-U
RNaseH WT
RNaseH Mutant
n=213
n=215
n=215
n=205
n=211
n=241
Relative frequency (%)
Number of TERRA-APBs per nucleus

Fig. S6

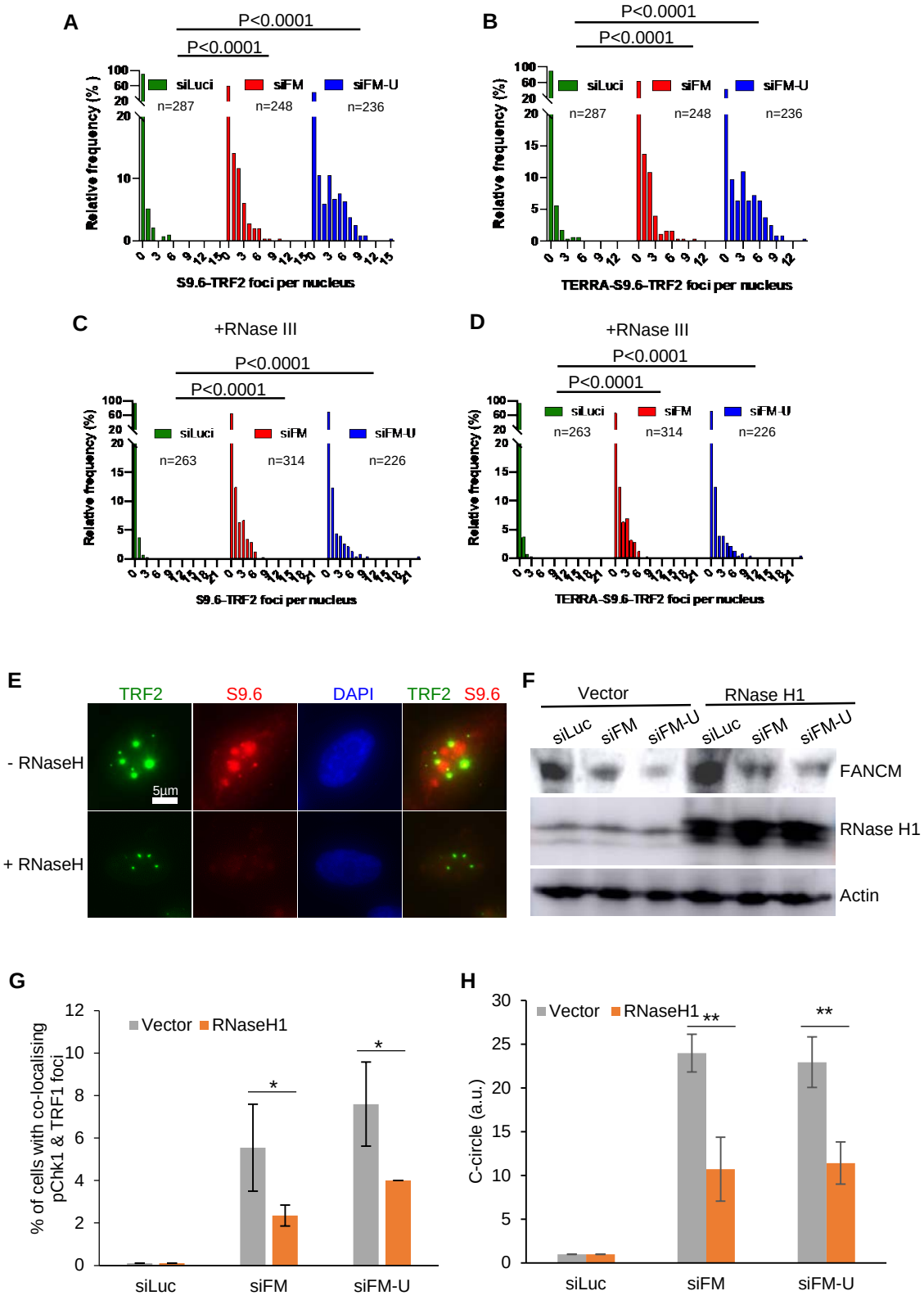
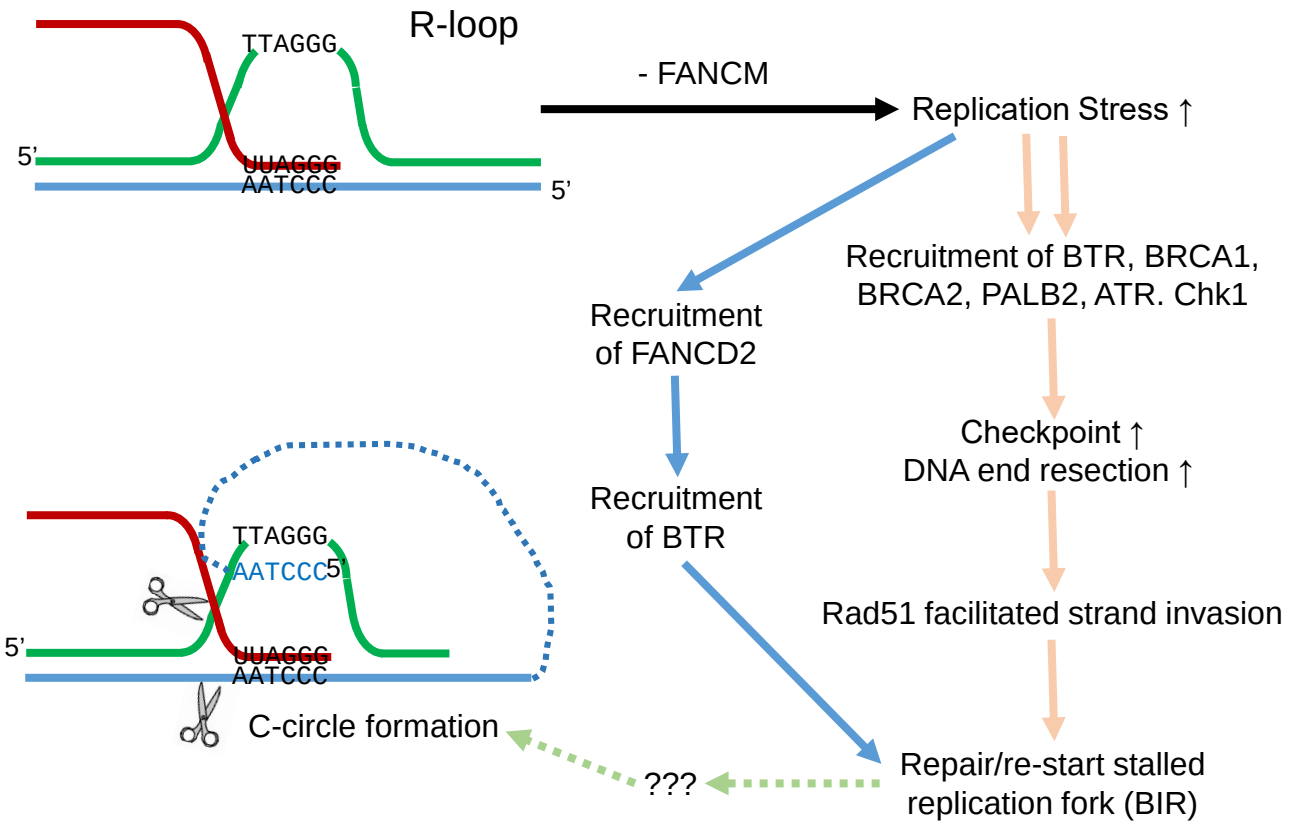

A
P<0.0001
P<0.0001
siLuci
siFM
siFM-U
n=287
n=248
n=236
Relative frequency (%)
S9.6-TRF2 foci per nucleus
B
P<0.0001
P<0.0001
TERRA-S9.6-TRF2 foci per nucleus
C
+RNase III
P<0.0001
P<0.0001
n=263
n=314
n=226
D
+RNase III
P<0.0001
P<0.0001
E
TRF2
S9.6
DAPI
TRF2 S9.6
- RNaseH
+ RNaseH
5µm
F
Vector
RNase H1
siLuc
siFM
siFM-U
FANCM
RNase H1
Actin
G
Vector
RNaseH1
% of cells with co-localising pChk1 & TRF1 foci
H
C-circle (a.u.)

Fig. S7


R-loop
TTAGGG
5'
UUAGGG
AATCCC
5'
- FANCM
Replication Stress ↑
Recruitment of BTR, BRCA1, BRCA2, PALB2, ATR. Chk1
Recruitment of FANCD2
Checkpoint ↑
DNA end resection ↑
Recruitment of BTR
TTAGGG
AATCCC
5'
5'
UUAGGG
AATCCC
Rad51 facilitated strand invasion
C-circle formation
???
Repair/re-start stalled replication fork (BIR)