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Supplemental Information

Mammalian RAD52 Functions in Break-Induced Replication Repair of Collapsed DNA Replication Forks

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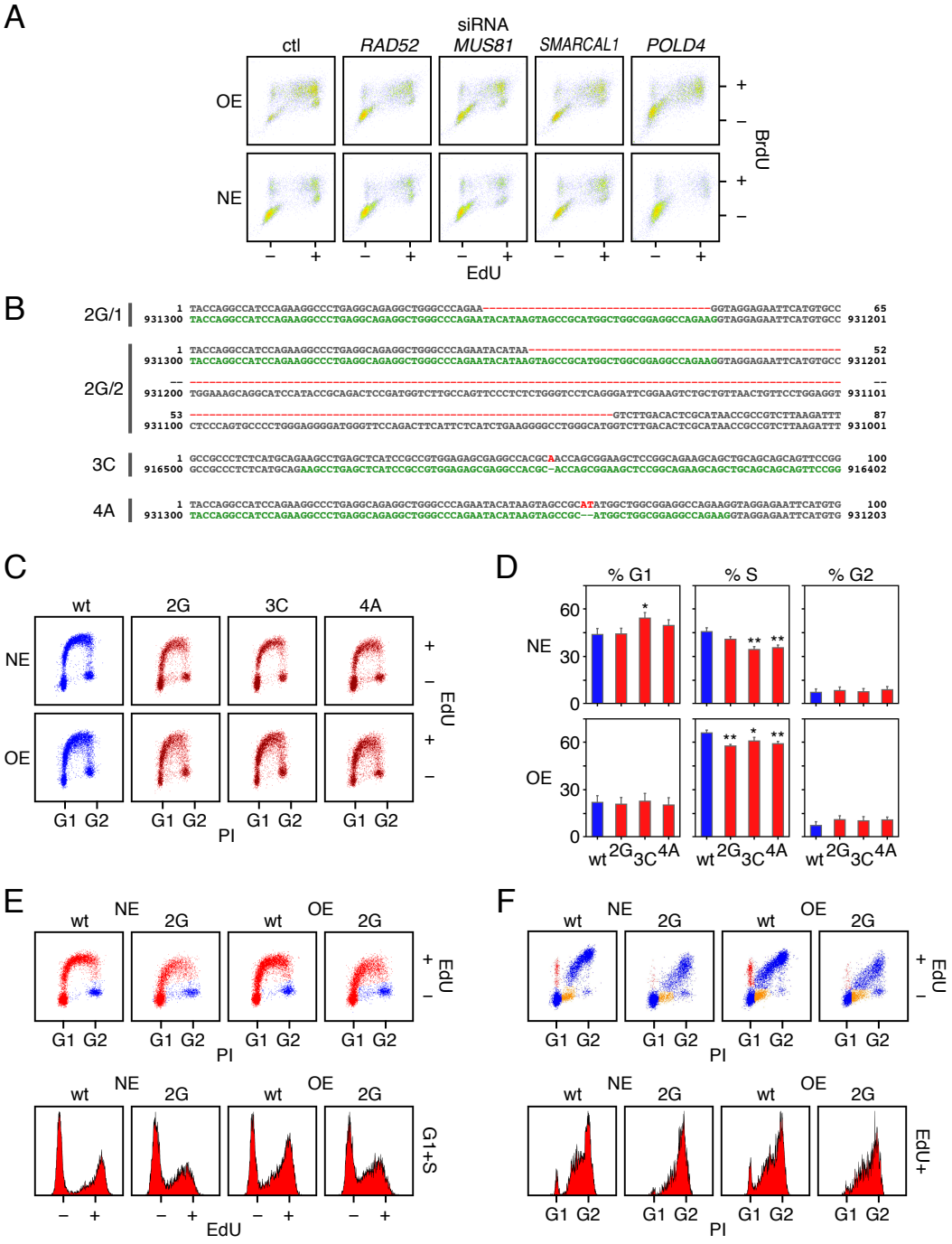
Fig. S1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siRNA
ctl
RAD52
MUS81
SMARCAL1
POLD4
OE
NE
BrdU
EdU
B
2G/1
2G/2
3C
4A
C
wt
2G
3C
4A
NE
OE
EdU
G1 G2
PI
D
% G1
% S
% G2
E
F
G1+S
EdU+

Fig. S2

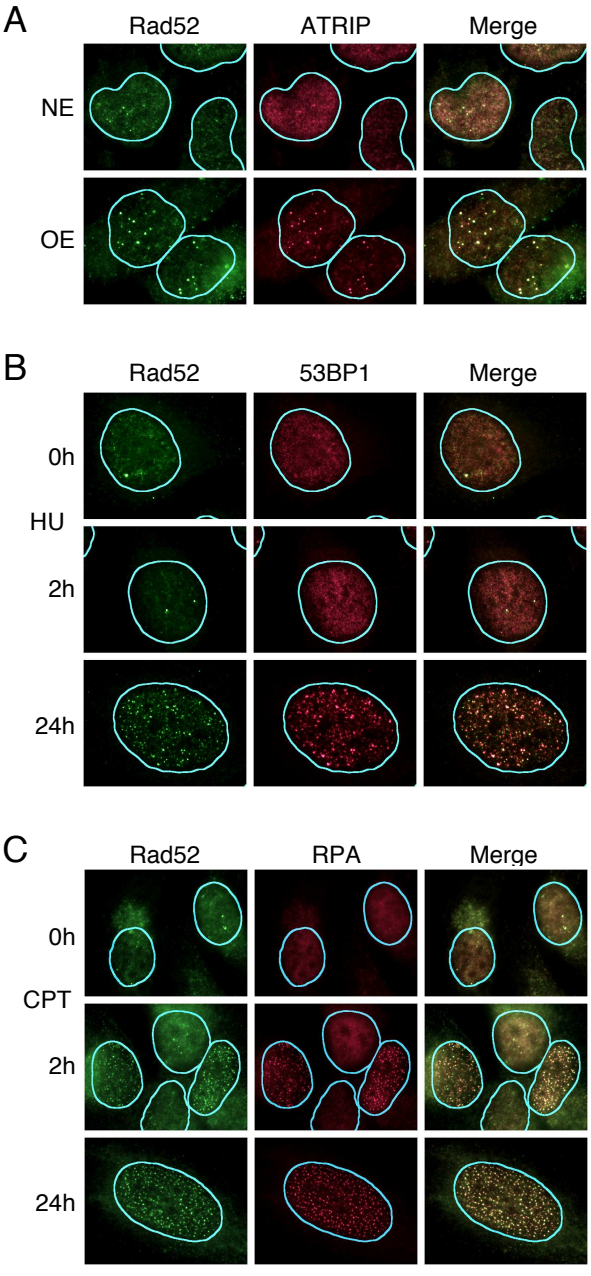

A
Rad52
ATRIP
Merge
NE
OE
B
Rad52
53BP1
Merge
0h
HU
2h
24h
C
Rad52
RPA
Merge
0h
CPT
2h
24h

Fig. S3

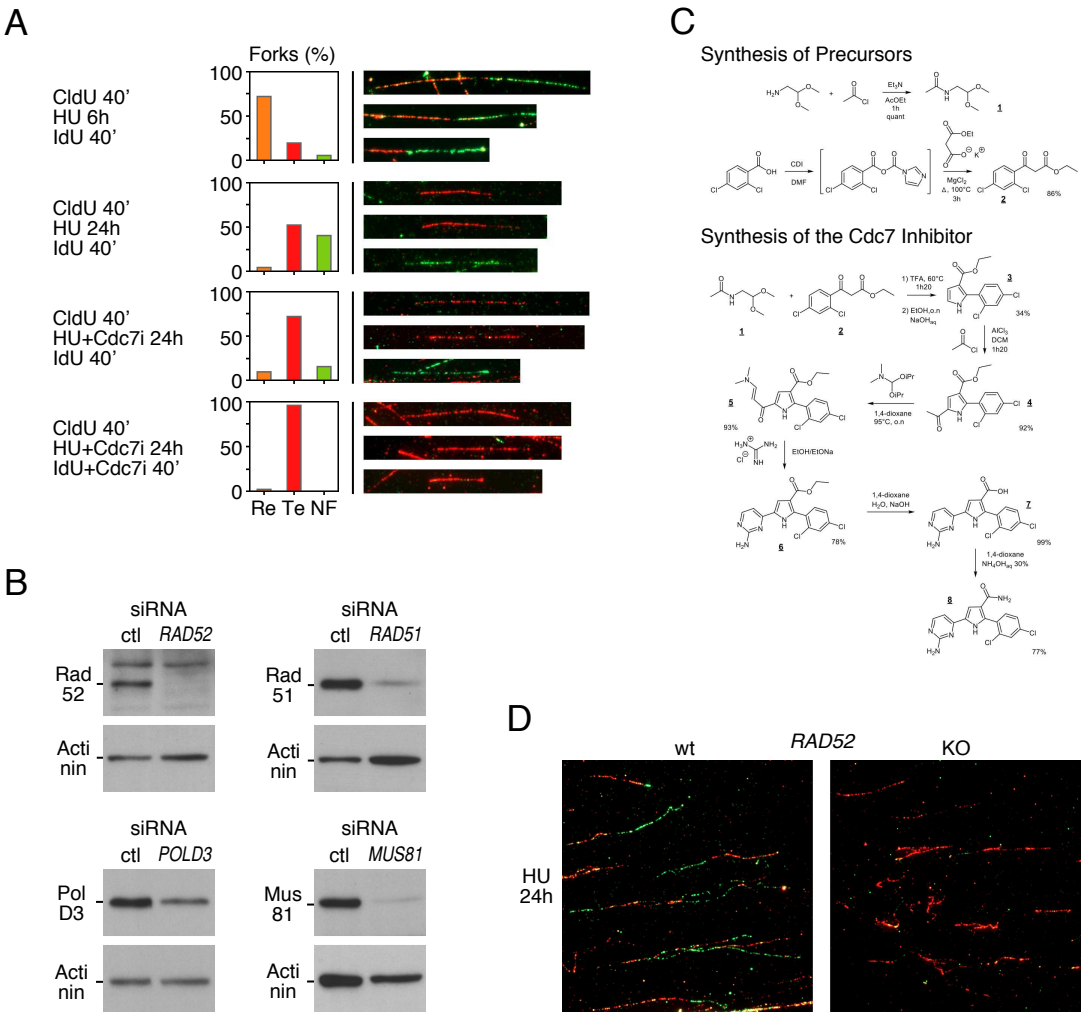
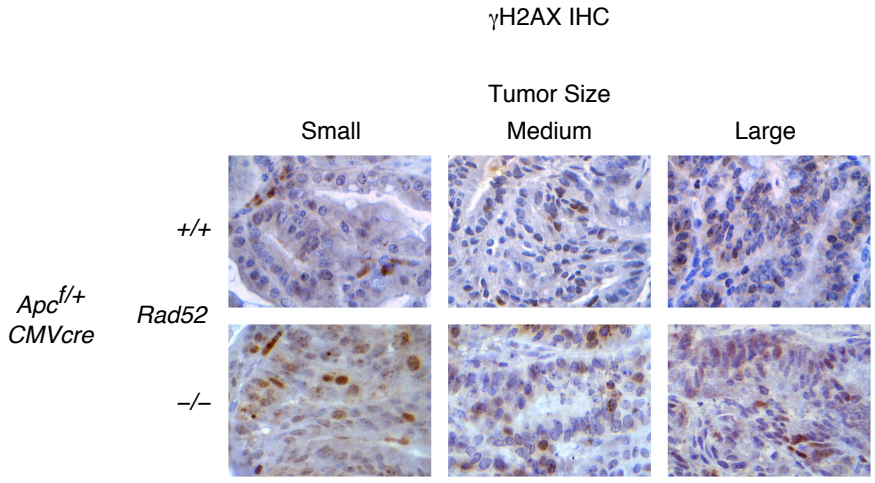

A
Forks (%)
CldU 40'
HU 6h
IdU 40'
CldU 40'
HU 24h
IdU 40'
CldU 40'
HU+Cdc7i 24h
IdU 40'
CldU 40'
HU+Cdc7i 24h
IdU+Cdc7i 40'
100
50
0
Re Te NF
B
siRNA
ctl RAD52
Rad 52
Acti nin
siRNA
ctl RAD51
Rad 51
Acti nin
siRNA
ctl POLD3
Pol D3
Acti nin
siRNA
ctl MUS81
Mus 81
Acti nin
C
Synthesis of Precursors
Synthesis of the Cdc7 Inhibitor
D
wt
RAD52
KO
HU 24h

Fig. S4



SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1. Depletion or knockout of the *RAD52* gene targets preferentially cells with oncogene-induced DNA replication stress.

(A) EdU/BrdU profiles of cells treated with control (ctl) siRNA or siRNAs targeting *RAD52*, *MUS81*, *SMARCAL1* or *POLD4*, as determined by flow cytometry. The cells were treated as shown in Fig. 1A and the results obtained were used for the plots shown in Figs 1A and 1B.

(B) Sequences of the genomic loci targeted by CRISPR/Cas9 in three different clones of U2OS-Cyclin E cells. Two different mutant alleles were detected in clone 2G, whereas clones 3C and 4A harbored a single mutant allele. In clone 3C, a wild-type *RAD52* allele was also detected.

(C) Inactivation of the *RAD52* gene has small to modest effects on the static cell cycle profile of U2OS cells inducibly overexpressing cyclin E. The flow cytometry profiles of the three clones, in which the *RAD52* gene was targeted by CRISPR/Cas9, were compared to the profile of the parental cells (wt). The cells expressed normal levels of cyclin E (NE) or had cyclin E overexpressed (OE) for four days prior to the flow cytometry analysis. EdU was added to the media 1 hour before harvesting the cells. PI, propidium iodide.

(D) Means and standard deviations of the percentages of cells in the G1, S and G2 phases of the cell cycle, as determined by flow cytometry profiles, like the one shown in panel C (experiment performed in triplicate). One and two asterisks denote statistical significance levels of $P<0.05$ and $P<0.01$, respectively, and relevant statistical parameters are listed in Table S6.

(E) Deletion of the *RAD52* gene leads to reduced levels of DNA synthesis, as assessed by EdU incorporation. U2OS parental cells (wt) and clone 2G with both alleles of *RAD52* inactivated were labeled with EdU for 30 min and then examined by flow cytometry. The cells in G1 and S (colored red in the genomic DNA content versus EdU incorporation plots) were gated and the degree of DNA synthesis was monitored by histogram plots showing the levels of EdU incorporation. The histograms show overall higher levels of EdU incorporation in the parental cells, than in the 2G clone. PI, propidium iodide.

(F) Deletion of the *RAD52* gene slows progression through S phase and the cell cycle in general. U2OS parental cells (wt) and clone 2G with both alleles of *RAD52* inactivated were pulsed with EdU for 30 min and then cultured for an additional 12 hours, before being examined by flow cytometry. The EdU-positive cells were gated and genomic DNA content was monitored by histograms showing the levels of propidium iodide (PI) staining. The parental cells have higher peaks corresponding to G1 DNA content than clone 2G cells, indicating faster overall progression through the cell cycle. The G2 DNA content peaks of the wt cells are also better defined than the corresponding peaks of clone 2G cells, indicating faster progression through S phase.

Figure S2, related to Figure 2. Rad52 localization to sites of DNA replication stress.

(A) Representative immunofluorescence images showing colocalization of Rad52 and Atrip foci in cells overexpressing cyclin E (OE), as compared to cells expressing normal levels of cyclin E (NE).

(B) Representative immunofluorescence images showing partial colocalization of Rad52 and 53BP1 foci in cells treated with HU for 24 hours.

(C) Representative immunofluorescence images showing colocalization of Rad52 and RPA foci in cells treated with CPT for 24 hours.

Figure S3, related to Figure 3. Rad52 functions in repair of collapsed DNA replication forks.

(A) Collapse of DNA replication forks after treatment of U2OS cells with HU and a Cdc7 inhibitor for 24 hours. Cells were pulse-labeled with CldU for 40 min, then incubated with HU or HU plus a Cdc7 inhibitor (NMS-1116354) for 6 or 24 hours and finally pulse-labeled with IdU for 40 min in the presence or absence of the Cdc7 inhibitor, as indicated. Fork status was monitored by DNA fiber analysis. Re, restarted forks; Te, terminated forks; NF, newly-fired forks. The Cdc7 inhibitor inhibits new origin firing. This would include origins near collapsed forks, whose firing may be misinterpreted as fork restart.

(B) Efficiency of siRNA-mediated depletion of Rad52, Rad51, PolD3 and Mus81. Immunoblot analysis of Rad52, Rad51, PolD3 and Mus81 protein levels in U2OS cells 72 hours after siRNA transfection. α-actinin served as loading control.

(C) Scheme for synthesis of the Cdc7 inhibitor NMS-1116354. Synthesis of the inhibitor involved first the synthesis of two precursors: N-(2,2-dimethoxyethyl)acetamide (1) and ethyl 3-(2,4-dichlorophenyl)-3-oxopropanoate (2). The Cdc7 inhibitor (5-(2-aminopyrimidin-4-yl)-2-(2,4-dichlorophenyl)-1H-pyrrole-3-carboxamide) was then synthesized using the steps shown.

(D) Rad52 facilitates restart of collapsed DNA replication forks. U2OS parental cells (wt) and the *RAD52* knockout (KO) clone 2G were pulse-labeled for 1 hour with CldU followed by a 24 hour treatment with 2 mM HU and 5 μM Cdc7 inhibitor. The cells were then released from the HU block into media containing the Cdc7 inhibitor and IdU for 1 hour. Representative DNA fiber images, like the ones used to calculate the frequencies of fork restart in Fig. 3D are shown. CldU, red; IdU, green.

Figure S4, related to Figure 4. Immunohistochemistry for γH2AX in mouse intestinal tumors.

Representative immunohistochemistry (IHC) examples for γH2AX in small (diameter < 1.5 mm), medium (diameter 2-2.5 mm) and large (diameter 3-7 mm) tumors from *Rad52*$^{+/+}$*;Apc*$^{f/+}$*;CMVcre* and *Rad52*$^{-/-}$*;Apc*$^{f/+}$*;CMVcre* mice.

Table S1, related to Figure 1A.

Results of the low throughput siRNA screen performed in cells expressing normal levels of cyclin E (NE) or overexpressing cyclin E (OE).

For each siRNA or siRNA pool, the percentage of EdU-/BrdU- cells is indicated. Selected genes were targeted by more than one siRNAs or siRNA pools.

	EdU-/BrdU- (%)			EdU-/BrdU- (%)	
siRNA	**NE**	**OE**	**siRNA**	**NE**	**OE**
Control	36.7	11.2	RAD54L #1	42.6	14.0
53BP1	56.6	17.0	RAD54L #2	60.6	30.6
APEX2	38.1	18.6	RAD54L #3	56.0	19.8
ATRX	46.7	11.4	RAD54L2	32.4	12.3
BLM	43.1	5.9	RAP80 #1	44.8	14.3
BRCA2	42.8	12.9	RAP80 #2	32.8	16.7
DDX11	43.7	16.6	RDM1	35.1	13.0
DHX36	49.9	7.5	RECQL5	40.4	15.8
DNA2L	37.7	20.0	REV1L	44.3	10.4
DUT	42.7	12.3	RING1 #1	58.0	4.6
EME1	51.4	13.2	RING1 #2	64.6	30.7
EME2	42.3	5.4	RING1 #3	74.2	30.0
EXO1	68.6	19.1	RMI1	43.1	13.4
FAN1	76.1	35.2	RTEL1	67.6	18.4
GEN1	44.0	21.0	SFPQ	68.9	9.2
HELQ	38.2	16.9	SFR1	49.2	21.7
HELZ	41.4	11.4	SLX1	41.3	13.0
HLAB	42.7	12.8	SLX4	66.8	40.7
HORMAD1	41.3	13.5	SMARCA3	54.8	9.6
MCM8	24.0	11.3	SMARCAL1	42.0	29.2
MCM9	45.9	14.9	SMC5	28.9	5.5
MRE11A	44.2	16.9	SMC6	68.1	25.2
MUS81	49.0	35.3	SPATA5	32.3	12.7
NOXIN	42.3	12.2	SUB1	46.2	18.4
PIF1 #1	34.9	14.7	SWI5	38.8	9.3
PIF1 #2	38.2	13.0	SWS1	58.6	25.5
POLD2	46.2	15.2	SWSAP1	52.8	23.5
POLD4	45.9	29.3	TIMELESS	40.8	26.1
POLH	37.9	12.4	TIPIN	38.8	34.3
POLN	60.6	17.9	TONSL	51.4	20.9
POLQ	38.8	11.1	TREX2	60.0	28.8
RAD51	46.4	24.1	WDHD1	30.8	14.8
RAD51B	31.7	18.8	WRNIP1	53.1	15.9
RAD51C	29.5	9.2	XRCC2	39.2	20.9
RAD51D	43.8	22.4	XRCC3	33.2	15.5
RAD52 #1	58.1	33.2	ZRANB3	46.8	22.6
RAD52 #2	53.6	32.9			

Table S2, related to Figure 1.

Statistical analysis parameters. N1, N2, number of replicates/samples for the two groups being compared; df, degrees of freedom; P, level of statistical significance.

Fig 1B - % EdU-/BrdU- Cells					
Group Names	N1	N2	t test	df	P
OE cells: siControl vs siRAD52 #1	31	7	9.43	6.9	0.00004
OE cells: siControl vs siRAD52 #2	31	7	11.06	7.3	0.00001
OE cells: siControl vs siMUS81	31	6	15.65	7	0.00001
OE cells: siControl vs siSMARCAL1	31	7	14.59	10	0.00001
OE cells: siControl vs siPOLD4	31	5	6.36	4.4	0.003
OE cells: siControl vs siPIF1 #1	31	4	0.82	3.2	NS
OE cells: siControl vs siPIF1 #2	31	4	1.17	4.5	NS
NE cells: siControl vs siRAD52 #1	31	7	12.96	32.9	0.00001
NE cells: siControl vs siRAD52 #2	31	7	9	23.6	0.00001
NE cells: siControl vs siMUS81	31	6	4.2	8.9	0.002
NE cells: siControl vs siSMARCAL1	31	7	1.8	10.4	NS
NE cells: siControl vs siPOLD4	31	5	1.88	5	NS
NE cells: siControl vs siPIF1 #1	31	4	0.5	4.6	NS
NE cells: siControl vs siPIF1 #2	31	4	0.6	7.5	NS
Fig 1D - % EdU-/BrdU- Cells					
Group Names	N1	N2	t test	df	P
OE cells: RAD52 wt vs KO clone 2G	4	4	3.91	6	0.008
OE cells: RAD52 wt vs KO clone 3C	4	4	5.63	6	0.002
OE cells: RAD52 wt vs KO clone 4A	4	4	4.85	6	0.003
NE cells: RAD52 wt vs KO clone 2G	4	4	0.51	6	NS
NE cells: RAD52 wt vs KO clone 3C	4	4	4.7	6	0.004
NE cells: RAD52 wt vs KO clone 4A	4	4	5.57	6	0.002
Fig S1D - Cell Cycle Profile of Rad52 wt vs KO clones					
Group Names	N1	N2	t test	df	P
% Cells in G1: Cyclin E NE; Rad52 wt vs clone 2G	3	3	0.1	4	NS
% Cells in G1: Cyclin E NE; Rad52 wt vs clone 3C	3	3	3.1	4	0.04
% Cells in G1: Cyclin E NE; Rad52 wt vs clone 4A	3	3	1.7	4	NS
% Cells in G1: Cyclin E OE; Rad52 wt vs clone 2G	3	3	0.28	4	NS
% Cells in G1: Cyclin E OE; Rad52 wt vs clone 3C	3	3	0.18	4	NS
% Cells in G1: Cyclin E OE; Rad52 wt vs clone 4A	3	3	0.4	4	NS
% Cells in S: Cyclin E NE; Rad52 wt vs clone 2G	3	3	2.69	4	NS
% Cells in S: Cyclin E NE; Rad52 wt vs clone 3C	3	3	6.08	4	0.004
% Cells in S: Cyclin E NE; Rad52 wt vs clone 4A	3	3	5.68	4	0.005
% Cells in S: Cyclin E OE; Rad52 wt vs clone 2G	3	3	6.96	4	0.003
% Cells in S: Cyclin E OE; Rad52 wt vs clone 3C	3	3	2.86	4	0.05
% Cells in S: Cyclin E OE; Rad52 wt vs clone 4A	3	3	5.04	4	0.008
% Cells in G2: Cyclin E NE; Rad52 wt vs clone 2G	3	3	0.61	4	NS
% Cells in G2: Cyclin E NE; Rad52 wt vs clone 3C	3	3	0.21	4	NS
% Cells in G2: Cyclin E NE; Rad52 wt vs clone 4A	3	3	0.95	4	NS
% Cells in G2: Cyclin E OE; Rad52 wt vs clone 2G	3	3	1.75	4	NS
% Cells in G2: Cyclin E OE; Rad52 wt vs clone 3C	3	3	1.35	4	NS
% Cells in G2: Cyclin E OE; Rad52 wt vs clone 4A	3	3	1.98	4	NS

Table S3, related to Figure 2.

Statistical analysis parameters. N1, N2, number of replicates/samples for the two groups being compared; df, degrees of freedom; P, level of statistical significance.

Fig 2 - % cells with foci					
Group Names	N1	N2	t test	df	P
Fig. 2A: Rad52 foci NE vs OE	3	3	13.57	4	0.0002
Fig. 2A: RPA foci NE vs OE	3	3	3.34	4	0.03
Fig. 2A: Atrip foci NE vs OE	3	3	6.8	4	0.003
Fig. 2A: Rad51 foci NE vs OE	2	2	1.24	2	NS
Fig. 2C: Rad52 foci HU 0h vs 2h	3	3	4.54	4	0.02
Fig. 2C: Rad52 foci HU 0h vs 24h	3	3	7.82	4	0.002
Fig. 2C: RPA foci HU 0h vs 2h	3	3	2.2	4	NS
Fig. 2C: RPA foci HU 0h vs 24h	3	3	51.6	4	0.00001
Fig. 2C: 53BP1 foci HU 0h vs 2h	3	3	3.42	4	0.03
Fig. 2C: 53BP1 foci HU 0h vs 24h	3	3	7.88	4	0.002
Fig. 2C: Rad51 foci HU 0h vs 2h	3	3	0.27	4	NS
Fig. 2C: Rad51 foci HU 0h vs 24h	3	3	5.01	4	0.008
Fig. 2C: Rad52 foci CPT 0h vs 2h	2	2	4.57	2	0.05
Fig. 2C: Rad52 foci CPT 0h vs 24h	2	2	7.85	2	0.02
Fig. 2C: RPA foci CPT 0h vs 2h	2	2	26.8	2	0.002
Fig. 2C: RPA foci CPT 0h vs 24h	2	2	23.43	2	0.002
Fig. 2C: 53BP1 foci CPT 0h vs 2h	2	2	2.94	2	NS
Fig. 2C: 53BP1 foci CPT 0h vs 24h	2	2	6.47	2	0.03
Fig. 2C: Rad51 foci CPT 0h vs 2h	2	2	0.94	2	NS
Fig. 2C: Rad51 foci CPT 0h vs 24h	2	2	30.2	2	0.002

Table S4, related to Figures 3 and 4.

Statistical analysis parameters. N1, N2, number of replicates/samples for the two groups being compared; df, degrees of freedom; P, level of statistical significance.

Fig 3B - % cells in indicated γH2AX gate					
Group Names	N1	N2	t test	df	P
Medium (blue) γH2AX gate - 2h HU: siControl vs siRAD52	3	3	5.55	4	0.006
Medium (blue) γH2AX gate - 2h HU: siControl vs siPOLD3	3	3	1.25	4	NS
Medium (blue) γH2AX gate - 2h HU: siControl vs siMUS81	3	3	11.15	4	0.0004
Medium (blue) γH2AX gate - 2h HU: siControl vs siRAD51	3	3	22.19	4	0.00003
Medium (blue) γH2AX gate - 24h HU: siControl vs siMUS81	3	3	3.6	4	0.03
Medium (blue) γH2AX gate - 24h HU: siControl vs siRAD51	3	3	15.75	4	0.0001
High (red) γH2AX gate - 24h HU: siControl vs siRAD52	3	3	4.14	4	0.02
High (red) γH2AX gate - 24h HU: siControl vs siPOLD3	3	3	7.29	4	0.002
Fig 3C - % cells in high gH2AX gate					
Group Names	N1	N2	t test	df	P
High γH2AX gate - 24h HU: siControl vs siPOLD3	2	2	11.72	2	0.008
High γH2AX gate - 24h HU: siControl vs siRAD52	2	2	10.95	2	0.009
High γH2AX gate - 24h HU: siControl vs siPOLD3+siRAD52	2	2	25.13	2	0.002
High γH2AX gate - 24h HU: siRAD52 vs siPOLD3+siRAD52	2	2	0.52	2	NS
Fig 3D - Restarted Forks					
Group Names	N1	N2	t test	df	P
6h HU Rad52 wt vs KO clone 2G	3	2	5.72	3.5	0.007
24h HU Rad52 wt vs KO clone 2G	4	4	5.79	6	0.002
Fig 3E - BIR GFP Assay					
Group Names	N1	N2	t test	df	P
siControl vs siPOLD3	4	3	3.41	4.9	0.02
siControl vs siPOLD4	4	3	4.34	5.4	0.006
siControl vs siRAD52	4	3	8.53	4.8	0.0005
siControl vs siPOLD3+siRAD52	4	3	10.01	4.4	0.0004
siControl vs siPOLD4+siRAD52	4	3	6.36	6.9	0.0004
siRAD52 vs siPOLD3+siRAD52	3	3	2.42	4	NS
siRAD52 vs siPOLD4+siRAD52	3	3	1.21	4	NS
Fig 4A - Number of Tumors according to Size or Histology					
Group Names	N1	N2	chi square	df	P
Tumor Size: Rad52 +/+ vs -/- (all tumor sizes: 2x4 table)	46	50	11.73	3	0.009
Tumor Size: Rad52 +/+ vs -/- (tumor size: 0.5 mm: 2x2 table)	46	50	3.72	1	0.05
Tumor Size: Rad52 +/+ vs -/- (tumor size: 5-7 mm: 2x2 table)	46	50	9.38	1	0.003
Tumor Histology: Rad52 +/+ vs -/- (all histology types: 2x4 table)	46	50	3.13	3	NS
Fig 4B - Ki67 and γH2AX Indices					
Group Names	N1	N2	t test	df	P
Ki67 Index: Tumor Size 0.5-1.5 mm Rad52 +/+ vs -/-	9	14	0.13	22.4	NS
Ki67 Index: Tumor Size 2-2.5 mm Rad52 +/+ vs -/-	12	12	0.17	22	NS
Ki67 Index: Tumor Size 3-7 mm Rad52 +/+ vs -/-	24	16	1.66	27.6	NS
γH2AX Index: Tumor Size 0.5-1.5 mm Rad52 +/+ vs -/-	9	14	2.66	20	0.02
γH2AX Index: Tumor Size 2-2.5 mm Rad52 +/+ vs -/-	12	13	0.17	24.3	NS
γH2AX Index: Tumor Size 3-7 mm Rad52 +/+ vs -/-	24	17	0.75	36.5	NS
Fig 4C - Survival of APCmin Rad52+/+ and -/- Mice					
Group Names	N1	N2	z score		P
Rad52 +/+ vs -/- Mice	8	8	2.66		0.008

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture

U2OS cells expressing cyclin E in a tetracycline-dependent manner (U2OS-CycE) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, 11960), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, 10500), penicillin 100 U/ml and streptomycin 0.1 mg/ml (Invitrogen, 15140), G418 400 μg/ml (Invitrogen, 10131-027), puromycin 1 μg/ml (Sigma, P8833) and tetracycline 2 μg/ml (Sigma, T7660). To induce cyclin E overexpression, tetracycline was removed from the medium. U2OS cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (FBS).

siRNAs and plasmids

The following siRNAs were used: *RAD52*, GGAUGGUUCAUAUCAUGAATT (Qiagen, SI03035123) or GGUCCAUGCCUUUAAUGUUTT (Qiagen, SI03041808) or a pool of 4 siRNAs: ACGAAAACGCGUACUAAAA, GGCAUUAUGUCUAGGACUA, CAAUUA-GUGGUUAGGGAAA, UGUAUAGCAAGCUGAGUAA (Dharmacon, L-011760-00-0005); *SMARCAL1*, an equimolar mixture of two siRNAs: GCUUUGACCUUCUUAGCAA (Thermoscientific, J-013058-06-0005) and GCUUUGACCUUCUUAGCAATT (Qiagen, SI00103180); *MUS81*, UCUACCGGGAGCACCUGAAUCCUAA (Invitrogen, HSS129459); *POLD3*, a pool of 4 siRNAs: ACGAAAACGCGUACUAAAA, GGCAUUAUGUCUAGGACUA, CAAUUAGUGGUUAGGGAAA, UGUAUAGCAA-GCUGAGUAA (Dharmacon, L-026692-01-0005); *POLD4*, an equimolar mixture of two siRNAs: CACUAAUGCUUAUCAAUAATT (Qiagen, SI00688695) and CCCAUGAUCUGGCAAGUUATT (Qiagen, SI04189276) or a pool of 4 siRNAs: CCUAUGAGGCACCACGUAA, AGUCAGACAUGGACAGUUG, GGAUCAAG-UCCUCGGAAGA, CAAGAAAGUCCUAGGCCGA (Dharmacon, L-014013-02-0005); *RAD51*, GGGAAUUAGUGAAGCCAAATT (Qiagen, SI02663682); negative control, AllStars Negative Control siRNA (Qiagen, 1027281) or Luciferase GL2 (Invitrogen) or ON-TARGETplus non targeting pool siRNA (Dharmacon, D-001810-10-05). The pSpCas9(BB)-2A-GFP (PX458) expression vector was purchased from Addgene (#48138).

Flow cytometry screen

U2OS-CycE cells were plated in 6-well plates in the presence or absence of tetracycline and 24 hours later were transfected with siRNAs. The siRNAs were used at a final concentration of 10 to 50 nM and trasfected using either the Hiperfect reagent (Qiagen, 301707) or the Interferin reagent (Polyplus transfection, 409-50) according to the manufacturer's instructions. 72 hours later, the cells were treated for 1 hour with 10 μM EdU, then for 6 hours with 0.1 mM nocodazole and finally with 10 μM BrdU for 1 hour, before being harvested and fixed overnight at 4°C in 90% ice-cold methanol. The flow cytometry staining was performed as previously described (Costantino et al., 2014) with the following modifications: before staining with the anti-BrdU antibody (BD Biosciences, 555627), cells were blocked in PBS containing 1% BSA for 1 hour. Moreover, both the primary and the secondary antibodies were diluted in PBS containing 1% BSA.

Generation of U2OS-Cyclin E-RAD52 knockout cells

The pX458_Rad52_Crispr2 (Guide Sequence Insert: CACCGCCGGAGCTTCCGCTG-GTGCG) construct targeting exon 9 and the pX458_Rad52_Crispr6 construct (Guide Sequence Insert: CACCGTACATAAGTAGCCGCATGGC) targeting exon 3 of the human *RAD52* gene were designed and generated as described in the MIT CRISPR tool (Cong et al., 2013). U2OS-CycE cells were transfected with the 2 CRISPR/Cas9 constructs and GFP-

positive cells were sorted by FACS in 96-well plates. Single clones were expanded and genomic DNA extracted and used for PCR-based amplification of the targeted loci. For the targeted loci in exons 9 and 3, the CATCCGCCGTGGAGAGCGAGGCC and GGAACTGCTGCTGCAGCTGCTTC or the CCCTGAGGCAGAGGCTGGGCCCAG and CTCCTACCTTCTGGCCTCCGCC primers were used, respectively. Clones that appeared to have PCR products longer or shorter compared to the PCR product from the control wild-type cells were tested for Rad52 expression by western blotting. The genomic PCR products from clones with defective Rad52 expression were cloned into pCR-Blunt II-TOPO vector using the Zero Blunt TOPO PCR Cloning kit (Invitrogen, 450245) and analyzed by Sanger sequencing.

γH2AX detection by flow cytometry

For γH2AX detection by flow cytometry, U2OS cells were seeded in 6-well plates and the next day transfected with the indicated siRNAs at a final concentration of 40 nM using the Interferin reagent. 48 hours after siRNA transfection, the cells were treated with 2 mM HU for 0, 2 or 24 hours. The cells were then fixed with 70% ice-cold ethanol and left overnight at -20°C. The staining was performed using the FlowCellect Histone H2AX Phosphorylation Assay Kit (Millipore, FCCS100182) according to the manufacturer's instructions. The genomic DNA was stained by incubating the cells in PBS containing RNase (Roche, 11119915001) and propidium iodide (PI) (Sigma, P4170).

Immunofluorescence

U2OS-CycE cells were seeded on glass coverslips and 4 days after cyclin E overexpression were fixed in ice-cold methanol for 15 min at -20°C. U2OS cells were also treated for 0, 2 or 24 hours with 2 mM HU or 2 μM CPT and fixed. Cells were permeabilized in PBS containing 0.2% Triton X-100 (Sigma-Aldrich). The permeabilized cells were blocked with PBS containing 1% BSA and then were incubated with the corresponding primary antibodies (listed on the table below) for 2 hours at room temperature followed by 1 hour incubation with the secondary-antibody conjugates Alexa Fluor 488 (Thermo Fischer Scientific, A11001 or A11008) and Alexa Fluor 594 (Thermo Fischer Scientific, A11016). More than 80 cells per replicate and per condition were counted. The threshold to determine if a cell was positive for Rad52 foci was set at 20 foci per nucleus and for Rad51 at 10 foci per nucleus.

Preparation of Chromatin Extracts

U2OS cells were exposed to 2 mM HU for 24 hours or to 9 Gy ionizing radiation 1 hour prior to harvesting. Where indicated, the cells were exposed to HU in the presence of 10 μM ATR inhibitor (VE-821). For subcellular fractionation, the cells were harvested, incubated in Buffer A [8 μM Zinc Acetate, 10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 10 mM NaPO4 pH 8.0, and protease inhibitor cocktail (Roche, 05892970001)] for 10 min on ice and then centrifuged for 5 min at 1000 rpm at 4°C. The cells were resuspended in Buffer A, lysed using a Dounce glass homogenizer and centrifuged for 15 min at 3000 rpm at 4°C. The pellets were washed once more in Buffer A and centrifuged for 5 min at 1000 rpm at 4°C. Subsequently, the pellets were resuspended in Buffer B [8 μM Zinc Acetate, 20 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 300 mM KCl, 0.2 mM EDTA pH 8, 10 mM NaPO4 pH 8, and protease inhibitor cocktail], incubated for 1 hour on ice and centrifuged for 15 min at 3000 rpm at 4°C. The pellets, which correspond to the chromatin fraction, were resuspended in sonication buffer [50 mM Hepes pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na deoxycolate, 0.1% SDS, protease inhibitor cocktail] and sonicated in a Bioruptor Pico sonicator (Diagenode). The samples were centrifuged for 15 min at 14000 rpm at 4°C and the supernatant which contained the chromatin fraction was collected and stored at -

20°C. For the phosphatase assay, the samples were treated as before but this time in buffers without EDTA and without phosphatase inhibitor cocktail. The samples were then treated with 800 units of λ-phosphatase, while in the control samples only the λ-phosphatase buffer was added without λ-phosphatase for 30 min at 30°C.

BIR-GFP reporter assay

U2OS cells with a stably integrated reporter construct for monitoring BIR were generated by transfecting the cells with the pBIR-GFP plasmid and then by selecting for stably-transfected clones (Costantino et al., 2014). The clone with the highest level of GFP induction after transfection with a plasmid expressing the I-SceI nuclease was selected for further experiments. In this clone, about 6% of the cells became GFP-positive, when I-SceI was expressed. For depletion of selected proteins, the following siRNAs were used: control, Dharmacon D-001810-10-05; *POLD3*, Dharmacon L-026692-01-0005; *POLD4*, Dharmacon L-014013-02-0005; *RAD52*, Dharmacon L-011760-00-0005. The cells were plated and two days later transfected with 20 ng of the indicated siRNAs and with 3 μg of the plasmid expressing I-SceI (pCMV-3xNLS-I-SceI) by Nucleofection using the Nucleofector Program X-01 (Amaxa-Lonza). Expression of GFP was monitored by flow cytometry 48 hours after transfection with the plasmid expressing I-SceI.

DNA fiber analysis

For the DNA fiber analysis, U2OS cells were pulse-labeled with 40 μM CldU for 40 min and then treated for 6 or 24 hours with 2 mM HU and with or without 5 μM Cdc7 inhibitor. Following the HU treatment, the cells were pulse-labeled with 400 μM IdU for 40 min or 1 hour in the presence or absence of 5 μM Cdc7 inhibitor. DNA fibers were prepared and stained as described previously (Ray Chaudhuri et al., 2012). Briefly, the cells were harvested, lysed and the DNA fibers were spread on APS-coated cover glass (Matsunami Trading, 7017.90000). The DNA fibers were denatured in 2.5 M HCl for 1 hour and blocked with PBS containing 0.2% Tween 20 and 2% BSA. The cover glasses were then incubated with primary antibodies against CldU (Abcam, ab6326) and IdU (BD Biosciences, 347580) for 2.5 hours. For visualization of CldU and IdU, the secondary antibodies Cy3 AffiniPure (Jackson ImmunoResearch, 712-166-153) and Alexa Fluor 488 (Thermo Fischer Scientific, A11001) were used, respectively. Finally, the fluorescence signal was amplified by using the secondary antibodies Dylight 550 conjugate (Thermo Fischer Scientific, SA5-10063) and the Alexa Fluor 488 (Thermo Fischer Scientific, A21467). More than 80 fibers per condition were counted.

Synthesis of the Cdc7 inhibitor

The synthesis strategy of the Cdc7 inhibitor (NMS-1116354; Montagnoli et al., 2010b) required a total of 8 steps including two steps for preparing precursors that are not commercially available (Fig. S5). The main steps were: i) the central aromatic pyrrole formation via a Knorr's reaction, ii) a regioselective electrophilic acylation, iii) the construction of the 2-amino-pyrimidine and iv) the primary amide formation. Except for the Knorr's reaction, which had a moderate yield of 34%, the yields of the other synthetic steps were good and reached at least 77%. The overall yield of the performed synthesis route was 17%. The identity and structural assignments of the intermediates and the final compound were assessed by ^{1}H-NMR, ^{13}C-NMR and LRMS. Spectral data were in total agreement with the structural formula of the synthesized compounds. Additionally, the identity and the quality of the final Cdc7 inhibitor were assessed by HRMS (HRMS (ESI+): expected m/z. 348.0413 for $C_{15}H_{11}N_5OCl_2$ [M+H]+; Found m/z: 348.0408) and High Performance Liquid Chromatography (purity >95%).

Mice

All mice were kept on a 12 hour light/dark cycle in an SPF room. The B6JIcoCrl.129P2-Rad52^{tm1Aps}/Cnrm mice were purchased from the European Mouse Mutant Archive (EMMA) (Rijkers et al., 1998). The C57BL/6-Apc^{tm1Tyj}/J mice were purchased from the Jackson Laboratory (Cheung et al., 2010). The CMVcre mice were obtained from the laboratory of Ivan Rodriguez at the University of Geneva (Dupe et al., 1997). The C57BL/6J-ApcMin/J mice were obtained from the laboratory of Joerg Huelsken at the University of Lausanne (Moser et al., 1990). Survival curves (euthanasia, as end-point) between Rad52$^{+/+}$;Apc$^{min/+}$ and Rad52$^{-/-}$;Apc$^{min/+}$ mice were analyzed in the Kaplan-Meier format using the log-rank (Mantel-Cox) test for statistical significance. All experiments involving mice were authorized by the Canton of Geneva and were performed according to accepted guidelines for animal handling.

Histological analysis

At the age of 8 months, Rad52$^{-/-}$;Apc$^{f/+}$;CMVcre and Rad52$^{+/+}$;Apc$^{f/+}$;CMVcre mice were euthanized according to approved animal protocols. The entire small intestine was rolled up into a "Swiss roll", fixed in formalin for 48 hours and embedded in paraffin for histological examination. A certified pathologist at the Medical School of the University of Athens performed the histopathological analysis.

Primary Antibodies used in this study

Antibody	Use	Dilution	Reference
α-Actinin	Western Blot	1/1000	Millipore, 05-384
H3 (phosphor T3)	Western Blot	1/5000	Abcam, ab78351
MCM5	Western Blot	1/500	Abcam, ab17967
MUS81	Western Blot	1/1000	Abcam, ab14387
POLD3	Western Blot	1/100	Abnova, H00010714-M01
RAD51	Western Blot	1/100	Santa Cruz, sc-8349
RAD52	Western Blot	1/200	Ochs et al., 2016
53BP1	Immunofluorescence	1/10	Schultz et al., 2000
ATRIP	Immunofluorescence	1/10	Venere et al., 2007
Cyclin E	Immunofluorescence/Western Blot	1/100	Novocastra, NCL-CYCLIN E
RAD51	Immunofluorescence	1/250	Abcam, ab63801
RAD51	Immunofluorescence	1/100	Santa Cruz, sc-8349
RAD52	Immunofluorescence	1/100	Ochs et al., 2016
RPA	Immunofluorescence	1/1000	GeneTex, GTX70258
γH2AX	Flow Cytometry	1/20	Millipore, CS208216
BrdU	Flow Cytometry	1/400	BD Biosciences, 555627
CldU	DNA Fiber Spreading	1/500	Abcam, ab6326
IdU	DNA Fiber Spreading	1/100	BD Biosciences, 347580
γH2AX	Immunohistochemistry	1/1000	Millipore, 05-636
Ki67	Immunohistochemistry	1/200	Abcam, ab16667

SUPPLEMENTAL REFERENCES

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