Supplemental Information

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

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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

CldU 40'
HU 6h
IdU 40'

CldU 40'
HU 24h
IdU 40'

CldU 40'
HU+Cdc7i 24h
IdU 40'

CldU 40'
HU+Cdc7i 40'

Re Te NF

Forks (%)

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

HU
24h

wt
RAD52
KD
Fig. S4

\[ \gamma \text{H2AX IHC} \]

| Tumor Size | Apo\textsuperscript{d/+} CMVcre | Rad52 |
|------------|---------------------------------|-------|
| Small      | \(+/+\)                         | \(-/-\) |
| Medium     |                                 |       |
| Large      |                                 |       |
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+/+;ApcFlox;CMVcre and Rad52-/-;ApcFlox;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.

| siRNA       | EdU-/BrdU-% (NE) | siRNA       | EdU-/BrdU-% (OE) |
|-------------|------------------|-------------|-----------------|
| Control     | 36.7             | RAD54L #1   | 42.6            | 14.0            |
| 53BP1       | 56.6             | RAD54L #2   | 60.6            | 30.6            |
| APEX2       | 38.1             | RAD54L #3   | 56.0            | 19.8            |
| ATRX        | 46.7             | RAD54L2     | 32.4            | 12.3            |
| BLM         | 43.1             | RAP80 #1    | 44.8            | 14.3            |
| BRCA2       | 42.8             | RAP80 #2    | 32.8            | 16.7            |
| DDX11       | 43.7             | RDM1        | 35.1            | 13.0            |
| DHX36       | 49.9             | RECQL5      | 40.4            | 15.8            |
| DNA2L       | 37.7             | REVI1       | 44.3            | 10.4            |
| DUT         | 42.7             | RING1 #1    | 58.0            | 4.6             |
| EME1        | 51.4             | RING1 #2    | 64.6            | 30.7            |
| EME2        | 42.3             | RING1 #3    | 74.2            | 30.0            |
| EXO1        | 68.6             | RMI1        | 43.1            | 13.4            |
| FAN1        | 76.1             | RTE1        | 67.6            | 18.4            |
| GEN1        | 44.0             | SFPQ        | 68.9            | 9.2             |
| HELQ        | 38.2             | SFR1        | 49.2            | 21.7            |
| HELZ        | 41.4             | SLX1        | 41.3            | 13.0            |
| HLAB        | 42.7             | SLX4        | 66.8            | 40.7            |
| HORMAD1     | 41.3             | SMARCA3     | 54.8            | 9.6             |
| MCM8        | 24.0             | SMARCAL1    | 42.0            | 29.2            |
| MCM9        | 45.9             | SMC5        | 28.9            | 5.5             |
| MRE11A      | 44.2             | SMC6        | 68.1            | 25.2            |
| MUS81       | 49.0             | SPATAS      | 32.3            | 12.7            |
| NOXIN       | 42.3             | SUB1        | 46.2            | 18.4            |
| PIF1 #1     | 34.9             | SW15        | 38.8            | 9.3             |
| PIF1 #2     | 38.2             | SWS1        | 58.6            | 25.5            |
| POLD2       | 46.2             | SWSAP1      | 52.8            | 23.5            |
| POLD4       | 45.9             | TIMELESS    | 40.8            | 26.1            |
| POLH        | 37.9             | TIPIN       | 38.8            | 34.3            |
| POLN        | 60.6             | TONSL       | 51.4            | 20.9            |
| POLQ        | 38.8             | TREP2       | 60.0            | 28.8            |
| RAD51       | 46.4             | WDHD1       | 30.8            | 14.8            |
| RAD51B      | 31.7             | WRNIP1      | 53.1            | 15.9            |
| RAD51C      | 29.5             | XRCC2       | 39.2            | 20.9            |
| RAD51D      | 43.8             | XRCC3       | 33.2            | 15.5            |
| RAD52 #1    | 58.1             | ZRANB3      | 46.8            | 22.6            |
| RAD52 #2    | 53.6             |             | 32.9            | 15.5            |
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 | 0.0004|
| OE cells: siControl vs siRAD52 #2  | 31 | 7  | 11.06  | 7.3 | 0.0001|
| OE cells: siControl vs siMUS81     | 31 | 6  | 15.65  | 7   | 0.0001|
| OE cells: siControl vs siSMARCAL1  | 31 | 7  | 14.59  | 10  | 0.0001|
| 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| 0.0001|
| NE cells: siControl vs siRAD52 #2   | 31 | 7  | 9      | 23.6| 0.0001|
| NE cells: siControl vs siMUS81      | 31 | 6  | 4.2    | 8.9 | NS    |
| NE cells: siControl vs siSMARCAL1   | 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.

| 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. 2A: Rad52 foci HU 0h vs 2h      | 3  | 3  | 4.54   | 4  | 0.02  |
| Fig. 2A: 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 γH2AX 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 GUCCAUUGCCUUUAUGUUTT (Qiagen, SI03041808) or a pool of 4 siRNAs: ACGAAAACCGCUACUAAPA, GGCAUUAGUCUGACUAA, CAUUUGUGGUAGGGAA, UGUAUAGCGACGAGACUA (Thermo Scientific, J-013058-06-0005) and GUUUAGACCCUUGAGCAAT (Qiagen, SI00103180); MUS81, an equimolar mixture of two siRNAs: GCUUUGACCUUCUAGCAA (ThermoScientific, J-013058-06-0005) and GGUCCAUGCCUUUAAUGUUTT (Qiagen, SI00103180); SMARCAL1, an equimolar mixture of two siRNAs: ACGAAAACGCGUACUAAAA, GGCAUUAUGUCUGACUAA, CAUUUGUGGUAGGGAA, UGUAUAGCGACGAGACUA (Thermo Scientific, J-013058-06-0005) and POLD4, an equimolar mixture of two siRNAs: CCAUAGUGCCUAAUUAATTTT (Qiagen, SI00688695) and POLD4, a pool of 4 siRNAs: CCAUAGUGCCUAAUUAATTTT (Qiagen, SI00688695) and GUUUAGACCCUUGAGCAAT (Qiagen, SI00103180); negative control, AllStars Negative Control siRNA (Qiagen, 1027281) or Luciferase GL2 (Invitrogen) or ON-TARGETplus non targeting pool siRNA (Dharmacon, L-014013-02-0005). 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 transfected 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: CACCGCCGAGCTTCCGCTG-GTGG) construct targeting exon 9 and the pX458_Rad52_Crispr6 construct (Guide Sequence Insert: CACCGTGATCATAAGTAGCCGCATGGC) targeting exon 3 of the human RAD52 gene were designed and generated as described in the MIT CRIPSPR 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 GGAACGTCTGACAGCTGTGCTTC or the CCCTGAGCCAGGCTGGGCCAG and CTCTACCTCTGTCCTCCGCC 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.

\( \gamma \text{H2AX detection by flow cytometry} \)

For \( \gamma \text{H2AX detection by flow cytometry} \), 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 Heps pH 7.9, 1.5 mM MgCl2, 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 1 min at 3000 rpm at 4°C. The pellets, which correspond to the chromatin fraction, were resuspended in sonication buffer [50 mM Heps pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na deoxycholate, 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 1H-NMR, 13C-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 C15H11N5OCl2 [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-Rad52tm1Ap+/Cnrm mice were purchased from the European Mouse Mutant Archive (EMMA) (Rijkers et al., 1998). The C57BL/6-Apctm1Tyj/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+/+;Apcmin/+ and Rad52-/-;Apcmin/+ 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-/-;Apcf+/;CMVcre and Rad52+/+;Apcf+/;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          |
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