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Supplementary Materials for

RAD51 bypasses the CMG helicase to promote replication fork reversal

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Materials and Methods

Cell lines

U2OS (RRID:CVCL_0042) and HEK293T (RRID:CVCL_0063) cells were cultured in DMEM with 7.5% fetal bovine serum (FBS). HCT116 (RRID:CVCL_0291) cells were cultured in McCoy's 5A supplemented with 7.5% FBS. HEK293T and U2OS cells for iPOND mass spectrometry experiments were cultured in SILAC-compatible DMEM + 7.5% dialyzed FBS (Thermo Fisher Scientific, catalog no. A33822; R&D Systems, catalog no. S12850H), supplemented with either isotopically light or heavy ^{13}C, ^{15}N lysine, and ^{13}C,^{15}N arginine. Cells were cultured at 37°C and 5% CO_2 with humidity. All cell lines were regularly tested for mycoplasma and verified using short tandem repeat profiling. U2OS, and HEK293T cells are female. HCT116 cells are male. HCT116 MCM2-AID2, MCM3-AID2, MCM4-AID2 and GINS4-AID2 cells were generated by CRISPR-Cas9 mediated genome editing using previously described methods (*15, 55*). Briefly, we designed a CRISPR-Cas9 plasmid for targeting the following gene locus.
MCM2: 5'-GAGGCCCTATGCCATCCATA(AGG)-3'
MCM3: 5'-CTACCTCCACCAAAGTCGCG(TGG)-3'
MCM4: 5'-GGTGTGGTCTGCATCTCAGT(TGG)-3'
GINS4: 5'-ATGCCTAGTTTTAAATTAGC(TGG)-3'
A donor plasmid harboring mAID and a selection marker with two homology arms (about 500 bp each) was constructed. A parental HCT116 cell line stably expressing sTIR1(F74G) from the AAVS1 locus was transfected with the CRISPR and donor plasmids. Subsequently, colonies were formed in selection medium. After isolation of potential clones, the biallelic insertion of the tagging construct was checked by genomic PCR, and then the expression of the fusion protein was confirmed by immunoblotting.

iPOND proteomics

Proteomics changes in U2OS cells, 293T cells and HCT116 cells were determined using iPOND-SILAC MS as described previously (*56*). Briefly, EdU labeled and indicated drug treated cells were harvested following cross-linking with 1% formaldehyde for 10 min and quenched with glycine. Cells were permeabilized in 0.25% Triton X-100/PBS for 30 min. Heavy labeled and light labeled cells were combined 1:1 prior to performing the click reaction for 2 hr. Streptavidin coupled C1 magnabeads were utilized to capture DNA-protein complexes, washed, and boiled in sample buffer to reverse the cross-links. The samples were separated by SDS-PAGE gel and digested with trypsin. MudPIT analysis was performed on the extracted peptides using an eight-step gradient. MaxQuant was utilized for peptide and protein identification. $5x10^8$ cells per sample were utilized for CMG level analysis in HEK293T cells and U2OS cells. To examine protein changes in MCM2 degron cells, cells were incubated for 10 min with 10 μM EdU followed by 4 hours of 4 mM HU and 2μM 5-ph-IAA leaving EdU in the media.

Retrovirus production

pLNCX2-RAD51 vectors were transfected with p-VSVG into GP2-293 cells with Fugene HD. Fresh media was added 24 hours after transfection. Virus was collected at 48 and 72

hours after transfection and used to infect HCT116 or U2OS cells. Cells are selected with 500μg/ml G418 to establish stable cell lines.

Nascent strand degradation assays

Nascent strand degradation assays were performed using DNA fiber spreading as described previously (*17*). Cells were labeled with nucleoside analogs as indicated in the figures. Following stretching and fixation on glass slides, DNA was denatured in 2.5 M HCl, washed three times with phosphate-buffered saline (PBS), and blocked in 10% goat serum/PBS with 0.1% Triton X-100 for 1 hour. Slides are incubated with mouse anti-BrdU (IdU) and rat anti-BrdU (CldU) antibodies followed by Alexa-Flour 594 anti-rat and Alexa-Flour 488 anti mouse secondary antibodies. Median of the IdU/CldU ratios are plotted, 100-300 fibers were measured for each sample. P values were calculated using a Kruskal-Wallis test.

DNA combing assays

Cells were labeled with the nucleoside analogs as described in the figures. DNA combing was then performed using a DNA combing instrument as previously described (*57*). The S1 nuclease digestion assay was also completed as previously described with the digestion happening in the low-melting agarose plugs (*57*).

Immunofluorescence

To measure the amount of proteins in the insoluble/chromatin fraction, cells were detergent extracted with 0.5% Triton X-100 prior to fixing with 3% paraformaldehyde/2% sucrose. Slides were blocked with 1% goat serum in PBST (0.1% triton-X100) and incubated with antibody. EdU was detected using click chemistry with an Alexa Fluor 488-conjugated azide. Immunofluorescent images were obtained and analyzed by ImageXpress microscopy and software.

PLA assay for MCM7 and EdU

The Sigma PLA kit protocol was used with the following additions. Cells were pre-extracted and fixed as stated in the immunofluorescence method. Then the cells were blocked with 3% BSA and processed via click chemistry with biotin-azide. MCM7 antibody (Santa Cruz SC-9966) (1:100 dilution) and biotin antibody (CST 5597) (1:200 dilution) were used in the assay.

Electron microscopy

Electron microscopy experiments were performed as previously described (*58*). $5x10^6$ cells transfected with the indicated siRNAs were collected immediately after treatment with 4 mM hydroxyurea, 20μM DNA2 inhibitor C5 and 25μM MRE11 inhibitor Mirin, with or without 2μM 5-ph-IAA for 4 hours. DNA was cross-linked by incubating with 10 μg/mL 4,5′,8-trimethylpsoralen followed by a 3-minute exposure to 366 nm UV light on a precooled metal block, for a total of three rounds. Cells were lysed and genomic DNA was isolated from the nuclei by proteinase K digestion and chloroform-isoamyl alcohol extraction. Genomic DNA was purified by isopropanol precipitation and digested with PvuII HF with the appropriate buffer for 4 hours at 37°C. Replication intermediates were enriched on a benzoylated naphthoylated DEAE-cellulose (Sigma-Aldrich) column. Samples were prepared for visualization by EM by spreading the purified, concentrated DNA on a carbon-coated grid in the presence of benzyl-dimethyl-alkylammonium chloride, followed by platinum rotary shadowing. Images were obtained on a JEOL JEM-1400 electron

microscope using a bottom mounted AMT XR401 camera. Analysis was performed using ImageJ software (National Institute of Health). EM analysis allows distinguishing duplex DNA—which is expected to appear as a 10 nm thick fiber after the platinum/carbon coating step necessary for EM visualization—from ssDNA, which has a reduced thickness of 5-7 nm. Criteria used for the assignment of a three-way junction, indicative of a replication fork, include the joining of three DNA fibers into a single junction, with two symmetrical daughter strands and single parental strand. Reversed replication forks consist of four DNA fibers joined at a single junction, consisting of two symmetrical daughter strands, one parental strand and the addition of a typically shorter fourth strand, representative of the reversed arm. The length of the two daughter strands corresponding to the newly replicated duplex should be equal (b = c), whereas the length of the parental arm and the regressed arm can vary ($a \neq b = c \neq d$). Conversely, canonical Holliday junction structures will be characterized by arms of equal length (a = b, c = d). Particular attention is paid to the junction of the reversed replication fork to observe the presence of a bubble structure, indicating that the junction is opened and that it is simply not the result of the occasional crossover of two DNA molecules. These four-way junctions of reversed replication forks may also be collapsed and other indicators such as daughter strand symmetry, presence of single-stranded DNA at the junction or the entire structure itself, all are considered during analysis.

Cell fractionation

Cells ($1x10^6$) were harvested and washed with PBS, then lysed on ice for 20 min with 100 µl H150 buffer, which contains 50 mM HEPES (pH7.4), 150 mM NaCl, 10% glycerol, 0.5% NP-40 and protease inhibitor cocktail (Roche). The lysate was centrifuged for 10 min at 5000g, and the supernatant is the soluble fraction. The pellet was washed two times with H150 lysis buffer, and the supernatant discarded. The pellet is then considered the insoluble nuclear fraction. The pellet was resuspended in PBS and 6xSDS loading buffer and boiled prior to SDS-PAGE and immunoblotting.

RAD51 expression vectors and mutagenesis

RAD51 was cloned to pLNCX2 retrovirus vector using Not1 and Sal1 digests. Primers are as follows: NotI-RAD51-F: ATAAGAATGCGGCCGCATGGCAATGCAGATGCA
SalI-RAD51-R: AGGCCTGTCGACTCAGTCTTTGGCATCTCCCACTC
RAD51 point mutation primers are as follows:
I287T
F: AACCTACAGGAGGAAATATCATCGCCC
R: TCCTGTAGGTTTTTTGGGATCAGCAGC
Y232A
F: CAGACGCCTCGGGTCGAGGTGAGCT
R: CGAGGCGTCTGTTCTGTAAAGGGCGGT
G151D
F: CGGGACGGAGGTGAAGGAAAG
R: TCCGtcCCGGTCAATGGGAA
K133R
TGGGCGTACCCAGATCTGTCATACGCT
TGGGTACGCCCAGTTCGGAATTCTCCAAA
II3A (Consists of three point mutations)

R130AF TTCGCAACTGGGAAGACCCAGATCT
R130AR CAGTTGCGAATTCTCCAAACATTTCTGTGAT
R303AF ATCTGGCGAAAGGAAGAGGGGAAACCAGA
R303AR TTTCGCCAGATACAATCTGGTTGTTGATGCATG
K313AF TCTGCGCAATCTACGACTCTCCCTGTCTT
K313AR AGATTGCGCAGATTCTGGTTTCCCCTCTTC
A293T
F: TCACACATGCATCAACAACCAGATTGTATCTG
R: ATGTGTGATGATATTTCCTCCAATAGGTTTTTTGGGA

T131P geneblock is synthesized from Genewiz, then inserted to the pLNCX2 vector.
All mutations are sequenced before use.

The miSIFTs geneblocks were synthesized by Genewiz and inserted into the pLNCX2-RAD51 vectors using Sal1 and Stu1 digests. Sequences of the miSFITs are as follows:
1xperfect:
GACTGAGTCGACCTAGCCTACCTGCACTGTAAGCACTTTGAAGGCCTTAATGG
2xperfect:
GACTGAGTCGACCTAGCCTACCTGCACTGTAAGCACTTTGTATCTACCTGCACT
GTAAGCACTTTGAAGGCCTTAATGG
8A:
GACTGAGTCGACCTAGCCTACCTGAACTGTAAGCACTTTGAAGGCCTTAATGG
18G:
GACTGAGTCGACCTAGCCTACCTGCACTGTAAGCGCTTTGAAGGCCTTAATGG
9G: GACTGAGTCGACCTAGCCTACCTGCGCTGTAAGCACTTTGAAGGCCTTAATGG
22G:
GACTGAGTCGACCTAGCCTACCTGCACTGTAAGCACTTGGAAGGCCTTAATGG
6C-16C:
GACTGAGTCGACCTAGCCTACCCGCACTGTAACCACTTTGAAGGCCTTAATGG
3C: GACTGAGTCGACCTAGCCTCCCTGCACTGTAAGCACTTTGAAGGCCTTAATGG
12C:
GACTGAGTCGACCTAGCCTACCTGCACTCTAAGCACTTTGAAGGCCTTAATGG
17A-18G:
GACTGAGTCGACCTAGCCTACCTGCACTGTAAGAGCTTTGAAGGCCTTAATGG

RNA interference
All siRNA transfections were performed using DharmaFECT reagents according to the manufacturer's instructions. Experiments were completed 3 days after transfection. See table S2 for siRNA sequences. Qiagen AllStars Negative Control Nontargeting (NT) siRNA was used in samples where a gene-selective siRNA is not indicated or to bring the total amount of siRNA to equal molar concentrations when comparing samples with one versus two siRNAs.

Quantification and statistical analysis
Statistical analyses were completed using Prism. A Kruskal-Wallis test was used for experiments with more than two samples, and P values were calculated by Prism for the multiple comparisons. A two-tailed t test was used to compare two samples with normally distributed data. No statistical methods or criteria were used to estimate sample size or to

include/exclude samples. Statistical details of individual experiments can be found in the figure legends and in Results. Experiments shown are representative of at least two biological replicates unless otherwise indicated in the figure legend.

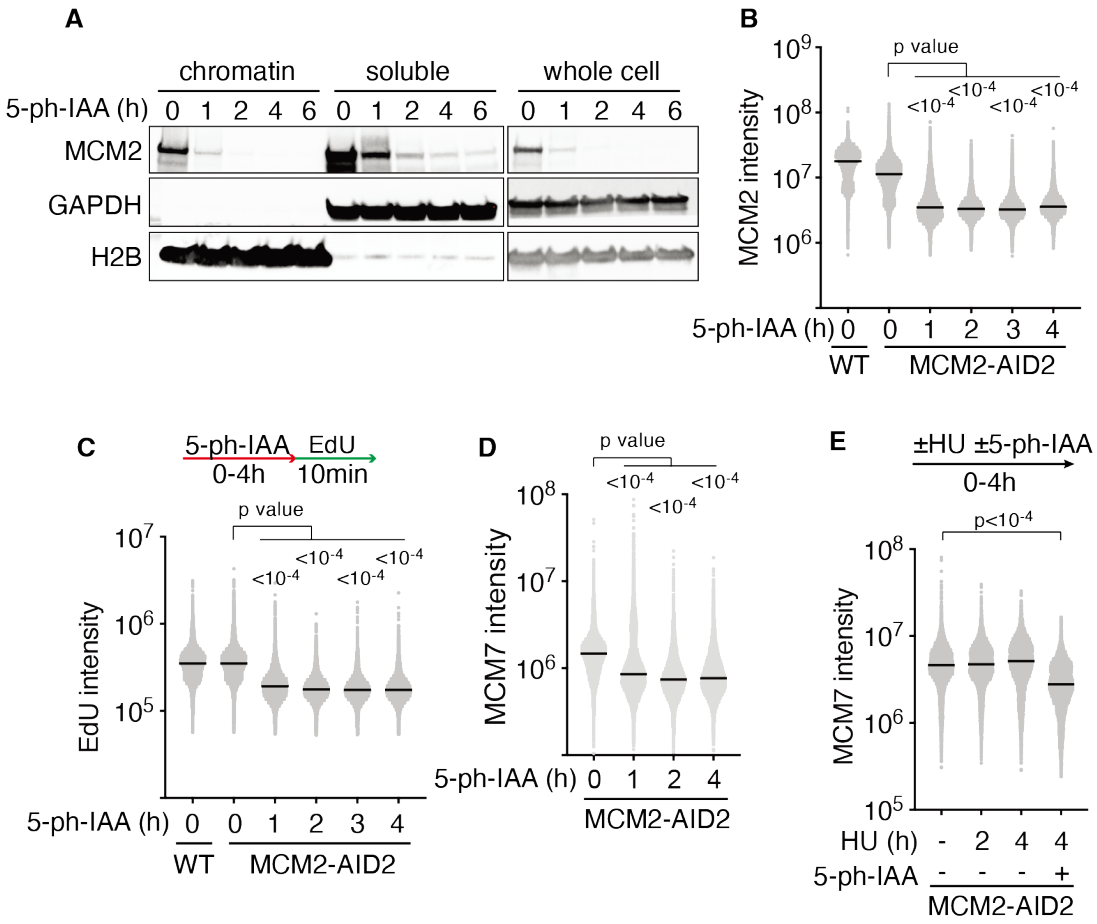


Fig. S1. Characterization of MCM2-AID2 degron cells.
(A) Immunoblot of MCM2-AID2 cells following 5-ph-IAA treatment and biochemical fractionation. Immunostaining of **(B)** insoluble MCM2, **(C)** EdU, **(D and E)** insoluble MCM7 in MCM2-AID2 cells treated as indicated. Graphs are representative of at least three experiments. P values were calculated using a Kruskal-Wallis test.

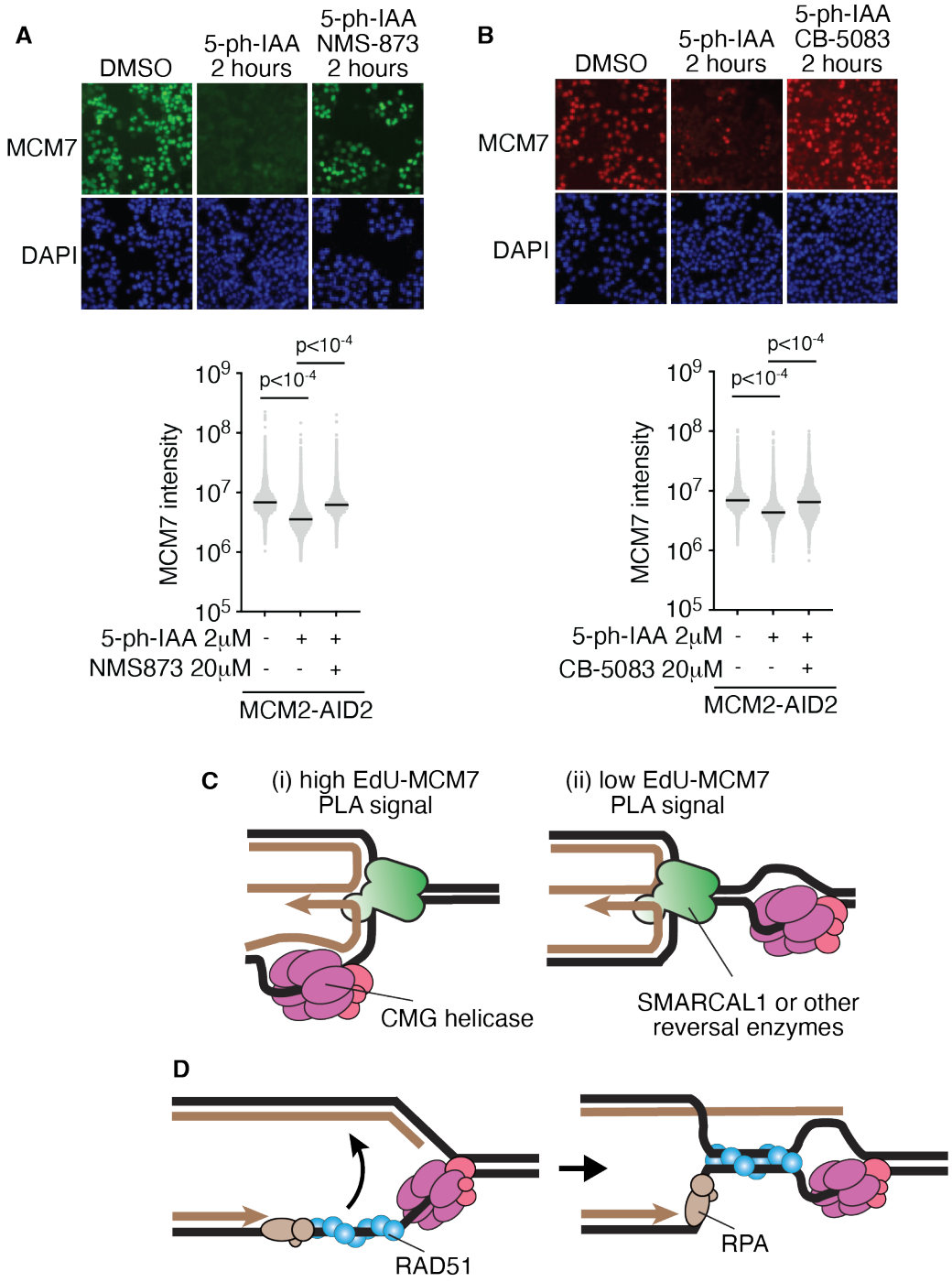
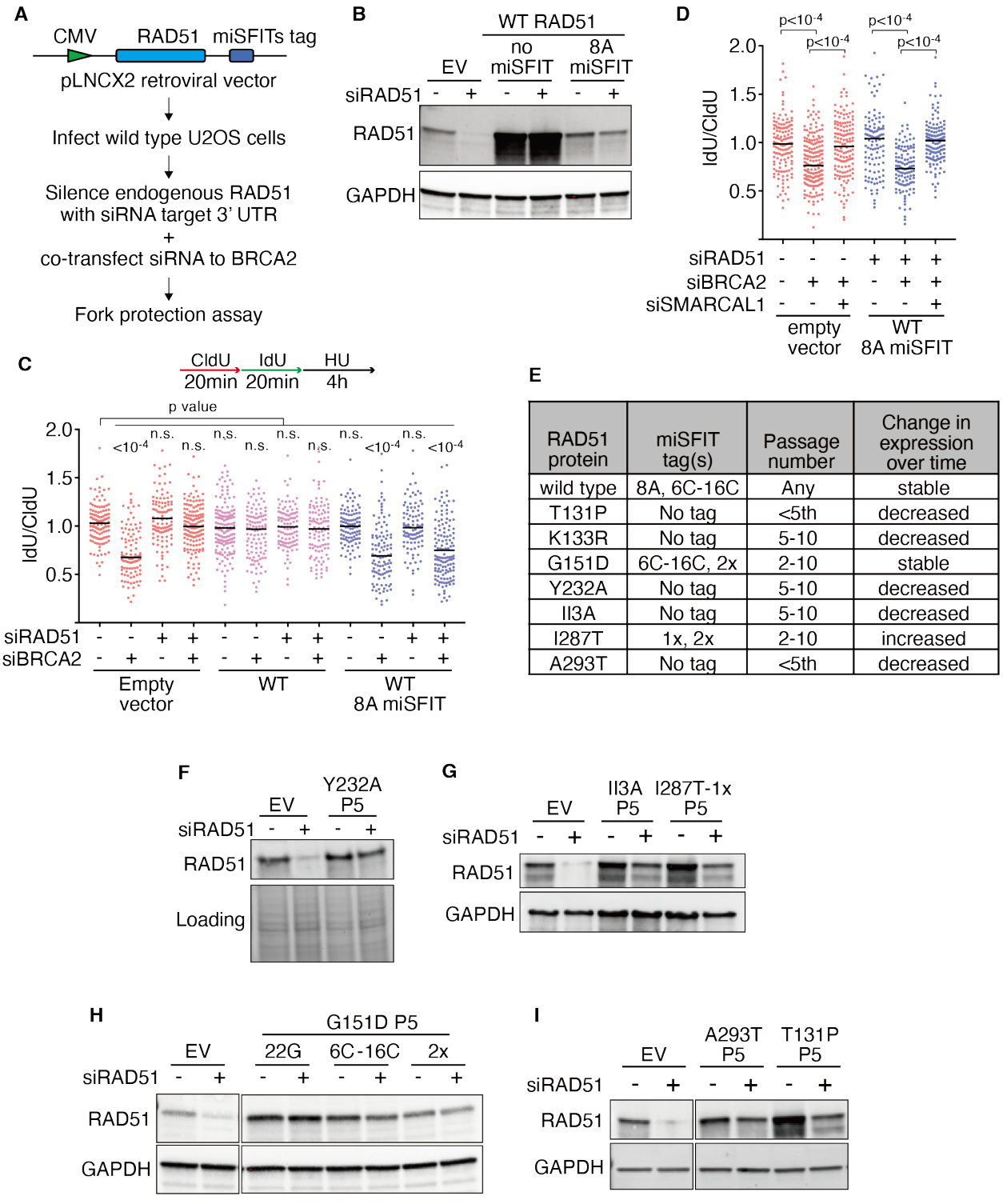


Fig. S2. Analysis of CMG localization.
(A-B) MCM2-AID2 cells were treated with 5-ph-IAA, and p97 inhibitor NMS-873 in **A)** and CB-5083 in **B)** for 2 hours to show that p97 inhibition blocks CMG disassembly. MCM7 was imaged by immunostaining in detergent pre-extracted cells. Representative images and quantitation from a representative experiment are presented. P values were calculated using a Kruskal-Wallis test. **(C)** Predictions of PLA assay between nascent DNA labeled with EdU and MCM7 depending on the position of the MCM complex during reversal. **(D)** Model for how

RAD51 strand exchange activity could generate a paranemic joint behind the CMG complex that could be a substrate for fork reversal enzymes.



RAD51 protein	miSFIT tag(s)	Passage number	Change in expression over time
wild type	8A, 6C-16C	Any	stable
T131P	No tag	<5th	decreased
K133R	No tag	5-10	decreased
G151D	6C-16C, 2x	2-10	stable
Y232A	No tag	5-10	decreased
II3A	No tag	5-10	decreased
I287T	1x, 2x	2-10	increased
A293T	No tag	<5th	decreased

Fig. S3. Establishment of RAD51 wild-type and mutant cell lines expressing near endogenous levels of RAD51 proteins.
(A) Strategy for creating and analyzing RAD51 wild-type and mutant expressing cell lines. **(B)** Immunoblot of U2OS cells containing the indicated wild-type RAD51 expression vector. **(C-D)** Fork protection assay for the indicated cell lines after transfection with siRNAs. All samples

were treated with HU. Graphs are representative of at least three experiments. P values were calculated using a Kruskal-Wallis test. **(E)** Summary table of the miSFIT vectors and passage numbers used to analyze each RAD51 mutant. **(F-I)** Immunoblots of the indicated cell lines. Passage numbers are indicated**.** The miSFIT microRNA tag inserted into the expression vectors are indicated. (WT, wild type; EV, empty vector)

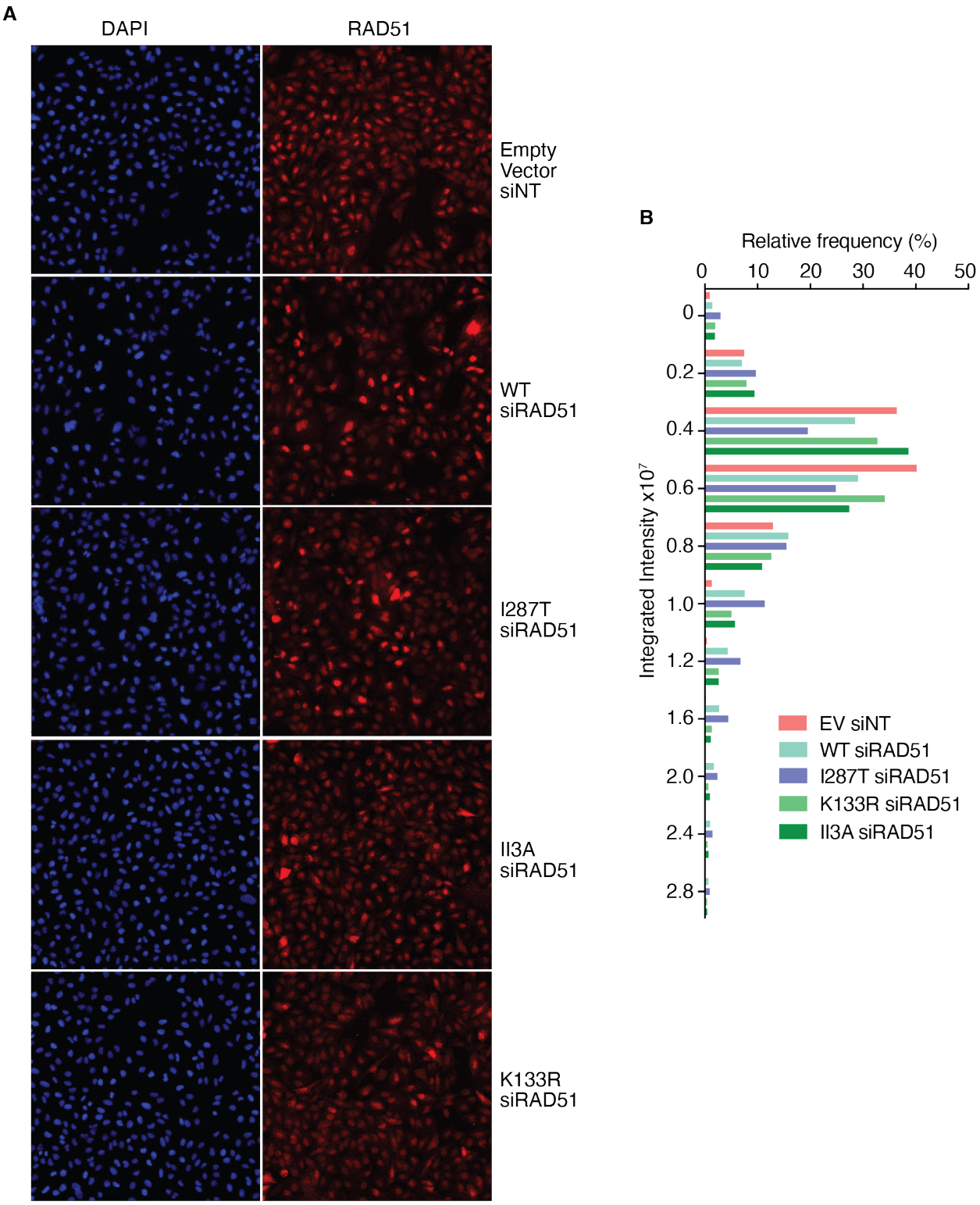


Fig. S4. Analysis of RAD51 expression heterogeneity in stable U2OS cell lines.
(A) Immunofluorescence imaging of RAD51 in the indicated cells. EV: empty vector, siNT: non-targeting siRNA. siRAD51, siRNA targeting endogenous RAD51. **(B)** Distribution of RAD51 expression level in the indicated cells after transfection with siRNA to deplete endogenous RAD51.

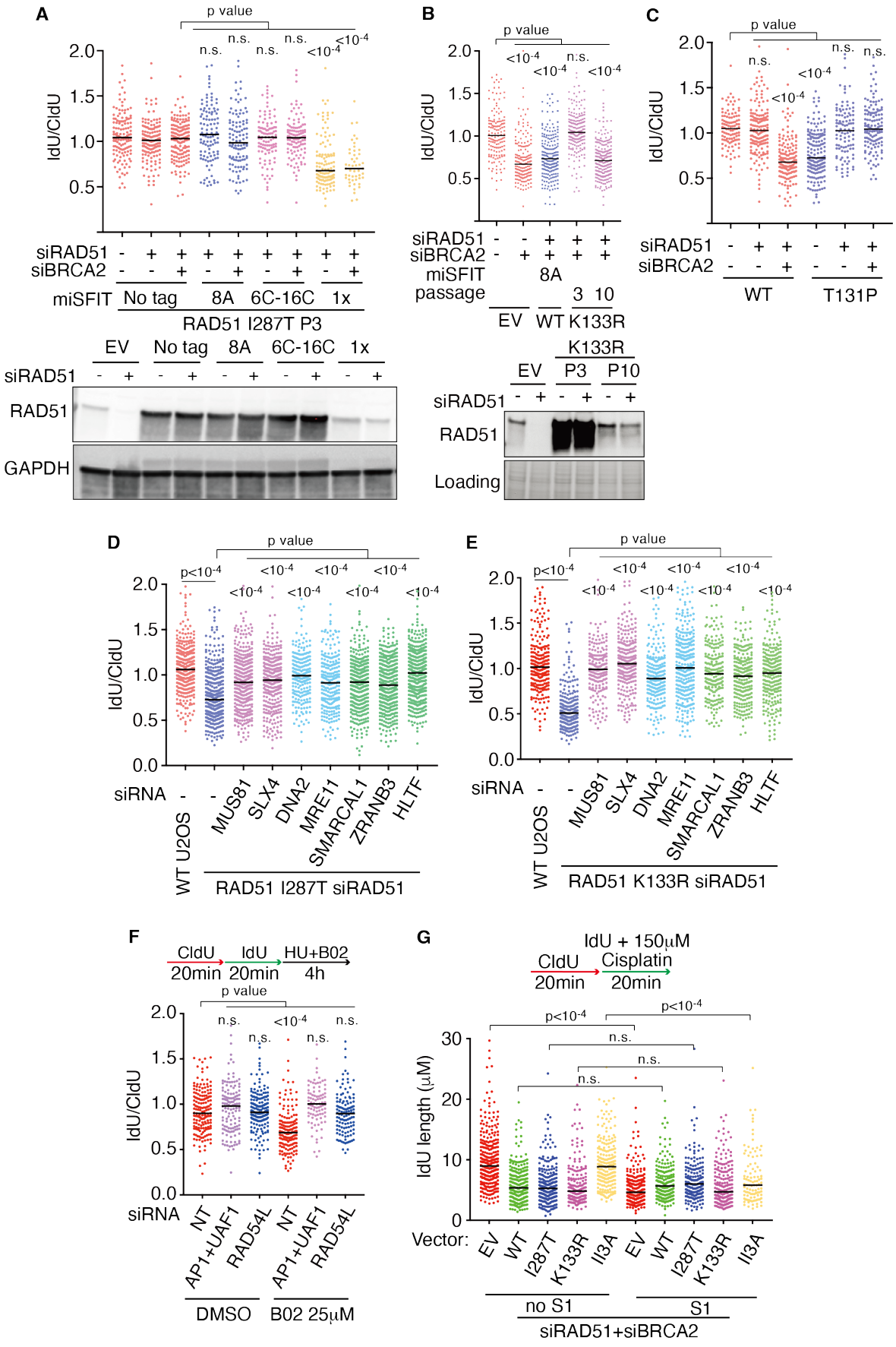

A
p value
IdU/CldU
siRAD51
siBRCA2
miSFIT
No tag
8A
6C-16C
1x
RAD51 I287T P3
EV
No tag
8A
6C-16C
1x
siRAD51
RAD51
GAPDH
B
p value
IdU/CldU
siRAD51
siBRCA2
miSFIT
8A
passage
3
10
EV
WT
K133R
K133R
EV
P3
P10
siRAD51
RAD51
Loading
C
p value
IdU/CldU
siRAD51
siBRCA2
WT
T131P
D
p value
IdU/CldU
siRNA
MUS81
SLX4
DNA2
MRE11
SMARCAL1
ZRANB3
HLTF
WT U2OS
RAD51 I287T siRAD51
E
p value
IdU/CldU
siRNA
MUS81
SLX4
DNA2
MRE11
SMARCAL1
ZRANB3
HLTF
WT U2OS
RAD51 K133R siRAD51
F
CldU
20min
IdU
20min
HU+B02
4h
p value
IdU/CldU
siRNA
NT
AP1+UAF1
RAD54L
NT
AP1+UAF1
RAD54L
DMSO
B02 25μM
G
IdU + 150μM
CldU
20min
Cisplatin
20min
IdU length (μM)
Vector:
EV
WT
I287T
K133R
I13A
EV
WT
I287T
K133R
I13A
no S1
S1
siRAD51+siBRCA2

Fig. S5. RAD51 recombinase activity is needed to generate reversed fork substrates for nascent strand degradation.
(A-E) Fork protection assays were completed in the indicated cell lines. All graphs are representative of at least three experiments. Immunoblots show the expression level of RAD51 proteins. (EV, empty vector; NT, non-targeting) **(F)** Fork protection assays were completed in the indicated cell lines as in Figure 2A after treatment with B02 or DMSO as a control. All graphs are representative of at least three experiments. P values were calculated using a Kruskal-Wallis test. **(G)** Replication elongation rates in the presence of cisplatin were measured in U2OS cells expressing the indicated RAD51 proteins after transfection with siRNAs. S1 nuclease was used where indicated to determine if ssDNA gaps were present in the IdU replication tracks. P values were calculated using a Kruskal-Wallis test.

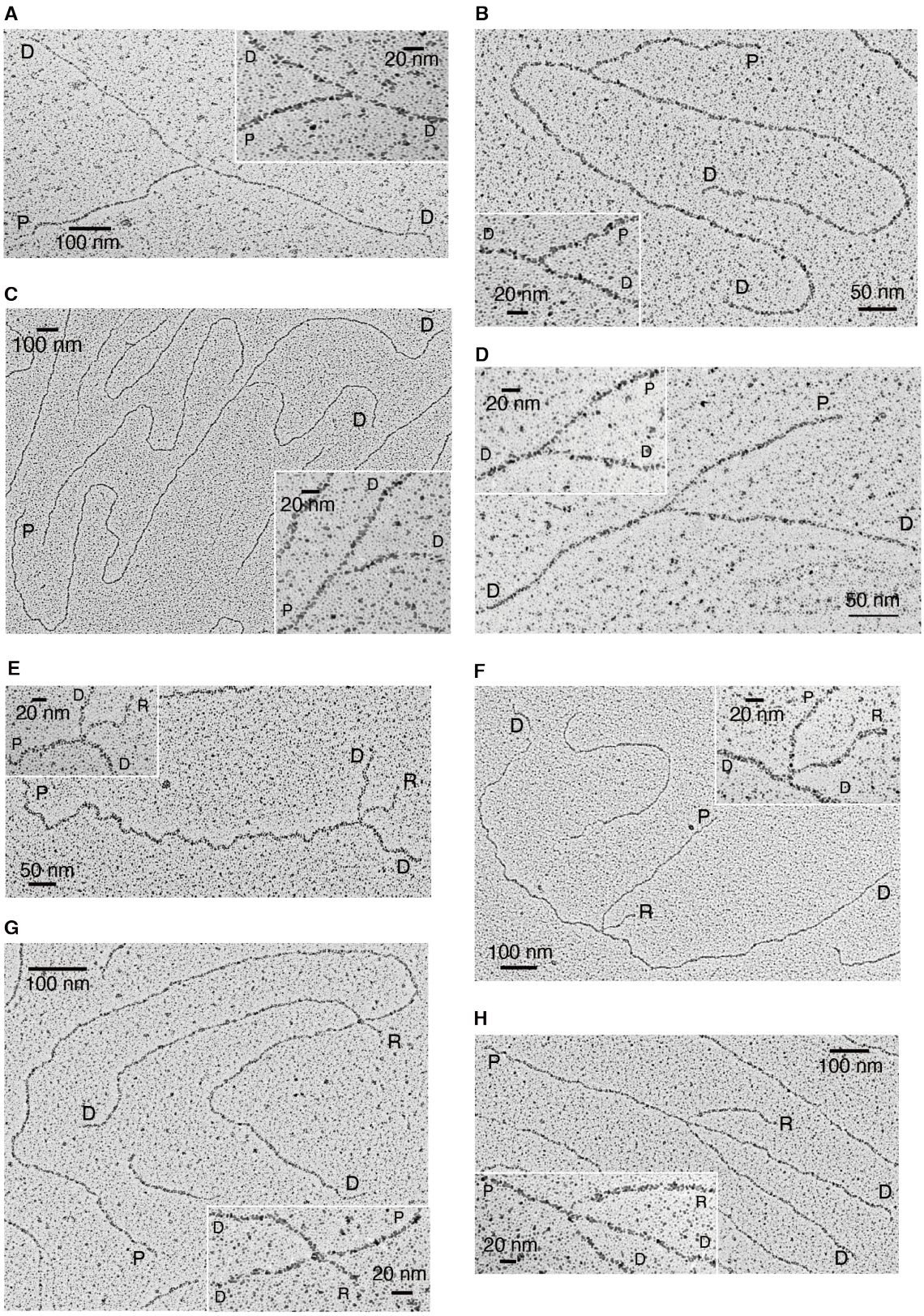


Fig. S6. Representative electron microscopy images.
Representative electron microscopy images of **(A-D)** three- and **(E-H)** four-way junction replication fork structures. P: parental strand, D: daughter strand and R: reversed arm

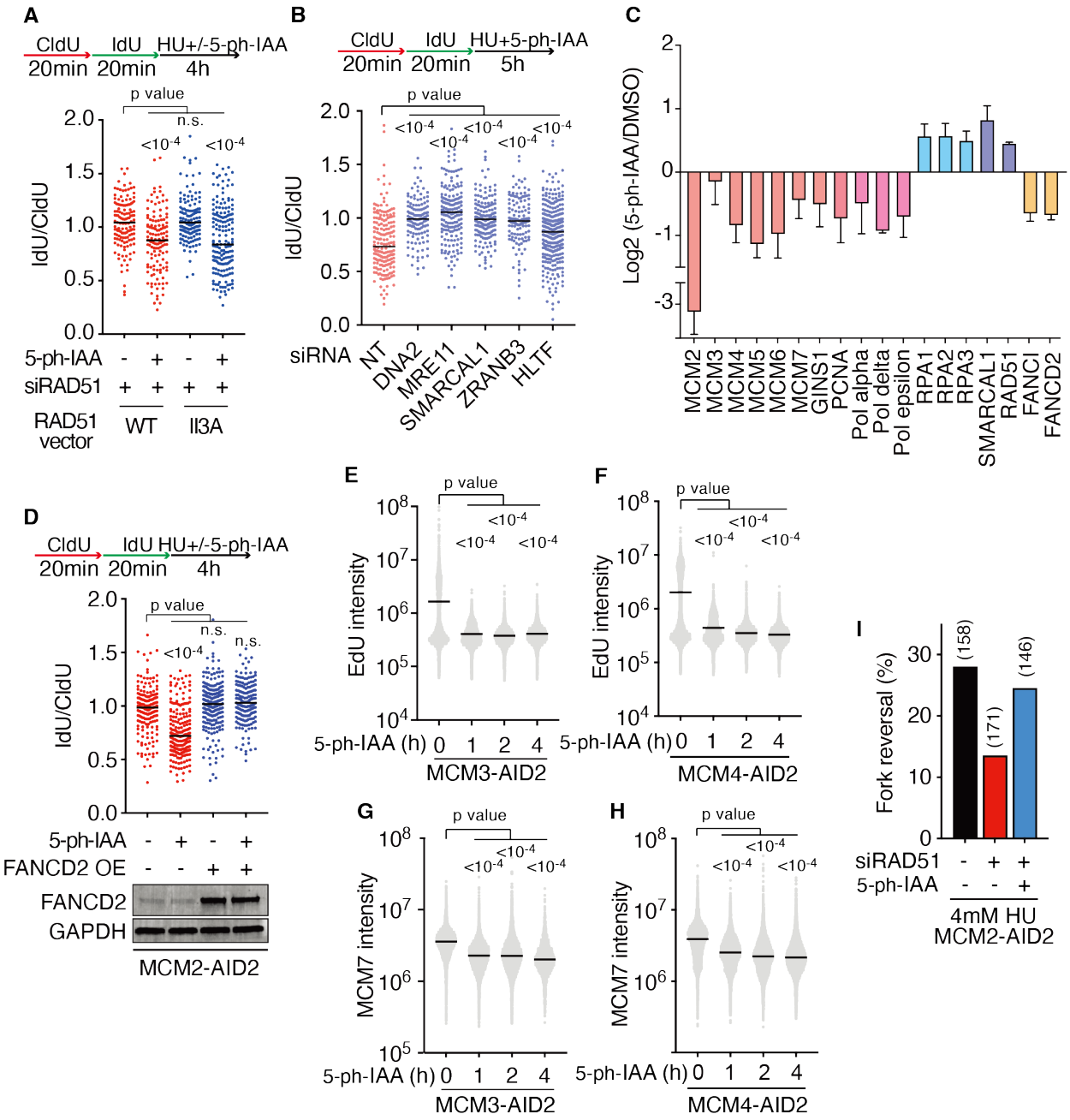


Fig. S7. RAD51 is dispensable for fork reversal when MCM proteins are removed from the stalled fork.

(A) Fork protection assay of MCM2-AID2 cells expressing wild type or II3A RAD51. Cells were transfected with siRNA to deplete endogenous RAD51 and treated with 2 μM 5-ph-IAA to degrade the MCM complex. The graph is representative of two experiments. P values were calculated using a Kruskal-Wallis test. **(B)** Fork protection assays in MCM2-AID2 cells transfected with indicated siRNAs. 2 μM 5-ph-IAA and 4mM HU were included in all samples. The graph is representative of at least three experiments. P values were calculated using a Kruskal-Wallis test. **(C)** iPOND-SILAC-MS was used to quantify the relative amounts of the indicated proteins bound to nascent DNA in the HU-treated MCM2-AID2 cells treated with 5-ph-IAA or DMSO vehicle control. **(D)** Fork protection assay in MCM2-AID2 cells

overexpressing (OE) FANCD2. The graph is representative of at least three experiments. P values were calculated using a Kruskal-Wallis test. The intensity of (**E and F**) EdU or (**G and H**) MCM7 was measured by immunofluorescence imaging in MCM3-AID2 or MCM4-AID2 cell lines. Cells were incubated with EdU for 20 minutes prior to staining. **(I)** Percentage of reversed replication forks in MCM2-AID2 cells transfected with the indicated siRNA and treated 72 hours later with DMSO or 5-ph-IAA together with HU, mirin, and C5 for 5 hours. The number of replication intermediates analyzed for each condition is indicated in parentheses.

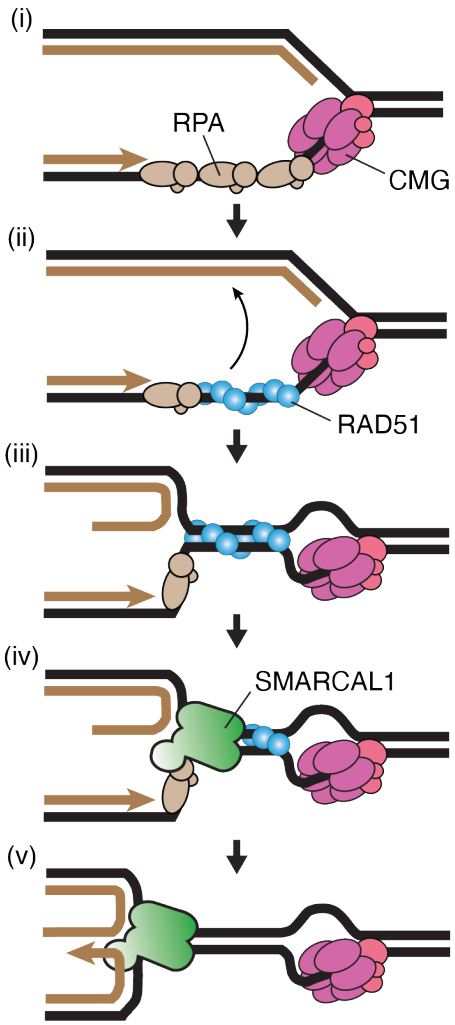


Fig. S8. Fork reversal model.
(i) Replication stress that causes fork reversal often involves uncoupling of helicase and polymerase activities to generate ssDNA that is bound by RPA at stalled forks. (ii) RAD51 accumulates at stalled forks and utilizes its strand exchange activity to promote invasion of the parental ssDNA into the sister chromatid duplex DNA. (iii) Strand invasion generates a paranemic joint that could be converted to a plectonemic joint by topoisomerases or other strand nicking enzymes. (iv) DNA translocases like SMARCAL1 binds the new fork junction and (v) catalyze branch migration to generate a reversed replication fork.

Table S1. RAD51 protein characteristics

Function	RAD51 Proteins							
	WT	K133R	I287T	G151D	T131P	A293T	II3A	Y232A
ssDNA binding	Yes	increased	increased	Yes	decreased	decreased	slightly decreased	decreased
dsDNA binding	Yes	increased	increased	Yes	decreased	decreased	slightly decreased	decreased
Filament formation	Yes	hyper stable	Yes	hyper stable	unstable	unstable	Yes	ND
ATPase activity	Yes	No	increased	decreased with DNA, increased without DNA	independent of DNA	decreased	ND	No DNA-dependent activity
BRCA2 binding BRC	Yes	ND	Yes	Yes	ND	ND	ND	ND
BRCA2 binding Exon27	Yes	ND	Yes	Yes	ND	ND	ND	ND
D-loop formation	Yes	Yes	ND	ND	No	No	No	ND
Strand exchange	Yes	Yes	Increased*	increased	No	ND	No	No
Homologous recombination in cells	Yes	decreased	decreased	increased	Yes, in presence of wild-type RAD51, ND on its own	ND	No	ND
References		(*33-35*)	(*31, 59, 60*)	(*36, 37*)	(*16, 18, 38, 40*)	(*40, 41*)	(*42, 44*) (*43*)	(*39, 61*)

ND, not determined; *increased strand invasion, but products may not resolve properly

Table S2. Antibodies, chemicals, and siRNAs utilized.

Reagent	Source	Catalog number/RRID
Antibodies		
Mouse monoclonal anti-GAPDH	Millipore	MAB374/AB_2107445
Mouse monoclonal anti-BrdU	BD Biosciences	347580/AB_10015219
Rat monoclonal anti-BrdU	Abcam	ab6326/AB_305426
Goat anti-rat Alexa Fluor 594	Thermo Fisher	A-11007/AB_10561522
Goat anti-mouse Alexa Fluor 488	Thermo Fisher	A-11029/AB_2534088
Mouse monoclonal anti-MCM7	Santa Cruz	SC-9966/AB_627235
Rabbit polyclonal anti-MCM3	Bethyl	A300-192A/AB_162726
Mouse monoclonal anti-mAID	MBL	M214-3/AB_2890014
Rabbit monoclonal anti-GINS4	Abcam	Ab139683/
Rabbit monoclonal anti-MCM2	CST	3619S/AB_2142137
Rabbit polyclonal anti-H2B	Abcam	Ab1790/AB_302612
Mouse monoclonal anti-RAD51	Abcam	ab213/AB_302856
Rabbit monoclonal anti-Biotin	CST	5597S/AB_10828011
Plasmid vectors		
pCMV-FANCD2	Campbell Lab	
pLNCX2	Clontech	631503
Chemicals		
Hydroxyurea	Millipore Sigma	H8627
CldU	Millipore Sigma	C6891
IdU	Millipore Sigma	I7125
CB-5083	Selleck Chemicals	S8101
NMS-873	ApexBio	B2168
MLN-4924	Sigma-Aldrich	5054770001
B02 inhibitor	Calbiochem	1290541-46-6
S1 nuclease kit	ThermoFisher	EN0321
5-ph-IAA	MCE	HY-134653
Mirin	Millipore Sigma	M9948
C5	MCE	HY-128729
Cisplatin	Millipore Sigma	P4394-250MG
Camptothecin	Sigma-Aldrich	7689-03-4

siRNAs		
siRNA	Sequence/Source	Catalog Number
SMARCAL1#6	GCUUUGACCUUCUUAGCAA	J-013058-06-0002
ZRANB3#2	GAUUCGAUCUAAUAACAGU	s38488
SLX4#2	GAAGUGGAAUUGUCUAGCA	s39054
MRE11#3	GCUAAUGACUCUGAUGAUA	D-009271-03-0010
DNA2#3	ACAGUUGCCUGCAUUCUAA	Custom
RAD51	AAGUGCUGCAGCCUAAUGAGAGUG	Custom
BRCA2 pool	Qiagen	S102653595
HLTF pool	Dharmacon SMARTpool	L-006448-00-0005
MUS81 pool	Dharmacon SMARTpool	L-016143-01-0005
RAD54L	Dharmacon SMARTpool	L-004592-00-0005
RAD51AP1	Dharmacon SMARTpool	L-017166-00-0005
UAF1	Dharmacon SMARTpool	L-016462-01-0005
PRIMPOL	Dharmacon SMARTpool	L-016804-02-0005