Science Advances

AAAS

Supplementary Materials for

Two replication fork remodeling pathways generate nuclease substrates for distinct fork protection factors

W. Liu, A. Krishnamoorthy, R. Zhao, D. Cortez*

*Corresponding author. Email: david.cortez@vanderbilt.edu
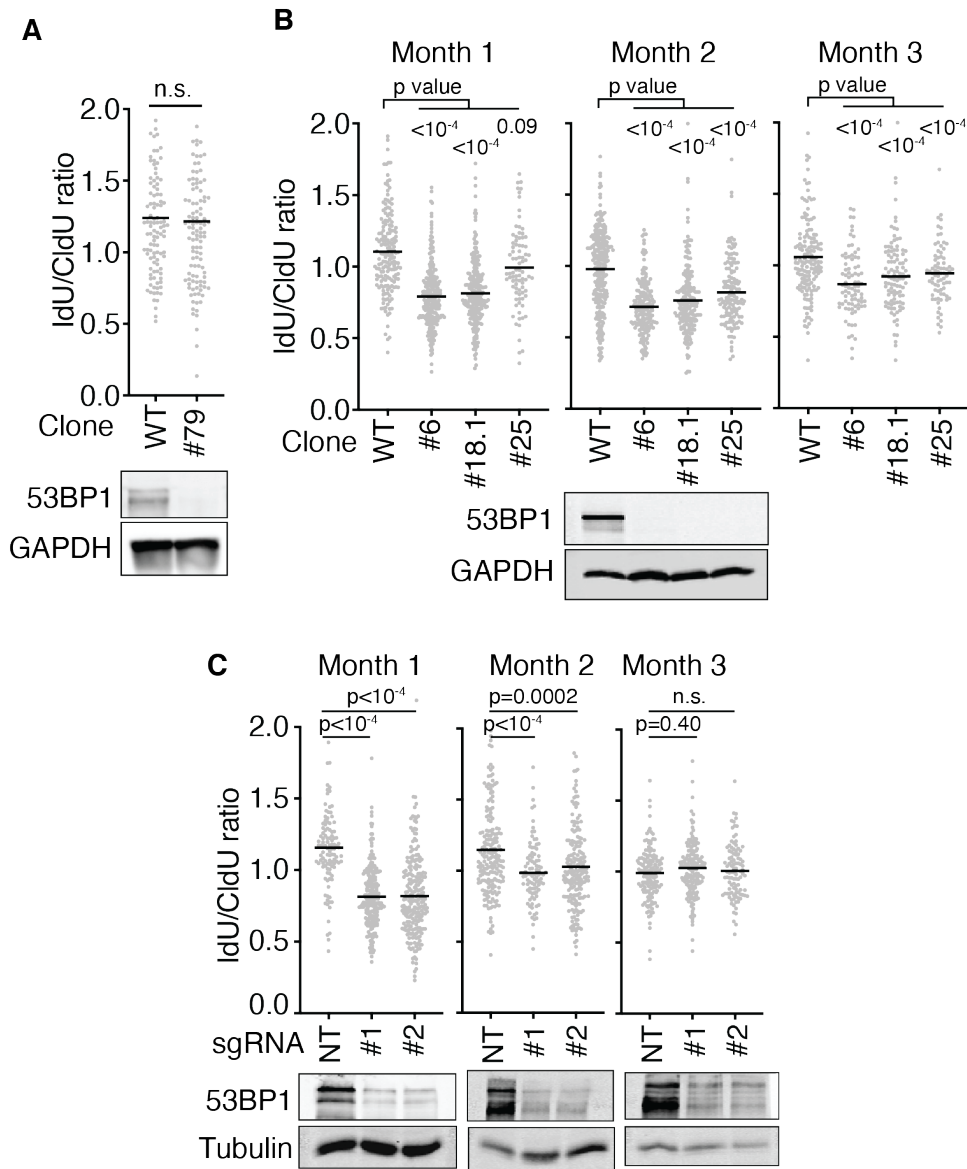
This PDF file includes:

Fig. S1. 53BP1 protects replication forks from nascent strand degradation in U2OS cells. Fork protection assays were completed as in Fig. 1. **(A)** 53BP1 knockout clone #79 obtained from Dr. Nima Mosammaparast was compared to parental U2OS cells. **(B)** U2OS 53BP1 knockout clones were isolated, verified by immunoblotting at first month, and examined for fork protection in the first, second, or third month after clonal cell lines were isolated. **(C)** Cas9 mRNA and sgRNAs targeting 53BP1 or non-targeting (NT) sgRNA were co-transfected into U2OS cells. Fork protection was analyzed in the first, second, and third months after transfection. A 53BP1 immunoblot was performed each month to measure 53BP1 expression levels.
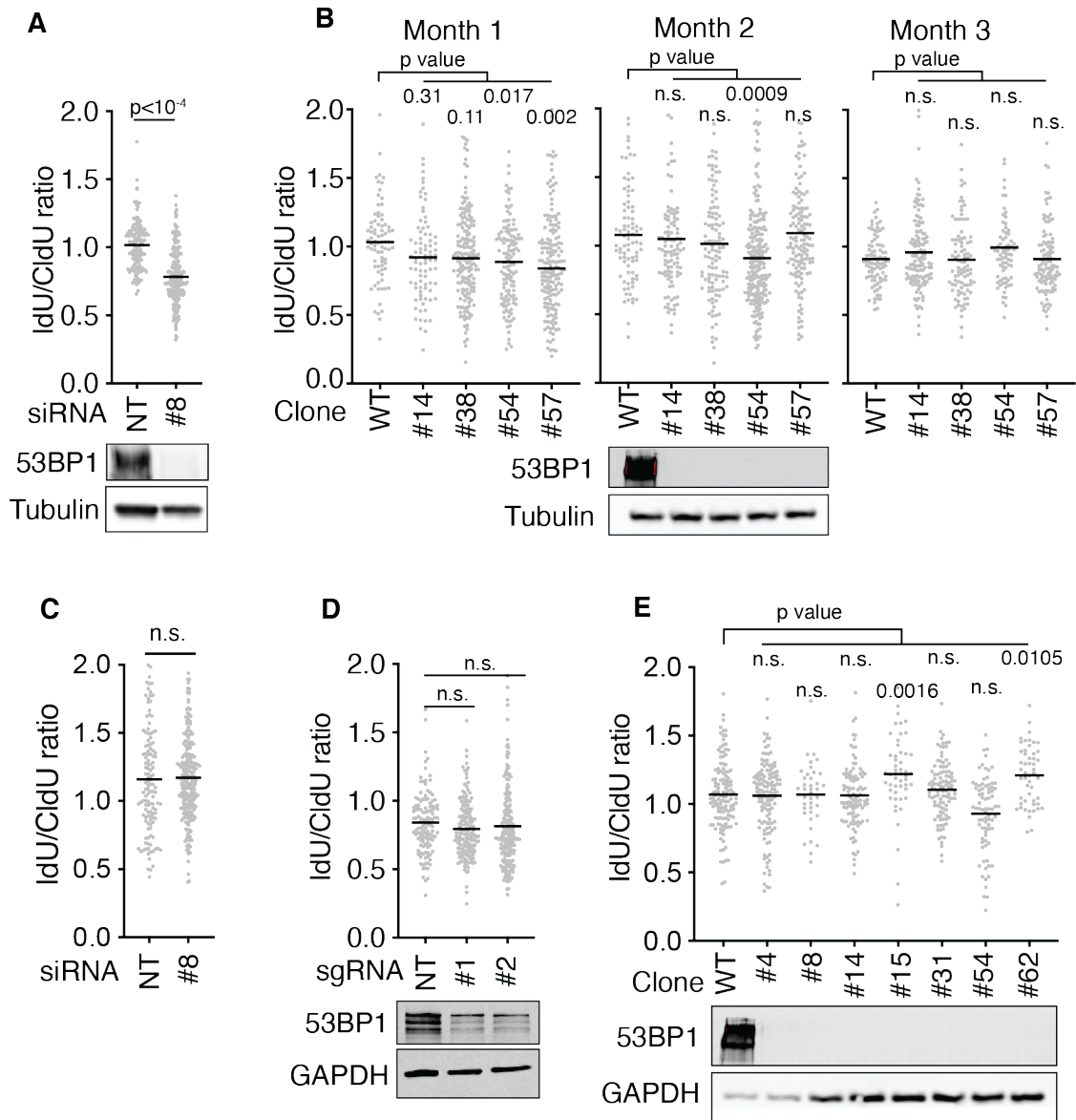
Fig. S2. 53BP1 mediated fork protection is cell type specific. (A) HEK293T cells transfected with non-targeting (NT) or 53BP1 siRNA were analyzed for fork protection and 53BP1 expression. **(B)** Four CRISPR-Cas9 generated 53BP1-deficient HEK293T cell lines were analyzed in the first, second, and third month after isolation for fork protection. Immunoblotting was performed in the first month. **(C)** hTERT-RPE1 cells transfected with the non-targeting and 53BP1 siRNA before analysis for fork protection and immunoblotting. **(D)** hTERT-RPE1 cells transfected with Cas9 expression vector and sgRNAs targeting 53BP1 or non-targeting sgRNA and analyzed for fork protection and 53BP1 expression. **(E)** hTERT-RPE1 53BP1 knockout cell clones were isolated and examined for fork protection and 53BP1 expression.
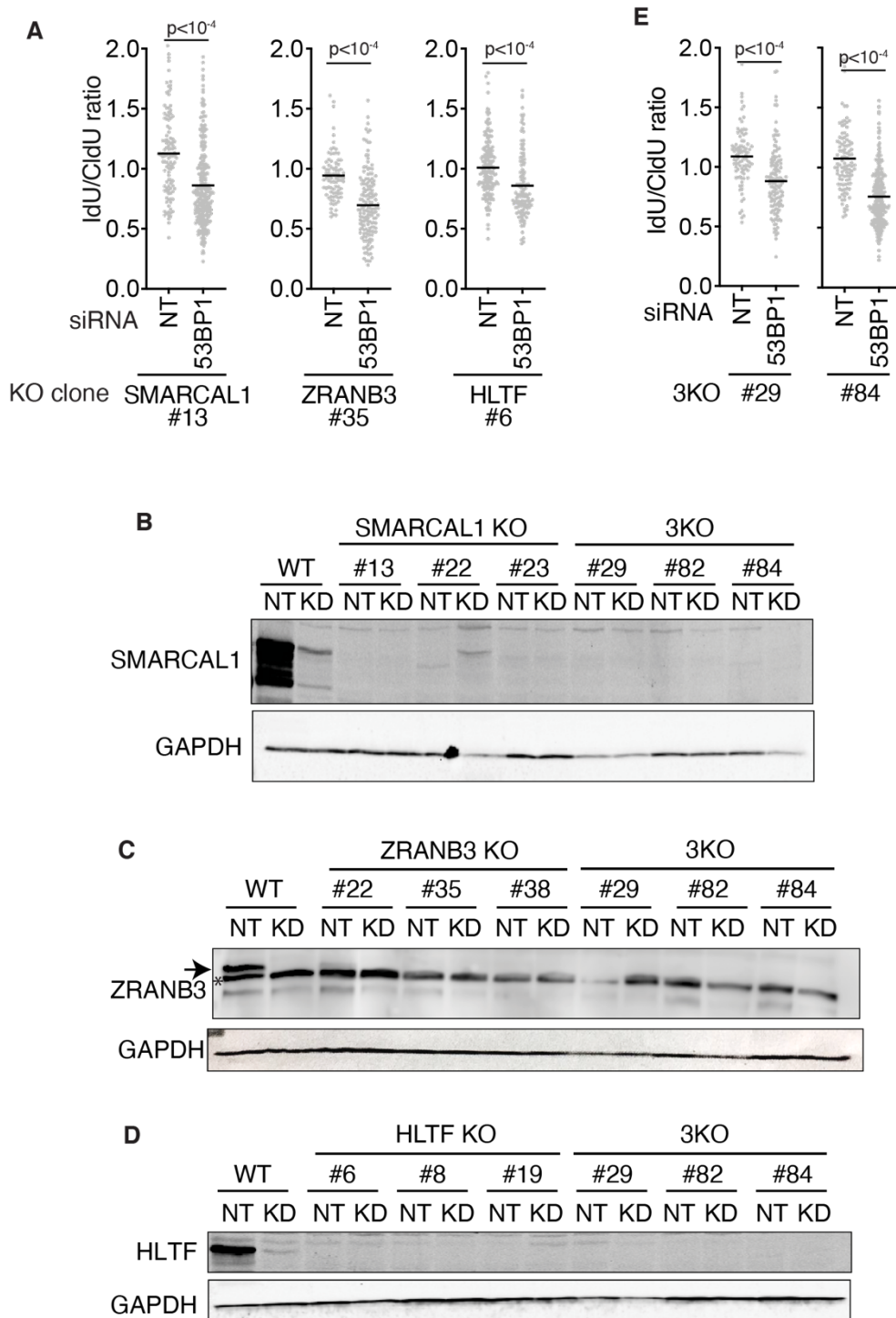
Fig. S3. SMARCAL1, ZRANB3, and HLTF are not required for nascent strand degradation in 53BP1-deficient U2OS cells. (A) SMARCAL1, ZRANB3 and HLTF single knockout U2OS clones were transfected with 53BP1 siRNA prior to performing the fork protection assay. **(B-D)** Immunoblots for SMARCAL1, ZRANB3 and HLTF single knockout and triple knockout (3KO) U2OS clones. Cells were transfected with non-targeting (NT) or SMARCAL1, ZRANB3, or HLTF siRNAs (KD) to confirm the knockouts did not retain protein expression and as a control for antibody specificity. The * in the ZRANB3 blot indicates a protein that cross-reacts with the ZRANB3 antibody. **(E)** 3KO U2OS clones were transfected with 53BP1 siRNA prior to performing the fork protection assay.
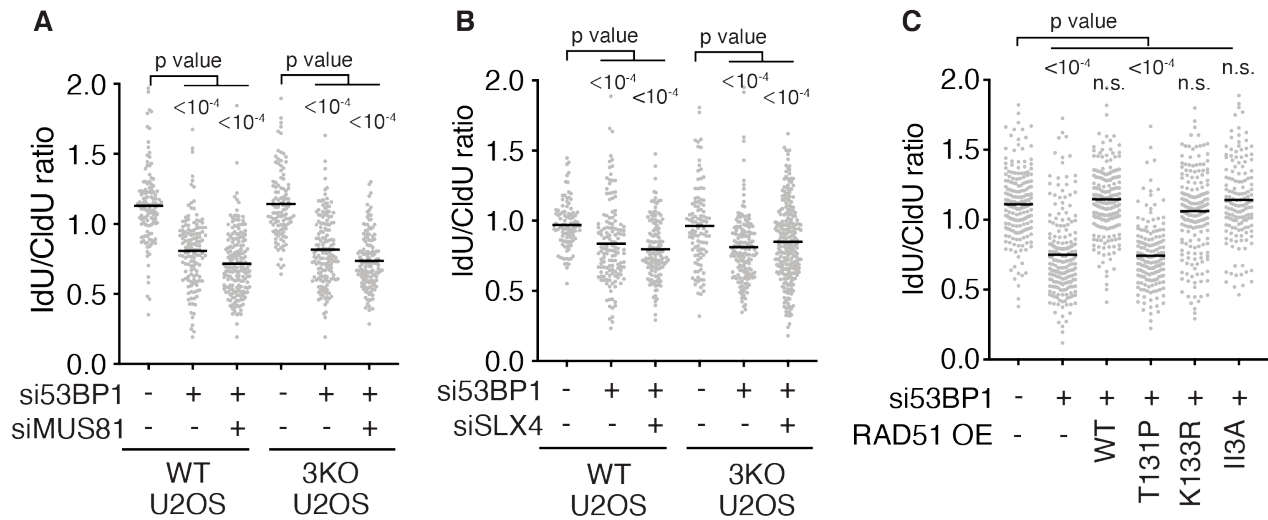
Fig. S4. SLX4 and MUS81 is not required for fork degradation in the absence of 53BP1, and RAD51 overexpression prevents degradation. (A-B) Wild-type (WT) or SMARCAL1, ZRANB3, and HLTF triple knockout (3KO) U2OS cells were transfected with the indicated siRNAs prior to analyzing fork protection. **(C)** U2OS cells transfected with 53BP1 or non-targeting siRNA and expression vectors encoding the indicated RAD51 wild type (WT) or mutant proteins were analyzed for fork protection.
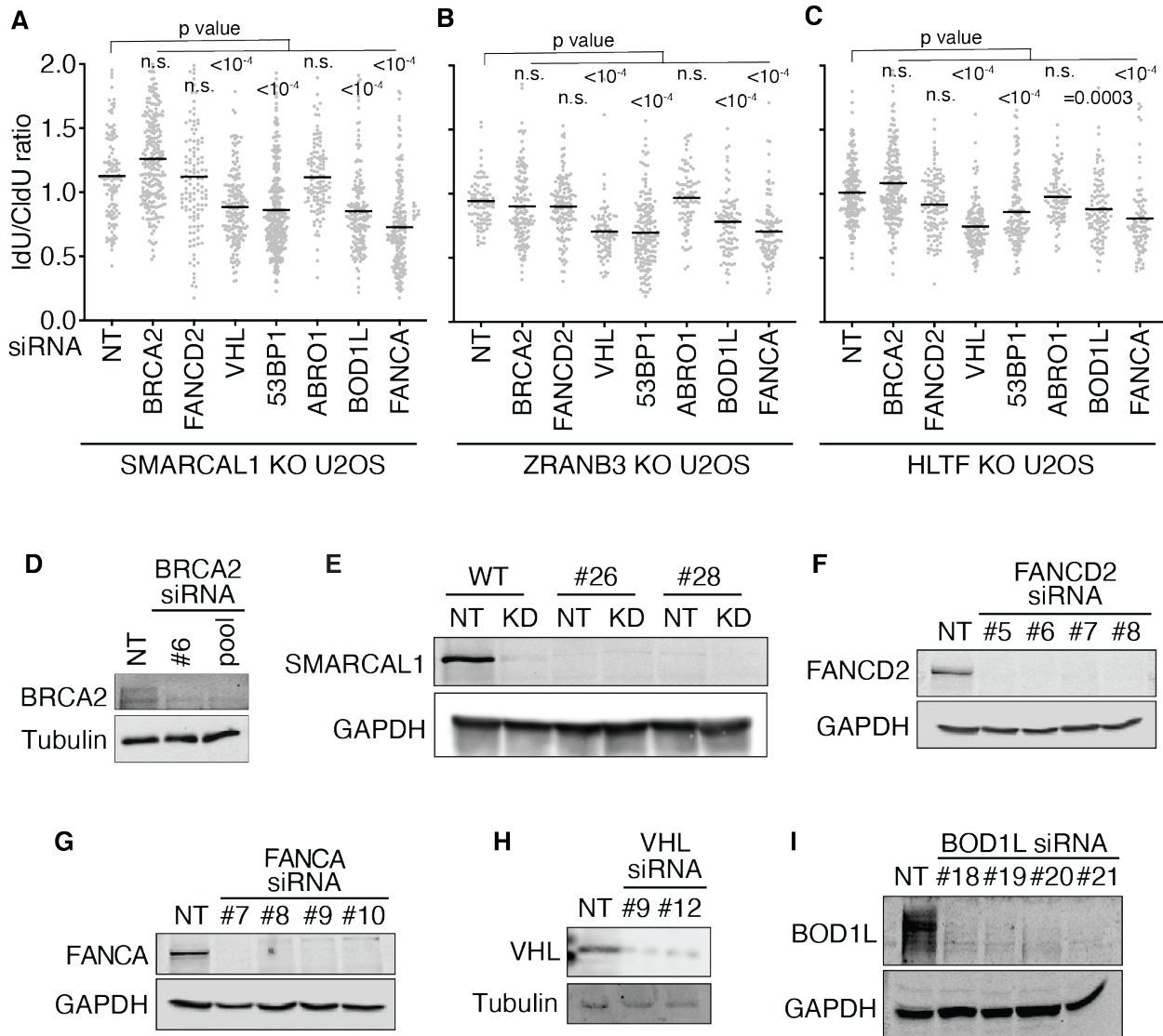
Fig. S5. SMARCAL1, ZRANB3, and HLTF are not required to generate the fork resection substrate in 53BP1, BOD1L, VHL, or FANCA-deficient cells. siRNAs to the indicated genes were transfected into **(A)** SMARCAL1, **(B)** ZRANB3, or **(C)** HLTF knockout U2OS cells prior to performing the fork protection assay. **(D-H)** Immunoblots were performed after transfection with the indicated siRNAs in U2OS cells to test knockdown efficiency. SMARCAL1 knockout clones were also examined for expression in panel **(E)**.
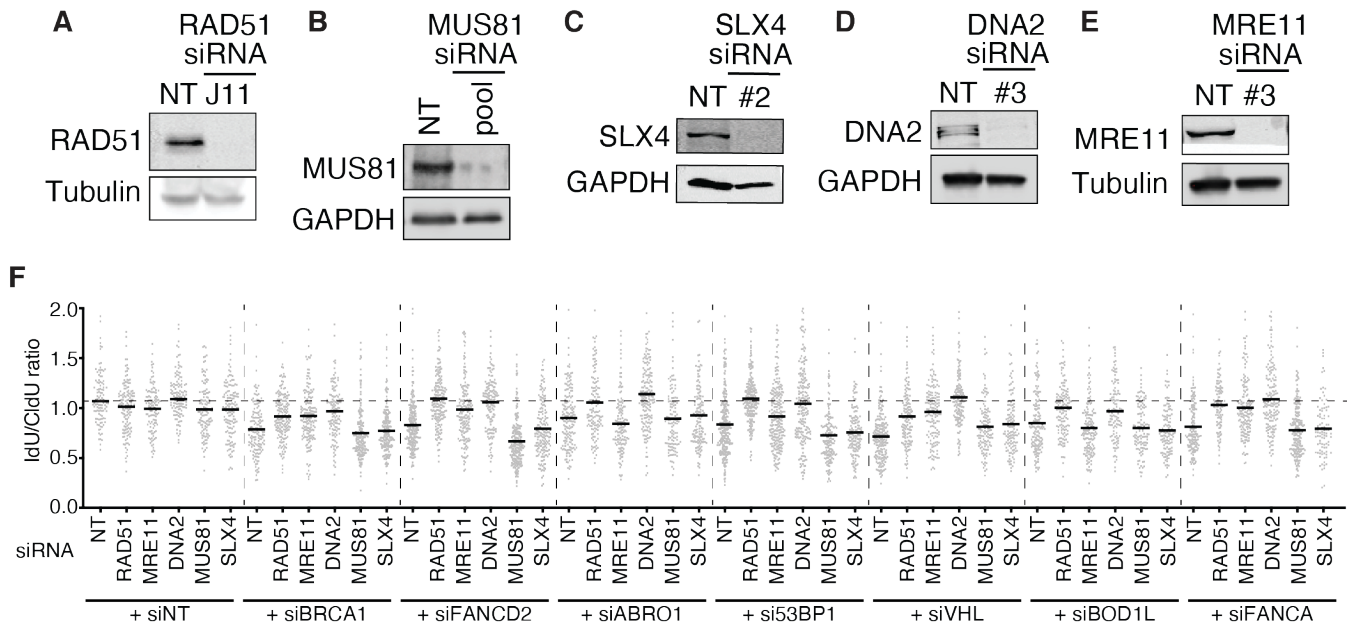
Fig. S6. Analysis of RAD51, MRE11, DNA2, SLX4, and MUS81 requirements for fork degradation in different genetic backgrounds. (A-E) Immunoblots were performed after transfection with the indicated siRNAs into U2OS cells to test knockdown efficiency. **(F)** The indicated siRNAs were transfected into U2OS cells prior to performing the fork protection assay. This is a representative experiment that was completed twice.
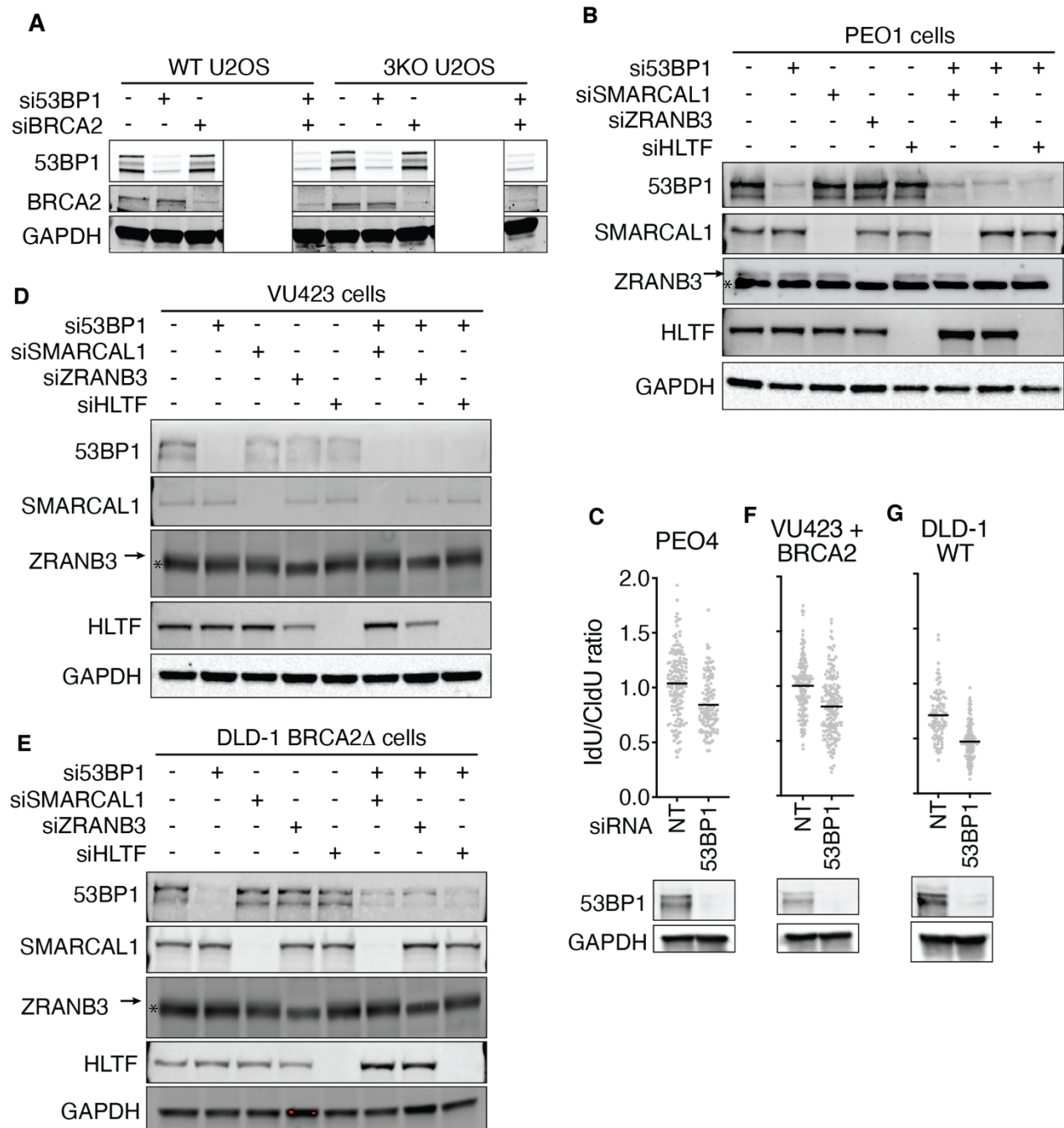
Fig. S7. Analysis of fork protection in response to 53BP1 inactivation in BRCA2-deficient and proficient cells. (A-B) and **(D-E)** Immunoblots examining the knockdown efficiencies. **(C, F, G)** Fork protection assays after 53BP1 knockdown in the indicated cell lines. All the figures represent at least two replicates.
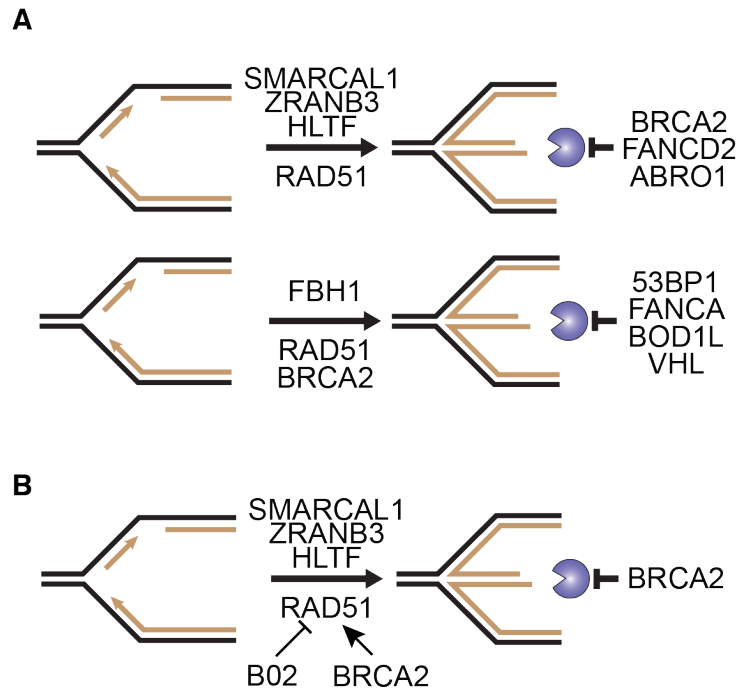
Fig. S8. Working model. (A) SMARCAL1, ZRANB3, and HLTF translocases work together with RAD51 to generate reversed forks that are protected by BRCA2, FANCD2 and ABRO1. FBH1 works with RAD51 and BRCA2 to generate a nascent strand degradation substrate that is protected by 53BP1, BOD1L, VHL, and the FA core complex. **(B)** BRCA2 is also required for generating a degradation substrate when RAD51 activity is partially inhibited by the B02 inhibitor.

Table S1. Description of cell lines generated with CRISPR-Cas9.

Cell Type	Gene	Clone	Mutation sequence	sgRNA, exon	Mutation type or reference (same in all alleles unless otherwise indicated)	Figures
U2OS	53BP1	#6	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	S1B
U2OS	53BP1	#18.1	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	S1B
U2OS	53BP1	#25	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	S1B
U2OS	53BP1	#1	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	1B
U2OS	53BP1	#2	TTAGAAGAATCGCACAGGGTCT	#2, exon 4	1bp insertion	1B
U2OS	53BP1	#3	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	1B-E
U2OS	53BP1	#7	TTAGAAGAATC(C/G)CACAGGGTCT	#2, exon 4	1bp insertion (one allele is C another allele is G)	1B
U2OS	53BP1	#8	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	1B-E
U2OS	53BP1	#12	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	1B-E
U2OS	53BP1	#15	TTAGAAGAATCGCACAGGGTCT	#2, exon 4	1bp insertion	1B
U2OS	53BP1	#18.2	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	1B
U2OS	53BP1	#22	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	1B-E
U2OS	53BP1	#79	N/A	N/A	(38)	S1A
293T	53BP1	#14	ACAGCTGGAGAAGGAACGAGG	#1, exon 3	1bp insertion	S2B
293T	53BP1	#38	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	S2B
293T	53BP1	#54	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	S2B
293T	53BP1	#57	AACAGCTGGAGAAGGAACGAGG	#1, exon 3	1bp insertion	S2B
RPE1	53BP1	#4	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	S2E
RPE1	53BP1	#8	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	S2E
RPE1	53BP1	#14	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	S2E
RPE1	53BP1	#15	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	S2E
RPE1	53BP1	#31	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	S2E
RPE1	53BP1	#54	TTAGAAGAATCCCACAGGGTCT	#2, exon 4	1bp insertion	S2E
RPE1	53BP1	#62	CCAGCTGGAGAAGAAACGAGG	#1, exon 3	1bp insertion	S2E
U2OS	FBH1	#2	CAGGAAGCTTGGTCCTCTGA	Exon 4	2bp deletion	4C
U2OS	FBH1 and HLTF	#2	AGGAAGCGGTTTGGTCCTCTGA	Exon 4	FBH1 1bp insertion	6E
			GTTGGGACTACGCTATTACAC	Exon 2	HLTF 1bp insertion	
U2OS	HLTF	#6	GGTTGGACTACGCTATTCACGGGA	Exon 2	1bp deletion	S3A 3C
U2OS	HLTF	#8	TGGTTGGACTACGCTATTCACGGG	Exon 2	1bp deletion	3C S5C
U2OS	HLTF	#19	TTGGACTACGCTATTCACGGGAGT	Exon 2	1bp deletion	3C
U2OS	SMARCAL1	#13	GCCCAGATTGCATCAACGCGTGGT	Exon 9	1bp deletion	S3A 3C S5A
U2OS	SMARCAL1	#26	TGCATCAACGTTCGTGGTGACT	Exon 9	1bp insertion	3C
U2OS	SMARCAL1	#22	GCCCAGATTGCATCAACGCGTGGT	Exon 9	1bp deletion	3C
U2OS	ZRANB3	#35	N/A	Exon 2	(14)	S3A S5B 3C
U2OS	ZRANB3	#38	N/A	Exon 2	(14)	3C
U2OS	SMARCAL1 ZRANB3 HLTF triple KO	#29	GACAAGACTAAGAGCAAAG	Exon 2	ZRANB3 1bp insertion	2B-C 2E S3E 4A-B 3C
			TTGCCTGACGACTAAGAGCA		ZRANB3 1bp deletion in another allele	
			CCACGACGTACGTGGGCCATCACCCAAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAAGGCGCTGGCAAGTGTAGCGTTGATG	Exon 9	SMARCAL1 176bp insertion in other alleles	
			CTCTGAGCCCAGATTGTCGTGGTGACTGG		SMA 8bp deletion	
			CTGAGCCCAGATTGCCGTCGTGGTGA		SMA 5bp deletion	
			GGACTACGCTATCACGGGAGT	Exon 2	HLTF 2bp deletion	
			GTGGTTGGACTATAGTAGATAATTA		HLTF 28bp deletion in other alleles	
U2OS	SMARCAL1 ZRANB3 HLTF 3KO	#84	GACAAGACTAAGAGCAAAG	Exon 2	ZRANB3 1bp insertion	3B-C S3E 5C 6A
			CAGATTGCATCAATCGTGGTGACTG	Exon 9	SMARCAL1 2bp deletion	
			TGGTTGGACTACGCTATTCACGGGAGT	Exon 2	HLTF 1bp deletion	

Table S2. Antibodies, chemicals, siRNAs, and sgRNAs utilized.

REAGENT	SOURCE	Catalog number
Antibodies		
Mouse monoclonal anti-BRCA2	Millipore	OP95
Mouse monoclonal anti-MUS81	Abcam	ab14387
Rabbit polyclonal anti-SMARCAL1	Custom antibody	
Rabbit polyclonal anti-ZRANB3	Bethyl	A303-033A
Mouse monoclonal anti-GAPDH	Millipore	MAB374
Mouse monoclonal anti-BrdU	BD Biosciences	347580
Rat monoclonal anti-BrdU	Abcam	ab6326
Goat anti-rat Alexa Fluor 594	Thermo Fisher	A-11007
Goat anti-mouse Alexa Fluor 488	Thermo Fisher	A-11029
Rabbit polyclonal anti-53BP1	Bethyl	A300-272A
Mouse monoclonal anti-tubulin	Santa Cruz biotechnology	sc-5286
Rabbit polyclonal anti-HLTF	Abcam	ab183042
Rabbit polyclonal anti-DNA2	Abcam	ab96488
Mouse monoclonal anti-MRE11	GeneTex	GTX70212
Mouse monoclonal anti-BRCA1	Calbiochem	OP-92
Mouse monoclonal anti-FANCD2	Santa Cruz biotechnology	sc-20022
Rat monoclonal anti-HA	Roche	11867423001
Chicken polyclonal GFP antibody	Abcam	ab13970
FANCA	Bethyl	A301-980A
BOD1L	Gift from Grant Stewart	
VHL	Cell Signaling technology	#68547
SLX4	Bethyl	A302-269A-1
Rabbit polyclonal anti-RAD51	Abcam	ab133534
Chemicals		
Hydroxyurea	Millipore Sigma	H8627
CldU	Millipore Sigma	C6891
IdU	Millipore Sigma	I7125
Mirin (used at 50μM)	Millipore Sigma	M9948
Olaparib (AZD2281)	AstraZeneca	
B02 inhibitor	Calbiochem	1290541-46-6
Combing assay kit	Genomic Vision	

siRNA	Sequence	Catalog Number (Dharmacon or Ambion)	Figures
TP53BP1#4	GAUAUCAGCUUAGACAAUU	D-003548-04-0002	3A 2A-E 3A-C S3A S3E S4A-B 4A-C S5A-C 5B-C S6F 6A-E S7C S7F-G
TP53BP1#6	GAGAGCAGAUGAUCCUUUA	J-003548-06-0002	1A
TP53BP1#7	GGACAAGUCUCUCAGCUAU	J-003548-07-0002	1A
TP53BP1#8	GAUAUCAGCUUAGACAAUU	J-003548-08-0002	1A 2A-E 3A-C S2A S2C S3A S3E S4A-C 4A-E 5B-C 6A-E S7C S7F-G
TP53BP1#9	GGACAGAACCCGCAGAUUU	J-003548-09-0002	1A
SMARCAL1#6	GCUUUGACCUUCUUAGCAA	J-013058-06-0002	2A-B S3B S5I 6B-D
ZRANB3#2	GAUUCGAUCUAAUAACAGU	s38488	2A-B S3C 6B-D
SLX4#2	GAAGUGGAAUUGUCUAGCA	s39054	S4B 3C S6C S6F

MRE11#3	GCUAAUGACUCUGAUGAUA	D-009271-03-0010	2E 3C S6E-F
BOD1L#18	AGUAGAAGGUUGUGCGAAA	J-017033-18-0002	S5H
BOD1L#19	GAUAAGAGCAGGAUCUAUA	J-017033-19-0002	3A-C 5B-C S5A-C S5H S6F
BOD1L#20	GAGUAGAAGACUUGAGCGA	J-017033-20-0003	S5H
BOD1L#21	UGAUAAAACCGAACGAAAA	J-017033-21-0003	S5H
FANCD2#5	UGGAUAAGUUGUCGUCUAU	J-016376-05-0002	3A-C 5A S5A-C S5E S6F
FANCD2#6	CAACAUACCUCGACUCAUU	J-016376-06-0002	3A-C 5A S5A-C S5E S6F
FANCD2#7	GGAUUUACCUGUGAUAAUA	J-016376-07-0002	3A-C 5A S5A-C S5E S6F
FANCD2#8	GGAGAUUGAUGGUCUACUA	J-016376-08-0002	3A-C 5A S5A-C S5E S6F
FANCA#7	GGGCCAUGCUUUCUGAUUU	J-019283-07	3A-C 5B-C S5A-C S5F S6F
FANCA#8	GCAGGUCACGGUUGAUGUA	J-019283-08	3A-C 5B-C S5A-C S5F S6F
FANCA#9	GUAGAAGGUCCACUGUGUA	J-019283-09	3A-C 5B-C S5A-C S5F S6F
FANCA#10	GUUAGAGUUUGCUCAGUAU	J-019283-10	3A-C 5B-C S5A-C S5F S6F
FBH1#5	CCUCAACGCUGGUCAAGUA	J-017404-05-0002	4A 5A-C
FBH1#6	AGGGAAGGGUGGAUUCAUA	J-017404-06-0002	4A 5A-C
FBH1#7	GUGCCUAUUUGGUGUAAGA	J-017404-07-0002	4A 5A-C
FBH1#8	AAACAAAACCUCGUCAUUA	J-017404-08-0002	4A 5A-C
FBH1Non-coding#1	GUGCCUAUUUGGUGUAAGA	Custom	4B
FBH1Non-coding#2	GGGAUGUUCUUUUGAUAAA	Custom	4B
DNA2#3	ACAGUUGCCUGCAUUCUAA	Custom	3E 3C S6D S6F
RAD51 J11		J-003530-11-0002	2C 3C S6D S6F
BRCA2 pool	Dharmacon SMARTpool	L-003462-00-0005	3A-C 5A 5C 6A 6E S5A-D S6F
HLTF pool	Dharmacon SMARTpool	L-006448-00-0005	2A-B 6B-D S3D
MUS81 pool	Dharmacon SMARTpool	L-016143-01-0005	S4A 3C S6B S6F
VHL pool (pool of #9 and #12)	Dharmacon SMARTpool	L-003936-00-0005	3A-C 5B-C S5A-C S5G S6F
ABRO1 pool	Dharmacon SMARTpool	L-016146-01-0005	3A-C 5A S5A-C S6F
FANCC pool	Dharmacon SMARTpool	L-011033-00-0005	5D
FANCG pool	Dharmacon SMARTpool	L-011899-00-0005	5D

sgRNA target	sgRNA Sequence	Figures
SMARCAL1	GCCCAGATTGCATCAACGTCG	All SMARCAL1 knockouts
HLTF	CACCGGTTGGACTACGCTATTACAC	All HLTF knockouts and HLTF/FBH1 double knockout
ZRANB3	AGCTTTGCTCTTAGTCTGTC	All ZRANB3 knockouts
TP53BP1#1	AAACAGCTGGAGAAGAACG	S1C S2B S2D-E
TP53BP1#2	TGTGGATTCTTCTAACTTGG	S1B-C S2B 1B S2D-E
FBH1	AGCGGTCTTGGTCCTCTG	All FBH1 knockouts and HLTF/FBH1 double knockout