Figure S1

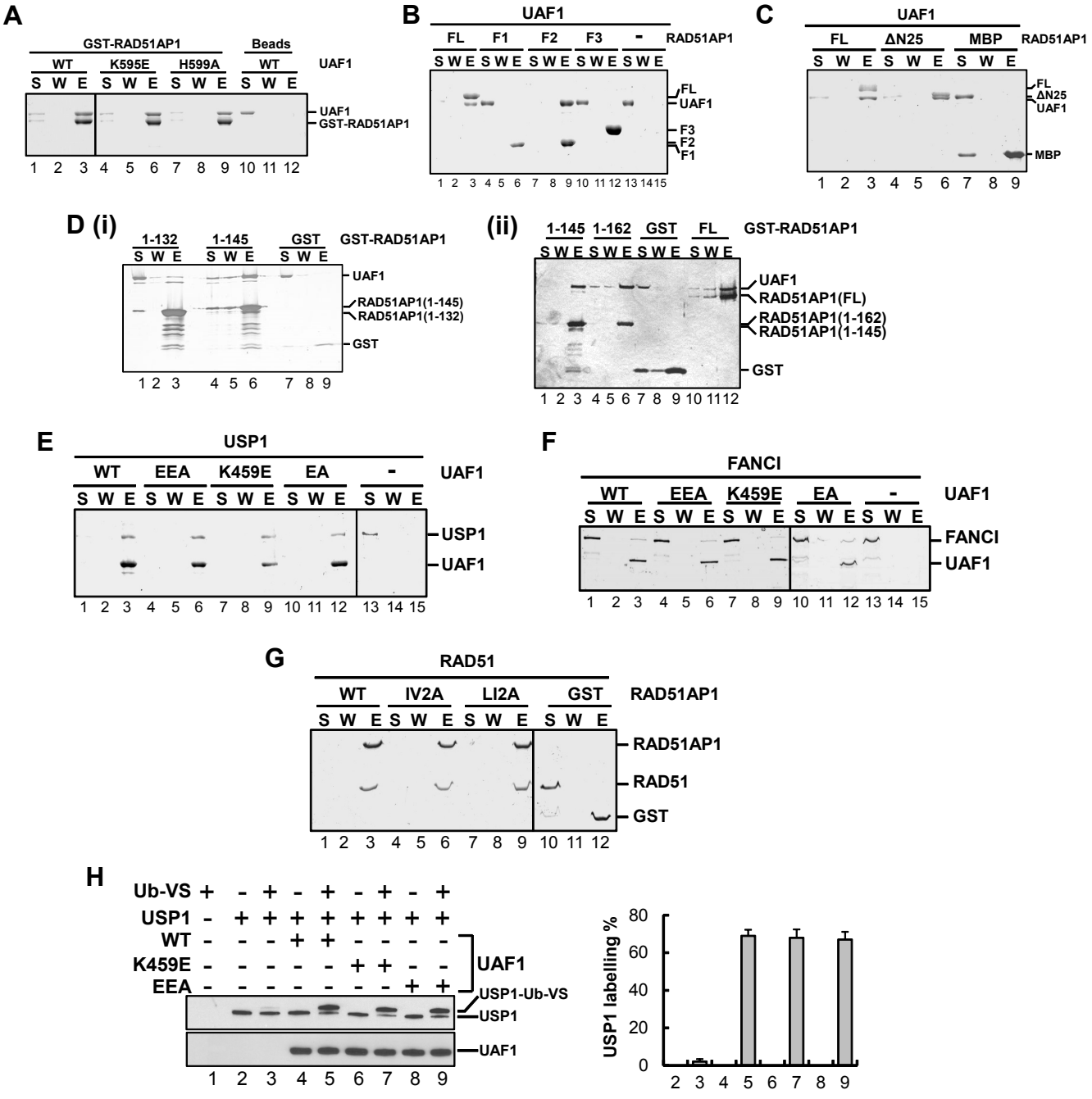
A
GST-RAD51AP1
Beads
WT
K595E
H599A
WT
UAF1
S W E S W E S W E S W E
UAF1
GST-RAD51AP1
1 2 3 4 5 6 7 8 9 10 11 12
B
UAF1
FL
F1
F2
F3
-
RAD51AP1
S W E S W E S W E S W E S W E
FL
UAF1
F3
F2
F1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
C
UAF1
FL
ΔN25
MBP
RAD51AP1
S W E S W E S W E
FL
ΔN25
UAF1
MBP
1 2 3 4 5 6 7 8 9
D (i)
1-132
1-145
GST
GST-RAD51AP1
S W E S W E S W E
UAF1
RAD51AP1(1-145)
RAD51AP1(1-132)
GST
1 2 3 4 5 6 7 8 9
(ii)
1-145
1-162
GST
FL
GST-RAD51AP1
S W E S W E S W E S W E
UAF1
RAD51AP1(FL)
RAD51AP1(1-162)
RAD51AP1(1-145)
GST
1 2 3 4 5 6 7 8 9 10 11 12
E
USP1
WT
EEA
K459E
EA
-
UAF1
S W E S W E S W E S W E S W E
USP1
UAF1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
F
FANCI
WT
EEA
K459E
EA
-
UAF1
S W E S W E S W E S W E S W E
FANCI
UAF1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
G
RAD51
WT
IV2A
LI2A
GST
RAD51AP1
S W E S W E S W E S W E
RAD51AP1
RAD51
GST
1 2 3 4 5 6 7 8 9 10 11 12
H
Ub-VS + - + - + - + - +
USP1 - + + + + + + + +
WT - - - + + - - - -
K459E - - - - - + + - -
EEA - - - - - - - + +
UAF1
USP1-Ub-VS
USP1
UAF1
1 2 3 4 5 6 7 8 9
USP1 labelling %
80
60
40
20
0
2 3 4 5 6 7 8 9

Figure S1. Protein interactions involving UAF1, RAD51AP1, and their mutants, related to Figure 1.

(A) GST-tagged RAD51AP1 was tested for interactions with Strep II-tagged UAF1 and the K595E or H599A mutant. Glutathione resin was used in the pulldown.
(B) Strep II-tagged UAF1 was tested for interaction with MBP-tagged full-length RAD51AP1 or the F1, F2 or F3 fragment. Amylose resin was used in the pulldown.
(C) MBP-tagged full-length and ΔN25 truncation of RAD51AP1 were tested for Strep II-tagged UAF1 interaction. Amylose resin was used in the pulldown.
(D) (i) GST-tagged RAD51AP1 fragments that harbor residues 1-132 or 1-145 were tested for Strep II-tagged UAF1 interaction. (ii) GST-tagged RAD51AP1 and the protein fragments that harbor residues 1-145 or 1-162 were tested for Strep II-tagged UAF1 interaction. Glutathione resin was used in the pulldown.
(E) Strep II-tagged UAF1 and mutants (K459E, EA and EEA) were tested for His-tagged USP1 interaction. Strep-Tactin resin was used in the pulldown.
(F) Strep II-tagged UAF1 and mutants were tested for FANCI interaction. Strep-Tactin resin was used in the pulldown.
(G) GST-tagged RAD51AP1 mutants (IV2A, LI2A) were tested for RAD51 interaction. Glutathione resin was used in the pulldown.
(H) The DUB activity of purified USP1 and UAF1-USP1 complex were monitored using the Ub-VS probe. The reactions were subject to Western blotting for UAF1 and USP1. The percentage of USP1 modification by Ub-VS from three independent experiments is shown as mean ± S.D.

Figure S2

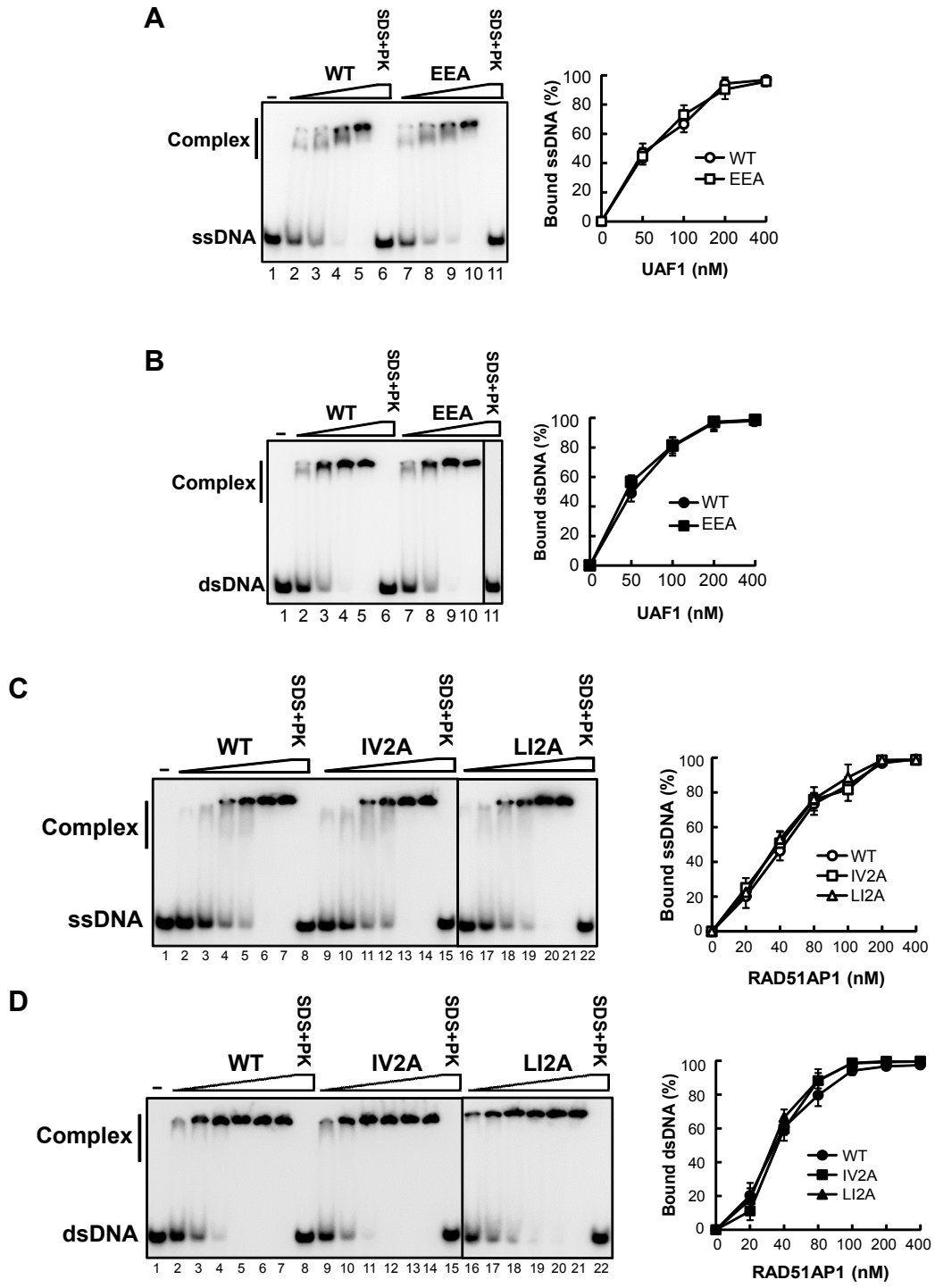
A
SDS+PK
SDS+PK
WT
EEA
–
Complex
ssDNA
1 2 3 4 5 6 7 8 9 10 11
Bound ssDNA (%)
100
80
60
40
20
0
0 50 100 200 400
UAF1 (nM)
WT
EEA
B
SDS+PK
SDS+PK
WT
EEA
–
Complex
dsDNA
1 2 3 4 5 6 7 8 9 10 11
Bound dsDNA (%)
100
80
60
40
20
0
0 50 100 200 400
UAF1 (nM)
WT
EEA
C
SDS+PK
SDS+PK
SDS+PK
WT
IV2A
LI2A
–
Complex
ssDNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Bound ssDNA (%)
100
80
60
40
20
0
0 20 40 80 100 200 400
RAD51AP1 (nM)
WT
IV2A
LI2A
D
SDS+PK
SDS+PK
SDS+PK
WT
IV2A
LI2A
–
Complex
dsDNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Bound dsDNA (%)
100
80
60
40
20
0
0 20 40 80 100 200 400
RAD51AP1 (nM)
WT
IV2A
LI2A

Figure S2. DNA binding by mutants of UAF1 and RAD51AP1 mutants, related to Figure 2.

(A, B) Strep II-tagged UAF1 and the EEA mutant (50-400 nM) were examined for ssDNA and dsDNA binding.
(C, D) RAD51AP1 and the IV2A and LI2A mutants (20-400 nM) were examined for DNA binding. The data plotted in all the panels were the mean ± S.D. from three independent experiments.

Figure S3

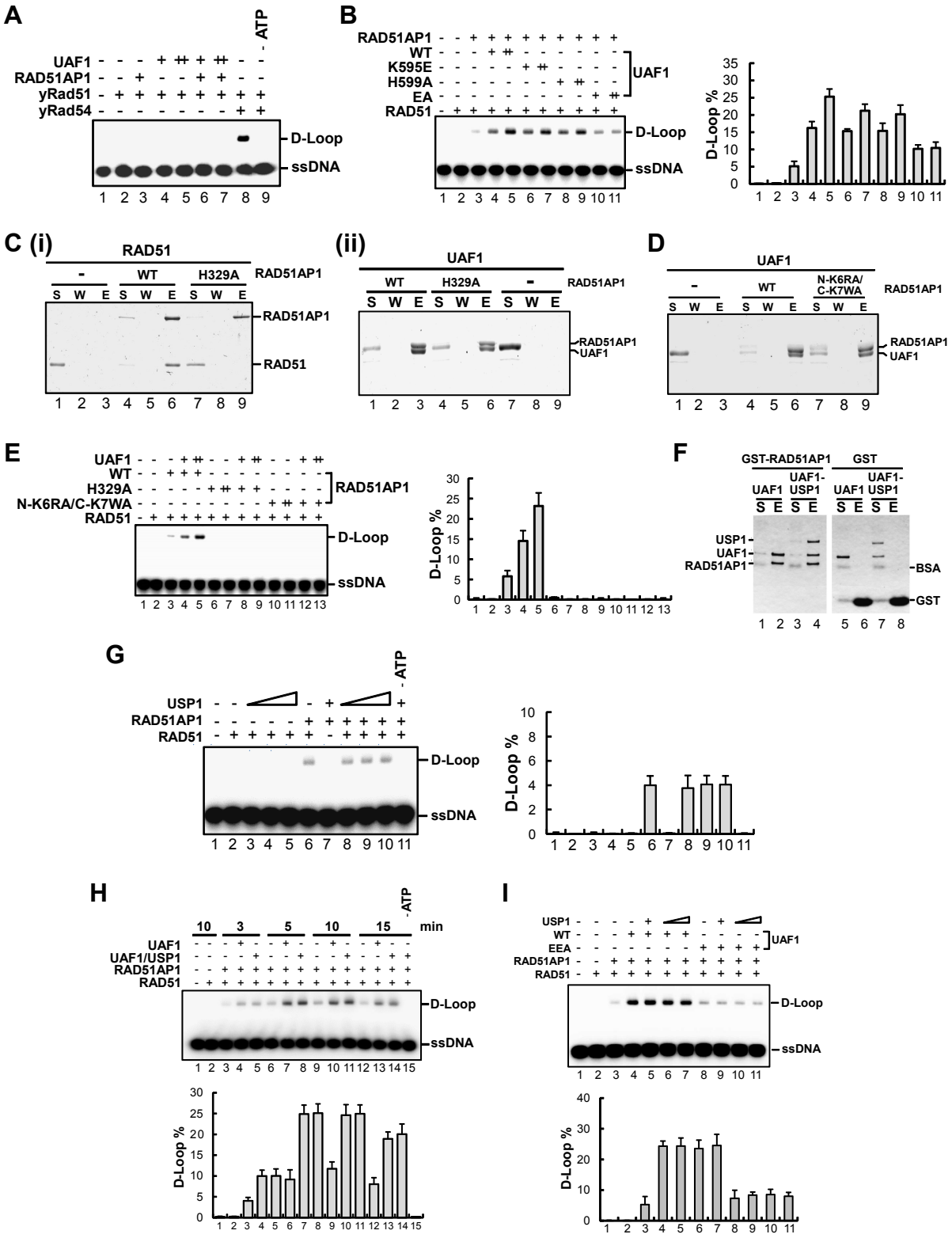
A
- ATP
UAF1
RAD51AP1
yRad51
yRad54
D-Loop
ssDNA
1 2 3 4 5 6 7 8 9
B
RAD51AP1
WT
K595E
H599A
EA
RAD51
UAF1
D-Loop
ssDNA
D-Loop %
1 2 3 4 5 6 7 8 9 10 11
C (i)
RAD51
-
WT
H329A
RAD51AP1
S W E S W E S W E
RAD51AP1
RAD51
1 2 3 4 5 6 7 8 9
(ii)
UAF1
WT
H329A
-
RAD51AP1
S W E S W E S W E
RAD51AP1
UAF1
1 2 3 4 5 6 7 8 9
D
UAF1
-
WT
N-K6RA/
C-K7WA
RAD51AP1
S W E S W E S W E
RAD51AP1
UAF1
1 2 3 4 5 6 7 8 9
E
UAF1
WT
H329A
N-K6RA/C-K7WA
RAD51AP1
RAD51
D-Loop
ssDNA
D-Loop %
1 2 3 4 5 6 7 8 9 10 11 12 13
F
GST-RAD51AP1
GST
UAF1
UAF1-
USP1
S E S E
S E S E
USP1
UAF1
RAD51AP1
BSA
GST
1 2 3 4 5 6 7 8
G
- ATP
USP1
RAD51AP1
RAD51
D-Loop
ssDNA
D-Loop %
1 2 3 4 5 6 7 8 9 10 11
H
- ATP
10 3 5 10 15 min
UAF1
UAF1/USP1
RAD51AP1
RAD51
D-Loop
ssDNA
D-Loop %
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
I
USP1
WT
EEA
RAD51AP1
RAD51
UAF1
D-Loop
ssDNA
D-Loop %
1 2 3 4 5 6 7 8 9 10 11

Figure S3. Activity of UAF1, RAD51AP1 and their mutants in the D-loop reaction, related to Figure 3.

(A) RAD51AP1-UAF1 has no effect on D-loop formation mediated by yeast Rad51.
(B) RAD51AP1 and the UAF1 K595E, H599A and EA mutants were examined for their activity in the D-loop reaction. The mean values of data ± S.D. from three independent experiments were plotted.
(C) (i) MBP-tagged RAD51AP1 (WT) and the H329A mutant were tested for RAD51 interaction. (ii) MBP-tagged RAD51AP1 (WT) and the H329A mutant were tested for Strep II-tagged UAF1 binding. Amylose resin was used in the pulldown.
(D) MBP-tagged RAD51AP1 (WT) and DNA-binding defective N-K6RA/C-K7WA mutant were tested for Strep II-tagged UAF1 interaction. Amylose resin was used in the pulldown.
(E) UAF1 was tested with the RAD51AP1 mutants in the D-loop reaction. The percentage of D-loop was quantified and plotted. The error bars represent mean values ± S.D. of data from three independent experiments.
(F) Pulldown of GST-tagged RAD51AP1 by glutathione resin revealed interactions with UAF1 and UAF1-USP1 complex.
(G) RAD51AP1, His-tagged USP1 and the combination of these proteins were tested in the D-loop reaction.
(H) RAD51AP1, Strep II-tagged UAF1, the UAF1-USP1 complex, and the indicated combinations of them were tested for the ability to enhance D-loop formation as a function of time.
(I) RAD51, RAD51AP1, Strep II-tagged UAF1, the UAF1 EEA mutant, His-tagged USP1 and the indicated combinations of these proteins were tested in the D-loop reaction.
The percentages of D-loop were quantified and plotted in (G), (H), and (I). The error bars represent mean values ± S.D. of data from three independent experiments.

Figure S4

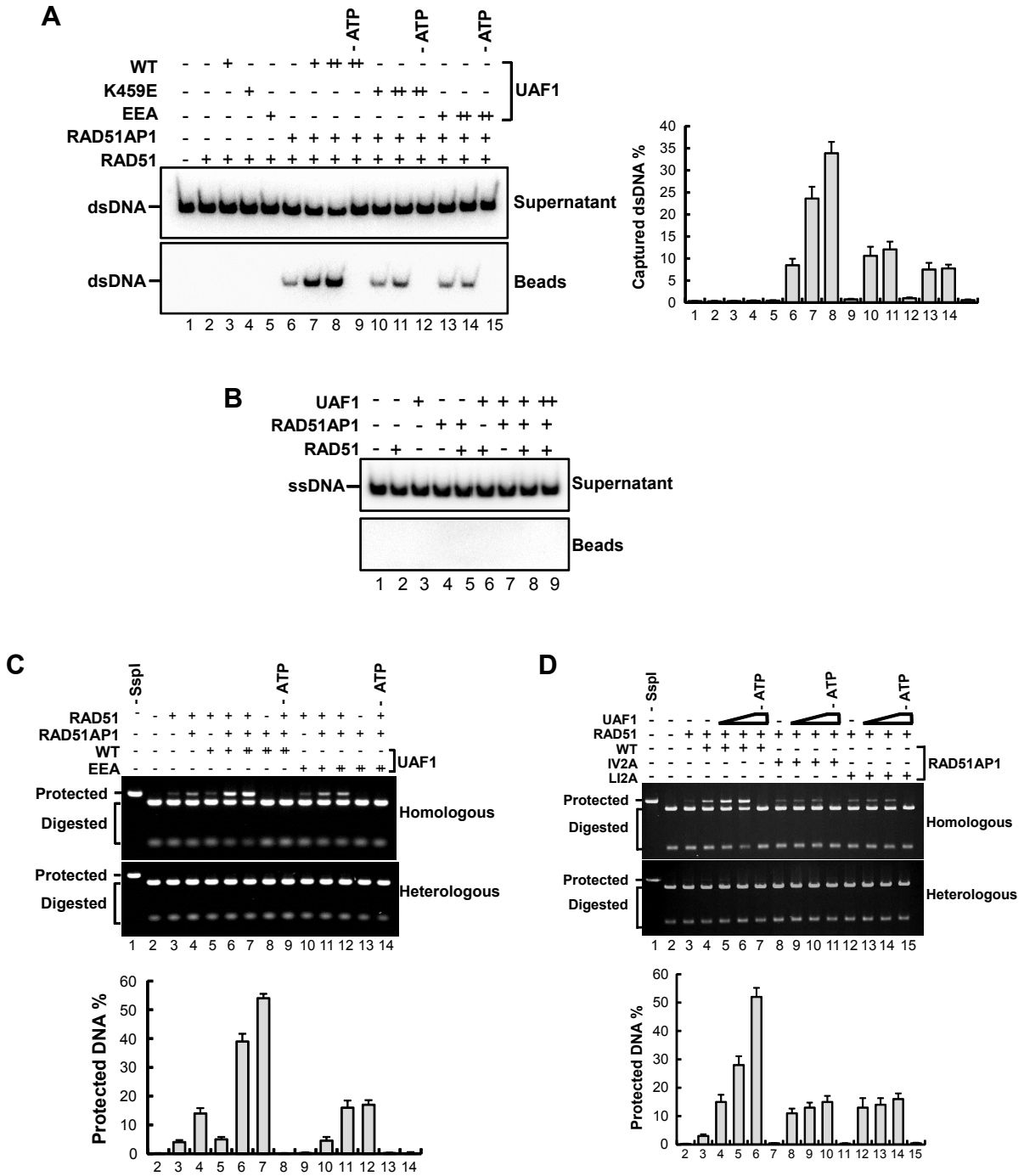
A
- ATP
- ATP
- ATP
WT
K459E
EEA
UAF1
RAD51AP1
RAD51
dsDNA
Supernatant
dsDNA
Beads
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Captured dsDNA %
40
35
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
UAF1
RAD51AP1
RAD51
ssDNA
Supernatant
Beads
1 2 3 4 5 6 7 8 9
C
- SspI
- ATP
- ATP
RAD51
RAD51AP1
WT
EEA
UAF1
Protected
Digested
Homologous
Protected
Digested
Heterologous
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Protected DNA %
60
50
40
30
20
10
0
2 3 4 5 6 7 8 9 10 11 12 13 14
D
- SspI
- ATP
- ATP
- ATP
UAF1
RAD51
WT
IV2A
LI2A
RAD51AP1
Protected
Digested
Homologous
Protected
Digested
Heterologous
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Protected DNA %
60
50
40
30
20
10
0
2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure S4. Duplex DNA capture and synaptic complex assembly by UAF1 and RAD51AP1 mutants, related to Figure 4.

(A) K459E and EEA mutants of UAF1 failed to enhance duplex capture mediated by RAD51-RAD51AP1. Data analysis was as in Figure 4B.
(B) RAD51AP1-UAF1 and RAD51 presynaptic filament are unable to capture ssDNA. Radiolabeled ssDNA (P1 in Table S2) was used.
(C) The UAF1-EEA mutant failed to enhance synaptic complex assembly by RAD51-RAD51AP1. Data analysis was as in Figure 4D.
(D) Enhancement by UAF1 of synaptic complex assembly was not seen with the RAD51AP1-IV2A or LI2A mutant. Data analysis was as in Figure 4D.

Figure S5

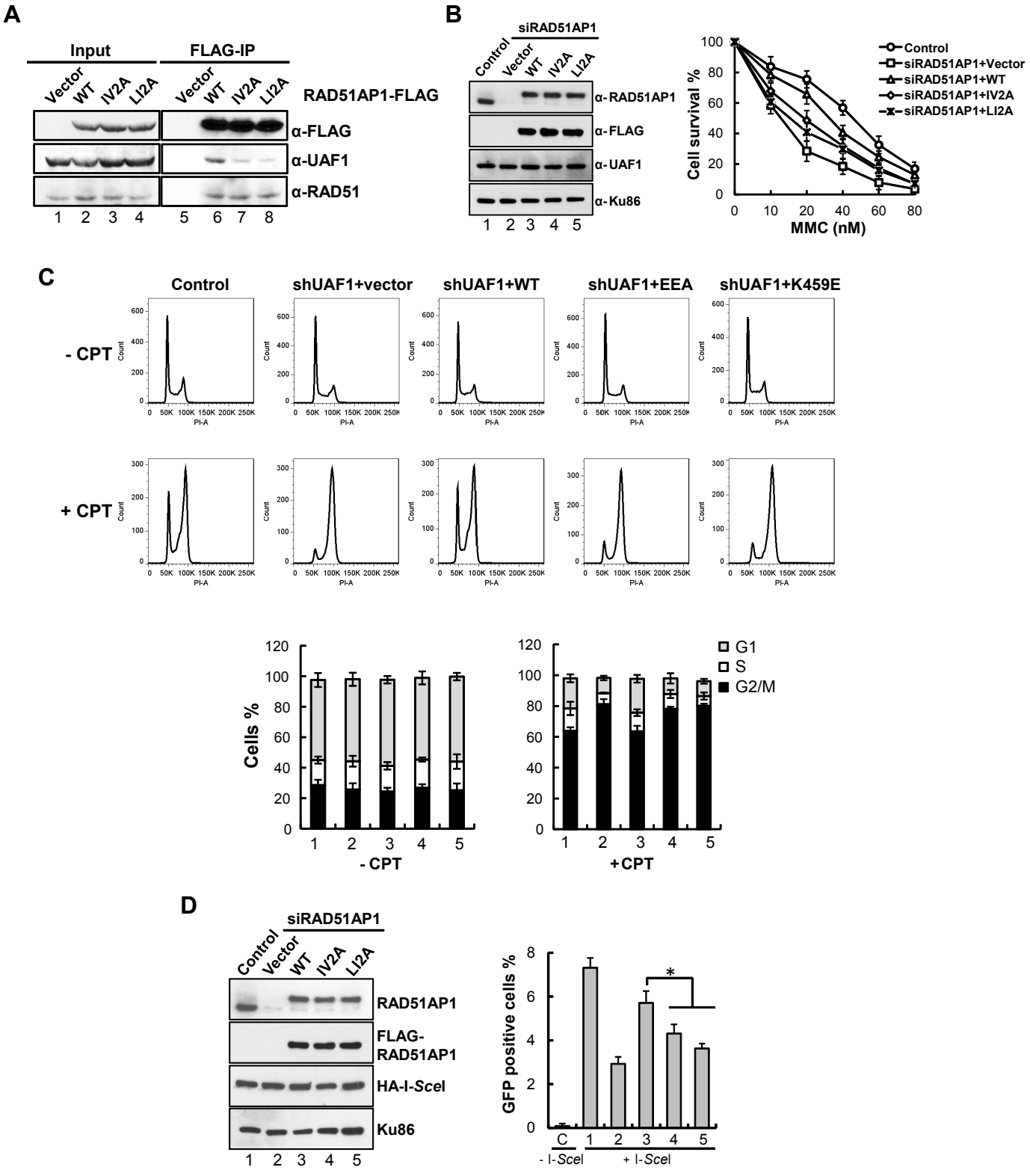
A
Input
FLAG-IP
Vector
WT
IV2A
LI2A
RAD51AP1-FLAG
α-FLAG
α-UAF1
α-RAD51
1 2 3 4 5 6 7 8
B
siRAD51AP1
Control
Vector
WT
IV2A
LI2A
α-RAD51AP1
α-FLAG
α-UAF1
α-Ku86
1 2 3 4 5
Cell survival %
MMC (nM)
Control
siRAD51AP1+Vector
siRAD51AP1+WT
siRAD51AP1+IV2A
siRAD51AP1+LI2A
C
Control
shUAF1+vector
shUAF1+WT
shUAF1+EEA
shUAF1+K459E
- CPT
+ CPT
Count
PI-A
Cells %
G1
S
G2/M
- CPT
+CPT
D
siRAD51AP1
Control
Vector
WT
IV2A
LI2A
RAD51AP1
FLAG-RAD51AP1
HA-I-SceI
Ku86
1 2 3 4 5
GFP positive cells %
C 1 2 3 4 5
- I-SceI
+ I-SceI
*

Figure S5

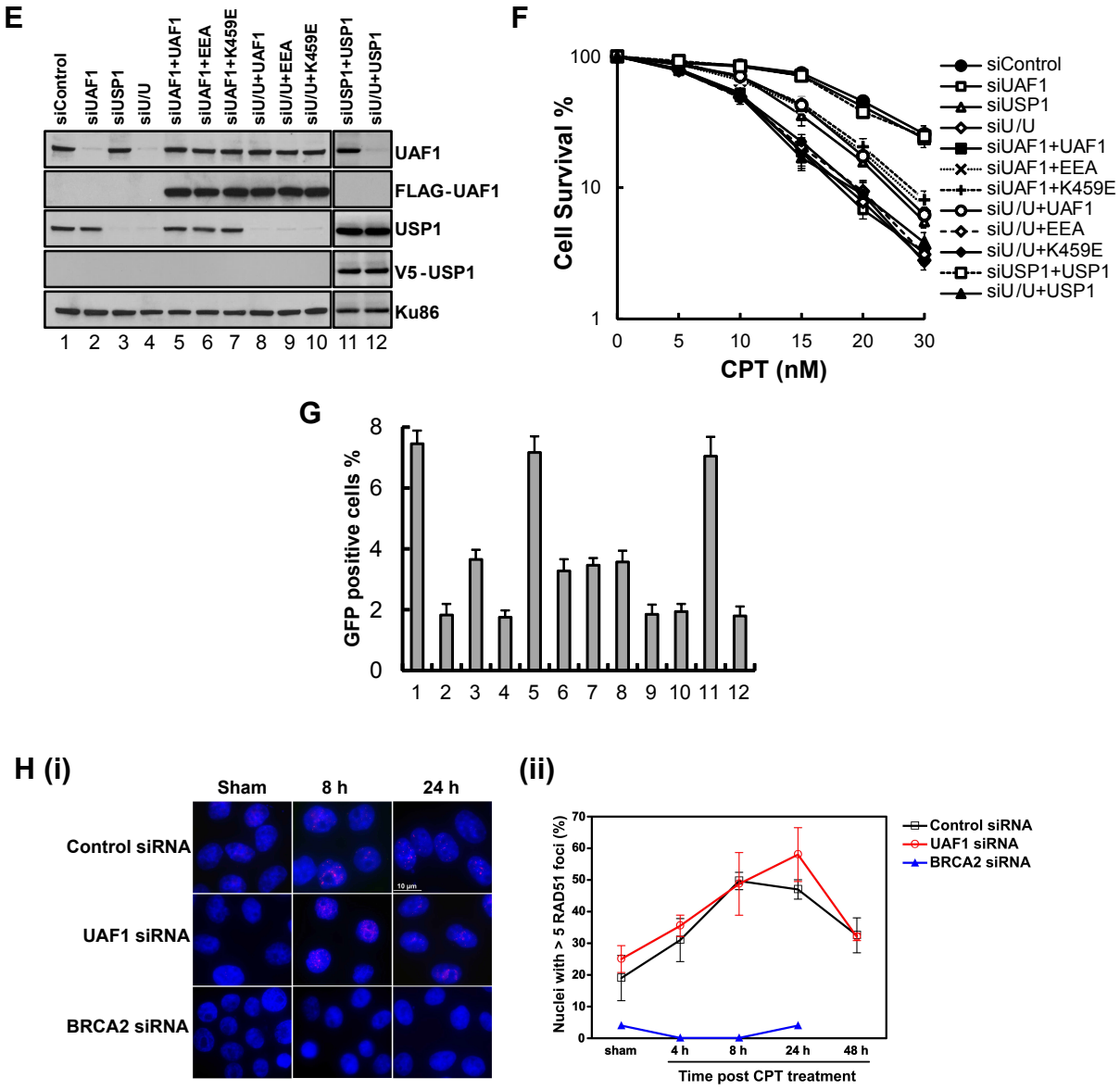
E
siControl
siUAF1
siUSP1
siU/U
siUAF1+UAF1
siUAF1+EEA
siUAF1+K459E
siU/U+UAF1
siU/U+EEA
siU/U+K459E
siUSP1+USP1
siU/U+USP1
UAF1
FLAG-UAF1
USP1
V5-USP1
Ku86
1 2 3 4 5 6 7 8 9 10 11 12
F
Cell Survival %
100
10
1
0 5 10 15 20 30
CPT (nM)
siControl
siUAF1
siUSP1
siU/U
siUAF1+UAF1
siUAF1+EEA
siUAF1+K459E
siU/U+UAF1
siU/U+EEA
siU/U+K459E
siUSP1+USP1
siU/U+USP1
G
GFP positive cells %
0 2 4 6 8
1 2 3 4 5 6 7 8 9 10 11 12
H (i)
Sham
8 h
24 h
Control siRNA
UAF1 siRNA
BRCA2 siRNA
(ii)
Nuclei with > 5 RAD51 foci (%)
0 10 20 30 40 50 60 70
sham
4 h
8 h
24 h
48 h
Time post CPT treatment
Control siRNA
UAF1 siRNA
BRCA2 siRNA

Figure S5. The function of RAD51AP1-UAF1 complex in DNA repair and HR, related to Figure 5.

(A) The IV2A and LI2A mutants of RAD51AP1 are impaired for association with UAF1. Extracts from HeLa cells stably expressing the wild type (WT) or mutant form of FLAG-tagged RAD51AP1 were prepared for co-IP analysis with anti-FLAG M2 agarose beads. Proteins were revealed with the indicated antibodies in Western blots.
(B) HeLa cells stably expressing a siRNA-resistant form of RAD51AP1 or the IV2A or LI2A mutant were transfected with RAD51AP1-siRNA. Cells without siRAD51AP1 were included as the control. Protein levels in cells are shown in the Western blots. Ku86 acts as the loading control. HeLa cells with the indicated genotype were treated with MMC, and cell survival was analyzed after incubation at 37℃ for 5 days. Cell survival values are shown in the right panel as mean ±S.D. from three independent experiments.
(C) Cells were treated with 30 nM CPT or left untreated for 24 hour and cell cycle profile was analyzed by flow cytometry.
(D) U2OS-DR-GFP cells stably expressing a siRNA-resistant form of either RAD51AP or the IV2A or LI2A mutant were transfected with RAD51AP1-siRNA. Cells were subsequently transfected with the I-*Sce*I expression plasmid and processed for flow cytometric analysis for GFP. Protein levels in cells are shown in the Western blots. Ku86 acts as the loading control. The DSB repair efficiency is shown in the right panel. C, Control cells without I-*Sce*I; 1, siRNA control cells; 2, siRAD51AP1 cells; 3, siRAD51AP1 cells with RAD51AP1; 4, siRAD51AP1 cells with RAD51AP-IV2A; 5, siRAD51AP1 cells with RAD51AP1-LI2A. Error bars indicate S.E.M. * indicates $P < 0.05$, unpaired t-test.
(E) U2OS-DR-GFP cells stably expressing WT or the indicated mutant form of UAF1 were transfected with siRNA targeting the 3' UTR of UAF1 or/and the coding region of USP1. A siRNA-resistant form of V5-tagged USP1 was expressed using the pLX304 vector as indicated. Cells without siUAF1 or siUSP1 were included as the control. Protein levels in cells are shown in the Western blots. Ku86 was included as the loading control. The symbol siU/U denotes siRNA treatment of cells to deplete endogenous UAF1 and USP1 simultaneously.
(F) The CPT sensitivity of the cells in (E) was analyzed as described in Figure 5C.
(G) The cells in (E) were transfected with the I-*SceI* expression plasmid and HR efficiency was analyzed as described in Figure 5D.
(H) Loss of UAF1 does not affect RAD51 focus formation and disassembly after CPT treatment. (i) Representative micrographs obtained from HeLa cells sham-treated or treated with 1 μM CPT for 1 h and fixed at 8 h and at 24 h post treatment. Red: RAD51; blue: DAPI. (ii) Quantification of RAD51 foci at various time points after a 1-h treatment with 1 μM CPT. Data points with error bars were from ≥ 3 independent experiments, except for the 48 h timepoint of the control siRNA, which was from 2 independent experiments. Errors: ± S.E.M. The results for BRCA2 knockdown are also shown.

Table S1 Summary of UAF1 and RAD51AP1 mutants

Mutants	Activity	Reference
UAF1-K459E	RAD51AP1-binding deficient	This study
UAF1-K595E	RAD51AP1-binding proficient	This study
UAF1-H599A	RAD51AP1-binding proficient	This study
UAF1-EA (K595E/H599A)	RAD51AP1-binding deficient	This study
UAF1-EEA (K459E/K595E/H599A)	RAD51AP1-binding deficient	This study
RAD51AP1-LI2A (L137A/I140A)	UAF1-binding deficient	This study
RAD51AP1-IV2A (I140A/V142A)	UAF1-binding deficient	This study
RAD51AP1-H329A	RAD51-binding deficient	Wiese et al., 2007
RAD51AP1-N-K6RA/C-K7WA	DNA-binding deficient	Dunlop et al., 2012

Table S2 Oligonucleotides used in this study

Name	Sequence	Assay
P1	5'-TTATATCCTTTACTTTGAATTCTATGTTTAACCTTTTACTTATTTT GTATTAGCCGGATCCTTATTTCAATTATGTTCAT-3'	DNA binding; Duplex capture
P2	5'-ATGAACATAATTGAAATAAGGATCCGGCTAATACAAAATAAGT AAAAGGTTAAACATAGAATTCAAAGTAAAGGATATAA-3'	DNA binding; Duplex capture
P3 (Homologous)	5'-AATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGC ATTTATCAGGGTTATT-3'	Synaptic assay
P4 (Heterologous)	5'-CAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAG GCCAGCAAAAGGCCAGGA-3'	Synaptic assay
90 mer	5'-AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTT ACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTT-3'	D-loop

Extended Experimental Procedures:

Mutant construction

All the point mutants of UAF1 and RAD51AP1 were generated with a site-directed mutagenesis Kit (Stratagene), and mutant proteins were expressed and purified following the same procedures developed for their wild type counterpart.

Protein expression and purification

Unless stated otherwise, cell lysate preparation and all the purification steps were carried out at 0-4^{o}C.

UAF1: The cDNAs for the wild type and mutant derivatives of UAF1 harboring a sequence coding for a C-terminal Strep II-tag were introduced into the pFastBac1 vector. Bacmids were generated in *E. coli* strain DH10Bac and used to infect Sf9 insect cells to produce recombinant baculoviruses. For protein expression, Hi5 insect cells were infected with the relevant recombinant virus, incubated at 27 ℃ for 48 hours, collected, and then stored at -80 ℃. A 5 g cell pellet (from 350 ml of insect cell culture) was thawed and resuspended in 50 ml cell breaking buffer (20 mM Tris-HCl, pH 7.5, 10% sucrose, 0.5 mM EDTA, 300 mM KCl, 0.1% Igepal CA-630, 1 mM DTT, 1 mM PSMF and a cocktail of protease inhibitors comprising aprotinin, chymostatin, leupeptin and pepstatin A at 3 μg/ml each) and subject to sonication to prepare crude lysate. After centrifugation (100,000 × g, 90 min), the clarified lysate was diluted with three volumes of buffer A (20 mM Tris-HCl, pH 7.5, 10% sucrose, 0.5 mM EDTA, 0.01% Igepal CA-630, and 1 mM DTT) and fractionated in a 25 ml Q Sepharose fast flow column using a 200-ml gradient of 50-500 mM KCl in buffer A. The fractions containing the peak of UAF1 (220 to 300 mM KCl) were combined and mixed with 2 ml Strep-Tactin resin (Qiagen) for 2 h. The resin was washed twice with 10 ml of buffer B (20 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT, and 1 M KCl), twice with 10 ml of buffer C (20 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT, and 300 mM KCl), before eluting the bound proteins by 10 ml of 2.5 mM desthiobiotin in buffer C. The eluate was concentrated in a Centricon 30 device (Millipore) to 0.5 ml and then fractionated in a 24 ml Superdex 200 gel filtration column in buffer C. The UAF1 peak fractions were collected and concentrated to 3 mg/ml before being frozen in small portions in liquid nitrogen and stored in small portions at -80 ℃.

UAF1-USP1 complex: A recombinant baculovirus that expresses N-terminally (His)6-tagged USP1 (Huang et al., 2006) was made following the procedure used in the generation of the Strep II-tagged UAF1 virus described above. Hi5 insect cells were co-infected with these recombinant viruses and incubated at 27 ℃ for 48 hours before being harvested. The preparation of clarified cell lysate from 5 g cell pellet (from 350 ml of insect cell culture) followed the same procedure as in the last section, but using a different cell breakage buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 150 mM KCl, 1 mM 2-mercaptoethanol and 0.01% Igepal, and protease inhibitors). The clarified lysate was diluted and fractionated in Q Sepharose fast flow as above. The UAF1-USP1 peak, eluting between 280 and 380 mM KCl, were pooled and incubated with 4 ml Nickel-NTA resin (Qiagen) for 2 h. The resin was washed twice with 20 ml of buffer C with 10 mM imidazole. The bound proteins were eluted by 15 ml of 200 mM imidazole in buffer C. The eluate was subject to Strep-Tactin affinity chromatography as before. After gel filtration, the purified UAF1-USP1 complex was concentrated in a Centricon-30 device to 3 mg/ml and stored in small portions at -80 ℃.

The UAF1 436X fragment: The cDNA encoding the N-terminal 436 residues of UAF1 and a C-terminal Strep II-tag was introduced into the pFastBac1 vector. The construction of recombinant baculovirus and protein expression in insect cells were carried out following the procedure described for full-length UAF1 above. Purification of the UAF1 436X fragment followed the same procedure as full-length UAF1 purification, except that SP Sepharose, instead of Q Sepharose, fast flow was used.

SLD1, SLD2 and SLD1-SLD2 domains of UAF1: The cDNAs encoding the SLD1, SLD2 and SLD1-SLD2 domains of UAF1 were introduced into the pMAT-9S vector to fuse them to the maltose binding protein (MBP). The resulting plasmids were introduced into Nico21 *E. coli* cells. Protein expression was induced by 0.5 mM IPTG when the cell culture reached an OD600 of 0.6-0.8, and cells were harvested after an overnight incubation at 16 ℃ and stored at -80 ℃. The cell pellet (10g) from three liters of culture was resuspended in 50 ml of buffer K (20 mM KH_2PO_4, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.1 mM TCEP, and 150 mM KCl), lysed in a micro fluidizer, and cleared by centrifugation (100,000 x g, 30 min). The clarified lysate was applied onto a 10 ml column of amylose resin. After

washing the column with 100 ml of buffer, bound proteins were eluted with the same buffer containing 10 mM maltose. The eluate was further fractionated in a 15 ml Q Sepharose fast flow column using a 200-ml gradient from 20 mM to 1 M KCl in K buffer. The protein pool was concentrated to 0.5 ml and then fractionated in a 24 ml Superdex 200 column in buffer D (50 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM TCEP and 0.5 mM EDTA). The protein preparations were concentrated to 4.5-12 mg/ml and stored in small portions at -80 ℃.

Other proteins: GST- and MBP-tagged RAD51AP1 (isoform 2), various mutants, and protein fragments were expressed in *E. coli* and purified as described previously (Wiese et al., 2007). Purification of human RAD51, *S. cerevisiae* Rad51 and Rad54 followed our published procedures (Chi et al., 2006; Petukhova et al., 1999). Purification of human FANCI was as described previously (Longerich et al., 2009).

Duplex capture assay

The assay was conducted at 37 ℃ and reaction mixtures were resolved by electrophoresis in 10% polyacrylamide gels as described previously (Dray et al., 2011). Briefly, magnetic resin containing 5'-biotinylated 83-mer oligo dT immobilized via streptavidin was incubated with RAD51 (3 μM) in 20 μl of reaction buffer (35 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM ATP, 4 mM MgCl2, 100 μg/ml BSA, and 1 mM DTT) for 5 min. The resin was captured with a magnetic separator, washed once with 20 μl buffer, and resuspended in 19 μl buffer. RAD51AP1 (200 nM) and UAF1 (100-400 nM) were added individually or in combination in 1 μl, followed by a 5-min incubation. The magnetic resin was again captured, washed, and resuspended in 19 μl buffer. Then, radiolabeled 80-mer dsDNA (4 μM base pairs, oligonucleotides P1/P2 in Table S2) was incorporated in 1 μl and the completed reaction was mixed gently for 10 min. The resin was captured, washed twice with 20 μl buffer, and the bound proteins and radiolabeled DNA were eluted with 20 μl of 2% SDS. Gels in which the supernatant from the last incubation and the SDS eluate had been resolved were dried and subject to phosphorimaging analysis to reveal and quantify the radiolabeled dsDNA. The reactions to examine the possible capture of radiolabeled ssDNA (Figure S4B) were conducted and analyzed in exactly the same way.

Assay for synaptic complex assembly

The assay was conducted at 37 ℃ and reaction mixtures were resolved by electrophoresis in 1% agarose gels as described previously (Dray et al., 2011). Briefly, the 60-mer oligonucleotide P3 (12 μM nucleotides; see Table S2 for sequence) complementary to the *Ssp*I restriction site of the pUC19 plasmid was incubated with 4 μM of RAD51 in 8 μl of reaction buffer (35 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM ATP, 4 mM MgCl2, 100 μg/ml BSA, and 1 mM DTT) for 5 min. After adding RAD51AP1 (300 nM), UAF1 (100-400 nM), or the combination of both proteins in 1 μl buffer, the reaction mixture was incubated for 5 min. Then, linear pUC19 plasmid DNA (85 μM nucleotides) was incorporated, followed by a 5-min incubation and the treatment with 2.5 units of *Ssp*I for 10 min. After electrophoresis, DNA species were stained with ethidium bromide. The heterologous oligonucleotide P4 (Table S2) was used as control.

Cell culture and transfection

The shRNA target sequence (5'-GGACCGAGATTATCTTTCA-3') at the 3' UTR of UAF1 was cloned into the pSUPER.retro.puro vector (Clontech). 293GPG cells were transfected with the UAF1-shRNA by FuGENE6 (Promega) to generate shUAF1 retroviral supernatants. HeLa and U2OS-DR-GFP cells (Nakanishi et al., 2005; Xia et al., 2006) were infected with the shUAF1 virus and selected in 3 μg/ml puromycin. FLAG-tagged cDNAs coding for the wild type and mutant forms of UAF1 were introduced into the pCMV(delta4) vector (Morita et al., 2012). The selected UAF1-depleted cells were transfected with the FLAG-tagged cDNAs using Lipofectamine 2000 (Invitrogen), and subsequently selected in medium with 800 μg/ml G418.

FLAG-tagged cDNAs coding for the wild type or mutant forms of UAF1 in the pCMV(delta4) vector were transfected into HeLa and U2OS-DR-GFP cells. Stable cell lines were selected in 800 μg/ml G418. HeLa and U2OS-DR-GFP cells were transfected with a siRNA-resistant form of FLAG-tagged cDNA coding for RAD51AP1 or mutant in the pCMV (delta4) vector, and selected in 800 μg/ml G418. For siRNA knockdown, 2×10^5 exponentially growing cells per well were seeded in 6-well plates and transfected with 2 μl siRNA (20 μM) using 9 μl Lipofectamine 2000. The siRNA target sequences are:

RAD51AP1, 5'- CCTCATATCTCTAATTGCA-3'; UAF1, 5'-GGACCGAGATTATCTTTCA-3'; USP1, 5'-TCGGCAATACTTGCTATCTTA-3'; BRCA2, 5'-TTGGAGGAATATCGTAGGTAA-3'.

Co-immunoprecipitation and Western blotting

The HeLa cells expressing FLAG-tagged wild type and mutant UAF1 or RAD51AP1 were cultured in 10 cm dishes. Following PBS buffer wash, whole cell lysate was prepared by adding 500 µl lysis buffer (50 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.5% NP-40, 1 mM DTT and protease inhibitors). After sonication for 10 s on ice, the cell lysate was cleared by centrifugation (14,000 × g, 15 min) at 4 ℃. The protein concentration was determined using the Bradford assay (BioRad) and 2 mg of protein was adjusted to 1 ml using lysis buffer, and then incubated with 50 µl anti-FLAG M2 affinity resin (Sigma) with gentle rocking at 4 ℃ for 12 h. The resin was washed 5 times with 500 µl ice cold lysis buffer and then treated with 2% SDS to elute bound proteins. The following antibodies were used in Western blotting: α-UAF1, α-USP1, α-RAD51, α-RAD51AP1, α-FANCD2, α-HA, α-ELG1 (Abcam), α-V5 (Invitrogen), α-FLAG (Sigma), and α-Ku86 (Santa Cruz Biotech).

Cell survival assay

Cells were seeded into 6-well plates at 1×10^4 cells/well and incubated at 37 ℃ for 24 h. After the addition of MMC (Sigma), CPT (Sigma), or olaparib (Selleck Chemicals), cells were incubated at 37 ℃ for 5 days. Surviving cells were fixed and stained with crystal violet dye, and absorbance was measured in a microplate reader (BioTek) as described (Williams et al., 2011).

Cell cycle analysis

Cells were stained with propidium iodide and analyzed by flow cytometry as described previously (Parplys et al., 2015). Briefly, 1×10^6 cells were collected and washed with PBS buffer. Then, cells were fixed in 10 ml 70 % ethanol/PBS at 4C for 48 hours. Fixed cells were washed with 5 ml 30% ethanol/PBS and 1 ml 0.05% BSA/PBS. Cells were then pelleted and stained with 30 µg/ml propidium iodide (Sigma) in PBS with 40 µg/ml RNase A for 30 min at 37 ℃ in the dark. Stained cells were analyzed in a LSRII flow cytometer (BD Biosciences) and with the FlowJo software.

Assay for DUB activity

Purified USP1 or UAF1-USP1 complex (0.5 µM) were incubated with Ub-VS (5 µM, Boston Biochem) in 30 µl of reaction buffer (50 mM Tris-HCl, pH 8.5, 50 mM NaCl, 1mM DTT) at 30 ℃ for 2 h. Modification of USP1 was detected by Western blot analysis.

Measurement of HR frequency

For HR assay, 2×10^5 exponentially growing U2OS-DR-GFP cells per well were seeded in 6-well plates. On the next day, the cells were transfected with 4 µg HA-tagged I-*Sce*I expression vector (pCBASce) using 10 µl Lipofectamine 2000. For some experiments, the U2OS-DR-GFP cells were transfected with siRNA against RAD51AP1, UAF1 or/and USP1 prior to I-*SceI* transfection. After 3 days, cells were trypsinized and processed for flow cytometric analysis of GFP in a BD LSRII Flow Cytometer (BD Bioscience).

Immunofluorescence microscopy and image analyses

Cells in exponential growth were transfected in MEM growth medium with 10% FBS using RNAiMAX (Invitrogen) with 20 nM UAF1, BRCA2 or control siRNA, as recommended by the manufacturer. On the next day, cells were seeded at 2×10^4 cells/well in four-well chamber slides. After 36 h, cells were treated with 1 µM CPT for 1 h in regular growth medium. Cells were then washed three times in PBS and incubated in regular growth medium until fixation. Immunocytohistochemistry, image acquisition and computational analysis of RAD51 foci were performed as described previously (Zhao et al., 2015).

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