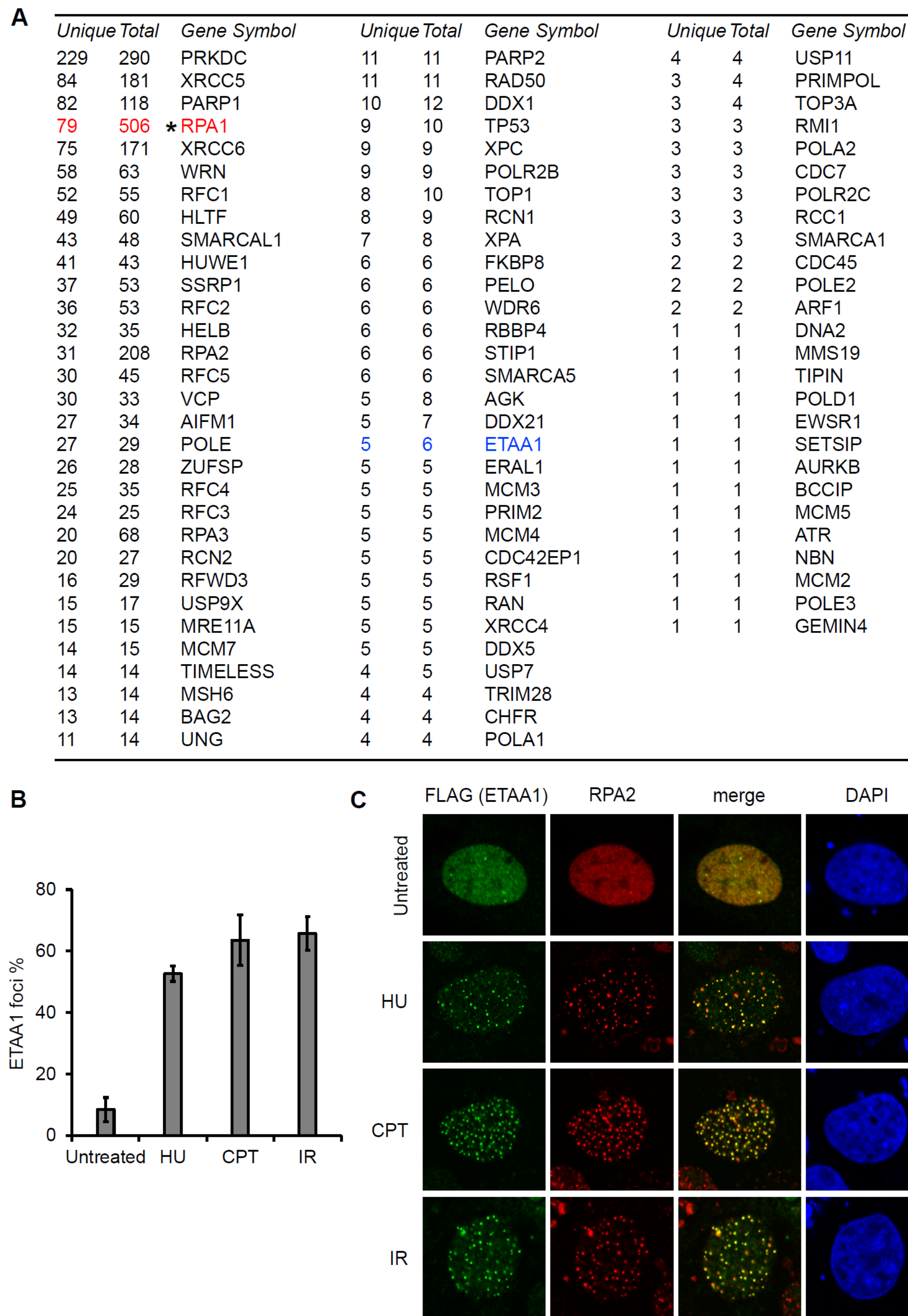
Supplemental Figures and Legends



A

Unique	Total	Gene Symbol	Unique	Total	Gene Symbol	Unique	Total	Gene Symbol
229	290	PRKDC	11	11	PARP2	4	4	USP11
84	181	XRCC5	11	11	RAD50	3	4	PRIMPOL
82	118	PARP1	10	12	DDX1	3	4	TOP3A
79	506	* RPA1	9	10	TP53	3	3	RMI1
75	171	XRCC6	9	9	XPC	3	3	POLA2
58	63	WRN	9	9	POLR2B	3	3	CDC7
52	55	RFC1	8	10	TOP1	3	3	POLR2C
49	60	HLTF	8	9	RCN1	3	3	RCC1
43	48	SMARCAL1	7	8	XPA	3	3	SMARCA1
41	43	HUWE1	6	6	FKBP8	2	2	CDC45
37	53	SSRP1	6	6	PELO	2	2	POLE2
36	53	RFC2	6	6	WDR6	2	2	ARF1
32	35	HELB	6	6	RBBP4	1	1	DNA2
31	208	RPA2	6	6	STIP1	1	1	MMS19
30	45	RFC5	6	6	SMARCA5	1	1	TIPIN
30	33	VCP	5	8	AGK	1	1	POLD1
27	34	AIFM1	5	7	DDX21	1	1	EWSR1
27	29	POLE	5	6	ETAA1	1	1	SETSIP
26	28	ZUFSP	5	5	ERAL1	1	1	AURKB
25	35	RFC4	5	5	MCM3	1	1	BCCIP
24	25	RFC3	5	5	PRIM2	1	1	MCM5
20	68	RPA3	5	5	MCM4	1	1	ATR
20	27	RCN2	5	5	CDC42EP1	1	1	NBN
16	29	RFWD3	5	5	RSF1	1	1	MCM2
15	17	USP9X	5	5	RAN	1	1	POLE3
15	15	MRE11A	5	5	XRCC4	1	1	GEMIN4
14	15	MCM7	5	5	DDX5			
14	14	TIMELESS	4	5	USP7			
13	14	MSH6	4	4	TRIM28			
13	14	BAG2	4	4	CHFR			
11	14	UNG	4	4	POLA1			

Figure S1, related to Figure 1. ETAA1 is an RPA-interacting protein involved in cellular response to DNA damage. (**A**) Tandem affinity purification (TAP) was performed using 293T cells stably expressing SFB-tagged RPA1. The data from mass spectrometry analysis were shown in the tables. ETAA1 was colored in blue. (**B**) HeLa cells were transfected with the plasmid encoding SFB-tagged ETAA1. Foci-positive transfected cells were quantified by counting a total of 100 transfected cells with positive staining. Data are presented as mean ± s.d. from 3 different experiments. (**C**) U2OS cells were transfected with the plasmid encoding SFB-tagged ETAA1. Immunostaining experiments were performed 6 hr after HU, CPT, or IR treatment using indicated antibodies.

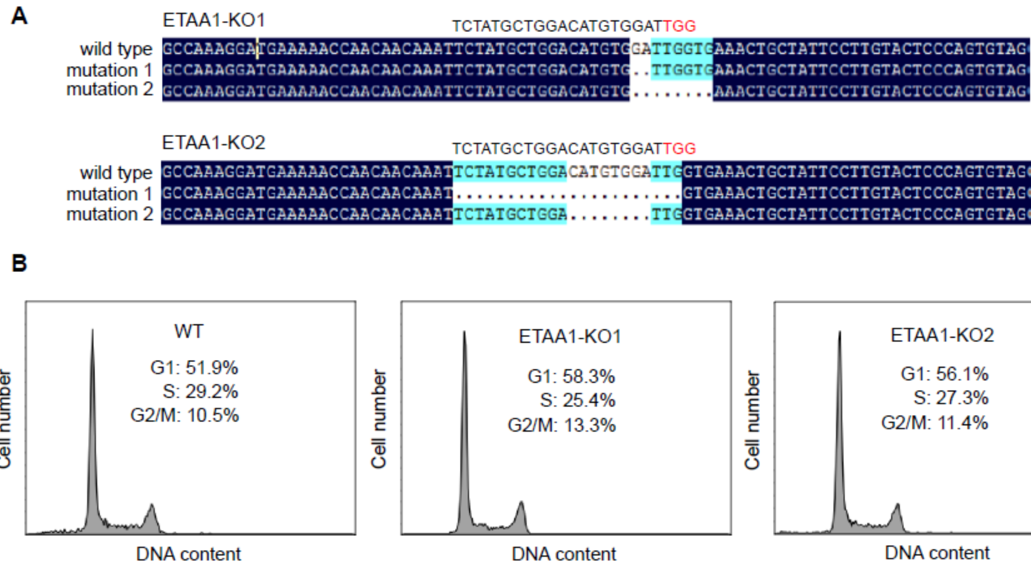


Figure S2, related to Figure 1. (**A**) Two isolated single colonies were subjected to DNA sequencing for verification of ETAA1 knockout. The frameshift mutations from both alleles were shown. The gRNA target sequence was indicated. (**B**) Cell cycle distributions of wild-type and ETAA1 knockout cells were verified by flow cytometry analysis.

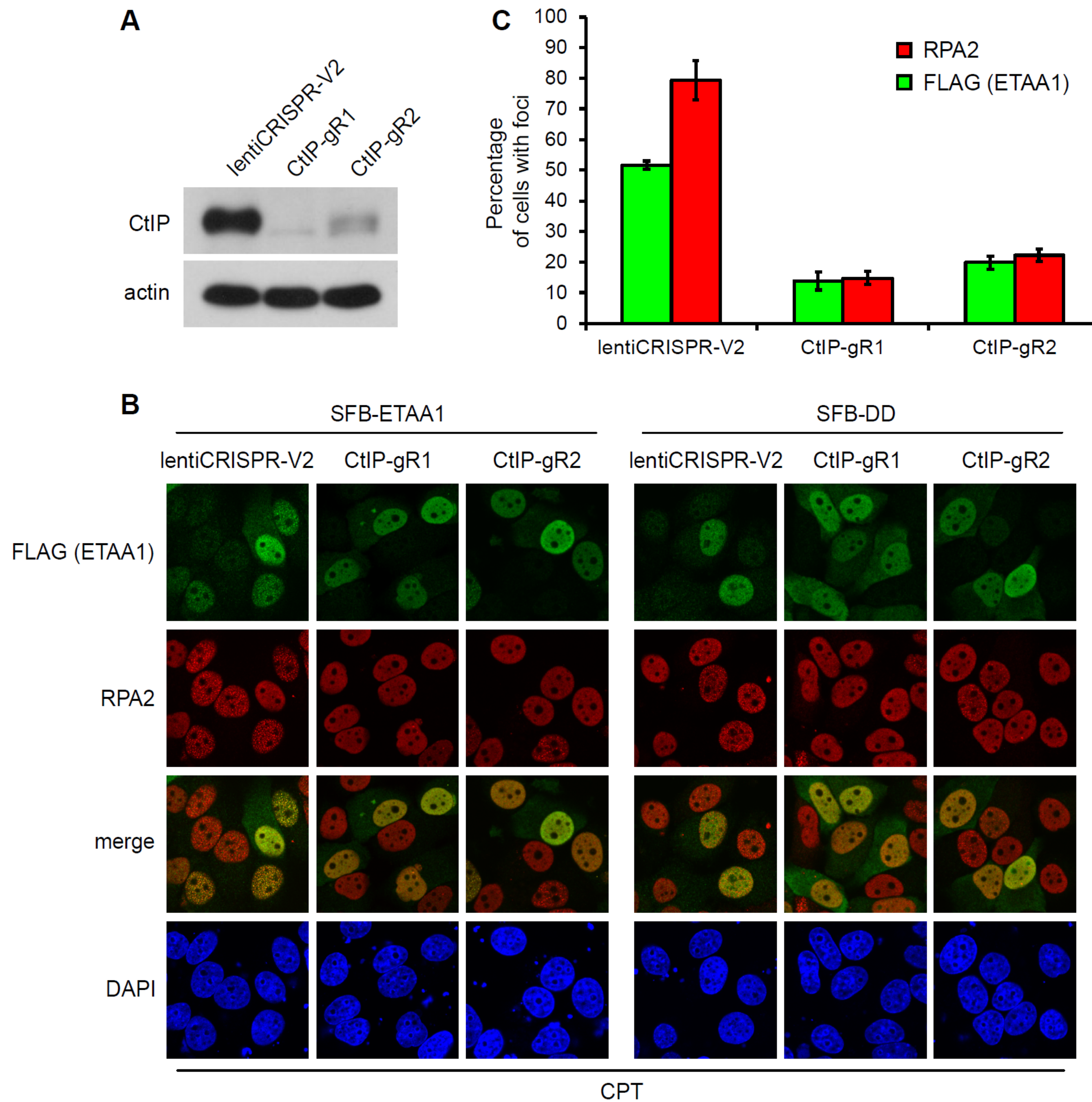


Figure S3, related to Figure 2. ETAA1 is recruited by RPA-bound ssDNA generated by DNA end resection mediated by CtIP. (**A**) lentiCRISPR-mediated CtIP depletion in HeLa cells was confirmed by immunoblotting. (**B**) Control and CtIP-depleted cells were transfected with the plasmid encoding SFB-tagged ETAA1 or a mutant lacking both of the RPA-binding domains (DD). Immunostaining experiments were performed 6 hr after CPT treatment using indicated antibodies. (**C**) ETAA1 foci-positive cells were quantified by counting a total of 200 transfected cells with positive staining. Similarly, RPA2 foci-positive cells were quantified by counting a total of 200 cells. Data are presented as mean ± s.d. from 3 different experiments.

ETAA1 homologues

human ETAA1

NLS AAD RBD1 RBD2

1 76 238 339 572 700 872 926

Hcmo
Bos
Mus
Rattus
Gallus
Danio
Xenopus

Figure S4, related to Figure 2. Comparison of ETAA1 amino acid sequences from different species. Black and gray shading indicate, respectively, identical and conserved residues. Four conserved regions were indicated with boxes in different colors and were also marked in the schematic representation of the ETAA1 protein.

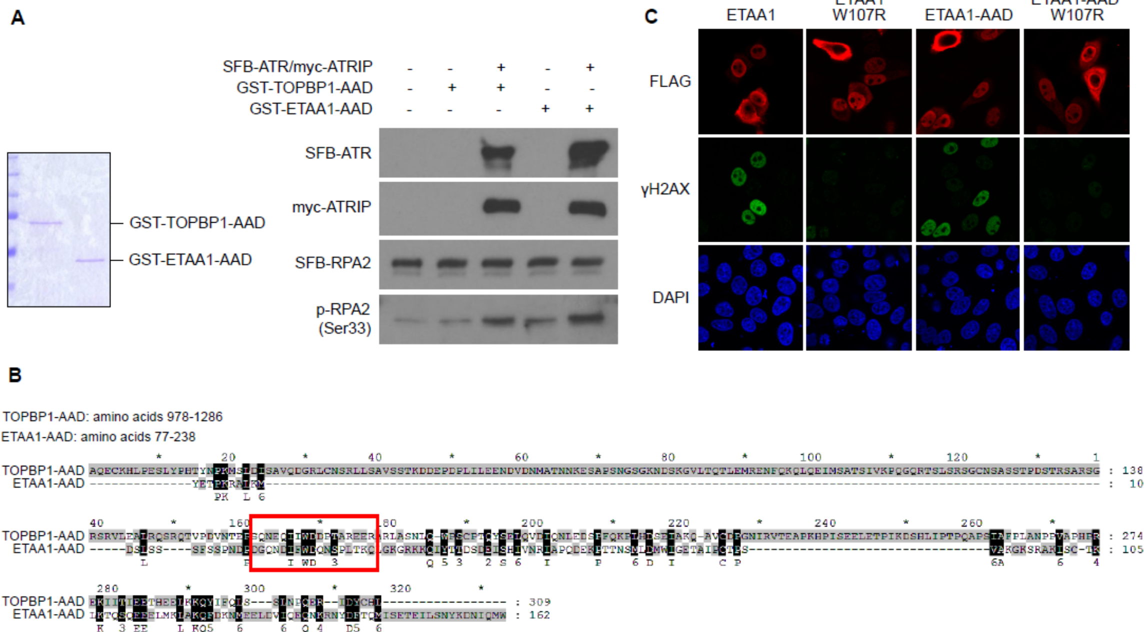


Figure S5, related to Figures 5 and 6. (**A**) Activation of human ATR by TOPBP1-AAD and ETAA1-AAD. Mock preparations (lanes 1, 2 and 4) and preparations containing ATR/ATRIP complex (lanes 3 and 5) were incubated with control buffer (lanes 1), GST-TOPBP1-AAD (lanes 2 and 3), or GST-ETAA1-AAD (lanes 4 and 5) in the presence of SFB-RPA2. Samples were subjected to SDS-PAGE and processed for immunoblotting with indicated antibodies. Purified GST-TOPBP1-AAD and GST-ETAA1-AAD from *E. coli* were subjected to SDS-PAGE and stained with Coomassie blue. (**B**) Alignments of ETAA1-AAD (amino acids 77-238) with TOPBP1-AAD (amino acids 978-1286). (**C**) W107R mutation disrupted the ability of ETAA1 in the induction of γH2AX. HeLa cells were transfected with indicated plasmids. Immunostaining experiments were performed using indicated antibodies.

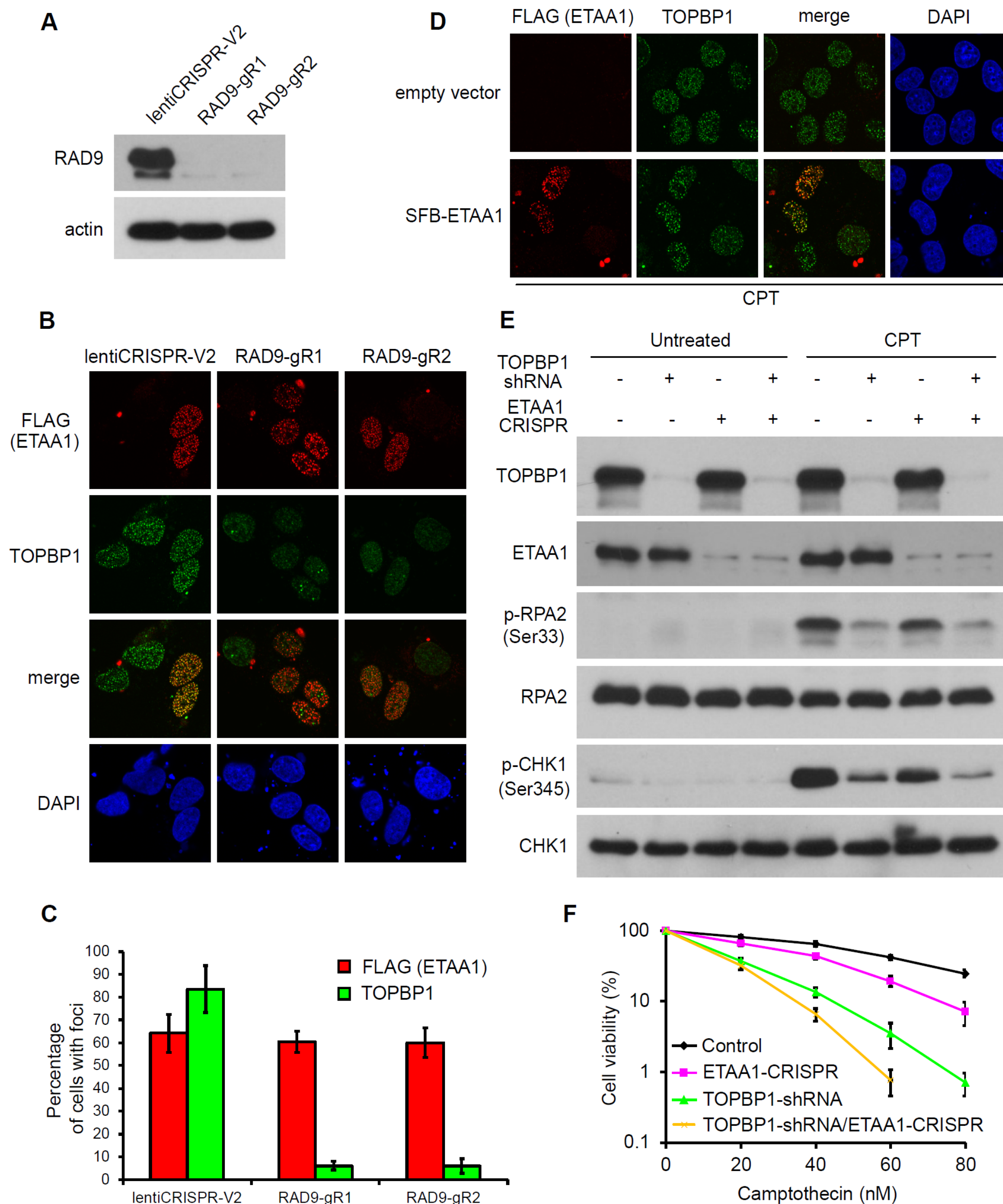


Figure S6, related to Figure 6. ETAA1 is located in a parallel pathway and activates ATR independent of 9-1-1 complex and TOPBP1. (**A**) lentiCRISPR-mediated RAD9 depletion in U2OS cells was confirmed by immunoblotting. (**B**) Control and RAD9-depleted cells were transfected with the plasmid encoding SFB-tagged ETAA1. Immunostaining experiments were performed 6 hr after CPT treatment using indicated antibodies. (**C**) ETAA1 foci-positive cells were quantified by counting a total of 200 transfected cells with positive staining. Similarly, TOPBP1 foci-positive cells were quantified by counting a total of 200 cells. Data are presented as mean ± s.d. from 3 different experiments. (**D**) U2OS cells were transfected with an empty vector or the plasmid encoding SFB-tagged ETAA1. Immunostaining experiments were performed 6 hr after CPT treatment using indicated antibodies. (**E**) HeLa cells were infected with lentiviruses expressing TOPBP1 shRNA or ETAA1 CRISPR to achieve single or double depletion of TOPBP1 and ETAA1. Cells were treated with CPT for one hour or left untreated and the cell lysates were collected for immunoblotting with indicated antibodies. (**F**) ETAA1 and TOPBP1 depletion has an additive effect on cell sensitivity following CPT treatment. Data are presented as mean ± s.d. from three different experiments.

Supplemental Experimental Procedures

Constructs

All cDNAs were subcloned into pDONR201 (Invitrogen) as entry clones and were subsequently transferred to gateway-compatible destination vectors for the expression of N or C-terminal-tagged fusion protein. All point or deletion mutants were generated using the QuickChange site-directed mutagenesis kit (Stratagene) and verified by sequencing.

Cell Culture and transfection

HeLa, 293T, and U2OS cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Plasmid transfection was performed using polyethylenimine (PEI) reagent.

Tandem affinity purification (TAP) of ETAA1-associated protein complexes

Tandem affinity purification was performed as previously described [S1, S2]. Briefly, 293T cells were transfected with plasmids encoding SFB-tagged ETAA1. Cell lines stably expressing tagged proteins were selected and the expression of exogenous proteins was confirmed by immunoblotting and immunostaining. For affinity purification, a total of twenty 10-cm dishes of 293T cells stably expressing SFB-ETAA1 were collected and lysed with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, containing 1 μg/ml of each pepstatin A and aprotinin) for 25 min. Crude lysates were cleared by centrifugation, and the supernatants were incubated with 150 μl streptavidin sepharose beads (Amersham Biosciences) for 2 hr at 4°C. The beads were washed three times with NETN and then eluted with 2 mg/ml biotin (Sigma) for 2 hr at 4°C. The eluates were incubated with 100 μl S-protein agarose beads (Novagen) for 2 hr at 4°C and then washed three times with NETN. The proteins bound to beads were eluted by boiling with SDS sample buffer, resolved by SDS-PAGE, visualized by Coomassie blue staining and subjected to mass spectrometry analysis for protein identification (performed by Taplin biological mass spectrometry facility, Harvard university).

CRISPR-cas9 mediated gene editing

ETAA1 knockout cell lines were generated using the CRISPR/Cas9 genome editing system. Briefly, CRISPR guide sequences were chosen and gene-specific guide RNA (gRNA) expression vectors were generated as previously described [S3]. hCas9 (plasmid 41815; Addgene) and gRNA cloning vector (plasmid 41824; Addgene) were obtained from Addgene. CRISPR guide sequence for targeting ETAA1: ETAA1_gR2: TCTATGCTGGACATGTGGAT. Cells were co-transfected with hCas9, gRNA expression vector, and a GFP-expressing vector. GFP-positive cells were sorted based on GFP fluorescence after 24 hours into 96-well plates for single colony isolation. The isolated single colonies were subjected to immunoblotting analysis first and DNA sequencing later for knockout verification.

To produce lentiviral particles, gRNA target sequences were cloned into lentiCRISPR-V2 (plasmid 52961; Addgene), and confirmed by DNA sequencing. The gRNA target sequences for CtIP are: CtIP_gR1: GAATGAAAGGAATACTCTAC; CtIP_gR2: TGACCAAAGTCAATCTCCAA. The gRNA target sequences for RAD9 are: RAD9_gR1: GGTAGAGCTCGTCCCCGATG; RAD9_gR2: GCGCTGTAAGATCCTGATGA. The gRNA target sequence for ETAA1 is: ETAA1_CRISPR: ATGGACAGAATGATATCTTT. The lentiCRISPR-V2 constructs were packaged into lentiviruses by co-transfecting with packaging plasmids pMD2G and pSPAX2 (kindly provided by Professor Songyang Zhou, Baylor College of Medicine) into 293T cells. 48 hr after transfection, the supernatant was collected and used for infection. Infection was repeated twice with an interval of 24 hr to achieve maximal infection efficiency. Infected cells were selected with media containing puromycin (2 μg/ml).

Immunoblotting

Cells were lysed with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 % Nonidet P-40) on ice for 30 min. Cleared cell lysates were then collected and boiled in 2× Laemmli buffer and separated by SDS-PAGE. Membranes were blocked in 5% milk in TBST buffer and then probed with antibodies as indicated.

Co-precipitation assay

For co-precipitation assays, constructs encoding SFB-tagged and myc-tagged proteins were transiently co-transfected into 293T cells. Cells were lysed in NETN buffer on ice for 30 min, cleared by centrifugation, and incubated with S-protein beads for 2 hours at 4°C. Beads were washed, boiled in 2× Laemmli buffer and separated on SDS-PAGE.

Immunostaining

Cells cultured on coverslips were washed with PBS, fixed with 3% paraformaldehyde for 12 min and then extracted with 0.5% Triton solution for 5 min. Coverslips were washed with PBS and then immunostained with primary antibodies in 5% goat serum for 60 min. Coverslips were washed and incubated with secondary antibodies conjugated with Rhodamine or FITC for 60 min. Cells were then stained with DAPI to visualize nuclear DNA. The coverslips were mounted onto glass slides with anti-fade solution and visualized using an A1 laser scanning confocal attachment on an Eclipse Ti microscope stand (Nikon Instruments, Melville, NY) using 60x/1.49 objective lens, and controlled by NIS Elements software (Nikon). The confocal pinhole was set at 1 Airy unit.

ATR kinase assay

The ATR kinase assay was performed as described previously [S4]. Briefly, samples were incubated in 20 µl kinase buffer (50 mM Tris-HCl [pH7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol and 100 µM ATP). The purified GST-TOPBP1-AAD or GST-ETAA1-AAD (50 µg/ml) was pre-incubated with ATR-ATRIP protein complex purified from 293T cells for 30 min at room temperature. After the pre-incubation, 1 mM ATP and SFB-RPA2 were added to the reaction, and the mixture was incubated for 20 mins at 30°C. The reaction was terminated by the addition of Laemmli buffer and the products were separated by SDS-PAGE. PRA2 phosphorylation was detected by immunoblotting using anti-RPA2 (Phospho-Ser33) antibody.

Cell survival assays

A total of 1×10^3 cells were seeded onto 60-mm dishes in triplicates. Twenty-four hours after seeding, cells were treated with HU or CPT at indicated concentrations. Medium with HU or CPT was replaced 24 hr later and cells were then incubated for 14 days. Resulting colonies were fixed and stained with Coomassie blue. Numbers of colonies were counted using a Gel Doc with Quantity One software (BIORAD).

Supplemental References

S1. Yuan, J., and Chen, J. (2013). FIGNL1-containing protein complex is required for efficient homologous recombination repair. Proc Natl Acad Sci U S A *110*, 10640-10645.

S2. Yuan, J., Ghosal, G., and Chen, J. (2012). The HARP-like domain-containing protein AH2/ZRANB3 binds to PCNA and participates in cellular response to replication stress. Mol Cell *47*, 410-421.

S3. Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-guided human genome engineering via Cas9. Science *339*, 823-826.

S4. Kumagai, A., Lee, J., Yoo, H.Y., and Dunphy, W.G. (2006). TopBP1 activates the ATR-ATRIP complex. Cell *124*, 943-955.