INVENTORY OF SUPPLEMENTAL INFORMATION

SUPPLEMENTAL DATA ITEMS

Figure S1 is Related to Figure 1. SIRT2 Interacts in a Complex with ATR-ATRIP. (A, B) 293T cells were transfected with FLAG-ATR and/or SIRT2-GFP **(A)**, or HA-ATRIP and/or FLAG-SIRT1 or FLAG-SIRT2. **(B)**, harvested and immunoprecipitated with anti-Flag M2 beads. Bound proteins were washed, separated by SDS–PAGE and immunoblotted with antibodies against Flag, GFP, HA and ATR. **(C)** HeLa cells were transfected with FLAG-SIRT1 or FLAG-SIRT2, harvested and immunoprecipitated with anti-Flag M2 beads. Bound proteins were washed, separated by SDS–PAGE and immunoblotted with antibodies against Flag and ATRIP.

Figure S2 is Related to Figure 2. SIRT2 Deacetylates ATRIP at Lysine 32 in Response to Replication Stress. (A, B, C) MS/MS peptide spectra of immunoprecipitated HA-ATRIP expressed in 293T cells shows site-specific ATRIP acetylation at lysine residues 32, 96, 101. **(D)** HCT116 cells were transfected with HA-ATRIP WT or K32R, and histone acetyltransferases, harvested in the presence of TSA, separated by SDS-PAGE, and immunoblotted with antibodies against Ac-ATRIP K32 and ATRIP. **(E)** HCT116 cells were transfected with ATRIP or NT siRNA and histone acetyltransferases, harvested in the presence of TSA, immunoprecipitated with an anti-ATRIP antibody, separated by SDS-PAGE, and immunoblotted with antibodies against Ac-ATRIP K32, ATRIP, and GAPDH. **(F)** HCT-116 cells stably transfected with HA-ATRIP were treated with or without TSA or nicotinamide, harvested, immunoprecipitated with HA antibody, separated by SDS-PAGE, and immunoblotted with antibodies against Ac-ACTRIP K32 and HA. **(G)** 293T cells were transfected with HA-ATRIP and/or SIRT2-FLAG, treated with or without HU in the presence of TSA, immunoprecipitated with anti-Flag M2 beads, separated by SDS-PAGE, and immunoblotted with antibodies against HA, acetyl-lysine, and FLAG. **(H)** 293T cells were transfected with HA-ATRIP and histone acetyltransferases, treated with 0, 2, 5, or 10 Gy IR with 1 hour recovery in the presence of TSA, harvested, separated by SDS-PAGE, and immunoblotted with antibodies against Ac-ATRIP K32, HA, and GAPDH. 293T cells transfected with HA-ATRIP and histone acetyltransferases and treated with TSA were synchronized with 0.5mM mimosine for 20 hours and released into G1, S, and S/G2 phases prior to treatment with or without 10 Gy IR with a 1 hour recovery before harvest. DNA content was measured by flow cytometry. Cells were lysed in presence of TSA, separated by SDS-PAGE, and immunoblotted with antibodies against Ac-ATRIP K32 and HA. Quantitation of bands was performed using LI-COR Odyssey Imaging software.

Figure S3 is Related to Figure 3. SIRT2 Deacetylation of ATRIP at Lysine 32 Promotes ATR Activation. (A) HCT-116 cells transfected with SIRT2 or NT siRNA were labeled with BrdU for 30 minutes, fixed, stained with fluorescently labeled antibodies against BrdU and PI, and analyzed by flow cytometry. **(B)** HCT-116 cells transfected with SIRT2 or NT siRNA were treated with or without HU, harvested, separated by SDS-PAGE, and immunoblotted with antibodies against P-ATR T1989, ATR, P-CHK1 S317, CHK1, SIRT2, and GAPDH. **(C)** HeLa cells cells were transfected with HA-ATRIP WT or K32Q, treated with or without HU, separated by SDS-PAGE, and immunoblotted with antibodies against P-ATR T1989, ATR, HA, pRAD17 S645, and RAD17. and GAPDH. **(D)** HCT-116 cells stably transfected with HA-ATRIP WT, K32R, or K32Q were labeled with or without BrdU for 30 minutes, fixed, stained with fluorescently labeled antibodies against BrdU and PI, and analyzed by flow cytometry.

Figure S4 is Related to Figure 4. ATRIP Lysine 32 Acetylation Impairs Recovery from Replication Arrest. (A) Western blot analysis of U2OS stable cell lines demonstrating expression of siRNA resistant HA-ATRIP WT, K32Q, K96Q, and K101Q. **(B)** Western blot analysis of U2OS stable cell line expressing empty vector and transfected with ATRIP or NT siRNA demonstrating efficiency of knockdown of endogenous ATRIP. **(C)** U2OS cells stably expressing an empty vector or siRNA resistant HA-ATRIP WT, K32Q, 96Q, or K101Q were transfected with NT of ATRIP siRNA, treated with 3 mM HU for 20 hours (arrested), and released into nocodazole for 10 hours (released). DNA content was analyzed by flow cytometry. **(D)** The percentage (mean and SEM) of cells that accumulated 4N DNA content in three replicate experiments is shown. **(E)** U2OS cells stably transfected with HA-ATRIP WT, K32R, or K32Q or an empty vector were silenced for endogenous ATRIP with siRNA, and treated with or without CPT at the indicated concentrations for 72 hours prior to assaying for cell viability. ** indicates $p<0.01$, *** indicates $p < 0.001$.

Figure S5 is Related to Figure 5. Lysine 32 Acetylation Impairs ATRIP Accumulation to Sites of DNA Damage. (A,B) HeLa cells were transfected with WT, K32R or K32Q GFP-ATRIP respectively. Cells were either treated with 10 Gy IR and allowed 4 h to recover or cultured with 3mM HU for 24 h before RPA70 staining for immunofluorescence analysis. The percentage of GFP-ATRIP positive cells with RPA foci from 3 replicate

experiments are separately shown for IR treatment **(A)** and HU treatment **(B)**. **(C)** HeLa cells transfected with GFP-ATRIP WT, K32R, or K32Q were harvested, fixed, stained with DNA content by PI, and analyzed by flow cytometry. **(D)** Western blot analysis demonstrating expression of GFP-ATRIP WT, K32R, or K32Q transfected in HeLa cells. **(E)** Representative images of HA-ATRIP foci MMT *SIRT2* KO cells complemented with or without SIRT2 WT and treated with or without HU.

Figure S6 is Related to Figure 6. SIRT2 Promotes the Interaction of ATRIP with RPA but not with ATR. (A) 293T cells were transfected with HA-ATRIP and histone acetyltransferases, together with vector or FLAG-SIRT2 in the presence of TSA, harvested, immunoprecipitated with anti-HA beads, separated by SDS-PAGE, and immunoblotted with antibodies against HA, ATR, RPA1, RPA2 and Flag. **(B)** HeLa cells transfected with HA-ATRIP K32R or K32Q, immunoprecipitated with anti-HA beads, separated by SDS-PAGE, and immunoblotted with antibodies against HA, ATR, and RPA70. **(C)** Coomasie blue stain of eluted fractions of RPA70, RPA32, and RPA14 purified from bacteria. **(D)** RPA-ssDNA binding assay for HA-ATRIP WT, K32R, or K32Q transfected into 293T cells treated with HU. **(E)** Coomasie blue stain of eluted fractions of GST-ATRIP aa 1-107 WT, K32R, and K32Q purified from bacteria.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Transfections

The siRNA duplexes were obtained from Dharmacon or Qiagen. Plasmid DNA and siRNA transfections were performed using Lipofectamine 2000 (Invitrogen) and HiPerfect (Qiagen), respectively, following the manufacturer's instructions.

Individual siRNAs were purchased from Dharmacon or Qiagen. These sequences were as follows:

NT (ATGAACGTGAATTGCTCAATT);
SIRT2-1 (GGAGAAAGCTGGCCAGTCG);
SIRT2-2 (TGGGCAGAAGACATTGCTTAT);
ATRIP (GGTCCACAGATTATTAGA);
shRNA SIRT2
(CCGGGCCAACCATCTGTCACTACTTCTCGAGAAGTAGTGACAGATGGTTGGCTTTTTG);

In vitro Deacetylation Assay

293T cells were transiently transfected with HA-ATRIP and histone acetyltransferase (p300/CBP, and pCAF), and treated with 0.5 μM TSA and 20 mM nicotinamide for overnight before harvesting. Harvested cells were lysed with IP buffer (20 mM Hepes pH 7.4, 180 mM KCl, 0.2 mM EGTA, 1.5 mM $MgCl_2$, 20% glycerol, 1.0% Nonidet P-40) supplemented with 1 μM TSA. The HA-ATRIP proteins were immunoprecipitated using anti-HA agarose beads (Sigma), and immunoprecipitants were washed with IP buffer containing 1 μM TSA to remove nicotinamide and then used for in vitro deacetylation assay. The agarose beads with conjugated HA-ATRIP (300ng) were re-suspended in 25 μL of deacetylation reaction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 10 mM NAD) along with the addition of purified SIRT2 (1 μg) at 30 °C for 3 h with constant agitation. For purification of SIRT2, Flag-SIRT2 was transiently transfected into 293T cells. After protein expression, the cells were lysed using IP buffer. The proteins lysates were immunoprecipitated using anti-FLAG M2 affinity beads (Sigma), and immunoprecipitants were washed with IP buffer followed by TBS (150mM NaCl, 50mM Tris, pH 7.5). Immunoprecipitated proteins were eluted with TBS supplemented with 0.15 mg/mL 3xFLAG peptide (Sigma) and then used for in vitro deacetylation assay. The reaction was stopped by the addition of 5x SDS-loading buffer, and samples were analyzed for acetylation by Western blot with an anti-pan acetyl antibody (Santa Cruz).

Cellular Deacetylation Assay

For cellular deacetylation analysis, HeLa cells were transiently co-transfected with HA-ATRIP, along with Flag-SIRT2-WT or Flag-SIRT2-HY, and cultured with 0.5 μM TSA for 12 hr. The cells were lysed with IP buffer containing 1 μM TSA, and protein lysates were immunoprecipitated using anti-FLAG M2 agarose (Sigma). The immunocaptured proteins were analyzed for deacetylation by immunoblotting with an anti-acetyl antibody.

Multi-Sequence Alignment

ATRIP protein sequences from different organisms were aligned using Clustal Omega and then processed in BoxShade to illustrate conserved amino acids.

Immunofluorescence Analysis

HeLa cells were transiently transfected with GFP-ATRIP (WT, K32R and K32Q respectively). MM2 S2KO cells stably complemented with or without SIRT2 WT were transiently transfected with HA-ATRIP (WT, K32R, and K32Q). Cells received 10 Gy of ionizing radiation (X-ray) or treated with 3mM HU. After the indicated recovery (for IR) or treatment (for HU) time, cells were fixed with 3% (wt/vol) paraformaldehyde for 10 min at room temperature, permeabilized with 0.5% Triton X-100 for 10 min, and then blocked with 5% BSA in PBS. Cells were immunostained with anti-RPA70 antibody (Cell Signaling), anti-HA antibody (Sigma, H9658), and Alexa Fluor 488 or 555 anti-mouse/rabbit secondary antibodies (Life technology). Images were captured on a Zeiss Observer Z1 microscope equipped with Axiovision Rel 4.8 software. Samples were scanned using a 60x oil objective. The percentage of cells with greater than five GFP foci or RPA foci in GFP expression cells or average number of HA-ATRIP foci was counted from three replicate experiments with at least 50 cells counted per replicate.

DNA Fiber Labeling Assay

293T cells transfected with ATRIP (WT, K32R or K32Q respectively) were labeled with 100uM IdU for 20 min, rinsed three times with 1XPBS, treated with 2mM HU for 1 hr, together with DMSO or 5uM VE-821 ATR inhibitor, and then labeled with 100uM CldU for 20 min. Cells were harvested and resuspended in ice-cold PBS. Two

microliters of cell suspension was deposited on a microscope slide, and 10 uL of spreading buffer (200 mM Tris-HCl at pH 7.4, 0.5% SDS, 50 mM EDTA) was added to the cells on the slide for 10 min. Next, the slides were tilted to 15 degree to stretch the DNA fibers. Following fixation in a 3:1 solution of methanol : acetic acid, the DNA was denatured with 2.5N HCl, blocked with 10% Normal Goat Serum blocking solution (Invitrogen), and stained with rat anti-CldU (Abcam) and mouse anti-IdU (BD Bioscience) diluted in 10% Normal Goat Serum blocking solution followed by secondary antibodies (Alexa-555 goat anti-rat IgG and Alexa-488 goat anti-mouse; Invitrogen). Images were captured on a Zeiss Observer Z1 microscope and analyzed with Axiovision Rel 4.8 software. In all experiments, data was collected from several replicates with high-quality DNA fibers with at least 100 fibers counted for lengths and 300 fibers counted for collapsed fork and origins.

Immunoblot

Cells were harvested in PBS and lysed for 30 min on ice in NP40 buffer (200 mM NaCl, 1% NP40, 50 mM Tris-Hcl pH 8.0) freshly supplemented with protease inhibitors. Lysates were clarified by centrifugation (13,000 rpm, 10 min at 4°C), and the supernatants were collected. Protein samples were then quantified with the Bradford assay and resolved by SDS-PAGE, transferred onto PVDF and probed using the appropriate primary antibodies. Detection was performed with the Licor Odyssey system. The antibodies used were as follows: SIRT2 (Millipore, 09-843; Santa Cruz, sc-20966); GAPDH (Santa Cruz, sc-47724); Flag (Santa Cruz, sc-51590); GFP (Abcam, Ab6556); HA (Sigma, H9658); Acetyl-lysine (Santa Cruz, sc-8649), ATRIP (Bethyl, A300-095A; Abcam, ab128529), RPA70 (Cell Signaling, 2267), ATR (Santa Cruz; sc-1887), Ac-ATRIP K32 (custom generated through Abcam), P-CHK1 S317 (Cell Signaling, 2344), P-P53 S15 (Cell Signaling, 9284), P-Rad17 S645 (Cell Signaling, 6981), P-RPA2 S33 (Bethyl, A300-246A), P-ATR T1989 (KeraFAST, EVU001), CHK1 (Santa Cruz, sc-7898), P53 (Santa Cruz, sc-263), Rad17 (Santa Cruz, sc-17761), RPA32 (Santa Cruz, sc-14692).

Immunoprecipitation

Cells were harvested in PBS and lysed for 20 min on ice in IP lysis buffer (0.75% CHAPS, 10% glycerol, 150 mM NaCl, 50 mM Tris pH 7.5) freshly supplemented with protease inhibitors. Lysates were clarified by centrifugation (13,000 rpm, 15 min at 4°C), the supernatants were then collected and diluted by same volume of dilution buffer (10% glycerol, 150 mM NaCl, 50 mM Tris pH 7.5) to adjust the CHAPS concentration to 0.375%. Protein concentration was then determined and lysates of 2 mg protein were used for immunoprecipitation reaction. For endogenous IP, the lysates were immunoprecipitated with primary antibody. For overexpression IP, target proteins were captured with anti-HA agarose beads (Sigma) or anti-FLAG M2 affinity beads (Sigma) for HA-tagged or Flag-tagged proteins. Complexes were washed 4 times with IP washing buffer (0.375% CHAPS, 10% glycerol, 150 mM NaCl, 50 mM Tris pH 7.5) supplemented with protease inhibitors. Immunoprecipitation with normal rabbit IgG or from cells that do not express epitope-tagged protein were used as negative controls.

Cell Cycle Recovery

U2OS HA-ATRIP stable cell lines were transfected with NT or ATRIP siRNA, treated with 3mM HU for 20 h (arrested), and released into 0.5 μg/ml nocodazole (Fisher) for 10 h (released). Both the suspended and adherent cells were harvested and fixed in ice-cold 70% ethanol and DNA was stained with 25 μg/ml propidium iodide (Sigma Aldrich) in PBS containing 100 μg/ml DNase-free RNase A (Qiagen). DNA content was measured by flow cytometry using a BD FACS Canto II flow cytometer and then analyzed by FlowJo software gating analysis tool (Tree Star, Inc). Stable cell lines were maintained in puromycin selection for the cell cycle recovery experiments.

Cellular Sensitivity Assay

U2OS cells stably expressing the indicated plasmid were transfected for 24 hours with ATRIP siRNA prior to plating on 96 well plates at a concentration of $2x10^3$ cells/well in triplicate. 24 hours after plating, cells were treated with media containing indicated concentrations of CPT continuously for 72 hours. After 72 hours drug exposure cell viability was indirectly determined by measurement of cellular metabolism using Alamar Blue Resazurin reagent, incubated at 1:11 dilution for 4 hours, and assayed for fluorescence according to manufacturer protocol. Fractions were normalized to vehicle treated cells for each plasmid expressing cell line. Error bars represent standard deviation from the mean, and Student's t-test p values calculated per data point as indicated from 3 replicas.

Nuclear Extraction

Nuclear extraction was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) following the manufacturer's instructions.

RPA-ssDNA Pull Down

To compare RPA-ssDNA binding affinities of WT and K32 mutant, 293T cells were transfected with WT, K32R or K32Q HA-ATRIP using Lipofectamine 3000 (Life Technologies). 48hr after transfection, cells were lysed in RPA-ssDNA binding buffer (Buffer A) (10mM Tris pH 7.5, 100mM NaCl, 10µg/ml BSA, 10% glycerol and 0.05% NP-40). Lysates were titrated to obtain a volume giving equal amount of HA-ATRIP. Trimeric RPA protein was purified by modifying a method previously described (Ball et al., 2007). His-RPA70, RPA32, and His_RPA14 or GST-ATRIP 1-107 expressing plasmids (both obtained from David Cortez) was transformed into BL21 *E. coli* and over-expressed in the presence of ampicillin. The culture was grown to an OD at 600nm of ~0.4 before induction with 1mM IPTG (Gold Biotechnology). 3hr after induction cells were pelleted, resuspended in Bugbuster protein extraction reagent (Novagen), and frozen at -80ºC after addition of 0.4kU/ml lysozyme (Novagen). The protein was purified using Ni^{2+} chelating chromatography as described previously (Daddacha et al., 2013; Weiss et al., 2002). GST-ATRIP 1-107 WT, K32R, and K32Q were purified using glutathione beads (Novagen). Purified His-RPA was analyzed by 10% SDS-PAGE. Fractions 5 and 6 were combined and concentration was determined using bovine serum albumin (Sigma) as a control. 20pmol biotinylated 69bp single stranded DNA (ssDNA) (synthesized by Invitrogen) was bound to streptavidine-conjugated beads (Novagen) by incubating in Buffer B (10mM Tris pH 7.5, 100mM NaCl) for 1hr with agitation every 5 min. Beads were washed two times with Buffer B and once with Buffer A. Purified His-RPA (8µg) was mixed with washed beads and incubated for 45 minutes in Buffer A at room temperature with constant agitation and washed three times with Buffer A. ssDNA-RPA-Streptavidin beads complex was then incubated with lysates for 2hr with constant agitation at 4ºC. The resulting complex was washed three times with buffer A and boiled in presence of SDS loading dye. The final product was resolved on 10% SDS-PAGE and analyzed by Western Blot.

Liquid Chromatography Coupled to Tandem Mass Spectrometry (LC-MS/MS)

Immunoprecipitated HA-ATRIP samples from 293T cells in the presence of TSA were reduced with 5 mM dithiothreitol (DTT) for 15 minutes at 37°C and then alkylated with 20 mM iodoacetamide (IAA) for 30 minutes at 37°C. The samples were resolved on a 10% polyacrylamide SDS gel and after staining with Coomassie G-250, the proteins were excised and subjected to in-gel digestion (12.5 ng/µl trypsin) overnight at 37°C. Extracted peptides were loaded onto a C_{18} column, eluted and detected by Orbitrap (300-1600 m/z, 1,000,000 automatic gating control (AGC) target, 1,000 ms maximum ion time, resolution 30,000). MS/MS scans in an LTQ linear-ion trap mass spectrometer (2 m/z isolation width, 35% collision energy, 5,000 AGC target, 150 ms maximum ion time; Thermo Finnigan, San Jose, CA) were acquired by data-dependent acquisition as previously described. All data were converted from raw files to the .dta format using ExtractMS version 2.0 (ThermoElectron) and searched against human reference database downloaded from the National Center for Biotechnology Information using the SEQUEST Sorcerer algorithm (version 3.11, SAGE-N). Searching parameters included mass tolerance of precursor ions (± 50 ppm) and product ion (±0.5 m/z), fully-tryptic restriction, with a dynamic mass shift for oxidized Met (+15.9949) and acetylated Lys (+42.0106), four maximal modification sites and a maximum of two missed cleavages. Only b and y ions were considered during the database match. To evaluate false discovery rate (FDR), all original protein sequences were reversed to generate a decoy database that was concatenated to the original database. The FDR was estimated by the number of decoy matches (nd) and total number of target matches (nt). FDR = 2*nd/nt, assuming mismatches in the original database were the same as in the decoy database. To remove false positive matches, assigned peptides were grouped by a precursor ion-charge state and each group was first filtered by mass accuracy (10 ppm for high-resolution MS), and by dynamically increasing correlation coefficient (Xcorr) and ΔCn values to reduce protein FDR to less than 1 percent. Peptide abundance was based on peptide extracted ion intensity (XIC) as previously reported (Herskowitz et al., 2010). Accurate peptide mass (±10 ppm) and retention time (RT) was used to derive signal intensity for each peptide across LC-MS/MS runs.

Brdu Labelling for S Phase Cells

Cells were labeled with or without 10uM BrdU for 30 minutes, harvested, fixed, stained with Alexa fluor488 conjugated anti-Brdu antibody and PI, and analyzed by flow cytometry.

SUPPLEMENTAL REFERENCES

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Herskowitz, J.H., Seyfried, N.T., Duong, D.M., Xia, Q., Rees, H.D., Gearing, M., Peng, J., Lah, J.J., and Levey, A.I. (2010). Phosphoproteomic analysis reveals site-specific changes in GFAP and NDRG2 phosphorylation in frontotemporal lobar degeneration. J Proteome Res *9*, 6368-6379.

Weiss, C., Niv, A., and Azem, A. (2002). Two-step purification of mitochondrial Hsp70, Ssc1p, using Mge1(His)(6) immobilized on Ni-agarose. Protein Expr Purif *24*, 268-273.

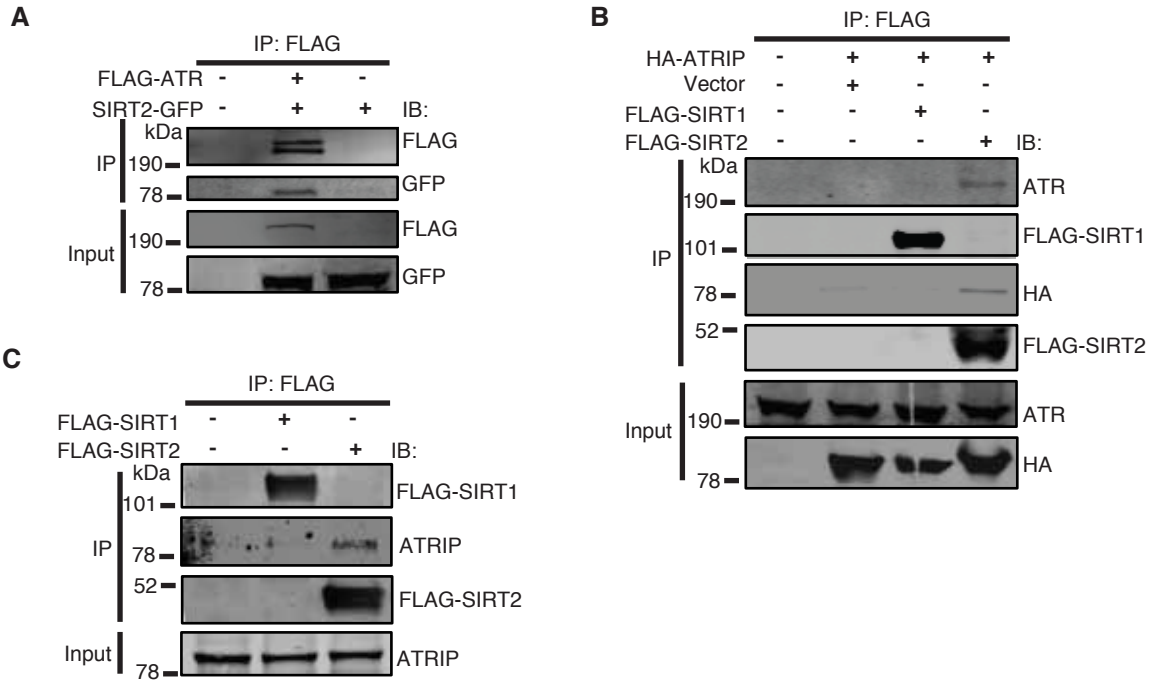
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Figure S1

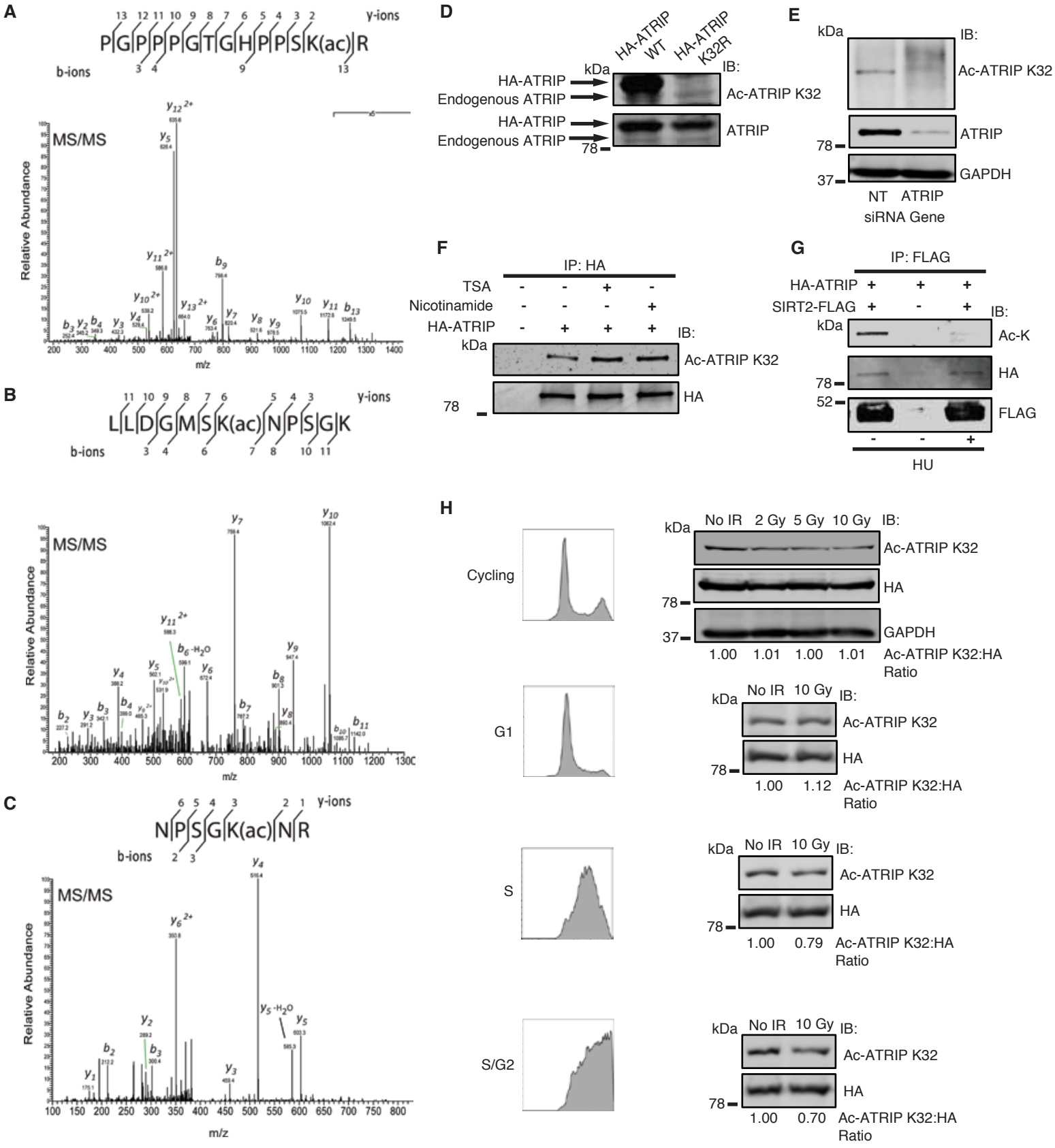
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Figure S2

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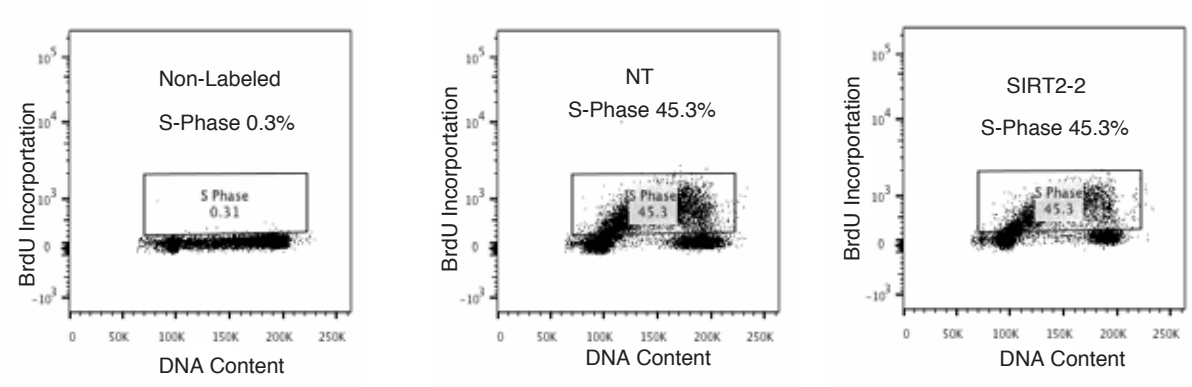

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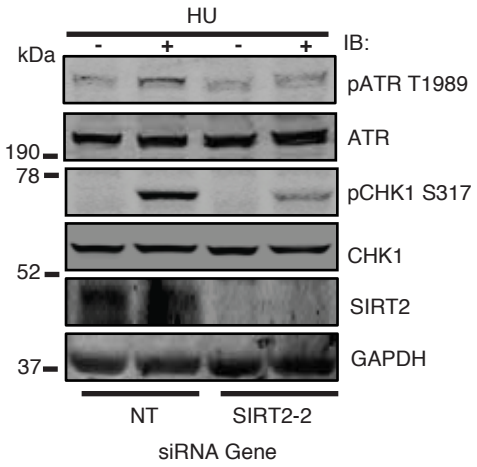

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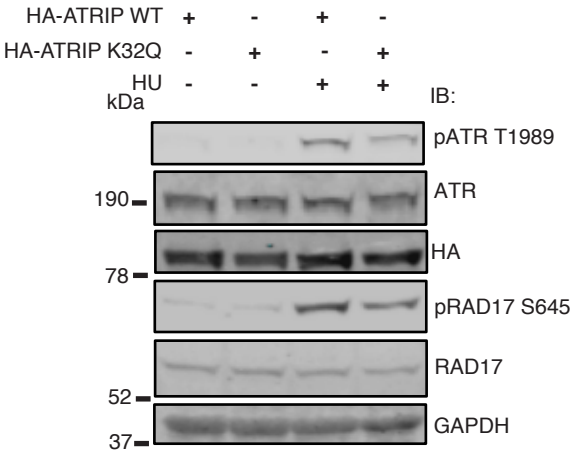

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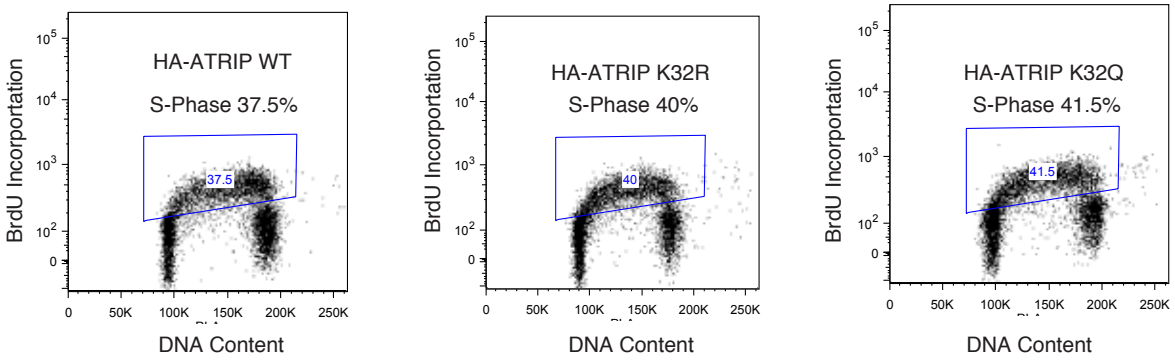
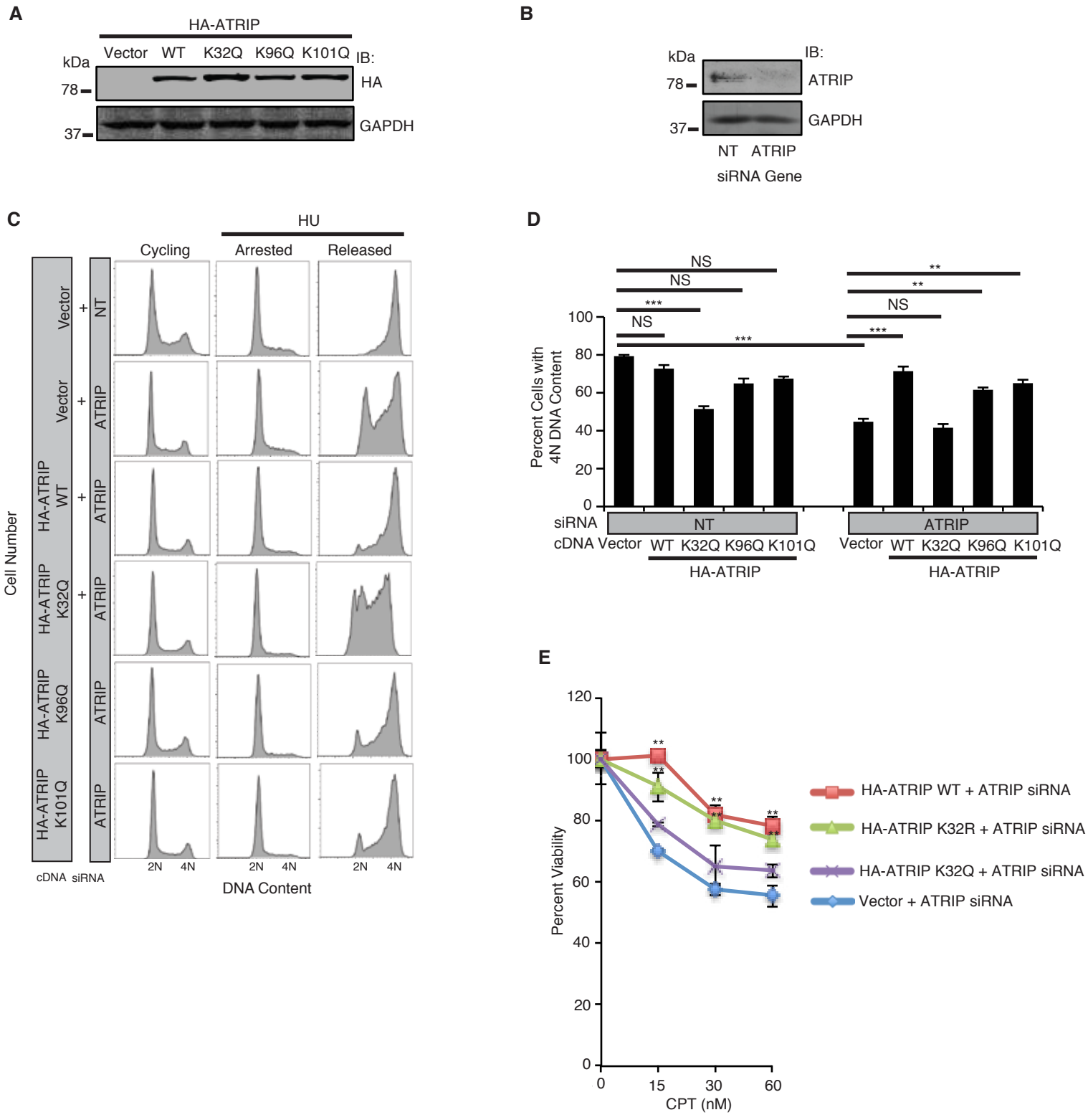


Figure S3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ATRIP
cDNA
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4N
DNA Content
D
NS
***
**
Percent Cells with 4N DNA Content
siRNA
cDNA Vector
WT
K32Q
K96Q
K101Q
HA-ATRIP
E
Percent Viability
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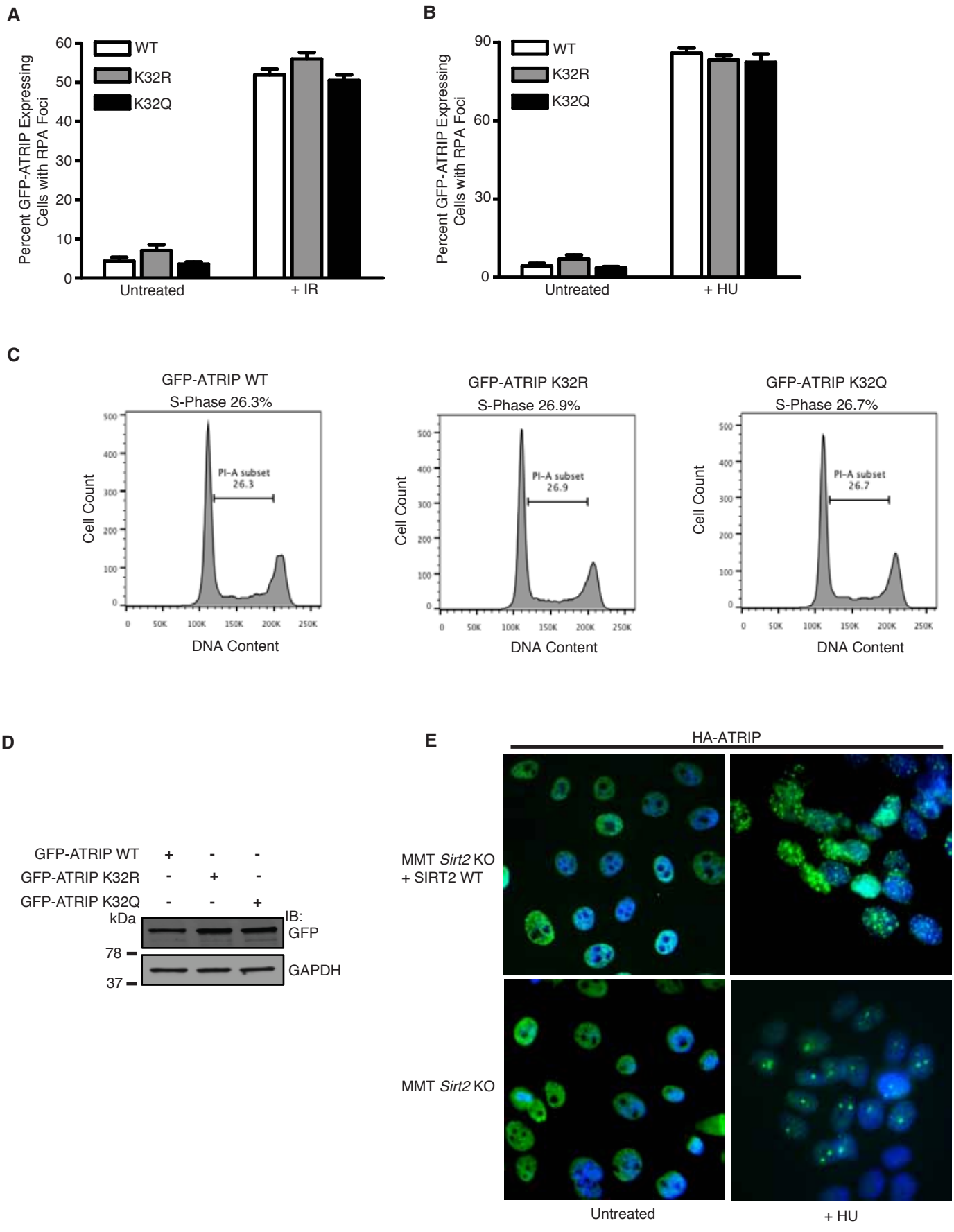
Figure S4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K32R
K32Q
Untreated
+ IR
B
Percent GFP-ATRIP Expressing Cells with RPA Foci
WT
K32R
K32Q
Untreated
+ HU
C
GFP-ATRIP WT
S-Phase 26.3%
PI-A subset 26.3
GFP-ATRIP K32R
S-Phase 26.9%
PI-A subset 26.9
GFP-ATRIP K32Q
S-Phase 26.7%
PI-A subset 26.7
Cell Count
DNA Content
D
GFP-ATRIP WT + - -
GFP-ATRIP K32R - + -
GFP-ATRIP K32Q - - +
kDa
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E
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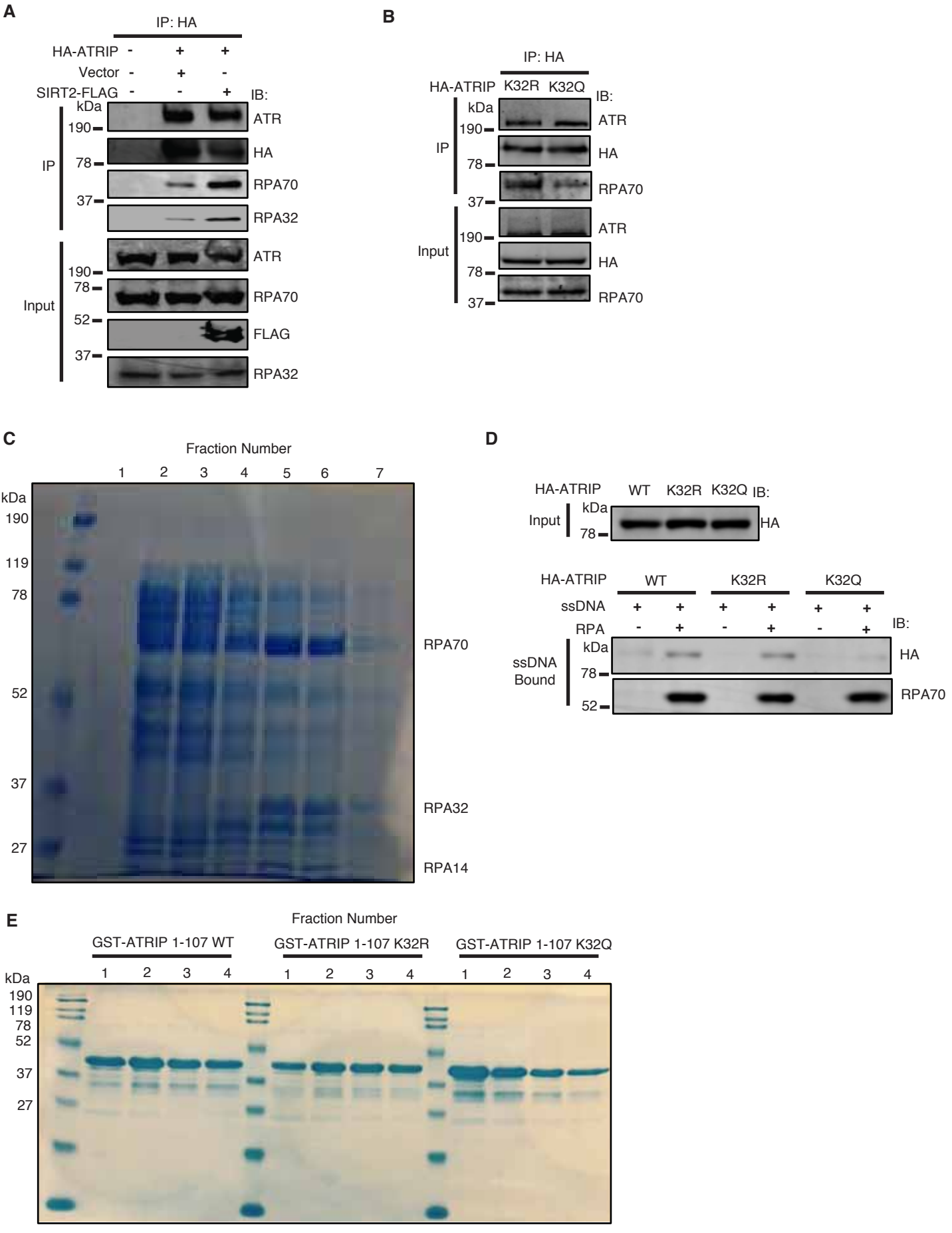
MMT Sirt2 KO
Untreated
+ HU

Figure S5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IB:
kDa
190
78
37
IP
ATR
HA
RPA70
Input
190
78
37
ATR
HA
RPA70
C
Fraction Number
1 2 3 4 5 6 7
kDa
190
119
78
52
37
27
RPA70
RPA32
RPA14
D
HA-ATRIP WT K32R K32Q IB:
Input
kDa
78
HA
HA-ATRIP WT K32R K32Q
ssDNA + + + + + +
RPA - + - + - +
IB:
ssDNA Bound
kDa
78
52
HA
RPA70
E
Fraction Number
GST-ATRIP 1-107 WT
GST-ATRIP 1-107 K32R
GST-ATRIP 1-107 K32Q
1 2 3 4 1 2 3 4 1 2 3 4
kDa
190
119
78
52
37
27

Figure S6