Supporting Information

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SI Materials and Methods

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 manufacturers' instructions.

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

SIRT2-1 (GAGATCAGCTATTTCAAGA);
SIRT2-2 (TAGATACCCTGGAGCGAAT);
SIRT2-3 (GGAGAAAGCTGGCCAGTCG);
SIRT2-4 (CGGCACGAATACCCGCTAA);
SIRT2-5 (TGGGCAGAAGACATTGCTTAT);
NT (ATGAACGTGAATTGCTCAATT);
ATR (CCTCCGTGATGTTGCTTGA);
ATRIP (GGTCCACAGATTATTAGA);
CHK1 (CTGAAGAAGCAGTCGCAGT);
CDK9 (GGCACAGTTTGGTCCGTTA).
CCNT1 (GACAAGCAACTTAGCACATAA)
CCNT2 (TGGGAATATGTGGATCCTACA)
CCNK (ATCCTGGATCTTTACTCACAA)
shRNA
SIRT2
(CCGGGCCAACCATCTGTCACTACTTCTCGAGAAGTAGTGACAGATGGTTGGCTTTTTG);

G2/M Checkpoint Assay. U2OS cells were transfected with ATR, ATRIP, NT, or indicated SIRT2 siRNA. Cells were then treated with 0 or 2 Gy IR after 72 h posttransfection, recovered for 1 h, and then placed in 15 μM of nocodazole containing media for an additional 7 h. Cells were analyzed by FASC by propidium iodide (PI) staining for DNA content and by phosphorhistone H3 ser10 (Cell Signaling; 9701) staining for mitotic cells.

Protein Stability. Cells were treated with cyclohexamide 75 μg/mL for indicated times before harvesting lysates for Western blot analysis.

Chromatin Fractionation. Chromatin fractionation was prepared as previously described (1).

Cell Survival Assay. U2OS cells were transfected with 25 nM siRNA using HiPerfect reagent, split 1:4 48 h later, treated 24 h later with or without 3 mM hydroxyurea (HU), 15 μM cisplatin, or 20 μM mitomycin C for 24 h, washed, and released into fresh media for 24–72 h before assaying for cell viability with WST-1 reagent (Roche). For gemcitabine, the drug was added for 72 h with no release. Absorbance was measured at 440 nm using a SpectraPlus plate reader. The ratio of treated compared with untreated cell viability relative to NT siRNA was determined. To control for the effect of SIRT2 depletion on NADH production, we have calculated a ratio of the viability of treated to nontreated cells, both having been depleted of SIRT2.

Colony Formation Assay. A total of 500 U2OS cells stably transfected with shSIRT2 or shLuc or MMT SIRT2 KO cells mock transfected or stably transfected with FLAG-SIRT2 WT or H187Y were seeded into six-well plates in triplicate. Cells were cultured overnight, challenged with various concentrations of HU for 24 h, washed, and released into fresh media for 8–12 d until cell colonies had grown to ~50 cells before staining with crystal violet.

Cell Cycle Recovery. U2OS cells were transfected with NT, ATR, ATRIP, or SIRT2 siRNA, treated with 3 mM HU or 15 μM aphidicolin (APH) for 20 h (arrested), and released into 0.5 μg/mL nocodazole (Fisher) for 8 h (released). Both the suspended cells and the 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). Stable cell lines were maintained in puromycin selection for the cell cycle recovery experiments.

Immunofluorescence. For RPA immunofluorescence, permeabilization was performed in preextraction buffer (10 mM Pipes pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 0.5% Triton X-100) for 10 min at room temperature. Cells were then fixed with 2% (wt/vol) paraformaldehyde for 15 min at room temperature and then blocked in 5% (wt/vol) BSA and immunostained with anti-RPA70 antibody (Cell Signaling; 2267) and anti-rabbit secondary antibody coupled to Alexa Fluor 488 (Invitrogen). Images were captured on a Zeiss Observer Z1 microscope equipped with AxiovisionRel 4.8 software. Samples were scanned using a 40× dry objective or 60× oil objective. The percentage of cells with positive RPA foci was counted from four replicate experiments with 50 cells counted per replicate. Positive RPA foci were defined as greater than five foci per cell.

Immunoblot. Cells were harvested in PBS and lysed for 30 min on ice in Nonidet P-40 buffer (200 mM NaCl, 1% Nonidet P-40, 50 mM Tris·HCl pH 8.0) freshly supplemented with protease inhibitors. Lysates were clarified by centrifugation (15,700 × *g*, 10 min at 4 °C), and the supernatants were then collected. Protein samples were then quantified with Bradford assay and resolved by SDS/PAGE, transferred onto PVDF, and probed using the appropriate primary antibodies. Detection was performed with the Odyssey system. The antibodies used were as follows: SIRT2 (Abcam; Ab67299), GAPDH (Millipore; MAB374), Flag (Santa Cruz; sc-51590), CDK9 (Abcam; Ab6544), CDK9 (custom generated through Open Biosystems) (1), GFP (Abcam; Ab6556), HA (Sigma; H9658), acetyllysine (Immunechem; ICP0380), ORC2 (BD Pharmingen; 559266), RPA70 (Cell Signaling; 2267), Ac-CDK9 K48 (custom generated through Epitomics), CCNK (custom generated through Open Biosystems) (1), CCNT1 (Santa Cruz; sc-8127), CCNT2 (Abcam; ab50979), SIRT6 (Sigma; S4322), Ac-tubulin (Sigma; T7451), tubulin (Sigma; T6074), and ATR (Santa Cruz; sc-1887).

Immunoprecipitation. For overexpression immunoprecipitation (IP), cells were harvested in PBS and lysed for 20 min on ice in IP lysis buffer [0.75% CHAPS, 10% (vol/vol) glycerol, 150 mM NaCl, 50 mM Tris pH 7.5] freshly supplemented with protease inhibitors. Lysates were clarified by centrifugation (15,700 × *g*, 15 min at 4 °C), and the supernatants were then collected and diluted by the 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 IP reaction. Target proteins were captured with anti-GFP antibody (Abcam; Ab6556) and protein A agarose beads (Invitrogen) or directly onto anti-Flag M2 beads (Sigma) for Flag-tagged proteins. Complexes were washed four times with IP washing buffer (0.375% CHAPS, 10% glycerol, 150 mM NaCl, 50 mM Tris pH 7.5) sup-

plemented with protease inhibitors. IP with rabbit serum or from cells that do not express epitope-tagged protein were used as negative controls.

For endogenous IP, cells were lysed in IP lysis buffer [20 mM Hepes, pH 7.9, 180 mM KCl, 0.2 mM EGTA, 1.5 mM MgCl2, 20% (vol/vol) glycerol, 0.1% Nonidet P-40, containing a mixture of protease inhibitors]. The lysates were IPed with primary antibody and were probed by Western blotting. The control for IP is normal rabbit IgG (Santa Cruz; sc-2027). Primary antibodies used for IP and immunoblotting were CDK9 (Santa Cruz; sc-8338), CDK9 (custom generated through Open Biosystems) (1), SIRT2 (Sigma-Aldrich; S8447), CCNT1 (Santa Cruz; sc-8127), and CCNK (custom generated through Open Biosystems) (1). Blots were incubated with peroxidase-conjugated light chain specific anti-rabbit IgG secondary antibody (Jackson ImmunoResearch; 211–032-171), using ECL (Amersham Biosciences; NA931) or IR Dye 800CW or 680RD antibodies (Li-Cor).

In Vitro or Cellular Deacetylation Assay. For in vitro deacetylation analysis of CDK9, 293T cells were transiently transfected with pCMV-Flag-CDK9 and histone acetyltransferase (p300/CBP, and pCAF) or pCMV-Flag SIRT2, and treated with 0.5 μM TSA and 20 mM nicotinamide for 12 h. Then, cells were lysed with IP buffer (20 mM Hepes pH 7.9, 180 mM KCl, 0.2 mM EGTA, 1.5 mM MgCl2, 20% glycerol, 0.1% Nonidet P-40) supplemented with 1 μM TSA. The acetyl-CDK9 proteins were immunoprecipitated using anti-FLAG M2 agarose beads (Sigma), and immunoprecipitants were washed with IP buffer containing 1 μM TSA to remove nicotinamide. Immunocaptured proteins were eluted with PBS supplemented with 0.25 mg/mL FLAG peptide (Sigma) and then used for in vitro deacetylation assay. The agarose-conjugated Flag-CDK9 was resuspended in 20 μL of deacetylation reaction buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1 mM NAD) along with the addition of purified SIRT2 (100 ng) at 30 °C for 3 h. The reaction was stopped by the addition of 5× SDS-loading buffer, and samples were analyzed for acetylation by Western blot with an antiacetyl antibody (Millipore). For cellular deacetylation analysis, HeLa cells were transiently cotransfected with Flag-CDK9, along with HA-SIRT2-WT or HA-SIRT2-H150Y, respectively, and cultured with 0.5 μM TSA for 12 h. 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 antiacetyl antibody.

In Vitro Kinase Assay. Cells were washed with PBS and lysed for 15 min at +4 with lysis buffer containing 25 mM Hepes (pH 8.0), 0.2 mM EDTA, 1.5 mM MgCl2, 0.3 M NaCl, 0.1% Triton X-100, 20 mM beta-glycerol phosphate, 0.1 mM Na3VO4, and protease inhibitors. Samples were centrifuged at 14,000 × *g* for 10 min, supernatant was collected and precleared with plain sepharose (50% slurry in lysis buffer) for 10 min at 4 °C and centrifuged again (14, 000 × *g*, 10 min, 4 °C). After addition of 2 μg of antibody, samples were incubated on the rotator for 2 h at 4 °C, then 70 μL of blocked protein G sepharose was added, followed by a second incubation for 2 h at 4 °C. After that, beads were collected (2,000 × *g*, 2 min, 4 °C), washed three times with lysis buffer without Triton, and resuspended in 35 μL of kinase buffer [50 mM Hepes (pH 7.5), 10 mM MgCl2, 6 mM EDTA, 100 μM ATP, 1 mM DTT, 0.1 mM Na3VO4, and protease inhibitors]. Kinase reaction was started by addition of GST-CTD or GST-UBE2A to beads, and samples were incubated at 30 °C for 1 h. The reaction was stopped by adding laemmli buffer and heating samples for 5 min at 95 °C. The antibodies were as follows: UBE2A (Bethyl; A300-282A), phospho-UBE2A (clone 7E4) (2), CDK9 (Santa Cruz; sc-484), P-Ser2-RNA polymerase CTD (Bethyl; A300-654A), RNA polymerase II CTD (Covance; MMS-126R), and HA (Covance; MMS-101P).

Liquid Chromatography Coupled to Tandem Mass Spectrometry. Immunoprecipitated FLAG-CDK9 samples with or without HU treatment were reduced with 5 mM DTT for 15 min at 37 °C and then alkylated with 20 mM iodoacetamide (IAA) for 30 min 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 C18 column, eluted, and detected by Orbitrap (300–1,600 *m/z*, 1,000,000 automatic gating control (AGC) target, 1,000 ms maximum ion time, resolution 30,000). Tandem mass spectrometry (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) 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%. Peptide abundance was based on peptide extracted ion intensity (XIC) as previously reported (3). Accurate peptide mass (±10 ppm) and retention time (RT) was used to derive signal intensity for each peptide across LC-MS/MS runs. An unmodified CDK9 peptide K.GSQITQQSTNQSR.N representing amino acids 346–358 was used to normalize the K44 single acetylated peptide, K.TGQK(Ac)VALK.K and K44/K48 diacetylated peptide, K.TGQK(Ac)VALK(Ac)K.V, intensity in control and HU samples. The data are representative of two replicate LC-MS/MS analyses.

1. Yu DS, et al. (2010) Cyclin-dependent kinase 9-cyclin K functions in the replication stress response. *EMBO Rep* 11(11):876–882.
2. Shchebet A, Karpiuk O, Kremmer E, Eick D, Johnsen SA (2012) Phosphorylation by cyclin-dependent kinase-9 controls ubiquitin-conjugating enzyme-2A function. *Cell Cycle* 11(11):2122–2127.
3. Herskowitz JH, et al. (2010) Phosphoproteomic analysis reveals site-specific changes in GFAP and NDRG2 phosphorylation in frontotemporal lobar degeneration. *J Proteome Res* 9(12):6368–6379.

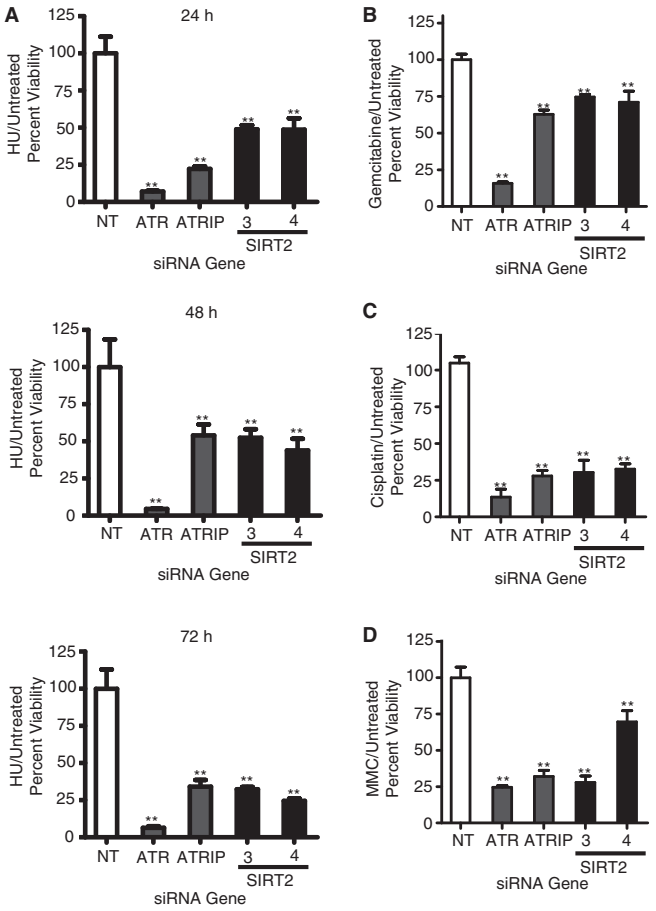

Fig. S1. SIRT2 silencing causes sensitivity to replication stress. (*A*) U2OS cells were transfected with NT, ATR, ATRIP, or SIRT2 siRNA, split 1:4 48 h later, and treated 24 h later with or without HU for 24 h. Cell viability was analyzed between 24 and 72 h after recovery from treatment. The ratio of HU-treated compared with untreated cell viability relative to NT siRNA was determined. (*B–D*) Cell viability following 24 h recovery from treatment with cisplatin or MMC or after 72 h of treatment with gemcitabine was analyzed in U2OS cells following NT, ATR, ATRIP, or SIRT2 silencing. For *A–D*, mean and SEM from at least three replicas are shown. $*P < 0.05$ and $**P < 0.01$.

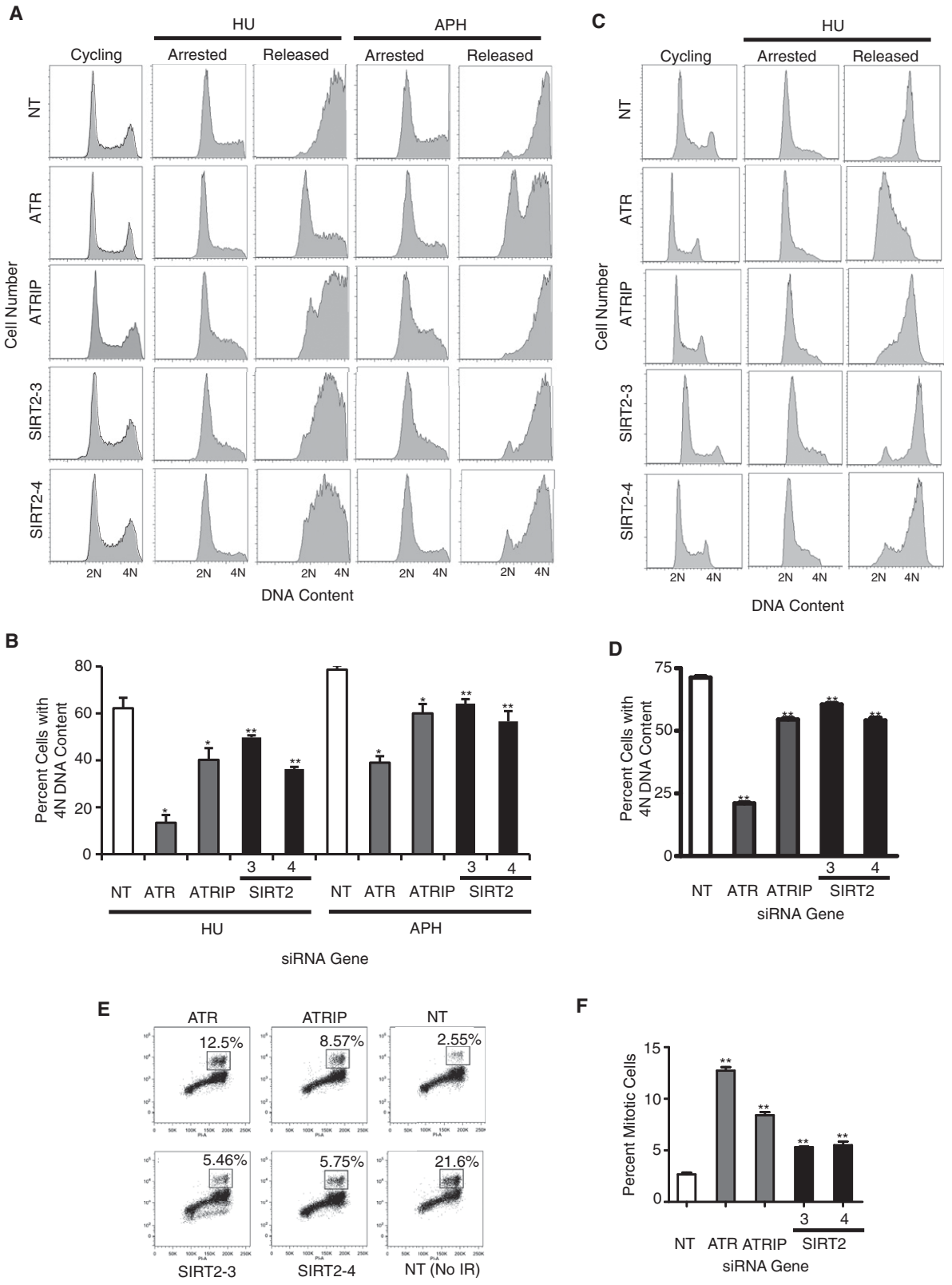

Fig. S2. SIRT2 is a replication stress response protein. (*A* and *B*) U2OS cells were transfected with NT, ATR, ATRIP, or SIRT2 siRNA, treated with 3 mM HU or 15 μM APH for 20 h (arrested), and released into nocodazole for 8 h (released). DNA content was analyzed by using flow cytometry. (*B*) The percentage (mean and SEM) of cells that completed DNA synthesis in three replicate experiments is shown. $*P < 0.05$ and $**P < 0.01$. (*C*) HCT-116 cells were transfected with NT, ATR, ATRIP, or SIRT2 siRNA, treated with 3 mM HU for 20 h (arrested), and released into nocodazole for 8 h (released). DNA content was analyzed by using flow cytometry. (*D*) The percentage (mean and SEM) of cells that completed DNA synthesis in three replicate experiments is shown. $**P < 0.01$. (*E* and *F*) U2OS cells were transfected with NT, ATR, ATRIP, or SIRT2 siRNA, treated with 2 Gy IR 72 h after transfection, recovered for 1 h, and released into nocodazole for 7 h. The percentage of cells progressing to mitosis was determined using flow cytometry for phosphohistone H3 Ser-10 staining. Mean and SEM from three replicas is shown. $**P < 0.01$.

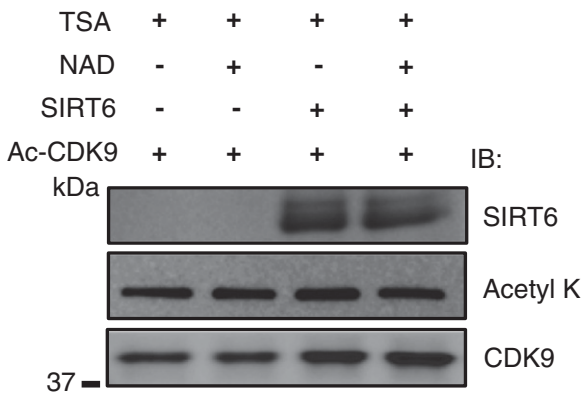

Fig. S3. SIRT6 does not deacetylate CDK9. Acetylated CDK9 was isolated from 293T cells transfected with FLAG-CDK9, p300/CBP, and pCAF and incubated in an in vitro deacetylation assay with FLAG-SIRT6 isolated from 293T cells in the presence of TSA with or without NAD. The reaction mixtures were separated by SDS/PAGE and immunoblotted with antibodies against acetyllysine, SIRT6, and CDK9.

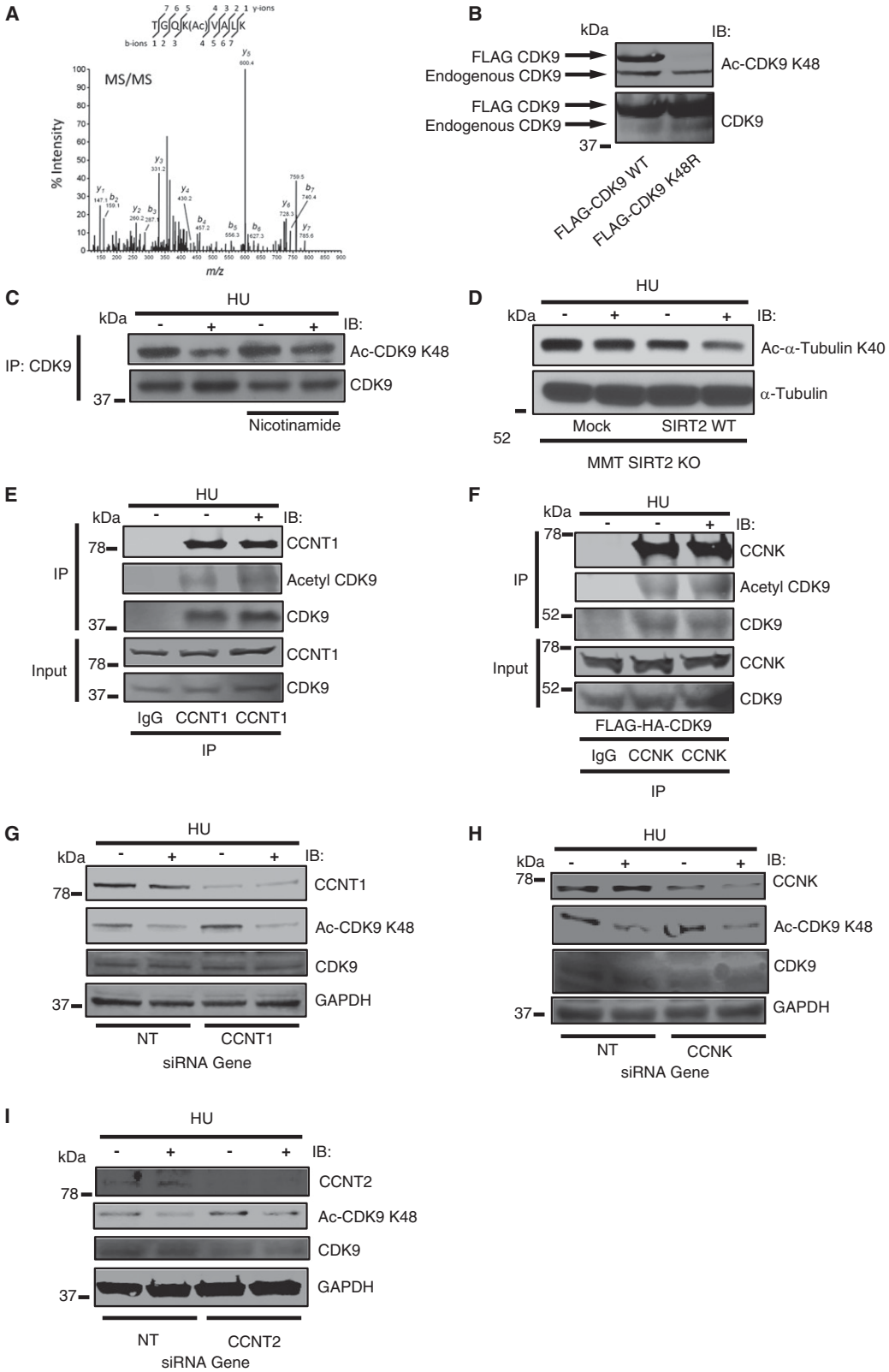

Fig. S4. CDK9 K48 deacetylation in response to replication stress is independent of its cyclin regulatory subunits. (*A*) MS/MS peptide spectra of immunoprecipated FLAG-CDK9 expressed in 293T cells shows site-specific CDK9 acetylation at lysine residue 44. (*B*) Validation of Ac-CDK9 K48 antibody. Western blot analysis of 293T cells transfected with FLAG-CDK9 WT or FLAG-CDK9 K48R shows Ac-CDK9 K48 antibody recognizing endogenous CDK9 and FLAG-CDK9 WT but not FLAG-CDK9 K48R. (*C*) HeLa cells treated with or without HU and nicotinamide were harvested, immunoprecipitated with an anti-CDK9 antibody, separated by SDS/PAGE, and immunoblotted with antibodies against Ac-CDK9 K48 and CDK9. (*D*) MMT *Sirt2* KO cells mock transfected or stably transfected with SIRT2 WT were treated with or without HU, harvested, separated by SDS/PAGE, and immunoblotted with antibodies against Ac–α-tubulin K40 and

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α-tubulin. (*E* and *F*) The 293T cells treated with or without HU were harvested, immunoprecipitated with an anti-CCNT1 or anti-CCNK antibody, respectively, separated by SDS/PAGE, and immunoblotted with antibodies against CCNT1, CCNK, acetyllysine, and CDK9. (*G*–*I*) The 293T cells were transfected with NT, CCNT1, CCNT2, or CCNK siRNA and treated with or without HU, harvested, separated by SDS/PAGE, and immunoblotted with antibodies against CCNT1, CCNT2, CCNK, Ac-CDK9 K48, CDK9, and GAPDH.

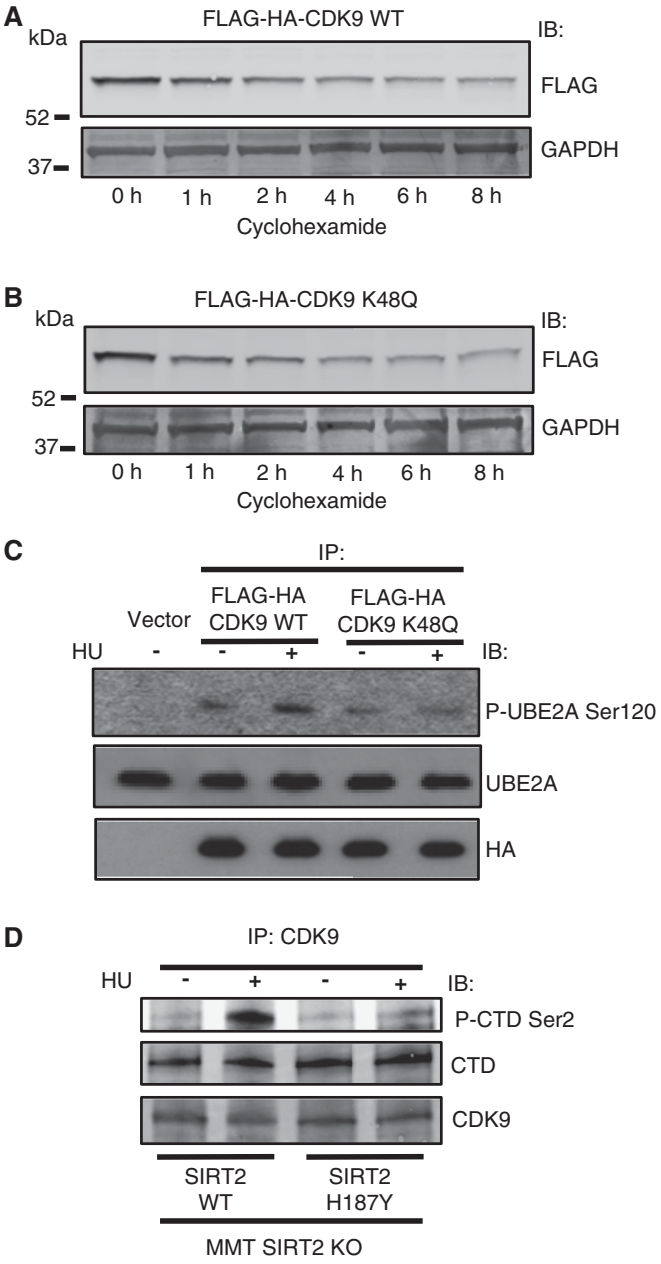

Fig. S5. Lysine 48 acetylation impairs CDK9 kinase activity but does not affect CDK9 protein stability. (*A* and *B*) U2OS cells stably expressing wild-type FLAG-HA CDK9 or FLAG-HA CDK9 K48Q were treated with 75 μg/mL cyclohexamide for the indicated time periods before harvesting for Western blot analysis with antibodies against FLAG and GAPDH. (*C*) FLAG-HA-CDK9 was purified from U2OS cells stably expressing an empty vector, wild-type FLAG-HA CDK9, or FLAG-HA CDK9 K48Q, treated with or without HU, and incubated in an in vitro kinase reaction with recombinant UBE2A purified from bacterial cells as substrate. The reaction mixtures were separated by SDS/PAGE and immunoblotted with antiphospho-UBE2A Ser120, antipan-UBE2A, and anti-HA antibodies. (*D*) SIRT2 deacetylation promotes CDK9 kinase activity. Endogenous CDK9 was purified from MMT S2KO cells stably expressing SIRT2 WT or SIRT H187Y, treated with or without HU, and incubated in an in vitro kinase reaction with recombinant GST-CTD purified from bacterial cells as substrate. The reaction mixtures were separated by SDS/PAGE and immunoblotted with antiphospho-CTD Ser2, antipan-CTD, and anti-CDK9 antibodies.

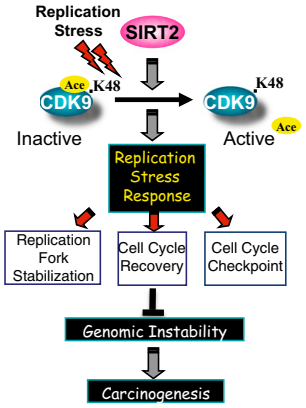

Fig. S6. Model of SIRT2 function in the replication stress response. In response to replication stress, SIRT2 deacetylates CDK9 at lysine 48, which stimulates CDK9 kinase activity and promotes recovery from replication stress.