Supporting Information

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SI Methods

Plasmids. pcDNA-mycBirAR118G, pCMV-Rep40 (pND229), pCMV-Rep52 (pND230), pCMV-Rep68 Y156F M225G (pND226), and pCMV-Rep78 Y156F M225G (pND227) have been previously described (45). BirA* and each of the Rep sequences were amplified by PCR, after which overlapping PCR was used to fuse the BirA* fragment to the N terminus of each Rep fragment. The resulting amplicons were cloned into pcDNA3.1+. FLAG- and T7-tagged Rep proteins were generated by cloning Rep PCR products into either the pEGFP-C1 vector (Clontech) containing an N-terminal FLAG tag or a T7 tag, respectively. K372A mutants were generated by site-directed mutagenesis. ZNF truncation mutants were generated by PCR amplification of amino acids 1–529 (Δ91/87), 1–558 (Δ63), or 1–577 (Δ44), using either pND230 or pND227 as a template. The amplified fragments were then cloned into the N-terminal T7 vector described above. FLAG-PP1α was generated by cloning PCR-amplified PP1α from an EST clone (IRAUp969F0817D) obtained from Genome Cube into the N-terminal FLAG vector described above. pC1-FLAG-wtKAP1 was provided by H.M.R. and was used for cloning PCR-amplified wild-type KAP1 into untagged pcDNA3.1+. PP1γ–NIPP1 and NIPP1-WT, -RATA, -FHAm, -amino acids 1–142, -amino acids 143–224, -amino acids 225–251, -amino acids 1–224, and -amino acids 143–351 fused to eGFP were provided by M.B.

Western Blot Analysis. Cells were lysed in RIPA buffer, and proteins were separated on a 6–12% SDS/PAGE gel and transferred to a nitrocellulose membrane (Hybond-C Extra nitrocellulose; Amersham Biosciences). Membranes were blocked with either 5% nonfat dry milk or 2.5% BSA (for phospho-antibodies) in PBS containing 0.5% Tween-20 (PBST) for 45 min at room temperature and then were incubated with primary antibody for 2 h at room temperature. The membranes were then washed three times (10 min each washing) in PBST and were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG (Bio-Rad) for 1 h at room temperature. After three 10-min washes in PBST, membranes were developed using West Pico ECL reagent (Thermo Scientific). The following primary antibodies were used: HSP90 (1:10,000; sc-69703; Santa Cruz), KAP1 (1:000; MAB3662; Chemicon), p-KAP1-S824 (1:2,000; A300-767A; Bethyl), VP (03-61058; 1:500; ARP), Rep (61069; 1:100; Progen), dsRed (632496; 1:2,000; Clontech), CHD3 (1:4,000; A301-219A; Bethyl), SETDB1 (1:1,000; ab12317; Abcam), FLAG (1:1,000; F1804; Sigma), T7 (69522-3; 1:10,000; Merck Millipore), pChk2 (1:1,000; 2661S; New England Biolabs), GFP (1:5,000; 11814460001; Roche), PP1α (1:1,000; A300-904A; Cambridge Bioscience), PP1β (1:1,000; A300-905A; Cambridge Bioscience), PP1γ (1:1,000; sc-6108; Santa Cruz), and avidin peroxidase (1:8,000; Sigma).

Lentiviral Transductions. pC-SIREN–based lentiviral vectors expressing either a hairpin targeting the 3′ UTR of KAP1 (shKAP1; GATCCGCCTGGCTCTGTTCTCTGTCCTTTCAAGAGAAGGA CAGAGAACAGAG CCAGGTTTTTTACGCGTG) or the corresponding empty vector (shEMPTY) were provided by H.M.R. pCSIG-eGFP lentiviral vectors provided by S.J.D.N. were modified to contain the truncated CMVΔ5 promoter (60) in place of the spleen focus-forming virus (SFFV) promoter and to express KAP1-WT, KAP1-S824D, or KAP1-S824A. Lentiviral vector-containing supernatants were produced by the common triple-transfection method using the VSV-G plasmid, HIV-Gag/Pol/Rev/Tat packaging plasmid, and the lentiviral transfer plasmid in a 3:2:1 molar ratio. Supernatants were harvested 48 and 72 h after transfection, pooled, filtered, and frozen at −80 °C until use. For transduction, 1×10^6 293T cells were transduced in a six-well format using 0.5–1.6 mL of particle-containing supernatant diluted with the appropriate amount of DMEM + 10% FBS. For complementation experiments, cells were transduced with pCSIG-based expression constructs and either shKAP1 or shEMPTY 72 h later. Cells were then infected with AAV and Ad5 48 h after knockdown.

Immunofluorescence. 293T cells were seeded at a density of 2×10^5/mL on poly-L-lysine (Sigma)–coated coverslips in 24-well plates the day before transfection. Four hours before transfection, DMSO or ATMi was added to the appropriate wells to a final concentration of 10 μM. Cells were then transfected with 20 ng of empty vector or pRep78-GFP using 2 μL Lipofectamine 2000 in 50 μL SF Opti-MEM. The next day, cells were infected with Ad5 (2 pfu per cell) in a total volume of 160 μL for 1 h, after which the medium was replaced with fresh DMEM + 10% FBS. Cells were fixed 24 h after Ad5 infection in 4% paraformaldehyde for 10 min at room temperature, washed in PBS, permeabilized in 0.1% Triton X-100 for 10 min at room temperature, and washed again in PBS. Cells were then incubated with primary antibody (1:1,000; α-p-KAP1-S824 antibody) diluted in PBS + 1% BSA for 2 h at room temperature, washed, and then incubated with the secondary antibody, rabbit IgG2b-AlexaFluor 594 (1 μg/mL) (1:1,000; BioLegend) diluted in PBS for 1 h at room temperature. Cells were then washed a final time and mounted in Prolong Gold Antifade Reagent (Invitrogen). Images were visualized using an Eclipse Ti-E inverted confocal microscope and analyzed with NIS Elements C software.

siRNA Transfections. In a 24-well format, 2×10^5 cells were transfected with 50 nM siKAP1.2 (GAAAUGUGAGCGUGU-ACUG) and siKAP1.4 (GAACGAGGCCUUCG GUGAC) or 100 nM siCHD3 (L-005046-00-0005; Dharmacon) and siSETDB1 (L-020070-00-0005; Dharmacon) siRNA using 2 μL DharmaFECT (Dharmacon) in 50 μL Opti-MEM (Gibco). Six hours later, the medium was replaced with fresh DMEM + 10% FBS. Twenty-four hours after transfection, cells were replated into a 12-well format. Thirty-six hours after transfection, cells were subjected to a second transfection as described above, using 4 μL DharmaFECT in 100 μL Opti-MEM. Four hours after the second transfection, cells were infected with AAV2 (10 IU per cell) and Ad5 (2 pfu per cell) as described for the viral replication experiments. For PP1 depletion experiments, cells were transfected only once with a total of 40 nM siPP1α (L-008927-00-0005; Dharmacon), siPP1β (L-008685-00-0005; Dharmacon), or both as described above. Twenty-four hours after transfection, cells were replated into a 12-well format for infection the next day as described above.

Real-Time PCR. For analysis of viral replication, total DNA was extracted using the Qiagen DNeasy Blood and Tissue DNA extraction kit. Viral DNA was quantified by real-time PCR using the SYBR Green JumpStart Taq ReadyMix for qPCR (Sigma-Aldrich) using an ABI PRISM system (Applied Biosystems). Cap and Ad5 100kd-specific primers and a pDG-based (61) standard curve were used for absolute quantification; the signal was normalized to cyclophilin. The primers were Cap forward (5′-TTCTCAGATGCTGC GTACCGGAAA-3′), Cap reverse (5′-TCTGCCATTGAGGTGGTACTTGGT-3′); Ad5 100kd forward (5′- TCATTACCCAGGGCCACATT-3′), Ad5 100kd reverse (5′-CCTCGTCCAAAACCTCCTCT-3′); and cyclophilin forward

(5′-GCTGGACCCAAC ACAAATG-3′), cyclophilin reverse (5′-TGCCATCCAACCACTCAGTCT-3′).

qRT-PCR. Total RNA was extracted using the RNeasy kit (Qiagen) after DNaseI (Qiagen) treatment for 15 min at 37 °C. Reverse transcription was performed using the High Capacity Reverse Transcription kit (Applied Biosystems). cDNA was quantified by real-time qPCR on an ABI PRISM system (Applied Biosystems) using TaqMan Universal PCR Master Mix (Life Technologies) and custom-designed primer-probe mixes (Eurofins). Primers were p5 forward (5′-AACAAGGTGGTGGATGAGT-3′), p5 reverse (5′-CGTTTACGCTCCGTGAGATT-3′) and p40 forward (5′-GAAGCAAGGCTCAGAGAAA-3′) and p40 reverse (5′-CCTCTCTGGAGGTTGG TAGATA-3′). Probes were p5 (5′-FAM-ACGTGGTTGAGGTGGAGCATGAT-TAM-3′) and p40 (5′-FAM-AGGAAATCAGGACAA CCAATCCCGT-TAM-3′). Relative expression levels were determined with the ΔΔCt quantification method using 18S ribosomal RNA (Taqman predeveloped assay reagents, human 18S rRNA; Applied Biosystems) as a housekeeping reference gene.

Analysis of p-KAP1-S824 Levels. Phosphorylation of KAP1-S824 was investigated in 293T cells that were either infected with AAV2/Ad5 or transfected with various Rep-expressing constructs using linear PEI or NIPP1-expressing constructs using TransIT-LT1 (Mirus). Where relevant, cells were pretreated with either DMSO or 10 μM ATMi 4 h before infection/transfection, and inhibitors were maintained throughout. Infections were performed as described above, and transfections were performed at ∼70% confluency using 1 μg DNA per 8×10^5 cells and 4 μL PEI per microgram of DNA. The medium was changed 6 h after transfection, and cells were harvested for Western blot 27 h after infection/transfection.

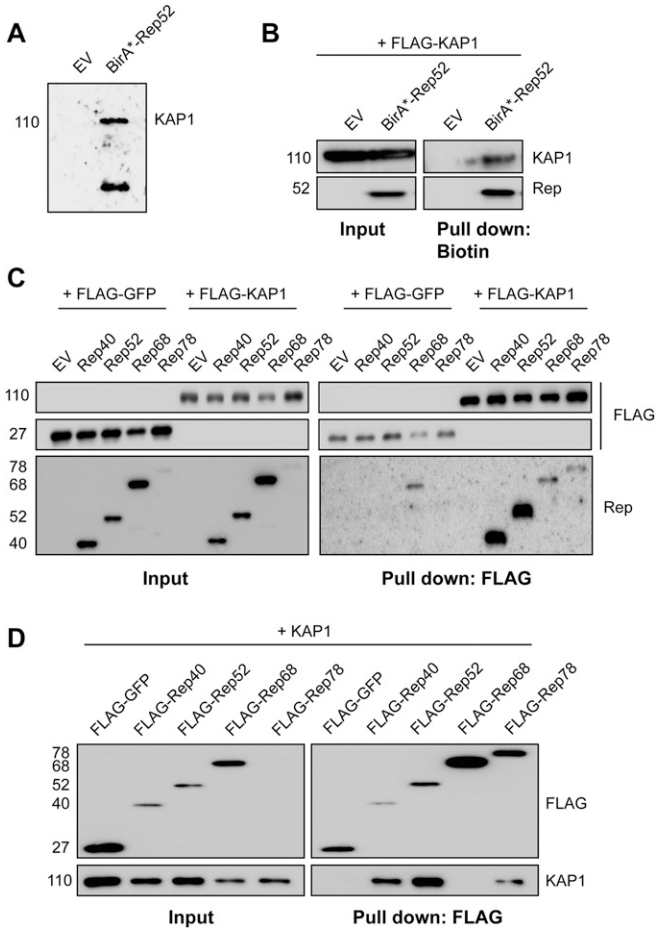

Fig. S1. The AAV2 Rep proteins physically interact with KAP1. (*A*) Immunoblot of biotinylated proteins purified from the BirA*-Rep52 BioID screen using anti-KAP1. EV, empty vector. (*B*) Verification of BioID using exogenous FLAG-KAP1; purified biotinylated proteins from 293T cells expressing FLAG-KAP1 with either empty vector or BirA*-Rep52 were analyzed for Rep and KAP1 by Western blot. (*C*) Cross-linked coimmunoprecipitation for FLAG-tagged proteins from 293T cells expressing FLAG-KAP1 or a FLAG-GFP control and each of the four Rep proteins. (*D*) Cross-linked coimmunoprecipitation for FLAG-tagged proteins from lysates of 293T cells expressing FLAG-GFP, FLAG-Rep40, FLAG-Rep52, FLAG-Rep68, or FLAG-Rep78 and KAP1.

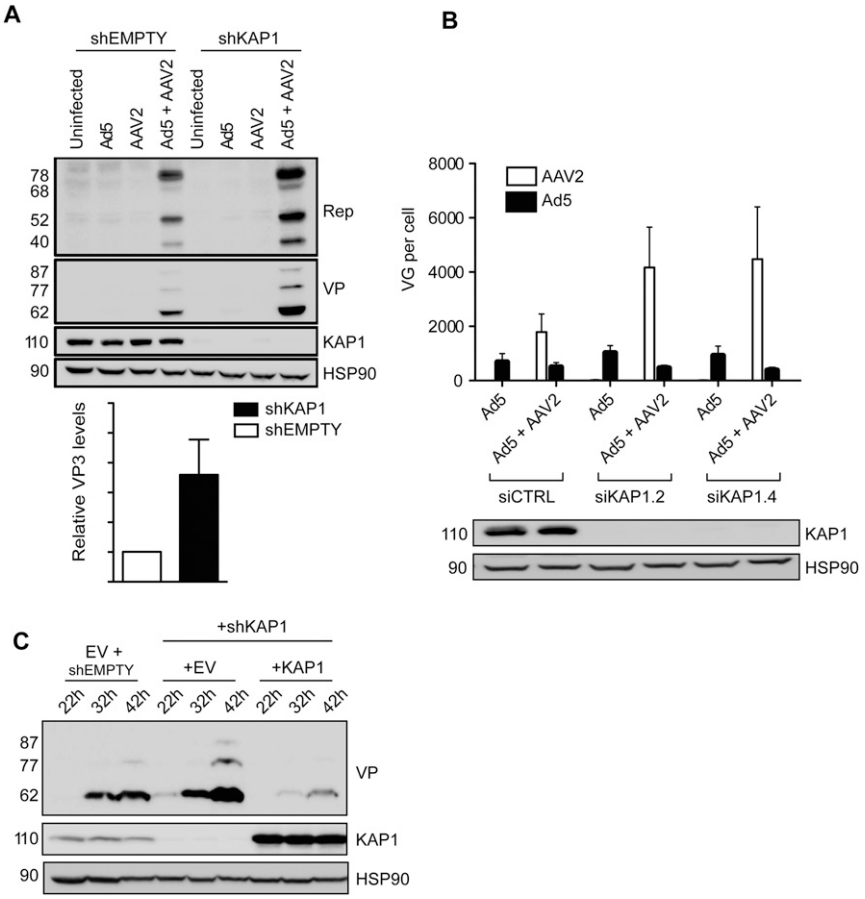

Fig. S2. AAV2 replication and protein expression in KAP1-depleted cells. (*A*) Rep and capsid protein (VP) expression and KAP1 knockdown efficiency in AAV2- and Ad5-infected control (shEMPTY) and KAP1-depleted (shKAP1) 293T cells. Data are reported as mean ± SEM, $n = 3$. (*B*) AAV2 replication in control (siCTRL) or KAP1-depleted (siKAP1.2/siKAP1.4) cells. Viral genome replication was analyzed by qPCR, and KAP1 knockdown efficiency was analyzed by Western blot. Data are reported as mean ± SEM, $n = 4$. (*C*) AAV2 capsid (VP) and KAP1 protein levels at different time points in control cells and KAP1-depleted cells complemented with shRNA-resistant *KAP1* or an empty vector (EV) control and coinfected with AAV2 and Ad5.

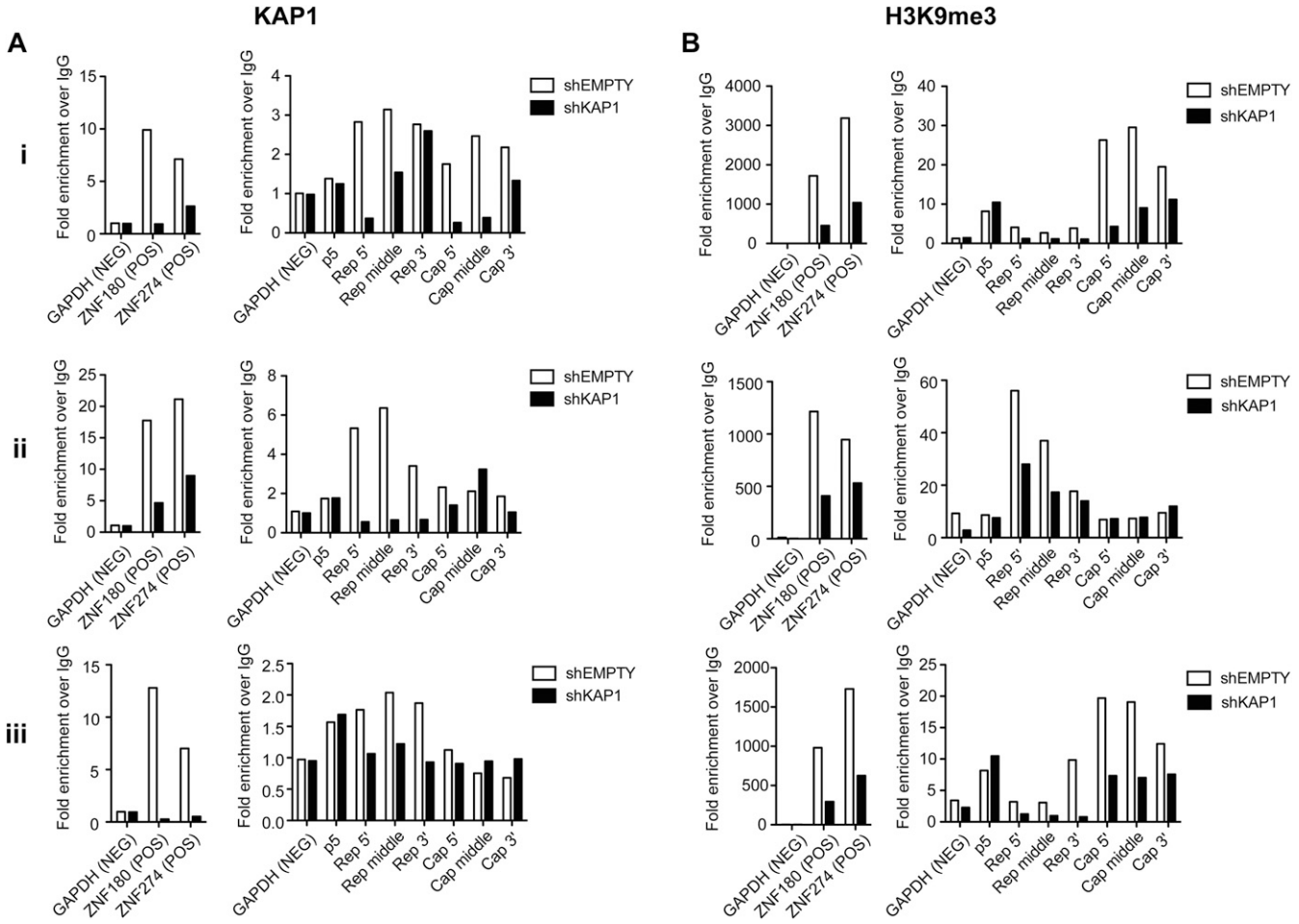

Fig. S3. ChIP-qPCR performed on control or KAP1-depleted 293T cells infected with AAV2 (100 IU per cell). Purified chromatin was analyzed by qPCR using primers for the viral p5 promoter or various regions of the *rep* and *cap* ORFs. *GAPDH* was used as a negative control, and the ZNF genes *ZNF180* and *ZNF274* were used as positive controls. Each of the three independent repeats (*i–iii*) is shown for (*A*) KAP1-specific and (*B*) H3K9me3-specific ChIP experiments (Fig. 1 *D* and *E*).

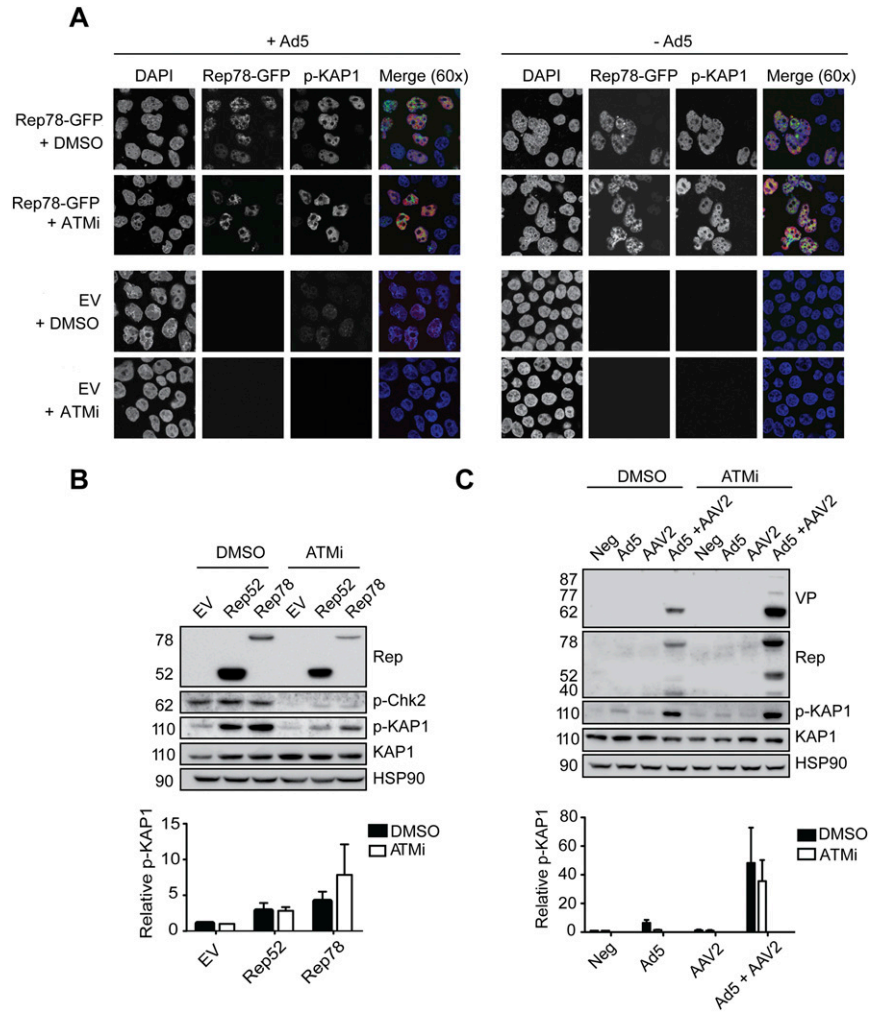

Fig. S4. Phosphorylation of KAP1-S824 by the Rep proteins is independent of ATM activation. (*A*) p-KAP1-S824 levels in 293T cells pretreated with ATMi and expressing Rep78-GFP with (*Left*) and without (*Right*) Ad5 infection. (*B* and *C*) p-KAP1-S824 levels analyzed in 293T cells pretreated with ATMi. (*B*) Cells were transfected with empty vector (EV), Rep52, or Rep78. p-Chk2 was monitored to assess the efficiency of ATM inhibition. p-KAP-S824 levels were normalized to Rep levels to correct for differences in transfection efficiency as a result of pretreatment with ATMi. (*C*) Cells were infected with AAV2 and Ad5. Values are reported as mean ± SEM, $n = 4$.

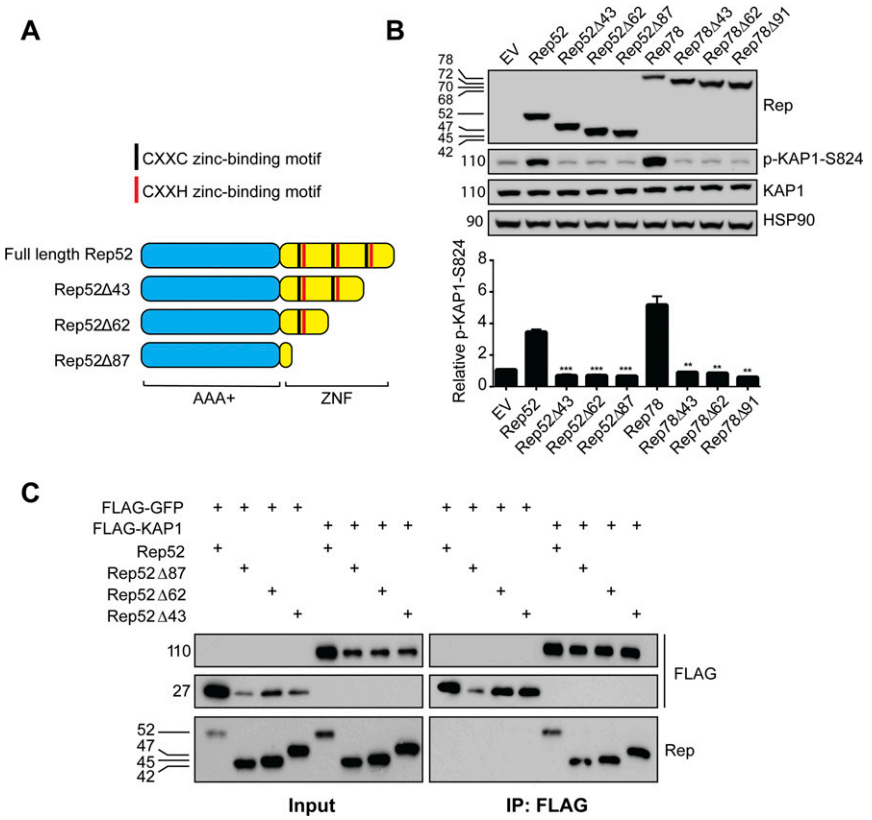

Fig. S5. Phosphorylation of KAP1-S824 in the presence of Rep52 and Rep78 is dependent on the Rep C-terminal ZNF domain. (*A*) Schematic diagram representing full-length Rep52, comprising an ATPase domain (AAA+) and a ZNF domain, and the C-terminal truncation mutants in which the ZNF domain is progressively removed. Black bars indicated a CXXC zinc-binding motif, and red bars indicate a CXXH zinc-binding motif. (*B*) p-KAP1-S824 in 293T cells transfected with full-length Rep52 and Rep78 or truncation mutants in which the C-terminal ZNF domain is progressively removed. Values are reported as mean ± SEM, $n = 3$. (*C*) Cross-linked coimmunoprecipitation for FLAG-tagged proteins from 293T cells expressing FLAG-KAP1 or a FLAG-GFP control with full-length Rep52 or each of the Rep52 C-terminal ZNF truncation mutants. Statistical significance was determined by unpaired *t* test, $^{**}P < 0.01$, $^{***}P < 0.001$.

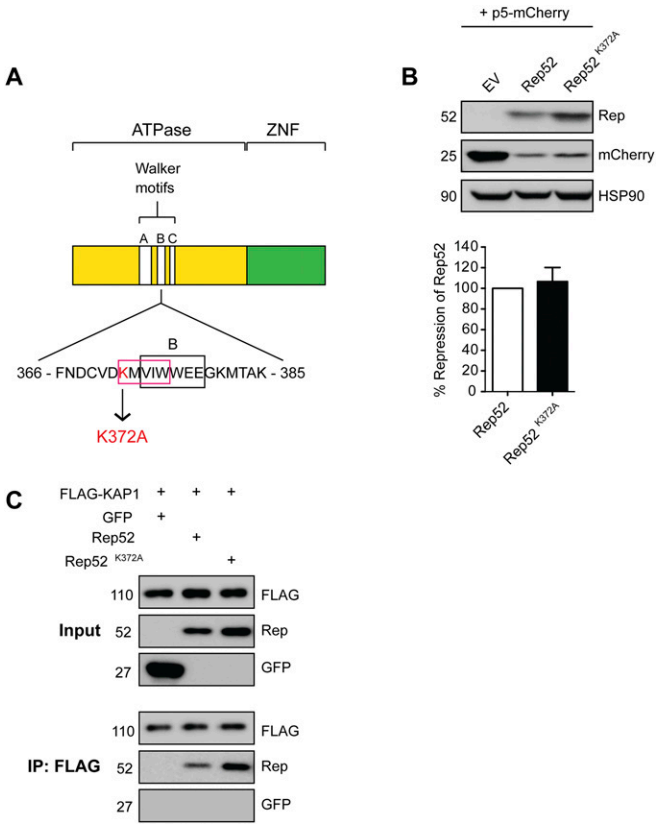

Fig. S6. Validation of Rep-K372A PP1-binding mutant. (*A*) Depiction of the PP1-binding site in the Rep ATPase domain. The Walker B motif is outlined in black, and the partially overlapping consensus binding site is outlined in pink. Lysine 372 alone was subjected to mutagenesis to preserve Rep ATPase/helicase function. (*B*) Rep-mediated repression of AAV2 p5 is dependent on a functional ATPase/helicase domain (62). To verify ATPase activity of Rep52^{K372A}, 293T cells cotransfected with a p5-mCherry reporter construct and Rep52 or Rep52^{K372A} expression plasmids were analyzed for p5 activity by Western blotting for mCherry (45). Protein levels were quantified using ImageJ software. Values are reported as mean ± SEM, $n = 3$. EV, empty vector. (*C*) Cross-linked coimmunoprecipitation for FLAG-tagged proteins from 293T cells expressing FLAG-KAP1 or a FLAG-GFP control with the various Rep proteins.

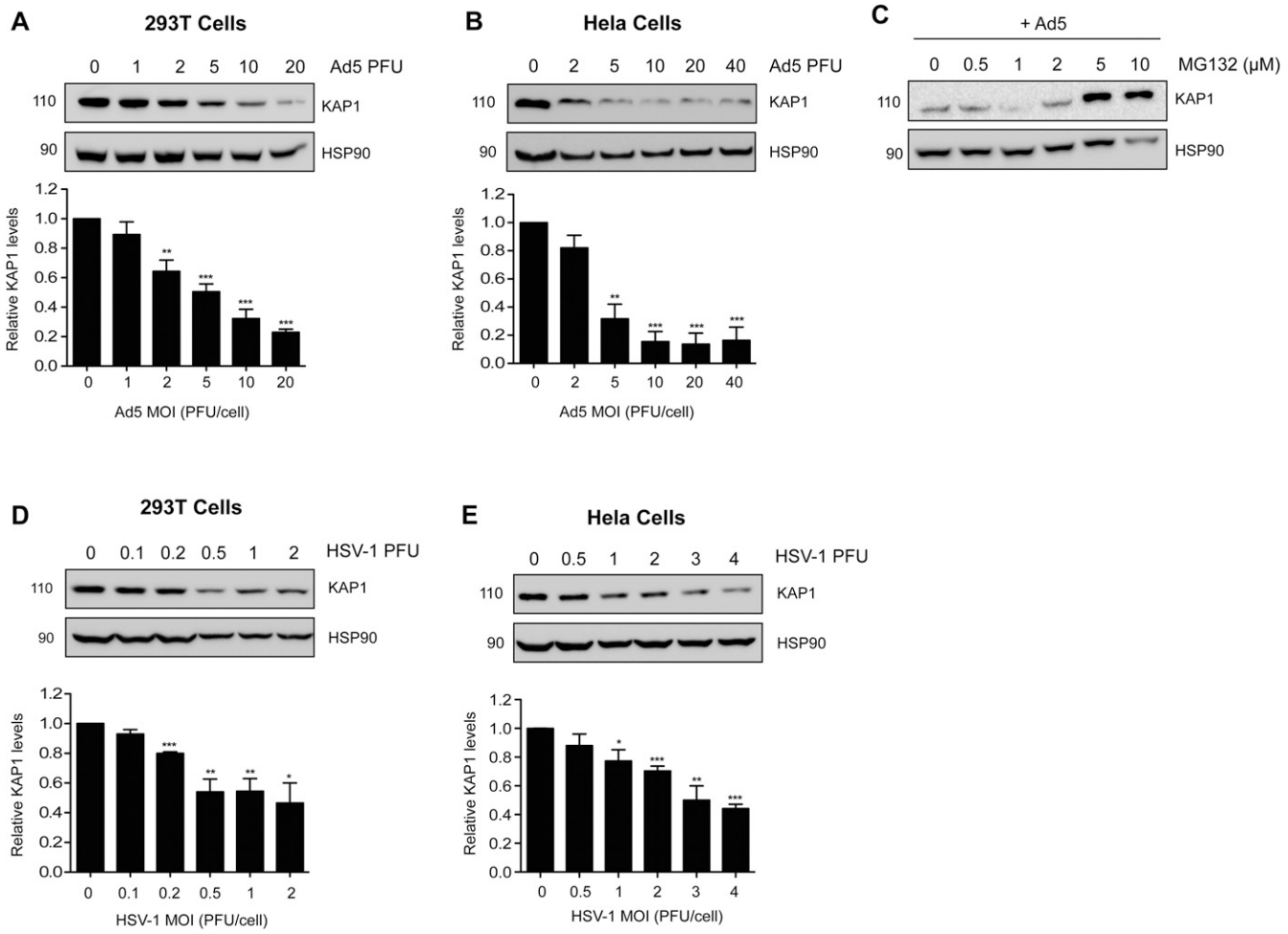

Fig. S7. Ad5 and HSV-1 infection leads to KAP1 degradation. (*A* and *B*) Immunoblot of KAP1 in 293T cells (*A*) and HeLa cells (*B*) infected with Ad5 at the stated MOI (stated as pfu per cell). Values are reported as mean ± SEM, $n = 3$. (*C*) Immunoblot of KAP1 in HeLa cells treated with MG132 at the stated concentrations and infected with Ad5 at 10 pfu per cell. (*D* and *E*) Immunoblot of endogenous KAP1 in 293T (*D*) and HeLa (*E*) cells infected with HSV-1. Values are reported as mean ± SEM, $n = 3$. Statistical significance was determined by unpaired *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

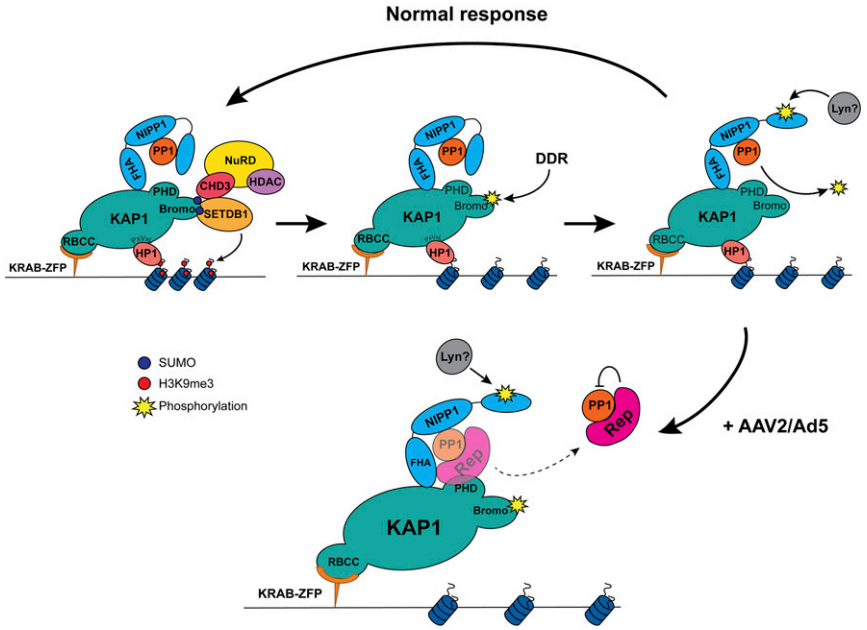

Fig. S8. Schematic representation of a model for the regulation of KAP1-S824 phosphorylation by NIPP1, PP1, and Rep. (*Upper*) Regulation in the absence of AAV2 infection. Inactive NIPP1-PP1 is recruited to KAP1 via the NIPP1 FHA domain. Initiation of a DDR leads to phosphorylation of KAP1-S824, allowing heterochromatin relaxation and repair. The DDR also leads to the inactivation of NIPP1 through phosphorylation of the C-terminal region, possibly through the tyrosine kinase Lyn, allowing PP1 to restore homeostatic levels of p-KAP1-S824. (*Lower*) In the event of AAV2/Ad5 coinfection, Rep interferes with this pathway by competing for PP1 and thus acts to maintain high levels of p-KAP1-S824 triggered by infection.

Table S1. Peptides identified by BioID for KAP1 and various known interaction partners of the Rep proteins

Identified protein	Accession number	Bait protein	Unique peptides	Sequence coverage, %	Protein ID probability, %
KAP1	Q13263	BirA*-Rep40	2	3.1	100
		BirA*-Rep52	8	12.9	100
		BirA*-Rep68	3	6.0	100
		BirA*-Rep78	1	1.3	100
RUVBL1	Q9Y265	BirA*-Rep40	2	7.2	100
		BirA*-Rep52	7	22.1	100
		BirA*-Rep68	1	2.9	99
		BirA*-Rep78	1	2.9	99
MRE11	P49959	BirA*-Rep40	1	2.0	99
		BirA*-Rep52	8	19	100
SNW1	Q5R7R9	BirA*-Rep40	5	23.7	100
		BirA*-Rep52	9	24.1	100
		BirA*-Rep68	1	2.8	100
		BirA*-Rep78	2	4.3	100
MDC1	Q14676	BirA*-Rep40	2	2.0	100
		BirA*-Rep52	4	3.3	100
		BirA*-Rep68	1	0.5	100
TAF1/SET	Q01105	BirA*-Rep40	1	4.8	100
		BirA*-Rep68	1	4.8	100
Nucleolin	P19338	BirA*-Rep40	1	2	99
		BirA*-Rep52	10	16.2	100
		BirA*-Rep68	13	18	100
		BirA*-Rep78	4	6.2	100

Table S2. ChIP-qPCR primers

Gene	Forward	Reverse
GAPDH	CACCGTCAAGGCTGAGAACG	ATACCCAAGGGAGCCACACC
ZNF180	TGATGCACAATAAGTCGAGCA	TGCAGTCAATGTGGGAAGTC
ZNF274	GGAGAAATCCCATGAGGGTAA	GGCTTTTGTGAGAATGTTTTCC
p5	CTGTATTAGAGGTCACGTGAGTG	TCAAACCTCCCGCTTCAAA
Rep 5′	CCGAGAAGGAATGGGAGTT	CCATTCCGTCAGAAAGTCG
Rep middle	GCCTTGGACAATGCGGGAAAGATT	TGTCGACACAGTCGTTGAAGGGAA
Rep 3′	TTCCCGTGTCAGAATCTCAA	CCAAATCCACATTGACCAGA
Cap 5′	GACAGTGGTGGAAGCTCAAA	TTGTACCCAGGAAGCACAAG
Cap middle	TTCTCAGATGCTGCGTACCGGAAA	TCTGCCATTGAGGTGGTACTTGGT
Cap 3′	GTCAGCGTGGAGATCGAGT	AGGCTCTGAATACACGCCAT