Supporting Information

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SI Materials and Methods

DNA Construction. The plasmid pcDNA4TO/myc-rZAP, which expresses myc-tagged full-length rat ZAP, has been described previously (1). pcDNA4TO/myc-hZAP-v1 and pcDNA4TO/myc-hZAP-v2 express myc-tagged hZAP-v1 and hZAP-v2, respectively. The coding sequence of hZAP-v1 was assembled from two overlapping fragments. The 5′ fragment was PCR-amplified from EST clone 5531412 (Invitrogen) using forward primer hZAP-FP bearing a *BamHI* site and reverse primer hZAP-mid-RP. The 3′ fragment was PCR-amplified from EST clone 4154337 (Invitrogen) using forward primer hZAP-mid-FP and reverse primer hZAP-v1-RP bearing a NotI site. The full-length hZAP-v1 was PCR-amplified from these two overlapping fragments using hZAP-FP and hZAP-v1-RP primers and cloned into expression vector pcDNA4 TO/myc-HisB (Invitrogen). The coding sequence of hZAP-v2 was PCR-amplified from hZAP-v1 using primers hZAP-FP and hZAP/1-699-RP and cloned into pcDNA4TO/myc-HisB (Invitrogen) using *BamHI* and *NotI* sites to generate pcDNA4 TO/myc-hZAP-v2.

To express myc-tagged NZAP fused with the zeocin resistance gene, pcDNA4TO/myc-HisB was first modified to generate pcDNA4TO/myc-Zeo by inserting the coding sequence of the zeocin resistance gene. The PCR-generated hNZAP/1-254 and rNZAP/1-254 fragments bearing *BamHI-NotI* sites were inserted into pcDNA4TO/myc-Zeo to generate pcDNA4TO/myc-hNZAP-Zeo and pcDNA4TO/myc-rNZAP-Zeo, respectively.

The retroviral vector expressing rNZAP-Zeo, pBabe-rNZAP-Zeo, and the control vector pBabe-HAZ have been previously reported (2). pBabe-hNZAP-Zeo, which expresses hNZAP-Zeo, was generated by inserting a PCR-generated fragment encoding the N-terminal 254 amino acids of hZAP into pBabe-HAZ.

To generate the hZAP-v1–expressing construct that cannot be targeted by hZAPi, silent mutations were introduced into the coding sequence of hZAP-v1. The PCR fragments generated using primers hZAP-FP/hZAPmm-RP and hZAPmm-FP/hZAP-RP were mixed and amplified using PCR primers hZAP FP and hZAP-RP. The resulting fragment was cloned into pcDNA4TO/myc-HisB.

The sequences of the primers are listed below.

hZAP-FP: 5′-AATAGGATCCGCCACCATGGCGGACCC-GGAGGTGTGC-3′
hZAP-mid-FP: 5′-CTGCCTTACCGGTGGCAGATG-3′
hZAP-mid-RP: 5′-GCATCTGCCACCGGTAAGGCAG-3′
hZAP-v1-RP: 5′-ATATAGCGGCCGCTCCGTTTGTCTTT-CTCTTCTC-3′
hZAP/1-699-RP: 5′-ATCTGAGCGGCCGCGGTCTGGCC-CTCTCTTCATC-3′
hZAPmm-FP: 5′-GAGAGAAACTTGTGCAAATATTCTC-ATGAGGTT C-3′
hZAPmm-RP: 5′-ATTTGCACAAGTTTCTCTCGGACTG-CGAATAGTTGC-3′
hNZAP-FP: 5′-GCTAGATCTGCCACCATGGCGGACCC-GGAGGTGTGC-3′
hNZAP254-RP: 5′-ATACGCGGCCGCCAAAGAACCGAT-CTCTACTCTTG-3′

The coding sequence of hRrp42 was PCR-amplified from a human fetal liver cDNA library using primers hRrp42-FP and hRrp42-RP. The coding sequences of rRrp42 and rRrp46 were PCR-amplified from a cDNA library from Rat2 cells. To express GST-Rrp42 and GST-Rrp 46 proteins, the coding sequences of these proteins were PCR-amplified and cloned into pGEX-5x-3 (Qiagen). The sequences of the primers are listed below.

hRrp42-FP: 5′-ATATGAATTCCCGCGTCCGTGACGCTG-AGCGAG-3′
hRrp42-RP: 5′-ATATCTCGAGTCACCCAGGAATCCAA-CTTTCTG-3′
hRrp42-GST-FP: 5′-ATAT*GGATCC*CCGCGTCCGTGAC-GCTGAGCGAG-3′
hRrp42-GST-RP: 5′-ACTT*CTCGAG*TCATCCCAGGAATC CAACTTTC-3′
rRrp42-GST-FP: 5′-ACTT*GGATCC*CCGCGTCGGTGTCG-CTAAGCGAGG-3′
rRrp42-GST-RP: 5′-ACTT*CTCGAG*TCACCCCAGGAACC-CGACTTTTGG-3′
rRrp46-GST-FP: 5′-ACTT*GGATCC*CCGAGGGAGCAAA-GCGTGCAGACG-3′
rRrp46-GST-RP: 5′-ACTT*CTCGAG*TCAGCTCTTGGAGT-AGCGCCTC-3′

pcDNA3-Flag-Dcp1a and pcDNA3-Flag-Xrn1, which express human Dcp1a and Xrn1, respectively, were kindly provided by Dr. Lykke-Andersen (University of California at San Diego, La Jolla, CA) and have been described previously (3). pCMV-HF-Dcp2, pCMV-HF-PARN, pCMV-HF-CCR4, and pCMV-HF-Pan2 express N-terminal Flag-tagged human Dcp2, PARN, CCR4, Pan2, and Rrp41, respectively. The ORFs of hDcp2 and hCCR4 were inserted into pCMV-HA-Flag vector between SalI and NotI sites. The ORF of hPARN was inserted into pCMV-HA-Flag between SalI and KpnI sites. The ORFs of hPan2 and hRrp41were inserted into pCMV-HA-Flag between EcoRI and KpnI sites.

The plasmid pNL4-3-luc was obtained from Dr. Nathaniel Landau through the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD). Plasmid pHR′-luc, which expresses HIV-1 vector HR′-Luc, was modified from pHR′-CMV-Luc (4, 5) by deleting the CMV promoter sequence. To construct pHR′-CMV-Luc plasmid containing different 5′ UTR of HIV-1 mRNAs, pHR′-CMV-Luc was modified by deleting the BamHI and XhoI sites, followed by insertion of XhoI and ClaI sites between CMV promoter and Luc sequence. This modification resulted in a plasmid named pHR′-CMV-MCS-Luc. The 5′ UTRs of *gag*, *vif*, *vpu*, *tat-1*, and *nef* were PCR-amplified and inserted into pHR′-CMV-MCS-Luc using XhoI and ClaI sites.

The plasmids expressing shRNAs were generated by annealing pairs of oligonucleotides and cloning into pSuper-retro-puro (OligoEngine) using *BglII* and *HindIII* sites according to the manufacture's protocol. The target sequences are listed below.

Ctrl: GCAAGCTGACCCTGAAG
hZAPi: AGCGGAATTGATGCAAC
hZAPi-A: GATTCTTTATCTGATGTCA
hZAPi-B: GTGTAAGGGTTGTCCGCTT
PARNi: AGTTGAAAGTGCCGAAGGT
CCR4i: CTGCAATGCTGATATCGTA
Pan2i: CCGATATCTTTCATGTGAA
Rrp41i: TATAGTTCAGCGACCTTCC
Dcp1ai: ACCGAGACTCTAGAAGAA
Dcp2i: GCAAAGCAGCAGAATTCTT
Xrn1i: GTCATGGCAAGGAGTTACC

siRNA and siRNA Transfection. Control siRNA (siCtrl.: catalog no. D-001810-10) and siRNA against hZAP (siZAP#1: catalog no. J-

017449-11, and siZAP#2: catalog no. J-017449-09) were obtained from Thermo Scientific. The siRNA against RIG-I (siRIG-I) was obtained from GenePharma. The target sequences of siRNAs are listed below.

siZAP#1: GGUAAAACCUGGACGGACU
siZAP#2: GUGUAAGGGUUGUCCGCUU
siRIG-I: GUUGGAGGAGUAUAGAUUA

siRNA oligos were transfected into cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Cell Culture, Plasmids Transfection, Virus Packaging, and Infection. All plasmid transfection assays were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

293TRex-rZAP, 293TRex-rZAP-Zm13, and 293TRex-rZAP-Zm63 cells have been described previously (1, 6). To establish cell lines expressing various versions of ZAP proteins in a tetracycline-inducible manner, 293TRex cells (Invitrogen) were stably transfected with constructs expressing myc-tagged hZAP-v1, hZAP-v2, rNZAP-Zeo, or hNZAP-Zeo and selected with 50 μg/mL zeocin. Individual clones were picked, expanded, and tested for tetracycline-inducible expression of the proteins by Western blotting.

To test the efficiency of shRNAs to knock down the expression of the target protein, HEK293 cells were transfected with the plasmid expressing the tagged protein together with the plasmid expressing the shRNA. The plasmid expressing myc-tagged GFP was included to control transfection efficiency and sample handling. At 48 h after transfection cells were lysed, and Western blot was performed to detect the expression levels of the target protein and myc-GFP.

Replication-competent HIV-1 strains NL4-3 and SF162 were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. VSV-G–pseudotyped NL4-3-luc virus was produced by transiently cotransfecting HEK293T cells with pVSV-G (a plasmid expressing VSV-G protein) and pNL4-3-luc. VSV-G–pseudotyped HR′-based viruses were packaged by transiently cotransfecting HEK293T cells with pVSV-G, pCMV-Delta8.2 (a plasmid expressing HIV-1 Gag-Pol) and HR′-based reporters.

To generate HOS-CD4-CCR5 stable cell lines expressing rNZAP-Zeo and hNZAP-Zeo, pBabe-rNZAP-Zeo and pBabe-hNZAP-Zeo were cotransfected into HEK293T cells with pVSV-G and pHIT60 (a plasmid expressing MLV Gag-Pol) to generate Babe-rNZAP-Zeo and Babe-hNZAP-Zeo pseudovirus. HOS-CD4-CCR5 cells were transduced with these viruses, selected with 50 μg/mL zeocin, and pooled. To generate Jurkat cells expressing hNZAP-Zeo, Jurkat cells were transduced with Babe-hNZAP-Zeo pseudovirus, selected in 4 μg/mL puromycin, and pooled.

VSV-G–pseudotyped SR-Ctrl, SR-hZiA, and SR-hZiB viruses were produced by transiently cotransfecting HEK 293T cell with pVSV-G and pHIT60 together with pSR-Ctrl, pSR-hZiA, and pSR-hZiB, respectively. Jurkat cells were transduced with the viruses, selected with 4 μg/mL puromycin, and pooled.

293TREx-hZAP2/NL4-3-luc #9 and 293TREx-hZAP2/NL4-3-luc #24 cell lines were constructed by infecting 293TREx-hZAP-v2 cells with VSV-G–pseudotyped NL4-3-luc virus, and single clones (#9 and #24) stably expressing luciferase were selected and expanded.

To evaluate the antiviral activity of ZAP against HIV-1 vectors, cells were seeded and infected with VSV-G–pseudotyped NL4-3-luc or HR′-based viruses on the next day. At 3 h after infection, the cells were mock treated or treated with 1 μg/mL tetracycline to induce ZAP expression. The cells were lysed, and luciferase activities were measured 48 h later.

To assay HIV-1 replication in ZAP-expressing cells, 5×10^4 HOS-rNZAP-Zeo or HOS-hNZAP-Zeo cells were seeded in 12-well plates and infected with HIV-1 strain SF162. Jurkat-hNZAP-Zeo cells were infected with HIV-1 strain NL4-3. The supernatants were collected, and the amounts of p24 in the supernatants were measured using the HIV-1 Antigen Microelisa System according to the manufacturer's instruction (Biomerieux).

Detection of Nuclear Circular Viral DNA. 293TRex-hZAP-v2 cells were seeded in 35-mm dishes and infected with VSV-G–pseudotyped NL4-3-luc viruses at varying dilution ratios on the next day. Heat-inactivated virus (80 °C for 30 min) was used as a DNA contamination control. Right after infection the cells were untreated or treated with tetracycline to induce hZAP-v2 expression. At 24 h after infection Hirt DNA was extracted, and the nuclear circular DNA was detected by PCR-amplification of the LTR–LTR junction using primers C-FP and C-RP. PCR conditions were 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for 40 cycles. The sequences of the primers are listed below.

C-FP: 5′-GGATGGGGTGCTTCAAGCTAGTACC-3′
C-RP: 5′-GCCTCAATAAAGCTTGCCTTGAGTG-3′

Coimmunoprecipitation. 293T cells in 35-mm dishes were transfeted with 4 μg total plasmids. At 48 h after transfection, cells were lysed in 500 μL Co-IP buffer [30 mM Hepes (pH 7.5), 100 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors mixture, with or without RNase A at a concentration of 50 μg/mL] for 10 min. The lysate was clarified by centrifugation at 4 °C for 15 min at 15,000 × *g*. The supernatant was mixed with the antibody and 10 μL protein G beads (Amersham Pharmacia), and the mixture was incubated at 4 °C for 4 h. The resin was washed with PBS three times, and the bound proteins were resolved by SDS/PAGE electrophoresis, transferred to PVDF membrane, and detected by Western blotting.

1. Guo X, Carroll JW, Macdonald MR, Goff SP, Gao G (2004) The zinc finger antiviral protein directly binds to specific viral mRNAs through the CCCH zinc finger motifs. *J Virol* 78:12781–12787.
2. Gao G, Guo X, Goff SP (2002) Inhibition of retroviral RNA production by ZAP, a CCCH-type zinc finger protein. *Science* 297:1703–1706.
3. Lykke-Andersen J, Wagner E (2005) Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1. *Genes Dev* 19:351–361.
4. Naldini L, et al. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 272:263–267.
5. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol* 15:871–875.
6. Wang X, Lv F, Gao G (2010) Mutagenesis analysis of the zinc-finger antiviral protein. *Retrovirology* 7:19.

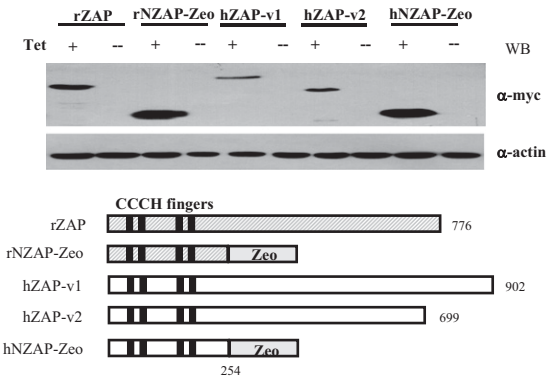

Fig. S1. Expression of different versions of ZAP in HEK293 cells in a tetracycline-inducible manner. The cells expressing the indicated myc-tagged ZAP proteins were mock treated (–) or treated with tetracycline (+) to induce ZAP expression. The cell lysates were subjected to Western blotting to detect the expression of the ZAP proteins (*Upper*). Schematic structures of the ZAP proteins are presented (*Lower*).

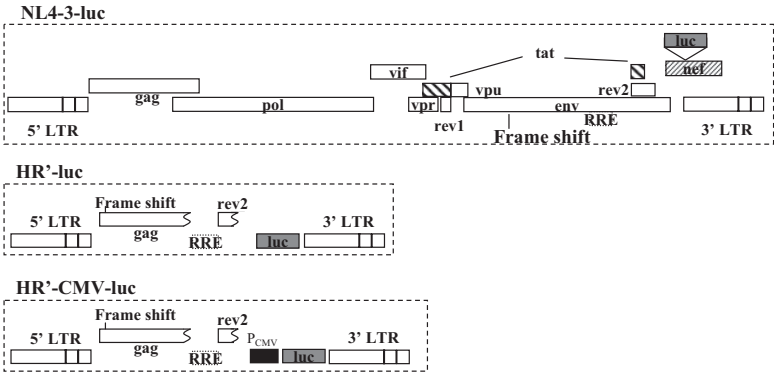

Fig. S2. Schematic structures of HIV-1 vectors.

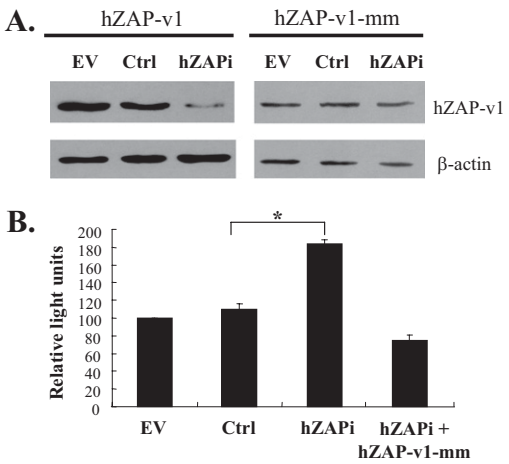

Fig. S3. Down-regulation of endogenous hZAP enhances HIV-1 infection in HEK293 cells. (*A*) The myc-tagged hZAP-v1–expressing plasmid or the rescue plasmid (hZAP-v1-mm) was cotransfected into HEK293 cells with the plasmid expressing the indicated shRNA. The expression levels of hZAP-v1 were judged by Western blotting using anti-myc and anti–β-actin antibodies. (*B*) HEK293 cells were transiently transfected with the plasmid expressing the indicated shRNA with or without the rescue hZAP-v1 expression plasmid bearing silent mutation that cannot be targeted by shRNA against wild-type hZAP. The cells were infected with VSV-G–pseudotyped NL4-3-luc virus at 6 h after transfection. At 48 h after infection the cells were lysed, and the luciferase activities were measured. Data are means + SE of four independent experiments. *$P < 0.05$. EV, empty vector; Ctrl, control shRNA; hZAPi, shRNA directed against hZAP.

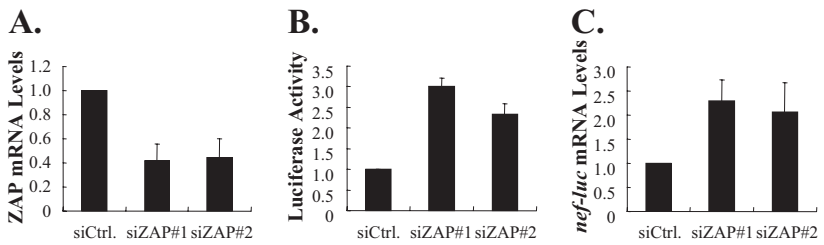

Fig. S4. Down-regulation of endogenous hZAP enhances HIV-1 infection in HOS cells. (*A*) HOS-CD4-CCR5 cells were transfected with control siRNA (siCtrl.) or siRNAs against hZAP (siZAP#1 and siZAP#2), and the endogenous hZAP mRNA levels were measured by real-time PCR. The relative ZAP mRNA level in cells transfected with control siRNA was set as 1. Data are means + SE of three parallel experiments. (*B* and *C*) HOS-CD4-CCR5 cells transfected with control siRNA (siCtrl.) or siRNAs against hZAP were infected with R5-tropic NL4-3-luc virus. At 48 h after infection, luciferase activities (*B*) and *nef-luc* mRNA levels (*C*) were measured. Data are means + SD of three independent experiments.

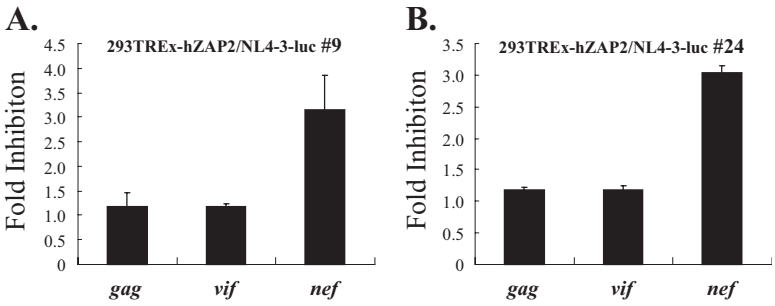

Fig. S5. ZAP inhibits the accumulation of *nef* mRNA but not *gag* or *vif* mRNA. 293TREx-hZAP2/NL4-3-luc clone 9 (#9) (*A*) or 293TREx-hZAP2/NL4-3-luc clone 24 (#24) (*B*) cells were mock treated or treated with 1 μg/mL tetracycline to induce ZAP expression for 24 h. Cytoplasmic RNA was extracted and subjected to real-time PCR to measure the mRNA levels of *gag*, *vif*, *nef-luc*, and *gapdh*. The mRNA levels of *gag*, *vif*, and *nef-luc* were normalized to that of *gapdh*. Fold inhibition was calculated as the ratio of normalized *gag*, *vif*, *nef-luc* mRNA levels in mock-treated cells to that in tetracycline-treated cells. Data are means + SD from three independent experiments.

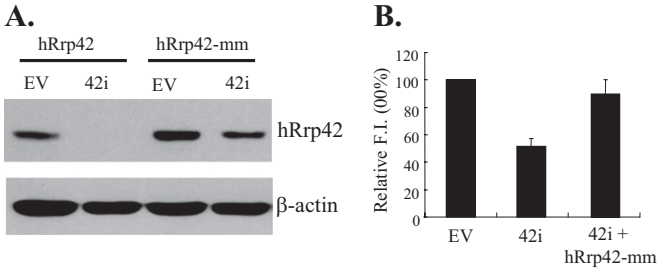

Fig. S6. Down-regulation of the exosome component Rrp42 reduces ZAP's activity. (*A*) The Flag-tagged hRrp42-expressing plasmid or the rescue plasmid (hRrp42-mm) was cotransfected into 293TRex cells with the plasmid expressing the shRNA directed against hRrp42 (R42i). The expression levels of hRrp42 or hRrp42-mm were measured by Western blotting using anti-Flag and anti–β-actin antibodies. (*B*) 293TRex-hZAP-v2 cells were transfected with the indicated plasmids. At 6 h after transfection, the cells were infected with VSV-G–pseudotyped NL4-3-luc. At 48 h after infection luciferase activity was measured, and fold inhibition was calculated as the ratio of luciferase activity in tetracycline mock-treated cells to that in tetracycline-treated cells. Relative fold inhibition was calculated as the fold inhibition in the presence of empty vector divided by the fold inhibition in the presence of shRNA. Data are means + SD of three independent experiments.

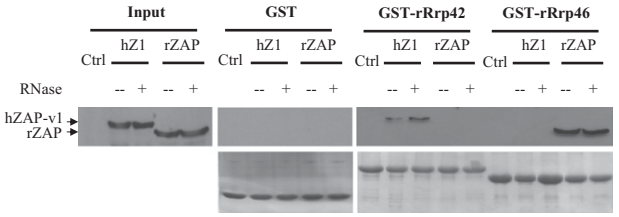

Fig. S7. Human ZAP interacts with rat Rrp42. The indicated bacterially expressed GST proteins were immobilized onto glutathione-Sepharose 4B resin and incubated with lysates of the cells expressing rZAP or hZAP-v1 in the presence of RNase A at 4 °C for 4 h. The resins were washed and boiled in sample loading buffer. The proteins were resolved by SDS/PAGE and detected by Western blotting with anti-myc antibody (*Upper*) or by Coomassie blue staining (*Lower*). Input, total cell lysates; Ctrl, 293TRex cells; hZ1, 293TRex-hZAP-v1 cells; hZ2, 293TRex-hZAP-v2 cells; rZAP, 293TRex-rZAP cells.

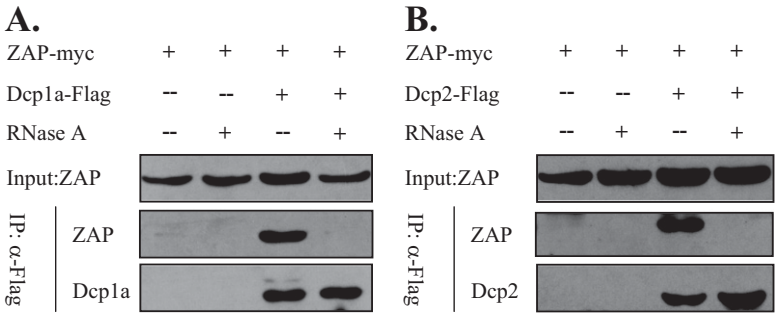

Fig. S8. ZAP interacts with Dcp1a and Dcp2 in an RNA-dependent manner. pcDNA4/TO-myc-ZAP was cotransfected with the plasmid expressing Flag-tagged Dcp1a (*A*) or Dcp2 (*B*) into HEK293T cells. Cell extracts were mock treated (RNase A -) or treated (RNase A +) with 200ug/mL RNase A and imunnoprecipitated with anti-Flag antibody, followed by Western blotting using anti-myc or anti-Flag antibody.

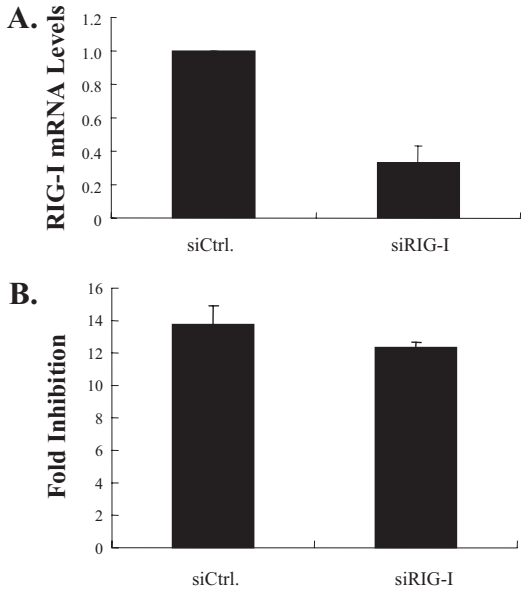

Fig. S9. Down-regulation of RIG-I by siRNA does not affect ZAP's antiviral activity against NL4-3-luc. (*A*) 293TREx-hZAP-v2 cells were transfected with control siRNA (siCtrl) or siRNA directed against RIG-I (siRIG-I). At 48 h after transfection, total RNA was extracted and reverse transcribed, and the mRNA levels of RIG-I and *gapdh* were measured by real-time PCR. The RIG-I mRNA level was normalized with that of *gapdh*, and the RIG-I mRNA level in siCtrl-transfected cells was set as 1. Data are means + SE of three parallel experiments. (*B*) 293TREx-hZAP-v2 cells were transfected with siCtrl or siRIG-I for 6 h and infected with VSV-G–pseudotyped NL4-3-luc for 3 h. Cells were mock treated or treated with tetracycline (1 μg/mL) for additional 36 h to induce ZAP expression and then harvested, and luciferase activities were measured. Fold inhibition was calculated as the luciferase activity in the mock-treated cells divided by the luciferase activity in the tetracycline-treated cells. Data are means + SE of two parallel experiments.