TNAP, a Novel Repressor of NF-κB-inducing Kinase, Suppresses NF-κB Activation*

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NF-κB-inducing kinase (NIK) has been implicated as an essential component of NF-κB activation. However, the regulatory mechanism of NIK signaling remains elusive. We have identified a novel NIK interacting protein, TNAP (for TRAFs and NIK-associated protein). In mammalian cells, TNAP physically interacts with NIK, TRAF2, and TRAF3 but not IKK1 or IKK2. TNAP specifically inhibits NF-κB activation induced by tumor necrosis factor (TNF)-α, TNF receptor 1, TRADD, RIP, TRAF2, and NIK but does not affect IKK1 and IKK2-mediated NF-κB activation. Knockdown of TNAP by lentiviral-mediated small interference RNA potentiates TNF-α-induced NF-κB activation. TNAP suppresses NIK kinase activity and subsequently reduces p100 processing, p65 phosphorylation, and IκB degradation. These data suggest that TNAP is a repressor of NIK activity and regulates both the classical and alternative NF-κB signaling pathways.

The NF-κB family of transcription factors is a key regulator of inflammation, immune responses, oncogenesis, apoptosis, and neuronal signaling (1–5). To date two principal pathways for NF-κB activation have been characterized: a classical and an alternative pathway (2, 6, 7). The classical pathway involves most forms of NF-κB, especially the p65 (RelA/p50) dimer. It is triggered by tumor necrosis factor (TNF)-α, 1 interleukin-1, lipopolysaccharide (LPS), and CD40 ligand (CD40L) and to a lesser extent by lymphotoxin-β (LT-β) and B cell-activating factor. Activation of this pathway depends on the IκB kinase (IKK) signalosome, which consists of at least two catalytic subunits (IKK1 or IKK-α and IKK2 or IKK-β) and a regulatory subunit (IKK-γ or NEMO). The IKK complex phosphorylates the inhibitor proteins of NF-κB (IκBs) to induce their ubiquitination and degradation, resulting in NF-κB translocation to the nucleus and transcriptional activation of specific target genes. This pathway is crucial for the activation of innate immunity and inflammation. The alternative pathway involves p100 processing and nuclear translocation of RelB/p52 dimers. It is activated by LT-β, CD40L, LPS, and B cell-activating factor and depends on IKK1 homodimers (8–12). This alternative pathway is crucial for secondary lymphoid organ development, maturation of B cells, and adaptive humoral immunity.

NF-κB-inducing kinase (NIK) has been implicated in activating both the classical and alternative pathways. NIK was originally identified as a mitogen-activated protein kinase kinase that interacts with TNF receptor-associated factor 2 (TRAF2) and activates NF-κB when expressed in mammalian cells (13). NIK has been characterized as a downstream component of the TNF signaling pathway (14, 15), which is activated directly or indirectly by cytoplasmic adaptor proteins like TNF receptor-associated death domain (TRADD) (16), receptor interacting protein (RIP) (17, 18), or TRAF2 (16, 19). A kinase inactive mutant of NIK can block NF-κB activation by various stimuli, such as TNF-α, interleukin-1, and LPS. In bone marrow, TNF-α and interleukin-1 direct pluripotent mesenchymal stem cell toward an osteoblast fate and suppress adipogenesis through the NIK signaling (20). NIK interacts with both IKK1 and IKK2 (14, 21). For the heterodimer of IKK complex, NIK activates IKK1 and IKK2 in a directional manner (22). Genetic studies with NIK mutant mice (23–25) and knockout mice (26, 27) have demonstrated that NIK plays an essential role in the signaling pathway of LT-β receptor but not TNF-α receptor, implying that NIK functions in a receptor- and cell type-specific manner. Further studies confirm that NIK is indispensable in the IKK1-mediated phosphorylation and processing of p100 and the activation of the alternative pathway of NF-κB activation (10, 12, 28–33). NIK also has been demonstrated to phosphorylate the p65 transactivation domain (34, 35), to activate mitogen-activated protein kinase pathways (36, 37), to be involved in signaling of epidermal growth factor receptor (38), to establish self-tolerance (39), and to promote neurite formation (40). These observations indicate that NIK exerts a wide range of biological functions. However, the mechanisms for NIK activation and regulation are not well understood. Using a yeast two-hybrid screen with NIK as bait, we have identified a novel protein, designated TNAP (for TRAFs and NIK-associated protein), which directly binds to NIK and inhibits NIK kinase activity. Studies via overexpression and knockdown of TNAP demonstrate that TNAP negatively regulates NF-κB activation upstream of the IKK complex and regulates both the classical and alternative NF-κB signaling pathways.

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The abbreviations used are: TNF, tumor necrosis factor; LPS, lipopolysaccharide; CD40L, CD40 ligand; LT, lymphotixin; IKK, IκB kinase; NIK, NF-κB-inducing kinase; TRAF, TNF receptor-associated factor; TRADD, TNF receptor-associated death domain; RIP, receptor interacting protein; RSV, Rous sarcoma virus; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; RT, reverse transcriptase; shRNA, short hairpin RNA.
REGULATION OF NF-κB SIGNS

The NF-κB transcription factors are essential for the expression of genes involved in inflammation and immune response. In this study, we investigated the regulation of NF-κB activity using TNAP (Tissue Nonspecific Alkaline Phosphatase).

**Experimental Procedures**

**Transfections and Generation of Stable Cell Lines**

- For each transfection, the cells were seeded in 10-cm dishes. For reporter gene assay and EMSA, the cells were seeded in 6-well dishes.
- For immunocytochemistry, the cells were seeded in 8-well chamber slides.

**Reporter Gene Assay**

- The dual-Light chemiluminescent reporter gene assay was developed for the detection of luciferase and β-galactosidase (Tropix) using a manufacturer's protocol.
- Each transfection was performed in triplicate, and when necessary, empty control plasmids added to each sample.

**Transfections using GFP-NIK**

- Three TFGR-NIK/NIK constructs were transfected to normalize luciferase activity.

**Luciferase Activity**

- The luciferase activity was normalized to inactive mutant IKK1(K44M), IKK2, and RSV-Luciferase.
- Mammalian expression vectors encoding TNFR1, TRADD, RIP1, TRAF2, TRAF3, NIK, and its mutants, IKK1, and its kinase-inactive mutant IKK1(K44M), IKK2, and RSV-β-galactosidase have been described previously (16, 41-45).

**RT-PCR and Northern Blot**

- Total RNA was isolated from TRV102-depleted (Reinvogen). Two micrograms of RNA were treated with DNase I and reverse transcriptase with random hexanucleotide as primers.

**Immunohistochemistry**

- The tissue-specific expression of TNAP was examined by hybridization of a human multiple tissue Northern blot containing 2 μg of poly(A) RNA (OriGene). A PCR-produced probe of 192 bp was [32P]dCTP-labeled with random priming.

**RESULTS**

**Cloning and Characterization of TNAP**

- By screening yeast two-hybrid and RT-PCR libraries of human adult tissues, we identified a novel clone, TNAP, which coincides with sequence 1431-437 of NIK, was inserted in frame into GAL4 DNA-binding domain in yeast expression vector pBridge (Clontech).

**Mammalian Expression Vector**

- The NF-κB-luciferase and interferon-response factor-luciferase reporter constructs were obtained from Clontech.

**GST-TNAP Fusion Protein Expression and Antibody Production**

- GST fusion proteins were expressed and purified according to standard procedures using expression vector pGEX-4T-2 (Amersham Biosciences).

**Immunoprecipitation**

- TNAP and its mutants, IKK1(K44M), IKK2, and RSV-β-galactosidase were translated and labeled with [32P]orthophosphoric acid (0.5 mCi) and added to each transfection.

**RESULTS**

- The TFGR-NIK/NIK construct was transfected to normalize luciferase activity.

**CONCLUSIONS**

- TNAP suppresses NF-κB activation.

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**DISCLOSURE**

- No conflicts of interest relevant to this study are disclosed.

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specifically recognizes the purified Myc-TNAP fusion protein. Double-labeled confocal image analysis confirms the colocalization of TNAP-like and Myc-like immunoreactivities (Fig. 1C). To determine the endogenous protein expression of TNAP, Western blot analysis of different cell lines was performed. As shown in Fig. 1D, the anti-TNAP antibody recognizes a band of ~16 kDa, the predicted size for TNAP in HL60 cells, which is consistent with the expression of TNAP mRNA demonstrated by RT-PCR (Fig. 1B). In the white matter of the spinal cord, TNAP-like immunoreactivity is detectable in glial cells (Fig. 1E).

Physical Interaction of TNAP with NIK—We cloned the open reading frame of TNAP into a mammalian expression vector to verify the interaction between TNAP and NIK in mammalian cells. Coimmunoprecipitation and Western blot analysis in transiently transfected HEK293T cells demonstrate that TNAP specifically interacts with NIK (Fig. 2A). TNAP also interacts with TRAF2 and TRAF3 but does not interact with
IKK1 or IKK2 (Fig. 2A). The direct interaction between TNAP and NIK, but not TNAP and IKK1, was confirmed by GST pull-down assay using bacterially expressed GST-TNAP fusion protein and in vitro translated NIK or IKK1 protein (Fig. 2B). To examine which region of TNAP interacts with NIK, we made several deletion mutants of TNAP and transiently expressed these mutants in HEK293T cells. The expression levels of TNAP and its mutants were confirmed by immunofluorescent cytochemistry with an anti-Myc antibody. As shown in Fig. 2C, the C-terminal fragment (amino acids 87–140) interacts with NIK, but the N-terminal fragment (amino acids 1–70) does not.

Expression of TNAP alone does not affect NF-κB-dependent gene expression but significantly inhibits TNF-α-induced NF-κB activation in HEK293T and Jurkat cells (Fig. 3A). However, TNAP does not affect interferon-γ-induced interferon response factor-1-dependent gene expression in either HEK293T or Jurkat cells (Fig. 3A), suggesting that the inhibition by TNAP of NF-κB-dependent gene expression is specific. Stable clones constitutively expressing TNAP also exhibit significant inhibition of TNF-α-induced NF-κB activation (Fig. 3A).

To support the reporter gene studies, the EMSA was used to determine whether TNAP reduces cytokine-induced NF-κB-DNA binding activity. In HEK293T cells, TNAP reduces constitutive levels of NF-κB-DNA binding activity (Figs. 3B and 4A) and attenuates TNF-α-induced NF-κB-DNA binding activity (Fig. 3B). In Jurkat cells, expression of TNAP reduces the NF-κB-DNA binding activity induced by TNF-α and 4-phorbol 12-myristate 13-acetate/ionomycin (Fig. 3B). In HEK293T stable clones, TNAP inhibits TNF-α-induced NF-κB-DNA binding activity (Fig. 3B).

To confirm the physiological function of endogenous TNAP in regulating NF-κB activation, we used lentiviral-mediated RNA interference (46, 57, 58) to confer stable knockdown (59, 60) of
the TNAP expression. The efficiency and specificity of lentiviral-mediated short hairpin RNA (shRNA) for TNAP were examined by Western blot analysis of the overexpressed Myc-tagged TNAP fusion protein and RT-PCR of the endogenous TNAP mRNA (Fig. 3C). Two of three constructs for TNAP shRNA efficiently reduce TNAP expression and one less efficiently (Fig. 3C). To examine the biological relevance of TNAP shRNA, we performed NF-\(\kappa\)B-dependent reporter gene assay. In addition, TNAP knockdown increases TNF-\(\kappa\)B-induced NF-\(\kappa\)B-dependent gene expression (Fig. 3C). Taken together, these data suggest that TNAP is an inhibitor of TNF-\(\kappa\)B-induced NF-\(\kappa\)B signaling.

**TNAP Functions Upstream of IKK**—It is well known that TNF-\(\alpha\) induces NF-\(\kappa\)B activation through the TNFR1-TRADD-RIP-TRAF2-NIK-IKK signaling pathway (1, 4, 49, 54–56). To test which step of the NF-\(\kappa\)B signaling pathway is the target of TNAP, we coexpressed these proteins together with TNAP and determined whether TNAP affects NF-\(\kappa\)B activation using luciferase reporter gene assay. As shown in Fig. 4A, transient expression of TNAP reduces TNFR1, TRADD, RIP, TRAF2, and NIK-mediated NF-\(\kappa\)B activation but does not affect IKK1 and IKK2-mediated NF-\(\kappa\)B activation. In addition, EMSA confirmed that TNAP reduces TRAF2 and NIK-mediated NF-\(\kappa\)B-DNA binding activity but does not affect IKK1-mediated NF-\(\kappa\)B-DNA binding activity (Fig. 4A). These results are consistent with the data presented in Fig. 2A and suggest that TNAP exerts its NF-\(\kappa\)B inhibitory effect by interfering with a component of NF-\(\kappa\)B signaling pathway upstream of IKK.

**TNAP Suppresses NF-\(\kappa\)B Activation**

**Fig. 3. TNAP specific inhibition of NF-\(\kappa\)B activation.** A, TNAP inhibits NF-\(\kappa\)B-dependent gene expression induced by TNF-\(\alpha\). HEK293T or Jurkat cells in a 6-well plate were cotransfected in triplicate with 2 \(\mu\)g of TNAP expression vector or empty pRK vector and 0.2 \(\mu\)g of NF-\(\kappa\)B-luciferase (left panel) or interferon response factor-luciferase (middle panel) and 0.1 \(\mu\)g of RSV-\(\beta\)-galactosidase vectors. Stable cell line of HEK293T (right panel) were transfected with 0.8 \(\mu\)g of NF-\(\kappa\)B-luciferase and 0.4 \(\mu\)g of RSV-\(\beta\)-galactosidase vectors. After 24 h, the cells were treated without (Control) or with TNF-\(\alpha\) (10 ng/ml) or interferon-\(\gamma\) (100 ng/ml) for 6 h, and then dual luciferase reporter gene assays were performed. The results were normalized and are representative of four independent experiments. The data shown are relative luciferase activities (fold changes) compared with pRK vector. The data represent the means \pm S.D. of triplicate determinations. B, TNAP suppresses NF-\(\kappa\)B-DNA binding activity. HEK293T or Jurkat cells 24 h after transfection with the indicated vectors or stable HEK293T cells were treated without (Control or Cont) or with TNF-\(\alpha\) (10 ng/ml) or combination of 100 ng/ml phorbol 12-myristate 13-acetate and 1 \(\mu\)g/ml ionomycin (P/I) for 30 min and EMSA were performed. The numbers under the panel represent the relative intensity (fold) compared with control pRK empty vector. C, TNAP knockdown enhances NF-\(\kappa\)B-dependent gene expression induced by TNF-\(\alpha\). HEK293T cells were infected with indicated lentivirus for 4 weeks (infection efficiency over 95%) and transfected with pRK-Myc-TNAP followed by Western blot with anti-Myc antibody (left panel) or with NF-\(\kappa\)B-luciferase and RSV-\(\beta\)-galactosidase vectors followed by TNF-\(\alpha\) (10 ng/ml) treatment and dual luciferase reporter gene assays (right panel). The endogenous mRNA level of TNAP after lentivirus infection for 4 weeks was examined by RT-PCR (middle panel).
Inhibition by TNAP of NIK Kinase Activity—Because TNAP interacts with NIK and blocks NIK-mediated NF-κB activation upstream of IKK complex, we hypothesized that TNAP affects the kinase activity of NIK. IKK1 is a preferential substrate for NIK kinase (14, 21, 22). Overexpression of IKK1 induces potent autophosphorylation (22). Therefore, we selected a kinase-inactive form of IKK1 (IKK1(K44M)) as a substrate for NIK. To verify the possible effect of TNAP on NIK kinase activity, we first performed an in vivo kinase assay using 32P-labeled inorganic phosphate and autoradiography (Fig. 4B, left upper panel), and then immunoprecipitation and autoradiography was performed (left upper panel), or cell lysates were directly subjected to Western blot with anti-phospho-IKK1 antibody (left and right lower panels). For in vitro kinase assay, the cell lysates were subjected to immunoprecipitation followed by in vitro kinase reaction and autoradiography (right upper panel). The amount of immunoprecipitated IKK1 was determined by Western blot (WB) to the same blot.

Suppression by TNAP of p100 Processing—NIK is essential for p100 processing (10, 12, 28–33). Because TNAP inhibits NIK kinase activity, we hypothesize that TNAP may affect NIK-mediated p100 processing. Consistent with previous reports, CD40L, LT-αβ, and LPS induce the processing of p100.
TNAP regulates both the classical and alternative pathways of NF-κB activation. A, TNAP inhibits NIK-mediated p100 processing. HEK293T cells were transfected with pRK empty vector or pRK-Myc-TNAP and treated with indicated stimuli for 24 h (left upper panel) or cotransfected with pRK-FLAG-NIK and pRK-Myc-TNAP or pRK empty vectors (right upper panel). Stable TNAP knockdown HEK293T cells were treated with indicated stimuli (left lower panel) or transfected with pRK-FLAG-NIK or pRK empty vectors (right lower panel). Then Western blot analysis was performed with anti-p52 and anti-β-actin antibodies. B, TNAP blocks TNF-α-induced NIK-mediated phosphorylation of p65. Wild-type HEK293T cells were cotransfected with indicated vectors (left panel) or TNAP knockdown HEK293T cells were treated with TNF-α for indicated period (right panel) followed by Western blot with indicated antibodies. C, TNAP reduces TNF-α-induced IκBα phosphorylation and degradation. Stable expression (left panel) or knockdown (right panel) HEK293T cells were treated with TNF-α 10 ng/ml for indicated period, and Western blot analyses were performed with anti-IκBα and anti-phospho-IκBα followed by anti-β-actin antibody. The numbers between the panels represents the relative intensity (percentage) of IκBα compared with control after β-actin normalization.

Inhibition by TNAP of p65 Phosphorylation—Phosphorylation of p65 has been shown to regulate NF-κB transactivation induced by cytokines, LPS, and LT-α/β (34, 64–68). Both IKK1 and IKK2 have been implicated in this process (34, 35, 66, 67, 69, 70). Recent studies demonstrate that NIK-MKK1-1 cascade plays a crucial role in p65 phosphorylation (35, 68). To address whether TNAP regulates p65 phosphorylation, we performed Western blot analysis of HEK293T cells that either overexpress or have a stable knockdown of TNAP. Overexpression of NIK induces p65 phosphorylation on Ser-536, which is reduced by transient overexpression of TNAP (Fig. 5B, left panel). TNF-α stimulation induces rapid phosphorylation of p65, consistent with several reports (65–67). Knockdown of TNAP increases TNF-α-induced phosphorylation of p65 (Fig. 5B, right panel). These data suggest that TNAP is an inhibitory regulator for TNF-α-induced NIK-mediated phosphorylation of p65.
Suppression by TNAP of TNF-α-induced Phosphorylation and Degradation of IκBα—IκBα phosphorylation and degradation is critical for NF-κB activation by cytokines (71, 72). To test whether TNAP affects TNF-α-induced IκBα degradation, we performed Western blot analysis in transient and stable TNAP cell lines. TNAP reduces TNF-α-induced IκBα degradation when transiently expressed in HEK293T cells (data not shown). Stable expression of TNAP stabilizes IκBα at 5 and 15 min after TNF-α stimulation, and delays IκBα degradation induced by TNF-α in all of the stable cell lines tested (Fig. 5C and data not shown). Stable knockdown of endogenous TNAP by lentiviral-mediated shRNA increases TNF-α-induced Ser32/36 phosphorylation of IκBα (Fig. 5C, right panel).

DISCUSSION

In most cells, two separate but overlapping pathways regulate NF-κB activation: the classical and alternative pathways. Both pathways rely on the IKK kinases for activation. However, each pathway has distinct activation requirements as well. For example, IKK2 and IKK-γ are essential for the classical pathway, but IKK1 and NIK are indispensable for the alternative pathway (2, 6, 7, 10, 12, 28–33). In this study we describe the cloning and initial characterization of a protein, TNAP, which regulates activation of both the classical and alternative pathways. We demonstrate that TNAP blocks NF-κB activation upstream of the IKK complex. TNAP physically associates with NIK and attenuates NIK kinase activity. Subsequently, TNAP blocks p100 processing, p65 phosphorylation, and IκBα degradation.

TNAP was cloned in a yeast two-hybrid screen of a human brain cDNA library using NIK as bait. The endogenous interaction between TNAP and NIK in mammalian cells was confirmed by coimmunoprecipitation studies. We also determined that TNAP interacts with TRAF2 and TRAF3 but not IKK1 or IKK2. Further studies using overexpression and small interference RNA knockdown of TNAP showed that TNAP negatively regulates NIK kinase activity. These observations suggest that TNAP suppresses NF-κB activation by interfering with NIK- and/or TRAF2-mediated signaling pathways.

Numerous in vitro studies have shown that NIK is an important intermediate in ligand-induced NF-κB activation (13, 14, 61, 62). However, genetic studies using a naturally occurring mutant, alv mice (23–25), and NIK gene knockout (26, 27) mice demonstrate that NIK is not essential for TNF-α-induced NF-κB activation but is required for lymphotoxin-induced NF-κB activation. It remains a possibility that NIK regulates NF-κB activation in a cell type- and stimulus-specific manner (26, 27, 73). Recent studies have determined that NIK is indispensable for p100 processing, mediated by IKK1 (10, 12, 28–33). However, the mechanism of NIK activation or inactivation has not been well elucidated. Our studies show that TNAP negatively regulates NIK signaling. TNAP is also an important addition to the family of negative regulators for NF-κB activation, such as A20 (74), TANK (75), TRAP2 (76), TRIP (77), ZINC (78), and CYLD (79–81). Although our data demonstrate that TNAP inhibits NIK kinase activity and p100 processing, we have not elucidated the mechanisms. TNAP does not have any known phosphatase domains, nor does it share any sequence similarity with other domains that may explain its inhibitory effects on NF-κB activation. Future studies will explore the mechanisms through which TNAP inhibits NIK kinase activity.

We provide evidence that TNAP retards TNF-α-induced and NIK-mediated IκBα degradation, suggesting that it also regulates the classical activation pathway. Because genetic studies strongly suggest that NIK is not an essential intermediate in TNF-α-induced NF-κB activation (26, 27), TNAP may inhibit another intermediate in this classical pathway. Previous studies have shown that TRAF2 is critically involved in NF-κB activation induced by TNF-α (16, 82) and CD40L (83–86). We demonstrate that TNAP associates with TRAF2 and that it significantly inhibits TRAF2-mediated NF-κB activation and CD40L signaling. Therefore, it is likely that TNAP-mediated inhibition of IκBα proteolysis is due in part to its interaction with TRAF2 and perhaps TRAF3 (87, 88). In addition to regulating TNF-α and TRAF2-dependent NF-κB activation, TNAP may regulate additional signaling pathways activated by TNF-α and TRAF2 such as c-Jun N-terminal kinase and p38 (89). Future studies are planned to determine whether TNAP inhibits TNF-α and CD40L signaling through a TRAF2-dependent mechanism.

We also found that TNAP negatively regulates TNF-α-induced and NIK-mediated phosphorylation of p65 on Ser536 using phosphorylation-specific antibody. Phosphorylation of p65 can generally potentiate NF-κB activation (90). Several kinases that have been implicated in p65 phosphorylation include protein kinase A (Ser-276) (91, 92), mitogen- and stress-activated protein kinase-1 (Ser-276) (93), casein kinase II (Ser-529) (94), protein kinase C (Ser-471), phosphatidylinositol 3-kinase/Akt (Ser-536) (95), p90 ribosomal S6 kinase-1 (Ser-536) (96), IKK2 (Ser-536) (64, 65, 70), NIK, and IKK1/Ser-536 (35, 68). TNF-α has been shown to induce p65 phosphorylation at Ser-529 via casein kinase II (94), and IKK1 (68) and IKK2 (64, 65, 70) phosphorylates p65 on Ser-536. Our data confirm that TNF-α induces rapid phosphorylation of p65 on Ser-536 and show for the first time that TNAP regulates TNF-α-induced and NIK-mediated p65 phosphorylation. Future studies will investigate the mechanisms through which TNAP regulates p65 phosphorylation by TNF-α, NIK, and possibly other kinases.

TNAP is a relatively small protein (16 kDa) with no known regulatory domains that could help explain its inhibitory mechanisms. TNAP shares a high degree of homology with several protein kinases related to inappropriate NF-κB activation such as cancer and inflammation (2). We thank Dr. Hong-Bing Shu (National Jewish Medical and Research Center) and Dr. Achim Leutz (Max-Delbrück-Center for Molecular Medicine) for reagents and comments. We thank the Vector Core Facility at the University of Miami for lentivirus preparation.

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