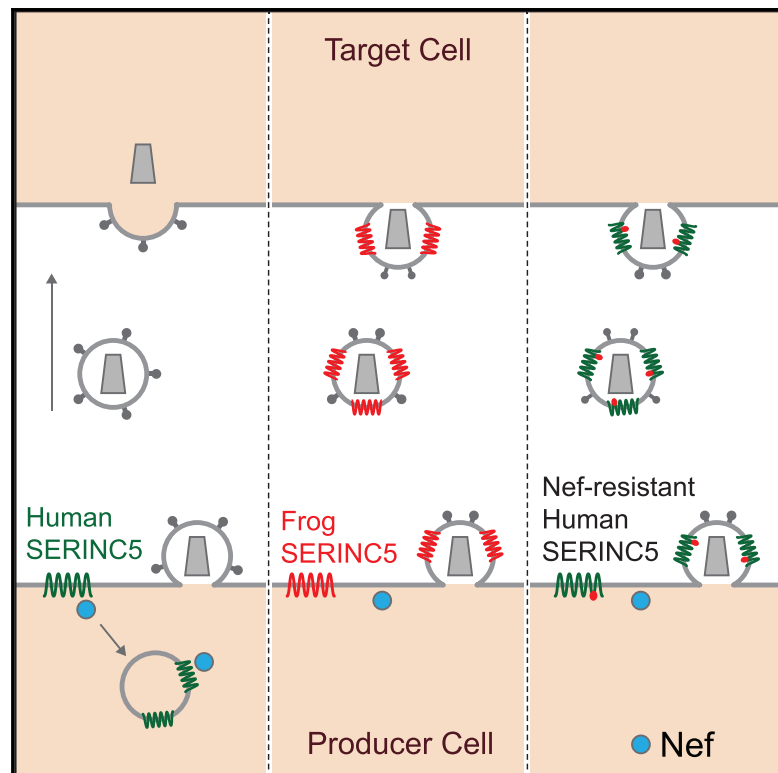


A Long Cytoplasmic Loop Governs the Sensitivity of the Anti-viral Host Protein SERINC5 to HIV-1 Nef

Graphical Abstract



Authors

Weiwei Dai, Yoshiko Usami, Yuanfei Wu, Heinrich Göttlinger

Correspondence

heinrich.gottlinger@umassmed.edu

In Brief

Human SERINC5 potently inhibits HIV-1 infectivity but is counteracted by HIV-1 Nef. Dai et al. show that an intracellular loop region of SERINC5 plays an important role in its sensitivity to Nef and that human SERINC5 can be modified to inhibit HIV-1 even in the presence of Nef.

Highlights

- Anti-HIV-1 activity is a conserved property of widely divergent SERINC5 proteins
- Sensitivity to HIV-1 Nef is not conserved
- Nef sensitivity can be transferred by exchanging an intracellular loop region
- Human SERINC5 can be modified to restrict HIV-1 even in the presence of Nef



A Long Cytoplasmic Loop Governs the Sensitivity of the Anti-viral Host Protein SERINC5 to HIV-1 Nef

Weiwei Dai,¹ Yoshiko Usami,¹ Yuanfei Wu,¹ and Heinrich Göttlinger^{1,2,*}

¹Department of Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA

²Lead Contact

*Correspondence: heinrich.gottlinger@umassmed.edu

<https://doi.org/10.1016/j.celrep.2017.12.082>

SUMMARY

We recently identified the multipass transmembrane protein SERINC5 as an antiviral protein that can potently inhibit HIV-1 infectivity and is counteracted by HIV-1 Nef. We now report that the anti-HIV-1 activity, but not the sensitivity to Nef, is conserved among vertebrate SERINC5 proteins. However, a Nef-resistant SERINC5 became Nef sensitive when its intracellular loop 4 (ICL4) was replaced by that of Nef-sensitive human SERINC5. Conversely, human SERINC5 became resistant to Nef when its ICL4 was replaced by that of a Nef-resistant SERINC5. In general, ICL4 regions from SERINC5s that exhibited resistance to a given Nef conferred resistance to the same Nef when transferred to a sensitive SERINC5, and vice versa. Our results establish that human SERINC5 can be modified to restrict HIV-1 infectivity even in the presence of Nef.

INTRODUCTION

Nef is an accessory protein of HIV-1 and other primate immunodeficiency viruses that is crucial for efficient virus replication in infected individuals and for virus pathogenicity (Deacon et al., 1995; Kestler et al., 1991). Although Nef is not an essential viral gene, it robustly enhances virus spreading in cultures of quiescent primary CD4⁺ T lymphocytes (Miller et al., 1994; Spina et al., 1994). Nef hijacks the clathrin-associated protein complex 2 (AP-2) adaptor to induce the internalization of the viral receptor CD4 (Aiken et al., 1994; Chaudhuri et al., 2007; Garcia and Miller, 1991). In addition, Nef downregulates numerous other cell surface proteins (Haller et al., 2014), including certain major histocompatibility class I molecules, which is thought to protect infected cells from cytotoxic T cells (Collins et al., 1998; Schwartz et al., 1996).

Nef also enhances the specific infectivity of progeny virions (Aiken and Trono, 1995; Chowers et al., 1994; Miller et al., 1995). This function of Nef requires its presence during virus production, but Nef has no detectable effect on the encapsidation of the genomic viral RNA, the incorporation of Env, or the morphology or stability of the mature core (Aiken and Trono,

1995; Forshey and Aiken, 2003; Schwartz et al., 1995). Nevertheless, Nef increases the efficiency of reverse transcription in target cells (Aiken and Trono, 1995; Chowers et al., 1995; Schwartz et al., 1995). The effects of Nef on HIV-1 infectivity are determined by variable HIV-1 Env regions (Usami and Göttlinger, 2013) and are dependent on clathrin-mediated endocytosis (Pizzato et al., 2007; Usami et al., 2014).

We and others have identified the multipass transmembrane proteins SERINC3 and SERINC5 as antiviral proteins that are incorporated into Nef-deficient HIV-1 virions and counteracted by Nef (Matheson et al., 2015; Rosa et al., 2015; Usami et al., 2015). SERINC5 in particular can dramatically inhibit the infectivity of Nef-deficient HIV-1 (Matheson et al., 2015; Rosa et al., 2015; Usami et al., 2015). Although primary HIV-1 Env proteins often confer significant resistance against SERINC5, virion-associated SERINC5 sensitizes even resistant Envs to some neutralizing antibodies (Beitari et al., 2017).

HIV-1 Nef removes SERINC3 and SERINC5 from the cell surface (Matheson et al., 2015; Rosa et al., 2015; Usami et al., 2015) and prevents their incorporation into progeny virions (Rosa et al., 2015; Usami et al., 2015). In contrast, Nef increased the cell surface expression of SERINC1 (Matheson et al., 2015), indicating that SERINC3 and SERINC5 are selectively downregulated. Furthermore, the effects of HIV-1 Nef proteins on SERINC3 and SERINC5 are strain dependent. For instance, the Nef proteins of the primary HIV-1 isolates 97ZA012 and 93BR020 strongly inhibit the incorporation of SERINC3 and SERINC5 into virions; in contrast, the Nef protein of the T cell line-adapted strain SF2 is only active against SERINC5 (Usami et al., 2015). Thus, Nef proteins can discriminate among SERINC family members. However, the determinants that govern sensitivity to Nef remain unknown.

We show that an intracellular loop of human SERINC5 confers sensitivity to widely divergent Nefs in the context of a Nef-resistant vertebrate SERINC5 and confers sensitivity to Nef_{SF2} in the context of human SERINC3, which is normally resistant to this particular Nef. Human SERINC5 acquired resistance against Nefs from different HIV-1 clades when this intracellular loop was replaced by the corresponding region from a Nef-resistant SERINC5. Altogether, our results identify a major determinant of Nef responsiveness and establish that human SERINC5 can be made resistant to Nef-induced downregulation.



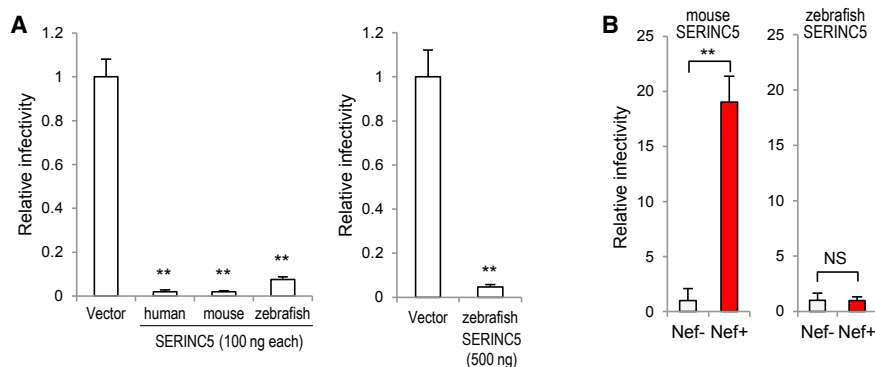


Figure 1. Anti-HIV-1 Activity, but Not Responsiveness to Nef, Is Conserved among SERINC5 Orthologs

(A) Human, mouse, and zebrafish SERINC5 proteins share the ability to inhibit the specific infectivity of HIV-1 progeny virions.

(B) Nef_{SF2} expressed *in trans* in virus producer cells counteracts the effect of exogenous mouse, but not zebrafish, SERINC5 on Nef⁻ HIV-1 progeny virion infectivity.

Bar graphs represent the mean + SD from 3 biological replicates.

RESULTS

Anti-HIV-1 Activity, but Not Sensitivity to Nef, Is Conserved among Vertebrate SERINC5 Orthologs

As little as 100 ng of a relatively weak (pBJ5-based) SERINC5 expression plasmid is sufficient to potently inhibit the single-cycle infectivity of Nef⁻ HIV-1 virions (Usami et al., 2015). Under the same conditions, mouse SERINC5 reduced the specific infectivity of Nef⁻ HIV-1 virions produced in 293T cells to a similar extent (40- to 50-fold) as human SERINC5 (Figure 1A). Zebrafish (*Danio rerio*) SERINC5, which is only 61% identical to human SERINC5, reduced progeny virus infectivity 13- or 21-fold when 100 or 500 ng of expression vector were used, respectively (Figure 1A).

Next, we examined the sensitivities of mouse and zebrafish SERINC5 to Nef_{SF2}, which selectively inhibits the incorporation of human SERINC5 into HIV-1 virions (Usami et al., 2015). Although the inhibitory effect of mouse SERINC5 on Nef⁻ HIV-1 produced in 293T cells was clearly counteracted by Nef_{SF2} expressed *in trans*, the effect of zebrafish SERINC5 was unaffected by Nef_{SF2} (Figure 1B). We infer that widely divergent SERINC5 proteins share the ability of human SERINC5 to inhibit HIV-1 infectivity, but not necessarily its sensitivity to Nef.

Transfer of Nef Sensitivity to a Nef-Resistant SERINC5

Because zebrafish SERINC5 was somewhat less active against Nef⁻ HIV-1 than human SERINC5, we also tested the SERINC5 protein of the Western clawed frog (*Xenopus tropicalis*). Frog SERINC5 expressed from pBJ5 inhibited the single-cycle infectivity of Nef⁻ HIV-1 progeny virions with a potency comparable to that of human SERINC5 (Figure 2A). However, whereas human SERINC5 is efficiently counteracted by Nef_{SF2} (Usami et al., 2015), the inhibitory effect of frog SERINC5 expressed from pBJ5 on Nef⁻ HIV-1 was unaffected by Nef_{SF2} (Figure 2C). Even when expressed from pBJ6, which lacks an SV40 origin required for episomal amplification, as well as SV40 enhancer sequences (Rosa et al., 2015), frog SERINC5 reduced the specific infectivity of Nef⁻ HIV-1 more than 40-fold, and this inhibitory effect largely persisted in the presence of Nef_{SF2} expressed *in trans* (Figure 2C). In the absence of exogenous SERINC5, the effect of Nef_{SF2} on the infectivity of Nef⁻ HIV-1 virions was quite modest in these experiments (Figure 2C), perhaps because 293T cells express only low levels of endogenous SERINC5 (Usami et al., 2015).

We subsequently used frog SERINC5 as a recipient of human SERINC5 sequences to identify determinants that confer sensitivity to Nef_{SF2}. The transmembrane hidden Markov model (TMHMM) membrane protein topology prediction method (Krogh et al., 2001) predicts that SERINC5 has 10 transmembrane helices, and our previous findings indicate that the loop connecting helices 7 and 8 is surface exposed (Usami et al., 2015). This in turn suggested that the loop connecting helices 8 and 9 represents the fourth and longest intracellular loop (Figure 2B). To examine the role of this region, we replaced intracellular loop 4 (ICL4) of frog SERINC5 (fS5) with that of human SERINC5 (hS5) (Figure 2B). The resulting fS5/hS5-ICL4 chimera largely retained the ability of frog SERINC5 to inhibit the specific infectivity of Nef⁻ HIV-1 progeny virions but lost much of its resistance to Nef_{SF2} expressed *in trans* (Figure 2C). Thus, the transfer of the ICL4 of human SERINC5 was sufficient to confer substantial sensitivity to Nef_{SF2}.

As previously reported (Usami et al., 2015), the incorporation of hemagglutinin (HA)-tagged human SERINC5 into progeny virions was strongly inhibited by Nef_{SF2} (Figure 2D, lanes 1 and 2). In contrast, the incorporation of frog SERINC5 was only slightly affected by Nef_{SF2} (Figure 2D, lanes 3 and 4). Finally, the effect of Nef_{SF2} on the incorporation of the fS5/hS5-ICL4 chimera resembled its effect on human SERINC5 (Figure 2D, lanes 5 and 6). We conclude that the transfer of the ICL4 was sufficient to allow Nef_{SF2} to significantly inhibit both the anti-HIV-1 effect and the virion incorporation of frog SERINC5.

The ICL4 of Human SERINC5 Confers Sensitivity to Widely Divergent Nefs

Unlike Nef_{SF2} (clade B), which selectively inhibits the incorporation of human SERINC5 into HIV-1 virions, Nef_{97ZA012} (clade C) also inhibits the incorporation of human SERINC3 (Usami et al., 2015). Nevertheless, the inhibitory effect of frog SERINC5 on HIV-1 infectivity was essentially unaffected by Nef_{97ZA012} (Figure S1A), as was the incorporation of frog SERINC5 into HIV-1 virions (Figure S1B). In contrast, Nef_{97ZA012} enhanced the specific infectivity of Nef⁻ HIV-1 produced in the presence of the fS5/hS5-ICL4 chimera about 13-fold (Figure S1A) and strongly inhibited the incorporation of the chimeric protein (Figure S1B). Thus, the ICL4 of human SERINC5 conferred sensitivity to HIV-1 Nefs from different subtypes.

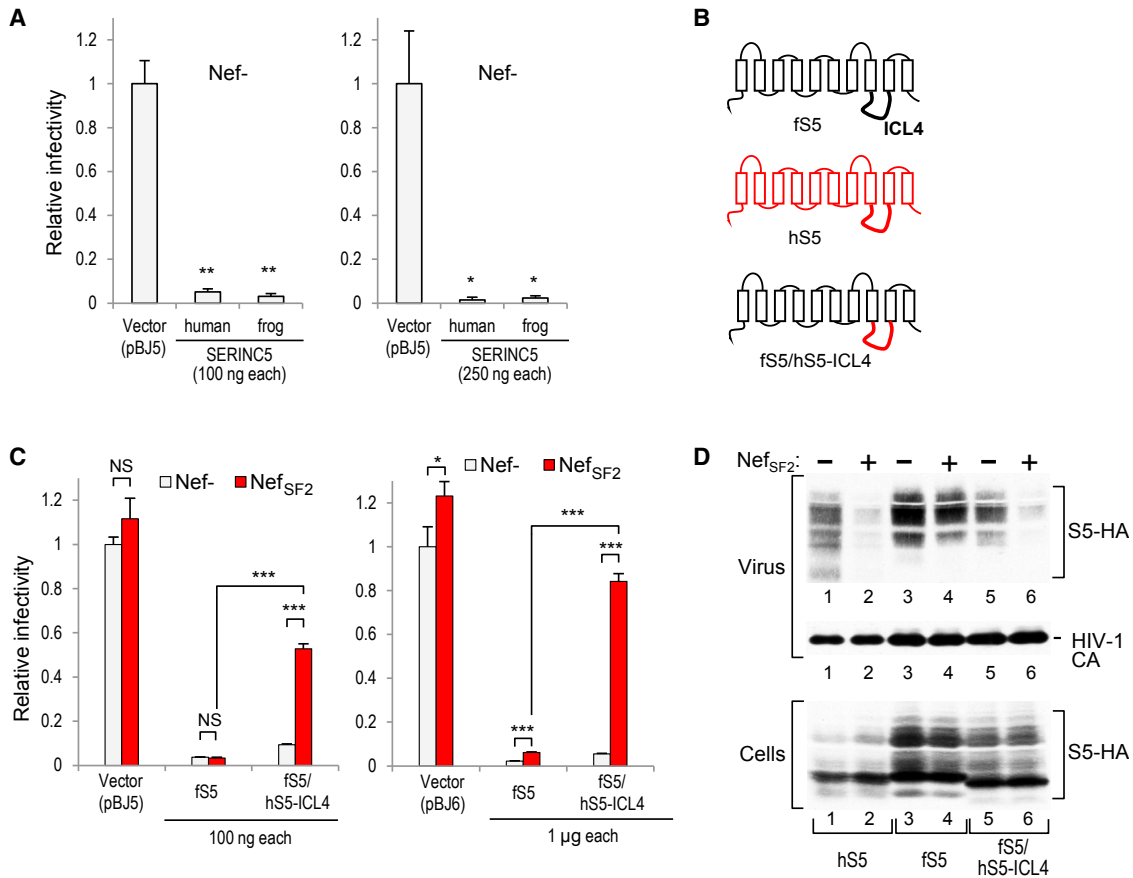


Figure 2. Transfer of Human SERINC5 ICL4 Is Sufficient to Confer Nef Responsiveness to Frog SERINC5

(A) Frog SERINC5 is as potent as human SERINC5 in inhibiting the single-round infectivity of Nef⁻ HIV-1 progeny virions.

(B) Schematic illustration of the parental SERINC5 proteins and of the chimera examined.

(C) Effect of frog SERINC5 on Nef⁻ HIV-1 progeny virion infectivity is largely resistant to Nef_{SF2} but becomes sensitive upon replacement of its ICL4 by that of human SERINC5. SERINC5s were expressed from pBJ5 or from the very weak pBJ6 expression plasmid.

(D) Western blots showing the effects of Nef_{SF2} on the incorporation of human, frog, and chimeric SERINC5 into Nef⁻ HIV-1 virions.

Bar graphs represent the mean + SD from 3 biological replicates.

See also Figures S1, S2, and S4.

We also observed that the LL_{164,165}AA AP-2 binding site mutant of Nef_{LAI}, which does not counteract SERINC5 (Rosa et al., 2015), was more abundant in HIV-1 virions produced in the presence of the fS5/hS5-ICL4 chimera than of frog SERINC5 (Figure S1C). Because frog SERINC5 and the fS5/hS5-ICL4 chimera were incorporated about equally in the absence of a functional Nef (Figure S1B), this finding provides indirect evidence for a physical interaction between Nef and the ICL4 of human SERINC5.

Because the ability to counteract SERINC5 is conserved among all primate lentiviral lineages (Heigle et al., 2016), we additionally examined the effects of simian immunodeficiency virus (SIV) Nefs. SIV_{mac239} Nef increased the specific infectivity of Nef⁻ HIV-1 produced in the presence of frog SERINC5 or of the fS5/hS5-ICL4 chimera 17- or 27-fold, respectively (Figure S1D). Thus, SIV_{mac239} Nef counteracted even unmodified frog SERINC5. In contrast, unmodified frog SERINC5 was largely resistant to the Nef proteins of SIV_{agm155} and SIV_{agm677}, and the

transfer of the ICL4 of human SERINC5 substantially increased its sensitivity to these Nefs (Figures S1E and S1F). Overall, these findings demonstrate that the ICL4 of human SERINC5 can confer sensitivity to Nefs from widely divergent primate lentiviruses.

Transfer of Sensitivity against Nef_{SF2} to SERINC3

Unlike other HIV-1 Nefs, Nef_{SF2} has no effect on the incorporation of SERINC3 into HIV-1 virions (Usami et al., 2015). To examine whether the ICL4 of SERINC5 confers sensitivity to Nef_{SF2} in the context of human SERINC3 (hS3), we generated the hS3/hS5-ICL4 chimera, which harbors the ICL4 of human SERINC5 in a human SERINC3 background (Figure S2A). Although the incorporation of authentic human SERINC3 was unaffected by Nef_{SF2}, as previously reported (Usami et al., 2015), the incorporation of the chimera was strongly inhibited (Figure S2B), confirming the role of the ICL4 in determining Nef sensitivity.

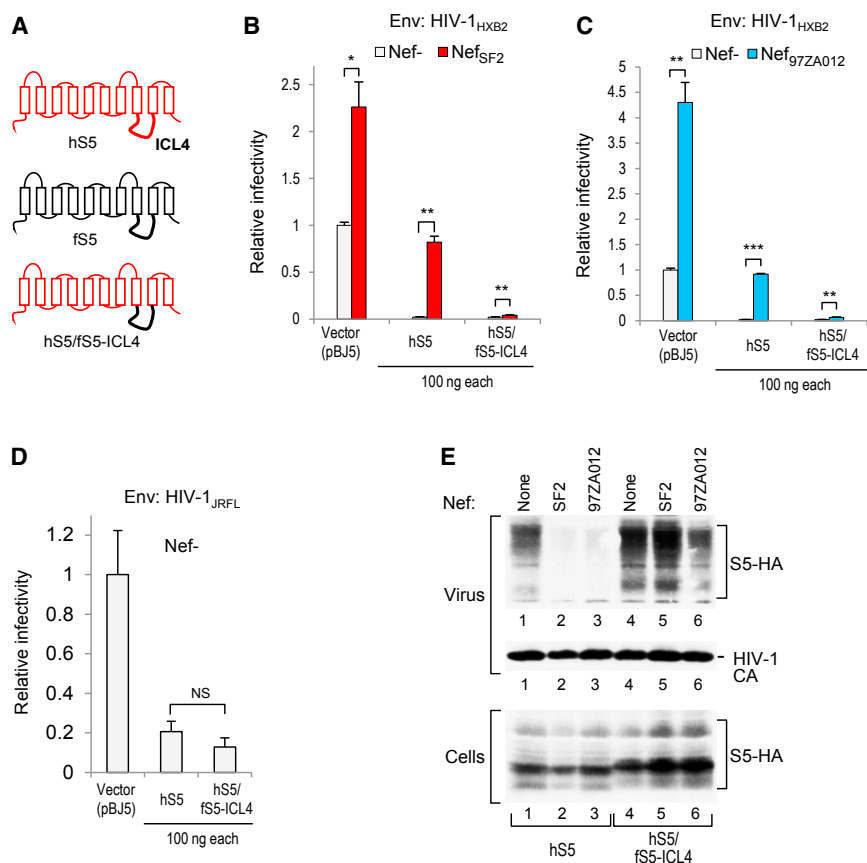


Figure 3. ICL4 of Frog SERINC5 Confers Resistance to Nef

(A) Schematic illustration of the parental SERINC5 proteins and of the chimera examined.

(B) Human SERINC5 bearing the ICL4 of frog SERINC5 remains fully capable of inhibiting the infectivity of Nef⁻ HIV-1 virions but exhibits strong resistance to a clade B HIV-1 Nef.

(C) SERINC5 chimera also exhibits resistance to a clade C HIV-1 Nef.

(D) SERINC5 chimera inhibits the infectivity of Nef⁻ HIV-1 virions carrying a relatively resistant HIV-1 Env at least as well as the parental human SERINC5.

(E) Western blots showing the effects of clade B and clade C HIV-1 Nefs on the incorporation of human SERINC5 and of the SERINC5 chimera into Nef⁻ HIV-1 virions.

Bar graphs represent the mean + SD from 3 biological replicates. See also Figure S4.

Transfer of Nef Resistance to Human SERINC5

The results described earlier showed that the fs5/hS5-ICL4 chimera gained sensitivity to various Nefs. We also made the reciprocal hS5/fs5-ICL4 chimera (Figure 3A), which inhibited the infectivity of Nef⁻ HIV-1 virions carrying Env_{HXB2} as potently as authentic human SERINC5 (Figures 3B and 3C). Although the infectivity of Nef⁻ HIV-1 virions carrying the relatively SERINC5-resistant Env_{JRFL} was inhibited to a lesser extent as expected (Rosa et al., 2015), the hS5/fs5-ICL4 chimera again was at least as potent as human SERINC5 (Figure 3D), confirming that it retained full anti-HIV activity. Crucially, whereas Nef_{SF2} increased the infectivity of progeny virions carrying Env_{HXB2} about 37-fold in the presence of exogenous human SERINC5, only a 2-fold increase was observed in the presence of the hS5/fs5-ICL4 chimera (Figure 3B). Furthermore, similar results were obtained with Nef_{97ZA012} (Figure 3C). Consistent with these observations, the incorporation of the hS5/fs5-ICL4 chimera into HIV-1 virions was not inhibited by Nef_{SF2} and was only moderately reduced by Nef_{97ZA012} (Figure 3E). As expected, both Nef proteins strongly inhibited the incorporation of authentic human SERINC5 (Figure 3E). We conclude that the hS5/fs5-ICL4 chimera exhibits marked resistance to HIV-1 Nef proteins.

We also replaced the ICL4 of human SERINC5 with that of human SERINC2 (hS2), which exhibits no sequence similarity in this region. The resulting hS5/hS2-ICL4 chimera (Figure 4A) inhibited

the specific infectivity of Nef⁻ HIV-1 virions, albeit less potently than authentic human SERINC5 (Figure 4B). In this experiment, Nef_{SF2} increased virion infectivity more than 50-fold in the presence of exogenous human SERINC5 but less than 2-fold in the presence of the chimera. Furthermore, the incorporation of the hS5/hS2-ICL4 chimera into HIV-1 virions was essentially resistant to Nef_{SF2} (Figure 4C). In addition, flow cytometry

revealed that the surface expression of SERINC5(iHA), which contains an internal HA tag within an extracellular loop, was not compromised when its ICL4 was replaced with that of SERINC2; it also revealed that the ICL4 swap conferred resistance to downregulation by Nef_{SF2} (Figure S3). Altogether, these results establish that the ICL4 swap markedly reduced the sensitivity of human SERINC5 to Nef_{SF2}.

During the course of these experiments, we observed that the incorporation of human SERINC2 into Nef⁻ HIV-1 virions, although unaffected by Nef_{SF2}, is inhibited by Nef_{97ZA012} (Figure 4D). Similarly, the incorporation of the hS5/hS2-ICL4 chimera, while unaffected by Nef_{SF2}, was clearly inhibited by Nef_{97ZA012} (Figure 4E). Because the hS5/fs5-ICL4 chimera, which differs only in the ICL4, was resistant to Nef_{97ZA012} (Figure 3C), these findings raise the possibility that Nef_{97ZA012} recognizes a determinant within the ICL4 of SERINC2. In addition, these findings suggest that only ICL4 regions derived from SERINC5 that exhibit resistance to a given Nef confer resistance to the same Nef when transferred to a sensitive SERINC.

Point Mutations in the ICL4 of Human SERINC5 Confer Resistance to Nef

Although Nef typically targets dileucine- or tyrosine-based sorting motifs, such motifs are not evident within the ICL4 of human or mouse SERINC5 (Figure S4A). To examine which region of the ICL4 determines sensitivity to Nef, we generated

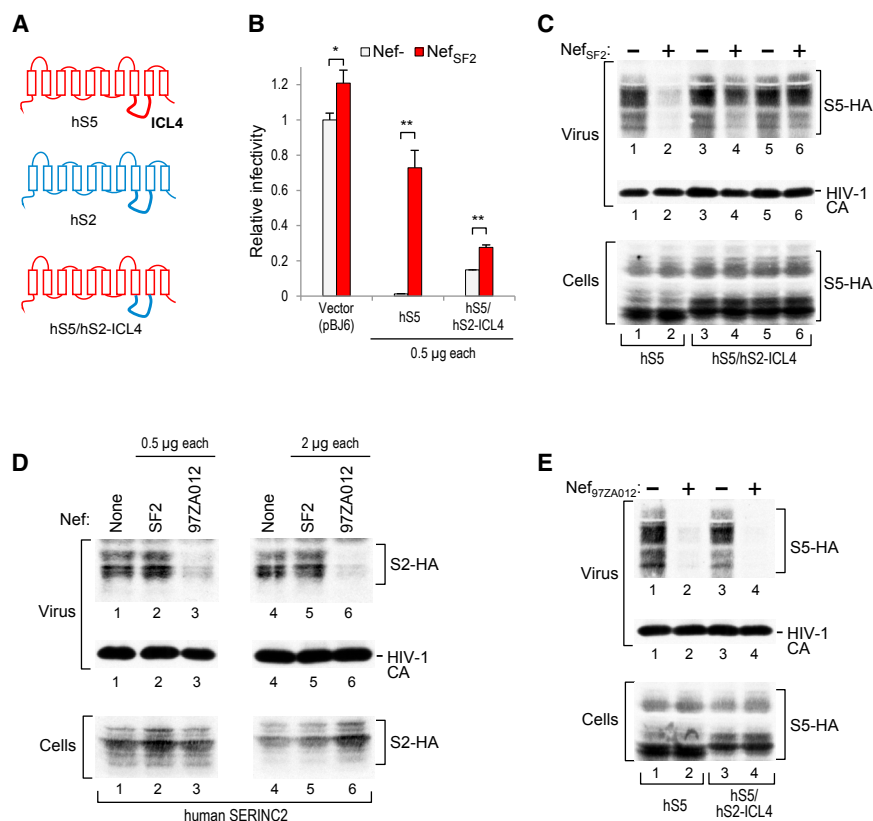


Figure 4. ICL4 of Human SERINC2 Confers Resistance to Nef_{SF2} but Not Nef_{97ZA012}

(A) Schematic illustration of the parental human SERINC proteins and of the chimera examined. (B) Inhibitory activity of human SERINC2 bearing the ICL4 of human SERINC2 on Nef⁻ HIV-1 progeny virion infectivity is largely resistant to Nef_{SF2}. Bar graphs represent the mean + SD from 3 biological replicates. (C) Western blots showing that the incorporation of the SERINC chimera into Nef⁻ HIV-1 virions is largely unaffected by Nef_{SF2} expressed *in trans*. The incorporation of the chimera was analyzed in duplicate to document reproducibility. (D) Western blots showing that the incorporation of human SERINC2 into Nef⁻ HIV-1 virions is unaffected by Nef_{SF2} but inhibited by Nef_{97ZA012}. (E) Western blots showing that Nef_{97ZA012} inhibits the incorporation of human SERINC2 bearing the ICL4 of human SERINC2 into Nef⁻ HIV-1 virions. See also Figures S3 and S4.

versions of Nef-resistant frog SERINC5 that have relatively poorly conserved segments replaced by the corresponding human SERINC5 sequences (Figure S4A). We observed that the transfer of ICL4 residues 9–26, but not of ICL4 residues 26–41, of human SERINC5 significantly increased the sensitivity of frog SERINC5 to Nef_{SF2} (Figure S4B). Within the 9–26 segment, a leucine residue (L350 in human SERINC5) is present in Nef-sensitive, but not in Nef-resistant, SERINC5 proteins (Figure S4A). Point mutations that targeted this residue (L350A) or another small hydrophobic residue nearby (I353A) reduced the sensitivity of human SERINC5 to Nef_{SF2} about 8- or 3-fold, respectively (Figure S4C). Together, these mutations (L350A/I353A) reduced the sensitivity of human SERINC5 more than 12-fold, and an 8-fold reduction was observed when L350 and I353 were replaced by the corresponding residues in frog SERINC5 (L350T/I353M) (Figure S4C). We also observed that the L350A and L350A/I353A mutations rendered the incorporation of human SERINC5 into HIV-1 virions largely resistant to Nef_{SF2} (Figure S4D). These findings confirm the crucial role of the ICL4 in Nef sensitivity.

DISCUSSION

This study shows that widely divergent SERINC5 proteins from different vertebrate species share the ability to markedly inhibit the infectivity of Nef⁻ HIV-1 virions. However, sensitivity to

Nef is not conserved and can be transferred to a Nef-resistant SERINC5 by exchanging the ICL4 region. In the same manner, human SERINC3 can be made sensitive to a particular Nef, supporting the crucial role of the ICL4 region in Nef sensitivity. Conversely, our results show that the anti-HIV-1 activity of human SERINC5 can be made to persist even in the presence of Nef, which might provide a novel strategy to combat HIV-1. However, the *in vivo* significance of the effect of Nef on SERINC5 remains to be established.

Nef is not conserved and can be transferred to a Nef-resistant SERINC5 by exchanging the ICL4 region. In the same manner, human SERINC3 can be made sensitive to a particular Nef, supporting the crucial role of the ICL4 region in Nef sensitivity. Conversely, our results show that the anti-HIV-1 activity of human SERINC5 can be made to persist even in the presence of Nef, which might provide a novel strategy to combat HIV-1. However, the *in vivo* significance of the effect of Nef on SERINC5 remains to be established.

Nearly the entire ICL4 region is predicted to be flexible by the PROFbval program (Schlessinger et al., 2006). The apparent flexibility of the ICL4 could conceivably facilitate interactions with widely divergent Nef proteins through a mechanism involving conformational selection (Boehr et al., 2009). Our data show that the ICL4 of human SERINC5 can confer sensitivity to Nefs from different primate lentiviral lineages.

The conservation of the anti-HIV activity among vertebrate SERINC5 proteins indicates that this activity is closely related to the biological function of SERINC5, which remains to be elucidated. SERINC5 has been named for their reported function as carrier proteins that facilitate the synthesis of serine-derived lipids (Inuzuka et al., 2005). This activity suggested a model in which SERINC5 inhibits HIV-1 infectivity by modifying the lipid content of progeny virions. However, a study argues strongly against this hypothesis by showing that SERINC5 does not alter the lipid composition of HIV-1 progeny virions or the exposure of phosphatidylserine on their surface (Trautz et al., 2017).

Usami et al. (2015) and Rosa et al. (2015) have presented evidence indicating that Nef counteracts SERINC5 by preventing its incorporation into HIV-1 virions. More recent work confirmed that Nef excludes SERINC5 from virions but suggested that Nef additionally inactivates the antiviral activity of any SERINC5 that becomes virion-associated even in the

presence of Nef as a consequence of overexpression (Trautz et al., 2016). In the present study, the ability of Nef proteins to reduce the incorporation of native or chimeric versions of frog or human SERINC5 correlated well with their ability to enhance HIV-1 infectivity, which supports the notion that virion exclusion is the primary mechanism by which Nef counteracts SERINC5.

The ability of Nef to counteract human SERINC5 depends on the AP-2 clathrin adaptor complex (Rosa et al., 2015), whose cargo-binding AP2M1 and AP2S1 subunits in particular are more than 95% identical in human, frog, and zebrafish. Nef also hijacks clathrin adaptors to downregulate CD4 and major histocompatibility complex class I (MHC class I) molecules, and in these cases, Nef:AP complexes recognize linear epitopes within the cytosolic domains of CD4 and MHC class I molecules (Jia et al., 2012; Ren et al., 2014). Our results indicate that certain small hydrophobic residues within the ICL4 of human SERINC5 are critical for its sensitivity to Nef, and it is tempting to speculate that the region containing these residues is also recognized by Nef:AP complexes.

EXPERIMENTAL PROCEDURES

Analysis of Virus Infectivity

Pseudovirions capable of a single round of replication were produced by transfecting 293T cells using a calcium phosphate precipitation method. The cells were co-transfected with HXB/Env⁻/Nef⁻ (1 μg in the experiments shown in Figures 1A, 1B, and 2A; 2 μg in all other experiments), a pSVIIIenv-based plasmid expressing Env_{HXB2} or, where indicated, Env_{JRFL} (0.1 μg in the experiments shown in Figures 1A, 1B, 2A, and 3D; 0.4 μg in all other experiments), and the indicated amounts of pBJ5- or pBJ6-based plasmids expressing wild-type or chimeric SERINC5 proteins, or with equimolar amounts of empty vector. In addition, equal amounts of the *nef*-deficient pNef_{FS} or of a plasmid expressing an HIV-1 or SIV Nef were co-transfected where indicated (2 μg in the experiments shown in Figure 1B and in the left panel of Figure 2C; 0.5 μg in all other experiments). Supernatants containing progeny virions were harvested 1 day post-transfection, clarified by low-speed centrifugation, filtered through 0.45-μm pore filters, and then used immediately to infect TZM-bl indicator cells in triplicate in 6-well plates. Aliquots of the virus stocks were frozen for HIV-1 capsid (p24) antigen quantitation by a standard ELISA. Three days post-infection, the indicator cells were lysed, and β-galactosidase activity induced as a consequence of infection was measured using a kit (E2000; Promega). Values were normalized for the amount of p24 antigen present in the supernatants used for infection.

Analysis of SERINC Incorporation

293T cells were co-transfected with 1 μg HXB/Env⁻/Nef⁻, pBJ5-based expression plasmids for C-terminally HA-tagged SERINC5s or SERINC5 chimeras (0.1 μg, unless indicated otherwise), and the *nef*-deficient control plasmid pNef_{FS} or a plasmid expressing an HIV-1 Nef (0.5 μg in the experiments shown in Figures 3E, S1B, S2B, and S4D; 2 μg in the experiments shown in Figures 2D, 4C, and 4E). Virions released into the medium were pelleted through 20% sucrose cushions, and virus- and cell-associated proteins were detected by western blotting as described (Accola et al., 2000). Because SERINC5 proteins are highly prone to aggregation (Usami et al., 2015), samples used for the detection of SERINC5s were not boiled before loading. The antibodies used were 183-H12-5C against HIV-1 CA, and HA.11 (BioLegend) against the HA epitope.

Statistical Analysis

Statistical analysis was performed using a two-tailed unpaired t test with Welch's correction in case of unequal variance. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, NS, not significant (p > 0.05).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <https://doi.org/10.1016/j.celrep.2017.12.082>.

ACKNOWLEDGMENTS

We thank M. Pizzato for pBJ6-SERINC5 and the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 for monoclonal antibody 183-H12-5C and for TZM-bl cells. This work was supported by NIAID/NIH grant R01AI127263.

AUTHOR CONTRIBUTIONS

W.D., Y.U., and Y.W. performed the experiments. W.D. and H.G. designed the experiments and analyzed the data. H.G. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: September 7, 2017

Revised: December 5, 2017

Accepted: December 22, 2017

Published: January 23, 2018

REFERENCES

- Accola, M.A., Strack, B., and Göttlinger, H.G. (2000). Efficient particle production by minimal Gag constructs which retain the carboxy-terminal domain of human immunodeficiency virus type 1 capsid-p2 and a late assembly domain. *J. Virol.* **74**, 5395–5402.
- Aiken, C., and Trono, D. (1995). Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. *J. Virol.* **69**, 5048–5056.
- Aiken, C., Konner, J., Landau, N.R., Lenburg, M.E., and Trono, D. (1994). Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell* **76**, 853–864.
- Beitari, S., Ding, S., Pan, Q., Finzi, A., and Liang, C. (2017). Effect of HIV-1 Env on SERINC5 antagonism. *J. Virol.* **91**, e02214–e02216.
- Boehr, D.D., Nussinov, R., and Wright, P.E. (2009). The role of dynamic conformational ensembles in biomolecular recognition. *Nat. Chem. Biol.* **5**, 789–796.
- Chaudhuri, R., Lindwasser, O.W., Smith, W.J., Hurley, J.H., and Bonifacino, J.S. (2007). Downregulation of CD4 by human immunodeficiency virus type 1 Nef is dependent on clathrin and involves direct interaction of Nef with the AP2 clathrin adaptor. *J. Virol.* **81**, 3877–3890.
- Chowers, M.Y., Spina, C.A., Kwok, T.J., Fitch, N.J., Richman, D.D., and Guatelli, J.C. (1994). Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact *nef* gene. *J. Virol.* **68**, 2906–2914.
- Chowers, M.Y., Pandori, M.W., Spina, C.A., Richman, D.D., and Guatelli, J.C. (1995). The growth advantage conferred by HIV-1 *nef* is determined at the level of viral DNA formation and is independent of CD4 downregulation. *Virology* **212**, 451–457.
- Collins, K.L., Chen, B.K., Kalams, S.A., Walker, B.D., and Baltimore, D. (1998). HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* **391**, 397–401.
- Deacon, N.J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D.J., McPhee, D.A., Greenway, A.L., Ellett, A., Chatfield, C., et al. (1995). Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* **270**, 988–991.
- Forshey, B.M., and Aiken, C. (2003). Disassembly of human immunodeficiency virus type 1 cores in vitro reveals association of Nef with the subviral ribonucleoprotein complex. *J. Virol.* **77**, 4409–4414.

- Garcia, J.V., and Miller, A.D. (1991). Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. *Nature* 350, 508–511.
- Haller, C., Müller, B., Fritz, J.V., Lamas-Murua, M., Stolp, B., Pujol, F.M., Keppler, O.T., and Fackler, O.T. (2014). HIV-1 Nef and Vpu are functionally redundant broad-spectrum modulators of cell surface receptors, including tetraspanins. *J. Virol.* 88, 14241–14257.
- Heigele, A., Kmiec, D., Regensburger, K., Langer, S., Peiffer, L., Stürzel, C.M., Sauter, D., Peeters, M., Pizzato, M., Learn, G.H., et al. (2016). The potency of Nef-mediated SERINC5 antagonism correlates with the prevalence of primate lentiviruses in the wild. *Cell Host Microbe* 20, 381–391.
- Inuzuka, M., Hayakawa, M., and Ingi, T. (2005). Serinc, an activity-regulated protein family, incorporates serine into membrane lipid synthesis. *J. Biol. Chem.* 280, 35776–35783.
- Jia, X., Singh, R., Homann, S., Yang, H., Guatelli, J., and Xiong, Y. (2012). Structural basis of evasion of cellular adaptive immunity by HIV-1 Nef. *Nat. Struct. Mol. Biol.* 19, 701–706.
- Kestler, H.W., 3rd, Ringler, D.J., Mori, K., Panicali, D.L., Sehgal, P.K., Daniel, M.D., and Desrosiers, R.C. (1991). Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell* 65, 651–662.
- Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E.L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* 305, 567–580.
- Matheson, N.J., Sumner, J., Wals, K., Rapiteanu, R., Weekes, M.P., Vigan, R., Weinelt, J., Schindler, M., Antrobus, R., Costa, A.S., et al. (2015). Cell surface proteomic map of HIV infection reveals antagonism of amino acid metabolism by Vpu and Nef. *Cell Host Microbe* 18, 409–423.
- Miller, M.D., Warmerdam, M.T., Gaston, I., Greene, W.C., and Feinberg, M.B. (1994). The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. *J. Exp. Med.* 179, 101–113.
- Miller, M.D., Warmerdam, M.T., Page, K.A., Feinberg, M.B., and Greene, W.C. (1995). Expression of the human immunodeficiency virus type 1 (HIV-1) nef gene during HIV-1 production increases progeny particle infectivity independently of gp160 or viral entry. *J. Virol.* 69, 579–584.
- Pizzato, M., Helander, A., Popova, E., Calistri, A., Zamborlini, A., Palù, G., and Göttlinger, H.G. (2007). Dynamin 2 is required for the enhancement of HIV-1 infectivity by Nef. *Proc. Natl. Acad. Sci. USA* 104, 6812–6817.
- Ren, X., Park, S.Y., Bonifacino, J.S., and Hurley, J.H. (2014). How HIV-1 Nef hijacks the AP-2 clathrin adaptor to downregulate CD4. *eLife* 3, e01754.
- Rosa, A., Chande, A., Ziglio, S., De Sanctis, V., Bertorelli, R., Goh, S.L., McCauley, S.M., Nowosielska, A., Antonarakis, S.E., Luban, J., et al. (2015). HIV-1 Nef promotes infection by excluding SERINC5 from virion incorporation. *Nature* 526, 212–217.
- Schlessinger, A., Yachdav, G., and Rost, B. (2006). PROFbval: predict flexible and rigid residues in proteins. *Bioinformatics* 22, 891–893.
- Schwartz, O., Maréchal, V., Danos, O., and Heard, J.M. (1995). Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell. *J. Virol.* 69, 4053–4059.
- Schwartz, O., Maréchal, V., Le Gall, S., Lemonnier, F., and Heard, J.M. (1996). Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nat. Med.* 2, 338–342.
- Spina, C.A., Kwok, T.J., Chowder, M.Y., Guatelli, J.C., and Richman, D.D. (1994). The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. *J. Exp. Med.* 179, 115–123.
- Trautz, B., Pierini, V., Wombacher, R., Stolp, B., Chase, A.J., Pizzato, M., and Fackler, O.T. (2016). The antagonism of HIV-1 Nef to SERINC5 particle infectivity restriction involves the counteraction of virion-associated pools of the restriction factor. *J. Virol.* 90, 10915–10927.
- Trautz, B., Wiedemann, H., Lüchtenborg, C., Pierini, V., Kranich, J., Glass, B., Kräusslich, H.G., Brocker, T., Pizzato, M., Ruggieri, A., et al. (2017). The host-cell restriction factor SERINC5 restricts HIV-1 infectivity without altering the lipid composition and organization of viral particles. *J. Biol. Chem.* 292, 13702–13713.
- Usami, Y., and Göttlinger, H. (2013). HIV-1 Nef responsiveness is determined by Env variable regions involved in trimer association and correlates with neutralization sensitivity. *Cell Rep.* 5, 802–812.
- Usami, Y., Popov, S., and Göttlinger, H.G. (2014). The Nef-like effect of murine leukemia virus glycosylated gag on HIV-1 infectivity is mediated by its cytoplasmic domain and depends on the AP-2 adaptor complex. *J. Virol.* 88, 3443–3454.
- Usami, Y., Wu, Y., and Göttlinger, H.G. (2015). SERINC3 and SERINC5 restrict HIV-1 infectivity and are counteracted by Nef. *Nature* 526, 218–223.

Cell Reports, Volume 22

Supplemental Information

**A Long Cytoplasmic Loop Governs
the Sensitivity of the Anti-viral Host
Protein SERINC5 to HIV-1 Nef**

Weiwei Dai, Yoshiko Usami, Yuanfei Wu, and Heinrich Göttlinger

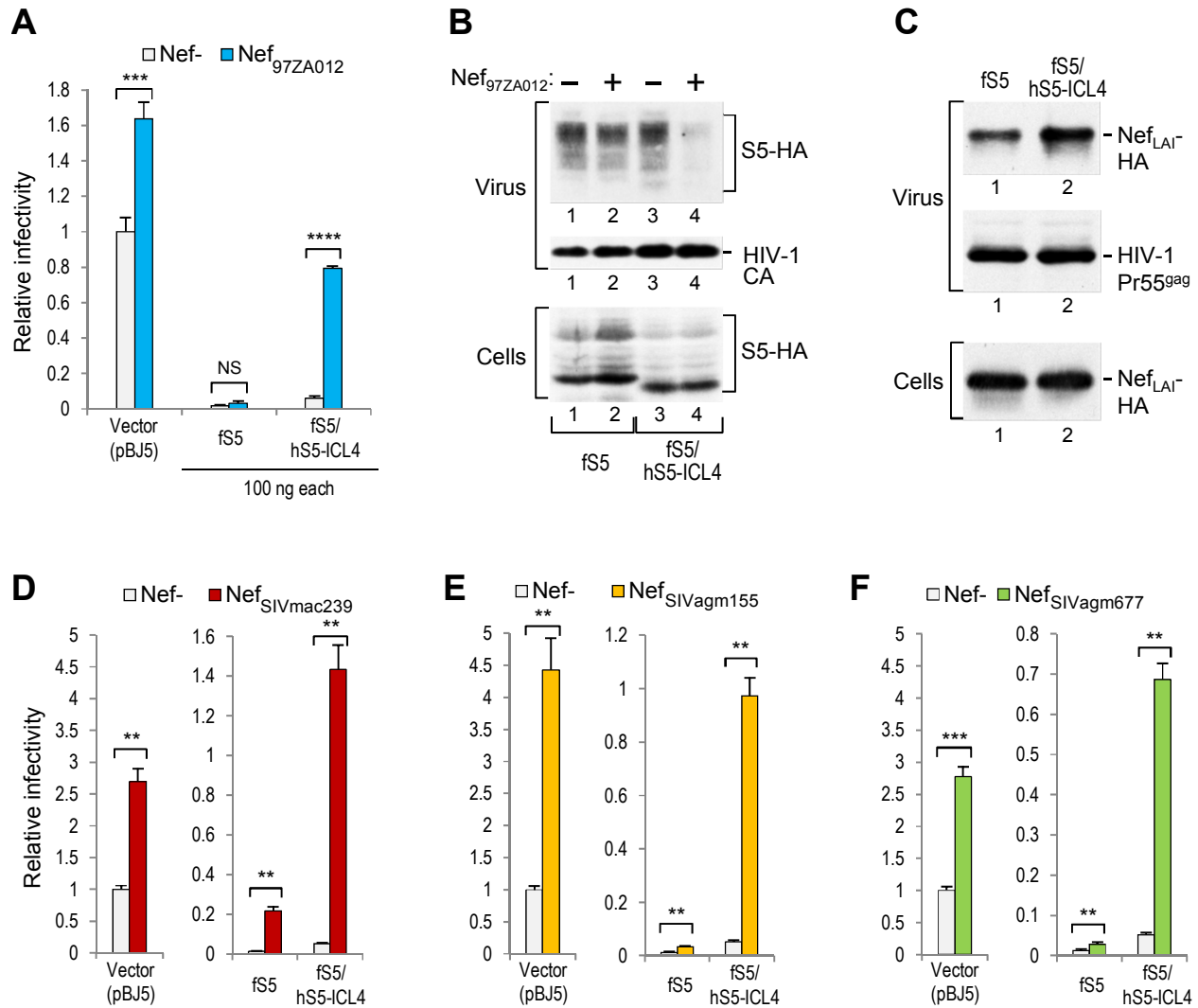


Figure S1 (related to Figure 2). The ICL4 of human SERINC5 confers sensitivity to widely divergent Nefs.

- (A) The inhibitory effect of frog SERINC5 on HIV-1 infectivity is resistant to HIV-1 subtype C Nef_{97ZA012} but becomes sensitive in the presence of the ICL4 of human SERINC5.
- (B) Nef_{97ZA012} does not affect the incorporation of frog SERINC5 into Nef⁻ HIV-1 virions but strongly inhibits the incorporation of a chimeric version that harbors the ICL4 of human SERINC5.
- (C) A Nef_{LAI} mutant unable to counteract SERINC5 (LL_{164,165}AA) is more abundantly incorporated into HIV-1 virions produced in the presence of frog SERINC5 bearing the ICL4 of human SERINC5. The virions lacked protease to prevent the cleavage of Nef.
- (D-F) Effects of SIV Nef proteins on the inhibition of HIV-1 infectivity by frog SERINC5 and by a version that harbors the ICL4 of human SERINC5.

The bar graphs represent the mean + SD from 3 biological replicates.

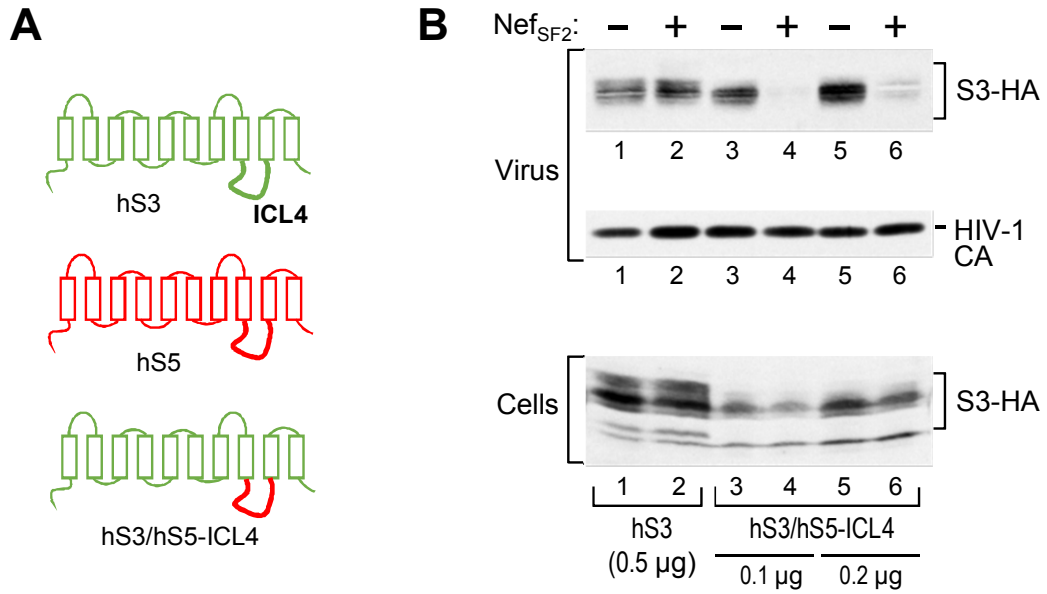


Figure S2 (related to Figure 2). Human SERINC3 acquires sensitivity to Nef_{SF2} in the presence of the ICL4 of human SERINC5.

(A) Schematic illustration of the parental human SERINC proteins and of the chimera examined.

(B) Western blots showing that Nef_{SF2} inhibits the incorporation of human SERINC3 into Nef⁻ HIV-1 virions only upon replacement of its ICL4 by that of human SERINC5.

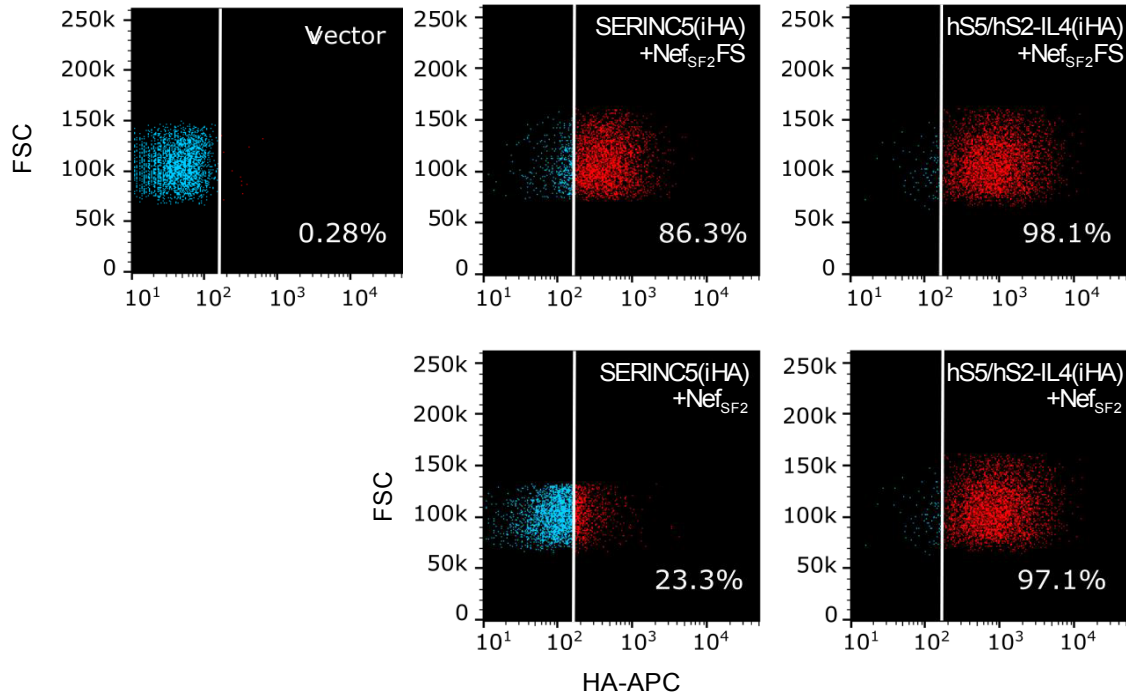


Figure S3 (related to Figure 4). Swapping the ICL4 of human SERINC5 for that of human SERINC2 confers resistance to down-regulation by Nef_{SF2}. JurkatTAg S5 ^{-/-} cells stably expressing SERINC5(iHA) or the S5/hS2-ICL4(iHA) chimera together with Nef_{SF2} were surface-stained with anti-HA antibody and analyzed by flow cytometry. Per cent fractions of cells expressing SERINC5(iHA) or the S5/hS2-ICL4(iHA) chimera on the surface are indicated.

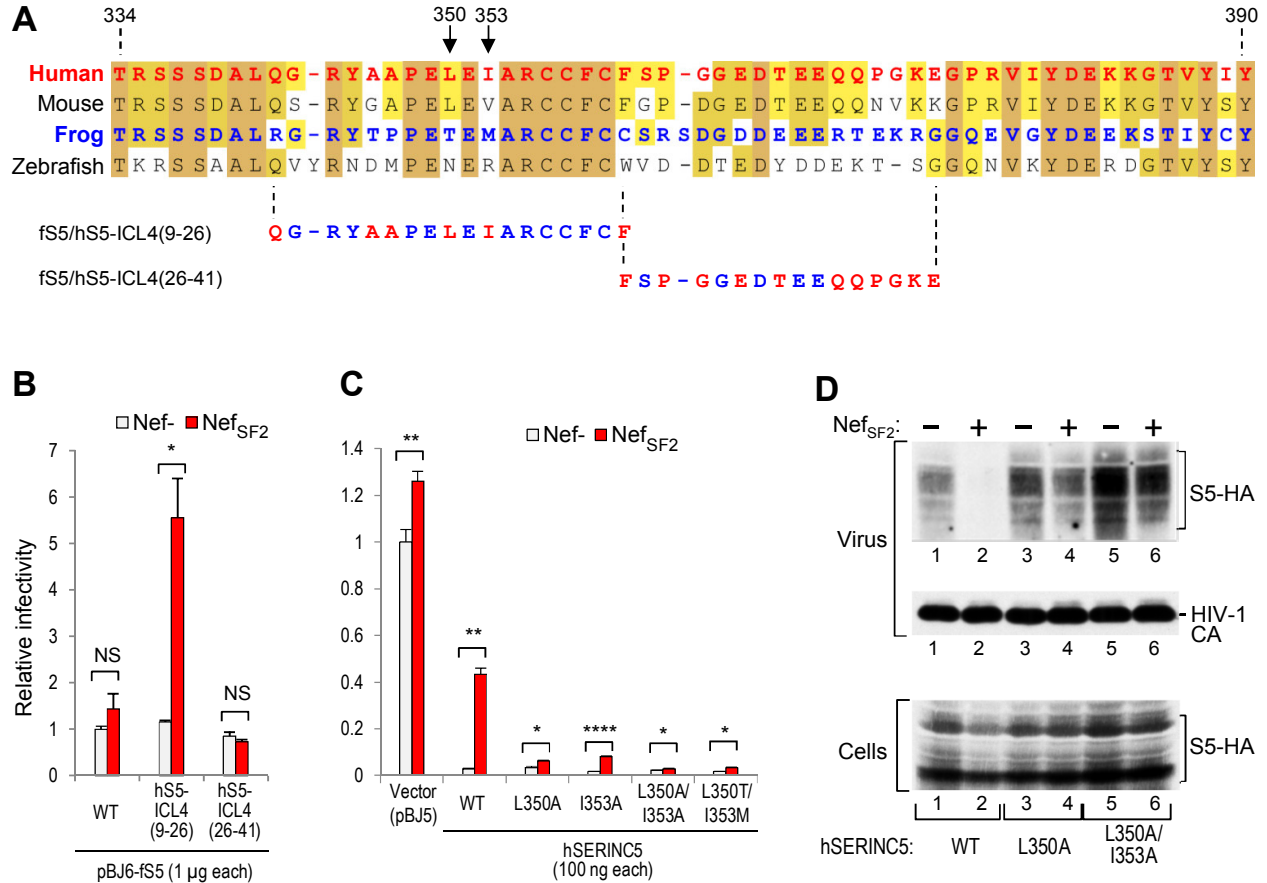


Figure S4 (related to Figures 2 through 4). Point mutations in the ICL4 of human SERINC5 confer resistance to Nef.

- (A) Alignment of the ICL4 regions of Nef-sensitive (human, mouse) and Nef-insensitive (frog, zebrafish) SERINC5 proteins, and schematic illustration of intra-ICL4 chimeras examined.
- (B) The transfer of human SERINC5 ICL4 residues 9-26, but not of residues 26-41, to the ICL4 of frog SERINC5 increases its sensitivity to Nef_{SF2}.
- (C) Point mutations targeting small hydrophobic amino acids within the ICL4 of human SERINC5 reduce the sensitivity of its effect on HIV-1 infectivity to Nef_{SF2}.
- (D) Western blots showing the effects of Nef_{SF2} on the incorporation of WT human SERINC5 and of ICL4 mutants into Nef⁻ HIV-1 virions.

The bar graphs represent the mean + SD from 3 biological replicates.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids. The *env*- and *nef*-deficient HIV-1 provirus HXB/Env⁻/Nef⁻, the pSVIIIenv-based plasmids expressing Env_{HXB2} or Env_{JRFL}, the pBJ5-based expression plasmids for the Nef proteins of HIV-1_{SF2}, HIV-1_{97ZA012}, and SIV_{mac239}, the *nef*-deficient control plasmid pNef_{FS}, and the pBJ5-based expression plasmids for human SERINC3-HA, human SERINC5, and human SERINC5-HA have been described (Dorfman et al., 2002; Helseth et al., 1990; Pizzato et al., 2007; Usami et al., 2015). A pBJ6-based expression plasmid for human SERINC5 was kindly provided by M. Pizzato. The *nef* genes of SIV_{agm155} and SIV_{agm677} were amplified from proviral clones and inserted into pBJ5. The coding sequences for mouse, frog, and zebrafish SERINC5 were amplified from cloned cDNAs purchased from Dharmacon (GenBank: BC062131, XM_002940195, and BC044159). DNAs encoding mutant and chimeric proteins without or with C-terminal HA tags were generated using an overlap extension PCR method (Horton et al., 1989) and inserted into pBJ5 or pBJ6 downstream of a Kozak sequence. The fS5/hS5-ICL4 chimera has residues 342-390 of frog SERINC5 (fS5) replaced by residues 342-389 of human SERINC5 (hS5). The intra-ICL4 chimeras fS5/hS5-ICL4(9-26) and fS5/hS5-ICL4(26-41) have residues 342-359 and 359-375 of frog SERINC5 replaced by the corresponding residues of human SERINC5. The hS5/fS5-ICL4 chimera has residues 342-389 of human SERINC5 replaced by residues 342-390 of frog SERINC5. The hS5/hS2-ICL4 chimera has residues 334-389 of human SERINC5 replaced by residues 343-388 of human SERINC2 (hS2). The hS3/hS5-ICL4 chimera has residues 358-404 of human SERINC3 (hS3) replaced by residues 335-389 of human SERINC5.

Retroviral vectors. The coding sequence for SERINC5(iHA) (Usami et al., 2015) preceded by a Kozak sequence was amplified from pBJ5-SERINC5(iHA) and inserted into the retroviral vector MSCVpuro (Clontech). Additionally, a version of the hS5/hS2-ICL4 chimera that harbors an internal HA tag at the same position as SERINC5(iHA) was cloned into MSCVpuro. The coding sequence for HIV-1 Nef_{SF2} preceded by a Kozak sequence was cloned into MSCVhyg (Clontech), yielding MSCVhygNef_{SF2}. A *nef*-deficient version (MSCVhygNef_{SF2}FS) was obtained by generating a frameshift at a unique *Xho*I site in *nef*.

Analysis of Nef incorporation. 293T cells were co-transfected with 2 µg of the protease-deficient HIV-1 proviral construct HXB/VPR⁺/PR⁻ (Bukovsky and Gottlinger, 1996), 2 µg of a pBJ5-based plasmid expressing C-terminally HA-tagged Nef_{LAI} with a LL_{164,165}AA mutation (Pizzato et al., 2007), and 2 µg of a pBJ5-based plasmid expressing native frog SERINC5 or a version harboring the ICL4 of human SERINC5 (fS5/hS5-ICL4). Virions released into the medium were pelleted through 20% sucrose cushions, and virus- and cell-associated proteins were detected by western blotting. The antibodies used for western blotting were 183-H12-5C against HIV-1 CA, and HA.11 (BioLegend) against the HA epitope.

Flow cytometry. Ectopic SERINC expression cassettes were introduced into JurkatTAG S5^{-/-} (1) cells (Usami et al., 2015) by retroviral transduction with

MSCVpuroSERINC5(iHA) or MSCVpurohS5/hS2-ICL4(iHA). Simultaneously, the cells were transduced with the *nef*-deficient control vector MSCVhygNef_{SF2}FS or with MSCVhygNef_{SF2}. After selection with puromycin and hygromycin, the doubly transduced bulk cultures were surface-stained with anti-HA antibody and APC-conjugated anti-mouse IgG (Jackson ImmunoResearch). Samples were then analyzed on a BD LSR II flow cytometer.

SUPPLEMENTAL REFERENCES

Bukovsky, A., and Gottlinger, H. (1996). Lack of integrase can markedly affect human immunodeficiency virus type 1 particle production in the presence of an active viral protease. *J Virol* 70, 6820-6825.

Dorfman, T., Popova, E., Pizzato, M., and Gottlinger, H. G. (2002). Nef enhances human immunodeficiency virus type 1 infectivity in the absence of matrix. *J Virol* 76, 6857-6862.

Helseth, E., Kowalski, M., Gabuzda, D., Olshevsky, U., Haseltine, W., and Sodroski, J. (1990). Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. *J Virol* 64, 2416-2420.

Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* 77, 61-68.

Pizzato, M., Helander, A., Popova, E., Calistri, A., Zamborlini, A., Palu, G., and Gottlinger, H. G. (2007). Dynamin 2 is required for the enhancement of HIV-1 infectivity by Nef. *Proc Natl Acad Sci U S A* 104, 6812-6817.

Usami, Y., Wu, Y., and Gottlinger, H. G. (2015). SERINC3 and SERINC5 restrict HIV-1 infectivity and are counteracted by Nef. *Nature* 526, 218-223.