Cul3-KLHL20 E3 ubiquitin ligase plays a key role in the arms race between HIV-1 and host restriction

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Abstract

HIV-1 must counteract various host restriction factors to establish productive infection. SERINC5 is a critical host restriction factor that potently blocks HIV-1 entry from virions, but its activity is counteracted by Nef. The SERINC5 and Nef activities are both initiated from the plasma membrane, where SERINC5 is packaged into virions and downregulated by Nef via lysosomal degradation. However, it is still unclear how SERINC5 is localized to the plasma membrane and how its expression is regulated on the plasma membrane. We now report that Cullin 3-KLHL20, a *trans*-Golgi network (TGN)-localized E3 ubiquitin ligase, polyubiquitinates SERINC5 at lysine 130 via K33- and K48-linked ubiquitin chains. The K130 polyubiquitination is required not only for the SERINC5 expression on the plasma membrane, but also the SERINC5 anti-HIV-1 activity and the Nef counteractive activity. Our study reveals an important role of K33/K48-branched ubiquitin chains in HIV-1 infection by regulating protein post-Golgi trafficking and degradation.

Introduction

Mammalian cells have evolved complex defense mechanisms to restrict viral infection, but HIV-1 has also evolved various antagonisms to counteract different host restriction factors to establish productive infection[1]. Serine incorporator 5 (SERINC5) is a potent host restriction factor that was discovered to function in the intrinsic immunity that defenses against retrovirus infection[2, 3]. SERINC5 is a ~ 45-kDa integral membrane protein that has ten transmembrane helices organized into two subdomains[4]. It is incorporated into budding virus particles from the cell surface of infected cells and subsequently disrupts the viral envelope (Env) glycoprotein trimmers in their open conformation, which prevents the fusion pore formation between virions and target cells, and thereby blocks virus entry[5, 6, 7, 8]. The SERINC5 antiviral activity is conserved across different species, and its important role in defending against retrovirus infection *in vivo* has been demonstrated in humans, monkeys, and mice[9, 10, 11].

To evade this powerful host restriction, retroviruses antagonize SERINC5 by expressing three structurally unrelated proteins: negative factor (Nef) from human immunodeficiency virus type 1 (HIV-1), glycoGag from murine leukemia virus (MLV), and S2 from equine infectious anemia virus (EIAV)[2, 3, 12, 13]. Nef, glycoGag, and S2 downregulate SERINC5 from the cell surface by directing SERINC5 to the endosomes and lysosomes for degradation, which prevents SERINC5 from incorporation into virions[14, 15, 16]. In particular, Nef interacts with SERINC5 via the largest intracellular loop 4 (ICL4) of SERINC5[17]. In addition, Nef directs the Cyclin K/Cyclin-dependent kinase 13 (CDK13) complex to phosphorylate a serine residue in ICL4, which bridges SERINC5 with the adaptor protein 2 (AP2) complex via Nef for endocytosis[18].

SERINC5 is expressed on the plasma membrane, where the SERINC5 antiviral activity and the Nef counteractive activity are initiated. When SERINC5 was dissociated from the plasma membrane by removal of its 10th transmembrane domain, its antiviral activity was lost[19]. In addition, when Nef was dissociated from the plasma membrane by removal of its myristoylation site, the SERINC5

downregulation was also blocked[16]. However, it is still unclear how SERINC5 trafficking is controlled at the post-Golgi level and how its expression is regulated on the plasma membrane.

Ubiquitination is catalyzed by three types of enzymes, including E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases[20]. Polyubiquitin chains are created when seven lysine residues K6, K11, K27, K29, K33, K48, and K63, or the N-terminal methionine (M1) of the 76-amino-acid (aa) ubiquitin itself are ubiquitinated by E1, E2, and E3. The substrate specificity of ubiquitination is determined by the large number of E3s. RING (Really Interesting New Gene) E3s are the most abundant ubiquitin ligases, in which the multi-subunit Cullin-RING ubiquitin ligases (CRLs) comprise the largest family[21]. CRLs are assembled from seven Cullin (Cul) proteins (Cul1, Cul2, Cul3, Cul4A, Cul4B, Cul5, and Cul7) that serve as a scaffold that engages substrates with E2s. With CRL3 E3 ligases, Cul3 recruits E2s via RING-box protein 1 (Rbx1), and substrates by Bric-a-brac/Tramtrack/Broad (BTB) proteins[22]. Among these BTB proteins, the Kelch-repeat domain subfamily is the most prevalent in metazoans, in which the *trans*-Golgi network (TGN)-localized, Kelch-like protein 20 (KLHL20), functions as an important Cul3 substrate adaptor[23]. Here, we identified $CRL3^{KLHL20}$ as a critical SERINC5 E3 ubiquitin ligase and have elucidated its critical role in SERINC5 expression and the arms race between HIV-1 and host restriction.

Results

Identification of Cul3-KLHL20 as a SERINC5 ubiquitin E3 ligase via mass spectrometry. To understand how SERINC5 is polyubiquitinated, we interrogated the presence of E3 ubiquitin ligases in SERINC5 protein complexes by mass spectrometry. FLAG-tagged SERINC5 was purified from HEK293T cells by an anti-FLAG affinity column and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS), as we reported[18]. Four independent experiments were conducted from which a total of 25 ubiquitin E3 ligase-associated proteins were identified (Fig. 1A). Cul1, Cul3, and Cul4B were found in this list. Because Cul4B is expressed in the nucleus and responsible for cell cycle regulation, only Cul1 and Cul3 were selected for our study.

We reported that ectopic ubiquitin (Ub) decreases SERINC5 expression at steady state[14, 15, 16]. Accordingly, we expressed SERINC5 with ubiquitin in HEK293T cells in the presence of Cul1 and/or its adaptor protein Skp1, or Cul3 and/or its adaptor protein KLHL20. The SERINC5 expression was then analyzed by western blotting (WB) (Fig. 1B). A SERINC5 lysine-free mutant with all 19 lysine residues mutated to arginine (1-19K/R), was included as a control. Ubiquitin decreased SERINC5 expression as expected (Fig. 1B, lanes 1, 2, 6, 7). Although this decrease was not affected by single expression of Cul1, Skp1, Cul3, or KLHL-20 and co-expression of Cul1 with Skp1 (lanes 3–5, 7–8), it was notably enhanced by co-expression of Cul3 with KLHL20 (lane 10). Neither ubiquitin nor Cul3/KLHL20 decreased the 1-19K/R mutant expression (lanes 11–15). In addition, KLHL20 decreased the amount of detectable ubiquitin (lanes 8, 10, 13, 15), indicating that Cul3-KLHL20 deceases the cellular ubiquitin pool. These results suggested that SERINC5 was likely targeted by the $CRL3^{KLHL20}$ ubiquitin E3 ligase.

Next, we interrogated the mechanism of how SERINC5 interacts with Cul3-KLHL20. We tried to knock out *Cul3* and *KLHL20* in HEK293T cells after expressing Cas9 with their specific guide (g) RNAs. Although these gRNAs effectively knocked out *Cul3* or *KLHL20* (Fig. 1C), we could not obtain stably knockout clones due to their necessity to cell survival. Thus, we used the same sets of Cas9 and gRNAs to transiently knock out their expression and studied their interaction with SERINC5.

To confirm that the SERINC5 interacts with Cul3/KLHL20, they were expressed in HEK293T cells and SERINC5 was subjected to immunoprecipitation (IP) (Fig. 1D). SERINC5 pulled down KLHL20 and Cul3, but not the GFP control (Fig. 1D, IP, lanes 2, 3, 5), indicating that SERINC5 interacts with Cul3-KLHL20. To test whether these interactions were dependent on each other, *KLHL20* and *Cul3* were knocked down by CRISPR/Cas9. The SERINC5-KLHL20 interaction was not affected by *Cul3*-knockdonw (KD), but the SERINC5-Cul3 interaction was disrupted by *KLHL2*-KD (IP, lanes 4, 6). These results demonstrate that SERINC5 interacts with Cul3-KLHL20 via KLHL20.

We then tested how SERINC5 interacts with KLHL20. KLHL20 has 609 amino acids that comprise three domains including Kelch-repeat (residues 1-316), BTB and C-terminal Kelch (BACK), and Bric-a-brac/tramtrack/broad complex (BTB) (residues 301–609). In addition, six residues at positions 109, 111, 113, 146, 148, and 150 are required for its binding to Cul3[23]. Two KLHL20 mutants, KLHL20ΔK that does not have the Kelch-repeat domain, and KLHL20m6 that has thosee six residues replaced with alanine, were tested for their interaction with SERINC5 by IP (Fig. 1E). Both the wild-type (WT) KLHL20 and its m6 mutant interacted with SERINC5 (Fig. 1E, IP, lanes 2, 4), whereas the ΔK mutant did not (IP, lane 3). These results demonstrate that SERINC5 binds to KLHL20 via the Kelch-repeat domain.

Next, we investigate the role of Cul3-KLHL20 in SERINC5 polyubiquitination. SERINC5 was expressed with ubiquitin in HEK293T cells in the presence of KLHL20/Cul3 expression or their knockdown by CRISPR/Cas9. Ectopic ubiquitin was subjected to immunoprecipitation and levels of SERINC5 polyubiquitination were analyzed by WB (Fig. 1F). To avoid SERINC5 degradation by ubiquitin, a ubiquitin mutant that had its K48 and K63 mutated to arginine ($Ub_{K48/63R}$) was used. Polyubiquitinated SERINC5 proteins were detected at over 90 kDa, that were increased by KLHL20 and/or Cul3 (Fig. 1F, IP, lanes 2–4). In addition, both *KLHL20*-KD and *Cul3*-KD notably reduced the SERINC5 polyubiquitination (IP, lanes 2, 6, 9). Importantly, this reduction by knockdown was rescued by ectopic KLHL20 or Cul3 expression (IP, lanes 7, 10).

To further explore this mechanism, the SERINC5 polyubiquitination was re-analyzed in the presence of ectopic ubiquitin, KLHL20 proteins, and the Cul3 E2-recruiting protein Rbx1 (Fig. 1G). When SERINC5 expression in cells was compared, its decrease by ubiquitin was enhanced by KLHL20 and Rbx1 (Fig. 1G, Input, lanes 3, 6), but not by the KLHL20ΔK and KLHL20m6 mutants (Input, lanes 4, 5). Consistently, when SERINC5 polyubiquitination was compared, it was increased by KLHL20 and Rbx1 (IP, lanes 3, 6), but was strongly decreased by these two KLHL20 mutants (IP, lanes 4, 5). These results demonstrate that like KLHL20, Rbx1 is also required for SERINC5 polyubiquitination. In addition, the SERINC5 polyubiquitination depends on the Cul3-KLHL20 interaction and the Kelch-repeat domain. Furthermore, it

was noticeable that the ΔK mutant was not detected from the pulldown sample (IP, lane 4), which further confirms that SERINC5 interacts with KLHL20 via the Kelch-repeat domain. Altogether, these results demonstrate that $CRL3^{KLHL20}$ is a SERINC5-associated ubiquitin ligase that is responsible for SERINC5 polyubiquitination.

Identification of lysine 130 (K130) as a critical polyubiquitination site on SERINC5. The 423 amino acids of human SERINC5 comprise 5 extracellular loops (ECLs), 10 transmembrane domains (TMDs), and 4 intracellular loops (ICLs) (Fig. 2A). They also include 19 lysine residues (K1 to K19) that are found spread throughout these regions. To test if these lysine residues are targeted for polyubiquitination, we generated SERINC5 mutants in which these lysine residues were replaced with arginine and determined how levels of SERINC5 polyubiquitination are affected.

Initially, we tested how ubiquitin affected expression and polyubiquitination of mutants 1-7K/R, 1-11K/R, 1-14K/R, 15-19K/R, and 1-19K/R, which have K1-K7, K1-K11, K1-K14, K15-K19, or K1-K19 replaced with arginine. These SERINC5 lysine mutants were expressed with ectopic ubiquitin and immunoprecipitated by anti-FLAG that targets SERINC5, and their polyubiquitination was detected by anti-HA that detects ectopic ubiquitin (Fig. 2B). Ubiquitin reduced expression of SERINC5 WT, 1-7K/R, and 15-19K/R in cells, and consistently, high levels of polyubiquitinated SERINC5 products were detected from these proteins (Fig. 2B, lanes 1–4, 9–10). On the contrary, ubiquitin did not affect expression of 1-11K/R, 1-14K/R, and 1-19K/R in cells, and consistently, their polyubiquitinated products were detected at much reduced levels (lanes 5–8, 11–12). These results suggest that there is a specific SERINC5 lysine residue(s) responsible for its polyubiquitination.

To further narrow down the specific site of polyubiquitination, we mutated K8 and K9, which are located at residue 130 or 179, to arginine, by generating mutants K130R and K179R. When these two mutants were expressed with ubiquitin, expression of K179R (lanes 17–18), but not K130R (lanes 15–16), was decreased in cells. In addition, K179R had a similar level of polyubiquitination as WT SERINC5 (lanes 13–14, 17–18), whereas K130R was poorly polyubiquitinated (lanes 15–16). These results demonstrate that K130 is the critical site for SERINC5 polyubiquitination.

To understand how K130 is polyubiquitinated, we generated a SERINC5 mutant, designated 130K, in which all the lysine residues, except K130, were replaced with arginine. In addition, we generated seven ubiquitin mutants, Ub_{K6R}, Ub_{K11R}, Ub_{K27R}, Ub_{K29R}, Ub_{K33R}, Ub_{K48R}, and Ub_{K63R}, where each of the seven lysine residues in ubiquitin were individually mutated to arginine. We also generated a ubiquitin mutant, Ub_{KO}, in which all seven lysine residues were mutated to arginine. When the SERINC5-130K mutant was expressed with either WT ubiquitin (Ub_{WT}) or each of the respective Ub mutants, its expression was only reduced in the presence of Ub_{WT} (Fig. 2C, Input, lane 2). Immunoprecipitation with anti-HA revealed that the polyubiquitination of K130 was detected with Ub_{WT}, but not Ub_{KO}, further confirming that K130 is the target for ubiquitination (IP, lanes 2, 10). Notably, although this SERINC5-130K mutant had a similar level of polyubiquitination with Ub_{K6R}, Ub_{K11R}, Ub_{K27R}, Ub_{K29R}, and Ub_{K63R} as Ub_{WT} (IP, lanes 3–6, 9), this level was significantly decreased with Ub_{K33R} and Ub_{48R} (IP, lanes 7–8).

To confirm the important role of K33 and K48 in SERINC5 polyubiquitination, we created another seven ubiquitin mutants, Ub_{K6}, Ub_{K11}, Ub_{K27}, Ub_{K29}, Ub_{K33}, Ub_{K48}, and Ub_{K63}, that only express each of those seven lysine residues of ubiquitin. We then repeated this experiment by expressing the SERINC5-130K mutant with these ubiquitin mutants. A similar level of SERINC5 polyubiquitination was detected from Ub_{WT}, Ub_{K33}, and Ub_{K48} (Fig. 2D, IP, lanes 2, 7, 8), but this level was significantly reduced from Ub_{K6}, Ub_{K11}, Ub_{K27}, Ub_{K29}, Ub_{K33}, Ub_{K63}, and Ub_{KO} (IP, lanes 3–6, 9, 10). These results demonstrate that SERINC5 is preferably polyubiquitinated at K130 via K33 and K48-linked ubiquitin chains.

To confirm that $CRL3^{KLHL20}$ plays a role in the K130 polyubiquitination, the SERINC5-K130 mutant was expressed with Ub_{WT}, Ub_{K33}, Ub_{K48}, and Ub_{KO} in the presence or absence of *KLHL20*-KD in HEK293T cells, and the K130 polyubiquitination state was determined exactly as described above. Again, we detected a similar level of SERINC5 polyubiquitination from Ub_{WT}, Ub_{K33}, and Ub_{K48}, and did not detect this polyubiquitination from Ub_{KO} (Fig. 2E, IP, lanes 2, 4, 6, 8). Importantly, KLHL20-KD completely disrupted this SERINC5 polyubiquitination (IP, lanes, 3, 5, 7). These results demonstrate that $CRL3^{KLHL20}$ is responsible for the K130 polyubiquitination via K33 and K48-linked ubiquitin chains.

K130 is required for SERINC5's localization to the plasma membrane. To understand how our lysine mutations affect SERINC5 subcellular localization, first, we tracked SERINC5 subcellular localization by confocal microscopy. SERINC5 was fused with a GFP tag at its C-terminus and similar lysine mutations were introduced into this SERINC5-GFP fusion protein. When these proteins were expressed in HeLa cells, WT SERINC5-GFP, 1-7K/R, and K179R were localized to the cell surface, whereas 1-14K/R, 1-19K/R, 1-11K/R, and K130R were found in the cytoplasm (Fig. 3A, HeLa, top panels). The 15-19K/R mutant was localized to both the cell surface and the cytoplasm. The colocalization of WT SERINC5-GFP, 1-7K/R, 15-19K/R, and K179R with $DiIC_{18}(5)$, confirmed that these proteins localize to the plasma membrane (Fig. 3A, HeLa, bottom panels). When WT SERINC5-GFP, K130R, and 1-19K/R were expressed in human Jurkat T cells, only the WT protein was found on the cell surface, consistent with the results observed from HeLa cells (Fig. 3A, Jurkat).

Next, we analyzed SERINC5 expression on the cell surface by flow cytometry. A FLAG-tag was inserted into the SERINC5 extracellular loop 3 region and similar lysine mutations were introduced into this SERINC5-iFLAG protein. These proteins were expressed in HEK293T cells, and their expression on the cell surface was determined via staining with anti-FLAG. WT SERINC5-iFLAG, 1-7K/R, 15-19K/R, and K179R were detected at much higher levels than 1-11K/R, 1-14K/R, 1-19K/R, and K130R (Fig. 3B). When this experiment was repeated in Jurkat cells, the poor expression of 1-19K/R and K130R was confirmed (Fig. 3B). These results are consistent with those from confocal microscopy.

Finally, we purified plasma membranes from cells to detect SERINC5 by WB. SERINC5 and its lysine mutants were expressed in HEK293T cells, and the plasma membrane fraction was isolated. CD4, a cell surface protein that is associated with the plasma membrane, was included as a control. Although WT SERINC5, 1-19K/R, K130R, K179R, and CD4 were detected at a similar level in total cell lysate (Fig. 3C,

total lysate), only WT SERINC5, K179R, and CD4 were found in the membrane fraction, whereas 1-19K/R and K130R were not (Fig. 3C, membrane). Collectively, these results demonstrate that K130 determines SERINC5's localization to the plasma membrane in a cell-type independent manner.

Polyubiquitination is required for SERINC5's localization to the plasma membrane. Although Cul3 and KLHL20 decreased SERINC5 expression in the presence of ectopic ubiquitin (Fig. 1A), their ectopic expression and knockdown did not affect SERINC5 expression at steady state (Fig. 4A). We then determined how Cul3 and KLHL20 affect SERINC5 subcellular localization by using the similar approaches. Notably, knockdown of *Cul3* and *KLHL20* expression by CRISPR/Cas9 in HeLa and Jurkat cells disrupted SERINC5 expression on the cell surface when detected by confocal microscopy (Fig. 4B) and flow cytometry (Fig. 4C, lanes 4, 6, 11, 13), and this cell surface expression was restored by ectopic Cul3 or KLHL20 expression (Fig. 4B; Fig. 4C, lanes 5, 7, 12, 14). In addition, these KDs also significantly reduced the SERINC5 levels in the plasma membrane fraction (Fig. 4D, lanes 4, 6), which was also restored by their ectopic expression (lanes 5, 7).

We detected SERINC5 interaction with ubiquitin in live cells via bimolecular fluorescence complementation (BiFC)[14, 15, 16]. In this assay, a basic yellow fluorescent protein Venus was divided into N-terminal (VN) and C-terminal (VC) fragments. HA-tagged VN and FLAG-tagged VC were fused to the C-terminus of ubiquitin or SERINC5, respectively. When Ub-VN and SERINC5-VC were co-expressed in HeLa cells, green fluorescence signals were detected, indicating that ubiquitin-SERINC5 interaction occurred (Fig. 4E). These BiFC signals co-localized with SERINC5, confirming the specificity of this interaction. Importantly, these signals primarily localized to the cell surface, consistent with the conclusion that ubiquitination is required for SERINC5's localization to the plasma membrane.

Next, the same lysine mutants were generated from SERINC5-VC and their interaction with Ub-VN was tested as above. BiFC signals were detected in cells expressing 15-19K/R, 1-7K/R, and K179R, but not 1-14K/R, 1-19K/R, 1-11K/R, 8-11K/R, 8-9K/R, and K130R (Fig. 4E). In addition, these signals primarily localized to the cell surface. Collectively, these results confirm the important role of polyubiquitination in SERINC5's localization to the plasma membrane.

Polyubiquitination is required for SERINC5 downregulation by HIV-1 Nef. To understand how SERINC5 polyubiquitination affects its antagonism by Nef, we expressed SERINC5 and its lysine mutants with Nef in HEK293T cells and analyzed SERINC5 downregulation by WB. Initially, we analyzed 9 lysine mutants and found that 1-14K/R, 1-19K/R, 1-11K/R, 7-11K/R, 7-14K/R, 8-9K/R, and 8-11K/R were resistant to Nef (Fig. 5A, lanes 3–4, 7–8, 13–16, 19–20, 23–24, 31–34), whereas 15-19K/R, 1-7K/R, and 11-14K/R were as sensitive to Nef as the WT SERINC5 (lanes 5–6, 11–12, 21–22). These results suggested that K8 and K9 residues should be required for SERINC5 downregulation by Nef. We then tested K130R and K179R and found that K179R was still sensitive, whereas K130R became resistant to Nef (lanes 27–30). These results demonstrate that K130 determines SERINC5 sensitivity to Nef.

Previously, we reported that ectopic ubiquitin synergizes the SERINC5 downregulation by Nef [16]. Therefore, we determined how these SERINC5 lysine residues affect such synergy. When SERINC5 proteins were expressed with Nef and ubiquitin in HEK293T cells, ubiquitin strongly promoted Nef downregulation of WT SERINC5 (Fig. 5B, lanes 1–4, 13–16). This ubiquitin synergy was also detected from Nef downregulation of the K179R mutant (lanes 21–24), but not the mutants containing the K130R mutation, such as mutants 1-19K/R, 8-11K/R, and K130R (lanes 5–12, 17–20). These results further confirm that K130 is critical for SERINC5 downregulation by Nef.

To understand how K130 determines the SERINC5 sensitivity to Nef, we analyzed the SERINC5-Nef interaction in live cells via BiFC as we and others reported[16, 24, 25]. When HA-tagged VN and FLAG-tagged VC were fused to the C-terminus of SERINC5 or Nef and expressed in HeLa cells, BiFC signals were detected, and co-localized with SERINC5, indicating an association of SERINC5-Nef in these cells (Fig. 5C). In addition, these signals were primarily detected in the cytoplasm, confirming the Nef-mediated downregulation of SERINC5 from the cell surface. When lysine mutations were introduced into SERINC5-VN, Nef interacted with 15-19K/R, 1-7K/R, and K179R, but not 1-14K/R, 1-19K/R, 1-11K/R, 8-11K/R, 8-9K/R, and K130R (Fig. 5C). These results demonstrate that K130 is required for SERINC5 interaction with Nef in cells and suggest that SERINC5 polyubiquitination plays an indispensable role in this interaction.

Polyubiquitination is required for SERINC5 anti-HIV-1 activity. To understand how lysine residues affect SERINC5 incorporation into virions, WT and ΔNef HIV-1 were produced from HEK293T cells in the presence of SERINC5 and its lysine mutants. Virions were purified from culture supernatants by ultracentrifugation and analyzed by WB. We again confirmed that Nef downregulates 1-7K/R, 15-19K/R, and K179R, but not 1-11K/R, 1-14K/R, 1-19K/R, and K130R in cells (Fig. 6A). Consistently, the 1-7K/R, 15-19K/R, and K179R mutants were detected in virions, and their incorporation was inhibited by Nef. In contrast, none of the 1-11K/R, 1-14K/R, 1-19K/R, and K130R mutants were detected in virions. Thus, K130 is required for SERINC5 incorporation into virions, which confirms its role in SERINC5 expression on the cell surface.

To further confirm the important role of polyubiquitination in SERINC5 antagonism by Nef, SERINC5 was expressed with HIV-1 Nef in HEK293T cells in the presence of *KLHL20*- or *Cul3*-KD by CRISPR/Cas9, and/or their ectopic expression. Nef effectively decreased SERINC5 expression (Fig. 6B, lanes 1–2), a phenotype which was further enhanced by ectopic KLHL20 and Cul3 expression (lanes 3–4). This phenotype was disrupted by *KLHL20*-KD and *Cul3*-KD (lanes 6, 9), and recovered upon complementation with their ectopic expression (lanes 7, 10). Thus, Nef downregulation of SERINC5 is dependent on Cul3-KLHL20 mediated SERINC5 polyubiquitination.

Finally, we determined how K130 and polyubiquitination affects the SERINC5 anti-HIV-1 activity. Initially, ΔNef HIV-1 was produced from HEK293T and Jurkat cells in the presence of SERINC5 and its lysine mutants, and viral infectivity was analyzed after infection of HIV-1 luciferase-reporter TZM-bl cells. 1-7K/R, 15-19K/R, and K179R inhibited the viral replication as strongly as WT SERINC5, whereas 1-11K/R, 1-14K/R, 1-19K/R, and K130R did not in both cell lines (Fig. 6C). Next, ΔNef HIV-1 was also produced

from HEK293T and Jurkat cells in the presence of SERINC5 and *KLHL20*- or *Cul3*-KD, and viral infectivity was analyzed again as above. Both *KLHL20*- and *Cul3*-KD disrupted the SERINC5 antiviral activity in both cell lines, a phenotype which was restored upon complementation with their ectopic expression (Fig. 6D). Thus, K130 and polyubiquitination are required for SERINC5 anti-HIV-1 activity.

Discussion

KLHL proteins normally have three functional domains: BTB, BACK, and Kelch. The BACK domain bridges the BTB domain with the Kelch domain and it also has a N-terminal structural motif called a 3-box that forms a cleft. In combination with the C-terminus of the BTB domain, the 3-box motif binds to the N-terminal tail of Cul3[26]. The Kelch domain has six Kelch repeats that serve as the substrate recognition domain, and this domain from KLHL20 binds to DAPK[23]. We now show that KLHL20 binds to SERINC5 via the Kelch domain and recruits SERINC5 to $CRL3^{KLHL20}$ for being polyubiquitinated, which potentiates the SERINC5 post-Golgi trafficking to the plasma membrane and regulates its expression.

TGN is a major soring station in the secretory pathway that is responsible for expression of secretory proteins and integral membrane proteins. It has been unclear how the SERINC5 anterograde trafficking from the Golgi to the plasma membrane is potentiated, which is a critical process for SERINC5 to exhibit its antiviral activity. We now show that this process is dependent on SERINC5 polyubiquitination at K130 by $CRL3^{KLHL20}$. Although ubiquitin chains can be homotypic that are linked through the same acceptor site of ubiquitin, they are also heterotypic that are linked through multiple sites. Heterotypic chains are further divided into a mixed single chain or at least two branched chains that have two linkages within the same polymer[27]. So far, four types of branched ubiquitin chains that have clear physiological functions have been identified, including K11/K48[28], K29/K48[29], K48/K63[30], K63/M1[31]. Several other branched ubiquitin chains with unidentified functions have also been detected. In fact, up to 20% ubiquitin chains detected from cellular proteins are branched chains, indicating that they play a critical role in cellular functions. Branched chains can be assembled via at least three mechanisms by 1) paired E3s that have distinct linkage specificities; 2) a single E3 with paired E2s that have distinct linkage specificities; 3) a single E3 with a single E2.

Notably, SERINC5 is polyubiquitinated at K130 via both K33- and K48-linked chains. These results strongly suggest that K33/K48-branched chains are assembled during polyubiquitination, which has not been described in any proteins so far to the best of our knowledge. These results further suggest that, in addition to homotypic K33- or K48-linked chain, $CRL3^{KLHL20}$ also generates K33/K48-branched chains. $CRL3^{KLHL20}$ generates K48-linked chains with different E2s, but its E2 for K33-linked chain assembly is still unknown. Thus, the mechanism of how $CRL3^{KLHL20}$ generates K33/K48-branched chains should be further investigated.

Our results suggest that K33/K48-branched chains have dual functions, which is distinctive from the single function of the other branched chains (Fig. 7). K11/K48- and K29/48-branched chains act as a

more powerful degradation signal and promote degradation in proteasomes[28, 29], whereas K29/K48- and K63/M1-branched chains serve as nondegradable signals and contribute to cell signaling events[30, 31]. In the case of SERINC5 polyubiquitination, the K33-linked ubiquitin chain should serve as a sorting signal that transports SERINC5 from the TGN to the plasma membrane. K33-linked ubiquitin chains may interact with a ubiquitin-binding protein such as Eps15 that is required for the post-Golgi trafficking, as implicated in the coronin 7 signaling cascade[32]. Once SERINC5 is localized to the plasma membrane, K48-linked ubiquitin chains should sort SERINC5 to the lysosomes via endocytic pathway for degradation. Although the K48-linked ubiquitin chain generated by $CRL3^{KLHL20}$ has been found to promote proteasomal degradation, it is clear that K48-linked ubiquitin chains also target proteins to lysosomes for degradation[33]. Collectively, K33/K48-branched chains should act as both nondegradable and degradable signals that potentiate and regulate SERINC5 expression on the cell surface.

It would be very interesting if K33/K48-branched chains play a very general role in regulating cellular protein expression. It is still unclear how expression and trafficking of integral membrane proteins are regulated at post-Golgi levels. However, transmembrane proteins are often downregulated from the plasma membrane via endocytic pathway for degradation. Thus, their expression must be tightly controlled at cellular levels by a negative mechanism. K33/K48-branched chains may act as a positive regulator by promoting their targeting to the plasma membrane via the K33-linked chain, and a negative regulator by promoting their downregulation from the plasma membrane via the K48-linked chain. It is noteworthy that HIV-1 is very adept to take advantage of this negative regulatory mechanism and antagonize SERINC5 by accelerating its degradation by Nef (Fig. 7). Thus, further understanding the biology of K33/K48-branched chains will collect new insights into the general mechanism for integral cellular membrane expression and the arms race between SERINC5 and Nef during HIV-1 infection.

Declarations

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Author Contributions

R.L. and I.A. performed all experiments except mass spectrometry analyses. L.S. conducted mass spectrometry analysis. S.F.J. created the model and provided insightful comments on the paper. S.L. and Y.H.Z. designed this study. Y.H.Z. wrote manuscript with input from all coauthors.

Declaration of Interests

The authors declare no competing interests.

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Materials And Methods

Cell Lines. The human HEK293T cells and HeLa cells were obtained from the American Type Culture Collection. TZM-bl cells were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. HEK293T and TZM-bl cells were maintained in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (Gibco), at 37°C and 5% CO_2. The human Jurkat-TAg (JTAg) cell line was provided by Heinrich Gottlinger and cultured in RPMI 1640 with 10% fetal bovine serum (FBS) (Sigma), at 37°C and 5% CO_2.

Bacterial strains. *Escherichia coli* HB101 (Promega) was used as the recipient strain for preparation of HIV-1 proviral vectors, whereas all the other plasmid vectors were prepared from *Escherichia coli* DH10α (Vazyme). All these bacteria were cultured in LB broth in a shaking incubator at 37°C.

Expression vectors. The Env-deficient HIV-1 proviral vector pNLΔE (pNLenCAT), its Nef-deficient version pNLΔEΔN (pNLenCAT-Xh), and HIV-1 Env expression vector pNLnΔBS were provided by Kenzo Tokunaga[34]. The followed plasmids pCMV6-Ser5-FLAG, pCMV-Ser5-eGFP, pCMV-HA-Ub, pCMV-His_6-Ub, pcDNA3.1-Ser5-FLAG-VC, pcDNA3.1-Ser5-VN-HA, pcDNA3.1-Ub-VN-HA, pcDNA3.1-SF2Nef-V5-VC, pcDNA3.1-SF2Nef-HA, and pCMV6-CD4-FLAG were reported previously[14, 16, 19]. pLVXm-N-FLAG-Skp1 and pLVXm-N-FLAG-Cul1 were provided by Yan Chen[35]. pcDNA3.1-iFLAG-Ser5 that has a FLAG tag inserted between residues 290 and 291 of Ser5 was created by overlapping PCR amplification. pCMV6-KLHL20-HA, pCMV6-ROC1-HA, and pCMV6-Cul3-HA were constructed from Comate Bioscience. The Kelch-repeat domain deletion mutant pCMV6-KLHL20ΔK-HA (KLHL20ΔK: residues 1–316) were generated by PCR. The pCMV6-KLHL20m6-HA mutant, in which the residues 109, 111, 113, 146, 148, and 150 were each replaced by an alanine residue, was generated by site-directed mutagenesis. pCMV6-KLHL20-His_6, pCMV6-ROC1-His_6, pCMV6-KLHL20ΔK-His_6, pCMV6-KLHL20m6-His_6, and pCMV6-Cul3-His_6 were generated by PCR. The Cas9 expression vector pMJ920 was obtained from Jennifer Doudna through Addgene. A *KLHL20 E2g1 gRNA (5'-GCGAACGTTTAGCTCATCACTGG-3')* targeting the 2nd exon, *KLHL20 E1g2 gRNA (5'- TGATCCGAGACATTGACGAGAGG – 3')* targeting the 1st exon; and *Cul3 E1g1 gRNA (5'-CGAGATCAAGTTGTACGTTATGG – 3'), Cul3 E1g2 gRNA (5'- GACCACTGTTATTCTTACGCTGG-3')* targeting the 1st exon, were expressed from pGEM-T (Promega) as we did before[36].

Ser5 1-14K/R, 15-19K/R, 1-19K/R, 1-7K/R, 1-11K/R, 1-14K/R, 7-11K/R, 11-14K/R, 7-14K/R, 8-9K/R, 8-11K/R, K130R, and K179R mutation in pCMV6-Ser5-FLAG, pCMV-Ser5-eGFP, pcDNA3.1-Ser5-VN-HA, pcDNA3.1-Ser5-VC-FLAG, and pcDNA3.1-iFLAG-Ser5 were created by a site-directed mutagenesis,

respectively. Ser5 130K mutation was created in pCMV6-Ser5-1-19K/R-FLAG vector. Primers and cloning methods are available upon request.

Western blotting (WB). According to experimental design requirements, HEK293T cells were seeded either in 6-well plates or in 6-cm dishes with an initial density of 5×10^5 cells per well or 1×10^6 cells per dish. After 24 h or 48 h of transfection, cells were lysed with RIPA buffer and the cytosolic cellular proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to Immun-Blot PVDF membrane, proteins were incubated with primary and secondary antibodies. The anti-KLHL20 was purchased from Novus; anti-CUL3 was purchased from CST; mouse anti-HA, anti-FLAG, HRP-conjugated anti-actin, HRP-conjugated anti-FLAG monoclonal antibodies, HRP-conjugated anti-HA, rabbit anti-Ub antibody, anti-FLAG(R) M2 Magnetic Beads antibody, anti-HA-Agarose, HRP-conjugated anti-rabbit or mouse immunoglobulin G secondary antibodies, HRP-conjugated anti-His_6, mouse anti-Nef monoclonal antibody and the mouse anti-p24Gag (183-H12–5C) monoclonal antibody were described previously[14, 15, 16]. The particles were purified from culture supernatants by ultracentrifugation and SERINC5 expression in virions were detected by WB. The total lysate and membrane protein were extracted by Membrane and Cytosol Protein Extraction Kit according to the experiment designment.

Immunoprecipitation (IP). To detect protein interactions in Fig. 1F, 1G, Fig. 2C, 2D, HA-tagged proteins were expressed with their target proteins in HEK293T cells cultured in 6-cm dishes. Proteins were pulled down by an Anti-HA-Agarose antibody and analyzed by WB. To detect protein interactions in Fig. 1D, 1E, Fig. 2B, FLAG-tagged proteins were expressed in HEK293T cells cultured in 6-cm dishes. Proteins were pulled down by an anti-FLAG M2 antibody and analyzed by WB.

Viral infectivity. Viruses were produced from HEK293T and Jurkat-TAg cells after transfection as described previously[16]. In brief, Ser5 and its indicated lysine mutant expression vector in the presence or absence of silencing vectors or expression vectors (KLHL20, Cul3) were transfected either in HEK293T or Jurkat-TAg cells. After 48 h, viruses were collected from the culture supernatants, and viral production was quantified by $p24^{Gag}$ ELISA. To determine viral infectivity, equal amounts of viruses were used to infect the HIV-1 luciferase reporter cell line TZM-bl in a 96-well plate at a density of 1×10^4 per well. 48 h later, the cells were lysed, and the intracellular luciferase activities were determined using the Bright-Glo Luciferase Assay System (Promega).

Flow cytometry. The HEK293T and Jurkat-TAg cells were transfected with Ser5 and its indicated lysine mutant expression vectors for 24 h. The cells were then fixed with 4 % paraformaldehyde for 5 min and blocked with 5% BSA for 2 h. The cells were incubated with an anti-FLAG monoclonal antibody at 4°C overnight. After washing, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse antibody for 1 h. Ser5 expression on the cell surface was determined by flow cytometry.

Confocal microscopy. $1.5 \sim 2.0\times10^5$ HeLa cells were seeded in coverslips and transfected with indicated vectors using Lipofectamine®3000 as a transfection reagent. After 24 h, cells were fixed with 4 % paraformaldehyde for 5 min and permeabilized with 0.1 % Triton X-100 for 10 min, and then blocked with

10 % FBS for 2 h. For immunofluorescence assay, cells were incubated with anti-HA or anti-FLAG antibodies overnight at 4°C. After washing, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse antibody for 1 h. Then incubated with DAPI(Sigma) for 30 s for nuclear staining. Finally, cells were observed under confocal microscope (ZEISS, LSM880). The Jurkat-TAg cells were prepared by centrifugation at 400 g for 5 min at 4°C. After fixation, permeabilization, blocking and staining cells with DAPI, the cells were mounted on a glass-slide and covered by a coverslip. Slides were left in dark at room temperature for 30 min before they were examined under Zeiss LSM 880 confocal microscope.

Statistical Analysis. Statistical tests were performed using GraphPad Prism 8. Variance was estimated by calculating the standard error of measurements (SEMs) and represented by error bars. Significance of differences between samples was assessed using two-way ANOVA with Bonferroni post-test. All experiments were performed independently at least three times, with representative experiment being shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns (not significant, $p > 0.05$).

Figures

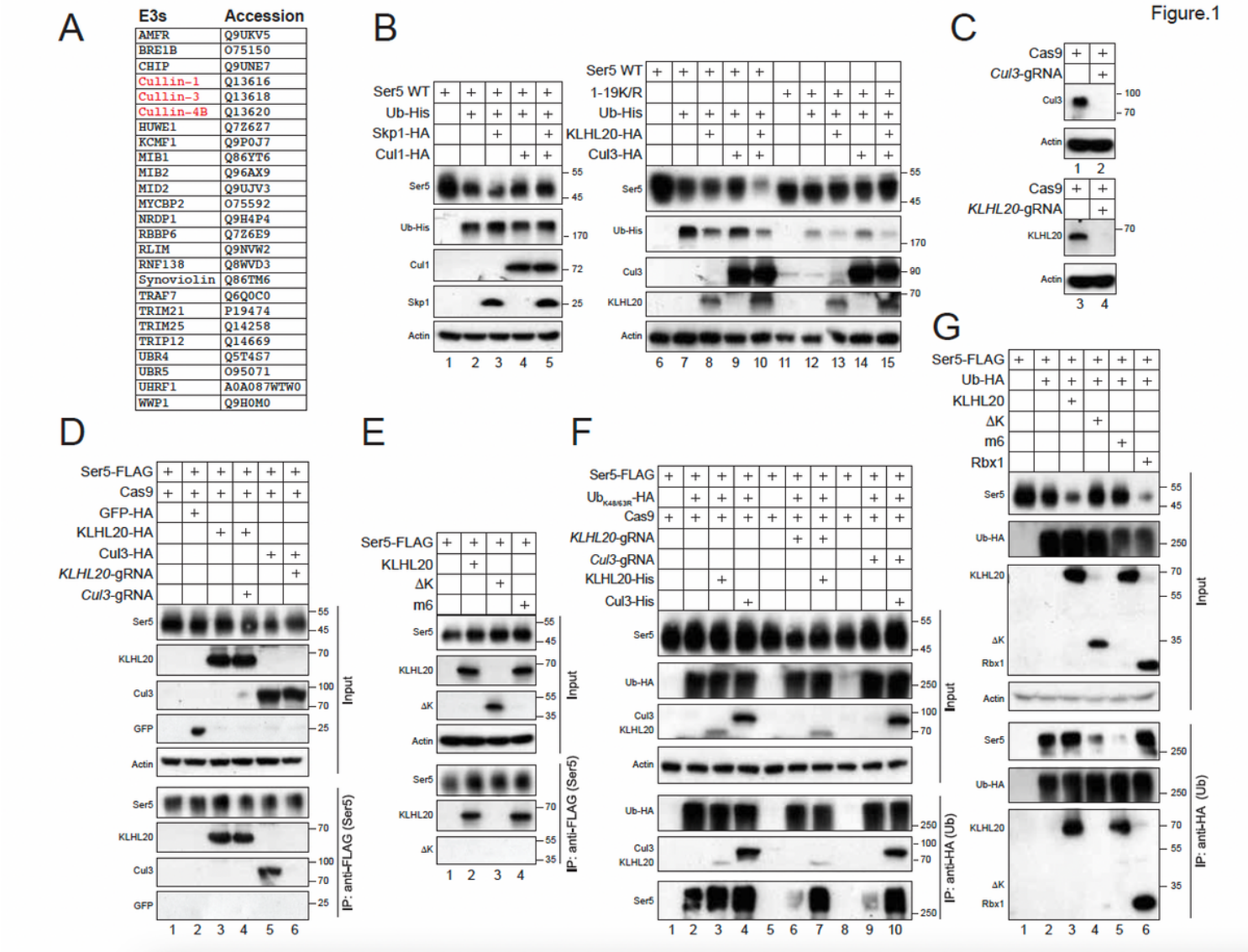

Figure 1

Identification of Cul3-KLHL20 as a SERINC5 ubiquitin E3 ligase via mass spectrometry. A) A list of E3 ubiquitin ligases that were identified from the SERINC5 protein complex by mass spectrometry. Cullin proteins are shown in red. B) SERINC5 (Ser5) was expressed with Ub in the presence of Skp1and/or Cul1 in HEK293T cells. Similarly, SERINC5 and its 1-19K/R mutant were expressed with Ub in the presence of KLHL20 and/or Cul3. Expression of these proteins was analyzed by WB using anti-epitope tag antibodies. C) Cas9 was expressed with Cul3- or KLHL20-specific gRNAs in HEK293T cells. Expression of Cul3 and KLHL20 was determined by WB using their specific antibodies. D) SERINC5 was expressed with Cas9, GFP, KLHL20, Cul3, and/or KLHL20-gRNA and Cul3-gRNA in HEK293T cells. SERINC5 was immunoprecipitated by anti-FLAG, and levels of GFP, KLHL20, and Cul3 in cell lysate (Input) and IP samples were compared by WB. E) SERINC5 was expressed with KLHL20 and its ΔK and m6 mutants in HEK293T cells. SERINC5 was immunoprecipitated and levels of these different KLHL20 proteins in cell lysate and IP samples were compared by WB. F) SERINC5 was expressed with ubiquitin, KLHL20, Cul3, and/or Cas9 plus KLHL20-gRNA and Cul3-gRNA in HEK293T cells. Ectopic ubiquitin was immunoprecipitated by anti-HA and levels of polyubiquitinated SERINC5 were compared by WB. G) SERINC5 was expressed with ubiquitin, KLHL20 or its mutants ΔK and m6, and Rbx1 in HEK293T cells. Ectopic ubiquitin was immunoprecipitated by anti-HA and levels of polyubiquitinated SERINC5 were compared by WB. All experiments were repeated at least twice, and similar results were obtained.

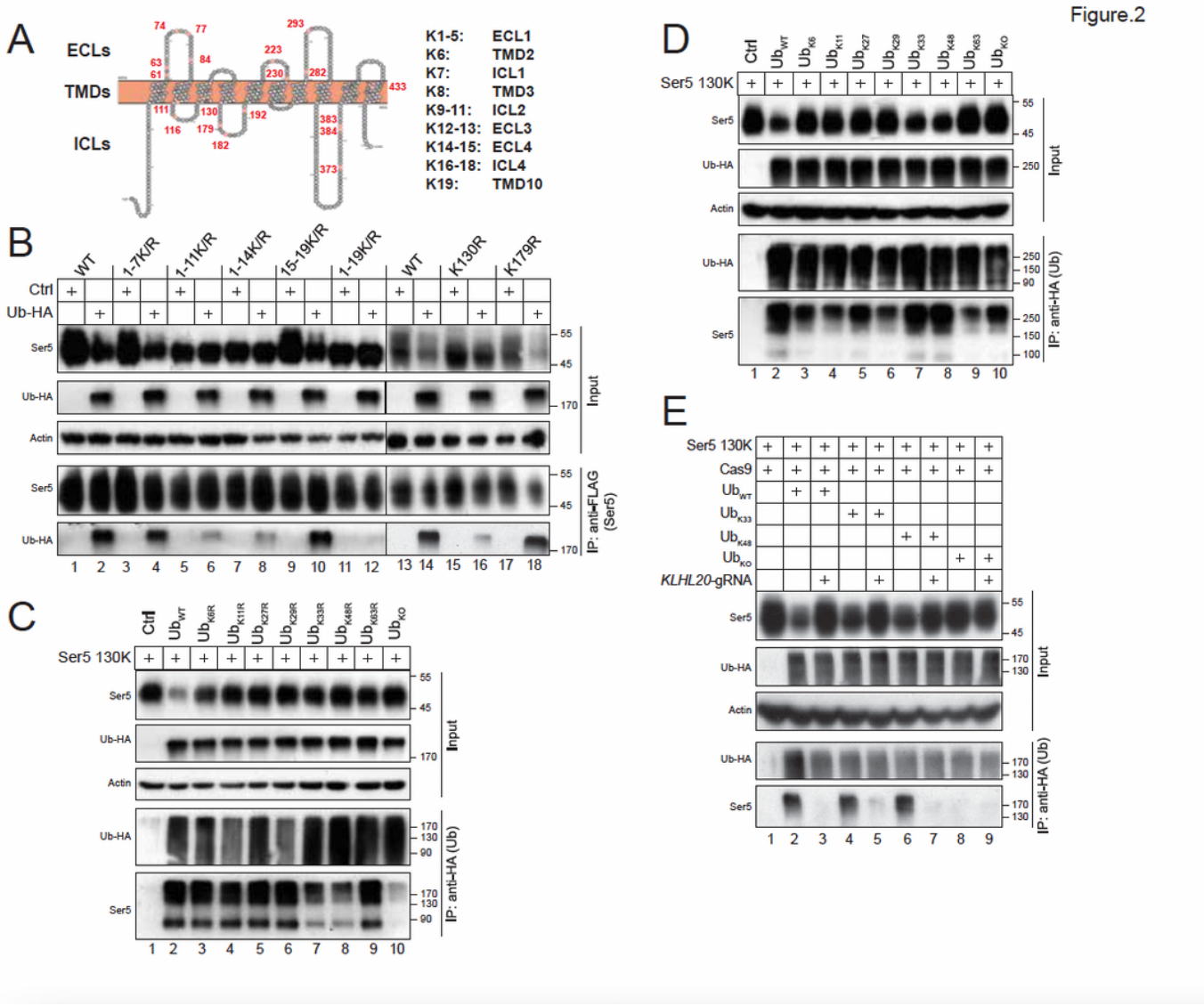

Figure 2

Identification of lysine 130 (K130) as a critical polyubiquitination site on SERINC5. A) SERINC5 membrane topology is presented. Five ECLs, 10 TMDs, and 4 ICLs are indicated. Nineteen lysine residues (K1 to K19) are numbered in red and their distributions into these regions are indicated. B) SERINC5 and its lysine mutants were expressed with ubiquitin in HEK293T cells. SERINC5 was immunoprecipitated by anti-FLAG and levels of polyubiquitinated SERINC5 were compared by WB. C) The SERINC5-130K mutant was expressed with ubiquitin and its mutants bearing indicated K-to-R mutations in HEK293T cells. Ectopic ubiquitin was immunoprecipitated by anti-HA and levels of polyubiquitinated SERINC5 were compared by WB. D) The SERINC5-130K mutant was expressed with indicated ubiquitin mutants in HEK293T cells. Ectopic ubiquitin was immunoprecipitated by anti-HA and levels of polyubiquitinated SERINC5 were compared by WB. E) The SERINC5-130K mutant was expressed with indicated ubiquitin mutants in HEK293T cells in the presence or absence of KLHL20-knockdown. Ectopic ubiquitin was immunoprecipitated by anti-HA and levels of polyubiquitinated SERINC5 were compared by WB. All experiments were repeated at least twice, and similar results were obtained.

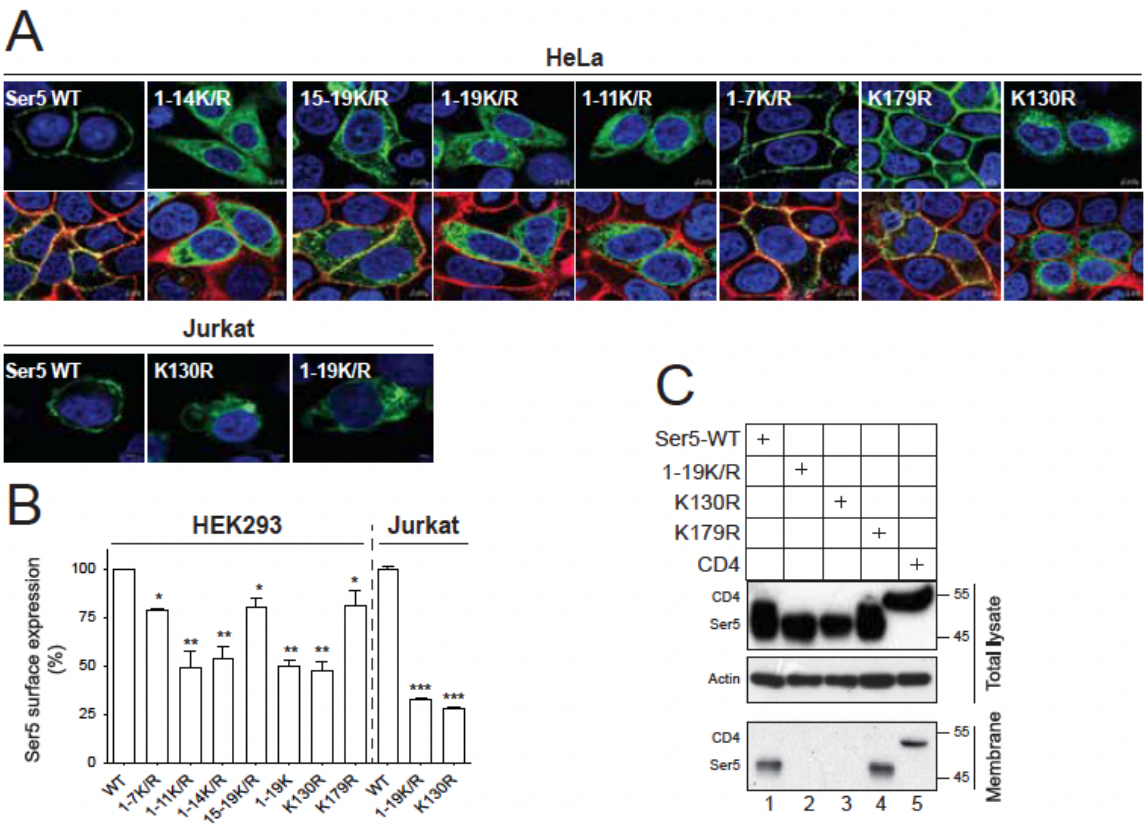

Figure 3

K130 is required for SERINC5's localization to the plasma membrane. A) SERINC5-GFP and its lysine mutants were expressed in HeLa cells. Cells were stained with DAPI for the nuclei (blue) and DiIC18(5) for the plasma membranes (far-red) (bottom panel) or only stained with DAPI (top panel). In addition, SERINC5-GFP and its K130R or 1-19K/R mutant were expressed in Jurkat cells, and cells were stained with DAPI. Subcellular localization of these SERINC5 proteins was detected by confocal microscopy (scale bar 2 or 5 μm). B) SERINC5-iFLAG and its lysine mutants were expressed in HEK293T and Jurkat cells. Cells were stained with anti-FLAG and levels of SERINC5 on the cell surface were analyzed by flow cytometry. Results are shown as relative values, with the surface expression of WT SERINC5 set as 100. Error bars indicate SEMs calculated from three experiments. Statistical analysis: *p<0.05, **p<0.01, ***p<0.001. C) SERINC5, its lysine mutants, and CD4 were expressed in HEK293T cells. The plasma membranes from these cells were purified and analyzed by WB. Experiments were repeated twice, and similar results were obtained.

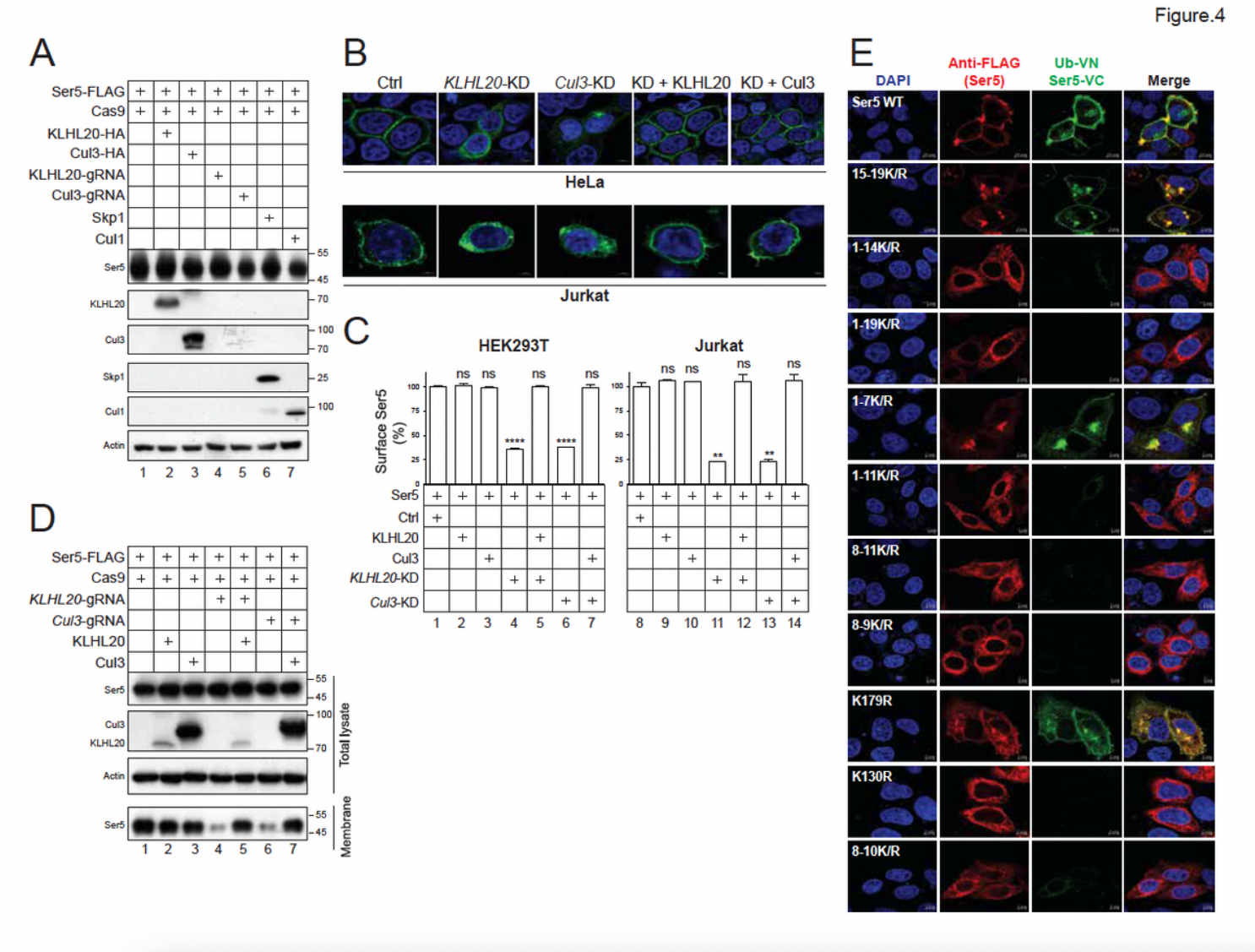

Figure 4

Polyubiquitination is required for SERINC5's localization to the plasma membrane. A) SERINC5 was expressed with Cas9, indicated proteins, and their gRNAs in HEK293T cells. SERINC5 expression was compared by WB. B) SERINC5-GFP was expressed in HeLa and Jurkat cells in the presence of KLHL20- or Cul3-KD by co-expressing Cas9 and their specific gRNAs. KLHL20 and Cul3 in these KD cells were also complemented by ectopic expression. After staining with DAPI, the SERINC5 subcellular localization was detected by confocal microscopy (scale bar 2 or 5 μm). C) SERINC5-iFLAG was expressed in HEK293T and Jurkat cells in the presence of ectopic KLHL20 and Cul3 expression and/or their KDs by CRISPR/Cas9. Cells were stained with anti-FLAG and levels of SERINC5 on the cell surface were analyzed by flow cytometry. Results are shown as relative values, with the surface expression of SERINC5 alone set as 100. Error bars indicate SEMs calculated from three experiments. Statistical analysis: **p<0.01, ****p<0.0001, ns (not significant, p>0.05). D) SERINC5 was expressed in HEK293T cells in the presence of ectopic KLHL20 and Cul3 expression and/or their KDs by CRISPR/Cas9. The plasma membranes from these cells were purified and analyzed by WB. E) Ub-VN was expressed with SERINC5-VC and its lysine

mutants in HeLa cells. Cells were stained with DAPI and anti-FLAG for SERINC5, and BiFC fluorescent signals were detected by confocal microscopy (scale bar 2 or 5 μm).

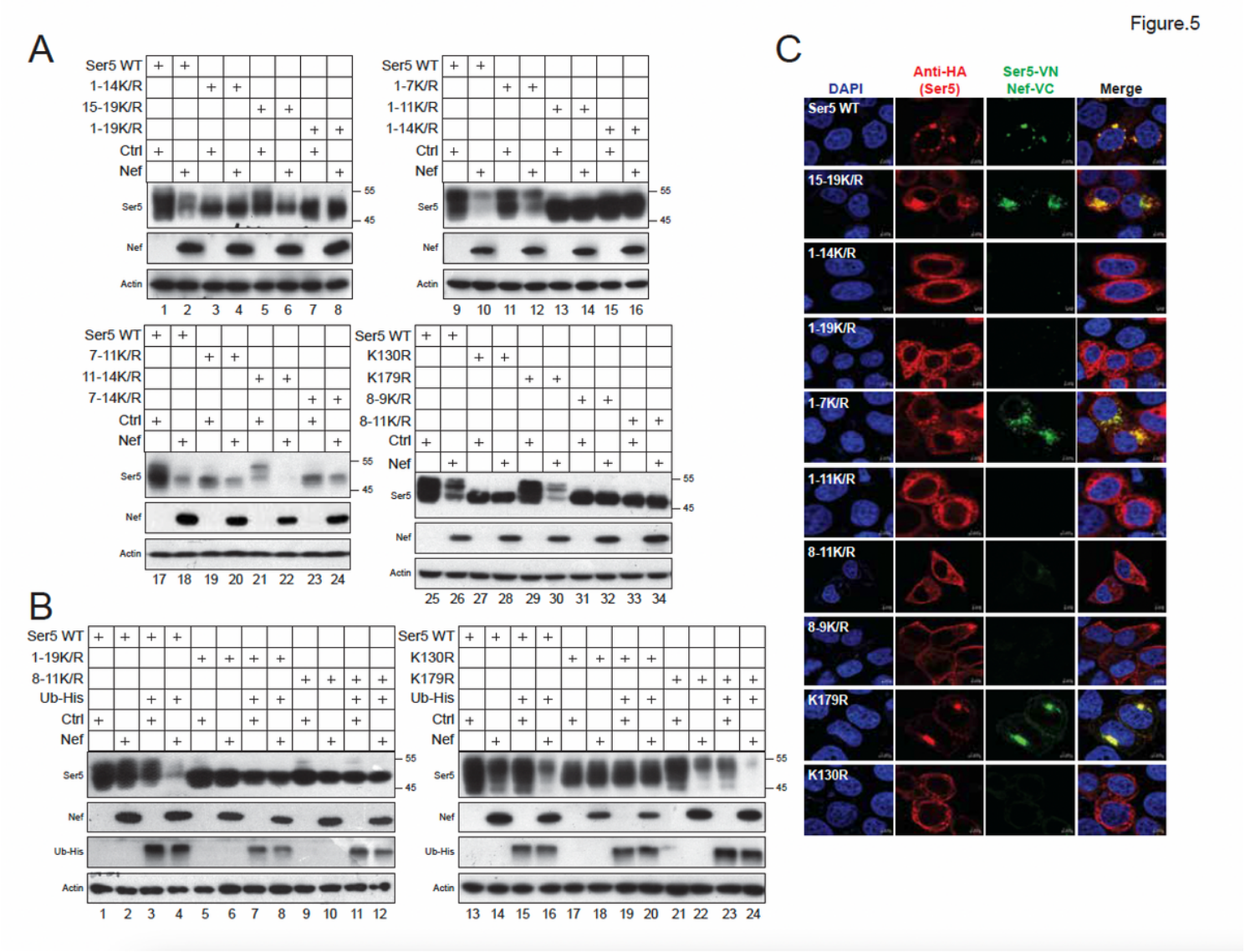

Figure 5

Polyubiquitination is required for SERINC5 downregulation by HIV-1 Nef. A) SERINC5 and its lysine mutants were expressed with HIV-1 Nef in HEK293T cells, and their expression was analyzed by WB. B) SERINC5 and its lysine mutants were expressed with ubiquitin and Nef in HEK293T cells, and their expression was analyzed by WB. C) SERINC5-VN and its lysine mutants were expressed with Nef-VC in HeLa cells. Cells were stained with DAPI and anti-HA for SERINC5, and BiFC fluorescent signals were detected by confocal microscopy (scale bar 2 or 5 μm). All experiments were repeated at least twice, and similar results were obtained.

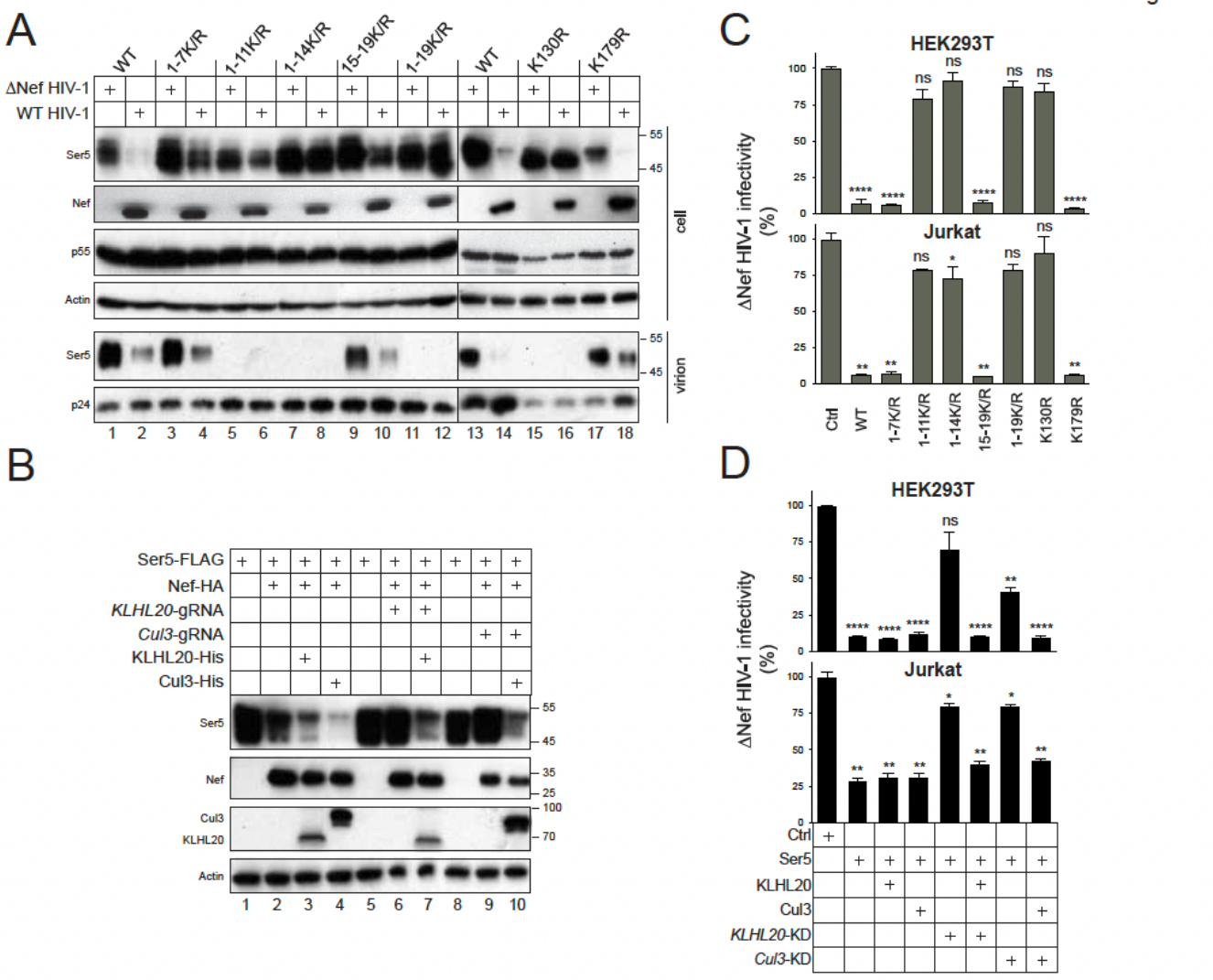

Figure 6

Polyubiquitination is required for SERINC5 anti-HIV-1 activity. A) WT and ΔNef HIV-1 were produced from HEK293T cells in the presence of SERINC5 and its lysine mutants. Virions were purified from culture supernatants via ultracentrifugation. SERINC5 proteins in cell lysate and virions were detected by WB. B) SERINC5 was expressed with Nef in HEK293T cells in the presence of ectopic KLHL20 and Cul3 expression and/or their KDs by CRISPR/Cas9. SERINC5 expression was compared by WB. C) ΔNef HIV-1 was produced from HEK293T and Jurkat cells in the presence of SERINC5 and its lysine mutants. After normalization by p24Gag ELISA, viral infectivity was analyzed after infection of HIV-1 luciferase reporter cell line TZM-bl. D) ΔNef HIV-1 was produced from HEK293T and Jurkat cells in the presence of SERINC5, ectopic KLHL20 and Cul3 expression, and/or their KDs by CRISPR/Cas9. Viral infectivity was analyzed similarly as in C). Results in C) and D) are presented as relative values, with the infectivity of viruses produced in the presence of a control (Ctrl) vector set as 100. Error bars indicate SEMs calculated from three experiments. Statistical analysis: **p<0.01, ****p<0.0001, ns (not significant, p>0.05).

Figure.7

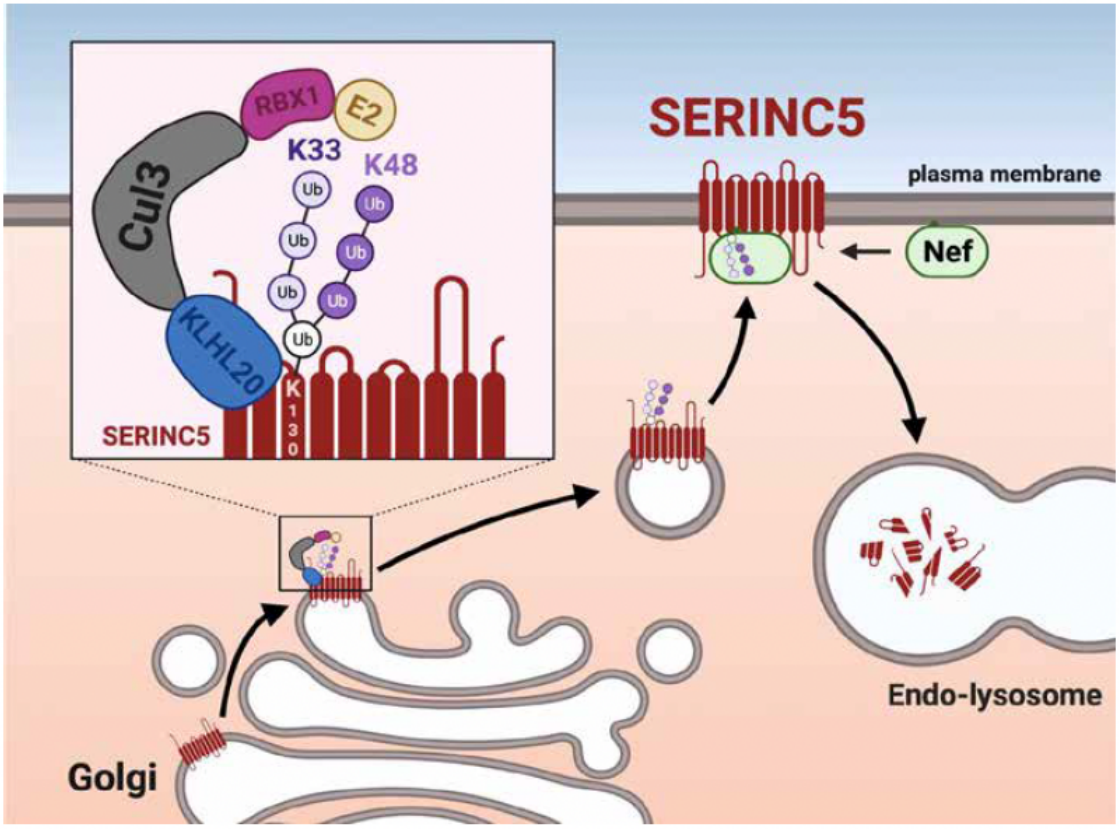

Figure 7

A model of how CRLKLHL20 determines SERINC5 trafficking and expression. CRLKLHL20 catalyzes SERINC5 polyubiquitination at the TGN and produces K33/K48-brached chains at K130 of SERINC5. The K33-linked ubiquitin chain (Ub-K33) potentiates SERINC5 trafficking to the plasma membrane, where it is packaged into HIV-1 virions to inhibit viral infection in the absence of Nef. However, when Nef is expressed, this viral accessory protein recruits CycK/CDK13 to phosphorylate the ICL4 of SERINC5. This phosphorylation triggers SERINC5 conformational change, resulting in recruitment of the AP2 complex to SERINC5 via Nef. SERINC5 is then targeted to the endocytic pathway and degraded in lysosomes via the K48-linked ubiquitin chain (Ub-K48).