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HIV-1 Nef-induced activation of the Src kinase Hck and its altered trafficking to the Golgi apparatus affecting the protein glycosylation process
(HIV-1 NefによるSrc kinase Hck の活性化とゴルジ体での局在がタンパク質の糖鎖付加過程に影響を及ぼす)

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２０１０年３月

2. Abstract

Human immunodeficiency virus type 1 (HIV-1) Nef accelerates the progression to AIDS by binding with and activating a Src tyrosine kinase Hck, but underlying molecular basis is not understood. A recently reported specific output of Nef-Hck binding was the inhibition of M-CSF function through inhibition of glycosylation maturation/intracellular trafficking of its cytokine receptor Fms, a possible trigger of uncontrolled immune system. In this study, we attempted to clarify the underlying molecular mechanism of the new function of HIV-1 pathogenetic protein Nef. A striking change in Hck induced by Nef other than activation was its skewed localization to the Golgi due to predominant Golgi-localization of Nef. Studies with different Nef alleles and their mutants showed a clear correlation among higher Nef-Hck affinity, stronger Hck activation, severe Golgi-localization of Hck and severe Fms maturation arrest. Studies with a newly-discovered Nef-Hck binding blocker 2c more clearly showed that skewed Golgi-localization of active Hck was indeed the cause of Fms maturation arrest. 2c blocked Nef-induced skewed Golgi-localization of an active form of Hck (Hck-P2A) and Fms maturation arrest by Nef/Hck-P2A, but showed no inhibition on Hck-P2A kinase activity. These results indicated that aberrant dual regulation of Hck, activation/localization at Golgi, was the direct cause of Fms maturation arrest by Nef. Our finding also establishes an intriguing link between the pathogenesis of Nef and a newly emerging concept that the Golgi-localized Src kinases regulate the Golgi function.

3. List of publication

1. **Ranya Hassan**, Shinya Suzu, Masateru Hiyoshi, Naoko Takahashi-Makise, Takamasa Ueno, Tsutomu Agatsuma, Hirofumi Akari, Jun Komano, Yutaka Takebe, Kazuo Motoyoshi and Seiji Okada. Dys-regulated activation of a Src tyroine kinase Hck at the Golgi disturbs *N*-glycosylation of a cytokine receptor Fms. ***Journal of Cellular Physiology*** 221 (2): 458-468, 2009

2. Masateru Hiyoshi, Shinya Suzu, Yuka Yoshidomi, **Ranya Hassan**, Hideki Harada, Naomi Sakashita, Hirofumi Akari, Kazuo Motoyoshi, Seiji Okada. Interaction between Hck and HIV-1 Nef negatively regulates cell surface expression of M-CSF receptor. ***Blood*** 111(1): 243-50, 2008

4. Acknowledgments

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Finally, I would like to dedicate this thesis to my mother, although she is not here to see the completion of my study but I know she would have been proud of me, to my beloved husband, my lovely daughters for believing in me and also for their love, patience, encouragement and understanding through the duration of my study.

5. Abbreviations

CSF-1	colony-stimulating factor 1
DAPI	4', 6-diamidino-2-phenylindole
ERK	extracellular signal-regulated kinases
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
GST	glutathione *S*-transferase
Hck	hematopoietic cell kinase
HIV-1	human immunodeficiency virus type 1
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
M-CSFR	macrophage colony-stimulating factor receptor
MHC I	major histocompitability complex I
Nef	negative factor
PE	phycoerythrin
PTyr	phospho-tyrosine
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
SFKs	Src family kinases

6. Introduction

Nef is a 25–30 KD myristoylated protein of primate immunodeficiency viruses (HIV-1), which plays an important role in the pathogenesis of AIDS (1- 4). For example, studies of HIV-1 transgenic (Tg) mice clearly revealed that Nef is the critical determinant of disease progression. Targeted expression of the entire coding sequences of HIV-1 in $CD4^+$ T cells and macrophages caused a severe AIDS-like disease, which was abolished by the deletion of Nef (5). Nef has no enzymatic activity, and instead functions as an adaptor bringing together different host cells proteins. A large number of proteins, mainly protein kinases and components of endocytic trafficking machinery, have been reported to bind to Nef through different motifs. A summary of Nef binding proteins for which the binding sites in Nef have been mapped is listed in Table 1, importantly, the disruption of the proline-rich region of Nef was sufficient to protect Tg mice from the disease (6). The region contains 4 proline residues repeated in every third position (PxxPxxPxxP), which is a canonical (Src homology) SH3-binding motif. Indeed, the Nef PxxP motifs mediate a strong binding to the SH3 domain of Hck (7-9), a Src family non-receptor tyrosine kinase expressed in macrophages (10, Fig 1). Other SH3-containing proteins including other Src kinases (Lyn, Fyn, Src and Lck) bind Nef but with lower affinities (11- 13). More importantly, Nef-induced disease progression is significantly delayed in *Hck*$^{-/-}$ mice (6), indicating that the Nef PxxP-Hck SH3 binding contributes to the disease progression. However, unresolved issue is how Nef-Hck binding followed by activation of Hck (8, 9) satisfactorily account for disease development and progression.

Table 1 Nef motifs and its corresponding functions

MHC I Down-regulation:	^{20}M	^{62}EEEE^{65}	^{69}PxxP^{78}
CD4 Down-regulation:	^{57}EL^{58}		^{164}LL^{165}
Virion-infectivity Enhancement:		(^{69}PxxP^{78})	^{164}LL^{165}
Pak2 Activation:		(^{69}PxxP^{78})	^{105}RR^{106}
Hck Activation:	(^{62}EEEE^{65})	^{69}PxxP^{78}	
AIDS-like Disease in Tg Mice:		^{69}PxxP^{78}	

An important clue to the issue is that Nef predominantly localized to the Golgi apparatus (14-16) and that Nef not only activated Hck but also induced skewed localization of Hck to the Golgi (17). The Golgi functions as a sorting hub and location of glycosylation for proteins, and several lines of evidence have revealed that Src kinases, shown to be involved in a wide array of intracellular signaling (10), also play a role in the regulation of the Golgi structure/function. First, a fraction of Src kinases, including Hck, is physiologically found at the Golgi (18-24). Second, fibroblasts lacking three ubiquitous Src kinases (c-Src/Yes/Fyn) exhibited an aberrant Golgi structure composed of collapsed stacks and bloated cisternae (25). Third, an increased protein load entering the *cis*-Golgi from the endoplasmic reticulum activated the Golgi-localized Src kinases, which in turn regulated overall protein trafficking activity in the secretory pathway (26). Importantly, the study by Pulvirenti et al. indicates that coordinated regulation of activity of the Golgi-localized Src kinases is crucial to maintain the Golgi function,

which raises an intriguing possibility that Nef affects protein trafficking process and thereby macrophage phenotype/function through skewed Golgi-localization of active Hck (26).

Indeed, we recently identified an aberrant function of Nef, which was possibly due to the skewed Golgi-localization of active Hck. We previously found that Nef inhibited the signal of M-CSF, a primary cytokine for macrophages which supports the survival, proliferation and differentiation of the monocyte lineage, interaction of M-CSF with its receptor (M-CSFR/encoded by c-fms) is associated with important biologic effects (27).

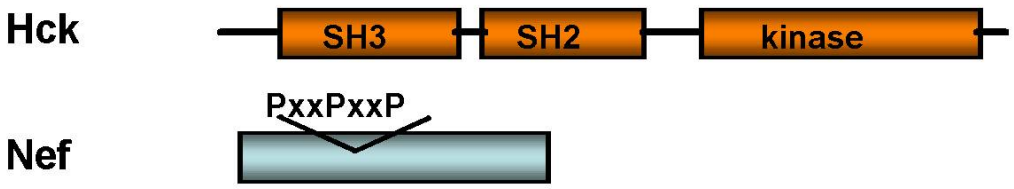

Fig.1 High affinity of Nef PxxP motif to bind to Hck SH3 domain.

The inhibition of M-CSF/Fms axis by Fms maturation arrest is therefore a possible trigger of uncontrolled immune systems in patients, as macrophages are normally exposed to sufficient levels of M-CSF to maintain them in an anti inflammatory M2-like polarized state with a relatively compromised ability to produce pro-inflammatory mediators (28 ,Fig 2). Of interest was the role of Hck in this inhibitory activity of Nef (29). Nef reduced cell surface expression of M-CSF receptor Fms in myeloid cells and macrophages which was the direct cause of the inhibitory activity of Nef on M-CSF signal in an Hck dependent pathway. Despite

its presumed pathologic significance, the molecular mechanism by which Nef down-regulates Fms via Hck is still unclear. Fms down-regulation was basically due to the inhibition of *N*-glycosylation maturation of nascent Fms and accumulation of immature fms at the Golgi apparatus and inhibiting trafficking to the cell surface (29). The question was the role of Hck, which is normally maintained in an inactive state by intramolecular interactions (30, 31). Importantly, studies with the kinase-dead and constitutive-active Hck mutants showed that the kinase activity of Hck was necessary but not sufficient for the Nef-induced Fms maturation arrest (29), implying the requirement of additional factor in Hck other than activation. The change in intracellular localization of active Hck appeared to explain the conflicting results, as the Nef-activated Hck mainly localized to the Golgi but not the plasma membrane as mentioned earlier (17,29). Given the presence of a fraction of Nef at the Golgi as well as the plasma membrane (14- 17), the Golgi-localization of active Hck by Nef is not surprising but previously unreported.

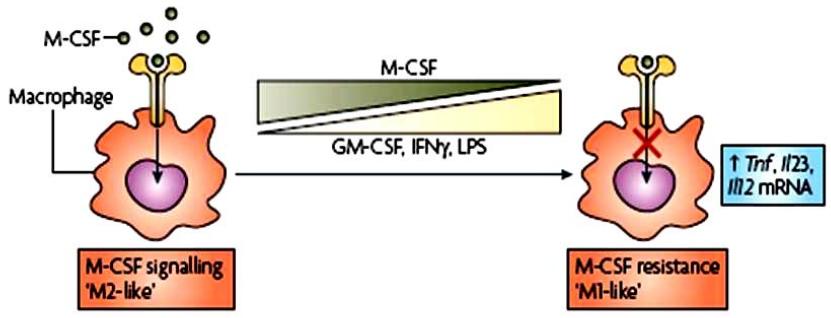

Fig.2 Macrophages are normally exposed to sufficient levels of M-CSF to maintain them in an anti inflammatory M2-like polarized state with a relatively compromised ability to produce pro-inflammatory mediators. Adapted from Chitu and Stanley (38).

In this study, we therefore sought to definitely conclude that skewed Golgi-localization of active Hck was indeed the direct cause of Fms maturation arrest by Nef. To this end, we employed two different approaches. First, we prepared various Nef proteins and compared their abilities to induce skewed Golgi-localization of Hck, Hck activation and Fms maturation arrest. Second and importantly, we discovered a small-molecule non-kinase inhibitor that effectively blocked Nef-Hck binding and performed mechanistic analyses with the newly-discovered compound.

7. Materials and methods

7.1 Expression Plasmids For the expression in HEK293 cells (Invitrogen, Carlsbad, CA), human Fms- and human p56Hck cDNA cloned into pCDNA3.1 vector (Invitrogen) were used (27,29). The constitutive-active Hck P2A mutant (29) was also used in selected experiments. The expression plasmid for human Lyn cloned in pME-puro vector was provided by Y. Yamanashi (Tokyo Medical and Dental University, Tokyo, Japan) and used in the pull-down assay with GST-Nef fusion proteins (see below). Nef cDNA derived from the NL43 or SF2 strain of HIV-1 was cloned into pRc/CMV-CD8 vector to express the extracellular/transmembrane regions of CD8-Nef fusion protein (29). NL43 Nef-M20A was prepared as described previously (32). NL43 Nef-AxxA and -ΔE mutant were provided by A. Adachi (University of Tokushima, Tokushima, Japan) and J.C. Guatelli (University of California, San Diego, CA), respectively. In this study, we prepared another NL43 Nef mutant (NL43 Nef-TR), by using QuikChange II Site-directed Mutagenesis Kits (Stratagene, La Jolla, CA). We also prepared Nef constructs expressing Nef-GFP fusion proteins (33). For the expression of GST-Nef fusion proteins, fragments containing the entire coding sequences of the wild-type NL43 Nef, NL43 Nef-TR mutant, wild-type SF2 Nef, and SF2 Nef-AxxA mutant were subcloned into pGEX-6P-1 vector (GE Healthcare, Buckinghamshire, UK). SF2 Nef-AxxA mutant was prepared by using Quick-change II Site-directed Mutagenesis Kits (Stratagene). The nucleotide sequences of the coding region of all Nef constructs were verified by using Big

Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

7.2 Chemicals PP2 (Sigma, San Diego, CA) was used as the Src kinase inhibitor. UCS15A and its synthetic derivatives, 2b and 2c, were prepared as described (34). All these inhibitors were dissolved in dimethyl sulphoxide (DMSO; Wako, Osaka, Japan).

7.3 Western Blotting HEK293 cells were maintained with DME medium (Wako) supplemented with 10% fetal calf serum (FCS). The maturation of Fms proteins or the activation of Hck was analyzed by the transient expression assay with the cells followed by Western blotting as described previously (27, 29). In brief, cells grown on a 12-well tissue culture plate were transfected with plasmid for Fms (0.4 μg), Nef (0.8 μg) or Hck (0.4 μg) in the combinations indicated using LipofectAMINE2000 reagent (Invitrogen), unless otherwise stated. Total amounts of plasmids were normalized with the empty vectors. After 6 hours, culture medium was replaced with complete medium and the transfected cells were cultured for an additional 42 hours. In selected experiments, chemicals such as PP2 and 2c were added to the culture at the same time of changing medium. Total cell lysate were prepared essentially as described (35). Primary antibodies used for Western blotting were as follows: anti-Fms (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD8 (H-160; Santa Cruz), anti-GFP (FL; Santa Cruz), anti-Hck (clone 18; Transduction Laboratories, Lexington, KY), anti-Hck

phosphorylated at tyrosine 411 (Hck-pTyr^{411}; Santa Cruz), anti-phosphotyrosine (PY99; Santa Cruz), and anti-ERK1/2 (K-23; Santa Cruz). The relative intensity of bands on scanned gel images was quantified using NIH Image software, and the Fms maturation arrest or Hck activation is also shown graphically on an arbitrary unit. The relative intensity of bands on Hck-pTyr^{411} blots was quantified and the degree of Hck activation was expressed as a fold-increase relative to the control. For Fms maturation arrest, we calculated the percentage of immature under-*N*-glycosylated Fms of total Fms protein amount, and compared the percentages among samples.

7.4 Immunofluorescence The signal of Nef-GFP was directly visualized with a BZ-8000 fluorescent microscope (Keyence, Osaka, Japan) equipped with Plan-Fluor ELWD 20x/0.45 objective lenses (Nikon, Tokyo, Japan) (29). To detect active Hck, cells were fixed in 2% paraformaldehyde, permeabilized with ethanol, and stained with goat anti-active Hck antibodies (Santa Cruz). Secondary antibodies were anti-goat IgG-AlexaFluo488 (Molecular Probes, Eugene, OR). Nuclei were stained with DAPI (Molecular Probes), and fluorescent signals were visualized as above. Image processing was performed using BZ-analyzer (Keyence) and Adobe Photoshop Software (Adobe Systems, San Jose, CA).

7.5 GST Pull-down The control GST or GST-Nef fusion proteins (wild-type NL43 Nef, NL43 Nef-TR, wild-type SF2 Nef and SF2 Nef-AxxA) cloned in pGEX-6P-1 vector was expressed in *E. coli* BL21 cells (GE Healthcare). Cells

were grown in LB media containing 50 μg/mL ampicillin followed by induction with 1 μM IPTG. The expression-induced cells were harvested and lysed with Bug Buster Protein Extraction Reagent containing 1 unit/ml rLysozyme and 25 units/ml Benzonaze Nuclease (Novagen, Madison, WI). The cleared lysate were then incubated with GST-Bind Resin (Novagen). After extensive washing with GST Bind/Wash Buffer (Novagen), the resin was incubated with the total cell lysate of HEK293 cells transfected with the expression plasmid for Hck or Lyn. In a selected experiment, 2c was added to the mixtures. After extensive re-washing, the resin was boiled with SDS-PAGE sample buffer and elutes were analyzed for the presence of Hck or Lyn by western blotting. Primary antibodies used were as follows (both from Transduction Laboratories): anti-Hck (clone 18) and anti-Lyn (clone 42). In a selected experiment, we also used GST proteins fused to the SH3 domain of Hck (86), which was provided by G. Swarup (Center for Cellular and Molecular Biology, Hyderabad, India).

7.6 Subcellular Fractionation The subcellular fractionation on sucrose gradients was performed exactly as reported (36). In brief, cells were swollen in hypotonic buffer containing protease inhibitors followed by homogenization. Then, the post-nuclear supernatants were fractionated by ultracentrifugation on discontinuous sucrose gradients. All steps were carried out on ice. The fractions obtained were subjected to Western blotting with antibodies to Hck (clone 18; Transduction Laboratories), Desmoglein (clone 62; Transduction Laboratories), annexin II (C-10; Santa Cruz), or Calnexin (H-70; Santa Cruz).

7.7 Flow cytometry Human myeloid TF-1-fms cells expressing Nef-ER fusion protein were maintained as described previously (27, 29). To activate the Nef-ER fusion protein, we used the estrogen analog 4-HT (Sigma) at a final concentration of 0.1 μM. The cells were stained with PE-labeled anti-Fms antibodies (Santa Cruz), and the level of cell surface Fms was analyzed by flow cytometry on a FACS Calibur using Cell Quest software (Becton Dickinson, Mountain View, CA).

8. Results

8.1 Analyses with Src kinase inhibitor and Hck mutant

As reported, Nef induces Fms maturation arrest when co-expressed with Hck in HEK293 cells (Fig. 3A). HEK293 cells did not endogenously express Hck, and the upper and lower band was the fully *N*-glycosylated and under-*N*-glycosylated Fms, respectively (29). The low molecular weight Fms was sensitive to Endo-H, which selectively cleaves high-mannose type oligosaccharide, and their increase was clearly associated with the intense staining of Fms mainly at the perinuclear region, which overlapped well with the signal of GM130 or Vti1a, the markers for the Golgi (29). These results strongly suggested that the low molecular weight Fms was the immature under-*N*-glycosylated form. The increase of the lower molecular weight species was obvious in the cells co-expressing Nef and Hck (Fig. 5A, left blot), and this Fms maturation arrest was blocked by a Src kinase inhibitor PP2 (Fig. 3A, right blot). However, the expression of a constitutive-active Hck mutant (Hck-P2A) was not sufficient to induce Fms maturation arrest when expressed alone (Fig. 3B, Fms blot), despite its strong kinase activity (total pTyr and $pTyr^{411}$ Hck blots). In this study, we monitored kinase activity of Hck by overall protein tyrosine-phosphorylation (total pTyr) and auto-phosphorylation of Hck ($pTyr^{411}$ Hck) .Nonetheless, Hck-P2A/Nef co-expression induced more severe Fms maturation arrest than wild-type Hck/Nef co-expression (Fig. 3B), and Nef did not enhance the kinase activity of Hck-P2A (Fig. 3C), confirming our previous finding that Hck activation was necessary but not sufficient for Nef-induced Fms maturation arrest.

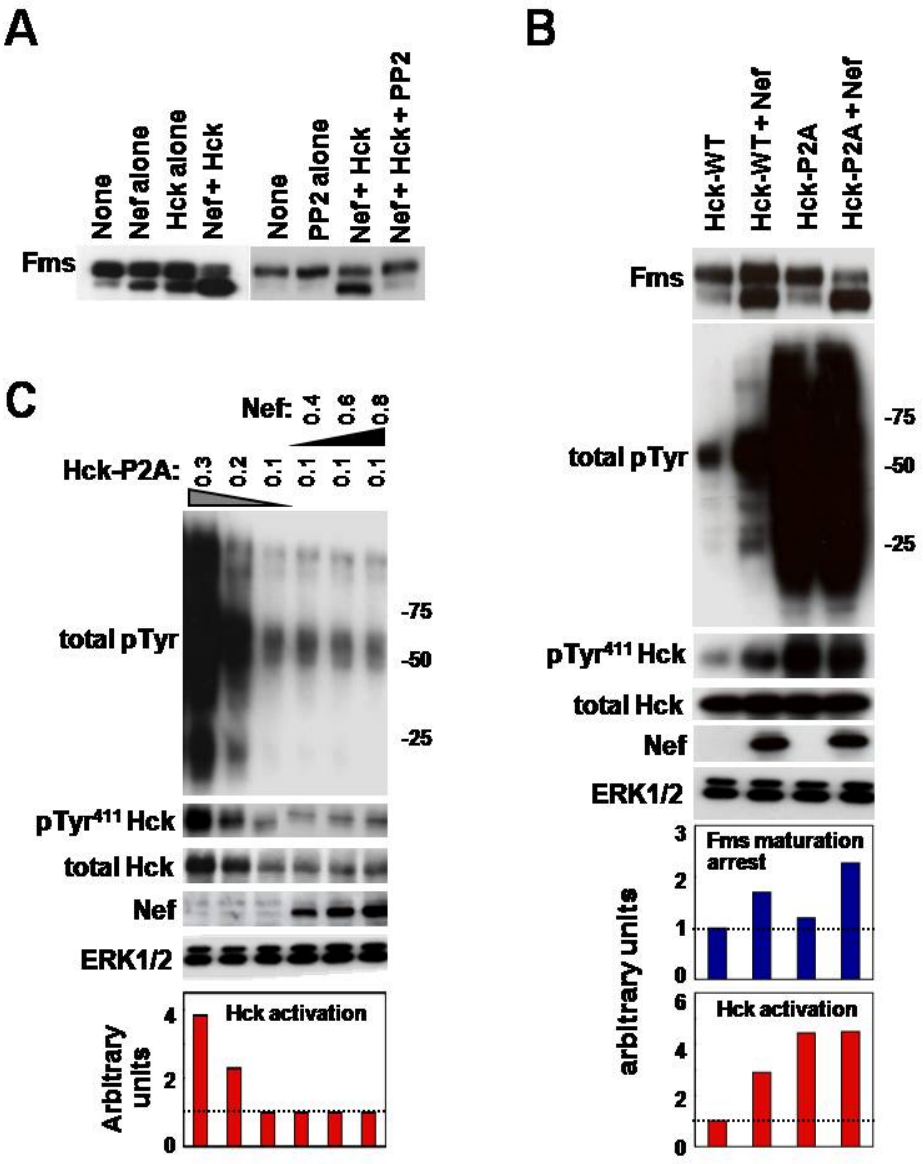

Fig. 3 Nef/Hck-induced Fms maturation arrest A: HEK293 cells were transfected with Fms plamid alone (None) or co-transfected with the plasmids for NL43 Nef and/or wild-type Hck as indicated. In the right blot, PP2 was added to selected wells at a final concentration of 10 μM after the transfection, Total cell lysate were subjected to Fms Western blotting. B: Cells were transfected with Fms plasmid alone (None) or in combination with the plasmids for Nef (NL43) and Hck (WT or constitutive-active P2A), as indicated. These cells were then analyzed for the expression of Fms, tyrosine-phosphorylated proteins (total pTyr), active-Hck ($pTyr^{411}$Hck), total Hck, CD8-Nef (Nef), or ERK by Western blotting. The ERK blot is a loading control. The quantified Fms maturation arrest and Hck activation are shown in the bar graphs. C: Cells were transfected with varying amounts (μg) of Hck-P2A and NL43 Nef plasmids as indicated, and analyzed as in B. The quantified Hck activation is shown in the bar graphs.

8.2 Analysis with different Nef alleles and their mutants

We first found that Nef derived from SF2 strain of HIV-1 induced more severe Golgi-localization of Hck-P2A than Nef derived from NL43 strain. Hck-P2A signal at the plasma membrane was still observed in some NL43 Nef-transfected cells, whereas such signal was not observed in SF2 Nef-transfected cells (Fig. 4A). The Nef-induced skewed Golgi-localization of Hck-2PA was confirmed by a quantitative analysis, i.e. subcellular fractionation on sucrose gradients. We used Desmoglein, annexin II and Calnexin as marker proteins for the plasma membrane, both the plasma membrane and the Golgi, and the endoplasmic reticulum, respectively. As shown (Fig. 4B), the plasma membrane was recovered in light fractions whereas the Golgi and the endoplasmic reticulum were recovered in heavy fractions, and the peak of Hck-P2A shifted to heavy fractions by the co-expression with NL43 Nef but not a Nef-AxxA mutant defective in the binding to Hck. The peal shift was also associated the appearance of many tyrosine-phosphorylated proteins in these fractions (Fig. 4B).Both NL43 Nef and SF2 Nef had intact PxxP motif (Fig. 5A), but SF2 Nef showed much higher affinity to Hck than NL43 Nef (Fig. 5B). In the control experiments, we confirmed that the binding of both Nef to Lyn remained low and the PxxP motif-disrupted SF2 Nef mutant (AxxA) bound neither Hck nor Lyn. Reflecting the higher affinity to Hck, SF2 Nef induced stronger Hck activation (Fig. 5C) and more severe Fms maturation arrest (Fig. 5D). However, SF2 Nef and NL43 Nef showed no obvious change in the pattern of predominant Golgi- localization (Fig. 5E). It was therefore likely that SF2

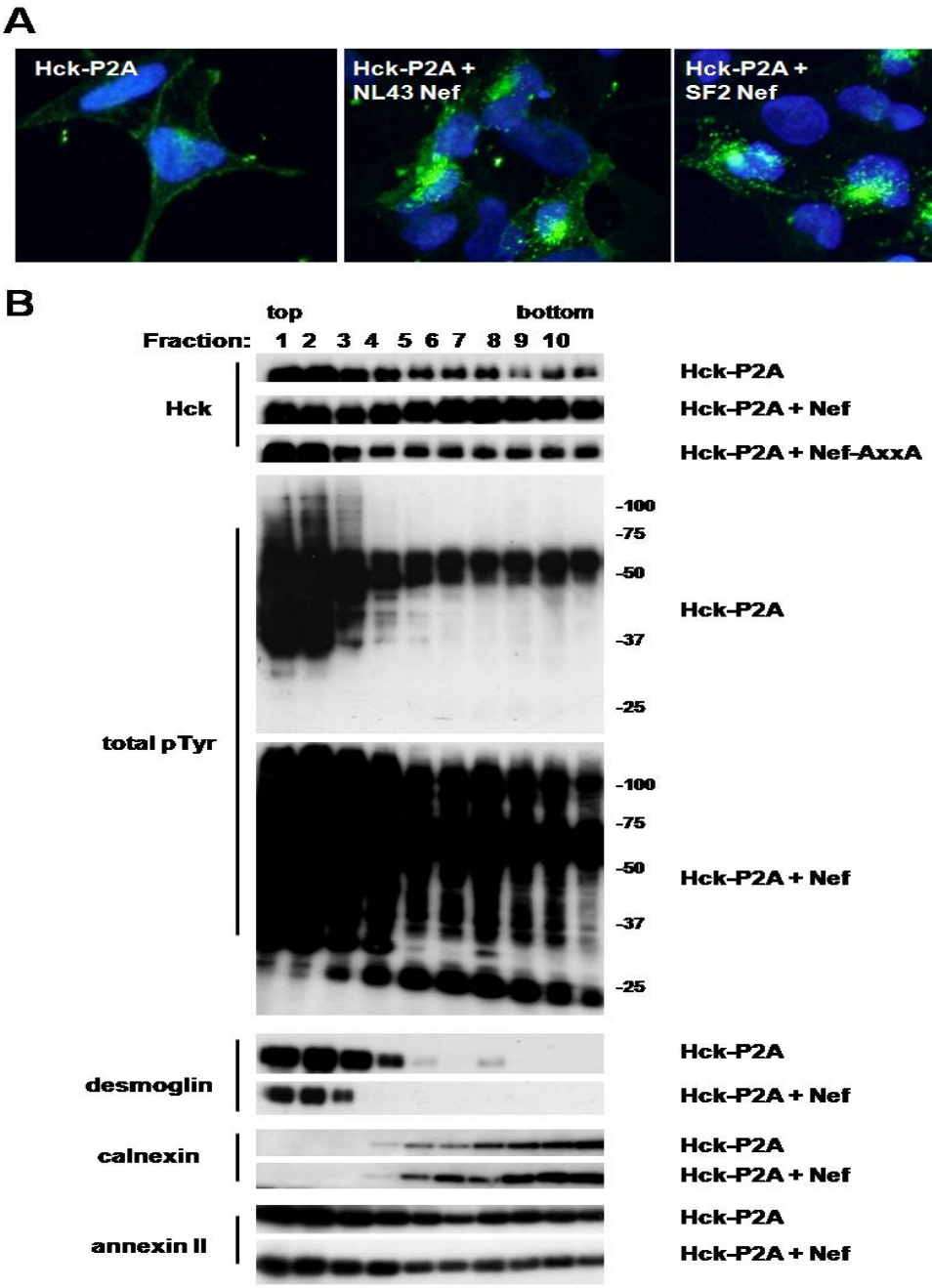

Fig. 4 Skewed Golgi-localization of Hck by Nef. A: HEK293 cells were transfected with Hck-P2A plasmid alone, or co-transfected with NL43 Nef or SF2 Nef plasmid. Cells were stained with antibody specific for active Hck (green) and DAPI (blue). B: Cells were transfected with Hck-P2A alone, or co-transfected with NL43 Nef. Then, cells were subjected to subcellular fractionation on sucrose gradients and Western blotting with antibodies against Hck, phosphotyrosine (pTyr), Desmoglein, Calnexin or annexin II.

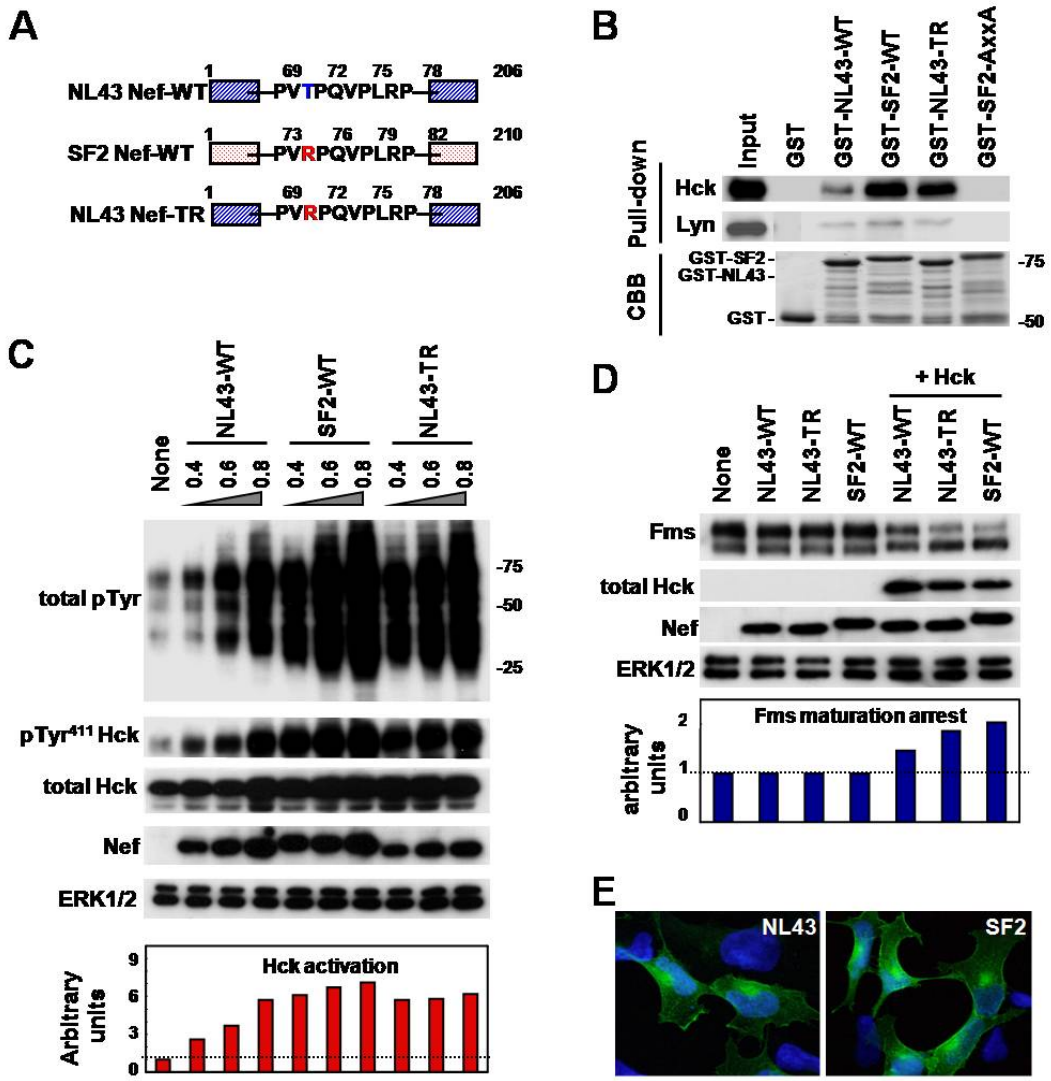

Fig. 5 Abilities of different Nef alleles to bind/activate Hck and to induce Fms maturation arrest. A: The NL43 Nef, SF2 Nef and NL43 Nef-TR mutant used are schematically shown. B: The resins, to which the control GST or indicated GST-Nef proteins were bound, were incubated with the lysate of HEK293 cells expressing Hck or Lyn. The amount of Hck or Lyn bound to the resins was determined by Western blotting (Pull-down). The amount of GST and GST-Nef fusion proteins bound to the resins was verified by the elution from the resins followed by SDS-PAGE/Coomassie brilliant blue (CBB) staining. C: HEK293 cells were co-transfected with the wild-type Hck and indicated Nef alleles. The amounts of Nef plasmids used are shown (0.4, 0.6 or 0.8 μg/ well). Total cell lysate were subjected to Western blotting with antibodies against phosphotyrosine (total pTyr), active-Hck ($pTyr^{411}$Hck), total Hck, CD8-Nef (Nef), or ERK by Western blotting. The quantified Hck activation is shown in the bar graph. D: Cells were transfected with Fms plasmid alone (None) or in combination with the plasmids for Nef and Hck, as indicated. Western blotting was done as in C. E: Cells were transfected with indicated GFP-Nef plasmid (green). Nuclei were stained with DAPI (blue).

Nef bound Hck at the Golgi with higher affinity and thereby induced stronger Hck activation and more severe Fms maturation arrest.

There was a single amino acid difference within the PxxP motif, Thr^{71} in NL43 Nef and Arg^{75} in SF2 Nef (Fig. 5A). We found that an NL43 Nef with $Thr^{71}Arg$ substitution (NL43 Nef-TR) showed higher affinity to Hck than wild-type NL43 Nef (Fig. 5B), and induced stronger Hck activation (Fig. 5C) and more severe Fms maturation arrest (Fig. 5D) than wild-type NL43 Nef. We also performed a complementary experiment with SF2 Nef mutants (Fig6A; 33). As a result, we found that mutants with $Arg^{75}Thr$ substitution (SF2 Nef-RT and SF2-RT/YF) induced moderate Hck activation/Fms maturation arrest (Fig. 6B). However, both showed no obvious change in the pattern of predominant Golgi-localization (Fig. 6C). These results indicated that the single amino acid difference (Thr to Arg) within the PxxP motif governed the higher ability of SF2 Nef to induce Golgi-localization and activation of Hck, and Fms maturation arrest.

Although PxxP motif is essential for Nef to bind Hck, a recent study showed that an acidic region of Nef facilitated Nef-Hck binding at the Golgi (17). Although an NL43 Nef mutant lacking this region (ΔE; Fig. 7A) bound GST-Hck SH3 fusion proteins as with wild-type NL43 Nef (Fig. 7B), ΔE mutant was indeed less active than wild-type in transfected HEK293 cells, i.e. in both Hck and activation Fms maturation arrest (Fig. 7C). Another mutant (M20; Fig. 7A), which was defective in the down-regulation of MHC I, another hallmark function of Nef (32), retained the ability to induce Hck activation and Fms maturation arrest (Fig. 7C). Both ΔE and M20A mutants showed no obvious change in the pattern of predominant Golgi-localization (7D.The mutant studies were summarized in Table 2.

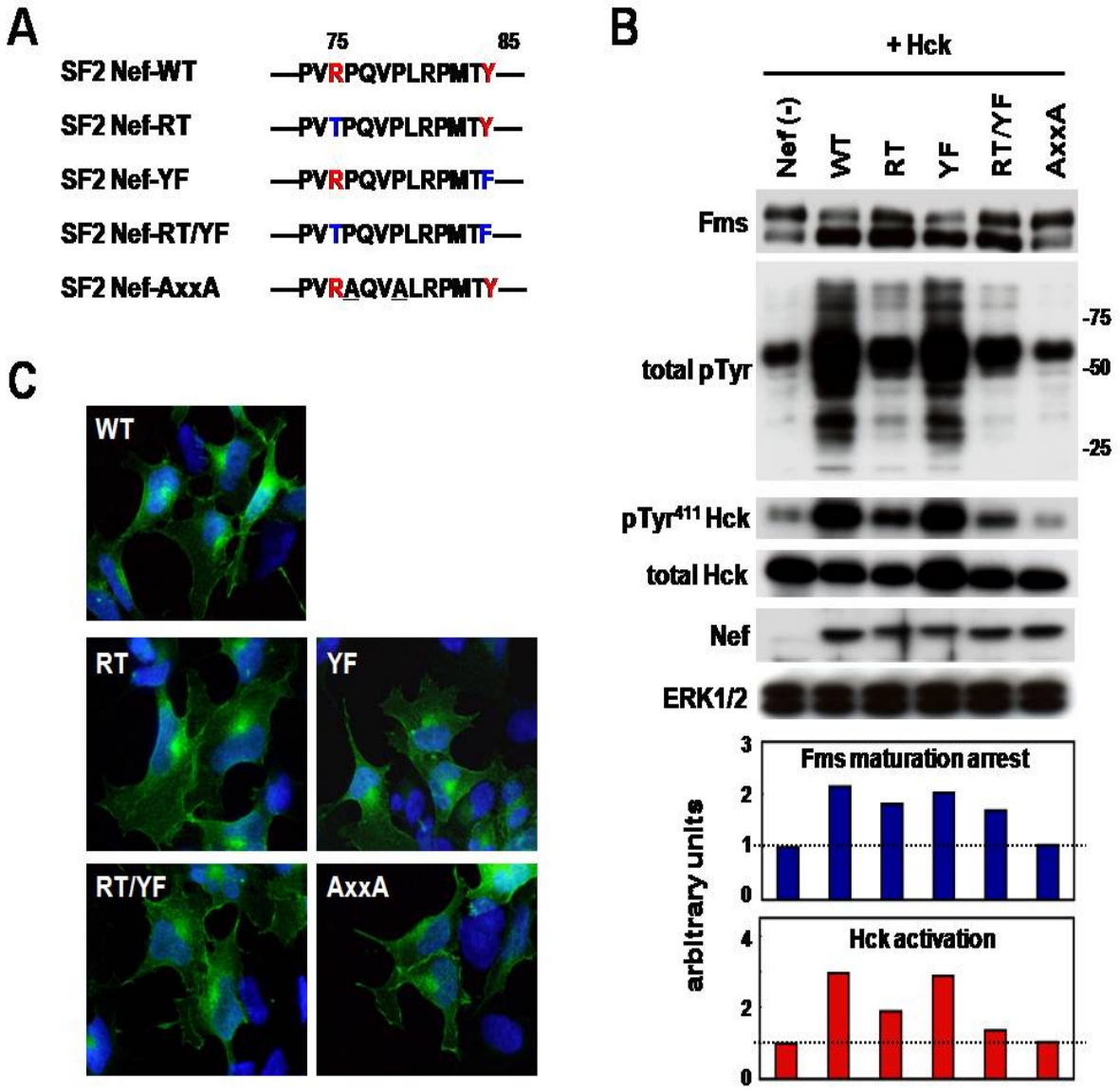

Fig.6 Abilities of SF2 Nef mutants to activate Hck and to induce Fms maturation arrest. A: The SF2 Nef mutants used (RT, YF, RT/YF and AxxA) are schematically shown. B: HEK293 cells were transfected with Fms plasmid alone (None) or in combination with the plasmids for Nef and Hck, as indicated. These cells were then analyzed for the expression of Fms, phosphotyrosine (total pTyr), active-Hck ($pTyr^{411}Hck$), total Hck, GFP-Nef (Nef), or ERK by Western blotting. The quantified Fms maturation arrest and Hck activation are shown in the bar graphs. C: Cells were transfected with indicated GFP-Nef plasmid (green). Nuclei were stained with DAPI (blue).

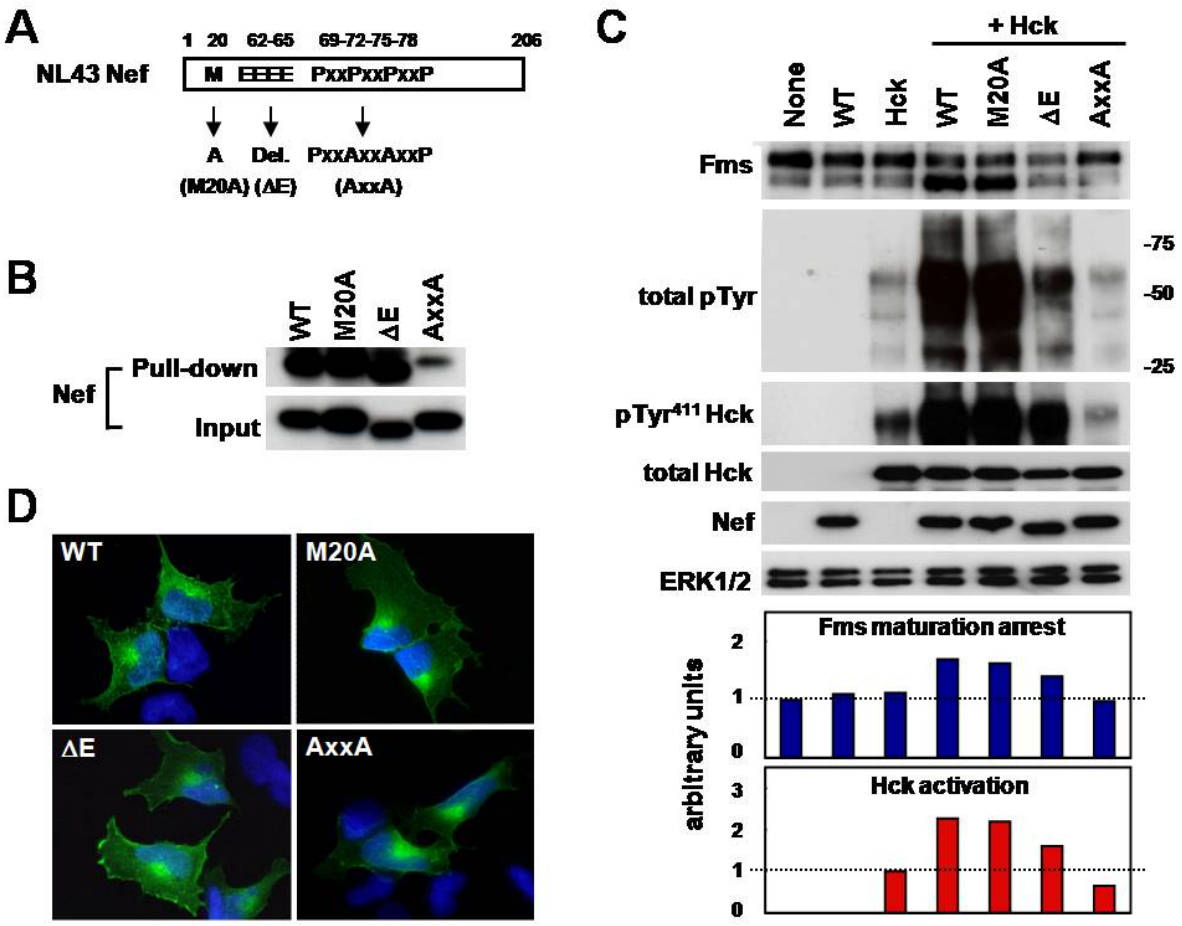

Fig. 7 Abilities of NL43 Nef mutants to activate Hck and to induce Fms maturation arrest. A: The NL43 Nef mutants used (M20A, ΔE and AxxA) are schematically shown. B: The resin, to which GST-Hck SH3 fusion proteins were bound, were incubated with the lysate of HEK293 cells expressing the indicated Nef proteins. The amount of Nef proteins in the lysate (Input) or bound to the resins (Pull-down) was verified by Western blotting. C: HEK293 cells were transfected with Fms plasmid alone (None) or in combination with the plasmids for Nef and Hck, as indicated. These cells were then analyzed for the expression of Fms, phosphotyrosine (total pTyr), active-Hck ($pTyr^{411}$Hck), total Hck, CD8-Nef (Nef), or ERK by Western blotting. The quantified Fms maturation arrest and Hck activation are shown in the bar graphs. D: Cells were transfected with indicated GFP-Nef (green). Nuclei were stained with DAPI (blue).

Table 2 Summary of mutant studies

Nef mutant	Hck activation	Fms maturation arrest
SF2wt	++++++	++++++
NL43wt	+++	+++
NL43TR	++++	++++
NL43AxxA	-	-
NL43M20A	+++	+++
NL43 E	++	++
SF2RT	++	++
SF2YF	++++++	++++++
SF2RT/YF	++	++

8.3 Analyses with a newly-discovered Nef-Hck binding blocker

To directly show that the Golgi-localization of active Hck determines Nef-induced Fms maturation arrest, we sought to discover Nef-Hck binding blockers. In this study, we focused on UCS15A and its analogs 2b and 2c (Fig. 8A), because these small-molecule compounds were shown to block several proline-rich motif-SH3 domain binding such as Sam68-Fyn binding (34) and AMAP1-cortactin binding (37). As they have not been used before for HIV-1 studies, we tested their capability to block Nef-Hck binding by the GST pull-down assay. As shown (Fig. 8B), all compounds blocked the binding of Hck to NL43 Nef or NL43 Nef-TR mutant (more potent than the wild-type, see Fig. 5), in a dose-dependent manner. Like the case of Sam68-Fyn binding (34), 2c was the most effective in blocking Nef-Hck binding (Fig. 8B), and showed no obvious toxicity to HEK293 cells (Fig. 8C). As shown (Fig. 8D), 2c indeed inhibited Hck activation by NL43 Nef and more potent SF2 Nef (see Fig. 5). Importantly, 2c had little inhibitory effect on kinase activity of the constitutive-active Hck P2A mutant, even when used at a concentration as high as 50 μM (Fig. 8E). These results indicated that 2c was not a kinase inhibitor but inhibited Nef-induced Hck activation by blocking Nef-Hck binding.

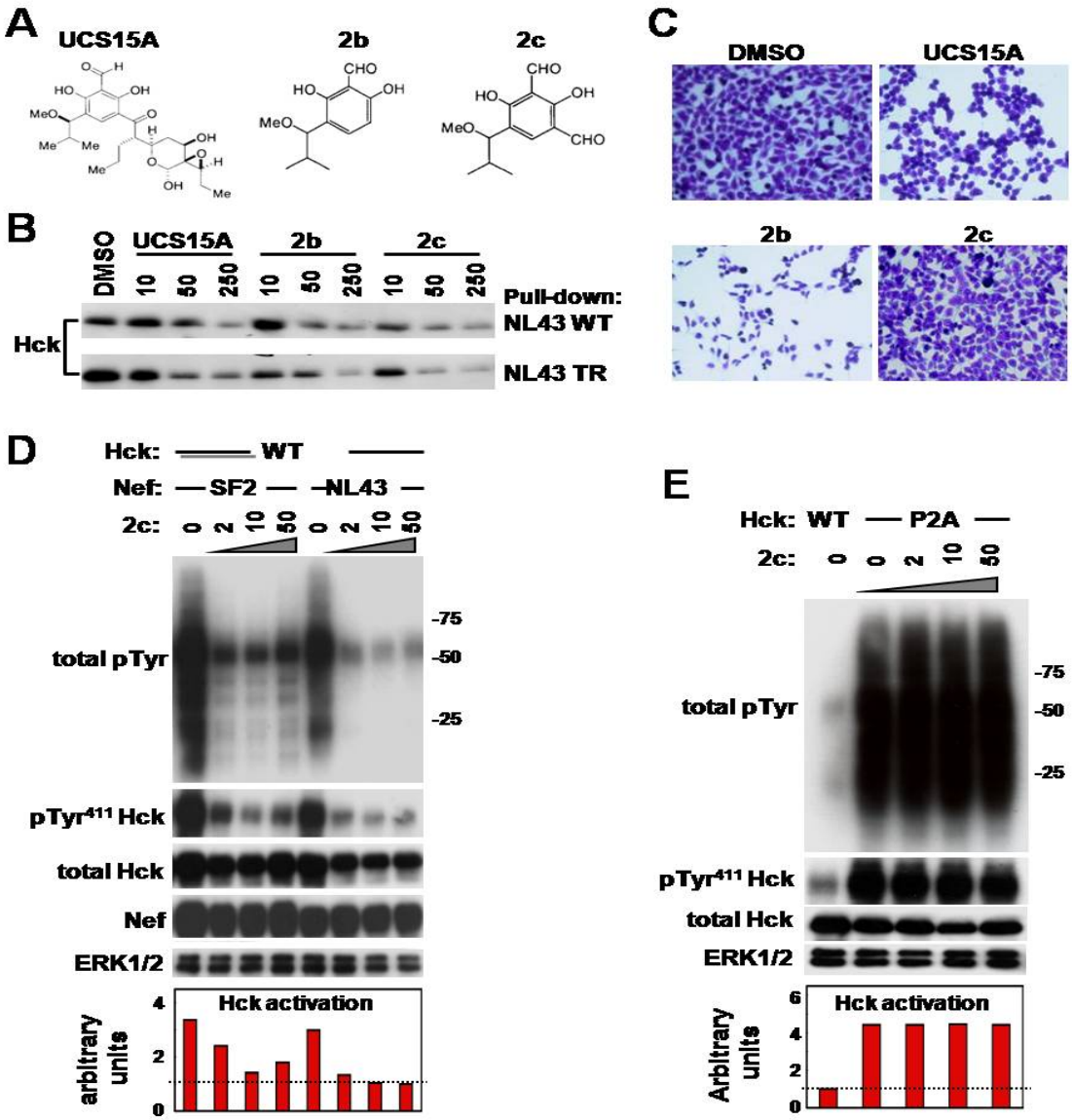

Fig. 8 Capability of 2c to block Nef-Hck binding and Nef-induced Hck activation A: Chemical structures of UCS15A, 2b and 2c are shown. (B) The resins, to which GST-Nef (NL43 wild-type or TR mutant) proteins were bound, were incubated with the lysate of Hck-expressing HEK293 cells in the absence (DMSO) or presence of the indicated concentration (0, 10, 50 or 250 μM) of UCS15A, 2b or 2c. The amount of Hck proteins bound to the resins was determined by Western blotting. C: HEK293 cells were cultured in the absence (DMSO) or presence of 50 μM of UCS15A, 2b or 2c for 2 days, and subjected to Wright-Giemsa staining. D: Cells were co-transfected with Hck-WT and indicated Nef alleles (SF2 or NL43), and cultured in the presence of increasing concentrations (μM) of 2c. Cells were then analyzed for the expression of (total pTyr), active-Hck ($pTyr^{411}$Hck), total Hck, CD8-Nef (Nef), or ERK by Western blotting. The quantified Hck activation is shown in the bar graphs. E: Cells were transfected with Hck-WT or Hck-P2A, and cultured in the presence of increasing concentrations (μM) of 2c. These cells were analyzed as in D.

This unique feature of 2c prompted us to examine whether 2c blocks Nef/Hck-induced Fms maturation arrest and Nef-induced skewed Golgi-localization of Hck. As shown (Fig. 9A), 2c completely blocked Fms maturation arrest induced by Nef and wild-type Hck as expected. However, of particular importance was that 2c also completely blocked severe Fms maturation arrest induced by Nef and the constitutive-active Hck P2A (Fig. 9B). Because 2c had little inhibitory effect on kinase activity of Hck-P2A (see Fig. 8E), these results strongly supported that the presence of Hck-P2A at the Golgi caused by its binding with Nef (see Fig. 4) was a direct cause of severe Fms maturation arrest. We therefore sought to verify that 2c indeed blocked Nef-induced skewed Golgi-localization of Hck-P2A. To this end, we employed the quantitative analysis, i.e. subcellular fractionation on sucrose gradients (see Fig. 4B). The peak of Hck-P2A shifted to heavier fractions by the co-expression with Nef, and such change in the intracellular localization of Hck-P2A was restored to normal by the addition of 2c (Fig. 9C). We also tested whether 2c blocked Nef-induced Fms abnormality in another culture system. We previously showed that the cell surface expression of Fms was impaired in human myeloid TF-1-fms cells expressing a conditionally active Nef-ER fusion protein when the Nef-ER in the cells was activated by the estrogen analog 4-HT (29). This impaired cell surface Fms expression was highly likely due to intracellular Fms maturation arrest (29). Finally, we found that the Fms down-regulation in Nef-active TF-1-fms-Nef-ER cells was also restored to normal by the addition of 2c (Fig. 9D). All taken together, our present study clearly demonstrated that skewed Golgi-localization of active Hck induced by Nef was indeed the direct cause of Fms maturation arrest (Fig .10).

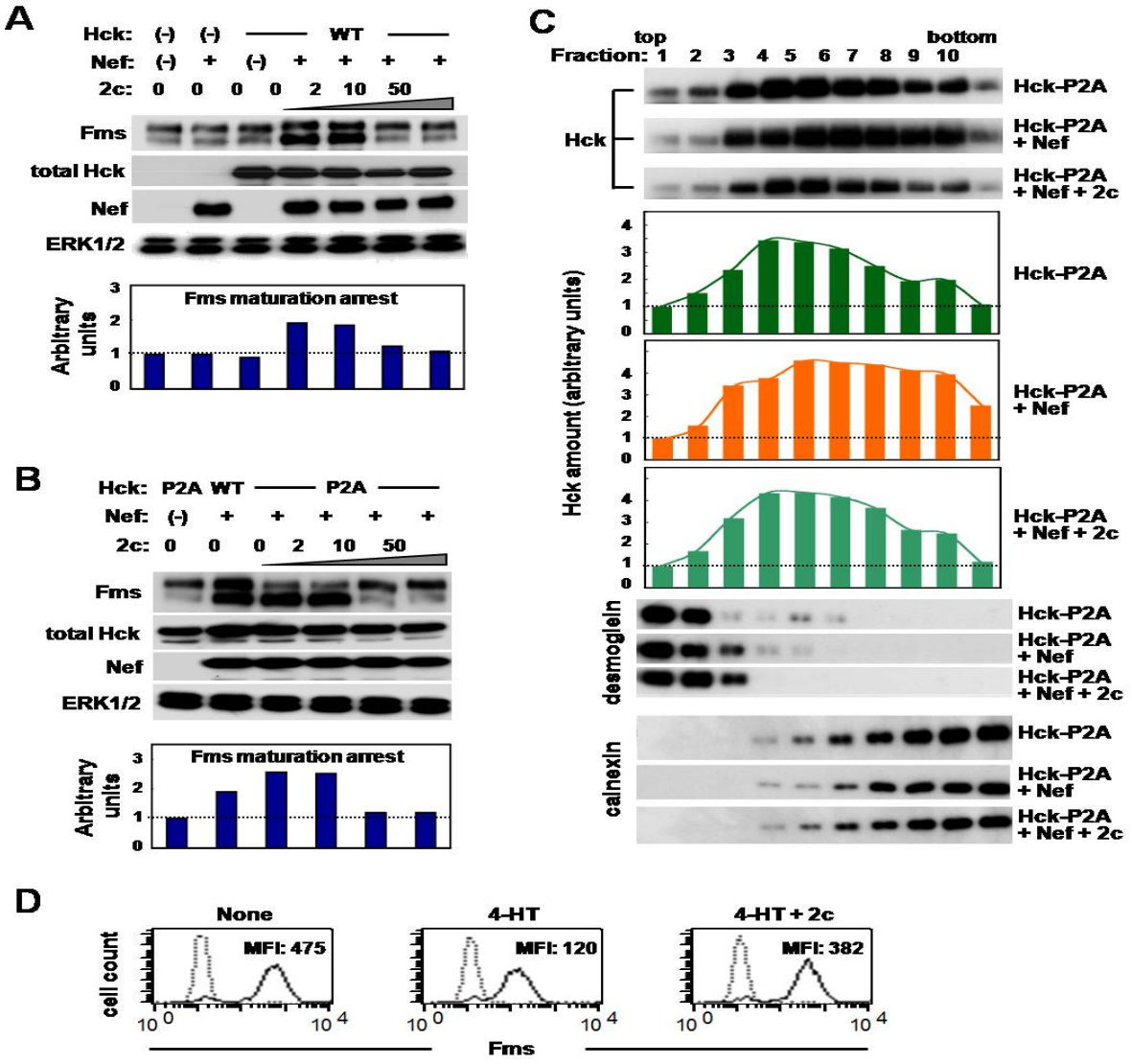

Fig. 9 Capability of 2c to block Fms maturation arrest and skewed Golgi-localization of Hck A: HEK293 cells were transfected with the plasmids (Fms, NL43 Nef and Hck-WT) in combination indicated, and cultured in the presence of increasing concentrations (μM) of 2c. These cells were then analyzed for the expression of Fms, total Hck, CD8-Nef (Nef), or ERK by Western blotting. The quantified Fms maturation arrest is shown in the bar graphs. B: Cells were transfected with the plasmids (Fms, NL43 Nef, Hck-WT and Hck-P2A) in combination indicated, and cultured in the presence of increasing concentrations (μM) of 2c. These cells were then analyzed as in A. C: Cells were transfected with Hck-P2A alone (top), or co-transfected with NL43 Nef (middle). 2c was added to a final concentration of 50 μM to selected wells (bottom). Then, cells were subjected to subcellular fractionation on sucrose gradients and Hck Western blotting. The quantified Hck amounts are shown in the bar graph. The fractions were also analyzed for the amount of Desmoglein and Calnexin. D: TF-1-fms-Nef-ER cells cultured with M-CSF-free media in the absence (left) or presence of 0.1 mM 4-HT (middle), or the co-presence of 0.1 μM 4-HT and 50 μM 2c (right) for 12 hours. The expression of Fms on the surface of treated cells was analyzed by flow cytometry with PE-labeled anti-Fms. The mean fluorescence intensity (MFI) of Fms expression is indicated

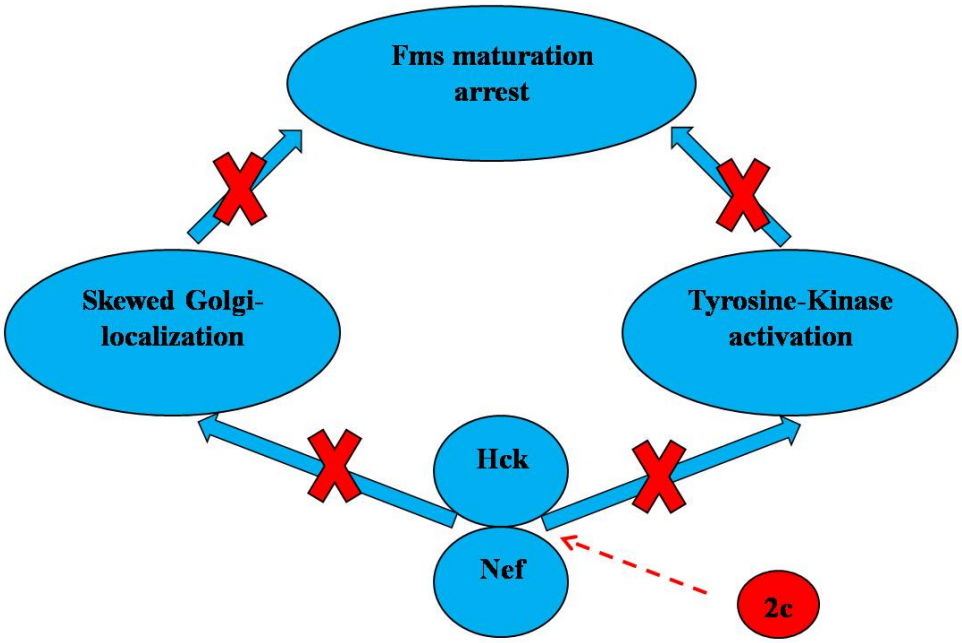

Fig.10 Explanation of Nef induced Fms maturation arrest and inhibitory effect of 2c.

9. Disscusion

The Nef protein unique to the primate lentiviruses HIV and SIV is essential for high-titer viral replication and AIDS progression. Despite its essential role, the molecular mechanisms by which Nef functions in HIV pathogenesis are not fully understood. Since Nef lacks intrinsic catalytic activity, many researches had been focused on analyzing interactions between Nef and cellular proteins in an attempt to understand the functions attributed to Nef. One of the important findings is that Nef binds to the macrophage-specific Src family member Hck through its SH3 domain with the highest affinity known for an SH3-mediated protein-protein interaction.

Previous studies from our laboratory have shown that Nef inhibited the signal of M-CSF, a primary cytokine for macrophages (27), essential not only for the survival of macrophages but also for the maintenance of macrophages at an anti-inflammatory state (28, 38). Thus, the inhibitory effect of Nef on M-CSF signal through Fms maturation arrest at the Golgi is a possible trigger to worsen uncontrolled immune systems in patients (27,29)., Of interest was the role of Hck in this inhibitory activity of Nef (29). Nef reduced cell surface expression of M-CSF receptor Fms in myeloid cells and macrophages, which was the direct cause of the inhibitory activity of Nef on M-CSF signal. Importantly, such reduced cell surface expression of Fms was reproduced in transfected 293 cells, but only in co-expression with Hck.

More importantly, the reduced cell surface expression was due to the accumulation of an immature under-*N*-glycosylated Fms at the Golgi. However,

constitutive-active Hck alone failed to induce such Fms maturation arrest. These results indicate that Nef inhibits M-CSF signal by arresting Fms *N*-glycosylation and trafficking at the Golgi and that such Fms maturation arrest was not caused just because of Hck activation. Thus, a most likely cause of Nef-induced Fms maturation arrest was skewed Golgi-localization of active Hck.

The purpose of this study was to define molecular basis of this newly discovered yet important function of Nef by using different Nef alleles, various Nef mutants, constitutive-active Hck mutant, and Nef-Hck binding blocker 2c,a small-molecule non-kinase inhibitor that effectively blocked Nef-Hck binding and performed mechanistic analyses with this newly-discovered compound.

The study with various Nef proteins supported the idea that high affinity Nef-Hck binding and subsequent stronger Hck activation, both of which took place mainly at the Golgi, determined Fms maturation arrest at the Golgi (Fig. 4-7). Moreover, the study with 2c enabled us to conclude that skewed Golgi-localization of active Hck by Nef was indeed the direct cause of Fms maturation arrest (Figs 8, 9). By analogy with the Sam68-Fyn binding inhibition (34), the inhibitory effect of 2c on Nef-Hck binding was supposed to be mediated by its interaction with Nef PxxP motif. It has been known for a long time that most members of Src kinases including Hck localize to the Golgi as well as to the plasma membrane. For example, it was shown that newly-synthesized Lyn initially localized and accumulated to the Golgi, and then moved toward the plasma membrane (24).

Importantly, Pulvirenti et al. recently revealed that coordinated regulation of activity of the Golgi-localized Src kinases is crucial to maintain intra-Golgi trafficking of proteins (26). Our present finding that skewed Golgi-localization of

active Hck leads to Fms maturation arrest at the Golgi is in line with the new concept. It appears that long-lasting and dys-regulated activation of the Golgi-localized Src kinases disturbs glycosylation and/or trafficking of proteins, exemplified by Fms maturation arrest. Moreover, it was reported that over-expression of an active form of Hck disturbed *N*-glycosylation of another cytokine receptor Flt3 even in the absence of Nef (39). These results may further support the idea that long-lasting and dys-regulated activation of the Golgi-localized Src kinases *per se* affect protein glycosylation and/or trafficking at the Golgi, which might be a potential way for the virus to divert macrophage functions.

Our system with Nef provides a useful model to elucidate how Src kinases regulate the Golgi structure/function. It will be important to identify which Golgi proteins are phosphorylated directly or indirectly by Hck activated at the Golgi and to clarify how such phosphorylation cascade leads to Nef-induced Fms maturation arrest.

Nef has been shown to affect protein trafficking and a well-characterized target is major histocompitability complex class I (MHC I). Nef reduced the cell surface expression of MHC I, which diminishes the recognition of infected cells by cytotoxic T cells (40, 41). However, it is still in intense debate whether Nef requires SH3 domain-containing proteins such as Hck to reduce the cell surface level of MHC I (14, 17, 32, 42, 43, 44). In this regard, Fms is not the direct target of Nef. However, as shown, Nef disturbed the cell surface expression of Fms, which is triggered by skewed Golgi-localization of active Hck. Moreover, it was shown that Nef disturbed the cell surface expression of another macrophage-specific protein HFE, an iron homeostasis regulator, which was blocked by a dominant-negative Hck (15). Although whether the reduced cell surface level of

HFE by Nef relates to skewed Golgi-localization of active Hck is unclear, it is conceivable that Nef acquires an additional machinery to manipulate protein trafficking in macrophages by exploiting the Golgi-localized Hck.

In this study, we showed that SF2 Nef had much higher affinity to Hck than NL43 Nef and thereby induced stronger Hck activation/severe Golgi-localization of Hck (Figs 4,5) and that the single amino acid difference (Thr^{71} in NL43 Nef and Arg^{75} in SF2 Nef) within PxxP motif largely governs the higher ability of SF2 Nef (Figs 5,6). This difference might reflect that the $Thr^{71}Arg$ substitution in NL43 Nef (NL43 Nef-TR, see Fig. 5) altered the flexibility of a loop containing the PxxP motif (45).

Importantly, for reasons not clearly understood, NL43 Nef-TR was more pathogenic in HIV-1 Tg mice than wild-type NL43 Nef and the pathogenicity of SF2 Nef in Tg mice was evident despite very low levels of expression (46). It is therefore possible that more severe Golgi-localization of active Hck followed by perturbed *N*-glycosylation and trafficking of proteins including Fms account for the high pathogenicity of SF2 Nef in Tg mice.

Despite the importance of Nef PxxP motifs and their binding with Hck proven by HIV-1 Tg mice studies (5, 6), no effective inhibitor against these motifs or Nef-Hck binding has been identified. In this study, we showed for the first time that recently-isolated compound 2c was the effective Nef-Hck binding blocker and proved that skewed localization of active Hck to the Golgi was the direct cause of Fms maturation arrest by using2c. 2c is the simple analog of UCS15A, the first and only non-peptide lead chemical that blocks the binding between proline-rich region and SH3 domain. Thus far, it has been shown that 2c and UCA15A block Sam68-

Fyn binding (34), and that UCS15A blocks AMAP1-cortactin binding and thereby prevents breast cancer metastasis in mouse model (37). By analogy with the Sam68-Fyn binding inhibition (34), the inhibitory effect of 2c on Nef-Hck binding/Nef-induced Hck activation is supposed to be mediated by its interaction with Nef PxxP motifs. Therefore, 2c is quite useful to elucidate essential significance of Nef PxxP motifs and/or Nef-Hck binding, and may provide a new approach for anti-HIV-1 therapy.

In summary, our present study clearly demonstrated that skewed Golgi-localization of active Hck was the direct cause of Fms maturation arrest by Nef. Our findings establish a novel small compound that can inhibit Nef/Hck binding and interactions which can be used a tool in investigating the role of Nef proline rich motif in many aspects in HIV-1 infection.

Our results also establish an intriguing link between the pathogenesis of HIV-1 Nef and the newly-emerging concept that the Golgi-localized Src kinases regulate the Golgi function. The identification of Golgi proteins phosphorylated by the Golgi-localized active Hck will provide novel insights into molecular mechanisms by which Nef functions as an HIV-1 pathogenetic factor through Hck and the Golgi-localized Src kinases regulate the Golgi function.

10. Conclusion

Our study revealed underlying mechanism of Fms maturation arrest by Nef and Hck showing that dual regulation of Hck by Nef (activation and skewed localization of active Hck to the Golgi) was indispensable; furthermore it suggests that long –lasting or dys-regulated Hck activation at the Golgi impairs its basic functions, the glycosylation of proteins.

Our study establishes a novel small compound 2c that can inhibit Nef/Hck binding / interactions which can be used as a tool in investigating the role of
Nef PxxP motif in many aspects of HIV-1 infection.

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