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# HIV-1 Nef-GCC185 interaction regulates assembly of cellular protein complexes at TGN targeting MHC-I downregulation

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## ABSTRACT

*Aim:* HIV-1 Nef downregulates surface MHC-I to protect the infected cells from CTLs-mediated killing. Although MHC-I downregulation has been extensively studied, the Nef-dependent assembly of the multi-protein complex and subsequent pathways activation has not yet been well explored. The present study is aimed for the identification of Nef-mediated sequential recruitment of cellular proteins that constitute the functional multi-protein complex, required for the downregulation of MHC-I.
*Main methods:* Different Cellular protein complexes were identified by co-immunoprecipitation in Nef or NefE4A mutant-expressing Jurkat T, and THP-1 cells followed by exposure to Nef-specific peptides 24 h post infection. The MHC-I downregulation was analyzed by confocal microscopy and flow cytometry.
*Key findings:* We found the association of Nef with PACS-2, GCC185, PI3K, AP-1, SFK, and MHC-I proteins that probably constitute a functional multi-protein complex. Furthermore, the immunoprecipitations with PACS-2 and GCC185 in the presence or absence of Nef, Nef E4A mutant and Nef with CP-inhibitor divide the functional complex of Nef into Nef-dependent (AP-1 and PI3K) and GCC185-dependent complex (MHC-I and SFK). The molecular mechanisms for activation of cellular pathways have been deciphered on the basis of these interactions that are brought in close proximity through Nef-GCC185 interaction. Knockdown of GCC185 using siRNA in Jurkat T cells showed a direct relationship between the assembly of functional multi-protein complex and MHC-I accumulation at GCC185.
*Significance:* Overall, our study elucidates that GCC185 is a focal point for the assembly of the Nef-mediated multi-protein complex at TGN.

## 1. Introduction

Human immunodeficiency virus-1 Nef (HIV-1 Negative Regulatory Factor) a 27 kDa protein, plays a fundamental role in the virus replication, pathogenesis, and disease progression. It has been reported previously that deletion of *nef* significantly reduces disease progression in humans [1–4]. Nef is characterized as an N-terminal myristoylated multifunctional protein, which regulates surface expression of multiple molecules, T-cell activation and maturation, viral infectivity and apoptotic machinery [5]. Nef-mediated CD4 downregulation avoids superinfection to facilitate the release of viral progeny [6,7]. To evade host CD8+ immune surveillance system, Nef modulates major histocompatibility complex-I (MHC-I) and Fas ligand (FasL) surface expression on infected cells [8–10]. Recently, studies have clarified that Nef obstructs Serine incorporator 5 (SERINC5) recruitment into newly formed virions results in increased virions infectivity [11–13].

One of the mysteries of HIV-1 infection is that the Nef interferes with surface expression of several critical immunoreceptors which ultimately contributes to the remodelling of the T-cell surface [14,15]. Previous evidence suggest that Nef exerts a distinct mechanism to disrupt the surface expression of MHC-I and CD4 [16–18]. Nef-directed MHC-I downregulation requires four conserved sites of HIV-1 Nef; Met at position 20 (M20), the acidic cluster$^{62}$EEEE$^{65}$, and the PXXP$^{75}$SH3-domain binding site (present in the N-terminal flexible region) and P78 [19–21] while the diacidic motif of Nef is needed for CD4 [17,18].

HIV-1 Nef action on MHC-I downregulation relies on two different

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*Abbreviations:* HIV-1, human immunodeficiency virus-I; Nef, Negative Regulatory Factor; GCC185, GRIP and coiled-coil domain-containing protein 185; *SFK*, *Src family kinase*; MHC-I, major histocompatibility complex-I; TGN, trans-Golgi network; PACS-2, phosphofurin acidic cluster sorting protein-2; AP-1, adaptor protein-1; PI3K, phosphoinositide 3-kinase; Co-IP, co-immunoprecipitation

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models: signaling mode and stoichiometric mode. During the early phase of HIV-I infection [20], MHC-I downregulated by the signaling mode in which Nef-host protein interaction subverts endocytic machinery through multikinase assembly. Evidence showed that HIV-1 Nef (EEEE65) interacts with both the phosphofurin acidic cluster sorting proteins-PACS-1 and PACS-2 [16,22–27] then delivered to the paranuclear region. At the trans-Golgi network (TGN) Nef PXXP75 region binds to the SH3 domain of TGN localized SFK (Src-Family kinase) which required for disease progression. This association promotes a multikinase cascade SFK-ZAP70/Syk-PI3K that accelerates Adenosine ribosylation factor 6 (ARF-6)-mediated cell surface MHC-I downregulation [16,28,29].

Besides that, Nef influences the dynamics of intracellular trafficking pathways through direct interaction with adaptin complexes: AP-1, AP-2 and AP-3 which are localized to TGN, cell surface and endosome respectively [30]. Knockdown of AP-1 was reported to impair Nef-mediated MHC-I downregulation. This interaction further assembles a ternary complex (MHC-1/Nef/AP-1) which is responsible to transport MHC-I for lysosomal degradation [31]. This pathway is defined as a *stoichiometric* mode of MHC-I downregulation and takes place at the later stage of infection [20]. Moreover, there is evidence that the inhibition of signaling mode by pharmacological small molecule inhibitor has shown to obstruct the stoichiometric mode of MHC-I downregulation [20]. Structural and dynamic measurements of HIV-1 Nef with vesicle adaptors showed that differences in cargo dynamics allows the same adaptor system to target cargos to different fates. MHC-I promotes formation of open trimers that directs MHC-I to lysosomes for degradation [32].

Hence, previous reports suggest that Nef mediates MHC-I downregulation from cell surface through two independent mechanisms [20,31]. In signaling mode, the Nef transports MHC-I from cell surface to TGN whereas in the stoichiometric mode the association of ternary complex occurs at TGN and transport MHC-I to lysosomes for degradation [16,31]. In both the pathways, the association of complexes has been reported at TGN but how the complex localized at TGN was not known [20,31]. Previously, we had reported that Nef physically interacts with GCC185, a TGN protein with its acidic domain. Nef protein disrupts GCC185–Rab9-mediated vesicular trafficking that is involved in recycling of mannose 6-phosphate receptor (M6PR) from late endosome to TGN [33]. This finding suggested that Nef localization at TGN was mediated by interaction with GCC185 and affects vesicular transport pathways. In the current study, we identified that Nef interaction with GCC185 is responsible for assembly of multi-protein complex and the sequential interaction of associated proteins, mediated by close proximity which is likely to activate both the signaling and stoichiometric pathways. The functional role of GCC185 was shown in knockdown studies in Nef-expressing Jurkat T cells where downregulation of GCC185 affected Nef-dependent MHC-I reprogramming, suggesting that GCC185 is the central point at TGN for recruiting complex that leads to regulate both pathways of MHC-I downregulation.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Antibodies for GCC185 (ab128173), PACS-2 (ab129402), HIV-1 Nef (ab42355), AP1 (ab230273), and HRP conjugated goat anti rabbit (ab6721) and anti-mouse (ab6789) were purchased from Abcam (USA). Antibodies SFK (2109S), PI3K (4292) and MHC-I (sc-32235), Golgin-245 (sc-514775) and beta-Actin (sc-47778) were purchased from CST and Santa Cruz biotechnology respectively. RPMI 1640, antibiotics, Trizma Base, SDS, Glycine and agarose were purchased from Sigma, USA. Protein A-Agarose beads (sc-2001) for IP were purchased from Santa Cruz Biotechnology. GCC185 siRNA purchased from IDT (Promega). N′-EEEE-C′ peptide used in this study as a competitive inhibitor against the acidic region of HIV-1 Nef.

### 2.2. Cell culture and transfection

THP-1 and Jurkat T cell lines used in our study were obtained from the Central Cell Culture Facility of our Institute (CSIR-CDRI, Lucknow). Jurkat T and THP-1 cells were grown in RPMI-1640 medium containing 20 mM L-Glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% Fetal Bovine Serum (FBS). Cells were grown in the humid $CO_2$ incubator at 37 °C in an atmosphere with 5% $CO_2$. Cell concentration was maintained between $10^5$ and $10^6$ cells/ml and regular monitoring were done. Jurkat T and THP-1 cells were transfected by electroporation. After 24 h of transfection, Nef-expressing cells were treated with 10 μM concertation of peptide.

### 2.3. Peptide synthesis

Peptides were synthesized manually via the solid phase method on Rink amide MBHA resin using standard Fmoc chemistry. Peptide cleavage from resin and its precipitation was finished utilizing standard procedures. Purification of the peptide was done by reverse phase HPLC on an analytical Waters Symmetry C18 column (5.0 μm, 300 Å, 4.6 mm × 250 mm) using a linear gradient of 20–80% acetonitrile for 40 min with a flow rate of 1.8 mL/min. The further purity of peptide was determined by reverse phase analytical chromatography to be ≥95. Experimental molecular mass of the peptides was evaluated by MALDI-TOF analysis which was very close to that calculated mass.

### 2.4. Immunoprecipitation

Jurkat T cells and THP1 cells were cultured and transfected with wild-type HIV-1 Nef and Nef E4A mutant. After 48 h of transfection, cells were harvested and lysed in I.P. buffer [20 mM Tris base pH 8, 137 mM NaCl, 10% Glycerol, 1–2% TritonX-100 and 2 mM EDTA]. The precleared solution of this lysate was obtained through centrifugation at 13000 rpm, 4 °C for 30 min. Protein solution quantified using Bradford method, equal concentration from all samples taken and incubated with the desired antibody (GCC185, Nef, and PACS-2) for overnight at 4 °C with gentle shaking. Negative control was immunoprecipitated with Rabbit IgG or Mouse IgG only. Further, this antibody-protein solution mixed with protein G-Agarose beads (Santa Cruz Biotechnology) for 4–5 h at 4 °C, washed 2–3 times with IP buffer and the desired co-immunoprecipitated proteins were detected by western blotting. All the mentioned proteins in an IP blot were analyzed from the same transfer (after stripping).

### 2.5. Western blotting

THP-1 and Jurkat T cells were harvested after 48 h of transfection (Nef and pYFP-N1 vector) and dissolved in 1× passive lysis buffer (Promega) containing phosphatase and protease inhibitor (10 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and 10 μM sodium orthovanadate,10 mg/ml leupeptin). Cells were lysed by sonication at 20 amplitude giving 4 pulses of 5 s on and 5 s off and centrifuged at 13,000rpmat 4 °C for 30 min to remove cell debris. Protein quantification was done using Bradford method, subjected to 8% SDS-PAGE and transferred to PVDF (Millipore, USA) membranes. Thereafter membrane blocked with 5% BSA (in 1× PBS plus 0.01% Tween-20) for 2 h at room temperature, incubated with the desired primary antibody with recommended dilution by respective company (1% BSA in 0.01%PBST) overnight at 4 °C with gentle shaking. The blot was washed 4–5 times with 0.05% PBST buffer (pH-7.4) and after that incubated with appropriate HRP (horseradish peroxidase) conjugated secondary antibody for 2 h at room temperature and again washed 5–6 times with PBST buffer. Peroxidase activity was analyzed using the ECL chemiluminescence substrate system (USA).

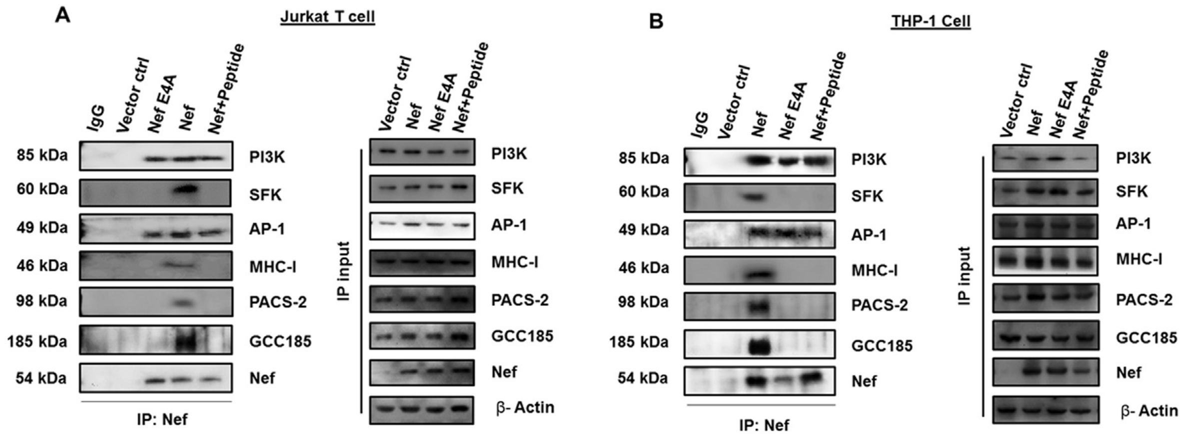

**Fig. 1.** Nef acidic domain is essential for the assembly of functional multi-protein complex.
Jurkat T (A) and THP1 (B) cells were transfected with vector control or wild type Nef or Nef E4A mutant and HIV-I Nef-expressing cells were treated with CP-inhibitor after 24 h of transfection. Nef was immunoprecipitated and proteins that co-precipitated with Nef were detected by western blotting as indicated. Input of all respective proteins in Jurkat T cells (A) and THP-1 (B) cells were represented. β-Actin used as a loading control. Results are typical of two independent experiments.

### 2.6. Confocal microscopy analysis

Jurkat T cells were transfected with vector control with siRNA control and with GCC185 siRNA, Nef with siRNA control and with GCC185 siRNA or mutant Nef E4A. After 48 h of post-transfection 20,000 cells were seeded on poly-L-lysine coated glass slides. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 3% BSA. For immunofluorescence assay, cells were incubated with primary antibodies against GCC185, MHC-I and Nef at recommended dilution in PBS with 3% BSA for 3–5 h with gentle shaking. After washing 3 times with PBS, cells were stained by respective Alexa-Fluor-647 or Texas red-conjugated secondary antibodies at 1:500 dilution for 2 h at RT. Cells were washed with PBS for 5 times, and then incubated with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min for nuclear staining. The images were taken by Zeiss confocal microscope using the 65× objective lens with 4× and 2× zoom and analyzed by Zeiss LSM Image viewer software.

### 2.7. Flow cytometry

Jurkat T cells were transfected with vector control with siRNA control and with GCC185 siRNA, Nef with siRNA control and with GCC185 siRNA or mutant Nef E4A. After 48 h of transfection of cells were harvested in PBS by centrifugation at 1500 rpm for 5 mins and washed twice. Fifty thousand cells were incubated with anti-human MHC I (W6/32) phycoerethrin conjugated antibody for 45 min at RT. The MHC class I counted on the cell surface by flow cytometer (BD Biosciences). The plasmid encoding pYFP-N1 was included to normalize for transfection efficiency. Dead cells were excluded by a gate in FSCySSC. Events corresponding to at least 5000 live cells YFP were accumulated per sample. The values plotted relate to the amount of MHC-I retained at the cell surface in each condition; therefore, each value is inversely proportional to Nef activity.

### 2.8. Statistical analysis

Numerical data were tested for statistical significance using paired Student's *t*-test (two-tailed). Differences were considered significant when $p \leq 0.05$(*), and very significant when $p \leq 0.01$(**). All experiments were performed independently at least two times. All data were analyzed by graph pad prism5.

## 3. Results

### 3.1. HIV-1 Nef acidic domain is crucial for the association of the functional multi-protein complex

Nef regulates diverse cellular pathways through the association of multi-protein complexes, among them one important is MHC-I downregulation which leads to promote immune evasion of the virus [34,35]. The molecular mechanism to form Nef-mediated multi-protein complex targeting MHC-I downregulation is not fully explored yet [16,20,31]. In the current study, we aimed to decipher the Nef-dependent recruitment of specific proteins in the multi-protein complex which probably participates in the MHC-I downregulation. The THP-1 and Jurkat T cells were transfected with vector control (absence of Nef) or Nef or Nef E4A mutant. Nef-expressing cells were treated with 10 μM of competitive peptide inhibitor (CP-Inhibitor) against the acidic domain of Nef after 24 h of transfection and the protein lysates of cells were prepared 48 h post transfection. The Nef antibody was used to pull down the proteins and the eluted proteins were immunoblotted with representative antibodies of desired proteins (proteins having a role in MHC-I downregulation and GCC185) [16,31]. In our results, we found that GCC185, PACS-2, AP-1 (μ1 subunit), PI3K, SFK, and MHC-I proteins were co-precipitated with Nef whereas AP-1 and PI3K proteins were identified with mutated Nef ($EEEE_{65} \rightarrow AAAA_{65}$) but GCC185, PACS-2, SFK and MHC-I could not be identified (Fig. 1A, B). Addition of competitive peptides inhibitor showed similar results as mutated Nef. These results are consistent with previous findings that the acidic domain of Nef is essential for interaction with GCC185 and PACS-2 proteins [9,33]. Moreover, identification of AP-1 and PI3K proteins with Nef E4A mutant or Nef with CP-inhibitor indicate that other domains of Nef are responsible to maintain interaction with AP-1 and PI3K.It has been reported that both SFK and MHC-I proteins interact with other than the acidic domain of Nef (SFK with PXXP domain) [16,36]. Surprisingly, we did not identify the SFK and MHC-I proteins (like GCC185 and PACS-2) in both Nef E4A mutant and CP-inhibitor condition, which suggest that these proteins may not form a direct interaction with Nef or require any other intermediate protein such as GCC185 and PACS-2.

Therefore, HIV-1Nef acidic region is essential to induce the association of the functional multi-protein complex which involved in the activation of signaling and stoichiometric pathways targeting MHC-I downregulation, prompting us to investigate the sequential recruitment of proteins in the complex and functional consequence of the functional multi-protein complex [16,20,31].

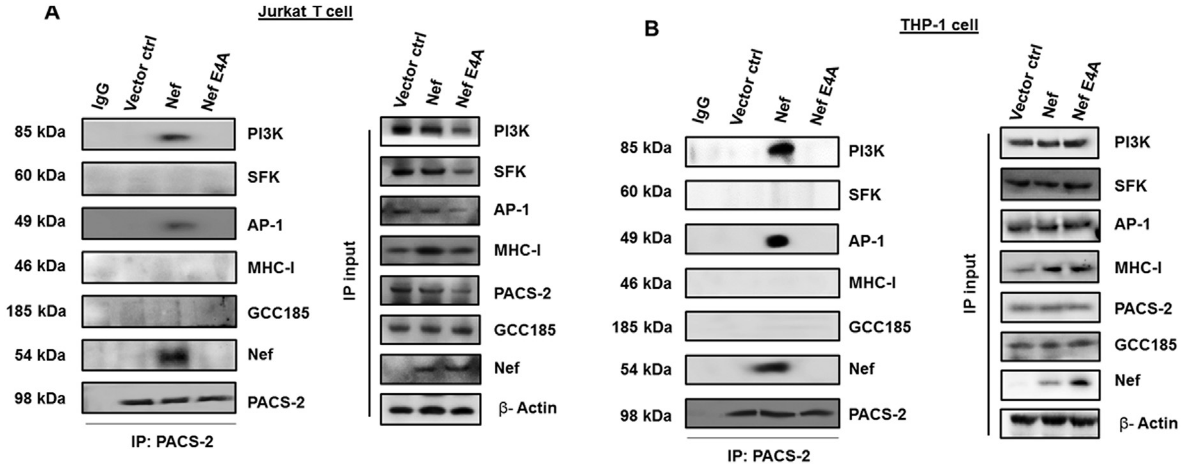

**Fig. 2.** Nef-PACS-2 interaction comprised only Nef-dependent complex.
Jurkat T (A) and THP1 (B) cells were transfected with vector control or wild type Nef, Nef E4A mutant. PACS-2 was immunoprecipitated and proteins that co-precipitated with PACS-2 were detected by western blotting as indicated. Input of all respective proteins in Jurkat T cells (A) and THP-1 (B) cells were represented. β-Actin used as a loading control. Results are typical of two independent experiments.

### *3.2. Identification of PACS-2-associated proteins in the presence of Nef*

Previous findings have shown thatPACS-2 interacts with the acidic region of Nef in the cells and involved in the downregulation of MHC-I from the cell surface, but the role of PACS2-in the Nef–mediated formation of the functional multi-protein complex is remains unexplored. To investigate whether Nef-PACS-2 interaction comprised all the essential proteins of afunctional multi-protein complex, we performed co-immunoprecipitation using PACS-2 antibody in the THP-1 and Jurkat T cells expressing vector control, Nef and mutant Nef. The co-precipitated proteins were eluted, subjected to SDS-PAGE and further examined by western blotting. As shown in the Fig. 2A, AP-1, PI3K and Nef proteins were co-precipitated with PACS-2 in the Nef-expressing cells whereas these proteins (Nef, AP-1 and PI3K) were not identified in the presence of Nef E4A mutant and absence of Nef, suggesting that PACS-2 interacts with the acidic domain of Nef, as also described previously [9,16]. In contrast, the SFK, MHC-I and GCC185 cellular proteins, which were observed in a Nef-associated functional protein complex (Fig. 1A, B), were not detected in the PACS-2 complex in the presence of Nef. Similar results were observed in both THP-1 and Jurkat T cells (Fig. 2A, B), indicating that PACS-2 is known as a sorting protein which might participate in the transport of Nef complex to TGN for the assembly of proteins in the functional multi-protein complex involved in the rerouting of MHC-I from the cell surface [16,20].

### *3.3. GCC185, a site of Nef-associated functional complex assembly regulating MHC-I downregulation*

Earlier evidence suggests that the paranuclear localization of Nef is crucial for signaling mode of MHC-I downregulation and also downregulated MHC-I is accumulated on TGN which is targeted to lysosomes by stoichiometric mode. However, the association of Nef and MHC-I with TGN is not well defined. We have previously shown that Nef acidic domain interacts with GCC185 which is a TGN specific protein and disrupt Rab9 mediated recycling of M6PR to TGN [33]. In the present study, we validated that GCC185 is a TGN protein by co-localization of GCC185 withGolgin-245, a knownmarker of TGN (Fig. 3A). Next, we determined whether the Nef-GCC185 interaction participates in the assembly of a Nef-dependent functional multi-protein complex or not? THP-1 and Jurkat T cells were transfected with vector control, Nef (presence and absence of CP-inhibitor) and Nef E4A mutant. A co-immunoprecipitation was performed with a GCC185 antibody, and the desired proteins were analyzed by western blotting with representative antibodies. Our results showed that GCC185 co-precipitated with SFK, AP-1, MHC-I, PI3K and Nef proteins in Nef-expressing cells. Presence of mutant Nef and addition of CP-inhibitor resulted in an identification of SFK and MHC-I with GCC185, suggesting that GCC185 interacts with SFK and MHC-I and are seen as independent complex at TGN. Interestingly, in all the cases the PACS-2 was not found with GCC185 indicating that both proteins did not interact with each other (Fig. 3B, C).

These results showed that there were two independent protein complexes, one is GCC185-SFK-MHC-I (Fig. 3B, C) and the second is Nef-AP-1-PI3K (Figs. 1A, 2A). Nef-GCC185 interaction could bring these two independent protein complexes in the close proximity to forms a functional multi-protein complex.

### *3.4. Knocked down of GCC185 reduces Nef-mediated MHC-I downregulation*

Further, we wanted to know whether the Nef-dependent functional multiprotein complex mediated MHC-I downregulation [20,31]was depended on GCC185 or not? Jurkat T cells were transfected with different concentrations of GCC185 specific siRNA or control siRNA. After 48 h, first we confirmed the expression of GCC185, and it was markedly reduced (70–80% compared to control siRNA) at 300 nM siRNA concentration (Fig. 4A).

Thereafter, we examined the importance of GCC185 in the formation of Nef-mediated functional multi-protein complex regulating MHC-I downregulation by confocal based immunofluorescence and flow cytometry studies with 300 nM GCC185 specific siRNA. As shown in the Fig. 4B (right panel), we observed a strong co-localization of Nef with GCC185 at TGN. This interaction increased MHC-I downregulation, as it was evident by observing MHC-I co-localization with GCC185 (left panel). However, in the presence of Nef E4A mutant, we were unable to detect co-localization of Nef with GCC185 that possibly resulted in the reduction of MHC-I downregulation as seen substantially reduced co-localization of MHC-I with GCC185 independently. Unexpectedly, in the presence of Nef E4A mutant and absence of Nef (vector control), we observed a similar co-localization pattern of MHC-I with GCC185 (Fig. 4B left panel). This co-localization might happen due to the recruitment of newly synthesized MHC-I on GCC185. These results showed that Nef-GCC185 association was essential for MHC-I re-routing from the surface to the GCC185 on TGN. Interestingly, in GCC185 knocked down cells, Nef-GCC185 interaction was decreased significantly with the reduction of GCC185 expression (Fig. 4B left panel). GCC185 knockdown proportionally hindered the co-localization of

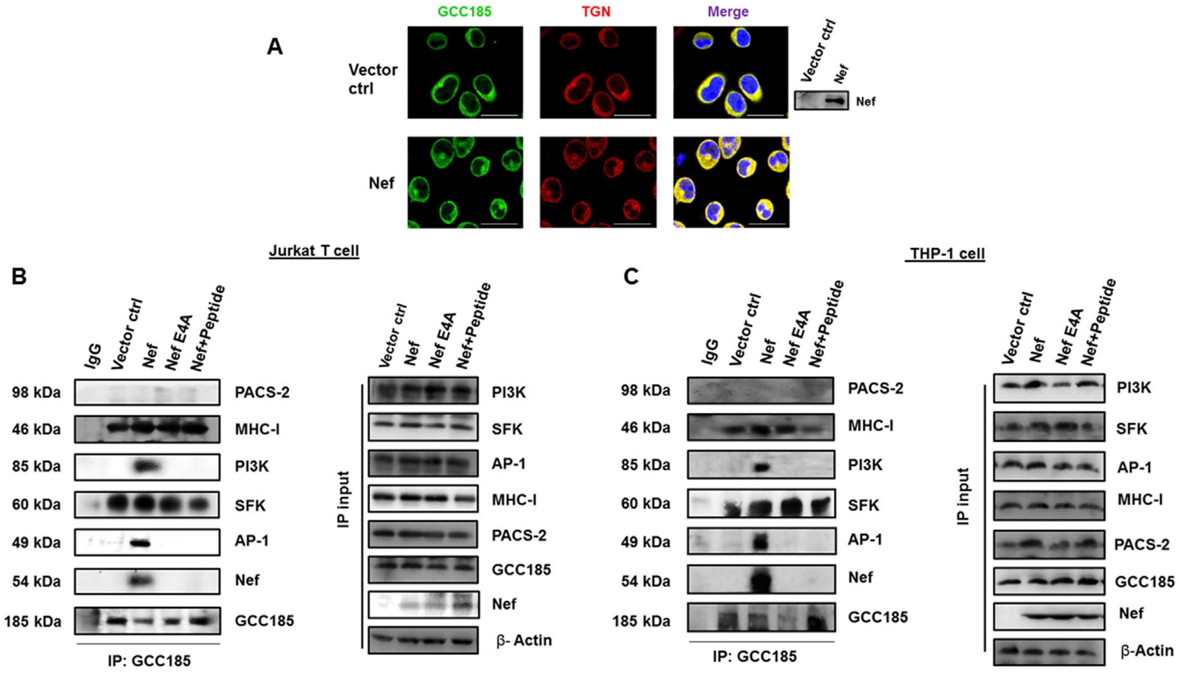

**Fig. 3.** GCC185 interacts with SFK and MHC-I in the presence and absence of Nef.

A. Jurkat T cell was transfected with vector control or wild type Nef. 48 h of post-transfection cells are harvested, fixed and stained with respective primary antibodies followed by conjugated secondary antibodies. The co-localization GCC185 (Green) and Golgin-245 (Red) in the cells (1 μm section) with their nucleus stained with DAPI (Blue) of fluorescent signals were visualized by confocal microscopy at 40 X and 63 X magnification.

Jurkat T (B) and THP1 (C) cells were transfected with vector control or wild type Nef, Nef E4A mutant and HIV-I Nef-expressing cells were treated with CP-inhibitor after 24 h of transfection. GCC185 was immunoprecipitated and proteins that co-precipitated with GCC185 were detected by western blotting as indicated. Input of all respective proteins in Jurkat T cells (A) and THP-1 (B) cells were represented. β-Actin used as a loading control. Results are typical of two independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

MHC-I with GCC185 (right panel). PACS-2 expression remained unaffected after GCC185 knockdown (Fig. 4C), confirming that PACS-2 is not involved in the formation of Nef-mediated functional multi-protein complex.

The role of GCCC185 in Nef-dependent MHC-I downregulation was further validated by the quantification of surface MHC-I by flow cytometry in the presence or absence of Nef under the GCC185 knocked down background. Nef-expressing cells showed a strong reduction in surface MHC-I level than the vector control. In GCC185 knocked down cells, the MHC-I downregulation was significantly inhibited compared to Nef-expressing cells. In combination with Co-IP evidence these data are suggesting that upon knockdown of GCC185, Nef was unable to form a functional multi-protein complex which leads to perturb MHC-I downregulation. Similarly, in the presence of Nef E4A mutant and Nef treated with CP-inhibitor, the level of MHC-I on the cell surface was found to be increased in comparison with Nef (Fig. 4D).

Overall, our results demonstrate that Nef-GCC185 interaction is essential for the formation of a multi-protein complex which activates both signaling and stoichiometric mode of MHC-I downregulation without involving PACS-2 (Fig. 4C).

## 4. Discussion

Downregulation of cell surface protein MHC-1 during HIV-1 infection is primarily accomplished by accessory protein Nef [15,37] which allows the virus to evade host immune surveillance system. Interaction of Nef with PACS-2 and AP-1 can activate both the signaling and stoichiometric pathways, that finally transport the MHC-I from the cell surface to TGN, and TGN to lysosomes, respectively [16,20,31]. The fact that both the signaling and stoichiometric pathways converge at TGN by various protein complexes, it drew our attention to understand the physical and functional interactions between Nef and TGN-specific GCC185 protein in the sorting and downregulation of MHC-I protein. Evidence from our experiments demonstrated that both Nef and GCC185 proteins form independent complexes and upon interaction constitute a functional multiprotein complex (Figs. 1, 2 and 3). The disruption of Nef-GCC185 interaction either by RNAi or CP-inhibitor against the acidic domain of Nef or by the use of Nef E4A mutant, reversed the Nef-dependent MHC-I downregulation (Fig. 4).

Co-immunoprecipitation data showed that Nef is associated with AP-1, PACS-2, SFK, MHC-I, PI3K, and GCC185 that indicates the formation of a functional multi-protein complex (Fig. 1). Previous studies have revealed that Nef downregulates MHC-I by two different pathways namely signaling and stoichiometric pathways. During signaling pathway, acidic domain of HIV-1 Nef interacts with PACS-2 resulting in its translocation to the paranuclear region where it binds to TGN localized SFK and assemble a multikinase complex which further downregulates MHC-I from the cell surface [16]. In stoichiometric pathway, the Nef–MHC-I interaction creates a binding site for AP-1 and this ternary complex targets newly synthesized MHC-I from TGN to lysosomes [31]. Overall, PI3K, SFK, MHC-I, AP-1, and PACS-2 proteins of the functional multi-protein complex are previously known while the presence of GCC185 has not yet been reported earlier in this multi-protein complex responsible for Nef-dependent MHC-I downregulation [9,16,31]. We and others have found that GCC185, a C-terminal GRIP domain-containing protein, bound to a region of TGN (Fig. 3A) that forms a highly tubular structure [33,38–40]. Previously, we have identified that Nef interacts with GCC185 and impairs GCC185-Rab9 interaction, which disrupts M6PR recycling from late endosomes to TGN [33].

Furthermore, in the presence of Nef E/A mutant, we have found only AP-1 and PI3K while SFK, MHC-I, PACS-2 and GCC185 were absent. This finding clearly demonstrates that AP-1 and PI3K possibly interact with Nef at a region other than acidic domain and named as

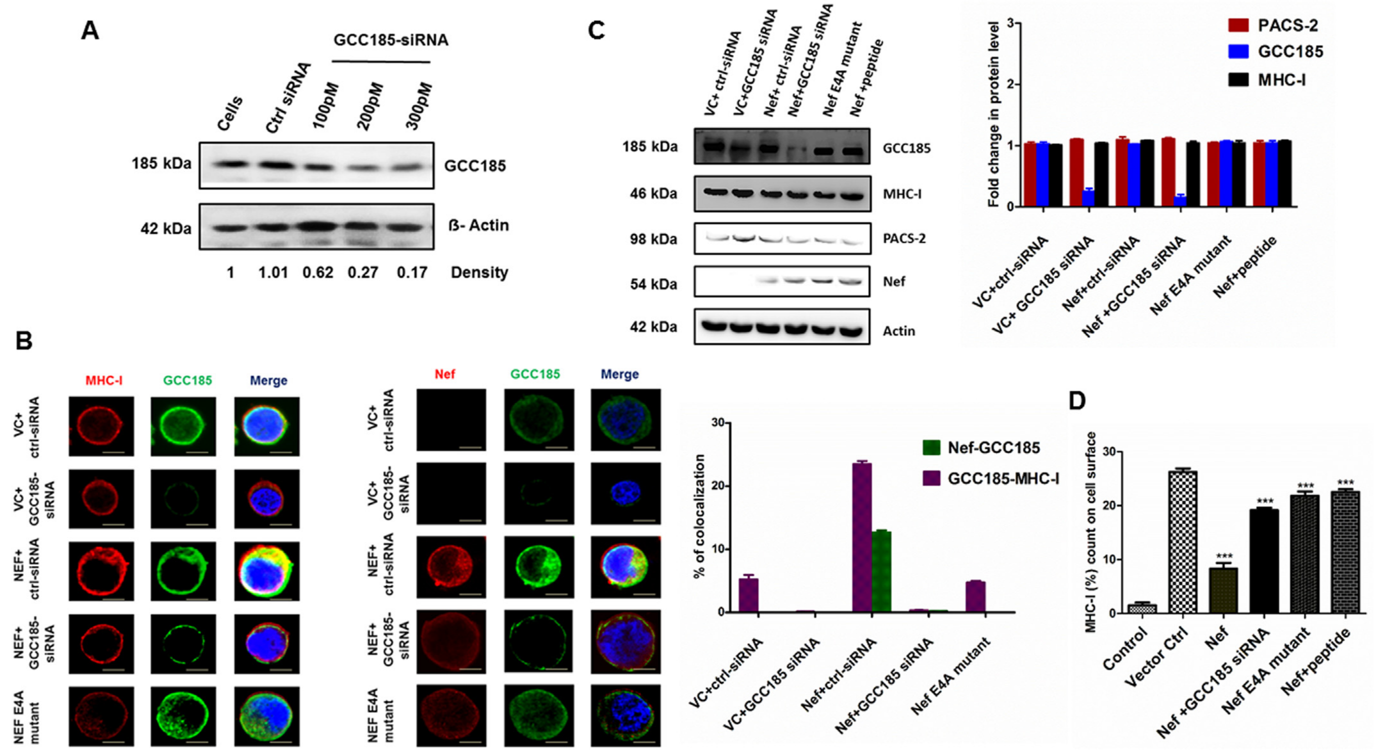

**Fig. 4.** Knocked down of GCC185 reduces Nef-mediated MHC-I downregulation.

A. Western blot of extracts from Jurkat T cells transfected with a control siRNA or different concentration of siRNA specific for GCC185. Protein band intensity was quantified using densitometry and normalized against β-actin. 300 nM siRNA knocked down the expression of GCC185 by 70–80% compared to control. Data are presented as the mean ± SD of two independent experiment.

B. Jurkat T cells were transfected with vector control with siRNA control or with GCC185 siRNA, Nef with siRNA control or with GCC185 siRNA and mutant Nef E4A. 48 h of post-transfection cells are harvested, fixed and stained with respective primary antibodies followed by conjugated secondary antibodies. In left panel, co-localization GCC185 (Green) and MHC-I (Red) in the cells (1 μm section) with their nucleus stained with DAPI (Blue) of fluorescent signals were visualized by confocal microscopy at 40 X and 63 X magnification. In right panel, GCC185 (Green) and Nef (Red) co-localization fluorescent signals in the cells (1 μm section) with their nucleus stained with DAPI (Blue) were visualized by confocal microscopy at 40 X and 63 X magnification. Error bars were calculated by quantification of at least 30 cells between 3 independent experiments (*Indicates p-value $< 0.05$).

C. Jurkat T cells were transfected with vector control with siRNA control or with GCC185 siRNA, Nef with siRNA control or with GCC185 siRNA and mutant Nef E4A. HIV-I Nef-expressing cells were treated with CP-inhibitor after 24 h of transfection. The level of PACS-2 and MHC-I were analyzed by western blotting after 48 h of transfection. Protein band intensity was quantified using densitometry and normalized against β-actin. Data are presented as the mean ± SD of two independent experiment.

D. Jurkat T cells were transfected with vector control with siRNA control or with GCC185 siRNA, Nef with siRNA control or with GCC185 siRNA or mutant Nef E4A. HIV-I Nef-expressing cells were treated with CI-peptide after 24 h of transfection. At 48 h posttansfection eYFP$^+$ cells were analyzed for cell surface MHC-I by flow cytometry (mAb W6/32). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Nef-dependent complex [39]. Our results support the earlier studies (18, 32) that mutation at the acidic domain of Nef disrupts its interaction with both GCC185 and PACS-2 proteins. Therefore, both proteins PACS-2 and GCC185 interact with same domain of Nef but in two distinct cellular compartments. Though, direct interactions of Nef with MHC-I [31,41], and Nef with SFK at TGN [16] have already been shown, we could not find these interactions with Nef-E4A mutant. The similar type of scenario was seen while using the CP-inhibitor against the acidic region of Nef, indicating that SFK and MHC-I might interact indirectly with Nef through GCC185 or PACS-2 partners (Fig. 1A, B).

Upon PACS-2 immunoprecipitation in Nef-expressing cells, we have found that Nef, PI3K, and AP-1 proteins were appeared with PACS-2 but could not find in the absence of Nef or Nef E4A mutant, which clearly indicates that Nef acts as a connector between AP-1, PI3K, and PACS-2 protein (Fig. 1A, B). However, MHC-I, SFK, and GCC185 were not detected with PACS-2 in presence or absence of Nef, demonstrating that Nef-PACS-2 interaction does not comprise all the essential components of functional multi-protein complex targeting MHC-I downregulation (Fig. 2A, B). Based on these observations we demonstrated that PACS-2 protein is only involved with the transport of Nef to TGN. Consistent with this finding, earlier studies reported that Nef mutants, defective in PACS-2 binding mislocalized in early and late endosomal compartments in the cell [9].

In order to confirm the possibility of the presence of GCC185 in Nef-associated multiprotein complex formation, co-immunoprecipitation using GCC185 antibody in Nef-expressing cells showed the presence of proteins like MHC-I, SFK, AP-1, PI3K and Nef. Interestingly, in the absence of Nef or in the presence of mutant Nef or Nef with CP-inhibitor, we found that only MHC-I and SFK proteins were associated with GCC185, suggesting that both of these proteins interact with GCC185 (Fig. 3B, C). Thus we have shown, for the first time, that GCC185 interacts with SFK and MHC-I proteins and named as a GCC185-dependent complex at TGN. Based on these results, we demonstrated that Nef-dependent complex (AP-1 and PI3K) and GCC185-dependent complex (SFK-MHC-I) are brought together by Nef-GCC185 interaction at TGN and constitute a functional multi-protein complex which activates both signaling and stoichiometric pathway of MHC-I downregulation [20]. In this complex PACS-2 protein was not identified, suggesting that PACS-2 is a cytosolic sorting protein which transports the Nef to GCC185 at TGN (Fig. 3B, C).

Further experiments highlight the importance of GCC185 in the formation of a functional Nef-mediated multi-protein complex involved in the MHC-I downregulation which was studied through immunofluorescence and flow cytometry upon knockdown of GCC185 in the cells (Fig. 4). We observed that in GCC185 knockdown cells, the association of Nef with GCC185 and also the co-localization of MHC-I with GCC185 reduced as compared to that of Nef-expressing cells (Fig. 4B left and right panel). Furthermore, flow cytometry analysis confirms that Nef interaction with GCC185 is essential for the MHC-I downregulation. The MHC-I surface expression was not reduced when the GCC185 level was down in Nef-expressing cells and remained almost similar to Nef with CI-peptide and Nef-E4A mutant condition. (Fig. 4D). Therefore, these data clearly demonstrate the functional significance of GCC185 in a Nef-dependent MHC-I downregulation, and also corroborated our immunoprecipitation-based complex association.

Overall, our results suggested that all the proteins namely SFK, AP-I, MHC-I, and PI3K are in close proximity due to the Nef-GCC185 interaction (Figs. 1, 3) which further clarified that how these proteins form an essential functional multiprotein complex for the activation of further signaling cascades. The possible cascade may initiate with the interaction of Nef with PACS-2 targeting it to the paranuclear region of TGN, thereby facilitating $\text{Nef}_{\text{EEEE65}}$-GCC185 binding. This interaction at TGN brings GCC185 bound SFK to close proximity with Nef resulting in Nef-SFK binding (mainly with PXXP domain of Nef) and leading to its phosphorylation. Activated SFK migrates to the cytosol and forms a multikinase assembly with ZAP70 and PI3K, which trigger ARF6 mediated surface MHC-1 downregulation as well as prevent recycling of MHC-I [9,16,29].

We have found that both the newly synthesized and downregulated MHC-I by signaling pathway are localized with GCCC185 at TGN (Figs. 3, 4) and Nef interaction with GCC185 restricts MHC-I transport to the cell surface and promotes its degradation in lysosomes. We are the first to show that Nef-GCC185 binding brings AP-1 to close proximity of GCC185 bound MHC-I and promote the formation of ternary complex (Nef-MHC-I-AP-1) (Fig. 1). As per Roeth et al. [31] this tertiary complex leads to the recruitment of MHC-I into AP-1 positive clathrin-coated vesicles destined for degradation in the lysosomes [31,36].

The present study provides the first evidence for the GCC185-Nef interaction to recruit cellular proteins in functional multi-protein complexes at TGN is responsible for MHC-I downregulation.

## 5. Conclusion

We identified a Golgi protein GCC185 as a critical component for MHC-I downregulation by HIV-1 Nef. It proposed that the interaction between HIV-1-Nef and GCC185 regulates the assembly of functional protein complex at TGN leading to MHC-I reduction. Overall, we address a strong relationship between signaling and stoichiometric pathways in a GCC185-dependent manner ultimately targeting the MHC-I downregulation at any pathogenic stage.

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## Authors contribution

SK: designed and conducted the experiments, analyzed the data and prepared the manuscript; MK: analyzed the data and prepared the manuscript; RV: Peptide synthesis; JKG: Peptide Synthesis; RKT: Conceptualize the study, designed experiments and analyzed data.

## Conflict of interest

There is no any conflict of interest occurs among authors.

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