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# The ABCA1 domain responsible for interaction with HIV-1 Nef is conformational and not linear

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ABSTRACT

HIV-1 Nef is an accessory protein responsible for inactivation of a number of host cell proteins essential for anti-viral immune responses. In most cases, Nef binds to the target protein and directs it to a degradation pathway. Our previous studies demonstrated that Nef impairs activity of the cellular cholesterol transporter, ABCA1, and that Nef interacts with ABCA1. Mutation of the $^{2226}$DDDHLK motif in the C-terminal cytoplasmic tail of ABCA1 disrupted interaction with Nef. Here, we tested Nef interaction with the ABCA1 C-terminal cytoplasmic fragment using yeast 2-hybrid system assay and co-immunoprecipitation analysis in human cells. Surprisingly, analysis in a yeast 2-hybrid system did not reveal any interaction between Nef and the C-terminal cytoplasmic fragment of ABCA1. Using co-immunoprecipitation from HEK 293T cells expressing these polypeptides, only a very weak interaction could be detected. The $^{2226}$DDDHLK motif in the C-terminal cytoplasmic tail of ABCA1 found previously to be essential for interaction between ABCA1 and Nef is insufficient to bestow strong binding to Nef. Molecular modeling suggested that interaction with Nef may be mediated by a conformational epitope composed of the sequences within the cytoplasmic loop of ABCA1 and the C-terminal cytoplasmic domain. Studies are now underway to characterize this epitope.



## 1. Introduction

Previously, we have demonstrated that HIV-1 protein Nef impairs activity of the main cellular cholesterol transporter, ABCA1, in part by inducing post-transcriptional downregulation [1]. ABCA1 inactivation by Nef likely contributes to low HDL levels and high risk of atherosclerosis documented in HIV-infected individuals [2–4]. Nef is a multifunctional protein responsible for many pathogenic effects of HIV infection. Nef downmodulates CD4, MHC-I, CD28, CXCR4 and several other receptors from the cell surface (reviewed in [5]), thus affecting the functional ability of the immune cells and benefiting the viral replication. The accepted mechanism of this activity is Nef binding to the target receptor with subsequent recruitment of adaptor proteins that mediate transfer of the Nef-receptor complex to the endocytic machinery and degradation pathways [6,7].

ABCA1 is a 12-transmembrane protein with ATP binding sites (Walker motifs) located in a large cytoplasmic central region (amino acids 899–1120 marked here as D1) and the C-terminal cytoplasmic domain (amino acids 1908–2261, marked here as D2) [8]. Our studies demonstrated that Nef binds to ABCA1 [1], and identified an DDDHLK motif in the C-terminal cytoplasmic domain of ABCA1 as essential for the Nef interaction with ABCA1 [9]. Indeed, $^{2226}$DDDHLK → AAAAAA substitution or truncation of the C-terminal 40 aminoacids greatly reduced ABCA1 binding to Nef [9]. Similar leucine-based motifs are contributing to Nef interaction with other receptors downregulated by Nef: mannose receptor [10] and CD4 [11]. Importantly, since all previous studies looked at the functional effect of the interaction motif mutations rather than at Nef binding, it remains unknown whether these leucine-based motifs are the actual binding sites for Nef. We therefore investigated the binding interactions between Nef and the C-terminal cytoplasmic domain of ABCA1 carrying the leucine-based motif: if interaction can be demonstrated, it would indicate that this motif is the linear epitope sufficient for Nef binding.

*Abbreviations:* ABCA1, ATP-binding cassette transporter A1; HIV-1, human immunodeficiency virus type 1; Nef, negative factor; HDL, high density lipoprotein; HEK, human embryonic kidney cells; Hcf1, host cell factor 1.

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## 2. Methods

### 2.1. Yeast 2-hybrid analysis

The interaction between Nef and the D2 domain of ABCA1 was tested in the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech). The Nef gene sequence was acquired from the HIV-1 NL4–3 clone (NCBI GenBank AF324493.2). The human ABCA1 gene sequence corresponds to RefSeq ID: NM_005502. The hydrophilic limits of the second cytoplasmic domain (D2′) of ABCA1 were predicted using PredictProtein Tool (https://www.predictprotein.org/) and TMHMM Server v. 2.0. (http://www.cbs.dtu.dk/services/TMHMM/). It comprises amino acids 1921–2261. Nef and D2′ coding sequences were fused to the DNA-binding domain of the GAL4 transcription factor (encoded in pGBKT7 vector) and the activation domain of GAL4 (encoded in pACT2 vector). pGBKT7 vectors expressing Nef or D2′ were transfected into yeast strain Y187, while pACT2 vectors were introduced into AH109 strain. Insertion of all gene sequences in the correct reading frame was verified by sequencing. Transformed yeasts were plated on selective media (leucine-deficient for yeast transformed with pACT2 constructs and tryptophan-deficient for yeast transformed with pGBKT7 constructs). Expression of fusion proteins was verified by Western blot analysis of yeast lysates. Anti-HA polyclonal antibody (Clontech) was used to detect constructs expressed in AH109 yeast carrying pACT2 which encodes a HA-tagged GAL4 activation domain, and anti-cMyc monoclonal antibody (Clontech) was used to detect constructs expressed in Y187 yeast carrying pGBKT7 which encodes a cMyc-tagged GAL4 DNA-binding domain. Secondary Peroxidase-AffiniPure Rabbit Anti-Mouse IgG antibody (Jackson ImmunoResearch) and ECL reagent (Amersham) were used for detection.

Yeast strains were mated and plated on leucine- and tryptophan-deficient medium for 5 days for mating control. To test the interaction between Nef and D2′, mated strains were replated on medium deficient in leucine, tryptophan and histidine. Interaction was confirmed by yeast growth on this media 7 days after plating the yeast. To prevent background activation of the reporter gene in the absence of the GAL4 activation domain, 2.5 mM of 3-aminotriazol was added to the media. To provide a positive control for interaction, the SV40 large T-antigen in fusion with the GAL4 activation domain in pTD1 vector and murine p53 in fusion with the GAL4 DNA-binding domain in pVA3 vector were used.

### 2.2. Co-immunoprecipitation

Co-immunoprecipitation was used to test the physical interaction of full-length ABCA1 or the fragment comprising the D2 cytoplasmic domain and adjacent transmembrane domain (amino acids 1823–2261) with Nef in human cells. HEK 293T cells were transfected with pcDNA3 expressing Nef (HIV-1 NL4–3) and FLAG-tagged ABCA1, FLAG-D2, or FLAG-Hcf1 as a control; 48 h after transfection, cells were washed with ice cold PBS and lysed in NP-40 Lysis Buffer (1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM Tris–HCl, pH 7.0, protease inhibitor cocktail) on ice for 30 min. Lysates were centrifuged to remove debris (10 min, 13,000 × *g*, 4 °C), the supernatants were transferred to fresh 1.5 mL Eppendorf tubes, and FLAG-tagged proteins were precipitated with M2 anti-FLAG affinity gel (Sigma) overnight at 4 °C with rotation. Murine IgG agarose gel was used in an isotype control precipitation. Precipitates were washed three times with TBS (50 mM Tris–HCl, pH 7.0, 150 mM NaCl) and FLAG-ABCA1 complexes were eluted with FLAG peptide (Sigma) (0.5 mg/ml, 1 h, 4 °C). Supernatants were separated on SDS–PAGE gels for immunoblotting with AE6 anti-Nef monoclonal antibody (NIH AIDS Research and Reference Program, cat. #1123).

### 2.3. Molecular modeling

The following experimental and modeled three-dimensional structures have been used to model Nef-ABCA1 interaction. Modbase [12] model covering residues 899–1120 was used to represent the cytoplasmic loop domain, designated as domain 1 (D1), and the Modbase model covering residues 1823–2261 was used for the C-terminal cytoplasmic structural domain, D2. The PDB [13] crystallographic structure of the HIV-1 protein Nef core domain (1AVV) has been used in the model. Initially the following conditions have been applied: the positions of D1 and D2 relative to the cell membrane and to each other were selected so as to ensure connection to the ABCA1 transmembrane domains, interaction D1−D2, and a possibility of interaction of both D1 and D2 with Nef. Docking has been performed to construct the final model, utilizing the GRAMM-X server [14]. To incorporate the known participation of $^{2226}$DDDHLK in the interaction domain [9] in the docking runs, the ABCA1 residues 2226–2231 have been used as the predefined potential interaction interface.

## 3. Results and discussion

To test whether Nef binds directly to the C-terminal cytoplasmic domain of ABCA1 (D2, amino acids 1908–2261) carrying the leucine-based motif, we used yeast two-hybrid system. The Matchmaker™ Gold Yeast Two-Hybrid System allows detecting interaction between two hydrophilic proteins. To avoid potential hydrophobic effects of membrane-adjacent region of D2, a truncated fragment comprising amino acids 1921–2261 (D2′) was used in the assay. This D2′ fragment was predicted to be hydrophilic, making it valid for testing in a two-hybrid system. The Nef protein undergoes myristoylation at the second glycine amino acid after cleavage of the N-terminal methionine [15]. The myristoylated form of Nef can be found in the cytoplasm, as well as being anchored to the membrane by the myristic group. For analysis in the yeast two-hybrid system, Nef was fused at its N-terminal end to the activation or DNA-binding domains of the GAL4 transcription factor, so the second glycine amino acid was located inside the amino acid chain and could not be myristoylated, allowing the nuclear localization of the Nef fusion protein.

The interaction was tested in two possible ways: (i) between Nef fused to the cMyc-tagged DNA-binding domain of GAL4 (BD) and D2′ fused to the HA-tagged activation domain (AD) of GAL4; and (ii) between Nef fused to the activation domain of GAL4 and D2′ fused to the DNA-binding domain of GAL4. Following yeast transformation with corresponding vectors, the expression of fusion proteins containing Nef and the D2′ domain of ABCA1 in the obtained strains was verified by Western blot analysis (Fig. 1). BD-fused Nef and D2′ were detected in the transformed Y187 yeasts using monoclonal anti-cMyc antibody (Fig. 1A). BD-Nef and BD-D2′ fusion proteins showed partial degradation, although a certain amount of the full-length proteins (arrows in Fig. 1A) was detected. Expression of fusion proteins AD-Nef and AD-D2′ containing the GAL4 activation domain was validated in transformed AH109 yeasts using polyclonal anti-HA antibody (Fig. 1B). Again, a number of non-specific bands were visible on a gel, but bands with expected molecular weight corresponding to fusion proteins were visible (arrows in Fig. 1B). Proteins fused to the DNA-binding domain of GAL4 may activate the promoter that induces the transcription of *HIS3* gene (the reporter gene for interaction) in the absence of the GAL4 activation domain by interacting with other transcription factors in the nucleus. This was the case for yeast strain expressing the BD-Nef fusion, but not for the BD-D2′ construct. Addition of 3-aminotriazol (a competitive inhibitor of the product of the HIS3 gene, imidazoleglycerol-phosphate

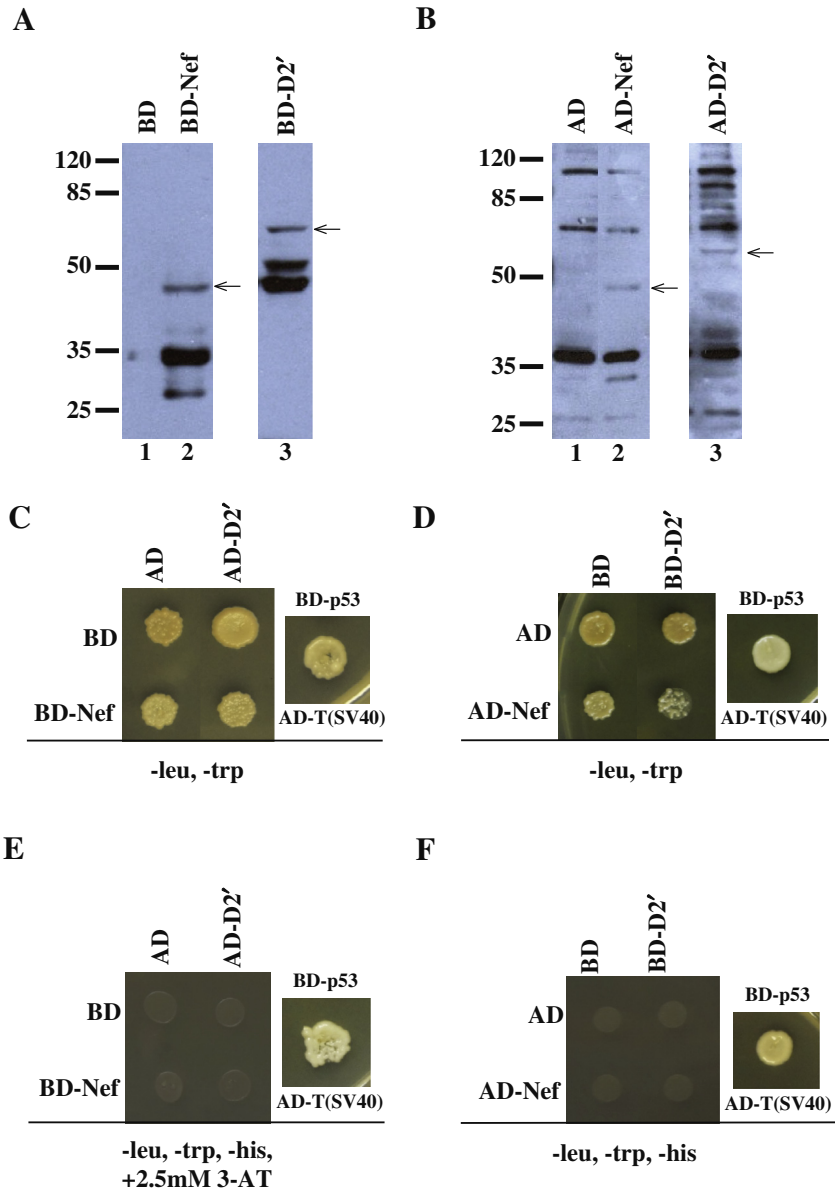


**Fig. 1.** Analysis of Nef-ABCA1 interaction using yeast 2-hybrid system. (A) Western blot analysis using anti-cMyc antibody of yeast lysates from strain Y187 transformed with pGBKT7 expressing the GAL4 DNA-binding domain (BD) alone (lane 1) or fused to Nef (lane 2) or to the D2′ domain of ABCA1 (lane 3). (B) Western blot analysis using anti-HA antibody of yeast lysates from strain AH109 transformed with pACT2 expressing the GAL4 activation domain (AD) alone (lane 1) or fused to Nef (lane 2) or to the D2′ domain of ABCA1 (lane 3). Arrows indicate the expected positions of fusion proteins. Positions of ladder marker proteins and their molecular weights in kDa are indicated in the left lanes of A and B. (C) Yeast strains Y187 expressing BD or BD-Nef were mated with each of the AH109 strains expressing AD or AD-D2′ and grown on leucine- and tryptophan-deficient medium for 5 days for mating control. (D) Yeast strains AH109 expressing AD or AD-Nef were mated with Y187 strains expressing BD or BD-D2′ and grown on leucine- and tryptophan-deficient medium for 5 days for mating control. (E) Mated yeast from C were grown on leucine-, tryptophan- and histidine-deficient medium supplemented with 2.5 mM 3-aminotriazol for 7 days. (F) Mated yeast from D were grown on leucine-, tryptophan- and histidine-deficient medium for 7 days. AH109 strain expressing AD-T (SV40) and Y187 strain expressing BD-p53 served as a positive control for mating (C, D) and interaction (E, F).

dehydratase) at 2.5 mM to the medium was sufficient to block the background expression of the *HIS3* gene and thus to prevent the strains expressing BD-Nef from growing on histidine deficient medium in the absence of the GAL4 activation domain, without affecting growth of yeast with strong binding between GAL4 domains (Fig. 1E).

Yeast strains expressing Nef fused with one of the GAL4 domains and D2′ fused with the other GAL4 domain were mated and plated on leucine- and tryptophan-deficient medium. All strains showed active growth on this medium ensuring the efficiency of mating (Fig. 1C and D). For testing the interaction between Nef and D2′, the mated yeasts were replated on medium deficient in leucine, tryptophan and histidine. For strains containing BD-Nef and the positive and negative controls, the medium was also supplemented with 2.5 mM of 3-aminotriazol. None of the mated strains showed growth on these selective media, whereas a clear growth was observed with yeast transfected with positive control vectors expressing BD-fused p53 (BD-p53) and AD-fused large T-antigen of SV-40 (AD-T(SV40)) (Fig. 1E and F). The SV40 T-antigen and p53 are known to interact [16] and they show strong binding in the yeast two-hybrid system [17]. Notably, the result was the same for both combinations of constructs tested, i.e. when Nef was fused to either the DNA-binding, or the activation domain of GAL4, with respective fusion of D2′ domain of ABCA1 to the DNA-activation or binding domain. Thus, we demonstrated that in the yeast two-hybrid system Nef did not interact with the second cytoplasmic domain of ABCA1.

A possible explanation for the lack of interaction in the 2-hybrid system is that folding of the second cytoplasmic domain in solution may be different from its native conformation, resulting in masking of the Nef-interacting epitope. To address this possibility, we expressed the D2 domain together with the transmembrane region (amino acids 1823–2261) and tested its interaction with Nef by co-immunoprecipitation in human cells. HEK 293T cells were co-transfected with vectors expressing Nef and FLAG-tagged D2 or full-length ABCA1 (or FLAG-tagged control protein, Hcf-1) (Fig. 2A), cell lysates were immunoprecipitated with anti-FLAG antibody, and blotted with anti-Nef antibody. While strong Nef-specific signal was observed in immunoprecipitates from FLAG-ABCA1-transfected cells (Fig. 2B), Nef signal in immunoprecipitate from FLAG-D2-transfected cells was only slightly above the background signal observed with negative control (cells co-transfected with FLAG-Hcf-1 and Nef).

Taken together, these results indicated that the $^{2226}$DDDHLK motif within the C-terminal cytoplasmic tail of ABCA1, while being necessary for Nef binding, was not sufficient. A possible explanation for this finding is that Nef interacts with a conformational epitope of which $^{2226}$DDDHLK is only one component. This possibility is illustrated by a model (Fig. 3) that shows a hypothetical mutual arrangement of two large ABCA1 cytoplasmic domains and Nef in three-dimensional space, indicating possible location of Nef interaction interfaces (shown in blue): the $^{2226}$DDDHLK motif in ABCA1 cytoplasmic domain 2 and a potential interface (amino acids 1009 – 1027) within the cytoplasmic domain 1. Future studies will identify the exact ABCA1 motifs composing the Nef interaction domain, but it is clear that the linear leucine-based motif is not sufficient for Nef binding to ABCA1. Of note, all previously characterized interactions between Nef and cellular proteins involved only linear motifs. If interaction between Nef and ABCA1 is proven to rely on a conformational interface, it would open new opportunities for designing drugs targeting this interaction.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

DJ, RH, TAS and TP performed biochemical experiments; VOC and AAA did molecular modeling; AAA, TSK and MB conceived the experimental plan, analyzed results, and wrote the manuscript.

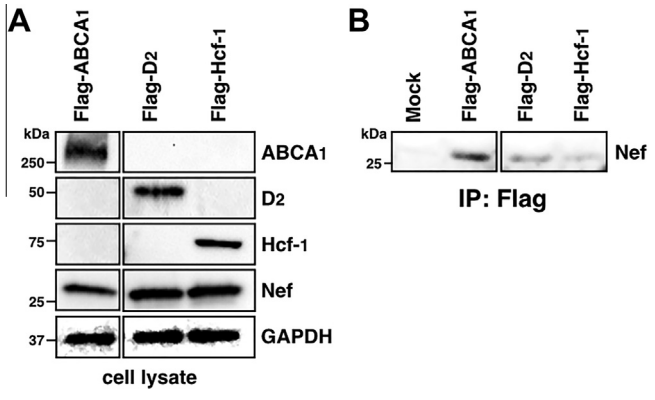


**Fig. 2.** Analysis of Nef-ABCA1 interaction using co-immunoprecipitation. (A) HEK 293T cells were transfected with vectors expressing Nef and either FLAG-tagged ABCA1, ABCA1 fragments D1 or D2, or Hcf1. Expression was tested in cell lysates with anti-FLAG or anti-Nef antibody. (B) Transfected cells from A were lysed, immunoprecipitated with anti-FLAG beads, and proteins eluted with FLAG peptide were analyzed by Western blotting using anti-Nef antibody.

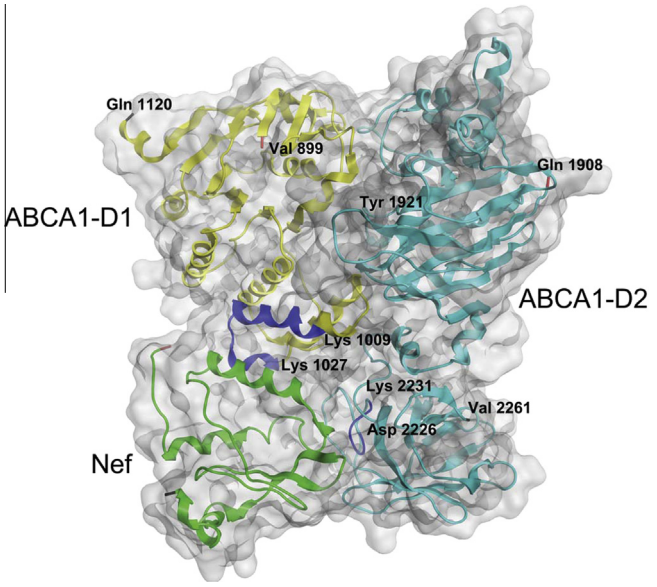


**Fig. 3.** Model of the hypothetical ABCA1–Nef complex. The model accounts for demonstrated interaction between ABCA1 and Nef [1], loss of this interaction when $^{2226}$DDDHLK is mutated [9], and weak interaction between cytoplasmic domains and Nef (this report) and can be used to discover other interaction interfaces. Yellow–ABCA1 D1 cytoplasmic domain ($^{899}$Val–$^{1120}$Gln), cyan– ABCA1 D2 cytoplasmic domain ($^{1908}$Gln–$^{2261}$Val), green – HIV-1 Nef. The D2 fragment $^{1908}$Gln–$^{1921}$Tyr was deleted in the construct used for the 2-hybrid assay. The D1 fragment $^{1009}$Lys-$^{1027}$Lys predicted to participate in the interaction with Nef, and the D2 motif $^{2226}$DDDHLK are shown in blue. Other color-coding: red – N-termini, black – C-termini of ABCA1 fragments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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