ACTIVATION OF TEC KINASES BY HIV-1 NEF AT THE CELL MEMBRANE REQUIRES DIMER FORMATION

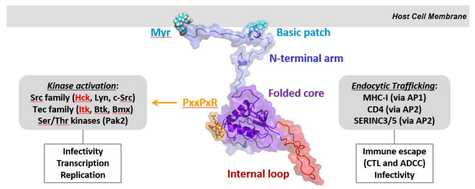
Li, W.F. [1, 2], Shu, S.T. [1], and Smithgall, T.E. [1]

[1] *Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA*

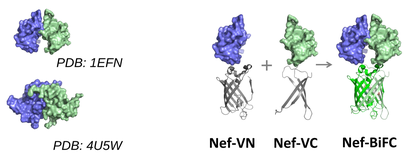
[2] *School of Medicine, Tsinghua University, Beijing, China*

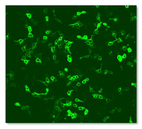
Introduction

- **HIV-1 Nef hijacks host cell signaling and trafficking pathways to promote viral replication and immune escape**



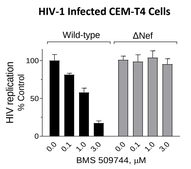
- **HIV-1 Nef forms dimers in both crystals and in cells**

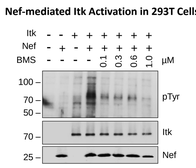


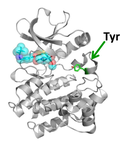


293T cells transfected with Nef-VN and Nef-VC

- **Itk Inhibitor BMS-509744 Blocks Nef-dependent HIV-1 Replication and Itk Activity**

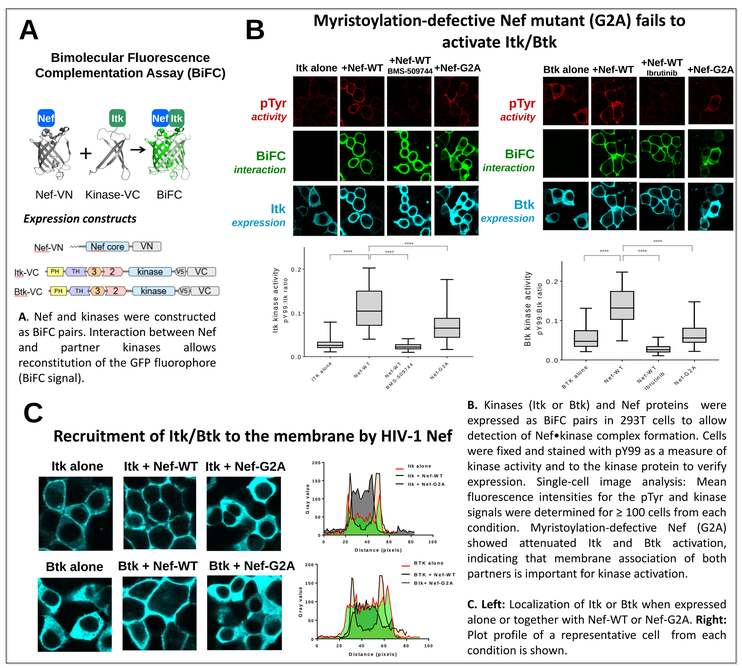






Itk kinase domain + BMS-509744
PDB: 3MJ2

Nef Recruits and Activates Itk and Btk at the Cell Membrane

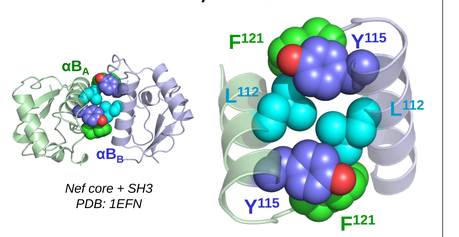


A. Nef and kinases were constructed as BiFC pairs. Interaction between Nef and partner kinases allows reconstitution of the GFP fluorophore (BiFC signal).

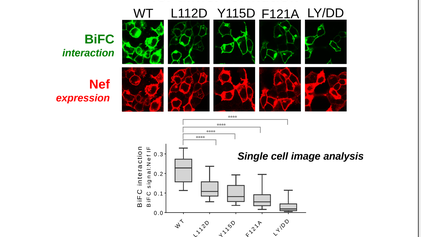
B. Kinases (Itk or Btk) and Nef proteins were expressed as BiFC pairs in 293T cells to allow detection of Nef•kinase complex formation. Cells were fixed and stained with pY99 as a measure of kinase activity and to the kinase protein to verify expression. Single-cell image analysis: Mean fluorescence intensities for the pTyr and kinase signals were determined for ≥ 100 cells from each condition. Myristoylation-defective Nef (G2A) showed attenuated Itk and Btk activation, indicating that membrane association of both partners is important for kinase activation.

C. Left: Localization of Itk or Btk when expressed alone or together with Nef-WT or Nef-G2A. **Right:** Plot profile of a representative cell from each condition is shown.

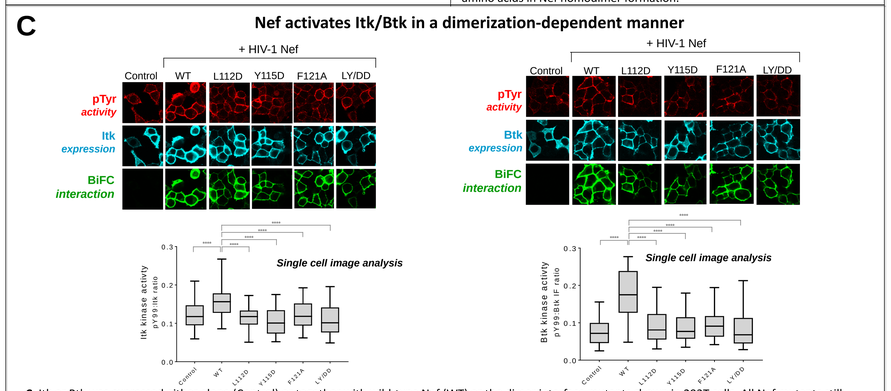
Nef Dimerization Mutants Bind but Fail to Activate Itk/Btk



A. Nef dimer interface is formed by the αB helices present in each Nef monomer (PDB: 1EFN). Side chains of Nef residues L112, Y115, and F121 from each monomer form the hydrophobic core of the interface.

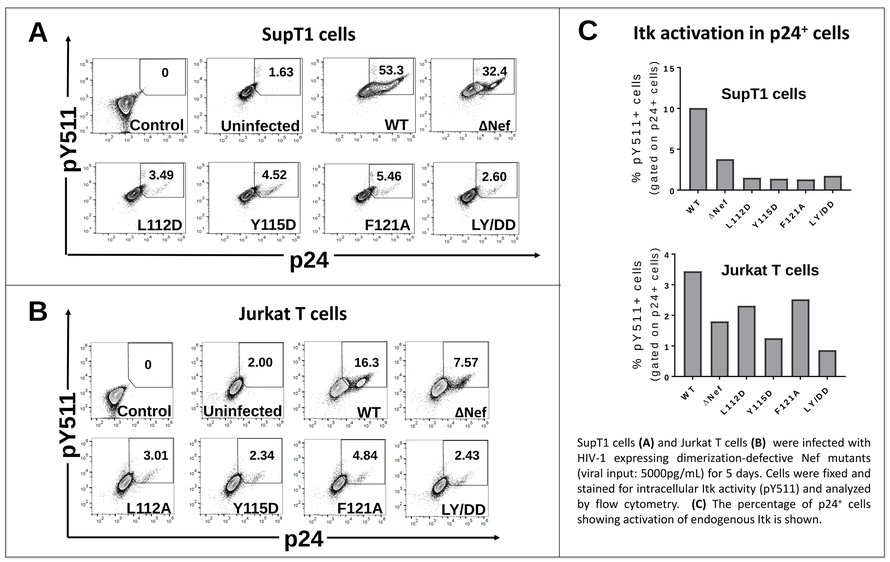


B. Nef proteins were expressed as BiFC pairs in 293T cells to allow detection of Nef homodimer formation. All Nef mutants show decreased interaction (BiFC signal) compared to the wild-type Nef protein, confirming the role of these amino acids in Nef homodimer formation.



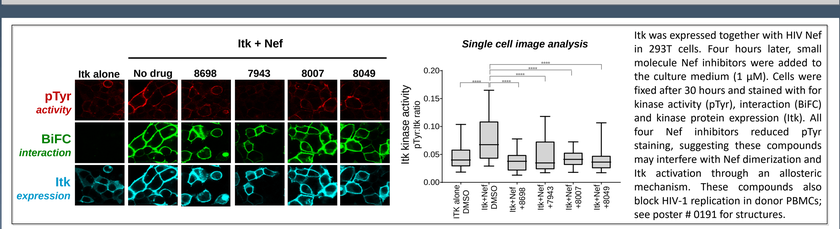
C. Itk or Btk was expressed either alone (Control) or together with wild-type Nef (WT) or the dimer interface mutants shown in 293T cells. All Nef mutants still interact with Itk (BiFC signals) but show reduced pY99 staining, indicating that Nef dimer formation is required for Itk/Btk activation. .

HIV-1 expressing Dimerization-defective Nef Fails to Activate Endogenous Itk activity



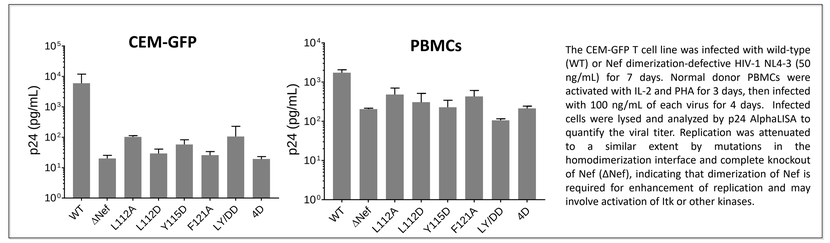
SupT1 cells **(A)** and Jurkat T cells **(B)** were infected with HIV-1 expressing dimerization-defective Nef mutants (viral input: 5000pg/mL) for 5 days. Cells were fixed and stained for intracellular Itk activity (pY511) and analyzed by flow cytometry. **(C)** The percentage of p24+ cells showing activation of endogenous Itk is shown.

Small molecule Nef inhibitors reverse Nef-mediated Itk activation



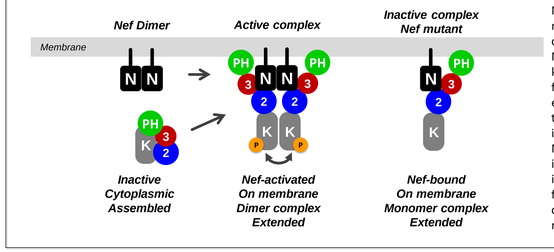
Itk was expressed together with HIV Nef in 293T cells. Four hours later, small molecule Nef inhibitors were added to the culture medium (1 μM). Cells were fixed after 30 hours and stained with for kinase activity (pTyr), interaction (BiFC) and kinase protein expression (Itk). All four Nef inhibitors reduced pTyr staining, suggesting these compounds may interfere with Nef dimerization and Itk activation through an allosteric mechanism. These compounds also block HIV-1 replication in donor PBMCs; see poster # 0191 for structures.

HIV-1 Expressing Dimerization-defective Nef Mutants Replicate poorly



The CEM-GFP T cell line was infected with wild-type (WT) or Nef dimerization-defective HIV-1 NL4-3 (50 ng/mL) for 7 days. Normal donor PBMCs were activated with IL-2 and PHA for 3 days, then infected with 100 ng/mL of each virus for 4 days. Infected cells were lysed and analyzed by p24 AlphaLISA to quantify the viral titer. Replication was attenuated to a similar extent by mutations in the homodimerization interface and complete knockout of Nef (ΔNef), indicating that dimerization of Nef is required for enhancement of replication and may involve activation of Itk or other kinases.

Model of Nef-dependent Itk and Btk Kinase Activation



Nef dimerizes at the cell membrane. Each Nef monomer interacts and recruits an inactive, cytoplasmic kinase protein to the membrane. Nef-mediated membrane association with the kinase induces changes in kinase conformation, from the assembled, inactive state to an extended, active conformation. Juxtaposition of the kinase domains drives autophosphorylation in *trans*, stabilizing an active complex formation. Nef dimer defective mutants or addition of Nef inhibitors impairs Nef dimer formation, resulting in recruitment of only one kinase molecule and forming an inactive complex. Under some conditions, this may produce a dominant negative effect on basal Itk kinase activity.

Summary and References

Summary:

- BiFC analysis shows that wild-type HIV-1 Nef recruits both Itk and Btk to the cell membrane, resulting in enhanced kinase kinase activity.
- Nef dimerization interface mutants retain interaction with both kinases, but fail to induce kinase activation.
- HIV-1 infection upregulated endogenous Itk activity in cell lines in a Nef dimerization-dependent manner.
- Addition of small molecule Nef inhibitors reversed Nef-dependent Itk autophosphorylation, suggesting that these compounds may interfere with Nef dimerization and Itk activation through an allosteric mechanism.
- Inhibition of Nef-dependent Itk activation may account in part for the antiretroviral activity of hydroxypyrazole Nef inhibitors in T cell lines and in primary CD4+ T cells.

References:

1. Readinger, J. A., Schiralli, G. M., Jiang, J. K., Thomas, C. J., August, A., Henderson, A. J., & Schwartzberg, P. L. (2008). Selective targeting of ITK blocks multiple steps of HIV replication. *Proceedings of the National Academy of Sciences*, *105*(18), 6684-6689.
2. Poe, J. A., & Smithgall, T. E. (2009). HIV-1 Nef dimerization is required for Nef-mediated receptor downregulation and viral replication. *Journal of molecular biology*, *394*(2), 329-342.
3. Tarafdar, S., Poe, J. A., & Smithgall, T. E. (2014). The accessory factor Nef links HIV-1 to Tec/Btk kinases in an Src homology 3 domain-dependent manner. *Journal of Biological Chemistry*, *289*(22), 15718-15728.