Integration Site-independent Enhancement of Latency Reversal By HIV-1 Nef

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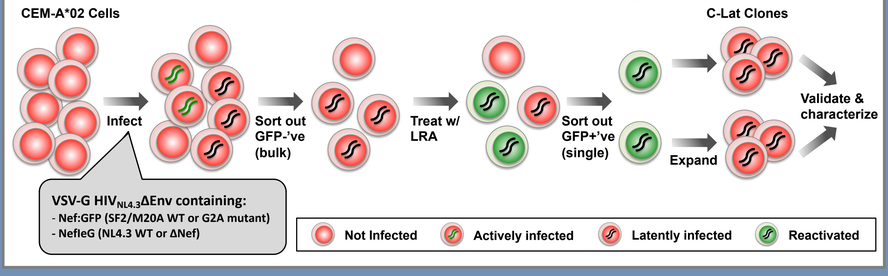
BACKGROUND

Nef is a crucial HIV-1 accessory protein that enhances viral pathogenesis, in part through its ability to evade host immunity. While Nef is reported to modulate T cell signaling events, which may alter cellular activation status, its contribution to the establishment and maintenance of viral latency remain unknown. To investigate this, we examined the proviral integration site and reactivation efficiency of clonal CEM T cell lines latently infected with HIV-1 strains encoding functional or defective Nef sequences (C-Lat). To avoid potential bias with respect to proviral DNA integration sites, functional Nef was knocked out in selected C-Lat clones using CRISPR/Cas9 system and reactivation efficiency was examined in progeny cells.

METHODS

Generation and characterization of C-Lat (Nef_{KO}) clones

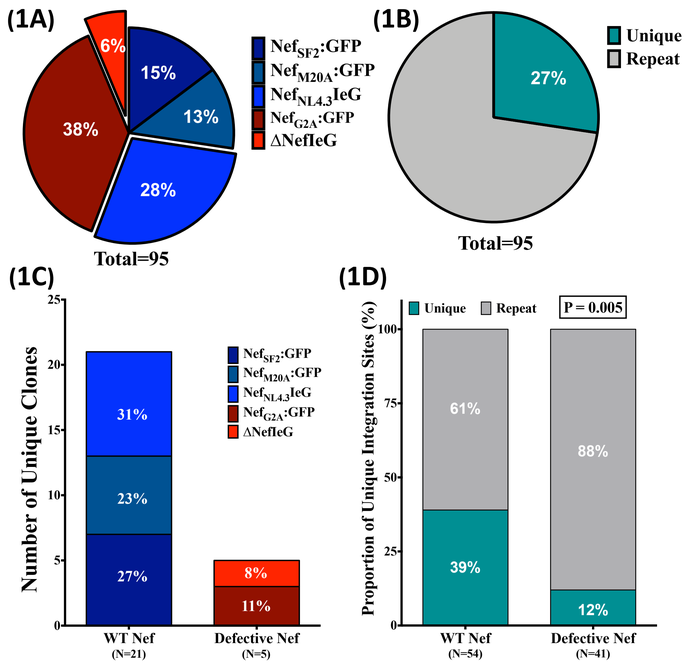


CONCLUSIONS

- Nef was associated with a broader repertoire of inducible proviral integration sites, including sites located in less transcriptionally active genomic regions.
- Nef contributed to HIV-1 reactivation efficiency following LRA stimulation. Latent T cell clones lacking Nef displayed lower Gag expression, suggesting impaired viral late gene expression.
- Nef's role in viral reactivation and Gag protein production was confirmed using CRISPR/Cas9-mediated knockout of Nef in latent T cell clones.

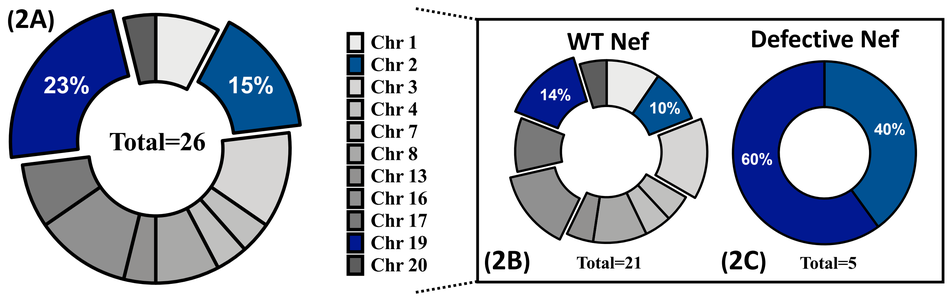
RESULTS (PART 1) – Distribution and Characteristics of HIV-1 Proviral Integration Sites in Inducible C-Lat Clones

Figure 1 – Higher frequency and proportion of unique clones/lineages in the presence of WT Nef



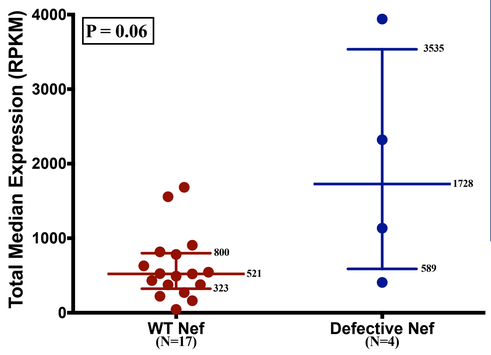
A total of 95 inducible C-Lat clones with linked HIV integration site and viral reactivation data were generated. **(A)** Pie chart displays Nef-GFP construct used for clone generation. WT and defective Nef variants are colored in blue and red, respectively. **(B)** Pie chart displays proportion of clones containing unique integration sites (repeated sites indicate expansion of clones). **(C)** Bar graph shows distribution of unique C-Lat clones/lineages for each Nef-GFP construct. Numbers represent the percent contribution of each Nef-GFP construct to the total unique C-Lat clones (N=26). **(D)** Bar graph displays the proportion of unique clones/lineages within C-Lat cells expressing WT vs. defective Nef.

Figure 2 – Chromosomal distribution of HIV-1 integration sites differed between WT Nef vs. defective Nef C-Lat clones



Pie charts display the distribution of chromosome locations for unique HIV integration sites identified in all C-Lat clones (N=26) **(A)**, or in clones that express WT Nef (N=21) **(B)** or defective Nef (N=5) **(C)**. Chromosomes colored in blue represent those with highest frequency of integration events in total C-Lat clones; pop-up/detached regions represent those with highest frequency in Nef-stratified data.

Figure 3 – Inducible proviruses lacking Nef tended to be integrated into protein-coding genes that are more transcriptionally active



*RKPM = read per kilobase per million mapped read

A list of genomic properties were examined near the HIV integration site:

- Percent GC content
- Distance to centromere
- Type of gene (coding, non-coding or pseudogene)
- Presence of transcription factor binding sites, DNase I hypersensitive sites, CpG islands and H3K27Ac histone modifications
- ***Expression of protein-coding gene (plotted on the left)***

Majority of genomic properties analyzed displayed comparable distribution between C-Lat clones encoding WT and defective Nef. Nonetheless, integration of inducible proviruses lacking Nef was modestly enriched in protein-coding genes that are expected to be more transcriptionally active.

RESULTS (PART 2) – HIV-1 Reactivation and Gag Protein Production in the Presence vs. Absence of Nef

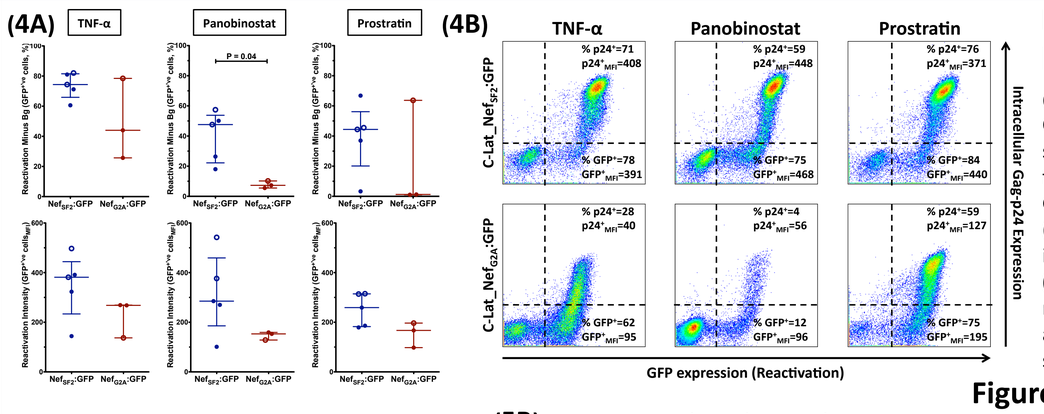


Figure 4 – Gag protein expression is lower in C-Lat clones harboring defective Nef_{G2A}

C-Lat clones with identical integration sites displayed similar viral reactivation phenotypes and were grouped for analysis. **(A)** Graphs display viral reactivation (percent GFP+ [top] and MFI/intensity [bottom]) for Nef_{SF2}:GFP (N=5) and Nef_{G2A}:GFP (N=3) C-Lat clones. Open circles indicate mean values from multiple independent clones. **(B)** Flow plots illustrate reactivation phenotypes for representative C-Lat clones, assessing early (Nef:GFP) and late (Gag-p24) protein expression following stimulation with TNF-α, panobinostat or prostratin.

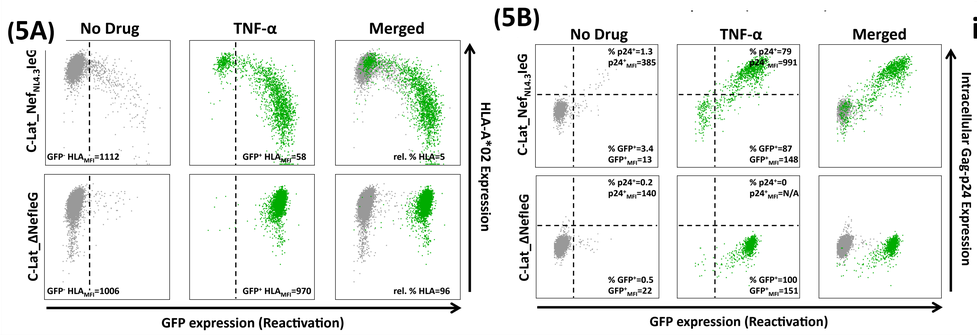
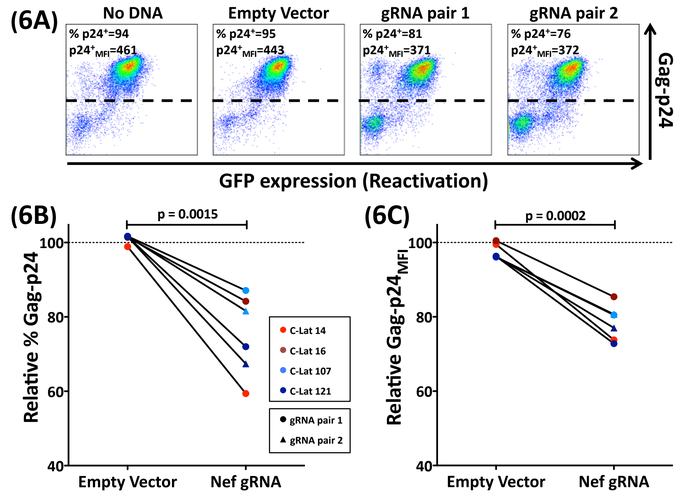


Figure 5 – Gag protein expression is lower in a Nef-IRES-GFP C-Lat clone lacking Nef

Results in Figure 4 were validated using C-Lat clones generated using HIV-1 strains encoding Nef-IRES-GFP (NefleG) **(A)** Flow plots display reactivation (GFP+) phenotypes for representative C-Lat clones in the absence (grey) or presence (green) of TNF-α. HLA class I downregulation (y-axis) was measured to confirm $Nef_{NL4.3}$ function. In merged plots, relative %HLA was calculated by dividing the MFI for HLA in GFP+ cells (TNF-α) by that in GFP- negative cells (no drug). **(B)** Flow plots display viral reactivation (GFP) and Gag-p24 protein expression in the same C-Lat clones. Percent and MFI for GFP+ and Gag-p24+ populations are indicated in the corresponding plots.

RESULTS (PART 3) – Effect of CRISPR/Cas9-mediated Nef KO on Reactivation and Gag Production

Figure 6 – Knocking out Nef in C-Lat cells reduces Gag protein expression



To overcome potential bias associated with proviral DNA integration site, we disrupted WT Nef alleles using the CRISPR/Cas9 system. Two different pairs of gRNAs were used to target Nef: pair 1 (KO1) recognizes Nef_{SF2} and $Nef_{NL4.3}$; pair 2 (KO2) recognizes only $Nef_{NL4.3}$. **(A)** Representative flow plots show TNF-α-induced viral reactivation and Gag expression in a representative C-Lat clone. **(B and C)** Graphs depict the % and MFI of Gag-p24 in bulk Nef_{KO} clones relative to the corresponding parental C-Lat cell line. Each color represents one parental cell line. Circles and triangles indicate Nef gRNA pair 1 and pair 2, respectively.

Figure 7 – Reactivation efficiency and Gag protein expression are impaired in C-Lat Nef_{KO} clones

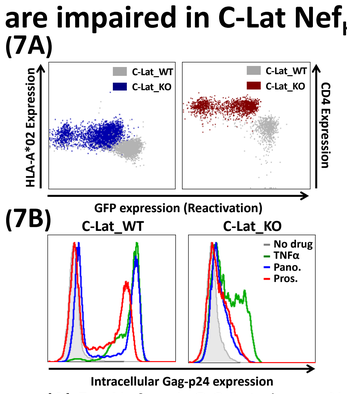


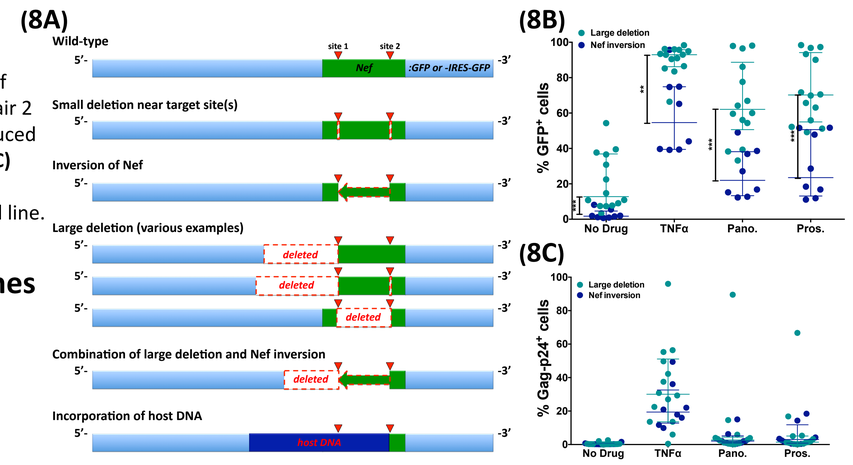
Table 1. Summary of detailed statistic results for all Nef_{KO} clones

		CLat-Nef$_{SF2}$GFP	CLat-Nef$_{NL4.3}$-IRES-GFP		
	Group	% Gag-p24	% Reactivation	Reac. Intensity	% Gag-p24
TNFα	WT	*79.0*	*97.2*	*236*	*96.9*
	KO$_1$	33.6 [5.0-74.9]	88.8 [57.3-96.0]	94.2 [43.6-126.8]	27.8 [12.7-51.1]
	KO$_2$	N/A	83.5 [44.0-93.0]	66.9 [55.0-114.0]	22.0 [16.0-36.1]
Pano.	WT	*65.2*	*62.6*	*246*	*55.5*
	KO$_1$	21.4 [0.6-44.8]	47.4 [21.3-69.1]	67.6 [38.8-94.8]	2.4 [1.0-8.8]
	KO$_2$	N/A	38.6 [16.8-64.2]	62.3 [51.7-102.0]	2.0 [0.8-3.3]
Pros.	WT	*72.9*	*62.7*	*102*	*51.9*
	KO$_1$	18.0 [1.0-23.0]	50.2 [34.8-73.5]	47.7 [39.2-60.4]	3.0 [0.4-16.9]
	KO$_2$	N/A	52.2 [18.4-71.7]	58.0 [44.1-79.2]	2.5 [1.0-5.3]

Note - CLat-Nef$_{SF2}$:GFP: N=6 for KO2. CLat-Nef$_{NL4.3}$-IRES-GFP: N=14 for KO$_1$ and N=15 for KO$_2$.

(A) For $Nef_{NL4.3}$IeG C-Lat clones, Nef_{KO} was confirmed by absence of CD4 and HLA-A*02 downregulation following TNF-α treatment. **(B)** Histograms depict Gag-p24 expression in a representative C-Lat parental cell line and its corresponding Nef_{KO} clone. Stimulation using TNF-α, panobinostat, and prostratin are indicated by corresponding colors.

Figure 8 – Various gene modifications in Nef_{KO} clones



(A) The effects of CRISPR/Cas9 on proviral DNA was assessed by sequencing individual clones. Various unexpected modifications were identified; most clones displayed inversion of Nef between the gRNA target sites or large deletions upstream of the target site(s). **(B and C)** Nef_{KO} clones with large deletions displayed higher GFP expression compared to those with Nef inversion **(B)**; but Gag-p24 expression **(C)** was reduced to a similar level.