

## RESEARCH ARTICLE

# Tetherin downmodulation by SIVmac Nef lost with the H196Q escape variant is restored by an upstream variant

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## Abstract

The H<sub>196</sub> residue in SIVmac239 Nef is conserved across the majority of HIV and SIV isolates, lies immediately adjacent to the AP-2 (adaptor protein 2) binding di-leucine domain (ExxxLM<sub>196</sub>), and is critical for several described AP-2 dependent Nef functions, including the downregulation of tetherin (BST-2/CD317), CD4, and others. Surprisingly, many stocks of the closely related SIVmac251 swarm virus harbor a *nef* allele encoding a Q<sub>196</sub>. In SIVmac239, this variant is associated with loss of multiple AP-2 dependent functions. Publicly available sequences for SIVmac251 stocks were mined for variants linked to Q<sub>196</sub> that might compensate for functional defects associated with this residue. Variants were engineered into the SIVmac239 backbone and in Nef expression plasmids and flow cytometry was used to examine surface tetherin expression in primary CD4 T cells and surface CD4 expression in SupT1 cells engineered to express rhesus CD4. We found that SIVmac251 stocks that encode a Q<sub>196</sub> residue in Nef uniformly also encode an upstream R<sub>191</sub> residue. We show that R<sub>191</sub> restores the ability of Nef to downregulate tetherin in the presence of Q<sub>196</sub> and has a similar but less pronounced impact on CD4 expression. However, a published report showed Q<sub>196</sub> commonly evolves to H<sub>196</sub> in vivo, suggesting a fitness cost. R<sub>191</sub> may represent compensatory evolution to restore the ability to downregulate tetherin lost in viruses harboring Q<sub>196</sub>.

## Introduction

The lentiviral Nef protein is a common target of CD8-T lymphocyte (CTL) responses in both HIV-1 infected persons and SIV infected rhesus macaques and readily evolves to evade these responses [1–6]. Nef is highly pleiotropic and mediates the downregulation of several cell surface molecules involved in innate and adaptive immune responses against virus infected cells such as TCR-CD3 (in most SIVs but not HIV-1) [7], CD4 [8–10], CD8αβ [11], CD28 [12], tetherin (BST2 or CD317; in most SIVs and in HIV-1 group O, but not HIV-1 group M) [13–15], MHC-I [16], MHC-II [17], CD1d [18], CD80/CD86 [19] and likely others as well as

enhancing viral infectivity by preventing virion incorporation of host serine incorporator 3 (SERINC3) and SERINC5 proteins [20–23]. Nef-mediated modulation of several of these molecules, including CD3, CD4, CD8 $\alpha\beta$ , CD28, tetherin, and SERINC3 and SERINC5 requires interactions between Nef and adaptor protein (AP)-2 complexes [11, 20, 24–29].

We used high throughput next generation sequencing to track evolution in SIV Nef [30, 31], with particular focus on viral escape from antiviral CTL responses, including CTL targeting the SIV Nef IW9 (IRYPKTFGW<sub>173</sub>, with subscript numbers representing the position in the SIVmac239 Nef protein) and MW9 (MHPAQTSQW<sub>203</sub>, hereafter referred to as MW9) epitopes in rhesus macaques that express Mamu-B\*017:01. MW9 overlaps the well-defined “di-leucine” ExxxLM<sub>195</sub> motif and lies immediately upstream of the DD<sub>205</sub> di-acidic motif also important for AP-2 binding [32]. Though selection eventually favored changes of the first position in MW9, specifically M<sub>195</sub>I or M<sub>195</sub>V, an H<sub>196</sub>Q (second position in MW9) substitution was initially favored in several animals. Since this variant was never fixed and generally lost soon after arising, we hypothesized it may have represented an effective escape mutation yet imparted a negative impact on Nef function. Specifically, we tested whether functions involving interactions with AP-2 would most likely be impacted, given the close proximity of this epitope with the ExxxLM<sub>195</sub> AP-2 interaction domain. Not surprisingly, the H<sub>196</sub>Q variant selectively disrupted Nef functions that rely on interactions with AP-2, such as downregulation of tetherin, CD4, and CD28 and disrupted Nef’s ability to reduce SERINC5-mediated reductions of viral infectivity, while having no impact on MHC-I downregulation, a function that does not rely on AP-2 interactions [33, 34]. In that study, we did not identify any potential compensatory mutations that allowed for regain of function in the presence of the H<sub>196</sub>Q variant leading to this variant being only fleetingly detected and eventually replaced by escape mutations with less significant impacts on important Nef functions.

Mining publicly available sequences from different isolates of SIVmac251, a commonly used strain in SIV studies, we found that many harbor a Q<sub>196</sub> in the viral Nef protein. In this study, we sought mutations linked to Q<sub>196</sub> that might compensate for loss of function associated with this residue. We identified an upstream variant, R<sub>191</sub> (E<sub>191</sub> in SIVmac239) that compensates for the loss of tetherin downregulation associated with Q<sub>196</sub>. However, we also found that Q<sub>196</sub> routinely mutated to H<sub>196</sub> in vivo, suggesting reduced fitness despite the maintenance of tetherin downregulation associated with the combination of Q<sub>196</sub> and R<sub>191</sub> residues.

## Materials and methods

### Ethics statement

Cells used in this study were taken from blood from six Indian-origin rhesus macaques (*Macaca mulatta*) that are part of the breeding colony at the Tulane National Primate Research Center. Animals were anesthetized as part of their routine semi-annual health assessment (SAHA) and additional blood was drawn for this study. Thus, animals were not anesthetized specifically for the studies described herein. All animals were housed in compliance with the NRC Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act. Blood draws were approved by the Institutional Animal Care and Use Committee of Tulane University (OLAW assurance #A4499-01) under protocol P0191. The Tulane National Primate Research Center (TNPRC) is fully accredited by AAALAC International [Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC#000594)], Animal Welfare Assurance No. A3180-01. Breeding colony animals at the TNPRC are housed outdoors in social groups and frequently monitored by veterinarians and behavioral scientists. The animals were fed commercially prepared monkey chow and supplemental foods were provided in the form of fruit, vegetables, and foraging treats as part of the TNPRC environmental enrichment

program. Water was available at all times through an automatic watering system. The TNPRC environmental enrichment program is reviewed and approved by the IACUC semiannually. Veterinarians at the TNPRC Division of Veterinary Medicine have established procedures to minimize pain and distress through several means. Monkeys were anesthetized with ketamine-HCl (10 mg/kg) or tiletamine/zolazepam (6 mg/kg) prior to all procedures. The above listed anesthetics were used in accordance with the recommendations of the Weatherall Report.

### Primary cell isolation, culture and infection

Primary CD4 T cells were magnetically isolated from PBMC from healthy rhesus macaques using nonhuman primate CD4 microbeads (Miltenyi) according to the manufacturer's protocol. Isolated cells were stimulated with concanavalin A for two days and cultured thereafter with R15/50 media, comprised of RPMI media with 15% FBS and 50U/ml IL-2. SIV infections were conducted using the spinoculation technique [35] with each 1ml aliquot of virus (approximately  $10^8$  viral RNA copies per milliliter) layered on 100ul of 20% sucrose solution and centrifuged for 1 hour at 4°C at 20,000xg. After removal of the supernatant, the concentrated virus was resuspended in 100ul of R15/50 media and gently dripped onto one million CD4 T cells plated at 1 million cells/ml in 48 well plates. Plates were then spun at 2000rpm for 2 hours at room temperature. After centrifugation, plates were placed in 37°C humidified incubators with 5% CO<sub>2</sub>. Cells were cultured for 36 hours before harvest for flow cytometry assays.

### Mutant virus and plasmid production

Mutants of the SIVmac239 virus were generated using site directed mutagenesis of the SIVmac239 3' hemiplasmid, using mutagenesis primers designed using web-based software (PrimerX from [bioinformatics.org](http://bioinformatics.org)). The same methods and primers were used to generate mutations on the Nef expressing plasmid pCGCG-Nef, which expresses both Nef and GFP from a bicistronic mRNA. To generate the H<sub>196</sub>Q mutation alone, we used the following mutagenesis primers using the QuikChange II Site-Directed Mutagenesis Kit (Agilent); F: GCA TTA TTT AAT GCA GCC AGC TCA AAC TTC CC, and R: GGG AAG TTT GAG CTG GCT GCA TTA AAT AAT GC and to generate the E<sub>191</sub>R mutation on the backbone that already contained the H<sub>196</sub>Q variant, we used the following mutagenesis primers; F: GGC ACA GGA GGA TGA GAG GCA TTA TTT AAT GCA GC, and R: GCT GCA TTA AAT AAT GCC TCT CAT CCT CCT GTG CC. After mutagenesis, plasmids were treated with DpnI to remove non-mutated parental plasmids and cloned into Stbl2 cells (Life Technologies) using the manufacturer's protocol. Mutations were sequence-verified, and successfully mutated plasmids were used for follow-up studies. For virus production, mutated 3' plasmids were ligated with the 5' hemiplasmid, transfected into Vero cells followed by harvest of the virus-containing supernatant. Of note, the Nef region of interest overlaps with the 3' long terminal repeat (LTR). Attempts to mutate the full-length SIV plasmid resulted in mutations in both the 5' and 3' LTR regions, which rendered the viruses replication incompetent, necessitating the need for mutating only the 3' hemiplasmid followed by ligation to the wild type 5' plasmid. Some viruses were further expanded in CEMx174 cells. All viral stocks and pCGCG plasmids were sequenced to ensure the presence of desired mutations.

### Generation of the A66 rhesus CD4+ Rh CCR5+ cells and Nef transfections

A human T cell line expressing rhesus CD4 and rhesus CCR5 was constructed using the A66 cell line, derived from the CD4+, CCR5- SupT1 cells by ablating CXCR4 using zinc-finger nucleases [36]. A66 cells were then electroporated with human CD4-specific CRISPR/Cas9

plasmids (Santa Cruz Biotechnology Cat# sc-400237-KO-2), and a CD4 negative clone was then isolated. Rh CD4 and Rh CCR5 were individually ligated into the pLenti6 (Invitrogen) lentiviral vector system and cells highly expressing both rhesus receptors isolated by FACS and single cell cloning.

For CD4 downmodulation experiments, A66 cells were transfected with Nef variants (wild-type, H196Q, and H196Q / E191R) using the Amaxa 4D-Nucleofector Protocol with the CA-137 program and SF cell line reagent in 20 ul Nucleocuvette strips. Briefly,  $2 \times 10^5$  cells were transfected with 1 ul plasmid DNA for each reaction. Average transfection efficiencies were as follows: 33.7% for wildtype, 33.5% for H196Q, and 36.9% for H196Q / E191R with background fluorescence of approximately 4% in the untransfected control. At least three independent experiments were performed with each vector.

### Flow cytometry

To measure surface tetherin expression in infected cells, thirty-six hours after infection, cells were stained with labeled antibodies to CD4 (BV421, clone L200, BD Biosciences) and tetherin (PE, clone RS38E, Biolegend), followed by fixation, permeabilization, and intracellular labeling with a FITC labeled antibody against the Gag p27 protein (clone 55-2F12). To measure surface CD4 expression in transfected Sup-T1 cells, twenty four hours after transfection, cells were stained with labeled antibodies to CD4 (BV421, clone L200, BD Biosciences), washed and fixed. Data was acquired on a BD LSRII instrument and analyzed using Flowjo v10 software.

### Structural analysis

Structures showing interactions between SIVsm Nef, Tetherin, and AP-2 subunits were recently published [37]. We used UCSF Chimera software [38] to probe potential interactions between our Nef residues of interest at positions 191 and 196 and host AP-2 and tetherin proteins. The Rotamers function in UCSF Chimera was used to predict impacts of mutations and the Match-maker function was used to assess positioning of Nef amino acids in SIV relative to HIV-1.

### Sequence analysis and alignments

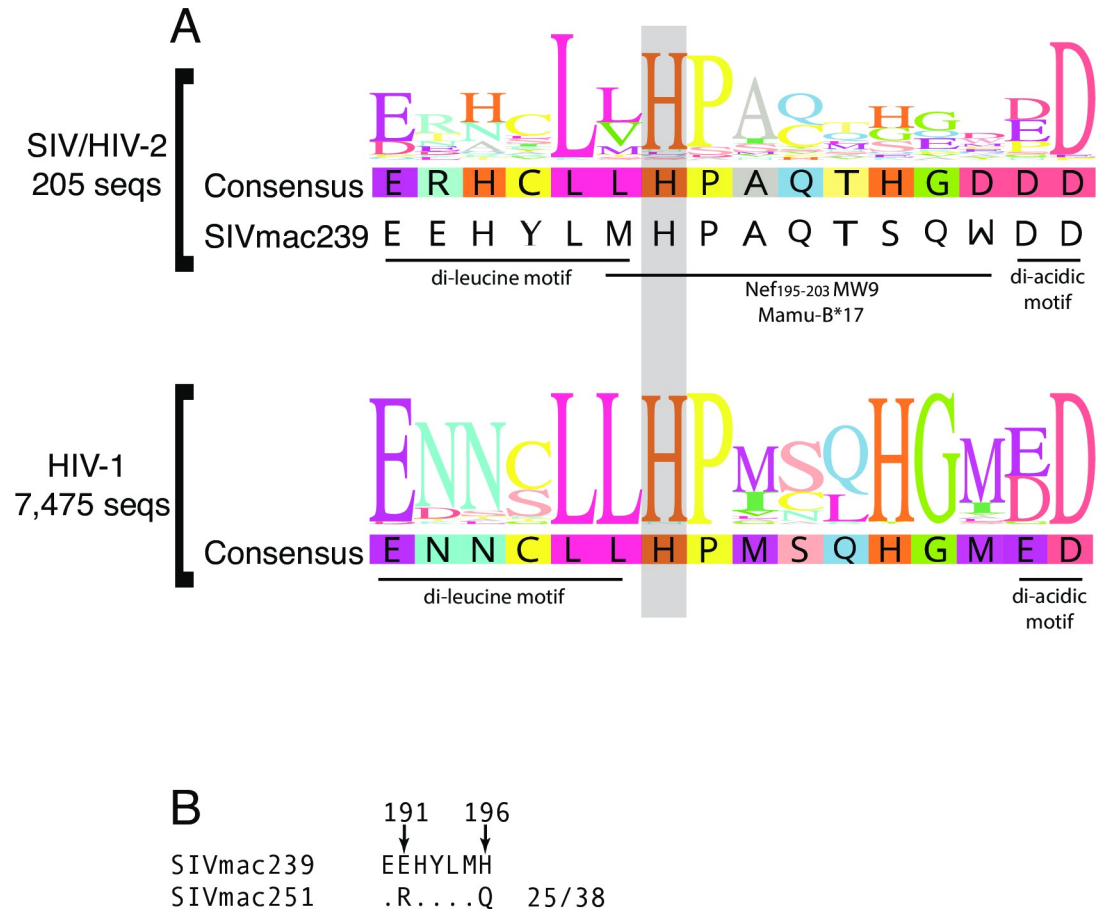
Nef sequences from a broad array of SIV isolates were identified from a published report [39] and downloaded from NCBI for amino acid alignments using Geneious Prime 2019.1.3 using the built-in Geneious Alignment algorithm with default settings. SIVmac251 sequences available from published reports [40, 41] were downloaded from NCBI into Geneious Prime 2019.1.3 and mapped to SIVmac239, used as the reference genome, followed by identification and quantification of variations using the Find Variations/SNPs function. Sequences published in the Lamers et al. report [41], were first divided into those extracted from the inoculum and from individual tissues, which were analyzed separately.

## Results

### Conservation of the Nef H<sub>196</sub> residue among primate lentiviruses

To assess conservation of the H<sub>196</sub> residue, we performed alignments of the Nef protein from all SIV and HIV-1 sequences available on the Los Alamos database. Although the flexible loop is, in general, far more variable than the core, we found the H<sub>196</sub> residue to be highly conserved among all isolates sequenced to date (Fig 1A), as conserved as important residues in the adjacent “di-leucine” E<sub>195</sub>LM<sub>195</sub> motif, E<sub>190</sub> and L<sub>194</sub>.

We next scanned publicly available sequences from a recent study that used single genome amplification to extensively examine SIVmac251 challenge stocks [40]. In this report, a Q<sub>196</sub>



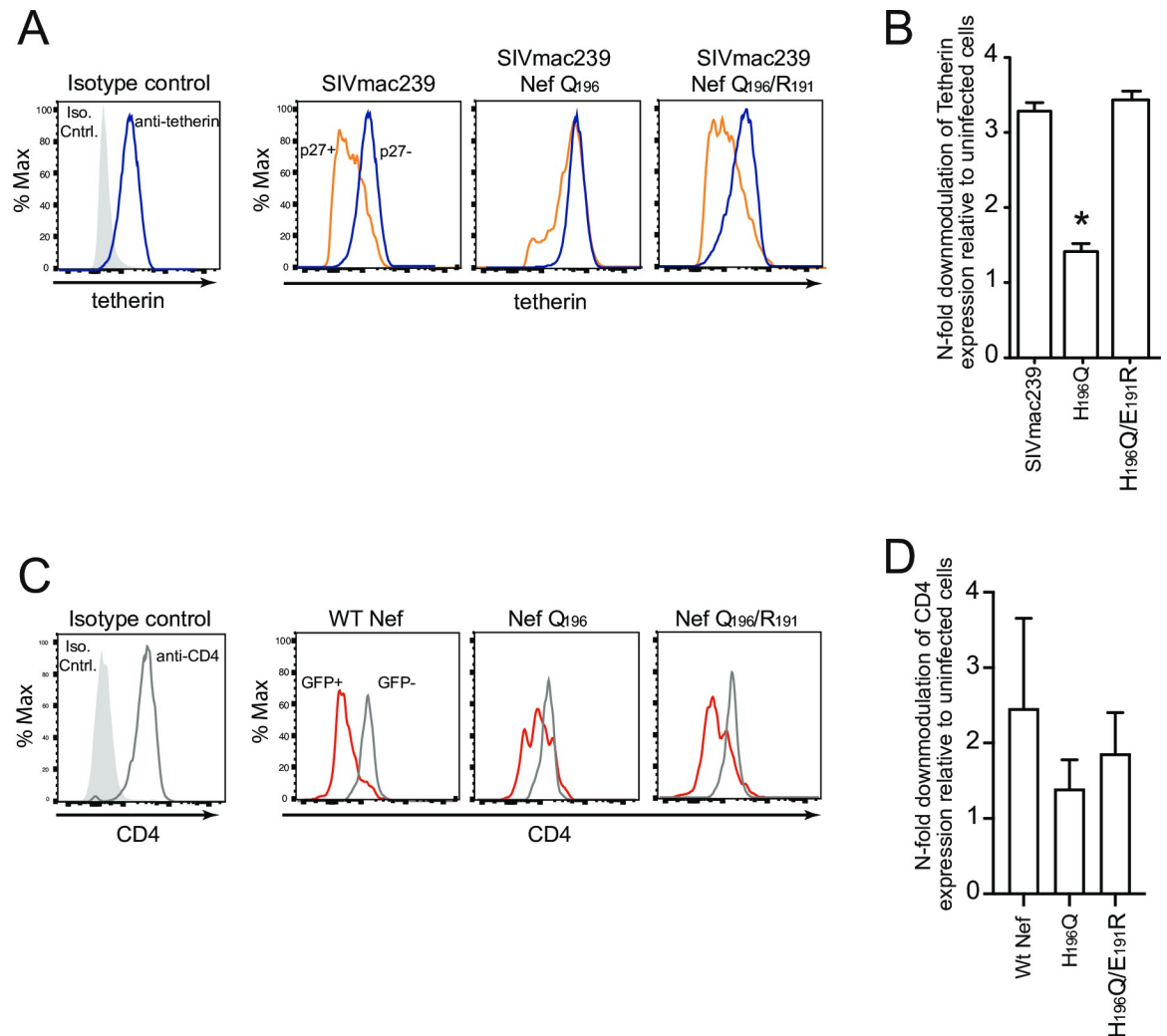
**Fig 1. Assessment of the conservation of residue 196 in SIV Nef.** Alignment of the region of Nef spanning the dileucine motif (ExxxLL) to the diacidic (DD) motifs (A). Sequences were derived from the Los Alamos database ([lanl.gov](http://lanl.gov)) and are shown in sequence logo format. The H<sub>196</sub> residue (based on SIVmac239 numbering) is encompassed by a grey box. SIVmac251 stock sequences from a published study [40] show a variant amino acid upstream of Q<sub>196</sub> (R<sub>191</sub>) that was always associated with Q<sub>196</sub>. (B). Alignments in both panels were performed using Geneious Prime 2019.1.3.

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residue was detected in a large fraction of sequences from SIVmac251 stock viruses from several labs. Interestingly, there was a perfect linkage between the Q<sub>196</sub> residue and an upstream R<sub>191</sub> residue, which is E<sub>191</sub> in SIVmac239 (Fig 1B). Of 38 total sequences that contained the region of interest, derived from three different challenge stocks, 25 sequences contained both R<sub>191</sub> and Q<sub>196</sub> while Q<sub>196</sub> was never found in the absence of R<sub>191</sub>. Other nearby variants relative to SIVmac239 were detected but only R<sub>191</sub> co-occurred with Q<sub>196</sub> in all sequences.

### Upstream potential compensatory variant restores tetherin downregulation

Given the strong linkage between the R<sub>191</sub> (E<sub>191</sub> in SIVmac239) variant and Q<sub>196</sub>, we tested whether R<sub>191</sub> allowed tetherin downregulation in the presence of Q<sub>196</sub>. The R<sub>191</sub> residue lies within the ExxxLM<sub>195</sub> motif (EEHYLM<sub>195</sub> in SIVmac239, ERHYLM<sub>195</sub> in many SIVmac251 isolates). We introduced the E<sub>191</sub>R variant along with the H<sub>196</sub>Q onto the SIVmac239 backbone to assess tetherin downregulation. Viruses harboring H<sub>196</sub>Q alone were largely deficient in tetherin downregulation, as expected. When E<sub>191</sub>R was introduced along with H<sub>196</sub>Q, the resulting virus showed full competency in tetherin downregulation, similar to SIVmac239 (Fig



**Fig 2. The E<sub>191</sub>R variant restores tetherin downregulation lost with the H<sub>196</sub>Q variant in SIVmac239.** (a) Representative flow cytometric analysis of surface expression of tetherin on primary CD4 T cells infected with wild type SIVmac239 or SIVmac239 harboring the H<sub>196</sub>Q variant alone or in combination with the E<sub>191</sub>R variant. Cells were identified as infected via intracellular Gag p27 staining, as we have described previously [30, 31]. Surface expression of tetherin compared between infected cells (orange line) and uninfected (blue line) are shown in the bottom panels. (b) N-fold analysis of tetherin downregulation from multiple experiments using cells derived from at least three different RM and compared by way of a two-tailed t-test. The asterisk indicates  $p < 0.05$  for the t-test. (c) Representative flow cytometry plots showing surface expression of rhesus CD4 in SupT1 cells with human CD4 removed by CRISPR and rhesus CD4 stably expressed, which were transfected with plasmids expressing wild type Nef or Nef with the aforementioned mutants. (d) N-fold analysis of CD4 downregulation in three separate experiments.

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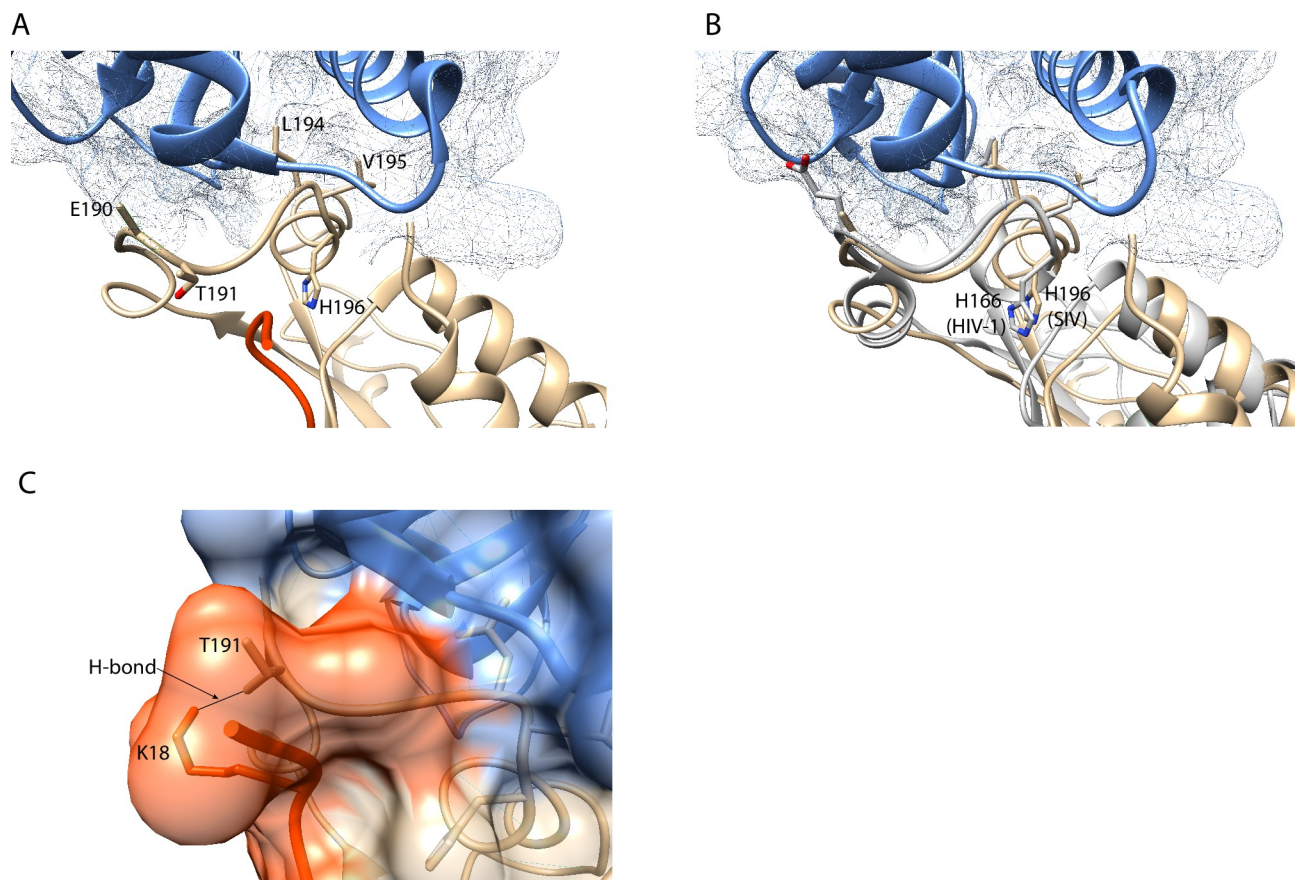
2A). N-fold analysis of tetherin downregulation in cells from multiple animals demonstrated significant loss of downregulation in the virus harboring only H<sub>196</sub>Q, while the addition of E<sub>191</sub>R restored this ability to wild type levels (Fig 2B).

We next assessed whether the R<sub>191</sub> residue also compensated for loss of other AP-2 dependent functions. Assessing Nef-mediated downregulation of CD4 in infected cells is complicated by the fact that Envelope also interacts with CD4 and reduces its surface expression. Thus, we transfected a Sup-T1 cell line that expresses rhesus CD4 and introduced Nef expression constructs with and without the Q<sub>196</sub> and R<sub>191</sub> residues. Although the results were not as clear as with tetherin in infected cells, we found that Q<sub>196</sub> compromises Nef's ability to downregulate CD4, as expected, and that the introduction of R<sub>191</sub> at least partially compensated for

this deficit (Fig 2C). This trend was not significant as measured by n-fold downregulation (Fig 2D).

### Structural insights

The structure of SIVsm Nef bound to simian AP-2 was recently published [37]. We used UCSF Chimera structural analysis software [38] to assess how the residues at positions 191 and 196 interact with host AP-2 and tetherin molecules. The critical residues in the dileucine motif [E<sub>190</sub>, L<sub>194</sub>, V<sub>195</sub> (M<sub>195</sub> in SIVmac239)] show clear interaction with AP-2, while H<sub>196</sub> is oriented in the opposite direction (Fig 3A), similar to H<sub>166</sub> in HIV-1 (Fig 3B) [29], which is homologous to H<sub>196</sub> in SIV. We next used the Rotamers function in UCSF Chimera to determine whether the H<sub>196</sub>Q variant impacted interactions with AP-2. Replacement of the H with a Q at this position resulted in a large number of possible rotamers for Q<sub>191</sub>, nearly all of which maintained a similar orientation as H<sub>191</sub>, directed away from AP-2, suggesting no obvious impact on the interaction between Nef and AP-2. However, the H<sub>196</sub>Q variant is predicted to disrupt a salt bridge between H<sub>196</sub> and tetherin residue D<sub>15</sub> as assessed using PISA (Proteins, Interfaces, Structures, and Assemblies) software [42], suggesting disruption of a direct interaction between Nef and tetherin may contribute to the selective disadvantage of this change.



**Fig 3. Structural insights into Nef, AP-2, and tetherin interactions.** (a) Although adjacent to the ExxxLM motif that directly binds Nef to AP-2, H196 is oriented away from this interaction. AP-2 is shown in blue, Nef in gold, tetherin in orange. (b) Alignment between SIVsm (gold) and HIV-1 Nef (PDB: 4NEE) (silver) with H<sub>166</sub> (HIV-1) and H<sub>196</sub> (SIVsm) highlighted. AP-2 is shown in blue. (c) T<sub>191</sub> in SIVsm directly interacts with K<sub>18</sub> in the DIWK motif of tetherin via a hydrogen bond.

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Position 191 is a T in SIVsm, as opposed to E<sub>191</sub> in SIVmac239. While this residue does not contact AP-2, intriguingly, it does interact directly with the K<sub>18</sub> residue in the DIWK motif of the tetherin protein itself via a hydrogen bond (Fig 3C). Replacement of T<sub>191</sub> with an E (as in SIVmac239) maintained the predicted hydrogen bond with K<sub>18</sub>, suggesting this interaction holds true between SIVmac239 and tetherin. Further, replacement of E<sub>191</sub> with an R resulted in only low probability orientations, preventing a meaningful analysis of this structural change.

### In vivo stability of the Q<sub>196</sub> residue

Finally, we wished to assess the in vivo stability of the Q<sub>196</sub> residue. We hypothesized that since the combination of Q<sub>196</sub> and R<sub>191</sub> residues allowed for efficient tetherin downregulation in vitro, that Q<sub>196</sub> would be stable in vivo when it exists in combination with R<sub>191</sub>. We used publicly available sequences from a recent study wherein macaques were infected with an stock of SIVmac251 that harbored virus with nearly 90% containing the combination of Q<sub>196</sub> and R<sub>191</sub> [41] based on our analysis of their deposited sequences. That study used a modified Single Genome Amplification (SGA) method to quantify viral variation in plasma throughout infection and multiple neurological tissues at necropsy. After infection, the Q<sub>196</sub> residue was detectable primarily at three weeks post infection, with the exception of a small number of reads that contained Q<sub>196</sub> at 3 months. This residue was thereafter lost in all three animals and was not detected in any neurological sites in any animals at necropsy (meninges, parietal lobe, temporal cortex) (data available in the cited manuscript and in their deposited sequences).

## Discussion

The SIVmac251 viral swarm is pathogenic in rhesus macaques and has been used in hundreds of studies to date. However, this swarm has been independently grown in many labs using multiple cell types and under a variety of conditions [40]. It stands to reason that there may be genetic differences between SIVmac251 viral stocks leading to unique biological differences, but few of these differences have been characterized for how they impact specific virologic properties, including the downregulation of host tetherin.

The ability to downregulate host tetherin is a feature of a wide variety of enveloped viruses ranging from Ebola to HIV [43, 44]. Most SIV isolates use the viral Nef protein to perform this task [13, 45, 46] but several isolates use alternate pathways, suggesting strong selection to maintain this function. Surprisingly, Nef encoded by SIVcpz cannot downregulate human tetherin and studies suggest that evolution of HIV-1 Vpu to gain the ability to downregulate tetherin was a critical event in the HIV-1 epidemic [45, 47]. Thus, countering tetherin likely is an important feature of all or nearly all SIV and HIV isolates.

SIV Nef mediated downregulation of several host proteins, including tetherin, involves interactions between Nef and AP-2 proteins. We previously showed that viral evolution to escape host CTL responses can lead to a compromised capacity to perform important functions, such as the downregulation of tetherin and other host proteins that require Nef interactions with AP-2. One of the Nef variants with the greatest impact on tetherin downregulation was the H<sub>196</sub>Q variant. We were surprised to find that many isolates of SIVmac251 harbor a Q<sub>196</sub> residue. In this study we sought additional variants in SIVmac251 that might allow for tetherin downregulation in the presence of Q<sub>196</sub>. We identified an upstream residue, R<sub>191</sub>, that was present in all sequences that encoded Q<sub>196</sub>. This was the only variant with a perfect correlation with the Q<sub>196</sub> residue. When introduced into SIVmac239 in the presence of Q<sub>196</sub>, R<sub>191</sub> restored the ability to downregulate tetherin and at least partially restored the ability to downregulate CD4, lost in the presence of Q<sub>196</sub>. Since we cannot determine the evolutionary



patterns that gave rise to both of these variants in SIVmac251, we cannot state with certainty that R<sub>191</sub> evolved specifically to compensate for functions lost with the Q<sub>196</sub> residue. However, our data clearly show that the presence of R<sub>191</sub> restores tetherin downregulation lost with the Q<sub>196</sub> residue and, thus, may have evolved in such a manner. Interestingly, the R<sub>191</sub> residue was detected in a small number of SIV isolates in the Los Alamos database. However, these diverse isolates represent thousands of years of evolution and the presence of the R<sub>191</sub> cannot be readily explained.

In addition to interactions with AP-2, SIV Nef is known to interact directly with the tetherin protein [48] and a subset of those interactions were recently verified structurally [37]. These structures show that H<sub>196</sub> does not directly interact with host AP-2 but is predicted to form a salt bridge with tetherin, which is predicted to be disrupted in the H<sub>196</sub>Q variant using PISA software [42]. However, our previous report showed that the H<sub>196</sub>Q variant disrupted multiple Nef functions that rely on AP-2 interactions suggesting that disruption of a direct interaction with tetherin likely does not fully explain the functional deficits identified in this variant. Here, we show that evolution of the E<sub>191</sub>R variant restored tetherin downregulation in the presence of Q<sub>196</sub>. Intriguingly, T<sub>191</sub> in SIVsm interacts directly with the lysine in the DIWK motif in the tetherin protein [37], suggesting variation at this residue may impact tetherin downregulation via a direct effect on this interaction. E<sub>191</sub> in SIVmac may also interact with this K<sub>18</sub> residue as these two amino acids are well known to form hydrogen bonds, although we cannot confirm without structural data.

Many strains of SIVmac251 encode a Nef protein with a Q<sub>196</sub> residue, which is always linked to an upstream R<sub>191</sub> residue. Here we show that the presence of R<sub>191</sub> fully restores competency in downregulation of tetherin in the presence of Q<sub>196</sub>. Nonetheless, our data also suggest that Q<sub>196</sub> is not stable in vivo and evolves to H<sub>196</sub>, the residue present in nearly all SIV isolates. These data beg the question of how the Q<sub>196</sub> residue arose in the first place. It's possible it arose during replication in cultured cells where selection pressures are undoubtedly different than those the virus experiences in vivo. Given our data suggesting the H<sub>196</sub>Q variant can arise in vivo in SIVmac239 infected macaques as a result of escape from CTL responses [31], these data may suggest that SIVmac251 was isolated from an animal that targeted this region with CTL, leading to viral escape, and prior to other escape variants becoming dominant, as happened in our previous study [31].

Taken together, our mutational and functional data combined with published structural and sequence data suggest the possibility that the E<sub>191</sub>R variant in SIVmac might enhance an interaction between Nef and tetherin thus restoring the ability of Nef to downregulate tetherin in the presence of the H<sub>196</sub>Q variant, but that this variant may not restore all functions that are impacted by the H<sub>196</sub>Q variant. Here, we show that E<sub>191</sub>R at least partially restores the ability to downregulate CD4, which is also lost with the H<sub>196</sub>Q variant suggesting that E<sub>191</sub>R does more than just enhance interaction with tetherin. However, we also found that Q<sub>196</sub> evolves to H<sub>196</sub> in vivo, even in the presence of R<sub>191</sub>, suggesting that R<sub>191</sub> does not fully compensate for all lost function associated with Q<sub>196</sub>. Our mining of publicly available sequences showed that R191 and Q196 were always detected together in isolates of SIVmac251, until Q196 was lost in vivo. However, sequences of diverse SIV isolates showed that R191 was present in some strains of SIV, even in the absence of Q196. These data may suggest that R191 is not a compensatory variant per se, but just a potential residue for this location in the protein. Nonetheless, our data clearly show that introduction of R191 restores tetherin downregulation function in the presence of Q196 whether it evolved to do so or not.

The existence of compensatory variation in viral proteins has been described in SIV and HIV-1 [49–52] but those descriptions are restricted to viral structural proteins, primarily Gag. Our data suggest R<sub>191</sub> in SIVmac251 may exist to compensate for loss of function associated

with Q<sub>196</sub>. If so, this may be the first report of compensatory variation in the viral Nef protein or any nonstructural viral protein. However, our analysis of published *in vivo* data clearly demonstrate that Q<sub>196</sub> evolves to H<sub>196</sub> *in vivo*. Finally, our data do not suggest that stocks of SIVmac251 that harbor a Q<sub>196</sub> residue are in any way less useful than stocks that do not. Instead, our data underscore the need to understand the evolutionary pressures that give rise to particular viral variants, which may be relevant in the choice of virus stock for animal model experiments.

## Author Contributions

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