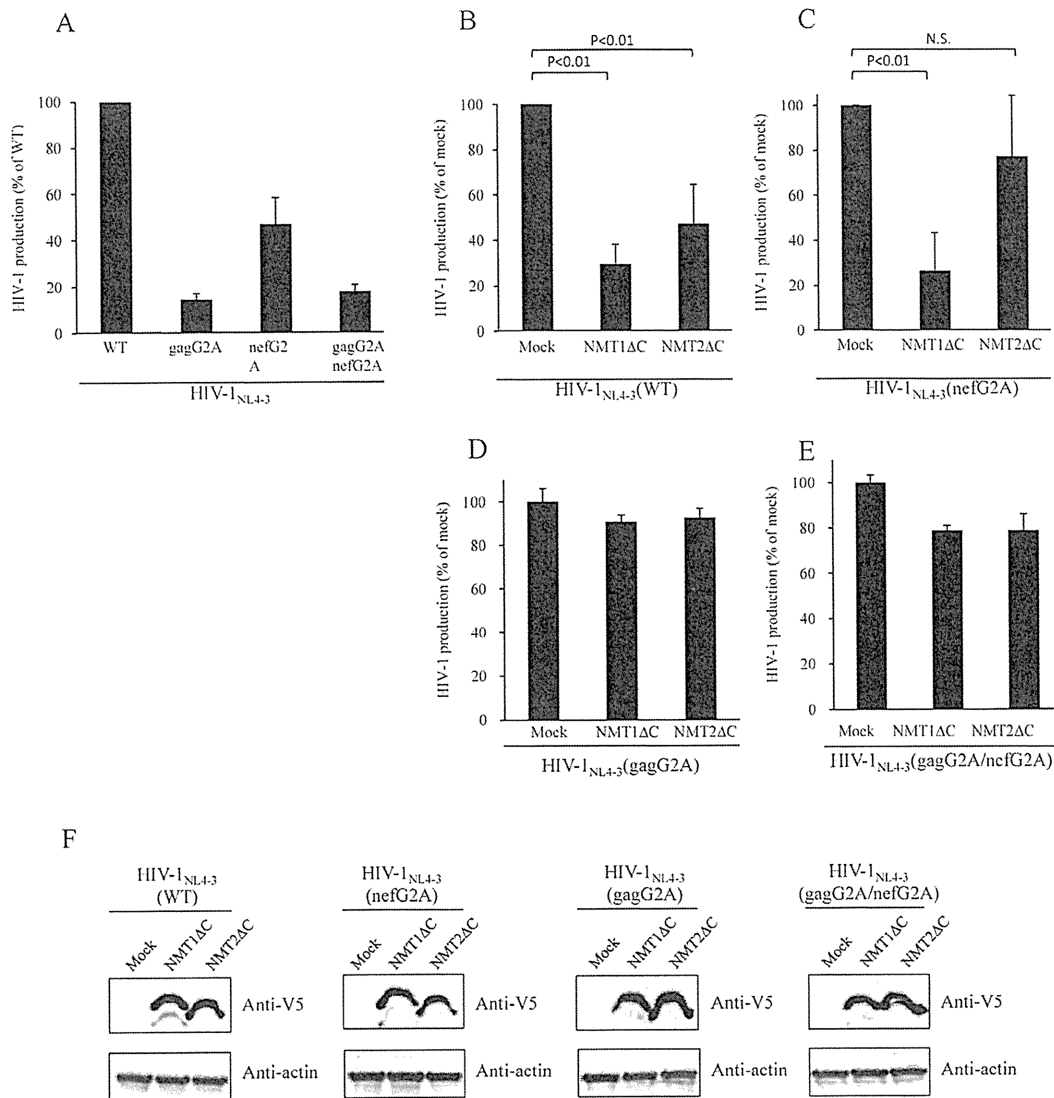

Fig. 4. Effect of NMT Mutants on WT and Nef-deficient HIV-1 Productions

HEK293 cells were transfected with proviral DNA for HIV-1 WT, HIV-1 gagG2A, HIV-1 nefG2A, or HIV-1 gagG2A/nefG2A. Forty-eight hours post-transfection, the level of virus production in the supernatant was measured by p24 ELISA (A). HEK293 cells expressing NMT1ΔC, NMT2ΔC, or a mock vector were transfected with proviral DNA for HIV-1 WT (B), HIV-1 nefG2A (C), HIV-1 gagG2A (D), or HIV-1 gagG2A/nefG2A (E). Forty-eight hours post-transfection, the level of virus production in the supernatant was measured by p24 ELISA. NMTΔC mutants and actin were detected by Western blot analysis (F). Each bar represents the mean standard deviation ($n=3$). $*p$ was calculated using Welch's t-test. N.S.: not significant.

proposed that the host factors required for HIV-1 replication could also become potential therapeutic targets.[23] The *N*-myristoylation of both $Pr55^{gag}$ and Nef is almost completely conserved in extremely diverse HIV-1, suggesting that the inhibition of NMT, especially the $Pr55^{gag}$-associated ribosomal NMT1 isozyme, is one of the most attractive targets against HIV-1. Since it is predicted that approximately 0.5% of all proteins in the human genome are *N*-myristoylated,[24] it should be investigated how the inhibition of targeting of NMT to ribosome could affect the other host *N*-myristoylated proteins, for example *src* family kinases and small G proteins. To accomplish such a strategy, the specific blockage of NMT isozyme targeting to the ribosome could become a novel therapeutic strategy for HIV-1 diseases.

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Title

Induction of extremely low protein expression level by fusion of *C*-terminal region of Nef

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Running title
Induction of extremely low protein expression level

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Synopsis

Nef is one of the accessory proteins of human immunodeficiency viruses. Here, we noted that the relative expression level of Nef_{NL4-3} is much lower than that of Nef_{JR-CSF} in HEK293 cells. By evaluating the expression level using a Nef mutant, it was indicated that amino acids 129-206 of Nef_{NL4-3}, i.e., the *C*-terminal region named NLAA129-206, could contain the region responsible for the induction of the low protein expression level. Additionally, the expression levels of the enhanced green fluorescent protein (EGFP) and *Renilla* luciferase (Rluc) became extremely low with the fusion of NLAA129-206, Interestingly, the NLAA129-206-corresponding sequences of other Nef variants with relatively high expression levels also induced the extremely low protein expression level by fusion. These results suggest that the *C*-terminal region of Nef can generally induce an extremely low protein expression level. Here, we propose that the *C*-terminal region of Nef could become an excellent tool for the induction of an extremely low expression level of arbitrary proteins by attachment as fusion proteins.

Footnotes

Key words: enhanced green fluorescent protein (EGFP), human immunodeficiency virus (HIV), Nef, protein degradation sequence, *Renilla* luciferase (Rluc).

Abbreviations used: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; HEK293, human embryonic kidney 293; EGFP, enhanced green fluorescent protein; Rluc, *Renilla* luciferase; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UPS, ubiquitin proteasome system

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Introduction

Nef, a 27-35-kDa protein, is one of the accessory proteins of human and simian immunodeficiency viruses (HIV and SIV, respectively) that enhance viral replication and is associated with the pathogenicity of these viruses. It has many functional motifs for contact to host proteins [1], by which it can serve as a molecular adaptor and exert multiple functions like CD4 downregulation and MHC class I downregulation [2]. Moreover, Nef can enhance viral infectivity, although the mechanism underlying such enhancement remains unclear [3]. *N*-myristoylation occurs at the *N*-terminus of Nef [4], and the posttranslational modification is essential for multiple functions [2].

Genetic diversity is one of the major characteristics of HIV and SIV [5]. We can find highly frequent mutations of amino acid substitution, insertion, and deletion among viral strains in the Los Alamos HIV database (http://hiv-web.lanl.gov). Such a genetic diversity can generate many viral phenotypes, resulting in, for example, CCR5 or CXCR4 usage [6], escape from the immune attack of the host [7], and the emergence of drug-resistant viruses [7].

The abundance of each protein is closely associated with the efficacy of its function, because protein activity is basically exerted in a dose-dependent manner. Protein expression level depends not only on mRNA level but also on translation rate and degradation rate [8, 9]. Protein degradation comprises two major systems: ubiquitin-mediated proteolysis and lysosomal degradation [10].

Some degradation signals conferring instability on proteins have been found, which include N-degrons [11], a murine ornithine decarboxylase (MODC) PEST region [12], and CL peptides [13]. These signals induce a rapid protein degradation mediated by a proteasome, in which the N-degron and CL peptides

require ubiquitination prior to degradation, while the PEST sequence is independent of ubiqutination [14]. The PEST sequence and CL peptides could convert stable proteins into unstable proteins by attachment as fusion proteins [13, 15, 16], of which the apparent expression levels could be much lower than those of the original proteins [15-17]. The feature of the instability induction of the PEST sequence and CL peptides has been applied to the development of a highly responsive reporter system [18-20] and to the improvement of the recombinant protein productivity of CHO cells [21]. In addition to protein degradation signals, mRNA-destabilizing elements [22-24] are utilized for such a system [18].

Here, we clarified that the *C*-terminal region (amino acids 129-206) of Nef_{NL4-3} with an extremely low expression level is necessary and sufficient for the induction of the low expression level of reporter proteins by attachment as fusion proteins. Additionally, it was indicated that the *C*-terminal regions of not only Nef_{NL4-3} but also other Nef variants, which even show relatively higher expression levels, have an ability to induce low protein expression levels by attachment. The mechanism of this induction has not been fully resolved yet. We propose that the *C*-terminal region of Nef is applicable to the development of a highly responsive reporter system and to the improvement of recombinant protein productivity.

Materials and Methods

Nef expression vectors

DNAs coding Nef proteins were amplified by PCR using the corresponding proviral DNA template and subcloned into pcDNA^{TM}3.1D/V5-His TOPO according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

DNA coding Nef_{JR-CSF}-V5 or Nef_{NL4-3}-V5 was amplified by PCR using pcDNA3.1/Nef_{JR-CSF}-V5 and pcDNA3.1/Nef_{NL4-3}-V5, respectively, and subcloned into the pcDNA4/HisMax vector (Invitrogen, Carlsbad, CA) without polyhistidine and the Xpress^{TM} epitope-coding region. The expression vector for the Nef chimera was generated by standard overlapping PCR techniques [25].

The expression vector for each Nef Gly 2-to-Ala 2 (G2A) mutant was constructed by site-directed mutagenesis, as previously described [26].

Expression vectors of enhanced green fluorescent protein (EGFP) or *Renilla* luciferase (Rluc) fusion protein

Each expression vector for EGFP appended with each amino acid sequence to the *N*-terminal end was constructed using pEGFP-N1 (Clontech, Mountain View, CA), in which a triple repeat of the linker Gly-Gly-Gly-Gly-Ser [$(GGGGS)_3$] [27] and Xpress-epitope-tag-coding DNAs were respectively inserted at the *Sal*I-*Apa*I and *Hin*dIII-*Pst*I sites. Each expression vector for Rluc was constructed by replacing the EGFP-coding DNA with the Rluc-coding DNA of the multiple cloning site (MCS) of each EGFP expression vector. Each expression vector for EGFP appended with each amino acid sequence to the *C*-terminal end was constructed using a pcDNA4/HisMax vector, in which EGFP, the $(GGGGS)_3$ linker, and DNAs coding each amino acid sequence were respectively inserted in the *Kpn*I-*Bam*HI, *Bam*HI-*Eco*RI, and *Eco*RI-*Pst* I sites of the MCS of the vector.

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were cultured and transfected using Lipofectamine LTX reagent, as previously described [26].

HEK293/CD4/Nef_{JR-CSF}, HEK293/CD4/Nef_{NL4-3}, HEK293/CD4/Nef_{JR-CSF} G2A, and HEK293/CD4/Nef_{NL4-3} G2A cells

HEK293/CD4 cells were transfected with the pcDNA4/HisMax vector for Nef_{JR-CSF}, Nef_{NL4-3}, Nef_{JR-CSF} G2A, and Nef_{NL4-3} G2A. 2 days after transfection, stable clones were selected in the presence of 500 μg/ml zeocin in the conditioned medium. Each clonal HEK293/CD4/Nef cell line was obtained by the limiting-dilution method.

Immunostaining analysis

The cells were rinsed and fixed with 1% fresh paraformaldehyde in PBS(-). After permeabilization with methanol, Nef was visualized using an anti-V5 antibody, followed by an anti-mouse-FITC secondary antibody. The nucleus was stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). The cells were observed using a Biozero digital microscope (Keyence, Osaka, Japan).

Cell lysis and western blot analysis

The cells were lysed in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, protease inhibitors [1 mM 4-amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF), 50 μg/ml aprotinin, 50 μg/ml leupeptin, 50 μg/ml pepstatin A, 50 μg/ml antipine]) and subjected to 5-20% polyacrylamide gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, separated proteins on the gel were transferred to the polyvinylidene fluoride (PVDF) membrane. The membranes blocked by 5% skim-milk were incubated with an anti-V5 antibody or anti-Xpress antibody (Invitrogen, Carlsbad, CA) diluted 1:5000 in immunoenhancer reagent A (Wako, Osaka, Japan) for 4 h, and then with peroxidase (POD) conjugated anti-mouse IgG in immunoenhancer reagent B (Wako, Osaka, Japan) for 1

h. Then, the specific signals were observed using chemiluminescent substrate (Thermo Fisher Scientific inc., Waltham, MA) and LAS4000 (GE Healthcare, Buckingham, England), as previously described [28]. The intensities of the bands were quantified with Fujifilm Image Gauge Software.

Rluc assay

HEK293 cells were transiently transfected with each Rluc-fusion-protein expression vector and cultured for 48 h. The harvested cells (1 x 10^5 cells) were lysed with the cell culture lysis reagent (Promega, Madison, WI). To measure Rluc activity, each lysate was transferred to a 96-well white microplate and coelenterazine h [29] was added at a final concentration of 5 μM. Then, luminescence was measured simultaneously using a Wallac ARVO™ SX 1420 luminometer (Perkin-Elmer, Waltman, MA).

Flow cytometry

HEK293/CD4/$Nef_{JR\text{-}CSF}$, HEK293/CD4/$Nef_{NL4\text{-}3}$, HEK293/CD4/$Nef_{JR\text{-}CSF}$ G2A, and HEK293/CD4/$Nef_{NL4\text{-}3}$ G2A cells were washed with PBS and then suspended in a cold washing buffer (PBS containing 2% FCS and 0.02% NaN_3) containing a phycoerythrin (PE)-conjugated anti-CD4 antibody. After 30 min of incubation at 4°C, the cells were washed three times and then analyzed using an EPICS XL flow cytometer (Beckman Coulter, Brea, CA).

Quantification of mRNA levels by RT-qPCR analysis

HEK293 cells were transfected with the $Nef_{NL4\text{-}3}$, $Nef_{JR\text{-}CSF}$, AA129-206-EGFP, CP-EGFP, or EGFP expression plasmid. Total RNA was extracted from these HEK293 cells using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) in accordance with to the manufacturer's instruction. First-strand cDNA synthesis was performed using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. DyNAmo™ HS SYBR®Green qPCR kit (FINNZYMES, Espoo, Finland) reagents were used as quantitative real-time PCR reagents according to the manufacturer's instruction. Thermocycling was carried out using the DNA Engine OPTICON®2 system (MJ Research, Inc, Waltham, MA). mRNA level was normalized to the transcript of the neomycin resistance gene, which is coded in the expression vector used. The oligonucleotide primers used for the PCR were as follows: a V5 tag sense primer, TCCTCGGTCTCGATTCTACG; a V5 tag antisense primer, TGGATCCTGGTACTCAATGGT; an EGFP sense primer, ACGTAAACGGCCACAAGTTC; an EGFP antisense primer, AAGTCGTGCTGCTTCATGTG; a NeoR sense primer, AGACAATCGGCTGCTCTGAT; and a NeoR antisense primer, AGTGACAACGTCGAGCACAG.

Results and Discussion

Detection of expression diversity of Nef proteins from small subset of HIV-1 and SIV

In the beginning of this study, we constructed expression vectors of Nef from HIV-$1_{NL4\text{-}3}$, HIV-$1_{JR\text{-}CSF}$, HIV-$1_{YU\text{-}2}$, HIV-$1_{89.6}$, and SIV_{mac239} for mammalian cells, which were appended to a V5 epitope tag at the *C*-terminal end for detection. The expression pattern of each Nef was examined at the same time. HEK293 cells transiently expressing each Nef were lysed and subjected to SDS-PAGE, followed by western blot analysis using an anti-V5 antibody, as described in Materials and Methods. As shown in Fig. 1A, the expressions of each Nef variant from HIV-$1_{NL4\text{-}3}$, HIV-$1_{JR\text{-}CSF}$, HIV-$1_{YU\text{-}2}$, HIV-$1_{89.6}$, and SIV_{mac239}, were observed, in which the molecular weights of the variants were expectedly detected as 27, 29, 28, 27, and 34 kDa, respectively. Then, in spite of the fact that the same conditions in terms of the type of cell, the amount of DNA for transfection, the expression vector with the CMV promoter, and the V5 epitope for detection were used, the diversity of the expression level among the five Nef's was observed (Figure 1A). We paid attention to the heterogeneity of the expression level. We again examined the expression properties of $Nef_{NL4\text{-}3}$ and $Nef_{JR\text{-}CSF}$, respectively showing the lowest and highest expression levels, using clones of HEK293/CD4 cells stably expressing each Nef. For the expression of these proteins, the pcDNA4/HisMax vector was used, which codes for a strong translational enhancer element [30] upstream of the ATG initiation codon of Nef, and by which an increase in Nef expression level is expected. As shown in Figure 1B, the marked differences between $Nef_{NL4\text{-}3}$ and $Nef_{JR\text{-}CSF}$ were reproducibly observed among the clones tested by western blot analysis; the expression levels of $Nef_{JR\text{-}CSF}$ were more than tenfold those of $Nef_{NL4\text{-}3}$. Relatively low expression levels of $Nef_{NL4\text{-}3}$ were still observed, although the vector with the translational enhancer element was used.

We also examined the expression property by immunostaining HEK293/CD4 cells stably expressing Nef. Nef and the nucleus were respectively stained with an anti-V5 antibody and DAPI, simultaneously. Then, it was verified at a glance that all the cells expressed Nef in both clones (Figure 2). $Nef_{NL4\text{-}3}$ was predominantly detected in some of the focused areas, especially in the perinuclear region (Figure 2, top panels), which is a typical pattern of the subcellular localization of Nef [31, 32]. The regions seemed to include the *trans*-Golgi network, as previously reported [31, 32], although we have not checked for the marker by double staining. On the other hand, $Nef_{JR\text{-}CSF}$ was detected in not only the perinuclear area but also other cytosolic areas with a relatively higher fluorescence (Figure 2, bottom panels). The difference in expression level in the western blot analysis between $Nef_{JR\text{-}CSF}$ and $Nef_{NL4\text{-}3}$ (Figure 1B) was reflected

in the result of the immunostaining of Nef_{JR-CSF} and Nef_{NL4-3} (Figure 2).

Altogether, it was indicated that the extreme difference in expression level between Nef_{JR-CSF} and Nef_{NL4-3} is not due to experimental artifacts but due to the property of each Nef. Furthermore, the diversity of the Nef expression level is plausible, although more Nef clones should be examined to draw an unequivocal conclusion. Previously, Hartz et al. have reported that Nef from the HIV-1_{lai} strain shows a low expression level in COS-1 cells [33], although they have not evaluated relative expression levels among some Nef variants. At the least, a considerable difference in the level between Nef_{NL4-3} and Nef_{JR-CSF} in HEK293 cells was confirmed in these experiments.

To confirm whether both Nef_{NL4-3} and Nef_{JR-CSF} expressed in HEK293/CD4 cells are functional, their CD4 downregulation activity, which is the main function of Nef, was evaluated. As shown in Figure 3, the CD4 downregulation activities of Nef_{NL4-3} and Nef_{JR-CSF} were clearly observed. The activity of Nef_{NL4-3} was less than that of Nef_{JR-CSF}, in which the mean fluorescent intensities (MFIs) indicating the CD4 level in two Nef_{NL4-3}-expressing cell clones were 0.65 and 0.63, whereas those in two Nef_{JR-CSF} expressing cell clones were 0.18 and 0.12. The *N*-myristoylation at the *N*-terminus of Nef is essential for the CD4 downregulation activity [2]. To verify whether CD4 downregulation in HEK293/CD4 cells is induced by the expressed Nef's, clones of HEK293/CD4 cells expressing non-myristoylated G2A mutants of Nef_{NL4-3} or Nef_{JR-CSF} were established and the CD4 levels were evaluated. Each G2A non-myristoylated mutant of Nef_{NL4-3} or Nef_{JR-CSF} expectedly showed no CD4 downregulation. It is clear that both Nef_{NL4-3} and Nef_{JR-CSF} are functional in HEK293/CD4 cells and that a high expression level of Nef is associated with efficient CD4 downregulation.

Characterization of low expression property of Nef

To determine which region of Nef_{NL4-3} is responsible for the low expression level, a chimera Nef expression vector was constructed. The primary structure of the chimera Nef tested was as follows: the first half-sequence, amino acids (AA) 1-139, was from Nef_{JR-CSF} and the second half-sequence, AA129-206, was from Nef_{NL4-3} (Figure 4A). HEK293 cells were transfected with each Nef expression vector and subjected to western blot analysis. As shown in Figure 4B, the expression level of the chimera Nef was almost the same as that of Nef_{NL4-3}. According to the quantification of the intensities of the bands with Fujifilm Image Gauge Software, the expression level of the chimera Nef was about 10% of that of Nef_{JR-CSF}. This result suggests that AA129-206 of Nef_{NL4-3}, named NLAA129-206, is required for the low expression level.

To examine whether the NLAA129-206 is not only required but also sufficient for the induction of the low protein expression level, the region was appended to the *C*- or *N*-terminal end of the enhanced green fluorescent protein (EGFP) [34, 35]. We used the combination of two protein degradation sequences, CL1 and PEST [12, 15, 36], namely, the CP sequence as a positive control of the induction of the low protein expression level. The CP sequence can induce a strong proteasome-mediated protein degradation by fusion, resulting in a low protein expression level [17, 37] We appended the NLAA129-206 to the *C*-terminal end of EGFP (Figure 4C). The $(GGGGS)_3$ linker was inserted between EGFP and each appended amino acid sequence (Figures 4C). It has been reported that the linker is sufficiently long and flexible to retain the function of two proteins at both ends [27]. HEK293 cells were transiently transfected with each expression vector and cultured for 48 h. Each cellular lysate was subjected to western blot analysis as described in Materials and Methods. As shown in Figures 4D and E, the expression levels of the NLAA129-206-fused EGFPs were extremely lower than that of wild-type EGFP in the *C*- and *N*-terminal fusions. Furthermore, the expression levels of the EGFPs fused by NLAA129-206 were clearly lower than those of the EGFP fused by the CP sequence (Figures 4D). The β-actin levels were almost the same among the lines, showing that the amounts of loaded proteins were almost the same among the lines. Another very small band in addition to the main one was observed in EGFP (asterisk in Figure 4D), which is not identified but thought to be EGFP with posttranslational modifications. These results suggest that NLAA129-206 has a very high potential for inducing the low protein expression level by fusion. Additionally, the potential of NLAA129-206 seems to be brought out more effectively in the case of fusion to the *N*-terminal end of EGFP than in the case of fusion to the *C*-terminal end of EGFP.

To examine whether the induction of the low protein expression level by the fusion of the sequence occurs in not only EGFP but also other proteins, Rluc [29] was chosen for the experiment. Since the fusion of NLAA129-206 to the *N*-terminal end of EGFP was more effective than that to the *C*-terminal end for the induction of the low protein expression level (Figures 4D and E), the NLAA129-206 and CP sequences were appended to the *N*-terminal of Rluc for examination (Figure 4C). The expression level of Rluc could be evaluated by measuring bioluminescent activity [29]. Transiently, HEK293 cells expressing each Rluc were lysed and their Rluc activity was measured, as described in Materials and Methods. As shown in Figure 4F, the activity of NLAA129-206-fused Rluc was much lower than that of wild-type Rluc, whose result was identical to those of EGFP (Figures 4D and E). These results suggest that the induction of the low protein expression level by NLAA129-206 fusion universally occurs in all arbitrary proteins.

To investigate the minimum sequence of NLAA129-206 for inducing the low protein expression level, the expression levels of the three deletion mutants (NLAA142-206, NLAA129-186, and NLAA142-186) shown in Figure 5A were examined. HEK293 cells were transiently transfected with each expression vector and cultured for 48 h. Each cellular lysate was subjected to western blot analysis for EGFP fusion proteins and to the measurement of bioluminescent activity for Rluc fusion proteins, as described in Materials and Methods. As shown in Figure 5B, partial recovery of the expression levels was observed in both the *N*- and *C*-terminal-region-deleted mutants, namely, NLAA142-206-EGFP and NLAA129-186-EGFP, respectively. The *N*- and *C*-terminal-region-deleted mutant NLAA142-186-EGFP showed almost full recovery of its expression level, similarly to the original EGFP. Almost identical profiles of the expression pattern in Figure 5B were observed in the comparison of activity among the deletion-mutant-fused Rluc's (Figure 5C). These results suggest that the complete sequence of NLAA129-206 is required for the induction of the lowest protein expression level. It is thought that the stepwise reduction levels established by the attachment of NLAA129-206 and the mutants would become a useful repertory for inducing the desired expression of arbitrary proteins.

The NLAA129-206 regions of the Nef_{NL4-3}-corresponding sequences of Nef_{JR-CSF} and Nef_{mac239} were named JRAA139-216 and macAA161-263, respectively. The expression levels of Nef_{JR-CSF} and Nef_{mac239} were high among the five Nef's (Figure 1A). Hence, do the JRAA139-216 or macAA161-263 fusion proteins also show relatively high expression levels? The sequence-fused EGFP and Rluc (Figure 6A) expression levels were respectively examined by western blot analysis and Rluc assay. As shown in Figures 6B and C, unexpectedly, but interestingly, JRAA139-216- or macAA161-263-fused EGFP and Rluc also showed extremely low expression levels as in the cases of NLAA129-206. These results suggest that all Nef variants generally have an extremely low expression property in the *C*-terminal region. Since the chimera Nef, i.e., AA1-139 of Nef_{JR-CSF} plus NLAA129-206, showed a low expression level similarly to that of Nef_{NL4-3} (Figures 4B), it is also speculated that the contribution of AA1-139 of Nef_{JR-CSF} to the entire Nef stabilization could be restrictive to the combination of JRAA139-216.

The case with the $(GGGGS)_3$ linker resulted in a much lower expression level of EGFP by the fusion of NLAA129-206 than the case without the linker (data not shown), suggesting that the independence of NLAA129-206 from EGFP generated by the flexibility of the linker is important for the induction of the low expression level. Taking this result into consideration, JRAA139-216 might not show independence from AA1-139 of Nef_{JR-CSF} or express the potential ability to induce the low expression level, which may result in a high expression level of Nef_{JR-CSF}.

Since macAA161-263 could also induce extremely low expression levels of both EGFP and Rluc, the low expression property might be universal in Nef of not only HIV-1 but also SIV. The examination of a more comprehensive set of Nef variants might be necessary to unequivocally conclude the low expression property of the *C*-terminal region of Nef.

Examination of mRNA levels and effect of proteasome inhibitor on expression levels

What is the mechanism underlying the induction of the low protein expression level by the fusion of NLAA129-206? First, the mRNA level in HEK293 cells transfected with each DNA was quantified by reverse-transcription real-time quantitative PCR analysis [38]. mRNA level was normalized to the transcript of the neomycin resistance gene, which is coded in the expression vector used. Almost comparable mRNA levels between Nef_{NL4-3} and Nef_{JR-CSF}, or between EGFP and NLAA129-206-fused EGFP were observed (Figures 7A and B). These results suggest that mRNA level cannot be associated with the induction of the extremely low protein expression level.

Then, we examined protein stability. The ubiquitin proteasome system (UPS) is one of the major protein degradation machineries in eukaryotic cells [39]. Nef_{NL4-3}- or NLAA129-206-EGFP-expressing HEK293 cells were treated with the proteasome inhibitor MG132 at 20 μM for 0, 3, or 6 h. The expression level at each time point was measured by western blot analysis, which was normalized to the actin level. As shown in Figures 8A and B, increases in the apparent expression level of both proteins were slightly observed upon treatment with the inhibitor in a time-dependent manner. The results suggest that the low protein expression property of NLAA129-206 is in part due to a high rate of protein degradation mediated by the proteasome. However, it still cannot be concluded whether the mechanism for the induction of low protein expression level is associated with protein degradation rate, since such proteasome-mediated degradation may generally occur in proteins [39]. There could be other mechanisms of inducing the low protein expression level by the *C*-terminal region of Nef. Now, we are investigating the mechanism of the induction of the low protein expression level by NLAA129-206 fusion from the viewpoint of not only protein destabilization but also protein translation.

Finally, we propose that the NLAA129-206 of Nef_{NL4-3} and the corresponding regions of other Nef variants, at least from $HIV\text{-}1_{JR-CSF}$ and SIV_{mac239}, are excellent tools for inducing extremely low expression levels of arbitrary proteins by attachment as fusion proteins. Furthermore, Nef variants from HIV and SIV viruses that are very rich in genetic diversity may become useful resources for the search of regions inducing low protein expression levels. Such induction of extremely low protein expression levels

is applicable to the development of highly responsive reporter systems [18, 19, 26], to the improvement in recombinant protein productivity [19], and to other technological research.

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Figure legends

Figure 1 Detection of heterogeneity of Nef expression levels. HEK293 cells transiently expressing each Nef using the pcDNA3.1 vector (A) or two clones of HEK293/CD4 cells expressing $\mathrm{Nef}_{\mathrm{NL4\text{-}3}}$ or $\mathrm{Nef}_{\mathrm{JR\text{-}CSF}}$ using the pcDNA4/HisMAX vector (B) were lysed and subjected to 5-20% SDS-PAGE, followed by western blot analysis using anti-V5 and anti-actin antibodies as described in Materials and Methods. The basic characteristics of the vector used in A and B are depicted in the bottom of the figure. The vector used in B has a translational enhancer sequence SP163 at upstream of the Nef coding region.

Figure 2 Immunostaining of $\mathrm{Nef}_{\mathrm{NL4\text{-}3}}$ and $\mathrm{Nef}_{\mathrm{JR\text{-}CSF}}$ expressed in HEK293/CD4 cells. The HEK293/CD4/$\mathrm{Nef}_{\mathrm{NL4\text{-}3}}$ (top row) and HEK293/CD4/$\mathrm{Nef}_{\mathrm{JR\text{-}CSF}}$ (bottom row) cells, which are stable Nef-expressing clonal cell lines, were immunostained using an anti-V5 antibody and an anti-mouse-FITC secondary antibody for Nef detection. The nucleus was stained with DAPI. The cells were observed using a Biozero digital microscope. Scale bar = 50 μm (rightmost panels).

Figure 3 Comparison of CD4 downregulation activities between $\mathrm{Nef}_{\mathrm{NL4\text{-}3}}$ and $\mathrm{Nef}_{\mathrm{JR\text{-}CSF}}$. HEK293/CD4/$\mathrm{Nef}_{\mathrm{NL4\text{-}3}}$ (top row panels) and HEK293/CD4/$\mathrm{Nef}_{\mathrm{JR\text{-}CSF}}$ (bottom row panels) cells, which are stable Nef-expressing clonal cell lines, were stained with a PE-conjugated anti-CD4 antibody and then analyzed using a flow cytometer. HEK293/CD4 cells are depicted with the gray filled histogram, Nef-expressing HEK293/CD4 cells are depicted by the solid black line, and HEK293 cells are depicted in the striped histogram. Each MFI value is of the CD4 level in the cells expressing each Nef.

Figure 4 Induction of low expression property by AA129-206 of $\mathrm{Nef}_{\mathrm{NL4\text{-}3}}$
HEK293 cells were transiently transfected with each Nef, EGFP, or the Rluc fusion protein expression plasmid. The expression level of each Nef after 48-h transfection was analyzed by western blot analysis using an anti-V5 (B) or anti-Xpress antibody (D and E), as described in Materials and Methods. (A) Schematic representations of $\mathrm{Nef}_{\mathrm{NL4\text{-}3}}$, $\mathrm{Nef}_{\mathrm{JR\text{-}CSF}}$, and chimera Nef tested in "B". "B" shows a comparison of $\mathrm{Nef}_{\mathrm{NL4\text{-}3}}$, $\mathrm{Nef}_{\mathrm{JR\text{-}CSF}}$, and chimera Nef. (C) Schematic representations of EGFP, EGFP-NLAA129-206, and EGFP-CP tested in "D", EGFP and NLAA129-206-EGFP tested in "E", and Rluc and NLAA129-206-Rluc tested in "F". "D" shows a comparison of EGFP and AA129-206 or CP fusion to the *C*-terminal end of EGFP. "E" shows a comparison of EGFP and AA129-206 fusion to the *N*-terminal end of EGFP. An asterisk marks an EGFP modified with unidentified posttranslational modification. "F" shows a comparison of Rluc and AA129-206 fusion to the *N*-terminal end of Rluc. Rluc level was evaluated by measuring bioluminescent activity using coelenterazine h as the substrate, as described in Materials and Methods. Each bar represents the mean standard deviation (n = 3).

Figure 5 Comparison of low expression property of each deletion mutant.
(A) Schematic representations of NLAA129-206-EGFP or Rluc, NLAA142-206-EGFP or Rluc, NLAA129-186-EGFP or Rluc, and NLAA142-186-EGFP or Rluc tested in "A" or "B". HEK293 cells were transiently transfected with each EGFP or the Rluc fusion protein expression plasmid. The expression level of each fusion protein after 48-h transfection was analyzed by western blot analysis using an anti-Xpress antibody (B) or by measuring the bioluminescent activity of Rluc using coelenterazine h as the substrate (C) as described in Materials and Methods. Each bar represents the mean standard deviation (n = 3). Asterisks mark EGFPs modified with unidentified posttranslational modification (B).

Figure 6 Induction of low expression property by *C*-terminal region of $\mathrm{Nef}_{\mathrm{mac239}}$ and $\mathrm{Nef}_{\mathrm{JR\text{-}CSF}}$.
(A) Schematic representations of EGFP or Rluc, macAA161-263-EGFP or macAA161-263-Rluc, and JRAA139-216-EGFP or JRAA139-216-Rluc tested in "B" or "C". HEK293 cells were transiently transfected with each expression plasmid of EGFP or Rluc and the macAA161-263 and JRAA139-216 fusion to the *N*-terminal end of EGFP or Rluc. The expression level of each fusion protein after 48-h transfection was analyzed by western blot analysis using an anti-Xpress antibody (B) or by measuring the

bioluminescent activity of Rluc using coelenterazine h as the substrate (C), as described in Materials and Methods. Each bar represents the mean standard deviation (n = 3).

Figure 7 Quantification of mRNA levels by RT-qPCR
HEK293 cells were transfected with the Nef_{NL4-3}, Nef_{JR-CSF}, AA129-206-EGFP, or EGFP expression plasmid. Total RNA was extracted from these HEK293 cells and subjected to reverse transcription reaction, followed by qPCR using the Syber Green method. mRNA level was normalized to the transcript of the neomycin resistance gene, which is coded in the expression vector used. Each bar represents the mean standard deviation (n = 3). (A) Comparison of mRNA level between Nef_{NL4-3} and Nef_{JR-CSF}. (B) Comparison of mRNA level between AA129-206-EGFP and EGFP.

Figure 8 Effect of proteasome inhibitor on expression levels of Nef_{NL4-3} and AA129-206-EGFP
HEK293 cells expressing Nef_{NL4-3} (A) or AA129-206-EGFP (B) were treated with 20 μM MG132 for 0, 3, and 6 h. The expression levels of Nef_{NL4-3} and AA129-206-EGFP in HEK293 cells were analyzed by western blot analysis. The intensities of the bands were semiquantified with Fujifilm Image Gauge Software. The intensities of Nef_{NL4-3} and AA129-206-EGFP were normalized to that of actin. Each bar represents the mean standard deviation (n = 3).

Figure 1

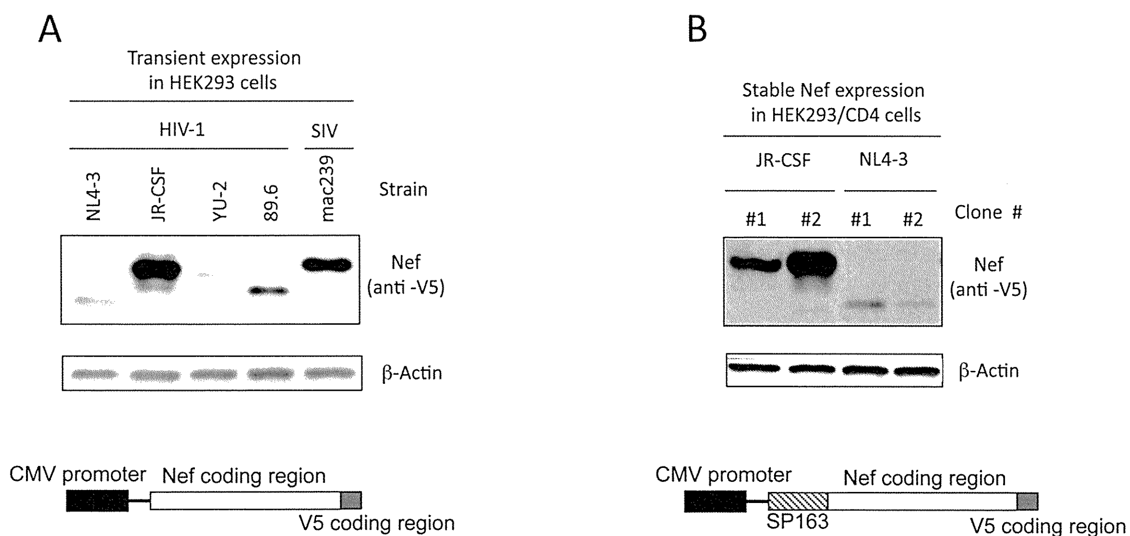
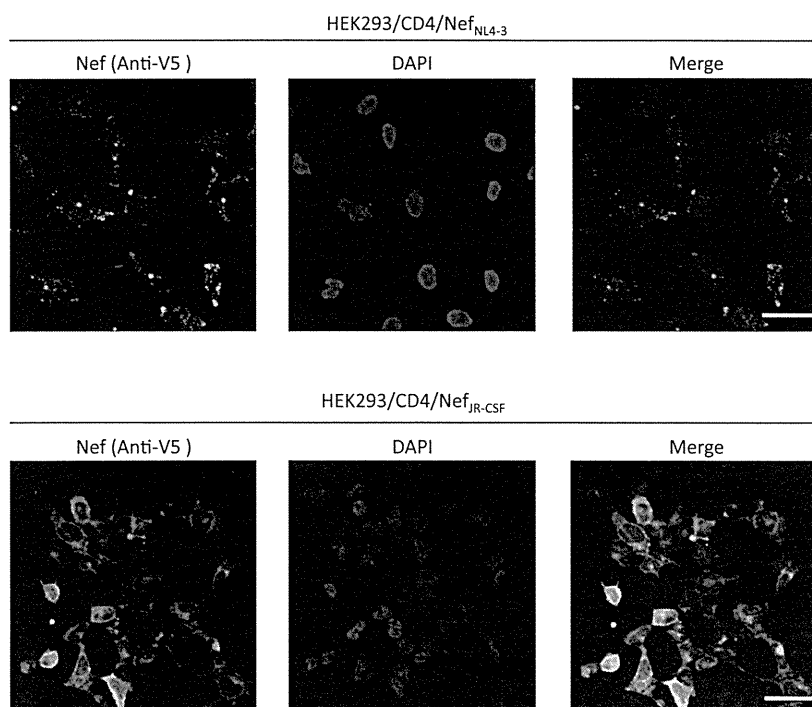


Figure 2



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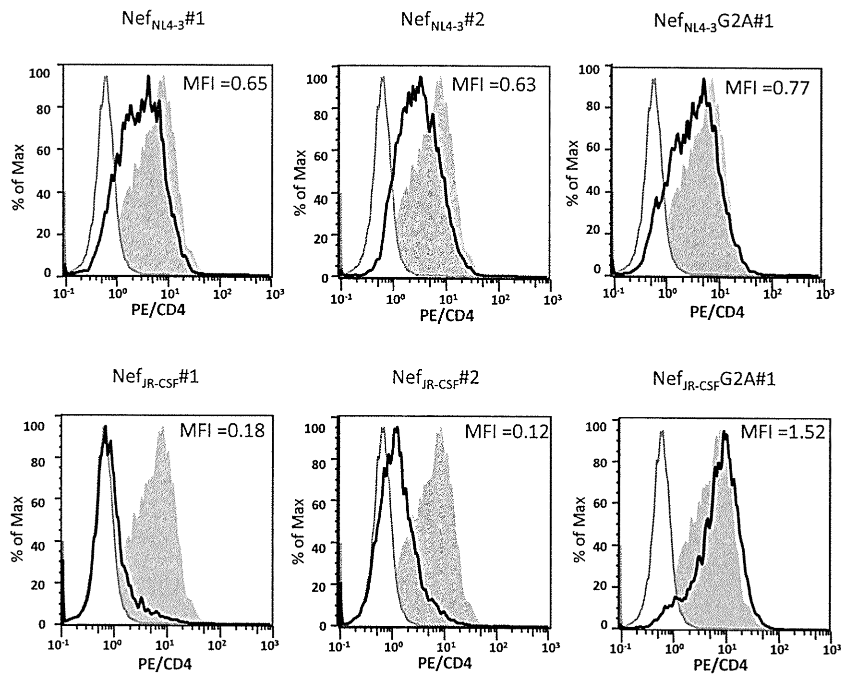

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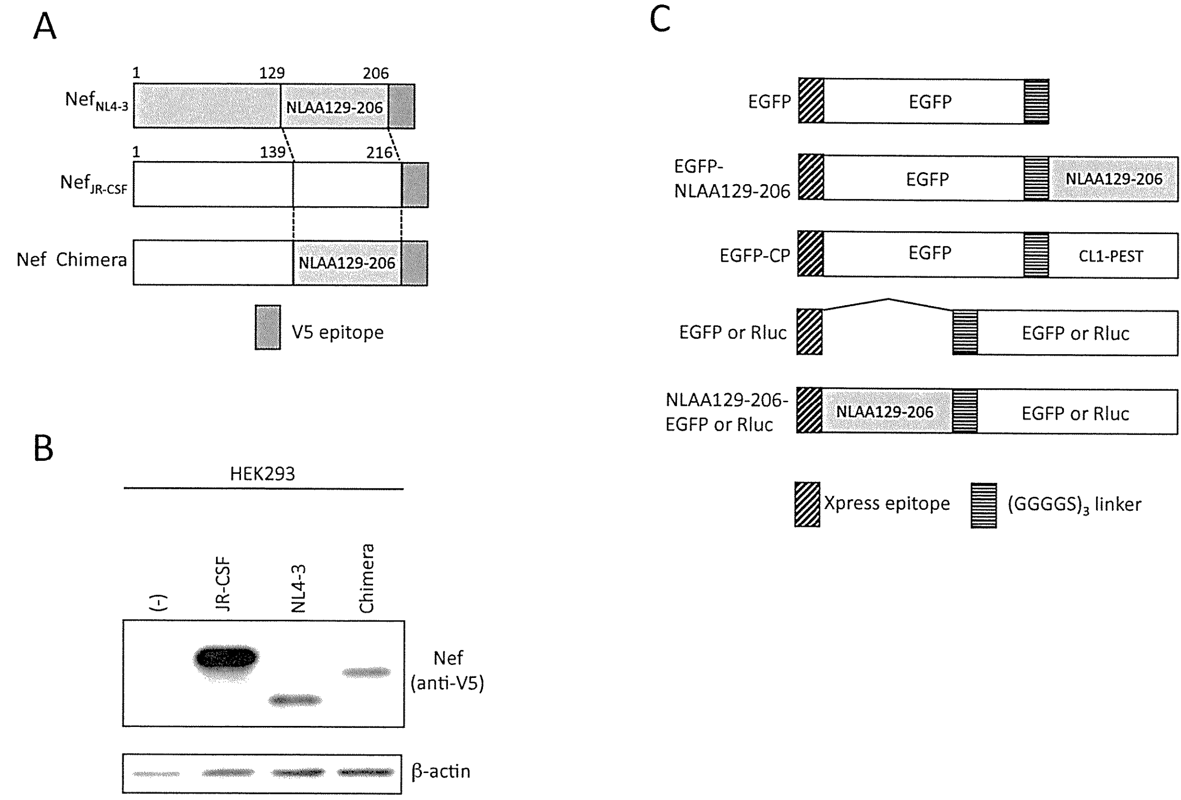
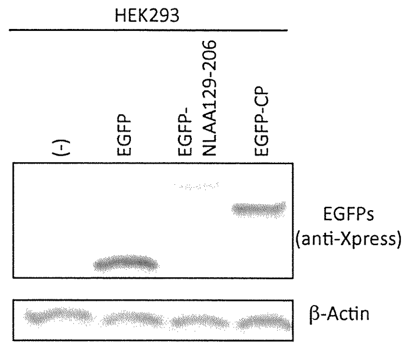


Figure 4

D



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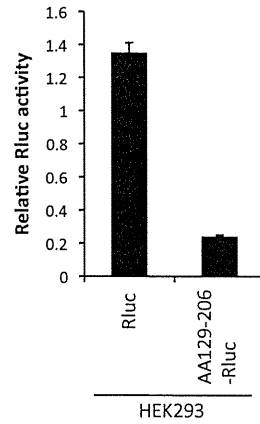

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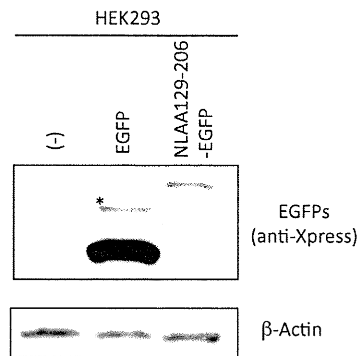
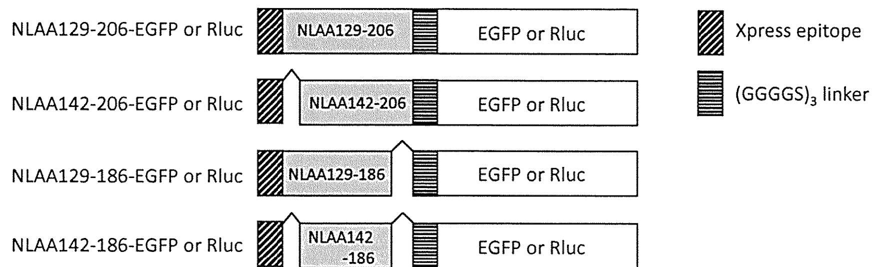


Figure 5

A



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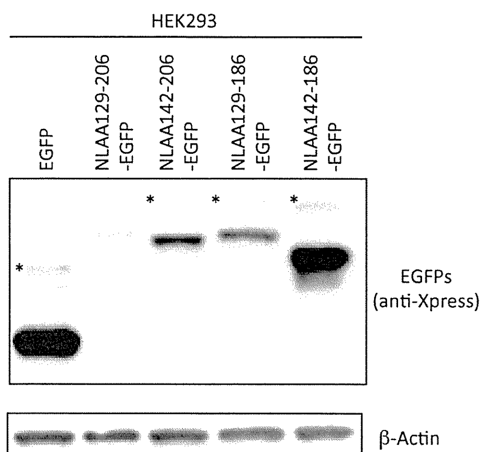

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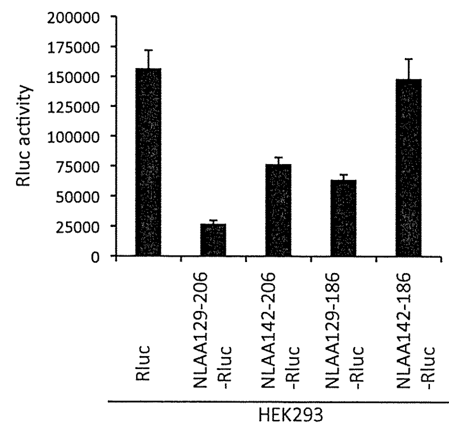
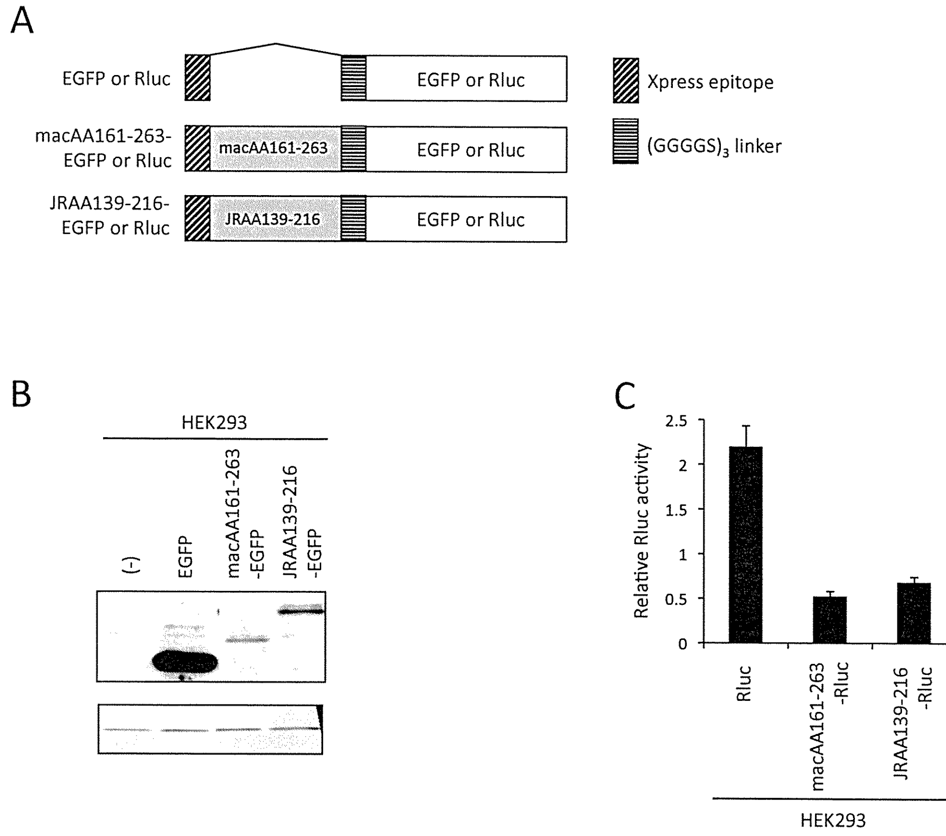


Figure 6



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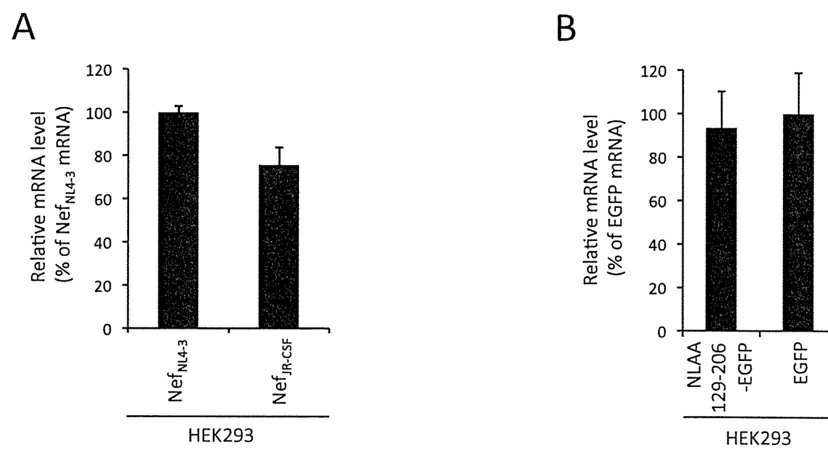

Figure 8