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-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 Nef_{NL4-3} or Nef_{JR-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 Nef_{NL4-3} and Nef_{JR-CSF} expressed in HEK293/CD4 cells. The HEK293/CD4/Nef_{NL4-3} (top row) and HEK293/CD4/Nef_{JR-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 Nef_{NL4-3} and Nef_{JR-CSF}. HEK293/CD4/Nef_{NL4-3} (top row panels) and HEK293/CD4/Nef_{JR-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 Nef_{NL4-3}
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 Nef_{NL4-3}, Nef_{JR-CSF}, and chimera Nef tested in "B". "B" shows a comparison of Nef_{NL4-3}, Nef_{JR-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 Nef_{mac239} and Nef_{JR-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

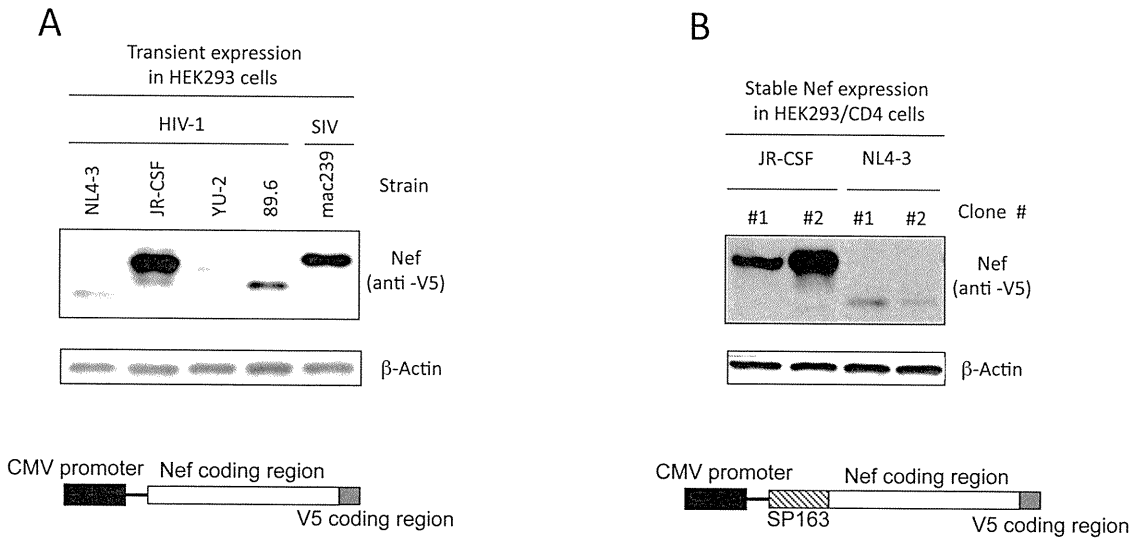
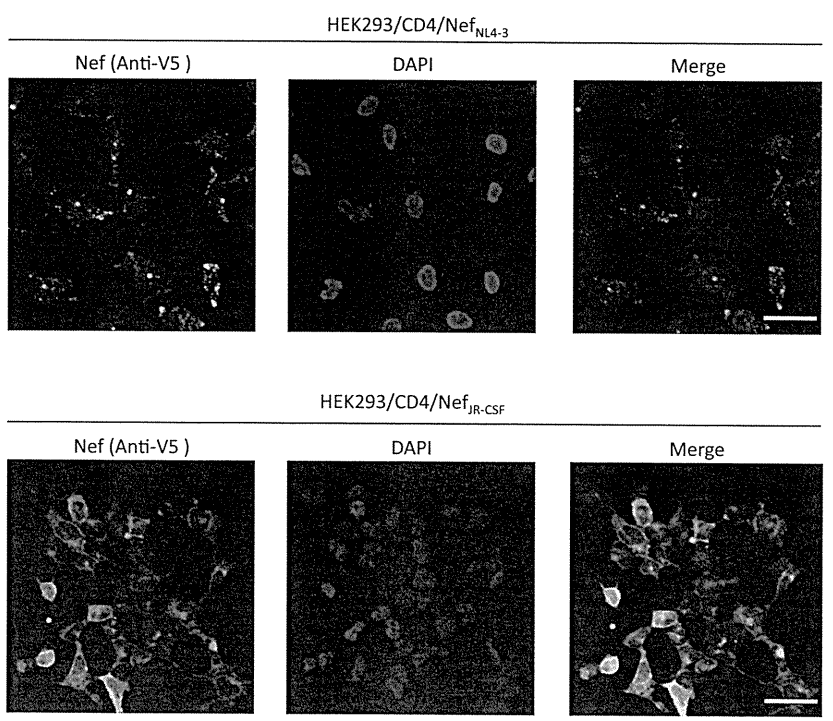


Figure 2



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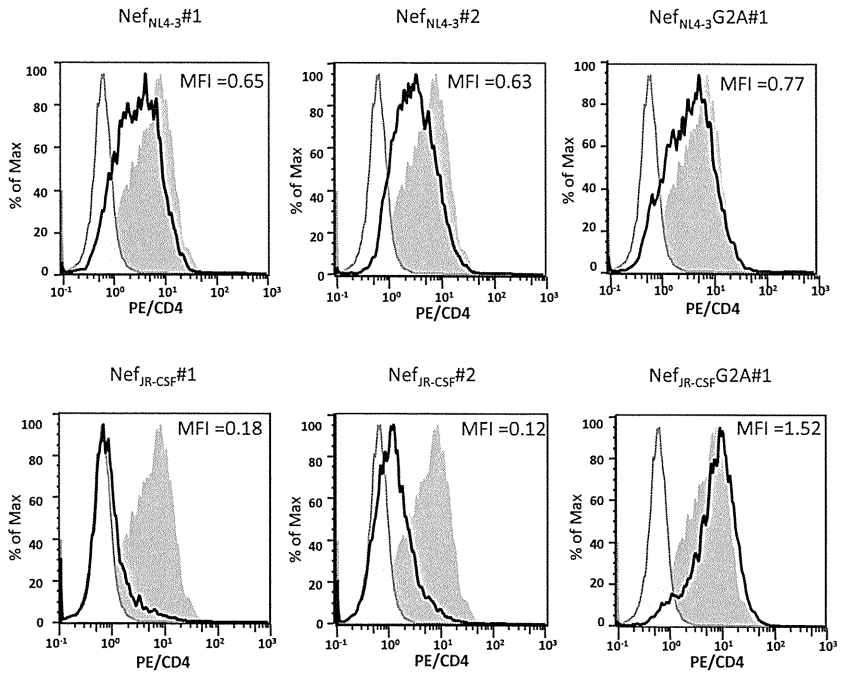


Figure 4

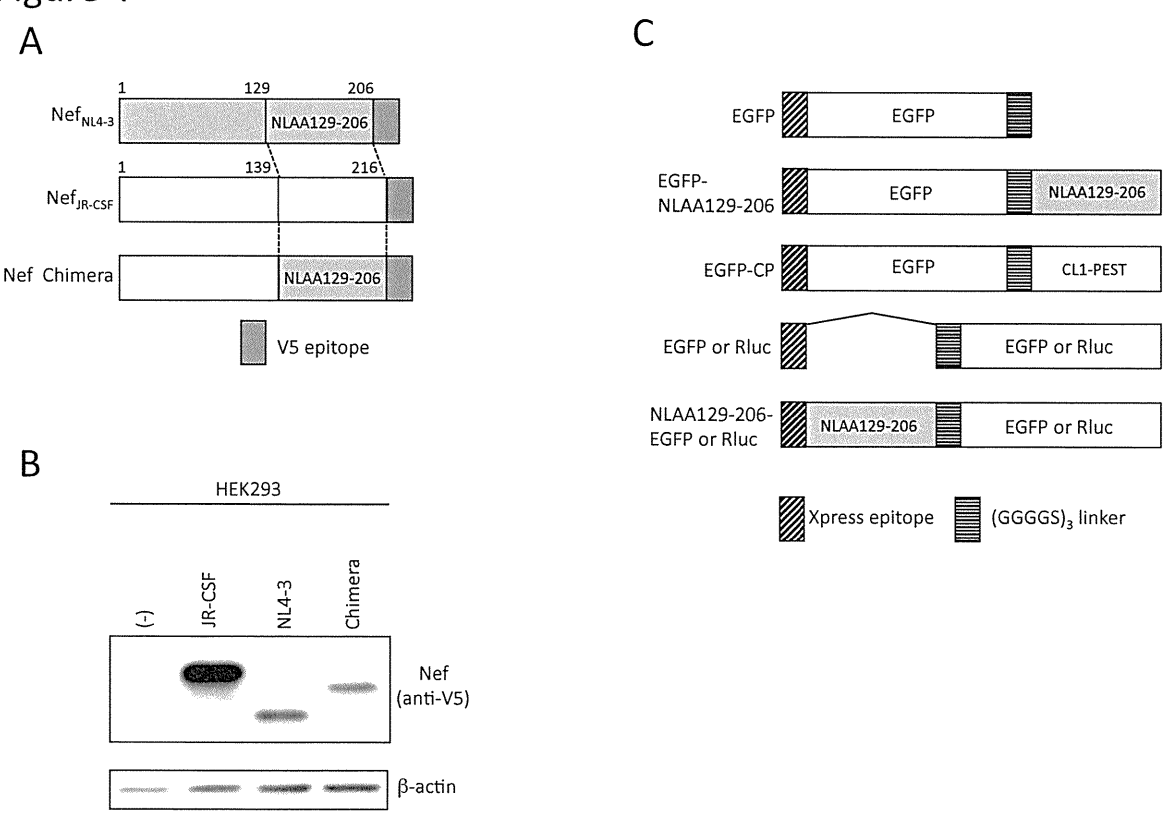


Figure 4

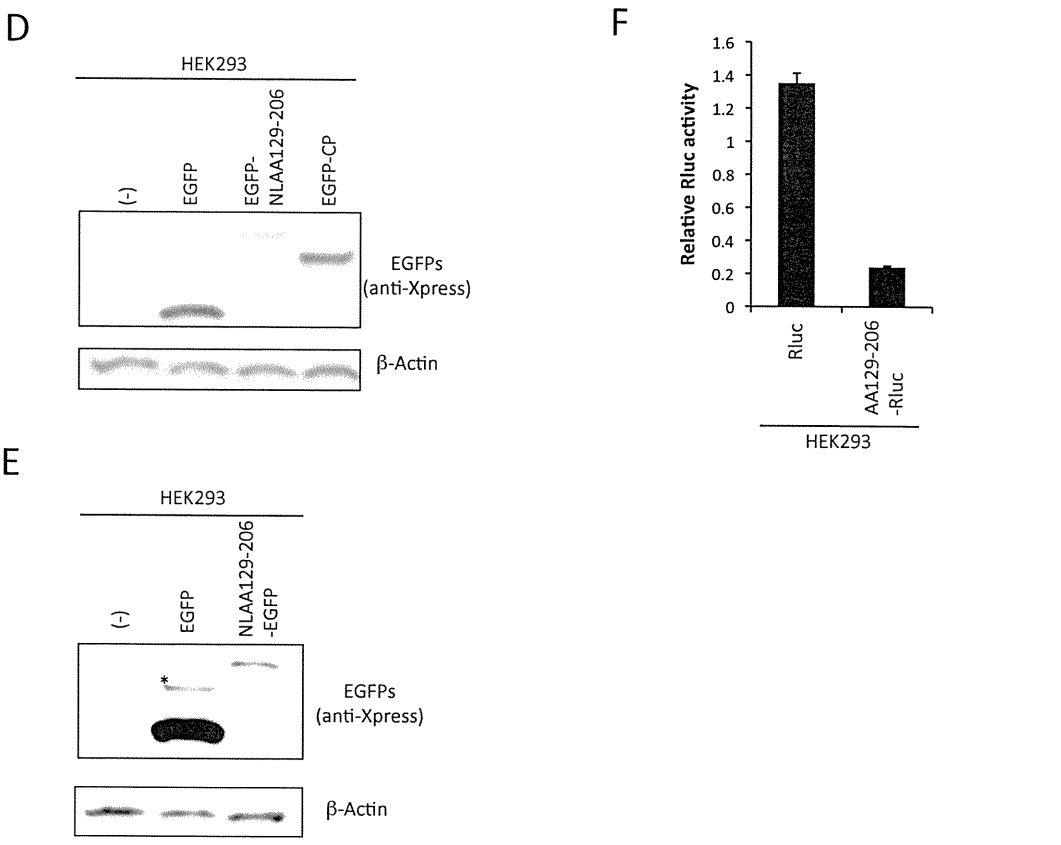
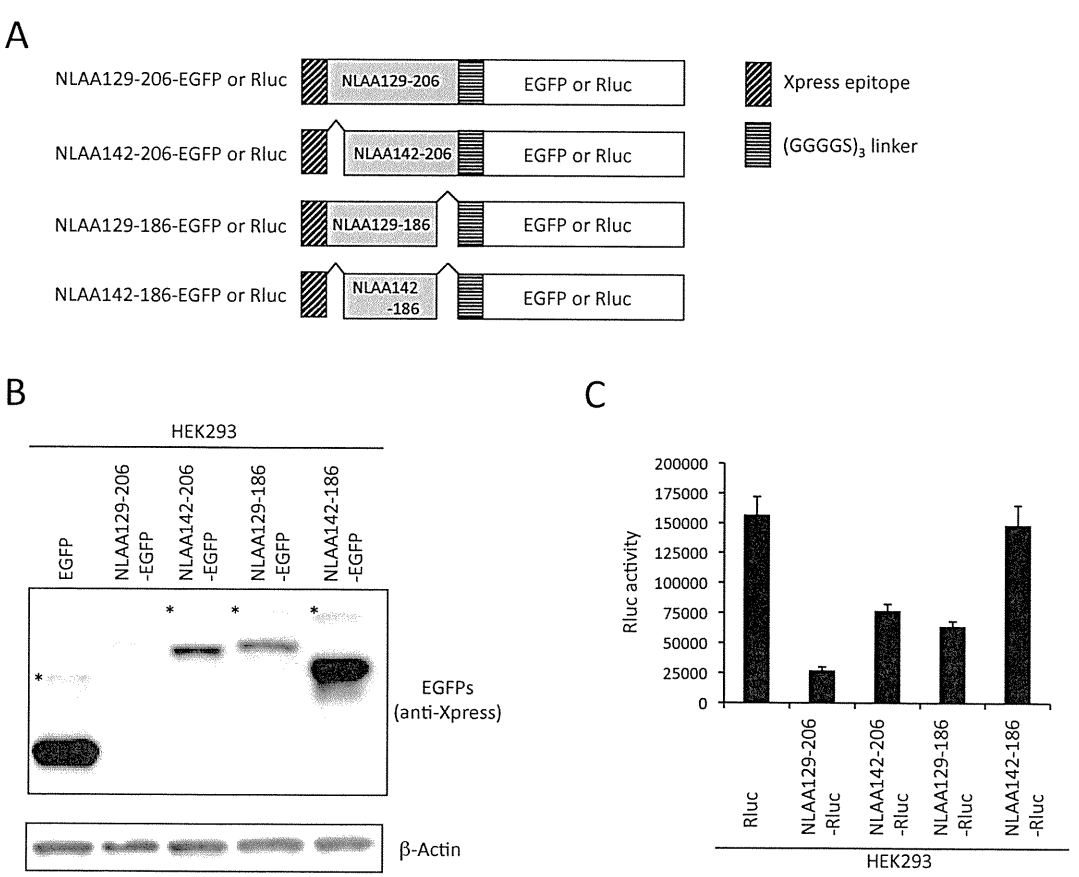


Figure 5



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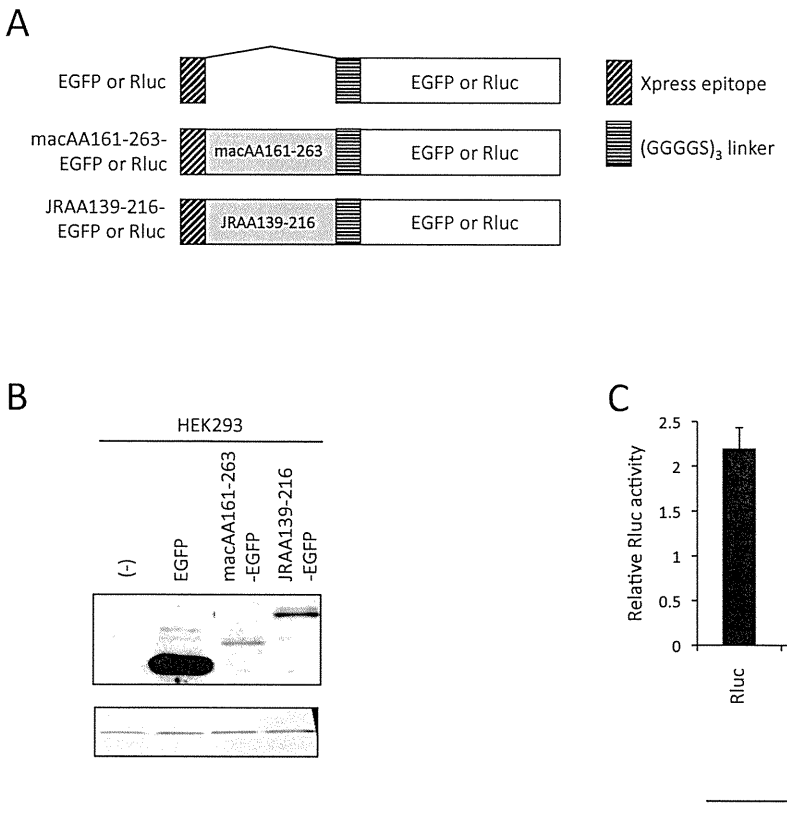


Figure 7

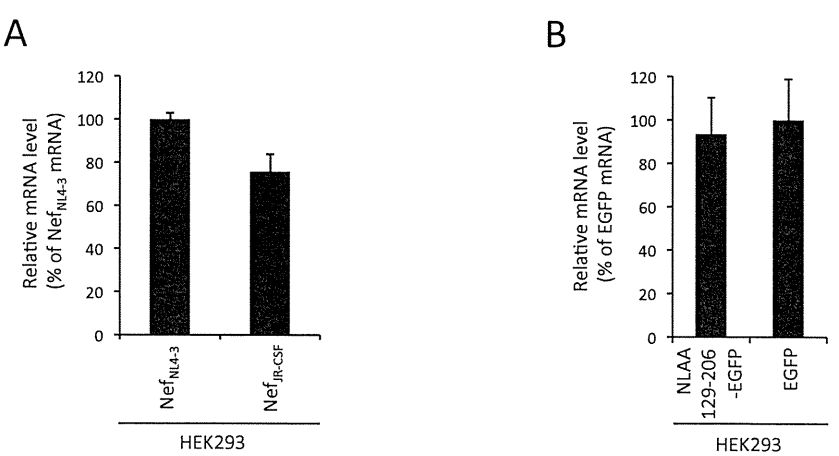
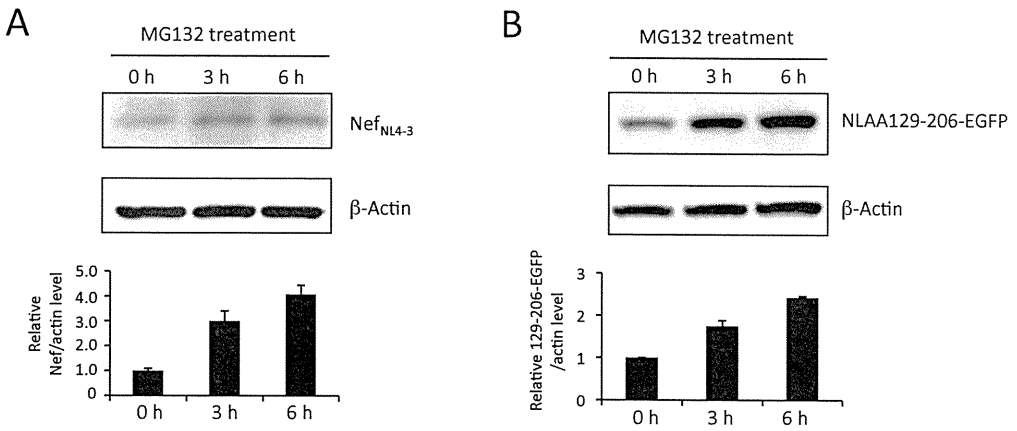


Figure 8


A
MG132 treatment
0 h
3 h
6 h
Nef$_{NL4-3}$
β-Actin
Relative Nef/actin level
5.0
4.0
3.0
2.0
1.0
0
0 h
3 h
6 h
B
MG132 treatment
0 h
3 h
6 h
NLAA129-206-EGFP
β-Actin
Relative 129-206-EGFP /actin level
3
2
1
0
0 h
3 h
6 h