NL43 and JRFL strain viruses (Fig. 3B, bar graph), and this effect was strongly associated with reduced Gag expression (Fig. 3B, Gag blot). Importantly, we found that IFITMs also reduced the expression of Nef and Vif (Nef and Vif blots). However, more important finding was that IFITM proteins did not induce any reduction in the expression of these viral proteins when they were co-transfected with the codon-optimized Gag expression plasmid (Fig. 4A, synGag–GFP blot), the Nef expression plasmid (Fig. 4A, Nef–GFP blot), or the codon-optimized Vif expression plasmid (Fig. 4B, HVif blot). It is well characterized that Gag and Vif RNAs contain a double-stranded region (Rev response element; RRE), and the incompletely spliced RNAs encoding these viral proteins require the binding of HIV-1 Rev protein to the RRE sequences for their nuclear export and subsequent expression [37,38]. Codon-optimization bypasses this complicated mechanism and allows these proteins to be expressed in a Rev-independent manner [31,37,38]. Thus, our results raised the possibility that IFITMs selectively interfere with the Rev/RRE-mediated expression of Gag and Vif. Indeed, IFITMs displayed an inhibitory effect on Gag expression in an artificial Rev-dependent expression system (Fig. 4C). In this system, the codon-unoptimized and RRE sequence-containing Gag–Pol and Gag expression plasmids (Gag–Pol–RRE and Gag-RRE) were expressed at normal levels only in the presence of the Rev plasmid (Fig. 5B, first 2 lanes). As a result, we

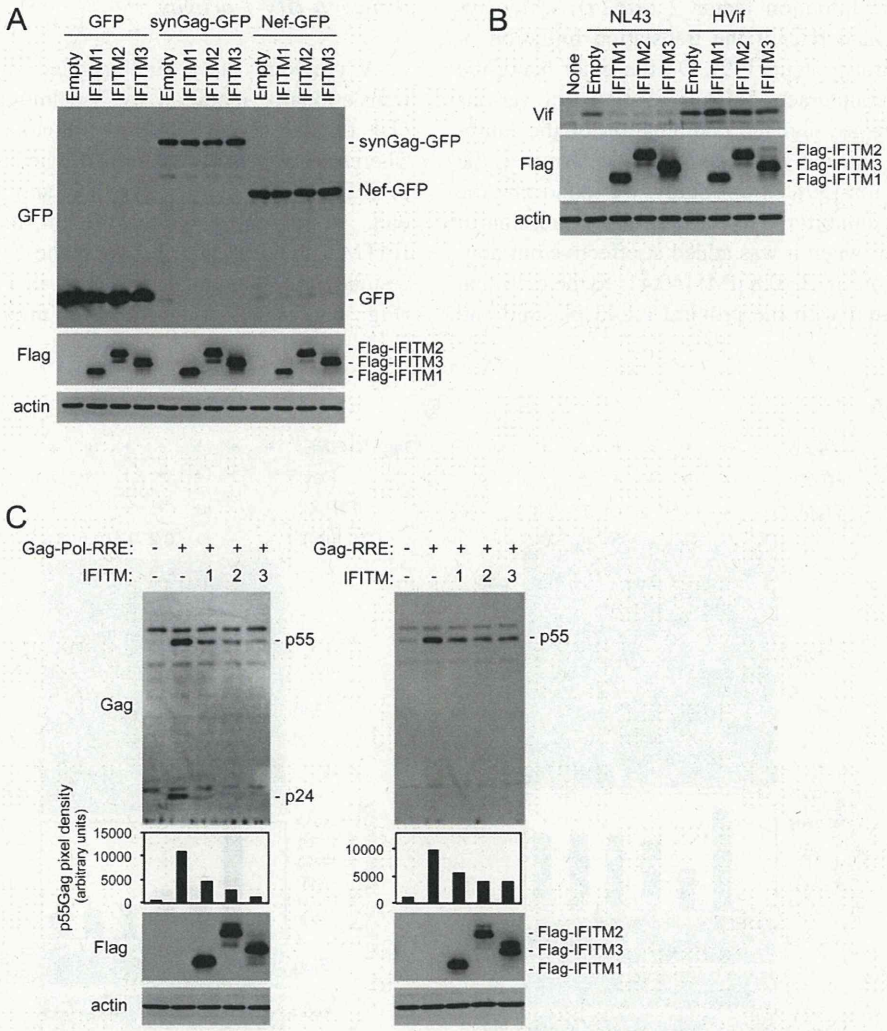

Fig. 4. The effects of IFITMs on the expression of the codon-optimized Gag, Nef and Vif, and the Rev/RRE-mediated expression of Gag. (A) The 293 cells were transfected with the GFP expression plasmid (0.1 μg), the codon-optimized Gag–GFP fusion expression plasmid (synGag–GFP, 0.2 μg), or the Nef–GFP expression plasmid, or co-transfected with the indicated IFITM expression plasmid (1.2 μg). (B) The 293 cells were transfected with the empty vector (None), the proviral NL43 plasmid (0.4 μg), or the codon-optimized Vif expression plasmid (HVif, 0.4 μg), or co-transfected with the indicated IFITM expression plasmid (1.2 μg). (A and B) The transfected cells were cultured for 2 days, lysed, and analyzed for the expression of GFP or GFP fusion proteins, Vif, and the Flag-tagged IFITM proteins by Western blotting. (C) The 293 cells were transfected with the Gag–Pol–RRE (0.3 μg) or Gag-RRE (0.3 μg) expression plasmid in combination with the Rev (0.1 μg) and/or IFITM (1.2 μg) expression plasmids, as indicated. The cells were cultured for 2 days, lysed, and analyzed for the expression of Gag and the Flag-tagged IFITM proteins by Western blotting. The profile created by quantifying the band pixel densities of p55Gag is also shown. (A–C) The actin blot is a loading control. Data shown are representative of two independent experiments with similar results.

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found that IFITM1, 2, and 3 significantly reduced Rev/RRE-mediated Gag expression (lanes 3–5), as they did in the proviral plasmid-mediated expression system (see Fig. 3B).

However, the finding that IFITMs interfere with Rev/RRE-mediated expression does not explain the fact that they also reduced the proviral plasmid-mediated expression of Nef (see Fig. 3B) because Nef RNA does not contain RRE, and therefore its expression is independent of the function of Rev [37,38]. Thus, we next examined whether another viral double-stranded RNA, i.e., the *trans*-activation response (TAR) element, was involved in the inhibitory activity of IFITMs. All of the HIV-1 RNAs including that encoding Nef contain the TAR element at their 5′ end, which binds and activates the double-stranded RNA-dependent protein kinase (PKR) [39]. Once activated, PKR phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α), which reduces the efficiency and rate of the translation initiation of proteins including viral proteins [39]. On the other hand, the HIV-1 Tat protein counteracts PKR activation via various complicated mechanisms, and the combination of the inhibitory pathways that prevent PKR activation determines the level of viral expression [39]. In this study, we found that the small molecule PKR inhibitor C16 restored the expression of p55Gag, Vif, and Nef, when it was added at effective but non-cytotoxic concentrations (0.2–0.8 μM) [40,41] to the cells that had been co-transfected with the proviral NL43 plasmid and IFITM3 (Fig. 5A). Meanwhile, C16 did not restore Gag expression in the Tat/TAR-independent but Rev/RRE-dependent expression system (Fig. 5B), which was consistent with the fact that TAR, but not RRE, induces strong PKR activation [39]. Therefore, our results (Figs. 3–5) suggested that IFITMs reduced the expression of Gag, and possibly Vif, by interfering with both Rev/RRE- and Tat/TAR-mediated expression, and reduced the expression of Nef by interfering with Tat/TAR-mediated expression. Again, the inhibitory effect was not non-specific because IFITMs did not affect the levels of these viral proteins when they were expressed via the system that bypassed the viral-specific machinery (see Fig. 4A and B).

3.4. The S-palmitoylation of IFITMs is not required for their anti-HIV-1 activity

A previous study demonstrated that IFITM3 was post-translationally modified by S-palmitoylation, which is crucial for its activity against influenza virus infection [7]. Therefore, we finally examined whether the S-palmitoylation of IFITMs is important for their anti-HIV-1 activity. To this end, we prepared three different mutants of Flag-tagged IFITM3 and IFITM2, in which the S-palmitoylated cysteine residues were mutated singly or in combination to alanine (Fig. 6A). Consistent with the finding that the first two

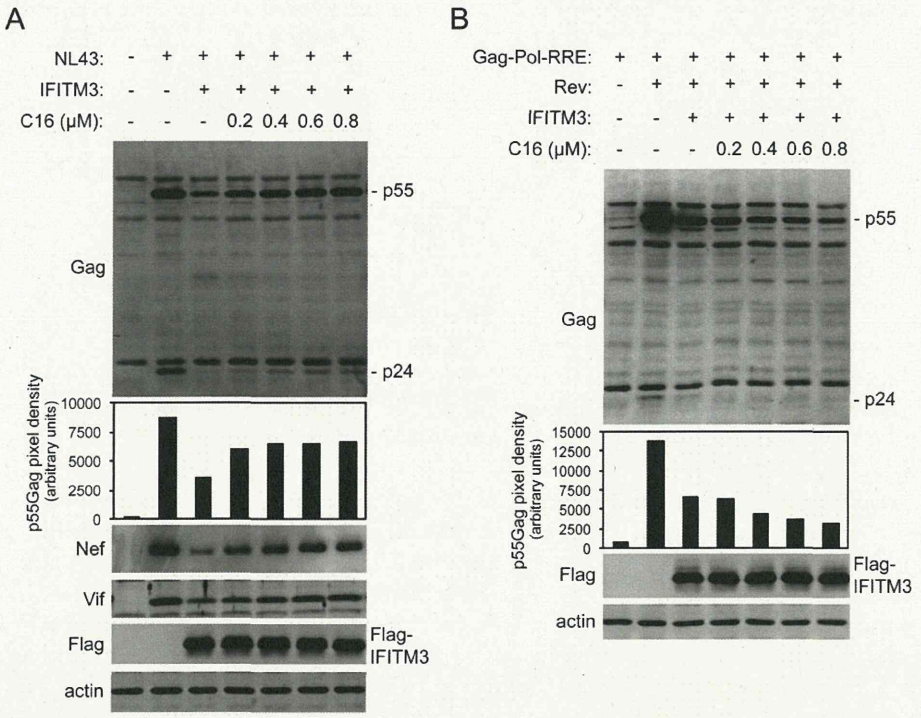

Fig. 5. The effect of the PKR inhibitor C16 on the suppression of Gag, Nef, and Vif expression by IFITM. (A) The 293 cells were transfected with the proviral NL43 plasmid (0.6 μg) and IFITM3 expression plasmid (1.0 μg), as indicated. (B) The 293 cells were transfected with the expression plasmids for Gag–Pol–RRE (0.3 μg), Rev (0.1 μg) and IFITM3 (1.0 μg), in the indicated combinations. (A and B) After 6 h transfection, the PKR inhibitor C16 was added to the culture at the indicated concentration (0.2–0.8 μM), and the cells were cultured for an additional 42 h. The cells were lysed and analyzed for the expression of Gag, Nef, Vif, and the Flag-tagged IFITM3 proteins. The actin blot is a loading control. The profile created by quantifying the band pixel densities of p55Gag is also shown. Data shown are representative of two independent experiments with similar results.

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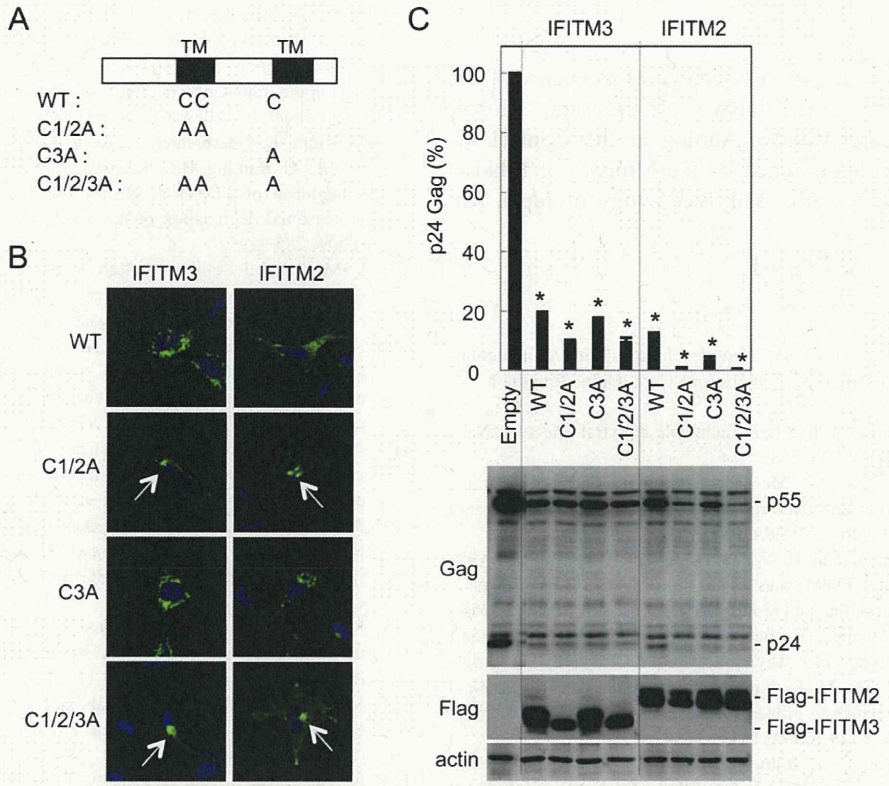

Fig. 6. The effects of S-palmitoylation-deficient IFITM mutants on viral production and Gag expression. (A) The IFITM mutants used are shown schematically. C, cysteine; A, alanine; TM, transmembrane. (B) The 293 cells were transfected with the expression plasmids (1.2 μg) for the wild-type (WT) or three mutants (C1/2A, C3A, and C1/2/3A) of IFITM3 or IFITM2. After 2 days transfection, the cells were fixed and co-stained with DAPI (blue) and anti-Flag antibody to detect Flag-tagged IFITM proteins (green). The C1/2A and C1/2/3A mutants displayed a distinct intracellular distribution (arrows) compared with the WT and C3A mutant. (C) The 293 cells were transfected with the proviral NL43 plasmid (1.0 μg), or co-transfected with the indicated IFITM expression plasmid (0.6 μg). The cells were cultured for 2 days, and the concentration of p24 Gag in the culture supernatants was determined by ELISA (bar graph). The results are expressed as percentages of the value for the sample on the far left. Data are shown as the mean ± SD of triplicate assays. Alternatively, the cells were lysed, and analyzed for the expression of Gag and the Flag-tagged IFITMs by Western blotting (lower blots). The actin blot is a loading control. (B and C) Data shown are representative of two independent experiments with similar results. $*p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cysteines were more heavily S-palmitoylated than the third cysteine [7], the mutants in which the first two cysteines were substituted (C1/2A and C1/2/3A) displayed more marked changes in their intracellular localization (Fig. 6B, arrows). However, unlike in the case of the anti-influenza virus activity of IFITM3, it was found that the S-palmitoylation was not necessary for the anti-HIV-1 activity of IFITMs because all of the mutants of IFITM3 and IFITM2 reduced the concentrations of p24 Gag in the supernatants (Fig. 6C, bar graph) and the expression levels of p55 and p24 Gag proteins in the cells (Gag blot). These results clearly indicated that IFITMs restrict HIV-1 and influenza viruses at distinct steps.

3.5. Conclusion

Both knockdown and enforced expression experiments demonstrated that IFITMs restrict HIV-1 replication [12,13]. In this study, we extended the findings of Lu et al. [12] and revealed that the enforced expression of IFITMs interfered with the production of HIV-1 proteins such as Gag, Vif, and Nef only when viral double-stranded RNAs (RRE and/or TAR) mediated their expression. These findings suggested that IFITM bind directly to viral double-stranded RNA. Indeed, a previous report raised the possibility that IFITM1 is an RRE-binding protein [P. Constantoulakis et al., Inhibition of Rev-mediated HIV-1 expression by an RNA binding protein encoded by the interferon-inducible 9–27 gene, Science 259 (1993) 1314–1318.]. However, as the report was retracted [M. Campbell et al., Science 264 (1994) 492.], careful studies will be necessary in order to clarify the exact mechanisms by which IFITMs interfere with the viral protein expression mediated by the double-stranded viral RNAs such as RRE and TAR. Studies will be also necessary to explain why there was no obvious difference in the anti-HIV-1 activity among three IFITM proteins in our transfection assay, in contrast to the study by Lu et al. [12]. Despite these unanswered questions, the present study demonstrated that IFITMs possess different characteristics from other anti-HIV-1 proteins such as tetherin and APOBEC3G and supported the idea that IFITMs restrict HIV-1 and influenza viruses at distinct steps.

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Acknowledgments

We thank H. Motoyama for her secretarial assistance. This work was supported by the Global COE program "Global Education and Research Center Aiming at the Control of AIDS", which was commissioned by the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to N.C. and S.S.).

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