

## VAP-B Binds to Rab3GAP1 at the ER: Its Implication in Nuclear Envelope Formation through the ER-Golgi Intermediate Compartment

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Received 3 July 2014/ Accepted 8 July 2014

**Key words:** VAP-B, Rab3GAP, ERGIC, Nuclear envelope

### ABSTRACT

The vesicle-associated membrane protein-associated protein B (VAP-B) is a tail-anchored protein in the endoplasmic reticulum (ER). VAP-B functions as an adaptor protein to recruit target proteins to the ER and execute various cellular functions, lipid transport, membrane traffic, ER stress etc. Recently, VAP-B has been shown to regulate the nuclear envelope protein transport through the ER-Golgi intermediate compartment (ERGIC). We showed here that VAP-B directly binds to Rab3 GTPase activating protein 1 (Rab3GAP1), the catalytic subunit of Rab3GAP, through the two phenylalanines (FF) in an acidic tract (FFAT)-like motif of Rab3GAP1. Rab3GAP consists of two subunits, the catalytic subunit Rab3GAP1 and the non-catalytic subunit Rab3GAP2. VAP-B binds to Rab3GAP1 even in the Rab3GAP1/2 heterodimer complex. A single amino acid substitution of the FFAT-like motif reduces the binding activity of Rab3GAP1 to VAP-B. On the other hand, the FFAT-like motif mutation increases the binding activity of Rab3GAP1 to ERGIC-53, the ERGIC marker protein. Overexpression of Rab3GAP1 affects nuclear envelope formation more potently than that of Rab3GAP1 FFAT-like motif mutant. These results suggest that the binding of VAP-B to Rab3GAP1 is implicated in the regulation of nuclear envelope formation through ERGIC.

### INTRODUCTION

Vesicle-associated membrane protein (VAMP) associated protein (VAP) was originally identified as a VAMP2 binding protein in a yeast two-hybrid screen of *Aplysia californica* cDNA library [27]. VAMP2 is a vesicular soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein (SNAP) receptor (*v*-SNARE) protein and essential for synaptic vesicle fusion [3,8]. Two mammalian homologs of VAP, VAP-A and VAP-B, have been identified. VAP-A and VAP-B are highly homologous proteins and conserved in all eukaryotic organisms [19,26,32]. VAP-A and VAP-B contain an N-terminal domain homologous to the nematode major sperm protein (MSP), a coiled-coil domain, and a C-terminal transmembrane domain, which contains a putative dimerization motif GxxxG [13]. Based on this topology, VAPs are defined as tail-anchored membrane proteins.

VAPs are ubiquitously expressed in various tissues and organs, and mainly localized in the ER [19,26,32]. VAPs bind to a number of lipid exchange and lipid-sensing proteins through the two phenylalanines (FF) in an acidic tract (FFAT) motif, <sup>1</sup>EFFDA-E<sup>7</sup> [9,13-15]. The bindings play a key role in the regulation of lipid metabolism and in non-vesicular lipid transfer between the ER and other organelles [7,22]. VAPs also bind to the FFAT-like motif-containing proteins similar to the FFAT motif-containing proteins [14,33]. FFAT-like motif is missing one or more of the conserved six amino acids of the FFAT motif. FFAT-like motif-containing proteins, including protrudin and the glycolipid transfer protein (GLTP), have been shown to bind to VAPs [24,31]. In addition, based on the FFAT-like motif key amino acids analysis with the VAP-dependent ER recruitment assay in yeast, the FFAT-like motif peptides of PKA anchoring proteins; AKAP220 and AKAP110, a family of plant lipid transfer protein, a GLTP family member phosphatidylinositol-four-phosphate adaptor-protein-2 (FAPP-2), and Rab3 GTPase-activating protein 1 (Rab3GAP1) are recruited to the ER in the yeast overexpressing Scs2p, the yeast homolog of VAP [16]. Through the FFAT motif and the FFAT-like motif, VAPs recruit a number of target proteins to the ER and are implicated in regulating various cellular functions including membrane traffic, organelle transport on microtubules, neurite extension, and the unfolded protein response [10,13,23,33].

A single missense mutation of the VAP-B gene, substituting of proline residue at position 56 by serine, causes three forms of familial motor neuron diseases, including a rare amyotrophic lateral sclerosis with slow progression (ALS8), typical severe ALS with rapid progression, and a late-onset spinal muscular atrophy

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[1,2,10,12,18]. Overexpression of the VAP-B P56S mutant forms intracellular inclusions and induces an altered ER structure, an organized smooth ER consisting of parallel ER cisternae [1,4,10,12,18,21,28,29]. Wild type VAP-A and VAP-B have a potency to form a heterodimer with VAP-B P56S, and are recruited to the VAP-B P56S inclusions [10,29]. VAP-B P56S is supposed to cause a loss of function of wild type VAP-A and VAP-B, leading to motor neuron death by a dominant negative effect [4,10,11,18,21,28,29]. On the other hand, VAP-B P56S mutant also causes a nuclear envelope defect characterized by separation of the outer and inner nuclear membrane [30]. This defect is caused by disruption of transport of the nuclear envelope proteins. Loss of VAP-B leads to accumulation of the nuclear envelope proteins in dilated ER-Golgi intermediate compartment (ERGIC) [30]. Nuclear envelope proteins are transported from the ERGIC to the nuclear envelope [30]. VAP-B is required for this final transport to the nuclear envelope [30]. Progressive abnormality of the nuclear envelope is a consequence of disrupting this transport and may contribute to ALS progression [30]. However, it is not known which of the many binding proteins of VAP-B facilitates this retrograde transport step from ERGIC to the nuclear envelope, and the full extent of VAP-B binding proteins have not been established.

In this study, to identify a novel VAP-B binding protein, we performed the yeast two-hybrid screening using a human pancreas cDNA library with the cytoplasmic domain of VAP-B as a bait. We isolated Rab3GAP1, the catalytic subunit of Rab3GAP [5] as a VAP-B binding protein. Rab3GAP1 interacts with VAP-B through its FFAT-like motif <sup>1</sup>EFFEC-S<sup>7</sup>. A single amino acid substitution of the FFAT-like motif <sup>1</sup>EFFAC-S<sup>7</sup> reduces the binding activity of Rab3GAP1 to VAP-B. On the other hand, the FFAT-like motif mutation increases the binding activity of Rab3GAP1 to ERGIC-53. Exogenously expressed Rab3GAP1 affects nuclear envelope formation more potently than Rab3GAP1 FFAT-like motif mutant. Together, we propose that the binding of VAP-B to Rab3GAP1 is involved in the regulation of nuclear envelope formation through ERGIC.

### MATERIALS AND METHODS

This study was approved by the Committee for Safe Handling of Living Modified Organisms in Kobe University (Permission number: 24-86) and carried out according to the guidelines of the committee.

#### Yeast two-hybrid

A bait vector, pGBD-c1-VAP-B-cyt, was constructed by subcloning the cDNA encoding the cytoplasmic domain of rat VAP-B (1–219 aa) (VAP-B-cyt) into the pGBD-c1 vector. A human pancreas yeast two-hybrid cDNA library was purchased from CLONTECH. The yeast strain PJ69-4A (MATa trp1–901 leu2–3, 112 ura3–52 his3–200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ) harboring pGBD-c1-VAP-B-cyt was transformed with the human pancreas cDNA library and screened as described previously [20].

#### Mammalian expression plasmids

To express proteins with an N-terminal FLAG tag or an N-terminal HA tag, the cDNAs encoding rat full-length VAP-B, VAP-B-cyt, the C-terminal transmembrane domain of rat VAP-B (209–243 aa) (VAP-B-TM), human Rab3GAP1 and human ERGIC-53 were subcloned into the pCMV-FLAG vector or the pCMV-HA vector. Rab3GAP1 E587A and VAP-B P56S were generated using the Quikchange II Lightning site-directed mutagenesis kit (Agilent Technologies).

#### Co-immunoprecipitation

Appropriate combinations of the mammalian expression plasmids were transfected into HEK293 cells with Lipofectamine LTX (Invitrogen) in accordance with the manufacture's manual. 24 h after transfection, the cells were lysed in a buffer containing 20 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 μM APMSF, 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.5 % TritonX-100 and subjected to ultracentrifugation at 100,000 x g at 4°C for 30 min. The Triton X-100 extracts were incubated with a mouse anti-FLAG mAb (SIGMA) or a mouse anti-HA mAb (Covance), followed by immunoprecipitation with Protein G sepharose (GE healthcare). The immunoprecipitates were subjected to SDS-PAGE followed by western blotting with a rabbit anti-FLAG pAb (SIGMA) and the mouse anti-HA mAb or a rabbit anti-HA pAb (SIGMA).

#### Immunofluorescence microscopy

Appropriate combinations of the mammalian expression plasmids were transfected into HeLa cells with Lipofectamine LTX. 24 h or 48 h after transfection, the cells were fixed with 4% paraformaldehyde, followed by permeabilization with 0.2% Triton X-100. After blocking with PBS supplemented with 1% BSA, the samples were incubated with various combinations of the rabbit anti-HA pAb, the mouse anti-HA mAb, the rabbit anti-FLAG pAb, a mouse anti-PDI mAb (Abcam), and a mouse anti-nuclear pore complex proteins mAb

(mAb414, Covance), followed by incubation with Alexa Fluor-conjugated secondary Abs (Invitrogen). After being washed with PBS, they were embedded and viewed using a confocal imaging system (ZEISS, LSM 510 Meta) or a fluorescent microscope (ZEISS, Axio Lab. A1).

As for quantification of the effect of Rab3GAP1 overexpression on the morphology of the nuclear envelopes, when the nuclear envelopes as detected with mAb414 lacked continuous outlines, they were defined as abnormally shaped nuclear envelopes.

### **Recombinant proteins**

For expression of GST-fused VAP-B-cyt (GST-VAP-B-cyt), the cDNA encoding VAP-B-cyt was subcloned into the pGEX-4T vector (GE healthcare). GST-VAP-B-cyt was expressed in *E.coli* and purified with glutathione sepharose resin (GE healthcare).

MBP-fused Rab3GAP1 (MBP-Rab3GAP1), MBP-fused Rab3GAP1 E587A (MBP-Rab3GAP1 E587A) and GST-fused Rab3GAP2 (GST-Rab3GAP2) were expressed in Sf21 cells using the Bac-to-Bac baculovirus expression system (Invitrogen) and purified with glutathione sepharose resin or amylose resin (New England Biolabs).

### **Pull-down assay using recombinant proteins**

To examine the direct binding between VAP-B and Rab3GAP1, 500 pmol of GST-VAP-B-cyt was immobilized on 50  $\mu$ l of glutathione sepharose resin and incubated with 100 pmol of MBP-Rab3GAP1, MBP-Rab3GAP1 E587A or MBP alone in buffer A containing 20 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1mM DTT, and 0.5% Triton X-100 at 4°C for 3 h. After being extensively washed with buffer A, the resins were boiled in an SDS sample buffer (60 mM Tris-HCl pH 6.7, 3% SDS, 2% 2-mercaptoethanol, and 5% glycerol) for 5 min. The eluted proteins were subjected to SDS-PAGE followed by coomassie brilliant blue (CBB) staining.

To assess the binding between VAP-B and the Rab3GAP1/2 heterodimer, 50 pmol of MBP-Rab3GAP1 or MBP-Rab3GAP1 E587A complexed with GST-Rab3GAP2 was immobilized on 50  $\mu$ l of amylose resin and incubated with increasing amounts of GST-VAP-B-cyt in buffer A. The bound proteins were analyzed in the same manner as described above.

## **RESULTS**

### **Identification of Rab3GAP1 as a VAP-B-binding protein.**

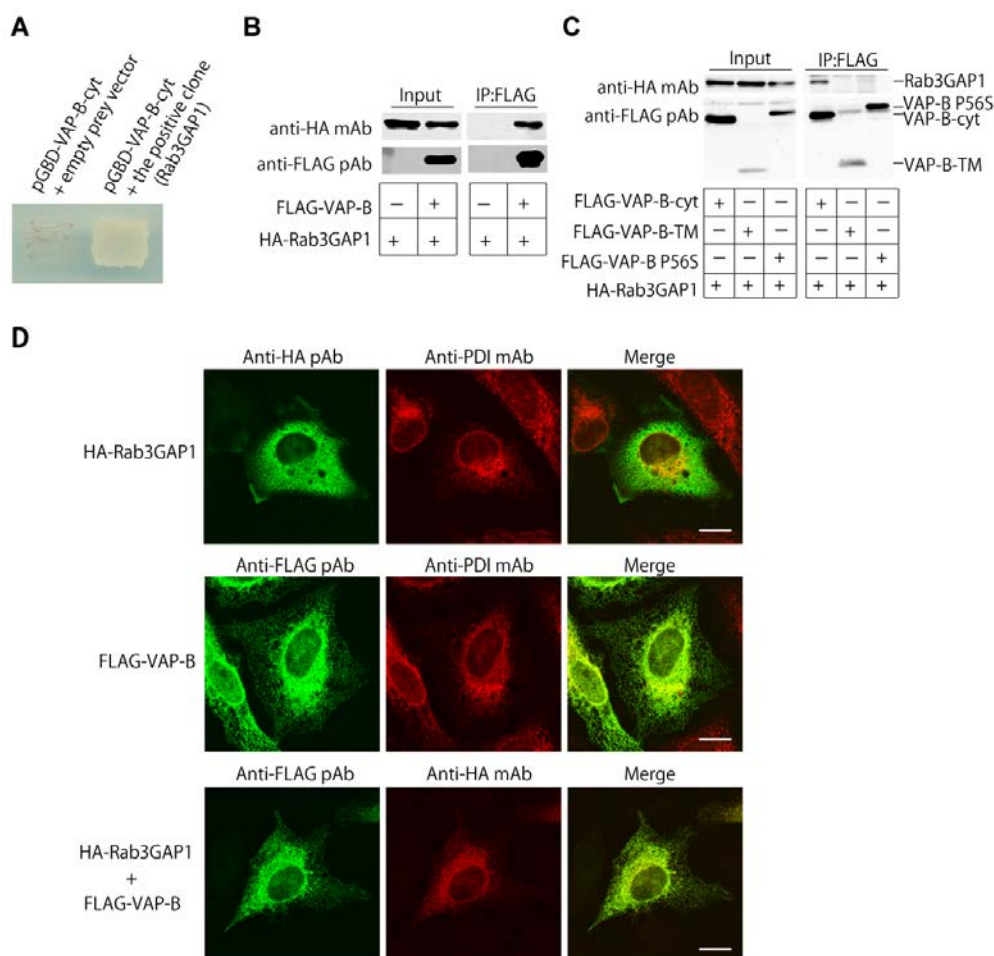
To identify a protein(s) that binds to VAP-B, we screened a human pancreas cDNA library by yeast two-hybrid system using the cytoplasmic domain of VAP-B as a bait. Eight positive clones were isolated and all of them encoded the same internal portion of Rab3GAP1 (573–850 aa) (Figure 1A). To validate the binding between VAP-B and Rab3GAP1, we next carried out immunoprecipitation analyses. FLAG-tagged full-length VAP-B (FLAG-VAP-B), FLAG-tagged cytoplasmic domain of VAP-B (FLAG-VAP-B-cyt) and FLAG-tagged C-terminal transmembrane domain of VAP-B (FLAG-VAP-B-TM) were transfected into HEK293 cells along with HA-tagged full-length Rab3GAP1 (HA-Rab3GAP1), followed by immunoprecipitation with the anti-FLAG mAb. HA-Rab3GAP1 was co-immunoprecipitated with FLAG-VAP-B and FLAG-VAP-B-cyt but not with FLAG-VAP-B-TM (Figures 1B and 1C). These results indicate that VAP-B binds to Rab3GAP1 through the cytoplasmic domain. To further confirm the binding, we carried out immunostaining of transfected HeLa cells. When HA-Rab3GAP1 alone was transfected, HA-Rab3GAP1 diffusely localized throughout the cytoplasm (Figure 1D). When FLAG-VAP-B alone was transfected, FLAG-VAP-B showed reticular staining pattern similar to PDI, an ER luminal enzyme, consistent with the earlier findings that VAP-B is an ER membrane protein [9,15,23,26,29]. By contrast, when HA-Rab3GAP1 was transfected with along with FLAG-VAP-B, HA-Rab3GAP1 showed reticular staining pattern and colocalized with FLAG-VAP-B (Figure 1D), indicating that Rab3GAP1 was recruited to the ER membrane through binding to VAP-B. Collectively, these results indicate that Rab3GAP1 is a VAP-B-binding protein and also suggest that VAP-B serves as an ER membrane receptor for Rab3GAP1.

### **The FFAT-like motif of Rab3GAP1 is responsible for binding to VAP-B.**

A point mutation of VAP-B that substitutes proline for serine at position 56 has been shown to be associated with motor neuron diseases including ALS [1,2,10,12,18]. Therefore, we examined the effect of the P56S mutation on binding to Rab3GAP1. FLAG-tagged VAP-B P56S mutant (FLAG-VAP-B P56S) and HA-Rab3GAP1 were transfected into HEK293 cells, followed by immunoprecipitation with the anti-FLAG mAb. HA-Rab3GAP1 was not co-immunoprecipitated with FLAG-VAP-B P56S (Figure 1C), indicating that, upon P56S mutation, VAP-B abolished Rab3GAP1 binding. Given that the P56S mutation is known to cause

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aggregation of VAP-B, resulting in abolishment of binding to FFAT-like motif-containing proteins [29], this result raises the possibility that VAP-B would directly bind to Rab3GAP1 through an FFAT-like motif. Indeed, Rab3GAP1 contains a potential FFAT-like motif (EFFEC-S<sup>7</sup>) at position 584–590. The internal portion of Rab3GAP1 as isolated by yeast two-hybrid (Figure 1A) encompassed the potential FFAT-like motif. Therefore, we next examined whether the potential FFAT-like motif of Rab3GAP1 was involved in binding to VAP-B. We generated a Rab3GAP1 mutant that disrupted the FFAT-like motif by substitution of glutamic acid for alanine at position 587 (Rab3GAP1 E587A) and transfected HA-tagged Rab3GAP1 E587A (HA-Rab3GAP1 E587A) or HA-Rab3GAP1 into HEK293 cells along with FLAG-VAP-B, followed by immunoprecipitation with the anti-FLAG mAb. HA-Rab3GAP1 E587A was co-immunoprecipitated with FLAG-VAP-B significantly less than HA-Rab3GAP1 (Figure 2A). We further assessed involvement of the FFAT-like motif by *in vitro* binding assay using recombinant proteins. GST-fused VAP-B-cyt (GST-VAP-B-cyt) immobilized on glutathione sepharose was incubated with MBP-fused Rab3GAP1 (MBP-Rab3GAP1) or Rab3GAP1 E587A (MBP-Rab3GAP1 E587A), followed by pull-down. MBP-Rab3GAP1 E587A bound to GST-VAP-B-cyt significantly less than MBP-Rab3GAP1 (Figure 2B). These results indicate that the FFAT-like motif of Rab3GAP1 is responsible for binding to VAP-B.

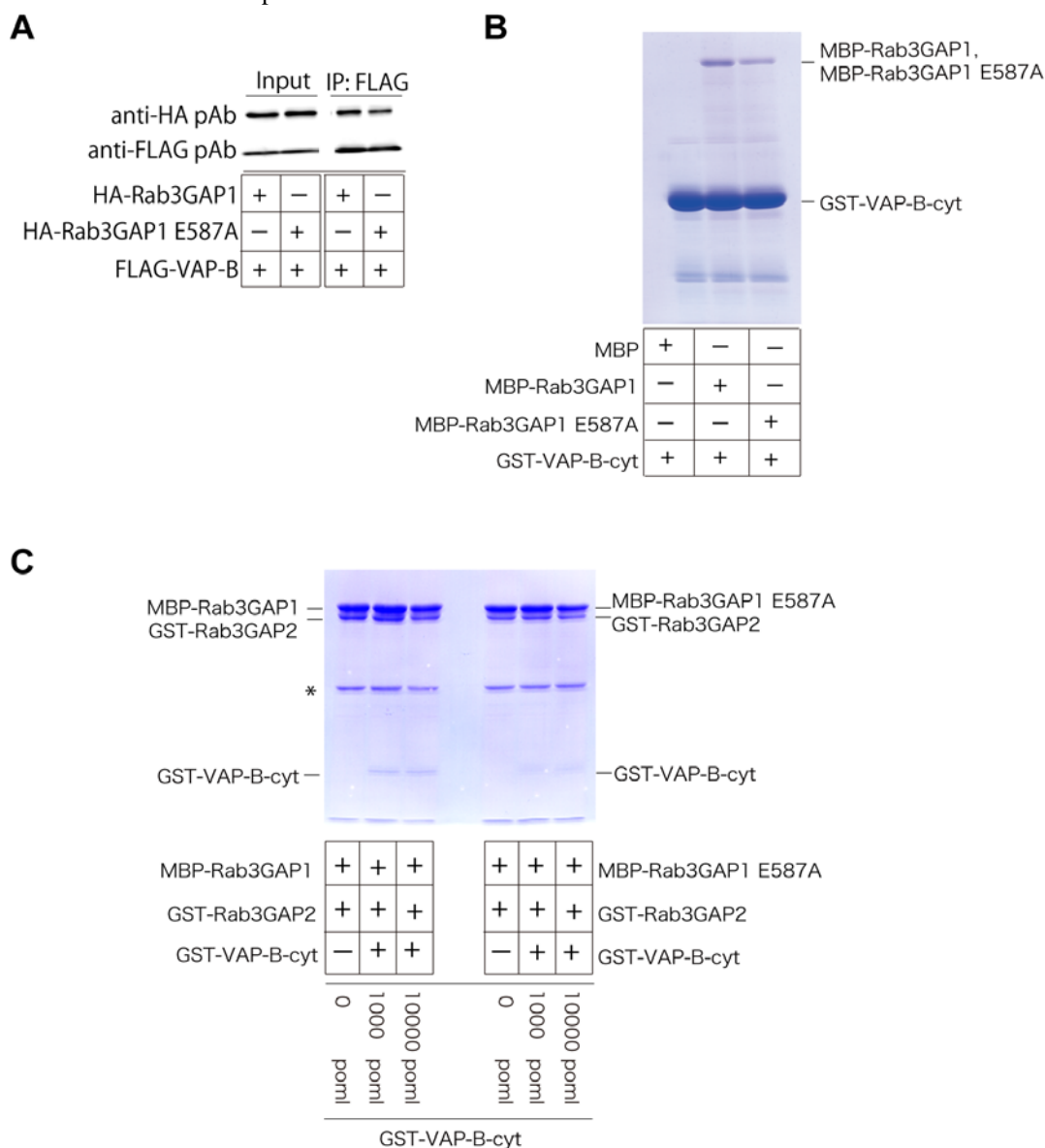


**Figure 1.** Identification of Rab3GAP1 as a VAP-B-binding protein.

(A) Identification of Rab3GAP1 by yeast two-hybrid using VAP-B-cyt as a bait. Yeast transformed with the indicated plasmids were grown on the synthetic complete medium lacking adenine at 30 °C for 3 days to score the ADE2 reporter activity. The isolated positive clone encoding the internal portion of Rab3GAP1 (573–850 aa) showed specific binding to VAP-B-cyt in the yeast two-hybrid system. (B) Co-immunoprecipitation of Rab3GAP1 and VAP-B. The indicated combinations of FLAG-VAP-B and HA-Rab3GAP1 were transfected into HEK293 cells, followed by immunoprecipitation with the mouse anti-FLAG mAb. The samples were subjected to SDS-PAGE followed by western blotting with the mouse anti-HA mAb and the rabbit anti-FLAG pAb. (C) VAP-B but not VAP-B P56S binds to Rab3GAP1 through the cytoplasmic region. The indicated combinations of FLAG-VAP-B-cyt, FLAG-VAP-B-TM, FLAG-VAP-B P56S and HA-Rab3GAP1 were transfected into HEK293 cells, followed by immunoprecipitation with the anti-FLAG mAb as in Fig. 1B. (D) Rab3GAP1 is recruited to the ER membrane though binding to VAP-B. HeLa cells were transfected with HA-Rab3GAP1 alone, FLAG-VAP-B alone or both of them. 24 h after transfection, the cells were doubly immunostained with the rabbit anti-HA pAb and the mouse anti-PDI mAb, the rabbit anti-FLAG pAb and the mouse anti-PDI mAb, or the rabbit anti-FLAG pAb and the mouse anti-HA mAb. The samples were analyzed by confocal microscope. Scale bars, 10 μm

**VAP-B binds to the Rab3GAP1/2 heterodimer.**

Since the previous studies have shown that Rab3GAP1 predominantly exists as a heterodimer complex with Rab3GAP2, a non-catalytic subunit [17], we next examined effect of VAP-B binding on Rab3GAP1/2 heterodimer status. MBP-Rab3GAP1 or MBP-Rab3GAP1 E587A complexed with GST-fused Rab3GAP2 (GST-Rab3GAP2) was immobilized on amylose resin and incubated with increasing amounts of GST-VAP-B-cyt, followed by pull-down. While GST-VAP-B-cyt bound to the MBP-Rab3GAP1/GST-Rab3GAP2 heterodimer more than the MBP-Rab3GAP1 E587A/GST-Rab3GAP2 heterodimer in good agreement with the result shown in Figure 2B, GST-Rab3GAP2 binding to MBP-Rab3GAP1 was comparable to GST-Rab3GAP2 binding to MBP-Rab3GAP1 E587A regardless of the amounts of GST-VAP-B-cyt (Figure 2C). This result indicates that VAP-B can bind to Rab3GAP1 in the Rab3GAP1/2 heterodimer complex.

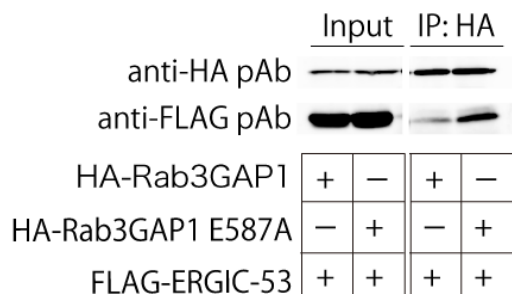


**Figure 2.** The FFAT-like motif of Rab3GAP1 is responsible for binding to VAP-B.

(A) Decreased co-immunoprecipitation of Rab3GAP1 with VAP-B upon point mutation of the FFAT-like motif. FLAG-VAP-B was transfected into HEK293 cells along with HA-Rab3GAP1 or HA-Rab3GAP1 E587A, followed by immunoprecipitation with the mouse anti-FLAG mAb. The samples were subjected to SDS-PAGE followed by western blotting with the rabbit anti-HA pAb and the rabbit anti-FLAG pAb. (B) Decreased binding of Rab3GAP1 to VAP-B upon point mutation of the FFAT-like motif. GST-VAP-B-cyt immobilized on glutathione sepharose was incubated with MBP alone, MBP-Rab3GAP1 or MBP-Rab3GAP1 E587A, followed by pull-down. The bound proteins were subjected to SDS-PAGE followed by CBB staining. (C) VAP-B binds to the Rab3GAP1/2 heterodimer. MBP-Rab3GAP1 or MBP-Rab3GAP1 E587A complexed with GST-Rab3GAP2 was immobilized on amylose resin and incubated with increasing amounts of GST-VAP-B-cyt, followed by pull-down as in Fig. 2B. The asterisk indicates non-specific bands.

**The FFAT-like motif of Rab3GAP1 is involved in the binding of Rab3GAP1 to ERGIC-53.**

A recent proteomic study identified Rab3GAP1 as an ERGIC-53-binding protein [6]. We therefore examined whether the FFAT-like motif of Rab3GAP1 was involved in the interaction between Rab3GAP1 and ERGIC-53. HA-Rab3GAP1 or HA-Rab3GAP1 E587A was transfected into HEK293 cells along with FLAG-tagged ERGIC-53 (FLAG-ERGIC-53), followed by immunoprecipitation with the anti-HA mAb. FLAG-ERGIC-53 was co-immunoprecipitated with HA-Rab3GAP1 E587A significantly more than HA-Rab3GAP1 (Figure 3), indicating that the FFAT-like motif of Rab3GAP1 is involved in the interaction between Rab3GAP1 and ERGIC-53.

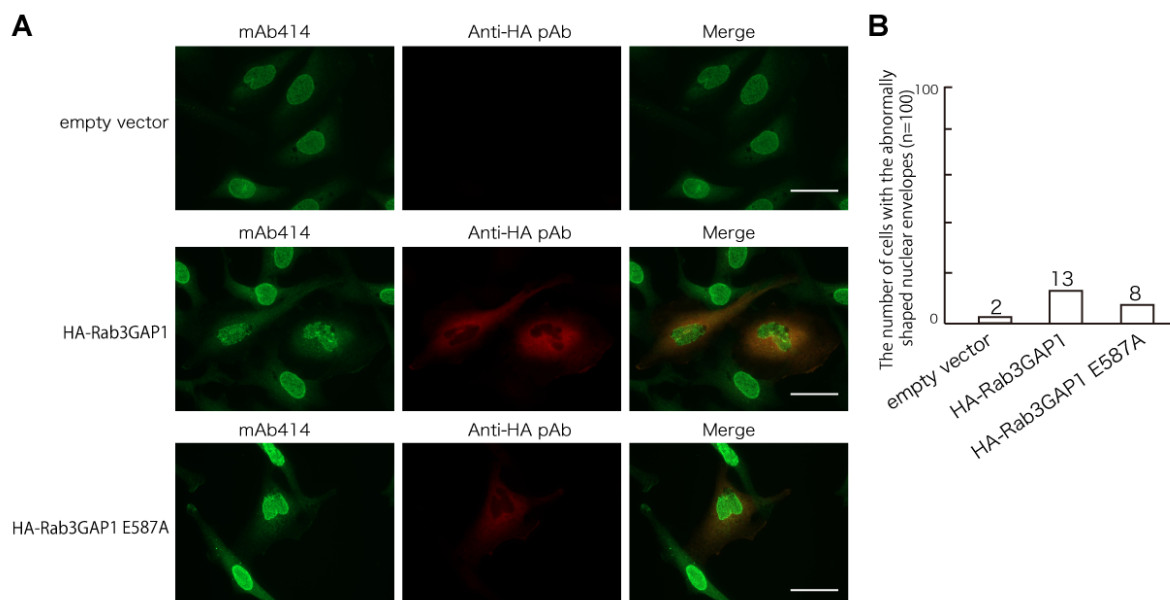


**Figure 3.** The FFAT-like motif of Rab3GAP1 is involved in the binding of Rab3GAP1 to ERGIC-53.

HA-Rab3GAP1 or HA-Rab3GAP1 E587A was transfected into HEK293 cells along with FLAG-ERGIC-53, followed by immunoprecipitation with the mouse anti-HA mAb. The samples were subjected to SDS-PAGE followed by western blotting with the rabbit anti-HA pAb and the rabbit anti-FLAG pAb.

**The FFAT-like motif of Rab3GAP1 is involved in the nuclear envelope formation.**

VAP-B is recently reported to regulate the retrograde transport of nuclear envelope membrane proteins from ERGIC to the nuclear envelope, thereby regulating the nuclear envelope formation [30]. We therefore examined whether the FFAT-like motif of Rab3GAP1 was involved in regulation of the nuclear envelope formation. HA-Rab3GAP1, HA-Rab3GAP1 E587A or the empty vector was transfected into HeLa cells, followed by immunostaining with the rabbit anti-HA pAb and the mouse anti-nuclear pore complex proteins mAb (mAb414). Compared with the transfection of the empty vector, the transfection of HA-Rab3GAP1 significantly increased the number of cells with the abnormally shaped nuclear envelopes as characterized by lack of the continuous outlines of mAb414 signals (Figure 4), indicating that overexpression of Rab3GAP1 induced the abnormal nuclear envelope. Importantly, the transfection of HA-Rab3GAP1 E587A decreased the number of cells with the abnormally shaped nuclear envelopes relative to that of HA-Rab3GAP1 (Figure 4). These results indicate that the FFAT-like motif of Rab3GAP1 is involved in regulation of the formation of the nuclear envelope presumably through binding to VAP-B.



**Figure 4.** Involvement of the FFAT-like motif of Rab3GAP1 in formation of the nuclear envelope.

(A) Overexpression of Rab3GAP1 induces abnormally shaped nuclear envelope. HA-Rab3GAP1, HA-Rab3GAP1 E587A or the empty vector was transfected into HeLa cells. 48 h after transfection, the cells were doubly immunostained with the rabbit anti-HA pAb and the mouse anti-nuclear pore complex proteins mAb (mAb414) and analyzed by fluorescent microscope. Scale bars, 20  $\mu$ m. (B) Quantification of the cells with abnormally shaped nuclear envelopes. One hundred of the transfected cells were randomly chosen, and the number of the cells with abnormally shaped nuclear envelopes was counted.

## DISCUSSION

Rab3GAP1 was originally identified as a GTPase-activating protein (GAP) toward the synaptic vesicle-associated Rab3 subfamily small GTPases [5] and has been characterized as a regulator of neurotransmitter release [25]. However, given that the Rab3GAP1 expression is not limited to nervous system [5], Rab3GAP1 is supposed to play ubiquitous roles other than regulation of neurotransmitter release. In the present study, we identified Rab3GAP1 as a VAP-B-binding protein and demonstrated that Rab3GAP1 was a potential regulator for formation of the nuclear envelope. The regulatory function for the nuclear envelope seems to be in agreement with the ubiquitous expression of Rab3GAP1.

Recently, VAP-B was reported to regulate formation of the nuclear envelope through mediating the retrograde transport of nuclear membrane proteins from ERGIC to nucleus [30], although the molecular mechanism, in particular a binding partner(s) for VAP-B, remains unclear. We showed here that VAP-B directly bound to the FFAT-like motif of Rab3GAP1. Furthermore, the FFAT-like motif of Rab3GAP1 was involved in the binding of Rab3GAP1 to ERGIC-53 and the nuclear envelope formation. Therefore, it seems likely that Rab3GAP1 would be the binding partner for VAP-B to regulate the nuclear envelope formation. Given that the mutation of the FFAT-like motif increased the binding of Rab3GAP1 to ERGIC-53 as shown in Figure 3, Rab3GAP1 might bind reciprocally to VAP-B and ERGIC-53. The reciprocal binding of Rab3GAP1 might mediate transport of ERGIC-53 from ER, leading to regulation of the formation of ERGIC. Eventually, Rab3GAP1 might be involved in regulation of the retrograde transport of the nuclear envelope membrane proteins from ERGIC. In good agreement with this reasoning, the previous study demonstrated that siRNA-mediated knockdown of VAP-B perturbed the localization of ERGIC-53 [30]. Excess free Rab3GAP1 due to overexpression of exogenous Rab3GAP1 would interfere with the reciprocal binding of endogenous Rab3GAP1 to VAP-B and ERGIC-53 more potently than overexpression of Rab3GAP1 E587A, thereby leading to the defects in the nuclear envelope formation as shown in Figure 4. On the other hand, we can not exclude the possibility that proteins other than VAP-B bind more preferentially to Rab3GAP1 than to the Rab3GAP1 E587A mutant and the bindings could be involved in the nuclear envelope formation. Therefore, further characterization of the interaction among Rab3GAP1, VAP-B, and ERGIC-53 will be required to address these concerns.

The previous study using the yeast homolog of VAP, Scs2p, has shown that Rab3GAP1 harbors a potential FFAT-like motif that Scs2p can bind [16], while yeasts do not have genes homologous to Rab3GAP1. Here we demonstrated that Rab3GAP1 was indeed a client of mammalian VAP-B and the FFAT-like motif was responsible for the binding. However, Rab3GAP1 binding to VAP-B is not so simple, because mutation of the FFAT-like motif (E587A) decreased but not abolished the binding as shown in Figure 2. This result suggests that, in addition to the FFAT-like motif, at least another region of Rab3GAP1 would be involved in binding to VAP-B, although the detailed mechanism of the binding remains unclear. The somewhat unusual binding mode between Rab3GAP1 and VAP-B might allow Rab3GAP1 to reciprocally bind to VAP-B and ERGIC-53, underlying the regulatory function of Rab3GAP1 for the nuclear envelope.

Given that Rab3GAP1 was recruited to the ER membrane through binding to VAP-B as shown in Figure 1D, one might be concerned whether Rab3GAP1 exerted the GAP activity on the ER membrane for regulation of the nuclear envelope. However, neither VAP-B binding nor mutation of the FFAT-like motif altered the GAP activity of Rab3GAP1 toward Rab3A in our preliminary experiments (data not shown), suggesting that the GAP activity was independent of regulation of the nuclear envelope formation. On the other hand, we can not exclude the possibility that there might exist an ER-localized Rab GTPase(s) other than Rab3 as a Rab3GAP1 substrate. Therefore, it still remains unclear whether the GAP activity of Rab3GAP1 is required for regulation of the nuclear envelope formation. Further studies will be required to address these concerns.

In summary, we propose that the interplay between Rab3GAP1 and VAP-B is implicated in nuclear envelope formation through ERGIC.

## ACKNOWLEDGEMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and by the grants from The Takeda Science Foundation, The Naito Foundation, and The Kanae Foundation for the Promotion of Medical Sciences.

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