

Na/K-ATPase beta1-subunit associates with neuronal growth regulator 1 (NEGR1) to participate in intercellular interactions

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Neuronal growth regulator 1 (NEGR1) is a GPI-anchored membrane protein that is involved in neural cell adhesion and communication. Multiple genome wide association studies have found that NEGR1 is a generic risk factor for multiple human diseases, including obesity, autism, and depression. Recently, we reported that *Negr1*^{-/-} mice showed a highly increased fat mass and affective behavior. In the present study, we identified Na/K-ATPase, beta1-subunit (ATP1B1) as an NEGR1 binding partner by yeast two-hybrid screening. NEGR1 and ATP1B1 were found to form a relatively stable complex in cells, at least partially co-localizing in membrane lipid rafts. We found that NEGR1 binds with ATP1B1 at its C-terminus, away from the binding site for the alpha subunit, and may contribute to intercellular interactions. Collectively, we report ATP1B1 as a novel NEGR1-interacting protein, which may help deciphering molecular networks underlying NEGR1-associated human diseases. [BMB Reports 2021; 54(3): 164-169]

INTRODUCTION

Sodium-potassium adenosine triphosphatase (Na/K-ATPase) is a member of the P-type family of active ion pumps that is found in the plasma membrane of all animal cells. Na/K-ATPase maintains the membrane voltage potential that is critical for many cellular processes (1). It has an oligomeric structure composed of essential α and β subunits and an additional tissue-specific regulator belonging to the FXYD protein family (2). The α -subunit possesses ten transmembrane (TM) domains, while the β and FXYD subunits each contain a single TM domain. Mammalian cells express multiple isoforms of each subunit:

four α , three β , and seven FXYD (3). Although different combinations of the α and β subunits are found in different tissues, the α 1 and β 1 subunits are the most widely expressed in mammals (3).

The beta subunit constitutes of a short N-terminal cytosolic domain, TM region, and a long and heavily glycosylated extracellular C-terminal domain. Interestingly, the β subunit has a unique function in cell-cell adhesion independent of its catalytic activity. The fact that β 2 isoform was originally identified as a neural adhesion molecule, AMOG (adhesion molecule on glia), may provide a link to another facet of the β subunit's role in intercellular interactions (4). The β 1 isoform is known to function as a cell-adhesion molecule. The overexpression of β 1 increased cell-cell association of nonpolarized cells and its expression is down-regulated in cancer cells, suggesting that it is a tumor suppressor (5). There are three disulfide bonds and three glycosylation sites in the extracellular domain of β 1; its N-glycans are important for cell adhesion.

Human neuronal growth regulator 1 (NEGR1) is a raft-associated glycosylphosphatidylinositol (GPI)-anchored protein containing three C2-type immunoglobulin domains (6). NEGR1 was originally named kilon, a kindred of IgLON (immunoglobulin LAMP, OBCAM and neurotrimin), neural cell adhesion molecules that functions in neural cell adhesion, neurite outgrowth, and synapse formation (7). NEGR1 was found to be down-regulated in multiple human tumors. NEGR1-overexpressing SKOV-3 cells exhibited enhanced cell-cell adhesion and impaired migration, demonstrating its tumor-suppressive activity (8). Recently, multiple genome-wide analyses have implicated the genetic alterations of *NEGR1* in multiple human pathologies including obesity, intellectual disability, and psychiatric disorders (9, 10), suggesting its importance in central nervous system function. In this study, we report a molecular interaction between NEGR1 and ATP1B1, that may provide a functional link between these two proteins.

RESULTS

ATP1B1 is a NEGR1-interacting protein

To identify novel NEGR1-binding partner proteins, we performed yeast two-hybrid screening using a human fetal testis cDNA library. Because the full-length NEGR1 showed self-activation

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in the Gal4-based system, we used a truncated N-terminal region of NEGR1 (residues 40-215) as bait. Among the positive clones obtained in the screen, we identified a clone containing approximately two-thirds (residues 93-303) of the Na/K-ATPase beta1-subunit (ATP1B1) (Supplementary Fig. S1A).

To validate the interaction between NEGR1 and ATP1B1, we obtained the human *ATP1B1* gene by PCR from a human fetal stomach cDNA library. The large extracellular domain (residues 51-303) of ATP1B1 ($\Delta 50$, also see Fig. 1F) was subcloned into the pcDNA3-3FLAG plasmid (11). Next, pcDNA3-3FLAG-ATP1B1 was transfected into 293T cells together with pEBG-NEGR1 (12) expressing three C2 domains (D1-3) (Fig. 1D). After GST-pull-down, we could observe that FLAG-ATP1B1 was co-isolated with GST-NEGR1, but not with GST control (Fig. 1A). Reciprocally, GST-ATP1B1 ($\Delta 50$) was constructed and GST-pull-down was performed with FLAG-NEGR1. NEGR1 was present in the ATP1B1-enriched fraction (Supplementary Fig. S1B), suggesting an *in vivo* interaction between these two pro-

teins.

To show the interaction between NEGR1 and ATP1B1 at an endogenous level, we performed immunoprecipitation (IP) using anti-NEGR1 antibody with HEK293 whole cell lysates and found that ATP1B1 co-fractionated with NEGR1 (Fig. 1B). Moreover, reciprocal IP using anti-ATP1B1 antibody also pulled down endogenous NEGR1 (Fig. 1C and Supplementary Fig. S1C), again consistent with an *in vivo* interaction between the two proteins.

NEGR1-ATP1B1 interaction is mediated by the C-termini of both proteins

To determine the domains critical for NEGR1-ATP1B1 interaction, we performed domain mapping with multiple domain constructs. In our previous study, we generated multiple GST-fused NEGR1 deletion constructs (12). We named three C2 domains D1, D2, and D3 from the N-terminus and designed constructs containing one or two C2 domains (Fig. 1D). Along with the positive control containing three domains (D1-3) of NEGR1, D2-3 and D3 constructs also exhibited high affinity for FLAG-ATP1B1 in GST-pull-down binding assay (Fig. 1E), suggesting that the C-terminal C2 domain (D3) may be important in ATP1B1 binding.

In addition to the previous FLAG-ATP1B1 construct containing the large extracellular compartment ($\Delta 50$, residues 51-303) describe above, we generated two more mutants that contained serially-deleted C-terminal region (residues 51-212 and 51-157), considering the location of putative disulfide bonds (Fig. 1F). GST pull-downs were then carried out after 293T cells were co-transfected with pEBG-NEGR1. Contrary to the positive control (ATP1B1 $\Delta 50$), two deletion mutants (residues 51-212 and 51-157) lacking the C-terminus failed to bind to NEGR1 (Fig. 1G). We then made an additional construct with only the C-terminal 94 residues from the C-terminus (210-303), and observed high-affinity binding to NEGR1 in GST pull-downs (Fig. 1G). Taken together, these data suggest that the C-terminus of each of these proteins is required for their interaction.

NEGR1 may form a complex with ATP1B1

To demonstrate that NEGR1 and ATP1B1 are present in a complex *in vivo*, we performed size-exclusion chromatography on a SKOV-3-NEGR1-FLAG stable cell lysate. Some NEGR1 proteins appeared in early eluents, possibly as components of large complexes. However, most cellular NEGR1-FLAG was detected in fractions 19-21, overlapping with the peak of endogenous ATP1B1 (Fig. 2A).

To validate the *in vivo* interaction between NEGR1 and ATP1B1, we performed an *in situ* proximity ligation assay (PLA) in Neuro-2a cells using Duolink PLA technology. After incubation with anti-NEGR1 and anti-ATP1B1, cells were further incubated with the PLA probes (anti-mouse MINUS and anti-rabbit PLUS) to produce signals when these two proteins were in close proximity. While no signals were observed in control samples in the presence of only one antibody (anti-NEGR1 or anti-ATP1B1), clear PLA signals were present in cells incubated

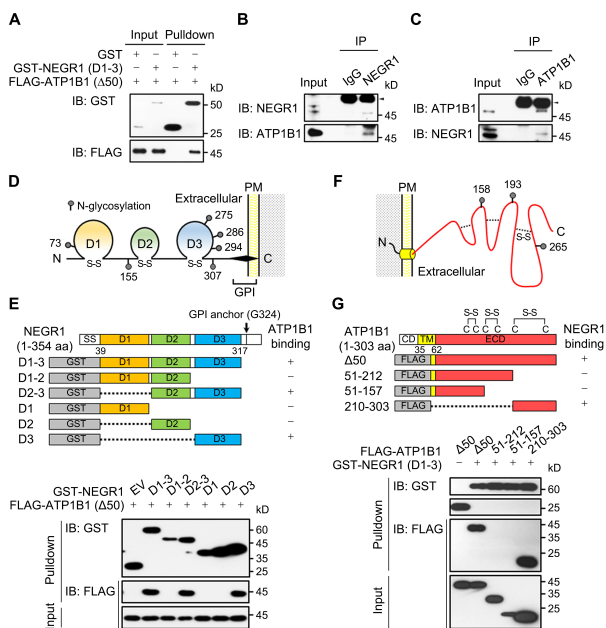


Fig. 1. ATP1B1 is a new binding partner of NEGR1. (A) GST-pull-down from 293T cells transfected with FLAG-ATP1B1 ($\Delta 50$) expressing the extracellular region of ATP1B1 (residues 51-303) and GST-NEGR1 (D1-3). (B) Interaction between endogenous NEGR1 and ATP1B1 assayed by IP using an anti-NEGR1 antibody with HEK293 cell lysates. (C) Reciprocal IP with an anti-ATP1B1 antibody using HEK293 cell lysates. Arrowheads, IgG bands. (D) NEGR1 structure. Three C2-type immunoglobulin domains had been named D1, D2, and D3 from the N-terminus. Filled dots represent predicted N-glycosylation sites. (E) Deletion mutants of NEGR1 were generated and GST-pull-down assay was performed after each construct was transfected into 293T cells with FLAG-ATP1B1 ($\Delta 50$) plasmid. (F) Domain structure of ATP1B1 with three predicted N-glycosylation sites. (G) Multiple FLAG-tagged ATP1B1 mutants were constructed and their binding to GST-NEGR1 was examined using GST-pull-down assays.

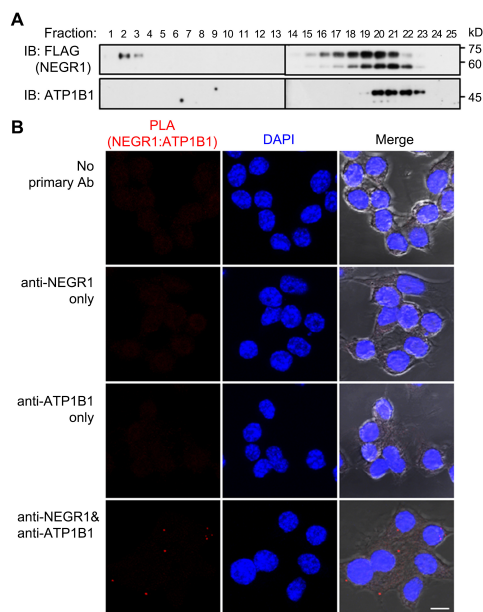


Fig. 2. NEGR1 may form a stable complex with ATP1B1 *in vivo*. (A) Gel filtration chromatography of a SKOV-3-NEGR1-FLAG stable cell lysate. Each fraction was used for immunoblotting with anti-FLAG and anti-ATP1B1 antibodies to visualize NEGR1 and ATP1B1, respectively. (B) *In situ* proximity ligation assay (PLA) performed on fixed Neuro-2a cells using Duolink PLA technology. Cells were incubated with a mouse anti-ATP1B1 antibody and/or a rabbit anti-NEGR1 antibody for 2 h, followed by incubation with PLA probes (anti-mouse MINUS and anti-rabbit PLUS). Cells were mounted with mounting solution containing DAPI. Bar = 10 μm.

with both antibodies (Fig. 2B). Overall, our data suggest that NEGR1 and ATP1B1 form a complex in cells.

NEGR1 co-localizes with ATP1B1 in membrane rafts

Since NEGR1 was identified as a novel raft-associated protein in rat brain (6) as well as ATP1B1 in many raft proteome analyses (13), we tested for their co-localization in membrane rafts. 293T cells were transfected with a plasmid expressing ATP1B1-MYC together with pEGFP-NEGR1 or pEGFP control vector. Then, floating lipid raft fractions were obtained by discontinuous density gradient centrifugation. The ganglioside GM1 was used as a marker for lipid rafts. When GFP-NEGR1 was co-expressed with ATP1B1, NEGR1 was mostly found in these raft fractions, with small amounts of ATP1B1 (Fig. 3A, right). Although the amount of raft-associated ATP1B1 was not affected by NEGR1 co-transfection, this result suggests that NEGR1 and ATP1B1 are co-localized in lipid rafts.

To visualize co-localization in cells, we performed immunofluorescence microscopy on U178-MG human astrocytoma cell. Upon permeabilization with Triton X-100, intracellular signals of both proteins were found to be strong and dispersed from the nucleus (top panel, Fig. 3B), suggesting that substantial

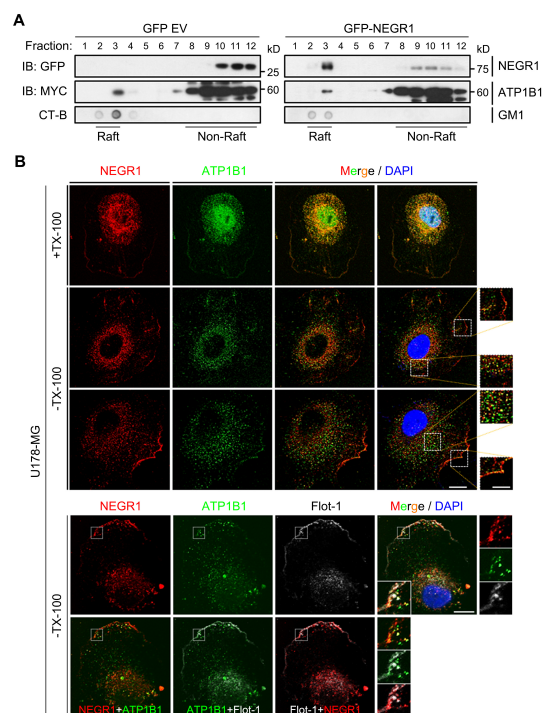


Fig. 3. NEGR1 co-localizes with ATP1B1 in the plasma membrane. (A) ATP1B1-MYC plasmid was co-transfected into 293T cells with pEGFP-NEGR1 (right) or pEGFP control (left). Membrane rafts were isolated by density-gradient centrifugation. GM1 was detected with HRP-conjugated cholera toxin B (CT-B) by dot blotting. (B) Immunofluorescence microscopy. U178-MG cells were either untreated or permeabilized with 0.1% Triton X-100 for 10 min. Next, cells were incubated with rat anti-NEGR1, mouse anti-ATP1B1, and rabbit anti-Flotillin-1 antibodies for 2 h, and further incubated with Alexa Fluor 568 anti-rat, Alexa Fluor 488 anti-mouse, and Alexa Fluor 633 anti-rabbit secondary antibodies. Imaging was performed by confocal laser scanning microscopy. Bar = 10 μm (5 μm in magnified images).

fractions of NEGR1 and ATP1B1 are localized in ER/Golgi and endosomal compartments. Without permeabilization, both NEGR1 and ATP1B1 appeared as weak small dots with partial overlap (second and third rows, also see Supplementary Fig. S2), suggesting their co-localization in the plasma membrane.

To clearly demonstrate their co-localization in membrane rafts, Flotillin-1, a conserved raft marker protein, was also visualized. When cells were immunostained with anti-NEGR1, anti-ATP1B1, and anti-Flotillin-1 antibodies, we could observe overlap in several punta (lower two panels, Fig. 3B). Collectively, these results suggest that NEGR1 and ATP1B1 are co-localized in the membrane lipid rafts.

NEGR1 and ATP1B1 participate in intercellular interaction

To assess the contributions of NEGR1 and ATP1B1 to cell-cell interaction, we performed IP after co-culturing SKOV-3-NEGR1-FLAG and SKOV-3-ATP1B1-MYC stable cells (Mix1) at high

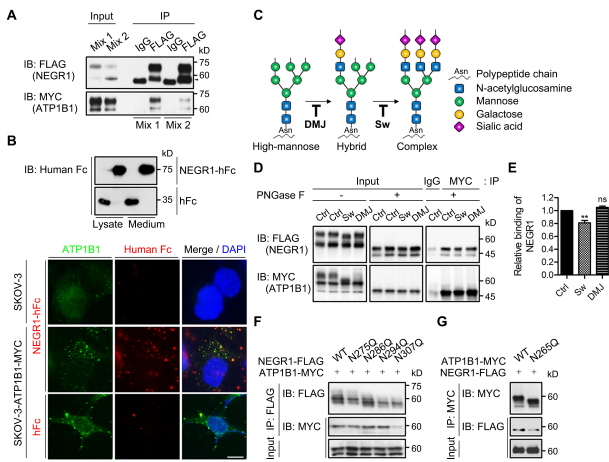


Fig. 4. NEGR1 may form a heterophilic trans-interaction with ATP1B1. (A) Co-IP from cocultures of SKOV-3-NEGR1-FLAG and -ATP1B1-MYC cells (Mix1) or mixed lysates from separate culture of each cell line (Mix2). (B) SKOV-3 or SKOV-3-ATP1B1 stable cells were incubated with the conditioned medium from 293T cells that expressed with NEGR1-hFc or hFc (control). After 1 h, cells were fixed and immunostained with anti-ATP1B1 and anti-hFc antibodies. Bar = 10 μ m. (C) DMJ and Sw suppress the synthesis of hybrid- and complex-type glycans, respectively. (D) Co-culture containing SKOV-3-NEGR1 and -ATP1B1 cells were treated with the N-glycosylation inhibitors, DMJ (100 μ M) or Sw (1 μ M) for 48 h. The immunoprecipitated sample was treated with PNGase F (500 units) for 2 h prior to western blotting. (E) The density of co-isolated NEGR1 protein was determined with ImageJ software. The data represent mean of three independent experiments \pm SD (**P < 0.01). (F) After the four putative N-glycosylation sites in the NEGR1 D3 domain were replaced with glutamine residues, the binding of each mutant to ATP1B1 was examined by IP. (G) One ATP1B1 N-glycosylation mutant (N265Q) was generated and IP was performed using co-cultured HEK293 cells that were separately transfected with plasmids encoding ATP1B1-MYC (N265Q) and NEGR1-FLAG.

confluency. As a control, IP was performed after mixing the cell lysates from separate cultures of each cell line (Mix2). Although we observed that some ATP1B1 coisolated with NEGR1 in Mix2, the ATP1B1-NEGR1 association was clearer in Mix1 (Fig. 4A), consistent with their intercellular binding. Then, we generated the secreted form of D3 (D3-hFc) and enriched D3 deletion construct using protein A beads. The co-isolated APT1B1 clearly decreased by the co-transfection of full-length NEGR1 plasmid (Supplementary Fig. S3), demonstrating the binding specificity of these two proteins.

To further assess this putative interaction, we collected conditioned medium from 293T cells expressing the secreted form of NEGR1 (NEGR1-hFc) (8) or hFc (control). SKOV-3-ATP1B1 cells were then incubated with the NEGR1-hFc-containing medium for 1 h at 4°C and stained with an anti-hFc antibody. Compared with the hFc control (bottom row, Fig. 4B), the Fc signals were stronger in the SKOV-3-ATP1B1 cells treated with NEGR1-hFc protein (middle row, Fig. 4B), consistent with trans-interaction.

Next, we examined the effect of N-glycan moieties on this interaction using co-cultured SKOV-3-NEGR1 and SKOV-3-ATP1B1 cells. During co-culture, cells were incubated with deoxymannojirimycin (DMJ) and swainsonine (Sw) for 48 h to suppress the synthesis of hybrid- and complex-type glycans, respectively (Fig. 4C). For more accurate quantification, we treated the immunoprecipitated samples with peptide N-glycosidase F (PNGase F) prior to immunoblotting. Although not dramatic, a slight decrease in NEGR1-ATP1B1 interaction was observed in Sw-treated cells (Fig. 4D & 4E).

To demonstrate the above results more clearly, we generated mutants at potential N-glycosylation sites. Since the C-termini of both NEGR1 and ATP1B1 are important for their binding (Fig. 1E & 1G), we focused on this region of each protein. To measure intercellular interactions, HEK293 cells separately transfected with each NEGR1 mutant construct and ATP1B1-MYC plasmid, mixed, and co-cultured with high confluency. Among the four putative N-glycosylation sites (N275, N286, N294, and N307) residing in the D3 domain of NEGR1, only the N307Q mutant protein had decreased binding to ATP1B1 (Fig. 4F). In case of ATP1B1, only one putative N-glycosylation site (N265) was identified in the C-terminal region, while N265Q mutant showed reduced binding to NEGR1 (Fig. 4G). Collectively, these results suggest that NEGR1-ATP1B1 association is partially influenced by their glycosylation status.

DISCUSSION

In the nervous system, cell adhesion plays a crucial role in the formation of functional neural networks, including axon guidance and synapse formation. Therefore, defects in cell adhesion molecules in the central nervous system are closely linked to many neurological conditions in humans (14). Although this study revealed the *in vivo* interaction between ATP1B1 and NEGR1 using diverse ways including endogenous IP and PLA assay, we were still unsure whether this interaction mainly occurs inside the intracellular compartments such as ER and secretory vesicles. To differentially assess the intercellular binding, we used the co-cultured stable cell lines that separately express NEGR1-FLAG and ATP1B1-MYC for IP. As shown in Fig. 4A, co-isolated ATP1B1 was easily detected in NEGR1-enriched fraction (Mix 1). Furthermore, exogenously added NEGR1-hFc proteins were clearly stained on the surface of ATP1B1-expressing cells. These results may demonstrate that these proteins contribute cell-cell interaction in neural network. Interestingly, although weak, we could still observe their binding in Mix 2, in which each stable cells were separately lysed for IP, suggesting a relatively high binding activity between two proteins.

N-linked glycans not only contribute in protein folding and stability, it can also modulate structure of interacting proteins and their functions (15). Abnormal glycosylation has been implicated in many neuropathological events (16). Our results showed that although blockage of complex- and hybrid-type glycans exerts little effect on ATP1B1-NEGR1 binding (Fig. 4D & E),

absence of glycosyl moiety on N307 of NEGR1 highly decreased affinity to ATP1B1 (Fig. 4F). Since the membrane trafficking is not impaired by this mutation (unpublished data), this potential membrane-proximal *N*-glycan may be critical for ATP1B1 binding.

The beta subunit of Na/K-ATPase shows a self-adhesive property and has the structure of a typical cell adhesion molecule with its long extracellular domain (17). Interestingly, the majority of the β ectodomain is not absolutely required for its binding with alpha subunit and its membrane targeting (18). The C-terminus of the β ectodomain contains an immunoglobulin-like fold that is important for β - β interaction. A previous study showed that the residues 198-207 are critical for the β 1- β 1 interaction (19). Another study identified that residues 222-229 were also important for the β 1 homodimer (18). In this study, we showed that ATP1B1 mutants devoid of their C-termini (51-212 and 51-157) completely lost the ability to bind NEGR1, while the C-terminal 94 residues (210-303) were sufficient for NEGR1 binding (Fig. 1G). The previously identified regions important for β 1- β 1 interaction (residues 198-207 and 222-229) did not completely overlap with the region for NEGR1 binding. However, since binding is commonly mediated by the C-terminus, there is a possibility that NEGR1 influences the β 1- β 1 interaction.

The beta subunit is also essential for Na/K-ATPase function as it facilitates a proper folding of the α subunit in ER and escorts it to the plasma membrane (20). The activity of Na/K-ATPase seems to be tightly regulated; its dysregulation has been linked to diverse neurological diseases, including depression, learning deficits, amnesia, and Alzheimer's disease (21). Furthermore, the function of Na/K-ATPase has linked to other clinical disorders, including cancer, cardiovascular diseases, and obesity (22). Recently, we reported that *NEGR1*^{-/-} mice had highly increased fat mass and muscle atrophy (12), in addition to a depression-like affective behavior (23). These studies collectively indicate that NEGR1 and ATP1B1 have roles in diverse biological and clinical aspects, which may imply a substantial overlapping of their cellular functions. Overall, we report that human NEGR1 is a novel binding partner of Na/K-ATPase β 1-subunit in the cell membrane, which may provide valuable insights in understanding the roles of both proteins in normal cell function and in several human disorders.

MATERIALS AND METHODS

Cell culture, transfection, and cloning

The cell lines HEK293, U178-MG, and Neuro-2a were maintained in DMEM (Welgene) supplemented with 10% FBS (Invitrogen), while SKOV-3 cells were cultured in RPMI 1640 medium. Transient transfections were performed using Effectene (Qiagen) or polyethylenimine (PEI, MilliporeSigma). The cDNA clone of ATP1B1 was obtained by PCR amplification from a human fetal stomach Marathon-ready cDNA library (Clontech). The ORF of ATP1B1 was subcloned into the pCS3-6MYC vector

(11) using BamHI and ClaI. To perform *in vivo* binding assays, the extracellular region (residues 51-303) of ATP1B1 was subcloned into pcDNA3-3FLAG or pEBG (11) to generate 3FLAG- or GST-fused expression constructs, respectively.

Yeast two-hybrid screening, protein binding, and antibodies

Screening was performed using the GAL4-based Matchmaker™ Two-Hybrid System on a pre-transformed human fetal testis library (Clontech) (12). The truncated N-terminal region containing two C2 domains (D1-2) of NEGR1 (residues 40-215) was inserted into pGBKT7 vector (Clontech) to produce a GAL4 DNA-binding domain (BD)-fused NEGR1 bait. Positive clones were isolated using the selective medium (SD/-Leu-Trp-His-Ade) containing 40 μ g/ml X- α -gal.

Immunoprecipitation and GST pulldown assays were carried out as previously described (12) using 1 μ g of appropriate antibody or glutathione-Sepharose 4B beads (GE Healthcare). Antibodies for MYC, FLAG, GST, and NEGR1 were purchased from Millipore Sigma. Anti-GFP, anti-Flot1, and anti-ATP1B1 antibodies were purchased from Santa Cruz Biotechnology. Anti-NEGR1 antiserum was obtained by immunizing rats with recombinant NEGR1 protein (8).

Rafts fractionation and size exclusion chromatography

Fractionation was performed using OptiPrep™ iodixanol (Sigma) as previously described (8). Briefly, cell lysates, which were adjusted to 32% OptiPrep (in 1.5 ml), were loaded into centrifuge tubes and overlaid with iodixanol solution (2 ml of 24% and 1.5 ml of 20% OptiPrep). After centrifugation at 76,000 \times g for 18 h at 4°C, fractions were collected from the upper layer and designated as No. 1. Gel-filtration chromatography was performed using a Sephacryl S-400 HR column (GE Healthcare, 1.0 \times 30 cm) with a size-exclusion buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM Na₃VO₄, 1 mM NaF, and 0.3% CHAPS) as described previously (24).

Immunofluorescence microscopy

Cells were grown on coverslips and fixed with 4% paraformaldehyde for 15 min, then were either not treated or permeabilized with 0.1% Triton X-100 in PBS for 10 min. After blocking with 10% CAS-Block™ (Invitrogen) in PBS, cells were incubated with appropriate primary antibodies. Next, cells were incubated with Alexa Fluor 568 anti-rat, Alexa Fluor 488 anti-mouse, or Alexa Fluor 594 anti-human IgG antibody (Invitrogen). Imaging was performed using an Olympus IX70 fluorescence microscope or Leica TCS SP5 AOBs confocal microscope equipped with 63 \times inverted NX oil lens, located at Gwangju Center, Korea Basic Science Institute.

In situ proximity ligation assay (PLA)

Assays were performed on fixed Neuro-2a cells using Duolink PLA technology probes and reagents (Merck). Briefly, cells were fixed and permeabilized with 0.1% Triton-X 100 in PBS

for 10 min, followed by incubation with anti-ATP1B1 and anti-NEGR1 antibodies for 2 h. After two washes, the cells were incubated with PLA probes (anti-mouse MINUS and anti-rabbit PLUS for ATP1B1 and NEGR1, respectively) for 1 h. Next, cells were treated with a ligation solution for 30 min and an amplification solution for 2 h. After two washes of 10 min, the cells were mounted with a mounting solution containing DAPI.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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