Mutations in genes encoding components of the dystrophin-associated glycoprotein complex (DGC) can cause various forms of muscular dystrophy such as Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). In addition to muscle-related symptoms, DMD and BMD patients show altered retinal responses to light/dark stimuli without an apparent loss of visual acuity.1-4 The DGC in skeletal muscles, which is composed of dystrophin, α- and β-dystroglycans, sarcoglycans, sarcospan, syntrophins, and dystrobrevins, is proposed to function not only as a molecular bridge between the intracellular cytoskeleton and the extracellular matrix but also as a molecular scaffold that recruits signaling molecules downstream of mechanical stimuli.5,6 Dystroglycan and dystrophin constitute the core components of the DGC. Dystroglycan itself is composed of α and β subunits cleaved from a single precursor protein: α-dystroglycan is an extensively glycosylated extracellular protein that functions as a receptor for extracellular matrix components, whereas β-dystroglycan is a transmembrane protein that is associated with α-dystroglycan extracellularly and with dystrophin intracellularly. Dystrophin exists in various isoforms (Dp427, Dp260, Dp140, Dp116, and Dp71) with tissue-specific expression patterns. In photoreceptors, dystrophin Dp427 and Dp260, dystroglycans, and β-dystrobrevin were immunohistochemically shown to localize in a specific microdomain of the plasma membrane of photoreceptor presynaptic terminals.7-10 Recently, pikachurin was identified as a novel physiologic ligand for α-dystroglycan and shown to regulate photoreceptor synaptic transmission and structure with the photoreceptor DGC.11,12 However, the exact molecular composition of the DGC differs depending on tissue and cell types,13 and we still have a poor understanding of the molecular components and functions of the DGC in nonmuscle tissues.

The ADP-ribosylation factors (Arfs) are critical small GTPases that regulate vesicle biogenesis and transport between various subcellular compartments.14,15 Arfs are divided into three classes based on structural similarity: class I (Arf1, Arf2, and Arf3), class II (Arf4 and Arf5), and class III (Arf6). Compared with the other Arfs, Arf6 is the most divergent in terms of its molecular structure and subcellular localization. Furthermore, in contrast to the primary function of other Arfs in the Golgi complex, Arf6 regulates endosomal trafficking between endo-
somes and the plasma membrane. Like other small GTPases, the interconversion of Arfs between GTP-bound active and GDP-bound inactive states is under tight control by two types of regulatory proteins: guanine nucleotide exchange factors (GEFs) that activate Arfs by catalyzing the exchange of GDP for GTP and GTPase-activating proteins (GAPs) that inactivate Arfs by triggering the hydrolysis of bound GTP to GDP. Brefeldin A–resistant Arf-GEF 2 (BRAG2), originally named Arf-guanine exchange protein 100 (Arf-GEP100) and also known as IQSEC1, was the first identified member of the BRAG/IQSEC family of Arf-GEFs, and it functions to activate Arf6 in most cellular contexts. There are at least two alternative splicing isoforms, BRAG2a and BRAG2b, which share a conserved domain structure among the BRAG/IQSEC family, consisting of an N-terminal calmodulin-binding IQ-like motif, a central catalytic Sec7 domain, and a pleckstrin homology (PH) domain, but differ by the alternative use of N-terminal and C-terminal regions (Fig. 1A). Particularly, BRAG2a is unique in terms of the presence of a long C-terminal region containing a proline-rich domain and a type I postsynaptic density protein-95 (PDS-95)/Disc large/Zonula occludens 1 (PDZ)-binding motif. In the retina, we recently demonstrated using immunohistochemical analyses with an anti-panBRAG2 antibody that recognizes all BRAG2 isoforms that BRAG2 colocalizes with the DGC at photoreceptor presynaptic terminals in contrast to its postsynaptic localization in retinal bipolar cells and hippocampal neurons. In the present study, we provide several lines of evidence for BRAG2a as a novel DGC component in photoreceptor presynapses with the use of independent assays including immunohistochemistry, pull-down, immunoprecipitation, and in situ proximity ligation assay (PLA). Furthermore, we show the effect of dystrophin (Dp427, Dp260, and Dp140) loss on the photoreceptor presynaptic localization of BRAG2a using dystrophin exon 52 knockout mice lacking dystrophin isoforms, Dp427, Dp260, and Dp140, which are expressed in photoreceptors.

**MATERIALS AND METHODS**

**Animals**

We purchased male C57BL/6N mice at postnatal week 10 from CLEA Japan (Tokyo, Japan). Dystrophin exon 52 knockout mice were kindly provided to us by Motoya Katsuki. Animals were kept on a 12-hour day/night cycle with free access to food and water. All experimental procedures were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine.

**Plasmids**

For bacterial expression vectors, the C-terminal region (amino acids 1014–1089) of mouse BRAG2a (BRAG2a [1014–1089]) and the 120-amino acid C-terminal intracellular region of β-dystroglycan (β-dystroglycan [C120aa]) were amplified by PCR and subcloned into pGEX4T-2 (GE Healthcare, Piscataway, NJ).
USA). For mammalian expression vectors, the coding regions of BRAG2a and BRAG2b lacking the N-terminal 121 amino acids (BRAG2bN121/GEPI100), which corresponded to the human counterpart of GEPI100, were amplified by PCR and subcloned into pCAGGS-FLAG. A mammalian expression vector for a BRAG2a mutant (P-PRD-A), in which proline residues that conform to the consensus WW domain-binding motif were replaced by alanine residues in the C-terminal proline-rich domain, was prepared as described elsewhere (Fukaya M, unpublished observations, 2017).

**Antibodies**

A glutathione S-transferase (GST) fusion protein of BRAG2a (1014–1089) was used as an antigen to immunize a rabbit. The serum was affinity-purified with the antigen. A guinea pig polyclonal anti-panBRAG2 antibody was characterized previously. Antibodies used in the present study were as follows: mouse anti-β-dystroglycan IgG2a (clone 43DA1/8D5; Novocastra Laboratories, Newcastle upon Tyne, UK); mouse anti-PSD-95 IgG1 (clone 16; BD Transduction Laboratories, San Jose, CA, USA); rabbit anti-dystrophin IgG (ab15277; Abcam, Cambridge, MA, USA); mouse anti-Bassoon IgG2a (clone SAP7F407; Stressgen, Victoria, BC, Canada); rabbit anti-RIBEYE IgG (C24; Sigma-Aldrich, St. Louis, MO, USA).

**Immunohistochemistry**

Immunohistochemical procedures were described previously. Briefly, eyeballs, from which the cornea and lens had been removed, were immersed in 4% paraformaldehyde for 10 minutes and cryoprotected in 30% (wt/vol) sucrose. Cryostat sections (20 μm) were treated with pepsin (1 g/L; Dako, Carpinteria, CA, USA) in 0.2 N HCl for 5 minutes at 37°C. For immunofluorescence staining, sections were incubated with anti-BRAG2a IgG and antibodies against panBRAG2 or β-dystroglycan overnight at room temperature. Immunoreactions were visualized with species-specific secondary antibodies conjugated with Alexa Fluor 488 or 594 (Invitrogen, Carlsbad, CA, USA). Sections were counterstained with 20 μg/ml of 1 mg/mL) were incubated with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and analyzed using a confocal laser scanning microscope (LSM 710; Carl Zeiss, Jena, Germany). For immunoelectron microscopy, after incubation with primary antibodies, sections were incubated with nanogold-conjugated secondary antibodies (1:100; Nanoprobes, Yaphank, NY, USA) and subjected to silver enhancement of gold particles (HQ kit; Nanoprobes). Ultrathin sections (70 nm) were analyzed using a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan). Data were acquired from five areas randomly selected in the OPL with digital zoom of 3.5 within the linear range below the saturation of detection signals. The intensities of immunoreactive puncta (100–200 puncta per mice) were measured using ZEN imaging software (Carl Zeiss), and statistical analyses were performed using Student’s t-test.

**Immuno blot Analysis**

Total lysates from the adult mouse brain and retina were prepared as described previously. HeLa cells were transfected with pcAGGS-FLAG-BRAG2a or pcAGGS-FLAG-BRAG2bN121/GEPI100 using Lipofectamine 2000 (Invitrogen) and harvested with loading buffer. The lysates (10 μg) were subjected to immunoblot analyses with anti-BRAG2a, anti-panBRAG2, or anti-FLAG antibodies as described previously. Experiments were repeated at least three times.

**Pull-Down**

HeLa cells were transfected with pcAGGS-FLAG vectors encoding BRAG2a, BRAG2bN121/GEPI100, or BRAG2a (P-PRD-A) using Lipofectamine 2000. Twenty-four hours after transfection, the cells were solubilized with lysis buffer consisting of 0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl, 1% Triton X-100, and a cocktail of protease inhibitors (Complete Mini; Roche, Mannheim, Germany). The retinal lysate was obtained by the solubilization of retinas from six to eight adult C57BL/6N mice in the lysis buffer by brief sonication and incubation for 30 minutes at 4°C. The lysates (500 μg) were incubated with 20 μg GST-dystrophin (2937–3685), GST-β-dystroglycan (C120aa), or GST alone, which had been immobilized on glutathione-Sepharose 4B, for 1 hour at 4°C. After the beads were washed with the lysis buffer, proteins bound to the beads were dissociated by boiling with loading buffer. Samples were subjected to immunoblotting with anti-FLAG or anti-panBRAG2 antibodies. Experiments were repeated at least three times.

**Immunoprecipitation**

The rabbit anti-dystrophin IgG and normal rabbit IgG were conjugated with protein G-coupled magnetic beads (Dynabeads Protein G, Life Technologies AS, Oslo, Norway) in phosphate-buffered saline (PBS; pH 7.4) containing 0.1% Triton X-100 overnight at 4°C. The complexes were cross-linked by incubation with 1 mM dithiobis(succinimidyl propionate) (DSP; Thermo Fisher Scientific, San Jose, CA, USA) in PBS for 2 hours at 4°C. After the cross-linking reaction was quenched by the addition of Tris-HCl (pH 7.5) for 15 minutes at room temperature, beads were washed with PBS containing 0.1% bovine serum albumin and stored at 4°C.

Retinas from six to eight adult C57BL/6N mice were solubilized in buffer consisting of 0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% digitonin, 0.05% Nonidet P-40, and a cocktail of protease inhibitors (Complete Mini; Roche) by brief sonication and subsequent incubation for 30 minutes at 4°C. The retinal lysates (500 μg of 1 mg/mL) were incubated with the cross-linked beads (5 μg IgG) overnight at 4°C. The beads were then washed three times with a buffer consisting of 0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1% digitonin, and 0.05% Nonidet P-40 and boiled with loading buffer. Samples were subjected to immunoblotting with antibodies against pan-BRAG2 and β-dystroglycan. Experiments were repeated three times.

**In Situ PLA**

In situ PLA was carried out in accordance with the manufacturer’s instructions (Olink Bioscience, Uppsala, Sweden). Briefly, cryostat sections of retinas from three mice were treated with pepsin for 5 minutes at 37°C, blocked with 5% normal donkey serum, and incubated overnight at 4°C with...
anti-BRAG2a IgG or β-dystroglycan IgG alone or with the combination of anti-BRAG2a IgG and anti-β-dystroglycan IgG or anti-Bassoon IgG. Sections were then incubated with species-specific secondary antibodies conjugated with oligo-nucleotides, anti-rabbit PLUS, and anti-mouse MINUS PLA probes (Sigma-Aldrich) for 1 hour at 37°C, followed by a rolling circle amplification reaction with the amplification-polymerase solution (Duolink In situ Detection Reagent Red; Sigma-Aldrich) for 100 minutes at 37°C. Fluorescent signals were analyzed using a confocal laser scanning microscope (LSM 710; Carl Zeiss). Experiments were repeated twice.

RESULTS

Expression of BRAG2a in the Mouse Retina

BRAG2 exists in at least two alternative splicing isoforms: BRAG2a and BRAG2b (Fig. 1A). In the present study, we produced an antibody specific for BRAG2a using a BRAG2a-specific C-terminal region (amino acids 1014–1089) as an antigen (Fig. 1A). In the immunoblot analysis, the anti-BRAG2a antibody recognized FLAG-BRAG2a but not FLAG-BRAG2bΔN121/GEP100 in the lysates of transfected HeLa cells and detected two major immunoreactive bands of 160 and 140 kDa in the lysates of mouse brain and retina (Fig. 1B). Meanwhile, an anti-panBRAG2 antibody, which had previously been raised against the common amino-terminal region20 (Fig. 1A), recognized both FLAG-BRAG2a and FLAG-BRAG2bΔN121/GEP100 and detected three immunoreactive bands of 160, 140, and 110 kDa in the mouse brain and retina lysates, as described previously20 (Fig. 1B). The sizes of the upper two panBRAG2-immunoreactive bands were consistent with those detected by the anti-BRAG2a antibody. Because the 140-kDa band detected by both anti-BRAG2a and panBRAG2 antibodies was consistent with FLAG-BRAG2a, it is likely that the 140- and 110-kDa bands correspond to BRAG2a and BRAG2b, respectively. The nature of the remaining 160-kDa species detected by the anti-BRAG2a remains unknown at present, but it could be explained by post-translational modification of BRAG2a such as phosphorylation or the presence of additional BRAG2 isoforms. Indeed, a previous proteomic analysis isolated BRAG2 as a phosphoprotein in the postsynaptic density fraction.28 In the present study, we examined the ability of BRAG2a to interact with dystrophin. HeLa cells were transiently transfected with BRAG2a and BRAG2a (P-PRD-A) (Fig. 4A), suggesting a specific interaction of BRAG2a with the GST fusion protein of the dystrophin C-terminal 749–3685. GST-dystrophin (2937–3685) efficiently pulled down wild-type FLAG-BRAG2a but not FLAG2bΔN121/GEP100 or BRAG2a (P-PRD-A) (Fig. 4B), suggesting a specific interaction of BRAG2a with dystrophin through the proline-rich domain. We further examined the possibility of an interaction between BRAG2 and β-dystroglycan using pull-down assays with GST fusion proteins of the β-dystroglycan C-terminal intracellular domain (GST-β-dystroglycan [C120]). GST-β-dystroglycan (C120) pulled down BRAG2a and BRAG2a (P-PRD-A) (Fig. 4B). In addition, a small amount of FLAG-BRAG2b (2937–3685) pulled down wild-type FLAG-BRAG2a but not FLAG2bΔN121/GEP100 or BRAG2a (P-PRD-A) (Fig. 4B), suggesting a specific interaction of BRAG2a with dystrophin through the proline-rich domain. We further examined the possibility of an interaction between BRAG2 and β-dystroglycan using pull-down assays with GST fusion proteins of the β-dystroglycan C-terminal intracellular domain (GST-β-dystroglycan [C120]). GST-β-dystroglycan (C120) pulled down BRAG2a and BRAG2a (P-PRD-A) (Fig. 4B).

Interaction of BRAG2a with Dystrophin and β-Dystroglycan

BRAG3, another member of the BRAG/IQSEC family, was previously shown to interact with a WW domain of dystrophin and utrophin through a proline-rich domain.26 Because BRAG2a also contains a similar proline-rich domain in the C-terminal region (Fig. 1A), we examined the ability of BRAG2a to interact with dystrophin. HeLa cells were transiently transfected with BRAG2a, BRAG2bΔN121/GEP100, or a BRAG2a mutant (P-PRD-A) in which proline residues that conform to the consensus WW domain-binding motif were mutated to alanine residues and subjected to pull-down assays with the GST fusion protein of the dystrophin C-terminal 749–3685. GST-dystrophin (2937–3685) pulled down wild-type FLAG-BRAG2a but not FLAG2bΔN121/GEP100 or BRAG2a (P-PRD-A) (Fig. 4A), suggesting a specific interaction of BRAG2a with dystrophin through the proline-rich domain. We further examined the possibility of an interaction between BRAG2 and β-dystroglycan using pull-down assays with GST fusion proteins of the β-dystroglycan C-terminal intracellular domain (GST-β-dystroglycan [C120]). GST-β-dystroglycan (C120) pulled down BRAG2a and BRAG2a (P-PRD-A) (Fig. 4B). In addition, a small amount of FLAG-BRAG2b (2937–3685) pulled down wild-type FLAG-BRAG2a but not FLAG2bΔN121/GEP100 or BRAG2a (P-PRD-A) (Fig. 4B), suggesting a specific interaction of BRAG2a with dystrophin through the proline-rich domain. We further examined the possibility of an interaction between BRAG2 and β-dystroglycan using pull-down assays with GST fusion proteins of the β-dystroglycan C-terminal intracellular domain (GST-β-dystroglycan [C120]). GST-β-dystroglycan (C120) pulled down BRAG2a and BRAG2a (P-PRD-A) (Fig. 4B). In addition, a small amount of FLAG-BRAG2b (2937–3685) pulled down wild-type FLAG-BRAG2a but not FLAG2bΔN121/GEP100 or BRAG2a (P-PRD-A) (Fig. 4B), suggesting a specific interaction of BRAG2a with dystrophin through the proline-rich domain. We further examined the possibility of an interaction between BRAG2 and β-dystroglycan using pull-down assays with GST fusion proteins of the β-dystroglycan C-terminal intracellular domain (GST-β-dystroglycan [C120]). GST-β-dystroglycan (C120) pulled down BRAG2a and BRAG2a (P-PRD-A) (Fig. 4B).
more efficiently pulled down than the upper band. On the other hand, GST-β-dystroglycan (C120) pulled down two species equally, although the efficiency of pull-down with GST-β-dystroglycan (C120) was much lower than that with GST-dystrophin (2937–3685). In the control, GST alone did not pull down any BRAG2 species from the retinal lysate.

To further confirm the in vivo interaction between BRAG2 and the DGC in the retina, we performed immunoprecipitation from the retina with anti-dystrophin IgG (Fig. 4D). Anti-dystrophin IgG precipitated β-dystroglycan and three BRAG2 species detected by the anti-panBRAG2 antibody, with the upper band being most densely labeled. On the other hand, normal rabbit IgG immunoprecipitated only a small amount of the lower BRAG2 species but not the two upper species. The reversed immunoprecipitation with the anti-panBRAG2 or anti-BRAG2a antibodies did not yield detectable immunoprecipitates for dystrophin or β-dystroglycan, because these antibodies for BRAG2 did not work well for the immunoprecipitation from the retinal lysate under the present condition (data not shown).

Finally, to verify the in situ interaction in photoreceptor synapses, we performed in situ PLA of the mouse retina. This is a sensitive method to visualize pairs of endogenous proteins that are localized in close proximity to form a protein complex in vivo.31 When retinal sections were subjected to in situ PLA with antibodies against BRAG2a and β-dystroglycan, discrete punctate fluorescent signals were detected in the OPL (Fig. 5A). In the control experiment, in situ PLA performed with the anti-BRAG2a or β-dystroglycan antibody alone, or the combination of antibodies against BRAG2a and Bassoon, an irrelevant presynaptic active zone protein, did not produce any fluorescent signals in the OPL (Figs. 5B–5D), thus confirming the specificity of the present in situ PLA.

**Synaptic Localization of BRAG2 in Photoreceptors of Dystrophin Exon 52 Knockout Mice**

The interaction of BRAG2a with the DGC led us to examine whether the presynaptic localization of BRAG2a in the OPL depends on the expression of dystrophin. Photoreceptors were shown to express three dystrophin isoforms, Dp426,
We examined the expression of BRAG2a in the OPL of dystrophin exon 52 knockout mice, in which the expression of these three isoforms were abolished. Although the density of immunofluorescent puncta was indistinguishable between wild and mutant retinas, the intensity of BRAG2a-immunoreactive puncta in the OPL was drastically decreased in mutant retinas by approximately 50% compared with wild-type retinas (Fig. 6; control, 100 ± 10.2%; mutant, 48.9 ± 6.2%; P = 0.00428). On the other hand, we also examined the expression of RIBEYE, a component of synaptic ribbon in photoreceptor terminals, and found that there were no significant differences in the pattern of distribution and the immunofluorescent intensity of puncta between wild-type and mutant retinas (Supplementary Fig. S3; control, 100 ± 22.3%; mutant, 107.0 ± 15.9%; P = 0.68), excluding the possibility that the loss of dystrophin isoforms could generally affect the expression of presynaptic components in photoreceptor terminals. Therefore, our results suggested that synaptic localization of BRAG2a in photoreceptors was partially dependent on dystrophin isoforms Dp426, Dp260, and Dp140, at the mRNA level. We examined the expression of BRAG2a in the OPL of dystrophin exon 52 knockout mice, in which the expression of these three isoforms were abolished.

**DISCUSSION**

Recent evidence of the existence of multiple Arf regulators in the photoreceptor terminal, including BRAG1, BRAG2, EFA6A, and ArfGAP3, suggests the functional importance of Arfs in the photoreceptor ribbon synapse. We previously demonstrated by immunohistochemistry using the anti-panBRAG2 antibody that BRAG2, a GEF specific for Arf6, colocalizes with the DGC at the photoreceptor presynaptic terminal. In the present study, we produced a novel antibody that recognized the C-terminal region of BRAG2a and extended our previous finding by showing that BRAG2, especially BRAG2a, is a novel component of the DGC at the photoreceptor terminal using independent assays. First, both immunofluorescence and immunolectron microscopy demonstrated that BRAG2a exhibited subcellular localization in the same subcompartment of the photoreceptor terminal as β-dystroglycan. Second, pull-down assays showed both GST fusion proteins of dystrophin and β-dystroglycan could interact with BRAG2a that was artificially overexpressed in HeLa cell and endogenously expressed in the mouse retina. Third, immunoprecipitation
from retinal lysates with the anti-dystrophin antibody demonstrated the formation of an immunoprecipitable protein complex between BRAG2a and the DGC. Finally, in situ PLA showed a close spatial relationship between BRAG2a and β-dystroglycan in the OPL. Taken together, these findings strongly supported the in vivo complex formation of BRAG2a and the DGC in the photoreceptor terminal.

Concerning the targeting mechanism for BRAG2a to the photoreceptor terminal, accumulating evidence for the existence of multiple BRAG2-interacting proteins suggested that BRAG2a can be recruited to various subcellular locations by partner proteins, thereby regulating the precise spatiotemporal activation of Arf6. In the present study, we demonstrated the dystrophin WW motif can interact specifically with BRAG2a through its proline-rich domain, whereas the β-dystroglycan intracellular domain can interact with both BRAG2a and BRAG2b. Thus, multiple interactions of BRAG2a with dystrophin and β-dystroglycan enable BRAG2a to build a more specific and stable complex with the DGC than BRAG2b. However, the present immunofluorescence showed that BRAG2a was significantly decreased but still present in the OPL of dystrophin exon 52 knockout mice lacking photoreceptor dystrophin isoforms, although this finding remains to be further verified by independent quantitative assays such as immunoblot and Northern blot analyses. Because this mutant retina was previously shown to contain very little β-dystroglycan in the OPL, it was suggested that dystrophin isoforms (Dp426, Dp260, and Dp140) and β-dystroglycan are not essential for the presynaptic localization of BRAG2a in the photoreceptor. Therefore, additional mechanisms are required to anchor BRAG2a to photoreceptor terminals. In the brain, BRAG2a is enriched at the postsynaptic density of excitatory synapses probably through the direct interaction of the C-terminal PDZ-binding motif of BRAG2a with PSD-95.

## Table. Summary of BRAG2-Interacting Proteins

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photoreceptors and present in the synaptic cleft of the photoreceptor ribbon synapse.11 Pikachurin-deficient knockout mice exhibit abnormal electroretinograms and improper apposition of bipolar dendritic tips to the photoreceptor presynapse, consistent with the retinal phenotypes observed in photoreceptor-specific dystroglycan conditional knockout mice.11,12 These findings suggested that the pikachurin–DGC pathway plays an essential role in the synaptic transmission and structure of the photoreceptor ribbon synapse. As summarized in the Table, one of the consequences of the association of BRAG2 with its interacting proteins is the enhancement of its GEF activity, thereby initiating the Arf6-dependent internalization of AMPARs during synaptic long-term depression.19 Thus, it is attractive to speculate that BRAG2 regulates the formation and maintenance of synaptic connections and transmission in the photoreceptor ribbon synapse through Arf6-dependent membrane traffic and actin cytoskeleton remodeling downstream of the pikachurin–DGC.

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