CRISPR/CasRx-Mediated Knockdown of Rab7B Restores Incomplete Cell Shape Induced by Pelizaeus-Merzbacher Disease-Associated PLP1 p.Ala243Val

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ABSTRACT: Pelizaeus-Merzbacher disease (PMD, currently known as hypomyelinating leukodystrophy type 1 [HLD1]) is a hereditary hypomyelinating and/or demyelinating disease associated with the proteolipid protein 1 (plp1) gene in the central nervous system (CNS). One of the major causes of this condition is incomplete or defective oligodendroglial cell myelin sheath formation triggered by endoplasmic reticulum (ER) stress and subsequent unfolded protein response (UPR). The HLD1-associated Ala-243-to-Val mutation (p.Ala243Val) of PLP1 is widely recognized to trigger defective oligodendroglial cell morphological differentiation, primarily due to ER stress. We have previously reported that knockdown of Rab7B (also known as Rab42), a small GTP/GDP-binding protein involved in intracellular vesicle trafficking around the lysosome, can recover chemical ER stress-induced incomplete cell shapes in the FBD-102b cell line, a model of oligodendroglial cell morphological differentiation. Here, we present findings indicating that incomplete cell shapes induced by PLP1 p.Ala243Val can be restored by knockdown of Rab7B using the clustered regularly interspaced short palindromic repeats (CRISPR) and CasRx (also known as Cas13d) system. Also, the knockdown promoted the trafficking of PLP1 p.Ala243Val to lysosome-associated membrane protein 1 (LAMP1)-positive organelles. These results highlight the unique role of Rab7B knockdown in modulating oligodendroglial cell morphological changes and potentially facilitating the transport of mutated PLP1 to LAMP1-positive organelles, suggesting its potential as a therapeutic target for alleviating HLD1 phenotypes, at least in part, at the molecular and cellular levels.

KEYWORDS: Rab7B, PLP1, HLD1, ER stress, oligodendrocyte, cell morphogenesis

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Introduction

The central nervous system (CNS) is composed of various types of tissues and cells, including neuronal and glial cells.[1-4] Oligodendrocytes, also known as oligodendroglial cells, are the glial cells responsible for forming myelin sheaths in the CNS.[1-4] These myelin sheaths around neuronal axons are derived of differentiated oligodendroglial cell plasma membranes[1-6] and play critical roles in the efficient transmission of nerve impulses and the protection of axons. Collected areas of differentiated oligodendroglial cell plasma membranes often reach more than fifty times the size of those of pre-myelinating cell plasma membranes at least in rodent model.[3-6] Therefore, improper formation or damage to myelin sheaths due to physiological and physical stresses can lead to serious neurodegenerative diseases.[1-8] Hypomyelinating leukodystrophy 1 [HLD1], also known as Pelizaeus-Merzbacher disease (PMD), is one such disease, characterized by X-linked recessive hypomyelination and/or demyelination.[9-14]

HLD1, often caused by duplication, multiplication, or mutation of the gene encoding PLP1, triggers significant endoplasmic reticulum (ER) stress and a subsequent unfolded protein response (UPR) throughout the cell body. This leads to incomplete oligodendroglial cell morphological differentiation and the generation of incomplete myelin sheaths.[11-14] It is therefore believed that mitigating ER stress and UPR contributes, at least in part, to normal morphological differentiation and the generation of mature myelin sheaths.[11-14]

Rab proteins, which belong to the small GTP/GDP-binding proteins of the Ras superfamily, exist in either the active GTP-bound or the inactive GDP-bound forms. They control the transportation of various intracellular vesicles.[15,16] Similar to other small GTP/GDP-binding proteins, Rab proteins are switching regulated by GDP/GTP exchange factors (GEFs) and GTPase-activating proteins (GAPs).[17] Each Rab protein localizes to specific cellular vesicles and intracellular membranes, essentially acting as a flag for the intracellular component.[18-20] Consequently, they control vesicular budding, trafficking, and fusion, impacting cell states in both health and disease.[18-20]

It is believed that Rab7A generally participates in the transport and degradation of proteins in the endosome and the lysosome, from yeast to mammalian cells.[21,22] Similarly, it is thought that Rab7B (also known as Rab42), a Rab7 subfamily branch molecule, is localized in vesicular organelles

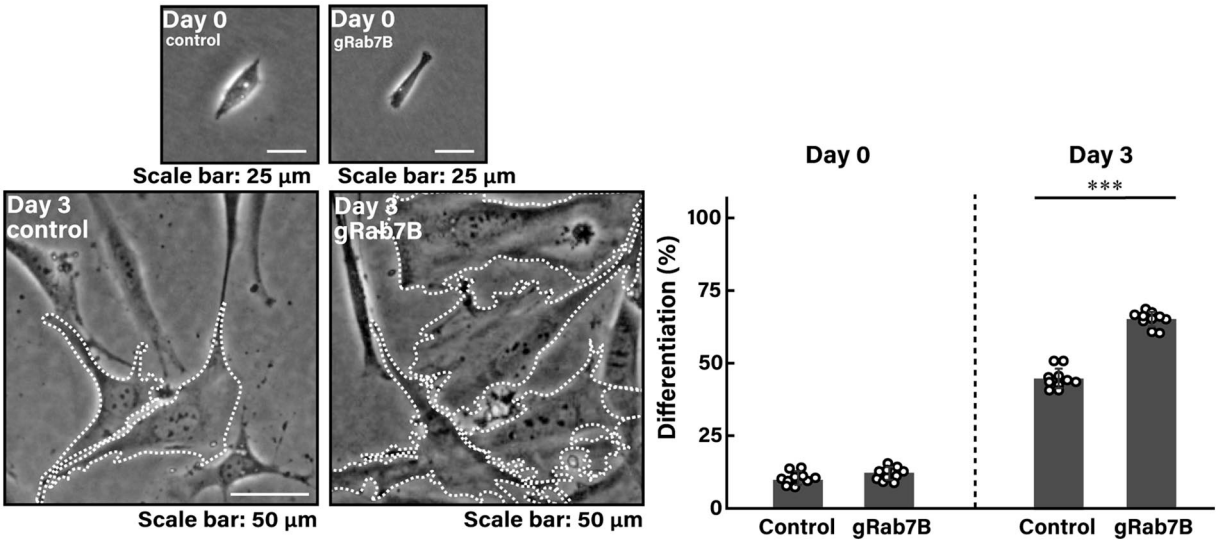

Figure 1. Knockdown of Rab7B can recover PLP1 p.Ala243Val-induced defective cell morphological changes. Cells were transfected with the plasmid encoding PLP1 with the A243V mutation together with gRNA for Rab7B or the control plus CasRx. Following the induction of differentiation, cell morphologies were photographed and cells exhibiting differentiated oligodendroglial cell-like widespread membranes were statistically depicted (**$P < .01$; n = 10 fields). Typical cell morphologies with differentiated oligodendroglial cell-like widespread membranes were outlined by white dotted lines.

around the lysosome and may participate in transporting proteins toward the direction of the trans-Golgi network.[23-26] We previously reported that knockdown of Rab7B using the specific small interfering (si)RNA restores morphological changes induced by a drug that triggers ER stress in an oligodendroglial cell line FBD-102b.[27] This cell line has the ability to undergo oligodendroglial cell-like morphological differentiation with widespread plasma membranes before myelination events.[28,29] As the next step, we analyzed the effects of Rab7B knockdown on cells harboring the HLD1-associated Ala-243-to-Val (A243V) mutation (also known as p.Ala243Val) of PLP1.[9-14] This mutation is known to induce ER stress and trigger UPR, leading to incompletely differentiated, undifferentiated, and/or hypomyelinating oligodendroglial cell phenotypes.[9-14,30-32] Using the clustered regularly interspaced short palindromic repeats (CRISPR) and CasRx (also known as Cas13d) system-mediated knockdown approach,[33,34] we observed the recovery of cell morphology in cells harboring PLP1 with the A243V mutation. These findings suggest that Rab7B could be a potential drug target molecule for HLD1, at least at the molecular and cellular levels.

Results

Transfection of PLP1 with the A243V mutation[9-14] into FBD-102b cells decreased oligodendroglial cell-like differentiated phenotypes with widespread membranes compared to the control transfection (Figure S1, *upper images*). Similar results were obtained in the case of cells stably harboring PLP1 with the A243V mutation (Figure S1, *lower images*). The small difference in differentiation potential between cells harboring PLP1 with the A243V mutation and the controls is likely due to the lack of a culture system in which pathological changes take years to develop, as occurs in the disease. On the other hand, transfection of wild type PLP1 into cells did not significantly affect their morphologies (Figure S2).

Under these experimental conditions, we examined whether cells harboring PLP1 with the A243V mutation could be recovered by knockdown of Rab7B, which negatively regulates morphological changes.[27] As a result, specific knockdown of Rab7B using CRISPR/CasRx system[33,34]-fitted gRNA was confirmed at the RNA and protein levels (Figure S3). The knockdown successfully recovered morphological changes in cells harboring PLP1 with the A243V mutation (Figure 1). Comparatively, knockdown of Rab7B slightly increased morphological changes in cells harboring wild type PLP1 (Figure 2) or mock-transfected cells (Figure 3).

The finding in cells harboring PLP1 with the A243V mutation in a Rab7B-knocked down background was supported by increased expression levels of oligodendroglial cell differentiation and myelination marker proteins 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) and the pi molecule of glutathione S-transferase (GSTpi), whereas the expression levels of actin as the internal control protein were comparable in Rab7B- and control-knocked down cells (Figure S4). We failed to use PLP1 as the differentiation marker, since we utilized cells harboring wild type or mutated PLP1. The expression levels of heat shock protein family A member 5 (HSPA5), a typical marker of ER stress response, were decreased following knockdown of Rab7B (Figure S4), suggesting that its knockdown has the ability to recover defective morphological changes.

As comparative experiments, we examined the effects of Rab7B knockdown on the expression levels of CNPase, GSTpi, and HSPA5 in cells harboring wild type PLP1 (Figure S5) or in mock-transfected cells (Figure S6). The expression levels of CNPase and GSTpi were slightly increased following Rab7B

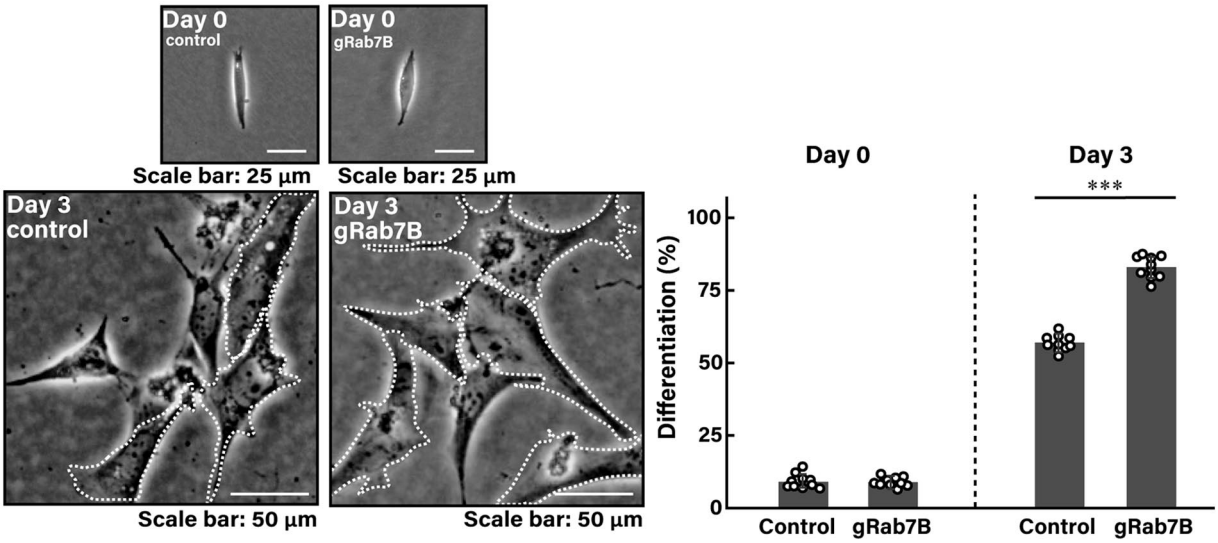

Figure 2. Effects of Rab7B knockdown on cells harboring wild type PLP1. Cells were transfected with the plasmid encoding wild type PLP1 together with gRNA for Rab7B or the control plus CasRx. Following the induction of differentiation, cell morphologies were photographed and cells exhibiting differentiated oligodendroglial cell-like widespread membranes were statistically depicted (**$P < .01$; n = 10 fields). Typical cell morphologies with differentiated oligodendroglial cell-like widespread membranes were outlined by white dotted lines.

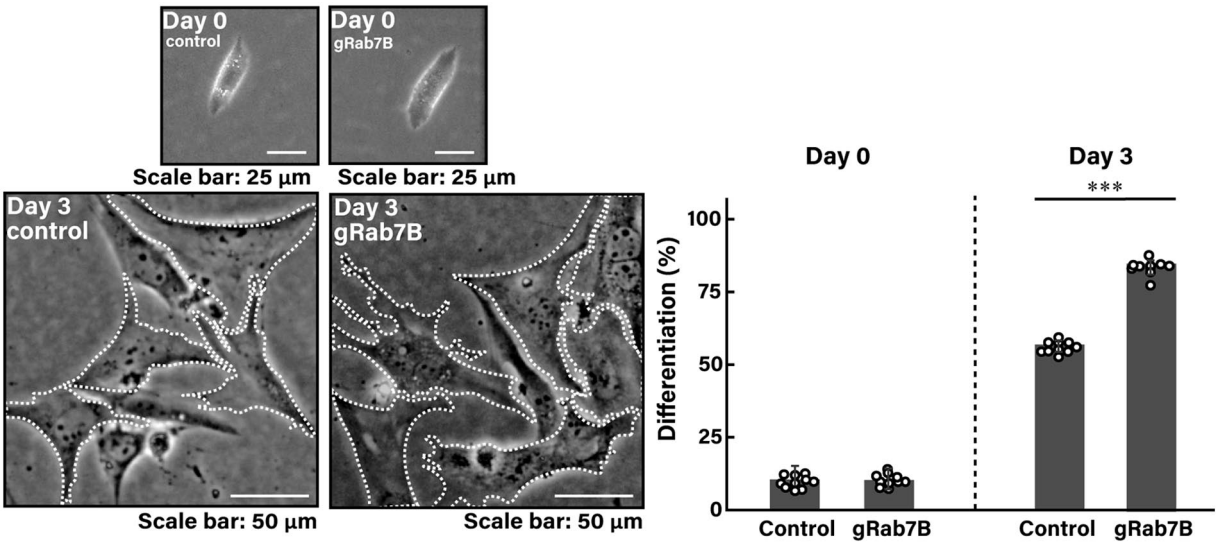

Figure 3. Effects of Rab7B knockdown on mock-transfected cells. Cells were transfected with the plasmid encoding gRNA for Rab7B or the control plus CasRx. Following the induction of differentiation, cell morphologies were photographed and cells exhibiting differentiated oligodendroglial cell-like widespread membranes were statistically depicted (**$P < .01$; n = 10 fields). Typical cell morphologies with differentiated oligodendroglial cell-like widespread membranes were outlined by white dotted lines.

knockdown, suggesting that its knockdown can promote morphological changes. The expression levels of HSPA5 did not significantly change following Rab7B knockdown.

To test the possibility that Rab7B knockdown promotes to transport into lysosomal-associated membrane protein 1 (LAMP1) lysosome marker protein-positive organelles, we carefully prepared cell extracts containing intact organelles from cells expressing PLP1 with the A243V mutation in an isotonic solution.[35,36] Organelle immunoprecipitation data illustrated that the mutated protein was present in a LAMP1-positive intracellular component in Rab7B-knocked down conditions (Figure S7), suggesting that Rab7B potentially plays a negative role in protein trafficking to LAMP1-positive organelles. In contrast, knockdown of Rab7A[27] decreased the amounts of mutated protein present in a LAMP1-positive intracellular component (Figure S8).

Discussion

Rab7 is generally involved in regulating the transport of vesicles around the lysosome in multiple types of cells.[18-20] Although it is commonly understood that Rab7 molecules participate in transporting various proteins in vesicles toward the lysosome, Rab7B appears to be included to direct vesicles to the trans-Golgi network.[18-20] Although these two directions of

vesicle transport may seem contradictory initially, Rab7 proteins likely oversee the overall transport of vesicles around the trans-Golgi network along the lysosome. In certain cell types rather than all cell types, Rab7B may preferentially mediate the regulation of transport from endosomes to the trans-Golgi network[23-26] whereas Rab7A primarily regulates transport to the lysosome in almost all cell types.[18-22]

Morphological differentiation in oligodendroglial cells represents dynamic changes occurring throughout tissues.[5-8] During the differentiation of oligodendroglial cells, their cell membrane surface expands significantly compared to oligodendroglial progenitor cells.[5-8] Knockdown of Rab7B potentially aids in escorting more proteins with low structural quality, such as PLP1 with the A243V mutation, in vesicles toward the lysosome, thereby facilitating efficient protein quality during differentiation.

It is likely that knockdown of Rab7B reduces ER stress, while other Rab family proteins like Rab10 and Rab18 generally control ER shapes by regulating fusion and/or fission. It is also known that overexpression of the dominant-negative form (Rab7A with the T22 N mutation) or knockdown of Rab7A with the specific siRNA leads to unusual enlargement of ER structures and abnormal spreading toward the cell periphery.[23-26] The dominant-negative form is believed to tightly bind to potential GEFs, the upstream regulators for Rab7, blocking the activation of Rab7A.[23-26] Furthermore, functional inhibition of Rab7A is directly associated with upregulation of ER stress and subsequent UPR.[21] In addition, Rab7A, acting through downstream effector molecules, positively regulates the endosomal sorting complex required for transport-III (ESCRT-III), which works alongside ESCRT-0, ESCRT-I, and ESCRT-II.[22] Through all ESCRTs, ubiquitinated proteins cargo is transported into vesicles to form multivesicular bodies for fusion with the lysosome. Therefore, deficiency of Rab7A results in incomplete or loss of protein homeostasis, indirectly leading to ER stress.[21] These cellular phenomena appear to be opposite to those observed in the case of Rab7B knockdown in oligodendroglial cell lines. Since Rab7B and Rab7A are homologous molecules, it is possible that Rab7B competes with the upstream or downstream of Rab7A. If true, knockdown of Rab7B might help enhance Rab7A's control of protein qualities from the ER to the lysosome, especially in possible pathological states. Molecular competition mechanisms among homologous molecules are widely conserved in small GTPases like the Ras and Rho families.[17,37-39]

In the human brain, transcripts of Rab7A and Rab7B display localization profiles in cells found in white matter and myelinating cells, specifically in differentiated oligodendroglial lineage cells (see the Human Protein Atlas website, https://www.proteinatlas.org). However, since the levels of Rab7A are much higher than those of Rab7B, it is suggested that Rab7A and Rab7B may cooperatively or independently play specific roles in the development of oligodendroglial lineage cells. Indeed, Rab7A promotes oligodendroglial cell morphological differentiation whereas Rab7B inhibits morphological differentiation.[27] It is likely that Rab7A functions as a housekeeping gene product, similar to other Rab proteins.[18-20] On the other hand, Rab7B may exhibit specific expression in some but not all cell types.[18-20] Further studies can help clarify whether Rab7A and Rab7B have similar or distinct roles in oligodendrocytes.

Rab7B, along with leucine-rich repeat containing 4 (LRRC4, also known as netrin-G ligand-2 [NGL-2]), is upregulated in the CNS following experimental autoimmune encephalomyelitis, a model for inflammatory demyelinating and hypomyelinating disease in the CNS.[40] It is predicted that during experimental autoimmune encephalomyelitis, the expression of Rab7B and LRRC4 does not exacerbate demyelinating states but partially contributes to the elongation and protection of neuronal axons.[40] Although decreased expression of Rab7B can protect oligodendroglial cells, CNS neuronal cells might be protected by the upregulation of Rab7B and/or LRRC4 at physiological levels.

In retinal ganglion cells (RGCs), Rab7A associates with various RNA-binding proteins and actively moves within their axons.[41] The role of Rab7A in real-time protein synthesis at local axonal sites also helps maintain mitochondrial homeostasis.[41] Rab7A likely contributes not only to neuronal axon elongation but also to axonal mitochondria homeostasis. It is of note that in the peripheral nervous (PNS) system, the Charcot-Marie-Tooth (CMT) disease type 2B–associated mutation of Rab7A disrupts mitochondrial homeostasis in the axons.[42,43] The fact that each CMT2B-associated mutation of Rab7A results in a constitutively active form[44,45] underscores the relationship between higher upregulation of the Rab7A activity and its functional defects. Subtle control of the activities and expression levels of both Rab7A and possibly Rab7B is thought to be essential for neuronal development and/or maintenance in both the CNS and the PNS.

Certain types of HLDs are caused by protein aggregation–induced ER stress and subsequent UPR.[30-32] For example, proteolipid protein 1 (PLP1), gap junction protein gamma-2 (GJA2, also known as gap junction protein alpha 12 [GJA12] or Cx47), and FAM126A are gene products responsible for HLD1, HLD2, and HLD5, respectively. Their gene mutations result in protein aggregation of the respective gene products and/or aggregates containing other proteins.[11,13,46] In HLD1, at the molecular and cellular levels, reducing ER stress signaling with specific drugs targeting ER stress signaling molecules helps maintain cellular phenotypes observed in HLD1.[30-32] Molecules targeting Rab7B could be included in the lists of factors reducing HLD phenotypes related to ER stress signaling.

In the present study, we describe for the first time that knockdown of Rab7B using CRISPR/CasRx-fitted gRNA can recover decreased morphological changes in cells harboring PLP1 with

the A243V mutation. Further studies will promote our understanding not only of the detailed mechanism by which knockdown of Rab7B can recover phenotypes of cells harboring PLP1 with the A243V mutation in FBD-102b as an oligodendroglial cell model, but also of whether or how its knockdown affects primary cells and mice. Such studies may elucidate how the molecular mechanism underlying Rab7B is directly or indirectly associated with phenotypes observed in HLD1 in oligodendroglial cells. These studies might lead to the development of therapeutic target molecule-specific medicines for HLD1 and HLD1-related diseases.

Materials and Methods

Materials

Key materials, including antibodies and nucleotides, are listed in Table 1.

Cell line culture and differentiation

The FBD-102b cell line (kindly provided by Dr. Yasuhiro Tomo-oka [Riken, Saitama, Japan and Tokyo University of Science, Chiba, Japan]) is a mouse oligodendroglial precursor cell one. Cells were cultured on Nunc brand's cell and tissue culture dishes (ThermoFisher Scientific, Waltham, MA, USA) in Dulbecco's modified Eagle medium (DMEM)/Ham's F-12 nutrient mixed medium (Nacalai Tesque, Kyoto, Japan or Fujifilm, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (FBS) and PenStrep mixed antibiotics (Nacalai Tesque or Fujifilm) in 5% carbon dioxide at 37°C.[27-29]

To induce differentiation, cells were cultured on polylysine-coated cell culture dishes (Nacalai Tesque) in a culture medium containing with a low concentration of serum in 5% carbon dioxide at 37°C. Cells exhibiting myelin membrane-like widespread membranes (cells large enough to contain a circle with a diameter exceeding 0.025 mm) were considered to have differentiated phenotypes.[27] Cell morphologies were captured using microscopic systems equipped with i-NTER LENS (Micronet, Saitama, Japan) and i-NTER SHOT ver.2 (Micronet). The images in the figures are representative of multiple images and were analyzed with Image J software (https://imagej.nih.gov/).

Polymerase chain reaction

For reverse transcription-polymerase chain reaction (RT-PCR), cDNAs were prepared using total cellular RNA extracted from Isogen (Nippon Gene, Tokyo, Japan)-extracted total cellular RNA with the PrimeScript RT Master Mix kit (Takara Bio, Kyoto, Japan) in accordance with the manufacturer's instructions.

PCR amplification from the RT products was performed using Gflex DNA polymerase (Takara Bio) for 35 cycles. Each cycle consisted of a denaturation reaction at 98°C (0.2 minutes), an annealing reaction at 56°C to 65°C (0.25 minutes) depending on the annealing temperature, and an extension reaction at 68°C (0.5 minutes). The resultant PCR products were loaded onto premade agarose gels (Nacalai Tesque).

Transfection

FBD-102b cells were transfected with the respective DNAs using the ScreenFect A transfection kit (Fujifilm) in accordance with the manufacturer's instructions. The medium was replaced 4 hours after transfection. Cells were typically used for biochemical experiments more than 48 hours post-transfection.[27] Additionally, G418-resistant clones were collected as stable clones. G418 was gradually increased in concentration from 0.5 to 1 mg/ml.

FBD-102b cells exhibited a transfection efficiency of 85.2% ± 2.15% with control GFP transfection using the ScreenFect A transfection kit,[47] although this efficiency was not evaluated with different plasmids encoding various genes.

Cell lysis, polyacrylamide gel electrophoresis, and immunoblotting

Cells were lysed in cell and tissue extraction buffer (50 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethane sulfonylfluoride, 1 µg/ml leupeptin, 1 mM EDTA, 1 mM Na_3VO_4, and 10 mM NaF) containing a mild detergent (0.5% NP-40).[27] For denaturing conditions, cell lysates were denatured in premade sample buffer (Nacalai Tesque or Fujifilm), and the denatured samples were then separated on premade sodium dodecyl sulfate-polyacrylamide gel (Nacalai Tesque or Fujifilm).

The electrophoretically separated proteins were transferred to a polyvinylidene fluoride membrane (Fujifilm), blocked with Skim Blocker (Fujifilm), and immunoblotted using primary antibodies. This was followed by incubation with peroxidase enzyme-conjugated secondary antibodies. The peroxidase-reactive bands were captured using a CanoScan LiDE 400 (Canon, Tokyo, Japan) and CanoScan LiDE 400 Scanner Driver Ver.1.01. Multiple sets of experiments were conducted in immunoblotting studies, and quantification of immunoreactive bands was performed using Image J software with another sample's immunoreactive band as 100%.

Immunoprecipitation of LAMP1-positive intact intracellular components

Cells were extracted in an isotonic extraction buffer (50 mM HEPES-NaOH, pH 7.5, 125 mM NaCl, 3 mM $MgCl_2$, 1 mM phenylethane sulfonylfluoride, 1 µg/ml leupeptin, 1 mM EDTA, 1 mM Na_3VO_4, and 10 mM NaF) with short-term sonication. Cell extracts were then used for immunoprecipitation with anti-LAMP1 antibody.[35,36] The denatured, immunoprecipitated samples containing intact intracellular components were separated on a premade sodium dodecylsulfate-polyacrylamide gel for subsequent immunoblots using specific antibodies.

Table 1. Key materials.

REAGENTS OR SOURCES	COMPANY OR SOURCE	CAT. NO.	LOT. NO.	CONCENTRATION USED
Antibodies				
Anti-CNPase (B-1)	Santa Cruz Biotechnology	sc-166019	2216	Immunoblotting (IB), 1:1,000
Anti-GSTpi pAb	MBL	312	067	IB, 1:500
Anti-actin (also called pan-beta type actin)	MBL	M177-3	007	IB, 1:5,000
Anti-Rab7B (also called Rab42)	Santa Cruz Biotechnology	sc-130482	A0824	IB, 1:100
Anti-heat shock protein family A member 5 (HSPA5)	PGI Proteintech Group, Inc.	11587-AP	85 813	IB, 1:1,000
Anti-lysosomal-associated membrane protein 1 (LAMP1)	Santa Cruz Biotechnology	sc-20011	J0919	Immunoprecipitation (IP), 500 ng for 0.5 mg of cell extracts; IB, 1:100
Anti-IgG (H + L chain) (Mouse) pAb-HRP	MBL	330	365	IB, 1:5,000
Anti-Mouse IgG (Goat), HRP-conjugated, Pre-absorbed	Nacalai Tesque	21860-61	L3E2991	IB, 1:25,000
Anti-IgG (H + L chain) (Rabbit) pAb-HRP	MBL	458	353	IB, 1:5,000
Key reagents				
ScreenFect TM A Transfection Reagent	FUJIFILM Wako Pure Chemical Corporation	294-73214	LEN4991	According to manufacturer's instructions
ScreenFect TM Dilution Buffer	FUJIFILM Wako Pure Chemical Corporation	291-73224	LEN4991	According to manufacturer's instructions
ImmunoStar TM Zeta Super	FUJIFILM Wako Pure Chemical Corporation	02230-30	L3H3671	According to manufacturer's instructions
Skim milk	FUJIFILM Wako Pure Chemical Corporation	190-12865	SKG4901	According to manufacturer's instructions
Western blotting stripping solution	Nacalai Tesque	05364-55	L5M5218	According to manufacturer's instructions
Fujifilm TM sample buffer	FUJIFILM Wako Pure Chemical Corporation	191-13282	WDP4995	According to manufacturer's instructions
Isogen	Nippon Gene	311-02501	75009K	According to manufacturer's instructions
5×PrimeScript master mix	TaKaRa Bio	RR036A	AIE0440A	According to manufacturer's instructions
Gflex DNA polymerase	TaKaRa Bio	R060A	AL80564A	According to manufacturer's instructions
2×Gflex PCR buffer (Mg2+, dNTP plus; with or without dye)	TaKaRa Bio	R060A	AL80564A	According to manufacturer's instructions

(Continued)

Table 1. (Continued)

REAGENTS OR SOURCES	COMPANY OR SOURCE	CAT. NO.	LOT. NO.	CONCENTRATION USED
TaKaRa Bio TM loading buffer	TaKaRa Bio	9157	A7201A	According to manufacturer's instructions
Pre-stained Protein Markers (Broad Range) for SDS-PAGE	Nacalai Tesque	02525-35	L9M9989	According to manufacturer's instructions
ExcelBand All Blue Regular Range Protein Marker	Cosmo Bio	PM1500	PM1500211500-5	According to manufacturer's instructions
ExcelBrand 3 Color Regular Range Protein Marker	Smo Bio	PM2500-2	PM25002112601-2	According to manufacturer's instructions
Cells used				
FBD-102b cells (mouse oligodendrocyte progenitor cells)	Dr. Yasuhiro Tomo-oka (Riken, Saitama, Japan/Tokyo University of Science, Chiba, Japan)	N/A	N/A	1 000 000 cells per 6-cm-dish culture
Plasmids and vectors				
CasRx (NLS-RfxCas13d-NLS)	Addgene	109049	N/A	
pcDNA3.1(+)-C-eGFP-PLP1	GenScript (Piscataway, NJ, USA)	N/A	N/A	
pcDNA3.1(+)-C-eGFP-PLP1-A243V	GenScript (Piscataway, NJ, USA)	N/A	N/A	
gRNA sequences (5′ to 3′) inserted into the plasmid (pSINmU6)				
Sense chain for gLuciferase-105th (control gRNA) gatccGCACCCGTGCAAAAATGCAGGGGTCTAA AAC-ATCCTCTAGAGGATAGAATGGCTTTTTTat Antisense chain for siLuciferase-105th cgatAAAAAAGCCATTCTATCCTCTAGAGGATGT TTTAGAC-CCCTGCATTTTTGCACGGGTGCg	Yamauchi, J. et. al. Exp. Cell Res. (2009) 315:2043-2052	N/A	N/A	50 nM per one transfection
Sense chain for gRab7B-102th gatccGCACCCGTGCAAAAATGCAGGGGTCTAA AAC-CGAGGAATACCAGACCACACTGTTTTTTat Antisense chain for gRab7B-102th cgatAAAAAACAGTGTGGTCTGGTATTCCTCGG TTTTAGAC-CCCTGCATTTTTGCACGGGTGCg	This manuscript	N/A	N/A	50 nM per one transfection

(Continued)

Table 1. (Continued)

REAGENTS OR SOURCES	COMPANY OR SOURCE	CAT. NO.	LOT. NO.	CONCENTRATION USED
Sense chain for gRab7B-168th gatccGCACCCGTGCAAAAATGCAGGGGTCTAA AAC-TTTGAAGCTGCAGATCTGGGACTTTTTTa Antisense chain for gRab7B-168th cgatAAAAAAGTCCCAGATCTGCAGCTTCAAAG TTTTAGAC-CCCTGCATTTTTGCACGGGTGCg	This manuscript	N/A	N/A	50 nM per one transfection
PCR primers (5′ to 3′)				
Sense primer for actin (internal control) ATGGATGACGATATCGCTGCGCTGGTC Antisense primer for actin CTAGAAGCACTTGCGGTGCACGATGGAG	Kato, Y. et. al. Exp. Cell Res. (2021) 405:112654	N/A	N/A	250 nM per one reaction
Sense primer for Rab7B CAAGCAGTCACTGCTACGGTGT Antisense primer for Rab9b CCTTGTCCAGCTTGATGACCCC	This manuscript	N/A	N/A	250 nM per one reaction

Statistical analysis

Values are expressed as means ± standard deviation (SD) from separate experiments. Intergroup comparisons were performed using the unpaired *t*-test with Student's correction or Welch's correction in Excel software (Microsoft, Redmond, WA, USA).

Differences were considered significant at $P < .05$. For all analyses, the investigator was blinded to the sample conditions.

Ethics statement

Techniques involving genetically modified cells and related procedures were performed in accordance with a protocol approved by the Tokyo University of Pharmacy and Life Sciences Gene and Animal Care Committee (Approval Nos. LS28-20 and LSR3-011).

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Author Contributions

Junji Yamauchi organized this study. Junji Yamauchi wrote the draft and edited the manuscript. Nana Fukushima carried out experiments and statistical analyses. Nana Fukushima and Yuki Miyamoto evaluated experimental and statistical data.

Data Availabilities

The datasets used for the current study are available from the corresponding author upon reasonable request.

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Supplemental Material

Supplemental material for this article is available online.

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