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Hypomyelinating leukodystrophy-associated missense mutation in HSPD1 blunts mitochondrial dynamics

Yuki Miyamoto [a, 1], Takahiro Eguchi [b, 1], Kazuko Kawahara [a], Nanami Hasegawa [a, c], Kazuaki Nakamura [a], Megumi Funakoshi-Tago [c], Akito Tanoue [a], Hiroomi Tamura [c], Junji Yamauchi [a, d, *]

[a] Department of Pharmacology, National Research Institute for Child Health and Development, Setagaya, Tokyo 157-8535, Japan
[b] The Institute of Medical Science, The University of Tokyo, Minato, Tokyo 108-8639, Japan
[c] Faculty of Pharmacy, Keio University, Minato, Tokyo 105-8512, Japan
[d] Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Bunkyo, Tokyo 113-8510, Japan

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ABSTRACT

Myelin-forming glial cells undergo dynamic morphological changes in order to produce mature myelin sheaths with multiple layers. In the central nervous system (CNS), oligodendrocytes differentiate to insulate neuronal axons with myelin sheaths. Myelin sheaths play a key role in homeostasis of the nervous system, but their related disorders lead not only to dismyelination and repeated demyelination but also to severe neuropathies. Hereditary hypomyelinating leukodystrophies (HLDs) are a group of such diseases affecting oligodendrocytes and are often caused by missense mutations of the respective responsible genes. Despite increasing identification of gene mutations through advanced nucleotide sequencing technology, studies on the relationships between gene mutations and their effects on cellular and subcellular aberrance have not followed at the same rapid pace. In this study, we report that an HLD4-associated (Asp-29-to-Gly) mutant of mitochondrial heat shock 60-kDa protein 1 (HSPD1) causes short-length morphologies and increases the numbers of mitochondria due to their aberrant fission and fusion cycles. In experiments using a fluorescent dye probe, this mutation decreases the mitochondrial membrane potential. Also, mitochondria accumulate in perinuclear regions. HLD4-associated HSPD1 mutant blunts mitochondrial dynamics, probably resulting in oligodendrocyte malfunction. This study constitutes a first finding concerning the relationship between disease-associated HSPD1 mutation and mitochondrial dynamics, which may be similar to the relationship between another disease-associated HSPD1 mutation (MitCHAP-60 disease) and aberrant mitochondrial dynamics.

1. Introduction

The myelin sheath consists of morphologically differentiated plasma membranes of myelin-forming glial cells [1–4]. Oligodendrocytes and Schwann cells contribute to producing myelin sheaths in the central nervous system (CNS) and peripheral nervous system (PNS), respectively. Myelin sheaths not only insulate axons to increase their nerve conduction velocity but also protect them from various external stresses such as physical stress. For this reason, myelin sheaths play essential roles in homeostasis of the nervous system [1–4]. Therefore, the diseases that affect them, triggering dismyelination and repeated demyelination, cause nerve damage and, in turn, severe CNS or PNS neuropathies [3,4]. One such disease is Pelizaeus-Merzbacher disease, a rare X-linked recessive disease [5,6]. This disease is the prototypic hereditary hypomyelinating leukodystrophy (HLD) and is now designated as HLD1 (OMIM No. 312080). The responsible gene is *plp1*, and the disease can be caused by a variety of alterations to it such as missense mutations and gene multiplication [5,6].

Through technological advances, including next-generation sequencing technology, nine or ten different somatic genes have

* Corresponding author. Molecular Pharmacology Group, Department of Pharmacology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya, Tokyo 157-8535, Japan. Fax: +81 3 5494 7057.
E-mail address: yamauchi-j@ncchd.go.jp (J. Yamauchi).
[1] These authors contributed equally to this work.

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been identified to date as the HLD responsible genes [3–7]; nevertheless, it still remains to be understood how their alterations affect the properties of their protein products. HLD4 (OMIM No. 612233) is an autosomal recessive lethal disease and is caused by a single missense mutation of the *heat shock 60-kDa protein 1* (*hspd1*, also known as *hsp60* or *cpn60*) gene [8]. Heat shock proteins, which have chaperonin functions, were originally identified as proteins responsible for preventing damage to other proteins in relation to temperature levels [9,10]. Classically, members of the heat shock protein family are referred to according to their molecular weight. For example, Hsp60, Hsp70, and Hsp80 are 60-, 70-, and 80-kDa, respectively. The *hspd1* gene encodes a bacterial GroEL-like heat shock protein in mitochondria. HSPD1 participates in the folding and assembly of newly-produced proteins imported into mitochondria [9,10]. At present, HSPD1-associated diseases are generally designated as MitCHAP-60 diseases, which include spastic paraplegia 13 (SPG13) and HLD4 [8]. In this study, we have set out to study the effect of a HSPD1 missense mutation (Asp-29-to-Gly) on mitochondrial morphological changes in living cells. Here, for the first time, we describe the relationship between a disease-associated mutation of HSPD1 and mitochondrial dynamics. HLD4-associated mutation of HSPD1 affects mitochondrial fission and fusion cycles and also mitochondrial membrane potential, probably due to disease. It may be worth exploring a possible link between MitCHAP-60 diseases and mitochondrial dynamics.

2. Materials and methods

2.1. Plasmid construction

Gene recombinations were carried out in accordance with a protocol approved by the Japanese National Research Institute for Child Health and Development Gene Recombination Committee. HSPD1 and a mitophagy/autophagy marker light chain 3β (LC3B, also called MAP1LC3B) cDNAs were amplified from human brain cDNAs or total RNAs (NipponGene, Tokyo, Japan). The D29G and N423A mutations of HSPD1 were produced by the method of overlapping PCR. They were ligated into the pTagGFP or pTagRFP vector (Evorgen, Moscow, Russia). All DNA sequences were confirmed by Fasmac sequencing service (Kanagawa, Japan). The pTagRFP-mit (mitochondrially-localized RFP) plasmid was purchased from Evorgen.

2.2. Cell culture and transfection

Monkey kidney fibroblast-like Cos-7 cells (Human Science Bio-Resource Bank, Tokyo, Japan) were cultured on cell culture dishes (Greiner Bio-One, Oberösterreich, Germany) in DMEM containing 10% heat-inactivated FBS and 1× PenStrep (Thermo Fisher Scientific, Waltham, MA, USA) in 5% CO_2 at 37 °C. Cells were transfected with plasmid DNA using a Lipofectamine LTX transfection kit (Thermo Fisher Scientific) or a CalPhos transfection kit (Takara Bio, Kyoto, Japan) according to the respective manufacturers' instructions. The medium was replaced 4 h after transfection for lipofection or 18 h after transfection for the calcium-phosphate method. Before experiments, cells were treated with or without 1x MitoTracker Red CM-H_2Xros reagent (Thermo Fisher Scientific). This MitoTracker dye is accumulated in active mitochondria depending upon their membrane potential.

2.3. Immunofluorescence and live imaging

Cells were fixed using an Image-iT Fixation/Permeabilization kit (Thermo Fisher Scientific), incubated with or without an antibody against pan-β type tubulin (MBL, Aichi, Japan), a microtubule component, or lysosomal-associated membrane protein 1 (LAMP1; abcam, Cambridge, UK), a lysosomal marker, in PBS containing 0.1% Tween-20, and treated with or without fluorescence-labeled secondary antibodies in PBS containing 0.1% Tween-20. The coverslips were mounted onto slides with Vectashield reagent with or without DAPI (Vector Laboratories, Burlingame, CA, USA) for observation using confocal microscopy. The confocal images were collected using an IX81 microscope with a laser-scanning FV500 or FV1000 system (Olympus, Tokyo, Japan) and analyzed using FluoView software (Olympus) [11,12]. For live imaging experiment, cells on CellView glass-bottom dishes (Greiner Bio-One) were cultured in a small CO_2 incubator (Tokai Hit, Shizuoka, Japan) in 5% CO_2 at 37 °C and maintained in a culture medium. Cells were scanned every 6 s for a duration of 12 min or 60 min (for movies or statistical data, respectively) using an IX81 microscope with a laser scanning FV1000 system. Values such as mitochondrial fission and fusion frequency and velocity were measured using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA) [11,12]. The values were also confirmed through naked-eye observation of the sequence of photographs that constitutes the AVI file formatted movie.

2.4. Statistical analysis

The values shown in the figure panels represent the means ± standard deviation from separate experiments. Comparisons between two experimental groups were made using the unpaired Student's t test (**, $p < 0.01$). A one-way analysis of variance (ANOVA) was followed by a Fisher's protected least significant difference (PLSD) test as a post hoc comparison (**, $p < 0.01$) using GraphPad Prism software (La Jolla, CA, USA). P values less than 0.05 were considered significant.

3. Results

The aim of this study was to determine whether and how HLD4-associated HSPD1 mutation affects mitochondrial morphological changes. First, we transfected the plasmid encoding GFP-tagged wild type HSPD1 or the D29G mutant together with mitochondrial RFP marker into Cos-7 cells. The N-terminal short amino acid sequence of HSPD1 contains a typical alternating pattern of hydrophobic and positively charged amino acids enabling it to be localized into mitochondria (see the gene website, http://www.ncbi.nlm.nih.gov/gene/3329). When a GFP tag was placed in the C-terminal position of HSPD1, wild type HSPD1 was indeed localized into mitochondria, as determined based on its colocalization with RFP proteins derived from the pTagRFP-mito plasmid (Fig. 1A and C). All mitochondrial morphologies were of the normal long and slender form. Mitochondria harboring the GFP-tagged D29G mutant, in contrast, assumed a short round form (Fig. 1B and C). In addition, we observed that the number of mitochondria harboring the D29G mutant was ~1.5-fold greater than the number harboring the wild type (Fig. 1D–F).

We thus asked whether mitochondrial fusion and fission cycles could account for these phenomena, that is to say, whether the D29G mutation in HSPD1 could cause slow fusion rates or fast fission rates. Mitochondria harboring the D29G mutant exhibited decreased rates of both fission and fusion compared to the wild type (Fig. 2A–C; Movie S2 in the D29G mutant compared to Movie S1 in the wild type). Movie data also suggested that the number of motile mitochondria was likely comparable in both cases. Also, while wild type HSPD1-harboring mitochondria exhibited fission and fusion cycles of equal lengths, fission rates in mutant-

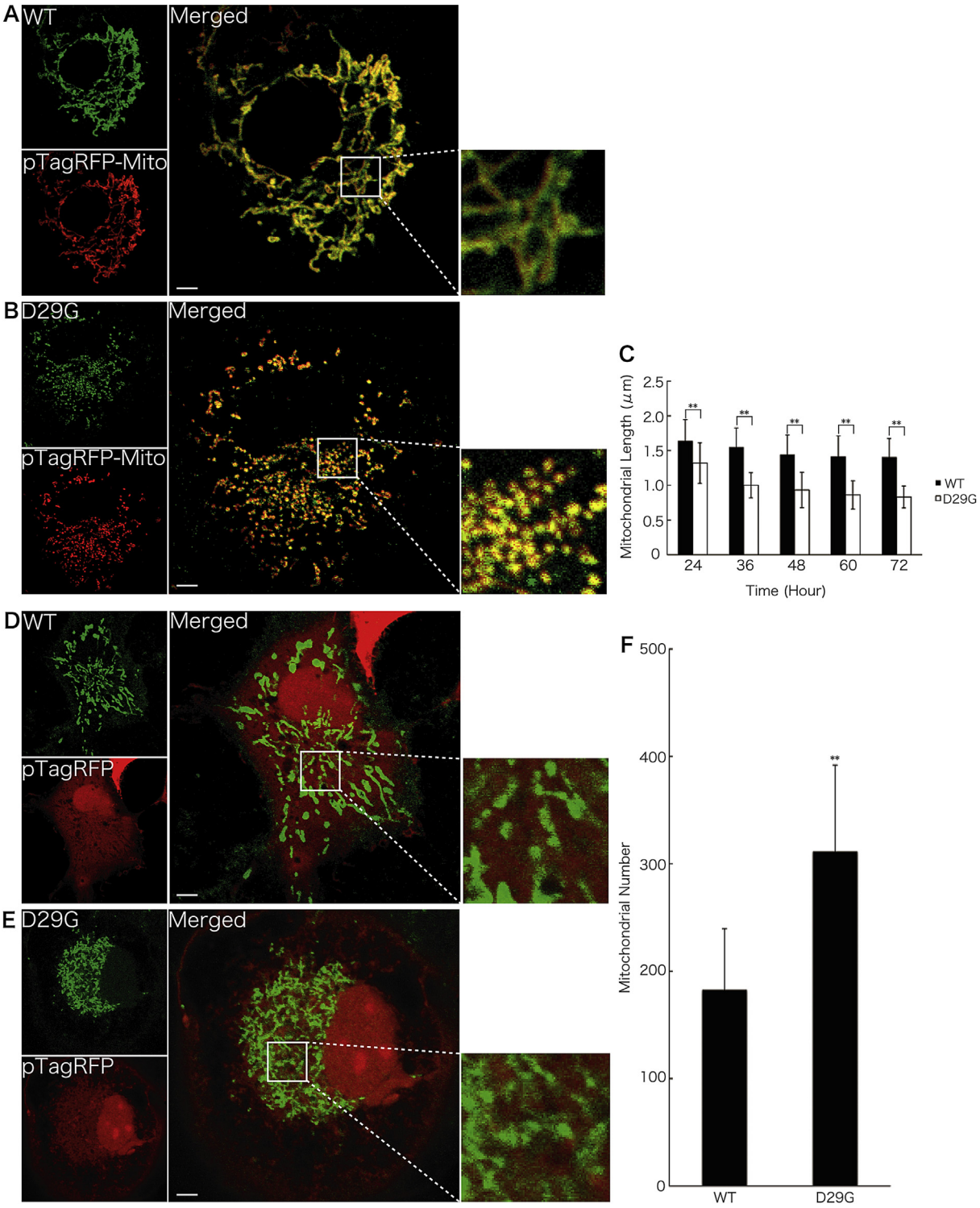

Fig. 1. Effect of HLD4-associated mutant on mitochondrial morphology. (A, B) Cos-7 cells were transfected with pTagGFP-wild type HSPD1 (WT, green) or the D29G mutant (D29G, green) together with pTagRFP-mito (red, mitochondrially-localized RFP). Data shown here were captured 48 h after transfection. Small panels on the left show the same data presented in the large central panel with the colors separated. Small panels on the right are 3.5-fold magnifications of the regions surrounded by the white squares in the large central panels. The scale bar in the large panel indicates 10 μm. (C) At 24–72 h after transfection, mitochondrial length was measured. (**, $p < 0.01$; $n = 70$ mitochondria). (D, E) Cells were transfected with pTagGFP-HSPD1 or the D29G mutant together with pTagRFP (empty vector). The scale bar indicates 10 μm. (F) At 48 h after transfection, the number of mitochondria in each cell was measured. (**, $p < 0.01$; $n = 12$ cells). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

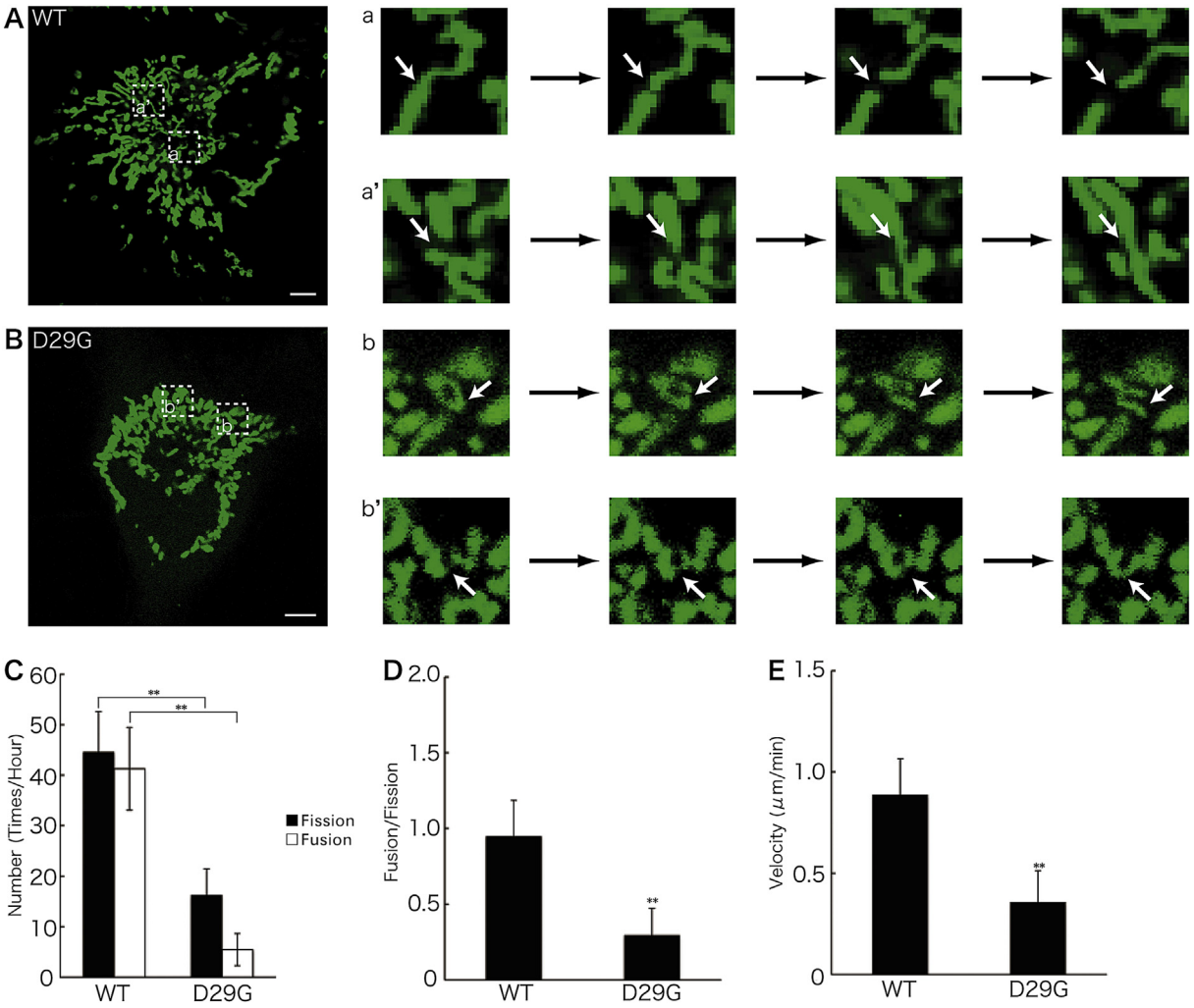

Fig. 2. HLD4-associated mutant affects mitochondrial fission and fusion cycles. (A, B) Cells were transfected with wild type or D29G mutant HSPD1 (green) for 48 h, then scanned every 6 s for a duration of 12 min using an IX81 microscope with a laser scanning FV1000 system. Photographs of typical morphological changes associated with fission (a and b in panels A and B) and fusion (a' and b' in panels A and B) in mitochondria are provided in the small panels on the right. The arrows indicate mitochondria undergoing fission or fusion. The scale bar indicates 10 μm. (C, D) Fission and fusion frequencies per cell are shown; the same data are also provided as ratios of fusion per fission (**, $p < 0.01$; $n = 12$ cells). (E) The velocity of motile mitochondria is shown (**, $p < 0.01$; $n = 50$ mitochondria). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

harboring mitochondria were ~3 times faster than their fusion rates (Fig. 2D). Thus mutation decreases mitochondrial fission and fusion rates, though the higher fission rates in mutant-harboring mitochondria suggest that the numbers of mutant-harboring mitochondria are elevated. We also noticed that the mutation decreased mitochondrial velocity by more than 50% (Fig. 2E), suggesting that the mutation drastically altered mitochondrial dynamics.

Supplementary video related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.132.

Next, we investigated the effect of the D29G mutation in HSPD1 on MitoTracker dye-incorporating activity. MitoTracker dye stains active mitochondria in live cells. Its accumulation is dependent upon membrane potential, and decreased dye-incorporating activity is one of the hallmarks of pathology in mitochondria [9,10,13]. Mitochondria harboring wild type HSPD1 had the ability to incorporate a typical quantity of MitoTracker dye (Fig. 3A). In contrast, the D29G mutant decreased dye-incorporating activity by more than 60% (Fig. 3B and D). Similarly decreased dye-incorporating activity was noted in mitochondria bearing the Gln-423-to-Ala (N423A) mutant of HSPD1 (Fig. 3C and D), which is already known to have this effect [9,10,13].

We examined mitochondrial distribution, as abnormal distribution is another hallmark of mitochondrial pathology. Compared to the wild type, the D29G mutant increased the number of mitochondria near DAPI-stained nuclei (Fig. 4A–C), consistent with previous reports stating that damaged mitochondria were transported into perinuclear regions [9,10,14]. Thus, we stained a tubulin network structure to determine mitochondrial localization with an anti-tubulin antibody [9,10,14]. In mutant-expressing cells, a tubulin network was primarily formed in perinuclear regions (Fig. S1), consistent with its mitochondrial localization. We further explored colocalization with the autophagy/mitophagy marker LC3B. Under the experimental conditions, LC3B was colocalized with neither the wild type- nor the D29G-harboring mitochondria; in contrast, the lysosome marker Lamp1 was indeed colocalized with LC3B (Fig. S2). It is unlikely that HLD4-associated HSPD1 mutant affects the autophagy system.

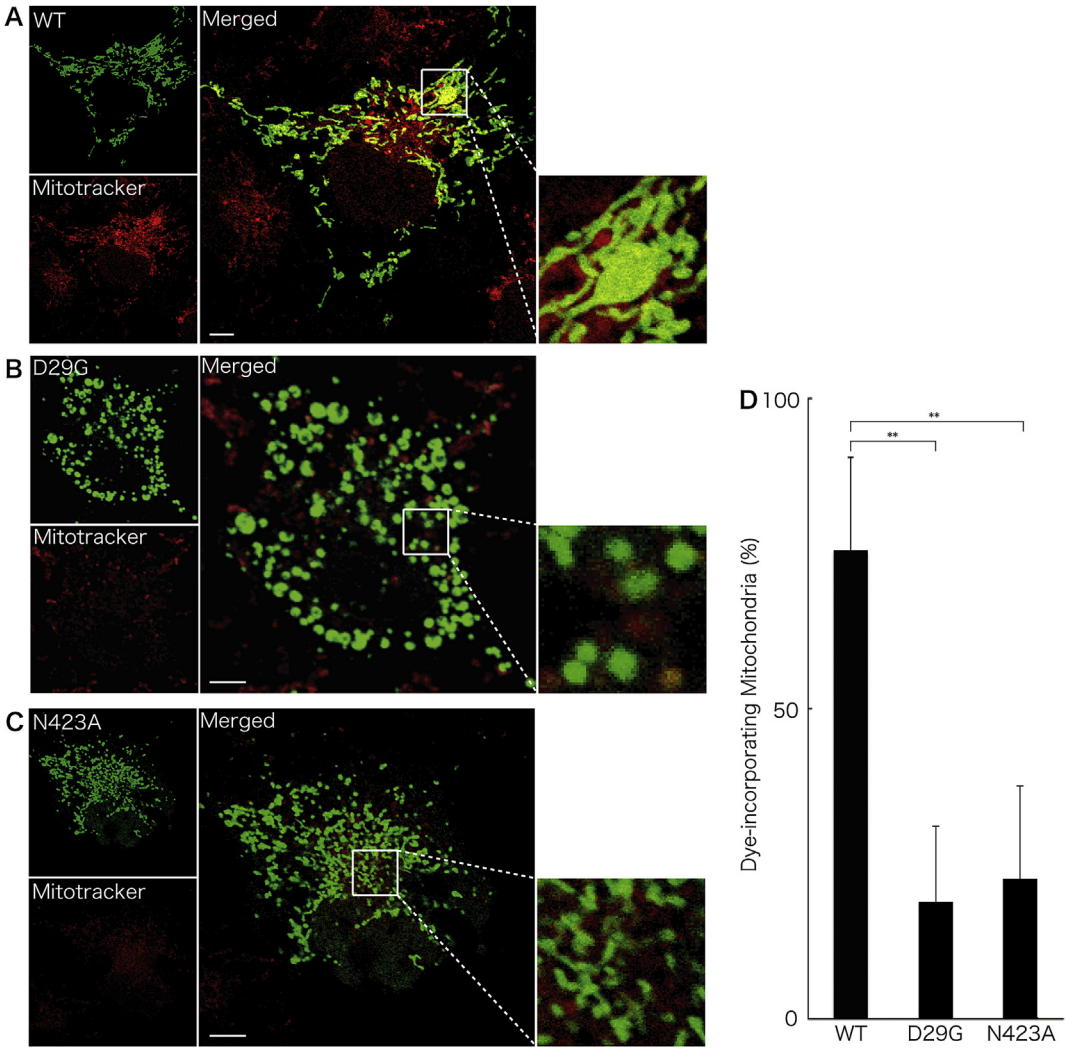

Fig. 3. HLD4-associated mutant decreases dye-incorporating activity into mitochondria. (A, B) Cells were transfected with wild type or D29G mutant HSPD1 (green), treated with or without MitoTracker dye (Red), which was incorporated at a rate dependent on mitochondrial membrane potential, and fixed for observation. The scale bar indicates 10 μm. (C) The N423A mutant was used as the positive control. The scale bar indicates 10 μm. (D) The percentage of dye-incorporating mitochondria is shown as a bar graph (**, $p < 0.01$; $n = 12$ cells). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Mitochondria are important bilayer membrane-containing organelles. They contribute not only to orchestrating cellular energy production and accumulating cellular calcium but also to maintaining cellular life as the gatekeepers of cell death signaling pathways such as that regulating apoptosis. Therefore, mitochondria play key roles in many aspects of cellular functions involved in the regulation of cell cycle, proliferation, differentiation, and cell death. Mitochondria have a complicated structure with inner and outer membranes that separate the aqueous region and the intermembrane space from each other as well as from the matrix [15,16]. However, mitochondria carry only a residual small genome in the form of the mitochondrial DNA, which encodes only 13 proteins essential for respiratory chain function. For this reason, it is crucial for the development and maintenance of mitochondrial function that somatic genome-encoded mitochondrial proteins are successfully transported from the cytoplasmic translational complexes into the mitochondria. This process also involves a step that helps these immature, cytoplasmically translated proteins to form accurate protein foldings. Therefore, mitochondrial production and maintenance essentially require chaperonins or chaperonin-like proteins. HSPD1 is such a mitochondrial chaperonin [9,10]. In the present study, we show that HLD4-associated HSPD1 mutation is related to aberrant fission and fusion cycles of mitochondria. Since the mitochondrial fission and fusion cycles are responsible for mitochondrial function, their aberrance often hinders an array of mitochondrial activities [15,16]. Consistently with this, disease-associated mutant-harboring mitochondria exhibit decreased mitochondrial membrane potential. As far as we could ascertain, this is the first report concerning the relationship between disease-associated mutation of HSPD1 and mitochondrial morphology and dynamics. Further studies in this vein will allow us to understand whether other MitCHAP-60 disease-associated mutations may affect changes in mitochondrial morphology and dynamics.

The HLD4-associated HSPD1 mutation is located in the outside of the amino acid region responsible for the mitochondrial localization signal (see the PSORT website, http://psort.hgc.jp/). Indeed, fluorescence protein-tagged, disease-associated HSPD1 mutant is not diffuse throughout the cytoplasm but is rather contained within the mitochondria. On the other hand, the

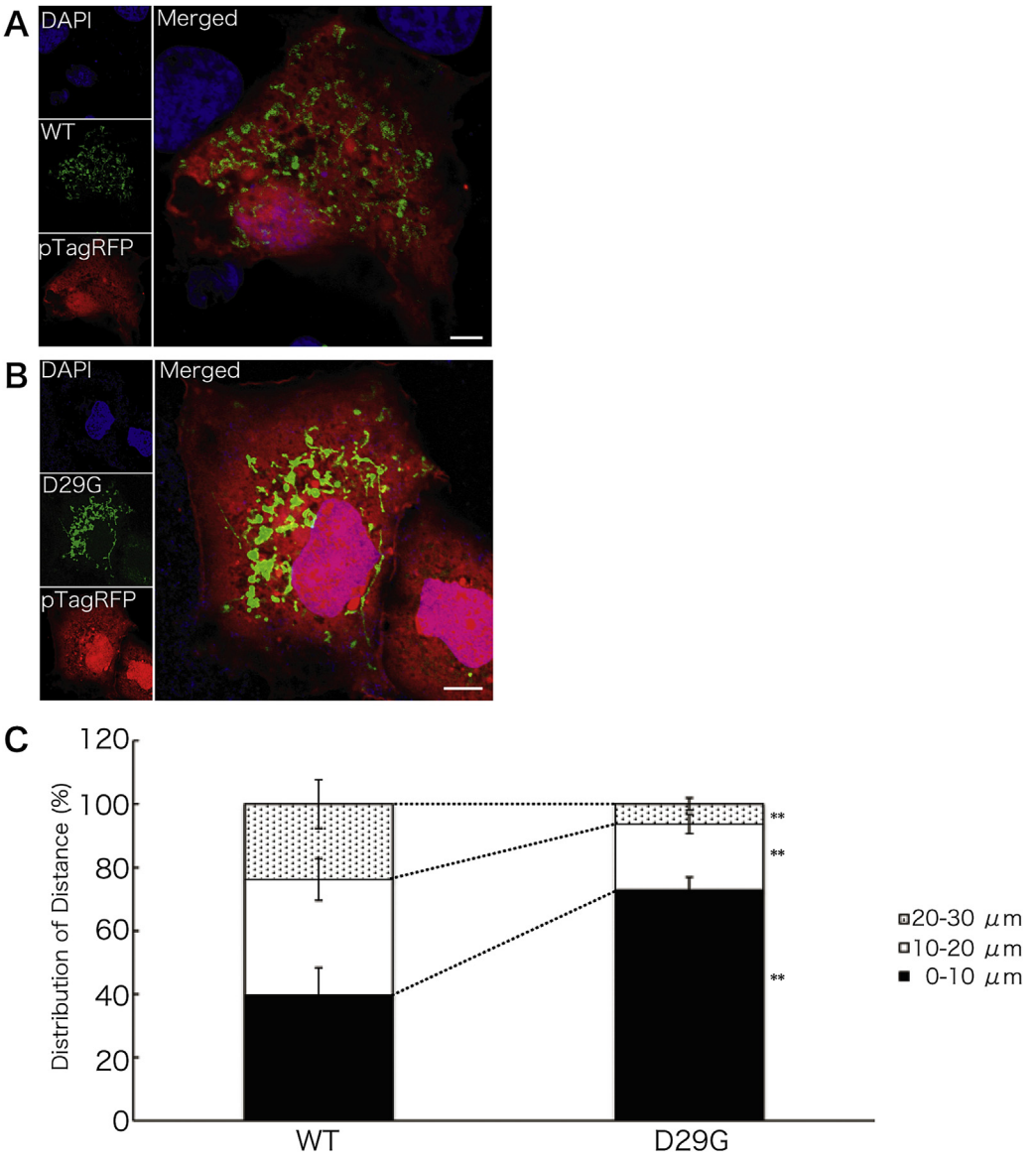

Fig. 4. HLD4-associated mutant-harboring mitochondria are preferentially localized in perinuclear regions. (A, B) Cells were transfected with wild type or D29G mutant (green) together with pTagRFP (red). Nuclei were stained with DAPI (blue). The scale bar indicates 10 μm. (C) The distribution of distances from DAPI-positive nucleus is shown (**, $p < 0.01$; $n = 12$ cells). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mutated position is far from the T-complex protein 1 subunit α (TCP-1)/Cpn60 chaperonin domain, which is needed for chaperonin's catalytic activity (see the Pfam website, http://pfam.xfam.org/), but the HLD4-associated HSPD1 mutant actually decreases MitoTracker dye-incorporating activity into mitochondria. The reason for this may be that the disease-associated mutant conformationally affects its chaperonin activity. Given that the fission and fusion cycles of mutant-harboring mitochondria are very slow, it is likely that the mutation decreases the folding of the mitochondrial proteins controlling these cycles, such as Drp1 and Fis1, which are involved in fission, and Mfn1 and Mfn2, which are involved in fusion [15,16]. Alternatively, the mutation may affect HSPD1 binding to the cochaperone HSPE1 (also known as Hsp10) or to some other known and as-yet-unknown binding proteins [15,16].

HLD1 is caused by various alterations in the *plp1* gene, including mutation and multiplication. This gene encodes a tetraspan-type, homophilic interactive protein present in CNS myelin membranes. Most missense mutations of PLP1 cause protein aggregation, resulting in accumulation of PLP1 mutant proteins in various subcellular compartments [5,6]. It is known that their accumulation often triggers an unfolded protein response [5,6] or mitogen-activated protein kinase (MAPK) activation [17] *in vitro* and *in vivo*. Increasing evidence indicates that some genes other than the *plp1* gene are associated with dismyelination and repeated demyelination. The gene responsible for HLD2 is a gap-junctional tetraspan membrane protein-encoding *gjc2* (also known as *gja12*) gene, in which many types of missense mutations cause protein aggregation [18]. While HLD5 is mostly due to deficiency of an unknown functional *fam126a* (also known as *hyccin* or *drctnnb1a*) gene, which is mostly caused by gene alterations such as mutations producing premature stop signals, one unique *fam126a* gene mutation (Leu-53-to-Pro) allows full-length FAM126A proteins to be produced [19]. A recent study reports that this mutation also causes protein aggregation, triggering an unfolded protein response *in vitro* and *in vivo* [20]. HLD4-associated HSPD1 mutation is unlikely to cause protein aggregation in cytoplasmic regions, but advanced genome sequencing technology may enable us to identify

mutation(s) causing protein aggregation in HSPD1, since protein aggregation is one of the hallmarks of neuropathies including HLD-associated mutations.

On the other hand, HLD3 may be associated with mutation of the aminoacyl-tRNA synthetase complex-interacting protein-encoding *aimp1* gene, but it remains unclear whether this mutation is due to AIMP1's functional deficiency [21]. However, some partially loss-of-functional alanyl-tRNA synthetase mutations have recently been identified in an unclassified heritable disorder characterized by severe neuropathic features and complete myelin defect [22]. Also, a possible loss-of-functional mutation of arginyl-tRNA synthetase causes HLD9 [23]. It is possible that mutation in proteins related to the aminoacyl-tRNA synthetase complex does not lead to protein aggregation but may instead decrease the respective protein activities. In addition to protein aggregation-mediated aberrant signaling activation, some types of HLDs may be caused by decreases in the activities of their responsible gene product proteins, as observed in this study.

Here we have shown that HLD4-associated HSPD1 mutation affects mitochondrial dynamics, which are linked to various mitochondrial functions. Further studies on HLD4-associated HSPD1 mutation *in vivo* as well as *in vitro* will further our understanding not only of how the mutation causes dismyelination and demyelination but also of whether and how MitCHAP-60 diseases modify mitochondrial fission and fusion cycles, resulting in disease. Such studies may shed light on a common disease-causing molecular mechanism in MitCHAP-60 diseases.

Conflict of interest

We have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.132.

Transparency document

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