Title

Drosophila model to clarify the pathological significance of OPA1 in autosomal dominant optic atrophy

Authors

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Abstract

Autosomal dominant optic atrophy (DOA) is a progressive form of blindness caused by degeneration of retinal ganglion cells and their axons, mainly caused by mutations in the OPA1 mitochondrial dynamin like GTPase (OPA1) gene. OPA1 encodes a dynamin-like GTPase present in the mitochondrial inner membrane. When associated with OPA1 mutations, DOA can present not only ocular symptoms but also multi-organ symptoms (DOA plus). DOA plus often results from point mutations in the GTPase domain, which are assumed to have dominant negative effects. However, the presence of mutations in the GTPase domain does not always result in DOA plus. Therefore, an experimental system to distinguish between DOA and DOA plus is needed. In this study, we found that loss-of-function mutations of the dOPA1 gene in Drosophila can imitate the pathology of optic nerve degeneration observed in DOA. We successfully rescued this degeneration by expressing the
human OPA1 (hOPA1) gene, indicating that hOPA1 is functionally interchangeable with dOPA1 in the fly system. However, we could not rescue any previously reported mutations known to cause either DOA or DOA plus. By expressing both WT and DOA plus mutant hOPA1 forms in the optic nerve of dOPA1 mutants, we observed that DOA plus mutations suppressed the rescue, facilitating the distinction between loss-of-function and dominant negative mutations in hOPA1. The fly model developed in this study can assist in the differential diagnosis between DOA and DOA plus and inform early treatment decisions in patients with mutations in hOPA1.

Introduction

Autosomal dominant optic atrophy (DOA) is a progressive form of blindness characterized by selective degeneration of retinal ganglion cells (RGCs) and the axons that form the optic nerve. Despite being a rare disease with a frequency of 1/12,000 to 1/50,000, DOA is the most commonly diagnosed form of hereditary optic neuropathy. In 2000, OPA1 mitochondrial dynamin like GTPase (OPA1) was identified as the causative gene for DOA (Alexander et al., 2000; Delettre et al., 2000). OPA1 encodes a dynamin-like GTPase located in the inner mitochondrial membrane (Olichon et al., 2002). It has various functions, including mitochondrial fusion, mtDNA maintenance, control of cell death, and resistance to reactive oxygen species (ROS) (Lenaers et al., 2021). The most
common pathogenic mutation in *OPA1* is the c.2708_2711 delTTAG deletion (Toomes et al., 2001).

Five pathogenic mutations, including the one above, result in a substantial decrease in OPA1 protein in the fibroblasts from affected patients (Zanna et al., 2008), supporting the theory that DOA is caused by haploinsufficiency.

Optic atrophy is a characteristic feature of *OPA1*-associated DOA; however, multisystem symptoms have been reported in up to 20% of *OPA1* mutation carriers (Yu-Wai-Man et al., 2010a). This is called DOA plus. DOA plus can include sensorineural deafness (Amati-Bonneau et al., 2008), multiple sclerosis (Verny et al., 2008; Yu-Wai-Man et al., 2016), parkinsonism and dementia (Carelli et al., 2015; Lynch et al., 2017), and cardiomyopathy (Spiegel et al., 2016). The R445H mutation is a well-known mutation associated with DOA plus (Amati-Bonneau et al., 2008, 2003; Shimizu et al., 2003). The proportion of mutated OPA1 locations differs between patients with DOA and DOA plus.

Missense mutations in the dynamin-featured structure, especially the GTPase domain of *OPA1*, are more likely to cause severe symptoms compared with loss-of-function (LOF) mutations such as deletions or splice site mutations (Yu-Wai-Man et al., 2010a). This is probably due to the dominant negative effect (DN) of a DOA plus mutation on the normal *OPA1* allele, since OPA1 functions as a homo-oligomer (Frezza et al., 2006; Olichon et al., 2006); in fact, its yeast ortholog, Mgm1, forms oligomers, increasing the GTPase activity (Meglei and McQuibban, 2009; Rujiviphat et al., 2009). Particularly, the dynamin-featured structure accounts for 70% of the 233 pathogenic variants of DOA
and DOA plus described in the locus-specific database (Ferré et al., 2015). However, mutations in these domains do not determine DOA plus, as it affects only 20% of all OPA1 mutation carriers. Thus, experimental models to determine whether a gene mutation is LOF or DN are required to distinguish between DOA and DOA plus. Although currently there is no effective treatment for DOA or DOA plus, distinguishing between them allows earlier planning of interventions such as hearing aids, rather than relying solely on visual aids, necessary only for those with DOA plus.

As OPA1 is conserved in various species, DOA models have been reported in vertebrates such as mice and zebrafish, simple organisms such as nematodes and fruit flies, and in vitro yeast and cultured cell models (Del Dotto and Carelli, 2021). In mouse models, focal RGC axons decrease in number with mitochondrial abnormalities at the electron microscope level, but the phenotype appears slowly, not allowing for analysis in a short time frame (Alavi et al., 2007; Davies et al., 2007; Nguyen et al., 2011; Sarzi et al., 2012). Zebrafish models have displayed developmental delays in eyes and heads, short length, and body axis abnormalities (Eijkenboom et al., 2019; Rahn et al., 2013) but have not been used to investigate optic nerve degeneration.

In C. elegans, most studies focused on muscle cells using mutants of eat-3, an OPA1 homolog (Kanazawa et al., 2008; Rolland et al., 2009). However, the mitochondria in the posterior lateral microtubule neurons did not show any difference in size from its wild-type (WT) counterparts.
Thus, LOF of eat-3 may have a limited impact on phenotypic outcomes in the nervous system. Nevertheless, a DOA model in which hOPA1K301A is expressed in GABAergic motor neurons, showed a decreased number of mitochondria in those cells (Zaninello et al., 2020). In a fly model, the dOPA1 somatic clone in the eye exhibited a rough-eye phenotype, eye structural abnormalities, and increased MitoSOX fluorescence, which was partially rescued by vitamin E or SOD1 expression (Yarosh et al., 2008). Heterozygous dOPA1 mutants showed shortened lifespan, elevated ROS levels, and irregular muscle tissue mitochondrial structures (Tang et al., 2009). Moreover, the Electroretinogram (ERG) pattern showed age-dependent decreases in the on transient response, heart rate, negative geotaxis response, and increased heart arrhythmia due to heat shock stress (Shahrestani et al., 2009). However, except for mice, the evidence of optic nerve axonal abnormalities is limited in model organisms such as zebrafish, worms, and flies.

The structure of the Drosophila visual system is similar to that of mammals (Sanes and Zipursky, 2010), with conserved mechanisms of synaptogenesis and neural circuit formation (Sanes and Zipursky, 2020). Drosophila photoreceptors type R7/8 extend their retinal axons from the retina directly into the brain, forming synapses in the second optic ganglion medulla via the chiasma (Figure 1A), having potential application as a model for mammalian photoreceptors and RGCs. Based on this, we aimed to develop an experimental model for observing and quantifying degeneration in the Drosophila retinal axon (Nitta et al., 2023; Richard et al., 2022).
In this study, a \textit{dOPA1} LOF could mimic the human DOA, in which mitochondrial fragmentation, increased ROS levels, and neurodegeneration occur in retinal axons. The axonal degeneration induced by the \textit{dOPA1} mutant was rescued by the expression of human \textit{OPA1} (\textit{hOPA1}). This demonstrated that the function of \textit{hOPA1} is the same as in the fly. As none of the previously reported mutations known to cause DOA or DOA plus were rescued, their function seems to be impaired. To distinguish between the effect of LOF and DN mutations, we expressed the \textit{hOPA1} gene of both WT and mutation in a genetic background in which \textit{dOPA1} was deleted in retinal axons. The DOA plus mutations D438V and R445H inhibited the rescue by the WT of \textit{hOPA1}. Taken together, we generated a new \textit{Drosophila} DOA model which served to isolate the LOF or DN effect of \textit{hOPA1} mutations.

\section*{Results}

\textit{dOPA1} depletion caused mitochondrial fragmentation in axon terminals

The OPA1 protein is located in the inner mitochondrial membrane. To confirm whether dOPA1 is also localized in the mitochondria of \textit{Drosophila} retinal axons, we used a Gal4/UAS system (Brand and Perrimon, 1993) and expressed HA-tagged dOPA1 proteins using the eye-specific \textit{GMR-Gal4} driver (Newsome et al., 2000a, 2000b). The outer membrane of the mitochondria was visualized with
mCherry-mito, which tagged the transmembrane domain of Miro with mCherry (Vagnoni and Bullock, 2016). Confocal microscopy revealed that the HA signals colocalized with mCherry-mito in the axon terminals of photoreceptor cells (Figure 1B). This indicated that dOPA1 colocalizes with mitochondria in retinal axons.

To examine the impact of dOPA1 on mitochondrial structure and density, we analyzed mitochondria in dOPA1 RNAi flies. The intensity of the mitochondrial signal was significantly reduced in the retinal axons of these flies (Figure 1C and D; quantification in 1E), suggesting a decreased number of mitochondria in retinal axons. However, whether this decrease is due to degradation resulting from a decline in mitochondrial quality or axonal transport failure remains unclear. Previously, impaired mitochondrial transport was reported in the LOF of marf (MFN 1/2 homolog), which is necessary for the fusion of the outer membrane of mitochondria (Sandoval et al., 2014). Thus, we investigated if the LOF of dOPA1 may cause transport defects, which may be responsible for the reduced mitochondrial density in retinal axons in dOPA1 RNAi flies. To this end, we observed the photoreceptor neurons after puparium formation at 24 h, which allowed us to simultaneously visualize the cell bodies and axon terminals of the photoreceptors (Figure S1A). Both dOPA1 knockdown and control flies showed mitochondrial signals in the axon terminals (Figure S1B and C). However, no mitochondrial signals were observed in the axon terminals of flies with a knockdown of milton, which is thought to act as an adaptor between kinesin to mitochondria,
promoting anterograde transport (Figure S1D). These findings suggest that trafficking defects are not
the main cause of the mitochondrial mislocalization observed in the retinal axons of the dOPA1
knockdown.

Nevertheless, the degradation resulting from mitochondrial fragmentation may have
decreased the mitochondrial signal. To further analyze the mitochondrial morphology of retinal axons
in the LOF of dOPA1, we conducted an electron microscopy analysis (Figure 1F and G). Although
Drosophila has eight types of photoreceptors, the axons of R1–6, which project to the lamina of the
first optic lobe, can be identified without markers; thus, we chose them for this experiment. The dOPA1
knockdown led to a significant decrease in mitochondria size in R1–6 axons (Figure 1F–H). In addition,
there was significant heterogeneity in healthy mitochondria, ranging from dense-matrix (Class A) to
swollen mitochondria with a hypodense matrix (Class B) (Figures 1I). Knocking down dOpal in R1–
6 axon terminals resulted in severely abnormal mitochondrial ultrastructure (22.8%; Figure 1J). These
results suggest that dOPA1 is involved in maintaining proper mitochondrial morphology by promoting
mitochondrial fusion in retinal axons. Previous studies have observed mitochondrial fragmentation in
S2 cells (McQuibban et al., 2006), muscle tissue (Deng et al., 2008), segmental nerves (Trevisan et
al., 2018), and ommatidia (Yarosh et al., 2008) due to LOF of dOPA1, which is consistent with the
results observed in the retinal axons.
To verify whether ROS is increased in the retinal axons in dOPA1 knockdown photoreceptors, we measured the ROS levels in the knockdown retinal axons using MitoSOX, a mitochondrial superoxide indicator (Yarosh et al., 2008). The results showed significantly elevated ROS levels in dOPA1 knockdown retinal axons compared to control (Figure 2A and B; quantification in 2C).

The dOPA1 LOF leads to progressive distal degeneration of Drosophila photoreceptors

We tested whether knocking down dOPA1 causes optic nerve degeneration in Drosophila. To this end, we performed an RNAi experiment using the GMR-Gal4 driver and evaluated the number of retinal axons projecting to the second optic ganglion medulla (Figure 3A). To compare the number of retinal axon terminals–R7 axons–between genotypes, we used a previously developed automated method, MeDUsA (method for the quantification of degeneration using fly axons) (Nitta et al., 2023). Our results showed that the dOPA1 knockdown caused axonal degeneration one day after eclosion, further decreasing one week later (Figure 3B–E, quantification in 3F). To determine whether the degeneration was limited to the axons, we counted the number of rhabdomeres in the cell bodies. Each compound eye comprises 700–800 ommatidia, with each ommatidium containing eight types of photoreceptors, designated as R1–R8. The distal region of an ommatidium reveals the R1–R7 photoreceptors with a
stereotypical arrangement of rhabdomeres (arrowheads in Figure 3G–J). Since it is easy to evaluate retinal degeneration by counting the number of rhabdomeres, a decrease in their number indicates degeneration of the photoreceptor cell body. The dOPA1 knockdown caused a significant decrease in the number of rhabdomeres per ommatidium in 1-week-old adults (Figure 3G–J, quantification in 3K).

Interestingly, although the retinal axons had already decreased on the day of eclosion, the ommatidia remained intact (Figure 3F and 3K). Note that the ommatidia in R7 remained intact after one week (Figure 3J). Our results indicate that the dOPA1 knockdown causes degeneration in both axonal terminals and cell bodies, with the neuronal degeneration starting from the axons and continuing in the cell bodies. This suggests that cell body loss precedes neurite retrograde degeneration, as described in many neurodegenerative diseases (Wang et al., 2012).

To test whether our RNAi results reflect dOPA1 downregulation, we performed mutant analysis of the dOPA1 gene in retinal axons. For this, we used the dOPA1s3475 hypomorphic mutant allele, which has a P-element insertion in exon 2 (Yarosh et al., 2008). Somatic mosaic clones were generated in the retinal axons using the FLP/FRT system (Golic and Lindquist, 1989) because homozygous mutant alleles are embryonic lethal (Sandoval et al., 2014; Yarosh et al., 2008). Using MeDUsA, we found a significantly lower number of dOPA1 mutant retinal axons than in controls one day after eclosion (Figure 4A and B, quantification in 4D). To determine if dOPA1 is responsible for axon neurodegeneration, we observed the dOPA1 mutant axons by expressing full-length versions of
dOPA1 in the photoreceptors at one day after eclosion and found that dOPA1 expression significantly rescued the axonal degeneration (Figure 4C, quantification in Figure 4D). Our results indicate that dOPA1 is necessary for maintaining the number of retinal axons in the Drosophila visual system. In conclusion, the neurodegeneration observed in the LOF of dOPA1 is due to the progressive loss of retinal axons, as well as the mammal optic nerve.

Investigating disease mutations in OPA1 using a fly DOA model to confirm their pathological significance

Our observations in dOPA1 LOF flies confirmed mitochondria fragmentation, increased ROS levels, and degeneration of retinal axons, as reported in mammals. These data indicated that the function of OPA1 may be conserved across Drosophila and humans. Thus, we tested whether hOPA1 could effectively replace dOPA1 in flies by generating a transgenic organism to express hOPA1 in Drosophila. Following the UAS sequence, that the yeast-derived transcription factor Gal4 binds, the construct included an HA tag, followed by the hOPA1 gene, and a myc tag (Figure 5A). This construct was expressed in Drosophila and protein expression was confirmed by Western blotting. The HA tag, attached to the N-terminus, was immunoblotted resulting in a band of the expected full-length size
For the myc tag at the C-terminus, a strong band and two weak bands were observed at the upper and lower positions (Figure 5B).

Using this UAS-hOpa1, we performed rescue experiments. hOpa1 expression in the retinal axons of the dOpa1 mutant, which generated somatic cell clones, significantly rescued the number of axons (Figure 5C). These results suggest that hOpa1 and dOpa1 are interchangeable. We also generated and expressed the hOpa1 mutation 2708_2711del, which is known to cause DOA, the I382M mutation, located in the GTPase domain and associated with DOA, as well as the D438V and R445H mutations, also located in the GTPase domain and associated with DOA plus. The 2708-2711del mutation was weakly detectable by HA but not detectable by myc due to the frameshift caused by the deletion (Fig. 5B). This appears to be a result from the mutation to generate a truncated protein, as previously reported (Zanna et al., 2008). The I382M, D438V, and R445H mutations had similar expression levels to the WT hOpa1 (Figure 5B). However, the expression of these mutations in retinal axons failed to rescue the dOpa1 deficiency to the same extent as the WT hOpa1 (Figure 5C). These results suggest that these mutations result in functional impairment, consistent with the established understanding (Zanna et al., 2008). Importantly, unlike the D438V and R445H mutations, the 2708-2711del and I382M mutations could be weakly rescued. This suggests that these mutations result in LOF, but may retain some residual activity. This is consistent with the characterization of I382M as a hypomorph (Del Dotto et al., 2018). These findings are also in line with the observed severity of the
associated diseases, DOA and DOA plus. As the D438V and R445H mutations were not significantly
rescued, unlike the 2708-2711del and I382M mutations. Our results demonstrate that our model can
quantitatively measure the degree of LOF.

**Distinction between LOF and DN mutations in hOPA1 linked to DOA or DOA plus**

Currently, the hOPA1 mutations that contribute to DOA plus have been primarily located in the
GTPase domain of hOPA1. The role of hOPA1 as a GTPase is facilitated by its ability to form a
d polymer via the GED domain and Middle domain (Li et al., 2019). This led to the hypothesis that
mutations in the GTPase domain can interact with WT hOPA1 but cannot activate the GTPase activity
to show a DN effect. However, it has been challenging to clarify whether these mutations are DN or
LOF, as there is a significant number of LOF mutations in the GTPase domain. Given that dOPA1 can
be substituted with hOPA1, we suspected that a DN effect could also be achieved by expressing the
D438V or R445H mutations in the WT background. However, the axons did not degenerate despite
expression and monitoring for two weeks in adult flies (Fig. 6A). The amino acid sequences of hOPA1
and dOPA1 are highly conserved (72% identity), particularly in the GTPase domain. The Middle and
GED domains are also relatively well-preserved, with 54% and 64% agreement, respectively (Figure
S2). These results imply that, although dOPA1 and hOPA1 are functionally interchangeable, they may
not interact with each other. Consequently, we expressed \( hOPA1 \) in the photoreceptor depleted of \( dOPA1 \) by somatic clone; under these conditions, we further expressed \( hOPA1 \) WT, 2708-2711del, and mutations in the GTPase domains, including R445H, I382M, and D438V. This allowed \( hOPA1 \) to interact with itself and to verify the DN effect. As a result, the expression of the WT, 2708del, and I382M did not result in any changes when rescued by \( hOPA1 \) substitution (Figure 6B). However, the rescue was significantly suppressed for D438V and R445H, which are known to cause DN; thus, the DN effect could be replicated (Figure 6B). In conclusion, we established an experimental model that can separate LOF and DN, the pathological significance of \( hOPA1 \) mutations.

Discussion

In this study, we found that LOF of \( dOPA1 \) in \( Drosophila \) can imitate human DOA, in which the optic nerve degenerates. We were successful in reversing this degeneration by expressing \( hOPA1 \) (Figure 6C). However, we could not rescue any previously reported mutations known to cause either DOA or DOA plus. In the context of rescuing the retinal axons of the \( dOPA1 \) mutant by expressing the WT of \( hOPA1 \), it was observed that only the DOA plus mutations suppressed the rescue (Figure 6C). This allowed us to distinguish between LOF and DN mutations in \( hOPA1 \). The fly model developed in this study will allow investigating new \( hOPA1 \) mutations for DOA or DOA plus.
Our established fly model is the first simple organism allowing observation of degeneration of axons in the optic nerve and fragmentation of mitochondria. Previously, the function of \( OPA1 \) in mice (\( mOPA1 \)) was substituted by \( hOPA1 \) (Del Dotto et al., 2017; Sarzi et al., 2018). Here we demonstrated that the function of \( dOPA1 \) can also be substituted by \( hOPA1 \). Thus, the function of \( OPA1 \) is conserved across a wide range of species but our fly model has the advantage of allowing rapid observation of phenotypes. In the mouse model of DOA with the heterozygous deletion of the \( mOPA1 \) gene at positions 329–355 (Alavi et al., 2007) or delTTAG mutant (Sarzi et al., 2012), a decrease in the number of RGC was observed after 17 months or 16 months of age, respectively. To save time, using a fly model for quick analysis and direct observation of retinal axons would be useful. Previous models using nematodes, flies, and zebrafish did not observe axonal degeneration directly, but our new model has made this possible. As a result, our model has the potential to be used for screening for modifiers of unclear molecular pathologies and drug screening.

Our model could also demonstrate the pathological significance of \( hOPA1 \) mutations and be used for genetic diagnosis to differentiate between LOF and DN mutations. Our results for LOF or DN segmentation were consistent with previously reported yeast models (Del Dotto et al., 2018) and fibroblasts obtained from patients with OPA1 mutations (Kane et al., 2017), and confirmed the conserved function of \( OPA1 \) across species. A major advantage of the \emph{Drosophila} model over yeast or fibroblasts, is that we can observe a phenotype that mimics the actual disease state of axonal
degeneration in retinal axons and analyze it not only at the molecular level, but also the structural level of axons and mitochondria. The transgenic *Drosophila* of disease-associated *hOpa1* mutations created in this study can also serve to understand the pathophysiology of these mutations.

Advances in genome analysis allowed the identification of many variants and genetic mutations associated with DOA using next-generation sequencing. This could confirm diagnoses and identify new forms of DOA. The widespread use of these sequencers has also created new challenges, such as the growing number of variants of unknown significance (VUS) in publicly available databases such as ClinVar for DOA. These variants are categorized as pathogenic, likely pathogenic, VUS, likely benign, or benign, and the number of VUS is increasing as more variants are registered. The ClinVar database had classified 110 out of 319 *Opa1* variants as VUS at the end of 2019. By the end of 2022, this number had increased to 357 out of 840, indicating an increase from 34% to 42% over the last three years (Henrie et al., 2018). This trend suggests that the number of VUS in *Opa1* may continue to increase in the future. Analyzing VUS requires testing the effects of a mutation *in vitro* or *in vivo*; this can be expensive and time-consuming without prior confirmation or high probability of pathological significance. However, our fly model can be used to easily and inexpensively determine the pathological effects of disease-causing gene mutations among the growing number of rare mutations identified in DOA.
In this study, we tested the effect of expressing the D438V and R445H mutations in the dOPA1 background and found that retinal axons did not degenerate. Regarding the interactions among OPA1 proteins, in yeast Mgm1, K854 in the region near the C-terminus is important for aggregation, and it becomes an oligomer to increase the activity of GTPase (Rujiviphat et al., 2009). Although hOPA1 normally has low GTPase activity, its interaction with negatively charged phospholipids such as cardiolipin causes hOPA1 to aggregate, increasing GTP hydrolysis activity (Ban et al., 2010). In the thermophilic fungus Chaetomium thermophilum, Mgm1 forms a dimer and then a tetramer at the C-terminal Stalk domain of Mgm1 (Faelber et al., 2019). Structural analysis also predicted that the Middle and GED domains are necessary for dimer and oligomer formation in hOPA1 (Li et al., 2019). In addition, peptide-binding assays have revealed that the coiled-coil domain, which is part of the GED domain, is required for hOPA1 interactions (Akepati et al., 2008). Comparing the amino acid sequences of hOPA1 and dOPA1, the Middle and GED domains, which are necessary for interactions between OPA1 molecules, exhibit concordance rates of 54.3% and 63.6%, respectively. These percentages are low compared to the 71.8% identity observed in the GTPase domains. These suggest that the OPA1 homologs of each species form oligomers with their unique OPA1. Note that the expression of dOPA1K273A, a presumably GTPase-negative form of dOPA1 (Tsuyama et al., 2017), degenerated retinal axons (data not shown). These results imply that, while dOPA1 and hOPA1 are interchangeable, they may not interact with each other. This could pave the way for the development...
of a chimeric hOPA1, which would retain its functional properties while avoiding the interaction with endogenous hOPA1. Such chimeric hOPA1 could potentially be used as gene therapy since induced pluripotent stem cells have been generated from a patient with R445H (Sladen et al., 2021).

OPA1 has been implicated in various other diseases as well, including normal tension glaucoma (Powell et al., 2003; Yu-Wai-Man et al., 2010b), multiple sclerosis (Verny et al., 2008; Yu-Wai-Man et al., 2016), Parkinson's disease and dementia (Carelli et al., 2015; Lynch et al., 2017), and cardiomyopathy (Spiegel et al., 2016). Although the connection between these diseases and OPA1 is not fully understood, further research on DOA may provide a deeper insight into this relationship. In the future, pathological analysis using the present model could have implications for understanding the mechanisms underlying these diseases.

DOA has also been reported to involve mitochondrial DNA (mtDNA) depletion (Amati-Bonneau et al., 2008), but our model, while useful for nuclear gene analysis, is limited for mtDNA analysis. The gene content in the Drosophila mtDNA genome is similar as that in vertebrates, but the gene order and distribution on both DNA strands differ. mOPA1 interacts with mtDNA nucleoids through exon 4b binding to mtDNA D-loops, independent of mitochondrial fusion (Yang et al., 2020). However, the homologous region of mOPA1 or hOPA1 to exon4b is not found in Drosophila, and the D-loop of mtDNA has not been found in cultured cells of flies or in mtDNA of embryos using electron
microscopy (Rubenstein et al., 1977). In addition, while the D-loop is involved in mtDNA replication in humans (Fish et al., 2004), the A + T region is the origin of replication in mtDNA replication in *Drosophila* (Goddard and Wolstenholme, 1978). Thus, there may be differences in the mechanism of mtDNA replication between humans and flies (Garesse and Kaguni, 2005). Therefore, the association of dOPA1 with mtDNA may differ from that in mammals and the regulatory mechanism of mtDNA homeostasis in which dOPA1 is involved requires further investigation.

Another limitation of our model is that the molecular mechanisms underlying the formation of long-form OPA1 (L-OPA1) and short-form OPA1 (S-OPA1), which are well analyzed in mammals (MacVicar and Langer, 2016), have not been elucidated in *Drosophila*. In hOPA1, S1 or S2 sites are processed by the i-AAA protease OMA1 and Yme1L respectively to produce S-hOPA1 (Anand et al., 2014). Despite the fact that Yme1L is conserved in *Drosophila*, it has been involved in the degradation of the dOPA1 protein rather than its cleavage by dYme1L (Liu et al., 2020). Moreover, the cleavage site for i-AAA does not exist in dOPA1 (Olichon et al., 2007). Regarding other proteases, yeast Mgm1 is processed by the rhomboid protease Pcp1 (Esser et al., 2002; Herlan et al., 2003). However, the Rhomboid-7, a Pcp1 ortholog of *Drosophila*, was not required for dOPA1 processing (Whitworth et al., 2008). Although hOPA1 expression in *Drosophila* has identified three different sizes of hOPA1 in our Western blotting result, it is unclear how they are cleaved. The upper band was expected as the full-length hOPA1 including the mitochondria import sequence (MIS). Since the Middle and the
The lowest bands were not present in HA-tagged samples, it is possible that the bands at the Middle size represent the long-form hOPA1 (L-hOPA1) in which probably MIS was processed. The band detected at the lowest position may represent a short form of hOPA1 (S-hOPA1). However, Drosophila does not have OMA1, an i-AAA protease that cleaves the S1 site. Although another i-AAA protease that cleaves the S2 site, YME1L, is conserved, our expressed hOPA1 is isoform 1 and lacks the S2 site (Fig. 5a). Therefore, it is not clear how S-hOPA1 is processed in Drosophila. The anti-dOPA1 antiserum detects three bands in the overexpression of a FLAG-tagged dOPA1 construct (Poole et al., 2010), suggesting a molecular mechanism by which dOPA1, like yeast and mammals, is cleaved after translation. However, the proteases involved in the mechanism are not yet clarified. Whether Drosophila’s endogenous mechanisms can be used to study how L-OPA1 and S-OPA1 are involved in DOA remains unclear.

In this study, we established a new model of DOA in Drosophila, in which we discovered the following: 1. We could replicate the human disease of optic nerve degeneration, allowing rapid genetic disease analysis. 2. We could distinguish the LOF and DN effects of hOPA1. These findings can reveal the pathological significance of de novo mutations and are useful in diagnosing DOA or DOA plus. Additionally, while hOPA1 is the major gene involved in DOA, other genes involved in DOA and interacting proteins have not been investigated yet. Our model can be used for pathological
modifier screening, exploring modifiers, and contributing to drug development. In the future, we hope
to provide insights that can be applied to the fundamental treatment of DOA.

**Materials and Methods**

**Fly strains**

Flies were maintained at a temperature of 25°C on standard fly food. For the knockdown experiments
(Figure 1C–E, 1F–H, 2A–C, 3B–K), flies were maintained at 29°C. Female flies were used in all
experiments to ensure consistency in the number of retinal axons. The following fly strains were
obtained from the Bloomington Drosophila Stock Center: *GMR-Gal4* (#1104), *UAS-dicer2(III)*
(#24651), *UAS-mito-HA-GFP* (#8443), *UAS-mCherry-mito* (#66532), *UAS-dOPA1 RNAi* (#32358),
*UAS-Milton RNAi* (#44477), *FRT42D*, *GMR-hid, l(2)cl-R11[1]/CyO*; ey-Gal4, *UAS-flp* (#5251), ey-
*FLP*; *FRT42D, l(2)cl-R11[1]/CyO* (#5617), and *Tub-Gal80TS* (#7017). The 40D-UAS (VDRC 60101)
fly strain was obtained from the Vienna Drosophila Resource Center. *FRT19* (Kyoto 101231),
*FRT42D* (Kyoto 101878), and *dOPA1* (Kyoto 111438) were obtained from the Kyoto Drosophila
Stock Center. The *UAS-dOPA1-HA* was generously provided by T. Uemura (Kyoto University, Japan).

**Generation of transgenic flies**
The cDNA for WT, I382M, D438V, R445H, and 2708-2711del forms of hOPA1 [NM_015560.3] were generated by Vectorbuilder Inc. (Yokohama, Japan). Then, the HA-hOPA1-myc sequence was amplified and inserted into pJFRC81-10XUAS-IVS-Syn21-GFP-p10 (ID36432, Addgene, Massachusetts, USA) using primers with the N-terminal containing the Kozak and HA sequences (TTACTTCAGGCAGCCGCGCAAAAATGTACCCTACGATGTTACATTAC) and the C-terminal containing the myc sequence (TTAAAAACGATTCATTCTAGTTACAGATCCTCTTGAGATGAGTTTTTGTTCTTTCTCCTGATGAAGAGCTTCAATG). To confirm production of the complete coding sequence, it was bidirectionally sequenced using the Sanger method. These plasmids were injected into embryos and integrated into the aTTP2 landing site (WellGenetics, Taipei, Taiwan).

**Immunohistochemistry and imaging**

Immunohistochemistry was performed as described previously (Sugie et al., 2017). The following antibodies were used: rat anti-HA (3F10, 1:400; Roche, Switzerland), mouse anti-Chaoptin (24B10, 1:25; Developmental Studies Hybridoma Bank, USA), rat anti-CadN (DN-Ex#8, 1:50; Developmental Studies Hybridoma Bank), anti-rat Alexa Fluor 488 (1:400; Thermo Fisher Scientific, USA), anti-mouse Alexa Fluor 488 (1:400; Thermo Fisher Scientific), anti-mouse Alexa Fluor 568 (1:400;
Thermo Fisher Scientific), and anti-rat Alexa Fluor 633 (1:400; Thermo Fisher Scientific). Specimens were mounted using a Vectashield mounting medium (Vector Laboratories, USA). Images were captured using an FV3000 confocal microscope (Olympus, Japan) or a C2 (Nikon, Japan) confocal microscope for Figure S1 and processed using the IMARIS software (Oxford Instruments, UK) or Fiji software, an open-source image analysis software (Schindelin et al., 2012).

Electron microscopy

Fly heads were fixed overnight with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer. Heads were then post-fixed with 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon. Ultra-thin tangential sections of the laminas (70 nm) were stained with uranyl acetate and lead citrate. Random retinas or lamina sections were imaged at a magnification of ×100 k with a VELETA CCD Camera (Olympus Soft Imaging Solutions) mounted on a JEM 1010 transmission electron microscope (JEOL). The area corresponding to the mitochondria in the R1–6 axons of the lamina was measured using the freehand line tool in NIS elements (Nikon).

Quantification of mito-GFP intensity in retinal axons and retina

To measure the relative intensity of mito-GFP in the photoreceptor R7/8 axons of the medulla, the
imaging analysis software IMARIS was used. First, the M1–6 layers of the medulla, including the
R7/8 axons, were manually covered using the surface function. Then, the surface area was masked,
the surface of the left axons was stained with anti-Chaoptin, and the mito-GFP signal was generated
automatically using the surface function. Finally, the voxel number of the mito-GFP surface was
divided by the voxel number of the R axon surface. These quantifications were performed by
experimenters blind to the genotype.

Mitochondrial superoxide detection and quantification

The ROS level was determined using the MitoSox Red Mitochondrial Superoxide Indicator
(Invitrogen). The procedure involved dissecting adult brains in phosphate-buffered saline (PBS),
transferring them to 5 µM MitoSox solution, and incubating them for 10 min in the dark at room
temperature. The brains were then washed with PBS containing 20% Vectashield (Vector Laboratories),
followed by sequential washes with PBS containing 40% and 60% Vectashield. The samples were
mounted in Vectashield and scanned using an FV3000 confocal microscope (Olympus). To quantify
the ROS level in photoreceptor R7/8 axons, the IMARIS software (Bitplane) was used for imaging
analysis. The software generated automatic surfaces of the MitoSox and mito-GFP signals. Then, the
ROS level was calculated by dividing the voxel number of the MitoSox surface on the mito-GFP
surface by the voxel number of the mito-GFP surface. These quantifications were performed by
experimenters blind to the genotype.

Western blot analysis of the whole Drosophila body

Protein expression was temporarily controlled in the whole body by combining Tub-Gal4 and Tub-GAL80<sup>TS</sup>. The flies crossed with each hOPA1 variant were raised at a permissive temperature (20°C), and newly eclosed females were held at the restrictive temperature (29°C). After two days, 10 whole bodies were sonicated (2 x 30 s) using a Q55 sonicator (Qsonica, Newtown, CT, USA) in lysis buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2% DDM) supplemented with 1:1000 (v/v) Protease Inhibitor Cocktail Set III (Calbiochem, La Jolla, CA, USA). Rat anti-HA (3F10, 1:5000; Roche, Mannheim, Germany), mouse anti-myc (9B11, 1:10000; Cell Signaling Technology, Danvers, MA, USA), and mouse anti-α-Tubulin (T9026, 1:100,000; Sigma-Aldrich, St. Louis, MO, USA) antibodies were used for Western blotting.

Pairwise alignment of hOPA1 and dOPA1

The amino acid sequences for hOPA1 and dOPA1 were referenced from NM_015560.3 and NM_166040.2, respectively, and aligned using the EMBOSS Needle. The GTPase, Middle, and GED domains in dOPA1 were identified by cross-referencing the previously described amino acid positions.
of these domains in hOPA1 (Liesa et al., 2009). The degree of identity between the aligned amino acid sequences was subsequently determined.

**Experimental design and statistical analyses**

Experimental analyses were performed using Prism 9 (GraphPad Software, San Diego, CA, USA). For Figures 3–5, data were analyzed using t tests (and nonparametric tests) and Mann-Whitney tests. For Figures 1, 2, 6, and 7, differences among multiple groups were examined using the Kruskal–Wallis test followed by the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli tests. The null hypothesis was rejected at a 0.05 level of significance. Sample sizes are indicated in each figure. The number of retinal axons and rhabdomeres, the intensity of mito-GFP and MitoSox, the mitochondrial area and the classification, and the corrected $P$ values are described in the Results section. All data are expressed as the mean ± SEM.

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association of autosomal dominant optic atrophy and moderate deafness may be due to the R445H


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**Figures with the corresponding legend**

**Figure 1:** Effect of dOPA1 knockdown on mitochondrial density and size in retinal axons.

(A) Schematic illustration of the *Drosophila* visual system from a dorsal view. (B) Visualization of
mitochondria and dOPA1 in a set of R7/8 axons. Mitochondria were visualized using the expression
of mCherry-mito driven by GMR-Gal4 (magenta) while HA-tagged dOPA1 was immunostained using
the anti-HA antibody (green). Scale bar: 3 µm. (C, D) Representations of mitochondria visualized
using the expression of Mito::GFP driven by GMR-Gal4 (green), one day after eclosion. Retinal axons
were labeled using the anti-Chaoptin antibody (magenta). Scale bar: 30 µm. (E) Quantification of
Mito::GFP intensity. Mito::GFP levels were calculated by dividing the Mito::GFP fluorescence
intensity by the anti-Chaoptin signal intensity. Control (n = 28 optic lobes) and dOPA1 RNAi (n = 28
optic lobes). The data are presented as mean ± SEM. (F, G) Electron micrographs of cross sections of
the R1–6 retinal axons in the lamina of the Control (F) and dOPA1 knockdown (G) on the day of
eclosion. Yellow circles and white dotted lines indicate mitochondria and a lamina column,
respectively. Scale bar: 2 µm. (H) Quantification of the mitochondrial area. Control (n = 96
mitochondria) and dOPA1 RNAi (n = 92 mitochondria). Data are expressed as mean ± SEM. (I)
Representative EM images showing mitochondria with a densely packed matrix structure (classified
as class A) and collapsed mitochondria (classified as class B). Scale bar: 500 nm. (H) Quantification
of the mitochondria classified into class A and class B. Control (n = 96 mitochondria) and dOPA1
RNAi (n = 92 mitochondria). Data are analyzed by Chi-square test.


Figure 2. Elevated reactive oxygen species levels in the dOPA1 knockdown.

(A, B) Mitochondrial reactive oxygen species (ROS) levels in a retinal axon visualized using mito-GFP (green) driven by GMR-Gal4 and MitoSOX (magenta). Representative images of a retinal axon in the Control (A, A’) and dOPA1 knockdown (B, B’). (C) Quantification of ROS levels for each genotype. ROS levels were determined by dividing the fluorescence intensity of MitoSOX by that of mito-GFP. Control (n = 20 optic lobes) and dOPA1 RNAi (n = 18 optic lobes). Scale bar: 3 µm. Data are presented as mean ± SEM.
Figure 3. Effect of dOPA1 knockdown on photoreceptor neurodegeneration in Drosophila.
Schematic of the visual system in *Drosophila*. A set of R7/8 photoreceptors project their axons to the second optic ganglion medulla. (B–E) All R7 and R8 axon terminals project to the medulla. *dOPA1* in the photoreceptor was knocked down by *GMR-Gal4. 40D-UAS* was used as a Control to match the number of *UAS* sequences recognized by Gal4. One day after eclosion in Control (B) and *dOPA1* knockdown (C), and one week after in Control (D) and *dOPA1* knockdown (E). The retinal axons were stained with anti-Chaoptin, a photoreceptor-specific antibody. Scale bar = 50 μm. (F) Number of axons at each time point in R7 neurons for each situation quantified by MeDUsA. At 1 day of Control (n = 34 optic lobes) and *dOPA1* RNAi (n = 23 optic lobes), and at 1 week of Control (n = 28 optic lobes) and *dOPA1* RNAi (n = 26 optic lobes). Data are expressed as mean ± SEM. (G–J) Electron micrographs of cross sections of the photoreceptor cell bodies in the ommatidia of the retina. Rhabdomeres are shown as yellow arrowheads. The day of eclosion in Control (G) and *dOPA1* knockdown (H), and 1-week-old adults of Control (I) and *dOPA1* knockdown (J). Scale bar = 2 μm. (K) Quantification of the number of rhabdomeres for each genotype and time point. At 0 day in Control (n = 50 ommatidia) and *dOPA1* RNAi (n = 127 ommatidia), and at 1 week in Control (n = 50 ommatidia) and *dOPA1* RNAi (n = 117 ommatidia). Data are expressed as mean ± SEM.
Figure 4. Role of dOPA1 in the retinal axon according to mutant analysis.

(A–C) Representations of retinal axons labeled with an anti-Chaoptin antibody (gray), one day after eclosion. The images show Control (A), dOPA1<sup>s3475</sup> somatic mosaic flies (B), and dOPA1<sup>s3475</sup> somatic mosaic flies expressing the eye-specific full-length dOPA1 (C). Scale bar: 50 µm. (D) Quantification of the number of axons for each genotype. Control (n = 41 optic lobes), mutant (n = 45 optic lobes), and rescue (n = 41 optic lobes). The data are presented as mean ± SEM.
Figure 5. Verification of the pathological significance of disease mutations in hOPA1.

(A) Schematic illustration of the hOPA1 gene construct with HA and myc tags in the UAS-based vector. hOPA1 includes a mitochondrial import sequence (MIS) cleaved by mitochondrial processing peptidase (MPP), a transmembrane region (TM), a coiled-coil region (CC1), a GTPase domain, a Middle domain, and a GTPase effector domain (GED) containing a coiled-coil region (CC2). The sites of variants (I382M, D438V, R445H, and a deletion from 2708 to 2711) are shown in red. S1 is the site cleaved by OMA1. (B) Western blot analysis to confirm the expression of hOPA1 variants. hOPA1_WT, hOPA1_D438V, hOPA1_I382M, hOPA1_R445H, and hOPA1_2708del were expressed...
in whole *Drosophila* bodies and detected using anti-HA and anti-myc antibodies. α-Tubulin was used as a loading control. (C) Rescue experiments were conducted to assess the expression of each *hOPA1* variant, including *dOPA1*, in the retina axons of *dOPA1* mutant somatic clones. The sample size is indicated (n). Data are presented as mean ± SEM.
Figure 6. LOF or DN effects of disease mutations in hOPA1.

(A) Impact of each human OPA1 variant on the axon number in the optic nerve of Drosophila as quantified using MeDUsA. (B) Expression of both hOPA1 WT and its respective variants was analyzed in photoreceptors lacking dOPA1, and the number of axons was quantified using the MeDUsA. The sample size is indicated (n). Data are expressed as mean ± SEM. (C) Schematic representation of
interspecies differences in OPA1 interactions and the interchangeability of OPA1 between human and fly.