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A proteomic screen with *Drosophila* Opa1-like identifies Hsc70-5/Mortalin as a regulator of mitochondrial morphology and cellular homeostasis

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ABSTRACT

Mitochondrial morphology is regulated by conserved proteins involved in fusion and fission processes. The mammalian Optic atrophy 1 (OPA1) that functions in mitochondrial fusion is associated with Optic Atrophy and has been implicated in inner membrane cristae remodeling during cell death. Here, we show *Drosophila* Optic atrophy 1-like (Opa1-like) influences mitochondrial morphology through interaction with 'mitochondria-shaping' proteins like Mitochondrial assembly regulatory factor (Marf) and *Drosophila* Mitofilin (dMitofilin). To gain an insight into Opa1-like's network, we delineated bonafide interactors like dMitofilin, Marf, Serine protease High temperature requirement protein A2 (HTRA2), Rhomboid-7 (Rho-7) along with novel interactors such as Mortalin ortholog (Hsc70-5) from *Drosophila* mitochondrial extract. Interestingly, RNAi mediated down-regulation of *hsc70-5* in *Drosophila* wing imaginal disc's peripodial cells resulted in fragmented mitochondria with reduced membrane potential leading to proteolysis of Opa1-like. Increased ecdysone activity induced dysfunctional fragmented mitochondria for clearance through lysosomes, an effect enhanced in *hsc70-5* RNAi leading to increased cell death. Over-expression of *Opa1-like* rescues mitochondrial morphology and cell death in prepupal tissues expressing *hsc70-5* RNAi. Taken together, we have identified a novel interaction between Hsc70-5/Mortalin and Opa1-like that influences cellular homeostasis through mitochondrial fusion.

1. Introduction

With two distinct structural and functional membranes, mitochondria reorganize into various shapes thereby influencing cellular metabolism, energy production and calcium homeostasis within a cell (Rizzuto et al., 2000; Danial and Korsmeyer, 2004). Mitofusins (Mfn-1 and -2) in the outer mitochondrial membrane (OMM) tether opposing mitochondria, following which mitochondrial targeted Phospholipase D (MitoPLD) functions *in trans* on the Cardiolipin present on opposing membranes to generate Phosphatidic acid (PA) leading to fusion of two opposing mitochondria (Choi et al., 2006). On the other hand, Dynamin related protein-1(Drp-1), a large cytosolic GTPase, is essential for scission of mitochondria (Imoto et al., 1998; Karbowski et al., 2002). The diverse inner mitochondrial membrane (IMM) compartmentalizes into boundary membrane and cristae separated by a junction, which harbor a growing family of 'mitochondria-shaping' proteins. Optic Atrophy 1 (OPA1), the only Dynamin family protein identified in the IMM is, however, involved in fusion (Olichon et al., 2002). OPA1 not only promotes mitochondrial fusion by cooperating with Mfn-1 (Cipolat et al., 2004), but also inhibits apoptosis through maintenance of cristae junctions by forming oligomers [preventing mobilization of Cytochrome c (Cyt c) on induction of apoptosis] which are

Abbreviations: OPA1, Optic atrophy 1; Cyt c, Cytochrome c; dMitofilin, *Drosophila* Mitofilin; IR, UAS driven inverted repeats, Serine protease; HTRA2, high temperature requirement protein A2; Marf, mitochondrial assembly regulatory factor; CCCP, Carbonylcyanide m-chlorophenylhydrazone; TMRM, Tetramethyl rhodamine methyl ester; IMM, Inner mitochondrial membrane; OMM, Outer mitochondrial membrane; AMP, Adult Midgut Progenitors of *Drosophila*; TORC, Target of Rapamycin Complex; cAMP-dependent PKA, cyclic Adenosine Mono Phosphate dependent protein kinase A; NMDA, N-methyl-D-aspartate.

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regulated by the function of Rhomboid protease (Frezza et al., 2006). The cristae harbor complexes of respiratory chain components: sites for oxidative phosphorylation. Apart from holding Cyt c within cristae, Mgm1p (OPA1 ortholog in *Saccharomyces cerevisiae*) functions in regulating ATP synthase assembly and cristae morphology (Amutha et al., 2004). The other newly identified IMM protein responsible for cristae junction maintenance is Mitofilin (Rabl et al., 2009).

Mostly OPA1's (and its orthologs) function has been studied in the context of neuronal cell/tissue physiology. For instance, extracellular glutamate levels acting on NMDA receptors yields enhanced calcium influx resulting in death of neurons, which is rescued by OPA1 gain-of-function (Jahani-Asl et al., 2011). Modulation of OPA1 and abnormal cristae structures linked to mitochondrial dysfunction are attributes of neuronal cells in mouse models for Parkinson's and Huntington's disease (Kieper et al., 2010). However, few studies implicate OPA1's role in determining mitochondrial morphogenesis in non-neuronal tissues (McQuibban et al., 2006; Dorn et al., 2011). Heterozygous mutations of *Drosophila* OPA1 (Opa1-like) in whole fly showed decreased life span due to increased Reactive Oxygen Species (ROS) production emphasizing the possible roles for OPA1 in non-neuronal tissues (Tang et al., 2009). Here, we show that Opa1-like maintains mitochondrial morphology in *Drosophila* through regulated interaction with Marf (Mitofusin ortholog in *Drosophila*) and dMitofilin (Mitofilin ortholog in *Drosophila*). We show *in vitro* that loss of membrane potential induces proteolytic cleavage of Opa1-like. Using mass spectrometry, we identify some known and novel interactors of Opa1-like from *Drosophila* larval mitochondrial extracts. *Drosophila* Mortalin ortholog Hsc70-5 (Heat shock 70 kDa protein cognate 5) identified in the proteomic screen, on depletion, yields fragmented, dysfunctional mitochondria which are susceptible to degradation. Treatment of peripodial cells depleted of *hsc70-5*, with ecdysone agonist triggers lysosome-mediated cell death. The severe phenotype of both increased lysosomal activity and cell death caused by depletion of *hsc70-5* is reversed by changing the mitochondrial morphology via manipulation of mitochondrial remodeling proteins.

2. Materials and methods

2.1. Drosophila stocks

Drosophila melanogaster stocks were grown in cornmeal agar bottles and vials in 12/12 h light/dark cycles at 25 °C or as otherwise indicated. UAS-OPA1 (C-terminally Flag tagged) was kindly gifted by Dr. Jongkyeong Chung (KIASK, Korea). *marfDf* [Df(1)dx81, BL#5281, (Park et al., 2009)], *opa1*^{EP} [(P{EPgy2}Opa1-like^{Ey09863}/Cyo), BL#20054, (Park et al., 2009)], *dmitofilinDf* [Df(3R)hh^{E23}/Tm3,Sb], esg-GAL4 (DGRC#104863), UAS-mito-GFP stocks (both on 2nd and 3rd chromosome) and TubGAL4/Tm6B, Tb were obtained from NCBS fly stock facility. UAS-dsRNA constructs were co-expressed together with UAS*Dicer2* to enhance RNAi activity in prepupal tissues (Dietzl et al., 2007).

UAS-IR lines: Opa1-like (KK106290); dMitofilin (KK106757); Hsc70-5 (KK106236); Drp-1 (GD44156); Rho-7 (GD45847); pka-C1 (KK108966). GD and KK lines are from the VDRC (Vienna) collection.

2.2. Imaging

2.2.1. Cells

Hemocytes were stained with primary antibodies, anti-OPA1 (abcam, 1:200) and anti-Mitofilin (abcam, 1:200) and imaged using a confocal microscope [63× objective, 1.4NA on LSM510 Meta; Carl Zeiss (Goyal et al., 2007)].

2.2.2. Drosophila wing imaginal disc

Late 3rd instar larval wing imaginal discs were dissected in 1× PBS (10 mM NaH_2PO_4/Na_2HPO_4, 175 mM NaCl, pH7.4) supplemented with 1.5 mg/ml BSA and 1:100 dilution of Protease inhibitor cocktail (Set III; Merck), further treated with or without 1 mM 20-hydroxyecdysone (Sigma) for 2 h (22 °C). The wing imaginal discs were stained with 100 μM LysoTracker Red DND-99 (Molecular Probes) after 3 min of fixation in 3% formaldehyde [(Polysciences, Inc.); Rusten et al., 2004] along with 16.2 μM Hoechst33342 [Molecular Probes] in 1× PBS for 10 min, rinsed in 1× PBS, mounted in a drop of 1× PBS, and immediately imaged. Images were obtained using 63× objective, 1.4NA on confocal microscope, LSM510.

2.2.3. Cell Death and Quantification

Late 3rd instar larval wing imaginal discs were treated with or without 20-hydroxyecdysone (4 h) in Schneider's insect medium (Invitrogen) and later stained with 16.2 μM Hoechst33342 in 1× PBS followed by 3 μg/ml acridine orange [AO; (5 min)] and manually counted from optical sections derived from confocal microscope (LSM710) images obtained using 20× objective/0.5NA.

The numbers of fixed anti-cleaved active Caspase-3 (1:100, Sigma) positive AMPs of different genotypes of 3rd instar larval guts were manually counted from optical sections. The mean score of the wild-type control group was set as 100%, and the values of the other genotype groups were expressed as percentage compared to wild-type. Images were obtained using 63× objective/1.4NA on confocal microscope, LSM510.

2.3. Mitochondrial morphology and membrane potential assay

Collagen-GAL4 (CgGAL4; Hemocytes) and Tubulin-GAL4 (TubGAL4; Wing Imaginal Disc) were used to express UAS-Mito-GFP (mitochondrial structures inheriting matrix-targeted GFP) for mitochondrial morphology characterization.

Hemocytes were incubated with 10 nM TMRM diluted in SCM (Schneider's insect medium supplemented with 10% non-heat-inactivated fetal bovine serum and 1 μg/ml of bovine pancreatic insulin) with or without 5 μM Oligomycin (15 min) and were exposed to 543 nm light on wide-field microscope [100×, 1.4 NA objective on Nikon TE2000-U inverted microscope equipped with Andor 512B EM-CCD camera (Andor Technology) controlled by Metamorph software (Molecular Devices Corporation)]. Time-lapse images were acquired every 2 s until all mitochondrial structures released TMRM.

Isolated wing imaginal disc were incubated with 25 nM TMRM diluted in 1× PBS (supplemented with 1.5 mg/ml BSA) for over 20 min at room temperature, allowing the dye to equilibrate in cytoplasm and mitochondria (Noguchi et al., 2011, Fig. S2C). Optical sections of mitochondria in peripodial cells ($n \sim 5$–8) were collected using 40×/1.3NA objective (Zoom = 6) LSM710 Meta with 568 nm laser yielding a RAW image. Thereafter, background correction revealed a mitochondrial staining pattern [background corrected image (BCI)]. BCI was converted into a binary (BIN) image by optimizing contrast [Linear contrast (LC) to reassign the gray values from 0 to 255] in BCI, followed by median filtering and thresholding (Th). Then, the BIN image was combined with the BCI to create a masked image (MI) using a Boolean AND operation which increases the number of black pixels and removes low-intensity TMRM pixels from the BCI. Normalized mean fluorescence intensity of TMRM within MI region for mitochondria were measured and compared among genotypes using Metamorph software. Control (Ctrl) wing imaginal discs were incubated with 2 μM CCCP (Sigma) for 20 min to quench mitochondrial membrane potential and subsequently mean fluorescence intensity of TMRM was measured as described above.