The Parkinson’s disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in Drosophila

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Mutations in PTEN-induced kinase 1 (pink1) or parkin cause autosomal-recessive and some sporadic forms of Parkinson’s disease. pink1 acts upstream of parkin in a common genetic pathway to regulate mitochondrial integrity in Drosophila. Mitochondrial morphology is maintained by a dynamic balance between the opposing actions of mitochondrial fusion, controlled by Mitofusin (mfn) and Optic atrophy 1 (opa1), and mitochondrial fission, controlled by drp1. Here, we explore interactions between pink1/parkin and the mitochondrial fusion/fission machinery. Muscle-specific knockdown of the fly homologue of Mfn (Marf) or opa1, or overexpression of drp1, results in significant mitochondrial fragmentation. Mfn-knockdown flies also display altered cristae morphology. Interestingly, knockdown of Mfn or opa1 or overexpression of drp1, rescues the phenotypes of muscle degeneration, cell death, and mitochondrial abnormalities in pink1 or parkin mutants. In the male germline, we also observe genetic interactions between pink1 and the testes-specific mfn homologue fuzzy onion, and between pink1 and drp1. Our data suggest that the pink1/parkin pathway promotes mitochondrial fission and/or inhibits fusion by negatively regulating mfn and opa1 function, and/or positively regulating drp1. However, pink1 and parkin mutant flies show distinct mitochondrial phenotypes from drp1 mutant flies, and flies carrying a heterozygous mutation in drp1 enhance the pink1-null phenotype, resulting in lethality. These results suggest that pink1 and parkin are likely not core components of the drp1-mediated mitochondrial fission machinery. Modification of fusion and fission may represent a novel therapeutic strategy for Parkinson’s disease.

Pink1 and Parkin Mutants Show Defects in Spermatogenesis Suggestive of Defects in Mitochondrial Fission, and Interact Genetically with Fzo and Drp1 in Testes. Both pink1- and parkin-null mutant adults show striking mitochondrial phenotypes in spermatids (10, 11, 13, 22). During spermatogenesis, stem-cell differentiation is followed by mitosis and meiosis with incomplete cytokinesis, creating syn...
cytoplasmic cysts of 64 spermatids (23). Mitochondria undergo significant morphological changes throughout spermatid development. During the “onion stage,” the mitochondria in each spermatid aggregate adjacent to the nucleus and undergo fusion to form a large spherical structure called a nebenkern, which is composed of two intertwined mitochondria. Under phase-contrast microscopy, each spermatid can be identified as containing two giant, adjacent spherical structures: the phase-light nucleus, and the phase-dark nebenkern (Fig. 1A and E). Subsequently, the spermatids begin to elongate, and the nebenkern unfurls to yield two mitochondrial derivatives (the “leaf blade stage”; Fig. 1A and I). These structures are maintained throughout subsequent spermatid elongation such that a cross-section through the sperm tail reveals two mitochondrial derivatives, known as the major and minor, adjacent to the microtubule-based axoneme (Fig. 1A and B) (23). pink1 mutant spermatids (Fig. 1 C, F, and J) showed vacuolated onion-stage nebenkerns, and, in subsequent stages, exhibited only one mitochondrial derivative rather than the normal two seen in WT spermatids (Fig. 1B and I). Similar phenotypes have been observed in parkin mutant testes (Fig. 1D) (22). These results suggest that pink1 and parkin mutants might have defects in mitochondrial dynamics, with pink1 or parkin loss of function reducing mitochondrial fission and/or increasing fusion in spermatids.

To explore this hypothesis, we searched for genetic interactions between pink1 and fzo, the fly Mfn homologue expressed exclusively in testes. Whereas an onion-staged nebenkern is composed of two giant intertwined mitochondria, the fusion defects in fzo-null mutants resulted in an onion-staged nebenkern composed of many small mitochondria. As a consequence of this, the borders of the nebenkern appeared irregular under phase-contrast microscopy (Fig. 1G) (18). When the nebenkern of fzo mutants unfurled at the leaf-blade stage, numerous small phase-dark mitochondria were seen adjacent to a single nucleus (Fig. 1K), rather than two mitochondrial derivatives seen in specimens (Fig. 1I). Double mutants with pink1 and fzo function removed exhibited nebenkerns that were still vacuolated, yet had smooth borders (Fig. 1H). Furthermore, leaf-blade spermatids of pink1/fzo double mutants showed a single elongated mitochondrial derivative (Fig. 1L). These results suggest that, in double mutants, fzo loss-of-function phenotypes are suppressed by pink1 loss of function, with double mutants showing pink1-like phenotypes. These results suggest a strong genetic interaction between pink1 and a fly homologue of mitofusin.

Next, we examined if pink1 and drp1 genetically interact in testes. Flies overexpressing drp1 specifically in testes (TMR-drp1) showed fzo-like phenotypes in a subset of nebenkerns [supporting information (SI) Fig. S1D]. Overexpression of drp1 in the pink1 mutant background resulted in suppression of the vacuolations in nebenkerns in a portion of the flies (Fig. S1 B–G). These results again implicate pink1 in promoting fission and/or inhibiting fusion.

A Balance Between Opposing Fusion and Fission Maintains Mitochondrial Morphology in Drosophila. Drosophila adult indirect flight muscle (hereafter referred to as “muscle”) is an ideal system in which to study mitochondrial dynamics because of numerous large mitochondria that fill the spaces between bundles of well-organized muscle fibers, as visualized on transmission electron microscopy (EM) (Fig. 2D) (10). A similar pattern can be visualized in muscle by fluorescence microscopy by using a version of GFP (mitoGFP) that specifically localizes to mitochondria while simultaneously labeling muscle fibers with phalloidin, which binds to filamentous actin (Fig. 2A). The function of the putative Mfn homologue, Marf, has not been previously characterized. Drosophila Marf shows 47% amino acid identity and 65 to 67% similarity to two human Mfn homologues. As there are no mutations available in Marf, we generated two RNAi constructs targeted to two independent regions of the Marf transcript (the coding region and the untranslated region, respectively). These transgenes were used to carry out tissue-specific silencing using the UAS-Gal4 system (24), and both Marf RNAi transgenes gave identical phenotypes. Whereas ubiquitous knockdown of Marf using tubulin-Gal4 resulted in lethality, muscle-specific knockdown of Marf using either Mei2-Gal4 or 24B-Gal4 resulted in viable adults in which muscles showed mitochondrial fragmentation (i.e., smaller and rounder size), as visualized by mitoGFP and EM (Fig. 2B and E). Abnormal cristae were also observed in these flies (Fig. 2E). These results indicate that Marf is a bona fide regulator of mitochondrial fusion in Drosophila. Similarly, muscle-specific knockdown of opal also resulted in mitochondrial fragmentation (Fig. 4B). Importantly, transgenic flies overexpressing drp1 specifically in muscle showed a similar, albeit weaker, phenotype of mitochondrial fragmentation (Fig. 2 C and F). In addition, overexpression of drp1 in the

![Fig. 1.](image-url)
**Wildtype**

**RNAi-Marf**

**drp1 Overexpr**

**Fig. 2.** Muscle-specific knockdown of Marf and overexpression of drp1 results in abnormal mitochondrial morphology. MitoGFP- (green) and phalloidin-labeled (red) muscle (A–C) and EM images (D–F) from 1- to 2-day-old flies. Compared with control (A and D), both Marf knockdown (B and E) and drp1 overexpression (C and F) result in mitochondrial fragmentation, with Marf knockdown flies also showing cristae irregularities and more severe mitochondrial fragmentation. The borders of mitochondria are marked with white dashed lines. Genotypes: (A and D) F6M/F6; Mef2-Gal4, UAS-mitoGFP+/+; (B and E) w; UAS-RNAi-Marf+/+; Mef2-Gal4, UAS-mitoGFP+/+; (C and F) w; UAS-drp1+/+; Mef2-Gal4, UAS-mitoGFP+/+. Note that, as controls, Mef2-Gal4, UAS-mitoGFP flies show similar mitochondrial phenotypes in backgrounds of w/Y, FM6/F6, or w/Y; UAS-LacZ. Scale bars: 0.5 μm in D–F.

**Wildtype**

**RNAi-Marf**

**drp1 Overexpr**

**p**

**ink1** and **parkin** Genetically Interact with Components of the Mitochondrial Fission–Fusion Machinery in Muscle

Indirect flight muscle from **p**ink1 or **parkin** mutant adults showed severe defects in mitochondrial morphology, including swollen mitochondria with broken cristae, as observed under EM (Fig. 3 J and P) (10–13). **p**ink1 and **parkin** mutants also displayed weak mitoGFP signal compared with (Fig. 3 B, G, and M). In addition, large clumps of intense GFP signal, which appeared beyond the space between muscle fibers as demarcated by phalloidin staining, were also observed. These mitochondrial morphological defects in **p**ink1 mutants could be completely suppressed by muscle-specific overexpression of **p**ink1 (Fig. 3 C) and partially rescued by overexpression of **parkin** (Fig. 3 D).

To test the hypothesis that **p**ink1 and **parkin** regulate mitochondrial dynamics, we searched for genetic interactions between **Marf**/drp1 and **p**ink1/ **parkin**. If loss of **p**ink1 or **parkin** function tips the fusion/fission balance toward fusion, we would expect silencing of **Marf** to suppress **p**ink1/ **parkin** mutant phenotypes. Consistent with this hypothesis, muscle-specific knockdown of **Marf** in the **p**ink1 or **parkin** mutant background resulted in a significant rescue of **p**ink1 and **parkin** mutant phenotypes: mitochondria were no longer elongated, and the intense accumulations of mitoGFP were no longer present (Fig. 3 E, H, and N). In addition, the broken cristae phenotypes observed in **p**ink1 and **parkin** mutants were significantly suppressed (Fig. 3 K and Q). Interestingly, however, most of the mitochondria still appeared fragmented—the phenotype resulting from **Marf** knockdown (compare with Fig. 2 B and E). Similar suppression of mitochondrial defects seen in **p**ink1 mutants could also be observed following muscle-specific knockdown of opa1 (Fig. 4 C–F). Importantly, overexpression of drp1 in the **p**ink1/**parkin** mutant background also resulted in significant suppression of the **p**ink1 or **parkin** mutant phenotypes, with mitochondria displaying drp1 overexpression-like phenotypes (Fig. 3 F, I, L, and R; compare with Fig. 2 C and F). These results suggest that **p**ink1/**parkin** function to promote fission and/or inhibit mitochondrial fusion, and that **Marf**/drp1 is genetically epistatic to **p**ink1/ **parkin**.

Next, we sought to determine if suppression of the mitochondrial morphological defects in **p**ink1 and **parkin** mutants by drp1 overexpression and **Marf** knockdown was functionally significant. Both **p**ink1 and **parkin**-null mutants showed wing posture defects associated with muscle degeneration as a result of extensive cell death (10–12). Remarkably, not only was normal wing posture restored in **p**ink1 and **parkin** mutants by drp1 overexpression or **Marf** knockdown (Fig. 5 A), but cell death (assayed by TUNEL-positive staining) and muscle degeneration (assayed by Toluidine blue staining) were also suppressed (Fig. 5 B–O). These data also suggest that apoptosis in **p**ink1 and **parkin** mutant muscle is secondary to defects in mitochondrial dynamics.

**p**ink1 Mutant Phenotypes Are Distinct from Those of Mitochondrial Dynamics Genes. Because our results indicate that the **p**ink1/**parkin** pathway promotes mitochondrial fission and/or inhibits fusion, we sought to determine if **p**ink1 and **parkin** serve as essential components of the Drp1-dependent mitochondrial fission machinery. If this were the case, we would expect **p**ink1 and drp1 mutants to show similar phenotypes. drp1 mutants were largely lethal, but rare escapers emerged. Muscles from drp1 mutant flies showed elongated mitochondria, but largely homogeneous mitoGFP signals, and no TUNEL-positive staining (Fig. S2 E and F). These observations stand in contrast to those associated with loss of **p**ink1 or **parkin** (Figs. 3 B, G, and M; Fig. 5 E and M). drp1 mutants also showed phenotypes distinct from those of **p**ink1 and **parkin** mutants in testes. Onion-stage nebenkerns of drp1 mutants were large, bizarrely shaped blobs that also contained irregular-shaped phase-light materials distinct from the phase-light nucleus (Fig. S2D). Finally, although some onion-stage nebenkerns from flies overexpressing drp1 showed nebenkerns with irregular borders reminiscent of the fzo mutant phenotype, overexpression of **p**ink1 did not affect nebenkern structure (data not shown). Together, these data suggest that the mitochondrial phenotypes associated with alterations of **p**ink1 and **parkin** are distinct from those of drp1 mutants, supporting the idea that **p**ink1 and **parkin** are not essential components of the canonical fission machinery controlled by drp1.

To further test this hypothesis, we sought to determine if loss of drp1 function could enhance the **p**ink1 mutant phenotype. Interestingly, we were unable to recover any **p**ink1 mutant flies that were heterozygous for each of three independent drp1-null or drp1-strong hypomorphic alleles under normal culturing conditions, whereas we had no difficulty recovering **p**ink1 mutant or drp1 heterozygous flies alone (Fig. S2A). The lethality prohibited us from examining the mitochondria of these animals. However, this striking synthetic lethal interaction between a drp1-null allele and a modest reduction in drp1 function suggests that the phenotype resulting from a complete lack of **p**ink1 function can be further enhanced through reduction of drp1 function. Collectively, these results suggest that **p**ink1 does not strictly function in a linear pathway to only regulate drp1.

**Discussion**

In yeast and mammals, mitochondrial morphology is maintained by a dynamic balance between fusion and fission. In *Drosophila*, although the functions of drp1 and opa1 in regulating mitochondrial morphology are known, the role of the main *mfn* homologue, Marf, was largely uncharacterized. Herein we show that **Marf** knockdown in muscle results in significant mitochondrial fragmentation and abnormal morphology of cristae, thereby indicating that **Marf** is a bona fide pro-fusion molecule. As would be expected for a dynamic opposing action between mitochondrial fusion and fission, overexpression of drp1 leads to similar mitochondrial fragmentation.

Previously, we and others have shown that flies lacking **p**ink1 or **parkin** function show similar mitochondrial phenotypes in the male germline, indirect flight muscle, and dopaminergic neurons (10–12). In these settings, **p**ink1 and **parkin** function in a common genetic pathway to regulate mitochondrial integrity and function (10–12). In this report, we have established that **p**ink1 and **parkin**...
mutants also show similar genetic interactions with molecules involved in mitochondrial dynamics. Specifically, muscle-specific Marf or opa1 knockdown or drp1 overexpression results in significant rescue of mitochondrial morphology phenotypes, and suppression of muscle cell death and degeneration in pink1 and/or parkin mutants. Furthermore, in tests, pink1 also genetically interacts with the testes-specific mfn homologue fzo. In this case, however, whereas loss of pink1 function strongly suppresses fzo mutant phenotypes, the pink1 mutant phenotype is not strongly suppressed. Because Marf is also expressed in testes (19), and may have partially redundant functions with fzo, it remains possible that removal of both Marf and fzo may result in rescue of the pink1 testes phenotype. These results are consistent with those of a recent report (26). Collectively, data from our work and Poole et al. provide compelling evidence that the function of the pink1/parkin pathway is to promote mitochondrial fission and/or inhibit fusion in Droso phila (Fig. 6).

Although the net action of the pink1/parkin pathway is to promote fission and/or inhibit fusion, it seems unlikely that Pink1 and Parkin are core components of the fission–fusion machinery. First, loss of function of key regulators of the mitochondrial dynamics machinery (Marf, opa1, drp1) causes lethality, whereas pink1- and parkin-null mutants are viable. Second, pink1 and parkin mutants show distinct phenotypes from drp1 mutants in both muscle and testes, and pink1 overexpression in testes results in different phenotypes from those caused by loss of fzo function or drp1 overexpression. In addition, as we have shown, pink1 and parkin mutants show synthetic lethality with a heterozygous mutant background (26). One likely possibility is that the pink1/parkin pathway acts in a tight linear pathway to regulate the mitochondrial dynamics machinery, at least for drp1. One likely possibility is that the pink1/parkin pathway regulates additional aspects of mitochondrial function that also impact mitochondrial morphology (Fig. 6).

How might Pink1 and Parkin regulate mitochondrial dynamics at the mechanistic level? Most literature suggests that Pink1 is present in the mitochondrial intermembrane space and may be anchored to
the inner membrane of the mitochondrion (27, 28), although a cytosolic localization of Pink1 has also been noted (29). Parkin, on the other hand, has largely been found located in the cytosol and endoplasmic reticulum (30). As for molecules mediating mitochondrial dynamics, Mfn is a membrane-spanning protein with domains exposed to the intermembrane space and cytosol (31, 32). Drp1 is localized to the outer membrane (33, 34), and in yeast, Drp1 localization to the outer membrane is facilitated by another pro-fission molecule, Fis1 (35). The role of Fis1 in mammals, however, is less clear, and it remains to be seen if Fis1 is involved in regulating fission molecule, Fis1 (35). The role of Fis1 in mammals, however, localization to the outer membrane is facilitated by another pro-fission molecule, Fis1 (35). Drp1 is exposed to the intermembrane space and cytosol (31, 32). Drp1 is localized to the outer membrane (33, 34), and in yeast, Drp1 localization to the outer membrane is facilitated by another pro-fission molecule, Fis1 (35). The role of Fis1 in mammals, however, is less clear, and it remains to be seen if Fis1 is involved in regulating fission in Drosophila. Based on the subcellular localization of these molecules, it is possible that Pink1 may directly phosphorylate Mfn and/or Opal1 to inhibit fusion, or phosphorylate Drp1 or Fis1 to promote fission. Alternatively, Parkin may act on Drp1 and/or Fis1 via non-degradative ubiquitination to facilitate mitochondrial localization of Drp1, or exert its function on Marf via degradative ubiquitination. As pink1 acts upstream of parkin, it is possible that the interface between the pink1/parkin pathway and the mitochondrial dynamics machinery occurs at the level of Parkin. Alternatively, both Pink1 and Parkin could be involved, i.e., with Pink1 acting, directly or indirectly, on the fusion machinery, and Parkin acting on the fission machinery, or vice versa. In any case, our studies suggest that manipulation of mitochondrial dynamics may provide a novel therapeutic target for PD.

Our results and those of Poole et al. suggest a need to investigate whether patients with PD manifest defects in mitochondrial dynamics. Interestingly, defects in mitochondrial morphology have been reported in mice overexpressing α-Synuclein (4). Dominant mutations or increased genetic dosage of α-Synuclein cause inherited forms of PD (36, 37), and α-Synuclein is a major component of Lewy bodies, the characteristic intracytoplasmic inclusions seen in most PD cases, including sporadic cases (1). Thus, it will be interesting to determine whether mitochondrial defects resulting from α-Synuclein overexpression are also mediated by defects in mitochondrial dynamics.
Molecular Biology. To silence Mar, two independent regions in the Mar transcript (coding region and UTR) were independently targeted using a microRNA-based technology (38, 39). To silence opa1, the coding region of opa1 transcript was targeted. PCR products of these microRNA precursors were cloned into pUAST. To silence drp1 and TMR-drp1, the drp1 cDNA (EST clone from Drosophila Genome Research Center, AT04516), was subcloned into each vector. All cloned PCR products were confirmed by DNA sequencing.

Drosophila Genetics and Strains. fzo1, fzo2, and fzo-deficiency (Df(3R)P2O) flies (18) were obtained from Margaret Fuller; drp1F and drp1F flies (21), from Patrik Verstreken and Hugo Bellen; and Me2-Ga4 from Leo Pallanc. pink1F (10) and parkinF (13) were previously described. drp1KG03185, UAS-mitoGFP, and 24B-Ga4 flies were obtained from the Bloomington Drosophila Stock Center. For experiments involving transgenic flies, multiple independent fly lines were generated (Rainbow Transgenic Flies) and tested for each transgene.

Phase-Contrast, Confocal, and Electron Microscopy. For light microscopic analysis of the male germline, testes were dissected from recently eclosed males, squashed in PBS buffer, and imaged using an Olympus BX51 microscope equipped with phase-contrast optics. For analysis of muscle, notums of 1- to 2-day-old adult flies were dissected, fixed in 4% paraformaldehyde, and stained with phalloidin, and indirect muscle fibers were isolated and imaged by a Zeiss LSM5 confocal microscope. For transmission EM, testes and muscle were dissected, fixed in paraformaldehyde/glutaraldehyde, postfixed in osmium tetroxide, dehydrated, and embedded in Epon. Tissue sections 1.5 μm thick were stained with Toluidine Blue. Sections 80 nm thick were stained with uranyl acetate and lead citrate and examined using a JEOL 100C transmission electron microscope (UCLA Brain Research Institute EM Facility). TUNEL assays were carried out using the In Situ Cell Death Detection Kit from Roche.

Note. While this article was in review, Yang et al. published a report (Proc Natl Acad Sci USA 105:7070–7075) suggesting that pink1 interacts with drp1, f1st, and opa1, findings that are consistent with this work.

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