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

Hansong Deng*, Mark W. Dodson*†, Haixia Huang‡, and Ming Guo*†§¶‖

Departments of *Neurology and §Pharmacology and ¶Brain Research Institute, David Geffen School of Medicine, and †Molecular Biology Institute, University of California, Los Angeles, CA 90095; and ‡Division of Biology, California Institute of Technology, Pasadena, CA 91125

Edited by Douglas C. Wallace, University of California Irvine College of Medicine, Irvine, CA, and accepted by the Editorial Board July 30, 2008 (received for review April 25, 2008)

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.

mitofusin | *drp1* | *opa1* | *parkin-pink1*

Parkinson's disease (PD), the second most common neurodegenerative disorder, is characterized by degeneration of dopaminergic neurons in the midbrain (1). Although the exact cause of PD is unclear, mitochondrial toxins such as 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) can selectively destroy dopaminergic neurons and cause clinical features similar to PD (2, 3). Moreover, mitochondrial respiratory dysfunction also occurs in sporadic PD (4). The most compelling evidence for a mitochondrial etiology of PD, however, derives from the study of genes mediating familial forms of the disease (4, 5). Mutations in *PTEN-induced kinase 1* (*Pink1*; *PARK6*), which encodes a serine–threonine kinase localized to mitochondria, and *parkin* (*PARK2*), which encodes a RING finger-containing E3 ubiquitin ligase, have been found in recessively inherited and sporadic PD cases (6–9). Previously, we and others have reported that *Drosophila pink1* and *parkin* function in the same genetic pathway, with *pink1* acting upstream of *parkin*, to regulate mitochondrial integrity in testes, muscle, and dopaminergic neurons (10–12). Flies lacking *pink1* or *parkin* function are viable and show muscle degeneration and TUNEL staining, indicative of cell death (10–13). Subsequent studies have shown that *parkin* can suppress mitochondrial defects caused by *pink1* knockdown in cultured human cells (14), and mitochondrial dysfunction also occurs in PD cases with *pink1* or *parkin* mutations (4). An understanding of how mutations in *pink1* and *parkin* cause mitochondrial dysfunction may lead to the development of novel therapeutic agents for PD.

Mitochondria undergo dynamic changes in morphology through fusion and fission. Although these processes have been extensively studied in yeast, only recently have molecules regulating mitochondrial dynamics been identified in mammals (15–17). These include the homologous GTPases Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2), which mediate fusion of the mitochondrial outer membrane, as well as Optic atrophy 1 (Opa1), a GTPase required for fusion of the inner membrane. Mitochondrial fission, conversely, requires Dynamin-related protein 1 (Drp1), which is also a GTPase (15–17) (Fig. 6). The *Drosophila melanogaster* genome encodes two homologues of Mfn, one being Fuzzy onion (Fzo), the first identified protein regulating mitochondrial dynamics in metazoans (18). The expression of Fzo is restricted to the testes, and mutations in *fzo* cause mitochondrial fusion defects in testes and male sterility (18). The second *Drosophila* Mfn homologue is a largely uncharacterized protein known as Mitochondrial assembly regulatory factor (Marf; CG3869), which is expressed in germline and somatic cells (19). The *Drosophila* genome also encodes single homologues of *opa1* (20) and *drp1* (21), both of which have been shown to function in mitochondrial dynamics in flies. Studies in yeast and mammals have demonstrated that defects in mitochondrial fission can be ameliorated by mutations in genes required for mitochondrial fusion and vice versa, indicating that a balance between fusion and fission is required to maintain proper mitochondrial morphology.

Careful studies of *pink1* and *parkin* mutant phenotypes in testes (as detailed later) suggest the possibility that *pink1* and *parkin* might regulate mitochondrial dynamics. To test this hypothesis, we examined genetic interactions between *pink1* or *parkin* and genes required for mitochondrial fusion and fission in *Drosophila*. Our data suggest that the net action of the *pink1*/*parkin* pathway is to promote mitochondrial fission and/or inhibit fusion.

Results

***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-

Author contributions: H.D., M.W.D., and M.G. designed research; H.D. and M.W.D. performed research; H.D., M.W.D., and M.G. analyzed data; H.H. contributed new reagents/analytic tools; and M.G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

‖To whom correspondence should be addressed. E-mail: mguo@mednet.ucla.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0803998105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA

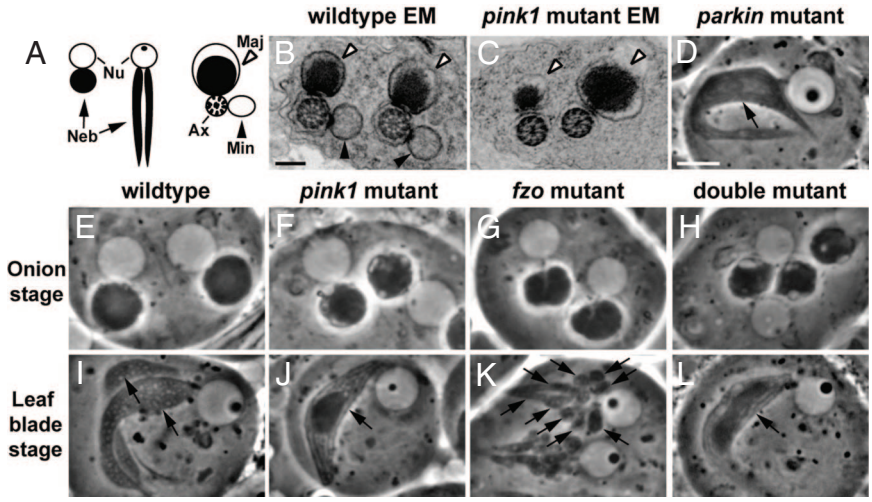

Fig. 1. *pink1* and *parkin* mutants show phenotypes in spermatogenesis suggestive of defects in mitochondrial fission; *pink1* genetically interacts with *fzo* in testes. (*A*) Schematic of an onion-staged (*left*) and a leaf-blade-staged (*middle*) spermatid showing the nucleus (Nu) and nebenkern (Neb, arrow), and a cross-section through a spermatid tail immediately before individualization (*right*). In these spermatid tails, an axoneme (Ax) is associated with two mitochondrial derivatives, the major (Maj, open arrowhead) and minor (Min, filled arrowhead). (*B* and *C*) EM images of WT (*B*) and *pink1*-mutant (*C*) spermatid tails show the presence of only the major mitochondrial derivative, but not the minor derivative in *pink1* mutants. (*D*) A *parkin*-mutant spermatid during the leaf-blade stage, as with a similarly staged *pink1*-mutant spermatid (*J*), contains only one mitochondrial derivative compared with two seen in WT specimens (*I*). (*E–L*) Genetic interactions between *pink1* and *fzo*. During the onion stage, *pink1* mutants show vacuolations of the nebenkern (*F*), which are not seen in WT specimens (*E*). $fzo^1/Df(3R)P2O$ mutants have nebenkerns with irregular borders (*G*). Similar phenotypes are also seen in fzo^1/fzo^2 and fzo^1-homozygous testes (data not shown). Double mutants removing both *pink1* and *fzo* function exhibit nebenkerns that have smooth borders, yet are still vacuolated (*H*). (*I*) WT leaf-blade–staged spermatids have two mitochondrial derivatives. During the leaf-blade stage, $fzo^1/Df(3R)P2O$ spermatids show fragmented mitochondria (*K*). Double mutants with *pink1* and *fzo* removed show a single mitochondrial derivative, the *pink1* mutant-like phenotype (*L*). Note that the cell membranes encapsulating spermatids often contain multiple spermatids. This is a result of the disruption of cytoplasmic bridges connecting spermatids during sample preparation, and is not a phenotype. Genotypes: (*B, E*, and *I*) *w*/Y; (*C, F*, and *J*) *w* $pink1^5$ (*G* and *K*) *w*/Y; *e* $fzo^1/Df(3R)P2O$; (*H* and *L*) *w* $pink1^5$/Y; *e* $fzo^1/Df(3R)P2O$; Scale bars: 200 nm in *B* and *C*; 10 μm in *D–L*.

cytial 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. 1 *A* and *E*). Subsequently, the spermatids begin to elongate, and the nebenkern unfurls to yield two mitochondrial derivatives (the "leaf blade stage"; Fig. 1 *A* 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. 1 *A* 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. 1 *B* and *I*). Similar phenotypes have been observed in *parkin* mutant testes (Fig. 1*D*) (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. 1*G*) (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. 1*K*), rather than two mitochondrial derivatives seen in specimens (Fig. 1*I*). Double mutants with *pink1* and *fzo* function removed exhibited nebenkerns that were still vacuolated, yet had smooth borders (Fig. 1*H*). Furthermore, leaf-blade spermatids of *pink1*/*fzo* double mutants showed a single elongated mitochondrial derivative (Fig. 1*L*). 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. S1*D*]. 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 it contains numerous large mitochondria that fill the spaces between bundles of well-organized muscle fibers, as visualized on transmission electron microscopy (EM) (Fig. 2*D*) (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. 2*A*). 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 Mef2-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. 2 *B* and *E*). Abnormal cristae were also observed in these flies (Fig. 2*E*). These results indicate that *Marf* is a bona fide regulator of mitochondrial fusion in *Drosophila*. Similarly, muscle-specific knockdown of *opa1* also resulted in mitochondrial fragmentation (Fig. 4*B*). 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

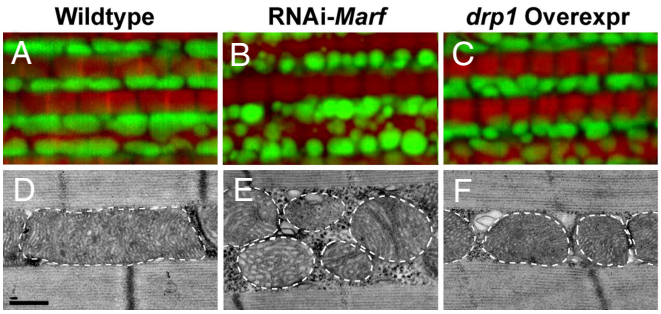

Fig. 2. Muscle-specific knockdown of *Marf* and overexpression of *drp1* results in abnormal mitochondrial morphology. MitoGFP- (green) and phalloidin-labeled (red) muscle (*A–C*) and EM images (*D–F*) from 1- to 2-day-old flies. Compared with control (*A* and *D*), both *Marf* knockdown (*B* and *E*) 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*) FM6/Y; 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/Y, or *w*/Y; UAS-*LacZ*. Scale bars: 0.5 μm in *D–F*.

background of muscle-specific *Marf* knockdown resulted in lethality. Together, these data indicate that mitochondrial morphology in *Drosophila*, like that in yeast and mammals, is regulated by a balance between the activities of canonical regulators of mitochondrial fusion–fission dynamics.

***pink1* and *parkin* Genetically Interact with Components of the Mitochondrial Fission–Fusion Machinery in Muscle.** Indirect flight muscle from *pink1* 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, 25). *pink1* 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 phenotypes in *pink1* mutants could be completely suppressed by muscle-specific overexpression of *pink1* (Fig. 3*C*) and partially rescued by overexpression of *parkin* (Fig. 3*D*).

To test the hypothesis that *pink1* and *parkin* regulate mitochondrial dynamics, we searched for genetic interactions between *Marf*/*drp1* and *pink1*/*parkin*. If loss of *pink1* or *parkin* function tips the fusion/fission balance toward fusion, we would expect silencing of *Marf* to suppress *pink1*/*parkin* mutant phenotypes. Consistent with this hypothesis, muscle-specific knockdown of *Marf* in the *pink1* or *parkin* mutant background resulted in a significant rescue of *pink1* 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 *pink1* 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 *pink1* mutants could also be observed following muscle-specific knockdown of *opa1* (Fig. 4 *C–F*). Importantly, overexpression of *drp1* in the *pink1*/*parkin* mutant background also resulted in significant suppression of the *pink1* 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 *pink1*/*parkin* function to promote fission and/or inhibit mitochondrial fusion, and that *Marf*/*drp1* is genetically epistatic to *pink1*/*parkin*.

Next, we sought to determine if suppression of the mitochondrial morphological defects in *pink* and *parkin* mutants by *drp1* overexpression and *Marf* knockdown was functionally significant. Both *pink1*- and *parkin*-null mutants showed wing posture defects associated with muscle degeneration as a result of extensive cell death (10–13). Remarkably, not only was normal wing posture restored in *pink1* 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 *pink1* and *parkin* mutant muscle is secondary to defects in mitochondrial dynamics.

***pink1* Mutant Phenotypes Are Distinct from Those of Mitochondrial Dynamics Genes.** Because our results indicate that the *pink1*/*parkin* pathway promotes mitochondrial fission and/or inhibits fusion, we sought to determine if Pink1 and Parkin serve as essential components of the Drp1-dependent mitochondrial fission machinery. If this were the case, we would expect *pink1* and *drp1* mutants to show similar phenotypes. *drp1* mutants were largely lethal, but rare escapers emerged. Muscles from *drp1* mutant fly escapers 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 *pink1* or *parkin* (Figs. 3 *B*, *G*, and *M*; Fig. 5 *E* and *M*). *drp1* mutants also showed phenotypes distinct from those of *pink1* and *parkin* mutants in testes. Onion-staged nebenkerns of *drp1* mutants were large, bizarrely shaped blobs that also contained irregular-shaped phase-light materials distinct from the phase-light nucleus (Fig. S2*D*). Finally, although some onion-stage nebenkerns from flies overexpressing *drp1* showed nebenkerns with irregular borders reminiscent of the *fzo* mutant phenotype, overexpression of *pink1* did not affect nebenkern structure (data not shown). Together, these data suggest that the mitochondrial phenotypes associated with alterations of *pink1* and *parkin* are distinct from those of *drp1* mutants, supporting the idea that *pink1* 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 *pink1* mutant phenotype. Interestingly, we were unable to recover any *pink1* 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 *pink1* mutant or *drp1* heterozygous flies alone (Fig. S2*A*). The lethality prohibited us from examining the mitochondria of these animals. However, this striking synthetic lethal interaction between a *pink1*-null allele and a modest reduction in *drp1* function suggests that the phenotype resulting from a complete lack of *pink1* function can be further enhanced through reduction of *drp1* function. Collectively, these results suggest that *pink1* 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 *pink1* or *parkin* function show similar mitochondrial phenotypes in the male germline, indirect flight muscle, and dopaminergic neurons (10–12). In these settings, *pink1* and *parkin* function in a common genetic pathway to regulate mitochondrial integrity and function (10–12). In this report, we have established that *pink1* and *parkin*

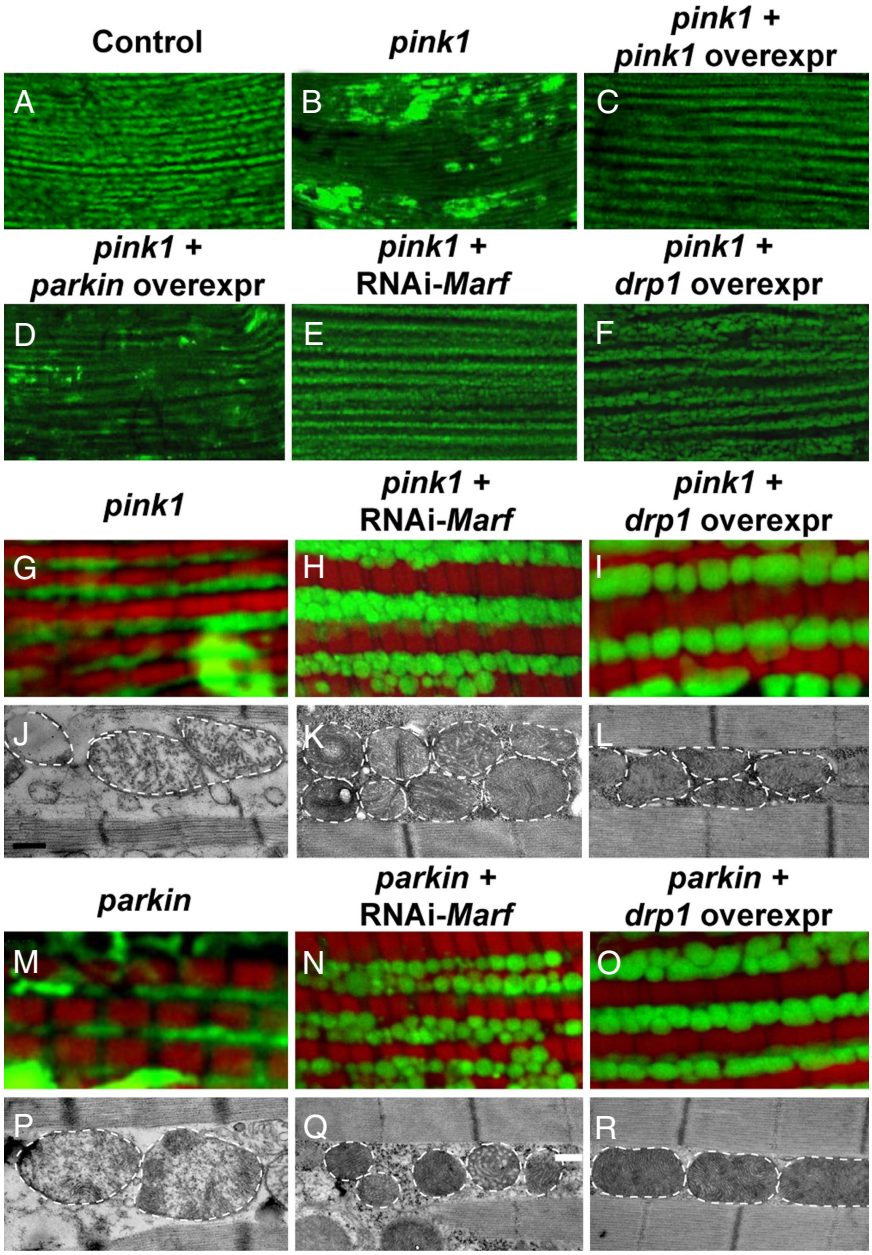

Fig. 3. Both *Marf* knockdown and *drp1* overexpression suppress mitochondrial phenotypes in *pink1*- and *parkin*-mutant muscle. MitoGFP-labeled muscle at low magnification (*A–F*), mitoGFP/phalloidin double-labeled muscle at higher magnification (*G–I* and *M–O*), and EM images (*J–L* and *P–R*) from 1- to 2-day-old flies. Both *pink1* (*B* and *G*) and *parkin* (*M*) mutants show weakened mitoGFP labeling and clumps of intense mitoGFP signal. In *pink1* mutants, these phenotypes can be completely suppressed by *pink1* overexpression (*C*) and partially rescued by *parkin* overexpression (*D*). Moreover, *pink1* and *parkin* phenotypes can also be suppressed by knockdown of *Marf* (*E, H,* and *N*) or by *drp1* overexpression (*F, I,* and *O*). At the EM level, *pink1* and *parkin* mutants show broken cristae (*J* and *P*), which can be suppressed by *Marf* knockdown (*K* and *Q*) or *drp1* overexpression (*L* and *R*). However, *pink1*- and *parkin*-mutant flies with *Marf* knockdown (*K* and *Q*) still show significant mitochondrial fragmentation and cristae abnormalities reminiscent of those seen in *Marf*-knockdown flies alone (Fig. 2*E*). The borders of mitochondria are marked with white dashed lines. Genotypes: (*A*) FM6/Y; (*B, G,* and *J*) *w pink1^{5} f*/Y; Mef2-Gal4, UAS-mitoGFP/+; (*C*) *w pink1^{5} f*/Y; Mef2-Gal4, UAS-*pink1*/+; UAS-mitoGFP/+; (*D*) *w pink1^{5} f*/Y; UAS-*parkin*/+; Mef2-Gal4, UAS-mitoGFP/+; (*E, H,* and *K*) *w pink1^{5} f*/Y; Mef2-Gal4, UAS-mitoGFP/UAS-RNAi-*Marf*; (*F, I,* and *L*) *w pink1^{5} f*/Y; Mef2-Gal4, UAS-mitoGFP/UAS-*drp1*; (*M* and *P*) *w*/Y; UAS-mitoGFP/+; 24B-Gal4 *park^{25}*/*park^{25}*; (*N* and *Q*) *w*/Y; UAS-mitoGFP/UAS-RNAi-*Marf;* 24B-Gal4 *park^{25}*/*park^{25}*; (*O* and *R*) *w*/Y; UAS-mitoGFP/UAS-*drp1*; 24B-Gal4 *park^{25}*/*park^{25}*. Scale bars: 0.5 μm in *J–L* and *P–R*.

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 testes, *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 *Drosophila* (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 mutation in *drp1* (26). Because a modest reduction in *drp1* levels can further worsen the phenotype as a result of compete absence of *pink1* or *parkin* function, it seems unlikely that the *pink1*/*parkin* pathway acts in a strict 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

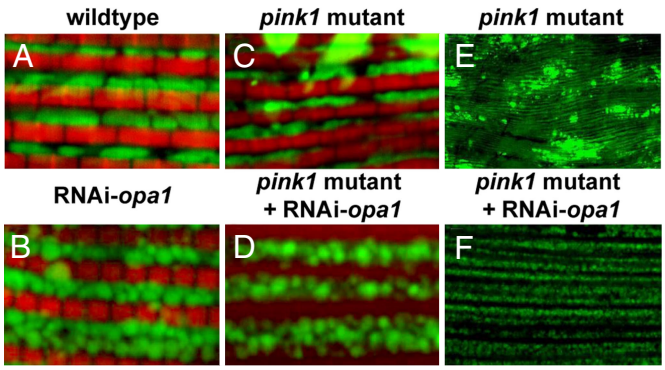

Fig. 4. Muscle-specific *opa1* knockdown results in mitochondrial fragmentation and suppression of mitochondrial defects observed in *pink1* mutants. Muscle from 1- to 2-day-old flies labeled with mitoGFP (green) and phalloidin (red) at high magnification (*A–D*) or labeled with mitoGFP (green) at low magnification. Compared with control (*A*), *opa1* knockdown results in smaller and rounder mitochondria (*B*), similar to what is observed in *Marf* knockdown (Fig. 2*B*). However, we note that the borders of *opa1* knockdown mitochondria appear fuzzy, whereas those of *Marf* knockdown do not. Compared with *pink1* mutants alone (*C* and *E*), *opa1* knockdown in *pink1* mutants displays striking rescue of mitochondrial morphology (*D* and *F*), Genotypes: (*A*) FM6/Y; Mef2-Gal4, UAS-mitoGFP/+; (*B*) *w*; UAS-RNAi-*opa1*/+; Mef2-Gal4, UAS-mitoGFP/+; (*C* and *E*) *w pink1*^{5} *f*/Y; Mef2-Gal4, UAS-mitoGFP/+; (*D* and *F*) *w pink1*^{5} *f*/Y; Mef2-Gal4, UAS-mitoGFP/UAS-RNAi-*opa1*.

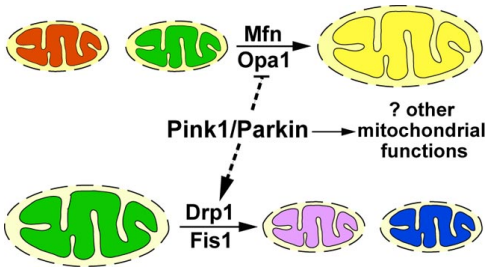

Fig. 6. Interactions of *pink1 and parkin* with genes regulating mitochondrial fusion and fission. Mitochondrial fusion requires Mfn and Opa1, and mitochondrial fission requires Drp1 and Fis1. Pink1 and Parkin promote fission and/or inhibit fusion, either directly or indirectly (dashed lines). In addition, Pink1 and Parkin are unlikely to be components of the canonical pathways regulating mitochondrial dynamics. Rather, Pink1 and Parkin may regulate other mitochondrial functions that also impact mitochondrial integrity.

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 in *Drosophila*. Based on the subcellular localization of these molecules, it is possible that Pink1 may directly phosphorylate Mfn and/or Opa1 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 vise 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.

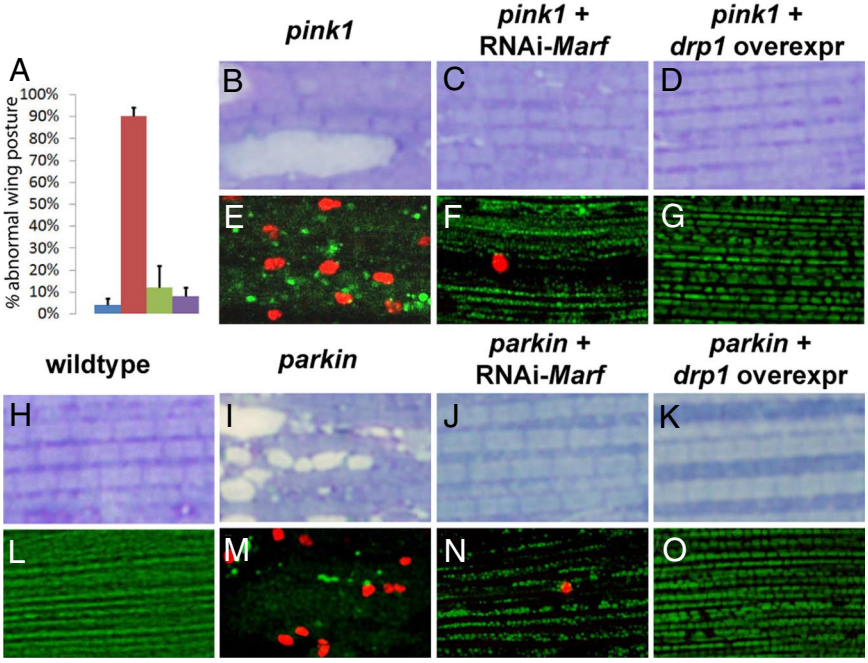

Fig. 5. *Marf* knockdown or *drp1* overexpression in muscle results in functional rescue of *pink1*- and *parkin*-mutant phenotypes. (*A*) Abnormal wing posture seen in *pink1* mutants (red) compared with WT specimens (blue) is significantly suppressed by *Marf* knockdown (green) or *drp1* overexpression (purple) in muscle. The *y* axis denotes the percentile of flies showing ''upheld'' or ''downheld'' wings, an indication of muscle degeneration. Toluidine blue (*B–D* and *H–K*) or TUNEL/mitoGFP stainings (*E–G* and *L–O*) of muscles. Compared with WT specimens (*H*), muscles from *pink1* and *parkin* mutants show vacuolations indicative of degeneration (*B* and *I*). These phenotypes can be suppressed by *Marf* knockdown (*C* and *J*) or *drp1* overexpression (*D* and *K*). WT muscle does not show any TUNEL-positive cell death (*L*), whereas *pink1* and *parkin* mutants show prominent TUNEL-positive staining (red; *E* and *M*). These phenotypes are suppressed by expression of RNAi-*Marf* (*F* and *N*) or *drp1* (*G* and *O*). Genotypes are as shown in Fig. 3.

Materials and Methods

Molecular Biology. To silence *Marf*, two independent regions in the *Marf* 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 generate UAS-*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.** fzo^1, fzo^2, and *fzo*-deficiency (*Df(3R)P2O*) flies (18) were obtained from Margaret Fuller; $drp1^1$ and $drp1^2$ flies (21), from Patrik Verstreken and Hugo Bellen; and Mef2-Gal4 from Leo Pallanck. $pink1^5$ (10) and $parkin^{25}$ (13) were previously described. $drp1^{KG03185}$, UAS-mitoGFP, and 24B-Gal4 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, fis1*, and *opa1*, findings that are consistent with this work.

ACKNOWLEDGMENTS. We thank G. Mohiddin Lone for generating one construct; Margaret Fuller, Hugo Bellen, and Leo Pallanck for fly stocks; Frank Laski for his microtome; Bruce Hay for comments on the manuscript; and the Guo lab members for discussions. This work was supported by the a National Institute of Health NRSA predoctoral fellowship (to M.W.D.), National Institute of Health Grants K02 and R01 (to M.G.), the Alfred P. Sloan Foundation, American Parkinson's Disease Association, Glenn Family Foundation, and the McKnight Endowment Fund for Neuroscience (to M.G.).

1. Dauer W, Przedborski S (2003) Parkinson's disease: Mechanisms and models. *Neuron* 39:889–909.
2. Langston JW, *et al.* (1983) Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* 219:979–980.
3. Sherer TB, Betarbet R, Greenamyre JT (2002) Environment, mitochondria, and Parkinson's disease. *Neuroscientist* 8:192–197.
4. Dodson MW, Guo M (2007) Pink1, parkin, DJ-1 and mitochondrial dysfunction in Parkinson's disease. *Curr Opin Neurobiol* 17:331–337.
5. Wood-Kaczmar A, Gandhi S, Wood NW (2006) Understanding the molecular causes of Parkinson's disease. *Trends Mol Med* 12:521–528.
6. Kitada T, *et al.* (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392:605–608.
7. Valente EM, *et al.* (2004) Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* 304:1158–1160.
8. Bonifati V, *et al.* (2005) Early-onset parkinsonism associated with PINK1 mutations: Frequency, genotypes, and phenotypes. *Neurology* 65:87–95.
9. Valente EM, *et al.* (2004) PINK1 mutations are associated with sporadic early-onset parkinsonism. *Ann Neurol* 56:336–341.
10. Clark IE, *et al.* (2006) Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature* 441:1162–1166.
11. Park J, *et al.* (2006) Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. *Nature* 441:1157–1161.
12. Yang Y, *et al.* (2006) Mitochondrial pathology and muscle and dopaminergic neuron degeneration caused by inactivation of Drosophila Pink1 is rescued by Parkin. *Proc Natl Acad Sci USA* 103:10793–10798.
13. Greene JC, *et al.* (2003) Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. *Proc Natl Acad Sci USA* 100:4078–4083.
14. Exner N, *et al.* (2007) Loss-of-function of human PINK1 results in mitochondrial pathology and can be rescued by parkin. *J Neurosci* 27:12413–12418.
15. Okamoto K, Shaw JM (2005) Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. *Annu Rev Genet* 39;503–536.
16. Detmer SA, Chan DC (2007) Functions and dysfunctions of mitochondrial dynamics. *Nat Rev Mol Cell Biol* 8:870–879.
17. Hoppins S, Lackner L, Nunnari J (2007) The machines that divide and fuse mitochondria. *Annu Rev Biochem* 76:751–780.
18. Hales KG, Fuller MT (1997) Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. *Cell* 90:121–129.
19. Hwa JJ, *et al.* (2002) Differential expression of the Drosophila mitofusin genes fuzzy onions (fzo) and dmfn. *Mech Dev* 116:213–216.
20. Yarosh W, *et al.* (2008) The molecular mechanisms of OPA1-mediated optic atrophy in Drosophila model and prospects for antioxidant treatment. *PLoS Genet* 4:e6.
21. Verstreken P, *et al.* (2005) Synaptic mitochondria are critical for mobilization of reserve pool vesicles at Drosophila neuromuscular junctions. *Neuron* 47:365–378.
22. Riparbelli MG, Callaini G (2006) The Drosophila parkin homologue is required for normal mitochondrial dynamics during spermiogenesis. *Dev Biol* 303:108–120.
23. Fuller MT (1993) Spermatogenesis. *The Development of Drosophila Melanogaster*, eds Martinez-Arias A, Bate M (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), pp 71–147.
24. Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118:401–415.
25. Pesah Y, *et al.* (2004) Drosophila parkin mutants have decreased mass and cell size and increased sensitivity to oxygen radical stress. *Development* 131:2183–2194.
26. Poole AC, *et al.* (2008) The PINK1/Parkin pathway regulates mitochondrial morphology. *Proc Natl Acad Sci USA* 105:1638–1643.
27. Silvestri L, *et al.* (2005) Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. *Hum Mol Genet* 14:3477–3492.
28. Gandhi S, *et al.* (2006) PINK1 protein in normal human brain and Parkinson's disease. *Brain* 129:1720–1731.
29. Beilina A, *et al.* (2005) Mutations in PTEN-induced putative kinase 1 associated with recessive parkinsonism have differential effects on protein stability. *Proc Natl Acad Sci USA* 102:5703–5708.
30. Shimura H, *et al.* (1999) Immunohistochemical and subcellular localization of Parkin protein: Absence of protein in autosomal recessive juvenile parkinsonism patients. *Ann Neurol* 45:668–672.
31. Rapaport D, *et al.* (1998) Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in Saccharomyces cerevisiae. *J Biol Chem* 273:20150–20155.
32. Rojo M, *et al.* (2002) Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo. *J Cell Sci* 115:1663–1674.
33. Bleazard W, *et al.* (1999) The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast. *Nat Cell Biol* 1:298–304.
34. Frank S, *et al.* (2001) The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. *Dev Cell* 1:515–525.
35. Mozdy AD, McCaffery JM, Shaw JM (2000) Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. *J Cell Biol* 151:367–380.
36. Polymeropoulos MH, *et al.* (1997) Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science* 276:2045–2047.
37. Singleton AB, *et al.* (2003) α-Synuclein locus triplication causes Parkinson's disease. *Science* 302:841.
38. Chen CH, *et al.* (2007) A synthetic maternal-effect selfish genetic element drives population replacement in Drosophila. *Science* 316:597–600.
39. Ganguly A, Feldman RM, Guo M (2008) Ubiquilin antagonizes presenilin and promotes neurodegeneration in Drosophila. *Hum Mol Genet* 17:293–302.