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Loss-of-Function Analysis Suggests That *Omi/HtrA2* Is Not an Essential Component of the *pink1/parkin* Pathway *In Vivo*

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Recently, a mutation in the mitochondrial protease Omi/HtrA2, G399S, was found in sporadic Parkinson's disease (PD) patients, leading to the designation of *Omi/HtrA2* as PD locus 13 (*PARK13*). G399S reportedly results in reduced Omi protease activity. *In vitro* studies have suggested that *Omi/HtrA2* acts downstream of *PINK1*, mutations in which mediate recessive forms of PD. We, as well as other, have previously shown that the *Drosophila* homologs of the familial PD genes, *PINK1* (*PARK6*) and *PARKIN* (*PARK2*), function in a common genetic pathway to regulate mitochondrial integrity and dynamics. Whether *Omi/HtrA2* regulates mitochondrial integrity and whether it acts downstream of *PINK1 in vivo* remain to be explored. Here, we show that *Omi/HtrA2* null mutants in *Drosophila*, in contrast to *pink1* or *parkin* null mutants, do not show mitochondrial morphological defects. Extensive genetic interaction studies do not provide support for models in which *Omi/HtrA2* functions in the same genetic pathway as *pink1*, or carries out partially redundant functions with *pink1*, at least with respect to regulation of mitochondrial integrity and dynamics. Furthermore, Omi/HtrA2 G399S retains significant, if not full, function of Omi/HtrA2, compared with expression of protease-compromised versions of the protein. In light of recent findings showing that G399S can be found at comparable frequencies in PD patients and healthy controls, we do not favor a hypothesis in which *Omi/HtrA2* plays an essential role in PD pathogenesis, at least with respect to regulation of mitochondrial integrity in the *pink1/parkin* pathway.

Key words: **Parkinson's disease; mitochondrial protease; Omi/HtrA2; Pink1; genetic interactions; *Drosophila***

Introduction

Parkinson's disease (PD) is characterized by degeneration of nigrostriatal dopaminergic neurons in the midbrain (Dauer and Przedborski, 2003), and genetic forms of the disease have provided insight into PD pathogenesis (Hardy et al., 2006). Mutations in *PINK1* (*PARK6*), a nuclear gene encoding a mitochondrial serine-threonine kinase, and *PARKIN* (*PARK2*) cause recessively inherited forms of PD/parkinsonism (Kitada et al., 1998; Valente et al., 2004). *Drosophila* homologs of *PINK1* and *PARKIN* act in a common genetic pathway (Clark et al., 2006; Park et al., 2006; Yang et al., 2006) to promote mitochondrial fission and/or inhibit mitochondrial fusion in multiple tissues, including dopaminergic neurons (Deng et al., 2008; Poole et al., 2008; Yang et al., 2008). Consistent with findings in *Drosophila*, patients with *PINK1* or *PARKIN* mutations have indistinguishable clinical features, and also show mitochondrial defects (Ibáñez et al., 2006; Dodson and Guo, 2007). Recent studies also suggest that *PINK1* and *PARKIN* regulate mitochondrial functions in mammals (Exner et al., 2007; Gautier et al., 2008; Piccoli et al., 2008; Wood-Kaczmar et al., 2008).

Omi/HtrA2 encodes a serine protease localized to mitochondrial intermembrane space. Although overexpression of *Omi/HtrA2* leads to apoptosis after its release into the cytosol (for review, see Vande Walle et al., 2008), mice lacking *Omi/HtrA2* or mice harboring a mutation in *Omi/HtrA2* disrupting protease function (Jones et al., 1993, 2003) show loss of nondopaminergic neurons in the striatum, but not loss of apoptosis (Rathke-Hartlieb et al., 2002; Martins et al., 2004). These studies underscore the importance of studying the *in vivo* functions of *Omi/HtrA2* using loss-of-function studies.

Recent reports have suggested links between *Omi/HtrA2* and PD (Strauss et al., 2005; Bogaerts et al., 2008). One mutation in Omi/HtrA2, G399S, was identified in sporadic PD patients and

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reportedly impairs activation of protease activity. In addition, Omi/HtrA2 can physically bind to PINK1 *in vitro*, and Omi/HtrA2 can be phosphorylated by a serine-threonine kinase, p38, with this phosphorylation being dependent on *PINK1*. Furthermore, substitution of a putative *PINK1*-dependent phosphorylation site with a nonphosphorylatable moiety (S400A) markedly reduced protease activity (Plun-Favreau et al., 2007). Thus, it has been suggested that *Omi/HtrA2* functions downstream of *PINK1*, with PINK1 positively regulating Omi/HtrA2 (Plun-Favreau et al., 2007). Based on these intriguing links between *Omi/HtrA2* and PD, *Omi/HtrA2* was recently designated as *Parkinson disease-13* locus (*PARK13*).

However, two recent human genetic studies report no association of *Omi/HtrA2* with PD (Ross et al., 2008; Simón-Sánchez and Singleton, 2008), with the G399S allele detected in both PD patients and healthy controls at a similar frequency. Because of these conflicting results, it is unclear whether *Omi/HtrA2* acts as a true PD gene and whether it performs a major function downstream of *PINK1*. Resolution of these questions is crucial for understanding PD pathogenesis. Studies on the endogenous function of *Omi/HtrA2* as it relates to *PINK1* function are required to address these questions. Here, we report studies on loss-of-function and disease-associated mutants of *Drosophila omi*, and the results of extensive genetic interaction studies between *pink1* and *omi*.

Materials and Methods

Molecular biology. To generate UAS-*omi*, GMR-*omi*, and TMR-*omi*, the *omi* cDNA (EST clone from *Drosophila* Genome Research Center, AT14262) was subcloned into each vector (Brand and Perrimon, 1993; Hay et al., 1994; Huh et al., 2004). To generate *Drosophila* Omi mutants analogous to human Omi/HtrA2 mutations, S276C, S306A, G399S, and S400A, site-specific mutagenesis of S236C, S266A, G363S, and S364A of Omi was carried out, and the altered cDNAs were subcloned into pUASt and pTMR vectors, respectively. A fly mutation corresponding to the human PINK1G309D mutation, Pink1G426D, was generated by site-specific mutagenesis (made by I.E.C. and Atsushi Yamaguchi, Novato, CA). To silence *omi*, the *omi* transcript corresponding to the coding region was targeted using a microRNA-based technology (Chen et al., 2007) (Ganguly et al., 2008), and PCR products of these microRNA precursors were cloned into pUASt. To generate CaSpeR-*pink1G426D*, site-specific mutagenesis in the backbone of CaSpeR-*pink1* was performed and the product subcloned into pCaSpeR4 vector. To generate CaSpeR-*omi*, a 2.5 kb PCR product, generated using the following primers, was subcloned into pCaSpeR4 vector (a gift from Nic Tapon, London, UK): 5′ primer: CAACTCGAGGAAGTACATTGGGCGGGTC; 3′ primer: GGGACTAGTGGGTTTGTCAGCGATTTC. All cloned PCR products were confirmed by DNA sequencing.

Drosophila genetics and strains. EMS mutations were recovered using the *Drosophila* Tilling Service (Fred Hutchinson Cancer Research Center). These alleles were generated in a previous screen (Koundakjian et al., 2004). omi^{NSO} is a nonsense allele resulting from a base substitution of C to T, leading to generation of a stop codon at Q196, and omi^{V110E} is a missense allele resulting from substitution of T to A. We independently confirmed these alleles by sequencing. Prosα6T-GFP flies (Zhong and Belote, 2007) were obtained from J. M. Belote (Syracuse, NY), and UAS-mitoGFP and *Df(3R)ED5644* flies were obtained from the Bloomington *Drosophila* Stock Center. $pink1^{5}$, TMR-*pink1*, UAS-*pink1*, and CaSpeR-*pink1–9myc* (Clark et al., 2006), UAS-*parkin* (Greene et al., 2003), and Mef2-Gal4 (Deng et al., 2008) flies have been previously described. For experiments involving transgenic flies, multiple independent fly lines were generated (Rainbow Transgenic Flies) and tested for each transgene. *Drosophila* strains were maintained in an 18°C, 25°C, or 29°C humidified incubator, or at room temperature.

Male and female fertility tests. Recently eclosed individual male flies were placed with four virgin females in vials. Single 0- to 3-d-old females were placed in a vial supplemented with dry yeast along with five sibling males and maintained at 25°C. Males or females were scored as sterile if they failed to produce progeny by day 6.

Phase-contrast, immunofluorescence, and confocal 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, nota of adult flies were dissected and fixed in 4% paraformaldehyde, and indirect muscle fibers were isolated and imaged by a Zeiss LSM5 confocal microscope. For analysis of dopaminergic neurons, anti-tyrosine hydroxylase (TH) (Immunostar) antibodies were used and imaged by a Zeiss LSM5 confocal microscope, and only clearly stained anti-TH-positive cells were counted. Wild-type, *pink1* mutant, or *omi* mutant brains were counted blindly. The Immunofluorescent staining was performed as previously described (Guo et al., 1996). Phalloidin was used 1:1000 for testes staining (Invitrogen). Anti-Omi antibodies were a kind gift from M. Miura (Tokyo, Japan) and were used 1:300 for immunocytochemistry.

Transmission electron microscopy. Testes and muscle were dissected, fixed in paraformaldehyde/glutaraldehyde, postfixed in osmium tetroxide, dehydrated, and embedded in Epon. Toluidine blue was used to stain 1.5-μm-thick tissue sections. Thin sections (80 nm thick) were stained with uranyl acetate and lead citrate, and examined using a JEOL 100C transmission electron microscope (University of California, Los Angeles Brain Research Institute Electron Microscopy Facility). At least three testes or thoraces of each genotype were examined by transmission electron microscopy (EM).

Scanning electron microscopy. Freshly killed flies were mounted on their sides, placed on a platform under vacuum, and imaged at 180× magnification and 100 psi using a Hitachi 2460N scanning electron microscope. Analysis of eye phenotypes was performed as previously described (Guo et al., 2003; Gross et al., 2008).

Stress and longevity assays. Zero- to three-day-old males were anesthetized on ice, aged for 48 h, starved for 6 h, and subjected to 5% sucrose plus each agent. Four vials of 30 flies were assayed simultaneously for each genotype. For longevity measurements, 120 males of each genotype were divided into six vials. Flies were maintained at 25°C and transferred to fresh food every 2 d.

Lysate preparation and Western blotting. Heads or testes from age- and sex-matched adults were disrupted in lysis buffer containing complete protease inhibitor mixture (Roche) using a sonicator-3000 from MISONIX. Samples were boiled and centrifuged, and total protein was analyzed by Western blotting. Antibodies used were anti-Myc (Millipore) and anti-Omi (Igaki et al., 2007).

Northern blotting. Standard protocols were used using a full-length *pink1* probe as previously described (Clark et al., 2006).

Results

Overexpression-based genetic interactions of *pink1* and *omi* in the eye

Omi/HtrA2 encodes a protein with a mitochondrial targeting sequence and a transmembrane domain, followed by a serine protease domain and a C-terminal PSD-95/DlgA/Zo-1 (PDZ) domain (Vande Walle et al., 2008). *Drosophila melanogaster* contains a single homolog of *Omi/HtrA2* (CG8464, hereafter called *omi*), with 50% amino acid sequence identity, 68% similarity, and a domain structure similar to that of human Omi/HtrA2. To test the hypothesis that *omi* and *pink1* function in the same pathway, we asked whether genetic interactions between these two genes could be observed in the *Drosophila* eye. The fly eye is dispensable for viability and fertility, and has been widely used as a system to study human neurodegenerative diseases [for review, see Bonini and Fortini (2003) and Marsh and Thompson (2006)]. We generated transgenic flies to carry out tissue-specific overexpression using the UAS-Gal4 system (Brand and Perrimon, 1993). When *omi* was overexpressed at high levels in the eye (25 or 29°C), small and rough eyes were observed (Fig. 1*K* com-

pared with *A*), similar to a previously report (Igaki et al., 2007). These small eyes likely result from the ability of Omi to activate cell death when overexpressed (Challa et al., 2007; Igaki et al., 2007; Khan et al., 2008). The eye phenotypes resulting from *omi* overexpression were very sensitive to the level of *omi* expression. Flies expressing lower levels of *omi* (18°C) exhibited wild-type-appearing eyes, providing a sensitized genetic background for interaction studies (Fig. 1*B*). Eye-specific *pink1* overexpression resulted in mild rough eyes (Fig. 1*C*) (Poole et al., 2008). However, flies overexpressing both *pink1* and *omi* at 18°C exhibited smaller and rougher eyes than those associated with *pink1* overexpression alone (Fig. 1*F*). This suggests that there is an overexpression-based interaction between *pink1* and *omi*, which is consistent with a recent report (Whitworth et al., 2008) and has been interpreted, in conjunction with other observations, as indicating that *omi* acts downstream of *pink1* in a common genetic pathway (Whitworth et al., 2008).

One possible explanation for the interaction observed when *pink1* and *omi* are coexpressed is that overexpression of two mitochondrially targeted proteins causes competition for limited amounts of mitochondrial import machinery. In such a model, increased import of Pink1 could lead to excess Omi in the cytosol, resulting in a rough eye. Overexpression of a mitochondrial matrix-targeted green fluorescent protein (mitoGFP) with either *pink1* or *omi*, however, did not lead to any enhancement of *omi* or *pink1* overexpression phenotypes, suggesting that mitochondrial import is not limited (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). The *omi/pink1* co-overexpression interaction was dependent on the protease activity of Omi, because flies overexpressing a protease-dead version of Omi, Omi-S266A (Fig. 1*D* and see below), failed to show enhanced eye phenotypes when in conjunction with *pink1* overexpression (Fig. 1*G*).

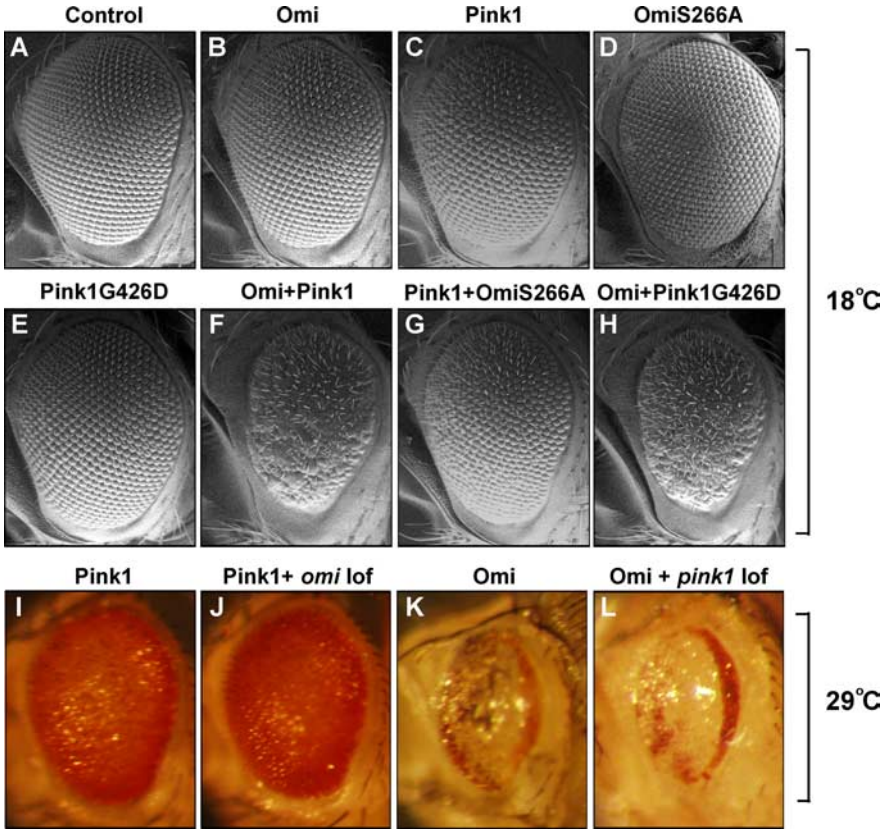

Figure 1. Overexpression-based genetic interactions between *pink* and *omi*. Scanning EM and light micrographs of *Drosophila* eyes are shown. At 18°C, *omi* overexpression (***B***) results in wild-type-appearing eyes, whereas overexpression of *pink1* (***C***) leads to mild rough eyes. Overexpression of both *omi* and *pink1* results in a small and rough eye (***F***). This *pink1-omi* overexpression interaction is abolished with expression of a protease-inactive version of Omi, S266A (***G***). Expression of OmiS266A by itself does not result in any eye phenotypes (***D***). Expression of a mutation analogous to the Pink1 disease mutant, G426D, has no phenotype (***E***); however, it still shows an interaction with *omi* overexpression (***H***). The phenotype of *pink1* overexpression cannot be suppressed by loss of *omi* function induced by RNAi-*omi*, even when raised at 29°C (***I***, ***J***). Silencing of *omi* function by RNAi-*omi* is strong because it completely suppresses the *omi* overexpression-induced eye phenotype (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Similarly, the eye phenotype resulting from *omi* overexpression cannot be modified by lack of *pink1* (***K***, ***L***). ***A–H*** are from flies raised at 18°C, whereas ***I–L*** are from flies raised at 29°C. Genotypes: Control: *w*; GMR-Gal4/+. Omi: *w*; GMR-Gal4, UAS-*omi*/+. Pink1: *w*; GMR-Gal4, UAS-*pink1*/+. Pink1G426D: *w*; GMR-Gal4/UAS-Pink1G426D. Omi+Pink1: *w*; UAS-*omi*/+; GMR-Gal4, UAS-*pink1*/+. Omi+Pink1G426D: *w*; GMR-Gal4, UAS-*omi*/UAS-Pink1G426D. Pink1+OmiS266A: *w*; UAS-OmiS266A/+; GMR-Gal4, UAS-*pink1*/+. Pink1+*omi* loss of function (lof): *w*; GMR-Gal4, UAS-*pink1*/UAS-RNAi-*omi*. Omi+*pink1* lof: *w pink1*^{5}; GMR-Gal4, UAS-*omi*/+.

Further exploring the hypothesis that *omi* acts downstream of *pink1*, we found that *pink1* overexpression-induced eye phenotypes could not be suppressed by loss of *omi* function (Fig. 1*I*,*J*). Similarly, the eye phenotype resulting from *omi* overexpression could not be modified by lack of *pink1* (Fig. 1*K*,*L*). Thus, these results do not provide support for *omi* functioning downstream of *pink1*. We next explored the relationship between these interactions observed in the eye and the well characterized functions of *pink* in regulating mitochondrial morphology. We generated transgenic flies expressing Pink1G426D, a *Drosophila* mutation analogous to the PINK1 PD-associated mutation G309D. G309D alters a residue in the kinase domain (Valente et al., 2004) and has a significant reduction of PINK1 kinase activity, as assayed by *in vitro* autophosphorylation (Beilina et al., 2005). *pink1* null mutant flies carrying G426D showed a largely abolished ability to rescue male sterility (<2% fertile, $n = 60$), muscle degeneration, and mitochondrial morphological defects of *pink1* null mutants (Fig. 2), indicating that this mutant protein is strongly compromised with respect to normal *pink1* function. Surprisingly, however, expression of Pink1G426D still led to a small and rough eye phenotype when combined with *omi* overexpression (Fig. 1*H*). These results suggest that *pink1* functions required to mediate *omi* overexpression-based interactions in the eye are distinct from *pink1* functions required to provide normal mitochondrial function. Together, although *omi* and *pink1* displayed genetic interactions in overexpression-based assays, these results do not provide evidence to support models in which *omi* plays a major role in transducing *pink1*-dependent signals to regulate mitochondrial function.

omi null mutants are male sterile, but show phenotypes distinct from those seen in *pink1* or *parkin* null mutants

To further explore the roles of *omi* as it relates to *pink1*, we performed loss-of-function studies of *omi* mutants. The endogenous functions of *omi* in *Drosophila* have not been fully studied be-

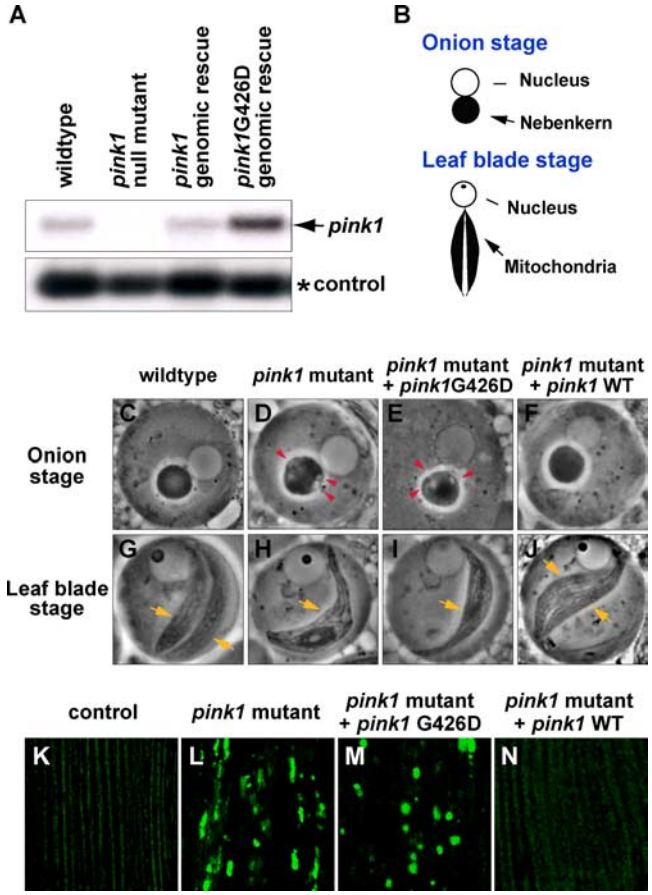

Figure 2. mRNA expression and phenotypic analysis of Pink1G426D, a mutation analogous to PD-associated mutation PINK1G309D. ***A***, Northern blot of whole flies using full-length *pink1* as a probe. Compared with wild type, *pink1* null mutant ($pink1^5$) flies do not show full-length mRNA. Transgenic flies expressing *pink1*G426D mutants show comparable or higher expression of *pink1* than is seen in flies expressing wild-type *pink1*. The arrow points to *pink1* expression, and *rp49* (*) serves as an RNA loading control. ***B–J***, Schematic (***B***) and phase-contrast micrographs of spermatid mitochondria during the "onion stage" (***C–F***) and "the leaf blade stage" (***G–J***). Compared with wild type, *pink1* mutants show vacuolation (red arrowheads) in the nebenkern during the onion stage (***D***), and one instead of the two mitochondrial derivatives (yellow arrows) seen in wild type at the leaf blade stage (***H***). A genomic rescue transgene carrying wild-type *pink1* (CaSpeR-*pink1*) completely rescues the male sterility resulting from lack of *pink1* (100% fertile, $n > 120$) (Clark et al., 2006), and spermatid phenotypes in both the onion stage and the leaf-blade stage (***F***, ***J***). In contrast, *pink1*G426D (CaSpeR-*pink1*G426D) fails to rescue the sterility of *pink1* males (<2% fertile, $n = 60$) or the spermatid phenotypes (***E***, ***I***). Red arrowheads point to vacuolation of the nebenkern, and yellow arrows mark each mitochondrial derivative. ***K–N***, Mitochondria of indirect flight muscle are labeled by mitoGFP. Compared with control (***K***), *pink1* mutants display overall reduced levels of mitoGFP signal and large clumps of intense GFP signal (***L***), which can be completely rescued by overexpression of Pink1WT (***N***), but not Pink1G426D (***M***).

cause of the absence of loss-of-function mutants. This analysis is more relevant to the role of *omi* as it relates to PD, because mutations in *omi* observed in PD patients are postulated to be loss-of-function or dominant-negative mutations, not resulting in increased activity. To obtain loss-of-function mutations in *omi*, we used TILLING (Till et al., 2003), a method for detecting ethyl methanesulfonate (EMS)-induced point mutations in a gene of interest after chemical mutagenesis. We obtained one nonsense mutation in *omi*, omi^{NSO}, and one missense mutation, V110E (see below). The truncated protein encoded by omi^{NSO} is predicted to lack the active site of the protease domain and the PDZ domain (Fig. 3*A*), and thus represents a null allele. Flies homozygous for omi^{NSO} were semilethal. However, flies carrying omi^{NSO} in trans to a deficiency in the region, *Df(3R)ED5644*, were fully viable, suggesting that the lethality associated with omi^{NSO} is caused by a background mutation. Flies with ubiquitous expression of RNAi-*omi* using a tubulin-Gal4 driver were also viable. The silencing effect of RNAi-*omi* was confirmed by its ability to completely suppress *omi* overexpression-induced eye phenotypes (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). As expected, Western blotting using an anti-Omi antibody revealed no detectable Omi-positive bands in omi^{NSO}/*Df(3R)ED5644* flies (supplemental Fig. S3, available at www.jneurosci.org as supplemental material).

omi^{NSO}/*Df(3R)ED5644* flies, hereafter called *omi* mutants, did not show any gross external defects. *omi* mutant females were fertile (96%, $n = 50$), but *omi* mutant males were sterile (100%, $n = 110$). In these males, seminal vesicles, which store mature sperm, were empty (Fig. 3*D*,*D*′), and no motile sperm were observed, suggesting defects in either production or transport to the seminal vesicles. To ensure that these phenotypes were attributable to lack of *omi*, we generated multiple transgenic fly lines expressing *omi* specifically in the male germline (TMR-*omi*). Overexpression of *omi* was confirmed using anti-Omi antibodies (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). Many of these lines were male sterile. However, three of 10 lines were fertile. Those fertile lines show weaker overexpression of *omi* than the sterile lines (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). This suggests that the sterility is caused by a high level of overexpression, likely resulting in promiscuous activity of the Omi protease. Introduction of any of the fertile *omi* overexpression lines into the *omi* mutant background resulted in the presence of motile sperm in the seminal vesicles (Fig. 3*E*,*E*′) and restoration of fertility (95%, $n = 100$). A single copy of a genomic rescue transgene containing *omi*, but not surrounding genes, also fully rescued the male sterility of *omi* mutants (100%, $n = 50$). Together, these results demonstrate that *omi* is essential for spermatogenesis.

We also analyzed the second *omi* EMS allele. V110 corresponds to V154 in human Omi/HtrA2, which is located in a highly conserved region (Fig. 3*A*,*B*) predicted by structural studies to mediate homo-trimerization of Omi/HtrA2, which is required to activate its protease activity (Li et al., 2002). omi^{V110E}/*Df(3R)ED5644* and omi^{V110E}/omi^{NSO} mutant flies were also male sterile (0%, $n = 45$; 0%, $n = 65$), displayed empty seminal vesicles, and had no motile sperm, indicating that omi^{V110} is likely a null or strong hypomorphic allele. These results provide *in vivo* support for an important role for the trimerization motif for Omi function, and suggest that the protease activity of Omi is crucially important for its role in regulating spermatogenesis.

Because *pink1* mutants also show male sterility, we asked whether *omi* mutant testes show defects in mitochondrial morphology, a prominent feature of *pink1* mutants (Clark et al., 2006; Deng et al., 2008). During *Drosophila* spermatogenesis, mitochondria undergo significant morphological changes (Fuller, 1993). Stem cell differentiation is followed by mitosis and meiosis with incomplete cytokinesis, creating syncytial cysts of 64 spermatids. Early spermatids undergo mitochondrial aggregation and fusion, creating two giant mitochondria that form a spherical structure known as the nebenkern (Fuller, 1993). Under phase-contrast microscopy, such "onion stage" spermatids can be identified as having two adjacent spherical structures: the nucleus and the nebenkern (Fig. 3*I*,*J*). During subsequent spermatid elongation, the nebenkern begins to unfurl, creating two mitochondria at this "leaf-blade" stage (Fig. 3*I*,*M*). After elongation, spermatids undergo a process known as individualization, in which the

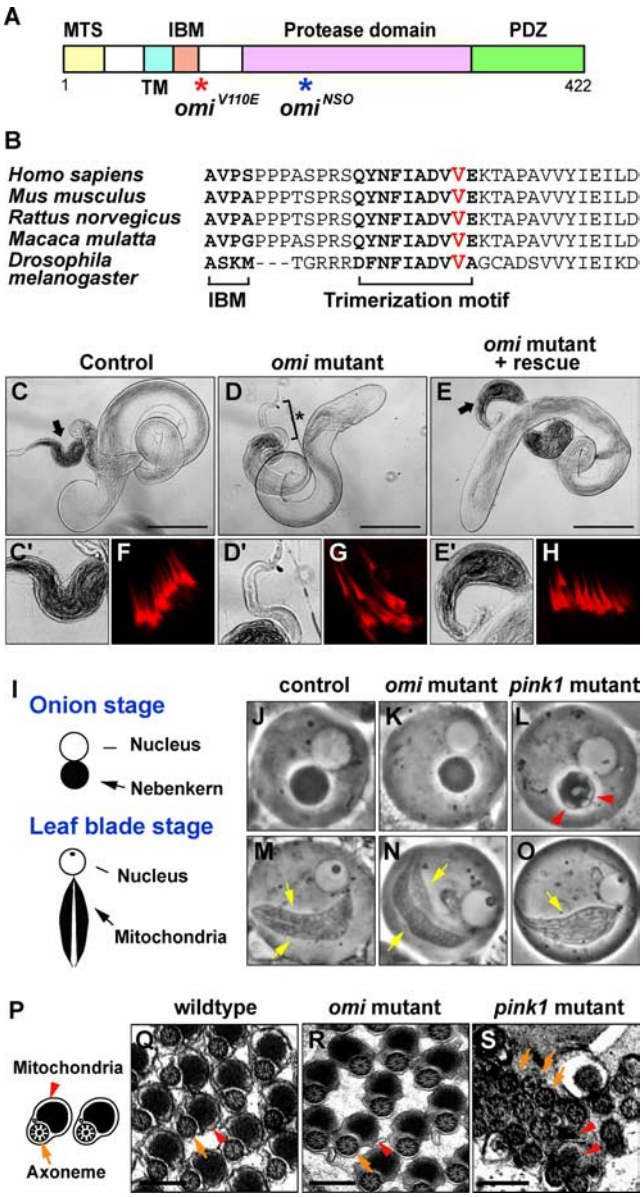

Figure 3. *omi* null mutants show defects in spermatogenesis, but have normal mitochondrial morphology in testes. ***A***, Schematic depicting domains of Omi. MTS, Mitochondrial targeting sequence. TM, Transmembrane domain. IBM, IAP-binding motif. The exact locations of omi^{NSO} and omi^{V110E} are depicted as blue and red asterisks, respectively. ***B***, Sequence alignment of Omi/HtrA2 in various species in a highly conserved region, in which the conserved valine, mutated in omi^{V110E}, is marked in red. ***C–E***, ***C′–E′***, Phase-contrast micrographs of testes. In contrast to a control fly (***C***, ***C′***), in which the seminal vesicle (arrow) is full of sperm (phase dark), *omi* mutants (***D***, ***D′***) show an empty seminal vesicle (bracket with an asterisk), which can be rescued by *omi* overexpression (***E***, ***E′***) (arrow pointing to the seminal vesicle). ***C′–E′*** are higher-magnification views of the seminal vesicle from ***C–E***. ***F–H***, Phalloidin staining of investment cones within one syncytial cyst. In contrast to controls, in which investment cones are well aligned, indicating synchronized movement (***F***), *omi* mutants show scattered investment cones in some of the cysts, indicative of a mild defect in individualization (***G***), which can be rescued by *omi* overexpression (***H***). ***I–O***, Schematics and phase-contrast micrographs of spermatid mitochondria during the "onion stage" (***I–L***) and "the leaf blade stage" (***I***, ***M–O***). Compared with wild type (***J***, ***M***), *omi* mutants do not show any defects in either stage (***K***, ***N***), whereas *pink1* mutants show vacuolation (red arrowheads) in the nebenkern during the onion stage (***L***), and one instead of the two mitochondrial derivatives (yellow arrows) seen in wild type at the leaf blade stage (***O***). ***P–S***, Schematic and transmission EM images of a portion of a postindividualization cyst. Each spermatid contains an axoneme (orange arrows) and mitochondrial derivative (red arrowheads) within an individual plasma membrane. The *omi* mutant cyst (***R***) shows disorganization of spermatids and occasional individualization defects (data not shown). However, compared with *pink1* mutants, which show severe impairment in the size and morphology of mitochondria (***S***), *omi* mutant cysts show normal-appearing mitochondria (***R***). Genotypes: wild type: *w*/Y; control: *w*/Y; omi^{NSO}/+; *omi* mutant: *w*/Y; omi^{NSO}/*Df(3R)ED5644*; *omi* mutant + rescue: *w*/Y; TMR-*omi*/+; omi^{NSO}/*Df(3R)ED5644*. Scale bars: ***C–E***, 500 μm; ***Q–S***, 500 nm.

cytoplasmic bridges that link the 64 spermatids within a cyst are broken and excess cytoplasm is extruded (Fuller, 1993). This individualization process requires synchronized movement of an actin-based structure known as the investment cone. After individualization, each spermatid tail consists largely of the axoneme, a microtubule-based structure required for motility, and mitochondrial derivatives (Fig. 3*P*,*Q*).

As expected for a protein with a mitochondrial targeting sequence, Omi localizes to nebenkerns (see Fig. 6*A–C*). In onion-stage spermatids, the nebenkerns of *pink1* mutants show significant vacuolation (Fig. 3*L*), and during the subsequent leaf blade stage *pink1* and *parkin* mutants contain only one mitochondrial derivative (Fig. 3*O*) rather than the two seen in wild type (Fig. 3*M*) (Clark et al., 2006; Riparbelli and Callaini, 2007; Deng et al., 2008). Surprisingly, mitochondria in *omi* mutants were indistinguishable from those in wild type. During the onion stage, the border of the nebenkern was smooth and no vacuolation was observed (Fig. 3*K*). At the leaf blade stage, *omi* mutant spermatids contained two mitochondrial derivatives instead of one observed in *pink1* or *parkin* mutants (Fig. 3*N*). *pink1* and *parkin* mutants also show dramatic defects in mitochondrial morphology during postindividualization stages, as visualized with transmission EM (Fig. 3*S*) (Greene et al., 2003; Clark et al., 2006; Riparbelli and Callaini, 2007; Deng et al., 2008). In contrast, mitochondria appeared normal in stage-matched *omi* mutants, although individual spermatids were somewhat disorganized within the cyst (Fig. 3*R*). In addition, investment cones in *omi* mutants were scattered (Fig. 3*G* compared with *F*), suggesting that movement of these structures is asynchronous. Such a phenotype is associated with individualization defects (Huh et al., 2004). Although the individualization defects were suppressed by testes-specific *omi* overexpression (Fig. 3*H*), we cannot rule out the possibility that defects in other postindividualization steps of spermatogenesis also contribute to sterility associated with *omi* mutants. This possibility seems particularly likely because the individualization defects observed in *omi* mutants appear relatively mild. In summary, *omi* mutant phenotypes in testes are distinct from those of *pink1* or *parkin* mutants, in which defects in mitochondrial morphology are observed.

In contrast to *pink1* mutants, *omi* mutants do not show dopaminergic neuronal loss, muscle degeneration, or defects in mitochondrial integrity

Next, we asked whether *omi* mutants show phenotypes similar to those of *pink1* mutants in other tissues and contexts. *omi* mutants were sensitive to treatments with multiple stress-inducing agents, including paraquat, a free radical inducer; rotenone, which impairs complex I activity in the mitochondrial respiratory chain (Przedborski and Ischiropoulos, 2005); protein folding inhibitors; and high concentrations of salt (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). Thus, rather than being specifically sensitive to oxidative stress, *omi* mutant flies are generally stress sensitive. These results may suggest a general sickness of *omi* mutants, particularly because *omi* mutants had a shortened life span (supplemental Fig. S5, available at www.jneurosci.org as supplemental material).

An age-dependent decrease in the number of dopaminergic neurons has been reported in *pink1* or *parkin* mutants (Meulener

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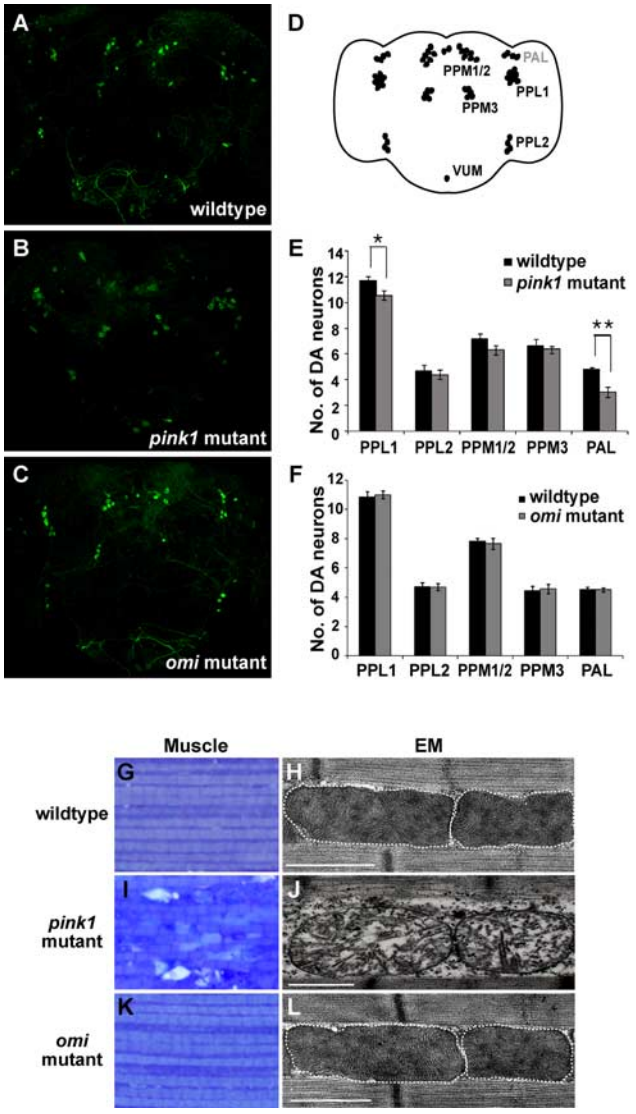

Figure 4. *omi* null mutants do not show dopaminergic neuronal loss, muscle degeneration, or mitochondrial morphological defects. ***A–C***, Anti-tyrosine hydroxylase immunostaining of whole-mount brains from 40-d-old wild-type (***A***), *pink1* mutant (***B***), and *omi* mutant (***C***) flies. Seventeen to twenty-seven individual flies (both hemispheres) were counted for each genotype. ***D***, Schematic depicting locations of the major dopaminergic neuron clusters in the adult brain as designated by abbreviations (Nässel and Elekes, 1992). The major dopaminergic neuron clusters are located near the posterior surface of the brain, with the exception of the protocerebral anterior lateral (PAL), which is located near the anterior surface (labeled in gray). PPL, Protocerebral posterior lateral; PPM, protocerebral posterior medial; VUM, ventral unpaired medial. ***E***, ***F***, Quantification of dopaminergic neurons in each cluster in *pink1* mutant flies and wild-type flies (***E***), and *omi* mutant flies and wild-type flies (***F***) aged for 40 d at 25°C. Error bars represent SDs, and Student's *t* test is used for statistical analysis. $*p < 0.05$; $**p < 0.01$. ***G–L***, Toluidine blue staining of indirect flight muscle fibers (***G***, ***I***, ***K***) and EM studies of these muscles (***H***, ***J***, ***L***). The borders of mitochondria are marked with white dashed lines. In contrast to *pink1* mutants (***I***, ***J***), *omi* mutants do not show muscle degeneration or mitochondrial morphological defects (***K***, ***L***), even when aged for 30 d. Genotypes: wild type: *w*/Y. *omi* mutant: *w*/Y; omi^{ISO}/*Df(3R)ED5644*. *pink1* mutant: *w* $pink1^{5}$/Y. Scale bars: ***H***, ***J***, ***L***, 1 μm.

et al., 2005; Park et al., 2006; Yang et al., 2006) (Fig. 4*A*,*B*,*D*,*E*). In contrast, *omi* mutants did not show any dopaminergic neuronal loss in the brains of flies aged for 40 d (Fig. 4*C*,*F*). *pink1* or *parkin* mutants also show striking indirect flight muscle degeneration and severely disrupted mitochondrial morphology with broken cristae, which are prominent in 1- to 2-d-old flies (Fig. 4*I*,*J* compared with *G*,*H*) (Greene et al., 2003; Pesah et al., 2004; Clark et al., 2006; Park et al., 2006; Yang et al., 2006). In contrast, although Omi is expressed in muscle and localized to mitochondria (data not shown), *omi* mutants did not exhibit any muscle degeneration, even when they were aged for 30 d (Fig. 4*K*). EM analysis of *omi* mutant muscle also failed to show any defects in mitochondrial integrity (Fig. 4*L*). Together, our data demonstrate that *omi* mutants, in contrast to *pink1* mutants, do not display mitochondrial morphological defects in multiple tissues, including spermatids and muscle. *omi* mutants also fail to show dopaminergic neuronal loss seen in *pink1* mutants.

Loss-of-function studies fail to detect any genetic interactions between *pink1* and *omi*

To further explore the hypothesis that *omi* and *pink1* work together to regulate mitochondrial integrity, we searched for genetic interactions based on loss of function of these genes. Genetic interactions between *pink1* and *parkin* provide an important reference for testing whether *omi* and *pink1* act in a common pathway (Clark et al., 2006; Park et al., 2006). We, as well as others, have previously shown that *Drosophila pink1* and *parkin* act in a common genetic pathway, with *pink1* functioning upstream of *parkin* (Clark et al., 2006; Park et al., 2006; Yang et al., 2006). This conclusion is based on several observations. Loss-of-function mutations in *pink1* and *parkin* result in highly similar, if not identical, defects in mitochondrial integrity (Clark et al., 2006; Park et al., 2006; Yang et al., 2006; Deng et al., 2008; Poole et al., 2008). Although overexpression of *parkin* rescues *pink1* null mutant phenotypes, overexpression of *pink1* fails to rescue *parkin* null mutant phenotypes (Clark et al., 2006; Park et al., 2006). In addition, double mutants removing both *pink1* and *parkin* show phenotypes identical to those of single mutants alone (Clark et al., 2006; Park et al., 2006).

In contrast to *pink1* (Fig. 5*C*,*H*) or *parkin* overexpression (Clark et al., 2006), testes-specific *omi* overexpression did not rescue the male sterility or the mitochondrial phenotype of *pink1* mutants (0% fertile, $n = 65$) (Fig. 5*D*,*I*). Expression of mutant versions of Omi analogous to S306A, S276C, G399S, or S400A (see below) also did not rescue male sterility of *pink1* mutants (0% fertile, $n > 30$). Similarly, *omi* overexpression, which leads to massive loss of muscle integrity, also failed to rescue the muscle degeneration phenotypes seen in *pink1* mutants. In addition, expression of a protease dead version of Omi, S266A, which does not result in loss of muscle integrity, also failed to rescue muscle phenotypes seen in *pink1* mutants (data not shown). Consistent with the hypothesis that *pink1* and *omi* function independently, neither the expression levels nor the mitochondrial localization of Omi was altered in *pink1* mutants (Fig. 6*D–F*).

To investigate whether *pink1* functions downstream of *omi*, we performed reverse rescue experiments. However, *pink1* overexpression failed to rescue male sterility seen in *omi* mutants (0% fertile, $n = 70$). In addition, neither the protein levels nor cleavage patterns of Pink1 were altered in *omi* mutants (Fig. 6). Thus, we failed to find any positive evidence that *omi* functions either upstream or downstream of *pink1* in a common pathway.

To test the hypothesis that *omi* might function in a parallel pathway with *pink1* in a partially redundant manner, we generated double mutants that remove both *pink1* and *omi*. These double mutant flies were viable and showed survival rates comparable to those of *pink1* mutants alone. These animals were male sterile and exhibited mitochondrial morphological defects in spermatids and muscle that were indistinguishable from those of *pink1* mutants alone, indicating that loss of *omi* function does not enhance *pink1* mutant phenotypes (Fig. 5*E*,*J*,*N*; compare with *B*,*G*,*L*). Together, our loss-of-function *in vivo* studies do not provide support for the hypothesis that *omi* functions either up-

stream or downstream of *pink1*, or in parallel with *pink1*, at least with respect to the regulation of mitochondrial integrity.

PD-associated mutations in Omi, and a mutation abolishing a putative Pink1-dependent phosphorylation site, show distinct phenotypes from mutations impairing Omi protease function

Because we failed to detect any loss-of-function-based genetic interactions between *pink1* and *omi*, we decided to examine the function of the PD disease-related *omi* mutations. The PD-associated polymorphism in Omi/HtrA2, A141S (detected in >1% of the normal population), and the mutation, G399S, have been reported to function as dominant-negative mutations, leading to a reduction of protease function of Omi/HtrA2 (Strauss et al., 2005). G399, which is located in the PDZ domain, is conserved in *Drosophila*, whereas A141, which is located in the IAP-binding domain of Omi/HtrA2, is not. Interestingly, S400, a residue next to G399, has been identified as a PINK1-dependent putative phosphorylation site for p38 (Plun-Favreau et al., 2007). This phosphorylation is reported to be important for Omi/HtrA2 activity, because S400A, a phosphorylation-incompetent mutation, markedly reduces protease activity (Plun-Favreau et al., 2007). To investigate whether these mutations affect Omi/HtrA2 function *in vivo*, we generated transgenic flies expressing Omi G363S or S364A, which are analogous to G399S or S400A in human Omi/HtrA2.

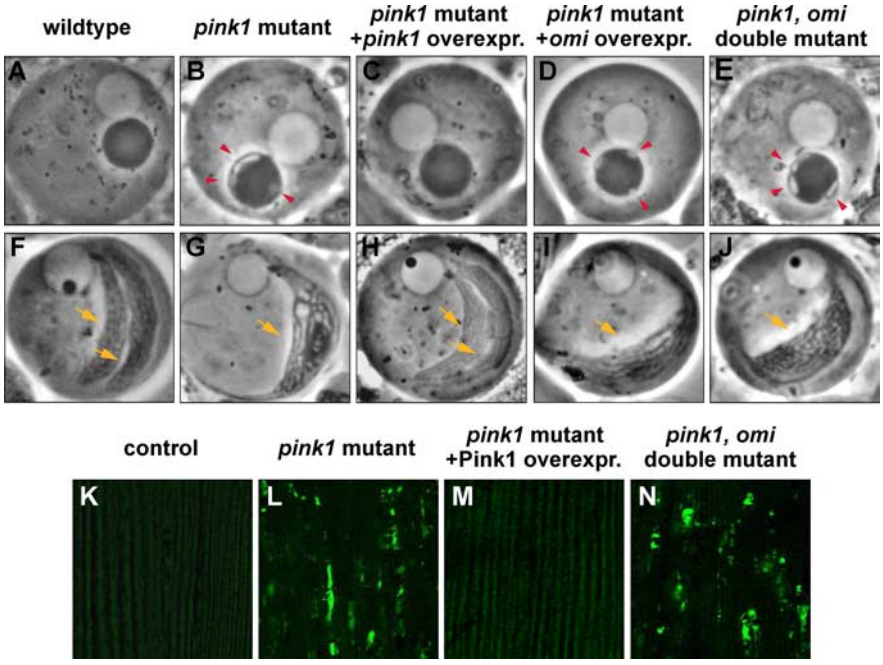

Figure 5. Lack of genetic interactions between *pink1* and *omi* in loss-of-function studies of mitochondrial morphology. ***A–J***, Phase-contrast micrographs of spermatid mitochondria during the "onion stage" (***A–E***) and "the leaf blade stage" (***F–J***). *pink1* null mutants show vacuolation of nebenkerns (***B***) and one single mitochondrial derivative (***G***). This phenotype can be completely suppressed by *pink1* overexpression (overexpr.) (***C***, ***H***), but not by *omi* overexpression (***D***, ***I***). Double mutants removing both *pink1* and *omi* function result in *pink1* mutant-like phenotypes without any enhancement (***E***, ***J***). Red arrowheads point to vacuolation of the nebenkern, and yellow arrows mark each mitochondrial derivative. ***K–N***, Mitochondria of indirect flight muscle are labeled by mitoGFP. Compared with control (***K***), *pink1* mutants display overall reduced levels of mitoGFP signal, and large clumps of intense GFP signal (***L***), which can be completely rescued by *pink1* overexpression (***M***). Double mutants of *pink1* and *omi* show *pink1* mutant-like phenotypes (***N***). Genotypes: ***A***, ***F***, *w*. ***B***, ***G***, *w pink1*^{5}/Y. ***C***, ***H***, *w pink1*^{5}/Y; TMR-*pink1*/+. ***D***, ***I***, *w pink1*^{5}/Y; TMR-*omi*/+. ***E***, ***J***, *w pink1*^{5}/Y; *omi*^{NSO}/*Df(3R)ED5644*. ***K***, *FM6*/Y; Mef2-Gal4, UAS-mitoGFP/+. ***L***, *w pink1*^{5}/Y; Mef2-Gal4, UAS-mitoGFP/+. ***M***, *w pink1*^{5}/Y; Mef2-Gal4, UAS-mitoGFP/UAS-*pink1*. ***N***, *w pink1*^{5}/Y; Mef2-Gal4, UAS-mitoGFP/UAS-RNAi-*omi*.

Both G399S and S400A reportedly compromise Omi protease activity *in vitro* (Strauss et al., 2005; Plun-Favreau et al., 2007). If this were true *in vivo*, G399S or S400A mutant forms of Omi would be expected to show similar phenotypes to protease-compromised Omi mutants. To test this hypothesis, we also generated two protease-impaired versions of *Drosophila* Omi, S266A and S236C. S266A is analogous to S306A in human Omi/HtrA2, which alters the active site serine in the protease domain and abolishes protease activity (Li et al., 2002), and S236C is analogous to the S276C mutation present in the *mnd2* mice, which significantly reduces the protease function of Omi/HtrA2 (Jones et al., 2003) (Table 1). These mutants were expressed and assayed in multiple somatic tissues using the UAS-GAL4 system (Brand and Perrimon, 1993), and in the male germline using the TMR promoter (Huh et al., 2004; Clark et al., 2006). The actions of G363S and S364A were compared with that of wild-type Omi (Omi WT), as well as the protease-deficient S266A and S236C mutant forms.

In contrast to overexpression of Omi WT, which resulted in male sterility in most transgenic lines, all transgenic lines expressing S266A or S236C were male fertile ($n > 13$ transgenic lines tested for each mutant). Expression of S266A also failed to rescue the male sterility (0% fertile, $n > 60$) and empty seminal vesicle phenotypes resulting from lack of *omi* (Fig. 7*B*, Table 1). These data suggest that protease-compromised mutations result in loss of Omi function. Further supporting this hypothesis, eye-specific overexpression of S266A or S236C, in contrast to overexpression of Omi WT, resulted in wild-type-appearing eyes (Fig. 7*E–G*, Table 1). Similarly, muscle-specific overexpression of S266A or S236C, in contrast to Omi WT, did not affect muscle integrity (Table 1, data not shown). Expression of S266A and S236C were confirmed using an anti-Omi antibody (Fig. 7*D*). These results, together with those described earlier with the missense mutation, *omi*^{V110E}, in the region responsible for activation of protease activity, suggest that Omi protease activity is important for its function *in vivo*.

In contrast, testes-specific expression of either OmiG363S or S364A resulted in significant male sterility, with only 3–4 lines of the 10–13 lines tested per construct giving fertile males, similar to what is seen with overexpression of Omi WT. These fertile lines likely represent those with lower expression levels. Using these fertile lines, we found that expression of G363S rescued the sterility and individualization phenotypes resulting from *omi* loss of function, as did those expressing Omi WT (Fig. 7*C*, Table 1). These results suggest that mutations analogous to G399S and S400A retain a significant amount of Omi activity. Further supporting this hypothesis, eye-specific overexpression of G363S or S364A resulted in small and rough eyes similar to those seen after overexpression of Omi WT (Fig. 7*H–J*, Table 1). Similarly, muscle-specific overexpression of G363S or S364A, or Omi WT, resulted in a massive loss of muscle integrity (Table 1, data not shown). Together, these observations (summarized in Table 1) suggest that Omi mutant proteins analogous to G399S and S400A

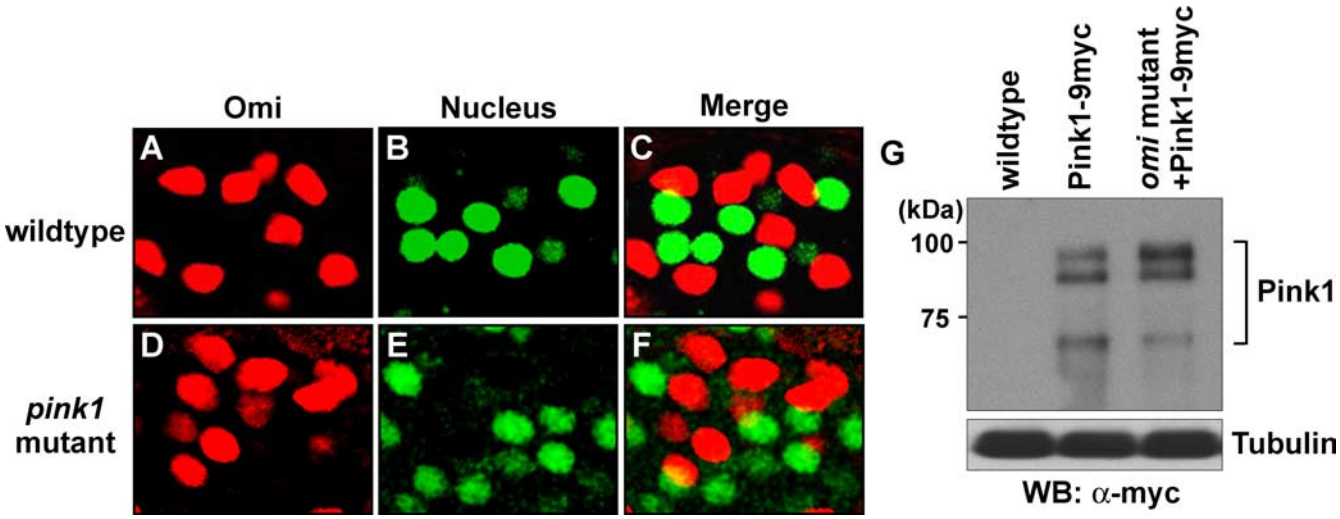

Figure 6. Omi is localized to mitochondria, and its expression is not altered in *pink1* null mutants; Pink1 expression is not altered in *omi* mutants either. ***A–F***, Double labeling of onion-staged spermatids in wild type (***A–C***) and *pink1* mutants (***D–F***) using Prosα6T-GFP (green), which labels the nucleus, and an anti-Omi antibody (red), which labels the nebenkern. In *pink1* null mutants, Omi is still localized to the nebenkerns of spermatids. ***G***, Western blotting (WB) of endogenous Pink1–9Myc expression using a genomic rescue transgene. Loss of *omi* function does not alter the cleavage pattern of Pink1.

Table 1. Summary of phenotypic effects of various Omi mutants reported in this study and by others

Genotype [fly (human)]	Molecular function from literature	Tissue-specific overexpression				Ability to rescue omi mutant sterility
		Protease activity from literature	Testes	Eye	Muscle	
Omi WT			Sterile	Small and rough	Disrupted	Rescue
S236C (S276C)	mnd2 mice	Reduced	Fertile	Wild type appearing	Wild type appearing	
S266A (S306A)	Protease active site	Protease dead	Fertile	Wild type appearing	Wild type appearing	Fail to rescue
G363S (G399S)	PD-associated mutation	Reduced	Sterile	Small and rough	Disrupted	Rescue
S364A (S400A)	pinkl-dependent phosphorylation site	Reduced	Sterile	Small and rough	Disrupted	

behave similarly to Omi WT, but differently from those with compromised protease activity *in vivo*.

Discussion

The *in vivo* function of *omi*

Omi/HtrA2 has been studied extensively for its role in apoptosis (for review, see Vande Walle et al., 2008). However, whereas overexpression of *Omi/HtrA2* induces apoptosis robustly in mammalian cells, mice lacking *Omi/HtrA2* fail to show decreased apoptosis, but instead show nondopaminergic neuronal loss in the striatum (Martins et al., 2004). *Omi/HtrA2* function is also implicated in regulating stress resistance (Vande Walle et al., 2008). Thus, determining the endogenous function of *Omi/HtrA2* is crucially important to understanding its roles in both health and disease. Using *Drosophila* as a model, we have dissected the *in vivo* function of Omi. We find that *omi* is essential for spermatogenesis, stress resistance, and maintaining a normal life span. Furthermore, the protease activity of Omi is crucial for its function.

We have identified an essential role of *omi* during spermatogenesis. However, although Omi is localized to mitochondria in both testes and muscle, no mitochondrial morphology defects are observed in *omi* null mutants in either of these tissues. It is possible that Omi is responsible for some aspects of mitochondrial function, such as chaperone activity or modulation of respiratory chain function, which do not affect mitochondrial morphology, and thus are not detected in our assays. It is also possible that *omi* is required only in certain contexts, such as during exposure to oxidative stress, and that mitochondrial defects may be revealed in *omi* mutants under these conditions. Alternatively, Omi may function in the cytosol rather than in the mitochondria, with mitochondria serving to regulate the release of Omi into the cytosol. Future studies are required to distinguish these possibilities.

Interaction of Omi and Pink1

The genetic interactions observed between *pink1* and *parkin* serve as an important reference for tests of the hypothesis that *omi* and *pink1* act in a common pathway. In contrast to *pink1* mutants, which show striking defects in mitochondrial integrity in muscle and testes, and a decrease in the number of dopaminergic neurons, *omi* mutants show normal mitochondrial morphology in both muscle and testes, and a normal number of dopaminergic neurons. Furthermore, in contrast to *parkin* overexpression, *omi* overexpression does not rescue *pink1* mutant phenotypes. Overexpression of *pink1* also fails to rescue male sterility resulting from *omi* loss of function. Lack of *pink1* does not affect the levels or the subcellular localization of Omi, and Pink1 levels and processing are not altered in *omi* mutants. In addition, double mutants removing both *pink1* and *omi* show identical phenotypes to *pink1* mutants alone, suggesting that *pink1* does not negatively regulate *omi* and that *omi* does not carry out partially redundant functions with *pink1*. Together, these data do not provide any *in vivo* evidence supporting the hypothesis that *omi* functions in the same pathway either upstream or downstream of *pink1*, or that it acts in a parallel manner to regulate mitochondrial morphology. These loss-of-function-based analyses are more relevant to PD than are *omi* overexpression-based analyses, because reported

Omi/HtrA2 mutations associated with PD are proposed to represent loss-of-function or dominant-negative mutations (Strauss et al., 2005).

Genetic interactions between *pink1* and *omi* have been observed by ourselves (see Results) and others (Whitworth et al., 2008) in eye-based overexpression studies: co-overexpression of *pink1* with *omi* results in small eye phenotypes not associated with expression of either protein alone. Although in isolation, these results could be explained by a model in which *omi* and *pink1* function in a common pathway (Whitworth et al., 2008), this model is difficult to reconcile with our loss-of-function data. The cellular basis of the *pink1* overexpression-induced eye phenotype, and its relationship to the normal endogenous roles of *pink1* in regulating mitochondrial function, is unclear. Although null mutants of *pink1* and *parkin* show highly similar, if not identical, phenotypes in almost all assays tested, overexpression of *pink1* results in a rough eye phenotype, whereas overexpression of *parkin* does not (data not shown) (Poole et al., 2008; Whitworth et al., 2008). Furthermore, a PD-causing, kinase-deficient mutant form of *pink1*, which fails to rescue *pink1* null mutant phenotypes in multiple tissues, still interacts with *omi* in the eye-based overexpression assay, suggesting that Pink1 kinase activity is required for its mitochondrial functions but not for the genetic interaction with *omi* in this assay. Based on our findings, one is led to conclude that the functions of *pink1* that mediate its co-overexpression interaction with *omi* are distinct from the functions of *pink1* and *parkin* in regulating mitochondrial morphology. Such a mitochondrial integrity-independent role of *pink* may be important, but has yet to be identified *in vivo*. Alternatively, it is possible that the *pink1-omi* interaction observed in the eye is not physiologically relevant. Overexpression studies, as well as *in vitro* studies, can identify interactions that are forced to happen, but that do not normally occur. For example, either protein, when overexpressed, may act on inappropriate targets or act in inappropriate subcellular compartments, thus generating cellular toxicity. In combination, this toxicity may be augmented. This possibility is further suggested by the results of overexpression-based observations that place Rhomboid 7 as an upstream positive regulator of Pink1 (Whitworth et al., 2008). This conclusion is difficult to reconcile with more physiological loss-of-function-based observations showing that *Drosophila rhomboid 7* functions to promote mitochondrial fusion (McQuibban et al., 2006), whereas both *pink1* and *parkin* function to promote mitochondrial fission (Deng et al., 2008; Poole et al., 2008; Yang et al., 2008). In any case, our loss-of-function studies demonstrate that *omi* does not play an essential role in regulating mitochondrial integrity in the *pink1/parkin* pathway. They leave open the possibility that interactions between *pink1* and *omi* are modulatory, or important in other contexts. However, these *in vivo* contexts remain to be identified.

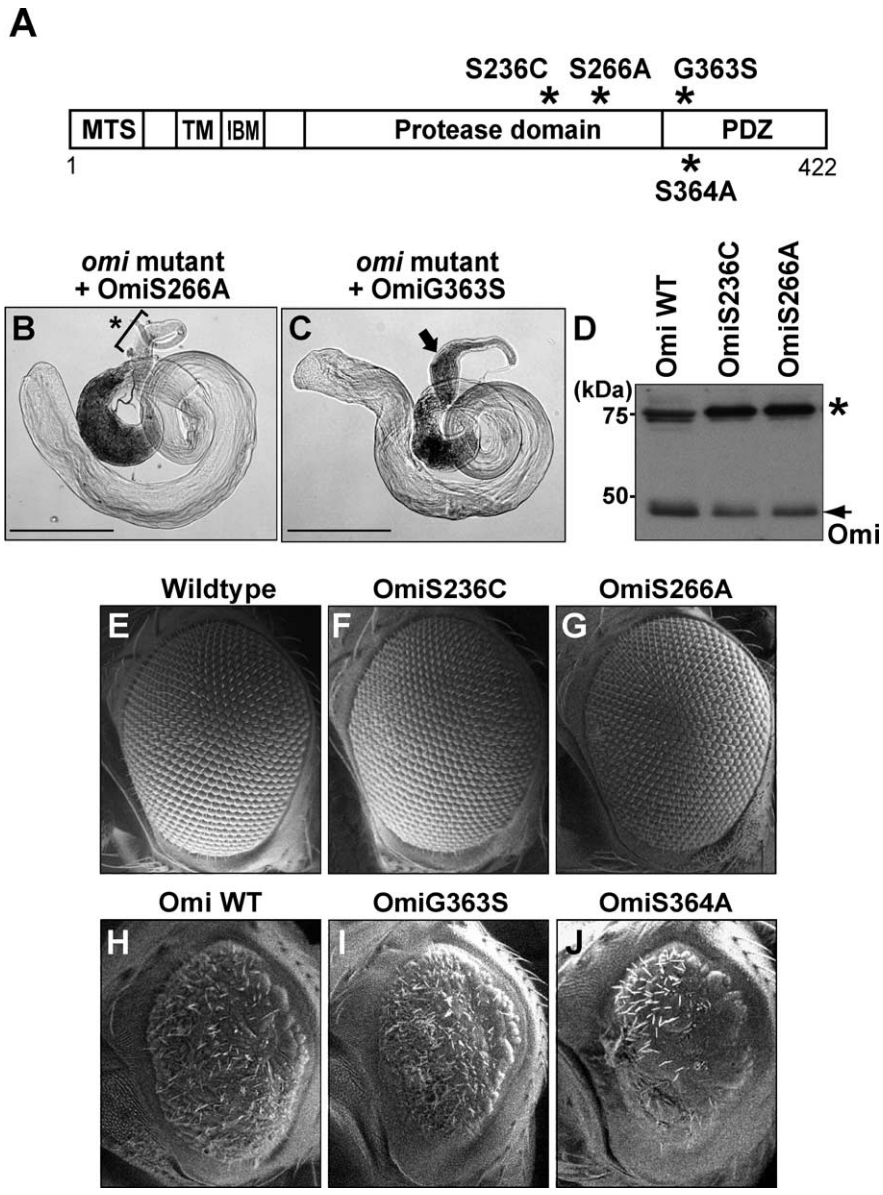

Figure 7. Functional analysis of Omi mutants. ***A***, Schematic depicting positions of 4 mutations (*) with respect to domains in Omi. ***B***, ***C***, Phase-contrast micrographs. As with overexpression of Omi WT (Fig. 3 *E*, *E′*), expression of G363S (***C***), but not S266A (***B***), restores the production of motile sperm in the seminal vesicle of *omi* mutants. An arrow in ***C*** indicates the presence of sperm (phase dark), whereas a bracket and an asterisk in ***B*** point to the absence of sperm. ***D***, Both OmiS236C and OmiS266A are expressed at comparable levels compared with Omi WT, as detected by an anti-Omi antibody. Western blots of head lysates from flies overexpressing OmiWT, OmiS236C, or OmiS266A using anti-Omi antibodies are shown. Overexpression is accomplished using the eye-specific driver (GMR-Gal4), and flies are raised at 18°C to avoid cell death compromising recovery of proteins. A nonspecific band (*) serves as protein loading control. ***E–J***, Scanning EM micrographs of *Drosophila* eyes. Compared with overexpression of Omi WT, which results in small and rough eyes at 25°C (***H***), overexpression of Omi G363S or S364A leads to similar rough eye phenotypes (***I***, ***J***), whereas overexpression of Omi S236C or S266A results in wild-type-appearing eyes (***F***, ***G***). GMR-Gal4 is used as an eye-specific driver. Scale bars: ***B***, ***C***, 500 μm.

Implications for *Omi/HtrA2* as a PD gene

Omi/HtrA2 was recently designated as *PARK13*, based on a report identifying G399S mutations in sporadic PD patients (Strauss et al., 2005). Mammalian cell culture studies suggest that G399S results in a significant reduction in Omi/HtrA2 protease activity,

providing a possible functional basis for disease association (Strauss et al., 2005). In contrast to previous *in vitro* observations (Strauss et al., 2005; Plun-Favreau et al., 2007), we find that both G399S and S400A retain significant, if not full, Omi function *in vivo*, leading to the conclusion that mutations previously thought to be associated with disease are functional in at least some contexts *in vivo*. Importantly, our conclusion is consistent with two recent reports showing that Omi G399S is found at similar frequencies in normal controls and PD patients (Ross et al., 2008; Simón-Sánchez and Singleton, 2008),

We cannot exclude the possibility that human *Omi/HtrA2* has a dopaminergic neuron-specific function that is revealed under certain circumstances, nor can we exclude the possibility that *Drosophila omi* acts differently from human *Omi/HtrA2*. However, the extensive homology and conservation of key domain structures between fly and human Omi/HtrA2 suggest that it is likely that studies in *Drosophila* Omi are relevant to the function of Omi/HtrA2 in humans. Together with the observations that *omi* mutant phenotypes are distinct from those associated with loss of *pink1* and *parkin* function, and that *pink1* and *omi* fail to interact in loss-of-function-based assays, we favor a hypothesis in which *Omi/HtrA2* does not play an essential role in PD pathogenesis.

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