Neurobiology of Disease

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
reportedly impairs activation of protease activity. In addition, 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-Favre et al., 2007). Thus, it has been suggested that Omi/HtrA2 functions downstream of PINK1, with PINK1 positively regulating Omi/HtrA2 (Plun-Favre 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 PINK1G399D 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-omiG1426D, site-specific mutagenesis in the backbone of CaSpeR-omi was performed and the product subcloned into pCaSpeR4 vector. To generate CaSpeR-omiG1426D, 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: CAACCTCGAGGAGATACATTGGGCGGGGTCC; 3′ primer: GGAGACATGTTGGTTGTCCAGCGATTTC. 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). omiNSO is a nonsense allele resulting from a base substitution of C to T, leading to the generation of a stop codon at Q196, and omiV110E is a missense allele resulting from substitution of T to A. We independently confirmed these alleles by sequencing. Pros+6T-GFP flies (Zhong and Belote, 2007) were obtained from J. M. Belote (Syracuse, NY), and UAS-mitoGFP and Dif3R/ED5644 flies were obtained from the Bloomington Drosophila Stock Center. pink1Δ, TMR-pink1, UAS-pink1, and CaSpeR-pink1-9myc (Clark et al., 2006), UAS-parkin (Greene et al., 2003), and Mef2-Gα (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 assay. 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/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. 1B). Eye-specific pink1 overexpression resulted in mild rough eyes (Fig. 1C) (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. 1F). 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 mitochondrionally 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. 1D and see below), failed to show enhanced eye phenotypes when in conjunction with pink1 overexpression (Fig. 1G).

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. 1I, J). Similarly, the eye phenotype resulting from omi overexpression could not be modified by lack of pink1 (Fig. 1K, 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. 1H). 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-
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\textsuperscript{NSO}, and one missense mutation, V110E (see below). The truncated protein encoded by omi\textsuperscript{NSO} is predicted to lack the active site of the protease domain and the PDZ domain (Fig. 3A), and thus represents a null allele. Flies homozygous for omi\textsuperscript{NSO} were semilethal. However, flies carrying omi\textsuperscript{NSO} in trans to a deficiency in the region, Df(3R)ED5644, were fully viable, suggesting that the lethality associated with omi\textsuperscript{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\textsuperscript{NSO}/Df(3R)ED5644 flies (supplemental Fig. S3, available at www.jneurosci.org as supplemental material).

omi\textsuperscript{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. 3D, 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. 3E, 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. 3A, 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\textsuperscript{V110E}/Df(3R)ED5644 and omi\textsuperscript{V110E}/omi\textsuperscript{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\textsuperscript{V110E} 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 pink\textsuperscript{1} mutants also show male sterility, we asked whether omi mutant testes show defects in mitochondrial morphology, a prominent feature of pink\textsuperscript{1} mutants (Clark et al., 2006; Deng et al., 2008). During Drosoidea spermatogenesis, mitochondria undergo significant morphological changes (Fuller, 1993). Stem cell differentiation is followed by mitosis and meiosis with incomplete cytokinesis, creating syncyntial 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. 3I, J). During subsequent spermatid elongation, the nebenkern begins to unfurl, creating two mitochondria at this “leaf-blade” stage (Fig. 3I, M). After elongation, spermatozoids undergo a process known as individualization, in which the

![Figure 2](image-url)
Mutations of Omi and Its Interaction with Pink1

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 et al., 2004). Although the individualization defects were suppressed by tests-specific omi overexpression (Fig. 3H), 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.
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 (J, L), *omi* mutants do not show muscle degeneration or mitochondrial morphological defects (K, L), even when aged for 30 d. Genotypes: wild type: w; *omi* mutant: w; *omi* [S306A, S276C, G399S, or S400A](Drosophila pink1) alone (Clark et al., 2006; Park et al., 2006). These animals were male sterile and exhibited mitochondrial morphological defects seen in *pink1* mutants (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. 5C,H) or *parkin* overexpression (Clark et al., 2006), tests-specific *omi* overexpression did not rescue the male sterility or the mitochondrial phenotype of *pink1* mutants (0% fertile, *n* = 65) (Fig. 5D,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. 5E,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.

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, in contrast to Omi WT, resulted in a massive loss of muscle integrity (Table 1, data not shown). Expression of S266A and S236C, in contrast to overexpression of S266A or S236C, in contrast to overexpression of Omi WT, resulted in wild-type-appearing eyes (Fig. 7E–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. 7D). These results, together with those described earlier with the missense mutation, omi<sup>V110E</sup>, in the region responsible for activation of protease activity, suggest that Omi protease activity is important for its function *in vivo*.

In contrast, testis-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. 7C, 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 S266A or S236C, in contrast to overexpression of G363S or S364A, resulted in small and rough eyes similar to those seen after overexpression of Omi WT (Fig. 7H–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...
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 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

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

<table>
<thead>
<tr>
<th>Genotype (fly [human])</th>
<th>Molecular function from literature</th>
<th>Protease activity from literature</th>
<th>Tissue-specific overexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype (WT)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S226C (S276C)</td>
<td>mmd2 mice</td>
<td>Reduced</td>
<td>Sterile, Small and rough</td>
</tr>
<tr>
<td>S266A (S306A)</td>
<td>Protease site</td>
<td>Protease dead</td>
<td>Fertile, Wild type appearing</td>
</tr>
<tr>
<td>G363S (G399S)</td>
<td>PD-associated mutation</td>
<td>Reduced</td>
<td>Sterile, Small and rough</td>
</tr>
<tr>
<td>S364A (S404A)</td>
<td>pink1-dependent phosphorylation</td>
<td>Reduced</td>
<td>Sterile, Small and rough</td>
</tr>
</tbody>
</table>

**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 ProscG6- 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.
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 pink1 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.

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.

References


