Loss-of-function analysis suggests that Omi/HtrA2 is not an essential component of the pink1/parkin pathway in vivo

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Abstract

Recently, a mutation in the mitochondrial protease Omi/HtrA2, G399S, was found in sporadic Parkinson 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 with 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.

Keywords

Parkinson disease; mitochondrial protease; Omi/HtrA2; Pink1; genetic interactions; Drosophila
Introduction

Parkinson 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 (Ibanez 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. While overexpression of Omi/HtrA2 leads to apoptosis, following its release into the cytosol (reviewed by Walle et al., 2008), mice lacking Omi/HtrA2 or mice harboring a mutation in Omi/HtrA2 disrupting protease function (Jones et al., 1993; Jones et al., 2003) show loss of non-dopaminergic 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 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 non-phosphorylatable 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; Simon-Sanchez 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 & 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 vector, 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). 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). PCR products of these microRNA precursors were cloned into pUASt. To generate CaSpeR-\textit{pink1G426D}, site-specific mutagenesis in the backbone of CaSpeR-\textit{pink1} was carried out and the product subcloned into pCaSpeR4 vector. To generate CaSpeR-\textit{omi}, a 2.5kb PCR product, generated using the following primers, was subcloned into pCaSpeR4 vector (a gift from Nic Tapon): 5’ primer: CAACCTCGAGGAAGTACATTGGGCGGGTC; 3’ primer: GGGACTAGTGGGTTTGTCAGCGATTTC. All cloned PCR products were confirmed by DNA sequencing.

\textbf{Drosophila Genetics and Strains}

EMS mutations were recovered using the \textit{Drosophila} Tilling Service (Fred Hutchinson Cancer Research Center). These alleles were generated in a prior screen (Koundakjian et al., 2004). \textit{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 \textit{omi}^{V110E} is a missense allele due to substitution of T to A. We independently confirmed these alleles by sequencing. Pros\textit{\alpha}6T-GFP flies (Zhong and Belote, 2007) were obtained from J.M. Belote, UAS-mitoGFP and \textit{Df(3R)ED5644} flies were obtained from the Bloomington \textit{Drosophila} Stock Center. \textit{pink1}^5, TMR-\textit{pink1}, UAS-\textit{pink1} and CaSpeR-\textit{pink1–9myc} (Clark et al., 2006), UAS-\textit{parkin} (Greene et al., 2003) and Me\textit{\textvisibles}2-Gal4 (Deng et al., 2008) flies were previously described. For experiments involving transgenic flies, multiple independent fly lines were generated (Rainbow Transgenic Flies) and tested for each transgene. \textit{Drosophila} strains were maintained in an 18°C, 25°C or 29°C humidified incubator, or at room temperature.

\textbf{Male and Female Fertility Tests}

Recently eclosed individual male flies were placed with four virgin females in vials. Single 0–3 day 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.

\textbf{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, fixed in 4% paraformaldehyde, and indirect muscle fibers 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. Wildtype, \textit{pink1} or \textit{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 and were used 1: 300 for immunocytochemistry.

\textbf{Transmission Electron Microscopy}

Testes and muscle were dissected, fixed in paraformaldehyde/glutaraldehyde, postfixed in osmium tetroxide, dehydrated and embedded in Epon. 1.5μm-thick tissue sections were stained with Toluidine Blue. 80nm thin sections were stained with uranyl acetate and lead citrate, and examined using a JEOL 100C transmission electron microscope (UCLA Brain Research).
Institute EM Facility). At least three testes or thoraces of each genotype were examined by TEM.

**Scanning Electron Microscopy**

Freshly sacrificed 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**

0–3 day-old males were anaesthetized on ice, aged for 48 hours, starved for 6 hours 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 days.

**Lysate Preparation and Western Blotting**

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

**Northern Blotting**

Standard protocols were utilized 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 PSD95/DlgA/Zo-1 (PDZ) domain (Walle et al., 2008). *Drosophila melanogaster* contains a single homolog of *Omi/HtrA2* (CG8464, hereafter called *omi*), with 50% amino acid sequence identity, and 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 if 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 (reviewed by (Bonini and Fortini, 2003; 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°C), small and rough eyes were observed (Fig. 1K compared 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 due to *omi* overexpression were very sensitive to the level of *omi* expression. Flies expressing lower levels of *omi* (18°C) exhibited wildtype-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 \textit{pink1} and \textit{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 \textit{pink1} or \textit{omi}, however, did not lead to any enhancement of \textit{omi} or \textit{pink1} overexpression phenotypes, suggesting that mitochondrial import is not limited (Supplementary Fig. S1). The \textit{omi}/\textit{pink1} co-overexpression interaction was dependent on the protease activity of Omi, since flies overexpressing a protease-dead version of Omi, Omi-S266A (Fig. 1D, see below), failed to show enhanced eye phenotypes when in conjunction with \textit{pink1} overexpression (Fig. 1G).

Further exploring the hypothesis that \textit{omi} acts downstream of \textit{pink1}, we found that \textit{pink1} overexpression-induced eye phenotypes could not be suppressed by loss of \textit{omi} function (Fig. 1I, J). Similarly, the eye phenotype due to \textit{omi} overexpression could not be modified by lack of \textit{pink1} (Fig. 1K, L). Thus, these results do not provide support for \textit{omi} functioning downstream of \textit{pink1}. We next explored the relationship between these interactions observed in the eye and the well characterized functions of \textit{pink} in regulating mitochondrial morphology. We generated transgenic flies expressing Pink1G426D, a \textit{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). \textit{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 \textit{pink1} null mutants (Fig. 2), indicating that this mutant protein is strongly compromised with respect to normal \textit{pink1} function. Surprisingly, however, expression of Pink1G426D still led to a small and rough eye phenotype when combined with \textit{omi} overexpression (Fig. 1H). These results suggest that \textit{pink1} functions required to mediate \textit{omi} overexpression-based interactions in the eye are distinct from \textit{pink1} functions required to provide normal mitochondrial function. Taken together, although \textit{omi} and \textit{pink1} displayed genetic interactions in overexpression-based assays, these results do not provide evidence to support models in which \textit{omi} plays a major role in transducing \textit{pink1}-dependent signals to regulate mitochondrial function.

\textbf{omi null mutants are male sterile, but show phenotypes distinct from those seen in \textit{pink1} or \textit{parkin} null mutants}

To further explore the roles of \textit{omi} as it relates to \textit{pink1}, we carried out loss-of-function studies of \textit{omi} mutants. The endogenous functions of \textit{omi} in \textit{Drosophila} have not been fully studied due to the absence of loss-of-function mutants. This analysis is more relevant to the role of \textit{omi} as it relates to PD, since mutations in \textit{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 \textit{omi}, we used TILLING (Till et al., 2003), a method for detecting ethyl methanesulfonate (EMS)-induced point mutations in a gene of interest following chemical mutagenesis. We obtained one nonsense mutation in \textit{omi}, \textit{omi}\textsuperscript{NSO}, and one missense mutation, V110E (see below). The truncated protein encoded by \textit{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 \textit{omi}\textsuperscript{NSO} were semi-lethal. However, flies carrying \textit{omi}\textsuperscript{NSO} in trans to a deficiency in the region, Df(3R)ED5644, were fully viable, suggesting that the lethality associated with \textit{omi}\textsuperscript{NSO} is due to a background mutation. Flies with ubiquitous expression of RNAi-\textit{omi} using a tubulin-Gal4 driver were also viable. The silencing effect of RNAi-\textit{omi} was confirmed by its ability to completely suppress \textit{omi} overexpression induced eye phenotypes (Supplementary Fig. S2). As expected, Western blotting using an anti-Omi antibody revealed no detectable Omi-positive bands in \textit{omi}\textsuperscript{NSO}/Df(3R)ED5644 flies (Supplementary Fig. S3).
omiNSO/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 due 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 (Supplementary Fig. S3). Many of these lines were male sterile. However, three of ten lines were fertile. Those fertile lines show weaker overexpression of omi compared with the sterile lines (Supplementary Fig. S3). This suggests that the sterility is due to 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). omiV110E/Df(3R)ED5644 and omiV110E/omiNSO mutant flies were also male sterile (0%, n=45; 0%, n=65), displayed empty seminal vesicles, and had no motile sperm, indicating that omiV110 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.

Since pink1 mutants also show male sterility, we asked if 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. 3L). During subsequent spermatid elongation, the nebenkern begins to unfurl, creating two mitochondria at this “leaf-blade” stage (Fig. 3M). Following elongation, spermatids undergo a process known as individualization, in which the 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. 3P,Q).

As expected for a protein with a mitochondrial targeting sequence, Omi localizes to nebenkerns (Fig. 6A–C). In onion-stage spermatids, the nebenkerns of pink1 mutants show significant vacuolation (Fig. 3L), and during the subsequent leaf blade stage pink1 and parkin mutants contain only one mitochondrial derivative (Fig. 3O) rather than the two seen in wildtype (Fig. 3M) (Clark et al., 2006; Riparbelli and Callaini, 2006; Deng et al., 2008). Surprisingly, mitochondria in omi mutants were indistinguishable from those in wildtype. During the onion stage, the border of the neberkern was smooth and no vacuolation was observed (Fig. 3K). At the leaf blade stage, omi mutant spermatids contained two mitochondrial derivatives instead of one observed in pink1 or parkin mutants (Fig. 3N). pink1 and parkin mutants also show dramatic defects in mitochondrial morphology during post-individualization stages, as
visualized with transmission electron microscopy (EM) (Fig. 3S) (Greene et al., 2003; Clark et al., 2006; Riparbelli and Callaini, 2006; Deng et al., 2008). In contrast, mitochondria appeared normal in stage-matched omi mutants, though individual spermatids were somewhat disorganized within the cyst (Fig. 3R). In addition, investment cones in omi mutants were scattered (Fig. 3G compared with F), suggesting that movement of these structures is asynchronous. Such a phenotype is associated with individualization defects (Huh et al., 2004). While the individualization defects were suppressed by testes-specific omi overexpression (Fig. 3H), we cannot rule out the possibility that defects in other post-individualization steps of spermatogenesis also contribute to sterility associated with omi mutants. This possibility seems particularly likely since 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 if 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 (Supplementary Fig. S4). 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 since omi mutants had a shortened life span (Supplementary Fig. S5).

An age-dependent decrease in the number of dopaminergic neurons has been reported in pink1 or parkin mutants (Meulener et al., 2005; Park et al., 2006; Yang et al., 2006) (Fig. 4A,B,D,E). In contrast, omi mutants did not show any dopaminergic neuronal loss in the brains of flies aged for 40 days (Fig. 4C,F). pink1 or parkin mutants also show striking indirect flight muscle degeneration, and severely disrupted mitochondrial morphology with broken cristae, which are prominent in 1–2 day old flies (Fig. 4I,J) (Greene et al., 2003; Pesah et al., 2004; Clark et al., 2006; Park et al., 2006; Yang et al., 2006). In contrast, though 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 days (Fig. 4K). EM analysis of omi mutant muscle also failed to show any defects in mitochondrial integrity (Fig. 4L). Taken 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). While 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), testes-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. 6D–F).

To investigate if pink1 functions downstream of omi, we carried out 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 upstream 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

Since 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 more than 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, while 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, since 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 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 germ line using the TMR promoter (Huh et al., 2004; Clark et al., 2006). The actions of G363S and S364A were compared with that of wildtype 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 due to lack of omi (Fig. 7B, 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 wildtype 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, omiV110E, 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 out 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 due to 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 G363S or S364A resulted in small and rough eyes similar to those seen following 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 (reviewed in Walle et al., 2008). However, while overexpression of Omi/HtrA2 induces apoptosis robustly in mammalian cells, mice lacking Omi/HtrA2 fails to show decreased apoptosis, but instead show non-dopaminergic neuronal loss in the striatum (Martins et al., 2004). Omi/HtrA2 function is also implicated in regulating stress resistance (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 \textit{omi} is required only in certain contexts, such as during exposure to oxidative stress, and that mitochondrial defects may be revealed in \textit{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.

\textbf{Interaction of Omi and Pink1}

The genetic interactions observed between \textit{pink1} and \textit{parkin} serve as an important reference for tests of the hypothesis that \textit{omi} and \textit{pink1} act in a common pathway. In contrast to \textit{pink1} mutants, which show striking defects in mitochondrial integrity in muscle and testes, and a decrease in the number of dopaminergic neurons, \textit{omi} mutants show normal mitochondrial morphology in both muscle and testes, and a normal number of dopaminergic neurons. Furthermore, in contrast to \textit{parkin} overexpression, \textit{omi} overexpression does not rescue \textit{pink1} mutant phenotypes. Overexpression of \textit{pink1} also fails to rescue male sterility due to \textit{omi} loss-of-function. Lack of \textit{pink1} does not affect the levels or the subcellular localization of Omi, and Pink1 levels and processing are not altered in \textit{omi} mutants. In addition, double mutants removing both \textit{pink1} and \textit{omi} show identical phenotypes to \textit{pink1} mutants alone, suggesting that \textit{pink1} does not negatively regulate \textit{omi}, and that \textit{omi} does not carry out partially redundant functions with \textit{pink1}. Together, these data do not provide any in vivo evidence supporting the hypothesis that \textit{omi} functions in the same pathway either upstream or downstream of \textit{pink1}, or that it acts in a parallel fashion to regulate mitochondrial morphology. These loss-of-function based analyses are more relevant to PD than are \textit{omi} overexpression based analyses, because reported \textit{Omi/HtrA2} mutations associated with PD are proposed to represent loss-of-function or dominant negative mutations (Strauss et al., 2005).

Genetic interactions between \textit{pink1} and \textit{omi} have been observed by ourselves and others (Whitworth et al., 2008) in eye-based overexpression studies: co-overexpression of \textit{pink1} with \textit{omi} results in small eye phenotypes not associated with expression of either protein alone. While in isolation, these results could be explained by a model in which \textit{omi} and \textit{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 \textit{pink1} overexpression-induced eye phenotype, and its relationship to the normal endogenous roles of \textit{pink1} in regulating mitochondrial function, is unclear. While null mutants of \textit{pink1} and \textit{parkin} show highly similar, if not identical, phenotypes in almost all assays tested, overexpression of \textit{pink1} results in a rough eye phenotype, whereas overexpression of \textit{parkin} does not (data not shown) (Poole et al., 2008; Whitworth et al., 2008). Furthermore, a PD-causing, kinase-deficient mutant form of \textit{pink1}, which fails to rescue \textit{pink1} null mutant phenotypes in multiple tissues, still interacts with \textit{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 \textit{omi} in this assay. Based on our findings, one is led to conclude that the functions of \textit{pink1} that mediate its co-overexpression interaction with \textit{omi} are distinct from the functions of \textit{pink1} and \textit{parkin} in regulating mitochondrial morphology. Such a mitochondrial integrity-independent role of \textit{pink1} may be important, but has yet to be identified in vivo. Alternatively, it is possible that the \textit{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 \textit{Drosophila rhomboid} 7 functions to promote mitochondrial fusion (McQuibban et al., 2006), while 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; Simon-Sanchez 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 suggests that it is likely that studies in Drosophila Omi are relevant to the function of Omi/HtrA2 in humans. Taken 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Overexpression based genetic interactions between *pink* and *omi*
Scanning EM and light micrographs of *Drosophila* eyes. At 18°C, *omi* overexpression (B) results in wildtype-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 since it completely suppresses the *omi* overexpression induced eye phenotype (Supplementary Fig. S2). Similarly, the eye phenotype due to *omi* overexpression cannot be modified by lack of
Fig. 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 wildtype, pink1 null mutant (pink1Δ) 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 wildtype pink1. Arrow points to pink1 expression and rp49 (*) serves as an RNA loading control.

(B–J) Schematic and phase contrast micrographs of spermatid mitochondria during the "onion stage" (C–F) and "the leaf blade stage" (G–J). Compared with wildtype, pink1 mutants show vacuolation (red arrowhead) in the nebenkern during the onion stage (D), and one instead of two mitochondrial derivatives (yellow arrow) seen in wildtype at the leaf blade stage (H). A genomic rescue transgene carrying wildtype pink1 (CaSpeR-pink1) completely rescues the male sterility due to 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, pink1G426D (CaSpeR-pink1G426D) 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 orange arrows mark each mitochondrial derivative.
Mitochondria of indirect flight muscle are labeled by mito-GFP. 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).
Fig. 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*\textsubscript{NSO} and *omi*\textsubscript{V110E} are depicted as a blue and a red asterisk, respectively. (B) Sequence alignment of Omi/HtrA2 in various species in a highly conserved region, in which the conserved Valine, mutated in *omi*\textsubscript{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 vesicles (arrow) is full of sperm (phase dark), *omi* mutants (D, D’) show 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–P) Schematics and phase contrast micrographs of spermatid mitochondria during the "onion stage" (I–L) and "the leaf blade stage" (I, M–O). Compared with wildtype (J,M), omi mutants do not show any defects in either stage (K,N), whereas pink1 mutants show vacuolation (red arrowhead) in the nebenkern during the onion stage (L), and one instead of two mitochondrial derivatives (yellow arrow) seen in wildtype at the leaf blade stage (O). (P–S) Schematic and transmission EM images of a portion of a post-individualization cyst. Each spermatid contains an axoneme (orange arrow) and mitochondrial derivative (red arrowhead) 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: wildtype: w/Y; control: w/Y; omi<sup>NSO</sup>+/+; omi mutant: w/Y; omi<sup>NSO</sup>/Df(3R)ED5644; omi mutant + rescue: w/Y; TMR-omi+/+; omi<sup>NSO</sup>/Df(3R)ED5644. Scale bars: 500µm in C–E; 500nm in Q–S.
Fig. 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-day old wildtype (A), *pink1* mutant (B) and *omi* mutant flies (C). 17–27 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 (Nassel and Elekes, 1992). The major dopaminergic neuron clusters are located near the posterior surface of the brain, with the exception of the PAL, which is located near the anterior surface (labeled in gray). (E,F) Quantification of dopaminergic neurons in each cluster in *pink1* mutant flies and wildtype flies (E), *omi* mutant flies and wildtype flies (F) aged for 40 days at 25°C. Error
bars represent standard deviations, and student T-test is used for statistical analysis. 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 days. Genotypes: wildtype: w/Y. omi mutant: w/Y; omiNSO /Df (3R)ED5644. pink1 mutant: w pink1/Y. Scale bars: 1µm in F,H,J.
Fig. 5. Lack of genetic interactions between pink1 and omi in loss-of-function studies of mitochondrial morphology

(A–H) 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 (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 orange arrows mark each mitochondrial derivative. (K–N) Mitochondria of indirect flight muscle are labeled by mito-GFP. Compared with control (K), pink1 mutants display overall reduced levels of mito-GFP 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/Y. (C,H) w pink1/Y; TMR-pink1+. (D,I) w pink1/Y; TMR-omi+. (E,J) w pink1/Y; omiNSD1E1DS(3R)E5644. (K) FM6/Y; Mef2-Gal4, UAS-mitoGFP/+ . (L) w pink1/Y; Mef2-Gal4, UAS-mitoGFP+. (M) w pink1/Y; Mef2-Gal4, UAS-mitoGFP/UAS-pink1. (N) w pink1/Y; Mef2-Gal4, UAS-mitoGFP/UAS-RNAi-omi.
Fig. 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 wildtype (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 of endogenous Pink1-9Myc expression using a genomic rescue transgene. Loss of omi function does not alter the cleavage pattern of Pink1.
Fig. 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. 3E,E’), expression of G363S (C), but not S266A (B), restores the production of motile sperm in the seminal vesicle of \textit{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 a comparable levels compared with Omi WT, as detected by an anti-Omi antibody. Western blots of head lysates from flies overexpressing OmiWT, OmiS236C or S266A using anti-Omi antibodies. 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 non-specific 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), while overexpression of Omi S236C or S266A results in wildtype-appearing eyes (F,G). GMR-Gal4 is used as an eye-specific driver. Scale bars: 500µm in B,C.
### 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>
<th>Ability to rescue omi mutant sterility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tests</td>
<td>Eye</td>
</tr>
<tr>
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<td>-</td>
<td>sterile</td>
<td>small and rough</td>
</tr>
<tr>
<td>S236C (S276C)</td>
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<td>fertile</td>
<td>wildtype appearing</td>
</tr>
<tr>
<td>S266A (S306A)</td>
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<td>protease dead</td>
<td>fertile</td>
<td>wildtype appearing</td>
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<td>small and rough</td>
</tr>
<tr>
<td>S364A (S400A)</td>
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