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Drosophila HtrA2 is dispensable for apoptosis but acts downstream of PINK1 independently from Parkin

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High temperature requirement A2 (HtrA2/Omi) is a mitochondrial protease that exhibits proapoptotic and cell-protective properties and has been linked to Parkinson's disease (PD). Impaired mitochondrial function is a common trait in PD patients, and is likely to play a significant role in pathogenesis of parkinsonism, but the molecular mechanisms remain poorly understood. Genetic studies in *Drosophila* have provided valuable insight into the function of other PD-linked genes, in particular *PINK1* and *parkin*, and their role in maintaining mitochondrial integrity. Recently, HtrA2 was shown to be phosphorylated in a PINK1-dependent manner, suggesting it might act in the PINK1 pathway. Here, we describe the characterization of mutations in *Drosophila HtrA2*, and genetic analysis of its function with *PINK1* and *parkin*. Interestingly, we find HtrA2 appears to be dispensable for developmental or stress-induced apoptosis. In addition, we found *HtrA2* mutants share some phenotypic similarities with *parkin* and *PINK1* mutants, suggesting that it may function in maintaining mitochondrial integrity. Our genetic interaction studies, including analysis of double-mutant combinations and epistasis experiments, suggest HtrA2 acts downstream of PINK1 but in a pathway parallel to Parkin.
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The mammalian HtrA (high temperature requirement) family of proteins show high homology to the bacterial chaperones DegS and DegP.[1,2] DegS, a serine protease, acts as a stress sensor by interacting with unfolded outer membrane porins via its PDZ domain.[3] This releases DegS proteolytic activity, triggering a cascade that results in activation of stress response genes via the transcription factor σE. Mammalian HtrA2/Omi, is a mitochondrial protein with both proapoptotic and cell-protective roles.[4] Its proapoptotic function is exerted through binding and cleavage of IAPs (inhibitor of apoptosis proteins) on its release into the cytoplasm following a proapoptotic stimulus.[5–8] In *Drosophila*, HtrA2 has also been reported to be released from the mitochondria on cellular insults such as UV irradiation, and to cleave DIAP1, the principal *Drosophila* IAP.[9–11]

However, genetic data have suggested that mammalian HtrA2, like its bacterial counterpart, normally functions as a stress response gene, preserving mitochondrial integrity. *mnd2* (motor neuron degeneration) mice, which have an inactivating mutation in the HtrA2 protease domain, show muscle wasting and neurodegeneration.[12] *HtrA2* knockout mice have neuronal degeneration in a subset of striatal neurons and exhibit a parkinsonian phenotype, abnormal mitochondria and reduced lifespan.[13]

In further support of a protective role, growing evidence suggests a link between HtrA2 and Parkinson's disease (PD), a progressive neurodegenerative disorder of unknown aetiology. Two mutant alleles of *HtrA2* (A141S and G399S) have been found in PD patients, leading to the classification of *HtrA2* as *PARK13* by OMIM.[14] Although one of these genetic variants was later found in non-PD controls,[15,16] Bogaerts *et al*.[17] identified a new mutation (R404W) in a large cohort of Belgian PD patients, confirming a role for HtrA2 in PD susceptibility. Importantly, recent studies have shown that HtrA2 forms a complex with the PD-related factor PINK1, a mitochondrial-targeted kinase.[18] Moreover, HtrA2 is phosphorylated in a PINK1-dependent manner in response to p38 stress-activated protein kinase (SAPK) pathway activation, suggesting that PINK1 can modulate HtrA2 activity as part of mitochondrial stress response. Overexpression studies in flies suggest PINK1 and HtrA2 may be functionally related,[19,20] although their precise relationship remains

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Abbreviations: HtrA2, high temperature requirement A2; PD, Parkinson's disease; PINK1, PTEN induced putative kinase 1; IAP, inhibitor of apoptosis protein; *mnd2*, motor neuron degeneration; SAPK, stress-activated protein kinase; MTS, mitochondrial targeting sequence; TM, transmembrane domain; PDZ, postsynaptic density protein (PSD95), disc large, zonula occludens-1; H2-Opt, HtrA2 optimal; mRpL11, mitochondrial ribosomal protein-like 11; STS, staurosporine; UV, ultraviolet; IFM, indirect flight muscle; WT, wild type; PARL, presenilin-associated rhomboid-like

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unclear. We took advantage of *Drosophila* genetics to examine *in vivo* the function of HtrA2 in the PINK1 pathway and assess its putative role in apoptosis.

Results

The *Drosophila* HtrA2 homologue, encoded by *CG8464*, contains a predicted N-terminal mitochondrial targeting sequence (MTS), a transmembrane domain (TM), a central protease domain, a C-terminal PDZ domain and an unconventional IAP-binding motif (Supplementary Figure S1a).[10] *HtrA2* encodes a full-length protein of ~46 kDa. On mitochondrial import, HtrA2 is cleaved to yield two products of 37 and 35 kDa. We have expressed and purified HtrA2 in bacteria and tested its activity towards an HtrA2 fluorescent peptide substrate (H2-Opt) as well as a control peptide as previously described.[8] These experiments revealed that HtrA2 efficiently cleaves the H2-Opt substrate but not a control peptide, suggesting that *Drosophila* HtrA2 has similar substrate specificity to its mammalian homologue (Supplementary Figure S1b).

To address the *in vivo* function of HtrA2, we generated a mutant allele by imprecise P-element excision of G4907 (Genexel Inc.), which is inserted between *HtrA2* and *mRpL11* (mitochondrial ribosomal protein-like 11; *CG3351*; Figure 1a). The G4907 stock is homozygous lethal and could be mutant for either *mRpL11* or *HtrA2*, or for both. We mobilized G4907 and generated a deletion removing 1037 bp from the insertion site to exon 1 of *HtrA2* leaving 8 bp of exon 1 (Figure 1a). This allele ($HtrA2^{\Delta 1}$) is homozygous lethal and lacks both the transcription and translation start sites of *HtrA2*, as well as the promoter region and transcription start site of *mRpL11,* and is therefore likely to be a null allele for both genes. We also recovered a precise excision as a control.

We generated genomic rescue constructs for both *HtrA2* and *mRpL11* (*gHtrA2* and *gmRpL11*; Figure 1a; Materials and Methods). The lethality of $HtrA2^{\Delta 1}$ was rescued by *gmRpL11* but not by *gHtrA2*, indicating that *mRpL11* is required for viability, as expected for a ribosomal component. $HtrA2^{\Delta 1}$ homozygotes bearing a *gmRpL11* rescue transgene represent null alleles solely for *HtrA2,* indicating *HtrA2* is non-essential.

Therefore, to further investigate the phenotypes of *HtrA2* mutant flies, we compared flies mutant for *HtrA2* and *mRPL11* bearing two copies of the gmRpL11 construct (*gmRpL11;* $HtrA2^{\Delta 1}$), which will be referred to as '$HtrA2^{\Delta 1}$', with precise excision control flies as a wild type. As an additional control, reversion of phenotypes was tested in flies carrying the $HtrA2^{\Delta 1}$ deletion and bearing two copies each of rescue constructs for both mRpL11 and HtrA2 (*gmRpL11*, *gHtrA2;* $HtrA2^{\Delta 1}$), which will be referred to as '$HtrA2^{\Delta 1}$ rescued'.

The *HtrA2* transcripts are expressed ubiquitously and uniformly in all the tested tissues (Supplementary Figure S2a–f). The precise excision, $HtrA2^{\Delta 1}$ and $HtrA2^{\Delta 1}$ rescued flies were tested by genomic PCR, RT-PCR and western blot to confirm the deletions and the absence of both transcript and HtrA2 protein in mutants (Figure 1b–d).

Several reports have suggested that HtrA2 can promote apoptosis by cleaving DIAP1, thus releasing the apical caspase Dronc in response to proapoptotic stimuli.[9–11] We therefore monitored cell death using an antibody directed against cleaved Caspase 3 in wing imaginal discs treated with γ-rays (4Gy), staurosporine (STS – 4 μM) and ultraviolet (UV) light (2.5 kJ/m^2). Apoptosis induction was identical in control (precise excision) and $HtrA2^{\Delta 1}$ mutant discs, both without treatment and on treatment (Figure 2a–f). In addition, the development of $HtrA2^{\Delta 1}$ animals is completely normal, in particular, showing normal eye morphology, indicating that HtrA2 is dispensable for retinal apoptosis during development (data not shown). This is consistent with findings in *HtrA2* knockout mice[13] and suggests that HtrA2 is dispensable for developmental and apoptosis induced by multiple stimuli *in vivo*.

To determine whether HtrA2 plays a role in maintenance of cellular homoeostasis by the PINK1 pathway, we assessed whether $HtrA2^{\Delta 1}$ flies share phenotypic similarities with *PINK1* mutants. *Drosophila PINK1* mutants exhibit locomotor defects in flight and climbing behaviours, widespread degeneration of indirect flight muscles (IFMs) with accompanying mitochondrial morphology defects, male sterility and partial loss of dopaminergic neurons.[21–23] Strikingly, all these phenotypes

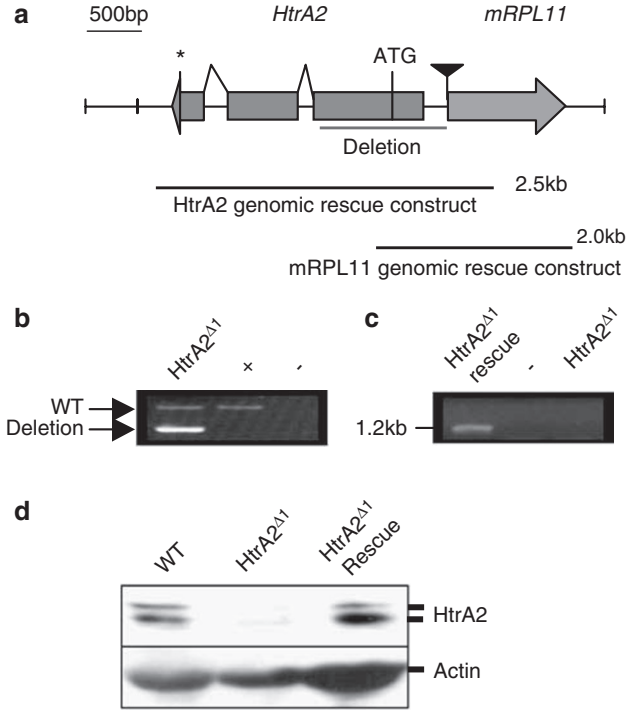

Figure 1 Generation of *HtrA2* mutants. (**a**) Schematic representation of the *HtrA2* genomic region. The P-element G4907 (black triangle) was excised to produce a deletion of 1037 bp (red line). Position of start (ATG) and stop (*) codons are indicated. Lengths of the genomic rescue constructs are indicated. (**b**) The $HtrA2^{\Delta 1}$ deletion mutant was balanced over TM3, primers from the genomic rescue construct were used to amplify a 2.5 kb (WT) product from TM3 and the resulting 1.8 kb deletion product (deletion). The positive control (+) was the amplification of the genomic product from w^{iso} flies; negative control (−, no DNA). (**c**) RT-PCR shows the absence of *HtrA2* transcript in mutants ($HtrA2^{\Delta 1}$). The *HtrA2* transcript can be amplified from the genomic rescued line ($HtrA2^{\Delta 1}$ rescue), negative control (−, no RNA). (**d**) Western blot shows the absence of HtrA2 protein in mutants ($HtrA2^{\Delta 1}$) and its presence in control and rescue flies

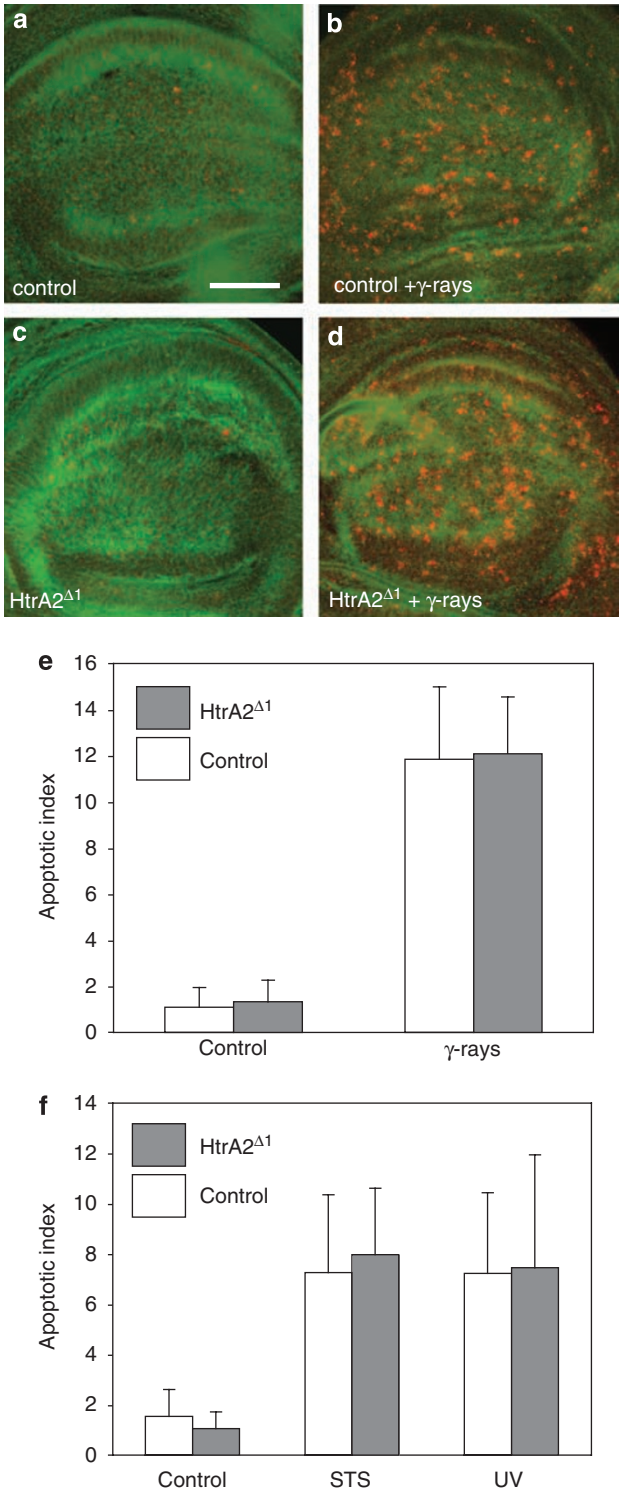

Figure 2 Apoptosis is normal in *HtrA2* mutants. (**a**–**d**) Confocal micrographs of the pouch region of wing imaginal discs stained with anti-cleaved caspase 3 (red) and phalloidin (green) after mock treatment or exposure to 4Gy γ-rays. No difference was detected between precise excision controls (**a**, **b**) and $HtrA2^{\Delta 1}$ mutants (**c**, **d**). Posterior is to the right. Scale bar = 50 μm. (**e**) Quantification of the apoptotic index from wing discs after treatment with γ-rays (see Materials and Methods). $n = 10$ for each genotype. (**f**) Quantification of the apoptotic index from wing discs after treatment with Staurosporine or UV light (see Materials and Methods). $n = 8$ for each genotype. Error bars represent standard deviations

are phenocopied in *Drosophila* mutants of another PD-linked gene, *parkin*,[24–27] which was shown to act genetically downstream of *PINK1*.[21–23] Thus, we investigated such phenotypes in *HtrA2* mutants.

Myopathology was investigated using toluidine blue staining of IFMs in longitudinal sections of adult thoraces (Figure 3a–c). Although marked myopathology akin to that described in *parkin* and *PINK1* mutants was not observed (in 5- or 30–day-old animals), more detailed investigation will be needed to determine whether *Htr2A* dysfunction leads to overt myodegeneration. However, examination of IFM ultrastructure from aged $HtrA2^{\Delta 1}$ mutants revealed increased numbers of defective mitochondria, displaying reduced electron density and open cristae, compared with wild-type and $HtrA2^{\Delta 1}$-rescued controls (Figure 3d–g; Supplementary S3). It should be noted that the mitochondrial abnormalities seen in $HtrA2^{\Delta 1}$ mutants are not widespread, suggesting this does not reflect a general mitochondrial defect. To confirm this, we measured mitochondrial activity by high-resolution respirometry in whole flies using 30-day-old males. Coupled mitochondria from WT and $HtrA2^{\Delta 1}$ showed no difference in oxygen consumption with substrates of complex I and complex II in the presence of ADP (Supplementary Figure S4a, b). This was confirmed by measuring respiratory activities of complex II and complex IV under uncoupled conditions (Supplementary Figure S4c, d). Thus, there is no systemic mitochondrial deficiency in $HtrA2^{\Delta 1}$ mutants compared with controls.

To determine if, like *PINK1* and *parkin* mutants, $HtrA2^{\Delta 1}$ mutant flies present locomotor deficits, $HtrA2^{\Delta 1}$ mutants were assayed for climbing and flight ability. $HtrA2^{\Delta 1}$ mutants showed wild-type flight and climbing ability at 5 days of age (Figure 3h and i). However, 30-day-old $HtrA2^{\Delta 1}$ flies show significantly reduced flight and climbing ability compared with wild-type flies. This defect was completely restored in $HtrA2^{\Delta 1}$ rescued flies (Figure 3h and i). Thus, the progressive mitochondrial morphological abnormalities seen in $HtrA2^{\Delta 1}$ mutants correlate with progressive motor deficits.

In further similarity to *parkin/PINK1* mutants, we also find that $HtrA2^{\Delta 1}$ males are sterile, whereas female fertility is unaffected. $HtrA2^{\Delta 1}$ males are almost completely sterile (4% fertility compared to 100% fertility in control flies; Figure 4a). Importantly, male fertility is restored to wild-type levels in $HtrA2^{\Delta 1}$ rescued lines (Figure 4a). However, although *PINK1/parkin* mutants exhibit distinct morphological defects during spermatogenesis,[21,22,27,28] the ultrastructural analysis of $HtrA2^{\Delta 1}$ testes revealed no observable defects (Figure 4b). Indeed, mature individualized sperm are formed properly, however, we noted that $HtrA2^{\Delta 1}$ sperm were completely immotile, as was the case for *PINK1* mutants (see Supplementary movies S1–3 in Supplementary materials).

Recent evidence indicates HtrA2 likely plays a role in cellular stress response mechanisms.[18] To test this we determined the effect of loss of $HtrA2^{\Delta 1}$ function on longevity and stress resistance. $HtrA2^{\Delta 1}$ mutants have a reduced lifespan compared to rescued or precise excision controls (Figure 5a). $HtrA2^{\Delta 1}$ flies were also sensitive to paraquat-induced oxidative stress and mitochondrial respiration deficit induced by complex I inhibitor rotenone (Figure 5b and c).

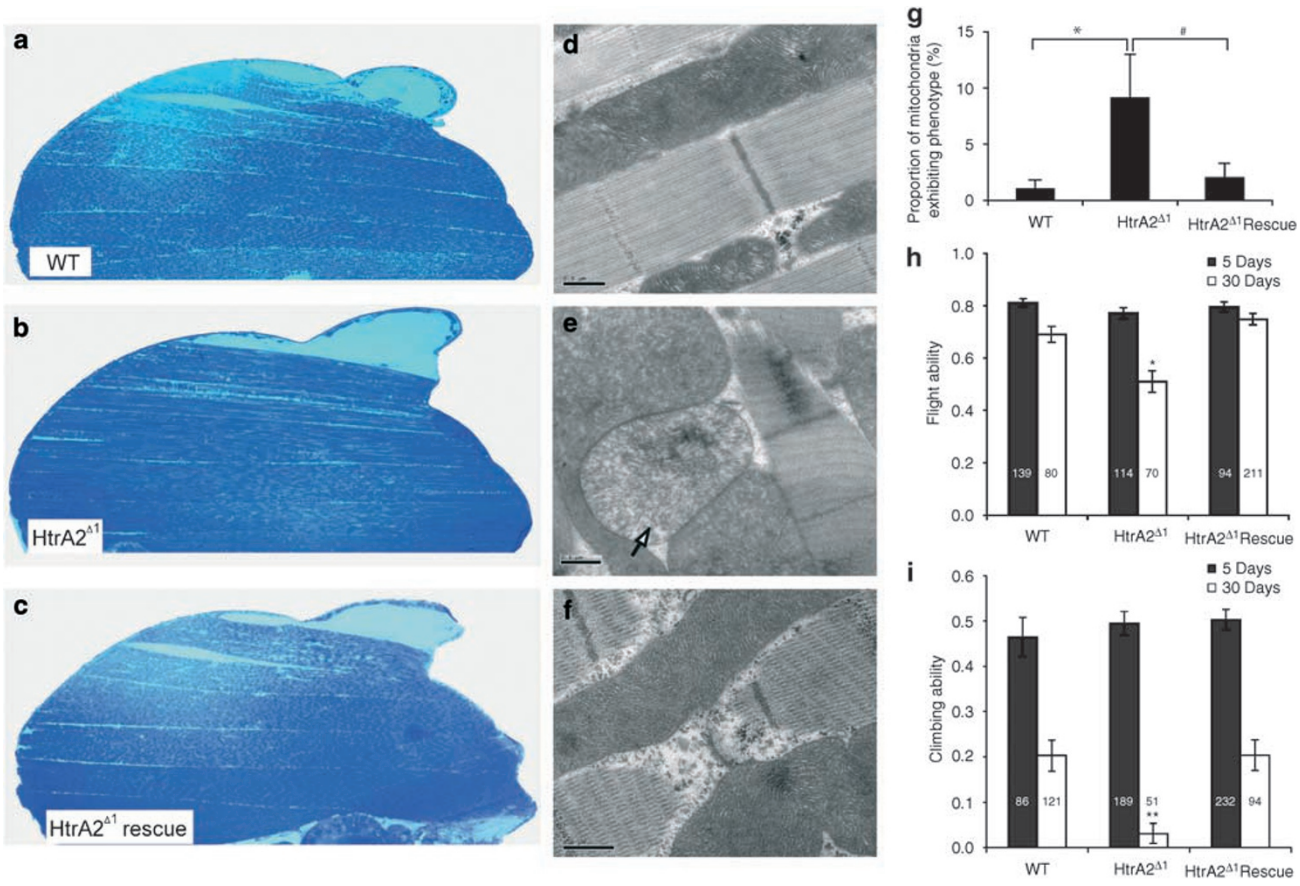

Figure 3 *HtrA2* mutants show mild mitochondrial disruption and progressive locomotor deficits. (**a–c**) Toluidine blue-stained, whole thorax sections of 30-day-old flies revealed that overall IFM structure is maintained in *HtrA2*$^{\Delta 1}$ mutant flies. (**d–f**) IFM mitochondria in TEM micrographs exhibit morphological abnormalities, typically reduced electron density and disrupted cristae in *HtrA2*$^{\Delta 1}$ mutant (arrow in **e**), compared to wild type (**d**) and *HtrA2*$^{\Delta 1}$ rescue flies (**f**). (**g**) Quantification of the mitochondrial phenotype from multiple individuals. Bars show mean and standard error (see Materials and Methods). *HtrA2*$^{\Delta 1}$ mutant flies have an increased proportion of mitochondria with reduced electron density, as determined by a Fisher's exact test (*$P < 0.01$, # = 0.058). *HtrA2*$^{\Delta 1}$ mutants show progressive deficits in flight (**h**) and climbing (**i**). Five-day-old mutant flies have wild-type flight and climbing ability. Aged (30–day-old) mutants show reduced flight and climbing, which is restored in *HtrA2*$^{\Delta 1}$ rescue flies. Bars show mean and standard error, inset numbers show *n*. **$P < 0.001$; *$P < 0.01$, by Kruskal–Wallis non-parametric analysis and Dunn's pair-wise comparison

Given the evidence linking HtrA2 to PD, we determined whether the dopaminergic neurons are affected in *HtrA2*$^{\Delta 1}$ flies. Although *parkin* and *PINK1* mutants show age-dependent dopaminergic neuron loss,[21,23,25] we found 30–day-old *HtrA2*$^{\Delta 1}$ flies showed no difference in number of dopaminergic neurons (Figure 6). However, this is also consistent with the weaker phenotypes in other tissues.

Thus, *HtrA2*$^{\Delta 1}$ mutant flies share a number of phenotypic similarities with *parkin/PINK1* mutants, though generally the *HtrA2*$^{\Delta 1}$ phenotypes are weaker. To address whether HtrA2 functions in a common or divergent pathways to PINK1/Parkin, we generated double mutant combinations and determined the effect on climbing ability. Previously, PINK1 and Parkin have been reported to act in a common pathway, consistent with the observation that *PINK1:parkin* double mutants do not have a more severe phenotype than either of the single mutants.[21–23] Corroborating these findings we find *PINK1:parkin* double mutants do not show a difference in climbing ability compared to single mutants of either *PINK1* or *parkin* (Figure 7a). Similarly, we find *PINK1:HtrA2* double mutants show no difference in climbing ability compared with *PINK1* mutants (Figure 7b), consistent with them acting in a common pathway. In contrast, we find that *HtrA2:parkin* double mutants show a dramatically enhanced climbing defect in comparison to *parkin* mutants (Figure 7c), suggesting that HtrA2 might act in a different pathway from Parkin. The *PINK1* mutant defects are rescued by overexpression of *parkin*, leading to the suggestion that Parkin acts downstream of PINK1.[21–23] We therefore generated transgenic animals expressing HtrA2 under GAL4/UAS control and tested its ability to rescue *PINK1* phenotypes. Interestingly, ubiquitous expression of the HtrA2 transgene significantly rescued the *PINK1* climbing defects (Figure 8). Together, these results suggest that HtrA2 likely plays a role in the PINK1 pathway.

Discussion

We have characterized the *Drosophila* homologue of the HtrA2 protease and found that *HtrA2* mutants share some phenotypic characteristics with previously described *Drosophila* models of PD. *HtrA2* null mutants are viable but exhibit mild mitochondrial defects, loss of flight and climbing ability,

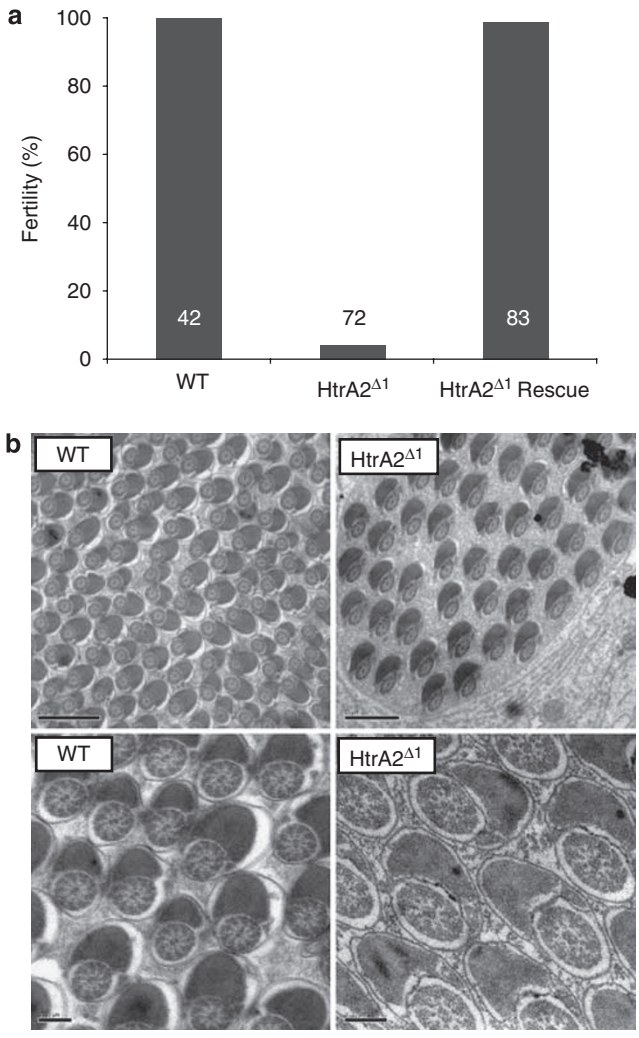

Figure 4 *HtrA2*$^{\Delta 1}$ have normal spermatogenesis but are male sterile. (**a**) *HtrA2*$^{\Delta 1}$ males have greatly reduced fertility compared to controls. Normal fertility is regained in *HtrA2*$^{\Delta 1}$ rescued flies. Inset numbers represent *n* value. (**b**) TEM micrographs of mature individualized spermatids show no structural defects in the axoneme or nebenkern. Scale bar shows 1 μm and 0.2 μm in upper and lower panels, respectively

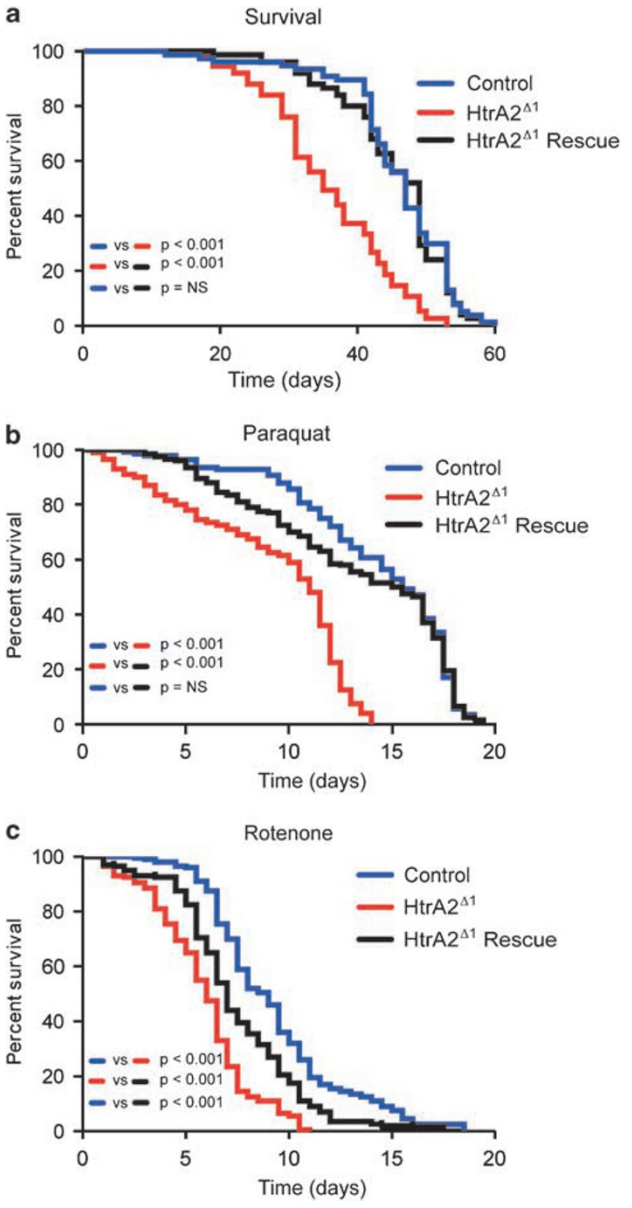

Figure 5 *HtrA2*$^{\Delta 1}$ have reduced survival under normal and stressed conditions. (**a**) Male *HtrA2*$^{\Delta 1}$ flies (red) have reduced lifespan compared with wild-type control (blue), which is restored with a genomic rescue construct (black). $n = 75$ for each genotype. (**b**) *HtrA2*$^{\Delta 1}$ mutant flies (red) are less resistant to oxidative stress resulting from paraquat ingestion compared to wild-type controls (blue) or rescued flies (black). $n = 200$ for each genotype. (**c**) *HtrA2*$^{\Delta 1}$ mutant flies are more sensitive to rotenone-induced oxidative stress (red) than wild-type controls (blue) or the *HtrA2*$^{\Delta 1}$ rescued line (black). $n = 200$ for each class. Data are presented as Kaplan–Meier survival distributions, Log-rank tests were performed to determine statistical significance. Individual *P*-values are shown with the figure

male infertility, and sensitivity to oxidative stress and mitochondrial toxins (Figures 3–6). In contrast, we find that HtrA2 is not required for developmental or stress-induced apoptosis (Figure 2). These data are consistent with findings in mice and humans, which suggest that, HtrA2 functions primarily to maintain mitochondrial integrity and protect cells against oxidative stress,[12,13] and contradicts previous reports that HtrA2 is a proapoptotic factor.[9–11]

Recently, Yun *et al*.[20] have reported a characterization of ethanemethylsulphonate-induced alleles of *Drosophila HtrA2/Omi*. In accordance with our results, they show that HtrA2 is required for male fertility, normal lifespan and stress resistance. They do not report a mitochondrial defect, though the mild nature of the defect observed in our hands and allelic or environmental differences might explain this discrepancy. Both of our reports suggest that *HtrA2* mutants have a markedly weaker phenotype than *PINK1*, which is consistent with the idea that HtrA2 does not play a critical role in the PINK1-Parkin interaction. However, our genetic interaction studies presented here, in conjunction with our previous evidence,[19] support the view that HtrA2 acts downstream of PINK1 in a divergent pathway parallel to Parkin.

We have previously shown that a *PINK1* overexpression phenotype is partially suppressed by loss of *HtrA2*, whereas conversely an *HtrA2* overexpression phenotype is not

suppressed by *PINK1* mutations,[19] suggesting that HtrA2 functions downstream of PINK1. In a complementary approach using a loss of function phenotype, we show here that overexpression of *HtrA2* can partially substitute for loss of PINK1 (Figure 8). These findings are consistent with our previous report and suggest that HtrA2 acts downstream of PINK1. Although the study of Yun *et al*. did not uncover evidence for HtrA2 acting downstream of PINK1, technical differences, particularly reflecting transgenic expression levels, may account for this discrepancy.

In agreement with Yun *et al*. we have shown that, similar to *PINK1:parkin* double mutants, *PINK1:HtrA2* double mutants display an identical phenotype to *PINK1* mutants alone, suggesting they act in a common pathway (Figure 7). However, we also showed that *parkin:HtrA2* double mutants display a stronger phenotype than either mutant alone (Figure 7), suggesting HtrA2 acts in parallel pathway to Parkin. These results are also consistent with our previous findings that loss of either *HtrA2* or *parkin* can partially suppress a PINK1 overexpression phenotype, whereas attenuating both genes together is sufficient to completely suppress the overexpression of PINK1.[19] Together these findings support the notion that HtrA2 and Parkin are acting as downstream effectors of PINK1 but in parallel pathways. The relative weakness of the *HtrA2* mutant phenotype suggests that it is not the predominant PINK1 effector.

The most pressing questions remain how this pathway is triggered and how its activation leads to protection from mitochondrial stress. By analogy to bacterial DegS, it is possible that the PDZ domain of HtrA2 acts as a sensor for unfolded proteins. In addition, we and others have shown that the mitochondrial protease Rhomboid-7/PARL can process both PINK1 and HtrA2,[19,29] which may represent a regulatory process. Lastly, the p38 SAPK pathway is known to be triggered by reactive oxygen species, which are produced when mitochondrial electron transport is perturbed.[30] Mitochondrial dysfunction may therefore modulate HtrA2 activity at several levels. Once activated, HtrA2, like DegS, may cleave unfolded protein and/or elicit a transcriptional response to clear the damaged proteins.[4] Further work will be required to fully elucidate the functional relationship between PINK1

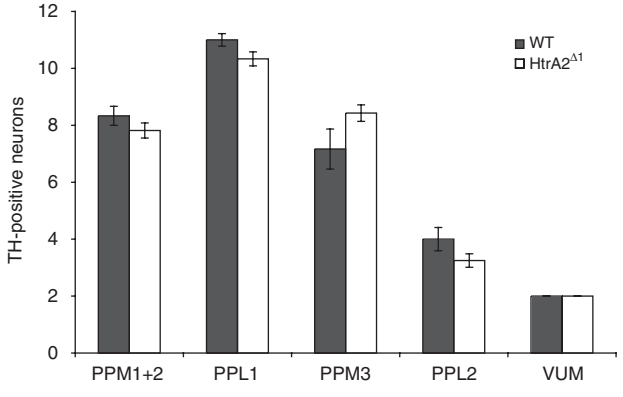

Figure 6 Loss of HtrA2 does not cause dopaminergic neurons loss. Number of neurons staining positive with anti-tyrosine hydroxylase antiserum from aged (30-day) precise excision control (black) and *HtrA2* mutants (grey). No difference is seen in any of the clusters. Bars show mean and standard error ($n \geqslant 10$)

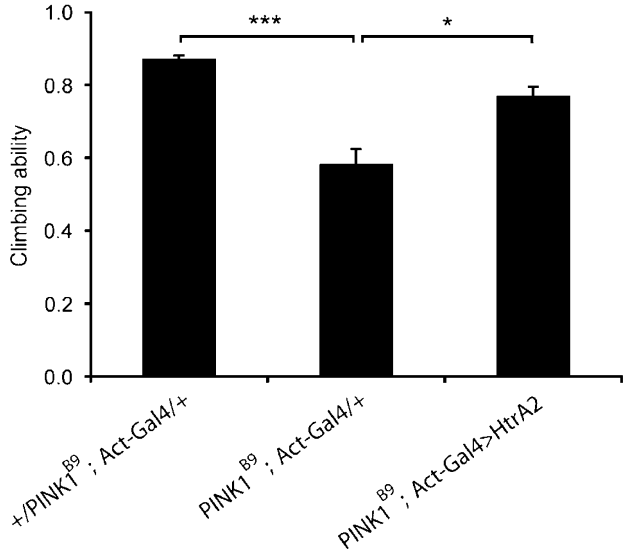

Figure 8 Overexpression of *HtrA2* can rescue *PINK1* climbing defect. Compared to heterozygous controls, *PINK1* mutants have reduced climbing ability, which is suppressed by overexpression of HtrA2 under the control of the *actin5c* promoter. Bars show mean and standard error; $n > 50$. Significance was established using a Kruskal–Wallis test and Dunn's comparison (***$P < 0.0001$; *$P < 0.01$)

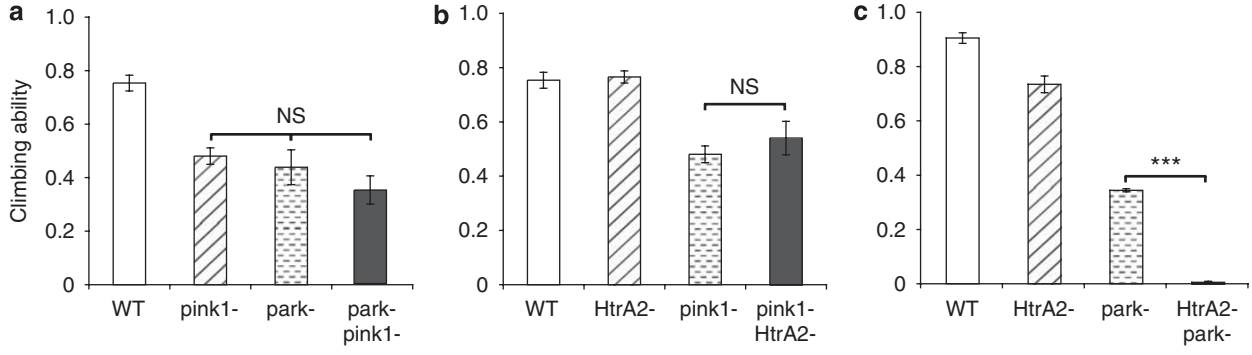

Figure 7 *HtrA2* mutants enhance the locomotor deficit exhibited by *parkin* mutants but not *PINK1* mutants. (**a**) Climbing ability of $PINK1^{B9}$, $park^{25}$ and $PINK1^{B9}$:$park^{25}$ double mutants. (**b**) Climbing ability of $HtrA2^{\Delta 11}$, $PINK1^{B9}$ and $PINK1^{B9}$:$HtrA2^{\Delta 11}$ double mutants. (**c**) Climbing ability of $HtrA2^{\Delta 11}$, $park^{25}$ and $park^{25}$:$HtrA2^{\Delta 11}$ double mutants. Bars show mean and standard error ($n \geqslant 50$). ***$P < 0.0001$

and HtrA2, and the involvement of this putative pathway in PD pathogenesis.

Materials and Methods

***Drosophila* genetics.** *HtrA2* mutants were created by imprecise excision of P-element G4907 (Genexel Inc.). Breakpoints were mapped by genomic PCR and sequencing. *PINK1*^{B9} mutants were a kind gift from J Chung.[21] *park*^{25} mutants have been described previously.[27] All fly experiments were carried out at 25 °C and on the same food batch for each experiment.

For crosses to combine *PINK1* mutants with GAL4/UAS transgenes, paternal males with marked X chromosomes (y^- or FM6) were used to ensure correct identification of *PINK1* mutant progeny.

Fertility assays. Single 1-day-old males of each genotype were individually placed in a vial with three virgin females. The number of vials with larvae was recorded after 5 days.

Flight/climbing assay. Flight/climbing assays were performed as previously described.[27] ($n \geqslant 30$ per genotype, unless otherwise stated within figures.) Statistical significance was calculated by Kruskal–Wallis non-parametric analysis and Dunn's pair-wise comparison.

Longevity assay. A total of 50, 0- to 4-h-old males and females of each genotype were placed into separate vials in groups of 25 for each gender. The flies were transferred to fresh food every 2 days and the number of live flies recorded. Data are presented as Kaplan–Meier survival distributions and significance determined by Log-rank tests.

Oxidative stress assay. The procedure used for longevity assay was repeated for oxidative stress assays using food containing 20 mM paraquat or 20 mM rotenone. Data are presented as Kaplan–Meier survival distributions and significance determined by Log-rank tests.

Molecular biology

RT-PCR. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed using the kit SuperScript III One-step RT-PCR with Platinum Taq (Invitrogen). The 5′ and 3′ sequences used to amplify the dHtrA ORF are *ATGGCTTTGCGCGGTTCC* and *CTAGGGATCTTCTGGCGTA*, respectively. Primers used to amplify 5′ and 3′ dPINK1 ORF are *ATGTCTGTG AGACTGCTGAC* and *CTACGCCACCACATTCTGGA*, respectively.

To detect transcripts by RT-PCR in mutants, 5′ primer *GTTGTGCCG CTGTCGTCTAT* and 3′ primer *CGTCGTGCGAAACGTACG* were used to amplify a product of 1 kb between exon 2 and exon 3, respectively.

Genomic rescue constructs. The predicted full-length HtrA2 gene, 700 bp of upstream promoter region (including the complete 3′ UTR), was amplified by PCR using 5′ primer *CAACTCGAGGAAGTACATTGGGCGGGTC* and 3′ primer *GGGACTAGTGGGTTTGTCAGCGATTTC*, sequenced and subcloned into pCaSpeR-HS using *Xho*I and *Spe*I restriction sites. mRPL11 was constructed by including 463 bp of upstream promoter region, the entire predicted gene and 108 bp of downstream genomic region using 5′ primer *GGGTCTAGAGCAGCTGAT TTCAATTTGGC* with *Xba*I restriction site and 3′ primer GGGGAATTCCACT GGAAAACTCAACAAGC with *Eco*RI restriction site, sequenced and subcloned into pCaSpeR-HS.

pUASp construct. The *dHtrA2* ORF was amplified using 5′ primer *TCACGCGGCCGCATGGCTTTGCGCGGTTCC* containing *Not*I restriction site, and 3′ primer *ACAGCTCGAGCTAGGGATCTTCTGGCGTA* containing *Xho*I restriction site. The sequenced, digested product was subcloned into pUASp. Western blotting was performed by standard procedures. The anti-HtrA2 antibody (used at 1:1000) was a kind gift from Dr. E. Alnemri (Kimmel Cancer Institute, USA).

Cell biology

Tissue sectioning. Thoraces and testes were prepared from 30- and 2-day-old adult flies, respectively, and fixed overnight in 2% paraformaldehyde, 2.5% gluteraldehyde in sodium cacodylate (0.1 M). Thoraces were rinsed in sodium cacodylate (0.1 M) and 1% tannic acid and postfixed in 1:1 2% OsO_4 and sodium cacodylate (0.2 M) for 1 h. After rinsing, thoraces were dehydrated in an ethanol series and embedded using Spurr's resin. Semithin sections were then taken and stained with toluidine blue, whereas ultrathin sections were examined using TEM. Quantification of the mitochondrial phenotype was carried out under blinded conditions for both imaging and analysis. Ten randomly selected fields of view were captured per individual and the number of mitochondria exhibiting a disrupted morphology phenotype and those with normal mitochondria were counted. Approximately 1500 mitochondria were examined per genotype. Pair-wise differences in proportion of mitochondria exhibiting the phenotype were made utilizing the conservative Fisher's exact test.

Dopaminergic neuron counts. Brains were dissected from 30-day-old flies and fixed in 4% paraformaldehyde, rinsed in PBS and incubated with anti-TH antibody (Immunostar Inc., Hudson, WI, USA) in PBS-T BSA (0.5%) overnight (4 °C). After washing with PBS-T, brains were incubated (2 h) with Alexafluor 488 secondary antibody (Invitrogen) in PBS-T BSA (0.5%). After washing, brains were mounted and TH-positive neurons imaged by confocal microscopy. In all cases, TH-positive neurons were counted with the investigator blinded to the genotypes.

Apoptosis assay. Third instar wing imaginal discs were exposed to 4 Gy γ-rays, fixed and stained with anti-caspase 3 after 2 h (Cell Signaling Technologies, Danvers, MA, USA) and FTIC-phalloidin (Sigma, Highland, IL, USA) as previously described.[31] For STS (4 μM) and UV treatment (2.5 kJ/m^2), larvae were dissected and discs were incubated in Schneider's medium (Invitrogen), then fixed and stained after 4 h. Images were acquired on a Zeiss LSM 510 confocal microscope. The apoptotic index corresponds to the percentage of caspase 3-positive pixels relative to the total area, and was determined as described using Image J.[31] For consistency, the analysis was performed on the pouch region of the wing discs.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)