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

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Abstract

High Temperature Requirement A2 (HtrA2/Omi) is a mitochondrial protease that exhibits pro-apoptotic and cell protective properties and has been linked to Parkinson 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.

Introduction

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

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stress response genes via the transcription factor σE . Mammalian HtrA2/Omi, is a mitochondrial protein with both pro-apoptotic and cell protective roles. 4 Its pro-apoptotic function is exerted through binding and cleavage of IAPs (Inhibitor of Apoptosis Proteins) upon its release into the cytoplasm following a pro-apoptotic stimulus. 5-8 In *Drosophila*, HtrA2 has also been reported to be released from the mitochondria upon 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 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. identified a new mutation (Arg404) in a large cohort of Belgian PD patients, confirming a role for HtrA2 in PD susceptibility. 17 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 SAPK (Stress Activated Protein Kinase) 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 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 trans-membrane domain (TM), a central protease domain, a C-terminal PDZ domain and an unconventional IAP-binding motif (Figure S1a and ¹⁰). *HtrA2* encodes a full-length protein of ~46kDa. Upon mitochondrial import, HtrA2 is cleaved to yield two products of 37 and 35kDa. 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 (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*, *HtrA2* or both. We mobilized G4907 and generated a deletion removing 1037bp from the insertion site to exon 1 of *HtrA2* leaving 8bp of exon 1 (Figure 1a). This allele (*HtrA2^{Δ1}*) 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 and methods). The lethality of *HtrA2^{Δ1}* was rescued by *gmRpL11* but

not by *gHtrA2*, indicating that *mRPL11* is required for viability, as expected for a ribosomal component. *HtrA2^{Δ1}* 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^{Δ1}*), which will be referred to as '*HtrA2^{Δ1}*', with precise excision control flies as a wild type. As an additional control, reversion of phenotypes was tested in flies carrying the *HtrA2^{Δ1}* deletion and bearing two copies each of rescue constructs for both *mRPL11* and *HtrA2* (*gmRPL11, gHtrA2; HtrA2^{Δ1}*), which will be referred to as '*HtrA2^{Δ1}* rescued'.

The *HtrA2* transcripts are expressed ubiquitously and uniformly in all tissues tested (Figure S2a-f). The precise excision, *HtrA2^{Δ1}* and *HtrA2^{Δ1}* 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 pro-apoptotic 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 μ M) and ultraviolet (UV) light (2.5 kJ/m²). Apoptosis induction was identical in control (precise excision) and *HtrA2^{Δ1}* mutant discs, both without treatment or upon treatment (Figure 2a-f). In addition, the development of *HtrA2^{Δ1}* 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 homeostasis by the PINK1 pathway, we assessed whether *HtrA2^{Δ1}* 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 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). While 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 *HtrA2* dysfunction leads to overt myodegeneration. However, examination of IFM ultrastructure from aged *HtrA2^{Δ1}* mutants revealed increased numbers of defective mitochondria, displaying reduced electron density and open cristae, compared with wild type and *HtrA2^{Δ1}* rescued controls (Figures 3c-f and S3). It should be noted that the mitochondrial abnormalities seen in *HtrA2^{Δ1}* 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^{Δ1}* showed no difference in oxygen consumption with substrates of complex I and complex II in the presence of ADP (Figure S4a, b). This was confirmed by measuring respiratory activities of complex II and complex IV under uncoupled conditions (Figure S4c, d). Thus, there is no systemic mitochondrial deficiency in *HtrA2^{Δ1}* mutants compared with controls.

To determine if, like *PINK1* and *parkin* mutants, *HtrA2^{Δ1}* mutant flies present locomotor deficits, *HtrA2^{Δ1}* mutants were assayed for climbing and flight ability. *HtrA2^{Δ1}* mutants

showed wild-type flight and climbing ability at 5-days of age (Figure 3h, i). However, 30-day-old *HtrA2^{Δ1}* flies show significantly reduced flight and climbing ability compared with wild type flies. This defect was completely restored in *HtrA2^{Δ1}* rescued flies (Figure 3h, i). Thus, the progressive mitochondrial morphological abnormalities seen in *HtrA2^{Δ1}* mutants correlates with progressive motor deficits.

In further similarity to *parkin/PINK1* mutants, we also find *HtrA2^{Δ1}* males are sterile, while female fertility is unaffected. *HtrA2^{Δ1}* 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^{Δ1}* rescued lines (Figure 4a). However, while *PINK1/parkin* mutants exhibit distinct morphological defects during spermatogenesis, 21,22,27,28 the ultrastructural analysis of *HtrA2^{Δ1}* testes revealed no observable defects (Figure 4b). Indeed, mature individualized sperm are formed properly, however, we noted that *HtrA2^{Δ1}* sperm were completely immotile, as was the case for *PINK1* mutants (see 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^{Δ1}* function on longevity and stress resistance. *HtrA2^{Δ1}* mutants have a reduced lifespan compared to rescued or precise excision controls (Figure 5a). *HtrA2^{Δ1}* flies were also sensitive to paraquat-induced oxidative stress and mitochondrial respiration deficit induced by complex I inhibitor rotenone (Figure 5b, c).

Given the evidence linking HtrA2 to PD, we determined whether the dopaminergic neurons are affected in *HtrA2^{Δ1}* flies. While *parkin* and *PINK1* mutants show age-dependent dopaminergic neuron loss, 21,23,25 we found 30 day old *HtrA2^{Δ1}* 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^{Δ1}* mutant flies share a number of phenotypic similarities with *parkin/PINK1* mutants, though generally the *HtrA2^{Δ1}* 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 single mutant. 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 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, 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 pro-apoptotic factor. 9-11

Recently, Yun et al have reported a characterization of ethanemethylsulfonate-induced alleles of *Drosophila HtrA2/Omi*.²⁰ 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 evidence¹⁹, support the view that *HtrA2* acts downstream of *PINK1* in a divergent pathway parallel to Parkin.

We have previously shown that a *PINK1* over-expression phenotype is partially suppressed by loss of *HtrA2*, while conversely an *HtrA2* overexpression phenotype is not suppressed by *PINK1* mutations¹⁹, 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*. While 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, while 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* may cleave unfolded protein and/or elicit a transcriptional response to clear the damaged proteins, like DegS.⁴ Further work will be required to fully elucidate the functional relationship between *PINK1* and *HtrA2*, and the involvement of this putative pathway in PD pathogenesis.

EXPERIMENTAL PROCEDURES

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 *park²⁵* 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 3 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 $n = 30$ per genotype, unless otherwise stated within figures. Statistical significance calculated by Kruskal-Wallis non-parametric analysis and Dunn's pair-wise comparison.

Longevity assay—50 0-4-hours-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 is 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 20mM paraquat or 20mM rotenone. Data is 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 *ATGTCTGTGAGACTGCTGAC* and *CTACGCCACCACATTCTGGA* respectively. 5' primer *GTTGTGCCGCTGTCGTCTAT* and 3' primer *CGTCGTGCCGAAACGTACG* were used to amplify a product of 1kb between exon 2 and exon 3 respectively to detect transcripts by RT-PCR in mutants.

Genomic Rescue Constructs—The predicted full-length *HtrA2* gene, 700bp of upstream promoter region (including the complete 3' UTR) was amplified by PCR using 5' primer *CAACTCGAGGAAGTACATTGGGCGGGTC* and 3' primer *GGGACTAGTGGGTTTGTACGCGATTTTC*, sequenced and subcloned into pCaSpeR-HS using XhoI and SpeI restriction sites. mRPL11 was constructed by including 463bp of upstream promoter region, the entire predicted gene and 108bp of downstream genomic region using 5' primer *GGGTCTAGAGCAGCTGATTTCAATTTGGC* with XbaI restriction site and 3' primer *GGGGAATTCCTACTGGAAAACCTCAACAAGC* with EcoRI restriction site, sequenced and subcloned into pCaSpeR-HS.

pUASp construct—the *dHtrA2* ORF was amplified using 5' primer *TCACGCGGCCGCATGGCTTTGCGCGGTTCC* containing *NotI* restriction site, and 3' primer *ACAGCTCGAGCTAGGGATCTTCTGGCGTA* containing XhoI 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-day old and 2-day old, adult flies, respectively and fixed overnight in 2% paraformaldehyde, 2.5% glutaraldehyde in sodium cacodylate (0.1M). Thoraces were rinsed in sodium cacodylate (0.1M) and 1% tannic acid and post-fixed in 1:1 2% OsO₄ and sodium cacodylate (0.2M) for 1h. After rinsing, thoraces were dehydrated in an ethanol series and embedded using Spurr's resin. Semi-thin sections were then taken and stained with Toluidine blue, while ultra-thin sections were examined using TEM. Quantification of the mitochondrial phenotype was carried out under blinded conditions for both imaging and analysis. 10 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 exact test.

Dopaminergic neuron counts—Brains were dissected from 30-day old flies and fixed in 4% para-formaldehyde, 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 (2h) 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 4Gy γ -rays, fixed and stained with anti-caspase 3 after 2h (Cell Signaling Technologies, Danvers, MA, USA) and FTIC-phalloidin (Sigma, Highland, IL, USA) as previously described 31. For STS (4 μ M) and UV treatment (2.5 kJ/m²), larvae were dissected and discs were incubated in Schneider's medium (Invitrogen, Carlsbad, CA, USA), then fixed and stained after 4h. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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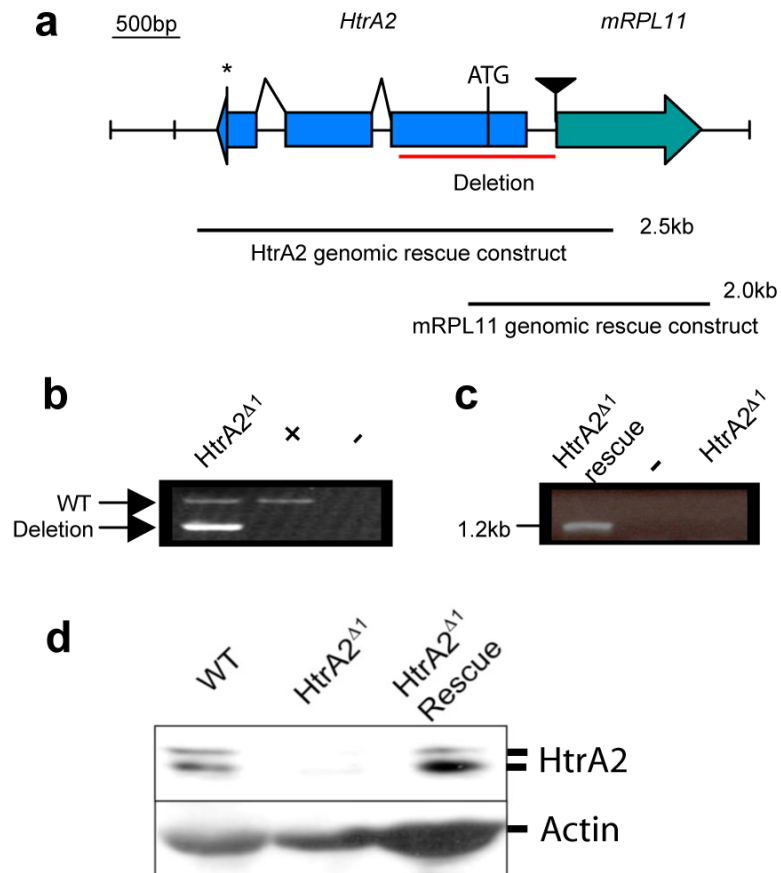


Figure 1. Generation of *HtrA2* mutants

(a) Schematic representation of the *HtrA2* genomic region. The P-element G4907 (black triangle) was excised to produce a deletion of 1037bp (red line). Position of start (ATG) and stop (*) codons are indicated. Lengths of the genomic rescue constructs are indicated. (b) The *HtrA2^{Δ1}* deletion mutant was balanced over TM3, primers from the genomic rescue construct were used to amplify a 2.5kb (WT) product from TM3 and the resulting 1.8kb 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^{Δ1}*). The *HtrA2* transcript can be amplified from the genomic rescued line (*HtrA2^{Δ1}* rescue), negative control (-, no RNA). (d) Western blot shows the absence of HtrA2 protein in mutants (*HtrA2^{Δ1}*) and its presence in control and rescue flies.

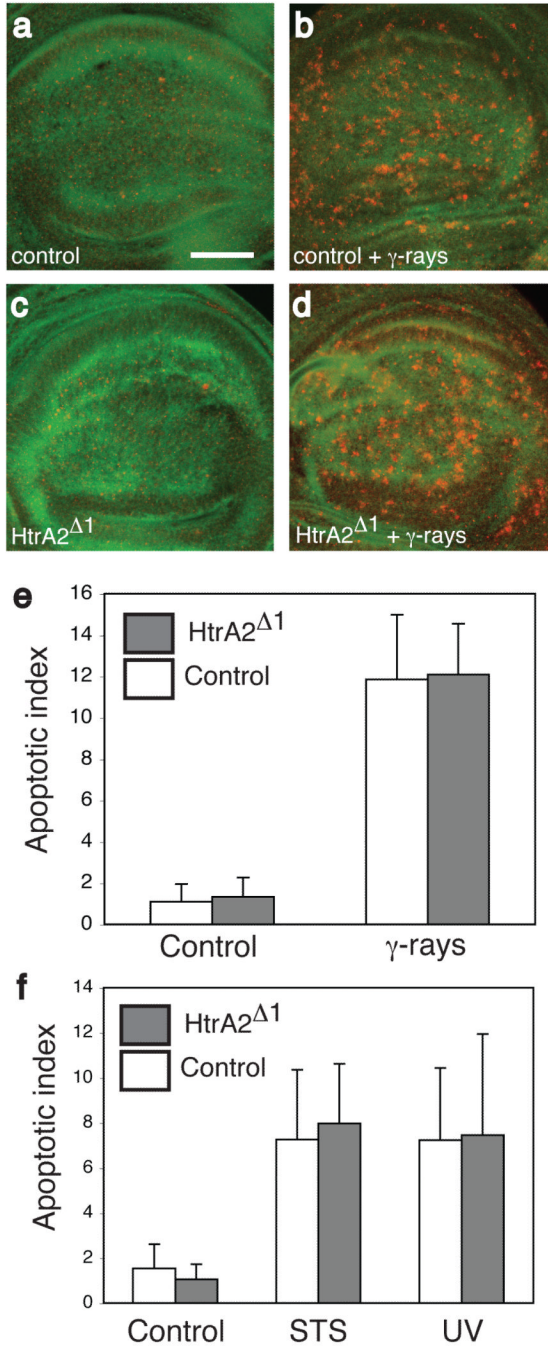


Figure 2. Apoptosis is normal in *HtrA2* mutants

(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* ^{Δ 1} mutants (c, d). Posterior is to the right. Scale bar = 50 μ m. (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.

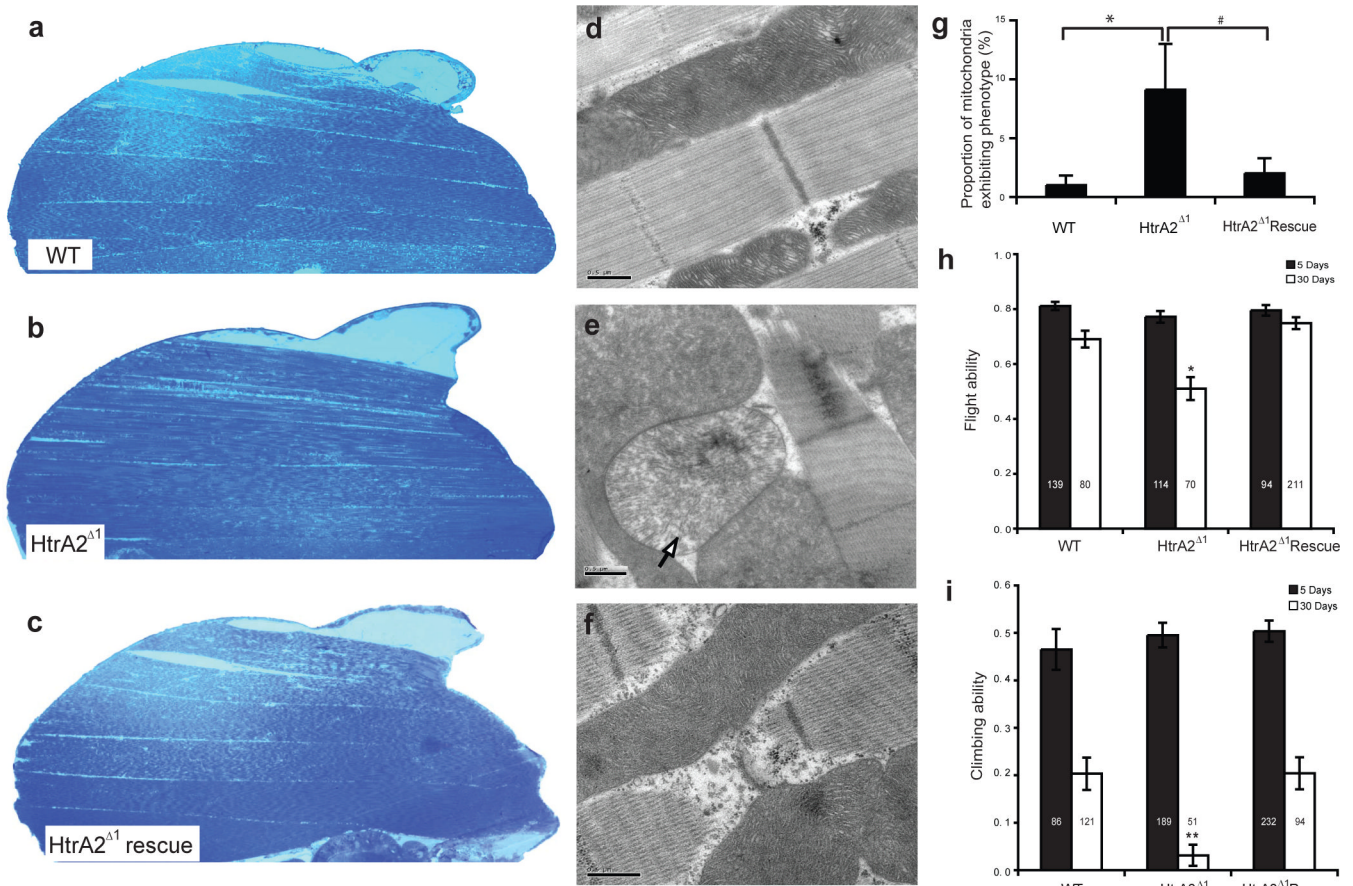


Figure 3. *HtrA2* mutants show mild mitochondrial disruption and progressive locomotor deficits (a-c) Toluidine blue stained, whole thorax sections of 30 day old flies revealed overall IFM structure is maintained in *HtrA2*^{Δ1} mutant flies. (d-f) IFM mitochondria in TEM micrographs exhibit morphological abnormalities, typically reduced electron density and disrupted cristae in *HtrA2*^{Δ1} mutant (arrow in e), compared to wild type (d) and *HtrA2*^{Δ1} rescue flies (f). (g) Quantification of the mitochondrial phenotype from multiple individuals. Bars show mean and standard error (see methods). *HtrA2*^{Δ1} mutant flies have an increased proportion of mitochondria with reduced electron density, as determined by a Fisher exact test (* $p < 0.01$, # = 0.058). *HtrA2*^{Δ1} mutants show progressive deficits in flight (h) and climbing (i). 5 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*^{Δ1} rescue flies. Bars show mean and standard error, inset numbers show *n*. ***, $p < 0.0001$; **, $p < 0.001$; * $p < 0.01$, by Kruskal-Wallis non-parametric analysis and Dunn's pair-wise comparison.

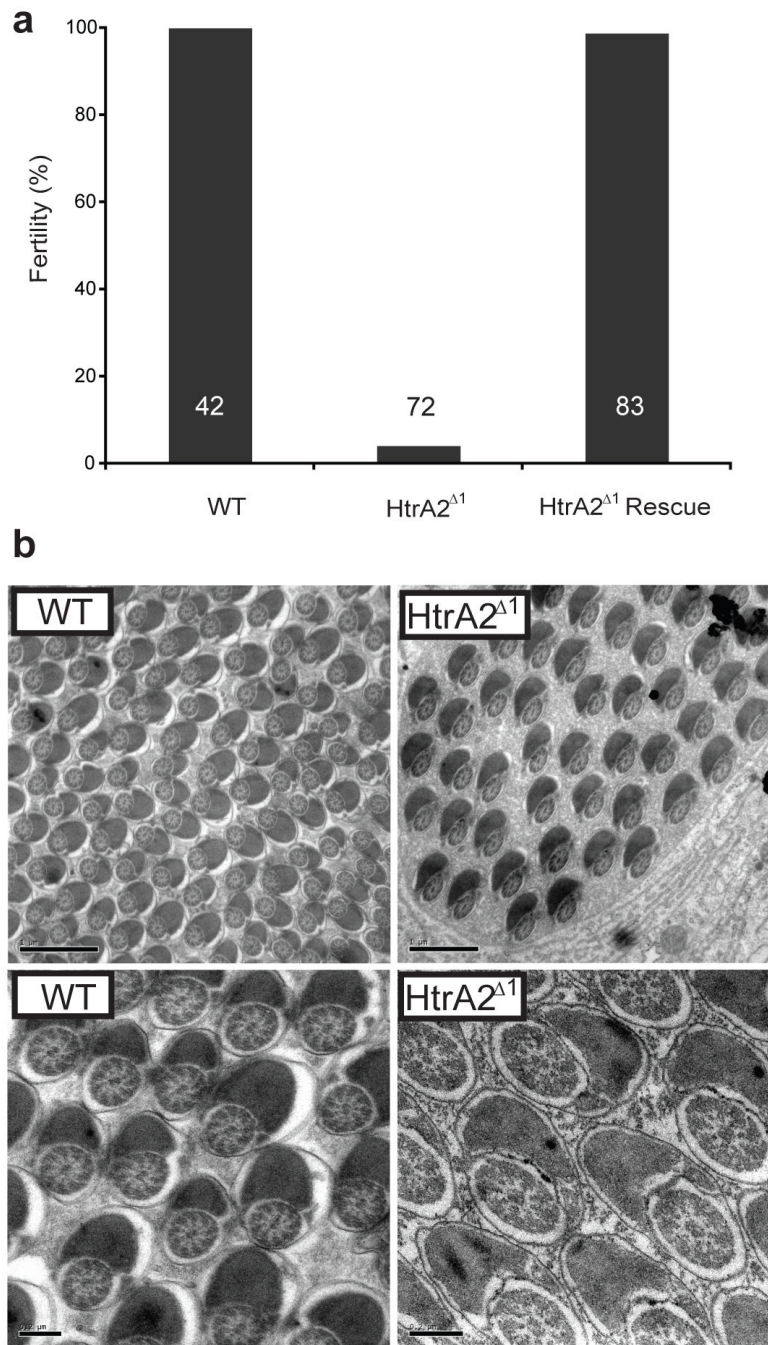


Figure 4. *HtrA2^{Δ1}* have normal spermatogenesis but are male sterile

(a) *HtrA2^{Δ1}* males have greatly reduced fertility compared to controls. Normal fertility is regained in *HtrA2^{Δ1}* 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.

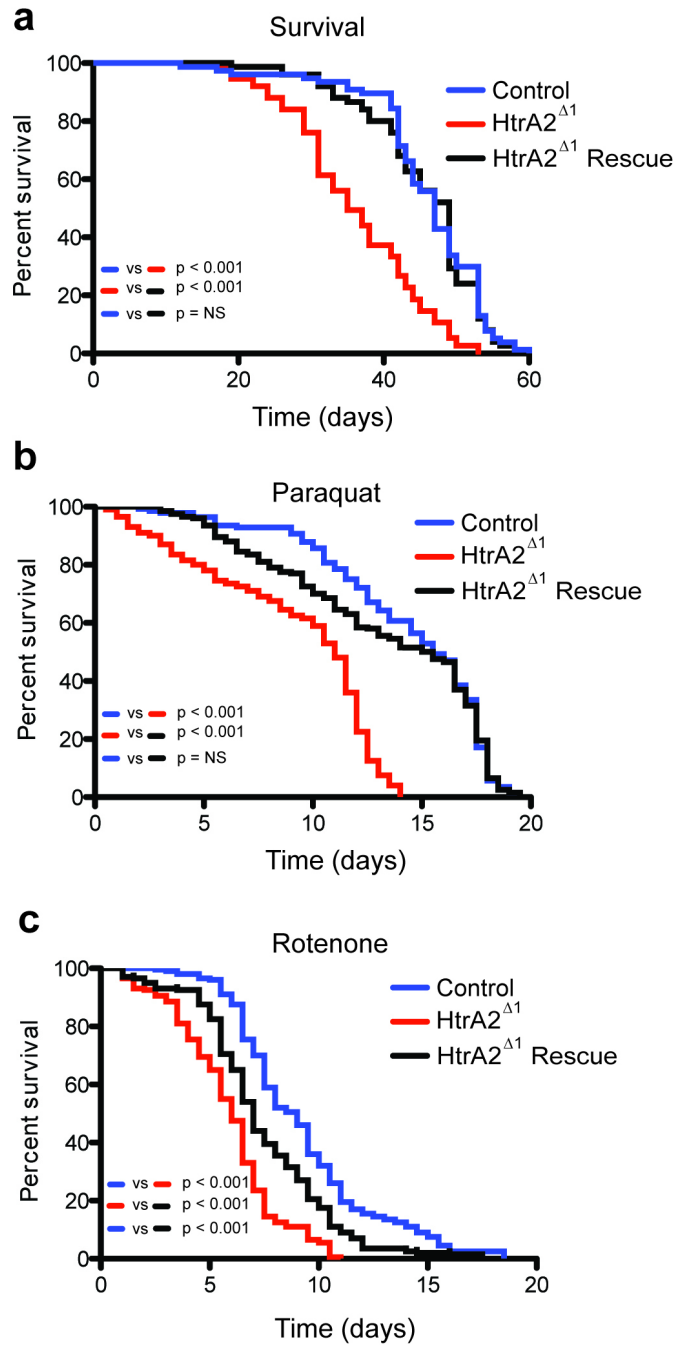


Figure 5. *HtrA2^{Δ1}* have reduced survival under normal and stressed conditions
(a) Male *HtrA2^{Δ1}* flies (red) have reduced lifespan compared with wild type control (blue), which is restored with a genomic rescue construct (black) (n=75 for each genotype). **(b)** *HtrA2^{Δ1}* mutant flies (red) 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^{Δ1}* mutant flies are more sensitive to rotenone-induced oxidative stress (red) than wild type controls (blue) or the *HtrA2^{Δ1}* rescued line (black). n= 200 for each class. Data is presented as Kaplan-Meier survival distributions, Log-rank tests were

performed to determine statistical significance. Individual p values are shown with the figure.

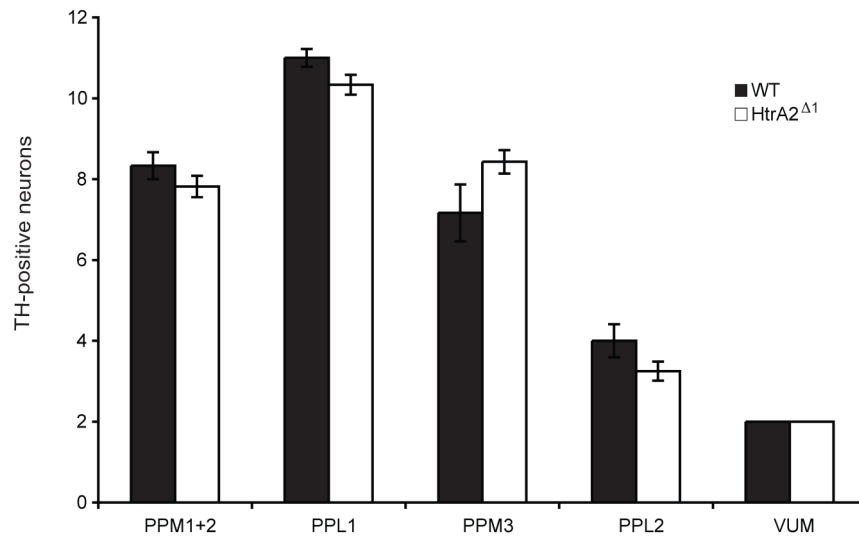


Figure 6. Loss of HtrA2 does not cause dopaminergic neurons loss

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 = 10).

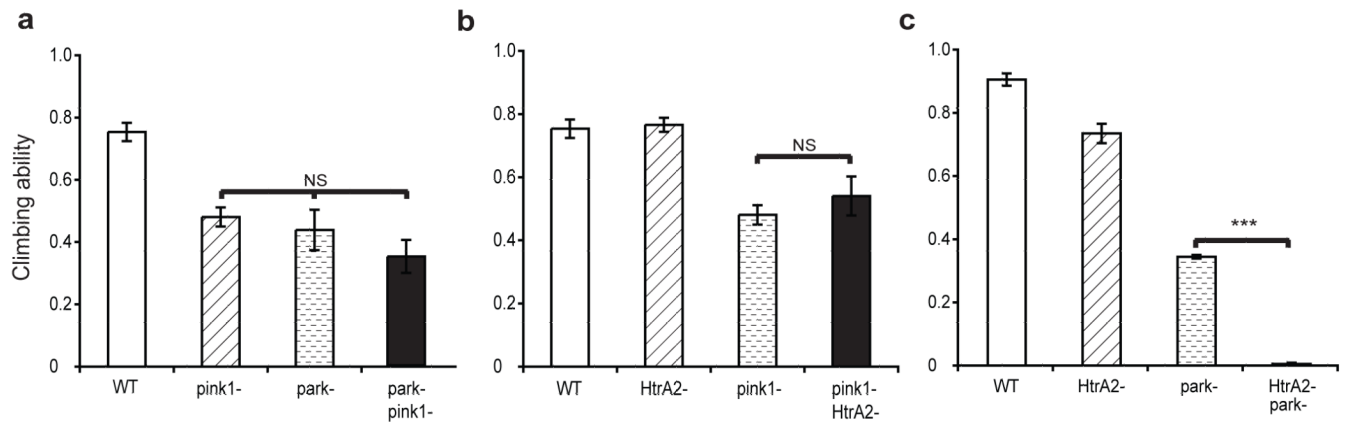


Figure 7. *HtrA2* mutants enhance the locomotor deficit exhibited by *parkin* mutants but not *PINK1* mutants

(a) Climbing ability of *PINK1*^{B9}, *park*²⁵ and *PINK1*^{B9};*park*²⁵ double mutants. (b) Climbing ability of *HtrA2*^{Δ1}, *PINK1*^{B9} and *PINK1*^{B9};*HtrA2*^{Δ1} double mutants. (c) Climbing ability of *HtrA2*^{Δ1}, *park*²⁵ and *park*²⁵;*HtrA2*^{Δ1} double mutants. Bars show mean and standard error. n = 50.

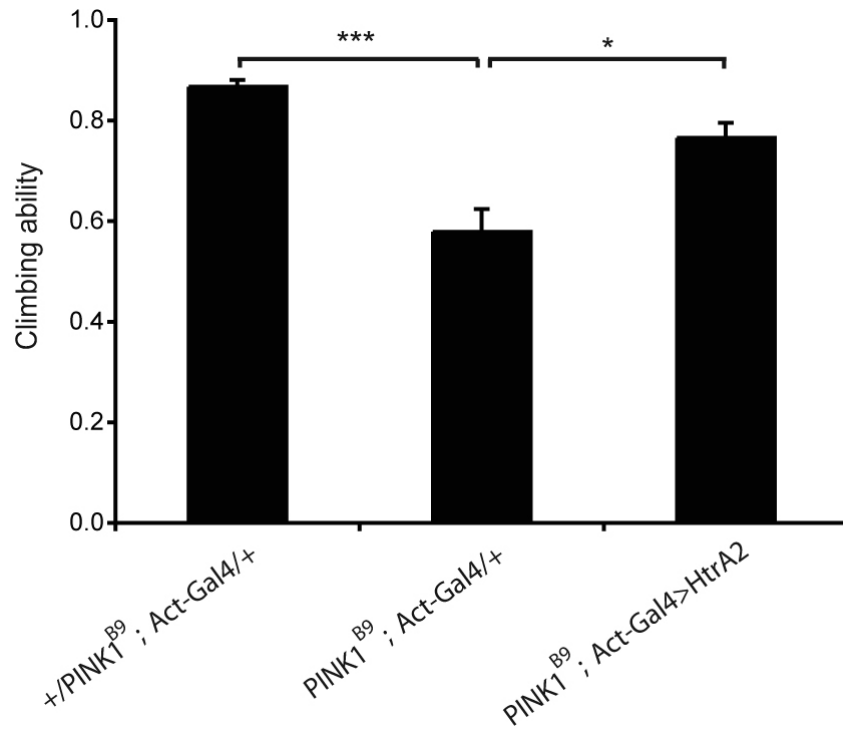


Figure 8. Overexpression of *HtrA2* can rescue *PINK1* climbing defect

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 (***, $p < 0.0001$; *, $p < 0.01$).