

# Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease

Karsten M. Strauss<sup>1</sup>, L. Miguel Martins<sup>5</sup>, Helene Plun-Favreau<sup>6</sup>, Frank P. Marx<sup>1</sup>, Sabine Kautzmann<sup>1</sup>, Daniela Berg<sup>1,4</sup>, Thomas Gasser<sup>1</sup>, Zbigniew Wszolek<sup>7</sup>, Thomas Müller<sup>8</sup>, Antje Bornemann<sup>2</sup>, Hartwig Wolburg<sup>3</sup>, Julian Downward<sup>6</sup>, Olaf Riess<sup>4</sup>, Jörg B. Schulz<sup>1,\*†</sup> and Rejko Krüger<sup>1,†</sup>

<sup>1</sup>Center of Neurology and Hertie-Institute for Clinical Brain Research, <sup>2</sup>Institute of Brain Research, <sup>3</sup>Institute of Pathology and <sup>4</sup>Department of Medical Genetics, University of Tübingen, Tübingen, Germany, <sup>5</sup>MRC Toxicology Unit, Leicester, UK, <sup>6</sup>Signal Transduction Laboratory, Cancer Research UK, London Research Institute, London, UK, <sup>7</sup>Department of Neurology, Mayo Clinic, Jacksonville, FL, USA, <sup>8</sup>Department of Neurology, Ruhr-University Bochum, Bochum, Germany and <sup>9</sup>CMPB and Center of Neurology, University of Göttingen, Göttingen, Germany

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**Recently targeted disruption of Omi/HtrA2 has been found to cause neurodegeneration and a parkinsonian phenotype in mice. Using a candidate gene approach, we performed a mutation screening of the *Omi/HtrA2* gene in German Parkinson's disease (PD) patients. In four patients, we identified a novel heterozygous G399S mutation, which was absent in healthy controls. Moreover, we identified a novel A141S polymorphism that was associated with PD ( $P < 0.05$ ). Both mutations resulted in defective activation of the protease activity of Omi/HtrA2. Immunohistochemistry and functional analysis in stably transfected cells revealed that S399 mutant Omi/HtrA2 and to a lesser extent, the risk allele of the A141S polymorphism induced mitochondrial dysfunction associated with altered mitochondrial morphology. Cells overexpressing S399 mutant Omi/HtrA2 were more susceptible to stress-induced cell death than wild-type. On the basis of functional genomics, our results provide a novel link between mitochondrial dysfunction and neurodegeneration in PD.**

## INTRODUCTION

Mitochondrial dysfunction is a common feature of Parkinson's disease (PD) (1). A specific and selective loss of mitochondrial complex I activity in the substantia nigra of PD patients reflects an important role of mitochondrial pathology in PD (2). Neuronal mitochondria function as integrators of diverse cellular stresses and mediators of apoptosis and therefore represent a major interface between endogenous or exogenous toxins and neurodegeneration. The serine protease Omi/HtrA2 is a nuclear encoded protein with a mitochondrial targeting sequence at its N-terminus. Once in the mitochondria, Omi/HtrA2 is thought to reside in the intermembrane space as a proteolytically processed form containing an N-terminal sequence related to that of *Drosophila* pro-apoptotic proteins

Reaper, Grim and Hid. During apoptosis, Omi/HtrA2 is released from the mitochondria into the cytosol where it binds inhibitor of apoptosis proteins (IAPs), relieving their inhibitory activity towards caspases (3–6). Binding of Omi/HtrA2 to IAPs also results in the activation of the proteolytic activity of this serine protease (7). Omi/HtrA2 is a member of a well-conserved family of PDZ domain containing serine proteases, which are found in most eukaryotes and prokaryotes. Well-characterized members of the family include DegS and DegP in *Escherichia coli*. The DegS stress sensor detects unfolded outer membrane porins in the periplasm, triggering a signalling protease cascade leading to adaptive changes in gene expression (8). Binding of specific peptides to the PDZ domain of both DegS and Omi/HtrA2 results in increased proteolytic activity (7,8). Activation of both DegS and

\*To whom correspondence should be addressed at: Department of Neurodegeneration and Restorative Research, Centers of Neurological Medicine and Molecular Physiology of the Brain, University of Göttingen, Waldweg 33, D-37073 Göttingen, Germany. Tel: +49 5513913540; Fax: +49 5513913541; Email: jschulz4@gwdg.de

†The authors contributed equally to this work.

Omi/HtrA2 seems to employ similar mechanisms at the structural level, for example, the turn structure of loop L1 that sets up the oxyanion hole is preformed in DegS and HtrA2, whereas it is entirely absent in DegP (9). A first link between Omi/HtrA2 and neurodegeneration has been established by the identification of Omi/HtrA2 as a presenilin-1-interacting protein in yeast two-hybrid assays (10). Mutations in presenilin-1 are responsible for inherited forms of early-onset Alzheimer's disease (AD). This interaction was subsequently confirmed and the regulation of Omi/HtrA2 protease activity via C-terminal presenilin was characterized (11). Further support for a possible role of Omi/HtrA2 in neurodegeneration was provided by the interaction between Omi/HtrA2 and AD-associated amyloid  $\beta$  (12). First insight into *in vivo* effects of Omi/HtrA2 dysfunction came from the characterization of *mnd2* mutant mice (13). A homozygous loss of function mutation (S276C) in the *Omi/HtrA2* gene was identified in *mnd2* mice leading to neurodegeneration, muscle wasting and death by 40 days of age (13). Recently, a neurodegenerative phenotype with parkinsonian features has been defined in Omi/HtrA2 knockout mice underscoring the physiological relevance of the protease activity of the protein (14). Taken together, the known protective stress response activities of bacterial Omi/HtrA2 homologues and the fact that the loss of proteolytic activity in both *mnd2* and Omi/HtrA2 knockout mice result in enhanced sensitivity to stress, indicate that this protease might be essential for the transduction of mitochondria related stress signals in cells not committed to apoptosis. Because loss of Omi/HtrA2 causes neurodegeneration with parkinsonian features and due to its functional relevance in terms of mitochondrial homeostasis, we decided to screen for mutations in the *Omi/HtrA2* gene in a large sample of German PD patients.

## RESULTS

### Mutation screening

Using denaturing, high performance liquid chromatography (dHPLC) analysis of amplified PCR fragments followed by direct sequencing, we identified three novel base pair substitutions in the coding region of *Omi/HtrA2* in our PD patients sample. A silent C447T substitution was identified in exon 1. Moreover, 2 bp substitutions that caused changes in the peptide sequence were identified in PD patients: a G421T substitution in exon 1 leading to an amino acid change from alanine to serine in position 141 (A141S) of the peptide sequence and a G1195A substitution in exon 7 resulting in a glycine to serine substitution in amino acid position 399 (G399S; Fig. 1A and B). The G421T substitution was subsequently defined as a polymorphism due to its frequency of >1% in the healthy control population. Performing an association study using the G421T polymorphism in 414 PD patients and 331 healthy controls, we identified 26 heterozygous individuals in the patients group (6.2%) and 10 heterozygous individuals in the control group (3%). No homozygous carriers of the mutation were observed. Thus, we found a significant overrepresentation of carriers of the T allele of the exon 1 polymorphism (S141) in patients compared with controls ( $\chi^2 = 4.25$ ,  $P = 0.039$ , OR = 2.15, CI = 1.02–4.52).

In contrast, the G1195A substitution, which was identified in four apparently sporadic PD patients, was not observed in 740 chromosomes of healthy control individuals. Interestingly, genotyping of 11 markers in the chromosomal region harbouring the *Omi/HtrA2* gene revealed that all carriers of the G399S mutation share the same haplotype based on six neighbouring single nucleotide polymorphisms (Fig. 2). The identified mutations leading to amino acid substitutions, A141S and G399S, localize to the N-terminus portion of the mature form of Omi/HtrA2 and the PDZ domain, respectively (Fig. 1C).

### Clinical phenotype of A141S and G399S mutation carriers

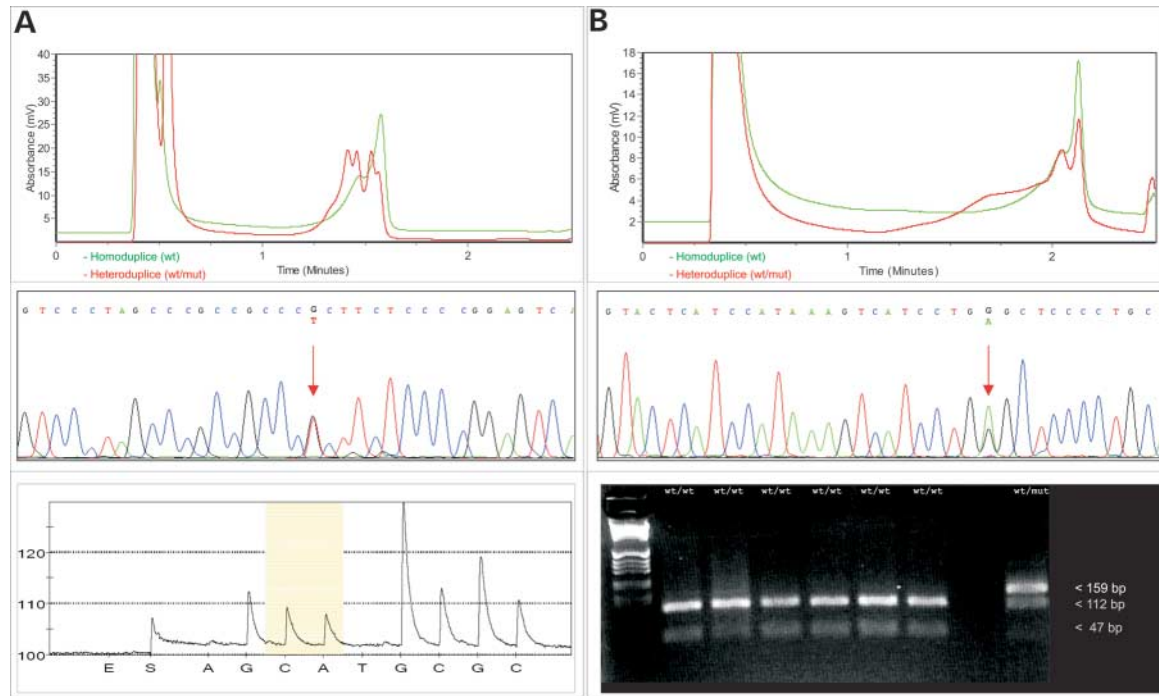
In total, we identified four PD patients carrying heterozygous point mutations leading to a G399S mutation in the Omi/HtrA2 peptide. All carriers of the G399S mutation were included as sporadic into the patients sample based on the reported absence of further affected family members. The age at disease onset ranged from 49 to 77 years (mean  $57.3 \pm \text{SEM } 13.3$  years). Clinical symptoms included typical features of idiopathic PD including bradykinesia, tremor and muscular rigidity. All symptoms responded well to levodopa therapy. In PD patients, structural lesions as causes of the disease were ruled out by computed tomography scans. Heterozygous carriers of the S141 mutant allele also reflect typical clinical features of PD including a positive and sustained response to levodopa therapy. The mean age at disease onset was  $55.3 \pm 11.0$  years, similar to that of the total study cohort ( $55.3 \pm 12.0$  years) not showing an age at disease onset modulating effect of the exon 1 polymorphism.

### Expression of WT and mutant Omi/HtrA2 in HEK293 and SH-SY5Y cells

Human embryonic kidney cells (HEK293) and human dopaminergic neuroblastoma cells (SH-SY5Y) were stably transfected with cDNA constructs encoding wild-type (WT) (A141 or G399) and mutant (S141 or S399) Omi/HtrA2–FLAG fusion protein, respectively. Similar levels of expression were assessed by western blotting analysis. The Omi/HtrA2–FLAG fusion protein produced  $\sim 35$  kDa band, corresponding to the processed form of the fusion protein (Fig. 3). Endogenous Omi/HtrA2 was detected as a band of  $\sim 33$  kDa (Fig. 3). This is the predicted size of mature Omi/HtrA2 deduced from the published peptide sequence (NCBI accession no. NP 037379). Our results show that both variants of Omi/HtrA2, S141 and S399, respectively, do not affect the processing of the precursor protein after transport into mitochondria, which is necessary to retain the active protein. Moreover, we found no obvious influence of the respective Omi/HtrA2 mutations on protein stability, as steady state levels were comparable in transiently transfected HEK293 and SH-SY5Y cells (data not shown).

### Effects of WT and mutant Omi/HtrA2 on the serine protease activity *in vitro*

To determine the effect of both detected mutations on the proteolytic activity, we produced recombinant mature



**Figure 1.** Identification of mutations in the human *Omi/HtrA2* gene in PD patients. (A) dHPLC profile (first row) and sequencing results (second row) of DNA of one PD patient revealing the G to T exchange in position 421 of the coding sequence. The mutation leading to an amino acid substitution from alanine to serine in position 141 of the peptide was confirmed by Pyrosequencing<sup>TM</sup> (reverse sequence; last row). (B) dHPLC profile and sequencing results of DNA of one carrier of the G1195A substitution of the coding sequence defining the G399S mutation. The mutation was subsequently confirmed by *Mva*I restriction. Presence of the mutation prevented restriction by *Mva*I and lead to an additional 159 bp band on agarose gel (last row). (C) Evolutionary conservation of the regions of *Omi/HtrA2* where amino acid substitutions were found to be associated with PD. Residue 134 corresponds to the N-terminal portion of mature *Omi/HtrA2* found to be essential for interaction with mammalian IAPs. Serine 276 found to be mutated in mice lacking *Omi/HtrA2* proteolytic activity is indicated as well as the active site, serine 306. MT, mitochondrial targeting sequence; TM, predicted transmembrane domain found in unprocessed *Omi/HtrA2*. The C-terminal PDZ domain is also indicated.

*Omi/HtrA2* and compared the activity to the WT enzyme using a previously described assay (7). Comparing the basal activity of the proteins, no significant differences in the reaction rates were measured (data not shown). This is consistent with the fact that both mutations are not localized near the protease domain on *Omi/HtrA2*. Both mutations are close to domains previously implicated in the regulation of the proteolytic activity of the enzyme, the IAP and PDZ binding domains. Therefore, we assayed the effect of these mutations on the ability of *Omi/HtrA2* to be activated by IAPs and PDZ-interacting peptides. For both S141 and S399 mutants, we observed a marked decrease in the ability of both BIR2 (X-linked inhibitor of apoptosis, XIAP) and PDZ-interacting peptides to activate the mutant enzymes (Fig. 4A and B). We therefore conclude that these mutations affect the regulation of the proteolytic activity of *Omi/HtrA2*.

#### Effects of WT and mutant *Omi/HtrA2* on complex formation

Functional protease activity of *Omi/HtrA2* protein requires trimerization, which is mediated by the serine protease domain of *Omi/HtrA2*. To screen for possible effects of the respective mutant S141 or S399 *Omi/HtrA2* proteins on trimer formation, we performed co-immunoprecipitation

assays as previously described (13) in cells transfected with WT HA-tagged *Omi/HtrA2* and the respective FLAG-tagged mutant. Both S141 and S399 mutants bind WT protein defining a prerequisite for complex formation. Thus, both variants retain the ability to allow heterotrimerization (Fig. 4C). For the S141 mutation, this was expected because deletion of the N-terminal portion of mature *Omi/HtrA2* including amino acid 134–141 did not affect trimer formation in previous studies (19). Thus, our results indicate that S141 and S399 mutant *Omi/HtrA2* monomers cause a decrease in protease function without interfering with complex formation, providing a possible explanation for the loss of function of this enzyme in patients heterozygous for this mutation.

#### Subcellular localization of WT and mutant *Omi/HtrA2* in HEK293 cells

Using immunocytochemistry, we investigated *Omi/HtrA2* protein in HEK293 cells stably overexpressing WT or mutant (S141 and S399) *Omi/HtrA2*. We confirmed the previously reported predominant mitochondrial localization of *Omi/HtrA2* protein in cells as assessed by immunofluorescence (4,6) (Fig. 5A–D). We observed no differential subcellular compartmentalization analysing WT *Omi/HtrA2* or S141 and S399 mutants. Interestingly, in ~40% of the

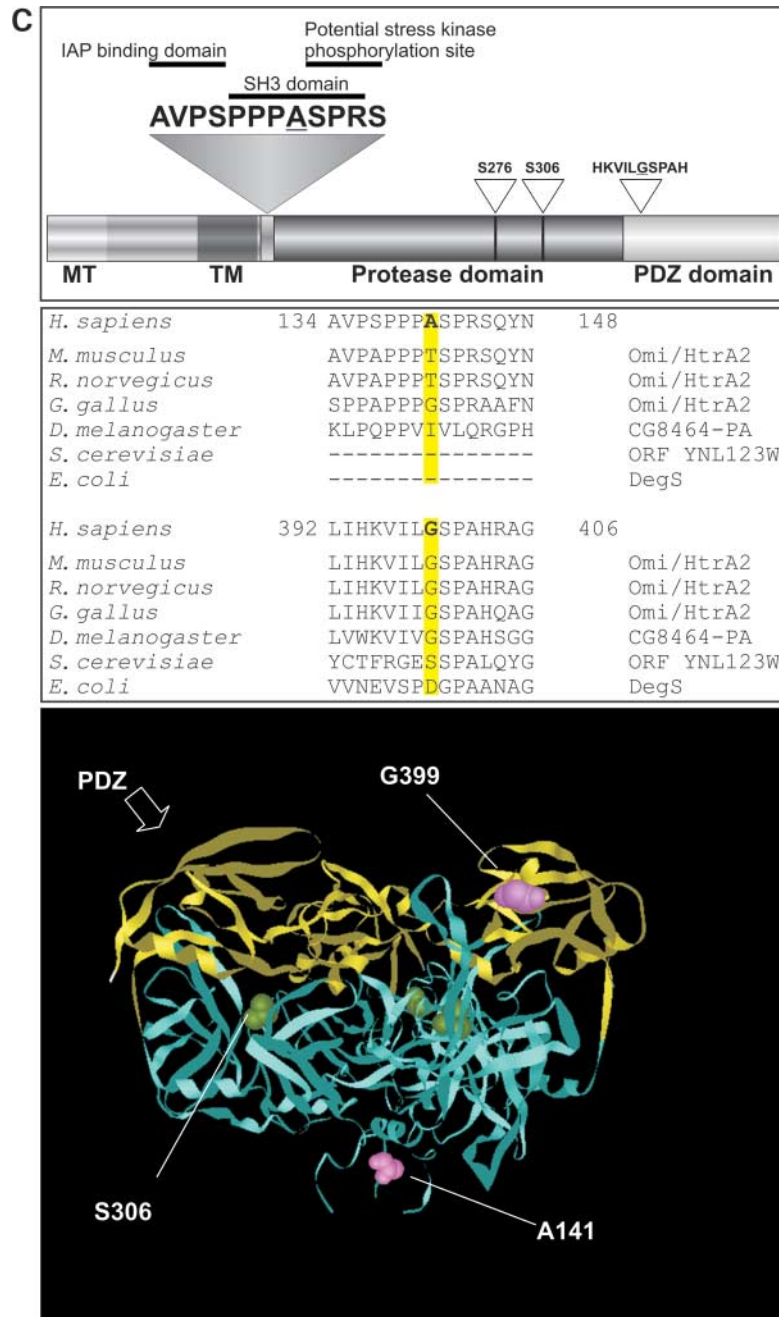


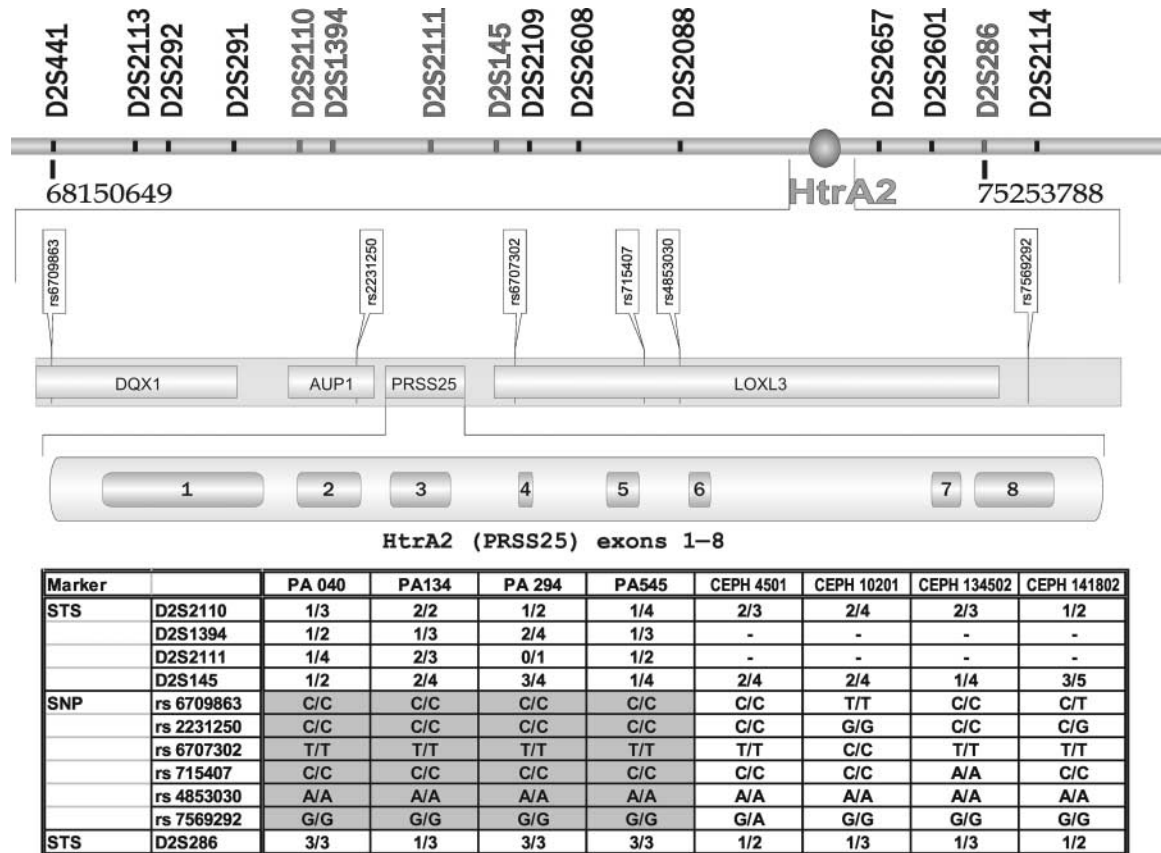
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cells expressing S399 mutant Omi/HtrA2 and in ~10% of cells expressing S141 mutant Omi/HtrA2, we observed characteristic large Omi/HtrA2-positive structures in the cytoplasm that were not observed in WT Omi/HtrA2. These structures stained positive with mitochondrial markers suggesting morphologically altered and swollen mitochondria (Fig. 5A–D). For some of these structures, we found co-localization of mitochondrial markers and antibodies staining lysosomal proteins (Fig. 5E–G). Similar observations were made in dopaminergic SH-SY5Y cells overexpressing S399 and S141 mutant Omi/HtrA2 protein (data not shown).

### Ultrastructural analyses in Omi/HtrA2 overexpressing cells

Using electron microscopy to study morphology of cells overexpressing WT and S141 and S399 mutant Omi/HtrA2 on the ultrastructural level, we identified characteristic changes of mitochondria in a subset of cells. When compared with WT Omi/HtrA2 (Fig. 5H), we observed enlarged mitochondria with disorganized cristae that were more pronounced in cells overexpressing S399 mutant Omi/HtrA2 (Fig. 5I and J). Some of these mitochondria were filled by dense, lamellar





**Figure 2.** Genomic representation of the *Omi/HtrA2* gene. Schematic view of the genomic representation and localization of the *Omi/HtrA2* gene on chromosome 2p13. Chromosomal localization of the *Omi/HtrA2* gene, neighbouring genes and the positions of genetic markers are indicated. The results of genotyping six single nucleotide polymorphisms in carriers of the G399S mutation are shown in Table 1. Concerning the analysed polymorphisms in the *Omi/HtrA2* gene all carriers of the G399S mutation (PA040, PA134, PA294 and PA545) shared the same alleles. CEPH are DNA samples from Caucasian controls provided by the Centre d'Etudes des Polymorphismes Humaines specified by the respective registration numbers.

structures (Fig. 5K). The latter fulfil criteria of involuting mitochondria including whorled membranous bodies that have been described previously (20). Sometimes disintegrating mitochondrial remnants were observed localized in lysosomal structures. This corresponds to the co-localization of mitochondria and lysosomes detected by laser scanning microscopy in HEK293 cells and might imply autophagy as a possible mechanism of cell death occurring in these cells (Fig. 5E–G).

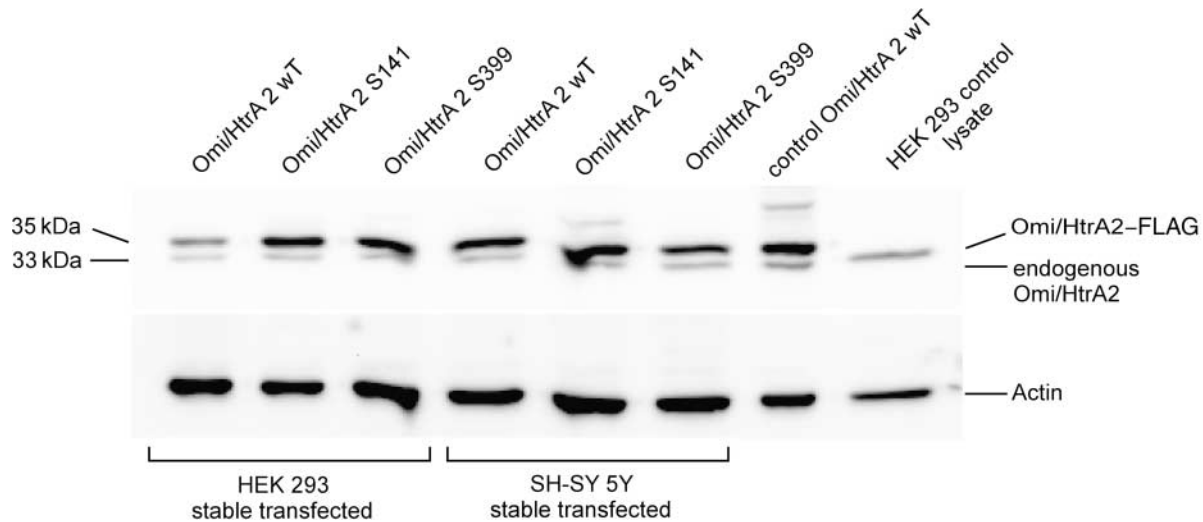
#### Effects of WT and mutant *Omi/HtrA2* on mitochondrial membrane potential

On the basis of morphological alterations, we next examined the effects of *Omi/HtrA2* overexpression on mitochondrial function. A sensitive marker for homeostasis of mitochondria is the mitochondrial membrane potential. In SH-SY5Y cells, stably expressing WT or mutant (S141 or S399) *Omi/HtrA2*, we monitored the mitochondrial membrane potential by flow cytometry using JC-1 as a probe. As a paradigm of cellular stress, we used the serine/threonine kinase inhibitor staurosporine known to induce apoptotic cell death by different mechanisms including loss of mitochondrial transmembrane potential. Analysis of JC-1 fluorescence demonstrated that

both S141 and S399 mutant *Omi/HtrA2* caused a decrease of mitochondrial membrane potential compared with WT *Omi/HtrA2*. After treatment with 0.5  $\mu$ M staurosporine for 4 h, the mitochondrial membrane potential was decreased in all cell types investigated (Fig. 6). However, this effect was more pronounced in cells overexpressing S141 and S399 mutant *Omi/HtrA2* compared with WT (Fig. 6). A similar effect on mitochondrial membrane potential was observed in HEK293 cells overexpressing WT or mutant *Omi/HtrA2* (data not shown).

#### Effects of WT and mutant *Omi/HtrA2* expression on cell viability

To test whether the effect of *Omi/HtrA2* overexpression on mitochondrial function might affect cell viability, we determined the LDH-release from HEK293 cells stably overexpressing WT or mutant *Omi/HtrA2* using staurosporine to induce cellular stress. Overexpression of both WT or mutant *Omi/HtrA2* in HEK293 cells without further treatment showed no difference in LDH release (Fig. 7). Addition of staurosporine to cells overexpressing WT, S141 or S399 *Omi/HtrA2* resulted in an increase in LDH-release in comparison with untreated cells. The G399S mutation in the



**Figure 3.** Expression of WT and mutant Omi/HtrA2 in HEK293 and SH-SY5Y cells. HEK293 (lanes 1–3) and SH-SY5Y cells (lanes 4–6) transfected with cDNA encoding WT or mutant (S141 or S399) Omi/HtrA2–FLAG fusion protein produced a band of expected size on western blots using anti-Omi/HtrA2 polyclonal antibodies and anti- $\beta$ -actin antibodies as a loading control (lanes 1–6). Untransfected HEK293 cells served as controls (lane 8). Endogenous Omi/HtrA2 produced a band of  $\sim$ 35 kDa in both HEK293 and SH-SY5Y cells. A transient transfection of the Omi/HtrA2 WT construct in HEK293 cells served as positive control. Equal amounts of protein were resolved on SDS–PAGE gel and subjected to western blot analysis using Omi/HtrA2 polyclonal antibodies (upper panel) and anti- $\beta$ -actin polyclonal antibodies as loading control (lower panel).

Omi/HtrA2 protein resulted in significantly increased sensitivity towards staurosporine-induced toxicity compared with WT and S141 Omi/HtrA2 ( $P < 0.001$ ) (Fig. 7). In this context, S141 mutant Omi/HtrA2 showed similar vulnerability as WT Omi/HtrA2. This underscores the toxic effect of the G399S mutation compared with A141S in terms of mitochondrial dysfunction and cell death.

#### Immunohistochemistry in brains of PD patients

Using polyclonal Omi/HtrA2 antibodies (Apotech, USA) raised against a synthetic human Omi/HtrA2 peptide, we screened brain stem sections and cortical slices from patients with pathologically confirmed idiopathic PD. We identified Omi/HtrA2 as a component of Lewy bodies (Fig. 8A). Regarding the local staining, we found Omi/HtrA2 predominantly present in the halo of Lewy bodies, a localization that has also been described for the major component of Lewy bodies, alpha-synuclein. In total,  $\sim$ 30–35% of Lewy bodies stained positive with antibodies against Omi/HtrA2. The granular material present in the indicated neuron is lipofuscin. The significance of the labelling of these structures by the anti-Omi/HtrA2 antibody is at present unknown. Neuronal lipofuscin contains proteins such as amyloid precursor protein (21). Interestingly, an interaction between Omi/HtrA2 and AD-associated amyloid beta has been described *in vitro* (12). Thus, it cannot be ruled out at the time that Omi/HtrA2 represents another component of neuronal lipofuscin. To confirm specificity of immunostaining, we omitted Omi/HtrA2 antibody in control sections of PD brain (Fig. 8B). Interestingly, the inhibitor of apoptosis XIAP, a known Omi/HtrA2 interacting protein, is also sequestered into a subset of 20% of Lewy bodies (data not shown). The staining parallels that of Omi/HtrA2 with a

predominant localization of XIAP in the halo of Lewy bodies.

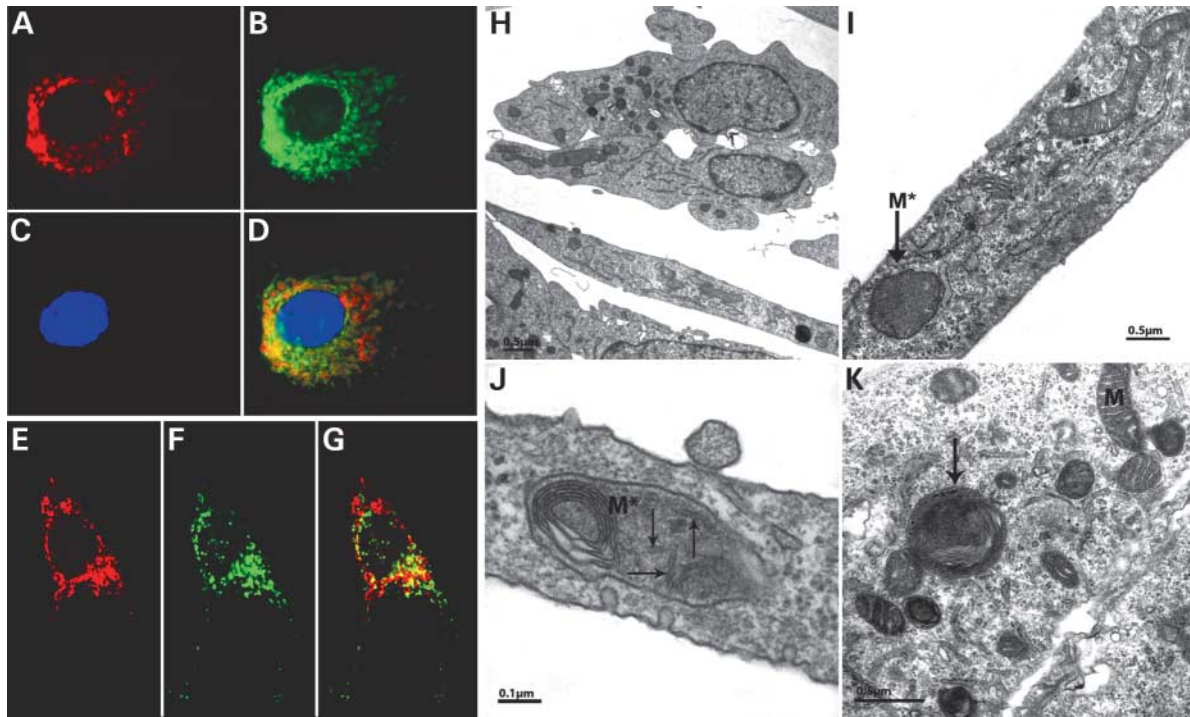
#### DISCUSSION

In this study, we report for the first time that mutations in the *Omi/HtrA2* gene are associated with a neurodegenerative disorder in humans. Data from animal models defined a role of the *Omi/HtrA2* gene in neurodegeneration with parkinsonian features (13,14). Omi/HtrA2 provides a functional link between proteolytic stress and mitochondrial function, making it an interesting candidate for mutation screening in PD. We identified two novel mutations in the *Omi/HtrA2* gene, defined the functional relevance of these mutations *in vitro* and detected Omi/HtrA2 in pathognomonic Lewy bodies in brains of idiopathic PD patients.

The absence of a family history in carriers of the G399S mutation in the *Omi/HtrA2* gene indicates a genetic trait with reduced penetrance and argues in favour of a susceptibility factor for PD. Recent studies on genetic susceptibility factors in the common late onset form of PD showed that the sporadic appearance of the disease does not preclude an involvement of genetic factors in the pathogenesis of PD (22). First support for the pathogenic relevance of the G399S mutation in the Omi/HtrA2 protein came from interspecies comparison of the amino acid sequence of the Omi/HtrA2 protein. Functional domains of the Omi/HtrA2 protein include the PDZ domain, which regulates the serine protease activity of Omi/HtrA2 thereby modulating cell death activity (7,19). The G399S mutation is located in the PDZ domain, which is highly conserved among different species suggesting functional implications. Indeed, we determined that S399 mutant Omi/HtrA2 displays a reduced serine protease activity *in vitro* following activating stimuli.







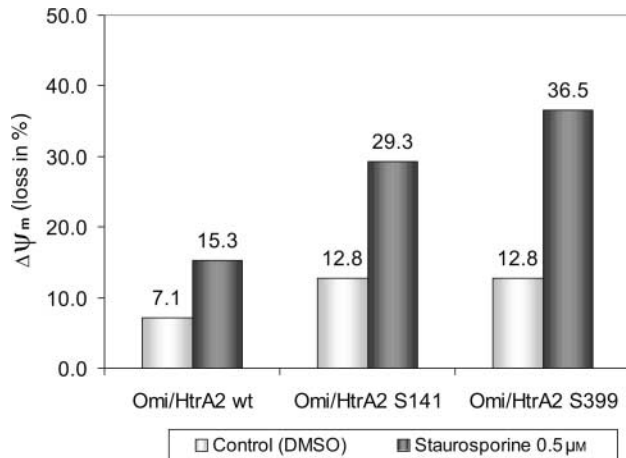
**Figure 5.** Immunocytochemical and electron microscopy studies of HEK293 cells stably overexpressing WT and mutant Omi/HtrA2. (A–D) HEK293 cells stably overexpressing S399 mutant Omi/HtrA2. Cells were stained with antibodies against Omi/HtrA2 (green, B), a mitochondrial marker (Mitotracker Red, A) and counterstained with Hoechst 33342 to identify nuclei (blue, C). (D) The respective stainings are merged, confirming the previously reported predominant mitochondrial localization of Omi/HtrA2 protein cells. In a subset of cells overexpressing S399 mutant Omi/HtrA2, we found enlarged, swollen mitochondria containing Omi/HtrA2 protein. (E–G) HEK293 cells stably overexpressing S399 mutant Omi/HtrA2. Cells were incubated with Mitotracker Red (E) and fixed as described earlier. Lysosomes were visualized using  $\alpha$ -LAMP-1 antibody (green, F). In merged panel G, co-localization of mitochondrial markers and lysosomal proteins is illustrated for some structures. (H–K) HEK293 cells stably overexpressing WT or S399 mutant Omi/HtrA2. Compared with WT Omi/HtrA2 (H) cells expressing S399 mutant Omi/HtrA2 often presented with enlarged or altered mitochondria (M\*) with disorganized cristae (arrows; I and J). In some cells, involuting mitochondria displayed dense, lamellar structures resembling whorled membranous bodies (K).

Although located outside the protease domain, either in the N-terminal part of mature Omi/HtrA2 (A141S) or in the PDZ domain (G399S), the mutations identified in PD patients also impact on the proteolytic function. As for the S276C mutant Omi/HtrA2, the A141S and G399S mutations heterotrimerize with WT Omi/HtrA2. Our results confirm data on S276C mutant Omi/HtrA2 indicating that a loss of protease function results in increased vulnerability towards cellular stress. In contrast to the autosomal recessive S276C mutation in *mnd2* mice, the A141S and G399S mutations in PD patients were not observed in the homozygous state. This might explain the typical late age of disease onset in affected PD patients, because recessive traits would be expected to cause early onset of the disease, i.e. as seen in parkin, PINK1 and DJ-1 mutations defining PARK2, PARK6 and PARK7, respectively (25,26,28). This suggests a dominant negative effect of the A141S and G399S mutation in neurodegeneration in PD. The fact that overexpression of mutant Omi/HtrA2 in HEK293 cells or dopaminergic SH-SY5Y cells lead to mitochondrial membrane dysfunction and increased susceptibility to cell death is in line with a dominant loss of protease function, because we found that both cell lines express WT Omi/HtrA2 (Fig. 3). Therefore, we speculate that the dominant loss of protease function might be mediated by the formation of

heterotrimers with WT Omi/HtrA2, which are more refractory to activation. Trimerization of Omi/HtrA2 is a prerequisite for its protease activity (19).

On the basis of functional analyses in *mnd2* and Omi/HtrA2 knockout mice, mitochondrial dysfunction has been suggested as a primary cause of neurodegeneration (13,14). We showed that S141 and S399 mutant Omi/HtrA2 display similar subcellular distribution like the WT protein including mitochondrial localization. Both mutations do not affect processing into mature Omi/HtrA2 and these variants exert their pathogenic role by loss of protease function resulting in increased mitochondrial susceptibility. Our results on a genetic and functional level are in line with existing biochemical data on the relevance of mitochondrial pathology in PD. Compromised mitochondrial function is a common feature of PD (29). Using electron microscopy and JC-1 staining, we demonstrate morphological and functional alterations in mitochondria following ectopic expression of mutant Omi/HtrA2. Biochemical data on a specific and selective loss of mitochondrial complex I activity in the substantia nigra of PD patients indicated mitochondrial pathology in PD (2). Neuronal mitochondria are important integrators of diverse cellular stresses and mediate cell death via apoptosis. Therefore, this interface between endogenous and exogenous





**Figure 6.** Mitochondrial membrane potential assay. In SH-SY5Y cells stably expressing WT or mutant Omi/HtrA2, we monitored the mitochondrial membrane potential by flow cytometry using JC-1 as a probe. The relative loss of mitochondrial membrane potential defined by the ratio of JC-1-monomers and -aggregates is indicated in bars. Analysis of JC-1 fluorescence demonstrated that overexpression of both S141 and S399 mutant Omi/HtrA2 caused a decrease of mitochondrial membrane potential compared with WT Omi/HtrA2. Treatment with 0.5 μM staurosporine for 4 h caused further decrease of mitochondrial membrane potential. This was more pronounced in cells overexpressing S141 and S399 mutant Omi/HtrA2 compared with WT. Experiments were replicated three times with similar results.

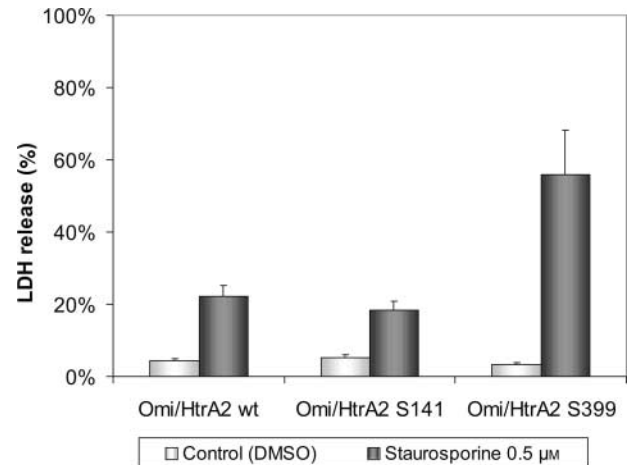
toxins is of special interest in deciphering molecular pathways of neurodegeneration.

## MATERIALS AND METHODS

### Mutation screening, genotyping and association studies

On the basis of the published genomic sequence of the human *Omi/HtrA2* gene (NCBI accession no. AC005041), a detailed screening for base exchanges was performed in the coding sequence and adjacent intronic sequences of the *Omi/HtrA2* gene. Our study included a total of 518 German PD patients (mean age at disease onset  $55.3 \pm 12.0$  years; males 55.4%, females 44.6%; 12.5% having a positive family history of PD) that were evaluated by experienced neurologists and were diagnosed as idiopathic PD based on the UK PD brain bank criteria. Moreover, we included index patients from four families with autosomal dominantly inherited PD previously linked to the PARK3 locus on chromosome 2p13 (15). All subjects signed an informed consent. As controls, we used DNA samples from 370 healthy German individuals matched for age and gender.

Primers to amplify the coding sequence and adjacent intronic sequences of the *Omi/HtrA2* gene were generated using the online software Primer3 ([http://www-genome.wi.mit.edu/cgi-bin/primer/primer3\\_www.cgi](http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)) based on the published genomic sequence of the *Omi/HtrA2* gene (NCBI accession no. AC005041) (Table 1). Each exon was amplified using the following conditions: 50 ng of DNA were amplified in a final volume of 30 μl in presence of 75 mM Tris-HCl (pH 8.8), 20(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Tween-20, 1.5 mM MgCl<sub>2</sub>, 200 μM of each dNTP, 7 pmol of each PCR primer and 2.5 U *Taq* polymerase (Genecraft, Germany). To allow the formation

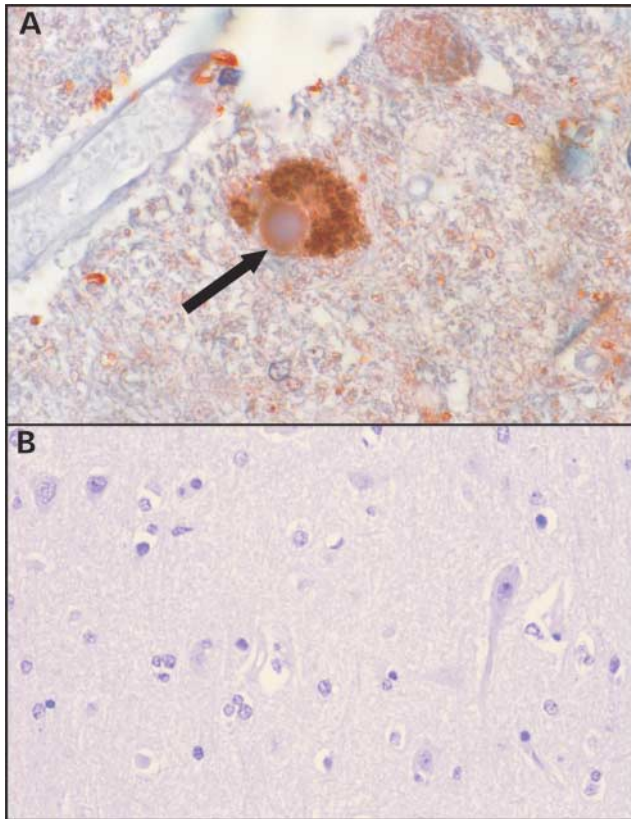


**Figure 7.** Cell viability assay. In HEK293 cells stably expressing WT or mutant Omi/HtrA2 were incubated in the presence of 0.5 μM staurosporine or DMSO for 6 h. LDH activity in medium relative to total cell lysate is shown in bars. Data are mean  $\pm$  SD of three independent experiments. Cells overexpressing S399 mutant Omi/HtrA2 were significantly more susceptible to staurosporine-induced cellular stress compared with WT Omi/HtrA2 ( $P < 0.0001$ , ANOVA followed by Tukey's *post hoc* test).

of heteroduplexes even in the presence of homozygous sequence variations, the resulting PCR products from two patients were pooled followed by a denaturation step at 95°C and subsequent slow renaturation. Prepared DNA samples were screened for mutations using dHPLC analysis on the WAVE™ DNA fragment analysis system (Transgenomic, USA) at column temperatures as described in Table 1. Column temperatures and running conditions were generated using the WaveMaker™ Software (Version 4.1.31, Transgenomic, USA) as indicated in Table 1. DNA samples exhibiting heteroduplexes were sequenced on a CEQ 8000 cycle sequencer using the CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckmann Coulter, Germany) with the primers used for exon amplification. To assess allele frequencies of identified single nucleotide substitutions, we used Pyrosequencing™ according to manufacturer's instructions (Pyrosequencing, Sweden) or restriction length polymorphism (RFLP) analysis, if adequate. High throughput analysis of the G421T mutation in exon 1 of the *Omi/HtrA2* gene was performed by Pyrosequencing using a biotin-labelled exon 1 forward primer for amplification and a reverse sequencing primer (5'-GACTCCGGGGAGAA-3'). Screening for the G1195A mutation was performed by a RFLP analysis on 2% agarose gels using *Mva*I (MBI, Germany) as restriction enzyme.

### Cloning of WT and mutant Omi/HtrA2

HtrA2 cDNA was cloned into *Eco*RV and *Xho*I sites of the FLAG-tagged pCMV-Tag4A vector (Clontech, USA) using the following primers: 5'-CTGATATCAAGGCGGAGCTG ATGGCT-3' and 5'-ATCCTCGAGTTCTGTGACCTCAGGG GTCA-3'. The G421T and G1195A mutations were inserted by the QuikChange™ site-directed mutagenesis kit (Stratagene, USA) using the primer pairs 5'-CTAGCCCGCCCTCTT



**Figure 8.** Immunohistochemistry in a patient with clinically and histopathologically proven sporadic PD. (A) Using Omi/HtrA2-specific antibodies, we identified this protein as a component of Lewy bodies (arrow). Positive Lewy body staining for Omi/HtrA2 was predominantly located at the halo. (B) To confirm specificity of the immunostaining, Omi/HtrA2 antibody was omitted in control sections.

CTCCCCGGAG-3', 5'-CTCCGGGGAGAAGAGGGCGGC GGGCTAG-3' for the G421T mutation and 5'-TCCATAAA GTCATCCTGTGCTCCCCTGCACACC-3', 5'-CCGGTGTG CAGGGGAGCACAGGATGACTTT-3' for the G1195A mutation, respectively. All cloned cDNA inserts were resequenced to rule out any PCR-generated errors. Full-length and  $\Delta$ 133Omi/HtrA2 tagged constructs (FLAG and HA) were generated by PCR and inserted using appropriate restriction enzyme sites into pCDNA3 vector. All constructs were verified by DNA sequencing.

#### Expression of recombinant proteins in *E.coli*

Mature, C-terminal His6-tagged human Omi/HtrA2 and point mutants were generated in pET-20b and subsequently expressed and purified as previously described (7). The BIR2 domain of XIAP (amino acids 159–256) was PCR-amplified and cloned into the pGEX-3X (Amersham Biosciences, Great Britain). GST and GST-BIR2 were expressed in *E.coli* strain BL21 Codon Plus (DE3)-RIL (Stratagene). Protein expression was induced by culturing cells at 37°C for 3 h in the presence of 0.5 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside. Protein was purified using glutathione–Sepharose beads and stored at –20°C as previously described (7).

#### Protease assays

Proteolytic activity of recombinant WT and mutant Omi/HtrA2 was determined using a fluorescent peptide assay as previously described (7). In brief, 100 nM Omi/HtrA2 pre-incubated or not with GST, GST-BIR2 or PDZ-Opt peptide, followed by addition of 10  $\mu$ M fluorescent substrate. Reactions were performed at 30°C in 50 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 1 mM dithiothreitol. Fluorescence was monitored on a CytoFluor multi-well plate reader, Series 4000 (PerSeptive Biosystems) using the filter pair Ex (360/40), Em (395/25). Reaction rates (V, arbitrary fluorescence units/minute) were determined by linear regression analysis of the data points corresponding to the maximum reaction rates for each assay condition. Assays are representative of at least three independent experiments done in triplicate.

#### Cell culture, transfection and generation of cell lines

HEK293 cells were cultured in a 5% CO<sub>2</sub> humidified atmosphere in Dulbecco's modified eagle's medium (DMEM, Invitrogen, Germany) containing penicillin and streptomycin (PAN, Germany) and 10% fetal bovine serum (FBS, Biochrom, Germany). Human SH-SY5Y neuroblastoma cells were cultured under same conditions with 15% FBS. Transfections were performed using FuGENE6 reagent according to the manufacturer's protocol (Roche, Germany). To generate polyclonal stable cell lines, 500 000 HEK293 or SH-SY5Y cells were transfected with pCMV-Tag4A containing the WT, S141 or S399 mutant Omi/HtrA2 cDNA and subsequently selected using medium containing G418 (concentrations for HEK293: 0.7 mg/ml; for SH-SY 5Y: 1.0 mg/ml).

#### Immunoprecipitation of Omi/HtrA2 complexes

HEK293 cells were co-transfected with constructs encoding C-terminal HA-tagged and FLAG-tagged processed or full-length Omi/HtrA2 variants (WT, A141S, G399S) using Efectene (QIAGEN). Forty-eight hours post-transfection, cellular lysates were immunoprecipitated with a FLAG-specific antibody (Sigma, USA). The immunoprecipitates were analysed by western blotting with a FLAG or a HA biotin conjugated antibody (Sigma, USA).

#### Immunocytochemistry

Stably transfected HEK293 or SH-SY5Y cells overexpressing WT or mutant Omi/HtrA2 were seeded on PLL-coated coverglasses (Assistent, Germany) or chambered coverglasses (Nalge Nunc, USA) and incubated over night in culture media. To visualize mitochondria, cells were incubated with 200 nM Mitotracker Red CMXRos (Molecular Probes, USA) and incubated for 30 min at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. After mitochondrial labelling, a 5 min wash step was performed in the appropriate culture medium without Mitotracker. For antibody staining, HEK293 or SH-SY5Y cells were fixed with cold 4% paraformaldehyde at room temperature for 12 min. After fixation cells were permeabilized with absolute methanol at –20°C for 7 min, washed three times with phosphate buffered saline (PBS) and blocked in

Table 1.

Exon	Primer sequence	Product length (bp)	Wave temperature (°C)	Wave gradient (% buffer B)	Sequence variation detected	Amino acid change
1	TGCCGCTCTGAGTAGGG CGGTCTACCCCCACCCCATTT	573	64.2	60–72	G421T G447T	Ala141Ser
1'	CCGGTTGTCTGTTGGGGTCA CGGTCTACCCCCACCCCATTT	350	64.2	59–71	G421T	Ala41Ser
2	TCTGTGCTTTCCTCCATTT TCATCTGAAGATGCGAGCAA	289	61.2 59.8	57–73 57–67		
3	GGTGGAGCTGCTTATTTGC TCCCCCATCATTTGTCATTT	298	61.5 58.0	55–65 58–68		
4	CCCAGACTAGAATCCCCAGA GGGATTCTTGGGAAGGAAGGA	116	60.0	46–60		
5	TAGGGAAC TGGGGCTGTAT CCACATTAAGGAACCCGTTT	174	61.0	51–63		
6	GGCTCATTTGTCCCTCTGTC CCCCCTCTGATTACTGGT	147	60.8 61.0	50–63 51–61		
7	GGGTTTGGCTAATAGGGTGA CCATATCACACTGCAGCCTCT	159	59.5	50–66	G1195A	Gly399Ser
8	TGTGTCCTTGAAC TAGGCTTGG GGAGCCTCATACTCTTGGTGA	251	60.5	56–66		

10% horse serum (PAA Laboratories GmbH, Germany). After blocking cells were incubated with Omi/HtrA2 (Apotech, USA) antibody over night at 4°C, washed three times with PBS and incubated with secondary FITC-labelled antibody (Dianova, Germany) for 1 h at room temperature. The first washing step was performed in PBS containing Hoechst 33342 (Molecular Probes, USA) in a 1:2000 dilution for 10 min. After a 4-fold wash step in PBS the coverglass was mounted in Mowiol (Sigma, Germany) containing additional DABCO as antibleach reagent and investigated using an epifluorescence microscope (Axioplan 2, Zeiss, Germany). To study fixed cells using on a laser scanning microscope (LSM 510, Zeiss, Germany), cells were incubated with Mitotracker Red CMXRos and fixed as described earlier. To visualize lysosomes, we used a  $\alpha$ -LAMP-1 antibody (30) detected by a FITC-labelled secondary antibody (Dianova, Germany).

### Electron microscopy

Cultured HEK293 cells stably expressing WT or mutant Omi/HtrA2 were fixed in 2.5% glutaraldehyde in Hank's modified salt solution, post-fixed in 1% OsO<sub>4</sub> in 0.1 M cacodylate buffer, scraped off, centrifuged and dehydrated in a series of ethanol. The 70% ethanol step was saturated with uranyl acetate for contrast enhancement. Dehydration was completed in propylene oxide and the specimens were embedded in Araldite (Serva, Germany). Ultrathin sections were produced on a FCR Reichert Ultracut ultramicrotome (Leica, Germany), mounted on pioloform-coated copper grids and contrasted with lead citrate. Specimens were analysed and documented with an EM 10A electron microscope (Zeiss, Germany).

### Mitochondrial membrane potential

Analysis of mitochondrial membrane potential in living cells was performed by a fluorescent-activated cell sorting (FACS)-based method. Stable polyclonal HEK293 or SH-SY5Y cells were treated with vehicle or 0.5  $\mu$ M staurosporine for 4 h in a

humidified 5% CO<sub>2</sub> atmosphere at 37°C. After incubation cells were harvested with trypsin, pelleted and washed in PBS (PBS; Invitrogen, Germany). Cells were incubated in PBS containing 5  $\mu$ g/ml JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; Molecular Probes, USA) for 15 min at 37°C, followed by three wash steps with pre-warmed PBS. For each sample, 10 000 cells were analysed for JC-1 monomers and JC-1-aggregates on a FACScalibur (BD Biosciences, USA) with a 488 nm argon laser. Results are representative of four independent experiments.

### Viability assay

To determine cell viability in different paradigms of cellular stress, we measured LDH release from cells using Cytotoxicity Detection Kit (Roche, Germany). HEK 293 cells were seeded into 24-well plates and cultured overnight to reach 70–80% confluency. The medium was exchanged with 500  $\mu$ l DMEM containing 1% FBS and 0.5  $\mu$ M staurosporine for 6 h. After a brief centrifugation step, 100  $\mu$ l conditioned medium was transferred to 96-well plate to measure the activity of released LDH. Triton X-100 was added to a final concentration of 1% to the remaining culture medium. An aliquot of 100  $\mu$ l of cell lysate was used after a brief centrifugation step to measure the total amount of LDH per well. Subsequently, LDH activity was determined according to the manufacturer, using blank medium with or without Triton X-100 as reference using an ELISA reader (MRX, Dynatech Laboratories; USA) at 490 and 650 nm for absorbance. Experiments were performed in triplicate and results reported are an average of three independent experiments.

### Immunohistochemistry

Immunohistochemistry was carried out on formalin-fixed, paraffin-embedded 8  $\mu$ m-thick sections using the avidin-bitotin-immunoperoxidase technique. Sections were deparaffinized and microwaved for 15 min at 400 W. Endogenous peroxidase



activity was blocked by incubating the sections with 2.4% H<sub>2</sub>O<sub>2</sub> in bidistilled water for 15 min. After rising in PBS sections were brought into 10% goat serum and 2% bovine serum albumine (Biogenex, CA, USA) for 20 min at room temperature. Sections were then incubated with the rabbit anti-human Omi/HtrA2 (Apotech, USA), diluted 1:100 in PBS containing 0.1% BSA overnight at 4°C. Next day, sections were washed in PBS, incubated in biotinylated goat anti-rabbit IgG diluted in PBS (StrAvidGen multi-Link Kit, Biogenex) for 30 min at room temperature, rinsed again in PBS and then incubated for 30 min at room temperature in peroxidase-conjugated streptavidin in PBS (StrAvidGen multi-Link Kit, Biogenex). After rinsing again in PBS, the enzymatic reaction was carried out for 2 min at room temperature with a solution containing 4% 3-amino-9-ethylcarbazole and H<sub>2</sub>O<sub>2</sub> substrate buffer (Biogenex). Subsequently, sections were counterstained with hematoxylin. To confirm specificity of the immunostaining, Omi/HtrA2 antibody was omitted in control sections.

### Statistics

Genetic data of the A141S polymorphism were evaluated for allele frequencies, genotype frequencies using the Genepop program originally designed by Michel Raymond and Francois Rousset (1995) (31). Differences in allele or genotype frequencies were evaluated by  $\chi^2$  analysis. A *P*-value <0.05 was accepted as statistically significant. For viability tests, statistical analysis was performed by analysis of variance (ANOVA) followed by Tukey's *post hoc* test to compare group means. Data are expressed as mean  $\pm$  SD values. Tests of variance homogeneity, normality and distribution were performed to ensure that the assumptions required for standard parametric analysis of variance were satisfied. In all analyses, the null hypothesis was rejected at the 0.05 level.

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