The mitochondrial protease HtrA2 is regulated by Parkinson's disease associated kinase PINK1

Hélène Plun-Favreau [1,7], Kristina Klupsch [1,7], Nicoleta Moisoi [4], Sonia Gandhi [5], Svend Kjaer [2], David Frith [3], Kirsten Harvey [6], Emma Deas [5], Robert J. Harvey [6], Neil McDonald [2], Nicholas W. Wood [5], L. Miguel Martins [4,8] and Julian Downward [1,8]

[1] Signal Transduction, [2] Structural Biology and [3] Protein Analysis Laboratories, Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK.

[4] Cell Death Regulation Laboratory, MRC Toxicology Unit, Lancaster Road, Leicester LE1 9HN, UK.

[5] Department of Molecular Neuroscience, Institute of Neurology, and National Hospital for Neurology and Neurosurgery, Queen Square, London, WC1N 3BG, UK.

[6] Department of Pharmacology, The School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX, UK.

[7] These authors contributed equally to this work

[8] Corresponding authors:

e-mail. julian.downward@cancer.org.uk

Tel. (44) 20 7269 3533, Fax. (44) 20 7269 3094

e-mail. lmm24@leicester.ac.uk

Tel. (44) 116 252 5533, Fax. (44) 116 252 5616

Keywords: HtrA2 / Omi / PINK1 / apoptosis / protease / parkinsonian syndrome

Running title: PINK1 targets HtrA2

SUMMARY

In mice, targeted deletion of the serine protease HtrA2/Omi causes mitochondrial dysfunction leading to a neurodegenerative disorder with parkinsonian features. In humans, point mutations in HtrA2 are a susceptibility factor for Parkinson's disease (PARK13 locus). Mutations in PINK1, a putative mitochondrial protein kinase, are associated with the PARK6 autosomal recessive, early-onset Parkinson's disease (PD) susceptibility locus. Here we determine that HtrA2 interacts with PINK1 and both are components of the same stress-sensing pathway. HtrA2 is phosphorylated upon activation of the p38 pathway, occurring in a PINK1-dependent manner at a residue adjacent to a position found mutated in PD patients. Importantly, HtrA2 phosphorylation is decreased in brains of PD patients carrying PINK1 mutations. We suggest that PINK1-dependent phosphorylation of HtrA2 might modulate its proteolytic activity, thereby contributing to increased resistance of cells to mitochondrial stress.

INTRODUCTION

The serine protease HtrA2 was first identified as a mammalian homologue of the *Escherichia coli* endopeptidases HtrA/DegP and DegS [1,2]. Like its bacterial orthologues, HtrA2 has a PDZ domain in addition to its serine protease domain. In mammalian cells, HtrA2 is localised to the mitochondria. A mature form of HtrA2 can be generated by proteolysis revealing an N-terminal sequence motif related to that of *Drosophila melanogaster* death-promoting proteins Reaper, Grim and Hid. During apoptosis, mature HtrA2 is released from the mitochondria into the cytosol, where it binds the Inhibitor of Apoptosis proteins (IAPs) via its N-terminal sequence. Binding of mature HtrA2 to IAPs relieves their inhibitory action towards caspases contributing to the induction of apoptosis [3-6]. In addition, this binding results in the increase of the proteolytic activity of HtrA2 [7], further contributing to apoptotic cell death [8]. HtrA2 has therefore been proposed to be a pro-apoptotic protein analogous to *Drosophila* Reaper.

However, this view of HtrA2 function has been thrown into doubt by the phenotype of mice with a homozygous deletion of the HtrA2 gene or a point mutation causing defective protease activity; these both die prematurely and display a neurodegenerative phenotype [9,10], with some similarity to parkinsonian neurodegeneration. It is possible that HtrA2 acts to protect mitochondria from certain stresses in a manner similar to the homologous stress-adaptive proteases DegP and DegS in bacteria [11,12]. We therefore studied whether HtrA2 is important in the aetiology of Parkinson's disease (PD) in humans and have identified and characterised two mutations in HtrA2 linked to the disorder which deregulate its serine protease activity [13]. The *HtrA2* gene has been designated as the Parkinson's disease-13 (PARK13) locus. These studies suggest that loss of function of HtrA2 leads to neuronal cell death, possibly due to mitochondrial dysfunction, both in mice and in man. Here we have set out to identify proteins interacting with HtrA2 and have identified PTEN-induced putative kinase 1 (PINK1) as a binding partner. We subsequently determined that HtrA2 is phosphorylated upon activation of p38, and that PINK1 is important for this phosphorylation event to occur. We also show that phospho-HtrA2 levels are decreased in the brains of patients with PD PINK1 mutations. Phosphorylation of HtrA2 is likely to modulate the proteolytic activity of HtrA2 and contribute to increased resistance of cells to mitochondrial stress.

RESULTS

PINK1 interacts with HtrA2

A C-terminally TAP-tagged version of HtrA2 was stably expressed in HEK293 cells, enabling the recovery and purification of tagged HtrA2 along with interacting proteins. Using 35 antibodies raised against a range of 29 mitochondrial proteins as well as an anti-XIAP antibody as a positive control, the purified fraction containing HtrA2 was analysed (Table 1). We co-purified HAX-1, a protein previously shown to interact with HtrA2 [14], as well as XIAP, demonstrating the validity of this approach. One other protein was found to co-purify with HtrA2, the putative mitochondrial kinase PINK1 (Fig. 1a). Mutations in the *PINK1* gene are associated with the recessive Parkinsonism locus PARK6. Initially, three pedigrees were described with identified mutations: a G309D point substitution in one family and a truncation mutation (W437X) in two additional families [15]. Subsequently, several studies have described other point mutations or truncations [16-20]. Given that dysfunction in both HtrA2 and PINK1 has been associated with parkinsonian neurodegeneration in mammals, we hypothesised that they might both be components of the same stress-sensing pathway. To confirm the interaction between endogenous PINK1 and HtrA2, we used an anti-HtrA2 antibody to immunoprecipitate endogenous HtrA2. When probed with an anti-PINK1 antibody, we were able to detect endogenous PINK1 in complexes purified from wild type (WT) mouse embryonic fibroblasts (MEFs) and SH-SY5Y human neuroblastoma cells but not in cells lacking HtrA2 (HtrA2 KO MEFs) or lysis buffer alone, further confirming the specificity of the interaction (Fig. 1b).

To determine which regions of PINK1 are involved in the interaction with HtrA2, various deletion mutants of PINK1 were created (Fig. 1c). The PINK1 mutant proteins were all at least partially co-localised to the mitochondria when corresponding constructs were over-expressed in U2OS cells. This was even the case for the PINK 150-507 construct, which encoded only the kinase domain (see Supplementary Information, Fig. S1). Co-purification of Myc-tagged PINK1 deletion mutants with FLAG-tagged HtrA2 showed that the kinase domain of PINK1 was sufficient for the interaction with HtrA2 (Fig. 1d). We next performed co-immunoprecipitation experiments from lysates prepared from cells subjected to sub-cellular fractionation. Such experiments showed that the interaction between HtrA2

and PINK1 is detected predominantly in mitochondria-enriched fractions (Fig. 1e). Therefore we would hypothesise that the interaction between PINK1 and HtrA2 is likely to occur in the mitochondria. However, it cannot be excluded that the interaction could occur initially in the cytosol before both proteins are imported into the mitochondria.

HtrA2 is phosphorylated upon MEKK3 activation

A mutation screen of the *HtrA2* gene performed in German PD patients recently led to the identification of a novel heterozygous G399S mutation as well as an A141S polymorphism that was associated with the disease [13]. The identified mutations localise to domains previously shown to be important in the regulation of the proteolytic activity of HtrA2 [7], namely the PDZ domain and the N-terminal portion of the mature form of HtrA2, respectively. An analysis using the Scansite algorithm [21] (www.scansite.mit.edu) indicates that Ser142 and Ser400, residues immediately adjacent to the mutations found in the PD patients, are putative phosphorylation sites for proline-directed serine/threonine kinases (Fig. 2a). Alignment of HtrA2 homologues of different species shows that Ser142 and Ser400 are conserved in human, rodents and chicken, and in addition Ser400 is further conserved in fly and baker's yeast (see Supplementary Information, Fig. S2a). According to Scansite, Ser142 is a putative phosphorylation site for CDC2, CDK5, ERK1 and p38, and Ser400 is a putative phosphorylation site for CDC2 and CDK5. Based on this, we tested whether recombinant HtrA2 could be phosphorylated by any of these kinases. These experiments revealed that HtrA2 could be phosphorylated by p38β, p38γ, p38δ, ERK1, CDC2 and CDK5 *in vitro* (see Supplementary Information, Fig. S2b). In order to determine the position of the phosphorylation sites on HtrA2, we subsequently performed mass spectrometric analysis using *in vitro* p38γ phosphorylated HtrA2. This analysis detected a phospho-serine at position 142 (data not shown). Due to the behaviour of the Ser400-containing peptide in mass spectrometric analysis, we were unable to address the phosphorylation state of this site directly. Subsequently, we raised an antibody that specifically recognises HtrA2 only when phosphorylated on Ser142.

To investigate the nature of the kinase phosphorylating HtrA2 on Ser142 in intact cells, using cell lines expressing 4-hydroxytamoxifen (4OH-Tx) inducible

versions of MEKK3, Akt and Raf (ΔMEKK3:ER, myrAkt:ER, ΔRaf-DD:ER). Lysates were then prepared from cells where MEKK3, Akt and Raf had been activated and probed with an antibody that specifically recognises HtrA2 that is phosphorylated on Ser142. These experiments showed that only activation of MEKK3 induced efficient phosphorylation of both full length and processed HtrA2 on Ser142 (Fig. 2b and see Supplementary Information, Fig. S2c). Activation of MEKK3 resulted in strong activation of endogenous p38, weaker activation of ERK or JNK and no activation of Akt (Fig. 2b).

To test if chemical inhibition of p38 affected MEKK3-dependent phosphorylation of HtrA2 on Ser142, we treated ΔMEKK3:ER expressing cells with increasing concentrations of BIRB796, a broad-spectrum inhibitor of all p38 MAPK isoforms *in vitro* and *in vivo* [22]. This partially decreased the levels of detected phospho-p38 and phopho-HtrA2, confirming the importance of p38 for the phosphorylation of HtrA2 (Fig. 2c). Accordingly, down regulation of MKK3 and MKK6, kinases upstream of p38 and activated downstream of MEKK3, led to a decrease in the levels of phospho-p38 and phospho-Ser142 HtrA2 (Fig. 2d).

To determine if p38γ phosphorylation could target either of the two identified Ser residues, we performed an *in vitro* kinase assay using S142A/S400A HtrA2. This mutant protein was considerably less phosphorylated by p38γ *in vitro* (Fig. 2e). Nevertheless, a slight increase in the incorporation of radioactive phosphate into HtrA2 remained, possibly explained by phosphorylation on Ser137, which was observed by mass spectroscopy using *in vitro* p38γ phosphorylated wild type HtrA2 (data not shown). This site was not further pursued, as it is not conserved between human and murine HtrA2 (see Supplementary Information, Fig. S2a).

In vitro, p38 is also able to phosphorylate a peptide containing the sequences around Ser142. As a control, we used a peptide where Ser142 is mutated into Ala, further confirming the specificity of the assay. The Ser142 peptide can only be phosphorylated by p38, not by ERK1, CDC2 or CDK5 (see Supplementary Information, Fig. S2d).

PINK1 modulates the levels of phosphorylated HtrA2

We next sought to determine if PINK1 could modulate the level of phosphorylated HtrA2 in cells. We first determined that siRNA-mediated suppression

of PINK1 resulted in a significant decrease of both PINK1 mRNA transcript and protein (see Supplementary Information, Fig. S3a and b). Transient down regulation of PINK1 resulted in a decrease in the levels of endogenous phospho-Ser142 HtrA2 (Fig. 3a) suggesting that PINK1 is capable of modulating HtrA2 phosphorylation. We next assessed the role of PINK1 in the modulation of MEKK3-induced phosphorylation of HtrA2. To achieve this, we looked at the levels of phosphorylated HtrA2 in 4OH-Tx treated ΔMEKK3:ER expressing HEK293 cells upon removal of endogenous PINK1 by RNA interference. PINK1 suppression resulted in a significant decrease in the levels of phospho-Ser142 HtrA2 when compared to controls (Fig. 3b and S3c), thus further confirming the role of PINK1 in an MEKK3/p38 pathway implicated in HtrA2 phosphorylation. Down regulation of MKK3 and MKK6 similarly resulted in a decrease in HtrA2 phosphorylation upon activation of MEKK3, confirming the activation of this specific pathway (Fig. 3c). However, siRNA-mediated knock down of CDK5 and CDC2 (see Supplementary Information, Fig. S3b) had minimal impact on MEKK3-induced phospho-Ser142 HtrA2 (Fig. 3c).

Another way to induce p38 phosphorylation is to stimulate the cells with $CoCl_2$, a hypoxia-mimetic agent that elevates reactive oxygen species levels [23]. Strikingly, when phospho-p38 levels were increased in HEK293 cells upon $CoCl_2$ treatment, phospho-Ser142 HtrA2 levels were also increased. Importantly, $CoCl_2$ stimulated HtrA2 phosphorylation is reduced by MKK3/6 and PINK1 siRNA (Fig. 3d, for quantitation see Supplementary Information, Fig. S3d and S3e).

The data reported so far suggest that PINK1 might be involved in a stress response at least in part by modulating the phosphorylation of HtrA2 on Ser142. Based on the importance of these two proteins in the pathogenesis of nigral neurodegeneration we investigated the PINK1/HtrA2 pathway in human brain with Parkinson's disease. We compared the level of phosphorylation of HtrA2 in control brain, brain with idiopathic PD (IPD), and brain with PD associated with mutations in the *PINK1* gene (Y431H and C575R). Western blot analysis performed using the HtrA2 phospho-specific antibody revealed a low level of phospho-Ser142 HtrA2 in control brain tissue. The level of phosphorylated HtrA2 was significantly higher in brains with IPD than in controls. Strikingly, the phosphorylation of HtrA2 was virtually abolished in brains with PD associated with PINK1 mutations (Fig. 3e). Co-expression of Myc-tagged PINK1 PD mutants with FLAG-tagged HtrA2 followed by purification of the complexes indicated that neither C575R, Y431H nor any of seven

other PD-associated mutant forms of PINK1 have lost their ability to interact with HtrA2 (see Supplementary Information, Fig. S3f). Thus it appears likely that HtrA2 phosphorylation is decreased in brains of PARK6 PD patients and that this requires an activity of PINK1 in addition to its physical interaction with HtrA2.

Mutations mimicking phosphorylated HtrA2 enhance its protease activity

To determine whether HtrA2 phosphorylation is likely to affect its proteolytic activity, we produced S142D and S400D phospho-mimetic HtrA2 mutants and compared their protease activity to the WT enzyme using a previously described assay [7]. Comparing the basal activity of WT, S142D and S400D mutants, we noticed that both phospho-mimic mutations increased the ability of HtrA2 to cleave an HtrA2-specific fluorogenic substrate peptide. The proteolytic activity of HtrA2 is increased about 2-fold in the S142D phospho-mimetic mutant and 3-fold in case of the S400D mutant (Fig. 4a). To control for the effect of introducing any mutation at residues Ser142 and Ser400 rather than an effect specific to the phospho-mimetic aspartate, these residues were additionally mutated to an alanine. Both, S142A and S400A, showed markedly lower protease activity when compared to their phospho-mimic counterparts (Fig. 4a). Quality control on the different HtrA2 mutants was performed by SDS-PAGE analysis (Fig. 4b). The S400D phospho-mimetic mutation within the PDZ domain may cause opening up of the PDZ domain or might reorient the flexible L3 loop as previously shown for WT HtrA2 [7,24,25], therefore leading to an increase in proteolytic activity of HtrA2. Similarly, the S142D site is close to the mature N-terminus of HtrA2, where XIAP has been shown to bind to and lead to increased HtrA2 activity [7]. It is thus likely that phosphorylation of HtrA2 increases its proteolytic activity.

It has been shown previously that the A141S polymorphism and the G399S mutation in HtrA2 identified in PD patients led to a decrease in the activation of HtrA2 protease activity by PDZ peptide and XIAP [13]. Therefore, we sought to determine whether these mutations could affect the ability of HtrA2 to be phosphorylated by p38. To achieve this, we performed an *in vitro* kinase assay with peptides containing either the Ser142 or the Ser400. The ability of p38γ to phosphorylate the peptide with the G399S PD mutation was decreased (Fig. 4c). Therefore, it is possible that the mutations in HtrA2 occurring in PD, especially

G399S, might impact on its protease activity at least in part by affecting the phosphorylation of Ser400.

Analysis of the contribution of HtrA2 and PINK1 to cell survival

Given that the data so far indicate that PINK1 could modulate the activity of HtrA2 through influencing its phosphorylation status, we assessed the effect of removing PINK1 by RNA interference on the survival of WT and HtrA2 KO cells in response to the mitochondrial toxin 6-hydroxydopamine (6-OHDA). We reasoned that if PINK1 acts upstream of HtrA2 in a mitochondrial pathway responsible for resistance to stress, removal of this kinase in cells devoid of HtrA2 might not be as significant as in WT cells. When 6-OHDA was added to WT and HtrA2 KO MEFs transfected either with control or PINK1 siRNA, we observed a decrease of mitochondrial membrane potential, measured with the potential-sensitive probe TMRE. We quantitated the mitochondrial potential based on TMRE fluorescence in viable cells and established that, even in the absence of any mitochondrial stress, down regulation of PINK1 in WT but not HtrA2 KO cells resulted in a significant decrease in mitochondrial potential when compared to cells transfected with control siRNA. Additionally, HtrA2 KO cells displayed a reduced TMRE fluorescence in the absence of any treatment (Fig. 5a).

Next, we proceeded to determine cell viability in these experimental settings. Our results indicate that down regulation of PINK1 sensitises WT MEFs to 6-OHDA, whereas removal of PINK1 from HtrA2 KO cells is unable to further sensitise these cells to 6-OHDA (Fig. 5b). To rule-out possible RNAi artefacts, these experiments were repeated with two different siRNA oligos targeting PINK1 (see Supplementary Fig. S4a and data not shown). In order to determine the ability of different HtrA2 mutants to protect from cell death induced by 6-OHDA and also the complex I inhibitor rotenone, we made use of HtrA2 KO MEFs where we transiently expressed WT HtrA2 or the phospho-mimetic mutants. Additionally, we included in these transfection experiments either control or PINK1 targeting siRNAs. We observed that re-introduction of HtrA2 into HtrA2 KO MEFs resulted in a significant protection from these toxic stimuli (Fig. 5c and see Supplementary Information, Fig. S4b). These levels of protection were decreased in cells where PINK1 was targeted by siRNA. We also observed that HtrA2 phospho-mimetic mutants have an enhanced ability to protect these cells from stress induced by either 6-OHDA or rotenone. Such

protection was also observed in cells where PINK1 expression was suppressed suggesting that the HtrA2 phospho-mimetic mutants confer enhanced protection to these stress stimuli and relieve the dependency on PINK1 for this process.

Further evidence that PINK1 plays a role upstream of HtrA2 in a stress protective pathway comes from results indicating that over-expression of PINK1 protects WT better than HtrA2 KO MEFs against stress-induced apoptosis (see Supplementary Information, Fig. S4c). Data presented so far indicates that PD associated PINK1 mutations (Y431H and C575R) result in a decrease in the levels of phospho-Ser142 HtrA2 *in vivo* (Fig. 3e). We therefore sought to determine the effect of co-expressing PINK1 containing PD associated mutations (Y431H and C575R) with HtrA2 in drug treated HtrA2 KO MEFs. Restoring HtrA2 expression partially rescued cell death triggered by oxidative stress stimuli (6-OHDA and rotenone) as well as the proteasome inhibitor MG132 (Fig. 5d). This protective effect of HtrA2 was further enhanced by co-expression of PINK1. However, co-expression of HtrA2 with PINK1 harbouring PD associated point mutations (Y431H and C575R) failed to rescue cell death to the same degree as HtrA2 alone, suggesting that such PINK mutant proteins suppress the ability of HtrA2 to protect cells from toxic stimuli. Taken together, these results suggest that the PD mutations associated with PINK1 used in this study might act as dominant negative inhibitors of HtrA2, preventing the productive interaction between endogenous WT PINK1 and HtrA2.

DISCUSSION

Seven nuclear genes have been found to carry mutations that are implicated in the pathogenesis of PD, encoding parkin (PARK2 locus), α-synuclein (PARK1 and PARK4 loci), UCHL1 (PARK5), DJ-1 (PARK7), dardarin (PARK8), ATP13A2 (PARK9) and PINK1 (PARK6) [26-29]. Mutations in these genes cause protein dysfunction which may lead to a range of different but overlapping pathological effects in neurons: protein aggregation, proteasomal stress, oxidative stress and mitochondrial impairment. On the basis of studying genetic models of PD, similar mechanisms have been proposed to contribute to the pathogenesis of sporadic forms of the disease. However, little is understood about putative direct or functional interactions between the genes that cause PD and a single pathway unifying these proteins has not been confirmed. Furthermore, the relevance of any such pathway in sporadic PD has not been studied.

Recently, HtrA2 has been added to this list (PARK13 locus) since two protease-inactivating mutations have been characterised that have been implicated in PD [13]. The location of these mutations adjacent to two putative phosphorylation sites in the protein, Ser142 and Ser400, suggested that these sites might play a role in the regulation of the enzymatic activity of HtrA2. In this study, we have identified PINK1 as a new binding partner of HtrA2. We have determined that HtrA2 can be phosphorylated upon MEKK3 activation on a serine residue adjacent to one mutated in PD, Ala141. We have also shown that PINK1 is important for this phosphorylation to occur optimally and that disease causing mutations in PINK1 decrease HtrA2 phosphorylation in PARK6 PD patient brain relative to idiopathic PD patient brain. Interestingly, HtrA2 mutant proteins mimicking the phosphorylation on these sites show an increase in their protease activity and this increase seems to be necessary to protect mitochondria under stressful conditions.

The role of PINK1 in HtrA2 regulation

It has been hypothesised that PINK1 may phosphorylate mitochondrial proteins in response to cellular stress, protecting against mitochondrial dysfunction [15]. Our data linking PINK1 to regulation of HtrA2 phosphorylation combined with the genetic analysis of HtrA2 and PINK1 function in *Drosophila,* which places HtrA2

downstream of PINK1 and Parkin (Begum et al., accompanying paper), suggest that both HtrA2 and PINK1 proteins take part in the same signalling pathway. From their observed interaction, it was a possibility that HtrA2 might be a PINK1 substrate, so we sought to measure the PINK1 kinase activity towards HtrA2 in an *in vitro* assay. Unfortunately, the PINK1 kinase activity demonstrated so far [30-32] is very weak and we were not able to improve on this; as a result we are not able to conclude whether or not PINK1 phosphorylates HtrA2 directly.

However, in this study we were able to show that PINK1 expression was important for HtrA2 phosphorylation to occur effectively, at least on Ser142. The most likely possibility is that HtrA2 phosphorylation is mediated by the p38 pathway, but is dependent on the interaction of HtrA2 with PINK1, possibly reflecting the existence of a trimeric complex. The precise cellular location where the phosphorylation of HtrA2 occurs is not known. One possibility is that it could require p38 recruitment to the mitochondria: p38 has been reported in mitochondrial fractions in some studies [33]. This could explain the prolonged time course required for MEKK3 activation to lead to HtrA2 phosphorylation. Alternatively, it is also possible that the phosphorylation event occurs in the cytosol prior to import of HtrA2 into the mitochondria. Being located just next to the cleavage site, phosphorylation on Ser142 could also impact on the processing of the protein. Alzheimer's amyloid precursor protein (APP) processing can be regulated by phosphorylation and it is now widely accepted that abnormal processing of APP can contribute significantly to Alzheimer's disease (for review [34]).

Is HtrA2 protease activity increased in stressed cells via p38? The evidence for activation of the p38 pathway in disease states and in models of neurodegenerative disorders is now widely accepted (for review [35]). In this study we determine that HtrA2 is phosphorylated and identify Ser142 as a phosphorylation site. Moreover, we show that PINK1 is implicated in the MEKK3/p38 pathway leading to HtrA2 phosphorylation and that this phosphorylation is likely to regulate the protease activity of HtrA2. Moreover, we show that prolonged hypoxic stress, mediated by $CoCl_2$ treatment, also induces p38 and HtrA2 phosphorylation. Nevertheless, the function of HtrA2 and the significance of its serine protease activity remain largely unclear. The notion that HtA2 is a pro-apoptotic protein analogous to *Drosophila* Reaper has been thrown into doubt by the parkinsonian phenotype of mice lacking HtrA2 function [9,10] and also by its neuroprotective function in cells subjected to stress.

It is therefore attractive to speculate that human HtrA2 might act more like its bacterial homologues DegS and DegP. It could degrade unfolded proteins, performing crucial functions with regard to protein quality control in the intermembrane space of the mitochondria, similarly to DegP. Alternatively, it could transduce a stress adaptive signal, like DegS (for review [36]).

The role of PINK1 and HtrA2 in Parkinson's disease

Our *in vitro* data suggest that p38, PINK1 and HtrA2 participate in a stress-sensing pathway in which the phosphorylation of HtrA2 increases its protease activity and results in protection from the causative stress. In order to validate the importance of this pathway in neurodegenerative disease, we compared control human brain tissue and PD brain. Interestingly we found that phosphorylation of HtrA2 at S142 was significantly increased in brain with idiopathic Parkinson's disease compared to control tissue. This suggests that the particular, albeit unknown, stress that results in sporadic PD is also associated with activation of a stress-sensing pathway resulting in the phosphorylation of HtrA2, possibly in an attempt to protect neurons from apoptosis.

In order to confirm that PINK1 modulates the phosphorylation of HtrA2 at S142 *in vivo*, we compared the phosphorylation of HtrA2 in idiopathic PD brain and PD brain with PINK1 mutations (C575R and Y431H). Both of these mutations are predicted to have a major pathogenic effect on PINK1 function. The Y431H mutation is predicted to affect the packaging of the APE motif at the end of the activation segment. The C575R mutation lies outside the kinase domain in the C-terminal tail, which contains regulatory motifs that may influence the kinase [32]. Over-expression of Y431H and C575R mutant forms of PINK1 results in a significant decrease in the mitochondrial membrane potential compared to WT PINK1 on exposure of the cells to proteasomal stress [37]. PD brain associated with these two heterozygous PINK1 mutations is morphologically indistinguishable from sporadic PD and exhibits the pathological hallmarks of dopaminergic neuronal cell loss in the substantia nigra and the presence of Lewy bodies [38]. We show that in brain, the phosphorylation of HtrA2 is virtually abolished in the presence of PINK1 mutations, further supporting our hypothesis that functional PINK1 is necessary for the phosphorylation of HtrA2. The protective function of HtrA2 that is conferred by phosphorylation in *in vitro* studies

would therefore be lost in brains with PINK1 mutations, increasing their vulnerability to stressful stimuli.

Our *in vitro* data suggests that the PINK1-HtrA2 pathway is a stress sensing pathway that is activated by particular stressful stimuli and acts to protect neurons from that insult. In keeping with this hypothesis, we find that there is increased phosphorylation of HtrA2, and thus activation of the pathway, in brains exposed to a stress leading to IPD. In brains that are exposed to a stress leading to nigral neuronal loss, but where PINK1 is not functional, we find that there is no activation of the PINK1-HtrA2 pathway. In such individuals, this potential protective response cannot be activated and thus these cases may be even more vulnerable to developing the pathology of PD.

Classically, as mutations in PINK1 cause an autosomal recessive form of PD the carriage of a single heterozygous mutation might be felt to be of no phenotypic significance. However there is accumulating clinical, radiological and genetic evidence that single heterozygous mutations represent a significant risk factor for developing disease in the presence of particular stress [37,39]. Our findings provide functional evidence that in the presence of a PD-inducing stress, a heterozygous mutation in the *PINK1* gene is sufficient to exert a significant effect on the phosphorylation of HtrA2, presumably via a haploinsufficiency mechanism. In the case of the heterozygous mutations in HtrA2 found in PD, these might have a greater than expected impact on overall HtrA2 activity as it functions as a trimer: if these PD mutant proteins were able to disrupt the function of a mixed mutant-wild type trimer then only one eighth of normal cellular activity would remain.

We have defined a mitochondrial stress responsive pathway involving HtrA2 and PINK1. We have shown a direct and functional interaction between two genes known to result in Parkinson's disease and suggested that this novel pathway may also be relevant to sporadic forms of this disease. The characterisation of this mitochondrial stress responsive pathway involving HtrA2 and PINK1 in neurodegeneration in humans, and also in flies (Begum et al., accompanying paper), will lead to improved understanding of the pathogenesis of Parkinson's disease and, ultimately, possibly to novel therapeutic approaches.

MATERIALS AND METHODS

Antibodies

For antibodies used to probe TAP purification eluate, please refer to Table1. Cell Signaling: anti-CDC2, anti-p38, anti-P-p38 (Thr180/Tyr182), anti-P-JNK (Thr183/Tyr185), anti-P-ERK1/2 (Thr202/Tyr204), anti-P-Akt (Ser473). Upstate: anti-CDK5. The anti-Smac antibody was generated as described [40]. HtrA2 polyclonal antibody was obtained from Alexis. M2 anti-FLAG and anti-FLAG agarose affinity gel were obtained from Sigma and 9E10 anti-Myc was developed and characterised as described [41]. The anti-Ser142 HtrA2 phospho-specific and total polyclonal antibody were generated against peptide SPPPASPRSQYC, based on the sequence surrounding Ser142 residue on human HtrA2. PINK1 polyclonal antibody was generated against peptide KSKPGPDPLDTRRLQ. Anti-PINK1 antibody was biotinylated using the EZ-link^{R} NHS-PEO Biotinylation kit (Pierce). Anti-mouse and -rabbit antibodies coupled to horseradish peroxidase were from Amersham. Streptavidin conjugated to peroxidase (DAKO) was required for the detection of biotinylated antibodies. All were visualised on X-ray film using ECL reagent (Amersham) according to the manufacturer's instructions.

Plasmids and reagents

Expression constructs encoding HtrA2-Flag, S306A HtrA2-Flag, S142D HtrA2-Flag, S400D HtrA2-Flag and S306A TAP-tagged HtrA2 were generated by adding the tag by PCR and sub-cloning the HtrA2 cDNA into the pcDNA3 vector (Invitrogen). S306A, S142A, S142D, S400D and S142A/S400A HtrA2, and PINK1 PD mutants were generated by site-directed mutagenesis using the QuickChange kit (Stratagene) and confirmed by DNA sequencing. Truncated PINK1 mutants were generated by PCR cloning.

The pEGFP-2 and pEGFP-F expression vectors were from Invitrogen

The pBP3:hbER* vector encoding human ΔMEKK3:ER* was a gift from Simon Cook [42]. BIRB796 was added 1 h prior to overnight (o/n) 4OH-Tx activation of MEKK3 in ΔMEKK3:ER stable cell line. BIRB796 was from Ana Cuenda [22]. ERK1, ERK2, CDK5, α, β, γ and p38δ were obtained from Upstate. Recombinant CDC2 was from New England Biolabs.

TAP-tagged protein purification

TAP purification was performed as described previously [4].

Protein biochemistry

Cell lines were lysed directly into 1x SDS gel sample buffer and resolved by SDS-PAGE. Densitometry analysis was performed using the gel analysis tool of the ImageJ image processing program (NIH, USA).

For immunoprecipitation, cells were lysed in 10 mM Tris–HCl pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate and 2 % CHAPS (v/v). After pelleting insoluble material, the supernatants were immunoprecipitated o/n. Complexes were then isolated using protein A sepharose beads and subjected to SDS–PAGE. Mitochondria-enriched and cytosolic fractions were obtained as described previously [43].

All gels were transferred onto Immobilon™ PVDF membranes (Millipore). The membranes were subsequently incubated with the indicated primary antibody before being incubated with the appropriate secondary antibody, or avidin labelled with peroxidase for 60 min. Where indicated, membranes were stripped in 0.1 M glycine pH 2.5, and neutralised in 1 M Tris-HCl pH 7.6 before re-blotting.

Human brains

Brain tissue was obtained from the Queen Square Brain Bank (where it had been donated with the informed consent of next of kin) with approval from the National Hospital for Neurology and Neurosurgery/Institute of Neurology Joint Research Ethics Committee. Two flash-frozen post-mortem brains with classical neuropathological appearances of PD, associated with heterozygous mutations in the *PINK1* gene were originally identified in an extensive mutation screen [37]. Four flash-frozen brains with idiopathic PD, and two control brains were used as age-matched and pH-matched controls. We initially screened a range of human brain regions (frontal cortex, temporal cortex, caudate, putamen, substantia nigra, cerebellum) for phospho-Ser142 HtrA2 and detected it most prominently in the caudate nucleus (data not shown). Frozen sections from the basal ganglia of eight brains were then cut to dissect the caudate nucleus. Tissue was homogenised in 10 mM Tris-HCl pH 7.6, 5

mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate and 2 % CHAPS, incubated on ice for 45 min, and centrifuged at 13,000 g for 15 min at 4ºC. Protein concentration was determined using the Bradford assay (Pierce).

***In vitro* kinase assay**

5 μg recombinant HtrA2 were incubated with 100 ng of the indicated kinase in a buffer containing 20 μCi of [γ-^{33}P] ATP, 50 mM Tris pH 7.6, 10 mM $MgCl_2$, 10 μM cold ATP, 100 nM okadaic acid for 30 min at 30°C in a final volume of 12 μl. The assays using recombinant HtrA2 or MBP as a substrate were resolved on a NuPAGE 4-12 % Bis-Tris gel (Invitrogen) and the assays using a peptide as a substrate were separated on 16% Tricine gels (Invitrogen). Following electrophoresis, gels were dried and incorporated radioactivity detected by autoradiography.

Peptide cleavage assay

Recombinant HtrA2 was prepared as previously described [7] and further fractionated by gel filtration chromatography (Superdex 75 prep grade column, GE Healthcare) to separate aggregated proteins from natively folded. Two fractions corresponding to the peak of WT HtrA2 were selected from each of the recombinant HtrA2 proteins. Purity and concentration of recombinant proteins were controlled by SDS-PAGE analysis and Bradford assay (Bio-Rad). Peptide cleavage was then assayed by incubating 56 and 209 nM HtrA2 with 10 μM fluorescent substrate as previously described [7]. Protease activity (arbitrary fluorescence units/min) was determined by linear regression analysis of the data points corresponding to the maximum reaction rates for each assay condition. The data presented are mean values +/- SD of 4 to 6 points.

Mitochondrial potential and survival assays

MEFs were reverse-transfected with siRNA oligos, and plated on 96 multi-well dishes (ParkerView). Following incubation at 37^{o}C for 36 hrs cells were treated with increasing concentrations of 6-OHDA (Sigma) for 14 hrs and stained with Hoechst33342 (0.3 μg/ml) and TMRE (50 nM). Live cell microscopy was performed using a KineticScan HCS Reader (KSR) automated microscope system. Data analysis

was performed using the KSR BioApplications software (Cellomics) with the molecular compartmental analysis algorithm [44].

For the rescue experiments employing HtrA2 expressing plasmids, HtrA2 KO MEFs were reverse transfected using Genejuice with 1.2 μg of HtrA2-encoding plasmid or vector control and 300 ng of pEGFP-F and either control or PINK1 siRNA at 50 nM final concentration (efficacy of siRNA mediated PINK1 down-regulation was confirmed in these experimental settings by quantitative RT-PCR analysis, see Supplementary Information, Fig. S4a). Cells were treated 48 hrs later with vehicle, 6-OHDA (25 μM) and rotenone (10 μM). Rescue experiments employing both HtrA2 and PINK1 expressing plasmids were performed similarly except that 600 ng of respective vectors were used. Additionally, 300 ng of pEGFP-F was added to the plasmid mixture and cells reverse transfected as described. For these experiments, cells were additionally treated with 0.1 μM MG132 before analysis. For viability analysis, 14 hrs after drug treatment, media was replaced with fresh culture media containing 1 μg/ml Hoechst33342 and live cell microscopy performed using the KSR system. Data analysis was performed using a compartmental analysis algorithm as before to select cells that were positive for GFP. All results are average +/- SD of at least 5 separate fields analysed using a 10x objective and are representative of 3 independent experiments.

Statistical analysis

Data are presented as mean values and error bars indicate the SD. The groups were compared by one- or two-way analysis of variance (ANOVA) using Bonferroni's post test using Prism statistical analysis software (www.graphpad.com). The significance is indicated as *** for $P < 0.001$, ** for $P < 0.01$, * for $P < 0.05$.

Further Materials and Methods can be found in the Supplementary Information online.

Acknowledgments

We thank Dave Hancock for valuable advice, Nick Totty for mass spectrometric analysis, Valina Dawson, Simon Cook, Giampietro Schiavo, Almut Schulze and Kai Dimmer for materials, Sam Loh for help with the Cellomics KSR microscopy system, and Annabel Borg and Tobias Simmonds for assistance in the preparation of recombinant HtrA2. K. Klupsch was funded by a PhD studentship from Boehringer Ingelheim Fonds. This work was supported by Cancer Research UK.

REFERENCES

1. Faccio, L. et al. Characterization of a novel human serine protease that has extensive homology to bacterial heat shock endoprotease HtrA and is regulated by kidney ischemia. *J Biol Chem* **275**, 2581-8 (2000).
2. Gray, C.W. et al. Characterization of human HtrA2, a novel serine protease involved in the mammalian cellular stress response. *Eur J Biochem* **267**, 5699-710 (2000).
3. Hegde, R. et al. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *J Biol Chem* **277**, 432-8 (2002).
4. Martins, L.M. et al. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. *J Biol Chem* **277**, 439-44 (2002).
5. Suzuki, Y. et al. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell* **8**, 613-21 (2001).
6. Verhagen, A.M. et al. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J Biol Chem* **277**, 445-54 (2002).
7. Martins, L.M. et al. Binding specificity and regulation of the serine protease and PDZ domains of HtrA2/Omi. *J Biol Chem* **278**, 49417-27 (2003).
8. Yang, Q.H., Church-Hajduk, R., Ren, J., Newton, M.L. & Du, C. Omi/HtrA2 catalytic cleavage of inhibitor of apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis. *Genes Dev* **17**, 1487-96 (2003).
9. Jones, J.M. et al. Loss of Omi mitochondrial protease activity causes the neuromuscular disorder of mnd2 mutant mice. *Nature* **425**, 721-7 (2003).
10. Martins, L.M. et al. Neuroprotective role of the Reaper-related serine protease HtrA2/Omi revealed by targeted deletion in mice. *Mol Cell Biol* **24**, 9848-62 (2004).
11. Spiess, C., Beil, A. & Ehrmann, M. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. *Cell* **97**, 339-47 (1999).
12. Walsh, N.P., Alba, B.M., Bose, B., Gross, C.A. & Sauer, R.T. OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. *Cell* **113**, 61-71 (2003).
13. Strauss, K.M. et al. Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. *Hum Mol Genet* **14**, 2099-111 (2005).
14. Cilenti, L. et al. Regulation of HAX-1 anti-apoptotic protein by Omi/HtrA2 protease during cell death. *J Biol Chem* **279**, 50295-301 (2004).
15. Valente, E.M. et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* **304**, 1158-60 (2004).
16. Bonifati, V. et al. Early-onset parkinsonism associated with PINK1 mutations: frequency, genotypes, and phenotypes. *Neurology* **65**, 87-95 (2005).
17. Hatano, Y. et al. Novel PINK1 mutations in early-onset parkinsonism. *Ann Neurol* **56**, 424-7 (2004).
18. Li, Y. et al. Clinicogenetic study of PINK1 mutations in autosomal recessive early-onset parkinsonism. *Neurology* **64**, 1955-7 (2005).
19. Rogaeva, E. et al. Analysis of the PINK1 gene in a large cohort of cases with Parkinson disease. *Arch Neurol* **61**, 1898-904 (2004).

20. Rohe, C.F. et al. Homozygous PINK1 C-terminus mutation causing early-onset parkinsonism. *Ann Neurol* **56**, 427-31 (2004).
21. Obenauer, J.C., Cantley, L.C. & Yaffe, M.B. Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Res* **31**, 3635-41 (2003).
22. Kuma, Y. et al. BIRB796 inhibits all p38 MAPK isoforms in vitro and in vivo. *J Biol Chem* **280**, 19472-9 (2005).
23. Chandel, N.S. et al. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc Natl Acad Sci U S A* **95**, 11715-20 (1998).
24. Li, W. et al. Structural insights into the pro-apoptotic function of mitochondrial serine protease HtrA2/Omi. *Nat Struct Biol* **9**, 436-41 (2002).
25. Wilken, C., Kitzing, K., Kurzbauer, R., Ehrmann, M. & Clausen, T. Crystal structure of the DegS stress sensor: How a PDZ domain recognizes misfolded protein and activates a protease. *Cell* **117**, 483-94 (2004).
26. Greenamyre, J.T. & Hastings, T.G. Biomedicine. Parkinson's--divergent causes, convergent mechanisms. *Science* **304**, 1120-2 (2004).
27. Paisan-Ruiz, C. et al. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron* **44**, 595-600 (2004).
28. Ramirez, A. et al. Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. *Nat Genet* **38**, 1184-91 (2006).
29. Zimprich, A. et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron* **44**, 601-7 (2004).
30. Beilina, A. et al. Mutations in PTEN-induced putative kinase 1 associated with recessive parkinsonism have differential effects on protein stability. *Proc Natl Acad Sci U S A* **102**, 5703-8 (2005).
31. Silvestri, L. et al. Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. *Hum Mol Genet* **14**, 3477-92 (2005).
32. Sim, C.H. et al. C-terminal truncation and Parkinson's disease-associated mutations down-regulate the protein serine/threonine kinase activity of PTEN-induced kinase-1. *Hum Mol Genet* (2006).
33. Baines, C.P. et al. Mitochondrial PKCepsilon and MAPK form signaling modules in the murine heart: enhanced mitochondrial PKCepsilon-MAPK interactions and differential MAPK activation in PKCepsilon-induced cardioprotection. *Circ Res* **90**, 390-7 (2002).
34. da Cruz e Silva, O.A. et al. Signal transduction therapeutics: relevance for Alzheimer's disease. *J Mol Neurosci* **23**, 123-42 (2004).
35. Harper, S.J. & Wilkie, N. MAPKs: new targets for neurodegeneration. *Expert Opin Ther Targets* **7**, 187-200 (2003).
36. Young, J.C. & Hartl, F.U. A stress sensor for the bacterial periplasm. *Cell* **113**, 1-2 (2003).
37. Abou-Sleiman, P.M. et al. A heterozygous effect for PINK1 mutations in Parkinson's disease? *Ann Neurol* **60**, 414-9 (2006).
38. Gandhi, S. et al. PINK1 protein in normal human brain and Parkinson's disease. *Brain* **129**, 1720-31 (2006).
39. Hedrich, K. et al. Clinical spectrum of homozygous and heterozygous PINK1 mutations in a large German family with Parkinson disease: role of a single hit? *Arch. Neurol.* **63**, 833-838 (2006).

40. Vyas, S. et al. Differentiation-dependent sensitivity to apoptogenic factors in PC12 cells. *J Biol Chem* **279**, 30983-93 (2004).
41. Evan, G.I., Lewis, G.K., Ramsay, G. & Bishop, J.M. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Biol* **5**, 3610-6 (1985).
42. Garner, A.P., Weston, C.R., Todd, D.E., Balmanno, K. & Cook, S.J. Delta MEKK3:ER* activation induces a p38 alpha/beta 2-dependent cell cycle arrest at the G2 checkpoint. *Oncogene* **21**, 8089-104 (2002).
43. Rytomaa, M., Lehmann, K. & Downward, J. Matrix detachment induces caspase-dependent cytochrome c release from mitochondria: inhibition by PKB/Akt but not Raf signalling. *Oncogene* **19**, 4461-8 (2000).
44. Abraham, V.C., Taylor, D.L. & Haskins, J.R. High content screening applied to large-scale cell biology. *Trends Biotechnol* **22**, 15-22 (2004).

TABLE

Company (clone)	Antigen	Detected in HtrA2-TAP eluate
SCBT (Q-18, N19)	ANT	No
SCBT (C-20), BD (cl 48)	Bad	No
Stressg (2-14), Calbio (Ab-1)	Bak	No
Mito (YTH-6A7), Imgenex	Bax	No
SCBT (N-19), Pharm (4D7)	Bcl-2	No
BD (cl 64)	Bcl-XL	No
Abcam (30A882.1.1)	Bif-1	No
SCBT (N-19, FL-60)	Bik	No
SCBT (N20)	Bim	No
Calbio (77-92)	Bnip3L	No
MolProbes (21C11)	ComplexI 17kD subunit	No
MolProbes (2E3)	ComplexIII (UQCRC1)	No
MolProbes (7H10)	ComplexV subunit alpha	No
MolProbes (3D5)	ComplexV subunit beta	No
MolProbes (7F9)	ComplexV subunit d	No
MolProbes (4C11)	ComplexV subunit OSCP	No
MolProbes (5E2)	ComplexV inh. Protein	No
Mito (12F4AD8AF8)	ComplexV	No
Pharm (7H8.2C12)	Cytochrome c	No
V. Dawson	DJ-1	No
Stressg (30A5)	Grp-75	No
BD (cl 52)	HAX-1	Yes
SCBT (N-20)	Hrk	No
Stressg (Map11-13)	Hsp-60	No
CRUK generated	PINK1	Yes
Abcam (II-14-10)	Prohibitin	No
CRUK generated	Smac	No
Calbio (Q-18)	VDAC	No
SCBT (C-17)	VDAC2	No
BD (cl 48)	XIAP	Yes

Table 1. Identification of proteins complexed with HtrA2.

TAP epitope-tagged HtrA2 was purified from HEK293 cells stably expressing the fusion protein, resolved by SDS-PAGE and probed with antibodies raised against mitochondrial proteins. Abbreviations: SCBT, Santa Cruz Biotechnology; BD, BD Transduction Laboratories; Stressg, Stressgen; MolProbes, Molecular Probes; Calbio, Calbiochem; Mito, Mitosciences.

FIGURE LEGENDS

Figure 1. HtrA2 binds PINK1.

(a) TAP purification of HtrA2 interacting proteins. TAP epitope-tagged HtrA2 or TAP alone were purified from HEK293 stable cell lines, resolved by SDS-PAGE and probed with anti-XIAP, anti-HAX-1 or anti-PINK1 antibodies. **(b)** Co-immunoprecipitation of endogenous PINK1 with endogenous HtrA2. Lysates prepared from MEFs and SH-SY5Y cells were subjected to immunoprecipitation with anti-HtrA2 antibody and purified proteins were subjected to Western blot (WB) with biotinylated anti-PINK1 antibody. Asterisk denotes IgG background band. **(c)** Schematic representation of three deletion mutant proteins of PINK1 (shaded area represents the putative kinase domain). **(d)** Co-immunoprecipitation of HtrA2 with the kinase domain of PINK1. HEK293 cell lysates expressing full length HtrA2-Flag and truncated PINK1-Myc constructs were subjected to immunoprecipitation with anti-Myc or anti-HtrA2 antibodies. Detection was performed with anti-Myc or anti-FLAG antibodies. **(e)** Co-immunoprecipitation of HtrA2 with PINK1 in the mitochondria-enriched fraction. PINK1-Myc was expressed in HEK293 cells. Cytosol and mitochondria-enriched fractions were subjected to immunoprecipitation with anti-HtrA2 antibody. Detection was performed with anti-Myc or anti-HtrA2 antibody.

Figure 2. HtrA2 is phosphorylated upon MEKK3 activation.

(a) Schematic representation of human HtrA2. MT, mitochondrial targeting sequence. TM, putative transmembrane domain of full length HtrA2. The IAP binding domain present in the N-terminus of mature HtrA2 is indicated. Also shown are residues mutated in PD (underlined) and potential Ser/Thr kinase phosphorylation sites (asterisks). **(b)** HtrA2 is phosphorylated on Ser142 upon activation of MEKK3. ΔMEKK3:ER, myrAkt:ER and ΔRaf-DD:ER stable cell lines were transfected with full length HtrA2. After 4OH-Tx activation of MEKK3, Akt and Raf in these cell lines, lysates were analysed by WB with the anti-phospho-Ser142 HtrA2 antibody. After stripping, the membrane was re-probed using anti-HtrA2 antibody. Lysates were also analysed by WB with anti-phospho-Akt, -Erk1/2, -JNK and -p38 antibodies. **(c)** BIRB796 inhibits 4OH-Tx-induced HtrA2 phosphorylation on Ser142. HEK293/ΔMEKK3:ER were transfected with full length HtrA2. Lysates were analysed by WB with anti-phospho-Ser142 HtrA2 and anti-phospho-p38 antibodies. After stripping, membranes were re-probed using anti-HtrA2 and anti-p38 antibodies. **(d)** Down regulation of MKK3 and MKK6 decreases endogenous Ser142 HtrA2 and p38 phosphorylation. HEK293 cells were transfected with siRNA specific for MKK3/6 and cell lysates were analysed by WB using anti-phospho-Ser142 HtrA2, anti-HtrA2, anti-phospho-p38 and anti-p38 antibodies. **(e)** Ser142 and/or Ser400 are required for HtrA2 phosphorylation by p38γ. Recombinant WT or S142A/S400A HtrA2 was incubated with p38γ in the presence of [γ-^{33}P] ATP. Proteins were then resolved by SDS-PAGE, the gel was dried and processed for autoradiography. In parallel experiments, gels were stained with colloidal Coomassie as a loading control. Images are from the same exposures. Results shown are representative of three independent experiments. Quantification of this blot indicated a 2.0 fold increased signal in lane 5 relative to lane 3 and a 2.5 fold increased signal in lane 6 relative to lane 4.

Figure 3. PINK1 is necessary for HtrA2 phosphorylation.

(a) HtrA2 phosphorylation on Ser142 is decreased by down regulation of PINK1. Mitochondrial fractions from control or PINK1 siRNA (Ambion) transfected SH-SY5Y cells were analysed by SDS-PAGE followed by WB with anti-phospho-Ser142 HtrA2 antibody. After stripping, the membrane was re-probed using anti-HtrA2 antibody. **(b)** MEKK3-induced phosphorylation of HtrA2 is decreased by down regulation of PINK1. HEK293/ΔMEKK3:ER cells were transiently transfected with full length WT or S142A HtrA2 and siRNA against PINK1 (Ambion). After stimulation of MEKK3 with 4OH-Tx cell lysates were analysed by WB with anti-phospho-Ser142 HtrA2 and anti-HtrA2 antibodies. **(c)** Down regulation of CDC2 and CDK5 had minimal impact on MEKK3-induced phosphorylation of HtrA2 Ser142. HEK293/ΔMEKK3:ER cells were transiently transfected with processed HtrA2 and indicated siRNAs. After stimulation of MEKK3 with 4OH-Tx cell lysates were analysed by WB with anti-phospho-Ser142 HtrA2 and anti-HtrA2 antibodies. **(d)** $CoCl_2$ induces Ser142 phospho-HtrA2 and phospho-p38 and this is decreased by down regulation of PINK1 (Dharmacon) and MKK3/6. HEK293 cells were transfected with processed HtrA2 and the respective siRNA. $CoCl_2$ stimulated cell lysates were subjected to WB using anti-phospho-Ser142 HtrA2 and anti-phospho-p38 as well as anti-HtrA2 and anti-p38 antibodies. Representative of three independent experiments. **(e)** HtrA2 phosphorylation is decreased in PD brains carrying mutant PINK1. Human brain tissue samples from the caudate region carrying a mutation in the *PINK1* gene (C575R and Y431H PINK1), samples from idiopathic PD (IPD) brain as well as normal control were analysed by WB with anti-phospho-Ser142 HtrA2, anti-HtrA2 and anti-PINK1 antibodies.

Figure 4. Phosphorylation of HtrA2 increases its proteolytic activity.

(a) Protease activity of WT HtrA2 as well as S142D and S400D phospho-mimetic mutants is displayed. Ser to Ala mutations at the phospho-sites show no significant effect compared to the WT protein. Mean values +/- SD of 4-6 data points are represented. Statistically significant values (one-way ANOVA with Bonferroni post-test, compared to WT HtrA2) are indicated: *** $P < 0.001$. **(b)** In parallel to (a), SDS-PAGE was performed and stained with colloidal Coomassie to show the recombinant HtrA2 proteins. **(c)** G399S peptide is phosphorylated to a lesser extend than WT HtrA2 peptide. A peptide surrounding Ser142 or Ser400, or mutant peptides with Ser to Ala mutation or peptides mimicking PD mutations were incubated with p38 *in vitro* in a buffer containing [γ-^{33}P] ATP. The peptides were resolved on a gel and exposed for autoradiography. This experiment shown is a representative of three independent experiments.

Figure 5. Depletion of PINK1 affects WT but not HtrA2 KO MEFs.

(a) Removal of PINK1 by siRNA decreases the mitochondrial potential in WT but not HtrA2 KO MEFs. **(b)** Removal of PINK1 by siRNA sensitises WT but not HtrA2 KO MEFs to 6-OHDA. Statistical analysis was performed for WT and HtrA2 KO MEFs and compared to control siRNA. **(c)** Mutations in HtrA2 mimicking constitutive phosphorylation (S142D and S400D) confer enhanced protection from toxic agents. Cells co-transfected with vector or full length HtrA2 expressing constructs and a membrane targeted GFP construct (pEGFP-F) together with control or PINK1 siRNAs were treated with the indicated drugs and the number of surviving GFP positive cells scored using an automated microscope system. Results represent the means +/- SD. Statistical analysis was performed for each drug treatment and compared to vector transfected cells. **(d)** Co-expression of PINK1 PD mutations suppresses HtrA2-mediated protection of HtrA2 KO MEFs treated with toxic agents. Cells co-transfected with vector, full length HtrA2, or full length HtrA2 and WT or PD-associated PINK1 mutants as well as pEGFP-F were treated with the indicated drugs and the number of surviving GFP positive cells were scored as mentioned above. Statistically significant values (two-way ANOVA with Bonferroni post-test, compared to control groups) are indicated: *, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

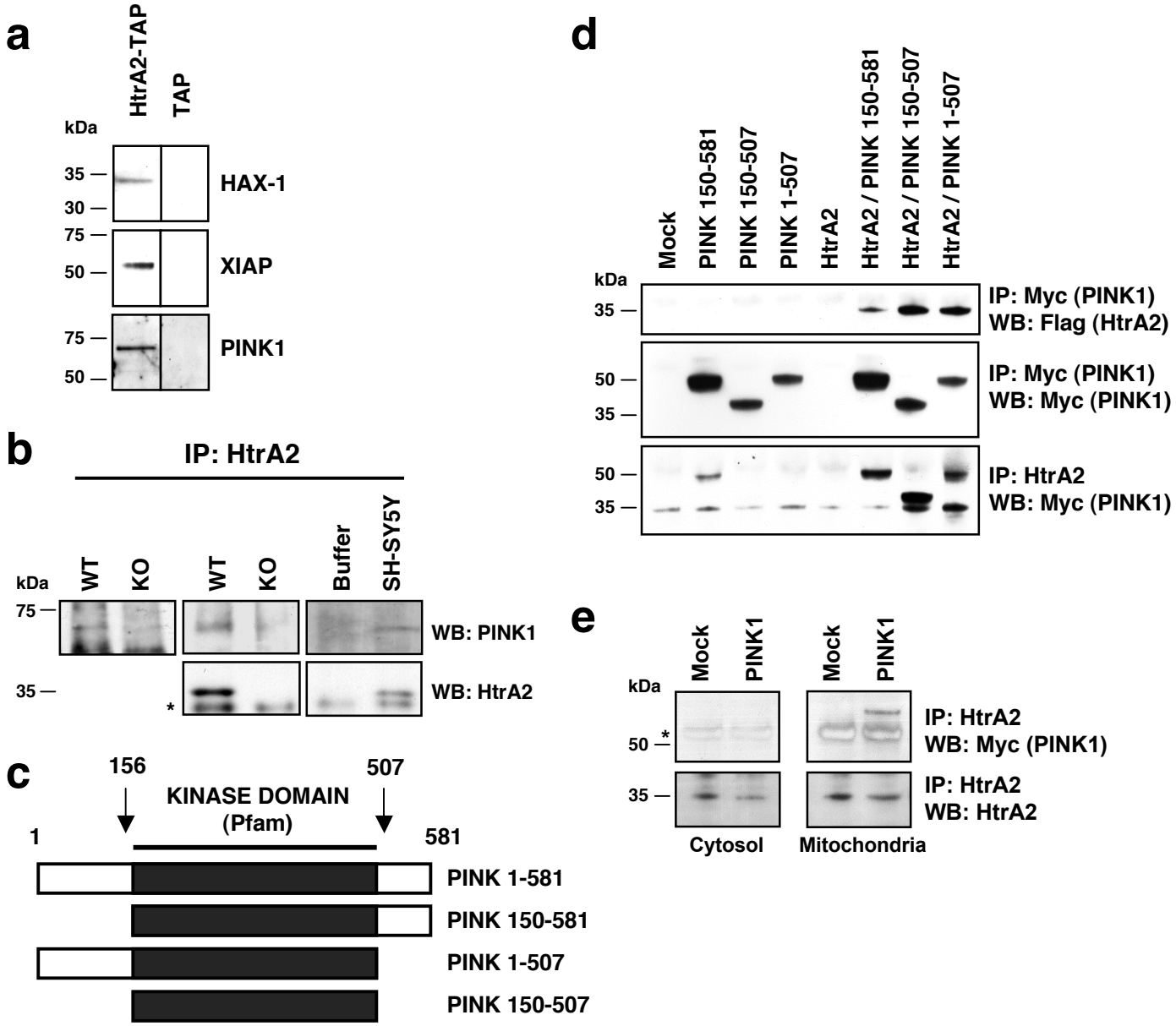


Figure 1, Downward

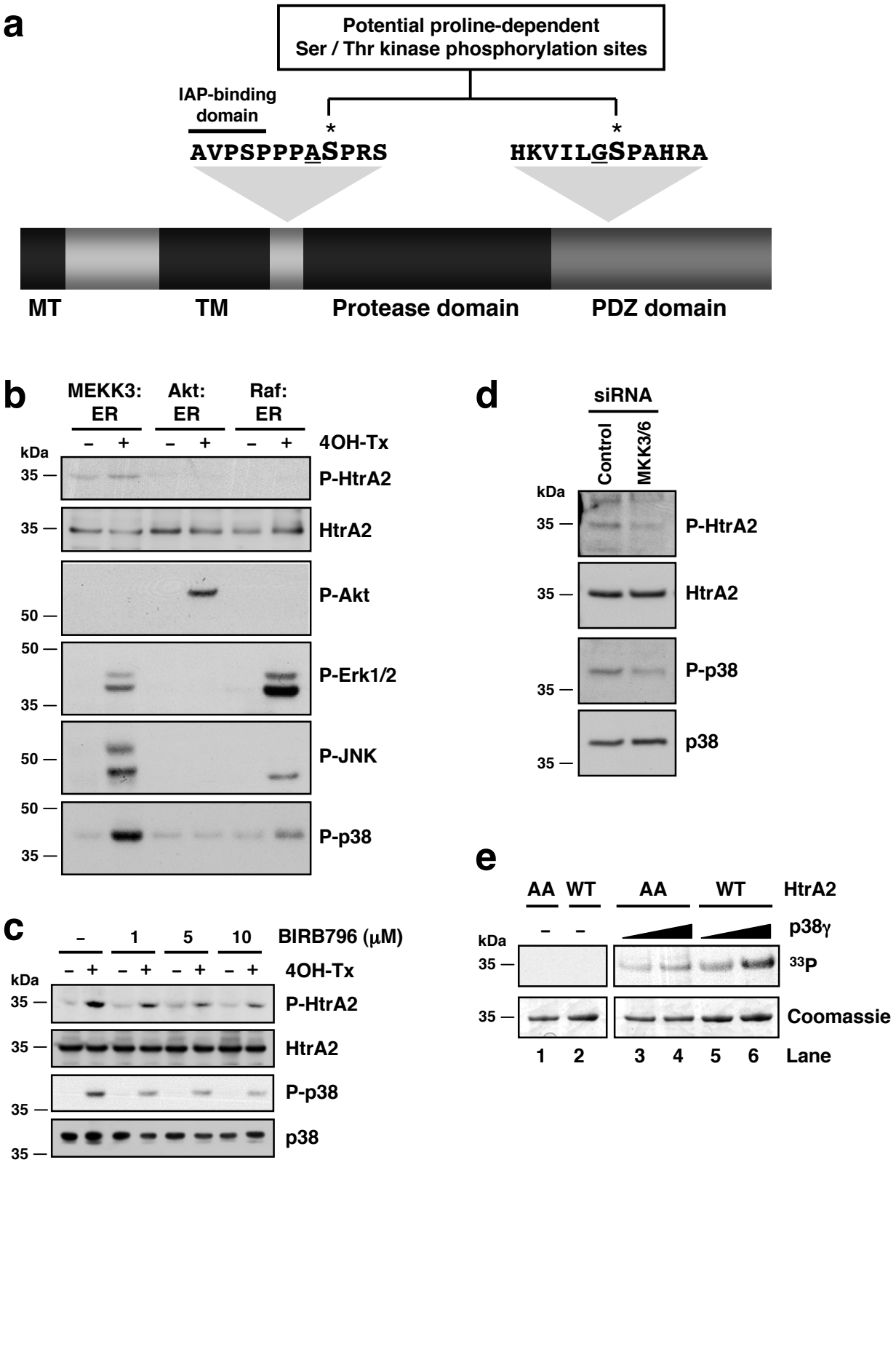


Figure 2, Downward

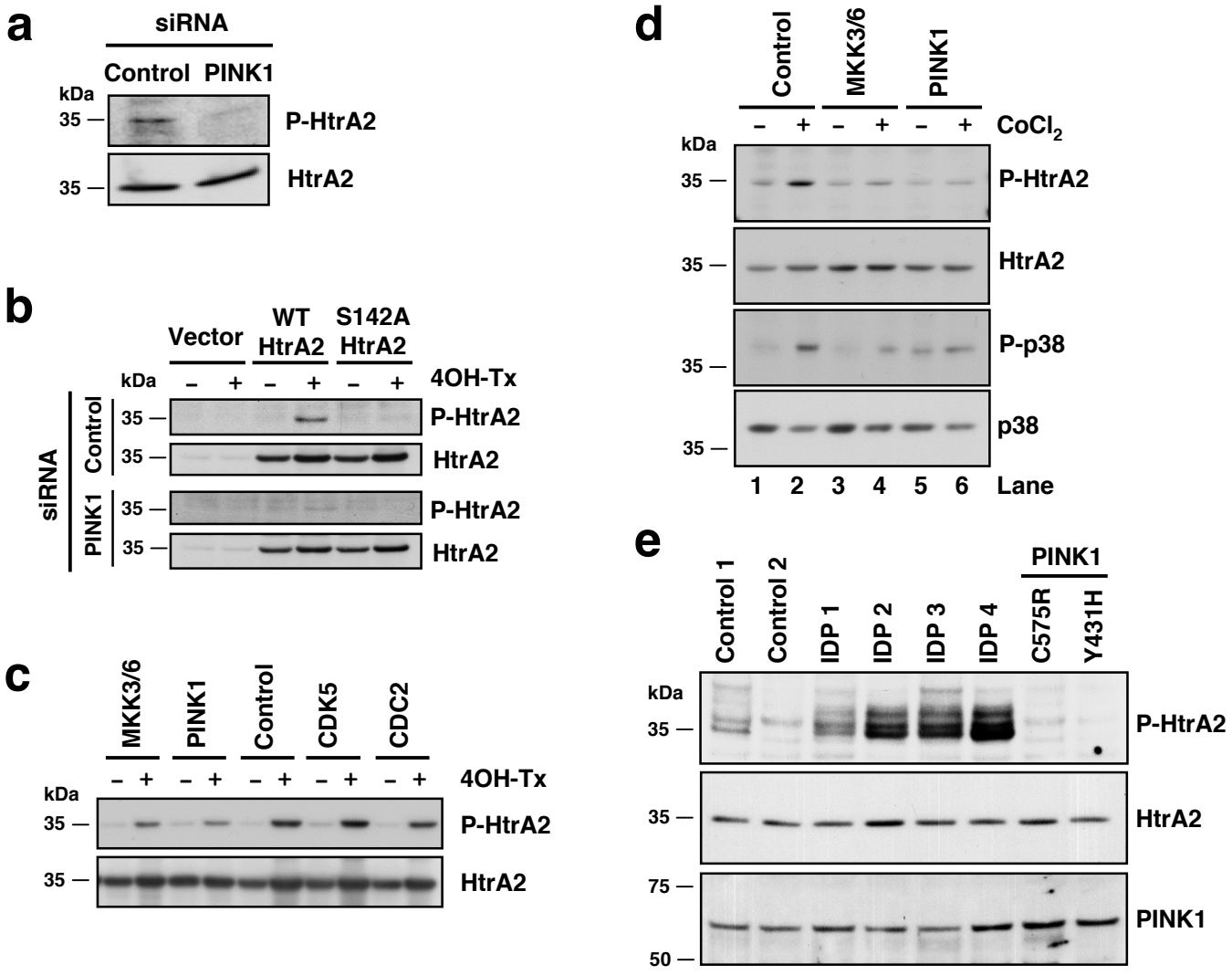


Figure 3, Downward

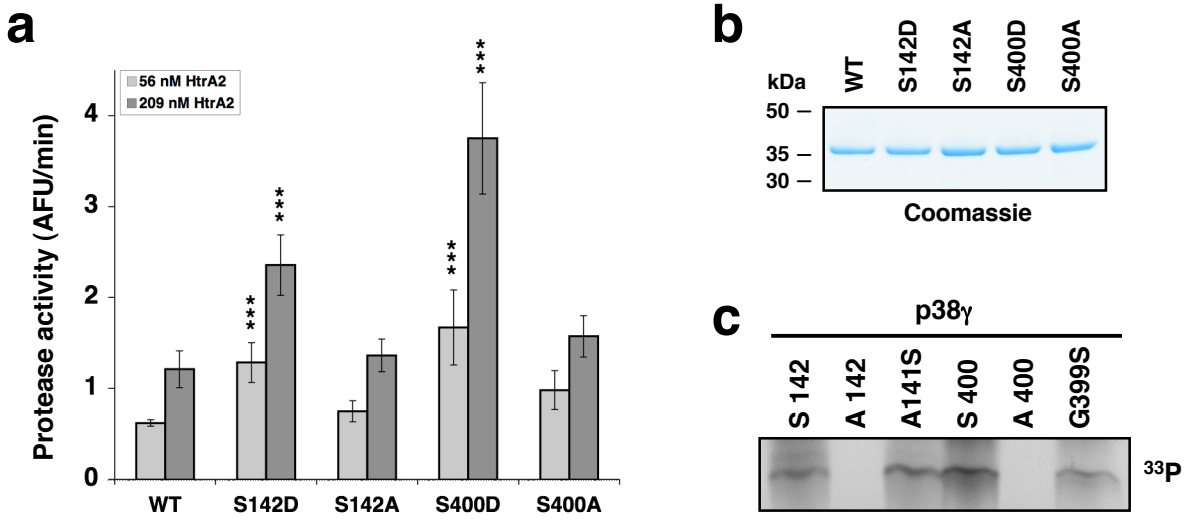


Figure 4, Downward

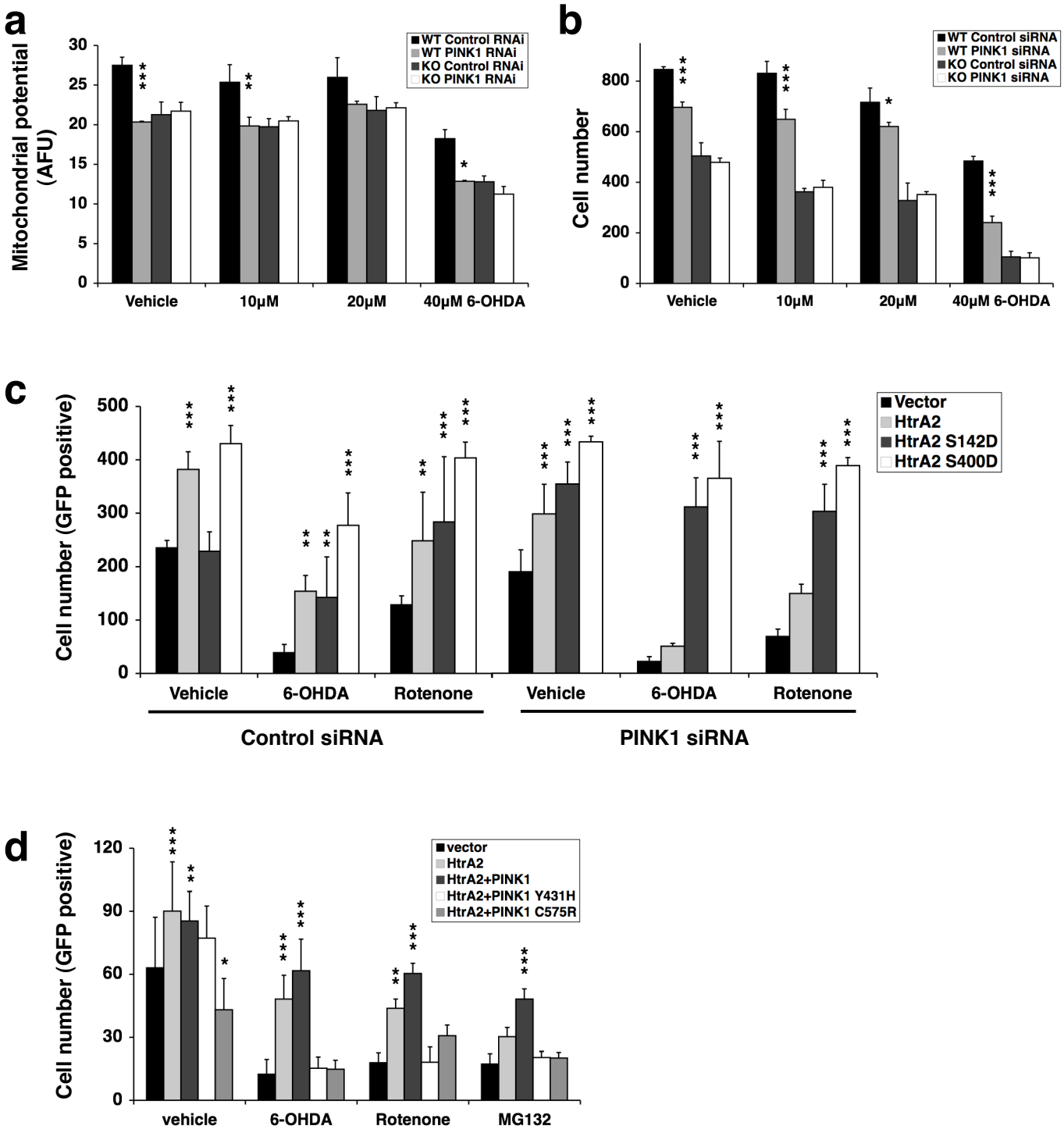


Figure 5, Downward

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY MATERIALS AND METHODS

Cell culture and transfections

SH-SY5Y cells, a gift of Giampietro Schiavo, were cultured in DMEM and F12 1:1 supplemented with 10 % foetal bovine serum (FBS). U2OS stably expressing mitochondria-targeted RFP (a gift from Dr. Kai Dimmer), HEK293 and NIH 3T3 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % FBS or donor calf serum (DCS), respectively. MEFs derived from WT and HtrA2 KO mice were immortalised by retroviral transduction using simian virus 40 large T antigen, selected with 2 μg/ml puromycin and cultured as previously described [10].

HEK293, SH-SY5Y, and MEFs were transiently transfected using Effectene (Qiagen), Lipofectamine2000 (Life Technologies), and Genejuice (Novagen), respectively, according to manufacturer's guidelines. The HEK293 HtrA2-TAP and ΔMEKK3:ER stable cell line were selected with 0.5 mg/ml G418 and 1 μg/ml puromycin, respectively. Single colonies were then assayed for Fc Immunoglobulin fragment expression or p38 phosphorylation upon 4OH-Tx treatment, respectively. The NIH 3T3 myrAkt:ER and ΔRaf-DD:ER cell lines were a gift from Almut Schulze (unpublished data).

RNA interference

SH-SY5Y cells were transfected with siRNA oligos against human PINK1 (5'–3': GGCAAUUUUUACCCAGAAAtt) or GFP as a control (Ambion) at a final concentration of 50 nM using Lipofectamine2000, according to the manufacturer's instructions. After 48 hrs, cells were harvested for Western blotting.

HEK293 and ΔMEKK3:ER expressing HEK293 cells were transfected with siRNA oligos against human PINK1, MKK3 (5'-3': GGAUCUACGGAUAUCCUGC) and MKK6 (5'-3': GGAAGAACAGAGGGUUUUG) or GFP, all from Ambion. siRNA against PINK1, CDC2 and CDK5 were from Dharmacon as SMARTpool® collections. As negative control we used si*CONTROL*™ Non-Targeting siRNA pool (scrambled). Cells were transfected with 50 nM siRNA using Dharmafect I reagent (Dharmacon). 24 hrs later,

cells were transfected with 50 nM siRNA and 500 ng plasmid per well using Effectene (Qiagen). In case of ΔMEKK3:ER expressing HEK293 cells, they were treated 24 hrs later with 100 nM 4OH-Tx o/n before being harvested. For hypoxia, cells were treated 24 hrs after the 2nd transfection with 200 nM $CoCl_2$ for 48 hrs and harvested for analysis.

Control experiments were performed with siRNA targeting GFP (Ambion) or si*CONTROL*™ Non-Targeting siRNA pool (Dharmacon), and both control oligos behaved identically in our experiments.

Delivery of siRNA into MEFs was performed using the Amaxa nucleofection system (Amaxa GmbH) or Genejuice (Novagen). Optimisation was performed with the Amaxa RNAi test kit using pmaxGFP™ plasmid and siRNA directed against maxGFP™. Following optimisation, cells were nucleofected with either si*CONTROL*™ Non-Targeting siRNA pool or PINK1 SMARTpool® siRNA (both Dharmacon) at a final concentration of 50 nM or a combination of 25 nM PINK1 siRNA ID#180640 (Ambion) and 25 nM siRNA ID#180642 (Ambion).

Total RNA was prepared using the RNeasy system in combination with the RNase-free DNase kit (Qiagen). Complementary DNA was synthesised with the Superscript III kit (Invitrogen) and used as a template for quantitative PCR analysis monitoring the real-time increase in fluorescence of SYBR Green (Applied Biosystems). Gene-specific primers for the target genes were designed using PrimerExpress software (Applied Biosystems), the primer sequences are available upon request. Relative transcript levels of target genes are normalised to Actin-γ RNA levels.

FACS analysis

pcDNA3 control and Myc-Pink1 expression plasmids were co-transfected with pEGFP-F. Cells were either treated with 0.5 μM MG132 (Calbiochem) the day after for 25 hrs or with 2 μM Staurosporine (Calbiochem) two days after for 3 hrs. Cells were then harvested, washed in PBS and stained in AnnexinV buffer containing 1 μl Alexa647-AnnexinV (Molecular Probes) / 100 μl buffer for 15 min at RT in the dark, propidium iodide (PI) was added immediately prior to analysis. Cells deemed to be early apoptotic within the EGFP positive population (AnnexinV positive and PI

negative) were analysed on a FACSCalibur (BD Biosciences). Results shown are representative of three independent experiments.

Confocal microscopy

U2OS expressing mitochondria-targeted RFP (a gift from Dr. Kai Dimmer) were plated onto glass coverslips and transfected with PINK1 expression constructs. Cells were fixed 48hrs post-transfection in 3% paraformaldehyde and processed for immunoflourescence. For immunostaining cells were probed with anti-myc antibody (1:200 dilution) and Alexa 488 fluorescently conjugated anti-rabbit secondary antibody. Cells were visualised on an LSM510 confocal microscopy (Zeiss).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1

PINK1 exhibits co-localisation with the mitochondria as well as diffuse cytosolic staining. WT or 150-507 mutant PINK1-Myc was expressed in U2OS cells stably expressing mitochondria-targeted RFP and processed for immunofluorescence. Confocal images are shown. mtRFP (red), PINK1 (green).

Supplementary Figure 2

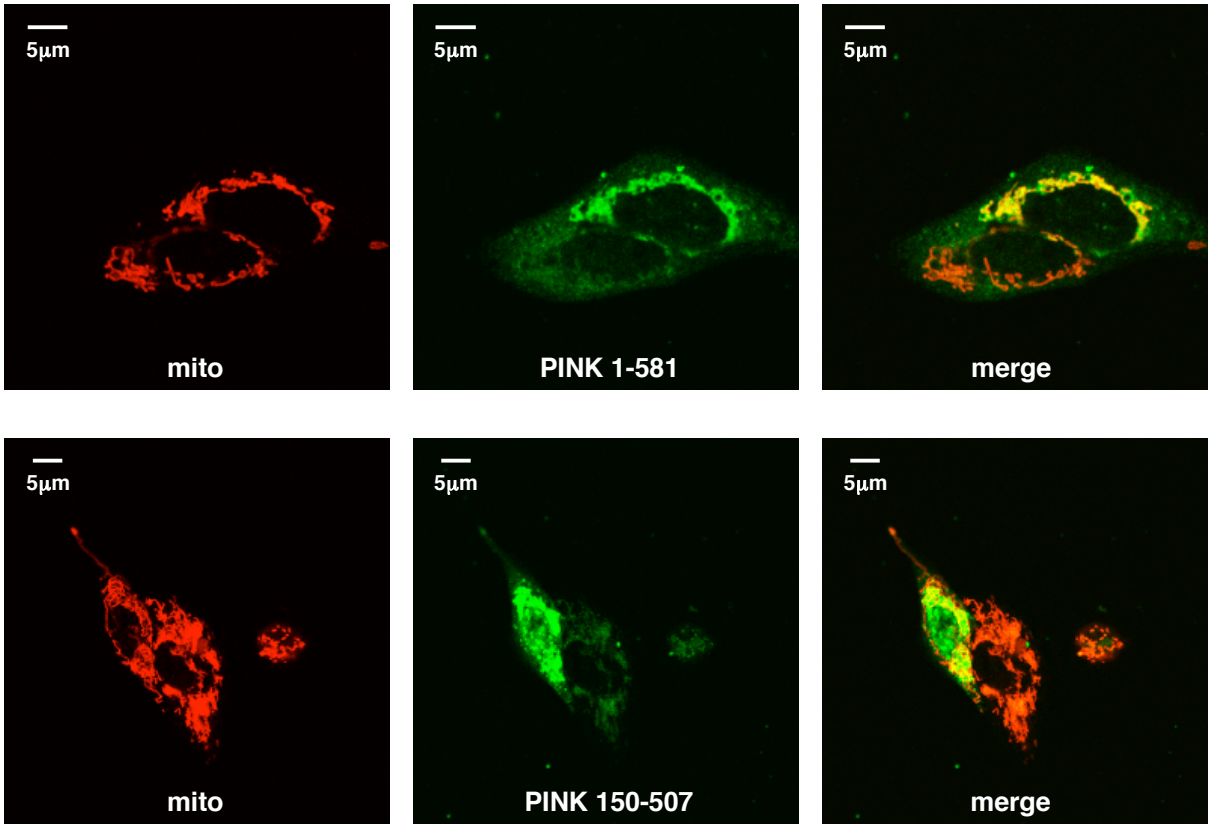
(a) Alignment of HtrA2 homologues in different species showing conservation of Ser142 and Ser400. **(b)** Kinases phosphorylating HtrA2 *in vitro*. Recombinant HtrA2 and Myelin Basic Protein (MBP) were incubated with p38α, β, γ or δ, ERK1, ERK2, CDC2, and CDK5 in the presence of [γ-^{33}P] ATP. Proteins were then resolved on SDS-PAGE. The gel was dried and processed for autoradiography. In parallel a WB for HtrA2 was performed. **(c)** HtrA2 is phosphorylated on Ser142 upon activation of MEKK3. ΔMEKK3:ER, myrAkt:ER and ΔRaf-DD:ER stable cell lines were transfected with processed HtrA2. After 4OH-Tamoxifen (Tx) activation of MEKK3, Akt and Raf in these cell lines, lysates were analysed by WB with anti-phospho-Ser142 HtrA2 antibody. After stripping, the membrane was re-probed using anti-HtrA2 antibody. **(d)** A peptide containing Ser142 is phosphorylated by p38β, γ, and δ. A peptide surrounding Ser142 or a mutant peptide containing S142A were incubated with recombinant kinases *in vitro* in a buffer containing [γ-^{33}P] ATP. Peptides were then resolved on a gel and exposed for autoradiography (upper panel). p38α, ERK1, CDC2 and CDK5 do not phosphorylate the Ser142 peptide. The Coomassie gel shown (lower panel) confirms equal input of the p38 isoforms.

Supplementary Figure 3

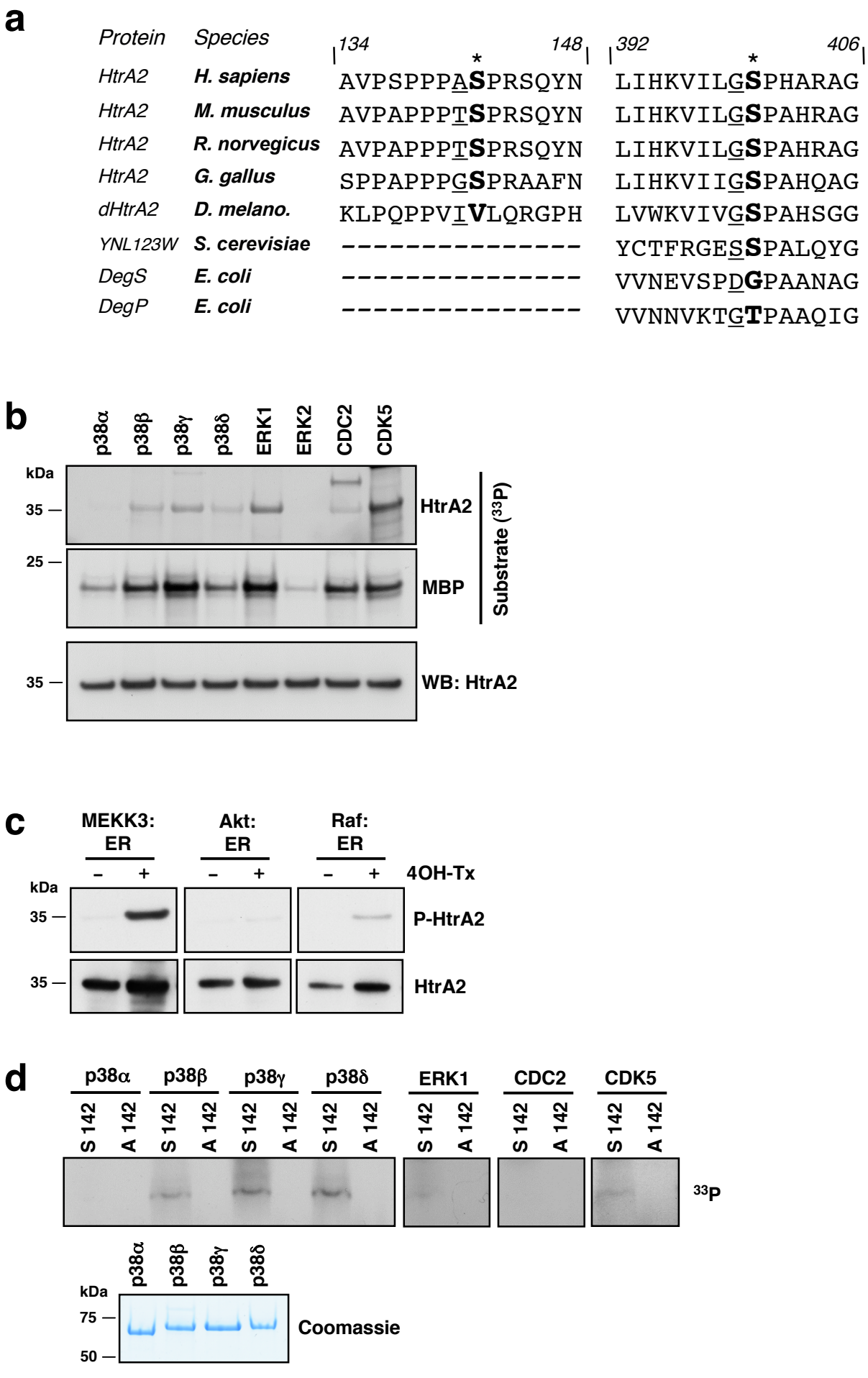
(a) Down regulation of PINK1, MKK3 and MKK6 mRNA by siRNA in HEK293 cells. The expression to appropriate control siRNA is shown. **(b)** Down regulation of PINK1, CDC2 and CDK5 protein by specific siRNA in HEK293 cells. Arrows indicate full length and processed forms of the PINK1 protein. **(c)** MEKK3-induced phosphorylation of HtrA2 is decreased by down regulation of PINK1. HEK293/ΔMEKK3:ER cells were transiently transfected with processed HtrA2 and siRNA against PINK1 (Ambion). After stimulation of MEKK3 with 4OH-Tx cell lysates were analysed with anti-phospho-Ser142 HtrA2 and anti-HtrA2 antibodies. **(d)** and **(e)** Densitometry analysis was performed on gel shown in Fig. 3d. Levels of phospho-HtrA2 and phospho-p38 were normalised to their respective loading control. The fold change of phospho-HtrA2 (d) and phospho-p38 (e) are shown relative to untreated control. Results correspond to three independent experiments. **(f)** Co-immunoprecipitation of HtrA2 with PINK1 PD mutants. Full length HtrA2-Flag and mutant PINK1-Myc constructs were co-expressed in HEK293 cells. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody. Detection was performed with anti-PINK1 antibody. Images are from the same exposures.

Supplementary Figure 4

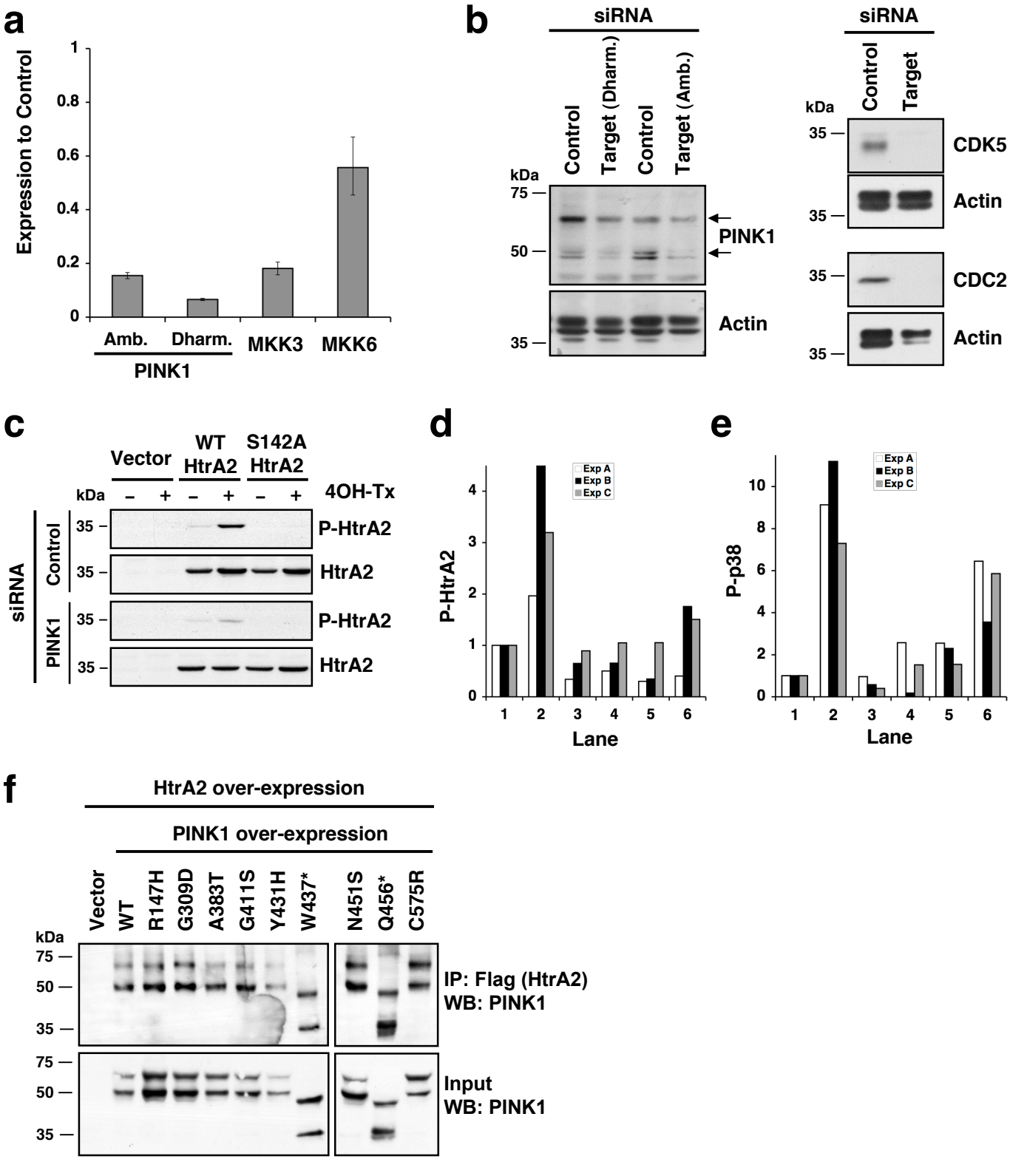
(a) Down regulation of PINK1 mRNA by siRNA in MEFs derived from HtrA2 KO mice using two independent cocktails of oligos targeting PINK1. Indicated is the fold-change in PINK1 transcript compared to cells transfected with appropriate control siRNAs. **(b)** Representative fluorescence microscopy images of HtrA2 KO MEFs co-transfected with either vector or full length HtrA2 expressing constructs and pEGFP-F together with control or PINK1 siRNAs. Nuclei of surviving cells are stained with Hoechst (magenta) and GFP positive cells are green. These images are representative of the experiment shown in Fig. 5c. **(c)** Over-expression of PINK1 fails to protect MEFs lacking HtrA2 against stress-induced apoptosis. Membrane targeted GFP was co-transfected with control or PINK1 vector, cells were treated with MG132 or Staurosporine (STS) and the number of Annexin V positive cells within the GFP positive population was determined by FACS. Results represent the means +/- SD of duplicates. The experiment shown is a representative of three independent experiments.



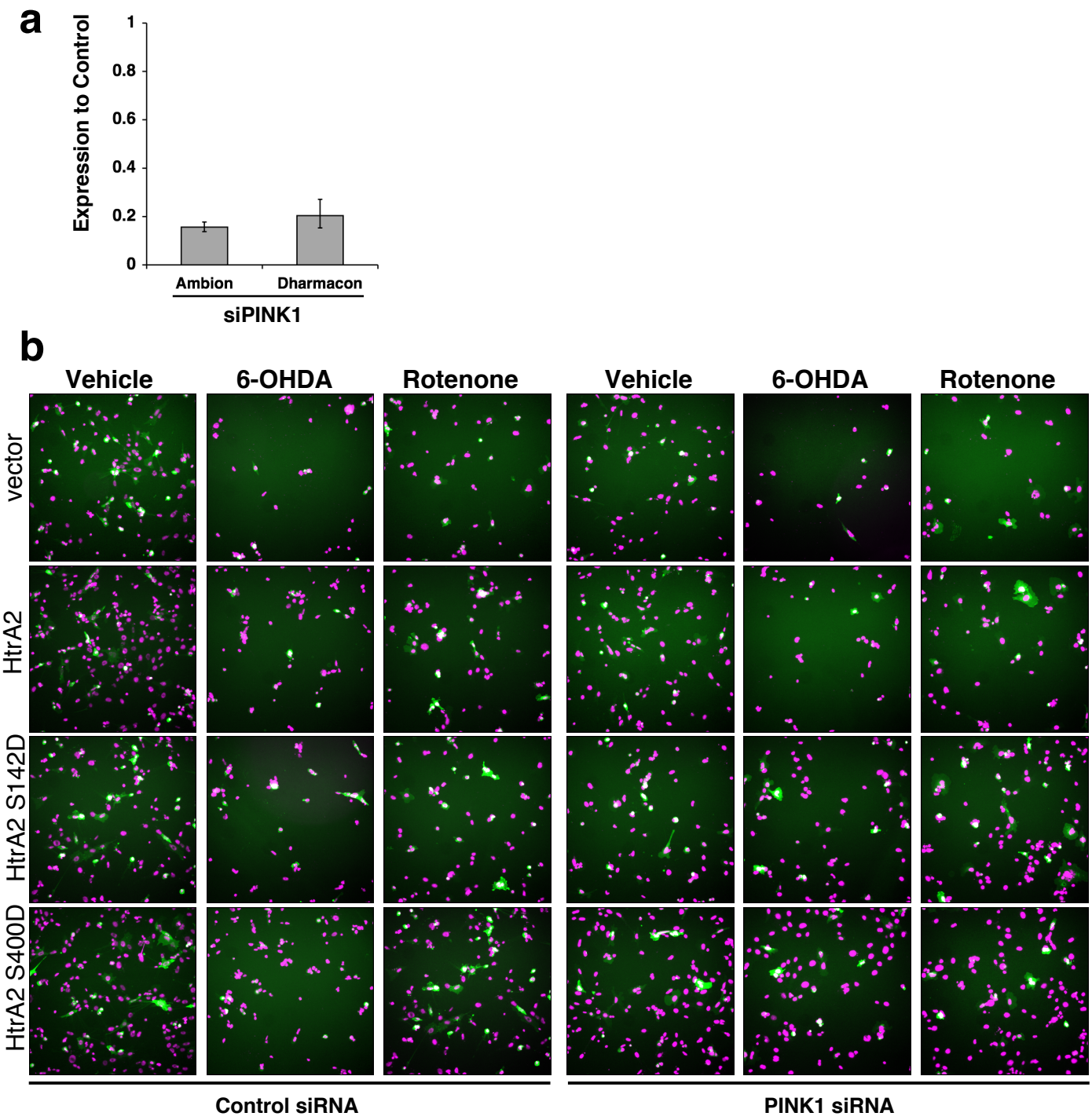
Supplementary Information, Figure 1, Downward



Supplementary Information, Figure 2, Downward



Supplementary Information, Figure 3, Downward



Supplementary Information, Figure 4, Downward