USP8 regulates mitophagy by removing K6-linked ubiquitin conjugates from parkin

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Abstract

Mutations in the Park2 gene, encoding the E3 ubiquitin-ligase parkin, are responsible for a familial form of Parkinson’s disease (PD). Parkin-mediated ubiquitination is critical for the efficient elimination of depolarized dysfunctional mitochondria by autophagy (mitophagy). As damaged mitochondria are a major source of toxic reactive oxygen species within the cell, this pathway is believed to be highly relevant to the pathogenesis of PD. Little is known about how parkin-mediated ubiquitination is regulated during mitophagy or about the nature of the ubiquitin conjugates involved. We report here that USP8/UBPY, a deubiquitinating enzyme not previously implicated in mitochondrial quality control, is critical for parkin-mediated mitophagy. USP8 preferentially removes non-canonical K6-linked ubiquitin chains from parkin, a process required for the efficient recruitment of parkin to depolarized mitochondria and for their subsequent elimination by mitophagy. This work uncovers a novel role for USP8-mediated deubiquitination of K6-linked ubiquitin conjugates from parkin in mitochondrial quality control.

Keywords: deubiquitination; mitophagy; parkin; ubiquitin; USP8

Subject Categories: Post-translational Modifications, Proteolysis & Proteomics; Membrane & Intracellular Transport; Neuroscience

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Introduction

Mutations in the Park2 gene are associated with a familial form of Parkinson’s disease (PD) and are believed to impair the normal function of the parkin protein as an E3 ubiquitin (Ub)-ligase (Shimura et al., 2000). These loss-of-function mutations impede the function of parkin in a variety of cellular pathways, including mitochondrial quality control, a process believed to be central to the pathogenesis of PD (Narendra et al., 2008; Geisler et al., 2010; McLelland et al., 2014). In this pathway, parkin collaborates with another PD gene, PTEN-Induced Kinase 1 (PINK1), to promote the removal of dysfunctional mitochondria by autophagy (mitophagy) (Narendra et al., 2010b). The PINK1 protein is a mitochondrial kinase that is rapidly degraded upon import into healthy mitochondria (Jin et al., 2010; Greene et al., 2012). When the mitochondrial membrane potential is abolished, PINK1 import is stalled leading to its accumulation on the mitochondrial surface (Lazarou et al., 2012), where it phosphorylates the parkin Ub-like domain (Ubl) and Ub at serine 65 (S65), a residue conserved in both proteins (Kondapalli et al., 2012; Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014). S65 phosphorylation, in turn, promotes the activation and translocation of parkin from the cytosol to mitochondria (Kondapalli et al., 2012; Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014), where it ubiquitinates a number of mitochondrial proteins (Chan et al., 2011; Sarraf et al., 2013), in addition to ubiquitinating itself (Matsuda et al., 2010; Lazarou et al., 2013). Parkin E3 ubiquitin-ligase activity is critical for the efficient elimination of dysfunctional mitochondria by mitophagy (Geisler et al., 2010; Matsuda et al., 2010). However, parkin activity is auto-inhibited at baseline (Trempé et al., 2013; Wauer & Komander, 2013), and understanding how PINK1-mediated phosphorylation of Ub and parkin triggers its activation and recruitment to mitochondria is incomplete (Kondapalli et al., 2012; Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014).

A common mode of regulation used by E3 ubiquitin ligases involves auto-ubiquitination. Depending on which of the seven lysines within Ub is used for conjugation, E3 Ub-ligases can assemble poly-Ub chains on themselves and on substrates, with different topologies, to mediate distinct biological functions (Komander & Rape, 2012; Kulathu & Komander, 2012). Chains linked via Lys48 (K48) in Ub, the best-characterized type of Ub conjugation, typically lead to degradation of the substrate by the proteasome (Voges et al., 1999). In contrast, chains linked via one of the other six lysines in Ub can protect modified substrates from proteasomal degradation and divert them toward functions in a variety of cellular pathways including trafficking, signaling, and autophagy (Komander & Rape, 2012; Kulathu & Komander, 2012). Thus, regulation of parkin by auto-ubiquitination has the potential to profoundly affect its function. Auto-ubiquitination can be antagonized by deubiquitinating enzymes (DUBs), which removeUb from the E3 (de Bie &
Ciechanover, 2011). This is typically believed to protect the E3 from proteasomal degradation (Wu et al, 2004; Nathan et al, 2008; Mei et al, 2011). However, certain DUBs have been shown to exhibit specificity in hydrolyzing non-K48-linked Ub chains (Komander et al, 2009a; Reyes-Turcu et al, 2009; Mevissen et al, 2013). Whether or how this could affect the stability and function of a cognate E3 partner is only beginning to be explored (Marfany & Denic, 2008; Ventii & Wilkinson, 2008; de Bie et al, 2010).

We recently reported that ataxin-3, a DUB responsible for Machado-Joseph’s disease, can deubiquitinate parkin (Durcan et al, 2011, 2012). However, the effects of ataxin-3 on parkin-mediated mitophagy were not investigated. Moreover, as E3s can be regulated by multiple DUBs (Daviet & Colland, 2008; Nathan et al, 2008), we used an unbiased siRNA-based approach to ask whether deubiquitination of parkin regulates its function in mitophagy. We report here that USP8, a DUB previously associated with endosomal trafficking (Mizuno et al, 2005; Row et al, 2006), but not mitochondrial quality control, is required for the efficient recruitment of parkin to depolarized mitochondria and subsequent mitophagy. Moreover, we find that USP8 preferentially removes K6-linked Ub conjugates from parkin but has little effect on the ubiquitination or stability of other known mitochondrial substrates of parkin. Whereas K6-linked Ub conjugates on parkin appear to protect it from proteasomal degradation and to impede mitophagy, USP8-mediated removal of K6-linked Ub from parkin promotes parkin turnover and is required for mitophagy to proceed efficiently. Taken together, our work identifies USP8 and K6-linked Ub conjugates as key players in the regulation of parkin-mediated mitophagy.

Results

Reducing USP8 levels impairs recruitment of parkin to depolarized mitochondria

Given that DUBs can regulate the function of E3 Ub-ligases, we surmised that parkin might also collaborate with a DUB during mitophagy. Using an unbiased siRNA-based approach, we knocked down 87 putative DUBs encoded by the human genome (Supplementary Table S1) (Nijman et al, 2005) in U2OS cells stably expressing GFP-parkin and screened for effects on CCCP-induced parkin recruitment to mitochondria. Expression of endogenous parkin in these cells was undetectable relative to other cell lines (Supplementary Fig S1A). Silencing of USP8, but not any of the other DUBs, impaired parkin recruitment to mitochondria after 1 h of treatment with CCCP (Fig 1A–C, Supplementary Fig S1B–D). Immunoblotting confirmed that the USP8 protein was indeed reduced in these cells (Fig 1C, Supplementary Fig S1C). Time-lapse microscopy showed that knockdown of USP8 delayed but did not abolish parkin recruitment to depolarized mitochondria (Fig 1D and E). Moreover, we find that USP8 preferentially removes K6-linked Ub conjugates from parkin but has little effect on the ubiquitination or stability of other known mitochondrial substrates of parkin. Whereas K6-linked Ub conjugates on parkin appear to protect it from proteasomal degradation and to impede mitophagy, USP8-mediated removal of K6-linked Ub from parkin promotes parkin turnover and is required for mitophagy to proceed efficiently. Taken together, our work identifies USP8 and K6-linked Ub conjugates as key players in the regulation of parkin-mediated mitophagy.

Figure 1. USP8 siRNA delays parkin recruitment onto mitochondria.

A, B USP8 siRNA impedes parkin recruitment onto mitochondria following CCCP treatment. U2OS-GFP-parkin cells were transfected with non-targeting or USP8 siRNA (10 nM) for 60 h (A). Untreated cells or cells treated with CCCP for 1 h were fixed, and images were acquired after staining for the mitochondrial protein TOM20. After 1-h CCCP treatment, cells were analyzed for GFP-parkin co-localization onto TOM20-positive mitochondria (B). Experiments were blinded and performed in triplicate with 100 cells analyzed for each condition. The vertical bars represent SEM for three independent experiments. For statistical analysis, a two-way ANOVA with Tukey post-test was performed, **P < 0.01.

C Validation of USP8 siRNA knockdown. U2OS-GFP-parkin cells were transfected with non-targeting or USP8 siRNA oligos (10 nM) for 60 h. Cells were lysed and analyzed by immunoblotting for USP8, parkin (long and short exposure), and actin.

D, E A delay in parkin recruitment onto mitochondria is observed in cells transfected with USP8 siRNA by live-cell microscopy. U2OS-GFP-parkin cells were transfected with non-targeting or USP8 siRNA (10 nM) for 60 h (D). 16 h prior to imaging, cells were infected with CellLight® mitochondria-RFP (Mito-RFP) to visualize mitochondria. Live-cell imaging was initiated 5 min after CCCP treatment, and images were acquired every 5 min over a 140-min period. Parkin recruitment upon membrane depolarization is visualized by the appearance of punctate GFP fluorescence superposed onto mitochondrial RFP fluorescence. Quantification of GFP-parkin recruitment to mitochondria (E) is facilitated by calculating the percentage of cells showing recruitment of GFP-parkin onto mitochondria at 5-min intervals over a period of 145 min. Experiments were performed in triplicate with 350 cells analyzed in each condition. The vertical bars represent the mean ± SEM for three independent experiments (see also Supplementary Video S3).

F, G Expression of FLAG-HA-USP8 rescues the effects of USP8 siRNA. U2OS-GFP-parkin cells were co-transfected with USP8 RNA (5 nM) and FLAG-USP8 (0.5 μg) for 60 h (F). Cells treated with CCCP for 1 h were fixed, and images were acquired after staining for the mitochondrial protein, TOM20. After 1-h CCCP treatment, cells were analyzed for GFP-parkin co-localization onto TOM20-positive mitochondria in cells either negative or positive for FLAG-USP8 (G). Experiments were blinded and performed in triplicate with 100 cells analyzed for each condition. For statistical analysis, a two-way ANOVA with Tukey post-test was performed, ***P < 0.0001. The vertical bars represent the mean ± SEM for three independent experiments (see also Supplementary Fig S3D and E).
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Figure 2. USP8 siRNA impairs parkin-mediated mitophagy.

A–E USP8 siRNA impairs parkin-mediated mitophagy. U2OS-GFP-parkin cells were transfected with non-targeting or USP8 siRNA (10 nM) for 48 h (A). Transfected cells were either left untreated or were treated with CCCP for 24 or 48 h before fixation. Immunofluorescence images of cells were acquired after staining for TOM20. Cells were outlined in white as a result of low GFP-parkin levels. The percentage of all U2OS-GFP-parkin cells after 48 h (B, C) or 48 h (D, E) CCCP treatment lacking TOM20-positive mitochondria or containing GFP-parkin puncta co-localizing with TOM20-positive mitochondria was quantified in cells transfected with non-targeting or USP8 siRNA. Experiments were blinded and performed in triplicate with 100 cells analyzed for each condition. The vertical bars represent SEM. For statistical analysis, a two-way ANOVA with Tukey post-test was performed, *P < 0.05, **P < 0.01, NS, not significant.

F USP8 siRNA has no effect on CCCP-induced mitochondrial depolarization. U2OS-GFP-parkin cells transfected with non-targeting or USP8 siRNA (10 nM) for 60 h were first incubated with the potentiometric dye TMRM (600 nM) 20 min prior to imaging. Prior to CCCP treatment, cells were imaged for 10 min to confirm TMRM staining. Following CCCP treatment, images of cells were acquired every minute for 10 min (see also Supplementary Video S2). The membrane potential in untreated cells was tested by imaging untreated cells for 1 h (see also Supplementary Video S3).

G USP8 siRNA has no discernible effect on CCCP-induced PINK1 accumulation. U2OS-GFP-parkin cells transfected with non-targeting, PINK1 siRNA, or USP8 siRNA (10 nM) for 60 h were either left untreated or were treated with CCCP for 1 or 3 h. Cells were lysed and immunoblotted for parkin, actin, and PINK1.

Source data are available online for this figure.

FLAG-USP8 expression plasmid (Fig 1F and G). Thus, knockdown of USP8 increases parkin levels and delays its recruitment onto mitochondria.

Silencing USP8 impairs mitophagy

Prolonged treatment with CCCP ultimately leads to the elimination of depolarized mitochondria by parkin-dependent mitophagy (Narendra et al., 2008). Given that silencing USP8 delayed parkin recruitment to mitochondria, we asked whether it also affected mitophagy. U2OS-GFP-parkin cells were transfected with either non-targeting or USP8 siRNA and treated with CCCP for 24 h. As expected, with transfection of non-targeting siRNA, the majority of cells showed a loss of TOM20 (Fig 2A upper), TIM23, and COX1 (data not shown) staining, indicating their mitochondria had been efficiently cleared by autophagy. In contrast, fewer cells transfected with USP8 siRNA lost TOM20 staining, indicating that mitophagy was impaired (Fig 2A and B). Prolonged CCCP treatment also led to a marked reduction in parkin staining in cells transfected with non-targeting siRNA, as has been noted previously (Fig 2A upper) (Geisler et al., 2010; Tanaka et al., 2010; Rakovic et al., 2013). In contrast, in USP8 siRNA-transfected cells, GFP-parkin staining persisted after 24 h of CCCP treatment and formed puncta that co-localized with mitochondrial markers (TOM20, COX1, and Tim23), providing a robust readout of parkin-mediated mitophagy (Fig 2A and C, Supplementary Fig S4A and B). However, as with parkin recruitment, the effect on mitophagy appears to be delayed rather than abolished, as no differences were detectable between non-targeting and USP8 siRNA-treated cells by 48 h of CCCP treatment (Fig 2D and E).

CCCP dissipates the proton gradient across the mitochondrial inner membrane, which blocks the import and degradation of PINK1 (Narendra et al., 2010b; Greene et al., 2012). This in turn leads to an accumulation of PINK1 at the mitochondrial outer surface, where it phosphorylates Ub and parkin at serine 65, thereby activating parkin and triggering its translocation from the cytosol (Kondapalli et al., 2012; Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014). Thus, we asked whether the effect of USP8 on parkin recruitment was upstream or downstream of mitochondrial depolarization and PINK1 accumulation. Staining with the potentiometric dye TMRM revealed that silencing USP8 affected neither basal mitochondrial membrane potential nor the ability of CCCP to abolish mitochondrial membrane potential (Fig 2F, Supplementary Videos S2 and S3). Similarly, USP8 knockdown affected neither the steady-state levels of endogenous PINK1 nor its accumulation in response to CCCP (Fig 2G).

Using time-lapse microscopy, we also demonstrate using mitochondrial targeted RFP that USP8 siRNA neither altered mitochondrial morphology, fusion, or fission at baseline (Supplementary Fig S4C, Supplementary Video S4) nor did it affect mitochondrial fragmentation in response to CCCP (Supplementary Video S5). Finally, steady-state levels of mitochondrial proteins appeared unchanged between non-targeting and USP8 knockdown cells (Supplementary Fig S4D). Thus, the effect of USP8 on parkin recruitment is downstream of mitochondrial depolarization and PINK1 accumulation and is not the result of off-target siRNA effects on PINK1 or on mitochondrial dynamics.

USP8 deubiquitinates parkin

We noticed an apparent increase in the levels of Ub conjugates on parkin in the absence of USP8 (Fig 1C). These findings were confirmed in HEK293T cells by overexpressing HA-Ub and FLAG-parkin, a paradigm that stimulates parkin auto-ubiquitination at steady state. Indeed, immunoprecipitation with FLAG followed by immunoblotting with HA showed that parkin Ub conjugates were significantly increased in cells transfected with USP8 siRNA (Fig 3A and B). Conversely, HA-Flag-USP8 overexpression reduced parkin auto-ubiquitination (Supplementary Fig S5A). To address whether USP8 could directly deubiquitinate parkin, we carried out in vitro ubiquitination reactions using recombinant and affinity-purified GST-parkin, Ub, ATP and E1 and E2 enzymes. Ubiquitinated GST-parkin, bound to glutathione sepharose beads, was then washed extensively to remove free Ub and other components of the reaction, followed by incubation with either full-length USP8 or one of several commercially available DUBs. These DUBs all exhibited enzymatic activity in Ub-AMC cleavage assays (Durcan et al., 2012). However, save for USP8 and USP2, none could hydrolyze Ub conjugates on parkin (Fig 3C). The DUB activity of USP8 appeared to be specific toward parkin, as it was much less efficient at deubiquitinating other E3s, including HHARI, an RBR family E3 Ub-ligase structurally very similar to parkin (Fig 3D) (Duda et al., 2013; Trempe et al., 2013). In addition to GST-parkin, USP8 could efficiently deubiquitinate untagged parkin, indicating that it targeted Ub conjugated to parkin per se rather than to the N-terminal GST-tag (Fig 3E). The effect of USP2 on parkin was not surprising, as it had been shown previously to disassemble Ub chains indiscriminately (Komander et al., 2009b). Indeed, USP2 was able to...
deubiquitate several E3s non-selectively in addition to parkin (Supplementary Fig S5B) (Komander et al, 2009b). However, USP2 siRNA showed no effect on parkin levels or on parkin recruitment to mitochondria (Fig 3F, Supplementary Fig S6). Similarly, knockdown of ataxin-3, the first identified DUB partner of parkin (Durcan et al, 2011), had no effect on the mitochondrial recruitment of parkin (Fig 3F, Supplementary Fig S6). Recently, USP15 and USP30 were shown to inhibit mitophagy by counteracting parkin-mediated ubiquitination (Bingol et al, 2014; Cornelissen et al, 2014). However, in contrast to USP8, these two DUBs were believed to deubiquitinate...
Figure 3. USP8 deubiquitinates parkin.

A, B USP8 knockdown causes Ub conjugates to accumulate on FLAG-parkin in HEK293T cells. HEK293T cells were co-transfected with FLAG-parkin (0.5 μg), HA-USP8 (0.5 μg) and non-targeting or USP8 siRNA (5 nM). Lysates were immunoprecipitated with FLAG resin and analyzed by immunoblotting for HA and FLAG. Input lysates (5% of total input) were analyzed by immunoblotting for USP8 and actin. The optical densities of the Ub-parkin conjugates were quantified using NIH ImageJ, and the data represent the mean ± SEM for three independent experiments. For statistical analysis, a two-way ANOVA with Tukey post-test was performed. ** * P < 0.01.

C The activity of USP8 toward parkin relative to other DUBs. GST-parkin bound to glutathione beads was left to ubiquitinate for 2 h alone at 37°C. After 2 h, the beads were washed to remove reaction components and ubiquitinated GST-parkin was then incubated in the presence or absence of the indicated DUB for 1 h at 37°C. Reactions were immunoblotted for Ub and Ponceau stained for GST-parkin.

D USP8 preferentially hydrolyzes preassembled parkin Ub conjugates. For these reactions, GST-parkin, GST-CHIP, GST-HHARI (RBR domain), or GST-cIAP1 bound to glutathione beads were left to ubiquitinate for 2 h alone at 37°C. After 2 h, the beads were washed to remove reaction components. The ubiquitinated E3 was then incubated in the presence or absence of the full-length His-USP8 for 1 h at 37°C. Reactions were analyzed by Ponceau S staining and immunoblotting for Ub.

E USP8 can hydrolyze preassembled Ub conjugates on untagged parkin. For these reactions, untagged parkin was left to ubiquitinate for 2 h alone at 37°C with UbCH7 as the E2. After 2 h, apryrase was added for 20 min to terminate the reaction. Ubiquitinated parkin was now incubated for 1 h at 37°C in the presence or absence of His-tagged full-length USP8. Reactions were immunoblotted for Ub and parkin.

F Knockdown of other DUBs compared to USP8 had no effect on the recruitment of parkin onto mitochondria following CCCP treatment. U2OS-GFP-parkin cells were transfected with either non-targeting, USP8, ataxin-3, USP30, or USP2 siRNA (10 nM) for 60 h. Cells treated with CCCP for 1 h were fixed, and images were acquired after staining for the mitochondrial protein, TOM20 (see also Supplementary Fig S6).

Source data are available online for this figure.

substances of parkin on mitochondria downstream of recruitment rather than deubiquitinating parkin itself. Consistent with this model, we find that USP15 cannot deubiquitinate parkin directly (Fig 3C) and that neither the knockdown of USP15 (not shown) nor USP30 (Fig 3F, Supplementary Fig S6) affects parkin recruitment to mitochondria. Taken together, the findings indicate that USP8 directly and specifically deubiquitinates parkin.

USP8 regulates parkin auto-ubiquitination during mitophagy

Next, we examined the effect of USP8 on the ubiquitination of parkin and mitochondrial proteins in cells during mitophagy. In cells transfected with non-targeting siRNA, CCCP treatment led to a transient increase followed by a profound reduction in parkin levels, as described previously (Fig 4A–C) (Chan et al., 2011; Rakovic et al., 2013). Concurrently with the changes in parkin levels, the electrophoretic mobility of parkin was transiently shifted upward, consistent with a CCCP-induced activation of parkin and parkin auto-ubiquitination, as reported previously (Matsuda et al., 2010; Lazarou et al., 2013). Indeed, in control conditions, Ub affinity chromatography using Ub binding Agarose (TUBEs) followed by immunoblotting for parkin confirmed that it was markedly but transiently ubiquitinated in response to CCCP (Fig 4B, upper). In comparison, the increase in parkin levels and ubiquitination after CCCP persisted several hours longer in USP8 knockdown cells (Fig 4A–C). No comparable differences were noted in total Ub levels, suggesting that the effect of USP8 on parkin ubiquitination was specific (Fig 4B, lower). Indeed, both steady-state levels (Supplementary Fig S4D) and CCCP-induced changes in levels and ubiquitination of other mitochondrial proteins, including known substrates of parkin such as the mitofusins and VDAC (Geisler et al., 2010; Lazarou et al., 2013; Sarraf et al., 2013), did not appear to be directly affected by USP8 knockdown (Fig 4A, lower). Finally, consistent with our findings in GFP-parkin U2OS cells, knockdown of USP8 in HEK293 cells also caused a marked increase in the ubiquitination of untagged parkin following treatment with CCCP (Fig 4D).

To determine which lysine residues in parkin were ubiquitinated in cells during mitophagy, we purified GFP-parkin using GFP-nAb Agarose, followed by mass spectrometry (Fig 5A). In untreated cells, no sites of ubiquitination were detected on parkin by LC-MS/MS, although phosphorylation at serine 131 was observed in both untreated and CCCP-treated cells (Fig 5B). Following treatment with CCCP, ubiquitination at three sites, K27, K48, and K76, and phosphorylation at S65 were identified by LC-MS/MS, all within the parkin UbI domain (Fig 5B) and consistent with previous findings (Kondapalli et al., 2012; Sarraf et al., 2013). Next, we performed Parallel Reaction Monitoring (PRM) to determine the relative abundance of Ub conjugates at each site, dynamically during mitophagy. These were normalized to total parkin levels, using a control, unmodified parkin peptide (Supplementary Table S2). Whereas Ub conjugates were undetectable by PRM at baseline, CCCP treatment led to an increase in ubiquitination at each of the three sites (Fig 5C). In contrast, silencing USP8 led to a detectable accumulation of Ub conjugates at two of the three sites (K48 and K76) on parkin at baseline and to a further increase in parkin ubiquitination after 4 h of CCCP at all three sites (Fig 5D–F). Indeed, LC-MS/MS did not reveal any additional lysines on parkin that were ubiquitinated upon knockdown of USP8, either at baseline or after CCCP. Thus, USP8 opposes parkin auto-ubiquitination during mitophagy. In the absence of USP8, Ub conjugates accumulate after several hours of parkin activity on depolarized mitochondria, predominantly on these sites within the parkin Ubl. In the absence of USP8, low levels of parkin Ub conjugates also accumulate at steady state, possibly due to constitutive parkin activity and ongoing mitophagy in these cells.

USP8 selectively removes K6-linked ubiquitin conjugates from parkin

What types of Ub chains are conjugated to parkin during mitophagy? Modification of proteins with K48-linked Ub chains typically leads to degradation of the substrate by the proteasome. In contrast, chains linked via one of the other six lysines in Ub can protect modified substrates from proteasomal degradation and divert them toward functions in variety of cellular pathways including trafficking, signaling, and autophagy (Komander & Rape, 2012; Kulathu & Komander, 2012). Given that collectively our findings so far indicated that, paradoxically, repressing USP8-mediated deubiquitination appears to protect parkin from degradation (Fig 1C, Supplementary Figs S1C and S3A–C), we asked whether such
alternative, non-K48-linked Ub modifications were involved. Indeed, affinity purification of GFP-parkin from CCCP-treated cells, followed by LC-MS/MS, revealed Gly-Gly modifications on K6, K11, K48, and K63 of Ub, indicating that Ub chains on parkin were linked via these residues (Fig 5B). We also noted phosphorylation at S65 of Ub, as recently reported (Fig 5B) (Kane et al, 2014; Kazlauskaite et al, 2014; Koyano et al, 2014). None of these modifications were present in the absence of CCCP. Thus, activation of parkin during mitophagy leads to the generation of K6-, K11-, K48-, and K63-linked Ub chains and the phosphorylation of Ub at S65. To determine the nature of the Ub conjugates on parkin more quantitatively, we carried out in vitro ubiquitination reactions followed by absolute
HA-UbK6 overexpression almost completely rescued parkin recruitment to levels seen in cells without USP8 knockdown. Thus, reducing the levels of K6-linked Ub conjugates in cells bypasses the need for USP8 to remove K6-linked Ub from parkin. To examine mitophagy, we again determined the loss of TOM20 staining and the presence of GFP-parkin puncta on mitochondria in USP8 knockdown cells after 24 h of CCCP (Supplementary Fig S7). Compared to wild-type HA-Ub, overexpression of HA-UbK6 only further impaired parkin-mediated mitophagy (Supplementary Fig S7). In contrast, HA-UbKQR overexpression again rescued mitophagy (Supplementary Fig S7). Taken together, these findings indicate that skewing Ub conjugation toward K6 potentiates the effects of USP8 knockdown on parkin function in mitophagy, whereas skewing it away from K6 rescues the effect of USP8 knockdown. Thus, we conclude that USP8-mediated removal of K6-linked Ub from parkin is required for mitophagy to proceed efficiently.

Finally, to test whether enhancing K6-linked Ub conjugation would be sufficient to impair parkin function in mitophagy, we overexpressed the HA-Ub mutants in the absence of USP8 siRNA. Overexpression of HA-UbK6 only impaired parkin recruitment, with only approximately 40% of HA-positive cells showing co-localization with mitochondria after 1 h of CCCP (Fig 7A and B). Remarkably, the degree of impairment was similar to the one seen with USP8 knockdown (Fig 1, Supplementary Fig S1). In contrast, neither wild-type HA-Ub nor HA-UbKQR had comparable effects on parkin recruitment (Fig 7A and B). Furthermore, HA-UbK6 only increased the number of HA-positive cells that had lost TOM20 staining and that contained GFP-parkin puncta after 24 h of CCCP (Fig 7C and D). In contrast, HA-UbKQR had the opposite effect. Thus, skewing Ub conjugation specifically toward K6 impairs parkin function in mitophagy, in effect phenocopying the effects of USP8 knockdown. Taken together, the findings further support a model whereby USP8-mediated removal of K6-linked Ub from parkin is critical for mitophagy to proceed efficiently (Fig 7E).

# Discussion

In this study, we identified USP8 as a novel DUB partner for parkin and demonstrated that it is required for parkin-mediated mitophagy to proceed efficiently. As dysfunctional mitochondria are an important source of reactive oxygen species to which neurons are particularly vulnerable, understanding the mechanisms that govern mitochondrial quality control will be essential to developing therapies for neurodegenerative diseases such as PD. Like parkin, USP8 is expressed throughout the brain and in particular, in the substantia nigra, which is particularly vulnerable to parkin loss in PD. Given these findings, inhibition of USP8 and possibly other USP family members could provide a target to rescue or protect against neurodegeneration.

**Figure 4. Parkin auto-ubiquitination is enhanced following USP8 siRNA.**

A–C USP8 siRNA prolongs parkin auto-ubiquitination following treatment with CCCP over 4 h. U2OS-GFP-parkin cells were transfected with non-targeting or USP8 siRNA (10 nM) for 60 h (A). Cells were either left untreated or were treated with CCCP for the indicated time intervals. Lysates were analyzed by immunoblotting for parkin, actin, USP8, Mfn2, PDH (E2), Tim23, TOM20, and VDAC1. From these lysates, ubiquitinated proteins were purified with Agarose-TUBES (B). Bound proteins were analyzed by immunoblotting for parkin and Ub. The optical densities of ubiquitinated parkin (parkin smear excluding unmodified parkin) and total actin were quantified using NIH ImageJ (C), and the data represent the mean ± SEM for three independent experiments. For statistical analysis, a two-way ANOVA with Tukey post-test was performed, *P < 0.05, **P < 0.001, ***P < 0.0001; NS, not significant.

D USP8 siRNA enhances the auto-ubiquitination of untagged parkin following treatment with CCCP. HEK293T cells were co-transfected with untagged parkin and either non-targeting or USP8 siRNA (10 nM) for 60 h. Cells were either left untreated or were treated with CCCP for the indicated time intervals. Lysates were analyzed by immunoblotting for parkin, actin, USP8, and Mfn1.

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Figure 5. Knockdown of USP8 leads to an increase in Ub conjugates on parkin.

A–C Mass spectrometry analysis of GFP-parkin in untreated and CCCP-treated U2OS-GFP-parkin cells. U2OS-GFP-parkin cells were left untreated or treated with CCCP for 1 h (A). Lysates (400 μg) were immunoprecipitated with GFP-nAb Agarose. Lysates and beads (5% of total bead volume) were analyzed by immunoblotting for parkin and actin. Following on-bead digestion, GFP-parkin samples were analyzed by LC-MS/MS mass spectrometry [B]. Identified sites of ubiquitination and phosphorylation on parkin and Ub are indicated. Peptides for each of the 3 sites of ubiquitination on GFP-parkin (K27, K48 and K76) and an unmodified control parkin peptide were selected for time-scheduled parallel reaction monitoring (PRM) [C]. The relative abundance of each peptide between samples was calculated, and the relative amount of Ub conjugates present at each individual lysine in parkin was normalized relative to total parkin levels. The data represent the mean value of two technical replicates.

D–F PRM mass spectrometry analysis of GFP-parkin following USP8 knockdown U2OS-GFP-parkin cells were transfected with either non-targeting or USP8 siRNA (10 nM). After 60 h, cells were either left untreated or were treated with CCCP for 4 h. The abundance of Ub conjugates at the indicated sites in GFP-parkin was analyzed by parallel reaction monitoring. The relative abundance of each peptide between samples was calculated, and the relative amount of Ub conjugates present at each individual lysine in parkin was normalized relative to total parkin levels. The data represent the mean value of two technical replicates.

C, H AQUA analysis and quantification of Ub linkages in parkin poly-Ub conjugates. In vitro ubiquitination reactions were carried out with GST-parkin bound to glutathione beads for 2 h at 37°C in the presence or absence of Ub, E1, Ubch7, and GST alone (C). Ubiquitinated GST-parkin was eluted, trypsinized, and analyzed on an LC-ESI-TOF. Internal peptides for Ub and K6, K11, K33, K48, and K63 Ub linkages were used in the analysis. Ub conjugates were not detected on GST alone. K27, K29, or K33 linkages were not detected in parkin poly-Ub conjugates. Poly-Ub conjugates did not form on parkin when Ub, E3, or E2 were absent (data not shown). The data represent the mean ± SEM for two independent experiments. For statistical analysis, a one-way ANOVA with Tukey post-test was performed, **p < 0.01, ***p < 0.005. AQUA analysis of parkin self-ubiquitination in the presence of different E2 Ub-conjugating enzymes (H). Ubch7, Ubch15b, and Ube7 were the E2s tested in the analysis.

Source data are available online for this figure.
efficiently. Parkin has been reported previously to conjugate Ub linked via a number of different lysines including K48, K63, K27, and K29 as well as linear Ub chains and mono-Ub (Lim et al., 2005; Matsuda et al., 2006; Geisler et al., 2010; Muller-Rischart et al., 2013). Most of these studies relied exclusively on overexpression of Ub mutants or on chain-specific antibodies. Remarkably, using quantitative AQUA mass spectrometry, we find that parkin preferentially assembles K6-linked Ub conjugates on itself, with K11-, K48-, and K63-linked Ub, together accounting for only 40% of all chain linkages. This predilection for assembling K6 chains is likely an intrinsic property of parkin as it occurred regardless of the E2 enzyme used in the assay.

Increasing K6-linked auto-ubiquitination (i.e. by silencing USP8) is likely to stabilize parkin by protecting it from degradation in the proteasome or lysosome (Shang et al., 2005; McLelland et al., 2014), in line with the role of K6 in stabilizing RING1b and BRCA1/BARD (Nishikawa et al., 2004; Ben-Saadon et al., 2006; de Bie et al., 2010), the only other mammalian E3s known to assemble K6-linked Ub.
USP8 regulates parkin-mediated mitophagy

**A**

USP8 siRNA + HA-Ub: CCCP 1h

% of HA-positive cells with mitochondrial localization of GFP-parkin

**B**

Ratio of Ub conjugates relative to GST-parkin

**C**

U2OS-GFP-parkin: USP8 siRNA + HA-Ub

**D**

USP8 siRNA + HA-Ub: CCCP 1h

% of HA-positive cells with mitochondrial localization of GFP-parkin

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chains. These E3s are believed to function in histone modification and DNA repair (Ben-Saadon et al., 2006). Interestingly, a recent study reported that parkin collaborates with Rad6a/Ub2a, an E2 involved in DNA repair, although K6 ubiquitination was not examined (Haddad et al., 2013; Zou et al., 2013). These findings do not preclude a role for the ubiquitination of parkin or parkin substrates via other linkages in distinct cellular pathways such as NF-kB signaling (K63, N-terminal/linear) (Henn et al., 2007; Muller-Rischart et al., 2013), endocytosis and trafficking (mono-Ub) (Fallon et al., 2006; Trempe et al., 2009), autophagy (K27, K48, K63) (Geisler et al., 2010; Narendra et al., 2010a; Okatsu et al., 2010; Chan et al., 2011; van Wijk et al., 2012), or proteasomal degradation (K11, K48) (Matsumoto et al., 2010; Chan et al., 2011). Recent work showing that parkin is auto-inhibited and uses a HECT-like conjugation mechanism now makes it possible to examine the structural basis for the types of Ub chains assembled by parkin and the specific E2(s) involved (Riley et al., 2013; Spratt et al., 2013; Trempe et al., 2013; Wauer & Komander, 2013).

Whether the specificity for removing K6-linked Ub from parkin extends to other USP8 substrates is unknown. However, as USP8 cleaves K6-, K11-, K48-, and K63-linked free di-Ub chains in vitro, our results suggest that the interaction with parkin may help restrict USP8’s activity toward K6. Indeed, no USP-family member DUB tested has shown K6-specificity toward free Ub chains (Hospenthal et al., 2013). Moreover, whether parkin also uses K6 for substrate ubiquitination or only for auto-ubiquitination is not known. However, as USP8 does not affect the ubiquitination or stability of the parkin substrates we tested during mitophagy, we suspect that these are not linked via K6. This point further highlights the idea that parkin auto-ubiquitination may serve a distinct role from substrate ubiquitination.

Beyond the role of parkin, our work manipulating the ratios of different Ub linkages in cells suggests, for the first time, a role for K6 ubiquitination per se in mitochondrial quality control. While we are not certain of the mechanisms, it must be kept in mind that parkin does not make K6-linked chains exclusively, as seen in our AQUA analysis. It is therefore plausible that other DUBs could eventually cleave mixed chains at other sites (i.e. K11, K48, and K63) or even at K6, albeit perhaps with lower efficiency. Such a process might explain the observed delay in recruitment and mitophagy, with an increased presence of these conjugates impeding parkin from interacting with substrates, PINK1, and/or phosphorylated Ub. The latter two have been shown to be essential for parkin activation and translocation onto the mitochondria (Kondapalli et al., 2012; Kane et al., 2013). As parkin auto-ubiquitination appears to occur predominantly on the Ub domain (K48, K76), it is also possible that these conjugates interfere with binding to Ub-interacting proteins (i.e. endophilin, Eps15, ataxin-3) (Fallon et al., 2006; Durcan & Fon, 2011; Trempe et al., 2013) or PINK1-mediated phosphorylation of the Ub at S65 (Kondapalli et al., 2012). Moreover, the presence of increased levels of Ub chains linked primarily via K6 appears to impair parkin-mediated mitophagy, perhaps by interfering with ongoing parkin E3 Ub-ligase activity or because of a reduced capacity of these chains to recruit p62, LC3, or other autophagy proteins. Regardless of the precise mechanism, our work uncovers a novel layer of regulation, mediated by USP8 and K6-linked auto-ubiquitination, critical for parkin-dependent mitophagy.

Materials and Methods

Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Sigma) was used at a final concentration of 20 μM. Tetramethylrhodamine, methyl ester (TMRM) (Invitrogen) was used at a final concentration of 600 nM. Antibodies in Western blots were used to detect: parkin (1:20K), USP8 (1:2K), TOM20 (1:20K), STAM1 (1:5K), USP30 (1:2K), mitofusin 1 and 2 (1:2K) (Santa Cruz); Ub (1:10K) (Covance); COX1 (1:5K), Complex 1 (NDUFS3) (1:10K), DRP1 (1:5K), VDAC1 (1:10K) (Abcam); Tim23 (1:10K), Opa1 (1:5K) (BD Biosciences); PINK1 (1:2K) (Novus Biologicals); anti-HA (1:20K) (Roche); FLAG (1:20K) (Sigma); STAM2 (1:20K), ataxin-3 (1:20K), anti-His, actin (Millipore); and USP2 (kind gift from Dr. Simon Wing, McGill). For immunofluorescence microscopy, TOM20 (Santa Cruz); PDH (E2), COX1 (Abcam), and anti-HA (Roche) antibodies were used at a 1:1K dilution. GST-parkin, FLAG-parkin, and HA-Ub plasmids were described previously (Durcan et al., 2011). FLAG-HA-USP8 (#22608) and His-USP8Catalytic domain (#28115) were purchased from Addgene. Di-ubiquitin conjugates, Ubch7, Ubch5b, His-USP8 (full-length), and all commercial DUBs were purchased from Boston Biochem, with the exception of USP15 and USP18 (Enzo Life Sciences). GST-CHIP was a kind gift from Dr. L. Beitel (Lady Davis Institute). GST-HHARI (RBR domain only) was kind gift of Dr. R. Klevit, (U. Washington). GST-clAP1 was kind gift of Dr. P. Barker (McGill).
siRNA oligos and libraries

A human ON-TARGETplus Smartpool siRNA library was used to knockdown 87 putative DUBs (G-104705-01, Thermo Scientific). The AllStars negative control siRNA (1027280, Qiagen) was used for non-targeting siRNA. The siRNAs used were USP8.1: SI00073017, USP8.2: SI00073024, USP8.3: SI00073031, USP8.4: SI03103604, SI00073024, ataxin-3: SI00071113, USP30: SI03122714, and USP2: SI03246698 (Qiagen). USP8.2 was the USP8 siRNA used in assays to knockdown USP8. The siRNA oligos used to knockdown STAM 1 and STAM 2 were as follows:

STAM1 siRNA: 5'-CAGCAUGAUUAAGAACCUUA-3'
STAM2 siRNA: 5'-CUGCUCAAAACUCAUAUUUA-3'

Cell culture

U2OS-GFP-parkin cells were a kind gift of Dr. R. Screaton, U. Ottawa. This cell line was created by stably infecting cells with a GFP-parkin viral vector, generating a pool of stable cells expressing similar levels of GFP-parkin. All U2OS, HEK293T, HeLa, and SH-SY5Y cell lines were maintained at 37°C in RPMI-1640 media composed of 5% fetal bovine serum, 5% CO2 in DMEM with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Wisent Life Sciences).

siRNA and plasmid transfection

For siRNA transfections, cells were passed into wells/plates containing the siRNA (final concentration of 10 nM), HiPerFect (Qiagen), and OptiMEM (Life Technologies) and incubated for 24 h. Fresh media was added the next day. At 60 h post-transfection, cells were either lysed, fixed on coverslips, or were used in live-cell microscopy experiments.

For co-transfection of siRNA with a plasmid, reactions containing the siRNA (final concentration of 5 nM), plasmid (1 μg), JetPrime (PolyPlus Transfections), and transfection buffer were added to cells in dishes plated 24 h prior to transfection and incubated overnight. For transfection of GFP or FLAG-parkin with other plasmids, 0.5 μg of the plasmid construct was incubated with other plasmids for a final DNA amount of 2 μg in reactions. Reactions containing DNA, JetPrime, and transfection buffer were added to cells in dishes plated 24 h prior to transfection and incubated overnight.

Control and USP8 microRNA constructs:

MicroRNA targeting sequences were designed using the Life Technologies algorithm Block-iT Designer.

**USP-8 (sense – 2911)**
5’-TGCTGATCATACGAGCGCTGAGGTGGTTTGGCCACCTGACTG
ACCCCTGGAGCTTCATGTACT-3’

**USP-8 (anti-sense – 2911)**
5’-CCTGACTACATGAAAGCTCCAGGACGTACGTGCCGGCCAAAC
CTTGAGGTCCTCATGTACTC-3’

Oligonucleotides encoding the shRNA were first cloned into a pCDNA6.2/G-EmFP.miR vector (Life Technologies). The USP-8 shRNAmiR expression cassette was next PCR-amplified using the following primers:

**miRGFP BglII sense**
5’-GCCGAATCTACGCGGCTGCACCATGGTGACGCAAAGGCAGAG
GC-3’

**miRXF Xhol antisense**
5’-GCCCTCGAAGTGCCGGGATCTGGGCAATTGTCCATCTG
GTGC-3’

The fragments generated were digested with BglII and Xhol and cloned into the pRLRinPPTeGF vector (Gift of Dr. Brigitte Ritter and Dr. Peter McPherson). The control construct was generated in an identical manner containing the scrambled sequence outlined below:

**Control (sense)**
5’-TGTCAGATCATGACGCTGAGGAATGGTTTGGCCACCTGACTG
ACAATTCTCCACGTTCAGGT-3’

**Control (anti-sense)**
5’-CCTGACGTAACGTGGGAATGGTCAGCTGGCAAAAACA
ATTCTCCGAAGTGTCACGTC-3’

MiRNA viruses were produced in HEK293T cells using the Miami system (Addgene), a lentiviral-based method requiring either the control or USP8 miRNA pRLRinPPTeGF vectors described above and a packaging mix composed of pRSV-Rev, pMD2.g, and pMDLg/pRRE.

Viral-mediated infection and lysis of cortical neurons

Mouse cortical neurons were dissected from day 15 C57 Black mouse embryos (Jackson Labs) and plated onto ploy-P-lysine
(Sigma) coated plates. Cells were maintained in Neurobasal medium supplemented with penicillin/streptomycin, B27, N2, and L-glutamate (Life Technologies). After 3 days, neurons were infected with viruses at a MOI of 10 or 15 for 3 h. After 3 h, virus-containing media was replaced with culture media supplemented with fresh Neurobasal medium. Neuronal cultures were harvested 6 days post-viral transduction in ice-cold lysis buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 1% NP-40, and protease inhibitors).
**Protein expression, binding and in vitro assays**

GST and His fusion proteins were expressed in *E. coli* strain BL21 (DE3) at 23°C for 3 h and affinity-purified using Glutathione Sepharose 4B beads (GE Life Sciences), or Ni-NTA Agarose (Qiagen), respectively.

Binding assays with Agarose-TUBEes (Life Sensors), FLAG-parkin immunoprecipitations, and *in vitro* ubiquitination/deubiquitination assays were performed as described previously (Durcan et al, 2011, 2012). For GFP-parkin immunoprecipitations, cells were lysed in ice-cold RIPA buffer, with 10 μl of GFP-nAb Agarose beads (Allele Biotech) incubated with 400 μg of protein lysate for 2 h at 4°C. GFP-parkin bound on beads was now washed with 50 mM ammonium bicarbonate, pH 8 (AMBIC) solution prior to mass spectrometry analysis. The E2 enzyme used in our ubiquitination assays was UbCh7, except where indicated as UbCh5b or UbC7.

**Mass spectrometry analysis**

For AQUA analysis of ubiquitinated GST-parkin, samples were washed twice for 5 min at 4°C with AMBIC solution. Following these washes, GST-parkin was eluted from the beads by eluting in 0.1% Rapigest (Waters) dissolved in AMBIC solution for 30 min at 50°C. AQUA analysis of Ub chain linkage was performed as described previously (Kirkpatrick et al, 2006).

For LC-MS/MS and PRM analysis, samples were diluted to 2 M urea/60 mM AMBIC and on-bead trypsin digestion was performed overnight at 37°C. The supernatant was reduced with 7.5 mM DTT at 37°C and, after cooling for 10 min, alkylated with 15 mM chloroacetamide at room temperature for 20 min in the dark. Chloroacetamide was used in order to avoid artifacts isobaric to di-glycine on lysine residues associated with iodoacetamide (Nielsen et al, 2008). Samples were dried under vacuum, reconstituted in 1% acetonitrile (ACN)/1% formic acid (FA), and acidified with trifluoroacetic acid for desalting and removal of residual NP-40 by MCX (Waters Oasis MCX 96-well Elution Plate). After elution in 10% ammonium hydroxide/90% methanol, samples were once again dehydrated and reconstituted in 1% ACN/1% FA.

A Thermo EASY-nLC system was coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific) equipped with a Proxeon nanoelectrospray ionization source (Thermo Fisher Scientific). The LC column was a PicoFrit fused silica capillary column (15 cm × 75 μm i.d.; New Objective, Woburn, MA), self-packed with C18 reverse-phase material (Jupiter 5 μm particles, 300 Å pore size; Phenomenex, Torrance, CA) using a high pressure packing cell. For LC-MS/MS identification of ubiquitination sites on GFP-parkin, peptides were loaded on-column and eluted with a three-slope gradient at a flow rate of 600 nl/min. Solvent B (0.1% FA in ACN) increased from 1% to 5% in 6 min, then from 5 to 35% in 50 min, and finally from 35 to 80% B in 9 min. Solvent A was 0.1% FA in water. MS data were acquired using a data-dependent top16 method with a dynamic exclusion window of 3 s. The scan range was from 300 to 1,800 m/z with an isolation window of 2.0 Th and resolution of 70,000 at m/z 200. The automatic gain control (AGC) target was set at 166, the maximum ion fill time (IT) at 100 ms, the intensity threshold at 1.4e4, and the underfill ratio at 0.7%. For MS2, the resolution was defined at 17,500, the AGC target at 1e5, and maximum IT at 50 ms. The normalized collision energy used was 27 eV. Nanospray and S-lens voltages were set to 1.2–1.8 kV and 50 V, respectively. Capillary temperature was 250°C. Protein database searches were performed using Mascot 2.3 (Matrix Science). The mass tolerance for precursor ions was set to 10 ppm and for fragment ions to 0.52 Da. The enzyme specified was trypsin; four missed cleavages were allowed. Cysteine carbamidomethylation was specified as a fixed modification, and methionine oxidation, serine and threonine phosphorylation, and lysine LeuArg-Gly-Gly and Gly-Gly modifications as variable modifications.

For Parallel Reaction Monitoring (PRM), peptides were also loaded on-column and separated using a three-slope gradient at a 600 nl/min flow rate. Solvent B (0.1% FA in ACN) first increased from 0 to 3% in 4 min, then from 3 to 30% in 20 min, and finally from 30 to 80% B in 4 min. Solvent A was 0.1% FA in water. All parkin peptides (K27, K48 and K76) observed in the LC-MS/MS analysis of GFP-parkin, as well as a control unmodified peptide for parkin, were selected for time-scheduled parallel reaction monitoring (targeted MS2 mode). Precursor ion m/z, normalized collision energies, and retention time windows are listed in Supplementary Table S2. The maximum IT was set to 100 ms and the AGC at 2e5. Nanospray and S-lens voltages and capillary temperature were as described above. Raw data were analyzed using Skyline (64 bit) 2.5.0.5675 (MacLean et al, 2010). Peaks were manually verified to be free of interference, for the overlap of fragment ion traces for a given precursor, and for peak shape. Each peptide was quantified by integrating the area under the curve for the four most intense fragment ions.

**Fixed-cell immunofluorescence**

Cells grown on coverslips were fixed with 4% formaldehyde-PBS (prepared from 16% formaldehyde stock, Thermo Fisher Scientific) for 15 min at 37°C. After fixation, cells were washed four times with 1× PBS, permeabilized with 1× PBS containing 0.25% Triton X-100 for 10 min, and blocked with 3% BSA + PBST (1× PBS + 0.1% Tween-20) for 1 h at RT. Cells were labeled with primary antibodies diluted in PBST with 3% BSA overnight at 4°C and then with Alexa Fluor (Life Technologies) secondary antibodies (1:1,000 dilution) in PBST with 3% BSA for 3 h at RT. Cells were washed three times in PBS, and coverslips were mounted with fluorescent mounting medium (Dako). Confocal images were acquired on an LSM 510 Meta confocal microscope (Zeiss) using a 25×, 0.8NA or 63×, 1.4 NA objective. Excitation wavelengths of 488 nm, 543 nm, and 633 nm were used.

**Time-lapse fluorescence microscopy**

U2OS-GFP-parkin cells were seeded onto 35-mm glass-bottom microwell dishes (MatTek Corporation) and transfected with non-targeting or USP8 siRNA using HiPerFect. After 48 h, cells were transferred to a heated stage maintained at 37°C and at 5% CO2 using a Zeiss temperature controller and cell perfusion system (Zeiss). To visualize mitochondria, cells were transduced with CellLight mitochondria-RFP (Life Technologies) 16 h prior to imaging. Microscopy was performed on a Zeiss AxioObserver.Z1 inverted fluorescence microscope. Fully automated multidimensional acquisition was controlled using the Zen 2012 software (Zeiss). Images were acquired using a 20× objective (Plan-Apochromat) with a
side-mounted Axiocam MRm camera. GFP or mito-RFP was excited using the Zeiss XBO75 Xenon illumination system and detected using the appropriate filters (62HE ExGFP in combination with a Hyper GFP reflector or CY3, respectively). Fixed exposure times were as follows: GFP 70 ms and RFP 180 ms. Fluorescent images were acquired at 5-min intervals for a total of 145 min and compiled into movie files using the Zen 2012 software.

For the live-cell analysis of GFP-parkin recruitment onto mitochondria, 350 cells were examined over three separate experiments to ascertain the time required for GFP-parkin to be recruited onto mito-RFP-positive mitochondria. Parkin recruitment upon membrane depolarization is visualized by the appearance of punctate GFP fluorescence superposed onto mitochondrial RFP fluorescence. Quantification of GFP-parkin recruitment to mitochondria is facilitated by calculating the percentage of cells showing recruitment of GFP-parkin on mitochondria at 5-min intervals over a period of 145 min. For measuring relative parkin levels in an individual cell, in the first recorded frame, the pixel intensity of GFP-parkin fluorescence in a region of interest (ROI) of defined size was recorded. Following the quantification of relative parkin pixel intensity, the cell was now followed over time following CCCP treatment until the initial appearance of GFP-parkin puncta that are present on the mitochondria in the cell of interest. For this analysis, 20 cells transfected with non-targeting siRNA and 15 cells transfected with USP8 siRNA were tested. Relative parkin pixel intensity was plotted against the initial time of parkin recruitment on mitochondria.

To visualize mitochondria membrane potential, cells were labeled with the membrane potential-dependent cationic fluorophore probe Tetramethylrhodamine, methyl ester (TMRM) (600 nM) (Life Technologies) for 20 min at 37°C. The labeling medium was removed and replaced with 150 nM TMRM to maintain the equilibrium distribution of the fluorophore. Cells were imaged as described above. Fixed exposure times were as follows: GFP 70 ms and RFP 20 ms. Fluorescent images were acquired at 1-min intervals for a total of 5 min and compiled using the Zen 2012 software.

Statistical analysis

For analysis of immunofluorescence coverslips, slides were blinded for three independent experiments. 100 cells were counted per experiment. Cells were analyzed for the number of cells with GFP-parkin puncta co-localizing on TOM20-positive mitochondria (CCCP 1 h), the number of cells with GFP-parkin puncta co-localizing with TOM20-positive mitochondria (CCCP 24 h), and the number of cells containing GFP-parkin puncta co-localizing with TOM20-positive mitochondria (CCCP 24 h). All statistical analysis was run on data obtained from three independent experiments. A one-way ANOVA analysis was run on data obtained from three independent experiments. 100 cells were counted per experiment. Cells were analyzed for the number of cells with GFP-parkin puncta co-localizing with TOM20-positive mitochondria (CCCP 24 h). All statistical analysis was run on data obtained from two independent experiments. A one-way ANOVA analysis was run on data obtained from three independent experiments. 100 cells were counted per experiment. Cells were analyzed for the number of cells with GFP-parkin puncta co-localizing with TOM20-positive mitochondria (CCCP 24 h).

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Author contributions

TMD and EAF conceived and planned the experiments, and interpreted the data. MYT acquired and analyzed all the live-cell analysis. JRP, DF, and BC performed and interpreted all the LC-MS/MS and PRM mass spectrometry analysis. MAA and TAS performed and interpreted all the AQUA mass spectrometry analysis. EAD generated USP8 miRNA viruses and cultured mouse cortical neurons, in addition to performing the USP8 knockdown experiments in neurons. PG assisted TMD in performing and interpreting data from Figs 6 and 7. TMD and EAF wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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