Parkin is an E3 ligase for the ubiquitin-like modifier FAT10, which inhibits Parkin activation and mitophagy

Highlights
- Parkin is an E3 ligase for the ubiquitin-like modifier FAT10
- Parkin mediates FAT10ylation and proteasomal degradation of mitofusin2
- FAT10 inhibits Parkin activation, translocation to damaged mitochondria, and mitophagy
- Death induced by rotenone in dopaminergic neuronal cells is elevated by FAT10

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In brief
Roverato et al. identify Parkin as an E3 ligase for the ubiquitin-like modifier FAT10. FAT10ylation of Parkin leads to its proteasomal degradation, to its inhibition, and to impairment of its translocation to damaged mitochondria resulting in a delay of mitophagy. FAT10 enhances the sensitivity of neuronal cells to mitochondrial damage.
Parkin is an E3 ligase for the ubiquitin-like modifier FAT10, which inhibits Parkin activation and mitophagy

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SUMMARY

Parkin is an E3 ubiquitin ligase belonging to the RING-between-RING family. Mutations in the Parkin-encoding gene PARK2 are associated with familial Parkinson’s disease. Here, we investigate the interplay between Parkin and the inflammatory cytokine-induced ubiquitin-like modifier FAT10. FAT10 targets hundreds of proteins for degradation by the 26S proteasome. We show that FAT10 gets conjugated to Parkin and mediates its degradation in a proteasome-dependent manner. Parkin binds to the E2 enzyme of FAT10 (USE1), auto-FAT10ylates itself, and facilitates FAT10ylation of the Parkin substrate Mitofusin2 in vitro and in cells, thus identifying Parkin as a FAT10 E3 ligase. On mitochondrial depolarization, FAT10ylation of Parkin inhibits its activation and ubiquitin-ligase activity causing impairment of mitophagy progression and aggravation of rotenone-mediated death of dopaminergic neuronal cells. In conclusion, FAT10ylation inhibits Parkin and mitophagy rendering FAT10 a likely inflammation-induced exacerbating factor and potential drug target for Parkinson’s disease.

INTRODUCTION

Autosomal recessive Parkinson’s disease (PD) is characterized by a selective loss of dopaminergic neurons of the substantia nigra pars compacta that leads to the early onset of Parkinsonism. Inherited familial PD is frequently caused by loss of function mutations in the PARK2 gene which encodes the ubiquitin E3 ligase Parkin. Parkin is a protein that acts as a protective agent in a broad variety of cellular stresses, and this pro-survival activity is generally linked to its function as an E3 ligase (Dawson and Dawson, 2010). In fact, Parkin is mainly known as a key component of the PINK1/Parkin axis that, after mitochondrial depolarization, triggers the selective degradation of damaged mitochondria by autophagy in a process known as “mitophagy.” Here, the loss of mitochondrial membrane potential leads to the localization of the kinase PINK1 on the outer mitochondrial membrane, where it phosphorylates several targets including the ubiquitin-like (UBL) domain of Parkin (Jin et al., 2010; Kondapalli et al., 2012; Shiba-Fukushima et al., 2012). This modification, in concert with the phosphorylation of ubiquitin, triggers the conformational opening of Parkin and the exposure of the catalytic domain, resulting in Parkin activation and subsequent polyubiquitylation of several substrate proteins on the mitochondrial outer membrane (Gladkova et al., 2018). These ubiquitin conjugates enable recruitment of autophagy adaptors, driving mitophagosome formation and lysosomal degradation of the damaged mitochondria (Dai et al., 2015; Wang et al., 2020). The efficient elimination of depolarized mitochondria that are a prominent source of reactive oxygen species (ROS) protects neurons from severe ROS-mediated damage and consequent apoptosis, thereby explaining the link between functional inactivation of Parkin and early onset of PD (Gao et al., 2017). Given its neuroprotective effect, efforts to elucidate the dysregulation of Parkin’s enzymatic activity are crucial to understand the onset and progression of Parkinsonism. The function of Parkin is fine-tuned by post-translational modifications such as phosphorylation, SUMOylation, ISGylation, and NEDDylation (Chakraborty et al., 2017). Nonetheless, the role of the inflammatory response in the striatum of PD patients in the regulation of Parkin function is poorly understood. Inflammation in the central nervous system (CNS) during pathogenesis of PD is accompanied by microglial activation and massive production of pro-inflammatory cytokines (Mogi et al., 1994). In particular, high levels of tumor necrosis factor alpha (TNF-α) and interferon (IFN)γ are expressed in the substantia nigra of PD patients, that stimulate dopaminergic neurons (McGuire et al., 2001) and are known to strongly and synergistically induce the ubiquitin-like modifier HLA-F adjacent transcript 10 (FAT10) (Liu et al., 1999).

FAT10 (also known as ubiquitin D [UBD]) is an 18-kDa ubiquitin-like modifier (UBL) that consists of two ubiquitin-like domains (UBDs) that are joined by a flexible linker (Aichem et al., 2018). FAT10 is encoded in the major histocompatibility complex
(MHC) class I locus (Fan et al., 1996) and is expressed mainly in organs and cells of the immune system (Lukasiak et al., 2008; Schregle et al., 2018). FAT10 gets isopeptide-linked via its C-terminal diglycine motif to hundreds of substrate proteins that label them for rapid degradation by the 26S proteasome independently of ubiquitin attachment (Aichem et al., 2012; Hipp et al., 2005; Schmidtke et al., 2009). In contrast to ubiquitin, FAT10 does not get cleaved from its substrates at the 26S proteasome but is most likely degraded along with its substrates thus explaining its short half-life of 1 h (Hipp et al., 2005). Similar to other ubiquitin-like modifiers, FAT10 possesses its private activation and conjugation cascade, which consists of the E1 activating enzyme UBA6 (Jin et al., 2007; Chiu et al., 2007; Pelzer et al., 2007), the E2 conjugating enzyme USE1 (Aichem et al., 2010), and putative, but so far unknown, E3 ligases. Here, we identify Parkin as an E3 ligase for FAT10 and the mitochondrial fusion protein mitofusin2 (Mfn2) as a Parkin-dependent FAT10ylation substrate in neuronal cells. Moreover, we show that FAT10 induction inhibits the ubiquitin-ligase activity of Parkin and the localization of Parkin to depolarized mitochondria leading to an impairment of mitophagy with likely adverse consequences for the pathogenesis of PD.

RESULTS

Parkin is a substrate for FAT10ylation

A previous proteomic analysis has described endogenous FAT10 to interact with 569 proteins and to covalently modify 176 of them. Unexpectedly, among these FAT10 binding partners there were various mitochondrial proteins (Aichem et al., 2012). Accordingly, we speculated that E3 ligases involved in the maintenance of mitochondrial homeostasis could promote the FAT10ylation of mitochondrial substrates. Therefore, we tested whether FAT10 interacts with one of these E3s. We overexpressed FLAG-tagged FAT10 in Hek293 cells together with ligases known to be involved in mitochondrial dynamics, such as MUL-1, MARCh5, and Parkin. After immunoprecipitating FLAG-FAT10, we aimed to detect putative interactors using antibodies against the co-expressed ligases. In addition, we treated the cells with the mitochondrial uncoupler CCCP (carbonyl cyanide m-chlorophenyl hydradrazine) for 1 h to create a state of mitochondrial dysfunction. Among the tested E3 ligases, we found a unique interaction between FAT10 and Parkin (Figures 1A, S1A, and S1B), but the conjugate formation was independent of the mitochondrial membrane potential (Figure 1A). To confirm the covalent nature of this interaction, we also co-expressed myc-tagged Parkin with an inactive variant of FLAG-FAT10 in which the C-terminal GG motif was replaced by AV (Figures 1A and 1B). Because FAT10-Parkin conjugates did not form with FLAG-FAT10–AV, and because FAT10 does not form chains, we conclude that Parkin is mono-FAT10ylated at two sites under overexpression conditions. As a further confirmation, the immunoprecipitation was performed in the other direction that validated our conclusion (Figures 1B and S1C). To further corroborate these results, we used SH-SY5Y cells to study the interaction between Parkin and FAT10 under fully endogenous conditions. SH-SY5Y is a dopaminergic neuroblastoma-derived cell line expressing endogenous Parkin and is commonly used to study neuronal function (Xicoy et al., 2017). Because these cells also express high amounts of endogenous FAT10 on 24 h of stimulation with TNF-α and IFNγ, we performed a co-immunoprecipitation of FAT10 with endogenous Parkin using an anti-Parkin antibody, followed by FAT10 western blot. We confirmed the formation of covalent FAT10-Parkin conjugates under endogenous conditions (Figure 1C). Curiously, in presence of TNF-α/IFNγ, we detected additional FAT10ylated forms of Parkin, suggesting that cytokine-induced factors might exist that enhance the formation of FAT10-Parkin conjugates. Such a discrepancy between overexpression and endogenous conditions was previously noticed also for the formation of FAT10-p62 conjugates (Aichem et al., 2012). Next, we asked whether the so far known conjugation machinery for FAT10 is involved in FAT10-Parkin conjugate formation (Chiu et al., 2007). To this aim, we investigated Parkin FAT10ylation in Hek293-UBA6 KO cells (Aichem et al., 2019b) or in Hek293-Use1 KO cells (Aichem et al., 2018) and confirmed that UBA6 and USE1 are essential for the formation of Parkin-FAT10 conjugates. Interestingly, we observed that FAT10 has a poor capacity to non-covalently bind Parkin in the absence of USE1 (Figure 1D). Finally, we analyzed the interaction between Parkin and FAT10 by chemical crosslinking coupled to mass spectrometry (XL-MS). FAT10 mainly formed inter-protein crosslinks with the ubiquitin-like (Ubl) domain of Parkin (Figure 1E; Table S1). Additionally, some inter-protein crosslinks with a region around the unique Parkin domain (UPD) and the catalytic RING2 domain were detected. Overall, these results describe Parkin as an interaction partner for FAT10 and as a substrate for oligo-FAT10ylation.

FAT10ylated Parkin can localize in both cytosol and mitochondria and is degraded by the proteasome

FAT10 is, besides ubiquitin, the only ubiquitin-like modifier that directly guides its substrates to proteasomal degradation (Hipp}

Figure 1. Parkin is a FAT10ylation substrate

(A and B) Immunoblot (IB) analysis of the co-immunoprecipitation (coIP) of Myc-Parkin with 3xFLAG-FAT10 or 3xFLAG-FAT10–AV from whole cell lysates of Hek293 cells transiently expressing the indicated constructs. Cells were treated with DMSO or, where specified, with 10 µM CCCP for 1 h prior to lysis. (C) Total cell extracts of untreated or TNF-α/IFNγ-stimulated SH-SY5Y cells were used to immunoprecipitate endogenous Parkin. An IgG with unrelated specificity was used as control. Immunoblotting was then performed with a polyclonal anti-FAT10 antibody to detect endogenous Parkin-FAT10 conjugate formation. Cells were treated with DMSO or, where specified, with MG132 prior to lysis. (D) Immunoblot analysis of the co-immunoprecipitation of transiently expressed Myc-Parkin with 3xFLAG-FAT10 from whole cell lysates of wild-type Hek293 cells, Hek293 UBA6 knockout (KO) cells, or Hek293 USE1 KO cells, after lysis in denaturing conditions (4% SDS lysis buffer). (E) Interaction of Parkin and Fat10 was analyzed by chemical cross-linking and mass spectrometry (XL-MS). Overall crosslinking pattern of mixtures of Parkin and FAT10 are shown (n = 3). Lysine residues, as potential targets of the crosslinking agent, are highlighted in gray. Parkin domains are indicated in different colors as indicated. Inter-protein crosslinks are shown in green and intra-protein crosslinks in purple (xPD n = 2, LD score ≥ 25, false discovery rate [FDR] ≤ 0.05). Shown results are representatives of at least three independent experiments with similar outcomes.
In fact, its intrinsic instability leads to a rapid degradation of both, FAT10 and its substrates, without the need for de-conjugation (Aichem et al., 2018). Therefore, we investigated the degradation of the FAT10-Parkin conjugate. We found that the FAT10-Parkin conjugate rapidly disappeared after 30 min of cycloheximide (CHX) treatment. Because monomeric FAT10 was degraded with the reported half-life of 1 h (Hipp et al., 2005), Parkin–FAT10 was degraded even faster than monomeric FAT10 (Figure 2 A). The proteasome inhibitor MG132 prevented degradation of the FAT10-Parkin conjugate whereas the autophagy-inhibitor bafilomycin A1 did not stabilize it (Figure 2 A). We then asked whether the FAT10–Parkin conjugate was accumulating as an insoluble aggregate and found that the level of FAT10ylated Parkin was not significantly altered over time in the insoluble fraction (Figure 2 A). Because Parkin is a protein involved in mitophagy, we examined whether mitochondrial uncoupling by CCCP has an effect on the degradation rate of the FAT10–Parkin conjugate. Strikingly, we found that the prolonged treatment of the cells with CCCP lead to a significant reduction of the steady-state level of FAT10, whereas the Parkin–FAT10 conjugate, after an initial reduction (1 h), was accumulating over 6 h of CCCP treatment (Figure 2 B). Interestingly, the degradation kinetic of FAT10 was not significantly altered by CCCP on CHX treatment (Figure 2 C). Next we investigated where the FAT10–Parkin conjugate is formed in cells during steady-state and mitochondrial depolarization conditions. We overexpressed myc–Parkin and FLAG–FAT10 in Hek293 cells, followed by 1 h CCCP and/or 5 h MG132 treatment to induce mitochondrial depolarization and inhibition of the proteasomal degradation of FAT10ylated Parkin, respectively. Subsequently, we separated the mitochondrial from the cytosolic fraction, and the FAT10–Parkin conjugate was immunopurified and visualized. The FAT10–Parkin conjugate was mainly found in the cytosol, and proteasome inhibition lead to its accumulation (Figure 2 D, lanes 4–7). Interestingly, FAT10 and FAT10ylated Parkin were detectable in the mitochondrial fraction on treatment with CCCP and MG132, demonstrating that the FAT10–Parkin conjugate could also be formed at mitochondria on their depolarization (Figures 2 D, lane 14, and S4 A). By immunostaining of FAT10 in TNF-α/IFN-γ-treated SH-SY5Y cells (validated in Figure S2 B), we found no detectable FAT10 translocation to mitochondria on CCCP treatment (Figure 2 E), but it was mainly cytosolic, in line with the results in Figure 2 D.

**Parkin auto-FAT10ylates itself in vitro**

Parkin is a primarily cytosolic protein and is known to ubiquitylate cytosolic substrates independently of PINK1 activation. For instance, Parkin promotes the clearance of cytosolic proteins such as tubulin α and β (Stevens et al., 2015). Furthermore, it ubiquitylates Bax in a PINK1-independent manner, whereas its activity in mediating K63-ubiquitylation of *M. tuberculosis* mediates resistance to intracellular pathogens (Johnson et al., 2012; Manzanillo et al., 2013). However, the exact mechanism regulating Parkin activity in the cytosol is still poorly understood. Parkin is a versatile E3 ligase and mediates Lys48 poly-ubiquitylation as well as mono-ubiquitylation or poly-ubiquitylations at Lys 6, Lys 11, and Lys 63 (Chew et al., 2011; Cunningham et al., 2015; Durcan et al., 2014). Considering that the FAT10–Parkin conjugate is formed both in the cytosol and mitochondria (Figures 1 A and 2 D), we asked whether Parkin might serve as an E3 ligase for FAT10. We first confirmed the formation of the FAT10–Parkin conjugate in vitro using immunopurified myc-tagged Parkin that was previously overexpressed in Hek293 cells (Figure 3 A). When FAT10 was added to the in vitro reaction in the presence of UBA6, we could detect the formation of a FAT10–Parkin conjugate (Figure 3 A, lane 5). The addition of USE1 slightly increased the FAT10ylation of Parkin, whereas the replacement of GG by AV at the C terminus of FAT10 abolished the covalent FAT10 attachment (Figure 3 A). To investigate the effect of Parkin activation on the Parkin–FAT10 conjugate formation, we compared the in vitro FAT10ylation of the unmodified recombinant Parkin protein to a Parkin variant in which the Serine65 residue was phosphorylated (Figure S3 A), a post-translational modification that opens and activates Parkin (Sauvé et al., 2018). Interestingly, we observed that phosphorylated Parkin undergoes enhanced FAT10ylation (Figure 3 B). To confirm this finding, we performed a similar experiment as in Figure 3 B but comparing a recombinant unmodified GST-tagged Parkin to its catalytically inactive recombinant C431A mutant. At the same time, we compared these proteins to a form that was previously phosphorylated in vitro by PINK1 (Figure S3 A). Remarkably, the exchange of the active site cysteine of Parkin to alanine strongly reduced FAT10–Parkin conjugate formation, whereas Parkin phosphorylation enhanced the formation of the FAT10–Parkin conjugates, demonstrating that Parkin uses its E3 ligase activity to auto-FAT10ylate itself (Figures 3 C, 3 D, and S3 B). Notably, the FAT10ylation of C431A GST-Parkin was not completely abolished, and this effect is likely due to the fact that the FAT10 E1/E2 enzymes (UBA6/USE1) are able to directly conjugate FAT10 to several substrates under in vitro conditions (Aichem et al., 2012; Bialas et al., 2015, 2019). In addition, our quantitative XL-MS analysis also suggests an intensified interaction of FAT10 with the UBL domain of phosphorylated Parkin, as indicated by significantly upregulated inter-protein crosslinks in samples containing Parkin–S65P/FAT10 compared to non-phosphorylated Parkin/FAT10 complexes (Figure 3 E; Table S2), in line with the results in Figures 3 C and 3 D. Finally, we evaluated the interaction between Parkin and USE1, the conjugating enzyme for FAT10. Interestingly, we found that in Hek293 cells endogenous USE1 non-covalently interacted with either EGFP- or myc-tagged Parkin (Figures 3 F and 3 G). These results describe Parkin as a multi-functional ligase that is able to auto-FAT10ylate itself.

**Parkin is a FAT10 E3 ligase that enhances the formation of the FAT10–Mfn2 conjugate**

Next, we asked whether there are proteins undergoing Parkin-dependent FAT10ylation. Mitofusin2 (Mfn2) is a GTPase involved in mitochondrial dynamics as an essential component of the mitochondrial fusion machinery (Santel and Fuller, 2001). Moreover, Mfn2 is a well-known PINK1/Parkin-dependent phospho-ubiquitylation substrate and is degraded in a proteasome-dependent manner after mitochondrial stress (McLelland et al., 2018). In a previous quantitative mass spectrometry analysis of FLAG–FAT10 conjugates, we identified Mfn2 as an interaction partner for
Figure 2. FAT10ylated Parkin can localize in both cytosol and mitochondria and is degraded by the proteasome

(A) Immunoblot analysis of the co-immunoprecipitation of Myc-Parkin with 3xFLAG-FAT10 in whole cell lysate from transiently transfected Hek293 cells expressing the indicated constructs. Cells were treated with DMSO or, where specified, with the protein synthesis inhibitor cycloheximide (CHX) at the indicated time points with or without autophagy inhibitor bafilomycin A1 (Baf-A1) or proteasome inhibitor MG132.

(B) Immunoblot analysis of the co-immunoprecipitation (co-IP) of Myc-Parkin with 3xFLAG-FAT10 in whole cell lysate from Hek293 cells expressing the indicated constructs. Cells were treated with DMSO or, where specified, with CCCP (10 μM) for the indicated time periods with or without bafilomycin A1 (Baf-A1, 100 nM) or MG132 (5 μM).

(C) Immunoblot analysis of the degradation rate of overexpressed FLAG-FAT10 in Hek293 cells after treatment with 50 μg/mL CHX in presence or absence of the mitochondrial uncoupler CCCP (10 μM).

(D) Untransfected Hek293 cells or Hek293 cells stably expressing Myc-Parkin were transiently transfected for 24 h with a plasmid expressing FLAG-FAT10 where indicated. DMSO or, where indicated, CCCP (10 μM) and/or MG132 (5 μM) were added for 1 h or 5 h prior to lysis, respectively. Cell lysates were fractionated into mitochondria and cytoplasm enriched samples followed by immunoprecipitation of FLAG-tagged FAT10, SDS-PAGE, and immunoblot with the indicated antibodies.

(E) SH-SY5Y cells were treated for 24 h with TNF-α/IFN-γ to induce FAT10 expression, followed by 2 h DMSO or CCCP treatment where indicated. Cells were immunostained with antibodies against TOM20 (red) and FAT10 (green). Scale bars, 10 μm.

Shown results are representatives of at least three independent experiments with similar outcomes.
FAT10 (data not shown). In fact, when expressed together in Hek293 cells, we detected a prominent stable conjugate when myc-tagged Mfn2 was transiently co-expressed with FLAG-tagged WT FAT10, but not with FLAG-FAT10-AV (Figure 4A). To confirm these data under endogenous conditions, we treated Hek293 wild-type (WT) or Hek293 FAT10 knockout (KO) cells (Aichem et al., 2019a) with TNF-α/IFNγ for 24 h to induce FAT10 protein expression, followed by an immunoprecipitation with the monoclonal anti-FAT10 antibody 4F1 and a western blot with an anti-Mfn2 antibody. We confirmed that the KO of FAT10 in Hek293 cells led to the complete disappearance of the Mfn2 interaction and conjugate formation (Figure 4B). We subsequently investigated the dynamics of the FAT10-Mfn2 conjugate degradation in a CHX chase experiment. In contrast to the Parkin-FAT10 conjugate, the FAT10-Mfn2 conjugate followed a kinetic of proteasome-dependent degradation that resembled the one of monomeric FAT10 (Figure 4C). To investigate whether mitochondrial depolarization stress, and the consequent Parkin activation, could influence the formation of the Mfn2-FAT10 conjugate, we examined the formation of this complex in SH-SYSY cells. Interestingly, mitochondrial depolarization led to a decrease in FAT10 steady-state level (Figure 4D), as we have reported for Hek293 cells (Figure 2B). We could not detect significant levels of FAT10-Mfn2 conjugates in TNF-α/IFNγ-stimulated SH-SYSY cells (Figure 4D), in contrast to what we had previously observed in Hek293 cells (Figure 4B), suggesting that the efficiency of the formation of the Mfn2-FAT10 conjugate is cell-type-specific. Nevertheless, the inhibition of proteasome activity with MG132 allowed us to detect the FAT10-Mfn2 conjugate (Figure 4D, lane 3). Strikingly, 2 h of mitochondrial depolarization with CCCP lead to an increase in the formation of the FAT10-Mfn2 conjugate, and this complex further accumulated after proteasome inhibition (Figure 4D, lanes 4 and 5). Moreover, we performed a co-immunoprecipitation analysis that validated that FAT10-Mfn2 interaction and conjugate formation takes place at mitochondria after treatment of SH-SYSY cells with MG132 and CCCP (Figure S4A). Given that in Hek293 cells the co-expression of EGFP-tagged Parkin followed by 1 h CCCP treatment led to significant enhancement of the FAT10ylation of Mfn2 (Figure 4E), we investigated whether the depletion of cellular Parkin in SH-SYSY leads to a reduction of the Mfn2-FAT10 complex. Remarkably, knocking down Parkin (Figures S4B and S4C) abolished the formation of the CCCP-dependent Mfn2-FAT10 conjugate in SH-SYSY cells, confirming Parkin as a FAT10 E3 ligase and Mfn2 as a Parkin-dependent FAT10ylation substrate under endogenous conditions (Figure 4F). To further confirm this data, we performed an in vitro FAT10ylation experiment with myc-tagged Mfn2 as substrate that was immunopurified from Hek293 cells after overexpression (Figure 4G). We detected the strongest FAT10-Mfn2 conjugate formation when the complete conjugation machinery UBA6, USE1, and active pS65-Parkin was present in the in vitro reaction. Overall, we confirmed Parkin to act as a FAT10 E3 ligase and discovered Mfn2 as a Parkin-dependent FAT10ylation substrate.

**FAT10 inhibits the activation of Parkin and the Parkin-dependent Mfn2 ubiquitylation**

Parkin interactors such as BAG5 and PICK1 inhibit the ubiquitylation activity of Parkin, whereas the ubiquitin-like modifiers NEDD8 and SUMO-1 have been described to activate Parkin (He et al., 2018; Kalia et al., 2004; Um and Chung, 2006; Um et al., 2012). Hence, we investigated whether the FAT10ylation of Parkin has an effect on its functions. As an assay to assess Parkin activity, we investigated its CCCP-induced auto-ubiquitylation (Chung et al., 2004). When we treated a stably transfected Hek293 clone expressing GFP-Parkin with CCCP, we observed Parkin polyubiquitylation after immunoprecipitation of overexpressed HA-ubiquitin in Parkin western blots (Figure 5A). Strikingly, when cells were transiently co-transfected with FLAG-tagged FAT10 WT, the CCCP-dependent auto-ubiquitylation of Parkin was strongly reduced, whereas this inhibitory effect was absent when FAT10-AV was co-expressed (Figure 5A). To investigate whether the same effect was visible under endogenous conditions, we evaluated the CCCP-induced auto-ubiquitylation of Parkin with or without pre-stimulation by TNF-α/IFNγ. Additionally, we compared Parkin auto-ubiquitylation in SH-SYSY WT cells to SH-SYSY FAT10 KO cells (validated in Figure S5A). To achieve that, we treated the cells with 10 μM CCCP for 1.5 h, followed by immunoprecipitation of endogenous ubiquitin and western blot analysis using a monoclonal anti-Parkin antibody. Strikingly, in SH-SYSY WT cells, the cytokine pre-treatment led to reduced auto-ubiquitylation of Parkin, and this negative effect was abolished by the knock out of the Fat10 gene (Figure 5B). Accordingly,
**Figure 4. Parkin FAT10ylates Mitofusin2**

(A) Immunoblot analysis of the coIP of Myc-Mfn2 with 3xFLAG-FAT10 or 3xFLAG-FAT10-AV from whole cell lysates of Hek293 cells transiently expressing the indicated constructs.

(B) Total cell extracts of untreated or TNF-α/IFN-γ-stimulated Hek293 WT or HEK293 FAT10 KO cells were used to immunoprecipitate endogenous FAT10. Immunoblotting was then performed with an anti-Mfn2 antibody to detect endogenous Mfn2-FAT10 conjugate formation.

(C) Immunoblot analysis of the coIP of Myc-Mfn2 with 3xFLAG-FAT10 from whole cell lysates of Hek293 cells expressing the indicated constructs. Cells were treated with DMSO or, where specified, with cycloheximide (CHX) for the indicated time periods. Where indicated, MG132 or bafilomycin A1 (Baf A1) have been added for the entire duration of the CHX-chase experiment.

(D) Total cell extracts of untreated or TNF-α/IFN-γ-stimulated SH-SY5Y cells were used to immunoprecipitate endogenous FAT10. Immunoblotting was then performed with a polyclonal anti-Mfn2 antibody to detect endogenous Mfn2-FAT10 conjugate formation. Where indicated, cells were treated for 2 h with CCCP (10 μM) and/or with MG132 (5 μM) prior to lysis.

(legend continued on next page)
we assessed the capacity of Parkin to ubiquitylate the mitochondrial substrate Mfn2. We detected a prominent reduction of Parkin-related Mfn2 ubiquitylation after CCCP treatment in the presence of FAT10 but not FAT10-AV (Figure 5G). Moreover, in a co-immunoprecipitation assay co-expression of WT FAT10 (but not FAT10-AV) with EGFP-Parkin abolished the interaction of the E3 ligase with Mfn2 on mitochondrial uncoupling (Figure 5D). To further confirm that the inhibitory effect of FAT10 is dependent on its conjugation, we tested whether FAT10 could inhibit Parkin activation in Hek293 UBA6 KO cells. Remarkably, the absence of UBA6 completely abolished the inhibitory effect of FAT10 on Parkin activation (Figure 5E). Finally, we reconstituted an in vitro system for Parkin auto-ubiquitylation and combined it with an in vitro FAT10ylation system to investigate whether the overall inhibitory effect of FAT10 was directly mediated by the impairment of Parkin activation. We first performed an in vitro FAT10ylation of Parkin, followed by the addition to the in vitro reaction of ubiquitin, UBE1, and UbC7 as indicated in Figure 5F. pSer65 Parkin was able to auto-ubiquitylate itself in vitro as previously reported, whereas the unmodified form was only poorly undergoing self-modification (Figure 5F, lane 7) (Ordureau et al., 2014). Of note, our in vitro data were in accordance with our in cellulo data: a pre-incubation of Parkin with FAT10 alone was not affecting its activation (Figure 5F, lane 8), whereas a reduction of its auto-ubiquitylation and accumulation of unmodified Parkin was evident when Parkin was pre-incubated with FAT10 and UBA6 or with a combination of UBA6 and USE1 (Figure 5F, lanes 9 and 10, respectively). Furthermore, FAT10-AV in the absence of UBA6 completely abolished the inhibitory effect of FAT10 on auto-ubiquitylation (Figure 5F, lane 11), suggesting that FAT10 directly impairs Parkin activation and that this effect requires FAT10 conjugation.

FAT10 delays Parkin translocation to mitochondria and inhibits mitophagy progression

To investigate the biological consequences of FAT10-dependent inhibition of Parkin activity, we established a cellular expression system based on the T-Rex-293 cell line in which EGFP-Parkin is stably expressed and FLAG-FAT10 expression can be induced by tetracycline (tet) (Figure 6A). In addition, this transfectant also stably expresses mtKeima, a fluorescent protein that is localized to mitochondria and that is widely used to quantify mitophagy (Figures 6B and S6A) (Sun et al., 2017). We first tested whether FAT10 expression has an effect on Parkin translocation to mitochondria on exertion of mitochondrial stress. Several studies have demonstrated that the integrity of the RING2 domain of Parkin is essential for Parkin ligase activity, and the loss of function mutation of the C431 active site cysteine abolishes the translocation of Parkin to mitochondria (Trempe et al., 2013; Zheng and Hunter, 2013). These studies have reported that EGFP-Parkin requires 1 h to localize to the surface of mitochondria on CCCP treatment, and the E3 ligase activity of Parkin is required for the translocation. Hence, we first ensured that the treatment of HeK293 cells stably expressing EGFP-Parkin with low concentrations of tet was not altering the translocation of the E3 ligase to the mitochondria (data not shown). Subsequently, we used the same time-lapse live cell imaging-based approach to evaluate the effect of overexpressed FLAG-FAT10 on Parkin translocation. Although we could not identify any significant difference after 1 h of CCCP treatment (Figure 6B), we noticed that the kinetics of Parkin translocation was slowed down in the presence of FAT10 (Figures 6C and 6D). To further validate the inhibitory effect of FAT10 on the progression of Parkin-dependent mitophagy, we tested the consequence of tet-induced FAT10 overexpression on mitochondrial turnover by evaluating the shift of mtKeima fluorescence excitation when the mitochondria are acidified in the autophagolysosomal compartment subsequent to mitophagy (Katayama et al., 2011). We could detect a progressive increase of mitochondrial acidification over 12 h post CCCP treatment by exciting mtKeima at a wavelength of 561 nm (Figure 6E). Next, we treated the cells with CCCP for 6 h and assessed the ratio of acidified mtKeima in single cells by flow cytometry as a quantitative marker for mitophagy progression (Katayama et al., 2011). When cells were pre-treated for 24 h with tet to induce FAT10 expression, we detected a significant decrease in acidified mtKeima-positive cells on 6 h of CCCP treatment (Figures 6F and 6G). To validate this data under endogenous conditions, we established two stable cell lines based on SH-SYSY WT and SH-SYSY FAT10 KO cells both stably expressing mtKeima. In this context, we could investigate the effect of TNF-α and IFNγ-induced endogenous FAT10 expression on mitophagy-dependent mitochondrial turnover. Although a previous report has shown that TNF-α is responsible for the induction of mitophagy in macrophages (Bell et al., 2013), we could observe the opposite effect in our neuronal cell line model. In fact, when SH-SYSY cells were pre-stimulated for 24 h with a combination of TNF-α and IFNγ, we found a significant reduction in the total number of acidified mitochondria after 18 h of CCCP treatment (Figures 6H and 6I, bars 3 and 4). However, pre-stimulations with TNF-α and IFNγ did not lead to a significant decrease in acidified mitochondria after 18 h of CCCP treatment in FAT10-deficient cells (Figures 6H and 6I, bars 7 and 8). These data demonstrate that under inflammatory conditions, FAT10 plays a role in inhibiting depolarization-induced mitophagy. Moreover, these results are in line with our previous experiments showing that FAT10 impedes the CCCP-induced activation of Parkin.
FAT10 promotes neuronal cell death in vitro

A pertinent question raised by our data so far is whether the inhibitory effect of FAT10 on mitophagy in neuronal cells will affect their susceptibility to agents known to cause mitochondrial depolarization and the induction of oxygen radicals. Accordingly, we investigated whether FAT10 influences the viability of SH-SY5Y cells (Xicoy et al., 2017). We treated SH-SY5Y cells for 32 h with 5 μM rotenone that is an inhibitor of mitochondrial complex I and impairs the electron transport chain, with consequent over-production of ROS (Li et al., 2003; Newhouse et al., 2004). Subsequently, we analyzed cell viability to investigate how the expression of Parkin and FAT10 influences the rotenone-induced cell death. First, we established by lentiviral transduction two SH-SY5Y cell lines that stably express FLAG-FAT10 WT or FLAG-FAT10-AV, followed by evaluation of cell viability after 32 h of rotenone treatment. Remarkably, cells overexpressing WT FLAG-FAT10 underwent a significant increase of cell death (Figure 7A, lanes 4 and 5). This toxic effect was not apparent when SH-SY5Y cells expressed the FLAG-FAT10-AV variant, implying that the toxic role of FAT10 is correlated to the FAT10-mediated inhibition of Parkin ubiquitin-ligase activity, which requires FAT10 conjugation (Figure 7A, lanes 5 and 6). Several works have described Parkin as an enzyme with cytoprotective effect in the neuronal cell line SH-SY5Y (Casarejos et al., 2006; Dai et al., 2015; Müller-Rischart et al., 2013). Other investigations describe autophagy as a key cellular process counteracting rotenone-induced production of ROS and cell death in the SH-SY5Y model (Deng et al., 2013; Zhang et al., 2019), with the degradation of mitochondria by PINK1/Parkin-dependent mitophagy exerting an essential pro-survival activity (Pan et al., 2009; Peng et al., 2019). In the light of these studies, we investigated whether Parkin overexpression has a cytoprotective effect in our model and whether FAT10 is involved in the modulation of Parkin function. To achieve that, we established a SH-SY5Y cell line which stably expresses mCherry-tagged Parkin and one which express both mCherry-Parkin and FLAG-FAT10. We found that the overexpression of Parkin leads to reduced cell death after rotenone treatment (Figure 7B, lines 2 and 4), confirming the cytoprotective role of the Parkin E3 ligase. Strikingly, the co-expression of FAT10 in Parkin-expressing cells significantly counteracted its pro-survival effect (Figure 7B, lines 4 and 8). To assess the effect of endogenous FAT10 expression on the susceptibility of SH-SY5Y cells to rotenone treatment, we compared SH-SY5Y WT cells to SH-SY5Y FAT10 KO cells (Figure S2B). The cells were pretreated with TNF-α/IFN-γ for 18 h to induce FAT10, followed by a 32-h treatment with rotenone, TNF-α/IFN-γ, or the combination of both stimuli. Then, we analyzed cell viability and mitochondrial ROS production using mitoSOX red (i.e., a fluorescent probe detecting superoxide radicals). The combination of rotenone and pro-inflammatory cytokines lead to a significantly stronger reduction in the viability of SH-SY5Y WT cells as compared to treated with rotenone alone (Figure 7C, lanes 3 and 4). Interestingly, the lack of FAT10 in the gene targeted SH-SY5Y cells (Figures S2B and S5A) abolished the TNF-α/IFN-γ-mediated negative effect on cell viability (Figure 7C, lanes 7 and 8). Thus, endogenously expressed FAT10 was essential for this cytokine-dependent effect in accordance with FAT10-mediated mitophagy inhibition described in Figures 6H and 6I. Using mitoSOX red as a detection system for mitochondrial ROS we confirmed the expected rotenone-mediated enhancement of ROS in mitochondria but this was only slightly but not significantly further enhanced by the cytokine treatment (Figure 7D, left side) and the latter tendency was not observed in FAT10-deficient cells (Figure 7D, right side). To confirm that the observed effect on cell viability is due to the lack of FAT10 expression, we reconstituted FLAG-FAT10 by stable expression in SH-SY5Y FAT10 KO cells (Figure 7C, lanes 9–12). Remarkably, the ectopic expression of FAT10 led to a significant increase in cell death, in line with Figures 7A and 7B. Taken together, we conclude that the negative effect of FAT10 on the viability of rotenone-treated SH-SY5Y cells is likely due to its inhibitory effect on Parkin-mediated mitophagy (Figure 7E).

DISCUSSION

Previous studies described several post-translational modifications to modulate Parkin activity, including SUMOylation and NEDDylation (Um and Chung, 2006). Here, we show that Parkin is a substrate for FAT10 on stimulation with TNF-α and IFN-γ (Figure 1). The Parkin-FAT10 conjugate is formed in the cytosol and is rapidly degraded by the proteasome as reported for other FAT10 substrates (Aichem et al., 2012; Bialas et al., 2019; Hipp...
A

EGFP-Parkin
Tet

IB: FLAG (FAT10)
FAT10 conjugates
FLAG-FAT10
IB: Parkin
IB: GAPDH

B

CCCP treatment (min)

| 0  | 60 |
|-----|-----|
| 20  | 60  |
| 30  | 60  |
| 40  | 60  |

EGFP-Parkin

mtKeima
405 nm excitation

- Tet
+ Tet

C

CCCP treatment (min)

| 20 | 25 | 30 | 35 | 40 |
|-----|-----|-----|-----|-----|
| EGFP-Parkin | EGFP-Parkin + Tet |

D

% cells with GFP-Parkin on mitochondria

0 20 40 60 80 100

t (min)

0 10 20 30 40 50 60

E

mtKeima pH7 (405 nm) mtKeima pH4 (561 nm)

Untreated
12h CCCP

F

EGFP-Parkin WT

CCCP

- Tet
+ Tet

G

% cells with high ratio acidified mtKeima (6h)

- Tet
+ Tet

H

SH-SYS WT

CCCP

mtKeima pH 4 (561 nm)

SH-SYS FAT10 KO

CCCP

mtKeima pH 4 (561 nm)

24h TNFα/IFNγ

I

% cells with high ratio acidified mtKeima (18h)

- Tet
+ Tet

FAT10 KO

WT

(legend on next page)
et al., 2005). Furthermore, we found that a fraction of FAT10ylated Parkin is present on depolarized mitochondria (Figure 2D). In the light of the extreme versatility of Parkin as an E3 enzyme (Chew et al., 2011; Cunningham et al., 2015; Durcan et al., 2014), we investigated whether it could act as a FAT10 E3 ligase. As criteria to identify Parkin as a ligase, we investigated its auto-FAT10ylation and its capacity to FAT10ylate a substrate protein (Huibregtse et al., 1995; Lee et al., 2017). Strikingly, we found that Parkin contributes to its own in vitro FAT10ylation and that it FAT10ylates Mfn2 in SH-SY5Y cells (Figures 3 and 4). Interestingly, phosphorylated Parkin could auto-FAT10ylate itself more efficiently than the WT protein, suggesting that the regulation of Parkin activity by FAT10 could take place at two distinct levels: in the cytosol and at mitochondria. In the first case, we observed that Parkin contributes to its own FAT10ylation while being in its “inactive” (cytosolic) form (Figures 3C and 3D). Here, a pertinent question would be how Parkin is activated in the cytosol. In fact, several works showed that cytosolic Parkin is in a repressed state and possesses no capacity to interact with cognate E2s through the RING1 domain, while also the catalytic RING2 domain is hindered and cannot perform the ubiquitin transmigration reaction (Trempe et al., 2013; Wauer and Komander, 2013). This notion led to the assumption that Parkin can act as a ligase exclusively at mitochondria upon their depolarization and PINK1 activation. However, these studies have been performed in the absence of TNF/IFN stimulation that is required for FAT10 expression. Moreover, several reports suggest that numerous Parkin substrates are not mitochondrial and do not require mitochondrial depolarization in order to become ubiquitylated by Parkin. For instance, Parkin exhibits ubiquitin-ligase activity toward FBP1 and Hsp70 in SH-SY5Y cells (Ko et al., 2006; Moore et al., 2008) and toward RanBP2 and RIPK1 in Hek293 cells (Um et al., 2006; Wang et al., 2018) that adds to the evidence of mitophagy-unrelated functions of Parkin (Johnson et al., 2012; Manzanillo et al., 2013). Apparently, other so far unknown factors could transiently activate Parkin in the cytosol. For instance, Figure 1E shows that FAT10 actively interacts with the Ubl domain of Parkin, a region that is strongly involved in the regulation of Parkin activation. In fact, several works have shown that Parkin activity is negatively regulated by its UBL domain, and the perturbation at this level influences Parkin function, leading to the activation of the E3 ligase by the disruption of its auto-inhibited conformation (Burchell et al., 2012; Chaugule et al., 2011). Accordingly, we speculate that the strong interaction between FAT10 and the UBL domain of Parkin could be involved in the perturbation of the structure of the N terminus of Parkin leading to a transient activation of cytosolic Parkin that can explain how Parkin is able to FAT10ylate itself independently of its phosphorylation status (Figures 3C and 3D). At mitochondria, activated Parkin possesses a high affinity for FAT10 (Figure 3E) and it accordingly auto-FAT10ylates itself and the mitochondrial substrate Mfn2 (Figures 2D and 4). Overall, we propose that Parkin contributes to its FAT10ylation at both cytosolic and mitochondrial level. At the cytosolic level, this modification sequesters a fraction of Parkin in the cytosol where it is degraded by the proteasome. At the mitochondrial level, activated Parkin FAT10ylates itself and mitofusin2. Simultaneously, Parkin FAT10ylation leads to a reduction in the ubiquitylation of Parkin itself and in the ubiquitylation of Mfn2 (Figure 5), suggesting that there exists a negative-regulatory mechanism by which the FAT10-ligase activity of Parkin directly alters its ubiquitin-ligase activity. Interestingly, this effect is dependent on FAT10 conjugation, and the FAT10 activating enzyme UBA6 is essential for the FAT10-dependent hindrance of Parkin activity (Figure 5), suggesting that the pharmacological inhibition of UBA6 could rescue the biological negative consequences that FAT10 has on Parkin activation. Remarkably, we found that UBA6 alone was not sufficient to FAT10ylate Mfn2 in vitro, whereas the combination of UBA6 and Parkin can exert this function, in line with previous work describing Parkin as a unique ligase that is able to catalyze in vitro mono-ubiquitylation of substrate proteins independently of E2 conjugating enzymes (Chew et al., 2011). Furthermore, we...
found that the combination of TNF-α and IFN-γ leads to FAT10 expression impairing the progression of mitophagy (Figure 6) and it aggravates the rotenone-induced ROS production, with consequent augmentation of cell death (Figure 7). This effect is counteracted by silencing the FAT10 gene, confirming that FAT10 plays an anti-survival role in this experimental setting by directly counteracting Parkin ubiquitin-ligase activity. Moreover, although our study focused on the effect of FAT10 on Parkin-dependent mitophagy, we do not exclude that FAT10 might interfere with the other pleiotropic functions of Parkin, such as the modulation of TNF-α signaling or its role as a putative tumor suppressor (Henn et al., 2007; Liu et al., 2018).

In conclusion, we report that during inflammation, FAT10 expression is induced and Parkin FAT10ylates itself and Mfn2 (see schematic in Figure 7E). Thereby, Parkin is inhibited and has a reduced capacity to trigger mitophagy. This inflammation-induced reduction of Parkin activity causes a decrease in its cytoprotective effect and, accordingly, leads to neuronal death. Hence, we identify FAT10 and its activation and conjunction cascade as likely exacerbating factors in the pathogenesis of PD that are druggable (e.g., by UBA6 inhibitors) (Hyer et al., 2018) and hold further promise as potential drug targets.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.108857.

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AUTHOR CONTRIBUTIONS

N.D.R. performed all experiments except the experiments in Figures 1E and 3E (performed by C.S and F.S). N.D.R. evaluated data and wrote the manuscript.

Figure 7. FAT10 sensitizes neuronal cells for death caused by mitochondrial ROS

(A) SH-SY5Y WT cells, SH-SY5Y FLAG-FAT10 cells, and SH-SY5Y FLAG-FAT10 AV cells were cultured in low glucose medium (1 g/L) and treated with DMSO or, where indicated, with 5 μM rotenone. After 32 h, cells were harvested and stained with SYTOX Blue dead cell dye to assess cell viability by fluorescence-activated cell sorting (FACS).

(B) SH-SY5Y WT cells, SH-SY5Y FLAG-FAT10 cells, SH-SY5Y mCherry-Parkin cells, and SH-SY5Y mCherry-Parkin FLAG-FAT10 cells were treated as in (A) and the cell viability has been quantified by FACS using the SYTOX Blue dead cell dye.

(C) SH-SY5Y WT cells, SH-SY5Y FAT10 KO, and SH-SY5Y FAT10 KO FLAG-FAT10 cells were pre-treated, where indicated, with TNF-α/IFN-γ, followed by the replacement with fresh low glucose medium (1 g/L) containing DMSO or, where indicated, 5 μM rotenone and/or TNF-α/IFN-γ as specified. After 32 h, cells where harvested, stained with SYTOX Blue dead cell dye as described in (A).

(D) SH-SY5Y WT cells and SH-SY5Y FAT10 KO were treated as in (C) and the intracellular ROS levels have been detected by flow cytometry after incubation of the cells with the MitoSOX dye for 10 min at room temperature.

(E) Schematic representation of the effect of FAT10 on mitochondrial and neuron survival. (1) FAT10 is expressed on TNF-α/IFN-γ stimulation, is activated by UBA6, and conjugated by USE1. (2) Parkin interacts with USE1, auto-FAT10ylates itself and is degraded by the 26S proteasome. (3) Parkin’s ability to translocate to depolarized mitochondria is reduced by the expression of FAT10. (4) At mitochondria, active Parkin gains FAT10-ligase activity and auto-FAT10ylates itself and the mitochondrial substrate Mfn2. Simultaneously, the ubiquitin-ligase activity of Parkin is impaired by FAT10 leading to reduced ubiquitylation of the mitochondrial substrate Mfn2. (5) FAT10 hinders the overall progression of mitophagy and autolysosomal degradation of damaged mitochondria and (6) contributes to accelerated neuronal cell death.

Error bars in (A)–(D) indicate SD (n = 3). *p < 0.05 (Student’s t test), n.s., not significant.
A.A. and N.C. generated and purified recombinant proteins and corrected the manuscript. M.G. conceived experiments, supervised the project, acquired resources, and refined the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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### Reagent or Resource Table

| Reagent or Resource                        | Source                        | Identifier                  |
|--------------------------------------------|-------------------------------|-----------------------------|
| Antibodies                                 |                               |                             |
| mouse monoclonal anti-FAT10 (4F1)          | Aichem et al., 2010           | BML-PW0765-0025; RRID:AB_11000253 |
| rabbit polyclonal anti-FAT10 (1069)        | This paper                    | N/A                         |
| mouse monoclonal anti-c-Myc antibody (9E10)| Invitrogen                    | Cat# 13-2500; RRID:AB_86583  |
| Rabbit polyclonal anti-c-Myc antibody      | Merck                         | Cat# C3956; RRID:AB_439680   |
| Mouse monoclonal anti-FLAG antibody (clone M2) | Merck                     | Cat# F1804; RRID:AB_262044   |
| Mouse monoclonal anti-HA antibody (clone HA-7) | Merck                      | Cat# H3663; RRID:AB_262051   |
| Mouse monoclonal anti-His antibody         | QIAGEN                        | Cat# 34660; RRID:AB_2619735  |
| Rabbit polyclonal anti-UBA6 antibody       | Aichem et al., 2010           | Cat# BML-PW0525-0025; RRID:AB_2052778 |
| Mouse monoclonal anti-Parkin antibody (PRK8)| Santa Cruz                   | Cat# sc-32282; RRID:AB_628104|
| Rabbit monoclonal anti-Parkin antibody     | Invitrogen                    | Cat# 21H24L9; RRID:AB_2724937|
| Rabbit monoclonal antiVDAC antibody (D73D12)| Cell signaling               | Cat# 4661; RRID:AB_10557420  |
| Mouse monoclonal anti β-actin antibody (AC-7)| Merck                     | Cat# A5316; RRID:AB_476743   |
| Mouse monoclonal anti-TOM20 antibody (F-10)| Santa Cruz                   | Cat# sc-17764; RRID:AB_628381|
| Mouse monoclonal anti-Ubiquitin antibody (FK2)| Enzo Life Sciences          | Cat# BML-PW8810-0100; RRID:AB_10541840 |
| Rabbit monoclonal anti-Mfn2 antibody (D2D10)| Cell Signaling              | Cat# 94825; RRID:AB_2716838  |
| Rabbit policlonal anti-GAPDH antibody      | Merck                         | Cat# G9545; RRID:AB_796208   |
| Rabbit monoclonal anti-γ-Tubulin antibody (GTU-88)| Merck          | Cat# T5326; RRID:AB_532292   |
| Rabbit polyclonal anti-Use1 antibody       | Enzo Life Science            | Cat# PW0770-0025; RRID:AB_10997992 |
| IRDye 800CW Goat anti-Rabbit IgG (H+L)     | LI-COR Biosciences           | Cat# 925-32211; RRID:AB_2651127|
| IRDye 680RD Goat anti-Mouse IgG (H + L)    | LI-COR Biosciences           | Cat# 925-68070; RRID:AB_2651128|
| Alexa Fluor 488 Goat anti-Rabbit IgG (H+L) | Thermo Fisher Scientific     | Cat# A-11008; RRID:AB_143165 |
| Alexa Fluor 633 Goat anti-Mouse IgG (H+L)  | Thermo Fisher Scientific     | Cat# A-21052; RRID:AB_2535719|
| Mouse Monoclonal ANTI-FLAG antibody (HRP)  | Merck                         | Cat# A6592; RRID:AB_439702   |
| Rabbit polyclonal anti-PINK1 antibody      | Thermo Fisher Scientific     | Cat# PA5-85930; RRID:AB_2802731|
| Chemicals, peptides, and recombinant proteins |                               |                             |
| Bafilomycin A1                              | Millipore                     | Cat# 19-148                  |
| MG132                                       | Enzo Life Sciences            | Cat# BML-P102                |
| Cycloheximide                               | Sigma                         | Cat# 01810                   |
| Protease inhibitor cocktail                 | Roche                         | Cat# 11697498001             |
| PhosSTOP (phosphatase inhibitor cocktail)  | Roche                         | Cat# 4906845001              |
| Tetracycline hydrochloride                  | Merck                         | Cat# T7660                   |
| Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) | Merck             | Cat# C2759                   |
| Rotenone                                    | Merck                         | Cat# R8875                   |
| SYTOX Blue                                  | Invitrogen                    | Cat# S34857                  |
| MitoSOX Red                                 | Invitrogen                    | Cat# M36008                  |
| DNAase I                                    | Thermo Fisher                 | Cat# EN0521                  |
| Recombinant human TNFz                      | PeproTech                     | Cat# 300-01A                 |
| Recombinant human IFNγ                      | PeproTech                     | Cat# 300-02                  |
| Puromycin dihydrochloride                   | Merck                         | Cat# P8833                   |
| Recombinant FAT10                           | Aichem et al., 2014           | N/A                         |
| Recombinant FAT10-AV                        | Aichem et al., 2019a          | N/A                         |
| Recombinant His-USE1                        | Aichem et al., 2010           | N/A                         |
| Recombinant HA-UBE1                         | Bialas et al., 2015           | N/A                         |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Recombinant MDYKDDDDK-tagged UBA6 | Enzo Life Sciences | Cat#BML-UW0350-0050 |
| Recombinant 6His-Ubiquitin | Enzo Life Sciences | Cat#BML-UW8610-0001 |
| Recombinant human Parkin | R&D systems | Cat#E3-160 |
| Recombinant human Parkin pS65 | R&D systems | Cat#E3-166 |
| Recombinant Human His6-UBE2G1 (His-Ubc7) | R&D systems | Cat# E2-700 |
| Recombinant human GST-Parkin | Wauer and Komander, 2013 | N/A |
| Recombinant human GST-Parkin C431A | This paper | N/A |
| Recombinant T.Castaneum 6His-MBP-PINK1 | R&D systems | Cat#AP-180-100 |
| DAPI | Abcam | Cat# ab228549 |
| HiPerFect Transfection Reagent | QIAGEN | Cat#301704 |
| TransIT-L1 Transfection Reagent | Mirus | Cat#2300 |
| MitoTracker Red CMXRos | Invitrogen | Cat#M7512 |

**Critical commercial assays**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| QuikChange II XL Site-Directed Mutagenesis Kit | Agilent | Cat#200521 |
| Pierce ECL Western Blotting Substrate | Thermo Fisher | Cat#32106 |
| ANTI-FLAG Affinity Gel | Merck | Cat#F2426 |
| Anti-HA Affinity Gel | Merck | Cat#E6779 |
| Anti-c-Myc Affinity Gel | Merck | Cat#E6654 |
| Protein A Affinity Gel | Merck | Cat#P6486 |
| GFP-Trap Agarose | Chromotek | Cat#GTA-10 |
| Phos-tag Acrylamide | Fujifilm | Cat# AAL-107M |
| Amylose resin | NEB | Cat#E8021S |
| BS3-H12/D12 (BisSulfoSuccinimidylSuberate) | Creative Molecules Inc. | Cat# 001SS |
| N,N-Dimethylformamide (DMF) | Sigma | Cat# D4551 |
| Ammonium bicarbonate | Sigma | Cat# 09830 |
| Urea | Sigma | Cat# U5378 |
| Trypsin (Sequencing Grade Modified, Frozen) | Promega | Cat# V5113 |
| DTT (1,4-Dithiothreitol) | Carl Roth | Cat# 6908.4 |
| TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) | Sigma | Cat# C4706-2G (CAS 51805-45-9) |
| Iodoacetamide | Sigma | Cat# l1149-5G (CAS 144-48-9) |
| Sep-Pak (C18) | Waters | Cat# WAT054960 |

**Deposited data**

The MS raw files, the crosslink database and original xQuest result files/ xTract input files as well as xTract result files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository.

| PRIDE | PXD022109 |

**Experimental models: cell lines**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Human: Hek293T | ATCC® | Cat# CRL-3216 |
| Human: Hek293 | ATCC® | Cat#CRL-1573 |
| Human Hek293 CRISPR/Cas9 FAT10 KO | Aichem et al., 2019a | N/A |
| Human Hek293 CRISPR/Cas9 UBA6 KO | Aichem et al., 2019b | N/A |
| Human Hek293 CRISPR/Cas9 USE1-ko cells | Aichem et al., 2018 | N/A |
| Human Hek293 EGFP-Parkin | This paper | N/A |
| Human T-REx-293 3XFLAG-FAT10 EGFP-Parkin mtKeima cells | This paper | N/A |
| Human SH-SY5Y cells | contributed by M. Leist, Univ. Konstanz | N/A |
| Human SH-SY5Y CRISPR/Cas9FAT10 KO | This paper | N/A |

(Continued on next page)
## REAGENT or RESOURCE SOURCE IDENTIFIER

| Human SH-SY5Y cells 3x FLAG-FAT10 | This paper | N/A |
| Human SH-SY5Y cells 3x FLAG-FAT10-AV | This paper | N/A |
| Human SH-SY5Y cells mCherry-Parkin | This paper | N/A |
| Human SH-SY5Y cells 3x FLAG-FAT10 mCherry-Parkin | This paper | N/A |
| Human SH-SY5Y CRISPR/Cas9FAT10 KO + FLAG-FAT10 | This paper | N/A |
| RetroPack PT67 Cell Line | MSC LTD | Cat#PT67 |

### Oligonucleotides

| Myc-Parkin C431A site directed mutagenesis primer F | This paper | 5'-cgacacgtcatgtgcat gccct tt ct cact-3' |
| Myc-Parkin C431A site directed mutagenesis primer R | This paper | 5’-agttggaaatagcctcgcgtcgcaggt gtcg-3 |
| GST-Parkin C431A site directed mutagenesis primer F | This paper | 5'-gactttcgtgcatgcccttt ct cact-3' |
| GST-Parkin C431A site directed mutagenesis primer R | This paper | 5’-gccctttcgtgcatgcccttt ct cact-3' |
| siRNA Parkin (1) | QIAGEN | Cat#HS PARK2 1 SI00677831 |
| siRNA Parkin (2) | QIAGEN | Cat#HS PARK2 2 SI00677838 |
| siRNA Parkin (3) | QIAGEN | Cat#HS PARK2 3 SI00677845 |
| siRNA Parkin (4) | QIAGEN | Cat#HS PARK2 4 SI01369620 |
| Control siRNA | QIAGEN | Cat#1027280 |

### Plasmids

| Plasmid:: pcDNA-His-3xFLAG-FAT10 (FLAG-FAT10) | Chiu et al., 2007 | N/A |
| Plasmid: pcDNA3.1-His-3xFLAG-FAT10- AV | Aichem et al., 2010; Chiu et al., 2007 | N/A |
| Plasmid: pcDNA4/TO-3xFLAG-FAT10 | This paper | N/A |
| Plasmid: pRK5-Myc-Parkin | Ted Dawson lab | Addgene plasmid # 17612 |
| Plasmid: pRK5-Myc-Parkin C431A | This paper | N/A |
| Plasmid: pEFP-parkin WT (EGFP-Parkin) | Edward Fon lab | Addgene plasmid # 45875 |
| Plasmid: pcDNA3.1/Myc-His-Mfn2 (Myc-Mfn2) | David Chan lab | Addgene plasmid # 23213 |
| Plasmid: pCHAC-mt-mKeima (mtKeima) | Richard Youle lab | Addgene plasmid # 72342 |
| Plasmid: pcDNA3.1-His/-A | Invitrogen | Cat#V38520 |
| Plasmid: pCMV6/Myc-Mul1 (Myc-Mul1) | Origene | Cat#RC204038 |
| Plasmid: pEFP-N2/MARCH5 (GF_P MARCH5) | Eric Schirmer lab | Addgene plasmid # 62039 |
| Plasmid: pET28a/6His-GST-3C-Parkin (GST-Parkin) | David Komander lab | Addgene plasmid # 110758 |
| Plasmid: pET28a/6His-GST-3C-Parkin C431A | This paper | N/A |
| Plasmid: pCDH/6His-3X FLAG-FAT10 | This paper | N/A |
| Plasmid: pCDH/6His-3X FLAG-FAT10-AV | This paper | N/A |
| Plasmid: pBMN-mCherry-Parkin | Richard Youle lab | Addgene plasmid # 59419 |
| Plasmid: pM2.G | Didier Trono lab | Addgene plasmids #12259 |
| Plasmid: psPAX2 | Didier Trono lab | Addgene plasmids #12260 |

### Software and algorithms

| ImageJ | Tree Star | https://imagej.nih.gov/ij/ RRID: SCR_003070 |
| GraphPad Prism | GraphPad software | https://www.graphpad.com RRID:SCR_002798 |
| FlowJo | FlowJo LLC | https://www.flowjo.com/ RRID: SCR_008520 |
| ImageStudioLite | LICOR Odyssey | https://www.licor.com/bio/image-studio-lite/ RRID: SCR_013715 |
| FACSDiva Software | BD Biosciences | https://www.bdbiosciences.com/en-us/instruments/research-instruments/research-software/flow-cytometry-acquisition/facsdiva-software |
| xQuest 2.1.3 | Leitner et al., 2014 | https://gitlab.ethz.ch/leitner_lab/xquest_xprophet |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marcus Groettrup (Marcus.Groettrup@uni-konstanz.de)

Materials availability
Cell lines and plasmids generated for this study are available from the lead contact.

Data and code availability
The MS raw files, the crosslink database and original xQuest result files/ xTract input files as well as xTract result files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (PXD022109).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture and cell lines
Hek 293T, Hek293, Hek293 CRISPR/Cas9 FAT10KO cells (Hek293 FAT10 KO), Hek293 CRISPR/Cas9 UBA6-ko cells, Hek293 CRISPR/Cas9 USE1-ko cells, Hek293 EGFP-Parkin cells, T-REx-293 3XFLAG-FAT10 EGFP-Parkin mtKeima cells, SH-SY5Y, SH-SY5Y CRISPR/Cas9FAT10 KO, SH-SY5Y cells 3x FLAG-FAT10, SH-SY5Y cells 3x FLAG-FAT10-AV, SH-SY5Y cells 3x FLAG-FAT10 mCherry-Parkin, SH-SY5Y cells mCherry-Parkin, SH-SY5Y CRISPR/Cas9FAT10 KO FLAG-FAT10 and RetroPack PT67 cells were cultivated in Dulbecco’s Modified Eagle Medium (DMEM) (Thermo Fisher) supplemented with 10% fetal calf serum (GIBCO/Thermo Fisher Scientific), 1% stable glutamine (100x, 200 mM), and 1% penicillin/streptomycin (100x) (both from Biowest/VWR). The sex of the cells has not been determined for not specified reasons.

Generation of a stable cell lines
The Hek293 EGFP-Parkin cell line was generated by transfection of Hek293 cells with a construct for EGFP-Parkin expression. One day after transfection, GFP-high cells were bulk sorted and cultivated as described above. The sorted cells were subjected to 5 expansion/GFP-bulk sorting cycles to select the cells stably expressing GFP-Parkin. A polyclonal cell population expressing EGFP-Parkin was hereby obtained. The Hek T-REx-293 3XFLAG-FAT10 EGFP-Parkin mtKeima cells were obtained as a polyclonal cell population stably expressing GFP-Parkin using the same procedure as for the Hek293 EGFP-Parkin cell line. For tet FLAG-FAT10 expression these cells were transiently transfected with the tetracycline (tet) inducible pcDNA4/TO-3xFLAG-FAT10 construct for 24 h, followed by single cell sorting and selection with puromycin (5 μg/μL). The clones with the best 3X-FLAG-FAT10 expression in presence of tet were selected and expanded. All the FLAG-FAT10-stably expressing cell lines that have been used in this work have been obtained by lentiviral transduction as previously described (Schregle et al., 2020). In brief, the lentiviral expression plasmid vector, the envelope plasmid vector pMD2.G, and the packaging plasmid vector psPAX2 were transfected into Hek293T cells using Mirus transfection reagent (See “Transfection of Plasmids” section). After 12 h, the culture medium was exchanged. The cell culture

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| xProphet 2.1.3      | Leitner et al., 2014 | [https://gitlab.ethz.ch/leitner_lab/xquest_xprophet](https://gitlab.ethz.ch/leitner_lab/xquest_xprophet) |
| xTract 1.0.2        | Walzthoeni et al., 2015 | [https://gitlab.ethz.ch/leitner_lab/xtract/-/wikis/](https://gitlab.ethz.ch/leitner_lab/xtract/-/wikis/) |
| xiNET               | Combe et al., 2015 | [http://crosslinkviewer.org/](http://crosslinkviewer.org/) |

Other

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| LSRFortessa flow cytometer | BD Bioscience | N/A |
| CellObserver HS spinning Disk microscope | Zeiss | N/A |
| AxioCamMRm camera | Zeiss | N/A |
| LSM700 confocal microscope | Zeiss | N/A |
| BD FACSAriaTMilu | BD Biosciences | N/A |
| Superdex™ Peptide 3.2/30 (SEC column for enrichment of crosslinked peptides) | GE Healthcare | Cat# 29-0362-31 |
| Acclaim PepMap™ RSLC (LC-MS/MS analysis) | Thermo Fisher Scientific | Cat# P/N 164943 |
| EASY-nLC 1200 system | Thermo Fisher Scientific | LC140 |
| Orbitrap Fusion™ Trivid™ Mass Spectrometer | Thermo Fisher Scientific | N/A |
supernatant containing the lentiviral particles was collected 48 h and 72 h after transfection and the remaining DNA in the supernatant was removed by digestion with DNase I and the lentivirus-containing medium was sterile-filtered. The target cells were transduced at a multiplicity of infection of 50 for 3 days prior to puromycin selection. All the mCherry expressing cell lines that have been used in this work have been obtained by retroviral transduction. The retroviral particles carrying the mCherry-Parkin genetic insert have been produced using the RetroPack PT67 cell line. In brief, the retroviral expression plasmid vector was transfected into Retropack PT67 cells using Mirus transfection reagent. After 12 h, the culture medium was exchanged. The cell culture supernatant containing the retroviral particles was collected 48 h and 72 h after transfection and the remaining DNA in the supernatant was removed by digestion with DNase I and the retrovirus-containing medium was sterile-filtered and added to the target. The target cells were transduced at a multiplicity of infection of 50 for 3 days prior to selection for high expression of mCherry by FACS sorting.

**Generation of CRISPR/Cas9 knockout mutants**

The SH-SYSY CRISPR/Cas9 FAT10 KO cell line was generated by transfection of SH-SYSY cells with pCMV-Cas9-GFP containing FAT10-specific gRNA (designed at https://www.sigmaaldrich.com/catalog/product/SIGMA/CRISPR?lang=en&region=US). 24 h after transfection, single GFP high cells were sorted using BD FACSAriaTMi lu (BD Biosciences) and cultivated as described above. Clones with successful FAT10 knockout were identified by western blot analysis, using a FAT10-specific polyclonal antibody (See Key Resources Table).

**Induction of endogenous FAT10**

Induction of endogenous FAT10 expression was performed using the above described medium with the addition of 300 U/mL human IFN-γ and 600 U/mL human TNF-α. The induction has been performed for 24 hours to achieve the highest level of endogenous FAT10 expression.

**METHOD DETAILS**

**Expression and purification of 6HIS-GST-Parkin / 6his-HIS-Parkin-C431A**

The expression construct pET28a-Parkin (resp. pET28a-Parkin-C431A) was transformed into competent E.coli BL21(DE3) by heat shock. Cells were grown at 37°C, 220 rpm until an OD600 of 0.6 was reached. The cells were then further incubated at 21°C for 30 min to allow the culture to reach the set temperature. Recombinant protein expression was induced by adding IPTG to a final concentration of 0.4 mM. Cells were further incubated overnight at 21°C, 220 rpm. After induction, the cells were harvested by centrifugation (10 min at 4000 x g, RT). The supernatant (SN) was discharged and the cell pellets were stored at −20°C until further processed. Cell pellets were lysed in 50 mL 25 mM Tris-HCl, pH7.5, 200 mM NaCl, 20 mM Imidazole, 1 mM TCEP (Binding Buffer IMAC). The homogenized cell pellets were then lysed twice in a Cell Disruptor TS (Constant System) at 1.5 kbar. After the second lysis step, PMSF was added to a final concentration of 1 mM. The lysate was then centrifuged for 30 min at 40 000 x g, 8°C (Sorvall, Thermo-Scientific). Subsequently, the SN was filtered through 2 μm, 1.2 μm, and 0.45 μm filters to remove aggregates. The SN was loaded onto a HisTrap FF 5 mL (Cytiva) column. Unspecifically bound proteins were washed out with Binding Buffer IMAC until the baseline was reached. A second wash step including 5% Elution Buffer IMAC (25 mM Tris-HCL, pH7.5, 200 mM NaCl, 500 mM Imidazole, 1 mM TCEP) was performed for 5 column volumes (CV). Recombinant fusion protein was eluted with 5 CV 50% Elution Buffer IMAC, fractionated in 1 mL fractions. Peak containing fractions were united and concentrated to a final volume of max. 5 mL by using VivaSpin columns, MWCO = 30 kDa (Sartorius). The concentrated sample was loaded onto a HiLoad 16/60 Superde 75 pg column (Cytiva) which was equilibrated in 25 mM Tris-HCl, pH 8.5, 200 mM NaCl, 1 mM TCEP. Resulting peaks were fractionated in 1 mL fractions with a flow of 1 mL/min. Protein containing peaks were united. Under these circumstances the recombinant protein containing peak can be observed at a retention volume of approx. 51.5 ml. Glycerol was added to the sample to a final concentration of 10%. Furthermore, the sample was aliquoted into 100 μL/500 μL portions, snap-frozen in liquid N₂ and stored at −80°C. The concentration of the recombinant protein was estimated by absorption at 280 nm (Nano-Drop 1000C, Thermo). For quality control samples of the whole purification process were separated on SDS-PAGE (10% polyacrylamide). Gels were analyzed by colloidal Coomassie staining (InstantBlue, Gentauer) and Western Blot analysis, using an anti-6his-POD antibody (1:5000, Sigma).

**In vitro FAT10ylation and ubiquitylation assay**

The in vitro FAT10ylation assay was performed for 45 minutes at 37°C in 1x in vitro buffer (20 mM Tris-HCl (pH 7.6), 50 mM NaCl, 10 mM MgCl₂, 4 mM ATP, 0.1 mM dithiothreitol (DTT) and 1x protease inhibitor mixture). The in vitro ubiquitylation assay (Parkin auto-ubiquitylation) was performed as follows: recombinant GST-Parkin, recombinant GST-pSer65-Parkin or recombinant GST-C431A-Parkin were subjected to an in vitro FAT10ylation assay; after 20 minutes, the in vitro buffer was added to the reaction tube together with HA-Ube1, His-Ubc7 and His-Ubiquitin. The protein amounts used were as follows: 1μg GST-Parkin, 1μg GST-pSer65-Parkin, 1μg GST-C431A-Parkin, 1μg FAT10, 1μg FAT10-AV, 120ng FLAG-UBA6, 2μg His-Ub, 1 μg His-USE1, 2 μg His-Ubc7, 500 ng HA-Ube1.

When the in vitro experiments were performed using substrate proteins that were previously immunopurified from cells, the expression constructs encoding for Myc-tagged Parkin (Myc-Parkin), Myc-tagged Parkin C431A (Myc-Parkin C431A) or Myc-tagged Mfn2 (Myc-Mfn2) were transfected in 8*10⁵ Hek293 cells for 24 h. The proteins were immunoprecipitated and the in vitro reactions were...
performed directly on the immunopurification beads after extensive washing with the NET-T and NET-TN buffers. The reactions were stopped by adding of 5x gel sample buffer with 4% 2-mercaptoethanol. Proteins were separated on 8% or 10% Laemmli SDS-PAGE gels and were subjected to western blot analysis.

**Parkin in vitro phosphorylation and Phos-tag gel**

For the in vitro phosphorylation of GST-Parkin, the recombinant MBP-PINK1 kinase was mixed together with recombinant GST-Parkin (molar ratio PINK1/Parkin 1:8) and resuspended in kinase buffer (50 mM Tris, 0.1 mM EGTA, 10 mM MgOAc, 10 mM DTT, 4 mM ATP). The kinase reaction took place at 30°C and 350 rpm for 2 h. Afterward MBP-PINK1 was removed from the solution by purification with an Amylose resin (NEB). The samples were used for the GST-Parkin-FAT10ylation assay or mixed with 10 μL 4x sample buffer and subsequently used for a control Phos-tag gel analysis. In brief, the Phos-tag gel analysis consists in the simultaneous analysis of a phosphoprotein isoform and its non-phosphorylated counterpart, with the proteins bound by a Phos-tagTM molecule showing a mobility shift in SDS-PAGE triggered by a retained running behavior. The Phos-tag gel analysis has been performed according to the manufacturer’s protocol.

**Plasmids**

pPK5-Myc-Parkin (Myc-Parkin) plasmid was a gift from Ted Dawson (Addgene plasmid # 17612); pM2.G and psPAX2 vectors were a kind gift from Didier Trono (Addgene plasmid #12259 and #12260); pET28α/6His-GST-3C-Parkin plasmid was a gift from David Komander (Addgene plasmid #110758); pCMV6/Myc-Mu1 (Myc-Mu1) was purchased from Origene. pEGFP-N2/MARCH5 (GFP-MARCH5) plasmid was a kind gift of Eric Schirmer (Addgene plasmid #62039), pEGFP-parkin WT (EGFP-Parkin) plasmid was a gift from Edward Don (Addgene plasmid # 45875); pcDNA3.1/Myc-His-M2n (Myc-M2n) plasmid was a gift from David Chan (Addgene plasmid # 23213), pBMN-mCherry-Parkin (mCherry Parkin) and pCHAC-mt-mKeima (mtKeima) was a gift from Richard Youle (Addgene plasmid # 72342). pPK5-Myc-Parkin C431A (Myc-Parkin C431A) plasmid was generated by site-directed mutagenesis of pPK5-Myc-Parkin plasmid with primers fwd 5’-cagacatcatgcatgcgcctctatttttctact-3’, rev 5’-aattttattatatagaggtggc-3’. pPK5-Myc-Parkin C431A plasmid was generated by site-directed mutagenesis of pET28α/6His-GST-3C-Parkin plasmid with primers fwd 5’-ggcagaaaaacgcccgtctctattttttctact-3’, rev 5’-ggcattctatcgtcatgacagagtccg-3’. pET28α/6His-GST-3C-Parkin C431A (GST-Parkin C431A) plasmid was generated by site-directed mutagenesis of pET28α/6His-GST-3C-Parkin plasmid with primers fwd 5’-gatagtacagggccctctctattttttctact-3’, rev 5’-gacatcgtcatgcatgcgcctctattttttctact-3’. To generate pcDNA4/TO-3xFLAG-FAT10, 3xFLAG-FAT10 was cloned from pcDNA-His-3xFLAG-FAT10 with HindIII and NotI into pcDNA4/TO plasmid (Thermo Fisher).

**Transfection of plasmids**

The transfection mixture used to transiently transfect the described cell lines was based on mixture of plasmid DNA and Mirus transfection reagent in a ratio of 1:3 (1 μg plasmid: 3 μL Mirus transfection reagent). Plasmid DNA and transfection reagent were dissolved in DMEM (without additives) and incubated at room temperature for 15 minutes. The mixture was added dropwise into the cell culture dish and incubated for 24 hours at 37°C and 5% CO2. When required, pcDNA3.1-His/-A (Invitrogen) was used to balance plasmid amounts.

**siRNA-mediated gene silencing of the Parkin gene**

Depletion of Parkin protein from SH-SY5Y cells was achieved with a siRNA-based strategy. In brief, 50% confluent SH-SY5Y cells were treated for 12 h with 10 μM of siRNA in the presence of 0.5 μM of MIRCOCCP to induce mitophagy-dependent removal of endogenous Parkin. Cells were then washed and medium was replaced with fresh DMEM medium (described above). After 1 h, cells were transfected with FlexiTube siRNA (Hs PARK2 2 SI00677838) or with control siRNA using the HiPerFect Transfection Reagent. After 72 hours, mitochondrial morphology status was determined by live-cell imaging using MitoTracker Red CMXRos (Figure S4C). Parkin-depleted cells were used to perform the described experiments (Figure 4F).

**Cell extracts, immunoprecipitation and cycloheximide (CHX) experiments**

Cells were harvested using trypsin and centrifuged for 5 minutes at 1000xg. The obtained cell pellet was lysed in Triton X-100 lysis buffer (20 mM Tris-HCL pH7.8, 50 mM NaCl, 10 mM MgCl₂, 1% Triton X-100, 1x protease inhibitor mix and 1x phosphatase inhibitor cocktail) and incubated for 20 minutes on ice, followed by direct addition of 250 μL of 4% SDS lysis buffer (20 mM Tris-HCL pH7.8, 50 mM NaCl, 10 mM MgCl₂, 4% SDS, 1x protease inhibitor mix and 1x phosphatase inhibitor cocktail). Afterward, the lysate was transferred to a 1.5 mL Eppendorf tube and sonicated to eliminate solid debris, followed by renaturation with 10 volumes of RIPA buffer prior to immunoprecipitation. Immunoprecipitations were performed for Myc-tagged proteins with 25 μL of EZView Red Anti-c-Myc Affinity Gel, for FLAG-tagged proteins with 25 μL of EZView Red ANTI-FLAG® M2 Affinity Gel, and for HA-tagged proteins with EZView Red Anti-HA Affinity Gel (all three from Sigma). GFP-tagged proteins were immunoprecipitated with GFP-trap Agarose (Chromotek). 30 μL of Protein A-Sepharose (Merck) and 5 μg of the monoclonal F10-10-reactive antibody 4F1 (Aichem et al., 2010) were used for immunoprecipitation of endogenous FAT10. Protein A-Sepharose (30 μL, Merck) and 5 μg of the monoclonal Parkin-reactive antibody PRK8 (Santa Cruz) were used for immunoprecipitation of endogenous Parkin. After incubation for 4 h at 8°C, beads were washed twice with NET-TN buffer (50 mM Tris, 650 mM NaCl, pH 7.8, 0.5% Triton-X) and subsequently twice with NET-T buffer.
(50 mM Tris, 150 mM NaCl, pH 7.8, 0.5% Triton-X), followed by addition of 5X Laemmli sample buffer and SDS-PAGE/Western blot analysis. For the CHX chase experiments, cells were treated with CHX (final concentration 10μg/mL in DMSO) for the indicated time periods prior to lysis. Where indicated, cells were additionally treated with 10μM CCCP, 5mM MG132 or 100 nm bafalomyacin A1.

**Triton X-100-based soluble/insoluble fractionation**

To assess the presence of the FAT10-Parkin conjugate in the insoluble fraction, cells overexpressing FLAG-FAT10 and Myc-Parkin were harvested and lysed as described above. Cells were subsequently centrifuged at 14000xg for 20 minutes. The supernatant (soluble fraction) was collected and the pellet (insoluble fraction) was washed with PBS and solubilized with a SDS-based lysis buffer (20 mM TRIS-HCl pH 7.8, 1 mM MgCl2, 1% Triton X-100, 4% SDS, 10% glycerol, protease inhibitor, phosphatase inhibitor). The soluble and insoluble samples were subjected to immunoprecipitation and western blot analysis.

**Preparation of crude mitochondrial fractions**

Crude mitochondrial fractions from Hek293 and SH-SY5Y cells were separated from the cytosolic fractions using differential centrifugation. In brief, cells from a confluent 10 cm² dish were trypsinized and centrifuged for 5 minutes at 500 x g at room temperature. Cells were washed with PBS, resuspended in freeze-thaw medium (225 mM mannitol, 0.1 mM EGTA, 30 mM Tris–HCl pH 7.4) and subjected to four freeze-thaw cycles. The resulting lysate was centrifuged for 5 minutes at 600 rpm at 4°C and the nuclear pellet was discarded. The postnuclear supernatant was then centrifuged for 10 minutes at 10000 rpm at 4°C and the cytosolic fraction in the supernatant was separated from the crude mitochondrial fraction in the pellet. The pellet was washed at least 5 times with PBS, centrifuged at 10000 rpm for 10 minutes and lysed with NP-40 lysis buffer as described above. Lysates from the two fractions were used for immunoblotting.

**Confocal microscopy and live cell imaging**

HeLa cells were seeded on glass slices in 12-well plates to a confluency of 25% and cultured at 37°C and 5% CO₂. After 24 hours, medium was replaced with fresh DMEM medium containing TNFα/FNβ. After 24 hours, cells were washed with PBS and fixed with 4% formaldehyde in PBS for 10 minutes. Cells were washed twice in PB buffer (PBS, 3%BSA) and permeabilized for 5 minutes with 0.2% Triton X-100/PBS. After two washing steps cells were incubated with primary antibodies for 2 hours. A polyclonal anti-FAT10 antibody (1069) and a monoclonal anti-TOM20 antibody (Santa Cruz, F-10) were used as primary antibodies. After 2 hours of primary antibody incubations, cells were washed three times with PB buffer and incubated for two hours with fluorescent-dye-coupled antibodies (see Key Resources Table), followed by 3 PB buffer washing steps. Nuclear staining was performed using mounting medium containing DAPI (Abcam). Images were acquired with a 63x Plan Apochromat objective using a Zeiss LSM700 confocal microscope. Live-cell imaging samples were prepared by culturing cells in µ-Slide 8 Wells (IBIDI, 80826) for 24 h in phenol-free equivalent DMEM (Thermo Fisher, 21063029) supplemented with 10% fetal calf serum (GIBCO/Thermo Fisher Scientific), 1% stable glutamine (100x, 200 mM), and 1% penicillin/streptomycin (100x) (both from BioWest/VWR). Live-cell imaging of cells expressing EGFP-Parkin and mtKeima was performed on a heated stage maintained at 37°C at 5% CO₂ using a Zeiss temperature controller. Cells were treated with CCCP at a final concentration of 10μM. Microscopy was performed on a Zeiss CellObserver HS spinning Disk microscope. Images were acquired with a 63x/1.40 objective (PlanApochromat) with a mounted AxioCamMRm camera and detected using appropriate lasers and filters. Cells were acquired with 5 minute intervals for 1 h to analyze the translocation of Parkin to mitochondria after addition of 10μM CCCP, and with 20 minutes intervals for 20 h to analyze the progression of mitophagy after addition of 10μM CCCP using mtKeima. Images were analyzed with ImageJ software.

**Chemical crosslinking coupled to mass spectrometry (XL-MS)**

Proteins were crosslinked and measured essentially as described (Aichem et al., 2019b). In short, a molar ratio of Parkin / Parkin-S65P and FAT10 of approximately 1:3.5 were incubated as follows: FAT10 (1.5 μg/μl stored in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM TCEP) and 50 μg of Parkin or Parkin-S65P, respectively (0.9 μg/μl or 0.65 μg/μl; stored in 25 mM Tris-HCl, pH 8.5, 200 mM NaCl, 0.03% Brj35, 10% glycerol, 5 μM TCEP), were incubated for 15 min on ice. Proteins were crosslinked by addition of H12/D12 BS3 (Creative Molecules) at a final ratio of 1 mmol BS3/1 μg protein for 30 min at 37°C while shaking at 650 rpm. After quenching by addition of ammonium bicarbonate to a final concentration of 50 mM and incubation for 10 min at 37°C, samples were dried, dissolved in 8M urea to a final concentration of 1 mg/ml, reduced with TCEP at a final concentration of 2.5 mM, alkylated with iodoacetamide at a final concentration of 5 mM and digested over night with trypsin (Promega V5113) in 1 M urea (diluted with 50 mM ammonium bicarbonate) at an enzyme-to-substrate ratio of 1:40. Digested peptides were separated from the solution and retained by a solid phase extraction system (SepPak, Waters) and then separated by size exclusion chromatography prior to liquid chromatography (LC)-MS/MS analysis on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific; details see below). Amounts of potential crosslinks were normalized prior to MS by measuring peptide bond absorption at 215 nm for each fraction. Crosslinked samples were prepared in triplicates and measured with technical duplicates. Data were searched using xQuest in ion-tag mode. Crosslinks which were identified with deltaS < 0.95, two high-confidence crosslinks per unique crosslinking site (uxID n = 2) with at least one Id-Score ≥ 25 and an assigned FDR as calculated by xProphet below 0.05 were visualized by xiNET software (Figure 1E; Tables S1 and S2).
Quantitative chemical crosslinking coupled to mass spectrometry (q-XL-MS)

Quantitative XL-MS analysis was carried out essentially as described (Sailer et al., 2018). Crosslinks which were identified with deltaS < 0.95 and ID-Score ≥ 20 were used as input for q-XL-MS analysis with xTract. Here, chromatographic peaks of identified crosslinks in the samples with Parkin-S65P/FAT10 versus Parkin/FAT10 (n = 3, each sample analyzed additionally as technical duplicate) were integrated and summed up over different peak groups (taking different charge states and different unique crosslinked peptides for one unique crosslinking site into account). Only high-confidence crosslinks that were identified consistently in both, light and heavy labeled states (xTract settings violations was set to 0), were selected for further quantitative analysis. If a peptide was detected in only one condition (e.g., only in the reference experiment), the fold change was estimated on the basis of the minimum detectable signal intensity (1e3 for Orbitrap Fusion Tribrid mass spectrometer), and instead of the area, the intensity of the first isotope was used for the comparision. This is indicated in Table S3 in the column ‘imputed values’. Changes in crosslinking abundance are expressed as log2 ratio (e.g., abundance state 1, Parkin-S65P/FAT10 was quantified versus abundance state 2, Parkin/FAT10). The p value indicates the regression between the two conditions. In this study, only links with an ID-Score ≥ 25 and an assigned FDR as calculated by xProphet below 0.056 that showed a change of log2ratio ≥ ± 1 and a p value of ≤ 0.05 were considered as significant changes in abundance and are shown in green and red in the 2D visualizations, respectively. All other changes were considered insignificant and are shown in gray. Crosslinks were visualized by xiNET software using additional in-house scripts for the analysis and representation of quantitative crosslink information (Figure 3E; Table S3).

Enrichment of crosslinked peptides by size exclusion chromatography (SEC)

Crosslinked peptides were enriched by size exclusion chromatography on an ÅKTAmero chromatography system (GE Healthcare) using a SuperdexTM Increase 3.2/30 column (GE Healthcare) at a flow rate of 50 μl/min of the mobile phase (water/acetonitrile/trifluoroacetic acid 70%/30%/0.1%, vol/vol/vol). UV absorption at a wavelength of 215 nm was used for monitoring the separation. The eluent was collected in fractions of 100 μl in a 96-well plate. The four fractions 1.0 - 1.1 ml, 1.1 – 1.2 ml, 1.2 – 1.3 mL and a pooled fraction of 1.3 – 1.5 mL were collected, dried and further analyzed by LC-MS/MS.

LC-MS/MS analysis

Samples fractionated by SEC were re-dissolved in an appropriate volume of MS buffer (acetonitrile/formic acid 5%/0.1%, vol/vol) according to their UV signal. Peptides were separated on an EASY-nLC 1200 (Thermo Scientific) system equipped with a C18 column (Acclaim PepMap 100 RSLC, length 15 cm, inner diameter 50 μm, particle size 2 μm, pore size 100 Å, Thermo Scientific). Peptides were eluted at a flow rate of 300 nl/min using a 60 min gradient starting at 94% solvent A (water/acetonitrile/formic acid 100%/0%/0.1%, vol/vol/vol) and 6% solvent B (water/acetonitrile/formic acid 20%/80%/0.1%, vol/vol/vol) for 4 min, then increasing the percentage of solvent B to 44% within 45 min followed by a 1 min step to 100% B for additional 10 min. The mass spectrometer was operated in data-dependent-mode with dynamic exclusion set to 60 s and a total cycle time of 3 s. Full scan MS spectra were acquired in the Orbitrap (120.000 resolution, 400-1500 m/z scan range, AGC target of 50%, 50 ms maximum injection time, profile’ data type). Most intense precursor ions with charge states 3-8 and intensities greater than 5e3 were selected for fragmentation using CID with 35% collision energy. Monoisotopic peak determination was set to peptide and MS/MS spectra were acquired in the linear ion trap (rapid scan rate, standard’ AGC target).

Flow cytometry and mtKeima measurement

Cells expressing the mtKeima constructs were treated with 10μM CCCP for 6 h (Hek T-Rex-293 3XFLAG-FAT10 EGFP-Parkin mtKeima cells) or 18 h (SH-SY5Y mtKeima-based cells), trypsinized and collected in FACS buffer (1 x PBS, 2% FCS, 2mM EDTA, 2 mM Na3VO4). Flow cytometry was performed on an LSRFortessa flow cytometer (BD Bioscience). The excitation wavelengths of 405 and 561 nm were used to detect mtKeima at pH 7.0 and 4.0, respectively, and 488 nm to detect GFP-parkin. For each condition, 106 cells, gated for single cells in suspension, were used for the analysis. Flow cytometry data were analyzed using the BD Diva Software. Representative graphs are shown after analysis with the FlowJo software (Tree Star).

Rotenone treatment, detection of ROS, and cell viability assay

Rotenone was dissolved in low glucose DMEM (Merck, D6046) supplemented with 10% fetal calf serum (GIBCO/Thermo Fisher Scientific), 1% stable glucose (100x, 200 mM), and 1% penicillin/streptomycin (100x) (Biowest/VWR) at the final concentration of 5μM. Where indicated, recombinant TNFα and IFNγ were added to the medium. SH-SY5Y cells and SH-SY5Y FAT10 KO cells were incubated with this medium for 32 h to induce ROS production and cell death. Where indicated, cellular ROS levels were stained with 5 μM mitoSOX Red at room temperature for 10 minutes. Cell viability was determined using SYTOX Blue (dead cell staining). mitoSOX and SYTOX Blue intensity were analyzed by flow cytometry using an LSRFortessa instrument (BD Biosciences). Flow cytometry data were analyzed using FlowJo (Tree Star).

QUANTIFICATION AND STATISTICAL ANALYSIS

The data are expressed as mean ± SD. Statistical analyses were performed using Student’s t tests (GraphPad Prism 6.0 software, San Diego, CA, United States). P-values < 0.05 were considered indicative of statistical significance.