




# MITOL promotes cell survival by degrading Parkin during mitophagy

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## Abstract

Parkin promotes cell survival by removing damaged mitochondria via mitophagy. However, although some studies have suggested that Parkin induces cell death, the regulatory mechanism underlying the dual role of Parkin remains unknown. Herein, we report that mitochondrial ubiquitin ligase (MITOL/MARCH5) regulates Parkin-mediated cell death through the FKBP38-dependent dynamic translocation from the mitochondria to the ER during mitophagy. Mechanistically, MITOL mediates ubiquitination of Parkin at lysine 220 residue, which promotes its proteasomal degradation, and thereby fine-tunes mitophagy by controlling the quantity of Parkin. Deletion of MITOL leads to accumulation of the phosphorylated active form of Parkin in the ER, resulting in FKBP38 degradation and enhanced cell death. Thus, we have shown that MITOL blocks Parkin-induced cell death, at least partially, by protecting FKBP38 from Parkin. Our findings unveil the regulation of the dual function of Parkin and provide a novel perspective on the pathogenesis of Parkinson's disease.

**Keywords** mitochondria; mitophagy; Parkin; MITOL/MARCH5; E3 ubiquitin ligase

**Subject Categories** Autophagy & Cell Death; Membranes & Trafficking

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## Introduction

Mutations in PARK2 (Parkin), a ubiquitin E3 ligase, and PARK6 (PINK1), a ubiquitin kinase, are associated with the familial form of Parkinson's disease (PD; Kitada *et al*, 1998; Shimura *et al*, 2000). Recent studies have revealed that recruitment of Parkin from the

cytosol to depolarized mitochondria is mediated by PINK1 to remove damaged mitochondria via autophagy (Narendra *et al*, 2008; Geisler *et al*, 2010; McLelland *et al*, 2014). PINK1 is constitutively degraded through the N-end rule pathway (Yamano & Youle, 2013). However, when mitochondrial depolarization occurs, PINK1 is stabilized on the outer mitochondrial membrane, where it phosphorylates ubiquitin and Parkin; therefore, PINK1 is known as a parkin activator and recruiter (Geisler *et al*, 2010; Matsuda *et al*, 2010; Narendra *et al*, 2010; Vives-Bauza *et al*, 2010; Kondapalli *et al*, 2012; Shiba-Fukushima *et al*, 2012; Kane *et al*, 2014; Kazlauskaite *et al*, 2014; Koyano *et al*, 2014). Hence, it is widely believed that the PINK1–Parkin pathway has a cell-protective function via mitophagy, and impaired mitophagy due to loss of function of PINK1 or Parkin is involved in the pathogenesis of PD.

On the other hand, widespread quantitative analysis of the ubiquitylome has led to the discovery of novel substrates for Parkin, such as cell survival-related proteins, including FK506-binding protein 38 (FKBP38; Sarraf *et al*, 2013; Rose *et al*, 2016). Some studies have indicated that Parkin induces cell death during mitophagy (Carroll *et al*, 2014; Zhang *et al*, 2014; Akabane *et al*, 2016), suggesting a dual role of Parkin in both cell survival and death signaling. However, the regulatory mechanism underlying the dual function of Parkin is unknown. In addition, although many studies have revealed the regulatory role of both the subcellular localization and ligase activity of Parkin in mitophagy, the quantitative aspects of Parkin regulation are still unknown.

FKBP38 is a membrane-bound chaperon protein that mainly resides on the outer membrane of mitochondria. It is a tail-anchored protein with a transmembrane domain at the carboxyl terminal. The known functions of FKBP38 include recruiting the anti-apoptotic proteins Bcl-2 and Bcl-xL into the mitochondria to inhibit apoptosis (Shirane & Nakayama, 2003). Interestingly, it has been reported that FKBP38 escapes mitochondrial degradation by translocating from the mitochondria to the ER during mitophagy, thereby inhibiting apoptosis (Saita *et al*, 2013). Since FKBP38 can inhibit apoptosis

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even in the absence of mitochondria, it is considered to contribute to the inhibition of mitochondria-dependent and ER-dependent apoptosis.

Previously, we identified MITOL/MARCH5 and found that it specifically polyubiquitinates and degrades misfolded mutant SOD1 and expanded polyglutamine in a proteasome-dependent manner to protect the cells (Yonashiro *et al*, 2009; Sugiura *et al*, 2011). With regard to its relationship with mitophagy, MITOL has been reported to influence the removal of mitochondria by controlling FUNDC1 in response to hypoxic stress (Liu *et al*, 2012). However, the function of MITOL in Parkin-mediated mitophagy cannot be fully excluded. In this study, we found that MITOL not only binds to Parkin in a mitophagy induction-dependent manner but also mediates the addition of K48-linked ubiquitin chain to its lysine 220 residue, and thereby degrading Parkin, particularly, its phosphorylated active form. Moreover, we reported that MITOL translocates to the ER with FKBP38 in an FKBP38-dependent manner and degrades Parkin to protect FKBP38 from Parkin for cell survival during the late phase of mitophagy. Our findings unveil the regulation of the dual role of Parkin and provide a novel perspective on the pathogenesis of PD.

## Results

### MITOL mediates ubiquitination of Parkin in Parkin-dependent mitophagy

It is well known that Parkin is selectively recruited to mitochondria with low membrane potential that is induced by an uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Narendra *et al*, 2008; Matsuda *et al*, 2010; Vives-Bauza *et al*, 2010). To investigate the physiological aspects of the interaction between MITOL and Parkin, a co-immunoprecipitation assay was first performed using HeLa cells with or without stable expression of HA-Parkin, which demonstrated that Parkin was co-immunoprecipitated with MITOL after CCCP treatment (Fig 1A). The interaction between endogenous MITOL and Parkin was also observed in a CCCP-dependent manner (Figs 1B and EV1A). To determine which domain of MITOL is necessary for interaction with Parkin, three glutathione *S*-transferase (GST)-fused fragments of MITOL were generated, which are considered to be localized on the cytosolic surface of the outer mitochondrial membrane (Fig EV1B). In the GST pull-down assay, the GST fragment containing the C-terminal of MITOL was able to pull Parkin down from the lysate of the CCCP-treated HeLa cells that stably expressed HA-Parkin (Fig EV1B). In addition, in an immunoprecipitation experiment, MITOL mutants lacking the C-terminal domain ( $\Delta$ C) did not bind to Parkin after CCCP treatment (Fig EV1C). These results demonstrated that MITOL interacts with Parkin via its C-terminal domain. To determine which domain of Parkin interacts with MITOL, GST-fused Parkin fragments were generated (Fig EV1D). In the GST pull-down assay, the GST-fused Parkin RING2 domain was able to pull MITOL down (Fig EV1D). PINK1 is essential for the translocation of Parkin to depolarized mitochondria (Lazarou *et al*, 2012; Okatsu *et al*, 2012; Okatsu *et al*, 2013). In the PINK1 knockdown cells, the binding between MITOL and Parkin was decreased due to reduced recruitment of Parkin to the mitochondria (Fig 1C). Additionally, binding was reduced in the case of the pathogenic mutant Parkin K211N (Fig EV1E; Geisler

*et al*, 2010; Matsuda *et al*, 2010), which is severely compromised the mitochondrial localization 8 h after CCCP treatment (Fig EV1F), indicating that Parkin is required for the mitochondrial recruitment to interact with MITOL. Thus, we conclude that MITOL binds to Parkin in a mitochondrial depolarization-dependent manner and that the binding domains responsible for the interaction are present in the C-terminal of MITOL and the RING2 domain of Parkin, respectively. To clarify the regulatory mechanism of Parkin by MITOL in detail, the ubiquitination of Parkin by MITOL was characterized. Upon CCCP treatment, overexpressed wild-type (WT) MITOL in the HeLa cells stably expressing HA-Parkin elevated the levels of ubiquitinated Parkin, compared with those of the control vectors or the MITOL CS (C65S, C68S) mutant lacking the E3 ligase activity (Fig 1D). On the other hand, as Parkin is also a ubiquitin ligase that is activated in a CCCP-dependent manner, we tried to detect the ubiquitination of MITOL upon mitochondrial depolarization using immunoprecipitation assay. Interestingly, the Parkin-dependent ubiquitination of MITOL was observed in the early phase of mitophagy consistent with a previous report (Koyano *et al*, 2019a); however, it could not be detected in the late stage (Fig EV2A). Next, to ascertain whether Parkin translocation to the mitochondria requires the ubiquitination of Parkin, we examined the ubiquitination of Parkin K211N mutant and found that MITOL-mediated ubiquitination did not occur 8 h after CCCP treatment (Fig EV2B), suggesting that Parkin is ubiquitinated by MITOL after its translocation to the mitochondria. We used UbPred, an *in silico* tool for ubiquitination analysis (Radivojac *et al*, 2010), to identify the lysine residue(s) essential for the ubiquitination of Parkin by MITOL and predicted several lysine residues that were potential ubiquitin linkage sites. Based on this, three site-specific KR mutants were prepared in which the candidate lysine residues of Parkin were mutated to arginine. Despite the overexpression of MITOL, the ubiquitination level of the K220R mutant was much lower than those of WT Parkin and other Parkin KR mutants (Fig 1E). The Lys48-linked polyubiquitin chain is mainly responsible for the degradation of ubiquitinated proteins by the 26S proteasome, whereas Lys63-linked polyubiquitination alters the activation and localization of substrates as well as the regulation of the binding proteins (Pickart & Fushman, 2004; Mukhopadhyay & Riezman, 2007). To determine the type of polyubiquitin chain, single KR ubiquitin mutants with a single point mutation (lysine to arginine) were generated. In the WT ubiquitin or the K63R ubiquitin mutant, Parkin ubiquitination was increased following the MITOL overexpression in a CCCP-dependent manner, but it was not altered in the K48R ubiquitin mutant (Fig EV2C). Previously, to identify the type of polyubiquitin chain, we prepared a variety of ubiquitin mutants with a single intact K residue. Consistent with the results obtained using the KR mutants, the ubiquitination of Parkin by MITOL was confirmed in the R48K ubiquitin mutant-transfected HeLa cells (Fig EV2D). Furthermore, we performed ubiquitin chain restriction assay (UbiCRest) to determine Parkin-attached endogenous ubiquitin (Hospenthal *et al*, 2015). Parkin-attached polyubiquitin chains were digested in the presence of the universal deubiquitinating enzyme USP2 and the K48-polyubiquitin-specific deubiquitinating enzyme OTUB1, whereas the K63-polyubiquitin-specific deubiquitinating enzyme AMSH did not alter the levels of the polyubiquitin chains in Parkin (Fig 1F). This indicates that K48 polyubiquitin linkages are attached to Parkin. We performed ubiquitination assay using K48-linked

polyubiquitin chain-specific antibody and found that the K48-linked ubiquitination in Parkin mediated by CCCP treatment was reduced in the mitochondrial and ER fraction due to MITOL deficiency (Fig 1G). Therefore, it was concluded that MITOL mediates the K48-linked polyubiquitination of Parkin at the K220 residue.

### MITOL regulates Parkin during mitophagy

As MITOL promotes the addition of K48-linked polyubiquitin to Parkin, we assumed that it induces proteasomal degradation of Parkin. To examine whether the degradation of Parkin depends on proteasomes or autophagy, we treated the cells with lactacystin, a proteasome-specific inhibitor, or bafilomycin A1, an autophagic flux inhibitor. Lactacystin strongly inhibited the degradation of Parkin in the CCCP-treated cells overexpressing MITOL (Fig 2A). Parkin degradation was also inhibited in the MITOL CS mutant and Parkin K220R mutant (Fig 2B and C). In contrast, MITOL overexpression downregulated the Parkin protein level in the mitochondrial fraction post CCCP treatment (Fig EV3A). Furthermore, cycloheximide (CHX) treatment reduced Parkin in CCCP-treated MITOL WT but not MITOL KO cells (Figs 2D and EV3B). As it is known that Parkin nonspecifically ubiquitinates mitochondrial proteins during mitophagy (Koyano *et al*, 2019b), we confirmed the specificity of MITOL for its substrate using Mito-GFP upon depolarization. While Parkin ubiquitinated Mito-GFP in a mitophagy-dependent manner, MITOL did not ubiquitinate it (Fig EV3C), indicating its substrate specificity during mitophagy. These findings suggest that MITOL ubiquitinates and subsequently degrades Parkin in a proteasome-dependent manner. To understand whether the ubiquitination of Parkin by MITOL has a functional effect on mitophagy, we performed quantitative analysis of mitophagy using the mitochondria-localized pH-dependent fluorescent protein mt-Keima (Katayama *et al*, 2011; Yamashita *et al*, 2016; Igarashi *et al*, 2020), which exhibits a short excitation in normal conditions (pH 8.0) and shifted to longer excitation wavelengths in mitophagy processes because of the acidic environment of the lysosome (pH 4.5). Flow cytometric analysis indicated that MITOL deletion significantly enhanced mitophagy, which was restored to normal levels by a low expression of MITOL. On the other hand, a high expression of MITOL strongly suppressed mitophagy through the intense degradation of Parkin by MITOL (Figs 2E and EV3E). Furthermore, MITOL overexpression failed to suppress the mitophagy induced by the Parkin K220R mutant (Fig EV3F), which exhibits normal translocation to mitochondria in mitophagy (Fig EV3D). Taken together, endogenous MITOL fine-tunes mitophagy by controlling the quantity of Parkin on mitochondria.

### MITOL translocates to the ER via FKBP38 in the later phase of mitophagy

Although MITOL is considered to localize in the mitochondria, the MITOL protein level was not reduced during the late phase of mitophagy when mitochondria were removed (Fig 3A). A previous study showed that the mitochondria-localized protein FKBP38 translocates from the mitochondria to the ER to escape the degradation together with the mitochondria during mitophagy (Saita *et al*, 2013). Thus, it was speculated that MITOL might also exhibit similar dynamic translocation. To monitor endogenous MITOL localization during

mitophagy, an EGFP-MITOL knock-in cell line was generated, and dynamic translocation from the mitochondria to the ER was observed in the later phase of mitophagy (Fig 3B and C). Consistently, immunostaining analysis revealed that MITOL was localized to the ER in the later phase of mitophagy, similar to FKBP38 (Fig EV4A), suggesting that MITOL translocates to the ER along with FKBP38. As expected, MITOL interacted with FKBP38 under both normal and mitochondrial depolarization conditions (Figs 3D and EV4B). As MITOL was bound to FKBP38, it was assumed that the translocation of MITOL to the ER might depend on FKBP38. Therefore, the effect of FKBP38 knockdown on MITOL localization was examined using siRNA in each organelle fraction. FKBP38 knockdown inhibited the translocation of MITOL to the ER and reduced MITOL expression under CCCP treatment conditions (Fig EV4C and D). To examine whether the ER-localized MITOL was indeed translocated from the mitochondria and not due to its *de novo* synthesis in the ER, the photoconverting fluorescent tag protein Kikume Green-Red (KikGR), which changes color from green to red following irradiation with ultraviolet rays (360–410 nm), was used. When MITOL-KikGR-transfected cells were exposed to ultraviolet rays before CCCP treatment, the MITOL-KikGR synthesized before mitophagy displayed red fluorescence in the mitochondria (Fig 3E) as well as in the ER during the late phase of mitophagy, suggesting that MITOL was transported from the mitochondria to the ER during mitophagy (Fig 3E). These results show that MITOL translocates to the ER in an FKBP38-dependent manner in the late phase of mitophagy.

### MITOL specifically degrades phosphorylated Parkin in mitophagy

Parkin and ubiquitin are phosphorylated by PINK1, which is stabilized on the outer membrane of the depolarized mitochondria. Subsequently, phosphorylated Parkin binds to phosphorylated ubiquitin to activate and induce mitophagy (Kondapalli *et al*, 2012; Kane *et al*, 2014; Kazlauskaitė *et al*, 2014; Koyano *et al*, 2014). However, the quantitative regulation between the active and inactive forms of Parkin in mitophagy remains largely unknown. Although endogenous MITOL mediates the K48-linked polyubiquitination of Parkin under mitochondrial depolarization, the entire pool of Parkin did not change dramatically, suggesting the possibility that endogenous MITOL specifically recognizes the activated form of Parkin. We examined whether endogenous MITOL regulates the amount of phosphorylated Parkin and found continuous accumulation of phosphorylated Parkin in the MITOL KO cells after mitochondrial depolarization (Fig 4A). In MITOL KO cells, Parkin was bound to ubiquitin, confirming the accumulation of activated Parkin in MITOL KO (Fig 4B). To determine the significance of the translocation of MITOL to the ER, we examined the phosphorylated Parkin in the ER fractions in the later phase of mitophagy and found a dramatic increase in Ser65-phosphorylated Parkin in the ER fraction of MITOL KO cells (Fig 4C). To examine whether the ER-localized Parkin was synthesized *de novo*, we used the photoconverting fluorescent tag protein KikGR. KikGR-Parkin exhibited red fluorescence in the ER at the late mitophagy phase, suggesting that Parkin was in the ER during this phase (Fig 4D). Parkin accumulation in the ER was observed in FKBP38-knockdown cells (Fig EV5A), suggesting that Parkin accumulates in the ER in an FKBP38-independent manner. Taken together, these data show that (i) endogenous

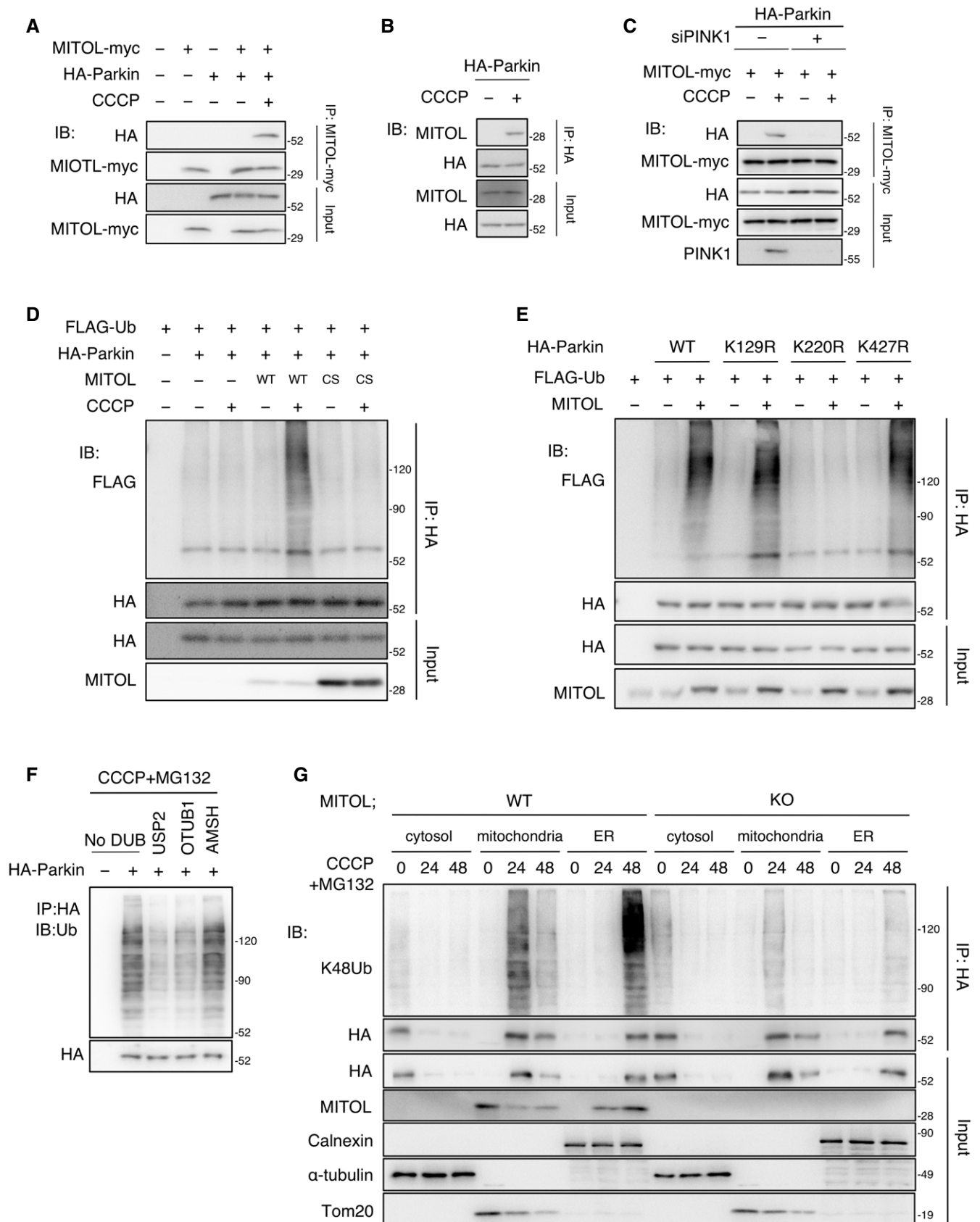


Figure 1.

**Figure 1. MITOL mediated the K48-linked polyubiquitination of Parkin at K220.**

- A MITOL interacts with Parkin under CCCP treatment. HeLa cells or HeLa cells stably expressing HA-Parkin were transfected with the indicated vectors and treated with DMSO or CCCP (10  $\mu$ M) for 4 h. Lysates of cells were subjected to an IP assay with anti-myc antibody and IB assay with the indicated antibodies.
- B Interaction of endogenous MITOL and Parkin in HeLa cells stably expressing HA-Parkin. HeLa cells stably expressing HA-Parkin were treated with DMSO or CCCP (10  $\mu$ M) for 12 h and subjected to an IP-IB assay with the indicated antibodies.
- C PINK1 knockdown decreases interaction between MITOL and Parkin. HeLa cells stably expressing HA-Parkin were transfected with PINK1 siRNA or control siRNA and treated with DMSO or CCCP (10  $\mu$ M) for 4 h. MG132 (30  $\mu$ M) was added 3 h after each treatment. Lysates of cells were subjected to an IP assay with anti-myc antibody and IB assay with the indicated antibodies.
- D MITOL overexpression enhances the ubiquitination of Parkin. HeLa cells or HeLa cells stably expressing HA-Parkin were transfected with the indicated vectors and treated with DMSO or CCCP (10  $\mu$ M) for 8 h. MG132 (30  $\mu$ M) was added 3 h after each treatment. Lysates of cells were subjected to an IP-IB assay with the indicated antibodies. WT, wild-type; CS, MITOL C65/67S mutant lacking ubiquitin ligase activity.
- E MITOL fails to ubiquitinate the Parkin K220R mutant. HeLa cells were transfected with the indicated vectors and treated with CCCP (10  $\mu$ M) for 8 h. MG132 (30  $\mu$ M) was added 3 h after CCCP treatment. Lysates of cells were subjected to an IP-IB assay with the indicated antibodies and compared ubiquitination levels of WT, K129R, K220R, and K427R HA-Parkin.
- F Parkin is ubiquitinated by K48-polyubiquitin in mitophagy. HeLa cells stably expressing HA-Parkin were treated with CCCP (10  $\mu$ M) for 48 h together with MG132 (30  $\mu$ M) for the final 45 h. Parkin was immunoprecipitated from cell lysates and then performed ubiquitin restriction assay with AMSH, OTUB1, and USP2 using UbiCRest kit. Lysates of cells were subjected to an IB assay with the indicated antibodies. DUBs, deubiquitinating enzyme.
- G Endogenous MITOL enhances the K48-polyubiquitin chains of Parkin. WT or MITOL KO HeLa cells stably expressing HA-Parkin were treated with CCCP (10  $\mu$ M) for indicated time. MG132 (30  $\mu$ M) was added 3 h after CCCP treatment. Lysates of cells were fractionated into cytosolic, mitochondrial, and ER fractions, and then subjected to an IP-IB assay with the indicated antibodies. K48-polyubiquitin chains were immunoblotted with K48-specific ubiquitin antibody.

MITOL also controls the degradation of Parkin, particularly phosphorylated Parkin, (ii) quantitative regulation of Parkin by endogenous MITOL affects mitophagy, and (iii) MITOL translocates to the ER in an FKBP38-dependent manner in the late phase of mitophagy.

#### MITOL protects FKBP38 by degrading phosphorylated Parkin for cell survival during mitophagy

Parkin degrades the Pael receptor (Pael-R) for the substrate in the ER (Imai *et al*, 2001), and based on this, we considered that the accumulation of Parkin in the MITOL KO cells might induce excessive degradation of its substrates in the ER. Therefore, we investigated the amount of FKBP38 in the MITOL KO cells and found that the amount of FKBP38 in the ER was markedly decreased by MITOL KO (Fig 4C). We examined whether FKBP38 is a specific substrate of Parkin by using phosphorylation-defective Parkin mutant (S65A; Kane *et al*, 2014) and Parkin catalytic cysteine mutant (C431S; Lazarou *et al*, 2013), and found that Parkin polyubiquitinated FKBP38 at its K271/K273 residue and induced FKBP38 degradation during mitophagy (Fig 5A–C). Furthermore, to determine the relationship between the subcellular localization and Parkin-induced degradation of FKBP38, we added either an ER targeting signal (IYFFT) or a mitochondria targeting signal (ActA) to FKBP38 (Zuchner *et al*, 2004; de Brito & Scorrano, 2008). In the MITOL KO cells, ER-targeted FKBP38 was markedly degraded after CCCP treatment compared to WT cells (Figs 5D and EV5B). In contrast, the degradation of FKBP38 targeted in the mitochondria was not strongly enhanced by MITOL deficiency as mitochondrial degradation ensued during this time (Fig 5D). These data suggest that MITOL inhibits the degradation of FKBP38, especially in the ER via the degradation of activated Parkin in the later phase of mitophagy. Therefore, we considered that MITOL KO cells become vulnerable to cell death due to FKBP38 degradation. Analysis of cell death by propidium iodide (PI) staining indicated that the cell death in MITOL KO cells increased compared to the WT cells after CCCP treatment and this enhanced cell death was rescued by the overexpression of WT MITOL (Fig 5E). Moreover, the FKBP38 mutant K271/273R, lacking the essential lysine residues for the degradation

modulated by Parkin, partly rescued the cell death in MITOL KO cells and CCCP-treated Parkin K220R cells (Fig 5F and G), indicating that FKBP38 inhibits Parkin-induced cell death at the ER during mitophagy. On the other hand, cell death in the FKBP38 K271/273R mutants was only partially rescued suggesting that activated Parkin targets not only FKBP38, but also other substrates in the ER. FKBP38 contributes to the localization of the anti-apoptotic protein Bcl-2 (Shirane & Nakayama, 2003). We hypothesized that the degradation of FKBP38 via Parkin causes mislocalization of Bcl-2 in the MITOL KO cells. Thus, we analyzed the localization of Bcl-2 between MITOL WT cells and MITOL KO cells during mitophagy. Consistent with previous reports (Saita *et al*, 2013), we found that Bcl-2 translocated to the ER in the HA-Parkin stably expressing MITOL WT cells in mitophagy while it was partially mislocalized to the cytosol in HA-Parkin stably expressing MITOL KO cells (Fig 6A). Finally, we verified whether this cell death pathway requires caspase activation or not and for this, we treated the cells with the pan-caspase inhibitors Z-VAD-FMK. Addition of Z-VAD-FMK to the MITOL KO cells prevented cell death in mitophagy (Fig 6B). Since Bcl-2, which regulates the Ca<sup>2+</sup> release from the ER to prevent excessive cell death (Lam *et al*, 1994), does not accurately localize at the ER in MITOL KO cells, we hypothesized that the mislocalization of Bcl-2 caused cell death via calcium leak. Thus, we treated the MITOL KO cells with BAPTA-AM, an intracellular calcium chelator and as expected, we observed that the cell death could be rescued in the MITOL KO cells (Fig 6C). Taken together, our findings demonstrated that MITOL inhibits Parkin-induced cell death, at least partially, by protecting FKBP38 from activated Parkin at the ER during mitophagy.

## Discussion

There have been recent advancements in the analysis of PINK1–Parkin-mediated mitophagy and its molecular mechanism has been elucidated in detail. It has also become clear that (i) PINK1 phosphorylates the S65 of ubiquitin during the lowering of the membrane potential in the mitochondria; (ii) this phosphorylated

ubiquitin is a Parkin-activating factor; and (iii) the conformation of Parkin changes based on its phosphorylation and binding with phosphorylated ubiquitin (Trempe *et al.*, 2013). *In vitro* binding studies

have revealed that MITOL binds to the RING2 domain of Parkin only when CCCP was added. Thus, it is considered that MITOL specifically binds to the activated Parkin that has already undergone

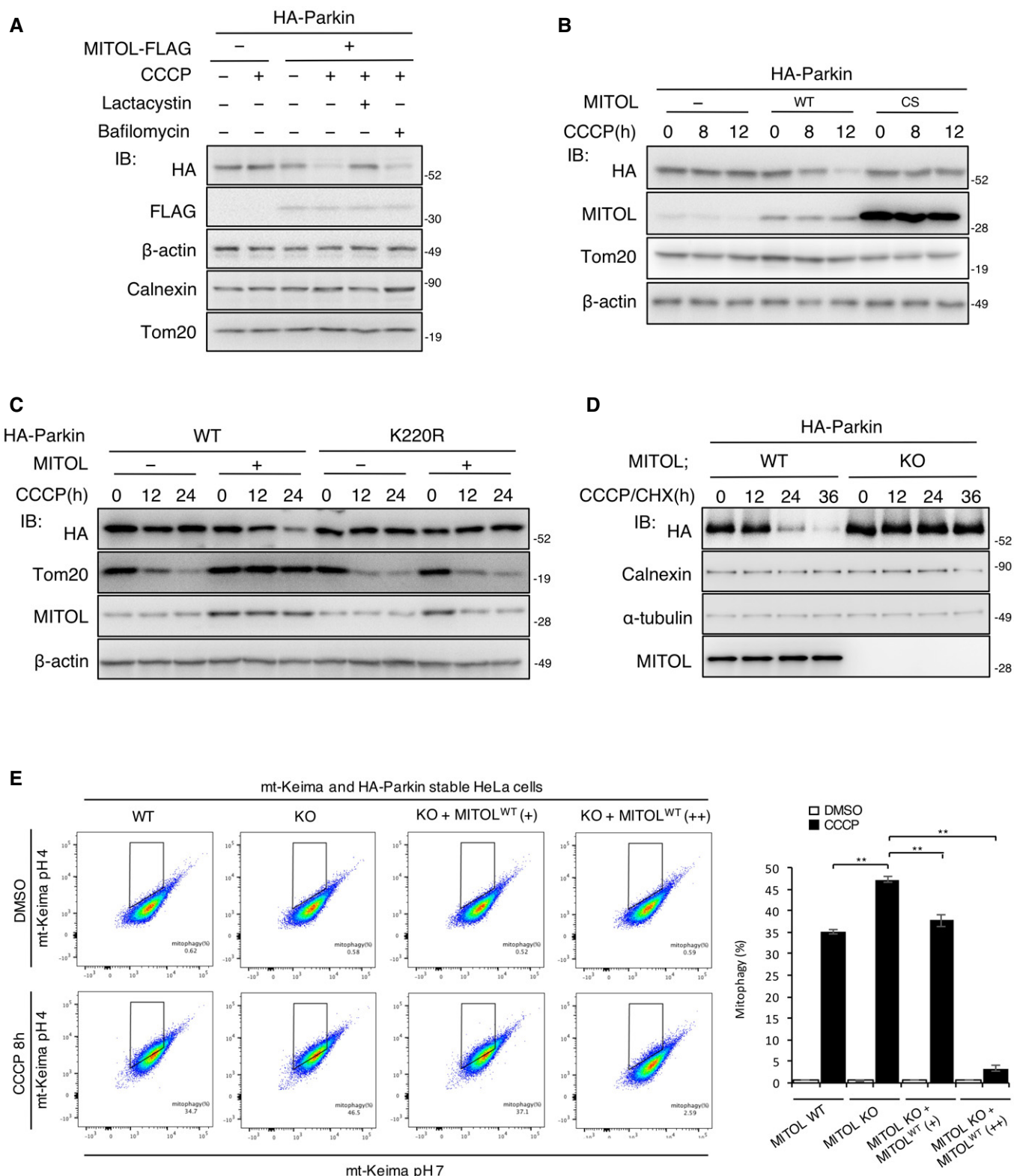


Figure 2.

**Figure 2. MITOL regulates mitophagy through the degradation of Parkin.**

- A MITOL degrades Parkin through the ubiquitin–proteasome system. HeLa cells stably expressing HA-Parkin were transfected with the indicated vectors and treated with DMSO or CCCP (10  $\mu$ M) for 8 h alone or either with bafilomycin A1 (10  $\mu$ M) or lactacystin (10  $\mu$ M) for the final 5 h. Lysates of cells were subjected to an IB assay with the indicated antibodies.
- B Overexpression of MITOL CS mutant fails to degrade Parkin. HeLa cells stably expressing HA-Parkin were transfected with the indicated vectors and treated with DMSO or CCCP (10  $\mu$ M) for the indicated time. Lysates of cells were subjected to an IB assay with the indicated antibodies. MITOL CS mutant; lacking ubiquitin ligase activity.
- C Parkin K220R mutant is not regulated by MITOL in mitophagy. HeLa cells were transfected with the indicated vectors and treated with DMSO or CCCP (10  $\mu$ M) for the indicated time. Lysates of cells were subjected to an IB assay with the indicated antibodies.
- D Endogenous MITOL attenuates Parkin in both CHX- and CCCP-treated conditions. WT or MITOL KO HeLa cells stably expressing HA-Parkin were treated with CCCP (10  $\mu$ M) as indicated times. CHX (30  $\mu$ M) was added 5 h after CCCP treatment. Lysates of cells were subjected to an IB assay with the indicated antibodies.
- E MITOL regulates mitophagy. MITOL WT HeLa cells, MITOL KO HeLa cells, and KO HeLa cells transfected with MITOL WT plasmids 0.5  $\mu$ g (+) and 5  $\mu$ g (++) stably expressing HA-Parkin and mt-Keima were treated with CCCP (10  $\mu$ M) for 8 h. Then, mKeima was measured at 488 (pH 7) and 561 (pH 4) nm lasers using Flow Cytometer. Percentages of mitophagy were calculated from 30,000 cells in each independent experiment. Data represent the mean  $\pm$  SD of three independent experiments. For statistical analysis, a one-way ANOVA with Tukey post-test was performed, \*\* $P$  < 0.01.

conformational changes. We found that MITOL mediates ubiquitination of Parkin at the K220 residue and promotes the degradation of Parkin. As MITOL degrades phosphorylated Parkin rather than unphosphorylated form, it can be considered that although it is not certain whether MITOL-mediated Parkin degradation is dependent on the structure of Parkin or not, MITOL can selectively recognize and degrade phosphorylated Parkin. Because the degradation of Parkin by endogenous MITOL is milder than that by overexpressed MITOL, the timing of degradation in endogenous MITOL is extremely slow. When Parkin is degraded at an early stage, mitophagy is strongly inhibited. This suggests that if the amount of phosphorylated Parkin does not surpass the threshold at the appropriate time, mitochondrial degradation might not occur. Based on this, we considered that endogenous MITOL mildly degrades phosphorylated Parkin at the appropriate timing to prevent any hindrance to quality control of the cells via Parkin. On the other hand, it has been recently reported that protein ubiquitination by MITOL is involved in Parkin recruitment and activation during the early phase of mitophagy (Koyano *et al*, 2019b), suggesting distinct roles of MITOL in the early and late phases. As mitophagy proceeded even when MITOL was completely depleted, it was concluded that although MITOL may be partially involved in Parkin recruitment, it is not essential for Parkin-dependent mitophagy.

In this study, we found that MITOL translocated to the ER in the latter phase of mitophagy. A previous study revealed that the

mechanism of the translocation of FKBP38 to the ER depends on the number of basic amino acids on the C-terminal domain of the protein as high basicity in the C-terminal domain disturbs the translocation to the ER (Saita *et al*, 2013). However, the C-terminal domain of MITOL possesses seven basic amino acids, suggesting that MITOL cannot leave the mitochondria by itself. Indeed, FKBP38 knockdown abolished MITOL translocation to the ER, suggesting that MITOL is transported by FKBP38. In the present study, we were unable to clarify how MITOL, which is tightly integrated into the mitochondrial outer membrane, is translocated to the ER by FKBP38, or the mechanism underlying the translocation of Parkin. As Parkin is phosphorylated by PINK1 on the mitochondria, phosphorylated Parkin accumulated in the ER might pass through the mitochondria once. MITOL is known to be abundant in the membrane contact sites between the mitochondria and ER, where it regulates the substrate IRE1 $\alpha$  (Sugiura *et al*, 2013; Takeda *et al*, 2019). However, as p97 reduces the contact sites between these two organelles, which are associated with mitophagy, translocation might not be mediated through the contact sites between ER and mitochondria (McLelland *et al*, 2018). Disruption of the mitochondrial outer membrane, which is triggered by the degradation of the substrates of Parkin, has been revealed to be essential for mitophagy (Yoshii *et al*, 2011; Wei *et al*, 2017). In a recent study, Koyano *et al* (2019a) reported that MITOL was translocated to the peroxisome in a Parkin

**Figure 3. MITOL translocates to the ER with FKBP38 in the late stage of mitophagy.**

- A MITOL was not degraded in mitophagy. HeLa cells stably expressing HA-Parkin were treated with DMSO or CCCP (10  $\mu$ M) for 48 h and subjected to an IB assay with the indicated antibodies.
- B Schematic diagram of EGFP knock-in for N-terminal tagging. MITOL-specific and PITCH-specific sgRNAs expressed from pX330A-MITOL/PITCH (not shown) individually target the MITOL exon 1 locus and the donor vector. This allows for both the cleavage of the genomic locus and the release of the EGFP-containing cassette. MMEJ leads to the repair of double-strand break via the insertion of the EGFP-containing cassette, resulting in endogenously EGFP tagged MITOL.
- C Endogenous MITOL translocates to the ER in later phase of mitophagy. EGFP-MITOL knock-in HeLa cells were transfected with HA-Parkin and treated with DMSO or CCCP (10  $\mu$ M) as indicated times. Cells were fixed, permeabilized, and subjected to immunofluorescence analysis with the indicated antibodies. Colocalization was quantified by Manders's coefficient. Means  $\pm$  SEM of more than 10 cells obtained from three independent experiments. For statistical analysis, a one-way ANOVA with Tukey's multiple comparisons test was performed, \*\*\*\* $P$  < 0.0001. Scale bar represents 1  $\mu$ m.
- D MITOL interacts with FKBP38. HeLa cells or HeLa cells stably expressing mCherry-Parkin were transfected with the indicated vectors and treated with DMSO or CCCP (10  $\mu$ M) for indicated times. Lysates of cells were subjected to an IP-IB assay with the indicated antibodies.
- E ER-localized MITOL is transported from the mitochondria and is not newly synthesized protein. HeLa cells stably expressing HA-Parkin were co-transfected with vectors for MITOL-KikGR and FLAG-FKBP38. After ultraviolet light (365 nm) exposure to the whole plate, cells were incubated with or without CCCP (10  $\mu$ M) for indicated time, and monitored for red and green KikGR fluorescence. Scale bar, 10  $\mu$ m. Higher magnification images of the boxed regions are shown in the small panel. Right panel is schematic experimental model for KikGR in this study.



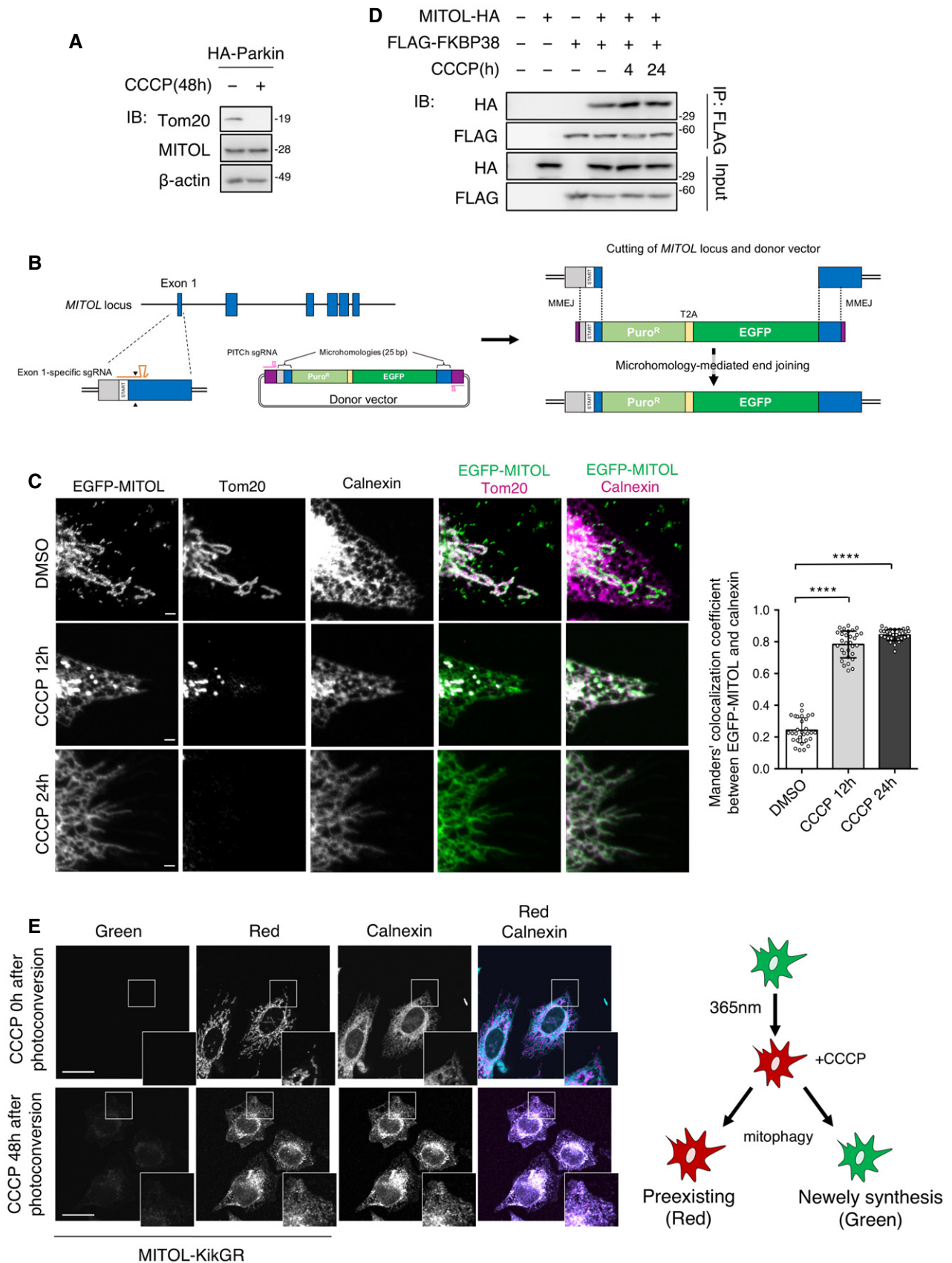


Figure 3.





**Figure 4. MITOL selectively recognizes phosphorylated form of Parkin.**

- A Phosphorylated Parkin accumulates in MITOL KO cells. HeLa cells stably expressing HA-Parkin were treated with DMSO or CCCP (10  $\mu$ M) for the indicated time and subjected to IB assay with the indicated antibodies. Other cell lysates were subsequently separated on a Phos-tag gel, followed by IB with anti-Parkin antibodies (Phos-tag). p-Parkin, phosphorylated Parkin.
- B Accumulation of activated Parkin in MITOL KO cells. WT or MITOL KO HeLa cells stably expressing HA-Parkin were treated with CCCP (10  $\mu$ M) for 30 h and then performed Duolink in situ proximity ligation (PLA) assay demonstrating the interaction between Parkin and Ubiquitin. Scale bar, 5  $\mu$ m. Higher magnification images of the boxed regions are shown in the small panel.
- C Phosphorylated Parkin accumulates in the ER fraction in the absence of MITOL. WT or MITOL KO HeLa cells stably expressing HA-Parkin were treated with DMSO or CCCP (10  $\mu$ M) for 48 h. Lysates of cells were fractionated into cytosolic, mitochondrial, and ER fractions, and then subjected to an IB assay with the indicated antibodies. Phospho-Ser65-Parkin is detected by using specific antibody. Right panels are the quantification of IB about Phospho-Ser65-Parkin protein levels of the ER fraction. The data represent the mean  $\pm$  SD for three independent experiments. For statistical analysis, a one-way ANOVA with Tukey post-test was performed,  $**p < 0.01$ .
- D Parkin is detected at the ER in the absence of MITOL. HeLa cells were transfected with vectors for KikGR-Parkin. After ultraviolet light (365 nm) exposure to the whole plate, cells were incubated with or without CCCP (10  $\mu$ M) for indicated time, and monitored for red and green KikGR fluorescence. Scale bar, 10  $\mu$ m. Higher magnification images of the boxed regions are shown in the small panel.

ubiquitination-dependent manner. We also found that the ubiquitination of MITOL by Parkin occurred in the early stage of mitophagy and thereafter disappeared in the late stage of mitophagy (Fig EV2). At present, it is not clear whether MITOL translocates to the ER via the peroxisome or directly, but it is considered that the loss of MITOL ubiquitination in the late stage of mitophagy might be key for this translocation.

Although previous studies using mass spectrometric analysis have suggested the possibility that the anti-apoptotic protein FKBP38 is one of the substrates of Parkin (Sarraf *et al*, 2013; Rose *et al*, 2016), it has not been experimentally proven yet. In this study, we have demonstrated that Parkin interacts with, ubiquitinates and degrades FKBP38, indicating that FKBP38 is a physiological substrate of Parkin during mitophagy. In addition, we have shown that the accumulation of activated Parkin in MITOL KO cells induced the degradation of FKBP38, resulting in enhanced cell death. However, complete mitigation of cell death by the FKBP38 mutant, which could not be degraded by Parkin, was not completely achieved, and thus, there may be other anti-apoptotic protein(s) that are ubiquitinated by Parkin in the ER. Further studies are required to clarify the cytotoxic pathway triggered by hyperactivation of Parkin in the ER. Our findings further

uncover the physiological significance of Parkin by showing that Parkin performs mitochondrial quality control as well as actively induces cell death via the degradation of FKBP38 when the mitochondria are severely damaged, thereby maintaining cellular homeostasis.

On the other hand, during mitophagy, MITOL may respond to mitochondrial damage functionally or quantitatively, and regulate cell death through Parkin degradation. The studies conducted so far have shown that physiologically, Parkin dysfunction can induce cytotoxicity by allowing the accumulation of the substrate (Xu *et al*, 2002). This phenomenon has also been demonstrated in a familial loss-of-function mutant of PD (Dawson & Dawson, 2010). However, in patients with sporadic PD, Parkin is mostly functionally normal. Furthermore, in Parkin KO mice, the loss of dopaminergic neurons, which is a pathological hallmark of PD, was not observed (Goldberg *et al*, 2003; Itier *et al*, 2003; von Coelln *et al*, 2004; Perez & Palmiter, 2005). Taking these facts into consideration, it is possible that Parkin itself might partially contribute to the pathology of PD by inducing cell death and the disruption of the Parkin degradation by MITOL may cause excessive Parkin activation, thereby inducing cell death and causing sporadic PD. If this is the case, MITOL could be considered as a potential therapeutic target for PD.

**Figure 5. MITOL protects FKBP38 by degrading phosphorylated Parkin for cell survival.**

- A Ubiquitination of FKBP38 depends on Parkin E3 activity. HeLa cells were transfected with the indicated vectors and treated with DMSO or CCCP (10  $\mu$ M) for indicated times. MG132 (30  $\mu$ M) was added 1 h before each treatment. Lysates of cells were subjected to an IP-IB assay with the indicated antibodies. WT, wild-type.
- B Parkin fails to ubiquitinate the FKBP38 K271/273R mutant. HeLa cells were transfected with the indicated vectors and treated with CCCP (10  $\mu$ M) for 30 h. MG132 (30  $\mu$ M) was added 5 h after CCCP treatment. Lysates of cells were subjected to an IP-IB assay with the indicated antibodies and compared ubiquitination levels of WT, K271R, K273R, and K271/273R FLAG-FKBP38.
- C Overexpression of WT FKBP38 is degraded in MITOL KO cells in mitophagy. MITOL KO HeLa cells stably expressing HA-Parkin were transfected with WT or K271/273R mutant FLAG-FKBP38 and treated with DMSO or CCCP (10  $\mu$ M) for 24 h. Lysates of cells were subjected to an IB assay with the indicated antibodies.
- D MITOL KO increases degradation of ER targeting FKBP38. HeLa cells stably expressing HA-Parkin were transfected with mitochondrial targeting FLAG-FKBP38 (FKBP38<sup>AC13</sup>) and ER targeting FLAG-FKBP38 (FKBP38<sup>YFF1</sup>), and treated with DMSO or CCCP (10  $\mu$ M) for 24 h. Lysates of cells were subjected to an IB assay with FLAG antibodies for organelle targeting FKBP38 or indicated antibodies. Another cell lysates were subsequently separated on a Phos-tag gel, followed by IB with anti-Parkin antibodies (Phos-tag).
- E–G MITOL partly prevents CCCP-induced cell death by reducing Parkin-mediated degradation of FKBP38. WT or MITOL KO HeLa cells stably expressing HA-Parkin and HeLa cells were transfected with the indicated vectors, and treated with DMSO or CCCP (10  $\mu$ M) for 24 h. Cells were stained with propidium iodide (PI) for counting the percentage of dead cells using fluorescence microscopy. Data represent the mean  $\pm$  SD of five independent experiments (>100 individual cells were counted). For statistical analysis, a one-way ANOVA with Tukey post-test was performed,  $**P < 0.01$ . KR, FKBP38 K271/273R.

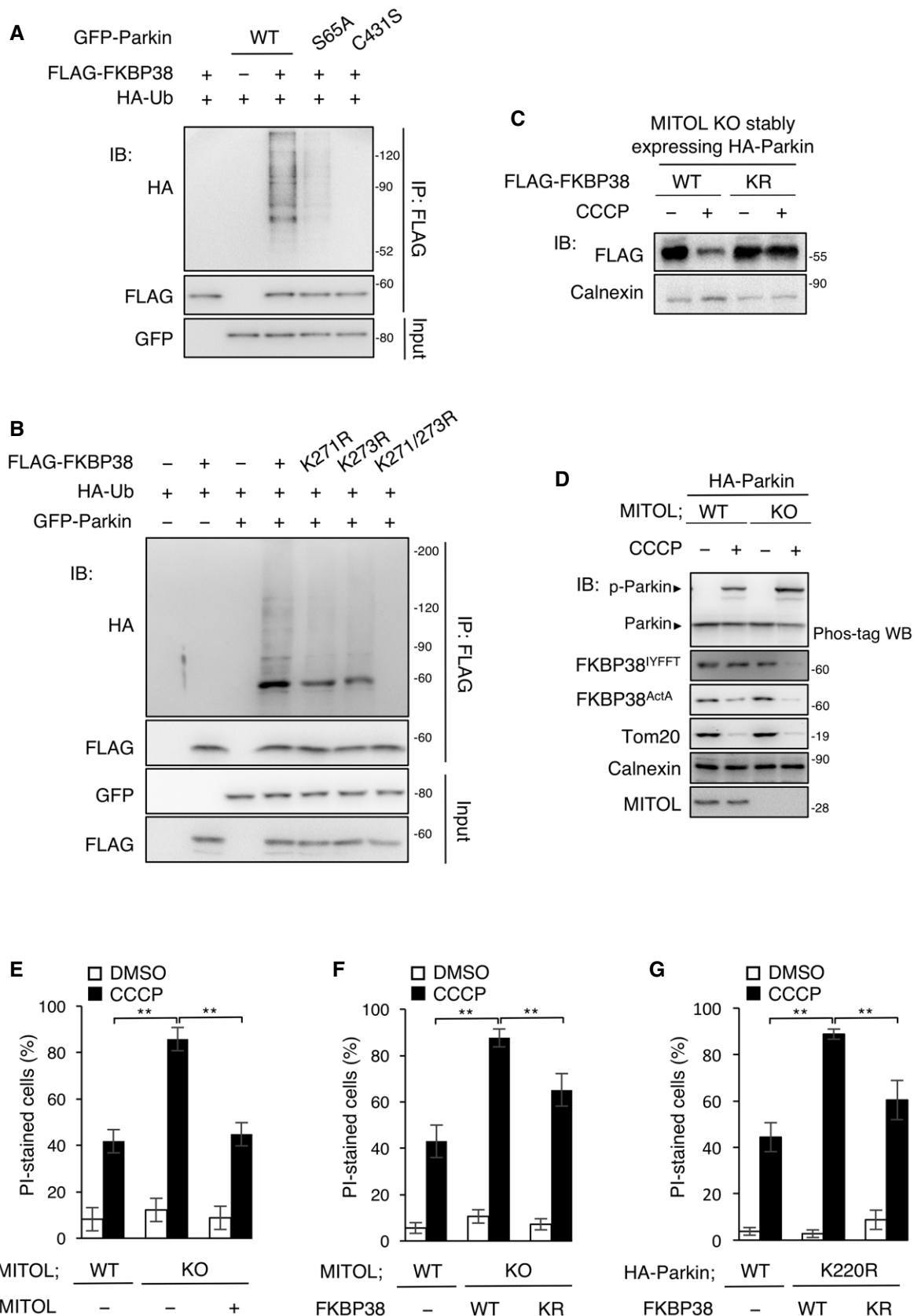
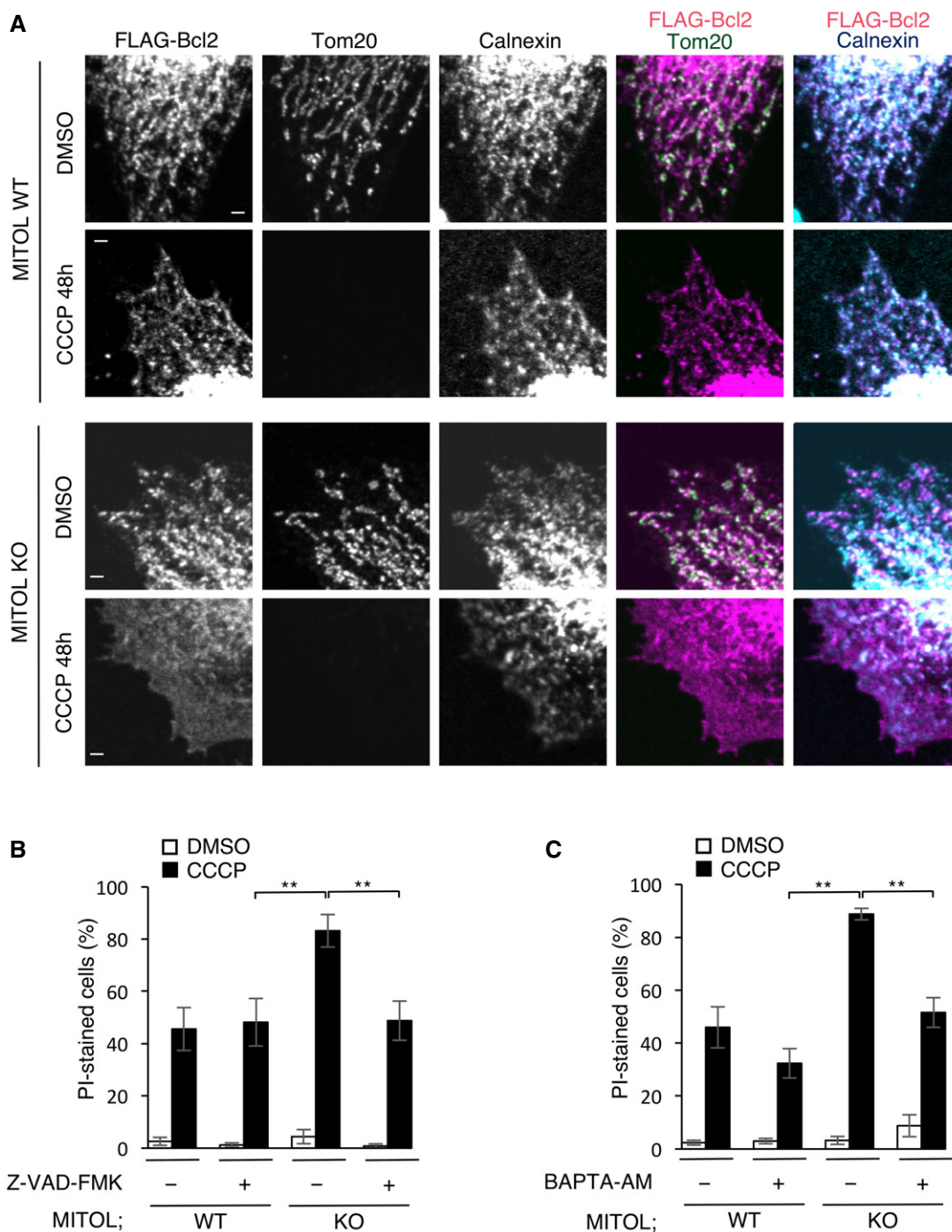


Figure 5.



**Figure 6. MITOL deletion causes mislocalization of Bcl-2 and leads to excessive cell death via calcium leak in mitophagy.**

- A** Mislocalization of Bcl-2 is observed in MITOL KO cells in mitophagy. WT or MITOL KO HeLa cells stably expressing HA-Parkin were transfected with FLAG-Bcl2 and treated with DMSO or CCCP (10  $\mu$ M) for the indicated times. Cells were immunostained with indicated antibody. Scale bar, 1  $\mu$ m.
- B** MITOL prevents caspase-dependent cell death in mitophagy. WT or MITOL KO HeLa cells stably expressing HA-Parkin and HeLa cells were treated with DMSO or CCCP (10  $\mu$ M) with or without Z-VAD-FMK (10  $\mu$ M) for 24 h. Cells were stained with propidium iodide (PI) for counting fluorescence microscopy of the percentage of dead cells. Data represent the mean  $\pm$  SD of three independent experiments (>100 individual cells were counted). For statistical analysis, a one-way ANOVA with Tukey post-test was performed, **\*\*** $P$  < 0.01.
- C** Cell death in mitophagy caused by MITOL deficiency depends on calcium leak. WT and MITOL KO HeLa cells stably expressing HA-Parkin or HeLa cells were treated with DMSO or CCCP (10  $\mu$ M) with or without BAPTA-AM (10  $\mu$ M) for 24 h. Cells were stained with propidium iodide (PI) for counting fluorescence microscopy of the percentage of dead cells. Data represent the mean  $\pm$  SD of three independent experiments (>100 individual cells were counted). For statistical analysis, a one-way ANOVA with Tukey post-test was performed, **\*\*** $P$  < 0.01.

## Materials and Methods

### Antibodies and reagents

Anti-MITOL rabbit polyclonal antibody was produced as described previously. Anti- $\beta$ -actin and anti-FLAG-M2 antibodies were purchased from Sigma. Anti-Parkin and anti-VDAC were purchased from Cell Signaling Technology. Anti-Parkin (phospho-Ser65) was purchased from UBIQUIGENT. Anti-Tom20 and anti-calnexin were purchased from Santa Cruz Biotechnology. Anti-ubiquitin and anti-GFP were purchased from MBL. Anti-myc was obtained from Recentec. Anti-HA was obtained from Proteintech. Anti-GST and BAPTA-AM were purchased from Wako. Anti-HSP60 was purchased from Enzo Life Sciences. Anti-FKBP38 and anti-K63-chain were purchased from Abcam. Anti-K48-chain was purchased from Millipore. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and chloroquine diphosphate were obtained from Wako. MG132, lactacystin, Z-VAD-FMK, and bafilomycin A1 were obtained from Peptide Institute. Cycloheximide (CHX) was purchased from Sigma.

### DNA constructs

Mitochondrial ubiquitin ligase (MITOL), ubiquitin mutant expression vector, and Mito-GFP were constructed as described previously (Sugiura *et al*, 2013). To generate stably expressing cells, HA-Parkin and mCherry-Parkin were subcloned into the pCX4 retrovirus vector (Akagi *et al*, 2000). The recombinant GST fusion proteins GST-MITOL and GST-Parkin were generated by subcloning into the pGEX-3X vector. Point mutations of Parkin and FKBP38 were generated with the site-directed mutagenesis kit (Stratagene). The expression plasmids for human Parkin and FKBP38 were generated by amplifying the corresponding cDNA by PCR and cloning it into pFLAG-CMV2, or pEGFP-C1 expression vectors. FLAG-Bcl2 (Addgene #18003) and mCherry-Sec61 $\beta$  (Addgene #49155) were purchased from Addgene. To generate localization-restricted FKBP38, mitochondrial FKBP38 (FKBP38<sup>ActA</sup>) and ER FKBP38 (FKBP38<sup>YFFT</sup>) were constructed as previously described (Zhu *et al*, 1996; Rojo *et al*, 2002). Complementary DNA was subcloned into the humanized-codon monomeric KikGR vector (MBL). Plasmid pX330A-MITOL/PITCh, an all-in-one CRISPR-Cas9 vector for cutting the genomic MITOL exon 1 locus and the donor vector, was constructed using the pX330A-1x2 (Addgene #58766) plasmid and pX330S-2-PITCh (Addgene #63670), in accordance with a previously described protocol (Sakuma *et al*, 2016). The oligonucleotides for the template of sgRNA targeting MITOL exon 1 are CRISPR-hMITOL\_sense (5'-caccGCAAGCCCTACAGCAGATGC-3') and CRISPR-hMITOL\_antisense (5'-aacGCATCTGCTGTAGGCTTGGC-3'). The donor vector, including PuroR, EGFP, and microhomologies, was constructed from pCRIS-PITChv2-FBL (Addgene #63672) using a standard PCR and a SLICE cloning method (Motohashi, 2015).

### Cell culture and transfection

HeLa cells and SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cells were transfected with either Lipofectamine 3000 (Invitrogen) or RNAiMax (Invitrogen) according to the manufacture's protocol. *siPINK1* has been

described previously (Villa *et al*, 2017). *siMITOL* was described previously (Yonashiro *et al*, 2009). Scrambled siRNA and *siFKBP38* were purchased from Qiagen.

### Generation of stable cell lines

Stable cell lines were generated using a retroviral expression system as previously described (Akagi *et al*, 2003). Briefly, Plat E cells were transfected with retroviral vectors using Lipofectamine LTX Reagent with PLUS Reagent (Invitrogen). After 48 h, the growth medium containing retrovirus was collected. HeLa cells were incubated with the collected virus-containing medium with 8  $\mu$ g/ml polybrene (Sigma) for 24 h.

### MITOL KO Cells

The CRISPR Design Tool (<http://www.genome-engineering.org/crispr/>) was used to select the genomic sequence target in *hMITOL* (5'-caccgcaagccctacagcagatgc-3'). Oligo pairs encoding 20-nt guide sequences were annealed and ligated into the plasmid pX330. HeLa cells were transfected using Lipofectamine 3000 (Invitrogen), and KO clones were selected by serial dilution.

### Generation of EGFP Knock-in cell line

HeLa cells were plated in a 10-cm dish and co-transfected with 1.5  $\mu$ g of the donor vector and 3  $\mu$ g of pX330A-MITOL/PITCh using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's instructions. After 72 h of transfection, cells were selected with 1  $\mu$ g/ml puromycin for 7 days.

### Subcellular fractionation

(Organelle fraction) HeLa cells or HeLa cells expressing HA-Parkin were homogenized in homogenization buffer (HB; 5 mM HEPES pH 7.4, 0.5 mM EDTA, 250 mM mannitol). The homogenate was centrifuged at 800 g for 10 min at 4°C, and the resulting supernatant was further centrifuged at 8,000 g for 10 min at 4°C. The new pellet (crude mitochondrial fraction) was collected, and the supernatant was centrifuged further at 100,000 g for 1 h at 4°C. The resulting pellet (light membrane fraction) and supernatant (cytosolic fraction) were collected. The crude mitochondrial fraction was centrifuged at 100,000 g for 30 min at 4°C. The resulting mitochondrial and ER bands were isolated and resuspended in an isolation buffer (mitochondria-rich membrane fraction). Cells were harvested and suspended in cell homogenization buffer. Cells were homogenized by 20 passages through a 27-gauge needle with a 1-ml syringe, on ice. The homogenates were separated into post-nuclear supernatant by centrifugation at 1,000 g for 5 min at 4°C. The heavy membrane fraction was separated by spinning down supernatant from PNS (post-nuclear supernatant) separation at 3,000 g for 10 min at 4°C. To wash the heavy membrane fraction, the pellet was resuspended in fresh buffer and spun back twice. The supernatant from the previous fractionation was spun down at 16,000 g for 15 min at 4°C to collect the light membrane fraction. The remaining supernatant was the cytosolic fraction (Sup). Light membranes and heavy membranes were combined to form the membrane fraction (Memb).

## Immunoprecipitation

To examine the protein interaction, cells were solubilized with NP-40 lysis buffer (1% NP-40, 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 10 mM NaF, and protease inhibitors) and centrifuged at 15,000 *g* for 20 min at 4°C. The supernatant was subjected to immunoprecipitation using the indicated antibodies. To evaluate ubiquitylation levels of protein, cells were solubilized with RIPA lysis buffer (0.1% SDS, 0.05% DOC, 1% Triton X-100, 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, and protease inhibitors) and centrifuged at 15,000 *g* for 20 min at 4°C. The supernatant was sonicated 10 s and subjected to immunoprecipitation using the indicated antibodies.

## Immunoblotting and Phos-tag PAGE

Whole lysates were separated by SDS-PAGE and transferred to the PVDF membranes (Millipore). The blots were probed with the indicated antibodies, and protein bands on the blot were visualized using the enhanced chemiluminescence reagent (Millipore). Phos-tag-PAGE was performed using 8% SDS-PAGE minigels containing 10  $\mu$ M Phos-tag (Wako) in the presence of 100  $\mu$ M MnCl<sub>2</sub> following the manufacturer's protocol.

## GST pull-down assay

To determine the specific domains required for the interaction between MITOL and Parkin, GST fusion deletion mutants were prepared as described previously (Yonashiro *et al*, 2006). Lysates from HeLa cells or HeLa cells stably expressing HA-Parkin were incubated with the purified GST fusion proteins. Precipitates were analyzed by immunoblotting.

## Morphological analysis by immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature, then washed twice with PBS, permeabilized with 0.2% Triton X-100 in PBS for 5 min, then washed four times with PBS, and blocked with 1% bovine serum albumin in PBS, all at room temperature. For double staining, the cells were incubated with appropriate primary Abs for 1 h at room temperature, washed three times with PBS, and then incubated with appropriate secondary Abs for 30 min. The samples were washed as described above, mounted using Fluorescent Mounting Medium (Dako), and analyzed using an Olympus FV1000 and FV3000 confocal laser scanning microscope.

## Photoconversion analysis

KikGR-labeled proteins expressed in cells were photoconverted with a 365-nm UV light for 5 min using the Benchtop UV Transilluminator, LM-26 (2UV; Funakoshi).

## In situ Proximity Ligation Assay

HeLa cells stably expressing HA-Parkin were 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature, then washed twice with PBS, permeabilized with 0.2% Triton X-100 in PBS for 5 min, then washed four times with

PBS, and blocked with 1% bovine serum albumin in PBS, all at room temperature. The Duolink in situ proximity ligation assay was carried out according to the manufacturer's instructions (Olink Bioscience, Sigma) using the following primary antibodies, anti-Parkin and anti-ubiquitin, and analyzed using an Olympus Fluoview FV1000 laser scanning confocal microscope.

## PI Staining

Plasma membrane integrity based on the exclusion of PI was measured as an index of dead cells. Cells were stained at 37°C for 10 min with PI (1  $\mu$ M final concentration). After incubation, stained cells were scored as dead cells under a fluorescence microscope.

## Mt-Keima-based mitophagy assay

For the detection of mitophagy, HeLa cells stably expressing mt-Keima transiently transfected with the plasmid as indicated, and MITOL WT and KO HeLa cells stably expressing HA-Parkin and mt-Keima were treated with 10  $\mu$ M CCCP for 8 h. For measurement of mKeima, a BD LSR II Flow Cytometer was used to detect the dual-excitation ratiometric pH at 488 (pH 7) and 561 (pH 4) nm lasers with 695/40 and 575/26 nm emission filters. For each sample, 30,000 cells were counted and analyzed using FlowJo software (version 10.7.1).

## Ubiquitin restriction assay

Ubiquitin restriction assay was performed using the UbiCRest kit (Boston Biochem) following the manufacturer's instructions. Recombinant AMSH, OTUB1, and USP2 were used for the reaction. Parkin was immunoprecipitated from HA-Parkin stably expressing cells treated with CCCP (10  $\mu$ M, 3 h) as described in the above section. After washing with NP-40 buffer three times, the beads were washed with reaction buffer for additional two times. The purified Parkin was then incubated with either with AMSH, OTUB1, Usp2, or buffer only (reaction buffer), in a 20  $\mu$ l reaction, for 1 h at 37°C. The reaction was terminated by the addition of SDS loading buffer. The samples were separated by SDS-PAGE and analyzed by Western blotting with the indicated antibody.

## Statistical analysis

Results are expressed as either mean  $\pm$  SD or  $\pm$  SEM. The obtained data were performed either two-tailed Student's *t*-test or a one-way ANOVA followed by *post hoc* Tukey test to determine significance.

## Data availability

No primary data sets deposited in a public database.

**Expanded View** for this article is available online.

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### Author contributions

IS, KT, NI, TT, SN, and SY conceived the project and designed the experimental strategy. IS, SN, KT, and NI performed the molecular biology experiments. S-IY, TKa, TKo, YU, and YF provided material and technical assistance. IS and SY wrote the manuscript with contribution from RI.

### Conflict of interest

The authors declare that they have no conflict of interest.

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