Optineurin is an autophagy receptor for damaged mitochondria in parkin-mediated mitophagy that is disrupted by an ALS-linked mutation

Yvette C. Wong and Erika L. F. Holzbaur[1]

Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6085

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Mitophagy is a cellular quality control pathway in which the E3 ubiquitin ligase parkin targets damaged mitochondria for degradation by autophagosomes. We examined the role of optineurin in mitophagy, as mutations in optineurin are causative for amyotrophic lateral sclerosis (ALS) and glaucoma, diseases in which mitochondrial dysfunction has been implicated. Using live cell imaging, we demonstrate the parkin-dependent recruitment of optineurin to mitochondria damaged by depolarization or reactive oxygen species. Parkin's E3 ubiquitin ligase activity is required to ubiquitinate outer mitochondrial membrane proteins, allowing optineurin to stably associate with ubiquitinated mitochondria via its ubiquitin binding domain; in the absence of parkin, optineurin transiently localizes to damaged mitochondrial tips. Following optineurin recruitment, the omegasome protein double FYVE-containing protein 1 (DFCP1) transiently localizes to damaged mitochondria to initialize autophagosome formation and the recruitment of microtubule-associated protein light chain 3 (LC3). Optineurin then induces autophagosome formation around damaged mitochondria via its LC3 interaction region (LIR) domain. Depletion of endogenous optineurin inhibits LC3 recruitment to mitochondria and inhibits mitochondrial degradation. These defects are rescued by expression of siRNA-resistant wild-type optineurin, but not by an ALS-associated mutant in the ubiquitin binding domain (E478G), or by optineurin with a mutation in the LIR domain. Optineurin and p62/SQSTM1 are independently recruited to separate domains on damaged mitochondria, and p62 is not required for the recruitment of either optineurin or LC3 to damaged mitochondria. Thus, our study establishes an important role for optineurin as an autophagy receptor in parkin-mediated mitophagy and demonstrates that defects in a single pathway can lead to neurodegenerative diseases with distinct pathologies.

mitophagy | autophagosome | optineurin | amyotrophic lateral sclerosis | Parkinson's disease

Damaged mitochondria are selectively turned over in eukaryotic cells via mitophagy, a process in which double-membraned autophagosomes sequester and ultimately degrade mitochondria via lysosomal fusion (1, 2). This process is regulated by phosphatase and tensin homolog-induced putative kinase protein 1 (PINK1) and parkin, two proteins linked to hereditary forms of Parkinson's disease (3, 4). PINK1 is stabilized on the outer membrane of damaged mitochondria and recruits the E3 ubiquitin ligase parkin, which ubiquitinates proteins on the outer mitochondrial membrane (OMM) (5–13). Parkin-mediated ubiquitination of damaged mitochondria is followed by autophagosome formation and engulfment of mitochondria (1, 2). However, the proteins involved in dynamically recruiting autophagic machinery to ubiquitinated damaged mitochondria still remain elusive.

Optineurin is an autophagy receptor, characterized by its ability to bind ubiquitin via its ubiquitin binding in ABIN (A20 binding and inhibitor of NF-κB) and NEMO (NF-κB essential modulator) (UBAN) domain (14) and the autophagosome-associated protein LC3 (microtubule-associated protein light chain 3) via its LC3 interacting region (LIR) domain (15). Optineurin regulates autophagosome maturation (16) and autophagic degradation of *Salmonella* and protein aggregates (15, 17). However, optineurin's role in mitophagy has not been previously studied. Mutations in optineurin lead to primary open-angle glaucoma (18) and amyotrophic lateral sclerosis (ALS) (19), two neurodegenerative diseases in which mitochondrial defects have been observed (20, 21). Thus, optineurin may play a role in regulating the autophagic turnover of damaged mitochondria during mitophagy.

Here, we use confocal live cell imaging to show that parkin is both necessary and sufficient to stabilize optineurin on the surface of damaged mitochondria. In the absence of parkin, optineurin puncta transiently localize to damaged mitochondria but do not remain stably associated. In cells expressing parkin, optineurin is recruited to mitochondria following parkin recruitment, and this recruitment is stabilized via the UBAN domain. Following optineurin recruitment, double FYVE-containing protein 1 (DFCP1) puncta transiently localize to parkin/optineurin-labeled damaged mitochondria to mark the initial site of autophagosome formation (22). This is followed by LC3 recruitment and subsequent autophagosome formation around optineurin-labeled damaged mitochondria. Importantly, we find that depletion of optineurin inhibits autophagosome recruitment to damaged mitochondria, leading to increased levels of the mitochondrial matrix protein Hsp60 and mtDNA content within cells. This defect in mitochondrial degradation is rescued by wild-type optineurin but not by the E478G UBAN mutant in optineurin causative for ALS (19) or by an optineurin LIR mutant unable to bind LC3 (15). Optineurin and p62, previously

Significance

In mitophagy, damaged mitochondria recruit parkin to ubiquitinate proteins on the outer mitochondrial membrane, targeting mitochondria for autophagosome engulfment and degradation. However, the proteins involved in mediating autophagosome formation to degrade damaged and ubiquitinated mitochondria remain unknown. We used live cell imaging to demonstrate that optineurin is actively recruited to parkin-labeled ubiquitinated mitochondria and is stabilized by its ubiquitin binding domain. Optineurin binds the autophagosome protein LC3 (microtubule-associated protein light chain 3), and this binding recruits autophagosome assembly around damaged mitochondria. We find that the E478G optineurin mutation, causative for the neurodegenerative disease amyotrophic lateral sclerosis, disrupts autophagosome recruitment. As mutations in parkin are linked to Parkinson's disease, this study indicates that defects in a single mitochondrial degradation pathway lead to neurodegenerative diseases with distinct pathologies.

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[1]To whom correspondence should be addressed. Email: holzbaur@mail.med.upenn.edu.

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implicated in mitophagy (23–26), are independently recruited to distinct domains on damaged mitochondria. In contrast to our observations with optineurin, depletion of p62 did not inhibit LC3 recruitment or efficient degradation of damaged mitochondria. Thus, our study shows an important role for the autophagy receptor optineurin in parkin-mediated mitophagy and provides support for the hypothesis that defective mitochondrial quality control may contribute to ALS pathogenesis.

Results

Optineurin Transiently Associates with Damaged Mitochondria in the Absence of Parkin. In untreated HeLa cells, GFP-labeled optineurin (GFP-Optn) is predominantly cytosolic (27) with few puncta per cell that rarely localize to mitochondria (Fig. 1*A*, *Upper* and Fig. S1*B*). We induced mitochondrial damage with the mitochondrial uncoupler carbonyl-cyanide *m*-chlorophenylhydrazone (CCCP), which depolarizes mitochondria. Mitochondria became more fragmented (Fig. 1*A*, *Lower* and Fig. S1*A*) but remained localized throughout the cell after 1 h of CCCP treatment. Following CCCP treatment, ~25% of optineurin puncta were observed to transiently associate with damaged mitochondria (Fig. 1 *A*, *Lower* and *B* and Fig. S1*C*). Following dissociation from mitochondria, optineurin puncta either rebind to a neighboring mitochondria or remain cytosolic (Fig. 1*C* and Movie S1).

Optineurin puncta preferentially localized to a single tip of an elongated mitochondria rather than along the length of the organelle (Fig. 1 *D–F*, Fig. S1*D*, and Movie S2). After 2 h of CCCP treatment, mitochondria became fragmented and punctate, whereas 6 h after treatment, the majority of mitochondria were circular rather than elongated (Fig. S2 *A* and *B*). Fewer optineurin puncta localized to mitochondrial tips following prolonged mitochondrial damage (Fig. 1*B*). However, even at 6-h CCCP, we observed optineurin puncta that transiently localized to a single point on damaged circular mitochondria or colocalized with extremely fragmented punctate mitochondria (Fig. S2*B*). Importantly, immunostaining of endogenous optineurin in CCCP-treated HeLa cells confirmed the specific localization of optineurin to the tips of damaged mitochondria (Fig. S2 *C* and *D*). Thus, in HeLa cells, which express a low level of endogenous parkin (28, 29), optineurin specifically but transiently localizes to the tips of damaged mitochondria.

Parkin Ubiquitination Stabilizes Optineurin Recruitment to the Outer Membrane of Damaged Mitochondria. Next, we used live cell microscopy to image the dynamics of parkin during mitophagy in CCCP-treated HeLa cells. The E3 ubiquitin ligase parkin is recruited from the cytosol to the OMM of damaged mitochondria via a PINK1-dependent pathway (5–8), where it ubiquitinates OMM proteins including mitochondrial fusion proteins Mfn1 and Mfn2 (9–13). We expressed YFP-Parkin in HeLa cells treated with CCCP for 1 h and showed that parkin was gradually recruited to the surface of mitochondria (DsRed2-mito), as previously noted (5) (Fig. S3*A* and Movie S3). Under confocal microscopy, we could visualize parkin rings forming around spherical mitochondrial fragments within a single *z* plane, indicative of parkin recruitment to the mitochondrial surface (Fig. 2*A*). About 50% of mitochondria were parkin-labeled by 30 min, and 95% of mitochondria were parkin-labeled by 1 h of CCCP treatment (Fig. S3 *B–D*). Prolonged mitochondrial damage (2-h CCCP) resulted in the aggregation of fragmented mitochondria, with parkin recruited to the surface of each fragment (Fig. 2 *B* and *C*), rather than to the surface of the entire mitochondrial aggregate. Further mitochondrial damage (6-h CCCP) resulted in the perinuclear localization of mitochondrial aggregates with parkin still stably localized to the surface of individual fragments (Fig. S3*E*).

Next, we examined the dynamics of GFP-Optn in CCCP-treated HeLa cells expressing parkin. In contrast to the transient localization of optineurin puncta to mitochondrial tips observed in the absence of parkin, parkin expression induced the stable recruitment of optineurin all around the surface of damaged mitochondria (Fig. 2*D*). Using confocal microscopy, we imaged a single *z* plane and observed optineurin rings surrounding spherical fragments of mitochondria (Fig. 2*E*). Using time-lapse video microscopy, we found that optineurin was gradually recruited from the cytosol to the surface of damaged mitochondria (Fig. S4*A* and Movie S4). Few mitochondria were labeled with optineurin after 30 min of CCCP treatment, but 60% of mitochondria were labeled with optineurin by 1 h of CCCP treatment (Fig. 2*F*) with optineurin rings forming around mitochondrial fragments

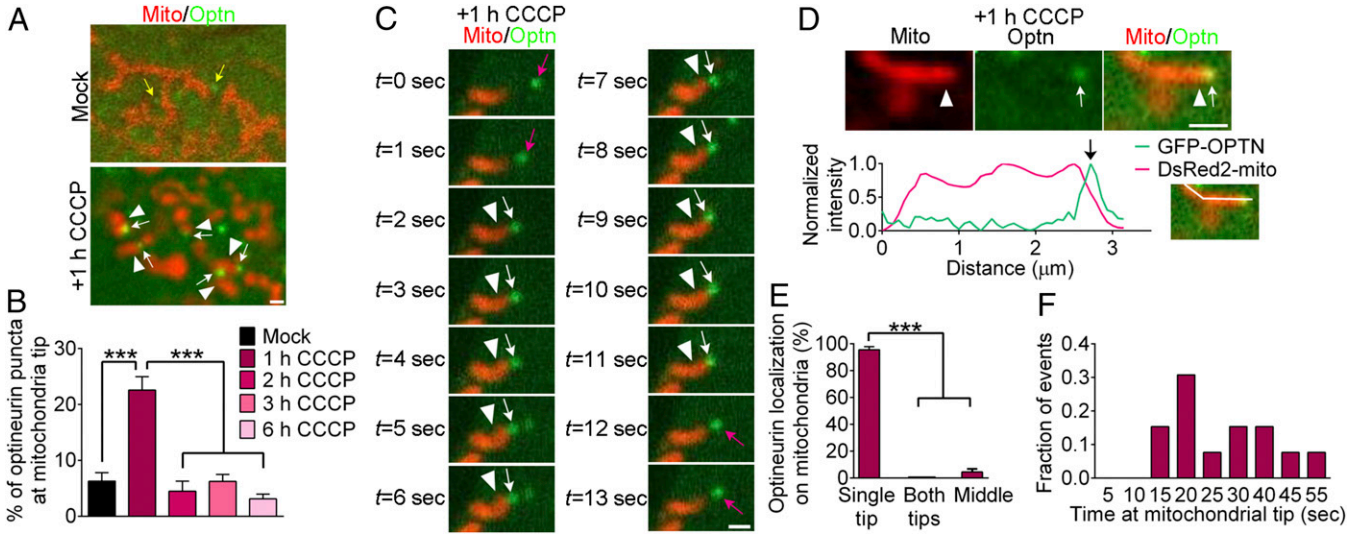

Fig. 1. Optineurin transiently associates with damaged mitochondria in the absence of parkin. (*A*) One hour of CCCP (20 μM) treatment of HeLa cells causes mitochondrial fragmentation. Optineurin puncta (GFP-Optn) in control cells do not associate with mitochondria (DsRed2-mito) (yellow arrows). In HeLa cells in the absence of parkin, CCCP-induced mitochondrial damage causes optineurin puncta to transiently associate (white arrows) with the tips of fragmented mitochondria (arrowheads). (*B*) Optineurin preferentially localizes to damaged mitochondria immediately following CCCP treatment. (*C*) Time series of optineurin recruitment from the cytosol (pink arrows) to a mitochondrial tip (white arrows, optineurin puncta; arrowheads, mitochondria). (*D*) Example of optineurin (arrow) localized to a mitochondrial tip (arrowhead). (*E* and *F*) After 1-h CCCP treatment of HeLa cells, optineurin preferentially localizes to the mitochondrial tip for ~30 s. [Scale bar, (*A*, *C*, and *D*) 1 μm.] Values represent means ± SEM; ***$P < 0.001$.

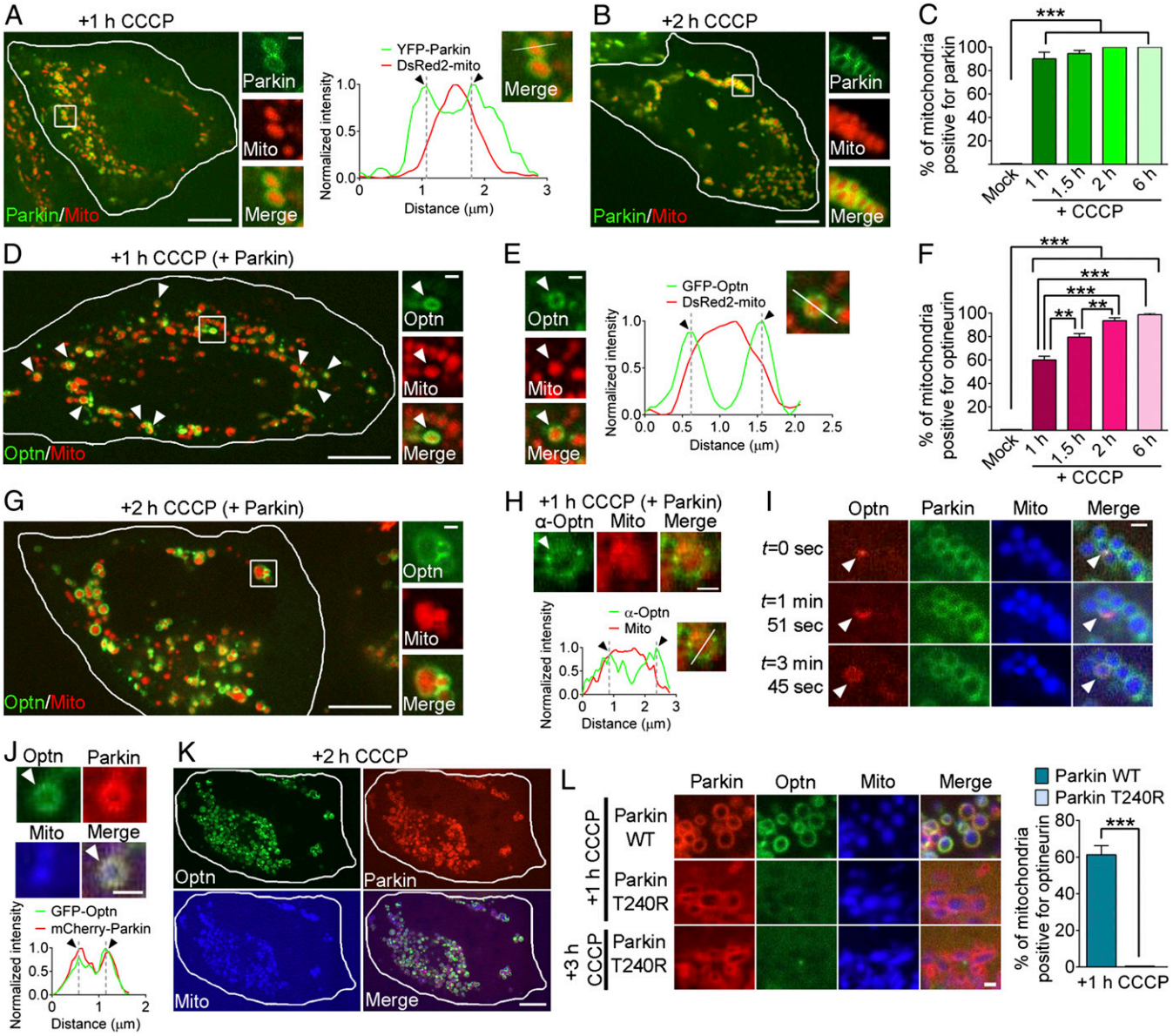

Fig. 2. Parkin ubiquitination stabilizes optineurin recruitment to the outer membrane of damaged mitochondria. (*A*) Confocal image of a HeLa cell expressing DsRed2-mito and YFP-parkin after 1 h of CCCP treatment. Parkin is recruited to the outer membrane of damaged mitochondria, seen as parkin rings around spherical mitochondrial fragments within a single *z* plane, with corresponding line scan (*Right*). (*B*) Two hours of CCCP treatment causes aggregation of parkin-labeled mitochondria. (*C*) The majority of mitochondria are parkin-positive after 1 h of CCCP treatment. (*D*) Confocal image of a HeLa cell expressing parkin, GFP-Optn, and DsRed2-mito treated with CCCP for 1 h. Optineurin is stably recruited to the surface of damaged mitochondria in the presence of parkin. (*E*) Optineurin localization on the outer membrane of damaged mitochondria with corresponding line scan (*Right*). (*F*) Optineurin recruitment lags parkin recruitment. (*G*) Optineurin is recruited to the majority of mitochondria following 2 h of CCCP treatment. (*H*) Immunostaining of endogenous optineurin in 1 h of CCCP-treated HeLa cells expressing parkin and BFP-mito (pseudocolored red) with corresponding line scan (*Lower*). Optineurin is recruited to the outer membrane of damaged mitochondria. (*I*) Confocal time series of a CCCP-treated HeLa cell expressing mCherry-Optn, YFP-parkin, and BFP-mito showing optineurin recruitment only occurs following parkin recruitment to damaged mitochondria. An optineurin puncta is first localized to the surface of parkin-labeled mitochondria and gradually grows into a ring. (*J*) CCCP-treated HeLa cells expressing GFP-Optn, mCherry-parkin, and BFP-mito. Confocal image and corresponding line scan (*Lower*) of damaged mitochondria with both optineurin and parkin localized to its outer membrane. (*K*) Both optineurin and parkin localize to mitochondria following 2 h of CCCP treatment. (*L*) A catalytically inactive Parkinson's disease-associated parkin mutant T240R is unable to recruit optineurin after either 1- or 3-h CCCP. [Scale bar, (*A*, *B*, *D*, *G*, and *K*) 10 μm, (*Insets* in *A*, *B*, *D*, *and G*) 1 μm, and (*E*, *H*, *I*, *J*, and *L*) 1 μm.] Values represent means ± SEM; $^{**}P < 0.01$, $^{***}P < 0.001$.

throughout the cell (Fig. S4*B*). After 2 h of CCCP treatment, the majority of mitochondria were surrounded by optineurin (Fig. 2*G*); this extensive colocalization remained after 6 h of CCCP treatment (Fig. S5*A*). Importantly, immunostaining of endogenous optineurin also showed a parkin-dependent stabilization of recruitment to the surface of damaged mitochondria, confirming our observations with the live cell GFP-Optn probe (Fig. 2*H*).

Next, we compared the dynamics of parkin and optineurin recruitment to damaged mitochondria, by coexpressing fluorescently tagged optineurin and parkin with blue fluorescent protein (BFP)-mito. We found that after 30 min to 1.5 h of CCCP treatment, some mitochondria were labeled with parkin only (Fig. 2*I*), but all optineurin-labeled mitochondria were parkin-positive (Fig. 2 *J* and *K*). For a given mitochondria, parkin was

recruited first, followed by optineurin (Fig. 2*L* and Movie S5). At 1-h CCCP, 67% of parkin-positive mitochondria were also positive for optineurin, and by 1.5-h CCCP, 84% of parkin-positive mitochondria were also positive for optineurin. By 2 h of CCCP treatment, most mitochondria were positive for both parkin and optineurin (Fig. S5 *B–D*) and remained so after 6 h of CCCP treatment (Fig. S5 *E–H*). Expression of mCherry-Optn (rather than GFP-Optn), YFP-parkin, and BFP-mito in HeLa cells provided similar results, with stepwise recruitment of parkin and then optineurin to damaged mitochondria (Fig. S5 *I* and *J*).

To test whether parkin's E3 ubiquitin ligase activity is necessary for optineurin recruitment, we compared the effect of wild-type parkin with a catalytically inactive Parkinson's disease-associated T240R mutant in parkin's RING1 domain (30). After 1 h of CCCP treatment, wild-type parkin was robustly recruited to damaged mitochondria (n = 87/87 cells), whereas parkin T240R showed low levels of recruitment to damaged mitochondria (n = 112/112 cells) (Fig. 2*L*). Optineurin was recruited to mitochondria in cells expressing wild-type parkin (61.3 ± 5.0% of mitochondria after 1-h CCCP), but was not recruited to any mitochondria in T240R parkin-expressing cells (0% of mitochondria after 1-h CCCP) (Fig. 2*L*). After 3-h CCCP, parkin T240R showed increased recruitment to damaged mitochondria, suggesting that T240R parkin can be recruited to damaged mitochondria but perhaps at a slower rate compared with wild-type parkin. However, even at this time point optineurin was not recruited to damaged mitochondria in T240R parkin-expressing cells (n = 54/54 cells) (Fig. 2*L*), demonstrating that optineurin's recruitment to damaged mitochondria is dependent on parkin's E3 ubiquitin ligase activity.

Optineurin Is Dynamically Recruited to Damaged Mitochondria. To spatially regulate the induction of mitochondrial damage, we expressed Mito-KillerRed (Mito-KR) in HeLa cells expressing parkin and GFP-Optn. Illumination of Mito-KR with 561-nm light induces generation of reactive oxygen species (ROS) within the mitochondrial matrix (31), resulting in damage to a spatially localized population of mitochondria within the cell (Fig. 3*A*) and parkin recruitment (32). Optineurin localization was predominantly cytosolic before bleaching (Fig. 3*B*, *Top*). Bleaching of the Mito-KR signal within the illuminated region (Fig. 3*B*, yellow box) led to the striking recruitment of optineurin to the surface of damaged mitochondria within 25 min, seen as the formation of optineurin rings around mitochondria labeled with BFP-mito (Fig. 3 *B* and *C*). After 30 min, optineurin was dynamically recruited to additional mitochondria surrounding the bleached region (Fig. 3 *B* and *C*). In contrast, optineurin was not recruited to mitochondria in a region of the cell distal to the point of focally induced mitochondrial damage (Fig. 3*D*). Optineurin remained localized to the surface of damaged mitochondria 1 h postbleaching proximal to the illuminated region, but was not recruited to mitochondria in the distal region (Fig. 3*E*). Importantly, in HeLa cells that did not express exogenous parkin, we did not observe recruitment of optineurin to damaged mitochondria even 30 min after bleaching (Fig. 3*F*). Thus, expression of parkin leads to the

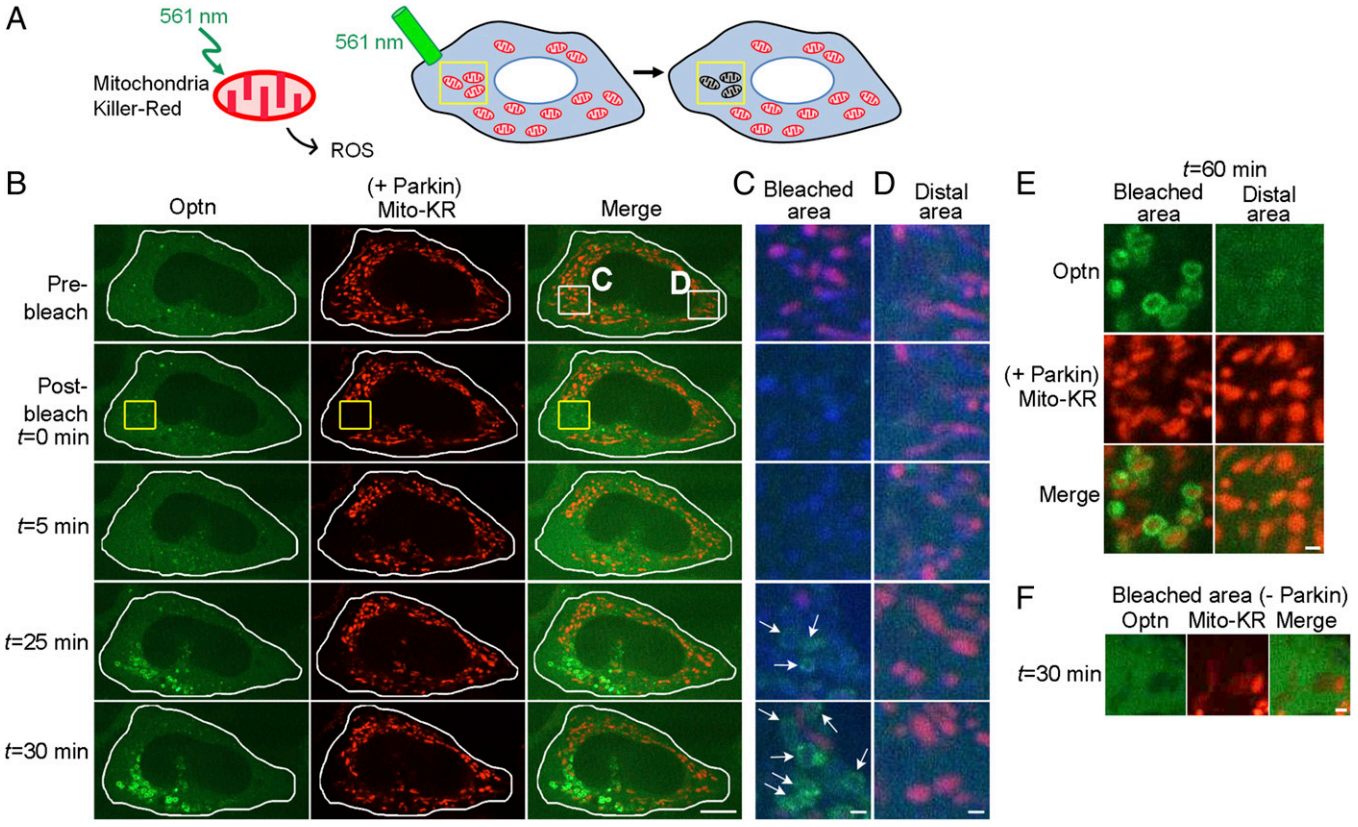

Fig. 3. Optineurin is dynamically recruited to damaged mitochondria via parkin. (*A*, Schematic) Illumination of mitochondrial-targeted mito-KR by 561-nm light leads to the generation of ROS within the mitochondrial matrix. Illumination of a defined region within the cell (yellow box) allows for spatiotemporally controlled damage of a specific mitochondrial population (black mitochondria). (*B*) Confocal image of a HeLa cell expressing parkin, GFP-Optn, mito-KR, and BFP-mito. A defined region was illuminated (yellow box) leading to Mito-KR bleaching and induced mitochondrial damage. Optineurin is recruited to the surface of damaged mitochondria beginning ~25 min after bleaching. (*C* and *D*) Optineurin recruitment is spatially restricted to mitochondria near the bleached region rather than to a distal unbleached region of the cell. (*E*) Optineurin remains localized to mitochondria in the bleached area 1 h after bleaching. (*F*) Optineurin is not recruited to damaged mitochondria in a HeLa cell not expressing exogenous parkin, demonstrating that optineurin recruitment to spatiotemporally restricted damaged mitochondria also requires parkin. [Scale bar, (*B*) 10 μm and (*C–F*) 1 μm.]

spatiotemporally restricted recruitment and stabilization of optineurin on damaged mitochondria.

Optineurin Stabilization on Damaged Mitochondria via Its UBAN Domain Is Disrupted by an ALS-Linked Mutation. We hypothesize that the parkin-mediated ubiquitination of proteins on the OMM (9–13) stabilizes the association of optineurin with damaged mitochondria, as optineurin can bind ubiquitin via its UBAN domain (14). To test this hypothesis, we expressed the ALS-associated E478G ubiquitin binding-deficient mutant (optn-E478G), which has a point mutation in the UBAN domain (15, 19). In HeLa cells expressing parkin and treated with CCCP, we found that optn-E478G was not recruited to the surface of damaged mitochondria, but instead remained predominantly cytosolic (Fig. 4*A*). Almost none of the mitochondria (1%) were surrounded by optn-E478G, compared with the 60% of mitochondria surrounded by wild-type optineurin following 1 h of CCCP treatment (Fig. 4*A*). Next, we performed confocal time-lapse video imaging and found that after >45 min of CCCP treatment, the majority of damaged mitochondria were parkin-positive but negative for GFP–Optn-E478G (Fig. S6*A*). We did observe transient interactions of GFP–Optn-E478G puncta with damaged mitochondrial fragments (Fig. 4*B*, white arrows and Fig. S6*A*). However, unlike wild-type optineurin, optn-E478G did not remain stably associated with damaged mitochondria, dissociating after ~6 min (Fig. 4*B*, yellow arrows and Movie S6). Strikingly, even after prolonged mitochondrial damage, optn-E478G remained cytosolic (Fig. 4*C*, yellow arrows), whereas wild-type optineurin had formed rings around parkin-positive mitochondria. These results indicate that ubiquitin binding is essential to stabilize the association of optineurin with damaged mitochondria and further support the hypothesis that optineurin binds parkin-ubiquitinated OMM proteins via its UBAN domain.

DFCP1 Omegasomes Form on Parkin/Optineurin-Labeled Damaged Mitochondria. Autophagy receptors such as optineurin have been proposed to recruit autophagic machinery to cargo (33). To test this hypothesis, we examined the omegasome-localized protein DFCP1, which marks the initial site of autophagosome formation

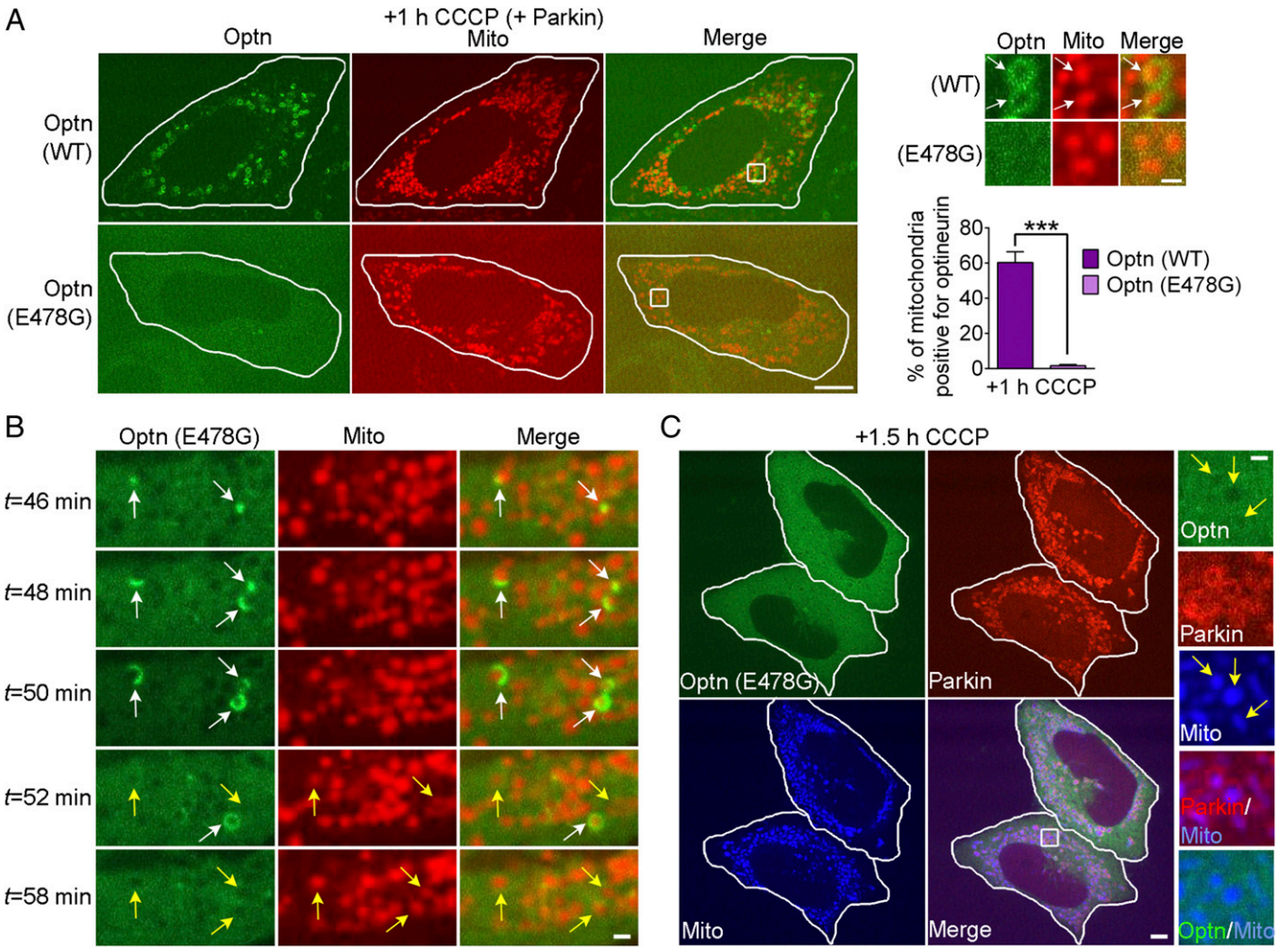

Fig. 4. Optineurin stably associates with damaged ubiquitinated mitochondria via its UBAN domain and is disrupted by an ALS-linked mutation. (*A*) Confocal image of a CCCP-treated HeLa cell expressing parkin, BFP-mito (pseudocolored red), and either wild-type mCherry-Optn or an ALS-associated UBAN mutant mCherry–Optn-E478G that has deficient ubiquitin binding (optn-E478G) (pseudocolored green). Wild-type optn shows clear recruitment to the surface of damaged mitochondria (white arrows on magnified image) after 1-h CCCP, whereas optn-E478G does not stably associate with damaged mitochondria. Quantification of optineurin recruitment to damaged mitochondria showing that wild-type optineurin is preferentially recruited and stabilized on mitochondria compared with the E478G mutant. (*B*) Time lapse showing optn-E478G puncta are transiently recruited to the outer surface of damaged mitochondria (white arrows). However, optn-E478G does not remain stably associated, resulting in mitochondria not labeled with optn-E478G (yellow arrows). (*C*) Optn-E478G remains cytosolic even after 1.5 h of CCCP treatment and does not localize to parkin-positive mitochondria (yellow arrows). [Scale bar, (*A*) 10 μm, (*C*) 5 μm, (*Insets* in *A* and *C*) 1 μm, and (*B*) 1 μm.] Values represent means ± SEM; ***$P < 0.001$.

(22, 34) and localizes to damaged mitochondria (32, 35). We expressed GFP-DFCP1 in CCCP-treated HeLa cells expressing parkin and, using time-lapse confocal imaging, found that DFCP1 puncta appeared dynamically on mitochondria at various time points from 45 min to 1.5 h of CCCP treatment (Movie S7). DFCP1 puncta transiently localized to parkin-positive mitochondria for ~3–5 min (Fig. 5 *A*, *B*, *E*, and *F*).

To examine whether optineurin recruitment occurs before omegasome formation, we coexpressed DFPC1 and optineurin in HeLa cells also expressing parkin. We observed optineurin recruitment to mitochondria after 1 h of CCCP treatment (Fig. S7*A*). DFCP1 puncta transiently localized to optineurin-labeled mitochondria (Fig. 5 *C*, *D*, and *G* and Movie S8) and remained on the surface of mitochondria for 3–5 min (see two examples—Fig. 5*C*, white and pink arrows). We also observed DFCP1 puncta that began as ring-like structures around optineurin-labeled mitochondria and formed extensions outwards from the optineurin ring (Fig. 5*D*). We found that DFCP1 localized to damaged mitochondria already positive for optineurin ($n = 54/54$ mitochondria), indicating that omegasome formation occurs downstream of optineurin recruitment. To test if optineurin is required for DFCP1 recruitment, we depleted optineurin using siRNA and found that DFCP1 could still localize to damaged mitochondria (Fig. S7*B*). In addition, the number of DFCP1 puncta on mitochondria per cell after 1-h CCCP was not affected by optineurin depletion (mock, 12.1 ± 1.1%; optn knockdown, 9.1 ± 0.7%; $P = 0.08$). Thus, DFCP1 omegasome formation occurs subsequent to optineurin recruitment but is localized to damaged mitochondria in an optineurin-independent pathway.

Optineurin Recruits Autophagosomes to Damaged Mitochondria via Its LIR Domain. We next examined autophagosome formation using the well-established marker GFP-LC3 (36). Autophagosomes form from omegasomes on damaged mitochondria (32), and we asked if this occurs downstream of optineurin recruitment to damaged mitochondria. We imaged GFP-LC3 and optineurin dynamics in HeLa cells expressing parkin. After 1 h of CCCP treatment, we saw the formation of LC3-positive autophagosomes around mitochondria surrounded by optineurin (Fig. 6*A*). As expected, we only observed autophagosome formation around mitochondria that had already recruited optineurin ($n = 185/185$ mitochondria). At this time point, not all optineurin-labeled mitochondria were engulfed by autophagosomes (Fig. 6*B*, yellow arrow), indicating that autophagosome engulfment of damaged mitochondria does not occur simultaneously throughout the cell but rather is a gradual, dynamic process that may depend on the depolarized state of each individual mitochondria. To observe the time frame of autophagosome formation, we examined HeLa cells treated after 1.5-h CCCP and found that by this time ~70% of optineurin-labeled mitochondria were engulfed by LC3-positive autophagosomes (Fig. 6*C* and Fig. S7*C*).

Using time-lapse video microscopy, we saw that autophagosome formation around optineurin-labeled mitochondria began as a small LC3 puncta on the surface of the mitochondria and grew into a sphere that enveloped the entire mitochondria, seen as an LC3 ring in a single *z* plane under confocal microscopy (Fig. S7*D*, white arrow and Movie S9). This process of LC3 puncta to ring formation lasted ~5 min, similar to the kinetics of basal autophagy observed in primary neurons (37).

Next, to test whether optineurin is an autophagy receptor for damaged mitochondria, we siRNA-depleted optineurin in parkin-expressing HeLa cells and examined whether GFP-LC3 autophagosomes could still be recruited to damaged mitochondria. siRNA depletion of optineurin led to >90% knockdown of endogenous optineurin (Fig. S8*A*). In control cells, we observed autophagosome formation around mitochondria after 1 h of

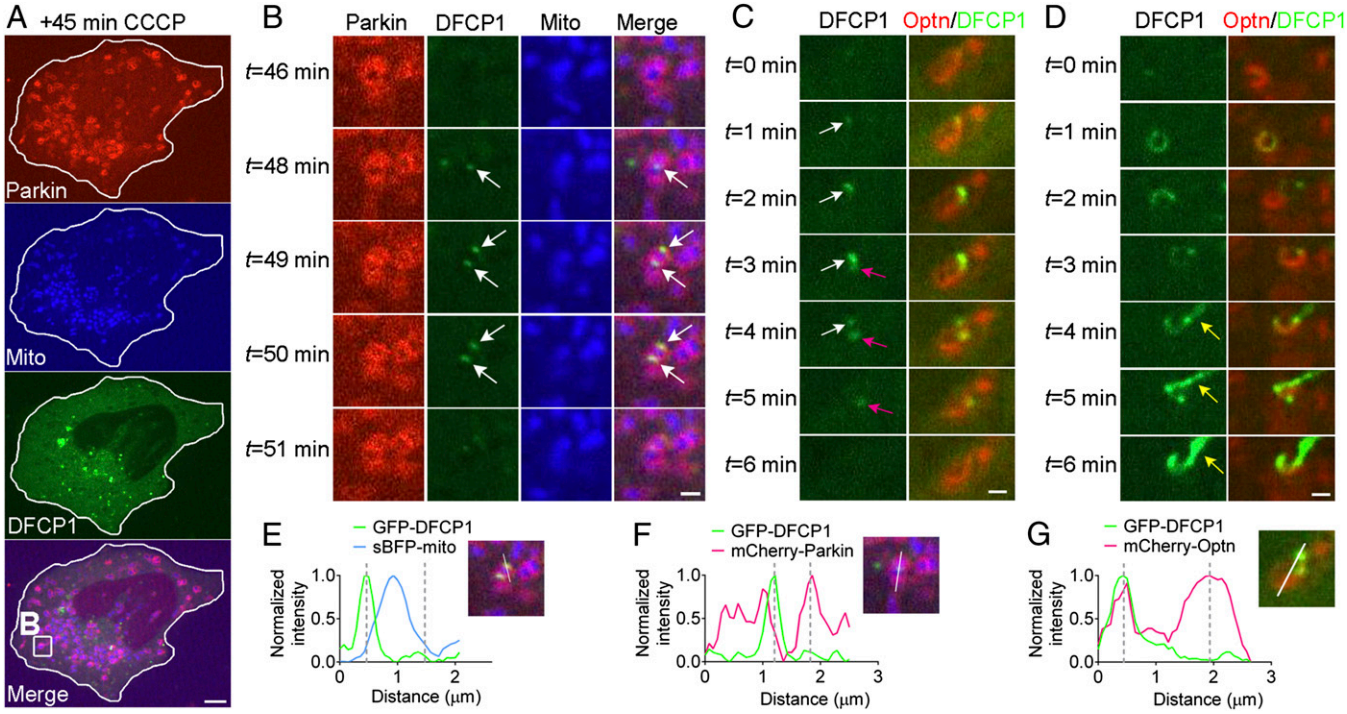

Fig. 5. DFCP1 omegasome formation occurs after parkin/optineurin recruitment to damaged mitochondria. (*A*) HeLa cell expressing BFP-mito, mCherry-parkin, and the omegasome marker GFP-DFCP1 treated with CCCP for 45 min. DFCP1 puncta are transiently recruited to parkin-positive mitochondria. (*B*) Time-lapse images of boxed area in *A* following CCCP treatment showing DFCP1 puncta recruited to parkin-labeled mitochondria for ~3 min before leaving (arrows). (*C*) Two examples of a DFCP1 puncta gradually forming on an optineurin-labeled mitochondria over ~3 min and then disappearing (white arrows and pink arrows). (*D*) Example of DFCP1 recruitment to the surface of an optineurin-labeled mitochondria and later forming DFCP1 tubules (yellow arrows) that extend outward. (*E*–*G*) Line scans showing DFCP1 localization with parkin and optineurin on the outer membrane of damaged mitochondria, from HeLa cells expressing GFP-DFCP1 with BFP-mito, mCherry-parkin, or mCherry-Optn. [Scale bar, (*A*) 5 μm and (*B*–*D*) 1 μm.]

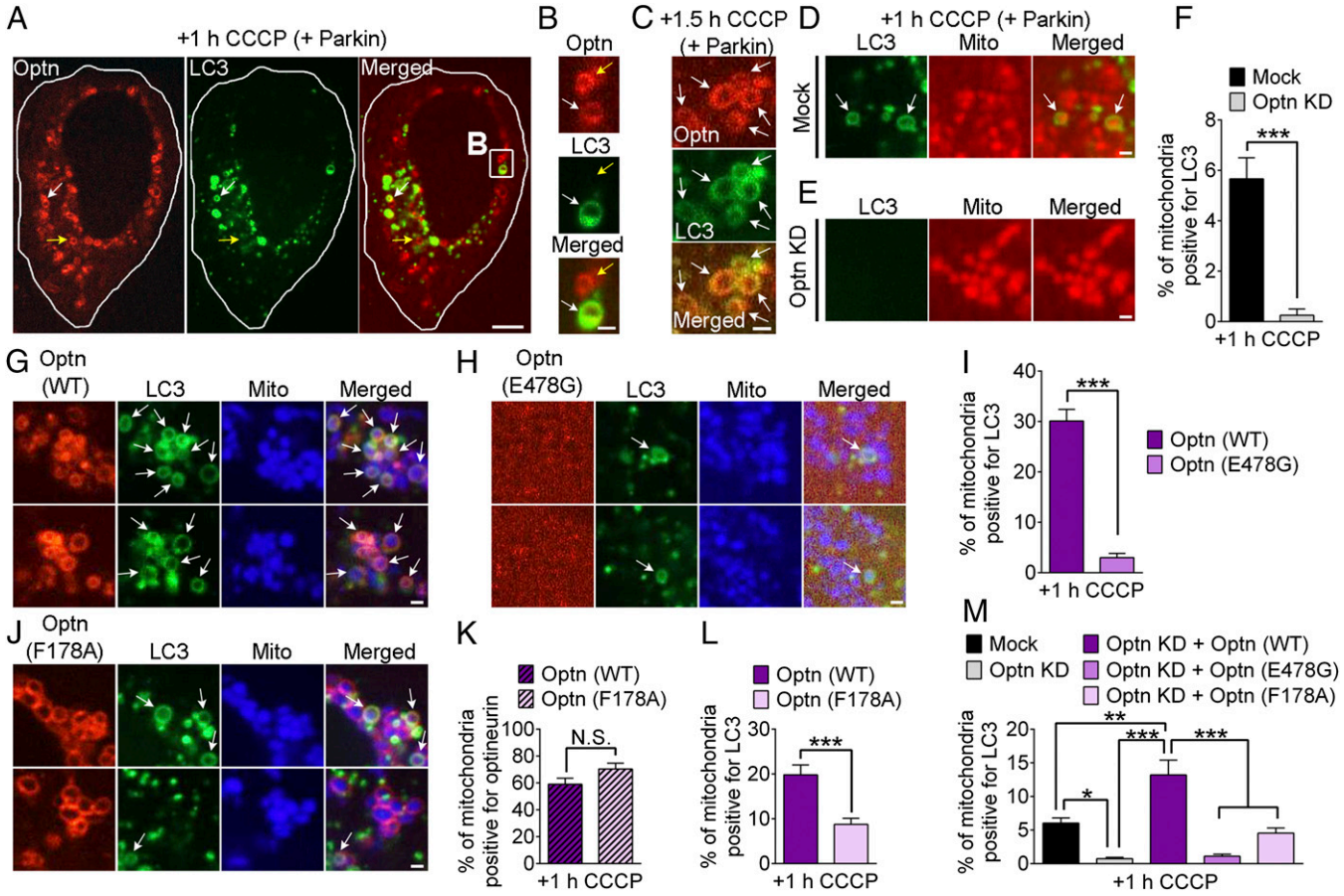

Fig. 6. Optineurin recruits LC3 autophagosomes to damaged mitochondria via its LIR domain. (*A* and *B*) Confocal images of a HeLa cell expressing parkin, mCherry-Optn, and the autophagosome marker GFP-LC3 treated with CCCP for 1 h. Optineurin is recruited to the surface of damaged mitochondria via parkin expression. LC3 autophagosomes dynamically form around optineurin-labeled damaged mitochondria (white arrows). Not all optineurin-labeled mitochondria are LC3-positive at this time point (yellow arrows). (*C*) Increased recruitment of LC3 to optineurin-labeled mitochondria after 1.5 h of CCCP treatment (white arrows). (*D–F*) HeLa cells expressing parkin, GFP-LC3, and BFP-mito (pseudocolored red). Formation of LC3 autophagosomes around damaged mitochondria (white arrows) in control cells with endogenous levels of optineurin (Mock) after 1 h of CCCP treatment is inhibited by siRNA knockdown (KD) of endogenous optineurin (Optn KD). (*G*) HeLa cell expressing parkin, mCherry-Optn, GFP-LC3, and BFP-mito after 1 h of CCCP treatment showing accelerated LC3 autophagosome engulfment of optineurin-labeled mitochondria in cells with increased optineurin expression (white arrows). (*H* and *I*) Expression of ALS-associated ubiquitin binding-deficient mCherry–Optn-E478G inhibits LC3 recruitment to mitochondria (white arrows). (*J–L*) Expression of LC3 binding-deficient mCherry–Optn-F178A that localizes to damaged mitochondria inhibits LC3 recruitment to mitochondria (white arrows). (*M*) Optineurin depletion disrupts LC3 autophagosome formation around mitochondria and is rescued by siRNA-resistant wild-type optineurin, but not siRNA-resistant optineurin E478G or F178A. [Scale bar, (*A*) 5 μm and (*B–E*, *G*, *H*, and *J*) 1 μm.] Values represent means ± SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. N.S., not significant.

CCCP treatment (Fig. 6*D*), in ∼80% of cells. However, in optineurin-depleted cells, almost none of the mitochondria were engulfed by autophagosomes (Fig. 6*E*); the percentage of total mitochondria that were engulfed by LC3 was significantly decreased compared with control cells (Fig. 6*F*). Indeed, the percentage of cells that demonstrated any autophagic engulfment of mitochondria after 1 h of CCCP treatment was dramatically decreased by optineurin depletion (mock, 78.5 ± 6.8%; optn knockdown, 11.8 ± 4.3%; $P = 0.001$).

After 2 h of CCCP treatment, optineurin-depleted cells still demonstrated a substantial lag in their ability to recruit LC3 to mitochondria, with less than 30% of cells containing any LC3-engulfed mitochondria. Even after 24 h of CCCP treatment, recruitment of LC3 autophagosomes to damaged mitochondria in optineurin-depleted cells was still strikingly inhibited. These results demonstrate that optineurin plays a crucial role as an autophagy receptor in parkin-dependent mitophagy by recruiting LC3 autophagosomes to damaged mitochondria.

We next examined whether increased levels of optineurin could enhance recruitment of autophagosomes to mitochondria. We overexpressed wild-type optineurin in parkin-expressing HeLa cells and found that this induced a striking increase in the percentage of mitochondria engulfed by autophagosomes (Fig. 6*G*). In contrast, expression of the ALS-associated optn-E478G mutant resulted in significantly slower rates of autophagosome formation, with only 3% of mitochondria engulfed after 1 h of CCCP treatment (Fig. 6*H*), compared with ∼30% of mitochondria in cells expressing wild-type optineurin (Fig. 6*I*).

To test if optineurin recruited LC3 to damaged mitochondria via its LIR domain, we expressed an optineurin F178A LIR mutant unable to bind LC3 (15). Optineurin F178A was robustly recruited to damaged mitochondria after 1 h of CCCP similar to wild-type optineurin (Fig. 6 *J* and *K*), but caused a defect in LC3 recruitment to mitochondria (Fig. 6 *J* and *L*), demonstrating that optineurin induces LC3 recruitment and autophagosome formation around mitochondria via its LIR domain.

Finally, we depleted optineurin using siRNA and tested whether we could rescue the defect of LC3 recruitment to damaged mitochondria by expressing siRNA-resistant optineurin in parkin-expressing HeLa cells. After 1-h CCCP, optineurin depletion decreased LC3 recruitment to damaged mitochondria; this defect was rescued by expression of the siRNA-resistant wild-type

optineurin. In contrast, expression of either an siRNA-resistant optineurin E478G or F178A mutant was unable to rescue the percentage of LC3-engulfed mitochondria (Fig. 6*M*). Taken together, these results demonstrate that optineurin is an autophagy receptor for damaged mitochondria that is capable of recruiting autophagosomes to mitochondria.

Optineurin and p62 Are Independently Recruited to Different Domains on Damaged Mitochondria and Have Distinct Roles in Mitophagy. There are currently five known autophagy receptors that bind both ubiquitin and LC3: p62/SQSTM1, NBR1, NDP52, T6BP, and optineurin (15, 16, 38–40). Among these proteins, only p62's role in parkin-mediated mitophagy has been investigated (23–26), but this work has suggested conflicting roles for p62 as either an autophagy receptor (23, 24) or a regulator of perinuclear clustering of depolarized mitochondria (25, 26). Thus, we asked whether optineurin and p62 act in the same pathway during parkin-dependent mitophagy.

In parkin-expressing cells, we found that optineurin was recruited to the entire surface of damaged mitochondria (Fig. 7*A*), whereas p62 preferentially localized to domains between adjacent mitochondria (Fig. 7 *B* and *C*) as previously observed (25, 26). Expression of p62 also accelerated the formation of mitochondrial aggregates after 1-h CCCP (Fig. 7*B*). These results further support a role for p62 as a regulator of mitochondrial aggregation and clustering during parkin-dependent mitophagy (25, 26).

Next, we examined whether p62 regulates optineurin recruitment to damaged mitochondria by using a p62 siRNA, which led to >90% knockdown of endogenous p62 (Fig. S8*B*). We found that p62 depletion had no effect on optineurin recruitment to mitochondria damaged with 1-h CCCP (Fig. 7 *D* and *E*). We also found that p62 depletion by siRNA did not disrupt LC3 recruitment to damaged mitochondria after 1-h CCCP (Fig. 7 *D* and *F*), further suggesting that p62 acts as a regulator of mitochondrial aggregation rather than as an autophagy receptor during parkin-dependent mitophagy. Interestingly, although depletion of p62 resulted in more tubular mitochondria after 1-h CCCP (Fig. 7*D*, *Right*), both optineurin and LC3 were still recruited to these elongated mitochondria. We also examined whether optineurin might regulate p62 recruitment to damaged mitochondria. In cells depleted of optineurin, p62 was still recruited to damaged mitochondria (Fig. S8*C*) and still preferentially localized between adjacent mitochondria (Fig. S8 *D* and *E*). Thus, our results suggest that although optineurin is an autophagy receptor in mitophagy recruiting LC3 to damaged mitochondria, p62 regulates mitochondrial aggregation following mitochondrial damage. Moreover, optineurin and p62 are recruited independently to separate domains on damaged mitochondria, potentially facilitating their distinct roles during parkin-dependent mitophagy.

The Autophagy Receptor Optineurin Is Necessary for Efficient Mitochondrial Degradation in Parkin-Mediated Mitophagy. To examine whether autophagosome recruitment and engulfment of damaged mitochondria leads to mitochondrial degradation, we analyzed levels of the mitochondrial matrix protein Hsp60 by immunoblot as a marker of mitochondrial protein content. We found that in parkin-expressing cells treated with 24-h CCCP, siRNA depletion of optineurin induced a significant increase in Hsp60 levels (normalized to loading control) compared with control cells (Fig. 8*A* and Fig. S9*A*). In contrast, siRNA depletion of p62 did not induce a significant increase in Hsp60 levels relative to control cells. We also observed a striking increase in the levels of the OMM protein Tom20 upon optineurin depletion, but not upon p62 depletion (Fig. S9*A*). Next, we immunostained for mtDNA as another measure of mitochondrial degradation. After 24-h CCCP, the majority of mitochondria in parkin-expressing cells were degraded, with only a few mitochondria remaining in each cell (Fig. S9*B*). Again, siRNA depletion of optineurin led to a striking increase in the number of mitochondria remaining, indicating defective mitochondrial degradation (optn knockdown, 2.4 ± 0.4-fold of mitochondria in control cells; $P < 0.01$) (Fig. 8*B* and Fig. S9*C*). This defect in mitochondrial degradation was rescued by expressing an siRNA-resistant wild-type optineurin (Fig. 8*B* and Fig. S9*D*). However, efficient mitochondrial degradation was not rescued by either an siRNA-resistant optineurin E478G ALS-linked UBAN mutant or an F178A LIR mutant (Fig. 8*B* and Fig. S9 *E* and *F*). In addition, p62 siRNA depletion did not significantly increase the number of mitochondria remaining (Fig. 8*B* and Fig. S9*G*). Together, these data further suggest that p62 is not a required receptor for the mitophagy of damaged mitochondria. In contrast, optineurin functions as an autophagy receptor for damaged mitochondria by binding to both ubiquitin and LC3, and this is critical for efficient mitochondrial clearance via mitophagy.

Discussion

In this study, we used live cell microscopy to image the dynamics of the parkin–optn–DFCP1–LC3 pathway for mitophagy. We found that optineurin recruitment to damaged mitochondria occurs subsequent to parkin recruitment but before autophagosome formation. The precise timing of optineurin's recruitment to mitochondria suggests a key role in linking parkin activity to the induction of autophagosome formation during mitophagy. Optineurin is recruited to parkin-ubiquitinated mitochondria

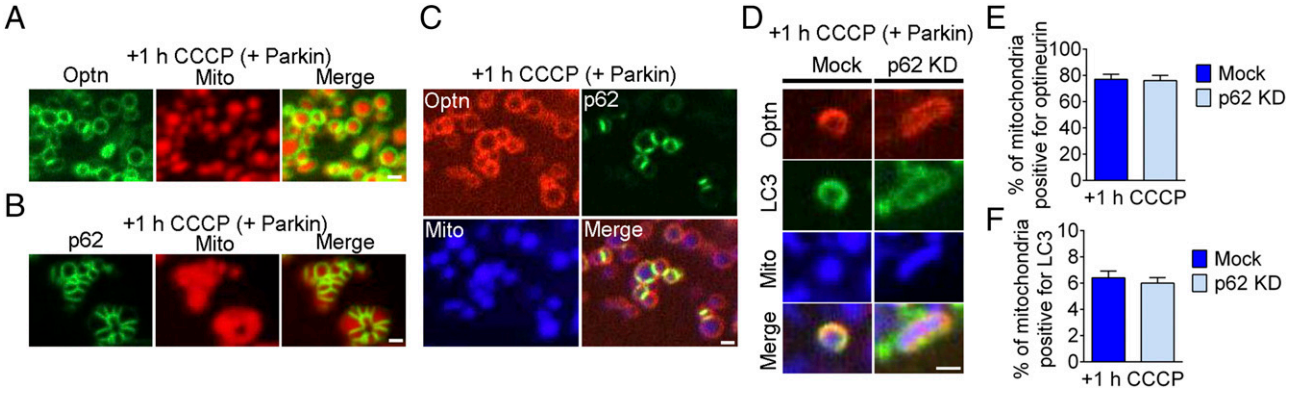

Fig. 7. Optineurin and p62 localize to different domains on damaged mitochondria and have distinct roles in mitophagy. (*A*) GFP-Optn is recruited to the entire surface of damaged mitochondria after 1-h CCCP in parkin-expressing HeLa cells. (*B*) GFP-p62 preferentially localizes to the domains between adjacent mitochondria and p62 expression accelerates mitochondrial aggregation after 1-h CCCP. (*C*) mCherry-Optn and GFP-p62 localize to different domains on damaged mitochondria. (*D–F*) p62 depletion by siRNA results in elongated mitochondria but does not inhibit optineurin or LC3 recruitment to mitochondria after 1-h CCCP (*D*, *Right*). [Scale bar, (*A–D*) 1 μm.] Values represent means ± SEM.

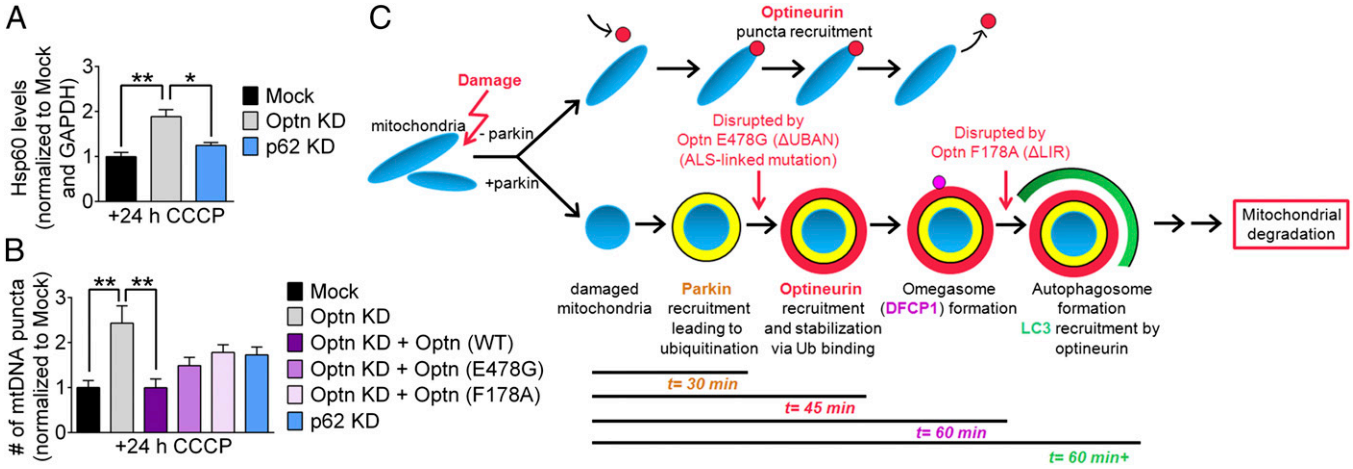

Fig. 8. Optineurin is an autophagy receptor for damaged mitochondria and regulates mitochondrial degradation. (*A*) Quantification of Hsp60 by immunoblot after 24-h CCCP in parkin-expressing HeLa cells normalized to control cells and loading control GAPDH. Optineurin siRNA knockdown (KD), but not p62 siRNA KD, leads to increased Hsp60 mitochondrial matrix protein content, indicating inefficient mitochondrial degradation. (*B*) Quantification of mtDNA by immunofluorescence after 24-h CCCP in parkin-expressing HeLa cells. Optineurin siRNA KD, but not p62 siRNA KD, results in the accumulation of inefficiently degraded mitochondria. siRNA-resistant wild-type optineurin, but not siRNA-resistant optineurin E478G or F178A, rescues efficient clearance of damaged mitochondria. (*C*, Model) In the absence of parkin, optineurin puncta transiently localize to the tips of damaged mitochondria but do not remain stably associated. In the presence of parkin, parkin is first recruited to the outer membrane of damaged mitochondria, followed by optineurin recruitment via its UBAN domain to parkin-ubiquitinated mitochondria. Next, the omegasome protein DFCP1 is transiently recruited to optineurin-labeled mitochondria, marking the initial site of autophagosome formation. Optineurin then recruits LC3 to mitochondria via its LIR domain, leading to autophagosome engulfment and mitochondrial degradation. A mutation in the UBAN domain (ALS-associated E478G) disrupts optineurin recruitment, whereas a mutation in the LIR domain (F178A) disrupts LC3 recruitment. Timeline (*Lower*) indicates approximate half-time for each step of the parkin–optineurin–DFCP1–LC3 pathway after induced mitochondrial damage. Values represent means ± SEM; $*P < 0.05$, $**P < 0.01$.

and is stabilized on damaged mitochondria by its UBAN domain. Optineurin subsequently recruits LC3 via its LIR domain, resulting in autophagosome formation around mitochondria (see Fig. 8*C*, model). Disruption of either of these steps leads to inefficient autophagic engulfment of mitochondria and the accumulation of damaged mitochondria within the cell.

Previous studies have shown that early autophagic machinery, including the omegasome marker DFCP1 (22, 34), is recruited independently and before LC3 during mitophagy (35). We found that although DFCP1 was recruited to damaged mitochondria independent of optineurin, DFCP1 recruitment temporally follows optineurin recruitment. Thus, optineurin might also play additional roles in regulating proteins involved in autophagosome formation before LC3 assembly. Interestingly, optineurin binds both huntingtin and myosin VI (41, 42), two proteins recently shown to regulate autophagosome motility (43) and maturation (16), further suggesting a scaffolding role for optineurin in autophagosome formation and dynamics.

There are currently five known autophagy receptors that bind both ubiquitin and LC3: p62/SQSTM1, NBR1, NDP52, T6BP, and optineurin (15, 16, 38–40). Here, we examined the interaction between two of these receptors: p62 and optineurin. We found that p62 and optineurin are independently recruited to different domains on damaged mitochondria, thereby allowing them to perform distinct functions during mitophagy. p62 preferentially localized to domains between adjacent mitochondria and accelerated mitochondrial aggregation, but did not regulate LC3 recruitment to mitochondria. These results are consistent with previous reports showing that p62 is not an autophagy receptor for damaged mitochondria but rather clusters damaged mitochondria via its PB1 oligomerization domain (25, 26). In contrast, we found that optineurin uniformly localized across the surface of damaged mitochondria and serves as a robust autophagy receptor for damaged mitochondria. Optineurin expression accelerated LC3 recruitment and facilitated autophagic engulfment and degradation of damaged mitochondria. Interestingly, p62 and optineurin have also been observed to localize to separate subdomains on ubiquitinated *Salmonella* (15), suggesting that p62 and optineurin may also play distinct roles in other types of selective autophagy. Because optineurin and NDP52 were found to localize to common subdomains on ubiquitinated *Salmonella* (15), it will be interesting to examine whether these other autophagy receptors have similar roles to optineurin in recruiting autophagic machinery to mitochondria during parkin-mediated mitophagy, which would provide further insight as to whether there are overlapping roles for autophagy receptors during selective autophagy.

Efficient turnover of damaged mitochondria is essential for maintaining cellular homeostasis, particularly in neurons, which have high energy demands (44). PINK1 and parkin, proteins linked with neurodegeneration in Parkinson's disease (3, 4), are key players in regulating mitochondrial degradation via mitophagy (1, 2). Here, we show that optineurin, mutations in which are causative for diseases including glaucoma and ALS (18, 19, 45), is recruited to damaged mitochondria downstream of PINK1 and parkin, but upstream of autophagosome formation and engulfment. Importantly, we find that an ALS-associated optineurin UBAN mutant is unable to stably associate with damaged mitochondria, leading to a reduced rate of autophagic engulfment and degradation of damaged mitochondria. Interestingly, ALS-associated valosin-containing protein was also recently implicated in PINK/parkin mitophagy (46). Thus, our results further highlight a potential role for mitophagy defects in ALS that may contribute to motor neuron death.

Materials and Methods

Reagents. Constructs used include DsRed2-mito (gift from T. Schwarz, Harvard Medical School, Boston, MA) recloned into pSBFP2-C1 (Addgene), pEGFP-OPTN and HA-OPTN E478G (gifts from I. Dikic, Goethe University, Frankfurt, Germany) recloned into pmCherry (Takara Bio Inc.), and pEGFP, YFP-parkin, and mCherry-parkin (gifts from R. Youle, National Institute of Health, Bethesda, MD) recloned into pSBFP2-C1 (Addgene) and an untagged parkin construct, DFCP1 (Addgene) recloned into pEGFP, pEGFP-LC3 (gift from T. Yoshimori, Osaka University, Osaka, Japan), GFP-p62 (Addgene), and pKillerRed-dMito (Evrogen). We generated mutant mCherry-parkin T240R and mCherry-OPTN F178A constructs by site-directed mutagenesis. We also generated siRNA-resistant mCherry-OPTN wild-type, E478G, and F178A constructs against the Optn siRNA described below. Antibodies used were against optineurin

(ab23666, Abcam), tubulin (T9026, Sigma), β-catenin (610154, BD Biosciences), p62 (ab56416, Abcam), Hsp60 (SPA-806, Enzo Life Sciences), GAPDH (ab9484, Abcam), Tom20 (sc-11415, Santa Cruz), DNA (61014, Progen), and an Alexa fluorophore-conjugated secondary antibody from Molecular Probes (Invitrogen). The siRNA to optineurin (5′-CCACCAGCTGAAAGAAGCC-3′) (42) and to p62 (5′-GCATTGAAGTTGATATCGAT-3′) (38) were obtained from Dharmacon (Thermo Scientific).

Cell Culture and Transfections. HeLa cells were cultured in DMEM (Corning) supplemented with 10% (vol/vol) FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin and maintained at 37 °C in a 5% CO_2 incubator. Cells were transfected using FuGENE 6 (Promega). CCCP (Sigma-Aldrich) was given at 20 μM. Knockdown experiments were performed with 60 nM siRNA using Lipofectamine RNAiMAX (Invitrogen). For KD and rescue experiments, cells were transfected with siRNA-resistant constructs 1 d after siRNA expression. For immunofluorescence analysis, cells were plated on coverslips and fixed in 4% (vol/vol) paraformaldehyde and permeabilized with 0.1% Triton X-100. Fixed cells were incubated in primary antibody for 1 h, washed 3 × 5 min, incubated in secondary antibody for 1 h, washed 3 × 5 min, and mounted on glass slides with ProLong gold (Invitrogen). For immunoblot analysis, cells were lysed and analyzed by SDS/PAGE and Western blot according to standard protocols. Tubulin, β-catenin, and GAPDH were used as loading controls for their varying molecular weights.

Live Cell Imaging and Analysis. All images were acquired on a spinning-disk confocal (UltraVIEW VoX; PerkinElmer) on a Nikon Eclipse Ti microscope using an Apochromat 100× 1.49 NA oil immersion objective (Nikon) in a temperature-controlled chamber (37 °C). Digital images were acquired with an EM charge-coupled device camera (C9100; Hamamatsu Photonics) using Volocity software (PerkinElmer) at 1 frame every 1–30 s or 1 frame every minute. Dual-color videos were acquired as consecutive green–red images, and tricolor videos were acquired as consecutive green–red–blue images. To induce spatiotemporally regulated mitophagy, we photobleached a region of a HeLa cell expressing pKillerRed-dMito with a 561-nm laser at 100% for 100 iterations. HeLa cells were transferred into phenol-red-free medium (Invitrogen; supplemented with FBS and penicillin/streptomycin) for live cell imaging. Line scans were generated using ImageJ [National Institutes of Health (NIH)] and normalized per protein and per condition. In line scans, dashed lines and arrows highlight peaks of protein fluorescent intensity. Cell and nucleus outlines were drawn using Photoshop (Adobe). Immunoblots were quantified using ImageJ (NIH). Anti-DNA preferentially stained mitochondrial DNA in HeLa cells as previously noted (47), and mtDNA nucleoids were counted per cell from a single confocal image. For each condition, >800 mtDNA puncta were counted from $n \geq 9$ cells ($n = 3$ experiments). All images were assembled using ImageJ (NIH) and Photoshop (Adobe). Statistics and graphing were performed using Prism (GraphPad) software. Data were analyzed using unpaired two-tailed Student *t* test (two datasets) or one-way ANOVA with Tukey's post hoc test (multiple data sets).

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