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COMMENTARY AND VIEWS

Two different axes CALCOCO2-RB1CC1 and OPTN-ATG9A initiate PRKN-mediated mitophagy

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ABSTRACT

PINK1 and PRKN, proteins mutated in Parkinson disease, selectively amplify ubiquitin signals on damaged mitochondria for elimination via mitophagy. Because all five macroautophagy/autophagy receptors in mammals possess domains binding to ubiquitin and Atg8-family proteins, they were thought to recruit Atg8-family protein labeled phagophores from a cytosolic pool. However, our recent findings show that, in addition to Atg8-family protein binding, two of the receptors CALCOCO2 and OPTN interact with RB1CC1 and ATG9A, respectively, indicating that two different axes, CALCOCO2-RB1CC1 and OPTN-ATG9A, can initiate de novo biogenesis of autophagic membranes on ubiquitin-coated damaged mitochondria. These results explain the critical roles of the autophagy receptors CALCOCO2 and OPTN in mitochondrial degradation, and their abilities to simultaneously bind multiple autophagy core proteins propose a new function, i.e. a scaffold to build multivalent interactions for the orchestrated assembly of autophagy proteins near the ubiquitinated cargo.

Abbreviations: ATG: autophagy-related; CALCOCO2/NDP52: calcium binding and coiled-coil domain 2; CRABP2: cellular retinoic acid binding protein 2; LIR: MAP1LC3/LC3-interacting region; MAP1LC3: microtubule associated protein 1 light chain 3; NBR1: NBR1 autophagy cargo receptor; OPTN: optineurin; PINK1: PTEN induced kinase 1; PRKN: parkin RBR E3 ubiquitin protein ligase; RB1CC1/FIP200: RB1 inducible coiled-coil 1; SNIPER: specific and nongenetic IAP-dependent protein eraser; SQSTM1/p62: sequestosome 1; ULK: unc-51 like autophagy activating kinase

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Eukaryotes eliminate unwanted intracellular materials including protein aggregates and depolarized mitochondria as well as invading microbes by Macroautophagy/autophagy. This process is called selective autophagy, which, in many cases, depends on ubiquitin signals. PINK1-PRKN-mediated mitophagy is one of the best characterized selective autophagy pathways. PINK1 and PRKN, the two gene products mutated in a familial Parkinson disease, cooperatively put ubiquitin on depolarized mitochondria. Following the dissipation of membrane potential across the mitochondrial inner membrane, the serine/threonine kinase PINK1 accumulates on the outer membrane of depolarized mitochondria where it phosphorylates ubiquitin. Phosphorylated ubiquitin then recruits a cytosolic E3 ligase PRKN to mitochondria where PRKN gets further activated by PINK1 phosphorylation. At the end of a positive feedback loop of PRKN-mediated ubiquitination, depolarized mitochondria become highly coated with mono-ubiquitin and poly-ubiquitin chains.

Autophagy receptors are thought to be critical for linking ubiquitin-coated mitochondria to the autophagy machinery, because they possess both a ubiquitin-binding domain and a LIR. Mammals encode at least five different autophagy receptors (CALCOCO2, OPTN, TAX1BP1 [Tax1 binding protein 1], SQSTM1 and NBR1), and all of them are recruited to ubiquitin-coated depolarized mitochondria. However, using cells knocked out of all autophagy receptors by a clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 technology, only two of them, CALCOCO2 and OPTN, were shown to be primarily essential for mitophagy, but the molecular mechanisms remained unclear.

A presumable regulator of the mitophagy process was phosphorylated ubiquitin. Accumulation of phosphorylated ubiquitin by ectopic placement of PINK1 on mitochondria in PRKN-null cells can induce mitophagy, while it is only slight, which suggested that phosphorylated ubiquitin facilitates the recruitment of particular autophagy receptors (i.e., CALCOCO2 and OPTN) or directly activates the autophagy machinery. However, because of the experimental limitation that the PINK1-PRKN system was the only way to induce the robust mitochondrial ubiquitination, the exact role of phosphorylated ubiquitin in mitophagy has not been dissected from the robust ubiquitination. To overcome this experimental difficulty, we recently established two different methods to coat mitochondria with ubiquitin chains in a PINK1-PRKN-independent manner: expression of mitochondria-targeted linear ubiquitin chains and activation of the BIRC2/cIAP1 (baculoviral IAP repeat containing 2) E3 ligase by a chemical compound to induce ubiquitination of mitochondria-targeted substrate [1].

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We first targeted tandem linear ubiquitin chains to mitochondria using a transmembrane segment of TOMM20 (translocase of outer mitochondrial membrane 20), but no degradation of mitochondria is detected, which is consistent with several previous reports. In sharp contrast, when the K0 version of the linear ubiquitin chain (in which all seven lysine residues of ubiquitin were replaced with arginine to prevent branched chain formation) was targeted to mitochondria, OPTN is recruited to these mitochondria, and mitochondrial matrix proteins such as MT-CO2 (mitochondrially encoded cytochrome c oxidase II) are drastically degraded, suggesting that mitophagy occurs. Keima-based fluorescence assisted cell sorting (FACS) analysis also indicated that this mitochondrial degradation depends on ATG5 and autophagy receptors, but not PINK1. Next, we utilized synthetic hybrid molecules known as SNIPERs to coat mitochondria with ubiquitin chains. SNIPER(CRABP)-11, a small hybrid compound connecting an E3 ligase BIRC2 and CRABP2, mediates BIRC2-dependent CRABP2 ubiquitination. We demonstrated that CRABP2-fused mitochondrial proteins including HK1 (hexokinase 1) and TOMM20 (translocase of outer mitochondrial membrane 20), but not cytosolic CRABP2 alone, could induce mitophagy, when treated the cells with a SNIPER, in a PINK1-PRKN-independent manner. From these two different ubiquitination approaches, we concluded that robust ubiquitination on the mitochondrial surface is sufficient for inducing mitophagy and that PINK1 and phosphorylated ubiquitin are not required for autophagy activation, whereas they are essential for PRKN activation. However, phosphorylation of ubiquitin may promote mitophagy by inhibiting deubiquitinase removal of mitochondrial ubiquitin chains.

Microscopy observation revealed that CALCOCO2 and OPTN assemble at the junction between the phagophore assembly site marked by MAP1LC3B and depolarized mitochondria during mitophagy, whereas other receptors such as SQSTM1 and NBR1 are uniformly recruited to mitochondria, suggesting that CALCOCO2 and OPTN possess abilities to strongly interact with the phagophore and/or phagophore assembly sites. Although the Atg8-family proteins (seven proteins; four of MAP1LC3 and three of GABARAP [GABA type A receptor-associated protein] subfamilies in mammalian cells) are the conceivable candidates, in-cell fluorescence-based protein-protein interaction analysis showed that the binding affinities of CALCOCO2 and OPTN with all Atg8-family proteins are lower than those of SQSTM1 and NBR1. Therefore, critical roles of CALCOCO2 and OPTN to assemble phagophores on depolarized mitochondria do not appear primarily derived from the affinity for Atg8-family proteins. This finding is consistent with previous result from Michael Lazarou's group [2] showing that phagophores can still encapsulate ubiquitinated mitochondria in the absence of all Atg8-family proteins.

How do CALCOCO2 and OPTN recruit phagophores to depolarized mitochondria? To address this issue, we first focused on CALCOCO2. By chemically induced dimerization assays, forced CALCOCO2 localization on mitochondria results in the recruitment of several ATG proteins, RB1CC1 and its downstream proteins, ATG14 and ATG16L1, to mitochondria. The ULK complex consisting of RB1CC1, ULK1/2, ATG13 and ATG101, is the most upstream unit and essential to initiate mitophagy. Chemical crosslinking followed by co-immunoprecipitation showed that CALCOCO2 associates with the ULK complex, which is enhanced during mitophagy. In RB1CC1 knockout cells, CALCOCO2 no longer bound to ULK1, indicating direct interaction between RB1CC1 and CALCOCO2 [3].

Next, we focused on OPTN. By creating artificial liquid-liquid phase separated foci consisting of oligomerized autophagy receptors and linear ubiquitin chains, OPTN, but not other autophagy receptors, was found to specifically recruit ATG9A-containing vesicles to the foci [1]. This indicates that OPTN forms a complex with ATG9A-containing vesicles. ATG9A is the sole multi-spanning transmembrane protein among the core autophagy proteins, and exists in cytosolic vesicles as well as on the trans-Golgi network and on endosomal membranes. Further mutational and truncation approaches demonstrated that the SKIP carboxyl homology (SKICH) domain in CALCOCO2 and the leucine zipper domain in OPTN are responsible for the interaction with RB1CC1 and ATG9A vesicles, respectively. Of note, disruptions of CALCOCO2-RB1CC1 or OPTN-ATG9A interactions inhibited mitophagy. These results indicate that, in addition to LIR binding, critical autophagy receptors CALCOCO2 and OPTN possess other sites for interaction with different autophagy core subunits.

Hierarchical analysis of ATG proteins during PINK1-PRKN-mediated mitophagy previously reported by Noboru Mizushima's group [4] uncovered that ULK complex, ATG9A-containing vesicles, and lipidated Atg8-family proteins associate with depolarized mitochondria independently of each other. Now it is apparent that, during PINK1-PRKN-mediated mitophagy, two different axes, CALCOCO2-RB1CC1 and OPTN-ATG9A, initiate de novo biogenesis of autophagic membranes on the ubiquitinated cargo, in addition to recruitment of Atg8-family protein lipidated membranes from a cytosolic pool (Figure 1).

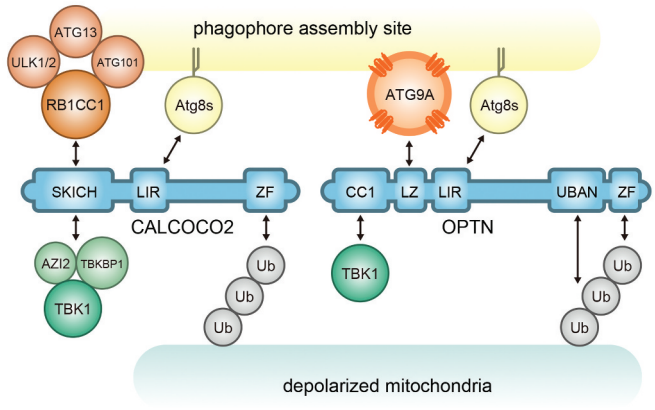

Figure 1. Model of multimeric interactions of CALCOCO2 and OPTN to link phagophore assembly site and ubiquitin (Ub)-coated depolarized mitochondria during PINK1-PRKN-mediated mitophagy. CALCOCO2 and OPTN are recruited to mitochondria via ubiquitin-binding domains, ZF and UBAN. CALCOCO2 recruits the ULK1 complex (RB1CC1, ULK1/2, ATG13, ATG101), the TBK1 (TANK binding kinase 1) complex (TBK1, AZI2 [5-azacytidine induced 2], TBKBP1 [TBK1 binding protein 1]), and Atg8-family proteins via the SKICH, and LIR domains, respectively. OPTN recruits TBK1, ATG9A-containing vesicles, and Atg8-family proteins via the coiled-coil 1 (CC1), leucine zipper (LZ), and LIR domains, respectively. UBAN, ubiquitin binding in ABIN and NEMO; ZF, zinc finger.

Furthermore, the multimeric ULK complex can interact with ATG16L1, and ATG9A (~30 molecules of which are contained in a vesicle) is also reported to bind ATG16L1 and ATG13. In this context, CALCOCO2 and OPTN function not only as receptors linking phagophores and ubiquitinated cargo, but also as scaffolds to build multivalent interactions for the assembly of different autophagy core machineries near the ubiquitinated cargo.

Disclosure statement

No potential conflicts of interest were disclosed by the authors.

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