Ulk1/Rab9-mediated alternative mitophagy confers cardioprotection during energy stress

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The heart relies on mitochondria-derived energy production for continuous contraction and relaxation; therefore, the maintenance of a pool of healthy mitochondria is essential for sustaining normal cardiac performance. Mitophagy serves as a critical process for maintaining mitochondrial quality control and involves the PTEN-induced kinase 1/Parkin (Pink1/Parkin) pathway and autophagosomes labeled with the autophagy proteins autophagy-related 7 (ATG) and light chain 3 (LC3). In this issue of the *JCI*, Saito and colleagues identify an alternative pathway for mitophagy that utilizes the serine/threonine protein kinase Unc-51–like kinase 1 (Ulk1) and the small GTPase Rab9 to clear damaged mitochondria independently of conventional autophagy proteins. Together, the results of this study reveal that Ulk1 phosphorylation of Rab9 at serine 179 is critical for alternative mitophagy and cardioprotection under energy stress conditions.

Conventional mitophagy

Autophagy is a highly conserved physiological process by which intracellular macromolecules, organelles, and proteins are degraded by a lysosome-regulated pathway. As an adaptive response mechanism, autophagy affords the cell the ability to avert the cytotoxic effects of damaged organelles and aggregated proteins during cellular stress, such as nutrient deprivation or ischemia. During general autophagy, the cytoplasmic constituents that are targeted for destruction are sequestered in a phagophore that is derived from the endoplasmic reticular membrane. The phagophore then undergoes a series of maturation steps that include nucleation, elongation, and conjugation. These processes are mediated by the autophagy protein complexes Beclin1-VPS34-VPS15 (Beclin1 is also known as ATG6), ATG12-ATG5, and ATG8–light chain 3 (ATG8-LC3), respectively, and lead to formation of the double-membraned autophagosome. The association of the autophagosome with adaptor proteins,

such as LC3-p62, facilitates fusion with acid-rich lysosomes and results in degradation of the autophagosome. Hence, autophagy is a quality control mechanism for maintaining cellular homeostasis by eliminating toxic biomolecules (1–3).

Damaged mitochondria are removed by a specialized form of autophagy, referred to as mitophagy. Mitophagy is best known to be regulated by the PTEN-induced kinase 1/Parkin (Pink1/Parkin) pathway (reviewed in refs. 4–6). The cellular kinase Pink1 and the ubiquitin E3-ligase Parkin were proposed to play a central role in mitochondrial clearance in Parkinson's disease, as mutations in the genes encoding Pink1, Parkin, or both have been found in patients with the disease (7). In this model, Parkin is retained in the cytoplasm but is phosphorylated and recruited to the outer membranes of damaged mitochondria following Pink1 stabilization. The Parkin-mediated polyubiquitination of outer mitochondrial membrane proteins targets depolarized mitochondria

for removal by the lysosome (4, 8, 9). In the context of the heart, mitophagy has been reported to increase during ischemia-reperfusion, myocardial infarction, and pressure-overload hypertrophy, supporting the notion that mitophagy is cardioprotective (10). Notably, in murine models, Parkin overexpression enhances mitophagy and ameliorates the decline in cardiac performance in the aged heart (11). Conversely, Parkin deficiency leads to the accumulation of dysfunctional mitochondria and cardiac dysfunction following myocardial infarction (12). Similarly, mice deficient for Pink1 are more susceptible to heart failure following pressure overload (13) and ischemia- reperfusion injury (14). These findings substantiate the importance of autophagic removal of mitochondria in the heart by the Pink1/Parkin lysosomal pathway. It is noteworthy that although mitophagy in the heart is a proven modality for mitochondrial clearance, the mechanisms of regulation remain unclear. For example, the role of Parkin for mitophagy in cardiac myocytes has recently been challenged, and Parkin-independent mechanisms have been proposed (5, 6). Further, mitochondria removal can be mediated by autophagy adapter proteins, LC3, p62, FUNDC, and Nix that target mitochondrial destruction by lysosomal pathways. There is a growing body of evidence that mitochondria can be discarded by an alternative autophagy pathway that utilizes autophagosomes derived from the *trans*-Golgi and late endosomes, which later associate with the small GTPase Ras-related protein 9 (Rab9) and not the conventional autophagy adapter protein LC3 (15).
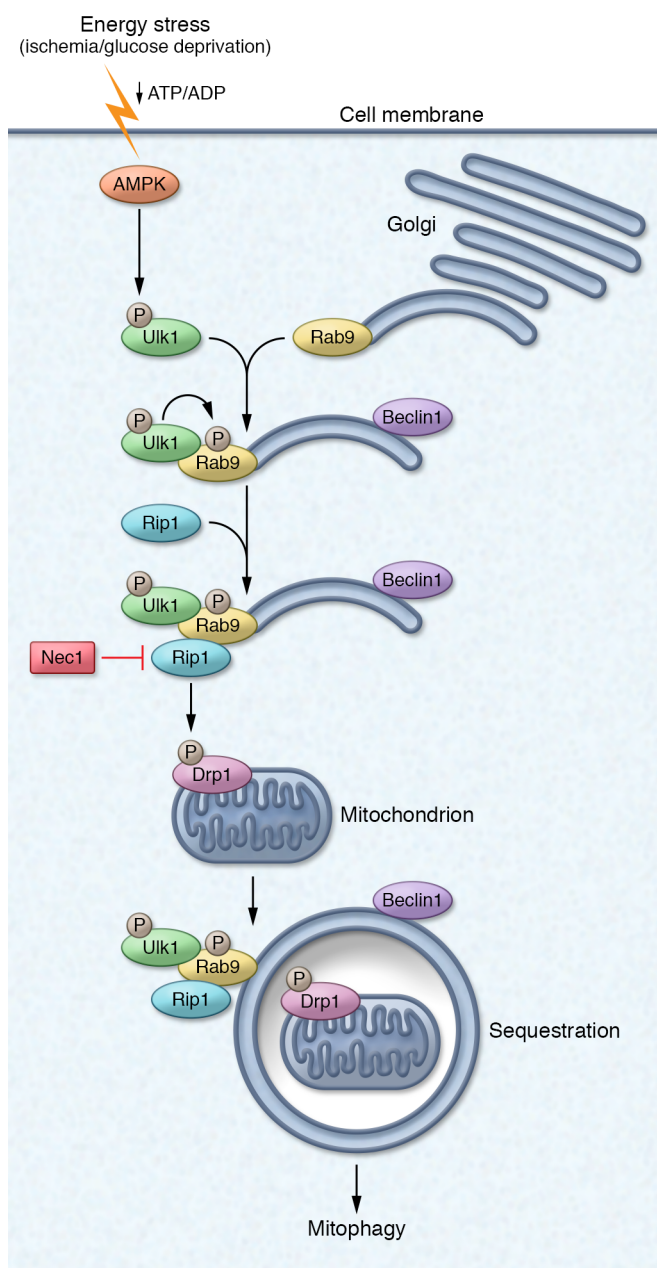
Unraveling an alternative mitophagy pathway

In 2009, Nishida et al. demonstrated the existence of an alternative mitophagy pathway involving Unc-51–like kinase 1 (Ulk1) during erythrocyte differentiation, but the underlying mechanism of this pathway was

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Figure 1. Ulk1 phosphorylation of Rab9 at serine 179 is essential for mitophagy and cardioprotection during energy stress. In this issue of the *JCI*, a study by Saito et al. provides molecular insight into an alternative mitophagy pathway activated in the heart under energy stress conditions (17). Energy stress provoked by glucose deprivation or hypoxia/ischemia activates AMPK, which then phosphorylates Ulk1. Ulk1 forms a complex with the Ras-related protein Rab9, which is associated with a Golgi-derived membrane. Ulk1 phosphorylates Rab9 at serine 179, and phosphorylated Rab9 then serves as a platform for the formation of a complex between Rip1 and Drp1. Rip1 phosphorylates Drp1 at serine 616 in a Ulk1- and Rab9-dependent manner. Mitochondria with phosphorylated Drp1 are recognized and seques-tered by Rab9-associated membranes, followed by fusion with lysosomes and digestion. The Rip kinase inhibitor necrostain-1 (Nec1) inhibits energy stress–induced mitophagy.

unclear (16). In this issue of the *JCI*, Saito et al. provide insight into the mechanisms that underlie alternative mitophagy in the heart imposed by energy stress (17). This study provides compelling evidence that Ulk1 phosphorylation of Rab9 at serine 179 is an essential step for the initiation of mitophagy under energy stress conditions. Through a variety of elegantly designed experiments, the authors show that mito-phagy in cardiac myocytes involves phos-phorylation of Rab9 by Ulk1, which then serves as a signaling platform for the for-mation of a complex between the receptor-interacting protein 1 (Rip1) kinase and dynamin-related protein (Drp1) (Figure 1).

To better understand the underlying mechanisms of mitophagy activation in the heart during energy stress, Saito et al. subjected WT and cardiac-specific *Atg7*–knockout (Atg7-cKO) mice to 48 hours of glucose starvation (17). As predicted, LC3 levels and autophagy flux were increased in WT mice but not in Atg7-cKO mice. Surprisingly, despite low LC3 levels, elec-tron micrographs of Atg7-cKO hearts dis-played numerous mitochondria surround-ed by double-membrane structures. Next, the authors generated Atg7-cKO/Mito-Keima–Tg mice, which allow reliable eval-uation of mitophagy, and subjected them to starvation to verify that mitochondria

surrounded by double membranes were actually sequestered within autophago-somes. Indeed, confocal and electron microscopic analyses revealed that starva-tion-induced mitophagy was independent of the conventional autophagy proteins Atg7 and LC3. Interestingly, the Atg7-cKo hearts displayed increased levels of Ulk1, which had been previously shown to trig-ger alternative mitophagy under different stress conditions (16). Saito et al. then explored the involvement of Ulk1 on mito-chondrial clearance by testing the effects of starvation on mitophagy activation in mice with cardiac-restricted knockout of *Ulk1* (Ulk1-cKO). Ulk1-cKO mice readily displayed increased levels of LC3 and pre-served autophagy flux; however, despite the presence of an intact LC3 autophagy clearance system, Ulk1-cKO/Mito-Keima–Tg mice exhibited a sharp decline in mito-phagy and mitochondrial clearance. These findings strongly supported the contention that activation of mitophagy during starva-tion requires Ulk1 and but not Atg7-LC3.

Finally, Saito and colleagues explored the mechanisms by which Ulk1 activates mitophagy in cardiac myocytes. The authors began by investigating whether the autophagosomal membranes in the Ulk1 pathway are derived from *trans*-Gol-gi and endosomes and associated with Rab9 (17). Specifically, inhibition of Golgi-derived membranes with brefeldin A resulted in a marked reduction in mito-phagy, without any effect on convention-

al autophagy markers. Moreover, Rab9, but not LC3, colocalized with autophagosomes and autolysosomes during energy stress, and knockdown of Rab9 disrupted energy stress–induced mitophagy. Interestingly, colocalization of Rab9 with autophagosomal or lysosomal membranes was highly dependent on Ulk1, and loss of Ulk1 or Rab9 impaired mitophagy and mitochondrial function during energy stress, demonstrating the importance of Ulk1 and Rab9 for mitophagy activation under these conditions. In addition, phosphorylation of Ulk1 by AMPK was critical for mitophagy during energy stress. As mitochondrial fission has previously been shown to be a requisite event for mitochondrial clearance by mitophagy, Saito et al. evaluated the contribution of the mitochondrial fission protein Drp1 in Ulk1/Rab9-mediated mitophagy. Indeed, Drp1 phosphorylation at serine 616 was increased and corresponded with increased mitochondrial fission during energy stress. Interestingly, Drp1 phosphorylation was contingent on Ulk1, as inhibition of Ulk1 in cardiomyocytes, or in Ulk1-cko mice, resulted in a marked decline in Drp1 phosphorylation. Interestingly, Rip1, a kinase typically involved in NF-κB signaling and necroptosis, directly phosphorylated Drp1 during energy stress, and mass spectroscopic analysis identified a protein complex composed of Ulk1, Rab9, Rip1, and Drp1. Further studies revealed that Ulk1 interaction and phosphorylation at serine 179 are critical for Rip1 association and phosphorylation of Drp1. Hence, mitochondria harboring phosphorylated Drp1 are sequestered and targeted by Rab9 for mitophagy. While alternative mitophagy by Ulk1 has been previously demonstrated, the activation of this pathway in the heart during energy stress, as described in this study, is relatively novel — especially the identification of Ulk1 as a critical kinase required for phosphorylation of Rab9 at serine 179 and the contribution of Drp1-mitochondrial fission for mitophagy activation and cardioprotection during energy stress.

Conclusions and future directions

While the study by Saito et al. highlights an alternative mode for mitophagy activation and mitochondrial clearance in cardiac myocytes, it also raises several issues for

future consideration. For instance, what are the signals that direct mitochondrial clearance by conventional mitophagy mediated by Parkin/Atg8-LC3 or by the alternative Ulk1/Rab9 pathway? Perhaps alternative autophagy provides a fail-safe–like mechanism to ensure that damaged mitochondria are removed at any cost in the event that the conventional pathway is blocked or inactivated. Alternatively, maybe the cells preferentially activate one pathway over the other on the basis of the type and severity of cellular stress. Another area of interest relates to how mitochondria that are undergoing Drp1-mediated fission become selectively removed by the alternative pathway, as Drp1-mediated fission is required for efficient mitochondrial clearance by the conventional pathway. At present, it is undetermined whether Ulk1/Rab9-mediated mitophagy is a universally conserved feature that serves as a mitophagy back-up system or is activated in response to other stress conditions, such as pressure-overload hypertrophy, inflammation, and/or drug-induced heart failure. Finally, what is the importance of the Ulk1/Rab9 mitophagy pathway in other organs, such as lung and brain, that may differ in mitochondrial abundance compared with cardiac myocytes and may therefore utilize different modes of autophagy for mitochondrial quality control?

Moreover, Rip1, which is involved in the regulation of necroptosis among other signaling pathways including that for NF-κB activation, was shown in the present study to interact with Rab9 in close proximity with mitochondria. However, it is unclear how Rip1, which otherwise mediates necroptosis signaling through associations with Rip3 and mixed-lineage kinase domain–like pseudokinase (MLKL), interacts with Rab9 to execute mitophagy. Generally, Rip1 dissociates from receptors in the necrosome in response to death receptor activation and necrosis signaling, but in the Saito et al. study, Rip1 was shown to be a part of the Rab9 alternative mitophagy pathway, which was cardioprotective (17). A better understanding of how Rip1 is regulated during cellular stress may provide important insight into how necrosis and mitophagy can be differentially influenced by Rip1. Nevertheless, the study by Saito et al. has revealed a novel signaling pathway for mitophagy in cardi-

ac myocytes that coordinates overlapping pathways between Golgi-endosomal trafficking and necroptosis pathways via the formation of Ulk1-Rab9-Rip1 complexes for cardioprotection.

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