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The TORC1 signaling pathway regulates respiration-induced mitophagy in yeast

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ABSTRACT

Mitophagy is an evolutionarily conserved autophagy process that selectively degrades mitochondria. This catabolic event is considered to be a mitochondrial quality control system crucial for cell homeostasis, however, mechanisms regulating mitophagy remain largely unknown. Here we show that the TORC1 (target of rapamycin complex 1) signaling pathway regulates mitophagy in budding yeast via SEACIT (Seh1-associated complex inhibiting TORC1) consisting of Iml1, Npr2, and Npr3. Cells lacking SEACIT displayed significant reductions in mitophagy during prolonged respiratory growth, while the other selective autophagy processes are less affected. Under the same conditions, mitophagy defects were strikingly rescued in the SEACIT mutants (1) treated with rapamycin, a specific TOR kinase inhibitor, (2) lacking Gtr1, a TORC1-stimulating Rag family GTPase downstream of SEACIT, and (3) devoid of Pib2, a phosphatidylinositol 3-phosphate-binding TORC1 activator. Notably, loss of Npr2 exacerbated mitophagy defects in cells lacking Atg13, a TORC1 effector crucial for activation of autophagy-related processes, suggesting additional mitophagy-specific regulator(s) downstream of TORC1. Finally, we found that *npr2*-null cells failed to stabilize the interaction of Atg32 with Atg11, a scaffold protein essential for mitophagy. Collectively, our data implicate SEACIT-mediated inactivation of TORC1 signaling as a critical step to promote respiration-induced mitophagy.

1. Introduction

Mitochondria play a central role in cell homeostasis, serving as a factory for many important reactions including energy conversion, phospholipid metabolism, and amino acid synthesis, and acting as a regulator for programmed cell death pathways and multiple signaling events [1]. During respiration, mitochondria concomitantly generate reactive oxygen species (ROS) from the electron transport chain, and accumulation of ROS eventually causes mitochondrial dysfunction that negatively affects energy-consuming cellular functions [2]. Accordingly, the maintenance of mitochondrial health is essential for cell fitness and dynamics. Studies in the last decade have established that mitochondrial quality control relies on selective degradation of mitochondria via macroautophagy (hereafter autophagy), an intracellular membrane transport process by which cytosolic components are sequestered into newly formed double-membrane vesicles called autophagosomes and delivered to lysosomes (or vacuoles in yeast) containing hydrolytic enzymes [3–5]. Malfunction of mitochondria-specific autophagy, termed mitophagy, is thought to cause a myriad of disorders such as neurodegeneration, heart/liver failure, aging, and cancer, underscoring the physiological relevance [6].

Basic principles underlying mitophagy are highly conserved from yeast to humans [7,8]. In the budding yeast *Saccharomyces cerevisiae*, mitophagy strictly depends on Atg32, a mitochondrial outer membrane protein of 59 kDa [9,10]. When cells are grown in a medium containing the non-fermentable carbon source glycerol, Atg32 is induced in response to oxidative stress and targets to the mitochondrial surface [9,10]. As a protein receptor, Atg32 directly interacts with Atg8, a ubiquitin-like modifier covalently linked to the lipid phosphatidylethanolamine (PE) and localized to the autophagosome [4], and Atg11, a selective autophagy-specific scaffold protein required for assembly of core Atg proteins mediating autophagosome formation [4], at the early stage of mitophagy [9–11]. In addition, Atg32-Atg11 interaction becomes stabilized through phosphorylation of Atg32 in a manner dependent on casein kinase 2 (CK2) [12,13]. Although the Atg32/8/11 complex and core Atg proteins are thought to cooperatively promote generation of autophagosomes surrounding mitochondria, how formation of

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this ternary complex is initiated remains poorly understood.

In this study, we demonstrate that mitophagy in respiring yeast is regulated through TORC1 (target of rapamycin complex 1), an evolutionarily conserved nutrient-responsive protein kinase complex [14]. When challenged with nutrient deprivation, yeast cells activate SEACIT (Seh1-associated complex inhibiting TORC1) consisting of Iml1, Npr2, and Npr3 that functions as a GAP (GTPase-activating protein) to negatively regulate Gtr1, a TORC1-stimulating Rag family GTPase, ultimately leading to inactivation of TORC1, a key step to trigger starvation-induced autophagy [15,16]. In spite of the significant defects in degradation of mitochondria, other selective autophagy processes are less affected in cells lacking SEACIT under prolonged respiration. Pharmacological and genetic suppressions of TORC1 almost completely restored mitophagy in *npr2*-null cells, indicating that SEACIT promotes mitophagy via TORC1 inactivation. Surprisingly, deletion of the *NPR2* gene further attenuated degradation of mitochondria in cells lacking Atg13, a key component of the autophagy initiation complex downstream of TORC1 [17], suggesting unknown TORC1 effector(s) crucial for mitophagy. Moreover, Atg32-Atg11 interaction, a prerequisite step for degradation of mitochondria, was labile in the absence of Npr2. We propose that mitophagy in respiring yeast is initiated through SEACIT-dependent TORC1 inactivation.

2. Materials and methods

2.1. Yeast strains and growth conditions

Yeast strains and plasmids used in this study are described in Supplementary Tables 1 and 2 Standard genetic and molecular biology methods were used for *S. cerevisiae*. Yeast cells were incubated in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose), synthetic medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate) with 0.5% casamino acids containing 2% dextrose (SDCA), or 0.1% dextrose plus 3% glycerol (SDGlyCA), supplemented with necessary amino acids. For mitophagy, pexophagy, ER-phagy, and the Cvt pathway under respiratory conditions, cells grown to mid-log phase in SDCA were transferred to SDGlyCA and incubated at 30 °C. For rapamycin treatment, cells grown to mid-log phase in SDCA were transferred to SDGlyCA, incubated at 30 °C for 18 h, and then treated with 1 μM rapamycin (1:10,000 dilution of the 10 mM stock dissolved in DMSO) until the time points for harvesting.

2.2. Microscopy

Cells were observed using an inverted microscope (Axio Observer. Z1; Carl Zeiss) equipped with differential interference contrast optics, epifluorescence capabilities, a 100X objective lens (αPlan-APOCHROMAT 100, NA: 1.46; Carl Zeiss), a monochrome CCD camera (AxioCam MRm; Carl Zeiss), and filter sets for green fluorescent protein (GFP) and mCherry, a monomeric variant of the red fluorescent protein DsRed (13 and 20, respectively; Carl Zeiss). Images were captured using acquisition and analysis software (Axio Vision 4.6; Carl Zeiss).

2.3. Immunoblotting

Protein samples corresponding to 0.1 OD_{600} units of cells were separated by SDS-PAGE followed by western blotting and immunodecoration with primary antibodies raised against mCherry (1:2,000, Abcam ab125096), HA (1:5,000, Sigma A2095), Pgk1 (1:10,000, Abcam ab113687), and Ape1 (1:5000), Atg8 (1:2000), and Atg11 (1:1000) (gift from Dr. Hitoshi Nakatogawa, Tokyo Institute of Technology, Japan). After treatment with the secondary antibodies, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (H + L) for anti-mCherry, anti-HA, and anti-Pgk1, and goat anti-rabbit IgG (H + L) for anti-Ape1, anti-Atg8, and anti-Atg11 (1:10,000, Jackson ImmunoResearch 315-035-003 and 111-035-003, respectively) followed by the enhanced chemiluminescence reagent Western Lightning Plus-ECL (NEL103001EA, PerkinElmer), proteins were detected using a luminescent image analyzer (LAS-4000 mini; GE Healthcare). Quantification of the signals was performed using ImageQuant TL (GE Healthcare).

2.4. Immunoprecipitation

Co-immunoprecipitation assays were performed using a vacuolar protease-deficient strain transformed with a low-copy plasmid vector encoding Atg32 (negative control) or Atg32-HA. 800-1000 OD_{600} units of cells grown in SDGlyCA for 24 h were collected by centrifugation, washed once with H_2O, resuspended in TD buffer (0.1 M Tris-SO4 [pH 9.4], 10 mM DTT), and incubated for 10 min at 30 °C. Cells were collected by centrifugation, resuspended in SP buffer (20 mM potassium phosphate buffer [pH 7.4], 1.2 M sorbitol) containing 0.125 mg/ml Zymolyase 100T (120493; Seikagaku), and incubated for 90 min at 30 °C. Spheroplasts were washed with SP buffer, resuspended in SH buffer (0.6 M sorbitol, 20 mM HEPES-KOH [pH 7.4]) containing a protease and phosphatase inhibitor cocktail (100X) (1861284; Thermo Scientific), and 1 mM phenylmethylsulfonyl fluoride (PMSF), and homogenized using a 40 mL dounce tissue grinder (357546; Wheaton) with a tight-fitting pestle (80 strokes) on ice. Whole cell homogenates were subjected to centrifugation (3,500 rpm) at 4 °C for 5 min. Membrane and soluble fractions were separated by centrifugation (12,000 rpm) at 4 °C for 5 min. Mitochondria-enriched fractions were then resuspended in lysis buffer (50 mMTris-HCl [pH 7.5], 100 mM NaCl, 0.1 mM EDTA, 0.4% Triton X-100, protease and phosphatase inhibitor cocktail, and 1 mM PMSF) at 4 °C for 15 min and subjected to centrifugation (12,000 rpm) at 4 °C for 5 min. The supernatant was incubated with 200 μL of anti-HA-agarose conjugate (014-23081; Wako) at 4 °C for 2 h with gentle agitation. The beads were washed twice with wash buffer (50 mM Tris-HCl [pH 7.5], 300 mM NaCl, 0.1 mM EDTA, 0.4% Triton X-100, and protease and phosphatase inhibitor cocktail), and once with lysis buffer. Immunoprecipitates were eluted with 100 mM Glycine HCl [pH2.5−3.5] for three times with gently upside down at room temperature for 5 min. Elution samples were precipitated with 100% (w/v) trichloroacetic acid solution for 30 min on ice and then subjected to centrifugation. Pellets were washed with acetone for three times at 4 °C, resolved in SDS-sample buffer, and analyzed by SDS-PAGE and western blotting.

3. Results and discussion

3.1. Mitophagy is significantly suppressed in respiring cells lacking SEACIT

Our previous genome-wide screen for non-essential, single gene deletion strains exhibiting mitophagy defects [9] has provided us with candidates for further investigation that included mutants lacking Npr3, a component of SEACIT. To clarify if the activity of SEACIT comprising Iml1, Npr2, and Npr3 is critical for mitochondrial degradation, we first examined *iml1*-, *npr2*-, and *npr3*-null cells by microscopy. Mitochondria and vacuoles were visualized using mito-dihydrofolate reductase (DHFR)-mCherry, a fusion protein marker located in the mitochondrial matrix, and Vph1-GFP, a vacuole-anchored marker, respectively [18]. Cells were grown in medium containing glycerol (Gly), a non-fermentable carbon

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