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The Nem1-Spo7 protein phosphatase complex is required for efficient mitophagy in yeast

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ABSTRACT

Mitochondria-targeted selective autophagy, termed mitophagy, is an evolutionarily conserved process that contributes to mitochondrial quantity and quality control. Mitophagy requires elaborate membrane biogenesis of autophagosomes surrounding mitochondria, although how this process is regulated remains obscure. We show here that mitophagy is strongly suppressed in yeast cells lacking Nem1 or Spo7, two proteins forming a heterodimeric protein phosphatase complex known to be important for proper shaping of the nucleus and endoplasmic reticulum (ER). Under the same conditions, selective degradation of the ER and peroxisomes was also suppressed strongly and to a lesser extent, respectively, whereas autophagy and the cytoplasm to vacuole targeting (Cvt) pathway were only slightly affected in those mutants. We also found that mitochondrial sequestration in the cytoplasm and their degradation in the vacuole, a lytic compartment in yeast, occurred poorly but did not completely arrest. Notably, deletion of the *INO2* gene in the *nem1*-or *spo7*-null mutant partially rescued nuclear/ER membrane shaping and mitophagy. Together, our data suggest that Nem1-Sop7-mediated regulation of membrane biogenesis is needed to promote mitophagy in yeast.

1. Introduction

Mitochondria are dynamic organelles that remodel their volume, shape, distribution, and activity in response to intra- and extracellular environmental changes, and play essential roles in a myriad of metabolic reactions and signaling events [1]. For example, they are highly developed in cells with high energy demands, and their quantity become reduced according to a decrease in ATP consumption or oxygen concentration. Since mitochondria generate their own byproducts, reactive oxygen species (ROS) from the electron transport chain, they are constantly challenged with ROS-derived oxidative stress that often leads to deterioration of mitochondrial quality [2]. Therefore, cells must survey mitochondrial fitness and maintain their activity. Numerous studies in the last decade reveal that mitochondrial quantity and quality is regulated in part through their autophagy-dependent degradation [3]. This catabolic process, called mitophagy, mediates selective sequestration of mitochondria into autophagosomes, newly formed double membrane structures, and their subsequent transport to the lysosome, a lytic organelle for degradation [4]. Mitophagy is a fundamental process conserved from yeast to humans whose defects are associated with various disorders including aging, cancer, heart failure, and neurodegeneration, highlighting the physiological significance [5].

Targeting of autophagosome formation to mitochondria is a key step in mitophagy that requires either mitochondrial outer membrane (OM) proteins acting as receptors for Atg8, an autophagosome-localized ubiquitin-like modifier conjugated to the phospholipid phosphatidylethanolamine, or ubiquitin molecules covalently linked to proteins on the surface of mitochondria and captured by adaptors for Atg8 [6]. In the yeast *Saccharomyces cerevisiae*, mitochondrial degradation is promoted by the mitophagy receptor Atg32, a 59 kDa single-pass OM protein [7,8]. When anchored ectopically to peroxisomes, Atg32 can facilitate peroxisomal degradation in an autophagy-dependent manner [9], suggesting that this protein contains necessary and sufficient activities to drive formation of autophagosomes surrounding mitochondria. Although it is evident that mitochondrial sequestration involves highly dynamic membrane biogenesis and deformation, molecular mechanisms regulating this elaborate process are poorly understood.

Here we report that mitophagy in yeast depends on normal morphology of the nucleus and ER. Loss of the Nem1-Spo7 protein phosphatase [10,11] causes alterations in the nucleus/ER shaping

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and defects in degradation of mitochondria, peroxisomes, and the ER, whereas it only slightly affects autophagy and the Cvt pathway, an autophagy-related process selective for vacuolar proteins. Our results also indicate that mitochondrial sequestration and degradation processes occur inefficiently but not completely blocked. Strikingly, deletion of the *INO2* gene, which encodes a global transcriptional activator for phospholipid biosynthetic genes [12], can partially rescue aberrant nucleus/ER morphology and mitophagy deficiency. Collectively, our findings implicate Nem1-Spo7 as a key regulator for efficient mitophagy through proper nucleus/ER membrane biogenesis.

2. Materials and methods

2.1. Yeast strains and growth conditions

Yeast strains used in this study are described in Supplementary Table 1. Standard genetic and molecular biology methods were used for *S. cerevisiae* strains. 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 assays under respiratory conditions, cells grown to mid-log phase in SDCA were transferred to SDGlyCA and incubated at 30 °C.

2.2. Fluorescence microscopy

Yeast cells were observed by 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). Cell images were captured using acquisition and analysis software (Axio Vision 4.6; Carl Zeiss).

2.3. Immunoblotting

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), Pgk1 (1:10,000, Abcam ab113687), and Ape1 (1:5,000, 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 and -Pgk1, and goat anti-rabbit IgG (H + L) for Ape1 (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. Electron microscopy

Ultrastructural analysis of yeast cells was performed by Tokai Electron Microscopy Inc. as described previously [8] with some modifications. Yeast cultures corresponding to 50 OD_{600} units of cells were collected by centrifugation. The sandwiched yeast pellets with copper disks were frozen in liquid propane at −175 °C, freeze-substituted with 2% OsO_4 in acetone and 2% distilled water at −80 °C for 48 h, and then transferred where they can keep −20 °C for 4 h and were warmed up to 4 °C for 1 h. The samples were dehydrated through the anhydrous acetone twice for 30 min each and twice changes of 100% ethanol for 30 min each at room temperature, and kept with 100% ethanol at room temperature overnight. The dehydrated samples were infiltrated with propylene oxide (PO) twice for 30 min each, put into a 70:30 mixture of PO and resin (Quetol-651; Nisshin EM Co., Tokyo, Japan) for 1 h, and kept open in air overnight. The samples were then transferred to a fresh 100% resin and polymerized at 60 °C for 48 h. The polymerized resins were ultra-thin sectioned at 70 nm with a diamond knife using an ultramicrotome (Ultracut UCT, Leica). The sections were mounted on copper grids, stained with 2% uranyl acetate at room temperature for 15 min, and washed with distilled water followed by being secondary-stained with Lead stain solution (Sigma) at room temperature for 3 min. The grids were observed by a transmission electron microscope (JEM-1400Plus; JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV. Digital images (2048 × 2048 pixels) were taken with a CCD camera (VELETA; Olympus Soft Imaging Solutions GmbH, Münster, Germany).

3. Results and discussion

3.1. Mitophagy is strongly suppressed in cells lacking Nem1 or Spo7

Based on our previous genome-wide screen for non-essential gene deletion mutants defective in degradation of mitochondria [8], we revisited several candidates exhibiting partial mitophagy deficiencies using mito-DHFR-mCherry, a mitophagy probe [13]. Cells pregrown in dextrose medium were shifted to glycerol medium, and observed using fluorescence microscopy. When transported to the vacuole by mitophagy, this mitochondrial matrix-localized probe was processed to be a free mCherry and kept fluorescent to exhibit spherical vacuolar patterns [13]. Although transport of mitochondria to the vacuole was hardly detected in mid log phase cells (Gly 24 h), it became visible in stationary phase cells (Gly 72 h) (Fig. 1A). Strikingly, this Atg32-dependent process was strongly suppressed in cells lacking Nem1 or Spo7 (Fig. 1A). To confirm and quantify these mitophagy defects, protein samples were analyzed by SDS-PAGE and western blotting. Consistent with their fluorescence images, Atg32-dependent generation of free mCherry, which indicates selective transport of mitochondria to the vacuole, was reduced to 30–35% in the *nem1*-or *spo7*-null mutant compared to wildtype cells (Fig. 1B and C). Together, these results raise the possibility that protein(s) modified by the Nem1-Spo7 phosphatase may be critical for efficient mitophagy.

3.2. ER degradation is strongly suppressed in the absence of Nem1 or Spo7

To investigate whether loss of the Nem1-Spo7 protein phosphatase causes defects in other autophagy-related processes, we performed western blotting and electron microscopy. For ER-specific autophagy (ER-phagy), processing of Sec63-mCherry was monitored in cells grown under respiratory conditions. Like mito-DHFR-mCherry, this ER membrane-anchored marker is processed to be a free mCherry in a manner dependent on Atg39 and Atg40, two proteins required for ER-phagy [14]. We found that ER-phagy was reduced to 20–30% in the *nem1*-or *spo7*-null mutant compared to wildtype cells (Fig. 2A and B). Similarly, degradation of peroxisomes was analyzed using Pot1-mCherry that is processed to be a free mCherry via pexophagy mediated by Atg36 [15]. Cells lacking Nem1 or Spo7 exhibited slower degradation kinetics with a reduction to 80% compared to wildtype cells at the 72 h time point (Fig. 2C and D). Moreover, we examined the Cvt pathway, a type of