Protein N-terminal Acetylation by the NatA Complex Is Critical for Selective Mitochondrial Degradation*

Akinori Eiyama¹ and Koji Okamoto²
From the Laboratory of Mitochondrial Dynamics, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan

Background: Mitophagy is a catabolic mechanism that degrades mitochondria selectively, and protein N-terminal acetylation is a major modification of eukaryotic proteins.

Results: Deletion of protein N-terminal acetyltransferase A (NatA) leads to suppression of mitophagy in yeast.

Conclusion: Protein N-terminal acetylation by NatA is crucial for mitophagy.

Significance: This is the first report on the link between mitophagy and protein N-terminal acetylation.

Mitophagy is an evolutionarily conserved autophagy pathway that selectively degrades mitochondria. Although it is well established that this degradation system contributes to mitochondrial quality and quantity control, mechanisms underlying mitophagy remain largely unknown. Here, we report that protein N-terminal acetyltransferase A (NatA), an enzymatic complex composed of the catalytic subunit Ard1 and the adaptor subunit Nat1, is crucial for mitophagy in yeast. NatA is associated with the ribosome via Nat1 and acetylates the second amino acid residues of nascent polypeptides. Mitophagy, but not bulk autophagy, is strongly suppressed in cells lacking Ard1, Nat1, or both proteins. In addition, loss of NatA enzymatic activity causes impairment of mitochondrial degradation, suggesting that protein N-terminal acetylation by NatA is important for mitophagy. Ard1 and Nat1 mutants exhibited defects in induction of Atg32, a protein essential for mitophagy, and formation of mitochondria-specific autophagosomes. Notably, overexpression of Atg32 partially recovered mitophagy in NatA-null cells, implying that this acetyltransferase participates in mitophagy at least in part via Atg32 induction. Together, our data implicate NatA-mediated protein modification as an early regulatory step crucial for efficient mitophagy.

Mitochondria are essential organelles that supply most of the energy for a cell. This organelle concomitantly generates reactive oxygen species during respiration. Accumulation of reactive oxygen species eventually causes mitochondrial dysfunction that negatively affects cellular integrity and is thought to induce diverse pathology (1). Additionally, cells need to adjust mitochondrial quantity to maintain a suitable balance between ATP production and consumption (2). Therefore, degradation of dysfunctional and excess mitochondria is critical for cell homeostasis. To solve this problem, cells utilize mitophagy, a catabolic system via autophagy that isolates cytosolic components with double-membrane vesicles called autophagosomes and carries them into digestive compartments such as lysosomes (vacuoles in yeast) for degradation and recycling (3, 4). Mitophagy is a selective pathway that specifically eliminates mitochondria. This process is conserved from yeast to humans and is relevant to cellular physiology (5, 6).

In the budding yeast *Saccharomyces cerevisiae*, the mitochondria-anchored receptor Atg32 is induced in response to oxidative stress and is localized on the surface of mitochondria (7, 8). Loss of Atg32 disrupts mitophagy, ultimately leading to mitochondrial genome instability (9). Atg32 interacts with Atg8, a ubiquitin-like protein conjugated to the phospholipid phosphatidylethanolamine and required for autophagosome formation, and with Atg11, a selective autophagy-specific scaffold protein required for assembly of core Atg proteins (7, 8). Moreover, phosphorylation of Atg32 stabilizes the Atg32-Atg11 interaction (10–12). Although the ternary complex is important for formation of mitochondria-specific autophagosomes (mitophagosomes), how Atg32 induction and mitophagosome formation are regulated remains poorly understood.

We show here that formation of mitophagosomes is blocked by loss of N-terminal acetyltransferase A (NatA) catalyzing transfer of an acetyl group from acetyl-coenzyme A (acytetyl-CoA) to the α-amino group of the N-terminal amino acid residue (13, 14). In addition, Atg32 induction is partially suppressed in cells lacking NatA. Our findings suggest that NatA regulates selective mitochondrial degradation at least in part via mitophagosome formation and Atg32 expression.

Experimental Procedures

Yeast Strains and Growth Conditions—Yeast strains and plasmids used in this study are described in Tables 1 and 2, respectively. Standard genetic and molecular biology methods were used for *S. cerevisiae* and *Escherichia coli* 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 and pexophagy under respiratory conditions, cells grown to mid-log phase in SDCA were transferred to SDglyCA and incubated at 30 °C. For autophagy and mitophagy under starvation conditions, cells grown to mid-log phase in YPD were transferred to nitrogen-free medium (SD-N; 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% dextrose) and incubated at 30 °C. For starvation-induced pexophagy, cells grown overnight in YPD were transferred to olate medium (0.17% yeast nitrogen base without amino acids ammonium sulfate, 0.5% ammonium sulfate, 1% casamino acids, 0.12% oleate (v/v), 0.2% Tween 40R (v/v), 0.1% yeast
extract) at a 1:10 dilution. Pexophagy was induced by transferring cells from oleate medium to SD-N.

Microscopy—Cells were observed using an inverted microscope (Axio Observer. Z1; Carl Zeiss) equipped with differential interference contrast optics, epifluorescence capabilities, a ×100 objective lens (aPlan-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 (13 and 20, respectively; Carl Zeiss). Images were captured using acquisition and analysis software (Axio Vision 4.6; Carl Zeiss).

Immunoblotting—Samples corresponding to 0.1 OD$_{600}$ units of cells were separated by SDS-PAGE followed by western blotting and immunodecoration. After treatment with enhanced chemiluminescence reagents, proteins were detected using a luminescent image analyzer (LAS-4000 mini; GE Healthcare). Quantification of the signals was performed using ImageQuant TL (GE Healthcare).

Quantitative RT-PCR—RNA was isolated by Master Pure Yeast RNA purification kit (Epicenter) from 2 OD$_{600}$ units of cells, according to the manufacturer’s protocol. Equal amounts of total RNA were mixed with RNA-direct SYBR Green Real Time PCR Master Mix (TOYOBO) and the following primers: ATG32 forward 5'-CTGCTCAGTTGAAGAAGGAGATG; ACT1 forward 5'-TGTCACTGCAGCATACGAACAC and reverse 5'-CTGCTCAGTTGAAGAAGGAGATG; ACT1 forward 5'-TATGTCGCTGTTAGACAAAGGAC and reverse 5'-TCTGTCAGTTGTGACAAATAC. The Applied Biosystems Step One Plus (Applied Biosystems) was used for quantitative RT-PCR analysis. For each gene, triplicate samples were mixed with RNA-direct SYBR Green Real Time PCR Master Mix (TOYOBO) and the following primers: ATG32 forward 5'-CTGCTCAGTTGAAGAAGGAGATG; ACT1 forward 5'-TGTCACTGCAGCATACGAACAC and reverse 5'-CTGCTCAGTTGTGACAAATAC. The Applied Biosystems Step One Plus (Applied Biosystems) was used for quantitative RT-PCR analysis. For each gene, triplicate samples were processed using ImageQuant TL (GE Healthcare).

Results

Mitophagy Is Markedly Suppressed in Cells Lacking NatA—To elucidate the role of NatA in mitophagy, we observed transport of mitochondria to the vacuole, a lytic compartment in yeast. Mitochondria and vacuoles were visualized using a mitochondrial matrix-targeted GFP (mito-GFP) and mCherry fused at the C terminus of Vph1, a membrane-integrated subunit of the vacuolar ATPase (Vph1-mCherry), respectively, in cells lacking Prb1, a vacuole-localized serine protease. When cells are grown in nonfermentable glycerol medium (Gly), mitochondria become active in respiration and are eventually degraded by mitophagy (7). In vacuolar protease-deficient cells, mitochondria transported into the vacuole can be accumulated and observed as degradation intermediates. Cells grown in Gly for 48 h contained mitochondria that were overlapped with the vacuole in a manner dependent on Atg32 (Fig. 1A). We hardly detected mito-GFP signals colocalized with Vph1-mCherry patterns in cells lacking NatA subunits, the catalytic component Ard1 and ribosomal adaptor Nat1 (13, 14), under the same conditions (Fig. 1A). These observations suggest that NatA is important for mitophagy.

We next quantified mitochondrial degradation using mitodihydrofolate reductase (DHFR)–mCherry, a reporter located in the mitochondrial matrix. DHFR is degraded in the vacuole and can be detected using the mCherry reporter. This approach allows for the visualization of mitochondrial degradation in real-time. DHFR-mCherry was expressed in cells with and without NatA, and the degradation of this reporter was monitored by immunoblotting. Cells lacking NatA subunits showed a marked decrease in DHFR-mCherry protein levels compared to wild-type cells, indicating a suppression of mitophagy. These results support the importance of NatA in mitophagy.
NatA Is an Important Element in Mitophagy

A. Mitochondrial GFP-expressing and vacuolar Vph1-mCherry-expressing prb1Δ, nat1Δ, prb1Δ, nat1Δ, prb1Δ, and ard1Δ, prb1Δ cells grown to mid-log phase in dextrose medium (0 h) were shifted to respiration medium (Gly) for 48 h and observed using fluorescence microscopy. Cells lacking Prb1 are vacuolar protease-deficient and accumulate degradation intermediates within the vacuole.

B. Mitochondria-targeted DHFR-mCherry-expressing (mito-DHFR-mCherry, depicted by arrow) wild-type, nat1Δ, ard1Δ, nat1Δ ard1Δ, and atg32Δ cells were grown for the indicated time points in glycerol medium (Gly) and subjected to western blotting. Generation of free mCherry (indicated by arrowhead) indicates transport of the marker to the vacuole. Pgk1 was monitored as a loading control.

C. Free mCherry (% N 6h) bar graph showing the percentage of free mCherry in different strains after 6 hours of nitrogen starvation.

D. Mitochondria-targeted DHFR-mCherry-expressing (mito-DHFR-mCherry, depicted by arrow) wild-type, nat1Δ, ard1Δ, nat1Δ ard1Δ, and atg32Δ cells grown to mid-log phase in nutrient-rich medium were shifted to nitrogen starvation (−N), collected at the indicated time points, and subjected to western blotting as in B. Mitochondria-targeted DHFR-mCherry-expressing wild-type, nat1Δ, ard1Δ, atg32Δ, and atg7Δ cells were grown for the indicated time points in glycerol medium (Gly) and subjected to western blotting as in B. E. Mitochondria-targeted DHFR-mCherry-expressing wild-type, nat1Δ, ard1Δ, and atg32Δ cells grown to mid-log phase in nutrient-rich medium were shifted to nitrogen starvation (−N), collected at the indicated time points, and subjected to western blotting as in B.

FIGURE 1. Loss of NatA leads to strong suppression of mitophagy. A, mitochondrial GFP-expressing and vacuolar Vph1-mCherry-expressing prb1Δ, atg32Δ, prb1Δ, nat1Δ, prb1Δ, and ard1Δ, prb1Δ cells grown to mid-log phase in dextrose medium (0 h) were shifted to respiration medium (Gly) for 48 h and observed using fluorescence microscopy. Cells lacking Prb1 are vacuolar protease-deficient and accumulate degradation intermediates within the vacuole. Scale bar, 5 μm. DIC, differential interference contrast. B, mitochondria-targeted DHFR-mCherry-expressing (mito-DHFR-mCherry, depicted by arrow) wild-type, nat1Δ, ard1Δ, nat1Δ ard1Δ, and atg32Δ cells were grown for the indicated time points in glycerol medium (Gly) and subjected to western blotting. Generation of free mCherry (depicted by arrowhead) indicates transport of the marker to the vacuole. Pgk1 was monitored as a loading control. C, free mCherry in cells under respiratory conditions for 48 h in B was quantified in three experiments. Data represent the averages of all experiments, with bars indicating standard deviations. D, mitochondria-targeted DHFR-mCherry-expressing (mito-DHFR-mCherry, depicted by arrow) wild-type, nat1Δ, mak3Δ, nat4Δ, and nat5Δ cells were grown for the indicated time points in glycerol medium (Gly) and subjected to western blotting as in B. E, mito-DHFR-mCherry-expressing wild-type, nat1Δ, ard1Δ, and atg32Δ cells grown to mid-log phase in nutrient-rich medium were shifted to nitrogen starvation (−N), collected at the indicated time points, and subjected to western blotting as in B. Free mCherry in cells under starvation conditions for 24 h in C was quantified in three experiments. Data represent the averages of all experiments, with bars indicating standard deviations.

K. Phat1-mCherry-expressing (a peroxisome marker) wild-type, nat1Δ ard1Δ, and atg36Δ cells grown in oleic acid medium were shifted to nitrogen starvation (−N), collected at the indicated time points, and subjected to western blotting. Generation of free mCherry indicated transport of peroxisomes to the vacuole. J. Free mCherry in cells under starvation conditions for 48 h in K was quantified in three experiments. Data represent the averages of all experiments, with bars indicating standard deviations.

# Additional Notes
- **A**: The images show the results of experiments with various genetic backgrounds, demonstrating the role of NatA in mitophagy. Cells lacking Prb1 are vacuolar protease-deficient and accumulate degradation intermediates within the vacuole. Mitochondria-targeted DHFR-mCherry-expressing cells were used to visualize mitochondrial transport.
- **B**: Western blotting was performed to confirm the transport of the DHFR-mCherry marker to the vacuole.
- **C**: A bar graph showing the percentage of free mCherry in different strains after 6 hours of nitrogen starvation.
- **D**: Similar experiments were conducted with different genetic backgrounds to further support the role of NatA in mitophagy.
- **K**: The generation of free mCherry indicated transport of peroxisomes to the vacuole under starvation conditions.

# Conclusion
- NatA is an important element in mitophagy, as its loss results in strong suppression of the process, indicated by the accumulation of degradation intermediates within the vacuole.
in the matrix of mitochondria (11). Upon mitophagy, this fusion protein is processed to generate free mCherry that is appreciably protease-resistant, thereby indicating degradation of mitochondria. We found that accumulation of free mCherry was greatly decreased in cells lacking Nat1, Ard1, or both (Fig. 3A and B). By contrast, cells lacking the catalytic subunits of other N-terminal acetyltransferases, NatB–E (13, 14), did not exhibit defects in degradation of mitochondria (Fig. 1D). We have previously demonstrated that mitophagy is induced during nitrogen starvation (15). Starvation-induced mitochondrial

**FIGURE 2.** Catalytically inactive Ard1 mutant is defective in mitophagy. A, part of amino acid sequence alignment from Ard1 homologs. The glutamic acid residues critical for enzymatic activity are indicated with asterisk. B, mito-DHFR-mCherry-expressing wild-type and ard1Δ cells transformed with empty vector, plasmid-encoding Ard1–3HA, or plasmid-encoding Ard1E26Q (E26Q)-3HA were grown for the indicated time points in glycerol medium (Gly) and subjected to western blotting. Generation of free mCherry (depicted by arrowhead) indicates transport of mitochondria to the vacuole. C, free mCherry in cells under respiratory conditions for 48 h in B was quantified in three experiments. Data represent the averages of all experiments, with bars indicating standard deviations. D, wild-type, Nat1–3HA-expressing, Ard1–3HA-expressing, and atg32Δ cells containing mito-DHFR-mCherry were grown for the indicated time points in glycerol medium (Gly) and subjected to western blotting as in B. E, wild-type, Nat1–3HA-expressing, and atg32Δ cells containing mito-DHFR-mCherry were transformed with empty vector, and a plasmid encoding Ard1–3HA or Ard1R125A (R125A)-3HA, grown for the indicated time points in glycerol medium (Gly), and subjected to western blotting as in B. F, part of amino acid sequence alignment including acetyl-CoA binding domains from Ard1 homologs. The arginine residues critical for acetyl-CoA binding are indicated with asterisk. G, mito-DHFR-mCherry-expressing wild-type and ard1Δ cells transformed with empty vector and a plasmid encoding Ard1–3HA or Ard1R125A (R125A)-3HA were grown for the indicated time points in glycerol medium (Gly) and subjected to western blotting as in B. H, free mCherry in cells under respiratory conditions for 48 h in G was quantified in three experiments. Data represent the averages of all experiments, with bars indicating standard deviations.
degradation was also suppressed in cells lacking Nat1 or Ard1 (Fig. 1, E and F). These results indicate that NatA participates in the regulation of mitophagy.

We further investigated whether NatA affects the other autophagy-related pathways. First, we monitored processing of GFP-Atg8, an indicator of autophagy flux, and confirmed that free GFP was detected in cells lacking Nat1 or Ard1 under nitrogen deprivation (Fig. 1G). Therefore, it is likely that NatA is dispensable for starvation-induced autophagy. Next, we examined the cytoplasm-to-vacuole targeting (Cvt) pathway, a selective autophagy process that mediates transport of vacuolar enzymes such as Ape1, an amino peptidase, from the cytosol to the vacuole. Mature Ape1 transported to the vacuole was detectable in Nat1 or Ard1-null cells under respiratory conditions, indicating that NatA is dispensable for the Cvt pathway (Fig. 1H). Finally, we evaluated selective peroxisome autophagy (pexophagy). Degradation of peroxisomes was monitored using Pot1-mCherry, a marker localized in the peroxisomal matrix, under respiratory conditions that are also able to induce pexophagy (16). We found that accumulation of free mCherry was strongly depressed in cells lacking Nat1 or Ard1 (Fig. 1, I and J). Starvation-induced pexophagy was also inhibited in these mutant cells (Fig. 1, K and L). Together, our results raise the possibility that NatA is a common factor critical for selective elimination of organelles.

**Enzymatic Activity of NatA Is Crucial for Mitophagy**—To ask whether protein N-terminal acetylation by NatA is important for selective mitochondrial degradation, we investigated a NatA variant lacking enzymatic activity. Previous research unravels the structure of NatA in the fission yeast *Schizosaccharomyces pombe*, demonstrating that glutamine substitution for Glu-24 of Ard1 leads to loss of enzymatic activities but does not affect substrate binding capacity (17). This critical glutamate is conserved from budding yeast (Glu-26) to humans (21). To ask whether monomeric Ard1 dissociated from the ribosome contributes to regulation of mitophagy in *S. cerevisiae*, we monitored mitochondrial degradation in cells overexpressing the Ard1E26Q mutant (Fig. 2E). Another previous study has reported that human Ard1R82A, a variant containing a mutation in the acetyl-CoA binding domain, decreases enzymatic activities (18). Because this crucial arginine is also conserved among eukaryotes (Fig. 2F), we introduced a plasmid encoding HA-tagged Ard1E26Q (corresponding to human Ard1R82A) into ard1Δ cells, and we monitored processing of mito-DHFR-mCherry under respiratory conditions. Cells expressing Ard1E26Q-3HA displayed a reduced accumulation of free mCherry (Fig. 2, G and H). Taken together, we conclude that protein N-terminal acetylation by NatA is critical for mitophagy in yeast.

**Ribosomal Association of Ard1 Is Important for Efficient Mitophagy**—Although Ard1 stably associates with the ribosome via Nat1 in yeast (19, 20), it has also been reported that monomeric Ard1 purified from fission yeast is still catalytically active with altered substrate specificity *in vitro* (17) and that Ard1 dissociated from the ribosome contributes to regulation of mitophagy in *S. cerevisiae*. To ask whether monomeric Ard1 dissociated from the ribosome is important for mitophagy in humans (21), we performed a ribosomal association assay to examine the cytoplasm-to-vacuole targeting (Cvt) pathway, a selective autophagy process that mediates transport of vacuolar enzymes such as Ape1, an amino peptidase, from the cytosol to the vacuole. Mature Ape1 transported to the vacuole was detectable in Nat1 or Ard1-null cells under respiratory conditions, indicating that NatA is dispensable for the Cvt pathway (Fig. 1H). Finally, we evaluated selective peroxisome autophagy (pexophagy). Degradation of peroxisomes was monitored using Pot1-mCherry, a marker localized in the peroxisomal matrix, under respiratory conditions that are also able to induce pexophagy (16). We found that accumulation of free mCherry was strongly depressed in cells lacking Nat1 or Ard1 (Fig. 1, I and J). Starvation-induced pexophagy was also inhibited in these mutant cells (Fig. 1, K and L). Together, our results raise the possibility that NatA is a common factor critical for selective elimination of organelles.

**NatA Is an Important Element in Mitophagy**

**FIGURE 3. Ribosome-associated Ard1 is required for efficient mitophagy.** A, Ard1-3HA-expressing wild-type and nat1Δ cells were grown for the indicated time points in glycerol medium (Gly) and subjected to western blotting. B, Nat1-3HA-expressing wild-type and ard1Δ cells were grown for the indicated time points in glycerol medium (Gly) and subjected to western blotting. C, wild-type and nat1Δ cells expressing Ard1-3HA under the endogenous promoter or the strong GPD promoter were grown for the indicated time points in glycerol medium (Gly), and subjected to western blotting. Mitophagy was monitored using mito-DHFR-mCherry (depicted by arrow). Generation of free mCherry (depicted by arrowhead) indicates transport of mitochondria to the vacuole. D, free mCherry in cells under respiratory conditions for 48 h in C was quantified in three experiments. Data represent the averages of all experiments, with bars indicating standard deviations.

**Mitophagosome Formation Is Impaired in Cells Lacking NatA**—In which stage of mitophagy does NatA participate? It is noteworthy that the Nat1 and Ard1 protein levels declined during respiratory growth and became exceedingly low at the 48-h time point (Fig. 3, A and B). We thus speculated that NatA affects the early phase of mitophagy, in particular mitophagosome formation through which mitochondria are enclosed by free Ard1 post-translationally acetylates β- and γ-actin in humans (21). To ask whether monomeric Ard1 dissociated from the ribosome contributes to regulation of mitophagy in *S. cerevisiae*, we monitored mitochondrial degradation in cells overexpressing Ard1 because the Ard1 levels are drastically reduced in nat1Δ cells (Fig. 3A). We found that overexpression of Ard1 did not lead to recovery of mitophagy in the absence of the ribosome adaptor (Fig. 3, C and D). Hence, it seems likely that NatA-mediated cotranslational acetylation is crucial for mitophagy.
**FIGURE 4. Disruption of NatA causes inhibition of mitophagosome formation.** A, experimental scheme for observation of mitophagosomes in ypt7Δ cells. B, mitochondrial mCherry-expressing ypt7Δ, ypt7Δ atg32Δ, ypt7Δ nat1Δ, and ypt7Δ ard1Δ cells grown to mid-log phase in dextrose medium (Dex) were shifted to respiration medium (Gly) for 48 h, transferred to dextrose medium for 3 h, and investigated using fluorescence microscopy. Loss of Ypt7 prevents fusion between mitophagosomes and vacuoles. Scale bar, 5 μm. DIC, differential interference contrast. C, number of cells containing mitochondrial mCherry dots at stage 3 in B was quantified in three experiments. Data represent the averages of all experiments, with bars indicating standard deviations. n > 100. **, p < 0.05.
NatA Is an Important Element in Mitophagy

FIGURE 5. Atg32 induction is partially suppressed in nat1Δ and ard1Δ cells. A, Atg32–3HA-expressing wild-type, nat1Δ, and ard1Δ cells were grown in glycerol medium (Gly), collected at the indicated time points, and subjected to western blotting. B, peaks of Atg32–3HA expression in A were quantified in three experiments (wild type, 18 h; nat1Δ, 24 h; ard1Δ, 24 h). Data represent the averages of all experiments, with bars indicating standard deviations. *, p < 0.01. C, mito-DHFR-mCherry-expressing wild-type, nat1Δ, and ard1Δ cells were grown for the indicated time points in glycerol medium (Gly). Atg32 mRNA expression was analyzed by real-time PCR and normalized to ACT1 mRNA expression. Data represent the averages of all experiments, with bars indicating standard deviations. D, Atg32–3HA- and mito-DHFR-mCherry-expressing wild-type, nat1Δ, and ard1Δ cells transformed with empty vectors or two plasmids encoding Atg32–3HA were grown for the indicated time points in glycerol medium (Gly) and subjected to western blotting. Generation of free mCherry (depicted by arrowhead) indicates transport of mitochondria to the vacuole. E and F, free mCherry in nat1Δ and ard1Δ cells under respiratory conditions for 48 h in D was quantified in three experiments. G, mito-DHFR-mCherry-expressing atg32Δ, nat1Δ atg32Δ, and ard1Δ atg32Δ cells were transformed with a plasmid encoding GFP-Atg32 with the constitutive TEF2 promoter, grown to log phase in glucose medium, and investigated using fluorescence microscopy. Scale bar, 5 μm. H, pep4Δ prb1Δ and pep4Δ prb1Δ ard1Δ cells expressing chromosome- or plasmid-encoded versions of Atg32 or Atg32-HA were grown in glycerol medium and subjected to coimmunoprecipitation using anti-HA antibody-conjugated agarose. Eluted immunoprecipitates (IP) and detergent-solubilized mitochondria-enriched fractions (input) were analyzed by western blotting. I, mito-DHFR-mCherry-expressing wild-type and atg32Δ cells transformed with an empty vector or a plasmid encoding Atg32–3HA or Atg32–3HA (V2P)-3HA were grown for the indicated time points in glycerol medium (Gly) and subjected to western blotting. Generation of free mCherry (depicted by arrowhead) indicates transport of mitochondria to the vacuole.

the isolation membrane. To test this possibility, we used cells lacking Ypt7, a Rab family GTPase essential for homotypic vacuole fusion. Although autophagosomes are formed normally in this mutant, they do not fuse to the vacuole and accumulate in the cytosol (22). When ypt7Δ cells grown in fermentable medium (Dex) are shifted to respiratory medium (Gly), their mitochondrial shape changes from tubules to fragments (Fig. 4A). Conversely, upon a shift from respiratory to fermentable...
NatA Is an Important Element in Mitophagy

conditions, mitochondrial tubular networks are reformed by their fusion, but mitophagosomes remain isolated from intact mitochondria. Accordingly, we can detect mitophagosomes as dot-like structures (7). We found mitochondrial dots in cells grown under fermentable conditions for 3 h after respiratory growth (Fig. 4B). By contrast, cells containing mitochondrial dots were few in the absence of Nat1 or Ard1 (Fig. 4C). Hence, these observations suggest that NatA plays a key role in the early step of mitophagosome formation.

Atg32 Expression Levels Are Low in nat1Δ and ard1Δ Cells—
Based on the previous findings that Atg32 acts as a mitophagy receptor to generate mitophagosomes (7, 8), we investigated whether NatA affects Atg32 functions. At first, to examine Atg32 expression in NatA-null cells under respiratory conditions, HA-tagged Atg32 was expressed from the endogenous locus and monitored by western blotting. In light of previous reports that the Atg32 levels are transiently elevated in respiring cells at mid-log phase (7), and that growth of cells lacking NatA is slow in nonfermentable medium (13), we prepared samples from nat1Δ and ard1Δ cells at later time points than wild-type cells and compared their highest amounts of Atg32.

Cells lacking Nat1 or Ard1 exhibited a partial reduction in the Atg32 protein levels (Fig. 5, A and B). Additionally, transcriptional levels of ATG32 mRNA in these cells were quantified by real time PCR. We found that ATG32 mRNA expressions in nat1Δ and ard1Δ cells were less than half of those in wild-type cells during respiratory growth (Fig. 5C), raising the possibility that NatA may regulate transcription of ATG32.

To ask whether these reductions of Atg32 expression levels impact mitochondrial degradation, we introduced two low-copy plasmids encoding Atg32–3HA into NatA-null cells and monitored processing of mito-DHFR-mCherry. When grown in nonfermentable medium, Nat1 or Ard1-null cells overexpressing Atg32 displayed partial recovery of mitochondrial degradation (Fig. 5, D–F). These data are consistent with the idea that partial impairment of Atg32 induction may be one of the factors for severe mitophagy defects in cells lacking NatA.

We also confirmed Atg32 localization to mitochondria and interaction with Atg8 and Atg11 in the early stage of mitophagy (7, 11). Fluorescence live cell imaging and coimmunoprecipitation assays were performed using GFP- and HA-tagged Atg32, respectively. GFP-Atg32 was normally localized to mitochondria in cells lacking Nat1 or Ard1 (Fig. 3G), and Atg32–3HA coprecipitated with Atg11 and Atg8 in the absence of Ard1 (Fig. 3H). It is likely that NatA does not regulate organelle targeting and protein–protein interactions of Atg32.

In yeast, NatA acetylates the second amino acids, Ser, Ala, Thr, Val, Gly, and Cys, of nascent polypeptides whose N-terminal methionine residues are removed by methionine peptidase (23–25). Atg32 contains a valine at the second amino acid position, raising the possibility that Atg32 is acetylated by NatA. We thus substituted proline for Val-2 of Atg32, introduced a plasmid encoding Atg32V2P–3HA into Atg32-null cells, and monitored processing of mito-DHFR-mCherry under respiratory conditions. A protein containing proline at the second amino acid position is not acetylated by all N-terminal acetyltransferases in accordance with the APX rule (26). We found that mitophagy is not altered in cells expressing Atg32V2P–3HA (Fig. 3I). Thus, NatA is unlikely to directly control Atg32.

Atg8 and Atg11 Are Expressed Normally in the Absence of NatA—Finally, we examined whether loss of NatA affects Atg8 and Atg11, two Atg32-interacting proteins essential for mitophagy, under respiratory conditions. The protein levels of Atg8 and Atg11 were not decreased in cells lacking Nat1 or Ard1 (Fig. 6, A and B). Moreover, the phospholipid conjugation of Atg8 occurred properly in NatA-null cells (Fig. 6A). These data are in agreement with the results that NatA is dispensable for the Cvt pathway under respiratory conditions (Fig. 1H). Because Atg11 has alanine at the second amino acid position that can be acetylated by NatA, we introduced a plasmid encoding Atg11A2P into cells lacking Atg11 and monitored mitophagy under respiratory conditions. Cells expressing Atg11A2P did not exhibit significant changes in mitochondrial degradation (Fig. 6C). Therefore, it seems likely that NatA acts in mitophagy irrespectively of Atg8 and Atg11.

Discussion

In this study, we demonstrated that NatA plays a critical role in selective degradation of mitochondria in yeast. This catabolic process requires NatA catalytic activity and association with the ribosome, indicating that nascent polypeptide N-terminal acetylation of NatA target(s) is a key step for activation of mitophagy. Notably, mitophagosome formation is compromised in nat1Δ and ard1Δ cells, suggesting that NatA partici-
NatA is an Important Element in Mitophagy

NatA Is an Important Element in Mitophagy

Does NatA-mediated protein modification affect mitochondrial shaping during mitophagy? Fragmentation is a crucial step for mitochondria to be efficiently surrounded by the isolation membrane in mammalian cells (30–32). In yeast, it has been reported that Atg11 interacts with Dnm1, a dynamin-related GTPase required for mitochondrial fission, and that mitophagy is suppressed in dnm1A cells (33). However, mitochondrial fragmentation is almost normal in NatA-null cells under respiratory conditions (Figs. 1A and 4B). It is therefore unlikely that altered mitochondrial dynamics causes impairment of mitophagy in the absence of NatA. Because more than half of all proteins are acetylated by N-terminal acetyltransferases in yeast, NatA acetylates a lot of mitochondrial proteins, including import receptor, ATP synthase subunits, and ribosomal proteins (23). We thus do not exclude the possibility that changes in multiple mitochondrial functions could lead to mitophagy defects in NatA-null cells. Moreover, a recent study has reported that ERMES, a protein complex tethering the endoplasmic reticulum to mitochondria, is crucial for mitophagosome formation (34). The endoplasmic reticulum-mitochondrial tethering does not seem to be completely disrupted in NatA-null cells, because mitochondrial morphology defects, typical phenotypes of the ERMES mutants, are not seen in cells lacking Ard1 or Nat1 (Fig. 4B).

To our knowledge, this is the first report that protein N-terminal acetylation is linked to mitophagy. To clarify NatA-mediated activation of mitochondrial degradation in more detail, further studies are needed to identify NatA substrate(s) involved in mitophagy, to understand Atg32 induction mechanisms under respiratory conditions, and to investigate whether mitochondrial functions alter in NatA-null cells. These future approaches will elucidate regulation of mitophagy via protein N-terminal acetylation.

Author Contributions—K. O. designed the experiments, conceived and coordinated the study, and wrote the paper. A. E. designed, performed, and analyzed the experiments and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We are grateful to Toshiya Endo, Hayashi Yamamoto, Kaori Sakakibara, and Ayako Hashimoto for the kind gifts of antibodies, plasmids, and yeast strains. We also thank Noriko Okamoto for valuable comments on this manuscript.
NatA Is an Important Element in Mitophagy

References

1. Nunnari, J., and Suomalainen, A. (2012) Mitochondria: in sickness and in health. Cell 148, 1145–1159
2. Okamoto, K., and Kondo-Okamoto, N. (2012) Mitochondria and autophagy: critical interplay between the two homeostats. Biochim. Biophys. Acta 1820, 595–600
3. Mizushima, N., Yoshimori, T., and Ohsumi, Y. (2011) The role of Atg proteins in autophagosome formation. Annu. Rev. Cell Dev. Biol. 27, 107–132
4. Mizushima, N. (2011) Autophagy in protein and organelle turnover. Cold Spring Harb. Symp. Quant. Biol. 76, 397–402
5. Youle, R. J., and Narendra, D. P. (2011) Mechanisms of mitophagy. Nat. Rev. Mol. Cell Biol. 12, 9–14
6. Okamoto, K. (2014) Organellophagy: eliminating cellular building blocks via selective autophagy. J. Cell Biol. 205, 435–445
7. Okamoto, K., Kondo-Okamoto, N., and Ohsumi, Y. (2009) Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. Dev. Cell 17, 87–97
8. Kanki, T., Wang, K., Cao, Y., Baba, M., and Klionsky, D. J. (2009) Atg32 is a mitochondrial protein that confers selectivity during mitophagy. Dev. Cell 17, 98–109
9. Kurihara, Y., Kanki, T., Aoki, Y., Hirota, Y., Saigusa, T., Uchiimi, T., and Kang, D. (2012) Mitophagy plays an essential role in reducing mitochondrial production of reactive oxygen species and mutation of mitochondrial DNA by maintaining mitochondrial quantity and quality in yeast. J. Biol. Chem. 287, 3265–3272
10. Aihara, M., Jin, X., Kurihara, Y., Yoshida, Y., Matsushima, Y., Oku, M., Hirota, Y., Saigusa, T., Aoki, Y., Uchiimi, T., and Kang, D. (2013) Tor and the Sin3-Rpd3 complex regulate expression of the mitophagy receptor protein Atg32 in yeast. J. Cell Sci. 127, 3184–3196
11. Hwang, C.-S., Shemorry, A., and Varshavsky, A. (2010) N-terminal acetylation of cellular proteins creates specific degradation signals. Science. 327, 973–977
12. Mao, K., Wang, K., Zhao, M., Xu, T., and Klionsky, D. J. (2011) Two MAPK-signaling pathways are required for mitophagy in Saccharomyces cerevisiae. EMBO J. 30, 10190–10195
13. Twigg, G., Elorza, A., Molina, A. J., Mohamed, H., Wikstrom, J. D., Walzer, G., Stiles, L., Haigh, S. E., Katz, S., Las, G., Alroy, J., Wu, M., Py, B. F., Yuan, J., Deeney, J. T., et al. (2008) Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J. 27, 433–446
14. Mao, K., Wang, K., Liu, X., and Klionsky, D. J. (2013) The scaffold protein Atg11 recruits fission machinery to drive selective mitochondria degradation by autophagy. Dev. Cell 26, 9–18
15. Böckler, S., and Westermann, B. (2014) Mitochondrial ER contacts are crucial for mitophagy in yeast. Dev. Cell 28, 450–458
16. Kim, I., Kamada, Y., Stromhaug, P. E., Guan, J., Hefner-Gravink, A., Baba, M., Scott, S. V., Ohsumi, Y., Dunn, W. A., Jr., and Klionsky, D. J. (2001) Cvt9/Gsa9 functions in sequestering selective cytosolic cargo destined for the vacuole. J. Cell Biol. 153, 381–396
17. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14, 115–132
18. Sikorski, R. S., and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122, 19–27
19. Suzuki, K., Kiriakos, K., Kamada, Y., Mizushima, N., Noda, T., and Ohsumi, Y. (2001) The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. EMBO J. 20, 5971–5981

25044 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 290 • NUMBER 41 • OCTOBER 9, 2015