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Hrr25 phosphorylates the autophagic receptor Atg34 to promote vacuolar transport of α-mannosidase under nitrogen starvation conditions

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ABSTRACT

In *Saccharomyces cerevisiae*, under nitrogen-starvation conditions, the α-mannosidase Ams1 is recognized by the autophagic receptor Atg34 and transported into the vacuole, where it functions as an active enzyme. In this study, we identified Hrr25 as the kinase that phosphorylates Atg34 under these conditions. Hrr25-mediated phosphorylation does not affect the interaction of Atg34 with Ams1, but instead promotes Atg34 binding to the adaptor protein Atg11, which recruits the autophagy machinery to the Ams1–Atg34 complex, resulting in activation of the vacuolar transport of Ams1. Our findings reveal the regulatory mechanism of a biosynthetic pathway mediated by the autophagy machinery.

Structured summary of protein interactions:
Hrr25 phosphorylates **Atg34** by protein kinase assay (View interaction)
Ams1 and **Atg34** colocalize by fluorescence microscopy (View interaction)
Atg34 physically interacts with **Ams1** by anti tag coimmunoprecipitation (View interaction)
Atg11 physically interacts with **Atg34** by cross-linking study (View interaction)

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1. Introduction

Autophagy is a lysosomal or vacuolar degradation pathway in which various cytoplasmic components are selectively or non-selectively sequestered by double-membrane vesicle autophagosomes and transported into the lytic compartments [1–3]. This process requires a unique set of proteins called autophagy-related (Atg) proteins, which play central roles in forming the autophagosomal membrane and determining cargo selectivity. The autophagy machinery is also utilized to transport enzymes that function in the vacuole. In the cytoplasm-to-vacuole targeting (Cvt) pathway of the budding yeast *Saccharomyces cerevisiae*, three vacuolar enzymes, the aminopeptidases Ape1 and Ape4 and the α-mannosidase Ams1, are recognized by the receptor protein Atg19, and these proteins form an aggregate-like structure called the Ape1 complex [4–8]. Atg19 also interacts with the adaptor protein Atg11. Atg11 targets the Ape1 complex to the vacuole and recruits the Atg proteins that mediate the formation of the autophagosomal membrane, which sequesters the Ape1 complex [7]. When the vesicle formed fuses with the vacuole, the Ape1 complex is released into the vacuolar lumen, where the complex is disassembled and the enzymes begin to function.

S. cerevisiae has a homolog of Atg19, Atg34, which also interacts with Atg11. Whereas Atg19 directly binds all three Cvt cargos, Atg34 specifically recognizes Ams1 [8–10]. A previous study revealed that Atg34 is not involved in the Cvt pathway, but instead serves as a receptor for Ams1 and is involved in its autophagic transport under nitrogen-starvation conditions [9]. That study also showed that Atg34 is phosphorylated under these conditions. This modification might regulate the function of Atg34, but to date the identity of the responsible kinase and how it affects Atg34 function have remained unknown. Here, we show that the casein kinase 1 homolog Hrr25 is the kinase that phosphorylates Atg34 under nitrogen-starvation conditions. Our data suggest that phosphorylation by Hrr25 promotes Atg34 interaction with Atg11 and thereby upregulates the initiation of Ams1 sequestration by the autophagosomal membrane.

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2. Materials and methods

2.1. Yeast strains and plasmids

The yeast strains and oligonucleotides used in this study are listed in Tables 1 and 2, respectively. Gene tagging and disruption were performed by a PCR-based method [11–13]. pFA6a-*EGFP-kanMX6*, pFA6a-*EGFP-hphNT1*, pFA6a-*mCherry-kanMX6*, pFA6a-*zeoNT3*, pFA6a-*CgHIS3*, and pFA6a-*CgTRP1* (donated by Dr. Hayashi Yamamoto) were used for protein tagging with EGFP and mCherry and for gene disruption with the cassettes of the zeocin-resistant gene, *CgHIS3*, and *CgTRP1*. Cells expressing *ATG34* under the control of the *CET1* promoter were constructed by integrating a DNA fragment containing the *CET1* promoter, which was obtained by PCR using the plasmid pFA6a-*kanMX6-pCET1-VN* [14] and the oligonucleotides CET1pro-ATG34-Fw and -Rv, into the upstream of the start codon of chromosomal *ATG34*. Cells expressing GFP-tagged Atg34 mutants were constructed as follows. The coding sequence of *ATG34* was amplified by PCR using the primers HindIII-ATG34-Fw and ATG34-HindIII-Rv and ligated into the *Hin*dIII site of pFA6a-*EGFP-kanMX6*, resulting in pFA6a-*ATG34–EGFP-kanMX6*. Site-directed mutagenesis was performed using Quick Change kit (Agilent Technologies) for the Ala replacement of Ser382 or Ser383 of Atg34 encoded in this plasmid. DNA fragments obtained by PCR using these plasmids and the primers ATG34-ORF-Fw and ATG34-Ctag-Rv were introduced into yeast cells to replace chromosomal *ATG34* with *ATG34–EGFP-kanMX6* containing the *ATG34* mutations or not by homologous recombination. Cells expressing non-tagged Atg34 mutants were constructed as follows. A DNA fragment encompassing the *ATG34* open reading frame and the promoter region was obtained by PCR using the primers XbaI-ATG34pro-Fw and ATG34-XhoI-Rv and cloned into the *Xba*I-*Xho*I site of the pRS303 vector, and the *ATG34* mutations were introduced into this plasmid by site-directed mutagenesis. DNA fragments encompassing *HIS3* and *ATG34* were amplified by PCR using these plasmids and the primers HIS3pro-Fw and MCS-HIS3ter-Rv and integrated into the chromosomal *HIS3* locus by homologous recombination in *atg34*Δ cells.

Table 2
Oligonucleotides used in this study.

Name	Sequence
CET1pro-ATG34-Fw	gaaactagttcctataggttgagtgtctatcaaaaatttacggagacgcg GAATTCGAGCTCGTTTAAAC
CET1pro-ATG34-Rv	tgatcgatgacaaagaagtcgaatagtgtcgtttctaccgcaattttcat AGTGGGAGGATAGAAATGCTAC
HindIII-ATG34-Fw	aaaAAGCTTatgaaaattgcggtagaaac
ATG34-HindIII-Rv	aaaAAGCTTtatttcttcccaagtaaatggc
ATG34-ORF-Fw	CTCTAAAATCTCCACTAGCC
ATG34-Ctag-Rv	ttaaataagtactatagccaaagaaactggaagaatataaaaaagcat TTAATCGATGAATTCGAGCTCG
XbaI-ATG34pro-Fw	aaaTCTAGAtcgatcaagaaattaaatga
ATG34-XhoI-Rv	aaaCTCGAGggaagaatataaaaaagcat
HIS3pro-Fw	CGTTTTAAGAGCTTGGTGAG
MCS-HIS3ter-Rv	tccatctcttttatatttttttttctcgagttcaagagaaaaaaaaaagaaa CTAAAGGGAACAAAAGCTGG

2.2. Media and growth conditions

Yeast cells were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) at 30 °C. To knock down Hrr25 using the auxin-inducible degron (AID) system, indole-3-acetic acid (IAA) in ethanol was added to a final concentration of 0.5 mM, and the same volume of ethanol was added to control samples [13]. Autophagy was induced by addition of 200 ng/ml rapamycin. For fluorescence microscopy, yeast cells grown to mid-log phase in YPD medium were shifted to SD + CA + ATU medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.5% casamino acid, 0.002% adenine sulfate, 0.002% tryptophan, 0.002% uracil, and 2% glucose) containing 200 ng/ml rapamycin and 0.5 mM IAA and incubated for the indicated time periods.

Table 1
Yeast strains used in this study.

Name	Genotype	Figures	Reference
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801*; *GAL*	–	[35]
YKM381	SEY6210 *ura3-52*::*ADH1pro-OsTIR1–9Myc-URA3*	1A	This study
YKM386	SEY6210 *ura3-52*::*ADH1pro-OsTIR1–9Myc-URA3 hrr25–aid-natNT2*	1A	This study
YKM409	YKM381 *atg19Δ*::*zeoNT3 ATG34–EGFP-kanMX6*	2B	This study
YKM410	YKM386 *atg19Δ*::*zeoNT3 ATG34–EGFP-kanMX6*	2B	This study
YKM424	YKM409 *atg1Δ*::*hphNT1*	1B, 2B and 3C	This study
YKM425	YKM410 *atg1Δ*::*hphNT1*	1B and 3C	This study
YKM422	YKM381 *atg19Δ*::*zeoNT3 atg1Δ*::*hphNT1*	3C	This study
YKM423	YKM386 *atg19Δ*::*zeoNT3 atg1Δ*::*hphNT1*	3C	This study
YKM620	YKM422 *kanMX6-CET1pro-ATG34*	1D	This study
YKM621	YKM423 *kanMX6-CET1pro-ATG34*	1D	This study
YKM578	YKM381 *atg19Δ atg34Δ*::*CgTRP1*	1D and 4E	This study
YKM405	YKM381 *atg19Δ*::*zeoNT3 AMS1–EGFP-kanMX6*	2A and 3A	This study
YKM406	YKM386 *atg19Δ*::*zeoNT3 AMS1–EGFP-kanMX6*	2A and 3A	This study
YKM417	YKM405 *atg34Δ*::*natNT2*	2A	This study
YKM443	YKM381 *atg19Δ*::*zeoNT3 atg1Δ*::*CgHIS3 AMS1–mCherry-kanMX6 ATG34–EGFP-hphNT1*	3B	This study
YKM444	YKM386 *atg19Δ*::*zeoNT3 atg1Δ*::*CgHIS3 AMS1–mCherry-kanMX6 ATG34–EGFP-hphNT1*	3B	This study
YKM441	YKM381 *atg19Δ*::*zeoNT3 atg1Δ*::*CgHIS3 AMS1–mCherry-kanMX6 ATG11–EGFP-hphNT1*	4B	This study
YKM442	YKM386 *atg19Δ*::*zeoNT3 atg1Δ*::*CgHIS3 AMS1–mCherry-kanMX6 ATG11–EGFP-hphNT1*	4B	This study
YKM480	YKM381 *atg19Δ*::*zeoNT3 pep4Δ*::*kanMX4*	4A	This study
YKM544	YKM480 *hphNT1-ADH1pro-yeGFP–ATG11*	4A	This study
YKM545	YKM386 *atg19Δ*::*zeoNT3 pep4Δ*::*kanMX4 hphNT1-ADH1pro-yeGFP–ATG11*	4A	This study
YKM557	YKM381 *atg19Δ atg34Δ*::*natNT2 pep4Δ*::*kanMX4 hphNT1-ADH1pro–yeGFP-ATG11*	4A	This study
YKM631	YKM381 *atg19Δ*::*zeoNT3 ATG34–EGFP-kanMX6*	4D	This study
YKM632	YKM381 *atg19Δ*::*zeoNT3* $ATG34^{S382A}$*–EGFP-kanMX6*	4D	This study
YKM633	YKM381 *atg19Δ*::*zeoNT3* $ATG34^{S383A}$*–EGFP-kanMX6*	4D	This study
YKM561	YKM405 *atg1Δ*::*hphNT1 atg34Δ*::*natNT2 his3-Δ200*::*HIS3-ATG34*	4E	This study
YKM562	YKM405 *atg1Δ*::*hphNT1 atg34Δ*::*natNT2 his3-Δ200*::*HIS3-*$ATG34^{S383A}$	4E	This study
YKM563	YKM406 *atg1Δ*::*hphNT1 atg34Δ*::*natNT2 his3-Δ200*::*HIS3-ATG34*	4E	This study

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