Selectivity of mRNA degradation by autophagy in yeast

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Synthesis and degradation of cellular constituents must be balanced to maintain cellular homeostasis, especially during adaptation to environmental stress. The role of autophagy in the degradation of proteins and organelles is well-characterized. However, autophagy-mediated RNA degradation in response to stress and the potential preference of specific RNAs to undergo autophagy-mediated degradation have not been examined. In this study, we demonstrate selective mRNA degradation by rapamycin-induced autophagy in yeast. Profiling of mRNAs from the vacuole reveals that subsets of mRNAs, such as those encoding amino acid biosynthesis and ribosomal proteins, are preferentially delivered to the vacuole by autophagy for degradation. We also reveal that autophagy-mediated mRNA degradation is tightly coupled with translation by ribosomes. Genome-wide ribosome profiling suggested a high correspondence between ribosome association and targeting to the vacuole. We propose that autophagy-mediated mRNA degradation is a unique and previously-unappreciated function of autophagy that affords post-transcriptional gene regulation.
Autophagy is a highly conserved eukaryotic pathway that isolates cellular components for degradation and recycling in response to nutrient starvation, thereby maintaining homeostasis in nutrient-limited environments. Upon autophagy induction, substrates are sequestered within a double-membrane vesicle called an autophagosome and subsequently delivered to the vacuole (yeast) or lysosome (mammals) where they are degraded by hydrolytic enzymes.

Autophagy is originally thought to be nonselective (i.e., bulk), which isolates cytoplasmic material in an apparently random manner. In contrast, selective autophagy eliminates harmful proteins and superfluous, damaged organelles, such as endoplasmic reticula (ER) and mitochondria. Substrates of selective autophagy, often marked for degradation by modifications such as ubiquitination, are recognized through their binding to receptors that facilitate localization to the site of autophagosome formation. In addition to the clear distinction of substrate, autophagy may show the broad spectrum of protein substrate preference. Acetaldehyde dehydrogenase and tRNA ligases are such examples. The mechanism underlining this preference has been still unknown.

Despite the many studies of protein and organelle degradation by autophagy, RNA degradation by autophagy has not been well examined. Historically, RNA degradation by autophagy was suggested in pioneering works examining amino acid-starved rat livers by Lardeux and Mortimore. More recently, our group showed that RNA delivered to vacuoles via autophagy is degraded by the T2-type RNase Rny1, and that the resulting nucleotides are further hydrolyzed to nucleosides by the vacuolar phosphatase/nucleotidase Pho8. The question of whether RNA degradation by autophagy occurs preferentially, however, remains unaddressed.

In this study, we examine the degradation of mRNAs by rapamycin-induced autophagy in yeast. mRNA sequencing reveals that autophagy preferentially delivers a subset of mRNA species to the vacuole for Rny1-mediated degradation. We further find that the selectivity of autophagy-mediated mRNA degradation is coupled with translation by reporter gene analysis. Genome-wide ribosome profiling further demonstrates that persistent ribosomal interaction is associated with selective mRNA delivery to vacuoles. The autophagic delivery of ribosome-mRNA complexes to the vacuole depends on Atg24 sorting nexin complex, which is required for autophagic degradation of multisubunit complexes, such as the proteasome and ribosome. We provide a foundational description of autophagy-mediated RNA degradation in yeast, as well as molecular insights into the selectivity of this hitherto underappreciated pathway.

**Results**

**Autophagy delivers mRNAs to the vacuole for degradation.** We began by identifying mRNAs species delivered to vacuoles by autophagy. As mRNA delivered to the vacuole is immediately degraded by the vacuolar ribonuclease Rny1 in wild-type (WT) cells, we constructed a strain (Δrny1) lacking this enzyme. To isolate vacuole-delivered mRNAs, we adopted a strategy employing a highly purified vacuolar fraction from cell lysates by flotation with ultracentrifugation (Fig. 1a). The purity of the vacuolar fraction was confirmed by vacuole protein enrichment and the apparent absence of ER, Golgi, and nucleolar proteins (Supplementary Fig. 1a).

Using rapamycin to induce autophagy through TORC1 suppression and the vacuole purification method described above, we examined the accumulation of RNA species in vacuoles. In addition to rRNAs, we also detected mRNAs that were clearly observed following rRNA subtraction (Fig. 1b). Importantly, we determined that the presence of mRNAs in vacuoles depends on autophagy; mRNAs were hardly detected in Δatg2Δ cells, which are completely defective for autophagy.

Next, we characterized the mRNAs in the vacuoles by RNA sequencing. The even distribution of vacuolar mRNA reads suggests that nontruncated, full length mRNAs are delivered to vacuoles in myΔ cells (Fig. 1c) and verifies that Rny1 is the sole vacuolar nuclease responsible for mRNA degradation. In contrast to nuclear genome-encoded mRNAs, mitochondrial genome-encoded mRNAs were clearly excluded from the vacuole fraction (Fig. 1d), suggesting only limited mitochondrial degradation under the employed conditions.

**Vacuolar delivery of mRNA has selectivity.** We next set out to determine unique features of mRNA species delivered to the vacuole while accounting for previously reported dramatic changes in the cellular transcriptome upon rapamycin treatment (Supplementary Fig. 2a and see Materials and Methods). A broad spectrum of mRNAs is delivered to the vacuole (Fig. 2a and Supplementary Fig. 2b). We identified quantitatively over- and under-represented subsets of mRNAs relative to the cellular transcriptome, which we refer to as vacuole enriched and vacuole depleted, respectively. Notably, this analysis further ensured that the delivery of vacuole-enriched mRNAs is dependent on Atg2 (Fig. 2b). Northern blotting of representative vacuole-enriched mRNAs further confirmed autophagy-dependent mRNA enrichment in the vacuole (Supplementary Fig. 2c).

Next, we conducted functional characterization of the vacuole-enriched and -depleted mRNAs by gene ontology analysis. Strikingly, housekeeping mRNAs associated with amino acid biosynthesis and ribosomal proteins were most likely to be delivered to vacuoles (Fig. 2c). In contrast, the vacuole-depleted fraction was characterized by mRNAs with regulatory roles, such as those encoding proteins with protein kinase, energy reserve metabolism, and transcriptional activator (Fig. 2c). We note that the mRNA selectivity is independent of basal mRNA abundance in cells (Fig. 2a). Taken together, these results reveal that autophagy-mediated mRNA delivery to vacuoles is selective, not random, in nature.

We quantified the proportion of mRNA delivered to the vacuole. To this end, we used the activity of vacuolar alkaline phosphatase as a standard to ascertain the recovery of vacuoles from cell lysates and thereby the relative quantity of mRNAs delivered to the vacuole. This strategy revealed that for vacuole-enriched mRNA species, the proportion of mRNAs accumulating in the vacuole increased dramatically upon autophagy induction (Fig. 2d and Supplementary Fig. 2c); more than 20% of vacuole-enriched HOM2 (aspartic beta semi-aldehyde dehydrogenase) and LYS1 (saccharopine dehydrogenase) mRNAs in cell lysates were delivered to vacuoles following 3 h of rapamycin treatment. In contrast, the fraction of vacuole-depleted species, such as APL5 (a subunit of the clathrin associated protein complex) and ASG1 (zinc cluster protein proposed to be a transcriptional regulator), was limited at most to a 5% increase in vacuolar localization (Fig. 2d and Supplementary Fig. 2d).

As vacuole-enriched mRNAs are preferentially delivered to the vacuole, we reasoned that the relatively high rate of degradation of these mRNAs should be observed in WT cells. This was verified by RNA-Seq analyses of WT total cell lysates: the total abundance of vacuole-enriched mRNAs was significantly decreased following rapamycin treatment (Supplementary Fig. 2e, f). To more directly monitor mRNA degradation, we labeled cellular mRNAs with 4-thiouracil (4-thioU) in vivo, after which we subjected cells to chase in uracil-containing media (Fig. 2e). For this experiment, we used Δura3Δ cells to enhance...
incorporation of modified uracil into mRNAs (Supplementary Fig. 3a). In addition, we ascertained that the deletion of ATG2 or RNY1 does not affect the RNA labeling rate and total mRNA levels with 4-thioU (Supplementary Fig. 3a, b). Following 1 h rapamycin treatment, labeled mRNAs were biotinylated and purified using streptavidin beads, followed by quantification of mRNA abundance. As the reliability of short-lived mRNA analysis is poor, we focused on relatively long-lived mRNAs. Two representative vacuole-enriched mRNAs engaged in amino acid biosynthesis, HOM2 and HIS5 (histidinol-phosphate aminotransferase), were detected at between 3- to 4-fold abundance in vacuoles following rapamycin treatment (3 h) versus transcript per million (TPM) in myΔ cells in growing condition. mRNAs identified as deviating significantly from total cell extracts were classified as “enriched” (log2-fold change ≥ 1 and q value < 0.01) or “depleted” (log2-fold change ≤ −1 and q value < 0.01), as indicated in red and blue, respectively. Autophagy dependency of vacuole-enriched mRNA delivery to the vacuole was determined by unpaired two-sided Mann-Whitney U-test. The median, IQR, and 1.5 IQR are represented by solid line, box, and whiskers, respectively. Significance was determined by unpaired two-sided Mann-Whitney U-test. A Gene ontology analysis of mRNAs by relative vacuolar enrichment, as determined by iPAGE. The representative vacuole-enriched (red) or -depleted (blue) genes analyzed in this study are shown. b Time course of vacuole-enriched (red) or -depleted (blue) mRNA accumulation in myΔ cells vacuoles following rapamycin treatment. c Overview of a labeling strategy employing 4-thiouracil (4-thioU) with rapamycin treatment to monitor mRNA degradation.
mRNA degradation is carried out via preferential vacuole delivery and subsequent Rny1-mediated hydrolysis.

Preferential mRNA delivery to vacuoles is coupled with translation. As for other forms of selective autophagy in protein degradation, the mRNA degradation pathway must employ a distinct mechanism. By comparing vacuolar mRNA delivery and mRNA stability, we did not find the correspondence between selectivity of mRNA delivery to the vacuole and the steady-state half-life of mRNAs (Supplementary Fig. 4a). Thus, the recognition and isolation of target mRNAs necessary for vacuolar degradation appeared to be different to canonical cytoplasmic mRNA turnover, leading us to investigate the mechanistic basis of vacuolar mRNA degradation.

Selective degradation of the ER has been reported, and many mRNAs are bound to the ER surface. We determined that concomitant degradation of ER-bound mRNAs is unlikely as ER-bound mRNAs were not enriched in vacuoles (Supplementary Fig. 4b). Another possibility is that mRNAs within ribonucleoprotein (RNP) granules, such as stress granules, are degraded by autophagy through their association with RNP granules. However, stress granule mRNAs were not enriched in the vacuole-depleted fraction, suggesting that stress granules are not degraded by autophagy under the conditions employed in this study (Supplementary Fig. 4c).

Autophagy is also known to sequester cytoplasmic ribosomes into autophagosomes for degradation in the vacuole. We observed the accumulation of rRNA in the vacuolar fraction following autophagy induction (Fig. 3a), giving rise to the possibility that translating mRNAs are delivered to the vacuole. To test this hypothesis, we designed a reporter assay to monitor mRNA enrichment by using the vacuolar delivery of HOM2 mRNA as a model (Fig. 2c, d, and Supplementary Fig. 2c, d). We generated and expressed a series of reporter plasmids under the control of the native HOM2 promoter (ensuring endogenous expression levels) in a hom2A background strain (Fig. 3b). Expression of WT HOM2 mRNA in this strain recapitulated vacuolar delivery. In contrast, delivery of reporter mRNA to the vacuole was inhibited by the insertion of a strong stem-loop just before the start codon, which blocks start codon scanning by the pre-initiation 43S complex and thus impedes translation (Fig. 3b, c). Further, blocking of translation by the elimination of the start codon (substitution to TTG or TAC) also reduced mRNA delivery into vacuoles while inhibiting translation (Fig. 3b, c). Reporter mRNA level in total cell lysate were comparable with WT HOM2 mRNA (Supplementary Fig. 5d). Alternative vacuole-enriched ARO2 (chorismate synthase/flavin reductase) mRNA, which was similarly engineered as HOM2 reporter, also showed translation dependency for vacuolar delivery (Supplementary Fig. 5a, b, e). These results suggest that mRNA delivery to the vacuole is closely coupled with translation.

To determine whether a protein-encoding region of vacuole-enriched mRNAs is responsible for autophagic delivery to the vacuole, we swapped the HOM2 ORF with an ORF encoding GFP. Substitution of the HOM2 ORF had little effect on vacuolar delivery (Supplementary Fig. 5c). In addition, the substitution of the 3′ UTR of HOM2 with that of PIG2 (putative type-1 protein phosphatase targeting subunit), a representative vacuole-depleted mRNA, had only a very weak effect on vacuolar delivery (Supplementary Fig. 5c, f). We next swapped the HOM2 5′ UTR with vacuole-depleted mRNA 5′ UTRs. We employed 5′ UTRs from vacuole-depleted mRNAs expressed at a similar level to HOM2 to obtain near equal amounts of reporter mRNA. As a result, we found that DEE1 ORF had a major contribution to vacuolar delivery. These findings suggest that the combination of a translation factor and a specific 5′ UTR are responsible for the preferential delivery of mRNA into the vacuole and subsequent degradation. The establishment of reporter assays that depend on a specific combination of the 5′ UTR and ORF will help to identify the RNA factors that mediate autophagic clearance in vivo.
mRNA level to WT HOM2 mRNA, since a low abundance of reporter mRNAs could result in the overestimation of vacuolar delivery (Supplementary Fig. 5g). The substitution of the HOM2 5' UTR with vacuole-depleted APL5 or ASG1 5' UTRs clearly reduced the efficiency of delivery (Fig. 3d). Importantly, the 5' UTR of vacuole-depleted mRNAs was associated with a marked decrease in HOM2 protein level (Fig. 3e), which is consistent with the coupling of translation and degradation. Thus, we propose that the 5’ UTR is critical for vacuolar delivery through its regulation of vacuole-enriched mRNA translation.

Ribosome-mRNA association enhances vacuolar delivery by autophagy. We next conducted an analysis of ribosome-mRNA interactions at the genome-wide level. To this end, we performed ribosome profiling and monitored associations upon rapamycin treatment, normalizing data to mRNA abundances obtained by RNA-Seq. Rapamycin treatment reduced global protein synthesis, as indicated by reduced polyomes formation (Supplementary Fig. 6a) and previously reported. Remarkably, ribosomal association with vacuole-enriched HOM2 and LYS1 mRNAs persisted during rapamycin treatment (Fig. 4a). In contrast, vacuole-depleted NTH2 and APL5 mRNAs exhibited a time-dependent decrease in ribosomal association (Fig. 4a). These trends were observed with a high degree of reproducibility throughout all vacuole-enriched and -depleted transcripts (Fig. 4b) and at all assessed time points following rapamycin treatment (1, 2, and 3 h) (Supplementary Fig. 6b). Taken together, these results strongly suggest that persistent ribosome-mRNA association is a key determinant of mRNA degradation by autophagy, even as global translation activity is inhibited during rapamycin treatment.

A previous study reported that Atg24 (also known as Snx4) is specifically required for the autophagic degradation of multi-subunit complexes such as ribosomes, but not of bulk proteins. Given that autophagic degradation of mRNA is coupled to ribosomal association, we hypothesized that Atg24 is required for autophagy-mediated RNA degradation. To test this, we assessed autophagic degradation of GFP-fused proteins by western blotting, whereby autophagic delivery to the vacuole and subsequent degradation of a protein is detected by the liberation of the vacuolar protease-resistant GFP moiety. As reported previously, the cleavage of free GFP from the bulk-autophagy reporter Pgk1-GFP was observed in both atg24Δ and WT cells (Supplementary Fig. 7a), whereas cleavage of GFP from the ribosomal protein Rpl37a-GFP was detected in WT but not atg24Δ cells (Supplementary Fig. 7b). Critically, accumulation of mRNAs in the vacuoles of myΔΔ and myΔΔ atg24Δ double mutant cells was not observed (Fig. 4c), indicating that ribosomal degradation by autophagy is largely dependent on Atg24. Quantification of the amount of mRNA delivered to the vacuole suggests that vacuolar mRNA delivery was severely inhibited in the absence of Atg24 following rapamycin treatment (Fig. 4d).

Atg24 consists of a sorting nexin complex with Atg20 or Snx4, and we also tested the impact of Atg20 and Snx4 for vacuolar delivery of mRNAs. Clearly, the deletion of these genes reduced the efficiency of mRNA delivery to the vacuole (Fig. 4d), suggesting that Atg24 functions as complexes with Atg20 or Snx4 for autophagy-mediated mRNA delivery into vacuoles. Overall, we conclude that ribosome-bound mRNAs are preferentially delivered to vacuoles by the autophagy machinery and facilitate their degradation (Fig. 4e).

Discussion
Whereas cytoplasmic RNA degradation pathways, including canonical mRNA decay and the mRNA surveillance system, have been well studied, vacuolar degradation of mRNAs mediated by autophagy remains poorly understood. In this study, we demonstrate the delivery of mRNAs to vacuoles by autophagy, and that this process is mRNA-selective in nature (Fig. 4e). Following TOR inhibition, a subset of mRNAs, including those implicated in amino acid biosynthesis and ribosomal protein-encoding mRNAs, are delivered by rapamycin-induced autophagy to the vacuole, where they are degraded by the vacuolar nuclease Rny1. The delivery of mRNAs requires Atg24 and is enhanced by their ribosomal association, the latter of which is
mediated by the 5′ UTR. The negative correlation between vacuole delivery and stress granule enrichment (Supplementary Fig. 4c) may reflect the relatively low translation rate of mRNAs within stress granules, thereby likely inhibiting autophagy-mediated vacuolar delivery.

Considering that mRNAs are apparently delivered to the vacuole in the form of a polysome, the selective degradation of ribosomes by autophagy (ribophagy) offers one potential explanation for the degradation of mRNAs observed in this study. Previous works reported that ribophagy occurs following a long period (~24 h) of nitrogen starvation.35

While ribophagy has been reported to depend on the Ubp3/Bre5 deubiquitination complex35, RNAs are still delivered into vacuoles even in the absence of these factors35. Although the relationship between translation status and ribophagy has not been well studied, the preferential mRNA degradation that we found in this study cannot be attributed to previously described ribophagy35. While the clear sequestration of ribosomes within vacuoles has been reported26, whether mammalian ribophagy occurs is currently a point of contention. While one study reported containing ribosomes within autophagosomes and attributed their sequestration into autophagosomes to the receptor protein NUFIP139, another showed only limited degradation of ribosomes by autophagy during nutrient stress.40 Meanwhile, the proportion of ribosomal protein in yeast is an order of magnitude higher than that of mammalian cells41. Ribophagy may therefore differently contribute to ribosomal turnover and mRNA degradation in various species.

There are two potential explanations for persisted ribosome-mRNA association during rapamycin treatment: increased translation initiation or reduced translation elongation, with the former being the most straightforward interpretation of the data presented in this study. In the latter case, reduced translation elongation would result in a high representation of ribosome footprints proximal to the 5′ end of ORFs42. We assessed the position of ribosome association on mRNAs by calculating polarity score42, but did not observed distribution of ribosome footprints proximal to the 5′ end of ORFs in vacuole-enriched mRNAs (Supplementary Fig. 8a). Enhanced translation initiation is also consistent with the important role of 5′ UTRs in translation: these regions possess important regulatory elements that determine the rate of translation initiation43. The upstream ORF (uORF), a region found within the 5′ UTR of genes such as GCN4 (transcriptional activator of amino acid biosynthetic genes)44,45, allows preferential translation under stress16 and may support such enhanced translation initiation (Supplementary Fig. 8b, c). However, ribosome profiling did not identify any uORFs among vacuole-enriched mRNAs (Supplementary Fig. 8d). We only observed that small differences in the length of 5′ UTR37 between vacuole-enriched and vacuole-depleted mRNAs (Supplementary Fig. 9a, b). RNA binding protein(s) that interact with the 5′ UTR of implicated mRNAs may also facilitate recognition by the autophagy machinery, although we did not find any consensus sequences by motif analysis of vacuole-enriched mRNAs.

Another question is how delivered translating ribosomes or ribosome-mRNA complexes (polysomes) are preferentially recognized by the autophagy machinery. Interaction with adaptor proteins, as observed in selective autophagy4,6, or specific, transient protein/RNA modifications18,19 may act as intermediaries that recruit the autophagy machinery for mRNA degradation. In any case, our data imply that the state of polysomes in the cytoplasm is not homogeneous and dynamically changes under cellular conditions.

In this study, we induced autophagy using rapamycin, a pharmacological inhibitor of TORC1 widely used in autophagy research, but the investigation of mRNA degradation selectivity under other conditions promises to shed further light on interplay between ribosomal association and mRNA delivery. Using this strategy to understand the global landscape of vacuole-enriched mRNAs under a range of autophagy-inducing conditions may provide a generalized rationale for mRNA preference.

We propose that autophagy acts as an mRNA degradation system at the translation step, adding a mechanism for transcriptional regulation and global translation inhibition during cellular adaptation to stress conditions. The autophagic degradation of ribosome-associated mRNAs in the early phase of the stress response may be required to facilitate the shift to translation of stress response genes. Degradation of mRNAs engaged in ribosome association may be counter-intuitive, as this may suggest inefficient adaptation to stress when the cell is least able to tolerate the waste of resources. However, such degradation likely reflects an intricate balance between the expression of target genes and the inhibition of needless expression of target mRNAs. This study demonstrates that autophagy is involved in the regulation of gene expression through mRNA degradation, thereby playing a critical role in cellular homeostasis.

### Methods

**Yeast Strains and media.** Strains used in this study are listed in Supplementary Table 1. Gene disruption and tagging were performed using a standard PCR-based method.40,41 Cells were grown in rich medium (1% yeast extract, 2% peptone, and 2% glucose) or synthetic defined casamino acid medium without uracil (SDCA-uracil: 0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% casamino acids, 0.5% ammonium sulfate, 0.002% thiamin, 0.002% adenine, and 2% glucose). Yeast cells were grown in liquid media at 30 °C to a density of OD400 = 1.0 before autophagy was induced by the addition of 0.2 μM rapamycin (LC Laboratories, R-5000). The strains prepared in this study will be distributed upon request.

**Vacuole isolation.** Yeast vacuoles were isolated from whole cells as described46,47 with some modifications. Cells were grown in rich medium or SDCA-uracil to a density of OD400 = 1.0 before supplementation of 0.2 μM rapamycin and incubation for further 1, 2, or 3 h. Spheroplasts were prepared by incubation of cells in spheroplast buffer (1.2 M sorbitol, 50 mM Tris–HCl pH 7.5, 50 mM 2-mercaptoethanol, and 5 U/ml Zymolyase 100 T [nacalai tesque, 07665-53]) for 30 min. Spheroplasts were then collected by centrifugation, washed with RNase I buffer (1.2 M sorbitol and 50 mM Tris–HCl pH 7.5), resuspended in ice-cold buffer A (12% w/v Ficoll 400, 0.1 mM MgCl2, 10 mM MES-Tris pH 6.9, and 1× complete, EDTA-free protease inhibitor cocktail [Roche, 5056489001]), and homogenized with Dounce homogenizer on ice. All the subsequent handling was performed on ice. The lysate was next transferrred to an ultracentrifuge tube and centrifuged B (8% w/v Ficoll 400, 0.1 mM MgCl2, and 10 mM MES-Tris pH 6.9) was layered on top, and centrifuged at 72,000 × g in a P28S swinging bucket rotor (Hitachi Koki) for 30 min at 4 °C. The top, white layer (crude vacuole) was collected in new ultracentrifuge tube and resuspended in buffer B. The crude vacuole was underlaid with buffer B′ (4% w/v Ficoll 400, 0.1 mM MgCl2, and 10 mM MES-Tris pH 6.9) and subjected to a further round of centrifugation at 72,000 × g in a P40ST swinging bucket rotor (Hitachi Koki) for 30 min at 4 °C. The top layer was collected (the ‘vacuole fraction’) and RNA or protein were isolated immediately. The vacuole fraction used in analysis in Figs. 2d, 3, 4d, and Supplementary Fig. 5 was treated with RNase I (Lucigen, NE89101). The RNase I was inactivated by dithiothreitol (DTT) for 20 min at 70 °C before RNA isolation.

**Vacuole recovery estimation.** The activity of a vacuolar enzyme, alkaline phosphatase (ALP), was employed as previously described35 to biochemically determine the yield of vacuoles recovered from cells by ultracentrifugation. Five microliter of vacuole fraction or whole-cell lysate were suspended in 500 μl of assay buffer (250 mM NaCl, 0.01 M Tris–HCl pH 9.0, 10 mM MgSO4, and 10 mM ZnSO4). The reaction was initiated by addition of 50 μl of 55 mM α-naphthyl phosphate. After incubation for 10 min at 30 °C, 500 μl of 2 M glycine-NaOH (pH 11.0) was added to stop the reaction. The fluorescence intensity was measured at 345 nm excitation and 472 nm emission. The vacuole recovery was calculated by the fluorescence intensity of the vacuole fraction normalized to that of whole-cell lysate.

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**Isolation, electrophoresis, northern blotting, and qPCR of RNA.** RNAs from yeast lysate and isolated vacuoles were extracted by TRIzol reagent (Thermo Fisher Scientific, 15596018) following the manufacturer’s instructions. RNA subtraction was performed using the Ribomix Transcriptome Isolation Kit (Yeast)
(Invitrogen, K155003) according to the manufacturer’s instructions. For electrophoresis, the extracted RNAs were separated on denaturing formaldehyde agarose gel (1% agarose, 1× TBE buffer, pH 8.0), and chemically digested mRNA reads were defined as transcripts with accumulation in vacuoles at more than or less than two-fold vacuolar enrichment, respectively, and with a q value below 0.01. Gene ontology analysis was performed using the iPAGE42 web interface (https://tavazoielab.c2b2.columbia.edu/iPAGE).

Ribosome profiling. Reads mapped to yeast noncoding RNAs (rRNAs, tRNAs, snRNAs, snoRNAs, and mitochondrial rRNAs) were excluded. The remaining reads were then aligned to the S288C yeast genome with STAR.43 PCR-duplicated reads with the same UMI in the linker sequence were removed from downstream analysis. Footprints with 26–30 nt were used for analysis. The distance of the A-site from the 5′-end of the reads was estimated for each footprint length as 15 nt for 26–28 nt reads and 16 nt for 29 and 30 nt reads. The change in ribosome association on mRNAs throughout time course of rapamycin treatment was calculated with DESeq using a generalized linear model to normalize overall mRNA expression change after rapamycin treatment. Polarity scores were calculated as a read distribution bias along CDS, as previously described.42 The reads located at the A-site in first and last 15 nt of CDS were excluded from analysis. Genes with ≥ 64 read counts in CDS were analyzed.

Statistical analyses of all data were performed using R software (https://www.r-project.org/) in the RStudio interface (https://www.rstudio.com/).

Preparation of in vitro transcribed 4-thioU-labeled spike-in RNA. A PCR fragment encoding Renilla reniformis luciferase was amplified from the psiCHECK plasmid using 5′-TAATACGACTCACTATAGGG-3′ and 5′-CACCACATATTGTTGTAG-3′ and 5′-GGTCAACAGTCAACATGGA-3′ oligonucleotides. DIG-labeled AR2 probe was prepared using 5′-CACCACATATTGTTGTAG-3′ and 5′-GGTCAACAGTCAACATGGA-3′ oligonucleotides. Anti-digoxigenin-AP (Roche, 11093274910, 1:10000) and CDP-Star (Roche, 11685627001) were used for signal detection. Chemiluminescence images were acquired using a FUSION-FX (Vilber-Lourmat) imaging systems. For qPCR, cDNAs were prepared using the PrimeScript RT reagent kit with gDNA eraser (Takara, RR047). A qPCR was conducted using TB Green Premix Ex Taq II (Tli RNase H Plus) (Takara, RR020) with the mRNA-specific primers listed in Supplementary Table 3. Serial dilutions of cDNA were used for qPCR calibrations. Melting-curve analyses confirmed the amplification of a single product for each mRNA. The proportion of each mRNA species in the vacuole (the "proportion in vacuoles") was calculated by the value of the vacuole fraction normalized to that of the total lysate, considering the vacuole recovery rate as described above.

Sequencing of RNAs isolated from total cell lysates and vacuolar fractions. Two hundred nanogram of RNA was isolated from total cell lysates or vacuolar fractions using TRIzol reagent (Thermo Fisher Scientific, 15596018) for extraction and Direct-zol RNA MiniPrep Kit (Zymo Research, R2052) for purification, both according to the manufacturer’s instructions. The RNA pellet was dehydrated performing the Ribo-Zero rRNA Removal Kit (yeast) (Illumina, MRZY1324), libraries were prepared using the TruSeq RNA Library Preparation Kit v2 (illumina, RS-122-1001), according to the manufacturer’s instructions. The multiplexed libraries were sequencing on a HiSeq 4000 sequencer (Illumina) for single-read sequencing. Following multiplexing, ribosome profiling and RNA-Seq libraries were sequencing on a HiSeq 4000 sequencer (Illumina) for single-read sequencing. The collected cells with drip of ribosome profiling and RNA-Seq libraries were applied for barcoding using the Oligo Clean & Concentrator 96 (Zymo Research, ZY9200) and then purified using the iPAGE62 web interface (https://tavazoielab.c2b2.columbia.edu/iPAGE). The multiplexed reads were then aligned to the S288C yeast genome with STAR. For DESeq analysis, reads were mapped to the S288C yeast genomic features using Tophat58 and then counted in the library using Cufflinks59. Reads belonging to snRNAs, snoRNAs, and mitochondrial rRNAs were excluded. The remaining reads were then aligned to the S288C yeast genome with STAR.43 PCR-duplicated reads with the same UMI in the linker sequence were removed from downstream analysis. The reads located at the A-site in first and last 15 nt of CDS were excluded from analysis. Genes with ≥ 64 read counts in CDS were analyzed. Statistical analyses of all data were performed using R software (https://www.r-project.org/) in the RStudio interface (https://www.rstudio.com/).

4-thiorauric labeling experiment. The 4-thiouracil (4-thioU) metabolic labeling and purification of 4-thioU-labeled RNA was performed by modifying a previously described method.44 Briefly, cells were grown in synthetic defined medium with low concentration of uracil (SD-low uracil: 0.2% Yeast Synthetic Drop-out Medium Supplements without uracil [Sigma-Aldrich, Y1501], 0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.0013% uracil, and 2% glucose) overnight (yielding a density of OD₅₆₀ 2–4). Next, cells were diluted into the SD-low uracil medium at OD₅₆₀ 0.1. Upon growth of cells to OD₅₆₀ 0.6, cells were collected to remove the presence of 1 mM of thioU (Sigma-Aldrich, 440736) for 2 h before washing out into fresh synthetic defined medium (SD: 0.2% Yeast Synthetic Drop-out Medium Supplements without uracil [Sigma-Aldrich, Y1501], 0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.002% uracil, and 2% glucose) without 4-thioU. Cells were then treated with rapamycin for another 30 min.

RNAs were extracted by a hot phenol method, as described previously.45 Frozen cells were resuspended in 400 µl of AE buffer (50 mM sodium acetate and 10 mM EDTA pH 5.0) with 1% SDS) for 20 min. Anti-streptavidin-HRP (Abcam, ab7403, 1:5000) was added to the suspected immediate linker and then precipitated with ethanol. Ten microgram of total RNA was mixed with 5 ng of Femtoglow HRP Substrate (Michigan Diagnostics, 21008) were acquired by a ChemiDoc XRS+ system (Bio-Rad). For in vitro transcribed 4-thioU-labeled Renilla reniformis luciferase mRNA, biotinylated using 0.2 mg/ml of EZ-Link HPPD-Biotin (Thermo Fisher Scientific, 21341) in 10 mM Tris-HCl pH 7.5 and 1 mM EDTA for 2 h at 23 °C in the dark, and then precipitated with isopropanol. The biotinylated RNAs were washed in 500 µl of AE buffer-saturated hot phenol and homogenized using 0.5 µl of RNase A (Yasui Kkai) with 0.5 µl of RNase Inhibitor (Enzo Life Sciences, 10000 units). The RNA precipitated with ethanol. Ten microgram of total RNA was mixed with 5 µl of the luciferase mRNA, Renilla reniformis (Thermo Fisher Scientific, 21341) in 10 mM Tris-HCl pH 7.5 and 1 mM EDTA for 2 h at 23 °C in the dark, and then precipitated with isopropanol. The biotinylated RNAs were washed in 500 µl of AE buffer-saturated hot phenol and homogenized using 0.5 µl of RNase A (Yasui Kkai) with 0.5 µl of RNase Inhibitor (Enzo Life Sciences, 10000 units). The RNA precipitated with ethanol.
Western blotting. Western blotting was performed with 0.2 OD_{600} units of cells harvested for sample preparation. Frozen cells were treated with 10% trichloroacetic acid (TCA) for 5 min on ice. After TCA was discarded by centrifugation, the pellet was washed with ice-cold acetone and resuspended in 1 x sample buffer (75 mM Tris-HCl pH 6.5, 10% glycerol, 25 mM DTT, and 0.6% SDS). The samples were homogenized using 0.5 mm zirconia beads (Yasui Kikai, YZB05) and FastPrep-24 (MP Biomedicals, 600-500) with a setting of 60 at 6.0 m/s, and then incubated at 65°C for 10 min. Samples were separated by SDS-PAGE followed by Western blotting. Anti-FLAG (Sigma–Aldrich, F3165, 1:1000), anti-Pho8 (Abcam, ab113688, 1:1000), anti-Ape1 (15000), anti-β-actin (Wako, 010-72841, 1:500), anti-DpnI (Invitrogen, A6429, 1:1000), anti-Gsp1 (ImmutQuest, IQ241, 1:10000), anti-Van1 (a gift from Koji Yoda, 1:3000), and anti-GFP (Roche, 11814460001, 1:1000) were used as primary antibodies. Chemiluminescence was generated by Fermentas HRPR Substrate (Michigan Diagnostics, 21008) and blots were visualized using LAS-4000 (GE Healthcare) or FUSION-FX7 (Vilber-Lourmat) imaging systems.

Polysome analysis. Two hundred OD_{600} units of yeast cells grown in rich medium with or without rapamycin were treated with 0.1 mg/ml of cycloheximide for 10 min at 30°C and centrifugation, the pellet was washed with ice-cold acetone and resuspended in 1 x polysome buffer (20 mM HEPES-KOH pH 7.4, 100 mM potassium acetate, and 2 mM magnesium acetate). The cell lysate was centrifuged at 1100 × g for 10 min to remove cellular debris. The supernatants containing 100 µg of total cellular RNA were layered on top of sucrose gradients (10–50% sucrose in 10 mM Tris-acetate pH 7.4, 70 mM ammonium acetate, and 4 mM magnesium acetate) and centrifuged for 2 h at 4°C. The gradients were collected in 1 ml volumes and an equal volume of SDS-PAGE sample buffer was added to each fraction. The samples were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with mouse anti-FLAG antibodies and then probed with horse anti-mouse IgG conjugated to alkaline phosphatase. Signals were visualized using NBT and BCIP. All samples were normalized to the region of 300 nucleotides upstream of the initiation codon for each gene and inserting each into pRS416- stem-loop-HOM2-FLAG-CYC1t using SacI and Sall sites.

Data availability
RNA-Seq and ribosome profiling data are deposited in NCBI as GEO: GSE149916. Custom codes used for this study will be provided upon request. Source data are provided with this paper.

Received: 23 June 2020; Accepted: 22 March 2021; Published online: 19 April 2021

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Acknowledgements

We are grateful to all the members of Ohsumi and Iwatsuki laboratories for helpful discussions and critical reading of the manuscript and the Biomaternal Analysis Division, Tokyo Institute of Technology for DNA sequencing. We would like to thank Alexander May for editing the manuscript and Shuluan Hotta-Ren for technical assistance; Yuihiro Mishima, Yoshitaka Matsu and Takashi Fukaya for critical comment on manuscript; Hideki Taguchi for helping polyoma analysis; Koji Yoda for providing antibodies. This work was supported in part by a Grant-in-Aid for Young Scientists (B) (PI17K13063 to S.M.), a Grant-in-Aid for Scientific Research (C) (PI15K00649 to T.K.), a Grant-in-Aid for Scientific Research (S) (PI16H05375 to Y.O.), a Grant-in-Aid for Young Scientists (A) (PI17H04998 to S.I.), and a Grant-in-Aid for Challenging Research (Experatory) (PI19K22406 to S.I) from the Japan Society for the Promotion of Science (JSPS) and by a Grant-in-Aid for Scientific Research on Innovative Areas “Multidisciplinary research on autophagy” (JP16H01197 to T.K.), a Grant-in-Aid for Scientific Research on Innovative Areas “nascent chain biology” (JP17H05679 to S.I.), and a Grant-in-Aid for Transformative Research Areas (B) (JP15H05744 to S.I) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT). S.M. was also supported by JST, AMED-CREST, AMED (JP20gm1410001), the RIKEN Pioneering Project (“Cellular Evolution”) and Aging Project, and the Takeda Science Foundation. RNA-Seq analyses was supported by the JSPS Platform for Advanced Genome Science (JP16H06279 to T.K.). DNA libraries were sequenced in part by the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley, supported by NIH S10 OD018174 Instrumentation Grant. Computations were supported by Manabu Iishi, Itoshi Nikaido, the Bioinformatics Analysis Environment Service on RIKEN Cloud, and HOKUSAI Sailing Ship in RIKEN.

Author contributions

S.M. performed experiments and analyzed the deep sequencing data; T.K., S.I., and Y.O. supervised the project; S.M., S.I., and Y.O. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-22574-6.

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Peer review information Nature Communications thanks Premal Shah and other, anonymous, reviewers for their contributions to the peer review of this work.

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