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Critical role for *Saccharomyces cerevisiae* Asc1p in translational initiation at elevated temperatures

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Abstract

The eukaryotic ribosomal protein RACK1/Asc1p is localized at the mRNA exit channel of the 40S subunit but lacks a defined role in mRNA translation. *Saccharomyces cerevisiae* deficient in *ASC1* exhibit temperature-sensitive growth. Using this null mutant, we evaluated potential roles for Asc1p in translation and ribosome biogenesis. We found that at the restrictive temperature the *asc1* null mutant had reduced polyribosomes. To test the role of Asc1p in ribosome stability, we used cryoelectron microscopy to examine the structure of 80S ribosomes in an *asc1* yeast deletion mutant at both the permissive and nonpermissive temperatures. cryoEM indicated that loss of Asc1p does not severely disrupt formation of this complex structure. We found no defect in rRNA processing in the *asc1* null mutant. We applied a proteomic approach to survey the effect of Asc1p loss on the global translation of yeast proteins. At the nonpermissive temperature, the *asc1* mutant had reduced levels of ribosomal proteins and other factors critical for translation. Collectively, these results are consistent with recent observations suggesting that Asc1p is important for ribosome occupancy of short mRNAs. Our results show the Asc1 ribosomal protein is critical in translation during heat stress.

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Keywords

yeast; *ASC1*; ribosome; translation; initiation

Introduction

The *Saccharomyces cerevisiae* *ASC1* gene, containing an intron encoding a U24 snoRNA, was first identified as an extragenic suppressor in a *hap1 hem1* yeast mutant [1]. The yeast Asc1p protein and its homologous RACK1 protein of higher eukaryotes were later discovered in a mass spectrometry screen of purified yeast and mammalian ribosomes and shown to be orthologous 40S ribosomal proteins [2–4]. Cryoelectron microscopy localized Asc1p to the head region of the 40S subunit near the mRNA exit channel [5]. High resolution x-ray crystallography confirmed the position of Asc1p near the head of the 40S subunit and illuminated interactions between Asc1p and the core ribosomal proteins RPS16e, RPS3e, and RPS17e [6]. The crystal structure of the 40S subunit showed interactions between Asc1p and the 18S rRNA and defined regions of Asc1p that were solvent accessible [6].

Structurally, Asc1p contains seven WD repeats that form a circularized β -propeller structure predicted to participate in a wide variety of functions and interactions [2, 7]. A large number of studies have reported Asc1/Rack1 interacting with proteins in various biological processes, especially signal transduction pathways [8, 9]. Asc1p is an obligate 40S ribosomal protein, with little or no ribosome-free Asc1p in the cytoplasm [6]. As a result, studies that have focused on Asc1p/RACK1-interacting proteins likely reflect interactions with ribosomal-bound Asc1p/RACK1 [2, 10]. Recently, two separate yeast studies have shown that Asc1p interacts with Nip1p and Rpg1p, subunits of the essential translation initiation factor eIF3 [9, 10]. Kouba *et al.* showed Asc1p interacts with the Proteasome, COP9, Initiation factor 3 (PCI) domain of the yeast Nip1p subunit of the essential yeast eIF3 translation initiation factor [9]. Optiz *et al.* showed that Asc1p interacts with the yeast mRNA translation initiation protein Rpg1p and other translation factors, suggesting a potential role as a tether for translation initiation factors to the 40S subunit [10]. In support of this model, an *asc1* yeast strain grown under standard conditions (30°C) showed a reduction in ribosome occupancy for short mRNAs that have the potential to form circular ribosome carousels [11].

Despite Asc1p's acceptance as a core *S. cerevisiae* 40S ribosomal protein and its reported interaction with eIF3 and eIF4, little is known about the precise function of Asc1p in the ribosome and translation. The fact that the *ASC1* locus is non-essential and an *asc1* mutant shows no apparent growth phenotype at 30°C frustrates efforts to establish a role for Asc1p in translation. However, an *asc1* yeast mutant shows a strong and consistent temperature-sensitive phenotype [3, 9]. In this report, we investigated the functional role of the *ASC1* locus and the Asc1p protein in translation by shifting an *asc1* and various *ASC1*-complemented yeast strains to the nonpermissive temperature (NPT). Our results show the *ASC1* gene's protein-coding region is critical for translational initiation when *S. cerevisiae* is grown at elevated temperatures. Structural analysis of yeast 80S ribosomes from *asc1* and WT yeast strains at the permissive and NPT shows no significant defects except for the loss of Asc1p. Our proteomics analysis of *asc1* yeast shows that the translational

machinery has a marked reduction at the NPT. Given the role of Asc1p's mammalian ortholog, RACK1, in various signal transduction processes these studies might highlight signal transduction mechanisms linking heat stress to translational initiation.

Materials and Methods

Strains, Media, and Plasmids

All *S. cerevisiae* media, growth, and genetic manipulation were performed using standard techniques [12]. The wild-type diploid yeast strain BY4743 (AL035) and the homozygous diploid *asc1* null strain, AL116, were obtained from the yeast gene deletion collection [13]. Plasmid p*ASC1* containing the entire *ASC1* genomic region cloned into the pRS416 vector has been previously described [3]. The *ASC1* rescue strain AL156 was generated by transforming the *asc1* strain AL116 with plasmid p*ASC1*. Plasmids pAJ100(pRS416), pAJ2562(pRS416-*ASC1*), pAJ2563(pRS416-*ASC1* ORF), pAJ2564(pRS413-ACT), and pAJ2565(pRS413-ACT/U24) were obtained from the Ed Marcotte laboratory and transformed into the *asc1* null diploid strain AL116.

Growth Analysis

For plate growth assays, yeast strains were grown in liquid SC medium from overnight cultures and adjusted to an O.D. 600 of 0.6. A 1:10 dilution series was made and spotted sequentially on SC agar plates and allowed to grow for 72 h before imaging. Plates were grown at either 30°C (permissive temperature) or 42°C (NPT).

Polyribosome Profiling

Our polyribosome profiling protocol was modified from previous methods [14]. Yeast strains were grown in SC at 30°C to mid-log phase. Strains were then transferred to fresh SC pre-warmed to 30°C or 39°C and growth continued at those temperatures. At 0, 1, 6, and 18h after transfer, cells were collected and lysed as previously described [14]. Protein concentration was measured, and an amount of lysate corresponding to 10 mg of protein was layered on top of 7%–47% sucrose gradients. The gradients were centrifuged and analyzed as previously described [14] to show densities representing ribosome particles including the 40S, 60S subunits, 80S ribosomes and polyribosomes. To reduce stochastic noise, the A₂₅₄ signal at each time point was averaged with signal over a two second period.

Ribosome Purification

Yeast strains were grown from overnight cultures in SC at 30°C and 39°C for 6 h. Cell pellets were washed with water and lysed with glass beads in Standard Buffer (10 mM Tris pH 7.5, 5 mM β-mercaptoethanol, 50 mM ammonium chloride, 5 mM magnesium acetate). The lysate was centrifuged at 20,000 x g in a microcentrifuge for 30 min at 4°C. The supernatant was centrifuged in a 50 Ti rotor (Beckman) at 150,000 x g for 2 h at 4°C. The pellet was re-suspended in Wash Buffer (10 mM Tris pH 7.5, 5 mM β-mercaptoethanol, 500 mM ammonium chloride, 100 mM magnesium acetate) and overlaid on a discontinuous sucrose gradient made from equal volumes of 20% sucrose in Wash Buffer and 5% sucrose in Wash Buffer. The gradient was centrifuged in a SW-41 rotor (Beckman) at 20,000 rpm (68,400 x g) for 18 h at 4°C. The pellet was resuspended in Standard Buffer and centrifuged

in a microcentrifuge at 10,000 x g for 10 min at 4°C. The pellet was discarded; purified ribosomes remained in the supernatant.

Cryo-Electron Microscopy

For cryo-EM experiments, wild-type strain AL035 and *asc1* null strain AL116 were grown at 30°C and 39°C for 6 h. Ribosomes were purified from each condition as described above. Four microliters of a sample was applied to holey carbon-coated Quantifoil copper grids, quickly frozen using the Vitrobot Mark IV freeze-plunger (FEI, Portland, Oregon), and then visualized using a 4k x 4k CCD camera (Gatan, Pleasanton, CA) in an FEI Tecnai F20 electron microscope at 200 kV acceleration voltage and 50,000x magnification, yielding a pixel size of 2.25 Å on the object scale [15]. Data were collected using the program *Legion* [16]. A total number of 75,925 particles were selected from 1,360 micrographs. Of these, 51,112 particles were verified using a work-flow written in Arachnid [17], and processed using the program *RELION* [18], which combines maximum likelihood-based classification with reconstruction, as well as a novel quantitative method [19], to analyze the classification results. For classification, data from all four conditions were pooled together so that differences among sub-populations could be effectively studied, and so the maximum number of particles was available for the 3D reconstruction of each class. The resolution was determined at FSC=0.143 under the “gold standard” regime. The *S. cerevisiae* ribosome ribbon structure used as reference was rigid body-fitted into cryo-EM density maps using UCSF Chimera (PDB ID: 3U5B, 3U5C, 3U5D, and 3U5E). Difference maps are shown at the threshold of ± 5 standard deviations of each difference map [20].

Quantitative Mass Spectrometry

For mass spectrometry analysis, the wild-type strain AL035 and the *asc1* null strain AL116 were grown at 30°C and 39°C. Quantitative mass spectrometry was performed on yeast whole cell extracts (WCE) as previously described [21]. Briefly, 10 µg of WCE protein from each condition was acetone precipitated, resuspended, and trypsin digested in triplicate. iTRAQ labelling was conducted, and replicates were pooled with a labeled WCE common standard. Samples were loaded on a strong cation exchange (SCX) column and 2D MuDPIT was performed to identify and quantitate specific proteins [21].

Proteomic Data Analysis

The *Multiplierz* software package was used to extract reporter ion intensities for each spectrum from the .raw data files for downstream quantitation [22]. Spectra were searched against *S. cerevisiae* forward and reverse protein databases using *X!Tandem* [23]. For quantitation, reporter ion intensities for each Peptide Spectrum Match (PSM) were retrieved, and signals for each gene's unique peptides were summed. Intensities were normalized separately for each iTRAQ labeling experiment. Normalized reporter intensities were then divided by the WCE common control included in each experiment to generate log₂ ratios. Only proteins identified in all conditions were included in the analysis. Sample processing issues for the second replicate of the wild-type 30°C condition meant it was excluded from the analysis. Heat maps were generated using *Cluster3.0* and *Java TreeView* [24]. A database of yeast proteins belonging to the GO terms listed in Figure 6B and C was obtained from

SGD^[25]. Box plots were generated using Prism from the average log2 ratios of quantified proteins across three biological replicates that matched to SGD GO terms ^[25].

Northern Blots

The wild-type strain AL035 and the *asc1* null strain AL116 were grown at 30°C and 39°C for 1 h before RNA was harvested. Membrane hybridization used single-stranded DNA ³²P-radiolabeled DNA probes prepared as previously described previously ^[26]. Probes 1–6 and 5S sequences have been previously described in Dong *et al*^[26]: probe 1, 5'-GGTCTCTCTGCTGCCGGA-3'; probe 2, 5'-CATGGCTTAATCTTTGAGAC-3'; probe 3, 5'-CGGTTTAAATTGTCCTA-3'; probe 4, 5'-AATTTCCAGTTACGAAAATTCTTG-3'; probe 5, 5'-GGCCAGCAATTTCAAGTTA-3'; probe 6, 5'-CTCCGCTTATTGATATGC-3'; probe 5S, 5'-CGGACGGGAAACGGTGCTTTCTGGTAGATATGGCCG-3'. Act1 CDS 2F, 5'-TCCTTCTGTTTTGGGTTTGGGAATC-3' for probe amplification; Act1 CDS 2R, 5'-CCAATCCAGACGGAGTACTTTCTTTC-3' for probe amplification/labeling; SCR1_F, 5'-AGGCTGTAATGGCTTTCTGGTGGGA-3' for probe amplification; SCR1_R 5'-ACGGGCTGCCCGCAAAGATCGA-3' for probe amplification/labeling; Rpl26b_1538_for 5'-ACTGCTCCATCCTCTGAACG-3' for probe amplification; Rpl26b_1675_rev 5'-TTGACCTTCTTGAACCAC-3' for probe amplification/labeling; RPL16A 4F 5'-CCACCTTGGGTAAGTTGTCTACTTCC-3' for probe amplification; RPL16A 4R 5'-TTTAAATTTCTTAGTAACCCAAAGCG-3' for probe amplification/labeling.

Measurement of Ribosome Biogenesis

Strains AL035+pRS416, AL116+ pRS416, and AL156 were grown to mid-log phase in SC-ura. Cultures were diluted in SC-met at 30°C for 2 h. Cell concentrations were calculated by hemocytometer and cells diluted to 1×10^7 cells/mL in fresh SC-met at either 30°C or 39°C. Cultures were allowed to incubate at their respective temperatures for 30 m before ³⁵S-methionine was added to a final concentration of 25 µCi/mL. Cultures were incubated at 30°C or 39°C for 30 min before ³⁵S-met incorporation was stopped by placing the cultures on ice and adding an excess (100 mM) of cold methionine. Ribosomes were purified using the above method. Purified ribosome pellets were resuspended in Standard Buffer and TCA precipitated. Dried proteins were resolubilized in SDS sample buffer and run by SDS-PAGE. Gels were transferred to nitrocellulose paper and ³⁵S-met incorporation visualized by autoradiography.

In Vitro WT and *asc1* Ribosome Stability Assay

50 mL yeast cultures from strains AL035+pRS416, AL116+pRS416, and AL156 were grown in SC-URA at 30°C to an OD₆₀₀ of 0.6. Cells were harvested by centrifugation and suspended in SC-met containing 300 µCi/mL of ³⁵S-cysteine and methionine (ICN). Cells were labelled for 4 h at 30°C. Cells were lysed in Standard Buffer (see ribosome purification). Lysates were incubated *in vitro* for 2 h at either 30°C or 39°C before ribosomes were purified as described above. Ribosome pellets were resuspended in ammonium bicarbonate and subjected to SDS-PAGE and transferred to nitrocellulose for visualization via autoradiography.

Results:

To test the functional role of different structural features in the *ASC1* gene locus, *asc1*⁻, *asc1*⁻ + pASC1 (complete genomic locus), *asc1*⁻ + pASC1-ORF (ASC1's protein coding ORF only), *asc1*⁻ + pU24snoRNA (ASC1's intron encoding a snoRNA element only), and wild-type diploid yeast strains were serially diluted and used for growth assays at the permissive or NPT (Fig. 1). The data showed the slow-growth phenotype of *asc1*⁻ was complemented by expression of both *ASC1*'s genomic locus and the gene's protein coding sequence (Fig. 1). However, expression of the *ASC1* gene's U24 intron alone failed to complement the temperature-sensitive (TS) phenotype (Fig. 1). The results strongly suggest that loss of the *ASC1* gene's protein-coding sequence, not the *ASC1*-intronic sequence encoding the snU24 snoRNA, is responsible for the TS growth phenotype of diploid *asc1*⁻ deficient yeast.

Next, we used polyribosome profiling to examine the relative abundances of the ribosomal 40S, 60S, 80S, and polyribosome complexes in wild-type, *asc1*⁻, *asc1*⁻ + pASC1, *asc1*⁻ + pASC1-ORF, and *asc1*⁻ + pU24snoRNA diploid strains grown at the permissive and NPT for 18 h (Fig. 2). The wild-type and the *asc1*⁻ strains complemented by either the *ASC1* genomic locus or the *ASC1* protein-coding element showed polyribosomes at both temperatures. However, the *asc1*⁻ and *asc1*⁻ + pU24snoRNA had no polyribosomes and demonstrated a reduction in the 80S peak at the NPT (Fig. 2).

Results from our polyribosome profiling experiments suggested that the diploid *asc1*⁻ deficient yeast had either a defect in ribosome biogenesis or translational initiation when grown at the NPT long-term (18 h)(Fig. 2). To experimentally test the potential role of *ASC1* in ribosome biogenesis, we analyzed the ribosomes in the diploid wild-type and *asc1*⁻ mutant strains grown at the permissive and NPT using cryoelectron microscopy. Other than the loss of the Asc1p protein from the 40S subunit, the cryo-EM images of the purified *asc1*⁻ and wild-type 80S ribosomes at the permissive and NPT showed no other loss of ribosomal proteins or obvious structural defects apart from a minor structural shift in the 40S subunit (Fig. 3). Consistent with our cryo-EM observations, ³⁵S-labeled ribosomes from *asc1*⁻ yeast were stable at both the permissive and NPT *in vitro* (Supplemental Figure 1B).

Next, we examined rRNA processing in the wild-type and *asc1*⁻ diploid mutant strains using a set of common DNA northern probes to detect rRNA processing intermediates (Fig. 4A). For the wild-type and *asc1*⁻ diploid strains, the 5S and 7S rRNAs showed similar reductions after 1 h at high temperature (Fig. 4B, probe 5/5S). The wild-type and *asc1*⁻ strains showed little difference in the levels of fully processed 18S and 25S rRNAs (Fig. 4B probe 1). Compared to the wild type, the *asc1*⁻ strain showed a slight accumulation of the rRNA processing intermediates 23S, 27SA2, and 27SA/B at the NPT (Fig. 4B, probe 4). Figure 4A shows that the 23S intermediate accumulates at the expense of 18S rRNA production. The various 27S intermediates accumulate at the expense of 25S production [37]. Overall, the data showed no significant defects in rRNA processing in the *asc1*⁻ strain at the NPT.

To test for a translation-initiation defect in *asc1* yeast, we performed a time-course experiment to analyze the dynamic changes in polyribosome profiles of the wild-type, *asc1*, and *asc1* + pASC1 diploid strains at the permissive and NPT. At the zero-hour time point, wild-type, *asc1*, and *asc1* + pASC1 diploid strains had similar polyribosome profiles at the permissive and NPT. Strikingly, between 2–6 h of growth at the NPT, we observed a loss of polyribosomes in the *asc1* diploid mutant (Fig. 5).

Given that we observed a reduction in polyribosomes in *asc1* yeast with no suppression of rRNA processing or major defects in ribosome structure after long-term growth of yeast at the NPT (Fig 3, 4, and 5), we studied changes in global protein levels using a quantitative proteomic analysis of whole-cell protein extracts from *asc1* and wild-type yeast grown at the permissive and NPT. An iTRAQ-based quantitative proteomics protocol was used to determine the relative abundances of ribosomal proteins (r-proteins) in the *asc1* strain compared to the wild-type diploid strain. The log₂ ratio of the change in protein abundance between the permissive and NPT for each strain was used to examine how the two strains responded to incubation at the NPT. Total protein levels showed no change in the experiment (Fig. 6A, 6B). Examination of the core components of ribosomal subunits showed that both wild-type and *asc1* strains had reductions in both 40S and 60S r-protein levels at the NPT compared to the permissive temperature. However, the reductions were significantly greater for both subunits in the *asc1* mutant (Fig. 6B). A similar analysis was conducted for proteins belonging to a set of Gene Ontology (GO) terms related to ribosomes. The *asc1* strain showed greater reductions than the wild type in all ribosome-associated GO terms at the NPT compared to the permissive temperature, except for proteins classified as “Ribosomal Export from Nucleus” (Fig. 6C, Supplemental Table 1). Consistent with the observed reduction in ribosome-associated proteins by iTRAQ, we observed a reduction in ribosomal protein synthesis in *asc1* yeast that were labeled with ³⁵S-methionine and shifted to the NPT (Supplemental Figure 1A). This defect was complemented by expression of the ASC1 protein-coding-sequence when yeast were grown at the NPT.

Discussion

In this study, we showed that the protein-coding region of *ASC1* is necessary and sufficient to complement the TS growth phenotype and the loss of ribosomes in *asc1* yeast. Additionally, we showed that loss of Asc1p does not induce obvious defects in ribosomal RNA processing and shows no structural defects in the ribosome, apart from the structural lesion caused by loss of Asc1p. Consistent with a strong translational initiation defect, *asc1* yeast shifted to the NPT lose polyribosomes. This polyribosome-deficient phenotype of *asc1* yeast is complemented by the *ASC1* protein-coding region, suggesting that ribosomal Asc1p is critical for translational initiation at elevated temperatures. Lastly, our proteomic studies of *asc1* yeast extracts highlight the translational machinery, including ribosomes, as the primary target of translational repression upon the shift to elevated temperatures. Our study expands on the observations of Thompson *et al.* [11] and suggests that Asc1p is critical for general translational initiation, especially at elevated temperatures. Future studies will decipher Asc1p's precise role in translational initiation by identifying the initiation factors that require Asc1p in the ribosome during temperature shift.

On the role of Asc1p in translation

The labs of Valasek and Gilbert have completed studies suggesting a potential role for Asc1p in translational initiation. Valasek's group showed that the C-terminus of Nip1p interacts with Asc1p in the 40S subunit [9]. Additionally, Opitz et al. found that Nip1p and Prt1p co-purified with Asc1p [10]. Much like the *asc1* yeast, mutations in individual subunits of the eIF3 complex lead to TS phenotypes [9, 27]. Additionally, the eIF3 TS mutants show reduced polyribosomes during growth at the NPT [9]. That Asc1p has been reported to interact with eIF3 subunit proteins in more than one study and both *asc1* yeast eIF3 TS mutants have similar translational defects suggests that some of the TS effects on translational initiation in *asc1* yeast could be the result of disruptions between eIF3 subunits and the 40S ribosome.

As a method to home-in on the potential role of ASC1 protein in translation, Thompson et al. used ribosome footprinting to characterize ribosome occupancy in yeast lacking the ASC1 ORF or the ASC1-derived U24 snoRNA [11]. The results of their study were striking. Short mRNAs, and specifically ribosomal-protein-coding mRNAs were less occupied by ribosomes in *asc1* yeast. The same effect was not observed in yeast lacking the ASC1-derived U24 snRNA. These results strongly suggest that Asc1p is necessary for translational initiation of the most abundant cellular mRNAs (including ribosomal proteins). While we observed no obvious growth or polyribosome defects when *asc1* yeast were grown at the permissive temperature, we found that *asc1* yeast shifted to the NPT displayed an immediate reduction in polyribosomes (Fig 5). The immediate reduction in polyribosomes was accompanied by an eventual reduction in both 80S ribosomes and polyribosomes among *asc1* yeast that were incubated at the NPT long-term (>18 h, (Fig 2)).

Our proteomics analysis of yeast extracts from *asc1* yeast were broadly consistent with the observations from the Gilbert lab [11] and suggest that loss of Asc1 protein disproportionately affects the translational initiation of mRNAs encoding components of the translational machinery. However, our results extend this observation and suggest that *asc1* yeast exhibit a stronger translational initiation defect at elevated temperatures. Additionally, our proteomic and metabolic labeling experiments show that components of the translational machinery have suppressed synthesis when *asc1* yeast are grown at the NPT.

In conclusion it is unlikely that the ASC1 locus has a strong role in ribosome biogenesis when yeast are grown at the NPT. Rather- the well documented TS phenotype of *asc1* yeast should be attributed to the loss of Asc1p. The absence of Asc1p in the 40S combined with elevated temperature leads to an immediate suppression of translation initiation especially among ribosomal protein-encoding mRNAs. In this sense Asc1p plays a critical role in translational initiation (first) and a role in regulating overall ribosomal levels (second). Hence the observation that 80S and polyribosomes are lost in cultures grown long-term at the NPT is most likely attributable to a defect in translational initiation. While heat stress can induce changes in the yeast transcriptome that are independent of translational control [28], it is unlikely that Asc1p plays a role in these processes from its cytoplasmic position as part of the ribosome core. Nevertheless, we cannot rule out the possibility that Asc1p has additional functional roles during heat stress.

Numerous studies have linked the Asc1p mammalian ortholog (RACK1) to signal transduction processes. Given the scaffolding role of (RACK1) in signal transduction processes, it is interesting to speculate that Asc1p might link the ribosome to kinases that phosphorylate initiation factors or specific mRNA-binding proteins during environmental stress. Future studies will illuminate the precise role of Asc1p in translational initiation during heat stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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Statement of significance

This work identifies the function of Asc1p (yeast ortholog of mammalian RACK1) in mRNA translation and shows that the protein is necessary to initiate mRNA translation during heat stress.

Figure 1. The *Asc1p* coding region is necessary for growth at NPT. Yeast strains were serially diluted at 1:10 on SC media and grown at the permissive temperature (30°C) or the NPT (42°C) for 72 h. Labels on the left show the genetics and plasmids transformed into each yeast strain. A key to the right shows the structure of the expressed *ASC1* components in the respective plasmid constructs.

Figure 2.

The *Asc1p* coding region is necessary for 80S and polyribosomes at NPT. Strains were grown in SC media at 30°C or 39°C for 18 h and ribosome dissociation was then blocked with cyclohexamide. Gradients were loaded with 1 mg of total protein from cleared lysate and profiles read at an absorbance of 254 nm. Diagrams to the right show the expressed *ASC1* components in the respective plasmid constructs.

Figure 3.

Ribosomes assembled in *asc1* yeast at NPT are structurally intact. (A) Cryo-EM maps of 80S ribosomes with P/E-site and A/P-site tRNAs. **Left:** WT strain AL035, 12.8Å from 7089 particles; **Right:** *asc1* mutant strain AL116, 10.4Å from 10,723 particles. Resolution determined at FSC = 0.143 under gold standard regime. Blue: large 60S subunit; yellow: small 40S subunit; green: P/E-site tRNA; magenta: A/P-site tRNA; orange: Asc1p. Note that tRNAs appear thinned because of nonstoichiometric occupancy. (B-E) the *S. cerevisiae* structure (PDB ID: 3U5B, 3U5C, 3U5D, and 3U5E) used as reference was rigid body fitted into density maps in UCSF Chimera [20]: (B) WT strain AL035 at 30°C, 14.5Å, from 3,656 particles; (C) WT strain AL035 at 39°C, 16.0Å, from 3,433 particles; (D) *asc1* null strain AL116 at 30°C, 11.2Å, from 7,916 particles; (E) *asc1* null strain AL116 at 39°C, 15.5Å, from 3,807 particles. Resolution determined at FSC = 0.143 under gold standard regime. Yellow ribbon: small 40S subunit; blue ribbon: large 60S subunit; orange ribbon: X-ray structure of Asc1p. The four density maps were compared after filtering to the same resolution, 16.0Å. (F-H) Comparisons between map (B) and (C-E), shown in difference maps in the lower panel: (F) (WT 30°C) – (WT 39°C), (G) (WT 30°C) – (*asc1* 30°C), and (H) (WT 30°C) – (*asc1* 39°C) respectively shown at the threshold of $\pm 5 \times$ standard deviations of each difference map. In (F-H) the density map of WT at 30°C is shown in light gray as a viewing aid. Red mass: positive difference; blue mass: negative difference. The blue arrow (bottom, H) indicates a minor difference in mass observed in the *asc1* yeast ribosomes at 39°C.

Figure 4.

asc1 at NPT carries out normal rRNA processing. (A) Diagram of rRNA processing steps and location of probes used for the analysis. Adapted from Dong *et al*^[26]. (B) Northern blot detection of various rRNA products and intermediates. WT Strain YSB194 and *asc1* null strain AL116 were grown at 30°C and then shifted to 39°C for 1h in SC media before RNA was harvested. The same membrane was iteratively probed with probes 1, 3, 4, and 5; *ACT1* RNA served as a loading control. For probe 5/5S, *SCR1* was used as a loading control. (n.s.): non-specific binding.

Figure 5.

Polyribosomes decrease over time in *asc1Δ* at NPT. WT strain AL035, *asc1Δ* strain AL116, and *asc1Δ* + pASC1 strain AL156 were grown at 30°C overnight in SC media to mid log phase then diluted 1:10 in fresh SC at 30°C or 39°C. Samples were taken at 0, 1, 2, and 6 h and ribosome dissociation then blocked with cycloheximide. Gradients were loaded with 1 mg of total protein from cleared lysate and profiles read at an absorbance of 254 nm. Results are representative of at least 2 replicates. The WT 30°C, 0 h profile includes labels for common features of polyribosome profiles.

Figure 6.

Ribosomal proteins are decreased in *asc1* at NPT. **(A)** Hierarchical clustering of log2 fold change protein expression values compared to yeast WCE common control as derived from normalized iTRAQ reporter ion intensities. Fold change values for each replicate condition were compared with the average intensity values of the WCE common control included in each set of experiments. **(B,C)** Box plot diagrams of various protein groups using average log2 ratio protein expression values from A. Ratios for each condition were determined by dividing the reporter signal by the average reporter signal across all channels. Individual data points are shown for the top and bottom 5% of the data. Results of t-test comparisons are shown. *****: $p < 0.0001$, ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, n.s.: $p > 0.05$. **(B)** All reproducibly detected proteins and the subset of those proteins belonging to the large and small ribosomal subunits. **(C)** Box plot diagram of log2 ratios as in B of quantified proteins belonging to various GO terms.