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A conserved domain important for association of eukaryotic J-protein co-chaperones Jjj1 and Zuo1 with the ribosome

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Abstract

J-proteins, obligate co-chaperones, provide specialization for Hsp70 function in a variety of cellular processes. Two of the 13 J-proteins of the yeast cytosol/nucleus, Zuo1 and Jjj1, are associated with 60S ribosomal subunits. Abundant Zuo1 facilitates folding of nascent polypeptides; Jjj1, of much lower abundance, functions in ribosome biogenesis. However, overexpression of Jjj1 substantially rescues growth defects of cells lacking Zuo1. We analyzed a region held in common by Zuo1 and Jjj1, outside the signature J-domain found in all J-proteins. This shared "zuotin homology domain" (ZHD) is important for ribosome association of both proteins. An N-terminal segment of Jjj1, containing the J-domain and ZHD , is ribosome-associated and, like full-length Jjj1, is competent to rescue both the cold- and cation-sensitivity of Δ*zuo1*. However, this fragment, when expressed at normal levels, cannot rescue the cytosolic ribosome biogenesis defect of Δ*jjj1*. Our results are consistent with a model in which the primary functions of Zuo1 and Jjj1 occur in the cytosol. In addition, our data suggest that Zuo1 and Jjj1 bind overlapping sites on ribosomes due to an interaction via their common ZHDs, but Jjj1 binds primarily to pre-60S particles and Zuo1 to mature subunits. We hypothesize that *ZUO1* and *JJJ1*, which are conserved throughout eukaryotes, arose from an ancient duplication of a progenitor J-protein gene that encoded the ZHD ribosome-binding region; subsequently, specialized roles and additional ribosome interaction sites evolved.

Keywords

J-protein; ribosome biogenesis; Hsp70; molecular chaperone; ribosome association

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1. Introduction

All Hsp70-based molecular chaperone machineries use the same fundamental biochemical mechanism of action, cycles of interaction with client proteins driven by ATP binding and hydrolysis, to function in many essential cellular processes [1, 2]. These processes range from folding of nascent chains as they emerge from ribosomes to driving protein translocation across membranes, protecting cells from heat stress, facilitating biogenesis of Fe/S clusters, and remodeling protein:protein complexes [3, 4]. Much of the capacity for such functional versatility is due to the fact that Hsp70s interact with an array of J-protein co-chaperones [5]. For example, the cytosol/nucleus of the budding yeast *S. cerevisiae* contains 13 J-proteins. All members of the J-protein superfamily possess a ~70 residue J-domain that binds Hsp70 and is responsible for stimulation of Hsp70's ATPase activity, an obligatory step for stabilizing Hsp70's interaction with client protein. However, outside their J-domains, J-proteins vary widely in sequence and structure [3]. These diverse regions often interact with client proteins, targeting them to Hsp70, or localize the J-protein to a particular site of action.

Eukaryotes contain two ribosome-associated J-proteins, called Zuo1 and Jjj1 in yeast (DNAJC2 and DNAJC21, respectively, in human cells). Both associate with the large ribosomal subunit [6-8]. Both have well-established roles: Zuo1 in chaperoning nascent chains and Jjj1 in a late step of subunit maturation, removing biogenesis factors. Zuo1 is present on approximately 1 of every 3 ribosomes [9, 10], Jjj1 is present at only about 1 per 1,000 ribosomes [10]. Cells lacking Zuo1 are slow-growing, particularly at low temperatures, cold-sensitive, and hypersensitive to cations [6, 11, 12], general defects likely reflecting the myriad of clients whose *de novo* folding requires ribosome-associated chaperones. As expected, loss of the ribosome-associated Hsp70:J-protein machinery results in aggregation of many newly-made polypeptides [13, 14]. Cells lacking Jjj1 are slow-growing and cold-sensitive, and exhibit hallmarks of inefficient 60S-maturation, such as decreased levels of 60S subunits and accumulation of aberrant polysomes [7, 15].

Jjj1's role in ribosome biogenesis is an example of involvement of Hsp70/J-protein chaperone machinery in remodeling protein complexes. A few of the many factors involved in 60S subunit biogenesis transit with pre-ribosomal particles to the cytosol [16]. These shuttling factors must be removed and recycled back to the nucleus. Jjj1 is required for removal of one such shuttling factor, Arx1 [7, 15, 17]. In doing so, Jjj1 partners not only with Hsp70, but also with another 60S-biogenesis factor, Rei1. In wild-type cells, Arx1 is largely associated with nuclear pre-60S particles due to efficient removal from cytosolic 60S particles and recycling to the nucleus. In the absence of Jjj1, however, Arx1 accumulates in the cytosol.

Consistent with their different roles, many regions outside the J-domain are quite disparate [6, 8, 17-20]. In Zuo1, an N-terminal region is required for interaction with its heterodimeric partner Ssz1, a positively-charged rRNA-binding region is required for stable interaction with ribosomes, and the extreme C-terminus forms a helical bundle that may regulate ribosome association. On the other hand, the C-terminus of Jjj1 is comprised of a largely charged region flanked by C_2H_2 zinc fingers, which facilitates binding to Rei1. Moreover, in

fungi Jjj1 and Zuo1 function with different Hsp70 partners, Jjj1 with the general Ssa class of Hsp70s, Zuo1 with the fungal-specific ribosome-associated Ssb Hsp70 [7, 21].

However, despite strong evidence that these two ribosome-associated J-proteins carry out distinct functions consistent with these sequence differences, there are intriguing hints of functional overlap. Overexpression of the relatively low-abundance Jjj1 can partially rescue the cold sensitivity and cation hyper-sensitivity of Δ*zuo1* [7, 22]. Here we report on our analysis of a second region of high similarity between Zuo1 and Jjj1, in addition to the J-domain, the ~80 zuotin homology domain (ZHD) [7, 18] . The ZHD is important for ribosome association of both proteins, suggesting that these proteins have overlapping ribosome-binding sites. The partial rescue of Δ*zuo1* phenotypes by overexpression of Jjj1 does not require its region specialized in ribosome biogenesis, suggesting that the tethering of a J-domain to an appropriate site on the 60S subunit may be sufficient for basal Zuo1-like activity.

2. Materials and methods

2.1 Yeast strains, plasmids and growth conditions

All yeast strains used in this study are isogenic with DS10, with the genotype *his3-11, 15 leu2-3, 112 lys1 lys2 trp1*Δ *ura3-52.* Deletion strains have been published as follows: Δ*zuo1::HIS* [6], Δ*jjj1::TRP* [7], Δ*arx1::KanMX* [7], Δ*jjj1::TRP* Δ*arx1::KanMX* [7], Δ*jjj1::TRP ARX1-GFP::HIS*$^+$ [7]. A list of yeast plasmids used in this study is shown in Supplemental Table 1; all plasmids used are centromeric plasmids based on the pRS plasmid series [23, 24]. Substitution of codons in *ZUO1* and *JJJ1* and deletion of codons for residues 340-590 in *JJJ1* was done by QuikChange PCR mutagenesis (Stratagene). Strains were grown in rich medium (YPD) or minimal medium as previously described [12]. For growth assays, approximately equal concentrations of cells were spotted onto minimal medium plates from 10-fold serial dilutions. Plates with paromomycin (250 μg/ml) were incubated for 3 days at 30°C, plates without paromomycin were incubated for 3 days at 23°C or for 2 days at 30°C.

2.2 Preparation of yeast extracts and analysis of ribosome association

For comparison of total protein levels, yeast cell extracts were prepared as follows. Δ*zuo1* or Δ*jjj1* cells containing the indicated plasmids were grown at 30°C in minimal medium to an OD_{600} of 0.4-0.5. The equivalent of 0.4 OD units of cells was harvested by centrifugation then resuspended in 50 μl of water. 50 μl of 0.2 M NaOH was added, incubated for 5 min at room temperature, then pelleted and resuspended in 50 μl of SDS sample buffer and boiled for 5 min [25]. Equivalent amounts of extract were subjected to SDS-PAGE and immunoblot analysis. Quantitation of band intensity was done using ImageJ software [26].

Lysate preparation and sucrose gradient centrifugation for polysome analysis were as follows. Δ*zuo1* or Δ*jjj1* cells containing the indicated plasmids were grown at 30°C, unless otherwise noted, to an OD_{600} between 0.5 and 0.7 in 50 ml of selective minimal medium, treated with 100 μg/ml of cycloheximide and harvested by centrifugation at 4°C. Cells were then washed with 10 ml of ice-cold Buffer I (20 mM Tris-HCl (pH 7.5), 50 mM KCl and 5

mM $MgCl_2$); pelleted by centrifugation at 4°C; and resuspended in 0.75 ml of ice-cold Buffer I plus 1.5 mM pepstatin; cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail (Roche) and 20 units of Recombinant RNasin Ribonuclease Inhibitor (Promega). 300 μl of ice-cold glass beads were added to the cell suspension, and lysates were prepared by vortexing at 4°C six times for 30 seconds with 30 seconds of cooling in between. Lysates were clarified by centrifugation at 14,000 rpm for 10 minutes. To fractionate polysomes, 10 OD_{260} units of lysate were applied to the top of a 4 ml 5–50% sucrose gradient in Buffer I and centrifuged for 80 minutes at 45,000 rpm at 4°C in a SW50.1 Ti rotor (Beckman). Gradients were monitored for absorbance at 254 nm to detect ribosomal subunits, monosomes and polysomes, and 0.4 ml fractions were collected. Proteins were precipitated by incubation with 86% acetone overnight at −20°C before SDS-PAGE and immunoblot analysis.

For RNase-treatment, lysates were prepared as above, then 250 μg/ml RNaseA was added to lysates on ice, incubated at 16° for 10 minutes, returned to ice and immediately applied to cold gradients and centrifuged, as above.

2.3 Antibodies and immunoblotting

To generate antibodies specific to the N-terminus of Jjj1, $Jjj1_{1\text{-}304}$ with a C-terminal 6x His tag was expressed from the pET3a vector (Novagen) in an *E. coli* strain, BL21 *dnaK*$^-$ *dnaJ*$^-$, and purified. The $Jjj1_{1\text{-}304}$ protein was used as an immunogen to generate anti-Jjj1 polyclonal antibodies in rabbits (Harlan). Anti-Rpl3 was a gift from Jon Warner (Albert Einstein College of Medicine, Bronx, NY). Anti-Jjj1 specific to the C-terminus (raised against $Jjj1_{304\text{-}590}$), anti-Zuo1 and anti-Ssc1 were produced as reported [6, 7, 27]. Immunoblot detection was done using Amersham ECL HRP-Linked Secondary Antibodies (GE Healthcare) and Western Lightning Plus ECL substrate (PerkinElmer).

2.4 Microscopy

JJJ1-encoding plasmids as indicated, as well as plasmid encoding RFP-Pus1, were transformed into Δ*jjj1* or Δ*jjj1 ARX1-GFP*. Overnight cultures grown in minimal medium at 30°C were diluted into fresh minimal medium to an OD_{600} of 0.2 and cultured at 30°C or 23°C as indicated to an OD_{600} between 0.5 and 0.7, then washed with ddH_2O prior to imaging. Fluorescence was visualized using a Zeiss Axio Imager.M2 epi-fluorescence microscope with a 100x oil immersion objective lens, and images were captured with an AxioCam MRm camera controlled with AxioVision software (Carl Zeiss Microscopy, LLC, Thornwood, NY).

2.5 Protein expression and purification

DNA encoding residues 166-303 of Zuo1 was amplified by PCR, and cloned into a modified pET-28a vector that contains an N-terminal His_6 tag, thioredoxin (TRX) tag and a tobacco etch virus (rTEV) protease cleavage site. Two arginine mutations (RR247,251AA) were introduced into wild type $ZUO1_{166\text{-}303}$ via QuikChange PCR mutagenesis (Stratagene). ^{15}N-labeled protein expression was conducted in Rosetta 2 (DE3) pLysS *E. coli* cells using 1 liter of auto-induction medium [28, 29]. Cells were resuspended in lysis buffer (50 mM Tris pH 7.5, 250 mM NaCl, 10 mM imidazole) with cOmplete, EDTA-free Protease Inhibitor

Cocktail (Roche), then lysed by French Press. Lysates were clarified by centrifugation. Tagged $Zuo1_{166-303}$ was purified using bench top nickel column chromatography and size-exclusion chromatography. Tags were removed by proteolysis with recombinant rTEV protease, for 14-18 hrs at 4°C. Tagged protein, cleaved His_6/TRX tag and His-tagged rTEV were separated from $Zuo1_{166-303}$ by bench top nickel chromatography. Protein was used immediately or stored at −80°C.

2.6 NMR spectroscopy and data processing

All 2D ^{15}N-^{1}H Heteronuclear Single-Quantum Correlation (HSQC) spectra were recorded at 4°C using a Varian 600 MHz spectrometer. NMR data were processed using NMRPipe [30] and analyzed using Sparky [31]. All NMR experiments were conducted in 25 mM sodium phosphate (pH 7.5), 200 mM NaCl, 5mM dithiothreitol and 7% D_2O.

3. Results

3.1 Two conserved ZHD region residues are important for function of both Jjj1 and Zuo1

An alignment of Zuo1 and Jjj1 from *S. cerevisiae* and their human orthologs, DNAJC2 and DNAJC21, was generated using ClustalW [32]. A preponderance of the conserved residues outside the J-domain were found in the ~81 residue ZHD. 15 are identical in the ZHD compared to 14 in the J-domain, with only 2 identical residues in the remainder of the alignment (Fig. 1A, Suppl. Fig. S1). Thus, the homology between the two proteins is minimal outside the J-domain and ZHD.

Since no region homologous to the ZHD region was found in other yeast J-proteins, we undertook a genetic analysis to understand its function in the two ribosome-associated J-proteins. To determine which of the 15 identical ZHD region residues are most important for function, we performed an alanine mutagenesis screen. Each of the codons for the identical amino acids was singly changed to an alanine codon. The resulting *ZUO1* and *JJJ1* mutants were expressed from their native promoter in Δ*zuo1* or Δ*jjj1* cells, respectively. None of the cells expressing a Zuo1 single-alanine variant had an observable growth defect in the presence of cation or at 23°C (Fig. 1B). However, cells expressing $Jjj1_{R221A}$ or $Jjj1_{R225A}$ were cold-sensitive, growing as poorly as Δ*jjj1*, even though the variants were expressed at normal levels (Fig. 1C, Suppl. Fig. S2A). Taking advantage of our previous observation that overexpression of Jjj1 partially rescues the growth defects of Δ*zuo1* [7], we also tested whether $Jjj1_{R221A}$ and $Jjj1_{R225A}$ could rescue Δ*zuo1* to the same extent. We placed *JJJ1*, $jjj1_{R221A}$ and $jjj1_{R225A}$ under the strong *GPD1* promoter. Though all three proteins were expressed at very similar levels, only wild-type Jjj1 rescued the cation-sensitivity of Δ*zuo1 cells* (Fig. 1D, Suppl. Fig. S2B). Since a low level of Zuo1 (<5% the level in wild-type cells) is sufficient to support growth indistinguishable from that of cells with normal Zuo1 levels [33], we considered that deleterious effects of the single-alanine substitutions in Zuo1 might be masked. To test more stringently whether the analogous arginines that are important for Jjj1 function are also important for Zuo1 function, we constructed a double substitution mutant, changing the codons for both arginine R247 and arginine R251 of *ZUO1* to alanine (called $zuo1_{RR\rightarrow AA}$ throughout). $zuo1_{RR\rightarrow AA}$ was both cation- and cold-sensitive (Fig. 1E), though $Zuo1_{RR\rightarrow AA}$ was expressed at a similar level to wild-type Zuo1 protein (Suppl. Fig.

S2C). Together these results suggest that the analogous residues of the ZHD, R247 and R251 in Zuo1, and R221 and R225 in Jjj1, are functionally important.

3.2 ZHD residues are required for stable association of Zuo1 with ribosomes

Having identified two functionally important ZHD residues in Zuo1 and Jjj1, we next asked whether they were important for ribosome association. We first assessed ribosome association by testing for co-migration of Zuo1/Jjj1 with ribosomes. Cell lysates were centrifuged through a sucrose density gradient to separate different-sized ribosomal complexes. Zuo1 co-migrated with monosomes and polysomes, as expected [6]. However, most of $Zuo1_{RR\rightarrow AA}$ remained near the top of the gradient (Fig. 2A), indicating destabilization of its interaction with ribosomes.

We considered that the lack of association with ribosomes for $Zuo1_{RR\rightarrow AA}$ might be due to structural changes of the ZHD, rather than loss of a specific interaction. To test this possibility we established a purification system for the ZHD of Zuo1. ^{15}N-labeled $Zuo1_{166\text{-}303}$ fragments, either with or without the RR→AA alterations, were isolated and ^{15}N-^{1}H HSQC NMR data collected. The ^{15}N-^{1}H HSQC spectra of the wild-type ZHD of Zuo1 and $ZHD_{RR\rightarrow AA}$ fragments had very similar patterns of peak distribution, indicating that the arginine to alanine alterations did not cause a change in the overall structure of the ZHD (Fig. 2B).

3.3 In the absence of its C-terminus, Jjj1 requires ZHD residues for stable ribosome association

We next tested whether the analogous RR→AA substitutions (R221A and R225A, called $Jjj1_{RR\rightarrow AA}$ throughout) affected Jjj1 ribosome association. As expected [7, 15], and consistent with its role in ribosome biogenesis, wild-type Jjj1 migrated at 60S in sucrose gradients. $Jjj1_{RR\rightarrow AA}$ also migrated at 60S (Fig. 2C). We next considered that Jjj1 may interact with ribosomes via multiple domains and that destabilization of the interaction of the ZHD with the ribosome might be masked by interaction of Jjj1's C-terminus with ribosomal complexes. Therefore, we tested whether the Jjj1 C-terminus is required for stable ribosome association. We made a construct encoding a Jjj1 variant lacking the C-terminal residues from 340-590, termed $Jjj1_{1\text{-}339}$. In a sucrose gradient, $Jjj1_{1\text{-}339}$ co-migrated not only with 60S-sized ribosome particles like Jjj1, but also with monosomes and polysomes (Fig. 3A). To determine whether this altered migration reflected association with 60S subunits or simply co-migration, cell lysates were treated with RNase to clip mRNA, thus collapsing polysomes into 80S monosomes. After RNase treatment, $Jjj1_{1\text{-}339}$ coincided with ribosomes, accumulating at 80S (Fig. 3B). The co-migration of $Jjj1_{1\text{-}339}$ with polysomes and with the 80S peak after polysome disruption strongly indicates that $Jjj1_{1\text{-}339}$ is ribosome-associated.

Having evidence that $Jjj1_{1\text{-}339}$ associates with ribosomes, we tested whether the RR→AA alteration affected $Jjj1_{1\text{-}339}$ ribosome association. $Jjj1_{1\text{-}339/RR\rightarrow AA}$ largely remained at the top of the sucrose gradient, indicating its destabilization from ribosomes (Fig. 3C). Together with the result that the Zuo1:ribosome interaction is destabilized by the RR→AA alterations, these data indicate that the ZHD contributes to ribosome association of both Zuo1 and Jjj1.

3.4 The Jjj1 C-terminus is important for Jjj1 function in 60S ribosomal subunit maturation

The ribosome association of Jjj1$_{1-339}$ raises the question of what sequences are required for Jjj1's major function in ribosome biogenesis. Therefore, we tested whether Jjj1$_{1-339}$ was able to rescue the effects of the absence of Jjj1, first testing for suppression of the Δ*jjj1* growth defect. We found that *jjj1*$_{1-339}$ is slow-growing and cold-sensitive, nearly indistinguishable from Δ*jjj1* (Fig. 4A, left). To facilitate accurate comparison of expression of Jjj1 and Jjj1$_{1-339}$, we generated polyclonal antibodies specific for residues 1-304. Using these antibodies, analysis of lysates made from cells expressing wild-type Jjj1 or Jjj1$_{1-339}$ revealed that Jjj1$_{1-339}$ was expressed at a slightly higher level than full-length Jjj1 (Fig. 4A, right), consistent with expression from a centromeric plasmid. We also inspected the cellular distribution of fluorescence signal from cells expressing Jjj1-GFP or Jjj1$_{1-339}$-GFP. Both Jjj1-GFP and Jjj1$_{1-339}$-GFP cells showed cytosolic signal (Fig. 4C), consistent both with previous reports of Jjj1 localization [7, 34] and with our observation that Jjj1$_{1-339}$ co-migrates with monosomes and polysomes (Fig. 3A). In some cells the fluorescence of Jjj1-GFP appeared somewhat less intense, while Jjj1$_{1-339}$-GFP fluorescence was consistently of similar intensity in the two compartments.

A more direct indicator of inefficient 60S subunit maturation than cold-sensitivity is the presence of half-mer peaks in polysome profiles, which are interpreted as 40S ribosomal subunits stalled in an initiation complex with mRNA because of a lack of mature 60S subunits [35, 36]. Consistent with previous reports, half-mers were apparent in polysome profiles of Δ*jjj1*, being most distinct in profiles of lysates from cells grown at a lower temperature, e.g. 23°C [7, 15]. Half-mer peaks were also present in *jjj1*$_{1-339}$ polysome profiles (Fig. 4B, also see Fig 3), consistent with a defect in 60S subunit biogenesis.

We also tested whether loss of residues 340-590 affected recycling of Arx1 from the cytosol into the nucleus. We inspected the distribution of Arx1-GFP signal between the nucleus and cytosol in wild-type, Δ*jjj1*and *jjj1*$_{1-339}$ cells. As expected, in Δ*jjj1* cells Arx1-GFP accumulated in the cytosol, rather than concentrating in the nucleus as in wild-type cells [7, 15]. In *jjj1*$_{1-339}$ cells Arx1-GFP was also present present in the cytosol (Fig. 5A), indicating that the C-terminus of Jjj1 is important for efficient recycling of Arx1. Further, deletion of *ARX1* has also been shown to suppress the growth phenotype of Δ*jjj1* [7, 15], consistent with the idea that the persistence of *ARX1* in the cytosol in the absence of Jjj1 is largely responsible for the Δ*jjj1* growth phenotype. We found that like Δ*jjj1*, the cold-sensitivity of *jjj1*$_{1-339}$ was suppressed by deletion of Arx1 (Fig. 5B). These results are consistent with Jjj1$_{1-339}$ being defective in the cytosolic function of Arx1 removal.

The C-terminal region of Jjj1 from residues 340-590 contains two zinc finger domains separated by a charged region, spanning residues 363-550, which is predominantly negative from 363-389 and predominantly positive from 390-550. To gain a better understanding of functionally important residues in the C-terminus, we analyzed the growth and Arx1-GFP fluorescence of cells expressing three truncations that removed smaller amounts of the C-terminal region than Jjj1$_{1-339}$, the focus of this study: Jjj1$_{1-362}$, Jjj1$_{1-389}$, and Jjj1$_{1-550}$ (see Fig 1A). Analysis of GFP fusions of all the truncations revealed dispersed cellular fluorescence (Suppl. Fig. 3A), indicating the presence of the Jjj1 truncations in the cytosol.

The two truncations removing the smaller segments of the C-terminus, Jjj1$_{1\text{-}550}$ and Jjj1$_{1\text{-}389}$, supported growth nearly as well as full-length Jjj1, while Jjj1$_{1\text{-}362}$ did not (Fig. 5C). Arx1 was concentrated in the nucleus in *jjj1*$_{1\text{-}550}$ and *jjj1*$_{1\text{-}389}$, like in wild-type cells, but was dispersed in the cytosol in *jjj1*$_{1\text{-}362}$, like in *jjj1*$_{1\text{-}339}$ (Fig. 5A). Jjj1$_{1\text{-}389}$, the smallest C-terminal truncation that supported Jjj1 function when expressed from its native promoter, co-migrated with ribosomal subunits, consistent with a ribosome-associated function of Jjj1 (Suppl. Fig. 3B).

3.5 The C-terminus of Jjj1 is not required for rescue of Δzuo1

That Jjj1$_{1\text{-}339}$ is ribosome associated, but defective in its ability to carry out its ribosome biogenesis function, raises two questions: (1) is Jjj1$_{1\text{-}339}$, like full-length Jjj1, competent in rescuing the deleterious effects of the absence of Zuo1 when overexpressed, and (2) does Jjj1$_{1\text{-}339}$ retain a level of competency that enables it to carry out Jjj1's ribosome biogenesis function if overexpressed. To answer the first question, we first placed *jjj1*$_{1\text{-}339}$ under the control of the strong *GPD1* promoter, as we had done previously for full-length Jjj1 ([7] and Fig 1D). Expression of Jjj1$_{1\text{-}339}$ and Jjj1 to the same level rescued Δ*zuo1* cation- and cold-sensitivity to the same extent, indicating that the C-terminus of Jjj1 is dispensable for Δ*zuo1* rescue (Fig. 6A).

Next we tested the ability of Jjj1$_{1\text{-}339}$ to rescue ribosome biogenesis defects when overexpressed. We placed *jjj1*$_{1\text{-}339}$ under promoters of varying strengths, and analyzed protein levels by immunoblotting with our antibody specific to the N-terminus of Jjj1. Modest overexpression of Jjj1$_{1\text{-}339}$ to 2-fold more than the normal level from the *CYC1* promoter had no obvious effect on growth of Δ*jjj1* cells. However, approximately 8-fold overexpression from the *ADH1* promoter (*pADH1-jjj1*$_{1\text{-}339}$), in which case the majority of Jjj1$_{1\text{-}339}$, like full-length Jjj1 co-migrated with ribosomes (Suppl. Fig. 4), resulted in significant suppression of cold-sensitivity and slow-growth (Fig. 6B).

To test whether rescue of growth by overexpression extended to rescue of Arx1 recycling, we examined Arx1-GFP localization in cells carrying *pADH1-jjj1*$_{1\text{-}339}$. Arx1-GFP is largely present in the nucleus in cells carrying *pADH1-jjj1*$_{1\text{-}339}$ (Fig. 6C), indicating that increased levels of Jjj1$_{1\text{-}339}$ can rescue both the growth defects and Arx1 recycling defect of Δ*jjj1*. But this rescue requires the conserved arginines at positions 221 and 225 (Fig 6; Suppl. Fig. 4). Overexpression of Jjj1$_{1\text{-}339}$ also rescued the aberrant polysome profiles of Δ*jjj1* (Fig. 6D). Together with the evidence described above, these results suggest that the C-terminus of Jjj1 is important for its cytosolic role in Arx1 removal, though the reduced function of Jjj1$_{1\text{-}339}$ can be rescued by increased cellular concentration. The data also indicates that the cold sensitivity and half-mer accumulation in Jjj1$_{1\text{-}339}$ cells are linked to Arx1 removal defects.

4. Discussion

Overall our results indicate that the ZHD is important for the association of both Zuo1 and Jjj1 with 60S ribosomal particles, with the primary functions of these proteins ribosome-associated activities in the cytosol. However, interaction of both proteins with the ribosome involves other unrelated sequences, in addition to the ZHD.

4.1 The ZHD is important for ribosome association

That the ZHD is important for ribosome association is supported by the fact that alteration of conserved arginine residues in full-length Zuo1 results in destabilization of its interaction with the ribosome. Although the analogous alterations in Jjj1 do not cause destabilization in full-length Jjj1, ribosome association of an N-terminal fragment containing the J domain and ZHD, $Jjj1_{1\text{-}339}$, is destabilized. Our biophysical analysis of the ZHD fragment of Zuo1 indicates that the alterations do not significantly affect its fold. Although it is possible that these arginine alterations affect the overall structure of the protein, we think it unlikely. More likely, the ZHD of both Zuo1 and Jjj1 interact with the ribosome at the same site, as other than the J-domain, sequences outside the ZHD of the two proteins have no obvious sequence similarity. This idea is also consistent with crosslinking and cryo-electron microscopic studies that localize Zuo1 and Jjj1 to the same general area of the 60S subunit near the ribosomal exit tunnel [37, 38].

However, in addition to the ZHD interaction, both Zuo1 and Jjj1 interact with ribosome complexes via their distinct C-terminal segments. Zuo1 and Jjj1 carry out different cellular roles, Zuo1 on translating ribosomes and the much less abundant Jjj1 on Arx1-containing 60S particles. Therefore, it is easy to envision that, although they utilize a shared ribosome interaction site, additional, distinct interactions determine their affinity for specific 60S ribosomal particles, and thus the ribosome particles with which they predominately associate. Such a model would explain the predominance of Jjj1 association with subunits migrating at 60S (i.e. immature pre-60S particles). For example, Rei1, Jjj1's partner in ribosome biogenesis with which it directly interacts, binds independently to the 60S subunit [17]. The Jjj1:Rei1 interaction could partially explain the predominant association of full-length Jjj1 with ribosomal particles migrating at 60S, compared to $Jjj1_{1\text{-}339}$, which associates with polysomes as well. In the case of Zuo1, recently reported cryo-electron microscopic studies [39] indicate that while the bulk of Zuo1 localizes near the exit site of the 60S subunit as does Jjj1, its extreme C-terminus, which has been shown to form a regulatory 4-helix bundle [20], interacts with the 40S subunit, thus potentially playing a role in translational regulation.

Our data also suggest that the ZHD does more than simply tether the J-proteins near the ribosome exit site. Even though $Jjj1_{RR\text{—}>AA}$ is ribosome-associated, it does not rescue either the cold sensitivity of Δ*jjj1* or the cold- or cation-sensitivity of Δ*zuo1* when overexpressed. In addition, that $jjj1_{RR\text{—}>AA}$ cells show half-mer accumulation and Arx1 mislocalization indicates that these alterations cause disruption of ribosome biogenesis. Why $Jjj1_{RR\text{—}>AA}$ is not functional, though ribosome-associated, is not clear. We speculate that the ZHD may play a role in positioning Jjj1 on the 60S subunit. For example, Jjj1 function requires that its J-domain interacts with its partner Hsp70. If not positioned correctly, such that this interaction can occur effectively, a null phenotype would result.

4.2 Jjj1 C-terminal regions are important for Arx1 recycling and efficient ribosome biogenesis

The results reported here indicate that the C-terminus of Jjj1 is important for Jjj1 function in ribosome biogenesis because it facilitates removal of the Arx1 biogenesis factor present on

cytosolic pre-60S ribosomal subunits, thus allowing its recycling to the nucleus. However, the C-terminus is not absolutely essential for the process, as overexpression of Jjj1$_{1\text{-}339}$ allows removal.

The exact mechanism of Arx1 release and Jjj1's role in it are not known. As mentioned above, however, it is known that a second cytosolic biogenesis factor, Reil is required [40, 41]. Jjj1 interacts with Rei1 via its C-terminus [17]. This interaction, though not essential for Arx1 removal, may facilitate it, thus explaining the requirement for higher levels of Jjj1$_{1\text{-}339}$ for function.

The alignment between growth defects and cytosolic 60S maturation defects reported here point to maturation of 60S subunits in the cytosol as the major function of Jjj1 that causes slow growth, especially at low temperatures. Both Δ*jjj1* and *jjj1*$_{1\text{-}339}$ displayed half-mers, cytosolic accumulation of Arx1 and suppression of the growth defect upon deletion of *ARX1*. However, our results do not exclude the possibility that Jjj1 also functions in the nucleus in ribosome biogenesis as previously suggested [18], as we do see some Jjj1-GFP fluorescence in the nucleus. But, our results are inconsistent with a nuclear role being Jjj1's major function, as was concluded previously [18]. That conclusion was based in good part on the observed lack of co-migration of Jjj1$_{1\text{-}389}$, with ribosomes and exclusively nuclear localization of Jjj1$_{1\text{-}389}$-GFP. Our results also indicate that this Jjj1$_{1\text{-}389}$ fragment is functional, as it could both support nearly wild-type growth and nuclear localization of Arx1-GFP. However, we found it in the cytosol, co-migrating with ribosomes, like the shorter Jjj1$_{1\text{-}339}$ fragment, which is the focus of this report. Thus, our results continue to be consistent with the major function of Jjj1 being in the cytosol, facilitating the removal of the Arx1 ribosome biogenesis factor.

Interestingly, our analysis of a set of C-terminal truncation mutants uncovered a phenotypic difference between the *jjj1*$_{1\text{-}339}$/*jjj1*$_{1\text{-}362}$ pair and the *jjj1*$_{1\text{-}389}$/*jjj1*$_{1\text{-}550}$ pair. The intervening region between these pairs, 363-389, though part of the larger "charged" region, contains a segment that is predominately negative in charge, compared to the more C-terminal positively charged region. Thus the difference in activity between these pairs raises the possibility that this 26 residue segment is particularly important for Jjj1 function. These results are also consistent with our previous data [17] that showed a deletion of the entire charged region (363-534) resulted in growth defects, as well as Arx1 cytosolic accumulation and half-mer formation, while disruption of both zinc fingers had minimal effects.

4.3 Conclusions

The results presented here strongly suggest that the ZHD of the eukaryotic-specific J-proteins Zuo1 and Jjj1 function as a ribosome-association region for both proteins. Yet both proteins contain sequences with specialized function. For example, the C-terminus of Jjj1 is important for ribosome biogenesis, a function that appears to be unique to Jjj1. We suggest that the specialized Zuo1 and Jjj1 proteins evolved from a progenitor J-protein that evolved early in the eukaryotic lineage and was tethered near the exit tunnel of the ribosome by its ZHD. Consistent with this idea and the eukaryotic specificity of ribosome-associated J-proteins, the predicted binding site of Zuo1 and Jjj1 near the ribosomal exit tunnel is in proximity to Rpl31 and Rpl22, two eukaryotic proteins that have no homolog in bacteria

[42] and reside in a region of the 60S subunit rich in eukaryote-specific rRNA expansion segments [37, 38]. It is likely that the duplication of the progenitor gene also occurred early in the lineage, as Zuo1 and Jjj1 orthologs are ubiquitous in eukaryotes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- J-protein Hsp70 chaperones Zuo1 and Jjj1 have conserved Zuotin Homology domain, ZHD
- ZHD is important for ribosome association of both Zuo1 and Jjj1
- Specialized C-terminus of Jjj1 is important for cytosolic ribosome biogenesis
- Partial rescue of Δ*zuo1* by overexpression of Jjj1 does not require its C-terminus
- ZHD likely a common ribosome association domain in specialized eukaryotic J-proteins

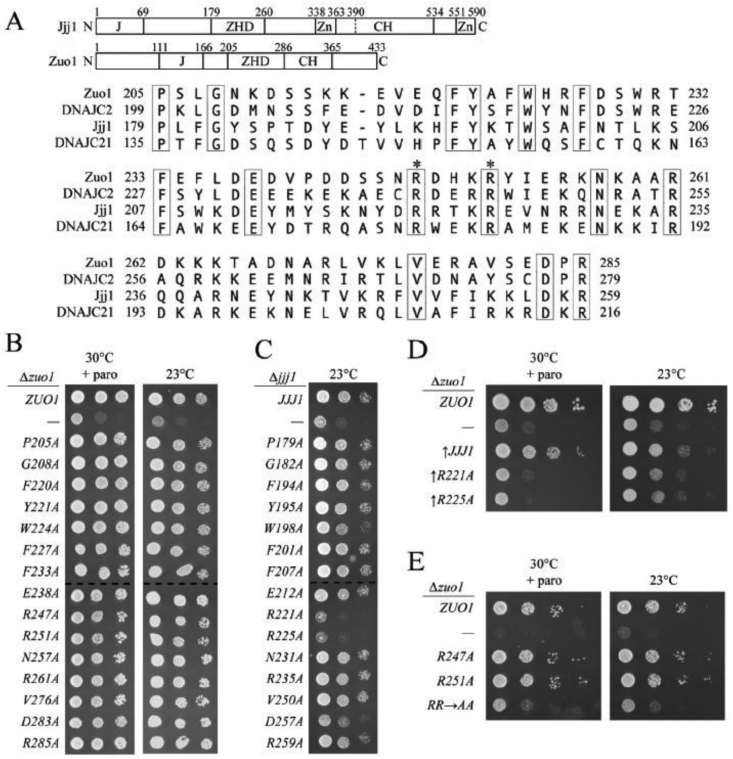

Fig. 1.

Two conserved arginines in the Zuotin homology domain (ZHD) are important for Jjj1 and Zuo1 function. A) (Upper) Diagram of Jjj1 and Zuo1 with vertical lines indicating region boundaries and numbers indicating the residue number at the boundary. J domain (J); Zuotin homology domain (ZHD); Zinc binding region (Zn); charged region (CH). (Bottom) Sequence alignment of *S. cerevisiae* Zuo1 and Jjj1 and their *H. sapiens* homologs (DNAJC2 and DNAJC21, respectively) was done with ClustalW; the ZHD sequences are shown with identical residues boxed. Residues of primary interest in this report are indicated (*). B-E) Analysis of deletion strains expressing wild-type or indicated variant proteins from the native promoter (exception noted in (D)) was as follows: strains were serially diluted, spotted on minimal medium plates and incubated at the indicated temperature for 3 days. Plates containing paromomycin (+ paro). B) Δ*zuo1* cells containing plasmid encoding wild-type *ZUO1*, no insert (—) or the indicated *ZUO1* mutant. C) Δ*jjj1* cells containing plasmid encoding wild-type *JJJ1*, no insert (—) or the indicated *JJJ1* mutant. D) Δ*zuo1* cells containing plasmid encoding wild-type *ZUO1* (WT) , no insert, or WT *JJJ1* or the indicated *JJJ1* mutant expressed from the *GPD1* promoter (↑). E) Δ*zuo1* cells containing plasmid encoding wild-type *ZUO1*, no insert (—) or the indicated *ZUO1* mutant ($Zuo1_{R247,251A}$ indicated by RR✧AA). Dotted lines in B-C indicate different plates from the same batch of media, analyzed at the same time.

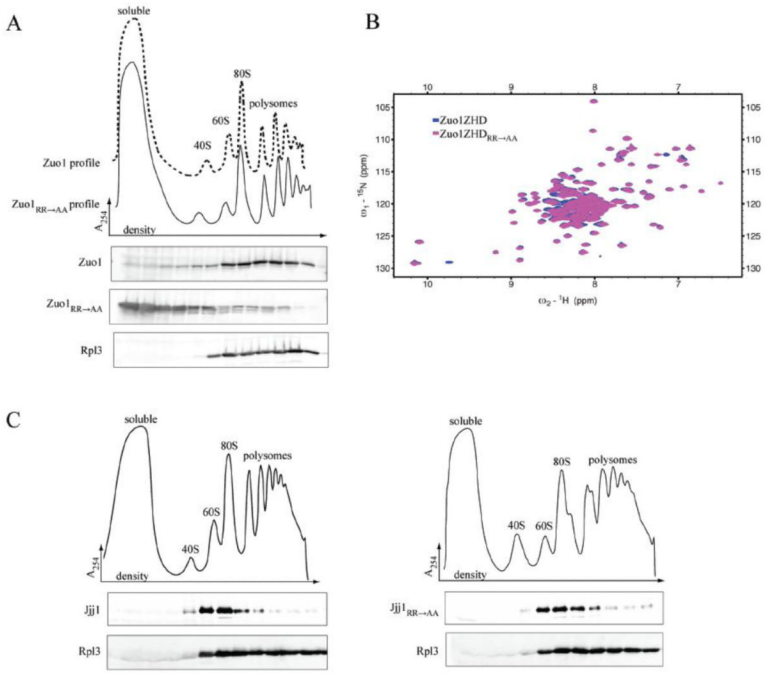

Fig. 2.

$Zuo1_{R247,251A}$ ($Zuo1_{RR\rightarrow AA}$) is largely dissociated from ribosomes. A) Lysate from Δ*zuo1* cells expressing wild-type Zuo1 or $Zuo1_{RR\rightarrow AA}$ from the native promoter was centrifuged through a sucrose gradient to separate ribosomal subunits, monosomes and polysomes. Fractions were collected. Upper, absorbance at 254 nm plotted versus the relative time of fraction collection (density); dotted line (Zuo1), solid line ($Zuo1_{RR\rightarrow AA}$). Lower, fractions were analyzed by immunoblotting using antibodies specific for Zuo1 and Rpl3 (Rpl3 distribution in both experiments was indistinguishable, the WT sample is shown for reference). B) 2D ^{15}N-^{1}H Heteronuclear Single-Quantum Correlation (HSQC) NMR spectra of WT $Zuo1_{166\text{-}303}$ (Zuo1ZHD), which includes the Zuotin homology domain (ZHD), and $Zuo1ZHD_{RR\rightarrow AA}$. C) Lysate from Δ*jjj1* cells expressing wild-type Jjj1 (left) or $Jjj1_{RR\rightarrow AA}$ (right) from the native promoter was subjected to sucrose gradient analysis as in (A). Upper, absorbance at 254 nm plotted versus the relative time of fraction collection (density). Lower, fractions were analyzed by immunoblotting using antibodies specific for the C-terminus of Jjj1 and Rpl3.

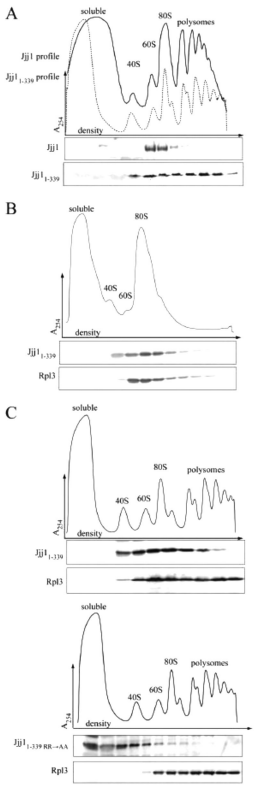

Fig. 3.

The N-terminus of Jjj1 is sufficient for ribosome association. (A-C) Lysate from Δ*jjj1* cells expressing wild-type Jjj1, Jjj1$_{1\text{-}339}$ or Jjj1$_{1\text{-}339\ R221,225A}$ (Jjj1$_{1\text{-}339\ RR\rightarrow AA}$) from the native promoter was centrifuged through a sucrose gradient. Upper, absorbance at 254 nm was monitored and plotted versus the relative time of fraction collection (density) Lower, fractions were analyzed by immunoblotting using antibodies specific for the N-terminus of Jjj1 and for Rpl3. A) Comparison of Jjj1 and Jjj1$_{1\text{-}339}$. Upper: dotted line (Jjj1), solid line (Jjj1$_{1\text{-}339}$). B) Lysate from cells expressing Jjj1$_{1\text{-}339}$ was briefly incubated with RNase before sucrose gradient analysis to clip the mRNA, accumulating monosomes. C) Comparison of Jjj1$_{1\text{-}339}$ (upper) and Jjj1$_{1\text{-}339RR\text{->}AA}$ (lower).

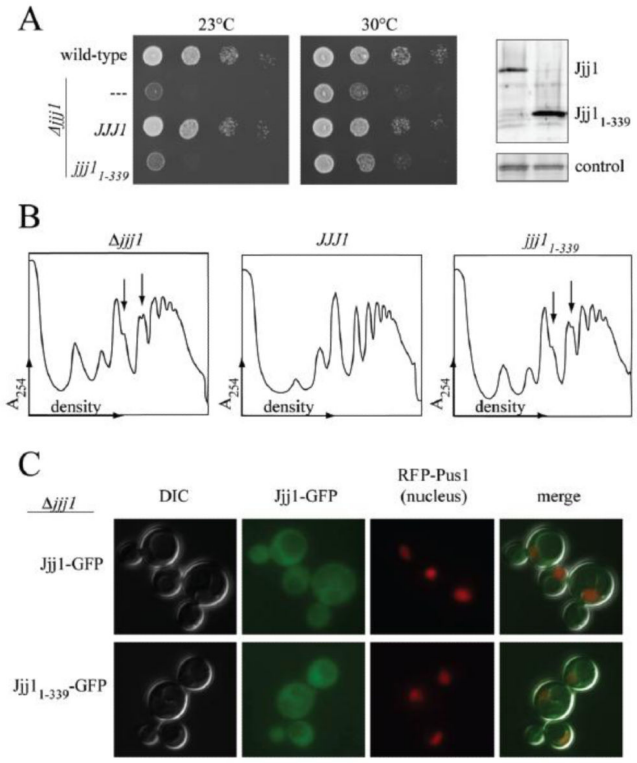

Fig. 4.

The Jjj1 C-terminus is important for the role of Jjj1 in ribosome biogenesis. A) Analysis of wild-type cells and Δ*jjj1* cells containing empty vector (—), or expressing Jjj1 or $Jjj1_{1\text{-}339}$ from the native promoter, was as follows: Left: strains were serially diluted, spotted on minimal medium plates, then incubated at 23°C for 3 days or 30°C for 2 days. Right: Cell extracts were prepared from Δ*jjj1+JJJ1* and Δ*jjj1+*$jjj1_{1\text{-}339}$ cultures used for serial dilutions and subjected to immunoblot analysis using antibody specific to the Jjj1 N-terminus and antibody specific to Ssc1 as a loading control. B) Δ*jjj1* cells containing empty vector and Δ*jjj1* cells expressing wild-type Jjj1 or $Jjj1_{1\text{-}339}$ from the native promoter were grown at 23°C, lysed. The lysate was then centrifuged through a sucrose gradient. The migration of ribosomal subunits, monosomes and polysomes was monitored by absorbance at 254 nm and plotted versus the time course of fraction collection. Arrows denote half-mer polysome peaks. C) Δ*jjj1* cells expressing either Jjj1-GFP or $Jjj1_{1\text{-}339}$ -GFP from the native promoter, and the nucleus-specific protein RFP-Pus1 as a nuclear marker, were grown at 30°C prior to imaging by fluorescence and differential interference contrast (DIC) microscopy. Representative images show DIC, localization of Jjj1-GFP or $Jjj1_{1\text{-}339}$ -GFP as indicated, localization of RFP-Pus1, a nuclear marker, and an image overlay (merge).

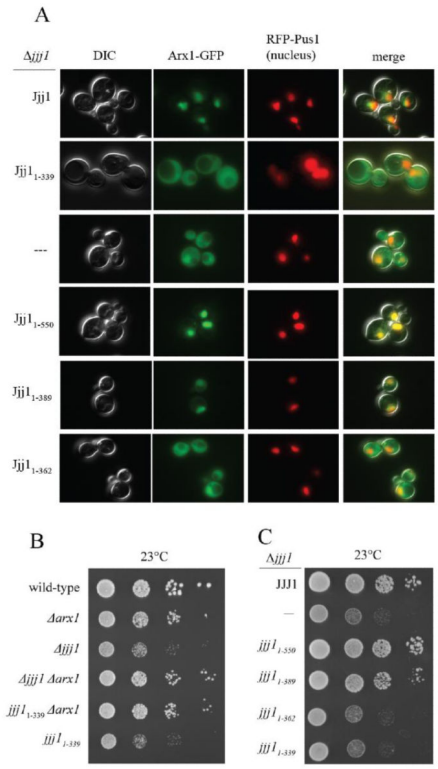

Fig. 5.

The Jjj1 C-terminus is important for the role of Jjj1 in Arx1 recycling. A) Δ*jjj1 ARX1-GFP* cells containing empty vector (—), or expressing Jjj1, $Jjj1_{1\text{-}339}$, $Jjj1_{1\text{-}550}$, $Jjj1_{1\text{-}389}$ or $Jjj1_{1\text{-}362}$ from the native promoter, were grown at 23°C prior to imaging by fluorescence and differential interference contrast (DIC) microscopy. Representative images show DIC, Arx1-GFP localization, localization of a nucleus-specific RFP fusion protein, RFP-Pus1, and an image overlay (merge). B) Wild-type, Δ*arx1*, Δ*jjj1*, and Δ*jjj1* Δ*arx1* cells containing empty vector, and Δ*jjj1* and Δ*jjj1*Δ*arx1* cells expressing $jjj1_{1\text{-}339}$ from the native promoter [($jjj1_{1\text{-}339}$) and ($jjj1_{1\text{-}339}$ Δ*arx1*), respectively], were serially diluted, spotted on a minimal medium plate, and incubated at 23°C for 3 days. C) Δ*jjj1* cells expressing either Jjj1, no Jjj1 (-) or the indicated Jjj1 truncation from the native promoter were serially diluted, spotted on a minimal medium plate, and incubated at 23°C for 2.5 days.

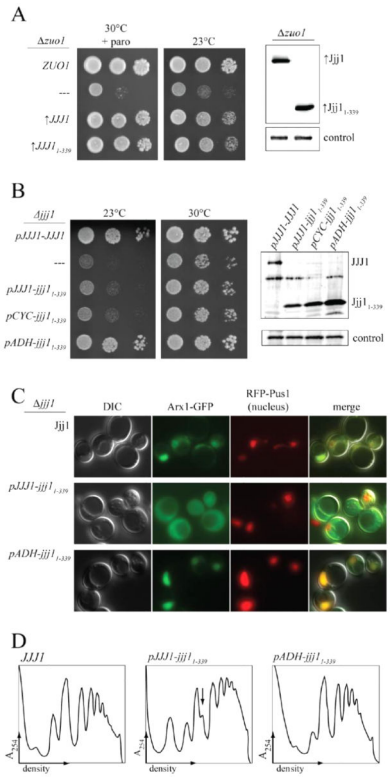

Fig. 6.

Effects of overexpression of *jjj1*$_{1\text{-}339}$. A) Left: Δ*zuo1* cells containing plasmid encoding *ZUO1* under control of the native promoter (ZUO1), no insert (—), or either *JJJ1* or *jjj1*$_{1\text{-}339}$ under control of the *GPD1* promoter (indicated by "↑"). Strains were serially diluted, spotted on minimal medium plates and incubated at the indicated temperature for 3 days. Plate containing paromomycin is indicated ("+paro"). Right: Cell extracts were prepared from cultures used for serial dilutions and subjected to immunoblot analysis using antibody specific to the Jjj1 N-terminus and antibody specific to Ssc1, as a loading control. B) Left: Analysis of Δ*jjj1* cells containing plasmid encoding *JJJ1* under control of the native promoter (*JJJ1*), no insert (—), or *jjj1*$_{1\text{-}339}$ under control of the indicated promoter: native (*pJJJ1*), *CYC* (*pCYC*) or *ADH* (*pADH*). Strains were serially diluted, spotted on minimal medium plates, then incubated at 23°C for 3 days or 30°C for 2 days. Right: Cell extracts were analyzed as described in (A). C) Δ*jjj1 ARX1-GFP* cells containing a plasmid encoding RFP-Pus1 and a plasmid encoding WT *JJJ1*, *jjj1*$_{1\text{-}339}$ under the native promoter or *jjj1*$_{1\text{-}339}$ under control of the *ADH* promoter (↑*jjj1*$_{1\text{-}339}$), were grown at 23°C prior to imaging by fluorescence and differential interference contrast (DIC) microscopy. Representative images show DIC, Arx1-GFP localization, localization of a nucleus-specific RFP fusion protein, RFP-Pus1, or an image overlay (merge). D) Δ*jjj1* cells expressing Jjj1, Jjj1$_{1\text{-}339}$ or Jjj1$_{1\text{-}339}$ from the *ADH* promoter were grown at 23°C and lysed, then centrifuged through a sucrose gradient. The migration of ribosomal subunits, monosomes and polysomes was monitored by absorbance at 254 nm and plotted versus the relative time of fraction collection. Arrows denote half-mer polysome peaks.