Bud23 promotes the progression of the Small Subunit Processome to the pre-40S ribosome in *Saccharomyces cerevisiae*

Joshua J. Black¹, Richa Sardana^{1, †}, Ezzeddine W. Elmir¹, Arlen W. Johnson^{1, *}

¹ Department of Molecular Biosciences, The University of Texas at Austin, Austin, Texas, United States of America

[†] Current Address: Department of Molecular Biology and Genetics, Weill Institute for Cell and Molecular Biology, Cornell University, Ithaca, NY

Running title: The role of Bud23 in 40S maturation.

*Corresponding author

Email: arlen@austin.utexas.edu (AWJ)

Abstract

The first metastable assembly intermediate of the eukaryotic ribosomal small subunit (SSU) is the SSU Processome, a large complex of RNA and protein factors that is thought to represent an early checkpoint in the assembly pathway. Transition of the SSU Processome towards continued maturation requires the removal of the U3 snoRNA and biogenesis factors as well as ribosomal RNA processing. While the factors that drive these events are largely known, how they do so is not well understood. The methyltransferase Bud23 has a role during this transition, but its function, beyond the nonessential methylation of 18S rRNA, is not characterized. Here, we have carried out a comprehensive genetic screen to understand Bud23 function. We identified 67 unique extragenic *bud23Δ*-suppressing mutations that mapped to genes encoding the SSU Processome factors *DHR1*, *IMP4*, *UTP2 (NOP14)*, *BMS1* and the SSU protein *RPS28A*. These factors form a physical interaction network that links the binding site of Bud23 to the U3 snoRNA and many of the suppressing mutations weaken protein-protein and protein-RNA interactions. Importantly, this network links Bud23 to the GTPase Bms1 and the RNA helicase Dhr1. Bms1 is thought to drive conformational changes to promote rRNA cleavage, and we previously showed that Dhr1 is required for unwinding the U3 snoRNA. Moreover, particles isolated from cells lacking Bud23 accumulated late SSU Processome factors and pre-rRNAs not cleaved at sites A1 and A2. We propose a model in which Bud23 dissociates factors surrounding its binding site to promote SSU Processome progression.

Author summary

Ribosomes are the molecular machines that synthesize proteins and are composed of a large and a small subunit which carry out the essential functions of polypeptide synthesis and mRNA decoding, respectively. Ribosome production is tightly linked to cellular growth as cells must produce enough ribosomes to meet their protein needs. However, ribosome assembly is a metabolically expensive pathway that must be balanced with other cellular energy needs and regulated accordingly. In eukaryotes, the small subunit (SSU) Processome is a metastable intermediate that ultimately progresses towards a mature SSU through the release of biogenesis factors. The decision to progress the SSU Processome is thought to be an early checkpoint in the SSU assembly pathway, but what drives this checkpoint is unknown. Previous studies suggest that Bud23 plays an uncharacterized role during SSU Processome progression. Here, we used a genetic approach to understand its function and found that Bud23 is connected to a network of factors that stabilize the particle. Interestingly, two of these factors are enzymes that facilitate structural rearrangements needed for progression. We conclude that Bud23 promotes the release of factors surrounding its binding site to drive rearrangements during the progression of the SSU Processome.

Introduction

Ribosomes are the molecular machines that translate the genetic code. Each ribosome is composed of a small subunit (SSU) that coordinates mRNAs and tRNAs for decoding and a large subunit (LSU) that catalyzes peptide bond formation. In the eukaryotic model organism *Saccharomyces cerevisiae*, the LSU, or 60S, contains three rRNAs (25S, 5.8S and 5S) and 46 ribosomal proteins (r-proteins), whereas the SSU, or 40S, has the 18S rRNA and 33 r-proteins [1].

The subunits are produced by an energetically expensive and dynamic assembly pathway requiring more than 200 *trans*-acting biogenesis factors (reviewed in [2–5]). Ribosome assembly begins in the nucleolus with the synthesis of the primary 35S rRNA transcript and the 5S rRNA. The primary transcript contains the 18S, 5.8S, and 25S rRNAs and four spacer regions that are removed during ribosome assembly: two external transcribed spacers (ETS) and two internal transcribed spacers (ITS) (Fig. S1). The r-proteins and the biogenesis factors assemble on the rRNA in a hierarchical order [6–10], most of which promote the correct architecture of the subunits by chaperoning and modifying the rRNA while others drive structural rearrangements and the removal of the spacer regions.

Because the pre-18S rRNA is encoded in the 5'-portion of the primary transcript the initial folding of the pre-rRNA is dedicated to SSU assembly. These co-transcriptional nucleolar events lead to assembly of the SSU Processome (sometimes referred to as a 90S pre-ribosome) [8,11,12]. The SSU Processome serves two primary roles: 1) promote the formation of the SSU architecture and 2) process the pre-rRNA at sites A0 and A1, to remove the 5' ETS, and at site A2, to separate the SSU and LSU precursors (reviewed in [13,14]). Recent high-resolution structures of the complete SSU Processome revealed a large metastable assemblage of 15 r-proteins and about 50 biogenesis factors on the 5' ETS and pre-18S rRNAs [15–17]. The 18S rRNA is divided into the 5', central, 3' major, and 3' minor domains (Fig. S2) that fold independently as the SSU Processome assembles [15–19]. In the SSU Processome, these RNA domains are scaffolded by a multitude of biogenesis factors, the 5' ETS ribonucleoprotein complex (RNP), and U3 snoRNA that prevent their collapse into the densely packed structure of the subsequent pre-40S and mature 40S particles. A correctly assembled SSU Processome ultimately transitions to the pre-40S [20] which

requires the release of most of these biogenesis factors, including U3 and 5' ETS RNP, as well as the aforementioned endonucleolytic cleavages of the pre-rRNA. This transition and the coordinated disassembly of the SSU Processome allows large architectural rearrangements to occur that yield the pre-40S and likely release it into the nucleoplasm for continued maturation. While recent structural and molecular analysis of the SSU Processome have brought its assembly into focus, there remains a dearth of understanding about the events and mechanisms driving its transition into the pre-40S.

Early recruitment of the U3 snoRNA is crucial for the formation and function of the SSU Processome [7,11,21–26]. Structural analyses of the SSU Processome show that the box C/D U3 snoRNA threads into the core of the complex where it spatially separates the rRNA domains and scaffolds biogenesis factors [15,16]. Two regions of U3, referred to as the 5' and 3' hinges, hybridize to the 5' ETS RNA while its Box A' and Box A regions hybridize to the pre-18S rRNA, henceforth U3-18S heteroduplexes. Notably, Box A hybridizes with helix 1 of 18S, precluding the formation of the Central Pseudoknot (CPK) of the SSU [15,17]. The CPK is a universally conserved feature of the SSU formed by long-range, non-canonical base-pairing between helices 1 and 2 that allows the four rRNA domains to compact onto one another (Fig. S2), and generates the environment necessary to establish the decoding center [1,27]. Because U3 blocks CPK formation, the release of U3 is a critical, irreversible step in the maturation of the SSU [14,28]. The unwinding of U3 is catalyzed by the DEAH/RHA helicase Dhr1 [29] which is activated by the SSU Processome factor Utp14 [30–32]. Mutational analysis identified a short loop of Utp14 that is necessary and sufficient for the activation of Dhr1 *in vitro* [30,32], and deletion or significant mutation of this loop phenocopies a catalytic *dhr1* mutant *in vivo* [30]. How Utp14 times the

activation of Dhr1 remains unknown, and neither Dhr1 nor the activation loop of Utp14 have been resolved in SSU Processome structures [15,16]. RNA crosslinking and structural analysis indicate that Utp14 binds simultaneously to the U3 and 5' ETS RNAs, as well as the central domain and 5'- and 3'-ends of the pre-18S rRNA, suggesting that Utp14 is uniquely positioned to time Dhr1 activation by monitoring completion of transcription of the pre-18S rRNA [15,16,33].

The endonucleolytic cleavages within the rRNA at sites A1 and A2 are also irreversible steps that occur around the time of the transition of the SSU Processome to pre-40S (Fig. S1). The current SSU Processome structures all contain rRNA cleaved at A0 but not at A1, indicating that A0 cleavage alone is not sufficient to trigger progression from the SSU Processome [15–17,19]. Around the time of Dhr1 function, cleavages at sites A1 within 5' ETS and A2 within ITS1 occur [29,34,35]. It is possible that cleavage at site A1 sets the transition in motion. Site A1 is cleaved by the PIN domain nuclease Utp24 [36–38]. However, it is positioned about 50 Å away from site A1 in the complete SSU Processome [15,16] indicating that some structural rearrangements must occur for it to access its substrate. It is proposed that the essential GTPase Bms1 acts as a molecular switch to drive such structural rearrangements [16], but what signals Bms1 activation is not known. Subsequent cleavage at site A2 separates the SSU precursor from the LSU precursor. When cleavage at site A2 is inhibited, cleavage at the downstream site A3 instead bifurcates the two maturation pathways.

Bud23 is a methyltransferase that acts with its cofactor Trm112 to modify guanosine 1575 (G1575) within the 3' major domain of 18S rRNA [39–42]. *BUD23* is a nonessential gene in yeast, but its deletion (*bud23Δ*) causes a significant growth defect that correlates with an approximate 70% reduction of 40S subunits [39]. Catalytically inactive *bud23* mutants fully complement the

growth defect of *bud23Δ* cells suggesting that the presence of the protein but not its methyltransferase activity is needed for ribosome assembly [39,40,43]. Bud23 is not a stable component of the complete SSU Processome and is usually thought to act on a nuclear pre-40S intermediate. Consistent with this notion, the human orthologs of Bud23 and Trm112 were resolved in an early pre-40S structure [44]. Moreover, its binding site is occupied by the assembly factor Emg1 in the SSU Processome structures, precluding Bud23 from assembling into this structure [15–17]. Despite these observations, there are multiple lines of evidence indicating that Bud23 joins the SSU assembly pathway during the transition of the SSU Processome to pre-40S. First, Bud23 coimmunoprecipitates with the late-acting SSU Processome factors Dhr1, Utp14, and Utp2 [45,46], and Trm112 copurifies several late SSU Processome factors including Dhr1 and Utp14 [42]. Second, Bud23 and Trm112 sediment at the positions of both 90S and 40S in sucrose density gradients, reflecting their association with the SSU Processome and pre-40S, respectively [40,42,46]. Third, *bud23Δ* cells are defective in A2 site cleavage [45] which releases the pre-40S particle. Finally, the growth and A2 site cleavage defects caused by the absence of Bud23 are suppressed by extragenic mutations in the SSU Processome factors *DHR1*, *UTP14*, *UTP2 (NOP14)*, and *IMP4* [30,45–47] suggesting that Bud23 acts concurrently with these factors. Despite the evidence that Bud23 enters the 40S biogenesis pathway during the transition of the SSU Processome to pre-40S, the specific function for Bud23 has not been described.

Here, we have carried out a comprehensive genetic analysis of extragenic suppressor mutations of *bud23Δ* and identified a genetic and physical interaction network that connects Bud23 to the transition of the SSU Processome to the pre-40S. We found novel extragenic mutations in *IMP4*, *RPS28A*, *UTP2*, *UTP14*, *DHR1*, and *BMS1* that acted as bypass suppressors of

bud23Δ. Recent SSU Processome structures provide the context to rationalize how these bypass suppressors disrupt SSU Processome structure and suggest a role for Bud23 in the SSU Processome transition. Bms1, Imp4, and Utp2 all interact with the 3' basal subdomain and have helical extensions that embrace the U3-18S substrate of Dhr1. We found that many of the mutations destabilized protein-protein or protein-RNA interactions connecting the 3' major domain to the U3-18S heteroduplexes. Finally, mass spectrometric and Northern blot analysis of particles isolated in the absence of Bud23 revealed an enrichment of late SSU Processome factors and rRNA species not yet cleaved at sites A1 and A2. Together, our data imply that Bud23 binding induces the disassembly of SSU Processome factors connecting the 3' basal subdomain to the U3-18S duplexes. We propose that Bud23 promotes the function of Bms1 and Dhr1 to drive rearrangements for compaction of the 3' basal subdomain in the pre-40S subunit.

Results

Extragenic suppressors of *bud23Δ* map to SSU Processome factors and connect Bud23 to the U3 snoRNA. Bud23 methylates G1575 in the 3' basal subdomain of the 18S rRNA [39,40], a region comprised of helices 28, 29, 30 41, 41es10, 42, and 43 (Figs. 1A, 1B, & S2) [17]. The deletion of *BUD23* severely impairs 40S production and cell growth, yet a catalytically inactive Bud23 fully complements *bud23Δ* [39,40], suggesting that 40S assembly requires Bud23 binding but not rRNA methylation. The slow growth defect of *bud23Δ* places strong selective pressure on cells for extragenic bypass suppressors. Our lab previously reported suppressing mutations in *DHR1*, *UTP14*, and *UTP2*, all coding for late acting SSU Processome factors [30,45,46]. We also found mutations in *IMP4* encoding an early SSU Processome factor [47]. These results connected

Bud23 to the late events of the SSU Processome, but they did not allow us to rationalize a mechanism for Bud23 function. The complete SSU Processome harbors nearly 70 ribosomal proteins and biogenesis factors, and we postulated that mapping the mutations to current high-resolution structures of the SSU Processome would help illuminate the function of Bud23. To expand the coverage, we screened for additional spontaneous suppressors by continuously passaging cultures of *bud23Δ* until they arose. We then amplified and sequenced the *IMP4*, *DHR1*, *UTP14*, and *UTP2* loci from these suppressed strains, and identified additional mutations in these genes. Suppressed strains that did not contain mutations in these genes were subjected to whole-genome sequencing and genome variant analysis. This revealed novel suppressing mutations in *RPS28A*, a ribosomal protein that binds the 3' basal subdomain, and *BMS1*, an essential GTPase of the SSU Processome. Mutations in *RPS28A* and *BMS1* were confirmed by Sanger sequencing and verified as suppressors of *bud23Δ* after reintroducing the mutations into *bud23Δ* cells (data not shown).

Portions of Bms1, Imp4, Rps28, Utp2, and Utp14, but not Dhr1, have been resolved in structures of the SSU Processome [15–17]. Remarkably, Bms1, Imp4, Rps28, and Utp2 all interact directly with the 3' basal subdomain containing the Bud23 binding site (Fig. 1C). These factors appear to contribute to the structural stability of the SSU Processome and form multiple protein-protein and protein-RNA contacts (Fig. 1D). Additionally, Bms1, Imp4, and Utp2 each contain extended alpha-helices that penetrate into the core of the SSU Processome where they embrace the U3 Box A and Box A'-18S duplexes (Fig. 1D). Although Dhr1 is not resolved in any of the SSU Processome structures published to date, we previously mapped its binding site on U3 to the 5'-hinge and Box A, immediately upstream of and overlapping the Box A and Box A'-18S duplexes

that we identified as the substrate for Dhr1 unwinding (Fig. S3A) [29]. Only a few segments of Utp14 are resolved in current SSU Processome structures, but Utp14 can be seen binding to pre-rRNA and U3 snoRNA immediately upstream of the duplexes Dhr1 unwinds (Fig. S3A) [15,33]. Intriguingly, Utp14 and the factors positioned at the 3' basal subdomain bookend the U3-18S heteroduplexes. Thus, Imp4, Utp2, and Bms1 provide a physical linkage between the 3' basal subdomain and the U3-18S heteroduplexes that are unwound by Dhr1.

We identified five novel mutations in *BMS1* and one in *RPS28A* as spontaneous suppressors of *bud23Δ* (Fig. 1E & Supplemental File 1). We also found an additional 15 mutations in *DHR1*, 13 mutations in *IMP4*, two mutations in *UTP14*, and one mutation in *UTP2* that were not isolated in our previous studies. Five additional mutations were identified in *UTP2* using error-prone PCR mutagenesis (discussed below). These observations revealed a network of SSU Processome factors that genetically interact with Bud23 and make multiple physical contacts amongst one another (Fig. 1F). Importantly, this interaction network physically connects the 3' basal subdomain with the U3-18S heteroduplex substrates of Dhr1, suggesting a functional linkage between these two sites. Many of the mutations that we report here and previously [30] are in protein-RNA or protein-protein interfaces where they would appear to weaken interactions within the SSU Processome. Because these mutations bypass the absence of Bud23, we propose that Bud23 binding to the 3' basal subdomain induces the release of factors from this region to promote progression of the SSU Processome to a pre-40S particle. In the following sections, we consider how the *bud23Δ* bypass suppressor mutations affect the dynamics of the particle.

The Imp4 and Rps28A mutations mainly cluster around their interfaces with the 3' basal

subdomain. We identified 21 unique mutations in *IMP4* and a single mutation within *RPS28A* that suppressed *bud23Δ* (Fig. 1E). All of these mutations partially restored growth in a *bud23Δ* mutant (Fig. 2A), although the *rps28A-G24D* mutation did not suppress as well as the *imp4* mutations, perhaps because expression of the wild-type paralog *RPS28B* partially masked its suppression phenotype. Imp4 is a component of the heterotrimeric Mpp10-Imp3-Imp4 sub-complex [48–52] which enters the SSU Processome at an early stage of its assembly, during transcription of the 5' ETS [6,7]. The Mpp10 complex may serve as an initial binding platform for several additional SSU Processome factors [53]. Recent structures show that Imp4 is positioned in the core of the SSU Processome where its N-terminal domain (NTD) contacts the U3-18S heteroduplexes while its RNA-binding Brix domain is cradled in the concave RNA fold of the 3' basal subdomain (Fig. 2B) [15–17]. On the other hand, the ribosomal protein Rps28 binds to the opposite, convex surface of the 3' basal subdomain (Fig. 2B), adjacent to but not occluding the Bud23 binding site (Fig. 2B; marked by G1575) [44].

The mutations in Imp4 primarily mapped to two regions of the protein (Fig. 2C). Most of the mutations were in the Brix domain of Imp4 at its interface with the 3' basal subdomain RNA, henceforth “rRNA interaction” mutations (Fig. 2C). These included mutations of residues S93, R94, S101, R116, N118, N121, and R146 which are all expected to form hydrogen bonds with the rRNA [15,16]. These mutations likely weaken the affinity of the protein for the rRNA. The single *Rps28-G24D* mutation maps to its rRNA interface with the 3' basal subdomain. Unlike Imp4, Rps28 is an integral component of the small subunit and remains associated with the mature ribosome. Consequently, it is unlikely that the glycine to aspartate substitution promotes release

of Rps28. More likely, this mutation may increase the flexibility of the RNA to facilitate release of Imp4 (Fig. 2C). Five of the mutations in Imp4, in residues D74, Y77, H156, H159 and H208, mapped to an intramolecular domain interface between the core of the protein and the NTD that interacts with the U3 Box A'-18S duplex, henceforth "NTD interaction" mutations (Fig. 2D). The NTD interaction mutations may alter the flexibility of the NTD, thereby destabilizing its interaction with the U3 Box A'-18S duplex. The observation that *imp4* mutations that suppress *bud23Δ* are predicted to weaken the affinity of Imp4 for the 3' basal subdomain, suggests that Bud23 binding to the 3' basal subdomain leads to disruption of the protein-RNA interactions in this region.

Bud23 is needed for efficient processing at the A2 cleavage site [39]. To ask if the suppressing mutations in *IMP4* and *RPS28A* bypass this rRNA processing defect in *bud23Δ* cells, we prepared total RNA from actively dividing wild-type (WT) cells or *bud23Δ* cells with or without a suppressing mutation in *IMP4* or *RPS28A* and probed for rRNA processing intermediates by Northern blotting (Fig. 2E). As we reported previously [45], *bud23Δ* cells showed a loss of the 27SA2 rRNA intermediate, indicating loss of A2 cleavage, and reduced levels of 18S rRNA compared to WT cells, but no concurrent accumulation of 23S rRNAs. Suppression of *bud23Δ* by the *imp4* and *rps28A* mutants partially restored levels of the 27SA2 rRNA intermediate and 18S rRNA indicating a restoration of cleavage at site A2 and 40S biogenesis. Surprisingly, *bud23Δ* cells also slightly accumulated the 22/21S intermediates (Fig. 2E). 22S represents rRNA cleaved at sites A0 and A3 but not A1 or A2 while 21S represents rRNA cleaved at sites A1 and A3 but not A2. (Fig. S1). Although the A2-A3 probe cannot distinguish between the 22S and 21S intermediates the A0-A1 probe gave a similar hybridization signal indicating that the 22S rRNA accounts for some of this signal (Fig. 2E). Suppression of *bud23Δ* partially alleviated the accumulation of this

species. This was most evident in the strains harboring the *imp4* mutants S93T, R94S, N121I, and H159R. These data indicate that Bud23 affects not only A2 processing, as we previously reported [39], but also cleavage at A1.

As a complementary approach to ask if *bud23Δ* suppressors restored 40S biogenesis, we analyzed ribosomal subunit levels on sucrose density gradients after separating free ribosomal subunits, 80S, and polysomes from wild-type cells and *bud23Δ* cells with or without a suppressing mutation. In wild-type cells, there was an appreciable steady-state level of free 40S and 60S subunits (Fig. 2F). In contrast, in *bud23Δ* cells in which 40S production is limited, the free 40S peak disappeared and the amount of free 60S was dramatically increased, at the expense of 80S (Fig. 2F). The introduction of suppressing mutations in *imp4* or *rps28a* partially restored the levels of 80S and free 40S, similar to the suppression of *bud23Δ* by mutations in *utp14* or *utp2* that we reported previously [45]. Taken together, the Northern blotting and sucrose density gradient data indicate that *imp4* and *rps28A* mutants partially alleviate the 40S biogenesis defects of *bud23Δ* cells.

Utp2 mutants that suppress *bud23Δ* destabilize Utp2 interaction with Imp4. Our lab previously identified *utp2-A2D* as a spontaneous and dominant suppressor of *bud23Δ* that partially restores 40S biogenesis and A2 processing of the primary rRNA transcript [45]. From our screen for additional spontaneous suppressors of *bud23Δ*, we found an additional Utp2 mutation, *utp2-L9S*, that suppressed *bud23Δ* (Fig. 3A). Utp2, also known as Nop14, assembles into the SSU Processome with its binding partners Emg1, Noc4, and Utp14 [54–56], once the 3' minor domain is fully transcribed [6,7]. The association of human Utp2 with human pre-40S

complexes indicates that Utp2 remains on nascent particles during the transition from the SSU Processome to pre-40S [44] suggesting that it has an active role in particle progression.

To gain further insight into the mechanism by which mutations in *UTP2* suppress *bud23Δ*, we performed random PCR mutagenesis of *UTP2* and identified six additional mutations in *UTP2* that suppressed *bud23Δ* to different degrees (Fig. 3B; left panel). In this screen we also reisolated the previously identified *utp2-A2D* mutation. All mutants fully complemented loss of Utp2 (Fig. 3B; right panel). The suppressing mutations all mapped to the N-terminal domain of Utp2; four clustered around the extreme N-terminus and another three clustered around residues 148-151. In an attempt to generate mutants with stronger phenotypes than the individual mutants, we generated the combinatorial mutants *utp2-DPE* containing the mutations A2D, L6P, and K7E, and *utp2-SSH* harboring the mutations L148S, F149S, and L151H. Both of the combinatorial mutants retained the ability to suppress *bud23Δ* and fully complemented loss of *UTP2*, but *utp2-SSH* was a stronger suppressor than *utp2-DPE* (Fig. 3B).

Based on recent partial structures of Utp2 within the SSU Processome [15,16] the globular domain of Utp2 directly contacts the Emg1 heterodimer, Enp1, and Noc4 within a region of the 3' major domain that will make up the beak of the mature SSU, while its extended N- and C-terminal arms pierce deep into the core of the SSU Processome. The C-terminal arm of Utp2 contacts the U3 Box A'-18S duplex while its NTD contacts the Brix domain of Imp4 (Figs. 1D & 3C). Notably, four of the residues (L6, K7, L9 and F58) mutated in our screen were resolved in current structures of the SSU Processome (Fig. 3C). Residues L6, K7, and L9 are within a small helix on the extreme N-terminus of Utp2 that interacts with Imp4, while K7 also appears to contact the phosphate backbone of C1623 of the 3' basal subdomain. Meanwhile, F58 of Utp2 makes an

additional nearby contact between these proteins. These observations prompted us to speculate that the *utp2* suppressors of *bud23Δ* perturb the interaction between Utp2 and Imp4. Previous large-scale yeast-two hybrid (Y2H) studies did not report an interaction between Utp2 and Imp4 [54,55]. However, those studies used Utp2 constructs harboring N-terminal fusions of GAL4 activating or DNA binding domain (AD and BD, respectively). Because the apparent interaction between Utp2 and Imp4 requires the extreme N-terminus of Utp2 (Fig. 3C), such a fusion protein could sterically hinder their interaction. To this end, we cloned a Utp2 Y2H construct harboring an HA-tagged GAL4 activating domain fused to its C-terminus (Utp2-AD-HA), which allowed us to detect an interaction between Utp2-AD-HA and BD-myc-Imp4 (Fig. 3D; left panel). Using this system, we assayed the Utp2-DPE, Utp2-F58S, and Utp2-SSH mutants for their ability to interact with Imp4. All of the mutants showed decreased interaction with Imp4 with the Utp2-F58S and Utp2-SSH mutants being the most severe. All the mutant Utp2 proteins were expressed to similar levels indicating that the reduced interaction was not due to differences in expression or degradation of the mutant proteins (Fig. 3D; right panel). The results from the analysis of Utp2-DPE and Utp2-F58S are consistent with the notion that the mutations in Utp2 that suppress *bud23Δ* disrupt the interaction between Utp2 and Imp4. The result of Utp2-SSH losing interaction with BD-Imp4 suggests that the flexible, unresolved region of Utp2 between R116 and P201 interacts with Imp4, but we cannot rule out the formal possibility that these mutations suppress *bud23Δ* by some other means.

F58 of Utp2 fits into a hydrophobic pocket of Imp4 (Fig. 3E; upper panel). Interestingly, two mutations in *IMP4*, V170F and P252L, that suppressed *bud23Δ* (Figs. 2A & 2B) map to residues that line this pocket (Fig. 3E; lower panel). The positions of these two mutations predict

that they could also disrupt the interaction between Imp4 and Utp2. To test this possibility, we introduced the V170F and P252L mutations into the BD-myc-Imp4 vector and assayed for their interaction with Utp2-AD-HA. Indeed, mutation of either of these residues caused a loss of interaction between Utp2 and Imp4 (Fig. 3F; upper panel), and the loss of interaction could not be explained by reduced protein expression of the mutant Imp4 constructs (Fig. 3F; lower panel). These results, together with the Y2H assays using mutant Utp2 strongly suggest that disrupting the interaction between Imp4 and Utp2 bypasses the 40S assembly defect in the absence of Bud23.

Bms1 mutants that suppress *bud23Δ* are poised to affect the conformational state of Bms1.

We found five *bud23Δ*-suppressing mutations within *BMS1* (Figs. 1F & 4A). Like the mutations in *DHR1*, *UTP14*, *UTP2*, *IMP4*, and *RPS28A* (Fig. 2E) [30,45,46], mutant *bms1* alleles partially alleviated the rRNA processing defects and restored 40S biogenesis (Figs. 4B & 4C) suggesting that these mutations overcome the same biogenesis defect in the absence of Bud23 that the other *bud23Δ* suppressors do. *BMS1* encodes a 136 kDa GTPase that is essential for 40S biogenesis [57,58]. Bms1 forms a subcomplex with Rcl1 [58–61] prior to its entry into the SSU Processome after completion of the 3' minor domain [6,7]. The GTPase activity of Bms1 has been confirmed *in vitro* [59,60], and its ability to bind GTP is essential [61] suggesting that it is a functional GTPase *in vivo*. However, the specific role of Bms1 within the SSU Processome has not been well explored. GTPases often serve as molecular switches that undergo conformational changes (reviewed in [62]). Due to its position in the SSU Processome, it has been suggested that Bms1 helps remodel the SSU Processome core so the endonuclease Utp24 can access and cleave

the A1 site [16]. Thus, this model predicts that Bms1 has a role in the progression of the SSU Processome to the pre-40S.

More than half of Bms1 has been resolved in structures of the SSU Processome and can be divided into five major domains and two small regions that bind its partner Rcl1 and the acetyltransferase Kre33 (Fig. 4D) [15–17]. Domain I contains the GTPase catalytic site and, together with the beta-barrels of domains II and III, forms a globular body. Domain IV protrudes from this globular body to interact with the 3' basal subdomain RNA and contacts the CTDs of Imp4 and Utp2 (Fig. 4E). Finally, the C-terminal domain V begins as an extended strand that lays on domain III before becoming an extended alpha-helix that inserts between the U3 Box A'-18S and U3 Box A-18S heteroduplexes (Fig. 4D). Although the five mutations that suppressed *bud23Δ* map to domains I, II, III and V (Fig. 4D; upper panel), in 3D structure the mutated residues D124, D843, A903, and S1020 lie immediately under the unstructured strand connecting domain IV to the extended C-terminus (Fig. 4F). Thus, four of the five suppressing mutations likely promote the flexibility of this connecting loop. G813 is located in the connector between Domains II and III where it could alter the relative positioning of these two domains and influence how domain III interacts with the unstructured strand of domain V.

Bms1 is structurally related to the translation elongation factor EF-Tu which delivers aminoacyl tRNAs to the ribosome (Fig. S4A) [57]. Comparison of the Bms1 structure from the SSU Processome to the crystal structures of EF-Tu bound to GDP or the non-hydrolysable GTP analog, GTPNP, suggests that Bms1 is in the GTP-bound state in the SSU Processome structures and allows us to speculate how Bms1 functions. The beta-barrels of domains II and III of Bms1 are conserved in EF-Tu (Fig. S4B). In the GTP-bound state, the beta-barrels of EF-Tu are positioned to

accommodate tRNA binding (Fig. S4C) [63]. In GDP-bound EF-Tu the beta-barrels are rotated to promote tRNA release (Figs. S4C & S4D) [64]. Interestingly, the comparison of the EF-Tu and Bms1 structures revealed that the space occupied by tRNA in EF-Tu (Fig. S4E) is occupied by an N-terminal helix of Mpp10 and the unstructured strand of Bms1 that connects domain IV to the extended C-terminus that interacts with U3 (Fig. S4F). This observation suggests that the GTP hydrolysis-induced conformational changes of Bms1 could facilitate undocking of the unstructured strand of Bms1 and Mpp10 from the conserved core of Bms1. Notably, four of the five mutations in *BMS1* that suppressed *bud23Δ* were either within or contact this strand of Bms1 (Fig. S4F). We suggest that these mutations facilitate release of this strand and, perhaps, Mpp10 from Bms1. As this C-terminal domain has been proposed to regulate the timing of Utp24-catalyzed cleavage of the A1 site [16], the functional interaction between Bud23 and Bms1 may explain why *bud23Δ* cells were deficient in A1 site cleavage (Figs. 2E & 4B), however we cannot exclude the possibility that this defect is indirect. Future molecular analysis is needed to better characterize the role of Bms1 in SSU Processome disassembly.

Disruption of Dhr1 and Utp14 interaction suppresses *bud23Δ*. Our lab previously reported 10 mutations in *DHR1* that suppress *bud23Δ* [46]. Here, we isolated an additional 15 suppressing mutations within *DHR1* (Figs. 1F & 5A). Dhr1 is the DEAH-box RNA helicase responsible for unwinding the U3 snoRNA from the SSU Processome [29,34]. The protein harbors a conserved helicase core containing two RecA domains, a Winged-helix (WH) domain, a Helical Bundle (HB) domain, and an OB-fold domain (Fig. 5A). Dhr1 also contains an N-terminal domain (NTD) that interacts with Bud23 [40,46] and a unique C-terminal domain (CTD) that enhances its interaction

with its activator Utp14 [30–32]. Although Dhr1 has yet to be resolved in SSU Processome structures, recent crystal structures of recombinant yeast Dhr1 [31] and its murine homolog DHX37 [32] lacking the NTD allow us to map most of the mutated residues to structure (Fig. 5B). Consistent with our previous report [46], the overwhelming majority of the mutations map to residues on the surface of the RecA1 and RecA2 domains (Fig. 5C). We previously reported that Utp14, the cofactor of Dhr1, binds the RecA1/2 domains [30]. Based on this, we hypothesized that the mutations within the RecA1/2 domains could affect its interaction with Utp14. To this end, we again turned to yeast 2-hybrid analysis between BD-myc-Dhr1 and AD-HA-Utp14. We made three Dhr1 Y2H constructs that combined several mutations within or proximal to the RecA1 domain (E360K, E397D, E402G, D408Y), within the RecA2 domain (H593Y, R596C, E831K), or in both the RecA1 and RecA2 domains (R563M, D566Y, E831K, F837L) and tested them for the ability to interact with Utp14. All three mutants showed a significant loss of interaction with Utp14 (Fig. 5D; left). All three of the mutant proteins expressed similarly (Fig. 5D; right), indicating that the loss-of-interaction was not due to differential expression. Thus, we conclude that mutations within the RecA1 and RecA2 of Dhr1 that bypass *bud23Δ* do so by weakening its interaction with Utp14. Several mutations mapped to the interface between the two RecA domains while glutamate 1037, which is mutated to lysine or glutamine in two of the mutants, coordinates residues important for RNA binding. Although additional *in vitro* analysis is required to understand how these mutations impact Dhr1 function, Dhr1-E1037K,Q likely impairs RNA binding.

Utp14 stimulates the unwinding activity of Dhr1 [30,32,65]. We previously reported five mutations within Utp14 that suppress the growth defect of *bud23Δ* [30]. Notably, these

mutations are within an unresolved region of Utp14 spanning residues 719 to 780 that interacts with Dhr1 (Fig. S3A & S3B), and extensive mutation or deletion of this region reduced Utp14 interaction with Dhr1, Utp14-dependent activation of Dhr1 unwinding activity *in vitro* and phenocopied catalytically null Dhr1 *in vivo* [30]. Here, we report two additional mutations in Utp14, W791L and W794L, that suppressed *bud23Δ* (Fig. S3B). These mutations are slightly downstream of those previously identified and affect two highly conserved tryptophan residues in a motif weakly reminiscent of a G patch, a motif common to activators of DEAH/RHA RNA helicases [30]. Determining how the mutations in Dhr1 and Utp14 bypass loss of Bud23 will require further work. However, the majority of the mutations in Dhr1 and Utp14 are predicted to diminish their interaction and consequentially reduce the activity of Dhr1 (Fig. 5) [30]. Thus, the simplest interpretation is that the bypass of *bud23Δ* by Dhr1 and Utp14 mutants is accomplished by reducing the activity of Dhr1, possibly providing increased opportunity for the helicase to work on a suboptimal substrate (see Discussion).

Depletion of Bud23 partially inhibits SSU Processome progression. The above genetic results suggest that disrupting protein-protein and protein-RNA interactions in the 3' basal subdomain of the SSU Processome can partially bypass the absence of Bud23. We interpret these results to indicate that Bud23 binding leads to disassembly events that promote the remodeling of this region the SSU Processome. To test the idea that Bud23 promotes disassembly events, we characterized pre-ribosomal particles in the absence of Bud23. We introduced a genomically encoded C-terminal Auxin-Induced Degron (AID) tag on Bud23 for rapid depletion of Bud23 upon the addition of the small molecule auxin without the need for shifting carbon sources [66]. The

BUD23-AID strain grew similar to wild-type cells on media lacking auxin, while it showed a growth defect comparable to the *bud23Δ* strain on media containing auxin indicating that the AID tag is functional and does not significantly impact Bud23 function (Fig. 6A). Bud23-AID was significantly depleted after 10 minutes and was undetectable after two hours of auxin treatment (Fig. 6B).

We first asked if Bud23 depletion impacted the association of Imp4 with pre-ribosomes. We depleted Bud23-AID for two hours and separated particles by ultracentrifugation on sucrose density gradients and fractionation. We then isolated the proteins from each fraction and performed western blot analysis for Imp4. In the presence of Bud23, Imp4 sedimented throughout the gradient with enrichment in the 40S to 80S fractions and near the top of the gradient (Fig. 6C). The Imp4 in fractions 2 and 3 likely reflects its association with the Mpp10 sub-complex [48,51,67] whereas the Imp4 in the 80S region reflects its association with the SSU Processome at 90S. We also monitored the sedimentation of the biogenesis factor Enp1 harboring a C-terminal Tandem Affinity Purification (TAP) tag which also sedimented throughout the gradient indicating its binding to both the SSU Processome and pre-40S particles [20]. In the absence of Bud23-AID, both Imp4 and Enp1-TAP showed reduced sedimentation in the 80S region but increased or maintained sedimentation in the 40S region of the gradient. The enrichment of these proteins in fractions 6 and 7 in the absence of Bud23 is reminiscent of the ~45S intermediate that accumulates in catalytically deficient Dhr1 mutants and are thought to represent a partially disassembled SSU Processome [29,30,46]. These results suggest that Imp4 and Enp1 are retained on a partially disassembled SSU Processome in the absence of Bud23, however we cannot exclude the possibility that the altered their sedimentation is due to degradation of unstable SSU Processome particles.

To further understand the nature of the 40S precursors that accumulate in the absence of Bud23, we affinity purified Enp1-TAP particles from WT and Bud23-AID strains after two hours of auxin treatment. Enp1-TAP is an ideal bait for these experiments as it binds to pre-ribosomes before Bud23 binding and is released after Bud23 function [20], and because its sucrose density gradient sedimentation was influenced by Bud23 (Fig. 6C). Following enzymatic elution, the particles associated with Enp1 were sedimented through sucrose cushions to separate them from any extraribosomal Enp1. The associated proteins were then separated by SDS-PAGE and analyzed by Coomassie staining. The depletion of Bud23 led to both the reduction of and accumulation of multiple protein species (Fig. 7A; black lines and blue lines, respectively). Mass spectrometry of these discrete species identified the depleted proteins as the pre-40S factors Tsr1, Rio2, and Nob1, while mostly late-acting, SSU Processome factors comprised the accumulated factors suggesting that the loss of Bud23 precluded their release from pre-ribosomes.

To further characterize the purified particles, we used mass spectrometry to analyze the entire proteomic compositions of the wild-type and Bud23-depleted particles. To approximate stoichiometry for each 40S assembly protein, we calculated its relative spectral abundance factor (RSAF) by dividing the spectral counts for each protein by its molecular weight then normalizing this value to the bait, Enp1 (Fig. S5 & Supplemental File 2). This analysis showed various proteins that were accumulated or depleted from these particles. Interestingly, the proteins that showed genetic interaction with *bud23Δ* remained relatively constant in the absence of Bud23, with the exception of Utp2 which increased in abundance. As Enp1 and Imp4 co-sedimented in the absence of Bud23 (Fig. 6C), we interpret this observation to indicate that these particles are

arrested upstream of the release of these factors where they remain stoichiometric with Enp1. To simplify our analysis, we calculated a log₂-fold change between Bud23-depleted and wild-type particles for each protein and considered a result significant if a protein showed a ± 0.5 -fold change with a difference of at least 10 spectral counts (Fig. S6). This analysis revealed a set of 26 proteins that altered upon Bud23-depletion. These results agreed with what we observed by SDS-PAGE in Fig. 7A in that there was an accumulation of mainly SSU Processome factors and a depletion of mostly pre-40S factors. Notably, Noc4, Utp2, and Rrp12 bind to the 3' major domain [15–17,19] while several factors, such as Bfr2, Enp2, Lcp5, Kre33, and Mrd1 have roles in the final assembly of the SSU Processome [19,68]. Strikingly, the pre-40S factors Slx9 and Rio1 [69–71] also accumulated in the absence of Bud23. These results suggest that without Bud23 the SSU Processome improperly disassembles in that certain regions are progressed to a pre-40S-like state whereas other regions appear arrested.

We also probed for the rRNA intermediates that co-precipitated with Enp1-TAP in the presence and absence of Bud23 (Fig. 7B). We observed that Enp1 decreased association with the 23S rRNA with a concomitant accumulation of both 21S and 22S RNAs in the absence of Bud23. By quantifying the amount of 22S relative to 35S detected with A0-A1 probe vs the amount of 21S/22S relative to 35S detected with the A2-A3 probe we calculate that the 21S and 22S species are approximately equally abundant in the Enp1-TAP sample from Bud23-depleted cells. The accumulation of 21S and 22S indicates that processing at A2 was inhibited upon Bud23 depletion, as we have previously reported [39]. The accumulation of 22S was unexpected as this indicates a defect in A1 cleavage as well. Furthermore, we saw a modest accumulation of the A0-cleaved 5' ETS rRNA (~1.5-fold) and U3 snoRNA (~1.2-fold). Consistent with what is seen in whole cell

extracts of *bud23Δ* cells (Figs. 2E & 4B) [45,46], we also saw that 27SA2 intermediate was present in the input and IP for the wild-type sample, but totally absent in the Bud23-depleted sample (Fig. 7B). These results reconfirm that Bud23 is needed for efficient processing at A2 and support the possibility that Bud23 also impacts cleavage at A1.

Discussion

Bud23 was identified as the methyltransferase that modifies G1575 in 18S rRNA and is thought to act at a relatively late stage of small subunit assembly in the nucleolus [39,42]. Although it is conserved from yeast to humans [40,72–74] and deletion of *BUD23* in yeast leads to severely impaired growth [39], its methyltransferase activity is dispensable for ribosome assembly [39,72]. This indicates that the primary function of Bud23 stems from its binding to SSU precursors, however the role of Bud23 in small subunit assembly is not well understood. Bud23 and its cofactor Trm112 co-sediment with both the pre-40S and the SSU Processome [40,42,45,46]. Consistent with this, Bud23 also co-purifies with late-acting Processome factors and early pre-40S factors [45,46]. Although Bud23 is not a component of the SSU Processome, as highlighted by the fact that its binding site is occupied by the assembly factor Emg1 in the SSU Processome structures [15–17], our work strongly implicates Bud23 in the disassembly of the SSU Processome as it transitions to a pre-40S. The absence of Bud23 impairs A2 site cleavage [39], an event that is tied to the transition. Prior to A2 cleavage, the U3 snoRNA is unwound from the 18S rRNA which is catalyzed by the RNA helicase Dhr1 and its co-factor Utp14 [29,30]. Dhr1 physically interacts with Bud23 [40,46], and we previously reported that mutations in Dhr1, Utp14, or other SSU Processome factors suppress the growth and A2 site cleavage defects in *bud23Δ* cells [30,45–47]. These results indicate that Bud23 enters the maturing SSU assembly pathway as the SSU

Processome transitions to the pre-40S. Here, we provide a significant body of additional evidence to build a case for the role of Bud23 in this transition.

Does Bud23 promote the compaction of SSU structure? The 3' basal subdomain forms a compact RNA unit whose structure remains relatively unchanged between the SSU Processome and Bud23-bound pre-40S, despite its dramatic repositioning during this transition. The majority of mutations that we found as suppressors of *bud23Δ* were in *IMP4* and *DHR1* with additional mutations in *BMS1*, *RPS28A*, *UTP2*, and *UTP14* (Fig. 1D & 1E). Imp4, Rps28, Utp2 and Bms1 all interact with the 3' basal subdomain of 18S rRNA. The Imp4 mutations clustered primarily in its interface with the RNA of the 3' basal subdomain (Fig. 2C), opposite the binding site of Bud23 and are predicted to be disrupting interactions. The Brix domain of Imp4 also contacts the NTD of Utp2 (Fig. 3C), and we showed that suppressing mutations on either side of this interface disrupted their interaction (Figs. 3D & 3F). Likewise, the mutations in the GTPase Bms1 are expected to affect intramolecular interactions in a manner that promotes a conformational change within Bms1 (Fig. 4F). As Bms1 simultaneously binds to the 5' domain and the 3' basal subdomain, a conformational change could induce large-scale repositioning of RNA elements within the SSU Processome. The picture that emerges is that the *bud23Δ* suppressing mutations define a network of protein-protein and protein-RNA contacts that stabilize the 3' basal subdomain within the SSU Processome structure. Disrupting this interaction network bypasses the requirement for Bud23, suggesting that the function of Bud23 is to destabilize these interactions to promote progression to the pre-40S. Consistent with this idea, pre-ribosomal particles accumulated late-acting SSU Processome factors in the absence of Bud23 (Figs. 7A &

S6). Thus, we propose that Bud23 binding to the 3' basal subdomain promotes large-scale rearrangements as the open structure of the SSU Processome collapses into the more compact structure of the pre-40S (Fig. 8A).

Is Dhr1 unwinding of the U3 snoRNA coordinated with the GTPase activity of Bms1? The network of genetic and physical interactions that we define in this work functionally link the RNA helicase Dhr1 and the GTPase Bms1. These two enzymes are thought to drive structural rearrangements critical to the transition of the SSU Processome to a pre-40S particle. Dhr1 catalyzes U3 snoRNA removal [29] while Bms1 is proposed to remodel structure to promote A1 site cleavage [16]. In addition to the functional linkage between Dhr1 and Bms1, the 3' basal subdomain connects directly to the U3-rRNA duplexes that Dhr1 unwinds via long alpha helical extensions of Imp4, Utp2, and Bms1, suggesting that the events at the 3' basal subdomain are coordinated with Dhr1 unwinding U3. The linkage of Dhr1 and Bms1 in the disassembly of the SSU Processome is reminiscent of the relationship between the DExH RNA helicase Brr2 and the EF-G-like GTPase Snu114 that regulate Spliceosome disassembly [75]. In the case of the spliceosome, it is believed that nucleotide-dependent conformational changes in Snu114 activate Brr2, mediated through the C-terminus of Snu114. It is possible that the actions of Dhr1 and Bms1 are similarly coordinated. Bms1 has a globular GTPase domain composed of domains I-III that binds helices 15-18 of the 5'-domain of 18S rRNA while domain IV binds to the 3' basal subdomain [15,16]. A long C-terminal helix of Bms1 extends into the SSU Processome core where it wedges between the U3 Boxes A and A' heteroduplexes with 18S rRNA (Figs. 1D & S3A), the substrates of Dhr1. In this way, Bms1 and Dhr1 can be mechanistically linked. We previously mapped the

binding site of Dhr1 to U3 snoRNA immediately downstream of the Boxes A and A' heteroduplexes [29]. As Dhr1 is a DEAH-box helicase it likely translocates in a 3' to 5' manner [32] and would be poised to disrupt the U3 heteroduplexes. However, the helical extension of Bms1 with those of Utp2 and Imp4 could act as obstructions to control Dhr1 translocation.

What is the role of Bud23 in SSU Processome disassembly? In the mature SSU Processome, Emg1 occupies the Bud23 binding site [15,16]. Thus, the release of Emg1 and possibly additional disassembly events must precede Bud23 recruitment. Consequently, we envision that Bud23 does not act directly on the intact SSU Processome but on a later, intermediate particle during the transition to pre-40S. The genetic linkage of Bud23 to Bms1 and Dhr1 as well as the physical linkage formed by Bms1 between the U3-18S heteroduplexes, that Dhr1 unwinds, and the 3' basal subdomain, where Bud23 binds, suggest that Bud23 may coordinate their activities. We propose a mechanism in which Bud23 binding facilitates SSU Processome progression. After A0 site cleavage but before A1 and A2 site processing Dhr1 binds to the SSU Processome [17,29,31,33–35], and Utp14 positions Dhr1 ahead of the U3-18S heteroduplexes where it is poised for unwinding [30,33]. However, the helical extensions of Bms1, Imp4, and Utp2 embrace and stabilize the U3-18S heteroduplexes thereby inhibiting Dhr1 unwinding. (Fig. 8B). After a partial disassembly of the SSU Processome, including the release of Emg1 from the 3' basal subdomain, Bud23 binding signals the GTPase activity of Bms1 to drive structural rearrangements to displace the helical extensions allowing Dhr1 to progress through its substrate.

How do the Dhr1 and Utp14 mutants bypass *bud23Δ*?

The activity of Dhr1 is stimulated by interaction with Utp14 both *in vivo* and *in vitro* [30,32,65]. This interaction is mediated by a short loop within Utp14, spanning residues 719-780 in the yeast protein [30], that is conserved from yeast to mammals [30,32,65]. Y2H and *in vitro* experiments show that mutation of this loop reduces the interaction between Utp14 and Dhr1 and decreases stimulation of Dhr1 activity [30,32]. Consistent with the *in vitro* results, mutation of this loop of Utp14 phenocopies a catalytically defective Dhr1 mutant *in vivo* [30]. Single point mutations in this loop were identified as suppressors of *bud23Δ*, suggesting that weakening the interaction between Utp14 and Dhr1 and likewise reducing the activation of Dhr1 by Utp14 suppresses *bud23Δ*. Similarly, the majority of the mutations in Dhr1 that suppress *bud23Δ* lay across the surface of the RecA domains (Fig. 5C) and combining multiple such mutations leads to reduced interaction with Utp14 (Fig. 5D). The ATPase activity of Dhr1 also depends on its interaction with RNA [29,30,65], and other *bud23Δ*-suppressing mutations, such as *dhr1-E1037K,Q*, affect residues that appear important for RNA binding. As with the Utp14 mutations, it appears that reducing Dhr1 activity leads to bypass of *bud23Δ*. We suggest that in the absence of Bud23, the structural context in which Dhr1 unwinds the U3-18S heteroduplexes is altered and wild-type Dhr1 activity promotes discard of a suboptimal substrate. Dhr1 unwinds U3 from the 18S to allows folding of the central pseudoknot [29,65]. If Bud23 promotes rearrangements within the transitioning processome that are necessary for folding of the CPK, then in the absence of Bud23, Dhr1 unwinding may be largely unproductive, acting before substrates are positioned for efficient folding of the central pseudoknot. Mutations in Dhr1, or in Utp14, that reduce its activity, may afford more time for rearrangements within the transitioning SSU Processome to allow productive unwinding by Dhr1.

In this model, mutations in factors binding the 3' basal subdomain (Imp4, Bms1, and Utp2) may help overcome the requirement of Bud23 to afford a more favorable substrate for central pseudoknot folding. Similar models of kinetic proofreading have been developed for RNA helicases involved in splicesosomal rearrangements [76] where, for example, mutations in Prp28 that reduce its ATPase activity enhance the splicing efficiency of a suboptimal substrate [77].

Materials and methods

Strains, growth media, genetic methods, and yeast two-hybrid (Y2H) analysis. All *S. cerevisiae* strains and sources are listed in Table 1. AJY2676 was generated by genomic integration of *EcoRI*-digested pAJ4339 [78] into AJY2161 to replace the *KanMX* marker with *CloNAT*. AJY3156 was generated by genomic integration of *bud23Δ::KanMX* into AJY2665. AJY4175 was generated by transforming pAJ4094 into a *UTP2/utp2Δ::KanMX* heterozygous diploid strain [79], sporulated, and dissected. AJY4395 was generated by genomic integration of *AID-HA::OsTIR1::LEU2* amplified from pJW1662 [80] into the *BUD23* locus of AJY2665. All yeast strains were cultured at 30°C in either YPD (2% peptone, 1% yeast extract, 2% dextrose), YPgal (2% peptone, 1% yeast extract, 1% galactose), or synthetic dropout (SD) medium containing 2% dextrose unless otherwise noted. When appropriate, media were supplemented with 150 to 250 μg/ml G418 or 100 μg/ml nourseothricin. All plasmids and sources are listed in Table 2. Y2H analysis was performed as previously described [33].

Identification of additional spontaneous suppressors of *bud23Δ*. AJY2676 cells were inoculated into 200 μL of YPD media in a 48-well format plate. Cells were cultured with

continuous shaking until saturation then diluted into fresh media. After each passage, cells were plated onto YPD plates to test for the presence of suppressors. This process was iterated for each culture until suppressors were observed. Single colonies of each suppressor strain were obtained and genomic DNA was prepped using MasturePure™ Yeast DNA Purification Kit (Lucigen). The *DHR1*, *IMP4*, *UTP2*, and *UTP14* loci were amplified and sequenced by Sanger to identify mutations in known suppressors [30,45–47].

Libraries for the six strains that did not carry suppressors in *DHR1*, *IMP4*, *UTP2* or *UTP14* were prepared and sequenced on an Illumina NextSeq 500 platform by the Genomic Sequencing and Analysis Facility at the University of Texas at Austin. The quality of the resultant reads was assessed using FastQC (v0.10.1) [<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>], and subsequently processed using TrimGalore (v1.14) [http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/] to discard low-quality sequences and adapters. The processed reads were aligned using Bowtie2 (v2.3.4) [81] using the default settings for paired-end reads. The resultant files were further processed with SAMtools (v0.1.18) [82] and BCFtools (v0.1.17) [83] to generate variant call format files. VCFtools (v0.1.16) [84] was used to filter out variants with low quality scores (Quality value < 100) and to compare samples pairwise to identify mutations unique to each suppressed strain. This analysis revealed single point mutations within *BMS1* and *RPS28A* that were confirmed by Sanger sequencing. The *bms1* and *rps28a* variants were subsequently cloned into centromeric vectors and, as with all other *bud23Δ* suppressors that we have identified, the *rps28a* and *bms1* mutants were dominant (data not shown). All mutant strains isolated in this screen are listed in Table 1.

Identification of mutations in *UTP2* that suppress *bud23Δ*. Random mutations in *UTP2*

were generated by error-prone PCR using *Taq* polymerase and pAJ2595 as the template and oligos that hybridize to the upstream and downstream sequences of *UTP2*. The restriction enzymes *EcoRI* and *SphI* were used to linearize the vector pAJ2595 and the linearized vector was, co-transformed with the mutant amplicon into AJY2161, and plated onto SD-Uracil media to allow recombination of the mutant amplicon into the pAJ2595 backbone. Colonies displaying a suppressed phenotype were isolated; vectors were rescued from yeast and sequenced after confirming that the vectors conferred suppression.

Affinity purification. Cells were cultured as described in the Northern blotting and mass

spectrometry subsections below. All steps were carried out on ice or at 4°C. Cells were washed with Lysis Buffer (50 mM Tris-HCl pH 7.6 (25°C), 100 mM KCl, 5 mM MgCl₂, 5 mM beta-mercaptoethanol (βME), 1 mM each of PMSF and Benzamidine, and 1 μM each of leupeptin and pepstatin) supplemented with EDTA-free Pierce Protease Inhibitor Mini Tablet cocktail (Thermo Scientific), then resuspended in 1 volume Lysis Buffer. Extracts were generated by glass bead lysis and clarified at 18,000g for 15 minutes. Clarified extracts were normalized according to A₂₆₀ and supplemented with 0.1% TritonX-100. Normalized extracts were incubated for 1.5 hours with rabbit IgG (Sigma) coupled to Dynabeads (Invitrogen), prepared as previously described [85]. Following binding, the beads were washed thrice with Wash Buffer (Lysis Buffer supplemented with 0.1% TritonX-100). The beads were resuspended in Elution Buffer (Wash Buffer supplemented with TEV protease and Murine RNase Inhibitor (New England Biolabs)) and the

bound Enp1-TAP containing complexes were eluted for 1.5 – 2 hours. The resultant eluates were handled as described in the Northern blotting and mass spectrometry subsections below.

Northern blot analysis. For analysis of rRNA processing in whole cell extract (WCE), strains were cultured overnight in YPD media to saturation. Cell cultures were diluted into YPD at a starting OD₆₀₀ of 0.1 and cultured to mid-exponential phase (OD₆₀₀ ~0.4-0.5) before collection and storage at -80°C prior to lysis. For analysis of affinity purified RNAs, strains AJY2665 and AJY4395 were cultured overnight in YPD media to saturation. Cells were diluted into YPD at a starting OD₆₀₀ of 0.05 and cultured for three hours. Cultures were treated with 0.5 mM auxin for 2 hours at 30°C, centrifuged, and frozen in liquid nitrogen. Affinity purification was performed as described above. Affinity purified and WCE RNAs were isolated using the acid-phenol-chloroform method as previously described (Zhu et al. 2016). RNAs were electrophoresed through 1.2%-agarose MOPS 6% formaldehyde gel. Northern blotting was performed as previously described (Li et al. 2009) using the oligo probes listed in the legends of Figures 2 and 7, and signal was detected by phosphoimaging on a GE Typhoon FLA9500.

Mass spectrometry and analysis. Strains AJY2665 and AJY4395 were cultured as described in the Northern blot analysis subsection. Affinity purifications were performed as described above. To isolate factors associated with only pre-ribosomal particles, the eluate was overlaid onto a sucrose cushion (15% D-sucrose, 50 mM Tris-HCl pH 7.6 (25°C), 100 mM KCl, 5 mM MgCl₂) then centrifuged at 70,000 rpm for 15 min in a Beckman Coulter TLA100 rotor. Following, the pellets were precipitated with 15% trichloroacetic acid (TCA), washed with acetone and dried,

and resuspended in 1X Laemmli buffer. Approximately equivalent amounts of protein were either fully separated on SDS-PAGE gels for excision of individual species or electrophoresed slightly into a NuPAGE Novex 4%–12% Bis-Tris gel for analysis of the entire affinity purification. Peptides were recovered from in-gel Trypsin digestion and prepared for identification by mass spectrometry as previously described [33]. The resultant peptides were identified at The University of Texas at Austin Proteomics Facility by LC-MS/MS on a Thermo Orbitrap Fusion 1 with either a 30 minute or 1 hour run time for identification of single species or complex sample, respectively. Mass spectrometry data were processed in Scaffold v4.8.3 (Proteome Software, Inc.). A protein threshold of 99% minimum with two peptides minimum and peptide threshold of 1% false discovery rate was applied. The data were exported, and custom Python 2.7 scripts were used to calculate the relative spectral abundance factor (RSAF) for each protein by dividing the total number of spectral counts by the molecular weight. Values for each protein were normalized to the bait, Enp1, to reflect relative stoichiometry. Supplemental File 2 contains relevant spectral counts and processed data from the mass spectrometry experiments.

Sucrose density gradient analysis. For polysome profile analysis of the suppressors of *bud23Δ*, BY4741, AJY2676, AJY3744, AJY4529, AJY4531, and AJY4535 were cultured overnight in YPD to saturation. Cultures were diluted into YPD at a starting OD₆₀₀ of 0.02 and cultured to early exponential phase (OD₆₀₀~0.10-0.13) and then treated with cycloheximide (CHX) at 100 µg/ml for 10 minutes at 30°C to inhibit translation. After centrifugation cells were frozen in liquid nitrogen and stored at -80°C. Cells were washed and resuspended in Lysis Buffer (50 mM Tris-HCl pH 7.6 (25°C), 100 mM KCl, 5 mM MgCl₂, 7 mM βME, 100 µg/mL CHX, 1 mM each of PMSF and

Benzamidine, and 1 μ M each of leupeptin and pepstatin). Extracts were prepared by glass bead lysis and clarified by centrifugation for 15 minutes at 18,000*g* at 4°C. 4.5 A₂₆₀ units of clarified extract were loaded onto 7-47% sucrose gradients made in the same buffer lacking protease inhibitors. Gradients were subjected to ultracentrifugation for 2.5 hours at 40,000 rpm in a Beckman SW40 rotor. The gradients were subjected to continuous monitoring at 254 nm using an ISCO Model 640 fractionator.

For analysis of the sedimentation of factors in the absence of Bud23, AJY2665 and AJY4395 were cultured overnight to saturation. Cells were diluted into YPD at a starting OD₆₀₀ of 0.05 and cultured for three hours (OD₆₀₀ $\approx$ 0.08-0.1). Cultures were treated with 0.5 mM auxin for 2 hours at 30°C, then treated with CHX at 100 μ g/mL for 10 minutes at 30°C. Cells were harvested and stored as described above. Cells were washed and resuspended in Lysis Buffer supplemented with an EDTA-free Pierce Protease Inhibitor Mini Tablet cocktail (Thermo Scientific). Extracts were generated, and nine A₂₆₀ units were loaded onto sucrose gradients and subject to ultracentrifugation as described above. Gradients were fractionated into 600 μ L fractions with continuous monitoring at 254 nm using an ISCO Model 640 fractionator. Each fraction was split in half to collect proteins and RNAs. Proteins were precipitated using 15% TCA as described previously [33]. One-fifth of protein from each half fraction was separated on SDS-PAGE gels and subjected to Western blotting. RNAs were precipitated as described previously [86] supplemented with 10 μ g of glycogen. One-third of RNA from each half fraction was subjected to Northern blotting as described above.

Western blot analysis. Primary antibodies used in this study were anti-c-Myc monoclonal 9e10 (Biolegend), anti-HA (Biolegend), anti-Bud23 (C. Wang), anti-Rps24 (our laboratory), anti-Glucose-6-phosphate dehydrogenase (Sigma ImmunoChemicals), and anti-Imp4 (S. Baserga). Secondary anti-bodies were goat anti-mouse antibody-IRDye 800CW (Li-Cor Biosciences), goat anti-rabbit antibody-IRDye 680RD (Li-Cor Biosciences), and goat anti-rabbit antibody-HRP (Jackson ImmunoResearch Laboratories). The blots in Figs. 3D, 3F, 5C, and 6B were imaged with an Odyssey CLx infrared imaging system (Li-Cor Biosciences) using Image Studio (Li-Cor Biosciences). The blots in Fig. 6C were imaged using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) and exposed to film.

Molecular visualization. All images of SSU Processome and Dhr1 structures are from PDB ascension codes 5WLC and 6H57, respectively. The structures of GDPNP- and GDP-bound EF-Tu are from PDB ascension codes 1B23 and 1EFC, respectively. Molecular visualizations were generated using MacPyMOL: PyMOL v1.8.2.1 Enhanced for Mac OS X (Schrödinger LLC).

Figure legends

Figure 1. Extragenic suppressors of *bud23Δ* reveal an interaction network that connects the 3' basal subdomain to the U3-18S heteroduplexes. (A) A secondary structure map of the 18S rRNA that indicates the position of the 3' basal subdomain (dark gray) and the central pseudoknot (CPK; deep olive). (B) The position of the 3' basal subdomain (dark gray) within the context of the SSU Processome structure (light gray). (C) Factors harboring mutations that suppress *bud23Δ* and are resolved in the SSU Processome (PDB 5WLC) cluster around the 3' basal subdomain and the U3-

Box A'-18S heteroduplex that Dhr1 unwinds. Shown are: Bms1 (forest green), Imp4 (blue), Rps28 (cyan), Utp2 (orange), Utp14 (brown), U3 (deep purple), 18S rRNA (deep olive), 3' basal subdomain (dark gray). (D) Zoomed view of factors in C showing contacts amongst each other, the 3' basal subdomain, and the U3-Box A'-18S heteroduplex. N- and C-terminal domains, NTD and CTD, respectively. The U3-18S heteroduplexes are shown as U3 Box A and U3 Box A'. Guanosine 1575 (G1575, red) is shown as a marker for the binding site of Bud23. (E) Tabulation of the number of unique mutations found in each extragenic suppressor of *bud23Δ*. Newly identified mutations (novel) and previously identified (known) [30,45–47]. The complete list of these mutations is available in Supplemental File 1. (F) Summary of the genetic and physical interactions amongst the suppressors of *bud23Δ*. Factors are indicated as nodes; genetic and physical interactions are shown as dashed and solid edges, respectively.

Figure 2. The Imp4 and Rps28A mutations primarily map to their interfaces with the 3' basal subdomain. (A) Point mutations within *imp4* and *rps28a* suppressed the growth defect of *bud23Δ* as shown by 10-fold serial dilutions of wild-type cells (BY4741), *bud23Δ* (AJY2676), and *bud23Δ*-suppressed cells spotted on YPD media and grown for two days at 30°C. (B) Rps28 and the Brix domain of Imp4 interact with the 3' basal subdomain RNA, while the NTD of Imp4 makes contacts with its Brix domain and the U3-18S heteroduplexes. G1575, the binding site of Bud23, is shown for reference. The regions where the mutated residues map are indicated by magenta and green dashed boxes for the rRNA interaction and the NTD interaction, respectively. Factors are colored the same as in Fig. 1. (C) Residues mutated in Rps28 and Imp4 Brix domain map to interaction interfaces with the 3' basal subdomain RNA (magenta sticks). (D) Several residues mutated in

Imp4 map to an intramolecular interaction between the Brix and NTD of Imp4 (green sticks). (E) Suppressing mutations in *imp4* and *rps28a* partially restored A2 processing and 18S rRNA levels in *bud23Δ* cells. RNA processing intermediates were detected by Northern blotting on RNAs extracted from wild-type (WT), *bud23Δ*, and *bud23Δ*-suppressed cells cultured to exponential phase at 30°C in liquid YPD. P32-radiolabeled probes (Table 3) hybridized to the indicated regions. The 25S and 18S rRNAs were detected by methylene blue staining of the RNAs prior to oligonucleotide hybridization. (F) The *imp4* and *rps28a* mutations partially restored 40S biogenesis as shown by polysome profiles after separation of extracts on sucrose density gradients from wild-type, *bud23Δ*, and *bud23Δ*-suppressed cells cultured to exponential phase at 30°C in liquid YPD media.

Figure 3. The mutations in Utp2 disrupted its interaction with Imp4. (A) Spontaneous point mutations within *utp2* partially suppressed the growth defect of *bud23Δ* as shown by 10-fold serial dilutions of wild-type cells (BY4741), *bud23Δ* (AJY2676), and *bud23Δ*-suppressed cells spotted on YPD media and grown for two days at 30°C. (B) Additional point mutations in *UTP2*, generated by error-prone PCR, also suppressed the growth defect of *bud23Δ* (left) and complemented loss of *UTP2* (right) as shown by 10-fold serial dilutions of *bud23Δ* (AJY2676) and *PGAL10-UTP2* (AJY4175) cells containing either empty vector, or vectors encoding the indicated alleles of *UTP2* spotted on SD-Leu media containing glucose and grown for two days at 30°C. (C) Several of the mutations in Utp2 map to residues (green sticks) located within its NTD (orange) that interacts with the Brix domain of Imp4 (blue) adjacent to the 3' basal subdomain RNA (gray). (D) Left: Yeast two-hybrid interaction assay between Imp4 and wild-type (WT) or mutant Utp2.

Strains carrying the indicated constructs were patched onto Leu- Trp- (L-W-) and Leu-Trip-His- (L-W-H-) media supplemented with 2 mM 3-Amino-1,2,4-triazole (3AT) (AD, Gal4 activation domain; BD, Gal4 DNA binding domain). Right: Western blot analysis of the wild-type and mutant Utp2-AD-HA proteins using equivalent amounts of total protein extracts. Glucose-6-phosphate dehydrogenase (G6PDH) was used as a loading control. (E) Top: F58 of Utp2 (green sticks) fits into a hydrophobic pocket in the Brix domain of Imp4 (surface representation). Bottom: The *bud23Δ*-suppressing mutations V170F and P252L of Imp4 (magenta sticks) line this pocket. Imp4 and Utp2 are colored blue and orange, respectively. (F) Top: Yeast two-hybrid interaction assay between Utp2 and wild-type or mutant Imp4. Strains carrying the indicated constructs were patched onto L-W- and L-W-H- media supplemented with 6 mM 3AT. Bottom: Western blot analysis of the wild-type and mutant BD-myc-Imp4 proteins in equivalent amounts of total protein extract is shown. G6PDH was used as a loading control.

Figure 4. The mutations in the GTPase Bms1 that suppress *bud23Δ* are poised to modulate its conformational state. (A) Spontaneous point mutations within *BMS1* suppressed the growth defect of *bud23Δ* as shown by 10-fold serial dilutions of wild-type cells (BY4741), *bud23Δ* (AJY2676), and *bud23Δ* cells carrying the indicated *bms1* mutations spotted on YPD media and grown for two days at 30°C. (B) The *bms1* mutations partially restored A2 processing and 18S rRNA production in *bud23Δ* cells as shown by Northern blotting of RNAs extracted from wild-type, *bud23Δ*, and *bud23Δ*-suppressed cells as described in Fig. 2E. (C) The *bms1* mutations partially restored 40S biogenesis as shown by the analysis of the polysome profiles from the indicated strains as described in Fig. 2F. (D) Top: Primary structure of Bms1 with domains (in

different shades of green), interacting regions and *bud23Δ* suppressing mutations annotated; regions not resolved in SSU Processome structures are indicated in light gray. Bottom: The partial structure of Bms1 (from PDB 5WLC) in the context of the SSU Processome is shown. Domains IV and V extend from its GTPase core (domains I – III) to contact the RNAs of the 3' basal subdomain (gray) and the U3-18S heteroduplexes (pink/gold), respectively. (E) At the 3' basal subdomain, Domain IV of Bms1 also contacts the CTDs of Utp2 (orange) and Imp4 (blue). (F) The mutated residues D124, D843, A903, and S1020 in Bms1 map to inter-domain contacts with the unstructured strand of domain V that connects it to domain IV.

Figure 5. Most of the Dhr1 mutations map to surface residues of its RecA domains. (A) A cartoon of the primary structure of Dhr1 is shown. The domains of Dhr1 are annotated by color: NTD, N-terminal domain (light gray); RecA1/2, Recombination protein A1/2 (blue/green); WH, winged-helix (yellow); HB, helical bundle (orange); OB, oligonucleotide-binding fold (brown); CTD, C-terminal domain (light red). Unstructured regions are colored as light gray. Mutations reported here (novel) and previously (known) [46] are indicated as black and magenta, respectively. Numbering indicates residue numbering of yeast Dhr1. (B) The structure of yeast Dhr1 (PDB 6H57) with relevant features colored as described in panel A. Catalytic residues involved in ATP hydrolysis are denoted as red sticks for reference. (C) The majority of the mutated residues map to the surfaces of the RecA domains. Mutated residues are shown as black and magenta sticks as described for panel A. The positions of the residues that were used to test loss-of-interaction with Utp14 in panel D are indicated. (D) Top: Yeast two-hybrid interaction data between AD-HA-Utp14 and wild-type (WT) or mutant BD-myc-Dhr1 are shown. Strains carrying the indicated

constructs were patched onto Leu- Trp- (L-W-) and Leu-Trip-His- (L-W-H-) media supplemented with 10 mM 3-Amino-1,2,4-triazole (3AT) (AD-HA, GAL4AD-HA; BD-myc, GAL4BD-myc). Bottom: Western blot analysis of the wild-type and mutant BD-myc-Dhr1 proteins using equivalent amounts of total protein extracts is shown. Glucose-6-phosphate dehydrogenase (G6PDH) was used as a loading control.

Figure 6. Imp4 and Enp1 accumulate with pre-40S upon Bud23 depletion. (A) The genomic fusion of an auxin-inducible degron (AID) to the C-terminus of Bud23 rendered cells sensitive to auxin, with a growth defect comparable to *bud23Δ*. 10-fold serial dilutions of wild-type (AJY2665), *BUD23-AID* (AJY4395), and *bud23Δ* (AJY3156) cells were spotted on YPD media with and without 0.5 mM auxin and grown for two days at 30°C. (B) Western blot of time-course of depletion of Bud23-AID, using equivalent amounts of total protein extract from AJY2665 or AJY4395 cells cultured to exponential phase then harvested prior to or after the addition of 0.5 mM auxin for the indicated time (WT; wild-type). G6PDH was used as a loading control. (C) The sucrose density gradient sedimentation of Enp1-TAP, Imp4, and Rps24 in the presence (upper panel) or absence (lower panel) of Bud23. Extracts were prepared from + Bud23 (AJY2665) and - Bud23 (AJY4395) cells treated with 0.5 mM auxin for two hours and separated on sucrose density gradients prior to fractionation. Proteins from each fraction were precipitated and subjected to Western blot analysis.

Figure 7. Composition of 40S precursors purified in the absence of Bud23. (A) Coomassie-stained gel of proteins that co-purified with Enp1-TAP in the presence (+) or absence (-) of Bud23.

Pre-ribosomal particles were enriched by overlaying eluate onto sucrose cushions followed by ultracentrifugation. Individual species that showed clear enrichment or depletion were excised and identified by mass spectrometry and are indicated in blue or black text, respectively. The asterisks (*) denote proteins that also appeared in the analysis described in Fig. S6. (B) The rRNA processing intermediates and U3 snoRNA that co-purified with Enp1-TAP in the presence (+) or absence (-) of Bud23 were detected by Northern blotting using the indicated probes. Oligonucleotides are listed in Table 3.

Figure 8. Models for Bud23 function in SSU Processome progression. (A) The Bud23-Trm112 subcomplex (light yellow and light blue, respectively) enters a partially disassembled SSU Processome to establish a state competent for productive unwinding of U3 snoRNA (gray) by Dhr1, to allow folding of the central pseudoknot and compaction of the 5' (blue), central (yellow), 3' major (magenta), and 3' minor (green) domains onto one another to yield an early pre-40S. Only the rRNA, U3 snoRNA, and Bud23-Trm112 subcomplex are shown for simplicity. The U3-18S rRNA heteroduplexes that Dhr1 unwinds are indicated by a dashed box. The rRNAs of the SSU Processome (left) are a composite of two structures (PDB: 5WLC, 5WYK), and the Bud23-Trm112 subcomplex and the rRNA of the pre-40S (right) are from an early human pre-40S (PDB: 6G4W). The middle image is an overlay of the left and right images with the partially disassembled SSU Processome grayed out. (B) A cartoon 2-dimensional projection of Fig. 1D showing factors that surround the 3' basal subdomain (gray) and U3-18S heteroduplexes (magenta/gold). During assembly of the SSU Processome, multiple contacts are formed amongst Bms1 (green), Imp4 (blue), and Utp2 (orange). These three proteins contain long helical extensions that support the

U3-18S heteroduplexes but block their access by Dhr1 (light blue). Bms1, Imp4, and Utp2 all have extensive interactions with the 3' basal subdomain rRNA that connect events here to the U3-18S heteroduplexes. After partial SSU Processome disassembly, Bud23 (yellow) binds to the 3' basal subdomain and signals to Bms1 to activate its GTPase domain. This, in turn, leads to conformational changes that displace the helical extensions of these proteins away from the U3-18S heteroduplexes allowing Dhr1 to gain access so that it can unwind it.

Supplemental figure legends

Figure S1. Schematic of rRNA processing relevant to 40S production in *S. cerevisiae*.

Endonucleolytic processing of the pre-18S rRNA at sites A0, A1, and A2 (or A3) occurs within the context of the SSU Processome. Events occurring co- or post-transcriptionally are denoted by blue and red scissors, respectively. Cleavage at either the A2 or A3 sites liberates the SSU precursors from the LSU precursors containing the 27SA2 and 27SA3 rRNA intermediates, respectively. In rapidly dividing cells processing of A0, A1, and A2 appear to occur co-transcriptionally in a sequential order to produce the 20S rRNA intermediate. The RNA Exosome exonucleolytically degrades the A0- and A1-cleaved 5' ETS fragments. When A1 or A2 cleavage is delayed, post-transcriptional cleavage at A3 occurs to produce the 22S or 21S rRNA, and A1 and A2 cleavage instead occur post-transcriptionally to generate the 20S rRNA. When SSU Processome function is entirely precluded, cleavage at A3 produces the 23S rRNA that becomes degraded. The 20S rRNA is a component of pre-40S intermediates that are exported to the cytoplasm where an endonuclease processes the D site to yield the 18S rRNA.

Figure S2. Secondary structure diagram of the 18S rRNA. The 18S rRNA is divided into four main domains: the 5' domain (blue), central domain (gold), 3' major domain (purple and black), and the 3' minor domain (green). The 3' basal subdomain (black) is a sub-region of the 3' major domain that forms during the assembly of the SSU Processome [17], and contains the binding site for Bud23. The base methylated by Bud23, guanosine 1575 (G1575, red) is indicated. The position of the Central Pseudoknot (CPK, gray) is also pictured.

Figure S3. The position of Utp14 and the binding site of Dhr1 within the SSU Processome. (A) The location of the resolved segments of Utp14 (brown) in the SSU Processome. A contour line indicates the unresolved region of Utp14 where the Dhr1-interaction surface and *bud23Δ*-suppressing mutations are located. The U3 snoRNA binding site of Dhr1 and U3 mutations that suppress a cold-sensitive Dhr1 mutant [29] are indicated by cyan and black sticks, respectively. Bms1, Imp4, Rps28, Utp2, and the 3' basal subdomain RNA are shown for reference. (B) A cartoon of Utp14 primary structure indicating the position of its resolved portions and the *bud23Δ*-suppressing mutations reported here (black) and previously (light blue) within its Dhr1-activation loop [30].

Figure S4. Comparison of the structure of Bms1 to the conformational states of EF-Tu. (A) Structural alignment of EF-Tu bound to the non-hydrolysable GTP analog, GDPNP (slate blue, PDB 1B23) to domain I of Bms1 (from PDB 5WLC) is shown. Bms1 is colored by domains as in Fig. 4D; the GTP analog and magnesium ion bound to EF-Tu are shown as orange sticks and green sphere. Structures are shown individually (left, middle) and as an overlay (right). (B) A view of domains II

and III of Bms1 compared to those of EF-Tu shows that the two domains adopt beta barrels in similar conformations. (C) GDPNP-bound EF-Tu forms a complex with tRNA, while GDP-bound EF-Tu (deep purple, PDB 1EFC) does not. (D) Conformational differences in the beta-barrel domains of GDP and GTP-bound EF-Tu suggest that these domains rotate away from one another upon GTP hydrolysis to promote tRNA release. (E) The amino-acyl tRNA contacts GDPNP-bound EF-Tu through its two beta barrel domains. (F) Bms1 in the same orientation as EF-Tu in panel E. The unstructured loop of domain V that connects it to domain IV (denoted by the black arrow) and an N-terminal helix of Mpp10 (red) contacts domains II and III of Bms1 in a manner reminiscent of how tRNA interacts with GDPNP-bound EF-Tu. The mutated residues that suppress *bud23Δ* are shown as magenta sticks.

Figure S5. Proteomic compositions of 40S pre-cursors from cells with or without Bud23. Related to Figure 7. A heatmap of SSU biogenesis proteins that co-immunoprecipitated with Enp1-TAP in the presence (+) or absence (-) of Bud23 is shown. The scale spanning from 0 (white) to 1 (cyan) reflects the relative spectral abundance factor (RSAF). The RSAF was calculated by first normalizing the total number of spectral counts identified for a given protein to its molecular weight; these values were further normalized to the bait, Enp1, to reflect stoichiometry. RSAF values for each protein are shown within each cell. For each protein, the number of spectral counts identified in the presence or absence of Bud23 are shown in parentheses, respectively. Proteins that showed a significant increase or decrease relative to the + Bud23 sample and are listed in Fig. S6 are denoted by an asterisks (*) colored blue or black, respectively. Proteins are grouped according to [7] or by known function. Heatmaps were generated in Graphpad Prism

version 8.3.0 (328) for Mac iOS. The complete data for this figure are available in Supplemental File 2.

Figure S6. 40S biogenesis factors whose association with 40S pre-cursors significantly changed upon Bud23-depletion. Related to Figures 7 and S5. Mass spectrometry analysis of total proteins that co-precipitated with Enp1. Proteins that showed a significant log2 fold-change difference in the absence or presence of Bud23 are shown. Total number of peptides identified for each protein was normalized to molecular weight then further normalized to the bait to generate RSAF values (see Methods) which were used to calculate the log2 fold-change between the mutant and wild-type samples. Proteins displaying a ± 0.5 -fold change or more with a difference of greater than 10 total spectral counts are plotted. Proteins are grouped according to when they first bind to pre-ribosomes [7]. The complete mass spectrometry data are available in Supplemental File 2.

Tables

Table 1: Yeast strains used in this study.

Strain	Genotype	Reference
AJY2665	<i>MATa his3Δ1 leu2Δ0 met15Δ0ura3Δ0 ENP1-TAP::HIS3MX6</i>	[87]
AJY2161	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::KanMX</i>	[39]
AJY2676	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT</i>	This study & [47].
AJY3156	<i>MATa his3Δ1 leu2Δ0 met15Δ0ura3Δ0 ENP1-TAP::HIS3MX6 bud23Δ::KanMX</i>	This study.
AJY3581	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::KanMX utp2-A2D</i>	[45]
AJY3512	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::KanMX</i>	This study & [47].

	<i>imp4-V170F</i>	
AJY3579	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::KanMX imp4-R94L</i>	This study & [47].
AJY3580	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::KanMX imp4-N118K</i>	This study & [47].
AJY3741	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::KanMX imp4-T92I</i>	This study & [47].
AJY3742	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::KanMX imp4-R116M</i>	This study & [47].
AJY3743	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::KanMX imp4-S93T</i>	This study & [47].
AJY3744	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::KanMX imp4-R94S</i>	This study & [47].
AJY3745	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::KanMX imp4-N118D</i>	This study & [47].
AJY4501	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT imp4-H159R</i>	This study.
AJY4502	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT utp2-L9S</i>	This study.
AJY4503	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT utp14-W794L</i>	This study.
AJY4504	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT imp4-R99L</i>	This study.
AJY4505	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT imp4-N121I</i>	This study.
AJY4506	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT imp4-Y77C</i>	This study.
AJY4507	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT imp4-S101W</i>	This study.
AJY4508	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT imp4-H208D</i>	This study.
AJY4509	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT imp4-R94C</i>	This study.
AJY4510	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT imp4-H156D</i>	This study.
AJY4511	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT</i>	This study.

	<i>imp4-R99H</i>	
AJY4512	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT imp4-R146G</i>	This study.
AJY4513	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT W791L</i>	This study.
AJY4514	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-D408Y</i>	This study.
AJY4515	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-G432R</i>	This study.
AJY4516	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-S511Y</i>	This study.
AJY4517	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-G434D</i>	This study.
AJY4518	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-E397D</i>	This study.
AJY4519	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-R596G</i>	This study.
AJY4520	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-D566Y</i>	This study.
AJY4521	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-A804D</i>	This study.
AJY4522	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-R596S</i>	This study.
AJY4523	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-M857I</i>	This study.
AJY4524	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-R563M</i>	This study.
AJY4525	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-R13G</i>	This study.
AJY4526	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-E1037Q</i>	This study.
AJY4527	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT dhr1-E1037K</i>	This study.
AJY4529	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT bms1-D843V</i>	This study.
AJY4530	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT</i>	This study.

	<i>dhr1-F594L</i>	
AJY4531	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT rps28a-G24D</i>	This study.
AJY4532	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT bms1-D124Y</i>	This study.
AJY4533	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT bms1-A903P</i>	This study.
AJY4535	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT bms1-G813S</i>	This study.
AJY4536	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT imp4-P252L</i>	This study.
AJY4537	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 met15Δ0 bud23Δ::CloNAT bms1-S1020L</i>	This study.
AJY4395	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ENP1-TAP::HIS3MX6 BUD23-AID-HA::OsTIR1::LEU2</i>	This study.
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Open Biosystems
PJ69-4a	<i>MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	[88]
PJ69-4alpha	<i>MATalpha trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	[88]

Table 2: Plasmids used in this study.

Plasmid	Description	Reference
pAJ2321	<i>GAL4AD-HA-UTP14 LEU2 2μ</i>	[30]
pAJ2595	<i>UTP2 URA3 CEN ARS</i>	[45]
pAJ2596	<i>utp2-A2D URA3 CEN ARS</i>	[45]
pAJ2922	<i>GAL4BD-c-myc-DHR1 TRP1 2μ</i>	[46]
pAJ2769	<i>GAL4BD-c-myc-IMP4 TRP1 2μ</i>	This study.
pAJ3332	<i>utp2-F149S URA3 CEN ARS</i>	This study.
pAJ3335	<i>utp2-L151H URA3 CEN ARS</i>	This study.
pAJ3347	<i>utp2-L148S, F149S, L151H URA3 CEN ARS</i>	This study.

pAJ3348	<i>utp2-L6P URA3 CEN ARS</i>	This study.
pAJ3349	<i>utp2-K7E URA3 CEN ARS</i>	This study.
pAJ3350	<i>utp2-L148S URA3 CEN ARS</i>	This study.
pAJ4093	<i>utp2-F58S URA3 CEN ARS</i>	This study.
pAJ4094	<i>PGAL10-UTP2 LEU2 CEN ARS</i>	This study.
pAJ4095	<i>utp2-A2D, L6P, K7E URA3 CEN ARS</i>	This study.
pAJ4188	<i>Utp2-GAL4AD-HA LEU2 2μ</i>	This study.
pAJ4192	<i>utp2-F58S-GAL4AD-HA LEU2 2μ</i>	This study.
pAJ4193	<i>utp2-L148S, F149S, L151H-GAL4AD-HA LEU2 2μ</i>	This study.
pAJ4194	<i>utp2-A2D, L6P, K7E-GAL4AD-HA LEU2 2μ</i>	This study.
pAJ4493	<i>GAL4BD-c-myc-imp4-V170F TRP1 2μ</i>	This study.
pAJ4494	<i>GAL4BD-c-myc-imp4-P252L TRP1 2μ</i>	This study.
pAJ4503	<i>GAL4BD-c-myc-DHR1-E360K, E397D, E402G, D408Y TRP1 2μ</i>	This study.
pAJ4513	<i>GAL4BD-c-myc-DHR1-R563M, D566Y, E831K, F837L TRP1 2μ</i>	This study.
pAJ4514	<i>GAL4BD-c-myc-DHR1-H593Y, R596C, E831K TRP1 2μ</i>	This study.
pGADT7	<i>GAL4AD-HA LEU2 2μ</i>	[89]
pGBKT7	<i>GAL4BD-c-myc TRP1 2μ</i>	[89]
pJW1662	<i>AtIAA17_71-114(AID*)-HA::LEU2 prADH1-OsTIR1</i>	[80]
pRS416	<i>URA3 CEN ARS</i>	[90]

983

984 **Table 3: Oligonucleotide probes used for Northern blotting.**

Target	Sequence
+1-A0	5' GGTCTCTCTGCTGCCGGAATG 3'
A0-A1	5' CCCACCTATTCCCTCTTGC 3'
A2-A3	5' TGTTACCTCTGGGCCCCGATTG 3'
U3 snoRNA	5' TAGATTCAATTTTCGGTTTCTC 3'

985

986 **Supporting information**

987 Supplemental File 1 – Cumulative list of the suppressors of *bud23Δ*.

988 Supplemental File 2 – Mass spectrometry data for the Enp1-TAP affinity purifications.

989 **Acknowledgments**

990 This work was supported by National Institutes of Health grants GM127127, GM053655,
991 and GM108823 to AWJ, a fellowship from the University of Texas at Austin Graduate School to
992 JJB, and a fellowship from the University of Texas at Austin Office of Undergraduate Research to
993 EWE.

994 We thank S. Baserga and C. Wang for the Imp4 and Bud23 antibodies, respectively. The
995 vector pJW1662 was a gift from J. Weissman (Addgene plasmid #112050). We thank J. Ream and
996 J. Zhu for assistance with cloning, and P. Sujita for assistance with preparing samples for Illumina
997 sequencing submission. We also thank the members of the A. Johnson and Sarinay-Cenik
998 laboratories for helpful comments regarding the manuscript.

999

1000 **Author contributions**

1001 JJB and AWJ conceptualized, analyzed and interpreted data, and wrote the manuscript.
1002 JJB performed experiments and bioinformatic analyses. JJB, RS, EWE, and AWJ isolated and
1003 identified the suppressors of *bud23Δ*.

References

1. Ben-Shem A, Garreau de Loubresse N, Melnikov S, Jenner L, Yusupova G, Yusupov M. The structure of the eukaryotic ribosome at 3.0 Å resolution. *Science*. 2011;334: 1524–9. doi:10.1126/science.1212642
2. Sloan KE, Warda AS, Sharma S, Entian K-D, Lafontaine DLJ, Bohnsack MT. Tuning the ribosome: The influence of rRNA modification on eukaryotic ribosome biogenesis and function. *RNA Biol*. Taylor & Francis; 2016;408: 1–16. doi:10.1080/15476286.2016.1259781
3. Tomecki R, Sikorski PJ, Zakrzewska-Placzek M. Comparison of preribosomal RNA processing pathways in yeast, plant and human cells - focus on coordinated action of endo- and exoribonucleases. *FEBS Lett*. 2017;591: 1801–1850. doi:10.1002/1873-3468.12682
4. Baßler J, Hurt E. Eukaryotic Ribosome Assembly. *Annu Rev Biochem*. 2019;88: 281–306. doi:10.1146/annurev-biochem-013118-110817
5. Klinge S, Woolford JL. Ribosome assembly coming into focus. *Nat Rev Mol Cell Biol*. Springer US; 2019;20: 116–131. doi:10.1038/s41580-018-0078-y
6. Chaker-Margot M, Hunziker M, Barandun J, Dill BD, Klinge S. Stage-specific assembly events of the 6-MDa small-subunit processome initiate eukaryotic ribosome biogenesis. *Nat Struct Mol Biol*. Nature Publishing Group; 2015;22: 920–3. doi:10.1038/nsmb.3111
7. Zhang L, Wu C, Cai G, Chen S, Ye K. Stepwise and dynamic assembly of the earliest precursors of small ribosomal subunits in yeast. *Genes Dev*. 2016;30: 718–32. doi:10.1101/gad.274688.115
8. Pérez-Fernández J, Román A, De Las Rivas J, Bustelo XR, Dosil M. The 90S preribosome is a multimodular structure that is assembled through a hierarchical mechanism. *Mol Cell Biol*. 2007;27: 5414–29. doi:10.1128/MCB.00380-07
9. Chen W, Xie Z, Yang F, Ye K. Stepwise assembly of the earliest precursors of large ribosomal subunits in yeast. *Nucleic Acids Res*. 2017;45: 6837–6847. doi:10.1093/nar/gkx254
10. Chaker-Margot M, Klinge S. Assembly and early maturation of large subunit precursors. *RNA*. 2019;25: 465–471. doi:10.1261/rna.069799.118
11. Dragon F, Gallagher JEG, Compagnone-Post PA, Mitchell BM, Porwancher KA, Wehner KA, et al. A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. *Nature*. 2002;417: 967–70. doi:10.1038/nature00769
12. Grandi P, Rybin V, Bassler J, Petfalski E, Strauss D, Marzioch M, et al. 90S pre-ribosomes include the 35S pre-rRNA, the U3 snoRNP, and 40S subunit processing factors but predominantly lack 60S synthesis factors. *Mol Cell*. 2002;10: 105–15. doi:10.1016/S1097-2765(02)00579-8
13. Barandun J, Hunziker M, Klinge S. Assembly and structure of the SSU processome-a nucleolar precursor of the small ribosomal subunit. *Curr Opin Struct Biol*. Elsevier Ltd; 2018;49: 85–93. doi:10.1016/j.sbi.2018.01.008
14. Chaker-Margot M. Assembly of the small ribosomal subunit in yeast: mechanism and regulation. *RNA*. 2018;24: 881–891. doi:10.1261/rna.066985.118

- 1046 15. Barandun J, Chaker-Margot M, Hunziker M, Molloy KR, Chait BT, Klinge S. The complete
1047 structure of the small-subunit processome. *Nat Struct Mol Biol.* Nature Publishing Group;
1048 2017; 1–36. doi:10.1038/nsmb.3472
- 1049 16. Cheng J, Kellner N, Berninghausen O, Hurt E, Beckmann R. 3.2-Å-resolution structure of
1050 the 90S preribosome before A1 pre-rRNA cleavage. *Nat Struct Mol Biol.* Nature
1051 Publishing Group; 2017;24: 954–964. doi:10.1038/nsmb.3476
- 1052 17. Sun Q, Zhu X, Qi J, An W, Lan P, Tan D, et al. Molecular architecture of the 90S small
1053 subunit pre-ribosome. *Elife.* 2017;6: e22086. doi:10.7554/eLife.22086
- 1054 18. Hunziker M, Barandun J, Buzovetsky O, Steckler C, Molina H, Klinge S. Conformational
1055 switches control early maturation of the eukaryotic small ribosomal subunit. *Elife.*
1056 2019;8: 1–16. doi:10.7554/eLife.45185
- 1057 19. Cheng J, Baßler J, Fischer P, Lau B, Kellner N, Kunze R, et al. Thermophile 90S Pre-
1058 ribosome Structures Reveal the Reverse Order of Co-transcriptional 18S rRNA Subdomain
1059 Integration. *Mol Cell.* 2019; 1–14. doi:10.1016/j.molcel.2019.06.032
- 1060 20. Schäfer T, Strauss D, Petfalski E, Tollervey D, Hurt E. The path from nucleolar 90S to
1061 cytoplasmic 40S pre-ribosomes. *EMBO J.* 2003;22: 1370–80. doi:10.1093/emboj/cdg121
- 1062 21. Hunziker M, Barandun J, Petfalski E, Tan D, Delan-Forino C, Molloy KR, et al. UtpA and
1063 UtpB chaperone nascent pre-ribosomal RNA and U3 snoRNA to initiate eukaryotic
1064 ribosome assembly. *Nat Commun.* 2016;7: 12090. doi:10.1038/ncomms12090
- 1065 22. Beltrame M, Henry Y, Tollervey D. Mutational analysis of an essential binding site for the
1066 U3 snoRNA in the 5' external transcribed spacer of yeast pre-rRNA. *Nucleic Acids Res.*
1067 1994;22: 4057–65. doi:10.1093/nar/22.20.4057
- 1068 23. Beltrame M, Tollervey D. Base pairing between U3 and the pre-ribosomal RNA is required
1069 for 18S rRNA synthesis. *EMBO J.* 1995;14: 4350–6. doi:10.1002/j.1460-
1070 2075.1995.tb00109.x
- 1071 24. Sharma K, Tollervey D. Base pairing between U3 small nucleolar RNA and the 5' end of
1072 18S rRNA is required for pre-rRNA processing. *Mol Cell Biol.* 1999;19: 6012–9.
1073 doi:10.1128/mcb.19.9.6012
- 1074 25. Dutca LM, Gallagher JEG, Baserga SJ. The initial U3 snoRNA:pre-rRNA base pairing
1075 interaction required for pre-18S rRNA folding revealed by in vivo chemical probing.
1076 *Nucleic Acids Res.* 2011;39: 5164–80. doi:10.1093/nar/gkr044
- 1077 26. Marmier-Gourrier N, Cléry A, Schlotter F, Senty-Ségault V, Branlant C. A second base pair
1078 interaction between U3 small nucleolar RNA and the 5'-ETS region is required for early
1079 cleavage of the yeast pre-ribosomal RNA. *Nucleic Acids Res.* 2011;39: 9731–45.
1080 doi:10.1093/nar/gkr675
- 1081 27. Petrov AS, Bernier CR, Gulen B, Waterbury CC, Hershkovits E, Hsiao C, et al. Secondary
1082 structures of rRNAs from all three domains of life. *PLoS One.* 2014;9: e88222.
1083 doi:10.1371/journal.pone.0088222
- 1084 28. Kressler D, Hurt E, Baßler J. A Puzzle of Life: Crafting Ribosomal Subunits. *Trends Biochem*
1085 *Sci.* 2017;42: 640–654. doi:10.1016/j.tibs.2017.05.005
- 1086 29. Sardana R, Liu X, Granneman S, Zhu J, Gill M, Papoulas O, et al. The DEAH-box helicase
1087 Dhr1 dissociates U3 from the pre-rRNA to promote formation of the central pseudoknot.
1088 *PLoS Biol.* 2015;13: e1002083. doi:10.1371/journal.pbio.1002083
- 1089 30. Zhu J, Liu X, Anjos M, Correll CC, Johnson AW. Utp14 Recruits and Activates the RNA

- 1090 Helicase Dhr1 To Undock U3 snoRNA from the Preribosome. *Mol Cell Biol.* 2016;36: 965–
1091 78. doi:10.1128/MCB.00773-15
- 1092 31. Roychowdhury A, Joret C, Bourgeois G, Heurgué-Hamard V, Lafontaine DLJ, Graille M.
1093 The DEAH-box RNA helicase Dhr1 contains a remarkable carboxyl terminal domain
1094 essential for small ribosomal subunit biogenesis. *Nucleic Acids Res.* 2019;
1095 doi:10.1093/nar/gkz529
- 1096 32. Boneberg FM, Brandmann T, Kobel L, van den Heuvel J, Bargsten K, Bammert L, et al.
1097 Molecular mechanism of the RNA helicase DHX37 and its activation by UTP14A in
1098 ribosome biogenesis. *RNA.* 2019;25: 685–701. doi:10.1261/rna.069609.118
- 1099 33. Black JJ, Wang Z, Goering LM, Johnson AW. Utp14 interaction with the Small Subunit
1100 Processome. *RNA.* 2018; rna.066373.118. doi:10.1261/rna.066373.118
- 1101 34. Colley A, Beggs JD, Tollervey D, Lafontaine DL. Dhr1p, a putative DEAH-box RNA helicase,
1102 is associated with the box C+D snoRNP U3. *Mol Cell Biol.* 2000;20: 7238–46.
1103 doi:10.1128/mcb.20.19.7238-7246.2000
- 1104 35. Granneman S, Bernstein KA, Bleichert F, Baserga SJ. Comprehensive mutational analysis
1105 of yeast DEXD/H box RNA helicases required for small ribosomal subunit synthesis. *Mol*
1106 *Cell Biol.* 2006;26: 1183–94. doi:10.1128/MCB.26.4.1183-1194.2006
- 1107 36. Bleichert F, Granneman S, Osheim YN, Beyer AL, Baserga SJ. The PINc domain protein
1108 Utp24, a putative nuclease, is required for the early cleavage steps in 18S rRNA
1109 maturation. *Proc Natl Acad Sci U S A.* 2006;103: 9464–9. doi:10.1073/pnas.0603673103
- 1110 37. Wells GR, Weichmann F, Colvin D, Sloan KE, Kudla G, Tollervey D, et al. The PIN domain
1111 endonuclease Utp24 cleaves pre-ribosomal RNA at two coupled sites in yeast and
1112 humans. *Nucleic Acids Res.* 2016; gkw213. doi:10.1093/nar/gkw213
- 1113 38. An W, Du Y, Ye K. Structural and functional analysis of Utp24, an endonuclease for
1114 processing 18S ribosomal RNA. *PLoS One.* 2018;13: e0195723.
1115 doi:10.1371/journal.pone.0195723
- 1116 39. White J, Li Z, Sardana R, Bujnicki JM, Marcotte EM, Johnson AW. Bud23 methylates
1117 G1575 of 18S rRNA and is required for efficient nuclear export of pre-40S subunits. *Mol*
1118 *Cell Biol.* 2008;28: 3151–61. doi:10.1128/MCB.01674-07
- 1119 40. Létoquart J, Huvelle E, Wacheul L, Bourgeois G, Zorbas C, Graille M, et al. Structural and
1120 functional studies of Bud23-Trm112 reveal 18S rRNA N7-G1575 methylation occurs on
1121 late 40S precursor ribosomes. *Proc Natl Acad Sci U S A.* 2014;111: E5518-26.
1122 doi:10.1073/pnas.1413089111
- 1123 41. Sardana R, Johnson AW. The methyltransferase adaptor protein Trm112 is involved in
1124 biogenesis of both ribosomal subunits. *Mol Biol Cell.* 2012;23: 4313–22.
1125 doi:10.1091/mbc.E12-05-0370
- 1126 42. Figaro S, Wacheul L, Schillewaert S, Graille M, Huvelle E, Mongeard R, et al. Trm112 Is
1127 Required for Bud23-Mediated Methylation of the 18S rRNA at Position G1575. *Mol Cell*
1128 *Biol.* 2012;32: 2254–2267. doi:10.1128/MCB.06623-11
- 1129 43. Lin J-L, Yu H-C, Chao J-L, Wang C, Cheng M-Y. New phenotypes generated by the G57R
1130 mutation of BUD23 in *Saccharomyces cerevisiae*. *Yeast.* 2012;29: 537–546.
1131 doi:10.1002/yea.2934
- 1132 44. Ameismeier M, Cheng J, Berninghausen O, Beckmann R. Visualizing late states of human
1133 40S ribosomal subunit maturation. *Nature.* 2018;558: 249–253. doi:10.1038/s41586-018-

- 1134 0193-0
- 1135 45. Sardana R, White JP, Johnson AW. The rRNA methyltransferase Bud23 shows functional
1136 interaction with components of the SSU processome and RNase MRP. *RNA*. 2013;19:
1137 828–40. doi:10.1261/rna.037671.112
- 1138 46. Sardana R, Zhu J, Gill M, Johnson AW. Physical and functional interaction between the
1139 methyltransferase Bud23 and the essential DEAH-box RNA helicase Ecm16. *Mol Cell Biol*.
1140 2014;34: 2208–20. doi:10.1128/MCB.01656-13
- 1141 47. Sardana R. From knobs to a central pseudoknot: understanding 40S ribosomal subunit
1142 biogenesis through Bud23. The University of Texas at Austin. 2013.
- 1143 48. Lee SJ, Baserga SJ. Imp3p and Imp4p, two specific components of the U3 small nucleolar
1144 ribonucleoprotein that are essential for pre-18S rRNA processing. *Mol Cell Biol*. 1999;19:
1145 5441–52. doi:10.1128/MCB.19.8.5441
- 1146 49. Dunbar DA, Wormsley S, Agentis TM, Baserga SJ. Mpp10p, a U3 small nucleolar
1147 ribonucleoprotein component required for pre-18S rRNA processing in yeast. *Mol Cell*
1148 *Biol*. 1997;17: 5803–12. doi:10.1128/mcb.17.10.5803
- 1149 50. Lee SJ, Baserga SJ. Functional separation of pre-rRNA processing steps revealed by
1150 truncation of the U3 small nucleolar ribonucleoprotein component, Mpp10. *Proc Natl*
1151 *Acad Sci U S A*. 1997;94: 13536–41. doi:10.1073/pnas.94.25.13536
- 1152 51. Gallagher JEG, Baserga SJ. Two-hybrid Mpp10p interaction-defective Imp4 proteins are
1153 not interaction defective in vivo but do confer specific pre-rRNA processing defects in
1154 *Saccharomyces cerevisiae*. *Nucleic Acids Res*. 2004;32: 1404–13. doi:10.1093/nar/gkh318
- 1155 52. Wehner KA, Gallagher JEG, Baserga SJ. Components of an interdependent unit within the
1156 SSU processome regulate and mediate its activity. *Mol Cell Biol*. 2002;22: 7258–67.
1157 doi:10.1128/MCB.22.20.7258-7267.2002
- 1158 53. Sá-Moura B, Kornprobst M, Kharde S, Ahmed YL, Stier G, Kunze R, et al. Mpp10
1159 represents a platform for the interaction of multiple factors within the 90S pre-ribosome.
1160 *PLoS One*. 2017;12: e0183272. doi:10.1371/journal.pone.0183272
- 1161 54. Baßler J, Ahmed YL, Kallas M, Kornprobst M, Calviño FR, Gnädig M, et al. Interaction
1162 network of the ribosome assembly machinery from a eukaryotic thermophile. *Protein*
1163 *Sci*. 2016;22: 1–50. doi:10.1002/pro.3085
- 1164 55. Vincent NG, Charette JM, Baserga SJ. The SSU processome interactome in *Saccharomyces*
1165 *cerevisiae* reveals novel protein subcomplexes. *RNA*. 2018;24: 77–89.
1166 doi:10.1261/rna.062927.117
- 1167 56. Liu PC, Thiele DJ. Novel stress-responsive genes EMG1 and NOP14 encode conserved,
1168 interacting proteins required for 40S ribosome biogenesis. *Mol Biol Cell*. 2001;12: 3644–
1169 57. doi:10.1091/mbc.12.11.3644
- 1170 57. Wegierski T, Billy E, Nasr F, Filipowicz W. Bms1p, a G-domain-containing protein,
1171 associates with Rcl1p and is required for 18S rRNA biogenesis in yeast. *RNA*. 2001;7:
1172 1254–67. doi:10.1017/S1355838201012079
- 1173 58. Gelperin D, Horton L, Beckman J, Hensold J, Lemmon SK. Bms1p, a novel GTP-binding
1174 protein, and the related Tsr1p are required for distinct steps of 40S ribosome biogenesis
1175 in yeast. *RNA*. 2001;7: 1268–83. doi:10.1017/S1355838201013073
- 1176 59. Karbstein K, Doudna JA. GTP-dependent formation of a ribonucleoprotein subcomplex
1177 required for ribosome biogenesis. *J Mol Biol*. 2006;356: 432–43.

- doi:10.1016/j.jmb.2005.11.052
60. Karbstein K, Jonas S, Doudna JA. An essential GTPase promotes assembly of preribosomal RNA processing complexes. *Mol Cell*. 2005;20: 633–43. doi:10.1016/j.molcel.2005.09.017
61. Delprato A, Al Kadri Y, Pérébasquine N, Monfoulet C, Henry Y, Henras AK, et al. Crucial role of the Rcl1p-Bms1p interaction for yeast pre-ribosomal RNA processing. *Nucleic Acids Res*. 2014;42: 10161–72. doi:10.1093/nar/gku682
62. Wittinghofer A, Vetter IR. Structure-function relationships of the G domain, a canonical switch motif. *Annu Rev Biochem*. 2011;80: 943–71. doi:10.1146/annurev-biochem-062708-134043
63. Nissen P, Thirup S, Kjeldgaard M, Nyborg J. The crystal structure of Cys-tRNA^{Cys}-EF-Tu-GDPNP reveals general and specific features in the ternary complex and in tRNA. *Structure*. 1999;7: 143–56.
64. Song H, Parsons MR, Rowsell S, Leonard G, Phillips SEV. Crystal structure of intact elongation factor EF-Tu from *Escherichia coli* in GDP conformation at 2.05 Å resolution. *J Mol Biol*. 1999;285: 1245–56. doi:10.1006/jmbi.1998.2387
65. Choudhury P, Hackert P, Memet I, Sloan KE, Bohnsack MT. The human RNA helicase DHX37 is required for release of the U3 snoRNP from pre-ribosomal particles. *RNA Biol*. Taylor & Francis; 2018;00: 1–15. doi:10.1080/15476286.2018.1556149
66. Nishimura K, Fukagawa T, Takisawa H, Kakimoto T, Kanemaki M. An auxin-based degron system for the rapid depletion of proteins in nonplant cells. *Nat Methods*. Nature Publishing Group; 2009;6: 917–22. doi:10.1038/nmeth.1401
67. Gérczei T, Correll CC. Imp3p and Imp4p mediate formation of essential U3-precursor rRNA (pre-rRNA) duplexes, possibly to recruit the small subunit processome to the pre-rRNA. *Proc Natl Acad Sci U S A*. 2004;101: 15301–6. doi:10.1073/pnas.0406819101
68. Lackmann F, Belikov S, Burlacu E, Granneman S, Wieslander L. Maturation of the 90S pre-ribosome requires Mrd1 dependent U3 snoRNA and 35S pre-rRNA structural rearrangements. *Nucleic Acids Res*. Oxford University Press; 2018;46: 3692–3706. doi:10.1093/nar/gky036
69. Fischer U, Schäuble N, Schütz S, Altvater M, Chang Y, Faza MB, et al. A non-canonical mechanism for Crm1-export cargo complex assembly. *Elife*. 2015;4: 1–20. doi:10.7554/eLife.05745
70. Ferreira-Cerca S, Kiburu I, Thomson E, LaRonde N, Hurt E. Dominant Rio1 kinase/ATPase catalytic mutant induces trapping of late pre-40S biogenesis factors in 80S-like ribosomes. *Nucleic Acids Res*. 2014;42: 8635–47. doi:10.1093/nar/gku542
71. Parker MD, Collins JC, Korona B, Ghalei H, Karbstein K. A kinase-dependent checkpoint prevents escape of immature ribosomes into the translating pool. Gilbert W V, editor. *PLOS Biol*. 2019;17: e3000329. doi:10.1371/journal.pbio.3000329
72. Zorbas C, Nicolas E, Wacheul L, Huvelle E, Heurgué-Hamard V, Lafontaine DLJ. The human 18S rRNA base methyltransferases DIMT1L and WBSCR22-TRMT112 but not rRNA modification are required for ribosome biogenesis. *Mol Biol Cell*. 2015;26: 2080–95. doi:10.1091/mbc.E15-02-0073
73. Öunap K, Käsper L, Kurg A, Kurg R. The human WBSCR22 protein is involved in the biogenesis of the 40S ribosomal subunits in mammalian cells. Wieden H-J, editor. *PLoS One*. 2013;8: e75686. doi:10.1371/journal.pone.0075686

- 1222 74. Haag S, Kretschmer J, Bohnsack MT. WBSR22/Merm1 is required for late nuclear pre-
1223 ribosomal RNA processing and mediates N7-methylation of G1639 in human 18S rRNA.
1224 RNA. 2015;21: 180–7. doi:10.1261/rna.047910.114
- 1225 75. Small EC, Leggett SR, Winans AA, Staley JP. The EF-G-like GTPase Snu114p regulates
1226 spliceosome dynamics mediated by Brr2p, a DExD/H box ATPase. Mol Cell. 2006;23: 389–
1227 99. doi:10.1016/j.molcel.2006.05.043
- 1228 76. Koodathingal P, Staley JP. Splicing fidelity. RNA Biol. 2013;10: 1073–1079.
1229 doi:10.4161/rna.25245
- 1230 77. Yang F, Wang X-Y, Zhang Z-M, Pu J, Fan Y-J, Zhou J, et al. Splicing proofreading at 5' splice
1231 sites by ATPase Prp28p. Nucleic Acids Res. 2013;41: 4660–70. doi:10.1093/nar/gkt149
- 1232 78. Tong AHY, Evangelista M, Parsons AB, Xu H, Bader GD, Pagé N, et al. Systematic genetic
1233 analysis with ordered arrays of yeast deletion mutants. Science. 2001;294: 2364–8.
1234 doi:10.1126/science.1065810
- 1235 79. Winzler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B, et al. Functional
1236 characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis.
1237 Science. 1999;285: 901–6. doi:10.1126/science.285.5429.901
- 1238 80. Costa EA, Subramanian K, Nunnari J, Weissman JS. Defining the physiological role of SRP
1239 in protein-targeting efficiency and specificity. Science. 2018;359: 689–692.
1240 doi:10.1126/science.aar3607
- 1241 81. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods.
1242 2012;9: 357–9. doi:10.1038/nmeth.1923
- 1243 82. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence
1244 Alignment/Map format and SAMtools. Bioinformatics. 2009;25: 2078–9.
1245 doi:10.1093/bioinformatics/btp352
- 1246 83. Narasimhan V, Danecek P, Scally A, Xue Y, Tyler-Smith C, Durbin R. BCFtools/ROH: a
1247 hidden Markov model approach for detecting autozygosity from next-generation
1248 sequencing data. Bioinformatics. 2016;32: 1749–51. doi:10.1093/bioinformatics/btw044
- 1249 84. Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, et al. The variant call
1250 format and VCFtools. Bioinformatics. 2011;27: 2156–8.
1251 doi:10.1093/bioinformatics/btr330
- 1252 85. Oeffinger M, Wei KE, Rogers R, DeGrasse JA, Chait BT, Aitchison JD, et al. Comprehensive
1253 analysis of diverse ribonucleoprotein complexes. Nat Methods. 2007;4: 951–6.
1254 doi:10.1038/nmeth1101
- 1255 86. Musalgaonkar S, Black JJ, Johnson A. The L1 stalk is required for efficient export of
1256 nascent large ribosomal subunits in yeast. RNA. 2019; rna.071811.119.
1257 doi:10.1261/rna.071811.119
- 1258 87. Ghaemmighami S, Huh W-K, Bower K, Howson RW, Belle a, Dephoure N, et al. Global
1259 analysis of protein expression in yeast. Nature. 2003;425: 737–741.
1260 doi:10.1038/nature02046\nnature02046 [pii]
- 1261 88. James P, Halladay J, Craig E a. Genomic libraries and a host strain designed for highly
1262 efficient two-hybrid selection in yeast. Genetics. 1996;144: 1425–36.
- 1263 89. Patel SS, Belmont BJ, Sante JM, Rexach MF. Natively unfolded nucleoporins gate protein
1264 diffusion across the nuclear pore complex. Cell. 2007;129: 83–96.
1265 doi:10.1016/j.cell.2007.01.044

- 1266 90. Sikorski RS, Hieter P. A system of shuttle vectors and yeast host strains designed for
1267 efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics. 1989;122: 19–27.
1268 doi:0378111995000377 [pii]
1269

Figure 1, Black, et al 2020

Figure 2, Black, et al 2020

Figure 3, Black, et al 2020

Figure 4, Black, et al 2020

Figure 5, Black, et al 2020

Figure 6, Black, et al 2020

Figure 7, Black, et al 2020

A **B**

Figure 8, Black, et al 2020

central domain

A 3D-based secondary structure, generated by RiboVision.

Figure S2, Black, et al 2020

Figure S3, Black, et al 2020

Figure S4, Black, et al 2020

Figure S5, Black, et al 2020

Figure S6, Black, et al 2020