# Ebp2 and Brx1 function cooperatively in 60S ribosomal subunit assembly in Saccharomyces cerevisiae

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## ABSTRACT

The yeast protein Ebp2 is required for early steps in production of 60S ribosomal subunits. To search for cofactors with which Ebp2 functions, or substrates on which it acts, we screened for mutants that were synthetically lethal (sl) with the ebp2-14 mutation. Four different mutant alleles of the 60S ribosomal subunit assembly factor Brx1 were found. To investigate defects of the double mutant, we constructed strains conditional for the ebp2-14 brx1- synthetic lethal phenotype. These ebp2-14 brx1 mutants were defective in processing of 27S pre-rRNA and production of 60S subunits, under conditions where each single mutant was not. Ebp2 and Brx1 exhibit a strong two-hybrid interaction, which is eliminated by some combinations of brx1 and ebp2 mutations. In one such mutant, Ebp2 and Brx1 can still associate with pre-ribosomes, but subunit maturation is perturbed. Depletion of either Ebp2 or Brx1 revealed that Brx1 requires Ebp2 for its stable association with pre-ribosomes, but Ebp2 does not depend on the presence of Brx1 to enter pre-ribosomes. These results suggest that assembly of 60S ribosomal subunits requires cooperation of Ebp2 with Brx1, together with other molecules present in pre-ribosomes, potentially including several found in assembly subcomplexes with Brx1 and Ebp2.

## INTRODUCTION

Yeast ribosomes contain four RNAs and 79 ribosomal proteins (r proteins). The mature 25S, 18S and 5.8S rRNAs are derived from a single long precursor rRNA, the 35S pre-rRNA, transcribed by RNA polymerase I. The 5S rRNA is transcribed from separate genes by RNA polymerase III (1). As these rRNAs are transcribed, they must fold into secondary and tertiary structures that enable modification of the RNA, removal of spacer sequences and binding of the ribosomal proteins. Thus, constructing these complex ribonucleoprotein particles requires the establishment and remodeling of RNA–RNA, RNA–protein and protein–protein interactions. Genetic and proteomic studies have revealed that there are more than 180 proteins, in addition to r proteins, required for these dynamic processes occurring during ribosome assembly (2,3). The effects on ribosome production and pre-rRNA processing have been examined when each of these factors was depleted or inactivated. Most factors have been assigned to function in production of one or the other ribosomal subunit, and to participate in one or more steps of pre-rRNA processing.

The challenge before us now is to elucidate precisely how each assembly factor (and r protein) facilitates accurate and efficient production of functional ribosomes. To understand in better detail the mechanisms of ribosome assembly, it will be critical to answer the following questions: When does each protein associate with pre-ribosomes, and when does each assembly factor dissociate? Which molecules are necessary for the stable

docking of each protein with pre-rRNPs, and for dissociation of each? Once bound to pre-ribosomes, with which proteins or RNAs does each factor and r protein interact? These pre-ribosomal ligands will include cofactors (both positive and negative regulators), as well as substrates upon which each factor might act. Where in pre-ribosomes is each factor located with respect to the others? How do these ligands and locations change as particles undergo maturation? The recent determination of the crystal structure of mature eukaryotic ribosomes (4,5) provides a valuable structural context to facilitate answering some of these questions.

One such assembly factor is Ebp2, which was previously shown to be essential for maturation of 25S rRNA and assembly of 60S ribosomal subunits (6–8). To investigate the function of Ebp2 in more detail, we carried out a genetic screen for mutations that are synthetically lethal (sl) with the *ebp2-14* mutation. Such a screen should identify proteins that functionally or physically interact with Ebp2. We found that mutations in the gene encoding 60S ribosomal subunit assembly factor Brx1 exhibit synthetic lethality with *ebp2-14*. We constructed *GAL-EBP2 ebp2-14 brx1* strains conditional for this synthetic lethality, and demonstrated that the *ebp2 brx1* double mutant strains are unable to assemble 60S subunits under conditions where each single mutant is functional in subunit biogenesis. Wild-type Ebp2 and Brx1 associate with each other tightly in a two-hybrid assay (9). However, three out of four *brx1* mutations (*brx1-52*, *brx1-102* and *brx1-227*) in combination with *ebp2-14* prevent this interaction. Interestingly, in the *ebp2-14 brx1-172* double mutant, the two proteins can still interact. Therefore, we studied in more detail changes in pre-ribosomal particles in one of the double mutants where the interaction is disrupted and compared them to the double mutant where the interaction is not abolished. Surprisingly, in both cases, both mutant proteins were able to assemble into pre-ribosomes. This result suggests that other molecules in pre-ribosomes help anchor Ebp2 and Brx1 within pre-rRNPs. Consistent with these findings, we also established that in the absence of Brx1, Ebp2 can stably associate with pre-ribosomes, but Brx1 cannot enter pre-ribosomes without Ebp2. Thus, these *ebp2* and *brx1* mutations may provide insight into functions of Ebp2 and Brx1 in 60S subunit maturation, independent of their recruitment into nascent ribosomes.

## MATERIALS AND METHODS

### Construction and growth of yeast strains

The yeast strains used in this study are listed in Supplementary Table S1. The *ebp2-14* conditional allele was generated by random-PCR mutagenesis (8). Strain 4795-408 (a gift from Lee Hartwell) was crossed twice with W303α, then subsequently crossed with the Ts⁻ *ebp2-14* strain containing Ycp50-*EBP2-ADE3* (see below), to generate KM822, the parental strain used to screen for synthetic lethal mutants (Figure 1A). A yeast strain for conditional expression of *EBP2* was constructed

by transformation of a plasmid containing *EBP2* expressed from the *GAL1* promoter. A yeast strain for conditional expression of *BRX1* was constructed as described in Longtine *et al.* (10). Briefly, sequences containing the *KANMX6* gene expressing resistance to geneticin (G418), plus the *GAL1* promoter sequence followed by an ATG and codons encoding 3HA were amplified by PCR. The PCR products were transformed into yeast strain JWY6147, and the strain was grown in selective media containing galactose. Transformants were screened for correct integration of the *GAL1* promoter and the 3HA tag upstream and in-frame with *BRX1,* by western blotting with anti-HA antisera. Yeast strains expressing C-terminal tandem-affinity purification (TAP)-tagged Nop7, Rpf2 or Rrp5, C-terminal triple hemagglutinin (3HA) tagged proteins or C-terminal 13Myc-tagged proteins were created by PCR of the tag sequence and a selectable marker (*URA3* for the TAP tag and *HIS3* or *TRP1* for other tags), transformation and selection, as described in Rigaut *et al.* (11) and Longtine *et al.* (10). Transformants were screened by western blotting for those expressing the tagged proteins.

Yeast cells were grown in YEPD (yeast extract, polypeptone, glucose) rich medium, YEPGal (yeast extract, polypeptone, galactose) rich medium, or synthetic complete medium containing 2% glucose (SC) or SC dropout medium, depending on the plasmid markers. Standard techniques were used for manipulation of yeast (12).

### Plasmid construction

To construct plasmid YCp50 (*CEN URA3*)-*EBP2-ADE3,* the SmaI–SalI fragment of pDK255 (13) containing *ADE3* was cloned between the NruI and SalI sites of YCp50-*EBP2* (6). pRS314-*GAL1-myc-BS*, a pRS314-based expression vector that contains the *GAL1* promoter, sequences encoding the three myc epitopes just downstream of the initiation codon, a multicloning site and the *TDH3* terminator, was kindly provided by Kazuma Tanaka. pRS314-*GAL1-myc-BRX1* was constructed by inserting a BamHI–SmaI PCR fragment of the *BRX1* ORF into pRS314-*GAL1-myc-BS*. A plasmid expressing myc-tagged Brx1 from its own promoter, pRS314-*myc-BRX1*, was constructed as follows. The NotI–EcoRI (blunt-ended) fragment containing the *GAL1* promoter from pRS314-*GAL1-myc-BRX1* was replaced with the NotI–SmaI PCR fragment of the *BRX1* promoter, produced from yeast genomic DNA by PCR. pRS304-*myc-BRX1* was generated by inserting the NotI–XhoI region from pRS314-*myc-BRX1* into pRS304. pRS304-*myc-BRX1* was digested with SpeI and used for integration into the genome to express myc-Brx1. Plasmids expressing mutant myc-brx1 were constructed similarly. PCR was performed with KOD plus (TOYOBO, Japan). The entire PCR products were sequenced and the structures of all plasmids were confirmed by restriction site analysis. Primers used for PCR are available upon request.

### Isolation of sl mutants

To generate mutations that cause synthetic lethality with the *ebp2-14* allele, $5.0 \times 10^5$ cells of strain KM822
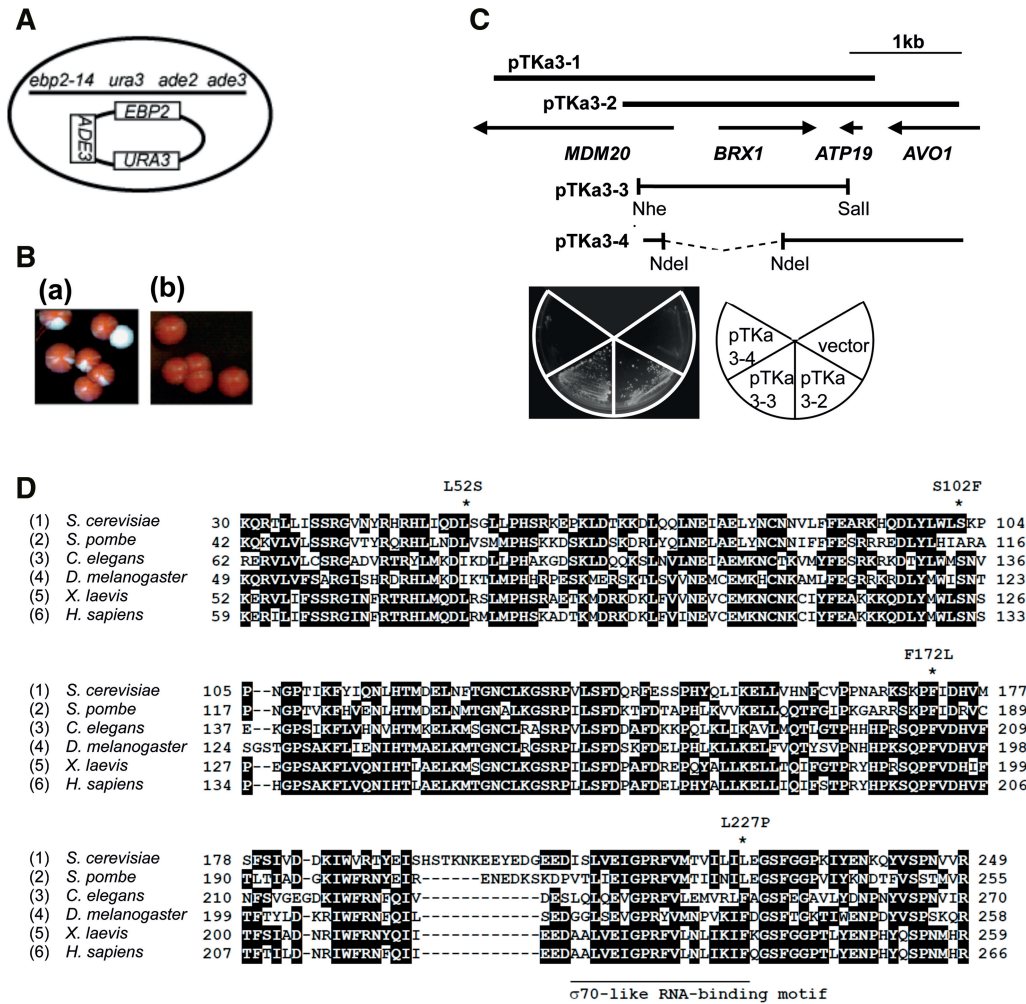
**Figure 1.** Isolation of mutants exhibiting synthetic lethality with *ebp2-14*. (**A**) Schematic representation of the strain used for the sl screen. (**B**) Non-sectoring phenotype of a mutant. The parent strain (a) and isolated mutants (b) were streaked on YEPD media and incubated at 30°C for 7 days. (**C**) Cloning of alleles responsible for synthetic lethality with *ebp2-14*. pKTa3-1 and pKTa3-2 were cloned from a yeast genomic library (cloned in plasmid vector YCplac111), and subcloned into pKTa3-3 (in pRS315) and pKTa3-4 (in YCplac111). The *m3* (KM635) strain was transformed with these plasmids or a vector plasmid (pRS315), and cultured on 5-FOA-containing medium at 25°C for 3 days. (**D**) Synthetic lethal *brx1* mutations are in conserved amino acid residues. The amino acid sequences are from the GenBank database. Restricted regions with a homologous sequence are shown in single-letter code; 1, *S. cerevisiae* (accession Q08235, 30–249 amino acids aligned from full length of 291 amino acids); 2, *Schizosaccharomyces pombe* (Q9HGL6, 42–255 amino acids from full length of 295 amino acids); 3, *Caenorhabditis elegans* (P34524, 62–270 amino acids from 352 amino acids); 4, *Drosophila melanogaster* (Q9VZE6, 49–258 amino acids from full length of 359 amino acids); 5, *Xenopus laevis* (Q8UVY2, 52–258 amino acids from 339 amino acids); 6, *Homo sapiens* (Q8TDN6, 59–266 amino acids from 353 amino acids). A sequence alignment was generated by BLAST and ClustalW. Deletions needed for alignment are indicated by dashes. Residues boxed in black are identical in at least three of the sequences compared. The amino acids substituted in the *brx1* alleles are indicated with asterisks above the sequence.

containing the *ebp2-14, ura3, ade2* and *ade3* mutations in the genome, plus plasmid YCp50 (*URA3*)-*EBP2-ADE3* (Figure 1A), were plated on YEPD, mutagenized with UV to 15–40% viability, and incubated at 30°C for 7–9 days. Colonies that could not grow without the plasmid, i.e. those showing a red non-sectoring phenotype, were isolated (Figure 1B). Of the 289 colonies that showed this phenotype, 121 colonies could not grow on 5-FOA medium at 30°C. These strains were subsequently transformed with plasmids pRS313-*EBP2* or pRS313-*ebp2-14*, which also contained the *HIS3* gene, to test whether pRS313-*EBP2*, and not pRS313-*ebp2-14*, could replace YCp50-*EBP2-ADE3* in cells grown on medium containing 5-FOA. Nine mutants (*m1–m9*) were obtained. After

crossing each mutant with an *EBP2* or *ebp2–14* strain, tetrad analysis revealed 8 alleles derived from single mutations of genomic DNA.

## Cloning and sequencing of the *BRX1* gene

In order to identify the gene defined by the single complementation group of sl mutants, we transformed the *m3* sl mutant strain (KM635) with a library of partial *Sau*3A fragments of S*accharomyces cerevisiae* genomic DNA constructed in the single copy yeast vector YCplac111 (a gift from Kouichi Funato). This plasmid contains *LEU2* as a selectable marker, and our parental sl mutant strain is Leu[+], because *EBP2* was disrupted by insertion of the *LEU2* gene. Therefore, we first replaced the *LEU2* gene

in the *m3* sl mutant strain with the *HIS3* gene. To do so, the strain was transformed with a DNA fragment prepared by digestion of plasmid pRS304 *ebp2::HIS3* with EcoRI and XbaI. The replacement of *ebp2::LEU2* by *ebp2::HIS3* was checked by PCR and DNA sequencing, and the strain was named KM823. We then transformed the Leu⁻ sl mutant strain KM823 with the plasmid library. Of $1.8 \times 10^4$ Leu⁺ transformants, 10 colonies grew at 25°C on medium containing 5-FOA. Four colonies grew well at 31°C but not at 37°C on SC–Leu medium, which is expected for complementation of the sl mutation but not of the Ts⁻ phenotype of *ebp2-14*. Each of the four plasmids that were isolated from these colonies complemented the synthetic lethality of the mutant cells when retransformed. Partial sequencing of the genomic DNA inserts and subcloning revealed that the complementing activity contained the *BRX1* gene (Figure 1C). In order to confirm that mutations(s) in *BRX1* are responsible for the synergistic temperature sensitivity of the *m3* mutant, DNA fragments containing the *BRX1* gene were amplified by PCR using chromosomal DNA extracted from the wildtype and the *m3* strains. The DNA fragment from wildtype cells, but not that from the *m3* mutant strain, could complement the temperature sensitivity of the *m3* cells (data not shown). This result confirms that *BRX1* is mutated in the *m3* strain and is responsible for the synthetic defect with *ebp2-14*. The wildtype *BRX1* gene and the mutated allele of the chromosomal *BRX1* gene from each of the five sl mutant strains (KM634, KM635, KM638, KM639, KM641) was isolated by PCR, using total chromosomal DNA isolated from wild type and each mutant. The PCR was done in duplicate and the products of each reaction were independently sequenced. PCR fragments were digested with BamHI and PstI and inserted into pRS315 (pRS315-*brx1*).

### Gene disruption and construction of *brx1* mutants

A deletion–insertion mutation of *BRX1* was constructed in the diploid W303. The *BRX1* gene was subcloned into pRS306 as a 1.9-kb NheI–SalI fragment. HpaI–XbaI fragments (amino acids 71–262) were deleted from the *BRX1* gene and a 1.8-kb long SmaI–XbaI fragment containing the *HIS3* gene was inserted at the site of the *BRX1* gene. The resulting *brx1Δ::HIS3* construct was excised with NotI and SalI as a 3.0-kb fragment and used to transform the diploid W303 strain to His⁺. Correct integration of the *brx1Δ::HIS3* gene at the homologous locus in His⁺ transformants was confirmed by PCR. The diploid *BRX1/brx1Δ::HIS3* was transformed with pRS316-*BRX1* (*URA3*) and sporulated, and tetrads were dissected to obtain the *brx1Δ* pRS316-*BRX1* (*URA3*) strain (KM642). To construct *brx1* strains, pRS315-*brx1-52*, pRS315-*brx1-102*, pRS315-*brx1-172* and pRS315-*brx1-227* were digested with BamHI and PstI, and each 2.1-kb BamHI-SalI fragment was subcloned into pRS304. The *brx1* genes were excised from the resulting plasmids with *Eco*RI and integrated in the genomic DNA of the KM642 strain. The *brx1-52, brx1-102, brx1-172* and *brx1-227* strains (KM1721–KM1724) were obtained by

selection on medium containing 5-FOA. The wildtype control strain KM1720 was constructed by similar means.

### Gradient assays of ribosomes and polyribosomes

Yeast whole cell extracts were overlaid on top of 11 ml 7–47% (wt/vol) sucrose gradients and centrifuged for 3.4 h at 35 000 rpm at 4°C in a Hitachi RPS40T rotor, as previously described (9). Gradients were collected by pumping up using a peristaltic pump, and fractions were monitored at 254 nm.

### Two-hybrid assays

To carry out yeast two-hybrid assays, plasmids pBTM116 and pACT2 encoding lexA binding domain-fusion proteins and Gal4 activation domain–fusion proteins, respectively, were co-transformed into the yeast L40 strain cells containing a *HIS3* reporter gene as previously described (6). Leu⁺ Trp⁺ transformants were selected. Five-fold serial dilutions of the cell cultures were stamped on SC–Leu, Trp, His plates containing various concentrations of 3-amino-1,2,4-triazole (3-AT), an inhibitor of the *HIS3* gene product, and incubated. A plasmid expressing the lexA binding domain-Adh1 fusion protein (a gift from David Shore) was used as a negative control.

### Primer extension analysis and northern blotting

Yeast strains expressing TAP-tagged assembly factors Ebp2, Brx1 or Nob1, were grown in rich medium containing galactose at 30°C to $3–5 \times 10^7$ cells/ml, harvested, and subjected to affinity purification, as described in Sahasranaman *et al.* (14). RNA enriched from these purified pre-ribosomes and ribosomes was extracted as described in Sahasranaman *et al.* (14). These pre-rRNAs and rRNAs were assayed by primer extension or northern blotting, as described in Horsey *et al.* (15). The sequences of primers used are available upon request.

### Affinity purifications of pre-ribosomes

Ribosome assembly intermediates were affinity-purified from whole cell extracts with magnetic Dynabeads (Invitrogen), using TAP-tagged assembly factors Nop7, Rpf2 or Rrp5, as described in Sahasranaman *et al.* (14).

### Western analysis

Western blotting was performed following standard techniques. Signals were visualized by Enhanced Chemiluminescence (Amersham), as instructed by the manufacturer, or otherwise using the colorimetric method (Promega). Mouse anti-myc monoclonal antibodies (9E10; BAbCO) and rabbit anti-Ebp2 polyclonal antibodies (8) were used. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (Amersham) were used in 1:1000 dilutions. TAP-tagged proteins were detected using alkaline phosphatase conjugated to IgG (Pierce). Otherwise, antibodies specific for r proteins or ribosomal assembly factors were used to detect proteins in purified pre-ribosomes. AP-conjugated anti-mouse or

anti-rabbit secondary antibodies (Promega) were used in 1:7500 dilutions in these experiments.

For SDS-PAGE, silver staining, and western blotting with samples from purified particles, proteins were recovered from affinity-purified pre-ribosomes by precipitation with 10% TCA, and suspended in SDS sample buffer. Proteins were resolved by SDS–PAGE on 4–20% Tris–Glycine Novex precast gels (Invitrogen), stained with silver by standard methods, and assayed by western blot analysis (16), with the following modification. To enable detection of multiple different proteins on one blot, and to conserve antiserum by using less volume of blotting buffer, nitrocellulose membranes were cut into smaller sections based on the previously established mobility of the different proteins.

## RESULTS

### *brx1* mutations cause a synthetic growth defect with the *ebp2-14* mutation

Previously we isolated temperature-sensitive *ebp2* mutants by random PCR mutagenesis of *EBP2*, and showed that they were defective in growth and 60S subunit biogenesis at 37°C, but not at 25°C (8). In order to obtain more details about the function of Ebp2, we sought yeast mutants that exhibit a synthetic growth defect with *ebp2-14* at temperatures permissive for its growth. We identified eight strains, each containing recessive mutations in a single gene (Figure 1A–C). Three strains were conditionally lethal (*m1, m4* and *m8*), and were not studied here. Five mutants (*m2, m3, m6, m7* and *m9*) were unconditionally synthetic lethal and formed one complementation group (data not shown), which upon cloning, proved to be the *BRX1* gene. *BRX1* encodes a ribosome assembly factor consisting of 291 amino acid residues. DNA sequence analysis revealed that each of the five sl *brx1*-mutants had a single point mutation in *BRX1*, leucine 52 to serine (*m9*; KM641), serine 102 to phenylalanine in two mutants (*m2*; KM634 and *m6*; KM638), phenylalanine 172 to leucine (*m3*; KM635), and leucine 227 to proline (*m7*; KM639). We named the alleles *brx1-52, brx1-102, brx1-172* and *brx1-227,* respectively. Brx1 is highly conserved in eukaryotes as a member of a superfamily that possesses a $\sigma^{70}$-like RNA-binding motif (17,18). Alignment of Brx1 homologs reveals that the mutated amino acids in *brx1-52, brx1-102* and *brx1-172* are highly conserved (Figure 1D). However, these amino acids are not conserved among the superfamily that consists of Brx1, Ssf1, Rpf1, Rpf2 and Imp4 (for alignment, see ref.19), suggesting that these residues are important for a specific function of Brx1. Leucine 227 is located at the C-terminal end of the $\sigma^{70}$-like RNA-binding motif in Brx1.

### Three of the four *brx1* single mutant strains exhibit no growth defect at any temperature, whereas *brx1-227* is temperature-sensitive

We constructed *brx1* single mutant strains, by integrating each of the four cloned mutant alleles into the genome of a *brx1* null strain, as described in 'Materials and Methods'.

The resulting *brx1-52, brx1-102* and *brx1-172* strains grew as well as wild-type *BRX1* strains at 16°C, 25°C, 30°C, 32°C, 35°C and 37°C, whereas the *brx1-227* strain exhibited weak temperature sensitivity at 37°C (Figure 2A). This result indicates that each *brx1* allele has little impact on cell growth, but is lethal in combination with *ebp2-14*.

To ascertain whether the *brx1-227* temperature-sensitive mutant is defective in 60S ribosome biogenesis at the restrictive temperature, we assayed levels of ribosomal subunits and polyribosomes in the mutant. The *brx1-227* strain was grown at 25°C and shifted to 37°C for 6 h, and extracts were prepared and subjected to centrifugation on 7–47% sucrose gradients. Amounts of free 60S subunits relative to 40S subunits decreased, and halfmer polysomes appeared in the extracts from the *brx1-227* mutant grown at 37°C (Figure 2B). In contrast, the *brx1-227* mutant maintained at 25°C or the wild-type *BRX1* strain shifted from 25°C to 37°C exhibited wild-type polysome profiles (Figure 2B). These results indicate that 60S subunit production is compromised in the *brx1-227* mutant at the restrictive temperature, 37°C, but not at temperatures permissive for growth.

### The synthetic growth defect with *brx1* is specific for the *ebp2* mutant

To further verify that *ebp2 brx1* double mutants are synthetically lethal, we constructed doubly heterozygous mutant diploids by crossing the *ebp2-14* mutant with each *brx1* mutant. These diploids were sporulated, and tetrads were dissected to obtain haploid spore clones. Each of the spores corresponding to *ebp2-14 brx1* double mutants, indicated by red triangles in Figure 2C, was inviable.

Then, we checked whether the synthetic lethality was specific for *brx1* mutants. To do so, we assayed whether a mutation in a different 60S ribosomal subunit assembly factor, Rrs1 (20), exhibits synthetic lethality with *brx1*. *EBP2* was first identified in a two-hybrid screen for interactions with Rrs1 (see Figure 3A; ref.6). In temperature sensitive *rrs1-124* yeast, 60S subunit biogenesis is compromised at the restrictive temperature (21). Tetrad analysis showed that *rrs1-124* did not exhibit synthetic lethality or a synthetic growth defect with any of the *brx1* alleles (Figure 2C). These results indicate that synthetic lethality is specific for *ebp2* and *brx1*.

### The combination of *ebp2-14* and *brx1* mutations eliminates the interaction of Ebp2 with Brx1 in three out of four double mutant strains

Consistent with the genetic interactions that we observe between *ebp2* and *brx1* mutations, we previously found that Ebp2 and Brx1 interact physically. *BRX1* was isolated in a two-hybrid screen using a yeast cDNA library, with *EBP2* as bait (9). In the screen, 55 out of 94 clones were identified as *BRX1*. The remaining clones included four other 60S assembly factor genes, *DBP9, LOC1, NOP12* and *RRP14*. *EBP2* and *RPF2* were isolated in the screen using *RRS1* as bait (6,22). The two-hybrid interaction of Ebp2 with Brx1 generated the
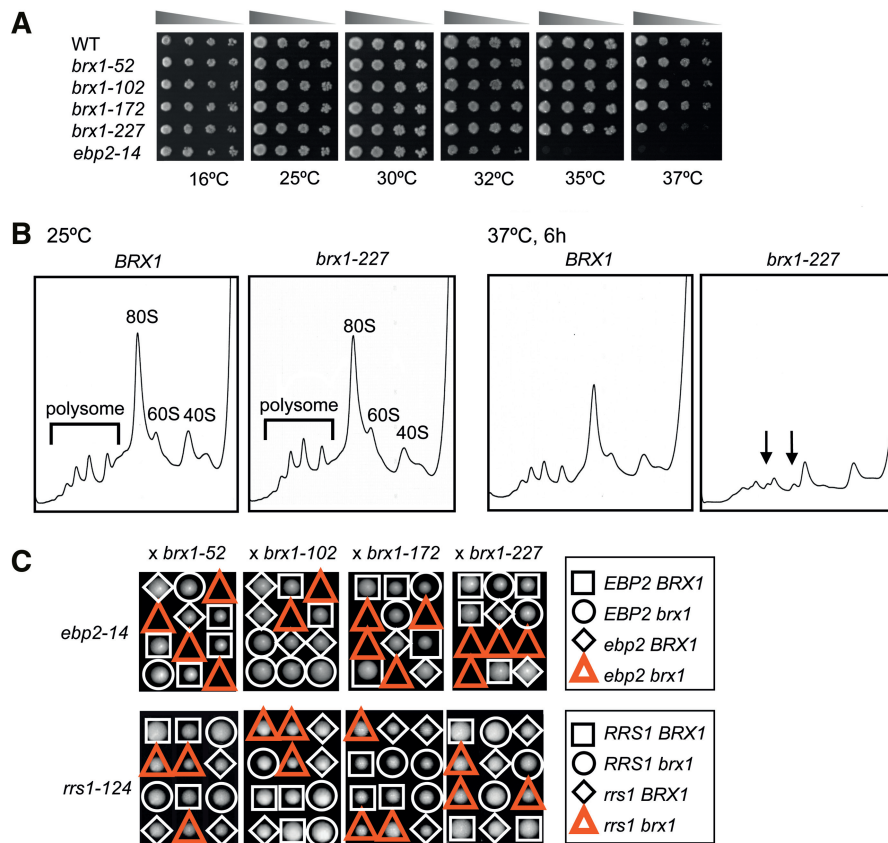
**Figure 2.** The synthetic lethality of *ebp2-14* with *brx1*. (**A**) *brx1* and *ebp2-14* single mutants grow under conditions that are lethal in the double mutants. The wild-type *BRX1* strain and *brx1-52, brx1-102, brx1-172, brx1-227* and *ebp2-14* single mutants were cultured in SC medium at 25°C overnight. Five-fold serial dilutions of the culture (OD$_{600}$ = ~1) were spotted onto YEPD medium, and incubated at 16°C for 10 days, and at 25°C, 30°C, 32°C, 35°C and 37°C for 3 days. (**B**) The temperature-sensitive *brx1-227* mutation leu227pro leads to a defect in 60S subunit biogenesis at 37°C. The KM1720 (*BRX1*) and KM1724 (*brx1-227*) strains were grown at 25°C overnight. The cultures were then shifted to 37°C and grown for 6 h. Cell extracts were used for polysome analysis by sucrose density gradient ultracentrifugation. Ribosomal profiles were determined by OD$_{254}$ measurement of the gradient (7–47%) fractions. Arrows indicate halfmer polysomes. (**C**) Specificity of the synthetic lethality of *ebp2-14* with *brx1*. Tetrad analysis of *ebp2 brx1* and *rrs1 brx1* diploids. Diploids constructed by crossing the *ebp2-14* or *rrs1-124* mutant with each *brx1* mutant were sporulated and meiotic progeny were analyzed by tetrad dissection. Colony formation was scored on plates containing glucose-medium incubated at 25°C for 7–8 days for *ebp2-14* (top) or 6 days for *rrs1-124* (bottom). Spores corresponding to double mutants are indicated by red triangles.

strongest response of reporter genes among all combinations (Figure 3A).

One possible explanation for the synthetic lethal phenotype of the *ebp2 brx1* double mutants is that the combination of mutations disrupts interaction between Ebp2 and Brx1. Therefore, we tested the effect of the *ebp2* and *brx1* mutations on the Ebp2-Brx1 two-hybrid interactions. A strong interaction, similar to that for wild-type proteins, was observed between wild-type Ebp2 and each mutant brx1 protein; these two-hybrid strains grew on 2 mM 3-AT (Figure 3B). However, when *ebp2-14* was combined with *brx1-52, brx1-102* or *brx1-227*, the interaction was abolished; none of these two-hybrid strains grew on medium even without 3-AT. Interestingly, the combination of *ebp2-14* and *brx1-172* did not affect their interaction.

### A combination of the *ebp2-14* and *brx1-102* or *brx1-172* mutations leads to synergistic defects in 60S subunit biogenesis and pre-rRNA processing

The observation that three of the *ebp2 brx1* sl mutations prevented physical interactions between Brx1 and Ebp2,

but the *ebp2 brx1-172* double mutation did not, led us to investigate the importance of the pairwise interaction between Brx1 and Ebp2 in ribosome biogenesis, downstream of docking of these proteins into pre-ribosomes. Because *ebp2 brx1* double mutants are lethal at all growth temperatures, we needed to construct a conditional lethal strain to investigate phenotypes of the double mutants in more detail. To do so, we built strains KM1732 and KM1733 containing *ebp2-14 brx1-102* or *ebp2-14 brx1-172*, respectively, plus a plasmid containing wild-type HA-tagged *EBP2* under the control of the galactose-dependent *GAL1* promoter (*GAL1-HA-EBP2:ADE2*). When these two strains were shifted from galactose-containing medium to glucose-containing medium, expression of *EBP2* was turned off, and the phenotypes of the *ebp2 brx1* double mutant could be revealed.

To examine effects on ribosome production, extracts were prepared from these strains grown at 25°C in galactose, and 16 and 24 h after shifting cultures from galactose to glucose medium. In the shifted strains, amounts of free 60S ribosomal subunits, 80S monoribosomes and
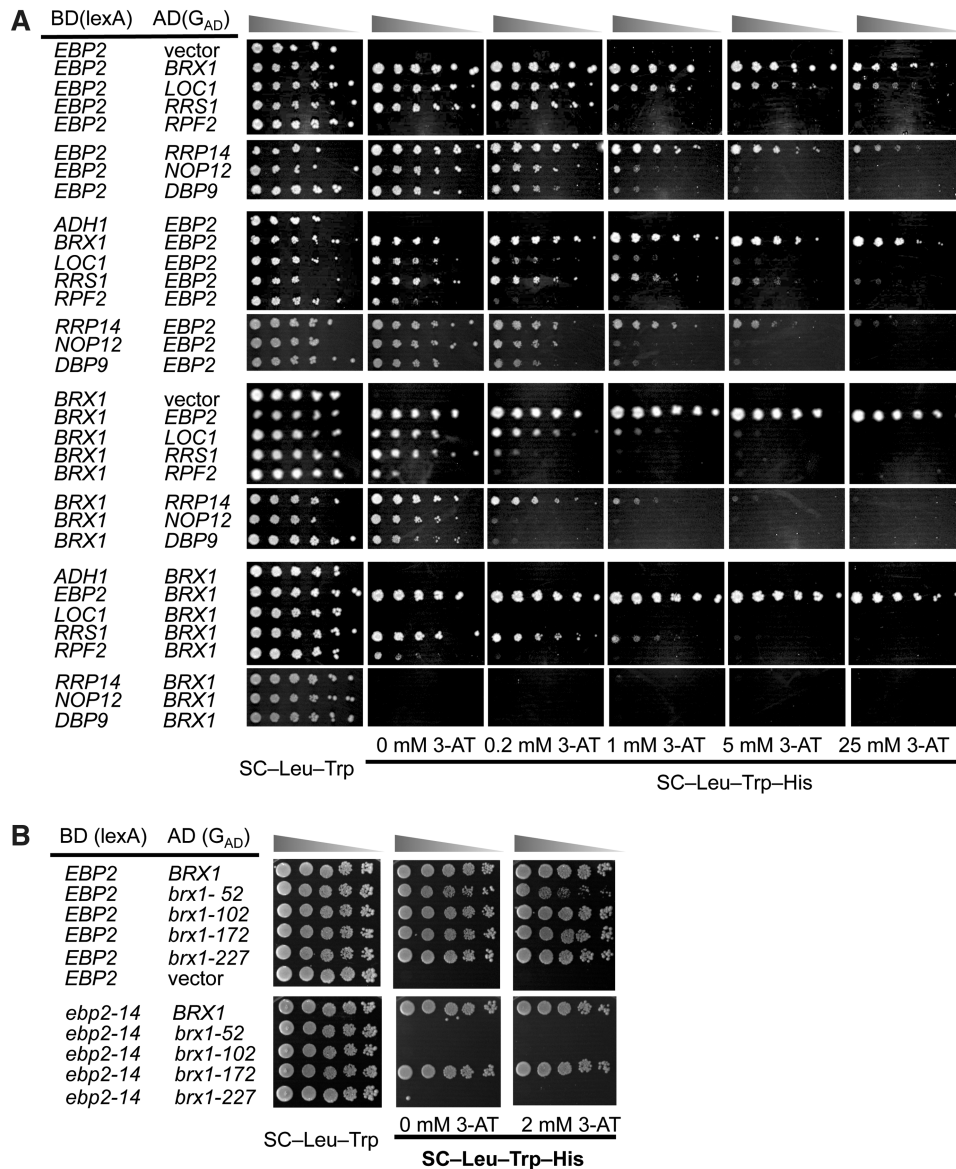
**Figure 3.** Wildtype Ebp2 and Brx1 exhibit a strong two-hybrid interaction, that is disrupted in some of the *ebp2 brx1* double mutants. (**A**) Protein–protein interactions were analyzed by the yeast two-hybrid system. Five-fold serial dilutions of cultures of L40 yeast transformed with lexA binding domain-fused and Gal4p activation domain-fused genes were plated on SC–Trp, Leu, or SC–Trp, Leu, His containing the indicated concentrations of 3-AT, and cultured at 25°C for 4–5 days. (**B**) Interactions were assayed as described in (A) above.

polyribosomes decreased, and halfmer polyribosomes appeared (Figure 4A). Polysome profiles of strains grown in galactose where *EBP2* was expressed but *brx1* is mutant, looked wild-type (Figure 4A, 0 h time point). Similarly, the original *ebp2-14* single mutant grown at 25°C had a wild-type polysome profile (8). Thus at 25°C, 60S subunit production is compromised in the *ebp2 brx1* double mutants, but not in any of the *ebp2* or *brx1* single mutants.

We examined pre-rRNA processing in the *brx1* and *ebp2* single mutants grown at 25°C and shifted to 37°C, as well as the conditional double mutants grown at 25°C, using primer extension assays to assay 5′-ends of pre-rRNAs. As shown in Figure 4B, lanes 1–3, amounts of pre-rRNAs containing $A_2$, $A_3$, $B_S$ or $B_L$ 5′-ends in the

*brx1-102* or *brx1-172* single mutants were similar to those in wild-type *BRX1* yeast. The *ebp2-14* mutant contained slightly higher levels of $27SA_2$ and $27SA_3$ pre-rRNAs relative to other pre-rRNAs, compared to the wild-type *EBP2* strain (Figure 4B, lanes 4 and 5). Lastly, both double mutant strains KM1732 and KM1733 shifted to glucose medium contained increased amounts of $27SA_2$ and $27SA_3$ pre-rRNAs compared to the unshifted strains expressing *EBP2* (Figure 4B, lanes 6–9).

## Brx1 and Ebp2 assemble into pre-ribosomes early, with pre-rRNPs containing 35S pre-rRNA

Before investigating the effect of the *ebp2* and *brx1* mutations on association of Ebp2 and Brx1 with pre-ribosomes,
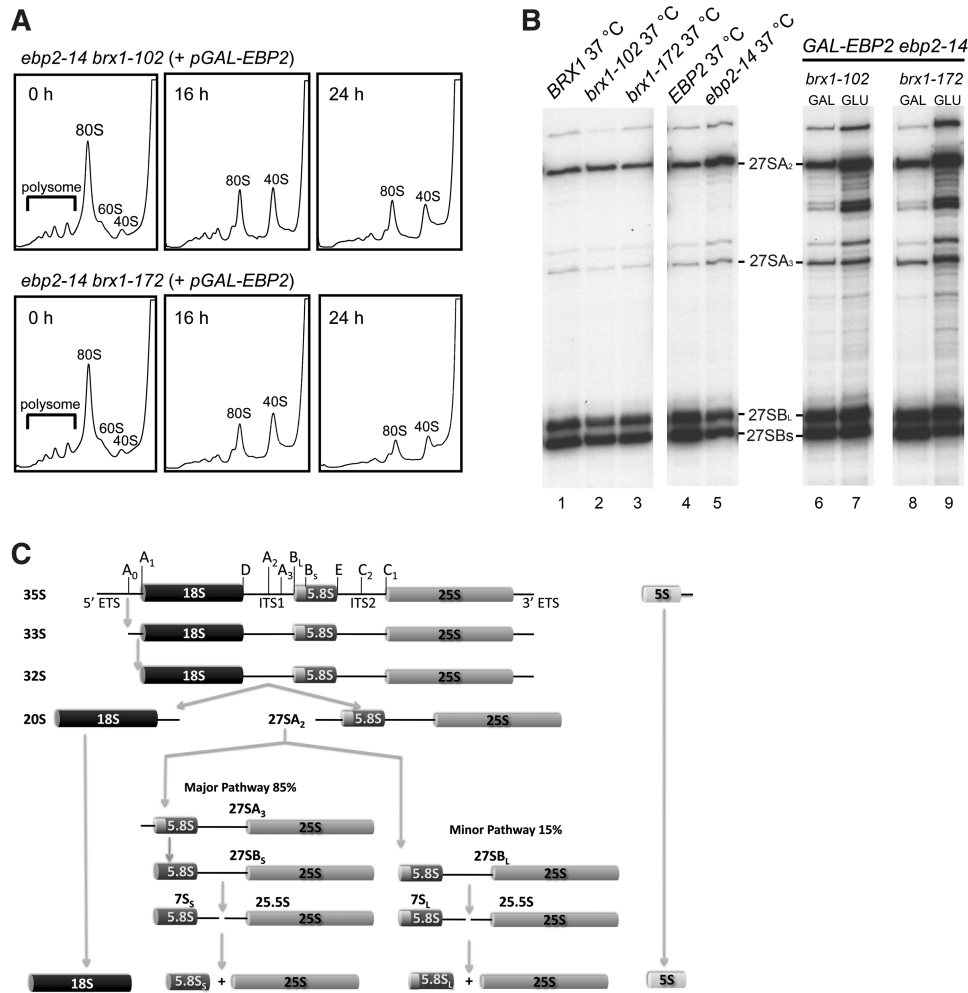
**Figure 4.** The synergistic defect of *ebp2* and *brx1* in biogenesis of 60S ribosomal subunits. The single mutant strains KM414 (*ebp2-14*), KM1722 (*brx1-102*) and KM1723 (*brx1-172*), were grown at 25°C and shifted to 37°C for 4 h. The KM1732 (*ebp2-14 brx1-102* p*GAL1-HA-EBP2*) and KM1733 (*ebp2-14 brx1-172* p*GAL1-HA-EBP2*) strains were grown in galactose medium at 25°C and shifted to glucose medium and grown at 25°C for 16 h and 24 h. (**A**) Cell extracts were used for polysome analysis by sucrose density gradient ultracentrifugation. (**B**) Pre-rRNA processing was assayed by primer extension. (**C**) Pre-rRNA processing pathway.

we wanted to determine with which pre-ribosomes they are associated. Brx1 and Ebp2 are routinely detected by mass spectrometry in pre-ribosomes affinity-purified using TAP-tagged early or middle assembly factors, but not with late assembly factors (reviewed in ref. 2). Sucrose gradients confirmed co-sedimentation of Brx1 and Ebp2 with 66S pre-ribosomes (Figure 5A). Myc-tagged Brx1 also sedimented in fractions at the top of the gradient.

To determine more precisely when Brx1 and Ebp2 enter the pathway of ribosome assembly, we assayed with which of the consecutive pre-rRNA processing intermediates TAP-tagged Brx1 and Ebp2 co-purify. Primer extension and northern blotting indicated that 35S, 27S and 7S pre-rRNAs co-purified with Brx1-TAP and Ebp2-TAP, whereas significantly smaller amounts of pre-rRNAs were detected with Nob1-TAP, a component of 43S precursors to 40S subunits, or with untagged control samples (Figure 5B). The 5.8S and 25S rRNAs also copurified with TAP-tagged Ebp2 or Brx1 above control levels, although the amount that copurified with Ebp2-TAP was much less

than with Brx1-TAP. This suggests that Ebp2 dissociates from pre-ribosomes before Brx1 or else that Ebp2 is less stably associated with late pre-ribosomes and fails to copurify with large amounts of mature rRNAs. Taken together, these results suggest that both Brx1 and Ebp2 assemble into 90S pre-ribosomal particles containing 35S pre-rRNA and are also present in all 66S pre-ribosomal particles (Figure 5B), consistent with their role in early steps of pre-rRNA processing (Figure 4B).

## Interaction between Ebp2 and Brx1 is not necessary for their assembly into pre-ribosomes

To determine the effect of sl mutations of *ebp2* and *brx1* on association of these proteins with pre-ribosomes, we began by assaying the ability of the *ebp2-14* mutant protein to compete with wild-type Ebp2 for association with pre-ribosomes. When myc-tagged *ebp2-14* was co-expressed with wildtype Ebp2 in a wild-type *BRX1* background, only wild-type Ebp2 protein cosedimented with pre-ribosomes on sucrose gradients, even though
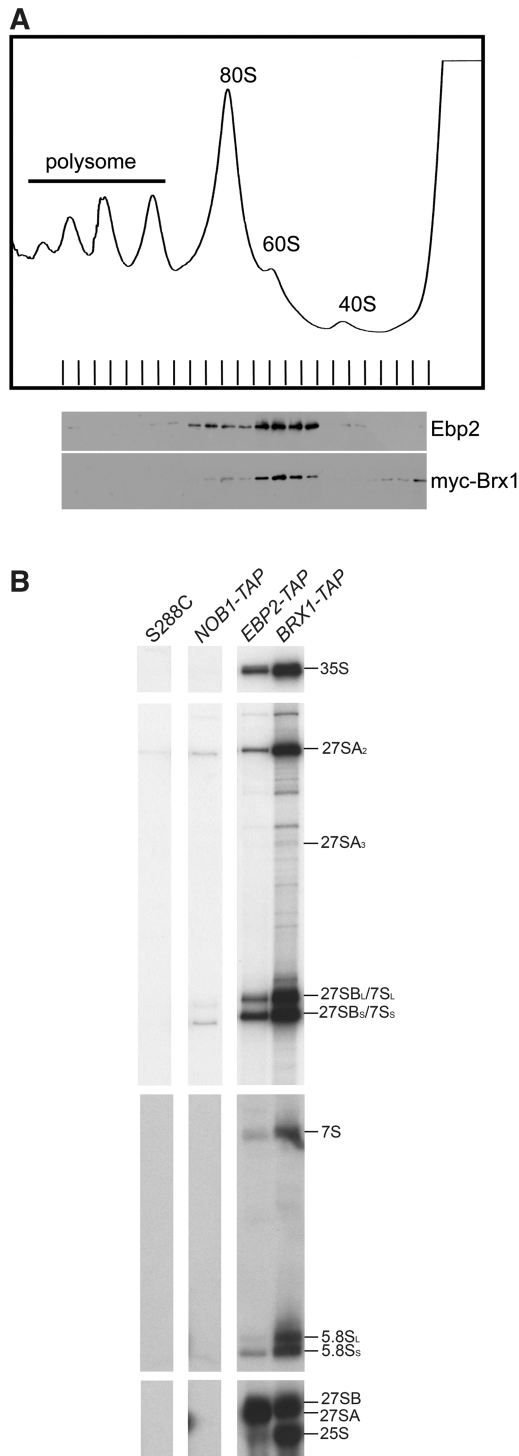
**Figure 5.** Both Brx1 and Ebp2 assemble early into pre-ribosomes. (**A**) KM1725 (*myc-BRX1*) cells were grown at 25°C to mid-log phase, then cell extracts were prepared and subjected to sucrose density gradient ultracentrifugation to resolve ribosomal subunits, monoribosomes and polyribosomes. A ribosomal profile was determined by measurement of $OD_{254}$ of gradient (7–47%) fractions. Each fraction was collected and subjected to SDS–PAGE and western analysis using antibodies against Ebp2 and the myc epitope. (**B**) Both Ebp2 and Brx1 associate with 90S pre-ribosomes containing 35S pre-rRNA, as well as 66S pre-ribosomes containing 27S pre-rRNAs. TAP-tagged Ebp2 and Brx1 were used to affinity-purify pre-ribosomes. Pre-RNAs contained in these pre-rRNPs were identified by primer extension and gel electrophoresis. The untagged parent strain S288c and Nob1-Tap were used as controls.

both forms of the Ebp2 protein were present in cells (Figure 6A). Upon depletion of Ebp2 only ebp2-14 was expressed, and it cosedimented with pre-ribosomes, although an equal amount sedimented at the top of gradients (Figure 6A). This result demonstrates that the ebp2 mutant protein does not stably associate with pre-rRNPs as strongly as the wild-type Ebp2 even at 25°C. However, when the wild-type Ebp2 protein is absent, the mutant ebp2 protein can enter pre-ribosomes.

We then investigated the effect of the synthetic lethal *ebp2 brx1* mutations on association of Ebp2 and Brx1 with pre-ribosomes. In order to purify pre-ribosomes, we TAP-tagged assembly factors Nop7 or Rpf2 in KM1732 (*GAL-HA-EBP2 ebp2-14 myc-brx1-102*) and KM1733 (*GAL-HA-EBP2 ebp2-14 myc-brx1-172*). Both Nop7 and Rpf2 co-purify with 90S and 66S pre-ribosomes (23,24), and thus can be used to purify the precursors to 60S subunits and survey changes in them. These strains were grown at 25°C in galactose-containing medium, and shifted to glucose-containing medium to deplete wild-type Ebp2 protein and uncover the sl interaction. Pre-ribosomes were affinity-purified from each strain and proteins in them were resolved by SDS–PAGE (Figure 6B). Western blotting was used to assay amounts of Ebp2 and Brx1 in each sample of pre-ribosomes (Figure 6C). The wild-type HA-tagged Ebp2 and the mutant ebp2-14 protein could be distinguished using anti-Ebp2 antisera, which cross-reacted with both proteins. The HA tag on wild-type Ebp2 causes it to migrate more slowly on gels than the mutant ebp2-14 protein. The mutant Brx1 proteins were identified using a myc tag. In both *ebp2-14 brx1-102* and the *ebp2-14 brx1-172* double mutant strains, amounts of brx1-102 and brx1-172 mutant proteins in pre-ribosomes were unchanged when wild-type Ebp2 was depleted and ebp2-14 could enter pre-ribosomes (Figure 6C). Since the interaction at permissive temperature between ebp2-14 and brx1-102 was shown to be disrupted (Figure 3), but was still intact between ebp2-14 and brx1-172, we conclude that the strong interaction between Ebp2 and Brx1 is not necessary for their assembly into pre-ribosomes. Alternatively, it is possible that the interaction seen between the mutant ebp2 and brx1 proteins in the context of the two-hybrid assay is disrupted, but within pre-ribosomal particles these two proteins are held in close proximity by the surrounding proteins or RNA structure.
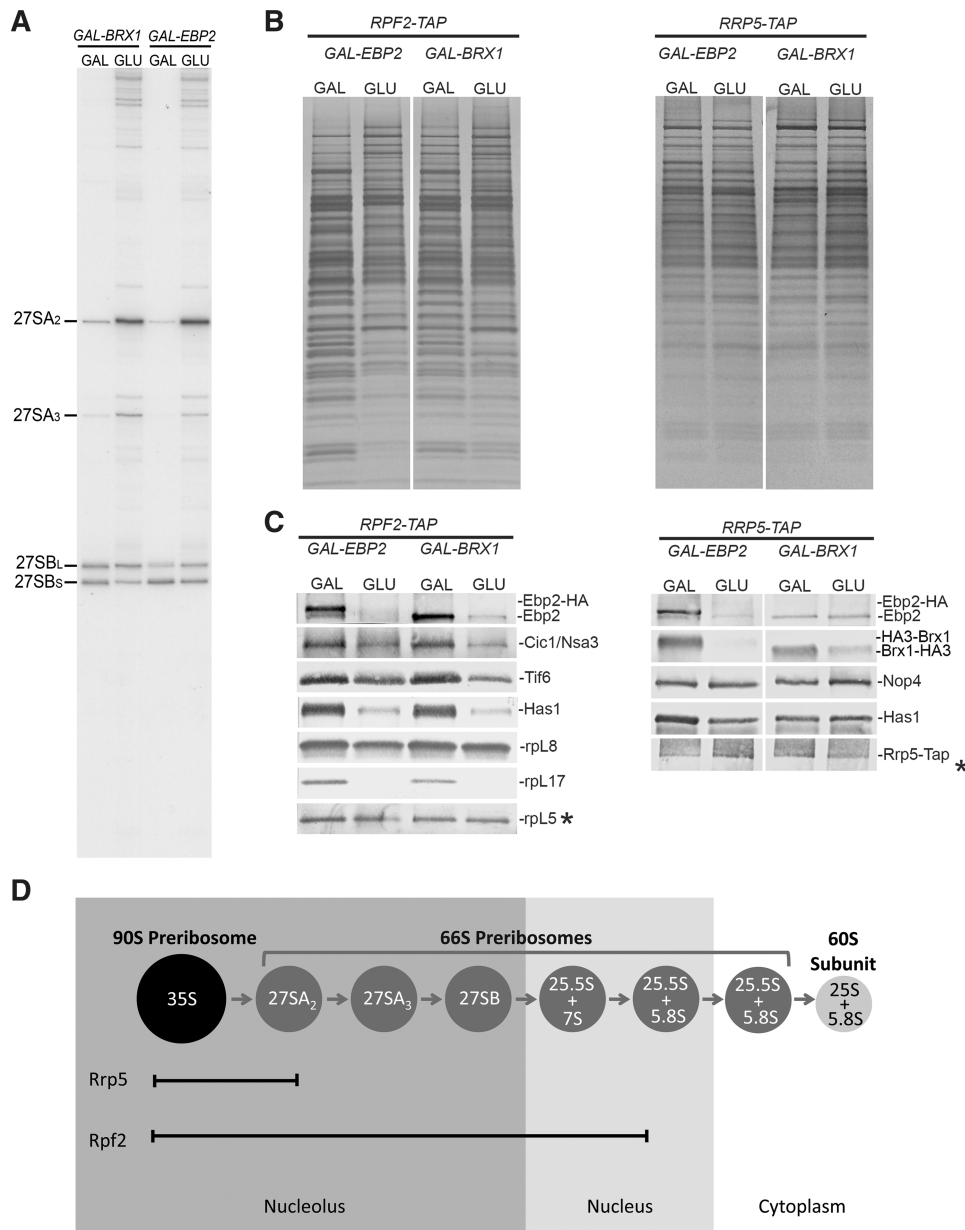
### Brx1 depends on Ebp2, but Ebp2 does not require Brx1 to assemble into pre-ribosomes

To further investigate the interdependence of Brx1 and Ebp2 for assembly into pre-ribosomes, we assayed the effects of depleting either protein, using *GAL* promoter fusions. As previously reported (6,25), depletion of either protein is lethal, causing a block in processing of $27SA_2$ and $27SA_3$ pre-rRNA to 27SB pre-rRNA (Figure 7A), and consequently decreased production of 60S ribosomal subunits. To purify pre-ribosomes, we TAP-tagged Rpf2, an assembly factor required for pre-rRNA processing steps after those involving Ebp2 or Brx1 (24). To assay association of Ebp2 and Brx1 with pre-ribosomes when
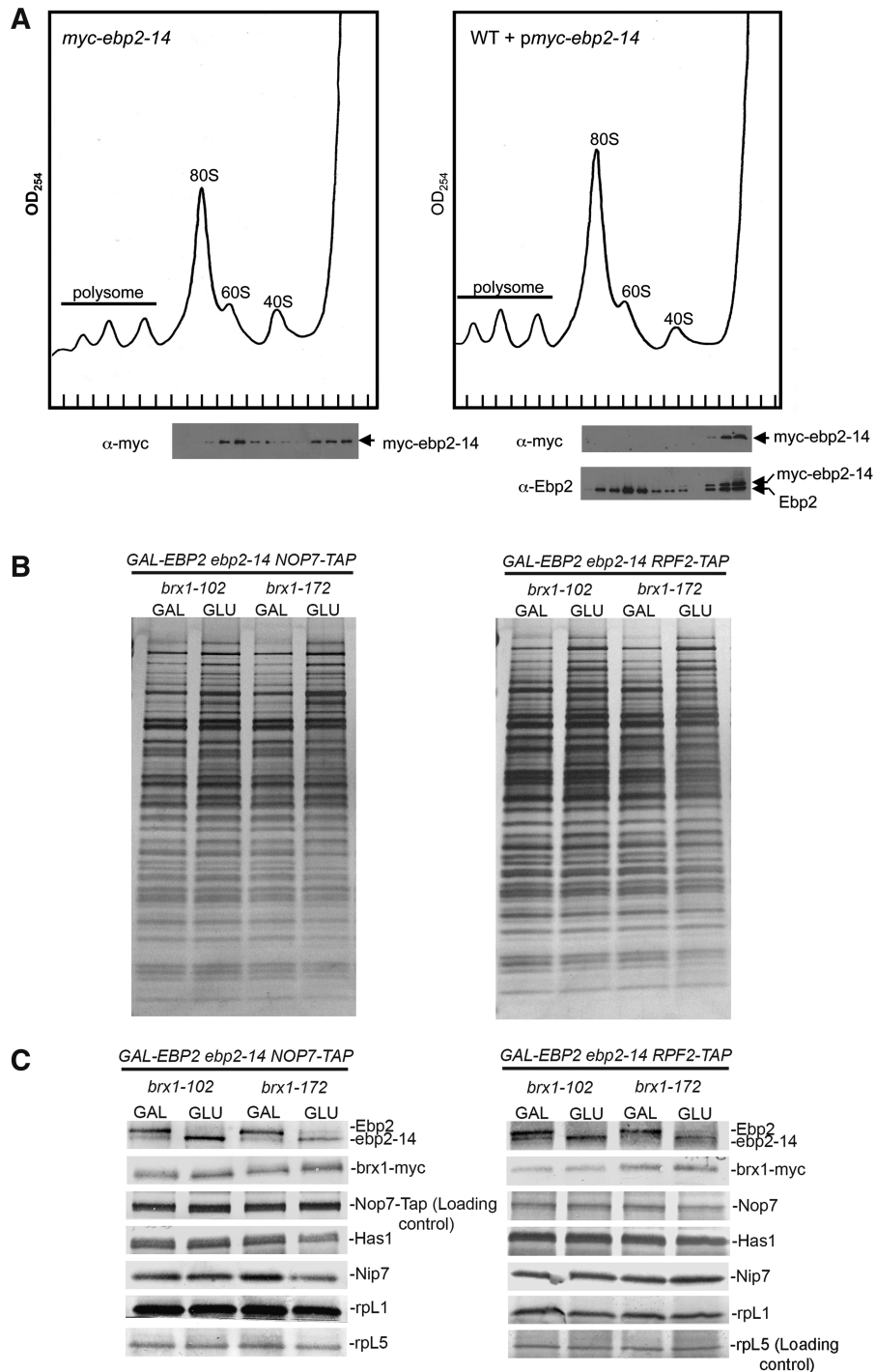
**Figure 6.** The synthetic lethal mutant ebp2-14 and brx1-102 proteins can assemble into pre-ribosomes, even though they have lost ability to interact with each other in the two-hybrid system. (**A**) A defect of the mutant ebp2-14 protein at 25°C is revealed by a competition assay. The ebp2–14 mutant protein can assemble into pre-ribosomes, but not when wild-type Ebp2 protein is present. KM1514 (*myc-ebp2*; left) and wild-type (*EBP2*) transformed with a plasmid expressing myc-ebp2-14 under the *EBP2* promoter (right) were cultured at 25°C. Extracts were prepared from the unshifted and shifted strains and subjected to centrifugation on 7–47% sucrose gradients. Proteins were precipitated from each fraction with 10% TCA. Amounts of wildtype Ebp2 or mutant ebp2 in pre-ribosomes were assayed by western blot analysis using anti-Ebp2 antisera. Anti-myc antibody was used to detect only myc-tagged ebp2-14 protein. (**B**) The *GAL-EBP2 ebp2-14 brx1-102* and *GAL-EBP2 ebp2-14 brx1-172* strains containing *NOP7-TAP* (left), and the *GAL-EBP2 ebp2-14 brx1-102* and *GAL-EBP2 ebp2-14 brx1-172* strains containing *RPF2-TAP* (right) were grown in galactose medium at 25°C and shifted to glucose-containing medium for 16 h. Pre-ribosomes were affinity-purified using TAP-tagged assembly factor Nop7 (left) or Rpf2 (right). Proteins present in purified pre-ribosomes were resolved by SDS–PAGE and stained with silver. (**C**) Amounts of HA-tagged wild-type Ebp2 protein, ebp2-14 mutant protein and myc-tagged brx1 mutant protein were assayed by western blotting using antibodies against Ebp2 and myc. Antibodies against other assembly factors and rp were used as controls. Loading controls are marked with an asterisk. Wild-type HA-tagged Ebp2 could be distinguished from mutant ebp2-14 due to the altered electrophoretic moblity caused by the HA tag.

**Figure 7.** Brx1 depends on Ebp2 to assemble into pre-ribosomes, but Ebp2 does not require Brx1. *GAL-EBP2* and *GAL-BRX1* yeast cells were grown in galactose-containing medium, or shifted to glucose-containing medium for 16 h. (**A**) The pre-rRNA processing defect in *GAL-EBP2* and *GAL-BRX1*. RNA was extracted from unshifted and shifted strains and assayed by primer extension. (**B**) SDS–PAGE of proteins in pre-ribosomes purified from strains containing or lacking Ebp2 and Brx1 are shown. Pre-ribosomes were affinity-purified from each culture, using TAP-tagged assembly factor Rpf2 (left) or Rrp5 (right), and proteins in them were resolved by SDS-PAGE and stained with silver. (**C**) Western blot analysis of association of HA-tagged Ebp2 and HA-tagged Brx1 with pre-ribosomes, upon depletion of Brx1 or Ebp2 respectively. Loading controls are marked with an asterisk. (**D**) Schematic representation of pre-ribosomal particles co-purifying with Rpf2-Tap and with Rrp5-Tap.

either protein was depleted, we epitope-tagged Brx1 in the *GAL-EBP2* strain, and used Ebp2 antibody in the *GAL-BRX1* strain. SDS-PAGE of proteins in pre-ribosomes purified from either depletion strain revealed significant changes compared to undepleted strains (Figure 7B, left, Figure 7C, left). Although many bands decreased upon depletion of Ebp2 and Brx1, we noticed an enrichment of early assembly factors relative to late factors and r proteins. Thus these effects, including those on Ebp2 and Brx1, may simply reflect the absence of

middle and late assembly intermediates in these two depleted strains blocked in the production of the later pre-ribosomes. Therefore, in order to be able to better compare pre-ribosomes from undepleted and depleted samples, we TAP-tagged Rrp5, an assembly factor that enters pre-ribosomes early, associating with 90S pre-ribosomes, and that leaves the pathway before the 27SA₃ pre-rRNA processing step (J. Talkish, unpublished). Thus using Rrp5-TAP, we could more specifically assay changes in early particles. When Rrp5-TAP was

used to purify pre-ribosomal particles in the presence and the absence of Ebp2 or Brx1, many fewer changes in pre-ribosomal proteins in depleted strains were visible by silver staining (Figure 7B, right). Western blotting of proteins in these pre-ribosomes demonstrated that HA-tagged Brx1 was absent upon depletion of Ebp2. However, when Brx1 was depleted, amounts of Ebp2 in pre-ribosomes were not changed (Figure 7C, right). Thus, Brx1 depends on Ebp2 to stably associate with early pre-ribosomes, but not vice versa. This result suggests that there may be molecules in pre-ribosomes other than Brx1 that are important for association of Ebp2 with pre-rRNPs, but which do not strictly depend on the presence of Brx1 to associate with pre-rRNPs or to maintain Ebp2 in pre-rRNPs.

## DISCUSSION

Previously Ebp2 and Brx1 were identified as phylogenetically conserved nucleolar proteins involved in early steps of 60S ribosomal subunit assembly in yeast. In the *ebp2-1* temperature-sensitive mutant, processing of $27SA_2$ pre-rRNA is perturbed (7). Upon depletion of Brx1, $27SA_2$ and $27SA_3$ pre-rRNAs accumulate and $27SB_S$ pre-rRNA is diminished (25). Here, we have identified *brx1* mutations that exhibit synthetic lethal interactions with the *ebp2-14* mutation. Three of these four *brx1* mutations also eliminate the strong two-hybrid interaction between Ebp2 and Brx1. Despite this inability to interact with each other, both mutant ebp2 and brx1 proteins can associate stably with pre-ribosomes. Nevertheless, these sl interactions result in defects in pre-rRNA processing. Thus, these interactions may enable us to investigate functions of Ebp2 and Brx1 after their initial docking into pre-rRNPs. We discuss a broader network of physical and functional interactions in which Ebp2 and Brx1 might participate to enable early steps of 60S subunit biogenesis.

We previously suggested that Ebp2 might enter 90S pre-ribosomal particles based on two-hybrid data demonstrating that Ebp2 associates with the 40S subunit assembly factors Utp11 and Faf1, and r protein S16 (9). Consistent with Ebp2 and Brx1 functioning early in assembly, both proteins copurify with ribosome assembly factors that function at early or middle steps in the pathway (2). Using the consecutive pre-rRNA processing intermediates as landmarks, we also found that Ebp2 and Brx1 are associated with the 35S pre-rRNA present in 90S pre-ribosomes, as well as each of the 27S pre-rRNAs found in 66S pre-rRNPs (Figure 5B). Neither of these experimental approaches distinguishes the order in which Ebp2 and Brx1 dock with nascent particles. However, two observations suggest that Ebp2 might assemble before Brx1: (i) Brx1 depends on Ebp2 to associate with pre-ribosomes, but not vice versa (Figure 7C, right). (ii) Depletion of Brx1 blocks $27SA_3$ pre-rRNA processing downstream of the step blocked by depletion of Ebp2 (Figure 7A). Nevertheless, a higher resolution view of the assembly hierarchy awaits better tools to investigate ribosome biogenesis.

The genetic interactions between *ebp2-14* and *brx1* could provide additional insight into the function of Ebp2 and Brx1. At 25°C, each of the single *ebp2* or *brx1* missense mutants has no apparent defect in growth or in production of 60S ribosomal subunits (Figure 2A, Figure 4A, see ref.8). Even the *brx1-227* mutant, which is temperature sensitive for growth, appears not to have a defect in production of 60S ribosomal subunits at 25°C (Figure 2B). In contrast, the *ebp2-14 brx1* double mutants are unable to grow, and are defective in production of 60S subunits and processing of $27SA_2$ and $27SA_3$ pre-rRNAs (Figure 2C, 4A). The *ebp2-14* mutation together with *brx1-52, brx1-102* or *brx1-227* mutations prevents stable interaction of ebp2 and brx1 mutant proteins with each other in a two-hybrid assay (Figure 3B). The combination of *ebp2-14* and *brx1-172* has little effect on the interaction. As the combination of *ebp2-12* (other allele, see ref. 8) and *brx1-172* also has no effect on the interaction (data not shown), we speculate that the brx1-172 mutant protein, similar to Brx1, might interact with ebp2 mutant proteins as well as with Ebp2. Despite the loss of interaction, both *ebp2-14* and *brx1-102* mutant proteins can efficiently assemble into nascent ribosomes in the double mutants, as well as the *ebp2-14 brx1-172* mutant proteins where the two-hybrid interaction is not abolished (Figure 6B). This result indicates that other protein–protein or protein–RNA interactions within assembling ribosomes must enable Ebp2 and Brx1 to stably associate with pre-ribosomes. A similar result for assembly factors Imp4 and Mpp10 was found previously by Gallagher and Baserga (26). Mutations in Imp4 that disrupt two-hybrid interactions with Mpp10, also cause a slow growth phenotype and perturb pre-rRNA processing, but nevertheless do not prevent either protein from associating with the SSU processome.

Although the ebp2-14 and the brx1-102 mutant proteins can assemble into pre-ribosomes, the *ebp2-14 brx1-102* double mutant is defective in processing of $27SA_2$ and $27SA_3$ pre-rRNAs, more so than either single mutant (Figure 4B). Furthermore, the *ebp2-14 brx1-172* double mutant in which the Ebp2–Brx1 interaction is not diminished, nevertheless produces fewer 60S subunits than either single mutant, and is defective in processing of $27SA_3$ as well as $27SA_2$ pre-rRNAs (Figure 4B).

The effects on ribosome assembly upon depleting either Ebp2 or Brx1 support the existence of a broader network of pre-ribosomal interactions with Brx1 and Ebp2 (Figure 8). Brx1 depends on Ebp2 to associate with early pre-ribosomes, but Ebp2 does not require Brx1 to do so (Figure 7C, right). Thus, there must be other molecules in pre-rRNPs not affected by the absence of Brx1 that help anchor Ebp2 in pre-rRNPs. On the other hand, Brx1 may not depend on direct interactions with Ebp2 to assemble, but on a third molecule in pre-ribosomes, whose presence or anchoring function is diminished by the absence of Ebp2 (Figure 8).

The best candidates for factors that help Ebp2 or Brx1 enter pre-ribosomes and function in these particles are four other ribosome assembly factors and r proteins found in subcomplexes together with Ebp2 or Brx1. Krogan *et al.* (27) found that Brx1 copurified with Nop12
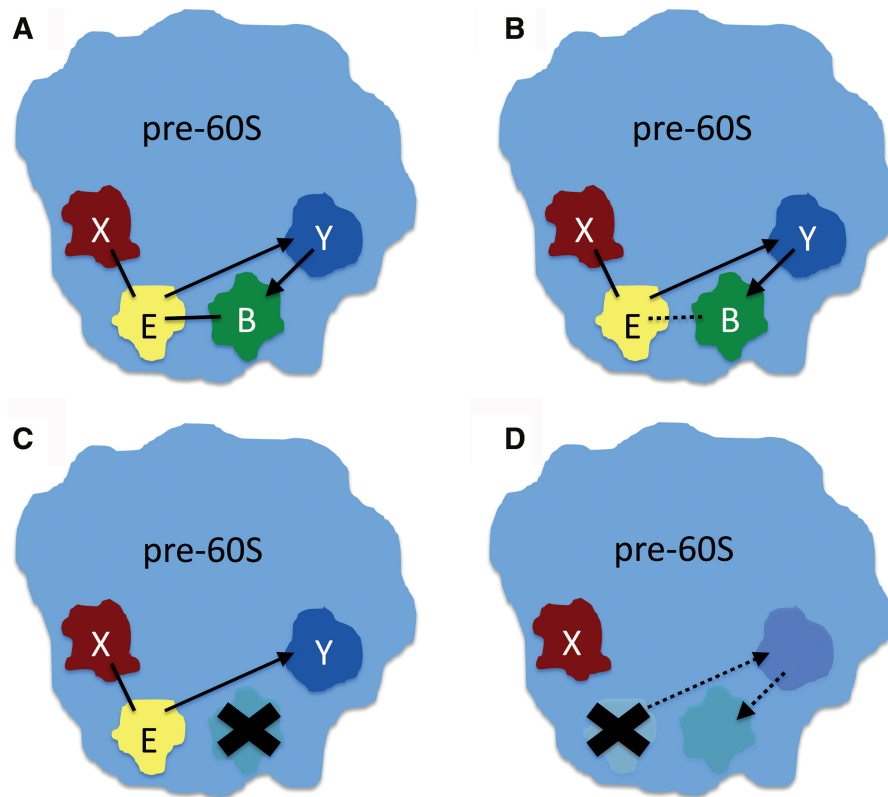
**Figure 8.** Model of dependence of Brx1 on Ebp2 for assembly into pre-ribosomes. Ebp2 (E) and Brx1 (B) are represented with yellow and green, respectively. X (red) and Y (blue) represent other proteins present in pre-ribosomes. Each X and each Y can represent one or more proteins. Black lines mark interactions. Loss of interaction is shown as dashed line. Arrows point FROM the protein required to recruit another protein into pre-ribosomes TO the protein dependent on the first protein to be in pre-ribosomes. (**A**) In the wild-type, Ebp2 and Brx1 interact with each other and each interacts with other proteins within pre-ribosomes (pre-60S). (**B**) Loss of interaction between Ebp2 and Brx1 does not cause either protein to be absent from pre-ribosomal particles, presumably due their interactions with other proteins. (**C**) In the absence of Brx1, Ebp2 is still present in pre-ribosomes. (**D**) The absence of Ebp2 affects one or more proteins that stabilize the association of Brx1 with pre-ribosomes. In their absence, Brx1 is absent from pre-ribosomes.

and Pwp1, when TAP-tagged Pwp1 was used for affinity purification from high speed supernatants of whole cell extracts from which intact pre-ribosomes and ribosomes were removed. By a similar approach, Zhang *et al.* (28) found that Ebp2, Brx1, Nop12 and r proteins L8 and L15 copurified with TAP-tagged Pwp1. Consistent with these observations, Ebp2 interacts not only with Brx1 in two-hybrid assays, but also with Nop12 (Figure 3A).

The association of Brx1 and Ebp2 with these four assembly factors and r proteins provides some hints about their possible location within pre-ribosomes. The r proteins L8 and L15 lie adjacent to each other in mature 60S ribosomal subunits, near the proximal stem formed by base-pairing of the 3′-end of 5.8S rRNA with the 5′-end of 25S rRNA (4,5). During biogenesis of the 60S subunit, these 5′-and 3′-ends of rRNA are formed by processing of the internal transcribed spacer 2 (ITS2) that lies between them. Nop12 cross-links to nucleotides immediately adjacent to the proximal stem, but distal from ITS2 (29). This physical neighborhood of six pre-ribosomal proteins, including Brx1 and Ebp2, is also functionally interconnected. The phenotype of *nop12*, *pwp1* and *rpl8* mutants is similar to that of *brx1* and *ebp2* mutants: $27SA_2$ and $27SA_3$ processing are perturbed (JJ and JT, unpublished).

The 'A3 cluster' of assembly factors required for processing of $27SA_3$ pre-rRNA (14) may also be part of a larger local neighborhood of proteins that interact with Ebp2 and Brx1 and function together with them. Erb1 and Nop7 crosslink to rRNA in domains I and III near the proximal stem (29), and Ytm1 binds directly to Erb1 (30). Nop15 and Cic1 cross-link to sequences in ITS2, which lies adjacent to the proximal stem (29). Thus, it seems likely that Ebp2 and Brx1 are located in pre-ribosomes near the proximal stem and function together with Nop12, Pwp1, r proteins L8 and L15, and perhaps other nearby $A_3$ factors, to create RNP structures necessary for early steps of 27S pre-rRNA processing.

How might the genetic and physical interactions between Ebp2 and Brx1, and between these two proteins and the other pre-ribosomal proteins described above, relate to their function in ribosome assembly and pre-rRNA processing? Their overlapping mutant phenotypes indicate that these proteins participate either directly or indirectly in processing of $27SA_2$ pre-RNA and subsequently $27SA_3$ pre-rRNA. This first processing step involves endonucleolytic cleavage at the $A_3$ site in the internal transcribed spacer 1 (ITS1) by the RNase MRP endonuclease (31–33). Subsequently, 77 nt at the 5′-end of

the resulting 27SA$_3$ pre-rRNA are removed by the 5′–3′ exonucleases Rat1, Xrn1 and Rrp17, stopping precisely at the B$_{1S}$ site, to produce 27SB$_S$ pre-rRNA (34,35). In both steps, the proper timing and efficiency of cleavage and processing of these sequences in ITS1 must depend upon the pre-rRNA substrates presenting the appropriate conformation to be recognized and acted upon by the nucleases. Curiously, five A$_3$ factors, as well as r proteins L8 and L15, are associated with pre-rRNA near the 3′-end of 5.8S rRNA, perhaps some distance from ITS1 located upstream of the 5′-end of 5.8S rRNA (29). It seems likely that these seven proteins, which bind RNA but lack any apparent enzymatic activities, may function in these processing steps by establishing conformations of pre-rRNA to create long-range interactions between the 5′-and 3′-ends of 5.8S rRNA, analogous to allosteric interactions that occur within the ribosome during protein synthesis (36). As pointed out by Granneman *et al.* (29), the A$_3$ factors may coordinate folding and processing at the 5′-and 3′-ends of 5.8S rRNA to ensure that ITS1 processing occurs after proper folding of ITS2, and before removal of ITS2.

We recently found that Ebp2 and Rrs1 are localized at the nuclear periphery as well as the nucleolus and play roles in telomere organization and silencing (37). Although it remains to be elucidated if Brx1 has similar functions, the synthetic lethality of *brx1* with *ebp2* reflects cooperative functions of the two proteins in ribosome biogenesis, not in other functions, because suppression of a defect in telomere organization of *ebp2-14* did not suppress either a defect in temperature sensitivity for growth or a defect in ribosome biogenesis (37).

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table S1.

## ACKNOWLEDGEMENTS

## FUNDING

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