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Bcp1 Is the Nuclear Chaperone of Rpl23 in *Saccharomyces cerevisiae*[*]

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Eukaryotic ribosomes are composed of rRNAs and ribosomal proteins. Ribosomal proteins are translated in the cytoplasm and imported into the nucleus for assembly with the rRNAs. It has been shown that chaperones or karyopherins responsible for import can maintain the stability of ribosomal proteins by neutralizing unfavorable positive charges and thus facilitate their transports. Among 79 ribosomal proteins in yeast, only a few are identified with specific chaperones. Besides the classic role in maintaining protein stability, chaperones have additional roles in transport, chaperoning the assembly site, and dissociation of ribosomal proteins from karyopherins. Bcp1 has been shown to be necessary for the export of Mss4, a phosphatidylinositol 4-phosphate 5-kinase, and required for ribosome biogenesis. However, its specific function in ribosome biogenesis has not been described. Here, we show that Bcp1 dissociates Rpl23 from the karyopherins and associates with Rpl23 afterward. Loss of Bcp1 causes instability of Rpl23 and deficiency of 60S subunits. In summary, Bcp1 is a novel 60S biogenesis factor via chaperoning Rpl23 in the nucleus.

The ribosome is a large macromolecular complex responsible for decoding cellular genetic information into proteins. There are two ribosomal subunits; in the eukaryotes, they are the (60S) large subunit and the small (40S) subunit, each composed of both rRNAs and ribosomal proteins. In eukaryotic cells, the assembly of ribosomes occurs in the nucleolus, a subcompartment of the nucleus devoted to ribosome biogenesis. rRNAs are transcribed by RNA polymerase I and III, and the initial assembly and processing events occur co-transcriptionally. The mRNAs of ribosomal proteins are transcribed in the nucleus by RNA polymerase II and translated in the cytoplasm. Consequently, the ribosomal proteins must be imported into the nucleus for assembly with the rRNAs. After ribosome assemblies have achieved a certain stage of maturation, export factors are loaded to direct these huge complexes to cross the nuclear membrane through the nuclear pore complexes (1–6). In the cytoplasm, ribosomes undergo additional steps in the maturation process; the trans-acting factors that are exported with nascent ribosomal subunits need to be stripped off, additional ribosomal proteins are added, and in the case of the 40S subunit, the final rRNA processing occurs (7, 8). The synthesis of ribosomes requires the coordination of many non-ribosomal trans-acting factors at different stages for this pathway to be completed. About 200 trans-acting factors are involved in rRNA processing, rRNA folding, protein loading, and export. To date, more trans-acting factors have continued to be identified (7, 9, 10).

BCP1 is an essential gene involved in the export of Mss4 (11), a phosphatidylinositol (PI)[2] 4-phosphate 5-kinase that catalyzes the phosphorylation of PI4-phosphate and acts with the PI4-kinase, Stt4p, at the plasma membrane to generate $PI4,5P_2$ (12, 13). Phosphoinositols are critical small signaling molecules that regulate cellular functions. The synthesis of $PI4,5P_2$ is essential for sporulation, endocytosis, membrane trafficking, and normal organization of the actin cytoskeleton (14, 15). In a *bcp1* mutant, Mss4 accumulated in the nucleus, resulting in reduced levels of $PI4,5P_2$ in the cell (11).

Bcp1 was further characterized as a 60S biogenesis factor in a high throughput screen of potential factors involved in ribosome biogenesis in yeast (16). In that work, Bcp1 was shown to be required for the synthesis and nuclear export of the 60S subunits (11, 16), and a fraction of Bcp1 protein co-sedimented with the 60S subunits in sucrose density gradients (16). This suggests that Bcp1 is physically involved in 60S biogenesis. However, the functional role of Bcp1 in 60S biogenesis is still not clear.

Tif6 is a shuttling factor but is found predominantly in the nucleus and nucleolus. Its homolog in higher eukaryotic cells is eIF6. Tif6 is required for 60S ribosome biogenesis (17), but it has not been found to be involved in translation initiation (18). The protein structures of Tif6/eIF6 (19) free and in complex with the 60S subunit have been determined, and the major interaction site is the C terminus of Rpl23 (20) (Rpl23 is called uL14 in the new nomenclature (21)). The binding of Tif6 on the 60S subunits blocks association between 60S and 40S subunits by preventing the formation of an intersubunit bridge (22). The presence of Tif6 is thought to prevent inappropriate interaction

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[2] The abbreviations used are: PI, phosphatidylinositol; $PI4,5P_2$, phosphatidylinositol 4,5-bisphosphate; 5FOA, 5-fluoroorotic acid; Ni-NTA, nickel-nitrilotriacetic acid; Kap, karyopherin; DIC, differential interference contrast.

with 40S subunits before 60S matures completely. After export from the nucleus bound to the large subunit, Tif6 is released by the GTPase, Efl1, and Sdo1 in the cytoplasm (23). The release of Tif6 is necessary for the downstream 60S export adapter, Nmd3, to separate from the joining face as the final step in the maturation of the 60S (24).

Several large scale proteomics studies reported that Bcp1 interacts with Rpl23. Interestingly, no other ribosomal proteins were identified in this complex (25–29). Furthermore, Bcp1 is reported to have negative genetic interaction with both *efl1* and *sdo1* mutants (30). BCCIPβ, the human homolog of Bcp1, was shown to interact with eIF6 and Rpl23 as a small complex, but depletion of BCCIPβ did not show deficiency in 60S biogenesis (31). These data point to a functional significance of Bcp1 with Rpl23 in the ribosome biogenesis pathway.

In this study, Bcp1 is identified as a chaperone of Rpl23 in yeast. Loss of Bcp1 results in the deficiency of Rpl23 and the 60S subunits. Bcp1 works as an escortin to dissociate Rpl23 from the karyopherins and interacts with Rpl23 as a complex, which facilitates the loading of Rpl23 into nascent 60S subunits.

Results

Bcp1 Is Required for Ribosome Biogenesis—To develop a deeper understanding of the role of Bcp1 in ribosome biogenesis, we assayed export and levels of 60S subunits in a *bcp1ts* mutant, with a single phenylalanine to serine substitution mutation at amino acid 241 (11). Rpl11B (uL5)-GFP was used to monitor the distribution of 60S subunits in *bcp1ts* mutant cells. Whereas Rpl11 was localized in the cytoplasm in *bcp1ts* at permissive temperature, it accumulated in the nucleus at 37 °C (Fig. 1*A*). This is consistent with previous reports that the 60S subunits, monitored by Rpl11 (uL5) or Rpl25 (uL23), were trapped in the nucleus if the function of Bcp1 was deactivated (11, 16). Rps2 (uS5)-GFP was also monitored in *bcp1ts* cells, and no change was observed (data not shown), indicating that the *bcp1ts* mutant affected 60S but not 40S export.

To further investigate ribosome biogenesis in *bcp1* mutant cells, cell extracts from *bcp1ts* and wild-type cells were fractionated by sedimentation through sucrose density gradients. Compared with wild-type cells (60S/40S ratio = 1.66), *bcp1ts* cells showed slight underaccumulation of 60S levels and halfmers at 30 °C (60S/40S ratio = 0.79), and these defects were enhanced at 37 °C (60S/40S ratio = 0.31) (Fig. 1*B*). Halfmers are formed when cells have insufficient levels of 60S subunits to efficiently associate with 40S subunits during translation initiation. These data suggest that Bcp1 is required for 60S biogenesis.

Because inactivation of Bcp1 trapped Rpl11 (uL5) in the nucleus, we suspected that Bcp1 might be involved in the ribosome export process. The *bcp1ts* mutant was crossed with a panel of characterized ribosome export factor mutants, including *nmd3*(*AAA*) (5, 6, 32), *arx1*Δ (1, 2), *ecm1*Δ (4), *mex67-5* (3), and *mtr2-33* (3). *Bcp1ts* showed synthetic sickness (Fig. 2*A*) and more severe 60S biogenesis defects (Fig. 2*B*) with all of these mutants compared with each single mutant. We anticipated that if Bcp1 was directly involved in the export process, then *BCP1* would be a high copy suppressor of these various export mutants. However, high copy *BCP1* did not suppress any of them (data not shown). Furthermore, *MEX67*, *MTR2*, *ARX1*, *NMD3*, and *CRM1* were not dosage suppressors of *bcp1ts* (data not shown). These data suggest that the nuclear accumulation of 60S subunits in *bcp1ts* mutant cells results from blocking ribosome biogenesis but is not directly linked to the export process.

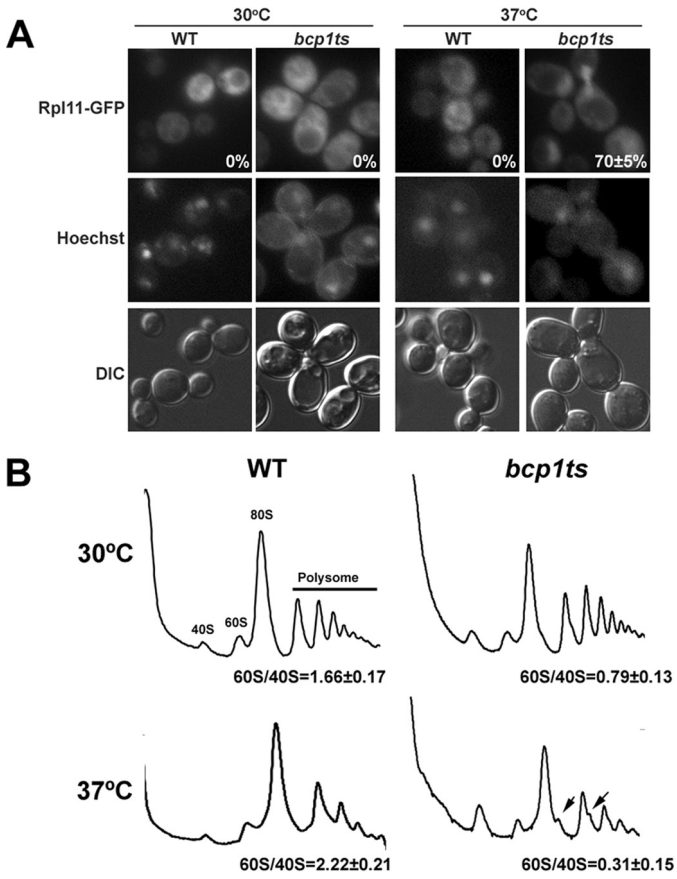

FIGURE 1. **Bcp1 is required for 60S biogenesis.** *A*, cultures of wild-type (BY4741) and *bcp1ts* (KLY106) cells carrying Rpl11-GFP (PKL228) were grown at 30 °C and held at 37 °C for 2 h. The localization of Rpl11-GFP was visualized by fluorescence microscopy. Hoechst staining identifies the DNA, and DIC was used for imaging whole cells. The fraction of cells with nuclear Rpl11 was shown. *B*, wild-type (BY4741) and *bcp1ts* (KLY106) cells were grown at 30 °C and shifted to 37 °C for 2 h before harvest. Protein extracts were prepared and fractionated by sedimentation through 7–47% sucrose density gradients by ultracentrifuge as described under "Materials and Methods." The halfmers are indicated by *arrows*.

Rpl23 Is a Dosage Suppressor of bcp1 Mutants—To further dissect the function of Bcp1 in the 60S maturation pathway, a high copy suppressor screen was performed. A 2μ yeast genomic library was transformed into *bcp1ts* (KLY106) strain, and colonies were screened for better growth at 37 °C. *RPL23A* was the only gene identified as a dosage suppressor of the *bcp1ts* mutant in this screen. This led us to consider that Bcp1 functionally interacts with Rpl23. There are two paralogs of *RPL23*, *RPL23A and RPL23B*, in *Saccharomyces cerevisiae* that encode proteins of identical sequences. First, we tested whether elevated levels of either *RPL23A* or *RPL23B* could suppress the impaired growth of *bcp1ts*. *RPL23A* or *RPL23B* was constructed on 2-μm plasmid and transformed to *bcp1ts.* Whereas *bcp1ts* was very slow growing at 37 °C, *RPL23A* and *RPL23B* each could partially rescue the growth of *bcp1ts* mutant at non-permissive temperature (Fig. 3*A*, 37 °C). However, high copy

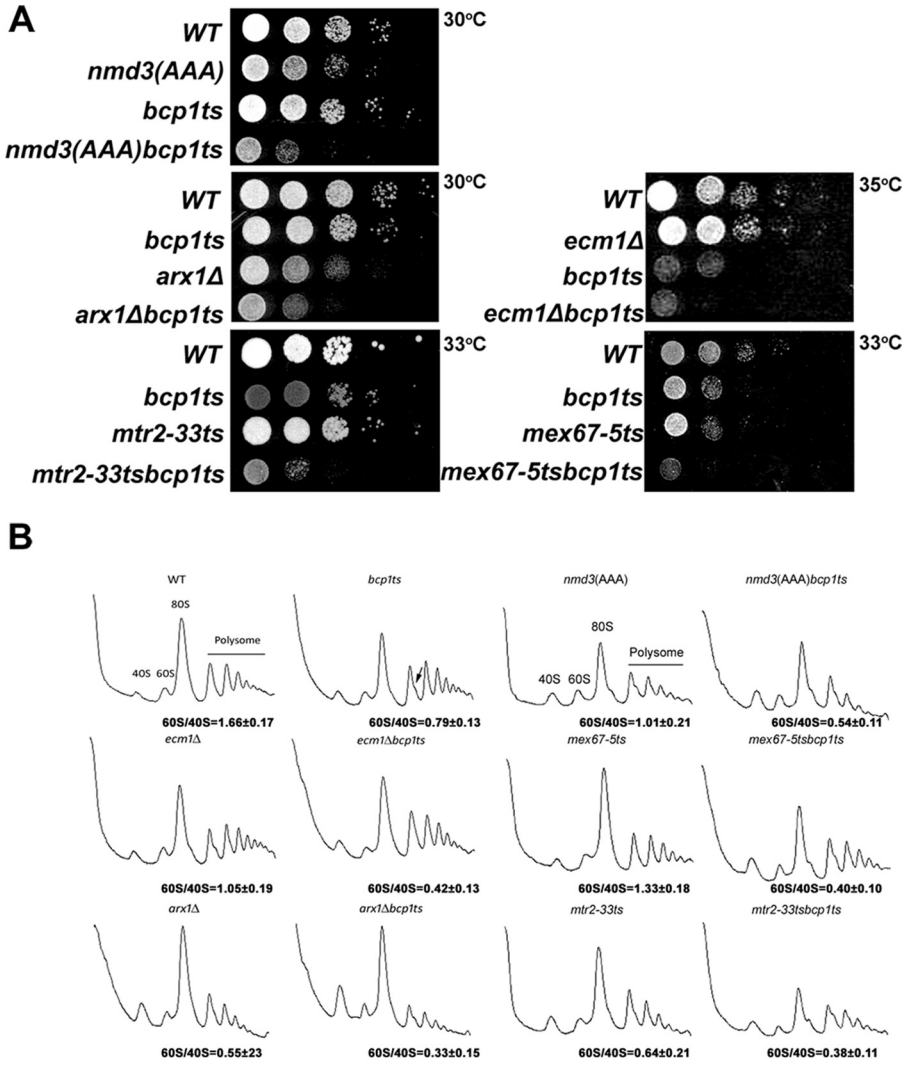

FIGURE 2. **The function of Bcp1 is tightly connected with many 60S biogenesis factors.** *A*, the normalized cell cultures were normalized and 10-fold serially diluted and spotted on the plates. A representative image for the indicated combination is shown. The sets of *bcp1ts* crossed with *nmd3*(*AAA*) or *arx1*Δ were incubated at 30 °C. The sets of *bcp1ts* crossed with *mtr2-33* or *mex67-5* were incubated at 33 °C. The set of *bcp1ts* crossed with *ecm1*Δ was incubated at 35 °C. The plates were incubated for 24–48 h before pictures were taken. *B*, sucrose density gradient (7–47% sucrose) sedimentation profiles were performed for each strain indicated on the figures. The halfmers are indicated by *arrows*.

RPL23 could not replace *BCP1* (Fig. 3*A*, 5FOA). This is consistent with the idea that Bcp1 has another function in exporting Mss4 and/or that Bcp1 is absolutely required for the proper function of Rpl23. Deletion of *RPL23A* or *RPL23B* resulted in a slight growth defect; however, combining the deletion of either *RPL23A* or *RPL23B* with the *bcp1ts* mutation resulted in a severe growth defect (Fig. 3*B*). Therefore, the genetic interaction results implied that the function of *BCP1* was related to *RPL23*.

We further tested whether suppression by overexpression of *RPL23* also rescued the 60S biogenesis defects of *bcp1ts* monitored by sucrose density gradient centrifugation. Protein extracts, prepared from *bcp1ts* cells expressing vector or *RPL23A*, were cultured for 1 h at 37 °C and were fractionated through sucrose gradients. Compared with the vector control (60S/40S ratio = 0.31), increasing levels of Rpl23 increased the levels of 60S ribosomal subunits (60S/40S ratio = 0.62) and decreased the levels of halfmers (Fig. 3*C*). We next examined 60S subunit export in *bcp1ts* cells expressing 2μ *RPL23* by monitoring Rpl11-GFP localization. Whereas about 60% of the *bcp1ts* cells exhibited nuclear accumulation of Rpl11-GFP, *bcp1ts* cells with dosage suppressor *RPL23* supported a higher level of export, with only 15% of cells showing nuclear Rpl11-GFP signal (Fig. 3*D*). In conclusion, overexpression of *RPL23* rescued the 60S biogenesis defects of *bcp1ts.*

Bcp1 and Rpl23 Interact Directly—Previous results of large scale protein interactions by mass spectrometric analyses (25–29) suggested that Bcp1 physically interacts with Rpl23. To test this assumption, Bcp1 and Rpl23 were fused to the GAL4 DNA binding domain (BD) or activation domain (AD), and potential interaction was tested using a yeast two-hybrid assay. Combinations of different bait and prey plasmids were then transformed to the reporter strain PJ69-4A. Interactions of the fusion proteins were monitored by streaking colonies on a histidine dropout plate. Whereas Bcp1 or Rpl23 fusion alone did not grow, cells co-expressing Bcp1-AD and Rpl23-BD could grow on the reporter plate and vice versa (Fig. 4*A*).

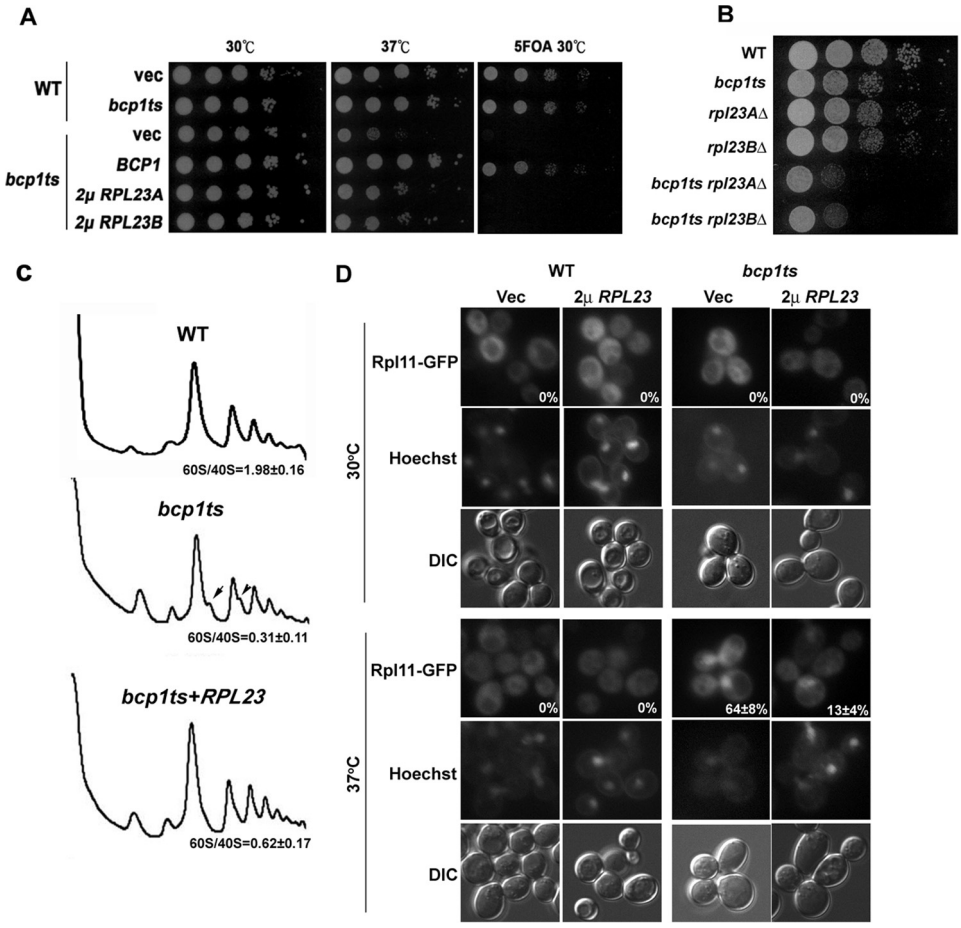

FIGURE 3. **RPL23 is a high copy suppressor of *bcp1ts*.** *A*, 10-fold serial dilutions of wild-type (BY4741) with vector (pRS413) or PKL483 (*CEN bcp1ts*) and *bcp1ts* (KLY106) cells with vector (pRS413), PKL478 (*CEN BCP1*), PKL194 (2μ *RPL23A*), or PKL195 (2μ *RPL23B*) were spotted onto a His^- dropout plate and 5FOA plate. The plates were incubated at 30 and 37 °C for 2 days. *B*, cultures of wild-type (BY4741), *bcp1ts* (KLY106), *rpl23A*Δ (AJY2132), *rpl23B*Δ (AJY2136), *rpl23A*Δ*bcp1ts* (KLY170), and *rpl23B*Δ*bcp1ts* (KLY173) were serially spotted on a YPD plate and incubated at 30 °C for 2 days. *C*, cultures of wild-type (BY4741) and KLY106 (*bcp1ts*) with vector (pRS413) or PKL194 (2μ *RPL23A*) were cultured at 30 °C until A_{600} 0.3 and then shifted to 37 °C for 1 h before cells were harvested. Protein extracts were prepared and fractionated by sedimentation through 7–47% sucrose density gradients. The halfmers are indicated by *arrows*. *D*, wild type (BY4741) and *bcp1ts* (KLY106) with vector (pRS413) or PKL194 (2μ *RPL23A*) were cultured in the complete synthetic defined drop-out medium to early log phase. The cells were incubated at 30 °C or shifted to 37 °C for 2 h before examination. The localization of Rpl11-GFP was visualized under fluorescence microscopy. The fraction of cells with nuclear Rpl11 was shown.

To further demonstrate that Bcp1 and Rpl23 interact directly, we overexpressed these two proteins in *Escherichia coli* and tested for *in vitro* interaction. Bcp1 was expressed as a recombinant protein with a C-terminal His tag, and Rpl23 was expressed as the native form, without any tag. After incubation with an Ni-NTA resin, Bcp1 was bound strongly onto the beads, whereas Rpl23 was not detectably bound (Fig. 4*B*, compare *lanes 3* and *4*). Once Bcp1 and Rpl23 were incubated together, Rpl23 was co-purified with Bcp1 together by the Ni-NTA resin and insensitive to RNase treatment (Fig. 4*B*, *lanes 5* and *6*). This suggests that Bcp1 and Rpl23 interact directly.

We next performed immunoprecipitation to test the interaction between Bcp1 and Rpl23 and determined whether this interaction occurred on or off pre-60S subunits. The ribosome biogenesis factors, Arx1, Tif6, and Nmd3, which bind to nascent 60S subunits containing Rpl23, were used as controls. Arx1, Tif6, and Nmd3 all pulled down both Rpl23-HA and Rpl8 (Fig. 4*C*), an independent marker for 60S subunits, demonstrating that they interact with the 60S subunits. In contrast, Bcp1 pulled down relatively more Rpl23, but Rpl8 was not detected (Fig. 4*C*). This result suggests that Bcp1 interaction with Rpl23 occurs primarily off the 60S subunit. Consistent with this interpretation, the majority of Bcp1 was shown as free form in sucrose density gradients (Fig. 4*D*). For additional evidence that Bcp1 bound free Rpl23, we took advantage of the fact that the Rpl23-GFP fusion protein did not interact with 60S subunits (Fig. 4*D*). Rpl23-GFP co-immunoprecipitated with Bcp1 but not with Arx1 or Tif6 and only modestly with Nmd3 (Fig. 4*C*). To avoid potential interference from the tag and plasmid expression system, immunoprecipitated was also carried out against Bcp1 and Tif6 from genomic TAP-tagged strains and detected by anti-Rpl23 and anti-Rpl8 antibodies for comparison. Consistent with Fig. 4*C*, whereas Tif6 interacted with both Rpl23 and Rpl8, Bcp1 interacted with only Rpl23 and not Rpl8 (data not shown).

As a final demonstration that Bcp1 interacts preferentially with Rpl23 off 60S, protein extracts were spun with ultracentrifugation (385,900 × *g* for 60 min) for separation of free and ribosome-containing fractions and immunoprecipitated Tif6, Nmd3, and Bcp1 and their associated proteins from two pools.

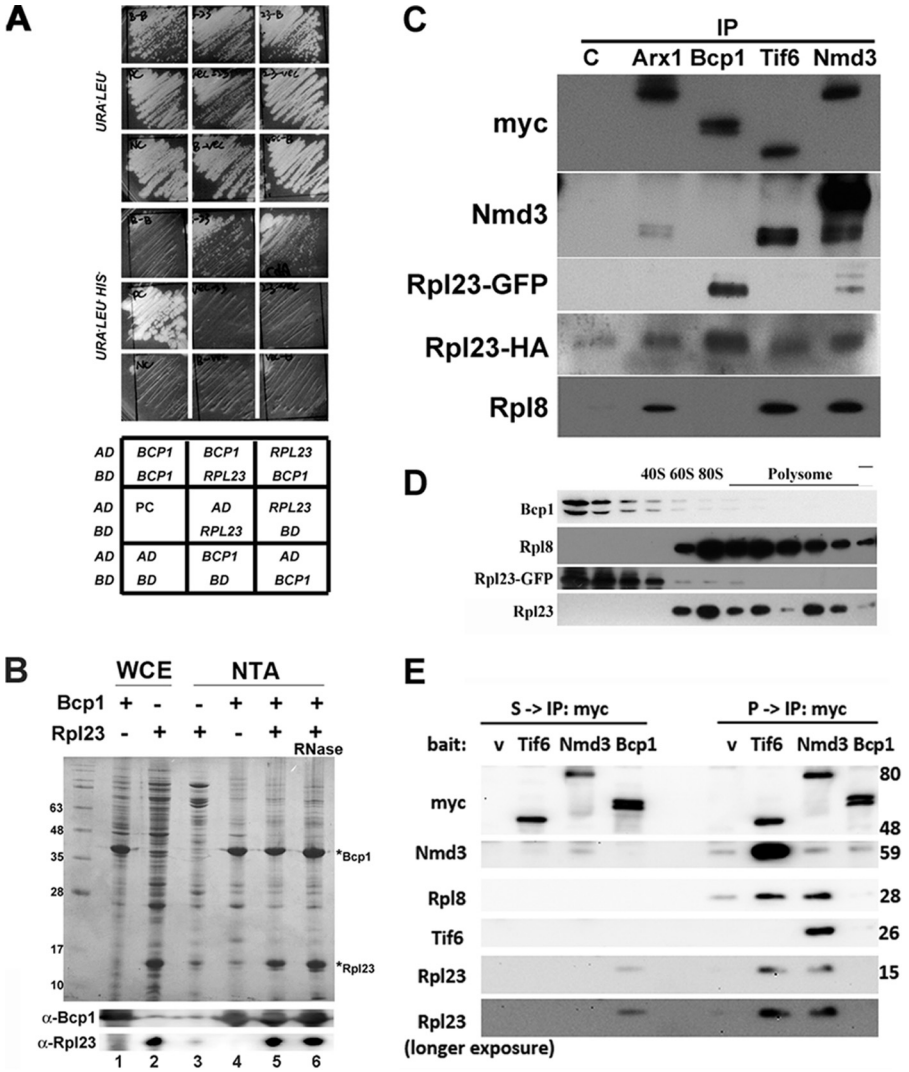

FIGURE 4. **Bcp1 interact directly with Rpl23.** *A*, in PJ69-4A, yeast two-hybrid interaction was carried between Bcp1 and Rpl23. Two of the plasmids, pGAD, PKL183 (*AD-BCP1*), PKL253 (*AD-RPL23*), pBDU, PKL187 (BD-BCP1), and PKL254 (BD-RPL23), were transformed in different combinations indicated. *AD*, GAL4 activation domain; *BD*, GAL4 DNA binding domain; *P.C.*, positive control. *B*, Ni-NTA resin was incubated with cell lysates (*WCE*) containing recombinant Rpl23, Bcp1-His, or both proteins. After washing, proteins were eluted, separated by SDS-PAGE, and visualized by Coomassie Blue staining or Western blotting. The positions of Bcp1 and Rpl23 are indicated with *asterisks*. *C*, Arx1-Myc (PAJ1026), Bcp1-Myc (PKL105), Tif6-Myc (PAJ1010), and Nmd3-Myc (PAJ538)-containing complexes were immunoprecipitated from wild-type strain containing Rpl23-HA (PKL327) and Rpl23-GFP (PKL329). The co-purified proteins were detected by Western blotting. *D*, cell extracts from BY4741 containing Rpl23-GFP (PKL329) were prepared and fractionated by sucrose gradient sedimentation. The positions of Bcp1, Rpl23, and Rpl8 in gradients were monitored by Western blotting using anti-Bcp1, anti-Rpl8, anti-GFP, and anti-Rpl23 antibodies, respectively. *E*, cell lysates from wild-type strain (BY4741) containing vector (pRS415), Tif6-Myc (PAJ1010), Nmd3-Myc (PAJ538), or Bcp1-Myc (PKL105) were spun at 385,900 × *g* for 60 min to separate ribosomes from free proteins. The pellets containing ribosomes were fully resuspended in lysis buffer. The separate ribosome-free (supernatant (*S*)) and ribosome-containing (pellet (*P*)) fractions were subjected to immunoprecipitation. Eluted proteins were resolved in SDS-PAGE and detected by Western blotting.

Tif6 and Nmd3 immunoprecipitated Rpl23 only from the ribosome-containing fractions (Fig. 4*E*). In contrast, whereas the majority of Rpl23 co-fractionated with the ribosomes (Figs. 4*D* and 5*A*), the Bcp1-Rpl23 interaction was detected only from the free protein pool (Fig. 4*E*). Unlike Tif6- and Nmd3-immunoprecipitated complexes containing ribosomal proteins, Rpl8 and Rpl23 in this case were not detected on Bcp1-containing complex isolated from the pellet fraction. Bcp1 may exhibit as a small complex and was separated to the ribosome-containing fraction during ultracentrifugation, or Bcp1 may associate with 60S very transiently. These data show that Bcp1 and Rpl23 preferentially interact as free proteins off 60S subunits.

Bcp1 Is Required to Stabilize Free Rpl23—Ribosomal proteins are typically highly positively charged to stably interact with the negative charge of rRNAs. It has been shown that karyopherins or chaperones interact with ribosomal proteins to maintain their solubility and also facilitate their loading into nascent ribosomal subunits (33–42).

Because Bcp1 has direct interaction with free Rpl23, we wanted to ask whether Bcp1 could be a potential chaperone of Rpl23. The half-life of ribosomes *in vivo* might be up to several days (43). To focus only on the free form and not yet incorporated Rpl23 proteins, we used ultracentrifugation to separate the newly synthesized Rpl23 (Fig. 5*S*) from the 60S-containing Rpl23 (Fig. 5*P*).

Bcp1 and Rpl23 were overexpressed individually or together under control of a galactose-dependent promoter. Overexpression of Bcp1 alone enhanced the level of free Rpl23 (Fig. 5*A*,

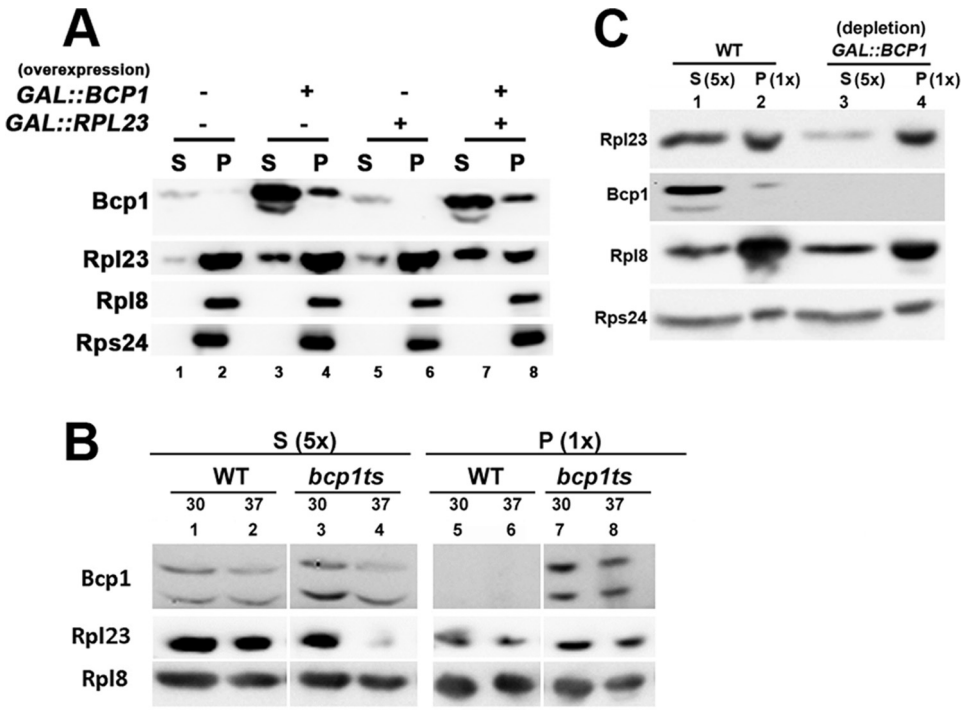

FIGURE 5. **Bcp1 is required to maintain the stability of free Rpl23.** Cells were cultured in the different conditions described below. Cell lysates were spun at 385,900 × *g* for 60 min at 4 °C to separate free (*S*) or ribosome-containing (*P*) fractions and examined by Western blotting. *A*, BY4741 was transformed with *GAL*::*BCP1* (PKL493) or *GAL*::*RPL23* (PKL300) alone or together. Cells were cultured in Ura$^-$His$^-$ raffinose medium, and 2% galactose was added for 4 h to overexpress the proteins. The pellets were resuspended with an equal volume of lysis buffer and loaded in a 1:1 ratio to supernatant. *B*, wild type and *bcp1ts* (KLY106) were cultured at 30 °C and shifted to 37 °C for 2 h. To enhance the level of the free form of Rpl23, the supernatant fractions were loaded with 5-fold more than the pellet fractions. *C*, wild-type and *GAL*::*BCP1* (KLY754) cells were cultured in YPD for 16 h to A_{600} 0.4–0.6. To enhance the level of the free form of Rpl23, the supernatant fractions were loaded with 5-fold more than the pellet fractions.

compare *lanes 1* and *3*). In fact, overexpression of Bcp1 alone yielded higher levels of free Rpl23 than did overexpression of Rpl23 alone (Fig. 5*A*, compare *lanes 3* and *5*). When Bcp1 and Rpl23 were co-overexpressed together, the level of free Rpl23 was enhanced even more (Fig. 5*A*, *lane 7*). Similarly, the level of Rpl23-GFP protein, the mutant form of Rpl23 that cannot be incorporated into 60S (Fig. 4*D*), was greatly increased when Bcp1 was overexpressed (data not shown).

If Bcp1 can stabilize the free form of Rpl23, we asked whether Rpl23 would be unstable in a *bcp1* mutant. Because the majority of Rpl23 signal is from the ribosome-bound pool (Fig. 5*A*), the fraction S was precipitated by 10% TCA and redissolved in 1× SDS sample buffer to enhance the signal of nascent Rpl23. After a 2-h incubation at non-permissive temperature of *bcp1ts* mutant, we did not observe any obvious differences in the levels of 60S-bound Rpl23, but free Rpl23 was greatly decreased (Fig. 5*B*, compare *lanes 2* and *4*). To rule out any potential side effects due to the mutant Bcp1 protein, we constructed the *GAL*::*BCP1* strain, in which *BCP1* transcription could be repressed by growth in glucose. Similar to the *bcp1ts* strain, depletion of Bcp1 caused slow growth, underaccumulation of 60S, and blockage of 60S export (data not shown). Most importantly, we also observed a decreased level of free Rpl23 upon depletion of Bcp1 (Fig. 5*C*, compare *lanes 1* and *3*).

Bcp1 Is an Escortin of Rpl23—Recently, many chaperones for specific ribosomal proteins have been identified. It has been shown that these chaperones bind nascent ribosomal proteins as soon as they emerge from the exit tunnel of the ribosome (39, 44) and accompany their transports. Because Bcp1 interacts with Rpl23 off 60S subunits, Bcp1 may interact with nascent Rpl23 in the cytoplasm and facilitate import of the complex into the nucleus. We tested whether free Rpl23 and/or the Rpl23-Bcp1 complex could interact with import karyopherins (Kaps). Kap121 and Kap123 are the major karyopherins for import of ribosomal proteins (45). Kap121 and Kap123 were expressed as GST fusions at the N terminus. The proteins were captured on GSH beads and challenged with extracts expressing Rpl23 and/or Bcp1. Rpl23 interacted with GST-Kap121 and GST-Kap123 but not with GST alone (Fig. 6*A*), indicating that Rpl23 could interact with Kaps independent of Bcp1. However, the levels of Rpl23 on Kap121 or Kap123 were decreased to background levels in the presence of Bcp1 (Fig. 6*A*, compare *lanes 2* and *3*).

Recently, Tsr2 was reported as an escortin to dissociate eS26 from karyopherins in a Ran-GTP-independent manner and for proper delivery of eS26 to nascent 40S subunits (40). Because Kaps do not interact efficiently with the complex of Bcp1 and Rpl23, we tested whether Bcp1 could compete with Kaps for Rpl23. GST-Kap121 and GST-Kap123 were first incubated with Rpl23 followed by the addition of purified Bcp1 or Gsp1Q71L-GTP to promote dissociation. Gsp1, the yeast Ran homolog, dissociates cargo from Kaps upon delivery into the nucleus, and Gsp1Q71L, equivalent to the human RanQ69L, is a mutant that cannot hydrolyze GTP efficiently, thereby stabilizing RanGTP (46, 47). The addition of 0.5 and 1.5 μM purified Bcp1 stimulated the dissociation of Rpl23 from Kaps. In contrast, the addition of RanQ71L did not dissociate Rpl23 from Kap121 or Kap123 (Fig. 6*B*).

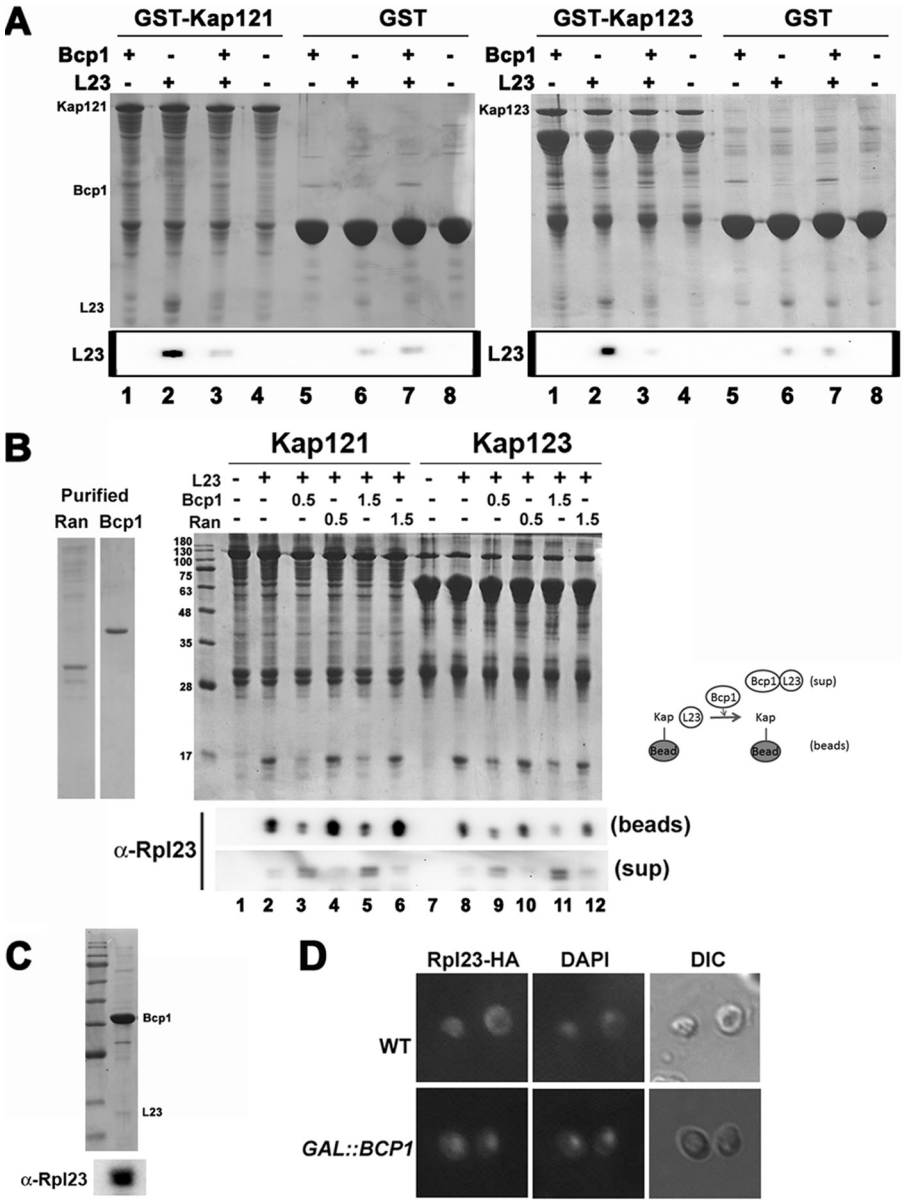

FIGURE 6. **Rpl23 is released by RanGTP from Kaps and interacts with Bcp1 afterward.** *A*, GST, GST-Kap121, or GST-Kap123 was immobilized on GSH-Sepharose resin. Beads were incubated with Bcp1, Rpl23, the complex of Rpl23 and Bcp1, or buffer alone at 4 °C for 1 h. After washing three times, bound proteins were eluted in 1× SDS sample buffer, separated by SDS-PAGE, and visualized by Coomassie Blue staining or Western blotting. *B* and *C*, GST-Kap121 or GST-Kap123 proteins were immobilized on GSH-Sepharose and incubated with Rpl23. After three washes, incubation with purified Bcp1 or Ran(Q71L)GTP was done at 4 °C for 60 min. The tubes were centrifuged shortly to separate supernatant and beads. Beads were washed three times, and the bound proteins were eluted with 1× SDS sample buffer. In parallel, the supernatant was collected, and NTA resin was added to purify Bcp1. Samples were analyzed with Coomassie Blue staining or Western blotting. The position of Rpl23 is indicated with an *asterisk*. *D*, vector (pRS413) or *GAL::BCP1* (PKL493) was transformed to wild-type cells expressing *RPL23-HA* (PKL78). Cells were cultured in the medium containing 2% raffinose overnight. After subculture, 2% galactose was added for 4 h to induce overexpression of Bcp1. Indirect immunofluorescence was used to detect the distribution of Rpl23-HA in the cells.

Because Bcp1 and Rpl23 interacted directly, Bcp1 may interact with free Rpl23 in the nucleus following dissociation of Rpl23. To further confirm this assumption, Bcp1 was affinity-purified from the supernatant derived from Fig. 5*B* with NTA resin, and Rpl23 was detected (Fig. 6*C*). In addition, Bcp1 was overexpressed under the GAL promoter to see whether that would drive Rpl23 accumulated in the nucleus. The localization of Rpl23 was evenly distributed in the wild type cells and became more nuclear upon overexpression of Bcp1 (Fig. 6*D*).

In conclusion, once Bcp1 dissociates Rpl23 from Kaps, Bcp1 binds to Rpl23 thereafter and is required to maintain the stability of Rpl23. Therefore, Bcp1 is the nuclear chaperone, an escortin, of Rpl23.

Discussion

Bcp1 Is a Chaperone of Rpl23 and Required to Maintain the Stability of Rpl23—In this study, Bcp1 and Rpl23 were shown to physically interact. Notably, Rpl23 tends to be unstable in *bcp1* mutant cells, and the level of free Rpl23 was stabilized by the overexpression of Bcp1. Ribosomal proteins are very basic proteins that aggregate readily with polyanions in cells. Karyopherins of ribosomal proteins play a dual role as import-

ers and chaperones to mask their basic nature (48). Many unique chaperones are reported to accompany ribosomal proteins. In addition to their classic role of protecting from proteolysis, chaperones are also involved in adjusting the loading or transport processes. Yar1 directly interacts with Rps3 (S3) and acts as an anti-aggregation factor (35, 37), and Sqt1 is a chaperone for Rpl10 (uL16) that facilitates loading of Rpl10 into 60S subunits (34, 42). Rrb1 has direct interaction with Rpl3 (uL3), and overexpression of Rrb1 increased the level and nuclear accumulation of Rpl3 (36). Syo1 facilitates coimport of Rpl5 (uL18) and Rpl11 (uL5) and chaperones 5S RNP assembly (33, 38). Acl4 is a chaperone of Rpl4 (uL4) and accompanies Rpl4 from the cytoplasm to the loading onto the pre-60S in the nucleus (39, 41). Tsr2 facilitates dissociation of Rps26 (eS26) from importin and binds the released Rps26 for proper delivery to 90S preribosome (40).

In this study, we identified a new pair of ribosomal proteins and their chaperone. Rpl23 was identified as a high copy suppressor of *bcp1* mutant. Whereas Bcp1 has direct interaction with Rpl23, overexpression of Bcp1 increased the level of free Rpl23, and the absence or mutation of Bcp1 resulted in the loss of Rpl23. We tested whether overexpression of other ribosomal proteins could also suppress the growth defects of *bcp1ts*. However, of the ribosomal proteins we had tested, including *RPL8*, *RPL12A*, *RPL12B*, *RPL25*, *RLP24*, *RPL43B*, and non-60S incorporated *RPL23-GFP*, none could support the growth of *bc1pts* mutant.[3] Although Rpl23 is the target of Bcp1, overexpression of Rpl23 could not replace the function of Bcp1, either in the shuffling assay of *RPL23* in the *bcp1ts* strain on a 5FOA plate (Fig. 3*A*) or in the complementation test of *RPL23* in the *GAL*::*BCP1* strain.[3] One explanation is that Bcp1 has an additional function to export the essential protein, Mss4, out of the nucleus (11). Alternatively, Bcp1 is required to maintain the proper function of Rpl23. Therefore, overexpression of Rpl23 could not complement the absence of Bcp1.

Although ribosomal proteins are highly basic and tend to aggregate, many chaperones have been shown to interact with accompanying ribosomal proteins right after their emergence from the ribosomal exit tunnels (44) and are imported together by karyopherins. It is unlikely that Kap121 and Kap123 can associate with Rpl23 independently on Bcp1, but noticeably, Kaps cannot bind Bcp1 and Rpl23 in complex. This suggests that Bcp1 binds Rpl23 either before or after importin association with Rpl23. We found that Bcp1 could dissociate Rpl23 from Kaps, and overexpression of Bcp1 drives Rpl23 accumulation in the nucleus. Based on these data, we propose that Bcp1 binds Rpl23 in the nucleus after it dissociates Rpl23 from Kaps (Fig. 7).

Ribosome Biogenesis and the Phosphoinositol Pathway—In this study, we have demonstrated the molecular function of Bcp1 in 60S biogenesis. Meanwhile, Bcp1 also has a dual role in regulating $PI4,5P_2$ synthesis, acting as an export factor for Mss4 (11). Thus, Bcp1 may coordinate the synthesis of the ribosomes to the PI signaling pathway. In ribosome biogenesis mutants, the disorder of cell morphology or polarity can often be

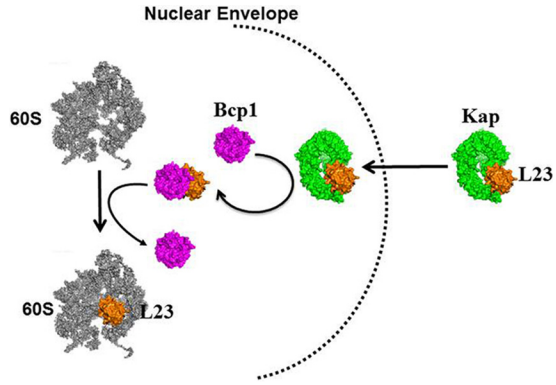

FIGURE 7. **Proposed function of Bcp1 in 60S biogenesis pathway.** After Rpl23 has been imported, Bcp1 releases Rpl23 from importins and interacts with Rpl23 directly for proper delivery to the assembly site on pre-60S.

detected (49). These phenotypes are tightly connected to cytoskeleton organization, which is controlled by PI signaling (50).

Rrs1 is an essential protein involved in the 60S biogenesis that together with Rpf2 is required for the recruitment of Rpl5, Rpl11, and 5S rRNA into pre-60S subunits (51). *Rrs1-1* mutant shows defects in the transcriptional repression of ribosomal genes, maturation of 25S rRNA, and 60S export (52). Intriguingly, disruption of an InsP6 (inositol 6-phosphate) kinase could suppress the cold sensitivity of an *rrs1-1* mutant. Therefore, the InsP6 metabolism pathway was proposed to affect 60S biogenesis (53). There is additional evidence of coupling between the inositol pathway and ribosome biogenesis; the addition of inositol to yeast cultures leads to an extremely fast transcriptional response, including genes involved in large ribosomal subunit assembly, rRNA transcription, processing, and modification (54). Additionally, mTORC2, a serine/threonine kinase conserved from yeast to humans, regulates the dynamics and organization of the cytoskeleton. Direct ribosome association is required to activate mTORC2 and PI3K signaling (55).

Although many studies suggest close links between ribosome biogenesis and the phosphoinositol signaling pathway, mechanistic studies of these links are still lacking. After Rpl23 is loaded, Bcp1 is released and is free to export Mss4, a PI4-phosphate 5-kinase. Whereas Bcp1 controls both 60S biogenesis and the synthesis of PI by regulating the export levels of Mss4, it may contribute the cross-talk between these two pathways. More work on this scenario is needed to determine the functional significance of these interconnections.

Tif6 Does Not Co-exist with Bcp1-Rpl23 Complex—BRCA2 and CDKN1A(p21,CIP1)- interacting protein (BCCIP) is the human homolog of Bcp1, sharing about 70% similarity with yeast Bcp1 (56). Due to alternative splicing, there are two isoforms of BCCIP, BCCIPα and BCCIPβ, that have identical N termini but unique C-terminal sequences (57). BCCIPα interacts with BRCA2, a tumor suppressor gene, and expression of BCCIPα is closely connected to cancer cell proliferation because its expression is found at significantly decreased levels in nine brain tumor cell lines and seven breast cancer cell lines (56). BCCIP was also shown to be required for the nuclear localization of the p21 protein, an inhibitor of cyclin-dependent kinase, which plays a critical role in regulation of the G_1-S tran-

[3] Y.-H. Ting and K.-Y. Lo, unpublished data.

TABLE 1
Yeast strains used in this study

Strain	Genotype	Source
AAY1341	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9bcp1Δ::HIS3 carrying bcp1ts (TRP CEN)*	Ref. 11
Y1029	*MATa leu2Δ ura3Δ ade2Δ his3Δ trp1Δ mtr2Δ::HIS3 (pRS316-MTR2)*	Ref. 3
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
PJ69–4A	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ Met2::GAL7-lacZ LYS2::GAL1-HIS3 GAL2-ADE2*	Ref. 59
AJY1901	*MATa his3Δ leu2Δ ura3Δ arx1Δ::KanMX*	Ref. 61
AJY2110	*MATa nmd3Δ::KanMX his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (PAJ112 NMD3 URA3 CEN)*	Ref. 2
AJY2132	*MATa ura3Δ0 his3Δ1 leu2Δ0 met15Δ rpl23AΔ::KanMX*	A.W. Johnson
AJY2136	*MAT α ura3Δ0 his3Δ1 leu2Δ0 lys2Δ0 rpl23BΔ::NatMX*	A.W. Johnson
AJY2494	*MATa leu2Δ ura3Δ ade2Δ his3Δ trp1Δ mtr2Δ::HIS3 (pAJ1425 mtr2-33 LEU2)*	A.W. Johnson
AJY2578	*MATa his3Δ leu2Δ ura3Δ ecm1Δ::KanMX*	A.W. Johnson
KLY69	*MATa nmd3Δ::KanMX his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 (PAJ752 nmd3AAA-myc LEU2)*	This study
KLY106	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bcp1Δ::KanMX carrying bcp1ts (URA3 CEN)*	This study
KLY110	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bcp1Δ::KanMX carrying bcp1ts (URA CEN)*	This study
KLY117	*his3Δ leu2Δ ura3Δ mtr2Δ::HIS3 bcp1Δ::KanMX (mtr2-33 LEU2) (bcp1ts URA3)*	This study
KLY122	*his3Δ leu2Δ ura3Δ mex67Δ:HIS3 bcp1Δ::KanMX (mex67-5 LEU2) (bcp1ts URA3)*	This study
KLY137	*his3Δ leu2Δ ura3Δ arx1Δ:KanMX bcp1Δ::KanMX (bcp1ts URA3)*	This study
KLY144	*his3Δ leu2Δ ura3Δ ecm1Δ::KanMX bcp1Δ::KanMX (bcp1ts URA3)*	This study
KLY153	*nmd3Δ::KanMX bcp1Δ::KanMX (nmd3AAA-myc LEU2) (bcp1ts URA3)*	This study
KLY170	*ura3Δ0 his3Δ1 leu2Δ0 met15Δ rpl23aΔ::KanMX bcp1Δ::KanMX (bcp1ts URA3)*	This study
KLY173	*ura3Δ0 his3Δ1 leu2Δ0 rpl23bΔ::NatMX bcp1Δ::KanMX (bcp1ts URA3)*	This study
KLY431	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rpl23BΔ::KanMX*	This study
KLY464	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rpl23AΔ::KanMX rpl23BΔ::KanMX carrying GAL::RPL23A plasmid (URA CEN)*	This study
KLY754	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bcp1Δ::KanMX carrying pRS413 GAL::Bcp1 (PKL493 HIS3 CEN)*	This study

sition of the cell cycle (58). BCCIPβ, but not BCCIPα, was shown to interact with Rpl23 and eIF6 (human homolog of Tif6), but depletion of BCCIPβ did not impair the 60S synthesis pathway (31).

Yeast Bcp1 is very different from human BCCIP, whereas 60S is severely impaired in the *bcp1* mutant (Fig. 1) and Bcp1 was not found to co-exist with Tif6 (Fig. 4, *C* and *E*) (data not shown). In our study, Rpl23 co-immunoprecipitated with both Bcp1 and Tif6 (Fig. 4, *C* and *E*). However, Tif6 could not be detected in Bcp1-containing complex (Fig. 4*E*), and Bcp1 could not be detected in Tif6-containing complex either (data not shown). To avoid potential artifacts from the C-terminal tag on Bcp1, we also immunoprecipitated native Bcp1 with anti-Bcp1 antibody but still did not detect Tif6 (data not shown). We also tested the interaction between Bcp1 and Tif6 by yeast two-hybrid analysis and did not see any positive interactions. In *in vitro* interaction assays, recombinant GST-Tif6 was used to test the interaction with Bcp1, Rpl23, or the complex of Bcp1 and Rpl23, and surprisingly, none of the tests showed positive results.

Rpl23 is the major binding site of Tif6 on the 60S subunits. However, we did not detect Tif6 interacting with free form Rpl23, only with the ribosome-containing Rpl23 (Fig. 4*E*). In addition, although both Bcp1 and Tif6 are found to interact with Rpl23, they are mutually exclusive (Fig. 4*E*). Moreover, the direct interaction between Tif6 and Rpl23 or Tif6 and Bcp1 was not detected in the *in vitro* assays (data not shown). Therefore, Bcp1 is assumed to stabilize and facilitate the loading of Rpl23 and is released right after accomplishing the incorporation of Rpl23. Tif6 may interact with Rpl23 only on the 60S subunits. This hierarchic event ensures the correct assembly of Rpl23 and proceeds with the loading of Tif6.

Materials and Methods

Strains, Plasmids, and Reagents—All *S. cerevisiae* strains used in this study are listed in Table 1. Unless otherwise indicated, all strains were grown at 30 °C in rich medium (yeast extract peptone) or synthetic dropout medium containing 2% glucose. KLY106 (*bcp1ts*) is a haploid strain derived from the heterozygous diploid deletion collection (Research Genetics) with *bcp1ts* (*URA3 CEN*) plasmid. *Bcp1ts* plasmid was derived from AAY1341 with amino acid 241 mutated from phenylalanine to serine (11). KLY754 (*GAL::BCP1*) was created by transforming PKL493 (*GAL::BCP1 HIS3*) to KLY106 and selecting the colonies viable on the 5FOA-Gal plate. *Mtr2-33*(*LEU2*) (3) was transformed to Y1029. This strain was streaked on the 5FOA plate and saved as AJY2494 (*mtr2-33*). AJY2110 (2) containing PAJ752 was streaked on the 5FOA plate and saved as KLY69 (*nmd3(AAA)*). KLY117 was derived from crossing KLY106 (*bcp1ts*) and AJY2494 (*mtr2-33*). KLY122 was derived from crossing KLY110 (*bcp1ts*) and PSY1687 (*mex67-5*). KLY137 was derived from crossing AJY1901 (*arx1*Δ) and KLY106 (*bcp1ts*). KLY144 was derived from crossing KLY106 (*bcp1ts*) and AJY2578 (*ecm1*Δ). KLY153 was derived from crossing KLY106 (*bcp1ts*) and KLY69 (*nmd3(AAA*)). The plasmids used in this study are listed in Table 2.

Anti-Bcp1, anti-Rpl23, anti-Tif6, and anti-Rpl8 (L8e) antibodies were generated in this laboratory. Briefly, individual genes were cloned in pET28a and overexpressed in *E. coli BL21*. Purified proteins were injected into rabbit for antibody generation. Anti-Myc antibody was obtained from MYC 1–9E10.2 (9E10) (ATCC® CRL1729™). Anti-Nmd3 was obtained from the laboratory of Dr. Arlen Johnson. Anti-HA and anti-GFP were purchased from Covance and Sigma, respectively. Signals were detected using Clarity™ Western ECL substrate (Bio-Rad) and scanned by MultiGel-21 (Top Bio, Taipei, Taiwan).

High Copy Suppressor Screen—A high copy number (2μ *URA3*) (gift from Dr. Phil Hieter) yeast genomic library was transformed into the *bcp1ts* (AAY1341) strain, and colonies were screened for better growth at 37 °C. Compared with empty vector control, cells showing bigger colony size contained the wild-type *BCP1* gene or genes that could suppress the growth defects of *bcp1ts*. Plasmids were isolated and checked with enzymatic digestion to eliminate *BCP1*. Those plasmids containing potential dosage-dependent suppressors

TABLE 2
Plasmids used in this study

Plasmid	Gene	Relevant markers or expression vector	Source
pGAD-C1		*2μ LEU2*	Ref. 62
pGBDU-C1		*2μ URA3*	Ref. 62
PAJ538	*NMD3-MYC*	*CEN LEU2*	Ref. 5
PAJ1003	*TIF6-GFP*	*CEN LEU2*	Ref. 24
PAJ1010	*TIF6-MYC*	*CEN LEU2*	A.W. Johnson
PAJ1014	*NOG2-MYC*	*CEN LEU2*	A.W. Johnson
PAJ1026	*ARX1-MYC*	*CEN LEU2*	Ref. 61
pKW586	*Gsp1(Ran)Q71L*	*pQE9*	Ref. 32
PKL78	*RPL23-HA*	*CEN URA3*	This study
PKL105	*BCP1-myc*	*CEN LEU2*	This study
PKL183	*AD-BCP1*	*2μ LEU2*	This study
PKL187	*BD-BCP1*	*2μ URA3*	This study
PKL194	*RPL23A*	*2μ HIS3*	This study
PKL195	*RPL23B*	*2μ HIS3*	This study
PKL228	*RPL11B-GFP*	*CEN LEU2*	This study
PKL253	*AD-RPL23*	*2μ LEU2*	This study
PKL254	*BD-RPL23*	*2μ URA3*	This study
PKL300	*GAL::RPL23*	*CEN URA3*	This study
PKL327	*RPL23A-HA*	*CEN HIS3*	This study
PKL329	*RPL23A-GFP*	*CEN URA3*	This study
PKL346	*RPL23*	*pET21*	This study
PKL478	*BCP1*	*CEN HIS3*	This study
PKL483	*bcp1ts*	*CEN HIS3*	This study
PKL493	*GAL::Bcp1*	*HIS3 CEN*	This study
PKL502	*BCP1-His*	*pET21*	This study
PKL547	*GST-KAP123*	*pGEX-4T3*	This study
PKL556	*GST-KAP121*	*pGEX-4T3*	This study

were retransformed to the *bcp1ts* strain to eliminate false positive ones. The suppressors were sequenced and searched in a database (the *Saccharomyces* genome database).

Sucrose Gradient Analysis—For sucrose density gradients, cultures were collected at an optical density at 600 nm (A_{600}) of 0.2–0.3. Cycloheximide was added to a final concentration of 50 μg/ml, and the mixture was continuously incubated for another 15 min. Cell extracts were prepared by vortexing (four times for 30 s with 1-min intervals of cooling on ice) with glass beads in polysome lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol, and 200 μg/ml cycloheximide). 10.5 A_{260} units of protein extracts were loaded onto linear 7–47% sucrose gradients in polysome lysis buffer. After 2.5 h of centrifugation at 284,000 × *g* in a P40ST rotor (Hitachi), gradient fractions were collected on a density gradient fractionator (BR-188, Brandel) continuously measuring absorbance at 254 nm. For detection of proteins, fractions were precipitated with 10% trichloroacetic acid. Pellets were suspended in 50 μl of 1× Laemmli buffer, separated on SDS-polyacrylamide gels, and detected by Western blotting. To calculate the ratio between 60S and 40S subunits, the area of the peak was calculated by ImageJ and counted as follows: ratio = (area of 60S subunit)/(area of 40S subunit).

Microscopy—Overnight cultures of yeast cells were diluted with fresh medium to an A_{600} of 0.2 and were incubated for another 2 h at 30 °C. For the *bcp1ts* mutant strain, cells were held at 37 °C for 2 h before the assay.

To monitor the distribution of Rpl23-HA under Bcp1 overexpression conditions, cells were cultured in the raffinose-containing medium, and 2% galactose was added to induce the overexpression of Bcp1. Cells were fixed with 3.7% formaldehyde and examined with indirect immunofluorescence. The anti-HA (Covance) antibody and FITC-conjugated anti-mouse IgG (Leinco Technologies) were used as primary and secondary antibodies, respectively. Fluorescence was visualized on a microscope (AxioScope A1; Zeiss) fitted with a Plan Apochromat ×100, numerical aperture 1.40 differential interference contrast (DIC) objective and a digital microscopy camera (AxioCam MRm Rev. 3) controlled with an AxioVision LE module Fluorescence Lite (Zeiss). Images were prepared using Photoshop version 7.0 (Adobe).

Immunoprecipitation—Cultures were grown to an A_{600} of ~0.5 in selective medium. The *bcp1* temperature-sensitive mutant was held at 37 °C for 2 h before cells were harvested. For the preparation of protein extracts, cells were resuspended in IP buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 6 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, and 1 mM leupeptin), lysed by vortexing with glass beads, and clarified by centrifugation. α-c-Myc antibody (9E10) and protein A-agarose beads were added to normalized samples and rocked at 4 °C. The beads were washed three times with IP buffer, and the proteins were eluted in 1× Laemmli sample buffer. Subsequently, proteins were separated in SDS-PAGE and observed by Western blotting.

In Vitro Protein Interaction Assays—Recombinant Bcp1, Rpl23, GST-Kap121, and GST-Kap123 were overexpressed in *E. coli* BL21 (DE3) upon isopropyl 1-thio-β-D-galactopyranoside induction. Cell extracts were prepared by sonication for 5 min (pulse on 9 s and pulse off 5 s)(Chrom Tech, Taipei, Taiwan). Bcp1 with a C-terminal His_6 tag was affinity-purified in TN100 buffer (20 mM Tris, pH 8, and 100 mM NaCl) with Ni-NTA resin, eluted in TN100 buffer containing 500 mM imidazole, and dialyzed in TN100 buffer. His-Gsp1(Ran)Q71L-GTP was expressed, and purification was performed as described previously (47). *E. coli* strain SG13009 containing pKW586 (*His_6-RANQ71L*) was expressed upon 0.5 mM isopropyl 1-thio-β-D-galactopyranoside induction. Cells were thawed and washed once in lysis buffer (30 mM KPO_4 buffer (pH 8.0), 300 mM KOAc, 20 mM imidazole, 2 mM $MgOAc_2$, 2 μM GTP, and protease inhibitors). Bound protein was eluted in lysis buffer containing 120 mM imidazole. EDTA was added to 6 mM and incubated on ice for 40 min in the presence of a 50-fold molar excess of GTP. $MgOAc_2$ was then titrated into the protein pool to a final concentration of 25 mM, and the mixture was incubated for an additional 10 min on ice. The purified proteins were dialyzed against 100 volumes of storage buffer (30 mM KPO_4 (pH 7.6), 50 mM KOAc, 7% glycerol, 2 mM $MgOAc_2$, 2 mM β-mercaptoethanol, 2 μM GTP) at 4 °C.

GST, GST-Kap121, or GST-Kap123 proteins were incubated with Rpl23 lysates in PBSKMT buffer (150 mM NaCl, 25 mM sodium phosphate, 3 mM KCl, 1 mM $MgCl_2$, 0.1% Tween 20, pH 7.3) with GSH-resin at 4 °C for 1 h. The beads were washed two times with buffer, and a fixed amount of purified Bcp1 or RanGTP was added and incubated at 4 °C for another 1 h. After three washes, bound proteins were eluted with 1× Laemmli sample buffer. Samples were separated in SDS-PAGE and detected with Coomassie Blue staining or Western blotting.

Separation of Free Form and Ribosome-bound Proteins Using Ultracentrifugation—Cultures were grown to an A_{600} of 0.4–0.5. Cell lysates were prepared by vortexing with glass beads in extraction buffer (50 mM NaCl, 20 mM Tris, pH 7.5, 6 mM $MgCl_2$, 10% glycerol, 0.1% Nonidet P-40, 1 mM PMSF, 1 μM leupeptin, and 1 μM pepstatin A). Normalized protein extracts

were centrifuged at 385,900 × *g* in a rotor (MLA130; Beckman Coulter) at 4 °C for 60 min. The top layer (free protein) and the pellet (ribosome-containing pool) were recovered. Pellets were fully resuspended with the same volume of extraction buffer. Free and ribosome-bound pools were further applied in immunoprecipitation if required.

Author Contributions—K.-Y. L. conceived and coordinated the study and wrote the paper. Y.-H. T., T.-J. L., A. W. J., J.-T. S., B.-R. C., and S. K. S. designed, performed, and analyzed the experiments. All authors reviewed the results and approved the final version of the manuscript.

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