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RESEARCH ARTICLE

Gpn2 and Rba50 Directly Participate in the Assembly of the Rpb3 Subcomplex in the Biogenesis of RNA Polymerase II

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ABSTRACT RNA polymerase II (RNAPII) is one of the central enzymes in cell growth and organizational development. It is a large macromolecular complex consisting of 12 subunits. Relative to the clear definition of RNAPII structure and biological function, the molecular mechanism of how RNAPII is assembled is poorly understood, and thus the key assembly factors acting for the assembly of RNAPII remain elusive. In this study, we identified two factors, Gpn2 and Rba50, that directly participate in the assembly of RNAPII. Gpn2 and Rba50 were demonstrated to interact with Rpb12 and Rpb3, respectively. An interaction between Gpn2 and Rba50 was also demonstrated. When Gpn2 and Rba50 are functionally defective, the assembly of the Rpb3 subcomplex is disrupted, leading to defects in the assembly of RNAPII. Based on these results, we conclude that Gpn2 and Rba50 directly participate in the assembly of the Rpb3 subcomplex and subsequently the biogenesis of RNAPII.

KEYWORDS Gpn2, RNA polymerase II, Rba50, assembly factor

RNA polymerase II (RNAPII) is one of the critical factors in gene expression, cell fate decision, and organizational development because it is directly responsible for catalyzing the synthesis of mRNAs and capped noncoding RNAs in eukaryotes (1–3). The core of this enzyme is composed of 10 subunits with a mass of approximately 520 kDa (4, 5). According to its crystal structure, the two largest subunits, Rpb1 and Rpb2, form the active catalytic center, while the other, smaller subunits associate with either the Rpb1 (Rpb5, Rpb6, and Rpb8) or Rpb2 (Rpb9) subcomplex (5, 6). The Rpb3, Rpb10, Rpb11, and Rpb12 subunits form a complex called the Rpb3 subcomplex (Rpb3-10-11-12), which bridges the two Rpb1 and Rpb2 subcomplexes to generate the core of RNAPII (6, 7). If eukaryotic RNAPII follows the assembling pathway of bacterial RNA polymerase, the general assembly process of the RNAPII core is proposed to consist of three steps, starting with the formation of the Rpb3 subcomplex, followed by the docking of the Rpb2 subcomplex, and finally the addition of the Rpb1 subassembly (8). In this working model, a few critical mechanisms related to RNAPII assembly require elucidation. First, how are these three subcomplexes assembled? Second, the mechanism of the assembly of the three subcomplexes into the 10-subunit catalytic core of RNAPII is unknown. Third, the assembly of large multisubunit complexes generally requires assembling factors, but the key assembling factors for the biogenesis of RNAPII remain to be demonstrated.

It is reported that the assembly of RNAPII takes place in the cytoplasm and the completely assembled RNAPII is then transported into the nucleus (9). Iwr1 and Rtp1p are required for transporting RNAPII into the nucleus (10, 11). Recently, Gpn1 and Gpn3 were also suggested to play an important role in transporting RNAPII into the nucleus (12–15). The GPN proteins contain a glycine-proline-asparagine motif (GPN) insertion in the G domain and are highly conserved (16). In eukaryotes, there are three members in

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the GPN family, named Gpn1, Gpn2, and Gpn3. Each member is essential for cell growth, indicating that they are not functionally redundant during cell growth. The functional defect of Gpn2 or Gpn3 in yeast cells, as well as knockdown of Gpn1 or Gpn3 in human cells, leads to cytoplasmic accumulation of RNAPII subunits (Rpb1 or/and Rpb3) (12, 13, 15, 17, 18). These results suggest that the GPN family may be required directly for transporting RNAPII into the nucleus. But another possibility also exists, namely, that Gpn1, Gpn2, and Gpn3 are essential assembling factors for the biogenesis of RNAPII and their functional defect causes disruption of RNAPII assembly and results in the cytoplasmic accumulation of RNAPII subunits.

Similar to RNAPII's requirement for specific factors for its transportation into the nucleus, the assembly of RNAPII *in vivo* also appears to require specific factors, particularly since the reconstitution of an active eukaryotic RNAPII *in vitro* has not been successful (8, 19, 20). Studies have indicated that the chaperone proteins of the heat shock protein 90 (HSP90)/HSP82, R2TP/prefoldin-like complex, and Bud27 are involved in the assembly of RNA polymerases (9, 21, 22). In general, chaperones are involved in the correct folding of newly synthesized polypeptides. And thus, they directly or indirectly participate in the assembly of some large macromolecular complexes.

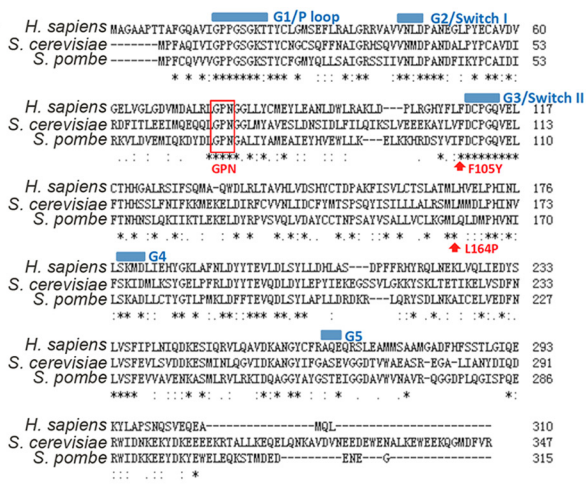
In this study, we obtained two temperature-sensitive mutants, the *gpn2*^{ts} and *rba50-3* mutants. By screening the genetic suppressors to these two mutants and conducting yeast two-hybrid assays, we established that Gpn2 interacts with Rpb12 and that Rba50 interacts with Rpb3. Both Rpb12 and Rpb3 are the subunits of the Rpb3 subcomplex. In addition, an interaction between Gpn2 and Rba50 was also demonstrated. Further studies showed that the assembly of the Rpb3 subcomplex was significantly impaired in the *gpn2*^{ts} and *rba50-3* cells, resulting in disruption of RNAPII biogenesis. These results demonstrated that Gpn2 and Rba50 are two essential factors that directly participate in the assembly of RNAPII.

RESULTS

***RPB12* and *RBA50* suppress the *gpn2*^{ts} mutant.** Gpn2 is a highly conserved protein from archaea to humans and is essential for cell growth. This suggests that Gpn2 may function in a central biological event. However, little is known about its biological function. We found that the immunoprecipitation against Gpn2 brought down some RNA polymerase subunits (data not shown). This suggests that the function of Gpn2 may be correlated with RNA polymerases. In order to determine the biological function of Gpn2, we first screened for mutants by random mutations of *GPN2* and obtained a temperature-sensitive mutation of *GPN2*: *gpn2*^{ts}. The *gpn2*^{ts} gene encoded two mutations at conserved residues, namely, Phe105Tyr and Leu164Pro. Phe105 is localized in the vicinity of the G3 motif, an essential GTPase-interacting domain for GTP binding and hydrolysis, while Leu164 is close to the G4 domain (23, 24) (Fig. 1A). The sensitivity of the *gpn2*^{ts} mutant to growth temperature and the transcription inhibitor mycophenolic acid (MPA) is shown in Fig. 1B. The temperature sensitivity of the *gpn2*^{ts} mutant is not due to a reduced protein level of Gpn2 at higher temperatures (Fig. 1C). The high sensitivity of the *gpn2*^{ts} mutant to MPA suggests that Gpn2 may function either in the transcription process or in the biogenesis of RNA polymerases.

To determine the biological function of Gpn2, we set to identify its genetically interacting proteins by screening the multicopy suppressors of the *gpn2*^{ts} mutants. Such a method has been successfully used in budding yeast in determining that Cdc6 and cyclins interact with the origin recognition complex (ORC) and Cdc28, respectively (25, 26). As expected, overexpressed Gpn1, Gpn2, and Gpn3 suppressed the temperature sensitivity of *gpn2*^{ts} cell growth (Fig. 1D and E). Unexpectedly, our results revealed that *RPB12* and *RBA50* are even better suppressors of the *gpn2*^{ts} mutant than are *GPN1* and *GPN3* (Fig. 1D and E). The extent to which these factors rescue the growth of the *gpn2*^{ts} mutant was consistent with the hit frequency in the table shown in Fig. 1D. Additionally, these suppressors also suppressed the sensitivity of the *gpn2*^{ts} mutant to the transcription inhibitor MPA (Fig. 1E). Rpb12 is the smallest subunit of RNA polymerase I, II, and III and is common to all three polymerase families (27, 28). Rpb12, Rpb3,

A



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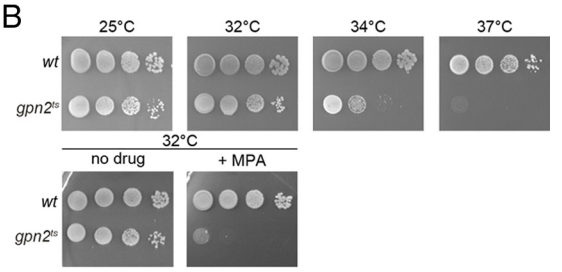

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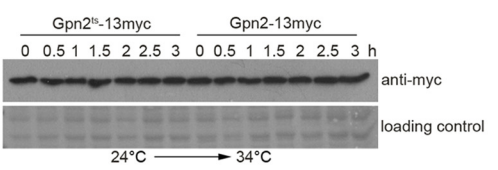
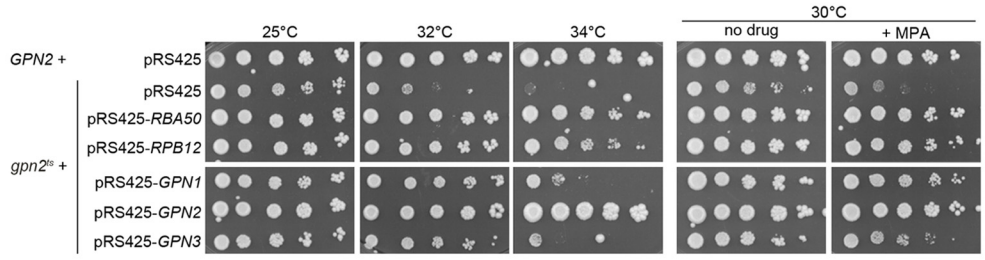


D

Suppressor	Systematic Name	Description	Hit
RBA50	YDR527W	RNA polymerase II (B) Associated protein	11
RPB12	YHR143W-A	RNA polymerase subunit ABC10-alpha, common subunit for RNA pol I, II, and III	10
GPN1	YJR072C	member of GPN-loop GTPase	2
GPN3	YLR243W	member of GPN-loop GTPase	1

E



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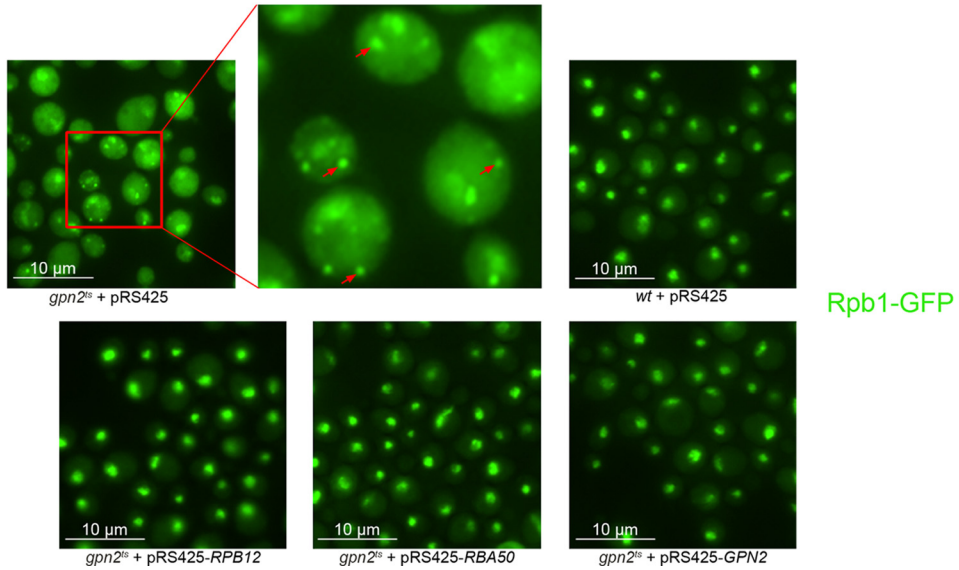

FIG 1 *RPB12* and *RBA50* suppress the *gpn2*^{ts} mutant. (A) Alignment of the Gpn2 amino acid sequences of the yeast (*S. cerevisiae* and *Schizosaccharomyces pombe*) and human (*Homo sapiens*) proteins. Gpn2 amino acid sequences of the budding yeast, fission yeast, and human proteins were aligned by ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/) using the default settings. The conserved G domains and the mutation sites of *gpn2*^{ts} are indicated. (B) Growth of wt (YFZ28) and *gpn2*^{ts} (YFZ 29) cells on YPD plates at different temperatures and sensitivity of *gpn2*^{ts} cells to 25 μg/μl MPA. (C) Exponentially growing wt (YFZ28) and *gpn2*^{ts} (YFZ 29) cells at 25°C were shifted to 34°C. Samples were taken at the indicated time points. TCA-processed whole-cell extracts were analyzed by immunoblotting with anti-myc (GPN2)

(Continued on next page)

Rpb10, and Rpb11 constitute the Rpb3 subassembly intermediate of RNAPII (Rpb3-10-11-12) (8). Rba50 is the yeast homolog of human RPAP1 (RNA polymerase II-associated protein 1) and was identified as an RNAPII-associated factor, but its exact function is unknown (29).

Studies by Minaker et al. showed that defective Gpn2 leads to cytoplasmic accumulation of Rpb1 (17). Accordingly, we examined whether overexpression of Rpb12 or Rba50 can correct the cytoplasmic accumulation of Rpb1 in the *gpn2*^{ts} cells. First, we confirmed that the *gpn2*^{ts} mutant also exhibited severe cytoplasmic accumulation of Rpb1 whereas Rpb1 localized to the nucleus in wild-type (wt) cells (Fig. 1F). Then, we found that, like the overexpression of Gpn2, the overexpression of Rpb12 or Rba50 also almost completely recovered the nuclear localization of Rpb1 in the *gpn2*^{ts} cells (Fig. 1F). Taken together, these results suggest that Gpn2 functions in the assembly of RNAPII rather than aiding in the transportation of RNAPII into the nucleus, since neither Rpb12 nor Rba50 has a nuclear localization motif.

Gpn2 is required for Rpb1 and Rpb2 association during the assembly of RNAPII. To determine the specific step at which Gpn2 is required for RNAPII biogenesis, we examined whether a functional defect of Gpn2 affects the addition of Rpb1 and Rpb2 to Rpb3. First, we verified the interactions between Rpb3 and Gpn2 or Rpb1 by immunoprecipitation (IP) against Rpb3 (30) (Fig. 2A). The cyclic peptide α-amanitin is used as an inhibitor of Rpb1 for the assembly of RNAPII (9). α-Amanitin binds to the Rpb1 subunit of RNA polymerase II with high affinity, which results in transcriptional arrest and the concomitant destruction of Rpb1, but the other subunits remain intact (9). Thus, the presence of α-amanitin inhibits the assembly of RNA polymerase II (31). In addition, assembling factors often associate with assembling intermediates of RNA polymerase II but dissociate from the completely assembled or whole RNA polymerase II. Thus, we used α-amanitin to inhibit the association of Rpb1 to RNAPII and examined whether the amount of Gpn2 in the Rpb3 subcomplex increases. In the presence of α-amanitin, there was a decrease in the amount of Rpb1 brought down by the IP of Rpb3, but the amount of Gpn2 in the brought-down sample was increased (Fig. 2A). Furthermore, we did not detect Rpb1 in the IP sample against Gpn2 (Fig. 2B). Thus, these results suggest that Gpn2 may only transiently associate with Rpb3 and that it dissociates from Rpb3 after Rpb1 is added onto Rpb3 or the Rpb3 subcomplex.

Next, a similar IP experiment was performed against Rpb3 in the extracts from wt or *gpn2*^{ts} cells. We found that the amounts of Rpb1 and Rpb2 in the IP sample were significantly reduced in the *gpn2*^{ts} cells compared with those in wt cells (Fig. 2C and D). These results suggest that Gpn2 is required for Rpb1 and Rpb2 addition onto Rpb3. Using a fluorescent assay, we found that the majority of the Gpn2 was located in the cytoplasm, whereas the Rpb3 was primarily in the nucleus (Fig. 2E), suggesting that the Gpn2 functions in the cytoplasm. This is consistent with the notion that the assembly of RNAPII takes place in the cytoplasm and a fully assembled RNAPII is then imported into the nucleus (9). Taken together, our data indicate that Gpn2 is required for the addition of Rpb1 and Rpb2 to the Rpb3 subunit or the Rpb3 subcomplex. Our data may also suggest that Gpn2 is essential for the formation of the Rpb3 subcomplex (Fig. 3E) and that Rpb1 and Rpb2 associate only with the Rpb3 subcomplex.

Gpn2 targets Rpb12 for the assembly of the Rpb3 subcomplex. The genetic suppression assay of the *gpn2*^{ts} gene illustrated in Fig. 1E suggests that Rpb12 interacts with Gpn2. To further examine the interaction between Gpn2 and Rpb12 or possibly other subunits of RNAPII, a yeast two-hybrid assay was performed with the human

FIG 1 Legend (Continued)
antibodies. A Ponceau S-stained region of the same membrane as that used for immunoblotting is shown as a loading control. (D) Multicopy suppressors of *gpn2*^{ts} identified in this screening. "Hit" indicates the number of clones obtained. (E) Fivefold serial dilutions of exponentially growing wt cells (YFZ28) carrying the pRS425 mock plasmid and the *gpn2*^{ts} mutant (YFZ29) carrying the indicated constructs were spotted onto SD-Leu plates with or without MPA and incubated at the indicated temperatures for 3 days. (F) *RPB12* or *RBA50* corrects the nuclear localization of RNAPII in the *gpn2*^{ts} cells. Wild-type cells (YFZ50) carrying empty plasmids (pRS425) and the *gpn2*^{ts} mutant (YFZ55) carrying the indicated constructs were grown to mid-exponential phase at 25°C, shifted to 32°C for 2 h, and then fixed for Rpb1-GFP distribution analysis by fluorescence microscopy. The arrows indicate the cytoplasmic foci of Rpb1-GFP in the *gpn2*^{ts} mutant at 32°C.

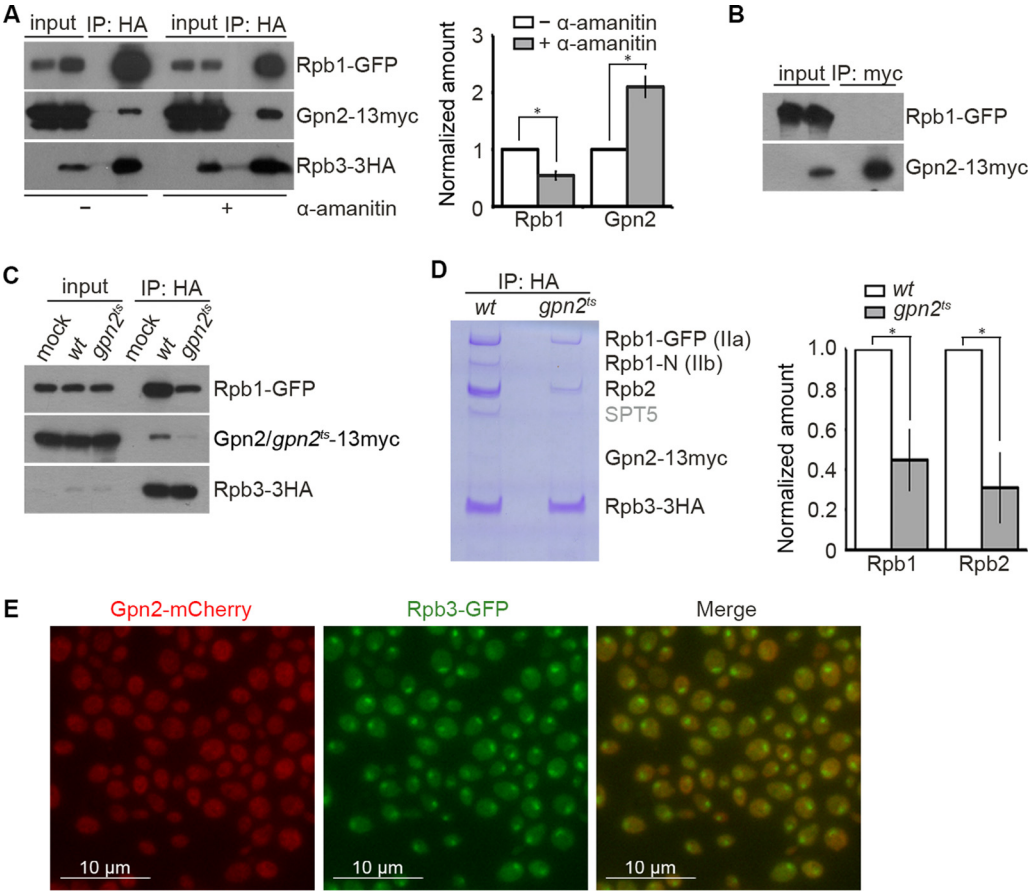

FIG 2 Gpn2 is required for the assembly of RNAPII at an early step. (A) Exponentially growing YFZ75 (Rpb3-3HA Gpn2-13myc Rpb1-GFP) and control YFZ50 (Gpn2-13myc Rpb1-GFP) cells, untreated or treated with 2 μg/ml α-amanitin for 1 h at 30°C, were collected and subjected to coimmunoprecipitation (Co-IP) using anti-HA beads (Rpb3). The coimmunoprecipitated proteins were analyzed by immunoblotting with either anti-GFP (Rpb1), anti-myc (Gpn2), or anti-HA (Rpb3) antibodies. The right panel shows the quantification of normalized Rpb1 and Gpn2 with Rpb3 immunoprecipitation with/without α-amanitin treatment. (B) Cell extracts from exponentially growing YFZ50 (Rpb1-GFP Gpn2-13myc) and the untagged control YFZ44 (Rpb1-GFP) were immunoprecipitated using anti-myc beads (Gpn2). The immunoprecipitated proteins were analyzed by immunoblotting with either anti-GFP (Rpb1) or anti-myc (Gpn2) antibodies. (C) Exponentially growing cultures of wt (YFZ75, Rpb1-GFP Gpn2-13myc Rpb3-3HA) and *gpn2*^{ts} (YFZ90, Rpb1-GFP Gpn2^{ts}-13myc Rpb3-3HA) cells as well as the mock control (YFZ50, Rpb1-GFP Gpn2-13myc) at 25°C were shifted to 36.5°C for 1.5 h and harvested. Rpb3 was immunopurified with anti-HA beads from the indicated extracts. Copurified Rpb1 and Gpn2 were analyzed with anti-GFP and anti-myc antibodies, respectively. (D) The copurified proteins from the sample in panel C were separated by SDS-PAGE and stained with Coomassie blue, followed by detection by mass spectrometry. The normalized quantification of copurified Rpb1 and Rpb2 is shown on the right-side panel. (E) Exponentially growing YFZ94 (Gpn2-mCherry Rpb3-GFP) cells were fixed and visualized on a Delta Vision Elite microscope.

Gpn2 and RNAPII subunits. Here, human Gpn2 and RNAPII subunits were used, since yeast Gpn2 shows artificial self-activation activity in the yeast two-hybrid assay. The results presented in Fig. 3A clearly show the interactions between Gpn2 with Rpb12 and Rpb3. A glutathione *S*-transferase (GST)–Rpb12 pulldown assay also demonstrated the interaction between Gpn2 and Rpb12 (Fig. 3B and C). As anticipated, Rpb3 was in the pulldown sample (Fig. 3C). Furthermore, we found that only Rpb12 overexpression could efficiently suppress the temperature sensitivity of *gpn2*^{ts} cells at restrictive temperatures or in the presence of MPA (Fig. 3D). These results prove that Gpn2 physically interacts with Rpb12 and also suggest that Gpn2 may participate in the assembly of the Rpb3 subcomplex.

To examine whether a defective Gpn2 affects the assembly of the Rpb3 subcomplex, an IP assay against Rpb3 was conducted with the extracts of wt and the *gpn2*^{ts} cells. In wt cells, the IP of Rpb3 brought down Rpb12, Rpb11, and Gpn2 (Fig. 3E), indicating a normal assembly of the Rpb3 subcomplex and the association of Gpn2 with this

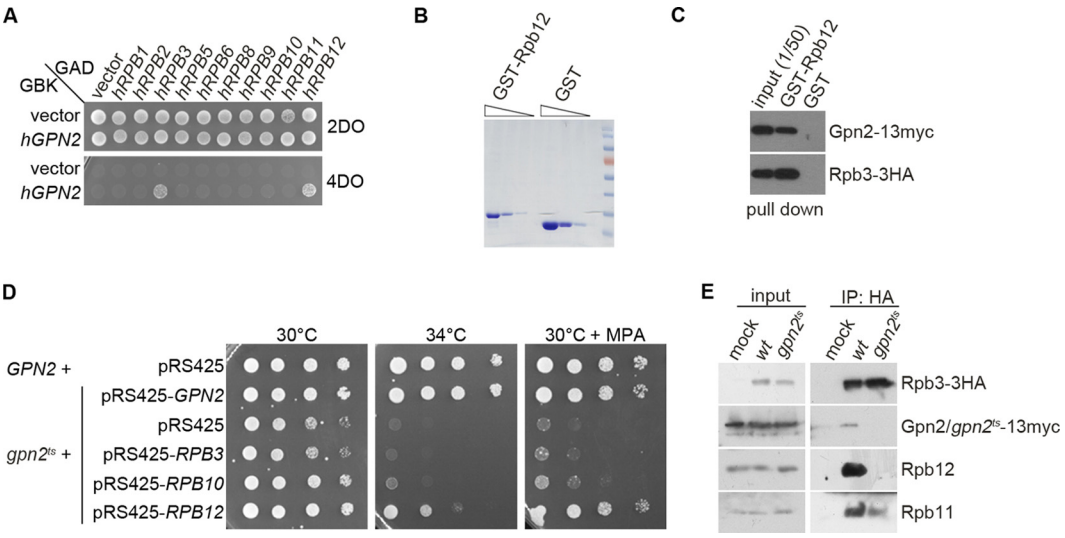

FIG 3 Gpn2 targets Rpb12 for the assembly of the Rpb3 subcomplex. (A) Two-hybrid interactions were judged by spot tests on two types of dropout (DO) plates with the corresponding proteins in human cells: high-stringency 4DO (SD-adenine, -histidine, -leucine, and -tryptophan) and the control 2DO (SD-leucine and -tryptophan). Reciprocal combinations of fusions with the GAL4 DNA-binding domain (BD) to hGpn2 and the GAL4 activation domain (AD) to each component of core human RNAPII were examined (the stalk part Rpb4/7 was not shown). (B) Coomassie blue-stained SDS-PAGE showing *Escherichia coli* purified recombinant GST-Rpb12 and GST. (C) Recombinant GST-Rpb12 or GST was used as bait in a pulldown assay with whole-cell extracts from exponentially growing wt cells (YFZ75). Proteins pulled down in the assay were analyzed by immunoblotting with anti-myc (Gpn2) or anti-HA (Rpb3) antibodies (1/50 lysate used in each pulldown was loaded as input control). (D) The genetic rescue assay of the indicated genes was examined following the assay described for Fig. 1E. (E) The same Co-IP experiment as that described for Fig. 2C was performed, and the copurified proteins were probed using antibodies against the indicated proteins.

complex. However, in the *gpn2*^{ts} cells, Rpb12 and Gpn2 were undetectable; the amount of Rpb11 in the brought-down sample was also reduced compared to that in wt cells (Fig. 3E). These results suggest that Gpn2 has a direct interaction with Rpb12 and that Gpn2 is required for the assembly of the Rpb3 subcomplex.

Rba50 coordinates with Gpn2 for the assembly of the Rpb3 subcomplex. Rba50 is essential for cell growth, but its biological function is not clearly elucidated according to the *Saccharomyces* genome database (SGD). This study revealed that *RBA50*, like *RPB12*, is an effective suppressor of the *gpn2*^{ts} mutant either in the presence or in the absence of the transcription inhibitor MPA (Fig. 1E), suggesting that Rba50 may play a critical role in the biogenesis of RNAPII. To test this possibility, we first screened and obtained a temperature-sensitive mutant, the *rba50-3* mutant (Fig. 4A). The *rba50-3* mutant harbors three point mutations that result in the changes of Ser288Pro, Asp293Val, and Ile344Arg. When the *rba50-3* mutant was crossed with the *GPN2-GFP* strain (where GFP stands for green fluorescent protein), we observed a severe growth defect at the semipermissive temperature of 28°C, suggesting that the functions of Rba50 and Gpn2 may be related and that the GFP tag in Gpn2-GFP has a negative effect on the growth of *rba50-3* cells (Fig. 4B). The subsequent IP and yeast two-hybrid assays indicated an interaction between Rba50 and Gpn2 in budding yeast (Fig. 4C) or between hRPRAP1 and hGpn2 in human cells (Fig. 4D), further indicating the functional relationship between Gpn2 and Rba50. In the brought-down sample against Rba50, Rpb1-GFP was not detected (Fig. 4C), suggesting that Rba50, like Gpn2, only transiently associates with the Rpb3 subcomplex and dissociates from it when Rpb1 is added to the complex.

In the *rba50-3* mutant cells, we observed a significant defect in the distribution of Rpb1-GFP (Fig. 4E), a phenomenon similar to that observed in the *gpn2*^{ts} cells (Fig. 1C). In addition, the cellular distributions of Gpn2 and Rba50 are similar (Fig. 4F). Furthermore, we found that overexpression of Rpb3 and Rpb12 but not Rpb10 can effectively suppress the temperature or MPA sensitivity of the *rba50-3* mutant (Fig. 4G), implying

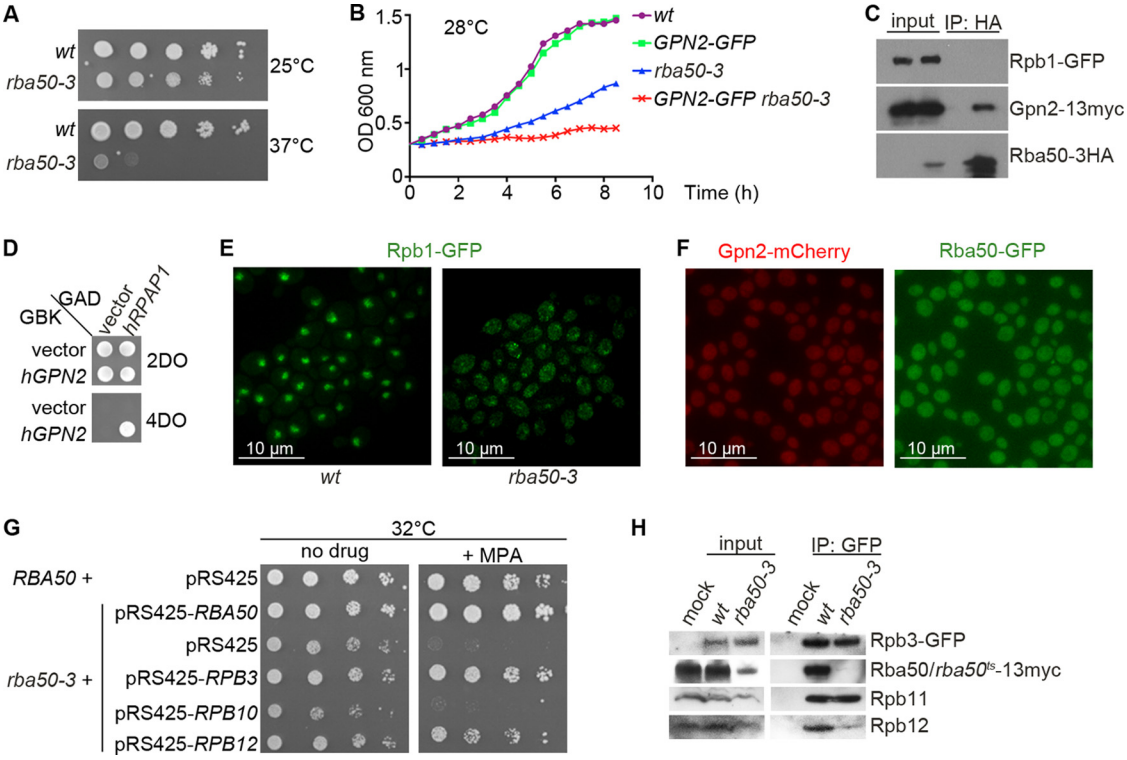

FIG 4 Rba50 is an essential assembly factor for the formation of the Rpb3 subcomplex. (A) Growth of wt (YFZ69) and *rba50-3* (YFZ77) on YPD plates at 24°C and 37°C. (B) Equal numbers of *GPN2-GFP*, *rba50-3*, or double mutant cells were grown at 28°C for 8.5 h. The data for the graph of the growth curves were the means for three replicates. OD_{600}, optical density at 600 nm. (C) Exponentially growing YFZ50 (Gpn2-13myc Rpb1-GFP) and YFZ74 (Rba50-3HA Gpn2-13myc Rpb1-GFP) cells were collected and subjected to Co-IP using anti-HA beads (Rba50). The coimmunoprecipitated proteins were analyzed by immunoblotting with either anti-GFP (Rpb1), anti-myc (Gpn2), or anti-HA (Rpb3) antibodies. (D) Two-hybrid interaction of human GPN2 and RPAP1 (the homolog of yeast Rba50). The procedure of the two-hybrid assay is the same as described for Fig. 3D. (E) Wild-type cells (YFZ44) and *rba50-3* mutants (YFZ96) expressing Rpb1-GFP were grown to mid-exponential phase at 24°C, shifted to 34°C for 2 h, and then fixed for Rpb1-GFP distribution analysis by fluorescence microscopy. (F) Distributions of Gpn2-mCherry and Rba50-GFP. Exponentially growing YFZ95 cells (Gpn2-mCherry Rba50-GFP) were fixed, and their fluorescence was visualized by fluorescence microscopy. (G) Fivefold serial dilutions of exponentially growing wt cells (YFZ28) carrying the pRS425 mock plasmid and the *rba50-3* mutant (YFZ77) carrying the indicated constructs were spotted onto SD-Leu$^-$ plates with/without MPA and incubated at the indicated temperatures for 3 days. (H) Wild-type cells (YFZ87, Rpb3-GFP Rba50-13myc), *rba50-3* mutant cells (YFZ97, Rpb3-GFP Rba50^{ts}-13myc), and mock cells (YFZ73, Rpb3-3HA Rba50-13myc) were grown to log phase at 30°C and then shifted to 36.5°C for an additional 1 h of incubation. The cells were harvested and subjected to the preparation of cell extracts and subsequent immunoprecipitation. Rpb3 and Rpb3-associated proteins were brought down with GFP-Trap beads. Rba50, Rpb11, and Rpb12 were detected by Western blotting with corresponding antibodies.

that Rba50 is involved in the assembly of the Rpb3 subcomplex. To directly demonstrate that Rba50 is essential for the assembly of the Rpb3 subcomplex, an IP assay against Rpb3 was conducted with the extracts of wt or *rba50-3* cells. In wt cells, the IP of Rpb3 brought down Rpb12, Rpb11, and Rba50 proteins (Fig. 4H). However, in the *rba50-3* cells, the amounts of Rba50 and Rpb12 in the brought-down sample were significantly reduced, although the amount of Rpb11 was not affected compared to that in wt cells. These results together indicate that Rba50 acts as an essential assembly factor to bring Rpb3 and Rpb12 together for the assembly of the Rpb3 subcomplex.

DISCUSSION

This study identified two factors, namely, Gpn2 and Rba50, that function as essential assembly factors for the biogenesis of RNAPII. We found that both Gpn2 and Rba50 are required for the assembly of the Rpb3 subcomplex. When Gpn2 was functionally defective, the formation of the Rpb3 subcomplex was disrupted (Fig. 3E); when Rba50 was defective, the assembly of the Rpb3 subcomplex was also inhibited (Fig. 4H), causing the accumulation of Rbp1 in the cytoplasm (Fig. 4E). Using the yeast two-hybrid assay and genetic suppressor screening, the interaction between Gpn2 and Rpb12 was determined (Fig. 1E and 3A and D). The interaction between Gpn2 and Rba50 or Rba50

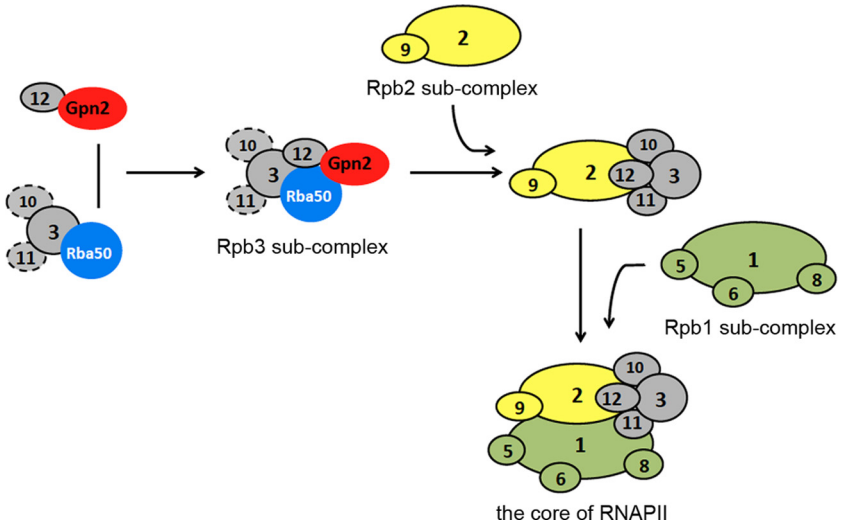

FIG 5 A working model of Gpn2 and Rba50 acting for the assembly of the Rpb3 subcomplex. Shown is a model for the recruitment of Rpb12 to Rpb3 in a Gpn2- and Rba50-coordinated manner. The Rpb3 subcomplex is followed by the Rpb2 subcomplex, finishing with the addition of the Rpb1 subcomplex.

and Rpb3 was also demonstrated (Fig. 1E and 4D and G). Taking these results together, we propose that the Rpb12, -3, -10, and -11 subunits are brought together through the physical interactions between Gpn2 and Rba50, Gpn2, and Rpb12, as well as Rba50 and Rpb3 (Fig. 5), which creates the Rpb3 subcomplex. The interaction among the Rpb10, -11, and -3 subunits was proposed in a previous study (32).

After the Rpb3 subcomplex is formed, the other subunits of RNAPII need to be recruited for the biogenesis of RNAPII. It is very likely that assembly factors are also required for the association of the Rpb2 and Rpb1 subcomplexes with the Rpb3 subcomplex. Currently, the identity of such assembly factors remains unknown. It is possible that Gpn1 and Gpn3 participate in this process. For instance, it has been reported that depletion of Gpn1 or Gpn3 led to a decreased level of RNAPII in the nucleus, suggesting that they may function in the transportation of RNAPII into the nucleus (13). However, an alternative explanation is that Gpn1 or Gpn3 may be required for the step of adding the Rpb1 and Rpb2 subcomplexes to the Rpb3 subcomplex. In line with this, a study by Minaker et al. suggested that the GPN proteins function upstream of Iwr1 (a protein required for nuclear importation of RNA polymerases) in the RNA polymerase II and III biogenesis (17). We found that both *GPN1* and *GPN3* can suppress the *gpn2*^{ts} mutant (Fig. 1E), suggesting that Gpn1 or -3 probably interacts with Gpn2. Previous studies also suggested that there are genetic and physical interactions among the three GPN members (17, 33, 34). Accordingly, it is very likely that Gpn1 and Gpn3 are also assembly factors for the biogenesis of RNAPII and they may act in subsequent steps in the formation of the core of RNAPII after the Gpn2- and Rba50-mediated assembly of the Rpb3 subcomplex. Further study will delineate their specific roles in the biogenesis of RNAPII.

MATERIALS AND METHODS

Constructs, strains, and culture conditions. The yeast strains used in this study are listed in Table 1; all these strains were derived from *Saccharomyces cerevisiae* W303-1a (35). *GPN2-13myc* and *gpn2-F105Y*, *L164P-13myc* mutants were generated at *GPN2* loci by integration of PCR-generated cassettes amplified from pRS306-GPN2-13myc:URA3 and pRS306-GPN2-F105Y, L164P-13myc:URA3, respectively, using oligonucleotides complementary to the sequences flanking the endogenous loci. *RPB2* and *RBA50* with a C-terminal *3HA* tag were generated at their genomic loci by integration of the PCR-generated cassettes amplified from the pFA6a-3HA-KanMX6 plasmid, using the oligonucleotides complementary to the sequences flanking the stop codon (30). *GPN2*, *RPB1*, *RPB3*, and *RBA50* with a *GFP* tag at the C-terminus-coding end were generated at their genomic loci by integration of the PCR-generated cassettes amplified from genomic DNA of the Yeast GFP Clone Collection (36), using oligonucleotides complementary to the sequences flanking the endogenous loci.

TABLE 1 Yeast strains used in this study

Strain	Relevant genotype	Source (reference)
W303-1a	*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*	Thomas and Rothstein (35)
YFZ28	W303-1a *GPN2-13myc::URA3*	This study
YFZ29	W303-1a *gpn2-del::gpn2-F105Y L164P-13myc::URA3*	This study
YFZ44	W303-1a *RPB1-GFP::HIS3MX6*	This study
YFZ50	W303-1a *GPN2-13myc::URA3 RPB1-GFP::HIS3MX6*	This study
YFZ55	W303-1a *gpn2-del::gpn2-F105Y L164P-13myc::URA3 RPB1-GFP::HIS3MX6*	This study
YFZ69	W303-1a *RBA50-13myc::URA3*	This study
YFZ73	W303-1a *RBA50-13myc::URA3 RPB3-3HA::KanMX6*	This study
YFZ74	W303-1a *RBA50-3HA::KanMX6 GPN2-13myc::URA3 RPB1-GFP::HIS3MX6*	This study
YFZ75	W303-1a *RPB3-3HA::KanMX6 GPN2-13myc::URA3 RPB1-GFP::HIS3MX6*	This study
YFZ77	W303-1a *rba50-del::rba50-S288P D293V I344R-13myc::URA3*	This study
YFZ82	W303-1a *GPN2-GFP::HIS3MX6*	This study
YFZ87	W303-1a *RBA50-13myc::URA3 RPB3-GFP::HIS3MX6*	This study
YFZ90	W303-1a *RPB3-3HA::KanMX6 gpn2-F105Y L164P-13myc::URA3 RPB1-GFP::HIS3MX6*	This study
YFZ94	W303-1a *GPN2-mCherry::KanMX6 RPB3-GFP::HIS3MX6*	This study
YFZ95	W303-1a *GPN2-mCherry::KanMX6 RBA50-GFP::HIS3MX6 RPB1-GFP::HIS3MX6*	This study
YFZ96	W303-1a *rba50-del::rba50-S288P D293V I344R-13myc::URA3*	This study
YFZ97	W303-1a *rba50-del::rba50-S288P D293V I344R-13myc::URA3 RPB3-GFP::HIS3MX6*	This study
YFZ100	W303-1a *rba50-del::rba50-S288P D293V I344R-13myc::URA3 GPN2-GFP::HIS3MX6*	This study

Yeast cells were cultured at the indicated temperatures in rich medium with 1% yeast extract, 2% peptone, and 2% glucose (YPD medium), except for the strains carrying pRS425 (2μm)-based multicopy constructs, which were grown in leucine-free SD medium (SD-Leu$^-$; 0.67% yeast nitrogen base [YNB], 2% glucose, 0.02% tryptophan, 0.02% histidine, 0.02% adenine, and 0.02% uracil). Mycophenolic acid (M-5255; Sigma, St. Louis, MO) was added at the indicated concentration. For serial dilution assays, exponentially growing cultures at 25°C were spotted onto the indicated plates to the same concentration using a 5-fold serial dilution. The cells were grown at the indicated temperatures for 3 or 4 days before imaging.

Isolation of multicopy suppressors of *gpn2^{ts}*. About 10^8 log-phase *gpn2^{ts}* cells (strain YFZ29) were transformed with 5 μg of plasmid DNA containing the Sau3AI partial digested genomic library, which was constructed in the pRS425 vector (2μm ARS LEU2) with an average insert size of about 10 kb. Cells were plated onto SD-Leu$^-$ plates and incubated at 25°C for 1 day and then at 36°C for 5 days. The transformation efficiency was about 1×10^4 transformants per 1 μg of DNA. Plasmid DNA was isolated from colonies that were able to grow at 36°C. Isolated plasmid was then recovered by transformation into *Escherichia coli* DH5α. The inserts were identified by sequencing with the T7 and T3 primers. The suppressor genes in the inserts were confirmed by subcloning into pRS425 and tested for suppression of *gpn2^{ts}*.

Co-IP and Western blotting. For coimmunoprecipitation (Co-IP), cultures were collected and extracted with glass bead beating in lysis buffer (50 mM Tris-HCl [pH 7.8], 150 mM NaCl, 1 mM EDTA, and 10% [vol/vol] glycerol, 0.1% [vol/vol] Nonidet P-40) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin) and phosphatase inhibitors (0.5 mM sodium pyrophosphate, 2 mM NaF). A 15-μl volume of antihemagglutinin (anti-HA) HA-7 (A7470, mouse monoclonal purified IgG; Sigma), anti-myc–HA-7–agarose matrix (A2095, mouse monoclonal purified IgG; Sigma), or anti-GFP (GFP-Trap_A; Chromotek) was added to extracts to immunopurify the indicated proteins, C-terminally tagged with either 3HA, 13myc, or GFP, respectively. The samples were gently rotated for 1 h at 4°C. The beads were then washed four times with 1 ml of cold lysis buffer. Finally, the precipitated proteins were released by boiling the beads in Laemmli sample buffer and then resolved by SDS-PAGE.

The primary antibodies used in this study are anti-HA (12CA5, monoclonal mouse hybridoma supernatant), anti-myc (9E10, monoclonal mouse hybridoma supernatant), anti-GFP (mouse monoclonal GF28R; Thermo Fisher Scientific, Waltham, MA), and anti-GST (ab19256; Abcam, Cambridge, UK) antibodies. The polyclonal rabbit antibodies against Rpb11 and Rpb12 were raised in rabbits immunized with the corresponding recombinant proteins. Whole-cell extracts for Western blotting were prepared by glass bead beating in trichloroacetic acid (TCA) and resolved by SDS-PAGE.

Yeast two-hybrid assay. Gal4-based Matchmaker yeast two-hybrid system 3 (Clontech Laboratories, Mountain View, CA) was used for the yeast two-hybrid assay according to the manufacturer's instructions. The indicated genes were subcloned to fuse the GAL4 activation domain (AD) in the pGADT7 vector or the GAL4 DNA-binding domain (BD) in pGBKT7 from a human cDNA library (37). The corresponding constructs were cotransformed into the tester strain AH109.

Fluorescence microscopy. For colocalization analysis of Gpn2-mCherry with Rpb3-GFP or Rba50-GFP, cells were cultured in rich medium (YPD) at 30°C. For Rpb1-GFP distribution analysis in *gpn2^{ts}* or *rba50-3* mutants, cells with the pRS425-based constructs were grown in SD-Leu$^-$ at 25°C and subsequently moved to 32°C for 3 h. The indicated cultures were then harvested and fixed with 70% ethanol. The fixed cells were washed and imaged using a Delta Vision Elite microscope (Applied precision Inc., Mississauga, ON, Canada) using Volocity software.

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