The poly(A)-binding protein Nab2 functions in RNA polymerase III transcription

L. Maximilian Reuter,1,2 Dominik M. Meinel,2,4 and Katja Sträßer1,2,3

1Institute of Biochemistry, Justus Liebig University Giessen, 35392 Giessen, Germany; 2Gene Center, 4Center for Integrated Protein Science Munich (CIPSM), Ludwig-Maximilians-University Munich, 81377 Munich, Germany

RNA polymerase III (RNAPIII) synthesizes most small RNAs, the most prominent being tRNAs. Although the basic mechanism of RNAPIII transcription is well understood, recent evidence suggests that additional proteins play a role in RNAPIII transcription. Here, we discovered by a genome-wide approach that Nab2, a poly(A)-binding protein important for correct poly(A) tail length and nuclear mRNA export, is present at all RNAPIII transcribed genes. The occupancy of Nab2 at RNAPIII transcribed genes is dependent on transcription. Using a novel temperature-sensitive allele of NAB2, nab2-34, we show that Nab2 is required for the occupancy of RNAPIII and TFIIIB at target genes. Furthermore, Nab2 interacts with RNAPIII, TFIIIB, and RNAPIII transcripts. Importantly, impairment of Nab2 function causes an RNAPIII transcription defect in vivo and in vitro. Taken together, we establish Nab2, an important mRNA biogenesis factor, as a novel player required for RNAPIII transcription by stabilizing TFIIIB and RNAPIII at promoters.

[Keywords: RNA polymerase III; Nab2; tRNA; ncRNA; gene expression; TFIIIB]

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PC4 and its *S. cerevisiae* ortholog, Sub1; and the RNAPII TFS Dst1/TFIIS, Yox1, Fkh1, Reb1, and Yap6 [White 2011; Acker et al. 2013; Gjidoda and Henry 2013 and references therein]. However, the molecular function in RNAPIII transcription of most of these proteins has not been determined. Interestingly, the activity of an in vitro reconstituted yeast RNAPIII transcription system with purified RNAPIII, TFIIC, and TFIIB is very low, supporting the argument that additional proteins other than the established RNAPIII TFS are involved in RNAPIII transcription [Ducrot et al. 2006].

In a genome-wide study to determine the occupancy of nuclear mRNA-binding proteins on the *S. cerevisiae* genome [Meinel et al. 2013], we serendipitously observed that Nab2 localizes to all genes transcribed by RNAPIII. Nab2 is a nuclear polyadenylated RNA-binding protein required for 3′ end formation and mRNA export [Soucek et al. 2012 and references therein]. It is present along the whole ORF of protein-coding genes as determined by chromatin immunoprecipitation [ChIP] experiments hybridized to high-density tiling arrays [ChIP-chip] [Meinel et al. 2013]. As a member of the poly[A]-binding protein (PAPB) family, Nab2 binds to the poly(A) tail of mRNAs during or shortly after their polyadenylation, regulating its length [Kelly et al. 2010; Soucek et al. 2012 and references therein]. In addition to poly[A] RNA [Kelly et al. 2010], Nab2 also shows nonspecific RNA binding [Kelly et al. 2007], consistent with its binding to the body of mRNAs in vivo (Tuck and Tollervey 2013) and its at least partially RNA-dependent occupancy at an RNAPII reporter gene [Meinel et al. 2013]. A second function of Nab2 in mRNP biogenesis is in mRNA export [Hector et al. 2002]. After the mRNA is correctly processed and packaged by proteins in an mRNP, it is exported from the nucleus to the cytoplasm through the nuclear pore complex by the conserved *S. cerevisiae* mRNA exporter Mex67-Mtr2. Nab2, the TREX components Hpr1 and Sub2, and the SR protein Npl3 have been proposed to act as mRNA adaptors that recruit Mex67-Mtr2 to the mRNP [Strasser and Hurt 2001; Strasser et al. 2002; Gilbert and Guthrie 2004; Gwizdek et al. 2006; Iglelias et al. 2010].

Here, we show that Nab2 functions in RNAPIII transcription. Nab2 occupies RNAPIII genes genome-wide in a transcription-dependent manner. Importantly, impairment of Nab2 function leads to lower occupancy of RNAPIII at genes and a concomitant RNAPIII transcription defect. Nab2 interacts directly with RNAPIII, RNAPIII transcripts, and TFIIB. Furthermore, the binding of TFIIB with the target promoter DNA is stabilized by Nab2. Thus, we established Nab2, the first mRNA biogenesis factor, as a novel player essential for efficient RNAPIII transcription.

**Results**

**Nab2 is recruited to RNAPIII genes**

The nuclear PABP Nab2 is well known for its function in poly[A] tail length control and nuclear mRNA export. In a recent study, we determined the genome-wide occupancy of Nab2 and other nuclear mRNP components in *S. cerevisiae* by ChIP-chip [Meinel et al. 2013]. As expected, Nab2 occupies protein-coding genes [Gonzalez-Aguilera et al. 2011; Meinel et al. 2013]. However, we discovered that, in addition to RNAPIII transcribed genes, Nab2 also localizes to genes transcribed by RNAPII. The occupancies of Nab2 were calculated separately for genes encoding mRNAs, sn/snoRNAs, tRNAs, and rRNAs. Their frequencies were then plotted against the signal intensities for each gene class [Fig. 1A]. The signal intensities for Nab2 are high for not only sn/snoRNA and protein-coding genes transcribed by RNAPII but also genes encoding tRNAs, which are transcribed by RNAPIII [Fig. 1A]. In contrast, there is no signal for Nab2 at tRNA genes [Fig. 1A]. Next, we calculated meta-tRNA gene occupancy profiles of Nab2 and Rpb160, the largest subunit of RNAPIII. The average nucleotide occupancy for Nab2 or Rpb160 was plotted for all intronless tRNA genes <76 base pairs [bp] after gene length normalization [Fig. 1B]. The meta-tRNA gene occupancy profiles of Nab2 and Rpb160 are very similar [Fig. 1B], suggesting co-occupancy of Nab2 and RNAPIII at tRNA genes. Inspection of the individual traces revealed that the meta-tRNA gene occupancy profile of Nab2 reflects its occupancy at all tRNA genes [Supplemental Fig. S1]. In contrast, meta-tRNA gene occupancy profiles of the RNAPII subunit Rpb3, the TREX component Tho2, and the SR protein Npl3 show that these proteins are absent from tRNA genes [Fig. 1B]. Thus, Nab2 recruitment to tRNA genes is most likely independent of RNAPII and nuclear mRNP-binding proteins.

We then correlated the peak occupancies of Nab2, RNAPII [Rpb3], and RNAPIII [Rpb160]. Scatter plots of the peak occupancies of Nab2 and the RNAPII subunit Rpb3 show that Nab2 and RNAPII occupancies are high at protein-coding genes [Fig. 1C, gray dots]. In contrast, tRNA genes show occupancy of Nab2 but not RNAPII [Fig. 1C, red dots]. Likewise, scatter plots of the peak occupancies of Nab2 and the RNAPIII subunit Rpb160 show that the Nab2 and RNAPIII peak occupancies are both high at tRNA genes [Fig. 1D, red dots], whereas protein-coding genes are occupied by Nab2 but not RNAPIII [Fig. 1C, gray dots]. The occupancy of RNAPIII at some protein-coding genes is probably caused by spillover effects due to tRNA genes located nearby [Fig. 1D, orange dots]. Consistently, Nab2 occupancies are strongly correlated with the RNAPIII occupancies but not with the RNAPIII occupancies at protein-coding genes, whereas Nab2 occupancies correlate strongly with the RNAPIII occupancies but not with the RNAPII occupancies at tRNA genes [Fig. 1E,F]. Thus, the occupancy of Nab2 at tRNA genes is specific and independent of RNAPIII. Furthermore, Nab2 occupies not only tRNA genes but all genes transcribed by RNAPIII, such as *RDN5, RPI1, SCR1, SNR6*, and *SNR52* [Supplemental Fig. S1]. Taken together, Nab2 is present at all genes transcribed by RNAPIII, indicating a general role of Nab2 in RNAPIII transcription.

**Recruitment of Nab2 to RNAPIII depends on active transcription**

We wanted to determine whether the presence of Nab2 at RNAPIII genes depends on active transcription. To impair
transcription by RNAPIII, we used the rpc25-S100P mutation, which impairs transcription initiation especially at the restrictive temperature (Zaros and Thuriaux 2005). We assessed the occupancy ofRpc160 (the largest subunit of RNAPIII) and Nab2 by ChIP at three exemplary RNAPIII genes: SNR6, tDNA<sup>57s</sup>, and RPR1.

When RNAPIII transcription was impaired by shifting the rpc25-S100P mutant to the restrictive temperature (37°C), the occupancy of RNAPIII expectedly decreased significantly at the three RNAPIII genes, whereas the RNAPIII occupancy stayed unchanged in wild-type cells at 37°C, the occupancy of RNAPIII expectedly decreased in the mutant (Fig. 2B), suggesting that Nab2 recruitment is required for full occupancy of RNAPIII at the three exemplary genes. The occupancy of RNAPIII in wild-type cells at 30°C (permissive temperature) was not significantly different from the occupancy at 25°C and 18°C, and are dead at the restrictive temperature of 37°C (Fig. 2C; Supplemental Fig. S2B).

We assessed the occupancy of Rpc160 (the largest subunit of RNAPIII) and Nab2 by ChIP at three exemplary RNA-P III genes: SNR6, tDNA<sup>57s</sup>, and RPR1. After mutagenesis of NAB2, we screened for alleles that grow well at 30°C and are not viable at 37°C. Two alleles already confers a slight growth defect at 30°C. nab2-34 and especially the nab2-1 allele to assess whether Nab2 functions in RNAPIII transcription.

Nab2 is required for full occupancy of RNAPIII

The transcription-dependent, genome-wide presence of Nab2 at RNAPIII genes suggests a function for Nab2 in RNAPIII transcription. To study this potentially novel function of Nab2 in RNAPIII transcription, we first generated a new conditional allele of NAB2 as a tool. Previously generated alleles of NAB2 are not optimal: They cause a growth defect already at the permissive temperature (such as nab2-1 and especially the nab2-1-GFP alleles) [Supplemental Fig. S2A], they do not cause pronounced temperature sensitivity (nab2-C437S), or they require a medium shift for depletion of Nab2 [nab2-td] [Marfatia et al. 2003; Gonzalez-Aguilera et al. 2011; Brockmann et al. 2012]. After mutagenesis of NAB2, we screened for alleles that grow well at 30°C and are not viable at 37°C and identified one temperature-sensitive allele, nab2-34. On plates, nab2-34 cells grow as wild-type cells at the permissive temperature (30°C), are weakly impaired in growth at 25°C and 18°C, and are dead at the restrictive temperature of 37°C (Fig. 2C; Supplemental Fig. S2B). Growth curves in liquid culture revealed that the nab2-34 allele already confers a slight growth defect at 30°C. At 37°C, a growth retardation is first visible after 4 h, but nab2-34 cells continue to grow slowly for at least 9 h [Supplemental Fig. S2B].

We used this novel NAB2 allele to assess whether Nab2 is required for full occupancy of RNAPIII at the three exemplary genes. The occupancy of RNAPIII in NAB2 and nab2-34 cells at 30°C (permissive temperature) was normalized to 100%. RNAPIII occupancy does not change in NAB2 cells after shifting to 37°C (nonpermissive
temperature (Fig. 2D). In contrast, when Nab2 function is impaired in nab2-34 cells by shifting for 3 h to 37°C, the occupancy of RNAPIII decreases to ∼50% (Fig. 2B). The fact that a decrease in RNAPIII occupancy was observed before the nab2-34 cells displayed a growth defect suggests that the effect of Nab2 on RNAPIII occupancy is direct. Thus, Nab2 is required for full occupancy of RNAPIII, and Nab2 function is thus required for RNAPIII transcription.

Nab2 interacts directly with RNAPIII

Nab2 is likely to interact with RNAPIII if it functions in RNAPIII transcription. To assess a possible physical interaction of Nab2 with RNAPIII in vivo, we purified TAP-tagged Nab2 from S. cerevisiae by tandem affinity purification and tested for copurification of RNAPIII by Western blotting against HA-tagged Rpc160. Rpc160-HA and thus likely the whole RNAPIII complex copurify with Nab2 (Fig. 3A, lane 5). A strain that expresses HA-tagged Rpc160 but no TAP-tagged protein served as a negative control (Fig. 3A, lane 4). Conversely, we purified RNAPIII by tandem affinity purification from an Rpc160-TAP strain and assessed copurification of Nab2 by Western blotting. Nab2 indeed copurifies with RNAPIII (Fig. 3A, lane 6). Since the whole-cell protein extracts were treated with RNase A and DNase, the observed in vivo interaction between Nab2 and RNAPIII is most likely independent of RNA and DNA.

In order to assess whether the in vivo interaction of Nab2 and RNAPIII is direct, we performed in vitro pull-down experiments. GST-Precission protease cleavage site–Nab2 was purified from Escherichia coli and bound to GST beads (Fig. 3B, lanes 1,3,7, Nab2). GST bound to beads served as a negative control (Fig. 3B, lanes 4,5). GST-Nab2 does not show high-molecular-weight bands without incubation with RNAPIII (Fig. 3B, lane 1). GST-Nab2 was then incubated with highly purified RNAPIII from S. cerevisiae, and bound proteins were eluted by cleavage with Precission protease. Importantly, purified RNAPIII (Fig. 3B, lane 2) bound to recombinant GST-Nab2 (Fig. 3B, lane 3). This interaction between Nab2 and RNAPIII is specific, as S. cerevisiae RNAPIII did not bind to GST alone (Fig. 3B, cf. lanes 4 and 5), and highly purified S. cerevisiae RNAPI did not bind to GST-Nab2 (Fig. 3B, cf. lanes 6 and 7). These experiments show that Nab2 interacts directly and specifically with RNAPIII, substantiating a function in RNAPIII transcription.

Nab2 binds RNAPIII transcripts in vivo

Since Nab2 binds poly[A] RNA and most likely functions in RNAPIII transcription, it could bind RNAPIII transcripts. Tuck and Tollervey (2013) recently determined all transcripts bound to 13 mRNA processing, export, and turnover proteins in S. cerevisiae in vivo—among them, Nab2. Analysis of these data revealed that Nab2 binds to RNAPIII transcripts [Fig. 4]. A metaprofile of all intronless tRNA transcripts shows that Nab2 binds to the body of tRNAs [Fig. 4A]. This metaprofile reflects binding to all tRNAs, as evident by the hit distribution for individual intronless and intron-containing tRNAs such as tRNA^{Ile}(AAU)E1, tRNA^{Leu}(UAG)L2, or tRNA^{Ile}(AAU)
L1 [Fig. 4B,C]. In addition to tRNAs, Nab2 also binds to all other known RNAPIII transcripts, such as the RNA of the signal recognition particle [Fig. 4B, SCtR1], the RNA component of RNase P [Fig. 4D], and the 5S rRNA [Fig. 4E]. Thus, Nab2 binds directly to all RNAPIII transcripts in vivo.

Interestingly, inspection of the traces of RNAPIII transcripts that are synthesized as precursors revealed that Nab2 already binds to the premature form of RNAPIII transcripts. For example, Nab2 binds to the intronic region of tRNA^L1ε(UAG)L2 and the 3′ region of tRNA^Aε(UAA)lL1 [Fig. 4C]. The ~84-bp 5′ extension of RPR1 [the RNA component of RNase P] that is cleaved off after transcription [Lee et al. 1991] is bound by Nab2 [Fig. 4D]. In addition, Nab2 binds sequences downstream from the 3′ end of the mature RPR1, indicating that 3′ extended transcripts occur. Together with the findings that Nab2 binds directly to RNAPIII and is present at its target genes during transcription, this indicates that Nab2 probably binds to RNAPIII transcripts as they are being synthesized.

Nab2 is required for RNAPIII transcription in vivo

The data above suggest that Nab2 functions in RNAPIII transcription. We thus investigated whether Nab2 is needed to maintain RNAPIII transcript levels in vivo by assessing the levels of selected RNAPIII transcripts: the intron-containing tRNA^L1ε(UAU)L, RPR1, and SNR6. The steady-state levels of these transcripts were determined by Northern blotting of total RNA extracted from wild-type and nab2-34 cells [Fig. 5A–C]. The rpc25-S100P mutant was used as positive control [Fig. 5A–C]. The amounts of intron-containing tRNA^L1ε(UAU)L precursors [Fig. 5A], the RPR1 precursor [Fig. 5B], and the U6 snRNA (SNR6) [Fig. 5C] were measured and normalized to the amounts of the respective RNA in wild-type cells. After 6 h at the restrictive temperature, the levels of all three transcripts decreased significantly in nab2-34 cells [Fig. 5A–C]. Since the levels of the pre-tRNA^L1ε(UAU)L primary transcript and the RPR1 precursor decrease, it is likely that the de novo synthesis of these RNAs—i.e., RNAPIII transcription—is affected in nab2-34 cells. Thus, Nab2 function is most likely needed for full transcriptional activity of RNAPIII in vivo.

Nab2 functions directly in RNAPIII transcription

To assess whether Nab2 is directly involved in RNAPIII transcription, we performed in vitro transcription assays with two RNAPIII templates. The de novo synthesis of tRNA^L1ε(UUC)E [Fig. 5D] and the U6 snRNA (SNR6) [Fig. 5E] in RNAPIII transcription-active extracts of wild-type and nab2-34 cells at 25°C and 37°C was determined. The synthesis of tRNA^L1ε(UUC)E and SNR6 is significantly decreased in nab2-34 extracts compared with the transcription activity in wild-type extracts at 37°C [Fig. 5D,E]. Thus, functional Nab2 is required for transcriptional activity in vitro, corroborating its function in RNAPIII transcription.

In order to assess whether the function of Nab2 in RNAPIII transcription is direct, we performed add-back experiments. In vitro transcription of the SNR6 gene was performed at 25°C and 37°C in NAB2 and nab2-34 transcription-active extracts. Increasing amounts of recombinant Nab2 were added to the transcription reactions at 37°C. Importantly, Nab2 stimulated the reduced transcriptional activity in nab2-34 extracts in a dose-dependent manner [Fig. 5F]. To corroborate the direct function of Nab2 in RNAPIII transcription suggested by this add-back experiment, we used a fully reconstituted in vitro transcription assay consisting of RNAPIII, TFIIIB, TFIIIC, and template DNA. Also, in this system, addition of increasing amounts of Nab2 stimulated RNAPIII transcription.
Supplemental Fig. S6C). Thus, Nab2 is needed for full occupancy decrease in occupancy is specific, as the occupancy of TFIIIC does not panel; Supplemental Fig. S6B). This reduction of TFIIIB occupancy occurs in cells at the restrictive temperature (Fig. 6A, left panel; Supplemental Fig. S6A). Interestingly, the occupancy of TFIIIB is also reduced along the SCRI gene and at selected RNAPIII transcribed genes in nab2-34 cells at the restrictive temperature [Fig. 6A, left panel; Supplemental Fig. S6B]. This reduction of TFIIIB occupancy is specific, as the occupancy of TFIIIC does not decrease in nab2-34 cells at 37°C [Fig. 6A, right panel; Supplemental Fig. S6C]. Thus, Nab2 is needed for full occupancy of TFIIIB in vivo. The reduced occupancy of TFIIIB at RNAPIII promoters in nab2-34 cells is most likely the reason for the decreased occupancy of RNAPIII and the decreased transcriptional activity in these cells [Figs. 2D, 5; Supplemental Fig. S6A]. In order to recruit TFIIIB to the promoter or stabilize its binding, Nab2 should interact with TFIIIB in vivo. To test this, we purified Nab2 from S. cerevisiae by tandem affinity purification and performed Western blots to determine the presence of TFIIIB or TFIIIC subunits. Indeed, Nab2 specifically copurifies the TFIIIB subunit Brf1 and thus most likely the whole TFIIIB complex but does not interact with the TFIIIC subunit Tfc8 [Fig. 6B].

These results indicate that Nab2 could increase the binding of TFIIIB to its cognate DNA. In order to test this directly, we performed gel shift assays [EMSAs]. As a DNA substrate, we used TA-30-B6, a 76-bp-long dsDNA template derived from the SUP4 gene with the 6-bp TATA-box TATAAA and two 2-nucleotide (nt) mismatches at which Tbp binds the kinked DNA [Kassavetis et al. 1998]. Since this DNA template requires specific interactions of the TFIIIB subunits [i.e., only a sequential binding of Tbp, Brf1, and Bdp1 is possible] in a TFIIIC-independent manner, we could test whether and, if so, at which step Nab2 increases the binding of TFIIIB subunits to the DNA. To first rule out an unspecific binding of Nab2 to DNA, we tested different amounts of Nab2 for binding to the cognate DNA template described above as well as four similar dsDNAs [dsDNA1+, dsDNA1−, dsDNA2+, and dsDNA2−], which are derived from TA-30-B6 with a scrambled nucleotide sequence with or without the 4-nt mismatches. Additionally, dsDNA1 contained no repeating nucleotides, whereas dsDNA2 contained double nucleotides such as AA or TT. Nab2 bound to these DNAs only at a high concentration of Nab2, and the least to the TFIIIB DNA template TA-30-B6 (Supplemental Fig. S7). Using this probe, we then tested whether Nab2 increases the binding of TFIIIB subunits to the cognate DNA. When Tbp is already bound to the dsDNA, Nab2 can also bind to the DNA and consequently induces a supershift [Fig. 6C, cf. lanes 1–3]. Similarly, Nab2 increases the amount of protein bound to the DNA and induces a supershift when either Tbp–Brf1 complexes or the whole TFIIIB complex are bound to the DNA [Fig. 6C, cf. lanes 4, 5, and 6, respectively]. Thus, Nab2 increases the amount of TFIIIB-bound DNA in vitro. Taken together, these results indicate that Nab2 increases TFIIIB binding to the promoter, thereby stimulating RNAPIII transcription.

**Discussion**

The PABP Nab2 has well-characterized roles in poly(A) tail length control and mRNA export. Here, we show that Nab2 also functions in RNAPIII transcription. Our genome-wide data on localization of several mRNA-binding proteins revealed that Nab2 occupies all RNAPIII transcribed genes [Fig. 1; Supplemental Fig. S1]. This finding is consistent with the recent observation that Nab2 localizes to five genes transcribed by RNAPIII [Gonzalez-
Aguilera et al. 2011). This work by Gonzalez-Aguilera et al. (2011) also implicated Nab2 in RNAPIII metabolism but excluded a role of Nab2 in RNAPIII transcription. However, nab2-1-GFP, the allele of NAB2 used in that study (encoded by plasmid pAC1038) [Marfatia et al. 2003], leads to extremely slow growth already at the permissive temperature [Supplemental Fig. S2A]. We believe that the extremely slow growth of the nab2-1-GFP mutant most likely has secondary effects, causing the function of Nab2 in RNAPIII transcription to be missed.

Using our novel temperature-sensitive allele, nab2-34, we show here that Nab2 is important for RNAPIII transcription [Fig. 7]. The function of Nab2 in RNAPIII transcription is most likely direct, since the in vitro RNAPIII transcription defect of nab2-34 extracts can be rescued by addition of recombinant Nab2 [Fig. 5F], and Nab2 stimulates transcription in a reconstituted in vitro transcription assay [Fig. 5G]. Nab2 is essential for efficient RNAPIII transcription initiation, as it is required for full occupancy of TFIIIB [Fig. 6A], most likely mediated by its direct interaction with TFIIIB [Fig. 6B]. Importantly, Nab2 increases the binding of Tbp and TFIIIB to promoter DNA in vitro [Fig. 6C]. The reduced occupancy of TFIIIB is most likely the reason for the decreased occupancy of RNAPIII in nab2-34 cells at the restrictive temperature (Fig. 2; Supplemental Fig. S6A). Thus, Nab2 functions to recruit TFIIIB and thus RNAPIII to the gene or increase their stability at the gene during transcription initiation [Fig. 7].

Since Nab2 interacts directly with precursors of RNAPIII transcripts [Fig. 4], it probably binds to RNAPIII transcripts already during transcription. This cotranscriptional binding of Nab2 to RNAPIII transcripts might be necessary for efficient transcription elongation, similar to the function of mRNA-binding proteins in RNAPII transcription. mRNA-binding protein complexes such as the TREX and THSC complexes prevent formation of so-called R loops [RNA:DNA hybrids plus a displaced template DNA. The amount of SNR6 was quantified, and the RNA amount without Nab2 was set to 1. Data (n ≥ 3) represent the mean ± SD. (B) In vitro transcription of tRNAAla(UAU) precursor (two highest bands), RPR1 precursor, and SNR6 levels in nab2-34 and rpa25-S100P normalized to the corresponding wild-type strains. Data (n ≥ 3) represent the mean ± SD. (C) Experiments as in B but with SNR6 as the reporter gene. Representative gels of the experiments quantified in D and E are shown in Supplemental Figure S4, A and B. Recombinant Nab2 stimulates deficient transcription in nab2-34 extracts at 37°C. In vitro transcription assays were carried out with NAB2 [lanes 1–5] and nab2-34 [lanes 6–10] extracts at 25°C [lanes 1,6] or 37°C [lanes 2–5 and 7–10] in the absence [lanes 1,2,6,7] or presence [lanes 3–5,8–10] of increasing amounts of recombinant Nab2 [0, 100, 200, and 400 ng]. The amount of SNR6 RNA was quantified, and the RNA amount at 25°C was set to 1. Data represent mean ± SD. (**) P < 0.01. (E) Experiments as in D but with SNR6 as the reporter gene. Representative gels of the experiments quantified in D and E are shown in Supplemental Figure S4, A and B. Recombinant Nab2 stimulates deficient transcription in nab2-34 extract at 37°C. In vitro transcription assays were carried out with NAB2 [lanes 1–5] and nab2-34 [lanes 6–10] extracts at 25°C [lanes 1,6] or 37°C [lanes 2–5 and 7–10] in the absence [lanes 1,2,6,7] or presence [lanes 3–5,8–10] of increasing amounts of recombinant Nab2 [0, 100, 200, and 400 ng]. The amount of SNR6 RNA was quantified, and the RNA amount without Nab2 was set to 1. Data (n = 3) represent mean ± SD. (**) P < 0.01. A representative gel of the experiments quantified in F is shown in Supplemental Figure S4C. (C) Recombinant Nab2 stimulates RNAPIII transcription in a fully reconstituted in vitro transcription system. Increasing amounts of Nab2 [0, 50, 100, and 250 ng] were added to RNAPIII, TFIIIC, and TFIIIB preassembled on the SNR6 template DNA. The amount of SNR6 RNA was quantified, and the RNA amount without Nab2 was set to 1. Data (n = 3) represent mean ± SD. (**) P < 0.01. A representative gel of the experiments quantified in G is shown in Supplemental Figure S4D.

Figure 5. Nab2 is required for full RNAPIII transcriptional activity in vivo and in vitro. (A–C) Nab2 is required for RNAPIII transcription in vivo. Northern blot analysis of tRNAAla(UAU) L, RPR1, and the U6 snRNA (SNR6) using total RNA from nab2-34, rpa25-S100P, and the corresponding wild-type strains shifted to 37°C for the indicated times. Neosynthesis of tRNAAla(UAU), RPR1, and SNR6 is decreased in nab2-34 cells at 37°C. Quantification of the tRNAAla(UAU) precursor [two highest bands], RPR1 precursor, and SNR6 levels in nab2-34 and rpa25-S100P normalized to the corresponding wild-type strains. Data (n ≥ 3) represent the mean ± SD. (**) P < 0.01. A representative gel of the experiments quantified in F is shown in Supplemental Figure S4C. (C) Recombinant Nab2 stimulates RNAPIII transcription in a fully reconstituted in vitro transcription system. Increasing amounts of Nab2 [0, 50, 100, and 250 ng] were added to RNAPIII, TFIIIC, and TFIIIB preassembled on the SNR6 template DNA. The amount of SNR6 RNA was quantified, and the RNA amount without Nab2 was set to 1. Data (n = 3) represent mean ± SD. (**) P < 0.01. A representative gel of the experiments quantified in G is shown in Supplemental Figure S4D.
DNA strand during transcription elongation by RNAPII, probably by binding to the mRNA (Aguilera and Garcia-Muse 2012 and references therein). Likewise, Nab2 could bind to the nascent RNA during transcription elongation by RNAPIII, thus reducing the ability of the RNA to rehybridize with the transiently opened DNA double strand. In contrast, Nab2 is most likely not necessary for the processing of RNAPIII transcripts, since precursors of pre-tRNAs or other RNAPIII transcripts do not accumulate in nab2-34 cells at the restrictive temperature (Supplemental Fig. S3). As tRNAs are spliced in the cytoplasm (Hopper and Huang 2015), it is also unlikely that Nab2 is required for nuclear tRNA export. Taken together, Nab2 might have two functions in RNAPIII transcription: One in initiation, as shown here, and a second one in elongation based on its interaction with precursors of RNAPIII transcripts. It will be interesting to determine the exact functions of Nab2 in RNAPIII transcription.

In recent years, several proteins involved in RNAPII transcription have been implicated in RNAPIII transcription (see above). In higher cells, RNAPII and RNAPII TFs localize to RNAPIII transcribed genes. However, this is not the case in S. cerevisiae (Barski et al. 2010; Raha et al. 2010; Venters et al. 2011), consistent with our finding that RNAPII, the TREX component Tho2, and the SR protein Npl3 are not present at RNAPIII genes (Fig. 1; Supplemental Fig. S1). Thus, the presence of Nab2 at RNAPIII genes is independent of its association with RNAPII. Importantly, Nab2 is the first mRNA biogenesis factor with a function in RNAPIII transcription. Interestingly, proteins involved in both RNAPII and RNAPIII transcription or metabolism, such as Nab2 and Dst1 in yeast (also see above), could coordinate these two important cellular pathways. Whether this is the case remains to be determined.

Nab2 is highly conserved throughout evolution and is named ZC3H14 in Homo sapiens, Msut-2 in Mus musculus, ZC3H14 in Rattus norvegicus, SUT-2 in Caenorhabditis elegans, dNab2 in Drosophila melanogaster, and nab2 in Schizosaccharomyces pombe. The functions of Nab2 homologs in these diverse organisms also seem to be conserved: Poly(A) binding has been shown in H. sapiens, M. musculus, R. norvegicus, and D. melanogaster (Pak et al. 2011; Kelly et al. 2014), and its requirement for correct poly(A) tail length has been shown in D. melanogaster and probably H. sapiens (Pak et al. 2011). Human ZC3H14 is ubiquitously expressed and exists in four different splice variants: Isoforms 1, 2, 3, and 3short contain a predicted classical nuclear localization signal (cNLS) and localize to the nucleus, whereas isoform 4 contains an alternative first exon lacking the NLS and consequently localizes to the cytoplasm (Leung et al. 2009).

Figure 6. Nab2 interacts with TFIIIB and stabilizes its binding to promoter DNA. (A) The occupancy of TFIIIB, but not TFIIIC, decreases when Nab2 function is impaired. ChIP analysis of the TAP-tagged TFIIIB subunit Bdp1 (left panel) and the TAP-tagged TFIIIC subunit Tfc1 (right panel) on selected RNAPIII transcribed genes in NAB2 and nab2-34 cells at 30°C and 37°C (see also Supplemental Figs. S5, S6). Data [n ≥ 3] represent the mean ± SD. (*) P < 0.05; (**) P < 0.01. (B) Nab2 interacts with TFIIIB but not TFIIIC in vivo. Tandem affinity purification of Nab2-TAP from a strain expressing C-terminally HA-tagged Brf1 (TFIIIB) or Tfc8 (TFIIIC). Strains without a TAP tag on Nab2 served as negative controls. [Left panel] Lysates and EGTA eluates were separated by SDS-PAGE and stained with Coomassie. Copurification of Brf1 (bottom panel) or Tfc8 (right panel) with Nab2 was assessed by Western blotting with antibodies directed against HA (Brf1/Tfc8). Pgk1 served as negative control. Tagged Nab2 is marked with an asterisk. (C) Nab2 increases the affinity of TFIIIB for DNA. EMSAs for Nab2-TFIIIB-DNA formation using TA-30-B6 DNA as template. Formed DNA–protein complexes are indicated at the right. Nab2 [500 ng] exclusively induces a supershift when Tbp is present on the promoter DNA and increases the affinity of Tbp/TFIIIB to target DNA (see also Supplemental Fig. S7).

Figure 7. Model of Nab2 function in RNAPIII transcription. See the text for details.
Interestingly, mutations in ZC3H14 cause a form of autosomal recessive intellectual disability in humans, and Nab2 is needed for normal neuronal function in *Drosophila* (Pak et al. 2011). Thus, Nab2 is important for proper cellular functions, especially in neuronal cells (Kelly et al. 2015). Since Nab2 is already known to have important and conserved cellular functions in 3′ end processing and mRNA export, it will be of great interest to assess whether its function in RNAPIII transcription is also conserved.

In conclusion, we show here that Nab2, an important protein in mRNP biogenesis but with no previous implication in transcription, has a second, pivotal role in RNAPIII transcription. Here, one function of Nab2 is to efficiently assemble the RNAPIII initiation complex by increasing the binding of TFIIIB to the promoter DNA.

**Materials and methods**

**Yeast strains and plasmids**

Yeast strains, plasmids, and DNA sequences used in this study are listed in Supplemental Tables S1–S3.

**Generation of a NAB2 temperature-sensitive allele**

The temperature-sensitive allele of *NAB2*, nab2-34, was generated essentially according to Chanarat et al. (2011). Briefly, the ORF of *NAB2* was randomly mutagenized by error-prone PCR, and the resulting PCR fragments were cotransformed with plasmid *pRS315-NAB2* cut with NotI and XhoI, which removes the ORF of *NAB2*, into the *NAB2*-shuffled strain (Δnab2 + *pRS316-NAB2*). Transformants were selected on SDC-β-leu plates, replica-plated on plates containing 5-fluoroorotic acid to shuffle out *pRS316-NAB2*, and tested for thermosensitivity at 37°C. One allele was identified and named nab2-34.

**ChIP experiments and analysis of the ChIP–chip and CRAC data**

The ChIP experiments were performed as in Rother et al. (2010), and the ChIP–chip experiments were performed as in Meinel et al. (2013). RPC25 and rpc25-S100P cells were grown for 10 h at 25°C or shifted to 37°C. NAB2 and nab2-34 cells were grown for 3 h at 30°C or shifted to 37°C. ChIP–chip data sets of Nab2–TAP, TAP-Npl3, TAP-Tho2, and Rpb3–TAP cells were used as published (Meinel et al. 2013), and the Rpc160–TAP ChIP–chip data are available at ArrayExpress [http://www.ebi.ac.uk/arrayexpress], accession number E-MTAB-3700.

The ChIP–chip data were normalized according to Meinel et al. (2013). Data normalization and analysis were carried out using R (CRAN). The metaprofiles for tRNA genes were calculated by averaging the occupancies of all intronless tRNA. To analyze the significance of the Pearson’s correlation coefficient for the peak occupancies at protein-coding or tRNA genes for Nab2 and Rpb3 or Nab2 and Rpc160, the correlation coefficients of 100,000 random permutations of the data sets were calculated and compared with the correlation coefficients of the nonpermuted data set.

The Nab2 CRAC data from Tuck and Tollervey (2013) were inspected for Nab2 binding to the different RNAPIII transcripts using Integrated Genome Browser (Nicol et al. 2009) and R (CRAN). For the CRAC tRNA metaprofile, the reads for all intronless tRNAs <76 bp were averaged and plotted versus the position relative to the first nucleotide of the tRNA gene.

**Protein purification**

RNAPI and RNAPIII were purified from *S. cerevisiae* by tandem affinity purification of Rpa190–TAP and Rpc160–TAP, respectively, according to Rother et al. (2006) with the following modifications: All whole-cell protein extracts were treated with RNase and DNase prior to the purification. For the in vivo interaction assay of Nab2 with RNAPIII, the proteins/protein complexes were purified to the EGA cluate. For in vitro interaction assays, proteins were purified with buffer containing 800 mM NaCl until the TEV eluate. GST- or His6-tagged recombinant Nab2 was produced in *E. coli* BL21 DE3 transformed with pGex6-P1-NAB2 or pET21a-NAB2 at 37°C by addition of 0.3 mM isopropyl-1-thio-β-D-galactopyranoside at an OD600 of 0.6 for 3 h. Cells were harvested, washed, resuspended in NETN buffer (1 M NaCl, 1 mM EDTA, 50 mM Tris-HCl at pH 7.5, 1% NP-40, 10 µM ZnCl2, 2 mM DTT, protease inhibitors) and lysed by sonication. Lysates were incubated with glutathione sepharose 4B (GE Healthcare) or Ni-NTA agarose (Qiagen) and purified according to the manufacturers’ protocols. His-tagged Nab2 was further purified by ion exchange chromatography using a MonoS 5/50 GL column (GE Healthcare) with a step elution from 0 M NaCl to 0.6 M NaCl, 50 mM MES (pH 6.5), 10 µM ZnCl2, and 2 mM DTT. To exchange buffers, a PD-10 desalting column (GE Healthcare), equilibrated with the respective assay buffer, was used. For the fully reconstituted in vitro transcription assay, proteins were purified as follows: Bdp1, Brl1, and Tbp were purified essentially as described in Kassavetis et al. (1998) and references therein. Nab2 was purified from *E. coli*, and RNAPIII (Rpc160–TAP) and TFIIIC (Tic1–TAP) were purified from *S. cerevisiae* as described above for RNAPIII under stringent conditions until the TEV eluate.

**In vitro interaction assay**

Purified GST or GST-Nab2 bound to beads and treated with 25 µg/mL DNase I and 100 µg/mL RNase A (Thermo Scientific) was incubated with equal amounts of TAP-purified RNAPI or RNAPIII in buffer containing 20 mM HEPES (pH 7.6), 1 mM DTT, 75 mM NaCl, and 2 mM MgCl2 for 1 h at 16°C. After washing, proteins were eluted by incubation with 5 µL of Precision protease for 2 h at 16°C in 150 µL of buffer and analyzed by SDS-PAGE.

**RNA extraction**

Total RNA was extracted from *S. cerevisiae* grown to 0.6–0.8 OD600 in YPD at 30°C or shifted to 37°C for the indicated times with 300 µL of buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% SDS, 2% Triton X-100), 300 µL of glass beads, and 300 µL of phenol by vortexing. After centrifugation at 13,200 rpm for 5 min at room temperature, the RNA was washed once with chloroform and precipitated using standard conditions. The RNA was dissolved in DEPC water, and the DNA was digested with 10 U/100 µL DNase I (Thermo Scientific) in the presence of 80 U/100 µL Ribonuclease inhibitor (Thermo Scientific) for 15 min at 37°C. RNA concentration and integrity were tested on 2% agarose denaturing formaldehyde gels.

**Northern blotting**

Five micrograms of total RNA was separated by electrophoresis on 6% or 9% polyacrylamide gels containing 6 M urea. After blotting onto a nylon membrane, radiolabeled oligonucleotides were hybridized in Church buffer. After washing three times with 2× SSC buffer and 0.1% SDS, the blots were exposed overnight to a storage phosphor screen and analyzed by a Typhoon 9400.
PhosphorImager (Amersham Bioscience). Signals were quantified with ImageQuant 5.2.

**In vitro transcription assay**

RNAPIII transcription-active extracts were generated, and in vitro transcription experiments were performed essentially as described in Schultz et al. [1991]. In brief, 30 µg of whole-cell extract was mixed with 4 µL of 5× transcription buffer [100 mM HEPES at pH 7.9, 400 mM KCl, 25 mM MgCl₂, 5 mM EDTA, 10% (v/v) glycerol], 1 mM DTT; 200 ng of α-amanitin; 20 µL of riboLock RNase inhibitor [Thermo Scientific]; 500 µM ATP, CTP, and GTP; 50 µM GTP; 100 ng of plasmid template, and 5 µCi [α-32P]GTP (3000 Ci/mmole, Hartmann Analytic). Reactions were incubated for 30 min at 25°C or 37°C. The fully in vitro reconstituted transcription experiments were carried out as described in Huet et al. [1996]. Reaction mixtures were set up with 20 mM HEPES (pH 7.9), 90 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 10% (v/v) glycerol, 1 mM DTT; 10 µL of riboLock RNase inhibitor; 0.6 mM ATP, CTP, and GTP; 0.03 mM UTP, 100 ng of plasmid template; and 10 µCi [α-32P]UTP (3000 Ci/mmole, Hartmann Analytic). Proteins were added in the described order and contained varying amounts of Nab2. Transcription mixes were incubated for 30 min at 25°C. After phenol/chloroform extraction and ethanol precipitation, the dried RNA pellets were resuspended in formamide loading buffer and separated on a 9% polyacrylamide gel with 7 M urea in TAE buffer. Dried gels were exposed overnight to a storage phosphor screen or autoradiographed and analyzed with a Typhoon FLA 9400 or FLA 9500 PhosphorImager (GE Healthcare). Signals were quantified with ImageQuant 5.2 or TL.

**EMSA**

EMSA were done as previously described in Kassavetis et al. [1998]. Briefly, protein–DNA complexes were formed in 40 mM Tris–HCl (pH 8.0), 7 mM MgCl₂, 3 mM DTT, 5% (v/v) glycerol, 100 µg/mL BSA, 50 mM NaCl, and 100 ng of ssDNA, and 5 pmol of 6-FAM 5'-labeled dsDNA was used instead of poly(dG–dC)–poly(dG–dC). Proteins were added as indicated. Reaction mixtures were incubated for 60 min at 25°C and separated on a 2% (w/v) agarose gel in 1× TAE. Gels were analyzed with a Typhoon FLA 9500 and ImageQuant TL software.

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