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## An Interaction between Two RNA Binding Proteins, Nab2 and Pub1, Links mRNA Processing/Export and mRNA Stability<sup>∇†</sup>

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**mRNA stability is modulated by elements in the mRNA transcript and their cognate RNA binding proteins. Poly(U) binding protein 1 (Pub1) is a cytoplasmic *Saccharomyces cerevisiae* mRNA binding protein that stabilizes transcripts containing AU-rich elements (AREs) or stabilizer elements (STEs). In a yeast two-hybrid screen, we identified nuclear poly(A) binding protein 2 (Nab2) as being a Pub1-interacting protein. Nab2 is an essential nucleocytoplasmic shuttling mRNA binding protein that regulates poly(A) tail length and mRNA export. The interaction between Pub1 and Nab2 was confirmed by copurification and in vitro binding assays. The interaction is mediated by the Nab2 zinc finger domain. Analysis of the functional link between these proteins reveals that Nab2, like Pub1, can modulate the stability of specific mRNA transcripts. The half-life of the *RPS16B* transcript, an ARE-like sequence-containing Pub1 target, is decreased in both *nab2-1* and *nab2-67* mutants. In contrast, *GCN4*, an STE-containing Pub1 target, is not affected. Similar results were obtained for other ARE- and STE-containing Pub1 target transcripts. Further analysis reveals that the ARE-like sequence is necessary for Nab2-mediated transcript stabilization. These results suggest that Nab2 functions together with Pub1 to modulate mRNA stability and strengthen a model where nuclear events are coupled to the control of mRNA turnover in the cytoplasm.**

The life cycle of an mRNA transcript is comprised of numerous highly coordinated steps, including RNA synthesis, processing, nuclear export, translation, and, ultimately, degradation (40). Although transcription has historically been the most extensively studied step in gene expression, more recent studies have successfully demonstrated that the modulation of expression levels can and does occur at all of these steps in the mRNA life cycle (40). Specifically, mRNA degradation is an important point for this modulation (19, 61). The half-life of an mRNA transcript can change in response to a variety of stimuli including environmental factors, mitogens, growth factors, and hormones (29). Interestingly, transcripts encoding proteins with housekeeping functions are typically characterized by long half-lives, while transcripts encoding proteins that are required transiently, such as during the cell cycle or differentiation, often have shorter half-lives (29). For example, in human cells, the mRNA transcript for the housekeeping glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has a half-life of over 24 h (34), whereas the mRNA transcript for the transient transcription factor *c-myc* (10) has a half-life of 10 min (13).

A number of studies that have analyzed mRNA turnover as a mode of regulating gene expression have exploited the budding yeast *Saccharomyces cerevisiae*, and those studies have uncovered crucial components of evolutionarily conserved mRNA decay mechanisms (28, 56, 61). The half-lives of yeast mRNAs vary substantially from 3 min to >100 min (24, 59), consistent with the idea that mRNA stability is a key regulatory mechanism in this model organism. In fact, certain groups of yeast mRNAs encoding functionally related proteins exhibit similar degradation profiles in response to environmental signals (24), suggesting that mRNA decay is coordinated with the biological requirements of the cell. These observations highlight the significant contribution of mRNA decay to the regulation of gene expression.

Throughout its biogenesis, mRNA is complexed with many RNA binding proteins that associate and dissociate at given times during the maturation of the transcript (14). The interplay between these RNA binding proteins and distinct *cis*-acting elements in the target mRNA transcripts regulates the mRNA life cycle (14). Significant evidence suggests that such steps are coordinated and mechanistically coupled rather than sequential (57). Some of the mechanisms that couple mRNA synthesis and export (57), and also translation and mRNA degradation (11, 32), have been characterized. However, the mechanistic basis for the coupling between mRNA processing/export and mRNA degradation is less well understood. Some examples of how nuclear events associated with mRNA processing can influence the fate of the mRNA in the cytoplasm have recently come to light. One example is poly(A) binding protein 1 (Pab1), which is localized in the cytoplasm at steady state (50) and is the key factor that controls the poly(A) tail-

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stimulated pathway for translation initiation and modulates mRNA stability in the cytoplasm (49). However, Pab1 can also shuttle into the nucleus (7, 15), where it can further regulate mRNA 3'-end processing (39) and overall poly(A) tail length (5). How these nuclear functions of Pab1 influence its well-characterized cytoplasmic functions is presently not understood; however, this role may involve interactions with other RNA binding proteins that associate with the transcript in the nucleus. Pab1 is only one of several RNA binding proteins that complex with mRNA transcripts in the nucleus and exert effects in the cytoplasm.

Some RNA binding proteins regulate gene expression by stabilizing or destabilizing particular target mRNAs (60). For example, yeast poly(U) binding protein 1 (Pub1) is a major nuclear and cytoplasmic poly(A) RNA binding protein (2) that has been implicated in the regulation of mRNA turnover (48, 56). HuR, the apparent mammalian orthologue of Pub1 (17, 45), stabilizes AU-rich element (ARE)-containing transcripts by protecting them from degradation via the deadenylation-dependent pathway (17, 41). Similarly, Pub1 binds to and stabilizes ARE and ARE-like sequence-containing transcripts (16). In addition, Pub1 can bind to a stabilizer element (STE) located in the 5'-untranslated region (UTR) of upstream open reading frame (ORF) (uORF)-containing transcripts to prevent their turnover via the nonsense-mediated decay (NMD) pathway (48). As described previously for the poly(A) binding protein Pab1 (7, 15), Pub1 is predominantly cytoplasmic, but it has also been detected in the nucleus (2). At present, it is unclear how Pub1 interfaces with other RNA binding proteins to stabilize target RNA transcripts.

Here, we report the identification of physical and functional interactions between Pub1 and the yeast heterogeneous nuclear ribonucleoprotein (hnRNP) nuclear poly(A) RNA binding protein 2 (Nab2). Nab2 is an essential, shuttling hnRNP required for both mRNA poly(A) tail length control and poly(A) RNA export (23, 26). Furthermore, we find that Nab2 can also modulate the stability of Pub1 target transcripts that contain ARE-like elements but not those transcripts that contain an STE. We propose a model in which Nab2, acting as a broad mRNA binding protein, could help recruit Pub1 to its specific targets.

## MATERIALS AND METHODS

**Strains, plasmids, and chemicals.** Yeast strains and plasmids used in this study are listed in Table 1. Methods for general yeast culture and DNA manipulations were performed according to standard protocols (4). All chemicals were obtained from Sigma, United States Biological, or Fisher unless otherwise noted.

**Two-hybrid screen.** The two-hybrid screen was performed using *S. cerevisiae* strain L40 (SVL86) that harbors the *HIS3* and *lacZ* reporter genes (30). The strain was transformed with the bait plasmid pSV424, encoding an in-frame fusion of the *lexA* DNA binding domain with the entire coding sequence of *PUB1*. Expression of the bait protein (LexA-Pub1) in L40 cells was confirmed by immunoblotting using an anti-LexA antibody (Santa Cruz). L40 cells containing the Pub1 bait plasmid were transformed with a yeast cDNA library fused to the Gal4 activation domain (ATCC 87002). Transformants were plated onto selective medium (lacking Leu, Trp, and His) and incubated for 5 days at 30°C. The His-positive transformants were further screened for the formation of blue colonies in the  $\beta$ -galactosidase lift assay with 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside (X-gal) (58) as a complementary test for interactions. The library plasmids from candidate positive clones were isolated, amplified in *Escherichia coli* HB101 cells, and retransformed into L40 cells, and assays were repeated for growth on selective medium and for blue colony formation. Plasmid DNA was

isolated from positive clones and sequenced to identify the genes encoding the interacting proteins.

**In vitro  $\beta$ -galactosidase assay.** The  $\beta$ -galactosidase activity from crude yeast extract was assayed by a protocol adapted from a method described previously by Rose and Botstein (46). Yeast cells were grown to an optical density at 600 nm ( $OD_{600}$ ) of 0.8 to 1.0 and then disrupted in breaking buffer (100 mM Tris-HCl [pH 8.0], 1 mM dithiothreitol, 20% glycerol, 5 mM phenylmethylsulfonyl fluoride) using glass beads and a bead beater. Cell extracts were clarified by centrifugation at  $16,000 \times g$  for 15 min, and 20  $\mu$ l of supernatant was added to 900  $\mu$ l of Z buffer (100 mM  $Na_2HPO_4$  [pH 7.0], 10 mM KCl, 1 mM  $MgSO_4$ , 50 mM  $\beta$ -mercaptoethanol). The final volume was adjusted to 1 ml with breaking buffer, and the mixture was preincubated at 28°C for 5 min. Following the preincubation, 200  $\mu$ l of *ortho*-nitrophenyl- $\beta$ -D-galactopyranoside solution (4 mg/ml in Z buffer) was added, and the reaction was processed until the solution acquired a pale yellow color. The reaction was terminated by the addition of 500  $\mu$ l of 1 M  $Na_2CO_3$ , and the  $OD_{420}$  was measured.  $\beta$ -Galactosidase activity was calculated using the function  $(OD_{420} \times 1.7)/(0.0045 \times \text{total protein [mg/ml]} \times \text{reaction time [min]})$ , where the total protein concentration was determined by using a Bio-Rad protein assay kit. Activity measurements were calculated from at least three independent experiments.

**Copurification assay.** Yeast cells expressing a specific tandem affinity purification (TAP)-tagged protein were grown in yeast extract-peptone-dextrose (YPD) medium at 30°C to an  $OD_{600}$  of  $\sim 0.6$ . The culture was centrifuged, and cell pellets were resuspended in IPP150 buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% NP-40, and 3  $\mu$ g/ml each of aprotinin, leupeptin, chymostatin, and pepstatin). One volume of glass beads was added to each sample, and cells were lysed using a bead beater. The lysate was clarified by centrifugation at  $16,000 \times g$  and assayed for total protein concentration by using a Bio-Rad protein assay kit. Samples of cell extract containing 3 mg of total protein were incubated with 25  $\mu$ l of immunoglobulin G (IgG)-conjugated Sepharose beads (Amersham Biosciences) in 1 ml for 2 h at 4°C. When required, the suspension was preincubated with 20  $\mu$ g of RNase A (Sigma) for 20 min at 25°C. After binding to beads, the unbound fraction was collected, and the beads were washed three times with 1 ml IPP150 buffer. To cleave the protein A domain of TAP-fused proteins and release the proteins from beads, the bead pellet was then incubated with 30  $\mu$ l of IPP150 buffer containing 1 mM dithiothreitol, 1 mM EDTA, and 0.5  $\mu$ g of tobacco etch virus protease (Invitrogen) for 1 h at 18°C. Supernatant consisting of material released by tobacco etch virus protease treatment was collected, and equal volumes of samples were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting using an anti-Pub1 antibody that we generated for these studies or anti-glutathione *S*-transferase (GST) antibody (Sigma) as a control.

**Protein purification.** Cell lysate preparation and purification of recombinant proteins were performed as recommended by the resin manufacturer (Amersham Biosciences). GST and GST fusion proteins were purified by affinity chromatography on glutathione-Sepharose. Six-histidine-tagged Pub1 (His<sub>6</sub>-Pub1) was purified with nickel-nitrilotriacetic acid-Sepharose. The purified His<sub>6</sub>-Pub1 was used for the immunization of rabbits to obtain polyclonal antibodies (see Fig. S1 in the supplemental material).

**In vitro binding assay.** For in vitro binding assays, 5  $\mu$ g of GST or GST-fused protein was bound to glutathione-Sepharose in phosphate-buffered saline for 30 min at 25°C. After three washes with 1 ml phosphate-buffered saline, 1  $\mu$ g His<sub>6</sub>-Pub1 fusion protein was added to a volume of 1 ml buffer A (20 mM Tris-HCl [pH 8.0], 0.5% Triton X-100) containing 10  $\mu$ g of RNase A, and the reaction mixtures were incubated for 20 min at 25°C to degrade any contaminating RNA. The reaction mixtures were then incubated for 1 h at 4°C. Unbound fractions were collected, and the beads were washed three times with 1 ml buffer A containing 300 mM NaCl. The bound fraction was eluted by incubation with SDS sample buffer for 5 min at 95°C and separated by 12% SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-GST (Sigma) or anti-Pub1 antibodies.

**FISH.** The intracellular localization of poly(A) RNA was assayed by a fluorescence in situ hybridization (FISH) protocol adapted from methods described previously (1, 63). *NAB2*-deleted cells (SVL544), maintained by plasmids carrying either wild-type *NAB2* (pSV572) or mutant *nab2-1* (deletion of the N-terminal domain) (pSV575) (37), and *PUB1*-deleted cells (SVL549) were grown in 2% glucose minimal medium to log phase at 25°C. Cells were fixed in 5.2% formaldehyde, digested with zymolyase, and subsequently permeabilized. A digoxigenin-labeled oligo(dT)<sub>50</sub> probe coupled with a fluorescein isothiocyanate-conjugated antidigoxigenin antibody (Roche Molecular Biochemicals) was used to detect poly(A) RNA. DAPI (4',6'-diamidino-2-phenylindole) was used to stain chromatin and indicate the position of the nucleus.

TABLE 1. Yeast strains and plasmids used in this study

Strain or plasmid	Designation	Description	Source or reference
<b>Strains</b>			
SVL86	L40	(L40) <i>MATa his3 trp1 leu2 ade2 LYS::(lexA op)<sub>s</sub>-lacZ URA3::(lexA op)<sub>s</sub>-lacZ</i>	Clontech
SVL129	L40-41	(L40-41) <i>MATa his3 trp1 leu2 ade2 LYS::(lexAop)<sub>s</sub>-lacZ URA3::(lexA op)<sub>s</sub>-lacZ [pBTM-NIP7] [pACT-NOP8]</i>	64
SVL296	<i>rpb1-1</i>	<i>MATα ura3 his4 rpb1-1</i>	43
SVL316	EGY48	<i>MATα trp1 ura3 his3 leu2::lexA-LEU2 [URA3 LacZ]</i>	Invitrogen
SVL467	Pub1-TAP	<i>MATa ura3 met15 leu2 PUB1-TAP::HIS3</i>	Open Biosystems
SVL538	Nab2-TAP	<i>MATa ura3 met15 leu2 NAB2-TAP::HIS3</i>	Open Biosystems
SVL544	$\Delta nab2$	<i>MATa ura3 trp1 leu2 nab2::HIS3 [pSV877]</i>	23
SVL679	$\Delta nab2 rpb1-1$	<i>MATa ura3 trp1 leu2 his3 rpb1-1 nab2::KAN MX4 [pSV877]</i>	This study
SVL549	$\Delta nab2 \Delta pub1$	<i>MATa ura3 trp1 leu2 pub1::KAN MX4 nab2::HIS3 [pSV877]</i>	This study
SVL565	Sec27-TAP	<i>MATa ura3 met15 leu2 SEC27-TAP::HIS3</i>	Open Biosystems
SVL678	$\Delta pub1 rpb1-1$	<i>MATa ura3 trp1 leu2 his3 rpb1-1 pub1::KAN MX4</i>	This study
SVL680	$\Delta pub1, \Delta upf1 rpb1-1$	<i>MATa ura3 trp1 leu2 rpb1-1 pub1::KAN MX4 upf1::HIS3</i>	This study
SVL682	$\Delta nab2 \Delta pub1 rpb1-1$	<i>MATa ura3 tr1 leu2 rpb1-1 nab2::KAN MX4 upf1::HIS3 [pSV877]</i>	This study
SVL688	<i>rat7-1 rpb1-1</i>	<i>MATa ura3 trp1 leu2 his3 rpb1-1 rat7-1</i>	This study
SVL690	$\Delta pub1 RPS16B \Delta ARE rpb1-1$	<i>MATa ura3 leu2 his3 rpb1-1 pub1::KAN MX4 RPS16B<math>\Delta ARE</math>-TRP1</i>	This study
SVL691	$\Delta nab2 RPS16B \Delta ARE rpb1-1$	<i>MATa ura3 leu2 his3 rpb1-1 nab2::KAN MX4 [pSV877] RPS16B<math>\Delta ARE</math>-TRP1</i>	This study
<b>Plasmids</b>			
pSV59	pRS315	<i>CEN6 LEU2</i>	52
pSV149	Gal4 AD	pACT; <i>GAL4 AD 2<math>\mu</math>m LEU2</i>	Clontech
pSV408	His <sub>6</sub> -Pub1	pQE30-PUB1	This study
pSV424	LexA-Pub1	pBTM116-PUB1 2 $\mu$ m TRP1	This study
pSV491	GST-Nab2	pGEX-4T-NAB2	This study
pSV545	B42-Pub1	pJG4-5-PUB1 2 $\mu$ m TRP1	This study
pSV567	LexA-Nab2	pEG202-NAB2 2 $\mu$ m HIS3	23
pSV568	LexA- $\Delta N$ Nab2	pEG202- $\Delta N$ NAB2 2 $\mu$ m HIS3	This study
pSV569	LexA- $\Delta RGG$ Nab2	pEG202- $\Delta RGG$ NAB2 2 $\mu$ m HIS3	This study
pSV570	LexA- $\Delta Q$ Nab2	pEG202- $\Delta Q$ NAB2 2 $\mu$ m HIS3	This study
pSV571	LexA- $\Delta CCH$ Nab2	pEG202- $\Delta CCH$ NAB2 2 $\mu$ m HIS3	This study
pSV572	Nab2	<i>NAB2 CEN6 LEU2</i>	37
pSV575	$\Delta N$ Nab2	<i><math>\Delta N</math>-NAB2 CEN6 LEU2</i>	37
pSV597	LexA-CCCH Nab2	pEG202-CCCH NAB2 2 $\mu$ m HIS3	This study
pSV607	GST- $\Delta CCH$ Nab2	pGEX-4T- $\Delta CCH$ NAB2	This study
pSV609	GST-CCCH Nab2	pGEX-4T-CCCH NAB2	This study
pSV645	LexA- $\Delta C3$ Nab2	pEG202- $\Delta C3$ NAB2 2 $\mu$ m HIS3	This study
pSV646	LexA- $\Delta CT$ Nab2	pEG202- $\Delta CT$ NAB2 2 $\mu$ m HIS3	This study
pSV741	LexA- $\Delta C4$ Nab2	pEG202- $\Delta C4$ -NAB2 2 $\mu$ m HIS3	This study
pSV859	GST-Nab2-67	pGEX-4T-nab2-67	This study
pSV864	LexA-CCCH-67	pEG202-ccch-67 NAB2 2 $\mu$ m HIS3	This study
pSV876	Nab2-67	<i>nab2-67 CEN6 LEU2</i>	This study
pSV877	Nab2	<i>NAB2 CEN6 URA3</i>	37
pSV878	LexA-CCCH-14	pEG202-ccch-14 NAB2 2 $\mu$ m HIS3	This study
pSV879	LexA-CCCH-5-7	pEG202-ccch-57 NAB2 2 $\mu$ m HIS3	This study
pSV880	LexA-CCCH-1-7	pEG202-ccch-17 NAB2 2 $\mu$ m HIS3	This study
pSV881	LexA-CCCH-5	pEG202-ccch-5 NAB2 2 $\mu$ m HIS3	This study
pSV882	LexA-CCCH-6	pEG202-ccch-6 NAB2 2 $\mu$ m HIS3	This study
pSV883	LexA-CCCH-7	pEG202-ccch-7 NAB2 2 $\mu$ m HIS3	This study
pSV884	LexA-CCCH-56	pEG202-ccch-56 NAB2 2 $\mu$ m HIS3	This study

**mRNA decay measurements.** mRNA decay rates were determined by either two-step quantitative real-time PCR (qRT-PCR) or Northern blot analysis of strains harboring a temperature-sensitive allele of RNA polymerase II (*rpb1-1*) (43). Briefly, 50 ml of cells was grown at 25°C to an OD<sub>600</sub> of ~0.6. The cells were centrifuged and resuspended in 50 ml of prewarmed medium (37°C) to shut off transcription. Aliquots of cells were removed at 0, 2.5, 5, 10, 15, and 20 min following the temperature shift and rapidly transferred to ice water, followed by centrifugation and freezing at -80°C. Total RNA was extracted from cells and used for either Northern blot or two-step qRT-PCR analysis. Northern blotting was performed as described previously by Ausubel et al. (4). For qRT-PCR, the High Capacity cDNA Archive kit (Applied Biosystems) was used to generate cDNA from RNA, and amplification reactions were performed with Power SYBR Green PCR Master Mix (Applied Biosystems) using the 7500 Real Time

PCR system (Applied Biosystems). Results were analyzed using 7500 system software v1.3, and data were normalized by the  $\Delta\Delta C_T$  method (36). *PGK1* mRNA, which is not affected by Pub1 (16), was used as a control transcript to normalize all samples. Each experiment was repeated at least three times to obtain an average half-life measurement for each transcript.

## RESULTS

**Identification of Pub1 binding proteins.** In order to gain insight into how Pub1 cooperates with other proteins to regulate the stability of specific target mRNA transcripts, we sought

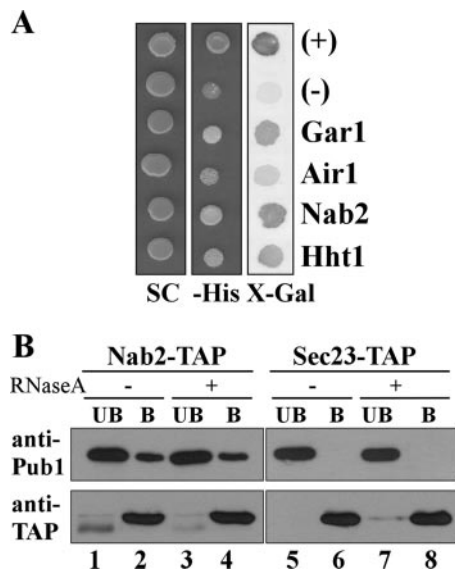


FIG. 1. Pub1 binds to proteins involved in RNA metabolism. (A) Yeast two-hybrid strain L40 (Clontech) containing a LexA-Pub1 plasmid (pSV424) was transformed with positive clones from the two-hybrid screen (*GAR1*, *AIR1*, *NAB2*, and *HHT1*) or with vector pACT (negative control). Transformants and positive control SVL129 cells (64) were grown on control synthetic complete (SC) plates or SC plates lacking histidine (-His) and also assayed for  $\beta$ -galactosidase activity (X-gal). Positive interactions are indicated by growth on plates lacking His and blue color on X-gal plates (darker grey in the right column). (B) Immunoblot analysis of unbound (UB) and bound (B) fractions from the copurification assay using cells expressing Nab2-TAP (lanes 1 to 4) or a control protein, Sec27-TAP (lanes 5 to 8). Proteins were purified with IgG-Sepharose in the presence or absence of 10  $\mu$ g RNase A, and fractions were probed with an anti-Pub1 antibody (top) or peroxidase antiperoxidase antibody complex (Sigma) (bottom).

to identify Pub1-interacting proteins. We employed a yeast two-hybrid system using a LexA-Pub1 fusion protein as bait and an *S. cerevisiae* cDNA library fused to the *GAL4* activation domain (AD) to screen for Pub1-interacting proteins based on their ability to activate the *HIS3* and *lacZ* reporter genes in the presence of LexA-Pub1. The screen led to the identification of several proteins involved in RNA metabolism. As shown in Fig. 1A, where growth on plates lacking His and blue color on plates containing X-gal are indicative of a positive interaction, we identified Gar1, Air1, Nab2, and Hht1 as being putative Pub1 binding partners. Gar1 is a component of the H/ACA snoRNP pseudouridylylase complex involved in the modification and cleavage of pre-rRNA (54). Air1 is a zinc knuckle protein component of the TRAMP complex required for the polyadenylation and degradation of a variety of RNA species (9). Hht1 is a core histone required for chromatin assembly and involved in heterochromatin-mediated telomeric and homothallic mating silencing (53). Finally, Nab2 is an essential shuttling hnRNP that is required for correct polyadenylation and poly(A) RNA export from the nucleus (3, 23, 26). The interaction between Pub1 and Nab2 was previously detected in a large-scale two-hybrid study (31).

Since Nab2 plays an essential role in mRNA metabolism (26) and shows the most robust two-hybrid interaction with Pub1, we decided to focus our efforts on the Pub1-Nab2 asso-

ciation. To confirm and extend the two-hybrid result, a copurification experiment was performed using yeast cells expressing TAP-tagged Nab2. To detect endogenous Pub1, we raised a polyclonal antibody to Pub1 that recognized a band of the predicted size (60 kDa) in wild-type cells but not in  $\Delta$ *pub1* cells (see Fig. S1 in the supplemental material). To determine whether Nab2 copurifies with Pub1, yeast lysates from cells producing TAP-tagged Nab2 or Sec27 (negative control) were incubated with IgG-Sepharose beads (Amersham Biosciences), and both the unbound and bound fractions were probed with the anti-Pub1 antibody (Fig. 1B). Immunoblot analysis showed that Nab2-TAP copurifies with Pub1, as indicated by the presence of Pub1 in the bound fraction (Fig. 1B, lane 2). This interaction did not depend on the presence of RNA, as similar results were obtained when lysates were pretreated with RNase A (Fig. 1B, lane 4). Pub1 did not copurify with the control protein Sec27-TAP (Fig. 1B, lanes 6 and 8). Probing with peroxidase antiperoxidase antibody complex confirmed that each TAP-fused protein was produced and bound to beads (Fig. 1B).

**Mapping the Pub1 binding domain of Nab2.** To map the interaction between Nab2 and Pub1, a series of Nab2 variants that precisely delete each of the major predicted domains (Fig. 2A) was generated. These Nab2 variants were expressed from two-hybrid vectors and tested for interactions with Pub1. The expression of all Nab2 variants was confirmed by immunoblotting with anti-Nab2 antibody (data not shown). Results of the binding analysis revealed that the C-terminal zinc finger domain of Nab2 (CCCH) is both necessary and sufficient to bind Pub1 (Fig. 2B). A closer analysis of this domain revealed that the seven CCCH repeats cluster into two groups: a set of four repeats that is followed by a set of three repeats (37). In an attempt to further dissect the Pub1 binding region in the zinc finger domain, we generated Nab2 mutants that deleted the first four repeats ( $\Delta$ C4), the last three repeats ( $\Delta$ C3), or the last 47 amino acids ( $\Delta$ CT) of Nab2, which lie outside of the zinc finger domain (Fig. 2A). Results of this analysis revealed that  $\Delta$ CT retains binding to Pub1 but that the other deletions within the zinc finger domain abolish the interaction (Fig. 2B). These results suggest that the full zinc finger domain, which is essential for Nab2 function (37), is necessary for Nab2 to retain the conformation required for binding to Pub1.

The above-described experiment establishes that Pub1 associates with Nab2 via the zinc finger domain of Nab2 but does not address whether this is a direct interaction. To determine whether the interaction is direct, an *in vitro* binding assay was performed (Fig. 2C). In this experiment, recombinant GST, GST-Nab2, GST-CCCH, or GST-Nab2  $\Delta$ CCCH produced in *E. coli* was bound to glutathione-Sepharose beads and incubated with purified, recombinant His<sub>6</sub>-Pub1. Results of this experiment show that Pub1 can bind directly both to Nab2 and to the CCCH domain as indicated by the presence of Pub1 in the bound fractions (Fig. 2C, lanes 4 and 6). However, Pub1 did not bind to Nab2 lacking the zinc finger domain ( $\Delta$ CCCH) or to GST alone (Fig. 2C, lanes 8 and 2, respectively). These results show that Pub1 binds directly to the zinc finger domain of Nab2 *in vitro* and suggest that Pub1 could interact directly with Nab2 *in vivo*.

**Analysis of the functional interaction between Nab2 and Pub1.** Our experiments link Nab2, an essential mRNA-pro-

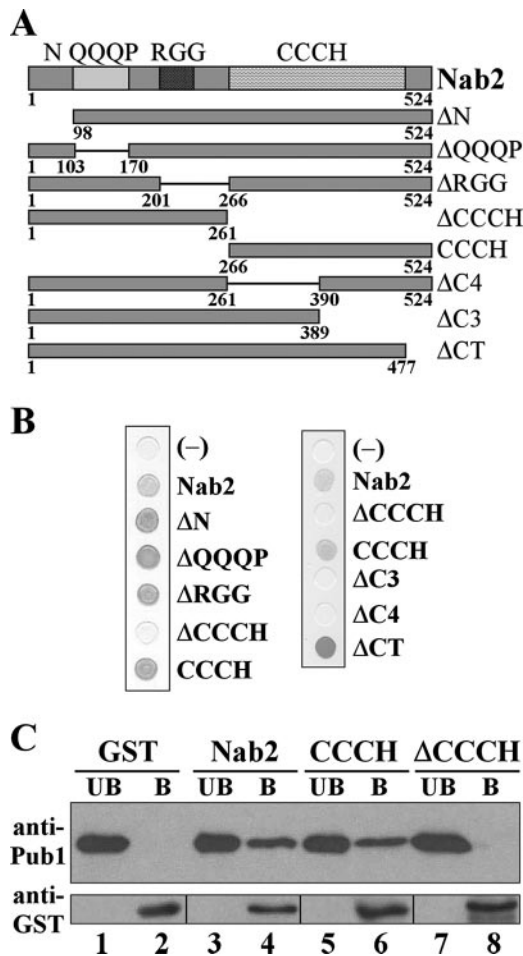


FIG. 2. Pub1 binds directly to the zinc finger domain of Nab2. (A) Schematic of Nab2 mutant proteins where the full length (amino acids 1 to 524) is shown at the top and where the four domains within the wild-type protein are indicated by the shading. The amino acids contained within the variant proteins are indicated. (B) Two-hybrid analysis to define the Pub1-interacting domain of Nab2. Two-hybrid SVL316 (EGY48; Invitrogen) cells expressing a transcription activation domain-Pub1 fusion (B42-Pub1; pSV545) transformed with vector (pEG202) (negative control) or plasmids expressing each Nab2 variant were grown on X-gal plates containing galactose to induce the expression of the Pub1 fusion protein. Positive interactions are indicated by the darker grey. (C) GST (lanes 1 and 2), GST-Nab2 (lanes 3 and 4), GST-CCCH (lanes 5 and 6), and GST-Nab2  $\Delta$ CCCH (lanes 7 and 8) were purified from *E. coli* and incubated with recombinant His<sub>6</sub>-Pub1. The unbound (UB) and bound (B) fractions were analyzed by immunoblotting with antibodies against Pub1 and GST, as described in Materials and Methods. The bottom panel, which shows each of the GST fusion proteins, displays the unbound and bound bands from different areas of the blot due to differences in protein sizes (26 kDa for GST alone, 95 kDa for GST-Nab2, 59 kDa for GST-CCCH, and 62 kDa for GST- $\Delta$ CCCH).

cessing and export factor (23, 26), to Pub1, a nonessential protein that modulates mRNA stability (16, 56). To analyze a possible functional interaction between these proteins, we hypothesized that if cells mutated in both genes show synthetic growth phenotypes, this would suggest that Pub1 may influence the function of the essential Nab2 protein. In contrast, if no synthetic growth phenotype was detected, this could suggest

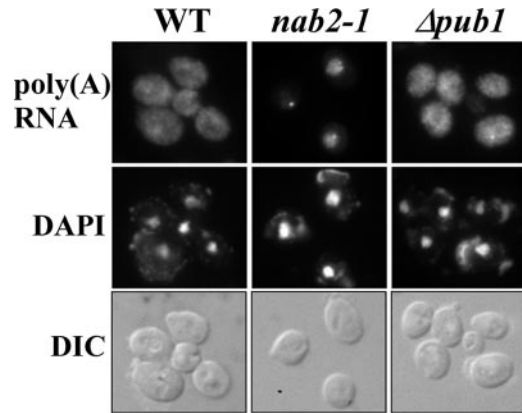


FIG. 3. Deletion of *PUB1* does not cause poly(A) RNA accumulation within the nucleus. *PUB1* deletion cells ( $\Delta$ *pub1*) were grown to log phase and subjected to FISH as described in Materials and Methods. As a control, we also analyzed *NAB2* deletion cells (SVL544) expressing either wild-type (WT) *NAB2* (pSV572) or the *nab2-1* allele (pSV575). Poly(A) RNA was detected using an oligo(dT) probe. The nucleus is indicated by DAPI staining of chromatin, and corresponding differential interference contrast images are shown.

that Nab2 contributes to the nonessential functions mediated by Pub1.

To test for synthetic growth defects, we deleted *PUB1* in cells previously deleted for *NAB2* but maintained by a plasmid expressing wild-type *NAB2*. We then combined several Nab2 mutants with the *PUB1* deletion by shuffling *nab2-1* ( $\Delta$ N),  $\Delta$ QQQP, and  $\Delta$ RGG *NAB2* plasmids (37) into the  $\Delta$ *pub1*  $\Delta$ *nab2* cells. We found that the deletion of *PUB1* did not exacerbate the growth defect of any of the Nab2 mutants (data not shown), suggesting that the essential functions of Nab2 are not affected by the deletion of *PUB1*.

To further probe whether Pub1 affects processes mediated by Nab2, we examined poly(A) RNA localization in  $\Delta$ *pub1* cells. In wild-type cells, poly(A) RNA localizes throughout the cell (Fig. 3), while *nab2-1* mutant cells show robust nuclear accumulation of poly(A) RNA (Fig. 3) (37). In contrast,  $\Delta$ *pub1* cells show no nuclear accumulation of poly(A) RNA (Fig. 3), confirming that Pub1 is not required for poly(A) RNA export. These results lead us to believe that Pub1 does not significantly affect the essential functions of Nab2.

We next tested whether Nab2 can influence mRNA stability, a known cellular function of Pub1 (16, 48, 56). Pub1 can bind to specific ARE-bearing transcripts, leading to their stabilization (56). In addition, Pub1 can selectively bind to STEs of uORF-containing transcripts, preventing their turnover via the NMD pathway (48). We selected two transcripts to analyze in detail, *RPS16B* and *GCN4* (Fig. 4A). The *RPS16B* mRNA is an ARE-like sequence-containing transcript, while the *GCN4* mRNA is an STE-containing transcript (16). Both of these transcripts are bound and stabilized by Pub1 (16, 48). To determine whether Nab2 could modulate mRNA stability, the half-lives of these two mRNA transcripts were determined in *nab2-1* mutant cells. We performed at least three independent experiments, as described in Material and Methods, and used the *PGK1* transcript as a control for a stable transcript, not affected by Pub1 (16, 56), to calculate an average half-life for each transcript in wild-type,  $\Delta$ *pub1*, *nab2-1*, and  $\Delta$ *pub1 nab2-1*

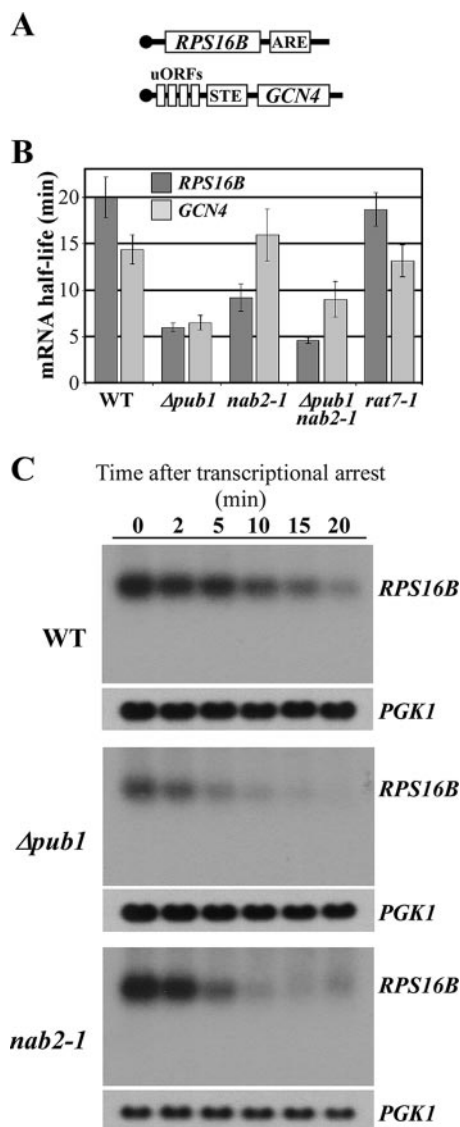


FIG. 4. Nab2 modulates mRNA stability of an ARE-like sequence-containing transcript. (A) Schematic of the ARE-like sequence-containing *RPS16B* and STE-containing *GCN4* transcripts. (B) The stability of the *RPS16B* transcript is decreased in *nab2-1* cells. Wild-type (WT),  $\Delta pub1$ , *nab2-1*,  $\Delta pub1 nab2-1$ , and *rat7-1* cells harboring a temperature-sensitive allele of RNA polymerase II (*rpb1-1*) (43) were grown to mid-log phase in YPD medium, and total mRNA was isolated at specific times following the inhibition of transcription by shifting the cells to the nonpermissive temperature. cDNA was prepared from each sample and subjected to qRT-PCR to analyze the *RPS16B*, *GCN4*, and *PGK1* (stable control) transcripts. Transcript half-lives were calculated by exponential fit, and a *P* value of  $<0.05$  was considered to be significant. Standard deviations are indicated. (C) Northern blot analysis of decay of the *RPS16B* transcript. Total mRNA samples isolated after shifting wild-type,  $\Delta pub1$ , and *nab2-1* cells harboring the *rpb1-1* allele to the nonpermissive temperature were probed for *RPS16B* and *PGK1* transcripts at the times indicated.

cells (Table 2). Interestingly, *nab2-1* cells showed a decreased half-life of the ARE-like sequence-containing *RPS16B* transcript compared to wild-type cells (Fig. 4B). As expected,  $\Delta pub1$  cells also showed a decrease in the half-life of *RPS16B* (Fig. 4B). The change in the half-life of the *RPS16B* transcript

is comparable to the change previously observed by Duttagupta et al. using the same method (16). The combination of  $\Delta pub1$  and *nab2-1* did not significantly decrease *RPS16B* mRNA stability compared to  $\Delta pub1$  alone (Fig. 4B), suggesting that Nab2 and Pub1 stabilize this transcript through the same pathway. In contrast to  $\Delta pub1$  cells, the stability of the STE-containing *GCN4* transcript was not decreased in *nab2-1* cells (Fig. 4B).

We performed Northern blot analysis to ensure that the real-time PCR method used in our study detects changes in the stability of full-length *RPS16B* mRNA. Each sample displayed a single specific band in this analysis (Fig. 4C), demonstrating that there is no significant decay intermediate which could confound the mRNA quantification by real-time PCR. Furthermore, the pattern of *RPS16B* transcript destabilization in both  $\Delta pub1$  and *nab2-1* cells is similar to that observed using the real-time PCR method (Fig. 4C). Finally, we verified that *PGK1* transcript stability is not altered in wild-type,  $\Delta pub1$ , or *nab2-1* cells, confirming that this transcript is an appropriate endogenous control.

The decreased stability of the ARE-like sequence-containing *RPS16B* transcript could be a secondary effect of mRNA accumulation within the nucleus since *nab2-1* cells show nuclear accumulation of poly(A) RNA (Fig. 3) (37). This scenario seems unlikely since the stability of both the control *PGK1* and the *GCN4* transcripts was not decreased in the *nab2-1* mutant cells. However, to eliminate this possibility, we examined the half-life of the *RPS16B* and *GCN4* transcripts in *rat7-1* cells, which express a variant of the Nup159 nuclear pore protein that causes nuclear accumulation of poly(A) RNA at the nonpermissive temperature (22). We observed only a small and not statistically significant decrease in the half-lives of the transcripts analyzed in this mutant (Fig. 4B and Table 2), suggesting that the decreased stability of ARE-like sequence-containing transcripts in the *nab2-1* mutant is not merely a secondary effect of the nuclear accumulation of poly(A) RNA.

**Identification of a new *NAB2* mutant.** The physical interaction between Pub1 and Nab2 as well as the specific RNA instability revealed in the *nab2-1* mutant suggest a role for Nab2 in the Pub1-mediated regulation of mRNA stability. However, there was no direct correlation between these two facts, as the *nab2-1* mutant lacks the N-terminal portion of Nab2 (37), which is a domain of Nab2 that is not necessary for its interaction with Pub1 (Fig. 2B). In order to obtain a *NAB2* variant that no longer interacts with Pub1, we performed site-directed mutagenesis of the Nab2 CCCH domain. As described above, this domain is composed of seven CCCH repeats clustered into two groups, a set of four repeats and a set of three repeats, all apparently necessary for the interaction with Pub1 (Fig. 2B). The first cysteine of each CCCH repeat was cumulatively replaced with arginine, generating three different CCCH mutants: *ccch-1-4*, with substitutions of Cys to Arg in all four CCCH repeats of the first set of zinc fingers; *ccch-5-7*, with substitutions of Cys to Arg in all three CCCH repeats of the second set of zinc finger; and *ccch-1-7*, with substitutions of Cys to Arg in all seven CCCH repeats (Fig. 5A). All amino acid substitutions are detailed in Table S1 in the supplemental material. Amino acid residue substitutions in the first CCCH mutant (*ccch-1-4*) did not cause any decrease in the interaction of Nab2 with Pub1 as assessed by two-hybrid

TABLE 2. Half-life of Pub1 mRNA targets

Strain	Half-life of target <sup>a</sup> :				
	<i>RPS16B</i>	<i>GCN4</i>	<i>RPL17A</i>	<i>RPS24B</i>	<i>WSC3</i>
Wild type	19.8 ± 2.1	14.4 ± 1.6	>20	>20	19.4 ± 0.9
$\Delta$ <i>pub1</i>	6.0 ± 0.5	6.5 ± 0.8	7.6 ± 1.2	8.1 ± 0.9	11.6 ± 3.1
<i>nab2-1</i>	9.2 ± 1.5	16.0 ± 2.8	12.5 ± 0.6	14.0 ± 0.4	>20
$\Delta$ <i>pub1 nab2-1</i>	4.6 ± 0.3	9.0 ± 1.9	ND	ND	ND
<i>nab2-67</i>	8.3 ± 0.5	15.1 ± 2.1	11.6 ± 0.9	15.3 ± 1.1	>20
$\Delta$ <i>pub1 nab2-67</i>	6.9 ± 0.6	7.1 ± 1.2	ND	ND	ND
<i>rat7-1</i>	18.7 ± 1.8	13.2 ± 1.7	ND	ND	ND
$\Delta$ <i>pub1</i> $\Delta$ <i>upf1</i>	5.6 ± 0.5	13.9 ± 1.4	ND	ND	ND
<i>nab2-1</i> $\Delta$ <i>upf1</i>	8.5 ± 1.3	15.3 ± 2.5	ND	ND	ND
<i>nab2-67</i> $\Delta$ <i>upf1</i>	9.1 ± 1.2	14.5 ± 2.1	ND	ND	ND

<sup>a</sup> ND, not determined.

assay (Fig. 5B). However, a slight but significant decrease was observed when substitutions were introduced into the second set of zinc fingers (*ccch-5-7* and *ccch-1-7*) (Fig. 5B). We then created additional amino acid substitutions in the *ccch-1-7* mutant, resulting in the substitution of the second cysteine of

the fifth (*ccch-5*), sixth (*ccch-6*), and seventh (*ccch-7*) CCCH repeats with arginine, and combined substitutions of the second cysteine to arginine within the fifth and sixth (*ccch-56*) or the sixth and seventh (*ccch-67*) CCCH repeats (Fig. 5A). As shown in Fig. 5B, the substitution of both cysteine residues of

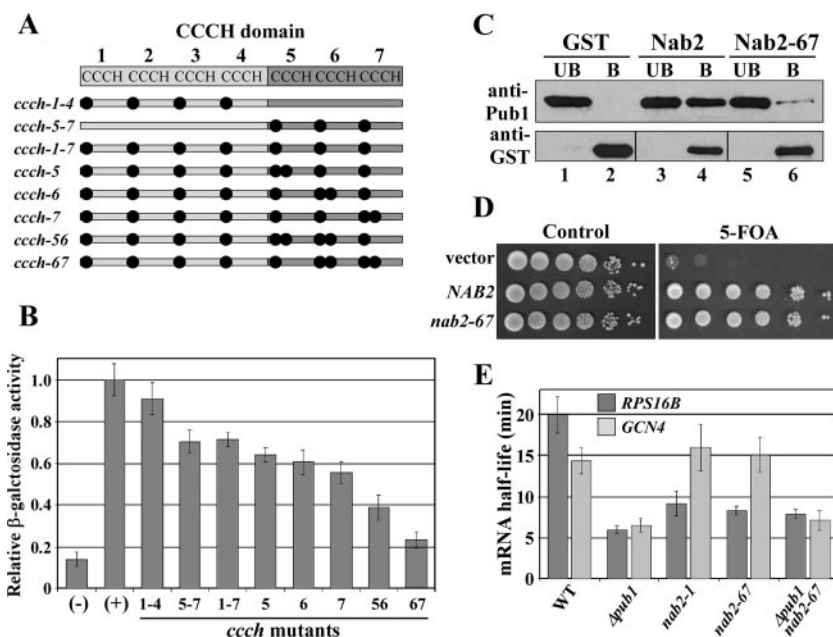


FIG. 5. Amino acid substitutions within the Nab2 zinc finger domain abolish the interaction with Pub1 and decrease *RPS16B* mRNA stability. (A) Schematic of amino acid changes within the Nab2 CCCH domain. The specific amino acid residues changed in each Nab2 variant are detailed in Table S1 in the supplemental material. (B) Amino acid substitutions in the Nab2 CCCH domain decrease binding to Pub1. Two-hybrid SVL316 (EGY48; Invitrogen) cells expressing a transcription activation domain-Pub1 fusion (B42-Pub1; pSV545) were transformed with vector (pEG202) (-) or plasmids expressing wild-type (+) or the indicated variant CCCH Nab2 domains. Cells were grown in liquid medium containing galactose, and quantification of *lacZ* reporter activity, as a measure of the Pub1 interaction with the CCCH domain of Nab2, was determined by an in vitro  $\beta$ -galactosidase assay. The bar graph displays  $\beta$ -galactosidase activity relative to the wild-type CCCH domain, which was set to 1.0. Standard deviations in the data are indicated. (C) Nab2-67 shows a greatly decreased interaction with Pub1. GST (lanes 1 and 2), GST-Nab2 (lanes 3 and 4), or GST-Nab2-67 (lanes 5 and 6) was purified from *E. coli* and incubated with recombinant His<sub>6</sub>-Pub1. The unbound (UB) and bound (B) fractions were analyzed by immunoblotting with anti-Pub1 and anti-GST antibodies. The bottom panel corresponds to the unbound and bound bands from different areas in the blot due to differences in protein sizes (26 kDa for GST alone and 95 kDa for GST-Nab2). (D) The *nab2-67* mutant allele is functional. A plasmid shuffle technique was used to assess the ability of the *nab2-67* mutant to replace the essential function of *NAB2*. *NAB2* deletion cells (SVL544) maintained by a *URA3 NAB2* plasmid (pSV877) were transformed with test plasmids carrying vector alone (pSV59), wild-type *NAB2* (pSV572), or *nab2-67* (pSV876). Samples were serially diluted and spotted onto a control plate (SC medium lacking Ura), where the wild-type *NAB2* plasmid is retained, or selective medium (5-fluoroorotic acid [5-FOA]), where the wild-type *NAB2* plasmid is lost and the only cellular copy of the essential *NAB2* gene is provided by the test plasmid. Plates were incubated for 3 days at 30°C. Vector alone and wild-type *NAB2* served as the negative and positive growth controls. (E) The stability of the *RPS16B* transcript is decreased in *nab2-67* cells compared to wild-type (WT) cells. The half-lives of the *RPS16B* and *GCN4* transcripts were determined using qRT-PCR as described in Materials and Methods.



adjacent zinc fingers (*ccch-56* and *ccch-67*) causes a dramatic decrease in the interaction between the Nab2 zinc finger domain and Pub1. All CCCH mutants showed approximately the same expression as the wild-type CCCH domain as assessed by immunoblotting (data not shown).

To assess the consequences of disrupting the interaction between Nab2 and Pub1, we introduced the *ccch-67* amino acid substitutions into the full-length Nab2 protein. To assay the interaction between the Nab2-67 mutant protein and Pub1, we performed an in vitro binding assay using recombinant GST, GST-Nab2, or GST-Nab2-67 bound to glutathione-Sepharose beads and purified His<sub>6</sub>-Pub1. We found that the interaction between Nab2-67 and Pub1 was almost abolished (Fig. 5C, lane 6) compared to the interaction between wild-type Nab2 and Pub1 (Fig. 5C, lane 4), confirming the results obtained by yeast two-hybrid analysis. A plasmid shuffle assay was then used to assess the function of the *nab2-67* allele (Fig. 5D). The positive control for growth is wild-type *NAB2*, and the negative control is the vector alone. Cells expressing *nab2-67* as the sole copy of *NAB2* showed no apparent growth defect at 30°C (Fig. 5D), indicating that this mutant can replace the essential function of the Nab2 protein. The same result was obtained when cells were grown at 14°C or 37°C (data not shown), demonstrating that *nab2-67* mutant cells do not display a temperature-sensitive phenotype. Finally, we tested whether the *nab2-67* mutant affects the stability of Pub1 target transcripts. The stability of both the *RPS16B* and *GCN4* transcripts was determined in *nab2-67* cells. As described above for *nab2-1* (Fig. 4B), the *nab2-67* mutant cells showed a decrease in the half-life of the *RPS16B* transcript, but the stability of the *GCN4* transcript was not altered in these cells (Fig. 5E and Table 2). Moreover, the combination of *Δpub1* and *nab2-67* did not significantly change *RPS16B* mRNA stability compared to *Δpub1* alone (Fig. 5E and Table 2). To test the generality of these results, we analyzed the stability of two additional ARE-like sequence-containing transcripts, *RPL17A* and *RPS24B*, and one additional STE-containing transcript, *WSC3* (16). Consistent with the *RPS16B* results, both the *RPL17A* and *RPS24B* transcripts were less stable in *nab2-1* and *nab2-67* cells than in wild-type cells (Table 2). In contrast, the stability of the STE-containing transcript *WSC3* (16), like *GCN4*, was not decreased in either *nab2-1* or *nab2-67* mutant cells (Table 2). Thus, as observed for two different *NAB2* mutants (*nab2-1* and *nab2-67*), our results indicate that the Nab2 protein affects the stability of ARE-like-sequence-containing transcripts but not transcripts containing an STE.

**Stabilization of the *RPS16B* transcript by Nab2 is dependent on the ARE-like sequence.** To test whether the *RPS16B* mRNA stability mediated by Nab2 is dependent on the ARE-like sequence, the half-life of an *RPS16B* mutant transcript lacking 16 nucleotides corresponding to its ARE-like sequence (*RPS16B-ΔARE*) was determined using *NAB2* mutants. The *RPS16B-ΔARE* mutant was generated by overlap PCR and cloned into an integrative vector. As shown in the schematic in Fig. 6A, the plasmid was integrated into the genome of both *Δpub1* cells and *Δnab2* cells maintained by a wild-type *NAB2* plasmid. This integration strategy resulted in gene duplication at the *RPS16B* locus such that cells express both the ARE-deleted transcript and the endogenous *RPS16B* transcript (Fig. 6A). With this experimental design, we could simultaneously measure the

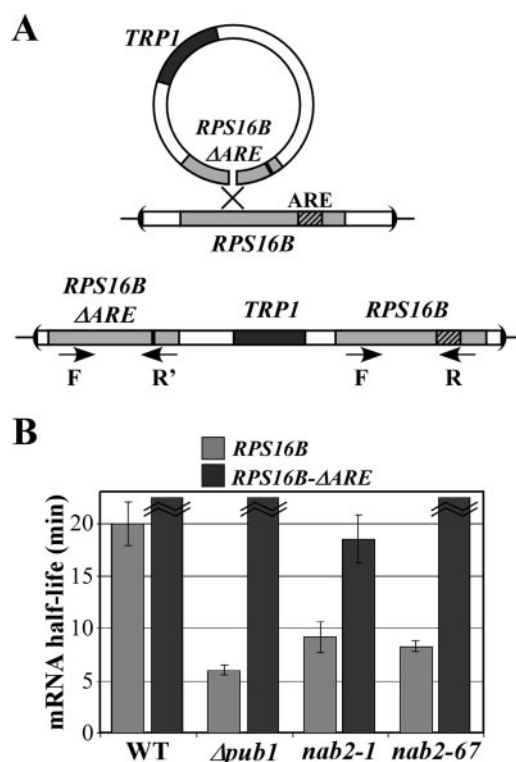


FIG. 6. Stabilization of *RPS16B* by Nab2 is dependent on the presence of the ARE-like sequence. (A) Schematic of integration at the *RPS16B* locus demonstrating how the integration of *RPS16B-ΔARE* was accomplished. The location of the primer pairs used to simultaneously amplify both endogenous *RPS16B* and *RPS16B-ΔARE* by qRT-PCR is also indicated. The F primer, which is used to amplify both endogenous *RPS16B* and the integrated *RPS16B-ΔARE* allele, hybridizes within the ORF region of both *RPS16B* sequences. The R primer, which specifically amplifies endogenous *RPS16B*, hybridizes with the ARE element. The R' primer, which specifically amplifies *RPS16B-ΔARE*, hybridizes to the junction created by the deletion of the ARE element. (B) The half-lives of both the *RPS16B* and *RPS16B-ΔARE* transcripts were simultaneously determined in wild-type (WT), *Δpub1*, *nab2-1*, and *nab2-67* cells harboring the *rpb1-1* allele. Cells were grown to mid-log phase in YPD medium and shifted to the nonpermissive temperature for 0, 2, 5, 10, 15, and 20 min. Total mRNA was isolated from each sample, reverse transcribed to cDNA, and subjected to qRT-PCR to determine *RPS16B*, *RPS16B-ΔARE*, and *PGK1* (control) transcript half-lives. Standard deviations in the data are indicated.

half-lives of both the control *RPS16B* transcript and the *RPS16B-ΔARE* transcript from the same cells by designing different real-time PCR reverse primers. A primer hybridizing in the ARE-like sequence (Fig. 6A) was used to amplify the endogenous *RPS16B* cDNA. A primer hybridizing to the junction created by deletion of the ARE element (Fig. 6A) was used to specifically amplify the *RPS16B-ΔARE* cDNA (Fig. 6A). Both reverse primers were paired with the same forward primer (F). To analyze the effect of Nab2 on the stability of both the endogenous *RPS16B* transcript and the *RPS16B-ΔARE* transcript, the wild-type *NAB2* plasmid covering the *Δnab2* cells was replaced by a *LEU* plasmid containing *NAB2*, *nab2-1*, or *nab2-67* (Fig. 6B) through a standard plasmid shuffle. As described above, the *RPS16B* transcript was less stable in *Δpub1*, *nab2-1*, and *nab2-67* cells than in wild-type cells (Fig.

TABLE 3. Half-lives of *RPS16B* and *RPS16B-ΔARE* transcripts in *NAB2* mutants

Strain	Half-life of target:	
	<i>RPS16B</i>	<i>RPS16B-ΔARE</i>
Wild type	19.3 ± 2.7	>20
$\Delta pub1$	6.5 ± 1.2	>20
<i>nab2-1</i>	9.5 ± 1.1	18.5 ± 2.3
<i>nab2-67</i>	7.9 ± 0.7	>20

6B and Table 2). In contrast, the stability of the *RPS16B-ΔARE* mRNA was comparable in wild-type,  $\Delta pub1$ , *nab2-1*, and *nab2-67* cells (Fig. 6B and Table 3). These results demonstrate that the ARE-like sequence is necessary for *RPS16B* transcript destabilization in both *nab2* mutants. Importantly, these data provide a link between mRNA stabilization mediated by Nab2 and the requirement for an ARE-like sequence, which is a binding site for Pub1 (16).

**Decay of the ARE-like sequence-containing *RPS16B* transcript is not NMD dependent.** Pub1 modulates mRNA stability by protecting transcripts from different decay pathways (48, 56). Based on a previous analysis of two endogenous yeast transcripts, *GCN4* and *YAP1*, it was suggested that STE-containing mRNAs are degraded via NMD because the stop codon of the upstream ORF is recognized as a premature stop codon (48). We found that the stability of the ARE-like sequence-containing transcript *RPS16B* is dependent on both Pub1 and Nab2, but it is not clear what mechanism leads to the degradation of endogenous ARE-like sequence-containing transcripts or whether Pub1 and Nab2 protect transcripts from the same degradation pathway. To directly test whether the ARE-like transcript that is modulated by Pub1 and Nab2 is degraded by the NMD pathway, we tested whether the deletion of *UPF1*, which encodes an essential component of the NMD pathway (20), could restore the stability of the *RPS16B* transcript, which is decreased in  $\Delta pub1$ , *nab2-1*, and *nab2-67* mutant cells. The disruption of the NMD pathway ( $\Delta upf1$ ) did not rescue the stability of the *RPS16B* transcript in  $\Delta pub1 \Delta upf1$ , *nab2-1 \Delta upf1*, or *nab2-67 \Delta upf1* cells, although, as previously reported (48), the deletion of *UPF1* did rescue the stability of the STE-containing *GCN4* transcript in  $\Delta pub1 \Delta upf1$  cells (Table 2).

## DISCUSSION

The interplay between RNA binding proteins and *cis*-acting regulatory elements within specific mRNA transcripts can regulate gene expression by stabilizing or destabilizing a particular transcript. Pub1 has been implicated in the stabilization of a group of mRNAs through binding to specific sequences in either the 5'- or 3'-UTR and thereby inhibiting different decay pathways (48, 56). In this report, we demonstrate that the poly(A) RNA binding protein Nab2 can bind directly to Pub1 and can also modulate the stability of a subset of Pub1 target mRNAs.

Since Pub1 is an mRNA binding protein and since we found that it can bind to Nab2, another RNA binding protein, we needed to determine if this interaction could be mediated by RNA. This point is particularly important because the essential

CCCH domain on Nab2 that mediates binding to Pub1 has also been implicated in RNA binding (3, 37). We found that TAP-tagged Nab2 copurifies with Pub1 from yeast extract even when the lysate is pretreated with RNase A, suggesting that this interaction is not mediated by RNA. An *in vitro* binding assay confirmed that Pub1 can bind directly to Nab2 and showed that the zinc finger domain of Nab2 is both necessary and sufficient to directly interact with Pub1.

Classical zinc finger proteins often employ multiple zinc fingers to bind to nucleic acids (38). Typically, aromatic residues within the zinc fingers interact with the nucleic acid to impart sequence specificity (38). However, in addition to binding to nucleic acids, zinc fingers can also bind to proteins (35). Furthermore, there are a number of proteins that contain multiple zinc fingers that mediate distinct binding interactions. For example, the transcription factor TFIIIA, a dual RNA/DNA binding protein, contains nine zinc fingers, and individual zinc fingers are used to recognize both RNA and DNA targets (6, 18, 44, 51). In addition, the transcription factor GATA-1 contains two zinc finger repeats, which not only act as the primary determinant of binding specificity for the DNA sequence GATA but also can mediate protein-protein interactions (38). Similarly, our results suggest that the CCCH domain of Nab2, which contains seven zinc fingers, is a multi-tasking zinc finger domain required for both RNA-protein and protein-protein interactions.

Previously characterized Pub1 target mRNAs contain two distinct *cis*-acting elements, the 5'-UTR STE or the 3'-UTR ARE (48, 56). A recent genome-wide survey identified a number of novel ARE-like and A-rich Pub1 binding motifs (16). We found that *nab2-1* cells show altered stability of the ARE-like Pub1 target transcripts but no effect on STE-containing Pub1 targets. It is not yet clear whether the effect on the stability of ARE transcripts is a consequence of the nuclear role of Nab2 or whether it reflects a distinct function for Nab2 in the cytoplasm. Given that the poly(A) RNA transcripts in *nab2-1* mutant cells accumulate longer poly(A) tails than in wild-type cells (26), it might be logical to assume that the mRNA half-life would be increased in *nab2-1* cells. Instead, we found that target RNAs containing an ARE-like element have decreased stability in *nab2-1* cells. Although this mutant does interact with Pub1, we suggest that the deletion of the Nab2 N terminus could impact Nab2 function in mRNA stabilization by affecting its interaction with another component of a multifunctional protein complex involved in mRNA export and/or mRNA decay. Consistent with this idea, the same defect in mRNA stability displayed by *nab2-1* mutant cells was observed in the novel *NAB2* mutant, *nab2-67*, which encodes a Nab2 variant with a substantially decreased interaction with Pub1 (Fig. 5C).

Based on our results with the *nab2-67* mutant, we suggest that the Nab2 function in stabilizing ARE-like sequence-containing transcripts is related to its interaction to Pub1, which acts by modulating the stability of this class of transcripts (16). Consistent with an independent role for Nab2 in modulating mRNA stability through interactions with Pub1, we find that cells expressing the Nab2 mutant *nab2-67*, which shows a decreased interaction with Pub1, do not display severe growth defects. However, these cells do show an altered stability of a subset of Pub1 target transcripts. These phenotypes for the

*nab2-67* allele are identical to the phenotypes, no growth defect and altered transcript stability, observed for cells lacking Pub1, strengthening the argument for a functional interaction between Nab2 and Pub1.

We propose that the specific effect of Nab2 on the ARE-like sequence-containing transcripts but not on the STE-containing transcripts could be due to the proximity of the Pub1 binding site, the ARE-like sequence within the 3'-UTR, to the poly(A) tail, which is where Nab2 associates with the transcript (25, 26; S. M. Kelly et al., unpublished data). Consistent with this idea, an interaction between the human poly(A) binding protein PABP and HuR, the apparent human Pub1 orthologue (45, 56), was recently described (42). This interaction increased the stability of the ARE-containing  $\beta$ -casein mRNA transcript through the interaction of the poly(A) tail and the ARE element (42), supporting the idea that these *cis*-acting elements could work together. In contrast, the STE is located in the 5'-UTR of the *GCN4* transcript, a site that should be physically distant from the poly(A) tail and thus not in close physical proximity to Nab2.

Although Nab2 is localized to the nucleus at steady state (3), it shuttles between the nucleus and the cytoplasm (23). Likewise, Pub1, which is primarily localized to the cytoplasm, can be detected in the nucleus (2). Recently, evidence has emerged that a number of RNA binding proteins play distinct roles in the nucleus and the cytoplasm (18, 21, 33, 62). For example, Hrp1 is an hnRNP which localizes to the nucleus at steady state (27), where it is required for proper mRNA 3'-end formation (33, 55). However, it can also shuttle to the cytoplasm, where it modulates the NMD pathway (21). Other examples include Gbp2 and Hrb1, which bind to mRNA in the nucleus but are also associated with polysomes in the cytoplasm (62). Additionally, eukaryotic translation initiation factor 4AIII, a nuclear protein that is loaded onto the mRNA during splicing in the nucleus, also functions in NMD and has properties related to the eukaryotic translation initiation factor 4A translation initiation factor (8, 18, 44, 51).

It is still not clear how Pub1 stabilizes transcripts containing 5'-UTR STE or 3'-UTR ARE elements. The NMD pathway is required for the degradation of upstream ORF-containing transcripts that contain the *cis*-acting STE element (47). In yeast, the major decay pathway for NMD involves 5' decapping followed by 5'-3' degradation bypassing deadenylation (12). Previous work showed that the endogenous uORF-containing *GCN4* transcript is protected from NMD-mediated decay by the binding of Pub1 to the STE sequence located in the 5'-UTR (48). In the case of ARE-containing transcripts, Pub1 protects a hybrid transcript harboring the tumor necrosis factor alpha ARE from decay via the deadenylation-dependent pathway (56). However, it is not clear whether endogenous ARE-containing transcripts are degraded by the same mechanism. Our results indicate that the stability of the *RPS16B* transcript is not recovered in  $\Delta$ *pub1*, *nab2-1*, or *nab2-67* cells where the NMD pathway is impaired by the deletion of *UPF1*. This result confirms that both Pub1 and Nab2 stabilize the ARE-like sequence-containing transcripts by modulating a pathway that is distinct from NMD.

Pub1 modulates *RPS16B* mRNA decay through a direct interaction with an ARE-like sequence at the transcript 3'-UTR (16). Our results indicate that the ARE-like sequence in

*RPS16B* mRNA is also necessary for the stabilization mediated by Nab2. The functional association of Nab2 with this specific *cis*-acting mRNA element could help to coordinate different events involved in the control of gene expression, coupling the maturation and nuclear export steps of mRNA biogenesis to the mRNA decay machinery. Taken together, our study suggests that Nab2, a protein with defined nuclear functions, together with Pub1, plays a role in determining the cytoplasmic fate of some mRNA transcripts, adding Nab2 to a growing list of nuclear RNA binding proteins that modulate the destiny of mRNA transcripts in the cytoplasm.

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