

A new yeast PUF family protein, Puf6p, represses *ASH1* mRNA translation and is required for its localization

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In yeast *Saccharomyces cerevisiae*, Ash1p, a protein determinant for mating-type switching, is segregated within the daughter cell nucleus to establish asymmetry of *HO* expression. The accumulation of Ash1p results from *ASH1* mRNA that is sorted as a ribonucleoprotein particle (mRNP or locasome) to the distal tip of the bud where translation occurs. To study the mechanism regulating *ASH1* mRNA translation, we isolated the *ASH1* locasome and characterized the associated proteins by MALDI-TOF. One of these proteins was Puf6p, a new member of the PUF family of highly conserved RNA-binding proteins such as Pumilio in *Drosophila*, responsible for translational repression, usually to effect asymmetric expression. Puf6p-bound PUF consensus sequences in the 3'UTR of *ASH1* mRNA and repressed the translation of *ASH1* mRNA both in vivo and in vitro. In the *puf6Δ* strain, asymmetric localization of both Ash1p and *ASH1* mRNA were significantly reduced. We propose that Puf6p is a protein that functions in the translational control of *ASH1* mRNA, and this translational inhibition is necessary before localization can proceed.

[Keywords: *ASH1*; RNA localization; translational regulation; *PUF6*]

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The asymmetric segregation of proteins is vital to cellular polarity and cell-fate determination. One mechanism for achieving protein asymmetry is to localize the mRNA encoding the protein. This mechanism is widely used in a number of cellular processes, such as fibroblast motility and synaptic plasticity in neurons (Bassell and Singer 1997, 2001; Bassell et al. 1999), embryogenesis in *Drosophila* (Bashirullah et al. 1998; Lipshitz and Smibert 2000), and mating-type switching in yeast *Saccharomyces cerevisiae* (Long et al. 1997; Takizawa et al. 1997).

The localization of *ASH1* mRNA in budding yeast provides a useful model to study how a cell-fate determinant is asymmetrically sorted (Darzacq et al. 2003). The asymmetric segregation of Ash1p within daughter cell nuclei negatively regulates the expression of *HO* endonuclease that is essential for mating-type switching (Jansen et al. 1996; Sil and Herskowitz 1996). This specific inheritance of Ash1p results from localizing the *ASH1* mRNA to the distal bud tip during anaphase of the cell cycle, where it is presumably translated near the bud nucleus (Long et al. 1997; Takizawa et al. 1997). The major components required for *ASH1* mRNA localization have been identified and were shown to form a large ribonucleoprotein particle ("locasome" or mRNP) containing She1p/Myo4p, a type V myosin that transports

ASH1 mRNA in the cytoplasm along cytoskeletal filaments to its final destination (Bertrand et al. 1998; Beach et al. 1999), She2p, identified as an RNA-binding protein that specifically interacts with the *ASH1* mRNA localization zipcodes, and She3p, which serves as a bridge protein between She1p and She2p (Bohl et al. 2000; Long et al. 2000; Takizawa and Vale 2000).

A major biological function of mRNA localization is to restrict the production of its encoded protein at the region of localization. To accomplish this, mRNA translation will likely be repressed during the localization pathway. For instance, translation of the maternal *Oskar* mRNA is restricted during transport to the posterior pole of the oocyte, where it is activated and effects the differentiation of abdominal structures (Macdonald and Smibert 1996). Ash1p segregation into daughter-cell nuclei relies on the efficiency of *ASH1* mRNA localizing at its distal tip before translation occurs (Chartrand et al. 2002; Irie et al. 2002). Translational repression is often mediated by regulatory proteins that interact with the *cis*-element(s) within the 3'untranslated region (3'UTR) of the mRNA. In *Drosophila*, Bruno protein prevents the translation of unlocalized *oskar* mRNA through binding to the Bruno responsive element (BRE) in the 3'UTR (Kim-Ha et al. 1995; Gunkel et al. 1998). Other translational regulatory proteins, the PUF family proteins (PUF for *Drosophila* Pumilio and *Caenorhabditis elegans* FBF), repress the translation of their target mRNAs by binding to *nanos*-responsive elements (NRE) within the

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of ~75 kDa corresponded to Puf6p, a novel PUF family protein in the yeast *S. cerevisiae* (Fig. 1B, lane 2). To determine whether affinity-purified Puf6p using the She2p-tap was specific, we performed the reciprocal immunoprecipitation from a strain in which *PUF6* was tap-tagged and *SHE2* was myc-tagged (Fig. 1C). After affinity precipitation with IgG-coated Sepharose, the corresponding precipitates were evaluated by immunoblotting with anti-myc antibodies (Fig. 1C). The result showed that the She2p-myc coprecipitated with Puf6p in the Puf6p-tap strain (Fig. 1C, lane 4) and was not present in the control strain in which *PUF6* was not tap-tagged (Fig. 1C, lane 3), indicating that Puf6p and She2p are in the same complex.

ASH1 mRNA contains conserved PUF recognition sequences in the 3'UTR

Analysis of structural motifs in Puf6p revealed that the protein contained seven putative pumilio-like repeats, first identified in the *Drosophila* Pumilio (Zhang et al. 1997), an aspartic and glutamic acid-rich region (D/E), and a putative bipartite nuclear localization signal (NLS; Fig. 1D). The presence of the NLS suggested that Puf6p may be a nuclear protein. PUF proteins have been identified to function through interaction with the 3'UTRs of *Drosophila hb* mRNA, *C. elegans fem-3* mRNA, and yeast *HO* mRNA (Wharton and Struhl 1991; Murata and Wharton 1995; Zhang et al. 1997; Wharton et al. 1998; Tadauchi et al. 2001; Wang et al. 2002). Although these 3'UTRs share little overall similarity to each other, they all contain the UUGU tetranucleotide, the conserved segment important for PUF protein function. The 3'UTR of *ASH1* mRNA also contains bipartite UUGU segments located at 54 and 70 nucleotides downstream of the stop codon (Fig. 1E), suggesting that the sequences could be the binding targets of Puf6p. Albeit that the UUGU sequences are within the E3 element, it is not likely that the UUGU sequences are involved in *ASH1* mRNA localization, because these nucleotides are not important for secondary structure formation (Chartrand et al. 1999, 2002). Furthermore, the UUGU tetranucleotide is not

present in the E1, E2a, and E2b elements that are also competent to localize the reporter RNA (Fig. 1E; Chartrand et al. 1999). An alignment of the partial 3'UTRs of *hb*, *fem-3*, cyclin b, *HO*, and *ASH1* mRNAs is shown in Figure 1F, which demonstrates that these 3'UTRs share similar putative UUGU sequence elements, and the surrounding nucleotides are generally AU-rich.

In a recent study, Shepard et al. (2003) have identified an additional 22 mRNAs that were associated with She2p, many of which localize. We therefore examined these genes and found 13 that contained UUGU consensus elements in the 3'UTR (Table 1).

Colocalization of Puf6p with *ASH1* mRNA requires the 3'UTR

To evaluate the possible role of Puf6p on *ASH1* mRNA localization, we analyzed whether the protein colocalized with *ASH1* mRNA in cells cotransformed with two plasmids, MS2-GFP fusion and MS2-*ASH1* RNA reporter. In the cells expressing MS2-*ASH1* reporter, its mRNA was marked with a MS2-GFP fusion protein that bound to the MS2 and displayed a single, large GFP particle (locasome), which localized to the distal tip of daughter cells (Bertrand et al. 1998). In this experimental system, we tap-tagged Puf6p and used immunofluorescence to detect the colocalization of Puf6p-tap with the GFP particle. We observed Puf6p mainly in the nucleus, as predicted by the presence of the NLS (Fig. 2). However, we also observed cytoplasmic Puf6p that was colocalized with the *ASH1* mRNA particle at the bud tip (Fig. 2, full *ASH1* mRNA), suggesting that Puf6p could play a role in *ASH1* mRNA localization. Because *ASH1* mRNA contains four *cis*-acting localization elements, that is, E1, E2A, E2B, and E3, and each is competent to localize a heterologous reporter RNA (Fig. 1E; Chartrand et al. 1999; Gonzalez et al. 1999), we constructed MS2-tagged LacZ RNA reporters containing either *ASH1* E1, E2A, E2B, or E3 elements to determine which were responsible for the colocalization with Puf6p. Whereas each of these constructs produced a bright particle (locasome) in

Table 1. The conserved UUGU elements are present in the 3'UTRs of 13 mRNAs associated with She2p

Genes	The conserved UUGU elements at the 3'UTRs		
<i>YLR190W/MMR1</i>	64	CUCCGUAUUGUAG	GCUUGUCG UAAAUAAA 391
<i>YOR247W/SRL1</i>	31	UUUUGUUU	AUUUGUUUGUUA AAAAUAAA 205
<i>YNL087W</i>	142	AAUUGUAA	UUUUGUUC AAAAUAAA 272
<i>YPL084W/BRO1</i>	23	UAUUGUAG	AUUUGUUG AUAAUAAA 261
<i>YLL028W/TPO1</i>	51	CAUUGUUA	GGUUGUUA UAAAUAAA 355
<i>YLR332W/MID2</i>	52	UAUUGUAA	AAUUGUAA AAAAUAAA 423
<i>YNL327W/EGT2</i>	83	GUUUGUAAUUGUAA	UCUUGUAC AAUAIAAA 304
<i>YGR056W/RSC1</i>	55	UUUUGUUA	UUUUGUUG AAUUGUAA CCAAUAAA 239
<i>YMR202W/ERG2</i>	97	UAUUGUUU UCAAUAAA 260
<i>YLL001W/DNM1</i>	117	UCUUGUUA UAAAUAAA 146
<i>YNL283C/WSC2</i>	79	CUUUGUAU AGAAUAAA 397
<i>YML072C</i>	73	GUUUGUUA CUAUUAAA 207
<i>YBR086C/IST2</i>	124	CCUUGUGC AAAUUAAA 342

The numbers show the position of nucleotides from the stop codon.

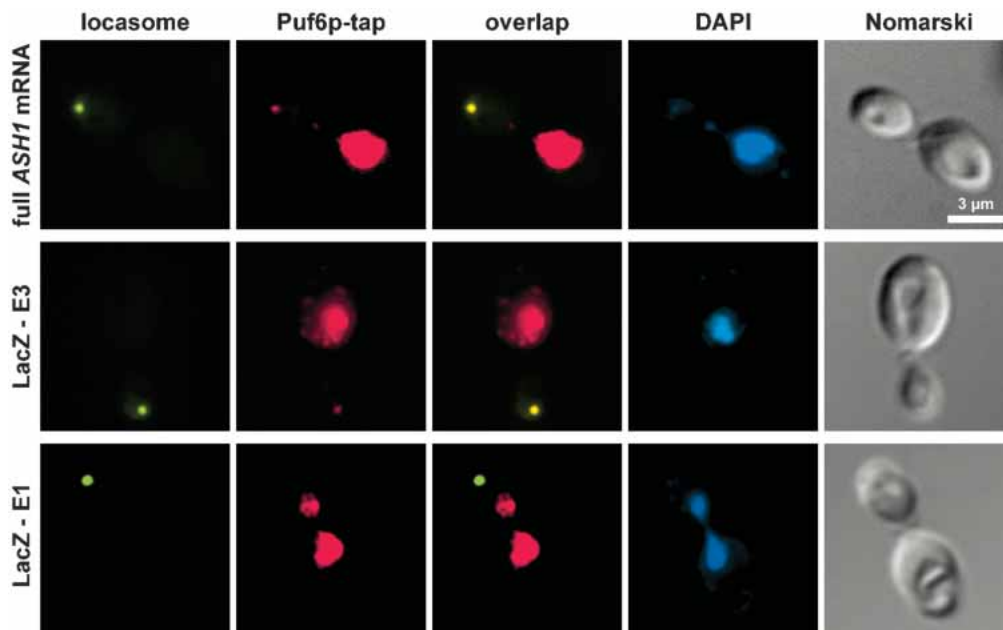


Figure 2. Colocalization of Puf6p and *ASH1* mRNP particles. The single, bright particle (locosome) localized to the bud tip of yeast cells expressing both GFP-MS2 fusion protein and the *ASH1* or LacZ reporter RNAs fused with MS2-binding sites. Immunofluorescent staining on Puf6p (red) visualizes the colocalization with the locosome (green). Note that because there are many GFP on the MS2-RNA reporter, the signal is considerably stronger than the immunofluorescence. (*Top*) Full-length *ASH1* mRNA was used as a reporter. (*Middle*) LacZ RNA fused with E3 (the *ASH1* mRNA localization element containing PUF consensus sequences) was used as a chimeric reporter. (*Bottom*) LacZ RNA fused with E1 *ASH1* mRNA localization element (does not contain PUF consensus sequences).

the bud, Puf6p only colocalized with the LacZ reporter containing the E3 element (Fig. 2, LacZ-E3, ~70% of the cells shown colocalized signals) and did not colocalize with the LacZ reporters fused with *ASH1* E1, E2A, or E2B localization elements (Fig. 2, LacZ-E1; data not shown). This result was in agreement with the observation that only E3 contained the conserved UUGU element identified for PUF protein binding. In contrast, tap-tagged She2p colocalized with each of the LacZ reporters (data not shown), indicating that Puf6p may have a more specific role than She2p in the RNA localization process.

Puf6p associates with ASH1 mRNA in vivo

To investigate whether Puf6p was colocalized with *ASH1* mRNA in the bud tip, we performed in situ hybridization for *ASH1* mRNA in a yeast strain in which Puf6p was labeled with GFP (Fig. 3A). Although Puf6p was predominantly present in cell nuclei, a small amount of the Puf6p-GFP was detected in the bud tip, where *ASH1* mRNA was localized (Fig. 3A, arrowheads; GFP fluorescence was reduced by the FISH technique). To further determine whether *ASH1* mRNA physically associated with Puf6p-tap in vivo, we immunoprecipitated the complex and tested for the presence of *ASH1* mRNA by RT-PCR. Tap-tagged She2p was used as a positive control, because it has been confirmed to interact with *ASH1* mRNA (Munchow et al. 1999; Long et al. 2000). We detected *ASH1* mRNA in the precipitates of

both Puf6-tap and She2-tap strains (Fig. 3B, lanes 1,3, respectively). *ASH1* mRNA was not detected in the immunoprecipitates from an untagged strain (Fig. 3B, lane 2), nor from an unrelated tap-tagged Pob3p strain (Fig. 3B, lane 4). In addition, the PCR product was not seen in a blank control (Fig. 3B, lane 10), indicating the formation of the PCR product requires RNA. These data, together with the colocalization with the *ASH1* mRNP, indicate that Puf6p associates with *ASH1* mRNA.

Puf6p is required for Ash1p segregation and asymmetric localization of ASH1 mRNA

The observation that Puf6p associated with *ASH1* mRNA raised the possibility that Puf6p could play a role in the asymmetric expression of *ASH1* mRNA. To address this, we constructed a *puf6::TRP* strain and examined the intracellular Ash1p segregation in *puf6* by immunofluorescence. We observed 69% ($\pm 5.9\%$) of budding cells in *puf6* that had Ash1p symmetrically distributed between mother and daughter nuclei compared with 19% ($\pm 2.4\%$) in wild type (Fig. 4A,B). In addition, the symmetric Ash1p was not evenly distributed in *puf6* cells; the concentration of the protein in the mother cell nuclei was slightly weaker than that in the bud nuclei. This result suggested a role for Puf6p in the asymmetric distribution of Ash1p into daughter cell nuclei. To determine whether the defect in Ash1p segregation in *puf6* cells resulted from *ASH1* mRNA mislocalization, we

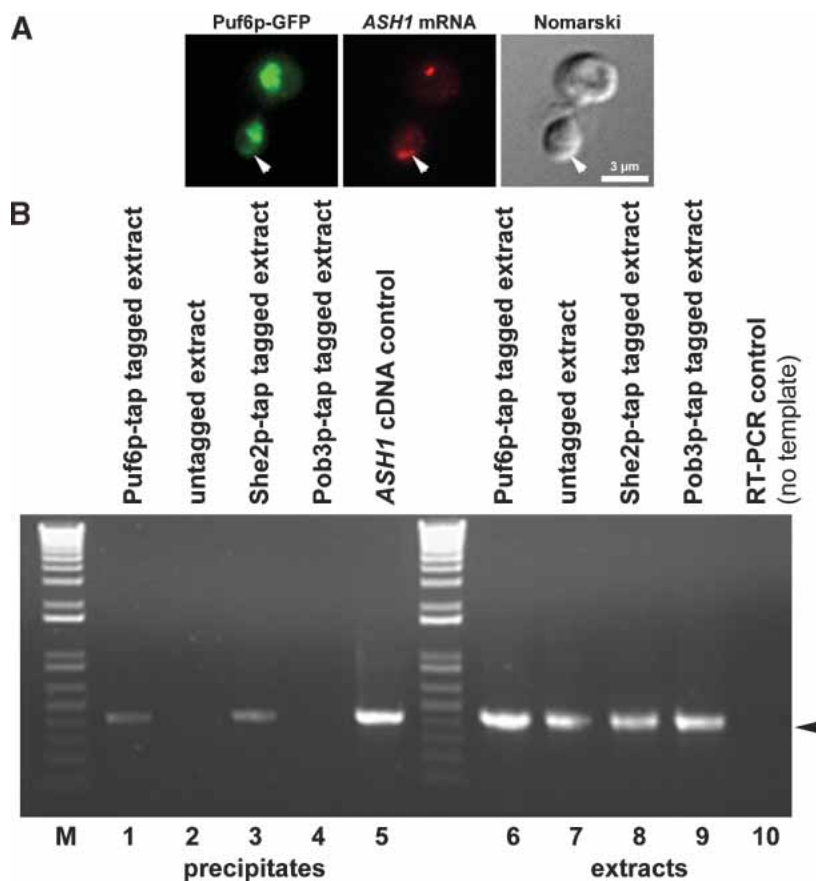


Figure 3. Puf6p is physically associated with *ASH1* mRNA in vivo. (A) Yeast cells with GFP-labeled Puf6p were cultured and fixed as described in the Materials and methods. The cells were in situ hybridized to *ASH1* mRNA (Cy3, red). Spots indicate the transcription sites of the *ASH1* mRNA in the nuclei. The green signal shows the GFP-labeled Puf6 proteins. The arrowheads indicate the position of *ASH1* mRNA and Puf6p. (B) Coprecipitation of *ASH1* mRNA with Puf6p-tap. IgG-coated Sepharose beads were used to immunoprecipitate a tap-tagged Puf6p extract. RT-PCR was used to detect the presence of *ASH1* mRNA in the precipitates (left) and in total cell extracts (right). Tap-tagged She2 yeast extract was used as a positive control (lane 3). The negative control used a wild-type extract and a tap-tagged Pob3p (a chromatin-associated protein) extract (lanes 2,4) and a no-template control (lane 10). The positive PCR control used *ASH1* cDNA (lane 5). (M) Molecular size of DNA. The arrow indicates the PCR product of *ASH1* mRNA.

analyzed the endogenous *ASH1* mRNA distribution by FISH analysis. Compared with the wild-type strain, in which 82% ($\pm 4.7\%$) of the budding cells showed localized *ASH1* mRNA, we observed a diminution of *ASH1* mRNA localization in *puf6*, where only 42% ($\pm 5.1\%$) of *ASH1* mRNA was properly localized at the bud tip (Fig. 4C,D). These results suggest that in *puf6* cells, the defect on Ash1p asymmetric segregation resulted from inefficient *ASH1* mRNA localization. Moreover, the observed phenotype for endogenous *ASH1* mRNA in *puf6* cells was less penetrant than the one observed in the *she2* deletion cells that completely disrupted the mRNA localization (Long et al. 1997; Chartrand et al. 2001). In those *puf6* cells, although *ASH1* mRNA was symmetrically distributed in both mother and bud, we still observed considerable amounts of the mRNA at the bud tip (Fig. 4C), implying that the *ASH1* mRNA was competent to localize in *puf6* cells, albeit inefficiently.

PUF6 deletion affects the HO promoter activity

To determine the physiological relevance of Puf6p on the ability of the cell to control mating-type switching, we tested the effect of a *puf6* deletion on the regulation of the *HO* promoter. *PUF6* was deleted in a strain in which the endogenous *CAN1* and *ADE2* genes were under the control of the *HO* promoter (Jansen et al. 1996). These

cells were sensitive to the arginine analog canavanine when the *CAN1* gene was expressed, whereas repression of *CAN1* leads to tolerance to the drug. In addition, expression of *HO-ADE2* will lead to white colonies, repression to the formation of red colonies. We observed that deletion of *PUF6* decreased the sensitivity to canavanine (Fig. 4E), although to a lesser extent than a mutation in *SHE3* that is known to more dramatically interfere with *ASH1* mRNA localization. Similarly, *she3-1440* (Jansen et al. 1996) and *puf6* formed red colonies in contrast to the wild-type strain, which remained white (data not shown). This confirms a role for *PUF6* in regulating *HO* expression and, therefore, mating-type switching.

Reduction of *ASH1* mRNA translation increases asymmetric distribution of Ash1p and its mRNA in the *puf6* strain

The symmetric distribution of Ash1 protein in the mother and bud of *puf6* could result from the premature translation of *ASH1* mRNA in mother cells during the localization process. This would inhibit the localization of the RNA, because the ribosomes may displace the transport complex. To test this hypothesis, we inserted a short stem-loop structure (SL) at the 5'UTR of the *ASH1* mRNA into the *puf6* strain that has been shown to slow mRNA translation, but not affect its localization (Char-

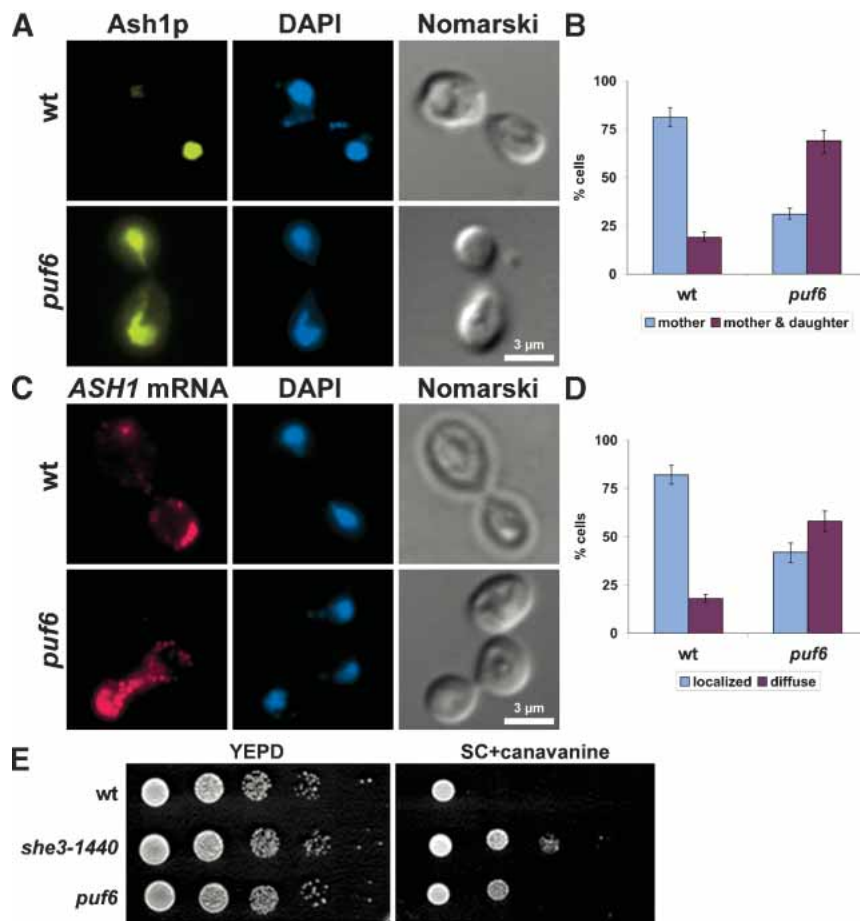


Figure 4. The *puf6* deletion decreases the asymmetric localization of *ASH1* mRNA and Ash1p. (A) Immunofluorescent detection of Ash1p-myc in wild-type and *puf6* yeast cells. Ash1p-myc accumulates within the bud nucleus in late anaphase of a wild-type cell (*top*), whereas the protein is distributed in both mother and bud cell nuclei of *puf6* cells (*bottom*). (B) Effect of *puf6* deletion on the percentage of cells with localized Ash1p. (Blue bars) Bud cell nuclei asymmetrically localized; (red bars) mother and bud symmetrically distributed. (C) Fluorescent in situ hybridization for *ASH1* mRNA in wild-type and *puf6* yeast cells. *ASH1* mRNA is localized at the tip of a wild-type budding cell (*top*), whereas it is diffuse in the cytoplasm of both mother and bud in the *puf6* cells (*bottom*). (D) Effect of Puf6p on the percentage of cells with localized *ASH1* mRNA in wild-type and *puf6* cells. (Blue bars) Bud tip localized; (red bars) delocalized. (E) Effect of *puf6* deletion on repression of the *HO* promoter. Tenfold serial dilutions of exponentially growing wild-type, *she3-1440* or *puf6* cells were spotted on YPD or SC medium containing 0.03% canavanine and incubated for 2 and 5 d at 30°C, respectively.

trand et al. 2002; *SL-ASH1 puf6*). We postulated that partially repressing Ash1p synthesis in *puf6* cells would, therefore, rescue the asymmetric distribution of both Ash1p and *ASH1* mRNA. As shown in Figure 5A, we observed an increase in the percentage of cells with asymmetrical segregation of Ash1p to bud nuclei ($66\% \pm 4.8\%$ of *SL-ASH1 puf6* budding cells compared with $32\% \pm 3.4\%$ of *puf6* budding cells) and an increased localization of endogenous *ASH1* mRNA at the bud tip (Fig. 5B, $68\% \pm 5.6\%$ of *SL-ASH1 puf6* budding cells compared with $43\% \pm 4.2\%$ of *puf6* budding cells). Therefore, reducing the rate of *ASH1* mRNA translation in *puf6* cells suppressed the phenotype caused by the *PUF6* deletion. This indicated that Puf6p could play a role in temporally regulating *ASH1* mRNA translation during its localization.

Mutagenesis of the UUGU Elements within the 3'UTR of *ASH1* mRNA

PUF family proteins are negative regulators that act by binding to the core component identified as UUGU tetranucleotides in the 3'UTRs of their target mRNAs (Murata and Wharton 1995; Zhang et al. 1997; Sonoda and Wharton 1999; Tadauchi et al. 2001). The 3'UTR of *ASH1* mRNA contains two UUGU sites located at 54

and 70 nucleotides downstream of the stop codon (Fig. 1E). This raised the possibility that Puf6p was involved in Ash1p and *ASH1* mRNA asymmetry by binding the 3'UTR of *ASH1*. To test this possibility, we generated *ASH1* mRNA mutations, in which the two UUGU elements in the 3'UTR were mutated to AACA. The 3'UTR mutations were created to disrupt the interaction with Puf6p, while still maintaining the capability for localization (Chartrand et al. 1999). The resulting *ASH1* mutant gene, named *ASH1-3'mut*, was integrated into an *ash1* yeast strain. The distribution of the *ASH1-3'mut* mRNA and Ash1 protein were determined by FISH and immunofluorescence, respectively. As shown in Figure 5C and D, the asymmetry of *ASH1-3'mut* mRNA and Ash1p localization were decreased. Whereas 83% ($\pm 3.4\%$) of the wild-type *ASH1* mRNA localized into the bud of late-anaphase yeast cells, 47% ($\pm 5.1\%$) of *ASH1-3'mut* mRNA was found localized in these cells. As a result of diminished mRNA localization, 59% ($\pm 4.9\%$) of Ash1 protein accumulated symmetrically between mother and daughter cell nuclei in comparison with the wild-type cells, in which only 16% ($\pm 2.3\%$) of Ash1p was symmetric. These data indicated that the lack of the UUGU sequences in the 3'UTR resulted in the predominantly symmetric distribution of *ASH1* mRNA and its encoded protein and was a phenocopy of the *puf6* deletion.

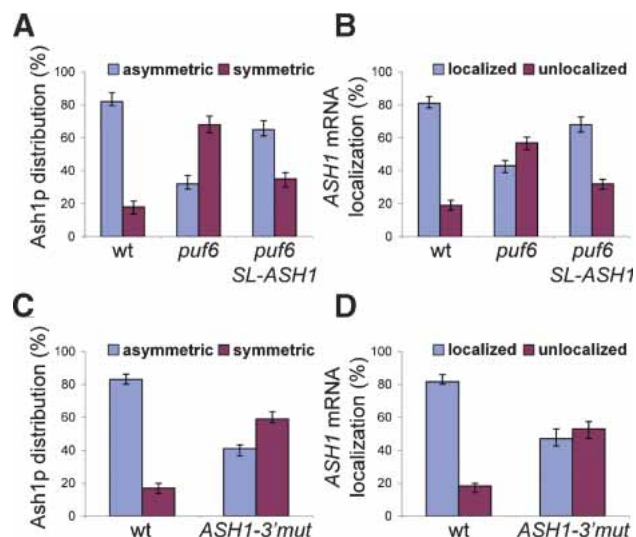


Figure 5. Translational inhibition of *ASH1* mRNA is required for asymmetry. (A) Effect of a stem-loop in the *ASH1*-5'UTR (*SL-ASH1*) on Ash1p asymmetry in *puf6*; reducing *ASH1* mRNA translation partially restored the asymmetric distribution of Ash1p in the *puf6* strain. (Blue bars) Bud cell nuclei asymmetrically localized; (red bars) mother and bud symmetrically distributed. (B) Effect of a stem-loop in the *ASH1*-5'UTR (*SL-ASH1*) on asymmetric distribution of *ASH1* mRNA in *puf6*, reducing *ASH1* mRNA translation increased the percentage of localized *ASH1* mRNA in the *puf6* strain. (Blue bars) Bud tip localized; (red bars) not localized. (C) Effect of deletion of Puf6p-binding sites on Ash1p distribution in *ASH1-3'mut* strain. (Blue bars) asymmetric; (red bars) symmetric. (D) Effect of deletion of Puf6p-binding sites on *ASH1* mRNA localization in *ASH1-3'mut* strain. (Blue bars) localized; (red bars) unlocalized.

The effect of Puf6p on intracellular Ash1p expression levels

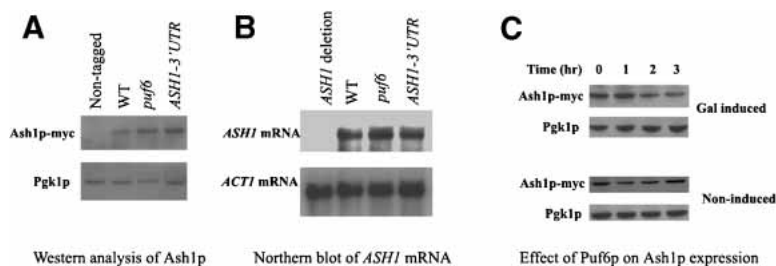
In *Drosophila* and *C. elegans*, PUF proteins function as translational repressors in concert with localization (Zhang et al. 1997; Wharton et al. 1998). To assess whether Puf6p affects Ash1p segregation through the regulation of *ASH1* mRNA translation, we examined intracellular Ash1p-myc expression in wild-type, *puf6*, and *ASH1-3'mut* strains by Western blot (Fig. 6A). Using Pgk1 protein as an internal control (Fig. 6A, bottom), we

found that Ash1p was markedly increased in the *puf6* strain and the *ASH1-3'mut* strain, where the UUGU sequences in the 3'UTR of *ASH1* mRNA were mutated (Fig. 6A, top). The levels of Ash1p in *puf6* and *ASH1-3'mut* cells were 2.4× (+0.4) and 2.2× (±0.3) that of the wild-type strain, respectively, indicating more Ash1 protein was translated in both *puf6* and *ASH1-3'mut* strains. These changes in the levels of Ash1p did not result from mRNA degradation, as the *ASH1* mRNA level was apparently unchanged in *puf6*, *ASH1-3'mut* and wild-type strains when normalized to *ACT1* mRNA (Fig. 6B). This was consistent with the results that either the Puf6p deletion or UUGU mutations affected the asymmetric distribution of Ash1p and *ASH1* mRNA (Figs. 4, 5) and supported the possibility that Puf6p, like PUF family proteins in other species, functioned as a translational repressor. To confirm this, we constructed a *puf6* deletion strain with Puf6p under the control of the galactose-inducible GAL1 promoter. After the strain was cultured in 2% raffinose overnight, galactose was added to induce Puf6p expression. Ash1p levels were analyzed by Western blots at 0, 1, 2, and 3 h after Puf6p induction. The expression of Puf6p in the *puf6* strain should progressively reduce Ash1p translation. The results revealed that a twofold reduction (±0.3) of Ash1 protein was observed over a 3-h induction of Puf6p expression (Fig. 6C, top). The reduction in Ash1p concentration was not due to the decrease in mRNA expression, as Northern blot analysis showed that the mRNA levels were not changed before and after galactose induction (data not shown). By comparison, the Ash1p levels did not change without Puf6p induction (Fig. 6C, bottom) nor did Pgk1p, even in the presence of galactose. Therefore, the in vivo translation of *ASH1* mRNA was inhibited by the presence of Puf6p.

Puf6p binds to the *ASH1*-3'UTR containing the PUF consensus UUGU segment and represses its translation in vitro

Because Puf6p associated with *ASH1* mRNA in vivo via the 3'UTR (E3), we reasoned that Puf6p could bind the 3'UTR of *ASH1* mRNA directly. We expressed and purified Puf6p in a baculovirus system and used purified Puf6p to test for RNA-binding activity using mobility-shift and

Figure 6. Puf6p suppresses the expression level of Ash1p. (A, top) Western blots showing the expression of myc-tagged Ash1p in wild-type, *puf6* and *ASH1-3'mut* strains. (Bottom) Pgk1p was used as an internal control. (B) Northern hybridization detecting intracellular levels of *ASH1* mRNA in wild-type, *puf6*, and *ASH1-3'mut* strains. *ACT1* mRNA was used as a control to normalize the relative signals. (C) Western blots of Ash1p concentrations in a *puf6* strain with *PUF6* driven by a galactose-inducible promoter. Samples were taken at 0, 1, 2, and 3 h after galactose induction (top) and without galactose induction (bottom). The Pgk1p antibody was used as an internal control.



UV cross-linking assays. When the ^{32}P -labeled 3'UTR of *ASH1* mRNA was incubated with recombinant Puf6p, we observed a distinct RNA–protein complex in a mobility-shift assay (Fig. 7A, lane 1). This binding was specific because the complex was effectively competed by excess amounts of the unlabeled 3'UTR (Fig. 7A, lanes 5–7). In contrast, incubation of the protein with excess amounts of nonspecific chicken β -actin mRNA 3'UTR (Fig. 7A, lanes 2–4), did not show any competition for complex formation. Therefore, Puf6p was able to specifically bind the 3'UTR of *ASH1* mRNA in vitro, supporting the in vivo colocalization assays of Puf6p with *ASH1* mRNA and the 3'UTR-LacZ reporter RNA (Fig. 2).

The PUF proteins in *Drosophila* and *C. elegans*, Pum and FBF, respectively, repress translation of their target mRNAs through binding to the 3'UTR containing the conserved UUGU segment (Zhang et al. 1997; Sonoda and Wharton 1999). The UUGU sequence in the *HO* 3'UTR is also required for MPT5/Puf5p-mediated repression of *HO* (Tadauchi et al. 2001). To determine whether

Puf6p-binding activity was mediated by the putative UUGU sequences within the 3'UTR of *ASH1* mRNA, we performed UV cross-linking assays using recombinant Puf6p, the ^{32}P -labeled 3'UTRs containing the UUGU sequences or the UUGU mutated to AACA and excess unlabeled RNA (Fig. 7B). We observed that Puf6p formed a complex with the ^{32}P -labeled 3'UTR containing the two UUGU sequences after UV cross-linking (Fig. 7B, lane 1). Puf6p still formed a complex with a mutant 3'UTR, in which one of the two UUGU segments was mutated (Fig. 7B, lane 4). However, the protein–RNA complex was not formed when both UUGU segments were mutated (Fig. 7B, lane 5). The UV cross-linking complexes were further confirmed by competition assays in which the complex was effectively competed in the presence of excess unlabeled RNA corresponding to the element (Fig. 7B, lane 3), whereas excess unlabeled chicken β -actin mRNA 3'UTR did not compete the complex formation (Fig. 7B, lane 2). These results imply that the interaction between Puf6p and the 3'UTR of *ASH1* mRNA is specific and is mediated by both the PUF consensus UUGU sequences.

To evaluate whether the consensus UUGU sequences could regulate repression in vitro, we performed cell-free translation assays in a rabbit reticulocyte lysate system using CAT reporter mRNAs. One reporter contained the wild-type 3'UTR of *ASH1* mRNA, the other contained the mutant 3'UTR, in which both UUGU sequences were mutated to AACA. When the reporters were incubated with increasing amounts of recombinant Puf6p, the wild-type reporter showed a progressive reduction of translation. When the molar ratio of the reporter RNA to Puf6p reached 1:64, the efficiency of translation was reduced to 30% of the level without the presence of Puf6p (Fig. 7C, top). In contrast, changes in translational efficiency were not observed when Puf6p was incubated with the reporter with the mutant 3'UTR (Fig. 7C, bottom). The translational inhibition did not result from selective RNA degradation, as the amount of the reporter RNA in the reactions did not change with increasing Puf6p levels (Fig. 7D). Thus, the UUGU consensus containing 3'UTR of *ASH1* mRNA confers Puf6p-regulated repression of translation.

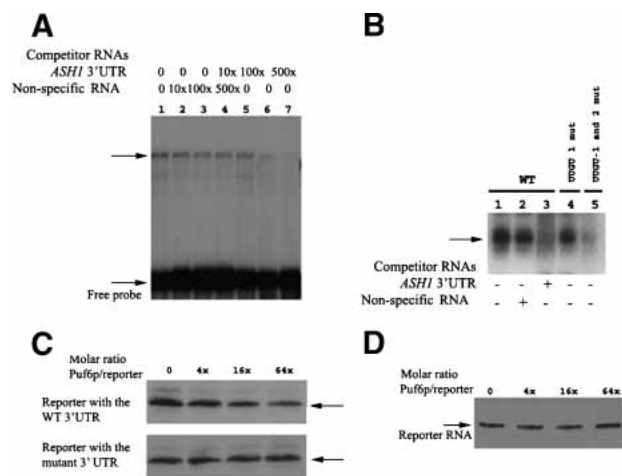


Figure 7. Puf6p binds the 3'UTR of *ASH1* mRNA containing conserved UUGU sequences and represses its translation in vitro. (A) Electrophoresis mobility shift assay using ^{32}P -labeled *ASH1*-3'UTR incubated with recombinant Puf6p in the absence or presence of specific and nonspecific competitor RNAs. The position of the RNA–protein complexes and the RNA free probe are indicated by two arrows. (B) Puf6p-binding activity assayed by UV cross-linking. Recombinant Puf6p was incubated with ^{32}P -labeled *ASH1* partial 3'UTR (130 nucleotides containing two conserved UUGU sequences) in the absence (lane 1) or presence of 500 \times nonspecific (lane 2) or specific (lane 3) competitor RNAs, and with one UUGU mutation (lane 4) or both UUGU mutations (lane 5). The UV cross-linked RNA–protein complex was resolved in 12% SDS-PAGE. The arrow indicates the position of the RNA–protein complex. (C) Autoradiography showing [^{35}S]methionine-labeled CAT reporter protein containing the *ASH1*-3'UTR as in B, translated in the presence of 0, 47times, 16 \times , and 64 \times excess molar ratio of recombinant Puf6p. (Top) CAT reporter mRNA fused with the wild-type 3'UTR. (Bottom) CAT mRNA reporter fused with the UUGU double mutated 3'UTR. (D) Reporter RNA was ^{32}P -labeled. A urea gel showing that the RNA reporter used for in vitro translation did not degrade over the course of the experiment in C.

Discussion

In budding yeast, localized translation of *ASH1* mRNA controls daughter cell-specific segregation of the transcriptional regulator Ash1p, which determines mating-type switching (Jansen et al. 1996; Sil and Herskowitz 1996). *ASH1* mRNA is transported as an mRNP, consisting of *ASH1* mRNA, She2p, She3p, and Myo4p/She1p and other proteins to the distal tip of the bud, where the Ash1 protein is translated and targeted to the daughter-cell nucleus (Bertrand et al. 1998; Bohl et al. 2000; Takizawa and Vale 2000; Irie et al. 2002). In this study, we used the TAP method to identify additional protein components associated with this *ASH1* mRNP. We show that one of the proteins is Puf6p, a novel PUF homology protein of *S. cerevisiae* and that this protein,

as with others in the PUF family, is a repressor of translation.

PUF is a large and evolutionarily widespread protein family that is found in *Drosophila*, *C. elegans*, human and yeast (Zhang et al. 1997; Zamore et al. 1999). PUF proteins usually contain eight conserved PUF repeats, each consisting of 36 amino acids (Zhang et al. 1997). *Drosophila* Pumilio protein (Pum) and *C. elegans* FBF (*fem-3* mRNA-binding factor) bind to the 3'UTRs of hunchback (*hb*) mRNA and *fem-3* mRNA, respectively, and repress their translation. (Wharton and Struhl 1991; Murata and Wharton 1995; Zhang et al. 1997). Yeast *S. cerevisiae* has six PUF family proteins, Jsn1p (Puf1p), Ypr042c (Puf2p), Yll013c (Puf3p), Ygl014w (Puf4p), Mpt5p (Puf5p; Zhang et al. 1997), and Puf6p (this work). The individual PUF proteins associate with functionally related mRNAs, indicating a clustering of function for the PUF proteins (Gerber et al. 2004). Using DNA microarray analysis, Shepard et al. (2003) also isolated 24 mRNAs, including *ASH1* and *IST2*, associated with the locosome components She2p, She1p, and She3p, many of which localize. Interestingly, 13 of these transcripts contain the UUGU sequence in their 3'UTR (Table 1), suggesting that Puf6p could define an additional class of translationally regulated transcripts, those localized by the She proteins. PUF proteins in yeast may, in general, be involved in translational regulation, as has been shown for Mpt5p/Puf5p, which repress *HO* mRNA translation (Tadauchi et al. 2001). Interestingly, Mpt5p/Puf5p and Puf6p, acting on different target mRNAs and functioning as translational repressors, are both involved in yeast sex determination by controlling the expression of *HO* gene expression.

Puf6p regulates the translation of *ASH1* mRNA during the transient localization process by interacting directly with *ASH1* mRNA. The binding and translational repression requires the interaction of Puf6 protein with the consensus UUGU segments, known to be recognized by PUF family proteins (Wang et al. 2002), in the 3'UTR of the *ASH1* mRNA. Deletion of *puf6* or mutation of the UUGU elements of the mRNA increased the intracellular Ash1p concentration and significantly affected asymmetric Ash1p segregation, evidently due to the disruption of the interaction between Puf6p and its target mRNA. This may be because unlocalized *ASH1* mRNA was prematurely translated in the mother cells. In support of this, the *ASH1* mRNA and Ash1p asymmetry could be rescued by reducing the translation rate either by inducing Puf6p from a Gal promoter or by inserting a stem loop at the 5'UTR of *ASH1* mRNA.

The processes of *ASH1* RNA transport and translational regulation need to be well coordinated (Chartrand et al. 2002; Irie et al. 2002). The proper delivery of *ASH1* mRNA to the bud tip requires a transport particle (the "locosome") containing She2p binding to the four zipcodes as well as to She3p, and assembly of the myosin motor, She1p/Myo4 (Bertrand et al. 1998; Bohl et al. 2000; Long et al. 2000; Takizawa and Vale 2000). However, to effect asymmetry, RNA translation must be temporally repressed before it anchors at the bud tip. If

translation begins prematurely, the localization process is abrogated, as translation can interfere with the binding of the She2p and, hence, the transport complex. In support of this is the fact that destabilizing the stem-loops (E1, E2A, E2B, E3) by making mismatches in the stem of all of the elements resulted in a delocalized RNA (Chartrand et al. 2002). This is because the She2p-binding complex requires these secondary structures (Long et al. 2000). It is axiomatic that ribosomes melt secondary structures upon transit, therefore, these stem-loops must be destabilized and the complex formed by She2p disrupted. In contrast to the more severe phenotype caused by the *she* mutations in which *ASH1* mRNA localization is completely disrupted, the mRNA in the *puf6* mutant can still be inefficiently localized to the bud tip by the localization machinery. Because *ASH1* mRNA translocation occurs rapidly (Bertrand et al. 1998; Chartrand et al. 2002), some mRNA may make it to the bud before Ash1p is fully synthesized, even in the absence of Puf6p. As a result, we observed a milder effect on the regulation of the *HO* promoter when compared with a mutation in *she3* (Fig. 4E). This could explain the partial penetrance of *puf6* and why this gene was not identified in the original genetic screens (Jansen et al. 1996).

Although *ASH1* mRNA requires translational inactivation during transport, rapid onset of translation has to occur after *ASH1* mRNA has been localized. Suppression of translation of *ASH1* mRNA, either by mutation of its AUG start codon or insertion of a stop codon directly after the start codon, impairs its tight localization at the bud tip (Gonzalez et al. 1999; Irie et al. 2002). This spatial component of Ash1 protein synthesis could be mediated by the dissociation of Puf6p from the RNA immediately after anchoring at the bud tip. Khd1p has been reported previously (Irie et al. 2002) as a regulator of *ASH1* mRNA translation. Khd1p interacts with the mRNA through binding to its 5' end (the "N" element, equivalent to E1). Possibly, Puf6p and Khd1p coordinate in order to regulate the translation of *ASH1* mRNA.

Unlike the other yeast PUF family proteins, Puf6p is a predominantly nuclear protein that may associate with *ASH1* transcripts in the nucleus, before the mRNA can come in contact with the translational mechanism, and is exported into the cytoplasm with the RNA. This would be consistent with increasing evidence that cytoplasmic mRNA localization is partly determined by a nuclear event. For example, in chicken fibroblasts, both zipcode-binding proteins, ZBP1, and the nuclear splicing factor, ZBP2/KSRP bind to the zipcode of β -actin mRNA and contribute to its localization (Gu et al. 2002). A common feature of these nuclear proteins is to interact directly with the *cis*-elements of the RNAs and shuttle between nucleus and cytoplasm. In *Drosophila* and *C. elegans*, PUF proteins have been shown to work coordinately with additional 3'UTR-binding proteins, such as the NOS proteins to assemble a distinct PUF protein complex (Wreden et al. 1997; Wharton et al. 1998; Kraemer et al. 1999; Sonoda and Wharton 1999; Subramaniam and Seydoux 1999; Wickens et al. 2002). The 3'UTR of *ASH1* mRNA has been shown to associate di-

rectly with both Puf6p (this study) and She2p (Bohl et al. 2000; Long et al. 2000). Puf6p recognizes the UUGU tetranucleotide segment within the same region that binds She2p. She2p is more dependent on the RNA structure (Chartrand et al. 1999, 2002). Although both proteins coprecipitate, whether they directly interact remains to be clarified. The fact that Puf6p can repress translation in vitro suggests that a second yeast protein may not be required.

In summary, a temporally ordered pathway is proposed. The newly transcribed *ASH1* mRNA is likely to be associated with Puf6p and other proteins, such as She2p, in the nucleus to form a protein-RNA complex prior to export (Kruse et al. 2002). Binding of Puf6p to the 3'UTR may repress the translation through interaction with translation initiation factors and prevention of ribosome transit. She2p on the zipcodes of the mRNA initiates She3p/Myo4p assembly on the mRNA (Bohl et al. 2000; Long et al. 2000; Kruse et al. 2002) and stabilizes the secondary structure of the zipcodes (Chartrand et al. 2002). At the bud tip, Puf6p dissociates from the mRNA as a result of an interaction with an unknown spatially segregated protein, and translation takes place. In concert with these other mechanisms, Puf6p is an important factor that integrates *ASH1* mRNA translation with localization.

Materials and methods

Growth medium, yeast strains, and plasmids

Yeast strains used in this study are listed in Table 2. The yeast cells were grown in either synthetic medium lacking the nutrients indicated, or rich medium (Rose et al. 1990). Transformation was performed according to the protocol of Gietz and Schiestl (1995). The C-terminal insertion cassette for tap-tagging of target genes and the disruption cassette for gene deletion were constructed by PCR amplification of the plasmid pBS1479 containing the TRP gene as a selective marker (Rigaut et al. 1999; Puig et al. 2001). Primers for PCR amplification were designed

such that 48 bases at the 5' end of the primers were complementary to those at the corresponding region on the target gene and 19 bases at their 3' end were complementary to the sequences of the pBS1479 outside of the TRP gene. The deletion was verified by colony-PCR analysis to confirm that the insertion and replacement was in the expected locus.

Plasmid construction

Plasmids used in this study were constructed using standard techniques (Table 3). The plasmid YCP111-GFP-MS2 was previously constructed (Bertrand et al. 1998). Plasmid YEP195-Gal-MS2-*ASH1*(f) contains six MS2-binding sites and full-length *ASH1* mRNA. Plasmids YEP195-Gal-MS2-LacZ-E1, YEP195-Gal-MS2-LacZ-E2A, and YEP195-Gal-MS2-LacZ-E2B were derived from plasmid YEP195-Gal-MS2-LacZ-E3 (Bertrand et al. 1998). Plasmid p3348-Puf6 was derived from p3348-*ASH1* (Chartrand et al. 2002), in which *ASH1* cDNA was replaced by *PUF6* cDNA. Plasmid pXR193-mut was derived from the plasmid pXR193 (Chartrand et al. 2002), in which the UUGU elements at the 3'UTR of *ASH1* cDNA were mutated to AACA by PCR-directed mutagenesis. For in vitro translation assays, the plasmid pC3.1-CAT-*ASH1*-3'UTR was generated by inserting a cDNA fusion of CAT coding sequences and the 3'UTR of *ASH1* mRNA to a plasmid pcDNA3.1 (Invitrogen). Plasmid pC3.1-CAT-*ASH1*-3'UTRmut was a mutation of pC3.1-CAT-*ASH1*-3'UTR, in which the UUGU sequences were mutated to AACA, using a PCR-directed mutagenesis method (Sambrook and Russell 2001). To construct pSP64-*ASH1*-3'UTR for in vitro transcription, a PCR product of 3'UTR of *ASH1* mRNA was cloned into plasmid pSP64 (Promega). The plasmid pSP64-*ASH1*-3'UTRmut was generated using the same PCR-directed mutagenesis method, the UUGU sequences in the 3'UTR were mutated to AACA.

Purification of the She2p-tap complex

Five liters of She2p tap-tagged yeast culture were grown in -Trp synthetic medium containing 2% glucose at 30°C to an optical density at 600 nm (OD₆₀₀) of ~1.5. Cells were harvested by centrifugation, resuspended in 30 mL of yeast SPH medium (Aris and Blobel 1991) containing 10 mM DTT (dithiothreitol) and treated with lyticase (Sigma) at a concentration of 1000 U/mL

Table 2. Yeast strains used in this study

Strain	Genotype	Source
K699	Mat α , <i>ura3</i> , <i>leu2-3</i> , <i>112</i> , <i>his3-11</i> , <i>trp1-1</i> , <i>ade2-1</i> , <i>ho can1-100</i>	Jansen et al. 1996
K699- <i>puf6</i>	K699, <i>puf6::TRP</i>	This study
K699-Puf6-tap	K699, <i>PUF6-tap:TRP</i>	This study
K699-Puf6-GFP	K699, <i>PUF6-GFP:HIS</i>	This study
K699,She2-tap	K699, <i>SHE2-tap:TRP</i>	This study
K4452	Mat α , <i>leu2-3</i> , <i>112</i> , <i>ura3</i> , <i>his3</i> , <i>ade2-1</i> , <i>ho can1-100</i>	Jansen et al. 1996
K4452- <i>ash1</i>	K4452, <i>ash1::KAN</i>	Jansen et al. 1996
K4452- <i>ash1</i> , Ash1-myc9	K4452- <i>ash1</i> , <i>ASH1-myc9:LEU</i>	Long et al. 1997
K4452- <i>ash1</i> , Ash1 _{3'mut} -myc9	K4452- <i>ash1</i> , <i>ASH1_{3'mut}-myc9:LEU</i>	This study
K4452- <i>ash1</i> , <i>puf6</i>	K4452- <i>ash1</i> , <i>puf6::TRP</i>	This study
K4535	MAT α , <i>his3 HO-ADE2</i> , <i>HO-CAN1</i> , <i>ura3::GAL1-10-O-lacZ</i>	Jansen et al. 1996
K4535 <i>she3-1440</i>	K4535, <i>she3-1440</i>	Jansen et al. 1996
K4535, <i>puf6</i>	K4535, <i>puf6::TRP</i>	This study
W303	Mat α , <i>ura-1-3</i> , <i>leu2-3</i> , <i>his3-11</i> , <i>trp1-1</i> , <i>ade2-1</i> , <i>ho can1-100</i>	Jansen et al. 1996
W303, she2-myc9	W303, <i>SHE2-myc9:KAN</i>	This study
W303, she2-myc9, Puf6-tap	W303, <i>SHE2-myc9:LEU</i> , <i>PUF6-tap:TRP</i>	This study

Table 3. *Plasmids used in this study*

Plasmid	Features	Source
YCplac111	yeast centromeric plasmid with <i>LEU2</i> selectable marker	Gietz and Sugino 1988
YCplac111-MS2-GFP	MS2-GFP fusion cDNA cloned into YCplac111	Bertrand et al. 1998
YEplac195	yeast multicopy plasmid with <i>URA3</i> selectable marker	Gietz and Sugino 1988
YEplac195-Gal- <i>ASH1</i> (f)	YEplac195 containing 6 MS2 binding sites wild-type <i>ASH1</i> gene under <i>GAL1</i> promoter	This work
YEplac195-Gal-LacZ-MS2-E1	YEplac195 containing LacZ reporter and 6 MS2 binding sites fused with <i>ASH1</i> E1 element under <i>GAL1</i> promoter	This work
YEplac195-Gal-LacZ-MS2-E2A	YEplac195 containing LacZ reporter and 6 MS2 binding sites fused with <i>ASH1</i> E2A element under <i>GAL1</i> promoter	This work
YEplac195-Gal-LacZ-MS2-E2B	YEplac195 containing LacZ reporter and 6 MS2 binding sites fused with <i>ASH1</i> E2B element under <i>GAL1</i> promoter	This work
YEplac195-Gal-LacZ-MS2-E3	YEplac195 containing LacZ reporter and 6 MS2 binding sites fused with <i>ASH1</i> E3 element under <i>GAL1</i> promoter	This work
pFastBacHT	Plasmid for protein expression in insect cells	Life Technology
pPuf6-exp	pFastBacHT expression vector containing entire Puf6p ORF	This work
pCDNA3.1	Vector for in vitro transcription	Invitrogen
pCAT- <i>ASH1</i> -3'W	pCDNA3.1 containing entire CAT ORF fused with <i>ASH1</i> 3'UTR	This work
pCAT- <i>ASH1</i> -3'M	pCDNA3.1 containing entire CAT ORF fused with mutant <i>ASH1</i> 3'UTR	This work
pSP64	Vector for in vitro transcription	Promega
pSP64- <i>ASH1</i> -3'W	pSP64 containing wild type <i>ASH1</i> 3'UTR	This work
pSP64- <i>ASH1</i> -3'M1	pSP64 containing mutant <i>ASH1</i> 3'UTR at UUGU-1	This work
pSP64- <i>ASH1</i> -3'M	pSP64 containing mutant <i>ASH1</i> 3'UTR at UUGU-1 and UUGU-2	This work
pBS 1479	Plasmid for tap-tagging and gene disruption	Rigaut et al. 1999
pXR193	YIplac128 containing wild-type <i>ASH1</i> gene with myc9-tag at the 3' end	Chartrand et al. 2002
pXR252	pXR193 plasmid with a stem-loop in the 5'UTR	Chartrand et al. 2002
pXR193-mut	pXP193 plasmid with the UUGU mutation at the 3'UTR of <i>ASH1</i> gene	This work

for 45 min at 30°C. After washing twice with buffer A (40 mM phosphate at pH 7.8, 120 mM NaCl, 40 mM KCl, 5 mM MgCl, 0.5 mM EDTA), the cells were suspended in 15 mL of Buffer A containing 1500 U RNasin (Promega), 0.1% NP-40, 1.0 mM PMSF (phenylmethylsulfonyl fluoride), 2 mM benzamidine, 2 mM leupeptin, and 1 mM aprotinin. The cells were homogenized in a dounce homogenizer for 15 strokes. The extract was centrifuged at 12,000g for 40 min, and the supernatant was transferred to a new tube. The subsequent steps were performed at 4°C, and the purification protocol was used as described previously (Puig et al. 2001). Briefly, the supernatant was applied to 0.4 mL of IgG Sepharose 6 Fast Flow (Amersham Biosciences) and incubated for 4 h at 4°C with gentle rotation. After three times washing with IPP150 (10 mM Tris-Cl at pH 7.8, 150 mM NaCl, 0.1% Nonidet P-40), the IgG beads with immobilized She2p-tap and associated proteins were incubated with 120 U of TEV protease (Invitrogen) for 2 h at room temperature. The eluate was recovered by gravity flow and was separated into a 10% SDS-polyacrylamide gel. The protein bands, after Coomassie blue staining, were cut from the gel and subjected to MALDI-TOF at Rockefeller University.

In situ hybridization and immunofluorescence

Yeast cells were grown in the appropriate culture medium to early or mid-log phase and were processed for in situ hybridization as described in Long et al. (2000). For in situ hybridization, yeast spheroplasts were hybridized with a pool of Cy3-conju-

gated *ASH1* DNA oligonucleotide probes. Immunofluorescence was performed using a protocol described previously (Chartrand et al. 2002) with modifications. For detecting myc-tagged proteins, the mouse anti-myc antibody (Roche Molecular Biochemical) was used in a 1:200 dilution in 1× PBS and 0.1% BSA. The secondary antibody was a FITC-conjugated donkey anti-mouse antibody (Jackson Laboratories) at a 1:400 dilution of the same buffer. For tap-tagged proteins, the Cy3-conjugated donkey anti-rabbit antibody (Jackson Laboratories) was used at a 1:400 dilution.

Western blots

Extracts were obtained from 2 ODs of yeast culture and processed with glass beads mixed with 40 µL of protein loading buffer, and heated to 95°C for 5 min. After vigorous vortexing, the beads and cell debris were removed by centrifugation at 14,000 rpm for 5 min. Cell extracts were resolved into a 10% SDS-PAGE, followed by electrophoresis onto a Hybond membrane (Amersham). The blots were blocked with 5% nonfat milk in PBS overnight at 4°C and incubated with a mouse anti-myc antibody in 1:1000 dilution for 2 h, followed with a HRP-conjugated donkey anti-mouse antibody in 1:5000 dilution for 1 h. The blots were then detected with an enhanced chemiluminescence detection kit (Amersham-Pharmacia). The Pgi1 protein was used as an internal control with a mouse anti-Pgi1p antibody (Molecular Probes). Western blots were quantified using the GeneTools software (SynGene Bio. Imaging) and the

c-myc signal intensities were normalized to Pgl1p signals (determined as relative signal intensities of c-myc to Pgl1p).

Northern blot analysis

Yeast RNA was prepared using an RNAeasy kit (Qiagen). Northern blot analysis was performed as described previously (Sambrook and Russell 2001). The probes used for hybridization were all gel-purified DNA fragments randomly labeled with ^{32}P using a RadPrime DNA Labeling system (Invitrogen). The probe for yeast *ACT1* mRNA is a PCR-amplified 0.4-kb coding sequence, and the probe for *ASH1* mRNA is 0.5 kb of coding sequence. Northern blots were quantified using an ImageQuant software 5.2 (Amersham Bioscience) and the *ASH1* mRNA signal intensities were normalized to *ACT1* signals (determined as relative signal intensities of *ASH1* to *ACT1*).

Expression and purification of recombinant Puf6p

Puf6p was cloned and expressed in a BAC-TO-BAC Baculovirus Expression Systems (GIBCO) according to the manufacturer's instruction. A cDNA encoding Puf6p was PCR amplified from yeast genomic DNA and cloned into a donor plasmid pFAST-BAC-HTb (His_6 -tag) into the BamHI and XhoI sites. The cloned plasmid, after DNA sequence analysis, was transformed into *Escherichia coli* DH10BAC cells to produce recombinant bacmid DNA containing the *PUF6* gene. The isolated bacmid DNA was then transfected to Sf9 insect cells. The viral stocks that expressed Puf6p-his₆ were collected and cultured for subsequent protein purification. To purify His₆-tagged Puf6p, 50 mL of transfected cells were cultured for 3 d and harvested. The cells were lysed in 4 mL of lysis buffer (50 mM NaH_2PO_4 at pH 8.0, 300 mM NaCl, 1% NP-40, 1 mM PMSF, 5 mM BME) and incubated on ice for 10 min. The supernatant, after centrifuging at 10,000g for 10 min at 4°C, was mixed with 200 μL 50% Ni-NTA slurry (Qiagen) and incubated with gentle shaking for 1–2 h at 4°C. The Ni-NTA beads were washed twice with PBS and once with PBS containing 10 mM imidazole. Bound Puf6p was eluted from the beads by incubation with 40 U of recombinant TEV protease (Invitrogen). The protein was then desalted and concentrated using an ultrafree-4 concentrator (Millipore).

Immunoprecipitation and *ASH1* mRNA detection by RT-PCR

Yeast extracts were prepared as described above (purification of the She2p-tap complex) from exponential growing cell cultures. A total of 200 μL of extracts were incubated with 10 μL of IgG-coated agarose for 2 h at 4°C with gentle shaking. The beads were washed extensively with DEPC-treated PBS, suspended in 100 μL of DEPC water, and boiled for 5 min. Total RNA was extracted from the supernatant using TRIzol RNA isolation reagent (GIBCO-BRL). RT-PCR was performed using Titan One Tube RT-PCR system from Roche Applied Science. One microliter of TRIzol extracted RNA was used as template and all PCR reactions were performed for 25 cycles using the primers specific for *ASH1* mRNA. The expected PCR product was an ~400-bp DNA fragment of *ASH1* mRNA at the position of 1451–1861 (numbers indicate nucleotide positions from *ASH1* ATG start codon).

HO promoter activity assay

Effect of PUF6 on *HO* expression was done as described by Jansen et al. (1996). Briefly, 10-fold serial dilutions of exponentially growing wild-type (K4535), *she3-1440* or *puf6* Δ cells were

spotted on YPD or SD medium contain 0.03% canavanine and incubated for 2 and 5 d at 30°C, respectively.

Gel mobility-shift assay and UV cross-linking

For the gel mobility-shift assay, ^{32}P -labeled RNA probe was generated by SP6 polymerase directed in vitro transcription from pSP64-*ASH1*-3'UTR and pSP64-*ASH1*-3'UTRmut constructs. The RNA transcribed from the construct was purified after resolving in a 6% denaturing gel. RNA-protein gel-shift assays were performed at room temperature as described previously (Gu et al. 2002). Briefly, 10^5 cpm of the ^{32}P -labeled RNA probe was incubated with 100 ng of recombinant Puf6p for 30 min in a 20- μL -binding solution containing 20 mM Hepes (pH 7.4), 50 mM KCl, 3 mM MgCl_2 , 2 mM DTT, and 5% glycerol. The nonspecific RNA-protein interactions were minimized by incubation with 5 mg/mL of heparin for 10 min. The RNA-protein complexes formed were separated by electrophoresis in a 4% native gel and visualized by autoradiography. To establish the specificity of RNA-protein interactions, competition assays were performed by preincubating the Puf6p with unlabeled RNA competitors.

For UV cross-linking, the protein and ^{32}P -labeled RNA were irradiated on ice in a UV Chamber (GS gene linker Bio-Rad) with 254-nm, 8-W UV bulbs at a distance of 6 cm for 5 min. After UV cross-linking, RNase A was added to 0.5 mg/mL and reactions were incubated at room temperature for 20 min. The UV cross-linked RNA-protein complexes was distinguished by autoradiography after electrophoresis in a 10% SDS-polyacrylamide gel.

In vivo analysis of *Ash1p* expression

Yeast strains carrying the Puf6p plasmid, under galactose promoter control, were grown in synthetic medium containing 2% raffinose. When the culture reached to OD_{600} of ~0.5, galactose was added to a final concentration of 3% to induce the expression of Puf6p. Cells were collected at 0, 1, 2, 3, and 4 h. Protein extracts were prepared from the cells as described previously and used for *Ash1p* analysis by Western blots.

In vitro translation assays

Capped mRNA was transcribed from the plasmids, pC3.1-CAT-*ASH1*-3'UTR and pC3.1-CAT-*ASH1*-3'UTRmut, using a T7 Message Machine kit (Ambion). In vitro translation was performed in 25 μL of rabbit reticulocyte lysate (Promega) containing 10 nM of the mRNA templates and [^{35}S]methionine as a tracer to monitor the translated proteins in the presence of increasing amounts of recombinant Puf6p. Translated proteins were detected by autoradiography after separation by a 12% SDS-polyacrylamide gel. Signal intensities were determined by a PhosphorImager screen (Molecular Dynamics) and quantified using an ImageQuant software.

Analysis of *ASH1* mRNA and *Ash1p* localization

The method of quantitative measurement on the localization of *ASH1* mRNA and *Ash1p* has been described previously (Chartrand et al. 2002). By microscopy, yeast cells in late anaphase were scored for localized or delocalized *ASH1* mRNA and *Ash1p* in each experiment. *ASH1* mRNA was considered as localized when it was predominantly in the bud tip (crescent localization). *ASH1* mRNA was considered as delocalized when it was distributed between bud and mother cell, or it was mainly in mother cells. To determine the asymmetric distribution of *Ash1p*, only anaphase cells were counted. For each ex-

periment, ~100 budding yeast cells were scored. The Ash1p was considered as distributed asymmetrically when it was predominantly present in the nucleus of the daughter cell. It was considered as symmetrically distributed when it was present in both mother and daughter cell nucleus. To obtain quantitative data on the localization of *ASH1* mRNA and Ash1p, three independent experiments were performed.

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