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The Khd1 protein, which has three KH RNA-binding motifs, is required for proper localization of *ASH1* mRNA in yeast

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RNA localization is a widespread mechanism for achieving localized protein synthesis. In *Saccharomyces cerevisiae*, Ash1 is a specific repressor of transcription that localizes asymmetrically to the daughter cell nucleus through the localization of *ASH1* mRNA to the distal tip of the daughter cell. This localization depends on the actin cytoskeleton and five She proteins, one of which is a type V myosin motor, Myo4. We show here that a novel RNA-binding protein, Khd1 (KH-domain protein 1), is required for efficient localization of *ASH1* mRNA to the distal tip of the daughter cell. Visualization of *ASH1* mRNA *in vivo* using GFP-tagged RNA demonstrated that Khd1 associates with the N element, a *cis*-acting localization sequence within the *ASH1* mRNA. Co-immunoprecipitation studies also indicated that Khd1 associates with *ASH1* mRNA through the N element. A *khd1*Δ mutation exacerbates the phenotype of a weak *myo4* mutation, whereas overexpression of *KHD1* decreases the concentration of Ash1 protein and restores *HO* expression to *she* mutants. These results suggest that Khd1 may function in the linkage between *ASH1* mRNA localization and its translation.
Keywords: *ASH1*/KH domain/mRNA localization/RNA-binding protein/translational control

Introduction

The asymmetric distribution of proteins is vital to cellular function and cell fate determination. One mechanism for achieving asymmetric distribution of a protein is by localizing its mRNA to a distinct site within the cell. Localization of mRNAs is specified by sequences generally found in the 3′ untranslated region (3′ UTR) of the mRNA, and is mediated by cytoskeletal filaments that are required for transport and subsequent anchoring of the mRNA at its final destination (Wilhelm and Vale, 1993; St Johnston, 1995; Nasmyth and Jansen, 1997; Oleynikov and Singer, 1998). The transport, anchoring and translational regulation of localized transcripts are governed by proteins that form large ribonucleoprotein complexes with the mRNAs (Wilhelm and Vale, 1993; Hazelrigg, 1998).

The asymmetric distribution of Ash1 in the budding yeast *Saccharomyces cerevisiae* provides an excellent opportunity to study the asymmetric segregation of cell fate determinants resulting from mRNA localization. Ash1 is a cell-type specific transcriptional repressor that determines proper mating-type switching by differentially regulating expression of the HO endonuclease (Bobola *et al*., 1996; Sil and Herskowitz, 1996). Ash1 is found in the nucleus of daughter cells, where it represses *HO* transcription and ultimately prevents mating-type switching in these cells (Bobola *et al*., 1996; Sil and Herskowitz, 1996). This transcriptional regulation of *HO* expression restricts mating-type switching to mother cells (Nasmyth, 1983; Herskowitz, 1988). The asymmetric distribution of Ash1 to daughter cell nuclei is a result of the localization of *ASH1* mRNA to the distal tips of daughter cells (Long *et al*., 1997; Takizawa *et al*., 1997).

Five genes have been identified that are required for *ASH1* mRNA localization; *SHE1–SHE5* (Jansen *et al*., 1996; Long *et al*., 1997; Takizawa *et al*., 1997). *SHE1* encodes a type V myosin motor, Myo4, which co-localizes with *ASH1* mRNA at the tip of daughter cells (Haarer *et al*., 1994; Bertrand *et al*., 1998; Munchow *et al*., 1999; Takizawa and Vale, 2000). Using a live-cell assay, particles containing Myo4 and *ASH1* mRNA were observed to move rapidly from mother cells to daughter cells, suggesting that Myo4 plays a direct role in transporting *ASH1* mRNA to the bud tip (Bertrand *et al*., 1998; Beach *et al*., 1999; Takizawa and Vale, 2000). Immunoprecipitation experiments have revealed that Myo4 associates with *ASH1* mRNA and that this association is dependent on *SHE2* and *SHE3* (Munchow *et al*., 1999; Takizawa and Vale, 2000). *SHE2* encodes an RNA-binding protein that directly binds to *ASH1* mRNA (Bohl *et al*., 2000; Long *et al*., 2000). The C-terminus of She3 interacts with She2, while its N-terminus interacts with Myo4 (Bohl *et al*., 2000; Long *et al*., 2000). Thus, She3 has the properties of an adapter that links Myo4 to the She2–*ASH1* mRNA complex. *SHE5* is identical to *BNI1*, which was shown to encode a protein involved in regulating the actin cytoskeleton (Jansen *et al*., 1996; Kohno *et al*., 1996; Evangelista *et al*., 1997). She4 is also hypothesized to be required for proper organization of the actin cytoskeleton (Jansen *et al*., 1996; Wendland *et al*., 1996). Taken together, these results suggest that *ASH1* mRNA is localized to the bud tip by actomyosin-based transport. Loc1, a nuclear RNA-binding protein, is also involved in *ASH1* mRNA localization (Long *et al*., 2001).

Based on these studies, the following model for *ASH1* mRNA localization has been proposed (Bohl *et al*., 2000; Long *et al*., 2000, 2001; Takizawa and Vale, 2000; Kwon and Schnapp, 2001). First, the *ASH1* mRNA is identified

by Loc1 in the nucleus. Secondly, the *ASH1* mRNA is transported through the nuclear pores to the cytoplasm, where it binds to the cytoplasmic RNA-binding protein She2. Thirdly, the She2–*ASH1* mRNA complex associates with Myo4 via the She3 adapter protein. Finally, the *ASH1* mRNA–She2–She3–Myo4 complex is transported to the distal tips of daughter cells along polarized actin filaments.

In cases where protein localization is determined by mRNA localization, it can be expected that translation of the mRNA would be blocked until its proper localization at the distant site. Thus, mRNA localization is likely to be tightly coupled to its translational control (Curtis *et al*., 1995; St Johnston, 1995; Preiss and Hentze, 1999). Indeed, several examples are known in which translational control is directly linked to protein localization. For example, in *Drosophila*, translation of maternal *oskar* mRNA is silenced during transport to the posterior pole of the oocyte and later activated when Oskar protein is required (Macdonald and Smibert, 1996). It has been shown that the protein Bruno binds to the 3′ UTR of *oskar* mRNA and prevents premature translation (Kim-Ha *et al*., 1995; Gunkel *et al*., 1998). It is therefore likely that additional components, such as RNA-binding proteins, contribute to efficient localization of *ASH1* mRNA through regulation of its translation.

During our studies on the identification and characterization of RNA-binding proteins required for *ASH1* mRNA localization, we identified a previously uncharacterized yeast protein, Khd1. In this study we show that Khd1 is required for the tight anchoring of *ASH1* mRNA to the distal tip of the daughter cell. Khd1 both co-localizes and physically associates with *ASH1* mRNA. Overexpression of Khd1 causes decreased Ash1 protein concentrations. These results suggest that Khd1 functions in the linkage between *ASH1* mRNA localization and its translation.

Results

A putative RNA-binding protein involved in proper localization of ASH1 mRNA

To identify proteins required for *ASH1* mRNA localization, we carried out a systematic survey of the different candidate RNA-binding proteins and their effects on *ASH1* mRNA localization. The yeast genome contains five genes, *PUF1/JSN1*, *PUF2*, *PUF3*, *PUF4/YGL014w* and *PUF5/MPT5*, that code for homologs of the Puf family of RNA-binding proteins (Zhang *et al*., 1997; Olivas and Parker, 2000; Tadauchi *et al*., 2001) and five genes, *MER1*, *MSL5*, *PBP2*, *SCP160* and *YBL032w*, which code for proteins that contain the KH RNA-binding motif (Engebrecht and Roeder, 1990; Van Dyck *et al*., 1994; Abovich and Rosbash, 1997; Weber *et al*., 1997; Mangus *et al*., 1998). We constructed mutants of each of these nine genes by PCR-mediated gene disruption, except for *MSL5*, which is an essential gene (Abovich and Rosbash, 1997) (see Materials and methods). Disruptants of each of the nine genes were viable, although *mpt5*Δ and *scp160*Δ mutants exhibited temperature-sensitive growth at 37°C. We examined *ASH1* mRNA localization in these deletions by *in situ* hybridization (Table I). *ASH1* mRNA was partially delocalized in *mpt5*Δ, *scp160*Δ and *ybl032w*Δ mutants, whereas *ASH1* mRNA was properly localized in the other six mutants. As *YBL032w* had not been characterized previously, we designated it *KHD1* (KH-domain protein 1).

Table I. *ASH1* mRNA localization in disruptants of genes encoding RNA-binding proteins

Genotype	% (n = 100)			
	Anchored	Delocalized in the bud	Delocalized in mother and bud	Neck
Wild type	87	12	1	0
*puf1*Δ*/jsn1*Δ	85	14	1	0
*puf2*Δ	69	29	2	0
*puf3*Δ	85	15	0	0
*puf4*Δ	83	16	1	0
*puf5*Δ*/mpt5*Δ	22	61	16	1
*scp160*Δ	23	61	16	1
*pbp2*Δ	79	20	1	0
*khd1*Δ*/ybl032w*Δ	53	40	7	0
*mer1*Δ	78	20	2	0

Anchored: tightly localized *ASH1* mRNA at the distal tip; delocalized in the bud: delocalized *ASH1* mRNA confined to the bud; delocalized in mother and bud: *ASH1* mRNA in both mother cell and bud; neck: *ASH1* mRNA at the bud neck.

To assess whether Khd1, Scp160 and/or Mpt5 play a direct role in *ASH1* mRNA localization, we analyzed whether these proteins co-localized with *ASH1* mRNA using a system in which U1A-tagged *ASH1* mRNA is marked with green fluorescent protein (GFP) (Takizawa and Vale, 2000). In this experimental system, cells are transformed with two plasmids, U1Ap-GFP and U1Atag-ASH1. U1Ap-GFP expresses a fusion protein of the RNA-binding domain of U1A and a variant of GFP (S65T) in which Ser65 is changed to threonine. U1Atag-ASH1 expresses *ASH1* mRNA containing the U1A-binding sequence downstream of the start codon under the control of the *GAL1* promoter. Cells expressing U1Ap-GFP and U1Atag-ASH1 display a single large GFP particle localized to the distal tips of daughter cells, and Myo4, She2 and She3 co-localize with this particle (Takizawa and Vale, 2000). We constructed strains harboring myc-tagged versions of Khd1, Scp160 and Mpt5 as described in Materials and methods. These tagged proteins carry 13 repeats of the c-myc peptide at the C-terminus of each protein. These strains displayed normal localization of *ASH1* mRNA, indicating that the addition of the myc-tag to the proteins did not impair their function (data not shown). Khd1myc, Scp160myc and Mpt5myc strains were transformed with U1Ap-GFP and U1Atag-ASH1 plasmids and tested for co-localization of the myc-tagged proteins with the GFP signal. Khd1myc co-localized with the GFP-tagged U1Atag-ASH1 RNA particle, whereas Scp160myc and Mpt5myc did not (Figure 1). These results suggest that Khd1 has a direct role in *ASH1* mRNA localization. Below, we further characterize the role of Khd1 in the regulation of *ASH1* mRNA localization.

The N element of ASH1 mRNA is responsible for co-localization with Khd1

ASH1 mRNA contains three or four *cis*-acting localization elements: N, C and U (Gonzalez *et al*., 1999), or E1, E2AB and E3 (Chartrand *et al*., 1999) (Figure 2). Each of these

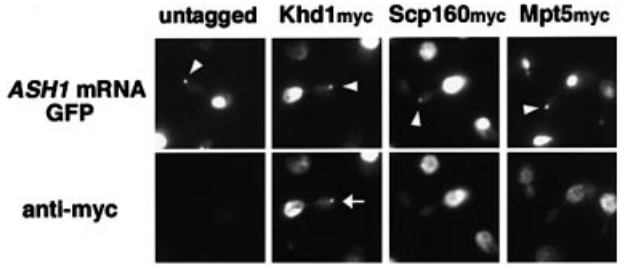

Fig. 1. Khd1 co-localizes with the U1Atag-ASH1 RNA particle. Thirteen repeats of the c-myc peptide sequence were inserted at the C-terminus of Khd1, Scp160 and Mpt5 in wild-type cells (10B). All samples expressed both U1Ap-GFP (pPT220) and U1Atag-ASH1 (pPT120). In untagged cells, GFP fluorescence from the U1A-ASH1 RNA particle is visible at the distal tip of the bud (arrowhead), but no staining was detected with the anti-myc antibody. In YKEN203 (Khd1myc) cells, GFP fluorescence from the U1A-ASH1 RNA particle co-localizes with anti-myc immunofluorescence (arrow). In YKEN202 (Scp160myc) and YKEN201 (Mpt5myc) cells, the U1A-ASH1 RNA GFP particle is visible at the distal tip of the bud (arrowhead) but does not co-localize with anti-myc fluorescence.

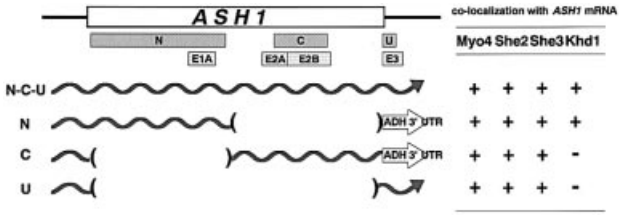

Fig. 2. Localization elements involved in *ASH1* mRNA localization and co-localization with Myo4, She2, She3 and Khd1. *ASH1* mRNA contains three or four localization elements [N, C and U in Gonzalez *et al*. (1999); E1, E2AB and E3 in Chartrand *et al*. (1999)]. U1Atag-N, U1Atag-C and U1Atag-U are U1A-tagged versions of each element. Right column indicates co-localization of Myo4myc, She2myc, She3myc and Khd1myc to GFP particles of U1A-tagged versions of U1Atag-Full, U1Atag-N, U1Atag-C and U1Atag-U. +, co-localization; –, no co-localization.

elements is sufficient for localization of a heterologous reporter mRNA to daughter cells. Two regions (N, C; E1, E2AB) are located in the *ASH1* open reading frame (ORF), whereas the U and E3 regions are located in the 3′ UTR. To determine which regions are responsible for the co-localization of *ASH1* mRNA with Khd1, we constructed U1A-tagged versions of each element, U1Atag-N, -C and -U, in addition to U1Atag-Full, which contains all of these elements (Figure 2). Each of these constructs produced a bright particle in buds when co-expressed with U1Ap-GFP, indicating that each of the three RNA elements is sufficient to form a particle and localize to buds in the U1Atag constructs that we used (Figure 3). We then tested co-localization of Khd1myc, Myo4myc, She2myc and She3myc to each element (Figures 2 and 3). We found that Myo4myc, She2myc and She3myc co-localized with the GFP signals from all three derivatives of the U1Atag-ASH1 RNA particle (Figures 2 and 3). In contrast, Khd1myc co-localized with U1Atag-N but not with U1Atag-C or U1Atag-U (Figures 2 and 3). These results suggest that Khd1 may have a role different from that of Myo4, She2 and She3, which function in *ASH1* mRNA transport (Bertrand *et al*., 1998; Munchow *et al*., 1999; Bohl *et al*., 2000; Long *et al*., 2000; Takizawa and Vale, 2000).

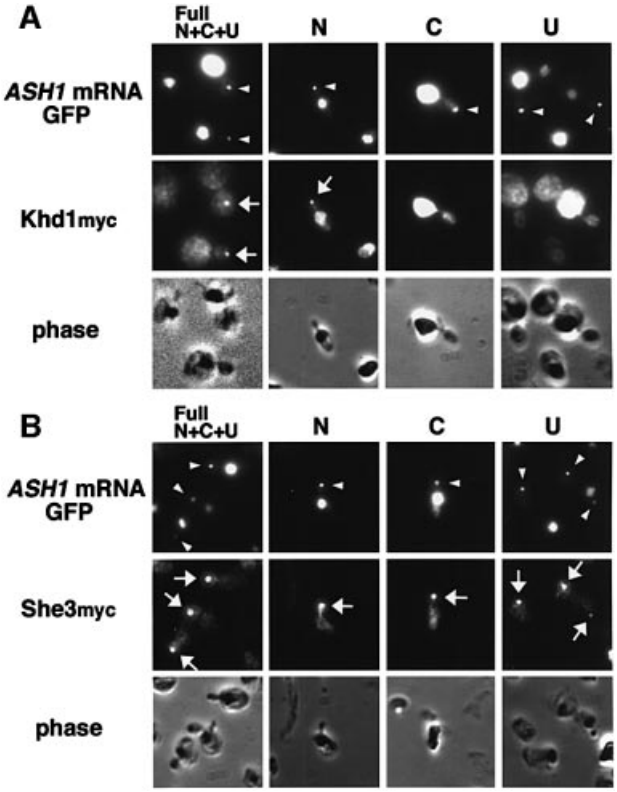

Fig. 3. The N element of *ASH1* mRNA is responsible for co-localization of Khd1. (**A**) Khd1myc co-localizes with U1Atag-N but not with U1Atag-C or U1Atag-U. (**B**) She3myc co-localizes with U1Atag-N, U1Atag-C and U1Atag-U. Arrowhead, GFP fluorescence from the U1A-ASH1 RNA particle visible at the distal tip of the bud; arrow, anti-myc immunofluorescence that co-localizes with the GFP fluorescence from the U1A-ASH1 RNA particle. Strains used: YKEN203 (Khd1myc), 134 (She3myc).

Khd1 associates with ASH1 mRNA in vivo

Co-localization of *ASH1* mRNA and Khd1 suggested that Khd1 is associated with *ASH1* mRNA *in vivo*. To test this possibility, we investigated whether *ASH1* mRNA co-immunoprecipitated with Khd1myc using immunoprecipitation and RT–PCR. We used myc-tagged She3 as a positive control, as Munchow *et al*. (1999) have shown that *ASH1* mRNA co-immunoprecipitates with She3myc. Khd1myc and She3myc strains were transformed with a control plasmid and YEpASH1. Cell lysates were prepared from these strains and used for immunoprecipitation with anti-myc monoclonal antibody. The anti-myc antibody efficiently precipitated Khd1myc and She3myc proteins from yeast extracts (Figure 4A). By RT–PCR analysis of the immunoprecipitates, we detected endogenous *ASH1* mRNA in immunoprecipitates from Khd1myc and She3myc strains. In contrast, we did not detect *ASH1* mRNA in immunoprecipitates from the untagged strain, even when *ASH1* was overexpressed (Figure 4A). The PCR product was not seen when reverse transcriptase was omitted, indicating that formation of this band is dependent on RNA (data not shown). These data indicate that Khd1 associates with *ASH1* mRNA *in vivo*.

To examine whether the association of Khd1myc with *ASH1* mRNA is mediated by the N element, Khd1myc proteins were immunoprecipitated from the Khd1myc strain co-expressing U1Atag-N or U1Atag-U, and U1Ap-

GFP. By RT–PCR analysis of the immunoprecipitates with the anti-myc antibody, the U1Atag-N mRNA was detected in the immunoprecipitates (Figure 4B, lane 2). U1Ap-GFP also co-immunoprecipitated with Khd1myc, suggesting that Khd1myc makes a complex with U1Ap-GFP through the U1Atag-N mRNA. In contrast, the U1Atag-U mRNA and U1Ap-GFP did not co-immunoprecipitate with Khd1myc in the Khd1myc strains co-expressing U1Atag-U and U1Ap-GFP (Figure 4B, lane 4). As a control, when She3myc was co-expressed with U1Atag-U and U1Ap-GFP, the U1Atag-U mRNA and U1Ap-GFP were detected in the She3myc immunoprecipitates (Figure 4B, lane 6). These results support the possibility that the association of Khd1myc with *ASH1* mRNA is mediated by the N element.

Genetic interaction between the KHD1 and SHE genes

Asymmetric expression of *HO* is ultimately determined by the localization of *ASH1* mRNA (Bobola *et al*., 1996; Sil and Herskowitz, 1996; Long *et al*., 1997; Takizawa *et al*., 1997). Delocalization of *ASH1* mRNA in *she* mutants causes a reduction in *HO* expression (Jansen *et al*., 1996; Long *et al*., 1997; Takizawa *et al*., 1997). Since the *khd1*Δ mutation partially affected *ASH1* mRNA localization (Table I), we examined the effect of *khd1*Δ on *HO* expression using an *HOp-ADE2* reporter gene to monitor expression of *HO*. *HOp-ADE2* was constructed by replacing the *ho* ORF with the *ADE2* ORF at the *ho* locus. Expression of the reporter can thus be assayed in an *ade2*Δ background by growth on medium lacking adenine (SC-Ade). *myo4*Δ and *she3*Δ mutants containing the *HOp-ADE2* reporter failed to grow on SC-Ade plates (Figure 5B), demonstrating that inactivation of *MYO4* or *SHE3* leads to delocalization of *ASH1* mRNA, resulting in repression of the *HOp-ADE2* reporter. In contrast, the *khd1*Δ mutation had little effect on *HO* expression (Figure 5A). The frequency of mating-type switching in the *khd1*Δ mutant was the same as that in the wild-type strain (data not shown). We then examined whether the *khd1*Δ mutation affected the phenotype associated with a weak *myo4-910* mutation, which by itself had little effect on *HO* expression. The *khd1*Δ *myo4-910* double mutant showed greatly reduced growth on the SC-Ade plate, indicating a reduced level of *HO* expression in these cells (Figure 5A). This reduced growth of the *khd1*Δ *myo4-910* double mutant on the SC-Ade plate was dependent on the *ASH1* gene, because disruption of the *ASH1* gene suppressed the growth defect (data not shown). Thus, the *khd1*Δ deletion enhanced the effect of the *myo4* mutation on *HO* expression, indicating that the *KHD1* gene genetically interacts with *MYO4*.

To analyze further the genetic interactions between *KHD1* and *SHE* genes, we examined the effect of *KHD1* overexpression on *HO* expression in *myo4*Δ and *she3*Δ mutants. Overexpression of *KHD1* from the *GAL1* promoter prevented the reduction in *HO* expression in *myo4*Δ and *she3*Δ mutants (Figure 5B). These results suggest a possible genetic interaction between the *KHD1* and *SHE* genes, and imply that Khd1 affects *ASH1* mRNA localization at a step different from that of the She proteins.

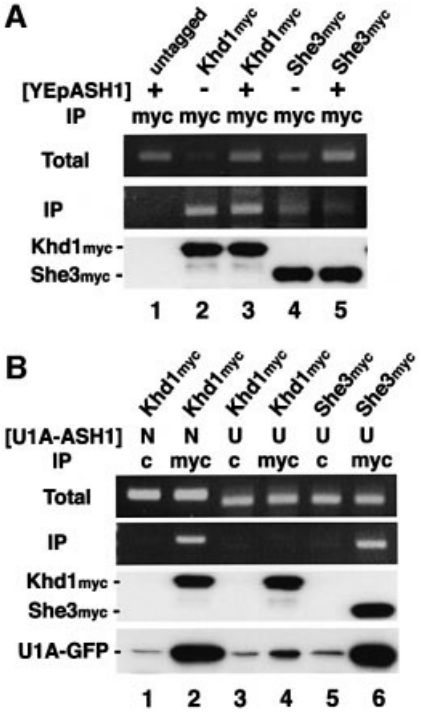

Fig. 4. Khd1 associates with *ASH1* mRNA. Khd1 and She3 tagged with the myc epitope were immunoprecipitated using anti-myc antibody 9E10 (myc) or control IgG (c) as described in Materials and methods. Each immunopellet was separated on a 10% SDS–PAGE gel, blotted and probed with anti-myc antibody or anti-GFP antibody for the presence of epitope-tagged proteins (Khd1myc, She3myc or U1Ap-GFP). RNA was extracted from cell extracts (Total) and immunoprecipitates (IP) and used as template for RT–PCR. (**A**) A 360 bp product was amplified using *ASH1*-specific primers. (**B**) PCR products of 420 and 380 bp were amplified using specific primers for U1Atag-N (N) and U1Atag-U (U), respectively. One-fifth of each reaction was separated on a 2% agarose gel and stained with ethidium bromide. (A) Lane 1, untagged (YEpASH1); lane 2, Khd1myc (YEplac181); lane 3, Khd1myc (YEpASH1); lane 4, She3myc (YEplac181); lane 5, She3myc (YEpASH1). (B) Lanes 1 and 2, Khd1myc (U1Atag-N + U1Ap-GFP); lanes 3 and 4, Khd1myc (U1Atag-U + U1Ap-GFP); lanes 5 and 6, She3myc (U1Atag-U + U1Ap-GFP). Total amounts of U1Ap-GFP were the same in each cell extract (data not shown). Strains used: 10B (untagged), YKEN203 (Khd1myc), 134 (She3myc).

Overexpression of KHD1 inhibits translation of ASH1 mRNA

How does *KHD1* overexpression suppress the effect of *she* mutations on *HO* expression? Since *ASH1* negatively regulates the *HOp-ADE2* reporter, disruption of the *ASH1* gene can suppress a defect in *HO* expression in *she* mutants (Figure 5B). This observation raises the possibility that overexpression of *KHD1* suppresses the decreased expression of *HO* observed in *she* mutations by decreasing Ash1 protein concentrations. To test this possibility, we measured the amounts of myc-tagged Ash1 protein after induction of *KHD1* expression from the *GAL1* promoter. Western blotting analysis revealed that *KHD1* overexpression reduced the concentration of Ash1myc 3.6-fold (Figure 6A). This reduction did not result from toxicity induced by *KHD1* overexpression, as the concentration of the unrelated Tub1 protein was not changed (Figure 6A). Overexpression of *KHD1* did not affect the concentration of *ASH1* mRNA (Figure 6B).

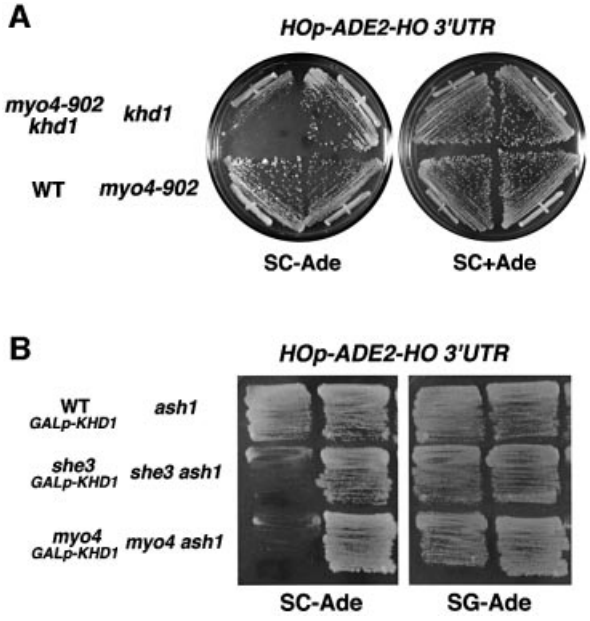

Fig. 5. Genetic interactions between *KHD1* and *SHE*. (**A**) Yeast strains YKEN251 (WT *HOp-ADE2-HO 3′ UTR*), YKEN252 (*myo4-910 HOp-ADE2-HO 3′ UTR*), YKEN254 (*myo4-910 khd1*Δ *HOp-ADE2-HO 3′ UTR*) and YKEN253 (*khd1*Δ *HOp-ADE2-HO 3′ UTR*) were streaked on SC-Ade or SC plates and incubated for 3 days at 30°C. (**B**) Yeast strains YKEN301 (WT *GAL1p-KHD1 HOp-ADE2-HO 3′ UTR*), YKEN302 (*she3*Δ *GAL1p-KHD1 HOp-ADE2-HO 3′ UTR*), YKEN303 (*myo4*Δ *GAL1p-KHD1 HOp-ADE2-HO 3′ UTR*), YKEN304 (*ash1*Δ *HOp-ADE2-HO 3′ UTR*), YKEN305 (*she3*Δ *ash1*Δ *HOp-ADE2-HO 3′ UTR*) and YKEN306 (*myo4*Δ *ash1*Δ *HOp-ADE2-HO 3′ UTR*) were streaked on SC-Ade or SG-Ade plates and incubated for 3 days at 30°C.

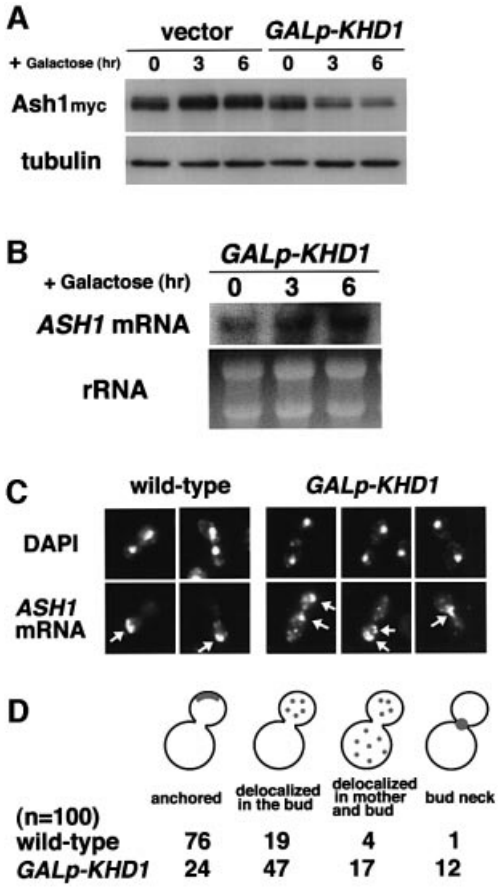

Fig. 6. Overexpression of *KHD1* inhibits translation of *ASH1* mRNA. (**A**) Effect of *KHD1* overexpression on Ash1myc protein concentration. Yeast cells were cultured in 2% raffinose medium at 30°C and treated with galactose (2%) to induce *KHD1* expression from *GAL1p-KHD1*. At the times indicated, cells were harvested and western blot analysis was performed to assay the concentration of Ash1myc protein. The concentration of tubulin protein was measured as a quantity control. Strain used: K5552 (Ash1myc) transformed with pK736 (*GAL1p-KHD1*). (**B**) Effect of *KHD1* overexpression on *ASH1* mRNA concentration. *ASH1* transcripts were quantitated by northern blotting as described in Materials and methods. rRNA was included as a quantity control. (**C**) Effect of *KHD1* overexpression on *ASH1* mRNA localization. Yeast cells were cultured in 2% raffinose medium at 30°C and treated with galactose (2%) for 3 h to induce *KHD1* expression from *GAL1p-KHD1*. *ASH1* mRNA was stained by digoxigenin-labeled *ASH1* antisense probe (*ASH1* mRNA; arrow), and DNA was stained by 4,6-diamino-2-phenylindole (DAPI). Strains used: K5552 (*ASH1myc*; wild type), YKEN307 (*ASH1myc*; *GAL1p-KHD1*). (**D**) The percentages of cells showing different patterns of *ASH1* mRNA localization. Localization was determined by RNA *in situ* hybridization and classified as follows: anchored: tightly localized *ASH1* mRNA at the distal tip; delocalized in the bud: delocalized *ASH1* mRNA confined to the bud; delocalized in mother and bud: *ASH1* mRNA in both mother cell and bud; neck: *ASH1* mRNA at the bud neck.

These results suggest that *KHD1* may be involved in translational control of *ASH1* mRNA.

We next examined the effect of *KHD1* overexpression on *ASH1* mRNA localization. *ASH1* mRNA was found to be delocalized in the strain overexpressing *KHD1* (Figure 6C and D). In the wild-type strain, 76% of *ASH1* mRNA was localized at the distal cortex of the bud. When *KHD1* was overexpressed, *ASH1* mRNA was localized diffusely within the bud (47%), or mother and bud (17%). These results suggest that the inhibition of *ASH1* mRNA translation by *KHD1* overexpression might result in a decrease in anchored *ASH1* mRNA.

Translation of ASH1 mRNA affects its proper localization

The observation that *KHD1* may regulate the localization of *ASH1* mRNA via regulation of *ASH1* translation raised the possibility that *ASH1* mRNA translation could in turn affect *ASH1* mRNA localization. *ASH1* mRNA is thought to be translated at the distal tips of daughter buds, with Ash1 protein then transported to the proximal, daughter nuclei. To address whether translation of *ASH1* mRNA affects its own localization, we compared localization of wild-type *ASH1* mRNA with that of an *ASH1* mRNA lacking its initiator ATG codon. Both versions of the *ASH1* transcript were placed under the control of the *GAL1* promoter to create the constructs *GAL1p-ASH1* and *GAL1p-ASH1*atg⁻. Western blot analysis confirmed that the mRNA derived from *GAL1p-ASH1*atg⁻ failed to produce Ash1 protein. RT–PCR analysis showed that this transcript was present at the same concentration as wild-type *ASH1* mRNA (Figure 7A). However, in comparison to wild-type *ASH1* mRNA, *ASH1*atg⁻ mRNA was found to be somewhat delocalized in the bud (Figure 7B and C). Whereas 60% of wild-type *ASH1* mRNA localized at the distal cortex of the bud, 74% of *ASH1*atg⁻ mRNA localized diffusely within the bud. These results suggest that translation of *ASH1* mRNA has a role in anchoring *ASH1* mRNA at the distal cortex of daughter cells.

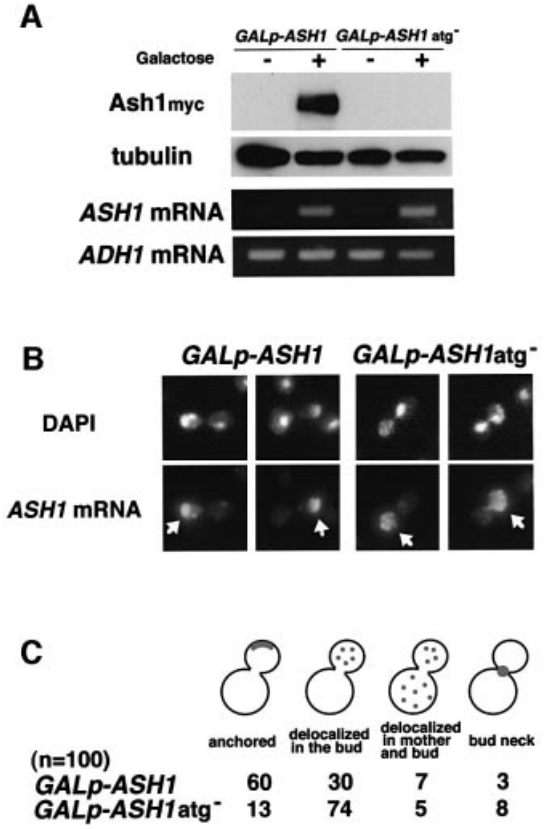

Fig. 7. Translation of *ASH1* mRNA is required for proper localization of *ASH1* mRNA. (**A**) Expression of Ash1myc protein and *ASH1* mRNA. Yeast cells were cultured in 2% raffinose medium at 30°C and treated with galactose (2%) to induce *ASH1* expression from *GAL1p-ASH1myc* and *GAL1p-ASH1*atg⁻*myc*. At the times indicated, cells were harvested and western blot analysis was performed to assay the concentration of Ash1myc protein. Tubulin protein was included as a quantity control. RNAs were also extracted from cell extracts and used as templates for RT–PCR. A 360 bp product was amplified using *ASH1*-specific primers. One-fifteenth of each reaction was separated on a 2% agarose gel and stained with ethidium bromide. *ADH1* mRNA was included as a quantity control. (**B**) Comparison of *ASH1* mRNA localization in TTC356 (*GAL1p-ASH1myc*; wild-type) and in TTC360 (*GAL1p-ASH1*atg⁻*myc*). Wild-type *ASH1* mRNA was localized at the distal cortex of the bud; *ASH1*atg⁻ mRNA was localized diffusely within the bud. *ASH1* mRNA was stained by digoxigenin-labeled *ASH1* antisense probe (*ASH1* mRNA; arrow), and DNA was stained by DAPI. (**C**) The percentages of cells showing different patterns of *ASH1* mRNA localization. Localization was determined by RNA *in situ* hybridization and classified as in Figure 6.

Discussion

Translation of ASH1 mRNA is required for proper localization of ASH1 mRNA

ASH1 mRNA is transported as part of a complex consisting of *ASH1* mRNA–She2–She3–Myo4 to the distal tips of daughter cells using polarized actin filaments (Bohl *et al*., 2000; Long *et al*., 2000, 2001; Takizawa and Vale, 2000; Kwon and Schnapp, 2001). *ASH1* mRNA is thought to be translated at the distal tips of daughter buds and then transported to the proximal daughter nuclei. It remains to be determined how *ASH1* mRNA is anchored at the bud tip and how translation of *ASH1* mRNA is regulated. In this study, we found that the tight anchoring of *ASH1* mRNA to the distal tip of the daughter cell requires translation of *ASH1* mRNA. Compared with wild-type *ASH1* mRNA, *ASH1*atg⁻ mRNA, which lacks the initiator ATG codon, was somewhat delocalized in the bud (Figure 7B and C). Overexpression of *KHD1* inhibits translation of *ASH1* mRNA and impairs the proper localization of *ASH1* mRNA (Figure 6C and D). Gonzalez *et al*. (1999) also demonstrated that the tight anchoring of *ASH1* mRNA to the distal cortex depends on the translation of *ASH1* mRNA, especially of the C-terminal sequences. They suggested that Ash1 protein might have a role in the tight anchoring of *ASH1* mRNA. Another possibility is that the translational machinery, including ribosomes co-localized with *ASH1* mRNA at the distal tip, might be involved in the tight anchoring of *ASH1* mRNA.

A novel RNA-binding protein, Khd1, is required for efficient localization of ASH1 mRNA

To identify RNA-binding proteins involved in translation, anchoring or transport of *ASH1* mRNA, we carried out a systematic survey of the different candidate RNA-binding proteins and their effect on *ASH1* mRNA localization (Table I). *ASH1* mRNA was found to be partially delocalized in *mpt5*, *scp160* and *khd1* deletion mutants. Khd1 co-localized with the GFP signal from the U1Atag-ASH1 RNA particle, whereas Scp160 and Mpt5 did not (Figure 1). In addition, co-immunoprecipitation studies indicated that Khd1 is associated with *ASH1* mRNA through the N element (Figure 4). These results suggest that Khd1 has a direct role in some aspect of *ASH1* mRNA localization.

ASH1 mRNA contains three or four localization elements, each of which is sufficient for localization of a reporter mRNA to daughter cells (Figure 2; Chartrand *et al*., 1999; Gonzalez *et al*., 1999). However, each element is not sufficient for the tight anchoring of *ASH1* mRNA at the distal tip (Gonzalez *et al*., 1999). When we tested each element independently (Figures 2 and 3), we found that Myo4, She2 and She3 co-localized with the GFP signals from all three elements of the *ASH1* RNA particle, whereas Khd1 co-localized with the N element (U1Atag-N) but not the C or U elements (U1Atag-C or U1Atag-U). In the *khd1*Δ mutant, Myo4 still co-localized with the GFP signals from U1Atag-N in the bud (data not shown), suggesting that *KHD1* is not required for localization of the N element in the bud and its co-localization with Myo4. Furthermore, localization of *ASH1* mRNA in the *khd1*Δ *myo4-910* double mutant was similar to that in the *myo4-910* single mutant (data not shown), suggesting that the *khd1*Δ mutation does not exacerbate partial defect in *ASH1* mRNA localization of a weak *myo4-910* mutation. Taken together, these results suggest that Khd1 might have a different role from Myo4, She2 and She3, which are thought to function in *ASH1* mRNA transport.

Khd1 contains three KH RNA-binding motifs. The KH domain was first identified in the human heterogeneous nuclear ribonucleoprotein K (hnRNP K) (Siomi *et al*., 1993; Krecic and Swanson, 1999). Overexpression of *KHD1* resulted in a decrease in Ash1p expression, suggesting that *KHD1* may be involved in the translational control of *ASH1* mRNA via interaction with its N element. This proposed site of Khd1 action contrasts with the fact that hnRNP-K1 governs translation by binding to the 3′ UTR of their target mRNAs (Ostareck *et al*., 1997; Shyu

and Wilkinson, 2000). Interestingly, chicken zipcode-binding protein (ZBP-1) and its *Xenopus* ortholog, Vera/Vg RBP, which have four KH domains in addition to one RNA recognition motif (RRM), are required for mRNA localizations of β-actin and Vg1 mRNAs (Ross *et al*., 1997; Deshler *et al*., 1998; Havin *et al*., 1998). Our finding that Khd1 is also involved in mRNA localization and its translation in yeast may provide a means to address functions of KH domain proteins in mRNA localization and their possible relationship to translation.

Khd1 may function in the linkage between ASH1 mRNA localization and its translation

We imagine that Khd1 inhibits the translation of *ASH1* mRNA during the time it is being transported. As Khd1 seems to localize around the nuclear membrane, possibly on the endoplasmic reticulum, Khd1 may associate with *ASH1* mRNA soon after its export from the nucleus, and this Khd1–*ASH1* mRNA complex is then transported by the She machinery. Overexpression of *KHD1* from the *GAL1* promoter resulted in decreased concentrations of Ash1p, perhaps due to increased inhibition in *ASH1* mRNA translation. Interestingly, strong overexpression of *KHD1* by the *TDH3* promoter is toxic to cell growth (data not shown), whereas deletion of *ASH1* is not. This suggests that Khd1 may be involved in translating other mRNAs in addition to *ASH1*. A recent report has identified other mRNAs, in addition to *ASH1*, that are transported by the She machinery (Takizawa *et al*., 2000). It would be interesting to test whether Khd1 also acts on the translation and/or localization of these mRNAs.

Inactivation of Khd1 in wild-type strains causes only partial delocalization of *ASH1* mRNA and has little effect on *HO* expression. This is in contrast to the much more severe phenotypes caused by the *she* mutations (Table I). Similarly, the frequency of mating-type switching is not affected in a *khd1*Δ strain, whereas it is greatly reduced in *she* mutants (Jansen *et al*., 1996). A requirement for Khd1 can be seen, however, in a strain containing a weak Myo4 mutation: combining a *khd1*Δ mutation with the weak *myo4-910* allele was found to cause reduced *HO* expression (Figure 5A). Thus, it appears that when the She machinery is intact and *ASH1* mRNA is rapidly transported (Bertrand *et al*., 1998), Khd1 function is not crucial for asymmetric localization of the *ASH1* mRNA. However, when there is some other defect in *ASH1* mRNA transport, the role of Khd1 in *HO* expression is manifested.

The weak phenotype of the *khd1*Δ mutant may also suggest the existence of a protein that functions redundantly with Khd1. The *PBP2* gene encodes a KH protein that has the greatest structural similarity to Khd1. We inactivated *PBP2* and examined the phenotype in a *khd1*Δ background. However, we found that the *pbp2*Δ mutation had no additive effect on *ASH1* mRNA localization or *HO* expression (data not shown). Furthermore, Pbp2 did not co-localize with the GFP signal from the U1Atag-ASH1 RNA particle (data not shown). These results indicate that Pbp2 is not redundant with Khd1 for *ASH1* mRNA localization. We have observed that another KH domain protein, Scp160, is required for optimal *ASH1* mRNA localization and *HO* expression (Table I). The Scp160 protein, however, did not co-localize with the GFP signal from the U1Atag-ASH1 RNA particle (Figure 1), and thus its role in ASH1 localization is probably indirect. It has recently been reported that Scp160 localizes to membrane-bound polysomes and that its deletion causes pleiotropic defects, including temperature-sensitive growth and increased ploidy (Weber *et al*., 1997; Lang and Fridovich-Keil, 2000; Frey *et al*., 2001). Scp160 might be a component of the general translational machinery involved in the translation of various mRNA including *ASH1*. In conclusion, our studies on localization of *ASH1* mRNA have begun to reveal the ways in which KH-domain proteins modulate mRNA localization and possibly translation.

Materials and methods

Strains and general methods

Escherichia coil DH5α was used for DNA manipulations. The yeast strains used in this study are described in Table II. Standard procedures were followed for yeast manipulations (Kaiser *et al*., 1994). The media used in this study included rich medium, synthetic complete medium with glucose (SC), synthetic minimal medium with glucose (SD) and sporulation medium (Kaiser *et al*., 1994). SC lacking amino acids or other nutrients (e.g. SC-Leu lacks leucine and SC-Ade lacks adenine) was used to select transformants and to score *ADE2* reporter activity. SG and SR are identical to SC except that they contain 2% galactose and raffinose, respectively, instead of 2% glucose. Recombinant DNA procedures were carried out as described previously (Sambrook *et al*., 1989).

Plasmids

Plasmids used in this study are described in Table III. Plasmid pPT120 expresses U1Atag-ASH1 from the *GAL1* promoter (Takizawa and Vale, 2000). Plasmid pPT220 expresses U1A-GFP-GST-NLS from the *TDH3* promoter (Takizawa and Vale, 2000). Plasmid pK404 is YEplac195 carrying *GAL1p*-U1Atag-*ASH1 3′ UTR*. The fragment containing *GAL1p*-U1Atag and the fragment containing *ASH1* 3′ UTR were inserted into YEplac195. Plasmid pK622 is pRS426 carrying *GAL1p*-U1Atag-*ASH1* coding region (1–804)-*ADH1* 3′ UTR. The fragment containing *GAL1p*-U1Atag-*ASH1* coding region (1–804) and the fragment containing *ADH1* 3′ UTR were inserted into pRS426. Plasmid pK852 is pRS426 carrying *GAL1p*-U1Atag-*ASH1* coding region (828–1764)-*ADH1* 3′ UTR. The fragment containing *GAL1p*-U1Atag-*ASH1* coding region (828–1764) and the fragment containing *ADH1* 3′ UTR were inserted into pRS426. Plasmid pK736 is YEpURA3 plasmid carrying *GAL1p-KHD1*. Plasmid pCgHIS3 is pUC19 carrying the *Candida glabrata HIS3* gene (Sakumoto *et al*., 1999).

Deletion of the genes encoding RNA-binding proteins

The deletions of *PUF1/JSN1*, *PUF2*, *PUF3*, *PUF4/YGL014w*, and *PUF5/MPT5*, *SCP160*, *PBP2*, *YBL032w* and *MER1* were constructed by the PCR-based gene deletion method (Baudin *et al*., 1993; Schneider *et al*., 1996; Sakumoto *et al*., 1999). Primer sets were designed such that 46 bases at the 5′ end of the primers were complementary to those at the corresponding region of the target gene, and 20 bases at their 3′ end were complementary to the pUC19 sequence outside the polylinker region in plasmid pCgHIS3 containing the *C.glabrata HIS3* gene as a selectable marker. Primer sets for PCR were designed to delete the ORF completely. The PCR products were used to transform strain 10B by selection for His^+. The disruption was verified by colony-PCR amplification (Huxley *et al*., 1990) to confirm that replacement had occurred at the expected locus.

Construction of Khd1myc, Scp160myc and Mpt5myc strains

Khd1myc, Scp160myc and Mpt5myc strains were prepared by the method of Longtine *et al*. (1998) using pFA6a-13Myc-kanMX6.

Construction of GAL1p-KHD1 strains

The *GAL1p-KHD1* strain was prepared by the method of Longtine *et al*. (1998) using pFA6a-kanMX6-GAL1p-3HA.

Localization of ASH1 mRNA

In situ RNA hybridization with digoxigenin-labeled *ASH1* antisense probe was performed as described previously (Takizawa *et al*., 1997).

Table II. Strains used in this study

Strain	Genotype	Source
W303	MAT**a** *ade2 trp1 can1 leu2 his3 ura3 GAL psi*$^{+}$	Sil and Herskowitz (1996)
K1107	MAT**a** *HOp-LacZ-HO 3′ UTR*	Nasmyth (1987)
K5552	MATα *ASH1-myc*	Jansen *et al*. (1996)
10B	MATα *HOp-ADE2-HO 3′ UTR*	Tadauchi *et al*. (2001)
TTC356	MAT**a** *GALp-ASH1-myc*	this study
TTC360	MAT**a** *GALp-ASH1-myc* (atg^{-})	this study
YKEN111	MATα *HOp-ADE2-HO 3′ UTR puf1Δ/jsn1Δ::CgHIS3*	this study
YKEN113	MATα *HOp-ADE2-HO 3′ UTR puf2Δ::CgHIS3*	this study
YKEN112	MATα *HOp-ADE2-HO 3′ UTR puf3Δ::CgHIS3*	this study
YKEN110	MATα *HOp-ADE2-HO 3′ UTR puf4Δ::CgHIS3*	this study
YKEN109	MATα *HOp-ADE2-HO 3′ UTR puf5Δ/mpt5Δ::CgHIS3*	this study
YKEN123	MATα *HOp-ADE2-HO 3′ UTR scp160Δ::CgHIS3*	this study
YKEN124	MATα *HOp-ADE2-HO 3′ UTR pbp2Δ::CgHIS3*	this study
YKEN125	MATα *HOp-ADE2-HO 3′ UTR khd1Δ::CgHIS3*	this study
YKEN126	MATα *HOp-ADE2-HO 3′ UTR mer1Δ::CgHIS3*	this study
YKEN201	MATα *HOp-ADE2-HO 3′ UTR PUF5/MPT5myc::kanMX6*	this study
YKEN202	MATα *HOp-ADE2-HO 3′ UTR SCP160myc::kanMX6*	this study
YKEN203	MATα *HOp-ADE2-HO 3′ UTR KHD1myc::kanMX6*	this study
YKEN204	MATα *HOp-ADE2-HO 3′ UTR SHE2myc::kanMX6*	this study
101	MATα *MYO4myc*	Jansen (1996)
134	MATα *SHE3myc*	Jansen (1996)
YKEN251	MATα *HOp-ADE2-HO 3′ UTR*	this study
YKEN252	MATα *HOp-ADE2-HO 3′ UTR myo4-910*	this study
YKEN253	MATα *HOp-ADE2-HO 3′ UTR khd1Δ::CgHIS3*	this study
YKEN254	MATα *HOp-ADE2-HO 3′ UTR khd1Δ::CgHIS3 myo4-910*	this study
YKEN301	MATα *HOp-ADE2-HO 3′ UTR kanMX6::GAL1p-KHD1*	this study
YKEN302	MATα *HOp-ADE2-HO 3′ UTR she3Δ kanMX6::GAL1p-KHD1*	this study
YKEN303	MATα *HOp-ADE2-HO 3′ UTR myo4Δ kanMX6::GAL1p-KHD1*	this study
YKEN304	MATα *HOp-ADE2-HO 3′ UTR ash1Δ*	this study
YKEN305	MATα *HOp-ADE2-HO 3′ UTR she3Δ ash1Δ*	this study
YKEN306	MATα *HOp-ADE2-HO 3′ UTR myo4Δ ash1Δ*	this study
YKEN307	MATα *ASH1-myc kanMX6::GAL1p-KHD1*	this study

Table III. Plasmids used in this study

Plasmid	Relevant markers	Source
YEplac181	*LEU2*, 2 μm	Gietz and Sugino (1988)
YEplac195	*URA3*, 2 μm	Gietz and Sugino (1988)
pRS426	*URA3*, 2 μm	Sikorski and Hieter (1989)
pAS191	*LEU2*, 2 μm, *ASH1*	Sil and Herskowitz (1996)
pPT120 (U1Atag-Full)	*HIS3*, 2 μm, *GAL1p*-U1Atag-*ASH1* (1–1764)-*ASH1 3′ UTR*	Takizawa and Vale (2000)
pPT220	*TRP1*, *CEN-ARS*, *TDH3p-U1A-GFP-GST-NLS*	Takizawa and Vale (2000)
pK114 (U1Atag-Full)	*URA3*, 2 μm, *GAL1p*-U1Atag-*ASH1* (1–1764)-*ASH1 3′ UTR*	this study
pK404 (U1Atag-U)	*URA3*, 2 μm, *GAL1p*-U1Atag-*ASH1 3′ UTR*	this study
pK622 (U1Atag-N)	*URA3*, 2 μm, *GAL1p*-U1Atag-*ASH1* (1–804)-*ADH1 3′ UTR*	this study
pK852 (U1Atag-C)	*URA3*, 2 μm, *GAL1p*-U1Atag-*ASH1* (828–1764)-*ADH1 3′ UTR*	this study
pK736	*URA3*, 2 μm, *GAL1p-KHD1*	this study
pFA6a-13Myc-kanMX6	*13MYC-ADH1 3′ UTR-kanMX6*	Longtine *et al*. (1998)
pFA6a-kanMX6-GAL1p-3HA	*kanMX6-GAL1p-3HA*	Longtine *et al*. (1998)
pCgHIS3	*C.glabrata HIS3* in pUC19	Sakumoto *et al*. (1999)

Induction and imaging of U1Atag-ASH1 RNA particles

Co-localization of myc-tagged proteins with U1Atag-ASH1 RNA particles was examined as described previously (Takizawa and Vale, 2000). Cells containing U1Ap-GFP and U1Atag-ASH1 were grown overnight at 30°C in synthetic media containing 2% raffinose. Overnight cultures were adjusted to an optical density (OD) of 0.5 (600 nm) in synthetic media containing 2% raffinose and incubated for 2 h at 30°C. Galactose was added to 0.2%, and the cultures incubated for 2 h at 30°C. Cells were examined by phase-contrast microscopy using a ×63/NA 1.4 lens. Images were captured with a cooled charged-coupled device and digital images displayed by using Adobe Photoshop. For co-localization experiments, samples were fixed after induction in 3.7% formaldehyde for 1 h. Cells were washed and made into spheroplasts in SP buffer (100 mM phosphate buffer pH 7.0, 1.2 M sorbitol containing 30 mM mercaptoethanol, 40 mg/ml zymolyase 100T) for 30 min at 37°C. Cells were washed and spread on polylysine-coated, multiwell test slides and then incubated with monoclonal anti-myc antibody 9E10 (Santa Cruz, CA) at a 1:1000 dilution in blocking buffer [phosphate-buffered saline (PBS), 1% bovine serum albumen (BSA)] for 1 h. After washing, cells were incubated with rhodamine-conjugated goat anti-mouse IgG (Boehringer Mannheim) in blocking buffer for 1 h. Cells were washed and mounted in mounting buffer (PBS, 90% glycerol, 1 mg/ml ρ-phenylenediamine, 0.1 μg/ml 4′,6-diamidino-2-phenylindole).

Immunoprecipitation and mRNA detection

Exponentially growing cells (3×10^8) were disrupted with glass beads in 200 ml extraction buffer [25 mM HEPES–KOH pH 7.5, 150 mM KCl, 2 mM $MgCl_2$ containing 20 mM vanadyl ribonucleoside complexes (Sigma), 200 U/ml RNasin (Life Technologies, Grand Island, NY), 0.1% NP-40, 1 mM DTT, 0.2 mg/ml heparin, 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin and pepstatin]. Extracts were cleared by centrifugation (10 min at 4000 *g*). Monoclonal anti-myc antibody 9E10 was added to the cleared extracts and incubated for 1 h on ice following incubation

with protein A agarose for 1 h at 4°C. Beads were washed four times in wash buffer (25 mM HEPES–KOH pH 7.5, 150 mM KCl, 2 mM $MgCl_2$) and were eluted in 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 1% SDS for 10 min at 65°C. Eluted samples were extracted with phenol-chloroform, ethanol precipitated, resuspended in RQ1 DNase buffer and treated with RQ1 DNase (Promega, Madison, WI). The remaining RNA was extracted, precipitated and resuspended in H_2O. RT–PCR was performed with 1 µl RNA as template using the 'Access'-RT–PCR kit (Promega) and the conditions suggested by the manufacturer. The number of amplification cycles was adjusted to avoid reaching a plateau phase during PCR.

Preparation of yeast extracts and western blot analysis

Yeast cells were grown to an OD_{600} of 0.5–1.0 and treated with 2% galactose to activate the *GAL1* promoter. After treatment, yeast cultures were quickly chilled, and cells were collected by rapid centrifugation. The pellet was washed twice and then suspended in breaking buffer (4% SDS, 40 mM Tris–HCl pH 7.0, 8 M urea, 0.1 mM EDTA, 1% 2-mercaptoethanol). Glass beads (0.4–0.6 mm diameter) were added to this suspension, and cells were broken by vigorous vortexing for 5 min at room temperature. Beads and cell debris were removed by centrifugation at 10 000 *g* at room temperature. Protein concentrations of the cell extracts were measured at OD_{280}. Cell extracts were subjected to SDS–PAGE on 7% acrylamide gels followed by electroblotting onto Hybond N^+ membrane (Amersham). Blots were blocked by incubation for 15 min at room temperature in TBS-M buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl with 4% non-fat dry milk). Blots were then incubated with monoclonal anti-myc antibody 9E10 diluted 1:2000 (to detect Ash1myc) or anti-tubulin antibody diluted 1:1000 (to detect tubulin) in TBS-M buffer overnight at 4°C. After three washes with TBS buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl), blots were incubated for 2 h with peroxidase-conjugated secondary antibody (Calbiochem) diluted 1:3000 with TBS-M buffer. After three final washes with TBS buffer, blots were detected using an enhanced chemiluminescence detection kit (Amersham).

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