Intracellular and intercellular polarity requires that specific proteins be sorted to discreet locations within and between cells. One mechanism for sorting proteins is through RNA localization. In *Saccharomyces cerevisiae*, *ASH1* mRNA localizes to the distal tip of the bud, resulting in the asymmetric sorting of the transcriptional repressor Ash1p. *ASH1* mRNA localization requires four *cis*-acting localization elements and the *trans*-acting factors Myo4p, She3p, and She2p. Myo4p is a type V myosin motor that functions to directly transport *ASH1* mRNA to the bud. She2p is an RNA-binding protein that directly interacts with the *ASH1* mRNA *cis*-acting elements. Currently, the role for She3p in *ASH1* mRNA localization is as an adaptor protein, since it can simultaneously associate with Myo4p and She2p. Here, we present data for two novel mutants of She3p, S348E and the double mutant S343E S361E, that are defective for *ASH1* mRNA localization, and yet both of these mutants retain the ability to associate with Myo4p and She2p. These observations suggest that She3p possesses a novel activity required for *ASH1* mRNA localization, and our data imply that this function is related to the ability of She3p to associate with *ASH1* mRNA. Interestingly, we determined that She3p is phosphorylated, and global mass spectrometry approaches have determined that Ser 343, 348, and 361 are sites of phosphorylation, suggesting that the novel function for She3p could be negatively regulated by phosphorylation. The present study reveals that the current accepted model for *ASH1* mRNA localization does not fully account for the function of She3p in *ASH1* mRNA localization.
**TABLE 1. Yeast strains used in this study**

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| K699   | MATα ade2-1 his3-11 leu2-3,112 ura3-1 trp1-1 ho can1-100 | 36 |
| PJ69-4a| MATα his3-200 leu2-3,112 trp1-90 ura3-52 gal4 gal80 GAL1-ADE2-2 gly3-GAL7-lacZ | 35 |
| YLM584 | MATα ade2 his3-200 leu2-3,112 trp1-1 ura3-52 LYS2::(LexAop)-lacZ LexA-MS2-MS2 coat (N55K) she1::KAN | 41 |
| YLM677 | MATα ade2-1 his3-11 leu2-3,112 ura3-1 trp1-1 ho can1-100 she2Δ silent she2Δ silent Myo4p-3HA::kanMX6 | This study |
| YLM923 | MATα ade2-1 leu2-3 trp1-1 ura3 HO-ADE2-2 HO-CAN1 she3::KAN | This study |
| YLM1320| MATα ade2-1 his3-11 leu2-3,112 trp1-1 ura3 ho can1-100 she2Δ silent she2Δ silent Myo4p-myc6::kanMX6 | 23 |
| YLM1406| MATα ade2-1 his3-11 leu2-3,112 trp1-1 ura3 ho can1-100 she2Δ silent she2Δ silent Myo4p-myc6::kanMX6 | 23 |

**MATERIALS AND METHODS**

**Yeast strains, media, and plasmids.** Yeast cells were grown in defined synthetic media lacking the indicated nutrients or rich YEP medium (53). All media contained 2% glucose unless indicated otherwise. Yeast cells were transformed by using lithium acetate (34). As described elsewhere, yeast strains deleted of specific genes were created by using PCR products generated from plasmid pUG6 (30). To label yeast cells with [32P]orthophosphate, minimal D3/5 high-Pi medium lacking leucine to an optical density at 600 nm (OD_{600}) of 0.600. Subsequently, the cultures were diluted into fresh SMD low-Pi medium to an OD_{600} of 0.4. Yeast cells were grown for 4 h at 30°C in SMD low-Pi medium containing 0.5 μCi of [32P]orthophosphate/ml. After labeling, the cells were harvested by centrifugation, cell lysates were prepared by breaking the cells with glass beads in 500 μl of breaking buffer (100 mM NaHPO₄ [pH 7.4], 1 M NaCl, 10% Triton X-100, 1% sodium dodecyl sulfate [SDS], 5% deoxycholate, 0.1 μg of chymostatin/ml, 2 μg of aprotinin/ml, 1 μg of pepstatin/ml, 0.5 μg of leupeptin/ml, 0.01 μg of benzamidine/ml, 1 mM Na₃VO₄, 50 mM NaF, 8.67 mg of β-glycerophosphate/ml), and the lysate was recovered from the glass beads. The glass beads were washed with 200 μl of fresh breaking buffer and combined appropriately. The lysates were cleared by centrifugation at 16,000 × g for 10 min at 4°C. The soluble fraction was recovered and stored at −80°C. For immunoprecipitation, the cell lysates were diluted into fresh breaking buffer for a total volume of 1 ml, and 5 μl of rabbit polyclonal She3p antiserum was added. She3p immune complexes were collected by protein A-agarose beads.

**TABLE 2. Plasmids used in this study**

| Plasmid | Features | Source or reference |
|---------|----------|---------------------|
| pACT2   | Yeast vector for expressing Gal4p activation domain fusion proteins | 39 |
| pET21b  | E. coli vector for generating His₆-tagged fusion proteins | Novagen |
| pGBDU-c2| Yeast vector for expression of Gal4p DNA-binding domain fusion proteins | 35 |
| pUG6    | Plasmid for generating KAN disruption cassettes by PCR | 30 |
| YCplac22| Yeast single-copy shuttle plasmid marked with TRP1 | 22 |
| YCplac111| Yeast single-copy shuttle plasmid marked with LEU2 | 22 |
| YCplac33| Yeast single-copy shuttle plasmid marked with URA3 | 22 |
| YEplac181| Yeast multicopy shuttle plasmid marked with LEU2 | 22 |
| YEplac195| Yeast multicopy shuttle plasmid marked with URA3 | 22 |
| C3431   | YEplac195 containing ASH1 | 43 |
| pHA/MS2-2| Three-hybrid vector for expressing MS2-fusion RNAs | 68 |
| pEP15   | pACT2 expressing wild-type She3p | 41 |
| pRL80   | plH1A/MS2-2 containing ASH1 E3 cis-acting localization element | 41 |
| pRL128  | YEplac181 expressing wild-type She3p | 41 |
| pRL174  | pGBDU-c2 expressing wild-type She2p | 41 |
| pRL189  | YEplac181 expressing She3p-myc6 | This study |
| pRL200  | YCplac111 expressing She3p-myc6 | 24 |
| pRL461  | pET21b expressing She3p amino acids 236 to 425 | This study |
| pRL519  | YCplac111 expressing wild-type She3p | This study |
| pRL676  | YCplac22 expressing She2p-myc6 | 23 |
| pRL1138 | YCplac111 expressing She3p-S343A S361A-myc6 | This study |
| pRL1139 | YCplac111 expressing She3p-S343E S361E-myc6 | This study |
| pRL1143 | YCplac33 expressing wild-type She2p | This study |
| pRL1182 | YCplac111 expressing She3p-S343A S361A | This study |
| pRL1183 | YCplac111 expressing She3p-S343E S361E | This study |
| pRL1206 | YCplac111 expressing She3p-S348A-myc6 | This study |
| pRL1207 | YCplac111 expressing She3p-S348E-myc6 | This study |
| pRL1221 | pACT2 expressing She3p-S343A S361A | This study |
| pRL1222 | pACT2 expressing She3p-S343E S361E | This study |
| pRL1225 | pACT2 expressing She3p-S348A | This study |
| pRL1226 | pACT2 expressing She3p-S348E | This study |
| pRL1252 | YCplac111 expressing She3p-S348A | This study |
| pRL1253 | YCplac111 expressing She3p-S348E | This study |

**In vivo labeling and immunoprecipitation.** In vivo labeling of yeast cells with [32P]orthophosphate was performed as described previously (42). Briefly, yeast cells were grown overnight at 30°C in minimal D3/5 high-Pi medium, and the lysate was recovered from the glass beads. The glass beads were washed with 200 μl of fresh breaking buffer and combined appropriately. The lysates were cleared by centrifugation at 16,000 × g for 10 min at 4°C. The soluble fraction was recovered and stored at −80°C. For immunoprecipitation, the cell lysates were diluted into fresh breaking buffer for a total volume of 1 ml, and 5 μl of rabbit polyclonal She3p antiserum was added. She3p immune complexes were collected by protein A-agarose beads.
beads were washed three times with 1 ml of fresh breaking buffer/wash. Bound proteins were eluted by boiling the protein A-antibody complexes in Laemmli buffer. The immunoprecipitations were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

**SHE genetic selection.** Plasmids expressing wild-type and mutant versions of She3p were transformed into yeast strain YLM923, and transformants were selected on synthetic medium lacking leucine. Subsequently, single yeast colonies were picked and grown overnight at 30°C in liquid synthetic medium lacking leucine. Then, 2 μl of the undiluted overnight culture and 10^4, 10^5, 10^6, and 10^7 dilutions were spotted onto synthetic medium devoid of leucine in the absence and presence of 0.03% canavanine. The plates were incubated at 30°C for 2 days, and images were captured. Subsequently, to ensure that the yeast cells produce the various She3p mutant proteins, cell lysates were prepared and analyzed by Western blotting with an anti-myc 9E10 monoclonal antibody (Roche), as well as with anti-Pgk1p (Molecular Probes).

**In situ hybridization.** Yeast cultures were grown in appropriate media to early log phase, fixed with formaldehyde, and converted to spheroplasts as described previously (9, 43). Subsequently, spheroplasts were hybridized with a pool of Cy3-conjugated ASH1 oligonucleotide probes and stained with DAPI (4′,6′-diamidino-2-phenylindole) (9, 43). Coverslips were mounted on slides with phenylmedihain mounting medium (9). Cells were visualized, and images were captured on a Nikon Eclipse 600 epifluorescence microscope equipped with a 60× N.A. 1.4 objective lens, connected to a Micromax-Interline transfer charge-coupled device camera (Princeton Instruments, Inc.), and using MetaMorph imaging software (Universal Imaging Corp.).

**Coinmunoprecipitation assays.** Exponentially growing cultures corresponding to 3 × 10^7 yeast cells were harvested, and soluble lysates were prepared in breaking buffer containing 50 mM HEPES-KOH (pH 7.3), 20 mM potassium acetate, 2 mM EDTA, 0.1% Triton X-100, 50 mM KCl, 1 mM MgSO_4_, and 50 mM NaCl, 10% glycerol, 0.1% NP-40, 0.5 μg of pepstatin/ml, 0.5 μg of leupeptin/ml, and 0.01 μg of benzamidine/ml as described previously (23). Protein complexes were purified by the addition of 7.5 μg of anti-myc 9E10 monoclonal antibody prebound to protein A-agarose beads (Pierce) and incubated for 2 h at 4°C. The immune complexes were collected by centrifugation at 500 × g for 4 min at 4°C. The matrix was washed four times with 500 μl of washing buffer (50 mM HEPES-KOH [pH 7.3], 20 mM potassium acetate, 2 mM EDTA, 0.1% Triton X-100, 5% glycerol). Bound proteins were eluted by boiling in Laemmli buffer. Equivalent amounts of cell extracts (input) and precipitated sample were separated by SDS-PAGE and analyzed by Western blotting. Proteins containing the hemagglutinin (HA) epitope were detected with anti-HA11 monoclonal antibody (Covalent), whereas She3p and She3p were detected with rabbit polyclonal anti-She3p and anti-She3p antisemur, respectively.

**Preparation of rabbit polyclonal She3p antiserum.** Polyclonal rabbit anti-serum was raised against She3p amino acids 236-425-His6 expressed and purified from *Escherichia coli*. Plasmid pRL461 was transformed into E. coli strain BL21(DE3)/pLysS, and expression of She3p-His6 was induced with 1 mM IPTG. Cell lysates were cleared by centrifugation at 4,000 × g for 4 min at 4°C. The supernatants were determined, and the OD_600 was calculated by the following formula: \( U = 1.000 \times [OD_{620} - (1.75 \times OD_{590})/\text{time (in min)} \times \text{volume of the aliquot of the cell suspension (in ml)} \times \text{OD}_{600}\). The OD_590 values reported represent the average of three independent experiments.

**Immunoprecipitation–reverse transcription-PCR (IP/RT-PCR) analysis.** Immunoprecipitations of She3p-myc, and detection of the associated mRNAs were performed essentially as described previously (23, 33). In summary, 3 × 10^6 exponentially growing yeast cells were harvested by centrifugation and disrupted with glass beads in 400 μl of breaking buffer containing 25 mM HEPES-KOH (pH 7.5), 150 mM KCl, 2 mM MgCl_2, 200 U of Superase-In (Ambion)/ml, 0.1% NP-40, 1 mM dithiothreitol, 0.2 mg of heparin/ml, 0.1 μg of chymostatin/ml, 2 μg of pepstatin/ml, 0.5 μg of leupeptin/ml, and 0.01 μg of benzamidine/ml. Cell lysates were cleared by centrifugation at 4,000 × g for 5 min, and the immunoprecipitation was performed at 4°C for 2 h with anti-myc monoclonal antibody 9E10 prebound to protein A-agarose beads. Immune complexes were recovered by centrifugation and washed four times in 300 μl of wash buffer (25 mM HEPES-KOH [pH 7.5], 150 mM KCl, 2 mM MgCl_2). Protein-RNA complexes were eluted from protein A-agarose beads at 65°C for 10 min in 100 μl of elution buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10 mM EDTA, 1% SDS). An aliquot of the elution was saved for Western blotting. RNA was extracted from the remaining portion of the sample by using phenol-chloroform–isoamyl alcohol and ethanol precipitation. The resulting RNA pellet was resuspended in nuclease-free water and treated with DNase. The DNase-treated RNA was subsequently analyzed with an Access/RT-PCR kit (Promega) using primers specific for ASH1 mRNA.

**RESULTS**

Identification of novel She3p mutants that are defective for ASH1 mRNA localization. She3p contains numerous consensus phosphorylation sites, implying that phosphorylation of She3p could regulate ASH1 mRNA localization activities. We investigated the in vivo phosphorylation state of She3p by labeling cells with [γ-32P]orthophosphate and immunoprecipitating She3p (Fig. 1). We observed a 32P-labeled protein corresponding to the molecular weight of She3p from an immunoprecipitation performed with a wild-type lysate, and this 32P-labeled protein is absent from an immunoprecipitation performed with a she3Δ lysate. These results suggest that either She3p is phos-
phosphorylation in vivo or a protein with a molecular weight similar to that of She3p is phosphorylated in a She3p-dependent manner. We further investigated whether She3p is phosphorylated in vivo by transforming a she3Δ strain with either a multicopy plasmid expressing She3p or a multicopy plasmid expressing She3p-myc₆ and labeling the transformants with [³²P]orthophosphate. After immunoprecipitation with the extract containing She3p (multicopy She3p), we observed a [³²P]-labeled protein migrating at the predicted molecular weight for She3p. Furthermore, after immunoprecipitation with the She3p-myc₆ extract (multicopy She3p-myc₆), we observed a slower-migrating [³²P]-labeled protein, corresponding to She3p-myc₆, and we observed an absence of a [³²P]-labeled protein at the molecular weight corresponding to She3p. From these results we conclude that She3p is phosphorylated in vivo.

To begin to map the sites of phosphorylation within She3p, we performed phosphoamino acid analysis and determined that She3p is phosphorylated in vivo predominantly on Ser residues (data not shown). Consistent with our in vivo labeling studies, global mass spectrometry approaches determined that Ser 28, 217, 343, 348, 361, 392, and 394 are sites of phosphorylation within She3p (1, 12, 27, 40, 59). In addition, Thr 393 and Tyr 123 have been reported to be sites of phosphorylation within She3p (1, 51). Ser 28 and Tyr 123 map to a region of She3p that interacts with Myo4p (Fig. 2A) (7). Ser 217 maps to a region between the domains of She3p that associate with either Myo4p or She2p, while Ser 343, 348, 361, 392, 394, and Thr 393 map to the region of She3p that interacts with She2p and ASH1 mRNA in a She2p-dependent manner (Fig. 2A) (7, 41).

Using the SHE genetic selection, we investigated whether Ser phosphorylation has a regulatory role in ASH1 mRNA localization by individually converting the Ser residues to Ala, which prevents phosphorylation, or Glu, which mimics constitutive phosphorylation. In the SHE genetic selection the asymmetric regulation of the HO promoter is monitored by the reporter gene HO-CAN1 (6, 36). Consequently, in the SHE genetic selection yeast colonies with a defect in ASH1 mRNA localization have the ability to grow in the presence of the toxic arginine analog canavanine. From our analysis, we observed that the She3p-S348E mutant is the only single Ser amino acid substitution that is insensitive to canavanine (Fig. 2B and Table 3 and data not shown). We also investigated whether multiple Ser-to-Ala and Ser-to-Glu substitutions alter the ability of She3p to localize ASH1 mRNA. We observed that the She3p-S343E S361E mutant also is insensitive to canavanine (Fig. 2B, Table 3, and data not shown). These results suggest that She3p-S348E and She3p-S343E S361E are defective for ASH1 mRNA localization activity. In contrast, we observed that cells expressing She3p-S348E or She3p-S343E S361E are unable to localize ASH1 mRNA as efficiently as wild-type She3p. Furthermore, we observed that cells expressing She3p-S348E or She3p-S343E S361E have a slight increase in the number of cells exhibiting the full budding phenotype which has been attributed to defects in anchoring ASH1 mRNA at the site of localization (Fig. 2C) (26, 33). The sum of our results from the SHE genetic selection and FISH indicate that She3p-S348E and She3p-S343E S361E cells are defective for ASH1 mRNA localization and Ash1p sorting.

A dramatic increase or decrease in the expression of She3p-S348E and She3p-S343E S361E would provide a trivial explanation for the inability of these She3 mutants to localize ASH1 mRNA. Consequently, we investigated the expression of the She3p mutants by Western blotting and determined that She3p-S348E and She3p-S343E S361E are expressed at equivalent levels to wild-type She3p, She3p-S348A, and She3p-S343A S361A (Fig. 3A and data not shown). The sum of our results is consistent with the hypothesis that phosphorylation of She3p at Ser 343, 348, and 361 negatively regulates an ASH1 mRNA localization activity. Furthermore, since we observed that the corresponding She3p-S348A and She3p-S343A S361A mutants retain the ability to localize ASH1 mRNA (Fig. 2B and C and Table 3), we hypothesize that phosphorylation at these Ser residues is not required for ASH1 mRNA localization.

She3p-S348E and She3p-S343E S361E retain the ability to associate with Myo4p and She2p. She3p possesses two known activities required for ASH1 mRNA localization: the ability to interact with Myo4p and the ability to interact with She2p (7, 41, 63). Consequently, we investigated the ability of She3p-S348E and She3p-S343E S361E to associate with Myo4p and She2p by coimmunoprecipitation (Fig. 3A). We observed that wild-type She3p (lane 3), She3p-S348A-myc₆ (lane 4), She3p-S348E-myc₆ (lane 5), She3p-S343A S361A-myc₆ (lane 6), and She3p-S343E S361E-myc₆ (lane 7) immunoprecipitate equivalent amounts of Myo4p-3HA and She2p. We further studied the She3p-She2p interaction by two-hybrid analysis. She3p-S348A, She3p-S348E, She3p-S343A S361A, and She3p-S343E S361E express She2p-dependent LacZ activity at levels comparable to that of wild-type She3p (Fig. 3B). Taken together, these results suggest that the observed ASH1 mRNA localization defect for She3p-S348E and She3p-S343E S361E is not due to defects in the Myo4p or She2p interactions.

In the proposed model for ASH1 mRNA localization, She3p simultaneously associates with Myo4p and She2p. While She3p-S348E and She3p-S343E S361E mutants retain the ability to individually associate with Myo4p and She2p, it remained a formal possibility that these She3p mutants may not retain the ability to simultaneously associate with Myo4p and She2p. Consequently, we investigated whether She3p-S348E and She3p-S343E S361E could simultaneously associate with Myo4p and She2p by coimmunoprecipitation (Fig. 4). We observed that each of the She3p mutants and She2p coimmunoprecipitate with Myo4p-myc₃. These results reinforce our assertion that the ASH1 mRNA localization defect in yeast strains expressing She3p-S348E or She3p-S343E S361E does not result from the inability of these She3p mutants to associate with Myo4p or She2p. Furthermore, these results imply...
that She3p contains an unidentified activity required for ASH1 mRNA localization. She3p-S348E and She3p-S343E S361E are defective for association with ASH1 mRNA. The current model for ASH1 mRNA localization postulates that She3p is tethered to ASH1 mRNA through a protein-protein interaction with She2p (7, 41, 63). Since She3p-S348E and She3p-S343E S361E retain the ability to associate with She2p, we reasoned that these She3p
TABLE 3. Summary for ASHI mRNA localization in cells expressing the various She3p mutants

| Amino acid(s) | Localizationa | Ala | Glu |
|---------------|---------------|-----|-----|
| S28           | L             | L   | L   |
| S217          | L             | L   | L   |
| S343          | L             | L   | L   |
| S348          | L             | D   | D   |
| S361          | L             | L   | L   |
| S392          | L             | L   | L   |
| T393          | L             | L   | L   |
| S394          | L             | L   | L   |
| S28, S343     | L             | L   | L   |
| S28, S361     | L             | L   | L   |
| S28, S392     | L             | L   | L   |
| S28, S394     | L             | L   | L   |
| S343, S361    | L             | D   | D   |
| S343, S392    | L             | L   | L   |
| S343, S394    | L             | L   | L   |
| S28, S361, S394 | L      | L   | L   |
| S28, S392, S394 | L     | L   | L   |
| S343, S361, S392 | L    | L   | L   |
| S343, S361, S394 | L   | L   | L   |
| S343, S361, S392 | L   | L   | L   |
| S28, S343, S361, S392 | L | L   | L   |
| S28, S343, S361, S394 | L | L   | L   |
| S28, S361, S394 | L | L   | L   |
| S343, S361, S392 | L | L   | L   |
| S28, S343, S361, S392, S394 | L | L   | L   |

a L, localized; D, delocalized.

mutants should also associate with ASHI mRNA. We investigated whether She3p-S348E and She3p-S343E S361E associate with ASHI mRNA by immunoprecipitation and RT-PCR (Fig. 5A). We observed no RT-PCR product for ASHI mRNA when the immunoprecipitation was performed with a cell lysate devoid of She3p (lane 1) or She2p (lane 2). We observed an RT-PCR product for ASHI mRNA when the immunoprecipitation was performed with cell lysates containing wild-type She3p-myc<sub>6</sub> (lane 3), She3p-S348A-myc<sub>6</sub> (lane 4), or She3p-S343A S361A-myc<sub>6</sub> (lane 6). In contrast, we observed a substantial reduction for the ASHI mRNA RT-PCR product when the immunoprecipitation was performed with a cell lysate containing She3p-S348E-myc<sub>6</sub> (lane 5) and no ASHI mRNA RT-PCR product when the immunoprecipitation was performed with a cell lysate containing She3p-S343E-myc<sub>6</sub> (lane 7). Given that She3p-S348E and She3p-S343E S361E associate with She2p in a manner indistinguishable from that of wild-type She3p, our results imply that the ability of She2p to tether ASHI mRNA to She3p through a protein-protein interaction does not fully explain the ability of She3p to associate with ASHI mRNA.

Given that the buffer used for the immunoprecipitation/RT-PCR analysis (Fig. 5A) (25 mM HEPES-KOH [pH 7.5], 150 mM KCl, 2 mM MgCl<sub>2</sub>, 200 U of Superase-In [Ambion]/ml, 0.1% NP-40, 1 mM dithiothreitol, 0.2 mg of heparin/ml, 0.1 μg of chymostatin/ml, 2 μg of aprotinin/ml, 1 μg of pepstatin/ml, 0.5 μg of leupeptin/ml, 0.01 μg of benzamidine/ml) is different from the buffer (50 mM HEPES-KOH [pH 7.3], 20 mM potassium acetate, 2 mM EDTA, 0.1% Triton X-100, 5% glycerol, 0.1 μg of chymostatin/ml, 2 μg of aprotinin/ml, 1 μg of pepstatin/ml, 0.5 μg of leupeptin/ml, 0.01 μg of benzamidine/ml) used to monitor the association of She3p with Myo4p and She2p (Fig. 3A and 4), we investigated whether She3p-S348E and She3p-S343E S361E remain associated with She2p under the immunoprecipitation conditions used for RT-PCR (Fig. 5A). After immunoprecipitation with wild-type She3p-myc<sub>6</sub> (lane 3), She3p-S348A-myc<sub>6</sub> (lane 4), She3p-S348E-myc<sub>6</sub> (lane 5), She3p-S343A S361A-myc<sub>6</sub> (lane 6), or She3p-S343E S361E-myc<sub>6</sub> (lane 7), we observed that She2p was undetectable after immunoprecipitation with any of these She3p alleles, and yet we detected the presence of ASHI mRNA by RT-PCR with the same immunoprecipitations. These results support our assertion that tethering of ASHI mRNA to She3p through a protein-protein interaction with She2p does not fully explain the ability of She3p to associate with ASHI mRNA.

To confirm the absence of the She2p-She3p interaction under the IP/RT-PCR conditions, we investigated whether She2p-myc<sub>6</sub> could coimmunoprecipitate She3p and ASHI mRNA using the IP/RT-PCR conditions (Fig. 5B). Consistent with our previous results, we observed that wild-type She3p (lane 3), She3p-S348A (lane 4), She3p-S348E (lane 5), She3p-S343A S361A (lane 6), and She3p-S343E S361E (lane 7) were not detected after immunoprecipitation of She2p-myc<sub>6</sub>, and we detected the presence of ASHI mRNA by RT-PCR with the same immunoprecipitations. Consequently, these results support our findings that the conditions used for IP/RT-PCR are inadequate for the detection of the She2p-She3p interaction.

We further studied the She3p-ASH1 mRNA interaction by three-hybrid analysis. As expected, She3p-S348A and She3p-S343A S361A express ASHI mRNA-dependent LacZ activity at levels comparable to those of wild-type She3p (Fig. 5C). Consistent with our immunoprecipitation/RT-PCR analysis, we observed that She3p-S348E expresses ASHI mRNA-dependent activity at lower levels than wild-type She3p. Unexpectedly, we observed that She3p-S343E S361E expresses ASHI mRNA-dependent activity at levels comparable to wild-type She3p. The combination of the IP/RT-PCR results (Fig. 5A) with the three hybrid results (Fig. 5C) suggests that the She3p-S348E and She3p-S343E S361E mutants are differentially defective for association with ASHI mRNA.

**DISCUSSION**

In this study we identify two novel mutants of She3p, S348E and S343E S361E, which are defective for ASHI mRNA localization. Given that ASHI mRNA localization requires that She3p associate with Myo4p and She2p, we investigated whether She3p-S348E and She3p-S343E S361E retain the ability to associate with Myo4p and She2p. Since S348E and S343E S361E are located outside the domain of She3p that interacts with Myo4p, we did not expect that these amino acid substitutions would affect the She3p-Myo4p interaction (7). However, since both S348E and S343E S361E are located in the domain...
of She3p that interacts with She2p, we anticipated that these amino acid substitutions could affect the She3p-She2p interaction (7, 41). As expected, we determined that these She3p mutants retain the ability to associate with Myo4p but, unexpectedly, we observed that these amino acid substitutions do not alter the She3p-She2p interaction. Although our assays, coimmunoprecipitation and two-hybrid analysis, indicate that She3p-S348E and She3p-S343E S361E associate with She2p at levels indistinguishable from those of wild-type She3p, we cannot be certain that in vivo the She3p mutants have the same affinity for She2p as wild-type She3p. Furthermore, if wild-type She3p induces a conformation change in She2p, She3p-S348E and She3p-S343E S361E might be defective for inducing the conformation change, while retaining the ability to associate with She2p. In any event, our results suggest that the ability of She3p to associate with Myo4p and She2p does not fully account for the function of She3p in \textit{ASH1} mRNA localization.

To begin to identify a novel function for She3p in \textit{ASH1} mRNA localization, we investigated whether She3p-S348E and She3p-S343E S361E can associate with \textit{ASH1} mRNA. Based on the current model for \textit{ASH1} mRNA localization, She3p is tethered to \textit{ASH1} mRNA through a protein-protein interaction with She2p (7, 41, 63). Since the She3p mutants retain the ability to associate with She2p, the current model for \textit{ASH1} mRNA localization predicts that the She3p mutants should retain the ability to associate with \textit{ASH1} mRNA. However, we determined that She3p-S348E and possibly She3p-S343E S361E are defective for association with \textit{ASH1} mRNA. This
The result implies that tethering of ASH1 mRNA through She2p does not fully account for the ability of She3p to associate with ASH1 mRNA. Although it has been previously demonstrated that She2p is required for the association of ASH1 mRNA with She3p, our results showing that the She2p-She3p interaction is not maintained under the conditions used to perform IP/RT-PCR imply that She2p may be necessary to establish the association between ASH1 mRNA and She3p, but She2p may not be necessary to maintain the interaction between ASH1 mRNA and She3p (7, 41).
She3p-S343E S361E. However, our data imply that She3p can associate with ASH1 mRNA through multiple, distinct interactions. For She3p-S343E and She3p-S343E S361E, some of these interactions are maintained in the three-hybrid assay, while the same interactions are not maintained in the IP/RT-PCR assay. Consequently, the results from these two assays may not entirely coincide. Furthermore, we observed a difference between She3p-S348E and She3p-S343E S361E in the three-hybrid assay. For She3p S348E, we observed a reduction in the association with ASH1 mRNA by the IP/RT-PCR assay and the three-hybrid assay, whereas for She3p-S343E S361E we observed a defect for the association with ASH1 mRNA with the IP/RT-PCR experiment but not with the three-hybrid experiment. These results suggest that the two She3p mutants differentially associate with ASH1 mRNA. We hypothesize that She3p-S348E is defective for association with individual cis-acting localization elements as determined by the IP/RT-PCR and three-hybrid assays. In contrast, She3p-S343E S361E might only be defective for the association with full-length ASH1 mRNA, which is limited to detection in the IP/RT-PCR assay. In any event, our results clearly show that She3p-S348E and She3p-S343E S361E are defective for ASH1 mRNA localization through a novel activity, since these mutants retain the ability to associate with Myo4p and She2p.

Previously, we demonstrated that a reporter mRNA artificially tethered to She2p could not localize in a manner analogous to ASH1 mRNA, and these results implied that mRNA artificially tethered to She2p could not properly anchor at the bud tip (24). From these results we hypothesized that a molecular reorganization event occurs that releases She2p from the Myo4p-She3p-ASH1 mRNA anchoring complex. Our results with She3p-S348E and She3p-S343E S361E support our previous hypothesis that a Myo4p-She3p-ASH1 mRNA complex devoid of She2p does exist. However, our results do not distinguish between at least two possible models. In the first model, She2p could identify ASH1 mRNA in the nucleus and escort ASH1 mRNA to the cytoplasm, where She2p functions to directly load ASH1 mRNA onto She3p (Fig. 6A). This model necessitates that She3p possess intrinsic RNA-binding activity, and further suggests that no additional proteins may be present in the Myo4p-She3p-ASH1 mRNA complex. However, homology searches reveal no obvious RNA-binding motifs within She3p. Consequently, if She3p directly contacts ASH1 mRNA, the She3p-ASH1 mRNA association most likely occurs through a novel RNA-binding domain.

In the second model She2p could identify ASH1 mRNA in the nucleus and escort ASH1 mRNA to the cytoplasm where She2p functions to directly load ASH1 mRNA onto an unidentified RNA-binding protein that directly associates with She3p (Fig. 6B). This model predicts that the protein-protein interaction between She3p and the unidentified RNA-binding protein would be stable under the IP/RT-PCR conditions, unlike the She2p-She3p interaction. Furthermore, this model predicts that additional novel proteins remain to be identified in the Myo4p-She3p-ASH1 mRNA complex. Global two-hybrid and affinity purification experiments have determined that She3p associates with at least 28 proteins, and the possible role for 23 of these proteins in ASH1 mRNA localization remains to be determined (14, 17, 20, 21, 31, 38, 46, 49, 52, 55, 64–67, 69). However, none of the 23 proteins appear to be RNA-binding proteins. Consequently, if any of the 23 proteins have a role in ASH1 mRNA localization, it would be interesting to determine whether these proteins possess RNA-binding activity.

We initiated the studies presented here to address the hypothesis that phosphorylation of She3p regulates ASH1 mRNA localization activities. Global mass spectrometry approaches have determined that Ser 343, 348, and 361 are sites of phosphorylation (1, 27, 40), and our studies demonstrating that She3p-S348E and She3p-S343E S361E, which mimic constitutive phosphorylation, are defective for ASH1 mRNA localization support the hypothesis that phosphorylation of She3p negatively regulates a novel ASH1 mRNA localization...
activity. If She3p possesses intrinsic RNA-binding activity, phosphorylation of She3p at Ser 343, 348, and 361 could negatively regulate She3p RNA-binding activity. There is precedent for RNA-binding activity being negatively regulated by phosphorylation. Khd1p and Puf6p are RNA-binding proteins that function to translationally repress ASH1 mRNA until it reaches the bud, and the RNA-binding activities for Khd1p and Puf6p are negatively regulated by phosphorylation (16, 50). Furthermore, the RNA-binding activity for Zbp1p, which is involved in the localization of β-actin mRNA in chicken embryo fibroblasts, is also negatively regulated by phosphorylation (32). However, if She3p indirectly associates with ASH1 mRNA through another unidentified RNA-binding protein, this She3p protein-protein interaction could be negatively regulated by phosphorylation. Analogous to RNA-binding activity, there are numerous examples of protein-protein interactions being negatively regulated by phosphorylation.

If phosphorylation at Ser 343, 348, and 361 is regulating She3p ASH1 mRNA localization activities, one might have expected that alanine substitutions at these amino acids would adversely affect ASH1 mRNA localization. However, our findings that alanine substitutions at these amino acids retain ASH1 mRNA localization activity support the hypothesis that phosphorylation at Ser 343, 348, and 361 is not required for ASH1 mRNA localization. Furthermore, other phosphorylation events exhibit a similar phenotype. In S. cerevisiae, the activity for iron regulatory protein 1 (Irp1p) is regulated by phosphorylation at Ser 138. Analogous to She3p, the Irp1p-S138A mutant exhibits wild-type activity, while the activity for the S138E mutant is substantially reduced compared to wild-type Irp1p (8). It is particularly intriguing that the She3p mutant containing Ala at all of the Ser and Thr sites of phosphorylation is active for ASH1 mRNA localization. If phosphorylation of She3p is required for ASH1 mRNA localization, possibly additional redundant sites need to be altered to observe an effect on ASH1 mRNA localization. Since She3p contains 69 Ser residues and 26 Thr residues, it remains a distinct possibility that additional sites of phosphorylation remain to be identified.

Global kinase studies indicate that She3p can be phosphorylated in vitro by at least 13 yeast kinases: Atg1p, Hsl1p, Ime2p, Ksp1p, Pko85p Pe1p, Prk1p, Rad53p, Snf1p, Ste20p, Swe1p, Tpk1p, Yck1p, and Yck2p (52). Using the yeast deletion collection we have examined if the absence of Atg1p, Hsl1p, Tpk1p, Yck1p, and Yck2p delocalize ASH1 mRNA and, consistent with our analyses of serine-to-alanine amino acid substitutions in She3p, we observed no evidence that phosphorylation of She3p is necessary for ASH1 mRNA localization. Based on our results with She3p-S348E and S348E S361E, we would predict that the absence of the phosphatases responsible for removing the phosphate group from Ser 343, 348, and 361 should exhibit a defect in ASH1 mRNA localization. However, the identity of candidate phosphatases remains elusive.

In conclusion, our studies provide compelling evidence that She3p possesses a novel function required for ASH1 mRNA localization and imply that the novel function is related to the ability of She3p to associate with ASH1 mRNA. Furthermore, our results suggest that phosphorylation could negatively regulate the novel She3p-RNA localization activity. In the future the identification and characterization of the novel She3p-RNA localization activity will provide new insight into the mechanism responsible for ASH1 mRNA localization.

ACKNOWLEDGMENTS

We thank Amy Hudson’s laboratory for generously providing the HA antibody. We also thank Claudia Kale for helpful discussions and critically reading the manuscript. This study was supported by NIH grant GM60392 and the Pew Scholars Program in the Biomedical Sciences.

REFERENCES

1. Albuquerque, C. P., R. B. Smolka, S. H. Payne, V. Bafna, J. Eng, and H. Zhou. 2008. A multidimensional chromatography technology for in-depth phosphoproteome analysis. Mol. Cell Proteomics 7:1389–1396.
2. Andoh, T., Y. Oshiro, S. Hayashi, H. Takeo, and T. Tani. 2006. Visual screening for localized RNAs in yeast revealed novel RNAs at the bud-tip. Biochem. Biophys. Res. Commun. 351:999–1004.
3. Bashdallah, A., R. L. Cooperstock, and H. D. Lipshitz. 1998. RNA localization in development. Annu. Rev. Biochem. 67:335–394.
4. Beach, D. L., E. D. Salmon, and K. Bloom. 1999. Localization and anchoring of mRNAs in budding yeast. Curr. Biol. 9:569–578.
5. Chartrand, P., R. P. Jansen, T. Minamitani, and K. Nasmyth. 1996. Asymmetric accumulation of Ash1p in a posterior body nucleolus depends on a myosin and restricts yeast mating-type switching to mother cells. Cell 84:699–709.
6. Bohl, F., C. Kruse, A. Frank, D. Ferring, and R. F. Jansen. 2000. She2p, a novel RNA-binding protein tethers ASH1 mRNA to the Myo10p myosin motor via She3p. EMBO J. 19:5514–5524.
7. Brown, N. M., S. A. Anderson, D. W. Steffen, T. B. Carpenter, M. C. Kennedy, W. E. Walden, and R. S. Eisenstein. 1998. Novel role of phosphorylation in Fe-S cluster stability revealed by phosphomimetic mutations at Ser-138 of an iron regulatory protein 1. Proc. Natl. Acad. Sci. USA 95:15235–15240.
8. Chartrand, P., E. Bertrand, R. H. Singer, and R. M. Long. 2000. Sensitive and high-resolution detection of RNA in situ. Methods Enzymol. 318:493–506.
9. Chartrand, P., X. H. Meng, S. Hurttemaier, D. Donato, and R. H. Singer. 2002. Asymmetric sorting of Ash1p in yeast results from inhibition of translation by localization elements in the mRNA. Mol. Cell 10:1319–1330.
10. Chartrand, P., X. H. Meng, R. H. Singer, and R. M. Long. 1999. Structural elements required for the localization of ASH1 mRNA and of a green fluorescent protein reporter particle in vivo. Curr. Biol. 9:333–336.
11. Choi, A., C. Hutterhoffer, L. Y. Geer, J. J. Coon, J. E. Syka, D. L. Bai, J. Shabanowitz, D. L. Burke, O. G. Tsylyshnyakaya, and D. F. Hunt. 2007. Analysis of phosphorylation sites on proteins from Saccharomyces cerevisiae by electron transfer dissociation (ETD) mass spectrometry. Proc. Natl. Acad. Sci. USA 104:2193–2198.
12. Collins, S. R., P. Kemmeren, X. C. Zhao, J. F. Greenblatt, F. Spencer, F. C. Holstege, J. S. Weissman, and N. J. Krogan. 2007. Toward a comprehensive atlas of the physical interactions of Saccharomyces cerevisiae. Mol. Cell Proteomics 6:439–450.
13. Darzacq, X., E. Powrie, W. Gu, R. H. Singer, and D. Zenklusen. 2003. RNA asymmetric distribution and daughter/mother differentiation in yeast. Curr. Opin. Microbiol. 6:614–620.
14. Deng, Y., R. H. Singer, and W. Gu. 2008. Translation of ASH1 mRNA is repressed by PufU-Pum200p-PUM2 interaction and released by CK2 phosphorylation. Genes Dev. 22:1037–1050.
15. Drees, B. L., B. Sundin, E. Brazeau, J. P. Caviston, G. C. Chen, W. Guo, K. G. Kozminski, M. W. Lau, J. J. Moskow, A. Tong, L. R. Schenkman, A. Mckenzie III, P. Brennwald, M. Longtine, E. Bi, C. Chan, P. Novick, C. Boone, J. R. Pringle, T. N. Davis, S. Fields, and D. G. Drabkin. 2001. A protein interaction map for cell polarity development. J. Cell Biol. 154:549–571.
16. Even, T. G., S. Jellbauer, M. Muller, M. Schmid, D. Niessing, and R. P. Jansen. 2008. Nuclear transit of the RNA-binding protein She2 is required for translational control of localized ASH1 mRNA. EMBO Rep. 9:781–787.
17. Dunn, B. D., T. Sakamoto, M. S. Hong, J. R. Sellers, and P. A. Takizawa. 2007. Myo10p is a monomeric myosin with motility uniquely adapted to transport mRNAs. J. Cell Biol. 178:1193–1206.
18. Gavin, A. C., P. Aloy, P. Grandi, R. Krause, M. Boesche, M. Marzioch, C. Rau, L. J. Jensen, S. Bastuck, B. Dempfleld, A. Edelmann, M. A. Heurtier,
40. Li, X., S. A. Gerber, A. D. Rudner, S. A. Beau soleil, W. Haas, J. Villen, J. E. Elias, and S. P. Gygi. 2007. Large-scale phosphorylation analysis of alpha-factor-arrested Saccharomyces cerevisiae. J. Proteome Res. 6:1190–1197.
41. Long, R. M., W. Gu, E. Lorimer, R. H. Singer, and P. Chartrand. 2000. Shc2p is a novel RNA-binding protein that recruits the Myo4p-Shc3p complex. EMBO J. 19:6562–6570.
42. Long, R. M., and J. E. Hopper. 1995. Genetic and carbon source regulation of phosphorylation of Sip1p, a Snf1p-associated protein involved in carbon response in Saccharomyces cerevisiae. Yeast 11:233–246.
43. Long, M., R. H. Singer, J. Gonzalez, K. Nasmyth, and R. P. Jansen. 1997. Mating type switching in yeast controlled by asymmetric localization of Ash1 mRNA. Science 277:383–387.
44. Lopez de Heredia, M., and R. P. Jansen. 2004. mRNA localization and the yeast cell cycle. Curr. Opin. Cell Biol. 16:85–90.
45. Mason, M. E., and I. Herskowitz. 2001. Ash1p is a site-specific DNA-binding protein that actively represses transcription. Proc. Natl. Acad. Sci. USA 98:1495–1500.
46. Millson, S. H., A. W. Truman, V. King, C. Prodromou, L. H. PEAR, and P. W. PIPER. 2005. A two-hybrid screen of the yeast genome for Hsp90 interactors uncovers a novel Hsp90 chaperone requirement in the activity of a stress-activated mitogen-activated protein kinase, Slit2p (Mpk1p). Eukaryot. Cell 4:489–490.
47. Munchow, S., C. Sauter, and R. P. Jansen. 1999. Association of the class V myosin Myo4p with a localized messenger RNA in budding yeast depends on She proteins. J. Cell Sci. 112(Pt 10):1511–1518.
48. Niesing, D., S. Huttelmaier, D. Zenklusen, R. H. Singer, and S. K. Burley. 2004. Shc2p is a novel RNA-binding protein with a basic helical motif. Cell 119:491–502.
49. Oeflting, M., K. E. Wei, R. Rogers, J. A. DeGrasse, B. T. Chait, J. D. Aitchison, and M. P. Rout. 2007. Comprehensive analysis of diverse ribonucleoprotein complexes. Nat. Methods 4:951–958.
50. Paquin, N., M. Menade, G. Poirier, D. Donato, E. Drouet, and P. Chartrand. 2007. Local activation of yeast ASH1 mRNA translation through phosphorylation of Kdh1p by the casein kinase Yck1p. Mol. Cell 26:795–809.
51. Peng, D. S., D. Schwartz, J. E. Elias, C. C. Thoreen, D. Cheng, G. Marsischky, J. Roelofs, D. Finley, and S. P. Gygi. 2003. A proteomics approach to understanding protein ubiquitination. Nat. Biotechnol. 21:921–926.
52. Pocsek, J., G. Devgan, G. Micheaud, H. Zhu, X. Zhu, J. Fasolo, H. Guo, J. Gona, J. Broitkruz, R. Supko, R. R. McCarty, M. C. Schmidt, N. R. Jansen, S. A. Lee, A. M. Mah, J. M. Stark, D. F. Stern, C. Del Greiguous, M. Tyers, B. Andrews, M. Gerstwein, B. Schweizer, P. F. Predki, and M. Snyder. 2005. Global mapping of protein phosphorylation in yeast. Nature 438:679–683.
53. Rose, M. D., F. Winston, and P. Hieter. 1990. Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
54. Sambrook, J., E. F. Fritch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
55. Sanders, S. L., J. Jennings, A. Canutescu, J. A. Link, and P. A. WEL. 2002. Protocemes of the eukaryotic transcription machinery: identification of proteins associated with components of yeast TFID by multidimensional mass spectrometry. Mol. Cell. Biol. 22:4723–4736.
56. Shen, Z., N. Paquin, A. Forget, and P. Chartrand. 2009. Nuclear shutting of Shc2p couples ASH1 mRNA localization to its translational repression by recruiting Loc1p and Pup1p. Mol. Biol. Cell 20:2260–2275.
57. She, K. A., A. A. Khrapko, O. U. Gnedin, M. E. Maxon, M. E., and I. Herskowitz. 2005. A two-hybrid screen of the yeast proteome for Hsp90 interactors identifies multiple secondary structural elements and Ash1 protein translation. Curr. Biol. 15:141–147.
58. Smolka, M. B., C. P. Albuquerque, S. H. Chen, and H. Zhou. 2007. Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases. Proc. Natl. Acad. Sci. USA 104:10364–10369.
59. St. Johnston, D. 2005. Moving messages: the intracellular localization of mRNAs. Nat. Rev. Mol. Cell Biol. 6:363–375.
60. Takizawa, P. A., J. L. DeRisi, J. E. Wilhelm, and R. D. Vale. 2005. Moving messages: the intracellular localization of mRNAs and the yeast cell cycle. Curr. Opin. Cell Biol. 17:85–90.
61. Takeda, T., T. Nakao, Y. Nakamura, T. Sugita, R. M. Long, and B. Andrews. 2007. Widespread cytoplasmic mRNA transport in yeast: identification of 22 bud-localized transcripts using DNA microarray analysis. Proc. Natl. Acad. Sci. USA 104:11429–11434.
62. Temple, J., and I. Herskowitz. 1996. Identification of asymmetrically localized determinant, Ash1p, required for lineage-specific transcription of the HO gene. Cell 84:711–717.
63. Smolka, M. B., C. P. Albuquerque, S. H. Chen, and H. Zhou. 2007. Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases. Proc. Natl. Acad. Sci. USA 104:10364–10369.
64. St. Johnston, D. 2005. Moving messages: the intracellular localization of mRNAs. Nat. Rev. Mol. Cell Biol. 6:363–375.
65. Takizawa, P. A., J. L. DeRisi, J. E. Wilhelm, and R. D. Vale. 2005. Moving messages: the intracellular localization of mRNAs and the yeast cell cycle. Curr. Opin. Cell Biol. 17:85–90.
66. Takizawa, P. A., A. S. Mah, J. R. Swedlow, I. Herskowitz, and R. D. Vale. 1999. Genetic and carbon source regulation of localization of the ASH1 mRNA ribonucleoprotein complex. RNA 5:1381–1389.
67. She, K. A., A. A. Khrapko, O. U. Gnedin, M. E. Maxon, M. E., and I. Herskowitz. 2005. A two-hybrid screen of the yeast proteome for Hsp90 interactors identifies multiple secondary structural elements and Ash1 protein translation. Curr. Biol. 15:141–147.
68. Smolka, M. B., C. P. Albuquerque, S. H. Chen, and H. Zhou. 2007. Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases. Proc. Natl. Acad. Sci. USA 104:10364–10369.
69. St. Johnston, D. 2005. Moving messages: the intracellular localization of mRNAs. Nat. Rev. Mol. Cell Biol. 6:363–375.
and C. Boone. 2001. Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science 294:2364–2368.

66. Uetz, P., L. Giot, G. Cagney, T. A. Mansfield, R. S. Judson, J. R. Knight, D. Lockshon, V. Narayan, M. Srinivasan, P. Pochart, A. Qureshi-Emili, Y. Li, B. Godwin, D. Conover, T. Kalbfleisch, G. Vijayadamodar, M. Yang, M. Johnston, S. Fields, and J. M. Rothberg. 2000. A comprehensive analysis of protein–protein interactions in Saccharomyces cerevisiae. Nature 403:623–627.

67. Uetz, P., and R. E. Hughes. 2000. Systematic and large-scale two-hybrid screens. Curr. Opin. Microbiol. 3:303–308.

68. Zhang, B., M. Gallegos, A. Puoti, E. Durkin, S. Fields, J. Kimble, and M. P. Wickens. 1997. A conserved RNA-binding protein that regulates sexual fates in the Caenorhabditis elegans hermaphrodite germ line. Nature 390:477–484.

69. Zhao, R., M. Davey, Y. C. Hsu, P. Kaplanek, A. Tong, A. B. Parsons, N. Krogan, G. Cagney, D. Mai, J. Greenblatt, C. Boone, A. Emili, and W. A. Houry. 2005. Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone. Cell 120:715–727.