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# Mother Cell–Specific *HO* Expression in Budding Yeast Depends on the Unconventional Myosin Myo4p and Other Cytoplasmic Proteins

**Ralf-Peter Jansen, Celia Dowzer,* Christine Michaelis, Marta Galova, and Kim Nasmyth**
Research Institute of Molecular Pathology
A-1030 Vienna
Austria

## Summary

**Certain cell types give rise to progeny that adopt different patterns of gene expression in the absence of any differences in their environment. Cells of budding yeast give birth to mother and daughter cells that differ in that only mother cells express the *HO* endonuclease gene and thereby switch mating types. We describe the identification of five genes, called *SHE1–SHE5*, that encode cytoplasmic proteins required for mother-specific *HO* expression. She1p, which is identical to the minimyosin Myo4p, and She3p are not, however, mother-specific proteins. On the contrary, they accumulate in growing buds. She proteins might be required for the transport of factors that promote *HO* repression from the mother cell into its bud. In an accompanying paper, we show that *SHE* genes are needed for the accumulation in daughter nuclei of Ash1p, a repressor of *HO*.**

## Introduction

The characteristics of different cell types in multicellular organisms are largely determined by differences in their patterns of gene expression. How cells give birth to daughter cells with differing gene expression during embryogenesis is a crucial problem. Many studies have shown that gene expression can be influenced by external factors. Sister cells frequently find themselves in different environments, in which one or the other is exposed to different factors secreted by neighboring cells. However, in the development of several organisms there are many examples of sister cells adopting different cell fates in the absence of any differences in their surroundings (Horvitz and Herskowitz, 1992). Whether this is due to differential segregation between sister cells of cytoplasmic or nuclear factors is in many cases not known. The problem of identifying such factors has so far only been amenable to genetic analyses, mainly in flies and worms.

Examples of cytoplasmic factors whose cellular distribution eventually influences gene expression are those that determine the major body axes in Drosophila. During oogenesis, *bicoid* and *oskar* mRNAs are localized to the anterior and posterior ends of the egg, respectively, and this later leads to different patterns of gene expression in nuclei that end up in the anterior or posterior of the cytoplasm (St Johnston, 1995). The factors whose location in Drosophila embryos determines subsequent cell fate are often mRNAs (e.g., *bicoid* and *oskar*) or proteins that regulate mRNA translation (e.g., Nanos; Curtis et al., 1995). Another good example is the different accumulation of GLP-1 protein in the two cell blastomeres of Caenorhabditis elegans; this asymmetry is also due to differences in mRNA translatability (Evans et al., 1994).

Studies using inhibitors have implicated the cytoskeleton in the segregation of developmental determinants. Destruction of the actin microfilament network of C. elegans with cytotochalasin B disrupts the asymmetric segregation of germline P granules (Hill and Strome, 1988), whereas disruption of microtubules in Drosophila oocytes by colchicine treatment abolishes the posterior localization of *oskar* mRNA (Ferrandon et al., 1994; Clark et al., 1994). However, the results obtained with such drastic treatments are hard to interpret. Cytoskeletal drugs could interfere with the cell fate of daughter cells either by directly affecting the distribution of determinants or by indirectly upsetting the structure of the cytoskeleton of the mother cell, upon which the asymmetry of the cell is dependent. Genetic studies in Drosophila have recently implicated filamentous actin in the segregation of determinants. Tropomyosin, which stabilizes actin filaments, is required for the posterior localization of *oskar* mRNA in oocytes (Erdelyi et al., 1995). Here we describe genetic evidence that determinants can be segregated by myosin-based motors. We have found that the minimyosin Myo4p and several other cytoplasmic proteins are required for mother cell–specific *HO* gene expression in the budding yeast Saccharomyces cerevisiae.

The *HO* endonuclease induces mating-type switching in S. cerevisiae by creating a double-stranded break at the *MAT* locus. The *HO* gene is only transcribed in "mother" cells, i.e., cells that have previously budded and given birth to a "daughter" cell. In mother cells, *HO* is transcribed transiently during the cell cycle, shortly before budding and DNA replication (Nasmyth, 1993). *HO* activation depends on at least ten genes, termed *SWI1* through *SWI10*. *SWI4* and *SWI6* encode subunits of a cell cycle–regulated transcription factor, SCB-binding factor (SBF), that activates a number of genes at the G1/S boundary (Koch and Nasmyth, 1994). Other *SWI* genes, for example *SWI1*, *SWI2*, *SWI3*, and *SWI10*, encode components of a large, multisubunit complex, which is needed for the expression of many yeast genes (Peterson and Herskowitz, 1992).

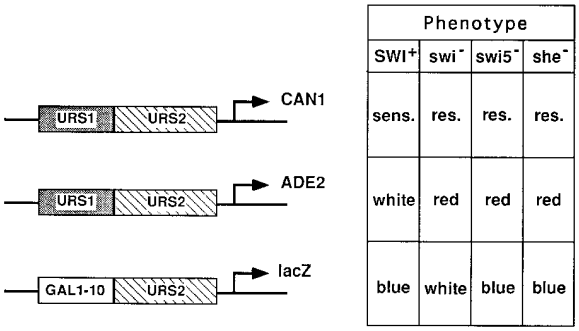
A crucial role in directing mother cell–specific *HO* expression has been established for the transcription factor encoded by *SWI5* (Nasmyth, 1993). Swi5p binds to two sites in a region of the *HO* promoter, called URS1, that directs mother-specific *HO* expression. However, Swi5p is not a mother cell–specific determinant, and its properties cannot explain the asymmetry of *HO* activation. It is synthesized during G2 and M phases, but remains in the cytoplasm until late anaphase, upon which it enters both mother and daughter nuclei and is rapidly degraded (Nasmyth et al., 1990; Tebb et al.,

*Present address: Asia Pacific Fermentation Industries, 5 Belvoir Road, Cambridge CB4 1JQ, United Kingdom.

1993). The transient accumulation of Swi5p in mother and daughter cell nuclei as cells enter G1 leads to the activation of several other genes whose transcription is Swi5p dependent (Koch and Nasmyth, 1994). *HO* expression, in contrast, does not occur at this time because it must await the subsequent activation of SBF at the G1 to S phase transition.

A second gene that is required for mother-specific *HO* expression, *SDI1/SIN3* is also unlikely to encode the determinant of asymmetric *HO* expression. Mutations that affect Sin3p allow *HO* to be expressed in the absence of Swi5p and cause *HO* to be expressed in daughter cells (Nasmyth et al., 1987; Sternberg et al., 1987). However, like Swi5p, Sin3 protein is found in both mother and daughter cell nuclei at the end of mitosis (Wang et al., 1990).

We describe here the identification of five new genes, called *SHE1–SHE5* (for Swi5p-dependent *HO* expression) that are specifically required for *HO* expression in mother cells. These *SHE* genes encode cytoplasmic proteins, which in the case of She1p and She3p accumulate preferentially in growing buds. She1p is identical to the minimyosin Myo4p. This observation leads us to propose that She proteins are involved in the transport from mother cells to their buds of a factor (or perhaps a repressor) that contributes to *HO* repression. *she* mutants might fail to express *HO* because the factor is now present in both mother and daughter cells. In an accompanying paper (Bobola et al., 1996 [this issue of *Cell*]), we show that the nuclear protein Ash1p (for asymmetric synthesis of *HO*), which is required to prevent *HO* expression in daughters, accumulates preferentially in daughter cell nuclei at the end of anaphase in a *SHE*-dependent manner.




| | Phenotype | | | |
|---|---|---|---|---|
| | SWI$^+$ | swi$^-$ | swi5$^-$ | she$^-$ |
| CAN1 | sens. | res. | res. | res. |
| ADE2 | white | red | red | red |
| lacZ | blue | white | blue | blue |

Mutants found in the *she* screen

| Gene | Number of alleles |
|---|---|
| *SWI* genes (except *SWI5*) | 3035 |
| *SWI5* | 222 |
| *SHE1* | 35 |
| *SHE2* | 9 |
| *SHE3* | 8 |
| *SHE4* | 1 |
| *SHE5* | 3 |

Figure 1. Screening Strategy

(Top) Three reporter constructs were used to identify *swi5*-like mutations. Owing to *HO* or *GAL–HO* promoter activity, the parental strain is sensitive to canavanine and gives rise to white colonies on adenine-free media and blue colonies on galactose medium. Mutants whose *HO* promoter does not function grow as red canavanine-resistant colonies. *she* and *swi5* mutants can be distinguished from mutants in other *SWI* genes by their ability to activate a lacZ reporter from a *GAL1-10–HO* hybrid promoter (see text) and give a blue colony color in a lacZ assay.

(Bottom) The number of mutant alleles identified in the *she* screen.

## Results

### Five Genes Needed for *HO* Expression

To identify factors that could be responsible for the differential activity of Swi5p at *HO* in mother and daughter cells, we designed a screen to isolate new mutants that, like *swi5* mutants, are specifically defective in URS1 function. We first replaced the promoters of *CAN1* and *ADE2* with that from *HO*. As a result of this exchange, *HO* promoter activity causes cells to be sensitive to the arginine analog canavanine and to form white colonies. Starting with a strain carrying both *HO–CAN1* and *HO–ADE2*, mutants whose *HO* promoter is inactive can be selected as red, canavanine-resistant colonies. However, the vast majority of mutations isolated by this means will be in genes involved in the SBF factor, the Swi–Snf complex, or repression of silent mating loci. To exclude these classes, we included a third reporter system: a *GAL–HO* promoter fusion driving the lacZ gene that is dependent on all *SWI* genes except *SWI5* and is repressed in diploid cells. Only mutants like *swi5*, which are specifically defective in the function of URS1, should still be capable of expressing lacZ in the presence of galactose (Figure 1).

Out of more than 3000 red canavanine-resistant colonies, only 315 expressed lacZ upon galactose induction; 222 recessive mutants failed to complement a *swi5* deletion and were assumed to be *swi5* alleles. We classified 46 mutants into five complementation groups, termed *she1–she5* (Figure 1). We were unable to classify 11 mutants. Out of 35 dominant mutations, 10 were shown by tetrad analysis to be *SHE1* alleles. Representative alleles of each *SHE* gene segregated 2:2 in crosses to strains with the parental genotype.

*she1*, *she2*, and *she3* mutants grow at wild-type rates and with a normal cell morphology at temperatures between 23°C and 37°C. *she5* mutants, in contrast, have a mild growth defect at all temperatures. Furthermore, about 30% of *she5* mutant cells have wide bud necks and often contain two large buds, which is indicative of a defect in separating daughter cells from their mothers (data not shown). The *she4* mutant has little or no growth defect, but a minor fraction (about 5%) of cells have wide bud necks resembling those of *she5* mutants.

### *she* Mutants Are Specifically Defective for *ho* Expression

To check whether *she* mutations affect expression of an authentic *ho* gene, we crossed representative *she* mutants with a wild-type strain carrying the *ho* allele and measured by Northern blotting the levels of *ho* RNA in *ho she* segregants. Little or no *ho* RNA can be detected in any of the *she* mutants (Figure 2A). In the case of *she4-4449* and two different mutant alleles of *SHE5*, larger than normal *ho* transcripts are found instead (Figure 2A, arrow). These abnormal transcripts were also detected using probes that hybridize to sequences between 1960 and 1270 bp upstream of the *ho* gene (Figure

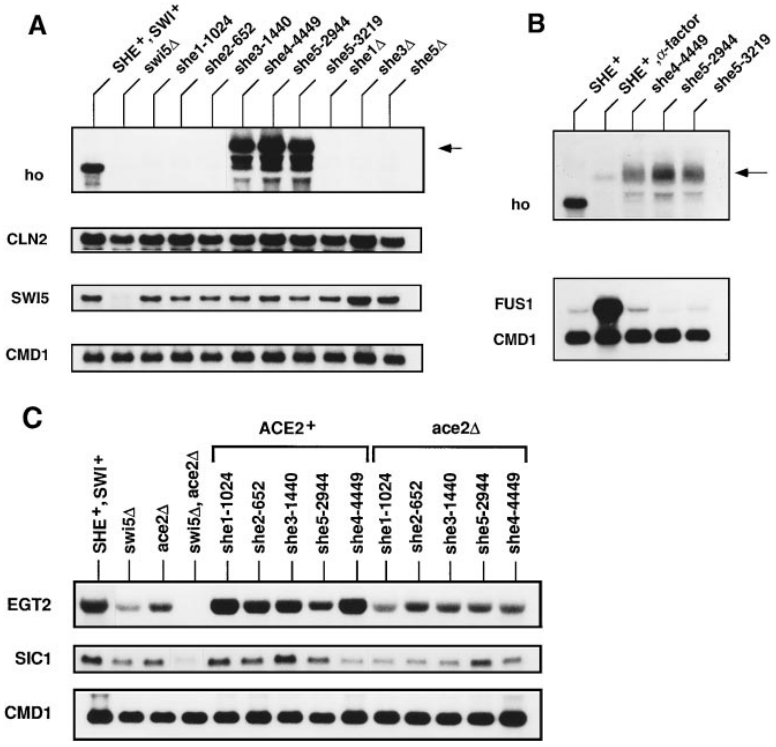



Figure 2. *she* Mutants Do Not Express *ho*

(A) RNA from exponentially growing *SHE*$^+$ (K699), *swi5*Δ (K1750), *she1-1024* (K4918), *she2-652* (K4919), *she3-1440* (K4917), *she4-4449* (K4925), *she5-2944* (K4913), *she5-3219* (K4914), *she1*Δ (K5209), *she3*Δ (K5234), and *she5*Δ (K5206) cells was analyzed by Northern blot. The filter was probed successively for *ho*, *CLN2*, *SWI5*, and *CMD1*. The arrow indicates the position of a large transcript that cross-hybridizes with probes against *ho*.

(B) RNA from wild-type (K699) cells, wild-type cells treated with the pheromone α factor, *she5-2944* (K4913) cells, and *she5-3219* (K4914) cells was probed successively with probes against the *ho* promoter (hybridizing to −1905 bp to −1221 bp of *ho*) and *FUS1*.

(C) RNA of exponentially growing *SHE*$^+$, *swi5*Δ, *ace2*Δ (K3772), *swi5*Δ *ace2*Δ (K3773), the indicated *she* mutant, and *ace2*Δ *she* double mutant cells was analyzed by Northern blotting. Filters were probed for *EGT2*, *SIC1*, and *CMD1*.

2B) and are due to transcription initiating far upstream of the normal *ho* initiation site. Similar transcripts appear in wild-type cells treated with pheromone (Figure 2B). Induction of the upstream transcripts in *she4* and *she5* mutants is not due to constitutive activation of pheromone signal transduction, because the *FUS1* gene is not induced in the absence of pheromone in *she* mutants.

*she* mutations did not affect expression of *CLN2*, another gene activated by SBF in late G1 (Koch and Nasmyth, 1994). More importantly, given that the mutants had been identified as having *swi5*-like defects in URS1 function, they did not affect expression of *SWI5* (Figure 2A). It seemed possible, nevertheless, that *SHE* genes might be needed for Swi5p activity. We therefore tested whether they were required for the expression of other *SWI5*-regulated genes. Transcription of *EGT2*, which is involved in cell separation, and *SIC1*, which encodes an inhibitor of cyclin B/Cdc28 kinases, depends on *SWI5* and a related gene, *ACE2* (Koch and Nasmyth, 1994; Kovacech and Schuster, submitted). *EGT2* and *SIC1* transcripts are modestly reduced in *ace2* and *swi5* single mutants, but are almost absent in *ace2 swi5* double mutants. We found that the levels of *EGT2* and *SIC1* transcripts were either only modestly or not at all affected by *she1*, *she2*, or *she3* mutations. The *she* mutations also had either little or no effect on *EGT2* or *SIC1* RNA levels in strains lacking *ACE2*, in which *EGT2* and *SIC1* expression is particularly dependent on *SWI5* (Figure 2C). The *ho* transcription defect in *she* mutants cannot therefore be due to a general reduction in the activity of Swi5p in the nucleus. In *she4* and *she5* mutants, the levels of *EGT2* and *SIC1* transcripts were, however, modestly reduced. Owing to the greater pleiotropy of these mutations, it is not clear whether this indicates a direct role for She4p or She5p in the activity of Swi5p or Ace2p.

### *SHE* Genes Affect Mating-Type Switching Only in Mother Cells

We introduced the *she* mutations into a *HO* strain and measured their effects on mating-type switching by pedigree analysis (Table 1). We found that 70% of mother cells and no daughters switch in wild-type cells. Mutations in *SHE1*, *SHE2*, *SHE3*, and *SHE4* reduced the frequency of mother cell switching to less than 6% and had no effect in daughters. Pedigree analysis of *she5* mutants was not possible owing to their cell separation defect. Although the effect of *she* mutations is not as extreme as that caused by a *swi5* mutation, the *SHE* genes are clearly essential for effective mating-type switching.

We next addressed whether *SHE* genes were involved in a process that is specific to mother cells. If the function of *SHE* gene products were mother cell specific, then *SHE* genes should not be necessary for mating-type switching in daughter cells caused by constitutive expression of *SWI5* from the *RP39* promoter (Lydall et al., 1991). We found that *she* mutations caused little or no reduction in daughter cell switching of *RP39–SWI5* mutant cells (Table 1). In *RP39–SWI5 she* double mutants, mothers and daughters switch with equal frequencies. This suggests that *SHE* genes might be important for generating the differences between mothers and daughters that restrict *HO* expression to mother cells. *RP39–SWI5* also causes germinating spores to switch mating types. None of the mutations *she1*, *she2*, or *she3* reduced the frequency of spore switching, but *she1* and *she2* mutations actually increased *RP39–SWI5*-induced

Table 1. Mating-Type Switching in *she* Mutants and *she RP39–SWI5* Double Mutants

| Strain | Mating-Type Switching (%) Mother | Daughter | Spores |
|---|---|---|---|
| *SHE⁺SWI⁺* | 70 (33 of 47) | 0 (0 of 42) | 0 (0 of 24) |
| *swi5-100* | 0 (0 of 88) | 0 (0 of 70) | 0 (0 of 30) |
| *she1-1024* | 1.5 (1 of 77) | 0 (0 of 68) | 0 (0 of 27) |
| *she2-652* | 5.5 (4 of 73) | 0 (0 of 58) | 0 (0 of 27) |
| *she3-1440* | 0.7 (1 of 151) | 0 (0 of 120) | 0 (0 of 36) |
| *she4-4449* | 3.0 (4 of 140) | 0 (0 of 104) | 0 (0 of 35) |
| *RP39–SWI5* | 70 (32 of 46) | 20 (8 of 42) | 50 (19 of 36) |
| *RP39–SWI5 she1-456* | 14 (16 of 112) | 15 (17 of 116) | 85 (62 of 72) |
| *RP39–SWI5 she2-652* | 18 (11 of 63) | 19 (11 of 58) | 81 (48 of 59) |
| *RP39–SWI5 she3-1440* | 28 (38 of 130) | 28 (35 of 128) | 55 (42 of 76) |

The frequency of switching in mother and daughter cells and in spores is shown. Percentages are at left, and the actual numbers of switches per division are within parentheses. Mating-type switching in *she* and *swi5* single mutants is shown in the upper group; the lower group is mating-type switching in *RP39–SWI5* and *RP39–SWI5 she* double mutants. The following homozygous diploid strains were used: H990 (*HO*), RPY34 (*HO swi5::URA3*), K1752 (*HO RP39–SWI5*), K4872 (*HO, she1-1024*), K4873 (*HO, she2-652*), K4874 (*HO she3-1440*), K4949 (*HO she4-4449*), K5108 (*HO RP39–SWI5 she1-456*), K5109 (*HO RP39–SWI5 she2-652*), and K5110 (*HO RP39–SWI5 she3-1440*).

spore switching. We currently have no explanation for this increase. Our results indicate that *she* mutations do not reduce *HO* expression either in daughter cells or in germinating spores when induced by *RP39–SWI5*, which contrasts with their dramatic effect of reducing *HO* expression in mother cells.

### *SHE1* and *SHE5* Genes Encode Cytoskeletal Proteins

To analyze further the function of She proteins, we isolated the *SHE1–SHE5* genes from a genomic library by virtue of their ability to complement the adenine auxotrophy of *she* mutants. This revealed that two genes, *SHE1* and *SHE5*, had previously been described, whereas *SHE2* and *SHE3* had been sequenced as part of the Yeast Genome Project, but had not been characterized.

We were surprised to find that none of the *SHE* genes encodes proteins that resemble transcription factors. The only plasmid complementing a *she1-456* mutation carried the *MYO4* gene encoding a class V minimyosin (Haarer et al., 1994) that is closely related to *MYO2* (57% amino acid identity), another class V myosin. Myo2p is required for vesicle transport from mother cells to their buds (Govindan et al., 1995). No function has hitherto been assigned to Myo4p. Disruption of *MYO4* has no apparent phenotype, either alone or in combination with other mutations that affect vesicle transport (Haarer et al., 1994).

We obtained two plasmids that complemented a *she5-2944* mutant. Both contained the *BNI1* gene. *bni1* mutations have been identified on the basis of their lethality in combination with a temperature-sensitive allele of the *CDC12* gene (Fares and Pringle, submitted) that encodes a septin protein required for cytokinesis (Sanders and Field, 1994). *bni* mutants, like *she5* mutants, are defective in some aspect of cytokinesis. *BNI1* encodes a 220 kDa protein that contains sequence motifs found in the FH1/2 protein family, which includes proteins involved in polarity establishment or cytokinesis (Castrillion and Wasserman, 1994; Emmons et al., 1995).

The *she2-652* mutation was complemented by plasmids containing sequences from chromosome XI (Dujon et al., 1994). The complementing activity resided in YKL130c, which we have renamed *SHE2*. *SHE2* has the potential to encode a 28 kDa protein with no homology to any other protein.

*she3-1440* was complemented by plasmids carrying a part of chromosome II with three open reading frames (ORFs) (Becam et al., 1994). Disruption of ORF YBR1005 destroyed the complementing activity. We renamed this ORF *SHE3*. It encodes a 47 kDa polypeptide with no significant homology to other proteins. The amino-terminal portion is predicted to form a coiled-coil domain extending over the first 200 amino acids.

Three plasmids were isolated that complemented the *she4-4449* mutant. The activity was localized to a 3 kb fragment carrying a 2376 bp ORF of chromosome XV adjacent to *PEP12*. *SHE4* encodes a 791 amino acid protein with no significant homology to other proteins.

To confirm that we had cloned the correct genes and to establish their null phenotypes, we replaced most of the coding sequences from each *SHE* gene with *URA3*. None of the five *SHE* genes is essential for mitotic growth. Tetrad analysis of crosses between *SHE* disruptions and the original *she* mutations confirmed that they were allelic. *ho* RNA is not detected by Northern blot analysis in cells deleted for *SHE1/MYO4*, *SHE2*, *SHE3*, *SHE4*, and *SHE5/BNI1* (Figure 2A; data not shown). In contrast with *she4-4449* and *she5-2944* mutant alleles, strains deleted for *SHE4* and *SHE5* did not cause the appearance of RNAs from upstream *ho* promoter sequences. The appearance of upstream *ho* transcripts in our *she4* and *she5* mutants is therefore specific for these alleles and cannot be responsible for their lack of *ho* expression.

### She1p, She3p, and She5p Localize to Buds

Given the mother cell specificity of *SHE* genes, we asked whether any of the *SHE* gene products accumulated preferentially in mother cells. We determined the cellular location of She proteins using epitope-tagged versions of *SHE1/MYO4*, *SHE2*, *SHE3*, and *SHE5/BNI1*. Endogenous *SHE* genes were replaced with versions that encoded three (in the case of *SHE2*) or six (in the case of *SHE1/MYO4*, *SHE3*, and *SHE5/BNI1*) copies of the c-Myc epitope tag (Evan et al., 1985) at their carboxyl termini. All four epitope-tagged *SHE* genes were fully functional, since they sustained wild-type levels of *ho* transcription or *HO–ADE2* function (data not shown).

*SHE2–myc3*, *SHE3–myc6*, and *SHE1/MYO4–myc6* produced proteins of around 34, 57, and 180 kDa, respectively, values consistent with their predicted molecular masses (Figure 3A). Several bands are visible in blots produced from *BNI1–myc6* cells, the uppermost

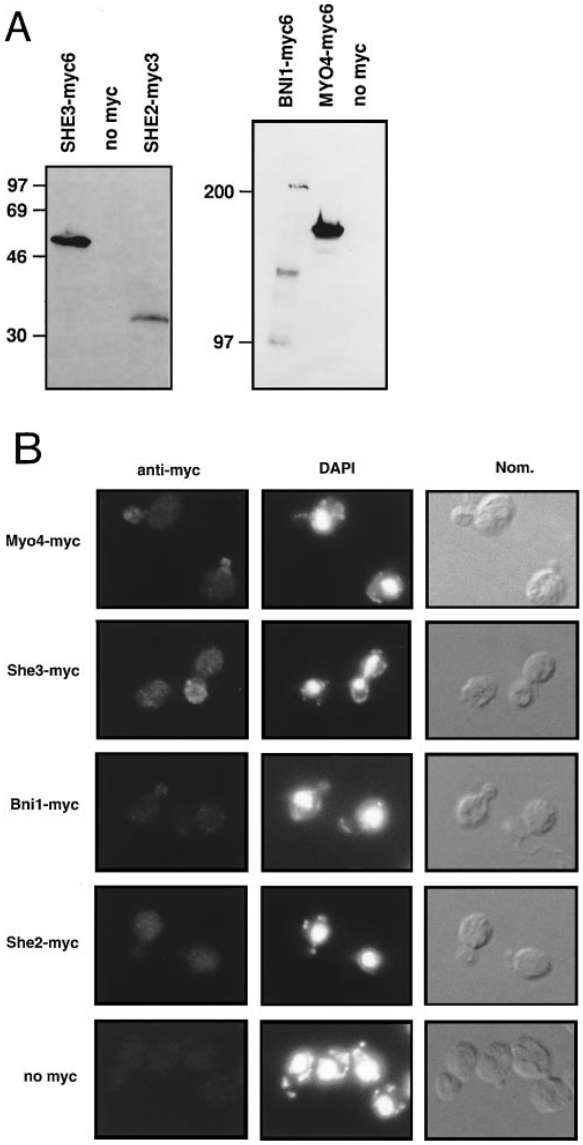



Figure 3. Myo4p and She3p Preferentially Accumulate in Buds

(A) Expression of Myc-tagged versions of *SHE1/MYO4*, *SHE2*, *SHE3*, and *SHE5/BNI1*. Proteins were extracted from strains expressing *MYO4–myc6* (K5543), *BNI1–myc6* (K5307), *SHE2–myc3* (K5542), *SHE3–myc6* (K5380), or no Myc epitope–tagged protein (K699) and separated on 6% (right) or 12% (left) SDS–polyacrylamide gels for Western blot analysis. Molecular mass standards are indicated on the left of each panel.

(B) Cells expressing the different Myc-tagged proteins as seen by indirect immunofluorescence with antibodies against the Myc epitope (diamidophenylindole [DAPI], DNA staining; Nom, Nomarski optics). Whereas She2p–Myc is seen mainly in the mother cell cytoplasm, She1p/Myo4p–Myc and She3p–Myc proteins are preferentially located in buds. Bni1p–Myc shows weak overall staining, but some preferential accumulation in only small buds is also visible.

of which corresponds to the expected size of an epitope-tagged Bni1 protein (230 kDa); the lower bands are probably degradation products.

We analyzed the intracellular distribution of the epitope-tagged proteins by indirect immunofluorescence. In all four cases, we detected cytoplasmic staining much greater than that of cells lacking a Myc epitope (Figure 3B). In the case of *SHE1/MYO4–myc6* and *SHE3–myc6*, fluorescence was always stronger in buds than in their mother cells, and this difference was particularly noticeable in cells with very small buds. In addition, some unbudded cells expressing She1p/Myo4p–Myc6 or She3p–Myc6 had a patch-like staining at one end of the cell, presumably at the site of the future bud (Figure 4B; data not shown). The overall cytoplasmic signal in most *BNI1–myc6* cells was very weak, but stronger staining in small buds was seen in some cells. Cytoplasmic She2p–Myc3 protein, in contrast, was mainly seen in the mother cell. Given their role in promoting *HO* transcription, it is remarkable that we could not detect any of the tagged She proteins in the nucleus. Indeed, signals due to She3p–Myc6 were sufficiently strong for its exclusion from the nucleus to be visible. It was also remarkable that none of the She proteins, with the possible exception of She2p, was mother cell specific in its distribution. On the contrary, She1p/Myo4p and She3p seem to accumulate preferentially in buds.

The preferential accumulation of She1p/Myo4p and She3p in buds is dependent both on the function of one another and on *SHE5/BNI1*, since disruptions of *SHE1*, *SHE3*, or *BNI1* result in a homogenous cytoplasmic staining of She1p/Myo4p–Myc6 or She3p–Myc6 (Figure 5). Disruption of *SHE2*, in contrast, does not affect accumulation of She1p and She3p in buds. This suggests that She1p/Myo4p, She3p, and She5p/Bni1p are involved in the same physiological process.

The distribution of She1p/Myo4p, She3p, and possibly She5p/Bni1p is reminiscent of that of "cap proteins," an unrelated group of proteins including Cdc42, Myo2p, Spa2p, and Rho1p (Lew and Reed, 1995). These proteins are named after their characteristic cap-like staining in unbudded and small-budded cells. Cap staining is lost as buds increase in size; many of the proteins become localized at the mother–daughter neck region at the end of mitosis (Lew and Reed, 1995). Some cap proteins (e.g., Cdc42, Myo2p, Bem2p, and Rho1p) are involved in budding, whereas others (e.g., Myo2p) are required for polarized growth.

To determine whether the distribution of She proteins is similar to that of cap proteins, we analyzed She3p–Myc6 distribution as a synchronous culture of daughter cells isolated by centrifugal elutriation progressed through the cell cycle. Western blotting (Figure 4A) showed that the cellular concentration of She3p–Myc6 protein remains constant during the cell cycle. In addition, *SHE3* transcripts (as well as *SHE1/MYO4*, *SHE2*, and *SHE5/BNI1* mRNAs; data not shown) did not vary during the cell cycle. She3p–Myc6 starts to accumulate, along with actin, at one side of a small daughter cell shortly before bud formation (Figure 4B, 75 min). It preferentially accumulates thereafter in growing buds; at this stage its distribution continues to resemble that of actin, although it is less patchy. Unlike actin, She3p–Myc6 does not form a ring at the bud neck in anticipation of cytokinesis, but continues to accumulate preferentially in buds until after anaphase (Figure 4C). However, it eventually redistributes equally between mother and daughter cell shortly before the mitotic spindle breaks down. She3p–Myc6 was asymmetric in 75 of 79 cells

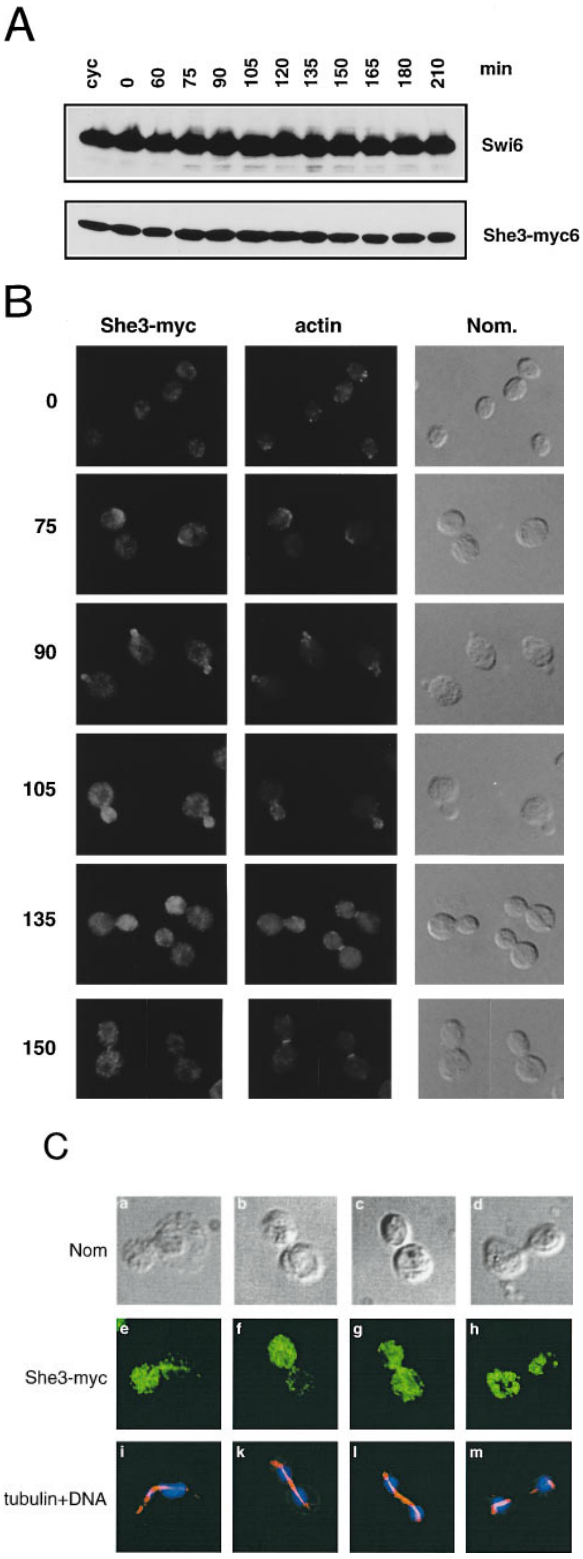



Figure 4. She3p Is Asymmetrically Distributed throughout Most of the Cell Cycle

(A) The ratio of She3p–Myc to total protein does not change during the cell cycle. Early G1 daughter cells (K5380, *SHE3–myc6*) were collected by centrifugal elutriation and incubated in YEP–raffinose at 30°C. A Western blot probed with anti-Myc and anti-Swi6p antibodies is shown. Numbers at the top indicate time.

with an elongated but undivided nucleus and in 67 of 118 binucleate cells with intact spindles, but was in only 5 of 60 binucleate cells lacking spindles. An analysis of asynchronous cultures suggests that She1p/Myo4p–Myc6 has a pattern similar to that of She3p–Myc6 (data not shown).

The changes in localization during the cell cycle of She1p/Myo4p and She3p resembles, but is not identical to, other cap proteins. To assess the specificity of the defect of the *she* mutants in *HO* transcription, we disrupted one of the two copies of *SPA2*, *RHO3*, and *RHO4* in a diploid strain homozygous for the *HO–ADE2* reporter gene. All four spores from each tetrad derived from strains heterozygous for any of the three disruptions were capable of growth in the absence of adenine. *HO* expression does not, therefore, depend on *SPA2*, *RHO3*, or *RHO4* (data not shown).

### Daughter, but Not Mother, Cell Cytoplasm Represses *HO*

Why should *HO* expression in mother cells depend on a myosin and other cytoskeletal proteins that accumulate in buds? One explanation is that *HO* is repressed in daughter cells by a factor that is transported from the mother cell cytoplasm into the bud by She1p/Myo4p and other She proteins. Thus, asymmetric *HO* expression may be due to the generation of differences between the cytoplasm of mothers and their daughters and not to differences between their nuclei.

This notion predicts that *HO* would not be expressed from a mother cell nucleus that finds itself in a daughter cell. The behavior of *esp1* mutants provides a means of testing this prediction. Despite a defect in anaphase, *esp1-1* mutant cells reenter the next cell cycle. Curiously, their single undivided nucleus is transported to the bud in 90% of these defective divisions, with the result that cytokinesis produces an "aploid" mother cell and a "diploid" daughter cell (McGrew et al., 1992). We compared *ho* RNA levels in wild-type and *esp1-1* mutants following incubation at 37°C of unbudded G1 daughter cells isolated by centrifugal elutriation (Figure 6). *ho* RNA accumulated to high levels in wild-type cells, but not in *esp1* mutants, as cells entered the second cell cycle (at 150 min for $ESP1^+$ and 240 min for *esp1-1* cells that are delayed for the entry into the second cell cycle). The failure of *esp1* mutants to express *ho* is not due to a general failure to reactivate SBF-regulated genes in the second cycle because *HCS26* RNAs reaccumulated to wild-type level upon entry into the cell

(B) Aliquots of cells were fixed at the indicated timepoints and analyzed by indirect immunofluorescence with anti-Myc antibodies and with tetramethylrhodamine B isothiocyanate–phalloidin (staining F-actin). Note that the asymmetric distribution of She3p–Myc disappears at the end of mitosis.

(C) Cells undergoing mitosis were simultaneously stained with anti-tubulin, anti-Myc, and DAPI to visualize the distribution of tubulin, DNA, and She3p–Myc at different stages of mitosis ([a], [e], and [i] are metaphase; [b], [c], [f], [g], [k], and [l] are anaphase; and [d], [h], and [m] are telophase). The asymmetric distribution of She3p–Myc is lost during anaphase (fully elongated spindle and separated nuclei). Note that some of the anti-Myc signal is the result of cross-reactivity with the anti-tubulin staining.

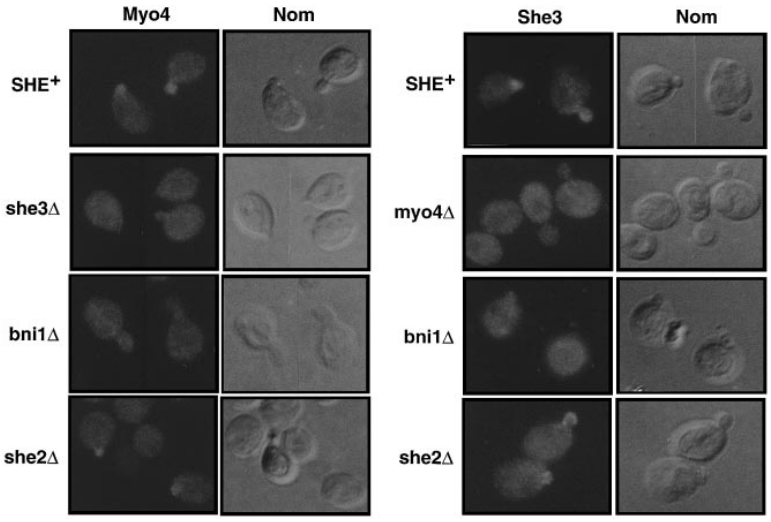



Figure 5. The Accumulation of She1p/Myo4p and She3p in Buds Is Dependent on Other *SHE* Genes

Cells of the following strains that express either *SHE1/MYO4–myc* or *SHE3–myc* and carry disruptions in different *SHE* genes were prepared for indirect immunofluorescence with antibodies against the Myc epitope: K5543 (*MYO4–myc*), K5706 (*she3*Δ *MYO4–myc*), K5707 (*bni1*Δ *MYO4–myc*), K5705 (*she2*Δ *MYO4–myc*), K5380 (*SHE3–myc*), K5458 (*myo4*Δ *SHE3–myc*), K5456 (*bni1*Δ *SHE3–myc*), and K5457 (*she2-652 SHE3–myc*). Disruption of *SHE1/MYO4*, *SHE3*, and *BNI1*, but not of *SHE2*, prevents the accumulation of She1p/Myo4p–Myc and She3p–Myc in buds.

cycle of diploid *esp1* daughter cells (data not shown; Surana et al., 1993).

We also performed the converse experiment; that is, we tested whether *ho* would be expressed when both mother and daughter nuclei find themselves in a mother cell. *tub2-401* mutants have a defect in cytoplasmic microtubules, which causes nuclear division to take place within the mother cell instead of the bud neck. As a consequence, both sets of sister chromatids remain in the mother cell after cytokinesis, which produces aploid daughter cells and dikaryotic mother cells (Sullivan and Huffaker, 1992). As shown in Figure 6, *ho* RNA accumulates to wild-type levels as *tub2-401* dikaryotic cells enter the second cell cycle (at 180 min for *TUB2*$^+$ and 210 min for *tub2-401* cells). This result shows, furthermore, that nuclei destined for daughter cells cannot act autonomously to make a *trans*-acting repressor of *HO*. Although this situation is not exactly the converse of the situation in *esp1* mutants, the pattern of *ho* expression in *tub2* and *esp1* mutants is consistent with the notion that it is determined by cytoplasmic factors.

## Discussion

### Asymmetric *HO* Expression

Cell division in S. cerevisiae occurs by budding and is therefore an inherently asymmetric process. Despite this, mother cells and their buds have in most regards similar developmental fates. One clear instance where mothers and their daughters have very different developmental fates is mating-type switching. Spores of homothallic yeast bud to produce a mother cell that switches its mating type and a daughter cell that does not. As a result, the progeny of mother and daughter cells express complementary sets of pheromones and receptors, which enables conjugation between the two sets of progeny (Nasmyth, 1993).

The ability of mothers, but not their daughters, to switch mating types is due to differential expression of the *HO* gene. *HO* is expressed transiently shortly before mothers bud, but never during the daughter cell cycle. Previous studies have highlighted the role of Swi5p in mother cell–specific *HO* expression. However, Swi5p accumulates to equal extents in mother and daughter nuclei at the end of anaphase, and it is not known why it is active at the *HO* promoter only in mothers. To identify factors that influence the activity of Swi5p on the *HO* promoter, we have isolated a new set of mutants with *swi5*-like defects in *HO* transcription. We identified mutations in five genes, *SHE1–SHE5*, that were not known to have a role in *HO* expression. The rarity of *she* mutants (none was identified in previous screens) is hard to explain, because many She proteins are not required for cell proliferation. We therefore suspect that mutations are more difficult to generate in *SHE* genes than in *SWI* genes.

### *SHE* Genes Encode Cytoplasmic Proteins, Including a Myosin

*SHE1–SHE5* have a very specific function in activating *HO* in mother cells. They are neither required for expression of other Swi5p-dependent genes nor for expression of *HO* in daughter cells when induced by ectopic *SWI5* expression. We cloned four of the genes, tagged them with Myc epitopes, and analyzed the location of their gene products. We found that She1p is identical to the minimyosin Myo4p and that She1p, She2p, She3p, and She5p are located in the cytoplasm. Surprisingly, both She1p/Myo4p and She3p accumulate preferentially in buds (in an interdependent manner).

*she1/myo4*, *she2*, and *she3* mutants have no obvious defects in budding, distribution of actin (data not shown), or cytokinesis. Moreover, other proteins that accumulate preferentially in growing buds, like Spa2p, or proteins necessary for bud growth, like Rho3p and Rho4p, are not needed for *HO* expression. This suggests that She proteins have a direct and specific role in this process.

### A Daughter Cell–Specific Repressor?

Our finding that Myo4p and She3p accumulate in buds indicates that these two proteins might be involved in the movement of factors from their predominant site of synthesis in the mother cell into growing buds. Myo4p,

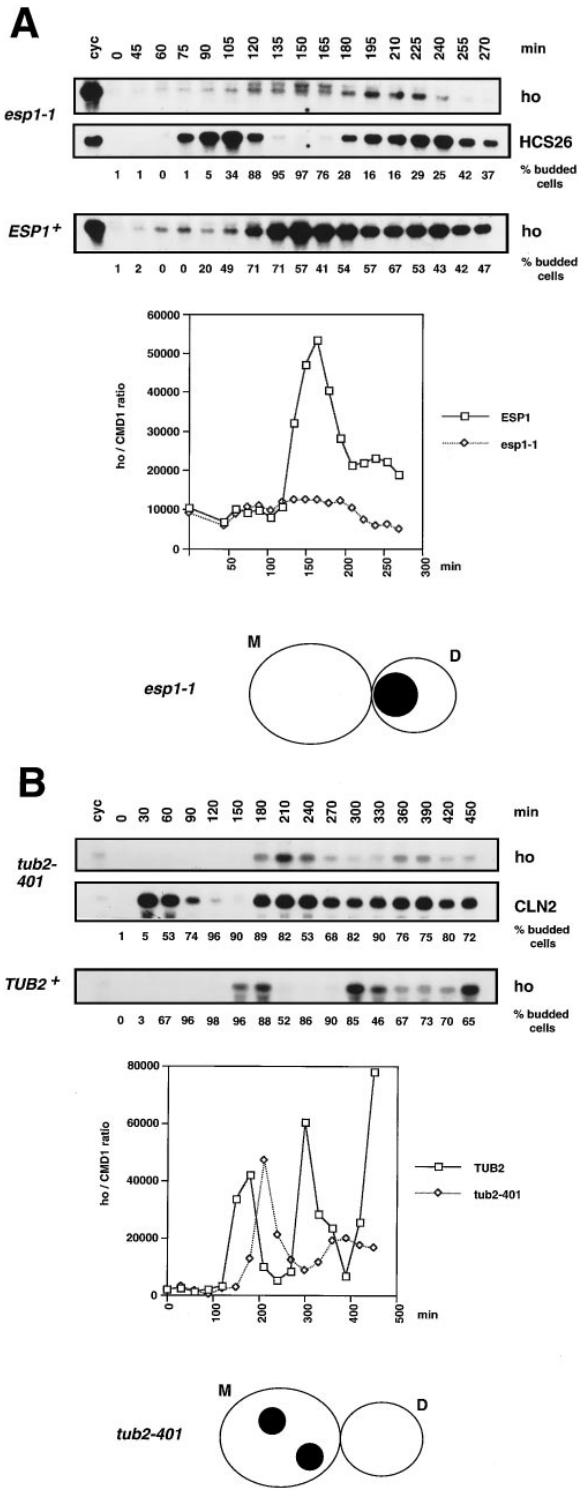



Figure 6. Nuclei Located in Daughter Cells Cannot Express *ho*

(A) Early G1 daughter cells of *esp1-1* (K2788) and *ESP1* (K699) strains were isolated by centrifugal elutriation and released into YEP–raffinose at 37°C (restrictive temperature). RNA was isolated at the indicated timepoints after release and probed for *ho*, *CMD1* (data not shown), and *HCS26* mRNAs. Shown are a Northern blot and budding index (top); the ratio of *ho* to *CMD1* as quantified by a Molecular Dynamics phosphoimager (middle); and a schematic presentation of the morphological phenotype of *esp1-1* cells at 37°C (bottom). The closed circle in the schematic (bottom) represents the nucleus. Note that the weak *ho* signal in the first cell cycle of the elutriated *ESP1* daughter cells (75 min) is due to a contamination with mother cells (5%).

(B) *tub2-401* (K5429) and *TUB2* (K1168) cells were synchronized by addition of α factor and released into YEPD at 18°C (restrictive temperature). Shown are a Northern blot, probed for *ho* and *CLN2* mRNAs, and a budding index (top); the ratio of *ho* to *CMD1* as quantified by a Molecular Dynamics phosphoimager (middle); and a schematic presentation of the morphological phenotype of *tub2-401* cells at 18°C (bottom). Closed circles (bottom) represent nuclei. Note that *ho* is not expressed in the first G1 phase (30 min) after release from an α factor arrest.

unlike its relative Myo2p, is not required for bud growth (Haarer et al., 1994). Myo2p (Lillie and Brown, 1994) is thought to use actin cables running from the mother to the bud to transport secretory vesicles from mother cells into their buds. Given the similarity of Myo2p and Myo4p, we suppose that Myo4p transports substances in a similar manner. If so, these two proteins would seem to transport very different cargos, because *HO* expression is highly dependent on *MYO4*, while the defective budding of *myo2-66* mutants is not exacerbated by deletion of *MYO4*.

To explain the dependence of *HO* transcription on *SHE* genes, we propose that the function of Swi5p as a transcription factor is inhibited by a protein whose exclusion from mothers depends on transport mediated by She1p/Myo4p. In an accompanying paper, we describe the identification of a protein with these properties. Ash1p is a protein necessary to repress *HO* in daughter cells and whose preferential accumulation in daughter cell nuclei at the end of mitosis is dependent on *SHE* genes (Bobola et al., 1996; Sil and Herskowitz, 1996 [this issue of *Cell*]). Our demonstration that *HO* cannot be expressed in *esp1* mutants in which both mother and daughter chromatids find themselves in the daughter cell suggests that the cytoplasm of daughter cells contains factors that promote *HO* repression. Such factors might be required for the synthesis or accumulation of Ash1p and might be transported into daughter cells by She1p/She3p.

### She Protein Functions

If Myo4p is used to move determinants from the mother to the daughter cell, what might be the role of the other She proteins? *she5/bni1* mutants have wide bud necks, are slow in undergoing cytokinesis, are altered in bud site positioning, and are lethal in combination with mutations in *CDC12* (Fares and Pringle, submitted). The *she5/bni1* mutant alleles that we isolated produce an abnormal transcript from the *HO* locus that initiates far upstream of the transcription start. Surprisingly, the abnormal *HO* transcripts are absent from *she5/bni1*Δ cells. This allele-specific phenotype suggests that the cytoplasmic She5p/Bni1p could have a more direct role in defining the transcription start site of *HO*. Alternatively, the abnormal transcript might merely be a coincidental pleiotropy that is unrelated to the defect in *HO* expression. We considered, but can reject, the notion that *HO* fails to be expressed in *she5* mutants for the trivial reason that mothers fail to separate their cytoplasm from that of daughters. We show in the accompanying paper

(Bobola et al., 1996) that Ash1p still accumulates asymmetrically and mothers still express *HO* in cells that fail to undergo cytokinesis owing to a *cdc12-1* mutation. Overexpression of *BNI1* in strains carrying mutations in the gene for the actin-binding protein profilin causes slow growth (J. Pringle, personal communication); She5p/Bni1p might, therefore, interact with the actin cytoskeleton. The functions of She2p, She3p, and She4p remain mysterious. The dependence of She1p/Myo4p accumulation in buds on *SHE3* and *BNI1* suggests that both might be involved in transport mediated by Myo4p.

## On the Mechanism of Segregating Developmental Determinants

During the development of most organisms, cell proliferation relies on the precise segregation of sister chromatids to daughter cells and the more or less equal division of cytoplasmic components. It is becoming increasingly clear that the unequal segregation of very specific cell components has an essential role in many cases of cell differentiation. How some cellular components are asymmetrically segregated while most are segregated evenly is a question of great interest. It was not known at the outset of this study whether nuclear or cytoskeletal processes were responsible for mother cell–specific *HO* expression. Our results clearly point to the latter.

The dependence of posterior *oskar* mRNA accumulation on tropomyosin in Drosophila oocytes (Erdelyi et al., 1995) suggests that actin cytoskeletal components have an important role in the asymmetric distribution of developmental determinants. The identification of a specific myosin subtype (Myo4p) in the asymmetric accumulation of Ash1p is direct evidence that actin-based motors have a crucial role in such processes. It will be intriguing to address whether Myo4p and other She proteins are involved in generating differences between mothers and daughters that affect processes besides *HO* expression. One candidate is the different life spans of mothers and their daughters.

## Experimental Procedures

### Culture Conditions, Strains, and Media

Unless otherwise stated, yeast strains are derived from W303a (which is equivalent to K699; Schwob et al., 1994). Strains K1168 (*MATa ura3-52 ho*) and K5429 (*MATa ura3-52 his4-539 lys2-801 tub2-401 ho*) are congenic to S288C. Only the relevant genotype is shown. Cells were grown in yeast extract–peptone (YEP) medium as described by Schwob et al. (1994).

### Mutant Isolation and Characterization

We generated yeast strains that allowed us to select for mutants that cannot express *HO*. The complete *HO* promoter was fused to both the *ADE2* and *CAN1* ORFs and integrated by targeted gene replacement into the *HO* and *CAN1* loci, respectively. *CAN1* causes cells to be sensitive to the arginine analog canavanine. Cells expressing *ADE2* form white colonies, whereas mutants that cannot express it form red colonies on media with limited amounts of adenine. Details concerning the construction of *HO–ADE2* and *HO–CAN1* are available upon request. A *GAL–HO–lacZ* fusion (Nasmyth, 1987) on a YIplac204 vector was integrated at the *URA3* locus, and the resulting strain was transformed either with the *HIS3* gene or with a *mat*Δ*:LEU2* construct to obtain strains K4535 (*MAT*α *HIS3 HO–ADE2 HO–CAN1 URA3::GAL1-10–HO–lacZ*) and K4570 (*mat*Δ*::LEU2 HO–ADE2 HO–CAN1 URA3::GAL1-10–HO–lacZ*) that were used for EMS mutagenesis. Deletion of the *MAT* locus in K4570 was necessary to allow complementation testing, since a *mat*Δ strain mates to *MAT*α, and the resulting diploid strain does not repress *HO*. After EMS mutagenesis to 75% survival (Cvrckova and Nasmyth, 1993), cells were grown for two generations in YEP–dextrose (YEPD) to avoid phenotypic lag. Cells ($6 \times 10^6$) were plated onto selective medium containing 0.03% canavanine, 10 mg/l adenine, 4% glucose, and all amino acids except arginine. After 3 days at 25°C, about 25,000 mutants had formed colonies, of which 3,400 showed a red color indicating a lack of both *CAN1* and *ADE2* expression. These were transferred onto YEP–Gal media to induce lacZ expression from the *GAL–HO–lacZ* reporter gene. Mutants that formed blue colonies in this assay were considered to carry mutations in genes involved in mother/daughter control of *HO*. We crossed 315 such mutants to a parent strain of the opposite mating type to test for dominance. We mated 280 recessive mutants with *MAT*α and *mat*Δ versions of a strain carrying a *SWI5* deletion. The corresponding diploids were tested for complementation of adenine auxotrophy. We found that 57 mutants complemented the *SWI5* deletion strains and placed 46 of them in complementation groups by testing the diploids for adenine auxotrophy. Using this screen, we identified five complementation groups, which were named *SHE1* to *SHE5*. Pairwise crosses between 10 out of 35 dominant mutants showed them to be allelic to each other and to *she1*. Two mutants from each complementation group (only one in the case of *she4*) were crossed back three times to nonmutagenized parent strains prior to further analysis.

### Mating-Type Switching Analysis

Strains K4824 (*HO–ADE2 HO–CAN1 she1-1024*), K4822 (*HO–ADE2 HO–CAN1 she3-1440*), K4819 (*HO–ADE2 HO–CAN1 she2-652*), and K4924 (*HO–ADE2 HO–CAN1 she4-4449*) were crossed to a $HO^+$ strain, and progeny were selected that carried a *she* mutation and the functional *HO* gene. Pedigree analysis on these strains was performed as described previously (Strathern and Herskowitz, 1979). Homothallic *she* mutant strains were crossed to a homothallic strain expressing *SWI5* from the constitutive RP39 promoter. Cells that carried both a *she* mutation and the *RP39–SWI5* fusion were chosen for pedigree analysis.

### Cloning and Disruption of the *SHE* Genes

The *SHE* genes were isolated by virtue of their ability to complement the adenine auxotrophy of *she* mutants. A genomic library (Cvrckova and Nasmyth, 1993) was transformed into K4793 (*she1-456*), K4819 (*she2-652*), K4822 (*she3-1440*), and K4795 (*she5-2944*), and transformants were selected on plates lacking adenine and leucine.

The only plasmid complementing a *she1-456* mutation contained an 8 kb insert. Sequencing showed that it carried the full *MYO4* gene. To verify that the complementing activity resided within the *MYO4* gene, we introduced a frameshift by filling in the unique XhoI site of *MYO4* with Klenow polymerase. The resulting plasmid could not complement a *she1-456* mutation. For disruption of *MYO4*, an internal SalI–HpaI fragment was replaced by the *URA3* gene. The disrupted allele was released from the vector and transformed into K842 (diploid wild-type strain) and K4452 (*MAT*α *HO–ADE2 HO–CAN1*).

The *she2-652* mutant was complemented by two plasmids. Sequencing of the 5 and 5.2 kb inserts revealed that both carried a part of chromosome XI containing the ORFs YKL130c to YKL133c. A 1.1 kb HindIII fragment with only ORF YKL130c and adjacent 5′ and 3′ untranslated region (UTR) sequences was able to restore *HO–ADE2* expression in K4819. We therefore refer to ORF YKL130c as *SHE2*. Disruption of *SHE2* was performed by replacing an internal NsiI–StuI fragment with *URA3* and transformation of the resulting construct into K4452 and K842.

A *she3-1140* mutant was complemented by four plasmids that carried different inserts with a common 2.2 kb SacI–XbaI fragment containing the complementing activity. Sequencing of this common fragment showed that it carried a part of yeast chromosome II with the full ORF YBR1005 and the amino-terminal third of YBR1004. Disruption of YBR1005 with a *URA3* marker at a unique HpaI site led to loss of the complementing activity, which indicates that YBR1005 is *SHE3*. For deletion of *SHE3*, a 1.8 kb PCR fragment with new XbaI and EcoRI sites at the 5′ and 3′ ends of *SHE3* was

subcloned into pUC19, and an internal 0.7 kb XhoI–HpaI fragment was replaced by *URA3*.

The three plasmids that complemented a *she4-4449* mutant carried inserts of different sizes, which contained a common 3 kb fragment. This fragment was sequenced and found to contain a part of chromosome XV with an unknown ORF of 2376 bp, which was designated *SHE4*. The gene was subcloned into pTZ18 and disrupted by replacing an internal 1.1 kb ClaI–XhoI fragment with *URA3*. The disruption construct was released from the vector and transformed into K4452 and K842.

Two plasmids were able to rescue a *she5-2944* mutant. The plasmids contained 6.5 and 12 kb inserts that showed several similar restriction fragments. The 12 kb insert carried the full *BNI1* gene and additional 5′ and 3′ sequence. The 6.5 kb insert carried the 5′ UTR and most of the *BNI1* coding sequence, lacking only the 3′ 176 bp of the *BNI1* ORF. For *BNI1* disruption, we used a construct according to Fares and Pringle (submitted).

To demonstrate linkage between the cloned *SHE* genes and the *she* mutations, we crossed strains carrying deletion alleles with the corresponding *she* mutant strains. Tetrad analysis showed that in all cases no spore of each tetrad could express the *HO–ADE2* reporter gene, demonstrating tight linkage of the *she* mutant and the corresponding *she* disruption.

To analyze the effect of deletions of cap protein genes, we transformed *spa2::URA3* (Gehrung and Snyder, 1990), *rho3::URA3*, and *rho4::HIS3* (Matsui and Toh-E, 1992) disruption constructs into K5044 (*HO–ADE2/HO–ADE2 HO–CAN1/HO–CAN1*). Tetrad dissection of diploids with one disrupted copy revealed that all spores could express *ADE2* and *CAN1* from the *HO* promoter.

### Localization of the She Proteins

To introduce an epitope tag at the carboxyl termini of She proteins, we created BamHI (in the case of *BNI1* and *SHE3*) or XbaI sites (in the case of *MYO4* and *SHE2*) in front of the corresponding stop codon by PCR-mediated mutagenesis. BamHI or XbaI fragments of the c-Myc epitope cassette (provided by S. Kron) that contained three Myc epitopes were cloned into the new site. *SHE2–myc3* and *SHE3–myc6* constructs were excised and integrated at the genomic locus by replacing the *she2::URA3* and *she3::URA3* disruptions in yeast. Positive clones were selected on 5-fluoroorotic acid–containing medium. To introduce the tagged versions of *MYO4* and *BNI1* at the genomic locus, we initially had to clone a *URA3* marker into the 3′ end of the genes (detailed construct description is available upon request). In a subsequent step, the marked alleles were replaced by the epitope-tagged versions. In all four cases, 5-fluoroorotic acid–positive clones were checked by Western blot analysis for the expression of epitope-tagged proteins (Schwob et al., 1994). Strains K5307 (*BNI1–myc6*), K5380 (*SHE3–myc6*), K5542 (*SHE2–myc3*), and K5543 (*MYO4–myc6*) were found to be positive and were used for indirect immunofluorescence microscopy. Cells were prepared for immunofluorescence according to Nasmyth et al. (1990), except that Zymolase 100T (75 μg/ml) was used for spheroplasting and the methanol–acetone fixation step was omitted when costaining with phalloidin anti-actin was done. The anti-Myc antibody used was purified from 9E10 hybridoma supernatant. Pictures were taken on Kodak T-MAX400 film or with a CCD camera mounted to a Zeiss Axiophot microscope.

### Synchronous Cultures, Northern Blot, and Yeast Transformation

RNA extraction from yeast and Northern blot analysis were performed as described previously (Tebb et al., 1993). Probes against *ho*, *CLN2*, *SWI5*, *SIC1*, and *CMD1* have been described elsewhere (Tebb et al., 1993; Schwob et al., 1994). The probe against *EGT2* was a 1.4 kb fragment (−215 bp to +1187 bp; obtained from B. Kovacech). The probe against the *HO* 5′ UTR was a 0.7 kb fragment (−1905 bp to −1221 bp). Yeast transformation was performed as described by Nasmyth et al. (1990). All of the above-mentioned gene replacements were confirmed by Southern blot analysis. Synchronization of yeast cells was performed by centrifugal elutriation under conditions described by Schwob et al. (1994) or by arrest–release experiments using α factor (Nasmyth et al., 1990).

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**GenBank Accession Number**

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