Journal of Cell Science 113, 1199-1211 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JCS0843

The *Saccharomyces cerevisiae SDA1* gene is required for actin cytoskeleton organization and cell cycle progression

Giacomo Buscemi, Francesca Saracino, Debora Masnada and Maria Luisa Agostoni Carbone*

Dipartimento di Genetica e di Biologia dei Microrganismi, Università degli Studi di Milano, Milano, Italy
*Author for correspondence (e-mail: pancar@mailserver.unimi.it)

Accepted 19 January; published on WWW 7 March 2000

SUMMARY

The organization of the actin cytoskeleton is essential for several cellular processes. Here we report the characterization of a *Saccharomyces cerevisiae* novel gene, *SDA1*, encoding a highly conserved protein, which is essential for cell viability and is localized in the nucleus. Depletion or inactivation of Sda1 cause cell cycle arrest in G_1 by blocking both budding and DNA replication, without loss of viability. Furthermore, *sda1-1* temperature-sensitive mutant cells arrest at the non-permissive temperature mostly without detectable structures of polymerized actin, although a normal actin protein level is maintained, indicating that Sda1 is required for proper organization of the actin cytoskeleton. To our knowledge, this is the first mutation shown to cause such a phenotype. Recovery of Sda1 activity restores proper assembly of actin structures, as well as budding and DNA replication. Furthermore we show that direct actin perturbation, either in *sda1-1* or in *cdc28-13* cells released from G_1 block, prevents recovery of budding and DNA replication. We also show that the block in G_1 caused by loss of Sda1 function is independent of Swe1. Altogether our results suggest that disruption of F-actin structure can block cell cycle progression in G_1 and that Sda1 is involved in the control of the actin cytoskeleton.

Key words: Actin, Cell cycle, *Saccharomyces cerevisiae*, Latrunculin-A

INTRODUCTION

For successful cell proliferation cell cycle events have to be coordinated. The proper order of these events is controlled by a family of serine/threonine protein kinases, called cyclin-dependent kinases (reviewed by Nasmyth, 1993), whose activity oscillates during the cell cycle, sequentially triggering nuclear and cytoplasmic events. Cell cycle progression also requires a number of morphogenetic events related to actin organization, whose molecular details are still partially unknown.

The actin cytoskeleton plays an essential role in different cellular processes, including polarized cell growth, cell motility, secretion and endocytosis, and is involved in preparing the cells for cytokinesis (Bi et al., 1998). Actin is a highly abundant protein in all eukaryotes, and physiological conditions favour the spontaneous assembly of polymerized (F) actin from monomeric (G) actin. In most eukaryotic cells, actin filaments undergo dynamic cycles of assembly and disassembly (Carlier and Pantaloni, 1997). In the budding yeast *Saccharomyces cerevisiae* the organization of actin structures changes during the cell cycle and forms the basis of the spatial control of cell surface growth, thereby determining cell division and morphology. In fact, the actin cytoskeleton, which in yeast consists of cables and patches, is polarized throughout most of the cell cycle. Actin patches are clustered in regions of active secretion, and actin cables are oriented along the mother-bud axis (for reviews see Madden and Snyder, 1998; Cid et al., 1995). This dynamic polarization relies on the capacity of the actin cytoskeleton to respond to cellular signals and reorganize spatially and temporally. Temperature-sensitive mutations in the yeast single actin gene, *ACT1*, cause phenotypes that vary considerably in their severity (for review see Ayscough and Drubin, 1996). The temperature-sensitive alleles *act1-1* and *act1-2* cause disorganization of actin cables and patches, cell lethality and also other phenotypes, like delocalized deposition of chitin and sensitivity to high osmolarity, which are probably caused by defects in polarized secretion (Novick and Botstein, 1985). In addition, the actin cytoskeleton has also been implicated in nuclear migration (Fujiwara et al., 1999). A large number of yeast genes are involved in controlling the dynamic turnover of actin filaments and their localization (for review see Botstein et al., 1997). Association of actin with actin-binding proteins makes the diversity of actin filament forms possible. Several actin binding proteins directly regulate the organization of actin filaments, other proteins affect the dynamic of filament turnover or allow reorganization of the actin cytoskeleton in response to different signals (for review see Ayscough, 1998).

Cytoskeletal events occur in a highly ordered fashion during cell division, suggesting that there are mechanisms that operate to coordinate them with the nuclear events of the cell cycle. Many of the components of the pathways leading to morphogenetic events so far identified are conserved throughout evolution, thus the identification of the molecular mechanisms regulating polarized growth in yeast could suggest a model for higher eukaryotes. Recently, actin organization has been directly implicated in a surveillance mechanism, called the morphogenesis checkpoint, which has been shown to delay cell cycle progression in response to perturbations of cell polarity that prevent bud formation (Lew and Reed, 1995). This checkpoint delays nuclear division, preventing accumulation of binucleate cells (Lew and Reed, 1995; Sia et al., 1996), and seems to directly monitor actin organization, although it is not yet clear how cells monitor the organization of their cytoskeleton (McMillan et al., 1998). The cell cycle delay has been shown to be due to inhibitory phosphorylation of the master cell cycle regulatory cyclin-dependent kinase Cdc28 by the Swe1 kinase (McMillan et al., 1998, 1999).

Here we describe the characterization of a novel *Saccharomyces cerevisiae* protein, which we named Sda1, that is the first discovered member of a family of conserved proteins and is essential for yeast cell viability. Sda1 is localized in the yeast nucleus. Several lines of evidence suggest that the lack of Sda1 function blocks the cell cycle in G_1, preventing bud formation and a new round of DNA replication. Construction and characterization of the *sda1-1* temperature sensitive mutant has allowed us to show that *sda1-1* cells arrest at the non-permissive temperature with a normal actin level, but mostly without detectable assembled actin structures, suggesting a role for Sda1 in controlling the actin cytoskeleton. We also show that direct perturbation of the actin cytoskeleton can arrest the cell cycle in G_1 and this block is independent of Swe1.

MATERIALS AND METHODS

Yeast strains, media and general methods

Yeast strains used in this work are listed in Table 1. Standard yeast genetic techniques and media were as described (Rose et al., 1990). When indicated the cultures were synchronized by α-factor treatment as described by Foiani et al. (1994). Lat-A was obtained by Phil Crews (University of California at Santa Cruz, USA), stored as a 20 mM DMSO stock solution at −80°C. Lat-A (or DMSO as a control) was used to 100 μM.

Strain and plasmid construction

To generate the *sda1Δ* allele, plasmid pLA666 was constructed by cloning into pGEM7Zf(+) vector the 3908 bp *Eco*RI fragment from cosmid pUGH1273 (Feroli et al., 1997), including the *SDA1* gene. Plasmid pLA666.D was obtained from pLA666, by substituting the *Pst*I-*Sal*I region internal to *SDA1* with the *Sal*I-*Sma*I fragment, including the *HIS3* gene, from pfl39-*HIS3* (Struhl and Davis, 1980). The *Eco*RI fragment from pLA666.D, containing the *sda1Δ::HIS3* allele (*sda1Δ*), was used for a one-step gene replacement (Rothstein, 1991) of *SDA1* genomic locus, by transforming the diploid strain W303 to histidine prototrophy. Tetrads from a strain heterozygous for *sda1Δ* showed a 2:2 segregation of lethality, linked to the *HIS3* marker. To generate the *swe1Δ::LEU2* allele (*swe1Δ*), W303/34c and Q133 strains were transformed with the *Hin*dIII-*Bam*HI fragment from plasmid pswe1-10g (Booher et al., 1993). *SWE1* disruption was tested by Southern analysis.

Plasmid pLA690 was generated by cloning the *SDA1* coding region, with 105 bp downstream of the stop codon, under the control of the *GAL1* promoter in pBM125 vector, *Bam*HI-*Nru*I digested. Plasmids pLA693 and pLA694, containing the *SDA1* gene, were obtained by subcloning the *Eco*RI fragment from pLA666, carrying *SDA1*, in the *Eco*RI site of pRS416 (*URA3*) and pRS314 (*TRP1*) vectors (Sikorski and Hieter, 1989), respectively. Plasmid pLA740, carrying the myc18-tagged version of *SDA1*, was constructed in two steps. A 1218 bp fragment generated by PCR, containing 759 bp upstream of the *SDA1* ATG, a *Not*I site immediately downstream and in frame with the ATG, and 459 bp of the *SDA1* open reading frame, was cloned into the YIplac128 vector. By cloning into the *Not*I site of this plasmid, in frame with ATG, two copies of a *Not*I myc9 cassette, containing 9 copies of the myc epitope (obtained by PCR of a myc9 cassette, a gift from S. Piatti), plasmid pLA740 was generated. Transformation of W303/34c with pLA740, linearized with *Pst*I, directed integration of the plasmid at the *SDA1* locus, generating a strain carrying a full length myc-tagged version (*myc18-SDA1*) and a truncated untagged version of *SDA1* (strain Q184).

In vitro mutagenesis of *SDA1*

To generate and isolate temperature-sensitive *sda1* mutations, a PCR random mutagenesis of the *SDA1* coding region was performed, followed by gap-repair (Rothstein, 1991) and plasmid shuffling (Sikorsky and Boeke, 1991). Temperature-sensitive mutants were identified by testing growth on YNB medium selective for Trp^+, at 25°C, 34°C and 37°C. Substitutive integration of the *sda1-1* allele into the *SDA1* chromosomal locus was obtained by transforming W303/34c strain with a YIp5 vector (*URA3*; Botstein et al., 1979), carrying the 3910 bp *Eco*RI fragment, containing the *sda1-1* allele, linearized with *Xba*I. Ura^- clones, derived from loss of a recombinant plasmid, carrying the *sda1-1* mutant allele as the only copy of the gene at the chromosomal locus, were selected by their ability to grow on 5-FOA plates, followed by growth analysis at 25°C, 34°C and 37°C. The presence of a single copy of the *SDA1* gene was checked by Southern analysis.

Viability assays

About 5×10^3 sonicated cells were plated on YPD and incubated at the

Table 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
W303/34c	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100*	
Q34	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 sda1Δ::HIS3* (pLA690 *URA3 GAL1-SDA1*) (pRS314 *TRP1*)	This study
Q35	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 sda1Δ::HIS3* (pLA690 *URA3 GAL1-SDA1*) (pLA694 *TRP1 SDA1*)	This study
Q32/2b	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100* (pLA690 *URA3 GAL1-SDA1*)	This study
Q47	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100* (pBM125 *URA3*)	This study
Q94	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 sda1Δ::HIS3* (pLA693 *URA3 SDA1*)	This study
Q133	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 sda1-1*	This study
Q184	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 sda1::myc18-SDA1::LEU2*	This study
Q224	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 swe1Δ::LEU2*	This study
Q225	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 sda1-1 swe1Δ::LEU2*	This study
38	*MAT**a** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 cdc28-13*	S. Piatti

All strains are isogenic to W303 (Thomas et al., 1989). Plasmids are indicated within parentheses. In the text the *sda1Δ::HIS3* allele is indicated as *sda1Δ*.

permissive temperature. After 24-48 hours, about 200 elements were analyzed under the microscope and considered viable if able to form a microcolony composed of ten or more cells.

Fluorescence staining of yeast cells

Cells grown in YPD were fixed directly in growth medium by addition of 37% formaldehyde to 3.7% final concentration, and incubation at room temperature for 2 hours. Staining with rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR) was performed as described (Guthrie and Fink, 1991). In some cases cells, before staining, were treated with 1% Triton X-100, for 1 minute at room temperature, or incubated with 0.1 mg/ml zymolyase 100T and 0.2 mg/ml β-glucoronidase in 1 M sorbitol, 0.1 M Na citrate and 0.06 M EDTA for 1 hour at 30°C. Indirect immunofluorescence staining of microtubules with rat anti-tubulin primary antibodies (YOL1/34, from Serotec, UK) and indirect immunofluorescence of myc18-Sda1 with monoclonal mouse anti-myc primary antibodies, followed by rhodamine-conjugated secondary antibodies, were carried out as described by Pringle et al., 1989. The DNA binding dye 4′,6′-diamidino-2-phenylindole (DAPI; 0.85 µg/ml in H_2O) was used to visualize the nucleus. The Calcofluor white or Fluorescent Brightener 28 (Sigma, St Louis, MO) was used to visualize-chitin rich structures in *S. cerevisiae* cell wall, as described (Pringle, 1991). Cells were viewed using a Zeiss Axioskop fluorescence microscope with a 100 W mercury lamp and a Zeiss ×100 Plan-NeoFluar oil immersion objective.

Other techniques

Cells were counted using the microscope. The Lucifer Yellow CH dilithium salt (Fluka, Buchs, Switzerland) was used to visualize fluid phase endocytosis (Dulic et al., 1991). Flow cytometric DNA quantitation was determined according to the method of Epstein and Cross (1992) on a Becton Dickinson FACScan. Photographs were taken using a Zeiss Axioskop fluorescence microscope or by Photometric Sensys CCD camera.

Western blot analysis

Total protein extracts, prepared by TCA precipitation (Muzi Falconi et al., 1993), were resolved by electrophoresis on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membranes (PROTRAN), incubated for two hours at room temperature with mouse anti-actin antibodies (clone C4, ICN) followed by incubation with peroxidase-conjugated anti-mouse antibodies (Pierce) for two hours. An ECL kit (Supersignal Pierce) was used according to the manufacturer's instructions for detection of peroxidase activity.

RESULTS

The Sda1 protein is conserved from yeast to humans

The *SDA1* (severe depolymerization of actin) gene of *Saccharomyces cerevisiae* (Yeast Genome Database ORF name: YGR245c) was identified during yeast genome sequencing as a previously uncharacterized ORF (Feroli et al., 1997), encoding a putative 767 amino acid protein (Fig. 1A), with a calculated molecular mass of 86.6 kDa.

Computer analysis of the amino acid sequence has identified three putative bipartite nuclear localization signals (Dingwall and Laskey, 1991) starting at residues 301, 713 and 737, respectively. A highly acidic region (53% of acid residues) is also present in the C-terminal part of the protein, from residue 546 to residue 657 (Fig. 1A and B). As depicted in Fig. 1B, comparison of the Sda1 amino acid sequence with peptide sequence and EST databases indicates that Sda1 is conserved from *Schizosaccharomyces pombe* to humans, showing a significant similarity with putative proteins of various organisms. The only completely sequenced proteins are from *Drosophila melanogaster* and *Arabidopsis thaliana*, showing 37% and 26% identity, respectively, with Sda1.

No indication of possible protein functions is available for any of these proteins. All of them have at least one nuclear localization signal and, when sequenced, the acidic region is conserved (Fig. 1B). Most cDNAs were from proliferating cells: for example *Drosophila melanogaster* cDNAs were from ovary and embryo, and human cDNAs were from colon cancer, germ line cancer and immature B lymphocytes.

The Sda1 protein is essential for yeast cell viability and is localized in the nucleus

Gene disruption analysis showed that Sda1 function is essential for yeast cell viability (see Materials and Methods). In order to gain further insights into the possible function of Sda1, we first determined its subcellular localization. To this end, we constructed a strain (Q184) which expressed, as the sole source of this protein, a functional version of Sda1, tagged at its amino terminus by 18 c-myc epitopes (myc18-Sda1, see Materials and Methods). This strain did not show any detectable difference in growth kinetics at different temperatures nor in cell cycle progression compared to wild type (data not shown), thus indicating that the myc18-Sda1 protein was not altered in Sda1 essential function(s). Western blot analysis of Q184 total protein extracts showed that monoclonal anti-myc antibodies specifically recognized a single protein species, which was not detected in extracts from strains not expressing the fusion protein (Fig. 2A). The Q184 strain was used to localize Sda1 by indirect immunofluorescence, using monoclonal anti-myc primary antibodies and FITC-conjugated secondary antibodies. As shown in Fig. 2B, myc18-Sda1 staining was localized in the nucleus and appeared to be uniform. Nuclear staining was observed in cells at every stage of the cell cycle, suggesting that the distribution of Sda1 within the cells does not change during the vegetative cell cycle. No staining above background was observed in cells not expressing the epitope tagged protein (Fig. 2B), thus confirming that the nuclear signal was specific for myc18-Sda1 and that the protein is indeed localized in the nucleus.

Both depletion and overproduction of Sda1 cause defects in cytokinesis

Our first approach to study the in vivo role of the *SDA1* gene was to test the effect of Sda1 depletion in yeast. For this purpose, we used the haploid strain Q34, carrying a lethal deletion of the *SDA1* chromosomal locus and a wild-type copy of the *SDA1* coding region under the control of the strong galactose-inducible *GAL1* promoter on the centromeric plasmid pLA690 (*GAL1-SDA1*, see Materials and Methods). The isogenic haploid strain Q35, carrying a plasmidic wild-type copy of *SDA1* in addition to *GAL1-SDA1*, was used as a control. The growth of the two strains on glucose and galactose containing solid medium is shown in Fig. 3B. Liquid cultures of both strains were grown in galactose-containing medium and shifted to glucose to switch off the *GAL1* promoter. The growth rates of the two strains were identical up to four generations, when the Q34 strain division time progressively increased until cells completely stopped dividing, eleven

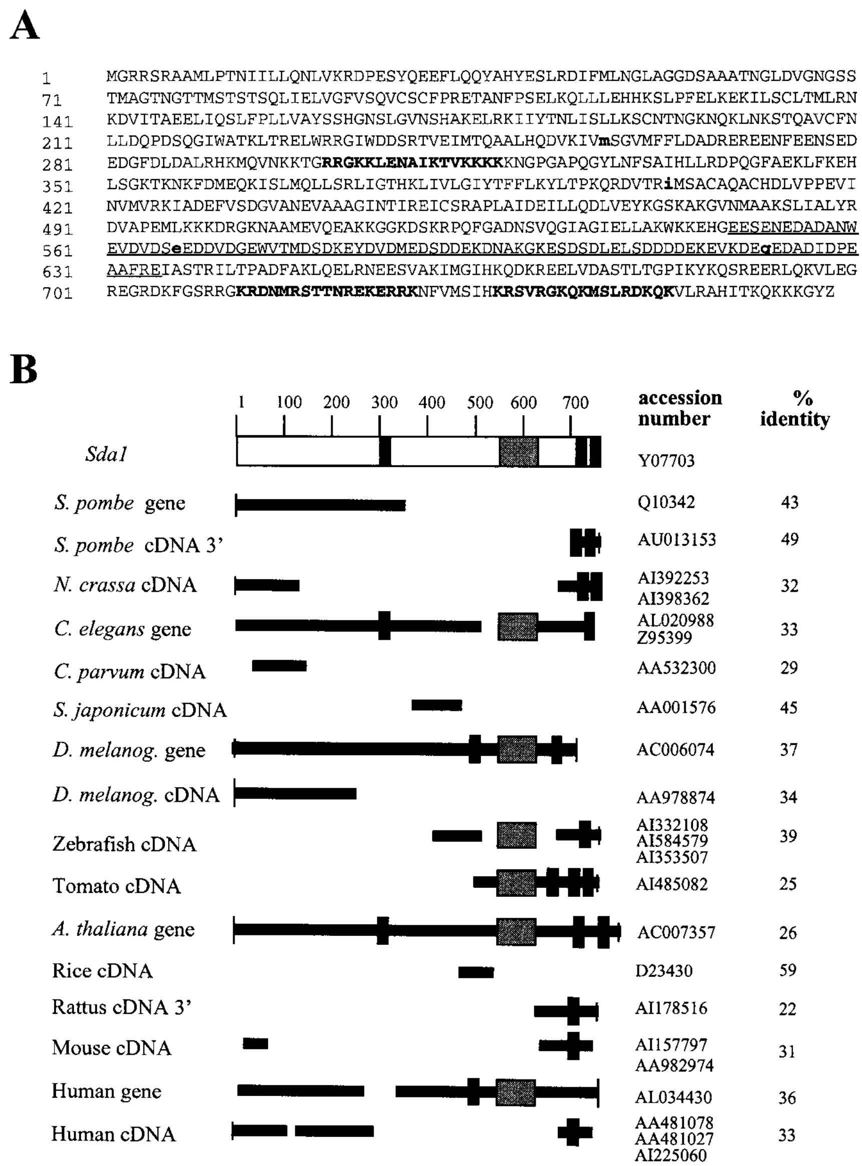


Fig. 1. Sequence analysis of Sda1. (A) The complete amino acid sequence of Sda1 (accession number Y07703). Underlined, acidic region; bold capital letters, nuclear localization signals; bold small letters, residues changed in the *sda1-1* mutant (see text). (B) Schematic diagram comparing the Sda1 amino acid sequence and the homologous sequences in other organisms, obtained by computer analysis with TBLASTN program. The nucleotide sequence accession numbers and the percentage protein identity with Sda1 are reported. Black rectangles, nuclear localization signals; grey rectangles, acidic regions.

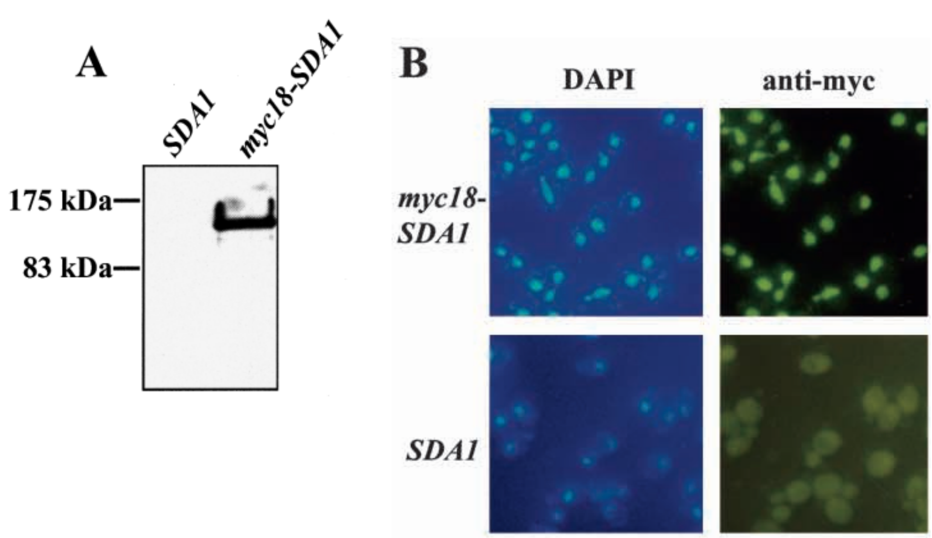


Fig. 2. (A) Western blot analysis of wild-type (W303/34c) and *myc18-SDA1* (Q184) protein extracts probed with anti-myc antibody. Extracts were obtained from the same amount of cells and equal amounts of total proteins were used. (B) Subcellular localization of the Sda1 protein. Samples of Q184 (*myc18-SDA1*), expressing N-terminal myc-tagged Sda1 as the sole source of the protein, and of W303/34c (*SDA1*), were withdrawn from cultures logarithmically growing in YPD at 28°C and fixed with formaldehyde before simultaneous staining with DAPI (left panel) and by indirect immunofluorescence, using monoclonal anti-myc primary antibodies and FITC-conjugated secondary antibodies, to visualize the myc18-Sda1 protein (right panel).

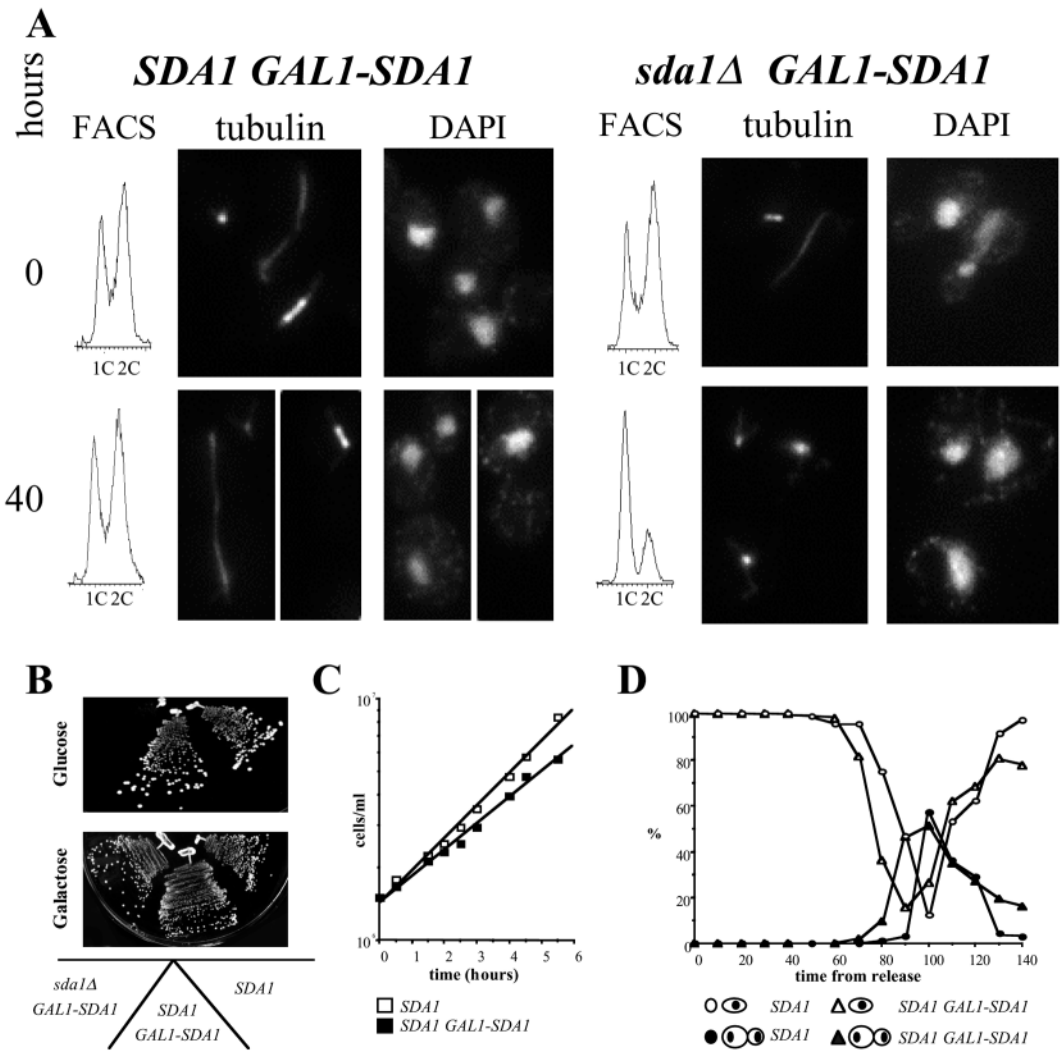


Fig. 3. Effects of Sda1 depletion and overexpression. (A) Q35 (*SDA1 GALI-SDA1*) and Q34 (*sda1Δ GALI-SDA1*) cultures, logarithmically growing in galactose medium (1×10^6 cells/ml) at 28°C, were shifted to glucose selective medium at time zero. Growth and cell morphology were monitored using the light microscope and cultures were diluted 1:10 with fresh medium when they reached a concentration of approximately 1×10^7 cells/ml. Samples taken at the indicated times after the shift were simultaneously stained by indirect immunofluorescence using rat anti-tubulin and rhodamine-conjugated secondary antibodies to visualize microtubules (left panel) and with DAPI to visualize the nucleus (right panel), and used for FACS analysis. (B) Growth of the indicated strains on solid medium. (C) Growth kinetics of strains Q47 (*SDA1*) and Q32/2b (*SDA1 GAL1-SDA1*) in galactose selective medium (*SDA1* overexpression in Q32/2b) at 28°C, monitored by cell counting. (D) W303/34c (*SDA1*) and Q32/2b (*SDA1 GAL1-SDA1*) cells synchronized with α-factor in galactose medium at 28°C and released from α-factor in fresh galactose selective medium at time zero. Cell samples at each indicated time points after release from G_1 arrest were sonicated and stained with DAPI to determine the percentage of cells with one or two nuclei (about 150 cells were scored).

generations after the shift (data not shown). Therefore, the Sda1 protein synthesized under induced conditions is sufficient to proceed through several cell divisions, indicating that Sda1 is a very stable polypeptide, which is inherited from mother to daughter cells and can be used for several cell generations. When cells stopped dividing (40 hours after the shift to glucose medium), the percentage of unbudded cells appeared increased from 30% at time zero (46% large budded and 24% small budded) to 76% (20% large budded and only 4% small budded), indicating defects in bud formation. Nuclei DAPI staining showed that the large budded cells had already segregated the nuclei and indirect immunofluorescence with anti-tubulin antibodies showed that they had disassembled the mitotic spindle (Fig. 3A); 95% of the large budded cells did not separate when treated with zymolyase. FACS analysis showed that most Sda1-depleted cells had a 1C DNA content (Fig. 3A). Therefore, when the amount of Sda1 is no longer sufficient to support cell growth, cells arrest in G_1, although some of them are presumably unable to complete cell separation in the previous cell cycle.

We also produced and characterized strains in which the gene was overexpressed. The effects of *SDA1* overexpression in the wild-type background were analyzed by shifting to galactose medium strain Q32/2b, which carries a *GAL1-SDA1* fusion on plasmid pLA690. *SDA1* overexpression caused a slight but reproducible increase of about 15 minutes in the doubling time compared to a congenic wild type (Fig. 3C). To better evaluate this effect, the cell cycle progression was monitored in synchronized cultures. The *GAL1-SDA1* and wild-type strains were arrested in G_1 with α-factor and released from the α-factor block in fresh medium with galactose. As Fig. 3D shows, while budding seemed to be slightly anticipated in the *SDA1* overexpressing strain compared to wild type, nuclear division was delayed with respect to budding in *GAL1-SDA1* cells, and a more pronounced delay was observed in the transition from large budded cells with segregated nuclei to single cells with one nucleus, suggesting a delay in completing cytokinesis.

Effects of the *sda1-1* temperature-sensitive mutation on cell cycle progression

To further study *SDA1* function we undertook random mutagenesis of the *SDA1* cloned coding region by PCR amplification, followed by gap-repair and plasmid shuffling (see Materials and Methods). By this procedure, we obtained four independent *sda1* temperature-sensitive alleles, one of which, called *sda1-1*, was further characterized. Sequence analysis of the *sda1-1* allele revealed four base-pair substitutions, generating the M257T, I403N, E567V and Q622P amino acid substitutions (Fig. 1A). Interestingly, the last two mutations cause the substitution of two acidic residues in the highly acidic Sda1 region. The *sda1-1* allele was used to replace the chromosomal *SDA1* gene in a haploid strain, thus giving rise to a stable *sda1-1* temperature-sensitive mutant strain (Q133), which was used for further analysis.

We first compared the growth kinetics of the *sda1-1* and isogenic wild-type strains at permissive (25°C) and non-permissive (37°C) temperature. As shown in Fig. 4 the growth rates of these two strains were very similar at 25°C. On the contrary, mutant and wild-type cells shifted at 37°C grew at identical rates up to 2.5 generations, at which point the mutant cells suddenly stopped growing, about 6 hours after the shift, while the wild-type cells continued growing until stationary phase. The *sda1-1* cells maintained a high viability (as determined by microcolony assay) both during growth and for many hours after growth arrest at 37°C. The arrested *sda1-1* cells were 83% unbudded with a single nucleus and 17% large budded cells with two segregated nuclei, and both cell types showed cytoplasmic microtubules, suggesting that spindles had been disassembled (Fig. 4B). Therefore, the *sda1-1* mutation prevents budding at the restrictive temperature. 90% of the large budded arrested cells did not separate when treated with zymolyase, and Calcofluor staining analysis (Pringle, 1991) showed a cytoplasmic bridge between mother and daughter cell in 75% of them. These cells, when maintained at the non-permissive temperature, did separate normally, but very slowly (data not shown).

To further characterize the *sda1-1* mutation, we analysed the effect of restrictive temperature on synchronized mutant cultures. Since our data had indicated a significant time requirement for inactivation of mutant Sda1 function, mutant cells exponentially growing at 25°C were preincubated at the non permissive temperature (37°C) for 20 minutes before synchronization, then maintained at 37°C in the presence of α-factor, to obtain a G_1 synchronized culture. Under these conditions, *sda1-1* cells could still progress through the cell cycle at 37°C, after release from the α-factor block, but they

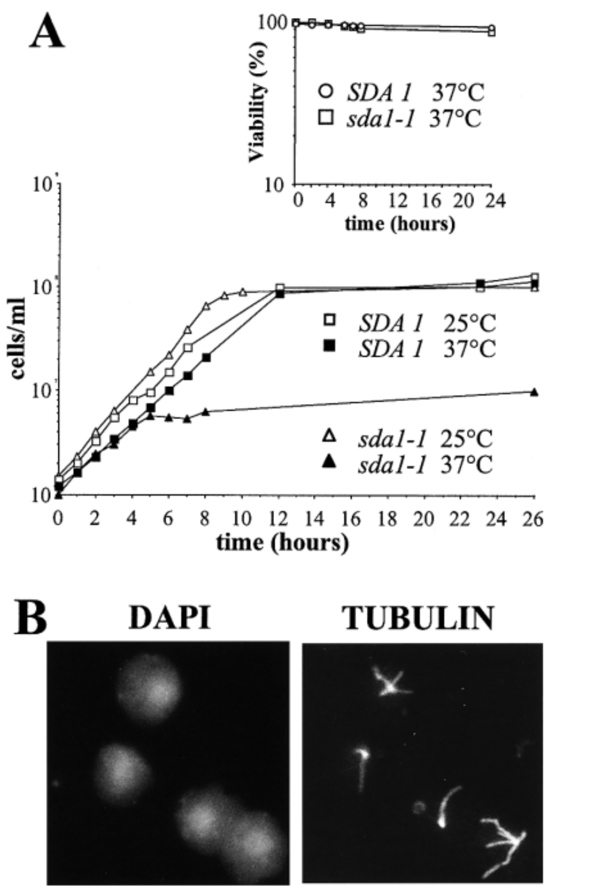


Fig. 4. Growth rate and arrest phenotype of asynchronous *sda1-1* temperature sensitive cell cultures. (A) Growth rate at 25°C and 37°C and viability at 37°C of strains W303/34c (*SDA1*) and Q133 (*sda1-1*). (B) Q133 cells after 16 hours in YPD at 37°C were simultaneously stained with DAPI to visualize the nucleus (left panel) and by indirect immunofluorescence using rat antitubulin and rhodamine-conjugated secondary antibodies to visualize microtubules (right panel).

showed a delay in budding and cell cycle progression, with a delay also in cytokinesis, compared to the wild type (Fig. 5A). A large fraction of the cells that separated through this very slow cytokinesis process were not able to bud, thus confirming our results on asynchronous cultures. FACS analysis (Fig. 5B) showed that *sda1-1* mutant cells underwent one cycle of DNA replication after α-factor release, and then mostly arrested with 1C DNA content. In fact, the fraction of cells arrested with 1C DNA content, 4 hours after release from α-factor block, was 51%, but, when maintained at restrictive temperature, the large budded cells separated very slowly, giving 80% of cells with 1C DNA content (16 hours after the release).

In summary, defective Sda1 causes bud formation and DNA replication block, after a delay in cytokinesis completion, and it does not affect cell viability.

Polymerized actin is not detectable in arrested *sda1-1* mutant cells at the non-permissive temperature

Since our findings indicated that the *sda1-1* mutation causes a delay in cytokinesis and actin is involved in this process, we verified whether the *sda1-1* mutant showed defects in actin

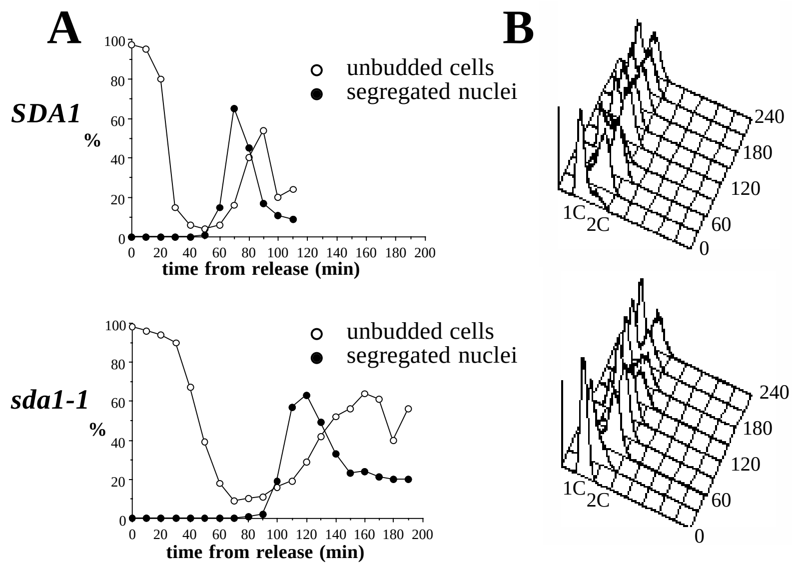


Fig. 5. Cell cycle progression of synchronized *sda1-1* cell cultures after release at 37°C. W303/34c (*SDA1*, A and B top) and Q133 (*sda1-1*, A and B bottom) cultures were shifted to 37°C for 20 minutes, then synchronized in G_1 with α–factor at 37°C in YPD (about 110 minutes) and shifted to fresh YPD medium. Cell samples were fixed with formaldehyde at each time point after the release from α-factor block. (A) The percentage of cells with segregated nuclei was monitored by direct visualization using DAPI staining. (B) FACS analysis of the samples in A.

organization. In yeast actin cables and cortical patches have polarized localization during cell cycle progression. In late G_1, the cortical actin patches congregate from a diffuse pattern into a ring at one pole of the cell, the pre-bud site, where the bud will later emerge. At the same time, the actin cables become oriented towards this site. Following bud emergence, actin patches are found almost exclusively in the bud. Bud growth, which occurs from late G_1 through S and G_2 phases, involves a switch from apical to isotropic growth. A corresponding switch from an apical pattern to a more diffuse distribution of actin patches is detectable in some genetic backgrounds (Mulholland et al, 1994; Botstein et al., 1997; Amberg, 1998). In these phases the cables are oriented along the mother/bud axis. When the bud is mature, actin patches are redistributed in both mother and bud, and they then congregate on both sides of the neck region during cytokinesis until cell separation (for review see Cid et al., 1995; Madden and Snyder, 1998).

The *sda1-1* temperature-sensitive mutant strain, after a shift to 37°C, shows an arrested phenotype with both unbudded and large budded cells. In these cells, when a correct sequence of morphogenetic events takes place, the actin patches are expected to congregate at precise sites, namely the bud site and the neck region. Therefore, we first analyzed the effect of the *sda1-1* mutation on actin structures in asynchronous wild-type and *sda1-1* cultures shifted to 37°C, where we observed the same growth arrest and phenotype shown in Fig. 4. Actin was visualized by the use of a fluorescent derivative of the F-actin binding toxin phalloidin (Wieland, 1977). After 5 hours at 37°C stained actin patches become progressively fewer and less bright in mutant cells compared to wild type: as Fig. 6A shows, actin patches were strongly reduced 7 hours after the shift, and almost no *sda1-1* cells contained phalloidin-stained actin 16 hours after the shift (about 10 hours after growth arrest), while over 90% of exponentially growing or late stationary phase wild-type cells were normally stained. The analysis of actin distribution in these cells by laser scanning microscopy (data not shown) confirmed these observations. Therefore inactivation of Sda1 leads both to disturbance of the actin cytoskeleton and to cell division arrest, although the complete disappearance of the stained patches is detectable only after the cell cycle block. A possible explanation

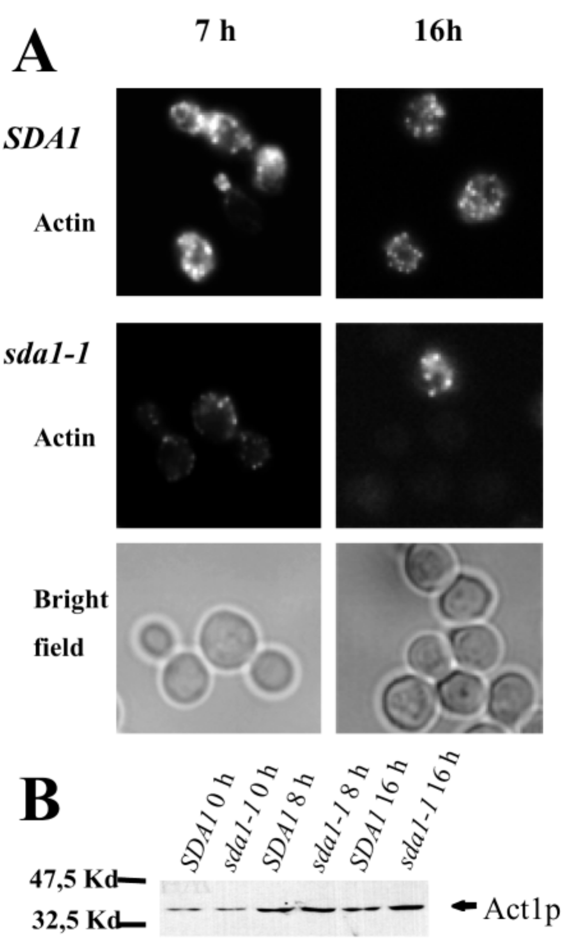


Fig. 6. Effects of *sda1-1* mutation on actin structures. (A) W303/34c (*SDA1*) and Q133 (*sda1-1*) cells, mantained at 37°C for 7 hours (about one hour after *sda1-1* growth arrest) and 16 hours, were fixed with formaldehyde and actin was visualized by rhodamine-phalloidin staining. A representative field of cells is shown. (B) Western blot analysis of wild-type (W303/34c) and *sda1-1* (Q133) protein extracts, prepared from cell samples taken at the indicated times after shift from 25°C to 37°C, and probed with anti-actin antibody.

is that the actin cytoskeleton is already perturbed when polymerized actin structures are still detectable: this interpretation is in accordance with the effect of Lat-A reported below. Moreover, similar results were obtained if samples were treated with Triton X-100 or zymolyase before staining with phalloidin (data not shown), thus indicating that modifications in the *sda1-1* cell wall did not interfere with actin detection. The same cells were normally stained by DAPI. Cells without detectable F-actin stayed viable for a long time (93% after 16 hours at 37°C, 88% after 30 hours, as measured by microcolony assay).

We then analyzed the effect of the *sda1-1* mutation on the actin cytoskeleton in synchronized cultures. To do this, the wild-type and the mutant strains were synchronized with α-factor at 37°C, followed by release in fresh medium at 37°C. Wild-type cells exhibited correct cellular localization of actin during the first cell cycle (Table 2) and then underwent asynchronous growth. Apical patch localization in the bud was clearly visible in this genetic background. As shown in Table 2, *sda1-1* cells had a clear delay in the localization of actin patches at the prebud site, compared to the wild type, after release from the α-factor block. Although consistently delayed, the cortical actin patches could localize properly throughout all the first cell cycle and during the second cycle up to the point where a large bud was present. At this stage, while actin patches were evenly redistributed between mother cell and bud in the wild type, redistribution did not take place in the *sda1-1* mutant, and the amount of actin patches decreased progressively. About 80% of the arrested *sda1-1* cells (both single or large budded) had strongly reduced (<5) or non-detectable actin patches and cables, while all wild-type cells displayed normally stained localized actin.

Table 2. Actin distribution in *sda1-1* mutant cells

	Actin (%)						
Time							Not stained
sda1-1							
0′	94.3						
20′	47.8	46.9					
60′		21.4	74.4				
90′			22.4	67.4			
110′				45.3	27.9	7.1	
120′				42.7	14.2	26.5	
130′	41.6			30.5	16.9	6	
150′	23.6	24.4	24.7	16.9			7.7
180′	37.2	23.9	9.7				20.3
210′	20.2	18.3					42.3
240′				9.2			80.1
SDA1							
0′	95						
20′	13.9	75.9					
40′			78	16			
60′				76	11.9		
80′					62.4	18.8	
100′	43.7					24.3	

Q133 (*sda1-1*) and W303/34c (*SDA1*) were analyzed for actin patch (black dots) localization after shift to 37°C for 20 minutes, followed by synchronization in G_1 with α-factor at 37°C in YPD (110 minutes) and release in fresh YPD medium (time zero) at the non permissive temperature. Values lower than 6% are not shown.

The absence of detectable actin in *sda1-1* mutant cells could be caused either by disassembling of F-actin or by the disappearance of monomeric actin. As shown in Fig. 6B, western blot analysis of yeast extracts from cells either grown at the permissive temperature or shifted to the restrictive temperature did not reveal any significant difference between wild-type and *sda1-1* cells in the amount of the 41.6 kDa polypeptide specifically recognized by anti-actin antibody. Therefore, although not detectable by phalloidin staining, actin is still present in *sda1-1* cells arrested at the non permissive-temperature, suggesting that *sda1* mutation affects the polymerization or the stability of polymerized actin rather than monomeric actin stability or synthesis.

The observation that stained actin starts decreasing in the *sda1-1* mutant at the stage where actin redistribution takes place in the wild-type strain suggests that the Sda1 protein might be involved in cytoskeletal organization and morphogenetic events during cell cycle progression. In accordance with the role of Sda1 in actin organization, we also observed that *MAT**a*** *sda1-1* mutant cells maintained for 16 hours at 37°C were not able to form mating projections in response to α-factor (data not shown).

The *sda1-1* mutation affects chitin deposition and endocytosis

Actin has been shown to be essential for polarized secretion, chitin deposition and endocytosis (Novick and Botstein, 1985; Kübler and Riezman, 1993). In order to further support the hypothesis of a possible involvement of Sda1 in cytoskeleton organization, we analyzed the effects of the *sda1-1* mutation on chitin deposition in the wall and endocytosis. Wild-type budding cells deposit a ring of chitin in the cell wall at the base of the neck (Hayashibe and Katohda, 1973; Cabib and Bowers, 1975), that remains on the mother cell wall as a bud scar after cell division and can be selectively stained with the fluorescent dye Calcofluor.

Wild-type and mutant *sda1-1* cells, exponentially growing in YPD at 25°C, were shifted to 37°C and cells were stained with Calcofluor white 8 hours after *sda1* growth arrest. As shown in Fig. 7, *sda1-1* cells exhibited an altered staining pattern compared to wild type. In fact, most mutant cells did not show normal chitin rings, but patches of fluorescence, which were often not appropriately located. One or more of these bright patches were frequently seen on the surface of

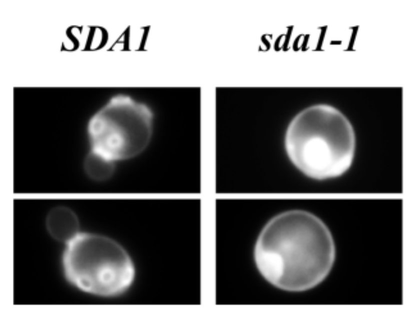


Fig. 7. Cell wall abnormalities in *sda1-1* mutant. W303/34c (*SDA1*, left panel) and Q133 (*sda1-1*, right panel) cells, mantained at 37°C for 16 hours, were fixed with formaldehyde and the cell wall visualized by Calcofluor white staining. A representative field of cells is shown.

single cells, but they were also found on buds, or covering the neck region of the few large budded cells observed in the arrested population. The mutant cells also showed generalized staining of the cell surface, a phenotype observed in mutants impaired in actin organization, which might be a secondary effect of perturbations in the actin cytoskeleton (Novick and Botstein, 1985; Novick et al., 1989).

Since endocytosis was also shown to be dependent on the presence of an intact actin cytoskeleton in yeast (Kübler and Riezman, 1993; Ayscough et al., 1997), we analyzed fluid phase endocytosis in our strains by monitoring the uptake of the fluid phase marker Lucifer Yellow (LY; Dulic et al., 1991). We observed a specific uptake of LY into the vacuoles in both wild-type cells, at 37°C and 25°C, and in mutant *sda1-1* cells at 25°C, whereas this was strongly reduced or abolished in *sda1-1* cells at 37°C, eight hours after arrest (data not shown). Therefore, *sda1-1* mutation inhibited endocytosis at a non-permissive temperature, further supporting the indication that Sda1 has a role in cytoskeleton organization.

Disassembly of the actin cytoskeleton in G_1 blocks budding and DNA replication

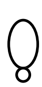
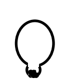
As previously shown (Fig. 4), mutant *sda1-1* cells, at the restrictive temperature of 37°C, arrest after 2.5 rounds of cell division as single cells with one nucleus or large budded cells with two segregated nuclei, and they stay viable for many hours following growth arrest. When the mutant cells stopped dividing, about 80% of them were unbudded and most of the cell population had a 1C DNA content. These cells maintained a 1C DNA content for many hours after the cell division arrest (data not shown), and a high viability (93% after 16 hours). When, after 16 hours at 37°C, *sda1-1* cells were shifted back to the permissive temperature of 25°C, the fraction of large budded cells (about 15%) completed cytokinesis after a lag period of 90 minutes, while cells arrested as unbudded began budding (Table 3) and replicating DNA (Fig. 8A). During the lag period and the subsequent cell cycle, cells progressively recovered the actin polarized structures, patches and cables, necessary to bud (Fig. 8B and Table 3). Only a small fraction of the newly budded cells did not show stained actin. These data indicate that the presence of a functional Sda1 protein is essential for the cells to properly organize actin structures and subsequently recover the ability to bud and replicate DNA.

This result suggests two alternative roles for Sda1: either the loss of Sda1 disturbs only the actin cytoskeleton and this in turn blocks cell cycle progression in G_1, or the loss of Sda1 directly affects budding and DNA replication as well as actin polymerization. To address this question, *sda1-1* mutant cells were shifted back from 37°C to 25°C in the presence of the drug latrunculin-A (Lat-A). Lat-A binds to monomeric actin, prevents polymerization and causes complete disruption of the yeast actin cytoskeleton within 2-5 minutes (Ayscough et al., 1997). The *sda1-1* mutant, based on both solid and liquid medium assays, was not significantly more resistant than the wild type to Lat-A. As shown in Fig. 8C and D, *sda1-1* mutant cells, arrested at 37°C for 20 hours, then shifted back to 25°C in 0.1 mM Lat-A, failed to replicate DNA and bud, and remained as unbudded cells, viable (>90% up to 6 hours), with 1C DNA content. In the conditions used (0.1 mM Lat-A) actin totally disappeared from the majority of the *sda1-1* cells: in a minority of cells patches could be stained with phalloidin, but polarization was not detectable at any time, and cables were not visible (data not shown). Therefore the absence of Sda1 causes perturbation of the actin cytoskeleton and arrest of cell cycle progression in G_1 and, when *sda1-1* cells are released from the G_1 block, direct depolymerization of actin with Lat-A during recovery prevents both budding and DNA replication.

While the budding defect was already described in cells treated with Lat-A (Ayscough et al., 1997; McMillan et al., 1998), the failure of replicating DNA in *sda1-1* cells treated with Lat-A was surprising. In fact, it has been reported that cells synchronized in G_1 using α-factor and released in 0.1 mM Lat-A were able to complete one cycle of DNA replication and arrested cell cycle progression before nuclear division (McMillan et al., 1998). Our observations suggest that Sda1 inactivation might cause a G_1 arrest which is different from the block caused by α-factor addition.

To investigate if the block of DNA replication depends on actin cytoskeleton perturbation in cells arrested in G_1, we decided to analyse the effect of disrupting the actin cytoskeleton integrity in cells arrested in G_1 by other means than α-factor. For this purpose, we carried out an experiment similar to the one reported above by using a mutant strain carrying the temperature sensitive allele *cdc28-13* (Reed and Wittenberg, 1990). *cdc28-13* mutant cells arrested in G_1 at the restrictive temperature of 37°C (96% of the cells were unbudded and 95% were viable after 2 hours 45 minutes at 37°C) were shifted back to the permissive temperature (25°C), with or without 0.1 mM Lat-A. As shown in Fig. 8C and D, cells without Lat-A, after a one hour lag, restarted budding

Table 3. Cell cycle and actin distribution after *sda1-1* shift back to the permissive temperature

	Budding (%)			Actin (%)						
Time										Not stained
sda1-1										
0′	84	2	14	16						82
60′	79	7.6	13.4	56						43
90′	83	14.8	2.2	38	9	12				40
120′	56.6	38.5	4.9	39	11	26	5			19
180′	26.3	60.2	13.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
270′	31.7	30	38.3	38	6	9	22	5		19

A Q133 (*sda1-1*) cell culture arrested at 37°C (16 hours after the shift at non-permissive temperature) was shifted back to 25°C (permissive temperature) and cells were analyzed for cell cycle distribution and actin patches (black dots) localization. Values lower than 6% were not shown. n.d.: not determined.

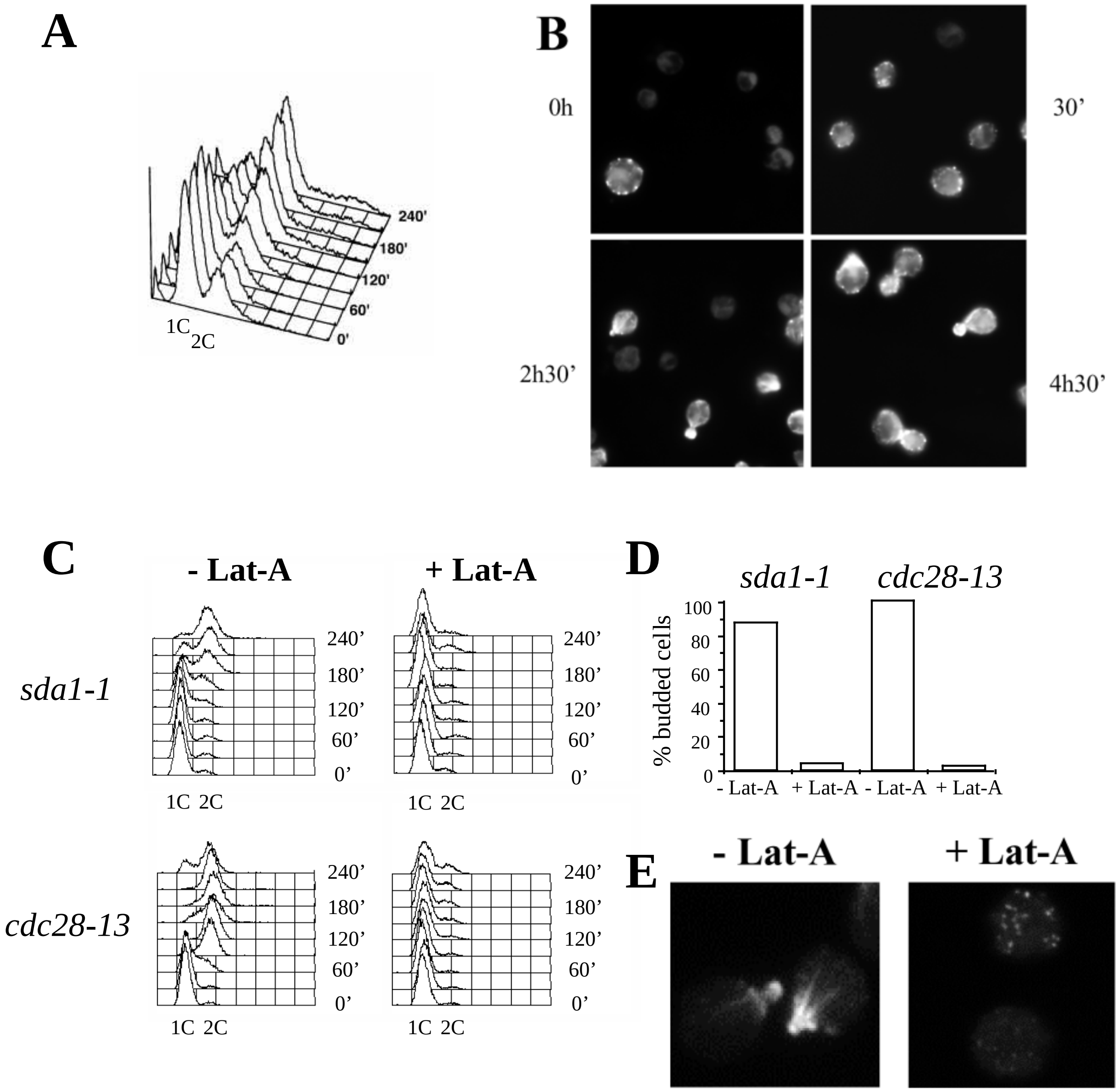


Fig. 8. DNA replication and actin structures analysis in *sda1-1* and *cdc28-13* mutant strains shifted back to permissive temperature, with or without Lat-A. (A) *sda1-1* cells maintained at 37°C for 16 hours were shifted back to 25°C (time zero). FACS analysis and (B) actin staining with rhodamine-phalloidin of samples taken at the indicated times after shift back. (C) DNA replication in *sda1-1* and *cdc28-13* mutants. *sda1-1* and *cdc28-13* cultures, arrested in G_1 at the restrictive temperature (37°C), were released at 25°C in the presence or absence of Lat-A and FACS analysis was performed. (D) Effect of Lat-A on budding: the percentage of budded cells in the same cultures of (C) is reported for *sda1-1* (4 hours after the shift back) and *cdc28-13* (2 hours after the shift back). (E) Actin visualization in *cdc28-13* cells with or without Lat-A.

(data not shown) and replicating DNA. On the contrary, treatment with Lat-A prevented DNA replication and budding (Fig. 8C and D). Actin cables, which were well visible in *cdc28-13* cells without Lat-A, were not detectable in the presence of Lat-A. In the conditions used, patches stained with phalloidin did not disappear, but they did not localize and progressively appeared as very small, poorly stained structures (up to 80% of the cells after 6 hours in Lat-A; Fig. 8E). Therefore, as in *sda1-1* mutant, in *cdc28-13* cells released from the G_1 arrest a perturbation in the actin cytoskeleton caused by Lat-A prevents not only budding but also DNA replication. Taken together these results demonstrate that actin integrity in G_1 is essential for cells to progress through the cell cycle and to start the S phase, and suggest that the primary effect of Sda1 inactivation or depletion might be the disruption of the actin cytoskeleton: monitoring of actin depolymerization might then cause arrest of the cell cycle in G_1.

The cell cycle arrest caused by *sda1-1* is not dependent on Swe1 protein

In *S. cerevisiae*, a morphogenesis checkpoint delays nuclear division when the actin cytoskeleton is perturbed (Lew and Reed, 1995; McMillan et al., 1998). This checkpoint prevents nuclear division, thereby providing time for recovery of actin

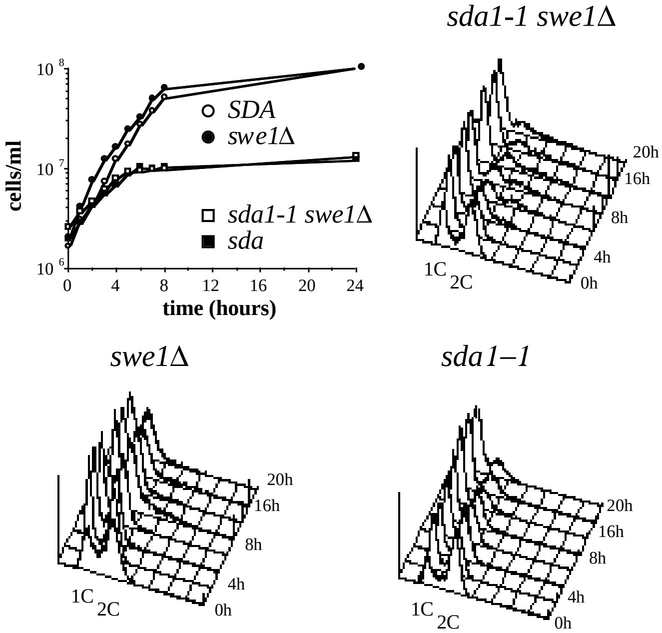


Fig. 9. *sda1-1* mutation causes a Swe1-independent cell cycle arrest. W303/34c (wild type), Q133 (*sda1-1*), Q224 (*swe1Δ*) and Q225 (*sda1-1 swe1Δ*) cultures, logarithmically growing (2×10^6 cells/ml) were shifted from 25°C to 37°C: growth rates and FACS analysis of 37°C cultures are reported. *swe1Δ* culture used for FACS analysis was diluted 1:10 with fresh medium when it reached a concentration of 1×10^7 cells/ml.

polarity and completion of bud formation prior to mitosis. During the checkpoint-induced delay, cell cycle progression is halted by inhibitory phosphorylation of Cdc28, catalyzed by the protein kinase Swe1. Cells lacking Swe1 are unable to delay mitosis in response to actin-perturbing conditions and most of them lose viability (Sia et al., 1996; McMillan et al., 1998). Cells lacking Sda1 exhibit actin cytoskeleton disruption and arrest cell cycle progression in G_1, by preventing budding and DNA replication, but maintaining high cell viability. We therefore investigated whether such arrest depends on Swe1 protein. To this end, we constructed the mutant strain Q225, carrying both *sda1-1* and *swe1Δ* mutations, and we analyzed the effect of Sda1 inactivation in this strain at the restrictive temperature. As a control we used the isogenic strains Q133, carrying the *sda1-1* mutation, and Q224, carrying the *swe1Δ* mutation. While growth rates of the four strains were very similar at 25°C (data not shown), the *sda1-1 swe1Δ* strain shifted to 37°C exhibited a phenotype analogous to *sda1-1*, as both stopped growing 2.5 generations after the shift (Fig. 9). The arrest phenotype of *sda1-1 swe1Δ* cells was similar to *sda1-1*, showing unbudded cells with a single nucleus and large budded cells with segregated nuclei (20 hours after the shift). Cells maintained high viability (as determined by microcolony assay) during growth and for many hours after growth arrest (94% after 24 hours at 37°C). *sda1-1* and *sda1-1 swe1Δ* cultures at 25°C, and *swe1Δ* cultures at 25°C and 37°C, used for FACS analysis, were diluted 1:10 with fresh medium when they reached a concentration of 1×10^7 cells/ml. FACS analysis (Fig. 9) showed that *sda1-1 swe1Δ* arrested with a 1C DNA content in most of the cells. The presence of cells with a DNA content >2C is in accordance with the observation that deletion of *SWE1* in our genetic background, independently of the presence of *sda1-1* mutation, causes about 10% of the cells to exhibit two or more buds, which separate slowly from the mother cell. Both in *sda1-1* and *sda1-1 swe1Δ* cells, phalloidin-stained actin progressively disappeared. Altogether these results indicate that the *sda1-1* mutation, at restrictive temperature, arrests cell cycle progression in G_1 independently of Swe1.

DISCUSSION

In this study we have characterized the novel Sda1 protein, which is essential for cell viability in budding yeast and appears to be conserved from yeast to humans

Indirect immunofluorescence microscopy of the tagged protein demonstrated that the Sda1 protein is located in the nucleus, and this location is maintained during cell cycle progression, since it was observed in all cell cycle phases. This finding is in agreement with the presence of nuclear localization signals, both in yeast and homologous proteins from other eukaryotes.

We showed that loss of Sda1 function causes a cell cycle arrest in G_1. In fact, cells carrying the temperature-sensitive *sda1-1* mutation, when shifted to the restrictive temperature, arrest mostly as unbudded, with unreplicated DNA and do not start a new nuclear division cycle. Growth arrest at the non-permissive temperature in *sda1-1* mutant cells does not irreversibly affect essential functions, since these cells maintain high viability for a long time and a shift back to the permissive temperature can rescue all the cell cycle processes. In fact, when shifted back to the permissive temperature, *sda1-1* cells, after a 90 minutes delay, concomitantly start a correct budding cycle and a new round of DNA replication. Therefore, the restoration of Sda1 activity is essential for both DNA replication and budding. On the contrary, in mutants specifically defective in budding, such as mutants in *CDC42* or *CDC24* genes, after growth arrest almost all cells replicate DNA, enter mitosis and many of them complete the nuclear division, producing multinucleate cells which are mostly not viable (Adams et al., 1990).

Both depletion/inactivation of the protein and overproduction of the Sda1 wild-type protein cause cell cycle delay. On the contrary, we did not observe any effect on the ability to segregate nuclei and to correctly assemble and disassemble the mitotic spindle during cell cycle progression, although overexpression of *SDA1* was found to confer to yeast hypersensitivity to benomyl, a drug which destabilizes microtubules (our unpublished observation). Actin is essential for polarized cell growth in yeast, with actin patches localizing at the cortex, near regions of active secretion and growth, such as the pre-bud site before bud emergence and the mother-bud neck at cytokinesis. A large number of proteins that physically or genetically interact with actin have been identified, and many of these components have homologues in other eukaryotes. We have shown that the temperature-sensitive *sda1-1* mutation causes defects in this reorganization of the actin cytoskeleton. In fact, when mutant cells stopped dividing at the non-permissive temperature, the majority of them showed strongly reduced or non-detectable actin patches and cables. Cells with a large bud failed to evenly redistribute actin patches from the bud to both the mother and the daughter cell, and unbudded cells did not show polarization of polymerized actin at a pre-bud site. Since western blot analysis of protein extracts from *sda1-1* mutant demonstrated a normal actin level in the cell, the disappearance of stained actin is probably dependent on perturbations in its polymerization. Moreover, when shifted back to the permissive temperature, *sda1-1* cells progressively reorganize the actin structures necessary to bud. Therefore, the Sda1 protein appears to be involved in actin cytoskeleton organization and morphogenetic events during cell cycle progression.

Cytokinesis is the final stage of the cell cycle leading to cells separation. Various proteins are thought to be involved in this process, based on their localization and on the phenotype associated with their alteration. Actin is involved in polarized processes in yeast, including septum deposition and cytokinesis. In this context, the delay in septation and cytokinesis observed when Sda1 function is perturbed in the cell, both in the *sda1-1* mutant strain at restrictive temperature and in Sda1 overproduction conditions, is likely to be a consequence of the Sda1 role in actin cytoskeleton organization. This hypothesis is further strengthened by the observation that other phenotypes of *sda1-1* mutant cells are associated with actin cytoskeleton perturbations. In fact, inappropriate location of chitin was observed in *sda1-1* mutant cells arrested at restrictive temperature, with chitin deposition over the entire surface of the cell and formation of patches of fluorescence either on mother or on daughter cells: a similar phenotype has previously been reported to be associated with perturbations of actin cytoskeleton (Novick and Botstein, 1985). Moreover, the inhibition of fluid phase endocytosis observed in *sda1-1* mutant cells at restrictive temperature can also be explained as an effect of actin cytoskeleton disruption.

Unlike the *sda1-1* mutation, the absence of functional actin due to *act1* (Novick and Botstein, 1985) conditional mutations causes lethality. The effects of the *sda1-1* mutation appear to be partially similar to those of Lat-A, as this drug has been shown to cause actin depolymerization, disrupting the actin cytoskeleton. Latrunculin-A causes in yeast cells disappearance of actin structures, defects in bud formation and arrest of growth without loss of cell viability. Similarly to *sda1-1* mutant cells, wild-type cells treated with Lat-A, when released in the absence of the drug, after a delay of about two hours, restore polymerized actin and recover growth ability (Ayscough et al., 1997).

The results we reported suggest that the *sda1-1* mutation directly or indirectly impairs the actin cytoskeleton, and this in turn activates a checkpoint control, which responds to perturbation of the actin cytoskeleton by arresting cell cycle progression in G_1. This hypothesis is supported by our observation that direct perturbation of the actin cytoskeleton by Lat-A in *cdc28-13* cells, previously arrested in G_1, triggers the same cell cycle block. A morphogenesis checkpoint dependent on the Swe1 kinase, has been recently described in budding yeast (Lew and Reed, 1995; McMillan et al., 1998): by using Lat-A it has been shown that this checkpoint directly, and possibly continuously, monitors actin organization, rather than (or in addition to) polarity establishment or bud formation, and affects cell cycle progression causing G_2 delay. Complete actin depolymerization blocks cell cycle progression, while less severe actin perturbation produces G_2 delays of various length. The conditions used in these experiments were very different from our conditions, since yeast cells were released from α-factor block in the presence of Lat-A. Our results suggest that the G_1 arrest observed either in the *sda1-1* mutant at the non-permissive temperature, and in the *cdc28-13* cells treated with Lat-A, might be due to a checkpoint mechanism activated by perturbation of actin organization. This checkpoint might couple actin cytoskeleton organization to budding and initiation of DNA replication, and should therefore be at least partly distinct from the above mentioned morphogenesis checkpoint. To further elucidate this point, we investigated the effect of Sda1 inactivation in cells lacking Swe1. We found that loss of Swe1 does not abolish the G_1 block caused by *sda1-1*. In fact, *sda1-1 swe1Δ* cells, shifted to the restrictive temperature, exhibit the same phenotype of *sda1-1* cells, since they arrest mostly as unbudded, with unreplicated DNA and do not enter mitosis. Moreover, these cells maintain high viability for a long time. Altogether these data suggest that perturbation of the actin cytoskeleton, either due to the direct effect of the drug Lat-A in cells arrested in G_1, or to defective Sda1, might activate a checkpoint mechanism which prevents transition to S phase. This checkpoint appears independent of Swe1.

While many proteins involved in actin cytoskeleton regulation have a cytoplasmic localization, we have shown that Sda1 is mainly localized in the nucleus. Further experiments will be necessary to shed light on the mechanism by which this nuclear protein can affect the actin cytoskeleton. The possibility that Sda1 is involved in transcriptional regulation has to be considered. Nuclear localization was demonstrated for Anc1/Tgf3 (Welch and Drubin, 1994), a protein implicated in the actin cytoskeleton function. This polypeptide has been shown to be a component of the yeast Swi/Snf, a multiprotein complex, involved in transcriptional activation, and also a member of two other yeast transcription factors, TFIID and TFIIF (Henry et al., 1994; Cairns et al., 1996). Since Sda1 is highly conserved in eukaryotes, the characterization of its role in cell cycle progression and in actin organization hopefully will provide an opportunity to obtain useful information on the function of homologous proteins in higher eukaryotes.

We thank Giovanna Lucchini and Simonetta Piatti for continuous support, fruitful discussions and critical reading of the manuscript. We also thank Lucia Panzeri for useful criticism and suggestions. We are grateful to J. N. McMillan and D. J. Lew for providing the *swe1Δ::LEU2* cassette, and to P. Crews for providing Latrunculin-A. The financial support of MURST-Università degli Studi di Milano Cofin. 1997 and Cofin. 1999 are gratefully acknowledged. This research was partially supported by MURST 60%. The NIH grant CA 47135 is acknowledged.

REFERENCES

Adams, A. E. M., Johnson, D. I., Longnecker, R. M., Sloat, B. F. and Pringle, J. R. (1990). *CDC42* and *CDC43*, two additional genes involved in budding and establishment of cell polarity in the yeast *Saccharomyces cerevisiae. J. Cell Biol*. **111**, 131-142.

Amberg, D. C. (1998). Three-dimensional imaging of the yeast actin cytoskeleton through the budding cell cycle. *Mol. Biol. Cell.* **9**, 3259-3262.

Ayscough, K. R. and Drubin, D. G. (1996). Actin. General principles from studies in yeast. *Annu. Rev. Cell Dev. Biol*. **12**, 129-160.

Ayscough, K. R., Styker, J., Pokala, N., Sanders, M., Crews, P. and Drubin,

D. G. (1997). High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor Latrunculin-A. *J. Cell Biol.* **137**, 399-416.

Ayscough, K. R. (1998). *In vivo* functions of actin-binding proteins. *Curr. Opin. Cell Biol*. **10**, 102-111.

Bi, E., Maddox, P., Lew, D. J., Salmon, E. D., McMillan, J. N., Yeh, E. and Pringle, J. R. (1998). Involvement of an actomyosin contractile ring in *Saccharomyces cerevisiae* cytokinesis. *J. Cell Biol.* **142**, 1301-1312.

Booher, N. R., Deshaies, R. J. and Kirschner, M. W. (1993). Properties of *Saccharomyces cerevisiae* wee1 and its differential regulation of $p34^{CDC28}$ in response to G_1 and G_2 cyclins. *EMBO J.* **9**, 3417-3426.

Botstein, D., Amberg, D., Mulholland, J., Huffaker, T., Adams, A., Drubin, D. and Stearns, T. (1997). The yeast cytoskeleton. In *The Molecular and Cellular Biology of the Yeast Saccharomyces cerevisiae*, vol. 3 (ed. J. R. Pringle, J. R. Broach and E. W. Jones), pp. 1-90. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Botstein, D., Falco, S. C., Stewart, S. E., Brennan, M., Scherer, S., Stinchcomb, D. T., Struhl, K. and Davis, R. W. (1979). Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. *Gene* **8**, 17-24.

Cabib, E. and Bowers, B. (1975). Timing and function of chitin synthesis in yeast. *J. Bacterol*. **124**, 1586-1593.

Cairns, B. R., Henry, N. L. and Kornberg, R. D. (1996). TFG3/TAF30/ANC1, a component of the yeast SWI/SNF complex that is similar to the leukemogenic proteins ENL and AF-9. *Mol. Cell. Biol*. **16**, 3308-3316.

Carlier, M. F. and Pantaloni, D. (1997). Control of actin dynamics in cell motility. *J. Mol. Biol*. **269**, 459-467.

Cid, V. J., Duran, A., del Rey, F., Snyder, M. P., Nombela, C. and Sanchez M. (1995). Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae. Microbiol. Rev.* **59**, 345-386.

Dingwall, C. and Laskey, R. A. (1991). Nuclear targeting sequences-a consensus? *Trends Biochem. Sci.* **16**, 478-481.

Dulic, V., Egleton, M., Elgundi, I., Raths, S., Singer, B. and Riezman, H. (1991). Yeast endocytosis essays. *Meth. Enzymol.* **194**, 697-710.

Epstein, C. B. and Cross, F. R. (1992). CLB5: a novel B cyclin from budding yeast with a role in S phase. *Genes Dev*. **6**, 1695-1706.

Feroli, F., Carignani, G., Pavanello, A., Guerreiro, P., Azevedo, D., Rodrigues-Pousada, C., Melchioretto, P., Panzeri, L. and Agostoni Carbone, M. L. (1997). Analysis of a 17. 9 kb region from the *Saccharomyces cerevisiae* chromosome VII reveals the presence of eight open reading frames, including the *BRF1* (*TFIIIB70*) and *GCN5* genes. *Yeast* **13**, 373-377.

Foiani, M., Marini, F., Gamba, D., Lucchini, G. and Plevani, P. (1994). The B subunit of the DNA polymerase alpha-primase complex in *Saccharomyces cerevisiae* executes an essential function at the initial stage of DNA replication. *Mol. Cell. Biol.* **14**, 923-933.

Fujiwara, T., Tanaka, K., Inoue, E., Mitsuhiro, K. and Takai, Y. (1999). Bni1p regulates microtubule-dependent nuclear migration through the actin cytoskeleton in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **19**, 8016-8027.

Guthrie, C. and Fink, G. R. (1991). Guide to yeast genetics and molecular biology. *Meth. Enzymol.* **194**, 729-731.

Hayashibe, M. and Katohda, S. (1973). Initiation of budding and chitin ring. *J. Gen. Appl. Microbiol.* **19**, 23-29.

Henry, N. L., Campbell, A. M., Feaver, W. J., Poon, D., Weil, P. A. and Kornberg, R. D. (1994). TFIIF-TAF-RNA polymerase II connection. *Genes Dev*. **8**, 2868-2878.

Kübler, E. and Rietzman, H. (1993). Actin and fimbrin are required for the internalization step of endocytosis in yeast. *EMBO J*. **12**, 2855-2862.

Lew, D. J. and Reed, S. I. (1995). Cell cycle control of morphogenesis in budding yeast. *Curr. Opin. Genet. Dev*. **5**, 17-23.

Madden, K. and Snyder, M. (1998). Cell polarity and morphogenesis in budding yeast. *Annu. Rev. Microbiol*. **52**, 687-744.

McMillan, J. N., Sia, R. A. L. and Lew, D. J. (1998). A morphogenesis checkpoint monitors the actin cytoskeleton in yeast. *J. Cell Biol*. **142**, 1487-99.

McMillan, J. N., Longtine, M. S., Sia, R. A. L., Theesfeld, C. L., Bardes, E. S. G., Pringle, J. R. and Lew, D. J. (1999). The morphogenesis checkpoint in *Saccharomyces cerevisiae*: cell cycle control of Swe1p degradation by Hsl1p and Hsl7p. *Mol. Cell. Biol.* **19**, 6929-6939.

Mulholland, J., Preuss, D., Moon, A., Wong, A., Drubin, D. and Botstein, B. (1994). Ultrastructure of the yeast actin cytoskeleton and its association with the plasma membrane. *J. Cell Biol.* **125**, 381-391.

Muzi Falconi, M., Piseri, A., Ferrari, M., Lucchini, G., Plevani, P. and Foiani, M. (1993). De novo synthesis of budding yeast DNA polymerase α and *POL1* transcription at the G_1/S boundary are not required for entrance into S phase. *Proc. Nat. Acad. Sci. USA* **90**, 10519-10523.

Nasmyth, K. (1993). Control of the yeast cell cycle by the Cdc28 protein kinase. *Curr. Opin. Cell Biol*. **5**, 166-179.

Novick, P. and Botstein, D. (1985). Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell* **40**, 415-416.

Novick, P., Osmond, B. C. and Botstein, D. (1989). Suppressors of yeast actin mutations. *Genetics* **121**, 659-674.

Pringle, J. R., Preston, R. A., Adams, A. M., Stearns, T., Drubin, D., Haarer, B. K. and Jones, W. E. (1989). Fluorescence microscopy methods for yeast. *Meth. Cell. Biol.* **1**, 357-435.

Pringle, J. R. (1991). Staining of bud scars and other cell wall chitin with calcofluor. *Meth. Enzymol.* **194**, 732-735.

Reed, S. I. and Wittenberg, C. (1990). Mitotic role for the Cdc28 protein kinase of *Saccharomyces cerevisiae*. *Proc. Nat. Acad. Sci. USA* **87**, 5697-5701.

Rose, M. D., Winston, F. and Hieter, P. (1990). *Methods in Yeast Genetics. A Laboratory Course Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Rothstein, R. (1991). Targeting, disruption, replacement and allele rescue: integrative DNA transformation in yeast. *Meth. Enzymol.* **194**, 281-301.

Sia, R. A. L., Herald, H. and Lew, D. J. (1996). Cdc28 tyrosine phosphorylation and the morphogenesis checkpoint in budding yeast. *Mol. Biol. Cell*. **7**, 1657-1666.

Sikorski, R. S. and Boeke, J. D. (1991). In vitro mutagenesis and plasmid shuffling from cloned gene to mutant yeast. *Meth. Enzymol.* **194**, 303-318.

Sikorski, R. S. and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae. Genetics* **1223**, 19-27.

Struhl, K. and Davis, R. W. (1980). A physical, genetic and transcriptional map of the cloned *HIS3* gene region of *Saccharomyces cerevisiae. J. Mol. Biol.* **136**, 309-332.

Wieland, T. (1977). Modification of actins by phallotoxins. *Naturwissenschaften* **64**, 303-309.

Welch, M. D. and Drubin, D. G. (1994). A nuclear protein with sequence similarity to proteins implicated in human acute leukemias is important for cellular morphogenesis and actin cytoskeletal function in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **5**, 617-632.