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Regulation of cytokinesis by the Elm1 protein kinase in *Saccharomyces cerevisiae*

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SUMMARY

A *Saccharomyces cerevisiae* mutant unable to grow in a *cdc28-1N* background was isolated and shown to be affected in the *ELM1* gene. Elm1 is a protein kinase, thought to be a negative regulator of pseudo-hyphal growth. We show that Cdc11, one of the septins, is delocalised in the mutant, indicating that septin localisation is partly controlled by Elm1. Moreover, we show that cytokinesis is delayed in an *elm1*Δ mutant. Elm1 levels peak at the end of the cell cycle and Elm1 is localised at the bud neck in a septin-dependent fashion from bud emergence until the completion of anaphase, at about the time of cell division. Genetic and biochemical evidence suggest that Elm1 and the three other septin-localised protein kinases, Hsl1, Gin4 and Kcc4, work in parallel pathways to regulate septin behaviour and cytokinesis. In addition, the *elm1*Δ morphological defects can be suppressed by deletion of the *SWE1* gene, but not the cytokinesis defect nor the septin mislocalisation. Our results indicate that cytokinesis in budding yeast is regulated by Elm1.

Key words: *Saccharomyces cerevisiae*, Cytokinesis, Division plane, Septin

INTRODUCTION

Cytokinesis is the process that partitions the cell surface and the cytoplasm of one cell to form two cells. This process requires selection of a specific division site where the cytokinetic machinery will be assembled. The selection of the division site has to be coordinated with the spindle position in order to ensure that each daughter cell has a full complement of chromosomes and all the essential organelles. Eukaryotes have evolved variable mechanisms to select the division plane. In animal cells, the correct positioning and assembly of the contractile ring requires the mitotic spindle (Field et al., 1999; Glotzer, 1997). In *Saccharomyces cerevisiae*, the selection of the division plane is coincident with bud emergence and does not depend on the spindle (Chant, 1996; Longtine et al., 1996). Rather, the spindle is positioned through the bud neck after cleavage plane selection. Once the division site has been determined, its location has to be marked on the cell surface. Thus, a precise zone has to be specified and has to be molecularly differentiated from the rest of the cell surface. In addition, cytokinesis has to be tightly coordinated with the other cell cycle events, so as not to occur before mitosis. Despite apparent differences, the cytokinesis molecular machinery is highly conserved amongst eukaryotes. In particular, an acto-myosin ring has been shown to provide the mechanical force necessary for cleavage in budding yeast (Bi et al., 1998; Lippincott and Li, 1998b) as well as in animal cells (Field et al., 1999).

A novel cytoskeletal structure, the 10-nm neck filaments, is formed by a homologous family of proteins called the septins (Longtine et al., 1996). The septins were originally identified in budding yeast (Hartwell, 1971) and homologs have since been identified in many other organisms, including humans (Nakatsuru et al., 1994; Nottenburg et al., 1990), mice (Hsu et al., 1998; Kinoshita et al., 1997), and flies (Fares et al., 1995; Field et al., 1996; Neufeld and Rubin, 1994). Analysis of budding yeast conditional alleles of the septin genes, *CDC3*, *CDC10*, *CDC11* and *CDC12*, revealed that they are required for cytokinesis and septum formation (Chant, 1996; Longtine et al., 1996). Similarly, a mutation in a *Drosophila* septin gene, *pnut* (Neufeld and Rubin, 1994), or microinjection of an antibody directed against a mouse septin (Kinoshita et al., 1997), have been shown to interfere with cytokinesis. In these organisms, the septins appear to recruit proteins and membrane to establish and/or maintain specialized domains on the plasma membrane. It is thought that they constitute a scaffold for the correct localisation of proteins directly involved in cytokinesis (Bi et al., 1998; Lippincott and Li, 1998a,b) and cell-surface growth (DeMarini et al., 1997). They are thus associated with the selection and the maintenance of the cleavage plane (Chant, 1996; Kinoshita et al., 1997; Longtine et al., 1996).

The septins form a multi-protein complex localised in a sub-membrane region in yeast (Chant, 1996; Longtine et al., 1996),

Drosophila (Fares et al., 1995; Neufeld and Rubin, 1994), mice (Kinoshita et al., 1997) and humans (Hsu et al., 1998). In *S. cerevisiae*, the septins assemble as a ring at the bud neck constriction. Behaviour of this ring is cell-cycle regulated. The ring appears at the presumptive bud site ≈15 minutes before bud emergence, is duplicated in G_2 and disappears after cytokinesis (Haarer and Pringle, 1987; Kim et al., 1991). Interestingly, proteins which are required for establishing cell polarity in yeast, such as Cdc42, are also localised at the future bud site before bud emergence. However, these proteins are later found at the apex of growing buds (Pringle et al., 1995), whereas the septin ring is maintained at the bud neck throughout the cell cycle. Moreover, recent papers suggest that correct bud-neck localisation is essential in budding yeast for septin function (Frazier et al., 1998; Longtine et al., 1998a). The nature of the proteins anchoring the septins at the bud neck is still unclear. Nevertheless, some proteins have been identified which are involved in this process. Cla4 is a Cdc42-dependent protein kinase, whose activity is required for keeping the septins at the bud-neck constriction (Cvrckova et al., 1995). Three genetically-redundant protein kinases, Hsl1, Gin4 and Kcc4, are also required for the maintenance of septin organisation (Barral et al., 1999; Carroll et al., 1998; Longtine et al., 1998a), as a *hsl1*Δ *gin4*Δ *kcc4*Δ triple mutant accumulates misshapen septin rings in budded cells (Barral et al., 1999). Interestingly, these three kinases are homologous to higher-eukaryote kinases, such as *Caenorhabditis elegans* PAR-1, implicated in cell polarity control (Drewes et al., 1997; Guo and Kemphues, 1995). Hsl1, Gin4 and Kcc4 associate with the septins at the bud neck (Barral et al., 1999; Longtine et al., 1998a; Tanaka and Nojima, 1996) and their kinase activities are dependent upon septin function (Barral et al., 1999; Carroll et al., 1998).

Passage through mitosis is a prerequisite for cytokinesis to occur. Dependency relationships between cell-cycle events are often established by checkpoints (Hartwell and Weinert, 1989). When actin is depolarised, a morphogenesis checkpoint delays mitosis through activation of the Swe1 kinase (Lew and Reed, 1995). It has recently been shown that a similar checkpoint delays the entry into mitosis as a result of septin defects and that it involves Hsl1, Gin4 and Kcc4 (Barral et al., 1999). These kinases are negative regulators of the Swe1 kinase (Ma et al., 1996) and link Swe1 activity to septin organisation (Barral et al., 1999). Thus, Hsl1, Gin4 and Kcc4 have a dual role in cytokinesis, maintaining cytoskeletal organisation and coordinating progression through mitosis with the preparation of cytokinesis.

The *ELM1* gene encodes a protein kinase and was originally identified on the basis of the growth hyperpolarisation associated with the inactivation of the gene, leading to the suggestion that it is a negative regulator of pseudohyphal differentiation (Blacketer et al., 1993). The hyperpolarised growth phenotype of *elm1*Δ is suppressed either by a dominant mutation in *HSL1* or a recessive mutation in *SWE1*. It was proposed that *ELM1* functions to regulate the morphogenesis checkpoint through direct activation of Hsl1, and possibly of Gin4 and Kcc4 too (Edgington et al., 1999). We have isolated a new *ELM1* allele in a screen for mutants colethal with *cdc28-1N*. We have shown that cytokinesis is specifically affected in *elm1* mutants; in particular, Elm1 function is required for proper septin localisation at the bud neck. However, both our genetic and biochemical evidence show that Elm1 works, at least in part, independently of Hsl1, Gin4 and Kcc4. Moreover, *SWE1* inactivation does not relieve *elm1*Δ cytokinesis defects. Thus, maintenance of the septins at the bud neck is achieved through at least two pathways, suggesting that this regulation is likely to be complex.

MATERIALS AND METHODS

Strains, media and reagents

All yeast strains used in this study are listed in Table 1. Strains were grown in YEPD (1% yeast extract, 2% Bactopeptone, 2% glucose) or, for diploid or plasmid selection, in synthetic minimal medium (0.67% yeast nitrogen base, 2% glucose, raffinose or galactose) supplemented with the appropriate amino acids at 40 μg/ml. The growth temperature was 30°C, unless otherwise stated. Yeast transformations were performed by a modification of the lithium acetate procedure (Gietz and Sugino, 1988).

Isolation of the *elm1-1N* mutant and cloning of the *ELM1* gene

A synthetic lethal screen was conducted with different *cdc28* mutants (Ghislain et al., 1993; Mazzoni et al., 1993; Zarzov et al., 1996). A mutation unable to grow in combination with *cdc28-1N*, a *CDC28* allele that is blocked at the G_2/M transition at the restrictive temperature, was isolated and designated *elm1-1N* (see below). The corresponding wild-type gene was isolated by selection of plasmids from a genomic DNA library in a centromere vector that complements the synthetic lethality. Sequencing of inserts from 8 complementing clones revealed that they carried the *ELM1* gene, suggesting that it might correspond to the wild-type gene. This was checked by integrating a *URA3* cassette at the *ELM1* locus and showing that it segregates 2:2 in crosses with our mutation. Thus, we have isolated a new allele of *ELM1* which is synthetically lethal with *cdc28-1N* (hence the name *elm1-1N*).

Plasmid and strain constructions

Standard genetic techniques were used to manipulate yeast strains (Sherman, 1991). Standard protocols were used to manipulate DNA (Sambrook et al., 1989). To clone *ELM1* under the control of the *GAL-1-10* promoter, *ELM1* was first amplified by PCR with the addition of a 5′ *Bam*HI site and a 3′ *Xba*I site and cloned into pYES2 (Invitrogen) digested with *Bam*HI and *Xba*I. A *SpeI*-restriction fragment containing a 9-myc epitope cassette (a kind gift from K. Nasmyth) was then inserted into the *Xba*I site of the resulting plasmid, pAB103, and the sequence of the *ELM1-myc*$_9$ fusion was checked. The fusion (without any promoter) was then cloned into the integrative pRS304 plasmid (Sikorski and Hieter, 1989). The resulting plasmid, pAB105, was cut by *Nde*I and inserted at the *ELM1* locus under the control of the endogenous *ELM1* promoter, giving strain NBY86. The morphology of the cells was wild type, indicating that the epitope-tagged protein was fully functional. In order to fuse *ELM1* to GFP in YYB64 or YYB214 (*cdc12-1*), a GFP cassette was inserted just before the stop codon of the gene, as previously described (Longtine et al., 1998b). For each strain, the morphology of the cells was wild-type at 25°C, indicating that the epitope-tagged protein was fully functional. A similar approach was used to tag *HSL1* and *KCC4* at the C terminus with three HA epitopes in YYB214.

Preparation of yeast crude extracts and protein analysis

Protein extractions were routinely carried out as previously described (Vialard et al., 1998), except for cell-cycle experiments, when they were made as described by Zarzov et al. (1996). Immunoprecipitation and H1 kinase assays were performed as described (Kramer et al., 1998).

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

Strain	Genotype	Reference
CMY715	*MAT***a** *ura3-52 trp1Δ1 his3Δ200 leu2Δ1 ade2-101 lys2-801 HIS3::cdc28-1N*	Lab collection
cim21	*MAT***a** *ura3-52 trp1Δ1 his3Δ200 leu2Δ1 ade2-101 lys2-801 elm1-1N*	This study
YPH499 background		
NBY74	*MAT***a** *ura3-52 trp1Δ1 his3Δ200 leu2Δ1 ade2-101 lys2-801 PDS1-HA:ura3*	This study
NBY75	*MAT***a** *ura3-52 trp1Δ1 his3Δ200 leu2Δ1 ade2-101 lys2-801 PDS1-HA:ura3 elm1::HIS3*	This study
NBY86	*MAT***a** *ura3-52 trp1Δ1 his3Δ200 leu2Δ1 ade2-101 lys2-801 PDS1-HA:ura3 ELM1myc9:: TRP1*	This study
NBY88	*MAT***a** *ura3-52 trp1Δ1 his3Δ200 leu2Δ1 ade2-101 lys2-801 PDS1-HA:ura3 elm1K117Rmyc9::TRP1*	This study
NBY100	*MAT***a** *ura3-52 trp1Δ1 his3Δ200 leu2Δ1 ade2-101 lys2-801 PDS1-HA:ura3 elm1::HIS3 swe1::LEU2*	This study
NBY103	*MAT***a** *ura3-52 trp1Δ1 his3Δ200 leu2Δ1 ade2-101 lys2-801 ELM1:GFPS65T-kanMX4*	This study
YYB64 background		
YYB64	*MAT***a** *ura3-52 trp1Δ1 his3Δ200 leu2Δ1 ade2-101 lys2-801*	(Barral et al., 1999)
YYB338	*MATα ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 kcc4::TRP1 gin4::HIS3 hsl1::URA3*	(Barral et al., 1999)
YYB339	*MATα ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 kcc4::TRP1 gin4::HIS3 hsl1::URA3 swe1::LEU2*	(Barral et al., 1999)
YYB443	*MAT***a** *ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 elm1::HIS3*	This study
YYB447	*MAT***a** *ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 elm1::HIS3 swe1::LEU2*	This study
YYB461	*MAT***a** *ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 elm1::HIS3 hsl1::URA3 kcc4::TRP1 gin4::HIS3*	This study
NBY95	*MAT***a** *ura3-52 his3Δ200 leu2Δ1 ade2-101 lys2-801 HA_3-HSL1*	This study
NBY107	*MAT***a** *ura3-52 his3Δ200 leu2Δ1 ade2-101 lys2-801 HA_3-HSL1 elm1::HIS3*	This study
NBY96	*MAT***a** *ura3-52 his3Δ200 leu2Δ1 ade2-101 lys2-801 HA_3-KCC4*	This study
NBY108	*MAT***a** *ura3-52 his3Δ200 leu2Δ1 ade2-101 lys2-801 HA_3-KCC4 elm1::HIS3*	This study
YYB214	*MAT***a** *ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 cdc12-1*	(Barral et al., 1999)
NBY130	*MAT***a** *ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 cdc12-1 HSL1-HA_3 kanMX4*	This study
NBY131	*MAT***a** *ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 cdc12-1 KCC4-HA_3 kanMX4*	This study
NBY132	*MAT***a** *ura3-52 trp1Δ63 his3Δ200 leu2Δ1 ade2-101 lys2-801 cdc12-1 ELM1:GFPS65T kanMX4*	This study

Other techniques

The isolation of small, unbudded G_1 cells was performed as described (Schwob and Nasmyth, 1993). FACS analysis was done using a Bio-Rad BRYTE cytometer. Immunofluorescence was performed as described (Munoz-Centeno et al., 1999) with an anti-Cdc11 rabbit polyclonal antibody (Santa Cruz) at a 1/100 dilution and Alexa 594 goat anti-rabbit IgG (Molecular Probes, ref: y-415) at a 1/100 dilution. Cells were observed with a Zeiss Axioplan microscope; photographs were taken with a cooled Micromax CCD camera (Princeton Instruments, Inc.); images were acquired with the MetaMorph software from Universal Imaging, Inc., and subsequently exported into Adobe Photoshop 5.0 for the preparation of figures.

RESULTS

Septins are delocalised in the *elm1*Δ mutant

A mutation unable to grow in combination with *cdc28-1N*, a *CDC28* allele that is blocked at the G_2/M transition at the restrictive temperature, was isolated and identified as an *elm1* mutant (see Materials and Methods). The *ELM1* (ELongated Morphology 1) gene encodes a novel type of protein kinase that was suggested to be a negative regulator of pseudohyphal growth (Blacketer et al., 1993). Although the *ELM1* gene is not essential, *elm1*Δ mutants display a hyperpolarised growth phenotype reminiscent of mutants defective in septin function (Barral et al., 1999; Cvrckova et al., 1995; Longtine et al., 1998a) and it was therefore possible that septins were affected in *elm1*Δ. We examined septin localisation in *elm1*Δ in the severely affected YPH499 background. In this strain, immunofluorescence analysis with polyclonal antibodies directed against Cdc11 showed that the protein did not form a straight ring at the bud neck as in the wild type. Rather, staining leaked into the bud, often localising at the tip of the bud (Fig. 1A). The same pattern of immunofluorescence was observed in a strain with an *elm1K117R* allele (Fig. 1Ad), encoding a catalytically inactive enzyme (Blacketer et al., 1993). Thus, Elm1 kinase activity is necessary for proper localisation of the septins in the YPH499 background. In the closely related YYB64 strain, Cdc11 delocalisation was not as pronounced as in the YPH background, correlating with the lesser degree of morphological abnormalities in this strain. Nevertheless, a Cdc11 signal at the bud tip could still be observed in most of the cells (Fig. 1B). Thus, even in a genetic background that is less affected by this mutation, the *ELM1* deletion clearly affects septin localisation. We also examined Cdc11 localisatin in three genetic backgrounds that were used in the initial *ELM1* characterisation, namely NY13, D273-10B and Σ1278b (Blacketer et al., 1993). In all three strains Cdc11 was delocalised into the bud (data not shown). We thus conclude that *ELM1* is required directly or indirectly for restricting septins to the bud neck.

The septin delocalisation defect observed in *elm1*Δ prompted us to investigate genetic interactions between *ELM1* and septin mutants. Overexpression of Elm1, either from an ectopic promoter or from a multicopy plasmid did not rescue *cdc12-1* temperature sensitivity (data not shown). On the other hand, defects conferred by *elm1*Δ and septin mutations were additive at the permissive temperature. In particular, an *elm1*Δ *cdc12-1* double mutant grows much more slowly than either single mutant at 25°C.

Cytokinesis is affected in *elm1*Δ

The synthetic lethality between *elm1-1N* and *cdc28-1N* suggested that *ELM1* could play a role in cell cycle progression. Indeed, it has been suggested that an *ELM1*

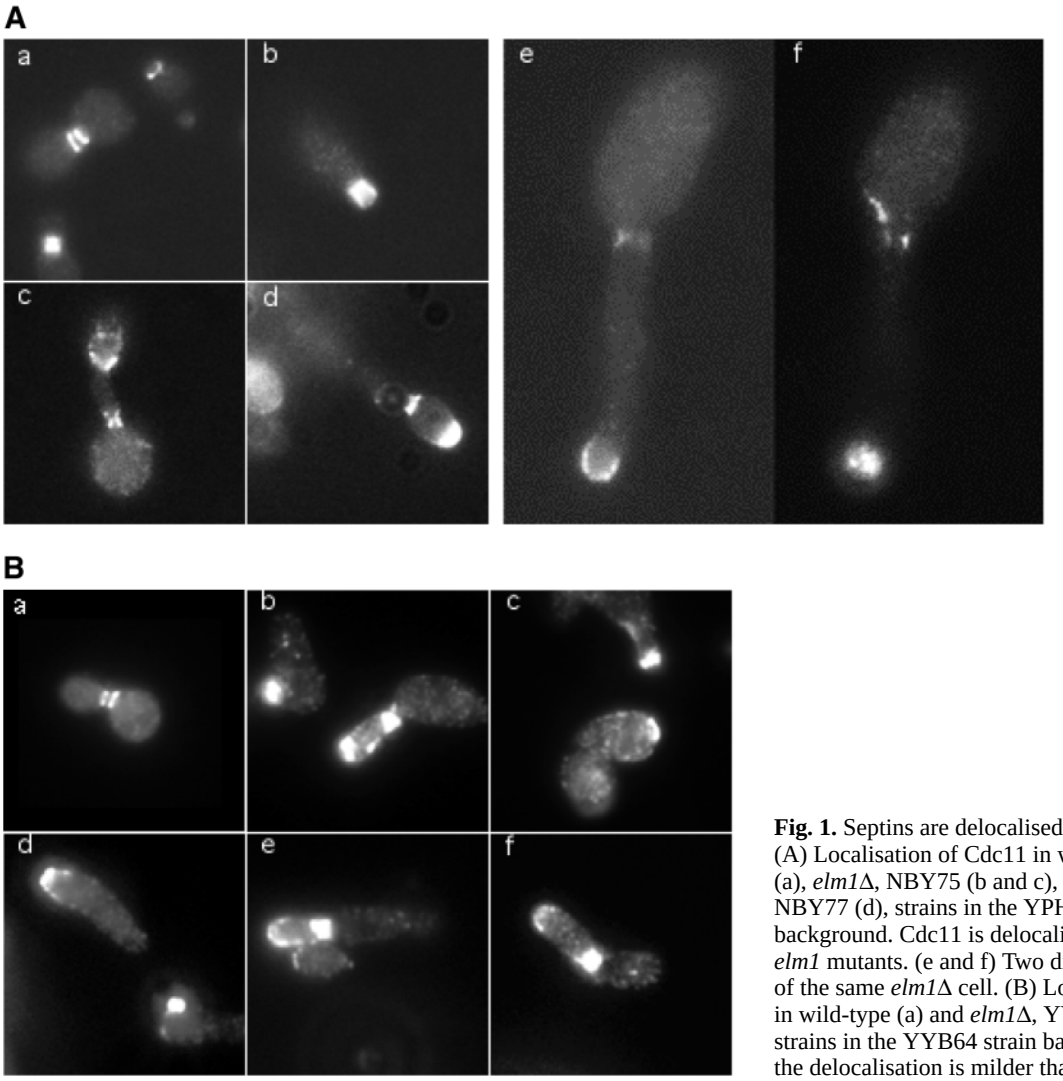


Fig. 1. Septins are delocalised in *elm1* mutants. (A) Localisation of Cdc11 in wild-type, NBY74 (a), *elm1*Δ, NBY75 (b and c), and *elm1K117R*, NBY77 (d), strains in the YPH499 strain background. Cdc11 is delocalised to the bud tip in *elm1* mutants. (e and f) Two different focal planes of the same *elm1*Δ cell. (B) Localisation of Cdc11 in wild-type (a) and *elm1*Δ, YYB443 (b,c,d,e,f), strains in the YYB64 strain background. Note that the delocalisation is milder than in YPH499.

deletion might affect cytokinesis, since it results in large elongated and multiply budded cells (Blacketer et al., 1993). However, mutants defective in other phases of the cell cycle, e.g. *cdc34* or *cdc4* (Schwob et al., 1994), display the same kind of morphological phenotype and it was thus not clear whether *elm1*Δ was affected in cytokinesis or in some previous cell-cycle event. In order to discriminate between these possibilities, we synchronised both *elm1*Δ and the isogenic wild type. We used the YYB64 background because the strong morphological defects of the *elm1*Δ mutant in YPH499 prevented us from obtaining properly synchronised cultures, either by centrifugal elutriation or α-factor arrest. Small G_1 cells of YYB64 and *elm1*Δ were isolated by centrifugal elutriation and incubated in YEPD at 30°C. Samples were taken at regular intervals and the timing of DNA replication (by FACS analysis), budding (frequency of budded cells) and of nuclear division (percentage of binucleate cells, i.e. cells showing two DAPI spots) were followed (Fig. 2). In both cultures, FACS analysis showed that DNA replication was not affected in *elm1*Δ (data not shown) and budded cells appeared at ca. 45-60 minutes. The budding pattern of both strains was identical until ≈85-90% of the cells were budded. Strikingly, whereas cytokinesis began at 120 minutes in the wild type, it

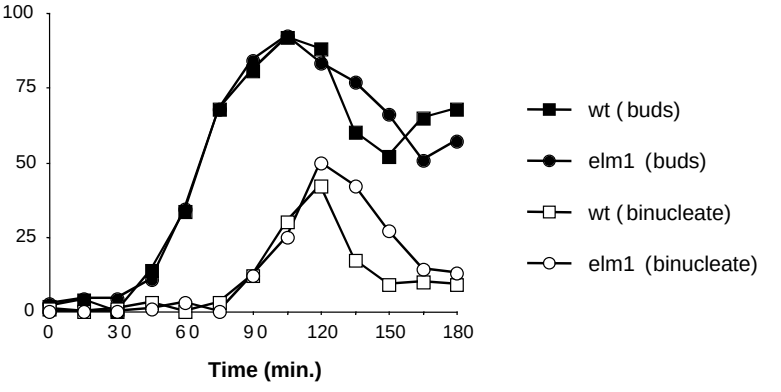


Fig. 2. Cytokinesis is delayed in *elm1*Δ cells. Wild-type (NBY74) and *elm1*Δ (NBY75) cells were synchronised by centrifugal elutriation and released at 30°C in YEPD. The percentage of budded cells and binucleate cells was determined microscopically.

was delayed for 15 minutes in *elm1*Δ, since the frequency of budded cells showed little decrease between 120 and 135 minutes compared to the wild type (Fig. 2). The drop in binucleate cells also showed a 15 minute lag in *elm1*Δ compared to the wild type, confirming that cytokinesis was delayed in *elm1*Δ. Importantly, anaphase onset (appearance of binucleate cells) was not affected by the *ELM1* deletion in this background, indicating that the delay in cytokinesis was not caused by a retarded entry into mitosis. These results suggest that the primary cell-cycle defect of *elm1*Δ lies in cytokinesis. Septin distribution through the cycle was also examined by immunofluorescence. Surprisingly, in contrast to YYB64 *elm1*Δ asynchronous cultures, no obvious defects in septin localisation could be detected in *elm1*Δ in this experiment. This difference may indicate that the effect of *elm1*Δ on septin localisation either is indirect or results from the accumulation of subtle defects in septin organisation over several generations in the YYB64 strain background.

Elm1 effects on cytokinesis are independent of Hsl1, Gin4 and Kcc4

Hsl1, Gin4 and Kcc4 are three redundant protein kinases controlling septin organisation (Barral et al., 1999; Longtine et al., 1998a). It was suggested, based on genetic data, that Elm1 could control Hsl1 (Edgington et al., 1999). In agreement with this idea, the *hsl1*Δ *gin4*Δ *kcc4*Δ triple mutant displays a hyperpolarised phenotype reminiscent of *elm1*Δ (Barral et al., 1999). To directly test the hypothesis that Elm1 is a regulator of Hsl1, and maybe of the other two kinases, we examined Hsl1 and Kcc4 phosphorylation status in *elm1*Δ. It was previously shown that phosphorylation of Hsl1 or Kcc4 results in retarded mobility of these proteins on SDS-polyacrylamide gels (Barral et al., 1999; Y. Barral, unpublished results). Western blotting of crude extracts of wild-type and *elm1*Δ strains (in the YYB64 background) expressing either HA_3-Hsl1 or Kcc4-HA_3 showed that the two proteins migrated faster in the mutant than in the wild type (Fig. 3A), indicating that they are hypophosphorylated in *elm1*Δ. However, dephosphorylation was only partial (Fig. 3A).

If Elm1 was a direct activator of Hsl1 and Kcc4, Elm1 overexpression might be able to bypass the septin requirement for their phosphorylation. On the other hand, septins are partially delocalised in *elm1*Δ (see above) and they are necessary for Hsl1 phosphorylation (Barral et al., 1999). Defects in Hsl1 and Kcc4 phosphorylation might therefore result from a defect in septin organisation. If this was the case, then full phosphorylation of the two proteins should not be restored by Elm1 overexpression in a septin mutant. We thus examined Hsl1 and Kcc4 phosphorylation by western blotting in a *cdc12-1* mutant carrying a plasmid with the *ELM1* gene under the control of the *GAL1-10* promoter. *cdc12-1 HSL1-HA*$_3$ and *cdc12-1 KCC4-HA*$_3$ strains were grown in a raffinose-containing medium until mid-log phase and half of each culture was then transferred to 37°C, whilst the other half remained at 25°C. Galactose or glucose was then added to induce or repress *ELM1* expression, respectively. At 25°C, the septin ring was correctly assembled and Hsl1-HA_3 and Kcc4-HA_3 presented their characteristic retarded migration. At 37°C, no septin ring could be observed (data not shown). On glucose, i.e. when *ELM1* transcription was repressed, only a fast-migrating form could be detected for each of the two proteins. On galactose, the mobility of these bands was not affected, despite the vast overexpression of the Elm1 protein (Fig. 3B). Thus, septin function is absolutely necessary for Hsl1 and Kcc4 phosphorylation and cannot be bypassed by Elm1 overexpression.

The relationship between *ELM1* and the three redundant kinase genes *HSL1*, *GIN4* and *KCC4* was also analysed genetically. We crossed the *hsl1*Δ *gin4*Δ *kcc4*Δ triple mutant with the *elm1*Δ mutant, in the YYB64 background, in which deletion of *ELM1* results in the mildest phenotype (see above). Combination of the four mutations was highly deleterious. Many spores predicted to be quadruple mutants did not germinate.

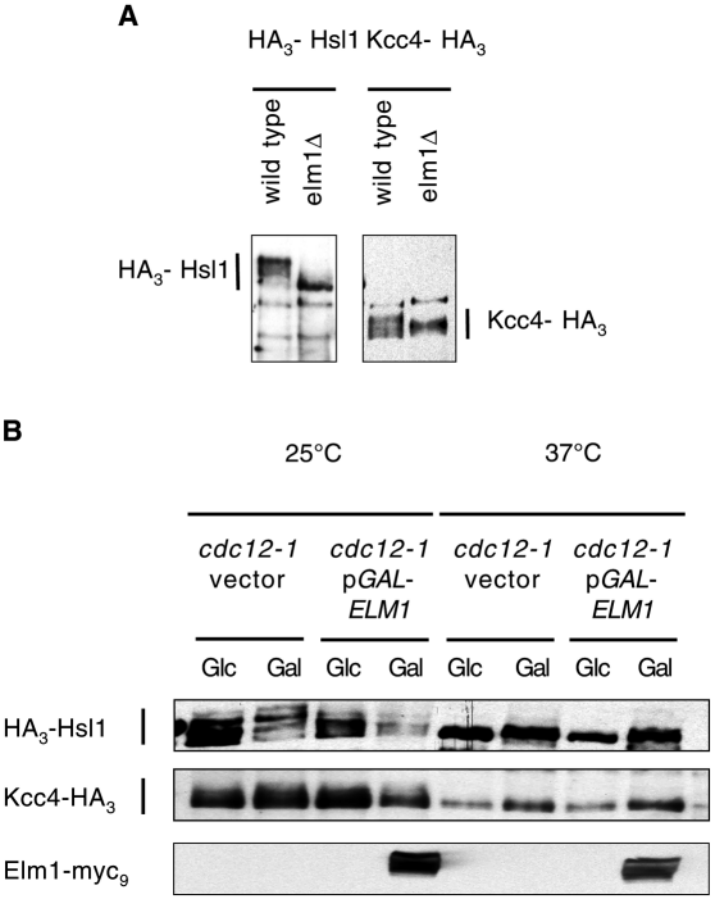


Fig. 3. (A) Hsl1 and Kcc4 phosphorylation is affected in *elm1*Δ. Immunoblot analysis with anti-HA antibody of extracts of *HA*$_3$-*HSL1* (NBY95), *HA*$_3$-*HSL1 elm1*Δ (NBY107), *HA*$_3$-*KCC4* (NBY96) and *HA*$_3$-*KCC4 elm1*Δ (NBY108) cells grown at 30°C. High molecular mass isoforms of each protein are detected in the corresponding wild type; they are absent in the isogenic *elm1*Δ mutants. (B) *cdc12-1 HSL1-HA*$_3$ (NBY130) and *cdc12-1 KCC4-HA*$_3$ (NBY131) carrying either a p*GAL-ELM1* plasmid (pAB103) or an empty vector (pYES2) were grown at 25°C in raffinose until mid-log phase and half of each culture was then transferred to 37°C, whilst the other half remained at 25°C. Galactose or glucose was then added to induce or repress *ELM1* expression, respectively. Immunoblot analysis was then performed on total protein extracts, with the anti-HA antibody to detect Hsl1-HA_3 and Kcc4-HA_3, and with the anti-myc antibody to detect Elm1-myc_9. The high molecular mass isoforms of each protein are detected at 25°C in all strains, but they are absent at 37°C, even when Elm1 is overexpressed.

Table 2. Cytological cell-cycle analysis of the single and multiple mutants

Genotypes	a	b	c	d	e	*n*
Wild type (YPH499)	41	13	26	3	17	233
*elm1*Δ (YPH499)	7	9	33	3	48	319
*elm1*Δ *swe1*Δ (YPH499)	10	13	31	2	44	323
Wild type (YYB64)	43	13	22	2	20	464
*hsl1*Δ *gin4*Δ *kcc4*Δ (YYB64)	1	5	36	4	54	547
*hsl1*Δ *gin4*Δ *kcc4*Δ *swe1*Δ (YYB64)	4	6	22	3	65	569
*elm1*Δ (YYB64)	10	9	44	4	33	971
*elm1*Δ *swe1*Δ (YYB64)	17	8	32	2	41	994
*hsl1*Δ *gin4*Δ *kcc4*Δ *elm1*Δ (YYB64)	1	5	44	4	46	240

Those which did were unable to grow at temperatures above 30°C, whereas both parental strains grew at 37°C (Fig. 4B). In addition, 61% of the cells were now multibudded, as compared to only 27% of *elm1*Δ and 51% of the triple mutant. Both misshapen Cdc11 rings, as in *hsl1*Δ *gin4*Δ *kcc4*Δ, and a delocalised Cdc11 signal in the bud, as in *elm1*Δ, was detected by immunofluorescence (Fig. 4A). The effects of the mutations on septin organisation and localisation were thus clearly additive. Our data indicate that Elm1 on the one hand, and Hsl1, Gin4 and Kcc4, on the other hand, work in different pathways to control septin assembly and localisation.

Indirect activation of the morphogenesis checkpoint by cytokinesis defects in *elm1*Δ

It was recently suggested that Elm1 functions upstream in a hierarchical cascade involving Hsl1, Swe1 and Cdc28 (Edgington et al., 1999). According to this model, activation of the morphogenesis checkpoint in *elm1*Δ would be responsible for the filamentous-growth characteristics and cell-cycle defects of the mutant. Indeed, in both YYB64 and YPH499, deletion of *SWE1* suppressed the hyper-polarised growth and the G_2/M accumulation of exponentially-growing *elm1*Δ cells (Table 2c). However, no delay in anaphase onset was detected in an elutriated *elm1*Δ culture, i.e. in a culture of *elm1*Δ cells which had successfully divided in the previous cell cycle (Fig. 2). This observation suggested that activation of the morphogenesis checkpoint might only be a secondary consequence of a primary cytokinesis defect in *elm1*Δ, rather than the cause of the cytokinesis defect. In agreement with this hypothesis, there was no significant reduction in the frequency

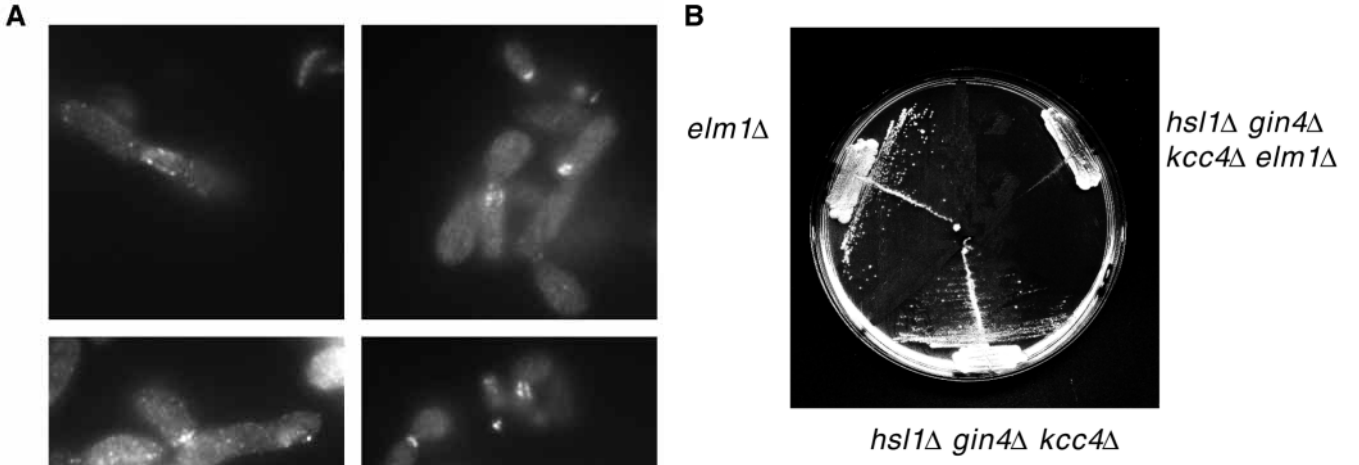


Fig. 4. (A) Localisation of Cdc11 in the *hsl1*Δ *gin4*Δ *kcc4*Δ *elm1*Δ quadruple mutant. The septins either form misshapen rings (as in *hsl1*Δ *gin4*Δ *kcc4*Δ) or are delocalised to the bud (as in *elm1*Δ). (B) The *hsl1*Δ *gin4*Δ *kcc4*Δ *elm1*Δ quadruple mutant is temperature sensitive. Cells of the indicated phenotype were incubated at 37°C.

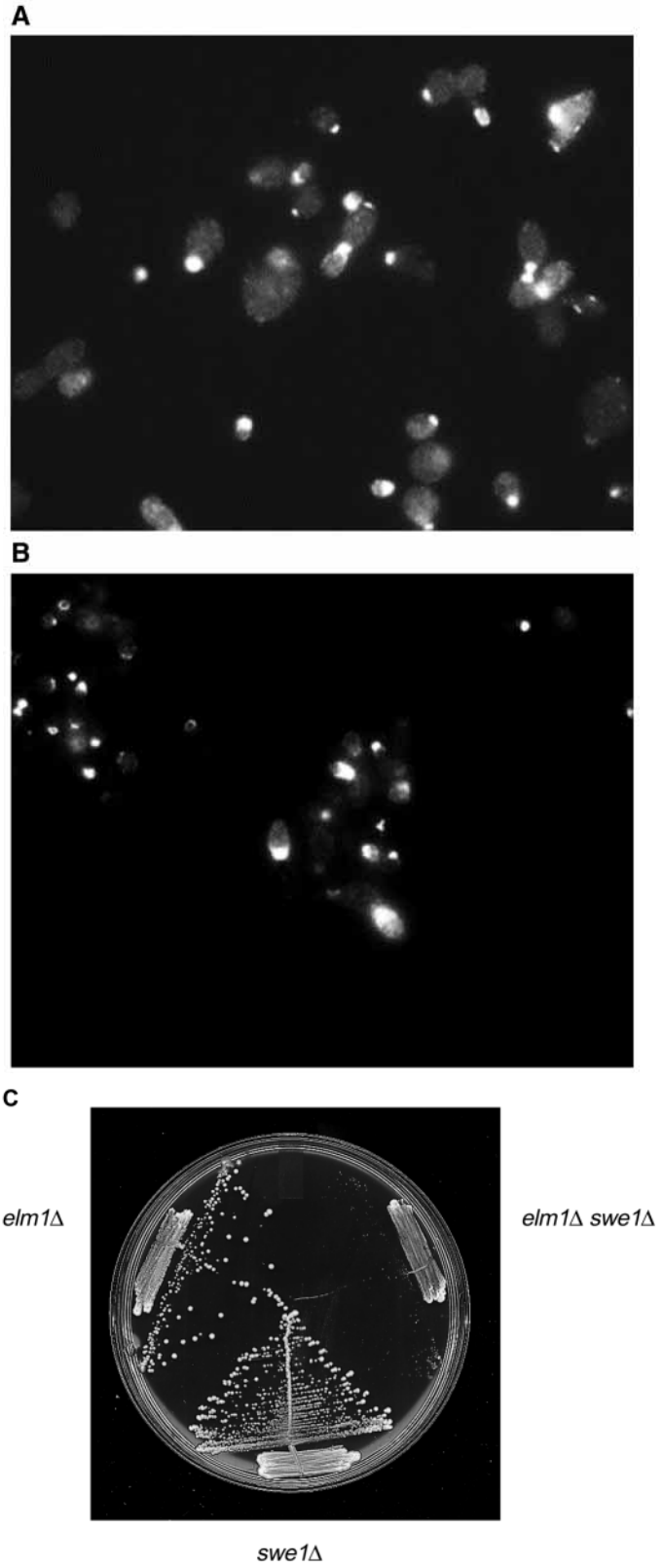


Fig. 5. (A) Cdc11 localisation in *elm1*Δ *swe1*Δ double mutant (NBY100) in the YPH499 strain background. (B) Cdc11 localisation in *elm1*Δ *swe1*Δ double mutant (YYB447) in the YYB64 strain background. (C) The *elm1*Δ *swe1*Δ double mutant (NBY100) in the YPH499 strain background is temperature sensitive. Cells of the indicated phenotypes were incubated at 37°C.

of cells undergoing cytokinesis in *elm1*Δ *swe1*Δ, as seen by cytological analysis of asynchronous populations (Table 2e). Moreover, immunofluorescent staining showed that Cdc11 was still delocalised, mostly to the bud tip, in the *elm1*Δ *swe1*Δ double mutant (Fig. 5A,B). This effect was stronger in the YPH background but, nevertheless, it was still apparent in the YYB64 strain. Identical results were obtained in a *elm1*Δ *CDC28Y19F* strain (data not shown). These results demonstrate that defects in septin ring organisation in *elm1*Δ are not due to activation of the morphological checkpoint. Finally, deletion of *SWE1* conferred temperature sensitive growth to the *elm1*Δ mutant, in both backgrounds (Fig. 5C and data not shown), adding even more strength to the idea that Elm1 and Swe1 work in distinct pathways. We therefore conclude that Elm1 is probably not a direct negative regulator of the morphological checkpoint pathway.

The accumulation of the Elm1 protein is cell-cycle regulated and peaks in mitosis

Our results strongly suggest that Elm1 is involved in cytokinesis regulation. In yeast, a plethora of genes is expressed in a cell cycle-dependent fashion. However, only a small subset of them, the most important cell-cycle regulators, is also cell-cycle regulated at the protein level. The *ELM1* gene is expressed at the end of the cycle (Cho et al., 1998; Spellman et al., 1998). To investigate whether Elm1 accumulation is also regulated through the cell cycle, *ELM1* was tagged with 9 myc epitopes and the resulting fusion was integrated at the *ELM1* locus under the control of the *ELM1* promoter. Small G_1 cells were isolated by centrifugal elutriation and cultured at 30°C. Samples were taken at regular intervals and cell cycle parameters, including H1 kinase activity, were followed. Levels of Elm1 and Clb2 were monitored by western blotting. Elm1 levels were low at the beginning of the experiment and increased afterwards, as cells progressed through the cell cycle. Elm1 levels were maximal just after Clb2/Cdc28 activation, approximately at cytokinesis onset (Fig. 6). This result is therefore supportive of a role of Elm1 in cytokinesis.

Elm1 localises as a ring to the bud neck

In order to obtain further information on the role of *ELM1*, we examined the protein's subcellular localisation. It was previously shown, based on overexpression studies, that an Elm1-GFP fusion protein localised to the bud neck (Moriya and Isono, 1999). We confirmed this result with a chromosomal Elm1-GFP fusion (Fig. 7A). The same staining pattern was obtained when indirect immunofluorescence was performed on a strain expressing Elm1-myc_9 (data not shown). No staining could be detected in cells in which Elm1 was untagged. Because the Elm1 levels are cell-cycle regulated, it was of interest to determine whether Elm1 localisation varied through

Table 3. Analysis of Elm1-GFP ring behaviour through the cell cycle

	 a	 b	 c	 d	 e
Hoescht	192	62	191	22	97
Elm1 ring	0	24	154	22	1
Elm1 ring (%)	0	39	81	100	1

the cell cycle as well. No signal was detected in unbudded cells. On the other hand, an Elm1-GFP ring could be seen in 39% of the small-budded cells (Table 3), suggesting that the ring formed after START. The frequency of cells exhibiting an Elm1-GFP ring at the bud neck increased with progression through the cell cycle. In particular, all the cells in anaphase displayed a ring of Elm1-GFP. The signal then vanished abruptly just after completion of mitosis (Table 3). The Elm1 protein thus appears at the bud neck shortly after START, is present during most of the cell cycle and disappears at about the time of cytokinesis.

The effect of disrupting septin structures on Elm1 localisation was analysed. A strain containing the *ELM1-GFP* allele and a temperature-sensitive *cdc12-1* allele was incubated either at the permissive or the restrictive temperature. The localisation of all septins is disrupted by the *cdc12-1* mutation (Kim et al., 1991). At the permissive temperature, Elm1 was localised at the bud neck in the *cdc12-1* mutant, as in wild-type cells, although the signal was significantly weaker in *cdc12-1* compared to the wild-type (Fig. 7B). On the other hand, no Elm1 ring was observed when the cells were shifted to 37°C (Fig. 7B), whereas Elm1 was still present at the bud neck in wild-type cells at high temperature (data not shown). Thus, septin function is required for correct localisation of Elm1 at the bud neck.

DISCUSSION

We isolated an allele of *ELM1* in a screen for mutations synthetically lethal with the *cdc28-1N* mutation, affecting the function of Cdc28 at the G_2/M transition. *ELM1* (ELongated Morphology 1) encodes a non-essential protein kinase (Blacketer et al., 1993), but *elm1* mutants form extremely elongated buds and we show here that such cells have cytokinesis defects (see below). The synthetic lethality between *elm1* and *cdc28-1N* may indicate that Cdc28 has an as yet undefined function in activating cytokinesis. Alternatively, it may reflect an indirect inhibition of cytokinesis due to the mitotic defects of *cdc28-1N* that is lethal when combined with an *elm1* mutant that is already defective in cytokinesis.

Because the *elm1* mutant forms elongated buds that resemble somewhat the morphology of cells during pseudohyphal growth, it was suggested that *ELM1* is a negative regulator of pseudohyphal differentiation (Blacketer et al., 1993). However, elongated buds are also seen in septin mutants that have defects in cytokinesis. Furthermore, the developmental switch in response to nitrogen starvation was not affected by changes in the *ELM1* gene dosage (Blacketer et al., 1993), as would have been expected of a genuine repressor of pseudohyphal growth. The behaviour of the Elm1 protein is rather highly suggestive of a role in cytokinesis. Accumulation of the Elm1 protein is cell-cycle regulated, peaking just before cytokinesis (Fig. 5) and Elm1 is localised to the bud neck, i.e. the division site, and this localisation is cell-cycle regulated, with the Elm1 ring appearing after bud emergence and vanishing at the time of cytokinesis. Our phenotypic analyses have indeed demonstrated that cytokinesis

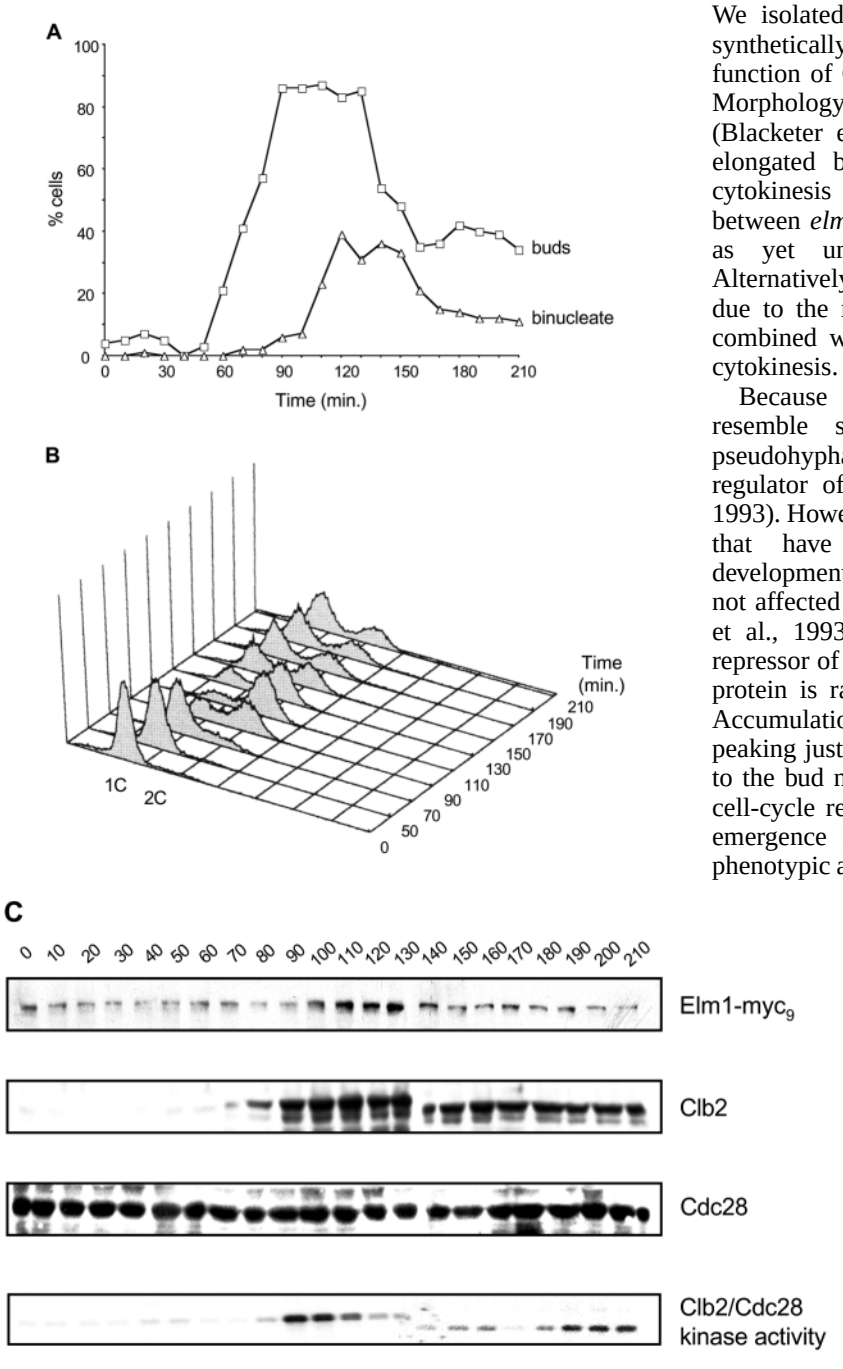
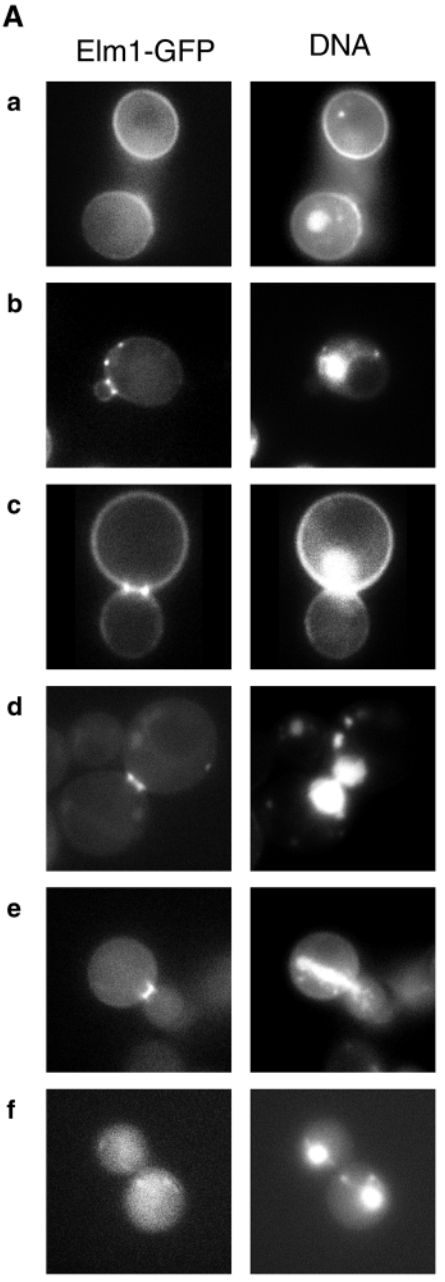


Fig. 6. Elm1 is expressed at the end of the cell cycle. *ELM1-myc*$_9$ cells (NBY86) were synchronised by centrifugal elutriation and released at 30°C in YEPD. (A) The percentage of budded cells and binucleate cells was determined microscopically. (B) DNA replication was followed by FACS analysis. (C) Samples for immunoblot analysis and Clb2/Cdc28 activity analysis were taken at the indicated time points. Levels of Clb2, Cdc28 and Elm1-myc$_9$ were determined by western blotting with antibodies against Clb2, Cdc28 and myc, respectively. For Clb2/Cdc28 kinase assays, anti-Clb2 immunoprecipitates were incubated with histone H1 and [γ-^{32}P]ATP.



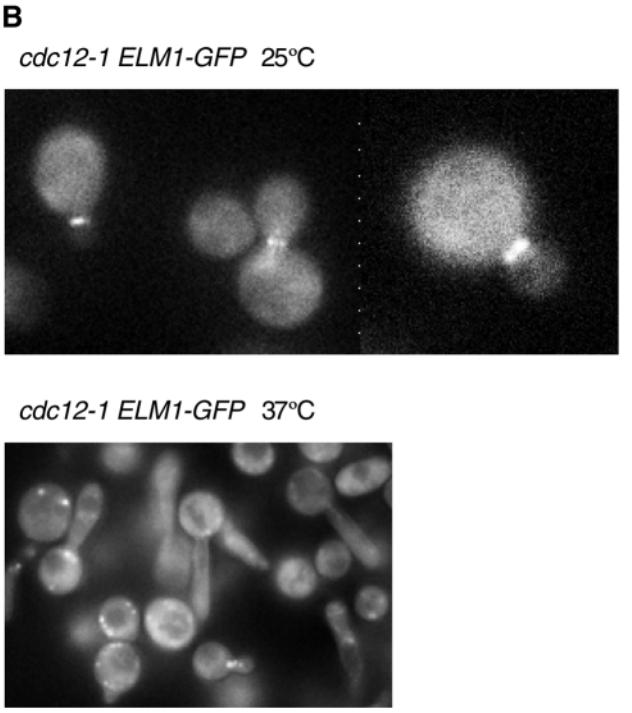


Fig. 7. Elm1-GFP localises to the bud neck. (A) No Elm1-GFP can be detected in unbudded cells (a). An Elm1-GFP ring at the bud neck is observed after bud emergence and for most of the cell cycle (b,c,d,e). This ring is absent after cells complete mitosis (f). The two dots in cell b were only observed in this unique cell; for this reason, we think they are not significant. The cellular DNA content was visualised with the vital dye Hoescht 33342. (B) Dependence of Elm1-GFP localisation to the bud neck upon septin function. At 25°C, an Elm1-GFP ring is present at the bud neck in *cdc12-1 ELM1-GFP* (NBY132); this ring cannot be detected at 37°C.

is affected in *elm1*Δ. Cytokinesis was delayed by 15 minutes in synchronised cultures of *elm1*Δ. Importantly, all the other cell-cycle parameters were identical in the mutant and in the wild type, indicating that the effect on cytokinesis was not a consequence of a delay in a previous cell-cycle phase. We thus conclude that the primary role of *ELM1* is in cytokinesis and that all the other *elm1*Δ phenotypes are probably a consequence of its cytokinesis defect.

We have shown that *ELM1* function is required for correct septin localisation at the bud neck. In asynchronous cultures of *elm1*Δ, septins were delocalised to the bud, often localising at the apex (Fig. 1). As a function of the strain background, deletion of *ELM1* leads to morphological defects ranging from mild to severe (Blacketer et al., 1993). The degree of septin delocalisation that we observed was correlated with the severity of the morphological and cytokinesis defects in each strain background. This result suggests that Elm1 is required for normal cytokinesis and septin localisation, but that mechanisms exist in some strain backgrounds to compensate for its loss.

Septin delocalisation in the *elm1* mutants is correlated with a strong hyperpolarisation phenotype reminiscent of septin mutant cells. It is worth noting that a *cdc12* septin mutant was isolated in the screen that originally uncovered *ELM1* (Blacketer et al., 1993). Moreover, deleting *ELM1* in *cdc12-1* enhances the cytokinetic and morphological defects of *cdc12-1* at the permissive temperature, emphasising that the functions of Elm1 and the septins are linked. Other mutants are affected in septin localisation (Barral et al., 1999; Cvrckova et al., 1995; Lippincott and Li, 1998a; Longtine et al., 1998a), but none of them show the striking presence of septins at the bud tip. Intriguingly, no septin localisation defect was detected in elutriated *elm1*Δ cells of the YYB64 background, a strain background in which the deletion of *ELM1* has a relatively mild effect (Fig. 2). In addition, all these cells had a wild-type morphology after one complete division cycle. One explanation for this difference is that in strain backgrounds such as YYB64 in which *elm1*Δ leads to mild phenotypes, only a subtle defect in septin localisation and organisation may be conferred by the absence of Elm1. The phenotype observed in asynchronous *elm1*Δ cultures would result from an

accumulation of small alterations in septin localisation. Another, non-exclusive possibility is that the effects of *elm1* mutations on the septins are indirect. In agreement with this hypothesis, we were unable to detect any co-immunoprecipitation between Elm1 and Cdc3 or Cdc11 (our unpublished results). Elm1 could affect the function or the localisation of a cytokinetic protein; this, in turn, would alter the septin localisation in the next cell cycle.

A checkpoint involving Swe1, the *S. cerevisiae* homolog of the fission yeast p107^{wee1} protein kinase, has recently been shown to monitor cytoskeletal defects such as actin disorganisation (Lew and Reed, 1995) or defects in septin assembly (Barral et al., 1999). Three negative regulators of Swe1, the protein kinases, Hsl1, Gin4 and Kcc4, interact, colocalise with, and depend on the septins for their activation. In the presence of septin defects, the three kinases remain inactive and Swe1 inhibits Clb/Cdc28, thereby delaying mitosis and the transition from apical to isotropic growth. The three kinases are therefore thought to link the regulation of the G_2/M transition by Swe1 to the proper organisation of septin structures (Barral et al., 1999). Cells in which all three kinases are deleted, leading to constitutive activation of the checkpoint, are extremely elongated, resembling *elm1*Δ cells. Because the hyper-polarised phenotype of *elm1* mutants is suppressed either by a dominant mutation in *HSL1* or by a recessive mutation in *SWE1*, it was suggested that Elm1 might act upstream of Hsl1 in the Swe1 pathway (Edgington et al., 1999). However, there is as yet no evidence of a direct activation of Hsl1 by Elm1.

Rather, it may be that the effects of *elm1*Δ upon Hsl1 and Kcc4 phosphorylation are indirect, perhaps because the septin defects would prevent activation of Hsl1 and Kcc4. Indeed, Elm1 overexpression cannot bypass the septin requirement for Hsl1 and Kcc4 phosphorylation. Furthermore, several results indicate that Elm1 acts at least partly independently of Hsl1, Gin4 and Kcc4. First, *elm1* mutant cells have stronger septin localisation defects than the *hsl1 gin4 kcc4* triple mutant. Notably, *elm1* mutants mislocalise septins to the bud tip, a defect that we did not observe in the *hsl1 gin4 kcc4* triple mutant strain. Second, the quadruple mutant *hsl1 gin4 kcc4 elm1* grows much more slowly and shows more severe morphological defects than either the *elm1* or the *hsl1 gin4 kcc4* strain. In particular, *elm1*Δ and *hsl1*Δ *gin4*Δ *kcc4*Δ show additive effects on morphology, cytokinesis and septin localisation defects. Finally, hyperactivating, with a triple deletion of *HSL1*, *GIN4* and *KCC4*, or inactivating, by deleting *SWE1*, the morphogenesis checkpoint causes temperature sensitivity at 37°C in *elm1*Δ. Our genetic and immunofluorescence data suggest that Elm1 effects on cytokinesis and septin localisation are not mediated by Hsl1, Gin4 and Kcc4, or by Swe1. Activation of the morphogenesis checkpoint in *elm1*Δ seems to be a secondary consequence of a primary defect in cytokinesis and septin assembly.

In budding yeast, the cleavage plane is determined by the formation of a septin ring at the bud neck that provides a scaffold for the correct localisation of the proteins that are actively involved in cytokinesis (Bi et al., 1998; Lippincott and Li, 1998a,b). However, what anchors the septins at the bud neck throughout the cell cycle is unknown. Hsl1, Gin4 and Kcc4 are required for proper septin assembly at the bud neck in budded cells (Barral et al., 1999; Longtine et al., 1998a). We have shown that Elm1 is involved in controlling septin ring localisation in a pathway distinct from Hsl1, Gin4 and Kcc4. The combination of these four mutations is highly deleterious, and no discrete, well-defined septin ring could be detected in *hsl1*Δ *gin4*Δ *kcc4*Δ *elm1*Δ quadruple mutants. Septins either formed misshapen rings as in *hsl1*Δ *gin4*Δ *kcc4*Δ or were delocalised to the bud as in *elm1*Δ (Fig. 4). This demonstrates that the anchoring of the septins to the division plane and their correct assembly into rings at the bud neck is severely compromised in the quadruple mutant. Identifying the targets of Elm1, Hsl1, Gin4 and Kcc4 should therefore help to understand how eukaryotes establish and maintain their division plane.

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