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Analysis of the *S. pombe* signalling scaffold protein Cdc11p reveals an essential role for the N-terminal domain in SIN signalling

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Abstract The initiation of cytokinesis in the fission yeast *Schizosaccharomyces pombe* is signalled by the septation initiation network (SIN). Signalling originates from the spindle pole body (SPB), where SIN proteins are anchored by a scaffold composed of cdc11p and sid4p. Cdc11p links the other SIN proteins to sid4p and the SPB. Homologues of cdc11p have been identified in *Saccharomyes cerevisiae* (Nud1p) and human cells (Centriolin). We have defined functional domains of cdc11p by analysis of deletion mutants. We demonstrate that the C-terminal end of cdc11p is necessary for SPB localisation. We also show that the N-terminal domain is necessary and sufficient for signal transduction, since tethering of this domain to the SPB will substitute for cdc11p in SIN function.

Keywords: Signal transduction; Cell cycle; Cytokinesis

1. Introduction

The fission yeast *Schizosaccharomyces pombe* divides by formation of a new cell wall, or division septum, between the separated nuclei at the end of mitosis. The position of the division site is marked by assembly of a contractile F-actin ring early in mitosis. At the end of anaphase, the ring contracts, guiding synthesis of the division septum. This process is triggered by the septation initiation network (SIN), which comprises the protein kinases plo1p, sid1p, cdc7p, and sid2p, the GTPase spg1p, the GTPase activating protein (GAP) byr4p-cdc16p, and the proteins mob1p and cdc14p. For a review of the organisation and function of the SIN see [1]. SIN signalling originates from the spindle pole body (SPB), where the proteins are anchored by a scaffold that includes a complex of sid4p and cdc11p [2,3]. Sid4p is required for cdc11p to associate with the SPB, while cdc11p is required for the binding of the remaining SIN proteins, with the exception of plo1p [2]. In the absence of SIN signalling, caused by loss of function mutations in *cdc7*, *cdc11*, *cdc14*, *mob1*, *sid1*, *sid2*, *sid4*, *spg1*, or *plo1*, the F-actin ring is formed but does not contract. Nuclear division and growth continue in the absence of cytokinesis, leading to the formation of multinucleate cells, the so-called "sid" phenotype. Deregulated SIN signalling, caused by loss of either *byr4* or *cdc16*, or through increased expression of *cdc7*, *spg1* or *plo1*, leads to the formation of multiple F-actin rings and septa, and can uncouple septation from the nuclear cycle, leading to "cutting" of the undivided nucleus, or an increase in ploidy. An understanding of how SIN signalling is regulated will therefore provide insights about how events of the cell cycle are coordinated properly.

Activators of SIN signalling associate with the SPB only during mitosis [1,4,5]. Cdc11p is a phosphoprotein, that becomes hyperphosphorylated during anaphase [2]. The mitotic hyperphosphorylation of cdc11p is mediated in vivo by the protein kinase cdc7p and is blocked in spindle assembly checkpoint arrested cells [6]. Hyperphosphorylation of cdc11p correlates with activation of the SIN and is promoted if SIN signalling is activated ectopically during interphase [6]. This has led to the hypothesis that phosphorylation of cdc11p during mitosis is important for activating signalling by the SIN.

The N-terminal half of cdc11p contains several serine-rich regions, while the C-terminal half contains multiple leucine rich repeat (LRR) domains, which may mediate interactions with other proteins [2]. The location of two thermosensitive mutants has been determined [2]. One is in the LRR region, which may mediate association of a subset of SIN proteins with the SPB [2], while the other lies at the extreme C-terminus of cdc11p, which defines the region required for SPB association [2,3]. Additional evidence for the C-terminus of cdc11p mediating its association with the SPB comes from the demonstration that a fusion of green fluorescent protein (GFP) to amino acids (aa) 631–1045 of cdc11p localises to the SPB [3]. Strong expression of this fragment inhibits SIN signalling and association of all SIN proteins except sid4p and plo1p with the SPB, by displacing full-length cdc11p from the SPB [3]. A fragment of cdc11p comprising GFP fused to aa 1–630 is cytoplasmic [3]. Cdc11p is also part of a complex that contains byr4p [6]. Proteins with significant sequence homology to cdc11p are present in other organisms. In *Saccharomyes cerevisiae* Nud1p is required for mitotic exit and cytokinesis [7], while in human cells, the centriolin protein, which is important for cytokinesis, shares a domain with homology to cdc11p [8].

In this study, we have attempted to identify domains important for cdc11p function through analysis of deletion mutants. We have also addressed the role of the protein kinase plo1p in regulating mitotic hyperphosphorylation of cdc11p.

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Abbreviations: aa, amino acids; DAPI, 4′,6′-diamidino-2-phenylindole; GAP, GTPase activating protein; GFP, green fluorescent protein; HU, hydroxyurea; LRR, leucine rich repeat; Sid, septation initiation defective; SIN, septation initiation network; SPB, spindle pole body

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2. Materials and methods

2.1. Yeast

Standard methods were used for growth and analysis of *S. pombe* [9]. All cell cycle mutants used have been described previously [6,10].

2.2. Molecular biology

Standard techniques [11] were used throughout. The restriction sites used to generate deletions are indicated in Fig. 1. The *Sma*I sites at aa 1 and 1045 have been created artificially [2]. Certain truncations were created using the following "forward" oligonucleotides VS640 ATACCCGGGGTCTATTTGAAGAGCTCACG, VS666 GTACCC-GGGTTCACTCCCTGAACCACGAAGACC, VS667 GTACCC-GGGTGCAAGGTTGGATTTGGAAAATATG, together with the "reverse" primer VS524 TTTTTAGACGACCTTCACGTTTCA-TTATGC. Fragments were cut with *spe*I, which was then repaired with Klenow polymerase. After digestion of the product with *Sma*I, the product was cloned and sequenced. All the cdc11p deletion proteins were fused to GFP at their N-terminus to permit their localisation. Expression of a protein of the expected size for each mutant was confirmed by western blotting with antiserum to GFP (not shown). Deletion mutants of *cdc11*, sid4, or the sid4–cdc11 fusion gene were expressed from the inducible *nmt1* promoter, inserted as a single-copy expression cassette replacing the *leu1* gene [12]. To test for rescue of mutant function, the *nmt1* promoter was induced to express for 16 h at 25 °C by growth in the absence of thiamine. Cells were then shifted to 36 °C for 5 h, fixed and examined as described. Cells were synchronised by hydroxyurea (HU) arrest–release as described. After 5 h incubation in HU, cells were washed in fresh medium, and incubated at 36 °C. Samples were taken as cells passed through anaphase [6].

2.3. Imaging

To determine the septation index, cells were fixed in ethanol, and stained with 4′,6′-diamidino-2-phenylindole (DAPI) and Calcofluor as described (Moreno 91). To assess the localisation of GFP-cdc11p fusion proteins, TILLvisION software (v3.3; TILL Photonics GmBH) was used to analyse data captured with an IMAGO CCD camera mounted on an Olympus IX70 microscope. Deconvolution was performed with BitPlane software. Images were assembled in Adobe Photoshop.

3. Results and discussion

3.1. The N-terminal region of cdc11p is essential for signalling septum formation

To determine which domains of cdc11p are important for its function and localisation, we analysed truncation mutants (shown in Fig. 1), assessing the following criteria: (1) subcellular localisation of a GFP fusion, (2) ability to rescue *cdc11* thermosensitive and null mutants, (3) the effect of increased expression of the protein upon cell cycle progression. The figures show representatives of each type of deletion.

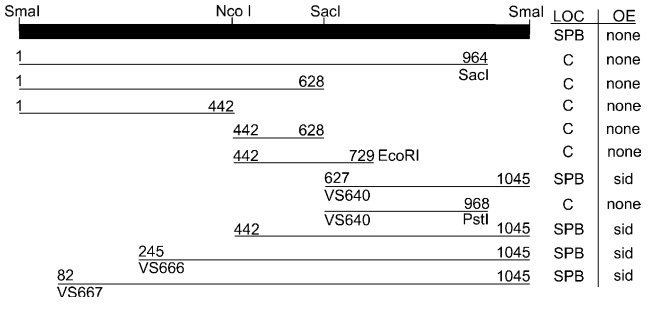


Fig. 1. Truncation mutants of *cdc11* analysed in this study. Mutants were created using appropriate restriction sites to generate truncations. The columns on the right show the observed localisation (LOC): C is cytoplasmic, SPB is spindle pole body, and the effect of overexpression (OE): sid indicates elongated, multinucleated cells, none indicates that cells had a wild-type appearance. The numbers indicate the aa of cdc11p present in the fusion protein. Where a restriction site has been used to generate multiple truncations, it is indicated above the full length protein for clarity.

A GFP fusion to the N-terminus of cdc11p did not affect its localisation (Fig. 2A) or function, as judged by its ability to rescue a null allele of *cdc11* (not shown), and increased expression had no effect upon cell cycle progression, in agreement with observations on the untagged, full length protein [2]. We conclude that the GFP tag does not impair cdc11p function. All the deletion mutants which lacked 81aa or more from the C-terminus failed to associate with the SPB (Fig. 2B, and data not shown), giving uniform staining through the cell. Aggregates of the protein in the cytoplasm were also observed. These data confirm that the C-terminal domain of the protein is required for SPB association and define sequences within the last 81aa as important for this. Expression of cdc11p fragments that lacked both the N-terminal and C-terminal domains were cytoplasmic or formed aggregates (Fig. 2C, and data not shown). Increased expression of fragments that were unable to associate with the SPB did not affect septum formation, and none was able to rescue mutant of *cdc11*. These data suggest that cdc11p must associate with the SPB to exercise its biological function.

Analysis of mutants that were deleted at the N-terminus, but had an intact C-terminal domain, showed that all of them localised to the SPB (Fig. 2D, and data not shown). As expected, increased expression of aa 626–1045 inhibited septation. However, we found that a protein lacking the N-terminal 245aa also inhibited septum formation (Fig. 2D), and a shorter truncation deleting only 82aa gave rise to elongated, multinucleated cells, which contained faint septa. Since these proteins are able to associate with the SPB, but do not permit septation to occur, we suggest that a domain in the N-terminal region of cdc11p is important for septum formation.

3.2. The N-terminal half of cdc11p will substitute for cdc11p function, if recruited to the spindle pole body

The observation that increased expression of cdc11p fragments lacking only the extreme C-terminus had no significant effect upon the cell when expressed at 25 °C, led us to entertain the hypothesis that the N-terminal region of cdc11p may only function in the context of the SPB. Therefore, we fused aa 1–628 of cdc11p to aa 133–660 of sid4p, which does not interact with cdc11p but is required for sid4p to associate with the SPB [3], with the aim of using the latter as a targeting module to recruit the N-terminus of cdc11p to the SPB. Expression of this fusion protein rescued the lethality of a *cdc11::ura4*$^+$ null mutant, permitting colonies to be formed at all temperatures (Fig. 2E), though the cells showed some deregulation of septation, including "cut" nuclei, mononucleate cells (which indicate the uncoupling of septum formation from mitosis) and multiple septa (Fig. 2F). This demonstrates that the N-terminal half of cdc11p is sufficient to provide the septation function of cdc11p, if it is directed to the SPB by sid4p. This fusion protein could not rescue mutations in *cdc7-A20*, indicating that it does not bypass the requirement for SIN signalling (Fig. 2E). These data also suggest that the crucial phosphorylation sites for cdc7p in cdc11p lie in the N-terminal domain. The reduction of restrictive temperature in a *cdc7-A20* background suggests that the cdc11-sid4 fusion protein may be toxic if SIN function is

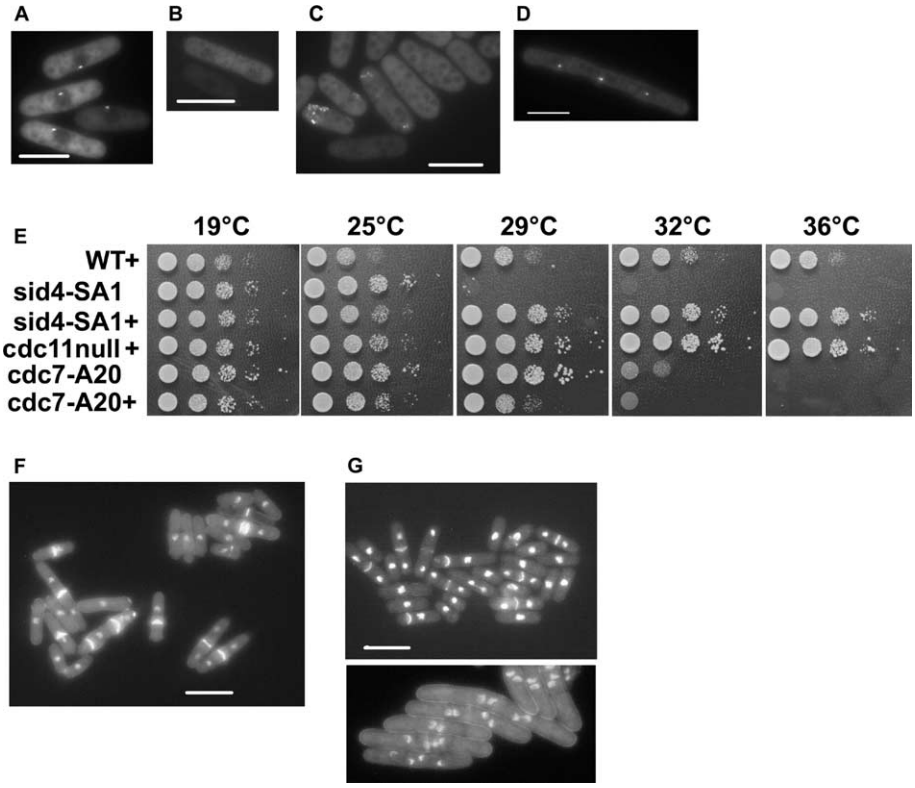


Fig. 2. Analysis of the cdc11p GFP tagged fragments and demonstration that the N-terminal region of cdc11p, fused to the C-terminal region of sid4p, will rescue a *cdc11* null mutant. Panels A–D show images of living cells. Panel A: cells expressing GFP-cdc11p: note the presence of the protein on the SPB associated with the nucleus. Panel B: Expression of GFP-cdc11 (1–964). Note the absence of the protein from the SPB. Panel C: Expression of GFP-cdc11 (442–628). Note the absence of the protein from the SPB and the presence of cytoplasmic aggregates in some cells. Panel D: Expression of GFP-cdc11 (442–1045). Note the presence of the protein on the SPB and the fact that the cell is elongated, indicating that septum formation is blocked. The scale bars represent 10 microns. Panel E: Cells of the indicated genotypes bearing either an empty vector, or a plasmid expressing a cdc11-sid4 fusion protein (+) were plated on minimal medium at the indicated temperatures, in the absence of thiamine. Cells were allowed to form colonies at the indicated temperatures. Panel F: *cdc11::ura4*$^+$ cells expressing the GFP-cdc11-sid4 fusion protein were grown at 25 °C, fixed and stained with DAPI and Calcofluor. Note that cells are dividing, though the regulation of septation is abnormal. Panel G: the mutant *sid4-SA1* expressing either the cdc11-sid4 fusion protein (upper photograph) or an empty vector (lower photograph) was grown at 25 °C and shifted to 36 °C for 6 h. Note that while cells expressing the cdc11-sid4 fusion continue to divide, those expressing the empty vector become elongated and multinucleated.

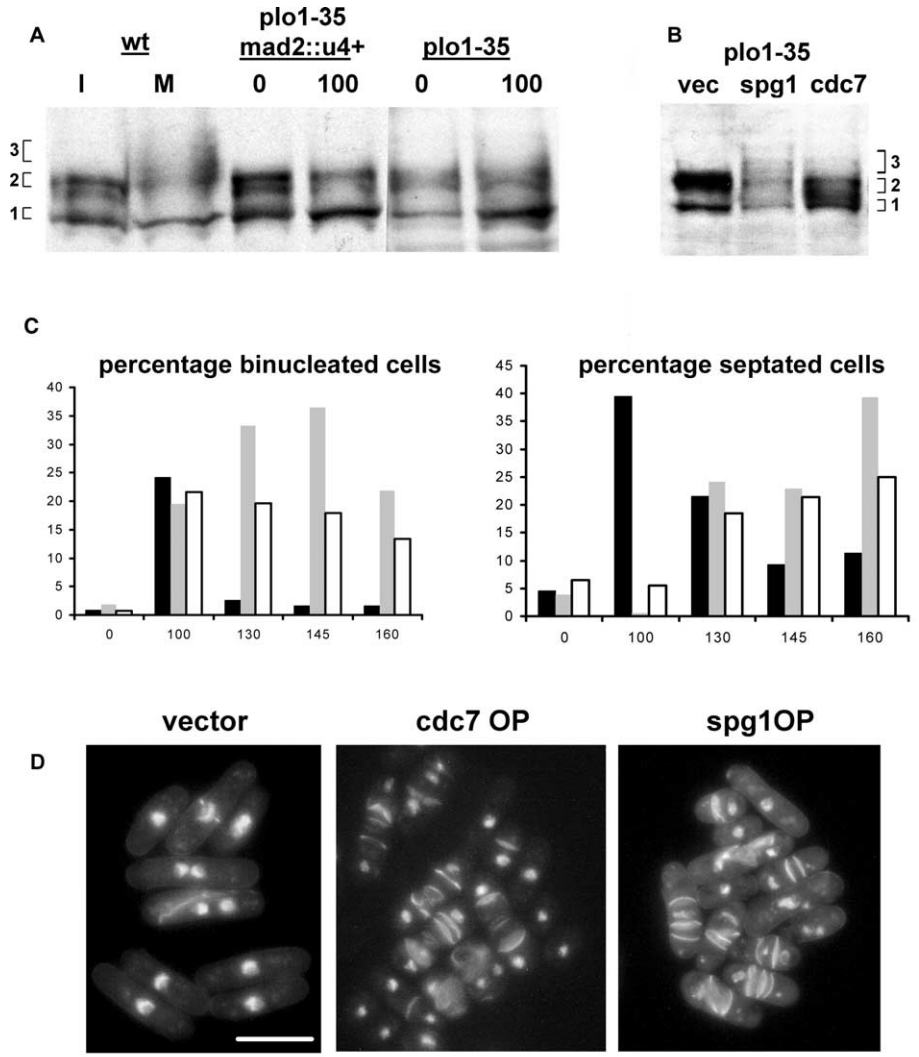
compromised. Sid4p is required for localisation of all the "core" SIN proteins including cdc11p [6,13]. However, expression of the sid4p-cdc11p fusion in a *sid4-SA1* mutant resulted in viable cells that could form a colony, showing that this protein can also substitute for sid4p in SIN signalling (Fig. 2E). Interestingly, a much higher percentage (36% compared to 15% for wt) of cells than normal had septa (Fig. 2G). Expression of the cdc11–sid4 fusion protein in wild-type cells also gave rise to an increased septation index (data not shown), but did not prevent colony formation (Fig. 2E). A fusion protein containing only GFP and aa 133–660 of sid4p did not rescue either cdc11 or sid4 mutants (data not shown).

Taken together, these data demonstrate that that aa 1–631 of cdc11p can rescue a *cdc11* null mutant, if this protein is recruited to the SPB, by fusion to its natural SPB anchor, sid4p. Since deletions of cdc11p lacking as little as 82aa from the N-terminus are located on the SPB, but do not permit SIN signalling, we conclude that the N-terminal domain of cdc11p is critical for mediating SIN signalling, while the C-terminal region is required for SPB association. Future studies will attempt to identify proteins that bind to this N-terminal domain.

Previous studies have shown that Nud1p is a phosphoprotein, and that overproduction of full-length Nud1p has no effect, while the C-terminal domain causes cell cycle arrest and defects in mitotic exit [14]. These data are consistent with those reported here, and suggest that cdc11p and Nud1p might be regulated in a similar manner. Increased expression of the cdc11-homology domain of centriolin blocks cytokinesis [8], though this does not displace full-length centriolin from the centrosome. As far as we are aware, no domain analysis of centriolin equivalent to those presented here, or in [14] have been published, so the identification of domains required for SPB localisation awaits further study.

3.3. *The protein kinase plo1p is required for mitotic hyperphosphorylation of cdc11p in vivo*

Our previous study indicated that mitotic hyperphosphorylation of cdc11p occurs normally in the mutant *plo1-ts4*, which is defective in SIN signalling [6]. However, *plo1-ts4* is not defective in spindle formation and so may retain significant activity. Subsequent to this study, a new *plo1* mutant, *plo1-35*, was described, which is also defective in spindle formation and has very low protein kinase activity in vitro [10]. In this mutant, no hyperphosphorylation of cdc11p occurred after release



	Mononucleated with septum	Binucleated with septum	Binucleated, no septum	Mononucleated no septum
Vector	0	9.10%	11.70%	79.20%
Cdc7OP	55.10%	30.10%	1.30%	13.50%
Spg1OP	71.20%	16.80%	0	12%

Fig. 3. The role of plo1 kinase in regulating mitotic hyperphosphorylation of cdc11p. Panel A: Cells were synchronised by HU arrest–release and the phosphorylation state of cdc11p was examined as cells passed through anaphase (100 min after release for wild-type cells). The interphase forms of cdc11p (1) and (2) and the mitotic hyperphosphorylated form (3) are indicated, as defined in [6]. Panel B: *plo1-35* cells transformed with an empty vector (vec), or plasmids expressing either *spg1* or *cdc7* were shifted to 36 °C, and expression from the *nmt1* promoter was induced as described in Section 2. The phosphorylation state of cdc11p was examined by western blotting. The shift in cdc11p induced by *cdc7* is less pronounced than that for *spg1*. The reason for this is unclear. Panel C: The cells from the experiment shown in panel A were fixed, and the number of binucleated cells and the septation index were determined at the indicated times after shift to 36 °C. The solid black boxes are wild-type cells, the grey boxes are *plo1-35* and the open boxes are *plo1-35 mad2Δ*. Panel D: *plo1-35* cells from the experiment shown in panel B were fixed and stained with DAPI and Calcofluor. Cdc7OP and spg1OP indicate increased expression of cdc7p and spg1p, respectively. A quantitative analysis of the cells is shown below the micrographs. More than 200 cells were counted.

from a HU block at a time when cdc11p is hyperphosphorylated in wild-type cells (Fig. 3A). The percentage of binucleated cells was similar in wild-type and *plo1-35*, indicating that similar numbers of cells had entered mitosis (Fig. 3C, 100 min). The same result was obtained in a *plo1-35 mad2::ura4*$^+$ background, indicating that the absence of phosphorylation was not due to activation of the spindle assembly checkpoint. Thus, plo1p is required for mitotic hyperphosphorylation of cdc11p in vivo.

To investigate whether *plo1-35* cells are capable of septation if the SIN is activated ectopically, cells were transformed with plasmids expressing either *cdc7* or *spg1* from the full-strength *nmt1* promoter. Expression was induced at the permissive temperature and then cells were shifted to 36 °C. Though approximately 10% of control cells transformed with the empty vector produced a septum, these were faint, and often misplaced, and no multiple septa were observed. In contrast, in cells expressing either *spg1* or *cdc7*, approximately 85% of cells

had septated (Fig. 3D). Further examination revealed the presence of many mononucleated septated cells, indicating that they had septated without completing mitosis (Fig. 3D, numeric data). Western blotting showed that hyperphosphorylated cdc11p (form 3) was present in cells expressing spg1p and cdc7p (Fig. 3B). Thus, ectopic activation of the SIN can bypass the requirement for plo1p to generate the hyperphosphorylated forms of cdc11p.

Previous studies have suggested that plo1p functions upstream of the SIN [15]. In a *plo1* mutant defective in spindle formation, no hyperphosphorylation of cdc11p was observed when the kinase was inactivated. Nonetheless, ectopic activation of the SIN in this mutant permits multiple rounds of septum formation and hyperphosphorylation of cdc11p. These data therefore support the previous suggestion that plo1p functions upstream of the SIN [15].

What is the role of plo1p in controlling cdc11p phosphorylation during mitosis? It is clear that polo-like kinases play multiple roles during mitosis (reviewed by [16,17]). Plk1p is required for proper maturation of the centrosome at the onset of mitosis in human cells [18]. By analogy with these findings, it is possible that plo1p is required to "activate" the SPB to receive SIN components, or to permit them to achieve maximal activity. For example, phosphorylation of SPB components by plo1p might be required for cdc7p to recognise cdc11p efficiently.

It is also possible that plo1p is part of a parallel pathway that controls septation in response to a different cue from those regulating the SIN (a "dual-key" control). In the absence of plo1p function, normal signal levels from the SIN alone are not effective in promoting septum formation, while a large continuous signal may be able to promote septum formation without the requirement for an input via plo1p.

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