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Essential Role for *Schizosaccharomyces pombe* *pik1* in Septation

Running Title: *Schizosaccharomyces pombe* *pik1*

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Abstract

Background: Schizosaccharomyces pombe pkl encodes a phosphatidylinositol 4-kinase, reported to bind Cdc4, but not Cdc4^{G107S}. Principle findings: Gene deletion revealed that pkl is essential. In cells with pkl deleted, ectopic expression of a loss-of-function allele, created by fusion to a temperature-sensitive dihydrofolate reductase, allowed normal cell proliferation at 25°C. At 36°C, cells arrested with abnormally thick, misplaced or supernumerary septa, indicating a defect late in septation. In addition to being Golgi associated, ectopically expressed GFP-tagged Pik1 was observed at the medial cell plane late in cytokinesis. New alleles, created by site-directed mutagenesis, were expressed ectopically. Lipid kinase and Cdc4-binding activity assays were performed. Pik1^{D709A} was kinase-dead, but bound Cdc4. Pik1^{R838A} did not bind Cdc4, but was an active kinase. Genomic integration of these substitutions in S. pombe and complementation studies in Saccharomyces cerevisiae pkl-101 cells revealed that D709 is essential in both cases while R838 is dispensable. In S. pombe, ectopic expression of pkl was dominantly lethal; while, pkl^{D709A,R838A} was innocuous, pkl^{R838A} was almost innocuous, and pkl^{D709A} produced partial lethality and septation defects. The pkl ectopic expression lethal phenotype was suppressed in cdc4^{G107S}. Thus, D709 is essential for kinase activity and septation.

Conclusions: We conclude that Pik1 is involved in septation and the R838 residue of Pik1 is required for important protein-protein interactions, possibly with Cdc4.
Introduction

Phosphoinositides are involved in signal transduction, regulation of actin cytoskeletal organization, cytokinesis, and membrane traffic in eukaryotic cells, including in yeasts [1, 2, 3, 4]. Phosphatidylinositol 4-kinases phosphorylate phosphatidylinositol (PtdIns) to produce PtdIns 4-phosphate, the precursor to other important phosphoinositides [5]. PtdIns 4-kinases have been implicated in regulation of cell division in Saccharomyces cerevisiae [6, 7] and Drosophila melanogaster [8]. Cytokinesis in S. pombe is a multistep process involving division site selection, contractile ring formation and contraction, membrane expansion/ingression and formation/dissolution of primary and secondary septa [9, 10, 11]. The specific aspects of cytokinesis that require PtdIns 4-kinase activity remain to be determined.

S. cerevisiae and S. pombe each appear to have 3 distinct PtdIns 4-kinases. In the budding yeast these are; Lsb6p, a type II enzyme; Stt4p, a type IIIα enzyme; and Pik1, a type IIIβ enzyme [3]. The functions of Stt4p and Pik1 are non-redundant as each is essential for viability [12, 13]. Stt4p is required for normal actin cytoskeletal organization [13]. Pik1p is required for maintenance of Golgi structures, protein secretion and endocytosis and for cytokinesis [7, 13, 14]. A conditionally lethal allele, pik1-101, arrests without completing cytokinesis [6, 7]. LSB6 is non-essential [15]; however, it is partially redundant with STT4 and is required for actin nucleation and endosome motility [15, 16].

In S. pombe, locus SPAC343.19 appears to encode a type II enzyme, locus SPBC577.06c appears to encode a type IIIα enzyme, and pik1 (SPAC22E12.16c) is a putative type IIIβ enzyme. Sequence comparisons suggest that S. cerevisiae PIK1 and S. pombe pik1 are orthologs; although, sequence similarities outside the lipid kinase domains are weak (27% identity, compared to 53% between kinase domains). The S. cerevisiae enzyme is in the nucleus and associated with the
Golgi [14]. Both pools of the protein are essential for cell viability [14]. The distribution of Pik1 between the nucleus and the cytosol requires association with two 14-3-3 proteins [17]. The S. pombe protein has been reported to be associated with the Golgi [18]. However, a nuclear localization of Pik1 in S. pombe has not been reported. The localization of S. pombe Pik1 during the cell cycle and the importance of this enzyme for cell division have yet to be determined. S. cerevisiae Pik1 has been reported to interact with a number of proteins, including Frq1 [19] and Bmh1 and Bmh2 [20]. Interactions between S. pombe Pik1 and the corresponding homologs of the S. cerevisiae proteins, Ncs1, Rad25 and Rad24, have not been reported. S. pombe Pik1 has been reported to interact with Cdc4 [21]. This interaction was abolished by only one of six conditionally lethal mutations of cdc4 (cdc4<sup>G107S</sup>) [21]. Cdc4 is a contractile ring component that is required for cytokinesis [22]. The contractile ring is a transient, dynamic structure made of actin, myosins and many other associated proteins [9, 23]. The timing and site of assembly of the ring and the regulation of its contractile activity are essential for cell division [24]. One established function of Cdc4 is that of a myosin essential light chain, bound to the first IQ domain in the neck region of type II myosins [25, 26, 27]. A putative pseudo-IQ motif has been identified in Pik1 [21, 28]. Cdc4 also interacts with Rng2, another contractile ring component, which is required for its assembly [29]. The rmg2 gene encodes a protein related to the human IQGAP1 protein, which binds actin and calmodulin and is a potential effector for the Rho family of GTPases. Cdc4 presumably interacts with Rng2 at one or several of its 6 IQ-motifs [29]. Thus, it is possible that Cdc4 has both a structural function, as a myosin essential light chain, and regulatory functions. There is much evidence that Cdc4 has functions in addition to that of a myosin essential light chain [21, 27, 30, 31]. Six cdc4 mutations have been identified that cause temperature-dependent
failure of cytokinesis [32, 33]. Five of those alleles, with highly penetrant, conditionally lethal phenotypes, were examined for interallelic complementation in diploid cells [21]. Strikingly, three of the ten possible heterozygous diploid strains were viable at the restrictive temperature. These observations are not consistent with Cdc4 functioning only as a myosin essential light chain. Structurally, two of the mutant proteins examined (\textit{cdc}4^{G19E} or \textit{cdc}4^{G107S}) were very stable, even at the restrictive temperature, suggesting that failure of cytokinesis is due to subtle changes in structure that may impair protein interactions [34]. Furthermore, only one of the six mutations (\textit{cdc}4^{R33K}) was shown to affect actin-myosin function in sliding filament motility assays at the non-permissive temperature [30]. In addition, strains carrying either of two temperature-sensitive \textit{cdc}4 alleles (\textit{cdc}4^{G19E}, \textit{cdc}4^{G107S}) and a modified myosin that lacked both IQ domains and thereby unable to bind Cdc4, still showed lethality at the non-permissive temperature [27]. These results suggest that failure of cytokinesis at the restrictive temperature is not due to failure of interaction of these two mutant forms of Cdc4 with myosins. Finally, the cell concentration of Cdc4 has been reported to be 10-fold higher than the concentrations of type II myosins (Myo2 and Myp2) or of the myosin regulatory light chain Rlc1 [31]. This is unusual, as regulatory and essential light chains are found in equimolar amounts in the neck region of type II myosins. These observations clearly suggest that Cdc4 has functions beyond its association with myosins.

It is not known if the essential functions of \textit{S. pombe} \textit{pik1} and \textit{S. cerevisiae} \textit{PIK1} are conserved. We hypothesized that in \textit{S. pombe}, Pik1 lipid kinase activity and possibly its Cdc4-binding activity are involved in a specific aspect of cytokinesis. To test this hypothesis, we created and characterized a number of new \textit{pik1} alleles; including, a chromosomal \textit{pik1} deletion allele, a conditional loss-of-function allele, a fluorescently tagged allele, and 3 point mutation
alleles. The latter 3 alleles were designed to affect Pik1 lipid kinase and Cdc4-binding activities. Alleles were assessed after genomic integration or ectopic expression, and for complementation of the conditionally lethal *S. cerevisiae* pik1-101 allele.

**Materials and Methods**

*Strains, media, genetic and molecular biology methods.* *S. pombe* strains (Table 1) were cultured in YES, ME or EMM with supplements, and transformed using lithium acetate or electroporation as described [35]. *S. cerevisiae* strains (Table 2) were cultured in synthetic minimal medium lacking leucine (SD-Leu; 0.67% nitrogen base, 2% glucose, and amino acid supplements as appropriate) in the presence or absence of 15 μM thiamine. Standard techniques for DNA manipulation were used. All recombinant sequences were verified with a model 370A automated sequencer (PE Applied Biosystems Inc.).

**Gene deletion.** The pik1 coding region was replaced with a *ura4* expression cassette by homologous recombination in diploid cells [36]. The diploid strain (N2 X N3) was transformed with a DNA fragment containing *ura4* flanked by 600 bp regions 5’ and 3’ to the pik1 coding region. Diploid transformants were selected on EMM – adenine – uracil. Sequencing of strain N1231 confirmed replacement of one copy of the pik1 coding sequence by *ura4*. N1231 spores from azygotic asci were incubated on YES at 19°C for 13 days, or at 25°C, 30°C, or 36°C for 5 days. Cells were transferred to EMM – uracil to assess for the presence of *ura4*. The presence of the pik1 coding sequence was determined by PCR. Similarly, the pik1 coding region was deleted in haploid cells [36] that carried pREP81-pik1, except a Kan<sup>R</sup> cassette was used to produce strain N1113.
pikl cDNA cloning and site directed mutagenesis. A pikl cDNA clone was isolated by reverse transcription PCR using primers H1280 and H1285 that incorporated Ndel and BamHI sites for cloning in pREP vectors [37, 38]. An internal Ndel site was removed by site-directed mutagenesis [39] using primer H1284, which introduced a silent C to T mutation at nucleotide 300 of the coding region (Genbank accession number FJ918571). The pikl\textsuperscript{D709A} and pikl\textsuperscript{R838A} alleles were similarly generated using primers H1340 and H1341 which introduced the mutations and restriction sites (PstI and MfeI, respectively) for screening (Genbank accession numbers FJ918572 and FJ918573, respectively). The double mutant pikl\textsuperscript{D709A, R838A} was generated by replacing the BamHI-AgeI fragment of pikl\textsuperscript{D709A} with the corresponding fragment from pikl\textsuperscript{R838A} (Genbank accession number FJ918574). Primer sequences are available upon request.

pikl allele replacement. Homologous recombination in diploid cells [36] was used to introduce missense mutations into pikl, producing substitution R838A, or D709A, or both. Allele replacement constructs consisted of the last 380 codons of pikl (pikl\textsuperscript{472-851}), the native stop codon, the nmt1 terminator region, ura4 gene cassette and 700 bp of genomic DNA downstream of the pikl coding region. As a result, the pikl locus was modified by the presence of nmt1 transcription termination sequences and by the presence of a downstream ura4 cassette. To control for these changes, the wild-type sequence was integrated into the pikl gene by the same method. Diploid cells (N250 X N253) were transformed, plated at 30°C on EMM – adenine – uracil. Colonies were tested for integration by colony PCR and growth on EMM – uracil. Tetrad analysis was used to establish the essentiality of each haploid pikl allele.

pikl-td allele. The N-degron approach fuses a protein of interest to one that is conditionally unstable. The latter consists of monoubiquitin, an arginine residue, and a thermolabile dihydrofolate reductase (Ub-R-DHFR\textsuperscript{86}). Long-lived at 25°C, at 36°C it is rapidly
degraded by the N-end rule pathway [40, 41]. The Ub-R-DHFR^R coding region from pPW66R
[40] was amplified by PCR as described [41] and fused, in frame, to the pikl cDNA coding
region in pREP41X [37]. Hemizygous N1231 cells were transformed. A haploid strain carrying
both pREP41X-Ub-R-DHFR^R-pikl and the disrupted chromosomal pikl allele (N1366, also
referred to as pikl-td), was selected by random spore analysis. In N1366, the sole source of Pikl
is episomal.

**Fluorescently tagged pikl allele.** The pikl coding region was cloned in pREP41-eGFP
[42]. A second eGFP coding region was inserted in frame as an NdeI fragment at the pikl
initiation codon to produce pREP41-2XeGFP-pikl which was used to transform hemizygous
strain N1231. Haploid strain N1369 carrying both the vector and the disrupted chromosomal pikl
allele was selected by random spore analysis.

**Synchronized cultures.** To examine cell cycle dependent changes in 2XeGFP-Pikl
localization, a cdc25-22 strain was transformed with pREP41-2XeGFP-pikl. Division
synchrony of the resulting strain, N1401, was induced by block and release. Cells grown at 25°C
in EMM –leucine –thiamine for 16 hours, were shifted to 36°C for 4 hours. Cells collected by
centrifugation (5 min., 3000 x g) were resuspended in 50 ml medium at 25°C and incubated for
220 min. Cells were sampled every 20 min. and examined immediately by microscopy to
evaluate the intracellular localization of 2XeGFP-Pikl or fixed with formaldehyde, incubated for
30 min. on a rotating wheel, washed 3 times in phosphate-buffered saline and kept at 4°C for
microscopic examination [35].

**Microscopy.** Cell morphology and mitotic index were examined by bright-field and
fluorescence microscopy after formaldehyde fixation and Calcofluor White (Fluorescent
Brightener 28, Sigma F3543-1G) and 4',6-diamidino-2-phenylindole (DAPI) staining [35]. Cell
numbers were estimated with a hemacytometer. F-actin-containing structures were visualized
with FITC-phalloidin [43]. Myo2 and Pik1 immunofluorescence microscopy of methanol-fixed
cells [35] used both primary antibodies and Texas red conjugated secondary antibodies at 1:100
dilution. Rabbit Myo2 antibodies were obtained from M. K. Balasubramanian (Temasek Life
Sciences Laboratory, Singapore). Rabbit Pik1 antiserum was already available [21].

An Olympus 1X70 inverted microscope with 60X 1.4NA Plan-apo objective, appropriate
filter sets and a RT-Slider (SPOT) CCD camera (Carsen Scientific Imaging Group, Markham,
Canada) was used for bright-field and fluorescence microscopy. Images were cropped and
processed for brightness and contrast with Spot32 Advanced software.

Potassium permanganate-fixed pik1-td cells were examined by transmission electron
microscopy as described [44, 45]. Cell preparations were done by P.A. Netto, Temasek Life
Sciences Laboratory, Singapore.

**Ectopic expression of pik1 and mutant alleles in S. pombe.** Recombinant pREP vectors
[37, 38] expressing pik1 alleles were introduced into cdc4 or cdc4⁴⁸₁₀⁷⁸ strains. Starter cultures in
EMM –leucine +thiamine were incubated overnight at 30°C or 25°C (for temperature-sensitive
strains). Cells were collected by centrifugation (700 x g, 10 min.), washed 3 times in sterile
distilled water, placed in 50-100 ml cultures at 10⁵ cells/ml, and incubated for 24 h. at 30°C or
25°C, + or - thiamine. Cell numbers were estimated with a hemacytometer. Cells were collected
by centrifugation, and either washed and fixed for microscopic examination, or lysed with a
‘mini’ French pressure cell at 900 p.s.i. for protein estimation, immunoblotting, lipid kinase
assays and ELISA.

**Colony formation assay.** Starter cultures in EMM –leucine +thiamine were incubated
to saturation (24-36 hours) at 30°C or 25°C. Cells were collected by centrifugation and washed 3
times in sterile distilled water. Aliquots (5 μl) from each of 4, 10-fold serial dilutions were
spotted onto EMM - leucine, + or - thiamine, + phloxin B plates and incubated at 30°C for 5-6
days.

**Phospholipid kinase assay.** Cells carrying pik1 expression vectors were cultured for 24
h., + or - thiamine, at 30°C or 25°C. Cells were harvested by centrifugation (700 x g for 5 min.),
resuspended in 25 mM HEPES, pH 7.4, 10 mM MgCl2, and passed through a ‘mini’ French
pressure cell 3 times at 900 p.s.i. Cell lysate protein content was estimated [46] and lysates were
diluted to 0.05 μg – 0.8 μg total protein per 50 μl assay. Each diluted cell lysate was incubated
with 10 μCi [γ-32P] ATP for 15 min. at room temperature and the reaction stopped by addition
of 6 M HCl to a final concentration of 1.7 M. Lipids were extracted with three volumes of
chloroform:methanol (1:1, vol:vol), vortexed for 10 s. and centrifuged in an Eppendorf
microcentrifuge at maximum speed for 5 min. [47]. The bottom organic layer was removed and
dispensed into a fresh tube. The acidic lipid fraction was further extracted with ½ volume
methanol: 1 N HCl (1:1, vol:vol), vortexed and centrifuged as previously described. The organic
phase was again retrieved and dried under N2 gas. The lipids were resuspended in 4 μl of
chloroform:methanol (1:1, vol:vol) and spotted onto a Silica gel 60 thin layer chromatography
(TLC) plate previously baked for 30 min. at 100°C. The TLC plate was placed in a
chromatography chamber pre-equilibrated for two hours with freshly made developing solution
(1-propanol:2 M acetic acid (13.7:7 vol:vol)). Lipid separation was for 6-8 h. The TLC plate
was dried overnight and either exposed to Kodak BioMax XAR film for 2-3 days or scanned
under 10% methane in argon using a BIOSCAN AR-2000 imaging scanner for radio-TLC.

WinSCAN 2D software version 1.05 was used to visualize the distribution of radioactivity on the
plate. The silica carrying the radiolabelled lipid was added to Aquasol (Perkin-Elmer) for liquid
scintillation counting. Data were corrected for counting efficiency and decay and expressed as
disintegrations per minute (DPM). Lipid standards purchased from Avanti Polar Lipids (50 µg
each of PtdIns, PtdIns(4)P, PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$) were run in parallel and visualized
using iodine vapour.

**Yeast two-hybrid assay.** Assays were performed as described [48] using the leucine
selectable pBI880 vector carrying the GAL4-DB (DNA binding domain) fused in-frame to a cdc4
cDNA sequence, and the tryptophan selectable pBI771 vector carrying sequences encoding the
GAL4-TA (trans-activating domain) fused in-frame to residues 507 through 851 of wild-type or
mutant pik1 alleles. Both vectors were introduced simultaneously into *S. cerevisiae* YPB2 cells
using a lithium acetate procedure. Cells were selected for the presence of both plasmids by
colony formation on SD–leucine–tryptophan plates at 30°C for 5 days. A positive protein
interaction was then identified by colony formation at 30°C for 7-9 days on SD–leucine–
tryptophan–histidine +3-amino-1',2',4'-triazole (3–AT). X-gal colony filter assays were also
performed to confirm positive yeast two-hybrid interactions [48].

**ELISA.** *S. pombe* cultures, initially at 1 × 10$^5$ cells/ml, carrying pREP1 plasmids
expressing wild-type or mutant pik1 alleles, were incubated + or – thiamine for 24 h. at 30°C.
Cell extracts were prepared using a French press ‘mini’ cell (3 passages at 900 p.s.i.) in
phosphate-buffered saline (PBS) with protease inhibitors (Sigma, P8215) at 4°C. Multiwell
plates were coated by incubating with purified Cdc4 protein [34] at 10 µg/ml overnight at 30°C.
The wells were blocked by incubating with 2% (w/vol) powdered skim milk in PBS at room
temperature for 1 h. Serial two-fold dilutions of cell lysates containing 56 µg to 14 µg protein
were added to the Cdc4-coated wells and incubated for 16 h. at 4°C. Wells were washed 4 times
with PBS-0.01% (vol/vol) Tween 20 and incubated with a 1:1000 dilution of rabbit anti-Pik1
serum [21] for 4 h. at room temperature followed by 1 h. at room temperature with goat anti-
rabbit IgG-HRP (horseradish peroxidase) at a dilution of 1:5000. Signal detection was with
TMB (3,3',5,5'-tetramethylbenzidine). Optical density was measured with a Molecular Devices
microplate reader at wavelengths 650 nm and 450 nm, and the data acquired using Softmax
Software, version 2.34.

Complementation analysis. *S. pombe* pikl wild-type and mutant cDNA coding regions
were cloned (*NdeI* – *BamHI*) in *S. pombe* expression vectors pREP1, pREP41, and pREP81 [37],
and pREP41-eGFP N [42]. The pikl coding regions flanked by the nmt1 promoter and
terminator sequences were then cloned (*PstI* – *SstI*) in *S. cerevisiae* expression vector,
YEplac181 [49]. *S. cerevisiae* cells carrying PIK1 or pikl-101 were transformed with each of
the resulting vectors using lithium acetate [50]. For colony formation assays, cells from
precultures were counted and cultures were started at 1 X 10^5 cells/ml at 25°C. After 18-20 h.,
cells were recovered by centrifugation, washed 3-times in sterile water, and resuspended at 2 X
10^7 cells/ml. Aliquots (5 μl) of serial 10-fold dilutions were spotted onto SD–Leu plates and
incubated at a permissive (25°C) or restrictive (37°C) temperature for 5 days.

Results

**pikl is essential.** Tetrad analysis was performed on the hemizygous diploid strain, pikl /
pikl::ura4. Analysis of the germination and colony formation potential of spores from azygotic
asci confirmed the essential nature of pikl at 30°C. Of the four spores from pikl / pikl::ura4
diploid cells, only two formed colonies at 30°C (Figure 1A, right panel; i). These cells were
wild-type, as determined by their growth requirement for uracil, and by the presence of pikl as
determined by PCR analysis. Of the spores that did not form colonies, some remained spherical,
most displayed some outgrowth and one germinated and divided once (Figure 1A, right panel; ii).
In contrast, and as expected, the four spores from homozygous diploid strain pikl/pikl germinated and formed colonies at 30°C (Figure 1A). To evaluate if the requirement for pikl is affected by temperature, spores were incubated at 19°C, 25°C and 36°C. Only two of four spores formed colonies at these temperatures (not shown). Most of the spores that did not form colonies at 19°C or 25°C germinated and divided a few times, in contrast to most of the spores at 36°C which did not germinate or germinated, but did not divide. Thus, pikl is essential for vegetative cell division. pikl also appears to be required for spore germination at the higher temperatures (30°C - 36°C).

Plasmid loss studies were carried out on a haploid pikl::KanR strain that carried an expressed episomal pikl cDNA sequence. To achieve this, the pikl coding region was replaced with a KanR gene cassette in haploid cells that carried an episomal pikl cDNA sequence under the control of a highly attenuated (pREP81) thiamine-repressible nmt1 promoter (strain N1113, Table 1). Cells were cultured without selection for the plasmid (+leucine). Repeated attempts to identify pikl::KanR cells that had lost the episome failed. In addition, cells whose only source of pikl was from the episome, proliferated and had normal morphology when incubated in the presence or absence of thiamine (not shown). The nmt1 promoter is leaky and some level of expression is observed, even in the presence of thiamine [51]. These results indicate that in cells carrying the pikl::KanR chromosomal allele, the level of pikl expression from a thiamine-repressed, highly attenuated nmt1 promoter is sufficient for cell growth and division.

**Pikl is required for septation and cell separation.** To create a loss-of-function allele, the pikl cDNA coding region was fused to sequences encoding a ubiquitin, an arginine residue, and a temperature-sensitive dihydrofolate reductase fusion protein (Ub-R-DHFRts) [40, 41]. This allele was designated pikl-td (pikl-temperature dependent). Ub-R-DHFRts is a thermolabile
protein that unfolds at elevated temperatures to expose a destabilizing N-end residue, making the
protein susceptible to degradation by ubiquitin-dependent proteolysis. At 25°C, the Ub-R-
DHFR<sup>ts</sup>-Pik1 fusion protein should be stable. At 36°C, the Ub-R-DHFR<sup>ts</sup> moiety should unfold
and cause degradation of Pik1. The effects of loss of Pik1 function were assessed by ectopic
expression of <i>pik1-td</i> under the control of an attenuated <i>rmt1</i> promoter in cells lacking the
chromosomal <i>pik1</i> coding region (<i>pik1::ura4</i>). The <i>pik1-td</i> cells were cultured at 25°C in the
presence of thiamine. These cells had a normal proliferation rate (division time = 3.6 h.; Figure
2A) and morphology (Figures 2B and C). F-actin was visualized at the cell tips and as a medial
band. About 13-17% of the cells were binucleate and had an F-actin ring and a septum. Septa,
visualized with calcofluor white (Figure 2C) or examined by transmission electron microscopy
(Figure 3), had morphologies similar to wild-type cells. Thus, fusion of Ub-R-DHFR<sup>ts</sup> to the N-
terminal end of Pik1 had no apparent effect on Pik1 function at 25°C and it was sufficient for cell
viability and proliferation. However, at 36°C, cell replication was suppressed with only a modest
increase in cell number even after 72 h. in culture (Figure 2A). Within 18-20 h. after the
temperature shift from 25°C to 36°C, cells appeared wider and many cells were elongated
(Figure 2B). About 50% of the cells were binucleate and 15% had 3 or more nuclei. F-actin
distribution was disrupted with F-actin patches dispersed throughout the cells rather than at one
or both ends (Figure 2C). F-actin rings were observed in approximately 16% of the cells. Some
cells had more than one F-actin ring. Apparent ring constriction was observed in some cells.
Approximately 74% of the cell population had one or more septa (Figure 2C). Approximately
33% of the cells seemed to be in the process of septum hydrolysis or to be arrested in the process
of septation as they remained attached in a V-shape form through some residual septum (Figure
2B). Only about 6% of the cells showed this phenotype at 25°C (Figures 2B and C). The septum
viewed by transmission electron microscopy is composed of three layers, one bright layer (primary septum) between two darker layers (secondary septa) [52]. Septum morphology of *pik1-td* cells incubated at 36°C was aberrant. These cells had one or more septa that were thickened, especially in the secondary septum layers, compared to the same cells at 25°C or wild type cells at either 25°C or 36°C (Figure 3). In addition, there was accumulation of intracellular membranous or vacuole-like structures in the *pik1-td* cells at 36°C (Figure 3). These structures were present to a much lesser extent at 25°C but were not observed in wild-type cells incubated at 25°C or 36°C. These observations indicate that temperature-dependent loss of Pik1 function arrests cells at a late stage in cytokinesis, preventing cell separation.

**Pik1, a Golgi associated protein, is also found at the medial region of dividing cells.** Sequences encoding two tandem eGFP (enhanced green fluorescent protein) proteins were fused to the 5’end of a *pik1* cDNA sequence in pREP41. Cells carrying this vector, pREP41-2XeGFP-*pik1*, and *pik1::ura4* were viable. The 2XeGFP-Pik1 protein fusion was stable, as western blots with anti-GFP and anti-Pik1 sera detected only the fusion protein in extracts from cells cultured in derepressed conditions (not shown). The fusion protein was below the detection limit in cells cultured under repressed conditions. Cells incubated in the presence or absence of thiamine had similar growth rates, morphology and septum and actin distributions (not shown). Thus, the fusion of 2XeGFP to Pik1 had no apparent effect on Pik1 functions. In the absence of thiamine, 2XeGFP-Pik1 fluorescence was observed as a punctate pattern throughout the cytoplasm and around the cell periphery. In about 8% of the cells, a fluorescent medial band was observed in addition to the dots (Figure 4A). No medial band or punctate fluorescence was observed within cells cultured under repressed conditions. The 2XeGFP-Pik1 punctate fluorescence was similar
to that observed with indirect immunostaining with Pik1 antiserum. The latter co-localized with
GFP-tagged Gma12p, a Golgi-associated galactosyltransferase (Figure 4B).

To evaluate the cell cycle dependence of recruitment of Pik1 to the medial region,
2XeGFP-pik1 was expressed in cdc25-22 cells synchronized by temperature block and release.
At the restrictive temperature (36°C), cdc25-22 cells arrest in G2/M [49]. After 4 h. at 36°C,
cells shifted to 25°C enter mitosis and divide over the following 3 h. (Figure 4C). The mitotic
index reached 80% in the synchronized cultures indicating a high level of induced synchrony.
2XeGFP-Pik1 first appeared at the medial region of the cells 100-120 min. after release. This
corresponds approximately to the time of appearance of the septum (80-120 min.). Formation of
the F-actin ring occurred earlier (60-80 min.). The proportion of cells showing recruitment of
2XeGFP-Pik1 to the medial region peaked at only 30% at 120 min. after release. 2XeGFP-Pik1
fluorescence intensity may have been one limiting factor. Another possibility is that Pik1
recruitment to the medial region is poorly synchronized by the cdc25-22 block and release
method. The punctate fluorescence throughout the cytoplasm was still observed at all time points
although it was quite faint. Thus, there is a cell cycle dependent recruitment of Pik1 to the medial
region of dividing cells, at about the same time as deposition of septum material occurs.

**Pik1 residues D709 and R838 are required for lipid kinase and Cdc4-binding activities, respectively.** Previously, the 345 C-terminal amino acids of Pik1 (a.a. 507-851) were
shown to interact with Cdc4 [21]. This region (Figure 5) includes the lipid kinase domain (a.a.
578-800) as identified by Pfam (http://pfam.sanger.ac.uk/) [53] or (a.a. 579-850) as identified by
Prosite (http://ca.expasy.org/prosite/). To evaluate the importance of Pik1 lipid kinase and Cdc4-
binding activities for cytokinesis, we created alleles that were impaired for each of these two
functions.
A conserved aspartic acid residue, D709, within a DRH motif unique to lipid kinases, is one of several residues involved in ATP binding and phosphate transfer [54]. This residue was replaced with alanine to create pik1<sup>D709A</sup> (Figure 5). Equivalent mutations in other lipid kinases were shown to abolish kinase activity [54, 55, 56].

Cdc4 is known to bind to IQ domains of type II myosins [25, 26, 27]. A pseudo IQ motif (I<sub>828</sub>KANCSVWTR<sub>838</sub>) is present in the C-terminal region of Pik1 (Figure 5). The first position of the IQ motif consensus sequence IQxxxxRGxxxR is somewhat variable. It is generally Ile, Leu or Val, but Met, Phe and Thr are also found at this position in the IQ motifs of myosins [57]. In Pik1 in many species, the first position of the pseudo IQ motif is either Val or Ile (Figure 5).

Furthermore, position 11 of the consensus sequence is typically occupied by an Arg or Lys, and this is also consistently observed within the pseudo IQ motifs of pik1 homologs (Figure 5). The central residues of the IQ motif are less conserved [57]. To evaluate if this pseudo IQ motif was a binding site for Cdc4, a conserved arginine residue (R838) was replaced with alanine to create pik1<sup>R838A</sup> (Figure 5).

A yeast two-hybrid assay, an ELISA and a lipid kinase assay were used to assess whether the R838A or D709A mutations affected binding of Pik1 to Cdc4, or Pik1 lipid kinase activity. For the yeast two-hybrid assay, S. cerevisiae cells were co-transformed with the ‘bait’ vector carrying the S. pombe cdc4 cDNA sequence fused to the GAL4-BD (DNA binding domain) and with the ‘prey’ vectors carrying the C-terminal region of S. pombe wild-type and mutant pik1 sequences fused to the GAL4-TA (transcriptional activation) domain. As observed previously [21], the C-terminal region of Pik1 interacted with Cdc4, as shown by colony formation on minimal medium plates lacking leucine, tryptophan and histidine, and containing 3-AT (Figure 6A; WT). In addition, these cells possessed β-galactosidase activity, also a positive indicator of
protein-protein interaction (Figure 6B; WT). Colony formation was not observed when cells
were transformed with the ‘prey’ vector without the pikl sequence indicating that the GAL4-TA
domain by itself did not result in a positive interaction phenotype (Figure 6A and B; -ve). When
cells carrying the GAL4-TA piklD709A fusion were examined for histidine prototrophy, colony
formation was similar to that observed for cells carrying the GAL4-TA pikl wild-type allele
(Figure 6A; D709A). These cells also possessed β-galactosidase activity (Figure 6B; D709A).
In contrast, cells carrying the GAL4-TA piklR838A fusion did not form colonies under these
conditions, indicating that the Cdc4 interaction with Pik1 did not occur (Figure 6A; R838A).
Doubly transformed cells carrying this pikl allele cultured in the presence of histidine formed
colonies that were negative for β-galactosidase activity (Figure 6B; R838A).
As Cdc4 interaction with Pik1 in the yeast two-hybrid assay involved only the C-terminal
end of Pik1, we wished to perform immunosorbent assays to assess the binding of Cdc4 to the
full-length protein. Western blot analysis was performed to determine if the wild-type and
mutant forms of Pik1 accumulated to detectable levels upon ectopic expression (Figure 6C).
These cells also carried the pikl genomic locus. Lysates were prepared from cells carrying the
ectopic pikl alleles and grown in repressed or derepressed conditions. Negative controls
included cells carrying the vector alone without the pikl sequence. A band migrating at
approximately 93 kDa was visible in all samples, including the negative control cells (Figure 6C,
-ve). This band was also observed in lysates from negative control cells grown under repressed
conditions (not shown). A band migrating at about 97 kDa, corresponding with the expected size
of Pik1, was visible when any of the pikl alleles were expressed (Figure 6C). The signal after
expression of the piklR838A allele was reproducibly stronger than after expression of the other
alleles. In lysates from cells grown under repressed conditions this band was either not visible or
barely visible (not shown). Thus, derepression of the ectopic expression of each of the \textit{pikl} alleles results in elevated levels of the corresponding protein in the cells.

For ELISA, wells were coated with purified Cdc4. Purified Cdc4 was obtained using a bacterial expression system and subsequent elution through an anion exchange column and gel filtration column [34]. One protein band was observed by SDS-PAGE and the protein identity was confirmed by mass spectrometry [34]. Subsequently the wells were incubated with serial dilutions of lysates of cells that carried \textit{pikl} alleles cultured in the presence or absence of thiamine. Binding of Pik1 to Cdc4-coated wells was monitored with a primary antiserum against Pik1 and a secondary antibody-HRP conjugate (Figure 6D). Lysates from cells carrying the empty vector under derepressed conditions produced a very low ELISA signal (Figure 6D, open square). Since these cells carried the intact \textit{pikl} locus this can be considered to be the background signal for this assay. Lysates from cells carrying the ectopic wild-type \textit{pikl} allele and grown under repressed conditions, produced a background level ELISA signal (Figure 6D, closed triangle). Lysates from cells carrying the wild-type \textit{pikl} allele and grown in the absence of thiamine, produced an increased Pik1 ELISA signal relative to that observed in the presence of thiamine (Figure 6D, open triangle). Pik1 with the D709A substitution also produced a positive ELISA signal that was reproducibly larger than that from Pik1 lacking this substitution (Figure 6D, open diamond). In contrast, Pik1 carrying the R838A substitution or carrying both the D709A and R838A substitutions, did not produce an ELISA signal that was above the background level. Thus, the Pik1\textsuperscript{R838} residue, which was shown to be required for the interaction with Cdc4 in the two-hybrid assay (Figure 6A) was also found to be required for interaction of the full length Pik1 protein with Cdc4-coated wells in ELISA (Figure 6D). We note that the
The abundance of the endogenous Pik1 protein is below or near the levels of detection in the ELISA and immunoblot assays.

To evaluate the effects of the D709A and R838A substitutions on Pik1 enzymatic activity, lysates of cells expressing episomal *pikl* alleles were assayed for lipid kinase activity (Figure 7). These cells all carried the intact chromosomal *pikl* locus. Cells carrying each allele were grown for 24 hours at 30°C in repressed or derepressed conditions. Cell lysates containing a constant amount of protein were pulse labeled with [γ-32P] ATP and phospholipids were extracted and separated by TLC. The radiolabeled lipid spot in Figure 7A was identified as PtdInsP as it co-migrated with PtdIns(4)P but not with PtdIns(4,5)P₂ or PtdIns(3,4,5)P₃ reference lipid-standards (data not shown). PtdIns(4)P and PtdIns(3)P migrate with the same Rf value and are not resolved with this assay system [58, 59]. Lysates from cells carrying the wild-type *pikl* allele had higher incorporation of ³²P label into PtdInsP when expression was derepressed (Figure 7A; WT, compare + and – thiamine). Lysates from cells carrying the *piklD709A* allele and grown under repressed conditions showed incorporation of ³²P label into PtdInsP that was similar to that of the wild-type allele under the same condition. Under derepressed conditions, incorporation appeared to be reduced (Figure 7A; D709A, - thiamine). In contrast, expression of the *piklR838A* allele showed a level of induced lipid kinase activity that was comparable to or higher than that of the wild-type allele under the same conditions (Figure 7A; R838A).

Serial dilutions of cell lysates were also assayed for lipid kinase activity (Figure 7B). Lysates of cells transformed with the vector alone and incubated with or without thiamine showed a linear increase in lipid kinase activity over the range of protein tested (Figure 7B; WT, dotted lines). This activity represents endogenous activities, including those of the three putative PtdIns 4-kinases in *S. pombe*. In lysates of cells carrying the wild-type allele cultured in the
presence of thiamine, an approximately 1.3-fold increase in lipid kinase activity was observed
(Figure 7B; WT, solid line, closed triangle). Derepression of the wild-type allele resulted in an
approximately 3-fold increase in lipid kinase activity (Figure 7B; WT, open triangle). These
observations are consistent with the known behavior of the nmt1 promoter under derepressed and
repressed conditions [51]. Under repressed conditions, ectopic expression of pik1D709A resulted
in lipid kinase activity that was comparable to that of the endogenous level (Figure 7B; D709A,
closed triangle). When the expression of this allele was derepressed, the apparent lipid kinase
activity was reproducibly reduced (Figure 7B; D709A, open triangle). It is likely that the
Pik1D709A protein that accumulated under these conditions competed with the endogenous
enzymes for the phosphatidylinositol substrate, reducing the apparent lipid kinase activity.
Ectopic expression of pik1R838A under repressed conditions resulted in lipid kinase activity that
was similar to that observed with ectopic expression of the wild-type allele under the same
condition (Figure 7B; R838A, closed triangle). Upon derepression, the activity increased
approximately 3-fold (Figure 7B; R838A, open triangle). Lipid kinase activity in lysates of cells
carrying pik1D709A,R838A was indistinguishable from that in lysates of pik1D709A cells (Figure 7B;
D709A, R838A).
In conclusion, Pik1 with the D709A substitution has little or no lipid kinase activity, but
retains its ability to interact with Cdc4. Pik1 with the R838A substitution retains lipid kinase
activity, but does not bind Cdc4. Pik1 with both substitutions has little or no lipid kinase activity
and does not bind Cdc4.

Functional analysis of pik1 alleles by ectopic expression. While performing the studies
described above it was observed that ectopic expression of pik1 was lethal under some
circumstances. To investigate this further, the effects of ectopic expression of pik1 alleles on
colony formation and proliferation in liquid cultures were evaluated relative to cells carrying the
vector alone (Figure 8A, B). Cells carrying the empty vector, or the wild-type or mutant alleles
of pkl on an episome under the control of the full-strength nmt1 promoter were serially diluted
and spotted onto minimal medium plates with or without thiamine, and supplemented with the
viability stain phloxin B. Phloxin B stains dead cells red [35]. After 6 days at 30°C in the
presence of thiamine, cells carrying each of the pkl alleles grew indistinguishably from cells
carrying the vector alone (Figure 8A, left panel). Similarly, the proliferation of these strains in
liquid culture in the presence of thiamine were similar to each other and to the cells carrying the
vector alone (Figure 8B). In the absence of thiamine, cells expressing the wild-type pkl
sequence had a markedly reduced ability to form colonies, and the colonies were red (Figure 8A;
WT, right panel). Proliferation of these cells after 24 hours in liquid culture in the absence of
thiamine, was markedly reduced (Figure 8B; WT, solid line, open triangle) compared to cell
cultures with thiamine (Figure 8B; WT, solid line, closed triangle) or to cells carrying the vector
alone in repressed or derepressed conditions (Figure 8B; dotted lines, open and closed triangles).
Cells expressing pkl^{D709A} formed colonies, but the colonies were red indicative of some cell
death. (Figure 8A; D709A, right panel). In liquid culture in the absence of thiamine, these cells
showed reduced proliferation (Figure 8B; D709A, open triangle). Colony formation of cells
expressing pkl^{R838A} was similar to that of cells carrying the vector alone (Figure 8A; R838A,
right panel). These cells in liquid culture in the absence of thiamine had a slightly reduced rate
of proliferation (Figure 8B; R838A, open triangle). Colony formation of cells ectopically
expressing the double-mutant pkl^{D709A,R838A} was also similar to that of cells carrying the vector
alone (Figure 8A; D709A, R838A, right panel). The proliferation of these cells in liquid cultures
was very similar under repressed and derepressed conditions and to cells carrying the vector
alone (Figure 8B; D709A, R838A, closed and open triangle).

Thus, ectopic expression of the wild-type pikl allele in cells carrying the intact pikl
chromosomal locus was lethal. The Pik1 activities that require the R838 residue accounted for
most of the lethal effect of ectopic expression of pikl, while the activities that require the D709
residue contributed to a lesser extent.

Morphological analysis of cells after ectopic expression of pikl alleles. In liquid
cultures after 24 hours at 30°C, with or without thiamine, about 10% of cells carrying the vector
alone showed actin and myosin medial bands, were binucleate and had normal septa (Figure 8C;
-ve; Table 3). Cells ectopically expressing the wild-type pikl allele had reduced cell length at
division relative to cells cultured in repressed conditions, or to cells carrying the vector alone
(Table 3). About 10% of the cell population had 2 nuclei and a septum, a frequency similar to
that of cells carrying the vector alone or cultured under repressed conditions (Table 3). However,
septum morphology was abnormal in some cells as defined by more intense calcofluor staining
and/or of aberrant shape (Figure 8C; WT). Remarkably, only about 1% of the cell population
ectopically expressing the wild-type pikl allele formed actin and myosin rings (Table 3). Actin
cytoskeletal structures visualized with FITC-phalloidin were disrupted with punctate actin
staining evident throughout the cell instead of at the cell poles or medial region as observed in
cells grown in the presence of thiamine or carrying the vector alone (compare Figure 8C; WT
with Figure 8C; -ve). Thus, ectopic expression of the wild-type pikl sequence, resulting in the
accumulation of an active lipid kinase (Figure 7), inhibits cell proliferation (Figure 8),
presumably by disrupting actin cytoskeletal and septum structures.
The most obvious effect of ectopic expression of pikl$^{D709A}$ was a marked increase in septation index as approximately 20% of the cell population had 2 nuclei and a septum (Table 3). The mean cell length at septation was also longer relative to cells grown under repressed conditions or carrying the vector alone (Table 3). In a third of these cells, calcofluor white staining showed intense fluorescence. Some cells had two or more septa (Figure 8C; D709A). The number of cells with actin or myosin rings was similar to that for pikl$^{D709A}$ cells grown in repressed conditions or cells carrying the vector alone (Table 3). Staining with FITC-phalloidin revealed some cells with disrupted F-actin structures (Figure 8C; D709A). These results suggest that expressing a pikl allele with no lipid kinase activity (Figure 7), interferes primarily with septation, and may have some effects on F-actin structures although contractile ring formation appeared to be unaffected.

Under derepressed conditions, cells carrying the pikl$^{R838A}$ allele had slightly reduced cell length at septation relative to cells cultured in repressed conditions or to cells with the vector alone (Table 3). The number of cells with actin rings, myosin rings and septa were similar to cells cultured in repressed conditions and to cells carrying the vector alone (Table 3 and Figure 8C). Similarly, septum appearance was unaffected (Figure 8C; R838A). Thus, the disruption of actin-myosin structures and the abnormal septum morphologies observed with the ectopic expression of the wild-type pikl sequence were not observed when the R838 residue was replaced with alanine.

Cells ectopically expressing pikl$^{D709A, R838A}$ were morphologically wild-type (Figure 8C, Table 3). These results emphasize the importance of both the D709 and R838 residues in the functions of Pik1.
The pikl ectopic expression lethal phenotype is suppressed in cdc4^{G107S} cells. The R838 residue of Pik1 is required to bind Cdc4 (Figure 6) and for pikl ectopic lethality (Figure 8). We reported previously that one mutation in cdc4, the G107S substitution, prevented the interaction of Pik1 with Cdc4 [21]. We hypothesized that ectopic expression of wild-type pikl might not be lethal in cells carrying the cdc4^{G107S} allele, just as ectopic expression of pikl^{R838A} had little effect in wild-type cells (Figure 8; Table 3). Ectopic expression of each of the pikl alleles in cdc4^{G107S} cells after 24 hours at 30°C resulted in accumulation of the corresponding protein as determined by immunoblotting (Figure 9A) and was similar to that observed when these alleles were expressed in cdc4 cells (Figure 6C). Of note, as in cdc4 cells, Pik1^{R838A} reproducibly accumulated in cdc4^{G107S} cells to higher levels than the other Pik1 variants (Figure 9A; R838A) and Pik1^{D709A, R838A} abundance appeared to be slightly reduced (Figure 9A; D709A, R838A).

In colony formation assays at 30°C in the presence of thiamine, cdc4^{G107S} cells carrying each of the episomal pikl alleles formed colonies indistinguishably from cdc4^{G107S} cells carrying the empty vector (Figure 9B, left panel). In the absence of thiamine, cells ectopically expressing the wild-type pikl sequence also formed colonies similarly to cells carrying the empty vector (Figure 9B; WT, right panel). This was in marked contrast to the lethal effect of pikl ectopic expression in cells carrying the wild-type cdc4 allele (compare WT in Figure 8A and 9B, right panels). Expression of pikl^{D709A} markedly reduced the ability of cdc4^{G107S} cells to form colonies (Figure 9A; D709A, right panel), also in contrast to the expression of the same allele in wild-type cdc4 cells (Figure 8A, right panel). Colony formation of cdc4^{G107S} cells expressing pikl^{R838A} was similar to that of cells carrying the empty vector (Figure 9B; R838A, right panel). Expression of pikl^{D709A, R838A} had some effect on colony formation in cdc4^{G107S} cells, intermediate between the
growth suppression seen with expression of pikl\textsuperscript{D709A} and the apparent viability of cells expressing pikl\textsuperscript{R838A} (Figure 9B; D709A, R838A, right panel). The same results were observed when the cells were grown at 25°C (not shown).

Cell proliferation by these strains in liquid cultures at 25°C was consistent with the results of the colony formation assays (Figure 9C). Namely, cdc4\textsuperscript{G107S} cells ectopically expressing wild-type pikl grew similarly to cells incubated in the presence of thiamine and to cells carrying the empty vector (Figure 9C; WT). Ectopic expression of each of the mutant alleles reduced cell proliferation to some extent compared to proliferation of cells ectopically expressing the wild-type pikl allele (Figure 9C). In both the colony formation assay and in liquid culture the greatest effect was from ectopic expression of pikl\textsuperscript{D709A} (Figures 9B and 9C).

Morphological analysis was performed on cdc4\textsuperscript{G107S} cells after ectopic expression of each of the pikl alleles (Table 4). Expression of the wild-type pikl allele resulted in a slight reduction in cell length at septation, but no effects were observed on the frequencies of cells with F-actin rings, with two nuclei or with septa (Table 4). This was in contrast to the marked reduction of the F-actin ring index in cdc4 cells after ectopic expression of wild-type pikl (Table 3). The suppression of the ectopic pikl phenotype in the cdc4\textsuperscript{G107S} strain was allele specific since it was not observed in the cdc4\textsuperscript{F12L}, cdc4\textsuperscript{G19F}, cdc4\textsuperscript{R33K} and cdc4\textsuperscript{G82D} backgrounds (not shown). Cells ectopically expressing the pikl\textsuperscript{D709A} allele had cell lengths at septation, and septum and contractile ring formation comparable to cells cultured under repressed conditions or carrying the empty vector (Table 4). Interestingly, expression of this allele had the greatest negative effect on cell proliferation and colony formation. The most pronounced morphological effect observed was in cells in which the pikl\textsuperscript{R838A} allele was ectopically expressed. Compared to cells carrying the empty vector with or without thiamine, under repressed conditions these cells were 10%
shorter at septation, and under derepressed conditions, 25% shorter (Table 4). Notably, the
proliferation and colony formation of these cells appeared to be normal. Cells in which pik1<sup>D709A</sup>,
<sup>R838A</sup> was ectopically expressed were slightly longer at septation and had increased binucleate and
septum indices (Table 4). The number of these cells with F-actin staining at the medial region
was similar to cells carrying the vector alone or cultured in repressed conditions.

Overall, ectopic expression of wild-type pik1 in cdc4<sup>G107S</sup> cells had little to no effect on
cell proliferation, in marked contrast to the lethality observed in cdc4 cells. In cdc4<sup>G107S</sup> cells, it
was ectopic expression of the kinase dead allele (pik1<sup>D709A</sup>) that caused dominant lethality
(Figures 9B and C).

Since the lethality caused by the ectopic expression of wild-type pik1 was not observed in
cdc4<sup>G107S</sup> cells, we evaluated if the lipid kinase activity of Pik1 was altered in this background.
Cells ectopically expressing wild-type pik1 or pik1<sup>R838A</sup> were incubated at 25°C for 24 h. In
accord with preceding results (Figure 7B), ectopic expression of either wild-type pik1 or
pik1<sup>R838A</sup> in cdc4 cells resulted in a 3 to 4-fold increase in lipid kinase activity in the cell lysates
relative to lysates of cells carrying the empty vector (Figure 9D; black bars). In contrast, this
was not observed with expression of the same alleles in cdc4<sup>G107S</sup> cells (Figure 9D; WT, open
bar). Some increase in cell lysate lipid kinase activity may have been observed with ectopic
expression of pik1<sup>R838A</sup> (Figure 9D; R838A, open bar). Thus, although both wild-type Pik1 and
Pik1<sup>R838A</sup> accumulated in cdc4<sup>G107S</sup> cells (Figure 9A), a corresponding increase in lipid kinase
activity was not observed (Figure 9D).

**Pik1 lipid kinase activity is essential.** We used two approaches to determine if Pik1<sup>D709</sup>
and Pik1<sup>R838</sup> have essential functions. First, we determined the phenotypes of cells carrying
these substitutions in the pik1 chromosomal locus. Second, we determined if *S. pombe* wild-type
and mutant pikl alleles could complement the conditional lethality of an S. cerevisiae strain that
carried pikl-101, an allele that has been shown to have little or no lipid kinase activity at the
restrictive temperature [7].

Heterozygous diploid strains (Table 1; N1550, N1565, N1582, N1596) were constructed
in which one copy of the pikl locus was fully wild-type, while the other was either wild-type
with respect to the coding sequence or carried either the R838A or D709A substitutions, or both.
We note that the modified loci also had the pikl 3'-untranslated region displaced by the nmt1
terminator sequence and a ura4 cassette (pikl:term

These diploid strains were
incubated on ME plates at 25°C for 2 days to obtain azygotic asci which were transferred to YES
plates. Spores from each of 8-10 asci were separated with a micromanipulator and incubated at
30°C for 5 days. As a control, strain N1550 was constructed in which one copy of the pikl locus
was fully wild-type, while the other copy was wild-type with respect to the coding sequence, but
had the same modifications to the 3'-untranslated region. In 8 of 10 tetrads, each of the 4 spores
formed a colony, although 2 colonies were much larger (Figure 1B; WT). The larger colonies
grew on plates lacking uracil indicating that they contained the 3' end modifications (not shown).

Uracil prototrophy may confer a growth advantage. Analysis of the germination and growth
potential of spores from 8 azygotic asci from the pikl/pikl

strain (N1565) revealed that the
D709 residue is essential, presumably reflecting a requirement for Pik1 lipid kinase activity at
30°C. In 6 of 8 azygotic asci dissected, only 2 of the 4 spores formed colonies (Figure 1B;
D709A). The cells of these colonies were wild-type for pikl, as confirmed by sequencing and
their growth requirement for uracil. Microscopic examination of the spores that did not form
colonies revealed that the majority had germinated, the cells elongated, but failed to divide (not
shown). In contrast, four colonies were observed from each tetrad in 8 of 10 azygotic asci
dissected from the pik1/pik1<sup>R838A</sup> strain (N1582) (Figure 1B; R838A), indicating that pik1
functions affected by this mutation are not essential. In 7 of 9 azygotic asci dissected from the
pik1/pik1<sup>D709, R838A</sup> strain (N1596), only 2 of the 4 spores formed colonies and these cells were
wild-type for pik1 (Figure 1B; D709A, R838A). Variable phenotypes were observed upon
microscopic examination of the spores that failed to form colonies. Some spores failed to
germinate, another grew into one cell that failed to divide while others grew into cells that
divided a few times (not shown). The functions affected by the R838A mutation are dispensable
for vegetative growth while Pik1 lipid kinase activity appears to be essential (Figure 1B).

We wished to perform complementation studies to determine if the essential functions of
*S. cerevisiae* PIK1, which are lost in pik1-101 cells at the restrictive temperature, can be
provided by expression of the *S. pombe* pik1 gene. However, *S. pombe* cells are highly sensitive
to changes in pik1 expression (Figure 8). If *S. cerevisiae* cells are similarly sensitive to changes
in PIK1 expression, and if *S. pombe* Pik1 is active and capable of providing the essential
functions of Pik1 in *S. cerevisiae*, then improperly regulated expression of pik1 might result in
lethality, thus confounding the experimental approach. Searching for a method to achieve a
range of levels of *S. pombe* pik1 expression in *S. cerevisiae*, we hypothesized that the thiamine-
repressible *S. pombe* nmt1 promoter might function in *S. cerevisiae* and provide such a method.
We therefore placed *S. pombe* pik1 cDNA sequences into the *S. cerevisiae* plasmid, YEplac181
[49], under the control of either the wild-type nmt1 promoter (P<sub>nmt1</sub>), or an attenuated (P<sub>nmt41</sub>) or a
highly attenuated (P<sub>nmt81</sub>) version of the promoter [38]. The nmt1 terminator sequence was
included in these expression cassettes. We performed all of the experiments described below
both in the presence and absence of thiamine. Since the results were indistinguishable only those
experiments in the absence of thiamine are shown (Figure 10). In initial studies with the wild-
type pikl coding sequence, we reproducibly observed a slight reduction in colony formation

efficiency at 37°C in PIK1 cells in which P_{nmt1} controlled S. pombe pikl expression (not shown).

This effect was not observed when the attenuated promoters were used. We observed
reproducible, but partial complementation of the lethal phenotype of pikl-101 at 37°C with P_{nmt1},
reduced partial complementation with P_{nmt41}, and no complementation with P_{nmt81} (not shown).

These results indicate that S. pombe pikl can provide essential functions of Pik1 in an
S. cerevisiae loss-of-function mutant and that S. pombe nmt1 promoter sequences are useful in
S. cerevisiae. Immunoblot analysis, using a polyclonal anti-S. pombe Pik1 serum that detected
this protein in S. pombe cell extracts (Figure 6C), failed to detect it in extracts from transformed
S. cerevisiae pikl-101 cells (not shown).

To study the subcellular distribution of S. pombe Pik1 in S. cerevisiae, we expressed an
eGFP-pikl fusion allele under the control of P_{nmt41} in S. cerevisiae PIK1 and pikl-101 cells.

Unfortunately, the fluorescent signal from the eGFP-S. pombe Pik1 fusion protein was
insufficient for the purpose of determining its subcellular distribution. Immunoblot analysis
again failed to detect this protein in extracts from transformed S. cerevisiae PIK1 cells (not
shown). Colony formation assays revealed that expression of eGFP-pikl in PIK1 cells was
innocuous at both 25°C and 37°C (Figure 10A), as it was in pikl-101 cells at 25°C (Figure 10B).

Remarkably, eGFP-pikl fully complemented the lethality of pikl-101 at 37°C (Figure 10B). We
then introduced the D709A and R838A substitutions into this construct. Expression of eGFP-
pikl^{D709A} in PIK1 cells appeared to impair colony formation at both temperatures assayed as
compared to the control cells that lacked the pikl sequences or the cells expressing the eGFP-
pikl allele (Figure 10A). This allele failed to complement pikl-101 at the restrictive temperature
(Figure 10B). In contrast, eGFP-\textit{pikl}^{R838A} had no apparent deleterious effect in \textit{PIK1} cells (Figure 10A) and it fully complemented \textit{pikl-101} at the restrictive temperature (Figure 10B).

\section*{Discussion}

\textit{Pik1 localizes to the medial region of dividing cells and is required for cell division.}

\textit{S. pombe} cells divide by medial fission. The contractile ring assembles at the onset of mitosis [24, 60]. A multilayered septum forms in concert with constriction of the ring. Cleavage of the primary septum, the inner layer, results in cell separation [61]. The exocyst is a multiprotein complex present in many cells and which appears to be involved in the recruitment of Golgi vesicles to the division site, using positioning cues provided by the contractile ring [45, 62]. In \textit{S. pombe}, the exocyst is involved in the recruitment of enzymes responsible for septum cleavage and is essential for cell separation [63]. \textit{S. pombe} Pik1 is associated with the Golgi [18]. We have shown here that \textit{pikl} is an essential gene, like its \textit{S. cerevisiae} orthologue [12]. Haploid \textit{pikl}::\textit{ura4} cells failed to divide (Figure 1), and \textit{pikl}::\textit{ura4} cells carrying the temperature-sensitive \textit{pikl-td} fusion stopped dividing when shifted to the restrictive temperature (Figure 2). These cells accumulated intracellular membranous and vacuolar materials (Figure 3). Similar structures were observed in the absence of the \textit{S. cerevisiae} \textit{PIK1} functions and were identified as abnormal Golgi structures [7, 13]. Likewise, expression of a kinase-dead allele of a mammalian \textit{pikl} homolog resulted in disrupted Golgi structures in mammalian cells [64]. Thus, some Pik1-dependent Golgi function is likely required for cell division in \textit{S. pombe}. These results also suggest that the essential functions of Pik1 are not redundant to the functions of the other two putative \textit{S. pombe} PtdIns 4-kinases.
S. pombe Pik1 was also found in the medial region of dividing cells at the time of septum formation (Figure 4). The Pik1 medial localization has not been reported previously [18], possibly because of low abundance of Pik1. Alternatively, fusion of GFP to the C-terminus of Pik1 used in that study may have interfered with its localization. Consistent with this, Pik1-GFP as a C-terminal fusion in S. cerevisiae is stable but not functional [14]. Our observation of Pik1 medial localization required the use of a 2XeGFP-Pik1 fusion protein in pik1: :ura4 cells. The fusion protein provided all essential functions, as cell proliferation and morphology were similar to wild-type. The appearance of Pik1 at the medial region corresponded to the time of septum material deposition, and was clearly after the onset of mitosis and formation of the F-actin ring (Figure 4).

S. cerevisiae Pik1 is found in the nucleus in addition to the Golgi, although its biological function in the nucleus is uncertain [14]. A nuclear localization of Pik1 in S. pombe was not observed in this study or in a global localization study [18]. It is possible that a nuclear pool of Pik1 is small and not detected under our experimental conditions. However, our complementation study in which expression of an S. pombe pik1 cDNA provided the essential functions that were lost in an S. cerevisiae pik1-101 strain may suggest that S. pombe Pik1 is capable of providing essential nuclear functions. The pik1-101 mutation is a serine to phenylalanine substitution at residue 1045. This residue is within the kinase domain as identified by Prosite (http://ca.expasy.org/prosite/). The pik1-101 allele appears to be either kinase-dead or greatly reduced for kinase activity [7]. Pik1 lipid kinase acitivity is required in both the Golgi and in the nucleus in S. cerevisiae [14]. Our observation of complete complementation suggests that either the fission yeast protein is reaching the nucleus in the budding yeast or that the pik1-
101 strain possesses sufficient residual kinase activity in the nucleus at the restrictive
temperature.

**Pik1 is required for septation.** Consistent with the timing of its appearance at the
division site (Figure 4), loss of Pik1 function in the pik1-td strain is associated with failure of
processes in late cytokinesis (Figures 2 and 3). Most obvious were the observations of
supernumerary septa, the accumulation of excessive amounts of secondary septum material and
the persistence of the primary septum (Figures 2 and 3). Thus, Pik1 activity may be required to
signal the termination of septum material deposition, to initiate the hydrolysis of the primary
septum or to suppress reinitiation of septation. The observations noted above may result from
loss of Pik1-dependent Golgi functions or from loss of Pik1 activity at the medial region, or both.
The importance of the lipid kinase activity of Pik1 was suggested by the observation that ectopic
expression of the kinase-dead allele pik1<sup>D709A</sup> also resulted increased septation index and failure
of cell separation (Figure 8 and Table 3). Examination of the phenotype of cells carrying the
chromosomally integrated kinase-dead allele revealed that its kinase activity is essential for
vegetative growth (Figure 1). The observation of an elongated cell, terminal phenotype by tetrad
analysis suggested a cytokinesis defect. Ectopic expression of pik1<sup>D709A</sup> resulted in a dominant
lethal phenotype with partial penetrance compared to that produced by ectopic expression of the
wild-type allele (Table 3). While actin structures, including the contractile ring, appeared to be
normal, defects in septation were observed. Thus, Pik1 lipid kinase activity is required for
septation.

Two possible functions of Pik1, its lipid kinase activity and its Cdc4-binding activity,
were examined in this study. We identified Pik1 residues, D709 and R838, that are required for
its lipid kinase activity and for its interaction with Cdc4, respectively. Our initial approach was
to attempt to create a *pik1* kinase-null allele and by ectopic expression of that allele to disrupt the
activity of the endogenous enzyme. As described above, this approach provided evidence for an
essential function of Pik1 lipid kinase activity in cell division. Similarly, we hoped to create an
allele that was impaired for binding to Cdc4 and to use ectopic expression of that allele to
identify functions related to the Pik1-Cdc4 protein interaction. Integration of this allele into the
chromosomal *pik1* locus was then used to assess the cell phenotypes when expressed under the
control of the native promoter.

Control experiments revealed that ectopic expression of wild-type *pik1* in cells that were
otherwise wild-type for *pik1* and *cdc4*, produced a dominant lethal phenotype. The terminal
phenotype revealed disruption of the actin cytoskeleton structure, including very few cells with
contractile rings (Table 3). In stark contrast, ectopic expression of the double mutant allele,
*pik1\textsuperscript{D709A, R838A}* , was apparently innocuous. Thus, the dominant lethal phenotype can be attributed
fully to Pik1 activities that depend on the two substituted residues. The observed lethal
phenotypes associated with ectopic expression of some *pik1* alleles cannot be attributed solely to
the increased levels of lipid kinase activity that we observed in cell lysates. As an example, cells
in which *pik1* or *pik1\textsuperscript{R838A}* were ectopically expressed both accumulated elevated levels of the
ectopic protein (Figure 6) and of lipid kinase activity (Figure 7); yet, the former died while the
latter were viable (Figure 8). Examination of the phenotype of cells carrying the chromosomally
integrated *pik1\textsuperscript{R838A}* substitution revealed that R838 is non-essential for vegetative growth. Cells
expressing only this allele were phenotypically wild-type. Notwithstanding, the R838 residue
appears to be important for Pik1 functions. Ectopic expression of *pik1\textsuperscript{R838A}* was almost
innocuous (Figure 8). Since ectopically expressed Pik1\textsuperscript{R838A} accumulated and was active as a
lipid kinase we can infer that this mutation does not cause folding or stability problems for the
protein. These observations demonstrate that the dominant lethal phenotype associated with
ectopic expression of pikl is largely attributable to a function requiring the R838 residue. One
such function may be an interaction between Pik1 and Cdc4, as suggested in this work based on
yeast two-hybrid and ELISA studies. Also consistent with this hypothesis is the observation that
the lethal phenotype associated with ectopic expression of wild-type pikl was almost completely
suppressed in cdc4G107S cells (Figure 9). We had shown previously that the G107 residue of
Cdc4 is required for the interaction between Cdc4 and the C-terminal 345 amino acids of Pik1
[21]. A peptide spanning the pseudo IQ motif of Pik1 including the R838 residue has also been
shown to bind purified Cdc4 by NMR spectroscopy (Escobar-Cabrera et al., personal
communication). The interaction between Pik1 and Cdc4 may be functionally important;
however, whether it affects the activity or localization of Pik1 is unknown.

Overall, these results suggest that Pik1 is a lipid kinase that is recruited to the medial cell
plane late in cytokinesis and is required for septation. These results also suggest that protein-
protein interactions involving the R838 residue of Pik1, possibly with Cdc4, are functionally
significant, although perhaps not essential.

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