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Characterization of temperature-sensitive alleles of anillin-like Mid1 and polo kinase Plo1 in *Schizosaccharomyces pombe*

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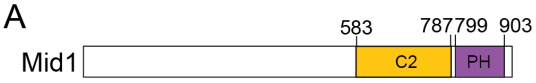
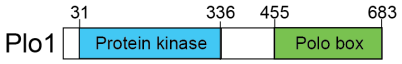
Abstract

The *Schizosaccharomyces pombe* anillin-like Mid1 is important for the correct positioning of the cell division site. A key regulator of Mid1 is the polo kinase Plo1 which is important for several mitotic and cytokinetic events including spindle formation and division site placement. Here, we defined the mutations within a set of temperature-sensitive *mid1* and *plo1* alleles and compared the growth and morphological defects of the strains. This work expands the repertoire of *mid1* and *plo1* mutants for studying cytokinesis and highlights the requirement of the Mid1 C2 domain and the Plo1 kinase domain C-terminal lobe as particularly important for cytokinesis.

A



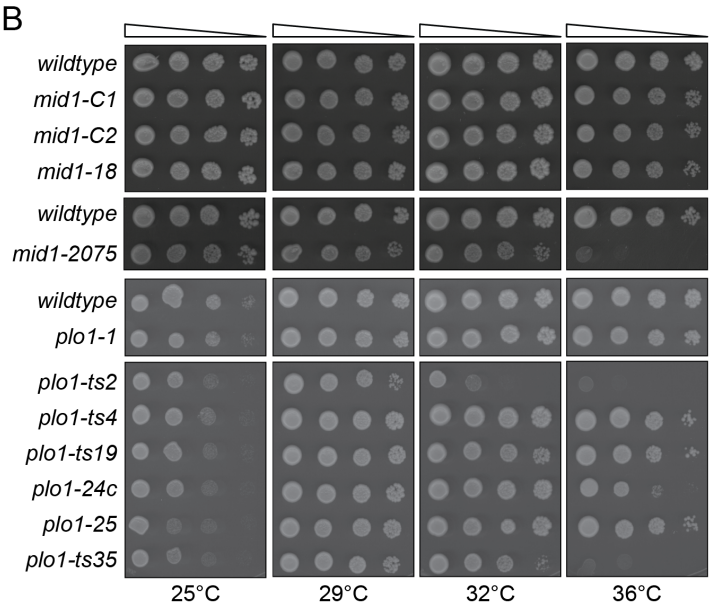
allele	amino acid change	nucleotide change*
mid1-18	S873F	C2618T
mid1-C1, mid1-C2	G764D	G2291A
mid1-2075	G584D	G1751A



allele	amino acid change	nucleotide change*
plo1-1, plo1-ts4	R132Q	G395A
plo1-24c	F295L	T883C
plo1-25	R141W	C421U
plo1-ts35	E139K	G415A

*The positions of nucleotide changes in open reading frames are indicated.

B



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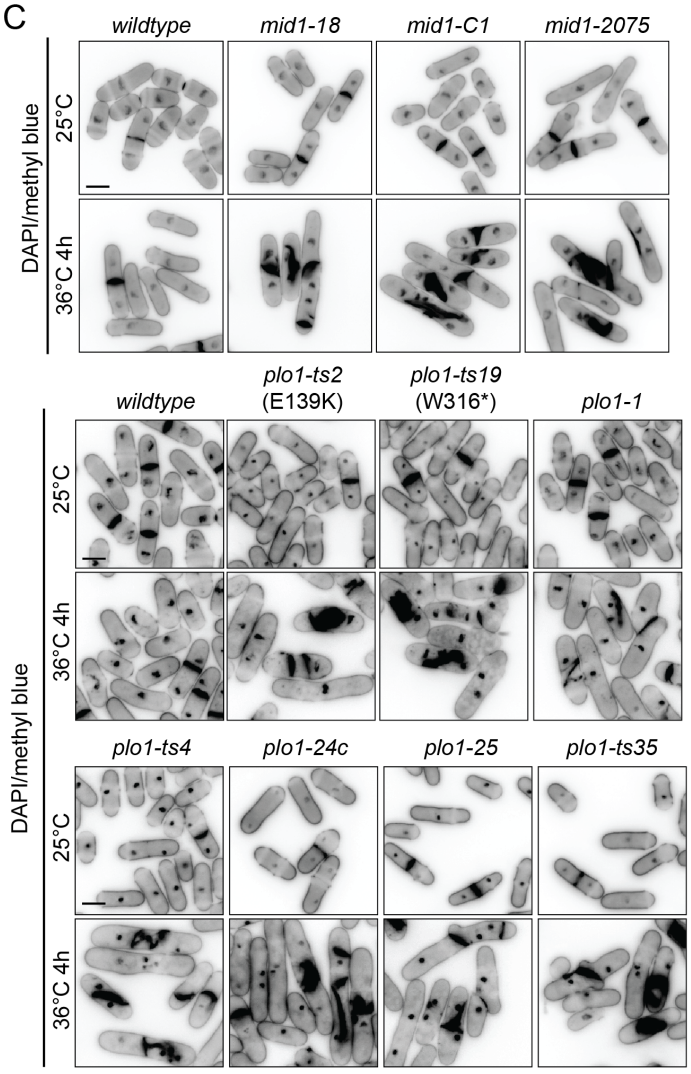
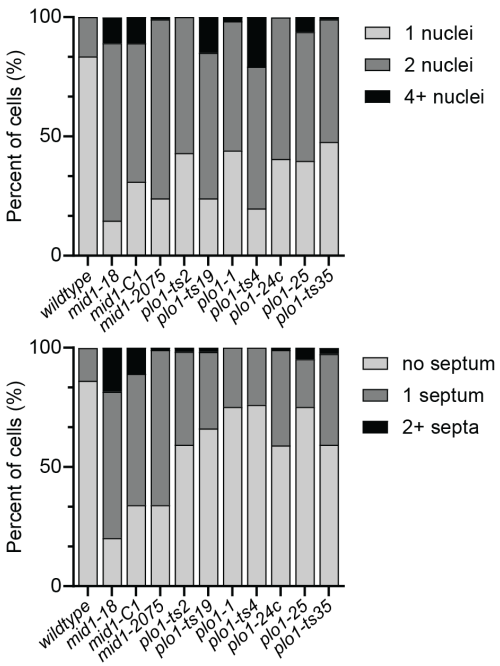


D



E

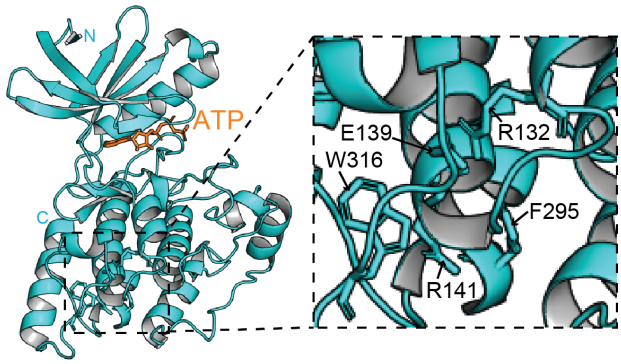


Figure 1. Characterization of *plo1* and *mid1* mutant alleles.:

(A) Schematics of Mid1 and Plo1, drawn to scale. The C2 and pleckstrin homology (PH) domains of Mid1 are indicated in yellow and purple, respectively. The protein kinase catalytic domain and polo box domain of Plo1 are indicated in blue and green, respectively. The amino acid and nucleotide substitutions in the indicated temperature-sensitive alleles are provided in the charts. (B) The indicated strains were grown in liquid YE media at 25°C until they reached mid-log phase and then adjusted to the same cell concentration measured by optical density (Moreno et al., 1991). Next, 10-fold serial dilutions were made and 2.5 µL of each was spotted on YE agar plates and incubated at the indicated temperatures for 2-3 days prior to imaging. (C) The indicated strains were grown in liquid YE media. Samples were collected after cells were grown at 25˚C and again after growing the cells for an additional 4 hours at 36˚C. The cells were then fixed and stained with DAPI and methyl blue. Scale bars, 5 µm. (D) The number of nuclei (top) or septa (bottom) per cell was quantified from the same experiment as in C. n ≥ 100 for each. (E) Ribbon diagram of a structural model of *S. pombe* Plo1 kinase domain bound to an ATP molecule using AlphaFold3 (Abramson et al., 2024). The positions of the N- and C-termini of the kinase domain are labelled. A section of the model containing the kinase domain C-terminal lobe is enlarged below and the positions of the mutated residues are indicated.

Description

The fission yeast *Schizosaccharomyces pombe* utilizes an actin- and myosin-based cytokinetic ring (CR) to accomplish cytokinesis (Cheffings et al., 2016; Glotzer, 2017; Mangione & Gould, 2019). The *S. pombe* CR is built from medial cytokinetic nodes established by the anillin-like Mid1 protein (reviewed in Rincon & Paoletti, 2012). These nodes condense into a coherent ring structure that will eventually constrict concomitant with septum ingression (reviewed in Willet et al., 2015). The precise spatial and temporal ordering of mitotic and cytokinetic events is critical for the faithful segregation of the genetic material into daughter cells. One mechanism by which mitosis and cytokinesis are coordinated is through the action of mitotic kinases that phosphorylate substrates in a cell cycle-dependent manner. One such enzyme is the *S. pombe* polo kinase Plo1 which operates downstream of Cdk1 to promote successful mitosis and cytokinesis (Ohkura et al., 1995; Tanaka et al., 2001). One of Plo1's substrates is Mid1 and Plo1-dependent phosphorylation of Mid1 promotes the nuclear export of Mid1 (Almonacid et al., 2011; Bähler et al., 1998; Ohkura et al., 1995). This ensures that Mid1 can localize to the medial cytokinetic nodes at mitotic entry to promote CR formation (Almonacid et al., 2009, 2011). Here, we examine *mid1* and *plo1* temperature-sensitive alleles obtained from various genetic screens, some previously uncharacterized and many lacking information as to the sites of mutations (Bähler et al., 1998; Balasubramanian et al., 1998; MacIver et al., 2003).

To better understand the nature of the *mid1* and *plo1* alleles, the respective open reading frames were amplified from nine strains (*mid1-18*, *mid1-C1*, *mid1-C2*, *mid1-2075*, *plo1-1*, *plo1-ts4*, *plo1-24c*, *plo1-25* and *plo1-ts35*) in which the relevant mutations have not been reported (Rutherford et al., 2024) and the PCR products sequenced. In each case, a single point mutation was detected (Figure 1A). *mid1-18* encodes a serine to phenylalanine substitution at position 873 (S873F), both *mid1-C1* and *mid1-C2* encode a glycine to aspartic acid substitution at position 764 (G764D) and *mid1-2075* encodes a glycine to aspartic acid substitution at position 584 (Figure 1A). All of the mutated amino acids reside in structured regions of the protein (Figure 1A). Another previously characterized *mid1* allele, *mid1-366*, encodes a different mutation within the C2 domain (G718D) (Chang et al., 1996; Sun et al., 2015). Thus, our results bring the total number of distinct *mid1* temperature-sensitive alleles to four.

Two *plo1* alleles (*plo1-1* and *plo1-ts4)* encode the same mutation causing an arginine to glutamine change at residue 132 (R132Q). *plo1-24c* encodes a mutation resulting in a change at residue 295 from phenylalanine to leucine, *plo1-25* encodes an arginine to tryptophan substitution at residue 141 (R141W) and *plo1-ts35* encodes a glutamic acid to lysine change at position 139 (E139K) (Figure 1A). E139K is the same mutation previously identified in the *plo1-ts2* allele (MacIver et al., 2003). Thus, in addition to two previously sequenced temperature-sensitive *plo1* alleles (*plo1-ts2* and *plo1-ts19*), there are three additional distinct temperature-sensitive *plo1* alleles.

To examine the *mid1* and *plo1* mutant cohorts further, we compared the temperature-sensitivity of each strain with a growth assay. *mid1-18, mid1-C1 and mid1-C2* grew similar to wildtype at all temperatures but *mid1-2075* showed almost no growth at 36°C (Figure 1B). Two *plo1* alleles, *plo1-ts2* and *plo1-ts35*, had almost no growth at 36°C and reduced growth at 32°C, and all other *plo1* alleles were slightly impaired in growth compared to wildtype at 36°C (Figure 1B). These results are consistent with previously published growth assays in which some of the mutants were examined (MacIver et al., 2003; Petersen & Hagan, 2005; Rachfall et al., 2014).

Finally, to visualize and compare the cell phenotypes, we examined each mutant by staining for nuclei and septa after the cells were grown at 25°C and then shifted to 36°C for 4 hours. We found that all temperature sensitive alleles looked similar to wildtype at 25°C, but at 36°C the cells were multinucleated with abnormal septa present (Figure 1C-D). Our data are

consistent with previous descriptions of the subset of previously studied alleles (Bähler et al., 1998; Balasubramanian et al., 1998; Bhutta et al., 2014; Huang et al., 2008; MacIver et al., 2003; Wachtler et al., 2006).

All *mid1* temperature-sensitive alleles displayed phenotypes similar to *mid1*Δ (Chang et al., 1996; Sohrmann et al., 1996), suggesting that they are loss-of-function alleles. *mid1-18* has a mutation in the PH domain while *mid1-C1*, *mid1-C2*, *mid1-2075* and *mid1-366* contain mutations in the C2 domain (Figure 1A). The C2 domain appears to directly bind the plasma membrane and is required for Mid1 function (Lee & Wu, 2012; Sun et al., 2015). Cells with a mutant Mid1 lacking the entire PH domain have only mild cell division site positioning defects, thus the function of the PH domain is not fully clear (Lee & Wu, 2012; Paoletti & Chang, 2000). It has been suggested that the PH domain may be important for protein stability and/or play a role in membrane binding in collaboration with the C2 domain, like human anillin (Hall et al., 2024; Liu et al., 2012; Sun et al., 2015). Taken together, we speculate that the *mid1-18* allele produces a less stable protein at high temperatures while the other alleles disrupt C2 domain function.

Plo1 has two essential domains, the N-terminal kinase domain and the C-terminal polo box domain (PBD) (Reynolds & Ohkura, 2003) (Figure 1A). While the kinase domain phosphorylates protein substrates, the PBD binds both substrates and protein partners to direct subcellular localization (Park et al., 2010). Interestingly, all mutations encoded by the *plo1* temperature sensitive alleles map within the kinase domain and none are within the PBD (Figure 1A). The one unique allele previously sequenced is *plo1-ts19* which contains an early stop codon at tryptophan 316 (MacIver et al., 2003). The W316* mutation truncates an α-helix within the kinase domain and eliminates an unstructured region and the PBD (MacIver et al., 2003) which is a surprising result given the reported requirement for the PBD for Plo1 function (Reynolds & Ohkura, 2003).

To follow up these observations, we used AlphaFold3 to model the Plo1 kinase domain with an ATP molecule (Abramson et al., 2024), and mapped the mutated residues encoded by the *plo1* alleles onto the predicted structure. We found that all of the mutated residues clustered in the C-terminal lobe of the kinase domain (Figure 1E). Deciphering if ATP binding, substrate binding or other protein partner binding is defective in these *plo1* alleles will be exciting direction for future studies. Further, how cells tolerate a lack of the Plo1 PBD will be an interesting avenue of study.

Methods

Yeast methods

S. pombe strains were grown in yeast extract (YE) and standard *S. pombe* mating, sporulation, and tetrad dissection techniques were used to construct new strains (Moreno et al., 1991).

Molecular biology methods

The *mid1* open reading frames from *mid1-C1* and *mid1-C18* cells were amplified by generating a PCR product with an oligonucleotide 74 bp upstream of the start site (GTTGTACTTCAGGGTGCTTA) and an oligonucleotide 380 bp downstream of the stop codon (AGGTTCTCCATCTCATGGCT) (Integrated DNA technologies). The *mid1* open reading frame was amplified from *mid1-C2* cells using two sets of overlapping oligonucleotides. The first PCR product was generated with the above-mentioned oligonucleotide 74 bp upstream of the start site and an oligonucleotide 1350 bp into the open reading frame (GTTGCATTGATGGGTGACGT). The second PCR product was produced with an oligonucleotide 1200 bp within the open reading frame (GTATGGTCATGGATCTGTAACG) and the above-mentioned oligonucleotide 380 bp downstream of the stop codon.

The *plo1* alleles were amplified by generating two PCR products. One PCR reaction used an oligonucleotide 40 bp upstream of the start site (GCAACCACTTTGTTTACCCTCA) and an oligonucleotide 1100 bp into the open reading frame of *plo1* (TGGACTTAAAACACTTGGTAATATTCG) (Integrated DNA technologies). The second PCR reaction used an oligonucleotide 900 bp into the open reading frame (TCCAGATGAAATTTTACATTCAATGCCT) with an oligonucleotide 20 bp downstream of the stop codon (GCATAGTAACTTAACGCCCAAGTA).

The PCR products were sequenced by Plasmidsaurus using Oxford Nanopore Technology with custom analysis and annotation.

Microscopy and image analysis

Strains for fixed-cell imaging experiments were grown at 25°C in YE and then shifted to 36°C for 3 hours. Cells were fixed with 70% ethanol for DAPI and methyl blue (MB) staining as described previously (Roberts-Galbraith et al., 2009). Images were acquired using a Zeiss Axio Observer inverted epifluorescence microscope with Zeiss 63× oil (1.46 NA) objective and captured using Zeiss ZEN 3.0 (Blue edition) software. A singular medial Z slice was obtained. All images were further processed using ImageJ (Schindelin et al., 2012).

AlphaFold3 structural prediction

Protein structure predictions were generated with the AlphaFold3 server (Abramson et al., 2024) and visualized using the PyMOL molecular graphics system (version 3.0, Schrodinger, LLC).

Reagents

The strains used in this study and their genotypes are listed below.

Strain	**Genotype**	**Source**
KGY246	*ade6-M210 leu1-32 ura4-D18 h*$^{-}$	Lab stock
KGY1001	*plo1-1 ura4-D18 leu1-32 ade6-M21X h*$^{+}$	*Bahler et al., 1998*
KGY846	*plo1-ts4:ura4*$^{+}$ *ura4-D18 leu1-32 ade6-M210 h*$^{+}$	*MacIver et al., 2003*
KGY1460	*plo1-24C leu1-32 ura4-D18 ade6-M216 his3-D1 h*$^{+}$	*Bahler et al., 1998*
KGY16150-2	*plo1-ts35:ura4*$^{+}$ *ura4-D18 leu1-32 ade6-M210 h-*	*Anderson et al., 2002*
KGY15085	*plo1-25 ade6-M21X leu1-32 ura4-D18 h*$^{+}$	*Bahler et al., 1998*
KGY16148-2	*plo1-ts2:ura4*$^{+}$ *ura4-D18 leu1-32 ade6-M210 h*$^{-}$	*MacIver et al., 2003*
KGY16149-2	*plo1-ts19:ura4*$^{+}$ *ura4-D18 leu1-32 ade6-M210 h*$^{-}$	*MacIver et al., 2003*
KGY1058	*mid1-C1 ura1 leu1-32 mam2::LEU2 ade6-216 h*^{90}	Balasubramanian et al., 1998
KGY1059	*mid1-C2 ura1 leu1-32 mam2::LEU2 ade6-216*	Balasubramanian et al., 1998
KGY19270	*mid1-18 ura4-D18 leu1-32 ade6-21X h*$^{-}$	Balasubramanian et al., 1998
KGY442-2	*mid1-C1 ura4-D18 h*^{90}	This study
KGY436-2	*mid1-C2 ura4-D18 h*$^{-}$	This study
KGY3717	*mid1-2075 ura4-D18 ade6-21X h*$^{-}$	This study

Acknowledgements: We are grateful to PomBase for compiling and making freely available a wealth of information on *S. pombe* proteins. Protein structure predictions were generated with the AlphaFold3 server.

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