Characterization of Mid1 domains for targeting and scaffolding in fission yeast cytokinesis

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Summary

Division-site selection and contractile-ring assembly are two crucial steps in cytokinesis. In fission yeast, the anillin-like Mid1 specifies the division site at the cell equator by assembling cortical nodes, the precursors of the contractile ring. Thus, Mid1 is essential for linking the positional cues for the cleavage site to contractile-ring formation. However, how Mid1 domains cooperate to regulate cytokinesis is poorly understood. Here we unravel the functions of different Mid1 domains/motifs by a series of truncations. The conserved PH domain stabilizes Mid1 in nodes by binding to lipids and is required for Mid1 cortical localization during interphase in the absence of Cdr2 kinase. Mid1 lacking an internal region that is ~1/3 of the full-length protein has higher nuclear and cortical concentration and suppresses the division-site positioning defects in cells with a deletion of the DYRK kinase Pom1. The N-terminus of Mid1 physically interacts with cytokinesis node proteins. When fused to cortical node protein Cdr2, Mid1(1-100) is sufficient to assemble cytokinesis nodes and the contractile ring. Collectively, our study recognizes domains regulating Mid1 cortical localization and reveals domains sufficient for contractile-ring assembly.
Introduction

At the final stage of the cell-division cycle, one mother cell is partitioned into two daughter cells by cytokinesis. The mechanism of cytokinesis, utilizing an actomyosin contractile ring, is conserved in amoebas, fungi, and animals (Balasubramanian et al., 2004; Barr and Gruneberg, 2007). The contractile ring is scaffolded and/or stabilized by a multidomain protein anillin. Identified as an actin-binding and bundling protein in Drosophila (Field and Alberts, 1995; Miller et al., 1989), anillins are at the hub of the organization and constriction of the cleavage furrow (Hickson and O'Farrell, 2008; Maddox et al., 2005; Oegema et al., 2000; Piekny and Maddox, 2010). In animal cells, anillins localize to the nucleus in interphase (Field and Alberts, 1995; Oegema et al., 2000; Straight et al., 2005). During mitosis, anillins interact with GTPase RhoA (Piekny and Glotzer, 2008) and RacGAP50C (D'Avino et al., 2008; Gregory et al., 2008) and are recruited to the cleavage furrow, where they organize the cytokinetic machinery by interacting with actin filaments, formins, myosin-IIs, septins, and other proteins (D'Avino et al., 2008; Field and Alberts, 1995; Goldbach et al., 2010; Gregory et al., 2008; Haglund et al., 2010; Kinoshita et al., 2002; Maddox et al., 2005; Oegema et al., 2000; Piekny and Maddox, 2010; Silverman-Gavrila et al., 2008; Straight et al., 2005; Watanabe et al., 2010). The domains interacting with actin filaments (Field and Alberts, 1995; Kinoshita et al., 2002; Oegema et al., 2000), myosin-IIs (Straight et al., 2005), and the formin mDia2 (Watanabe et al., 2010) reside in the N-termini of anillins, while the C-terminal PH domain interacts with and recruits septins (Kinoshita et al., 2002; Oegema et al., 2000; Silverman-Gavrila et al., 2008).

The fission yeast Schizosaccharomyces pombe is an excellent model organism to study division-site selection and contractile-ring assembly (Bathe and Chang, 2010; Goyal et al., 2011; Laporte et al., 2010; Pollard and Wu, 2010). While human and Drosophila have a single anillin gene with different splicing isoforms, two anillin-related genes, mid1/dmf1 and mid2, encode proteins with non-overlapping functions in S. pombe cytokinesis. Mid2 interacts with and regulates septins starting from late anaphase B, while Mid1 mainly functions at early mitosis (Berlin et al., 2003; Paoletti and Chang, 2000; Sohrmann et al., 1996; Tasto et al., 2003). The importance of Mid1 in division-site specification and contractile-ring assembly is well established (Almonacid et al., 2009; Almonacid et al., 2011; Bähler et al., 1998a; Celton-Morizur et al., 2004; Paoletti and Chang, 2000; Sohrmann et al., 1996). Deletion of mid1 abolishes cytokinesis nodes and results in randomly-positioned contractile rings and septa (Sohrmann et
In interphase, Mid1 localizes to both the nucleus and cortical nodes that are organized by Cdr2 kinase and contain several other proteins including Cdr1 and Wee1 kinases, Bt1, kinesin Klp8, and a putative Rho guanine exchange factor (GEF) Gef2 (Almonacid et al., 2009; Moseley et al., 2009; Paoletti and Chang, 2000). At the G2/M transition, the Polo kinase Plo1 phosphorylates Mid1 and triggers its further release from the nucleus to cortical nodes at the cell equator (Almonacid et al., 2011; Bähler et al., 1998a). Mid1 then recruits other proteins to assemble the cytokinesis nodes and contractile ring (Almonacid et al., 2011; Laporte et al., 2011; Padmanabhan et al., 2011).

Significant efforts have been made to identify functional domains/motifs of Mid1. Two nuclear localization sequences (NLS) and two nuclear export sequences (NES) regulate nuclear shuttling of Mid1, and an amphipathic helix and the adjacent NLS mediate lipid interaction and Mid1 localization on the plasma membrane (Celton-Morizur et al., 2004; Paoletti and Chang, 2000). However, functions of large portions of Mid1 including the conserved Pleckstrin Homology (PH) domain have never been uncovered. Two partially overlapping regions of Mid1 are known to interact with the kinase Cdr2 and Cdc14 family phosphatase Clp1 (Almonacid et al., 2009; Clifford et al., 2008). Although Mid1 is essential for the assembly of cytokinesis nodes (Laporte et al., 2011; Padmanabhan et al., 2011; Pollard and Wu, 2010), which Mid1 domains interact with other cytokinesis node proteins were largely unknown.

Here we systematically investigate functions of different Mid1 domains. We recognize domains involved in localizing Mid1 and scaffolding cytokinesis-node assembly. The PH domain and the internal region, amino acids (aa) 101-400, regulate Mid1 localization. The PH domain of Mid1 directly interacts with lipids. The N-terminal 100 aa is sufficient to assemble cytokinesis nodes and the contractile ring with the help of a localizing protein. Taken together, our analyses provide a thorough understanding of cytokinesis regulation by Mid1 in fission yeast.
Results

Domain analyses reveal important Mid1 domains for division-site specification

Mid1 contains multiple domains/motifs (Fig. 1A). We investigated functions of different domains in Mid1 with high resolution by constructing a series of strains expressing truncated mid1 at the native mid1 locus and under the control of mid1 promoter (Fig. 1B, left). The expressions of these constructs were verified and quantified by western blotting and localized fluorescent signals (Table 1; supplementary material Fig. S1). To assess whether these truncations are sufficient for division-site specification, we quantified the percentage of centered (Fig. 1B, middle) and orthogonal septa (Fig. 1B, right) in septating cells (Table 1).

Consistent with previous reports (Paoletti and Chang, 2000; Sohrmann et al., 1996), <20% septa were centered in mid1Δ while all were centered and orthogonal in wild type (wt) cells (Fig. 1B,C). Truncating the conserved PH domain [mid1(1-800)] did not affect the septum position (Paoletti and Chang, 2000), but we did find ~10% septating cells with tilted septa (P = 0.02 compared to wt; Fig. 1B,C). No obvious defects in septum positioning were observed in mid1(1-580), although the percentage of tilted septa increased to 30% (Fig. 1B,C). However, further truncations significantly compromised Mid1 function in cytokinesis, as 60-80% septa formed in mid1(1-420), mid1(1-200), and mid1(1-100) cells were misplaced and/or tilted (Fig. 1B,C; Table 1).

By contrast, all N-terminal truncations we generated except Mid1(41-920) displayed severe defects in septum positioning and angle (Fig. 1B,D; Table 1). Cells with centered and orthogonal septa decreased to 40–50% when aa(1-100) was truncated. Thus, aa(41-100) plays an important role in division-site specification.

mid1(581-920) had similar phenotypes as mid1(101-920) cells (P = 0.48 and 0.70 for septum position and angle, respectively; Fig. 1B,D; Table 1). Because the severe defects observed in mid1(101-920) might mask the functions of other domains in the N-terminal half, we truncated the internal region aa(101-400) to reveal its role. Neither cell morphology nor septum position of mid1[(1-100)-(401-920)] was distinguishable from wt (Fig. 1B,E), suggesting that aa(101-400) is not essential for division-site selection. Even when the internal truncation was combined with C-terminal truncations, most septa were centered (Fig. 1B,E), although the percentage cells with off-centered or tilted septa were higher than the corresponding C-terminal truncations alone (Table 1).
Collectively, our results confirm that the N-terminal half of Mid1 is sufficient for division-site specification (Celton-Morizur et al., 2004; Paoletti and Chang, 2000). We find that aa(1-100), especially aa(41-100), are essential for Mid1 functions. Although the PH domain and the internal region aa(101-400) do not appear to be essential for cytokinesis, it is possible that their functions are masked by redundant pathways. The behavior of truncated Mid1 constructs and how these truncations affect contractile-ring assembly are further analyzed below.

**The PH domain and the internal region aa(101-400) regulate Mid1 localization and dynamics**

We tagged full length (FL) Mid1 and the truncations at their C-termini with monomeric YFP (mYFP) or monomeric enhanced Citrine (mECitrine; a more photo- and thermal-stable variant of mYFP; Griesbeck et al., 2001) at the *mid1* locus and under the control of *mid1* promoter (Table 1). These truncations were expressed at a level comparable to the endogenous Mid1(FL) (Fig. 1C; Table 1; supplementary figure Fig. S1A). Mid1(1-800), Mid1[(1-100)-(401-920)], and Mid1[(1-100)-(401-800)] all localized to the nucleus, cortical nodes, and the contractile ring (Fig. 2A). However, their fluorescence intensities at each location were not identical to FL Mid1. To directly compare the global and local protein levels, cells expressing each mECitrine-tagged Mid1 truncation were compared with cells expressing *mid1*-mECitrine *sad1*-mCFP in the same image (Fig. 2B,C). While the total and nuclear fluorescence intensities were not affected, Mid1(1-800) intensity in nodes was 20% lower than FL Mid1. In contrast, in *mid1*[1-(1-100)-(401-920)] the nuclear intensity doubled, and the signal in nodes was 37% higher than FL Mid1, but the cytoplasmic intensity decreased (supplementary material Fig. S2A), resulting in only 28% increase of the total cellular fluorescence intensity. Mid1[(1-100)-(401-800)] retained the strong nuclear signal as Mid1[(1-100)-(401-920)], but had a 14% decreased cortical node signal similar to Mid1(1-800), indicating that the increase of cortical signal in *mid1*[1-(1-100)-(401-920)] depends on the PH domain.

The changed intensities in cortical nodes suggest that the PH domain and the internal region aa(101-400) regulate Mid1 localization. We used Fluorescence Recovery After Photobleaching (FRAP) assays to investigate Mid1 dynamics (Fig. 2D). While the recovery halftime in interphase nodes for FL Mid1 was 3.0 ± 1.3 min (Fig. 2E, gray) (Laporte et al., 2011), Mid1(1-800) was more dynamic (τ_{1/2} = 1.6 ± 0.6 min; Fig. 2E, left), suggesting that the
PH domain stabilizes Mid1 in nodes. Mid1(1-800) was also more dynamic in cytokinesis nodes (supplementary material Fig. S2B). In contrast, the recovery curve of Mid1[(1-100)-(401-920)] was superimposable to FL Mid1, although the recovery halftime was slightly different (τ_{1/2} = 3.8 ± 1.4 min; Fig. 2E, middle). Mid1[(1-100)-(401-800)] has a halftime of 1.7 ± 1.1 min (Fig. 2E, right), similar to that of Mid1(1-800) (Fig. 2F). Together, these data suggest that the PH domain stabilizes cortical Mid1.

Nodes are precursors of the contractile ring (Bähler et al., 1998a; Coffman et al., 2009; Wu et al., 2003; Wu et al., 2006). We investigated whether the changes in cortical abundance and node dynamics affect contractile-ring assembly. However, in cells expressing mid1(1-800), mid1[(1-100)-(401-920)], or mid1[(1-100)-(401-800)], cytokinesis nodes condensed into a compact ring with normal kinetics (Fig. 2G,H). We conclude that neither the PH domain nor the internal region of Mid1 is essential for contractile-ring assembly from cytokinesis nodes under normal growth conditions.

Cdr2-independent cortical localization of Mid1 is coordinated by the PH domain and the internal region

Because Cdr2 kinase is the major organizer of interphase nodes and it interacts with Mid1, we investigated whether the increase in cortical signals of Mid1[(1-100)-(401-920)] depends on Cdr2. Like the FL protein, Mid1[(1-100)-(401-920)] co-immunoprecipitated with Cdr2 (supplementary material Fig. S3A). However, the weaker band detected did not suggest a stronger interaction. In addition, the level of Cdr2 tagged with monomeric enhanced GFP (mEGFP) in interphase nodes was not obviously altered in mid1[(1-100)-(401-920)] (supplementary material Fig. S3B). Thus, we hypothesized that the increase in cortical signals is independent of Cdr2.

To test this hypothesis, we examined the localizations of FL Mid1, Mid1(1-800), Mid1[(1-100)-(401-920)], and Mid1[(1-100)-(401-800)] in cdr2Δ cells (Fig. 3A,B). Most septa were centered and orthogonal in these double mutants (supplementary material Fig. S4A). Only 28% cdr2Δ cells expressing FL mid1 had a few node-like structures during interphase, consistent with previous studies (Almonacid et al., 2009; Moseley et al., 2009). To our surprise, Mid1[(1-100)-(401-920)] was found in a broad band on the cortex in 75% interphase cdr2Δ cells. Like the FL protein in cdr2+ cells (Almonacid et al., 2009; Celton-Morizur et al., 2006; Daga and Chang,
the cortical distribution of Mid1[(1-100)-(401-920)] in cdr2Δ followed the misplaced nucleus when MBC-treated cells were centrifuged (Fig. 3C).

Interestingly, the cortical localizations of Mid1 and Mid1[(1-100)-(401-920)] in cdr2Δ during interphase were abolished when the PH domain was deleted (Fig. 3A,B), suggesting that the PH domain does play a role in Mid1 localization to the plasma membrane.

The PH domain of Mid1 directly interacts with lipids
While a previous study concludes that the PH domain is not required for Mid1 localization and function (Paoletti and Chang, 2000), our results indicate that the PH domain regulates Mid1 localization. The PH domain alone was not sufficient for cortical localization (Table 1). A polybasic region aa(681-710) in Mid1 C-terminus (containing an amphipathic helix and an NLS) anchors Mid1 to the cortex (Celton-Morizur et al., 2004). More mid1(1-800, Helix*) cells exhibited division-site specification defects than mid1(Helix*) (supplementary material Fig. S4B,C), suggesting that the amphipathic helix and PH domain of Mid1 have overlapping functions in Mid1 cortical binding.

Many PH domains bind to lipids directly. We purified bacteria-expressed 6His-Mid1(PH) (Fig. 3D) and tested its binding with lipids spotted on the nitrocellulose membrane. Compared to the positive 6His-Ksg1(PH) (Mitra et al., 2004) and negative 6His-mEGFP controls, we found that 6His-Mid1(PH) interacts with several lipids (Fig. 3E). Taken together, our results indicate that Mid1 PH domain plays a role in anchoring Mid1 to the plasma membrane.

Mid1[(1-100)-(401-920)] suppresses cytokinesis defects of pom1Δ cells
When the DYRK kinase pom1 is deleted, Mid1 cortical localization uncouples from the nuclear location, as Mid1 binds to Cdr2 that shifts toward the non-growing tip (Almonacid et al., 2009; Celton-Morizur et al., 2006; Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009). In addition, Mid1 localizes to the nucleus less efficiently and misplaced septa are observed at high frequency (Bähler and Pringle, 1998; Celton-Morizur et al., 2006). Interestingly, the internal truncation mid1[(1-100)-(401-920)] alleviated the pom1Δ defects in division-site specification (Fig. 4A-C). When grown on YE5S + Phloxin B (which accumulates in dead cells) plate, mid1[(1-100)-(401-920)] pom1Δ cells exhibited a lighter color compared to pom1Δ cells expressing FL mid1 (Fig. 4A). Consistently, ~90% centered septa was found in the former
compared to ~50% in pom1Δ (P = 6.5 × 10⁻⁵), indicating that the defects in septum positioning were suppressed when the internal region aa(101-400) of Mid1 was deleted (Fig. 4B,C).

Next we explored molecular mechanisms of the suppression (Fig. 4 and supplementary material Fig. S5). A strain expressing two copies of FL mid1 both under the mid1 promoter did not suppress pom1Δ (P = 0.19; Fig. 4A,C), therefore the suppression was not due to the increase in global Mid1[(1-100)-(401-920)] protein level (Fig. 2C and Table 1). Increased Mid1[(1-100)-(401-920)] in nucleus may make nuclear export to the equator dominant over Cdr2-dependent Mid1 mislocalization. This hypothesis was tested using mid1[(1-100)-(401-800)], a mutant with stronger nuclear but not cortical localization during interphase (Fig. 2C). Indeed, we observed ~20% less cells with off-centered septa in mid1[(1-100)-(401-800)] pom1Δ than in pom1Δ (P = 8.4 × 10⁻⁴; Fig. 4A,C), thus the increased nuclear concentration is partially responsible for the suppression in mid1[(1-100)-(401-920)] pom1Δ. In addition, Mid1[(1-100)-(401-920)] could bypass the mislocalized Cdr2 in pom1Δ cells via a weaker interaction with Cdr2. Indeed, although some cdr2Δ pom1Δ cells have morphological defects (data not shown), the rod-shaped cells exhibited milder defects in division-site specification (P = 0.015). Furthermore, Gef2 is a putative Rho GEF that interacts with Mid1(300-350) (Y. Ye, I-J. Lee, K.W. Runge, and J.-Q. Wu, unpublished data) and also shifts toward one cell end in pom1Δ (Fig. 4D). Consistently, septum positioning defects in pom1Δ were partially suppressed by gef2Δ (P = 0.002) or mid1(Δ300-350) (P = 0.002), and rod-shaped gef2Δ cdr2Δ pom1Δ resembled mid1[(1-100)-(401-920)] pom1Δ in septum positioning (Fig. 4C). Together, we propose that several factors lead to the observed suppression (see Discussion) and our results underlined the complexity of the regulation of division-site specification.

Consistent with the suppression, Mid1[(1-100)-(401-920)] spread along the cell side on the cortex in pom1Δ cells instead of localizing to the non-growing half like FL Mid1 (Fig. 4E,F). The band of nodes was broader than that in pom1Δ cells (Fig. 4F). This cortical Mid1 was capable of mediating contractile-ring assembly at or near the cell equator (Fig. 4E,G; supplementary material Movies 1 and 2).

**Major physical interactions with node proteins reside in Mid1(1-580)**

The morphology of mid1(1-580) is very similar to that of wt cells (Fig. 1C). Mid1(1-580)-mECitrine localized to the nucleus, cortical nodes, and the contractile ring even during its
constriction (supplementary material Fig. S6A and Movie 4). In mid1(1-580), both Mid1(1-580)-mEYFP and Rlc1-tandem Tomato (tdTomato) appeared in fewer nodes (and/or with lower intensity) and condensed into a contractile ring much slower (Fig. 5A-D; supplementary material Movies 3 and 4).

Although contractile-ring assembly was slower in mid1(1-580), the ring still initiated from cytokinesis nodes. Thus, we hypothesized that the major interactions required for assembling cytokinesis nodes reside in Mid1(1-580). Mid1 physically interacts with four cytokinesis node proteins: myosin-II essential light chain Cdc4, IQGAP Rng2, myosin-II heavy chain Myo2, and F-BAR protein Cdc15 (Almonacid et al., 2011; Laporte et al., 2011; Motegi et al., 2004; Padmanabhan et al., 2011). We therefore tested whether Mid1(1-580)-Myc interacts with these proteins. Mid1(1-580)-Myc was detected in co-immunoprecipitation (IP) with mYFP-Rng2, mYFP-Cdc4 and mYFP-Cdc15 (Fig. 5E,F), suggesting that they interact in vivo.

Consistent with its localization to interphase nodes, Mid1(1-580) also co-immunoprecipitated with Cdr2-mEYFP (Fig. 5A,F).

Mid1 phosphorylation is cell-cycle regulated and a hyperphosphorylated form is detected during cytokinesis (Almonacid et al., 2011; Bähler et al., 1998a; Sohrmann et al., 1996). When beads after co-IP were treated with λ phosphatase, FL Mid1 underwent a mobility shift on SDS-PAGE, indicating that the hyperphosphorylated Mid1 interacts with cytokinesis node proteins Cdc4, Rng2, Cdc15, and Myo2 (Fig. 5G). The slow-migrating forms of Mid1(1-580) co-immunoprecipitated with Rng2 (Fig. 5E), suggesting Mid1(1-580) can still be phosphorylated.

Nodes and contractile-ring assembly are rescued by fusing Mid1 N-terminal domains to Cdr2 kinase

Because Mid1(1-580) co-immunoprecipitated with cytokinesis node proteins (Fig. 5E,F) and Mid1(1-100) but not the internal region aa(101-400) is critical for division-site specification (Figs 1 and 2; supplementary material Fig. S6B-D) (Almonacid et al., 2011), we hypothesized that Mid1[(1-100)-(401-580)] or even Mid1(1-100) is sufficient for cytokinesis-node assembly. However, this hypothesis was difficult to test because neither Mid1(1-100) nor Mid1[(1-100)-(401-580)] localized to nodes, and the contractile ring assembled from linear structures at random locations in these cells (Fig. 6A,B). Thus, we tested our hypothesis in the presence of a
localization signal by fusing Cdr2 to the C-terminus of Mid1[(1-100)-(401-580)] or Mid1(1-100) (Fig. 6C). As expected, the fusion proteins localized to cortical nodes (Fig. 6F,G).

To validate that the excess Cdr2 could not restore cytokinesis-node assembly, we constructed a strain expressing cdr2 at the mid1 locus under the control of mid1 promoter besides the native cdr2, while the mid1 ORF was deleted (midΔ::Pmid1-cdr2). In this strain, no Rlc1 nodes were detected (Fig. 6D) and the phenotype was similar to midΔ (Fig. 6E). By contrast, when Cdr2 was fused to Mid1[(1-100)-(401-580)], 99% of septa were centered and 90% orthogonal (Fig. 6E). Furthermore, while mid1(1-100) cells exhibited only 21% centered and 33% orthogonal septa (Figs 1B and 6B; Table 1), Mid1(1-100)-Cdr2 rescued the defects (100% centered and 90% orthogonal septa, Fig. 6E).

To test whether Mid1[(1-100)-(401-580)]-Cdr2 and Mid1(1-100)-Cdr2 are functionally equivalent to FL Mid1 in cytokinesis-node and contractile-ring assembly, localizations of Rlc1 and Cdc15, proteins representing two modules in cytokinesis-node assembly (Laporte et al., 2011), were investigated. Both Mid1[(1-100)-(401-580)]-Cdr2 and Mid1(1-100)-Cdr2 co-localized with Rlc1 in cortical nodes and the contractile ring before the departure of Mid1[(1-100)-(401-580)]-Cdr2 from the contractile ring (Fig. 6F). All the contractile rings were assembled from nodes in these strains (Fig. 6H, n = 9 and 15 cells, respectively; supplementary material Movie 6). The kinetics of contractile-ring assembly (31.8 ± 5.3 and 35.9 ± 6.8 min from Rlc1 node appearance to a compact ring, respectively) was significantly faster than that of midΔ::Pmid1-cdr2, mid1[(1-100)-(401-580)], and mid1(1-100) cells (Fig. 6H; P < 0.05 for each pairwise comparison; supplementary material Movies 5 and 6) although still slower than in FL Mid1 cells (Fig. 2H). Interestingly, while Cdc15 co-localized with Mid1[(1-100)-(401-580)]-Cdr2 in a band of nodes and the contractile ring, it only appeared at the division as a contractile ring in mid1(1-100)-cdr2 (Fig. 6G). Latrunculin-A treatment confirmed the difference in Cdc15 recruitment, as Cdc15 was detected in a broad band of nodes in mid1[(1-100)-(401-580)]-cdr2 cells but absent from nodes in mid1(1-100)-cdr2 cells (Fig. 6I).

Collectively, our results suggest that both Mid1[(1-100)-(401-580)] and Mid1(1-100) are sufficient for contractile-ring assembly by recruiting downstream proteins when they are localized to cortical nodes.
Discussion

In this study, we discovered that the PH domain and the internal region aa(101-400) regulate the localization of Mid1 on the plasma membrane. In addition, Mid1(1-580) interacts with cytokinesis node proteins and Mid1 (1-100) is sufficient for directing the assembly of a well-positioned contractile ring when localized properly. The findings are summarized in Fig. 6J.

The role of the PH domain in different anillins

The PH domain is the most conserved domain of different anillins (D'Avino, 2009; Piekny and Maddox, 2010). It is necessary but not sufficient for localizing human anillin to the cleavage furrow (Oegema et al., 2000; Piekny and Glotzer, 2008). In Drosophila, it is dispensable for anillin’s furrow localization (D'Avino et al., 2008), but required for recruiting septins (Field et al., 2005). In S. pombe, while it is required for the localization and function of anillin-related Mid2 (Berlin et al., 2003; Tasto et al., 2003), we confirm that the PH domain is not essential for Mid1 localization (Paoletti and Chang, 2000). However, decreased cortical Mid1 localization in mid1(1-800) (Table 1; Figs 2C and 3A) and the fast recovery in FRAP analyses suggest that the PH domain stabilizes Mid1 on the cortex (Fig. 2E,F).

Although best known for its interaction with phosphoinositides, many PH domains are not sufficient for cortical targeting and showed promiscuous interaction with phosphoinositides (Kavran et al., 1998; Yu et al., 2004). For Mid1 PH domain, the weak interactions in protein-lipid overlay assay are consistent with the lack of cortical localization of Mid1(801-920) (Table 1). Mid1 is known to oligomerize (Celton-Morizur et al., 2004), which may enhance the affinity between the PH domain and the plasma membrane.

The internal region and the regulation of Mid1 localization

Although the biochemical properties of the internal region aa(101-400) of Mid1 are not clear, our data show that it regulates Mid1 localization. Deleting aa(101-400) enhances Mid1 nuclear and cortical localization, and the latter is more obvious in cdr2Δ cells (Figs 2 and 3). Moreover, in pom1Δ cells, Mid1[(1-100)-(401-920)] localizes to a broad, centered cortical zone and suppresses the cytokinesis defects of pom1Δ by assembling a contractile ring at the cell equator (Fig. 4). Both increased Mid1 concentration in nucleus and the weaker interactions with Cdr2 or Gef2 could contribute to the suppression of pom1Δ phenotypes (Fig. 4). The weaker interaction
between Mid1[(1-100)-(401-920)] and Cdr2 (supplementary material Fig. S3A) suggests that
either the structure of aa(400-450), the indicated binding site to Cdr2 (Almonacid et al., 2009), is
affected in mid1[(1-100)-(401-920)], or the internal region is also involved in the Mid1-Cdr2
interaction. The synthetic defects in septum position in pom1Δ mid1(Δ400-450) cells (Fig. 4C;
supplementary material Fig. S5) might result from the loss of phosphatase Clp1 binding site in
Mid1(Δ400-450) (Clifford et al., 2008). Although two PEST domains (Soehrmann et al., 1996) in
the internal region aa(101-400) might mediate proteolysis, the protein level of Mid1[(1-100)-
(401-920)] only increases slightly (Fig. 2C; Table 1), thus their missing is not likely the main
cause of the changes in Mid1[(1-100)-(401-920)] localization.

Because Mid1(FL) only localizes to the non-growing end of pom1Δ cells, it has been
speculated from modeling that there is another inhibitor of Mid1 at the growing end (Padte et al.,
2006, Celton-Morizur et al., 2006). However, the existence of such inhibitor has not been
confirmed yet. Mid1[(1-100)-(401-920)] can form a centered broad band of nodes spreading
toward both ends of pom1Δ cells (Fig. 4F), suggesting the inhibition of Mid1 localization at the
growing end can also be overcome by the mechanisms discussed above.

**Overlapping mechanisms regulating the cortical localization of Mid1**

Given that Mid1 is essential for division-site specification, it is not surprising that its localization
is regulated by several overlapping mechanisms. Nuclear shuttling regulates the balance between
nuclear and cytoplasmic Mid1 (Almonacid et al., 2009; Almonacid et al., 2011; Paoletti and
Chang, 2000). The cortical ER network limits the lateral diffusion of Mid1 on the plasma
membrane (Zhang et al., 2010). Pom1 kinase inhibits Mid1 from localizing to the non-growing
cell end through inhibiting Cdr2 (Almonacid et al., 2009; Celton-Morizur et al., 2006; Martin
and Berthelot-Grosjean, 2009; Moseley et al., 2009; Padte et al., 2006). With the findings in this
study, we now have a more thorough understanding of Mid1 localization.

Cdr2 kinase is critical for Mid1 localization to interphase nodes, but it was unknown why
deleting cdr2 only partially affected cortical Mid1 localization in interphase (Almonacid et al.,
2009; Moseley et al., 2009). Our findings suggest that the Cdr2-independent cortical targeting of
Mid1 in interphase depends on the PH domain (Fig. 3). Moreover, the PH domain also affects
the localization and dynamics of Mid1 in the presence of Cdr2 (Fig. 2) and has overlapping
function with the polybasic region aa(681-710) that anchors Mid1 to the cortex (supplementary
A similar cooperation between a polybasic region and the PH domain was reported in ARNO, a GEF of ADP-ribosylation factor (Macia et al., 2000).

The Polo kinase Plo1 targets Mid1 to cortical nodes at the G2/M transition (Almonacid et al., 2011; Bähler et al., 1998a). The regulation by Plo1 in mitosis is independent of the PH and the internal region aa(101-400) of Mid1 (Fig. 2G,H). This is consistent with the recent identifications of the Plo1 binding site (T517) and phosphorylation sites in Mid1 (Almonacid et al., 2011), as these sites are still present in Mid1[(1-100)-(401-800)].

Together, the balance of these positive and negative regulations on Mid1 ensures the fidelity of division-site selection and contractile-ring assembly in fission yeast cytokinesis.

**Physical interactions between Mid1 and other cytokinesis proteins**

Besides Cdr2 kinase, Polo kinase Plo1, and phosphatase Clp1, Mid1 is known to physically interact with four cytokinesis node proteins: Rng2, Cdc4, Myo2, and Cdc15 (Almonacid et al., 2011; Laporte et al., 2011; Motegi et al., 2004; Padmanabhan et al., 2011). We find that Mid1(1-580) physically interacts with Rng2, Cdc4, and Cdc15 and assembles cytokinesis nodes (Fig. 5). Moreover, when Mid1[(1-100)-(401-580)], a truncation that cannot localize to cortical nodes, is fused to Cdr2, cytokinesis-node and contractile-ring assembly are restored (Fig. 6). Two modules for cytokinesis-node assembly have been identified in *S. pombe* (Laporte et al., 2011). Mid1[(1-100)-(401-580)]-Cdr2 can recruit Rlc1, the most downstream protein in the module I, suggesting Rng2, Cdc4, and Myo2 are also present; and F-BAR protein Cdc15, the core protein in the module II, providing strong evidence that the interactions mediated by Mid1[(1-100)-(401-580)] are sufficient to scaffold cytokinesis-node and contractile-ring assembly. Consistently, Almonacid et al. (2011) recently found that Mid1(1-100) interacts with Rng2 C-terminus in vitro at high protein concentrations.

Rlc1 co-localizes with Mid1(1-100) at cytokinesis nodes and the contractile ring (Fig. 6F). However, the co-localization between Mid1(1-100) and Cdc15 was mainly detected in the contractile ring but not in cytokinesis nodes (Fig. 6G), suggesting a delay in Cdc15 recruitment. The lack of dephosphorylation of Cdc15 by Clp1 is a possible explanation for the difference since the fusion protein does not have aa(431-481), the Clp1 binding site (Clifford et al., 2008). Indeed, we found that *mid1(1-100)-cdr2* exhibited negative genetic interactions with *cdc15-140* and *cdc12-112*, temperature sensitive mutants that are synthetic with *mid1Δ(431-481)* (Clifford...
et al., 2008). However, it remains possible that Mid1(401-580) contributes to the recruitment by physically interacting with Cdc15.

Our co-IP showed that the hyperphosphorylated Mid1 interacts with cytokinesis node proteins, supporting the role of phosphorylation in Mid1’s scaffolding property, as eight Plo1 phosphorylation sites are identified in Mid1 (Almonacid et al., 2011). Whether phosphorylation of Mid1-related anillins in other systems is required for interacting with and recruiting cytokinesis proteins remains unknown.

Taken together, by domain analyses, we demonstrate how different Mid1 domains cooperate to ensure correct positioning of the division site and the timely assembly of the contractile ring. Similar to anillins (see Introduction), the N-terminus of Mid1 mainly mediates its scaffolding functions besides its role in Mid1 recruitment, and the C-terminus regulates its localization to the plasma membrane. Thus, the roles of anillins and the anillin-related protein Mid1 in cytokinesis are highly conserved during evolution.
Materials and Methods

Strain constructions and yeast methods

Table S1 lists the *S. pombe* strains used in this study. Standard genetic methods were used (Moreno et al., 1991). All tagged or truncated genes are under the control of endogenous promoters and integrated at their native chromosomal loci unless stated otherwise. PCR-based gene targeting was performed as described (Bähler et al., 1998b). All constructs were checked by PCR and/or DNA sequencing. mECitrine was made from mYFP (S65G, V68L, Q69K, S72A, T203Y, and A206K) by introducing F64L and Q69M. The functionalities of the tagged strains expressing FL proteins were validated by normal cell morphology and growth from 23 to 36°C and the lack of negative genetic interactions with *pom1Δ* or *clp1Δ*.

The C-terminal truncations of Mid1 were generated by transforming wt strain JW81 with *mECitrine-kanMX6* or *mYFP-kanMX6* flanked with homologous sequences from the designated position at *mid1* locus. The 5' untranslated region of *mid1* (-1108 to -1 bp upstream of the ATG according to [http://old.genedb.org/genedb/pombe/](http://old.genedb.org/genedb/pombe/)) was cloned and used as *Pmid1*. *mid1(41-920)* and *mid1(101-920)* were generated by transforming the wt strain JW81 with *kanMX6-Pmid1-mYFP* flanked with homologous sequences from the designated position at *mid1* locus. Strains *mid1(581-920)*, *mid1(681-920)*, and *mid1(801-920)* were generated by making strains carrying *ura4+*-Purg1-mYFP-(truncated mid1) (Purg1, the urg1 promoter; Watt et al., 2008) and then replacing *ura4+*-Purg1 with *kanMX6-Pmid1*.

To construct the internal truncations in plasmids, two primers with their 5'-ends separated by the Mid1 sequences to be deleted were utilized to amplify the pTOPO-mid1(FL) plasmid using iProof DNA polymerase (Bio-Rad, #172-5302). After blunt-end ligations, the resulting plasmids were sequenced. The desired Mid1 fragment was released from the plasmid by digestion with SacI and XhoI and transformed into *mid1Δ::ura4+*-mECitrine-kanMX6 cells (JW2349), which deletes +66 to +2694 bp of *mid1* ORF from strain *mid1-mECitrine-kanMX6* (JW1790) using the plasmid KS-ura4 (Bähler et al., 1998b). Cells plated onto YE5S were incubated at 25°C for 36–48 h before being selected by 5-FOA resistance.

To fuse *cdr2* to *mid1* truncations, *mid1(1-100)-linker*, *mid1[(1-100)-(401-580)]-linker* and *cdr2-mEGFP-kanMX6* were first cloned into the TOPO vector. *mid1(1-100)-linker* and *mid1[(1-100)-(401-580)]-linker* were amplified from the genomic DNA of *mid1(1-100)-6His-mYFP* (JW1303) and *mid1[(1-100)-(401-580)]-6His-mECitrine* (JW2704), respectively, using a
forward primer starting 25 bp before the ATG of mid1 ORF and a reverse primer complementary to the canonical C-terminal gene targeting linker CGGATCCCCGGGTTAATTAAC (Bähler et al., 1998b). SalI and PmeI sites were added to the reverse primer for further construction. cdr2-mEGFP-kanMX6 was amplified from the genomic DNA of cdr2-mEGFP (JM346) using a forward primer with a SalI site followed by the first 20 bp of cdr2 and a reverse primer GAATTCGAGCTCGTTTAAAC that is complementary to the C-terminal targeting module of pFA6a and has a PmeI site (Bähler et al., 1998b). cdr2-mEGFP-kanMX6 was then inserted to the 3'-end of the mid1 fragments after SalI and PmeI digestion and ligation. The linker between Mid1 fragments and Cdr2 is RIPGLINVD. mid1(1-100)-linker-cdr2-mEGFP-kanMX6 or mid1[(1-100)-(401-580)]-linker-cdr2-mEGFP-kanMX6 was released from the final plasmid using SpeI and XhoI and transformed into mid1-6His-13Myc-hphMX6 (JW3206) or mid1[(1-100)-(401-920)]-6His-13Myc-hphMX6 (JW2701), respectively. Transformants that were G418-resistant and hygromycin-sensitive were screened visually. The fusion gene is inserted into the mid1 locus under the control of the endogenous mid1 promoter. The resulting strain was JW3718 and JW3506.

mid1(Helix*)-mECitrine and mid1(1-800, Helix*)-mECitrine were made from AP583 (mid1Δ::ura4 + pAP159 Pmid1-mid1(Helix*)-GFP-leu1*). The GFP-leu1* fragment was first replaced by 13Myc-hphMX6. For making mid1(Helix*)-mECitrine, 13Myc-hphMX6 was replaced by mECitrine-kanMX6. For mid1(1-800, Helix*)-mECitrine, mECitrine-kanMX6 was amplified using the same forward primer used to generate mid1(1-800)-mECitrine so that the PH domain was truncated while replacing 13Myc-hphMX6 with mECitrine-kanMX6.

Latrunculin-A treatment at a concentration of 100 μM was performed as described (Coffman et al., 2009). To displace the nucleus by centrifugation, cells at exponential phase was centrifuged at 2,000 × g for 30 s and washed 2x with EMM5S, then incubated with 25 μg/ml MBC or DMSO at 25°C for 10 min. Cells were then transferred to a 1.5 ml microcentrifuge chamber filled with 1 ml of EMM5S agar with 25 μg/ml MBC or DMSO prepared as described (Daga and Chang, 2005), centrifuged at 18,000 × g for 2 min, and incubated in 1 ml EMM5S with 25 μg/ml MBC or DMSO for 1 h at 25°C before imaging.
Microscopy and data analysis

Cells were usually grown in liquid medium at exponential phase for ~48 h at 25°C before microscopy as described (Wu et al., 2006) at 23-24°C unless otherwise stated. mid1Δ cells and truncations with severe cytokinesis defects were grown for 24 to 36 h in liquid medium to prevent adaptation or second-site suppressor(s). Cells were prepared and imaged using the spinning disk confocal microscope as previously described (Coffman et al., 2009; Laporte et al., 2011; Wu et al., 2011). For maximum intensity projections of fluorescence images, a stack spanning 4 μm and spaced at 0.4-1.0 μm was taken, and the projection was generated in UltraVIEW. For single slice fluorescence images shown in figures, 10 consecutive images of the middle focal plane were taken without delay, and the average intensity projection was generated using ImageJ (http://rsb.info.nih.gov/ij/). Line-scans of cortical fluorescence intensities were generated using ImageJ.

Fluorescence intensity was quantified as described (Coffman et al., 2011; Laporte et al., 2011). For total fluorescence intensity in a cell, the corrected sum intensity projections of stacks were used. For local fluorescence intensity, the corrected average intensity projection of 10 consecutive pictures of the focal plane was used. For the nuclear intensity, a circular region of interest (ROI) with a diameter of 2.6 μm was used. For the cortical intensity, a rectangular ROI with an area of 1.9-2.5 μm² was used. To compare the intensities of FL Mid1 and each truncation, the mean intensity of the truncation was normalized to the mean intensity of the FL Mid1 in the same image.

FRAP analysis

FRAP analysis was performed using the photokinesis unit on the UltraVIEW ERS confocal system (Coffman et al., 2009; Laporte et al., 2011). A ROI was selected at sites with Mid1 signal in cortical nodes. Four pre-bleach images were collected, followed by 60 post-bleach images. The interval between images was 20 s. Data analyses were performed as described (Coffman et al., 2009; Laporte et al., 2011).

IP and immunoblotting

IP and immunoblotting were performed as described (Laporte et al., 2011) with the following modifications: (a) 200 mg lyophilized cells were used; (b) anti-Myc antibody (9E10; Santa Cruz
Biotechnology, Inc) was used in 1:1,000 dilution. The monoclonal JL-8 antibody against
GFP/YFP (Clontech, #632381, 1:5,000 dilution) was used to blot mYFP-Rng2, mYFP-Cdc4,
mYFP-Cdc15, and mYFP-Myo2. A polyclonal rabbit anti-GFP antibody (Novus, NB600-308,
1:20,000 dilution) was used to detect Cdr2-mEgfp. To quantify the level of different truncations
in cells, total proteins extracted from ~0.5 mg lyophilized cells were loaded in duplicates on the
SDS-PAGE. Monoclonal antibodies against GFP/YFP (Roche, #11814460001, or JL-8) and
tubulin (TAT1; Woods et al., 1989) were used. The intensities of Mid1 truncations were
corrected by levels of tubulins from the same extract and normalized to FL Mid1 with the same
tag.

Phosphatase treatment was carried out by splitting one sample into two tubes at the last
washing step of IP. After removing supernatant, beads in both tubes were resuspended in 19 μl
1x phosphatase buffer (50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35, pH 7.5) with
2 mM MnCl2. One tube is incubated with 1 μl ddH2O (mock treatment), and the other with 1 μl
λ phophatase (NEB, P0753S) at 30°C for 30 min. 5 μl 5x sample buffer was then added to each
tube, and samples were boiled for 5 min before loading to SDS-PAGE.

Expression and purification of the PH domains

The encoding sequences of the PH domains of Mid1 [aa(801-920)] and Ksg1 [aa(434-592)] were
amplified from genomic DNAs using primers with BamHI and SalI restriction sites and cloned
into the TOPO vector and sequenced. After released by BamHI and SalI, the fragments were
cloned into pQE80L to obtain the expression constructs pQE80L-6His-Mid1(801-920)
(JQW470) and pQE80L-6His-Ksg1(434-592) (JQW471).

The PH domains were expressed and purified as described for the purification of 6His-
mEGFP (our negative control) and 6His-mYFP using TALON metal affinity resin (Clontech,
#635502; Wu and Pollard, 2005; Wu et al., 2008) with the following modifications: (a) protein
expression was induced with 1 mM IPTG at 20°C for 20 h; (b) the lysates were centrifuged at
107,200 × g for 15 min and then 247,600 × g for 30 min at 4°C and bound to the resin were
washed with washing buffer (50 mM Na2PO4, 300 mM NaCl, 10 mM β-mercaptoethanol, and
20 mM imidazole; pH8.0); (c) the fractions with purified proteins were dialyzed into storage
buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 1 mM NaN3, 1 mM EDTA, 1 mM
DTT, pH7.5) at 4°C using the SnakeSkin dialysis tubing with a cut-off size of 7 kDa (Thermo
Scientific, #68700); (d) protein concentrations were determined using the absorbance at 280 nm and extinction coefficients obtained using ProtParam [http://web.expasy.org/protparam/; 21,500 M\(^{-1}\)cm\(^{-1}\) for 6His-Mid1(PH) and 25,000 M\(^{-1}\)cm\(^{-1}\) for 6His-Ksg1(PH)].

**Protein-lipid overlay assay**

We used commercial PIP membrane strips (Invitrogen, P23751) to perform the protein-lipid overlay assay as described by the manufacturer. Blocking was performed by immersing the membrane in TBS (20 mM Tris-HCl, 150 mM NaCl) + 3% fatty acid-free BSA (Sigma, A7030) at room temperature (RT) for 1 h with shaking. The membrane was then incubated with 50 nM purified proteins at 4°C overnight with shaking. After the removal of the protein, the membrane was washed 5 times using TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20), and immunoblottings were performed using anti-His antibody (Clontech, #631212, 1:10,000 dilution) in TBS + 3% fatty acid-free BSA.
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Definition of individual components within the cytoskeleton of Trypanosoma brucei by a 


**FIGURE LEGENDS**

**Fig. 1. Serial truncations of Mid1 reveal functions of different domains/motifs in division-site specification.** (A) Schematic representation of Mid1 domains/motifs. Known domains involved in localizing Mid1 or mediating interactions with other proteins are shown in darker gray. The PH domain identified from homology is shown in lighter gray. Numbers indicate the corresponding aa. (B) Quantifications of septum position/angle in septating cells of the truncations made in this study. (Left) Numbers indicate aa present in the schematics of truncations; (middle) a septum within the central 1/5 of the cell is defined as centered/normal (gray), a cell with >1 septa is defined as abnormal; (right) a septum at 90 ± 10° relative to the long axis of the cell is defined as orthogonal/normal (gray). FL Mid1 tagged at either the C- (C-tag; JW1790) or N-terminus (N-tag; JW1513) and mid1Δ (JW1604) are included as controls. For each strain, the mean and s.d. from 3 independent experiments are shown. n > 48 septating cells per strain in each experiment. This panel corresponds to the data and strains in Table 1. (C-E) Representative Differential Interference Contrast (DIC) images of cells in a series of C-terminal (C), N-terminal (D), and other (E) truncations as indicated. Bars, 5 μm.

**Fig. 2. The PH domain and the internal region aa(101-400) affect Mid1 localizations and dynamics.** (A) Localizations of mECitrine-tagged FL Mid1 (JW1790), Mid1(1-800) (JW2601), Mid1[(1-100)-(401-920)] (JW2391), and Mid1[(1-100)-(401-800)] (JW2702). The average intensity projection of the middle focal plane is shown. Images are taken using the same parameters and inverted. Asterisks indicate the nuclei; arrows, nodes; and arrowheads, contractile rings. (B) Merged image of Mid1-mECitrine (green) and Sad1-mCFP (magenta). Cells expressing Mid1-mECitrine (example with white outlines; JW1790) were imaged together with cells expressing Mid1-mECitrine Sad1-mCFP (orange outlines; JW1834). (C) Quantification of global mean protein levels (gray) and local levels in nodes (blue) and the nucleus (pink) of the strains in (A). The differences of fluorescence intensity between the truncations and the FL Mid1 are plotted. n > 60 cells for each strain. Error bars, s.d. from different images. (D) Illustration of FRAP analysis of Mid1 nodes. Top, a cell marked with two ROIs. Bottom, kymographs showing the change in fluorescence intensity over 15 min in the bleached (green box) and unbleached (yellow box) ROI. Images were collected every 20 s. Arrowhead, the time point of the bleaching. (E,F) Fluorescence recovery curves (E) and
recovery rates (F) of strains in (A). Gray, recovery of FL Mid1 (Laporte et al., 2011). Error bars, s.e.m. (F) $k_{\text{off}}^* t$ is plotted as a function of time to illustrate the differences in recovery rates (slope). (G,H) Rlc1-tdTomato contractile-ring assembly in wt (JW2433), mid1(1-800) (JW1779), mid1[(1-100)-(401-920)] (JW2435), and mid1[(1-100)-(401-800)] (JW3173). (G) Maximum intensity projections are shown. Yellow dashed lines outline the cells. Arrowheads indicate appearance of Rlc1 nodes. The time point just before Rlc1 node appearance is defined as time zero. (H) Scatter plots of the time from Rlc1 node appearance to the formation of a compact ring in mid1(1-800), mid1[(1-100)-(401-920)], and mid1[(1-100)-(401-800)] ($P > 0.3$, each compared to FL Mid1). Bars, 5 μm.

**Fig. 3. The internal region aa(101-400) and the PH domain regulate the localization of Mid1.** (A) Localization of indicated mECitrine-tagged Mid1 constructs in cdr2Δ during interphase (JW2160, JW3097, JW2672, and JW3034). The average of 10 consecutive images of the middle focal plane is shown. Arrowheads indicate cortical localization of Mid1. (B) Quantification of mononucleated cells with cortical signals of strains in (A). Error bars, s.d. from different experiments. $n > 36$ cells per strain for each experiment. (C) Cortical localization of Mid1[(1-100)-(401-920)]-mECitrine in cdr2Δ cells (JW2672) depends on the nuclear location. Cells were incubated with either MBC or DMSO and centrifuged to move the nuclei (see Materials and Methods). Dashed lines span the cortical signal at the cell equator and arrowheads indicate signal at cell tips. Bars, 5 μm. (D) SDS-PAGE of purified 6His-Ksg1(PH) and 6His-Mid1(PH) stained with Coomassie blue. Two eluted fractions for each protein are shown. Expected sizes of the PH domains were indicated on the right. (E) 6His-Mid1(PH) has weak interactions with several lipids. (Left) Protein-lipid overlay assay with 6His-Ksg1(PH), 6His-Mid1(PH), and 6His-mEGFP. (Right) Layout of the PIP strip.

**Fig. 4. Mid1 lacking the internal region aa(101-400) suppresses the cytokinesis defects of pom1Δ.** (A) mid1[(1-100)-(401-920)] pom1Δ cells are healthier (a lighter color) on YES5 + Phloxin B plate. Similar amount of cells from indicated strains were plated and incubated at 30°C for 42 h. (B) DIC images of pom1Δ (JW2425) and pom1Δmid1[(1-100)-(401-920)] (JW2426) cells. Open arrowheads indicate cell equators and closed arrowheads, misplaced septa. (C) mid1[(1-100)-(401-920)] and other mutants suppress septum-position defects in pom1Δ cells.
(JW2425, JW2426, JW2663, JW2732, JW3700, JW3701, JW2673, JW4059, and JW4039). The same criteria as in Fig. 1B are used. The vertical dashed lines are for aiding comparisons. For each strain, the mean and s.d. from 3 independent experiments are shown. \( n > 48 \) septating cells per strain in each experiment. (D) mECitrine-Gef2 localizes to the equator of wt cells (left, JW3825) but shifts toward one cell end in \( pom1\Delta \) (right, JW4060). (E) Localization of FL Mid1 and Mid1[(1-100)-(401-920)] in \( pom1\Delta \) cells (JW2425 and JW2426). The average of 10 consecutive images of the middle focal plane is shown. Arrowheads indicate contractile rings. (F) Cortical nodes in \( pom1\Delta \) mid1[(1-100)-(401-920)] cells spread farther along the cortex. Cortical Mid1 intensities of 3 representative cells from strains JW1790, JW2391, JW2425, and JW2426 are measured as indicated by the diagram. (G) Contractile-ring assembly in \( rlc1\,-\,tdTomato \, pom1\Delta \) cells expressing FL mid1 (JW2555) or mid1[(1-100)-(401-920)] (JW2596). Maximum intensity projections are shown. The time point just before the appearance of Rlc1 cytokinesis nodes is defined as time zero. Bars, 5 \( \mu \)m.

Fig. 5. The N-terminal half of Mid1 physically interacts with several node proteins for contractile-ring assembly. (A) The localization of FL Mid1 (JW1790) and Mid1(1-580) (JW2603) during cytokinesis. Maximum intensity projections are shown. Time zero is arbitrary. (B) Contractile ring of Rlc1-tdTomato assembles from cytokinesis nodes more slowly in mid1(1-580) cells (JW3338) than in wt (JW2433). Maximum intensity projections are shown. Arrowheads, Rlc1 node appearance. The time point just before the appearance of cytokinesis nodes is defined as time zero. Dashed lines outline the cells. Bars, 5 \( \mu \)m. (C) Scatter plots of the time from Rlc1-tdTomato node appearance to the formation of a compact contractile ring. (D) Number of Rlc1-tdTomato nodes in wt (JW2178) and mid1(1-580) (JW3337). (C,D) Asterisks indicate a significant difference (\( P < 0.05 \)) from wt. (E-G) Physical interactions between Mid1 and node proteins. IP was performed using antibody against YFP (see Materials and Methods). (E) Mid1(1-580)-Myc co-IP with mYFP-Rng2 (right; JW909, JW2548, and JW2345). (Left) the presence of mYFP-Rng2 and/or Mid1(1-580)-Myc before IP. (F) Mid1(1-580)-Myc co-IP with mYFP-Cdc4 (left; JW910-2 and JW2625), mYFP-Cdc15 (middle; JW1052 and JW2549), and Cdr2-mEGFP (right; JM346 and JW2551). (G) Mobility shifts of FL Mid1 treated with \( \lambda \)-phosphatase after IP (JW2233, JW2226, JW2231, and JW2227). The 50 kDa non-specific band is used as a loading control.
**Fig. 6. Mid1(1-100) is sufficient to assemble cytokinesis nodes and the contractile ring.**

(A,B) Localizations of (A) Mid1[(1-100)-(401-580)] (left; JW2704) and Rlc1 in mid1[(1-100)-(401-580)] (right; JW3175), and (B) Mid1(1-100) (left; JW1303) and Rlc1 in mid1(1-100) (right; JW1738). Asterisk indicates nuclear localization and closed arrowheads, aberrant contractile ring/filaments. (C) Schematic representation of the fusion proteins. (D) Localization of Rlc1-tdTomato in mid1Δ::Pmid1-cdr2-mEGFP (JW3471). Closed arrowheads indicate aberrant contractile ring/filaments. (E) Quantifications of septum position and angle in the indicated strains (JW3449, JW2704, JW3506, JW1303, and JW3718). The same criteria as in Fig. 1B are used. For each strain, the mean and s.d. from 3 independent experiments are shown. \( n > 50 \) septating cells per strain in each experiment. (F) Rlc1 nodes and contractile-ring assembly are restored in mid1[(1-100)-(401-580)]-cdr2 (JW3525) and mid1(1-100)-cdr2 (JW3736) cells. Arrows indicate cytokinesis nodes; open arrowheads, condensing nodes; and the closed arrowhead, a compact contractile ring. (G) Cdc15 localizes to nodes in mid1[(1-100)-(401-580)]-cdr2 (JW3603) but not in mid1(1-100)-cdr2 (JW3738) cells. Arrows indicate cytokinesis nodes; closed arrowhead, contractile rings. (H) Fusing Mid1[(1-100)-(401-580)] or Mid1(1-100) to Cdr2 partially rescued the kinetics of contractile-ring assembly. The mean time from Rlc1 signal appearance to the formation of a compact ring is shown (JW3471, JW3175, JW3525, JW1738, and JW3736). \( P \)-values in pair-wise comparisons are shown. Error bars, s.d. (I) A broad band of Cdc15 nodes (arrow) was detected in mid1[(1-100)-(401-580)]-cdr2 (JW3603) but not in mid1(1-100)-cdr2 (JW3738) cells upon treatment with 100 \( \mu \)M Latrunculin-A. (J) Summary of the findings in this study (below the diagram) in the context of what is known about Mid1 (see text for details). Bars, 5 \( \mu \)m.
Table 1. Summary of phenotypes and localizations of Mid1 truncations.

<table>
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<tr>
<th>Genotype</th>
<th>Strain</th>
<th>Protein level$^a$</th>
<th>% Normal septum in septating cells</th>
<th>Localization$^d$</th>
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<td></td>
<td></td>
<td>Septum position$^b$</td>
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<td>FL (N-tag)$^f$</td>
<td>JW1513</td>
<td>1.0</td>
<td>100 ± 0</td>
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<td>JW1537</td>
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<td>(1-40)-(101-920)$^e$</td>
<td>JW2390</td>
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<td>43 ± 7</td>
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<td>581-920$^f$</td>
<td>JW1640</td>
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<td>JW1610</td>
<td>(+)$^g$</td>
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<tr>
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$^a$Protein level: quantified by western blotting (supplementary material Fig. S1) and normalized to the level of FL Mid1. The mean and s.d. from 2 to 6 experiments are shown.

$^b$Definition of normal septum position: single septum in the central 20% of the cell.

$^c$Definition of normal septum angle: septum at 90 ± 10° to the long axis of the cell.

$^d$Localization: +, localized; -, not localized; nd, not determined. Interphase and cytokinesis nodes: discrete protein clusters close to the equatorial cortex during interphase and the G2/M transition, respectively.

$^e$Tagged with mECitrine.

$^f$Tagged with mYFP.

$^g$The expression of this truncation is confirmed by localized signal.

$^h$This truncation localizes to both the full-size and the constricted ring.

$^i$This truncation localizes all over the cell cortex.
Fig. 2. Lee and Wu, 2012

A

mECtome

FL

1-800

(1-100)-(401-920)

(1-100)-(401-800)

B

C

D

E

F

G

H
Fig. 3. Lee and Wu, 2012
Fig. 6. Lee and Wu, 2012

A mid1[1-100]—mYFP
B mid1[1-100]—mYFP
C mid1[1-100]—mYFP
D mid1[1-100]—mYFP
E mid1[1-100]—mYFP
F mid1[1-100]—mYFP
G mid1[1-100]—mYFP
H mid1[1-100]—mYFP
I mid1[1-100]—mYFP
J mid1[1-100]—mYFP

Assembles cytokinesis nodes
Modulates cortical stability
Fig. S1. Western blotting of mYFP or mECitrine tagged Mid1 truncations. Asterisks between duplicated samples mark the sizes of different truncations. Blottings of anti-tubulin are used as a loading control. Strains used are listed in Table S1. (A,B) The expression levels of mECitrine-tagged Mid1 C-terminal (A) and internal (A,B) truncations are shown. The truncations are compared to Mid1-mECitrine (FL) (JW1790; the first two lanes). (C–E) The expression levels of mYFP-tagged Mid1 C-terminal (C) and N-terminal (D,E) truncations are shown. The nonspecific bands near 50 and 70 kDa in (E) are due to using the JL-8 antibody. The C-terminal truncations are compared to Mid1-mYFP (FL) (JW1089; the first two lanes). The N-terminal truncations are compared to mYFP-Mid1(FL) (JW1513; the first two lanes).
**Fig. S2. Fluorescence intensity of Mid1[(1-100)-(401-920)] and dynamics of Mid1(1-800).** (A) The nuclear (white), cortical (within the black boxes in the middle), and total fluorescence intensities of Mid1(FL)-mECitrine (JW1834) and Mid1[(1-100)-(401-920)]-mECitrine (JW2391) in the middle focal plane were measured and normalized to the total Mid1(FL)-mECitrine in the same plane. Cytoplasmic intensity (gray) was obtained by subtracting the nuclear and cortical intensities from the total. Colors in the plot correlates with the location in the cell shown above the plot. (B) The FRAP recovery rate of Mid1(FL) and Mid1(1-800) cytokinesis nodes in cdr2Δ cells (JW2160 and JW3097). $k_{\text{off}}^*t$ is plotted as a function of time to illustrate the differences in recovery rates (slope).

**Fig. S3. Mid1[(1-100)-(401-920)] interacts with Cdr2-mEGFP.** (A) Mid1[(1-100)-(401-920)] co-IP with Cdr2-mEGFP. Antibody against EGFP was used to immunoprecipitate Cdr2-mEGFP, and antibody against Myc was used to detect Mid1. (left) Protein levels before IP. (right) Mid1[(1-100)-(401-920)] co-IP with Cdr2-mEGFP. Co-IP between Cdr2 and FL Mid1 was shown as a positive control (3rd lane). Strains used were (from left to right): JM346, JW3206, JW3267, JW2701, and JW2727. (B) Truncating aa(101-400) does not obviously affect the localization of Cdr2-mEGFP (strains JW3267 and JW2727). Scale bar, 5 μm.
Fig. S4. Mid1 PH domain has overlapping function with the amphipathic helix. (A) Mid1 PH domain has no obvious overlapping function with Cdr2. Quantifications of septum position/angle in septating cells of the indicated strains (JW2160, JW3097, JW2672, and JW3034). The same criteria as in Fig. 1B are used. For each strain, the mean and s.d. from 3 independent experiments are shown. *n* > 44 septating cells per strain in each experiment. (B,C) Mid1 PH domain has overlapping function with the amphipathic helix. Strains used are JW3820 and JW3837. (B) DIC images of mid1(Helix*) and mid1(1-800, Helix*) cells. Mid1(Helix*) has mutations in the helix and severely affects Mid1 cortical binding (Celton-Morizur et al., 2004). (C) Quantifications of septum position/angle in septating cells. For each strain, the mean and s.d. from 3 independent experiments are shown. *n* > 38 septating cells per strain in each experiment. Scale bar, 5 μm.

Fig. S5. mid1 mutants alleviate division-site-specification defects in pom1Δ. Representative DIC images of the cells quantified in Fig. 4C. Strains used are JB109, JW2663, JW2732, JW3700, JW3701, JW2673, JW4059, and JW4039. Scale bar, 5 μm.
Fig. S6. Localization of Mid1(1-580), Mid1(41-920), and Mid1[(1-40)-(101-920)]. (A) Maximum intensity projection (top) and middle slice (bottom) of Mid1(1-580)-mECitrine cells (JW2603). Open arrowheads indicate full-size contractile rings and closed arrowheads, constricting rings. (B,C) Maximum intensity projections are shown. Asterisks indicate nuclear localization; open arrowheads, cortical nodes; arrows, normal contractile rings; and closed arrowheads, aberrant contractile ring/filaments. (B) Truncating aa(1-40) does not obviously affect Mid1 localization (left; JW1537) or contractile-ring assembly (right; JW1554). (C) Truncating aa(41-100) results in significant defects in Mid1 cortical localization (left; JW2390) and contractile-ring assembly (right; JW2434). (D) Time courses showing localization of Mid1 (JW1790) and Mid1[(1-40)-(101-920)] (JW2390) during mitosis. Only the middle focal plane is shown. Time zero is arbitrary. Cell equators are indicated by arrows. Scale bar, 5 μm.
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<th>Notes</th>
</tr>
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<tr>
<td>JW3820</td>
<td>h− mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 + Pmid1-mid1(Helix*)-mECitrine-kanMX6 integrated at leu1 locus (but not leu1(^+))</td>
<td>This study</td>
</tr>
<tr>
<td>JW3825</td>
<td>h kanMX6-Pgef2-mECitrine-4Gly-gef2 ade6-M216 leu1-32 ura4-D18</td>
<td>Yanfang Ye</td>
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<td>JW3837</td>
<td>h mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 + Pmid1-mid1(1-800, Helix*)-mECitrine-kanMX6 integrated at leu1 locus (but not leu1(^+))</td>
<td>This study</td>
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<td>JW4039</td>
<td>cdr2Δ::kanMX6 gef2Δ::hphMX6 pom1-Δ1::ura4+ ade6 leu1-32 ura4-D18</td>
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<td>JW4059</td>
<td>gef2Δ::hphMX6 pom1-Δ1::ura4+ ade6 leu1-32 ura4-D18</td>
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<td>JW4060</td>
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<td>AP528</td>
<td>h mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 + pAP146 Pmid1-mid1-GFP integrated at leu1(^+)</td>
<td>Celton-Morizur et al., 2004</td>
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<td>AP583</td>
<td>h mid1Δ::ura4+ ade6-M216 leu1-32 ura4-D18 + pAP159 Pmid1-mid1(Helix*)-GFP integrated at leu1(^+)</td>
<td>Celton-Morizur et al., 2004</td>
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<td>AP1943</td>
<td>h mid1Δ::kanMX4 ade6-M216 leu1-32 ura4-D18 + pMA21 [pJK148-Pmid1-mid1(Δ400-450)-4GFP-stopnmt] integrated at leu1(^+)</td>
<td>Almonacid et al., 2009</td>
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<td>AP1977</td>
<td>h mid1Δ::kanMX4 ade6-M216 leu1-32 ura4-D18 + pMA23 [pJK148-Pmid1-mid1(Δ300-350)-4GFP-stopnmt] integrated at leu1(^+)</td>
<td>Almonacid et al., 2009</td>
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<td>JB109</td>
<td>h+ pom1-Δ1::ura4+ ade6-M216 ura4-D18</td>
<td>Bähler and Pringle, 1998</td>
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<td>JM346</td>
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<td>Moseley et al., 2009</td>
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<td>YDM821</td>
<td>h+ clp1Δ::ura4+ ade6-M216 leu1-32 ura4-D18</td>
<td>Jin et al., 2007</td>
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Table S1. *S. pombe* strains used in this study