Pom1 regulates the assembly of Cdr2–Mid1 cortical nodes for robust spatial control of cytokinesis

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Proper division plane positioning is essential to achieve faithful DNA segregation and to control daughter cell size, positioning, or fate within tissues. In Schizosaccharomyces pombe, division plane positioning is controlled positively by export of the division plane positioning factor Mid1/anillin from the nucleus and negatively by the Pom1/DYRK (dual-specificity tyrosine-regulated kinase) gradients emanating from cell tips. Pom1 restricts to the cell middle cortical cytokinetic ring precursor nodes organized by the SAD-like kinase Cdr2 and Mid1/anillin through an unknown mechanism. In this study, we show that Pom1 modulates Cdr2 association with membranes by phosphorylation of a basic region co-operating with the lipid-binding KA-1 domain. Pom1 also inhibits Cdr2 interaction with Mid1, reducing its clustering ability, possibly by down-regulation of Cdr2 kinase activity. We propose that the dual regulation exerted by Pom1 on Cdr2 prevents Cdr2 assembly into stable nodes in the cell tip region where Pom1 concentration is high, which ensures proper positioning of cytokinetic ring precursors at the cell geometrical center and robust and accurate division plane positioning.

Introduction

Close coordination between chromosome segregation and cytokinesis is crucial for proper cell division and inheritance of the genetic material. For example, division plane positioning defects can lead to chromosome segregation anomalies and aneuploidy and can also perturb cell organization or cell lineage during early development of multicellular organisms.

In animal cells, division plane positioning is regulated by positive and negative spatial cues emanating from the spindle midzone and spindle poles, respectively, ensuring a direct spatial coordination between chromosome segregation and cytokinesis (Balasubramanian et al., 2012; Fededa and Gerlich, 2012; Green et al., 2012; White and Glotzer, 2012). Similarly, in the fission yeast Schizosaccharomyces pombe, a combination of positive and negative spatial cues act to establish the position of the division plane in the middle of this rod-shaped single-celled organism (Oliferenko et al., 2009; Almonacid and Paoletti, 2010; Rincon and Paoletti, 2012).

Positive signaling of the division plane involves the nuclear export of the anillin-like protein Mid1 (Sohrmann et al., 1996), which spatially couples the position of the nucleus during interphase to the assembly site of the cytokinetic ring (Daga and Chang, 2005; Almonacid et al., 2009). This mechanism leads to Mid1 accumulation on juxtanuclear regions of the cortex, where Mid1 forms cytokinetic ring precursor nodes and, upon activation by the polo-like kinase Plo1, promotes the sequential recruitment of essential components of the contractile ring (Bathe and Chang, 2010; Laporte et al., 2010; Pollard and Wu, 2010; Almonacid et al., 2011; Goyal et al., 2011; Lee et al., 2012).

This mitotic recruitment phase starts with the recruitment of myosin II in an IQGAP (IQ domain GTPase-activating protein)/Rng2-dependent manner followed by the F-BAR (FER/CIP4 homology domain-Bin-Amphiphysin-Rvs–like protein) protein...
Cdc15 and F-actin nucleator Cdc12 (Almonacid et al., 2011; Laporte et al., 2011; Padmanabhan et al., 2011). Compaction of mature ring precursors driven by actomyosin forces then leads to the formation of a medially placed contractile ring (Vavylonis et al., 2008; Ojkic et al., 2011).

In mid1Δ cells, actomyosin strands or contractile rings assemble at random positions within the cell. Contractile ring assembly then depends on the septation initiation network (Hachet and Simanis, 2008; Huang et al., 2008), which regulates Cdc15 conformation through Clp1-dependent dephosphorylation and inhibits Cdc12 multimerization by Sid2-dependent phosphorylation (Roberts-Galbraith et al., 2010; Bohnert et al., 2013). Nevertheless, mid1 mutation or deletion yields extensive cell death in the cell population (Chang et al., 1996; Sohmann et al., 1996), indicating that Mid1-dependent regulation of the contractile ring position contributes to cell survival and proper segregation of chromosomes between sister cells.

A parallel pathway regulating division plane positioning in fission yeast relies on a negative cue that emanates from cell tips in the form of spatial gradients of the dual-specificity tyrosine-regulated kinase (DYRK) family kinase Pom1 (Bähler and Pringle, 1998; Celton-Morizur et al., 2006; Padte et al., 2006). Pom1 membrane diffusion-based gradients are nucleated by microtubule-dependent delivery of the Tea protein complexes to the cell tips where they anchor a PPI phosphatase (Alvarez-Tararés et al., 2007; Martin, 2009; Hachet et al., 2012). PP1 reverses Pom1 autophosphorylation on its membrane-anchoring domain, which otherwise promotes a productive detachment of Pom1 from the plasma membrane as Pom1 diffuses away from the cell tip region (Hachet et al., 2011; Saunders et al., 2012). Pom1, in turn, has been shown to control the distribution of Cdr2 (Breeding et al., 1998; Kanoh and Russell, 1998), a conserved member of the Brsk/SAD (synapses of the amphid defective)/Septin kinase subfamily of AMP kinase–like kinases that assembles into nodes on the cortex (Morrell et al., 2004). In pom1Δ cells, growth is monopolar, and Cdr2 nodes, normally restricted to the medial cortex, invade the nongrowing cell tip (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009). Surprisingly, Cdr2 nodes remain excluded from the growing tip, but the mechanism of this additional Pom1-independent regulation remains unknown.

Importantly, Cdr2 nodes recruit Mid1 to the medial cortex during interphase and gradually collect a series of nonessential ring components (e.g., Bti1, Gef2, and Nod1) released from the previous division site (Almonacid et al., 2009; Moseley et al., 2009; Ye et al., 2012; Guzman-Vendrell et al., 2013; Jourdan et al., 2013; Zhu et al., 2013; Akamatsu et al., 2014). As a result, in pom1Δ cells, the distribution of contractile ring precursors is perturbed, leading to asymmetric division (Celton-Morizur et al., 2006; Padte et al., 2006). Thus, Pom1-dependent regulation of Cdr2 node distribution on the cortex allows prepositioning of Mid1 to the geometric center of the cell. This pathway, which is necessary to promote the formation of equally sized sister cells when Mid1 shuttling in the nucleus is impaired, contributes to the robustness and accuracy of division plane positioning in wild-type cells.

Interestingly, Pom1 and Cdr2 regulate not only division plane positioning but also division timing by modulation of Cdk1 activity (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009). Cdr2 cortical nodes indeed contain several regulators of mitotic entry, including Cdr2 itself, which acts as a Wee1-inhibitory kinase (Breeding et al., 1998; Kanoh and Russell, 1998), the Cdr1/Nim1 kinase homologous to Cdr2 (Russell and Nurse, 1987a; Coleman et al., 1993; Parker et al., 1993; Wu and Russell, 1993), and the Cdk1 inhibitor Wee1 (Russell and Nurse, 1987b; Moseley et al., 2009). Collectively, these constitute a Wee1-inhibitory network. In the proposed models, Pom1 maintains this Wee1-inhibitory network inactive in short cells, preventing mitotic entry until a critical length has been reached (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009). Whether the Pom1–Cdr2 pathway constitutes an active cell size sensor has nevertheless been recently challenged (Wood and Nurse, 2013). Recent data indicate that this role of Pom1 in regulating division timing is genetically separable from its role in division plane positioning (Bhatia et al., 2014) and involves Pom1 indirectly regulating the kinase activity of Cdr2 by phosphorylation of the C-terminal tail, which antagonizes the phosphorylation of Cdr2 T loop by the calcium/calmodulin-dependent protein kinase kinase Ssp1 (Bhatia et al., 2014; Deng et al., 2014).

In contrast, the molecular mechanisms underlying how Pom1 gradients negatively regulate Cdr2 node distribution are not understood. Here, we have deciphered the mechanisms by which Cdr2 assembles into nodes on the cell cortex and dissected how the spatial information of Pom1 gradients is transduced into functional outputs for division plane positioning. Similar to Pom1 gradients, several dynamic gradients are known to provide spatial subcellular information to establish stable domains with specific biochemical activities (Fuller, 2010). Yet, in most cases, how the spatial information is transduced to target activities remains unclear. Our study provides a first mechanistic model that might be relevant to other spatial regulatory events involving membrane gradients.

**Results**

The Cdr2 KA-1 domain and a neighboring basic region cooperate to promote Cdr2 membrane binding

To understand how Pom1 constrains Cdr2 node distribution at the cell medial cortex to predefine the division plane, we first studied how Cdr2 associates with the cortex. A large region of Cdr2 of ~500 amino acids C terminal to the kinase domain was previously shown to be necessary and sufficient for Cdr2 cortical localization (Morrell et al., 2004). This region includes a predicted structured domain of ~130 amino acids composed of three putative α helices and five β sheets (Fig. 1 A) with low similarity to the recently described lipid-binding KA-1 domain of Kcc4, a kinase homologous to Cdr2 in *Saccharomyces cerevisiae* (Moravecic et al., 2010). Mutations in any of the three α helices completely detached Cdr2 from the cortex (Fig. S1 A) and induced Cdr2 relocation to the nucleus, indicating that the lipid-binding function of this domain may be conserved.

Surprisingly, homology modeling of the Cdr2 KA-1 domain, based on the crystal structure of Kcc4 KA-1 (Fig. 1 B),
Figure 1. C-terminal KA-1 domain and basic motif mediate Cdr2 membrane binding. (A) Schematic representation of full-length Cdr2 (top) and Cdr2 C-terminal cortex-anchoring domain (Cdr2-Cter, residues 591–747; bottom) composed of a basic region (blue) and a KA-1 domain (green on top and rainbow on the bottom). Predicted α helices and β sheets of the KA-1 domain are shown. (B) Structural model of Cdr2 KA-1 domain derived from the \textit{S. cerevisiae} Kcc4 KA-1 crystal structure (Protein Data Bank accession no. 3OST). Seven positively charged residues sticking out of the domain are highlighted in blue. (C) Epifluorescence medial plane images of mEGFP-tagged Cdr2 mutants of the positively charged residues of Cdr2 KA-1 shown in B (Cdr2\textsuperscript{RR*}: R624Q and R628Q; Cdr2\textsuperscript{RKRKR*}: R682Q, K684Q, R685Q, K692Q, and R695Q; Cdr2\textsuperscript{RR*-RKRKR*}: combination of all mutations), in the basic region upstream of Cdr2 KA-1 (Cdr2\textsuperscript{bsc*}: K598N, H599Q, R600Q, R602Q, K612N, K613N, and K614N), or in both (Cdr2\textsuperscript{bsc*-RKRKR*}). Bar, 5 µm. (D) Mean FRAP of mEGFP-tagged Cdr2 or Cdr2\textsuperscript{RR*} and Cdr2\textsuperscript{bsc} mutants on the medial cortex as indicated. Error bars show SDs. Cdr2 \( t_{1/2} = 3 \) min; Cdr2\textsuperscript{RR*} \( t_{1/2} = 3 \) min; Cdr2\textsuperscript{bsc} \( t_{1/2} < 1 \) min. B, bleach.
revealed that surface-exposed basic residues mediating the electrostatic interactions with acidic phospholipids in Kcc4 KA-1 (Moravcevic et al., 2010) were not conserved in Cdr2 KA-1 (Fig. S1 B). In addition, some hydrophobic residues of the β3–β4 hydrophobic loop proposed to insert into the lipid bilayer were replaced by nonhydrophobic residues. Instead, we identified a cluster of surface-exposed basic residues in β3, β4, and the β3–β4 loop (R682, K684, R685, K692, and R695). Mutagenesis of these basic residues to noncharged polar amino acids (N or Q) showed that these residues have an important role in targeting Cdr2 to the cortex (Fig. 1 C). Mutations of two other basic residues from helix α1 (R624 and R628) did not affect Cdr2 association with the cortex on their own but led to a complete detachment when combined with mutations of the aforementioned five basic residues. Thus, a series of surface-exposed basic residues in Cdr2 KA-1 may cooperatively establish electrostatic interactions with negatively charged phospholipids.

The Cdr2 KA-1 domain is preceded by a basic motif (8 out of 17 positively charged residues; Fig. 1 A), whose deletion, or mutation of basic residues, resulted in a partial delocalization of Cdr2 from the cortex and appearance of a nuclear pool (Figs. 1 C and S1 A). In combination with mutation of the five basic residues close to the β3–β4 loop, this led to complete detachment of Cdr2 from the cortex. Thus, the basic motif cooperates with Cdr2 KA-1 to promote Cdr2 anchoring at the cortex.

FRAP experiments further revealed that the localization defects observed in the various cortex-anchoring mutants described in this paper correlated with increased dynamic exchange of Cdr2 on the cortex (Fig. 1 D). These mutants also produced longer cells, with stronger Cdr2 localization defects correlating with longer delays in mitotic entry (Table S3), indicating that Cdr2 cortical localization is crucial for its function.

Cdr2 clustering involves both the KA-1 domain and Cdr2 N-terminal interaction with Mid1

A C-terminal region composed of the Cdr2 basic motif and KA-1 domain (residues 591–747), hereafter called Cdr2-Cter, was sufficient for cortex binding (Fig. 2 A). Similar to the Kcc4 KA-1 domain (Moravcevic et al., 2010), recombinant Cdr2-Cter also bound in vitro on lipid strips to phosphatidylserine, a major acidic phospholipid of the plasma membrane (Fig. S1 C). We also observed minor binding to phosphatidic acid and cardiolipin but not to phosphoinositides. Thus, Cdr2-Cter is necessary and sufficient for localization to the plasma membrane.

Interestingly, Cdr2-Cter formed small clusters on the medial cortex in contrast to the equivalent C-terminal domain of in input and pull-down fractions with anti-GST or antimyc antibodies. (right) Normalized Cter-myc signals in GST pull-down measured in two independent experiments. Molecular masses are indicated. (D) Cdr2 KA-1 model highlighting the two hydrophobic residues of the β4–β5 loop (F704 and F705) and the Kcc4 KA-1 structure 3OST (Moravcevic et al., 2010), in which the equivalent loop contains a charged residue (D1001) surrounded by two neutral residues (G1000 and G1002; not depicted). A.U., arbitrary unit; WB, Western blot.
Comparing our Cdr2 KA-1 structural model to the Kcc4 crystallographic structure, we found that the β4–β5 loop of the Cdr2 KA-1, opposite to the membrane-binding surface, is very hydrophobic, whereas the same loop is negatively charged in Kcc4 (Fig. 2 D). Remarkably, mutations of the hydrophobic residues F704 and F705 to aspartic acid (D) abolished Cdr2-Cter clustering (Fig. 3, A and B). Moreover, the interaction between differentially tagged Cdr2-CterFF* mutants was strongly impaired in pull-down experiments (Fig. 3 C). Intriguingly, Kcc4, which was distributed homogeneously on the cortex when expressed in fission yeast (Fig. 2, A and B). This suggested to us that Cdr2 KA-1 may have unique oligomerization properties, not shared with Kcc4 KA-1, which may contribute to Cdr2 assembly into nodes and also allow multivalent membrane binding with high avidity (Lemmon, 2008). Accordingly, we found that differentially tagged wild-type Cdr2-Cter molecules interacted in pull-down experiments, whereas corresponding Kcc4-Cter failed to do so (Fig. 2 C).
Cdr2-CterFF* was distributed evenly along the cortex (Fig. 3 A), suggesting that clustering is necessary to maintain a differential distribution between the medial cortex and the cell tips.

To confirm the specificity of these mutations on Cdr2-Cter oligomerization properties, Cdr2-CterFF* was next compared with Cdr2-CterKbsc, which lacks the basic region preceding the KA-1 domain. In this case, Cdr2-CterKbsc still formed clusters (Fig. 3, A and B), and similar to wild-type Cdr2-Cter, differentially tagged Cdr2-CterKbsc mutants interacted in pull-down experiments (Fig. 3 C).

Finally, we introduced the mutations of hydrophobic residues of the β4–β5 loop in full-length Cdr2 (Cdr2FF*). This led to fewer medial nodes and a small pool of the mutant protein at the cell tips (Figs. 3 E, 4 A, and S2 A). The mutations also resulted in a slightly increased cytoplasmic pool and increased dynamics on the cortex, consistent with a reduced clustering ability (Fig. 3, D and E). Combination of these mutations with mutations in the basic region preceding the KA-1 domain enhanced Cdr2 detachment from the cortex and confirmed that these two regions of Cdr2 cooperate to establish Cdr2 nodes at the cell medial cortex (Fig. S2 A).

In summary, Cdr2 KA-1 is not only essential for membrane binding but also plays a role in Cdr2 clustering. Based on our homology modeling, this specific property of Cdr2 KA-1 compared with Kcc4 KA-1 depends on a hydrophobic loop positioned on the surface opposite to that binding the membrane.

Because the mutations of F704 and F705 disrupted clustering of Cdr2-Cter much more than it disrupted clustering of full-length Cdr2 (Fig. 3, A and E), we examined whether Cdr2 sequences N terminal to Cdr2-Cter also contribute to Cdr2 node assembly. We constructed a Cdr2N-Kcc4C chimera, in which Cdr2-Cter (residues 591–775) was replaced by Kcc4-Cter (residues 917–1,037), to target the Cdr2 N terminus to the cortex. Whereas the Kcc4-Cter does not assemble into nodes on its own (Fig. 2 A), the Cdr2N-Kcc4C chimera formed nodes, though with much lower efficiency compared with full-length Cdr2 (Fig. 4 A). Differentially tagged Cdr2N-Kcc4C chimeras could also interact with one another in immunoprecipitation experiments, although the interactions were weaker than with full-length Cdr2 molecules (Fig. 4 B). Thus, Cdr2 N-terminal regions exhibit clustering properties in addition to the C-terminal KA-1 domain.

We showed previously that Cdr2 associates with the anillin-like protein Mid1 in medial nodes (Almonacid et al., 2009; Moseley et al., 2009). Moreover, Mid1 can self-interact, and a fragment that includes the Cdr2 interaction domain was recently reported to form octamers in vitro (Celton-Morizur et al., 2004; Almonacid et al., 2009; Saha and Pollard, 2012). We thus tested whether Mid1 could play a role in Cdr2 clustering and found that the clustering of Cdr2-N terminus was dependent on interactions with Mid1, as Cdr2N-Kcc4C nodes were abolished in mid1400–450, which lacks the Cdr2 interaction site (Fig. 4 A). Node assembly was also largely disrupted when mid1400–450 was combined with the Cdr2FF* mutation, which was deficient for KA-1–dependent clustering (Fig. 4 A). In contrast, a control experiment showed that the clustering of Cdr2-Cter was maintained in the mid1400–450 mutant (Fig. S2 B). We conclude that Cdr2 clustering relies on self-interactions of the C-terminal KA-1 domain and on N-terminal interactions with Mid1.
We reasoned that to prevent node assembly, Pom1 may regulate Cdr2 interaction with the cortex through modulation of Cdr2 membrane binding and/or clustering. To test this, we expressed a Pom1-C-Mid1C chimera (called Pom1-chimera hereafter) in which the Mid1 C-terminal amphipathic helix targets Pom1 kinase domain to the medial cortex independently of Cdr2 (Celton-Morizur et al., 2004; Almonacid et al., 2009). This construct was previously shown to induce Cdr2 redistribution on the cortex and to delay mitotic entry (Moseley et al., 2009), demonstrating that the Pom1 kinase domain is active. We found that the expression of Pom1-chimera strongly increased Cdr2 dynamics on the cortex as measured by FRAP (Fig. 5, C, F, and G).

In contrast, we did not detect significant changes in Cdr2 exchange rate on the medial cortex upon pom1 deletion (Fig. 5 D). In these cells, Cdr2 was detected in the nongrowing cell tip region as expected (Fig. 5 E; Martin, 2009; Moseley et al., 2009). We reasoned that to prevent node assembly, Pom1 may regulate Cdr2 interaction with the cortex through modulation of Cdr2 membrane binding and/or clustering. To test this, we expressed a Pom1-C-Mid1C chimera (called Pom1-chimera hereafter) in which the Mid1 C-terminal amphipathic helix targets Pom1 kinase domain to the medial cortex independently of Cdr2 (Celton-Morizur et al., 2004; Almonacid et al., 2009). This construct was previously shown to induce Cdr2 redistribution on the cortex and to delay mitotic entry (Moseley et al., 2009), demonstrating that the Pom1 kinase domain is active. We found that the expression of Pom1-chimera strongly increased Cdr2 dynamics on the cortex as measured by FRAP (Fig. 5, C, F, and G).

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We measured slightly faster exchange rates at nongrowing cell tips than on the medial cortex (Fig. S3 A).

We conclude that Pom1 may normally restrict Cdr2 nodes to the medial cortex by increasing Cdr2 dynamic exchange at the cell tips, thereby restricting node assembly to the cell middle where Cdr2 can associate more stably with the cortex. Pom1-independent factors could also contribute to a minor extent to the differential regulation of Cdr2 exchange on the cortex between nongrowing cell tips and the cell middle.

**Pom1 down-regulates Cdr2 clustering by modulation of Cdr2-Mid1 interaction**

To increase Cdr2 turnover on the cortex, Pom1 could reduce Cdr2 affinity for the plasma membrane or down-regulate its clustering properties. Using the Pom1 chimera, we first tested a possible effect of Pom1 on Cdr2 N-terminal clustering. Strikingly, neither Cdr2N-Kcc4C chimera nor Cdr2FF* mutant formed nodes in the presence of Pom1-chimera and redistributed toward the cell tips in a smoother pattern (Figs. 6 A and S3 C). We also found after careful quantification that Pom1-chimera reduced by ~50% the level of Mid1 coimmunoprecipitation with Cdr2N-Kcc4C (Fig. 6 B) and by ~25% the degree of colocalization between Cdr2 and Mid1 nodes at the medial cortex (Fig. 6 C). In contrast, the degree of colocalization between Cdr2 and Mid1 nodes remained similar upon pom1 deletion (Fig. S3 B). Together, these results indicate that Pom1 negatively regulates the Mid1-dependent clustering of Cdr2 N terminus.

It has been shown that Cdr2 association with Mid1 partially depends on Cdr2 kinase activity (Almonacid et al., 2009; Moseley et al., 2009). Accordingly, we found that disrupting Cdr2 kinase activity by introduction of a kinase-dead mutation (E177A referred to as Cdr2KΔ, Morrell et al., 2004) abolished Cdr2N-Kcc4C node formation (Fig. 6 A). A similar result was obtained with the Cdr2FF* mutant (Fig. S3 C). Disrupting Cdr2 kinase activity also reduced both Mid1 coimmunoprecipitation with Cdr2N-Kcc4C chimera and the degree of Mid1 colocalization with Cdr2 cortical nodes, mimicking the effect of Pom1 (Fig. 6, B and C).

In contrast, as reported previously (Almonacid et al., 2009; Moseley et al., 2009), Cdr2KΔ mutation alone or in combination with mid1Δ400-450 was neither sufficient to affect node formation by full-length Cdr2 nor to mimic Pom1-chimera effect on its dynamic exchange on the cortex (Figs. 6 A and S3, D and E). Thus, inhibiting the Mid1-dependent clustering of the Cdr2 N terminus is not sufficient to recapitulate Pom1 effect on Cdr2. This indicates that Pom1 may regulate additional properties of Cdr2 besides Mid1-dependent clustering.

**Pom1 phosphorylates Cdr2 C-terminal basic motif**

We thus tested whether Pom1 also acts on the Cdr2 C terminus (Fig. 7, A and B). Analysis of Cdr2-Cter distribution on the cortex revealed that it was largely excluded from both cell tips in most wild-type cells but enriched at one or both cell tips in the majority of pom1Δ cells, reminiscent of the localization of full-length Cdr2 in pom1Δ cells (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009). In addition, Cdr2-Cter was more enriched at both tips upon expression of Pom1-chimera. Cdr2-Cter clustering was not affected in these conditions. Finally, deletion of the Cdr2-Cter basic motif also induced a redistribution of Cdr2-Cter toward the cell tips, suggesting that this basic motif could be a Pom1 target.

Proteomic analysis of phosphopeptides from Cdr2-mEGFP immunoprecipitated from wild-type cells revealed four sites phosphorylated in vivo within the Cdr2-Cter basic motif (S804, S807, S616, and S618; Figs. 7 C and S4 A). In addition, recombinant GST-Pom1 phosphorylated a maltose-binding protein (MBP)-Cdr2 fragment comprising the Cdr2-Cter basic motif (aa 518–620) in vitro (Figs. 7 D and S4 B). Mutation of these four sites to nonphosphorylatable residues (Cdr2KΔC) reduced 32P incorporation (shorter Cdr2 fragments within this region could not be tested as substrates as they were unfortunately unstable or insoluble; Figs. 7 D and S4 B). These results suggest that the four sites represent some of the Pom1 phosphosites on Cdr2, although other as-yet-unidentified phosphosites may also be targeted by Pom1 in vitro.

Substitution of S618 by aspartic acid, alanine, or arginine in Cdr2 expressed from its endogenous locus led to cell elongation at division in all cases (unpublished data), suggesting that mutations of this amino acid may unfold the region and alter Cdr2 interaction with the cortex nonspecifically, precluding a functional interpretation of these results. We therefore produced a triple substitution of serines 604, 607, and 616 to aspartic acid (Cdr2-CterKΔC) and Cdr2KΔC mutants) to mimic phosphorylation or to alanine to inhibit phosphorylation (Cdr2-CterKΔC and Cdr2KΔC mutants). These mutations affected the cortical distribution of Cdr2-Cter only mildly (Figs. 7 E and 8 A). Nevertheless, intensity measurements revealed that Cdr2-CterKΔC and Cdr2KΔC were less concentrated in the medial region compared with cell tips (Fig. 7 F). The localization of full-length Cdr2KΔC and Cdr2KΔC mutants was largely similar to wild-type Cdr2, although a small pool of these mutants could be detected at the cell tips in ~40% of cells (Fig. S5 A). FRAP analysis also revealed faster exchange rates for Cdr2KΔC on the cortex compared with wild-type Cdr2 (Fig. S5 B). In contrast, exchange rates were slightly slower when serines 604, 607, and 616 were converted to basic residues (Cdr2KΔC; Fig. S5 B). This confirms that the basic region establishes electrostatic interactions with acidic phospholipids of the plasma membrane that can be negatively modulated by negative charges mimicking phosphorylation or artificially enhanced by addition of basic residues. Collectively, these results suggest that the Cdr2 C-terminal basic motif, adjacent to the KA-1 domain, is a direct target of Pom1 to modulate Cdr2 affinity for acidic phospholipids of the plasma membrane.

**Combining mutations in the basic regions with inhibition of N-terminal clustering recapitulates Pom1 regulation**

As described thus far, the two relatively mild effects of Pom1 on Cdr2 (effect on clustering mediated through the Cdr2 N-terminal region and effect on membrane binding involving the C-terminal basic motif adjacent to the KA-1 domain) are
individually insufficient to promote Cdr2 node disassembly. We thus tested next whether their combination could recapitulate Pom1 inhibition of node assembly. To do so, we produced a double cdr2bsc-3D mid1\(^{400–450}\) mutant to mimic the dual regulation performed by Pom1. In this context, Cdr2 node assembly was strongly affected specifically in the number of nodes (Fig. 8, A and B).
Figure 7. Pom1 phosphorylates the Cdr2 C-terminal basic domain to modulate Cdr2 association with membranes. (A) Localization of GFP–Cdr2-Cter in wild-type (wt) and pom1Δ cells or in cells expressing the Pom1-chimera and of GFP–Cdr2-Cterbsc in wild-type cells. (B) GFP–Cdr2-Cter fluorescence intensity ratio between the cell tip and the medial cortex in strains shown in A: Cdr2-Cter (n = 31), Cdr2-Cter pom1Δ (n = 39), Cdr2-Cter Pom1-chimera (n = 37), and Cdr2-Cterbsc (n = 34) from two experiments. T1, tip of lowest intensity; T2, tip of highest intensity. Horizontal bars are means. (C) Scheme highlighting the sequence of Cdr2 basic domain and residues found to be phosphorylated in vivo (red). (D) In vitro kinase assay of GST-Pom1 on MBP-Cdr2(518–620) with the indicated mutations. (Top) Phosphorimager detection of 32P incorporation. (Bottom) Silver-stained gel. The top band represents Pom1 autophosphorylation. Molecular masses are indicated. Black bars indicate that intervening lanes have been spliced out. (E) Localization of GFP–Cdr2-Cter bsc-3D (S604D, S607D, and S616D) or GFP–Cdr2-Cterbsc-3A (S604A, S607A, and S616A). (F) GFP–Cdr2-Cter fluorescence intensity ratio between the cell tip and the medial cortex in strains shown in E: Cdr2-Cter (n = 45), Cterbsc-3D (n = 39), and Cterbsc-3A (n = 44) from one experiment representative of two repeats. Horizontal bars are means. Gray bars represent t tests: *, P < 5 × 10−2; **, P < 5 × 10−3. Bars, 5 µm.
In contrast, combining cdr2*bssc-3A with mid1Δ400-450 did not impair node assembly. Similar results were obtained when cdr2*bssc-3D or cdr2*bssc-3A was combined to the cdr2KD mutation (Fig. 8, A and B).

We finally tested whether the cdr2*bssc-3A mutant presented functional defects in division plane positioning similar to pom1Δ cells. Because Cdr2-dependent positioning of Mid1 is corrected by Mid1 export from the nucleus at mitotic entry (Almonacid et al., 2009), we combined the cdr2*bssc-3A mutation with the mid1nsm mutation preventing Mid1 shuttling in the nucleus (Almonacid et al., 2009). Measuring septum position, we
found that the double mutant divided more asymmetrically than either single mutant (Fig. 8 C), although the degree of asymmetry was less pronounced than in pom1Δ cells. We conclude that the cdr2<sup>2bsc-3A</sup> mutant exhibits some Pom1-resistant properties for division plane positioning.

We conclude that Pom1 restricts Cdr2 node assembly to the cell middle by a dual modulation of its clustering and affinity for membrane lipids. The fact that similar effects are elicited by preventing Mid1-dependent clustering of the Cdr2 N terminus and by inhibition of Cdr2 kinase activity suggests that the Pom1 effect on Cdr2 clustering could possibly rely on the down-regulation of Cdr2 kinase activity (Fig. 9).

**Discussion**

Fission yeast assembles its cytokinetic contractile ring in two major steps: first, by recruitment of ring components on precursors nodes in a medial region of the cortex and second, by compaction of mature nodes into a tight and well-centered ring. Such a mechanism may be evolutionarily conserved because two genetically independent steps of assembly have also been described in Caenorhabditis elegans (Lewellyn et al., 2010).

A key feature of this assembly mechanism is that precursor nodes are heterooligomeric assemblies of membrane-binding proteins. A detailed description of the composition and number of molecules present in these nodes before and after their mitotic maturation has been obtained (Wu et al., 2003; Wu and Pollard, 2005; Almonacid et al., 2009; Laporte et al., 2011; Akamatsu et al., 2014), and node motion studies combined with mathematical modeling have revealed that transient actomyosin-dependent attraction forces between nodes can promote their compaction into a functional ring with the contribution of F-actin–bundling proteins (Vavylonis et al., 2008; Ojkic et al., 2011; Laporte et al., 2012). In particular, modeling highlighted that the total number of cortical nodes and the width of their distribution must remain in a narrow range for an efficient assembly process.

Cdr2 has been shown to be the major node scaffolding component, which initiates node assembly during interphase (Almonacid et al., 2009; Moseley et al., 2009). Nevertheless, how Cdr2 controls node assembly and how the Pom1 gradients function to influence node distribution and limit node number and node spreading to a narrow region of the medial cortex remain unclear. In this work, we established some of the key molecular properties by which Cdr2 can assemble into cortical nodes and deciphered molecular mechanisms of Pom1-dependent control of node assembly.

Cdr2 membrane binding and clustering properties drive node assembly

Cdr2 ability to assemble into cortical nodes relies on (a) Cdr2 membrane binding properties and (b) Cdr2 propensity to assemble into clusters of molecules. Membrane binding is ensured primarily by a C-terminal lipid-binding KA-1 domain, which functions similarly to Kcc4 KA-1 (Moravecic et al., 2010) and may bind to phosphatidylinerine, an abundant acidic phospholipid of the inner leaflet of the plasma membrane. Accordingly, using a model of Cdr2 KA-1 based on Kcc4 crystal structure, we could show that Cdr2 KA-1 possesses several likely surface-exposed basic residues that are required for Cdr2 targeting to the cortex and likely establish electrostatic interactions with acidic phospholipids of the plasma membrane.

Membrane binding of Cdr2 is reinforced by a basic motif that lies a few residues before Cdr2 KA-1 along the Cdr2 sequence. This motif is of particular interest, as we found that it constitutes a modulator of Cdr2 membrane binding affinity that is targeted by Pom1 kinase (see Pom1 controls Cdr2 node assembly and distribution in Discussion).

Cdr2 clustering depends on a dual mechanism. It involves primarily Cdr2 KA-1. This additional and unique clustering property of Cdr2 KA-1 compared with previously characterized KA-1s (Moravecic et al., 2010) involves the hydrophobic β4–β5 loop containing two phenylalanine residues, not conserved in Kcc4 KA-1 and located opposite to the putative membrane binding surface in the Cdr2 KA-1 model. Additional work will be necessary to establish how this loop confers oligomerization properties to Cdr2 KA-1. Interestingly, this feature of Cdr2 KA-1 may be shared with other fission yeast species and other filamentous fungi because the β4–β5 loop of Cdr2 orthologues in Schizosaccharomyces japonicus or Aspergillus nidulans also contain hydrophobic residues (unpublished data).

The second mechanism of clustering depends on Cdr2 N-terminal region and requires Mid1. This result was unexpected because Cdr2 was previously shown to assemble into nodes in the absence of Mid1 or when Mid1 cannot interact with Cdr2 (Mid1<sup>400→450</sup> mutant; Almonacid et al., 2009; Moseley et al., 2009). Indeed, the Mid1–Cdr2 interaction was shown to recruit Mid1 to Cdr2 nodes (Almonacid et al., 2009; Moseley et al., 2009). Our results now show it also contributes to reinforcing these nodes through Cdr2 clustering, in addition to the KA-1 domain. Clustering mediated by a partner protein is reminiscent
of the reported septin-dependent clustering of Kcc4 at the bud neck (Moravcevic et al., 2010) and may rely on Mid1 oligomerization properties (Celton-Morizur et al., 2004; Saha and Pollard, 2012). Because the Mid1–Cdr2 interaction is reinforced by Blt1 and Gef2 (Guzman-Vendrell et al., 2013), these components of medial cortical nodes may also contribute to Cdr2 clustering. Similar to the nonessential membrane-binding basic motif, Mid1-dependent Cdr2 clustering is of particular physiological interest because it can be modulated by Pom1 (see next section).

Pom1 controls Cdr2 node assembly and distribution

Our work shows that Pom1 controls the distribution of Cdr2 nodes in two distinct ways: modulation of Cdr2 membrane binding and modulation of Mid1-dependent clustering (Fig. 9). Mass spectrometry (MS) and in vitro phosphorylation experiments identified four phosphorylation sites within the Cdr2 basic motif. Among them, S604 but not the others, matches the R–(X)1–3,S/T/P/V/L DYRK2 consensus. However, we note that DYRK kinases phosphosites are sometimes loosely related to the consensus, in particular Pom1 autophosphorylation sites identified from bacterially expressed Pom1 (Campbell and Proud, 2002; Aranda et al., 2011; Hachet et al., 2011). Functional analysis showed Pom1-dependent phosphorylations on the C-terminal basic motif increase slightly Cdr2 exchange rate on the cortex without important effect on node assembly on their own.

Whereas Pom1 does not appear to modulate the unique clustering properties of Cdr2 KA-1 domain, we found that it acts on Mid1-dependent clustering of Cdr2 in addition to the aforementioned modulation of Cdr2 affinity for lipids. Thus, Mid1, which represents the physiological target of Pom1-dependent spatial regulation of Cdr2 nodes for division plane positioning, appears to contribute to its own spatial regulation rather than bind passively to Cdr2 nodes prepositioned in the cell middle by Pom1.

It is interesting that the effect of Pom1 on Mid1-dependent clustering of Cdr2 can be mimicked by a down-regulation of Cdr2 kinase activity, which has been shown to modulate Mid1 recruitment to Cdr2 nodes (Almonacid et al., 2009; Moseley et al., 2009). It is thus possible that Pom1 controls Cdr2 clustering by modulating its activity. One recently proposed mechanism by which Pom1 may inhibit Cdr2 activity, without a noted effect on Cdr2 node localization or assembly, involves Pom1-dependent phosphorylation of the short C-terminal tail of Cdr2 after the KA-1 domain (Bhatia et al., 2014; Deng et al., 2014). This phosphorylation was shown to antagonize the phosphorylation of Cdr2 T loop by the calcium/calmodulin-dependent protein kinase Ssp1, necessary for the activation of Cdr2 (Deng et al., 2014).

However, this C-terminal tail phosphorylated by Pom1 is absent in the Cdr2N-Kcc4–KA-1 chimera used to establish that Pom1 regulates Mid1-dependent clustering of Cdr2. Future experiments will thus be necessary to address if and how Pom1 controls Cdr2 kinase activity independently of the C-terminal tail.

Although neither of the two additional effects exerted by Pom1 on Cdr2 that we describe here is sufficient on its own, we show that mimicking them simultaneously strongly reduces the efficiency of node assembly. We propose that, by combining two mild effects on membrane binding and clustering, Pom1 renders Cdr2 node assembly less favorable at the cell tips compared with other regions of the cortex, leading to accumulation of stable nodes at the cell middle. The Cdr2 clustering properties may further reinforce this localization by increasing the avidity of Cdr2 molecules for regions with established nodes compared with Cdr2-free regions of the cortex, thus creating a positive feedback focusing the concentration of Cdr2 on the medial cortex.

We note that the assembly of Cdr2 nodes is not fully abolished upon modulation of Cdr2 membrane binding and Mid1-dependent clustering. It is thus possible that Pom1 has additional effects on Cdr2, through as-yet-unidentified phosphorylation sites or on Cdr2 partners within medial cortical nodes (e.g., Mid1, Blt1, or Gef2) to further inhibit node assembly.

Additional factors are also likely to contribute to Cdr2 node spatial restriction to the cell middle besides Pom1. Indeed, it is well established that in the absence of Pom1, Cdr2 node distribution depends on the cell’s growth pattern (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009), though how growth prevents Cdr2 node assembly remains elusive. Possible factors contributing to Cdr2 exclusion from growing cell tips include lipid microdomains, sterol-rich microdomains in particular because they are enriched at cell tips (Wachtler et al., 2003), and filipin treatment was shown to disrupt Cdr2 nodes (Morrell et al., 2004). Interestingly, in pom1Δ cells, the Cdr2 domain, which spans approximately two thirds of the cell length, keeps enlarging with cell length, indicating that Cdr2 domain enlargement with cell size may be largely driven by cell extension. This growth pattern–dependent control may thus further reinforce Cdr2 medial localization, in addition to the Pom1-dependent mechanisms described here.

Finally, the presence of Cdr2 at the cell cortex is important not only for division plane positioning but also for the timing of cell division (Morrell et al., 2004; Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009). Accordingly, we found that mutants with important cortex anchoring defects displayed a longer cell size at division, suggesting that minimum amounts of cortical Cdr2 are necessary for timely entry in mitosis. Nevertheless, we also showed recently that a 50% reduction of Cdr2 amounts does not modify cell length at division in diploids, whereas mild overexpression of Cdr2 in haploids strongly advances mitosis only if pom1Δ is deleted (Bhatia et al., 2014). These data indicate that mitotic commitment is fairly robust to alterations in Cdr2 levels when Cdr2 activity is under Pom1 control. Thus, the Pom1-dependent regulation of Cdr2 node assembly described here may primarily serve as an input for division plane placement, whereas the timing of cell division is modulated by Pom1-dependent regulation of Cdr2 kinase activity (Bhatia et al., 2014; Deng et al., 2014).

In conclusion, our work provides a first mechanistic model for how Pom1 gradients can delineate the medial region of the cell where Cdr2 cooperates with Mid1 to assemble precursor nodes for the actomyosin ring to determine the position of the division plane accurately and robustly and favor the production of equally sized daughter cells. This model may be conceptually relevant to spatial regulatory events involving membrane-based gradients during cell polarity establishment in other eukaryotes.
Materials and methods

Yeast genetics and culture

Standard S. pombe media and genetic manipulations were used (Moreno et al., 1991). All strains used in the study were isogenic to wild-type 972D and are described in Table S1. Strains from genetic crosses were selected by random spore germination or tetrad dissection and replica in plates with appropriate supplements or drugs. Transformations were performed using the lithium acetate–DMSO method as previously described (Bähler et al., 1998).

Production of mutant and tagged strains

Cdr2 deletion strain, a gift from J. Moseley (Geisel School of Medicine, Dartmouth College, Hanover, NH; Moseley et al., 2009) was produced by homologous recombination using the pFA6a-NatMX6 plasmid (Hentges et al., 2005) as previously described (Bähler et al., 1998). All cdr2 mutant alleles were integrated at the cdr2 endogenous locus, except those cloned in pJK148- or pk210-derived plasmids, as described in Table S1. These constructs were, respectively, integrated at the leu1 and urad locus under the control of their own promoter unless stated otherwise.

Mutants of Cdr2 KA-1 domain, basic region, or Cdr2 kinase-dead mutant (Cdr2 K2-234 carrying the E177A substitution) were produced by site-directed mutagenesis of sP3R, a plasmid-based plasmid carrying Cdr2 ORF, or by double PCR. Most mutants were then subcloned between BamHI and PacI sites in pSR34 (Guzman-Vendrell et al., 2013), a pA6a-mEGFP-KanMX6-derived plasmid carrying cdr2 promoter and terminator for integration at cdr2 locus in a cdr2::KanMX6 strain, in replacement of the NatMX6 cassette by homologous recombination. Plasmids were digested with NotI before transformation.

Cdr2N-Kcc4C and Cdr2N-Kcc4C chimeras containing Cdr2 residues 1–590 and Kcc4 residues 971–1,037, Cdr2 terminally tagged with GFP, were inserted between BamHI and Ascl sites of pSR34. Plasmids were digested with SpeI before transformation.

The sequence encoding Cdr2-Cter (residues 591–747) N-terminally tagged with GFP, carrying or not carrying mutations (FF, J3sc, bscl-3c, and bscl-3a), was cloned between PacI and Ascl sites in pSR25. GFP-Kcc4-Cter (residues 893–1,037) was cloned in pSR25 between BamHI and Ascl sites.

Cdr2Ndr1*, Cdr2Ndr2*, and Cdr2Ndr3* mutants C-terminally tagged with GFP were cloned between Xhol and SalI sites in an Xhol-derived plasmid (Kennedy and Boeke, 1999) containing cdr2 promoter and nmt1 terminator. Plasmids were digested with NruI before transformation.

Cdr2, Cdr2-Cter, Cdr2-Cterns*, Cdr2-Cter11*, and Kcc4-Cter were also cloned between Xhol and NolI sites in a pJK148-plasmid containing the cdr2 promoter, a C-terminal myc12 tag derived from pWV01-myc (Iacovoni et al., 1999), and the nmt1 terminator. Plasmids were digested with NruI before transformation.

Cdr2N-Kcc4C chimera was subcloned between Xhol and NolI sites in a pk210-derived plasmid (Kennedy and Boeke, 1994) containing the cdr2 promoter, a C-terminal myc12 tag, and the nmt1 terminator. This plasmid was digested with StuI before transformation.

To produce a Cdr2-TagRFP fusion, Cdr2 was amplified by PCR from pTagRFP-PN (Evrogen) and cloned between PacI and Ascl sites in pFA6a-GFP:NatMX6 (Hentges et al., 2005) in replacement of GFP to obtain the pFA6a-TagRFP-NatMX6 plasmid. This plasmid was used to integrate TagRFP in the JCB • VOLUME 206 • NUMBER 1 • 2014
of 1 mM IPTG and incubation for 1 h. Bacterial pellets were resuspended for 4 h at 30°C. Protein expression was induced by the addition of tagged strains. In brief, cells were grown overnight in LB (Luria–Bertani) medium supplemented with 100 µg/ml ampicillin at 37°C. Soluble extract was incubated with 200 µl glutathione–Sepharose beads at 50% slurry for 2 h at 4°C. Finally, beads were washed 3 x with cold PBS and eluted in four steps at 100 µl elution buffer (15 mM reduced glutathione and 50 mM Tris-HCl, pH 8). Eluted proteins were incubated with lipid strips (Echelon, Inc.) following the manufacturer’s protocol. Lipid strips were probed with a rabbit affinity-purified anti-GST antibody (1:100; gift from J. Dumont).

Expression of GST-Cdr2 fragments from pGEX-4T-1-derived plasmids was performed as described in the “Recombinant protein production and lipid-binding assay” section. MBP-Acryl–TEV-Cdr2(518–620) and MBP–TEV–Cdr2(518–620) fragments were also induced in BL21 cells from pMAL–TEV-derived plasmid as described in the “Recombinant protein production and lipid-binding assay” section. In brief, cells were grown overnight in LB supplemented with 100 µg/ml ampicillin at 37°C. 250 ml of LB-ampicillin was inoculated with 6.25 ml of the saturated culture, grown 3 h at 37°C. Protein expression was induced by the addition of 100 µM IPTG for 5 h at 18°C. Bacterial pellets were resuspended in 10 ml of cold resuspension buffer (50 mM Tris–Cl, pH 8, 1 mM EDTA, 100 mM KCl, and PMSF), sonicated 3 x for 30 s (50% amplitude), incubated with 1% Triton X-100 at 4°C, and centrifuged 15 min at 4°C at 10,000 g. Soluble extract was incubated with 400 µl amyllose resin (New England Biolabs, Inc.) for 2 h at 4°C. Finally, beads were washed 3 x with cold resuspension buffer and eluted in three steps with 100 µl elution buffer (50 mM Tris–Cl, pH 8, 1 mM EDTA, 100 mM KCl, and 10 mM maltose).

For kinase assays, recombinant GST–Pom1 or GST–Pom1T40 fusion proteins were expressed in BL21 cells and purified with glutathione–Sepharose 4B (GE Healthcare) columns according to the manufacturer’s protocol (Hachet et al., 2011). Kinase assays were performed in 30 mM Tris, 100 mM NaCl, 10 mM MgCl2, 1 mM EGTA, 10% glycerol, 1 mM DTT, 20 µM ATP, and 2 µCi [32P]ATP (PerkinElmer) in a 15-µl final volume reaction. After a 30-min incubation at 30°C, the reaction was stopped by boiling in sample buffer and analyzed by SDS-PAGE. 32P incorporation was detected by autoradiography with phosphorimager (Typhoon FLA 7000, GE Healthcare). Silver staining was performed according to the manufacturer’s protocol (Silver Stain Kit; Thermo Fisher Scientific) to check for equivalent amounts of substrates.

Online supplemental material
Fig. S1 reports additional properties of Cdr2 C-terminal domain. Fig. S2 shows the localization of the Cdr2bsc*-FF* mutant and of Cdr2-Cter in mid1Δ/Δ cells. Fig. S3 analyses the role of Pom1, Mid1, and Cdr2 kinase activity in Cdr2 node assembly. Fig. S4 defines how Pom1 phosphorylates the Cdr2 basic region. Fig. S5 analyses the dynamic exchange on the cortex of Cdr2 phosphoinhibitory or phosphomimetic mutants in the basic region. Tables S1 and S2 list strains and plasmids used in this study, respectively. Table S3 reports cell length at division in Cdr2 mutants in the C-terminal–anchoring domain. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201311097/DC1.

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