Title: Stable Pom1 clusters form a glucose-modulated concentration gradient that regulates mitotic entry

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Abstract: Control of cell size requires molecular size sensors that are coupled to the cell cycle. Rod-shaped fission yeast cells divide at a threshold size partly due to Cdr2 kinase, which forms nodes at the medial cell cortex where it inhibits the Cdk1-inhibitor Wee1. Pom1 kinase phosphorylates and inhibits Cdr2, and forms cortical concentration gradients from cell poles. Pom1 inhibits Cdr2 signaling to Wee1 specifically in small cells, but the time and place of their regulatory interactions were unclear. We show that Pom1 forms stable oligomeric puncta that dynamically sample the cell cortex. Binding frequency is patterned into a concentration gradient by the polarity landmarks Tea1 and Tea4. Pom1 puncta colocalize with Cdr2 nodes, forming a glucose-modulated inhibitory threshold against node activation. Our work reveals how Pom1-Cdr2-Wee1 operates in multiprotein clusters at the cell cortex to promote mitotic entry at a specific size that can be modified by nutrient availability.
**Introduction:**

Many cell types display a remarkable ability to maintain a constant size during rapid cycles of growth and division (Fantes and Nurse, 1977; Ginzberg et al., 2015; Jorgensen and Tyers, 2004; Lloyd, 2013). Such cell size control is a systems-level property that emerges from the integration of multiple size-dependent signal transduction pathways. Each signaling pathway is comprised of tunable biochemical parameters, including gene-expression and post-translational modifications such as protein phosphorylation (Alberghina et al., 2009). One major challenge in cell size research is to understand the biochemical mechanisms of signal transduction in each pathway, and what makes them size-dependent. These control systems can generate size homogeneity for a given cell type, but cell size is also an adaptable property. For example, nutritional cues and other environmental factors can alter cell size (Fantes and Nurse, 1977; Kelkar and Martin, 2015; Shiozaki, 2009; Yanagida Mitsuhiro et al., 2011). Thus, a second major challenge in cell size research is to understand how size-dependent signaling pathways respond to changes in cell metabolism and stress.

In eukaryotic cells, these signaling pathways lead to regulated activation of the conserved cyclin-dependent kinase Cdk1 (Harashima et al., 2013). Activated Cdk1 triggers mitotic entry and the cascade of events that lead to cell division (Gould and Nurse, 1989; Simanis and Nurse, 1986). The fission yeast *Schizosaccharomyces pombe* represents an excellent model system to study size-dependent signaling pathways that regulate Cdk1. Genetic screens performed in past decades have identified many conserved factors that regulate Cdk1, but how these factors form size-dependent signaling pathways remains less clear. Fission yeast cells have a simple geometry that facilitates cell size studies. These cylindrical cells maintain a constant cell width, and grow by linear extension during interphase (Fantes and Nurse, 1977; Moreno et al., 1989). A network of cell polarity proteins positioned at cell tips restricts growth to these sites and maintains proper cell morphology (Chang and Martin, 2009). As a result, cell length doubles in one cell cycle, and many aspects of cell geometry scale with this increase in cell length (Gu and Oliferenko, 2019; Neumann and Nurse, 2007). Recent studies used cell shape mutants to show that fission yeast cells primarily measure surface area, not
length or volume, for cell size control (Facchetti et al., 2019; Pan et al., 2014). A critical next step is to understand how signaling pathways that regulate Cdk1 might operate at the cell cortex in a size-dependent manner.

In all eukaryotes, Cdk1 activity is established by the opposing activities of the inhibitory protein kinase Wee1, and the counteracting phosphatase Cdc25 (Gautier et al., 1991; Gould and Nurse, 1989; Kumagai and Dunphy, 1991; Russell and Nurse, 1986, 1987; Strausfeld et al., 1991). In fission yeast, mutations in Wee1, Cdc25, and their upstream regulators lead to changes in cell size that correspond to changes in the signaling pathways. Separate mechanisms appear to link cell size with regulation of Wee1 versus Cdc25. For Cdc25, its cellular concentration increases through unknown mechanisms as cells grow during interphase (Keifenheim et al., 2017; Moreno et al., 1990). In contrast, the concentration of Wee1 remains constant during interphase, but it is progressively phosphorylated by the conserved inhibitory kinases Cdr1 and Cdr2 (Aligue et al., 1997; Breeding et al., 1998; Kanoh and Russell, 1998; Keifenheim et al., 2017; Lucena et al., 2017; Opalko and Moseley, 2017; Russell and Nurse, 1987; Wu and Russell, 1993; Young and Fantes, 1987). cdr2 mutants fail to divide at a constant surface area, and instead dividing according to cell volume or length (Facchetti et al., 2019). This change suggests that Cdr2-Cdr1-Wee1 signaling underlies the primary size-sensing pathway that relates to surface area, while additional pathways related to volume and length are engaged in its absence. The localization of Cdr2, Cdr1, and Wee1 supports this model. Cdr2 forms punctate oligomeric structures called nodes that stably bind to the medial cell cortex, and recruits Cdr1 to these sites (Akamatsu et al., 2014, 2017; Guzmán-Vendrell et al., 2015; Martin and Berthelot-Grosjean, 2009; Morrell et al., 2004; Moseley et al., 2009). Wee1 localizes primarily in the nucleus and spindle-pole body, where it encounters Cdk1 to prevent mitotic entry (Masuda et al., 2011; Moseley et al., 2009; Wu et al., 1996). In addition, Wee1 transiently visits cortical Cdr1/2 nodes in bursts that lead to inhibition of Wee1 kinase activity (Allard et al., 2018). The frequency and duration of these Wee1 bursts at Cdr1/2 nodes increase approximately twenty-fold as cells double in size during interphase (Allard et al., 2018). This mechanism leads to size-dependent inhibition of Wee1 by a signaling pathway at the cell surface.
This size-dependent change in Wee1 bursting dynamics is encoded into the Cdr1-Cdr2-Wee1 pathway at two proposed levels. First, increased Wee1 bursting depends upon the doubling in number of Cdr1/2 nodes, which occurs as growth results in a doubling of cell surface area, but this increase is smaller than the magnitude of change in Wee1 bursting (~2-fold vs ~20-fold) (Allard et al., 2018; Deng and Moseley, 2013; Pan et al., 2014). Second, activation of Cdr2 increases as cells increase in size (Deng et al., 2014). Cdr2 kinase activity is required for Wee1 localization to nodes, and is controlled by the upstream kinase Pom1 (Allard et al., 2018; Moseley et al., 2009). Pom1 directly phosphorylates and inhibits the activation of Cdr2 (Bhatia et al., 2014; Deng et al., 2014; Kettenbach et al., 2015; Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009). Pom1 also phosphorylates a separate set of sites on Cdr2 to disrupt oligomerization and membrane binding (Bhatia et al., 2014; Rincon et al., 2014). This regulation of Cdr2 by Pom1 changes the size dependency of Wee1 bursts. Specifically, the frequency and duration of Wee1 bursts at Cdr1/2 nodes increases, but only in small cells (Allard et al., 2018). Consistent with this defect, pom1Δ cells divide at a small size due to dysregulation of the Cdr2-Cdr1-Wee1 pathway (Bähler and Pringle, 1998; Bhatia et al., 2014; Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009; Wood and Nurse, 2013). Taken together, these results suggest a size-dependent interaction between Pom1 and its substrate Cdr2. However, the location and timing of Pom1 interactions with Cdr2 have remained poorly defined.

Pom1 localizes in a spatial gradient that is enriched at cell tips, with a lower concentration at the medial cell cortex, where its substrate Cdr2 forms nodes (Bähler and Pringle, 1998; Bhatia et al., 2014; Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009; Pan et al., 2014). Thus, the majority of Pom1 and Cdr2 molecules in a cell are spatially separated, raising the question of when and where they interact. Several lines of evidence suggest that the spatial distributions of Pom1 and Cdr2 are critical for the size-dependent signaling properties of this pathway, and suggest the lateral cell cortex as the key interface. For example, ectopic targeting of Pom1 to the medial cell cortex inhibits Cdr2 node formation and Cdr2-dependent cell size signaling (Martin and
Additionally, the Pom1 gradient is dissipated as part of a cellular response to glucose deprivation, which leads to increased Pom1 concentration at the lateral cortex where it delays mitotic entry (Kelkar and Martin, 2015). This result also demonstrates that Pom1-Cdr2 signaling responds to environmental input to coordinate cell size with nutrient availability. In this system, Pom1 functions analogously to an intracellular morphogen, acting as a concentration-dependent and localization-controlled inhibitor of Cdr2 nodes. However, levels of Pom1 at the medial cell cortex in wild-type cells growing under steady state conditions are low, constant, and do not vary with cell size (Bhatia et al., 2014; Pan et al., 2014). The mechanism by which Pom1 provides size specific input to Cdr2 is unclear and requires analysis of their molecular dynamics at the lateral cell cortex.

Past studies have led to a model for Pom1 gradient formation driven by the binding and diffusion of individual Pom1 molecules in the plasma membrane (Hachet et al., 2011; Saunders et al., 2012). Pom1 binds directly to anionic phospholipids at the cell cortex, and these interactions are disrupted upon intramolecular autophosphorylation (Hachet et al., 2011). Polarity factors including Tea1 and Tea4 are enriched at the cell tips by microtubules, where they recruit the protein phosphatase 1 (PP1) Dis2, which dephosphorylates Pom1 to promote membrane attachment specifically at the cell tip (Hachet et al., 2011). Cortical Pom1 molecules are then thought to diffuse in the plane of the membrane away from the tip, until multiple autophosphorylation events disrupt membrane binding (Hachet et al., 2011; Hersch et al., 2015; Saunders et al., 2012). Tip-concentrated Pom1 occludes Cdr1/2 node formation, restricting them instead to the medial cell cortex (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009). Intriguingly, dynamic clusters of Pom1 have also been observed and are proposed to form by unstable oligomerization of molecules diffusing in the plane of the membrane (Hachet et al., 2011; Saunders et al., 2012). These clusters are thought to form and decay rapidly, temporarily sequestering Pom1 molecules and thereby slowing molecular diffusion rates and reducing noise in the gradient (Saunders et al., 2012). These puncta represent nanoscale pockets of increased Pom1 concentration, but whether these clusters contribute to regulation of Cdr2 nodes is unknown.
Here, we use biochemical approaches in combination with quantitative TIRF and confocal microscopy to show that the Pom1 gradient itself is formed by these punctate clusters. Pom1 puncta are stable oligomers that bind and release the membrane with minimal lateral diffusion. The frequency of Pom1 puncta binding is higher at cell tips than on cell sides. Thus, the gradient is formed because the membrane-association frequency of Pom1 puncta is patterned along the long axis of the cell. A portion of Pom1 puncta in the medial cell cortex colocalize with Cdr2 nodes, and the ratio of Pom1 to Cdr2 at the medial cell cortex changes as a function of cell size. When glucose availability is restricted, Pom1 clusters redistribute to the medial cell cortex to prevent dramatic dysregulation of Wee1 bursts. Our work reveals that the Pom1-Cdr2-Wee1 signaling pathway is organized as a series of cortical clusters. The relative distribution of these clusters at the plasma membrane changes with cell size and glucose availability, thus relaying cell surface area to the core cell cycle machinery in a nutrient-controlled manner.

Results:

The Pom1 gradient is formed by transient cortical puncta

We sought to examine the molecular dynamics of Pom1-Cdr2 signaling at the cell cortex. As a starting point, we imaged Pom1 by TIRF microscopy, which selectively excites fluorophores near the coverslip. Pom1 was tagged at the endogenous locus with the bright and photostable fluorophore mNeonGreen (mNG). Surprisingly, along the lateral cell cortex Pom1-mNG was localized almost exclusively in discreet puncta, with no apparent diffuse signal (Figure 1A). In images from a single time-point, Pom1 puncta formed a noisy concentration gradient (Figure 1B). Time-averaging produced a smoother gradient dotted by occasional clusters, similar to the gradient observed by confocal microscopy (Figure 1A-B). Similar results were obtained by confocal microscopy (Figure S1A-H). Pom1 puncta were highly dynamic. They appeared on the lateral cortex with a frequency of ~2 puncta*µm^{-2}*s^{-1}, and remained bound for less than 2 seconds on average (Figure 1C,F,H). During this brief cortical attachment, Pom1 puncta exhibited minimal diffusion which lacked directionality, and instead appeared to diffuse randomly in sub-micron patches of cortex (Figure 1C,G).
Similar puncta were previously observed by confocal microscopy, but they were not thought to form the Pom1 concentration gradient per se (Hachet et al., 2011; Saunders et al., 2012). Our findings suggested the distinct possibility that the cortical gradient itself is comprised of dynamic puncta. To test this possibility, we needed to image Pom1-mNG at cell tips, which are inaccessible to visualization using standard slide preparation for TIRF microscopy because this region is outside of the TIRF illumination field. Therefore, we performed “head-on” TIRF imaging of cell tips. Pom1-mNG localized at cell tips almost entirely in puncta, similar to cell sides (Figure 1D-E). We also used “head-on” Airyscan confocal imaging to confirm that Pom1 localization in puncta at tips was not an artifact of the TIRF approach (Figure S1I), a conclusion also supported by work using high resolution wide-field microscopy (Dodgson et al., 2013). Compared to cell sides, Pom1 puncta appeared twice as frequently at cell tips but with a similar cortical duration, again exhibiting minimal and non-directional diffusion away from their binding site (Figure 1F-H). Fluorescence intensity of individual puncta at cell tips and cell sides were similar, although we detected a low number of brighter puncta at cell tips, likely representing multiple diffraction-limited puncta (Figure 1I, Figure S1I-K). From these data, we conclude that the Pom1 gradient is formed by patterning the membrane binding frequency of cortical puncta along the long axis of the cell, rather than by diffusion of Pom1 molecules from the cell tips to the cell side.

*In vitro* analysis shows Pom1 exists in large, stable puncta

Past work suggested that Pom1 puncta assemble and disassemble at the cortex through oligomerization of individual molecules diffusing in the plane of the membrane (Saunders et al., 2012). However, our high-speed (20ms/frame) continuous TIRF videos suggested that Pom1 puncta bind and release the cortex as a pre-formed unit (Figure S2A). Consistent with this possibility, we observed rare cell lysis events in which Pom1 puncta remained intact in extruded cytoplasm (Figure 2A). This apparent stability while removed from the plasma membrane led us to test the existence and properties of Pom1 puncta in detergent cell extracts. Using TIRF microscopy, we observed puncta of Pom1-mNG in extracts made from Pom1-mNG cells, but not from untagged wild-type cells.
We next examined the size of these Pom1 puncta by velocity sucrose gradient sedimentation. By fractionating detergent cell extracts from pom1-3xHA cells, we found that most Pom1 exists in a large 60S complex, consistent with Pom1 puncta observed in cells. Fractions 6-8 containing this peak were dialyzed and centrifuged on a second sucrose gradient. These pooled fractions again sedimented at 60S, indicating that they represent a stable complex (Figure 2C-D, S2B-C).

Pom1 complexes present in detergent extracts resemble the cortical puncta observed in cells. To test this connection further, we examined the membrane-binding capacity of Pom1 complexes in vitro using artificial supported lipid bilayers (SLBs). Pom1-mNG puncta were isolated by sucrose gradient centrifugation, incubated with fluid SLBs on coverslips, and then imaged by TIRF microscopy (Figure 2E, S2E). In this cell free-system, Pom1-mNG puncta bound and released SLB lipids with strikingly similar dynamics as in cells (Figure 2E-F). Dwell times were increased for the kinase-dead mutant Pom1(K728R)-mNG, which was previously shown to increase cortical Pom1 levels in cells (Figure S2D) (Bähler and Pringle, 1998; Hachet et al., 2011). Collectively, our TIRF microscopy and in vitro analysis of Pom1 puncta support a model whereby the majority of cellular Pom1 protein is contained within discreet, highly-stable oligomers that bind and release membranes with kinetics dictated by their catalytic activity in a geometric pattern that resembles a concentration gradient.

Formation of clusters could represent an intrinsic property of Pom1 protein, or alternatively might require additional cellular factors. To distinguish between these possibilities, we expressed and purified GST-Pom1 from bacteria, and then performed sucrose gradient centrifugation experiments on this recombinant protein. Recombinant GST-Pom1 sedimented in a low molecular weight peak unlike Pom1 puncta from cells. Remarkably, purified GST-Pom1 was assembled into a puncta-sized high molecular weight complex by incubation with wild-type yeast detergent extract (Figure 2G). Complex formation was also induced by incubation of GST-Pom1 with pom1Δ cell extracts, meaning that additional cellular factors drive this assembly process. (Figure 2G). Once assembled, Pom1 puncta are stable oligomeric complexes with intrinsic
membrane-binding properties that can be reconstituted in vitro. Additional cellular regulatory proteins are likely to promote the binding of Pom1 puncta at cell tips, leading to the spatial gradient.

**Polarity landmarks pattern cortical dynamics of Pom1 puncta to shape the gradient**

The key role of puncta in forming the Pom1 gradient led us to reexamine the role of cell polarity landmark proteins Tea1 and Tea4. Both of these proteins localize at cell tips and are required for proper Pom1 gradient formation (Hachet et al., 2011). However, past studies have shown that Tea1 and Tea4 have distinct mechanistic roles: Tea4 is required for localization of Pom1 to the cortex, whereas Tea1 is required for enrichment of cortical Pom1 to the cell tip (Hachet et al., 2011). We first used sucrose gradient centrifugation to test if either protein is required for assembly of Pom1 into stable biochemical complexes. The sedimentation pattern of Pom1 puncta isolated from tea1Δ and tea4Δ cells in velocity sucrose gradients is unchanged from wild-type cells, suggesting that neither Tea1 nor Tea4 are required for assembly of Pom1 into stable biochemical complexes (Figure 3A). Rather, these regulators are likely to act downstream of puncta assembly.

We next tested the localization and dynamics of Pom1-mNG in tea1Δ and tea4Δ cells. Past work showed that membrane bound Tea4 is both necessary and sufficient to nucleate cortical Pom1 gradients, and this relationship is non-stoichiometric (Hachet et al., 2011). We confirmed that Pom1 was absent from the cell cortex in confocal micrographs of tea4Δ cells (Figure 3B). Surprisingly, we did observe Pom1-mNG puncta at the cortex of tea4Δ cells by TIRF microscopy (Figure 3C). These binding events occurred at lower frequency and had a shorter dwell time than in wild-type cells (Figure 3C-E). Pom1-mNG puncta in tea4Δ cells were distributed evenly around the cell cortex with no detectable enrichment at tips. These infrequent, transient binding events do not result in accumulation of enough cortical Pom1 to be detected by confocal microscopy. Their rapid and position-independent association with the cortex leads to the absence of a cortical Pom1 gradient in tea4Δ cells.
In contrast, confocal micrographs of Pom1-mNG in *tea1Δ* cells confirmed even distribution around the entire cell cortex (Figure 3B). TIRF microscopy of *tea1Δ* cells revealed that this distribution arises from Pom1-mNG puncta, which bound the cell cortex at a frequency in between the tips versus sides of wild-type cells (Figure 3C-D). The duration of individual binding events was longer than in *tea4Δ* cells but shorter than in wild-type cells (Figure 3E, S3A). Thus, in *tea1Δ* cells Pom1 puncta bind at an intermediate frequency and for an intermediate duration, when compared to wild type and *tea4Δ* cells. These binding events are independent of spatial cues at the cell ends versus cell sides, resulting in enrichment of cortical Pom1 but not a gradient. Thus, Tea1 and Tea4 cooperate to promote localized membrane binding but not assembly of Pom1 puncta. These spatial cues pattern the frequency of Pom1 puncta cortical interaction along the long axis of the cell.

**Pom1 puncta interact with Cdr2 nodes at the lateral cortex**

It has been unclear when and where Pom1 interacts with its inhibitory target Cdr2, which localizes in cortical nodes positioned in the cell middle. Since Pom1 puncta appear to bind throughout the medial cell cortex, we used simultaneous two-color TIRF microscopy to test colocalization of Pom1 puncta and Cdr2 nodes. We observed unambiguous overlap between Cdr2 nodes and some Pom1 puncta, as well as Pom1 puncta that bound to the cortex without encountering a Cdr2 node (Figure 4A). Thus, Pom1 puncta bind to the medial cortex in a stochastic pattern that can generate overlap with Cdr2 nodes. These patterns of colocalization were apparent in both static images and time-lapse movies (Figure 4A-B). The frequency, dwell time, and displacement of Pom1 cortical puncta were unaffected in *cdr2Δ* cells (Figure S4A-DC). These data suggest that Pom1 interacts with its inhibitory phosphorylation target Cdr2 when Pom1 clusters associate with Cdr2 nodes at the medial cell cortex.

Phosphorylation by Pom1 prevents activation of Cdr2 in small cells, thereby contributing to cell size-dependent regulation of Wee1 and mitotic entry (Allard et al., 2018). To place this pathway in the context of Pom1 puncta and Cdr2 nodes, we analyzed how these structures accumulate and colocalize as cells grow. Intriguingly, the total
number of both Cdr2 nodes and Pom1 puncta detected in TIRF images all along cell sides were equivalent for cells of a given size (Figure 4C-D). The numbers of both structures show a similar size-dependent doubling. We next restricted our analysis to a 2μm x 2μm square ROI positioned at the cell middle, where Cdr2 nodes concentrate. The local density of Cdr2 nodes in this region increased as a function of cell size, consistent with past studies (Deng and Moseley, 2013; Pan et al., 2014). In contrast, the density of Pom1 puncta in this region was largely independent of cell size (Figure 4E). In both TIRF and confocal images, the concentration of Pom1 protein and Pom1 clusters decreased slightly as cells increase in size, but this trend was dwarfed by the cell size-dependent increase in Cdr2 node density (Figure 4E, S4D). Thus, as cells grow larger, the ratio of Pom1 to Cdr2 in the medial cell cortex changes to favor Cdr2 because the densities of these two structures scale differently with cell size (Figure 4G).

We next tested how this density scaling affects colocalization of Pom1 clusters and Cdr2 nodes as cells grow. Due to the increasing density of Cdr2 nodes, we observed an increased number of colocalized Pom1 clusters and Cdr2 nodes as cells increase in size (Figure SE-F). The increased colocalization, combined with the constant density of Pom1 clusters, means that there are less “free” Pom1 clusters as cells increase in size (Figure S4G). Perhaps more importantly, the number of “free” Cdr2 nodes increased as a function of cell size (Figure S4G). These free Cdr2 nodes are not occupied by an inhibitory Pom1 cluster, and thus have increased potential to promote mitotic entry by inhibiting Wee1. In this manner, Pom1 sets an inhibitory threshold that must be overcome by an increase in Cdr2 node density. The inhibitory threshold decreases slightly as cells grow, and functions most effectively in small cells, where Pom1 was previously shown to inhibit downstream signaling to Wee1 (Allard et al., 2018).

Control of Pom1 puncta levels in the medial cell cortex

Our results suggest that Pom1 sets an inhibitory threshold that can be tuned by altering the concentration of Pom1 cortical clusters in the cell middle. We sought to test this model by increasing the abundance of Pom1 clusters in the cell middle. Low glucose media and tea1Δ are two independent conditions that disperse Pom1 throughout the
cortex, thereby increasing its concentration in the cell middle (Hachet et al., 2011; Kelkar and Martin, 2015) (See also Figure S7A-C). As a starting point, we tested the effect of mislocalized Pom1 in tea1Δ cells (Figure 5A-B). Surprisingly, higher Pom1 levels at the medial cortex did not impact the number of Cdr2 nodes or their recruitment of Wee1 (Figure 5C-F). Past work showed that Pom1 retains kinase activity in tea1Δ cells (Bähler and Nurse, 2001), but several experiments suggested that this residual activity may be reduced. First, we compared in vitro kinase activity of Pom1 immunoprecipitated from tea1Δ cells or wild type cells. Pom1 from tea1Δ cells showed less kinase activity towards itself (autophosphorylation) and its substrate Cdr2 (Figure S5A-C). Second, phosphorylation of the Pom1 substrate Rga7 was reduced in tea1Δ cells (Figure S5D-E). Third, Tea4 is completely absent from the cortex of tea1Δ cells (Figure S6D). Catalytically active Pom1 autophosphorylates and requires Tea4-dependent dephosphorylation for cortical enrichment. Thus, in tea1Δ cells, Pom1 likely binds to the membrane because it cannot autophosphorylate properly. Finally, we note that Pom1 localization in tea1Δ mimics the localization of kinase-dead pom1-m2 mutant (Figure S6A-B). Therefore, we focused on the activity and regulation of Pom1 clusters in wild type cells grown in low glucose.

Fission yeast cells respond to low glucose in part by depolymerizing their microtubule cytoskeleton, which leads to Pom1 redistribution to the lateral cell cortex where it delays mitotic entry (Kelkar and Martin, 2015). In time-lapse TIRF microscopy experiments, we observed increased numbers of discreet Pom1 puncta at the lateral cell cortex under low glucose conditions (Figure 6A). These puncta bound to the lateral cortex more frequently than under normal glucose conditions, but the average dwell time was unaffected (Figure 6B-C). This increased on-rate leads to accumulation of Pom1 clusters at the medial cell cortex.

We next investigated how increased numbers of Pom1 puncta at the lateral cell cortex affect Cdr2 nodes using TIRF microscopy. In low glucose conditions, the number of Cdr2 nodes was largely unchanged, but the fluorescence intensity of individual Cdr2 nodes and the overall concentration of Cdr2 at the medial cortex was significantly lower.
In *pom1Δ* cells, which grow only from one tip, Cdr2 nodes form normally but invade the non-growing tip. Low glucose conditions do not measurably affect Cdr2 nodes in *pom1Δ* cells, so the effects that we observed are Pom1-dependent (Figure 6D-F). These results indicate that Pom1 relocalization to the lateral cortex induces partial disassembly of Cdr2 nodes under low glucose. To quantify how this effect scales with glucose availability, we varied the concentration of media glucose over four orders of magnitude. Then, we measured the concentrations of both Pom1 and Cdr2 at both the cell sides and the cell tips. As glucose was reduced, Pom1 concentration decreased at cell tips and increased at cell sides, consistent with redistribution (Figure 7A,C-D). In contrast, Cdr2 concentration increased at cell tips and decreased at cell sides (Figure 7B-D). Plots of glucose availability versus Pom1 or Cdr2 concentration produced sigmoidal curves with opposite orientations (Figure 7C-D). Correlation analysis of Pom1 and Cdr2 concentrations at cell sides show they are nearly perfectly anti-correlated (Pearson’s *r*=−0.9352, *p*=0.0006) (Figure 7E-F). We conclude that the increasing frequency of Pom1 cluster binding at the lateral cell cortex leads to progressive disruption of Cdr2 nodes.

**Pom1 disrupts Wee1 bursting under glucose deprivation**

We next examined the downstream effects of altered Pom1-Cdr2 signaling by using TIRF microscopy to monitor the previously described bursts of Wee1-mNG localization at Cdr2 nodes (Allard et al., 2018). In wild-type cells, both the frequency and duration of Wee1 bursts increased linearly with cell size, consistent with increased inhibition of Wee1 as cells grow (Figure 8A,B). In low glucose, the frequency of Wee1-mNG bursts still scaled with cell size as in normal glucose. However, the duration of each burst was uniformly short and independent of cell size (Figure 8A-B). These same burst properties were seen for Wee1-mNG bursts in kinase-dead *cdr2(E177A)* mutant cells grown in normal glucose (Figure S8A-B). These results indicate that Cdr2 kinase activity is required for retention of Wee1 at nodes, but not for the initial binding event. Further, they demonstrate that low glucose phenocopies the kinase-dead mutant, consistent with Pom1-dependent inhibition of Cdr2 kinase activity.
These results suggested that attenuation of Wee1 bursting dynamics in low glucose might require Pom1 redistribution. We tested this idea by examining the glucose dependency of Wee1 node localization in pom1Δ cells. In pom1Δ cells growing under high glucose, the Wee1 burst frequency scales with cell size but Wee1 burst duration is uniformly high and independent of cell size (Figure 8A,C). Unlike wild type cells, which suppress Wee1 bursts in low glucose, pom1Δ cells show a striking increase in Wee1 bursts under low glucose. This increase is not cause by burst duration, which was independent of both cell size and glucose concentration in pom1Δ cells. Rather, we measured increased Wee1-mNG burst frequency in low glucose pom1Δ cells (Figure 8A,C). The reason for this increase is unknown, and suggests the existence of additional glucose-regulated pathways that operate on Cdr2-Wee1 signaling.

Wee1 localization at Cdr2 nodes leads to its inhibitory phosphorylation by Cdr2 and the related kinase Cdr1. To test how our TIRF-based localization results connect with Wee1 phosphorylation status, we analyzed phosphorylation-dependent shifts of Wee1 migration using western blots (Figure 8D). In high glucose, Wee1 migrates as a smear of phosphorylated isoforms (Allard et al., 2018; Lucena et al., 2017). The upper, hyperphosphorylated forms are increased in pom1Δ cells but absent in cdr2Δ cells. This hyperphosphorylated Wee1 is lost in wild-type cells grown in low glucose, consistent with reduced bursts of Wee1 localization to nodes. In contrast, Wee1 appears even more hyperphosphorylated in pom1Δ cells grown under low glucose. This result suggests that Pom1 prevents phospho-inactivation of Wee1 in response to low glucose. These combined experiments support a model where Pom1 redistribution to the lateral cell cortex inactivates Cdr2 nodes to relieve inhibition of Wee1 under low glucose conditions.

Discussion:

In this study, we have shown that the Pom1 concentration gradient is generated by oligomeric Pom1 puncta that rapidly bind and release from the plasma membrane. These puncta bind more frequently at cell tips versus cell sides in high glucose media, while their binding rate is increased at cell sides in low glucose media. At the medial cell cortex, these puncta overlap with their inhibitory target Cdr2, which localizes in static...
oligomeric nodes. More Cdr2 nodes are free from Pom1 inhibition as cells grow larger due to different density scaling of these two structures at the medial cell cortex. Our TIRF-based colocalization experiments on these structures were limited to short periods of time to avoid photobleaching, partly caused by the imaging conditions needed to observe these highly dynamic Pom1 clusters. Nevertheless, even over these short timescales of observation, the stochastic positioning of Pom1 puncta resulted in spatiotemporal colocalization with Cdr2 nodes. Given the high frequency of Pom1 puncta binding, we expect that many more colocalization events occur between Cdr2 nodes and Pom1 clusters during the course of a full cell cycle, with the potential for Pom1 clusters to visit each Cdr2 node multiple times. Thus, these rapid dynamics of Pom1 puncta interacting with Cdr2 nodes must be integrated in time throughout the cell cycle, leading to the G2/M transition. It appears likely that the most critical temporal window for Pom1-Cdr2 interactions occurs during early in G2, as pom1Δ show defects in Cdr2-Wee1 signaling specifically in small cells (Allard et al., 2018). It will be interesting to determine how these rapid interactions in small cells are integrated into the mitotic entry decision, which occurs later in the cell cycle when cells are longer.

**Molecular clusters are widespread in signal transduction**

The Pom1-Cdr2-Wee1 signaling pathway appears to function entirely within the confines of oligomeric protein clusters at the plasma membrane. The development of imaging technologies with increased spatial and temporal resolution has enabled the discovery of protein clustering as a paradigm in signaling. As a result, a growing number of signaling events and proteins have been found in similar clustered structures that are referred to as nanodomains, clusters, nanoclusters, and nodes, among other names. For example, recent studies have shown how PAR-3 polarity complexes in the *C. elegans* zygote form clusters to reduce random diffusion and promote vectorial transport (Dickinson et al., 2017; Munro, 2017; Rodriguez et al., 2017; Wang et al., 2017). In addition, major signal transduction pathways in plants are organized into protein clusters on plasma membrane nanodomains (Gronnier et al., 2017). Similarly, different Ras isoforms partition into distinct and exclusive clusters that digitize downstream signals in animal cells (Zhou and Hancock, 2015). In immune cells, both B-cell receptors and T-cell
receptors form oligomeric compartments at the cell cortex (Douglass and Vale, 2005; Liu et al., 2016; Maity et al., 2015). These T cell receptor clusters enrich downstream kinases while excluding phosphatases (Su et al., 2016), a mechanism that could relate to concentrated clusters of the kinases Pom1 and Cdr2. Clustering can have a range of critical impacts on the signaling properties of these pathways, such as the clustering of bacterial chemotactic receptors to generate signal amplification (Bray et al., 1998; Duke and Bray, 1999; Falke, 2002). Theoretical work has suggested that clustering can reduce noise by generating reaction bursts (Kalay et al., 2012), which have the capacity to overcome inhibitory thresholds, such as those imparted by phosphatases, more effectively than a system driven by gradual accumulation of signal. Insight into the functional role of clustering for Pom1-Cdr2-Wee1 will require additional in vivo analysis combined with in vitro reconstitution. We have shown that Pom1 clusters remain stable through biochemical enrichment, and isolated clusters exhibit the same membrane-binding properties in vitro as in cells. Past work has demonstrated Cdr2 nodes also remain stable through biochemical fractionation (Allard et al., 2018), providing the necessary tools for future dissection of the pathway in a reconstituted system.

A new model for cortical gradient formation

Our work has shown that Pom1 clusters bind to the cell cortex with different frequencies along the long axis of the cell, resulting in a concentration gradient. Past work suggested single Pom1 molecules (or small oligomers) bind to the membrane at tips, and then diffuse directionally away from the tip before dissociating (Hachet et al., 2011; Saunders et al., 2012). Clusters were proposed to dynamically assemble and disassemble at the cortex from this diffusing population (Saunders et al., 2012). Our findings suggest a new model for gradient formation, which is distinct from this previous model in four ways. First, we found that clusters themselves form a concentration gradient that emanates from the cell tips. Second, our combined imaging and biochemical approaches demonstrated that clusters are stable complexes that bind and rapidly release from the membrane. Third, we did not observe extensive diffusion by complexes bound to the membrane. It remains possible that smaller, diffusing Pom1 complexes or single molecules were not detected by our imaging approach, but we favor a model where
Diffusion is limited to short, non-directional movements that do not contribute to gradient formation. Fourth, the key step in gradient formation is a positional system that increases the on-rate for Pom1 clusters at cell tips versus the cell middle. Concentration gradients are found across vast size scales in biology, so the mechanism of gradient formation by Pom1 clusters may have broad implications. It is also important to note that the spatial gradient of on-rates for Pom1 clusters binding to the cortex may depend on a concentration gradient of specific lipids in the plasma membrane. In support of this hypothesis, Pom1 binds to phosphatidylserine in vitro (Hachet et al., 2011), and this lipid is enriched at the tips of growing fission yeast cells (Haupt and Minc, 2017). Thus, further investigation of these lipids and their localization dynamics could reveal additional layers of this morphogen gradient.

We found that the polarity landmarks Tea1 and Tea4 provide the positional information for Pom1 clusters to form a concentration gradient. Past work has shown that Pom1 localizes homogenously throughout the cortex in tea1Δ, and does not localize to the cortex in tea4Δ (Bähler and Pringle, 1998; Hachet et al., 2011). Based on these and other results, Tea1 was proposed to recruit Tea4 to cell tips, where it promotes cortical loading of Pom1 molecules. Several results suggest that additional mechanisms may contribute to gradient formation. For example, we found that Pom1 clusters still bind to the cortex in tea4Δ cells but for very short durations. Past work has also shown that Tea1 and Tea4 form cortical clusters that co-localize at cell tips, but these Tea1-Tea4 clusters do not co-localize with Pom1 clusters (Dodgson et al., 2013). Thus, Tea1-Tea4 may position binding of Pom1 clusters to the cortex through an additional mechanism. For example, Tea1 and Tea4 have been shown to assemble sterol-enriched lipid domains at cell tips when cells initiate polarization following exit from starvation (Makushok et al., 2016). Sterols and phosphatidylserine are thought to form a common membrane domain in fission yeast, suggesting that these proteins could act through the recruitment and organization of lipids to generate cortical domains permissive for Pom1 cluster binding (Haupt and Minc, 2017; Makushok et al., 2016).
Adjustable Pom1 gradients regulate mitotic entry

We propose an integrated mechanism for fission yeast cell size sensing in which Cdr2 acts as an important sensor of cell size that is dynamically antagonized by cortical Pom1 (Figure 9). Pom1 puncta bind to the medial cell cortex in a manner that can overlap with Cdr2 nodes. These dynamic Pom1 puncta and stable Cdr2 cortical nodes suggest a system that monitors surface expansion, consistent with the notion that fission yeast cells monitor surface area as the primary determinant of size at division (Facchetti et al., 2019; Pan et al., 2014). As cells grow, the density of Pom1 clusters in the cell middle decreases slightly due to their binding preference at cell tips. In contrast, the density of Cdr2 nodes doubles to overcome this inhibitory Pom1 threshold. The density of Pom1 clusters and the resulting inhibitory threshold can be modulated by glucose availability, consistent with past work (Kelkar and Martin, 2015). As fission yeast cells begin to starve due to depletion of nutrients such as glucose, their length at division progressively decreases and the length of the cell cycle increases (Pluskal et al., 2011; Yanagida Mitsuhiro et al., 2011). The Pom-Cdr2 network has a very prominent role in this nutrient modulation, suggesting that it may represent a key physiological role for the pathway (Kelkar and Martin, 2015). We found that the size dependence of Wee1 localization bursts to Cdr2 nodes, which facilitate Wee1 inhibition, was dampened in low glucose conditions. Re-localization of Pom1 clusters to the medial cortex likely mediates this response. However, for pom1Δ mutants in low glucose, Wee1 localization to nodes exceeds even wild type cells in high glucose. This result indicates that additional mechanisms contribute to Wee1 regulation in low glucose, for example Wee1 nuclear transport could be altered under these conditions.

The Pom1 gradient as a read-out for cell polarity

More broadly, our study reveals the adjustable nature of the concentration gradient formed by Pom1 clusters. Concentration of Pom1 clusters at cell tips reads out the polarity state of the cell for downstream signaling by Cdr2 and Wee1. Conditions that alter the polarized growth state of the cell would lead to changes in the gradient of Pom1 clusters, resulting in altered cell size at division. This dynamic system appears to operate in a manner that depends on both cell size and nutrient availability. Identification of additional
growth and environmental conditions that alter the relative distributions of Pom1 clusters and Cdr2 nodes, and determination of how they connect with other cell cycle signaling pathways, may reveal new mechanisms for nutrient modulation of cell size control.

**Integration of the Pom1-Cdr2-Wee1 pathway with other sizing mechanisms**

Pom1-Cdr2-Wee1 signaling is cell size dependent but is not the only pathway that contributes to the overall cell size control network. Intriguingly, a recent study reported that cdr2Δ cells no longer divide based on accumulation of a threshold surface area and instead revert to a secondary mechanism of cell size control based on volume sensing. These results demonstrate that cells use different signal transduction networks to measure different aspects of their size. Whereas the Pom1-Cdr2 network is well suited to measure cortical surface area, size-control systems that measure other aspects of size would require different signaling logic. Theoretically, mechanisms for cell volume sensing could depend on proteins whose concentrations do not scale linearly with cell size. Such mechanisms are thought to underlie cell size control in other organisms, such as the budding yeast, where growth-dependent dilution of the transcriptional repressor Whi5 is thought to underlie G1/S size control (Schmoller et al., 2015). One possible candidate for cellular volume sensing in fission yeast is Cdc25, which increases in concentration with cell size (Keifenheim et al., 2017; Moreno et al., 1990). These mechanisms are attractive candidates for volume sensors because cytoplasmic concentration is independent of cell geometry. The existence of sizing mechanisms that measure other aspects of cell size, and function independently of the Pom1-Cdr2 surface area sensing network, underscores that cell size is a systems level property controlled by multiple signaling pathways. Thus, it remains important to discover size-dependent signaling mechanisms within each individual pathway, as a step towards understanding the integration of multiple pathways into a larger size control system.
Materials and Methods:

Strain Construction and Media

Standard *S. pombe* media and methods were used (Moreno et al., 1991). Strains used in this study are listed in Supplementary Table S1. Gene tagging and deletion were performed using PCR and homologous recombination (Bähler et al., 1998). The mNeonGreen (mNG) sequence was used under license from Allele Biotechnology.

TIRF Microscopy and Analysis

Pom1 puncta and node components were imaged using simultaneous dual-color total internal reflection fluorescence microscopy to limit excitation of fluorophores to those nearest to coverslip. Imaging was performed on a commercially available TIRF microscope (Micro Video Instruments) composed of a Nikon Eclipse Ti microscope base equipped with a 100x Nikon Apo TIRF NA 1.49 objective and a two-camera imaging adaptor (Tu-CAM, Andor Technology) containing a dichroic and polarization filters (Semrock FF580-FDi01-25x36, FF02-525/40-25, FF01-640/40-25) to split red and green signal between two aligned Andor iXon electron-multiplied CCD cameras (Andor Technology). Red/green beam alignment was performed prior to imaging using a TetraSpeck Fluorescent Microsphere size kit (Thermofisher).

Standard #1.5 glass coverslips were RCA cleaned before use to remove fluorescent debris. Cells were grown in EMM4S, and washed into fresh EMM4S immediately before imaging to remove auto-fluorescent debris resulting from overnight culture. Cells were imaged in EMM4S media on glass slides at ambient temperature. Individual slides were used for no more than five minutes to prevent cells from exhausting nutrients or oxygen. Agar pads were not used due to increased background fluorescence.

Image analysis and processing was performed using ImageJ2 (NIH). Cdr2 node numbers and Pom1/Wee1 puncta number, frequency and binding duration were quantified using the Trackmate plugin (Tinevez et al., 2017). Due to variable fluorescence intensity in different TIRF fields and images, thresholding parameters were determined separately for each image, and accuracy was confirmed by visual inspection to ensure
that only nodes/puncta were counted and that no nodes/puncta were omitted. For Wee1
burst and Pom1 puncta tracking, Particle Diameter was set to 0.3 microns (approximate
lateral resolution), with Maximum Gap Linking set to 2 frames, and Linking Range for
particle tracking was set to 0.15 microns. Lookup table was adjusted to fire for some
images to emphasize signal intensities.

“Head-On” Tip Imaging

“Head-On’ or “Tip” imaging was performed using a protocol modified from
(Dodgson et al., 2013). Custom micro-well coverslips or Ibidi Sticky-Slide VI channels
(Ibidi, Martinsried, Germany) were coated with BS-I lectin (1mg/mL in water) by incubation
for 30 minutes and washed 3X with water to remove non-adherent lectin. Cells were then
added to the imaging chambers and allowed to settle to the cover-slip bottom where they
adhere to the lectin. Cells oriented with the long axis perpendicular to the coverslip were
identified using brightfield prior to fluorescence imaging.

Spinning Disc Microscopy and Analysis

Spinning-disc confocal imaging was performed using a commercially available
system (Micro Video Instruments, Avon, MA) featuring a Nikon Eclipse Ti base equipped
with an Andor CSU-W1 two-camera spinning disc module, dual Zyla sCMOS cameras
(Andor, South Windsor, CT) an Andor ILE laser module, and a Nikon 100X Plan Apo λ
1.45 oil immersion objective. Cells were imaged in EMM4S media on glass slides at
ambient temperature unless otherwise noted.

Airyscan Super-Resolution Microscopy and Analysis

To achieve maximum resolution and sensitivity, fluorescence intensity of Pom1
puncta on both cell sides and cell tips was measured using a Zeiss Airyscan microscope
(Figure S1I-K), composed of a Zeiss LSM-880 laser scanning confocal microscope
(Zeiss, Oberkochen, Germany) equipped with 100X alpha Plan-Apochromat/NA 1.46 Oil
DIC M27 Elyra objective, Airyscan super-resolution module and GaAsP Detectors, and
Zen Blue acquisition software using the Super-resolution mode with pin-hole size of 1.2
airy-units to prioritize resolution. Z-volumes of 16 slices with 0.19µm spacing for high
spatial resolution in all dimensions were centered on the cell cortex closest to the coverslip. Airyscan images were processed in Zeiss Zen Blue software, and quantification was performed on sum projections of Airyscan reconstructed stacks.

Pom1 and Cdr2-CTD Purification

The full length Pom1 sequence, and the Cdr2-CTD fragment were purified as follows: Each sequence was subcloned into pGEX6P1 vector using the Xho1 restriction site and expressed as a GST-fusion protein in Escherichia coli strain BL21(DE3). Transformants were cultured to log-phase at 37°C, followed by a shift to 25°C for 30 minutes. Expression was then induced by addition of 1-thio-β-D-galactopyranoside to 200µM, followed by growth for an additional 3 hours at 25°C. Cells were harvested by centrifugation and lysed by passing them twice through a French press in lysis buffer (1xPBS, 100mM NaCl, 1mM DTT, and complete EDTA-free protease inhibitor tablets (1 tablet/50mL Buffer) (Roche, Basel, Switzerland)). Following lysis, Triton-X 100 was added to 1% V/V. Lysates were then cleared by centrifugation for 10 minutes at 12,000 x g at 4°C in a Sorval SS-34 fixed-angle rotor (Thermo Scientific, Waltham, MA). The supernatant was then incubated with glutathione-agarose (Sigma Aldrich, St. Louis, MO) for 2h at 4°C. 20 mM glutathione (pH 8.0) was used to elute purified protein, and the eluate was dialyzed overnight at 4°C into 10mM Tris-HCl, pH8 + 5% V/V glycerol.

Preparation of Yeast Extracts

Fission yeast detergent extracts were prepared by growing 1.5L cells to mid-log phase (OD ~0.3), and then washed twice with 50mL Node Isolation Buffer (NIB – 50mM HEPES, 100mM KCl, 1mM MgCl2, 1mM EDTA, pH 7.5) (Allard et al., 2018). Next, we resuspended the pellet in an equal volume of 2X NIB (W/V) containing a protease/phosphatase inhibitor cocktail (10µL/mL 200x PI, 50µL/mL 1M β-glycerol phosphate, 50µL/mL 1M NaF, 2.5 µL/mL 200mM PMSF, 1mM DTT), and snap froze the resuspension as pellets by pipetting drop-wise into a liquid nitrogen bath. Then, yeast pellets were ground using liquid-nitrogen chilled coffee-grinders for 2 min, and collected into chilled falcon tubes and stored at -80°C. 1.5g of frozen yeast powder was then thawed on ice, and Triton X-100 was added to a final concentration of 1%, and the extracts were
mixed by gentle pipetting. Extracts were then centrifuged at 4˚C for 10min at 20,000 x g to yield a low speed supernatant, which were then used for subsequent experiments.

**Sucrose Gradient Ultracentrifugation**

Discontinuous sucrose gradients were prepared in 14 x 89mm Ultra Clear Ultracentrifuge tubes (Beckman Coulter, Brea, CA) by layering 5 – 23% (top to bottom) sucrose in NIB + 1% Triton X-100 in 0.37mL steps of 2% sucrose increments. 700μL of yeast extract or recombinant protein diluted in NIB Buffer was then added to the top of the gradient. Sucrose gradients were centrifuged at 100k x g (50kRPM) in a Beckman L8-M ultracentrifuge for 2hr at 4˚C in a chilled SW60ti swinging bucket rotor. 0.5mL gradient fractions were collected from the top by hand, vortexed, and 100μL of each fraction was mixed 2:1 in 3X SDS-PAGE sample buffer (65 mM Tris pH 6.8, 3% SDS, 10% glycerol, 10% 2-mercaptoethanol, 50 mM NaF, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate) and boiled for 5 min. Samples were then subjected to SDS-PAGE and western blotting or Coomassie staining. To calculate S-values of sedimentation peaks from western blot signal intensities, mean band intensities were measured using Image Studio Lite (LICOR, Lincoln, NE), and Gaussian Curves were fit to these values. The peak of the Gaussian for each sedimentation peak of each protein was used to assign the known or interpolated S-value.

**Western Blotting**

For western blots, cells were lysed in 150 µl of 3X SDS-PAGE sample buffer with glass beads in a Mini-beadbeater-16 (Biospec, Bartlesville, OK) for 2 minutes. Gels were run at a constant 20 mAmperes until 75kDa marker was at the bottom of the gel. Blots were probed with anti-HA (Covance, Princeton, NJ) or homemade anti-GST. For monitoring Wee1 phosphorylation, samples were run on an SDS-PAGE gel containing 6% acrylamide and 0.02% bisacrylamide, and then probed with a homemade anti-Wee1 antibody (Allard et al., 2018).

**Supported Lipid Bilayers**
To prepare supported lipid bilayers, we first prepared small unilamellar vesicles (SUVs) composed of three lipids: (1) DOPC (18:1 (Delta9) Cis PC - 1,2 Dioleoyl-sn-glycero-3-phosphocholine), (2) DOPS (18:1 PS - 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine]), and (3) fluorescent 18:1 Liss Rhod PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl). All lipids were dissolved in chloroform (Avanti, Alabaster, AL). 2µmol total lipid with the desired molar ratios were mixed in glass vials with PTFE coated caps, which had been RCA cleaned and rinsed three times with chloroform before use. Excess chloroform was evaporated in a vacuum desiccator for 1 hour. Lipids were resuspended in 400µL SUV Buffer (50mM HEPES, 100mM KCl, 1mM EDTA, 1mM MgCl$_2$, pH7.5) for 5mM lipid mixture stocks. The lipid mixtures were vortexed until cloudy, and then transferred to 1.5mL Eppendorf tubes and subjected to ten freeze-thaw cycles using a liquid nitrogen bath and 32°C hot plate, with 2 minutes of sonication in a sonicating water bath following each thaw cycle. Stocks were stored at -80°C in 20µL aliquots.

Supported lipid bilayers were made by adding 10µL of SUVs to RCA cleaned custom microwells built on 22x40mm #1.5 coverslips. Chambers were then incubated at 37°C for one hour to induce vesicle fusion, and unincorporated vesicles were rinsed away using five rinses with 100µL SUV buffer. Yeast extract was added to these chambers by removing 90µL of buffer, leaving just enough to cover the chamber bottom, and then adding extract.

**Pom1 Immunoprecipitation**

Cultures were grown in EMM4S media to log phase, and a volume equivalent to 200 optical density units were harvested by centrifugation. The pellet was washed in Wash Buffer (1X PBS, 250mM NaCl, 1mM EDTA, 1mM PMSF, 50mM NaF, 50mM β-glycerophosphate, 1mM sodium orthovanadate, and 1% Triton X-100, and complete EDTA-free protease inhibitor cocktail (1 tablet/50mL) (Roche)). The pellet was then resuspended in 5mL of wash buffer, and divided into 1.5mL screw-cap tubes each with 200µL glass beads for lysis by bead-beading for 2 minutes using a mini-Beadbeater-16 (Biospec) at 4°C. Lysates were cleared by centrifugation at 16,000 x g for 10 minutes at
4°C in an Eppendorf 5415C centrifuge. The supernatant was then incubated with rotation at 4°C for 1 hour with Pierce anti-HA magnetic beads (Thermo Scientific). Beads were washed five times for 15 minutes each in Wash Buffer prior to use. Beads were resuspended in Kinase Assay Buffer (see below) for immediate use in kinase assays.

**In vitro kinase assays**

For *in vitro* kinase assays, immunoprecipitated Pom1 proteins and purified Cdr2-CTD were combined at a 1:1 ratio in Kinase Assay Buffer (20mM Tris pH 8, 100mM NaCl, 50mM NaF, 50mM β-glycerophosphate, 1mM sodium orthovanadate, 10mM MgCl₂, 20µM ATP-γ-S) for 30 minutes at 30°C. The reaction was halted by addition of EDTA to 20mM. For subsequent analysis by anti-thiophosphate western blot, proteins in *in vitro* kinase assays were then alkylated by addition of p-nitrobenzyl mesylate to 2.5mM, and incubation at 25°C for one hour. Samples were then prepared for western blot as described above. Thiophosphate western blots were probed with an antibody against thiophosphate-ester (Abcam 92570) (Abcam, Cambridge, United Kingdom).

**Figure Legends:**

**Figure 1:** The Pom1 gradient is formed by time-averaging of puncta that transiently bind the cortex with different dynamics along the long axis of the cell. (A) Individual frame (top panel) and sum projections (bottom panel) of high-speed TIRF microscopy movie. Scale bar 1µm. Movie was continuous 15s time-lapse acquisition of 200ms exposures. Blue arrows mark position of line scans performed for data in panel B. (B) Line scans of fluorescence intensity along the long axis of the snap shot and projection images in panel (A). Note that time-averaging of Pom1 puncta smoothens the concentration gradient. (C) Traces of single-particle diffusion paths generated from a 15s time-lapse TIRF microscopy acquisition of Pom1-mNeonGreen. Scale bar 1µm. (D-E) Pom1-mNeonGreen forms puncta both at the cell tip and cell side. Images are sum projections of three consecutive 200ms exposures from continuous time-lapse TIRF
microscopy acquisitions of either the cell tip or side as depicted in the cartoon diagrams. Scale bar 1µm. (F-H) Particle-tracking analysis of 2µm x 2µm ROIs from 30s continuous time-lapse TIRF acquisitions of Pom1-mNeonGreen at either the cell tip or side was used to measure and compare (F) the binding frequency of Pom1 puncta (****, p=<0.0001), (G) the average displacement of individual puncta (***, p=0.0002), (H) the binding duration of individual puncta (n.s., p=0.6747). Each data point represents a single cell mean, and line and error bars represent mean and standard deviation of all cells. Statistical significance was tested using a Student’s T-test. (I) Histogram of the fluorescence intensity of individual Pom1 puncta at the cell tip and cell side. Puncta were binned by fluorescence intensity and displayed as a population frequency.

Figure 2: Pom1 puncta are stable structures that can be isolated in vitro. (A) TIRF microscopy image reveals fluorescent puncta in the extruded cytoplasm (white dotted line) from a lysed Pom1-mNeonGreen cell (yellow dotted line). Scale bar 5µm. (B) TIRF microscopy images of cell extracts prepared from wild-type (no tag) or Pom1-mNeonGreen cells. Images are 50 frame sum projections of continuous 200ms time-lapse exposures. The two images were contrasted equally. Scale bar 1µm. (C) Cytoplasmic extracts of pom1-3HA cells were subjected to velocity sucrose gradient sedimentation, and fractions were probed against the HA tag (upper blot). Fraction 1 corresponds to the top of the gradient and contains smaller structures; fraction 12 corresponds to bottom of the gradient and contains larger structures. Fractions 6-8 were pooled, sucrose was removed by dialysis, and then the sample was subjected to a second identical round of sucrose gradient sedimentation and western blotting of the resulting fractions (lower blot). (D) Quantification of Pom1-3HA band intensities from western blot of yeast extracts in panel C. Each apparent peak was fit to a gaussian curve, and S-values were determined using size-standards run on identical gradients. (E) TIRF microscopy of Pom1-mNG puncta from cytoplasmic extracts on supported lipid bilayers. Scale bar 1µm. Left panel is single time point image. Right panel is kymograph taken from line scan of time-lapse TIRF experiment. (F) Quantification of binding duration of Pom1-mNG puncta on supported lipid bilayers by TIRF microscopy as in panel F. Values are compared to cellular measurements of pooled tip and side localized Pom1 puncta (n.s., p=0.0514). (G)
Purified GST-Pom1 was subjected to sucrose gradient sedimentation and the fractions were probed against the GST tag (upper blot). Purified GST-Pom1 was also added into wild-type cell or pom1Δ cell extracts and incubated for 1hr at 4°C in the presence of ATP before velocity sucrose sedimentation and western blotting (bottom blots).

Figure 3: Polarity landmarks pattern cortical dynamics of Pom1 puncta to shape the gradient. (A) Western blots of sucrose gradient fractions after sedimentation analysis as in Figure 1C of extracts prepared from tea1Δ or tea4Δ cells expressing Pom1-3HA. (B) Confocal micrographs of Pom1-mNG in tea1Δ or tea4Δ cells. Images are from the middle focal plane; scale bar 5µm. (C) TIRF micrographs of Pom1-mNG localization at the tip or side cortex in tea1Δ or tea4Δ cells. Scale bar 1µm. (D) The mean binding frequency of Pom1-mNG puncta at the tips and sides of wild-type, tea1Δ or tea4Δ cells, measured by TIRF microscopy. (E) The mean binding duration of Pom1-mNG puncta at the tips and sides of wild-type, tea1Δ or tea4Δ cells, measured by TIRF microscopy. For D-E, wild-type data are replotted from Figure 1. Each data point represents a single cell mean, and line and error bars represent mean and standard deviation of all cells. Comparisons are 1-way ANOVA with Tukey’s multiple comparisons tests. **** indicates p < 0.0001, n.s. indicates p > 0.05.

Figure 4: Scaling of Pom1 puncta and Cdr2 nodes at the lateral cell cortex. (A) A subset of Pom1 puncta colocalize with Cdr2 nodes. Images are dual-channel simultaneously-acquired TIRF microscopy images of cells expressing Pom1-mNG and Cdr2-tagRFP-t. Yellow dashed brackets outline the ROI of the lower zoomed panels. Orange arrows point to Pom1 puncta colocalized with Cdr2 nodes. Scale bar is 1µm. (B) Kymographs generated using the ROI drawn on the merged zoom image in panel A. Cyan arrows indicate prominent Pom1 puncta, magenta arrows indicate prominent Cdr2 nodes, and orange arrows adjacent to the merged kymograph indicate colocalization. (C) Localization of Cdr2-mEGFP (upper panels) or Pom1-mNG (lower panels) in representative cells of increasing size by TIRF microscopy. Scale bar is 1µm. (D) Total number of Cdr2-mEGFP nodes (blue circles) or Pom1-mNG puncta (red squares) measured per cell by TIRF microscopy. Quantification is limited to puncta detected and
resolvable in the TIRF illumination field. The slopes of the corresponding linear
regressions are not significantly different ($p=0.3757$). (E) Density of Cdr2-mEGFP nodes
(blue circles) or Pom1-mNG puncta (red squares) in 2x2µm square ROIs at the cell middle
using TIRF microscopy. (F) Example of colocalization analysis for Pom1 puncta and Cdr2
nodes. The spot detection algorithm in TrackMate plugin for ImageJ2 was used to assign
0.3 µm diameter circular ROIs to Cdr2 nodes (cyan) and Pom1 puncta (magenta) in
images such as panel A. Structures were counted as colocalized if the ROI assigned to
one channel overlapped with the middle of the ROI of the other channel (right panel,
yellow circles). Adjacent structures that failed this criterion were deemed not to be
colocalized (right panel, grey circles). (G) Ratio of free Cdr2 nodes to free Pom1 puncta,
plotted as a function of cell size.

Figure 5: Properties of Pom1 puncta in tea1Δ cells. (A) Localization of Pom1-mNG in
wild-type or tea1Δ cells imaged by confocal microscopy at focal planes in the cell middle.
Scale bar, 5µm. (B) Comparison of Pom1 concentration along 2µm cortical line scans
positioned at the cell middle of wild-type and tea1Δ cells, from images as in panel A.
Significance was tested using a Student’s T-test (***, $p<0.0001$). (C) Localization of
Cdr2-mNG in tea1Δ cells by TIRF microscopy. Scale bar is 5µm. (D) Number of Cdr2-
mNG nodes as a function of cell length in wild-type (blue circles) or tea1Δ cells (red
squares), measured using TIRF microscopy images as in panel C and Figure 5A. The
slopes of the linear regression of each data set do not differ significantly from each other
($p=0.2029$). (E) Localization of Wee1-mNG in tea1Δ cells by TIRF microscopy. Scale bar is 5µm. (F) Number of Wee1-mNG bursts as a function of cell length in wild-type (blue
circles) or tea1Δ cells (red squares), measured using TIRF microscopy images as in panel
E and Figure 7A. There is a small but significant difference in the slopes of the linear
regressions ($p=0.0158$) such that the number of Wee1-mNG bursts scales more slowly
in tea1Δ cells than in WT. This discrepancy may result because tea1Δ cells can be bent
or T-shaped, rendering length an imperfect proxy for size.

Figure 6: Redistribution of Pom1 puncta to the lateral cortex disrupts Cdr2 nodes
during glucose restriction. (A) Localization of Pom1-mNG in wild-type cells grown in
either high (2%) or low (0.03%) glucose media. Images were collected using TIRF microscopy. Scale bar, 5µm. (B) Comparison of the binding frequency of Pom1 puncta at the lateral cell cortex in cells grown under high and low glucose measured using TIRF microscopy (***, p=0.0003). Statistical significance was tested using a Student’s T-test. (C) Comparison of the binding duration of Pom1 puncta at the lateral cell cortex in cells grown under high and low glucose measured using TIRF microscopy (n.s., p=0.6833). Statistical significance was tested using a Student’s T-test. (D) Localization of Cdr2-mNG in wild-type or pom1Δ cells grown in either normal (2%) or low (0.03%) glucose media. Images were collected using TIRF microscopy. Scale bar, 1µm. (E) Comparison of the fluorescence intensity of individual Cdr2-mNG nodes in wild-type or pom1Δ cells grown in either normal (2%) or low (0.03%) glucose media. Cdr2 nodes in wild-type cells become significantly smaller under low glucose conditions (****, p<0.0001), but there is no significant difference in Cdr2 nodes size in pom1Δ cells grown in normal vs low glucose (n.s., p=0.4015). Measurements were taken from Airyscan Super-Resolution confocal micrographs. (F) Quantification and comparison of the total number of Cdr2-mNG nodes visible in TIRF micrographs of wild-type or pom1Δ cells grown in either normal (2%) or low (0.03%) glucose media. There is no significant difference in any condition (p>0.05). Statistical significance was tested using a one-way ANOVA.

Figure 7: Pom1 and Cdr2 concentrations at the lateral cell cortex are anti-correlated. (A) Localization of Pom1-mNG in confocal micrographs at middle cell focal planes. Regions where line-scans were used to measure fluorescence intensity are marked at the tip and side (orange lines). Scale bar, 1µm. (B) Localization of Cdr2-mNG in confocal micrographs at middle cell focal planes. Scale bar, 1µm. Regions where line-scans were used to measure fluorescence intensity are marked at the tip and side (orange lines). (C) Concentration of Pom1 (red squares) or Cdr2 (blue circles) at cell sides in a range of glucose concentrations, measured using ‘Side ROI’ denoted in panels A and B. (D) Concentration of Pom1 (red squares) or Cdr2 (blue circles) at cell tips in a range of glucose concentrations, measured using ‘Tip ROI’ denoted in panels A and B. (E) Correlation of Cdr2 vs Pom1 concentrations at cell sides in each glucose concentration
from panel C. Concentrations are anticorrelated across all tested media glucose concentrations ($p=0.0006$, Pearson $r=-0.9352$). (F) Correlation of Cdr2 vs Pom1 concentrations at cell tips in each glucose concentration from panel D. Concentrations show weak anticorrelation across the tested media glucose concentrations, but the correlation is not statistically significant ($p=0.09$, Pearson $r=-0.65$).

Figure 8: Pom1 redistribution under glucose restriction disrupts Wee1 regulation at cortical nodes. (A) Localization of Wee1-mNG in wild-type or pom1Δ cells grown in either high (2%) or low (0.03%) glucose media. Images were collected using TIRF microscopy. Scale bar, 5µm. (B) Quantification of Wee1 bursting kinetics in wild-type cells grown under high (2%, blue circles) and low (0.03%, red squares) glucose conditions. The top panel is a plot of the total number of Wee1 bursts as a function of cell length, counted in single time point TIRF micrographs. The slopes of the linear regressions are significantly different ($p<0.0001$, $R^2_{2\%}=0.66$, $R^2_{0.03\%}=0.45$). The middle panel is a plot of the frequency of Wee1 bursts as a function of cell length, measured using the TrackMate particle tracking algorithm on TIRF microscopy movies. The slopes of the linear regressions are not significantly different ($p=0.8426$, $R^2_{2\%}=0.50$, $R^2_{0.03\%}=0.51$). The bottom panel is a plot of Wee1 burst duration as a function of cell length, measured using the TrackMate particle tracking algorithm on TIRF microscopy movies. The slopes of the linear regressions are not significantly different ($p=0.09$, $R^2_{2\%}=0.28$, $R^2_{0.03\%}=0.05$), but the values of the elevation of the regressions are significantly different ($p=0.0005$). (C) Quantification of Wee1 bursting kinetics in pom1Δ cells grown under normal (2%, green circles) and low (0.03%, magenta squares) glucose conditions. The top panel is a plot of the total number of Wee1 bursts as a function of cell length, counted in single time point TIRF micrographs. The slopes of the linear regressions are not significantly different ($p=0.09$, $R^2_{2\%}=0.45$, $R^2_{0.03\%}=0.54$), but the values of the elevation of the regressions are significantly different ($p<0.0001$). The middle panel is a plot of the frequency of Wee1 bursts as a function of cell length, measured using the TrackMate particle tracking algorithm on TIRF microscopy movies. The slopes of the linear regressions are not significantly different ($p=0.3125$, $R^2_{2\%}=0.45$, $R^2_{0.03\%}=0.56$) but the values of the elevation of the regressions are significantly different ($p=0.009$). The bottom panel is a plot of Wee1
burst duration as a function of cell length, measured using the TrackMate particle tracking algorithm on TIRF microscopy movies. The slopes of the linear regressions are not significantly different ($p=0.8352$, $R^2_{2\%}=0.3$, $R^2_{0.03\%}=0.002$), and the values of the intercepts of the regressions are not significantly different ($p=0.5392$). For (B-C), $b_1$ denotes slope and $b_0$ denotes the Y-intercept. (D) Western blot of Wee1 in wild-type, $pom1\Delta$, and $cdr2\Delta$ cells grown in high (2%) or low (0.03%) glucose media showing phosphorylation dependent band-shifts. Whole cell extracts were separated by SDS-PAGE, and blots were probed with an $\alpha$-Wee1 antibody.

**Figure 9:** A model for the glucose-modulated control of Wee1 bursting by Pom1 and Cdr2. See text for discussion.

**Supplemental Figure S1** (Figure 1): Analysis of the Pom1 gradient by confocal microscopy. (A) Similar to TIRF microscopy, at middle-cell focal planes, discreet Pom1-mNeonGreen puncta are apparent at the lateral cell cortex of cells in individual frames (200ms) of spinning-disc microscopy movies. Scale bar 1µm. (B) Individual Pom1 puncta become averaged out in a projection of the entire continuous spinning-disc confocal microscopy movie. Sum projection of a continuous 15s time-lapse acquisition of 200ms exposures. (C) A line scan along the cell-perimeter in the direction indicated by the arrow of the single time-point micrograph of the cell in (A) reveals that Pom1 puncta form a noisy concentration gradient that begins at the cell tip and decays toward the cell middle. (D) The same line scan as in panel C, but of the time-lapse sum projection of the cell in panel B. This plot demonstrates that time-averaging of Pom1 puncta smoothen the concentration gradient. (E) Spinning-disc confocal micrographs of single 200ms acquisitions and (F) sum-projections of time lapse movies as in panels A and B respectively, but using a top-focal plane. (G-H) Line scan using a straight line across the long axis of the cell indicated by the arrow head on both the single time-point (G) and sum projection (H) of a 15s continuous time-lapse movie. (I-J) Representative images of Airyscan super-resolution micrographs of Pom1-mNeonGreen at either the cell tips (I) or cell side (J). Images are sum projections of sequential five-frame Z-stacks spaced 0.17µm
apart, positioned to include all detected cortical Pom1-mNeonGreen signal. Scale bar, 1µm. (K) Fluorescence intensities of all single puncta resolved in Airyscan images as in panels I-J. Data points represent individual puncta values, error bars are the mean and standard deviation.

**Supplemental Figure S2 (Figure 2): Controls and supporting in vitro analysis of Pom1 puncta.** (A) Fluorescence intensity trace of a single Pom1 puncta binding to the cell cortex, measured by high speed TIRF microscopy of a cell expressing Pom1-mNG (~50 frames per second with continuous acquisition). Rapid intensity changes indicating binding and unbinding are indicated by green upward and magenta downward pointing arrows respectively. (B) Size standards for velocity sucrose gradients, as in main Figure 2C-E. Data are quantified from single Coomassie-stained gels, or using spectrophotometry data. (C) Plot of S-value versus sedimentation pattern. Line is linear regression of the three size standards, with the S-value for Pom1 peaks interpolated. (D) Quantification and comparison of binding duration of either wild-type or kinase-dead Pom1-mNG to supported lipid bilayers in TIRF microscopy movies (**p<=0.0001). Statistical significance was tested with a Student’s T-test. (E) FRAP analysis of supported lipid bilayers. Upper panel: A circular ROI (teal dashed line) was used to bleach fluorescent PE in the bilayers. Lower panel: Kymograph at white dashed line in upper panel of fluorescence recovery as monitored by TIRF microscopy, indicating fluidity.

**Supplemental Figure S3 (Figure 3): Representative single-cell data used to compute mean cellular values in Figure 3.** (A) Quantification of binding duration of individual Pom1-mNG puncta at the tips or sides of representative wild-type, tea1Δ or tea4Δ cells, measured by TIRF microscopy. (B) Quantification of total displacement of individual Pom1-mNG puncta at the tips or sides of representative wild-type, tea1Δ or tea4Δ cells, measured by TIRF microscopy. For A-B, line and error represent single cell mean and standard deviation.

**Supplemental Figure S4 (Figure 4): Supporting analysis of Cdr2 nodes and Pom1 puncta at the medial cell cortex.** (A) Comparison of Pom1 puncta binding frequency on
the cell sides of wild-type and \textit{cdr2\Delta} cells (n.s., \(p=0.1076\)). (B) Comparison of Pom1 puncta binding duration on the cell sides of wild-type and \textit{cdr2\Delta} cells (n.s., \(p=0.371\)). (C) Comparison of Pom1 puncta displacement on the cell sides of wild-type and \textit{cdr2\Delta} cells (n.s., \(p=0.8191\)). In A-C, significance was tested using a Student's T-test. (D) Schematic and quantification of Pom1 concentration at the lateral cell cortex measured by confocal microscopy, using 2\(\mu\)m line scans at the cell middle from images such as Figure 5A. The slope of the linear regression is negative, but not significantly non-zero (\(p=0.2603\), \(R^2=0.03\)). (E) Plot of the number of colocalized structures counted in a middle cell 2\(\times\)2\(\mu\)m ROI as a function of cell size. The slope of the linear regression is significantly non-zero (\(p=0.0006\), \(R^2=0.3\)). (F) Plot of free, non-colocalized Cdr2 nodes (blue circles) and Pom1 puncta (red squares). The slope of the linear regression of \#Pom1 puncta versus cell length is negative, and significantly non-zero (\(p=0.001\), \(R^2=0.27\)). The slope of the linear regression of \#Cdr2 nodes versus cell length is positive, and significantly non-zero (\(p=0.021\), \(R^2=0.15\)).

\textbf{Supplemental Figure S5 (Figure 5): In vitro analysis of Pom1 activity in \textit{tea1\Delta} cells.} (A) Western blot of an \textit{in vitro} thiophosphate kinase assay to test Pom1 autophosphorylation activity in \textit{pom1-3HA}, \textit{pom1-AS-3HA}, and \textit{pom1-AS-3HA tea1\Delta} cells. Blots were probed with an \(\alpha\)-thiophosphate antibody. Only analog-sensitive (AS) alleles can use ATP\(\gamma\)S to thio-phosphorylate substrates. (B) Comparison of western blot band intensities from four replicate gels, derived from two separate experiments as in panel A. Statistical significance was tested using a paired T-test between \textit{Pom1-AS-3HA} and \textit{Pom1-AS-3HA tea1\Delta} cells (*, \(p=0.02\)). (C) Western blot of an \textit{in vitro} thiophosphate kinase assay to measure phosphorylation of purified Cdr2 by Pom1 in \textit{pom1-3HA}, \textit{pom1-AS-3HA}, and \textit{pom1-AS-3HA tea1\Delta} cells. Blots were probed with an anti-thiophosphate antibody. Excess Pom1-3HA was used in lanes 1 and 3 to ensure absence of signal in negative controls lacking the analog-sensitive mutation. Cdr2(712-775) is a fragment that contains known Pom1 phosphorylation sites. (D) Western blot of the Pom1 substrate Rga7-3HA in wild-type, \textit{tea1\Delta}, and \textit{pom1\Delta} cells. Whole-cell extracts were separated by SDS-PAGE, and probed with \(\alpha\)-HA antibodies. The upper band represents a Pom1-dependent phospho-isoform. (E) Comparison of band intensities from four replicate gels
as in (D), expressed as the ratio of the grey-value of the top band/bottom band. Line and
error are mean and standard deviation. Statistical significance was tested using a one-
way ANOVA and Tukey’s multiple comparisons test (WT vs tea1Δ, ***p=0.003, WT vs
pom1Δ, ****p<0.0001, tea1Δ vs pom1Δ, ****p<0.0001).

Supplemental Figure S6 (Figure 5): **Control of Pom1 concentration at the cell side.**

(A) Localization of Pom1-mNG in wild-type, tea1Δ, and pom1-KD (kinase-dead) cells at
the indicated concentration of glucose. Images are confocal micrographs taken at a
middle cell focal plane. Scale bar, 5µm. (B) Quantification of Pom1 concentration along
2µm line scans as in figure S4D, measured using images as in panel A. The means of
each column are significantly different from every other column (p<0.005). Statistical
significance was tested using a one-way ANOVA and Tukey’s multiple comparison’s test.
(C) Plot of Pom1 concentration at the cell side as a function of glucose concentration in
the growth media. Data were fit to an exponential curve showing a negative sinusoidal
relationship reaching half saturation at ~0.05% glucose. The concentration of Pom1-mNG
at the lateral cell cortex in a tea1Δ mutant was fit to this curve, and is the approximate
equivalent of wild-type cells grown in 0.03% Glucose. Line and error are mean and
standard deviation. (D) Localization of Tea4-mNG in wild-type or tea1Δ cells grown in the
indicated concentration of glucose for one hour. Images are confocal micrographs taken
at middle cell focal planes. Arrows point to lateral puncta of Tea4-mNG. Scale bar, 5µm.

Supplemental Figure S7 (Figure 6): **Redistribution of Pom1 puncta to the lateral
cortex disrupts Cdr2 nodes during glucose restriction, supporting data.** (A) Analysis
of Pom1 and Cdr2 redistribution by spinning-disc confocal microscopy, as in (Kelkar and
Martin, 2015). Images are confocal micrographs of cells co-expressing Pom1-tdTomato
and Cdr2-mNG in high (2%) or low (0.03%) glucose media. Scale bar, 5µm. (B) Cortical
fluorescence intensity of Cdr2 and Pom1 was measured using line scans drawn around
the circumference of the representative cell marked with an arrowhead in the 2% glucose
portion of panel A. (C) Cortical fluorescence intensity of Cdr2 and Pom1 was measured
using line scans drawn around the circumference of the representative cell marked with
an arrowhead in the 0.03% glucose portion of panel A.
Supplemental Figure S8 (Figure 8): Re-plotting of data from Main Figure 8 for additional comparison. (A) Plot of the frequency of Wee1 bursts as a function of cell length in wild-type cells grown in high (2%) glucose (blue circles) and low glucose (red squares), and kinase-dead cdr2(E177A) mutant cells grown in 2% glucose (green triangles). Measurements were made using the TrackMate particle tracking algorithm on TIRF microscopy movies. The slopes of the linear regressions are not significantly different ($p=0.7651$, $R^2_{2\%}=0.5$, $R^2_{0.03\%}=0.51$, $R^2_{Cdr2(E177A)}=0.51$) and the elevations of the regressions are also not significantly different ($p=0.4397$). (B) Plot of Wee1 burst duration as a function of cell length in wild-type cells grown in low glucose (red squares) and kinase-dead cdr2(E177A) mutant cells grown in 2% glucose (green triangles). Measurements were made using the Trackmate particle tracking algorithm on TIRF microscopy movies. The slopes of the linear regressions are not significantly different ($p=0.8048$, $R^2_{0.03\%}=0.05$, $R^2_{Cdr2(E177A)}=0.2$) and the elevations of the regressions are also not significantly different ($p=0.8048$).

Supplemental Table S1: Fission yeast strains used in this study.

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Figure 1

A. Pom1-mNG – TIRF Microscopy
- 200ms Snap Shot
- Sum Projection 15s Movie

B. Fluorescence (A.U.) vs. X-Position (µm)
- 200ms snap
- 15s projection

C. Pom1-mNeonGreen Particle Traces
- Duration: 0.4s, 12s
- 570nm

D. Pom1-mNG
- Cell Tip
- Cell Side

E. Pom1-mNG

F. Frequency (punctae/s) vs. TIP vs. SIDE
- ****

G. Avg. Displacement (µm) vs. TIP vs. SIDE
- ***

H. Avg. Dwell Time (s) vs. TIP vs. SIDE
- n.s.

I. Frequency (% Total) vs. Fluorescence Bin Center (A.U.)
- TIP
- SIDE
Supplemental Figure 1 in support of Figure 1

A. Spinning Disc – Middle Focal Plane
   200ms Snap Shot
   Pom1-mNG

B. Sum Projection of 15s Movie

C. Graph: X-Position (μm) vs. Fluorescence (A.U.)
   (Middle Focal Plane)

D. Graph: X-Position (μm) vs. Fluorescence (A.U.)
   (Middle Focal Plane)

E. Spinning Disc – Top Focal Plane
   200ms Snap Shot
   Pom1-mNG

F. Sum Projection of 15s Movie

G. Graph: X-Position (μm) vs. Fluorescence (A.U.)
   (Top Focal Plane)

H. Graph: X-Position (μm) vs. Fluorescence (A.U.)
   (Top Focal Plane)

I. Cell Tip
   Pom1-mNG

J. Cell Side
   Pom1-mNG

K. Graph: Fluorescence (A.U.) vs. TIP vs. SIDE

Supplemental Figure 1 in support of Figure 1
Figure 2

A. Pom1-mNG Burst Cell

B. No Tag Cell Extract vs. Pom1-mNG Cell Extract

C. Yeast Extract

D. Sucrose Gradient Fraction

E. Pom1-mNG - Supported Lipid Bilayer

F. Pom1-mNG Puncta Dwell Time (s)

G. Purified Protein

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**Figure 2**

A. Pom1-mNG Burst Cell

B. No Tag Cell Extract vs. Pom1-mNG Cell Extract

C. Yeast Extract

D. Sucrose Gradient Fraction

E. Pom1-mNG - Supported Lipid Bilayer

F. Pom1-mNG Puncta Dwell Time (s)

G. Purified Protein
Supplemental Figure 2 in support of Figure 2

A. Single Puncta Trace
   TIRF Microscopy -20ms/frame

B. Size Standards
   BSA  Thyroglobulin  Ferritin

C. Sedimentation Peak Fraction

D. Pom1-mNG Puncta Dwell Time (s)
   WT (SLB)  Kinase-Dead (SLB)

E. 0.01% Liss-Rhod-PE
   75% PC 25%PS
Figure 3

A. Sucrose Gradient Fraction

| Fraction | tea1Δ | tea4Δ |
|----------|-------|-------|
| 1        |       |       |
| 2        |       |       |
| 3        |       |       |
| 4        |       |       |
| 5        |       |       |
| 6        |       |       |
| 7        |       |       |
| 8        |       |       |
| 9        |       |       |

150 kDa αHA
150 kDa αHA

B. Confocal Microscopy

tea1Δ

C. TIRF Microscopy

Cell Side

Pom1-mNG

D. Frequency (punctuals)

E. Avg. Dwell Time (s)
Supplemental Figure 3 in support of Figure 3
Figure 4

A. 
Pom1-mNG  Cdr2-tagRFP-t  Merge

B. 
Kymograph ROI

C. 
Cdr2-mEGFP

D. 
Cdr2 Nodes  Pom1 Puncta

E. 
Cdr2 Nodes  Pom1 Puncta

F. 
Cdr2-mNG  Pom1-tdTomato  Merge

G. 
# Free Cdr2/Pom1 Structures

Figure 4
Supplemental Figure 4 in support of Figure 4
Figure 5

A. Pom1-mNG

WT

tea1Δ

Confocal Microscopy – Middle Focal Plane

Cdr2-mNG

tea1Δ

TIRF Microscopy

B. [Pom1] at cell sides

Fluorescence (A.U.)

WT

tea1Δ

D. WT

tea1Δ

# Cdr2 Nodes

Cell Length (μm)

E. Wee1-mNG

tea1Δ

TIRF Microscopy

F. WT

tea1Δ

# Wee1 Bursts

Cell Length (μm)
Supplemental Figure 5 in support of Figure 5
Supplemental Figure 6 in support of Figure 5

A. Confocal Microscopy images showing the effect of glucose concentrations on Pom1-mNG, Pom1-mNG tea1Δ, and Pom1-KD-mNG cells. The images compare 2% Glucose and 0.03% Glucose conditions.

B. Graph showing the average fluorescence intensity in cell sides with different glucose concentrations.

C. Graph showing the fluorescence of Pom1 at cell sides with varying glucose concentrations. The interpolation for the glucose concentration is indicated.

D. Additional Confocal Microscopy images showing Tea4-mNG expression under different conditions (2% Glucose, 0.03% Glucose, tea1Δ 2% Glucose).
Figure 6

A. Pom1-mNG

2% Glucose

0.03% Glucose

TIRF Microscopy

B. Frequency (puncta/s)

2% Glucose Media

0.03% Glucose Media

C. Avg. Dwell Time (s) n.s.

2% Glucose Media

0.03% Glucose Media

D. Cdr2-mNG

2% Glucose

0.03% Glucose

TIRF Microscopy

E. Single Node

Fluorescence Intensity (A.U.)

WT 2% Glucose

WT 0.03% Glucose

pom1Δ 2% Glucose

pom1Δ 0.03% Glucose

**** n.s.

F. Avg. # Cdr2 Nodes / Cell

WT 2% Glucose

WT 0.03% Glucose

pom1Δ 2% Glucose

pom1Δ 0.03% Glucose

n.s. n.s.
Supplemental Figure 7 in support of Figure 6

A. Confocal Microscopy

| 2% Glucose | 0.03% Glucose | Merge |
|------------|---------------|-------|
| Pom1-tdTomato | Cdr2-mNG | Merge |

B.

C.

Supplemental Figure 7 in support of Figure 6
Figure 7

A. Pom1-mNG

B. Cdr2-mNG

C. Lateral [Cdr2] vs. Lateral [Pom1]

D. Tip [Cdr2] vs. Tip [Pom1]

E. [Cdr2] Cell Side vs. [Pom1] Cell Side

F. [Cdr2] Cell Tip vs. [Pom1] Cell Tip

Pearson r = -0.9352
P=0.0006 (***)

Pearson r = -0.65
P=0.090 (n.s.)
A. TIRF Microscopy

B. Graph showing the relationship between cell length and Wee1 bursts, frequency, and burst durations for WT and pom1Δ strains in 2% and 0.03% glucose media.

C. Graph showing the relationship between cell length and Wee1 bursts, frequency, and burst durations for WT and pom1Δ strains in 2% and 0.03% glucose media.

D. Western blot analysis of αWee1 protein levels in WT, pom1Δ, and cdr2Δ strains with 2% and 0.03% glucose.
Supplemental Figure 8 in support of Figure 8

A. WT 2% Glucose
   WT 0.03% Glucose
   cdr2(E177A) 2% Glucose

b₁ = n.s.
b₀ = n.s.

B. WT 0.03% Glucose
   cdr2(E177A) 2% Glucose

b₁ = n.s.
b₀ = n.s.
Figure 9
| Strain   | Genotype                          | Origin              | Figure |
|----------|-----------------------------------|---------------------|--------|
| JM4496   | pom1-yomNeonGreen::hphR           | This Study          |        |
| JM797    | pom1::hphR ura4::D18 leu1::2 h+   | PN948 (Paul Nurse Lab) |        |
| JM966    | pom1::natR h-                     | Lab Stock           | 2G, 8D |
| JM5412   | pom1-m2-yomNeonGreen::hphR ura4::D18 leu1::2 h- | This Study | 5A-D, 5A-B, 56A-C, 6A-C, 7A-C-F |
| JM5414   | pom1::hphR tea1::ura4+ ura4::D18  | This Study          | 3A     |
| JM5415   | pom1::hphR tea1::kanMX6           | This Study          | 3A     |
| JM4792   | pom1-yomNeonGreen::hphR tea1::kanMX6 | This Study   | 3B-F, 5A-B, 5A-C, 6A-C |
| JM4791   | pom1-yomNeonGreen::hphR tea1::kanMX6 | This Study   | 3B-F, 5A-B |
| JM5373   | pom1-yomNeonGreen::hphR cd2-1::tagRFP::hphR | This Study | 4A-B |
| JM4493   | cd2-yomNeonGreen::hphR h-         | Lab Stock           | 4C-E, 6D-F, 7B-F |
| JM4525   | cd2-yomNeonGreen::hphR pom1::cd2::natR | Lab Stock | 5A-C |
| JM5135   | cd2-yomNeonGreen::hphR pom1::cd2::natR | Lab Stock | 4F-G, 5A-E, 57A-C |
| JM374    | cd2-yomNeonGreen::hphR tea1::kanMX6 | This Study | 5C-D |
| JM372    | wee1-yomNeonGreen::hphR tea1::kanMX6 | This Study | 5E-F |
| JM2209   | rga7::hphR h-                     | Lab Stock           | 5SD-D |
| JM5612   | rga7::hphR tea1::kanMX6           | Lab Stock           | 5SD-E |
| JM2210   | rga7::hphR pom1::kanMX6           | Lab Stock           | 5SD-E |
| JM4211   | pom1::c2-H4::hphR                 | Lab Stock           | 5SA-C |
| JM5565   | pom1::c2-H4::hphR tea1::kanMX6   | This Study          | 5SA-C |
| JM5132   | tea1-yomNeonGreen::hphR h-        | Lab Stock           | 5ED    |
| JM373    | tea1-yomNeonGreen::hphR tea1::kanMX6 | This Study | 5ED |
| JM359    | cd2-yomNeonGreen::hphR pom1::natR | This Study          | 6D-F   |
| JM4495   | wee1-yomNeonGreen::hphR h-        | Lab Stock           | BA-B, 5BA-B |
| JM4527   | wee1-yomNeonGreen::hphR pom1::kanMX6 | Lab Stock | BA-C |
| JM366    | 972 h-                            | PN1 (Paul Nurse lab) | BD     |
| JM360    | 972 h-                            | Lab Stock           | BD     |
| JM4578   | wee1-yomNeonGreen::hphR cd2(E177A) | Lab Stock | 5BA-B |