**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

32  length or volume, for cell size control (Facchetti et al., 2019; Pan et al., 2014). A critical

33  next step is to understand how signaling pathways that regulate Cdk1 might operate at

34  the cell cortex in a size-dependent manner.

35

36      In all eukaryotes, Cdk1 activity is established by the opposing activities of the

37  inhibitory protein kinase Wee1, and the counteracting phosphatase Cdc25 (Gautier et al.,

38  1991; Gould and Nurse, 1989; Kumagai and Dunphy, 1991; Russell and Nurse, 1986,

39  1987; Strausfeld et al., 1991). In fission yeast, mutations in Wee1, Cdc25, and their

40  upstream regulators lead to changes in cell size that correspond to changes in the

41  signaling pathways. Separate mechanisms appear to link cell size with regulation of Wee1

42  versus Cdc25. For Cdc25, its cellular concentration increases through unknown

43  mechanisms as cells grow during interphase (Keifenheim et al., 2017; Moreno et al.,

44  1990). In contrast, the concentration of Wee1 remains constant during interphase, but it

45  is progressively phosphorylated by the conserved inhibitory kinases Cdr1 and Cdr2

46  (Aligue et al., 1997; Breeding et al., 1998; Kanoh and Russell, 1998; Keifenheim et al.,

47  2017; Lucena et al., 2017; Opalko and Moseley, 2017; Russell and Nurse, 1987; Wu and

48  Russell, 1993; Young and Fantes, 1987). *cdr2* mutants fail to divide at a constant surface

49  area, and instead dividing according to cell volume or length (Facchetti et al., 2019). This

50  change suggests that Cdr2-Cdr1-Wee1 signaling underlies the primary size-sensing

51  pathway that relates to surface area, while additional pathways related to volume and

52  length are engaged in its absence. The localization of Cdr2, Cdr1, and Wee1 supports

53  this model. Cdr2 forms punctate oligomeric structures called nodes that stably bind to the

54  medial cell cortex, and recruits Cdr1 to these sites (Akamatsu et al., 2014, 2017; Guzmán-

55  Vendrell et al., 2015; Martin and Berthelot-Grosjean, 2009; Morrell et al., 2004; Moseley

56  et al., 2009). Wee1 localizes primarily in the nucleus and spindle-pole body, where it

57  encounters Cdk1 to prevent mitotic entry (Masuda et al., 2011; Moseley et al., 2009; Wu

58  et al., 1996). In addition, Wee1 transiently visits cortical Cdr1/2 nodes in bursts that lead

59  to inhibition of Wee1 kinase activity (Allard et al., 2018). The frequency and duration of

60  these Wee1 bursts at Cdr1/2 nodes increase approximately twenty-fold as cells double in

61  size during interphase (Allard et al., 2018). This mechanism leads to size-dependent

62  inhibition of Wee1 by a signaling pathway at the cell surface.

63

64      This size-dependent change in Wee1 bursting dynamics is encoded into the Cdr1-

65   Cdr2-Wee1 pathway at two proposed levels. First, increased Wee1 bursting depends

66   upon the doubling in number of Cdr1/2 nodes, which occurs as growth results in a

67   doubling of cell surface area, but this increase is smaller than the magnitude of change

68   in Wee1 bursting (~2-fold vs ~20-fold) (Allard et al., 2018; Deng and Moseley, 2013; Pan

69   et al., 2014). Second, activation of Cdr2 increases as cells increase in size (Deng et al.,

70   2014). Cdr2 kinase activity is required for Wee1 localization to nodes, and is controlled

71   by the upstream kinase Pom1 (Allard et al., 2018; Moseley et al., 2009). Pom1 directly

72   phosphorylates and inhibits the activation of Cdr2 (Bhatia et al., 2014; Deng et al., 2014;

73   Kettenbach et al., 2015; Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009).

74   Pom1 also phosphorylates a separate set of sites on Cdr2 to disrupt oligomerization and

75   membrane binding (Bhatia et al., 2014; Rincon et al., 2014). This regulation of Cdr2 by

76   Pom1 changes the size dependency of Wee1 bursts. Specifically, the frequency and

77   duration of Wee1 bursts at Cdr1/2 nodes increases, but only in small cells (Allard et al.,

78   2018). Consistent with this defect, *pom1Δ* cells divide at a small size due to dysregulation

79   of the Cdr2-Cdr1-Wee1 pathway (Bähler and Pringle, 1998; Bhatia et al., 2014; Martin

80   and Berthelot-Grosjean, 2009; Moseley et al., 2009; Wood and Nurse, 2013). Taken

81   together, these results suggest a size-dependent interaction between Pom1 and its

82   substrate Cdr2. However, the location and timing of Pom1 interactions with Cdr2 have

83   remained poorly defined.

84

85      Pom1 localizes in a spatial gradient that is enriched at cell tips, with a lower

86   concentration at the medial cell cortex, where its substrate Cdr2 forms nodes (Bähler and

87   Pringle, 1998; Bhatia et al., 2014; Martin and Berthelot-Grosjean, 2009; Moseley et al.,

88   2009; Pan et al., 2014). Thus, the majority of Pom1 and Cdr2 molecules in a cell are

89   spatially separated, raising the question of when and where they interact. Several lines

90   of evidence suggest that the spatial distributions of Pom1 and Cdr2 are critical for the

91   size-dependent signaling properties of this pathway, and suggest the lateral cell cortex

92   as the key interface. For example, ectopic targeting of Pom1 to the medial cell cortex

93   inhibits Cdr2 node formation and Cdr2-dependent cell size signaling (Martin and

94    Berthelot-Grosjean, 2009). Additionally, the Pom1 gradient is dissipated as part of a
95    cellular response to glucose deprivation, which leads to increased Pom1 concentration at
96    the lateral cortex where it delays mitotic entry (Kelkar and Martin, 2015). This result also
97    demonstrates that Pom1-Cdr2 signaling responds to environmental input to coordinate
98    cell size with nutrient availability. In this system, Pom1 functions analogously to an
99    intracellular morphogen, acting as a concentration-dependent and localization-controlled
100   inhibitor of Cdr2 nodes. However, levels of Pom1 at the medial cell cortex in wild-type
101   cells growing under steady state conditions are low, constant, and do not vary with cell
102   size (Bhatia et al., 2014; Pan et al., 2014). The mechanism by which Pom1 provides size
103   specific input to Cdr2 is unclear and requires analysis of their molecular dynamics at the
104   lateral cell cortex.

105

106         Past studies have led to a model for Pom1 gradient formation driven by the binding
107   and diffusion of individual Pom1 molecules in the plasma membrane (Hachet et al., 2011;
108   Saunders et al., 2012). Pom1 binds directly to anionic phospholipids at the cell cortex,
109   and these interactions are disrupted upon intramolecular autophosphorylation (Hachet et
110   al., 2011). Polarity factors including Tea1 and Tea4 are enriched at the cell tips by
111   microtubules, where they recruit the protein phosphatase 1 (PP1) Dis2, which
112   dephosphorylates Pom1 to promote membrane attachment specifically at the cell tip
113   (Hachet et al., 2011). Cortical Pom1 molecules are then thought to diffuse in the plane of
114   the membrane away from the tip, until multiple autophosphorylation events disrupt
115   membrane binding (Hachet et al., 2011; Hersch et al., 2015; Saunders et al., 2012). Tip-
116   concentrated Pom1 occludes Cdr1/2 node formation, restricting them instead to the
117   medial cell cortex (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009).
118   Intriguingly, dynamic clusters of Pom1 have also been observed and are proposed to form
119   by unstable oligomerization of molecules diffusing in the plane of the membrane (Hachet
120   et al., 2011; Saunders et al., 2012). These clusters are thought to form and decay rapidly,
121   temporarily sequestering Pom1 molecules and thereby slowing molecular diffusion rates
122   and reducing noise in the gradient (Saunders et al., 2012). These puncta represent nano-
123   scale pockets of increased Pom1 concentration, but whether these clusters contribute to
124   regulation of Cdr2 nodes is unknown.

125

126     Here, we use biochemical approaches in combination with quantitative TIRF and
127     confocal microscopy to show that the Pom1 gradient itself is formed by these punctate
128     clusters. Pom1 puncta are stable oligomers that bind and release the membrane with
129     minimal lateral diffusion. The frequency of Pom1 puncta binding is higher at cell tips than
130     on cell sides. Thus, the gradient is formed because the membrane-association frequency
131     of Pom1 puncta is patterned along the long axis of the cell. A portion of Pom1 puncta in
132     the medial cell cortex colocalize with Cdr2 nodes, and the ratio of Pom1 to Cdr2 at the
133     medial cell cortex changes as a function of cell size. When glucose availability is
134     restricted, Pom1 clusters redistribute to the medial cell cortex to prevent dramatic
135     dysregulation of Wee1 bursts. Our work reveals that the Pom1-Cdr2-Wee1 signaling
136     pathway is organized as a series of cortical clusters. The relative distribution of these
137     clusters at the plasma membrane changes with cell size and glucose availability, thus
138     relaying cell surface area to the core cell cycle machinery in a nutrient-controlled manner.
139

140     # Results:

141     **The Pom1 gradient is formed by transient cortical puncta**

142     We sought to examine the molecular dynamics of Pom1-Cdr2 signaling at the cell
143     cortex. As a starting point, we imaged Pom1 by TIRF microscopy, which selectively
144     excites fluorophores near the coverslip. Pom1 was tagged at the endogenous locus with
145     the bright and photostable fluorophore mNeonGreen (mNG). Surprisingly, along the
146     lateral cell cortex Pom1-mNG was localized almost exclusively in discreet puncta, with no
147     apparent diffuse signal **(Figure 1A)**. In images from a single time-point, Pom1 puncta
148     formed a noisy concentration gradient **(Figure 1B)**. Time-averaging produced a smoother
149     gradient dotted by occasional clusters, similar to the gradient observed by confocal
150     microscopy **(Figure 1A-B)**. Similar results were obtained by confocal microscopy **(Figure
151     S1A-H)**. Pom1 puncta were highly dynamic. They appeared on the lateral cortex with a
152     frequency of ~2 puncta*$\mu m^{-2}s^{-1}$, and remained bound for less than 2 seconds on average
153     **(Figure 1C,F,H)**. During this brief cortical attachment, Pom1 puncta exhibited minimal
154     diffusion which lacked directionality, and instead appeared to diffuse randomly in sub-
155     micron patches of cortex **(Figure 1C,G)**.

156

157   Similar puncta were previously observed by confocal microscopy, but they were

158  not thought to form the Pom1 concentration gradient *per se* (Hachet et al., 2011;

159  Saunders et al., 2012). Our findings suggested the distinct possibility that the cortical

160  gradient itself is comprised of dynamic puncta. To test this possibility, we needed to image

161  Pom1-mNG at cell tips, which are inaccessible to visualization using standard slide

162  preparation for TIRF microscopy because this region is outside of the TIRF illumination

163  field. Therefore, we performed "head-on" TIRF imaging of cell tips. Pom1-mNG localized

164  at cell tips almost entirely in puncta, similar to cell sides **(Figure 1D-E)**. We also used

165  "head-on" Airyscan confocal imaging to confirm that Pom1 localization in puncta at tips

166  was not an artifact of the TIRF approach **(Figure S1I)**, a conclusion also supported by

167  work using high resolution wide-field microscopy (Dodgson et al., 2013). Compared to

168  cell sides, Pom1 puncta appeared twice as frequently at cell tips but with a similar cortical

169  duration, again exhibiting minimal and non-directional diffusion away from their binding

170  site **(Figure 1F-H)**. Fluorescence intensity of individual puncta at cell tips and cell sides

171  were similar, although we detected a low number of brighter puncta at cell tips, likely

172  representing multiple diffraction-limited puncta **(Figure 1I, Figure S1I-K)**. From these

173  data, we conclude that the Pom1 gradient is formed by patterning the membrane binding

174  frequency of cortical puncta along the long axis of the cell, rather than by diffusion of

175  Pom1 molecules from the cell tips to the cell side.

176

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

178   Past work suggested that Pom1 puncta assemble and disassemble at the cortex

179  through oligomerization of individual molecules diffusing in the plane of the membrane

180  (Saunders et al., 2012). However, our high-speed (20ms/frame) continuous TIRF videos

181  suggested that Pom1 puncta bind and release the cortex as a pre-formed unit **(Figure**

182  **S2A)**. Consistent with this possibility, we observed rare cell lysis events in which Pom1

183  puncta remained intact in extruded cytoplasm **(Figure 2A)**. This apparent stability while

184  removed from the plasma membrane led us to test the existence and properties of Pom1

185  puncta in detergent cell extracts. Using TIRF microscopy, we observed puncta of Pom1-

186  mNG in extracts made from Pom1-mNG cells, but not from untagged wild-type cells

187   **(Figure 2B)**. We next examined the size of these Pom1 puncta by velocity sucrose

188   gradient sedimentation. By fractionating detergent cell extracts from *pom1-3xHA* cells,

189   we found that most Pom1 exists in a large 60S complex, consistent with Pom1 puncta

190   observed in cells. Fractions 6-8 containing this peak were dialyzed and centrifuged on a

191   second sucrose gradient. These pooled fractions again sedimented at 60S, indicating that

192   they represent a stable complex **(Figure 2C-D, S2B-C)**.

193

194          Pom1 complexes present in detergent extracts resemble the cortical puncta

195   observed in cells. To test this connection further, we examined the membrane-binding

196   capacity of Pom1 complexes *in vitro* using artificial supported lipid bilayers (SLBs). Pom1-

197   mNG puncta were isolated by sucrose gradient centrifugation, incubated with fluid SLBs

198   on coverslips, and then imaged by TIRF microscopy **(Figure 2E, S2E).** In this cell free-

199   system, Pom1-mNG puncta bound and released SLB lipids with strikingly similar

200   dynamics as in cells **(Figure 2E-F)**. Dwell times were increased for the kinase-dead

201   mutant Pom1(K728R)-mNG, which was previously shown to increase cortical Pom1

202   levels in cells **(Figure S2D)** (Bähler and Pringle, 1998; Hachet et al., 2011). Collectively,

203   our TIRF microscopy and *in vitro* analysis of Pom1 puncta support a model whereby the

204   majority of cellular Pom1 protein is contained within discreet, highly-stable oligomers that

205   bind and release membranes with kinetics dictated by their catalytic activity in a geometric

206   pattern that resembles a concentration gradient.

207

208          Formation of clusters could represent an intrinsic property of Pom1 protein, or

209   alternatively might require additional cellular factors. To distinguish between these

210   possibilities, we expressed and purified GST-Pom1 from bacteria, and then performed

211   sucrose gradient centrifugation experiments on this recombinant protein. Recombinant

212   GST-Pom1 sedimented in a low molecular weight peak unlike Pom1 puncta from cells.

213   Remarkably, purified GST-Pom1 was assembled into a puncta-sized high molecular

214   weight complex by incubation with wild-type yeast detergent extract **(Figure 2G)**.

215   Complex formation was also induced by incubation of GST-Pom1 with *pom1∆* cell

216   extracts, meaning that additional cellular factors drive this assembly process. **(Figure**

217   **2G).** Once assembled, Pom1 puncta are stable oligomeric complexes with intrinsic

218    membrane-binding properties that can be reconstituted *in vitro*. Additional cellular

219    regulatory proteins are likely to promote the binding of Pom1 puncta at cell tips, leading

220    to the spatial gradient.

221

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

223         The key role of puncta in forming the Pom1 gradient led us to reexamine the role of

224    cell polarity landmark proteins Tea1 and Tea4. Both of these proteins localize at cell tips

225    and are required for proper Pom1 gradient formation (Hachet et al., 2011). However, past

226    studies have shown that Tea1 and Tea4 have distinct mechanistic roles: Tea4 is required

227    for localization of Pom1 to the cortex, whereas Tea1 is required for enrichment of cortical

228    Pom1 to the cell tip (Hachet et al., 2011). We first used sucrose gradient centrifugation to

229    test if either protein is required for assembly of Pom1 into stable biochemical complexes.

230    The sedimentation pattern of Pom1 puncta isolated from *tea1Δ* and *tea4Δ* cells in velocity

231    sucrose gradients is unchanged from wild-type cells, suggesting that neither Tea1 nor

232    Tea4 are required for assembly of Pom1 into stable biochemical complexes **(Figure 3A)**.

233    Rather, these regulators are likely to act downstream of puncta assembly.

234

235         We next tested the localization and dynamics of Pom1-mNG in *tea1Δ* and *tea4Δ* cells.

236    Past work showed that membrane bound Tea4 is both necessary and sufficient to

237    nucleate cortical Pom1 gradients, and this relationship is non-stoichiometric (Hachet et

238    al., 2011). We confirmed that Pom1 was absent from the cell cortex in confocal

239    micrographs of *tea4Δ* cells **(Figure 3B)**. Surprisingly, we did observe Pom1-mNG puncta

240    at the cortex of *tea4Δ* cells by TIRF microscopy **(Figure 3C)**. These binding events

241    occurred at lower frequency and had a shorter dwell time than in wild-type cells **(Figure**

242    **3C-E)**. Pom1-mNG puncta in *tea4Δ* cells were distributed evenly around the cell cortex

243    with no detectable enrichment at tips. These infrequent, transient binding events do not

244    result in accumulation of enough cortical Pom1 to be detected by confocal microscopy.

245    Their rapid and position-independent association with the cortex leads to the absence of

246    a cortical Pom1 gradient in *tea4Δ* cells.

247

248    In contrast, confocal micrographs of Pom1-mNG in *tea1Δ* cells confirmed even
249    distribution around the entire cell cortex **(Figure 3B)**. TIRF microscopy of *tea1Δ* cells
250    revealed that this distribution arises from Pom1-mNG puncta, which bound the cell cortex
251    at a frequency in between the tips versus sides of wild-type cells **(Figure 3C-D)**. The
252    duration of individual binding events was longer than in *tea4Δ* cells but shorter than in
253    wild-type cells **(Figure 3E, S3A)**. Thus, in *tea1Δ* cells Pom1 puncta bind at an
254    intermediate frequency and for an intermediate duration, when compared to wild type and
255    *tea4Δ* cells. These binding events are independent of spatial cues at the cell ends versus
256    cell sides, resulting in enrichment of cortical Pom1 but not a gradient. Thus, Tea1 and
257    Tea4 cooperate to promote localized membrane binding but not assembly of Pom1
258    puncta. These spatial cues pattern the frequency of Pom1 puncta cortical interaction
259    along the long axis of the cell.

260

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

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

274

275    Phosphorylation by Pom1 prevents activation of Cdr2 in small cells, thereby
276    contributing to cell size-dependent regulation of Wee1 and mitotic entry (Allard et al.,
277    2018). To place this pathway in the context of Pom1 puncta and Cdr2 nodes, we analyzed
278    how these structures accumulate and colocalize as cells grow. Intriguingly, the total

279    number of both Cdr2 nodes and Pom1 puncta detected in TIRF images all along cell sides

280    were equivalent for cells of a given size **(Figure 4C-D)**. The numbers of both structures

281    show a similar size-dependent doubling. We next restricted our analysis to a 2μm x 2μm

282    square ROI positioned at the cell middle, where Cdr2 nodes concentrate. The local

283    density of Cdr2 nodes in this region increased as a function of cell size, consistent with

284    past studies (Deng and Moseley, 2013; Pan et al., 2014). In contrast, the density of Pom1

285    puncta in this region was largely independent of cell size **(Figure 4E)**. In both TIRF and

286    confocal images, the concentration of Pom1 protein and Pom1 clusters decreased slightly

287    as cells increase in size, but this trend was dwarfed by the cell size-dependent increase

288    in Cdr2 node density **(Figure 4E, S4D)**. Thus, as cells grow larger, the ratio of Pom1 to

289    Cdr2 in the medial cell cortex changes to favor Cdr2 because the densities of these two

290    structures scale differently with cell size **(Figure 4G)**.

291

292    We next tested how this density scaling affects colocalization of Pom1 clusters and

293    Cdr2 nodes as cells grow. Due to the increasing density of Cdr2 nodes, we observed an

294    increased number of colocalized Pom1 clusters and Cdr2 nodes as cells increase in size

295    **(Figure SE-F)**. The increased colocalization, combined with the constant density of Pom1

296    clusters, means that there are less "free" Pom1 clusters as cells increase in size **(Figure**

297    **S4G)**. Perhaps more importantly, the number of "free" Cdr2 nodes increased as a function

298    of cell size **(Figure S4G)**. These free Cdr2 nodes are not occupied by an inhibitory Pom1

299    cluster, and thus have increased potential to promote mitotic entry by inhibiting Wee1. In

300    this manner, Pom1 sets an inhibitory threshold that must be overcome by an increase in

301    Cdr2 node density. The inhibitory threshold decreases slightly as cells grow, and

302    functions most effectively in small cells, where Pom1 was previously shown to inhibit

303    downstream signaling to Wee1 (Allard et al., 2018).

304

**Control of Pom1 puncta levels in the medial cell cortex**

306    Our results suggest that Pom1 sets an inhibitory threshold that can be tuned by

307    altering the concentration of Pom1 cortical clusters in the cell middle. We sought to test

308    this model by increasing the abundance of Pom1 clusters in the cell middle. Low glucose

309    media and *tea1Δ* are two independent conditions that disperse Pom1 throughout the

310   cortex, thereby increasing its concentration in the cell middle (Hachet et al., 2011; Kelkar

311   and Martin, 2015) (See also **Figure S7A-C**). As a starting point, we tested the effect of

312   mislocalized Pom1 in *tea1Δ* cells **(Figure 5A-B)**. Surprisingly, higher Pom1 levels at the

313   medial cortex did not impact the number of Cdr2 nodes or their recruitment of Wee1

314   **(Figure 5C-F)**. Past work showed that Pom1 retains kinase activity in *tea1Δ* cells (Bähler

315   and Nurse, 2001), but several experiments suggested that this residual activity may be

316   reduced. First, we compared *in vitro* kinase activity of Pom1 immunoprecipitated from

317   *tea1Δ* cells or wild type cells. Pom1 from *tea1Δ* cells showed less kinase activity towards

318   itself   (autophosphorylation)   and   its   substrate   Cdr2   **(Figure   S5A-C)**.   Second,

319   phosphorylation of the Pom1 substrate Rga7 was reduced in *tea1Δ* cells **(Figure S5D-E).**

320   Third, Tea4 is completely absent from the cortex of *tea1Δ* cells **(Figure S6D)**. Catalytically

321   active Pom1 autophosphorylates and requires Tea4-dependent dephosphorylation for

322   cortical enrichment. Thus, in *tea1Δ* cells, Pom1 likely binds to the membrane because it

323   cannot autophosphorylate properly. Finally, we note that Pom1 localization in *tea1Δ*

324   mimics the localization of kinase-dead *pom1-m2* mutant **(Figure S6A-B)**. Therefore, we

325   focused on the activity and regulation of Pom1 clusters in wild type cells grown in low

326   glucose.

327

328        Fission yeast cells respond to low glucose in part by depolymerizing their

329   microtubule cytoskeleton, which leads to Pom1 redistribution to the lateral cell cortex

330   where it delays mitotic entry (Kelkar and Martin, 2015). In time-lapse TIRF microscopy

331   experiments, we observed increased numbers of discreet Pom1 puncta at the lateral cell

332   cortex under low glucose conditions **(Figure 6A)**. These puncta bound to the lateral cortex

333   more frequently than under normal glucose conditions, but the average dwell time was

334   unaffected **(Figure 6B-C)**. This increased on-rate leads to accumulation of Pom1 clusters

335   at the medial cell cortex.

336

337        We next investigated how increased numbers of Pom1 puncta at the lateral cell

338   cortex affect Cdr2 nodes using TIRF microscopy. In low glucose conditions, the number

339   of Cdr2 nodes was largely unchanged, but the fluorescence intensity of individual Cdr2

340   nodes and the overall concentration of Cdr2 at the medial cortex was significantly lower

341    **(Figure 6D-F, S7A-C)**. In *pom1Δ* cells, which grow only from one tip, Cdr2 nodes form

342    normally but invade the non-growing tip. Low glucose conditions do not measurably affect

343    Cdr2 nodes in *pom1Δ* cells, so the effects that we observed are Pom1-dependent **(Figure**

344    **6D-F)**. These results indicate that Pom1 relocalization to the lateral cortex induces partial

345    disassembly of Cdr2 nodes under low glucose. To quantify how this effect scales with

346    glucose availability, we varied the concentration of media glucose over four orders of

347    magnitude. Then, we measured the concentrations of both Pom1 and Cdr2 at both the

348    cell sides and the cell tips. As glucose was reduced, Pom1 concentration decreased at

349    cell tips and increased at cell sides, consistent with redistribution **(Figure 7A,C-D)**. In

350    contrast, Cdr2 concentration increased at cell tips and decreased at cell sides **(Figure**

351    **7B-D)**. Plots of glucose availability versus Pom1 or Cdr2 concentration produced

352    sigmoidal curves with opposite orientations **(Figure 7C-D)**. Correlation analysis of Pom1

353    and Cdr2 concentrations at cell sides show they are nearly perfectly anti-correlated

354    (Pearson's r=-0.9352, p=0.0006) **(Figure 7E-F)**. We conclude that the increasing

355    frequency of Pom1 cluster binding at the lateral cell cortex leads to progressive disruption

356    of Cdr2 nodes.

357

358    **Pom1 disrupts Wee1 bursting under glucose deprivation**

359    We next examined the downstream effects of altered Pom1-Cdr2 signaling by

360    using TIRF microscopy to monitor the previously described bursts of Wee1-mNG

361    localization at Cdr2 nodes (Allard et al., 2018). In wild-type cells, both the frequency and

362    duration of Wee1 bursts increased linearly with cell size, consistent with increased

363    inhibition of Wee1 as cells grow **(Figure 8A,B)**. In low glucose, the frequency of Wee1-

364    mNG bursts still scaled with cell size as in normal glucose. However, the duration of each

365    burst was uniformly short and independent of cell size **(Figure 8A-B)**. These same burst

366    properties were seen for Wee1-mNG bursts in kinase-dead *cdr2(E177A)* mutant cells

367    grown in normal glucose **(Figure S8A-B)**. These results indicate that Cdr2 kinase activity

368    is required for retention of Wee1 at nodes, but not for the initial binding event. Further,

369    they demonstrate that low glucose phenocopies the kinase-dead mutant, consistent with

370    Pom1-dependent inhibition of Cdr2 kinase activity.

371

372    These results suggested that attenuation of Wee1 bursting dynamics in low

373    glucose might require Pom1 redistribution. We tested this idea by examining the glucose

374    dependency of Wee1 node localization in *pom1Δ* cells. In *pom1Δ* cells growing under high

375    glucose, the Wee1 burst frequency scales with cell size but Wee1 burst duration is

376    uniformly high and independent of cell size **(Figure 8A,C)**. Unlike wild type cells, which

377    suppress Wee1 bursts in low glucose, *pom1Δ* cells show a striking increase in Wee1

378    bursts under low glucose. This increase is not cause by burst duration, which was

379    independent of both cell size and glucose concentration in *pom1Δ* cells. Rather, we

380    measured increased Wee1-mNG burst frequency in low glucose *pom1Δ* cells **(Figure**

381    **8A,C)**. The reason for this increase is unknown, and suggests the existence of additional

382    glucose-regulated pathways that operate on Cdr2-Wee1 signaling.

383

384    Wee1 localization at Cdr2 nodes leads to its inhibitory phosphorylation by Cdr2

385    and the related kinase Cdr1. To test how our TIRF-based localization results connect with

386    Wee1 phosphorylation status, we analyzed phosphorylation-dependent shifts of Wee1

387    migration using western blots **(Figure 8D)**. In high glucose, Wee1 migrates as a smear

388    of phosphorylated isoforms (Allard et al., 2018; Lucena et al., 2017). The upper,

389    hyperphosphorylated forms are increased in *pom1Δ* cells but absent in *cdr2Δ* cells. This

390    hyperphosphorylated Wee1 is lost in wild-type cells grown in low glucose, consistent with

391    reduced bursts of Wee1 localization to nodes. In contrast, Wee1 appears even more

392    hyperphosphorylated in *pom1Δ* cells grown under low glucose. This result suggests that

393    Pom1 prevents phospho-inactivation of Wee1 in response to low glucose. These

394    combined experiments support a model where Pom1 redistribution to the lateral cell

395    cortex inactivates Cdr2 nodes to relieve inhibition of Wee1 under low glucose conditions.

396

# Discussion:

398    In this study, we have shown that the Pom1 concentration gradient is generated

399    by oligomeric Pom1 puncta that rapidly bind and release from the plasma membrane.

400    These puncta bind more frequently at cell tips versus cell sides in high glucose media,

401    while their binding rate is increased at cell sides in low glucose media. At the medial cell

402    cortex, these puncta overlap with their inhibitory target Cdr2, which localizes in static

403    oligomeric nodes. More Cdr2 nodes are free from Pom1 inhibition as cells grow larger

404    due to different density scaling of these two structures at the medial cell cortex. Our TIRF-

405    based colocalization experiments on these structures were limited to short periods of time

406    to avoid photobleaching, partly caused by the imaging conditions needed to observe

407    these highly dynamic Pom1 clusters. Nevertheless, even over these short timescales of

408    observation, the stochastic positioning of Pom1 puncta resulted in spatiotemporal

409    colocalization with Cdr2 nodes. Given the high frequency of Pom1 puncta binding, we

410    expect that many more colocalization events occur between Cdr2 nodes and Pom1

411    clusters during the course of a full cell cycle, with the potential for Pom1 clusters to visit

412    each Cdr2 node multiple times. Thus, these rapid dynamics of Pom1 puncta interacting

413    with Cdr2 nodes must be integrated in time throughout the cell cycle, leading to the G2/M

414    transition. It appears likely that the most critical temporal window for Pom1-Cdr2

415    interactions occurs during early in G2, as *pom1Δ* show defects in Cdr2-Wee1 signaling

416    specifically in small cells (Allard et al., 2018). It will be interesting to determine how these

417    rapid interactions in small cells are integrated into the mitotic entry decision, which occurs

418    later in the cell cycle when cells are longer.

419

**420    Molecular clusters are widespread in signal transduction**

421         The Pom1-Cdr2-Wee1 signaling pathway appears to function entirely within the

422    confines of oligomeric protein clusters at the plasma membrane. The development of

423    imaging technologies with increased spatial and temporal resolution has enabled the

424    discovery of protein clustering as a paradigm in signaling. As a result, a growing number

425    of signaling events and proteins have been found in similar clustered structures that are

426    referred to as nanodomains, clusters, nanoclusters, and nodes, among other names. For

427    example, recent studies have shown how PAR-3 polarity complexes in the *C. elegans*

428    zygote form clusters to reduce random diffusion and promote vectorial transport

429    (Dickinson et al., 2017; Munro, 2017; Rodriguez et al., 2017; Wang et al., 2017). In

430    addition, major signal transduction pathways in plants are organized into protein clusters

431    on plasma membrane nanodomains (Gronnier et al., 2017). Similarly, different Ras

432    isoforms partition into distinct and exclusive clusters that digitize downstream signals in

433    animal cells (Zhou and Hancock, 2015). In immune cells, both B-cell receptors and T-cell

434  receptors form oligomeric compartments at the cell cortex (Douglass and Vale, 2005; Liu

435  et al., 2016; Maity et al., 2015). These T cell receptor clusters enrich downstream kinases

436  while excluding phosphatases (Su et al., 2016), a mechanism that could relate to

437  concentrated clusters of the kinases Pom1 and Cdr2. Clustering can have a range of

438  critical impacts on the signaling properties of these pathways, such as the clustering of

439  bacterial chemotactic receptors to generate signal amplification (Bray et al., 1998; Duke

440  and Bray, 1999; Falke, 2002). Theoretical work has suggested that clustering can reduce

441  noise by generating reaction bursts (Kalay et al., 2012), which have the capacity to

442  overcome inhibitory thresholds, such as those imparted by phosphatases, more

443  effectively than a system driven by gradual accumulation of signal. Insight into the

444  functional role of clustering for Pom1-Cdr2-Wee1 will require additional *in vivo* analysis

445  combined with *in vitro* reconstitution. We have shown that Pom1 clusters remain stable

446  through biochemical enrichment, and isolated clusters exhibit the same membrane-

447  binding properties *in vitro* as in cells. Past work has demonstrated Cdr2 nodes also remain

448  stable through biochemical fractionation (Allard et al., 2018), providing the necessary

449  tools for future dissection of the pathway in a reconstituted system.

450

451  **A new model for cortical gradient formation**

452  Our work has shown that Pom1 clusters bind to the cell cortex with different

453  frequencies along the long axis of the cell, resulting in a concentration gradient. Past work

454  suggested single Pom1 molecules (or small oligomers) bind to the membrane at tips, and

455  then diffuse directionally away from the tip before dissociating (Hachet et al., 2011;

456  Saunders et al., 2012). Clusters were proposed to dynamically assemble and

457  disassemble at the cortex from this diffusing population (Saunders et al., 2012). Our

458  findings suggest a new model for gradient formation, which is distinct from this previous

459  model in four ways. First, we found that clusters themselves form a concentration gradient

460  that emanates from the cell tips. Second, our combined imaging and biochemical

461  approaches demonstrated that clusters are stable complexes that bind and rapidly

462  release from the membrane. Third, we did not observe extensive diffusion by complexes

463  bound to the membrane. It remains possible that smaller, diffusing Pom1 complexes or

464  single molecules were not detected by our imaging approach, but we favor a model where

465    diffusion is limited to short, non-directional movements that do not contribute to gradient

466    formation. Fourth, the key step in gradient formation is a positional system that increases

467    the on-rate for Pom1 clusters at cell tips versus the cell middle. Concentration gradients

468    are found across vast size scales in biology, so the mechanism of gradient formation by

469    Pom1 clusters may have broad implications. It is also important to note that the spatial

470    gradient of on-rates for Pom1 clusters binding to the cortex may depend on a

471    concentration gradient of specific lipids in the plasma membrane. In support of this

472    hypothesis, Pom1 binds to phosphatidylserine *in vitro* (Hachet et al., 2011), and this lipid

473    is enriched at the tips of growing fission yeast cells (Haupt and Minc, 2017). Thus, further

474    investigation of these lipids and their localization dynamics could reveal additional layers

475    of this morphogen gradient.

476

477          We found that the polarity landmarks Tea1 and Tea4 provide the positional

478    information for Pom1 clusters to form a concentration gradient. Past work has shown that

479    Pom1 localizes homogenously throughout the cortex in *tea1Δ*, and does not localize to

480    the cortex in *tea4Δ* (Bähler and Pringle, 1998; Hachet et al., 2011). Based on these and

481    other results, Tea1 was proposed to recruit Tea4 to cell tips, where it promotes cortical

482    loading of Pom1 molecules. Several results suggest that additional mechanisms may

483    contribute to gradient formation. For example, we found that Pom1 clusters still bind to

484    the cortex in *tea4Δ* cells but for very short durations. Past work has also shown that Tea1

485    and Tea4 form cortical clusters that co-localize at cell tips, but these Tea1-Tea4 clusters

486    do not co-localize with Pom1 clusters (Dodgson et al., 2013). Thus, Tea1-Tea4 may

487    position binding of Pom1 clusters to the cortex through an additional mechanism. For

488    example, Tea1 and Tea4 have been shown to assemble sterol-enriched lipid domains at

489    cell tips when cells initiate polarization following exit from starvation (Makushok et al.,

490    2016). Sterols and phosphatidylserine are thought to form a common membrane domain

491    in fission yeast, suggesting that these proteins could act through the recruitment and

492    organization of lipids to generate cortical domains permissive for Pom1 cluster binding

493    (Haupt and Minc, 2017; Makushok et al., 2016).

494

495

**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

527  growth and environmental conditions that alter the relative distributions of Pom1 clusters
528  and Cdr2 nodes, and determination of how they connect with other cell cycle signaling
529  pathways, may reveal new mechanisms for nutrient modulation of cell size control.
530
531  **Integration of the Pom1-Cdr2-Wee1 pathway with other sizing mechanisms**
532  Pom1-Cdr2-Wee1 signaling is cell size dependent but is not the only pathway that
533  contributes to the overall cell size control network. Intriguingly, a recent study reported
534  that $cdr2\Delta$ cells no longer divide based on accumulation of a threshold surface area and
535  instead revert to a secondary mechanism of cell size control based on volume sensing.
536  These results demonstrate that cells use different signal transduction networks to
537  measure different aspects of their size. Whereas the Pom1-Cdr2 network is well suited to
538  measure cortical surface area, size-control systems that measure other aspects of size
539  would require different signaling logic. Theoretically, mechanisms for cell volume sensing
540  could depend on proteins whose concentrations do not scale linearly with cell size. Such
541  mechanisms are thought to underlie cell size control in other organisms, such as the
542  budding yeast, where growth-dependent dilution of the transcriptional repressor Whi5 is
543  thought to underlie $G_1/S$ size control (Schmoller et al., 2015). One possible candidate for
544  cellular volume sensing in fission yeast is Cdc25, which increases in concentration with
545  cell size (Keifenheim et al., 2017; Moreno et al., 1990). These mechanisms are attractive
546  candidates for volume sensors because cytoplasmic concentration is independent of cell
547  geometry. The existence of sizing mechanisms that measure other aspects of cell size,
548  and function independently of the Pom1-Cdr2 surface area sensing network, underscores
549  that cell size is a systems level property controlled by multiple signaling pathways. Thus,
550  it remains important to discover size-dependent signaling mechanisms within each
551  individual pathway, as a step towards understanding the integration of multiple pathways
552  into a larger size control system.
553
554
555
556
557

## 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 Technlogy) 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

589    that only nodes/puncta were counted and that no nodes/puncta were omitted. For Wee1

590    burst and Pom1 puncta tracking, Particle Diameter was set to 0.3 microns (approximate

591    lateral resolution), with Maximum Gap Linking set to 2 frames, and Linking Range for

592    particle tracking was set to 0.15 microns. Lookup table was adjusted to fire for some

593    images to emphasize signal intensities.

594

595    **"Head-On" Tip Imaging**

596    "Head-On' or "Tip" imaging was performed using a protocol modified from

597    (Dodgson et al., 2013). Custom micro-well coverslips or Ibidi Sticky-Slide VI channels

598    (Ibidi, Martinsried, Germany) were coated with BS-I lectin (1mg/mL in water) by incubation

599    for 30 minutes and washed 3X with water to remove non-adherent lectin. Cells were then

600    added to the imaging chambers and allowed to settle to the cover-slip bottom where they

601    adhere to the lectin. Cells oriented with the long axis perpendicular to the coverslip were

602    identified using brightfield prior to fluorescence imaging.

603

604    **Spinning Disc Microscopy and Analysis**

605    Spinning-disc confocal imaging was performed using a commercially available

606    system (Micro Video Instruments, Avon, MA) featuring a Nikon Eclipse Ti base equipped

607    with an Andor CSU-W1 two-camera spinning disc module, dual Zyla sCMOS cameras

608    (Andor, South Windsor, CT) an Andor ILE laser module, and a Nikon 100X Plan Apo ƛ

609    1.45 oil immersion objective. Cells were imaged in EMM4S media on glass slides at

610    ambient temperature unless otherwise noted.

611

612    **Airyscan Super-Resolution Microscopy and Analysis**

613    To achieve maximum resolution and sensitivity, fluorescence intensity of Pom1

614    puncta on both cell sides and cell tips was measured using a Zeiss Airyscan microscope

615    **(Figure S1I-K)**, composed of a Zeiss LSM-880 laser scanning confocal microscope

616    (Zeiss, Oberkochen, Germany) equipped with 100X alpha Plan-Apochromat/NA 1.46 Oil

617    DIC M27 Elyra objective, Airyscan super-resolution module and GaAsP Detectors, and

618    Zen Blue acquisition software using the Super-resolution mode with pin-hole size of 1.2

619    airy-units to prioritize resolution. Z-volumes of 16 slices with 0.19μm spacing for high

620   spatial resolution in all dimensions were centered on the cell cortex closest to the

621   coverslip. Airyscan images were processed in Zeiss Zen Blue software, and quantification

622   was performed on sum projections of Airyscan reconstructed stacks.

623

624   **Pom1 and Cdr2-CTD Purification**

625   The full length Pom1 sequence, and the Cdr2-CTD fragment were purified as

626   follows: Each sequence was subcloned into pGEX6P1 vector using the Xho1 restriction

627   site and expressed as a GST-fusion protein in *Escherichia coli* strain BL21(DE3).

628   Transformants were cultured to log-phase at 37˚C, followed by a shift to 25˚C for 30

629   minutes. Expression was then induced by addition of 1-thio-β-D-galactopyranoside to

630   200μM, followed by growth for an additional 3 hours at 25˚C. Cells were harvested by

631   centrifugation and lysed by passing them twice through a French press in lysis buffer

632   (1xPBS, 100mM NaCl, 1mM DTT, and complete EDTA-free protease inhibitor tablets (1

633   tablet/50mL Buffer) (Roche, Basel, Switzerland)). Following lysis, Triton-X 100 was added

634   to 1% V/V. Lysates were then cleared by centrifugation for 10 minutes at 12,000 x g at

635   4˚C in a Sorval SS-34 fixed-angle rotor (Thermo Scientific, Waltham, MA). The

636   supernatant was then incubated with glutathione-agarose (Sigma Aldrich, St. Louis, MO)

637   for 2h at 4˚C. 20 mM glutathione (pH 8.0) was used to elute purified protein, and the

638   eluate was dialyzed overnight at 4˚C into 10mM Tris-HCl, pH8 + 5% V/V glycerol.

639

640   **Preparation of Yeast Extracts**

641   Fission yeast detergent extracts were prepared by growing 1.5L cells to mid-log

642   phase (OD ~0.3), and then washed twice with 50mL Node Isolation Buffer (NIB – 50mM

643   HEPES, 100mM KCl, 1mM MgCl$_2$, 1mM EDTA, pH 7.5) (Allard et al., 2018). Next, we

644   resuspended the pellet in an equal volume of 2X NIB (W/V) containing a

645   protease/phosphatase inhibitor cocktail (10μL/mL 200x PI, 50μL/mL 1M β-glycerol

646   phosphate, 50μL/mL 1M NaF, 2.5 μL/mL 200mM PMSF, 1mM DTT), and snap froze the

647   resuspension as pellets by pipetting drop-wise into a liquid nitrogen bath. Then, yeast

648   pellets were ground using liquid-nitrogen chilled coffee-grinders for 2 min, and collected

649   into chilled falcon tubes and stored at -80˚C. 1.5g of frozen yeast powder was then thawed

650   on ice, and Triton X-100 was added to a final concentration of 1%, and the extracts were

651    mixed by gentle pipetting. Extracts were then centrifuged at 4˚C for 10min at 20,000 x g

652    to yield a low speed supernatant, which were then used for subsequent experiments.

653

654    **Sucrose Gradient Ultracentrifugation**

655    Discontinuous sucrose gradients were prepared in 14 x 89mm Ultra Clear

656    Ultracentrifuge tubes (Beckman Coulter, Brea, CA) by layering 5 – 23% (top to bottom)

657    sucrose in NIB + 1% Triton X-100 in 0.37mL steps of 2% sucrose increments. 700µL of

658    yeast extract or recombinant protein diluted in NIB Buffer was then added to the top of

659    the gradient. Sucrose gradients were centrifuged at 100k x g (50kRPM) in a Beckman L8-

660    M ultracentrifuge for 2hr at 4˚C in a chilled SW60ti swinging bucket rotor. 0.5mL gradient

661    fractions were collected from the top by hand, vortexed, and 100µL of each fraction was

662    mixed 2:1 in 3X SDS-PAGE sample buffer (65 mM Tris pH 6.8, 3% SDS, 10% glycerol,

663    10% 2-mercaptoethanol, 50 mM NaF, 50 mM β-glycerophosphate, 1 mM sodium

664    orthovanadate) and boiled for 5 min. Samples were then subjected to SDS-PAGE and

665    western blotting or Coomassie staining.  To calculate S-values of sedimentation peaks

666    from western blot signal intensities, mean band intensities were measured using Image

667    Studio Lite (LICOR, Lincoln, NE), and Gaussian Curves were fit to these values. The peak

668    of the Gaussian for each sedimentation peak of each protein was used to assign the

669    known or interpolated S-value.

670

671    **Western Blotting**

672    For western blots, cells were lysed in 150 µl of 3X SDS-PAGE sample buffer with

673    glass beads in a Mini-beadbeater-16 (Biospec, Bartlesville, OK) for 2 minutes. Gels were

674    run at a constant 20 mAmps until 75kDa marker was at the bottom of the gel. Blots were

675    probed with anti-HA (Covance, Princeton, NJ) or homemade anti-GST. For monitoring

676    Wee1 phosphorylation, samples were run on an SDS-PAGE gel containing 6%

677    acrylamide and 0.02% bisacrylamide, and then probed with a homemade anti-Wee1

678    antibody (Allard et al., 2018).

679

680    **Supported Lipid Bilayers**

681        To prepare supported lipid bilayers, we first prepared small unilamellar vesicles

682    (SUVs) composed of three lipids: (1) DOPC (18:1 (Delta9) Cis PC - 1,2 Dioleoyl-sn-

683    glycero-3-phosphocholine), (2) DOPS (18:1 PS - 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-

684    Serine]), and (3) fluorescent 18:1 Liss Rhod PE (1,2-dioleoyl-sn-glycero-3-

685    phosphoethanolamine-N-(lissamine rhodamine B sulfonyl). All lipids were dissolved in

686    chloroform (Avanti, Alabaster, AL). 2µmol total lipid with the desired molar ratios were

687    mixed in glass vials with PTFE coated caps, which had been RCA cleaned and rinsed

688    three times with chloroform before use. Excess chloroform was evaporated in a vacuum

689    desiccator for 1 hour. Lipids were resuspended in 400µL SUV Buffer (50mM HEPES,

690    100mM KCl, 1mM EDTA, 1mM MgCl$_2$, pH7.5) for 5mM lipid mixture stocks. The lipid

691    mixtures were vortexed until cloudy, and then transferred to 1.5mL Eppendorf tubes and

692    subjected to ten freeze-thaw cycles using a liquid nitrogen bath and 32˚C hot plate, with

693    2 minutes of sonication in a sonicating water bath following each thaw cycle. Stocks were

694    stored at -80˚C in 20µL aliquots.

695

696        Supported lipid bilayers were made by adding 10µL of SUVs to RCA cleaned

697    custom microwells built on 22x40mm #1.5 coverslips. Chambers were then incubated at

698    37°C for one hour to induce vesicle fusion, and unincorporated vesicles were rinsed away

699    using five rinses with 100µL SUV buffer. Yeast extract was added to these chambers by

700    removing 90µL of buffer, leaving just enough to cover the chamber bottom, and then

701    adding extract.

702

703    **Pom1 Immunoprecipitation**

704        Cultures were grown in EMM4S media to log phase, and a volume equivalent to

705    200 optical density units were harvested by centrifugation. The pellet was washed in

706    Wash Buffer (1X PBS, 250mM NaCl, 1mM EDTA, 1mM PMSF, 50mM NaF, 50mM β-

707    glycerophosphate, 1mM sodium orthovanadate, and 1% Triton X-100, and complete

708    EDTA-free protease inhibitor cocktail (1 tablet/50mL) (Roche)). The pellet was then

709    resuspended in 5mL of wash buffer, and divided into 1.5mL screw-cap tubes each with

710    200µL glass beads for lysis by bead-beading for 2 minutes using a mini-Beadbeater-16

711    (Biospec) at 4˚C. Lysates were cleared by centrifugation at 16,000 x g for 10 minutes at

712    4˚C in an Eppendorf 5415C centrifuge. The supernatant was then incubated with rotation

713    at 4˚C for 1 hour with Pierce anti-HA magnetic beads (Thermo Scientific). Beads were

714    washed five times for 15 minutes each in Wash Buffer prior to use. Beads were

715    resuspended in Kinase Assay Buffer (see below) for immediate use in kinase assays.

716

717    *In vitro* **kinase assays**

718        For *in vitro* kinase assays, immunoprecipitated Pom1 proteins and purified Cdr2-

719    CTD were combined at a 1:1 ratio in Kinase Assay Buffer (20mM Tris pH 8, 100mM NaCl,

720    50mM NaF, 50mM β-glycerophosphate, 1mM sodium orthovanadate, 10mM MgCl$_2$,

721    20µM ATP-γ-S) for 30 minutes at 30˚C. The reaction was halted by addition of EDTA to

722    20mM. For subsequent analysis by anti-thiophosphate western blot, proteins in *in vitro*

723    kinase assays were then alkylated by addition of p-nitrobenzyl mesylate to 2.5mM, and

724    incubation at 25˚C for one hour. Samples were then prepared for western blot as

725    described above. Thiophosphate western blots were probed with an antibody against

726    thiophosphate-ester (Abcam 92570) (Abcam, Cambridge, United Kingdom).

727

728

729

730    # Figure Legends:

731

732    Figure 1: **The Pom1 gradient is formed by time-averaging of puncta that transiently**

733    **bind the cortex with different dynamics along the long axis of the cell. (A)** Individual

734    frame (top panel) and sum projections (bottom panel) of high-speed TIRF microscopy

735    movie. Scale bar 1µm. Movie was continuous 15s time-lapse acquisition of 200ms

736    exposures. Blue arrows mark position of line scans performed for data in panel B. **(B)**

737    Line scans of fluorescence intensity along the long axis of the snap shot and projection

738    images in panel (A). Note that time-averaging of Pom1 puncta smoothens the

739    concentration gradient. **(C)** Traces of single-particle diffusion paths generated from a 15s

740    time-lapse TIRF microscopy acquisition of Pom1-mNeonGreen. Scale bar 1µm. **(D-E)**

741    Pom1-mNeonGreen forms puncta both at the cell tip and cell side. Images are sum

742    projections of three consecutive 200ms exposures from continuous time-lapse TIRF

743 microscopy acquisitions of either the cell tip or side as depicted in the cartoon diagrams.

744 Scale bar 1μm. **(F-H)** Particle-tracking analysis of 2μm x 2μm ROIs from 30s continuous

745 time-lapse TIRF acquisitions of Pom1-mNeonGreen at either the cell tip or side was used

746 to measure and compare (F) the binding frequency of Pom1 puncta (****, *p*=<0.0001),

747 (G) the average displacement of individual puncta (***, *p*=0.0002), (H) the binding

748 duration of individual puncta (n.s., *p*=0.6747). Each data point represents a single cell

749 mean, and line and error bars represent mean and standard deviation of all cells.

750 Statistical significance was tested using a Student's T-test. **(I)** Histogram of the

751 fluorescence intensity of individual Pom1 puncta at the cell tip and cell side. Puncta were

752 binned by fluorescence intensity and displayed as a population frequency.

753

754 Figure 2: **Pom1 puncta are stable structures that can be isolated *in vitro.* (A)** TIRF

755 microscopy image reveals fluorescent puncta in the extruded cytoplasm (white dotted

756 line) from a lysed Pom1-mNeonGreen cell (yellow dotted line). Scale bar 5μm. **(B)** TIRF

757 microscopy images of cell extracts prepared from wild-type (no tag) or Pom1-

758 mNeonGreen cells. Images are 50 frame sum projections of continuous 200ms time-lapse

759 exposures. The two images were contrasted equally. Scale bar 1μm. **(C)** Cytoplasmic

760 extracts of *pom1-3HA* cells were subjected to velocity sucrose gradient sedimentation,

761 and fractions were probed against the HA tag (upper blot). Fraction 1 corresponds to the

762 top of the gradient and contains smaller structures; fraction 12 corresponds to bottom of

763 the gradient and contains larger structures. Fractions 6-8 were pooled, sucrose was

764 removed by dialysis, and then the sample was subjected to a second identical round of

765 sucrose gradient sedimentation and western blotting of the resulting fractions (lower blot).

766 **(D)** Quantification of Pom1-3HA band intensities from western blot of yeast extracts in

767 panel C. Each apparent peak was fit to a gaussian curve, and S-values were determined

768 using size-standards run on identical gradients. **(E)** TIRF microscopy of Pom1-mNG

769 puncta from cytoplasmic extracts on supported lipid bilayers. Scale bar 1μm. Left panel

770 is single time point image. Right panel is kymograph taken from line scan of time-lapse

771 TIRF experiment. **(F)** Quantification of binding duration of Pom1-mNG puncta on

772 supported lipid bilayers by TIRF microscopy as in panel F. Values are compared to

773 cellular measurements of pooled tip and side localized Pom1 puncta (n.s., *p*=0.0514). **(G)**

774    Purified GST-Pom1 was subjected to sucrose gradient sedimentation and the fractions

775    were probed against the GST tag (upper blot). Purified GST-Pom1 was also added into

776    wild-type cell or *pom1Δ* cell extracts and incubated for 1hr at 4°C in the presence of ATP

777    before velocity sucrose sedimentation and western blotting (bottom blots).

778

779    Figure 3: **Polarity landmarks pattern cortical dynamics of Pom1 puncta to shape the**

780    **gradient. (A)** Western blots of sucrose gradient fractions after sedimentation analysis as

781    in Figure 1C of extracts prepared from *tea1Δ* or *tea4Δ* cells expressing Pom1-3HA. **(B)**

782    Confocal micrographs of Pom1-mNG in *tea1Δ* or *tea4Δ* cells. Images are from the middle

783    focal plane; scale bar 5µm. **(C)** TIRF micrographs of Pom1-mNG localization at the tip or

784    side cortex in *tea1Δ* or *tea4Δ* cells. Scale bar 1µm. **(D)** The mean binding frequency of

785    Pom1-mNG puncta at the tips and sides of wild-type, *tea1Δ* or *tea4Δ* cells, measured by

786    TIRF microscopy. **(E)** The mean binding duration of Pom1-mNG puncta at the tips and

787    sides of wild-type, *tea1Δ* or *tea4Δ* cells, measured by TIRF microscopy. For D-E, wild-

788    type data are replotted from Figure 1. Each data point represents a single cell mean, and

789    line and error bars represent mean and standard deviation of all cells. Comparisons are

790    1-way ANOVA with Tukey's multiple comparisons tests. **** indicates $p < 0.0001$, n.s.

791    indicates $p > 0.05$.

792

793    Figure 4: **Scaling of Pom1 puncta and Cdr2 nodes at the lateral cell cortex. (A)** A

794    subset of Pom1 puncta colocalize with Cdr2 nodes. Images are dual-channel

795    simultaneously-acquired TIRF microscopy images of cells expressing Pom1-mNG and

796    Cdr2-tagRFP-t. Yellow dashed brackets outline the ROI of the lower zoomed panels.

797    Orange arrows point to Pom1 puncta colocalized with Cdr2 nodes. Scale bar is 1µm. **(B)**

798    Kymographs generated using the ROI drawn on the merged zoom image in panel A. Cyan

799    arrows indicate prominent Pom1 puncta, magenta arrows indicate prominent Cdr2 nodes,

800    and orange arrows adjacent to the merged kymograph indicate colocalization. **(C)**

801    Localization of Cdr2-mEGFP (upper panels) or Pom1-mNG (lower panels) in

802    representative cells of increasing size by TIRF microscopy. Scale bar is 1µm. **(D)** Total

803    number of Cdr2-mEGFP nodes (blue circles) or Pom1-mNG puncta (red squares)

804    measured per cell by TIRF microscopy. Quantification is limited to puncta detected and

805    resolvable in the TIRF illumination field. The slopes of the corresponding linear

806    regressions are not significantly different ($p$=0.3757). **(E)** Density of Cdr2-mEGFP nodes

807    (blue circles) or Pom1-mNG puncta (red squares) in 2x2μm square ROIs at the cell middle

808    using TIRF microscopy. **(F)** Example of colocalization analysis for Pom1 puncta and Cdr2

809    nodes. The spot detection algorithm in TrackMate plugin for ImageJ2 was used to assign

810    0.3 μm diameter circular ROIs to Cdr2 nodes (cyan) and Pom1 puncta (magenta) in

811    images such as panel A. Structures were counted as colocalized if the ROI assigned to

812    one channel overlapped with the middle of the ROI of the other channel (right panel,

813    yellow circles). Adjacent structures that failed this criterion were deemed not to be

814    colocalized (right panel, grey circles). **(G)** Ratio of free Cdr2 nodes to free Pom1 puncta,

815    plotted as a function of cell size.

816

817    <u>Figure 5</u>: **Properties of Pom1 puncta in *tea1Δ* cells. (A)** Localization of Pom1-mNG in

818    wild-type or *tea1Δ* cells imaged by confocal microscopy at focal planes in the cell middle.

819    Scale bar, 5μm. **(B)** Comparison of Pom1 concentration along 2μm cortical line scans

820    positioned at the cell middle of wild-type and *tea1Δ* cells, from images as in panel A.

821    Significance was tested using a Student's T-test (****, p<0.0001). **(C)** Localization of

822    Cdr2-mNG in *tea1Δ* cells by TIRF microscopy. Scale bar is 5μm. **(D)** Number of Cdr2-

823    mNG nodes as a function of cell length in wild-type (blue circles) or *tea1Δ* cells (red

824    squares), measured using TIRF microscopy images as in panel C and Figure 5A. The

825    slopes of the linear regression of each data set do not differ significantly from each other

826    ($p$=0.2029). **(E)** Localization of Wee1-mNG in *tea1Δ* cells by TIRF microscopy. Scale bar

827    is 5μm. **(F)** Number of Wee1-mNG bursts as a function of cell length in wild-type (blue

828    circles) or *tea1Δ* cells (red squares), measured using TIRF microscopy images as in panel

829    E and Figure 7A. There is a small but significant difference in the slopes of the linear

830    regressions ($p$=0.0158) such that the number of Wee1-mNG bursts scales more slowly

831    in *tea1Δ* cells than in WT. This discrepancy may result because *tea1Δ* cells can be bent

832    or T-shaped, rendering length an imperfect proxy for size.

833

834    <u>Figure 6</u>: **Redistribution of Pom1 puncta to the lateral cortex disrupts Cdr2 nodes**

835    **during glucose restriction. (A)** Localization of Pom1-mNG in wild-type cells grown in

836   either high (2%) or low (0.03%) glucose media. Images were collected using TIRF

837   microscopy. Scale bar, 5µm. **(B)** Comparison of the binding frequency of Pom1 puncta at

838   the lateral cell cortex in cells grown under high and low glucose measured using TIRF

839   microscopy (***, *p*=0.0003). Statistical significance was tested using a Student's T-test.

840   **(C)** Comparison of the binding duration of Pom1 puncta at the lateral cell cortex in cells

841   grown under high and low glucose measured using TIRF microscopy (n.s., *p*=0.6833).

842   Statistical significance was tested using a Student's T-test. **(D)** Localization of Cdr2-mNG

843   in wild-type or *pom1Δ* cells grown in either normal (2%) or low (0.03%) glucose media.

844   Images were collected using TIRF microscopy. Scale bar, 1µm. **(E)** Comparison of the

845   fluorescence intensity of individual Cdr2-mNG nodes in wild-type or *pom1Δ* cells grown

846   in either normal (2%) or low (0.03%) glucose media. Cdr2 nodes in wild-type cells become

847   significantly smaller under low glucose conditions (****, *p*<0.0001), but there is no

848   significant difference in Cdr2 nodes size in *pom1Δ* cells grown in normal vs low glucose

849   (n.s., *p*=0.4015). Measurements were taken from Airyscan Super-Resolution confocal

850   micrographs. **(F)** Quantification and comparison of the total number of Cdr2-mNG nodes

851   visible in TIRF micrographs of wild-type or *pom1Δ* cells grown in either normal (2%) or

852   low (0.03%) glucose media. There is no significant difference in any condition (*p*>0.05).

853   Statistical significance was tested using a one-way ANOVA.

854

855

856   Figure 7: **Pom1 and Cdr2 concentrations at the lateral cell cortex are anti-correlated.**

857   **(A)** Localization of Pom1-mNG in confocal micrographs at middle cell focal planes.

858   Regions where line-scans were used to measure fluorescence intensity are marked at

859   the tip and side (orange lines). Scale bar, 1µm. **(B)** Localization of Cdr2-mNG in confocal

860   micrographs at middle cell focal planes. Scale bar, 1µm. Regions where line-scans were

861   used to measure fluorescence intensity are marked at the tip and side (orange lines). **(C)**

862   Concentration of Pom1 (red squares) or Cdr2 (blue circles) at cell sides in a range of

863   glucose concentrations, measured using 'Side ROI' denoted in panels A and B. **(D)**

864   Concentration of Pom1 (red squares) or Cdr2 (blue circles) at cell tips in a range of

865   glucose concentrations, measured using 'Tip ROI' denoted in panels A and B. **(E)**

866   Correlation of Cdr2 vs Pom1 concentrations at cell sides in each glucose concentration

867    from panel C. Concentrations are anticorrelated across all tested media glucose

868    concentrations ($p$=0.0006, Pearson r=-0.9352). **(F)** Correlation of Cdr2 vs Pom1

869    concentrations at cell tips in each glucose concentration from panel D. Concentrations

870    show weak anticorrelation across the tested media glucose concentrations, but the

871    correlation is not statistically significant ($p$=0.09, Pearson r=-0.65).

872

873    <u>Figure 8:</u> **Pom1 redistribution under glucose restriction disrupts Wee1 regulation at**

874    **cortical nodes. (A)** Localization of Wee1-mNG in wild-type or *pom1Δ* cells grown in

875    either high (2%) or low (0.03%) glucose media. Images were collected using TIRF

876    microscopy. Scale bar, 5μm. **(B)** Quantification of Wee1 bursting kinetics in wild-type cells

877    grown under high (2%, blue circles) and low (0.03%, red squares) glucose conditions.

878    The top panel is a plot of the total number of Wee1 bursts as a function of cell length,

879    counted in single time point TIRF micrographs. The slopes of the linear regressions are

880    significantly different ($p$<0.0001, $R^2_{2\%}$=0.66, $R^2_{0.03\%}$=0.45). The middle panel is a plot of

881    the frequency of Wee1 bursts as a function of cell length, measured using the TrackMate

882    particle tracking algorithm on TIRF microscopy movies. The slopes of the linear

883    regressions are not significantly different ($p$=0.8426, $R^2_{2\%}$=0.50, $R^2_{0.03\%}$=0.51). The

884    bottom panel is a plot of Wee1 burst duration as a function of cell length, measured using

885    the TrackMate particle tracking algorithm on TIRF microscopy movies. The slopes of the

886    linear regressions are not significantly different ($p$=0.09, $R^2_{2\%}$=0.28, $R^2_{0.03\%}$=0.05), but the

887    values of the elevation of the regressions are significantly different ($p$=0.0005). **(C)**

888    Quantification of Wee1 bursting kinetics in *pom1Δ* cells grown under normal (2%, green

889    circles) and low (0.03%, magenta squares) glucose conditions. The top panel is a plot of

890    the total number of Wee1 bursts as a function of cell length, counted in single time point

891    TIRF micrographs. The slopes of the linear regressions are not significantly different

892    ($p$=0.09, $R^2_{2\%}$=0.45, $R^2_{0.03\%}$=0.54), but the values of the elevation of the regressions are

893    significantly different ($p$<0.0001). The middle panel is a plot of the frequency of Wee1

894    bursts as a function of cell length, measured using the TrackMate particle tracking

895    algorithm on TIRF microscopy movies. The slopes of the linear regressions are not

896    significantly different ($p$=0.3125, $R^2_{2\%}$=0.45, $R^2_{0.03\%}$=0.56) but the values of the elevation

897    of the regressions are significantly different ($p$=0.009). The bottom panel is a plot of Wee1

898   burst duration as a function of cell length, measured using the TrackMate particle tracking

899   algorithm on TIRF microscopy movies. The slopes of the linear regressions are not

900   significantly different ($p$=0.8352, $R^2_{2\%}$=0.3, $R^2_{0.03\%}$=0.002), and the values of the

901   intercepts of the regressions are not significantly different ($p$=0.5392). For (B-C), $b_1$

902   denotes slope and $b_0$ denotes the Y-intercept. **(D)** Western blot of Wee1 in wild-type*,*

903   *pom1Δ*, and *cdr2Δ* cells grown in high (2%) or low (0.03%) glucose media showing

904   phosphorylation dependent band-shifts. Whole cell extracts were separated by SDS-

905   PAGE, and blots were probed with an $\alpha$-Wee1 antibody.

906

907

908   Figure 9: **A model for the glucose-modulated control of Wee1 bursting by Pom1 and**

909   **Cdr2.** See text for discussion.

910

911   Supplemental Figure S1 (Figure 1): **Analysis of the Pom1 gradient by confocal**

912   **microscopy**. **(A)** Similar to TIRF microscopy, at middle-cell focal planes, discreet Pom1-

913   mNeonGreen puncta are apparent at the lateral cell cortex of cells in individual frames

914   (200ms) of spinning-disc microscopy movies. Scale bar 1μm. **(B)** Individual Pom1 puncta

915   become averaged out in a projection of the entire continuous spinning-disc confocal

916   microscopy movie. Sum projection of a continuous 15s time-lapse acquisition of 200ms

917   exposures. **(C)** A line scan along the cell-perimeter in the direction indicated by the arrow

918   of the single time-point micrograph of the cell in (A) reveals that Pom1 puncta form a noisy

919   concentration gradient that begins at the cell tip and decays toward the cell middle. **(D)**

920   The same line scan as in panel C, but of the time-lapse sum projection of the cell in panel

921   B. This plot demonstrates that time-averaging of Pom1 puncta smoothens the

922   concentration gradient. **(E)** Spinning-disc confocal micrographs of single 200ms

923   acquisitions and **(F)** sum-projections of time lapse movies as in panels A and B

924   respectively, but using a top-focal plane. **(G-H)** Line scan using a straight line across the

925   long axis of the cell indicated by the arrow head on both the single time-point (G) and sum

926   projection (H) of a 15s continuous time-lapse movie. **(I-J)** Representative images of

927   Airyscan super-resolution micrographs of Pom1-mNeonGreen at either the cell tips (I) or

928   cell side (J). Images are sum projections of sequential five-frame Z-stacks spaced 0.17μm

929　apart, positioned to include all detected cortical Pom1-mNeonGreen signal. Scale bar,

930　1μm. **(K)** Fluorescence intensities of all single puncta resolved in Airyscan images as in

931　panels I-J. Data points represent individual puncta values, error bars are the mean and

932　standard deviation.

933

934　Supplemental Figure S2 (Figure 2): **Controls and supporting *in vitro* analysis of Pom1**

935　**puncta. (A)** Fluorescence intensity trace of a single Pom1 puncta binding to the cell

936　cortex, measured by high speed TIRF microscopy of a cell expressing Pom1-mNG (~50

937　frames per second with continuous acquisition). Rapid intensity changes indicating

938　binding and unbinding are indicated by green upward and magenta downward pointing

939　arrows respectively. **(B)** Size standards for velocity sucrose gradients, as in main Figure

940　2C-E. Data are quantified from single Coomassie-stained gels, or using

941　spectrophotometry data. **(C)** Plot of S-value versus sedimentation pattern. Line is linear

942　regression of the three size standards, with the S-value for Pom1 peaks interpolated. **(D)**

943　Quantification and comparison of binding duration of either wild-type or kinase-dead

944　Pom1-mNG to supported lipid bilayers in TIRF microscopy movies (***$p$=<0.0001).

945　Statistical significance was tested with a Student's T-test. **(E)** FRAP analysis of supported

946　lipid bilayers. Upper panel: A circular ROI (teal dashed line) was used to bleach

947　fluorescent PE in the bilayers. Lower panel: Kymograph at white dashed line in upper

948　panel of fluorescence recovery as monitored by TIRF microscopy, indicating fluidity.

949

950　Supplemental Figure S3 (Figure 3): **Representative single-cell data used to compute**

951　**mean cellular values in Figure 3. (A)** Quantification of binding duration of individual

952　Pom1-mNG puncta at the tips or sides of representative wild-type, *tea1Δ* or *tea4Δ* cells,

953　measured by TIRF microscopy. **(B)** Quantification of total displacement of individual

954　Pom1-mNG puncta at the tips or sides of representative wild-type, *tea1Δ* or *tea4Δ* cells,

955　measured by TIRF microscopy. For A-B, line and error represent single cell mean and

956　standard deviation.

957

958　Supplemental Figure S4 (Figure 4): **Supporting analysis of Cdr2 nodes and Pom1**

959　**puncta at the medial cell cortex. (A)** Comparison of Pom1 puncta binding frequency on

960    the cell sides of wild-type and *cdr2Δ* cells (n.s., *p*=0.1076). **(B)** Comparison of Pom1

961    puncta binding duration on the cell sides of wild-type and *cdr2Δ* cells (n.s., *p*=0.371). **(C)**

962    Comparison of Pom1 puncta displacement on the cell sides of wild-type and *cdr2Δ* cells

963    (n.s., *p*=0.8191). In A-C, significance was tested using a Student's T-test. **(D)** Schematic

964    and quantification of Pom1 concentration at the lateral cell cortex measured by confocal

965    microscopy, using 2μm line scans at the cell middle from images such as Figure 5A. The

966    slope of the linear regression is negative, but not significantly non-zero (*p*=0.2603,

967    $R^2$=0.03). **(E)** Plot of the number of colocalized structures counted in a middle cell 2x2μm

968    ROI as a function of cell size. The slope of the linear regression is significantly non-zero

969    (*p*=0.0006, $R^2$=0.3). **(F)** Plot of free, non-colocalized Cdr2 nodes (blue circles) and Pom1

970    puncta (red squares). The slope of the linear regression of #Pom1 puncta versus cell

971    length is negative, and significantly non-zero (*p*=0.001, $R^2$=0.27). The slope of the linear

972    regression of #Cdr2 nodes versus cell length is positive, and significantly non-zero

973    (*p*=0.021, $R^2$=0.15).

974

975    Supplemental Figure S5 (Figure 5): ***In vitro* analysis of Pom1 activity in *tea1Δ* cells.**

976    **(A)** Western blot of an *in vitro* thiophosphate kinase assay to test Pom1

977    autophosphorylation activity in *pom1-3HA*, *pom1-AS-3HA*, and *pom1-AS-3HA tea1Δ*

978    cells. Blots were probed with an α-thiophosphate antibody. Only analog-sensitive (AS)

979    alleles can use ATPγS to thio-phosphorylate substrates. **(B)** Comparison of western blot

980    band intensities from four replicate gels, derived from two separate experiments as in

981    panel A. Statistical significance was tested using a paired T-test between *Pom1-AS-3HA*

982    and *Pom1-AS-3HA tea1Δ* cells (*, *p*=0.02). **(C)** Western blot of an *in vitro* thiophosphate

983    kinase assay to measure phosphorylation of purified Cdr2 by Pom1 in *pom1-3HA*, *pom1-*

984    *AS-3HA*, and *pom1-AS-3HA tea1Δ* cells. Blots were probed with an anti-thiophosphate

985    antibody. Excess Pom1-3HA was used in lanes 1 and 3 to ensure absence of signal in

986    negative controls lacking the analog-sensitive mutation. Cdr2(712-775) is a fragment that

987    contains known Pom1 phosphorylation sites. **(D)** Western blot of the Pom1 substrate

988    Rga7-3HA in wild-type, *tea1Δ,* and *pom1Δ* cells. Whole-cell extracts were separated by

989    SDS-PAGE, and probed with α-HA antibodies. The upper band represents a Pom1-

990    dependent phospho-isoform. **(E)** Comparison of band intensities from four replicate gels

991    as in (D), expressed as the ratio of the grey-value of the top band/bottom band. Line and

992    error are mean and standard deviation. Statistical significance was tested using a one-

993    way ANOVA and Tukey's multiple comparisons test (WT vs *tea1Δ*, ***p=0.003, WT vs

994    *pom1Δ*, ****p<0.0001, *tea1Δ* vs *pom1Δ*, ****p<0.0001).

995

996    <u>Supplemental Figure S6 (Figure 5)</u>: **Control of Pom1 concentration at the cell side.**

997    **(A)** Localization of Pom1-mNG in wild-type, *tea1Δ*, and *pom1-KD* (kinase-dead) cells at

998    the indicated concentration of glucose. Images are confocal micrographs taken at a

999    middle cell focal plane. Scale bar, 5μm. **(B)** Quantification of Pom1 concentration along

1000    2μm line scans as in figure S4D, measured using images as in panel A. The means of

1001    each column are significantly different from every other column (p<0.005). Statistical

1002    significance was tested using a one-way ANOVA and Tukey's multiple comparison's test.

1003    **(C)** Plot of Pom1 concentration at the cell side as a function of glucose concentration in

1004    the growth media. Data were fit to an exponential curve showing a negative sinusoidal

1005    relationship reaching half saturation at ~0.05% glucose. The concentration of Pom1-mNG

1006    at the lateral cell cortex in a *tea1Δ* mutant was fit to this curve, and is the approximate

1007    equivalent of wild-type cells grown in 0.03% Glucose. Line and error are mean and

1008    standard deviation. (D) Localization of Tea4-mNG in wild-type or *tea1Δ* cells grown in the

1009    indicated concentration of glucose for one hour. Images are confocal micrographs taken

1010    at middle cell focal planes. Arrows point to lateral puncta of Tea4-mNG. Scale bar, 5μm.

1011

1012    <u>Supplemental Figure S7 (Figure 6)</u>: **Redistribution of Pom1 puncta to the lateral**

1013    **cortex disrupts Cdr2 nodes during glucose restriction, supporting data. (A)** Analysis

1014    of Pom1 and Cdr2 redistribution by spinning-disc confocal microscopy, as in (Kelkar and

1015    Martin, 2015). Images are confocal micrographs of cells co-expressing Pom1-tdTomato

1016    and Cdr2-mNG in high (2%) or low (0.03%) glucose media. Scale bar, 5μm. **(B)** Cortical

1017    fluorescence intensity of Cdr2 and Pom1 was measured using line scans drawn around

1018    the circumference of the representative cell marked with an arrowhead in the 2% glucose

1019    portion of panel A. **(C)** Cortical fluorescence intensity of Cdr2 and Pom1 was measured

1020    using line scans drawn around the circumference of the representative cell marked with

1021    an arrowhead in the 0.03% glucose portion of panel A.

1022

1023     Supplemental Figure S8 (Figure 8): **Re-plotting of data from Main Figure 8 for**

1024     **additional comparison. (A)** Plot of the frequency of Wee1 bursts as a function of cell

1025     length in wild-type cells grown in high (2%) glucose (blue circles) and low glucose (red

1026     squares), and kinase-dead *cdr2(E177A)* mutant cells grown in 2% glucose (green

1027     triangles). Measurements were made using the TrackMate particle tracking algorithm on

1028     TIRF microscopy movies. The slopes of the linear regressions are not significantly

1029     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

1030     regressions are also not significantly different (*p*=0.4397). **(B)** Plot of Wee1 burst duration

1031     as a function of cell length in wild-type cells grown in low glucose (red squares) and

1032     kinase-dead *cdr2(E177A)* mutant cells grown in 2% glucose (green triangles).

1033     Measurements were made using the Trackmate particle tracking algorithm on TIRF

1034     microscopy movies. The slopes of the linear regressions are not significantly different

1035     (*p*=0.8048, $R^2_{0.03\%}$=0.05, $R^2_{Cdr2(E177A)}$=0.2) and the elevations of the regressions are also

1036     not significantly different (*p*=0.8048).

1037

1038     Supplemental Table S1: Fission yeast strains used in this study.

1039

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1237

A.

**Pom1-mNG – TIRF Microscopy**

**200ms
Snap Shot**

**Sum Projection
15s Movie**

C.

570nm

570nm

Particle Traces

Pom1-mNeonGreen

0.4s  **Duration**  12s

B.



D.

**Pom1-mNG**

**Cell Tip**

E.

**Pom1-mNG**

**Cell Side**

F.

G.

H.

I.

**Figure 1**

# A.
**Spinning Disc – Middle Focal Plane**
**200ms Snap Shot**



**Pom1-mNG**

# B.
**Sum Projection of 15s Movie**

# C.



# D.



# E.
**Spinning Disc – Top Focal Plane**
**200ms Snap Shot**



**Pom1-mNG**

# F.
**Sum Projection of 15s Movie**



# G.



# H.



# I.
**Cell Tip**

Pom1-mNG



# J.
**Cell Side**



# K.
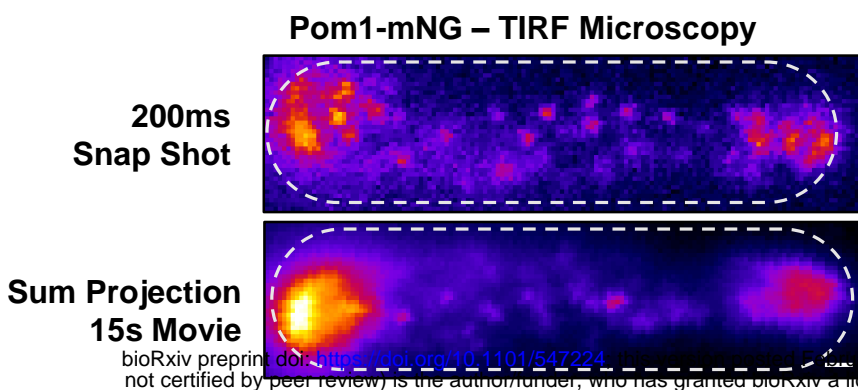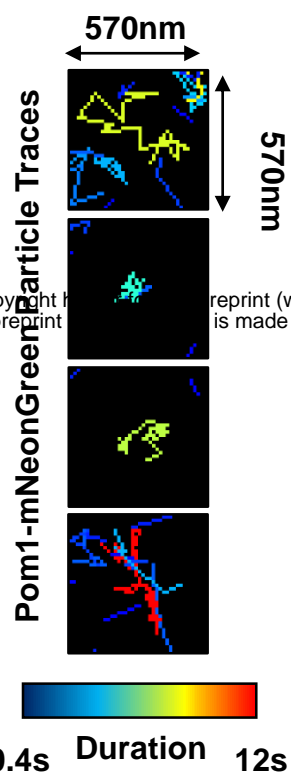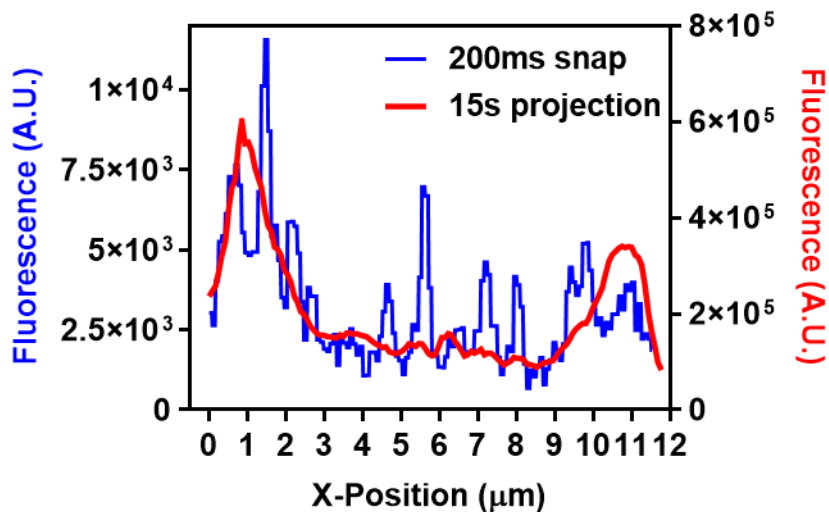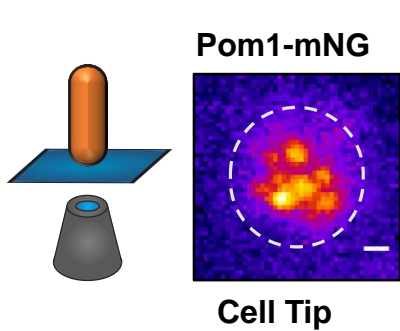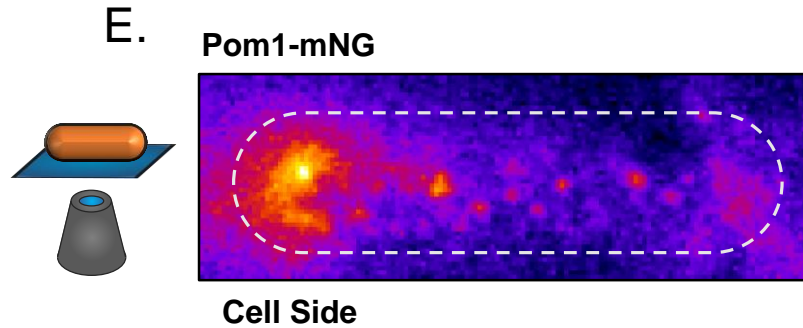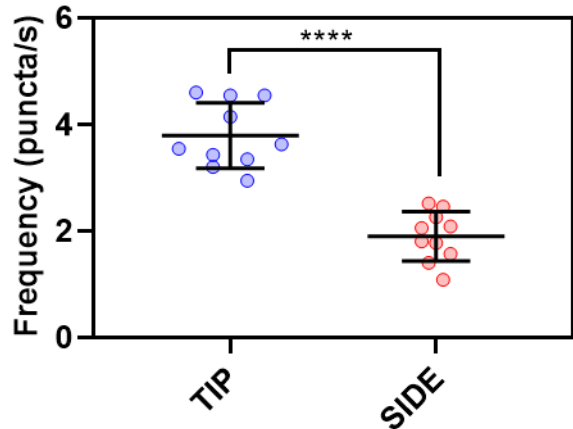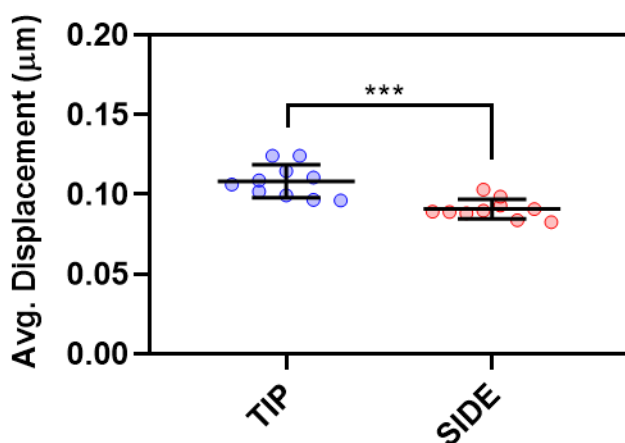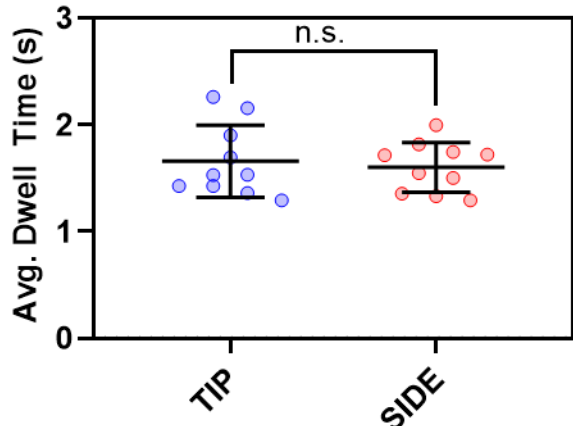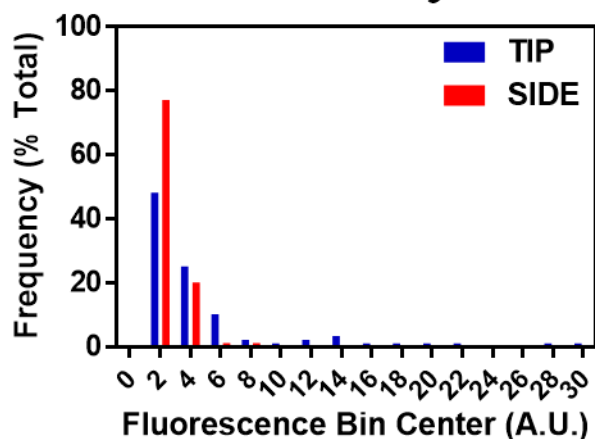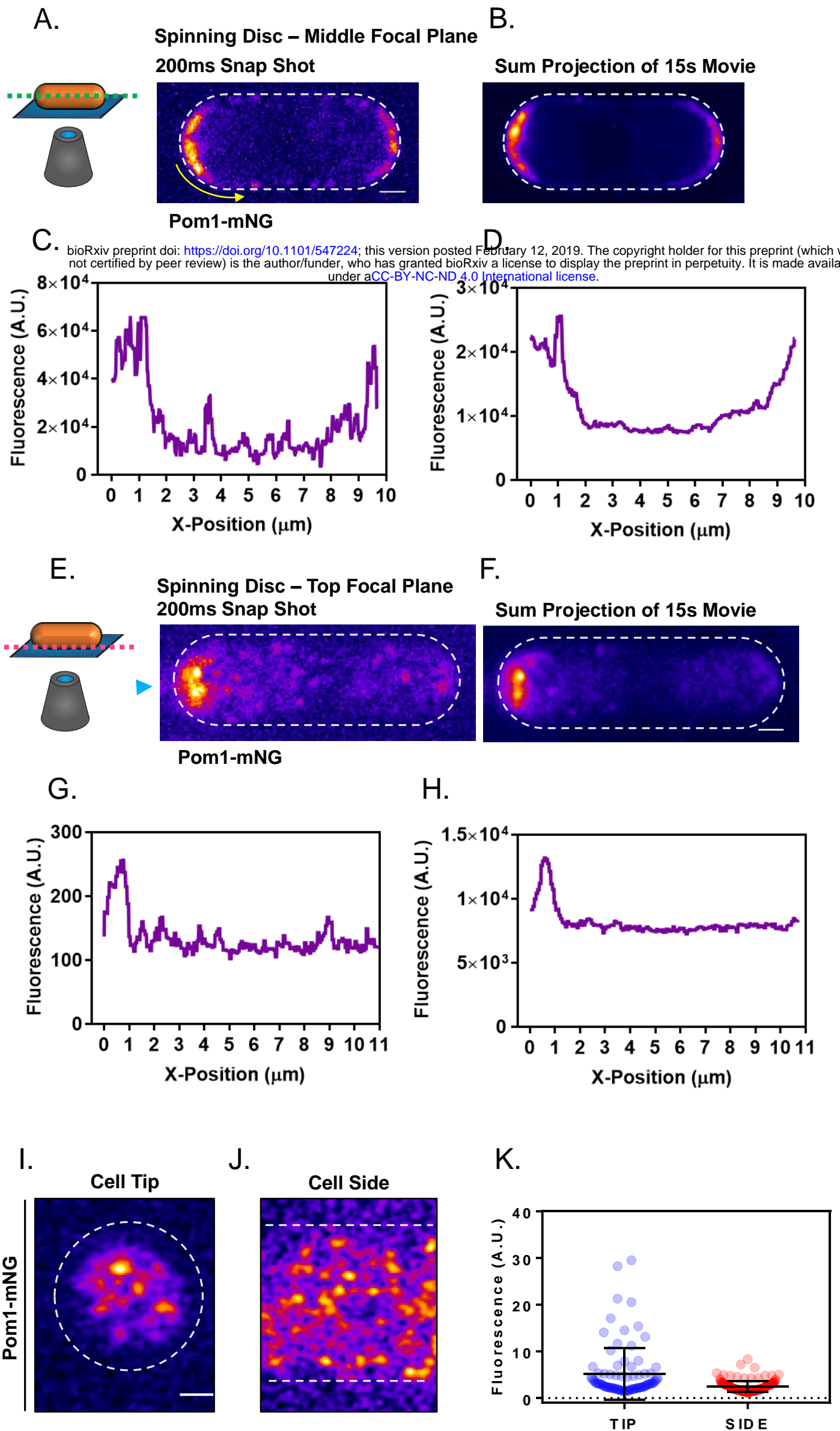


**Supplemental Figure 1 in support of Figure 1**

A.



**Pom1-mNG Burst Cell**

B.



**No Tag
Cell Extract**          **Pom1-mNG
Cell Extract**

C.



| *Yeast Extract* | **Sucrose Gradient Fraction** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |

Pom1-3HA — 150kDa αHA

Fractions 6-8 Re-spin — 150kDa αHA

**Increasing Particle Size** →

D.



Peak 1 2.6

Peak 2 7.4

% Western Blot Signal

**Sucrose Gradient Fraction**

E.

**Pom1-mNG - Supported Lipid Bilayer**



0  5  10  15  20  25  30  35  40  45  50  55

**Time (s)**

F.



n.s.

Pom1-mNG Puncta Dwell Time (s)

*in vivo* (Cell Side)    *in vitro* (SLB)

G.



| *Purified Protein* | **Sucrose Gradient Fraction** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |

GST-Pom1 — αGST 150kDa

GST-Pom1 + Yeast Extract — αGST 150kDa

GST-Pom1 + *pom1*Δ Extract — αGST 150kDa

**Increasing Particle Size** →

**Figure 2**

**A.**

**Single Puncta Trace**
*TIRF Microscopy -20ms/frame*

**B.**

**Size Standards**
**BSA   Thyroglobulin   Ferritin**

**C.**

**D.**

**E.**

**0.01% Liss-Rhod-PE**
**75% PC 25%PS**

**Supplemental Figure 2 in support of Figure 2**

## A.

**Pom1-HA**

**Sucrose Gradient Fraction**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| *tea1Δ* | | | | | | | | | | 150 kDa αHA |
| *tea4Δ* | | | | | | | | | | 150 kDa αHA |

## B.

**Confocal Microscopy**



Pom1-mNG

*tea1Δ*   *tea4Δ*

## C.



**Cell Side**   **Cell Tip**

Pom1-mNG    *tea4Δ*    *tea1Δ*

**TIRF Microscopy**

## D.



## E.



# Figure 3

# A.

**Single Cell Data**



B.

**Single Cell Data**

**Supplemental Figure 3 in support of Figure 3**

A.

Pom1-mNG    Cdr2-tagRFP-t    Merge

Kymograph ROI

B.

Merge

Cdr2-tagRFP-t

Pom1-mNG

Time (s)

C.

Cdr2-mEGFP

Pom1-mNG

D.

Cdr2 Nodes    Pom1 Puncta

Puncta/Cell

Cell Length (µm)

E.

Cdr2 Nodes    Pom1 Puncta

Medial Density (puncta/µm²)

Cell Length (µm)

F.

Cdr2-mNG    Pom1-tdTomato    Merge
Co-localized
Free

G.

# Free Cdr2/Pom1 Structures

Cell Length (µm)

Figure 4

**A.**

Frequency (puncta/s)

n.s.

WT  cdr2Δ

**B.**

Avg. Dwell Time (s)

n.s.

WT  cdr2Δ

**C.**

Avg. Displacement (μm)

n.s.

WT  cdr2Δ

**D.**

[Pom1] in TIRFM

Medial Fluorescence (A.U.)

Cell Length (μm)

**E.**

Colocalized Structures

Cell Length (μm)

**F.**

Cdr2 Nodes — Pom1 Puncta

\# Free Structures

Cell Length (μm)

**Supplemental Figure 4 in support of Figure 4**

# A.

Pom1-mNG

WT     *tea1Δ*

**Confocal Microscopy – Middle Focal Plane**

# B.

**[Pom1]
at cell sides**

****

Fluorescence (A.U.)

WT     *tea1Δ*

# C.

Cdr2-mNG
*tea1Δ*

**TIRF Microscopy**

# D.

WT    *tea1Δ*

#Cdr2 Nodes

Cell Length (µm)

# E.

Wee1-mNG
*tea1Δ*

**TIRF Microscopy**

# F.

WT    *tea1Δ*

#Wee1 Bursts

Cell Length (µm)

**Figure 5**

**A.**



α-Thio-phosphate

α-HA

Pom1-3HA
Pom1-AS-3HA
Pom1-AS-3HA *tea1*Δ

150kDa
150kDa

**B.**



Thiophosphate Band Intensity

150

100

50

0

WT            *tea1*Δ

Experimental Replicates

*

**C.**



| | | | | | |
|---|---|---|---|---|---|
| + | - | + | - | - | Pom1-3HA |
| - | - | - | + | - | Pom1-AS-3HA |
| - | - | - | - | + | Pom1-AS-3HA *tea1*Δ |
| - | + | + | + | + | GST-Cdr2(712-775) |

α-HA                                   150kDa

α-Thiophosphate                       25kDa

α-GST                                 25kDa

**D.**



WT        *tea1*Δ    *pom1*Δ

Rga7-3HA

αHA

75kDa

**E.**



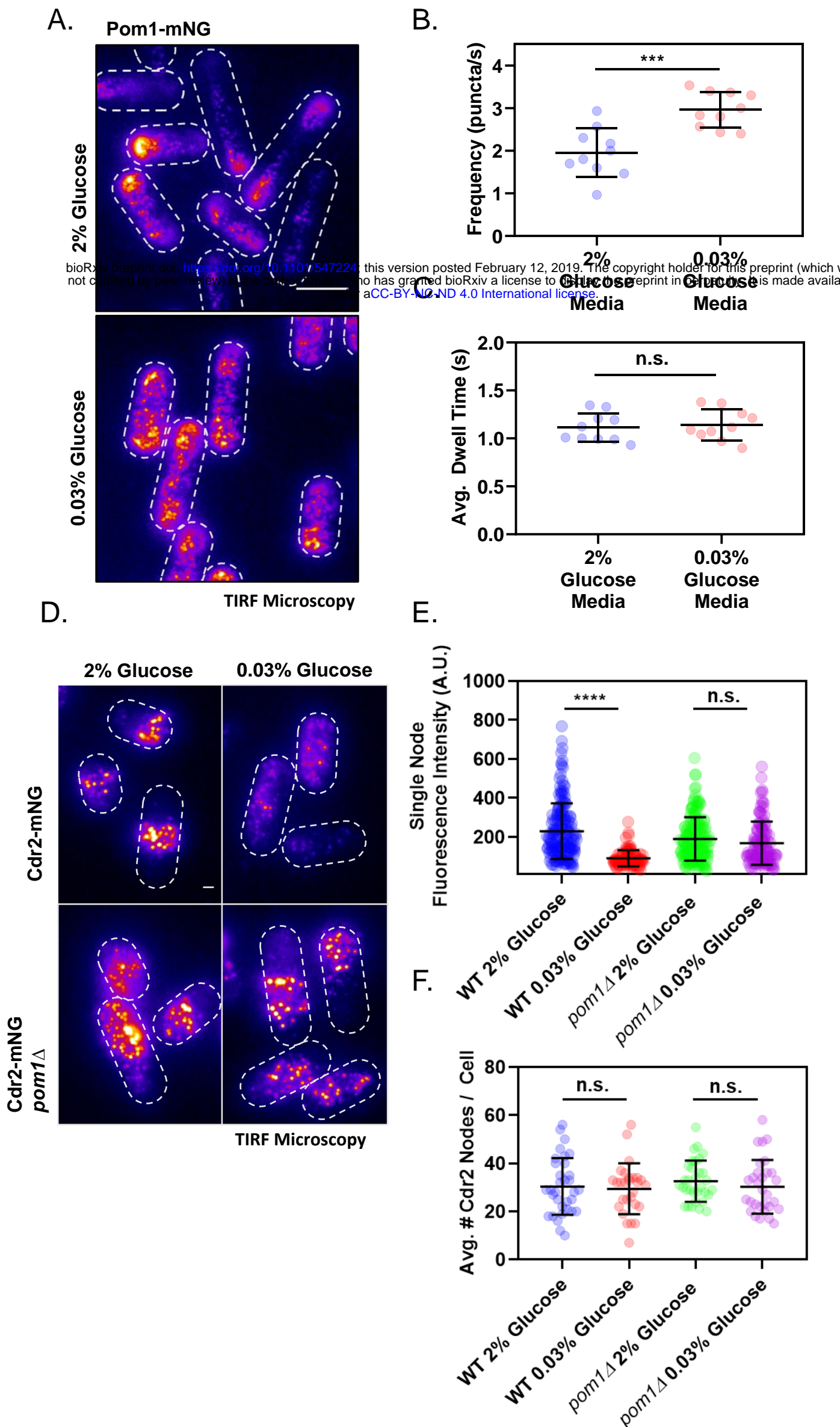Rga7 Top/Bottom Band Intensity

0.6

0.4

0.2

0.0

WT        *tea1*Δ    *pom1*Δ

***        ****

# Supplemental Figure 5 in support of Figure 5

A.

Pom1-mNG  |  Pom1-mNG tea1Δ  |  Pom1-KD-mNG

2% Glucose

0.03% Glucose

B.

Cell Side Avg. Fluor. Intensity (A.U.)

2 / 0.03 : pom1-mNG
2 / 0.03 : pom1-mNG tea1Δ
pom1-KD-mNG

C.

[Pom1] at cell sides

Fluorescence (A.U.)

*tea1Δ*
interpolation = 0.02-0.03% glucose

% Glucose Media

D.

Tea4-mNG

2% Glucose  |  0.03% Glucose  |  tea1Δ 2% Glucose

Confocal Microscopy

**Supplemental Figure 6 in support of Figure 5**

**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.**

2% Glucose    0.03% Glucose

Cdr2-mNG

Cdr2-mNG
*pom1Δ*

TIRF Microscopy

**E.**

Single Node
Fluorescence Intensity (A.U.)

****    n.s.

WT 2% Glucose
WT 0.03% Glucose
*pom1Δ* 2% Glucose
*pom1Δ* 0.03% Glucose

**F.**

Avg. # Cdr2 Nodes / Cell

n.s.    n.s.

WT 2% Glucose
WT 0.03% Glucose
*pom1Δ* 2% Glucose
*pom1Δ* 0.03% Glucose

**Figure 6**

# A.
## Confocal Microscopy

| Pom1-tdTomato | Cdr2-mNG | Merge |



2% Glucose

0.03% Glucose

## B.



2% Glucose

Pom1 Intensity (A.U.) vs Cdr2 Intensity (A.U.)
Distance from Tip (μm)

## C.



0.03% Glucose

Pom1 Intensity (A.U.) vs Cdr2 Intensity (A.U.)
Distance from Tip (μm)

# Supplemental Figure 7 in support of Figure 6

A. Pom1-mNG

B. Cdr2-mNG

C.

D.

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

F. Pearson r = -0.65
P=0.090(n.s.)

**Figure 7**

**A.**

**TIRF Microscopy**

**B.**

**C.**
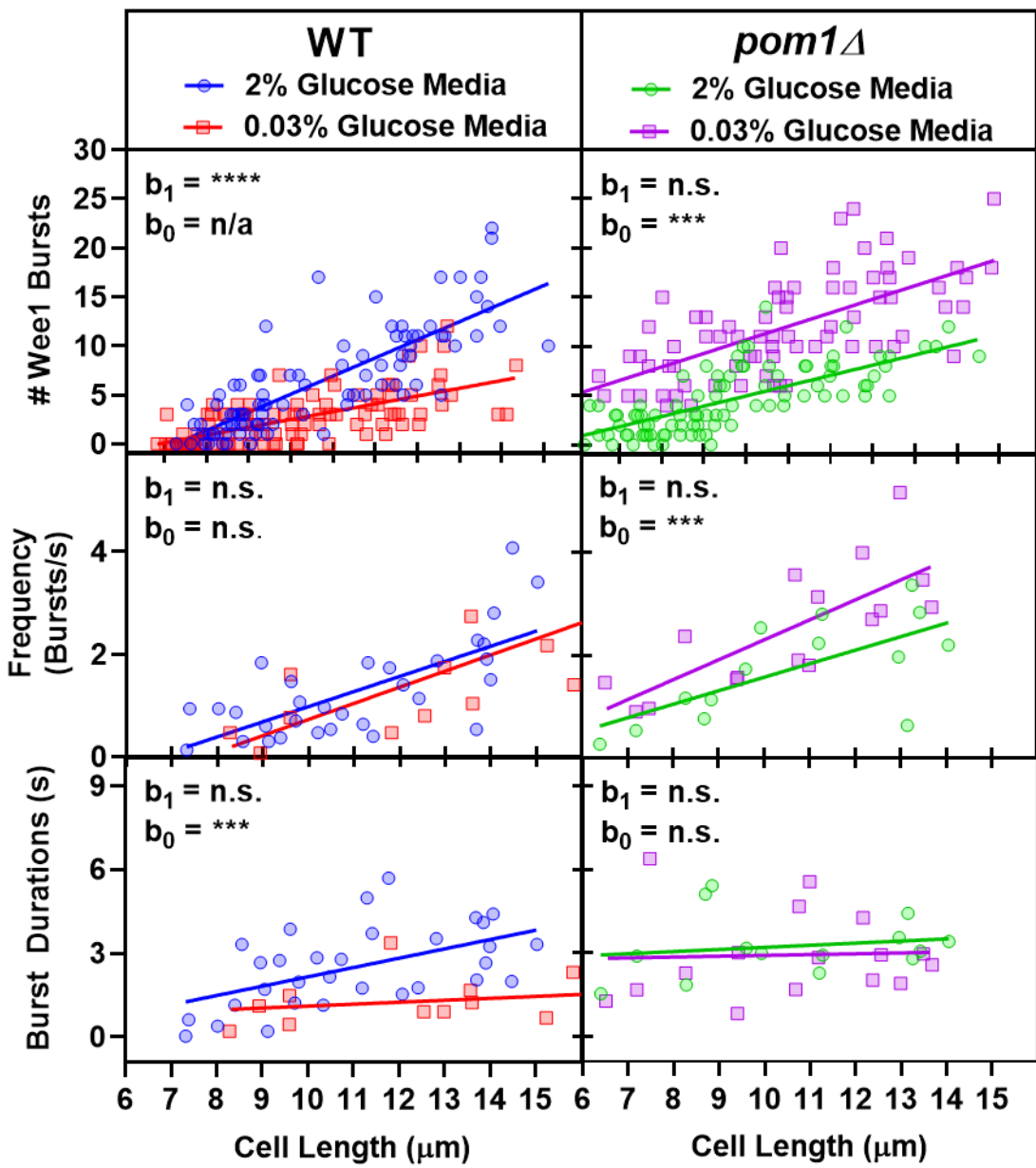


**D.**
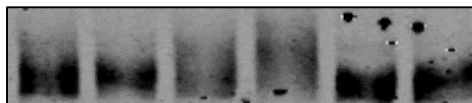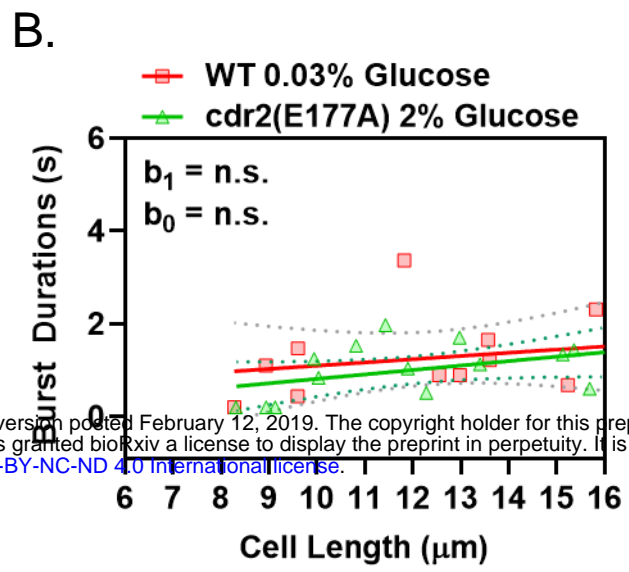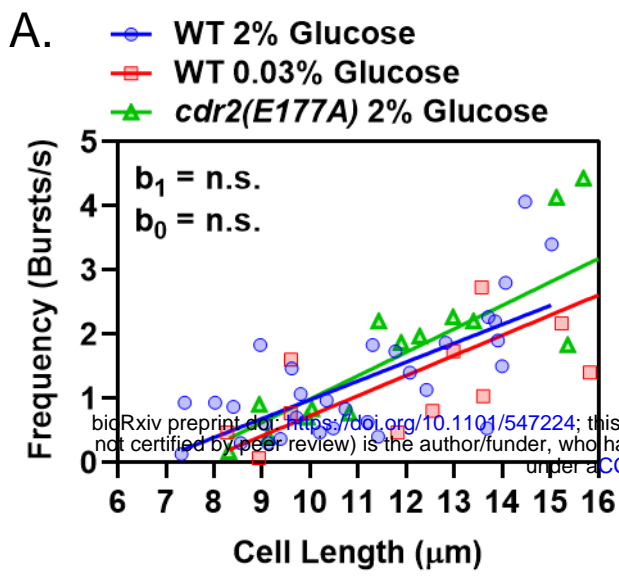


**Figure 8**

**A.**

Legend:
— WT 2% Glucose (blue circle)
— WT 0.03% Glucose (red square)
— *cdr2(E177A)* 2% Glucose (green triangle)

Y-axis: Frequency (Bursts/s), scale 0 to 5
X-axis: Cell Length (μm), scale 6 to 16

$b_1$ = n.s.
$b_0$ = n.s.

**B.**

Legend:
— WT 0.03% Glucose (red square)
— *cdr2(E177A)* 2% Glucose (green triangle)

Y-axis: Burst Durations (s), scale 0 to 6
X-axis: Cell Length (μm), scale 6 to 16

$b_1$ = n.s.
$b_0$ = n.s.

**Supplemental Figure 8 in support of Figure 8**

**CELL TIP**

| Pom1 Puncta | Cdr1/2 Node | Wee1 |
|---|---|---|

**Small Cell**

**Large Cell**

**Glucose Starvation**

**CELL SIDE**

**Figure 9**

## Strains used in this study:

| Strain | Genotype | Origin | Figure |
|--------|----------|--------|--------|
| JM4496 | *pom1-yomNeonGreen::hphR* | This Study | 1A-I, S1A-K, 2A-B,E-F, S2A,D, 3D-F, S3A-B, 4C-E, S4A-D, 5A-B, S6A-C, 6A-C, 7A,C-F |
| JM797 | *pom1-3HA::hphR ura4-D18 leu1-32 h+* | PN948 (Paul Nurse Lab) | 2C-D,G, S5A,C |
| JM966 | *pom1Δ::natR h-* | Lab Stock | 2G, 8D |
| JM5412 | *pom1-m2-yomNeonGreen::hphR ura4-D18 leu1-32 h-* | This Study | S2D, S6A-B |
| JM5414 | *pom1-3HA::hphR tea1Δ::ura4+ ura4-D18* | This Study | 3A |
| JM5415 | *pom1-3HA::hphR tea4Δ::kanMX6* | This Study | 3A |
| JM4792 | *pom1-yomNeonGreen::hphR tea1Δ::kanMX6* | This Study | 3B-F, S3A-B, 5A-B, S6A-C |
| JM4791 | *pom1-yomNeonGreen::hphR tea4Δ::kanMX6* | This Study | 3B-F, S3A-B |
| JM5373 | *pom1-yomNeonGreen::hphR cdr2-tagRFP-t::hphR* | This Study | 4A-B |
| JM4493 | *cdr2-yomNeonGreen::hphR h-* | Lab Stock | 4C-E, 6D-F, 7B-F |
| JM4525 | *wee1-yomNeonGreen::hph cdr2Δ::natR* | Lab Stock | S4A-C |
| JM513 | | | |
| JM5374 | | | |
| JM5372 | *wee1-yomNeonGreen::hphR  tea1Δ::kanMX6* | This Study | S4C |
| JM2209 | *rga7-3HA::hphR h-* | Lab Stock | S5D-E |
| JM5612 | *rga7-3HA::hphR tea1Δ::kanMX6* | This Study | S5D-E |
| JM2210 | *rga7-3HA::hphR pom1Δ::kanMX6* | Lab Stock | S5D-E |
| JM4211 | *pom1-as-3HA::hphR* | Lab Stock | S5A-C |
| JM5565 | *pom1-as-3HA::hphR tea1Δ::kanMX6 ura4-D18* | This Study | S5A-C |
| JM5132 | *tea4-yomNeonGreen::hphR h-* | This Study | S6D |
| JM5733 | *tea4-yomNeonGreen::hphR tea1Δ::kanMX6* | This Study | S6D |
| JM5359 | *cdr2-yomNeonGreen::hphR pom1Δ::natR* | This Study | 6D-F |
| JM4495 | *wee1-yomNeonGreen::hphR h-* | Lab Stock | 8A-B, S8A-B |
| JM4527 | *wee1-yomNeonGreen::hphR pom1Δ::kanMX6* | Lab Stock | 8A,C |
| JM366 | *972 h-* | PN1 (Paul Nurse lab) | 8D |
| JM600 | *cdr2Δ::natR ura4-D18 leu1-32 h+* | Lab Stock | 8D |
| JM4578 | *wee1-yomNeonGreen::hphR cdr2(E177A)* | Lab Stock | S8A-B |

# Supplemental Table 1