Integration of cell wall synthesis activation and chromosome segregation during cell division in *Caulobacter*

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ABSTRACT

To divide, bacteria must synthesize and remodel their peptidoglycan (PG) cell wall, a protective meshwork that maintains cell shape. FtsZ, a tubulin homolog, dynamically assembles into a midcell band, recruiting division proteins including the PG synthases FtsW and FtsI. FtsWI are activated to synthesize PG and drive constriction at the appropriate time and place, however their activation pathway remains unresolved. In *Caulobacter crescentus*, FtsWI activity requires FzlA, an essential FtsZ-binding protein. Through time-lapse imaging and single-molecule tracking of *C. crescentus* FtsW and FzlA in perturbed genetic backgrounds, we demonstrate that FzlA is a limiting constriction activation factor that converts inactive, fast-moving FtsW to an active, slow-moving state. We find that FzlA interacts with the DNA translocase FtsK, and place FtsK genetically in a pathway with FzlA and FtsWI. Misregulation of the FzlA-FtsK-FtsWI pathway leads to heightened DNA damage and cell death. We propose that FzlA integrates the FtsZ ring, chromosome segregation, and PG synthesis to ensure robust and timely constriction during *Caulobacter* division.

Introduction

Bacterial cell division is a robust process, evolved over billions of years, that requires tight regulation of multiple essential events to ensure survival. These events include marking the division site, recruiting division proteins, segregating the chromosome, remodeling and synthesizing cell wall, and separating the daughter cells (Dewachter et al., 2018; Mahone and Goley, 2020). The first step in division is the assembly of FtsZ, an essential and conserved tubulin homolog, into a cytokinetic "Z-ring" at the incipient site of division (Mahone and Goley, 2020; Barrows and Goley, 2021). Once the Z-ring is established, dozens of proteins, coined the divisome, are directly or indirectly recruited by FtsZ to the division site (Mahone and Goley, 2020; McQuillen and Xiao, 2020). FtsZ polymers within the Z-ring are highly dynamic, driven by FtsZ's GTPase activity, and exhibit treadmilling motion about the inner circumference at the division site (Bisson-Filho et al., 2017; Yang et al., 2017).

After divisome assembly, cells constrict inward via envelope remodeling. In Gram-negative bacteria, the cell envelope is composed of an inner membrane, cell wall, and outer membrane external to the cell wall. The cell wall is a macromolecular meshwork made of peptidoglycan (PG) that protects against turgor pressure and other stresses, and dictates bacterial morphology (Daitch and Goley, 2020). New PG is synthesized by glycosyltransferases that polymerize lipid II, a lipid-linked disaccharide made of N-acetylmuramic acid and N-acetylglucosamine with a pentapeptide side chain. The peptide stems are crosslinked by transpeptidases, including D,D-transpeptidases of the penicillin-binding protein (PBP) family (Daitch and Goley, 2020). During division, the PG synthases FtsW and FtsI (PBP3) are the primary glycosyltransferase and transpeptidase, respectively (Daitch and Goley, 2020; Mahone and Goley, 2020). These two enzymes work together as a cognate pair (FtsWI) to synthesize cytokinetic PG that provides the constrictive force (Coltharp and Xiao, 2017).

51   Single molecule tracking studies show that the PG synthases move dynamically about

52 the division site. In bacteria where dynamics have been characterized (*Escherichia coli, Bacillus*

53 *subtilis, Staphylococcus aureus, Streptococcus pneumoniae*), the dynamics of FtsZ and the PG

54 synthases are associated, but range from FtsWI requiring FtsZ treadmilling for movement to

55 requiring FtsZ for placement at midcell, but moving independently (Yang et al., 2017, 2021;

56 Bisson-Filho et al., 2017; Perez et al., 2019; Yang and Liu, 2022). In *E. coli,* the only Gram-

57 negative organism in which dynamics have been studied, the moving PG synthases can be

58 differentiated into fast- and slow-moving populations. Fast-moving PG synthases depend on

59 FtsZ for movement and are inactive for PG synthesis. Slow-moving molecules depend on PG

60 synthesis for locomotion and are thought to be actively synthesizing new PG (Yang et al., 2017,

61 2021; McCausland et al., 2021).

62   In most model bacteria, divisome components upstream of FtsWI activation have been

63 identified, but their precise functions and mechanisms of signaling remain unclear. In *E. coli*,

64 FtsN has been proposed as a trigger for constriction initiation and is last to localize to the

65 division plane (Weiss, 2015; Mahone and Goley, 2020; Lyu et al., 2022). Hyperactivating

66 mutations in *ftsB* and *ftsL*, which encode subunits of the FtsQLB complex, result in shorter cells

67 (Tsang and Bernhardt, 2015; Liu et al., 2015). These mutants, when combined with other

68 genetic perturbations, can bypass requirements of otherwise essential divisome components,

69 suggesting they are critical mediators of activation (Park et al., 2020; Li et al., 2021; Du et al.,

70 2016). Consistent with this idea, purified FtsQLB from *Pseudomonas aeruginosa* can activate

71 FtsWI *in vitro* (Marmont and Bernhardt, 2020). While the final stages of activation are becoming

72 clear, the FtsZ-proximal partners and how they ultimately signal for FtsWI activation remain

73 elusive.

74   In *Caulobacter crescentus,* an α-proteobacterium in which divisome assembly has been

75 well-defined, FtsW is the last divisome component to arrive at the division plane prior to

76 constriction initiation (Goley, Yeh et al., 2011). However, localization of FtsW is not sufficient for

77 cells to constrict. Recently, we implicated FzlA, an essential division protein conserved across

78 α-proteobacteria, as a regulator of constriction (Lariviere et al., 2019, 2018; Goley, 2010). FzlA

79 binds to FtsZ and co-localizes with FtsZ in stalked and pre-divisional cells (Goley, 2010).

80 Previous work identified residues on FzlA required for two essential activities: binding to FtsZ

81 and an unknown activity in the C-terminal tail (Lariviere et al., 2018). When FzlA is depleted, or

82 either essential activity is disrupted, cells do not activate constriction, despite the remainder of

83 the divisome localizing to midcell rings (Lariviere et al., 2018; Goley, 2010). A hyperactive

84 mutant of FtsW (FtsW^{A246T}) renders *fzlA* non-essential, albeit with a slower constriction rate than

85 in the presence of FzlA (Lariviere et al., 2019). An *ftsWI* triple mutant (*ftsW$^{F145L/A246T}$ftsI^{I45V}*,

86 termed *ftsW**I**) constricts faster than the *ftsW^{A246T}* mutant and also renders *fzlA* non-essential,

87 with minimal effects on cell length (Lariviere et al., 2019; Lambert et al., 2018). We concluded

88 that FzlA acts upstream of FtsWI activation, as an FtsZ-proximal step in the FtsZ-FtsWI

89 activation pathway.

90   While we have established that FzlA is required for FtsWI activation in *Caulobacter*, the

91 mechanism by which FzlA signals for FtsWI activation and the functional importance of

92 regulation by FzlA is unknown. Here, we employed time-lapse imaging, genetics, biochemistry,

93 and single-molecule tracking (SMT) to dissect the relationship between FzlA and FtsWI activity.

94 We discovered that FzlA activates constriction by converting inactive, fast-moving molecules of

95 FtsW to an active, slower-moving state. Overproducing FzlA leads to hyperconstriction by

96 converting a greater proportion of FtsW into an active state, and this hyperactivation is toxic,

97 particularly in the *ftsW**I** background. The toxicity of FzlA overproduction is in part due to

98 enhanced DNA damage via misregulation of a novel interaction between FzlA and FtsK, a

99 division protein that segregates the chromosomal termini during division (Wang and

100 Lutkenhaus, 1998; Wang et al., 2006). These results unify divisome assembly, chromosome

101   segregation, and activation of cytokinetic PG synthases into a single pathway, which requires

102   proper regulation to ensure envelope and chromosomal integrity.

103

104 **Results**

105 **Overproduction of FzlA hyperactivates constriction**

106   To better understand how FzlA regulates FtsWI, we sought to characterize the effects of

107 FzlA overproduction on cell division and viability. We hypothesized that FzlA may be a limiting

108 activator of FtsWI. If that is the case, cells with an overabundance of FzlA should be hyperactive

109 for constriction and therefore constrict faster than control cells (Yang et al., 2021; Lambert et al.,

110 2018; Lariviere et al., 2019). To test this hypothesis, we performed timelapse phase contrast

111 microscopy on synchronized cells overexpressing *fzlA* (EG3637) or bearing an empty vector

112 (EV) control (EG1644) (Fig. 1 A). We induced *fzlA* overexpression for one hour prior to and

113 throughout the timelapse experiments, and isolated newborn swarmer (G1) cells by density

114 centrifugation prior to imaging. We determined that *fzlA*-overexpressing cells increase FzlA

115 levels to roughly 20-fold higher than WT, as measured by western blotting with an anti-FzlA

116 antibody (Fig. S1, A-B). For each cell, we calculated the rates of constriction and elongation as

117 well as the time from birth to initiation of constriction (pre-constriction time).

118   When FzlA was overproduced, cells constricted significantly faster on average (20.0 ±

119 0.5 nm/min, N = 154) than the EV control (16.8 ± 0.3 nm/min, N = 182) using conventional

120 statistical analysis (Fig. 1 B, Video 1, p < 0.0001). To ensure that the measured differences

121 were not due to sample size effects, we performed a more strenuous analysis based on

122 Superplots, a method of statistical analysis that treats the averages of independent biological

123 replicate experiments (rather than individual cell values) as data points for each condition (Lord

124 et al., 2020). With Superplots analysis, the constriction rate was still significantly faster when

125 *fzlA* was over-expressed (19.8 ± 1.6 nm/min) compared to an EV (16.9 ± 0.6 nm/min), using an

126 α-cutoff of 0.1, but not when using an α-cutoff of 0.05 (Fig. 1 B, p = 0.1). In *Caulobacter*, the

127 elongation rate is generally inversely proportional to the constriction rate. That is, as cells

128 constrict faster, they elongate slower and vice versa (Lambert et al., 2018; Lariviere et al.,

129   2019). Superplots analysis of the elongation rates showed no significant difference between *fzlA*

130   over-expressing (15.2 ± 0.6 nm/min) and EV control cells (16.8 ± 1.7 nm/min) (Fig. 1 C). In

131   contrast, the ratio of constriction rate to elongation rate by Superplots analysis was significantly

132   shifted in favor of constriction in strains overproducing FzlA (1.33 ± 0.10) when compared to EV

133   control cells (1.06 ± 0.08), signifying a shift from elongation to constriction, similar to

134   observations made for hyperactive strains (Fig. 1 D, p = 0.049) (Lariviere et al., 2019). These

135   results demonstrate that *fzlA* overexpression enhances constriction, consistent with the

136   hypothesis that FzlA is a limiting factor in FtsWI activation. We used the amount of time elapsed

137   post-synchrony to measure the time from birth to constriction initiation. There was no significant

138   difference in time to constriction initiation between *fzlA* overexpressing (92 ± 2 min, N = 154)

139   and EV control cells (86 ± 2 min, N = 182), even by conventional statistics (Fig. 1 E, p = 0.071),

140   indicating that excess FzlA does not pre-maturely initiate constriction, but rather causes FtsWI

141   to build PG inwards at an increased rate.

142   **Active Halo-FtsW molecules move more slowly on average**

143   We hypothesized that *fzlA* overexpressing cells constrict faster because FtsWI are

144   synthesizing PG more frequently or at an increased rate. To test these hypotheses, we

145   performed single molecule tracking (SMT) of FtsW. In *E. coli,* the phylogenetically closest

146   relative in which PG synthase dynamics have been measured, inactive PG synthases move with

147   similar speeds (32 nm/s) to the FtsZ-treadmilling speed (28 nm/s) but move more slowly (8

148   nm/s) when activated (Yang et al., 2021). We therefore sought to characterize the relationship

149   between FtsWI activation state and dynamics in *C. crescentus* by performing SMT.

150   We generated strains expressing either *halo-ftsW or -ftsW*** as the sole copy at the

151   native locus, in a WT (EG3052) or *ftsW**I** (EG3053) background, respectively. We labeled and

152   measured single molecules of FtsW or FtsW** by titrating the levels of Janelia Fluor 646 Halo

153   ligand (Grimm et al., 2017). While tracking FtsW, we observed single molecules that had

154   processive movement, with some molecules changing direction and speed, implying that FtsW

155   can dynamically switch between movement modes, similar to what was observed in *E. coli*

156   (Yang et al., 2021). To focus on FtsW molecules during division, we synchronized cells prior to

157   analysis and imaged after approximately 50 minutes of growth to ensure we were imaging pre-

158   divisional and constricting cells. We measured speeds of mobile molecules of FtsW at the

159   division site by dividing the distances covered by the time each molecule took to cover that

160   distance (Fig. 2 A, Fig. S2, A-B, Video 2-4). In some cases, a single molecule exhibited changes

161   in the speed of movement and/or transitioned from moving to stationary or vice versa during

162   measurement (Fig. S2, A-B, Video 3-4). Additionally, a proportion of molecules existed at the

163   division plane without movement, which we define as the stationary population. We measured

164   the number of molecules in a population that were stationary, as well as how long they

165   remained stationary.

166   We observed that hyperactivated FtsW** molecules move more slowly on average (16.6

167   ± 0.7 nm/s, N = 403) than WT FtsW (24.9 ± 1.1 nm/s, N = 318) (Fig. 2 B, Table 1), consistent

168   with observations in *E. coli* that active FtsWI molecules move at a slower rate than inactive, but

169   mobile, molecules in *E. coli* (Yang et al., 2021). To compare the processivity of mobile FtsW

170   molecules between the *ftsW**I** and WT backgrounds, we plotted the speeds of moving

171   molecules against the time those molecules spent on moving. For this analysis, we excluded

172   molecules moving faster than 20 nm/s as they are unlikely to be actively synthesizing PG. We

173   found that slow-moving FtsW** molecules travel for longer periods of time than slow-moving WT

174   FtsW on average (Fig. 2 C). Since slow-moving PG synthases are more likely to be active,

175   these data suggest active FtsW** molecules are more processive than active WT FtsW

176   molecules. Interestingly, we did not observe a change in the percentage of stationary FtsW

177   molecules when FtsW was hyperactivated (roughly 50% in both WT and *ftsW**I**), but instead

178   observed longer stationary lifetimes for FtsW** molecules (16.2 ± 0.8 s, N = 249) when

179 compared to WT (9.4 ± 0.5 s, N = 210) (Fig. 2 D, Table 1). Based on observations in *E. coli*, we

180 hypothesize that stationary molecules are not active for cell wall synthesis but may be poised for

181 activation (Yang et al., 2021). Our data indicate that *ftsW**I** cells constrict faster due to both a

182 greater proportion of active (slow moving) PG synthase complexes and because these

183 complexes polymerize longer stretches of cytokinetic PG than their WT counterparts.

Absence of FzlA decreases the proportion of active Halo-FtsW in a *ftsW**I** background**

185 To understand the role FzlA plays in FtsWI activation, we deleted *fzlA* in the *ftsW**I**

186 background producing Halo-FtsW** and replaced it with a gentamycin resistance cassette,

187 confirmed by western blotting (Fig. S1 C). Notably, in the absence of FzlA (EG3207), moving

188 FtsW** molecules (26.1 ± 1.6 nm/s, N = 198) had average speeds similar to FtsW in a WT

189 background (24.9 ± 1.1 nm/s, N = 318) and significantly faster than FtsW** in the presence of

190 FzlA (16.6 ± 0.7, N = 403) (Table 1, Fig. 2 B). However, the proportion of stationary PG

191 synthases significantly increased when compared to either FtsW** or FtsW in the presence of

192 FzlA (Table S1, 75% in *ΔfzlA* vs ~50% in WT or *ftsW**I**). These data suggest that there are

193 significantly fewer active, slow-moving synthases in a *ΔfzlA* background than WT or *ftsW**I**

194 with FzlA and are consistent with the cell length and constriction rate defects associated with

195 *fzlA* deletion we previously reported (Lariviere et al., 2019). These data further indicate that

196 FtsW**I** can still receive input from FzlA even though *fzlA* is no longer essential.

197 Interestingly, FtsW** in the *ΔfzlA* background appeared to have processive movement

198 similar to FtsW** in the presence of FzlA, suggesting that FzlA does not affect the processivity

199 of PG synthases, but rather the fraction of active PG synthases (Fig. 2 C). Finally, absence of

200 FzlA reduced the amount of time FtsW** molecules remained stationary when compared to

201 stationary time in the presence of FzlA (Table S1, Fig. 2 D, 10.4 ± 0.4 s, N = 366 for *ΔfzlA;*

202 *ftsW**I** vs 16.2 ± 0.8 s, N = 249 for *ftsW**I**), to an average stationary time similar to WT FtsW

203 (Table S1, Fig. 2 D, 9.4 ± 0.5 s, N = 210). Since the absence of FzlA increases the stationary

204   proportion of FtsW** molecules and these molecules are shorter-lived than when FzlA is

205   present, we propose that short-lived stationary FtsW are poised, awaiting activation by a FzlA-

206   dependent pathway.

207   **FzlA moves with active FtsWI**

208   Considering that FzlA is an FtsWI activator, we sought to test if FzlA moves with

209   activated FtsWI or functions through a "kiss-and-run" mechanism, dissociating shortly after

210   activation. To do this, we assessed FzlA dynamics using SMT. We constructed strains

211   expressing *halo-fzlA* at the native *fzlA* locus in WT (EG3619) and *ftsW***I** (EG3617)

212   backgrounds. We observed similar average FzlA speeds in the WT (Table S1, 17.3 ± 1.1 nm/s,

213   N = 104) and *ftsW***I** backgrounds (Table S1, 20.3 ± 2.5 nm/s, N = 71). However, the

214   distribution of Halo-FzlA speeds was broader in a *ftsW***I** background than in WT, with more

215   very slow- and very fast-moving molecules than in WT (Fig. 2 E). The presence of slow-moving

216   FzlA molecules suggests that it can remain associated with activated FtsWI as it synthesizes

217   PG. In the *ftsW***I** background, FzlA was stationary more often than in WT cells (Table S1,

218   72% stationary in *ftsW***I** vs 59% stationary in WT). The activation state of the PG synthases

219   impacted the slow-moving population of FzlA (Fig. 2 F), but not the stationary life-time of FzlA

220   (Fig. 2 G), leading us to conclude that FzlA likely remains associated with active, slow-moving

221   FtsWI complexes after activation.

222   **Absence of FzlA does not impact the proportion of stationary Halo-FtsW in a WT**

223   **background**

224   To examine the effects of loss of FzlA on FtsW dynamics in a WT background, we

225   generated a strain dependent on xylose for *fzlA* expression with natively expressed WT *ftsWI*

226   (EG3523). When the xylose inducer was removed, FzlA dropped to undetectable levels after

227   seven hours and cells began to filament (Fig. S3, Fig. S1 D). We optimized xylose concentration

228   to produce FzlA at WT levels and avoid FtsWI hyperactivation by overexpression of *fzlA* before

229   depletion (Fig. S1 E). To ensure that we measured FtsW molecules that are associated with a

230   Z-ring in these filamentous cells, we replaced *zapA* at the native locus with *zapA-mNeonGreen*

231   (*zapA-mNG*) (Fig. S3). ZapA is an FtsZ-binding protein that reliably reports on Z-ring positioning

232   (Woldemeskel et al., 2017).

233   When comparing FtsW dynamics between induced and depleted conditions, we

234   observed that FtsW moves faster, on average, when FzlA is absent (Table S1, Fig. 3 A, 23.0 ±

235   1.9 nm/s during FzlA depletion, N = 117 vs 19.6 ± 1.1 nm/s with FzlA present, N = 230),

236   consistent with our Δ*fzlA* results and suggesting fewer active PG synthase complexes are

237   present without FzlA. FtsW processivity was not affected by FzlA in this background (Fig. 3 B),

238   consistent with our observations using the *ftsW**I** strain with and without FzlA.

239   We next assessed the stationary FtsW population in our depletion strain with and without

240   FzlA. Surprisingly, depletion of FzlA in a WT background did not change the proportion of

241   moving FtsW molecules (Table S1, ~50% in both deplete and induced), unlike in the Δ*fzlA*

242   background (Table S1, 25%). These results indicate that the increase in stationary FtsW**

243   molecules observed in the Δ*fzlA* background is likely due to binding of the PG synthases to a

244   stationary target rather than an inability to move dynamically about the Z-ring in the absence of

245   FzlA. The amount of time FtsW molecules remain stationary decreased when FzlA was

246   depleted in a WT background (Table S1, Fig. 3 C, 8.8 ± 0.7 s during FzlA depletion, N = 107 vs

247   10.9 ± 0.6 s at WT FzlA levels, N = 244). These tracking data are consistent with FzlA-mediated

248   signaling acting to convert fast-moving, inactive molecules of FtsW into an activated, slow-

249   moving state. Moreover, our data indicate that FtsW molecules may become stationary when

250   they are waiting to receive activating signals (i.e., via FzlA) or when they are poised to

251   synthesize PG but have not yet begun to do so (i.e., for FtsW**I*).

Overabundant FzlA converts Halo-FtsW from a fast-moving and inactive state to a slow and active PG-synthesis state

Having assessed the effects of loss of FzlA on FtsWI dynamics and activity, we next turned to cells overproducing FzlA. To identify changes to PG synthase activation during hyperconstriction caused by FzlA overproduction, we compared Halo-FtsW speeds from strains with the *fzlA* overexpression construct (EG3519) or EV (EG3537) in the presence of 0.3% xylose to induce *fzlA* overexpression for one hour prior to synchrony and during the experiment (Fig. S1 F). With FzlA overproduction, FtsW speeds were reduced compared to EV (Table S1, Fig. 4 A, 23.2 ± 0.9 nm/s with FzlA overproduction, N = 300 vs 32.3 ± 3.6 nm/s for the EV control, N = 472). As expected from earlier results, additional FzlA altered neither the processivity nor the proportion of moving molecules (Table S1, Fig. 4 B). These results reinforce our conclusion that FzlA-mediated signaling converts inactive PG synthases into an active state.

Our prior results demonstrated that FzlA-mediated signaling still impacts FtsW**, which suggests that FzlA-mediated hyperactivation can occur in a *ftsW**I** background as well. In line with this hypothesis, we observed that FzlA overproduction in this background (EG3525) further stimulates FtsW**, as speeds decreased to the slowest observed average speed when FzlA was overproduced compared to EV (EG3538) (Table S1, Fig. 4 C, 13.5 ± 0.8 nm/s for overproduced FzlA, N = 205 vs 16.3 ± 1.2 nm/s for EV, N = 160). Again, there was no change to processivity or the proportion of moving PG synthases when FzlA was overproduced (Table S1, Fig. 4 D). These results further demonstrate that in the *ftsW**I** background, the PG synthases are primed for activation, but FtsW**I* can still receive activation signals from FzlA. However, excess FzlA again had no effect on the proportion of stationary FtsW or FtsW** or their lifetimes (Table S1, Fig. 4 E).

FtsW and FzlA molecules exhibit two moving populations

276   In *E. coli*, the PG synthases move with two dynamic modes: 1) fast, inactive PG

277   synthases driven by FtsZ cluster movement and 2) slow, actively synthesizing PG synthases

278   driven by synthesis (Yang et al., 2021). Due to the small size of *C. crescentus*, we were unable

279   to resolve FtsZ clusters sufficiently to determine their treadmilling speeds. To better understand

280   PG synthase dynamics in *C. crescentus*, we employed a computational approach to simulate

281   the contributions of two populations of moving molecules to the distributions of FtsW and FzlA

282   speeds we observed. In a WT background, FtsW speeds appear to move as a major population

283   with an average speed of 22.2 nm/s and a small portion moving much faster at an average

284   speed of 54.6 nm/s (Fig. 5 Ai, Table S1), although the two-populations were not well-

285   distinguished. However, when observing FtsW** in the hyperactive background, slow- (Fig. 5

286   Aii, Table S1, 10.8 ± 1.1 nm/s) and fast-moving (Fig. 5 Aii, Table S1, 51.4 ± 11.4 nm/s)

287   populations are better resolved, suggesting that there are two-populations of FtsW and that the

288   actively synthesizing population moves slower on average. To understand if FzlA modulates

289   FtsW dynamics by shifting FtsW from one population to the other, we compared the proportions

290   of each FtsW population in *ftsW***I** backgrounds with and without FzlA. In a *ΔfzlA* background,

291   a greater proportion of FtsW moved at faster speeds (Fig. 5 Aiii, Table S1, 84.2% fast-moving)

292   than when FzlA was present (Fig. 5 Aiii, Table S1, 17.1% fast-moving), suggesting less active

293   FtsW when FzlA was not present. This trend was observed when FzlA was not present in a WT

294   background as well, and the inverse was observed when FzlA was overproduced, solidifying the

295   ability of FzlA to convert fast-moving FtsW to a slow-moving and active state (Fig. S4, Table

296   S1).

297   After confirming FzlA can shift FtsW from an inactive, fast-moving state to a slow-moving

298   active state, we sought to understand the effects of FtsW activation state on FzlA dynamics. In

299   the WT background, FzlA appears to move primarily in a slow population (Fig. 5 Bi, Table S1,

300   13.4 ± 1.0 nm/s, 76% slow-moving FzlA). When FtsW is hyperactivated, the slow-moving

301   population was observed moving even slower on average (Fig. 5 Bii, Table S1, 7.2 ± 0.5 nm/s).

302   This is consistent with our hypothesis that moving FzlA travels with active FtsW (Fig. 5 Ai and

303   ii).

Hyperactivated constriction via FzlA overproduction causes lethal division events

305   Previous work demonstrated that hyperactivating FtsWI has consequences on the cell

306   wall and envelope integrity (Modell et al., 2014; Lariviere et al., 2019). These observations led

307   us to ask if there are phenotypic consequences of FtsWI hyperactivation through an over-

308   abundance of FzlA. While analyzing timelapse images of *fzlA* overexpressing cells, we noticed

309   that cells died during or immediately after division at a significant rate, though a majority of cells

310   were still viable. The lethal division events we observed were pleiotropic, with three major

311   distinguishable categories; 1) both daughters halt growth post-division, 2) one daughter halts

312   growth post-division, and 3) one daughter lyses post-division (Fig. 6, A-B, Video 5-7). These

313   lethal division events were rare in the EV control strain, suggesting that the hyperconstriction

314   caused by FzlA over-abundance is deleterious to cells.

315   Interestingly, without FzlA overproduction in a *ftsW**I** background (EG3466), we did not

316   observe lethal divisions, suggesting hyperactive PG synthases are not sufficient to cause a

317   significant increase in lethal division events on this timescale. FzlA overproduction in *ftsW**I**

318   (EG3467) increased lethal division events compared to the EV control, but not to a greater

319   extent than in a WT background. To understand how these division failures affect long-term

320   viability, we performed spot dilutions and growth curves in both WT and *ftsW**I** backgrounds

321   with and without FzlA overproduction. Overexpression of *fzlA* in a WT background caused a 2-

322   log reduction in colony-forming units and a moderate reduction in doubling time in liquid media

323   (Fig. 6, C-D). Strikingly, long-term overexpression of *fzlA* was lethal in a *ftsW**I** background,

324   both on solid and liquid medium (Fig. 6, C-D).

325   FzlA has two essential surfaces: an FtsZ-binding surface and the C-terminal tail

326   (Lariviere et al., 2018). We next sought to test whether either function is required for the

327   observed toxicity. To do this, we assessed the effects of overexpression of a mutant *fzlA* with a

328   disrupted FtsZ-binding site (*fzlA^{NB2}*) or a charge-reversal mutation in the penultimate residue of

329   the C-terminal tail (*fzlA^{D227K}*) in the WT or *ftsW**I** backgrounds. Neither *fzlA^{D227K}* nor *fzlA^{NB2}*

330   overexpression was lethal in WT or *ftsW**I** backgrounds, demonstrating that FzlA must retain

331   both essential activities for toxicity (Fig. 6 C, Fig. S1, A-B).

332   To better understand how FzlA overproduction in a *ftsW**I** background causes toxicity,

333   we imaged cells after 20 hours of overproduction of FzlA. We observed cells that had lysed

334   during division and remained attached (red arrows), similar to observations from the earlier

335   time-lapse imaging, as well as cells that were significantly shorter (green arrows) than *ftsW**I**

336   cells with an EV (Fig. 6, E-F). Interestingly, we also observed cell chaining (blue arrows). The

337   cells that chained frequently had multiple deep constrictions with cell bodies remaining

338   connected, suggesting that division failed to complete at a late stage then initiated again at a

339   second site.

340   **FtsK is a potential binding partner of FzlA**

341   Cell chaining has been observed in cells with mutations to cell wall hydrolase-related

342   proteins, outer-membrane components, and factors that regulate genome integrity and

343   segregation (Meier et al., 2017; Bernhardt and de Boer, 2004; Peters et al., 2011; Heidrich et

344   al., 2001; Wang et al., 2006; Uehara et al., 2010; lo Sciuto et al., 2014). To better understand

345   both the mechanism for the cell division defects that result from FzlA overproduction and how

346   FzlA signals to activate FtsWI, we sought to identify downstream interactors of FzlA. To identify

347   FzlA binding partners, we performed co-immunoprecipitation (coIP) in a strain (EG2217) that

348   carries *3x-FLAG-fzlA* as the sole copy at the native locus and subjected the eluate to mass

349   spectrometry (MS)-based protein identification, using WT cells (not producing Flag-tagged FzlA)

350   as a negative control. MS identified FtsZ, a known interactor for FzlA, in the 3xFLAG-FzlA

351   eluate, validating this approach (Table S2). From the proteins identified and enriched more than

352   5-fold in the 3xFLAG-FzlA eluate, we identified FtsK, a known cell division protein, as a putative

353   binding partner (Table S2).

354   FtsK is an essential, bifunctional cell division protein with an N-terminal domain (FtsK$_N$:

355   residues 1-213) containing four transmembrane passes and a cytoplasmic C-terminal domain

356   (FtsK$_C$: residues 315-825), connected by a disordered linker (residues 213-315) (Wang et al.,

357   2006). FtsK$_N$ is responsible for localizing FtsK to the divisome and plays an essential role in

358   constriction, while FtsK$_C$ is a hexameric DNA translocase that segregates the chromosomal

359   termini into each daughter cell during division. To confirm our MS results, we generated a strain

360   in which *mChy-ftsK* was expressed at the *ftsK* locus as the sole copy, in a WT (EG2427) and

361   *3xFLAG-fzlA* background (EG2428) and performed anti-FLAG coIP followed by immunoblotting.

362   Western blot analysis revealed that 3xFLAG-FzlA interacted with mChy-FtsK *in vivo* (Fig. 7 A).

363   To pursue the interaction further, we performed reciprocal coIP in a strain which harbored

364   *FLAG-ftsK* at the *ftsK* locus as the sole copy (EG742) and compared to WT. Western blot

365   analysis demonstrated that FLAG-FtsK interacts with native, untagged FzlA *in vivo* as well (Fig.

366   7 B).

367   **The FzlA-FtsK interaction occurs between the FzlA C-terminal tail and FtsK$_C$**

368   To narrow down the domains responsible for the FzlA-FtsK interaction, we performed

369   Bacterial Two-Hybrid (BTH) analysis. We first sought to confirm our coIP-MS results for full-

370   length, WT FzlA and FtsK using BTH. FzlA forms a homodimer and exhibits self-interaction by

371   BTH, allowing FzlA self-interaction to serve as an internal positive control (Fig. 7 C). Indeed,

372   FzlA and full-length FtsK (FtsK$_{FL}$) were able to interact by BTH, suggesting a direct interaction in

373   this heterologous system (Fig. 7 C).

374   In narrowing down the interacting surfaces on FzlA, we sought to test residues

375   previously determined to be essential for FzlA function. We hypothesized that if the FzlA-FtsK

376   interaction is essential, the downstream signaling would likely occur through the essential C-

377   terminal tail (Lariviere et al., 2018). To test this hypothesis, we used a point mutant in the

378   penultimate residue of FzlA (FzlA^{D227K}), a variant that is unable to function in division despite

379   retaining its ability to bind to and colocalize with FtsZ (Lariviere et al., 2018, 2019). Consistent

380   with our hypothesis, FzlA^{D227K} was unable to interact with FtsK$_{FL}$ by BTH, but was still able to

381   self-interact, suggesting the protein is properly folded but that the C-terminus is required to bind

382   FtsK. (Fig. 7 C).

383   FtsK$_N$ and FtsK$_C$ have independent, separable, essential functions *in vivo* (Wang et al.,

384   2006). To determine which domain of FtsK interacts with FzlA, we tested each separately for its

385   ability to interact with FzlA by BTH. The FtsK$_N$ construct included the disordered linker up to

386   residue 333, while FtsK$_C$ included the remainder of the protein. FzlA was able to interact with

387   FtsK$_C$, but not with FtsK$_N$ (Fig. 7 C). To determine if the FzlA C-terminal tail is required for the

388   interaction, we tested the ability of FtsK$_C$ and FzlA^{D227K} to interact. FzlA^{D227K} and FtsK$_C$ were

389   able to interact by only one orientation, suggesting reduced affinity when the C-terminal tail is

390   mutated (Fig. 7 C, Fig. S5). (Fig. S4). Collectively our coIP-MS and BTH approaches indicate

391   that FzlA and FtsK directly interact via the C-terminal tail of FzlA and the DNA translocase

392   domain of FtsK.

393   **FtsK functions upstream of FtsWI**

394   Prior work characterizing *C. crescentus* FtsK demonstrated that depleting the C-terminal

395   domain of FtsK causes cell chaining, with multiple cell bodies connected by unresolved

396   constrictions, whereas depleting full-length FtsK causes cell filamentation without constriction

397   (Wang et al., 2006). We hypothesized that if FtsK acts downstream of FzlA in an FtsWI

398   activation pathway, in the *ftsW**I** background where *fzlA* is non-essential FtsWI should also be

399   less dependent on FtsK-mediated activation. While *ftsK* remains essential in a *ftsW**I**

400   background based on comparative transposon sequencing (Lariviere et al., 2019), we reasoned

401   that examining the phenotype associated with depletion of FtsK in a *ftsW**I** background could

402   reveal a genetic relationship between *ftsK* and *ftsWI*. We generated strains with xylose-

403   dependent expression of *ftsK* in which $ftsK_{FL}$ was placed at the *xylX* locus and native *ftsK* was

404   replaced with a spectinomycin resistance cassette at the native locus, in both the WT (EG3332)

405   and *ftsW**I** (EG3333) backgrounds. We also examined the phenotype of the previously

406   characterized $FtsK_C$ depletion strain (LS4202/EG2996) (Wang et al., 2006).

407   We confirmed the previously observed depletion phenotypes for $FtsK_{FL}$ and $FtsK_C$, which

408   were predominantly filamentous and chained cells, respectively (Fig. 8 A, chaining: blue

409   arrows). In liquid media, cells depleted of full-length FtsK in a WT background still experienced

410   an increase in absorbance, likely owing to continued growth of filamentous cells. However, FtsK

411   depletion was lethal on solid media (Fig. 8, B-C). We reasoned that if FtsK plays a role

412   downstream of FzlA in activating FtsWI, we might observe constrictions or even completed

413   division events in cells depleted of FtsK in the *ftsW**I** background. This expectation would be

414   similar to our observations that FzlA depletion in a WT background leads to filamentation,

415   whereas *ftsW**I** cells lacking FzlA still divide (Lariviere et. al, 2019). Indeed, cells depleted of

416    $FtsK_{FL}$ in a *ftsW**I** background were not filamentous. Instead, they were short, with pleiotropic

417   phenotypes (Fig. 8 A) including thin, extended constrictions (magenta arrows); chaining (blue

418   arrows); and/or lysis (orange arrows). The phenotypes of cells depleted of $FtsK_{FL}$ in the *ftsW**I**

419   background were reminiscent of those associated with long-term *fzlA* over-expression in the

420   *ftsW**I** background. Despite the presence of cells of normal length, FtsK depletion in the

421   *ftsW**I** background was still lethal, indicating an essential function of FtsK in addition to its role

422   in regulating the activation state of FtsWI. These results collectively indicate that FtsK lies

423   between FzlA and FtsWI in a constriction activation pathway and that regulatory input from FtsK

424   may couple chromosome segregation to constriction activation.

425   **Proper regulation of FzlA-mediated signaling is critical to prevent DNA damage**

426   Cell chaining is observed in strains that have either cell separation (Meier et al., 2017) or

427   chromosome segregation defects (Wang et al., 2006; Yu et al., 1998). Identification of a FzlA-

428   FtsK interaction led us to hypothesize that the cell chaining observed during both FtsK depletion

429   and *fzlA* overexpression in the *ftsW**I** background is due to defects in coupling chromosome

430   segregation to division. Specifically, we hypothesized that misregulation of FzlA-FtsK-mediated

431   signaling in a background with hyperactive constriction traps DNA at the division site, preventing

432   the completion of division and causing DNA damage. To test this, we leveraged a previously

433   characterized P_{sidA}-*egfp* reporter construct (Modell et al., 2014). The *sidA* promoter is sensitive

434   to DNA damage, leading to production of EGFP when DNA damage occurs. To obtain a

435   baseline of DNA damage in *Caulobacter*, we introduced the reporter construct into a WT

436   background (EG3653). Since DNA damage is rare in WT cells without stress, expression was

437   relatively low. To compare among different strains, we set a threshold for "high GFP" as above

438   the 99th percentile of WT GFP values. Any cell with a value higher than that threshold was

439   considered to have high GFP and, by proxy, DNA damage.

440   To understand how hyperconstriction and the presence of FzlA influence DNA damage,

441   we introduced the DNA damage reporter construct into *ftsW**I** (EG3655) and Δ*fzlA, ftsW**I**

442   (EG3667) backgrounds. Consistent with our hypothesis, the hyperconstricting *ftsW**I**

443   population had a modest increase in DNA damage (Table S3, Fig. 9 A, 4.59%) (Modell et al.,

444   2011, 2014). Interestingly, the Δ*fzlA, ftsW**I** population had WT levels of DNA damage (Table

445   S3, Fig. 9 A, 0.79%), which suggests that hyperconstriction is the underlying cause of DNA

446   damage (since Δ*fzlA* cells constrict more slowly than WT or *ftsW**I**), not insensitivity of

447   FtsW**I* to the DNA damage inhibitors SidA and DidA (Modell et al., 2014, 2011).

448   To determine if hyperconstriction due to an overabundance of FzlA also leads to DNA

449   damage, we introduced the DNA damage reporter into our xylose-dependent *fzlA* over-

450   expressing and EV strains in the WT and *ftsW***I** backgrounds. The EV control in a WT

451   (EG3658) background had WT levels of DNA damage when supplemented with 0.3% xylose for

452   eight hours (Table S3, Fig. 9 B, 2.1%). The FzlA-overproducing WT strain (EG3664)

453   experienced a modest increase in the amount of EGFP expression, starting at 3.3% at four

454   hours of over-production and increasing to near 4.6% at six and eight hours (Table S3, Fig. 9

455   B). The maximum levels of DNA damage we observed for FzlA overproduction were similar to

456   the levels observed in the *ftsW***I** background.

457   We next assessed DNA damage in cells overproducing FzlA (EG3672) compared to the

458   EV control (EG3666) in the *ftsW***I** background. Considering that this is the background in

459   which FzlA overproduction led to cell chaining, we hypothesized the DNA damage may be much

460   greater than in the WT background. In line with this hypothesis, four hours of FzlA over-

461   production in the *ftsW***I** background significantly increased DNA damage when compared to

462   the EV control (Table S3, Fig 9 B, 9 vs 4.6%). The amount of DNA damage increased with

463   longer induction times (Table S3, Fig 9 B, 11.94% at 6 hr and 14.56% at 8 hr), consistent with

464   our observations of cell chaining at six hours post-induction. We conclude that hyperactivation

465   of constriction by FzlA overproduction leads to DNA damage.

466   We next tested whether misregulating cell division through modulation of FtsK, itself,

467   causes DNA damage. To obtain a baseline of DNA damage for this set of experiments, we

468   introduced the DNA damage reporter construct into the xylose-dependent FtsK$_{FL}$ depletion

469   strain (EG3332) described above (Fig. 8). We set a threshold for "high GFP" as above the 99th

470   percentile of GFP values observed in this strain grown in the presence of xylose to induce *ftsK$_{FL}$*

471   expression. We then introduced the DNA damage reporter construct into the FtsK$_{FL}$ (EG3904)

472   and FtsK$_C$ (EG3900) depletion strains in a WT background. We hypothesized that depleting

473   FtsK$_{FL}$ in a WT background would not result in DNA damage since these cells fail to initiate

474   constriction and become filamentous. Supporting our hypothesis, we observed little to no

475   increase in DNA damage at 4 (Table S3, Fig. 9C, 0.647%), 6 (Table S3, Fig. 9C, 0.360%) and 8

476   hours of depletion (Table S3, Fig. 9C, 2.10%) when compared to the replete condition (Table

477   S3, Fig. 9C, 1.08%). We next examined the effect of depleting FtsK$_C$ as this results in cell

478   chaining. Indeed, there were higher levels of DNA damage compared to our replete control

479   strain (Table S3, Fig. 9C, 1.08%) at 6 (Table S3, Fig. 9C, 3.88%) and 8 hours (Table S3, Fig.

480   9C, 7.80%) of depletion, with DNA damage increasing over time as FtsK$_C$ was depleted.

481   Finally, we tested the effect of depleting FtsK$_{FL}$ in an *ftsW**I** background since these

482   cells experience chaining and extended constrictions. We introduced the DNA damage reporter

483   construct into a *ftsW**I** strain with the only copy of *ftsK$_{FL}$* under the control of the xylose

484   promoter (EG3902). In contrast to FtsK depletion in a WT background, when we depleted FtsK

485   in this background we observed an increase in DNA damage compared to our control strain

486   (Table S3, Fig. 9C, 1.08%) at 4 (Table S3, Fig. 9C, 4.00%) 6 (Table S3, Fig. 9C, 6.62%), and 8

487   hours of depletion (Table S3, Fig. 9C, 12.2%). Collectively, these data indicate that

488   misregulating the FzlA-FtsK-FtsWI pathway results in DNA damage due to improper coupling of

489   chromosome segregation to constriction, and reinforces our hypothesis that FtsK plays a central

490   role in this constriction activation process.

491

Discussion

The specifics of bacterial constriction activation can vary from species to species, but the functional requirements to select the division plane, dynamically position the division components, segregate the chromosomes, and activate constriction in a controlled manner are essential and conserved. Our work here describes a key role for FzlA in signaling to activate FtsWI in coordination with chromosome segregation. FzlA activates constriction during division by converting fast-moving FtsW molecules into an active, slow-moving state (Figs. 1-5). The FzlA activation pathway signals through FtsK, an essential protein that segregates sister chromosomes during division (Figs. 7-8). When this signaling is misregulated through hyperactivating mutations or over-production of FzlA, the cell suffers from cell envelope defects (Lariviere et al., 2019; Modell et al., 2014) (Fig. 6) and DNA damage (Fig. 9), likely due to defects in coordinating chromosome segregation with division. We propose that, prior to activation, inactive FtsWI is either stationary or is fast-moving through indirect association with treadmilling FtsZ clusters (Fig. 10 A). FzlA binds FtsZ and may infrequently move by dynamic association with FtsZ filament ends or, more frequently, remain stationary (but treadmilling) by association with the interior of FtsZ filaments. Upon binding the C-domain of FtsK through its C-terminal tail, FzlA signals through FtsK to license FtsWI activation, likely acting through additional factors. Ultimately FtsWI is converted from a fast-moving, inactivate state to an active state that moves slowly, guided by PG synthesis (Fig. 10 B). FzlA can move together with the activated FtsWI complex.

Our findings provide a deeper understanding of the mechanisms of FtsWI hyperactivation and its consequences. We observed that active FtsW** synthesized PG for longer periods than FtsW. Conversely, FzlA did not impact the processivity of active FtsW or FtsW**, suggesting increased processivity results from a mechanism distinct from that of FzlA-mediated activation. The mutations of FtsW** may perturb association with a regulator that

517 modulates processivity. An obvious candidate is the FtsQLB complex, based on the

518 observations made in *E. coli* and *P. aeruginosa* (Tsang and Bernhardt, 2015; Marmont and

519 Bernhardt, 2020; Park et al., 2020), though further experiments are required to test this

520 hypothesis. Alternatively, mutation of FtsWI may directly impact processivity of the enzyme.

521 Our study also illuminates characteristics of stationary PG synthases. In the presence of

522 FzlA, stationary FtsW** remain stationary for longer times than FtsW. Because FzlA is present

523 and FtsW** has a greater propensity for the active state, we do not believe that these long-lived

524 molecules are poised for activation. One hypothesis is that FtsW** has increased affinity for the

525 cell wall, which is not moving. It is also possible that FtsW** has increased affinity for a divisome

526 component that remains stationary. In *B. subtilis,* the FtsZ-binding proteins have been observed

527 to be largely stationary (Squyres et al., 2021), so a candidate for this role is one of the FtsZ

528 membrane anchors. The proportion of stationary FtsW or FtsW** was also largely unaffected by

529 depletion or overproduction of FzlA, except in the case of FtsW** in the absence of FzlA. Since

530 FtsW** is primed for activation, we hypothesize that being poised for activation, but awaiting

531 FzlA for a prolonged period results in inactive, moving FtsW shifting to a stationary state.

532 Moreover, in the absence of FzlA, both FtsW (FzlA depletion) and FtsW** (*ΔfzlA*) have shorter

533 stationary times than when FzlA is present, suggesting that short-lived stationary molecules

534 may be those that are poised to be activated. Collectively, our results suggest one stationary

535 population of FtsW is short-lived and awaiting activation by FzlA and another population has

536 longer-lived associations with a stationary fixture at the division plane.

537 We discovered that FzlA converts inactive, fast-moving FtsW to an active, slow-moving

538 state and that upstream signaling from FzlA is limiting for FtsW activation. Our observations for

539 FzlA are, in some ways, similar to observations made for FtsN in hyperactive *ftsB* mutant

540 (*ftsB^{E56A}*) and WT backgrounds in *E. coli* (Yang et al., 2021). Expression of *ftsB^{E56A}*

541 hyperactivates the PG synthases, causes cell shortening, and renders *ftsN* non-essential. The

542   double mutant of $\Delta ftsN$, $ftsB^{E56A}$, results in cell lengths more similar to WT *E. coli* and the PG

543   synthases are active less often than in the $ftsB^{E56A}$ background with FtsN. FtsN depletion in an

544   otherwise WT background also results in cell filamentation and the PG synthases are more

545   often inactive, consistent with FtsN being an FtsW activator. These results mirror many of our

546   FzlA results and may be a common feature of factors that serve to activate FtsWI. However,

547   there are notable differences between FzlA and FtsN and we propose that they act at distinct

548   stages of divisome activation in α-proteobacteria to couple division to other cellular processes.

549   FtsN is transmembrane protein with a PG-sensing SPOR domain that is proposed to couple PG

550   synthesis to amidase activity at the division site (Yahashiri et al., 2015; Gerding et al., 2009).

551   Based on genetics in *E. coli*, FtsN is thought to bind directly to the FtsQLB complex which

552   directly interacts with and activates FtsWI (Park et al., 2021; Gerding et al., 2009; Marmont and

553   Bernhardt, 2020). Conversely, FzlA is a soluble FtsZ-binding protein that also binds the

554   chromosome-segregation factor FtsK. Considering that *C. crescentus* has essential homologs of

555   FtsN and FtsQLB, we propose that FzlA and FtsK provide an additional layer of regulation to

556   FtsN-FtsQLB-mediated activation that serves to integrate information from the Z-ring and

557   chromosome with constriction.

558   In *C. crescentus,* the essential divisome components include FtsZ, FtsA, FzlA, FtsK,

559   FtsN, FtsQLB, and FtsWI (Goley et al., 2011). FzlA is restricted to the α-proteobacteria and

560   FtsN is restricted to proteobacteria, but the remainder of these proteins are widely conserved

561   across bacterial phyla. Our work characterizing the FzlA-FtsK interaction identifies, for the first

562   time, where FtsK integrates in the constriction activation pathway in α-proteobacteria. The FzlA-

563   FtsK interaction is likely conserved throughout α-proteobacteria, as residues 223, 227, and 228

564   of the FzlA C-terminal tail are invariant in FzlA homologs (Lariviere et al., 2018, 2019). FzlA

565   homologs are even found in the Rickettsiales, which have streamlined genomes and are often

566   outliers among the α-proteobacteria in conservation of factors that are essential in *C.*

567 *crescentus*. Outside of α-proteobacteria, FtsK and the family of FtsK/SpoIIIE/HerA homologs are

568 widespread and often essential, even extending to archaea (Bigot et al., 2007). In *E. coli,* only

569 FtsK$_N$ is essential, which suggests that its role in the constriction activation pathway is

570 conserved, but that redundant chromosome maintenance mechanisms support growth in the

571 absence of FtsK$_C$ (Draper et al., 1998). Notably, *E. coli* encodes a nucleoid occlusion system as

572 an additional chromosome maintenance mechanism. SlmA, the protein responsible for nucleoid

573 occlusion in *E. coli*, binds to the nucleoid and prevents proper Z-ring assembly wherever it is

574 present, which prevents the cell from constricting onto the nucleoid (Bernhardt and de Boer,

575 2005). *C. crescentus* and most other α-proteobacteria lack nucleoid occlusion systems. We

576 therefore propose that FzlA functions in this group to robustly couple constriction to

577 chromosome segregation through FtsK.

578   Our findings highlight distinct mechanisms of chromosome maintenance that ultimately

579 regulate constriction in different bacteria. Our work suggests that, in α -proteobacteria, the FtsZ-

580 FzlA-FtsK pathway coordinates constriction with chromosome dimer resolution and segregation

581 of termini, while other bacteria rely more heavily on negative regulators of FtsZ at the earliest

582 stages of Z-ring assembly. Once chromosomes are segregated, the FzlA-FtsK interaction

583 promotes a shift of FtsWI to an active state. FzlA, and presumably FtsK (likely when it is not

584 associated with the chromosome), travel with FtsW for a time, but there are likely additional

585 mechanisms influencing FtsWI processivity. Future experiments will identify how other

586 components contribute to regulation of constriction activation and will further clarify the

587 mechanistic underpinnings of the FtsZ-FzlA-FtsK activation pathway to FtsWI.

588 **Methods**

589 ***C. crescentus* and *E. coli* growth media and conditions:**

590 　*C. crescentus* NA1000 cells were grown at 30°C in peptone-yeast extract (PYE) media.

591 *E. coli* NEB Turbo (NEB Catalog #C2986K) and BTH101 cells were grown at 37°C in Luria-

592 Bertani (LB) medium. Xylose was used at final concentrations of either 0.001% or 0.3% (w/v) as

593 indicated. Glucose was used at final concentrations of either 0.001% or 0.2% (w/v). For

594 depletion strains, cells were grown in PYE supplemented with xylose as indicated prior to being

595 washed with plain PYE medium three times and resuspended in the PYE medium

596 supplemented with the stated concentrations of glucose or xylose. Solid media included 1.5%

597 (w/v) of agar. Antibiotics were used in liquid (solid) medium at the following concentrations for *C.*

598 *crescentus* growth: kanamycin, 5 (25) µg/mL; gentamycin, 1 (5) µg/mL; spectinomycin, 25 (100)

599 µg/mL; and oxytetracycline, 1 (2) µg/mL. Streptomycin was used at 5 µg/mL in solid media. *E.*

600 *coli* antibiotics were used in liquid (solid) medium as follows: kanamycin, 30 (50) µg/mL;

601 gentamycin 15 (20) µg/mL; spectinomycin, 50 (50) µg/mL; ampicillin, 50 (100) µg/mL; and

602 oxytetracycline, 12 (12) µg/mL. For growth curves, a Tecan Infinite M200 Pro plate reader

603 measured absorbance every 30 minutes at OD_{600} of a 100 µL culture volume, at a starting OD_{600}

604 of 0.05, in a 96-well plate in biological and technical triplicate with intermittent shaking. In the

605 growth curve of Figure 2.3 C, cultures were pre-induced for 6 hours before spotting. For spot

606 dilution assays, mid-log cells were diluted to an OD_{600} and serially diluted up to 10^{-5} before

607 spotting 5 µL onto a PYE plate with indicated inducer and/or antibiotic. Plates were incubated at

608 30°C for 48 hours. For time-lapse microscopy or single-molecule tracking experiments,

609 induction with 0.3% xylose occurred for one hour before synchrony and throughout the length of

610 the experiment.

611

612 **Synchrony**

613   Synchrony was performed as previously described (Goley et al., 2011). Cells were

614   grown overnight in 15 mL of PYE medium to an OD_{600} between 0.1 and 0.5, harvested by

615   pelleting at 6,000 rpm at 4°C, resuspended in 750 µL of ice-cold M2 salts, and combined with

616   750 µL of Percoll (Fisher Scientific Catalog #45-001-748). The cell suspension was centrifuged

617   at 20,000 x g for 20 minutes at 4°C. The swarmer (lower) band was isolated, transferred to a

618   fresh tube, and pelleted at 8,000 x g for 2 minutes. The swarmer pellet was washed twice with 1

619   mL of ice-cold M2 salts, and pelleted each time at 8,000 x g for 2 minutes. The swarmer cells

620   were resuspended in PYE with appropriate additives and grown at 25°C. Samples were

621   analyzed by phase-contrast or fluorescence microscopy.

622

623   **Phase-contrast and standard epifluorescence microscopy**

624   Cells in exponential phase of growth were spotted on pads made of 1% agarose

625   resuspended in water or PYE medium, supplemented with 0.3% xylose when appropriate, and

626   imaged using a Nikon Eclipse Ti inverted microscope equipped with a Nikon Plan Fluor 100X

627   (NA1.30) oil Ph3 objective and Photometrics CoolSNAP HQ^2 cooled CCD camera. Timelapse

628   imaging was performed as previously described (Lariviere et al., 2019), using Gene Frames

629   (Thermo Fisher Scientific Catalog #AB0577) to ensure a tight seal during imaging. After

630   synchrony, cells were resuspended in PYE with the appropriate additives and a stopwatch was

631   started to begin measuring pre-constriction time. The cell suspension was spotted on agarose

632   pads. After the pad was allowed to dry sufficiently, the top coverslip was adhered to the gene

633   frame and timelapse imaging was initiated. The timelapse imaging proceeded by taking an

634   image every five minutes for 4 hours. Images were processed using FIJI with the MicrobeJ

635   plugin or using Adobe Photoshop.

636

637   **Timelapse microscopy analysis for growth metrics comparison**

638   To determine dimensions of log-phase cells, cell length and width were measured using

639   FIJI (Schindelin et al., 2012) and MicrobeJ software, similar to as previously described (Lariviere

640   et al., 2018). Constriction rate and elongation rate were also determined using MicrobeJ (Ducret

641   et al., 2016). MicrobeJ software allowed for tracking of cells imaged by time-lapse microscopy

642   throughout the division process, with automatic detection of constriction initiation and manual

643   determination of cell separation. Cell length was found for cells at each time point, cell width

644   was found at the site of constriction, and constriction time was calculated by multiplying the

645   number of frames in which constriction was detected by 5 (since images were acquired every 5

646   minutes), allowing for calculation of constriction and elongation rates. Lethal division events

647   were quantified manually by counting dividing cells that had 1) one daughter halt growth, 2) both

648   daughters halt growth, and 3) one daughter lyse post-division. Prism was used for graphing and

649   statistical analysis of calculated terms. Statistical analysis for Superplots involved one-tailed

650   Mann-Whitney tests, based on the hypothesis that FzlA overproduction would enhance

651   constriction. All cells that were in focus were included in these analyses.

652

653   **Immunoblot analysis**

654   Immunoblot analysis was performed using standard procedures, with a 1:6,666 dilution

655   of affinity-purified α-FzlA antibodies (Goley et al. 2010), a 1:2,500 dilution of α-MreB antisera

656   (Beaufay et al 2015), a 1:2500 dilution of α-RFP antisera (Chen et al. 2005), a 1:1000 dilution of

657   α-FLAG M2 monoclonal antibodies and/or 1:10,000 dilution of HRP-labeled α-rabbit secondary

658   antibodies (BioRAD Catalog #170-6515) on nitrocellulose membranes. Clarity Western

659   Electrochemiluminescent substrate (BioRAD Catalog #170-5060) was added to facilitate protein

660   visualization via an Amersham Imager 600 RGB gel and membrane imager (GE).

661

662   **Advanced epifluorescence imaging and single-molecule tracking of Halo-FtsW and Halo-**

663   **FzlA**

664   Except for the FzlA depletion experiment, all single-molecule tracking (SMT)

665   experiments were performed after synchrony. After synchrony, cells were labeled with Janelia

666   Fluor 646 (a gift from Dr. Luke Lavis, Howard Hughes Medical Institute) at an empirically

667   determined concentration for each sample to obtain single-molecule level labeling, ranging from

668   0.2 nM to 10 nM in PYE with additives as required (xylose or glucose). The cell suspension was

669   incubated in the dark with shaking at 25°C for 15 minutes before washing away the dye with

670   three washes of 1 mL PYE. Cells were resuspended in fresh PYE and allowed to continue

671   growing until the time of an experiment. Cells were imaged no later than 90 minutes after

672   synchrony, with the imaging beginning one-hour post-synchrony.

673   SMT experiments were performed as previously described (Yang et al., 2021). Briefly,

674   single JF646-labeled Halo-FtsW or Halo-FzlA molecules were detected on an Olympus IX-81

675   inverted microscope equipped with an UPlanApo 100x TIRF (1.50NA/oil) objective and an

676   Andor iXon 897 Ultra EM-CCD camera in epifluorescence-illumination mode using a Coherent

677   647-nm laser at ~2 W cm^{-2} power density. Brightfield and fluorescent images were acquired

678   using Metamorph 7.8.13.0 software. The Camera's emGain was turned to 300 with a pre-

679   amplifier gain setting on 3 and digitizer set to 16-bit. Baseline clamp was activated, with a

680   baseline offset set to 100. The focal plane was placed at the middle of the cell to capture the

681   most moving molecules at the division plane. SMT images were taken continuously with 500-ms

682   exposure time for 200 s.

683   Fluorescent spots of single FtsW or FzlA molecules were first localized using two-

684   dimensional Gaussian fitting in an ImageJ (1.52p) (Schneider et al., 2012) plug-in,

685   ThunderSTORM (dev-2016-09-10-b1) (Ovesný et al., 2014). A bandpass filter (80–250 nm) for

686   sigma was applied to remove the single-pixel noise (small sigma) and out-of-focus (big sigma)

687   molecules. To focus on the FtsW or FzlA involved during cell division, especially cytokinetic PG

688   synthesis, we only analyzed the molecules located at mid-cell or the places with observable

689   invagination. In the case of the FzlA depletion strain (EG3523) without obvious constrictions,

690   ZapA-mNeonGreen-marked Z-rings were used to identify divisome-associated FtsW

691   trajectories. Those localizations were then linked to trajectories using a home-built Matlab

692   2020a script (https://github.com/XiaoLabJHU/SMT_Unwrapping) adopting the nearest neighbor

693   algorithm as previously described (Sbalzarini & Koumoutsakos, 2005)

694   https://www.nature.com/articles/s41564-020-00853-0 - ref-CR49. For the movement of each

695   molecule, the rate for each segment was measured and recorded, but for calculating the

696   proportion of moving molecules, the molecule was counted once.

697   The unwrapped and corrected trajectories described above were then segmented to

698   determine whether Halo-FtsW or Halo-FzlA molecules in a segment are stationary or moving

699   directly as previously described (Yang et al., 2021). The cumulative probability density function

700   of the directional moving speeds was further calculated and fit to a two-population model (of log-

701   normal distribution) as described previously (Yang et al., 2021). In this work, we did not fix the

702   fast-moving speed since the FtsZ treadmilling speed is undetermined in *Caulobacter*.

703

704   **Co-immunoprecipitation**

705   Co-immunoprecipitation was performed as previously described (Bowman et al., 2008;

706   Lariviere et al., 2018). All coIP steps, unless designated, were performed at 4°C. CoIP buffer

707   consists of 20 mM HEPES pH 7.5, 100 mM NaCl, and 20% glycerol and one Pierce Protease

708   Inhibitor Mini Tablet, EDTA Free, (Fisher Scientific, A32955) per liter of coIP buffer. Cells were

709   harvested during exponential growth by centrifugation at 6,000 x g at 4°C. Cells were washed

710   with coIP buffer, pelleted at 6,000 x g, and resuspended in coIP buffer. Dithiobis(succinimidyl

711   propionate) (DSP) was used to crosslink free amines. DSP was resuspended at 10 mg/mL in

712   DMSO and added to the cell suspension to reach a final concentration of 2 mM of DSP in 0.08%

713   DMSO for 1 hr at 4°C and 10 min at room temperature. The reaction was quenched by adding 1

714   M Tris-HCl, pH 7.5 to a final concentration of 20 mM for 10 minutes. After quenching, cells were

715   lysed by three passes an EmulsiFlex-C3 at 16,000 psi. Cell membranes were solubilized by

716   adding sodium deoxycholate to 0.5% w/v, Igepal CA-630 to 1% w/v, and EDTA to a 2 mM final

717   concentration. Insoluble cellular debris was removed by pelleting at 4,500 x g for 10 min. For

718   each sample, up to 40 mL of supernatant of each sample was transferred to a fresh tube and

719   normalized based on OD to the lowest density sample. To each tube, 100 µL of washed α-

720   FLAG resin (Sigma-Aldrich, A2220-5ML) was added before incubating on a rotator overnight.

721   Afterwards, the resin was pelleted at 6,000 x g and washed to remove non-specific bound

722   proteins. FLAG-fusion proteins were eluted by incubating the resin with 100 µL of free FLAG

723   peptide at a concentration of 0.5 mg/mL for 15 min, three times. The pooled elution was either

724   provided to the Mass Spectrometry Core Facility at Johns Hopkins University or prepared for

725   western blot analysis.

726

727   **Bacterial Two-Hybrid**

728   Plasmids containing fusions to T18 and T25 were co-transformed into BTH101 competent cells

729   and plated onto LB agar supplemented with ampicillin (100 µg/µL) and kanamycin (50 µg/µL)

730   and grown overnight at 30°C. Biological triplicates of each co-transformation were grown

731   overnight at 30°C in LB including with ampicillin (100 µg/µL), kanamycin (50 µg/µL), and IPTG

732   (0.5 mM). After overnight incubation, 2 µL of each was spotted on LB agar plates supplemented

733   with ampicillin (100 µg/µL), kanamycin (50 µg/µL), X-gal (40 µg/mL), and IPTG (0.5 mM) and

734   incubated for 2 days at 30°C. Blue colonies were considered positive protein-protein

735   interactions.

736

737   **DNA damage quantification using the P_{sidA}-*egfp* reporter construct**

738   To quantify DNA damage using the P_{sidA}-*egfp* reporter, average fluorescence intensity for each

739   cell in three biological replicate experiments per strain was measured using FIJI with the

740   MicrobeJ plugin. We calculated "High GFP" intensity, which we considered above the 99th

741   percentile of the specified control strain GFP values, by first sorting the average fluorescent

742   intensities for each replicate from lowest to highest. For the *fzlA* over-expression or deletion and

743   *ftsW**I** strains, we compared fluorescent intensity values to the 99th percentile of WT GFP

744   values. For the FtsK depletion strains we compared fluorescent intensity values to the 99th

745   percentile of the replete condition of the FtsK$_{FL}$ depletion strain in a WT background grown for 4

746   hours in the presence of 0.3% xylose. We then multiplied the total number of cells analyzed for

747   each control replicate by 0.99. The average intensity of that resulting cell number was then

748   recorded. After obtaining the fluorescence intensity of the 99th percentile of each control

749   replicate, we averaged the fluorescence intensity of the three replicates. For all of the strains

750   analyzed we looked for fluorescence intensity values above the calculated 99th percentile of the

751   control strain average. The number of cells that had higher values were counted and then

752   divided by the total number of cells analyzed for each replicate of each strain. The average

753   percentage of cells above the 99th percentile of the control strain fluorescent intensity was then

754   calculated by averaging the percentages of each replicate for each strain. These average

755   percentages were then used for graphing and subsequent analysis.

756

757

767

768 **Supplemental Materials Summary**

769 Fig. S1 contains western blots that confirm the phenotypes and protein levels of various strains

770 used throughout this article. Fig. S2 demonstrates example trajectories and speed

771 measurements generated by SMT of Halo-FtsW. Fig. S3 illustrates that ZapA-mNeongreen is

772 produced and localizes to the division plane, validating its use for locating Z-rings in FzlA

773 depleted cells. Fig S4 is a reanalysis of the single molecule tracking from Fig. 3-4, similar to the

774 re-analysis displayed in Fig. 5, to demonstrate that modulating FzlA levels can modulate the

775 state of the two-populations of FtsW dynamics. Fig. S5 summarizes the tested combinations of

776 FzlA and FtsK variants in each orientation for interaction by BTH. Table S1 summarizes the

777 SMT results of Halo-FtsW and Halo-FzlA in the various backgrounds and conditions referred to

778 throughout the text. Table S2 summarizes the results from mass spectrometry-based protein

779 identification of eluent from the 3xFLAG-FzlA and wild-type co-immunoprecipitation samples.

780 Table S3 summarizes the DNA-damage results from the various strains and conditions referred

781 to in Fig. 9. Video 1 is an example timelapse of a *Caulobacter* cell constricting and dividing.

782 Videos 2-4 are examples of single molecules of FtsW** (Video 2) and FtsW (Videos 3-4) that

783 illustrate example movements by single-molecule tracking. Videos 5-7 are examples of the three

784 constriction defects (Video 5, both daughters halt growth post-division. Video 6, one daughter

785 halts growth post-division. Video 7, one daughter lyses post-division) observed during FzlA

786 overproduction.

787

Figure legends.

Figure 1. Overproduction of FzlA hyperactivates constriction. A. Representative phase contrast timelapse microscopy of a wild-type constricting *C. crescentus* cell. The timepoints of constriction initiation and completion are denoted. Scale bars are all 2 µm. The orange lines are drawn for ease of visualizing the approximate measures of length or width for each cell. T_i: Time of initiation. T_f: Final timepoint of constriction. **B-E.** Superplots comparing the **B.** constriction rate **C.** elongation rate **D.** ratio of constriction to elongation rate or **E.** time to constriction initiation for strains harboring either an empty vector (EV; EG1644) control or *fzlA* overexpression (OE; EG3637) construct. The strains were induced with 0.3% xylose for 1 h prior to and throughout the timelapse experiment. Each circle is the value of a single cell, while the large squares are the average of a biological replicate. The bar is the average of the three means and statistics were performed using the means of the three replicates. EG1644 – replicate 1, 67 cells; replicate 2, 63 cells; replicate 3, 54 cells. EG3637 – replicate 1, 60 cells; replicate 2, 40 cells; replicate 3, 55 cells. * represents a p-value < 0.05.

Figure 2. FtsW and FzlA influence each other's movement. **A. i.** Brightfield image of a *Halo-ftsW**I** cell. **ii.** Representative maximum fluorescence intensity projection (Fluor) for Halo-FtsW**. **iii.** Kymograph (Kymo) of the fluorescence signal of a line scan across the division plane for a Janelia Fluor 646-labeled single Halo-FtsW**. **iv.** Plot of the molecule position within the line scan over the course of timelapse imaging and speeds of movement for each segment. X indicates the short axis of the cell. **B.** Histogram comparing single molecule speeds of FtsW or FtsW** in *halo-ftsW* (WT; EG3052, N = 318), *halo-ftsW***; *ftsI** (*ftsW**I**; EG3053, N = 403), or *ΔfzlA*; *halo-ftsW***; *ftsI** (*ΔfzlA, ftsW**I**; EG3207, N = 198) backgrounds. **C.** Plot comparing the lifetime of single FtsW or FtsW** molecules move at slow speeds (< 20 nm/s) in *halo-ftsW* (WT; EG3052, N = 318), *halo-ftsW***; *ftsI** (*ftsW**I**; EG3053, N = 403), or *ΔfzlA*; *halo-ftsW***, *ftsI** (*ΔfzlA, ftsW**I**; EG3207, N = 198) backgrounds. Error bars are the standard error of the

813 mean for the speeds in the move-times of those molecules. **D.** Plot comparing the lifetimes of

814 stationary single FtsW or FtsW** molecules in a *halo-ftsW* (WT; EG3052, N = 210), *halo-ftsW***;

815 *ftsI** (ftsW***I**; EG3053, N = 249), or *ΔfzlA; halo-ftsW***; ftsI** (ΔfzlA, ftsW***I**; EG3207, N = 366)

816 backgrounds. **E.** Histogram comparing speeds of FzlA molecules in *halo-fzlA*; WT (EG3619, N =

817 104) or *halo-fzlA; ftsW***I** (EG3617, N = 71) backgrounds. **F.** Plot comparing the length of time

818 single FzlA molecules move at slow speeds (< 20 nm/s) in *halo-fzlA; WT* (EG3619, N = 318) or

819 *halo-fzlA; ftsW***I** (EG3617, N = 403) backgrounds. Error bars are the standard error of the

820 mean for the speeds in the move-times of those molecules. **G.** Plot comparing the lifetimes of

821 stationary FzlA molecules in a *halo-fzlA; WT* (EG3619, 153 lifetimes) or *halo-fzlA; ftsW***I**

822 (EG3617, 174 lifetimes) background. **** represents a p-value < 0.0001.

823 **Figure 3. Depletion of FzlA increases average FtsW speeds and decreases stationary**

824 **FtsW lifetimes in a WT background. A.** Histogram comparing single molecule speeds of FtsW

825 in cells (EG3523) producing FzlA (+FzlA, 0.001% xylose, N = 230) or depleted of FzlA for seven

826 hours (-FzlA (7 h), 0.001% glucose, N = 117). **B.** Plot comparing the lifetime of single FtsW

827 molecules moving at slow speeds (< 20 nm/s) in cells producing FzlA (+FzlA, 0.001% xylose, N

828 = 230) or depleted of FzlA for seven hours (-FzlA (7 h), 0.001% glucose, N = 117). **C.** Plot

829 comparing the lifetimes of stationary single FtsW molecules in cells producing FzlA (+FzlA,

830 0.001% xylose, N = 244) or depleted of FzlA for seven hours (-FzlA (7 h), 0.001% glucose, N =

831 107). * represents a p-value < 0.05.

832 **Figure 4. FtsW and FtsW** molecules move more slowly when FzlA is overabundant. A**

833 **and C.** Histogram comparing speeds of FtsW or FtsW** in cells with an empty vector (EV)

834 control or with a *fzlA* overexpression (OE) construct in either **A.** a *halo-ftsW* (EV; EG3537, N =

835 472. *fzlA* OE; EG3519, N = 300) or **C.** *halo-ftsW***; ftsI** (EV; EG3538, N = 160. *fzlA* OE;

836 EG3525, N = 205) background. **B and D.** Plot comparing the lifetime of single FtsW and FtsW**

837 molecules moving at slow speeds (< 20 nm/s) with and without FzlA overproduction in a **B.**

838   *halo-ftsW* or **D.** *halo-ftsW***; *ftsI** background. **E.** Plot of the lifetimes of stationary single FtsW or

839   FtsW** molecules during *fzlA* overexpression (OE) compared to an empty vector (EV) control in

840   a *halo-ftsW* or *halo-ftsW***; *ftsI** background (WT; *fzlA* OE, EG3519, N = 291. *ftsW**I**; *fzlA* OE,

841   EG3525, N = 159. WT; EV, EG3537, N = 381. EG3538, N = 123). 0.3% xylose was used to

842   overexpress *fzlA*. WT: wild-type apart from *halo-ftsW*. **** represents a p-value < 0.0001. ***

843   represents a p-value < 0.001.

844   **Figure 5. Active FtsW moves at a slower rate than inactive FtsW, while FzlA moves with**

845   **active FtsW. A-D.** Histograms of SMT data for Halo fusion proteins with calculated two-

846   population curves that best fit the data for **A.** FtsW or FtsW** in a **i.** *halo-ftsW* (WT; EG3052), **i.**

847   *halo-ftsW***; *ftsI** (*ftsW**I**; EG3053), or **iii.** *ΔfzlA*; *halo-ftsW***; *ftsI** (*ΔfzlA, ftsW**I**; EG3207)

848   background (a re-analysis of the data presented in Fig. 2 B). **B.** FzlA in **i.** a WT (EG3619) and **ii.**

849   *ftsW**I** (EG3617) background (a re-analysis of the data presented in Fig. 2 E). Red = slow

850   population. Blue = fast population. Black: total based on two-population.

851   **Figure 6. Overproduction of FzlA is toxic. A.** Representative phase contrast timelapse

852   images of constricting cells that result in lethal division events due to FzlA overproduction. **B.**

853   Quantification of lethal divisions in the wild-type (WT) and *ftsW**I** background comparing

854   empty vector (EV) controls (WT: EG1644, n = 182. *ftsW**I**: EG3466, n = 145) and to *fzlA* over-

855   expression (WT: EG3637, n = 155. *ftsW**I**: EG3467, n = 140). **C.** Spot dilutions of wild-type

856   (WT) or *ftsW**I** strains harboring an empty vector (EV) control or *fzlA* variant overexpression

857   construct, plated with 0.3% xylose (+ Inducer) or 0.2% glucose (- Inducer). Cultures were diluted

858   as indicated. **D.** Growth curves of wild-type (WT) or *ftsW**I** strains harboring an empty vector

859   (EV) control or *fzlA* overexpression construct. Strains were pre-induced for six hours before

860   growth curves with 0.3% xylose and continued to be cultured with 0.3% xylose during the

861   growth curve. Points are the mean of three technical replicates at that timepoint, error bars are

862   the standard error of the mean. Shown is a representative replicate of a biological triplicate. **E.**

863   Phase contrast time course (0, 7, 20 hr post-induction) of wild-type (WT) or *ftsW**I** cells

864   harboring an empty vector (EV) control or *fzlA* overexpression construct. **F.** Example phase

865   contrast images of cells after 20 hours of FzlA overproduction in a *ftsW**I** background. Arrow

866   colors: Green = very short, Orange = one half of the constricted cell is lysed, Blue = cell

867   chaining. *fzlA* OE, roughly 20-fold overproduced FzlA.

868   **Figure 7. FzlA interacts with FtsK$_C$ via the FzlA C-terminal tail. A-B.** Western blots of whole

869   cell lysates or eluates from an immunoprecipitation (IP) using α-FLAG resin. Ladder values to

870   the left of the blots are in kDa. Red arrows to the right of the blots indicate to what protein the

871   band is attributed. Presence or absence of the fusion proteins in the strains used are indicated

872   above the blots by plus (+) or minus (-) signs, respectively. **A.** 3xFLAG-FzlA

873   immunoprecipitation. Blots were incubated with primary antibodies recognizing either mCherry

874   (αRFP) or FLAG (αFLAG). Lane 1 and 4, *fzlA::3xFLAG-fzlA* (EG2217). Lane 2 and 5,

875   *ftsK::mCherry-ftsK* (EG2427). Lane 3 and 6, *fzlA::3xFLAG-fzlA*; *ftsK::mCherry-ftsK* (EG2428). **B.**

876   FtsK-FLAG immunoprecipitation. Blots were incubated with primary antibodies recognizing

877   either FzlA (αFzlA) or FLAG (αFLAG). Lane 1 and 3, WT (EG865). Lane 2 and 4, *ftsK::ftsK-*

878   *FLAG* (EG742) **C.** Bacterial Two-Hybrid results for interaction between FzlA variants and full-

879   length FtsK or its domains. The adenylyl cyclase subunits T18 (left) and T25 (top) are fused to

880   proteins via the N-terminus or C-terminus, respectively. A green box around the representative

881   spot image means that the three biological triplicates were positive for induction of the cAMP-

882   dependent β-galactosidase reporter, indicating a positive interaction. EV, empty vector. FtsK$_{FL}$,

883   Full-length FtsK. FtsK$_N$, FtsK N-domain. FtsK$_C$, FtsK C-domain.

884   **Figure 8. FtsK acts upstream of FtsWI and is required for constriction initiation. A.**

885   Representative phase contrast of strains during depletion of the FtsK C-domain (FtsK$_C$) or full-

886   length FtsK (FtsK$_{FL}$) in either wild-type or *ftsW**I** backgrounds. Cells were grown in the

887   presence of 0.3% xylose (top row, *ftsK* induced) or 0.2% glucose (bottom row, FtsK or FtsK$_C$

888   depleted) with images taken at six and 20 hours post depletion. $FtsK_C$ depletion, EG2996.

889    $FtsK_{FL}$ depletion in WT, EG3332. $FtsK_{FL}$ depletion in *ftsW**I**, EG3333. **B.** Spot dilutions of

890   strains with xylose-inducible *ftsK* expression in the wild-type or *ftsW**I** background, in the

891   absence or presence of native *ftsK*. Plates contain either 0.2% glucose (- Inducer) or 0.3%

892   xylose (+ Inducer). Row 1, EG3299. Row 2, EG3300. Row 3, EG3332. Row 4, EG3333. **C.**

893   Growth curves of strains dependent on xylose for FtsK expression (same as A). Solid lines

894   indicate grown in the presence of 0.3% xylose. Dashed lines indicate grown in the presence of

895   0.2% glucose. $FtsK_C$ depletion, EG2996. $FtsK_{FL}$ depletion in WT, EG3332. $FtsK_{FL}$ depletion in

896   *ftsW**I**, EG3333. Points are the mean of three technical replicates at that timepoint, error bars

897   are the standard error of the mean. Shown is a representative replicate of a biological triplicate.

898   **Figure 9. Misregulation of the FzlA-FtsK-FtsWI pathway results in DNA damage. A-C.** Plot

899   of the percentage of cells containing high levels of EGFP (greater than the 99th percentile of the

900   specified control strain) from the P_{sidA}-*egfp* reporter. Plots comparing high EGFP producing cells

901   between **A.** wild-type (WT, EG3653), *ftsW**I** (EG3655), and *ΔfzlA; ftsW**I** (EG3667)

902   backgrounds or **B.** strains containing either an empty vector (EV) control or *fzlA* overexpression

903   (OE) construct in WT (EV: EG3658, *fzlA* OE: EG3664) or *ftsW**I** (EV: EG3666, *fzlA* OE:

904   EG3672) backgrounds in the presence of 0.3% xylose for four, six, and eight hours. * represents

905   a p-value < 0.05. Averages of biological triplicates are shown and were used for statistical

906   analysis. **C.** Plot comparing high EGFP-producing cells grown in 0.3% xylose for 4 hr of a full-

907   length $FtsK_{FL}$ depletion strain in a WT background (EG3904 + xyl 4hr) to various depletion

908   strains grown in the presence or absence of 0.3% xylose (EG3904: FtsK depletion in WT,

909   EG3900: $FtsK_C$ depletion in WT, EG3902: FtsK depletion in *ftsW**I**) at four, six, and eight

910   hours. * represents a p-value < 0.05. Averages of biological triplicates are shown and were used

911   for statistical analysis.

912 **Figure 10. FzlA binds to FtsZ and signals through FtsK to shift inactive FtsW to a slow-**

913 **moving, active state. A.** Inactive FtsW molecules either move with FtsZ clusters as they

914 treadmill about the division plane or are stationary, either because they are poised to activate or

915 bound to a stationary partner at the division plane. FtsK begins segregating chromosomal

916 termini after localizing. **B.** FzlA binds to FtsZ through the FtsZ-binding residues while the FzlA

917 C-terminal tail binds to the $FtsK_C$ to signal downstream for FtsWI activation, after properly

918 ensuring the division plane is free of obstruction by DNA.

919

Supplementary figure, table, and video legends.

Figure S1. Western blots to confirm FzlA levels during strain characterization. A-F.
Western blot analysis using primary antibody recognizing either FzlA (αFzlA) or the loading
control MreB (αMreB). Ladder values next to blots are in kilodaltons (kDa). **A-B.** *fzlA* variant
overexpression in both wild-type (WT) and *ftsW**I** backgrounds with empty vector (EV)
controls. **A.** Strains with high FzlA were diluted 1:20 before loading as indicated to enable
detection of all samples at a single exposure time. **B.** Same as **A** but samples were all
undiluted. **C.** FzlA is not detected in the *ΔfzlA; ftsW**I** strain. **D.** *fzlA* induction in the *halo-ftsW*
background (EG3523) as a function of xylose concentration to match FzlA levels in the wild-type
halo-ftsW (EG3052) background. We selected 0.001% xylose as the closest approximation of
FzlA production without underproducing. **E.** FzlA depletion in the *halo-ftsW* background
(EG3523). **F.** FzlA over-production in the *halo-ftsW* (EG3519) and *halo-ftsW**; ftsI** (EG3525)
backgrounds, as well as the respective empty vector (EV) controls (WT: EG3537. *ftsW**I**:
EG3538).

Figure S2. Example trajectories of single molecules of Halo-FtsW showing FtsW may
change speeds. A-B. A. i. Brightfield image of a *halo-ftsW* background cell (EG3052). **ii.**
Representative maximum fluorescence intensity projection image (Fluor) for single labeled
Halo-FtsW. **iii.** Kymograph (Kymo) of the fluorescence signal of a line scan across the division
plane that encapsulates a labeled single molecule of Halo-FtsW**. **iv.** Plot of the molecule
position within the line scan over the course of single molecule tracking.

Figure S3. ZapA localization reflects Z-ring position in filamentous cells depleted of FzlA.
Phase contrast, epifluorescence, and merged images of a strain expressing *zapA-mNG*
dependent on xylose for production of FzlA (EG3523). The strain was either continuously
induced (top) or depleted for seven hours (bottom) by being grown in the presence of 0.001%
xylose or glucose, respectively.

945 **Figure S4. FzlA converts fast-moving FtsW into a slow-moving, active state. A.**

946 Histograms of SMT data for Halo-FtsW (WT or FtsW**) with calculated two-population curves

947 that best fit the data for **i.** FtsW in cells producing FzlA (+FzlA, 0.001% xylose) or **ii.** depleted of

948 FzlA for seven hours (-FzlA (7 h), 0.001% glucose) (re-analysis of data presented in Fig. 3 A).

949 **Bi-ii.** FtsW in **i.** a *halo-ftsW* (WT EV): EG3537), **ii.** *fzlA* OE (WT +FzlA: EG3519), (re-analysis of

950 data presented in Fig. 4 A) **Biii-iv.** FtsW** in **iii.** *halo-ftsW***; *ftsI** (*ftsW**I** EV: EG3538), **iv.** *fzlA*

951 *OE* (*ftsW**I** +FzlA: EG3525) background (re-analysis of data presented in Fig. 4 C). Red =

952 slow population. Blue = fast population. Black: total based on two-population.

953 **Figure S5. Complete results of bacterial two-hybrid analysis.** Bacterial Two-Hybrid results

954 for interaction between FzlA variants and full-length FtsK or its domains. The adenylyl cyclase

955 subunits T18 (left) and T25 (top) are fused to proteins at the indicated terminus for each row or

956 column. A green box around the representative spot image means that the three biological

957 triplicates were positive for induction of the cAMP-dependent β-galactosidase reporter,

958 indicating a positive interaction. $FtsK_{FL}$, Full-length FtsK. $FtsK_N$, FtsK N-domain. $FtsK_C$, FtsK C-

959 domain.

960 **Table S1. Summary of SMT of Halo-FtsW and Halo-FzlA variants with varying states of**

961 **activation.** Average FtsW or FzlA molecule speeds (nm/s) are indicated with standard error of

962 the mean (SEM), proportion of moving molecules (%), and the life-time of stationary FtsW (s).

963 N: sample size. WT: wild-type. OE: overexpressed. EV: empty vector control. ++FzlA: ~20-fold

964 overproduced FzlA. +FzlA: induced to wild-type levels. -FzlA: without induction.

965 **Table S2. Proteins identified by mass-spectrometry that are five-fold enriched in relative**

966 **abundance in 3xFLAG-FzlA eluate compared to WT.** Proteins identified by Mass-

967 spectrometry with accession numbers, and information about relative abundance between

968 3xFLAG-Fzla and WT eluate. WT: wild-type. kDa: kilodalton. --: not detected in WT sample.

969   **Table S3. Summary of DNA damage reporter experiments.** Percentage (%) of cells with high

970   levels of P_{sidA}-driven EGFP production in backgrounds with varying FzlA or FtsK levels and

971   states of FtsWI activation. WT: wild-type. NA: Not applicable (not an inducible strain). OE:

972   overexpression. EV: empty vector control. ++FzlA: ~20-fold overproduced FzlA. FtsK$_C$: FtsK C-

973   terminus. FtsK$_{FL}$: FtsK full-length.

974   **Table S4. Strains and plasmids used in this study.**

975   **Video 1. A wild-type division that completes successfully.** Representative timelapse of a

976   constricting wild-type cell with an empty vector control. Each frame is 5 minutes apart and the

977   total timelapse is 4 hours.

978   **Video 2. FtsW** movement about the division plane in a *ftsW**I** background.**

979   Representative timelapse of fluorescent JF646-labeled Halo-FtsW** in a *ftsW**I** background

980   over 140 s. This timelapse corresponds to the kymograph present in Figures 2aiii. Each frame is

981   500 ms.

982   **Video 3. FtsW moving at multiple speeds in a wild-type background.** Representative

983   timelapse of fluorescent JF646-labeled Halo-FtsW in a wild-type background over 40 s. This

984   timelapse corresponds to the kymograph present in Figure S2aiii. Each frame is 500 ms.

985   **Video 4. FtsW moving at multiple speeds in a wild-type background.** Representative

986   timelapse of fluorescent JF646-labeled Halo-FtsW in a wild-type background over 29 s. This

987   timelapse corresponds to the kymograph present in Figure S2aiii. Each frame is 500 ms.

988   **Video 5. A division in which both daughter cells halt growth post-constriction.**

989   Representative timelapse of a FzlA overproducing cell in a WT background for which both

990   daughter cells halt growth post-division. Each frame is 5 minutes apart and the total timelapse is

991   4 hours.

992   **Video 6. A division in which one daughter cell halts growth post-constriction.**

993   Representative timelapse of a FzlA-overproducing cell in a WT background for which one

994   daughter cell halts growth post-division. Each frame is 5 minutes apart and the total timelapse is

995   4 hours.

996   **Video 7. A division in which one daughter cell lyses growth post-constriction.**

997   Representative timelapse of a FzlA overproducing cell in a WT background for which one

998   daughter cell lyses post-division. Each frame is 5 minutes apart and the total timelapse is 4

999   hours.

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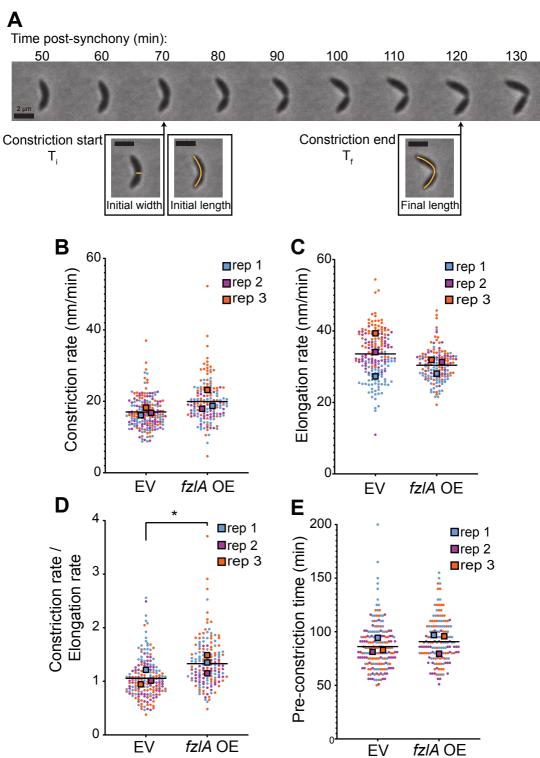# Figure 1

A Time post-synchony (min):

Constriction start T_i

Initial width | Initial length

Constriction end T_f

Final length

B

C

D

E

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A Time post-synchrony:

Both halt post-division

One halts post-division

Cell lyses post-division

2 µm

B

	WT	ftsW**I*
EV		
fzlA OE		

Proportion of division events
- Successful division
- Cells lysed post division
- Both daughters halt post division
- One daughter halts post division

C

+ Inducer   − Inducer

EV | WT
     ftsW**I*

fzlA^WT | WT
          ftsW**I*

fzlA^C-term | WT
             ftsW**I*

fzlA^NB | WT
         ftsW**I*

D

OD600 vs Time (hr)

- EV | WT
- fzlA OE | WT
- EV | ftsW**I*
- fzlA OE | ftsW**I*

E

WT   ftsW**I*

Time (h): 0   7   20   0   7   20

2 µm

EV   EV

fzlA OE   fzlA OE

F

fzlA OE in *ftsW**I** - 20 hr

Short cells   Lysis   Chaining

2 µm

Figure 7

A 3x-FLAG-FzlA IP

	Input			Eluted		
3xFLAG-FzlA	+	–	+	+	–	+
FtsK-mChy	–	+	+	–	+	+

150 —
100 —
← FtsK-mChy

WB: αRFP

37 —
25 —
← 3xFLAG-FzlA

WB: αFLAG

B FtsK-FLAG IP

	Input		Eluted	
FtsK-FLAG	–	+	–	+

25 —
← FzlA

WB: αFzlA

100 —
75 —
← FtsK-FLAG

WB: αFLAG

C

T25 C \ T18 N	FzlA	FzlA^{D227K}	EV
FzlA$^{WT\ or\ D227K}$			
FtsK$_{FL}$			
FtsK$_{N}$			
FtsK$_{C}$			
EV			

Figure 8

A

FtsK_C depletion WT — 6, 20

FtsK_FL depletion WT — 6, 20

FtsK_FL depletion ftsWI*** — 6, 20

2 µm

+ Xylose

Depleted

$ftsK::ftsK\Delta aa334$-806
$xylX::ftsK$
$ftsWI^{WT}$

$ftsK::specR$
$xylX::ftsK$
$ftsWI^{WT}$

$ftsK::specR$
$XylX::P_{xyl}\ ftsK$
$ftsW**I*$

B

− Inducer

$ftsK::ftsK$; WT
$ftsK::ftsK$; $ftsW**I*$
$ftsK::specR$; WT
$ftsK::specR$; $ftsW**I*$

+ Inducer

$ftsK::ftsK$; WT
$ftsK::ftsK$; $ftsW**I*$
$ftsK::specR$; WT
$ftsK::specR$; $ftsW**I*$

All strains contain $xylX::P_{xyl} ftsK$

C

FtsK^C depl - WT
+ inducer   deplete

FtsK^FL depl - WT
+ inducer   deplete

FtsK^FL depl - $ftsW**I*$
+ inducer   deplete

OD_600 vs Time (hr)

Figure 9

Figure 10

Figure S1

A

P_{xyl} EV or fzlA variant	EV				WT					D227K		NB2			
ftsWI variant	WT		hyp		WT		hyp			hyp		WT		WT		hyp
Inducer	-	+	-	+	-	+	-	+		-	+	-	+	-	+	-	+
Dilution	undil.	undil.	undil.	undil.	undil.	1:20	undil.	1:20		undil.	1:20	undil.	1:20	undil.	1:20	undil.	1:20

25kDa ——— αFzla   25kDa

37kDa ——— αMreB   37kDa

B

P_{xyl} EV or fzlA variant	EV				WT					D227K		WT		WT		hyp
ftsWI variant	WT		hyp		WT		hyp			hyp		WT		WT		hyp
Inducer	+	-	-	+	-	+	-	+		-	+	-	+	-	+	-	+

25kDa ——— αFzla   25kDa

37kDa ——— αMreB   37kDa

C

fzlA^{WT} or ΔfzlA	WT	WT	Δ
ftsWI variant	WT	hyp	

HALO-ftsW

25kDa αFzlA

37kDa αMreB

D

	WT	fzlA::gentR xylX::P_{xyl}fzlA				
		% Xylose				0.2% Glucose
		0.001	0.0005	0.0001	0.00005	

25kDa αFzlA

37kDa αMreB

E

	WT	Time depleted (h)				
		0	4	7	12	23

25kDa αFzlA

37kDa αMreB

F

P_{xyl} EV or fzlA^{WT}	EV	WT	EV	WT	EV	WT	EV	WT
ftsWI variant	WT		hyp		WT		hyp	
Inducer	-				+			

HALO-ftsW

25kDa αFzlA

37kDa αMreB

Figure S2

A

i Brightfield

ii Fluor

iii Kymo

0s

40s

iv

V = 12.5 nm/s

V = 25.6 nm/s

V = 37.8 nm/s

X (nm)

Time (s)

500 nm

B

i Brightfield

ii Fluor

iii Kymo

0s

29s

iv

V = 28.7 nm/s

V = 50.1 nm/s

V = 40.1 nm/s

X (nm)

Time (s)

500 nm

Figure S3

	Phase	ZapA-mNG	Merge
+FzlA			
-FzlA (7 h)			

Figure S4

Figure S5