Supplementary Information for

Single-molecule imaging reveals that Z ring condensation is essential for cell division in *Bacillus subtilis*

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Supplementary Table 1: Strains used in this study

Description	Strain	Genotype	Source
FtsZ single molecule imaging			
FtsZ	bAB309	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ*	this study
2 colour	bGS104	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, pbp2B::mNeonGreen-15aa-pbp2B*	this study
FtsZ(T111A)	bGS109	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, ftsZΩftsZ(T111A) (tet)*	this study
MciZ expression	bGS328	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, yvbJ::PxylA-mciZ::erm, pbp2B::mNeonGreen-15aa-pbp2B*	this study
Δ*ezrA*	bGS167	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, ezrA::cat*	this study
Δ*sepF*	bGS304	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, sepF::scar*	this study
Δ*zapA*	bGS141	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, zapA-yshBD::tet*	this study
↑*ezrA*	bGS157	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, ycgO::cat-pXyl-ezrA*	this study
↑*sepF*	bGS158	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, ycgO::cat-pXyl-sepF*	this study
↑*zapA*	bGS159	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, ycgO::cat-pXyl-zapA*	this study
Δ*sepF* Δ*zapA*	bGS318	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, zapA-yshBD::tet, sepF::scar*	this study
Δ*ezrA* ↓*sepF*	bGS204	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, ezrA::scar, sepF::cat-pXyl-sepF*	this study
↓*ezrA* Δ*sepF*	bGS316	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, sepF::scar, ezrA::cat-pXyl-ezrA*	this study
Δ*ezrA* ↓*zapA*	bGS206	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, ezrA::scar, zapA::cat-pXyl-zapA*	this study
↓*ezrA* Δ*zapA*	bGS306	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, zapA-yshBD::tet, ezrA::cat-pXyl-ezrA*	this study
↓*ezrA* Δ*sepF* Δ*zapA* (ΔZBPs)	bGS331	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, zapA-yshBD::tet, sepF::scar, ezrA::cat-pXyl-ezrA*	this study
FtsZ filament and Z ring imaging			
FtsZ	bAB219	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ*	[5]
FtsZ	bGS630	*ycgO::cat-pXyl-mNeonGreen-15aa-ftsZ*	this study
FtsZ(T111A)	bAB281	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, ftsZΩftsZ(T111A) (tet)*	[5]
MciZ expression	bGS326	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, yvbJ::PxylA-mciZ::erm*	this study

Δ*ezrA*	bGS256	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, ezrA::cat*	this study
Δ*sepF*	bGS254	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, sepF::tet*	this study
Δ*zapA*	bGS250	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, zapA-yshBD::tet*	this study
Δ*ftsA*	bGS639	*ftsAZ::spec, amyE::pHyperspank-ftsZ, ycgO::cat-pXyl-mNeonGreen-15aa-ftsZ*	this study
↓*ezrA*	bGS588	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, ezrA::cat-pXyl-ezrA*	this study
↓*sepF*	bGS590	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, sepF::cat-pXyl-sepF*	this study
↓*zapA*	bGS586	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, zapA::cat-pXyl-zapA*	this study
↑*ezrA*	bGS263	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, ycgO::cat-pXyl-ezrA*	this study
↑*sepF*	bGS260	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, ycgO::cat-pXyl-sepF*	this study
↑*zapA*	bGS259	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, ycgO::cat-pXyl-zapA*	this study
Δ*sepF* Δ*zapA*	bGS368	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, sepF::scar, zapA-yshBD::tet*	this study
Δ*ezrA* ↓*sepF*	bGS290	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, ezrA::scar, sepF::cat-pXyl-sepF*	this study
↓*ezrA* Δ*sepF*	bGS298	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, sepF::tet, ezrA::cat-pXyl-ezrA*	this study
Δ*ezrA* ↓*zapA*	bGS293	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, ezrA::scar, zapA::cat-pXyl-zapA*	this study
↓*ezrA* Δ*zapA*	bGS297	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, zapA-yshBD::tet, ezrA::cat-pXyl-ezrA*	this study
↓*ezrA* Δ*sepF* Δ*zapA* (ΔZBPs)	bGS308	*amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ, sepF::scar, zapA-yshBD::tet, ezrA::cat-pXyl-ezrA*	this study
FtsZ(K86E)	bGS432	*ftsZ::ftsZ(K86E), amyE::erm-Phyperspank-FtsA-mNeonGreen-15aa-FtsZ(K86E)*	this study
FtsZ(K86E) Δ*ezrA* Δ*zapA*	bGS463	*ftsZ::ftsZ(K86E), amyE::erm-Phyperspank-FtsA-mNeonGreen-15aa-FtsZ(K86E), ezrA::cat, zapA-yshBD::tet*	this study
Other divisome proteins: single molecule imaging			
EzrA	bMH42	*ftsZ::mNeonGreen-15aa-ftsZ multicopy ezrA::ezrA-30aa-HaloTag-cat*	this study
SepF	bMH372	*ftsZ::mNeonGreen-15aa-ftsZ multicopy amyE::erm-Phyperspank-sepF-30aa-HaloTag*	this study
ZapA	bMH560	*ftsZ::mNeonGreen-15aa-ftsZ multicopy zapA::zapA-30aa-HaloTag-cat*	this study
DivIB	bAB366	*ftsZ::mNeonGreen-15aa-ftsZ multicopy divIB::erm-Pxyl-HaloTag-15aa-divIB*	this study

DivIC	bAB367	*ftsZ::mNeonGreen-15aa-ftsZ multicopy divIC::erm-Pxyl-HaloTag-15aa-divIC*	this study
FtsL	bGS165	*ftsZ::mNeonGreen-15aa-ftsZ multicopy ftsL::erm-Phyperspank-HaloTag-15aa-ftsL*	this study
FtsW	bAB368	*ftsZ::mNeonGreen-15aa-ftsZ multicopy ftsW::erm-Pxyl-HaloTag-15aa-ftsW*	this study
Pbp2B	bGS31	*ftsZ::erm-mNeonGreen-15aa-ftsZ-cat multicopy pbp2b::erm-pHyperSpank-HaloTag-15aa-pbp2b*	[5]
Other divisome proteins: single molecule lifetime measurements			
FtsA	bAB213	*ftsAZ::erm-ftsA-HaloTag(sw)-ftsZ-cat multicopy*	[5]
EzrA	bMH03	*ezrA::ezrA-30aa-HaloTag-cat*	this study
SepF	bMH332	*amyE::erm-Phyperspank-sepF-30aa-HaloTag*	this study
ZapA	bMH559	*zapA::zapA-30aa-HaloTag-cat*	this study
Pbp2B dynamics			
Control	bMH512	*pbp2B::erm-Phyperspank-HaloTag-15aa-pbp2B, amyE::kan-Paz-ftsA-mNeonGreen-15aa-ftsZ*	this study
↓*ezrA* Δ*sepF* Δ*zapA* (ΔZBPs)	bMH443	*pbp2B::erm-Phyperspank-HaloTag-15aa-pbp2B, amyE::kan-Paz-ftsA-mNeonGreen-15aa-ftsZ, sepF::scar, zapA-yshBD::tet, ezrA::cat-pXyl-ezrA*	this study
Pbp2B colocalization			
Control	bGS104	*see above*	this study
Δ*ezrA* ↓*zapA*	bGS644	*pbp2B::mNeonGreen-15aa-pbp2B, amyE::Phyperspank-ftsA-HaloTag-15aa-ftsZ, zapA::pXyl-zapA, ezrA::cat*	this study
↓*ezrA* Δ*sepF* Δ*zapA* (ΔZBPs)	bMH445	*pbp2B::mNeonGreen-15aa-pbp2B, amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, sepF::scar, zapA-yshBD::tet, ezrA::cat-pXyl-ezrA*	this study
FtsZ(K86E)	bGS618	*ftsZ::ftsZ(K86E), pbp2B::mNeonGreen-15aa-pbp2B, amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ(K86E)*	this study
FtsZ(K86E) Δ*ezrA* Δ*zapA*	bGS628	*ftsZ::ftsZ(K86E), pbp2B::mNeonGreen-15aa-pbp2B, amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ(K86E), zapA-yshBD::tet, ezrA::cat*	this study
Cell wall synthesis labelling			
Control	bMH510	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, dacA::kan*	this study
↓*ezrA* Δ*sepF* Δ*zapA* (ΔZBPs)	bMH508	*amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ, sepF::scar, zapA-yshB::tet, ezrA::cat-pXyl-ezrA, dacA::kan*	this study
Cell length measurements			
WT	PY79	*wild type*	[63]
HT-FtsZ	bAB309	*see above*	this study
FtsA-HT(sw)	bAB213	*see above*	[5]
EzrA-HT	bMH03	*see above*	this study

SepF-HT	bMH332	*see above*	this study
ZapA-HT	bMH559	*see above*	this study
HT-DivIB	bAB352	*divIB::erm-Pxyl-HaloTag-15aa-divIB*	this study
HT-DivIC	bAB347	*divIC::erm-Pxyl-HaloTag-15aa-divIC*	this study
HT-FtsL	bMH47	*ftsL::erm-Phyperspank-HaloTag-15aa-ftsL*	this study
HT-FtsW	bAB350	*ftsW::erm-Pxyl-HaloTag-15aa-ftsW*	this study
HT-Pbp2B	bGS28	*pbp2b::erm-pHyperSpank-HaloTag-15aa-pbp2b*	[5]
Δ*ezrA*	bMH45	*ezrA::cat*	this study
Δ*sepF*	bSW234	*sepF::tet*	this study
Δ*zapA*	RL2638	*zapA-yshBD::tet*	[18]
Δ*divIB*	bMH92	*divIB::cat*	this study
Δ*zapA* EzrA-HT	bMH221	*zapA-yshBD::tet, ezrA::ezrA-30aa-HaloTag-cat*	this study
Δ*ezrA* SepF-HT	bMH542	*ezrA::cat, amyE::erm-Phyperspank-sepF-30aa-HaloTag*	this study
Δ*ezrA* ZapA-HT	bMH565	*ezrA::scar, zapA::zapA-30aa-HaloTag-cat*	this study

Abbreviations: ↑: overexpression, ↓: depletion, 15aa: 15 amino acid linker, 30aa: 30 amino acid linker, (sw): sandwich fusion, scar: genomic scar remaining after an antibiotic cassette has been looped out, Paz: promoter of *ftsA-ftsZ* operon, multicopy: genotype of bAB185[5].

Supplementary Table 2: Constructs used in this study

Constructs from other studies

Construct	Reference
amyE::erm-Phyperspank-ftsA-mNeonGreen-15aa-ftsZ	[5]
amyE::erm-pHyperspank-ftsZ	[5]
ftsZ::mNeonGreen-15aa-ftsZ multicopy	[5]
ftsAZ::erm-ftsA-HaloTag(sw)-ftsZ-cat multicopy	[5]
pbp2B::mNeonGreen-15aa-pbp2B	[5]
pbp2b::erm-pHyperSpank-HaloTag-15aa-pbp2b	[5]
ftsZΩftsZ(T111A) (tet)	[5]
yvbJ::PxylA-mciZ::erm	Gift from D. Z. Rudner
zapA-yshBD::tet	[18]
dacA::kan	[64]

Constructs created in this study

Amplicon	Primer: Sequence (5' to 3')
amyE::erm-Phyperspank-ftsA-HaloTag-15aa-ftsZ	
amyE(up)-erm-Phyperspank-ftsA	oMD191: TTTGGATGGATTCAGCCCGATTG oAB13: ccagtaccgatttctgccatGCTAAATCCTCCTAATCTGCCGAATG
HaloTag-15aa	oJE32: ATGGCAGAAATCGGTACTGG oAB14: tggcctgagcccggtccctggccagatccctcgagGCCGCTGATTTCTAAGGTAGAAAG
15aa-ftsZ-amyE(down)	oAB140: ggaccgggctcaggccaaggaagcggcATGTTGGAGTTCGAAACAAACATAGACG oMD197: TCACATACTCGTTTCCAAACGGATC
amyE::kan-Paz-ftsA-mNeonGreen-15aa-ftsZ	
amyE(up)-kan	oMD191: TTTGGATGGATTCAGCCCGATTG oSW42: TTCTGCTCCCTCGC
pAZ-ftsA(partial)	oAB76: gaacggtactgagcgagggagcagaaGTATTTGTTTCCGGTTTCT oAB38: GCGAAGCTCTTCTGA
this assembly was transformed directly into bAB219 to complete the construct	
ycgO::cat-pXyl-mNeonGreen-15aa-ftsZ	
ycgO(up)-cat-pXyl	oMD247: ATCGAACTGGCAAAAGGCAAAC oMD226: GGTAGTTCCTCCTTAATCGATCCATTCAAATACAGATGCATTTTATTTC

mNeonGreen-15aa-ftsZ	oGS35: tcgattaaggaggaactaccATGGTTTCGAAAGGAGAGGAGGATAATATG oGS40: gggacagccccttcctcctcctttcgatctTTAGCCGCGTTTATTACGGTTTC
ycgO(down)	oMD257: AGATCGAAAGGAGGAGGAAGG oMD252: CAAGGTTTTGAGCAGCTCAGTG
ftsAZ::spec	
ftsA(up)	oAB23: GCGGGTGAAATAGATTGAAAATAAAGC oAB72: atgctatacgaacggtagttgaccagtgctccctgTCTATGGCACCTCCTCACAT
spec	oSW40: CAGGGAGCACTGGTC oSW42: TTCTGCTCCCTCGC
ftsZ(down)	oAB73: acattatacgaacggtactgagcgagggagcagaaTGTAAAGGACAAAATCGTTT oAB30: CCATCCTCATATGTCTGACC
this assembly was transformed into a strain containing a second copy of ftsAZ under inducible control	
ftsZ::ftsZ(K86E)-kan	
ftsZ(up)-ftsZ(K86E)	oWM20: ATGAACAACAATGAACTTTACGTC oWM66: cagggagcactggtcaactaccgttcgtatTTAGCCGCGTTTATTACGGT
kan	oSW40: CAGGGAGCACTGGTC oSW42: TTCTGCTCCCTCGC
ftsZ(down)	oAB73: acattatacgaacggtactgagcgagggagcagaaTGTAAAGGACAAAATCGTTT oAB30: CCATCCTCATATGTCTGACC
amyE::erm-Phyperspank-FtsA-mNeonGreen-15aa-FtsZ(K86E)	
amyE(up)-erm-Phyperspank	oMD191: TTTGGATGGATTCAGCCCGATTG oMD232: GGTAGTTCCTCCTTAAAGCTTAATTGTTATCCGCTCACAAT
ftsA-mNeonGreen-15aa	oAB78: agcggataacaattaagctttaaggaggaactaccATGAACAACAATGAACTTTACGTC oZB34: tggcctgagcccggtccctggccagatccctcgagCTTATAGAGTTCATCCATACCCATC
15aa-FtsZ(K86E)	oAB140: ggaccgggctcaggccaaggaagcggcATGTTGGAGTTCGAAACAAACATAGACG oAB94: ctttcggtaagtcccgtctagccttgcccTTAGCCGCGTTTATTACGGTTTC
amyE(down)	oMD196: GGGCAAGGCTAGACGGG oMD197: TCACATACTCGTTTCCAAACGGATC
amyE::erm-Phyperspank-FtsA-HaloTag-15aa-FtsZ(K86E)	
amyE(up)-erm-Phyperspank	oMD191: TTTGGATGGATTCAGCCCGATTG oMD232: GGTAGTTCCTCCTTAAAGCTTAATTGTTATCCGCTCACAAT
ftsA-HaloTag-15aa	oAB78: agcggataacaattaagctttaaggaggaactaccATGAACAACAATGAACTTTACGTC oAB14: tggcctgagcccggtccctggccagatccctcgagGCCGCTGATTTCTAAGGTAGAAAG
15aa-FtsZ(K86E)	oAB140: ggaccgggctcaggccaaggaagcggcATGTTGGAGTTCGAAACAAACATAGACG oAB94: ctttcggtaagtcccgtctagccttgcccTTAGCCGCGTTTATTACGGTTTC

amyE(down)	oMD196: GGGCAAGGCTAGACGGG oMD197: TCACATACTCGTTTCCAAACGGATC
sepF::tet	
sepF(up)	oMH43: TATTGGCCCGTCTATCAG oMH98: gcgagggagcagaaCTCATTGCTGTACACCCC
tet	oSW40: CAGGGAGCACTGGTC oSW42: TTCTGCTCCCTCGC
sepF(down)	oMH20: tgaccagtgctccctgAGCGAGATGATCCTTTATCAAG oMH21: CTATGTATGAAGGATCTTCAACCA
ezrA::cat	
ezrA(up)	oMH53: GACATCTCCCGCTTGATG oAB99: cgaacggtactgagcgagggagcagaaAATGAGCCCCCTTGCTGT
cat	oJM28: TTCTGCTCCCTCGCTCAG oJM29: CAGGGAGCACTGGTCAAC
ezrA(down)	oMH05: tgaccagtgctccctgATAATCACGACCATGAAAAAGAG oMH06: GTTGTGGATCGAGTCGGA
ycgO::cat-pXyl-ezrA	
ycgO(up)	oMD247: ATCGAACTGGCAAAAGGCAAAC oMD248: tacgaacggtagttgaccagtgctccctgTCCCGCCATATAAATACAAATCGAAATAATC
cat-pXyl	oSW40: CAGGGAGCACTGGTC oMD226: GGTAGTTCCTCCTTAATCGATCCATTCAAATACAGATGCATTTTATTTC
ezrA	oMH14: atcgattaaggaggaactaccATGGAGTTTGTCATTGGATTATTA oGS37: acagccccttcctcctcctttcgatctCTAAGCGGATATGTCAGCTT
ycgO(down)	oMD257: AGATCGAAAGGAGGAGGAAGG oMD252: CAAGGTTTTGAGCAGCTCAGTG
ycgO::cat-pXyl-sepF	
ycgO(up)	oMD247: ATCGAACTGGCAAAAGGCAAAC oMD248: tacgaacggtagttgaccagtgctccctgTCCCGCCATATAAATACAAATCGAAATAATC
cat-pXyl	oSW40: CAGGGAGCACTGGTC oMD226: GGTAGTTCCTCCTTAATCGATCCATTCAAATACAGATGCATTTTATTTC
sepF	oGS38: atggatcgattaaggaggaactaccATGAAAAATAAACTGAAAAACTTTTTCTCAATGG oGS39: gggacagcccctcctcctcctttcgatctTTAGCCGCGTTTATTACGGTTTC
ycgO(down)	oMD257: AGATCGAAAGGAGGAGGAAGG oMD252: CAAGGTTTTGAGCAGCTCAGTG
ycgO::cat-pXyl-zapA	
ycgO(up)	oMD247: ATCGAACTGGCAAAAGGCAAAC oMD248: tacgaacggtagttgaccagtgctccctgTCCCGCCATATAAATACAAATCGAAATAATC

cat	oSW40: CAGGGAGCACTGGTC oSW42: TTCTGCTCCCTCGC
pXyl-zapA	oSW38: cattatacgaacggtactgagcgagggagcagaaGAATTCGAGCTTGCATG oGS36: acagccccttcctcctcctttcgatctTCAATCCTTTTCTTTAAGCTGACGC
ycgO(down)	oMD257: AGATCGAAAGGAGGAGGAAGG oMD252: CAAGGTTTTGAGCAGCTCAGTG
ezrA::cat-pXyl-ezrA	
ezrA(up)	oMH35: GAATATGTCCGTCTCGCT oMH54: tgaccagtgctccctgAATGAGCCCCCTTGCTG
cat-pXyl	oSW40: CAGGGAGCACTGGTC oMD226: GGTAGTTCCTCCTTAATCGATCCATTCAAATACAGATGCATTTTATTTC
ezrA(partial)	oMH14: atcgattaaggaggaactaccATGGAGTTTGTCATTGGATTATTA oMH56: CTTAGTACGGATTGACCGG
sepF::cat-pXyl-sepF	
sepF(up)	oAB109: GCCCGTGAGTATCACACG oAB110: gctatacgaacggtagttgaccagtgctccctgACTCATTGCTGTACACCCCC
cat-pXyl	oSW40: CAGGGAGCACTGGTC oMD226: GGTAGTTCCTCCTTAATCGATCCATTCAAATACAGATGCATTTTATTTC
sepF-sepF(down)	oGS38: atggatcgattaaggaggaactaccATGAAAAATAAACTGAAAAACTTTTTCTCAATGG oAB112: GCCAAAACCTCTGATAGACAGC
zapA::cat-pXyl-zapA	
zapA(up)	oMH22: AATGGCTTCAGGCTTTACTC oMH58: tgaccagtgctccctgCGTTTCTCCTCCATTCCG
cat-pXyl	oSW40: CAGGGAGCACTGGTC oMD226: GGTAGTTCCTCCTTAATCGATCCATTCAAATACAGATGCATTTTATTTC
zapA-zapA(down)	oAB152: gtatttgaatggatcgattaaggaggaactaccTTGTCTGACGGCAAAAAAACA oMH31: AGAGATTCTGCATCGTGT
ezrA::ezrA-30aa-HaloTag-cat	
ezrA(partial)	oMH01: GATTGCAAAGCTCAAGGATG oMH02: AGCGGATATGTCAGCTTTGA
30aa-HaloTag	oMH03: caaagctgacatatccgctCTTGAGGGTAGCGGACAAG oMH04: agcgagggagcagaaTTAGCCGCTGATTTCTAAGGTAG
cat	oJM28: TTCTGCTCCCTCGCTCAG oJM29: CAGGGAGCACTGGTCAAC
ezrA(down)	oMH05: tgaccagtgctccctgATAATCACGACCATGAAAAAGAG oMH06: GTTGTGGATCGAGTCGGA
amyE::erm-Phyperspank-sepF-30aa-HaloTag	

amyE(up)-erm-pHyperSpank	oMD191: TTTGGATGGATTCAGCCCGATTG oSW28: GGTAGTTCCTCCTTAAAGC
SepF-15aa-HaloTag	oMH45: ttaagctttaaggaggaactaccATGAGTATGAAAAATAAACTGAAAAACTT oAB257: cggtaagtcccgtctagccttgcccTTAGCCGCTGATTTCTAAGG
amyE(down)	oMD196: GGGCAAGGCTAGACGGG oMD197: TCACATACTCGTTTCCAAACGGATC
zapA::zapA-30aa-HaloTag-cat	
zapA(up)	oMH22: AATGGCTTCAGGCTTTACTC oMH24: gtccgctaccctcaagATCCTTTTCTTTAAGCTGACGC
30aa-HaloTag-cat	oMH25: CTTGAGGGTAGCGGACAA oSW40: CAGGGAGCACTGGTC
zapA-zapA(down)	oMH29: tgaccagtgctccctgacaactATGCTAGATATCATCATC oMH31: AGAGATTCTGCATCGTGT
divIB::cat	
divIB(up)	oAB235: GCCTGAGTATTTAAAGGCCATTG oAB236: gtagttgaccagtgctccctgTGCCTGTTCACCTCATTCAA
cat	oJM28: TTCTGCTCCCTCGCTCAG oJM29: CAGGGAGCACTGGTCAAC
divIB(down)	oMH100: tgagcgagggagcagAATTGAGGGGCAAATCAGC oAB238: CGCAAGCGATAAATAGTTTGAG
divIB::erm-Pxyl-HaloTag-15aa-divIB	
divIB(up)	oAB235: GCCTGAGTATTTAAAGGCCATTG oAB236: gtagttgaccagtgctccctgTGCCTGTTCACCTCATTCAA
erm-Pxyl-HaloTag-15aa	oJM29: CAGGGAGCACTGGTCAAC oAB14: tggcctgagcccggtccctggccagatccctcgagGCCGCTGATTTCTAAGGTAGAAAG
15aa-divIB-divIB(down)	oAB237: ctggccagggaccgggctcaggccaaggaagcggcATGAACCCGGGTCAAGAC oAB238: CGCAAGCGATAAATAGTTTGAG
divIC::erm-Pxyl-HaloTag-15aa-divIC	
divIC(up)	oAB239: CGGCGTACACTAGCGAA oAB240: gtagttgaccagtgctccctgACCAGACGGTCCTCCTTTC
erm-Pxyl-HaloTag-15aa	oJM29: CAGGGAGCACTGGTCAAC oAB14: tggcctgagcccggtccctggccagatccctcgagGCCGCTGATTTCTAAGGTAGAAAG
15aa-divIC-divIC(down)	oAB241: ctggccagggaccgggctcaggccaaggaagcggcTTGAATTTTTCCAGGGAACG oAB242: CAGTGAATGCAAATGATGAGTC
ftsL::erm-Phyperspank-HaloTag-15aa-ftsL	

ftsL(up)	oMH49: CTTCTTCGTGAAACCGTAGA oMH50: tgaccagtgctccctgaGGCTGATGACCTCCTTTTA
erm-Phyperspank-HaloTag-15aa	oSW40: CAGGGAGCACTGGTC oAB14: tggcctgagcccggtccctggccagatccctcgagGCCGCTGATTTCTAAGGTAGAAAG
15aa-ftsL	oMH61: agggaccgggctcaggccaaggaagcggcATGAGCAATTTAGCTTACCAACC oMH52: CGCTCCTTCAAATACTTATCCA
ftsW::erm-Pxyl-HaloTag-15aa-ftsW	
ftsW(up)	oME1: GAGAGACTTGATTATTTGCTTTCTTTTATC oAB234: gtagttgaccagtgctccctgAACATCCTCTTCCCTGCTTC
erm-Pxyl-HaloTag-15aa	oJM29: CAGGGAGCACTGGTCAAC oAB14: tggcctgagcccggtccctggccagatccctcgagGCCGCTGATTTCTAAGGTAGAAAG
15aa-ftsW	oME6: ctcgagggatctggccagggaccgggctcaggccaaggaagcggcATGTTAAAAAAAATGCTAA AATCTTATGATTACTCAC oME7: GTACACACTTGTTTTTTACAGATAAACAGoME6: ctcgagggatctggccagggaccgggctcaggccaaggaagcggcATGTTAAAAAAAATGCTAA AATCTTATGATTACTCAC oME7: GTACACACTTGTTTTTTACAGATAAACAG

Abbreviations: 15aa: 15 amino acid linker, 30aa: 30 amino acid linker, (sw): sandwich fusion, (up): homology region upstream of the indicated gene, (down): homology region downstream of the indicated gene, multicopy: genotype of bAB185[5]. For primers, uppercase sequence indicates annealing region, and lowercase sequence indicates overhang.

Supplementary Table 3: FtsZ treadmilling velocity and velocities of other divisome proteins

Condition	Strain	Velocity (nm/s)	N
FtsZ treadmilling velocities			
Control	bAB219	28.5 ± 9.8	119
Δ*ezrA*	bGS256	26.2 ± 8.1	132
Δ*sepF*	bGS254	26.3 ± 7.5	120
Δ*zapA*	bGS250	28.2 ± 6.9	125
↑*ezrA* 100 µM xyl	bGS263 + 100 µM xyl	28.4 ± 8.5	134
↑*ezrA* 500 µM xyl	bGS263 + 500 µM xyl	28.9 ± 9.9	128
↑*ezrA* 5 mM xyl	bGS263 + 5 mM xyl	28.7 ± 9.3	128
↑*sepF*	bGS260 + 30 mM xyl	27.7 ± 7.3	118
↑*zapA*	bGS259 + 30 mM xyl	24.1 ± 6.6	110
Δ*sepF* Δ*zapA*	bGS368	26.3 ± 7.3	132
Δ*ezrA* ↓*sepF*	bGS290	28.2 ± 8.6	143
↓*ezrA* Δ*sepF*	bGS298	27.4 ± 7.6	121
Δ*ezrA* ↓*zapA*	bGS293	28.2 ± 7.3	137
↓*ezrA* Δ*zapA*	bGS297	27.2 ± 8.0	126
↓*ezrA* Δ*sepF* Δ*zapA* (ΔZBPs)	bGS308	27.6 ± 7.1	136
FtsZ(K86E)	bGS432	29.2 ± 9.2	123
FtsZ(K86E) Δ*ezrA* Δ*zapA*	bGS463	27.8 ± 6.9	140
Velocities of other divisome proteins			
DivIB	bAB366	26.2 ± 4.8	270
DivIC	bAB367	26.7 ± 5.1	285
FtsL	bGS165	25.4 ± 4.7	261
FtsW	bAB368	24.1 ± 7.5	120
Pbp2B	bGS31	25.6 ± 6.8	98

Velocity: mean ± standard deviation. N: number of trajectories analysed. Abbreviations: ↑: overexpression, ↓: depletion, xyl: xylose.

Supplementary Table 4: Single-molecule lifetimes

Condition	Strain	Lifetime (s)	N	p-value
FtsZ lifetimes				
Control	bAB309, bGS104	8.1 (7.6, 8.7)	1897	
↑*ftsAZ*	bAB309 + 100 µM IPTG	8.8 (6.7, 13)	455	ns (0.19)
FtsZ(T111A)	bGS109	16.5 (12.6, 23.8)	337	**** (3.04e-18)
MciZ expression	bGS328 + 60 mM xyl	3.9 (3.1, 5.1)	442	**** (3.35e-25)
Δ*ezrA*	bGS167	11.3 (7.8, 20.2)	820	**** (2.44e-10)
Δ*sepF*	bGS304	7.4 (6.5, 8.5)	446	ns (0.18)
Δ*zapA*	bGS141	7.3 (6.4, 8.5)	348	ns (0.24)
↑*ezrA* 100 µM xyl	bGS157 + 100 µM xyl	6.1 (5.5, 6.8)	461	*** (7.4e-04)
↑*ezrA* 500 µM xyl	bGS157 + 500 µM xyl	4.6 (4.2, 5)	163	**** (1.54e-06)
↑*ezrA* 5 mM xyl	bGS157 + 5 mM xyl	4 (3.6, 4.4)	285	**** (2.34e-19)
↑*sepF*	bGS158 + 30 mM xyl	6.1 (5.7, 6.7)	338	**** (1e-6)
↑*zapA*	bGS159 + 30 mM xyl	8.7 (7, 11.4)	531	* (0.01)
Δ*sepF* Δ*zapA*	bGS318	7.8 (6.9, 8.9)	324	ns (0.3)
Δ*ezrA* ↓*sepF*	bGS204	7.8 (6.7, 9.3)	146	ns (0.68)
↓*ezrA* Δ*sepF*	bGS316	8.1 (6.8, 10.2)	399	ns (0.41)
Δ*ezrA* ↓*zapA*	bGS206	8.4 (5.8, 15.3)	654	ns (0.9)
↓*ezrA* Δ*zapA*	bGS306	9.4 (7.4, 13)	281	ns (0.63)
↓*ezrA* Δ*sepF* Δ*zapA* (ΔZBPs)	bGS331	7.4 (5.7, 10.3)	193	ns (0.26)
2 colour: outside of Z ring	bGS104	5.6 (5.2, 6.1)	383	

2 colour: inside of Z ring	bGS104	11 (8, 17.4)	238	
1 second intervals	bAB309	8.2 (7.3, 9.4)	418	ns (0.53)
Lifetimes of other ZBPs				
FtsA	bAB213	4.5 (3.9, 5.5)	222	**** (2.74e-08)
EzrA	bMH03	4.7 (4.1, 5.4)	1160	**** (1.88e-22)
SepF	bMH332	8 (6, 12)	642	*** (2.27e-4)
ZapA	bMH559	6.7 (5.4, 8.7)	647	ns (0.68)

Lifetime: mean (95% confidence interval) from single exponential fit. N: number of particles analysed. Abbreviations: ↑: overexpression, ↓: depletion, xyl: xylose. P-value computed from two-sided Wilcoxon rank-sum test vs control. ns $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; p-value is written underneath in parenthesis.

Supplementary Table 5: Z ring peak widths

Condition	Strain	Z ring width (nm)	N
Control	bAB219	330 ± 40	2427
FtsZ(T111A)	bAB281	360 ± 40	962
↓*ezrA*	bGS588	390 ± 40	1685
Δ*ezrA*	bGS256	490 ± 50	1651
↓*sepF*	bGS590	320 ± 40	667
Δ*sepF*	bGS254	300 ± 40	657
↓*zapA*	bGS586	330 ± 40	885
Δ*zapA*	bGS250	320 ± 40	590
Δ*sepF* Δ*zapA*	bGS368	310 ± 40	341
Δ*ezrA* ↓*sepF*	bGS290	590 ± 60	668
↓*ezrA* Δ*sepF*	bGS298	510 ± 50	304
Δ*ezrA* ↓*zapA*	bGS293	610 ± 60	1555
↓*ezrA* Δ*zapA*	bGS297	470 ± 50	822
↓*ezrA* Δ*sepF* Δ*zapA* (ΔZBPs)	bGS308	550 ± 50	436
FtsZ(K86E)	bGS432	340 ± 40	888
FtsZ(K86E) Δ*ezrA* Δ*zapA*	bGS463	450 ± 50	208

Z ring width: Full width at half maximum of the average Z ring intensity peak ± bootstrapped standard error. N: number of Z rings analysed. Abbreviations: ↓: depletion.

Supplementary Table 6: Index of strains and experimental conditions

Figure	Label	Strain	Induction and labelling conditions
1b	EzrA	bMH42	300 pM JF549-HTL, 15 min
1b	SepF	bMH372	no IPTG 200 pM JF549-HTL, 15 min
1b	ZapA	bMH560	600 pM JF549-HTL, 15 min
1c	DivIB	bAB366	1 mM xyl, continuous 400 pM JF549-HTL, 15 min
1c	DivIC	bAB367	5 mM xyl, continuous 500 pM JF549-HTL, 15 min
1c	FtsL	bGS165	30 µm IPTG, continuous 40 pM JF549-HTL, 15 min
1c	FtsW	bAB368	8 mM xyl, continuous 300 pM JF549-HTL, 15 min
1c	Pbp2B	bGS31	20 µM IPTG, continuous 200 pM JF549-HTL, 15 min
1d	DivIB	bAB366	1 mM xyl, continuous 400 pM JF549-HTL, 15 min
1d	DivIC	bAB367	5 mM xyl, continuous 500 pM JF549-HTL, 15 min
1d	FtsL	bGS165	30 µm IPTG, continuous 40 pM JF549-HTL, 15 min
1d	FtsW	bAB368	8 mM xyl, continuous 300 pM JF549-HTL, 15 min
1d	Pbp2B	bGS31	20 µM IPTG, continuous 200 pM JF549-HTL, 15 min
2b		bAB309	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
2c		bAB309 & bGS104	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
2d		bGS104	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
2e	Slower	bGS109	20 pM JF549-HTL,1 hr
2e	Control	bAB309 & bGS104	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
2e	Faster	bGS328	~65 mM xyl, during imaging 20 pM JF549-HTL,1 hr
2f	FtsZ	bAB309 & bGS104	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
2f	FtsA	bAB213	50 pM JF549-HTL, 15 min
2f	EzrA	bMH03	300 pM JF549-HTL, 15 min
2f	SepF	bMH332	no IPTG 200 pM JF549-HTL, 15 min

2f	ZapA	bMH559	600 pM JF549-HTL, 15 min
3a, left	Control	bAB219	20 µM IPTG,1 hr
3a, left	Δ*ezrA*	bGS256	20 µM IPTG,1 hr
3a, left	Δ*sepF*	bGS254	20 µM IPTG,1 hr
3a, left	Δ*zapA*	bGS250	20 µM IPTG,1 hr
3a, right	Control	bAB309 & bGS104	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
3a, right	Δ*ezrA*	bGS167	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
3a, right	Δ*sepF*	bGS304	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
3a, right	Δ*zapA*	bGS141	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
3b, left	Control	bAB219	20 µM IPTG,1 hr
3b left	↑*ezrA*	bGS263	xyl as indicated, 2 hr 20 µM IPTG,1 hr
3b, right	Control	bAB309 & bGS104	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
3b, right	↑*ezrA*	bGS157	xyl as indicated, 2 hr 20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
3c	Control	bAB219	20 µM IPTG,1 hr
3c	↑*ezrA*	bGS263	xyl as indicated, 2 hr 20 µM IPTG,1 hr
3d, left	Control	bAB219	20 µM IPTG,1 hr
3d, left	ΔZBPs	bGS308	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
3d, right	Control	bAB309 & bGS104	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
3d, right	ΔZBPs	bGS331	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
3e	Control	bAB219	20 µM IPTG,1 hr
3e	ΔZBPs	bGS308	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
3f	Control	bAB219	20 µM IPTG,1 hr
3f	ΔZBPs	bGS308	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
3g	ΔZBPs	bGS308	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
4a		bAB219	20 µM IPTG,1 hr
4b		bAB219	20 µM IPTG,1 hr

4c	Control	bAB219	20 µM IPTG,1 hr
4c	Δ*ezrA* ↓*zapA*	bGS293	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
4c	FtsZ(K86E) Δ*ezrA* Δ*zapA*	bGS463	20 µM IPTG,1 hr
4d		bMH443	1 mM xyl overnight, withdrawn for 7 hr 100 µM IPTG overnight, reduced to 20 µM for 7 hr 100 pM JF549-HTL, 15 min
4e	Control: Pbp2B colocalization	bGS104	20 µM IPTG,1 hr 5 nM JF549-HTL,1 hr
4e	Control: FDAA labelling	bMH510	20 µM IPTG,1 hr 5 nM JF549-HTL,1 hr 1 mM FDL, 3 min, washed out
4e	ΔZBPs: Pbp2B colocalization	bMH445	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr 5 nM JF549-HTL,1 hr
4e	ΔZBPs: FDAA labelling	bMH508	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr 5 nM JF549-HTL,1 hr 1 mM FDL, 3 min, washed out
ED1a	WT	PY79	
ED1a	HT-FtsZ	bAB309	20 µM IPTG, 2 hr
ED1a	FtsA-HT(sw)	bAB213	
ED1a	EzrA-HT	bMH03	
ED1a	SepF-HT	bMH332	no IPTG
ED1a	ZapA-HT	bMH559	
ED1a	HT-DivIB	bAB352	1 mM xyl, continuous
ED1a	HT-DivIC	bAB347	10 mM xyl, continuous
ED1a	HT-FtsL	bMH47	50 µm IPTG, continuous
ED1a	HT-FtsW	bAB350	10 mM xyl, continuous
ED1a	HT-Pbp2B	bGS28	50 µM IPTG, continuous
ED1b	WT	PY79	
ED1b	Δ*ezrA*	bMH45	
ED1b	Δ*sepF*	bSW234	
ED1b	Δ*zapA*	RL2638	
ED1b	Δ*divIB*	bMH92	
ED1c	Δ*zapA*	RL2638	
ED1c	Δ*zapA* EzrA-HT	bMH221	
ED1c	Δ*ezrA*	bMH45	
ED1c	Δ*ezrA* SepF-HT	bMH542	no IPTG

ED1c	Δ*ezrA* ZapA-HT	bMH565	
ED2a		bAB309 & bGS104	20 μM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED2b	HMM	bAB309 & bGS104	20 μM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED2b	Manual	bAB309	20 μM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED2c	1 colour	bAB309	20 μM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED2c	2 colour	bGS104	20 μM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED2d	0.5 s	bAB309 & bGS104	20 μM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED2d	1 s	bAB309	20 μM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED2e	Control	bAB219	20 μM IPTG,1 hr
ED2e	↑*ftsAZ*	bAB219	100 μM IPTG, 1 hr
ED2f	Control	bAB309 & bGS104	20 μM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED2f	↑*ftsAZ*	bAB309	100 μM IPTG, 2 hr 20 pM JF549-HTL,1 hr
ED3	Control	bAB219	20 μM IPTG,1 hr
ED3	Δ*ezrA*	bGS256	20 μM IPTG,1 hr
ED3	Δ*sepF*	bGS254	20 μM IPTG,1 hr
ED3	Δ*zapA*	bGS250	20 μM IPTG,1 hr
ED3	FtsZ(T111A)	bAB281	20 μM IPTG,1 hr
ED4a	↑*sepF*	bGS260	30 mM xyl, 2 hr 20 μM IPTG,1 hr
ED4a	↑*zapA*	bGS259	30 mM xyl, 2 hr 20 μM IPTG,1 hr
ED4b, left	Control	bAB219	20 μM IPTG,1 hr
ED4b, left	↑*sepF*	bGS260	30 mM xyl, 2 hr 20 μM IPTG,1 hr
ED4b, left	↑*zapA*	bGS259	30 mM xyl, 2 hr 20 μM IPTG,1 hr
ED4b, right	Control	bAB309 & bGS104	20 μM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED4b, right	↑*sepF*	bGS158	30 mM xyl, 2 hr 20 μM IPTG,1 hr 20 pM JF549-HTL,1 hr

ED4b, right	↑*zapA*	bGS159	30 mM xyl, 2 hr 20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED4c	Control	bAB219	50 µM IPTG,1 hr
ED4c	↑*ezrA*	bGS263	xyl as indicated, 2 hr 50 µM IPTG,1 hr
ED5	Control	bAB219	20 µM IPTG,1 hr
ED5	Δ*ezrA* ↓*sepF*	bGS290	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED5	↓*ezrA* Δ*sepF*	bGS298	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED5	Δ*ezrA* ↓*zapA*	bGS293	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED5	↓*ezrA* Δ*zapA*	bGS297	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED5	↓*ezrA* Δ*sepF* Δ*zapA*	bGS308	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6a, left	Control	bAB219	20 µM IPTG,1 hr
ED6a, left	Δ*ezrA* ↓*sepF*	bGS290	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6a, left	↓*ezrA* Δ*sepF*	bGS298	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6a, left	Δ*ezrA* ↓*zapA*	bGS293	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6a, left	↓*ezrA* Δ*zapA*	bGS297	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6a, left	↓*ezrA* Δ*sepF* Δ*zapA*	bGS308	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6a, right	Control	bAB309 & bGS104	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED6a, right	Δ*ezrA* ↓*sepF*	bGS204	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED6a, right	↓*ezrA* Δ*sepF*	bGS316	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED6a, right	Δ*ezrA* ↓*zapA*	bGS206	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED6a, right	↓*ezrA* Δ*zapA*	bGS306	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED6a, right	↓*ezrA* Δ*sepF* Δ*zapA*	bGS331	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr

ED6b	Control	bAB219	20 µM IPTG,1 hr
ED6b	Δ*ezrA* ↓*sepF*	bGS290	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6b	↓*ezrA* Δ*sepF*	bGS298	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6b	Δ*ezrA* ↓*zapA*	bGS293	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6b	↓*ezrA* Δ*zapA*	bGS297	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6b	↓*ezrA* Δ*sepF* Δ*zapA*	bGS308	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6c, left	Control	bAB219	20 µM IPTG,1 hr
ED6c, left	Δ*ezrA*	bGS256	20 µM IPTG,1 hr
ED6c, left	↓*sepF*	bGS590	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6c, left	↓*zapA*	bGS586	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6c, left	Δ*ezrA* ↓*sepF*	bGS290	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6c, left	Δ*ezrA* ↓*zapA*	bGS293	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6c, right	Control	bAB219	20 µM IPTG,1 hr
ED6c, right	↓*ezrA*	bGS588	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6c, right	Δ*sepF*	bGS254	20 µM IPTG,1 hr
ED6c, right	Δ*zapA*	bGS250	20 µM IPTG,1 hr
ED6c, right	↓*ezrA* Δ*sepF*	bGS298	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6c, right	↓*ezrA* Δ*zapA*	bGS297	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED6c, right	↓*ezrA* Δ*sepF* Δ*zapA*	bGS308	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
ED7, top	Control	bAB219	20 µM IPTG,1 hr
ED7, top	Δ*sepF* Δ*zapA*	bGS368	20 µM IPTG,1 hr
ED7, bottom left	Control	bAB219	20 µM IPTG,1 hr
ED7, bottom left	Δ*sepF* Δ*zapA*	bGS368	20 µM IPTG,1 hr
ED7, bottom centre	Control	bAB309 & bGS104	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
ED7, bottom centre	Δ*sepF* Δ*zapA*	bGS318	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr

ED7, bottom right	Control	bAB219	20 µM IPTG,1 hr
ED7, bottom right	Δ*sepF* Δ*zapA*	bGS368	20 µM IPTG,1 hr
ED8a	FtsZ(K86E)	bGS432	20 µM IPTG,1 hr
ED8a	FtsZ(K86E) Δ*ezrA* Δ*zapA*	bGS463	20 µM IPTG,1 hr
ED8b	Control	bAB219	20 µM IPTG,1 hr
ED8b	FtsZ(K86E)	bGS432	20 µM IPTG,1 hr
ED8b	FtsZ(K86E) Δ*ezrA* Δ*zapA*	bGS463	20 µM IPTG,1 hr
ED8c	Control	bGS104	20 µM IPTG,1 hr 5 nM JF549-HTL,1 hr
ED8c	FtsZ(K86E)	bGS618	20 µM IPTG,1 hr 5 nM JF549-HTL,1 hr
ED8c	Δ*ezrA* ↓*zapA*	bGS644	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr 5 nM JF549-HTL,1 hr
ED8c	FtsZ(K86E) Δ*ezrA* Δ*zapA*	bGS628	20 µM IPTG,1 hr 5 nM JF549-HTL,1 hr
ED9a	Control	bGS104	20 µM IPTG,1 hr 5 nM JF549-HTL,1 hr
ED9a	ΔZBPs	bMH445	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr 5 nM JF549-HTL,1 hr
ED9b	Control	bMH510	20 µM IPTG,1 hr 5 nM JF549-HTL,1 hr 1 mM FDL, 3 min, washed out
ED9b	ΔZBPs	bMH508	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr 5 nM JF549-HTL,1 hr 1 mM FDL, 3 min, washed out
ED9c		bGS104	20 µM IPTG,1 hr 5 nM JF549-HTL,1 hr
ED9d	Control	bMH512	100 µM IPTG overnight, reduced to 20 µM for 7 hr 100 pM JF549-HTL, 15 min
ED9d	ΔZBPs	bMH443	1 mM xyl overnight, withdrawn for 7 hr 100 µM IPTG overnight, reduced to 20 µM for 7 hr 100 pM JF549-HTL, 15 min
ED10a	Control	bGS630	30 mM xyl, 1 hr
ED10a	Δ*ftsA*	bGS639	10 µM IPTG, continuous 30 mM xyl, 1 hr
ED10b	Control	bGS630	30 mM xyl, 1 hr
ED10b	Δ*ftsA*	bGS639	10 µM IPTG, continuous 30 mM xyl, 1 hr

ED10c	Control	bGS630	30 mM xyl, 1 hr
ED10c	Δ*ftsA*	bGS639	10 µM IPTG, continuous 30 mM xyl, 1 hr
Video 1		bAB219	20 µM IPTG,1 hr
Video 2	EzrA	bMH42	300 pM JF549-HTL, 15 min
Video 2	SepF	bMH372	no IPTG 200 pM JF549-HTL, 15 min
Video 2	ZapA	bMH559	600 pM JF549-HTL, 15 min
Video 3	DivIB	bAB366	1 mM xyl, continuous 400 pM JF549-HTL, 15 min
Video 3	DivIC	bAB367	5 mM xyl, continuous 500 pM JF549-HTL, 15 min
Video 3	FtsL	bGS165	30 µm IPTG, continuous 40 pM JF549-HTL, 15 min
Video 3	FtsW	bAB368	8 mM xyl, continuous 300 pM JF549-HTL, 15 min
Video 3	Pbp2B	bGS31	20 µM IPTG, continuous 200 pM JF549-HTL, 15 min
Video 4		bAB309	20 µM IPTG,1 hr 20 pM JF549-HTL,1 hr
Video 5	Control	bAB219	50 µM IPTG,1 hr
Video 5	↑*ezrA*	bGS263	xyl as indicated, 2 hr 50 µM IPTG,1 hr
Video 6	Control	bAB219	20 µM IPTG,1 hr
Video 6	ΔZBPs	bGS308	1 mM xyl overnight, withdrawn for 7 hr 20 µM IPTG,1 hr
Video 7	Control	bGS630	30 mM xyl, 1 hr
Video 7	Δ*ftsA*	bGS639	10 µM IPTG, continuous 30 mM xyl, 1 hr

Abbreviations: ↑: overexpression, ↓: depletion, xyl: xylose, JF549-HTL: JF549-HaloTag linker, FDL: fluorescent D-lysine.

Supplementary Discussion 1

Understanding EzrA's function has been complicated by EzrA's apparently contradictory effects on the Z ring. On one hand, EzrA has been repeatedly characterized as a negative regulator of FtsZ polymers[13,30-33,65,66]. On the other hand, EzrA is known to be synthetically lethal with SepF and ZapA, which are positive regulators of FtsZ[18,29]. It has also been shown that EzrA's role in inhibiting polar Z ring formation and EzrA's role at midcell are separable: mutants in EzrA can be made that disrupt one of these functions but not the other[34,67].

The results presented here also indicate that EzrA has two separable functions. We find that EzrA plays a positive role in condensing the Z ring *in vivo*, working together with SepF and ZapA to promote lateral bundling of the Z ring. Z ring condensation is essential for cell division, and this function explains the synthetic lethalities of Δ*ezrA* mutants with Δ*sepF* and Δ*zapA* mutants[18,29]. We additionally find that EzrA decreases the length of FtsZ filaments. This is consistent with the inhibition of Z ring formation by *ezrA* overexpression[30] and the fact that Z rings in Δ*ezrA* cells recover more slowly after photobleaching[13]. We find that this length decrease does not change FtsZ's treadmilling dynamics, and thus the concentration of free FtsZ monomer. This agrees with *in vitro* results that EzrA has no effect on FtsZ's GTPase activity[30,32]. Because EzrA has also been shown to increase the amount of FtsZ needed to form polymer[30,31,33], and because EzrA is highly expressed in the cell (10,000-20,000 molecules per cell, versus 5,000 for FtsZ)[30,68], this length decrease is likely due to monomer sequestration, as previously proposed[31].

Our results also indicate that these two functions are separable. In cells missing both EzrA and one or both of its synthetically lethal partners Δ*sepF* or Δ*zapA*, Z ring condensation is disrupted but we no longer observe a change in filament length. There are several possible mechanisms by which EzrA's effect on length may be absent in these conditions. One possibility is that this effect happens specifically during Z ring constriction, as has been previously suggested for EzrA; because cytokinesis is inhibited in these conditions, such effects would be lost[18,69]. More generally, inhibition of cell division may trigger stress responses that affect FtsZ polymer equilibria. Regardless, the observation that EzrA's effects are separable is consistent with previous studies[34,67], and our results indicate that EzrA's role in bundling is of primary importance for cell division.

Supplementary Video Legends

Supplementary Video 1: FtsZ filaments treadmill around the cell. Cells expressing FtsZ-mNeonGreen (strain bAB219) were induced with 20 µM IPTG for 2 hours, then imaged at 1-second intervals for 100 seconds by TIRFM. The video is displayed at 30 frames per second (30x actual speed). Scale bar: 5 µm.

Supplementary Video 2: Single molecules of EzrA, SepF, and ZapA are stationary. Each protein was expressed as a HaloTag fusion and labelled with JF549-HTL dye for single-molecule imaging using TIRFM. Videos begin with an image of the Z rings in each cell, visualized by FtsZ-mNeonGreen. Cells were imaged at 1-second intervals for 200 seconds; every other frame is displayed here. The video is displayed at 30 frames per second (60x actual speed). Scale bar: 5 µm.

Supplementary Video 3: Single molecules of DivIB, DivIC, FtsL, FtsW, and Pbp2B move directionally around the division site. Each protein was expressed as a HaloTag fusion and labelled with JF549-HTL dye for single-molecule imaging using TIRFM. Videos begin with an image of the Z rings in each cell, visualized by FtsZ-mNeonGreen. Cells were imaged at 1-second intervals for 200 seconds; every other frame is displayed here. The video is displayed at 30 frames per second (60x actual speed). Scale bar: 5 µm.

Supplementary Video 4: Single-molecule imaging of FtsZ for lifetime analysis. Cells expressing FtsZ-HaloTag were imaged at 500-ms intervals for 50 seconds by TIRFM. The video is displayed at 30 frames per second (15x actual speed). Scale bar: 5 µm.

Supplementary Video 5: EzrA overexpression decreases FtsZ filament length, visualized by SIM-TIRF microscopy. A second copy of *ezrA* was expressed from a xylose-inducible promoter. Panel labels indicate the amount of xylose added in each experiment. Cells expressed FtsZ-mNeonGreen to visualize FtsZ filaments and were imaged at 1-second intervals for 100 seconds by SIM-TIRF microscopy. The video is displayed at 30 frames per second (30x actual speed). Scale bar: 5 µm.

Supplementary Video 6: FtsZ filament treadmilling is unaltered in the absence of ZBPs. ΔZBPs cells have *sepF* and *zapA* knocked out, and *ezrA* depleted from a xylose-inducible promoter for 7 hours prior to imaging. Cells expressing FtsZ-mNeonGreen were imaged at 1-second intervals for 100 seconds by TIRFM. The video is displayed at 30 frames per second (30x actual speed). Scale bar: 5 µm.

Supplementary Video 7: FtsA filaments show less directional treadmilling in a Δ*ftsA* strain. Δ*ftsA* cells express FtsZ under the pHyperSpank promoter induced with 10 µM IPTG. Cells expressing FtsZ-mNeonGreen were imaged at 1-second intervals for 100 seconds by TIRFM. The video is displayed at 30 frames per second (30x actual speed). Scale bar: 5 µm.