Supporting Information for

Design of a histidine kinase FRET sensor to detect complex signal integration within living bacteria

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Supplementary Figures

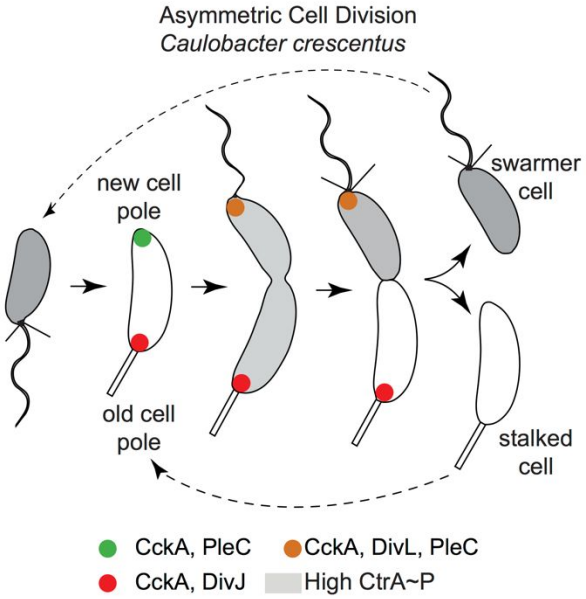


Figure S1: Asymmetric cell division in Caulobacter crescentus
Asymmetric cell division in *C. crescentus* is a tightly regulated process facilitated by the new cell pole signaling complex that includes the histidine kinases CckA and PleC and the pseudokinase DivL. Collectively, this multikinase network regulates CtrA phosphorylation levels in order to turn on gene transcription and block the origin of replication. The old cell pole signaling complex histidine kinases of DivJ and CckA work as a system to downregulate CtrA phosphorylation levels.

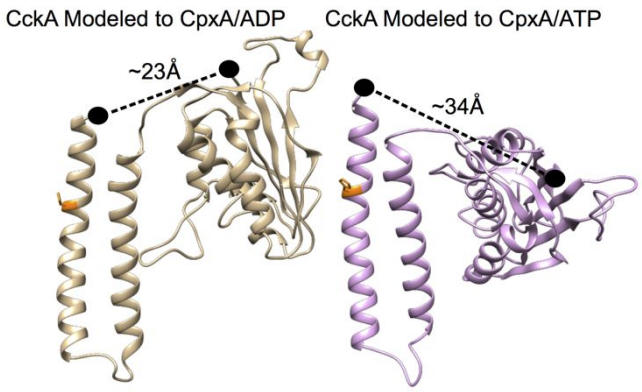


Figure S2: CckA modeling to CpxA to identify domain conformational changes
CckA DHp/CA modeled to CpxA crystal structures bound to ADP (PDB: 4BIX) and ATP (PDB: 4BIV) with residues at the top of the bundle and CA domain measured to signify the distance change that occurs between histidine kinase states.

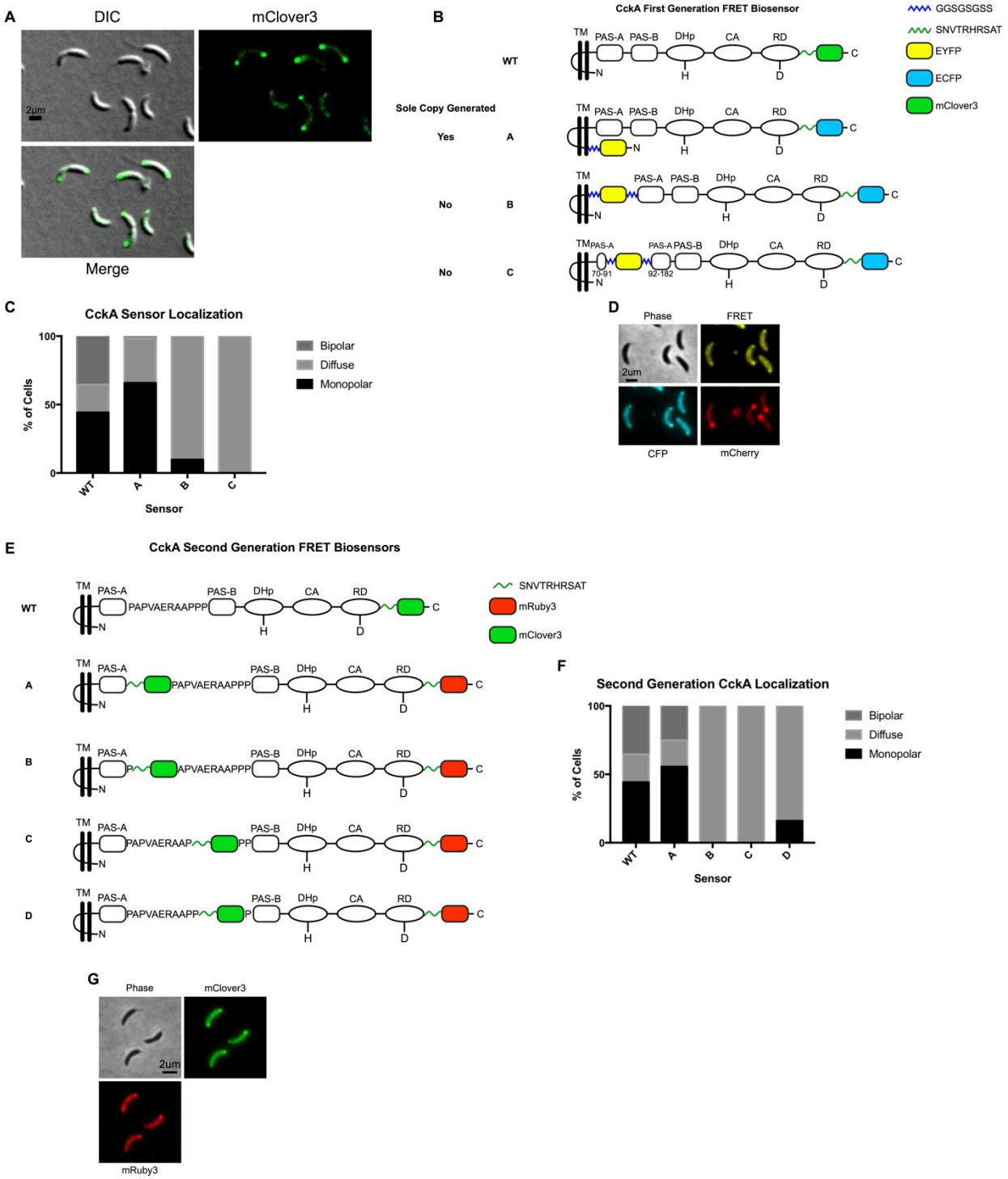


Figure S3: CckA FRET sensor designs and localization screens

A) *cckA::cckA-mClover3* cells imaged under DIC and GFP filter settings. **B)** Panel of first-generation FRET sensors designed using ECFP and EYFP as the fluorescent protein pair. The C-terminal ECFP remained static across all designs while the N-terminal EYFP was moved to generate three sensors. Sensor A was the only sensor functional as the wild-type copy. **C)** Localization pattern of all three sensors compared to NA1000 *cckA::cckA-mClover3* cells, all unsynchronized populations. **D)** Sensor A cells imaged under FRET imaging conditions also

expressing *tipN::tipN-mCherry* in order to visualize the new cell pole. **E)** Second-generation FRET sensor design panel using mClover3 and mRuby3 as the fluorescent protein pair. The C-terminal mRuby3 position remained fixed and the mClover3 was inserted at varied positions with the linker between PAS-A and PAS-B. **F)** Localization of second-generation FRET sensor panel compared to NA1000 *cckA::cckA-mClover3*. **G)** Second-generation Sensor A imaged under GFP and mCherry non-FRET imaging conditions to visualize localization of sensor.

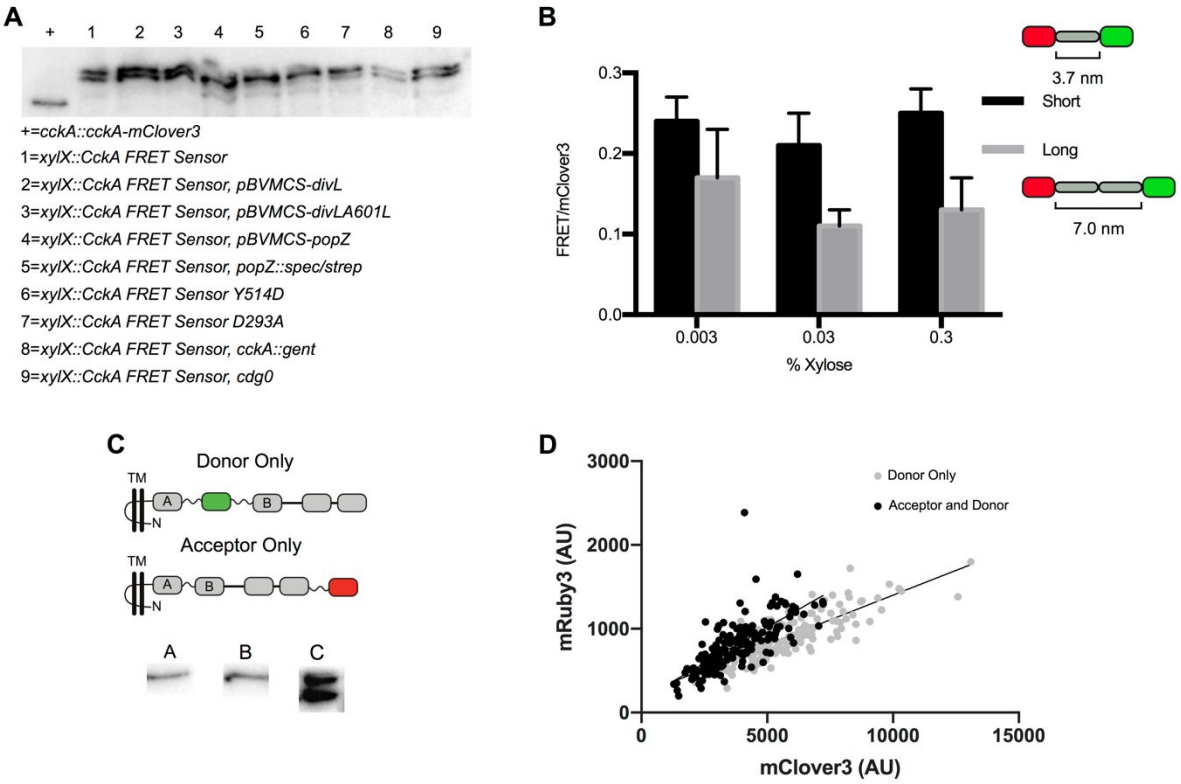


Figure S4: Characterization of FRET standards that utilize fibronectin to rigidly separate mRuby and mClover3 at a distance of 3.7 nm or 7.4 nm and CckA FRET sensor western blot

A) Western blot using anti-GFP antibody to detect CckA FRET sensor (MW ~123kDa) and *cckA::cckA-mClover3* (~96kDa). **B)** Comparison of short and long FRET standards expressed from the xylose locus in *C. crescentus* after 3 hours. Standards were created using domain f fibronectin (FN) 7 for the short standard and FN8-10 for the long standard. **C)** Cartoon representations of donor only (green represents mClover3) and acceptor only (red represents mRuby3. The donor only construct was blotted using an anti-GFP antibody. The acceptor only contained a Flagtag and was blotted using an anti-Flagtag antibody. Western blots: A is the donor only, B is the donor in a strain expressing donor and acceptor, and C is the acceptor in the strain expressing both donor and acceptor constructs. **D)** Plot of acceptor (mRuby3) intensity vs. donor (mClover3) intensity for strains expressing donor only (gray) R^2=0.6723, donor and acceptor (black) R^2=0.5329.

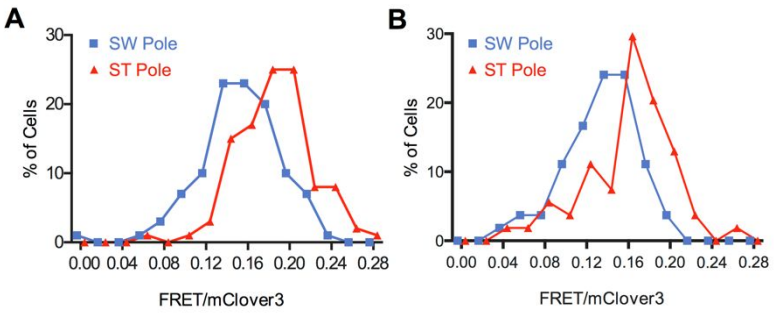


Figure S5: Histograms of wild-type and sole copy CckA FRET sensor
A) Histogram of swarmer (new) and stalk (old) cells pole average FRET/mClover3 of CckA FRET sensor in wild-type *C. crescentus*, n=106. **B)** Histogram of CckA FRET sensor in *cckA::gent* cells, n=190.

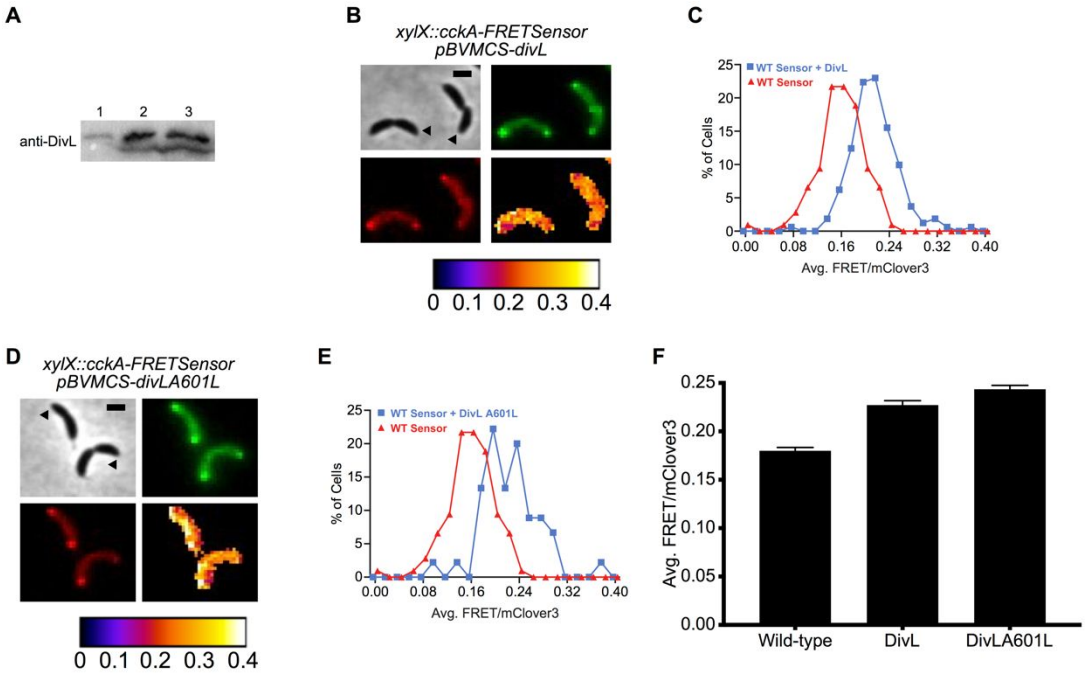


Figure S6: DivL overexpression leads to increase in average FRET/mClover3 in vitro
A) Western blot for DivL in wild-type cells (1), DivL overexpression (2), and DivLA601L (3) using the anti-DivL antibody. **(B)** CckA FRET sensor expressed with DivL overexpressed in wild-type cells. **C)** Histogram of CckA FRET sensor in cells overexpressing DivL compared to CckA FRET sensor with wild-type levels of DivL, n=160. **(D)** CckA FRET sensor expressed with DivLA601L overexpressed in wild-type cells. **E)** Histogram of CckA FRET sensor in cells overexpressing DivL compared to CckA FRET sensor with wild-type levels of DivLA601L, n=102. **F)** Old cell pole average FRET/mclover3 for cells expressing CckA FRET sensor in *C. crescentus* wild-type strain, DivL overexpression cells, and DivL A601L overexpression cells.

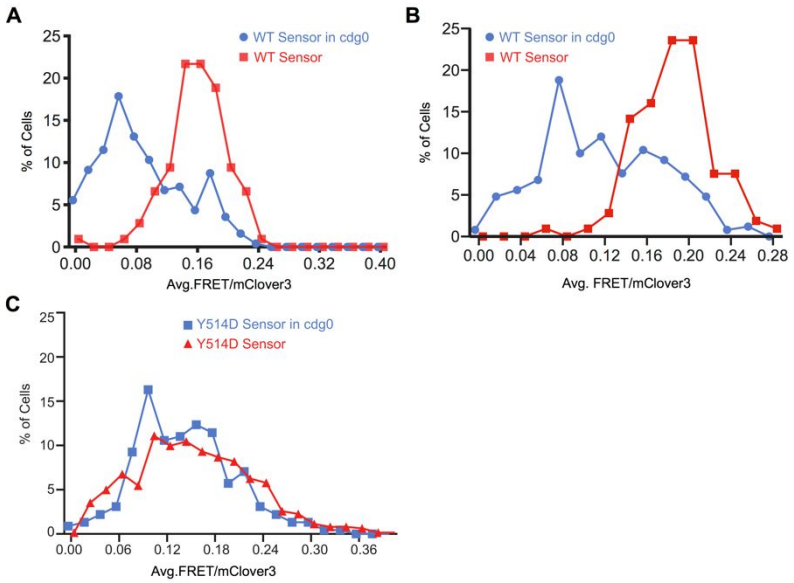


Figure S7: Histograms of CckA FRET sensor in cdg^0 and CckAY514D FRET sensor.
A) Histogram of CckA FRET sensor in wild-type cells and cdg^0 cells (n=252) at the new cell pole. **B)** Histogram of CckA FRET sensor in wild-type cells and cdg0 cells (n=250) at the old cell pole. **C)** Histogram of CckAY514D FRET sensor in wild-type cells (n=721) and cdg^0 (n=227).

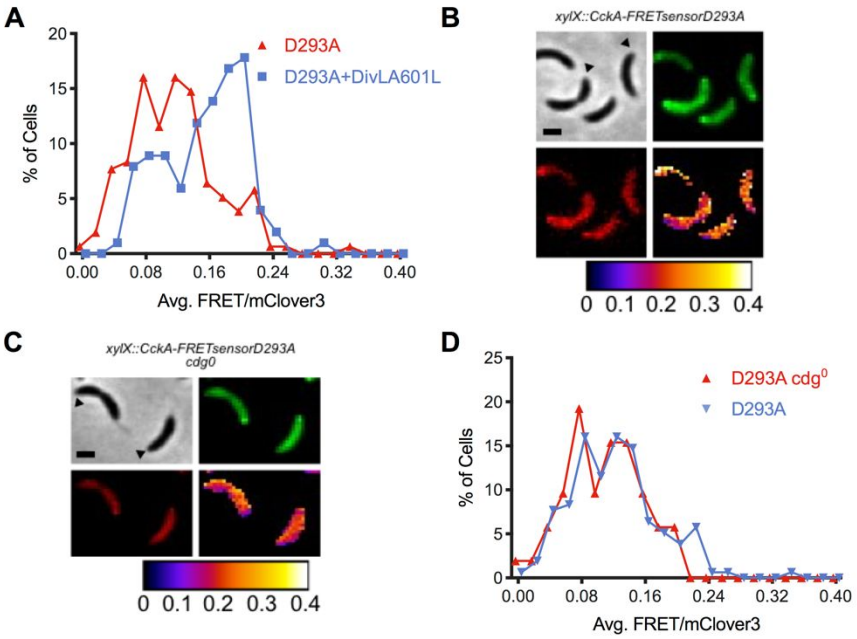


Figure S8: Histograms of CckA FRET sensor in cdg^0 and CckAY514D FRET sensor.
A) CckAD293A FRET sensor in wild-type cells. **B)** CckAD293A FRET sensor in cdg^0 cells. Histogram of CckA FRET sensor in wild-type cells and cdg0 cells (n=252) at the new cell pole. **C)** CckAD293A FRET sensor in wild-type cells (n=156) compared to CckAD293A in cells overexpressing DivLA601L (n=101). **D)** Histogram of CckAD293A FRET sensor in wild-type cells (n=156) and cdg^0 (n=52).

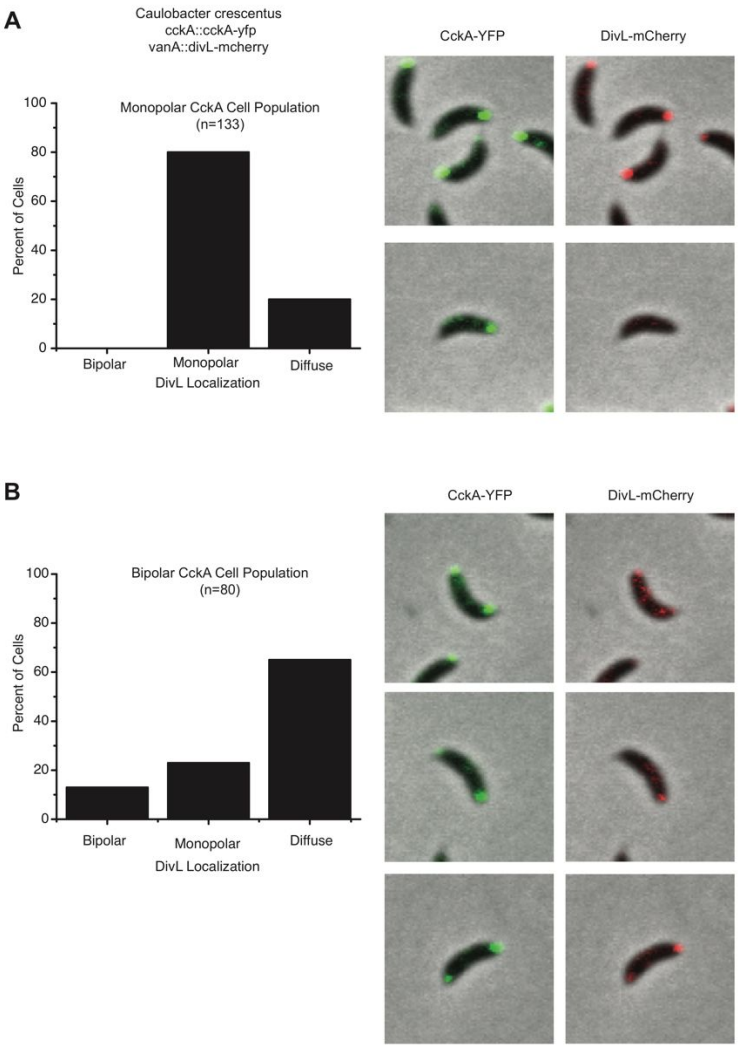


Figure S9: DivL localization in different CckA localization backgrounds
A) DivL-mCherry expressed from an integrated vanillate promoter is ~80% monopolar when CckA-EYFP is monopolar. **B) In contrast,** when CckA-EYFP is bipolar, we observed that ~80% of cells exhibit diffuse DivL with smaller bipolar and monopolar populations.

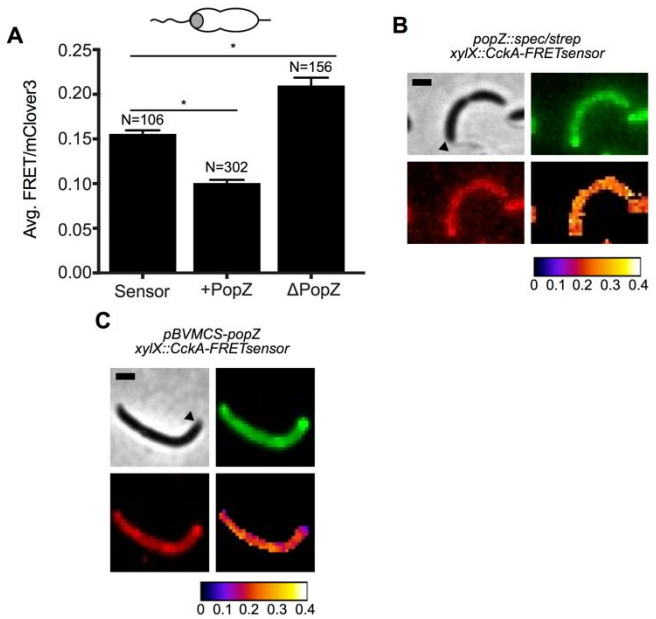
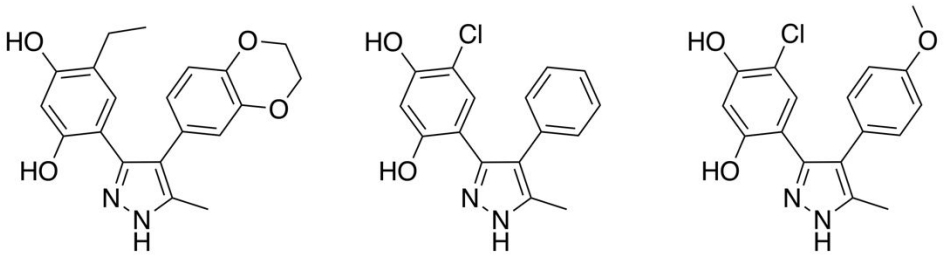


Figure S10: PopZ scaffold protein levels impact CckA FRET
A) CckA FRET sensor in cells where *popZ* has been deleted compared with a PopZ overexpression from a vanillate inducible replicating plasmid (+PopZ) and the CckA FRET sensor in wild-type cells shows a significant difference ($p<0.05$). **(B)** CckA FRET sensor in cells where *popZ* has been deleted. **(C)** Cells expressing the CckA FRET sensor and overexpressing PopZ from a replicating vanillate inducible plasmid.



CCT018159 CDV008 CDV009

Figure S11: Diaryl pyrazole scaffold for ATP binding competitive inhibitors
Structures of CCT018159, CDV008, and CDV009.

SI Methods Growth Conditions

E. coli DH5α strains were grown at 37 °C with shaking overnight in LB media supplemented with antibiotics (SI Table 6). *C. crescentus* strains were grown in PYE or M2G media supplemented with antibiotics (SI Table 6) at 28 °C with shaking overnight or for specified times for imaging.

Electroporation

Plasmids introduced into *C. crescentus* by centrifuging 4 mL for 5 minutes at 21,100 x g. Supernatant was removed and cells were washed in 100 µL of cold, sterile water and centrifuged for 1 minute at 21,100 x g. This step was repeated 2 more times, followed by suspension of cells in a final volume of 80 µL. Integrating plasmids (12.5 µL) or replication plasmids (3 µL) were then added to the cells and electroporated on the *E. coli* setting of a BioRad GenePulser using 0.1 cm electroporation cuvettes (BioRad). Cells were then resuspended in 1 mL of 2xPYE and shaken at 28 °C for 3 hours, then 100 µL of cells were then plated on PYE/antibiotic plates. The remaining cells were pelleted, resuspended in 100 µL of 2xPYE, and plated on PYE/antibiotic plates. Cells were grown at 28 °C for 3 days and colonies were streaked onto a PYE/antibiotic plate. Colonies that grew after streaking were screened for using colony PCR (DreamTaq Green) using primers for integration at the xylose locus (RecUni-1, RecXyl-2), vanillate locus (RecUni-1, RecVan-2), or for replicating plasmids (Pvan-for, M13-for) according to the manufacturer's protocol with 5 minute extension times.

Phage Transduction

Phage transduction followed previously published protocol from Bert Ely and co-workers[1]. In order to delete *cckA* from strain WSC1413 containing the CckA-FRET sensor under the xylose promoter, a strain containing a gentamycin replacement of CckA was grown overnight in PYE (WSC1149). 5 µL of three concentrations of phage (1x, 10x dilution, 100x dilution) were each added to 0.5 mL of cells and incubated at room temperature for 15 minutes. Cells were then added to 2 mL of PYE in a 15 mL conical. 500 µL of warm PYE top agar (1.5% PYE agar) was then added to cells and dumped and spread on a PYE plain agar plate. Plates were incubated

overnight at 28 °C right-side-up. Plates with confluent lysis were recovered by scraping top agar layer into 50mL conical, washing plate with 5 mL of PYE, and adding 100 µL of chloroform followed by vortexing. The tube was shaken for 10 minutes at room temperature. After centrifuging for 30 minutes at 8000 rpm, supernatant was transferred to a new tube. 100 µL of chloroform was added and the tube was vortexed. Phage was aliquoted and stored at 4 °C. The strain WSC1413 was grown in PYE/chlor overnight. 100 µL of prepared phage was added to 500 µL of cells. Cells were incubated at room temp for 45 minutes to allow phage to attach to cells. Cells were centrifuged at 21,100 x g for 5 minutes, supernatant was removed, and cells were resuspended in 100 µL of PYE. After 2 hours of incubation at room temperature cells were plated on PYE/gent/chlor/0.003%xylose media to select for the deletion of *cckA.* Replacement was confirmed using colony PCR (DreamTaq) using primers nSWD35 and nSWD36.

Imaging Conditions

C. crescentus strains for single time point imaging were grown overnight in M2G media supplemented with antibiotics to an OD_{600} 0.1-0.2. Cells were then induced with either xylose (0.03% w/v) or vanillate (0.5 mM) or both for 3 hours. 2 µL of cells were deposited on a 1% agarose/M2G pad on a glass slide and covered with a glass coverslip. For intermolecular experiments, 0.03% xylose was used and 0.15 mM van was used to avoid increasing levels of CckA too much. For experiments evaluating histidine kinase directed antibiotics, cells were then grown in 0.03% xylose for 2 hours and an image was taken (Time 0). The culture was then split and either DMSO, CCT018159 (28 µM) , Kanamycin (2.5 µg/mL), CDV008 (17.5 µM), or CDV008 (12.5 µM) were added to cultures. Cells were then imaged as a time-course for 210 minutes.

Time-lapse and Time-course Synchrony

Method adapted from Schrader, et al[2]. Synchronized *C. crescentus* cells for time-lapse or time-course imaging were grown overnight in M2G media supplemented with antibiotics to a cell density between OD_{600} 0.1 and 0.2 before being induced with either xylose (0.03% w/v) or vanillate (0.5 mM) or both for 3 hours. Cells were then spun down at 4000 rpm for 17 minutes at 4 °C. Supernatant was removed and pellet was resuspended in 1mL of cold 1xM2. Cells were transferred to a 2mL Eppendorf tube and spun for 3 minutes at 4 °C at 13000 rpm. Supernatant was removed and the pellet was resuspended in 900 µL of cold 1xM2. 900 µL of Percoll was then added. Cells were then spun at 11000 rpm for 20 minutes at 4 °C in order to density centrifugate stalk from swarmer cells. The top band (stalk cells) was then removed and the bottom band (swarmer cells) was isolated and washed with 1 mL of 1xM2. Cells were spun at 7500rpm for 3 minutes at 4 °C. Cells were then washed again with 1xM2. Pelleted cells were resuspended in M2G and incubated with shaking at 28 °C for 5 minutes. 2 µL of cells were deposited on a 0.75% agarose/M2G pad on a glass slide and covered with a glass coverslip. Wax was then used to seal pad to prevent evaporation for time-lapses. For time-courses 2 µL of cells were deposited on a 1% agarose/M2G pad on a glass slide and covered with a glass coverslip. Cells were incubated at 28 °C with shaking during the duration of the time-course.

Microscopy

Images were taken on a Nikon TiE inverted setup using an APO Lambda 100X oil objective with Phase Contrast or Differential Interference Contrast (DIC) using Zeiss Immersol 518F Immersion Oil using Nikon Element AR software to control the setup. A halogen lamp was used for the white light source. Spectra X light engine was used for all fluorescence excitation. An Andor Ixon Ultra 897 EMCCD camera was used for all imaging. All filter sets used were purchased from Chroma. CFP/YFP/mCherry (77074157) and GFP (77074160) filter cubes were used with emission filter sets GFP (77074161) and CFP/YFP/mCherry (77074158).

FRET Microscopy and Image Processing

Simultaneous FRET imaging was done by placing a Hamamatsu W-View Gemini Image Splitter in the light path between the Nikon TiE and the camera. A GFP/mCherry dichroic mirror, GFP emission filter, and mCherry emission filter were used. Alignment of the channels was done using the supplied alignment slide. Alignment of the channels was done before each imaging event. Each strain was imaged under the same conditions (600 millisecond Autoexposure, 100 Gain, 2x2 binning for capture, and 15% power). Background from the fluorescent channels was subtracted in the Nikon software and images were exported at Tiffs for processing in ImageJ.[3] In ImageJ a mask was generated by thresholding cell signal above background. The original image was then multiplied by the masked image to remove background signal and the mCherry emission channel image was divided by the GFP emission channel (32 bit image) to generate a ratiometric FRET image.

Image Analysis

For each experiment, 4 images were uploaded into MicrobeJ[4] (Phase, GFP channel, mCherry channel, and ratiometric image). The phase image was used to generate a cell outline using the Medial Axis setting. Cell poles were outlined using the MicrobeJ automatic pole settings which to draw a shape around the cell pole. MicrobeJ was preset to mark the stalk pole as the pole with higher intensity in the GFP channel. After software chose the poles, manual adjustment was done by choosing cells with a visible stalk and cells with incorrect pole alignment were corrected manually. MicrobeJ's data analysis tool was then used to average the intensity of the cell pole and body. Data was exported to Prism in order to generate histograms and average data. Student's t-test was used to determine significant difference in populations. Error bars are reported as standard error of the mean.

Western Blot

Western blots were done by centrifuging 1 mL of cells of a known OD_{600}, removing the supernatant and resuspending in 100 µL of 4x Sample Buffer. Samples were then heated at 75 °C for 10 minutes. Samples were loaded onto a 10% resolving SDS-PAGE gel with a 4%

stacking gel. Loading volumes were normalized using OD_{600}. The ladder used was ThermoFisher Page Ruler Pre-Stained Ladder. Transfer was done using ThermoFisher Mini Blot Module with a PVDF transfer membrane for 2 hours at 20 V and 4 °C. Blotting for DivL was done by blocking overnight at 4 °C in blocking buffer (0.1% Tween, 1xTBS, 5% milk). The blot was rinsed 3x5 min in TBST (1xTBS, 0.1% Tween-20). It was then incubated in primary antibody solution (TBST, 1:10000 anti-DivL antibody dilution) for 1 hour at 4 °C. The blot was then rinsed 3x5 min in TBST and incubated in secondary antibody solution (TBST, 1:10000 goat anti-rabbit IgG HRP antibody, Sigma) for 1 hour at 4 °C. The blot was then rinsed 1x15 min, 2x5 min in TBST, brought to room temperature and exposed to chemiluminescent substrate (SuperSignal West Pico PLUS Chemiluminescent Substrate) and imaged using the Chemi Hi Resolution setting on a BioRad ChemiDoc with auto exposure for intense bands.

Blotting for mClover3 was done using the same procedure for SDS-PAGE gel and transfer. The resulting blot was blocked in 1xTBST (0.2% Tween-20) with 5% milk for 2 hours at 4 °C. After two hours the solution was changed to 1xTBST (0.2% Tween-20) with 5% milk with a 1:2000 dilution of GFP Rabbit Polyclonal Antibody (ProteinTech) and incubated overnight at 4 °C. The blot was then washed in 1xTBST (0.2% Tween-20) 3x10 minutes. It was then incubated for 1 hour in 1xTBST (0.2% Tween-20) with 5% milk with 1:5000 dilution of goat anti-rabbit IgG HRP antibody for 1 hour at 4 °C. The blot was then washed 3x10 minutes in 1xTBST (0.2% Tween-20) and treated with chemiluminescent substrate the same way as the DivL blot.

Blotting for Flagtag was completed with same SDS-PAGE gel and transfer as above. Blots were incubated in 1xTBST (0.2% Tween-20), 5% milk overnight. The blot was then washed in 1xTBST (0.2% Tween-20) 3x5 minutes. It was then incubated for 1 hour in 1xTBST (0.2% Tween-20) with 5% milk with 1:5000 dilution of anti-Flagtag antibody (Sigma) for 1 hour at 4 °C. The blot was then washed 3x5 minutes in 1xTBST (0.2% Tween-20) and 1xTBST (0.2% Tween-20) with 5% milk with 1:10000 dilution of goat anti-rabbit IgG HRP was added and incubated for

one hour at 4ºC. The blot was then washed 3x5 minutes in 1xTBST (0.2% Tween-20) and treated with chemiluminescent substrate the same way as the DivL blot.

Construction of plasmids

The plasmids used in this study can be found in the Plasmids section with details on their construction. Screening primers for each plasmid are listed with the plasmid and the sequence is included in the Primer Table (Table S2, S4) The lists of oligonucleotides used in this study can be found in Table S4. All oligos were ordered from IDT. The strains used can be found in Table S6 and methods to generate strains are listed below. Phusion polymerase (ThermoFisher) was used for amplification of PCR products used in Gibson assembly and isolated using gel electrophoresis using a 1% agarose, 1xTAE gel with ethidium bromide added for visualization by UV light. Band sizes were determined using a GeneRuler 1 kb DNA Ladder (ThermoFisher). Bands were excised from the gel and extracted using a GeneJet Gel Extraction Kit (ThermoFisher) following manufacturer's protocols, with the addition of a 100µL binding buffer wash step following the spin through of DNA/agarose/binding buffer. Water was used for elution. DNA concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). All reactions were done according to the manufacturer's protocols with annealing temperature at 55 °C for 30s, unless otherwise noted. KOD Hot Start Polymerase (Millipore) was used for site-directed mutagenesis PCR according the manufacturer's protocols with annealing temperatures noted (Table S3) and 10 minute extension times. DpnI (Thermofisher) was used to degrade template after reaction was finished. 5µg of vector was digested at a time using the manufacturer's protocol and isolated the same way as PCR products (above). If a single restriction enzyme was used, FastAP (ThermoFisher) was used to decrease occurrence of reannealing.

Plasmids

Below is an example of generation of plasmid using the Gibson assembly method and the corresponding table. This can be used as a guide to understand the remainder of plasmids

constructed using the Gibson assembly method. Plasmids for Gibson assembly method were designed using J5.[5] All Gibson reaction buffers were made using Phusion polymerase (ThermoFisher), Taq ligase (NEB), and T5 exonuclease (NEB).[6] The plasmid pSWD95 encodes a xylose inducible copy of CckA-mRuby with mClover3 inserted between amino acids 182 and 183. mClover3 was linked to the CckA N-terminal piece using a GGSGS linker and was directly fused to the C-terminal piece of CckA. mRuby3 was linked to CckA using an SNVTHRSAT linker. The vector pXCFPC-6 was linearized by double digestion with the restriction endonucleases NheI and NdeI. PCR products with overhangs for Gibson assembly were generated following the schematic below at a 100 ng scale Gibson reaction scale. Four inserts were generated by PCR that include codons 1-182 of *cckA* were amplified by PCR using plasmid pMT383 as a template with primers SWD160 and SWD305, and codons 183-691 were amplified as a separate PCR product using primers SWD308 and SWD309. Additionally *mclover3* was amplified by PCR using plasmid pSWD14 as a template with primers SWD306 and SWD307. The c-terminal insert, *mruby3* was amplified by PCR using plasmid pSWD14 as a template with primers SWD310 and SWD311. The linearized pXCFPCp-6 and each of the four inserts were combined at 100 ng reaction scale and incubated at 50°C for 60 min in a reaction mixture containing T5 exonuclease, Phusion DNA polymerase, and Taq ligase (NEB). 5 µL of the reaction mixture was transformed into KCM competent DH5α *E. coli* cells and plated on LB/agar supplemented with the appropriate antibiotic. Colonies were screened using primers Pxyl-for Thanbichler, et al. [7] and M13-for[7] using DreamTaq pcr with a 5 minute extension time. The first generation CckA-FRET sensor contained EYFP that was joined to the CckA residue 69 using a GGSGSGSS linker (in primer) and to CckA residue 70 using a GGSGSGSS linker. CckA residue 691 was joined using and SNVTRHRSAT.

Table S1: Gibson assembly of plasmids for this study.

Plasmid	Insert Description	Forward Primer	Reverse Primer	Template	Screening primers
pSWD95 CckA-FRET Sensor	cckA_1-182	SWD160	SWD305	pMT383	Pxyl-for Thanbichler, et al. [7], M13-for[7]
	mClover3	SWD306	SWD307	pSWD14	
	cckA_183-691	SWD308	SWD309	pMT383	
	mRuby3	SWD310	SWD311	pSWD14	
	pXCFPC6, Digested with NheI and NdeI	n/a	n/a	pXCFPC-6	
pSWD155 DivL overexpression	pVMCS-1	SWD485	SWD486	pVMCS-1	Pvan-for[7], M13-for
	DivL	SWD487	SWD488	divL	
pSWD156 DivLA601L overexpression	pVMCS-1	SWD489	SWD486	pVMCS-1	Pvan-for[7], M13-for
	DivL1-600	SWD487	SWD490	divL	
	DivL602-769	SWD491	SWD488	divL	
pSWD95Y514D CckA Y514D sensor	cckA_1-182	SWD160	SWD305	pMT383	Pvan-for[7], M13-for
	mClover3	SWD306	SWD307	pSWD14	
	cckA_183-691(Y514D)	SWD308	SWD309	pMT383(Y514D)	
	mRuby3	SWD310	SWD311	pSWD14	
	pXCFPC6, Digested with NheI and NdeI	n/a	n/a	pXCFPC-6	

Plasmid	Insert Description	Forward Primer	Reverse Primer	Template	Screening primers
pSWD95D293A D293A sensor	cckA_1-182	SWD160	SWD305	pMT383	Pxyl-for[7] and M13-for[7]
	mClover3	SWD306	SWD307	pSWD14	
	cckA_183-691(D293A)	SWD308	SWD309	pMT383(D293A)	
	mRuby3	SWD310	SWD311	pSWD14	
	pXCFPC-6, digested with NheI and NdeI	n/a	n/a	pXCFPC-6	
pSWD94 PopZ overexpression	popZ	SWD303	SWD304	popZ	Pvan-for[7], M13-for
	pBVMCS-2 , digested with NdeI_FastAP			pBVMCS-2	
pSWD109 CckA 183/184 FRET Sensor	cckA_1-183	SWD357	SWD358	pMT383	Pxyl-for and M13-for
	mClover3	SWD213	SWD359	pSWD14	
	cckA_184-691	SWD319	SWD360	pMT383	
	mRuby3	SWD361	SWD189	pSWD14	
	pXCFPC-6, digested with SacI and NdeI	n/a	n/a	pXCFPC-6	

Plasmid	Insert Description	Forward Primer	Reverse Primer	Template	Screening primers
pSWD110 CckA 192/193 FRET Sensor	cckA_1-192	SWD357	SWD362	pMT383	Pxyl-for and M13-for
	mClover3	SWD213	SWD323	pSWD14	
	cckA_193-691	SWD324	SWD348	pMT383	
	mRuby3	SWD294	SWD189	pSWD14	
	pXCFPC6 digested with SacI and NdeI	n/a	n/a	pXCFPC-6	
pSWD111 CckA 193/194 FRET Sensor	cckA_1-193	SWD357	SWD325	pMT383	Pxyl-for and M13-for
	mClover3	SWD213	SWD363	pSWD14	
	cckA_194-691	SWD327	SWD348	pMT383	
	mRuby3	SWD294	SWD189	pSWD14	
	pXCFPC-6_SacI_NdeI	n/a	n/a	pXCFPC-6	
pWSC10011 CckA 69/70 FRET Sensor	cckA_1-69	WSC10026	WSC10027	pMT383	Pxyl-for and M13-for
	EYFP	WSC10028	WSC10029	pXYFPC-1	
	cckA_70-691	WSC10030	WSC10031	pMT383	
	pXCFPC-2_EcoRI	n/a	n/a	pXCFPC-2	
pWSC10012 CckA 91/92 FRET Sensor	cckA_1-91	WSC10026	WSC10032	pMT383	Pxyl-for and M13-for
	EYFP	WSC10028	WSC10033	pXYFPC-1	
	CckA	WSC10034	WSC10035	pMT383	
	pXCFPC-2_EcoRI	n/a	n/a	pXCFPC-2	

Plasmid	Insert Description	Forward Primer	Reverse Primer	Template	Screening primers
pWSC10013 CckA N-terminal FRET Sensor	EYFP	WSC10036	WSC10037	pXYFPC-1	Pxyl-for and M13-for
	cckA_1-691	WSC10038	WSC10031	pMT383	
	pXCFPC-2_EcoRI	n/a	n/a	pXCFPC-2	
pSWD55 Short FRET Standard	mClover3	SWD184	SWD185	pSWD14	Pxyl-for and M13-for
	FN_10	SWD186	SWD187	FN_7-8_FN_10	
	mRuby3	SWD188	SWD189	pSWD14	
	pXCFPC-6_NheI_SacI	n/a	n/a	pXCFPC-6	
pSWD56 Long FRET Standard	mClover3	SWD184	SWD190	pSWD14	Pxyl-for and M13-for
	FN_7-8	SWD191	SWD192	FN_7-8_FN_10	
	mRuby3	SWD193	SWD189	pSWD14	
	pXCFPC-6_NheI_SacI	n/a	n/a	pXCFPC-6	
pSWD275 CckA mRuby3 intermolecular	CckA	SWD160	SWD309	pMT383	Pxyl-for and M13-for
	mRuby3	SWD361	SWD523	pSWD14	
	flagtag	SWD542	SWD528	flagtag	
	pXCFPC-6_NdeI_NheI	n/a	n/a	pXCFPC-6	
pSWD276 Ccka mClover3 intermolecular	CckA(1-182)	SWD818	SWD305	pMT383	Pvanl-for and M13-for

	mClover3	SWD306	SWD307	pSWD14	
	CckA(183-691)	SWD308	SWD815	pMT383	
	flagtag	SWD816	SWD528	flagtag	
	pVCFPC-1_NdeI_NheI	n/a	n/a	pVCFPC-1	

Site Directed Mutagenesis (SDM)

Site directed mutagenesis was performed to generate point mutants that alter CckA function. Each reaction was performed at a 10ng scale using pMT383 as a template. KOD PCR reactions were performed using the manufacturer's protocol with a 10-minute extension time. Reactions were DpnI treated for 1 hour at 37 °C followed by 80 °C for 5 minutes for enzyme inactivation. 20 µL of reaction were transformed into KCM competent DH5α *E. coli* competent cells. Colonies were isolated, grown, and miniprepped. Plasmids were sequenced to determine correct mutation. Plasmids were then used as templates for PCR product amplification for Gibson assembly reactions.

Table S2: Site directed mutagenesis primers for introduction of CckA point mutants

Template	**Mutation**	**Forward Primer**	**Reverse Primer**	**Annealing Temperature (°C)**
pMT383	Y514D	nSWD98	nSWD99	68
pMT383	D293A	nSWD104	nSWD105	65

Table S3: Plasmids utilized in this study

Name	Description	Reference/Addgene #
pSWD95	2nd Gen CckA-FRET Sensor	This work
pSWD155	DivL overexpression plasmid	This work
pSWD156	DivL A601L overexpression plasmid	This work
pSWD95Y514D	CckA FRET Y514D Mutant sensor	This work
pSWD95D293A	CckA FRET D293A Mutant sensor	This work
pSWD94	PopZ overexpression plasmid	This work
pSWD109	2nd Gen CckA 183/184 FRET Sensor	This work
pSWD110	2nd Gen CckA 192/193 FRET Sensor	This work
pSWD111	2nd Gen CckA 193/194 FRET Sensor	This work
pWSC10011	1st Gen CckA CckA 69/70 FRET Sensor	This work
pWSC10012	1st Gen CckA 91/92 FRET Sensor	This work
pWSC10013	1st Gen CckA N-terminal FRET Sensor	This work
pSWD55	Short Fibronectin FRET Standard	This work
pSWD56	Long Fibronectin FRET Standard	This work
pSWD275	mRuby3 Intermolecular Sensor	This work
pSWD276	mClover3 Intermolecular Sensor	This work
pSWD14	mClover3/mRuby3	Bajar, et al. [8], Addgene# 74252
pSWD63	cckA::cckA-mClover3	This work
pMT383	CckA template	Shapiro Lab
pXCFPC-6	Xylose inducible Vector	Thanbichler, et al. [7]
pVMCS-1	Vanillate inducible Vector	Thanbichler, et al. [7]

pBVMCS-2	Vanillate inducible high copy plasmid	Thanbichler, et al. [7]
pXYFPC-1	Xylose inducible Vector	Thanbichler, et al. [7]
pXCFPC-2	Xylose inducible Vector	Thanbichler, et al. [7]
FN_7-8_FN_10	Fibronectin Gene Block	Ohashi, et al. [9]

Table S4: DNA primers utilized in this study.

Name	**Sequence(5'-3')**
SWD160	CGCTCGAGTTTTGGGGAGACGACCATATGGCCGACTTGCAGCTCCAG
SWD184	ATATGCATGGTACCTTAAGATCTCGAATGAGCAAGGGCGAGGAGC
SWD185	GGACGTCCGACACGCTCACCATCGGATCTTCGCCG
SWD186	TCCGATGGTGAGCGTGTCGGACGTCCCGCGC
SWD187	GCTCTTCGCCCTTAGAGGTGCGATAGTTGATGGAGATCGGC
SWD188	CCATCAACTATCGCACCTCTAAGGGCGAAGAGCTGATCAAGGA
SWD189	ACTAGTGGATCCCCCGGGCTGCAGCTTTACTTGTACAGCTCGTCCATGCC ACCA
SWD190	GGGGCGACAGCGGGCTCACCATCGGATCTTCGCCG
SWD191	TCCGATGGTGAGCCCGCTGTCGCCCCCCACC
SWD192	CTTCGCCCTTAGAGGTCTTTTGGCGCCCGCG
SWD193	GGGCGCCAAAAGACCTCTAAGGGCGAAGAGCTGATCAAGGA
SWD213	CCGGTCGGCCACCAGCAAGGGCGAGGAGCTGTTCACC
SWD294	CCGGTCGGCCACCTCTAAGGGCGAAGAGCTGATCAAGGA
SWD303	CGAGGAAACGCATATGTCCGATCAGTCTCAAGAACCTACAATGG
SWD304	GGCCCGTTTTCATTTAGGCGCCGCGTCCCCG
SWD305	GCGTAACGTTCGACGACGCGTCTTCCACAACCGG
SWD306	GGAAGACGCGTCGTCGAACGTTACGCGTCACCGG

SWD307	CCTTGCTCATCTCGGTGGCCGACCGGTGACG
SWD308	CCGGTCGGCCACCGAGATGAGCAAGGGCGAGGAGC
SWD309	ACCGGGGCCGGGCTCACCATCGGATCTTCGCCG
SWD310	GATCCGATGGTGAGCCCGGCCCCGGTCGCCGAG
SWD311	CGTAACGTTCGACGCCGCCTGCAGCTGCTG
SWD319	CGAAGATCCGATGGTGAGCGCCCCGGTCGCCGAGCGC
SWD323	GCTGGGTGGGCTCACCATCGGATCTTCGCCGC
SWD324	CGAAGATCCGATGGTGAGCCCACCCAGCTCGCTGGACGC
SWD325	CCTCGCCCTTGCTGGTGGCCGACCGGTGACGCGTAACGTTCGATGGCGG GGCGGCGCGCTC
SWD327	CGAAGATCCGATGGTGAGCCCCAGCTCGCTGGACGCC
SWD348	CTTCGCCCTTAGAGGTGGCCGACCGGTGACGCGTAACGTTCGACGCCGC CTGCAGCTGCTG
SWD357	ATATGCATGGTACCTTAAGATCTCGAATGGCCGACTTGCAGCTCCAGG
SWD358	CCTCGCCCTTGCTGGTGGCCGACCGGTGACGCGTAACGTTCGACGGCGA CGCGTCTTCCACAACC
SWD359	ACCGGGGCGCTCACCATCGGATCTTCGCCGC
SWD360	TTCGCCCTTAGAGGTGGCCGACCGGTGACGCGTAACGTTCGACGCCGCC TGCAGCTGCTGC
SWD361	ACCGGTCGGCCACCTCTAAGGGCGAAGAGCTGATCAAGGA
SWD362	CCTCGCCCTTGCTGGTGGCCGACCGGTGACGCGTAACGTTCGACGGGGC GGCGCGCTCGGC
SWD363	CGAGCTGGGGCTCACCATCGGATCTTCGCCGC
SWD485	GCCCGAACTCGGCTTCTAGCTCTTAAGATCTACGTACCGGTGCTAGCTG

SWD486	CGTACGAAGTCATCTCACCACGTGGTACCTCGAGAATTCATATGC
SWD487	GGTACCACGTGGTGAGATGACTTCGTACGACCTGATCCTCGCG
SWD488	CGTAGATCTTAAGAGCTAGAAGCCGAGTTCGGGCTGC
SWD489	CGAACTCGGCTTCTAGCTCTTAAGATCTACGTACCGGTGCTAGCTG
SWD490	CGGCGTCGATCTGCAGCATGTCCAGCACGTCGTCGATCG
SWD491	GCTGGACATGCTGCAGATCGACGCCGGCGAAATGG
SWD523	CGTGGTCCTTGTAGTCCTTGTACAGCTCGTCCATGCCACC
SWD528	GAACTAGTGGATCCCCCGGGCTGCAGCTTCACTTATCGTCGTCGTCCTTG
SWD542	GGACGAGCTGTACAAGGACTACAAGGACCACGACGGC
SWD818	CCGAACCACGATGCGAGGAAACGCATATGGCCGACTTGCAGCTCCAGG
SWD815	GTCCTTGTAGTCCGCCGCCTGCAGCTGCTGCTTGACG
SWD816	AGCTGCAGGCGGCGGACTACAAGGACCACGACGG
WSC10026	CCTTAAGATCTCGAGCTCCGGAGAATATGGCCGACTTGCAGCTC
WSC10027	CCTTGCTCACCATCGAGCTCCCGCTACCACTGCCACCAATGGCGACAAGG CCCAAC
WSC10028	TAGCGGGAGCTCGATGGTGAGCAAGGGCGAGG
WSC10029	GCTGAGCCGCGCGAGCTCCCGCTACCACTGCCACCCTTGTACAGCTCGTC CATGC
WSC10030	GGTAGCGGGAGCTCGCGCGGCTCAGCGCTTTCC
WSC10031	GCCGACCGGTGACGCGTAACGTTCGACGCCGCCTGCAGCTGCTG
WSC10032	CCTTGCTCACCATCGAGCTCCCGCTACCACTGCCACCCTCGGCCAGCGCC TCGATG
WSC10033	AGGGCGGCCGGCGAGCTCCCGCTACCACTGCCACCCTTGTACAGCTCGT CCATGC
WSC10034	GGTAGCGGGAGCTCGCCGGCCGCCCTGGCCGCC

WSC10035	GCCGACCGGTGACGCGTAACGTTCGACGCCGCCTGCAGCTGCTGC
WSC10036	CCTTAAGATCTCGAGCTCCGGAGAATATGGTGAGCAAGGGCGAGG
WSC10037	GCAAGTCGGCCATCGAGCTCCCGCTACCACTGCCACCCTTGTACAGCTCG TCCATGC
WSC10038	TAGCGGGAGCTCGATGGCCGACTTGCAGCTC
nSWD084	GCCGCCGACCTCCCGCGCAAGCTCTTG
nSWD085	CAAGAGCTTGCGCGGGAGGTCGGCGGC
nSWD086	CCAATCCGGCGCCGACCACGATGAC
nSWD087	GTCATCGTGGTCGGCGCCGGATTGG
nSWD088	CTGCAGCTGTCCCGCGCCCAGAAGATG
nSWD089	CATCTTCTGGGCGCGGGACAGCTGCAG
nSWD090	GAGGTCTTGCTGTGCCGCCTGCTGC
nSWD091	GCAGCAGGCGGCACAGCAAGACCTC
Pxyl-for	CCCACATGTTAGCGCTACCAAGTGC
M13-for	GCCAGGGTTTTCCCAGTCACGA
Pvan-for	GACGTCCGTTTGATTACGATCAAGATTGG
RecUni-1	ATGCCGTTTGTGATGGCTTCCATGTCG
RecVan-2	CAGCCTTGGCCACGGTTTCGGTACC
RecXyl-2	TCTTCCGGCAGGAATTCACTCACGCC
nSWD035	CCAAGGTCATCAGCTTCATCC
nSWD036	CGCTGGTGCGTAATCCG

Table S5: Strains used in this study.

Name	Genotype	Plasmid	Reference
WSC1119	*E. coli,* DH5α	none	
WSC1411	*C. crescentus*, NA1000	none	
WSC1412	*E. coli,* DH5α	pSWD95	This work
WSC1413	NA1000	pSWD95	This work
WSC1414	NA1000, *cckA::gent*	pSWD95	This work
WSC1415	*E. coli,* DH5α	pSWD155	This work
WSC1416	NA1000	pSWD155, pSWD95	This work
WSC1417	*E. coli,* DH5α	pSWD156	This work
WSC1418	NA1000	pSWD156, pSWD95	This work
WSC1419	*C. crescentus*, cdg^0	none	Abel, et al. [10]
WSC1420	cdg^0	pSWD95	This work
WSC1421	cdg^0	pSWD95, pSWD156	This work
WSC1422	*E. coli,* DH5α	pSWD94	This work
WSC1423	NA1000	pSWD94, pSWD95	This work
WSC1424	NA1000, *popZ::spec/strep*	none	Bowman, et al. [11]
WSC1425	NA1000, *popZ::spec/strep*	pSWD95	This work
WSC1426	*E. coli,* DH5α	pSWD95D293A	This work
WSC1427	NA1000	pSWD95D293A	This work
WSC1428	cdg^0	pSWD95D293A	This work
WSC1429	*E. coli,* DH5α	pSWD95Y514D	This work

WSC1430	NA1000	pSWD95Y514D	This work
WSC1431	cdg^{0}	pSWD95Y514D	This work
WSC1432	*E. coli,* DH5α	pSWD63	This work
WSC1433	NA1000, *cckA::cckA-mClover3*	pSWD63	This work
WSC1434	*E. coli,* DH5α	pWSC10011	This work
WSC1435	NA1000	pWSC10011	This work
WSC1436	*E. coli,* DH5α	pWSC10012	This work
WSC1437	NA1000	pWSC10012	This work
WSC1438	*E. coli,* DH5α	pWSC10013	This work
WSC1439	NA1000	pWSC10013	This work
WSC1440	*E. coli,* DH5α	pSWD109	This work
WSC1441	NA1000	pSWD109	This work
WSC1442	*E. coli,* DH5α	pSWD110	This work
WSC1443	NA1000	pSWD110	This work
WSC1444	*E. coli,* DH5α	pSWD111	This work
WSC1445	NA1000	pSWD111	This work
WSC1446	*E. coli,* DH5α	pSWD55	This work
WSC1447	NA1000	pSWD55	This work
WSC1448	*E. coli,* DH5α	pSWD56	This work
WSC1449	NA1000	pSWD56	This work
WSC1450	*E. coli,* DH5α	pSWD14	This work
WSC1027	*E. coli,* DH5α	pWSC1027	This work
WSC1149	NA1000, *cckA::gent*	pMR10cckAover	Jacobs, et al. [12]

WSC1451	*E. coli,* DH5α	pXCFPC-6	Thanbichler, et al. [7]
WSC1452	*E. coli,* DH5α	pVMCS-1	Thanbichler, et al. [7]
WSC1453	*E. coli,* DH5α	pBVMCS-2	Thanbichler, et al. [7]
WSC1454	*E. coli,* DH5α	pXYFPC-1	Thanbichler, et al. [7]
WSC1455	*E. coli,* DH5α	pXCFPC-2	Thanbichler, et al. [7]
WSC1459	*E. coli,* DH5α	pSWD275	This work
WSC1460	*E. coli,* DH5α	pSWD276	This work
WSC1461	NA1000	pSWD275	This work
WSC1462	NA1000	pSWD276	This work
WSC1463	NA1000	pSWD275,pSWD276	This work

Table S6: Antibiotic used in this study.

Antibiotic	**Final concentration in *C. crescentus* (liquid, solid)**	**Final concentration in *E. coli* (liquid, solid)**	**Manufacturer/Product Number**
Kanamycin	5, 25 µg/mL	30, 50 µg/mL	Fisher M150025
Gentamycin	1, 5 µg/mL	15, 20 µg/mL	Alfa Aesar J62834-09
Chloramphenicol	1, 20 µg/mL	20, 30 µg/mL	Fisher Scientific AC227920250
Spectinomycin	25, 100 µg/mL	50, 50 µg/mL	Fisher AAJ6182006
Streptomycin	5, 5 µg/mL	30, 30 µg/mL	ThermoFisher 11860038

CCT018159	30μM		Cayman Chemical, Item No. 10012591
CDV008	17.5μM		5a, Vo et al., 2017[13]
CDV009	12.5μM		5b, Vo et al., 2017[13]

Table S7: Raw materials used in this study.

Material	**Used for**	**Supplier**	**Product #**
Difco Peptone	PYE media (Caulobacter)	VWR	90000-264
BD Yeast Extract	PYE media (Caulobacter)	VWR	90000-726
$MgSO_4$	PYE media (Caulobacter)	Fisher	M65-500
$CaCl_2$	PYE media (Caulobacter)	Fisher	AC349610250
Xylose	Caulobacter	Fisher	BP708-250
Vanillic Acid	Caulobacter	Alfa Aesar	A12074
D-Glucose	M2G Media (Caulobacter)	VWR	97061-168
$FeSO_4$	M2G Media (Caulobacter)	FisherSci	AC423730050
Na_2HPO_4	M2G Media (Caulobacter)	FisherSci	S373-500

KH_2PO_4	M2G Media (Caulobacter)	FisherSci	P285-500
NH_4Cl	M2G Media (Caulobacter)	Fisher	A661-500
Percoll	Synchrony	GE	17089102
Difco Bacto Agar	PYE/Agar Plates	Fisher	DF0140010
Fisher Agar	LB/Agar Plates	Fisher	BP1423500
LB Broth	LB media (Ecoli)	Fisher	BP1426-2
Ultrapure Agarose	Imaging Agarose Pads	FisherSci	16500100
Microscope Slides	Imaging	VWR	16004-368
Coverslips	Imaging	VWR	48366-227
Zeiss Immersol 518F Immersion Oil	Imaging	Fisher	1262466A
PVDF Membrane	Western Blot	ThermoFisher	88520
Anti-DivL antibody	Western Blot		
Goat anti-rabbit IgG HRP secondary antibody	Western Blot	Sigma	A0545
SuperSignal West Pico PLUS Chemiluminescent Substrate	Western Blot	FisherSci	PI34577

Tween 20	Western Blot	FisherSci	BP337-100
Milk	Western Blot	FisherSci	NC0166631
GFP Rabbit Polyclonal Antibody	Western Blot	ProteinTech	50430-2-AP
Rabbit anti-FLAG	Western Blot	Sigma	F7425
NdeI FastDigest	Plasmid linearization	ThermoFisher	FD0583
NheI FastDigest	Plasmid linearization	ThermoFisher	FD0973
SacI FastDigest	Plasmid linearization	ThermoFisher	FD1133
EcoRI FastDigest	Plasmid linearization	ThermoFisher	FD0274
FastAP	Cloning	ThermoFisher	EF0651
KOD Hot Start	PCR	MilliporeSigma	71806
Phusion	PCR and Gibson Assembly	ThermoFisher	F530S
DpnI	Plasmid linearization	ThermoFisher	FD1703
DreamTaq Green	Colony PCR	ThermoFisher	K1081
T5 Exonuclease	Gibson Assembly	NEB	M0363S
Taq Ligase	Gibson Assembly	NEB	M0208L

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