Supporting Information

The response threshold of *Salmonella* PilZ domain proteins is determined by their binding affinities for c-di-GMP

Ingrid Swanson Pultz[1], Matthias Christen[1], Hemantha Don Kulasekara[1], Andrew Kennard[1], Bridget Kulasekara[3], Samuel I. Miller[1, 2, 4 *]

Departments of [1]Microbiology, [2]Medicine, [3]Molecular and Cellular Biology, and [4]Genome Sciences,

University of Washington, Seattle, WA 91895, USA.

*For correspondence, email millersi@uw.edu.

Supporting Materials and Methods

Analysis of BcsA protein expression

To compare BcsA protein expression between strains growing at different temperatures, cells of a *S.* Typhimurium strain harboring a FLAG-tagged BcsA were scraped off of LB plates incubated either 37°C for 24 hours, or at 24°C for 48-72 hours, into pre-weighed Eppendorf tubes, immediately frozen at -80°C for at least 15 minutes, then resuspended in PBS pH 7.4 at a concentration of 0.2 µg wet weight $µl^{-1}$with chlormphenicol (20 ng $µl^{-1}$) to inhibit any protein translation that might occur before and during the sonication process. To verify that the observed band that reacted with the anti-FLAG antibody represented FLAG-tagged BcsA protein, cells expressing the native untagged BcsA protein grown at 24°C were also included in this analysis. Cells were disrupted by sonication, centrifuged for 10 min at 4°C at full speed in a tabletop Eppendorf centrifuge, and the supernatant containing soluble protein was discarded. The pellet was resuspended in PBS with 1% lauryldimethylamine-oxide to solubilize membrane proteins. The mixture was centrifuged again to remove whole cells. The supernatant, containing membrane proteins, was obtained, and protein content was quantified using a Bradford assay. 5 mg total protein was run on an SDS-PAGE gel (6% acrylamide), transferred to nitrocellulose membranes, and blocked using PBST containing 0.5% milk. Blots were incubated with a primary antibody (polyclonal anti-FLAG 1:500, Immunology Consultants Laboratory), washed, incubated with secondary antibody (1:5000, donkey anti-rabbit conjugated to HRP, GE Healthcare) and imaged using X-ray film.

Cell lysate assay for production of cellulose

Strains were inoculated into 1 liter of LB and shaken at 37°C overnight. The next morning, chloramphenicol was added (20 ng $µl^{-1}$) to stop any new protein translation, and cells were allowed to incubate for another hour. Cells were then rapidly cooled in a dry ice/ethanol bath, pelleted, and stored at -80°C for at least 15 minutes. Pellets were resuspended in 25 ml of 70 mM Tris pH 7.7 with 50 mM NaCl, 10 mM MgCl2, 5 mM CaCl2, 1 mM EDTA, 5 mM β-mercaptoethanol, protease inhibitors, 20 ng $µl^{-1}$ chloramphenicol, and 1 µg ml^{-1} lysozyme. After 30 minutes of incubation on ice, cells were lysed in an Avestin™ EmulsiFlex-C3, and cell lysate was centrifuged 4 times for 15 min at 4C at 4500**g* to get rid of whole cells. At this point, 100 µl of cell lysate was plated onto LB-agar plates to verify that all live cells had been removed. Lysate was then incubated in 5% glycerol, 100 µM GTP, 20 mM MgCl2, 1 mM UDP-glucose, and µg ml^{-1}calcofluor, with or without 6 µg ml^{-1} purified DgcA of *Caulobacter crescentus,* at 37°C for 15 min. 12 replicates of 100 µl each were performed for each sample. After incubation, samples were centrifuged, washed twice in Tris buffer, resuspended in 100 µl Tris buffer, and transferred to a 96-well black plate (Greiner). Calcofluor fluorescence was measured on an Envision multilabel reader (PerkinElmer). The data was analyzed using a Student's two-tailed T test.

Measurement of in vivo FRET/CFP ratios in ara::DGC and ara::PDE control strains

To characterize all biosensors for use as *in vivo* c-di-GMP measurement tools, all strains containing biosensor plasmids were grown at 30°C to prevent homologous recombination between the *mYPet* and *mCYPet* genes. To determine FRET/CFP ratios in *ara::DGC* or *ara::PDE* strains expressing biosensors,

strains were inoculated into one ml LB with 15 $\mu g\ ml^{-1}$ gentamicin and 75 μM IPTG, and incubated overnight at 30°C with shaking. The next day, strains were diluted to an OD_{600} of 0.04 in 1 ml modified 0.5X M63 media (11 mM KH_2PO_4, 20 mM K_2HPO_4, 7.5 mM $(NH_4)_2SO_4$, 25 mM glycerol, 5 μM $Fe^{2+}NH_4SO_4$, 2 mM $MgCl_2$, 10 mM NaCl, 15 $\mu g\ ml^{-1}$ gentamicin, 500 μM IPTG, 1.3 mM arabinose). M63 media was used in these experiments since LB media demonstrates autofluorescence at the wavelengths used for imaging. Strains were then incubated for 5 hours and 25 minutes at 30°C with shaking. At this point, cells were prepared for fluorescence microscopy.

Fig S1: Binding curves of PA3353 for c-di-GMP suggest two binding sites. Shown are binding curves of the PA3353 FRET construct for c-di-GMP at temperatures ranging from 24°C to 37°C at 1°C intervals as indicated by the legend.

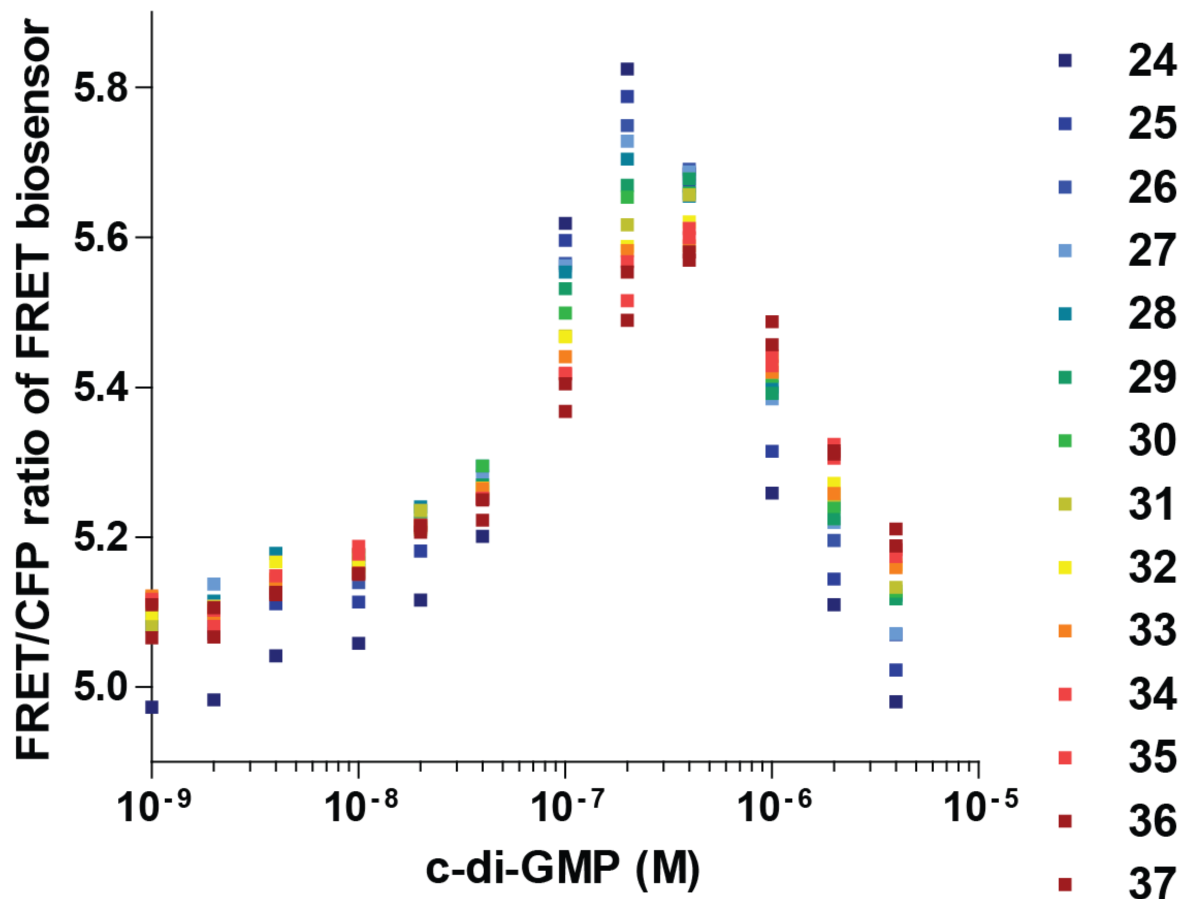

Fig S2: Cellulose is predominantly regulated on a post-translational level by intracellular c-di-GMP. A. A C-terminal FLAG tag on the BcsA protein does not affect its ability to produce cellulose. Shown is a calcofluor agar plate inoculated with the indicated strains and incubated for 72 hours at 24°C, after which the plate was exposed to UV light with a wavelength of 365 nm. B. Western blot using anti-FLAG antibodies on membrane proteins isolated from *S.* Typhimurium *bcsA-FLAG* or a wild-type strain with untagged BcsA that had been incubated at either 37°C or 24°C. 5 mg total protein was added to each lane. C. Fold increase in calcofluor fluorescence of lysate generated from *S.* Typhimurium, or a *ΔbcsA* deletion strain, at 37°C, incubated *in vitro* with a DGC, compared to the amount of calcofluor fluorescence of lysate without DGC addition. n.s. = not significant.

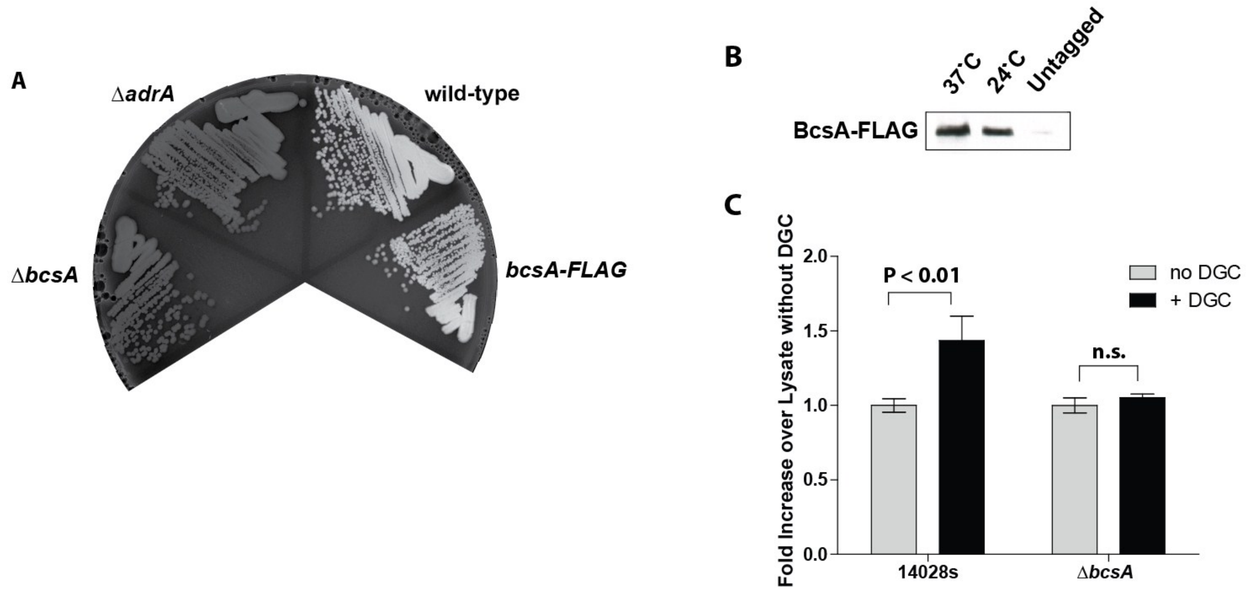

Fig S3: A *S.* Typhimurium strain harboring a chromosomal arabinose-inducible DGC generates cellulose in the presence of arabinose. Cellulose production at 37°C on calcofluor-agar plates with the indicated concentrations of arabinose for *S.* Typhimurium *ara::DGC,* or *ara::DGC* in which the *bcsA* gene has been deleted. As the concentration of arabinose is increased, calcofluor fluorescence is also increased.

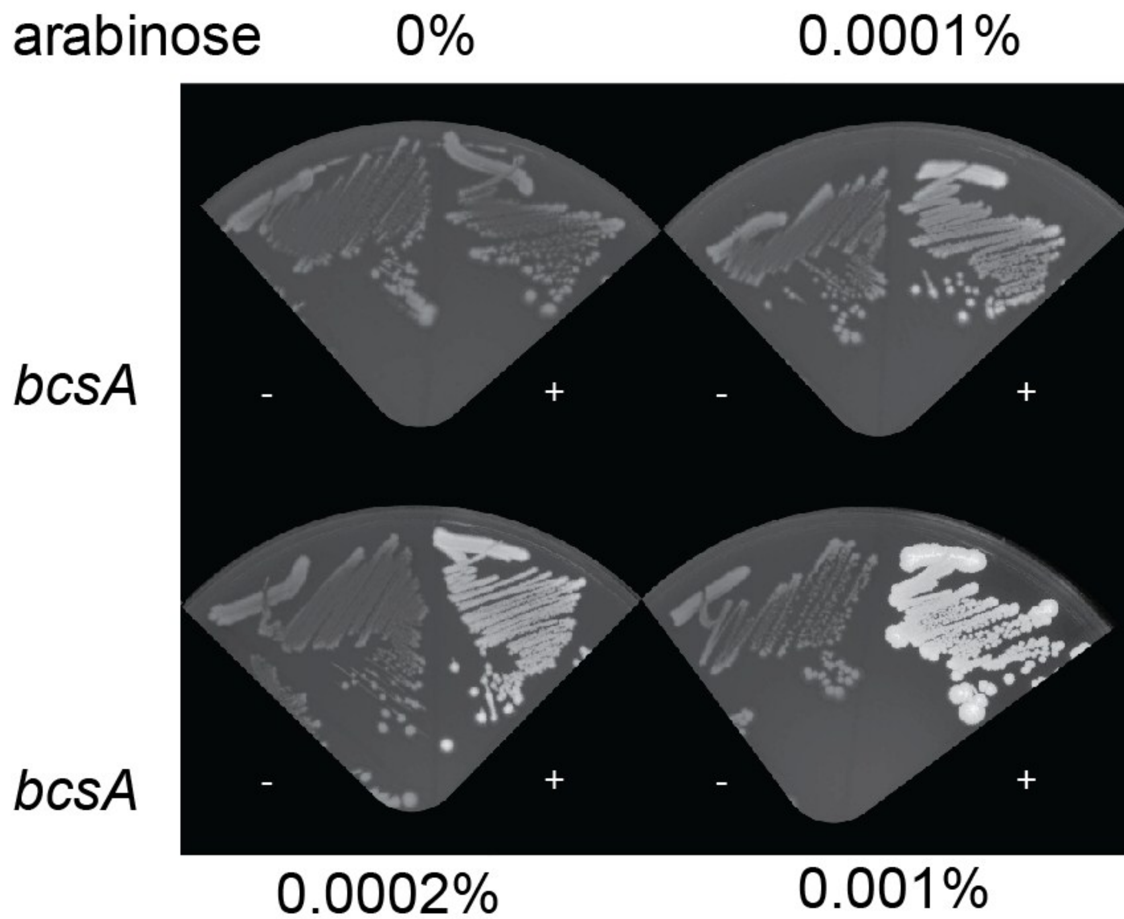

Fig S4: Expression of the YcgR biosensor differentiates between cells overexpressing a DGC and cells overexpressing a PDE. A. Dual-emission ratio microscopic images (FRET/CFP) of cells expressing a DGC (*ara::DGC*) or a PDE (*ara::PDE*) and the YcgR biosensor. Pseudocolors represent emission ratios (527/480 nm) of the FRET-based biosensor as indicated by the figure legend to the left. B. Dot plot illustrating the nFRET/CFP ratios of cells expressing either a DGC or a PDE in addition to the YcgR biosensor. Each dot represents the FRET and CFP emission intensities of an individual cell. Expression of the biosensor separates the strains into two distinct populations based on their nFRET/CFP ratios. C. Histogram showing the fraction of cells that demonstrate the indicated nFRET/CFP ratios for strains expressing either a DGC or a PDE. nFRET: net FRET intensity, calculated by subtracting bleedthrough coefficients and intensity of the YFP channel as described in Materials and Methods. This experiment was performed at 30°C.

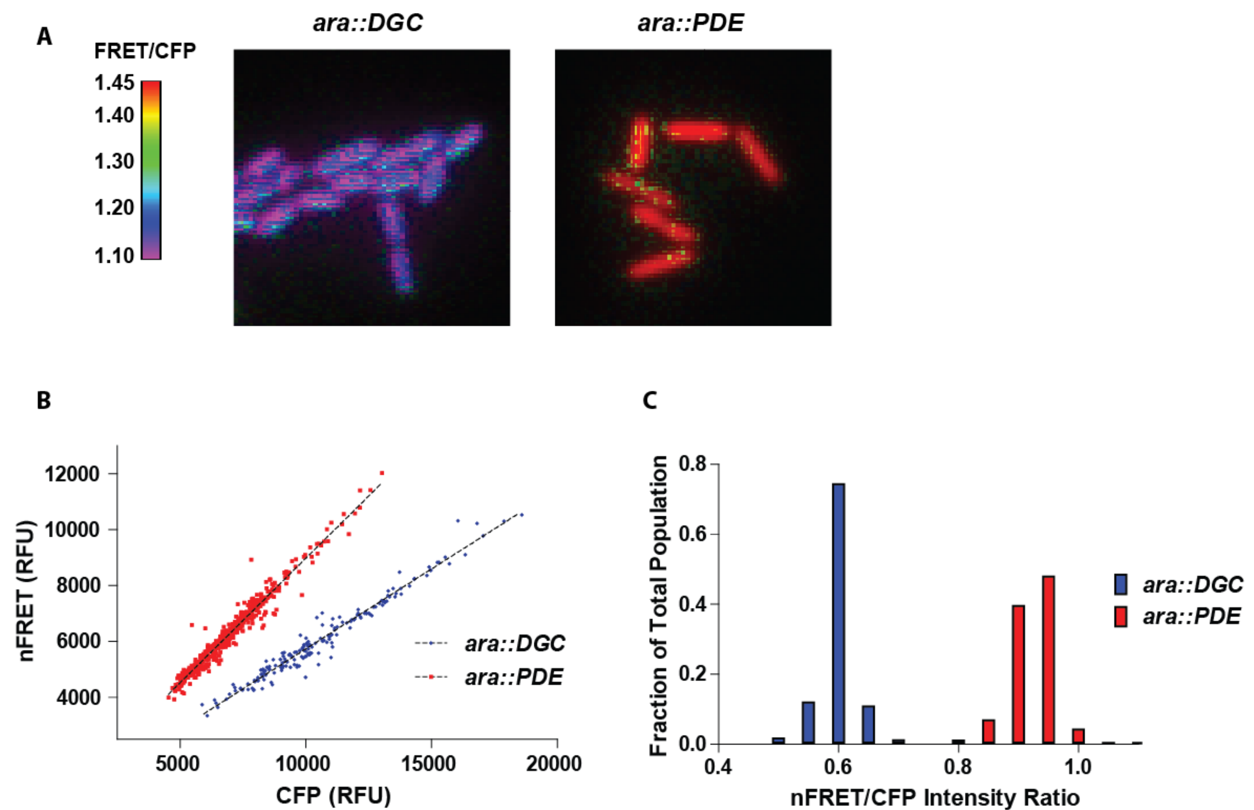

Fig S5: Expression of the BcsA PilZ biosensor differentiates between cells overexpressing a DGC and cells overexpressing a PDE. A. Dual-emission ratio microscopic images (FRET/CFP) of cells expressing a DGC (*ara::DGC*) or a PDE (*ara::PDE*) and the BcsA PilZ biosensor. Pseudocolors represent emission ratios (527/480 nm) of the FRET-based biosensor as indicated by the figure legend to the left. B. Dot plot illustrating nFRET/CFP ratios of cells expressing either a DGC or a PDE, in addition to the BcsA PilZ biosensor. Each dot represents the FRET and CFP emission intensities of an individual cell. Expression of the biosensor separates the strains into two distinct populations based on their nFRET/CFP ratios. C. Histogram showing the fraction of cells that demonstrate the indicated nFRET/CFP ratios for strains expressing either a DGC or a PDE. nFRET: net FRET intensity, calculated by subtracting bleedthrough coefficients and intensity of the YFP channel as described in Materials and Methods. This experiment was performed at 30°C.

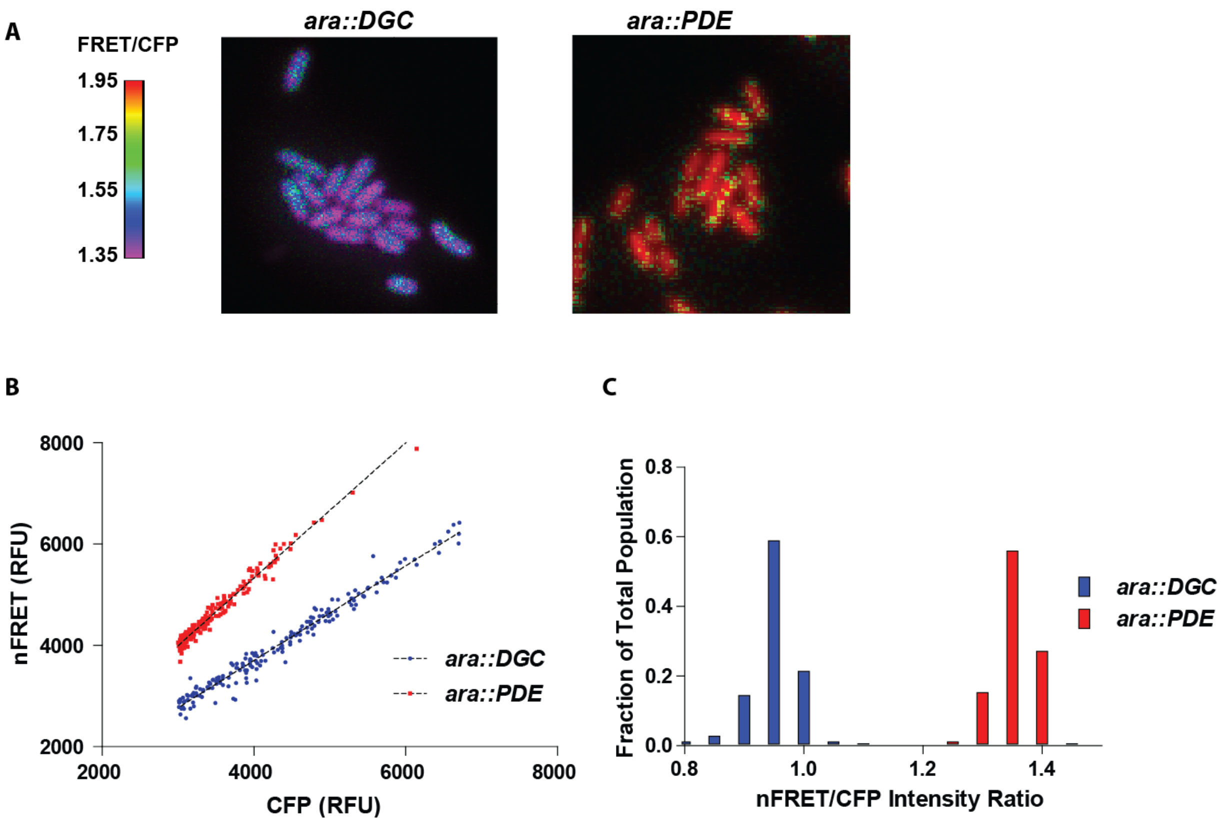

Table S1: Binding affinities and Hill coefficients of YcgR and the BcsA PilZ domain at various temperatures

	Kd (nM)		Hill Coefficient	
Temp (°C)	YcgR	BcsA PilZ	YcgR	BcsA PilZ
24	125 ± 11	5606 ± 226	1.6 ± 0.2	2.4 ± 0.2
25	191 ± 18	8240 ± 238	1.5 ± 0.2	2.2 ± 0.1
26	220 ± 24	10350 ± 220	1.5 ± 0.2	2.2 ± 0.1
27	266 ± 16	11640 ± 345	1.8 ± 0.2	2.1 ± 0.1
28	286 ± 13	13200 ± 400	1.9 ± 0.1	2.1 ± 0.1
29	291 ± 16	15330 ± 610	1.9 ± 0.2	2.1 ± 0.1
30	320 ± 19	17400 ± 740	1.8 ± 0.2	2.0 ± 0.1
31	371 ± 13	18930 ± 1030	1.9 ± 0.1	2.1 ± 0.2
32	406 ± 24	22130 ± 1620	2.2 ± 0.2	1.9 ± 0.2
33	437 ± 30	25250 ± 1980	1.7 ± 0.2	1.9 ± 0.2
34	476 ± 27	29360 ± 3120	1.9 ± 0.2	1.9 ± 0.2
35	522 ± 53	36080 ± 4540	2.2 ± 0.4	1.9 ± 0.3
36	571 ± 39	38670 ± 5960	1.6 ± 0.1	2.0 ± 0.3
37	621 ± 32	41830 ± 6400	1.6 ± 0.1	2.0 ± 0.3