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Supplementary Information for

Monitoring Glycolytic Dynamics in Single Cells Using a Fluorescent Biosensor for Fructose 1,6-Bisphosphate

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This PDF file includes:

Other supplementary materials for this manuscript include the following:

Supplementary Information Text

Supplemental Information for Materials and Methods

Biosensor library cloning. pHO_pTEFmut7_CggR_R250A_ble was a gift from Matthias Heinemann (Addgene plasmid # 124585 ; http://n2t.net/addgene:124585; RRID:Addgene_124585). The coding sequence for CggR (89-340) was amplified from pHO_pTEFmut7_CggR_R250A_ble using primers CggR-p7-GG-F and CggR-p7-GG-R to add BsaI restriction sites and compatible overhangs for Golden Gate assembly into the plasmid HC_Kan_RFP-p7. All Golden Gate reactions were carried out using 40 fmol of vector (HC_Kan_RFP-p7), 40 fmol of purified insert (CggR amplicon), 10 units of BsaI (NEB), 800 units of T4 DNA ligase (NEB), and 1 × T4 DNA Ligase Reaction Buffer (NEB) in a total volume of 20 µL. The reaction was incubated 2 min at 37°C and 5 min at 16°C for 50 cycles and followed by 20 min at 60°C and 20 min at 80°C. The R250A mutation in the resulting plasmid HC_Kan_CggR-R250A-p7 was reverted to wildtype using the primers CggR-A250R-Q5-F and CggR-A250R-Q5-R and Q5 site-directed mutagenesis.

A holding plasmid for cloning CggR-180 linker libraries was generated by Gibson Assembly. HC_Kan_CggR-EBD-WT was linearized at amino acid 180 by PCR using primers CggR-180-Dest-GA-F2 and CggR-180-Dest-GA-R2 which added homologous ends to a lacZ cassette amplified from pATT-Dest with primers CggR-180-Dest-GA-F1 and CggR-180-Dest-GA-R1. pATT-Dest was a gift from David Savage (Addgene plasmid # 79770 ; http://n2t.net/addgene:79770 ; RRID:Addgene_79770). The resulting plasmid HC_Kan_CggR-180-Dest was used to clone linker libraries by Golden Gate Assembly using the BsaI sites added with lacZ cassette. Golden Gate compatible cpGFP was amplified from pTKEI-Mal-B2 with primers CggR-cpGFP-VSTx4-GG-F and CggR-cpGFP-VSTx4-GG-R for the VST library or CggR-cpGFP-NNKx4-GG-F and CggR-cpGFP-NNKx4-GG-R for the NNK library. pTKEI-Mal-B2 was a gift from David Savage (Addgene plasmid # 79756 ; http://n2t.net/addgene:79756 ; RRID:Addgene_79756).

Transposon-mediated domain-insertion profiling (DIP) was used to construct a library of cpGFP insertion into CggR (1). The Mu-BsaI transposon was digested from pUCKanR-Mu-BsaI (Addgene plasmid # 79769) with BglII and HindIII in Buffer 3.1 (NEB) at 37°C overnight and purified by gel extraction (NucleoSpin Gel and PCR Clean-up). pUCKanR-Mu-BsaI was a gift from David Savage (Addgene plasmid # 79769 ; http://n2t.net/addgene:79769 ; RRID:Addgene_79769). Transposition was performed using 100 ng of purified MuA-BsaI transposon, pATT-CggR plasmid DNA at a 1:2 molar ratio relative to transposon, 4 µL of 5× MuA reaction buffer, and 1 µL of 0.22 µg/µL MuA transposase (Thermo Fisher) in a total volume of 20 µL. Reactions were incubated at 30°C for 18 h, followed by 75°C for 10 min. Reactions were cleaned up using the DNA Clean & Concentrator-5 Kit (Zymo Research Corp.) and eluted in 6 µL of water. Transformation was performed using 2 µL of reaction in 25 µL of E. Cloni 10G ELITE cells (Lucigen) in 1.0 mm Bio-Rad cuvettes using a Gene Pulser Xcell Electroporation System (settings: 10 µF, 600 Ω, 1.8 kV). Cells were immediately resuspended in 975 µL Recovery

Media and shaken at 250 rpm for 1 h at 37°C. A 10 µL aliquot of transformed cells was plated on carbenicillin (100 µg/mL) and chloramphenicol (25 µg/ mL) to select for the presence of pATT plasmid backbone and transposon insertion to assess library coverage. The remaining transformed cells were pelleted and resuspended in 50 mL of LB with 100 µg/mL carbenicillin and 25 µg/mL chloramphenicol. Cultures were grown at 250 rpm, at 37°C overnight, followed by plasmid DNA purification using a HiSpeed Plasmid Midi Kit (Qiagen).

The CggR-DIP and CggR-180 linker libraries were moved to a recombination plasmid, EMMA-attB-Dest, by Golden Gate Assembly as described previously (2) using Esp3I in place of BsaI. The domain-insertion library was amplified using primers CggR-BsmbI-GG-F and CggR-BsmbI-GG-R to add compatible overhangs. The PCR product was purified by gel extraction (NucleoSpin Gel and PCR Clean-up) and used as the insert for the Golden Gate Assembly reaction. Reactions were cleaned up using the DNA Clean & Concentrator-5 Kit and eluted in 6 µL of water. Bacterial transformation was performed using 25 µL of E. Cloni 10G ELITE Chemically Competent cells combined with 2 µL of purified Golden Gate reaction and heat shock for 45 seconds in a 42°C water bath. A small aliquot (5 µL) of the outgrowth media was plated on LB agar with carbenicillin to assess transformation efficiency. The remaining outgrowth media was diluted into 100 mL LB supplemented with 50 µg/mL carbenicillin and grown overnight at 37°C. Plasmid DNA for transfection was purified using a Qiagen Plasmid Maxi Kit.

HEK293T Landing Pad Transfection. The HEK293T Lentiviral Landing Pad (LLP-iCasp9-Blast) cell line (3) obtained from Kenneth Matreyek and Doug Fowler was used to express libraries for sort-seq experiments. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and 4 mM L-glutamine (Gibco 11965092) supplemented with 10% fetal bovine serum (FBS). BFP expression was induced with 2 µg/mL doxycycline (Sigma-Aldrich), which was removed 1-2 days prior to transfection. Recombination was achieved by transfecting cells with a total of 3 µg of plasmid DNA (1.5 µg each of attB recombination plasmid and pCAG-NLS-HA-Bxb1) and 6 µL of FuGENE 6 (Promega) in 300 µL of Opti-MEM (Gibco). pCAG-NLS-HA-Bxb1 was a gift from Pawel Pelczar (Addgene plasmid # 51271 ; http://n2t.net/addgene:51271 ; RRID:Addgene_51271). The DNA transfection reagent mixture was incubated for 15 minutes at room temperature before adding to a six-well plate containing 1×10^6 freshly seeded cells per well. Cells were incubated with the transfection mixture for 24-48 hours before expanding each well to a 10 cm plate. After 1 day of growth to reach approximately 90% confluency in the 10 cm plate, 2 µg/mL of doxycycline was added to induce expression. Selection for recombined cells was accomplished by adding 1 nM AP1903 approximately 24 hours after adding doxycycline. Recombined cells were grown for an additional 7 days after induction before FACS was performed.

Fluorescence activated cell sorting. Three sort-seq experiments were conducted, characterizing a single DIP library and two linker libraries, with minor experimental differences. Cells at 50% confluency in a 15 cm plate were detached by trypsinization,

pelleted, and resuspended in 2 mL of DMEM supplemented with 50 µg/mL of gentamicin. Sorts were performed using a BD Influx instrument equipped with a 488 nm laser for excitation and a 530/40 nm emission filter for GFP measurements and a 405 nm laser with 460/50 filter for mTagBFP measurements. Cells transfected with Bxb1 recombinase but no attB plasmid were used to adjust instrument voltages and establish a baseline of autofluorescence in the green channel and the presence of mTagBFP expression. Cells transfected with an attB plasmid but without expression of Bxb1 recombinase were used to determine the level of green fluorescence derived from plasmid expression as opposed to genomic integration and doxycycline induced expression. Cells transfected with the biosensor library plasmid (CggR-DIP or CggR-180-VST/NNNK) and Bxb1 recombinase were sorted to collect cells positive for GFP and negative for mTagBFP fluorescence to enrich for recombined cells.

The CggR-DIP library was initially sorted for fluorescence above the background to enrich for productive insertions with a total of 70,000 cells collected. Cells were collected in a 15 cm tube containing 5 mL of DMEM supplemented with 10% FBS and 50 µg/ mL gentamicin. Sorted cells were pelleted, resuspended in 800 µL of media, and plated in a 12-well plate with 2 µg/mL doxycycline. Cells were expanded over 8 days to reach 50% confluence in two 15 cm plates before the second round of sorting. The CggR-180-NNK was initially sorted to bottleneck the number of unique combinations, collecting a total of 2,000 cells above the 95th percentile brightness. Cells were collected in a single well of a 96-well plate containing 100 µL of DMEM supplemented with 10% FBS, 50 µg/ mL gentamicin and 2 µg/mL doxycycline. Cells were expanded over 14 days to reach 50% confluence in two 15 cm plates before the second round of sorting.

For all three sort-seq experiments, the two samples for the second round of FACS were detached from 15 cm plates by trypsinization, pelleted, and resuspended in 2 mL of DMEM supplemented with 50 µg/mL gentamicin and either 25 mM glucose or an equal volume of water. Cells transfected with Bxb1 recombinase but no attB plasmid were used to adjust instrument voltages and establish a baseline of autofluorescence in the green channel. Four equal width gates on the log scale were set to span the range of log(AFU) covered by the distribution of each library. The ± glucose samples were sorted using the same gates for a duration of 1.5 h each. The cells for each bin were collected in 5 mL tubes containing 1 mL of DMEM supplemented with 10% FBS and 50 µg/mL gentamicin. The +glucose samples contained 25 mM glucose. The -glucose samples for the CggR-DIP and CggR-180-VST libraries were supplemented with 10 mM pyruvate assuming an additional carbon source would be beneficial, while CggR-180-NNK did not include added pyruvate to the -glucose sort-seq sample. Cells were sorted into each bin at approximately proportional amounts to the relative density of cells in each bin (Table S2). The collected cells for each bin were individually pelleted, resuspended, and plated in either a 24-well (<200,000 cells), 12-well (>200,000 and <500,000 cells), six-well plate (>400,000 and <2M cells) or 10 cm dish (>2M cells). All samples were expanded to 50% confluent in a 10 cm plate before harvesting by trypsinization and centrifugation. Cell

pellets were washed with PBS and stored at −20°C. Genomic DNA was extracted from cell pellets containing approximately 5 M cells using a Qiagen DNeasy Blood & Tissue Kit.

Domain-insertion library DNA Sequencing. The ORF to be sequenced was PCR amplified from the Landing Pad genomic integration site using primer specific to the genome LP-Ptet-F and one specific to the integrated plasmid sequence LP-STOP-R (the sequences for all primers used for DNA sequencing can be found in Table S1). Each 50 µL reactions was prepared with a final concentration of 10 ng/µL of genomic DNA, 0.25 µM forward and reverse primer, 1 × SeqAmp PCR buffer, 1 × SeqAmp Polymerase (Clontech), and 1 × SYBR Green (Invitrogen). Amplification was monitored by qPCR with cycling conditions: [94°C 60s, (98°C 10s, 55°C 15s, 68°C 60s, plate read) × 29 cycles]. The number of cycles was determined such that reactions were in the exponential phase of amplification upon completion of the program. Reactions were cleaned with a NucleoSpin Gel and PCR Clean-up kit and eluted in 15 µL of water. All primer sequences can be found in Table S1.

Amplicons were fragmented and tagged (tagmented) in a 20 µL reaction containing 2.5 ng/µL amplicon, 1 × TD buffer, and 0.5 µL TDE1 enzyme from the Nextera DNA Sample Prep Kit (Illumina). Tagmentation reactions were cleaned up using a NucleoSpin column and eluted in 15 µL of water. Tagmented DNA was amplified with primer i5-Nex2p and a unique indexed primer per sample (i7- TXX-NEX2p). The 25 µL reactions were prepared containing 1 µL of tagmented DNA, 0.5 µM forward and reverse primer, 1 × KAPA HiFi Hotstart ReadyMix, and 1 × SYBR Green. Amplification was monitored by qPCR with cycling conditions: [72°C 3 min, 95°C 20 s, (98°C 20s, 52°C 15 s, 72°C 30 s, plate read, 72°C 8s) × 13 cycles]. Reactions were removed during the exponential phase of amplification.

PCR products were run on a 1.5% agarose gel to visualize distribution of tagmented DNA size and to estimate relative concentrations using FIJI gel analysis. Indexed samples were pooled while normalizing for relative concentration. Pooled products were run on a 1.5% agarose gel, cutting out a band at approximately 500bp, which was then purified using the NucleoSpin Gel and PCR Clean-up column. The concentration of the pooled library was quantified using a Qubit fluorometer, and size distribution was assessed using a HS DNA chip on the Bioanalyzer 2100 instrument (Agilent). The library was sequenced using 2 × 75bp paired-end reads on an Illumina MiSeq (v3 Reagent kit).

Linker library DNA Sequencing. The entire ORF was initially PCR amplified from the Landing Pad genomic integration site using a forward primer specific to the genome, LP-Ptet-F, and a reverse primer specific to the integrated plasmid sequence, LP-STOP-R, to limit amplification of any residual plasmid DNA. Each 50 µL reactions was prepared with a final concentration of 20 ng/µL of genomic DNA, 0.25 µM forward and reverse primer, 1 × SeqAmp PCR buffer, 1 × SeqAmp Polymerase (Clontech), and 1 × SYBR Green (Invitrogen). Amplification was monitored by qPCR with cycling conditions: [94°C 60s,

(98°C 10s, 55°C 15s, 68°C 60s, plate read) × 14 cycles]. Reactions were cleaned with a NucleoSpin Gel and PCR Clean-up kit and eluted in 15 µL of water.

The linker regions flanking cpGFP were PCR amplified in a second round using primers CggR-180-seq-F2 and CggR-180-seq-R2. Two replicate 50 µL reactions were prepared for each sample with a final concentration of 5 ng/µL genomic DNA, 0.25 µM forward and reverse primer, 1 × SeqAmp PCR buffer, 1 × SeqAmp Polymerase (Clontech), and 1 × SYBR Green (Invitrogen). Amplification was monitored by qPCR with cycling conditions: [94°C 60s, (98°C 10s, 55°C 15s, 68°C 60s, plate read) × 10 cycles]. The number of cycles was determined such that reactions were in the exponential phase of amplification upon completion of the program. Replicate reactions were pooled and cleaned with a NucleoSpin column and eluted in 15 µL of elution buffer (5 mM Tris/HCl, pH 8.5).

Second-round PCR products were amplified a third time with primer i5-IPE2p and a unique indexed primer per sample (i7- iPE2p-XX). Then, 25 µL reactions were prepared containing 1 µL of round 2 DNA, 0.5 µM forward and reverse primer, 1 × KAPA HiFi Hotstart Readymix (KHF), and 1 × SYBR Green. Amplification was monitored by qPCR with cycling conditions: [95°C 3 min, (98°C20 s, 60°C15s, 72°C 30 s, plate read, 72°C8s) × 8 cycles]. Reactions were removed during the exponential phase of amplification. PCR products were run on a 1.5% agarose gel to ensure only a single band had been produced and to estimate relative concentrations using FIJI gel analysis. Indexed samples were pooled, normalizing for relative concentration. Pooled products were run on a 1.5% agarose gel, cutting out a band at 941 bp, which was then purified using the NucleoSpin Gel and PCR Clean-up column. The concentration of the pooled library was quantified using a Qubit fluorometer, and size distribution was assessed using a HS DNA chip on the Bioanalyzer 2100 instrument (Agilent). The library was sequenced using 2 × 75 bp paired-end reads on an Illumina MiSeq (v3 Reagent kit) loaded at a final concentration of 14 pM with 15% PhiX spiked in.

Sort-seq data analysis. Raw sequencing reads were deposited into the NCBI Sequence Read Archive (SRA) with the BioProject accession code PRJNA792029 (4). Paired end reads were merged using BBMerge (5). CggR-cpGFP insertion sites were counted using the dipseq analysis pipeline developed by the Savage lab available at (https://github.com/SavageLab/dipseq). This Python package identifies reads that contain sequences originating from both CggR and cpGFP. Junction reads are then trimmed to remove the cpGFP sequence plus transposon scar before aligning the remaining sequence to CggR to identify the site of insertion. The output is a file containing counts for each insertion site (including out-of-frame and reverse insertions) in each sample, which was used for enrichment and sort-seq analysis.

Sort-seq data analysis was performed as previously described using functions written in R available at: https://github.com/jnkoberstein/biosensor-sort-seq (2, 6). Raw sequencing data were processed to obtain read counts of each variant in each bin using the dipseq python package for the CggR-DIP library or the custom R package for the

CggR-180 library. Mean fluorescence was estimated for both samples using a maximum likelihood estimator and then used to calculate the dynamic range as $\Delta F/F = (F_I - F_0) / F_0$ where F_I is the fluorescence intensity in the sample with added glucose and F_0 is the fluorescence intensity in the absence of glucose. The CggR-DIP and CggR-180 libraries were filtered to keep only variants in which an estimate of more than 500 cells were collected for each sample and variance in each sample was less than 0.3.

Protein Purification. A protein expression destination vector was constructed by moving the CggR-180-Dest sequence from HC_Kan_CggR-180-Dest into the plasmid pSMT3. pSMT3 was a gift from Arthur Glasfeld that consists of a pET-28b vector modified to include an N-terminal 6xHIS fused to *S. cerevisiae* Smt3 (yeast homolog of SUMO). Briefly, the CggR-180-Dest sequence was amplified using primers pSMT3-CggR-EBD-GA-F2 and pSMT3-CggR-EBD-GA-R2 while the pSMT3 vector was amplified and linearized using pSMT3-CggR-EBD-GA-F1 and pSMT3-CggR-EBD-GA-R1. The two PCR fragments were purified and combined in a 20 µL Gibson Assembly reaction using 50 ng total of backbone amplicon and a 3-to-1 molar backbone:insert ratio. HYlight (CggR-180-PPKE) was cloned into pSMT3-CggR-180-Dest by amplifying cpGFP from pTKEI-Mal-B2 using primers CggR-180-PP-F and CggR-180-KE-R to add the linker sequences, complementary overhangs and BsaI restriction sites for Golden Gate Cloning.

pSMT3-CggR-180-PPKE was transformed into E. cloni EXPRESS BL21(DE3) Competent Cells and plated on LB agar with kanamycin. A single colony was picked into 40 mL LB with kanamycin and grown overnight at 37°C while shaken at 250rpm. The following day 2 mL of the overnight culture was used to inoculate 400 mL LB with kanamycin which was incubated at 37°C for approximately 2 hours until reaching an OD600 between 0.5 and 0.6. Protein expression was then induced by adding IPTG to a concentration of 750 µM. Induced cultures were grown overnight at 30°C. Cultures were pelleted by centrifugation at 5000g for 20 minutes.

Cell pellets were resuspended in 8 mL lysis buffer (50 mM Na_2HPO_4, 350 mM NaCl and 20% sucrose, pH 8) in a 15 mL Falcon tube and a dash of lysozyme was added. Cells were lysed by sonication using a Fisher Scientific 60 Sonic Dismembrator with 20s pulses at the maximum setting, 19 watts (RMS), followed by 60s on ice, for five repetitions. Lysates were centrifuged at 15,000g for 20 minutes to pellet the insoluble fraction. Cleared lysate was added to a column containing TALON Metal Affinity Resin (equilibrated with Wash Buffer) and incubated while nutating at 4°C for at least 1 hour. The flow through was drained and the beads bound to HIS-tagged protein were washed 2 times with Wash Buffer (50 mM Na_2HPO_4 and 350 mM NaCl, pH 8) before eluting in Wash Buffer with 200 mM imidazole. The SMT3 domain was removed by incubation with the Ulp1 protease. 0.2mg of Ulp protease was added to the samples and dialyzed overnight in Wash Buffer containing 1mM DTT at room temperature, which was subsequently removed from the solution the following day through 3 rounds of dialysis in Wash Buffer at 4°C, as DTT is incompatible with the TALON resin. The protein containing solution was then run over a column containing TALON Metal Affinity Resin

to separate the 6xHIS-tagged SMT3 from the now untagged protein which was captured in the flow through. The concentration of collected protein was measured by bradford assay prior to further characterization of biosensor function.

HYlight (and various binding and fluorescence mutations) were tested for affinity and dynamic range in a 96-well plate format measured by a BMG ClarioStar plate-reader. Purified protein diluted to approximately 200 nM in PBS in a black-bottom 96-well plate at a volume of 100 µL per well. Excitation scans were collected at 550 nm emission with a 20 nm bandwidth, and emission scans were performed with 405- and 488-nm excitation. FBP solutions were prepared at 10X final concentration in PBS and pH measured using NaOH and pH paper to pH7.5. Tenfold dilutions of 5, 2, and 1 mM FBP were tested in triplicate to generate FBP binding curves.

Biosensors were tested for affinity and dynamic range in a 96-well plate format measured by a BMG ClarioStar plate-reader. Purified protein was diluted to approximately 200 nM in PBS in a black-bottom 96-well plate at a volume of 100 µL per well. FBP solutions were prepared at 10X final concentration in PBS, the pH adjusted to 7.5 using NaOH and pH paper. Tenfold dilutions of 5, 2, and 1 mM FBP were tested in triplicate to generate FBP binding curves. Excitation scans were collected at 550 nm emission and a 20 nm bandwidth, from 320 nm to 520 nm in 1 nm increments. Emission scans were performed using 405- and 472-nm wavelengths with a 16 nm bandwidth, from 500 nm to 600 nm, in 1 nm increments. Endpoint fluorescent assays used either 405- or 488-nm with a 15 nm bandwidth excitation wavelength, a 504 nm low pass dichroic filter, and emission intensity collected at 530 nm with a 40 nm bandwidth.

Cell culture. HEK293T cells were maintained in DMEM supplemented with 10% FBS and transfected using lipofectamine 2000 as per the manufacturer's protocol. MIN6 cells were maintained in DMEM containing 15% FBS, 1% Pen/Strep and 70 µM β-mercaptoethanol. MIN6 cells were transduced with an insulin promoter driven, β-cell specific adeno-associated virus (7) encoding HYlight for 2 hours and then returned to MIN6 culture media.

Imaging and Quantification. MIN6 or HEK293T cells were plated onto glass bottom 35 mm pie dishes at $2x10^5$ or $1x10^5$ cells per quadrant, respectively and either transfected or transduced as described above. One hour prior to imaging, culture media was changed to 500 µL of 0 mM or low glucose in media containing 145 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, and 10 mM HEPES, pH 7.4. Live cell imaging was performed on a Nikon Eclipse TiE inverted microscope encased in a Okolab H101 stage-top incubator with temperature, humidity and CO_2 control with a Yokogawa CSU-W1 spinning disk confocal unit. Images were taken using a 60X oil-based objective (NA 1.4, Plan Apo VC OFN 25) and using the perfect focus system (PFS) for automatic correction of drift and to aid in stability during long term time-lapse imaging. Cells were maintained in 5% CO_2 at 37˚C for the duration of the experiment. Cells were excited at 488 nm and 405 nm with emission wheel 525/25 nm. Images were acquired every 10-15 seconds. To

test sensor responses, glucose or drugs were applied directly to the media during the imaging session. For the glycolytic stress test cells were glucose starved for 1 hour prior to imaging. Starved cells were imaged for 5 minutes, followed by 11 mM glucose for 30 minutes, 2.5 µM Oligomycin for 30 minutes and lastly 2-deoxyglucose for 30 minutes. For the imaging of whole islets, a peristaltic pump perfusion system was used to deliver media which was alternated between low and high glucose. Images were processed using CellPose (8) to segment individual cells and generate ROIs, which were quantified using FIJI. Background fluorescence intensity was calculated from a region containing no cells and this value was subtracted from all pixel values. Mean intensity values for both the 488 and 405 channels were measured for each ROI and used to calculate the excitation ratio ($\mathrm{R} = \mathrm{F}_{488}/\mathrm{F}_{405}$). The change in excitation ratio was calculated as $\Delta\mathrm{R}/\mathrm{R} = (\mathrm{R}_{\mathrm{t}} - \mathrm{R}_{\mathrm{t=0}})/\mathrm{R}_{\mathrm{t=0}}$ where $\mathrm{R}_{\mathrm{t=0}}$ is the excitation ratio as the start of experiment.

Flow cytometry. MIN6 cells were seeded in 6 well plates (10^6 per well). The following day MIN6 cells were transduced with a β-cell specific AAV encoding HYlight. Cells were incubated with 5% CO_2 at 37˚C for two days. HEK293T cells were plate in 6 well plates ($7.5x10^6$ per well). The following day HEK293T cells were transfected using Lipofectamine 2000 per the manufacturer's protocol. Cells were incubated with 5% CO_2 at 37˚C for 24 hours. One hour prior to harvest, MIN6 or HEK293 culture media was exchanged for media containing 145 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, 10 mM HEPES and glucose ranging from 0-25 mM. For experiments in MIN6 cells using the glucokinase activator dorzagliatin (Selleckchem, Cat. #S6921), cells were treated with 10 µM dorzagliatin for 1 hour in media containing 145 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, 10 mM HEPES and glucose ranging from 0-25 mM. Following the 1-hour incubation, cells were trypsinized for 2 minutes, spun down at 2,000rpm for 3 minutes and resuspended in 500ul of media containing the same concentration of glucose prior to harvest, with or without dorzagliatin. Cells were kept at room temperature for the remainder of the experiment. Data were collected on a BD Symphony Flow Cytometer using lasers 488-1 (Ex. 488 nm, Em. 530/30 nm) and 405-2 (Ex. 405 nm, Em. 525/50 nm). Cells were gated to exclude dead cells and debris (using forward and side scatter area) followed by a standard doublet-exclusion (using forward scatter area and width). Fluorescent cells were gated for on the diagonal of a plot of 405-2 vs 488-1 excluding all non-transfected cells. At least 10^4 fluorescent cells were evaluated per sample and three samples (independent wells) were prepared per glucose concentration. The excitation ratio ($\mathrm{R} = \mathrm{F}_{488} - \mathrm{F}_{405}$) was calculated per cell on log(AFU) values. Michaelis-Menten curves were fit to the median ratio calculated per sample.

Primary islet isolation, culture, and imaging. Pancreatic islets were imaged in solution containing (mM) 140 NaCl, 4.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 1 NaH_2PO_4, 5 $NaHCO_3$, 10 HEPES (pH 7.4, with NaOH). All animal experiments were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986) and the University of Oxford ethical guidelines. Pancreatic islets were isolated from humanely killed C57Bl6/J mice (Charles River, UK) by injecting cold liberase solution into the bile duct, with subsequent

digestion of the connective and exocrine pancreatic tissue. Islets were cultured for 36h in RPMI medium containing 11mM glucose, supplemented with 10% FBS, 100IU/mL penicillin and 100µg/mL streptomycin (all reagents from Life Technologies, Paisley, UK). HYlight was delivered via an AAV vector at 10^5 infectious units per islet, followed by 24-36h culturing to express the sensors.

Intracellular FBP was reported in pancreatic islet cells by exciting the HYlight fluorescence at 485 nm and the emission recorded at 515nm using a Zeiss Axiozoom.V16 microscope (2.3/0.56 objective) or Zeiss Axioskop microscope (20x/1.0 objective). $[FBP]_i$ was imaged at a frequency of 33 mHz. All imaging at 34°C was performed using an open chamber (9). The bath solution containing various stimuli was perfused continuously at a rate of 60 µl/min. Images were acquired using Zen (Carl Zeiss) or MicroManager software. Image sequences were analyzed (registration, background subtraction, intensity vs time analysis) using open-source FIJI software (http://fiji.sc/Fiji). The numerical data were then analyzed using IgorPro package (Wavemetrics), as detailed in Draper et al. (10).

Sequences for HYlight (CggR-180-PPKE).

Color legend
CggR 96-180
Linkers
cpGFP (148-146, mutations relative to EGFP: H148Y, Y151F, V163A, T203V, V93I, Y145F)
CggR 181-340

Amino Acid sequence:
MGSKDVLGLTLLEKTLKERLNLKDAIIVSGDSDQSPWVKKEMGRAAVACMKKRFSGKN
IVAVTGGTTIEAVAEMMTPDSKNRELLFVPARGGLGEPPYNVFIMADKQKNGIKANFKIR
HNIEDGGVQLAYHYQQNTPIGDGPVLLPDNHYLSVQSKLSKDPNEKRDHMVLLEFVTA
AGITLGMDELYKGGTGGSMVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATY
GKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYIQERTIF
FKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNKEDVKNQANTIC
AHMAEKASGTYRLLFVPGQLSQGAYSSIIEEPSVKEVLNTIKSASMLVHGIGEAKTMAQR
RNTPLEDLKKIDDNDAVTEAFGYYFNADGEVVHKVHSVGMQLDDIDAIPDIIAVAGGSS
KAEAIEAYFKKPRNTVLVTDEGAAKKLLRDESG*

DNA sequence:
ATGGGCAGCAAGGATGTTTTGGGTTTGACCTTGTTGGAAAAGACTTTGAAAGAAAGA
TTGAACTTGAAGGACGCCATCATCGTTTCTGGTGATTCTGATCAATCTCCATGGGTCA
AAAAAGAAATGGGTAGAGCTGCTGTTGCTTGCATGAAGAAAAGATTTTCTGGTAAGA
ACATCGTTGCTGTTACTGGTGGTACTACTATTGAAGCTGTTGCTGAAATGATGACCCC
AGATTCTAAGAACAGAGAATTGTTGTTTGTTCCAGCTAGAGGTGGTTTGGGTGAACC
GCCTTATAACGTCTTTATCATGGCCGACAAGCAGAAGAACGGCATCAAGGCGAACTT
CAAGATCCGCCACAACATCGAGGACGGCGGCGTGCAGCTCGCCTATCACTACCAGCA
GAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCGT
GCAGTCCAAACTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGG
AGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGGGCGGTA
CCGGAGGGAGCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATC
CTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGG
CGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAA
GCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTC
AGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAA
GGCTACATTCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTATAAGACACGC
GCTGAGGTTAAGTTCGAGGGCGACACTCTGGTTAACCGCATCGAGCTGAAGGGCATC
GACTTCAAGGAGGACGGCAACATCCTGGGCCATAAGCTTGAATATAACTTCAACAAG
GAGGATGTTAAGAATCAAGCTAACACCATTTGCGCTCATATGGCTGAAAAAGCTTCA
GGTACTTACAGATTGTTATTCGTCCCAGGTCAATTGTCTCAAGGTGCTTACTCTTCCA
TTATCGAAGAACCATCTGTCAAAGAAGTCTTGAACACCATCAAATCCGCTTCCATGT
TGGTTCACGGTATTGGTGAAGCTAAAACTATGGCTCAACGCAGAAACACCCCATTGG
AAGATTTGAAAAAGATCGATGATAACGATGCTGTTACCGAAGCTTTCGGTTACTACT
TTAATGCTGATGGTGAAGTTGTTCACAAGGTTCATTCAGTTGGTATGCAATTGGATGA
TATTGATGCCATCCCAGATATTATTGCAGTTGCTGGTGGTTCTTCTAAGGCTGAAGCA
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GCCAAAAAGTTGTTGAGAGATGAATCAGGCTAA

Fig. S1.

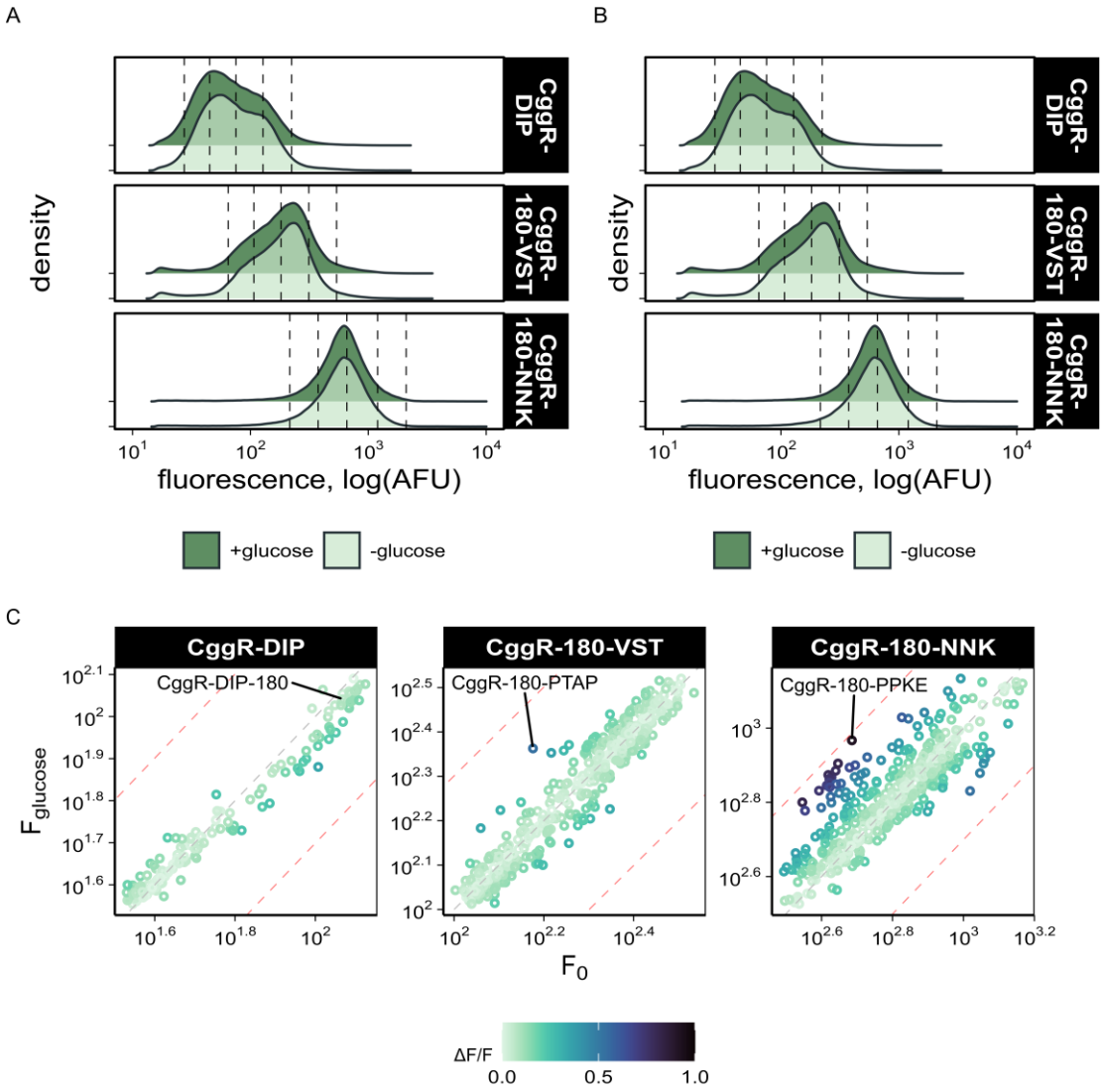
A
B
CggR-DIP
CggR-180-VST
CggR-180-NNK
density
fluorescence, log(AFU)
+glucose
-glucose
C
CggR-DIP
CggR-180-VST
CggR-180-NNK
CggR-DIP-180
CggR-180-PTAP
CggR-180-PPKE
$F_{glucose}$
F_0
ΔF/F
0.0
0.5
1.0

Fig. S1. Discovery of a high-dynamic-range FBP biosensor by sort-seq assay. (*A*) A transposon based cloning strategy was used to construct a domain-insertion profiling (DIP) library with variable insertion of cpGFP into CggR. The BsaI restriction sites flanking the transposon sequence produce short Ala-Ser linkers on either side of cpGFP, along with a duplication of the first two amino acids preceding cpGFP (N-1 and N). Two additional libraries consisting of cpGFP insertion at CggR residue 180 with variable linkers were constructed using PCR with primers containing degenerate nucleotides. These libraries are referred to as CggR-180-VST and CggR-180-NNK. (*B*) Cellular fluorescence distributions and FACS gate configurations for the three separate libraries evaluated by sort-seq. The CggR-180-NNK library was initially sorted for a limited number of bright cells to bottleneck the number of unique variants present while also enriching especially bright variants. CggR-DIP was initially sorted for variants with fluorescence intensity above un-recombined cells, while the naive CggR-180-VST library was used as input for sort-seq. (*C*) Sort-seq estimates of the mean fluorescence for each variant in glucose starved (F_0) and fed ($F_{glucose}$) conditions. Points are colored by dynamic range with $\Delta F/F$ calculated as $(F_{max} - F_{min}) / F_{min}$ to produce a symmetrical metric. See also dataset 1.

Fig. S2.

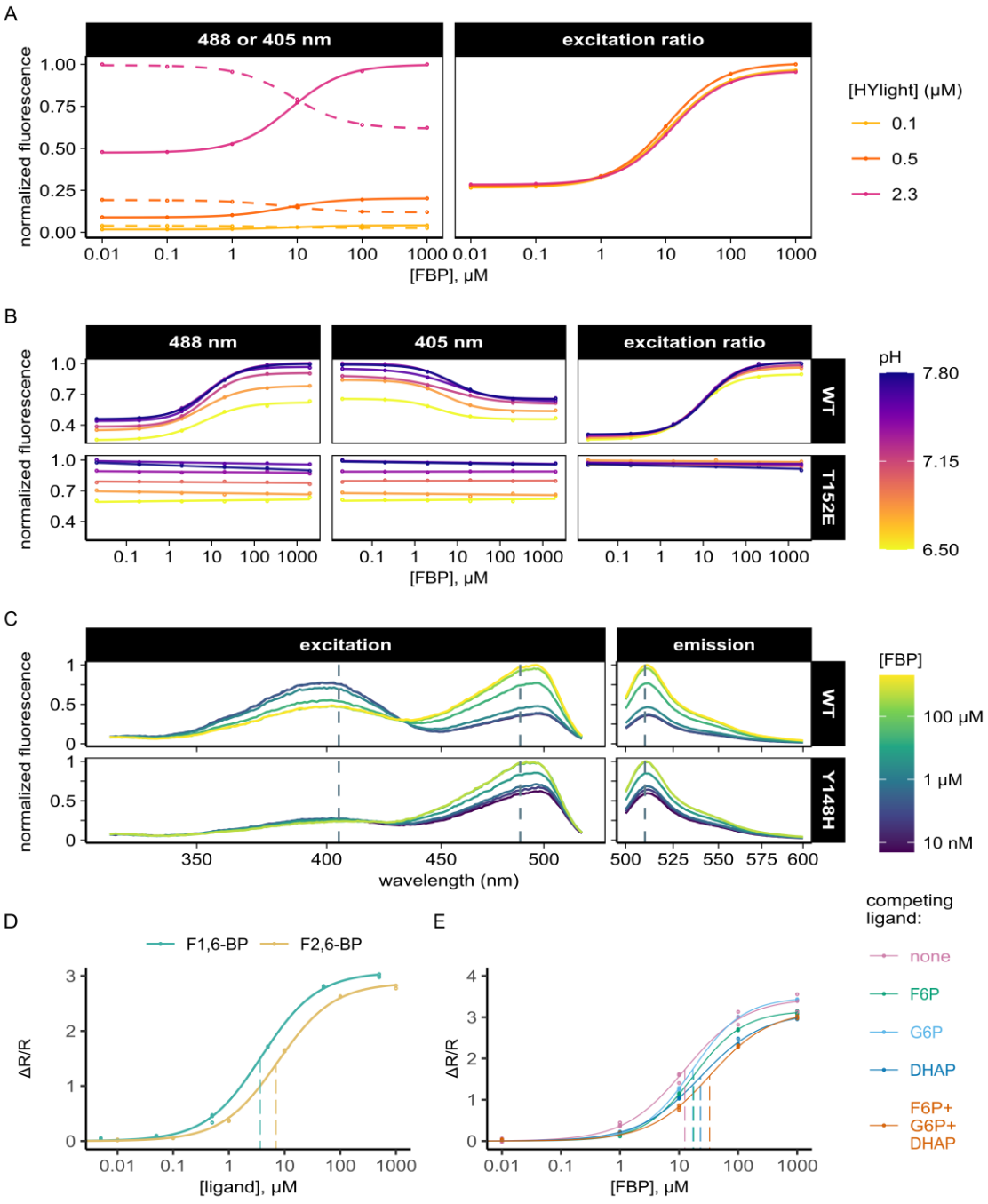
A
488 or 405 nm
excitation ratio
normalized fluorescence
[FBP], µM
[HYlight] (µM)
0.1
0.5
2.3
B
488 nm
405 nm
excitation ratio
WT
T152E
pH
7.80
7.15
6.50
C
excitation
emission
WT
Y148H
wavelength (nm)
[FBP]
100 µM
1 µM
10 nM
D
F1,6-BP
F2,6-BP
ΔR/R
[ligand], µM
E
competing ligand:
none
F6P
G6P
DHAP
F6P+ G6P+ DHAP

Fig. S2. In vitro characterization of HYlight. (*A*) HYlight excitation ratio consistently reports [FBP] across varied protein concentrations. Dashed lines in left panel correspond to 405 nm and solid line to 488 nm excitation. (*B*) HYlight exhibits pH dependent changes for both 488 nm and 405 nm excitation, however the ratio of these two parameters is largely pH insensitive. Mutation of Thr^{152} to Glu (numbered according to CggR) results in a loss of FBP sensitivity while retaining similar pH sensitivity across both 488 and 405 nm excitation. (*C*) The prominent neutral fluorophore excitation peak (405 nm) and red-shifting of the anionic fluorophore excitation peak (maximum at 495 nm) observed for HYlight can be attributed to two specific mutations. Substitution of Tyr^{148} back to wildtype His (numbered according to wtGFP) results in a loss of 405 nm excitation. Other biosensors including ratiometric pericam (11), Perceval (12), FGBP (13), GEX-GECO1 (14) and ExRai-AKAR (15) exhibit similar dual excitation capabilities. None of these other ratiometric biosensors contain H148Y. However, ratiometric-pericam, Perceval, and FGBP contain the mutation H148D, which may function similarly. Substitution of Val^{203} to Thr results in a blue-shift of anionic peak to a maximum at 488 nm compared to 495 nm for HYlight. (*E*) Competing ligands shift the affinity of HYlight for FBP from 12.5 µM (no competition) to 17.6 µM (100 µM F6P), 17.1 µM (675 µM G6P), 23.0 µM (1.67 mM DHAP) and 33.0 µM (100 µM F6P + 675 µM G6P + 1.67 mM DHAP).

Fig. S3.

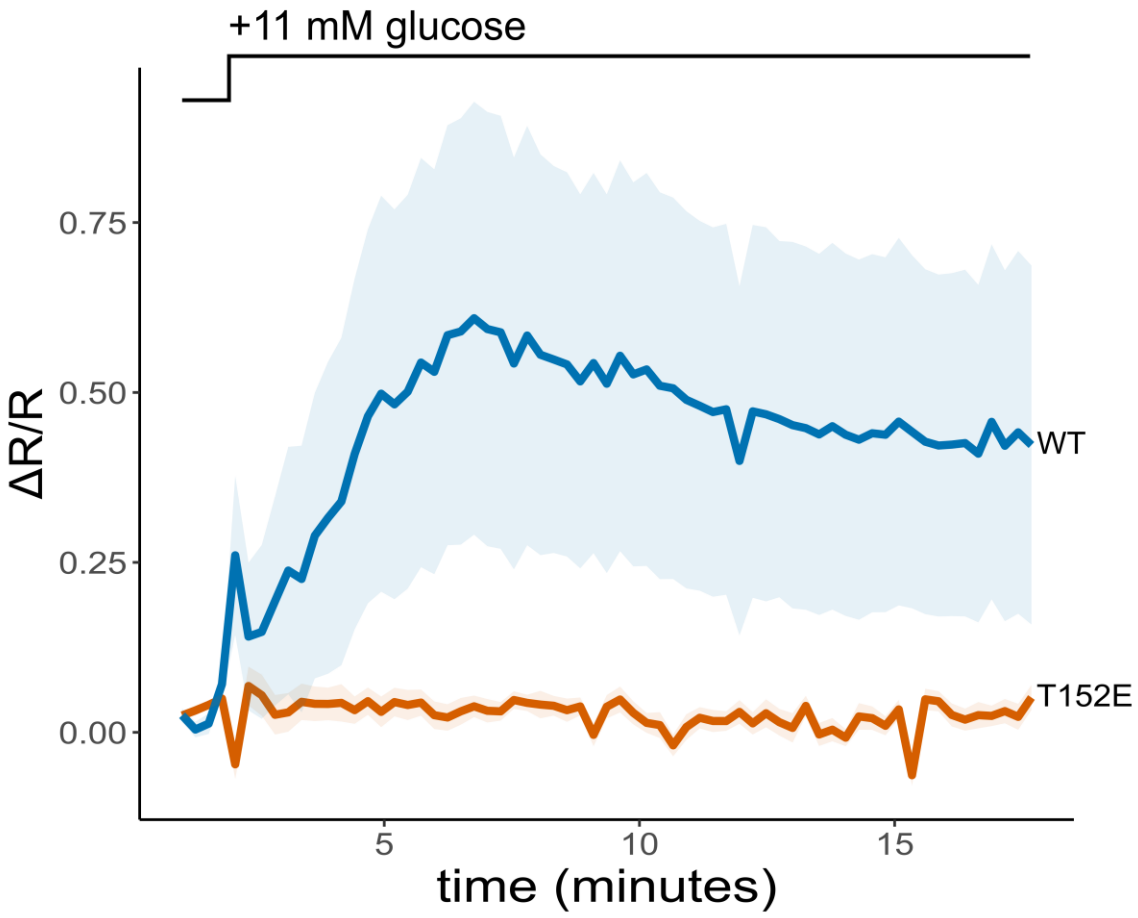

Fig. S3. The HYlight-T152E "binding dead" mutant expressed in MIN6 cells exhibits no change in fluorescence ratio upon addition of 11mM glucose. ΔR/R was normalized to the glucose starved state at the beginning of each experiment. Solid line represents the mean across cells while shaded ribbon represents the mean ± standard deviation.

Table S1. Primer sequences

Name	Sequence
CggR-p7-GG-F	TCGCTAGGTCTCCCAGCAAGGATGTTTTGGGTTTG
CggR-p7-GG-R	TCGCTAGGTCTCCGCCTGATTCATCTCTCAACAACTT
CggR-A250R-Q5-F	TATGGCTCAACGCAGAAACACCCCATTG
CggR-A250R-Q5-R	GTTTTAGCTTCACCAATAC
CggR-180-Dest-GA-F1	CTAGAGGTGGTTTGGGTGAAAGAGACCATTAATGCAGCTG
CggR-180-Dest-GA-R1	TTAGCTTGATTCTTAACATCAGAGACCAGCTTGTCTGTAA
CggR-180-Dest-GA-F2	TTACAGACAAGCTGGTCTCTGATGTTAAGAATCAAGCTAACA
CggR-180-Dest-GA-R2	CAGCTGCATTAATGGTCTCTTTCACCCAAACCACCTCTAG
CggR-cpGFP-VSTx4-GG-F	GTCATCGGTCTCCTGAAVSTVSTTATAACGTCTTTATCATGGC
CggR-cpGFP-VSTx4-GG-R	GTCATCGGTCTCCCATCASBASBGTTGAAGTTATATTCAAGCT
CggR-cpGFP-NNKx4-GG-F	TGCACAGGTCTCGTGAANNKNNKTATAACGTCTTTATCATGGC
CggR-cpGFP-NNKx4-GG-R	TGCACAGGTCTCGCATCMNNMNNGTTGAAGTTATATTCAAGCT
CggR-BsmbI-GG-F	CAGACTCGTCTCCCAGCAAGGATGTTTTGGGTTTG
CggR-BsmbI-GG-R	CAGACTCGTCTCCGCCTGATTCATCTCTCAACAAC
pSMT3-CggR-EBD-GA-F1	ATTCCCCGTCTCGGGTGAAGATGTTAAGAATCAAGCTAA
pSMT3-CggR-EBD-GA-R1	ATTCCCCGTCTCGGCTTCACCCAAACCACCTCT
pSMT3-CggR-EBD-GA-F2	GAGAACAGATTGGTGGATCCAAGGATGTTTTGGGTTTGAC
pSMT3-CggR-EBD-GA-R2	ATTCCCCGTCTCCCACCTGACGCGTTGAAGTTATA
CggR-180-PP-F	TTGGCAGGTCTCGTGAACCGCCTTATAACGTCTTTATCATGGC
CggR-180-KE-R	TTGGCAGGTCTCGCATCCTCCTTGTTGAAGTTATATTCAAGCT
CggR-180-seq-F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTTGTTTGTTCCAGCTAGAGGT
CggR-180-seq-R2	TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNTATGAGCGCAAATGGTGTTAGC
LP-Ptet-F	CCTGGAGCAATTCCACAACAC
LP-STOP-R	GTAGGGATGTTCTAGAGTCCGG
i5-IPE2p	AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGAC
i7-TXX-iPE2p	CAAGCAGAAGACGGCATACGAGAT[index]GTGACTGGAGTTCAGACGTGTGC

Table S2. Number of sorted cells for each sample and condition in each bin

sample	condition	bin	number of sorted cells	lower bound log(AFU)	upper bound log(AFU)
CggR-180-NNK	-glucose	1	1,342,014	2.3338	2.5745
		2	3,000,000	2.5745	2.8182
		3	3,000,000	2.8182	3.0791
		4	445,088	3.0791	3.3213
	+glucose	1	801,400	2.3338	2.5745
		2	2,850,616	2.5745	2.8182
		3	3,000,000	2.8182	3.0791
		4	463,896	3.0791	3.3213
CggR-180-VST	-glucose	1	186,989	1.4891	1.7066
		2	303,642	1.7066	1.9363
		3	444,897	1.9363	2.1723
		4	149,643	2.1723	2.4083
	+glucose	1	129,363	1.4891	1.7066
		2	215,152	1.7066	1.9363
		3	319,049	1.9363	2.1723
		4	91,353	2.1723	2.4083
CggR-DIP	-glucose	1	706,159	1.3849	1.6126
		2	527,182	1.6126	1.8403
		3	452,703	1.8403	2.0680
		4	460,600	2.0680	2.2957
	+glucose	1	569,654	1.3849	1.6126
		2	624,135	1.6126	1.8403
		3	467,017	1.8403	2.0680
		4	260,761	2.0680	2.2957

Dataset S1 (separate file). Sort-seq estimates of brightness and dynamic range for domain-insertion and linker variant libraries.

Sort-seq measurements for 156 out of 251 possible domain-insertion variants are presented in sheet 1. Variants are identified by the nucleotide site and direction of transposon insertion (“variant” column), as well as the corresponding amino acid number in CggR proceeding the first amino acid of cpGFP insertion (“AA” column). In sheet 2, sort-seq measurements for 900 linker combinations are compiled for the two libraries. Variants are identified by the codon sequences (“variant” column) as well as the amino acid translation (“AA” column). The “F0.bin1-4” and “Fl.bin1-4” columns represent the estimated number of sorted cells for each variant in each bin (1-4) in the -glucose (F0) and +glucose (Fl) conditions. F0.mu and Fl.mu are the estimated mean fluorescence intensities for each variant after log transformation. F0.sigma and Fl.sigma are the estimated lognormal standard deviations. F0.mean and Fl.mean are the estimated mean fluorescence intensity prior to log transformation. DynamicRange is $\Delta F/F$ calculated as (Fl.mean – F0.mean) / F0.mean.

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