Supplementary Information

A modular approach for dCas9-mediated enzyme cascading via orthogonal bioconjugation

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MATERIALS AND METHODS

Materials

All primers were purchased from IDT (Coralville, IA). All ingredients for culturing media were purchased from Fisher Scientific (Pittsburgh, PA), all ingredients for SDS-PAGE were purchased from BIO-RAD (Hercules, CA), all enzymes related to DNA manipulation and cloning were purchased from New England Biolabs (Ipswich, MA), and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Strains and Cloning

Escherichia coli NEB5α (New England Biolabs, Ipswich, MA) [*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17]* was used as the host for all genetic manipulations and plasmid maintenance. *E. coli* strain BLR (DE3) (EMD Millipore, Madison, WI) *[F- ompT hsdS$_B$(r_B- m_B-) gal dcm (DE3) Δ(srl-recA)306::Tn10 (Tet^R)]* was used as the production host for all proteins.

The nuclease-null Cas9 proteins *S. pyogenes* (SpdCas9), *S. thermophilus* (ST1dCas9) and *S. aureus* (SadCas9) were purchased from Addgene. The dCas9 protein sequences were PCR amplified, double digested, and inserted into pET24a using SpeI and bamHI. SpyTag and SnoopTag peptides were added into the vectors using the restriction sites bamHI and MfeI for the following constructs: pET24a SpdCas9-SpyTag-6xhis and St1dCas9-SnoopTag-6xhis. For the pathways enzymes, bamHI and XhoI restriction sites were used to add the catcher proteins to the vectors for the following constructions: pET24a StrepII-CBD-Spycatcher and pET24a SnoopCatcher-CelA-Flagtag-StrepII.

Protein Expression

All constructs were transformed into *E. coli* BLR [F- ompT hsdSB (r-B m-B) gal dcm(DE3) Δ(srl-recA)306::Tn10(TetR); Novagen, Madison,WI] cells for protein expression. Expression cultures were grown in Luria-Bertani (LB) media (10.0 g/L tryptone, 5.0 g/L yeast extract, 10.0 g/L NaCl) supplemented with 100 µg/mL ampicillin (dCas9-Tag fusions) or kanamycin (enzyme-catcher fusions).

Expression of dCas9-Tag Constructs

Overnight cultures were used to inoculate 30 mL of fresh LB to an initial OD_{600} of ~0.05 and grow to mid-exponential phase (OD_{600} ~0.75) at 37°C. Protein expression of SpdCas9-SpyTag-6xhis was induced with 200 µM isopropyl-β-thiogalactopyranoside (IPTG) and incubated at 20°C for ~16hrs. Expression of ST1dCas9-SnoopTag-6xhis protein fusion was optimal with induction of 100 µM IPTG and incubation at 37°C for 5 hours.

Induced cultures were pelleted using centrifugation at 3000xg for 10 minutes at 4°C, and the pellet was suspended in 1x TBS (Tris Buffered Saline) to an OD600 = 30. The cell suspension was sonicated to release protein and clarified using centrifugation at 15,000xg for 15 min at 4°C. Soluble proteins were immediately purified using affinity chromatography.

Purification of dCas9-Tag Constructs

Soluble proteins were mixed with imidazole to a concentration of 5 mM. Meanwhile, gravity columns were packed with HisPur™ Ni-NTA Resin (ThermoFisher Scientific, Waltham, MA). The column was used as instructed using TBS + 10 mM imidazole as the equilibration buffer, TBS + 25 mM imidazole as the wash buffer, and TBS + 250 mM imidazole as the elution buffer. Eluates were dialyzed against TBS overnight. The cell lysates and the purified proteins were loaded onto a 7.5% SDS-PAGE gel and stained with coomassie blue for analysis.

Expression of Cellulosomal Enzyme-Catcher Constructs

Overnight cultures were used to inoculate 30 mL of fresh LB to an initial OD_{600} of ~0.05 and grow to mid-exponential phase (OD_{600} ~0.75) at 37°C. Protein expression of both StrepII-CBD-SpyCatcher and SnoopCatcher-CelA-FlagTag-StrepII proteins were induced with 200 µM isopropyl-β-thiogalactopyranoside (IPTG) and incubated at 20°C for ~16 hours.

Induced cultures were pelleted using centrifugation at 3000xg for 10 minutes at 4°C, and the pellet was suspended in 1x TBS (Tris Buffered Saline) to an OD600 = 30. The cell suspension was sonicated to release protein and clarified using centrifugation at 15,000xg for 15 min at 4°C. Soluble proteins were immediately purified using affinity chromatography.

Purification of Cellulosomal Enzyme-Catcher Constructs

Soluble StrepII-CBD-Spycatcher proteins were added to gravity columns packed with Strep-Tactin® (IBA,Germany) The column was used as instructed washing the column with the proprietary wash buffer (buffer W) and eluting with 2.5 mM desthiobiotin (Buffer E). Eluates were dialyzed against TBS overnight. The cell lysates and the purified proteins were loaded onto a 7.5% SDS-PAGE gel and stained with coomassie blue for analysis.

StrepTag purification interfered with the SnoopCatcher-SnoopTag reaction post purification. Due to this conflict, SnoopCatcher-CelA-FlagTag-StrepII was purified by incubating soluble lysate in a 60°C water bath for 15 minutes followed by centrifugation at 15,000xg for 15 min at 4°C to separate purified SnoopCatcher-CelA-FlagTag-StrepII proteins from aggregated proteins. The cell lysates and the purified proteins were loaded onto a 12% SDS-PAGE gel and stained with coomassie blue for analysis.

RNA *In Vitro* Transcription and Purification

All RNAs were transcribed in vitro via HiScribe T7 Quick High Yield RNA Synthesis Kit (New England BioLabs, Inc., Ipswich, MA, USA) and purified via phenol-chloroform extraction and ethanol precipitation. The purity, quality and quantity of RNA was analyzing by spectrophotometry using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Then subsequently using a denaturing urea PAGE electrophoresis with a 6% polyacrylamide gel containing 8 M urea.

Tag/Catcher Lysate Reactions

Soluble lysates were mixed together at a 1:1 ratio, SpdCas9-SpyTag-6xhis and StrepII-CBD-SpyCatcher as well as ST1dCas9-SnoopTag-6xhis and SnoopCatcher-CelA-FlagTag-StrepII. The improper Tag/Catcher pairs were also mixed to confirm orthogonality. Reactions were incubated overnight at 4°C with rotation. Overnight reaction concentrations were quantified by measuring A595 after incubation with Bradford reagent and were loaded onto a 7.5% SDS-PAGE gel and stained with coomassie blue for analysis.

Tag/Catcher Purified Protein Reactions

Purified proteins were mixed together at a total concentration of 24 μM in a 1:1 ratio, SpdCas9-SpyTag-6xhis and StrepII-CBD-SpyCatcher as well as ST1dCas9-SnoopTag-6xhis and SnoopCatcher-CelA-FlagTag-StrepII. Reactions were incubated overnight at 4°C with rotation. Overnight reaction concentrations were quantified by measuring A595 after incubation with Bradford reagent and were loaded onto a 7.5% SDS-PAGE gel and stained with coomassie blue for analysis.

Tag/Catcher Reaction Time Course

Purified proteins were mixed together at a total concentration of 24 μM in a 1:1 ratio, SpdCas9-SpyTag-6xhis and StrepII-CBD-SpyCatcher as well as ST1dCas9-SnoopTag-6xhis and SnoopCatcher-CelA-FlagTag-StrepII. Reactions were incubated at room temperature and samples were taken at time 0, .5, 1 and 2 hours. Samples were quenched by addition of SDS-PAGE loading dye and boiling. Samples were loaded onto a 7.5% SDS-PAGE gel and stained with coomassie blue for analysis.

Electrophoretic Mobility Shift Assay

Binding of ST1dCas9-SnoopTag with a large truncation was qualitatively verified using electrophoretic mobility shift experiments carried out in 4.5% non-denaturing acrylamide gels. Final assay mixtures contained 10 nM hybridized beacon target, 100 nM dCas9 protein and 100 nM sgRNA. The fully hybridized beacon DNA only, as well as the beacon DNA bound by the dCas9:gRNA complex were loaded and run for 90 minutes at a constant 90V. The gel was then imaged using an Amersham™ Typhoon™ Biomolecular Imager (GE Healthcare Bio-Sciences Corporation, Marlborough, MA).

Beacon Assay

All DNA probes were prepared using unmodified and fluorophore or quencher labeled oligonucleotides synthesized and purified by the commercial vendor Integrated DNA Technologies (Coralville, IA, USA). Target Cas9 beacon DNA shown in Table 2.1 were created by mixing equimolar amounts of complementary DNA strands at a final concentration of 1 µM in a beacon hybridization buffer (40 mM Tris, pH 7.9, 100 mM NaC); heating for 5 minutes at 90°C then cooled to 12°C at a rate of 0.1 /s using a S1000 Thermal Cycler (Bio-Rad Laboratories, INC., Hercules, CA, USA). All beacon binding assays were carried out in beacon binding buffer (20 mM Tris–HCl, 120 mM NaCl, 5% v/v glycerol, 0.1 mM DTT, 1 mM MgCl2, 0.02% v/v Tween 20, pH 7.9) and fluorescence measured using a Synergy H4 Hybrid microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Final assay mixtures contained 1 nM hybridized beacon target DNA, 10 nM dCas9 protein(s), and 10 nM sgRNA(s). Excitation and emission wavelengths

were dictated by the fluorophore in use for each target (A: FAM, ex: 498nm, em:520 nm; B: Cy5 ex: 648nm, em: 668nm).

Step-wise Cellulosome Assembly and Reducing Sugar Assay

Phosphoric acid-swollen cellulose (PASC) was prepared as described previously.[1] Tag/Catcher reactions were performed in separate tubes at total concentrations of 24 µM in a 1:1 ratio, SpdCas9-SpyTag-6xhis and StrepII-CBD-SpyCatcher as well as ST1dCas9-SnoopTag-6xhis and SnoopCatcher-CelA-FlagTag-StrepII. Reactions were incubated overnight at 4°C with rotation or at room temperature for 1-2 hours. Full length chimeric proteins were then diluted added together to final concentrations of 20 nM each and added to 20 nM sgRNA(s) with or without 20 nM target DNA in the presence of PASC. Synthetic cellulosome assembly was assayed at 30°C in 20 nM Tris-HCl buffer pH 6.0 with shaking. Samples were collected periodically over a 40 hour time period and immediately mixed with 0.25 mL DNS reagent (10 g/L dinitrosalicylic acid, 10 g/L sodium hydroxide, 2 g/L phenol, 0.5 g/L sodium sulfite) and incubated at 95°C for 10 minutes. After incubation, 0.5 mL of 40% Rochelle salts were added to fix the color before measuring the absorbance using a spectrophotometer at 575 nm.

One Pot Cellulosome Assembly and Reducing Sugar Assay

Tag/Catcher reactions were performed in the same test tube each at a total concentration of 24 µM in a 1:1 ratio, SpdCas9-SpyTag-6xhis and StrepII-CBD-SpyCatcher as well as ST1dCas9-SnoopTag-6xhis and SnoopCatcher-CelA-FlagTag-StrepII. Reactions were incubated at room temperature for 1-2 hours and diluted to final concentrations of 20 nM each and added to 20 nM sgRNA(s) with or without 20 nM target DNA in the presence of PASC. Synthetic cellulosome

assembly was assayed at 30°C in 20 nM Tric-HCl buffer pH 6.0 with shaking. Samples were collected periodically over a 40-hour time period and immediately mixed with 0.25 mL DNS reagent (10 g/L dinitrosalicylic acid, 10 g/L sodium hydroxide, 2 g/L phenol, 0.5 g/L sodium sulfite) and incubated at 95°C for 10 minutes. After incubation, 0.5 mL of 40% Rochelle salts were added to fix the color before measuring the absorbance using a spectrophotometer at 575 nm.

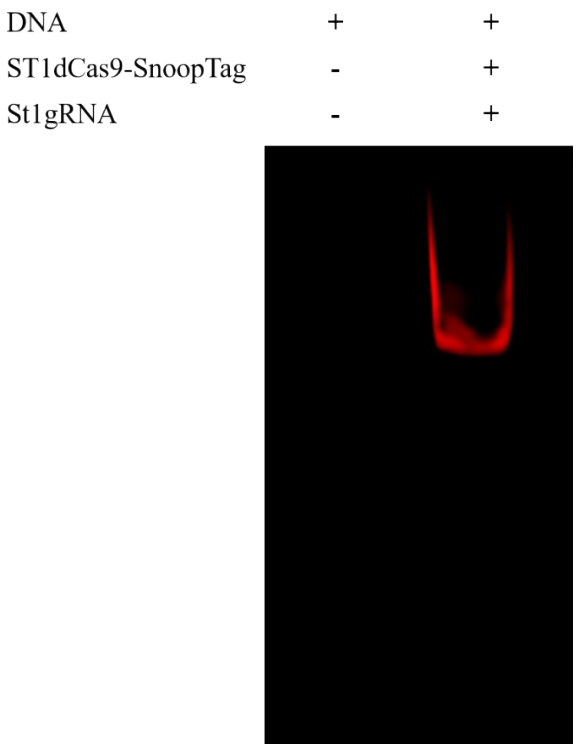

Figure S1. ST1dCas9-SnoopTag truncation is not capable of binding DNA. EMSA analysis of ST1dCas9-SnoopTag:ST1gRNA binding to our synthetic DNA beacon shows only one product band indicating the N-terminal truncation does not retain binding capabilities.

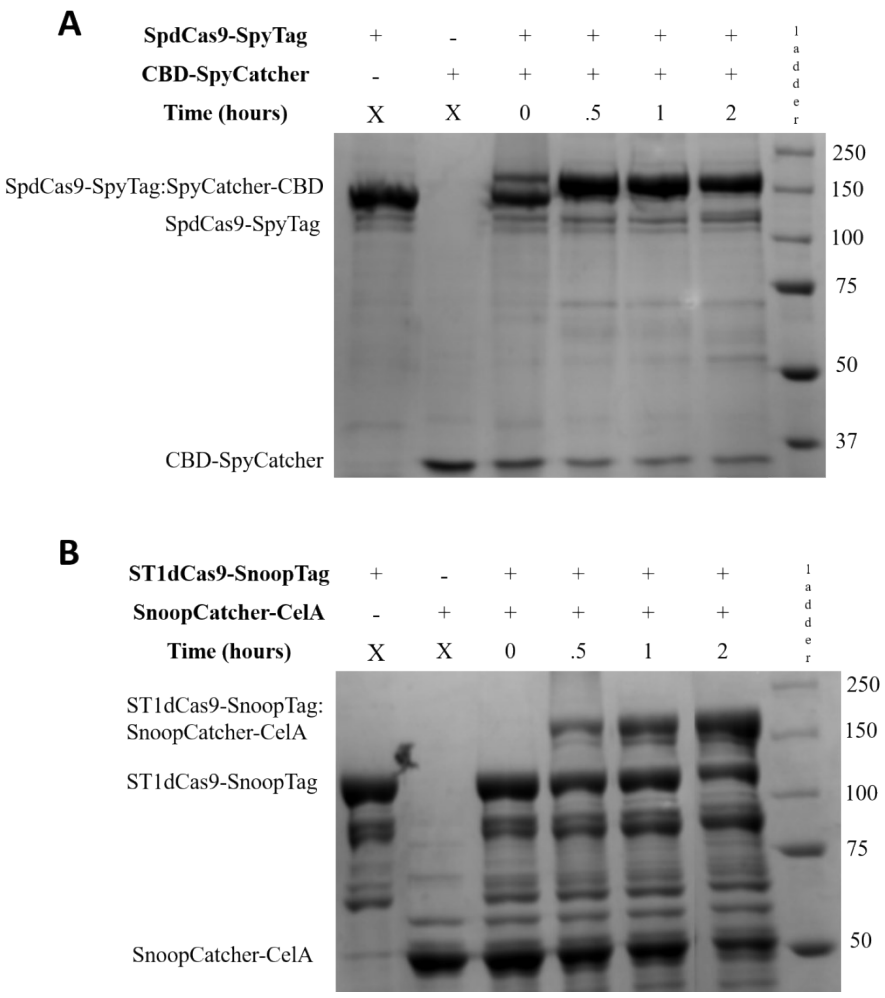

Figure S2. Time Course of Tag/Catcher Reactions. (A) SpdCas9-SpyTag began reacting with CBD SpyCatcher immediately upon mixing and was completely reacted by 30 minutes. (B) St1dCas9-SnoopTag was completely reacted with SnoopCatcher-CelA at 2 hours post mixing.

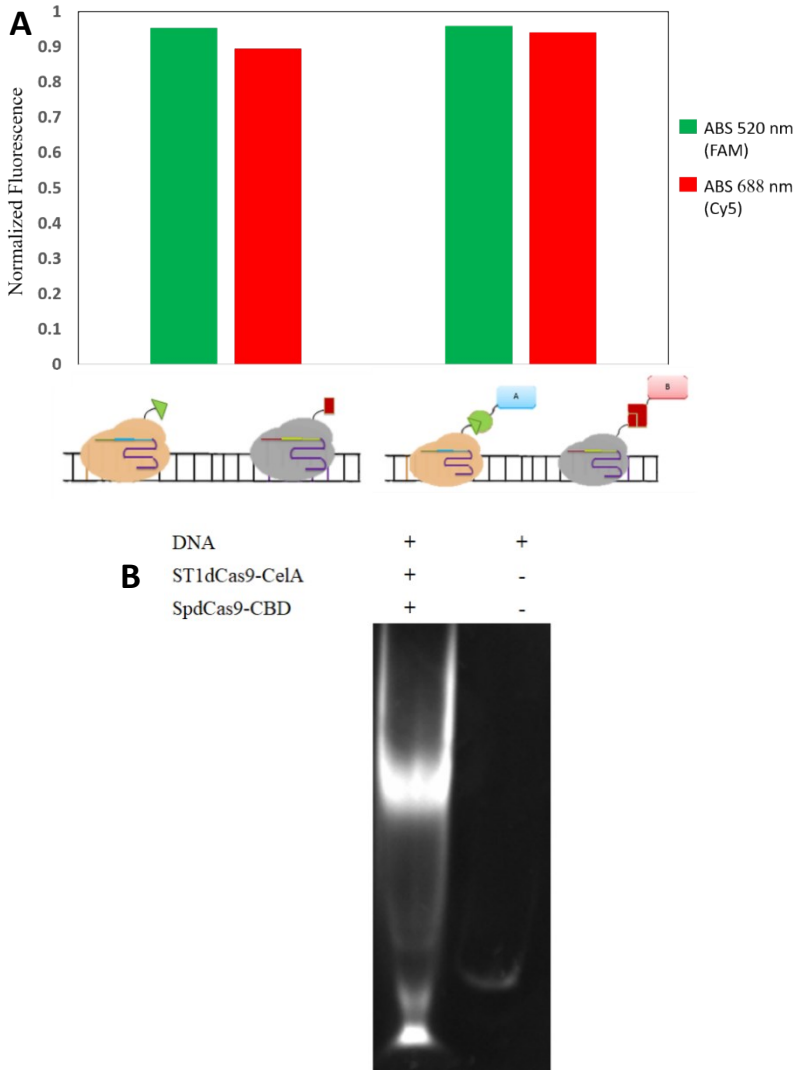

Figure S3. Binding Efficiency of dCas9-Tag proteins pre and post ligation reactions in a stepwise fashion. (A) A Cas beacon assay was used to verify dual binding. Left bars indicate SpdCas9-SpyTag and ST1dCas9-SnoopTag are capable of binding the dual beacon with high efficiency. Right bars show similar levels of dual dCas9-Tag binding after the ligation reactions have occurred in separate reactions. (B) Physically binding of both dCas9-enzyme conjugates was confirmed using the electro-mobility shift assay and a slower mobility band was detected/

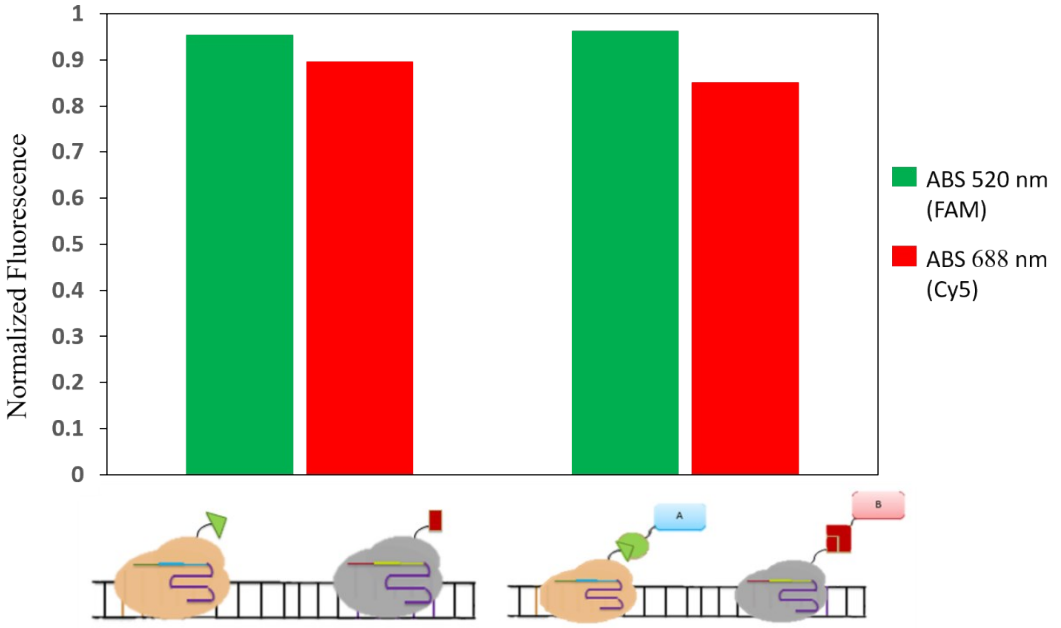

Figure S4. Binding Efficiency of dCas9-Tag proteins pre and post ligation reactions in one pot reaction. Left bars indicate SpdCas9-SpyTag and ST1dCas9-SnoopTag are capable of binding the dual beacon with high efficiency. Right bars show similar levels of dual dCas9-Tag binding after the ligation reactions have occurred in a single reaction

1. Mali P., Esvelt K.M. & Church G.M. Cas9 as a versatile tool for engineering biology. *Nature Methods* **10**, 957-963 (2013).