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Employing 25-Residue Docking Motifs from Modular Polyketide Synthases as Orthogonal Protein Connectors

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Abstract

The proteins of *trans*-acyltransferase modular polyketide synthases (PKSs) self-organize into assembly lines, enabling the multienzyme biosynthesis of complex organic molecules. Docking domains comprised of ~25 residues at the C- and N-termini of these polypeptides (^CDDs and ^NDDs) help drive this association through the formation of four-helix bundles. Molecular connectors like these are desired in synthetic contexts, such as artificial biocatalytic systems and biomaterials, to orthogonally join proteins. Here, the ability of six ^CDD/^NDD pairs to link non-PKS proteins is examined using green fluorescent protein (GFP) variants. As observed through size-exclusion chromatography and Förster resonance energy transfer (FRET), matched but not mismatched pairs of Venus+^CDD and ^NDD+mTurquoise2 fusion proteins associate with low micromolar affinities.

Graphical Abstract

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Author Contributions

J.L.M., A.J.S., and D.T.W. are cofirst authors. A.J.S., D.T.W., A.D.E., and A.T.K. conceived the project. A.J.S. and D.T.W. designed the Venus+^CDD and ^NDD+Turquoise2 constructs. J.L.M., A.J.S., D.T.W., B.M., and S.Y. expressed and purified the proteins. J.L.M. and D.T.W. performed the size-exclusion chromatography and ran the SDS-PAGE gels. A.J.S. acquired and analyzed the FRET measurements. J.L.M. and A.J.S. wrote the paper with input from A.D.E. and A.T.K.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.9b00047.

Primer sequences, size-exclusion traces, SDS-PAGE analysis, data processing methods, fluorescence spectra, FRET efficiency plots, and titrations at differing pH and salt concentrations (PDF)

The authors declare no competing financial interest.

Keywords

docking domain; four-helix bundle; orthogonal connectors; polyketide synthase; fluorescent proteins; FRET

Engineering macromolecular complexes that assemble into specific structures has been a long-standing goal in materials science, chemical engineering, and synthetic biology.¹ These assemblies could facilitate such processes as the synthesis of medicines, the delivery of drugs, and the transport of energy. To help engineer these complexes, researchers have harnessed modular connectors such as oligonucleotides,^{2–4} metal-binding domains,^{5–7} and leucine zippers.^{8,9} However, these connectors each have their limitations: connecting proteins with oligonucleotides can be cumbersome, metalbinding domains have limited orthogonality, and leucine zippers require the interaction of long α -helices that can geometrically constrain the bound domains.

A promising yet largely unexplored class of connectors are the recently discovered four-helix bundle docking domains that commonly join polypeptides within the *trans*-acyltransferase modular polyketide synthases (*trans*-AT PKSs)(Figure 1).^{10–13} The enzymes that reside in these polypeptides mediate the synthesis of polyketide natural products.^{14–17} For groups of these enzymes known as modules (the calyculin assembly line contains thirty-three),¹⁸ to correctly elongate growing intermediates, the polypeptides of the assembly line need to be precisely ordered.

While the polypeptides of the *cis*-AT PKSs,^{16,17,19} such as the erythromycin synthase, rely on homodimeric docking motifs C-terminal to the acyl carrier protein (ACP) domain and N-terminal to the ketosynthase (KS) domain,^{20–22} the polypeptides of *trans*-AT PKSs are connected through split domains at various locations within the module. These domains can be enzymes such as ketoreductases (KRs) or dehydratases (DHs); however, the most common split domain is a four-helix bundle formed by two helices at the C-terminus of an upstream polypeptide and two helices at the N-terminus of a downstream polypeptide (the C- and N-terminal portions of the docking domain are referred to as ^{CDD} and ^{NDD}).¹³ ^{CDD} and ^{NDD} have been shown to interact in a pseudosymmetric manner with low micromolar affinity.^{12,13} While the docking domains of *cis*-AT and *trans*-AT PKSs both naturally operate

in the context of homodimers, only those of *trans*-AT PKSs are functional as monomers and thus suited for (but not limited to) connecting monomeric proteins.

Cognate $^{\text{CDD}}/^{\text{NDD}}$ pairs within *Bacillus amyloliquefaciens* FZB42 (a.k.a. *Bacillus velezensis* FZB42) help mediate the self-organization of the assembly lines that produce the polyketides bacillaene, difficidin, and macrolactin.¹⁴ In a previous study from our group, the association of portions of *trans*-AT PKS polypeptides containing cognate $^{\text{CDD}}$ s and $^{\text{NDD}}$ s was observed.¹³ Through swapping the $^{\text{CDD}}$ and $^{\text{NDD}}$ motifs new connections between PKS enzymes could be engineered. While these experiments indicated that $^{\text{CDD}}$ and $^{\text{NDD}}$ are portable between the polypeptides of *trans*-AT assembly lines, their ability to connect non-PKS proteins was not investigated.

Here, we report the use of *B. amyloliquefaciens* $^{\text{CDD}}/^{\text{NDD}}$ pairs to orthogonally connect non-PKS proteins, demonstrating their ability to mediate the association of the monomeric green fluorescent protein (GFP) variants Venus and mTurquoise2.^{23,24} $^{\text{CDD}}$ and $^{\text{NDD}}$ motifs from the bacillaene, difficidin, and macrolactin assembly lines of *B. amyloliquefaciens* FZB42 appended to the termini of Venus and mTurquoise2 enabled orthogonal connectivities with low micromolar affinities. The association of Venus+ $^{\text{CDD}}$ and $^{\text{NDD}}$ +mTurquoise2 cognates, visualized by size-exclusion chromatography and measured using Förster resonance energy transfer (FRET), demonstrates the utility of these docking domains in generating engineered complexes.

RESULTS AND DISCUSSION

Four helix-bundle docking domains connect polypeptides in each of the three *trans*-AT PKSs from *B. amyloliquefaciens* FZB42: once in the bacillaene assembly line ($^{\text{CDD}}_{\text{BaeM}}/^{\text{NDD}}_{\text{BaeN}}$ for polypeptides BaeM/BaeN), once in the difficidin assembly line ($^{\text{CDD}}_{\text{DifF}}/^{\text{NDD}}_{\text{DifG}}$ for polypeptides DifF/DifG), and four times in the macrolactin assembly line ($^{\text{CDD}}_{\text{MlnB}}/^{\text{NDD}}_{\text{MlnC}}$ for polypeptides MlnB/MlnC, $^{\text{NDD}}_{\text{MlnC}}/^{\text{NDD}}_{\text{MlnD}}$ for polypeptides MlnC/MlnD, $^{\text{NDD}}_{\text{MlnD}}/^{\text{NDD}}_{\text{MlnE}}$ for polypeptides MlnD/MlnE, and $^{\text{NDD}}_{\text{MlnF}}/^{\text{NDD}}_{\text{MlnG}}$ for polypeptides MlnF/MlnG)(Figure 1b). Each of these $^{\text{CDD}}$ and $^{\text{NDD}}$ motifs was fused to the yellow GFP variant Venus and the cyan GFP variant mTurquoise2, respectively.^{23,24} Venus + $^{\text{CDD}}$ fusion proteins were engineered by linking the Venus domain and $^{\text{CDD}}$ motif through a glycine, a serine, and 4–5 residues that naturally precede the $^{\text{CDD}}$. Fusion $^{\text{NDD}}$ +Turquoise2 proteins were engineered by linking the $^{\text{NDD}}$ motif and mTurquoise2 domain with 5–6 residues naturally downstream of $^{\text{NDD}}$. To minimize interference with $^{\text{CDD}}/^{\text{NDD}}$ interactions, vector-encoded hexahistidine purification tags were positioned on the terminus opposite of the docking motif (N-terminal in Venus+ $^{\text{CDD}}$ constructs and C-terminal in $^{\text{NDD}}$ +mTurquoise2 constructs). The 12 fusion proteins were expressed in *Escherichia coli* BL21(DE3) from pET28b-derived plasmids and purified. Each construct showed the expected fluorescent properties, with the peak excitation and emission wavelengths at ~510 and ~530 nm for the Venus+ $^{\text{CDD}}$ constructs and ~420 and ~480 nm for the $^{\text{NDD}}$ +mTurquoise2 constructs (Figure S1).

Orthogonal interactions were first observed between Venus+ $^{\text{CDD}}$ and $^{\text{NDD}}$ +mTurquoise2 constructs by size-exclusion chromatography (Figures 2 and S2). When loaded individually

onto a Superdex 200 column, Venus+^{CDD} and ^{NDD}+mTurquoise2 constructs elute at 17–18 mL. However, when loaded together in equimolar amounts, matched pairs of Venus+^{CDD} and ^{NDD}+mTurquoise2 constructs primarily elute earlier, at ~15 mL, indicating the formation of a larger complex (with smaller peaks at 17–18 mL from residual, uncomplexed protein). The behavior of each Venus+^{CDD} construct in the presence of each of the six ^{NDD}+mTurquoise2 constructs was also observed through a series of Coomassiestained SDS-PAGE gels. These gels indicated that for five of the six pairs tested, only cognate pairs of Venus+^{CDD} and ^{NDD}+mTurquoise2 interact. For example, while Venus+^{CDD}_{BaeM} forms a complex with ^{NDD}_{BaeN}+mTurquoise2, it does not form a complex with any of the other ^{NDD}+mTurquoise2 constructs (Figure 2c). The other constructs show similar orthogonality, with the exception of Venus+^{CDD}_{MlnB}, which complexes weakly with the ^{NDD}_{MlnD}+mTurquoise2, ^{NDD}_{MlnE}+mTurquoise2, and ^{NDD}_{MlnG}+mTurquoise2 constructs (Figure S3).

Measurements of Förster resonance energy transfer (FRET) between Venus+^{CDD} and ^{NDD}+mTurquoise2 constructs enabled characterization of the concentration-dependent nature of their interactions. FRET is an energy transfer mechanism in which an excited donor fluorophore nonradiatively transfers energy to a nearby acceptor fluorophore and is commonly harnessed to detect bimolecular interactions by fusing interacting proteins or domains of interest to a complementary pair of donor and acceptor fluorescent proteins.^{25,26} Interactions that bring the donor and acceptor into close proximity enable FRET between the donor and acceptor, which results in increased acceptor and decreased donor fluorescence in response to donor excitation. The FRET efficiency E depends on the distance between the acceptor and donor, r

$$E = \frac{1}{1 + r^6/R_0^6} \quad (1)$$

where R_0 is the Förster radius, the distance between the acceptor and donor at which FRET efficiency is half its maximum value. In the experimental system described here, Venus and mTurquoise2 serve as both test proteins to assess the ability of ^{CDD}/^{NDD} pairs to connect non-PKS proteins and as the acceptor/donor FRET pair to enable detection of ^{CDD}/^{NDD} interactions. Because the Förster radius between Venus and mTurquoise2 (5.8 nm)²⁷ is larger than the expected distance between chromophores upon docking domain-mediated connection, the FRET efficiency between ^{CDD}/^{NDD}-complexed Venus and mTurquoise2 proteins is expected to be relatively high.

Consistent with the size-exclusion chromatography binding assays, FRET measurements indicated specific, orthogonal interactions between matched Venus+^{CDD} and ^{NDD}+mTurquoise2 constructs (Figures 3 and S4–10). Normalized fluorescent spectra obtained from a solution containing 3 μ M of Venus+^{CDD}_{BaeM} mixed with an equivalent amount of its cognate construct, ^{NDD}_{BaeN}+mTurquoise2, showed increased Venus fluorescence at ~530 nm, and decreased mTurquoise2 fluorescence at ~480 nm in response to excitation at 420 nm compared to spectra obtained from solutions containing ^{NDD}_{BaeN}+mTurquoise2 alone (processing and normalization are described in Figure S4). Conversely, fluorescence spectra obtained from combining noncognate constructs, such as Venus+^{CDD}_{BaeM} with ^{NDD}_{DifG}

+mTurquoise2, were similar to those obtained from solutions containing the ^NDD +mTurquoise2 construct alone (Figure 3a). The extent of fluorescence increase at ~530 nm and decrease at ~480 nm is dependent on the Venus+^CDD concentration (Figures 3b and S5–10). For each Venus+^CDD/^NDD+mTurquoise2 combination, containing 3 μM of the ^NDD +mTurquoise2 construct, the ~530 nm peak is evident at ~1 μM of the cognate Venus+^CDD and grows to the same height as the ~480 nm peak at ~6 μM of the cognate Venus+^CDD. Noncognate Venus+^CDD constructs do not produce the ~530 nm peak unless present at significantly higher concentrations (due to either lower-affinity interactions between the noncognate pairs or weak fluorescence from excess Venus+^CDD) (Figure 4).

Plotting FRET efficiency as a function of Venus+^CDD concentration further indicated the orthogonality of cognate Venus+^CDD and ^NDD+Turquoise2 constructs. FRET efficiencies are most simply quantified as the ratio of the acceptor to either the donor or total fluorescence.^{25,26} However, accurate comparisons of such ratios generally require simple, often unimolecular, systems in which donor and acceptor concentrations remain constant. Bimolecular studies in which the concentrations of the donor or acceptor change have employed alternative methods.^{28–31} Here, a method^{25,29} was employed in which the FRET efficiency *E* at varying acceptor concentrations is quantified as

$$E = 1 - \frac{F_{da}}{F_d} \quad (2)$$

where the intensity of the donor emission is measured (at 480 nm) in the absence (*F_d*) and presence (*F_{da}*) of an acceptor (Figure 5a). Plotting *E* as a function of acceptor concentration for each of the six combinations of matched Venus+^CDD and ^NDD+mTurquoise2 constructs shows that FRET efficiency increases with increasing acceptor concentration, and an analysis of all 36 combinations of Venus+^CDD and ^NDD+mTurquoise2 constructs reveals sharper increases for matched pairs compared to mismatched pairs (Figures 5b and S11).

Measurements of the FRET efficiency between Venus+^CDD and ^NDD+mTurquoise2 constructs as a function of Venus+^CDD concentration also enabled estimations of binding affinities. While the spectral overlap between Venus and mTurquoise2 precluded precise quantitation at concentrations of Venus+^CDD much higher than ^NDD+mTurquoise2 required for the accurate determination of affinities (Figure S12),³² the obtained data were fit to binding isotherms to estimate *K_d* values^{25,29}

$$E = E_{\max} \times \frac{[\text{acceptor}]}{[\text{acceptor}] + K_d} \quad (3)$$

Fitting the FRET efficiency versus Venus+^CDD concentration data to this equation yielded *K_d* values in the low micromolar range with acceptable *R*² values: Venus+^CDD_{BaeM}/^NDD_{BaeN}+mTurquoise2, *K_d* = 7.4 ± 3.8 μM, *R*² = 0.96; Venus+^CDD_{Diff}/^NDD_{Diff}+mTurquoise2, *K_d* = 6.3 ± 2.2 μM, *R*² = 0.98; Venus+^CDD_{MlnB}/^NDD_{MlnC}+mTurquoise2, *K_d* = 2.9 ± 1.1 μM, *R*² = 0.90; Venus+^CDD_{MlnC}/^NDD_{MlnD}+mTurquoise2, *K_d* = 9.4 ± 7.5 μM, *R*² = 0.85; Venus+^CDD_{MlnD}/^NDD_{MlnE}+mTurquoise2, *K_d* = 4.8 ± 2.3 μM, *R*² = 0.73; Venus+^CDD_{MlnF}/^NDD_{MlnG}+mTurquoise2, *K_d* = 14 ± 7.8 μM, *R*² = 0.84 (Figure 5c and 5d). These values are similar to previously determined isothermal titration calorimetry measurements of

the binding affinities of three of these cognate docking motifs in the context of their native polypeptides: ${}^{\text{C}}\text{DD}_{\text{MlnC}}/{}^{\text{N}}\text{DD}_{\text{MlnD}}$, $K_{\text{d}} = 1.8 \mu\text{M}$; ${}^{\text{C}}\text{DD}_{\text{MlnD}}/{}^{\text{N}}\text{DD}_{\text{MlnE}}$, $K_{\text{d}} = 0.8 \mu\text{M}$; ${}^{\text{C}}\text{DD}_{\text{MlnF}}/{}^{\text{N}}\text{DD}_{\text{MlnG}}$, $K_{\text{d}} = 9.0 \mu\text{M}$.¹³

Measurements were also made to determine whether salt concentration, pH, or small impurities from the Ni-NTA purifications had a significant impact on Venus+ ${}^{\text{C}}\text{DD}/{}^{\text{N}}\text{DD}$ +mTurquoise2 interactions. FRET titrations were conducted at 37.5, 75, 150, and 300 mM NaCl for each combination of Venus+ ${}^{\text{C}}\text{DD}_{\text{BaeM}}$ and Venus+ ${}^{\text{C}}\text{DD}_{\text{MlnF}}$ with ${}^{\text{N}}\text{DD}_{\text{BaeN}}$ +mTurquoise2 and ${}^{\text{N}}\text{DD}_{\text{MlnG}}$ +mTurquoise2 (Figure S13). These titrations indicated similar orthogonality of the matched Venus+ ${}^{\text{C}}\text{DD}/{}^{\text{N}}\text{DD}$ +mTurquoise2 pairs, with similar K_{d} values for the matched pairs (Venus+ ${}^{\text{C}}\text{DD}_{\text{BaeM}}/{}^{\text{N}}\text{DD}_{\text{BaeN}}$ +mTurquoise2 and Venus+ ${}^{\text{C}}\text{DD}_{\text{MlnF}}/{}^{\text{N}}\text{DD}_{\text{MlnG}}$ +mTurquoise2) at each salt concentration (Figure S14). FRET titrations also showed that the Venus+ ${}^{\text{C}}\text{DD}_{\text{BaeM}}/{}^{\text{N}}\text{DD}_{\text{BaeN}}$ +mTurquoise2 and Venus+ ${}^{\text{C}}\text{DD}_{\text{MlnF}}/{}^{\text{N}}\text{DD}_{\text{MlnG}}$ +mTurquoise2 pairs demonstrate specific, orthogonal binding from pH 6.0 to 7.4 (Figure S15). Further purification of Ni-NTA-purified constructs by gel filtration chromatography did not result in significant changes in the measured FRET efficiencies (Figure S16).

Currently, the most utilized protein connectors are synthetic leucine-zipper coiled coils known as SYNZIP pairs.^{8,33,34} These can associate more tightly than the ${}^{\text{C}}\text{DD}$'s and ${}^{\text{N}}\text{DD}$'s of four helix bundle docking motifs, often with low nanomolar affinities. However, the binding orientations and oligomerization states are not known for many of these pairs, and orthogonality is a concern when several are used in the same system. The structure of the four helix docking domain complex has been characterized (PDB Codes 2N5D and 5D2E), and its ${}^{\text{C}}\text{DD}$ and ${}^{\text{N}}\text{DD}$ components have been naturally selected for orthogonality within organisms such as *B. amyloliquefaciens* FZB42.^{12,13} The smaller size of the four helix bundle docking motifs, approximately half that of SYNZIP motifs, also confers an advantage in many applications.

Engineered macromolecular complexes are desired in diverse synthetic biotechnologies; however, general strategies for their construction remain elusive. Here, we demonstrate the use of four-helix bundle docking domains from *trans*-AT assembly lines to connect synthetic proteins with low micromolar binding constants. They may be used to daisy-chain enzymes in a designed biosynthetic pathway or to connect arrays of light harvesting or metal templated proteins. The small size, portability, and orthogonality of these docking motifs augurs well for their broad utility as molecular connectors to generate desired synthetic biomolecular assemblies.

METHODS

Cloning and Protein Expression.

The DNA encoding each docking domain was amplified from the genomic DNA of *B. amyloliquefaciens* FZB42, and the genes encoding the GFP variants Venus and mTurquoise2 (F46L/F64L/S65G/V68L/S72A/D133G/M153T/V163A/S175G/T203Y and F64L/Y66W/S72A/N146F/H148D/M153T/V163A/S175G/A206K/H231L) were amplified from the YTK kit plasmids pYTK033 and pYTK057, respectively (Table S1). Amplicons were gel extracted and Gibson assembled into the pET28b expression vector (New England Biolabs)

such that the hexahistidine tag is encoded opposite the docking domain. pET28b was digested with NdeI and XhoI to assemble Venus+^CDD constructs and NcoI and XhoI to assemble ^NDD+mTurquoise2 constructs. The histidine-tagged fusion proteins were expressed in *E. coli* BL21(DE3) (6 L of LB media) that were grown to an OD₆₀₀ of 0.6 at 37 °C, induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside, and left overnight at 15 °C for ^NDD+mTurquoise2 constructs or 5 h at 37 °C for Venus+^CDD constructs. Protein was purified from cell lysate using HisPur nickel-NTA resin (Thermo Scientific), flash frozen, and stored at -80 °C. Additional preparations of Venus+^CDD_{BaeM}, ^NDD_{BaeN}+mTurquoise2, Venus+^CDD_{MlnF}, and ^NDD_{MlnG}+mTurquoise2 were polished via gel filtration for salt- and pH-dependent FRET assays (GE Superdex 200 Increase 10/300 column equilibrated in 150 mM NaCl, 10 mM HEPES pH 7.5, and 10% (v/v) glycerol).

Size-Exclusion Chromatography Docking Assays.

Pairs of Venus+^CDD and ^NDD+mTurquoise2 constructs were combined ~1:1 (75 μ M for each protein) in a 1 mL volume and injected onto a Superdex 200 Increase gel filtration column (GE Healthcare Life Sciences) equilibrated with 150 mM NaCl, 15 mM HEPES (pH 7.5). Protein elution was monitored by absorbance at 280 nm, and fractions were collected every 0.5 mL after 9.5 mL had eluted. Individual domains were also run (75 μ M in 1 mL). Fractions 12–23 from each run were analyzed with Coomassie-stained SDSPAGE gels loaded with 15 μ L of each fraction.

FRET Measurements.

Fluorescence measurements were conducted in a solution containing 75 mM NaCl and 25 mM NaPO₄ (pH 7.4) in all experiments, except where noted, and in a clear bottom 96-well plate. Fluorescence spectra were recorded between 460 and 600 nm (420 nm excitation, 5 nm intervals, ~25 °C). To obtain a titration curve, a Venus+^CDD construct was successively added to solutions initially containing 3 μ M of ^NDD+mTurquoise2 constructs and 220 μ L total volume. Spectra taken at each concentration of the Venus+^CDD construct were normalized by multiplying the ratio of current to starting volume to correct for dilution of the ^NDD+mTurquoise2 construct and subtracting a spectrum of a sample containing no ^NDD+mTurquoise2 construct but an equivalent amount of the Venus+^CDD construct in response to 420 nm excitation. To correct for signal decrease over time due to changes in solution refractive index due to increasing protein concentration, spectra were further normalized by dividing the fluorescence at each point by the sum of the fluorescence values taken between 470 and 540 nm. To estimate affinities, the normalized estimated FRET efficiencies were plotted as a function of Venus+^CDD concentration in GraphPad Prism and fit to the standard single-site binding isotherm to yield E_{max} and K_d values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Co-opting docking domains from biosynthetic assembly lines. (A) A comparison of enzymatic assembly line docking domains (PDB IDs 4MYV, 2N5D) shows that, while *cis*-AT assembly line docking domains (class 2 shown, but class 1 is also dimeric)²² are obligate dimers, *trans*-AT assembly line docking domains are not and could be useful in complexing proteins of various oligomerization states. KS, ketosynthase; AT, acyltransferase; ACP, acyl carrier protein. (B) C- and N-terminal docking domain (^CDD and ^NDD) motifs were appended to the C-terminus of Venus and the N-terminus of mTurquoise2. The sequences of the six pairs used in this study from the bacillaene (Bae), difficidin (Dif), and macrolactin

(Mln) assembly lines are displayed along with their numbering.¹³ BaeM/BaeN, bacillaene, *Bacillus amyloliquefaciens*, CAG23959/CAG23960; DifF/DifG, difficidin, *Bacillus amyloliquefaciens*, CAG23977/CAG23978; MlnB/MlnC/MlnD/MlnE/MlnF/MlnG, macrolactin, *Bacillus amyloliquefaciens*, CAG23964/CAG23965/CAG23966/CAG23967/CAG23968/CAG23969.

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Figure 2.

Size-exclusion chromatography docking assay. Data for Venus+ CDD_{BaeM} are shown here (data for all Venus+ CDD constructs are reported in Figures S2 and S3). (A) Individually, the cognate constructs Venus+ CDD_{BaeM} and NDD_{BaeN} +mTurquoise2 elute at 17 mL. A coinjection results in a species that elutes at 15 mL. (B) A coinjection of the noncognate constructs Venus+ CDD_{BaeM} and NDD_{MlnC} +mTurquoise2 does not generate an earlier-eluting species. (C) Venus+ CDD_{BaeM} paired with each of the NDD +mTurquoise2 constructs shows that only the coinjection with NDD_{BaeN} +mTurquoise2 results in complex formation (NDD_{BaeN} +mTurquoise2, red; NDD_{DifG} +mTurquoise2, orange; NDD_{MlnC} +mTurquoise2, green; NDD_{MlnD} +mTurquoise2, light blue; NDD_{MlnE} +mTurquoise2, navy; NDD_{MlnG}

+mTurquoise2, purple). Coomassie-stained SDS-PAGE gels confirm that both the Venus +^CDD_{BaeM} and ^NDD_{BaeN}+mTurquoise2 constructs are present in the early eluting species (boxed in green). Legend for all SDS-PAGE gels: Lane 1, ladder (PageRuler Prestained Protein, Thermo Scientific); lanes 2–13, fractions 12–23; lane 14, Venus+^CDD construct; lane 15, ^NDD+mTurquoise2 construct. Matched ^NDD_{BaeN}+mTurquoise2 and Venus +^CDD_{BaeM} appear in lanes 3–5 (corresponding to fractions 13–15), while mismatched constructs do not.

Figure 3.

FRET from complexes. (A) Emission spectra of an $^{\text{NDD}}$ +mTurquoise2 construct alone (gray), an equimolar matched Venus+ $^{\text{CDD}}$ / $^{\text{NDD}}$ +mTurquoise2 pair (red), and an equimolar unmatched Venus+ $^{\text{CDD}}$ / $^{\text{NDD}}$ +mTurquoise2 pair (blue) in response to direct excitation of $^{\text{NDD}}$ +mTurquoise2 at 420 nm. While excitation of an $^{\text{NDD}}$ +mTurquoise2 construct alone and in solution with an unmatched Venus+ $^{\text{CDD}}$ construct produces a single peak at ~480 nm, excitation of a matched Venus+ $^{\text{CDD}}$ / $^{\text{NDD}}$ +mTurquoise2 pair produces a second emission peak at ~530 nm. (B) Emission spectra of increasing concentrations of Venus+ $^{\text{CDD}}\text{DD}_{\text{BaeM}}$ with 3 μM of each $^{\text{NDD}}$ +mTurquoise2 variant in response to 420 nm excitation. Increasing concentrations of Venus+ $^{\text{CDD}}\text{DD}_{\text{BaeM}}$ in the presence of its cognate, $^{\text{NDD}}\text{BaeN}$ +mTurquoise2 (red), produces decreased donor and increased acceptor fluorescence. Increasing concentrations of Venus+ $^{\text{CDD}}\text{DD}_{\text{BaeM}}$ in the presence of noncognate $^{\text{NDD}}$ +mTurquoise2 proteins produce little effect until 10 μM , when acceptor fluorescence

increases due to low-affinity interactions between mismatched $^CDD/^NDD$ pairs or due to off-maximum excitation becoming too large to accurately subtract. $^NDD_{BaeN}+mTurquoise2$, red; $^NDD_{DifG}+mTurquoise2$, orange; $^NDD_{MlnC}+mTurquoise2$, green; $^NDD_{MlnD}+mTurquoise2$, light blue; $^NDD_{MlnE}+mTurquoise2$, blue; $^NDD_{MlnG}+mTurquoise2$, purple. Because the spectra of the mismatched pairs are extremely similar, the $^NDD_{DifG}+mTurquoise2$ (orange), $^NDD_{MlnC}+mTurquoise2$ (green), and $DD_{MlnE}+mTurquoise2$ (dark blue) spectra are mostly obscured.

Figure 4.

Emission spectra of 3 μM of each Venus+^{CDD} with 3 μM of each mTurquoise2+^{NDD}.

Thicker traces correspond to matched pairs. While matched pairs produce decreased donor fluorescence (480 nm) and increased acceptor fluorescence (530 nm), mismatched pairs do not. ^{NDD}_{BaeN}+mTurquoise2, red; ^{NDD}_{DifG}+mTurquoise2, orange; ^{NDD}_{MlnC}+mTurquoise2, green; ^{NDD}_{MlnD}+mTurquoise2, light blue; ^{NDD}_{MlnE}+mTurquoise2, blue; ^{NDD}_{MlnG}+mTurquoise2, purple.

Figure 5.

Estimating binding constants from the titration curves of matched pairs. (A) Plot showing normalized fluorescence curves obtained from titrating an increasing amount of Venus+^CDD to its cognate ^NDD+mTurquoise2. The trace obtained from ^NDD+mTurquoise2 is shown as the thick blue line; traces obtained with increasing Venus+^CDD concentration are shown as thinner purple to red lines. The turquoise box outlines the peak ^NDD+mTurquoise2 (donor) fluorescence at 475–480 nm. (B) Plots of the FRET efficiency E by Venus+^CDD concentration for all matched pairs. Each color corresponds to a different matched pair: Venus+^CDD_{BaeM}/^NDD_{BaeN}+mTurquoise2, red; Venus+^CDD_{Diff}/^NDD_{DiffG}+mTurquoise2, orange; Venus+^CDD_{MlnB}/^NDD_{MlnC}+mTurquoise2, green; Venus+^CDD_{MlnC}/^NDD_{MlnD}+mTurquoise2, light blue; Venus+^CDD_{MlnD}/^NDD_{MlnE}+mTurquoise2, blue; Venus+^CDD_{MlnF}/^NDD_{MlnG}+mTurquoise2, purple. The data were fit well by a single-site binding curve function, as shown by the solid lines. Each point is the average of three replicates. Error bars correspond to standard deviations. (C) Plots of estimated fraction of ^NDD+mTurquoise2 bound at each Venus+^CDD concentration using the data shown in panel B. (D) Estimated dissociation constant (K_d) values of matched pairs and R^2 of fit to eq 3. Standard deviations are indicated.