SI Appendix

Approaching infinite affinity through engineering of peptide-protein interaction

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A

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                    1         10        20        30        40        50        60
SpyCatcher          VDTLSGLSSEQGQSGDMTIEEDSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWISD
SpyCatcher002       VTTLSGLSGEQGPSGDMTTEEDSATHIKFSKRDEDGRELAGATMELRDSSGKTISTWISD
SpyCatcher003       VTTLSGLSGEQGPSGDMTTEEDSATHIKFSKRDEDGRELAGATMELRDSSGKTISTWISD

                              70        80        90        100       110
SpyCatcher          GQVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGKATKGDAHI
SpyCatcher002       GHVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGEATKGDAHT
SpyCatcher003       GHVKDFYLYPGKYTFVETAAPDGYEVATPIEFTVNEDGQVTVDGEATEGDAHT
                      110       120
SpyTag              ---AHIVMVDAYKPTK 13
SpyTag002           --VPTIVMVDAYKRYK 14
SpyTag003           RGVPHIVMVDAYKRYK 16
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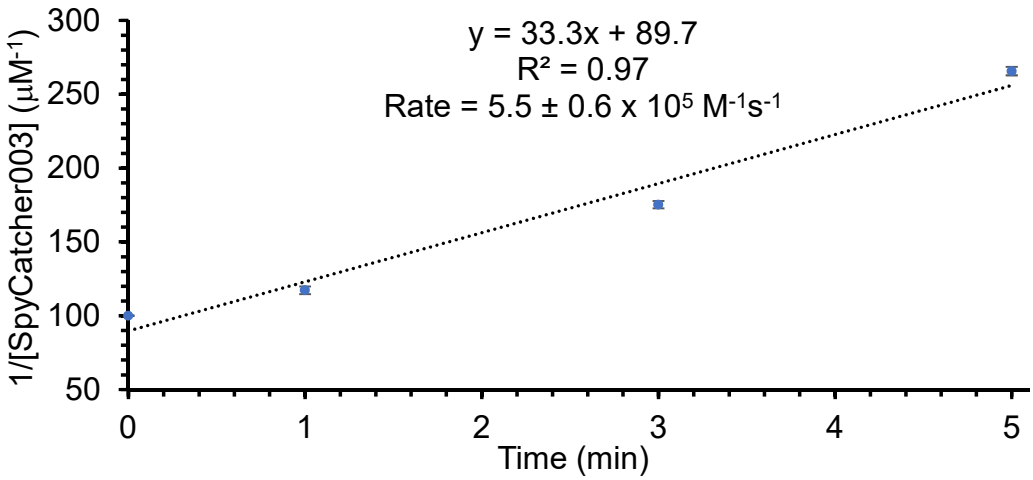
B



C

Rate constant ($M^{-1}s^{-1}$)	SpyTag-MBP	SpyTag002-MBP	SpyTag003-MBP
SpyCatcher003-sfGFP	$2.4 \pm 0.2 \times 10^4$	$8.3 \pm 0.2 \times 10^4$	$5.5 \pm 0.6 \times 10^5$

D

Rate constant ($M^{-1}s^{-1}$)	SpyCatcher-sfGFP	SpyCatcher002-sfGFP	SpyCatcher003-sfGFP
SpyTag003-MBP	$3.9 \pm 0.3 \times 10^4$	$1.3 \pm 0.1 \times 10^5$	$5.5 \pm 0.6 \times 10^5$

Figure S1. Sequence and reaction rates for Spy003 variants. **(A)** Amino acid sequence alignment of original, 002 and 003 versions of SpyCatcher and SpyTag. Red represents changes from original to 002 and green represents changes from 002 to 003. The length of SpyTag variants is indicated. Numbering is based on PDB 2X5P. **(B)** Spy003 reaction followed second-order kinetics. 10 nM SpyTag003-MBP was reacted with 10 nM SpyCatcher003-sfGFP for various times as in Fig. 1C (mean ± 1 s.d., *n* = 3). The equation for the best fit-line, the correlation coefficient, and the derived second-order rate constant (mean ± 1 s.d., *n* = 3) are shown. **(C)** SpyCatcher003 is backwards-compatible. Rate constants for reaction of SpyCatcher003-sfGFP with SpyTag003 and earlier versions linked to MBP (mean ± 1 s.d., *n* = 3). **(D)** SpyTag003 is backwards-compatible. Rate constants for reaction of SpyTag003-MBP with SpyCatcher003 and earlier versions linked to sfGFP (mean ± 1 s.d., *n* = 3).

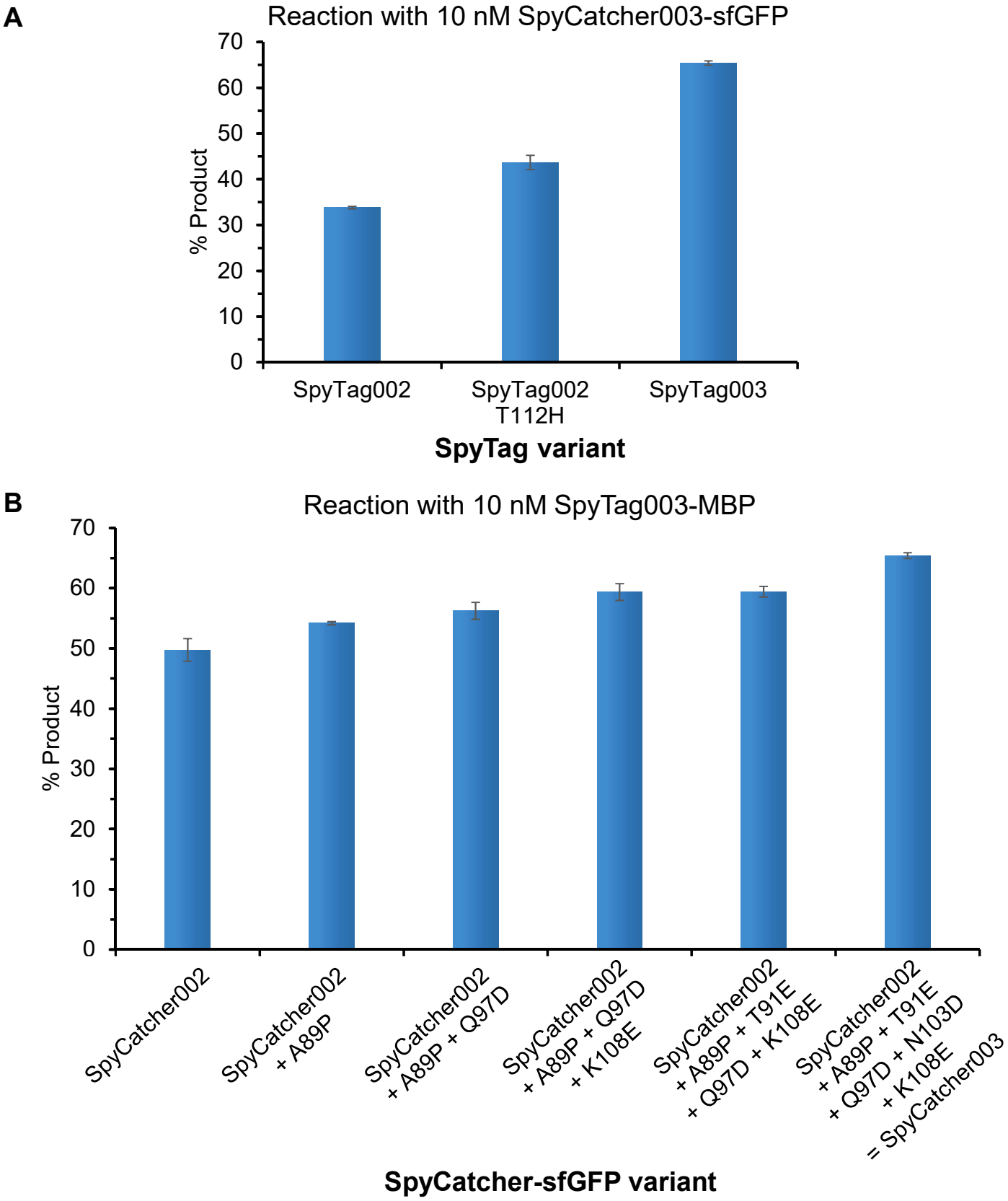
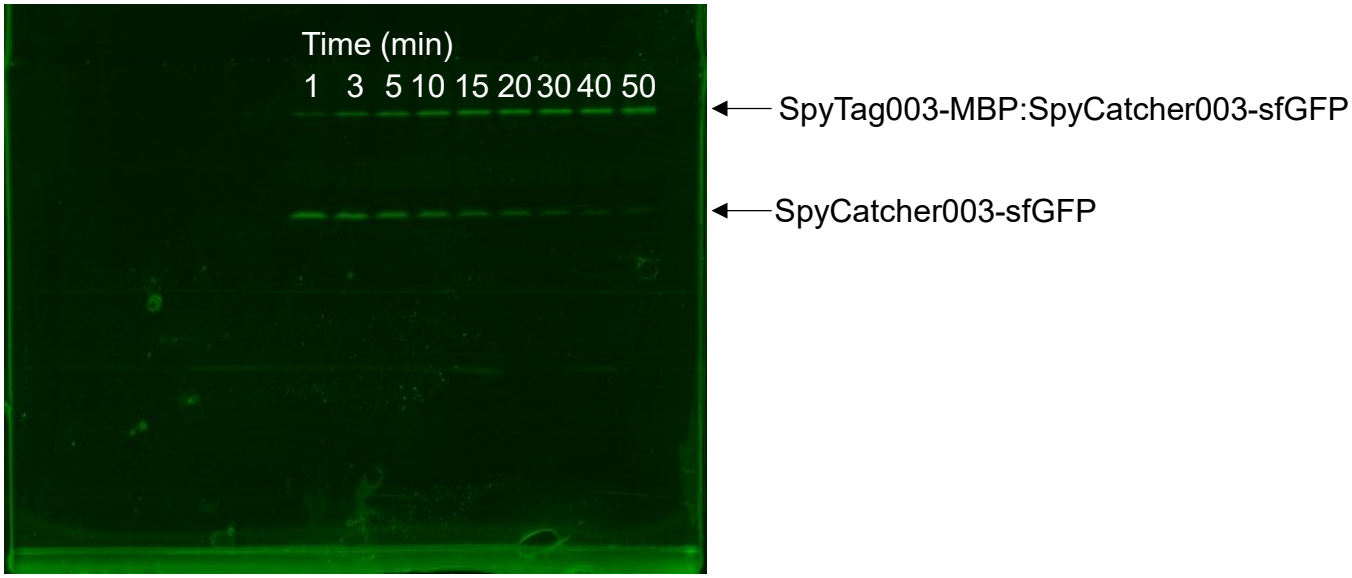


Figure S2. Step-wise improvement of SpyTag002/SpyCatcher002 to SpyTag003/SpyCatcher003 illustrated by product formation after 10 min reaction in SPG buffer pH 7.0 + 0.2% BSA at 25 °C, with each partner at 10 nM. (**A**) Comparison of reactivity of SpyTag002 (Tag=VPTIVMVDAYKRYK), SpyTag002 T112H (Tag=VPHIVMVDAYKRYK), and SpyTag003 (Tag=RGVPHIVMVDAYKRYK) with SpyCatcher003-sfGFP. (**B**) Comparison of reactivity of development intermediates between SpyCatcher002 and SpyCatcher003. All SpyCatcher variants were expressed as sfGFP fusions and reacted with SpyTag003-MBP. (Mean ± 1 s.d., *n* = 3)

A



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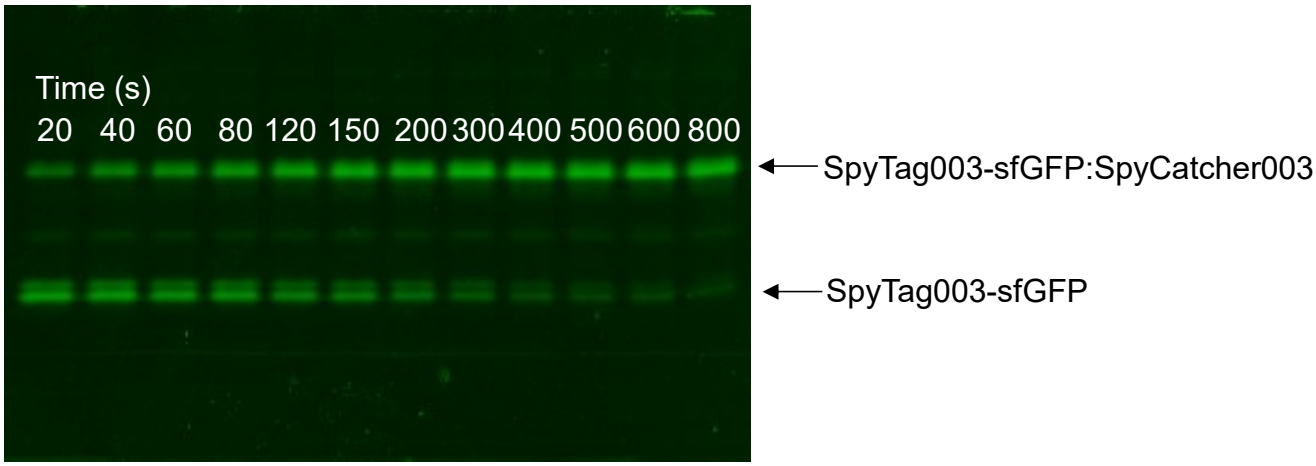


Figure S3. (**A**) Example of gel for 10 nM SpyCatcher-sfGFP reacting with 10 nM SpyTag003-MBP, as used in Fig. 1C. Reaction was quenched after the indicated time, run on SDS-PAGE, and imaged by fluorescence scanning in the GFP channel. (**B**) Example of gel assay for 100 nM SpyTag003-sfGFP reacting with 400 nM SpyCatcher003, as used in Fig. 2D, by SDS-PAGE and fluorescence scanning in the GFP channel.

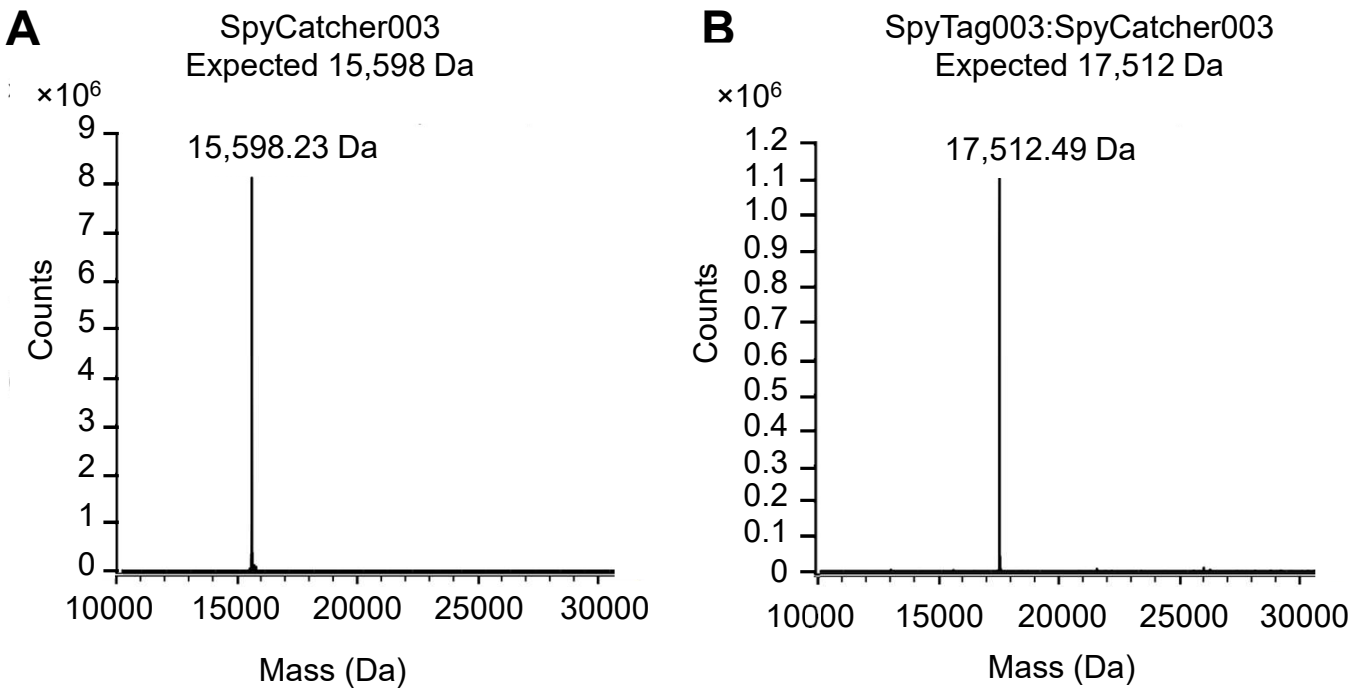


Figure S4. Mass spectrometry validation of SpyCatcher003 and reacted conjugate. **(A)** Electrospray ionization mass spectrometry of SpyCatcher003, with observed and expected molecular weight shown. **(B)** Electrospray ionization mass spectrometry of SpyCatcher003 reacted with SpyTag003 peptide, with observed and expected molecular weight shown. Expected mass for SpyTag003 + SpyCatcher003 = 17,530 Da minus 18 Da (H_2O released upon isopeptide bond formation) = 17,512 Da.

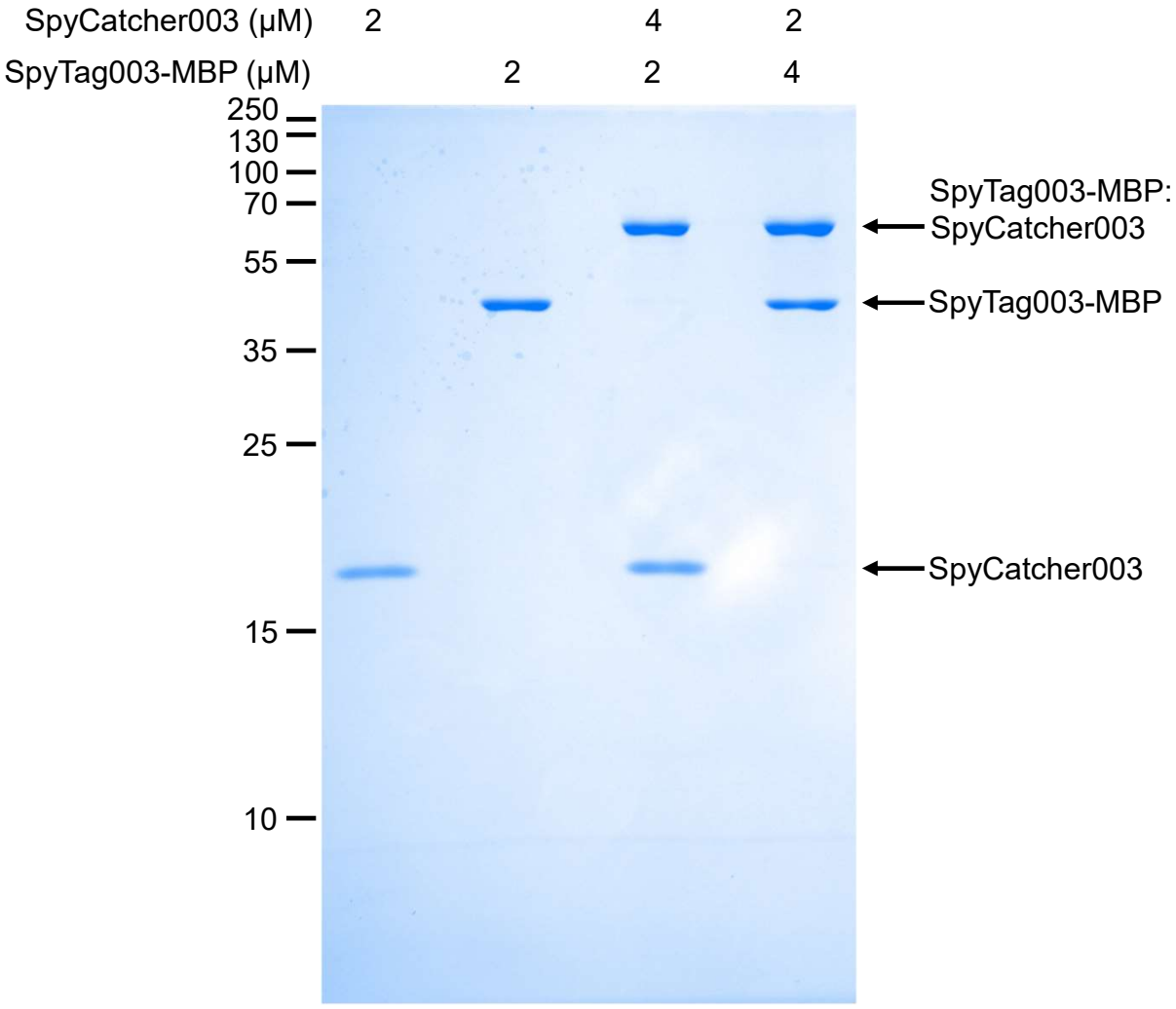


Figure S5. SpyTag003 and SpyCatcher003 reacted to high yield and 99% completion. SpyCatcher003 was incubated with SpyTag003-MBP, with either component in two-fold excess, for 1.5 h and analyzed by SDS-PAGE with Coomassie staining.

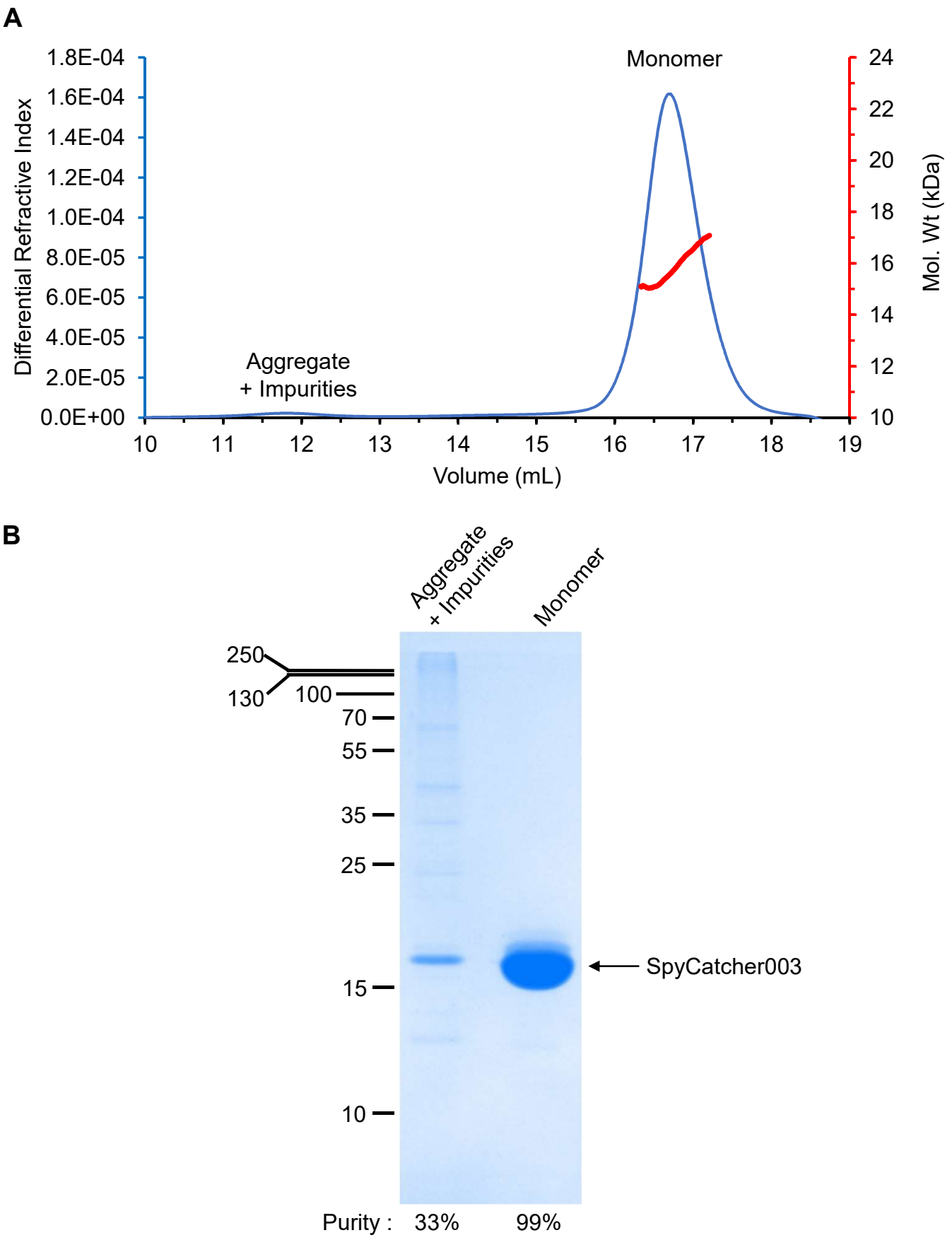
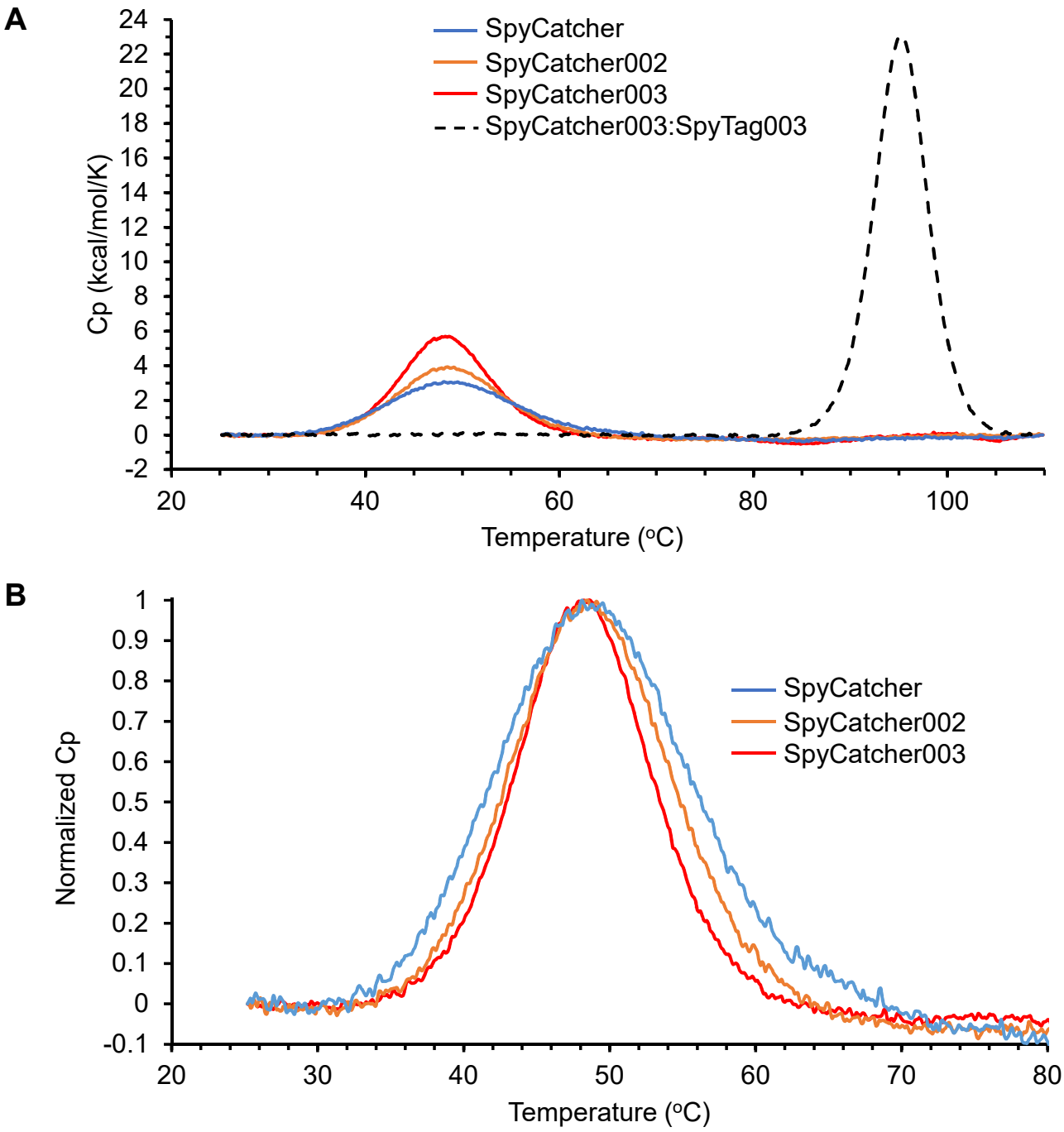


Figure S6. Size exclusion chromatography-Multiple Angle Light Scattering showed the Ni-NTA-purified SpyCatcher003 was mainly monomeric with a solution molecular weight of 15.8 kDa, close to the expected 15.6 kDa based on the sequence. (**A**) Column elution profile and fitted molecular weight. (**B**) Sample of aggregate/impurities and monomer peaks run on 16% SDS-PAGE with Coomassie staining. Purity was assessed by gel densitometry.



C

Protein	T_m (°C)	Enthalpy change (kcal/mol)	FWHM (°C)
SpyCatcher	48.8	48.7	16.1
SpyCatcher002	48.4	51.7	13.1
SpyCatcher003	48.3	65.2	11.0
SpyCatcher003:SpyTag003	95.2	165	6.6

Figure S7. Thermal unfolding of SpyCatcher variants. (**A**) DSC trace for each version of SpyCatcher or for SpyCatcher003 pre-reacted with SpyTag003 peptide. (**B**) DSC for SpyCatcher variants with the peak in each trace normalized to a value of 1. (**C**) Fitted thermodynamic values based on (A). FWHM = Full width half maximum.

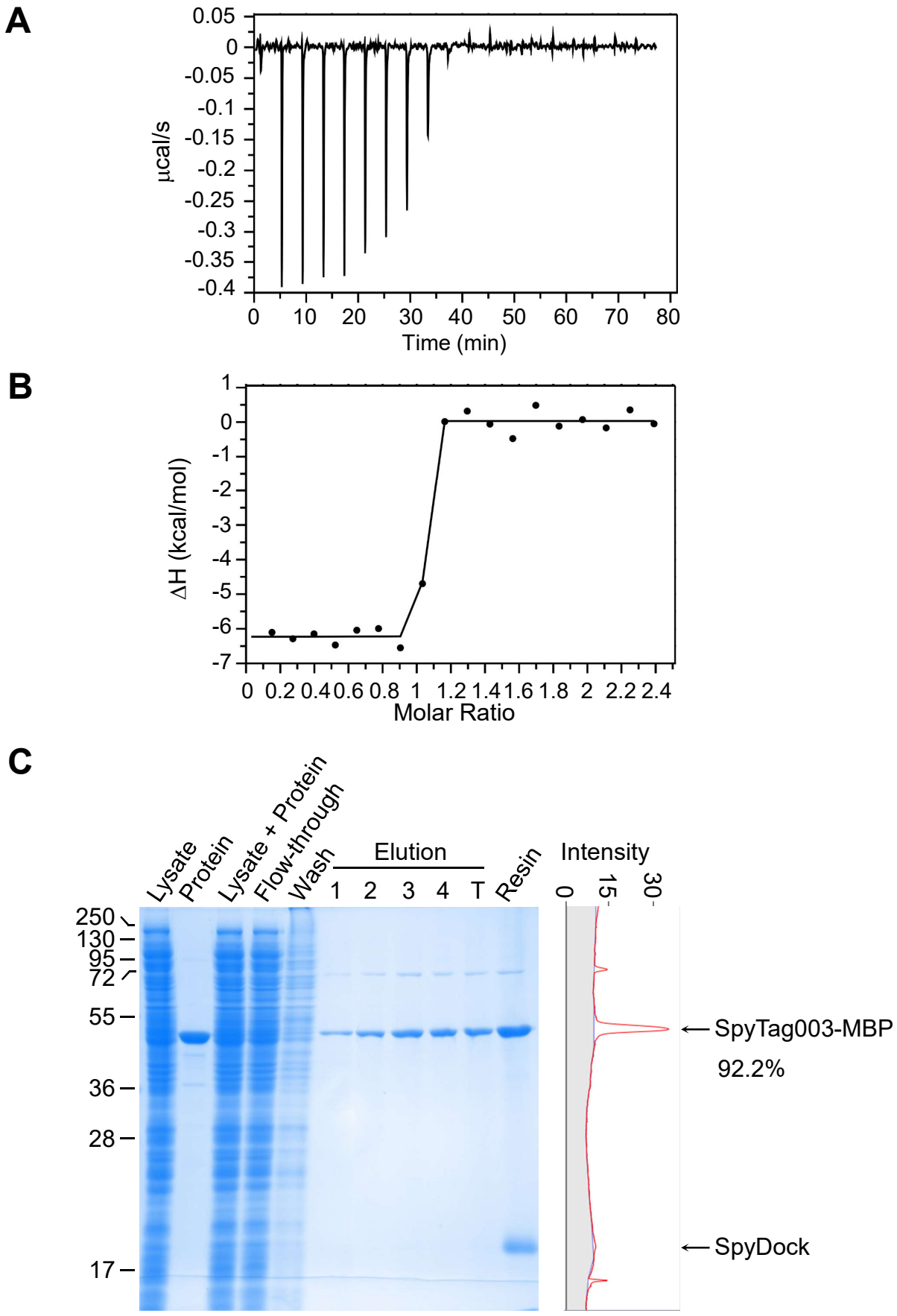


Figure S8. Non-covalent 003 interaction. **(A)** Raw ITC trace of SpyCatcher003 interaction with SpyTag003 D117A-MBP. **(B)** ITC titration from (A). **(C)** Spy&Go purification. SpyTag003-MBP was purified from *E. coli* lysate using the non-reactive SpyDock-resin and analyzed by SDS-PAGE with Coomassie staining. Lysate: clarified cell lysate, Protein: pure SpyTag003-MBP, Lysate + Protein: cell lysate mixed with SpyTag003-MBP, Flow-through: flow-through from resin binding, Wash: total washes with 500 mM imidazole in TP buffer, Elution 1 – 4: elution fractions with 2.5 M imidazole in TP buffer, T: total pooled elutions, Resin: resin post-elution. Scan of lane T shows relative purity of Spy&Go-purified SpyTag003-MBP.

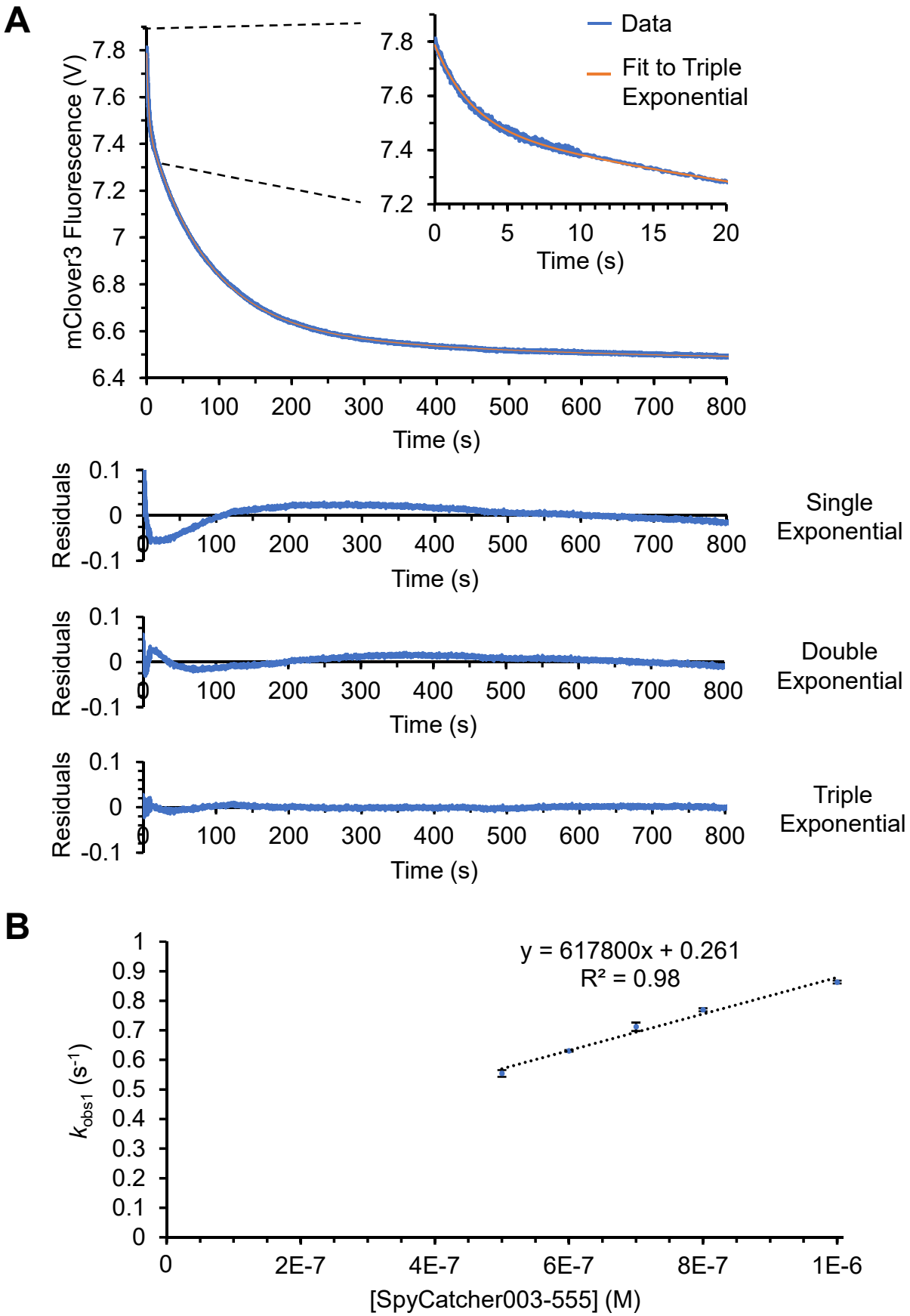


Figure S9. Kinetic analysis of SpyTag003/SpyCatcher003 interaction. (**A**) Stopped-flow analysis of 100 nM SpyTag003-mClover3 associating and reacting with 500 nM SpyCatcher003-555 results in a fluorescence trace described by a sum of three exponentials. The lower three panels show the residuals (difference between the data and the fit) for single, double, and triple exponential fits to the data. The inset shows the first 20 s of the trace. (**B**) Pseudo-first order analysis of the concentration-dependence of k_{obs1}. The mean k_{obs1} is shown ± 1 s.d., n = 4. The equation of the best-fit line and R^2 of the fit to the data are shown.

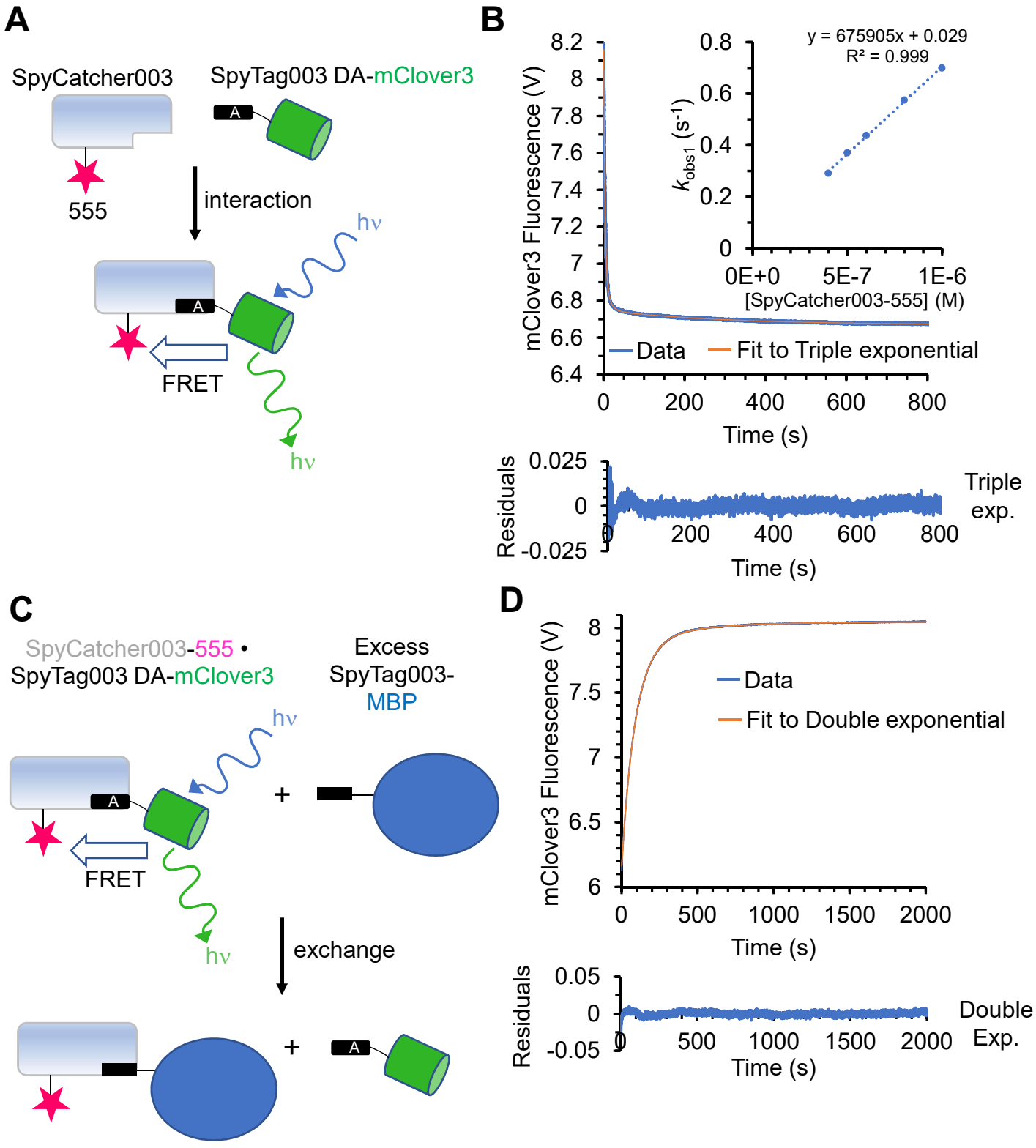


Figure S10. Stopped-flow kinetic analysis of SpyTag003 DA-mClover3 interacting with SpyCatcher003-555. (**A**) Schematic of association kinetics experiment. (**B**) Change in fluorescence upon 100 nM SpyTag003 DA-mClover3 interacting with 500 nM SpyCatcher003-555. A triphasic quench was observed, with a dominant rapid first phase followed by two slower phases. The lower panel shows the residuals (difference between data and fit) for a triple exponential fit. *Inset*: pseudo-first order analysis of the concentration-dependence of k_{obs1}. (**C**) Schematic of dissociation kinetics experiment for SpyCatcher003. The excess SpyTag003-MBP traps the SpyCatcher003-555 after SpyTag003 DA-mClover3 dissociates. The white A indicates the D117A mutation in SpyTag003. (**D**) Analysis of dissociation rate-constants for SpyCatcher003. SpyTag003 DA-mClover3 was equilibrated with SpyCatcher003-555. Excess SpyTag003-MBP was added at time 0 and mClover3 fluorescence was monitored (blue line). We fit the data to a double exponential (orange line) and the residuals from this fit are plotted in the lower panel.

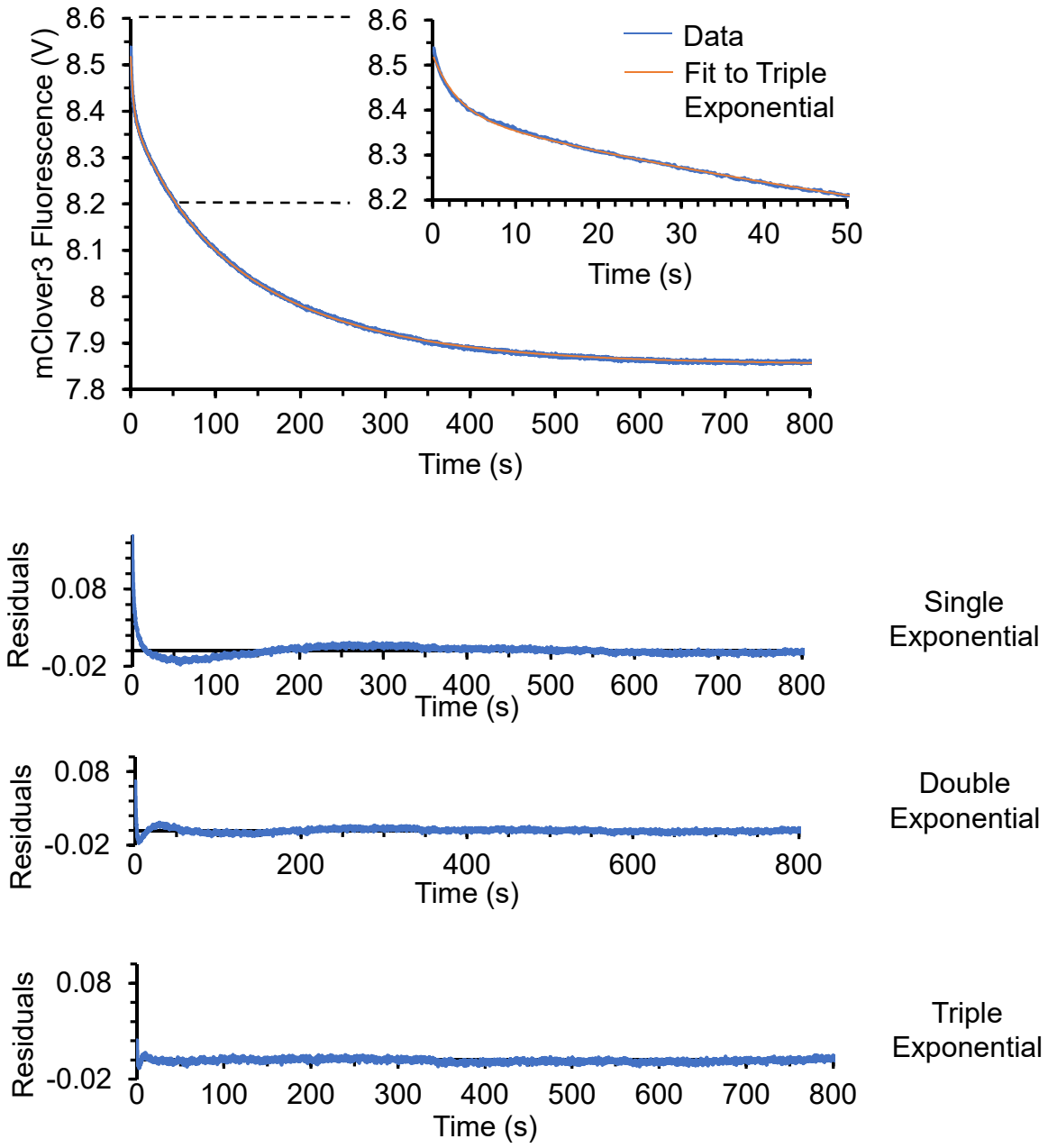


Figure S11. Stopped-flow kinetic analysis for mixing of 500 nM SpyTag003-mClover3 with 100 nM SpyCatcher003-555. The inset shows a zoom on the first 50 seconds. The lower panels shows the residuals for the fits of single, double, or triple exponentials to the data. Triple exponential kinetics, as previously observed when SpyCatcher003-555 was in higher concentration than SpyTag003-mClover3, supports induced fit conformational changes being coupled to binding.

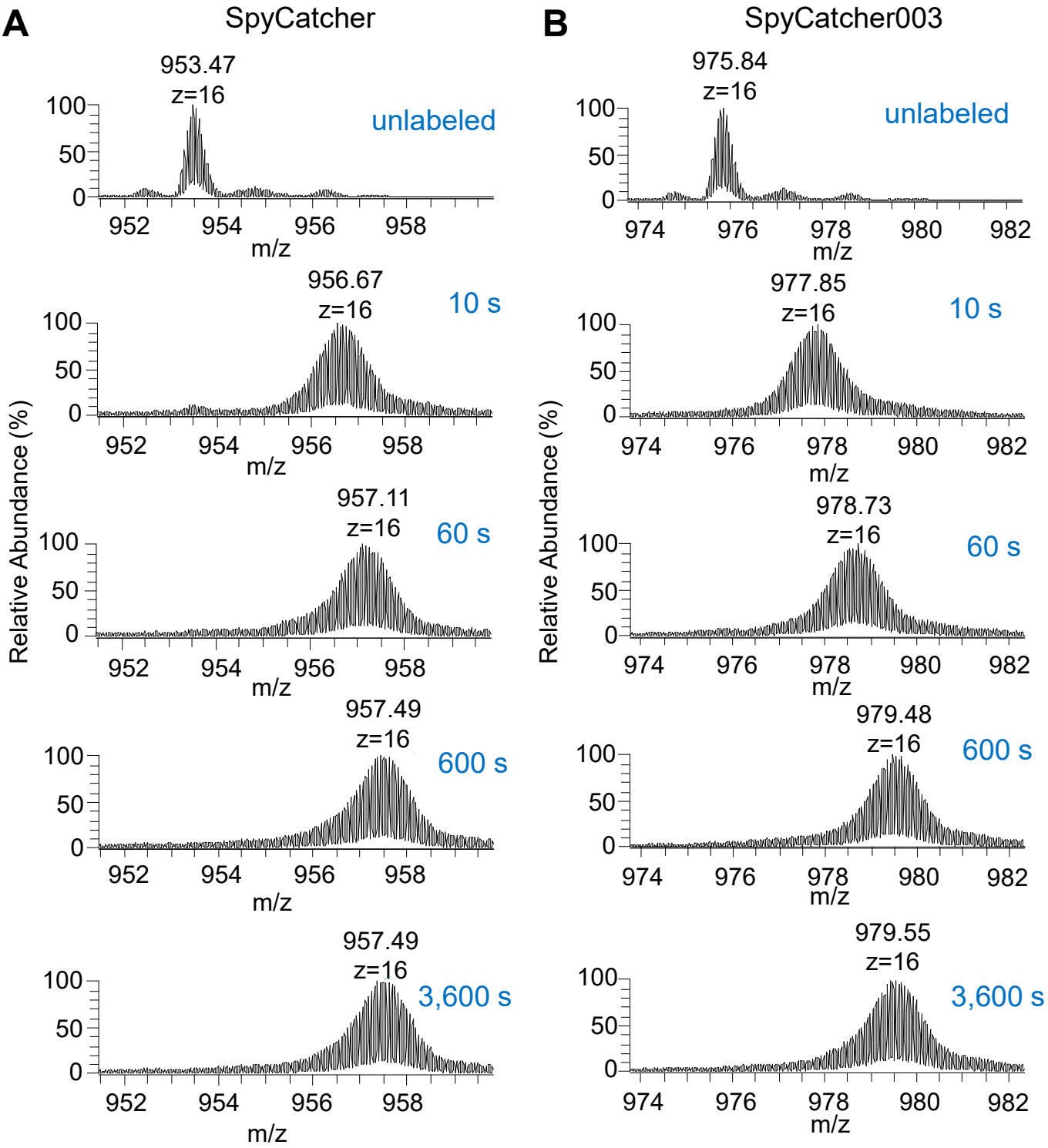


Figure S12. HDX mass spectra for (**A**) SpyCatcher and (**B**) SpyCatcher003 after mixing with D_2O for the indicated time at 25 °C. The m/z peak and the assigned charge-state (z) are marked (histogram plotted in Fig. 3A).

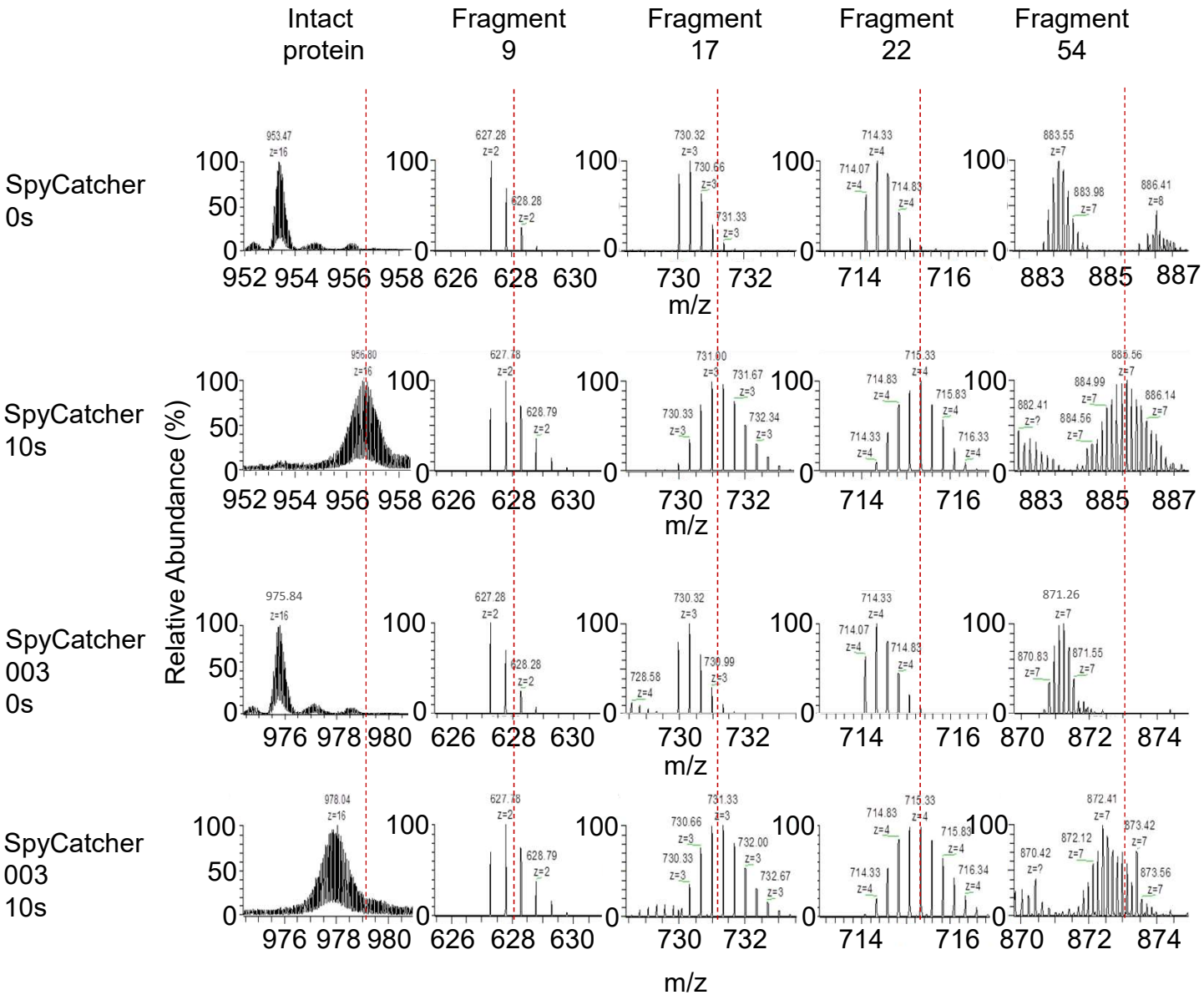


Figure S13. Representative mass spectra for the intact protein or sample fragments after online ETD fragmentation of SpyCatcher or SpyCatcher003. The mass of peaks and charge (z) are indicated. The red lines are placed manually as a visualization guide, based on the central m/z of the SpyCatcher HDX after 10s, to help comparison with the SpyCatcher003 data.

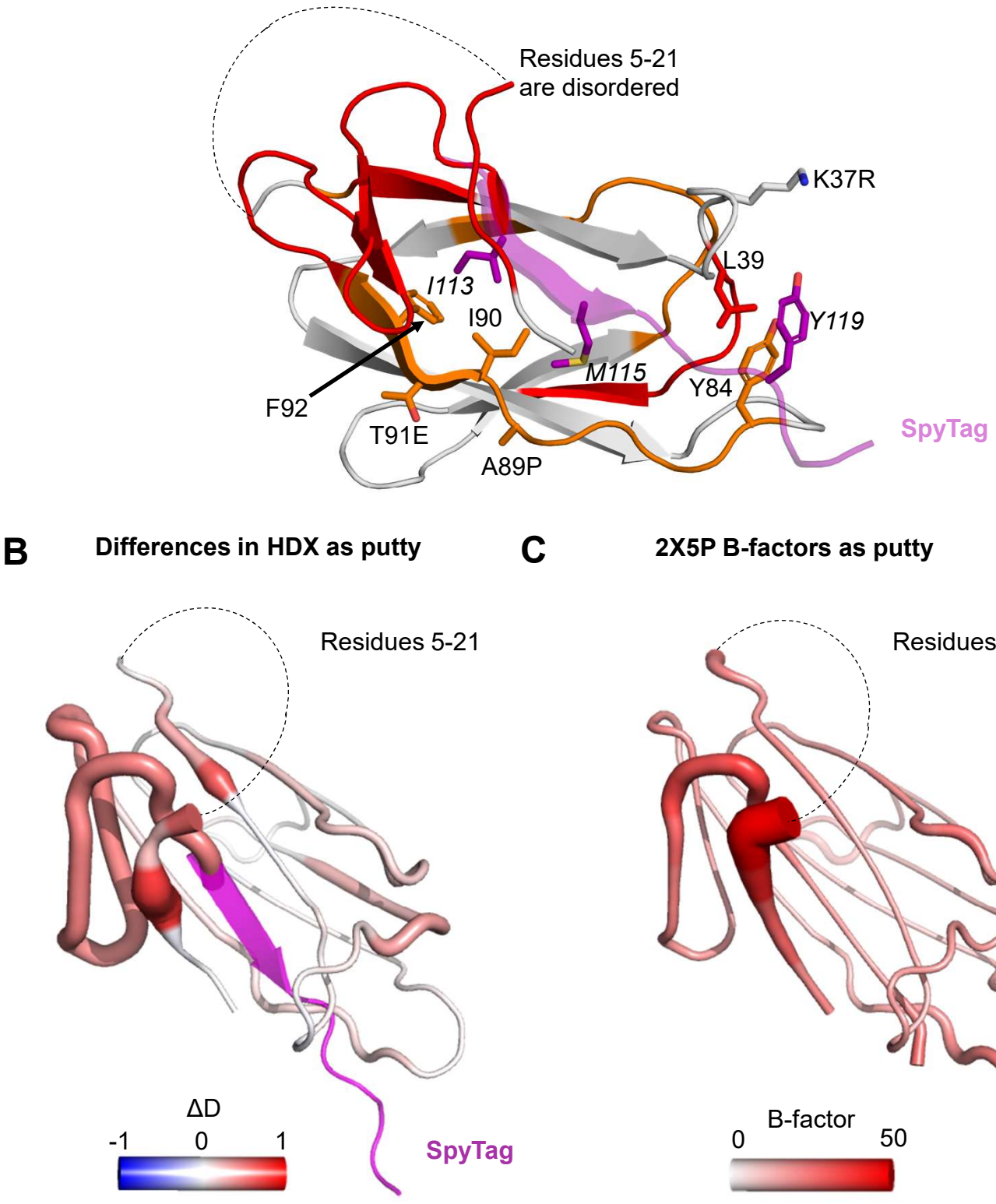


Figure S14. HDX and crystallographic representation of dynamics. (**A**) HDX differences related to important SpyTag003/SpyCatcher003 residues. SpyCatcher domain structure (represented by PDB 2X5P, with SpyTag overlaid in transparent purple from PDB 4MLI). SpyCatcher residues that are referred in the main text are shown and where they are mutated from SpyCatcher to SpyCatcher003 the mutations are shown. SpyTag residues are in italics. Orange: more stable in SpyCatcher003 (0.1-0.2). Red: much more stable in SpyCatcher003 (>0.2) (**B**) HDX difference in deuterium uptake as color-coded putty (based on PDB 2X5P with the SpyTag overlaid in transparent purple from PDB 4MLI), where width corresponds to divergence from ΔD 0. (**C**) Relative B-factors for the α-carbon of each amino acid in the CnaB2 domain (PDB 2X5P) shown as color-coded putty, where residues with higher B-factor are wider.

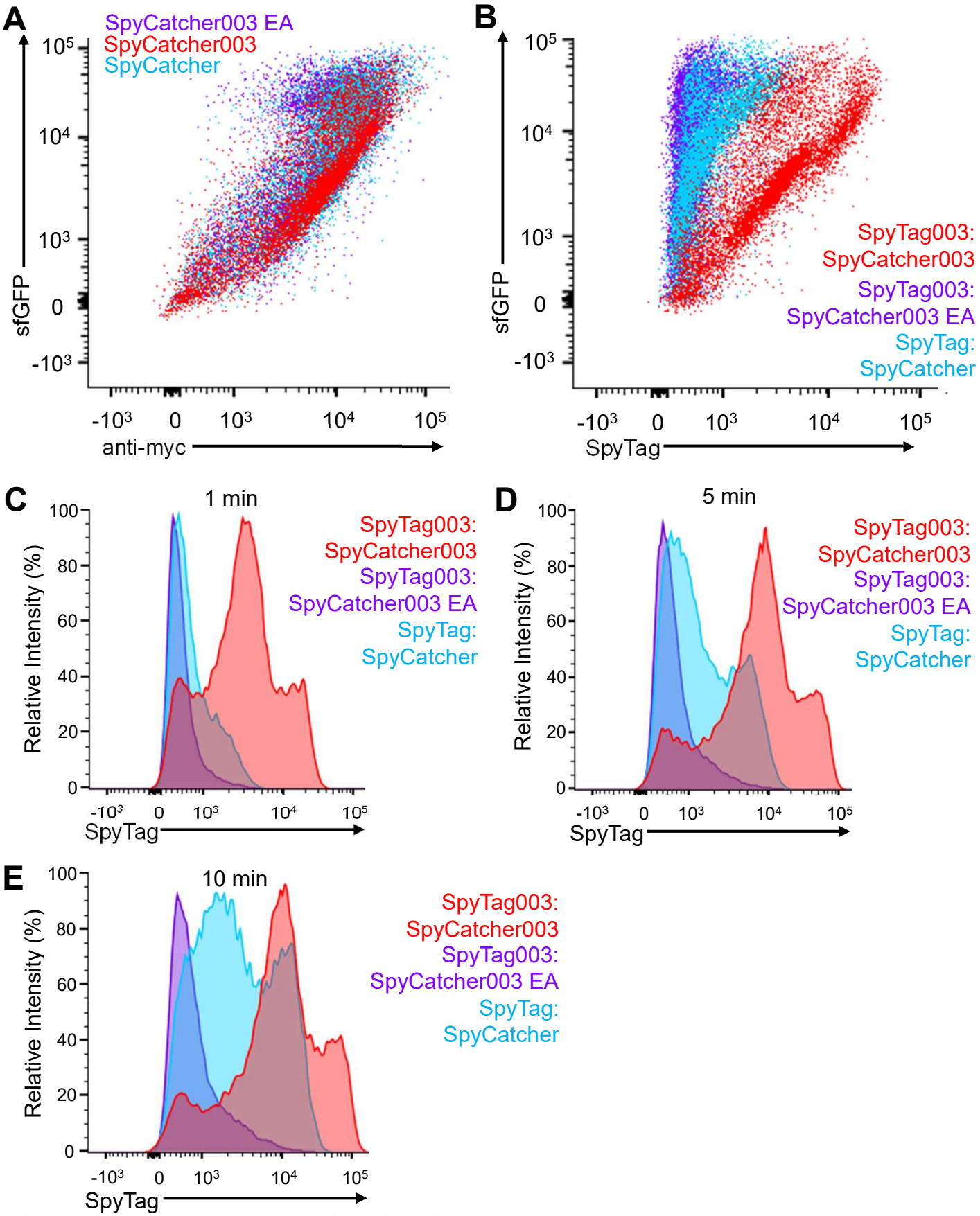
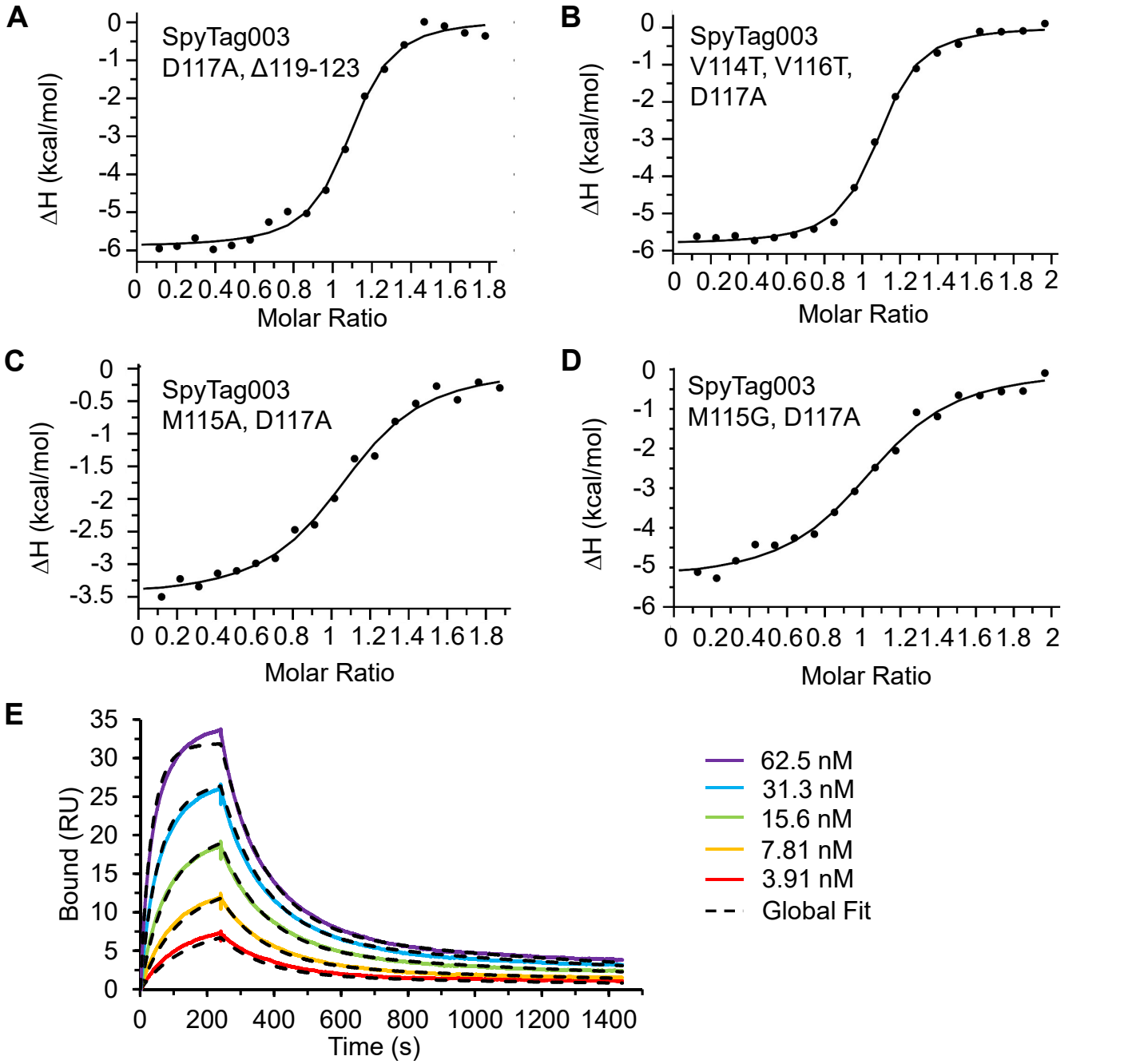


Figure S15. Flow cytometry testing of SpyCatcher003. (**A**) Flow cytometry showing expression of TfR-sfGFP-myc tag-SpyCatcher variants in Expi293 cells by sfGFP expression versus anti-myc-Alexa Fluor 647 binding. Sample dot plot as for Fig. 4B. (**B**) Flow cytometry analysis showing expression of TfR-sfGFP-myc tag-SpyCatcher variants in Expi293 cells versus SpyTag variant-mKate2 reaction, as in Fig. 4C after 1 min staining. (**C**) Time-dependence of labeling. Cells expressing TfR-sfGFP-myc tag-SpyCatcher were labeled for 1 min with SpyTag-mKate2, while SpyCatcher003 or SpyCatcher003 EA cells were labeled with SpyTag003-mKate2, before flow cytometry. (**D**) As in (C) but reaction was stopped after 5 min. (**E**) As in (C) with reaction stopped after 10 min. All steps were performed at 4 °C.



F

Binding Type	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (nM)	% Population
High Affinity	$3.1 \pm 0.6 \times 10^{5}$	$7.3 \pm 0.5 \times 10^{-4}$	2.4	20.1 ± 0.04
Low Affinity	$3.7 \pm 0.5 \times 10^{5}$	$7.8 \pm 0.2 \times 10^{-3}$	21	79.9 ± 0.04

G

SpyTag003 mutant	K_d (nM)
D117A	21 ± 4
V114T, V116T, D117A	220 ± 4
D117A, Δ119-123	240 ± 17
M115A, D117A	690 ± 84
M115G, D117A	1,140 ± 50

Figure S16. Affinity of SpyTag003 non-covalent series for SpyCatcher003. SpyTag003 D117A-MBP variants were tested for binding to SpyCatcher003 in PBS pH 7.4 at 25 °C. ITC for (**A**) SpyTag003 D117A, Δ119-123-MBP, (**B**) SpyTag003 V114T, V116T, D117A-MBP, (**C**) SpyTag003 M115A, D117A-MBP, and (**D**) SpyTag003 M115G, D117A-MBP. (**E**) SPR for SpyTag003 D117A-MBP binding to SpyCatcher003 on the chip. (**F**) Quantification of the two populations from (E). (**G**) Table summarizing affinities. Values are mean ± 1 s.d., n = 3.

SI Methods

Plasmids and cloning

PCR-based cloning and site-directed mutagenesis were carried out using Q5 High-Fidelity Polymerase (NEB) or KOD polymerase (EMD Millipore) and Gibson assembly. All constructs were confirmed by Sanger sequencing. pDEST14-SpyCatcher (GenBank JQ478411, Addgene plasmid ID 35044) and pET28a-SpyTag-MBP (Addgene plasmid ID 35050) were described (60). pDEST14 SpyCatcher002 (GenBank MF974388, Addgene plasmid ID 102827), pET28a SpyTag002-MBP (GenBank MF974389 Addgene plasmid ID 102831), pJ404-SpyCatcher-sfGFP, and pJ404-SpyCatcher002-sfGFP were described previously (61). Residue numbers for SpyTag and SpyCatcher variants are based on PDB 2X5P (62). pDEST14 SpyCatcher003 (*SI Appendix*, Fig. S1A; GenBank Accession no. MN433887, Addgene plasmid ID 133447) was derived from pDEST14 SpyCatcher002 (61) incorporating the following mutations: A89P, T91E, Q97D, N103D, and K108E. pJ404-SpyCatcher003-sfGFP (GenBank Accession No. MN433889, Addgene plasmid ID 133449) was derived by incorporating SpyCatcher003 in place of SpyCatcher002 in pJ404-SpyCatcher002-sfGFP (61). pDEST14-SpyCatcher S49C, pDEST14-SpyCatcher002 S49C and pDEST14-SpyCatcher003 S49C (Addgene plasmid ID 133448) include a Ser to Cys mutation enabling labeling with maleimide-dyes. pET28a-SpyTag003-MBP (SpyTag003 sequence RGVPHIVMVDAYKRYK; GenBank Accession no. MN433888, Addgene plasmid ID 133450) was derived from pET28a-SpyTag002-MBP (SpyTag002 sequence VPTIVMVDAYKRYK) (61). pET28a-SpyTag003 DA-MBP was derived from pET28a-SpyTag003-MBP by mutation of the reactive Asp117 to Ala (RGVPHIVMV<u>A</u>AYKRYK). SpyCatcher002-sfGFP variants were derived from pDEST14 SpyCatcher002-sfGFP (62) by Gibson assembly.

pET28-SpyTag002 T112H (VPHIVMVDAYKRYK)-MBP was derived from SpyTag002-MBP by Gibson assembly. pET28a-SpyTag003 DA Δ119-123 (RGVPHIVMVAA)-MBP was derived from pET28a-SpyTag003 DA-MBP by Gibson assembly. pET28a-SpyTag003 V114T V116T DA (RGVPHI<u>T</u>M<u>T</u>AAYKRYK)-MBP was derived from pET28a-SpyTag003 DA-MBP by Gibson assembly. pET28a-SpyTag003 M115A DA (RGVPHIV<u>A</u>VAAYKRYK)-MBP was derived from pET28a-SpyTag003 DA-MBP by Gibson assembly. pET28a-SpyTag003 M115G DA (RGVPHIV<u>G</u>VAAYKRYK)-MBP was derived from pET28a-SpyTag003 DA-MBP by Gibson assembly.

Mammalian surface expression of SpyCatcher variants was carried using pENTR4-TfR-sfGFP-myc tag-SpyCatcher, where TfR is the Transferrin receptor transmembrane domain and cytosolic region incorporating Y20C and F23A mutations that block internalization (63). This template was used to generate pENTR4-TfR-sfGFP-myc tag-SpyCatcher003 (GenBank Accession No. MN433890 and Addgene plasmid ID 133451) and pENTR4-TfR-sfGFP-myc tag-SpyCatcher003 E77A (where the mutation prevents isopeptide bond formation) (64). pET28-SpyTag003-mKate2 has the organization of SpyTag003-linker-mKate2-linker-His_6 (Addgene plasmid ID 133452). pET28-SpyTag-mKate2 has the same arrangement with SpyTag in place of SpyTag003. pET28-SpyTag003-sfGFP (Addgene plasmid ID 133454) and pET28-SpyTag003-mClover3 (Addgene plasmid ID 133453) have the organization SpyTag003-linker-fluorescent protein-linker-His_6. pET28-SpyTag003 DA-mClover3 was derived from pET28a-SpyTag003-mClover3 by mutation of the reactive Asp117 to Ala. pET28-SpyTag003-MBPa has His_{10}, SpyTag003 and a linker to the N-terminal side of MBP (GenBank Accession No. MN433891).

EGFP- or mCherry-tagged mouse Talin-1 expression constructs were created by subcloning DNA fragments encoding full-length mouse Talin-1 (amino acid residues 1-2541) or Talin-1 head (1-433) and EGFP or mCherry (65) into a modified pEGFP-C1 vector backbone (Clontech). Expression constructs for modified Talin-SpyTag003 proteins were created by subcloning mouse Talin-1 and SpyTag003 or SpyCatcher003 fragments into a modified pEGFP-C1 backbone with Talin-SpyTag003 DNA constructs being ordered from Genscript. Constructs were pEGFP-C1

SpyTag003-Talin rod (434-2541)-mCherry (GenBank Accession No. MN527524 and Addgene plasmid ID 133567); pEGFP-C1 EGFP-Talin head (1-433)-SpyCatcher003 (GenBank Accession No. MN527523 and Addgene plasmid ID 133566); and pEGFP-C1 WT Talin-mCherry (1-2541). SpyTag003 mutations blocking covalent bond formation (DA) and altering the binding affinity were created in the SpyTag003-Talin rod (434-2541)-mCherry construct by standard PCR methods and Gibson cloning. SpyTag003 variants were preceded by a glycine, to avoid an arginine at the N-terminus of the protein that could promote N-end rule degradation.

Bacterial protein expression and purification by Ni-NTA

pDEST14-SpyCatcher003 and pDEST14-SpyCatcher003 S49C were transformed into chemically-competent *Escherichia coli* C41 DE3 (a gift from Anthony Watts, University of Oxford), while all other constructs were transformed into *E. coli* BL21 (DE3) RIPL (Agilent). Single colonies were picked into 10 mL LB containing either 100 μg/mL ampicillin (pDEST14 and pJ404) or 50 μg/mL kanamycin (pET28a) and grown overnight at 37 °C with shaking at 200 rpm. 1 L LB supplemented with 0.8% (w/v) glucose and appropriate antibiotic in ultra-yield baffled flasks (Thomson Instrument Company) was inoculated with 1/100 dilution of the saturated overnight culture and grown at 37 °C with shaking at 200 rpm. After reaching A_{600} 0.5-0.6, the cultures were inoculated with 0.42 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 30 °C with shaking at 200 rpm for 4-5 h. For pET28a-SpyTag003-mKate2, a single colony was used to inoculate 1 L of autoinduction media plus trace elements (Formedia) supplemented with 50 μg/mL kanamycin and grown at 30 °C for 24 h with shaking at 200 rpm. Cells were harvested and lysed by sonication in 1× Ni-NTA buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.8) containing mixed protease inhibitors (cOmplete mini EDTA-free protease inhibitor cocktail, Roche) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were centrifuged at 30,000 *g* for 25 min before purification using Ni-NTA resin (Qiagen) using standard procedures (66). After elution, proteins were dialyzed into PBS pH 7.5 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) with three buffer changes using 3.5 kDa molecular weight cut-off Spectra/Por dialysis tubing (Spectrum Labs). Protein concentrations were determined from A_{280} using the extinction coefficients from ExPASy ProtParam.

Isothermal titration calorimetry

Experiments were carried out using a Microcal PEAQ-ITC calorimeter (Malvern) at 25 °C in PBS pH 7.4. 20 μM SpyCatcher003 was used in the cell and titrated with 20 injections of 210 μM SpyTag003 DA-MBP (or related mutants) in the syringe. Analysis was carried out using a 1:1 binding model with MicroCal PEAQ-ITC Analysis software version 1.1.0.1262.

Isopeptide bond formation assays

For the comparison of different stages of SpyTag development (*SI Appendix*, Fig. S2A), 10 nM MBP fusion was reacted with 10 nM SpyCatcher003-sfGFP for 10 min in SPG buffer (12.5 mM succinic acid, 43.75 mM NaH_2PO_4, 43.75 mM glycine) pH 7.0 with 0.2% w/v bovine serum albumin (BSA) at 25 °C.For the comparison of reactivities of different stages of SpyCatcher development (*SI Appendix*, Fig. S2B), 10 nM SpyCatcher variant was reacted with 10 nM SpyTag003-MBP for 10 min in SPG pH 7.0 with 0.2% w/v BSA at 25 °C.

Size exclusion chromatography-multiple angle light scattering (SEC-MALS)

100 μL Ni-NTA purified SpyCatcher003 at 7.5 mg/mL in PBS pH 7.4 was injected into a Superdex 200 HR 10/30 column (GE Healthcare) at 25 °C at 0.5 mL/min connected to a Shimadzu HPLC system comprising LC-20AD pump, SIL-20AC autosampler and SPD20A UV/Vis detector with PBS pH 7.4 as running buffer. Light scattering was detected by a Wyatt Dawn HELEOS-II 8-angle light scattering detector and Wyatt Optilab rEX refractive index monitor. The resulting light scattering, refractive index and UV traces were processed in ASTRA 6 (Wyatt Technologies).

Surface plasmon resonance
SPR experiments were carried out using a Biacore T200 (GE Healthcare). The SpyCatcher003 binding surface was created by coupling SpyCatcher003 S49C to a Sensor Chip CM5 using the thiol coupling kit (GE Healthcare). SpyTag003 DA-MBP was injected at 3.91, 7.81, 15.6, 31.3 and 62.5 nM at 30 µL/min in running buffer PBS pH 7.4 + 0.005% v/v Tween-20. Protein was injected for 240 s, followed by a 1,200 s dissociation time. The binding surface was regenerated with 30 s flow of 10 mM glycine pH 2.0. Measurements were performed at 25 °C, with double referencing subtraction to observe only specific binding. Data were fitted to a heterogeneous ligand binding mechanism using the Biacore T200 Evaluation software (GE Healthcare) where SpyCatcher003 forms two classes of complexes with SpyTag003 DA, with high and low affinities. $K_d = k_{off}/k_{on}$ in each case, with K_d being the dissociation constant, k_{off} being the dissociation rate constant, and k_{on} the association rate constant.

Differential scanning calorimetry
Experiments were performed with 32 µM SpyCatcher, SpyCatcher002, SpyCatcher003 or SpyCatcher003 pre-reacted with the SpyTag003 peptide (sequence RGVPHIVMVDAYKRYK, solid-phase synthesized by Insight Biotechnology at >95% purity) in PBS pH 7.4 on a MicroCal PEAQ-DSC (Malvern). 32 µM SpyCatcher003 was reacted with 60 µM SpyTag003 for 1 h at 25 °C in SPG buffer pH 7.0 followed by dialysis with three changes of PBS pH 7.4. Thermal transitions were monitored from 20 to 110 °C at a scan rate of 3 °C/min at 3 atm. Data were analyzed using MicroCal PEAQ-DSC analysis software version 1.22. The buffer blank (PBS pH 7.4) was subtracted from the experimental sample and corrected for concentration and volume, followed by the baseline subtraction. Subsequently the observed transition was fitted to a two-state model, to obtain the melting temperature (T_m), area under the peak (the enthalpy of unfolding, ΔH_m), and Full Width Half Maximum using the MicroCal PEAQ-DSC analysis software version 1.22 and Origin 2015 (OriginLab).

Electrospray Ionization Mass Spectrometry
30 µM SpyCatcher003 was reacted with 60 µM SpyTag003 peptide for 1 h at 25 °C in SPG buffer pH 7.0. The reaction was dialyzed against 10 mM ammonium acetate pH 7.5 using 3.5 kDa cut-off Spectra/Por dialysis tubing (Spectrum labs) prior to analysis.

An Agilent RapidFire 365 platform was coupled to the Agilent 6550 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) mass spectrometer. This system was used to perform intact protein mass spectrometry in positive ion-mode employing a jet-stream electrospray ion source (Agilent). Samples at 10 µM in 50 µL volume were prepared on a 384-well polypropylene plate (Greiner) and acidified to 1% (v/v) formic acid. The samples were aspirated under vacuum for 0.4 s using the RapidFire sampling platform. Samples were loaded onto a C4 solid-phase extraction cartridge. After washes with 0.1% (v/v) formic acid with 1.5 mL/min flow-rate for 5.5 s, the samples were eluted with deionized water containing 85% (v/v) acetonitrile and 0.1% (v/v) formic acid at 1.25 mL/min for 5.5 s. The cartridge was then equilibrated with deionized water for 0.5 s. Nitrogen drying gas for the ionization source was operated at 13 L/min at 225 °C. The jet stream sheath gas was at a flow-rate of 12 L/min at 350 °C and the nozzle voltage was 1,500 V. The data were analyzed using Mass Hunter Qualitative Analysis software version 7.0. The protein ionization data were deconvoluted using the maximum entropy algorithm. The predicted mass came from ExPASy ProtParam, based on cleavage of the N-terminal formylmethionine.

Fluorophore conjugation to cysteine-containing SpyCatcher proteins
Labeling took place with tubes wrapped in foil to minimize light exposure. DyLight 680-maleimide (Thermo Fisher) or Alexa Fluor 555-maleimide (Thermo Fisher) was dissolved in anhydrous

dimethylsulfoxide (DMSO) to a final concentration of 10 mg/mL. Samples were aliquoted and stored at -80 °C until use. SpyCatcher-S49C, SpyCatcher002-S49C or SpyCatcher003-S49C in PBS pH 7.4 was incubated with a 10-fold molar excess of dye:protein. Samples were rapidly pipetted to mix the samples thoroughly, prior to rotating end-over-end at 25 °C for 4 h. Samples were then centrifuged at 16,000 *g* for 5 min to remove any aggregates. 800 µL swollen Sephadex G-25 resin (Sigma-Aldrich) was added to a Bio-Rad Poly-Prep column and washed with 4 mL PBS pH 7.4 to remove residual storage ethanol. The PBS was allowed to drain and the samples were added to the column to remove unconjugated dye. A further 1 mL PBS pH 7.4 was added to the top of the column and 300 µL fractions were collected. Fractions 1 and 2 were pooled and dialyzed thrice for at least 3 h in PBS pH 7.4 at 4 °C.

Western Blotting

Untransformed *E. coli* Turbo cells (NEB) were grown for 8 h, pelleted and resuspended in PBS pH 7.4 in the presence of cOmplete mini EDTA-free protease inhibitor cocktail (Roche) and 1 mM PMSF. 1.5×10^7 human Expi293 cells were pelleted and lysed in 5 mL lysis buffer [150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% (v/v) Triton X-100, 1mM EDTA, in the presence of cOmplete mini EDTA-free protease inhibitor cocktail (Roche) and 1 mM PMSF]. *S. cerevisiae* strain K699 was a kind gift of the Nasmyth laboratory, University of Oxford. The *Drosophila melanogaster* sample was GenLysate (G-Biosciences). Lysate was aliquoted and stored at -80 °C until use. For testing by Western blot, 3 pmol SpyTag003-MBPa was doped into selected samples of the cell lysate. Samples were mixed with 6× SDS loading buffer, and heated for 3 min at 99 °C in a Bio-Rad C1000 thermal cycler. 5 µg cell lysate was loaded per lane, except for *D. melanogaster* where 10 µg was loaded to counteract the weak anti-GAPDH recognition.

Protein samples and cell lysate were resolved by 16% SDS-PAGE using the XCell SureLock system (Thermo Fisher) at 200 V. Samples were transferred onto equibrated nitrocellulose membrane (Bio-Rad; 162-0112) between filter paper (Fisherbrand; FB59025) in transfer buffer [10% (v/v) MeOH, 25 mM Tris base, 192 mM glycine) using the XCell II Western blot module (Thermo Fisher) at 35 V for 90 min. Membranes were blocked in 5% (w/v) skimmed milk made in PBS pH 7.4 with 0.05% (v/v) Tween-20 (PBST) for a minimum of 1 h. Membranes were then probed with 13 nM SpyCatcher003-680 and 1:500 anti-GAPDH-DyLight 800 (Thermo Fisher) in PBST with 5% (w/v) skimmed milk for 2 h at 25 °C. The membrane was washed while protected from light for a minimum of 3× 30 min in PBST, 1× 30 min in PBS pH 7.4, and rinsed in MilliQ H_2O. Western blots were imaged using a Li-Cor Odyssey Fc and image analysis was conducted using Image Studio Lite 5.2 (Li-Cor).

Spy&Go purification

Affinity purification of SpyTag003-fusion was performed as previously described (64). 0.09 mg SpyTag003-MBP (previously purified by Ni-NTA) was added into ~0.25 g wet cell weight of *E. coli* BL21 (DE3) RIPL cleared lysate dissolved in 500 µL TP buffer (25 mM orthophosphoric acid adjusted to pH 7.0 with Tris base) in an Eppendorf tube containing 50 µL packed Spy&Go resin (SpyCatcher2.1 S49C E77A coupled to SulfoLink resin). This sample was mixed for 1 h, tumbling end-over-end at 4 °C. Resin was washed 4 times with 10 resin volumes of wash buffer (500 mM imidazole in TP buffer, pH 7.0), with incubation at 4 °C and shaking at 1,200 rpm for 3 min. The resin was centrifuged at 4,000 *g* for 3 min at 4 °C. Protein was eluted with 4 times with 1.5 resin volume of 2.5 M imidazole in TP buffer. After SDS-PAGE, the gel was stained using InstantBlue (Expedeon) and analyzed with Gel Doc XR imager and Image Lab 5.2 software (Bio-Rad). Percentage purity was defined as 100 × [target protein Band % in lane T/target protein Band % in lane Protein].

Structure visualization

Protein structures were rendered in PyMOL version 2.0.6 (DeLano Scientific), based on Protein Data Bank files 2X5P (62) and 4MLI (67). Relative α-carbon B-factors were shown using the cartoon putty function of PyMOL, such that the chain is wider and redder with higher B-factor. For the representation of HDX differences, B-factors were replaced with the ΔD value for each residue and the cartoon putty function of PyMOL was used. Fig. 1A was generated from PDB 2X5P, except PDB 4MLI was used to represent SpyTag.

SI References

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