Supporting Information:

SnoopLigase catalyzes peptide-peptide locking and enables solid-phase conjugate isolation

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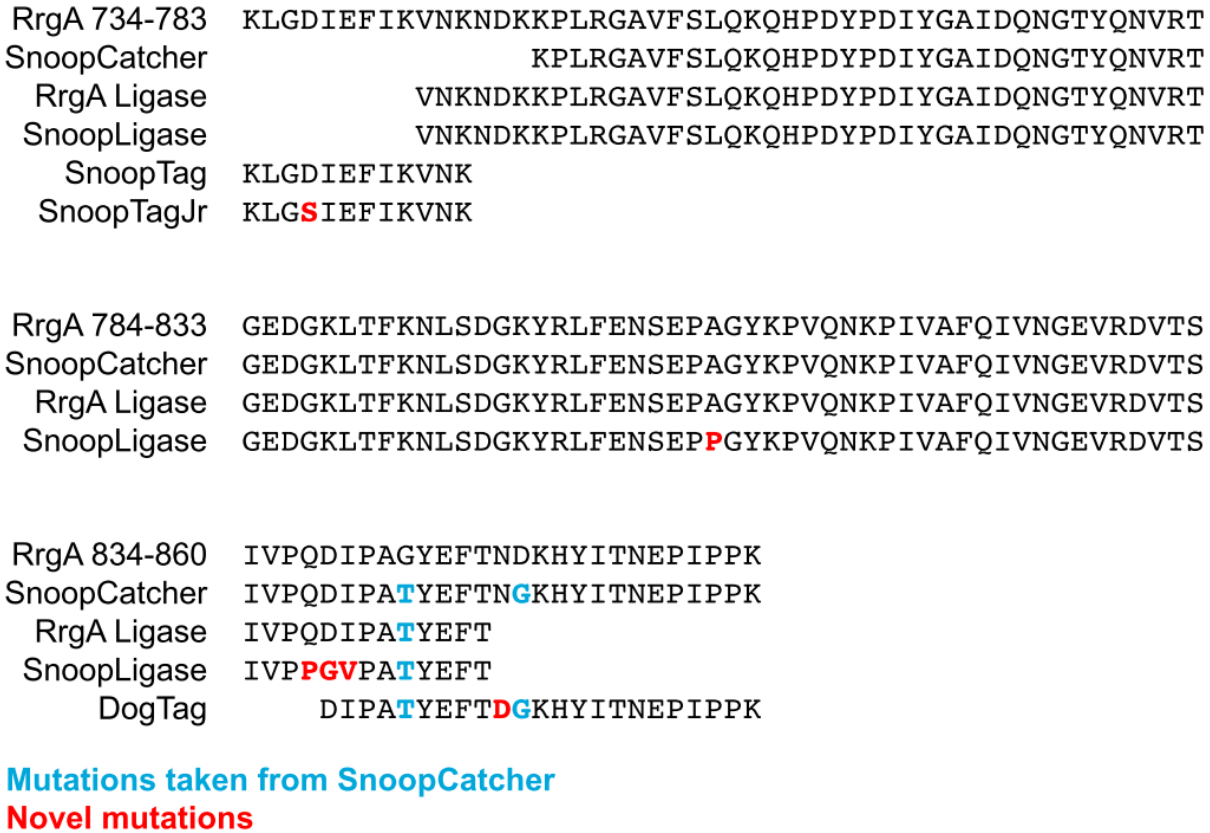


Figure S1. Amino acid sequences of partners. Sequence alignment of the C-terminal domain of RrgA and proteins/peptides derived from this domain (SnoopCatcher, RrgA Ligase, SnoopLigase, SnoopTag, SnoopTagJr and DogTag). Previously published mutations are highlighted in cyan and novel mutations in red.

a

Mutation	R.E.U.	∆R.E.U.
Parent, RrgA (734-860 G842T N847D D848G)	-267.2	+0.0
D737S	-268.6	-1.4
A820E	-268.5	-1.3
D830N	-266.6	+0.6
D838G	-267.5	-0.3
I839V	-267.6	-0.4

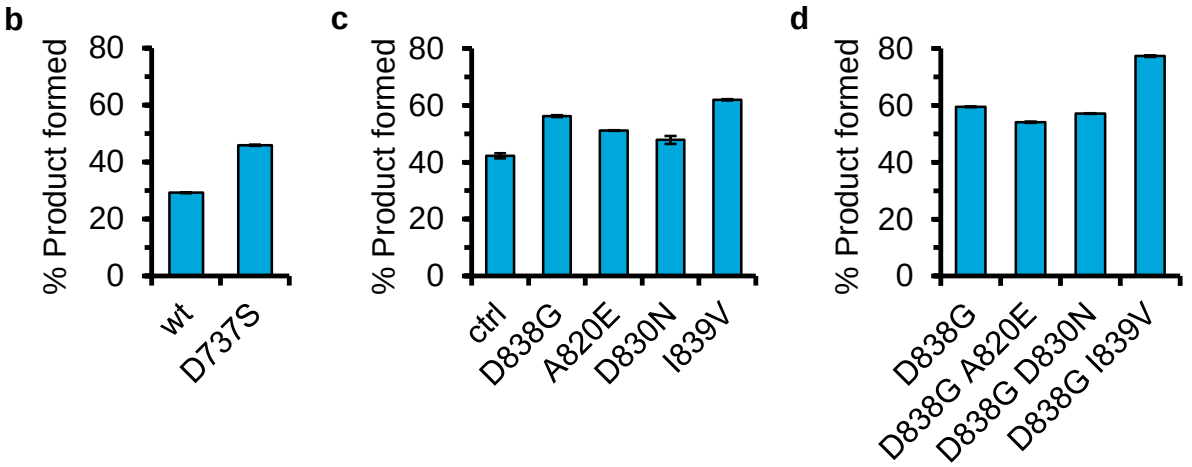
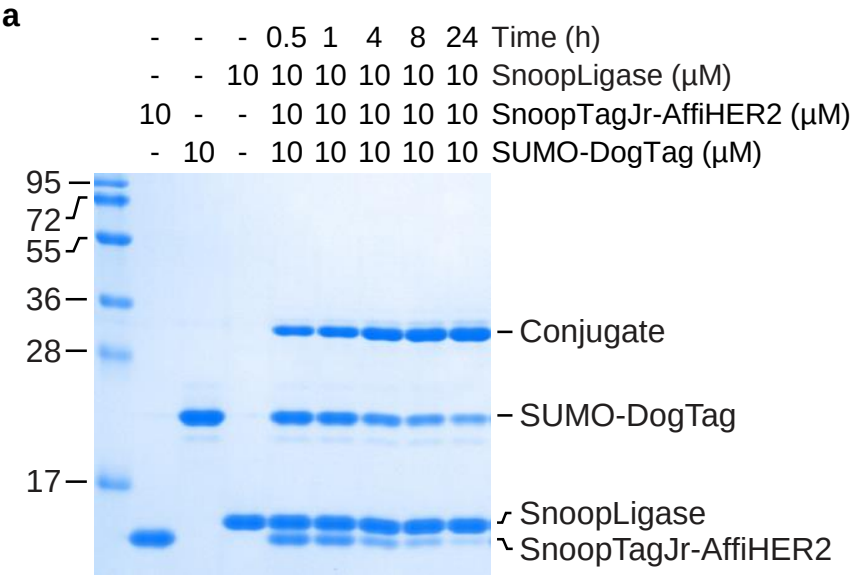


Figure S2. Point mutations to enhance SnoopLigase reactivity. (a) Rosetta Energy units (R.E.U.) of mutations in RrgA suggested by PROSS. The change in R.E.U. is shown relative to the parent protein. **(b)** Reactivity of wild-type (wt) SnoopTag and SnoopTag D737S with +6RrgALigase+9 A808P and SUMO-DogTag in 50 mM boric acid with 1 M TMAO pH 9.0 and 30% (v/v) glycerol for 3 h at 4 °C. **(c)** Reactivity of +6RrgALigase+9 A808P (ctrl) and single point mutants thereof with SnoopTagJr-AffiHER2 and SUMO-DogTag in 50 mM Tris borate with 0.5 M TMAO pH 8.0 and 30% (v/v) glycerol for 4 h at 4 °C. **(d)** Reactivity of +6RrgALigase+9 A808P Q837P D838G (D838G) and point mutants thereof with SnoopTagJr-AffiHER2 and SUMO-DogTag in 50 mM Tris borate with 0.75 M TMAO pH 8.0 and 7.5% (v/v) glycerol for 1.5 h at 4 °C. Results are mean of triplicate ± 1 SD; some error bars are too small to be visible.

Mutation	R.E.U.	∆R.E.U.	Relative reaction rate
control (RrgALigase)	-268.6	+0.0	1.0
A808P	-269.8	-1.2	3.1
A808P Q837P	-269.5	-0.9	6.9
A808P Q837P D838G	-270.5	-1.9	28
A808P Q837P D838G I839V (SnoopLigase)	-270.9	-2.3	66

Figure S3. Rosetta Energy units (R.E.U.) and relative reaction rates of RrgALigase mutants. The change in R.E.U. and relative reaction rate with SnoopTagJr-AffiHER2 and SUMO-DogTag is given relative to RrgALigase. 10 µM RrgA Ligase or point mutants thereof was incubated with equimolar SnoopTagJr-AffiHER2 and SUMO-DogTag for 15 min at 4 °C and ligated product was determined by SDS-PAGE with Coomassie staining. Relative reaction rate for each mutant was calculated as the amount of product formed from the mutant divided by the amount of product formed from the control (RrgALigase).

a



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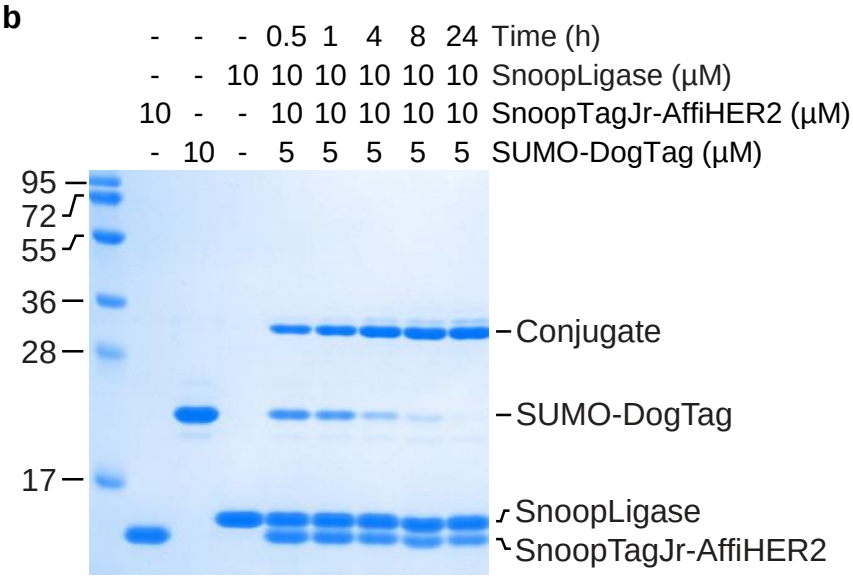

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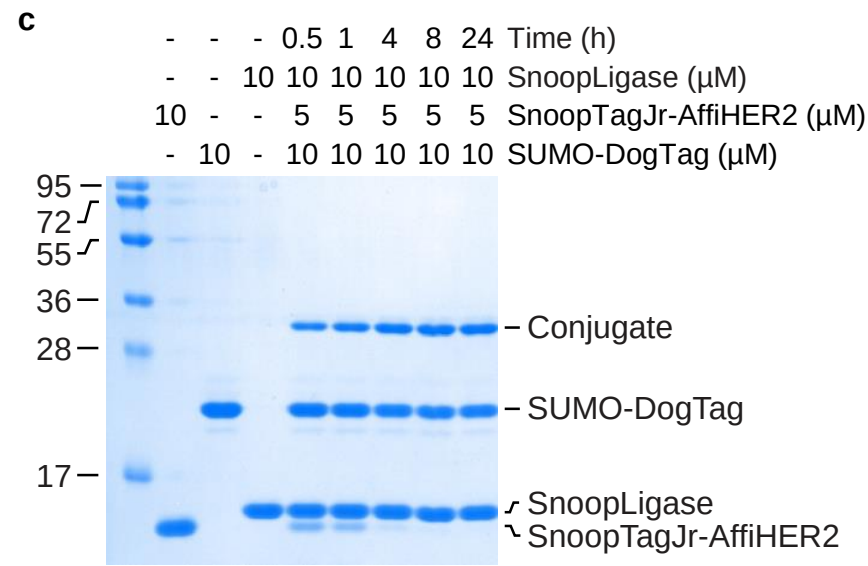

Figure S4. Maximizing SnoopLigase conjugation yield. **(a)** Equimolar reaction. 10 µM SnoopTagJr-AffiHER2 and 10 µM SnoopLigase were incubated with 10 µM SUMO-DogTag at 4 °C. **(b)** Maximizing SUMO-DogTag conjugation. 10 µM SnoopTagJr-AffiHER2 and 10 µM SnoopLigase were incubated with 5 µM SUMO-DogTag at 4 °C. **(c)** Maximizing SnoopTagJr-AffiHER2 conjugation.10 µM SUMO-DogTag and 10 µM SnoopLigase were incubated with 5 µM SnoopTagJr-AffiHER2 at 4 °C. Analysis by SDS-PAGE with Coomassie staining.

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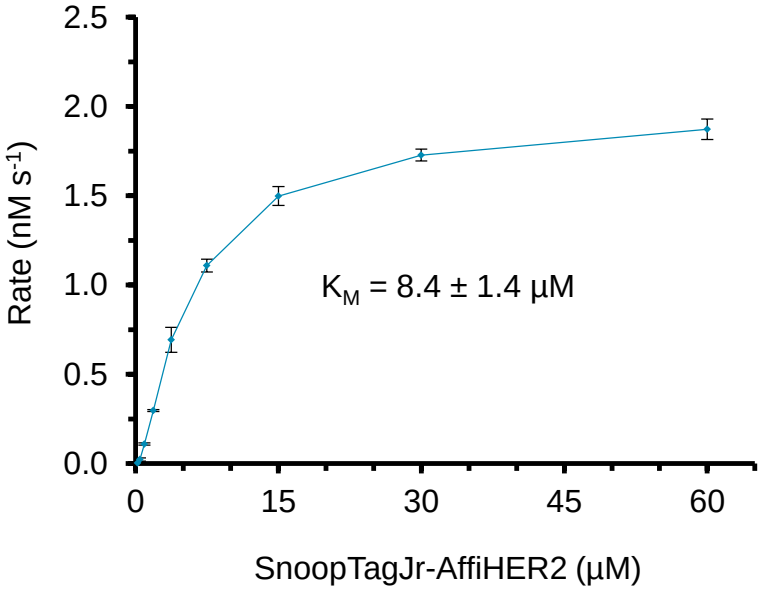

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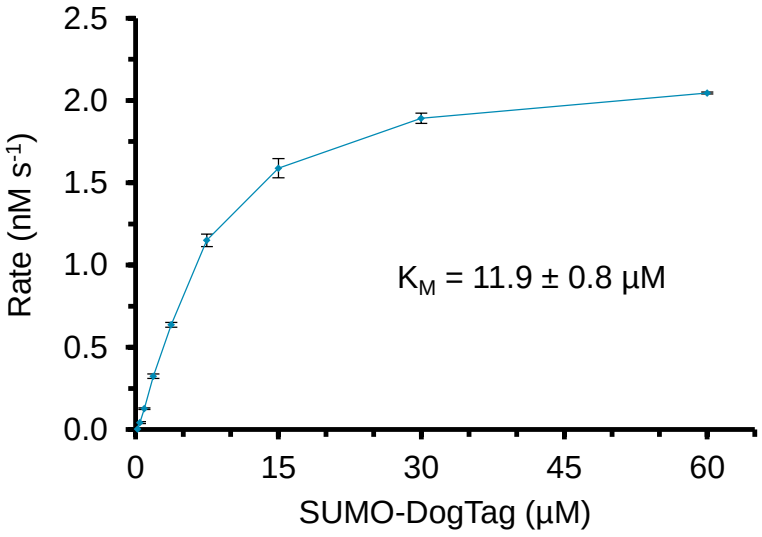

Figure S5. SnoopLigase Michaelis constants. **(a)** Determining SnoopLigase's Michaelis constant for SnoopTagJr-AffiHER2. 40 µM SUMO-DogTag and 5 µM SnoopLigase were incubated with indicated concentrations of SnoopTagJr-AffiHER2 for 7.5 min at 4 °C. The initial rate of formation of SUMO-DogTag:SnoopTagJr-AffiHER2 conjugate was plotted. **(b)** Determining SnoopLigase's Michaelis constant for SUMO-DogTag. 40 µM SnoopTagJr-AffiHER2 and 5 µM SnoopLigase were incubated with indicated concentrations of SUMO-DogTag for 7.5 min at 4 °C and initial rate of conjugate formation was plotted. Analysis by SDS-PAGE with Coomassie staining. Results are mean of triplicate ± 1 SD.

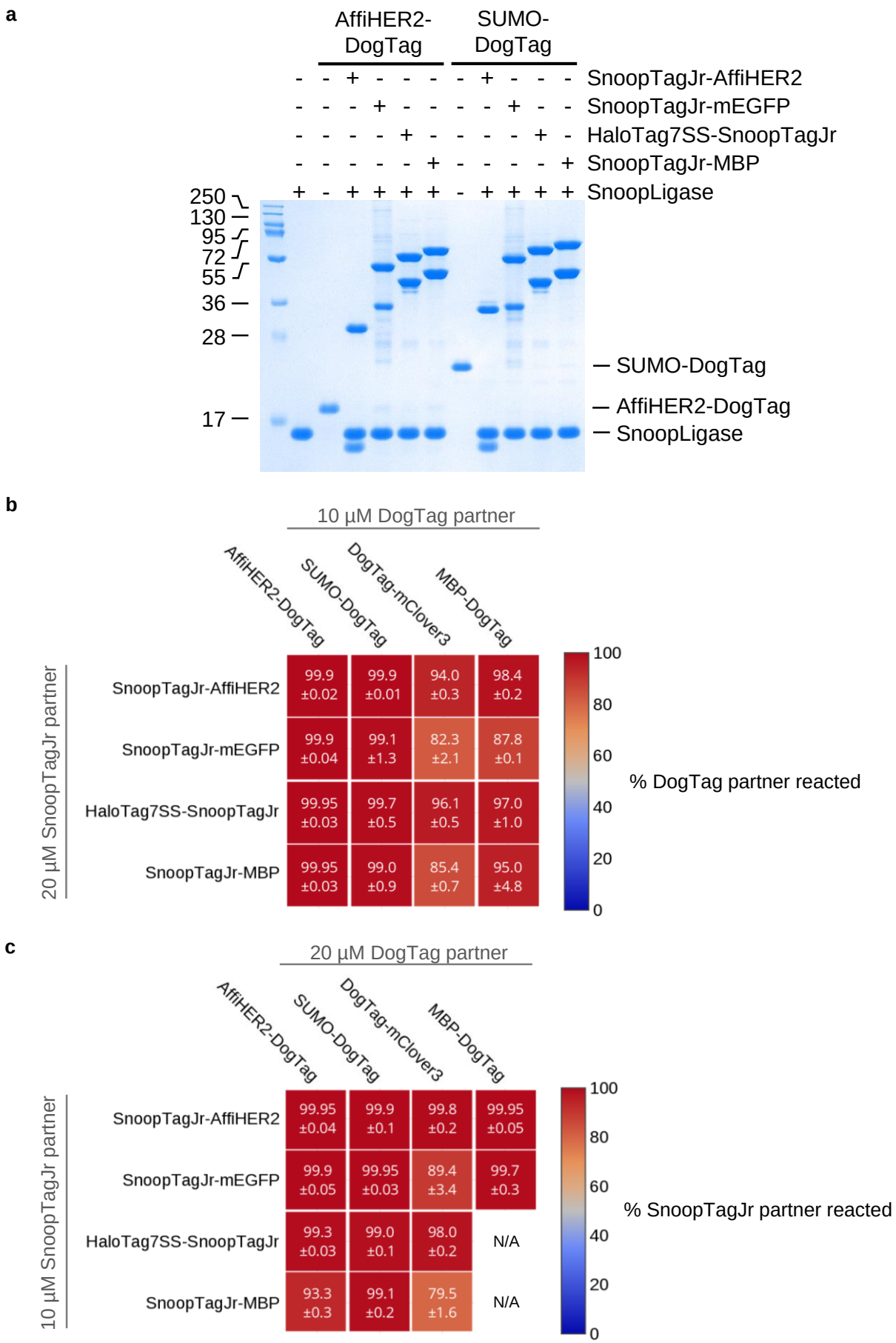

Figure S6. DogTag or SnoopTagJr could be conjugated to high yield at the N- or C-terminus. (a) Representative gel testing high yield conjugation. 10 µM AffiHER2-DogTag or SUMO-DogTag was incubated with 20 µM SnoopLigase and 20 µM SnoopTagJr-linked proteins for 24 h at 4 °C. **(b)** 10 µM DogTag-linked protein was reacted with 20 µM SnoopLigase and 20 µM SnoopTagJr-linked protein for 24 h at 4 °C. % DogTag partner reacted is shown in each box (mean ± 1 SD, n = 3), along with color-coding. **(c)** 10 µM SnoopTagJr-linked protein was reacted with 20 µM SnoopLigase and 20 µM DogTag-linked protein for 24 h at 4 °C. % SnoopTagJr partner reacted is shown in each box (mean ± 1 SD, n = 3), with color-coding. Analysis by SDS-PAGE + Coomassie staining. N/A where band overlap prevented quantification.

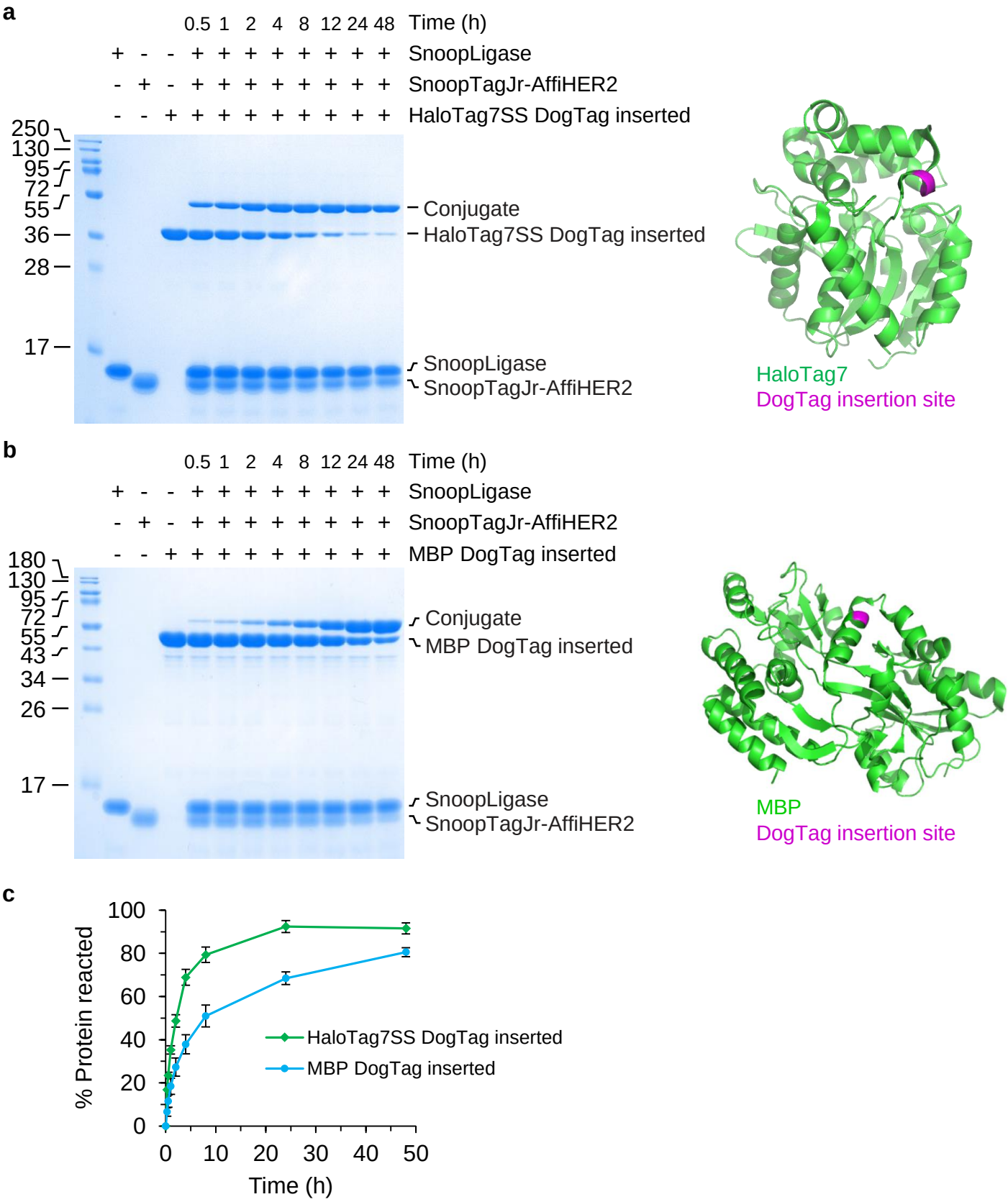

Figure S7. SnoopTagJr reacted with DogTag inserted internally into proteins. (a) 10 µM HaloTag7SS with DogTag inserted between residues D139 and E140 was incubated with 20 µM SnoopLigase and 20 µM SnoopTagJr-AffiHER2 for 0.5 – 48 h at 4 °C. The crystal structure of HaloTag7 (PDB code 5Y2Y) indicates the site for DogTag insertion in magenta. **(b)** 10 µM MBP with DogTag inserted between residues R317 and A319 (I318 deleted) was incubated with 20 µM SnoopLigase and 20 µM SnoopTagJr-AffiHER2 for 0.5 – 48 h at 4 °C. The crystal structure of MBP (PDB code 1OMP) indicates the site for DogTag insertion in magenta. Analysis by SDS-PAGE with Coomassie staining. **(c)** Analysis of reaction extent in (a) and (b) by densitometry. Results are mean ± 1 SD, n = 3.

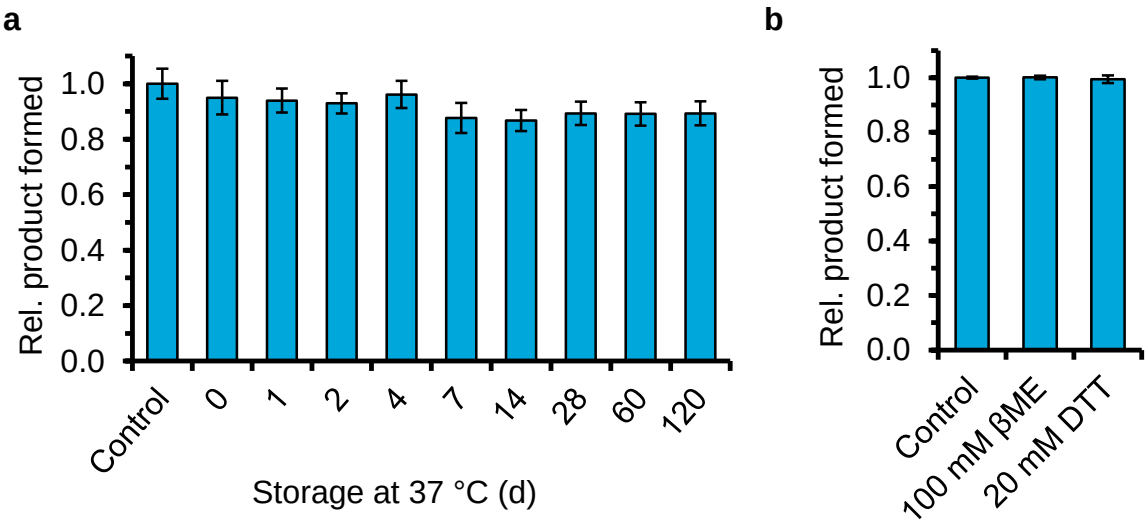

Figure S8. SnoopLigase reactivity was tolerant to lyophilization and reducing agent. (a) SnoopLigase was stable after lyophilization. SnoopLigase was lyophilized and stored for the indicated number of days at 37 °C. SnoopLigase was then reconstituted in reaction buffer with SnoopTagJr-AffiHER2 and SUMO-DogTag (10 µM each) for 2 h at 4 °C in TB pH 7.25 with 15% (v/v) glycerol. Product formation is shown relative to the non-lyophilized control. **(b)** SnoopLigase reaction was unaffected by reducing agent. AffiHER2-DogTag and SUMO-DogTag were conjugated using SnoopLigase (10 µM each) for 2 h at 4 °C with or without the reducing agent β–mercaptoethanol (βME) or dithiothreitol (DTT). Product formation is compared relative to the control without reducing agent. Analysis by SDS-PAGE with Coomassie staining. Results are mean of triplicate ± 1 SD.

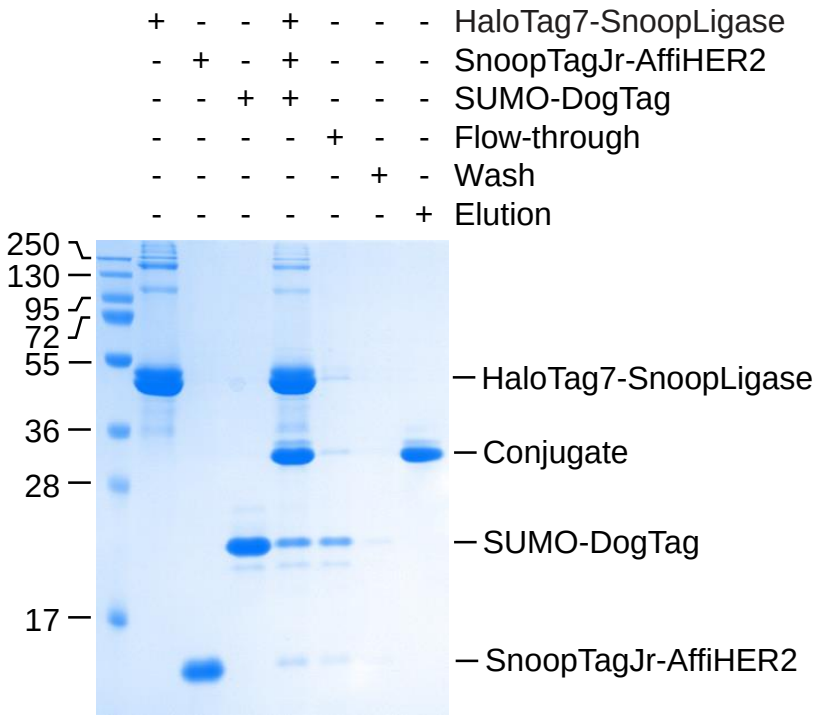

Figure S9. Purification of SnoopLigase reaction product by imidazole elution. SnoopTagJr-AffiHER2 and SUMO-DogTag were ligated using HaloTag7-SnoopLigase (15 µM each) for 24 h at 4 °C. SnoopLigase was captured with HaloLink resin, resin was washed, and the product was eluted with 2 M imidazole. Analysis was performed by SDS-PAGE with Coomassie staining.

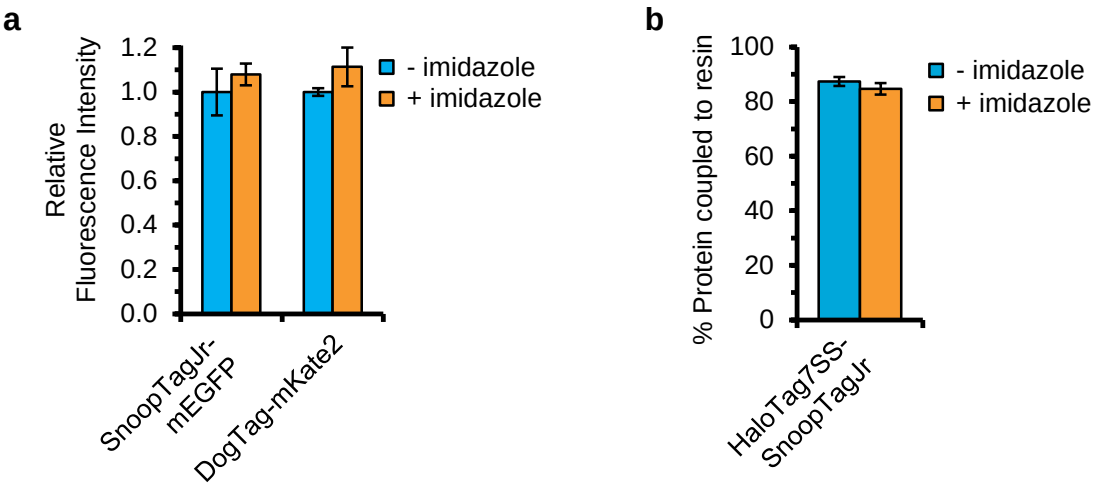

Figure S10. Imidazole exposure had minimal effect on protein activity. SnoopTagJr-mEGFP, DogTag-mKate2 or HaloTag7SS-SnoopTagJr (25 µM) were incubated in buffer with or without 2 M imidazole pH 7.0 for 10 min at 25 °C, followed by dialysis to remove the imidazole. **(a)** Imidazole exposure did not affect the brightness of fluorescent proteins. Fluorescence of dialyzed proteins with or without imidazole exposure was recorded at λ_{ex} = 485 nm and λ_{em} = 538 nm for SnoopTagJr-mEGFP. Fluorescence of dialyzed proteins was recorded at λ_{ex} = 544 nm and λ_{em} = 612 nm for DogTag-mKate2. Relative fluorescence is plotted, with the – imidazole value set to 1. **(b)** Imidazole exposure did not affect HaloTag's ligand binding. Dialyzed HaloTag7SS-SnoopTagJr, with or without imidazole exposure, was incubated with HaloLink resin for 5 min at 25 °C, prior to centrifugation and collection of the supernatant containing unbound protein. The HaloTag7SS-SnoopTagJr remaining in the supernatant was quantified by SDS-PAGE with Coomassie staining. Results are mean of triplicate ± 1 SD.

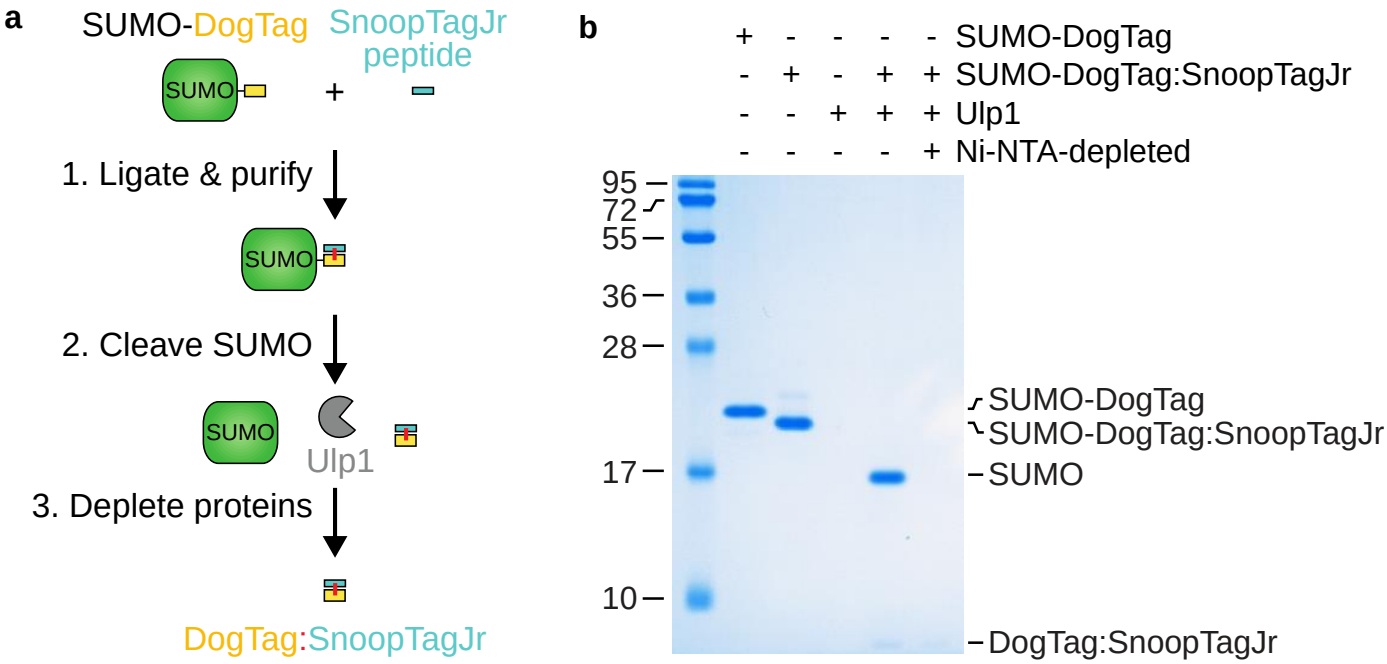

Figure S11. Generation of DogTag:SnoopTagJr competitor. (a) Cartoon of peptide competitor production. SUMO-DogTag and SnoopTagJr peptide are covalently conjugated using HaloTag7-SnoopLigase, followed by purification of the conjugate with imidazole elution. SUMO-protease Ulp1 (gray) cleaves the conjugated SUMO-DogTag:SnoopTagJr. Incubation with Ni-NTA resin depletes the His-tagged SUMO and Ulp1, yielding purified DogTag:SnoopTagJr peptide. **(b)** Production of competitor. HaloTag7-SnoopLigase was coupled to HaloLink resin. The resin was incubated with 50 µM SUMO-DogTag and 75 µM SnoopTagJr, followed by elution of the conjugate using imidazole. The purified conjugate was incubated with Ulp1, followed by Ni-NTA resin to deplete His-tagged proteins. Analysis was performed by SDS-PAGE with Coomassie staining. A faint band corresponding to DogTag:SnoopTagJr is seen, although peptide is not reliably observed by fixation and Coomassie staining and was more robustly quantified by its UV absorbance.

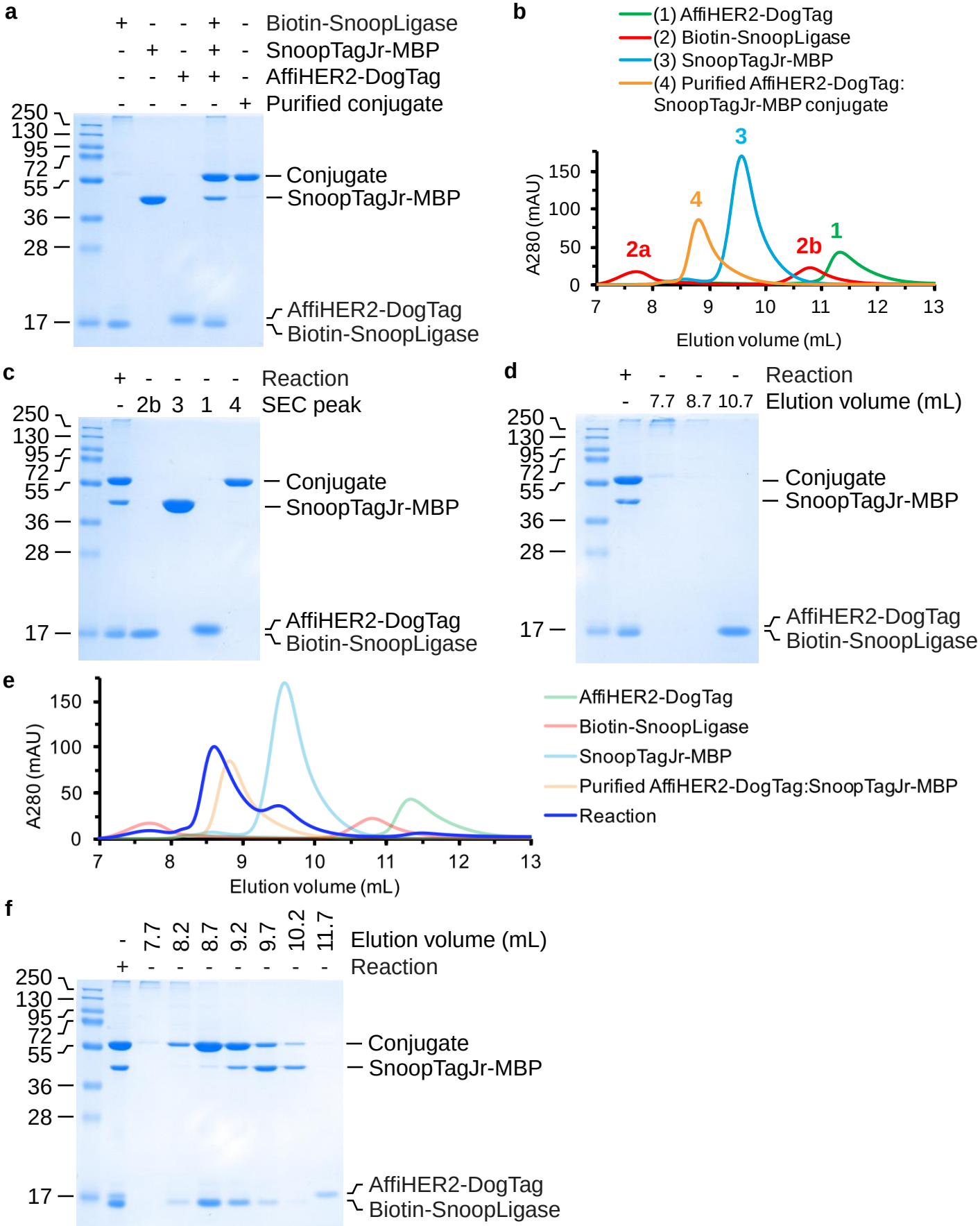

Figure S12. Size exclusion chromatography (SEC) of SnoopLigase reaction. **(a)** SnoopLigase reaction and conjugate purification. SnoopTagJr-MBP and AffiHER2-DogTag were reacted with biotin-SnoopLigase (each 50 μM) for 24 h at 4 °C. The conjugate was purified using imidazole elution. **(b)** SEC of individual proteins from (a). **(c)** SDS-PAGE of the conjugation reaction or SEC peaks from (b). **(d)** SDS-PAGE of the conjugation reaction or peaks from running biotin-SnoopLigase on SEC from (b). Peak 2a contains high molecular weight impurities, while peak 2b represents biotin-SnoopLigase. **(e)** SEC of the reaction sample from (a). Traces from (b) are shown in faint colors for comparison. **(f)** SDS-PAGE of fractions from SEC separation of the reaction in (e). Biotin-SnoopLigase was exclusively found in fractions containing AffiHER2-DogTag:SnoopTagJr-MBP, indicating interaction with the conjugate.

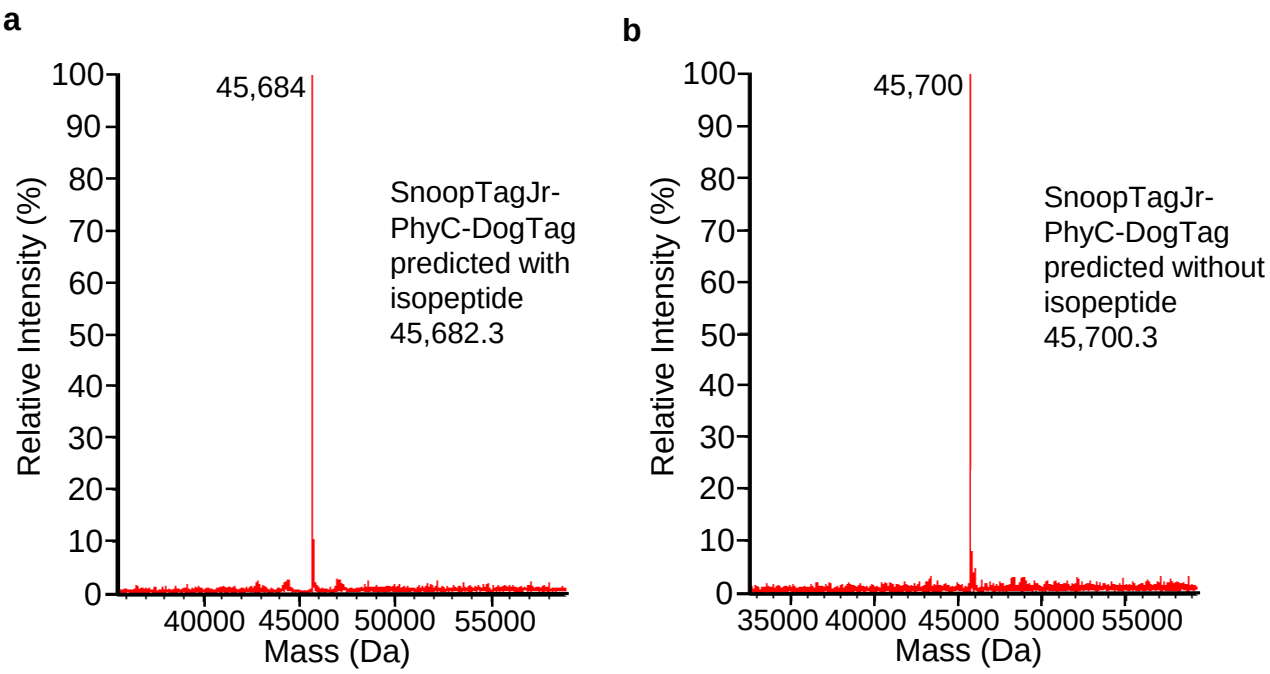

Figure S13. Mass spectrometry analysis of isopeptide bond formation of SnoopTagJr-PhyC-DogTag. Electrospray ionization mass spectrometry of **(a)** SnoopLigase-cyclized SnoopTagJr-PhyC-DogTag following SnoopLigase removal and **(b)** SnoopTagJr-PhyC-DogTag not exposed to SnoopLigase. The principal peak is marked and annotated with the mass predicted by the ProtParam online tool.

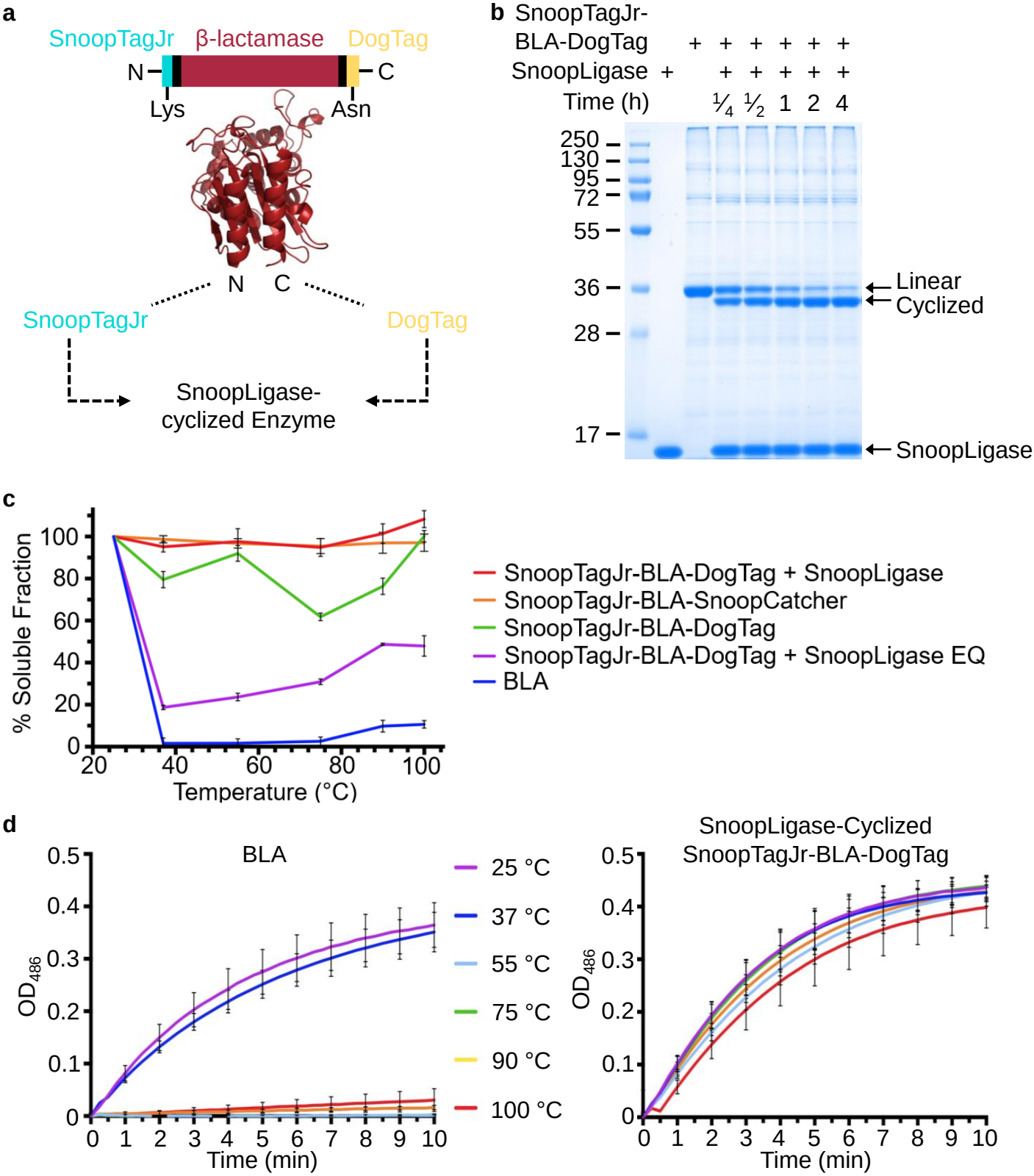

Figure S14. SnoopLigase cyclization enhancing BLA thermal resilience. **(a)** Schematic of SnoopLigase cyclization of BLA, based on PDB code 1BTL. **(b)** SnoopLigase efficiently cyclized BLA. SnoopLigase and SnoopTagJr-BLA-DogTag (10 μM each) were incubated at 4 °C for 0.25-4 h, before SDS-PAGE with Coomassie staining. **(c)** Effect of tags and inactive SnoopLigase on heat-induced aggregation of BLA. BLA or variants were incubated for 10 min at indicated temperatures. Samples were centrifuged to remove aggregates and soluble protein in the supernatant was quantified. **(d)** SnoopLigase cyclization improved the catalytic activity of BLA after heating. BLA or SnoopLigase-cyclized SnoopTagJr-BLA-DogTag were incubated for 10 min at the indicated temperatures and cooled to 25 °C. Then nitrocefin hydrolysis was monitored colorimetrically over time at 20 °C. All are mean of triplicate ± 1 SD; some error bars are too small to be visible.

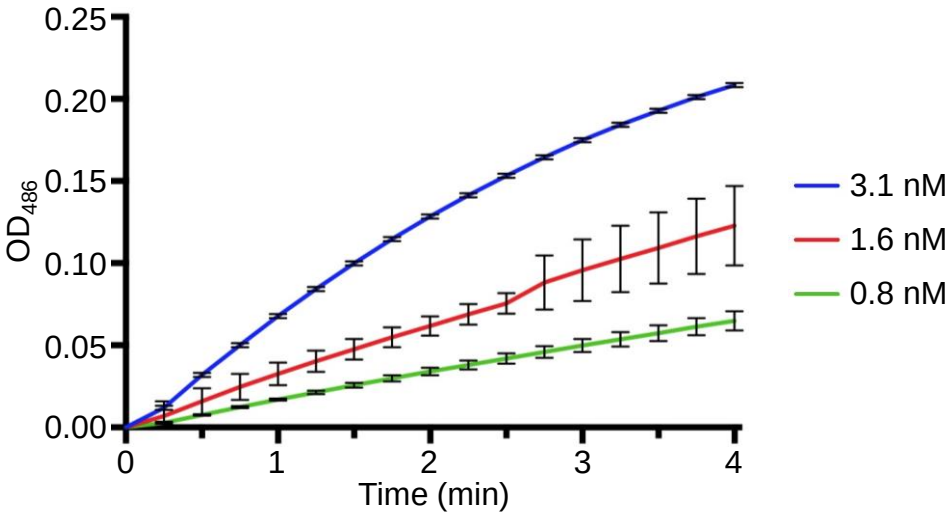

Figure S15. The BLA activity assay correlated to enzyme concentration. To validate that the amount of BLA enzyme was limiting in our activity assays, so that the assay reported on functional enzyme, wild type BLA at the indicated concentrations was reacted with 95 μM nitrocefin. Nitrocefin hydrolysis was monitored colorimetrically over time at 20 °C. Results are mean of triplicate ± 1 SD.

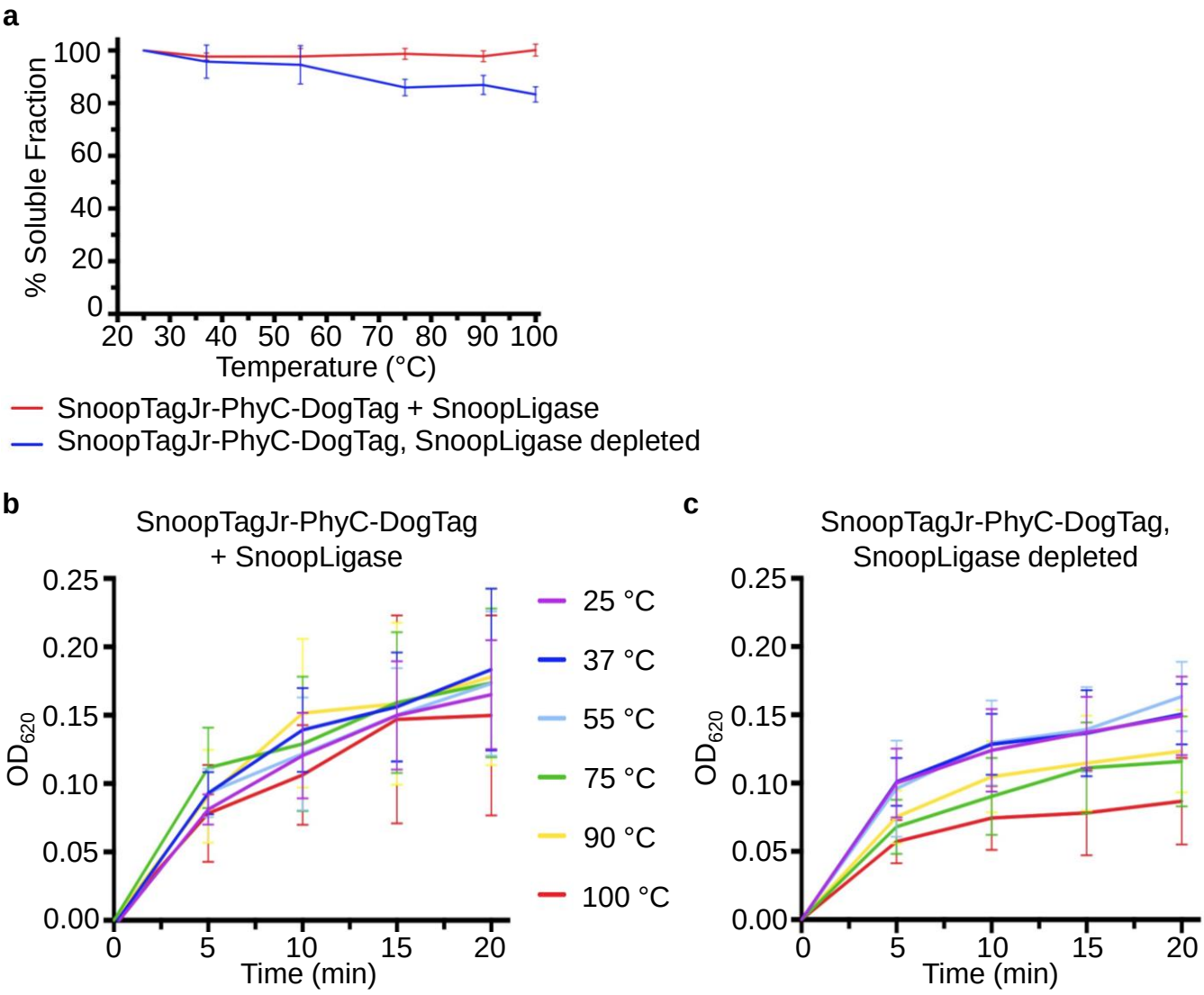

Figure S16. Effect of SnoopLigase removal on resilience of cyclized PhyC. (a) SnoopLigase-cyclized PhyC retained solubility following SnoopLigase removal. Cyclized PhyC before and after SnoopLigase removal was incubated for 10 min at the indicated temperature. Samples were centrifuged to remove aggregates and soluble protein in the supernatant was quantified. Results are mean ± 1 SD, n=6. **(b)** Cyclized PhyC without SnoopLigase removal was incubated at the indicated temperature for 10 min, cooled to 10 °C and used in colorimetric assays to quantify phosphate release from the hydrolysis of phytic acid. Results are mean ± 1 SD, n=9. **(d)** SnoopLigase removal compromised the thermal resilience of enzymatic activity of cyclized PhyC. Cyclized PhyC following SnoopLigase removal by imidazole was incubated at the indicated temperature for 10 min, cooled to 10 °C and used in colorimetric assays to quantify phosphate release from the hydrolysis of phytic acid. Results are mean ± 1 SD, n=9.

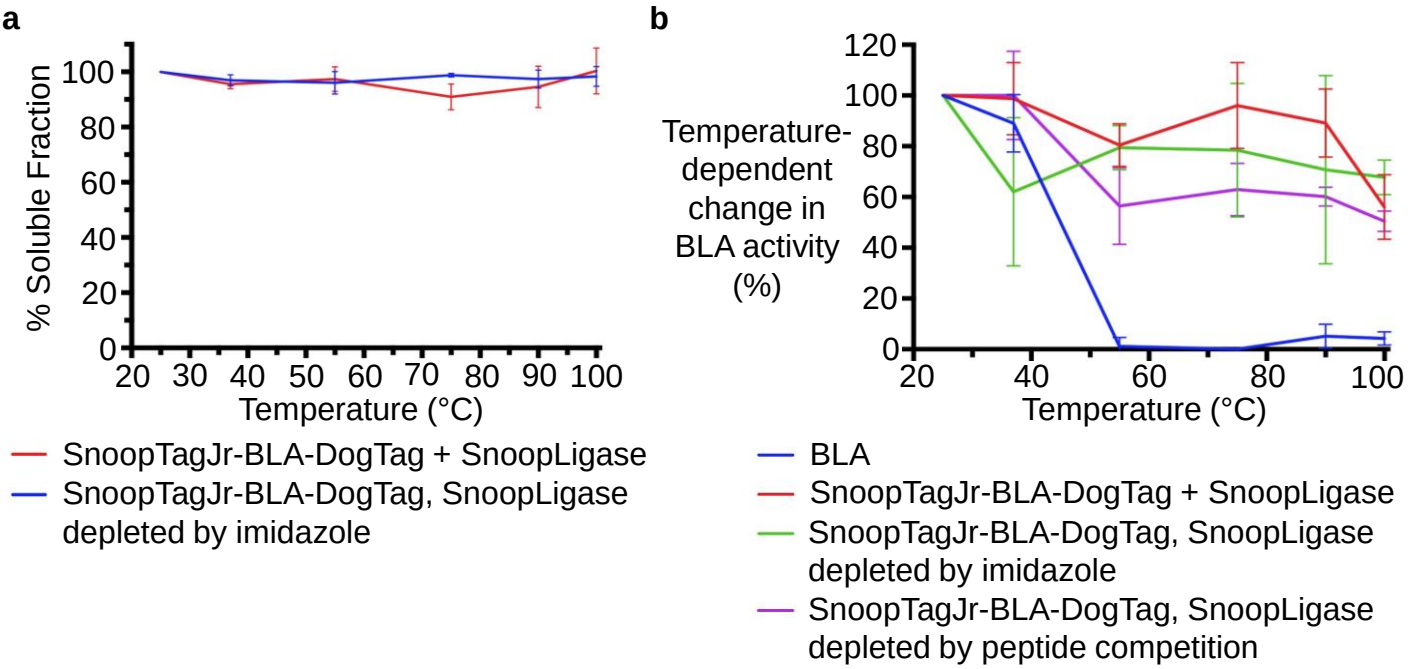

Figure S17. Effect of SnoopLigase removal on solubility and activity of cyclized BLA. (a) SnoopLigase-cyclized BLA remained soluble following SnoopLigase removal, even after boiling for 10 min. Cyclized BLA before and after SnoopLigase removal was incubated for 10 min at the indicated temperature. Samples were centrifuged to remove aggregates and soluble protein in the supernatant was quantified. Results are mean ± 1 SD, n=6. **(b)** BLA constructs were incubated at the indicated temperatures for 10 min, cooled and used in a colorimetric assay to determine the initial rate for cleavage of nitrocefin. Constructs incubated at 25 °C were defined as 100% active. Results are mean of triplicate ± 1 SD.