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## Supplemental information

## **DogCatcher allows loop-friendly**

## protein-protein ligation

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Figure S1: Structural comparison of the different Tag/Catcher pairs, related to Figure 1. (A) SpyTag/SpyCatcher has the Tag as a linear single  $\beta$ -strand (PDB: 2X5P and 4MLI). The isopeptide-forming residues of SpyTag (Asp117) and SpyCatcher (Lys31) are shown as spheres. (B) Model of SnoopTag/SnoopCatcher based on RrgA domain 4 (PDB: 2WW8). SnoopTag has the Tag derived from a linear single  $\beta$ -strand. The isopeptide-forming residues of SnoopTag (Lys742) and SnoopCatcher (Asn854) are shown as spheres. (C) Model of DogTag/DogCatcher based on RrgA domain 4 (PDB: 2WW8). DogTag/DogCatcher has the Tag derived as a  $\beta$ -hairpin. The side-chains for the isopeptide-forming residues of the DogTag (Asn854) and DogCatcher (Lys742) are shown as spheres. The dark blue sphere indicates the nitrogen atom in the isopeptide bond.

A R2Catcher DogCatcher	734 74   Klgdief: Klg <b>e</b> ief:	10 75 I <b>K</b> VNKNDKKP I <b>K</b> V <b>D</b> K <b>T</b> DKKP	50 7 Irgavfslgk Lrgavfslgk	60 7 QHPDYPDIYG QHPDYPDIYG	70 GAIDQNGTYG GAIDQNGTYG	780 J NVRTGEDGK DVRTGEDGK
R2Catcher DogCatcher Reactive Lysir	790   LTFKNLSE LTF <b>T</b> NLSE	800   OGKYRLF <b>E</b> NSE OGKYRL <b>IE</b> NSE	810 I Epagykpvqne Ep <mark>p</mark> gykpvqni	820 I KPIVAFQIVN KPIV <b>SFR</b> IV <b>D</b>	830 I GEVRDVTSI GEVRDVTSI	VPQ- VPQ-

Catalytic Glutamate Mutations to create DogCatcher

		840	850	860
В	R2Tag	I DIPAGYEFI	I NDKHYIT <b>n</b> ei	PIPPK
	DogTag	DIPATYEFI	T <b>DG</b> KHYIT <b>N</b> EI	PIPPK

Reactive Asparagine Mutations to create DogTag

С	Domain 4 SnoopCatcher SnoopLigase DogCatcher	734 74 KLGDIEFI KLGEIEFI	0 7 I KVNKNDKKP NDKKP VNKNDKKP	50 LRGAVFSL LRGAVFSL LRGAVFSL	760 I QKQHPDYPD: QKQHPDYPD: QKQHPDYPD: QKQHPDYPD:	770 IYGAIDQNG IYGAIDQNG IYGAIDQNG IYGAIDQNG	780 I TYQNVRTGED TYQNVRTGED TYQNVRTGED TYQDVRTGED	GK GK GK GK
	Domain 4 SnoopCatcher SnoopLigase DogCatcher	790 I LTFKNLSD LTFKNLSD LTFKNLSD LTFTNLSD	800 I GKYRLFENS GKYRLFENS GKYRLFENS GKYRLIENS	810 I EPAGYKPV EPAGYKPV EPPGYKPV	820 J QNKPIVAFQ QNKPIVAFQ QNKPIVAFQ QNKPIVSFR	83 I IVNGEVRDV IVNGEVRDV IVNGEVRDV IVDGEVRDV	0 841 TSIVPQDIPA TSIVPQDIPA TSIVPPGVPA TSIVPQ-	0 GY TY TY
	Domain4 SnoopCatcher SnoopLigase DogCatcher	85 I EFTNDKHY EFTNGKHY EFT-	0 8 I IT <b>N</b> EPIPPK IT <b>N</b> EPIPPK	60  :-				

Reactive Lysine Catalytic Glutamate Reactive Asparagine

**Figure S2: Amino acid sequences of the key protein variants, related to Figure 1. (A)** Sequence alignment of R2Catcher with DogCatcher. **(B)** Sequence alignment of R2Tag with DogTag. **(C)** Sequence alignments of the ways that RrgA domain 4 has been split to create different protein coupling reagents. Numbering is based on PDB 2WW8.



Figure S3: Comparison of solubility and reaction rate, related to Figures 1 and 5. (A) Yield of soluble protein for Catcher variants per liter of *E. coli* culture, determined after Ni-NTA elution (blue) or after dialysis (orange) (mean of duplicate with error bars signifying variation between measurements). (B) Time-course of reaction for DogTag/DogCatcher or R2Tag/R2Catcher. 5  $\mu$ M AviTag-DogTag-MBP and 5  $\mu$ M DogCatcher or 5  $\mu$ M AviTag-R2Tag-MBP and 5  $\mu$ M R2Catcher were incubated in PBS pH 7.5 at 25 °C, with quantification by SDS-PAGE/Coomassie (mean ± 1 s.d., n=3). Some error bars are too small to be visible. The resultant second-order rate constant is marked (mean ± 1 s.d., n=3). (C) Zoom of the y-axis from (B), to make the data clearer for R2Tag/R2Catcher.



Figure S4: Electrospray ionization mass spectrometry validation of DogCatcher and reaction with DogTag, related to Figure 1. (A) SDS-PAGE with Coomassie staining, illustrating reaction of DogCatcher with DogTag peptide to approximately 50%, so that preand post-reacted DogCatcher can be compared. (B) Electrospray ionization mass spectrometry of DogCatcher and the reaction product of DogCatcher with DogTag peptide. Expected mass for DogTag peptide + DogCatcher = 17,524.5 Da minus 17 Da (NH<sub>3</sub>) released upon isopeptide bond formation) = 17,507.5 Da.

Α



**Figure S5: DogTag/DogCatcher reacted efficiently at nanomolar concentrations, related to Figure 3.** Time-course of reaction for 100 nM HaloTag7SS-DogTag with 100 nM DogCatcher-sfGFP in PBS pH 7.5 + 0.2% (w/v) BSA at 25 °C, with quantification by SDS-PAGE/Coomassie and densitometry.



## Figure S6: DogTag/DogCatcher orthogonality, as described in STAR Methods

(Isopeptide Bond Formation Assays). (A) DogTag reacted with DogCatcher but not SnoopCatcher or SpyCatcher003. 15  $\mu$ M DogCatcher, Affi-SnoopCatcher or SpyCatcher003 was incubated with 10  $\mu$ M HaloTag7SS-DogTag for 24 h in PBS pH 7.5 at 25 °C, before SDS-PAGE with Coomassie staining. (B) DogCatcher reacted with DogTag and SnoopCatcher. 15  $\mu$ M DogCatcher was incubated with 10  $\mu$ M HaloTag7SS-DogTag, SpyTag003-MBP, SnoopTagJr-Affi, Affi-SnoopCatcher or SpyCatcher003 for 24 h in PBS pH 7.5 at 25 °C, before SDS-PAGE with Coomassie staining. M = molecular weight markers.

Gre2p Variant	Specific activity (µmol <sub>NAPDH</sub> .min <sup>-1</sup> .µmol <sub>protein</sub> <sup>-1</sup> )
WT	1,892 ± 294
SpyTag003 Loop A	2,391 ± 347
SpyTag003 Loop B	1,572 ± 372
SpyTag003 Loop C	1,814 ± 83
DogTag Loop A	3,087 ± 259
DogTag Loop B	3,268 ± 361
DogTag Loop C	1,484 ± 223

**Table S1:** Specific enzyme activities for Gre2p variants, related to Figure 6. Each Gre2p variant was incubated with isovaleraldehyde and NADPH in phosphate buffer at 25 °C and reaction was monitored spectrophotometrically (mean ± 1 s.d., n=3 biological replicates).