

## Supplementary Information

Genetically encoded FRET sensors containing a fluorescent unnatural amino acid  
as a FRET donor

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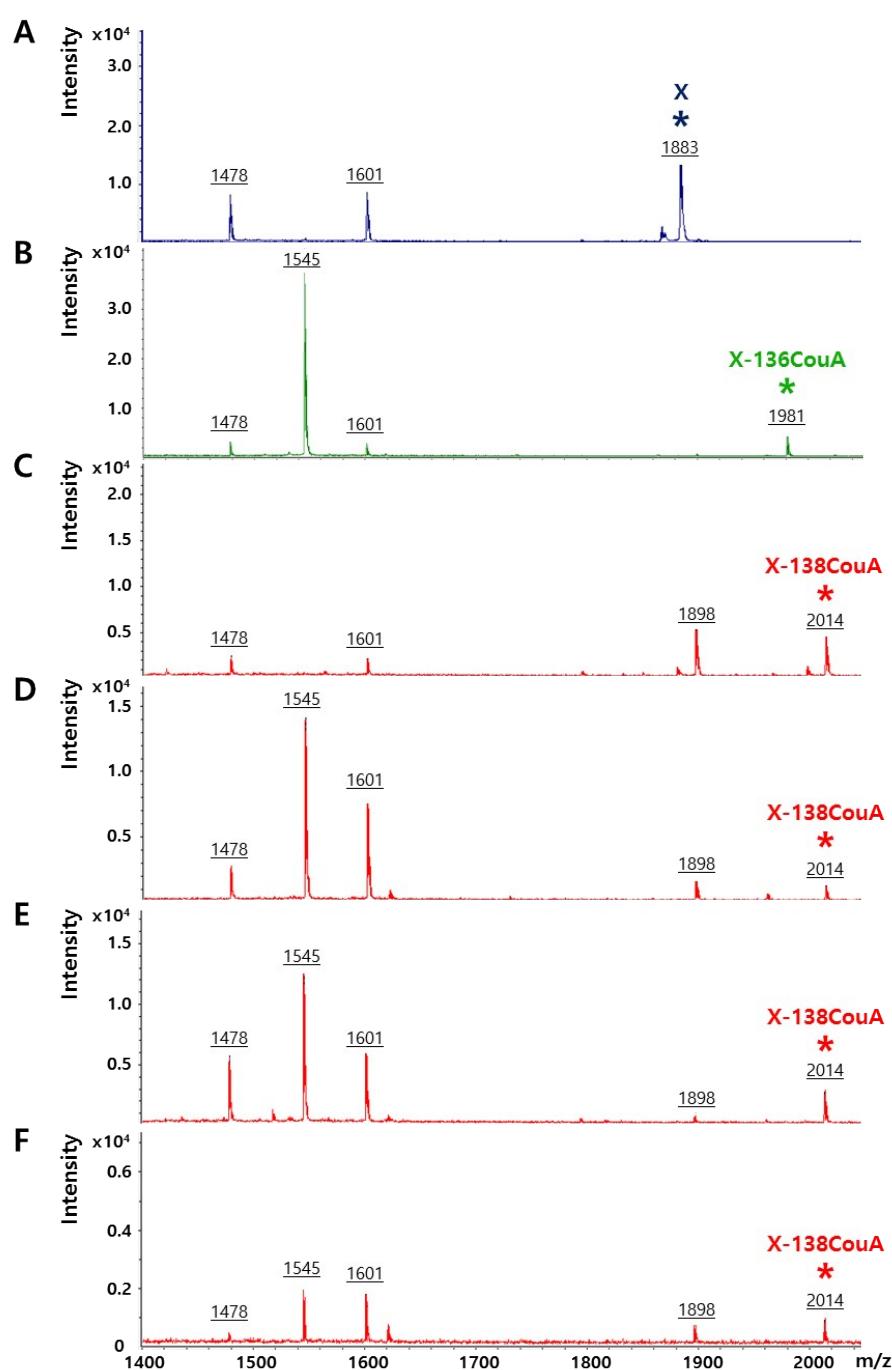
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**Table S1.** Measurements of the distance of V104 and L155 from Leu5 in GlnBP, and calculation of % changes in the distances upon ligand binding. The distances between the  $\alpha$ -carbons were measured using *PyMOL*. Five residues (S116, G117, F136, P137, and N138) showing the largest distance change are boldfaced. PDB IDs used for the measurements are 1GGG (*apo*-form) and 1WDN (ligand-bound form).

Residue	Distance from Leu5 in the ligand-free form (Å)	Distance from Leu5 in the ligand-bound form (Å)	% change of the distance (%)
V104	48.03	40.21	16.28
K105	48.81	43.34	11.21
D106	51.00	42.18	17.29
L107	54.82	42.92	21.71
D108	55.62	45.87	17.53
G109	55.08	45.50	17.39
K110	51.27	43.23	15.68
V111	49.26	40.61	17.56
V112	45.50	37.20	18.24
A113	43.64	34.18	21.68
V114	40.52	30.66	24.33
K115	38.40	27.12	29.38
<b>S116</b>	<b>39.50</b>	<b>26.15</b>	<b>33.80</b>
<b>G117</b>	<b>37.20</b>	<b>23.82</b>	<b>35.97</b>
T118	34.97	24.33	30.43
G119	35.10	26.40	24.79
S120	37.29	28.99	22.26
V121	40.21	30.08	25.19
D122	40.20	30.17	24.95
Y123	39.96	32.28	19.22
A124	42.83	34.58	19.26
K125	45.08	35.25	21.81
A126	44.96	36.07	19.77
N127	44.78	38.02	15.10
I128	46.82	39.67	15.27
K129	50.43	42.30	16.12
T130	49.96	41.18	17.57
K131	53.20	43.39	18.44
D132	50.95	40.31	20.88
L133	47.43	36.55	22.94
R134	46.61	34.54	25.90
Q135	43.85	30.82	29.71
<b>F136</b>	<b>42.37</b>	<b>28.82</b>	<b>31.98</b>
<b>P137</b>	<b>40.38</b>	<b>25.23</b>	<b>37.52</b>

<b>N138</b>	<b>37.90</b>	<b>25.01</b>	<b>34.01</b>
I139	37.38	27.26	27.07
D140	38.33	28.86	24.71
N141	41.72	30.50	26.89
A142	41.65	31.90	23.41
Y143	41.00	33.21	19.00
M144	44.06	34.95	20.68
E145	46.03	36.48	20.75
L146	45.32	37.95	16.26
G147	45.84	39.08	14.75
T148	49.53	40.87	17.48
N149	51.03	42.56	16.60
R150	50.77	41.03	19.18
A151	47.78	39.7	16.91
D152	48.01	41.29	14.00
A153	44.33	38.26	13.69
V154	41.16	34.52	16.13
L155	38.72	32.8	15.29

**Figure S1.** MALDI-TOF MS analyses of trypsin-digested EGFP-GlnBP-WT and EGFP-GlnBP mutant proteins containing CouA. Peptide X (QFPNIDNAYMELGTNR) contains F136 and N138; each residue is substituted by CouA in Peptide X-136CouA or X-138CouA. MALDI TOF MS data for the peptide products of (a) EGFP-GlnBP-WT, (b) EGFP GlnBP F136CouA, (c) EGFP-GlnBP-N138CouA, (d) EGFP-4-GlnBP-N138CouA, (e) EGFP-7-GlnBP-N138CouA, and (f) EGFP-10-GlnBP-N138CouA.



**Figure S2.** SDS-PAGE analyses of purified sensor proteins. EGFP-GlnBP mutant proteins with linkers of different length were expressed in the presence of 1 mM CouA and the required evolved tRNA/aaRS pair, and then purified by Ni-NTA affinity chromatography. The purified proteins were analyzed by SDS-PAGE, and visualized by Coomassie-staining (left) and fluorescence (right).

