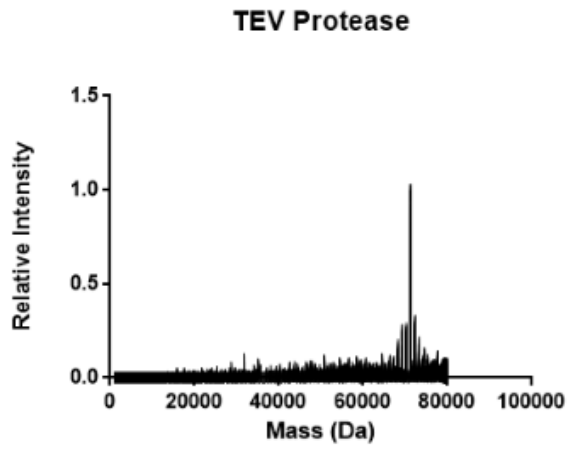


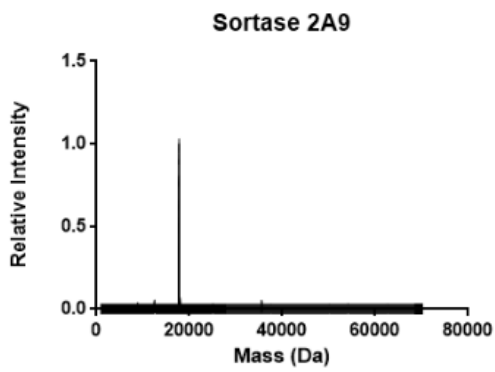
Supplemental Information:

Figure S1. Protease verification:

a.



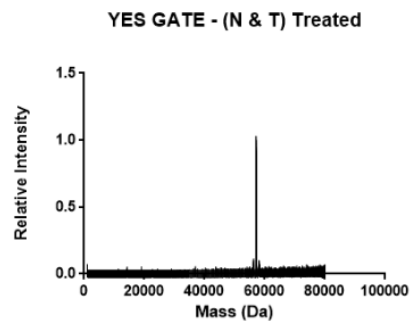
b.



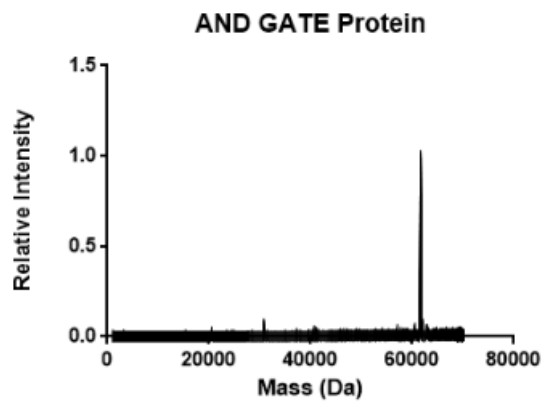
Mass spectrometry analysis confirming the molecular weights of Sortase A29 (a) and TEV protease (b).

Figure S2 Protein verification:

a.



b.

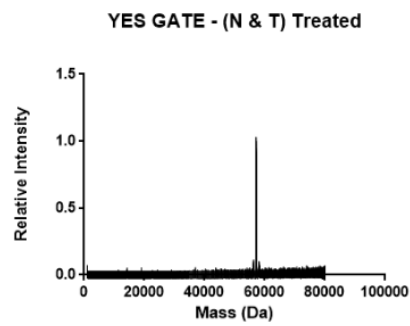


Mass spectrometry analysis confirming the molecular weights of YES-Gate (a) and AND-Gate protein (b).

S3. Protease Treated FRET Analysis

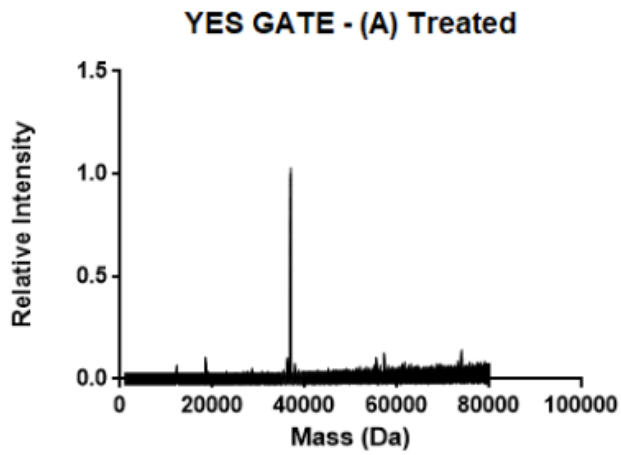
YES-GATE Protease conditions:

a.



YES-GATE construct of the non-enzyme exposed group (N) and the TEV exposed-only group (T). They both exhibited the same molecular weight.

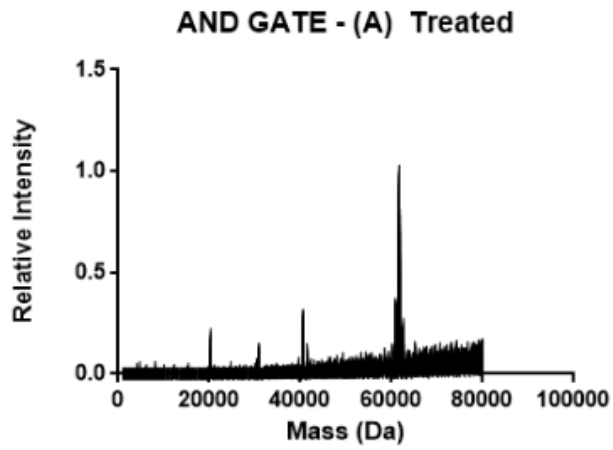
b.



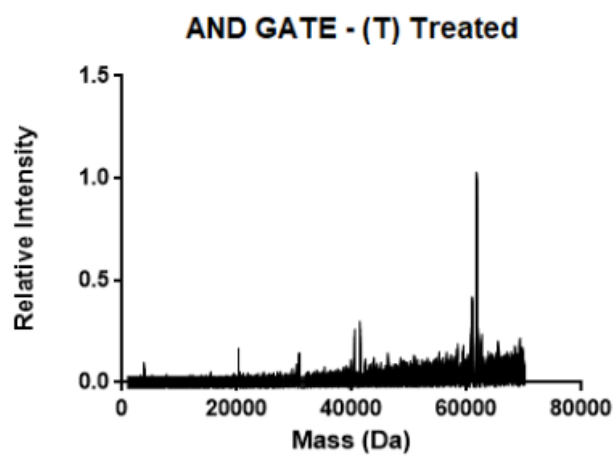
YES-GATE construct of the 2A9-only condition. The 2A9 with TEV condition (not shown) yielded highly similar spectra, indicating comparable cleavage properties.

AND-GATE Protease conditions:

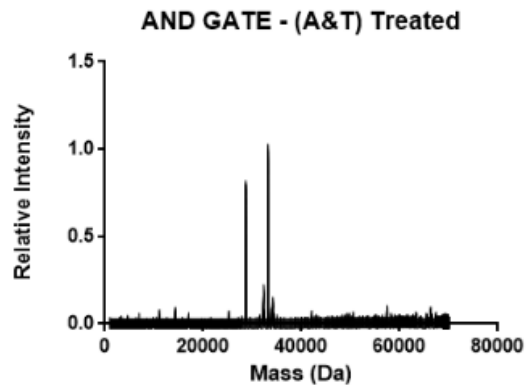
c.



d.



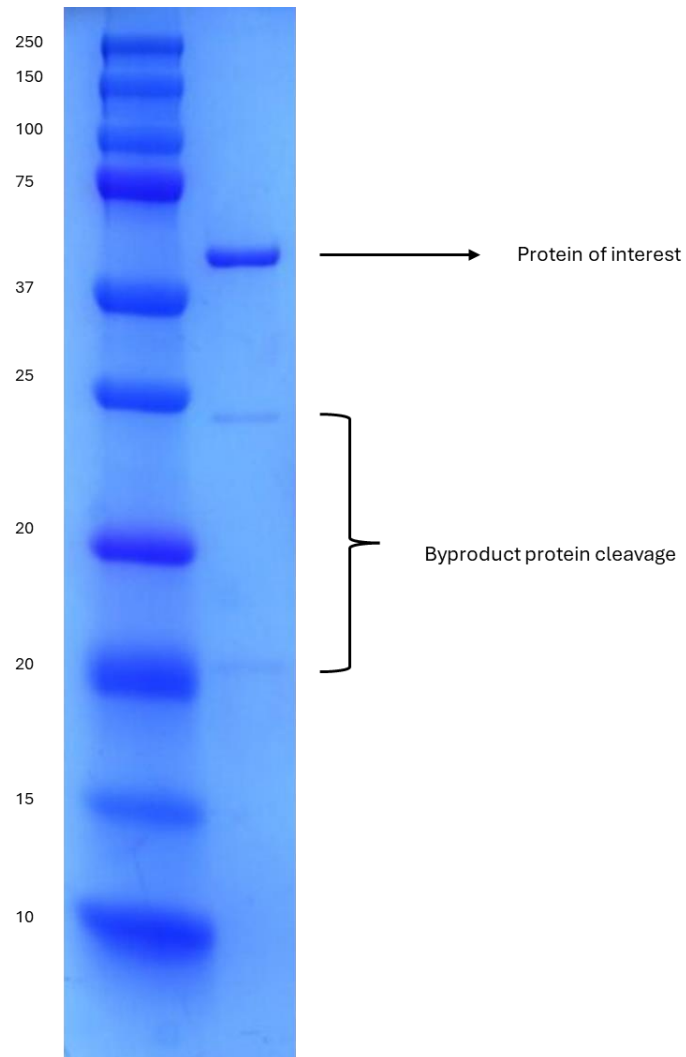
e.



AND-gate construct analyzed under different protease treatment conditions (c-e).

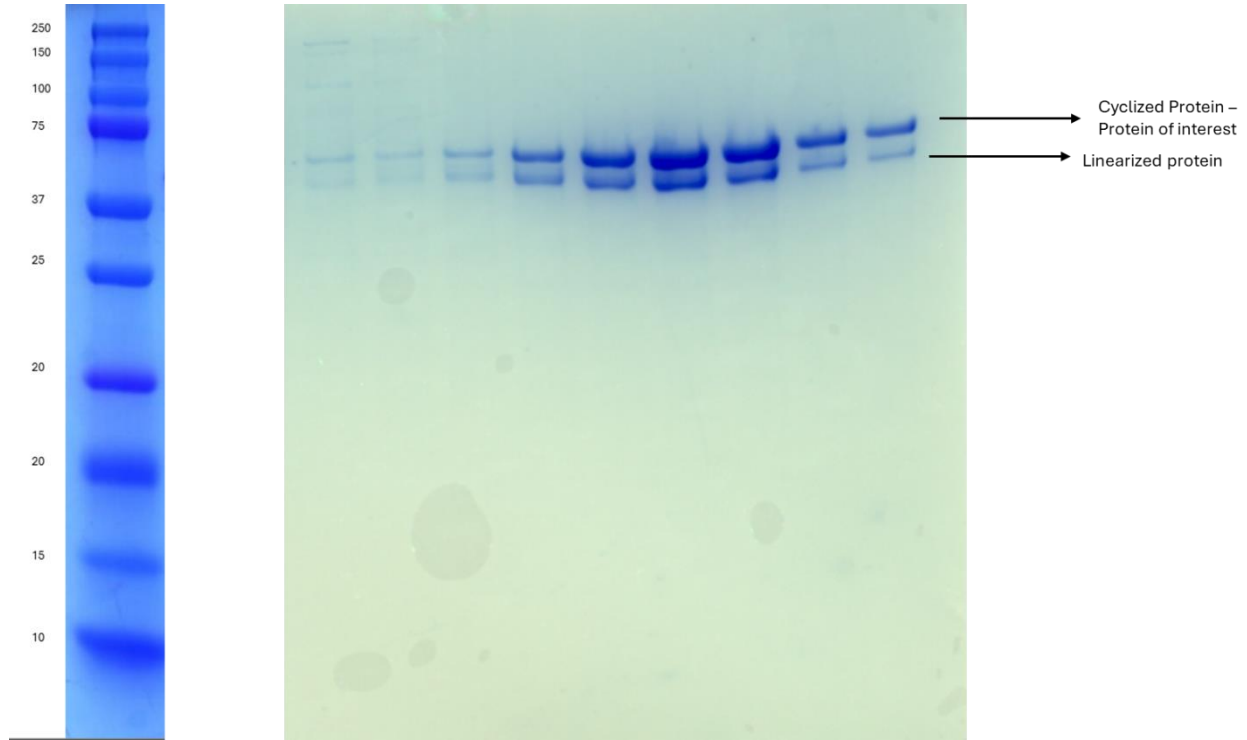
S4. SDS-PAGE analysis

a. YES-GATE Protein:



Protein Purification of the YES-GATE (A) construct. Cleavage byproducts were observed, suggesting detachment due to conformational changes during protein folding and maturation.

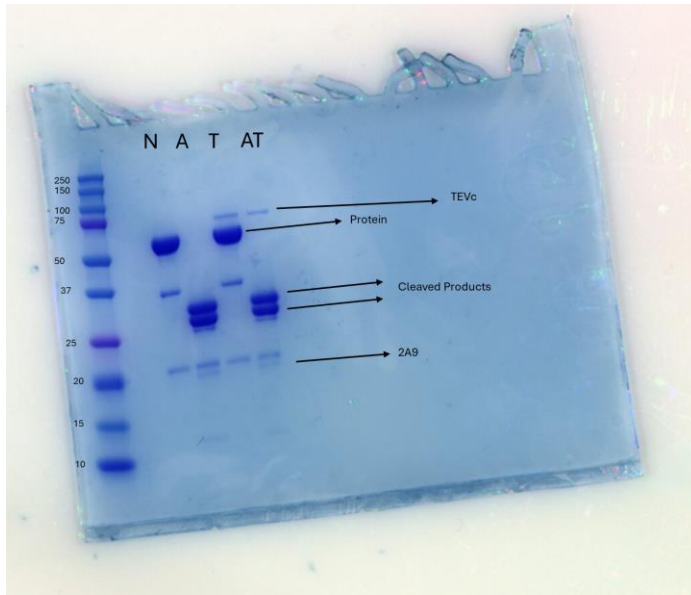
b. AND-GATE Protein:



SEC purification of the AND-GATE (2A9 & TEVc) construct. The desired cyclic protein generated via SICLOPPS was observed. A linear byproduct was also observed, likely resulting from N- to C- terminal cleavages rather than intein mediated trans-splicing.

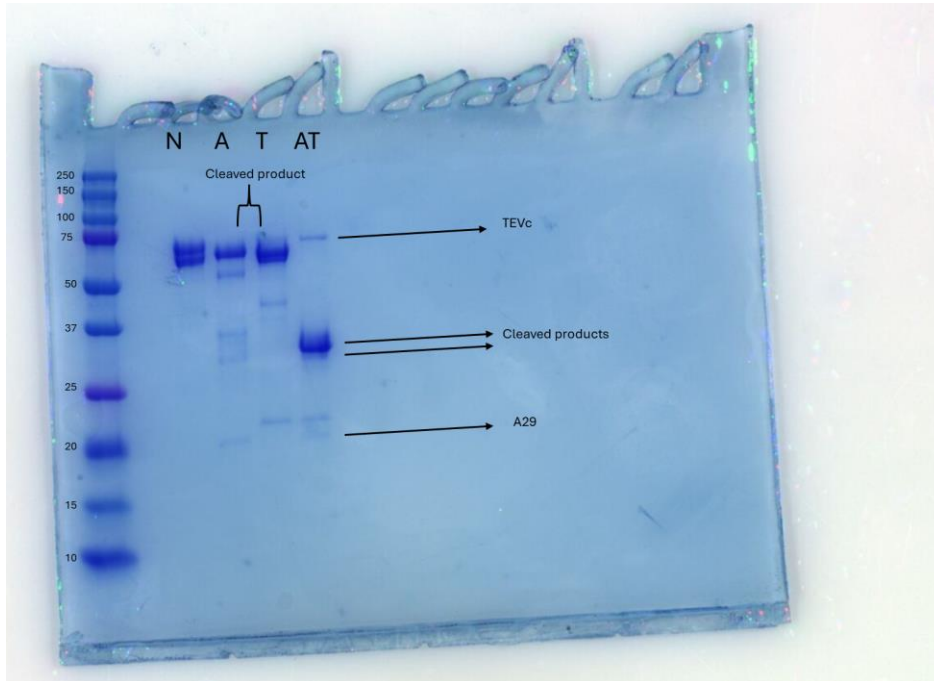
S5. Protease Treatment

a. YES-GATE:



Protease treatment for the YES-GATE (2A9) construct. Lane N represents the untreated control with no protease exposure. Lanes A, T, A&T were treated with Sortase 2A9, TEVc protease, and the combination of both enzymes, respectively. A selective response to Sortase 2A9 was observed in lanes A and A&T. Additional protein fragments were detected, likely resulting from detachment due to conformational changes during protein folding and maturation.

b. AND-GATE Protease Treatment:



Protease treatment for the AND-GATE (2A9 & TEVc) construct. Lane N represents the untreated control with no protease exposure. Lanes A, T, A&T were treated with Sortase 2A9, TEVc protease, and the combination of both enzymes, respectively. Distinct cleavage events were observed in each of the lanes. Additional protein fragments were detected, likely due to conformational changes during protein folding and maturation and/or sample impurities.

Tables:

S6. FRET Statistical Analysis

a. YES-GATE

A - YES GATE Relative FRET				
Protease Treatments	N	A	T	AT
Trial 1	1.015420366	0.6887111385	1.021628859	0.6777667065
Trial 2	0.9947784688	0.6827879825	1.003941071	0.6665604488
Trial 3	0.9898011654	0.6712490661	0.9875830228	0.6707863044
Average FRET	1	0.6809160624	1.004384318	0.6717044865

1way ANOVA Multiple comparisons						
1	Number of families	1				
2	Number of comparisons per family	6				
3	Alpha	0.05				
4						
5	Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
6						
7	N vs. A	0.3191	0.2875 to 0.3507	Yes	****	< 0.0001
8	N vs. T	-0.004384	-0.03601 to 0.02725	No	ns	0.9690
9	N vs. AT	0.3283	0.2967 to 0.3599	Yes	****	< 0.0001
10	A vs. T	-0.3235	-0.3551 to -0.2918	Yes	****	< 0.0001
11	A vs. AT	0.009212	-0.02242 to 0.04084	No	ns	0.7890
12	T vs. AT	0.3327	0.3010 to 0.3643	Yes	****	< 0.0001

FRET analysis of the YES-gate construct showing three technical replicates of EGFP (donor) to mScarlet3 (acceptor) fluorescence ratios, with average FRET values calculated. One-way ANOVA was used to determine statistical significance between treatment groups.

b. AND-GATE

A&T - AND GATE Relative FRET				
Protease Treatments	N	A	T	AT
Trial 1	1.00238776	0.7802060042	0.9312655897	0.6407142012
Trial 2	0.9980900535	0.7683296089	0.9090327872	0.6478828915
Trial 3	0.9995221861	0.7817876296	0.9142510815	0.6472562011
Average FRET	1	0.7767744142	0.9181831528	0.6452844313

1	Number of families	1				
2	Number of comparisons per family	6				
3	Alpha	0.05				
4						
5	Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
6						
7	N vs. A	0.2232	0.2043 to 0.2422	Yes	****	< 0.0001
8	N vs. T	0.08182	0.06288 to 0.1008	Yes	****	< 0.0001
9	N vs. AT	0.3547	0.3358 to 0.3737	Yes	****	< 0.0001
10	A vs. T	-0.1414	-0.1603 to -0.1225	Yes	****	< 0.0001
11	A vs. AT	0.1315	0.1126 to 0.1504	Yes	****	< 0.0001
12	T vs. AT	0.2729	0.2540 to 0.2918	Yes	****	< 0.0001

FRET analysis of the AND-gate construct showing three technical replicates of EGFP (donor) to mScarlet3 (acceptor) fluorescence ratios, with average FRET values calculated. One-way ANOVA was used to determine statistical significance between treatment groups.