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Supplementary Materials for

Cln5 represents a new type of cysteine-based *S*-depalmitoylase linked to neurodegeneration

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Figs. S1 to S4
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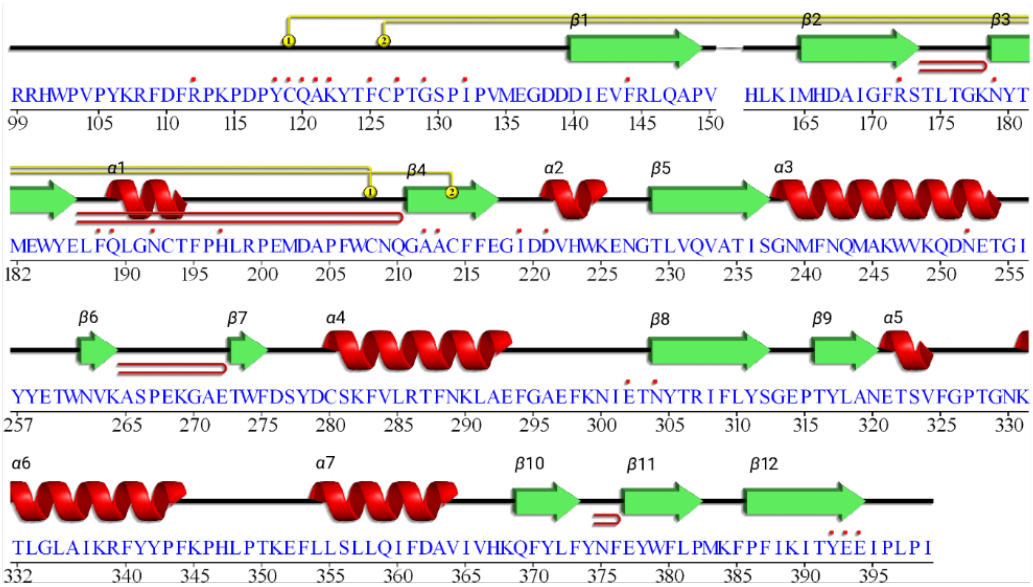
A


β1
β2
β3
RRHWPVPYKRFDFRPKPDPYCQAKYTFCPTGSPIPVMEGDDDIEVFRLQAPV
HLKIMHDAIGFRSTLTGKNYT
99 105 110 115 120 125 130 135 140 145 150 165 170 175 180
α1
β4
α2
β5
α3
MEWYELFQLGNCTFPHLRPEMDAPFWCNQGAACFFEGIDDVHMKENGTLVQVATISGNMFNQMAKWVKQDNETGI
182 190 195 200 205 210 215 220 225 230 235 240 245 250 255
β6
β7
α4
β8
β9
α5
YYETWNVKASPEKGAETWFDSYDCSKFVLRTFNKLAEFGAEFKNIETNYTRIFLYSGEPTYLANETSVFGPTGNK
257 265 270 275 280 285 290 295 300 305 310 315 320 325 330
α6
α7
β10
β11
β12
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B

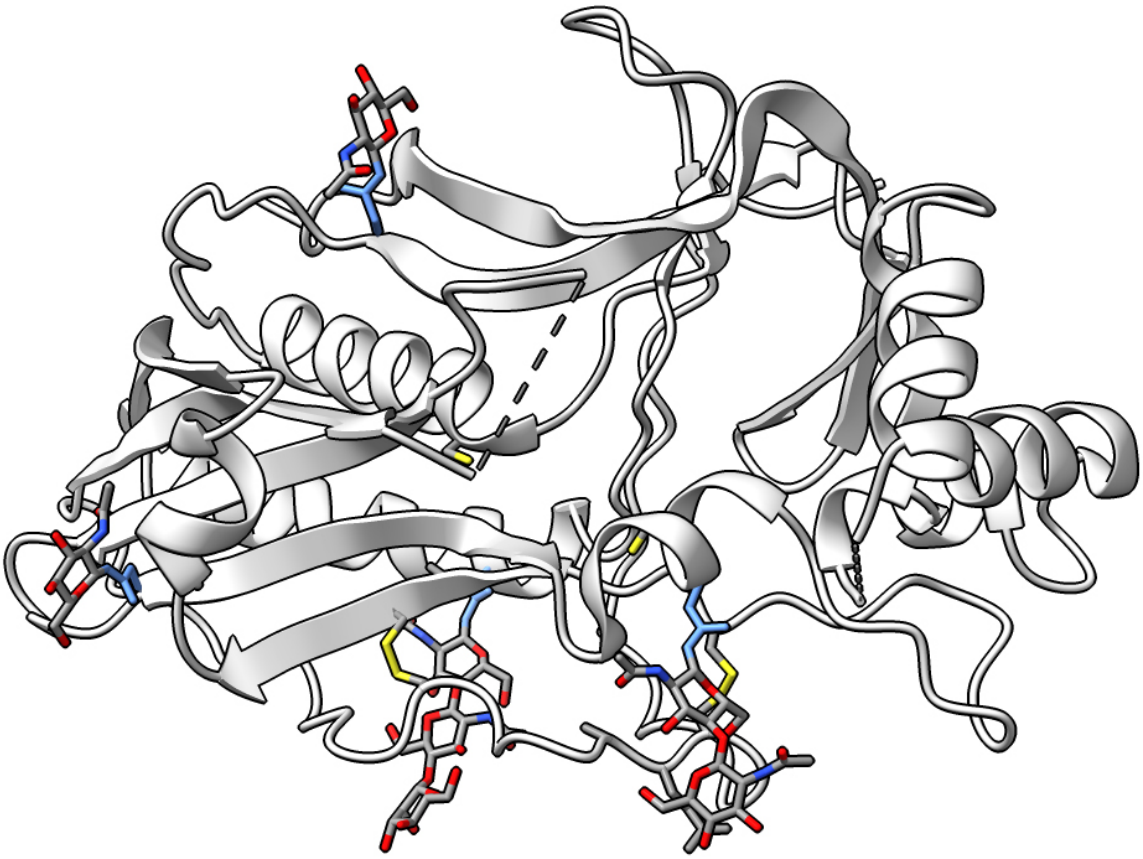

Fig. S1. Structural features and sequence alignment of Cln5
(A) The sequence of Cln5 annotated with secondary structure elements, α-helices are shown in red and β-strands are displayed as green arrows. The numbering of the secondary structure elements is consecutive from the N-terminus, β-hairpins are illustrated with red lines, disulfide bridges are marked in yellow and amino acids with contact to ligands/sugar residues are tagged with a red dot. **(B)** Ribbon model of Cln5 displaying the disulfide bridges (yellow) as well as the glycosylation sites (blue).

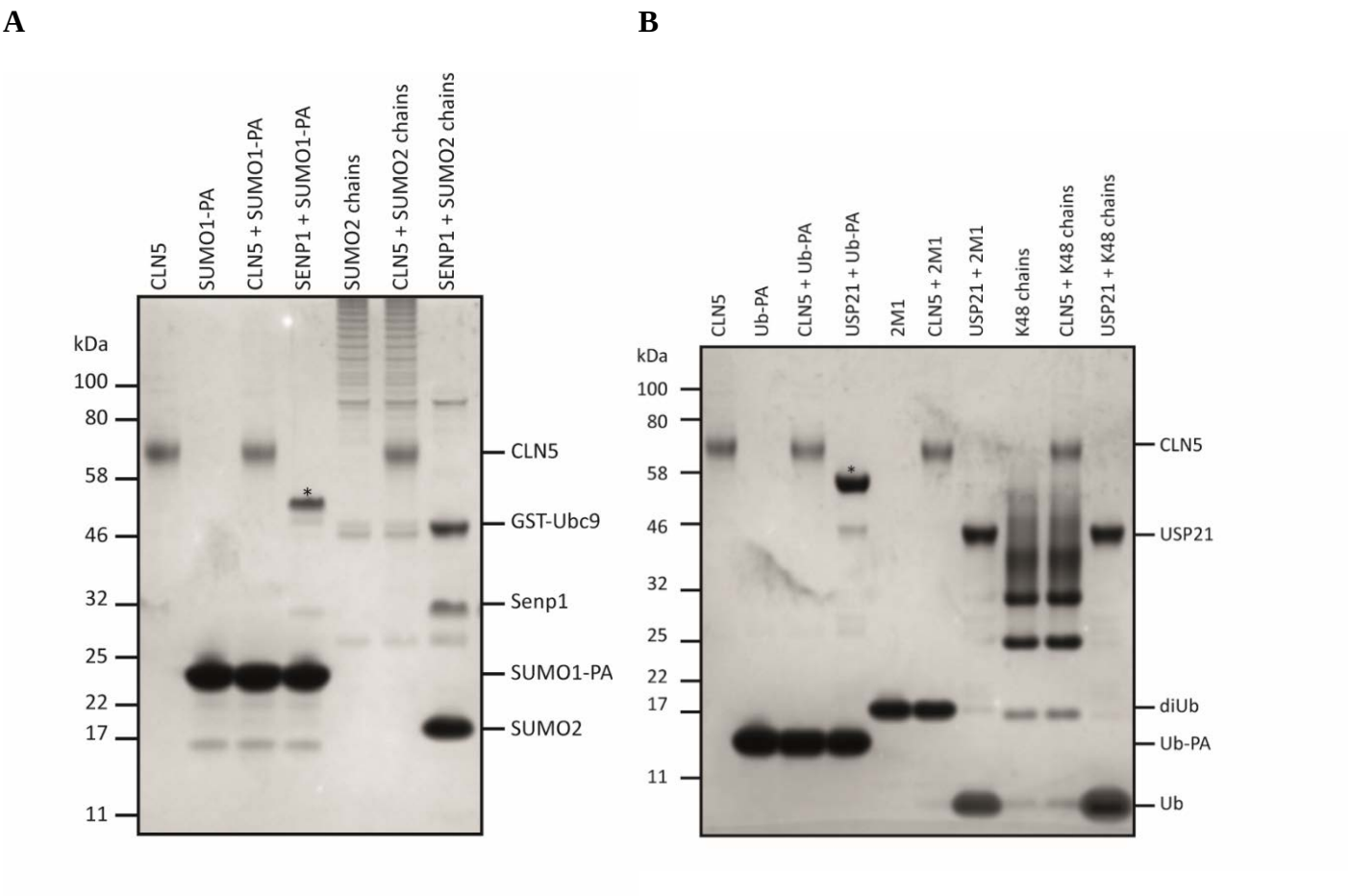

Fig. S2. Probing Cln5 for deubiquitinating and deSUMOylating activity
(A) Cln5 shows no activity against SUMO1-PA or SUMO2 chains at pH 7.5. SENP1 was used as a positive control. The asterisk (*) marks the shifted band after reaction with the activity-based probe. **(B)** Cln5 does not react with Ub-PA or ubiquitin chains 2M1 and K48 at pH 7.5. USP21 was used as a positive control. The asterisk (*) marks the shifted band after reaction with the activity-based probe.

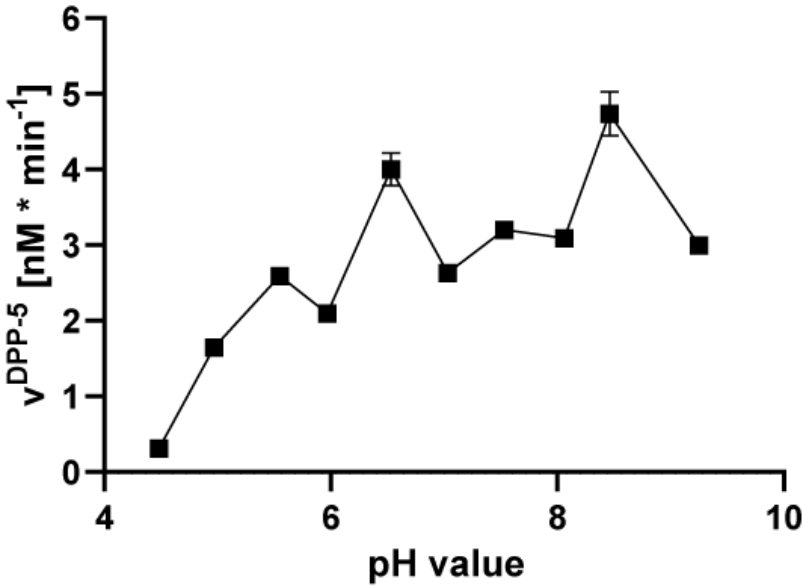

Fig. S3. pH dependent Cln5 thioesterase activity. 0.25 µg of Cln5 protein was assayed with 10 µM DPP-5 substrate at different pH values ranging from pH 4.5 to pH 9. Cln5 showed its highest activity at pH 6.5 and 8.5.

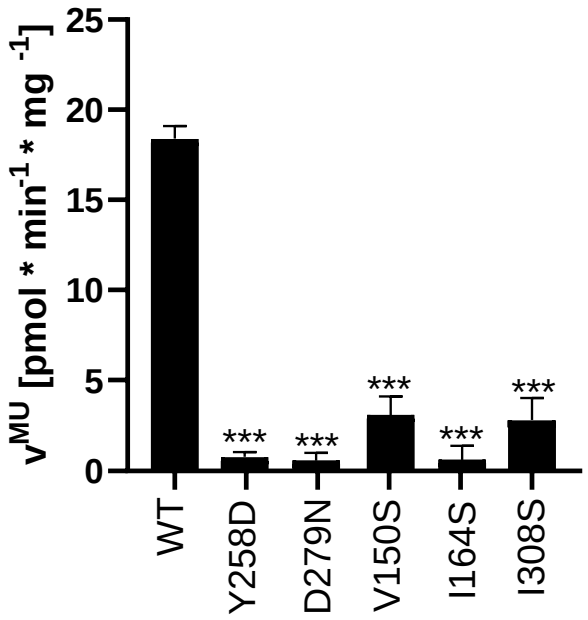

Fig. S4. Thioesterase activity of Cln5 variants. S-depalmitoylase activities of Cln5 variants Y258D, D279N, V150S, I164S, and I308S were compared with Cln5 WT. Reaction includes 1 µg of each purified enzyme with 10 µM MU-6S-palm-βGlc plus 20 µg β-glucosidase for 60 minutes. Values represent means ± SD (***$p \leq 0.001$).

Table S1. Summary of the data collection statistics for the averaged data set. Seven datasets were merged for structure solution and refinement. Values in parentheses refer to outer resolution shell.

Source	SLS PXII-X10SA
Unit cell dimensions (Å)	a = 58.406, c = 178.235
Space group	$P3_221$
Wavelength (Å)	0.9790
Resolution range (Å)	48.67-2.70 (2.79-2.70)
Number of Reflections	
Measured	540369
Unique	10352 (1013)
Redundancy[a]	52.2 (19.0)
Completeness[a]	100 (100)
Mean I/σ(I)	52.2 (2.35)
R_{rim}	0.2343 (1.255)
R_{pim}	0.0305 (0.2873)
$CC_{1/2}$	99.9 (83.5)
CC*	100 (95.5)

[a] Friedel pairs merged.

Table S2. Refinement statistics. Values in parentheses refer to outer resolution shell. CC(work) and CC(free) refer to the correlation of the observed intensities to the model-based intensities for the work and test sets, respectively.

Refinement statistics	
Resolution range	48.59-2.70 (2.79-2.70)
Completeness (%)	99.94 (100)
Number of reflections in working set	10352 (1013)
Number of reflections in test set	522 (45)
Solvent content (%)	48.4
Number of protein atoms	2406
Number of water molecules	35
Protein molecules per ASU	1
R value	0.2207 (0.2983)
R_{free}	0.2472 (0.3741)
CC_{work}[a]	49.2
CC_{free}[a]	52.4
Average B factors (Å²)	
Overall	57.05
Protein atoms	56.93
Water molecules	53.54

Observed r.m.s.d. from ideal geometry[b]	
bond length (Å)	0.004
Bond angles (°)	0.74
Ramachandran plot, residues in[c]	
Favored regions	94.62
Allowed regions	4.66

[a]calculated with phenix_refine (*53*).
[b]REFMAC output.
[c]calculated with MolProbity.