

Supplementary Information

Reprogramming of bacterial virulence by lysine acetylation

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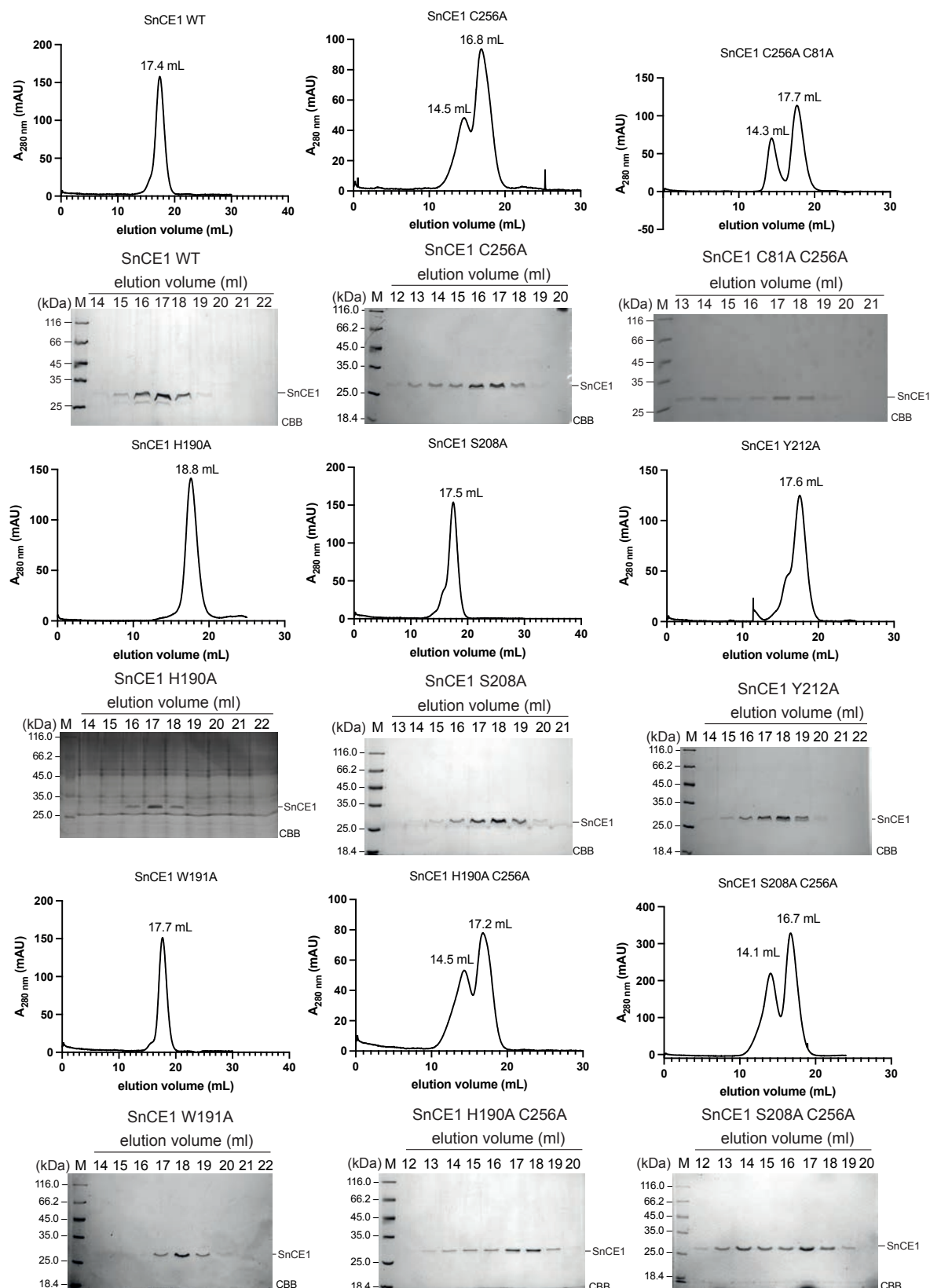
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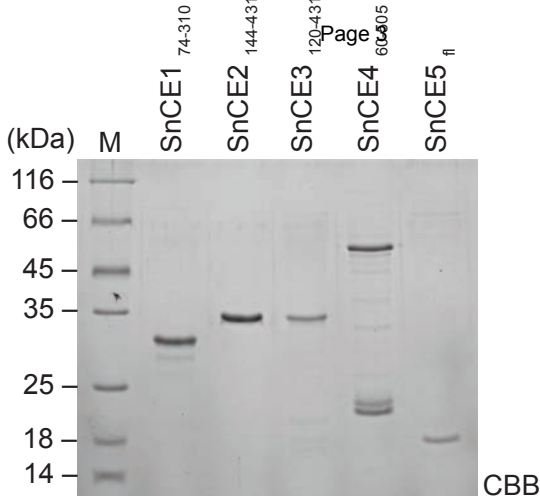
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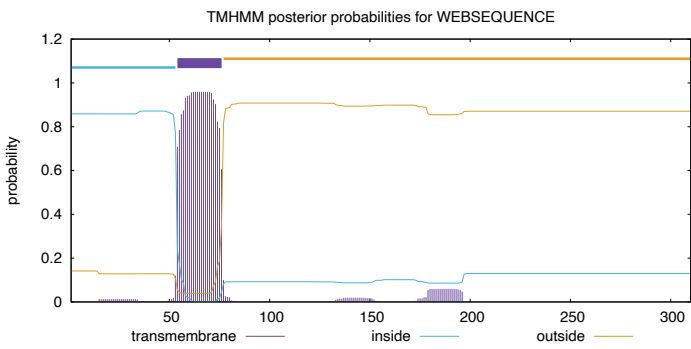
Supplementary Figure 1: Purification and analytical size-exclusion chromatography (SEC) of SnCE1 proteins used in this study.

Analytical size-exclusion chromatography (SEC) of all proteins studied here. 0.1 to 0.2 mg of protein was analysed on a Superdex 200 Increase 10/300 GL column. Below the SEC elution profiles showing the absorbance at 280 nm ($A_{280 \text{ nm}}$) in mAU (milli absorbance units) the SDS-PAGE gels were shown analysing fractions of the observed absorption peaks. SDS-PAGE gel was stained using Coomassie brilliant blue (CBB). Source data are provided as Source Data file.

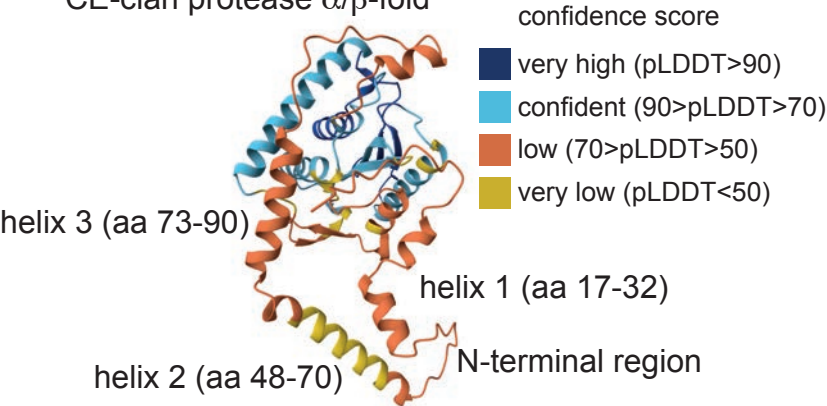
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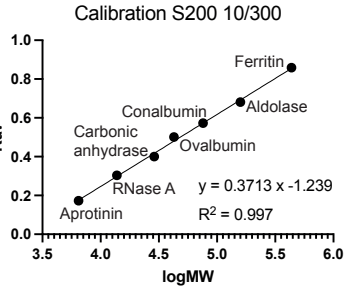
b



CE-clan protease α/β -fold



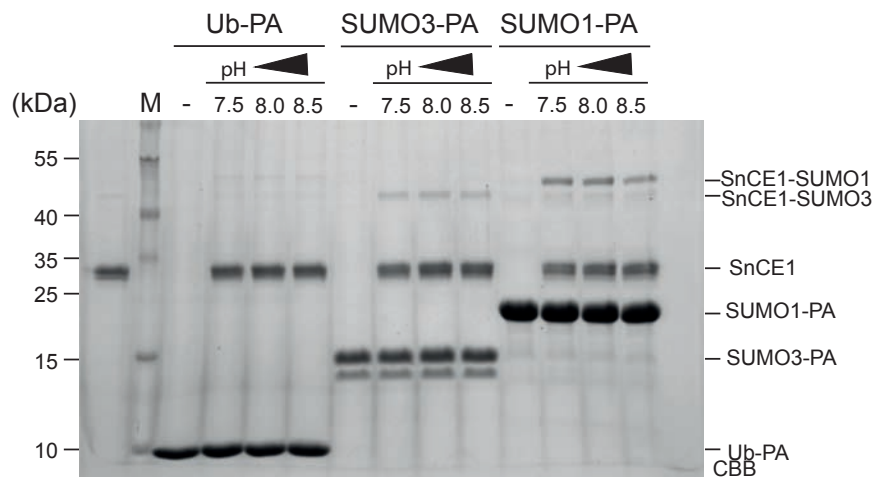
c



protein	exp. MW [Da]	peak 1			peak 2		
		elution volume [mL]	calc. MW [Da]	oligomeric state [calc. MW/exp. MW]	elution volume [mL]	calc. MW [Da]	oligomeric state [calc. MW/exp. MW]
SnCE1 ₇₄₋₃₁₀	28354				17.4	36178	1.3
SnCE1 ₁₀₄₋₃₁₀	25079				18.5	22260	0.9
SnCE1 ₁₀₄₋₃₁₀ C256A	25047	15.0	99715	4.0	18.4	24034	1.0
SnCE1 ₇₄₋₃₁₀ C256A	28322	14.5	124443	4.3	16.8	46714	1.7
SnCE1 ₇₄₋₃₁₀ Y212A	28262				17.6	33223	1.2
SnCE1 ₇₄₋₃₁₀ S208A	28338				17.5	34669	1.2
SnCE1 ₇₄₋₃₁₀ C81A/C256A	28290	14.3	135510	4.8	17.7	31837	1.1
SnCE1 ₇₄₋₃₁₀ S208A/C256A	28306	14.3	124443	4.8	17.0	42899	1.5
SnCE1 ₇₄₋₃₁₀ H190A	28288				18.8	19926	0.7
SnCE1 ₇₄₋₃₁₀ H190A/C256A	28256	14.5	124443	4.3	17.2	39395	1.4
SnCE1 ₇₄₋₃₁₀ W191A	28239				17.7	31837	1.1
SnCE1 ₇₄₋₃₁₀ C256A AcK78	28364	14.4	129858	4.6	17.8	30510	1.1
SnCE1 ₇₄₋₃₁₀ C256A AcK94	28364	14.3	135510	4.8	17.8	30510	1.1
SnCE1 ₇₄₋₃₁₀ C256A AcK98	28364	14.4	129858	4.6	17.9	29237	1.0
SnCE1 ₇₄₋₃₁₀ C256A AcK103	28364	14.2	141407	5.0	17.6	33223	1.2
SnCE1 ₇₄₋₃₁₀ C256A AcK106	28364	14.3	135510	4.8	17.9	29237	1.0
SnCE1 ₇₄₋₃₁₀ C256A AcK209	28364	14.4	129858	4.6	17.7	31837	1.1
SnCE1 ₇₄₋₃₁₀ Y212A AcK231	28304				18.3	24656	0.9
SnCE1 ₇₄₋₃₁₀ C256A AcK231	28364				17.6	33223	1.2
SnCE1 ₇₄₋₃₁₀ C256A AcK248	28364	14.2	141407	5.0	17.5	34669	1.2
SnCE1 ₇₄₋₃₁₀ C256A S/T/CtoA	28164	14.0	153983	5.5	17.4	36178	1.3
SnCobB (S200 10/300)	30524	13.7	174975	5.7	17.7	31837	1.0

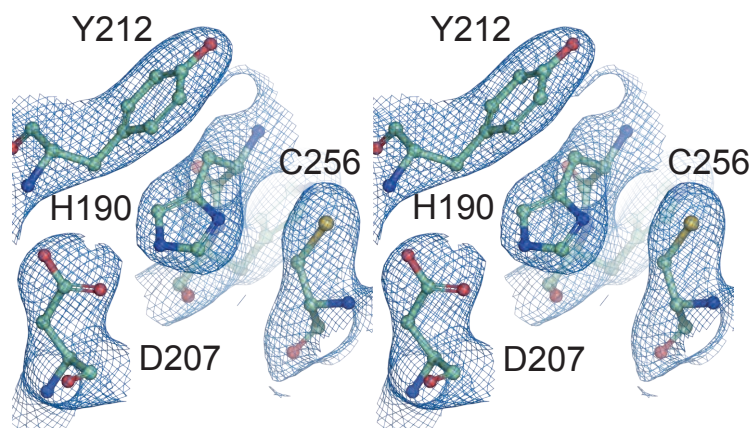
Supplementary Figure 2: Final purity obtained for SnCE1-5 and determination of oligomeric states by analytical SEC of proteins expressed and purified in this study.

- a. SDS-PAGE gel showing the final purity obtained for *Simkania negevensis* SnCE1-5. 1 µg of recombinantly expressed and purified protein was loaded on the SDS-PAGE gel. Staining was done with Coomassie brilliant blue (CBB).
- b. Prediction of a potential N-terminal transmembrane helices in SnCE1. The prediction of transmembrane helices in SnCE1 was done with TMHMM2.0 (<https://services.healthtech.dtu.dk/services/TMHMM-2.0/>). It predicts the presence of a transmembrane helix with high probability covering residues 54 to 74. This agrees in part with the AlphaFold3 model obtained for full length SnCE1 (<https://alphafoldserver.com/>) showing the presence of α -helices in the N-terminal part covering residues 17-32 with very low confidence (pLDDT<50), residues 48-70 with low confidence (50>pLDDT>70) and residues 73-90 again with very low confidence (pLDDT<50)¹⁻³.
- c. The elution volumes and calculated molecular weights (calc. MW) were used in comparison to expected molecular weights (exp. MW) to calculate the apparent oligomeric state of the proteins analysed in this study. The calculated molecular weights were obtained based on the elution volume using a calibration curve. Source data are provided as Source Data file.

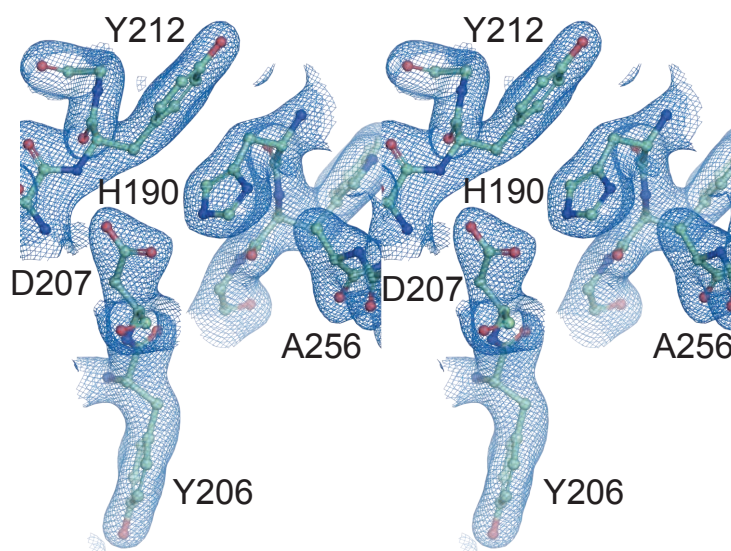


Supplementary Fig. 3: Changes in the pH-value from pH 7.5 to 8.5 have no effect on the reactivity of SnCE1 with the activity-based Ub-PA, SUMO1-PA and SUMO3-PA probes. The SDS-PAGE was stained with Coomassie brilliant blue (CBB).

a



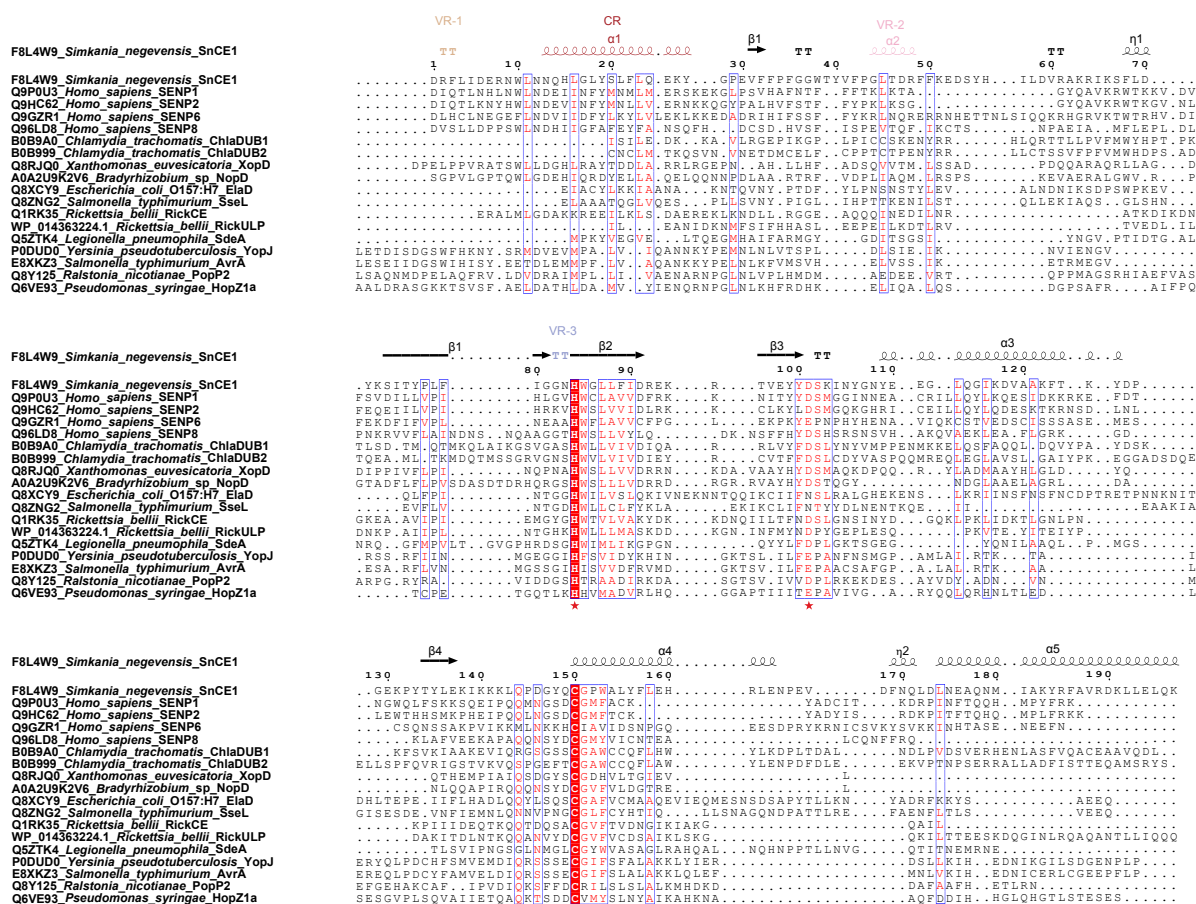
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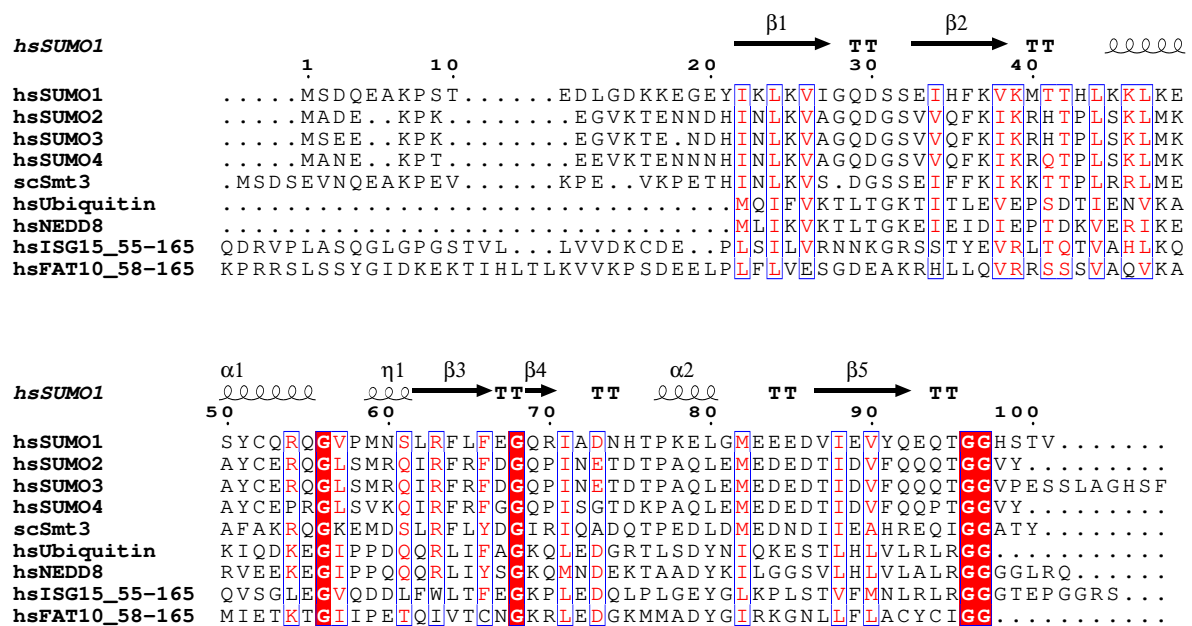


Supplementary Figure 4: Representative electron density for the apo structures of SnCE1 wildtype and SnCE1 C256A solved in this study.

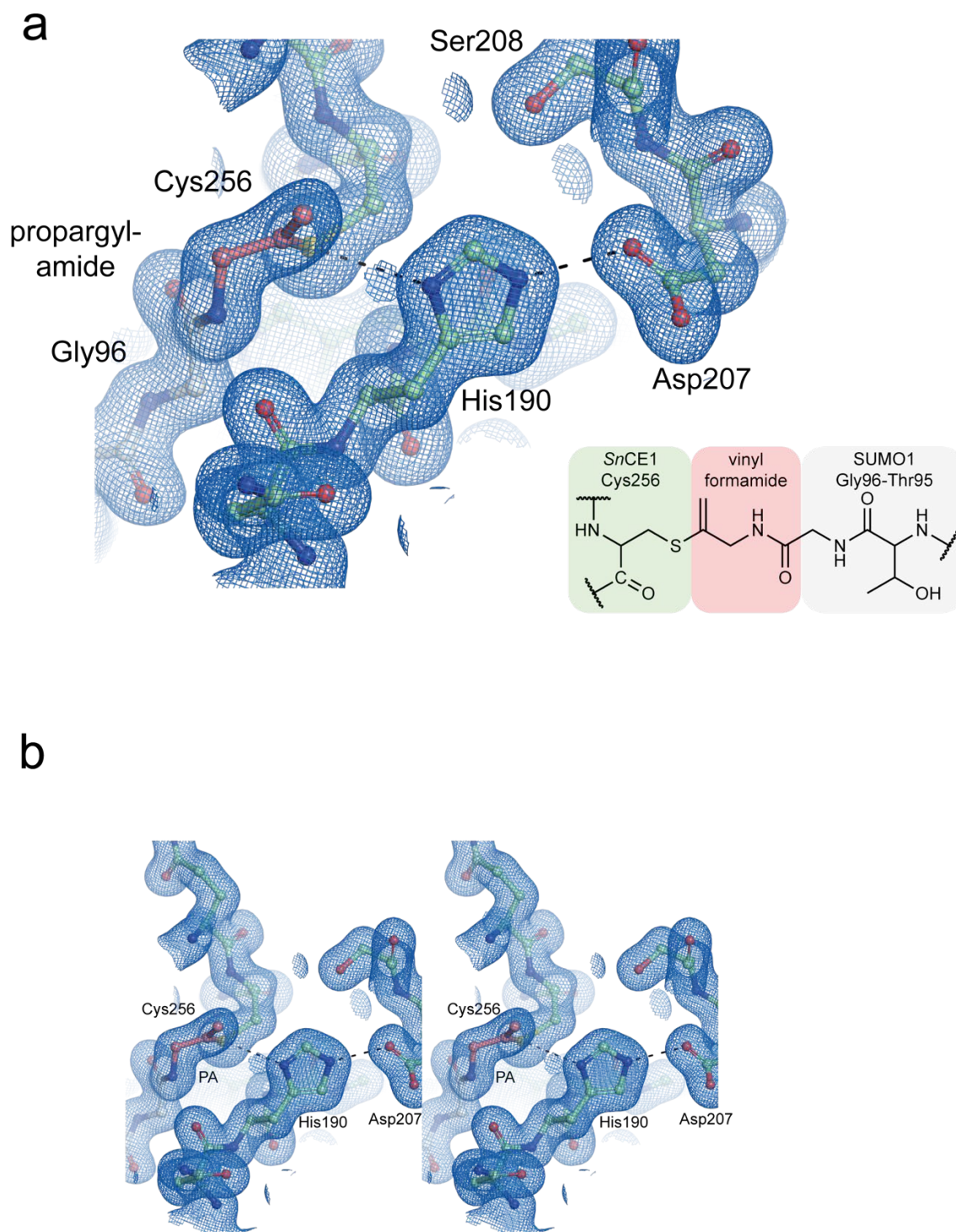
Shown is a closeup of the active site including the catalytic triad residues, i.e. the nucleophile Cys256 or the replacement of Cys256 with Ala, the His190 base and the acid Asp207. SnCE1 backbone trace is coloured in green. The figure was prepared with PyMOL version 2.3.4⁴

- Stereo figure of the active site in wall-eyed-viewing mode. Shown is the 2F_o-F_c electron density map of a closeup of the active site for SnCE1 wildtype contoured at 1σ (PDB: [9QTE](https://doi.org/10.2210/pdb9QTE/pdb), [<https://doi.org/10.2210/pdb9QTE/pdb>]).
- Stereo figure of the active site in wall-eyed-viewing mode. Shown is the 2F_o-F_c electron density map of a closeup of the active site for catalytic mutant SnCE1 C256A contoured at 1σ (PDB: [9QTF](https://doi.org/10.2210/pdb9QTF/pdb), [<https://doi.org/10.2210/pdb9QTF/pdb>]).





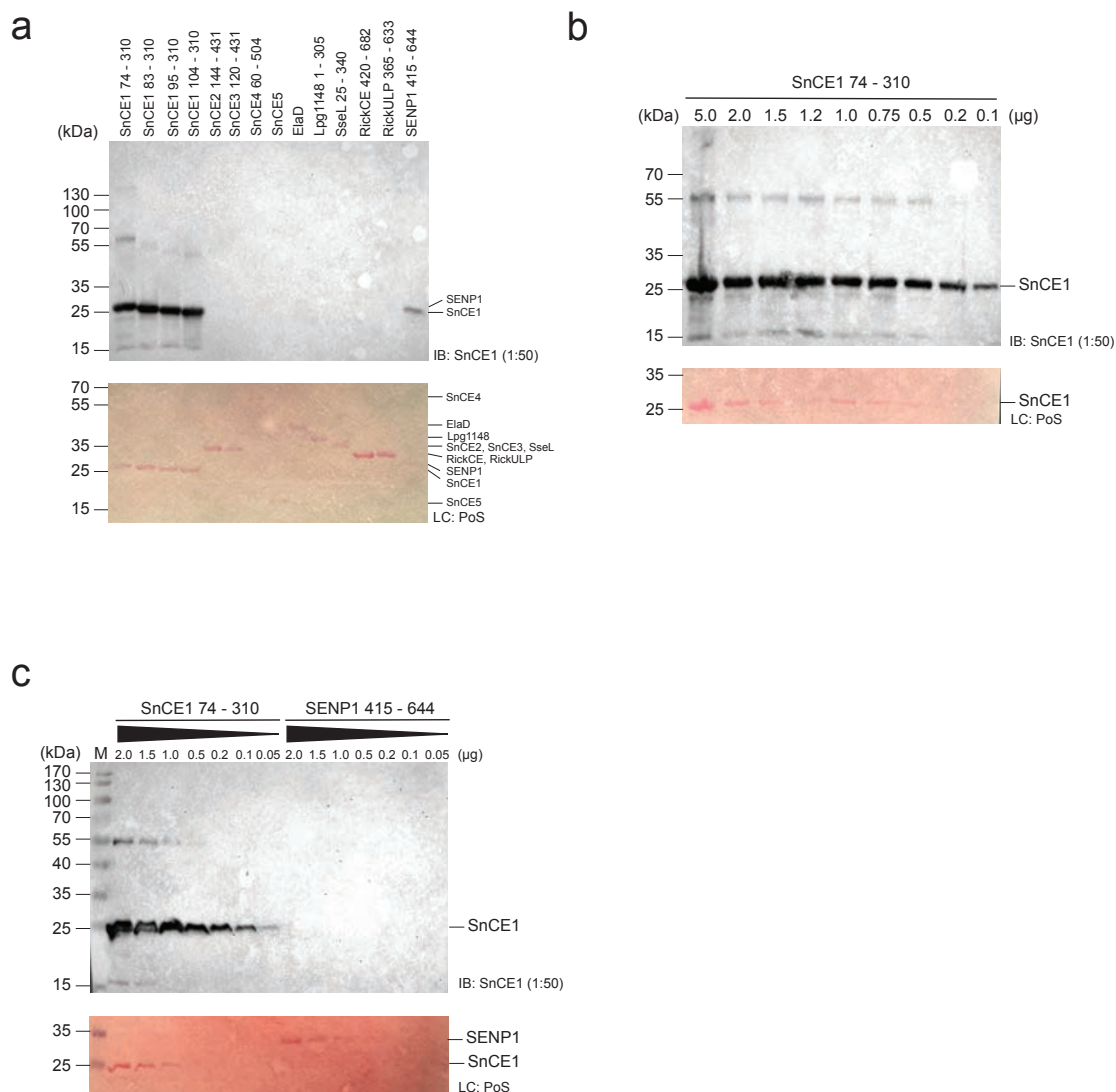
Supplementary Figure 6: Sequence alignment of human ubiquitin, SUMO1, SUMO2, SUMO3, SUMO4, NEDD8, ISG15 and FAT10. The alignment was conducted to uncover conserved and diverse regions in human ubiquitin and Ubl-proteins. The following sequences were used in the alignment: P63165: *Homo sapiens* SUMO1, hsSUMO1; P61956: *Homo sapiens* SUMO2, hsSUMO2; P55854: *Homo sapiens* SUMO3, hsSUMO3; P63165: *Homo sapiens* SUMO4, hsSUMO4; Q12306: *Saccharomyces cerevisiae* SMT3, scSMT3; P0CG47: *Homo sapiens* Ubiquitin, hsUb; Q15843: *Homo sapiens* NEDD8; hsNEDD8; P05161: *Homo sapiens* ISG15, hslSG15; O15205: *Homo sapiens* FAT10, hsFAT10. The secondary structure elements are given on top for SUMO1 from the SnCE1-SUMO1-PA structure solved here. The sequence alignments were conducted with ClustalW and ESPript 3.0 was used to render sequence similarities and information on secondary structure elements derived from the experimental structure of SnCE1 wildtype determined here (PDB: [9QTE](https://doi.org/10.2210/pdb9QTE/pdb))⁵.



Supplementary Figure 7: Representative electron density for the structure SnCE1-SUMO1-PA solved in this study.

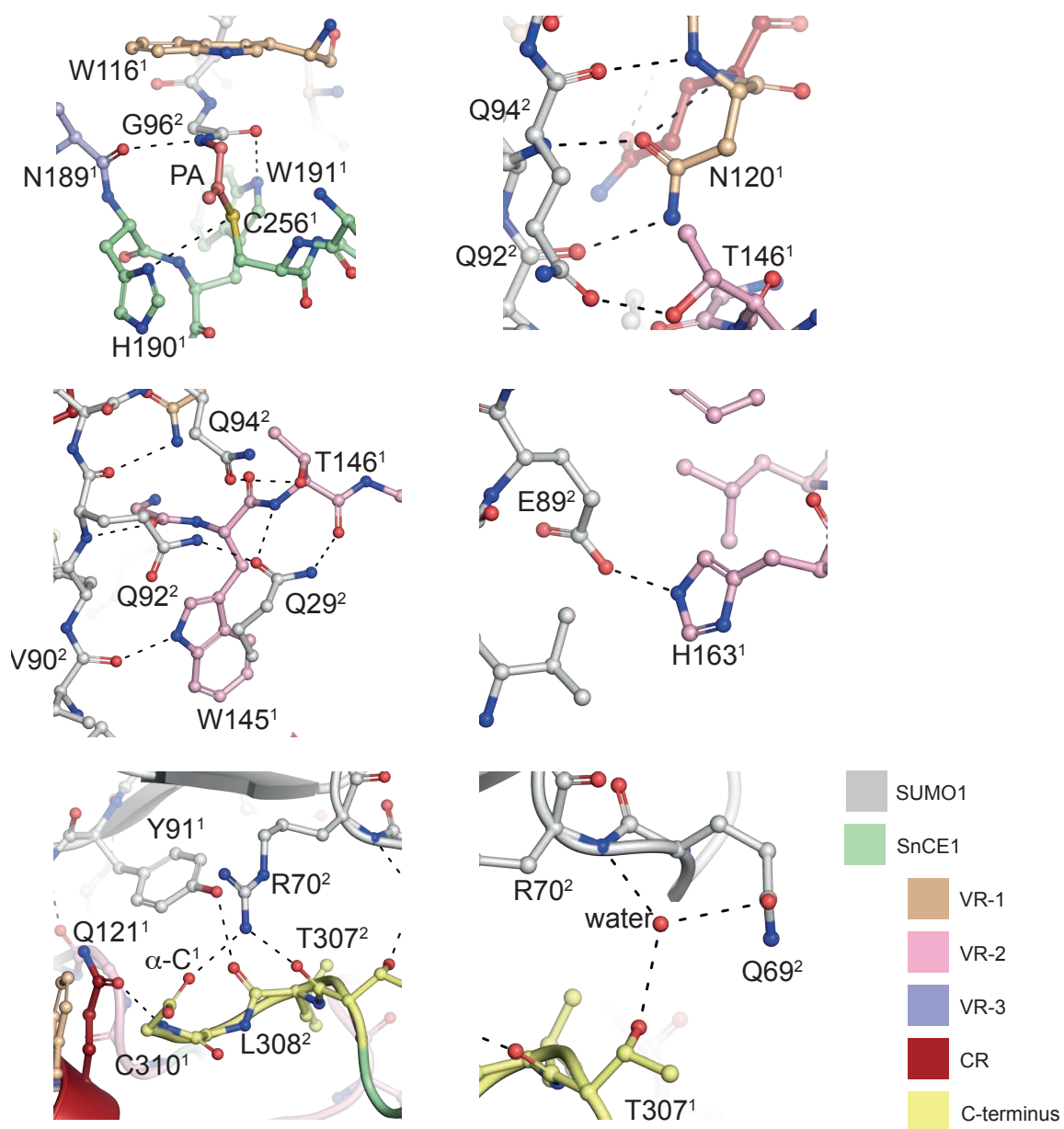
Shown is a closeup of the active site including the catalytic triad residues, i.e. the nucleophile Cys256 modified with propargylamide (PA) forming an amide bond to the C-terminal G96 of the SUMO1, the base His190 and the acid Asp207. The SUMO1 is coloured in grey, SnCE1 is coloured in green and the propargylamide moiety in red (PDB: [9QTG](https://doi.org/10.2210/pdb9QTG/pdb), [<https://doi.org/10.2210/pdb9QTG/pdb>]). The figures were prepared with PyMOL version 2.3.4⁴

- Electron density $2F_o - F_c$ map is contoured at 1σ and shown in blue. The scheme shows the active site architecture with the Cys256 linked by a vinyl formamide derived by reaction of the propargylamide bound to the C-terminal Gly96 of SUMO1 with the Cys256 thiol group forming a vinyl thioether.
- Stereo figure of the active site in wall-eyed-viewing mode. Shown is the $2F_o - F_c$ electron density map contoured at 1σ .



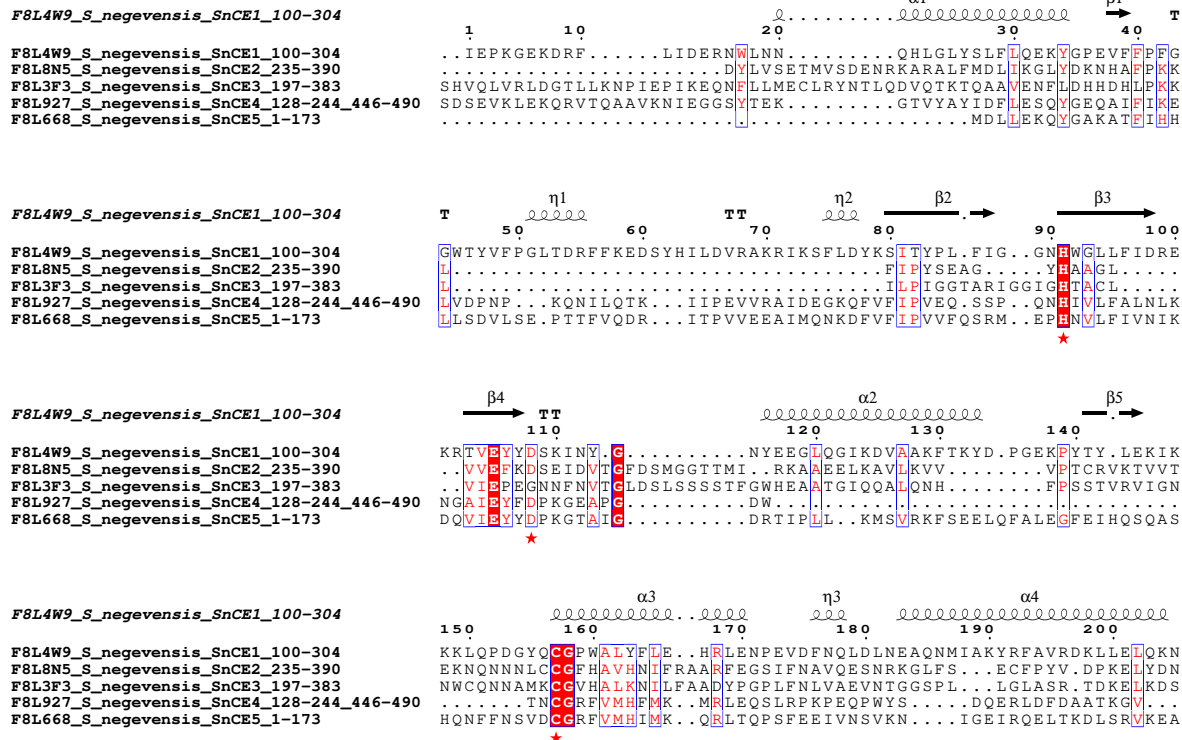
Supplementary Figure 8: Validation of antibody raised against recombinant SnCE1₁₀₄₋₃₁₀ protein regarding selectivity and sensitivity.

- a. Selectivity of the SnCE1 antibody:** Validation of the selectivity of the antibody raised against recombinantly expressed and purified *Simkania negevensis* SnCE1₇₄₋₃₁₀, SnCE1₈₃₋₃₁₀, SnCE1₉₅₋₃₁₀, SnCE1₁₀₄₋₃₁₀, *S. negevensis* SnCE2₁₄₄₋₄₃₁, *S. negevensis* SnCE3₁₂₀₋₄₃₁, *S. negevensis* SnCE₄₆₀₋₅₀₄, *S. negevensis* SnCE5, *Escherichia coli* ElaD, *Legionella pneumophila* Lpg1148₁₋₃₀₅, *Salmonella enterica* Thyphimurium, SseL₂₅₋₃₄₀, *Rickettsia bellii* RickCE₄₂₀₋₆₈₂, *Rickettsia* sp. RickULP₃₅₆₋₆₃₃ and *Homo sapiens* SENP1₄₁₅₋₆₄₄. The validation shows selectivity towards the *S. negevensis* SnCE1 constructs while the other *S. negevensis* proteins were not recognized. The N-terminal region of SnCE1 N-terminally from Gly104 is not recognized by the antibody. The antibody shows cross-reactivity with human SENP1 requiring pre-clearing for studies in human cells. Immunoblotting was done with the anti-SnCE1 antibody (IB: SnCE1), loading control was done by Ponceau S red staining of the membrane (LC: PoS). Dilution of the SnCE1 antibody was 1:50.
- b. Sensitivity of the SnCE1 antibody.** The antibody detects less than 100 ng of SnCE1 protein. Immunoblotting was done with the anti-SnCE1 antibody (IB: SnCE1), loading control was done by Ponceau S red staining of the membrane (LC: PoS). Dilution of the SnCE1 antibody was 1:50.
- c. Pre-clearing of the SnCE1 antibody.** To prevent cross-reactivity of the SnCE1 antibody with human SENP1 in cellular studies, a pre-clearing step was done by incubation of the antibody with recombinantly expressed human SENP1 protein. This enables to increase the specificity of the SnCE1 antibody. The dilution row shows sensitivity of the SnCE1 antibody of detecting less than 50 ng SnCE1 protein. Immunoblotting was done with the anti-SnCE1 antibody (IB: SnCE1), loading control was done by Ponceau S red staining of the membrane (LC: PoS). Dilution of the SnCE1 antibody was 1:50.

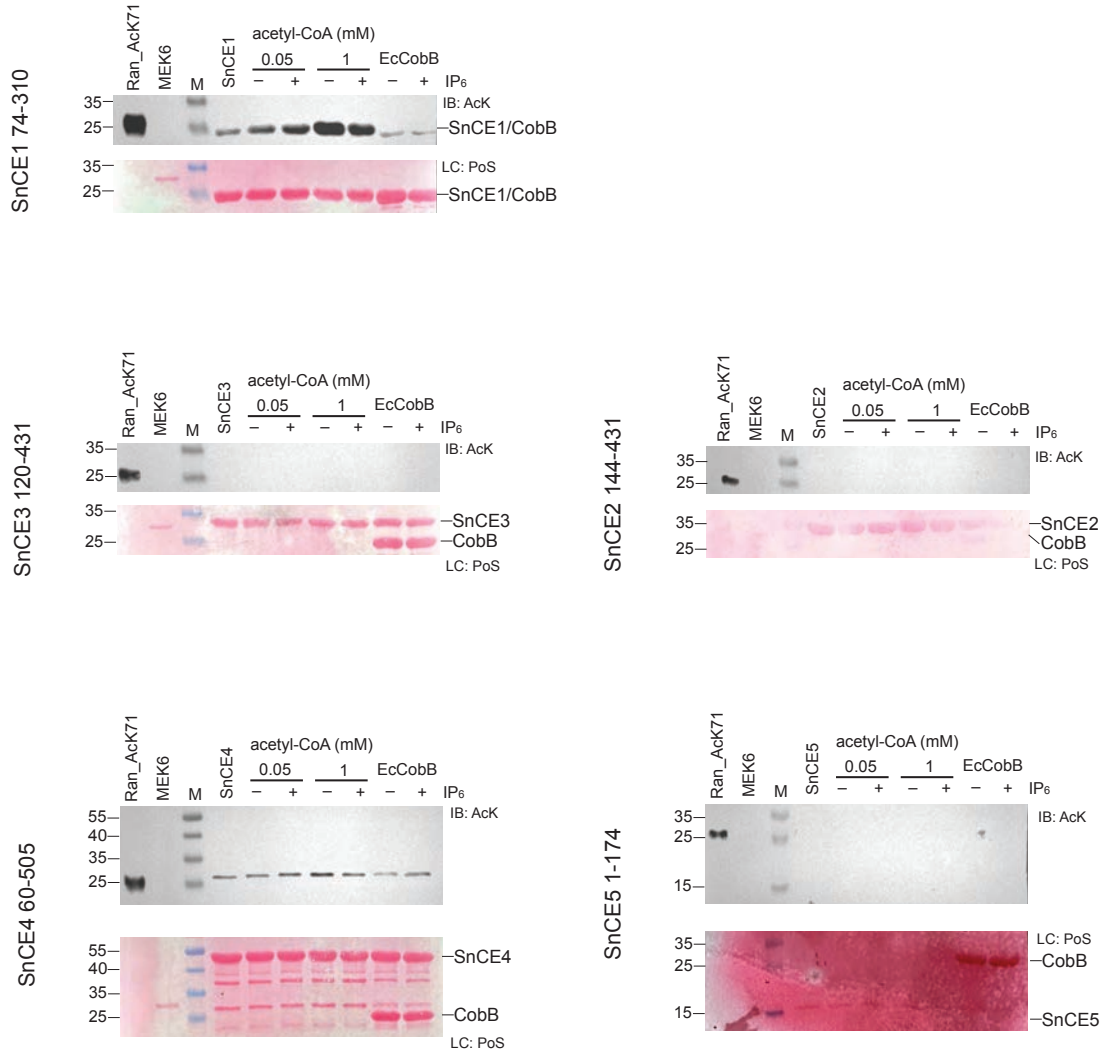


Supplementary Fig. 9: Closeup views of variable regions 1-3 (VR-1-3) of SnCE1 contributing to SUMO1 binding at S1.

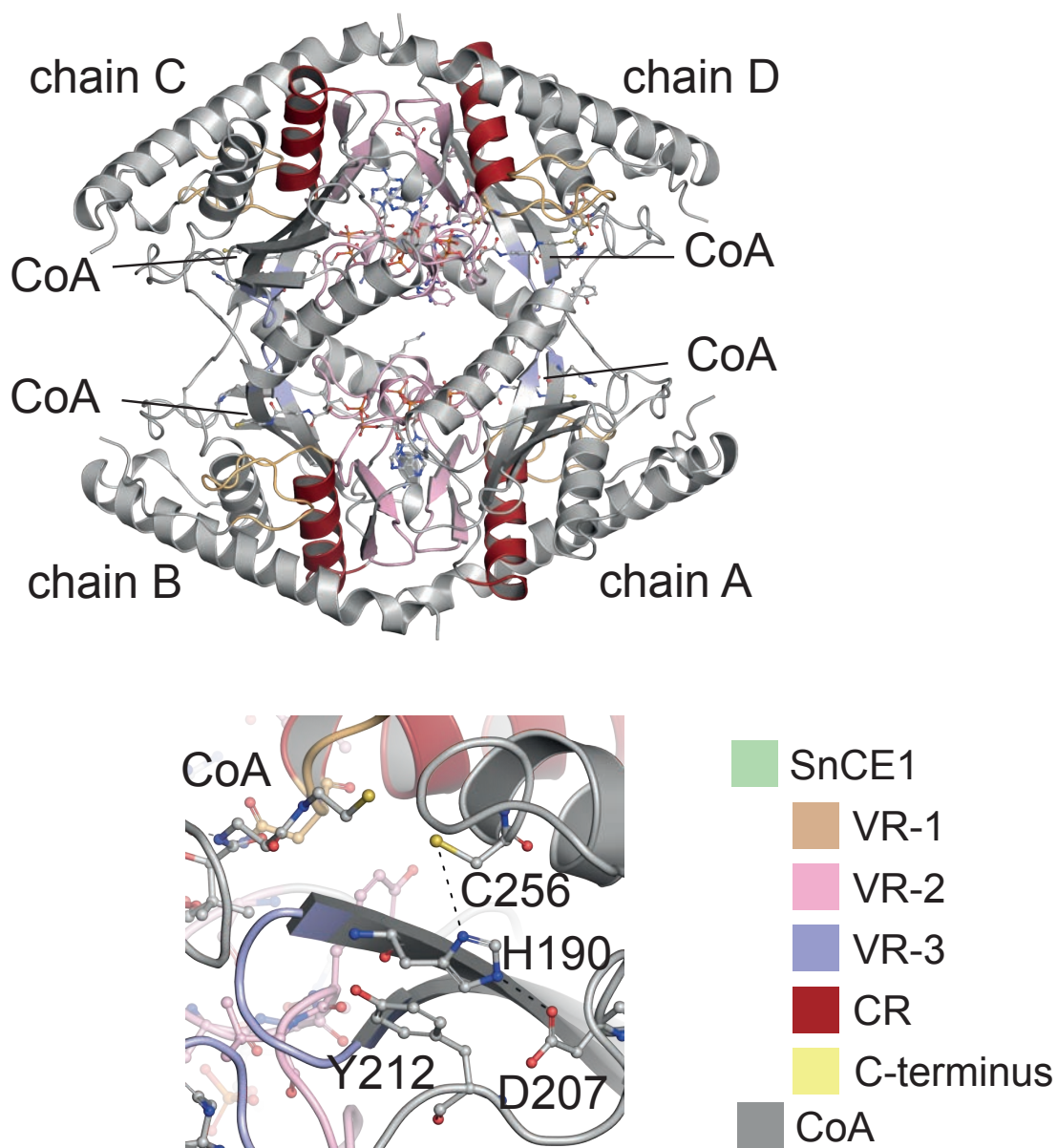
Shown are closeups of the interactions of SUMO1 with residues of VR-1 (orange), VR-2 (magenta) and VR-3 (blue). Apart from these VRs, SnCE1 C-terminal residues (yellow) including the α -carboxylate contribute to SUMO1 binding. Details are described in the manuscript text.



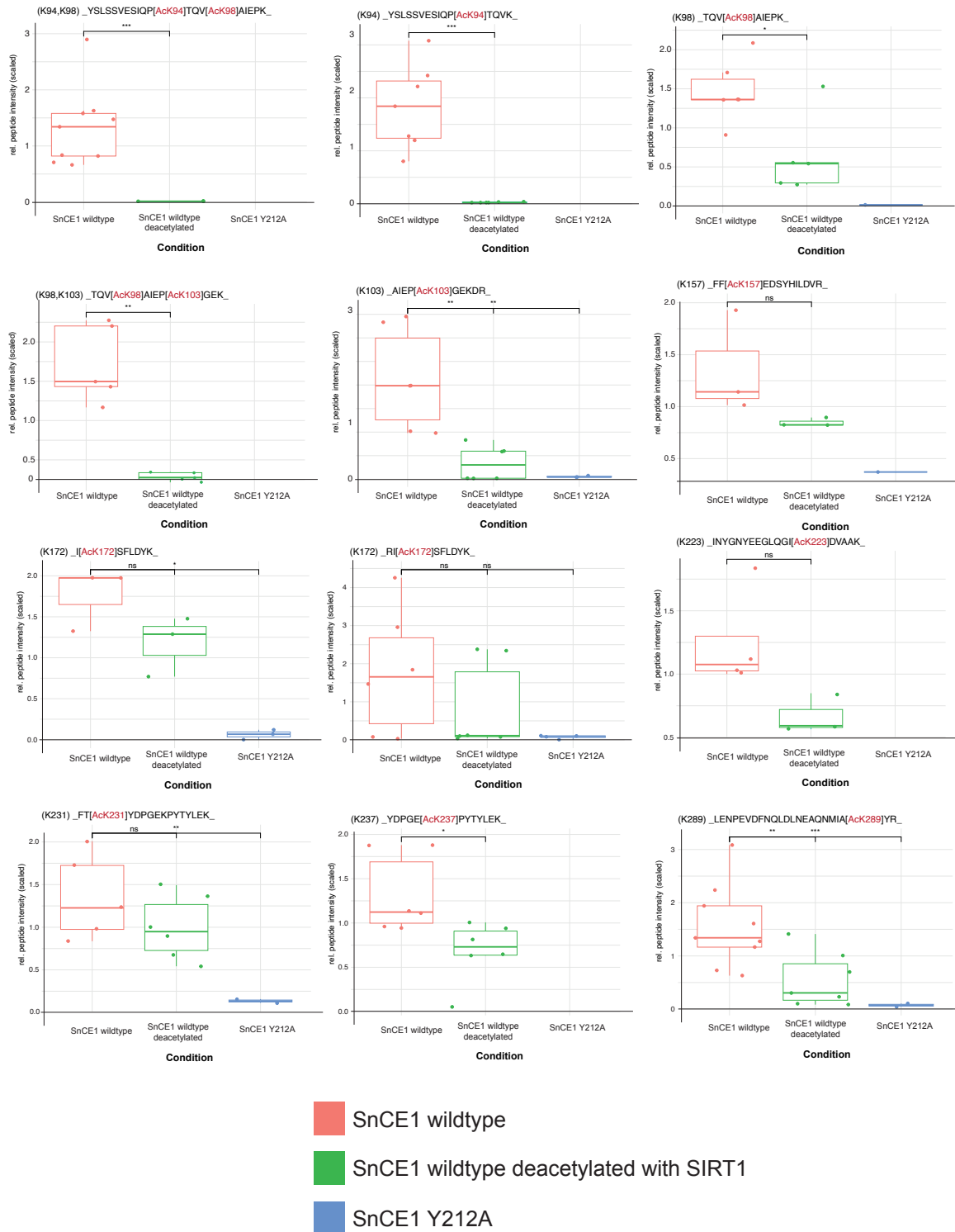
Supplementary Figure 10: Sequence alignment of the *S. negevensis* CE-clan protease-related proteins SnCE1-5. The alignment was conducted to uncover conserved regions in *S. negevensis* CE-clan protease-related proteins. The following sequences were used in the alignment: F8L4W9: *S. negevensis* SnCE1, F8L8N5: *S. negevensis* SnCE2; F8L3F3: *S. negevensis* SnCE3; F8L927: *S. negevensis* SnCE4; F8L668: *S. negevensis* SnCE5. Red asterisks show the conserved residues of the catalytic triad, i.e. His190, Asp207, and Cys256 in SnCE1. In SnCE3 the catalytic triad Asp is replaced by Gly. The secondary structure elements are given on top for SnCE1 structure solved here. The numbering is shown for SnCE1 and Ile1 corresponds to Ile100 in full length SnCE1. The sequence alignments were conducted with ClustalW and ESPript 3.0 was used to render sequence similarities and information on secondary structure elements derived from the experimental structure of SnCE1 wildtype determined here (PDB: [9QTE](https://doi.org/10.2210/pdb9QTE/pdb)) [<https://doi.org/10.2210/pdb9QTE/pdb>]⁵.



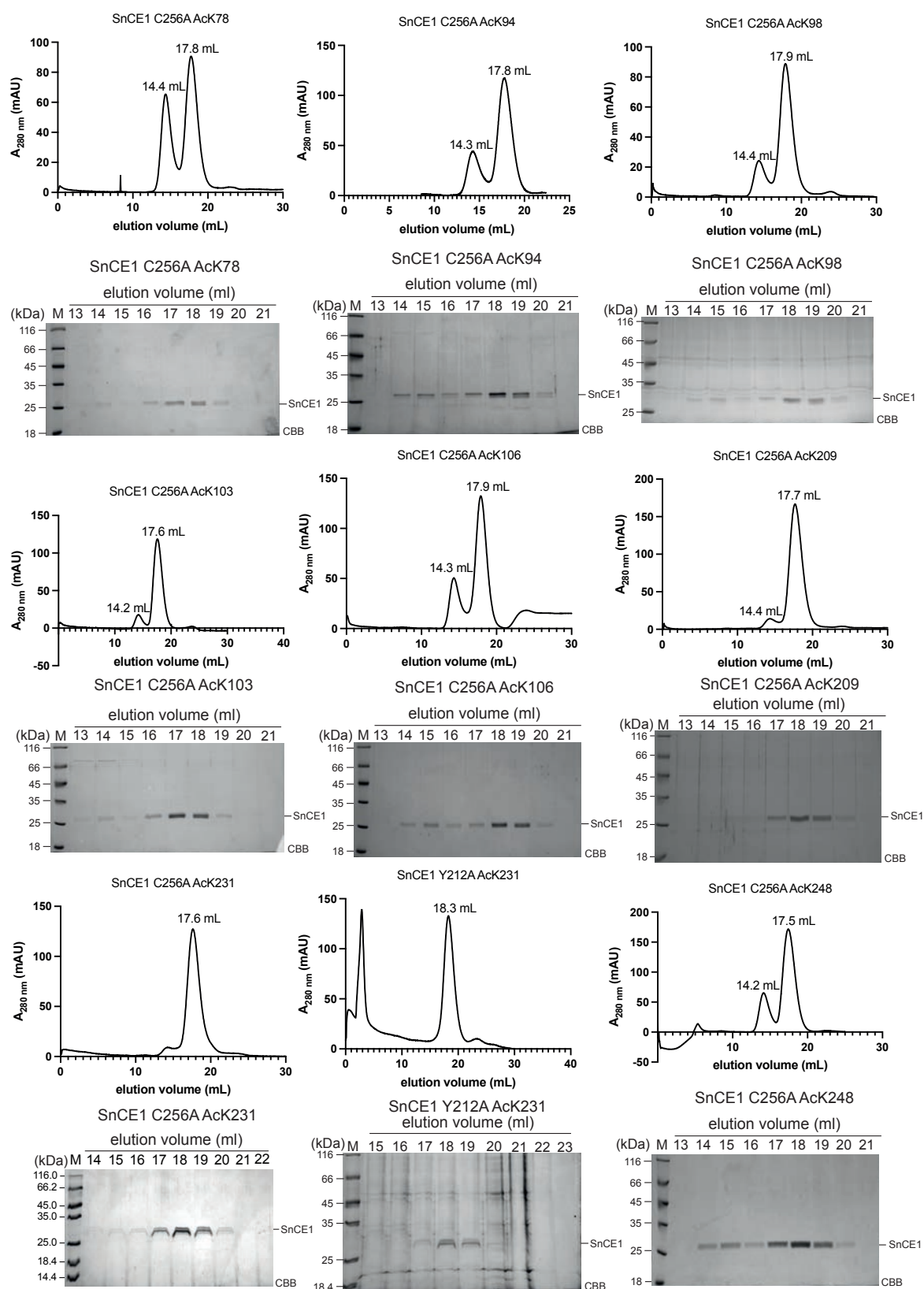
Supplementary Fig. 11: Analysis of the acetylation state of *S. negevensis* SnCE1-SnCE5. From the five CE-clan protease-related DUBs/ULPs, i.e. SnCE1-SnCE5, in *S. negevensis* only SnCE1 is lysine acetylated. Addition of acetyl-CoA increases the acetylation signal for SnCE1. *E. coli* CobB, EcCobB, is not capable to deacetylate SnCE1. Presence of the activator inositol hexakisphosphate (IP₆) does not enhance acetyltransferase activity as described for YopJ-family acetyltransferases, i.e. YopJ, AvrA, PopP2⁶⁻⁸. Immunoblotting was done with the anti-acetyl-lysine antibody (IB: AcK), loading control was done by Ponceau S red staining of the membrane (LC: PoS). Source data are provided as Source Data file.



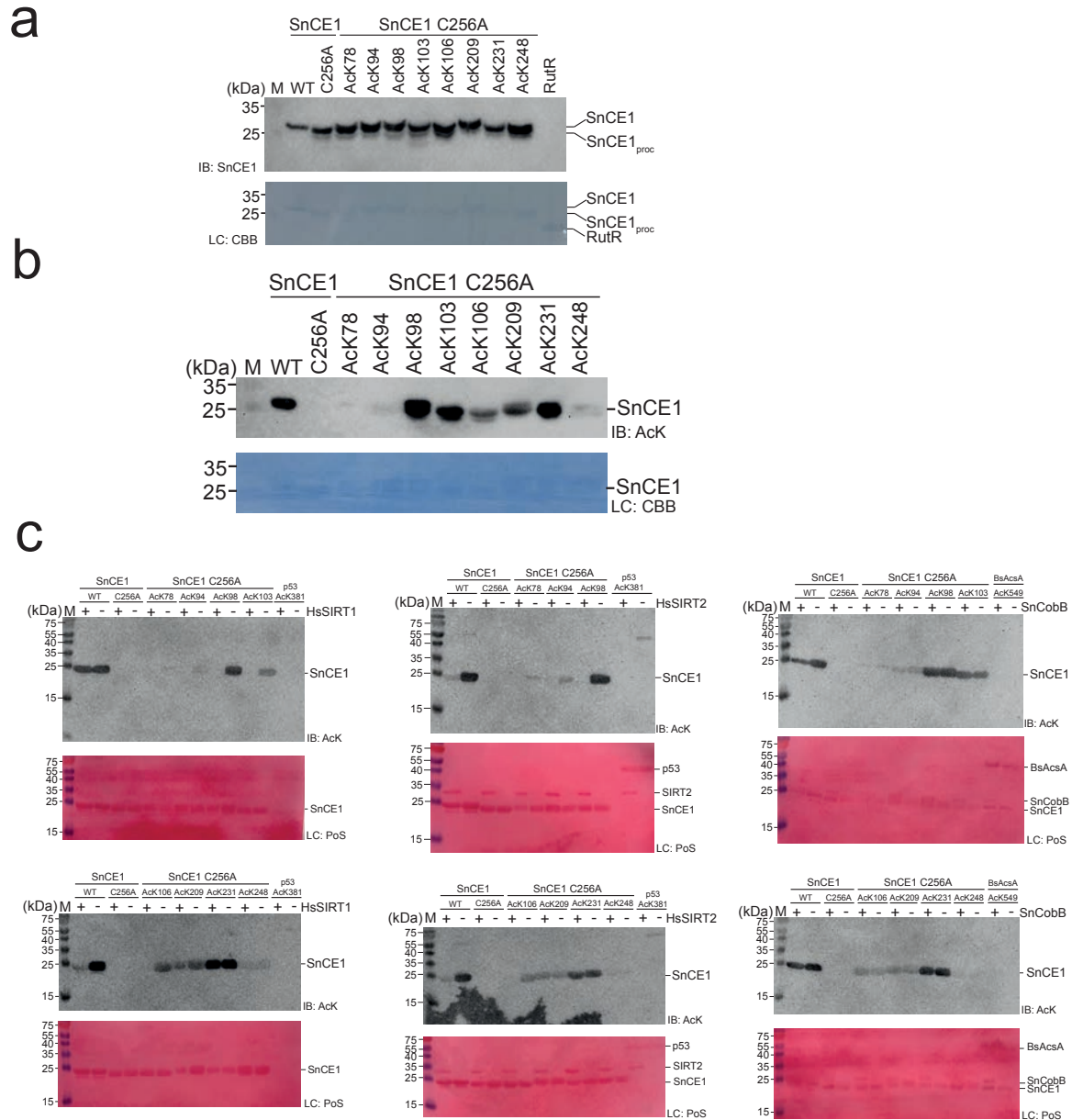
Supplementary Fig. 12: AlphaFold3 structure prediction of a SnCE1•CoA tetramer. The prediction has pTM+ipTM value of 0.38+0.48 suggesting this prediction is not representing the true tetramer¹⁻³. In contrast to the experimental structure, the AlphaFold3 model, the CoA is bound by two neighboring chains of the tetramer. The CoA molecule is not directly interacting with Tyr212 and some residues sterically clash with the CoA molecule, i.e. the side chain of Asn119, and the main chain carbonyl oxygen of L164 of the neighboring chain and the exocyclic amino group of the CoA adenine base¹⁻³.



Supplementary Fig. 13: Lysine acetylation sites identified in SnCE1 by mass spectrometry. Recombinant SnCE1 wildtype protein was analysed by tryptic digest and LC-MS/MS to identify the acetylation sites. Besides, SnCE1 wildtype was deacetylated by human SIRT1 to discover which sites are accessible to enzymatic deacetylation. AcK94, AcK98, AcK103, AcK237 and AcK189 are deacetylated by SIRT1, AcK157, AcK172, AcK223 and AcK231 are not. Non-acetylated SnCE1 Y212A served as control. Shown are whisker plots showing a box indicating the upper and lower quartiles and the median values. The maximum and minimal values are shown. Statistical significance between the different samples was determined using unpaired Student's t-tests with p-values < 0.05 considered significant.

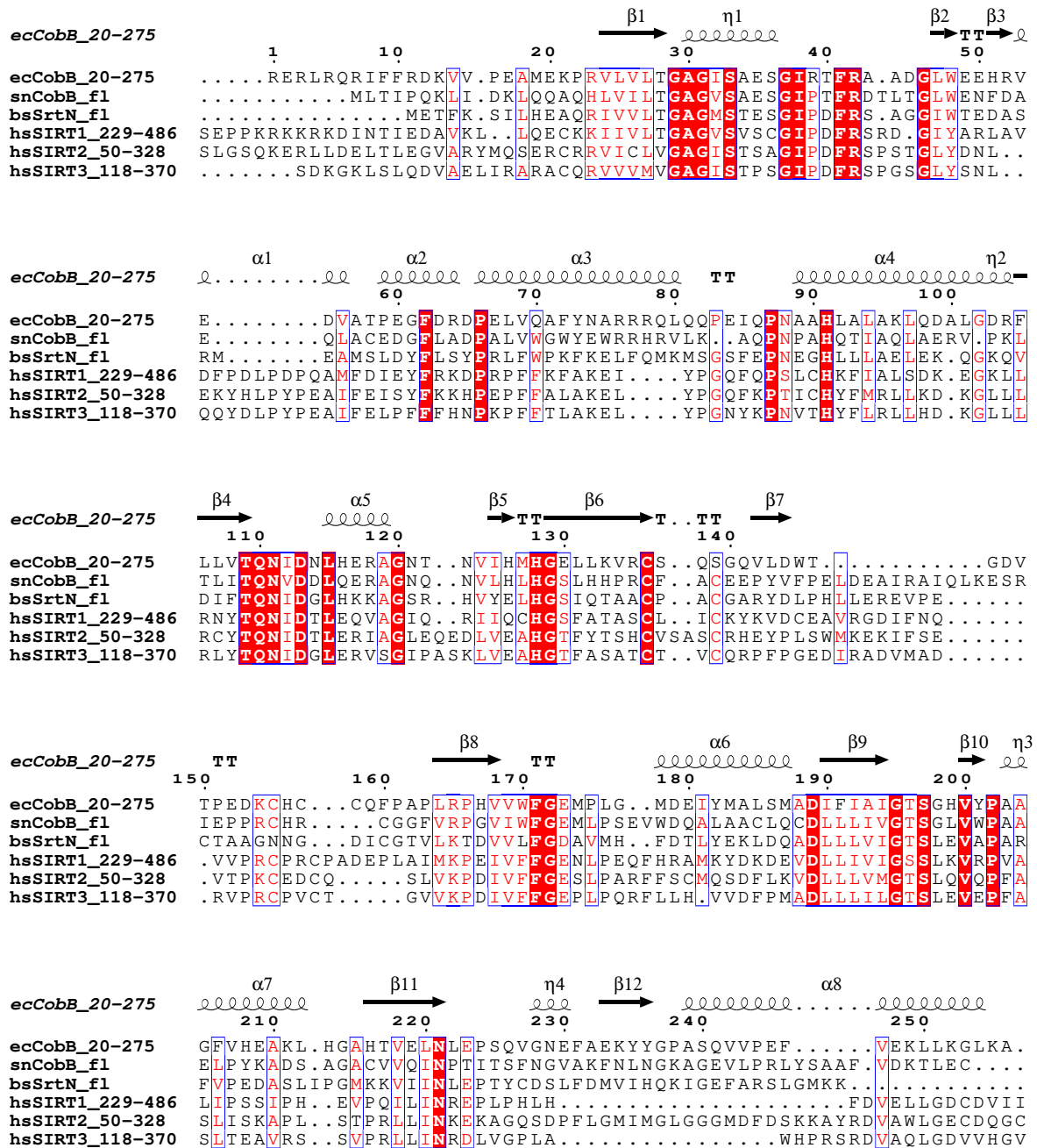


Supplementary Fig. 14: Analytical size-exclusion chromatography (SEC) of site-specifically lysine acetylated SnCE1 C256A proteins prepared by GCEC. Apart from SnCE1 C256A AcK231, all other proteins elute in a monomer-tetramer equilibrium. SnCE1 C256A AcK231 elutes as monomer. The elution volumes and calculated molecular weights (calc. MW) were used in comparison to expected molecular weights (exp. MW) to calculate the apparent oligomeric state of the proteins. The calculated molecular weights were obtained based on the elution volume using a calibration curve shown in Supplementary Fig. 2. A_{280} is the absorption at 280 nm. mAU: milli absorbance units. The SDS-PAGE gels were stained with Coomassie brilliant blue (CBB). Source data are provided as Source Data file.



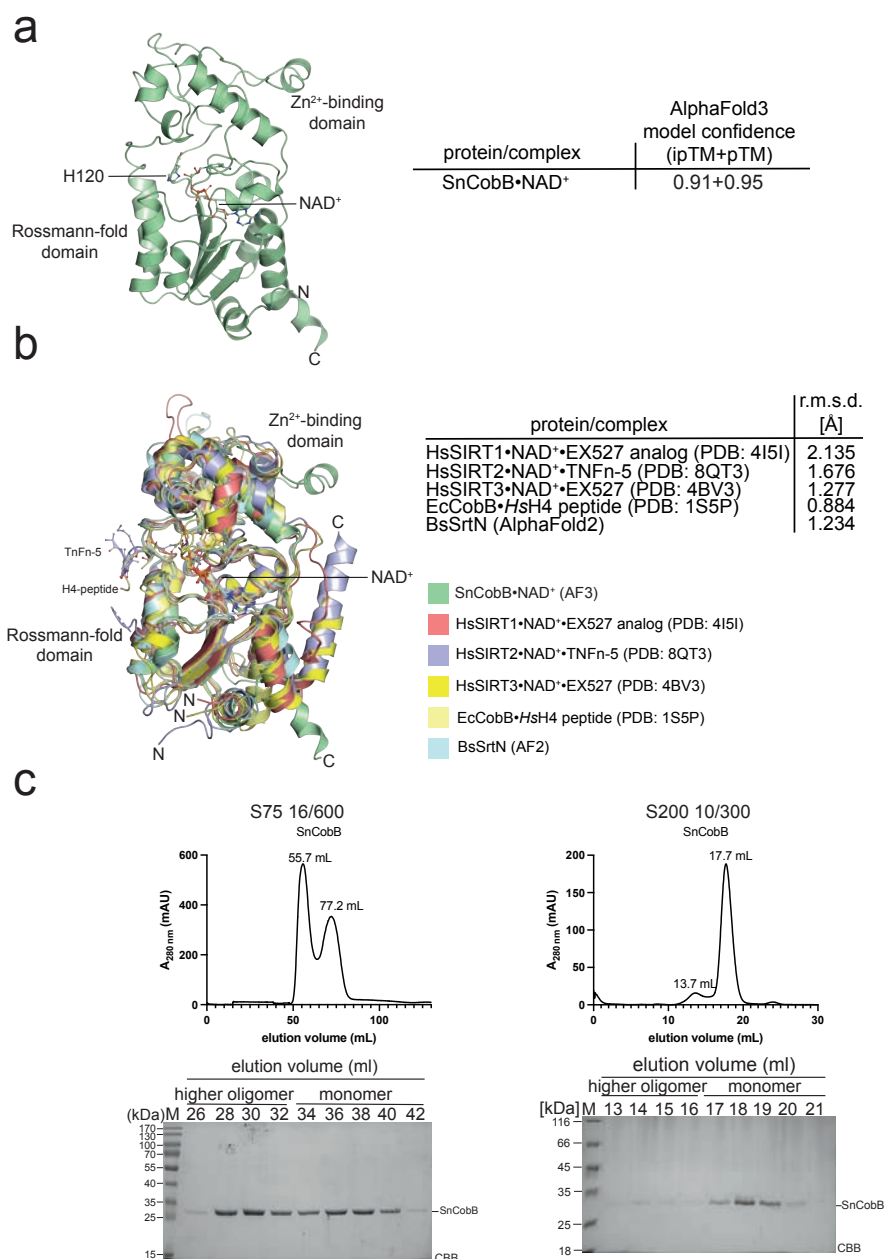
Supplementary Figure 15: Site-specific incorporation of acetyl lysine into SnCE1 using the genetic code expansion concept (GCEC).

- a** Site-specifically lysine acetylated SnCE1 C256A proteins prepared by GCEC were analysed by immunoblotting using a SnCE1 antibody (IB: SnCE1). Shown are SnCE1 C256A AcK78, SnCE1 C256A AcK94, SnCE1 C256A AcK98, SnCE1 C256A AcK103, SnCE1 C256A AcK106, SnCE1 C256A AcK209, SnCE1 C256A AcK231, SnCE1 C256A AcK248. Coomassie brilliant blue (CBB) staining was done as loading control (LC: CBB). Source data are provided as Source Data file.
- b** Immunoblotting of site-specifically lysine acetylated SnCE1 C256A proteins prepared by GCEC, expression in *E. coli* and purification. Shown are SnCE1 C256A AcK78, SnCE1 C256A AcK94, SnCE1 C256A AcK98, SnCE1 C256A AcK103, SnCE1 C256A AcK106, SnCE1 C256A AcK209, SnCE1 C256A AcK231, SnCE1 C256A AcK248. Staining of the immunoblot was done using an anti-acetyl lysine antibody (IB: AcK). The staining with the anti-AcK antibody shows some sites are only weakly detected by the antibody. Coomassie brilliant blue (CBB) staining was done as loading control (LC: CBB). Source data are provided as Source Data file.
- c** Deacetylation of SnCE1 by human SIRT1, SIRT2 and by *S. negevensis* SnCobB. SnCE1 and mutants thereof were deacetylated with SIRT1 and the acetylation state was assessed by immunoblotting with anti-acetyl-lysine antibody (IB: AcK). The acetylated proteins SnCE1 C256A AcK209, SnCE1 C256A AcK231 and SnCE1 C256A AcK248 were not deacetylated by any deacetylase tested supporting acetylation at K231 being irreversible. Loading control was done with Ponceau S red staining of the membrane (LC: PoS). Source data are provided as Source Data file.



Supplementary Fig. 16: *Simkania negevensis* encodes for a single sirtuin deacetylase CobB (SnCobB).

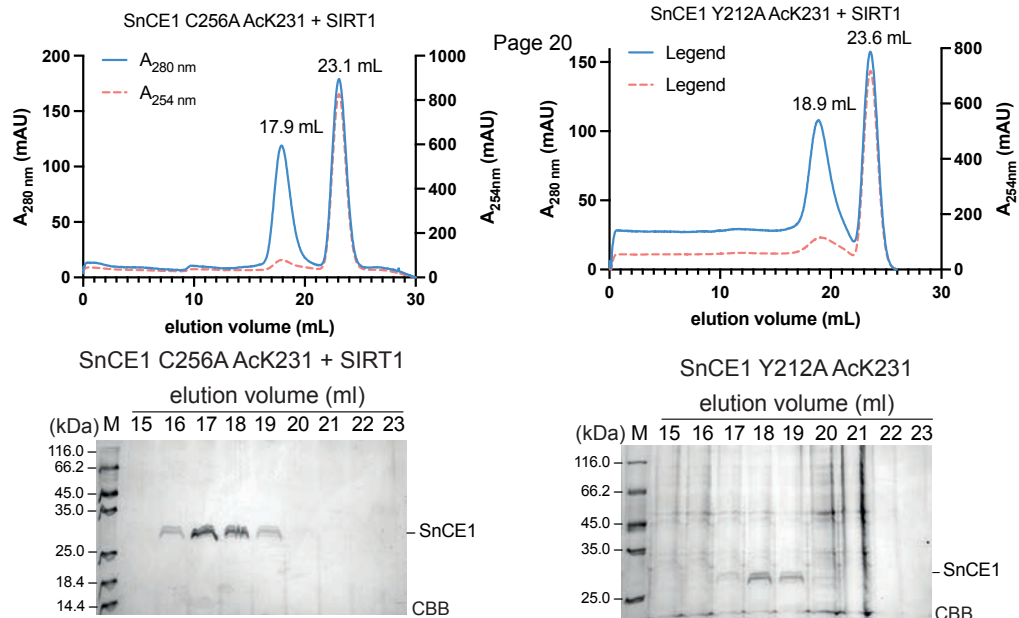
Sequence alignment of bacterial and human sirtuins, i.e. human SIRT1, SIRT2, SIRT3, *B. subtilis* SrtN, *E. coli* CobB and *S. negevensis* CobB. The alignment covers the active site uncovering conserved residues. The following sequences were used in the alignment: P0CG47: *Homo sapiens* Ubiquitin, hsUb; Q96EB6: *Homo sapiens* SIRT1, hsSIRT1; Q8IXJ6: *Homo sapiens* SIRT2, hsSIRT2; Q9NTG7: *Homo sapiens* SIRT3, hsSIRT3; A0A6M4JHV4: *Bacillus subtilis* SrtN: bsSrtN; P75960: *Escherichia coli* CobB, ecCobB; A0A942ELM6: *Simkania* sp. CobB, snCobB. The secondary structure elements are given on top for the AlphaFold3 model of SnCobB+NAD⁺ from the SnCE1(pTM+iPTM: 0.91+0.95). The sequence alignments were conducted with ClustalW and ESPrpt 3.0 was used to render sequence similarities and information on secondary structure elements derived from the AlphaFold3 model for SnCobB.



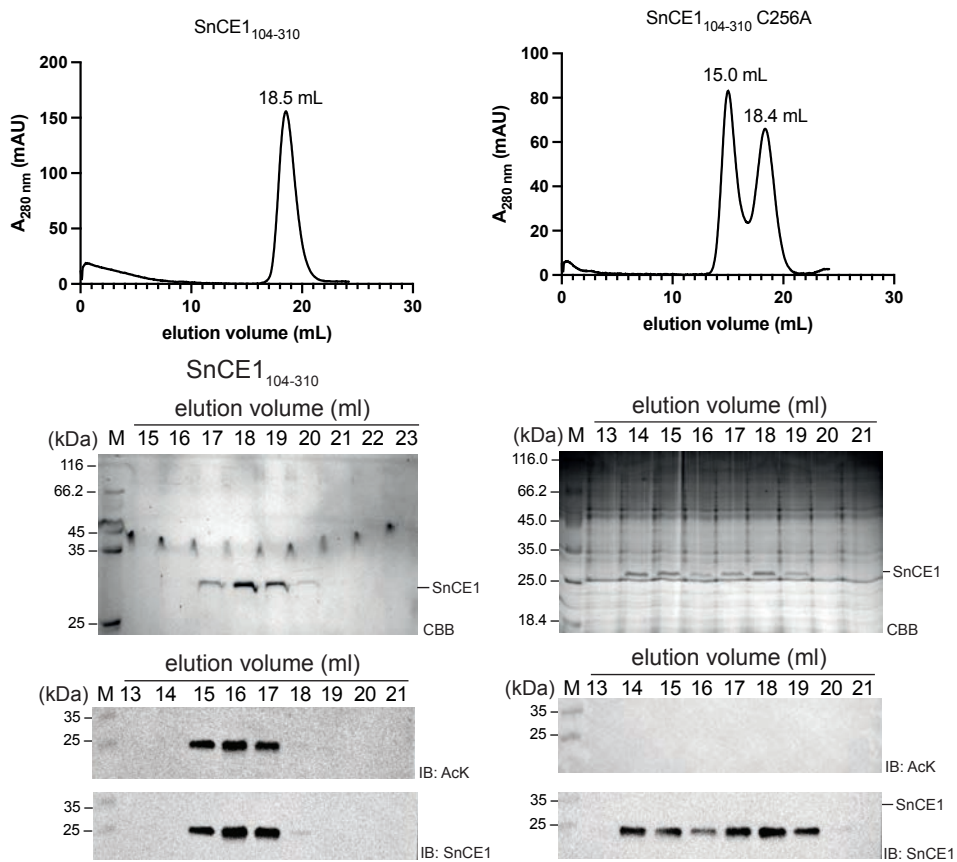
Supplementary Fig. 17: AlphaFold3 structure prediction for *Simkania negevensis* CobB (SnCobB).

- a.** AlphaFold3 model of SnCobB•NAD⁺ from the SnCE1(pTM+iPTM: 0.91+0.95). the model shows a typical domain organization with a Rossmann-fold domain and a Zn²⁺-binding domain with the substrate and NAD⁺-binding site in the interface¹⁻³.
- b.** Superposition of the AlphaFold3 model of SnCobB•NAD⁺ with selected sirtuins as indicated. The highest structural similarity of SnCobB•NAD⁺ is observed towards the bacterial sirtuins CobB from *E. coli* (*EcCobB*) (r.m.s.d.: 0.884 Å) and SrtN from *B. subtilis* (*BsSrtN*) (r.m.s.d.: 1.234 Å). Shown are the following structures: *HsSIRT1*•NAD⁺•EX527 (PDB: [4I5I](https://doi.org/10.2210/pdb4I5I/pdb)) [<https://doi.org/10.2210/pdb4I5I/pdb>], *HsSIRT2*•NAD⁺•TNFn-5 (PDB: [8QT3](https://doi.org/10.2210/pdb8QT3/pdb)) [<https://doi.org/10.2210/pdb8QT3/pdb>], *HsSIRT3*•NAD⁺•EX527 (PDB: [4BV3](https://doi.org/10.2210/pdb4BV3/pdb)) [<https://doi.org/10.2210/pdb4BV3/pdb>], *EcCobB*•HsH4 peptide (PDB: [1S5P](https://doi.org/10.2210/pdb1S5P/pdb)) [<https://doi.org/10.2210/pdb1S5P/pdb>], AlphaFold2 of *BsSrtN*.
- c.** SnCobB elutes as higher oligomer and monomer from SEC. The preparative SEC (S75 16/600) suggests CobB forms a higher oligomer and a monomer. This is confirmed on a calibrated analytical S200 10/300 column. The elution volumes and calculated molecular weights (calc. MW) were used in comparison to expected molecular weights (exp. MW) to calculate the apparent oligomeric state (Supplementary Fig. 2). A₂₈₀ is the absorption at 280 nm. mAU: milli absorbance units. SDS-PAGEs were stained with Coomassie brilliant blue (CBB). Source data are provided as Source Data file.

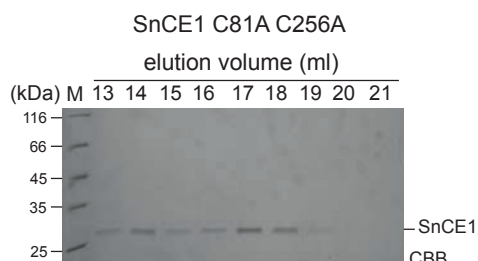
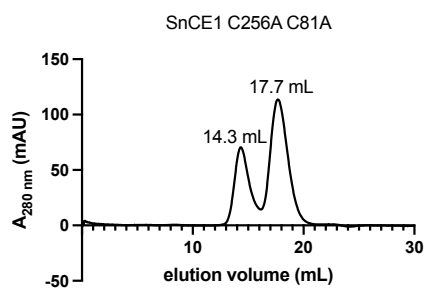
a



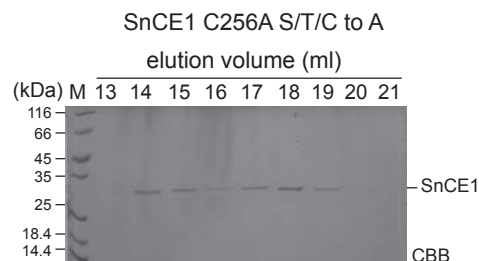
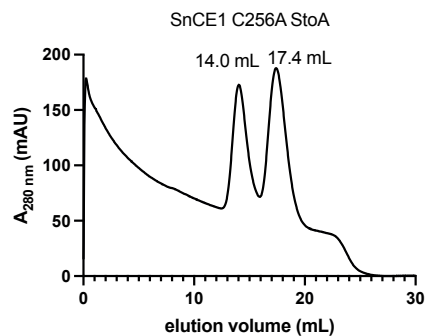
b



c

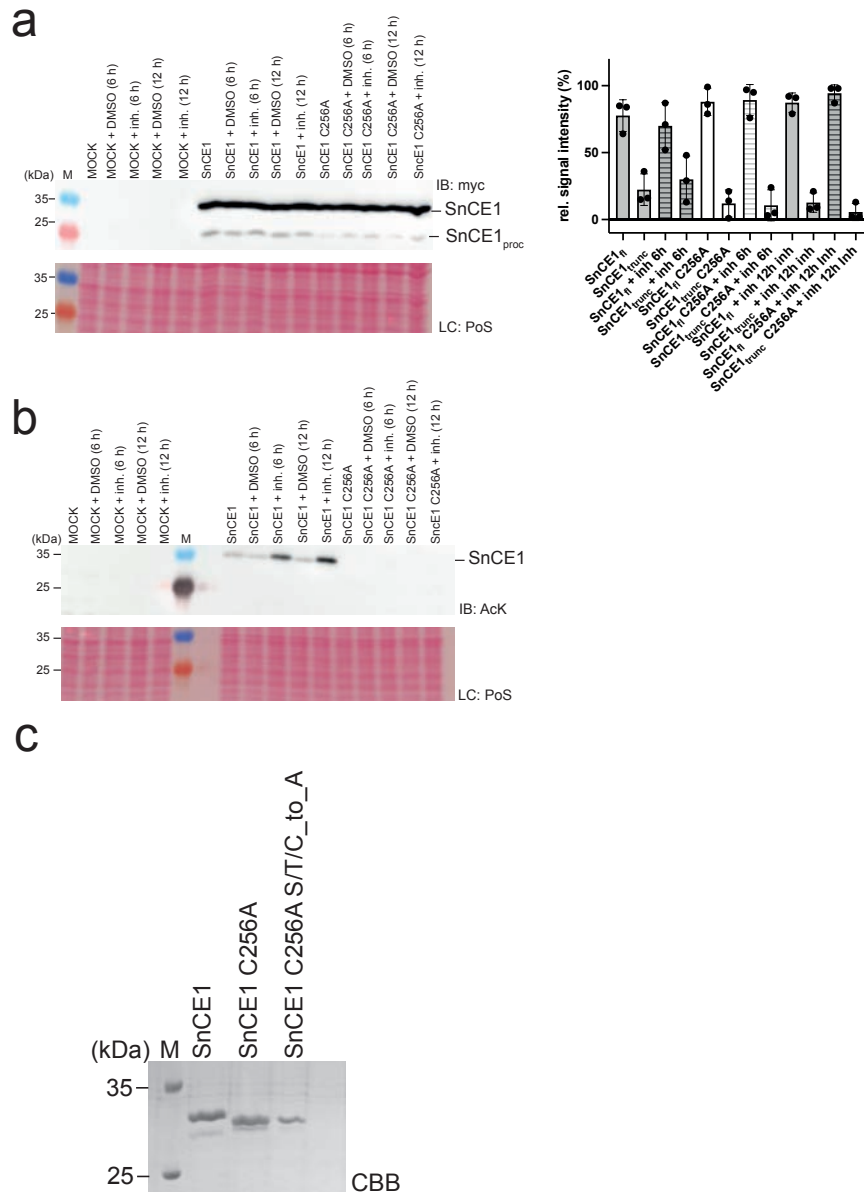


d



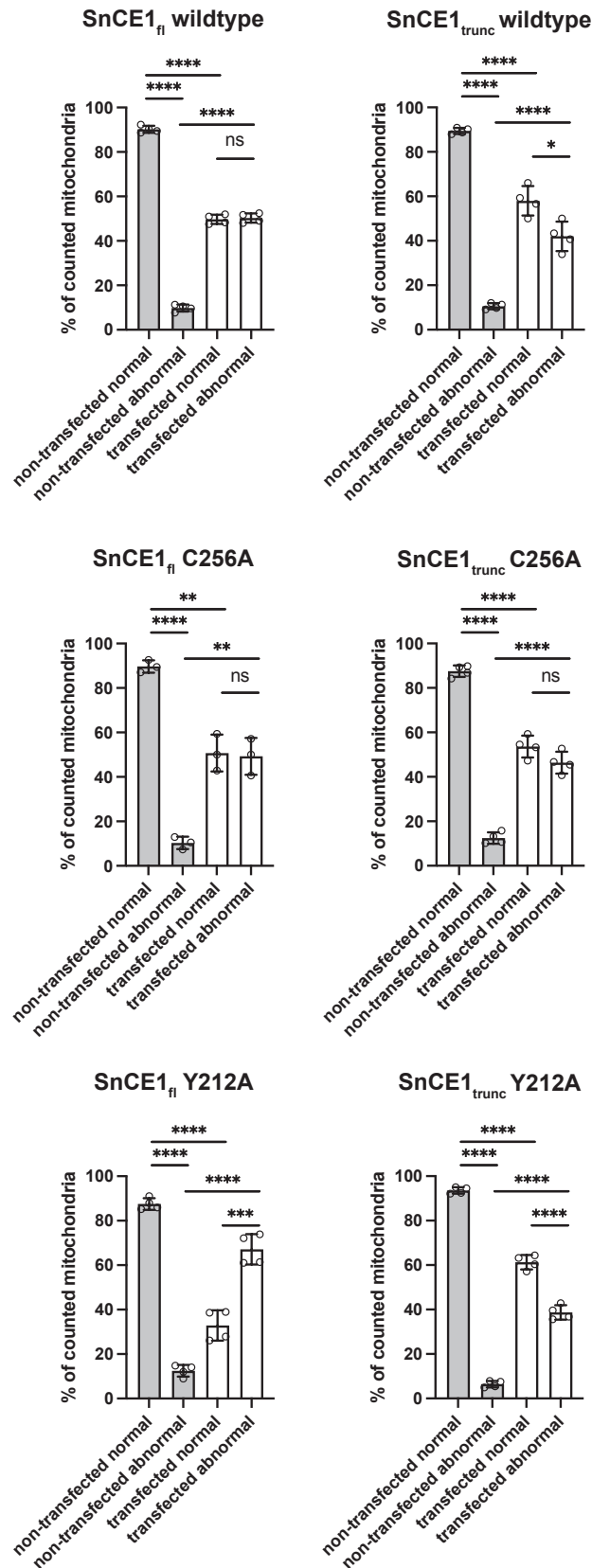
Supplementary Figure 18: Analytical SEC on a S200 10/300 column of deacetylated SnCE1 C256A AcK231 and SnCE1 Y212A AcK231, SnCE1 C256A C81A and SnCE1₁₀₄₋₃₁₀ C256A.

- a. The treatment of SnCE1 C256A AcK231 and SnCE1 Y212A AcK231 with human SIRT1 in presence of NAD⁺ (peak at 23.1 mL; absorbance $A_{254\text{ nm}}$) does not affect its oligomeric state, i.e. both proteins elute as pure monomers. A_{280} is the absorption at 280 nm. mAU: milli absorbance units. Fractions were analysed by SDS-PAGE and gels were stained with Coomassie brilliant blue (CBB). The experiment was performed in two independent replicates ($n=2$). Source data are provided as Source Data file.
- b. Analytical size-exclusion chromatography (SEC) of SnCE1₁₀₄₋₃₁₀ C256A shows that it elutes as monomer and SnCE1₁₀₄₋₃₁₀ C256A as tetramer. The SDS-PAGE gel shows no truncation due to proteolytic processing suggesting in SnCE1₁₇₄₋₃₁₀ a truncation from the N-terminus. The elution volumes and calculated molecular weights (calc. MW) were used in comparison to expected molecular weights (exp. MW) to calculate the apparent oligomeric state (Supplementary Fig. 2b). A_{280} is the absorption at 280 nm. mAU: milli absorbance units. SDS-PAGE gels were stained with Coomassie brilliant blue (CBB). Source data are provided as Source Data file.
- c. Analytical SEC of SnCE1 C256A/C81A shows it elutes in a tetramer-monomer equilibrium. The elution volumes and calculated molecular weights (calc. MW) were used in comparison to expected molecular weights (exp. MW) to calculate the apparent oligomeric state (Supplementary Fig. 2b). A_{280} is the absorption at 280 nm. mAU: milli absorbance units. The SDS-PAGE gel was stained with Coomassie brilliant blue (CBB). Source data are provided as Source Data file.
- d. Analytical SEC of SnCE1 multiple S to A mutant shows it elutes in a tetramer-monomer equilibrium. The elution volumes and calculated molecular weights (calc. MW) were used in comparison to expected molecular weights (exp. MW) to calculate the apparent oligomeric state (Supplementary Fig. 2b). A_{280} is the absorption at 280 nm. mAU: milli absorbance units. The SDS-PAGE gel was stained with Coomassie brilliant blue (CBB). Source data are provided as Source Data file.



Supplementary Fig. 19: SnCE1 full length is proteolytically processed at the N-terminus in eukaryotic HEK-293 cells and the mutant SnCE1 C256A S/T/CtoA is proteolytically processed.

- SnCE1 full length (SnCE1_{f.l.}) and SnCE1_{f.l.} C256A were transiently expressed in HEK-293 cells as C-terminal myc-His₆ fusion protein. Immunoblotting using an anti-myc-antibody shows the signals representing SnCE1_{f.l.} and the proteolytically processed form were stained suggesting processing occurs from the N-terminus (IB: myc). Ponceau S red staining was done as loading control (LC: PoS). Experiments were performed in three biological replicates and bars depict means $\pm$ standard deviations. Statistical significance was tested using t-tests. All comparisons did not show statistical significance. Source data are provided as Source Data file
- SnCE1 full length (SnCE1_{f.l.}) and SnCE1_{f.l.} C256A were transiently expressed in HEK-293 cells as C-terminal myc-His₆ fusion protein as described in a. Immunoblotting using an anti-acetyl-lysine antibody (IB: AcK) shows the acetylation of SnCE1_{f.l.}. Treatment with lysine deacetylase inhibitor cocktail, i.e. TSA and SAHA were used to inhibit classical HDACs and nicotinamide (NA) to inhibit sirtuins for 6 h or 12 h as indicated. DMSO was used as control. Ponceau S red staining was done as loading control (LC: PoS). Experiments were performed in three biological replicates and bars depict means $\pm$ standard deviations. Source data are provided as Source Data file.
- The mutant SnCE1 C256A S/T/CtoA, in which several Ser/Thr/Cys residues in the sequence range of Ser74-Ser90, were mutated to Ala, i.e. S74A/T76A/S77A/C81A/ S84A/S86/S87A/S90A, was recombinantly expressed and purified. The protein shows proteolytic processing as SnCE1 C256A indicating the mutations did not affect proteolytic processing. For comparison the non-processed SnCE1 wildtype was loaded on the SDS-PAGE gel. The gel was stained with Coomassie brilliant blue (CBB).



Supplementary Fig. 20: Quantification of the mitochondrial fragmentation observed upon expression of SnCE1 in HEK-293T cells. The fluorescence images shown in Fig. 8c were quantified and the number of non-fragmented and fragmented number was expressed as % of all mitochondria. Experiments were performed in two biological replicates each consisting of two technical replicates ($n=4$). Bars depict means $\pm$ standard deviations of determined miller units. Statistical significance (*: $p < 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$; ns: not significant) was tested using t-tests. Source Data are provided as Source Data file.

a

SnCE1

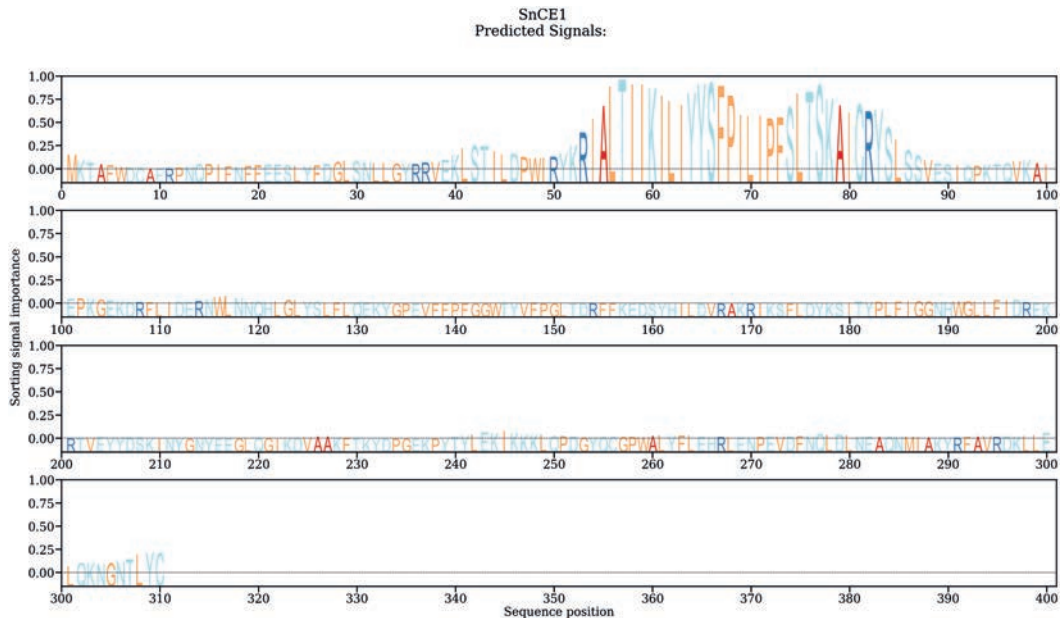
Predicted localizations: Cytoplasm, Endoplasmic reticulum
Predicted signals:

Localization	Cytoplasm	Nucleus	Extracellular	Cell membrane	Mitochondrion	Plastid	Endoplasmic reticulum	Lysosome/Vacuole	Golgi apparatus	Peroxisome
Probability	0.5856	0.4577	0.1875	0.2335	0.4139	0.0721	0.6866	0.1530	0.3577	0.0384

Probability thresholds for the subcellular localizations. A localization is predicted if its probability is above the threshold shown below:

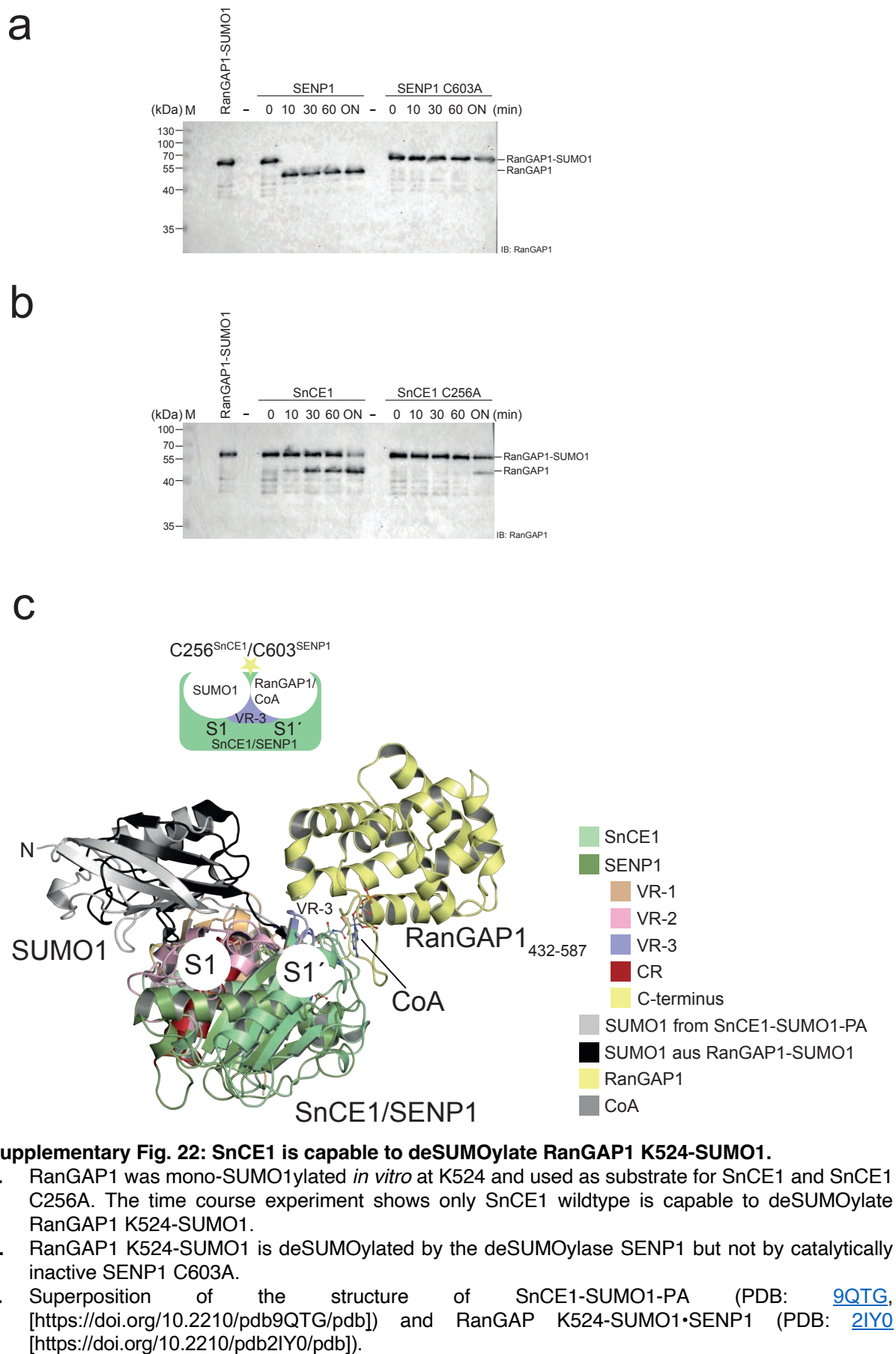
Localization	Cytoplasm	Nucleus	Extracellular	Cell membrane	Mitochondrion	Plastid	Endoplasmic reticulum	Lysosome/Vacuole	Golgi apparatus	Peroxisome
Threshold	0.4761	0.5014	0.6173	0.5646	0.6220	0.6395	0.6090	0.5848	0.6494	0.7364

b



Supplementary Fig. 21: Analyses of SnCE1 regarding its subcellular localization by DeepLoc (<https://services.healthtech.dtu.dk/services/DeepLoc-2.0/>)⁹.

- a. The sequence of full length SnCE1 was analysed by DeepLoc. This analysis reveals SnCE1 localizing with high probability to the ER and to the cytosol, whereas localization to the mitochondria is not predicted with high probability. The table at the bottom shows the thresholds indicating a localization to the compartments.
- b. Localization to the ER is derived from the presence of a predicted ER localization signal in the amino acid region covering residues 55 to 83 with sorting signal importance scores >0.50.



Supplementary Table 1: Data collection and refinement statistics (molecular replacement) for the structures of *Simkania negevensis* CE1 (SnCE1) wildtype, SnCE1 C256A and SnCE1-SUMO1-PA.

	SnCE1 wildtype ^{1*} (PDB: 9QTE) [https://doi.org/10.2210/pdb9QTE/pdb]	SnCE1 C256A ^{2*} (PDB: 9QTF) [https://doi.org/10.2210/pdb9QTF/pdb]	SnCE1-SUMO1-PA (PDB: 9QTG) [https://doi.org/10.2210/pdb9QTG/pdb]
Data collection			
Space group	I4	P12 ₁ 1	P2 ₁ 2 ₁ 2 ₁
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	105.645, 105.645, 122.086	78.626, 151.173, 80.777	34.967, 56.164, 148.651
α , β , γ (°)	90, 90, 90	90, 105.07, 90	90, 90, 90
Resolution (Å)	47.27 (2.82) ^{3*}	48.55 (2.20)	49.55 (1.55)
<i>R</i> _{merge}	0.228 (0.941)	0.108 (1.715)	0.117 (1.397)
<i>I</i> / σ <i>I</i>	8.9 (2.0)	9.7 (0.6)	11.9 (1.7)
Completeness (%)	98.4 (89.1)	96.0 (44.3)	100 (100)
Redundancy	12.9 (7.5)	6.9 (3.5)	12.6 (11.4)
Refinement			
Resolution (Å)	2.82	2.20	1.55
No. reflections	205043	608615	551740
<i>R</i> _{work} / <i>R</i> _{free}	0.247 / 0.269	0.155 / 0.191	0.153/0.216
No. atoms			
Protein	5057	13537	2360 (1728 SnCE1, 632 hsSUMO1)
Ligand/ion	14 (MLI)	68 (TRS, EDO)/3	12 (AYE, BME)/1
Water	10	881	268
<i>B</i> -factors			
Protein (main chain/side chain)	63.12 (61.42/64.64)	53.6 (50.7/56.18)	31.05 (28.46/33.38)
Ligand/ion	65.79	63.33/64.54	37.94/25.81
Water	33.34	50.63	37.16
R.m.s. deviations			
Bond lengths (Å)	0.0088	0.0141	0.0166
Bond angles (°)	1.57	1.90	1.92

^{1*/2*}: for each structure one crystal was used.

^{3*}: values in parentheses are for highest-resolution shell.

Supplementary Table 2: Prediction of SUMO-interaction motifs (SIMs) and SUMOylation

ID	Position	Peptide	Score	Cut-off	Type	sites in
SnCE1	209	TVEYYDSKINYGNYE	0.8946	0.82	SUMOylation	
SnCE1	298-302	RFAVRDKLLELQKNGNTLY	0.8608	0.85	SUMO interaction	
SnCE1	228	GIKDVAAKFTKYDPG	0.8429	0.82	SUMOylation	
SnCE1	231	DVAAKFTKYDPGEKP	0.8327	0.82	SUMOylation	
SnCE1	178	IKSFLDYKSITYPLF	0.8233	0.82	SUMOylation	

SnCE1. The online tool GPS-SUMO 2.0 (<https://sumo.biocuckoo.cn/index.php>) was used to predict potential SIMs and SUMOylation sites in SnCE1. Red: SIMs and lysines predicted to be SUMOylated.

Supplementary Table 3: Primers for site-directed mutagenesis of SnCE1 and Gibson assembly of truncated SnCE1 variants.

Primer	Sequence 5' -> 3'
Gibson assembly	
Gib_pOPIN-S_SnCE1_83-310_f	CGCGAACAGATCGGTGGTTATTCACTATCTTCTGTTGAAAG
Gib_pOPIN-S_SnCE1_1-310_r	GGTGTTTAAATGGTCTAGAAAGCTTTAACAATACAAAGTATTTCCATTT TTTTG
Gib_pGEX_SnCE1_fl_r	GTCAGTCACGATGCGGCCGCTCGAGTTAACAATACAGGGTATTGC
Gib_pGEX_SnCE1_95-310_f	TCTGTTCCAGGGGCCCTGGGATCCACCCAGGTTAAAGCCATTG
Gib_pGEX_SnCE1_104-310_f	TCTGTTCCAGGGGCCCTGGGATCCGGTGAAAAAGATCGTTTTTC
Site-directed mutagenesis	
QC_pOPIN-S_SENP1_C603A_f	GCAGATGAATGGAAGTGACGCTGGGATGTTTGCCTGCAAATATG
QC_pOPIN-S_SENP1_C603A_r	CATATTTGCAGGCAAACATCCCAGCGTCACTTCCATTTCATCTGC
QC_pGEX_SnCE1_Y146A_f	CGGAAAAACAGCGGTCCAACCG
QC_pGEX_SnCE1_Y146A_r	CGGTTGGACCGCTGTTTTTCCG
QC_pGEX_SnCE1_H190A_f	GTTTATTGGTGGCAATGCTTGGGGCCTG
QC_pGEX_SnCE1_H190A_r	CAGGCCCCAAGCATTGCCACCAATAAAC
QC_pGEX_SnCE1_W191A_f	CGCTGTTTATTGGTGGCAATCATGCGGGCCTGCTGTT
QC_pGEX_SnCE1_W191A_r	AACAGCAGGCCCGCATGATTGCCACCAATAAACAGCG
QC_pGEX_SnCE1_S208A_f	CGCACCGTTGAATATTATGATGCTAAAAATTAACACG
QC_pGEX_SnCE1_S208A_r	CTTCGTAGTTACCGTAGTTAATTTTAGCATCATAATATTCAACG
QC_pGEX_SnCE1_K209Q_f	GTTGAATATTATGATAGTCAAATTAACACGTTAACTAC
QC_pGEX_SnCE1_K209Q_r	GTAGTTACCGTAGTTAATTTGACTATCATAATATTCAAC
QC_pGEX_SnCE1_Y212A_f	GTAAAATTAACGCCGGTAACTACG
QC_pGEX_SnCE1_Y212A_r	GTTACCGGCGTTAATTTTACTATC
QC_pGEX_SnCE1_K247R_f	CTGGAAAAAATTAAGAGAAAGCTGCAGCC
QC_pGEX_SnCE1_K247R_r	GGCTGCAGCTTTCTCTTAATTTTTTCCAG
QC_pGEX_SnCE1_C256A_f	GATGGTTATCAGGCCGGTCCGTGGGC
QC_pGEX_SnCE1_C256A_r	GCCCACGGACCGGCCTGATAACCATC
QC_pcDNA_SnCE1_Y212A_f	GCAAGATCAATGCCGGCAACTACGAG
QC_pcDNA_SnCE1_Y212A_r	CTCGTAGTTGCCGGCATTGATCTTGC
QC_pcDNA_SnCE1_C256A_f	CGGCTACCAGGCTGGCCCTTG
QC_pcDNA_SnCE1_C256A_r	CAAGGGCCAGCCTGGTAGCCG

Supplementary Table 4: Bacterial and mammalian expression vectors used in this study.

Vector	Insert	Origin/Source/Manufacturer
Bacterial expression vectors		
pGEX6P.1	SnCE1 (74 – 310)	BioCat GmbH, Heidelberg
pGEX6P.1	SnCE1 (74 – 310) Y146A	this study
pGEX6P.1	SnCE1 (74 – 310) H190A	this study
pGEX6P.1	SnCE1 (74 – 310) H190A C256A	this study
pGEX6P.1	SnCE1 (74 – 310) W191A	this study
pGEX6P.1	SnCE1 (74 – 310) S208A	this study
pGEX6P.1	SnCE1 (74 – 310) S208A C256A	this study
pGEX6P.1	SnCE1 (74 – 310) K209Q	this study
pGEX6P.1	SnCE1 (74 – 310) Y212A	this study
pGEX6P.1	SnCE1 (74 – 310) K247R	this study
pGEX6P.1	SnCE1 (74 – 310) C256A	this study
pGEX6P.1	SnCE1 (95 – 310)	this study
pGEX6P.1	SnCE1 (95 – 310) C256A	this study
pGEX6P.1	SnCE1 (104 – 310)	this study
pGEX6P.1	SnCE1 (104 – 310) C256A	this study
pOPIN-S	SnCE1 (83 – 310)	this study
pOPIN-S	SnCE2 (144 – 431)	Reference: ¹⁰
pOPIN-S	SnCE3 (120 – 431)	Reference: ¹⁰
pOPIN-S	SnCE4 (60 – 504)	Reference: ¹⁰
pOPIN-S	SnCE5 (1 - 174)	Reference: ¹⁰
pOPIN-S	SENP1 (415 – 644)	Reference: ¹⁰
pRSF-Duet1	GST-SnCE1 (74 – 310) C81A C256A	BioCat GmbH, Heidelberg
pRSF-Duet1	GST-SnCE1 (74 – 310) Ack78 C256A	BioCat GmbH, Heidelberg
pRSF-Duet1	GST-SnCE1 (74 – 310) Ack94 C256A	BioCat GmbH, Heidelberg
pRSF-Duet1	GST-SnCE1 (74 – 310) Ack98 C256A	BioCat GmbH, Heidelberg
pRSF-Duet1	GST-SnCE1 (74 – 310) Ack103 C256A	BioCat GmbH, Heidelberg
pRSF-Duet1	GST-SnCE1 (74 – 310) Ack106 C256A	BioCat GmbH, Heidelberg
pRSF-Duet1	GST-SnCE1 (74 – 310) Ack209 C256A	BioCat GmbH, Heidelberg
pRSF-Duet1	GST-SnCE1 (74 – 310) Ack231 Y212A	BioCat GmbH, Heidelberg
pRSF-Duet1	GST-SnCE1 (74 – 310) Ack231 C256A	BioCat GmbH, Heidelberg
pRSF-Duet1	GST-SnCE1 (74 – 310) Ack248 C256A	BioCat GmbH, Heidelberg
pRSF-Duet1	GST-SnCE1 (74-310) C256A S/T/C_to_A	BioCat GmbH, Heidelberg
pRSF-Duet1	SnCobB (1 – 262)	BioCat GmbH, Heidelberg
pTXB1	ubiquitin (1 – 75)	Reference: ¹⁰
pTXB1	3xFLAG-SUMO1 (1 – 96)	Reference: ¹⁰
pTXB1	SUMO1 (20 – 96)	BioCat GmbH, Heidelberg
pTXB1	SUMO3 (1 – 91)	Reference: ¹⁰
Mammalian expression		
pcDNA3.1(+)	SnCE1 (1 – 310)	BioCat GmbH, Heidelberg
pcDNA3.1(+)	SnCE1 (74 – 310)	BioCat GmbH, Heidelberg
pcDNA3.1(+)	SnCE1 (1 – 310) K78Q C256A	BioCat GmbH, Heidelberg
pcDNA3.1(+)	SnCE1 (1 – 310) C81A C256A	BioCat GmbH, Heidelberg
pcDNA3.1(+)	SnCE1 (1 – 310) S208A	BioCat GmbH, Heidelberg
pcDNA3.1(+)	SnCE1 (1 – 310) S208A C256A	BioCat GmbH, Heidelberg
pcDNA3.1(+)	SnCE1 (1 – 310) Y212A	this study
pcDNA3.1(+)	SnCE1 (74 – 310) Y212A	this study
pcDNA3.1(+)	SnCE1 (1 – 310) C256A	BioCat GmbH, Heidelberg
pcDNA3.1(+)	SnCE1 (74 – 310) C256A	this study
pcDNA3.1(+)	SnCE1 (74-310) C256A S/T/C_to_A	BioCat GmbH, Heidelberg

Supplementary Table 5: Antibodies used in this study.

Manufacturer/Source	Cat no.	Antigen	Clonality	Dilution	Host
Primary antibodies					
abcam, Cambridge UK	ab21623	acetyl-L-lysine	polyclonal	1:5000 1:10000	rabbit
provided by Prof. Frauke Melchior, Dr. Annette Flotho, Heidelberg University	non-commercial	RanGAP1	polyclonal	1:1000	goat
Davids Biotechnologie, Regensburg Germany	non-commercial	SnCE1	polyclonal	1:50 1:100	rabbit
Thermo Fisher	MA5-37627	SUMO2	monoclonal	1:2000	mouse
Santa Cruz	sc-23954	Calnexin	monoclonal	1:100	mouse
Proteintech	16686-1-AP	CKAP4	polyclonal	1:1000	rabbit
Abcam	ab48139	Mitofilin/Mic60	polyclonal	1:1000	rabbit
GeneTex	GTX102150	Rieske FeS	polyclonal	1:500	rabbit
Santa Cruz	sc-5274	Tubulin beta	monoclonal	1:1000	mouse
GeneTex	GTX18184	His	monoclonal	1:1000	mouse
Gramsch laboratories	CM-100	Myc	polyclonal	1:5000	rabbit
Elabscience	E-AB-20007	Myc-Tag	Monoclonal	1:1000	mouse
Secondary antibodies (HRP-coupled)					
abcam, Cambridge UK	ab97110	goat IgG H&L	polyclonal	1:1000	donkey
abcam, Cambridge UK	ab6728	mouse IgG H&L	polyclonal	1:2000	rabbit
abcam, Cambridge UK	ab6721	rabbit IgG H&L	polyclonal	1:5000 1:10000	goat
Thermo Fisher Scientific	A16066	mouse IgG H&L - HRP	polyclonal	1:3000	goat
Jackson ImmunoResearch	111-035-144	rabbit IgG H&L - HRP	polyclonal	1:3000	goat
Thermo Fisher Scientific	A-11001	anti-mouse-IgG H&L-Alexa488	polyclonal	1:100	goat
Thermo Fisher Scientific	A-21422	anti-mouse-IgG H&L-Alexa555	polyclonal	1:100	goat
Jackson ImmunoResearch	115-225-146	Anti-mouse-IgG H&L-Cy2	polyclonal	1:100	goat
Cell Signaling	7076S	mouse IgG	polyclonal	1:15.000	horse
Cell Signaling	7074S	rabbit IgG	polyclonal	1:15.000	goat
LI-COR bioscience	925-68072	IRDye 680RD mouse IgG	polyclonal	1:10.000	goat
LI-COR bioscience	925-32213	IRDye 800CW rabbit IgG	polyclonal	1:5.000	goat

Supplementary Table 6: Reversed phase liquid chromatography (RPLC).

TRAP COLUMN	75 µm inner diameter, packed with 3 µm C18 particles (Acclaim PepMap100, Thermo Scientific)
ANALYTICAL COLUMN	Accucore 150-C18, (Thermo Fisher Scientific) 25 cm x 75 µm, 2,6 µm C18 particles, 150 Å pore size
BUFFER SYSTEM	binary buffer system consisting of 0.1% acetic acid in HPLC-grade water (buffer A) and 100% ACN in 0.1% acetic acid (buffer B)
FLOW RATE	300 nl/min
GRADIENT	0 min 2% B → 2 min 5% B → 18 min 25% B → 20 min 40% B → 22 min 90% B → 25 min 90% B → 26 min 2% B → 29 min 2% B → 30 min 2% B
GRADIENT DURATION	30 min
COLUMN OVEN TEMPERATURE	40°C

Supplementary Table 7: Mass spectrometry.

INSTRUMENT	Q Exactive Plus mass spectrometer (Thermo Scientific)
ELECTROSPRAY	Nanospray Flex Ion Source
OPERATION MODE	Data dependent acquisition
METHOD DURATION	30 min
FULL MS	
MS SCAN RESOLUTION	70000
AGC TARGET	3e6
MAXIMUM ION INJECTION TIME FOR THE MS SCAN	120
SCAN RANGE	333 to 1650 m/z
POLARITY	positive
SPECTRA DATA TYPE	profile
INTENSITY THRESHOLD	8300
CHARGE STATE	include 2-6
DYNAMIC EXCLUSION	30 s
DD-MS2	
NUMBER OF DEPENDENT SCANS	10
RESOLUTION	17,500
MAXIMUM ION INJECTION TIME MODE	120
DATA TYPE	centroid
DYNAMIC EXCLUSION	30 s
MICROSCANS	1
ISOLATION WINDOW	13 m/z overlap
FIRST MASS	100
DISSOCIATION MODE	higher energy collisional dissociation (HCD)
MINIMUM AGC TARGET	1000
APEX TRIGGER	1 to 40 s
HCD NORMALIZED COLLISION ENERGY	27.5

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