



493
 SENP1 ITEEMEKE IKNVFRNGNQDEV LSEA-----FRLTITRK⁵⁶⁸DIQTLNHLNWLND⁵⁸²EI⁵⁸⁵INFYMNMLMERSKEKGL
 SENP2 LTEDMEKE ISNALGHGPQDEI LSSA-----FKLRITRGDIQTLKNYHWLND⁵⁸²EV⁵⁸⁵INFYMNLLVERNKKQGY
 SENP3 LVLQLIQSYQRMFGNAMVRGFRVAY-----KRHVL⁵⁶⁸TMD⁵⁸²DLGTL⁵⁸⁵YGQNW⁵⁸²LNDQVMNMYGDLVMDTVPE---
 SENP5 **PFINREITNYRARHQKCNFRIFYNK-----HMLDMD⁵⁶⁸DLAT⁵⁸²LDGQNW⁵⁸⁵LNDQVINMYGELIMDAVPDK-**
 SENP6 -FDEEETGENHTIFIGPVEK⁵⁶⁸LIVYPPPPAKGGISV⁵⁸²TNE⁵⁸⁵DLHCLNEGEF⁵⁸²LNDVI⁵⁸⁵IDFY⁵⁸²LKYLVLLEKLKED
 SENP7 -ITSNPDEEWREVRHTGLVQK⁵⁶⁸LIVYPPPTKGG⁵⁸²LV⁵⁸⁵TNE⁵⁸²DLECL⁵⁸⁵EEGEF⁵⁸²LNDVI⁵⁸⁵IDFY⁵⁸²LKYLILEKASDEL
 SENP8 -----MDPVVLSYMD-----SLLRQSD⁵⁶⁸VSIL⁵⁸²DPPSW⁵⁸⁵LNDHI⁵⁸²IGFAFEYFANS-QFHDHC
 Ulp1 ELNEKDDDQVQKALASRENTQLM-----NRDNIET⁵⁶⁸VRD⁵⁸²FKTLAPRRW⁵⁸⁵LNDTI⁵⁸²IEFFMKYIEKST-----
 Ulp2 ATTEFETPEL⁵⁶⁸FKPSLCY---KFN-----DGSSYTITN⁵⁸²QD⁵⁸⁵FKCLFNKDWV⁵⁸²ND⁵⁸⁵SILDF⁵⁸²FTKFYIES⁵⁸⁵IEKSI



607 609 613
 SENP1 --P-SVHAFNTFFFTKLKTA-----GYQAV⁶²⁴KRWT⁶²⁷KKVD⁶⁴²VSVDIL⁶⁴⁶LVP⁶⁴²IHL---GVH⁶⁴⁶WCL
 SENP2 --P-ALHVFSTFFYPK⁶⁰⁷LKSG-----GYQAV⁶²⁴KRWT⁶²⁷KG⁶⁴²VNL⁶⁴⁶FEQEII⁶⁴²LVP⁶⁴⁶IHR---KVH⁶⁴⁶WSL
 SENP3 ---KVHFFNSFFYDK⁶⁰⁷LRTK-----GYDGV⁶²⁴KRWT⁶²⁷KN⁶⁴²VD⁶⁴⁶IFNKE⁶⁴²LLI⁶⁴⁶PIHL---EVH⁶⁴⁶WSL
 SENP5 -----VHFFNSFFH⁶⁰⁷RQL⁶⁰⁹VTKG-----YNGV⁶²⁴KRWT⁶²⁷KK⁶⁴²VD⁶⁴⁶LEFKS⁶⁴²LLI⁶⁴⁶PIHL---EVH⁶⁴⁶WSL
 SENP6 --ADRIHIFSSFFYK⁶⁰⁷RLN⁶⁰⁹Q⁶¹³RER-RNHETT⁶²⁴NLSIQ⁶²⁷QKRHGR⁶⁴²VKT⁶⁴⁶WTRH⁶⁴²VD⁶⁴⁶IFEKDF⁶⁴²IFVP⁶⁴⁶LNE---AAH⁶⁴⁶WFL
 SENP7 --VERSHIFSSFFYK⁶⁰⁷CLTRKEN⁶⁰⁹NLTEDN⁶¹³PNLSMAQRRH⁶²⁴KRV⁶²⁷RT⁶⁴²WTRH⁶⁴⁶INI⁶⁴²FNKDY⁶⁴⁶IFVP⁶⁴²VNE---SSH⁶⁴⁶WYL
 SENP8 --SDHVSFISPEVTQ⁶⁰⁷FIK⁶⁰⁹CTSN-----PAEIAM⁶²⁴FLEPL⁶²⁷DL⁶⁴²PNKRV⁶⁴⁶VFLAIND⁶⁴²NSNQAAG⁶⁴⁶TH⁶⁴²WSL
 Ulp1 --PNTVA-FNSFFY⁶⁰⁷TN⁶⁰⁹LSE-----RGYQGV⁶²⁴VR⁶²⁷WMK⁶⁴²RKKT⁶⁴⁶QID⁶⁴²KL⁶⁴⁶DKI⁶⁴²FTP⁶⁴⁶INLNQ⁶⁴²SH⁶⁴⁶WAL
 Ulp2 IKREQVH⁶⁰⁷LMS⁶⁰⁹SFFY⁶¹³TK⁶¹³LISNPA-----DYYSN⁶²⁴VK⁶²⁷KW⁶⁴²VNNT⁶⁴⁶DL⁶⁴²FSK⁶⁴⁶YVVI⁶⁴²FINI---SYH⁶⁴⁶WFS
 *** ** *



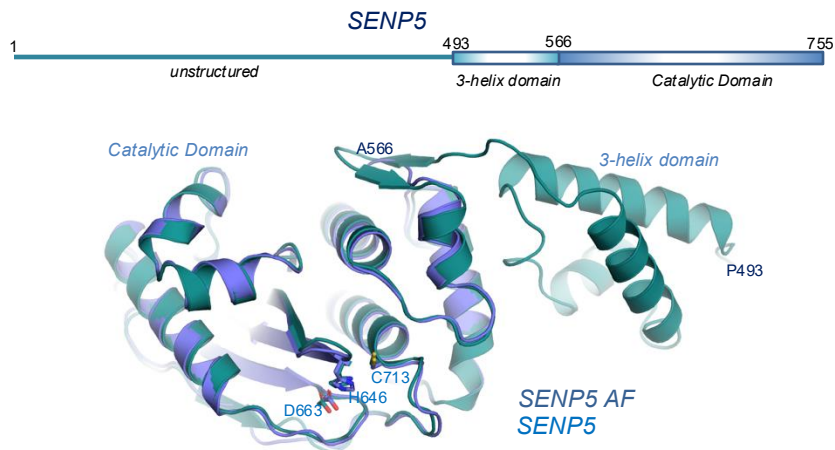
663
 SENP1 AVVDF-----RKKNITYYDS⁶⁶³SMGG-INNEACR⁶⁶³IL⁶⁶³LQYL⁶⁶³KQESID⁶⁶³KKRKEFD⁶⁶³T--NGWQ⁶⁶³LFSK
 SENP2 VVIDL-----RKKCLKYLDS⁶⁶³SMGQ-KGHRICE⁶⁶³IL⁶⁶³LQYL⁶⁶³QDESK⁶⁶³TKRNSDLNLL--EWTH⁶⁶³HS
 SENP3 ISVDV-----RRRTITYYDS⁶⁶³QRTLNRRCPKH⁶⁶³IAK-⁶⁶³YLQAEAVKK⁶⁶³DR⁶⁶³LDF-----HQGW⁶⁶³KGY
 SENP5 **ITVTL-----SNRIISFYDS⁶⁶³QGIHFKFCVEN⁶⁶³IRK-⁶⁶³YLLTEAREKNRPEF-----LQGW⁶⁶³QTA**
 SENP6 AVVCFPGLEKPKYE//PTICKQPCILLMDS⁶⁶³SLRGPSRSNVV⁶⁶³KIL⁶⁶³REY⁶⁶³LEV⁶⁶³EW⁶⁶³EV⁶⁶³KK⁶⁶³SK⁶⁶³RS⁶⁶³FS⁶⁶³KD⁶⁶³VM⁶⁶³KG⁶⁶³SNP
 SENP7 AVICFPWLEEAVYE//KKMCKRPCILILDS⁶⁶³SLKAASVQNTVQ⁶⁶³NIL⁶⁶³REY⁶⁶³LEV⁶⁶³EW⁶⁶³EV⁶⁶³KL⁶⁶³TH⁶⁶³RQ⁶⁶³FS⁶⁶³KTN⁶⁶³MVD⁶⁶³LC
 SENP8 LVYLQ-----DKNSFFHYDS⁶⁶³SHSRNSVHAKQVA-EKLEAFLGR⁶⁶³KGD⁶⁶³KLAF-----
 Ulp1 GIIDL-----KKKTIGYVDS⁶⁶³LSNGPNAMSFA⁶⁶³IL⁶⁶³TDLQKYVMEESKHTIGEDF----DLIHL
 Ulp2 CIITNLD//AINSDEISINNPLVNILTFDS⁶⁶³SLRQT-HSREID⁶⁶³PIKE⁶⁶³FLISYALDK⁶⁶³YSIQ⁶⁶³LDK⁶⁶³TQ⁶⁶³IK⁶⁶³MKT---



713
 SENP1 KSQEI⁷¹³PQQMNGSD⁷¹³CGMFACKYADCITKDRPI-----NFTQ⁷¹³QHMPYFRKRMVWEIL⁷¹³HRK⁷¹³IL-----
 SENP2 KPHEI⁷¹³PQQLNGSD⁷¹³CGMFTCKYADYISRDKPI-----TFTQ⁷¹³QHMPYFRKRMVWEIL⁷¹³HQ⁷¹³QIL-----
 SENP3 FKMNVARQNNDS⁷¹³CGAFV⁷¹³LQYCKHLALSQPF-----SFTQ⁷¹³QDM⁷¹³PKLR⁷¹³RQ⁷¹³IYKELCHCK⁷¹³ITV-----
 SENP5 **VTKCI⁷¹³PQQKNDSD⁷¹³CGV⁷¹³FLQYCKCLALEQPF-----QFSQEDMPVR⁷¹³KRIYKELCECR⁷¹³IMD-----**
 SENP6 ---KV⁷¹³PQQNNFSD⁷¹³CGV⁷¹³YV⁷¹³LQYVESFFENPILSFELPMNLANW⁷¹³PPPR⁷¹³MRTKREEIRN⁷¹³IL⁷¹³KLQEDQSKEKRK
 SENP7 ---KV⁷¹³PKQDNSSD⁷¹³CGVYLLQYVESFFKDPIVNFELPIHLEK⁷¹³W⁷¹³PRHVIKTKREDIREL⁷¹³IL⁷¹³KLHL⁷¹³QQQKGS
 SENP8 VEEKA⁷¹³PAQQNSYD⁷¹³CGMY⁷¹³ICNTEALCQNF⁷¹³FRQTE-----SL⁷¹³LQ⁷¹³LLTPAYITKKRG⁷¹³EWD⁷¹³LDIT⁷¹³T⁷¹³LAKK---
 Ulp1 ---DC⁷¹³PQQPN⁷¹³GYD⁷¹³CGIY⁷¹³VMNTLYGSADAPLD-F-----DY-KDAIRM⁷¹³R-FIAHL⁷¹³IL⁷¹³TDAL⁷¹³K-----
 Ulp2 --CPV⁷¹³PQQPN⁷¹³MSD⁷¹³CGVH⁷¹³VILNIRKFFE-NPVETIDV-----WK-NSKIKSKH-FTAKM⁷¹³INKYFDK⁷¹³NERNSAR

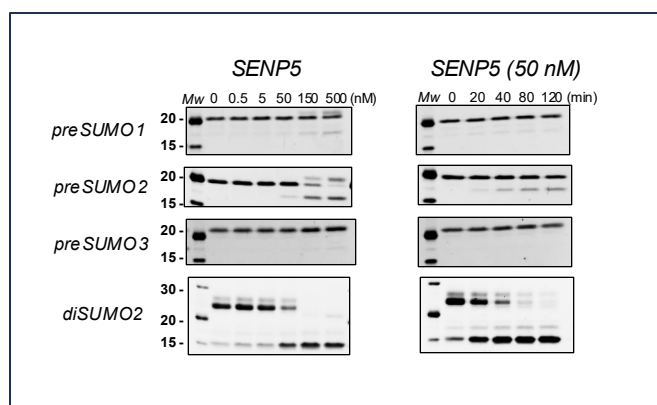
Supplementary Figure 1

Structural alignment of the SENP5 catalytic domain with the catalytic domains of SENP1, SENP2, SENP3, SENP6, SENP7, SENP8, Ulp1 and Ulp2. SENP5 secondary structures (α -helix and β -strands) are numbered and labelled. A yellow backdrop indicates side chain identity with 70% conservation in the alignment. Red residues indicate the three catalytic residues. SENP5 residues involved in the interface with SUMO1 and SUMO2 are marked with an asterisk and the SENP5 residues mutated are colored in bold green.



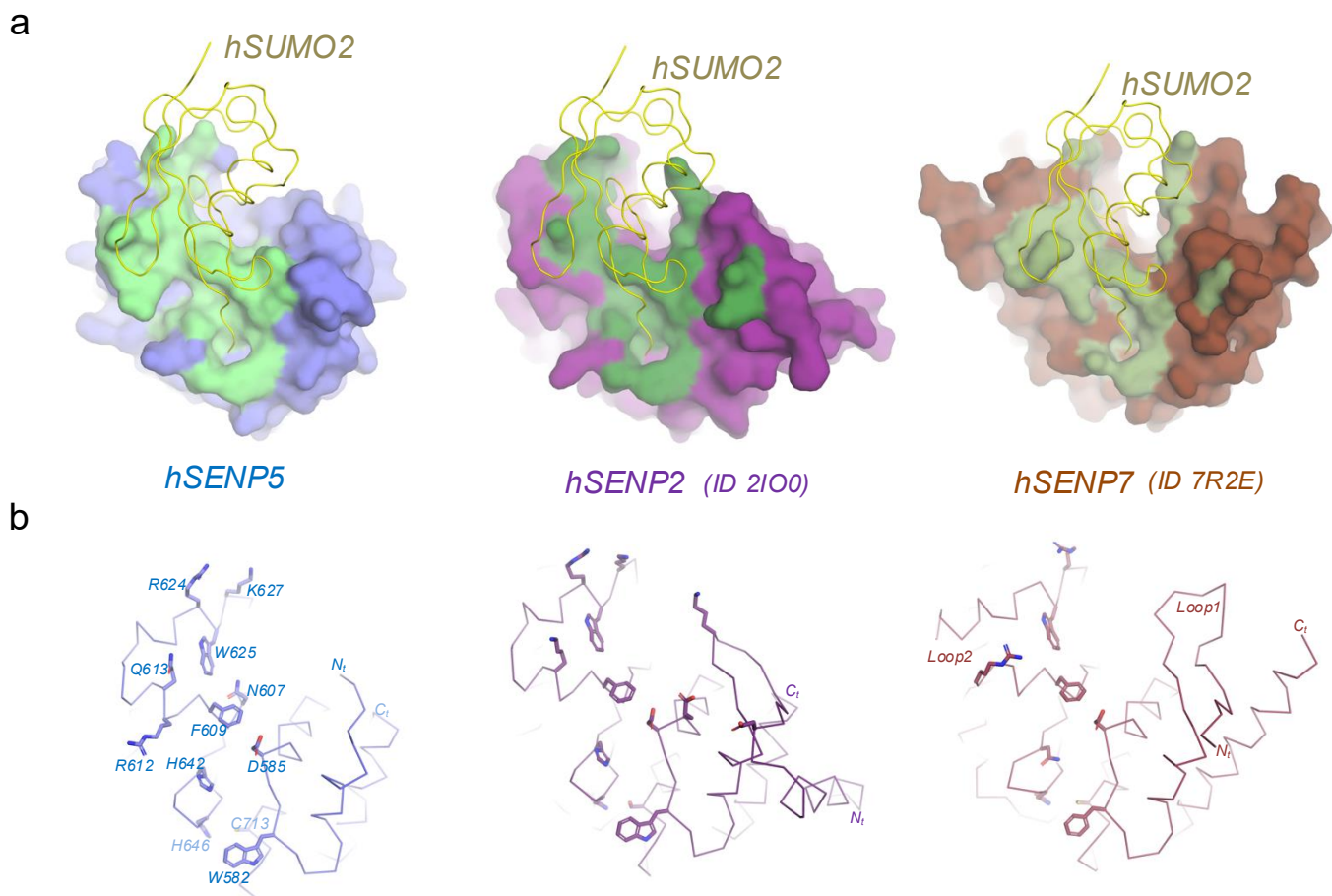
Supplementary Figure 2

Structural alignment of the crystal structure of the catalytic domains of SENP5 (blue cartoon) with the AlphaFold-2 model of the catalytic domain of SENP5 plus a predicted three-helix bundle domain. Active site residues are labelled and shown in stick representation.



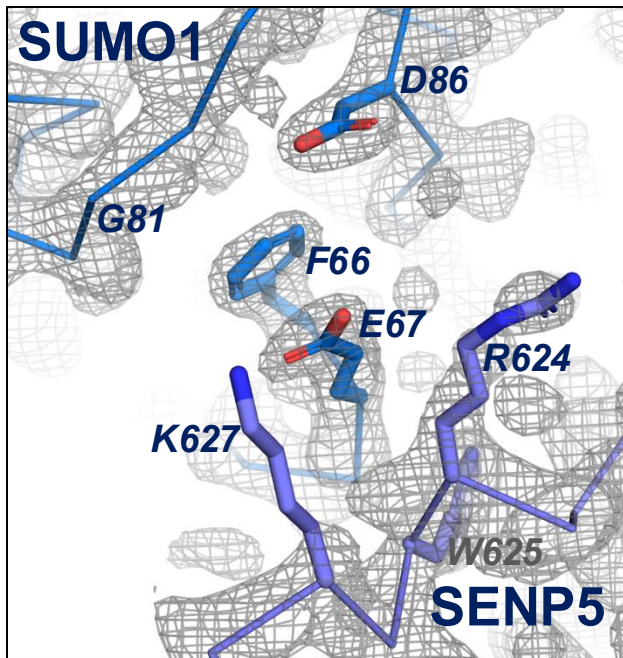
Supplementary Figure 3

(*Left*) In vitro processing reactions of the three SUMO precursors (*preSUMO1*, *preSUMO2* and *preSUMO3*) and *diSUMO2* substrates at increasing SENP5 concentrations (0.5 to 500 nM). (*Right*) Time course processing reactions of the three SUMO precursors (*preSUMO1*, *preSUMO2* and *preSUMO3*) and *diSUMO2* substrates at 50 nM SENP5 concentration. Uncropped gels can be seen in the Source Data File.

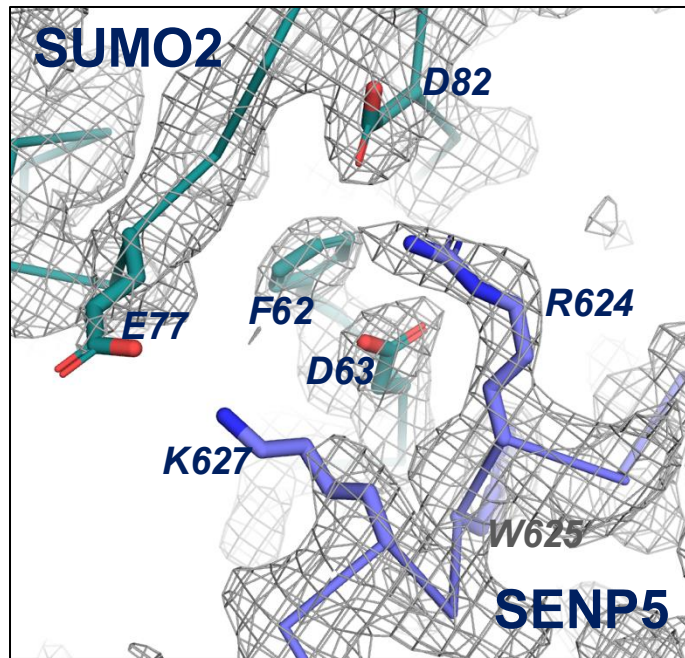


Supplementary Figure 4

a) Structural surface representation of the interface (green) between SENP5 (blue), SENP2 (violet) and SENP7 (brown) with SUMO2 (yellow ribbon representation). **b)** Stick representation of the interface residues of SENP5 (blue), SENP2 (violet) and SENP7 (brown) with SUMO2. SENP5 residues are labelled and shown in stick representation.



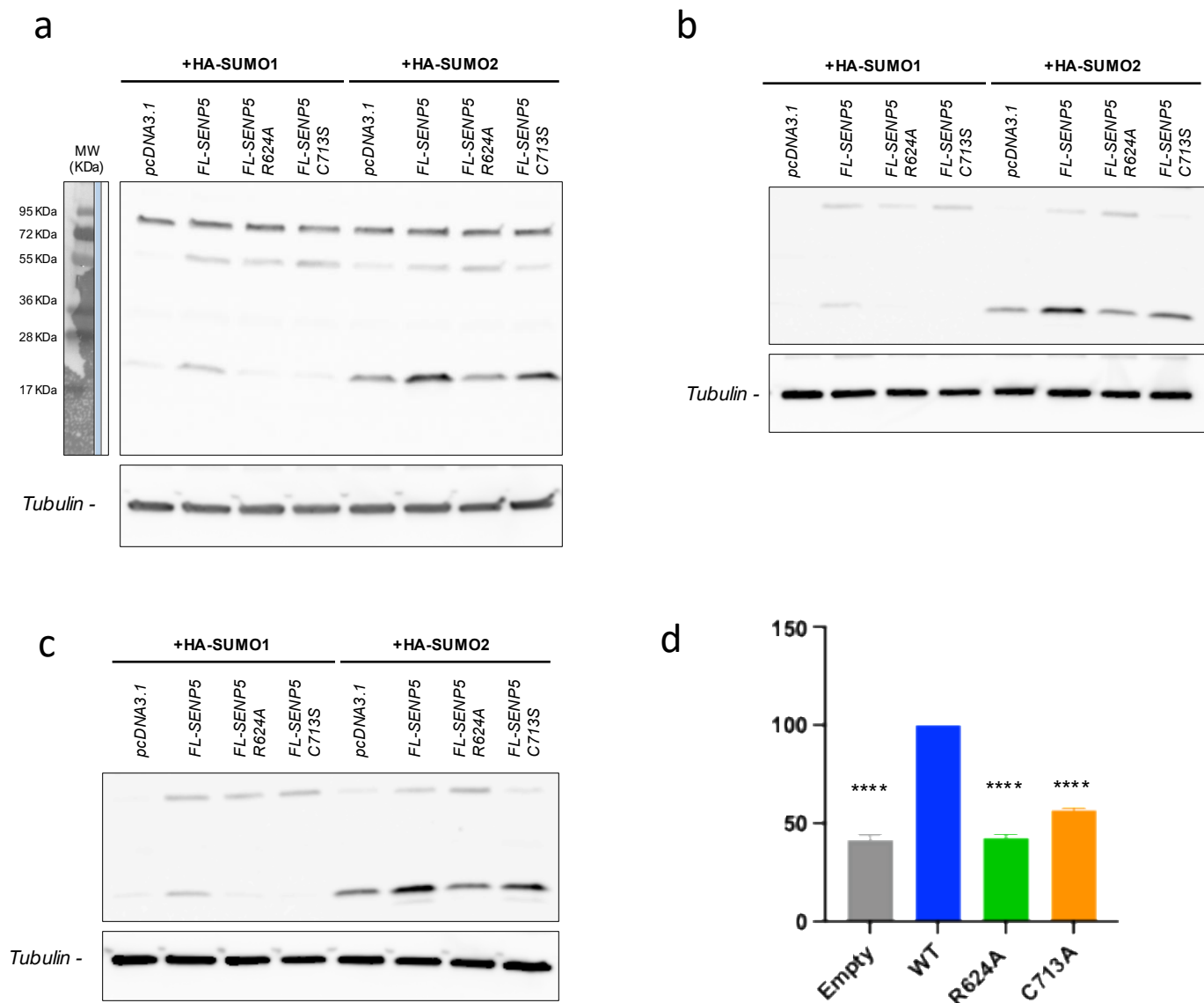
SENP5-SUMO1



SENP5-SUMO2

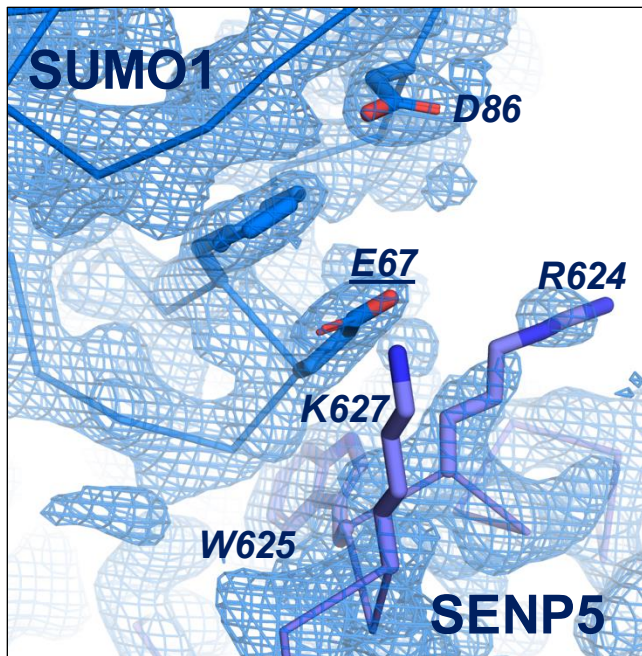
Supplementary Figure 5

Structural representation of the 2FoFc electron density maps contoured at 1.0σ of the interface of the complex between SENP5 and either SUMO1 or SUMO2 around the Arg624/Lys627 basic patch of SENP5. Major interface residues are labelled and shown in stick representation.

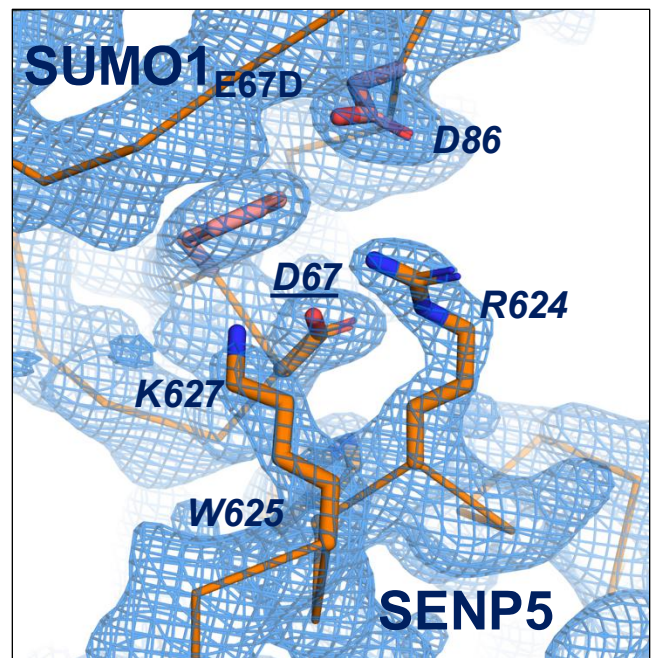


Supplementary Figure 6

a,b,c) Triplicate of the Western Blot (WB) analysis of COS-7 cells co-transfected with HA-SUMO1 and HA-SUMO2 and either the empty pcDNA3 (-) or the plasmids expressing SENP5, SENP5-R624A, or SENP5-C713A. Immunoblot showing the levels of mono HA-SUMO2 or HA-SUMO1 in the absence and presence of the wild-type or mutated forms of the SENP5 protease. Anti-HA antibody was used to detect both HA-SUMO1 and HA-SUMO2 by WB. Anti-tubulin antibody was used as a loading control. *d*) Plot of the WB triplicate showing the fraction of HA-SUMO2 in each experiment regarding SENP5 wild type (100%). A two-tailed unpaired t-test was used to determine significance in comparison to wild-type. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$.



SENP5-SUMO1_{WT}

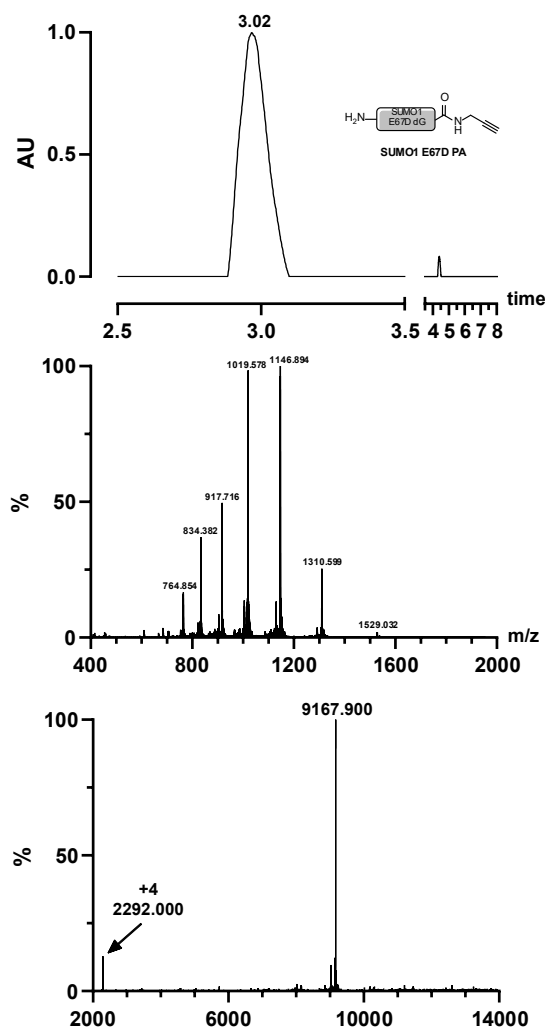


SENP5-SUMO1_{E67D}

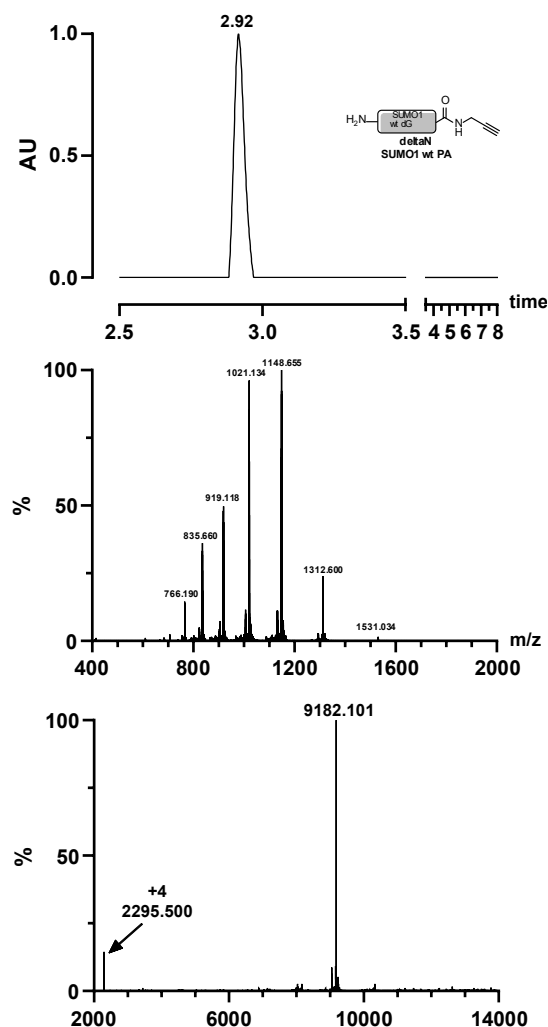
Supplementary Figure 7

(Left) Structural representation of the 2FoFc electron density maps contoured at 1.0σ of the interface of the complex between SENP5 and SUMO1 wild type around the Arg624/Lys627 basic patch of SENP5. Major interface residues are labelled and shown in stick representation. (Right) Similar figure with the structure of SENP5 with the SUMO1 E67D point mutant.

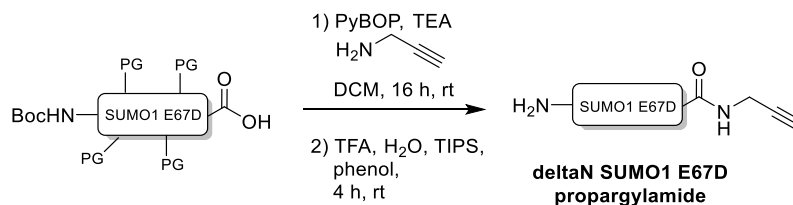
a



b



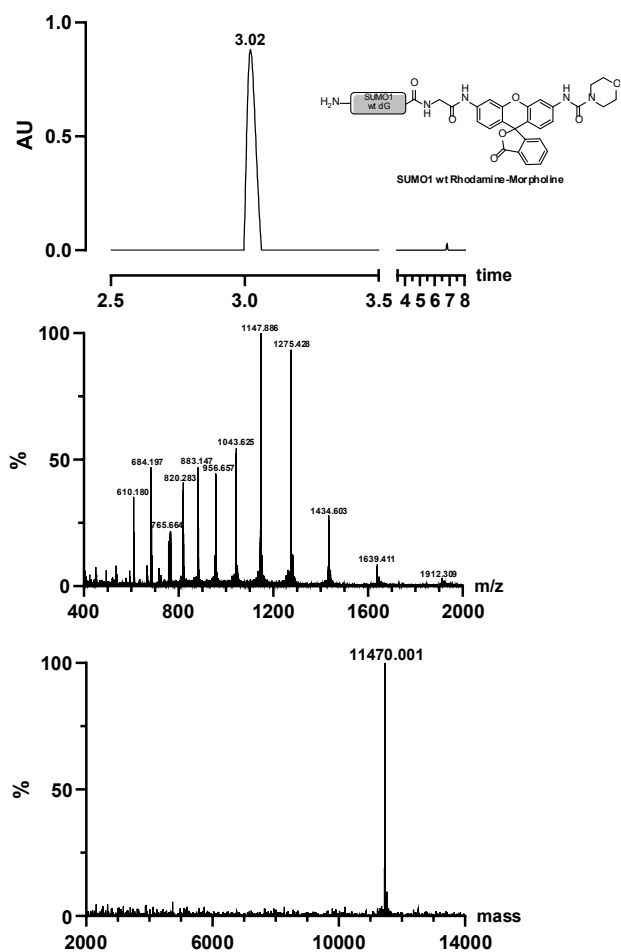
c



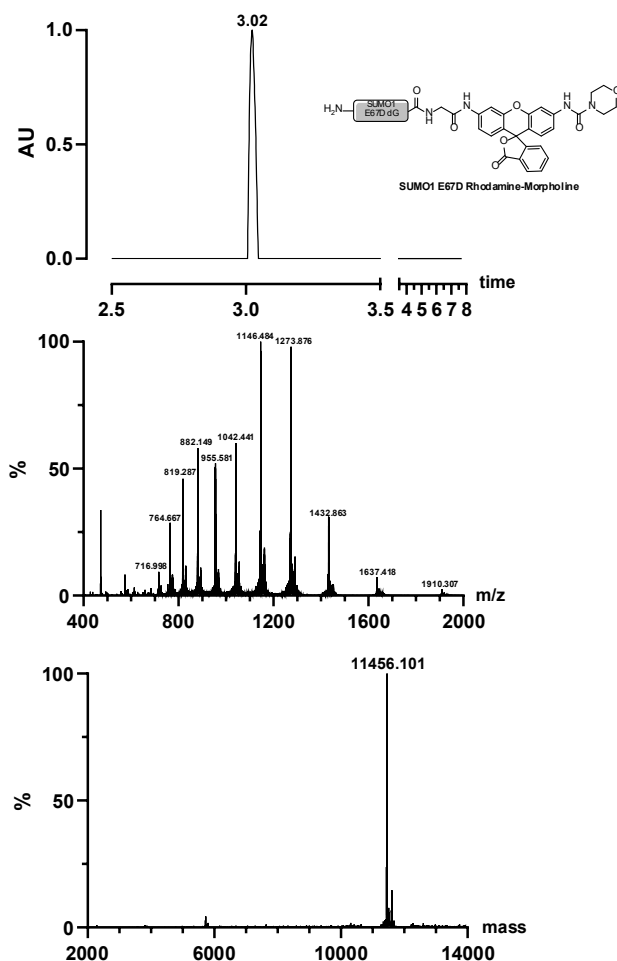
Supplementary Figure 8

a) Δ N₁₋₁₇ SUMO1 E67D propargylamide. Diode array chromatogram (top). MS spectrum (middle). Deconvoluted mass of product peak (bottom). ESI [M+H]⁺ Expected: 9168, found 9168. b) Δ N₁₋₁₇ SUMO1 wild type propargylamide. Diode array chromatogram (top). MS spectrum (middle). Deconvoluted mass of product peak (bottom). ESI [M+H]⁺ Expected: 9182, found 9182. c) Scheme of the chemical synthesis.

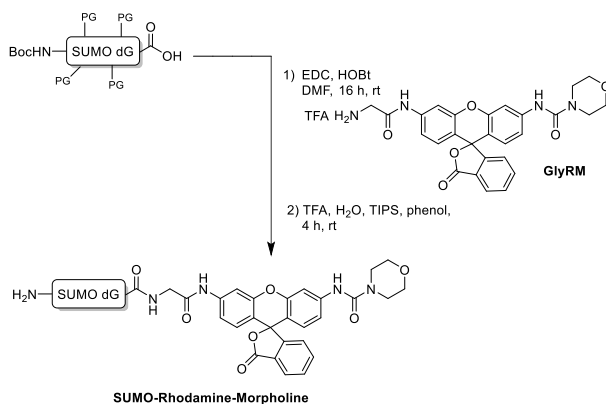
a



b



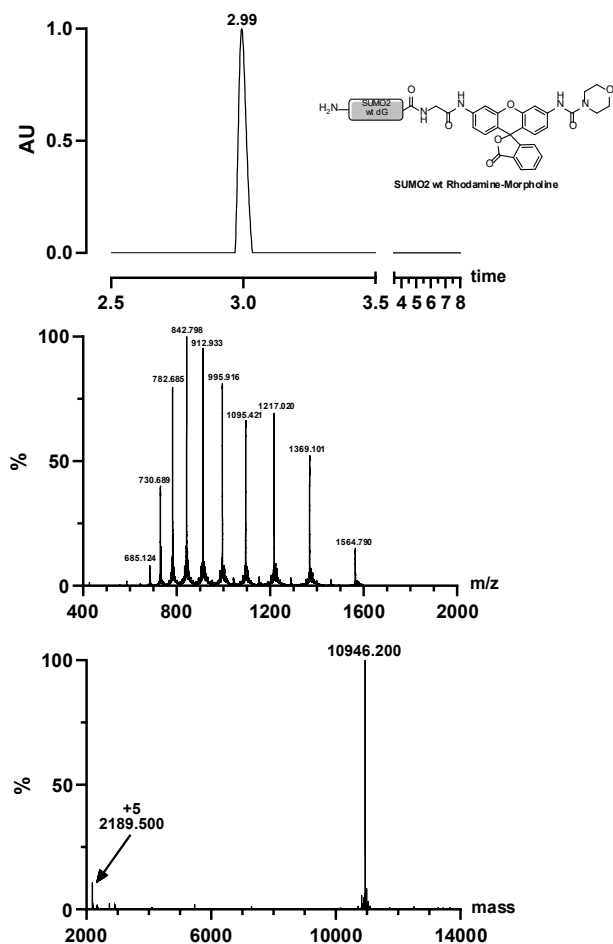
c



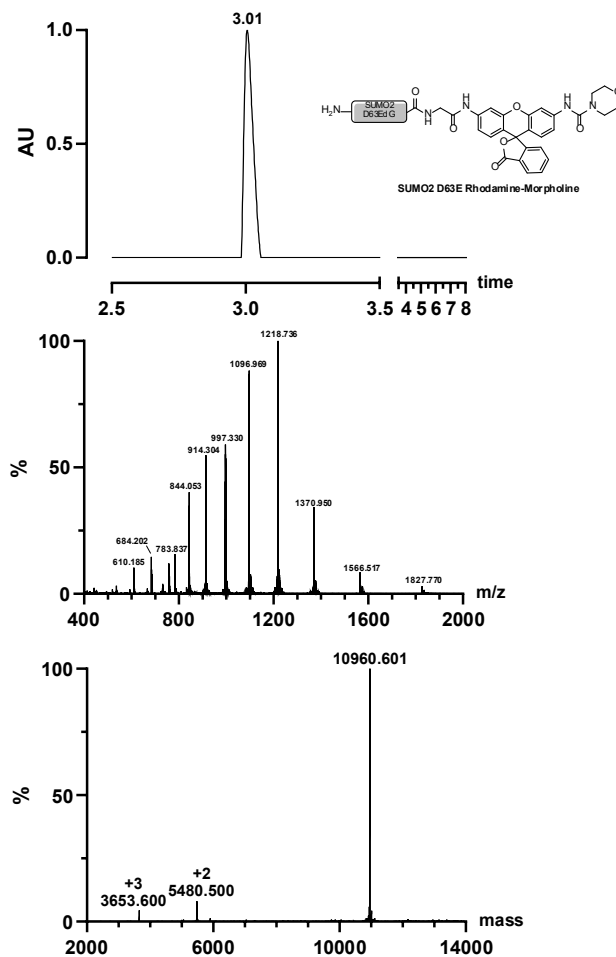
Supplementary Figure 9

a) SUMO1 wild type Rhodamine Morpholine. Diode array chromatogram (top). MS spectrum (middle). Deconvoluted mass of product peak (bottom). ESI [M+H]⁺ Expected: 11471, found 11470. b) SUMO1 E67D Rhodamine Morpholine. Diode array chromatogram (top). MS spectrum (middle). Deconvoluted mass of product peak (bottom). ESI [M+H]⁺ Expected: 11457, found 11456. c) Scheme of the chemical synthesis for SUMO1 and SUMO2.

a



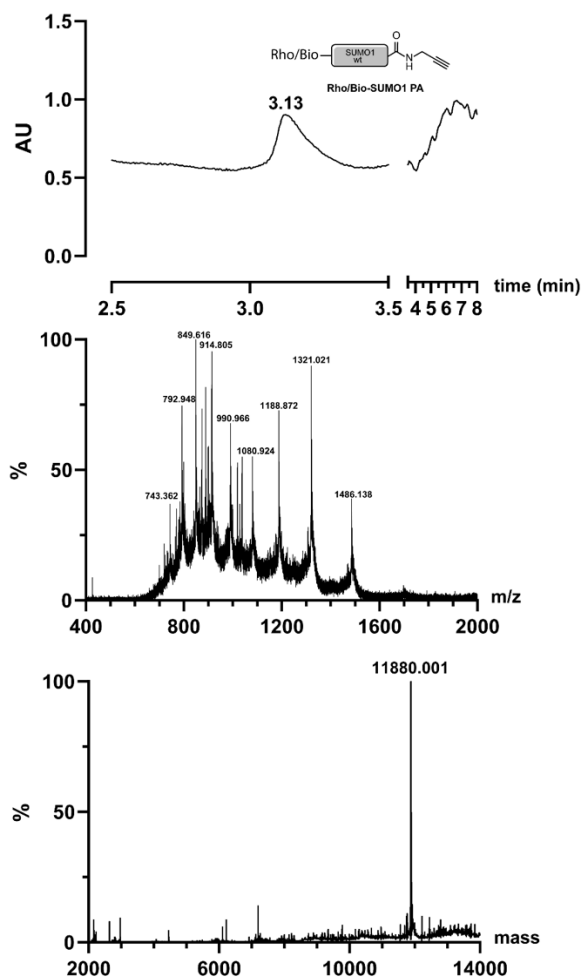
b



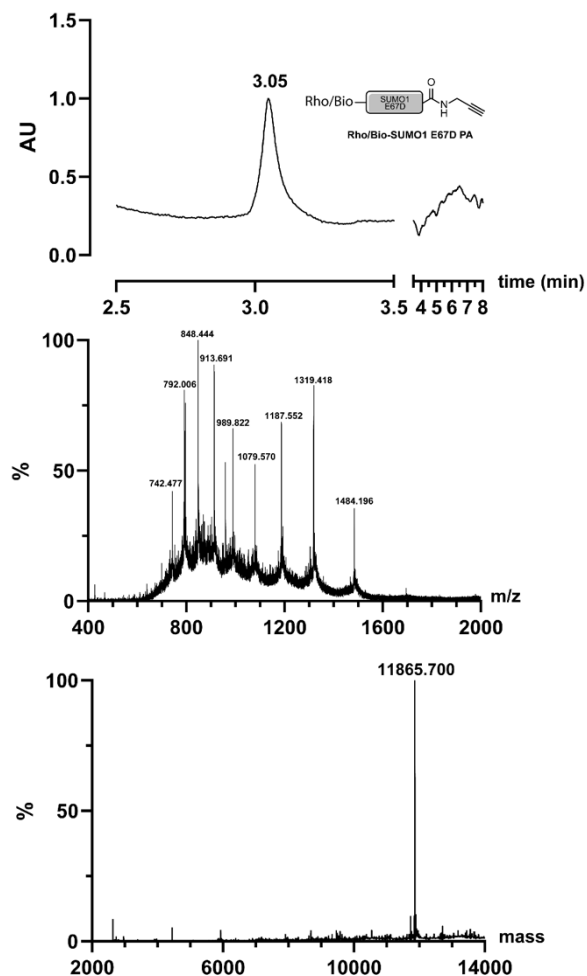
Supplementary Figure 10

a) SUMO2 wild type Rhodamine Morpholine. Diode array chromatogram (top). MS spectrum (middle). Deconvoluted mass of product peak (bottom). ESI $[M+H]^+$ Expected: 10947, found 10946. b) SUMO2 D63E Rhodamine Morpholine. Diode array chromatogram (top). MS spectrum (middle). Deconvoluted mass of product peak (bottom). ESI $[M+H]^+$ Expected: 10961, found 10961.

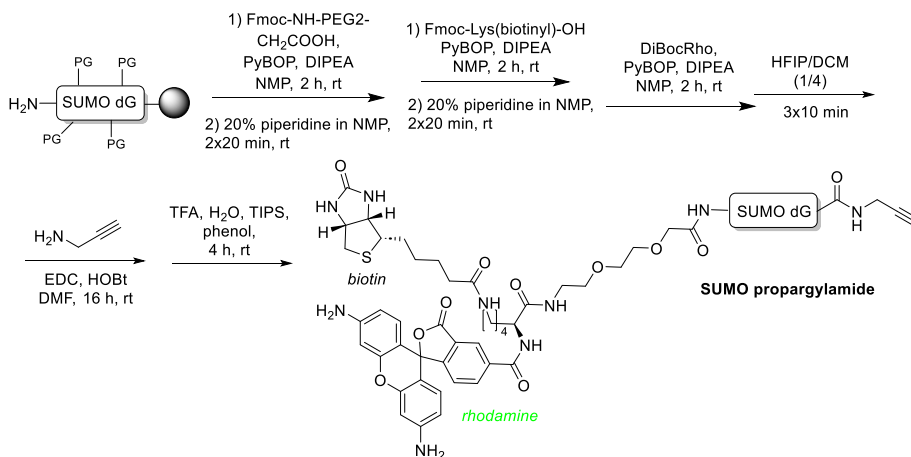
a



b



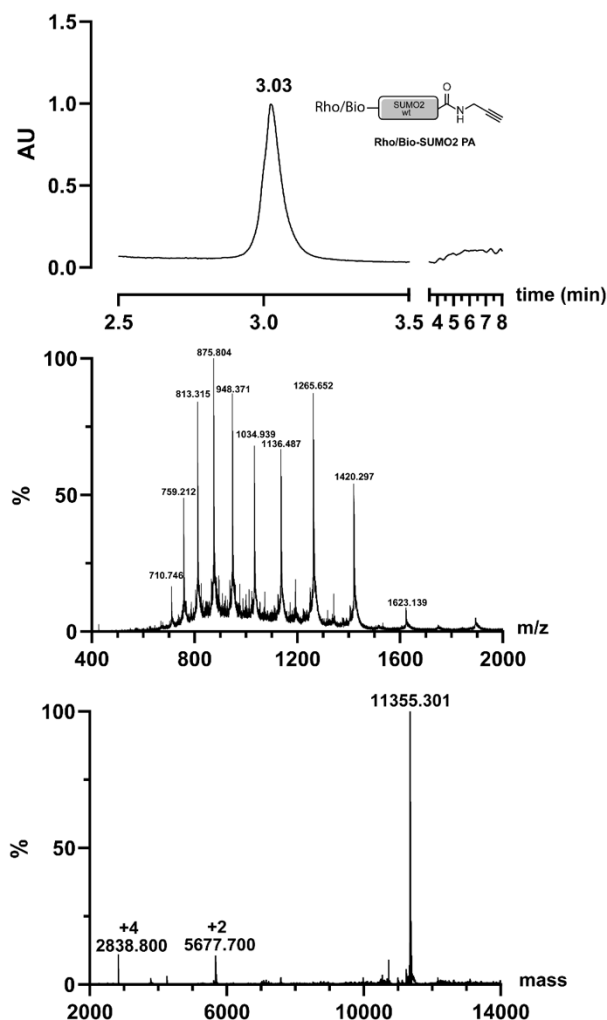
c



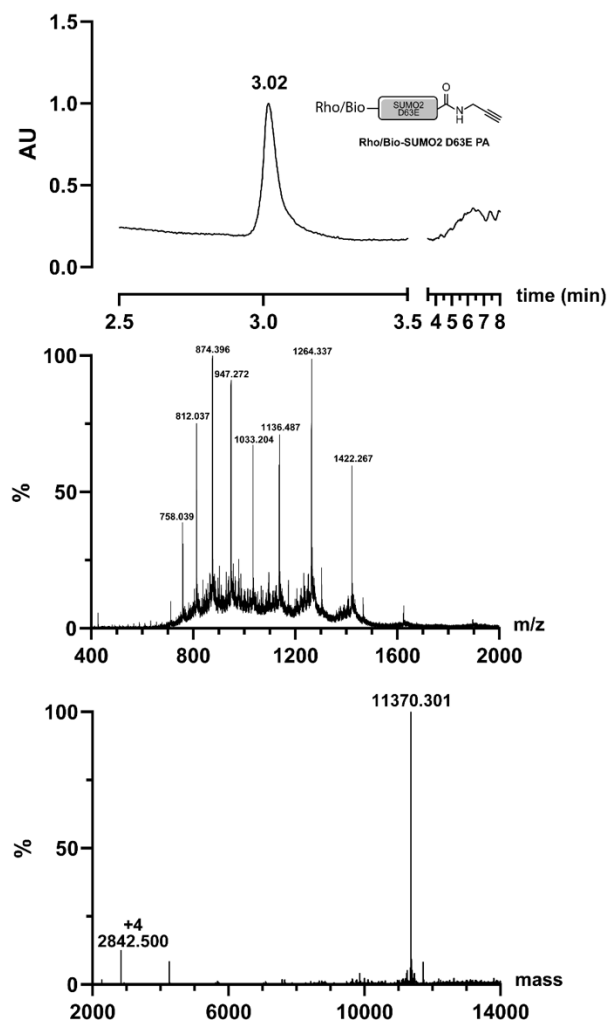
Supplementary Figure 11

a) Rho-Bio SUMO1 wild type propargylamide. Diode array chromatogram (top). MS spectrum (middle). Deconvoluted mass of product peak (bottom). ESI [M+H]⁺ Expected: 11881, found 11880. b) Rho-Bio SUMO1 E67D propargylamide. Diode array chromatogram (top). MS spectrum (middle). Deconvoluted mass of product peak (bottom). ESI [M+H]⁺ Expected: 11867, found 11866. c) Scheme of the chemical synthesis for SUMO1 and SUMO2.

a



b



Supplementary Figure 12

a) Rho-Bio SUMO2 wild type propargylamide. Diode array chromatogram (top). MS spectrum (middle). Deconvoluted mass of product peak (bottom). ESI $[M+H]^+$ Expected: 11358, found 11355. b) Rho-Bio SUMO2 D63E propargylamide. Diode array chromatogram (top). MS spectrum (middle). Deconvoluted mass of product peak (bottom). ESI $[M+H]^+$ Expected: 11372, found 11370.

Supplementary Table 1 – List of the used primers.

PTXB1-Δ14SUMO2G fw	CTTTAAGAAGGAGATATACATATGAACGATCATATTAATTTGAAGGTGGCG
PTXB1-Δ14SUMO2G rv	CAACTAGTGCATCTCCCGTGATGCATCCCGTCTGCTGTTGGAACACATCA
SEN5 Δ568 fw	GTTGATTGTATATGGATTAGGAGTAACTAATGAA
SEN5 Δ568 rv	TTACTCCTAATCCATATACAATCAACTTCTGAACAA
SEN5 C713A fw	AAAAACGACAGTGACGCTGGAGTCTTTGTGCTC
SEN5 C713A rv	GAGCACAAAGACTCCAGCGTCACTGTCGTTTTT
SEN5 R624A fw	TATAATGGAGTAAAAGCATGGACTAAAAAGGTG
SEN5 R624A rv	CACCTTTTtagtccatgcttttactccattata
SEN5 N607A fw	AAAGTTCATTCTTCGCCAGCTTTTTCATAGA
SEN5 N607A rv	TCTATGAAAAAGCTGGCGAAGAAGTGAAC TT
SEN5 K627A fw	GTAAAAAGATGGACTGCCAAGGTGGATTGTTT
SEN5 K627A rv	AAACAAATCCACCTTGGCAGTCCATCTTTTAC
SEN5 K627A R624A fw	GTAAAAGCATGGACTGCCAAGGTGGATTGTTT
SEN5 K627A R624A rv	AAACAAATCCACCTTGGCAGTCCATGCTTTTAC
SUMO2 D63E fw	GATCAGATTCCGATTTGAGGGGCAACCAATCAATG
SUMO2 D63E rv	CATTGATTGGTTGCCCTCAAATCGGAATCTGATC
SUMO1 E67D fw	CTCAGGTTTCTCTTTGACGGTCAGAGAATTGCT
SUMO1 E67D rv	AGCAATTCTCTGACCGTCAAAGAGAAACCTGAG