

Staphylococcal SplA and SplB serine proteases target ubiquitin(-like) specific proteases

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SplB autohydrolysis of C-terminal Twin-Strep-tag

The native SplB construct used in this study shows an additional ~28 kDa band in SDS-PAGEs, which is not present in preparations of the inactive SplB mutant or SplA (Figure S1B). This ~28 kDa band increases in intensity with incubation times (e.g. during cleavage assays). MS-analysis of the lower band revealed a C-terminal truncation of the SplB construct, specifically inside the Twin-Strep-tag. The cleavage was confirmed by MS to occur after the glutamine residues in the first and second Strep-repeat (WSHPQ[↓]FEK, [↓] = cleavage site). Since glutamine was previously determined to be accepted in the P1 position (nomenclature according to Schechter and Berger (Schechter and Berger 1967)) of the SplB cleavage motif (Dubin et al. 2008), this is likely due to the recombinant SplB construct targeting its own C-terminal Twin-Strep-tag leading to autohydrolysis.

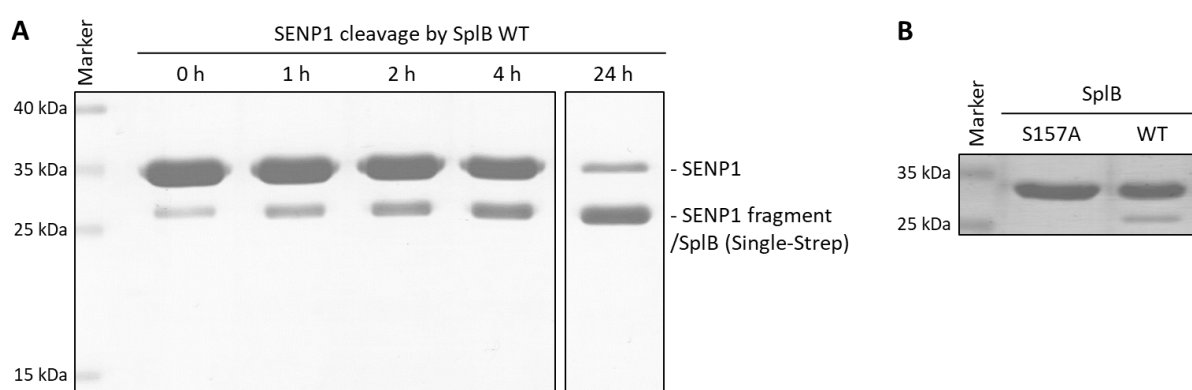


Fig. S1 SENP1 cleavage by SplB and SplB autohydrolysis. **A:** Time-dependent cleavage of SENP1 by SplB (construct with Single-Strep-tag). SENP1 was incubated with SplB (~10:1 ratio of SENP1 to SplB) at 37 °C in PBS buffer. The depletion of the SENP1 protein band (~35 kDa) and simultaneous increase of the lower band (~28 kDa, SplB and SENP1 fragment signals overlaid) signifies the time-dependent cleavage of SENP1 catalyzed by SplB. **B:** The recombinant SplB construct cleaved-off its own Twin-Strep-tag after the Gln residues present in both Strep repeats (WSHPQ[↓]FEK, [↓] = cleavage site). The enzymatically inactive SplB construct was unable to cleave its Twin-Strep-tag. Both protein samples shown were taken directly after purification. SplB_S157A = inactive SplB mutant; WT = native SplB. Additional band appearing for SplB WT at ~28 kDa.

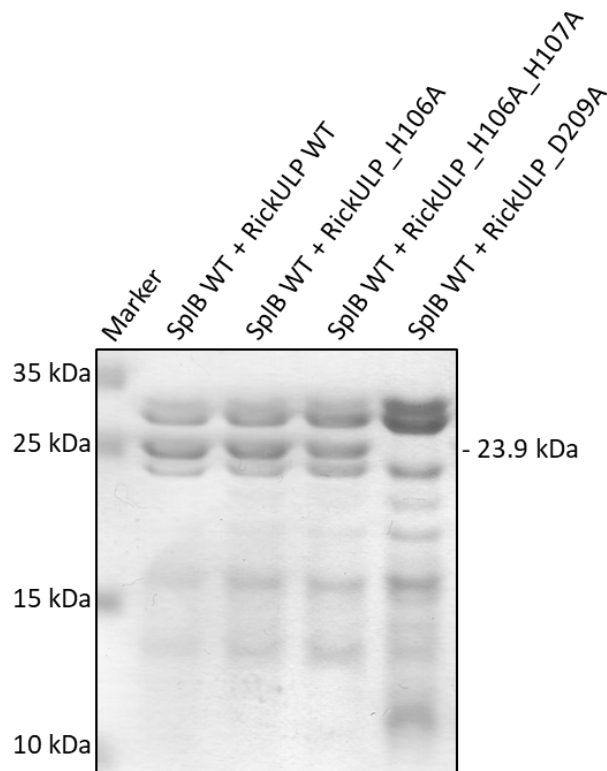


Fig. S2 Cleavage patterns of RickULP variants digested by SplB. Confirmation of SplB cleavage sites in RickULP identified using the HUNTER method by site-directed mutagenesis. SDS-PAGE of recombinantly expressed and purified RickULP variants after incubation with native SplB (WT) for 24 h at 37 °C. Cleavage of RickULP at residue D209 would result in a 23.9 kDa fragment, which was not observed for the D209A mutant.

Table S1 Amino acid sequences of the protein constructs.

Construct	Amino acid sequence
ElaD	MVTVVSNYCQLSQTQQLSQTFAEKFTVTEELLQSLKKTALSGDEESI ELLHNIALGYDKFGKEAEDILYHIVR TPTNETLSIIRLIKNACLKLYNLAHIAITNSPLKSHSDDLLFKKLFSPSKLMTIIGDEIPLISEKQSLSKVLL NDENNELSDGTNFWDKNRQLTTDEIACYLQKIAANAKNTQVNYPTGLYVPYSTRTHLEDALNENIKSDPSWPN EVQLFPINTGGHWILVSLQKIVNKKNNKLQIKCVIFNSLRALGYDKENSLKRVINSFNSELMGEMSNNNIKVH LNEPEIIIFLHADLQQYLSQSCGAFVCMQAQEVIEQRESNSDSAPYTL LKNHADRFKKYSAAEQYEIDFQHRLA NRNCYLDKYGDANINHYRNL EIKHSQPKNRASGKRVS
Lpg1148 (1-305)	MYMDLGSDNMALSKEIQNKSLRSLIQKYARQLNETDKKLI ELINNLAAADDIPLYAQNQNVINLLIKTIKDSQ SEHNPDLEGTSYFYGLITAEMEAQSEINRQRHLMQGRFDETALHLRTERDQM QDHDMTLLMPKSQGRIVVMA VLNRYDSHSANAI IETLASDVFNPEVHYIMIPVGPGRHWRGVYLSKPQGGTSDTAYDLELFDPYGPEGAAVLDD YVLDLLNQCGVPKELVNIRHTGPKHPQGDAYS CGDFTCAYSHKKMKEFGAPEGSYNPILIDTLDNLGNEDNVL RMTTREETRALVD
Lpg1621/ Ceg23 (1-368)	MHTKKDKKVISLQERVENAVDVSGAFDNCFFHNFALYLLTNNLPLPDDL FHFKSI INRNSKAEQLFEFFHNPE SLNFLSILDKENDVSEPSGYLFEKSLILGFLLEWFPQTQLVNNSAVKAEMLEGEKGVSAFKNYKEYRSFMSK EELKSTEFGALYEANAEFLYFYNRSESTLINKDSPFEKYFVGSSSDEEAIKNYWD AEGYTLYCQHAKPQVK LSYIEIMTMMKVINQPLTIYDRSTSSIVAEYVNPKNLPDFEVAIDALQGHYFLLKTEETEKELEEYERSY AQ YKRDRSEILAHSDKPVSSLLVRATCPKGHLDEDPFIALIESLSEINSLSQIDTNLKNENTDIPNCNFFLLKVGA SVV
OTUD7B	MAHHHHHTLDMDAVLSDFVRSTGAEPGLARDLLEGKNWDVNAALSDFEQLRQVHAGNLPPSFSEGGSGSRTP EKGFS DREPTRPRPILQRQDDIVQEKRLSRGISHASSSIVSLARSHVSSNGGGGGSNEHPLEMPCAFQLPD LTVYNEDFRSFIERDLIEQSMLVALEQAGRLNWWVSVDPTSQRLLPLATTGDGNCLLHAASLGMWGFHDRDLM LRKALYALMEKGVEKEALKRRWRWQQTQONKESGLVYTEDEWQKEWNELIKLASSEPRMHLGTNGANC GGVES SEEPVYESLEEFHVFLAHVLRPIVVVADTMLRDSGGEAFAPIPFGGIYLPLEVPA SQCHRSPVLVAYDQAH FSALVSMEQKENTKEQAVIPLTDSEYKLLPLHFAVDPGKGWEWGKDDSDNVRLASVILSLEVKLHLLHSYMN KWIFLSSDAQAPLAQPESPTASAGDEPRSTPESGSDSKESVGSSSTSNEGGRRKEKSKRDREKDKKRADSVAN KLGSGFKTLGSKLKNMGLMHSGSKPGGVGTGLGGSSGTETLEKKKNSLSKSWKGKKEEAAGDGPVSEKPP AESVGNNGGSKYSQEVMSLSILRTAMQGEKGFIFVGT LKMGRHQYQEEMI QRYLSDAEERFLAEQKQKEAER KIMNGGIGGGPPPAKKPEPDAREEQPTGPPAESRAMAFSTGYPGDFTIPRPSGGGVHCQEP RRLAGGFCVGG

	LPPTYATFPRQCPPGRPYPHQDSIPSLPESHKSDGLHRGALLPPPYRVADSYSNGYREPPEPDGWAGGLRGLP PTQTCKQCPNCSFYGHPETNNFCSCCYREELRRREREPDGEELLVHRE
PLpro	MAHHHHHHGADVTKIKPHVNHEGKTFVFLPSDDTLRSEAFEYYHTLDESFLGRYMSALNHTKKWKFPQVGGLT SIKWADNNCYLSSVLLALQQLVEKFNPALQEAYYRARAGDAANFCALILAYSNKTVELGDVRETMTLLQH ANLESARKVLNVCKHCQKTTTLTGVEAVMYMGTLSDNLKTGVSI PCVCGRDATQYL VQQESSFVMM SAPP AEYKLQOGTFLCANEYTGNYQCGHYTHITAKETLYRIDGAHLTKMSEYKGPVTDVFYKETS YTTTTIKPVSYKL DGVTYTEIEPKLDGYK
RavD	MNLKKEVFLNQCAEMMIKKAARLILGSDLDFFEYTRDVQDIQIDLGP AFMFSPDEEKT LWVSGKNQETLERDL ATLNKSSAYFFRTGTQGGAGHWQVLYEEAAKSGWVSYSQSNHFQVTD SNGKLTASGKGLLVPHANWGKENG YAFLLVNASAQNI IHAANFVYIFRTQNE DAAIQYCALNQALHPEIKRITITKVTQTTRLTPEIHCPRGDLSYA NLKNVYSKHLALLHVINRLEAGSKSYNPYWMGSQEKLNKIFEALGHCYQKGLDVDNELNNSNSKLSKAINMRR LPSLFSFWCEKNESFVESRKIIEEHRICRLNNL
RickCE	MAHHHHHHVGT EIKDYWTENEITHLLTAQLDEKFKSVQPAITFRNTALTEEMLKDYTAKGEEKNKILAEVQE TIKIANLIPDKEERALMLGDAKKREEILKLSDAEREKLNKNDLLRGGEAQQQINEDI LN RATKDIKNGKEAAV IPIEMGYGHWTVLVAKYDKDNQIILTFNDSLGN SINYDQKLPKLIKDTLGNLPNKPII IDEQTKQQT DQSA CGVFTVDNGIKIAKGQAILSTEEKGEKGLRLREHHAQILTDAMFKQDAQWIRQQ
RickULP	MAHHHHHHVGTQQQAPANNQKPWEKLGIPQEMYKESLAEQQQAKPIIEPKQQIPEKKSSSLVINTEDQVGVYN TGNIKQPTYLYTEDDIKNILEANIDKNMFSIFHHASLEEPEILKDTLRVTVEDLILDNKPAPIPLNTGHKHWL LLMASKDDKGNINFMYNDPYGEPLSQPKVTEYITEIYPDAKITDLNKTQQANVYDCGVFVCD SAIKLSKGQK ILTEESKDQGINLRQAQANTLLIQQAITIGHE
SENp1	MSYHHHHHHHDYDIPTTENLYFQGGAMADIGSDSEDEFPEITEEMEKEIKNVFRNGNQDEV LSEAFRLTITRKD IQTLNHLNWLND EINFYMNMLMERSKEKGLPSVHAFNTFFFTKLKTAGYQAVKRWTKKVDVFSVDILLVPIH LGVHWCLAVVD FRKNITYYDSMGGINNEACRILLQYLKQESIDKKRKEFD TNGWQLFSKKSQEI PQQMNGSD CGMFACKYADCITKDRPINF TQQHMPYFRKRMVWEILHRKLL
SnCE1	GPLGSSLTSKAICRYSLSSVESIQPKTQVKAIEPKGEKDRFLIDERNWLNNQHLGLYSLFLQEKYGPVEVFFPF GGWTVYVFPGLTDRFFKEDSYHILDVRAKRIKSF LDYKSITYPLFIGGNHWGLLFI DREKRTVEYYDSKINYN YEEGLQG IKDVAAKF TKYDPGEKPYTYLEKIKKKLQPDGYQCGPWALYFLEHRLNPEVDFNQLDLNEAQNMI AKYRFAVRDKLLELQKNGNTLYC
SplA WT	EKNVKEITDATKEPYN SVVAFVGGTG VVVGKNTIVTNKHI AKSNDIFKNRVSAHSSSKGKGGGNYDVKDIVEY PGKEDLAIVHVHETSTEGLNFNKNVSYTKFADGAKVKDRISVIGYPKGAQTKYKMFESTGTIN HISGTFMEFD AYAQPNGSGSPVLNSKH ELIGILYAGSGKDESEKNFGVYFTPQLKEFIQNNIEKGSWSHPQFEKGGGSGGGSG GSSAWSHPQFEK
SplA-SUMO	MGHHHHHHGSGLVPRGSASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAK RQKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIEAHREQIGGEKNVKEITDATKEPYN SVVAFVGGTG VVV GKNTIVTNKHI AKSNDIFKNRVSAHSSSKGKGGGNYDVKDIVEYPGKEDLAIVHVHETSTEGLNFNKNVSYTK FADGAKVKDRISVIGYPKGAQTKYKMFESTGTIN HISGTFMEFDAYAQPNGSGSPVLNSKH ELIGILYAGSGK DESEKNFGVYFTPQLKEFIQNNIEKGSWSHPQFEKGGGSGGGSGGSSAWSHPQFEK
SplB WT	ENNVTKVKDTNIFPYTG VVAFK SATGFVVGKNTILTNKHVSKNYKVGDRITAH PNSDKNGGGIYSIKKI INYP GKEDVSVIQVEERA IERGPKGFNFNDNVT PFKYAAGAKAGERIKVIGYPHYPKNKYVLYESTGPVMSVEGSSI VYSAHTESGNSGSPVLNSNNELVGIHFASDVKNDDNRNAYGVYFTPEIKKFFIAENIDKGSWSHPQFEKGGGSG GGSGGSSAWSHPQFEK
SplB-SUMO	MGHHHHHHGSGLVPRGSASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAK RQKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIEAHREQIGGENVTKVKDTNIFPYTG VVAFK SATGFV GKNTILTNKHVSKNYKVGDRITAH PNSDKNGGGIYSIKKI INYPGKEDVSVIQVEERA IERGPKGFNFNDNVT PFKYAAGAKAGERIKVIGYPHYPKNKYVLYESTGPVMSVEGSSI VYSAHTESGNSGSPVLNSNNELVGIHFAS DVKNDDNRNAYGVYFTPEIKKFFIAENIDKGSWSHPQFEKGGGSGGGSGGSSAWSHPQFEK
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USP48	MAHHHHHHAPRLQLEKAAWRWAETVRPEEVSQEH IETAYRIWLEPCIRGVCRRNCKGNPNCLVGIGEH IWLGE IDENSFHNIDDPNCERRKKN SFVGLTNLGATCYVNTFLQVWFLNLELRQALYLCPS TCS DYM LGDGIQEEKDY EPQTICEHLQYLFALLQNSNRRYIDPSGFVKALGLDTGQQQDAQEFSKLFMSLLED TLSKQKNPDVRNIVQQQ FCGEYAYVTVCNQCGRESKLLSKFYELELNIQGHKQLTDCISEFLKEEKLEGDNR YFCENCQSKQNATRKIRL LSLPCTLNLQLMRVFVDRQTGHKKLNTYIGFSEILDMEPYVEHKGGSYVYELS AVLIHRGVSAYSGHYIAHV KDPQSGGEWKFNDEIDIEKMEGKKLQLGIEEDLAEPSKSQTRKPKCGKGT HCSRNAYMLVYRLQTQEKENTTVQ VPAFLQELVDRDNSKFEEWCIEAEMRKQSVDKGKAKHEEVKELYQRLPAGAEPEYFVSLEWLQKWLDESTPT KPIDNHACLC SHDKLHPDKISIMKRIS EYAADIFYSRYGGGPR LTVKALCKE CVVERCRILRLKNQLNEDYKT VNNLLKAAVKSGDGFVWGSSLSRWRQLALEQLDEQDGAEQSNGKMNGSTLNKDESKEERKEEEE LNFNEDI LCPHGELCISENERRLVSKEAWSKLQQYF PKAPEFPSYKECCSQCKILEREGEENEALHKMIANEQKTSLPNL FQDKNRPCLSNWPEDTDVLYIYSQFFVEEWRKFVRKPTRCSPVSSVGN SALLCPHGGLMFTFASMTKEDSKLI ALIWPSEWQMIQKLFVVDHVIKITRIE VGDVNPSETQYI SEPKLCEPCREGLLCQQQRDLREYTQATIVYHKV

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Nucleotide sequences of the synthetic genes

ElaD (GenBank accession number: Q47013):

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RavD (GenBank accession number: Q5ZZ51):

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RickCE (GenBank accession number: Q1RK35):

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RickULP (GenBank accession number: ABV76659.1):

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SENPI (GenBank accession number: Q9P0U3):

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SnCE1 (GenBank accession number: F8L4W9):

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SplA-SUMO (GenBank accession number: WP_399659753.1, N-terminal SUMO-tag):

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SplB-SUMO (GenBank accession number: WP_271285242.1, N-terminal SUMO-tag):

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SseL (GenBank accession number: Q8ZNG2):

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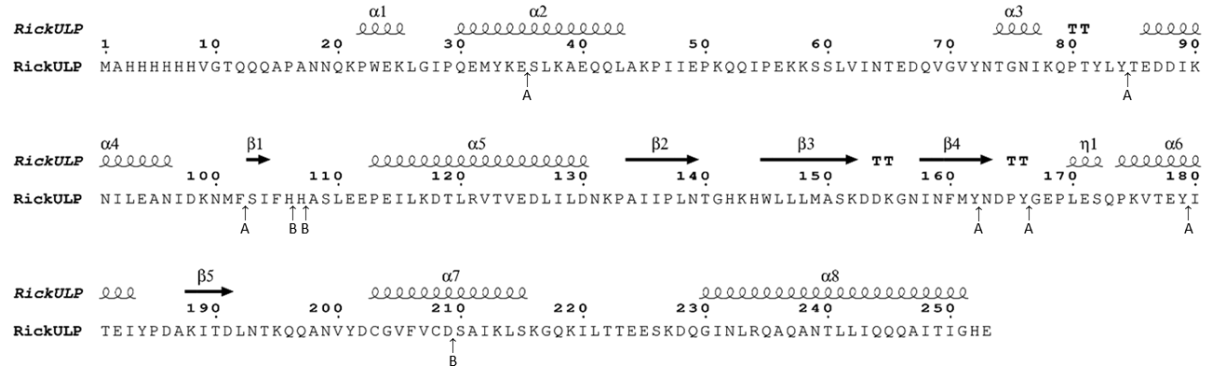
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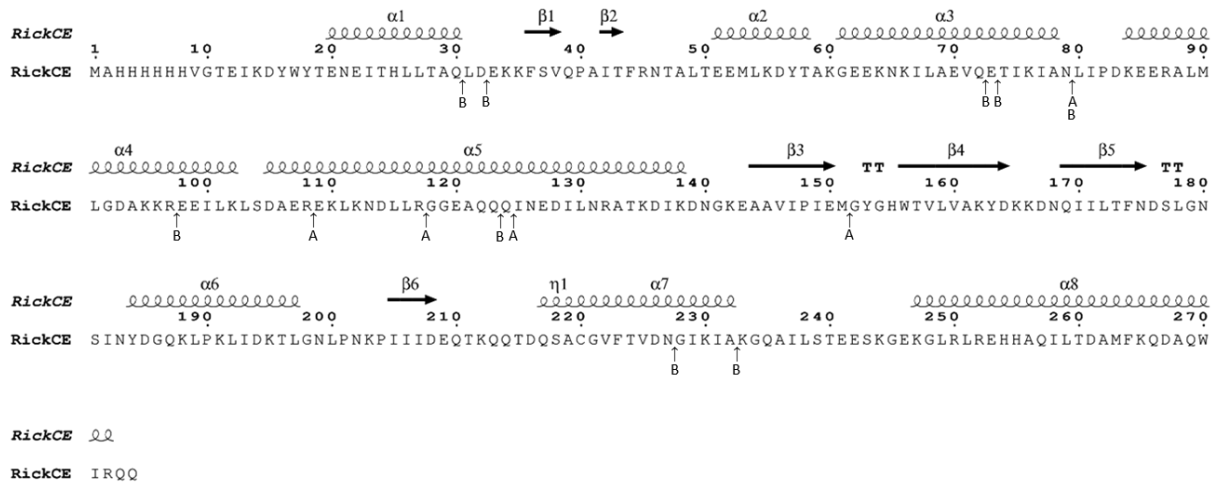
Topological maps of the target proteins

Topological maps of the four candidate proteins that were used to determine Spl cleavage sites (Table 2) were generated using ESPrict 3.0 (Robert and Gouet 2014). The putative cleavage sites (identified by MS) and confirmed cleavage sites (by site-directed mutagenesis) for SplA and SplB are marked with arrows and the type of Spl (A or B).

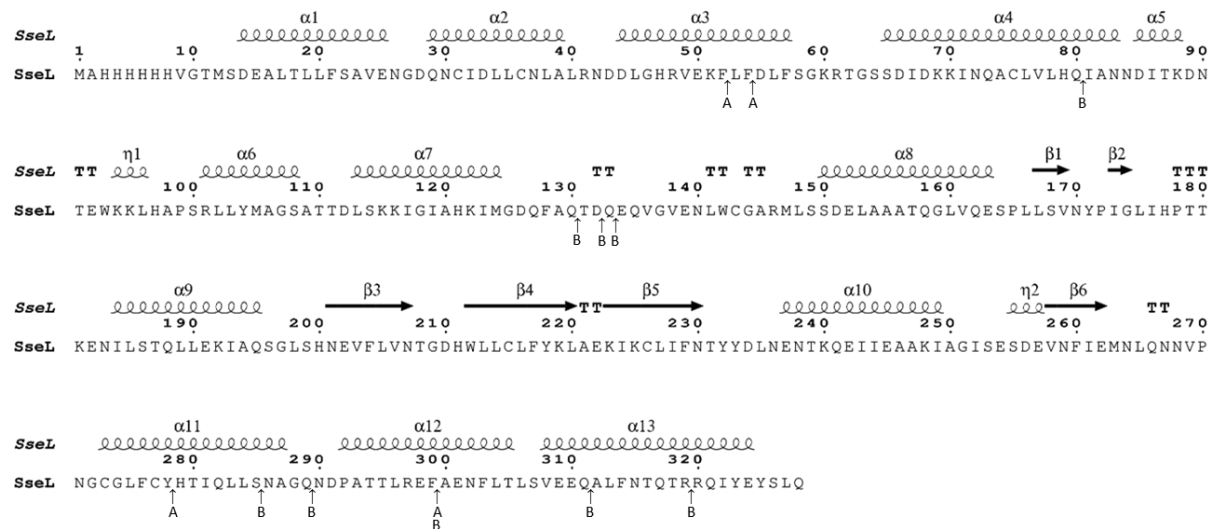
RickULP cleavage by Spls



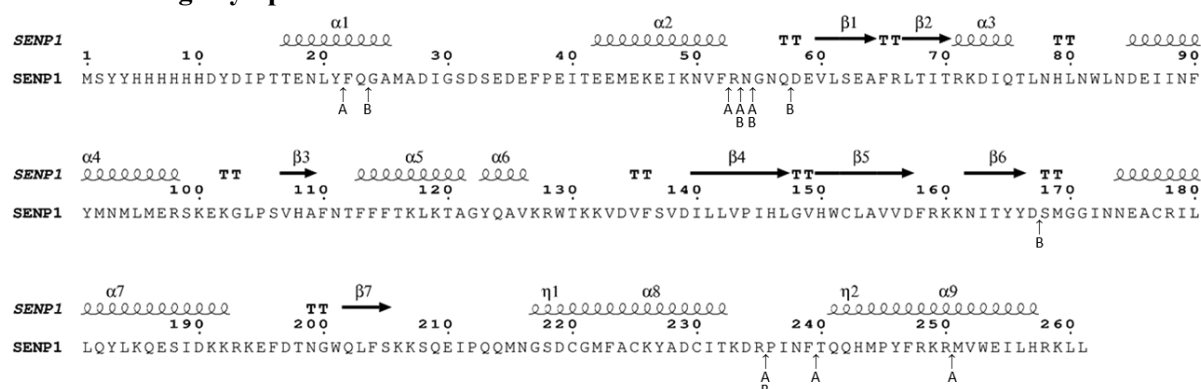
RickCE cleavage by Spls:



SseL cleavage by Spls:



SENP1 cleavage by Spl:



Instrument settings for mass spectrometry analysis

Table S2 Reverse phase liquid chromatography (RPLC) settings.

Instrument	Ultimate 3000 RSLC (Thermo Scientific)	
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	HUNTER samples 0 min 2% B → 2 min 5% B → 10 min 7% B → 40 min 25% B → 45 min 40% B → 47 min 90% B → 53 min 90% B → 55 min 2% B → 65 min 2% B	gel slices 0 min 2% B → 2 min 5% B → 10 min 5% B → 70 min 25% B → 75 min 40% B → 77 min 90% B → 82 min 90% B → 83 min 2% B → 90 min 2% B
Gradient duration	65 min	90 min
Column oven temperature	40°C	

Table S3 Mass spectrometry settings.

	HUNTER samples	gel slices
Instrument	Exploris 480 mass spectrometer (Thermo Scientific)	Q Exactive Plus mass spectrometer (Thermo Scientific)
Electrospray	Nanospray Flex Ion Source	
Operation mode	Data dependent acquisition	
Method duration	60 min	90 min
Full MS		
MS scan resolution	120000	70,000
AGC target	300 %	3e6
RF Lens	40 %	
maximum ion injection time for the MS scan	auto	120 ms
Scan range	350 to 1200 m/z	300 to 1650 m/z
Spectra data type	profile	
dd-MS2		
Number of dependent scans	15	10
Resolution	15,000	17,500
Maximum ion injection time mode	auto	120 ms
Scan range	350 to 1200 m/z	200 to 2000 m/z
Data type	centroid	
Time	22 ms	none
Microscans	1	
Isolation window	1.4 m/z overlap	3 m/z
First mass	110 m/z	100 m/z
Dissociation mode	higher energy collisional dissociation (HCD)	
HCD normalized collision energy	30	27,5
Dynamic exclusion	10 s	30 s
Apex trigger	none	1 to 40 s
Charge state	include 2-6	
Intensity threshold	5000	none
Multiple charge states	None	all
Peptide Match	None	preferred

References

- Dubin G, Stec-Niemczyk J, Kisielevska M, Pustelny K, Popowicz GM, Bista M, Kantyka T, Boulware KT, Stennicke HR, Czarna A, Phopaisarn M, Daugherty PS, Thøgersen IB, Enghild JJ, Thornberry N, Dubin A, Potempa J (2008) Enzymatic activity of the *Staphylococcus aureus* SplB serine protease is induced by substrates containing the sequence Trp-Glu-Leu-Gln. *J Mol Biol* 379:343–356. doi: 10.1016/j.jmb.2008.03.059
- Robert X, Gouet P (2014) Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Res* 42:W320–4. doi: 10.1093/nar/gku316
- Schechter I, Berger A (1967) On the size of the active site in proteases. I. Papain. *Biochem Biophys Res Commun* 27:157–162. doi: 10.1016/S0006-291X(67)80055-X