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SUPPLEMENTARY INFORMATION

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Two-site recognition of *Staphylococcus aureus* peptidoglycan by lysostaphin SH3b

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Supplementary Table 1. CSPs associated with amino-acid side-chains.

G5			P4		
Side chain	**Normalized Δδ[a] (ppm)**	**Δδ/averageΔδ[b] (0.016)[c]**	**Side chain**	**Normalized Δδ[a] (ppm)**	**Δδ/averageΔδ[b] (0.079)[c]**
Ws402	0.006	0.383	Ws402	0.039	0.489
N405s	0.122	7.643	N405s	0.028	0.350
Ws460	0.005	0.288	Rs427	0.168	2.122
Ws478	0.004	0.258	Rs433	0.048	0.610
Ws489	0.001	0.068	Ws460	0.143	1.796
			Rs470	0.038	0.484
			Rs476	0.015	0.194
			Ws478	0.014	0.175
			Ws489	0.157	1.982
P4-G5			**GM-P4-G5**		
Side chain	**Normalized Δδ[a] (ppm)**	**Δδ/averageΔδ[b] (0.069)[c]**	**Side chain**	**Normalized Δδ[a] (ppm)**	**Δδ/averageΔδ[b] (0.121)[c]**
Ws402	0.010	0.143	Ws402	0.126	1.045
N405s	*	*	N405s	0.174	1.446
Rs427	*	*	Rs427	0.226	1.873
Rs433	0.024	0.348	Rs433	0.096	0.794
Ws460	0.129	1.879	Ws460	0.199	1.648
Rs470	0.231	3.367	Rs470	0.216	1.786
Rs476	0.012	0.175	Rs476	0.071	0.588
Ws478	0.004	0.058	Ws478	0.021	0.171
Ws489	0.097	1.417	Ws489	0.143	1.188
P5-G5-P4-G5			**GM-P5-G5-GM-P4-G5**		
Side chain	**Normalized Δδ[a] (ppm)**	**Δδ/averageΔδ[b] (0.119)[c]**	**Side chain**	**Normalized Δδ[a] (ppm)**	**Δδ/averageΔδ[b] (0.057)[c]**
Ws402	0.059	0.499	Ws402	0.015	0.256
N405s	0.078	0.653	N405s	0.191	3.343
Rs427	0.244	2.059	Rs427	0.071	1.247
Rs433	0.132	1.108	Rs433	0.013	0.235
Ws460	0.189	1.597	Ws460	0.066	1.165
Rs470	0.083	0.703	Rs470	0.019	0.334
Rs476	0.032	0.272	Rs476	0.029	0.513
Ws478	0.172	1.452	Ws478	0.053	0.925
Ws489	0.095	0.802	Ws489	0.039	0.689

[a] Nor malized ^{1}H and ^{15}N chemical shifts in ppm.
[b] Value for the ^{1}H ^{15}N chemical shift divided by the average chemical shift.
[c] Average chemical shift value from all residues when titrated with the corresponding ligand.
*Slow conformational exchange was reported for the backbone and side-chain of these residues showing line broadening and disappearance of the signal at the highest ligand concentrations.

Supplementary Table 2. Peptidoglycan binding activities of WT SH3b-NG and derivatives showing the amount of PG required for 50% binding (PG_{50}) and the fold change as compared to the wild-type domain.

	SH3b	PG_{50}	Fold change
Fig. 3a	WT [c]	69.2	1.00
	femB	118.3	1.71
	femAB	152.6	2.20
Fig. 3a	WT	105.8	1.00
	N405A	163.0	1.54
	T409V	167.3	1.58
	Y411S	193.5	1.83
	T429V	129.5	1.22
	D450N	164.8	1.56
	E451M	177.9	1.68
	V452A	129.7	1.23
	M453A	171.5	1.62
	Y472S	168.0	1.59
Fig. 3c	WT	69.2	1.00
	F418V	102.4	1.48
	N421L	107.9	1.56
	V461A	118.9	1.72
	I425A	128.1	1.85
	R427M	170.6	2.47
	L473A	139.0	2.01
	W489L	151.0	2.18

Table 3. Bacterial strains and plasmids used in this study

Strains, plasmids	Relevant properties or genotype[a]	Source or reference
Strains		
Staphylococcus aureus		
SH1000	8325-4 derivate with a restored *rsbU* allele	46
NCTC8325	Wild type strain	46
Escherichia coli		
Lemo21(DE3)	BL21 derivative for protein production	NEB
NEB5α	Host strain for DNA cloning	NEB
Plasmids		
pET15b	Plasmid for the production of proteins with an N-terminal His-tag	Novagen
pET21a	Plasmid for the production of proteins with a C-terminal His-tag	Novagen
pET2818	Plasmid for the production of proteins with a C-terminal His-tag	lab stock
pET2817-TEV	Plasmid for the production of proteins with an N-terminal cleavable His-tag	lab stock
pET- SH3b	pET15b derivative encoding Lss SH3b domain for NMR experiments	38
pET-Lss	pET21a derivative for the expression of the full length Lss lysostaphin	39
pET-SH3b-TEV	pET2817-TEV derivative for the expression of the Lss-SH3b domain for X-ray crystallography	This study
pET-SH3b-NG	pET2818 derivative for the expression of SH3b-mNeonGreen fusions	This study

[a] Amp^R, resistant to ampicillin; Erm^R, resistant to erythromycin

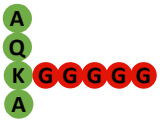
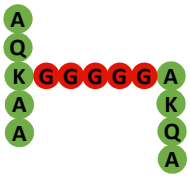
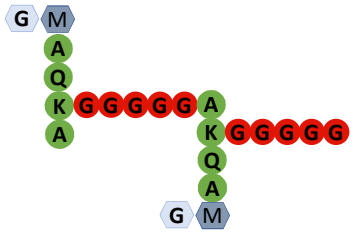
Supplementary Table 4. Oligonucleotides used in this study

Name	Sequence
N405A_Fw	CATGGGATGGAAAACAGCCAAATATGGCACACTATATAAATC
N405A_Rev	TAGTGTGCCATATTTGGCTGTTTTCCATCCCATGGTATATC
N405A_Fw_Lss	TACAGGTTGGAAAACAGCCAAATATGGCACACTATATAAATC
N405A_Rev_Lss	AGTGTGCCATATTTGGCTGTTTTCCAACCTGTATTCGGCGTTG
T409V_Fw	ACAAACAAATATGGCGTGCTATATAAATCAGAGTCAG
T409V_Rev	CTCTGATTTATATAGCACGCCATATTTGTTTGTTTTC
Y411S_Fw	AAATATGGCACACTATCAAAATCAGAGTCAGCTAGCTTC
Y411S_Rev	AGCTGACTCTGATTTTGATAGTGTGCCATATTTGTTTG
F418V_Fw	CAGAGTCAGCTAGCGTCACACCTAATACAGATATAATAAC
F418V_Rev	ATCTGTATTAGGTGTGACGCTAGCTGACTCTGATTTATATAGTG
N421L_Fw	GCTAGCTTCACACCTCTTACAGATATAATAACAAGAACGAC
N421L_Rev	GTTATTATATCTGTAAGAGGTGTGAAGCTAGCTGACTC
I425A_Fw	ACCTAATACAGATATAGCAACAAGAACGACTGGTCCATTTAG
I425A_Rev	CCAGTCGTTCTTGTTGCTATATCTGTATTAGGTGTGAAGCTAG
T429V_Fw	ATAATAACAAGAACGGTTGGTCCATTTAGAAGCATG
T429V_Rev	CTTCTAAATGGACCAACCGTTCTTGTTATTATATCTG
D450N_Fw	CAAACAATTCATTATAATGAAGTGATGAAACAAGAC
D450N_Rev	GTTTCATCACTTCATTATAATGAATTGTTTGACCTG
E451M_Fw	ACAATTCATTATGATATGGTGATGAAACAAGACGGTCATG
E451M_Rev	GTCTTGTTTCATCACCATATCATAATGAATTGTTTGAC
V452A_Fw	TTCATTATGATGAAGCGATGAAACAAGACGGTCATG
V452A_Rev	ACCGTCTTGTTTCATCGCTTCATCATAATGAATTGTTTG
M453A_Fw	CATTATGATGAAGTGGCAAAACAAGACGGTCATGTTTG
M453A_Rev	ATGACCGTCTTGTTTTGCCACTTCATCATAATGAATTG
M461A_Fw	ACGGTCATGTTTGGGCAGGTTATACAGGTAACAGTG
M461A_Rev	TTACCTGTATAACCTGCCCAAACATGACCGTCTTGTTTC
Y472S_Fw	AGTGGCCAACGTATTTCCTTGCCTGTAAGAACATGGAAT
Y472S_Rev	TGTTCTTACAGGCAAGGAAATACGTTGGCCACTGTTAC
L473A_Fw	GGCCAACGTATTTACGCGCCTGTAAGAACATGGAATAAATC
L473A_Rev	CATGTTCTTACAGGCGCGTAAATACGTTGGCCACTGTTAC
W489L_Fw	ACTTTAGGTGTTCTTCTGGGAACTATAAAGGGATCCGGAG
W489L_Rev	TCCCTTTATAGTTCCCAGAAGAACACCTAAAGTATTAGTAG
W489L_Fw_Lss	TACTTTAGGTGTTCTTCTGGGAACTATAAAGCTCGAGCAC
W489L_Rev_Lss	GAGCTTTATAGTTCCCAGAAGAACACCTAAAGTATTAGTAG

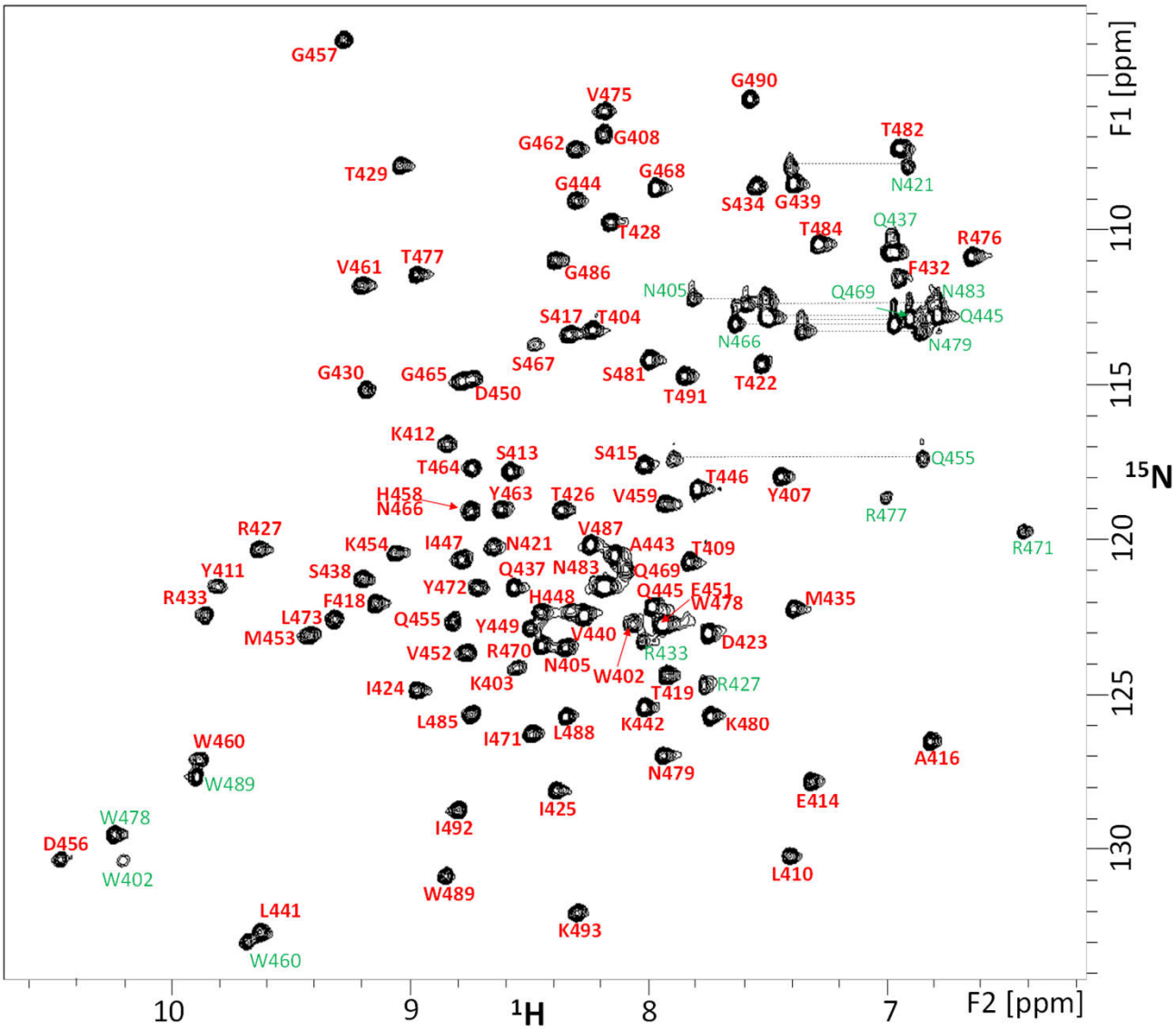
Table 5. Data collection and refinement statistics

	Home source set	Synchrotron set
Data collection		
Space group	$P4_12_12$	$P4_12_12$
Cell dimensions		
a, *b*, *c* (Å)	47.2, 47.2, 123.1	47.1, 47.1, 122.4
α, β, γ (°)	90, 90, 90	90, 90, 90
Resolution (Å)	2.5(2.6-2.5)*	1.43 (1.47-1.43)
R_{merge}	12.8(21.5)	5.9 (341.3)
$I / \sigma I$	14.3(6.0)	21.6(1.1)
Completeness (%)	99.6(98.7)	98.9(98.4)
Redundancy	11.9(8.9)	24.3(24.7)
Refinement		
Resolution (Å)	2.5	1.43
No. reflections	5270	26342
R_{work} / R_{free}	25.4/29.0	19.9/23.0
No. atoms		
Protein	741	741
Ligand/ion	49	52
Water	3	24
B-factors		
Protein	27.7	43.4
Ligand/ion	29.7	44.2
Water	17.9	44.3
R.m.s. deviations		
Bond lengths (Å)	0.003	0.008
Bond angles (°)	1.18	1.49

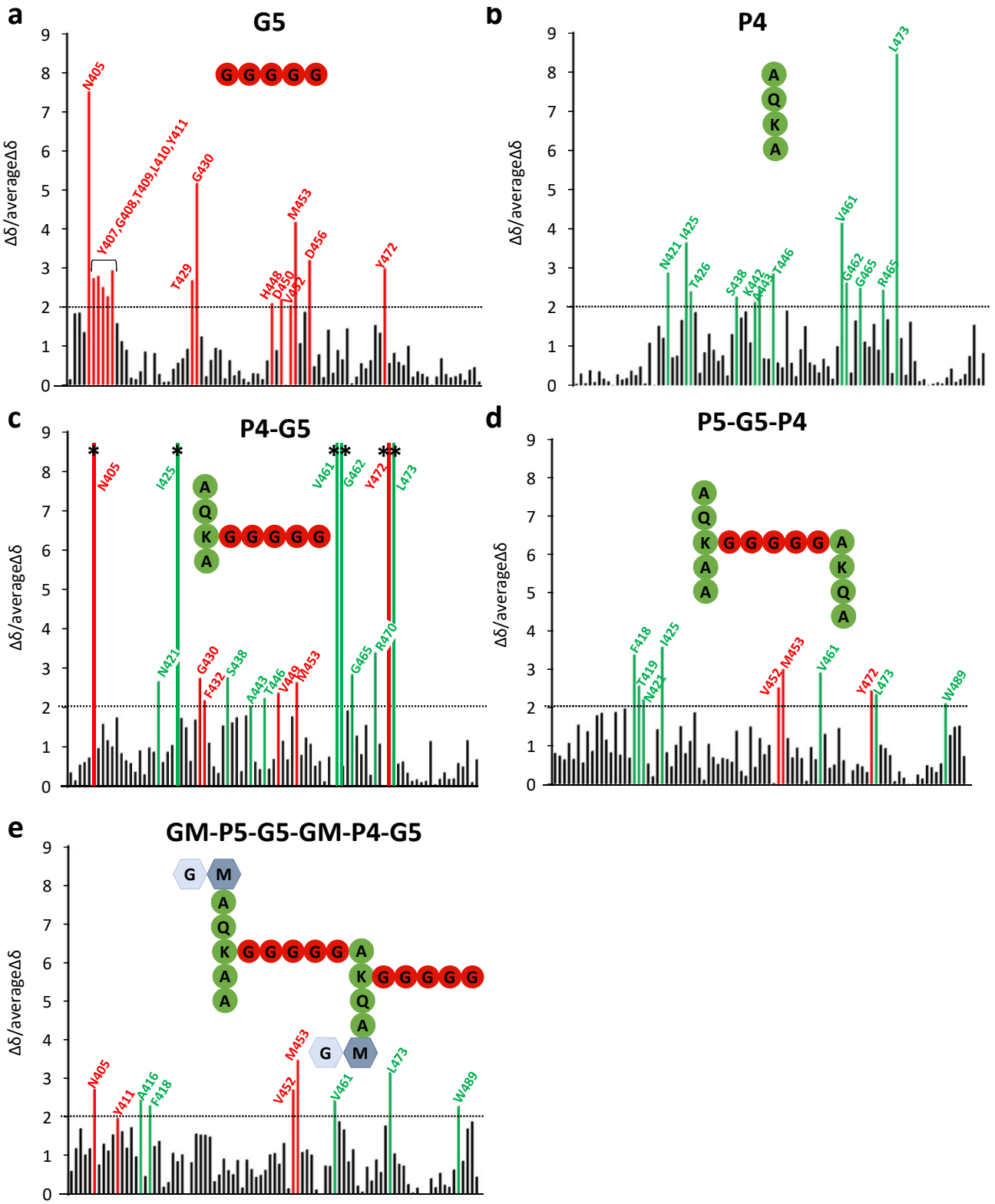
A single crystal was used for both data collections.
*Values in parentheses are for highest-resolution shell.

Ligand		Source
	GMGM	Synthetic compound
	G5	Synthetic compound
	P4	Synthetic compound
	P4-G5	Synthetic compound
	P5-G5-P4	Synthetic compound
	GM-P5-G5-GM-P4-G5	Peptidoglycan digestion (mutanolysin)

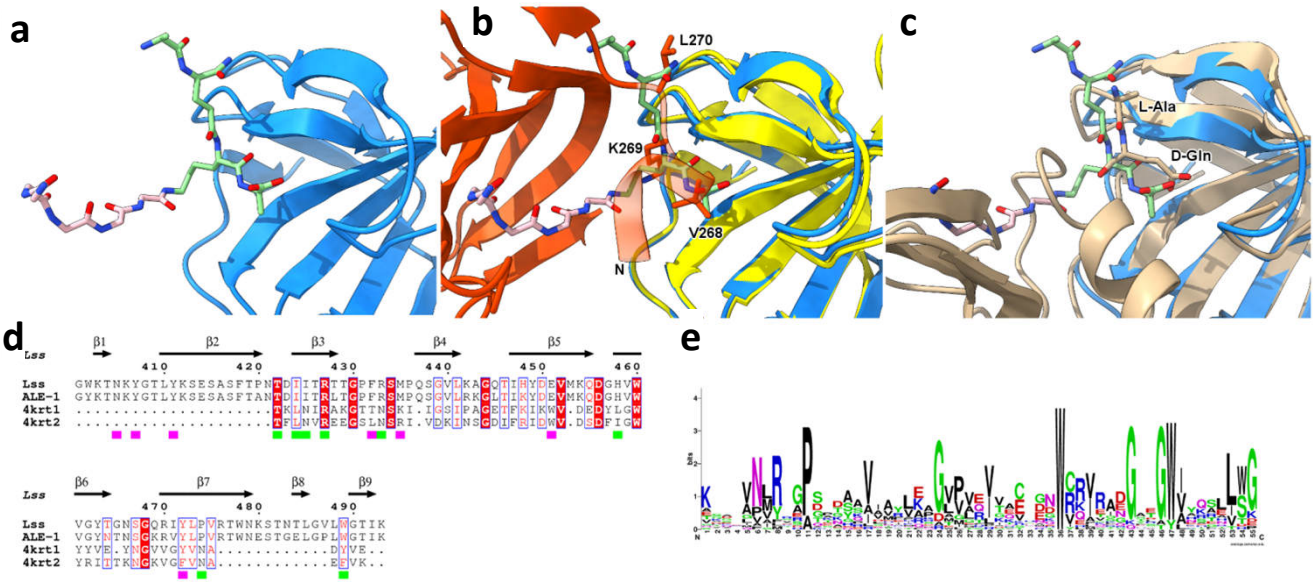
Supplementary Fig 1. *S. aureus* PG fragments used as SH3b ligands. The structure of each ligand, its name and method of preparation are indicated.



Supplementary Fig 2. Fully assigned ^{1}H- ^{15}N HSQC spectrum of the lysostaphin SH3b apo domain (residues 402-493). Assigned backbone amide resonances are shown. Side-chain resonances of tryptophan, glutamine and asparagine residues are indicated in green.

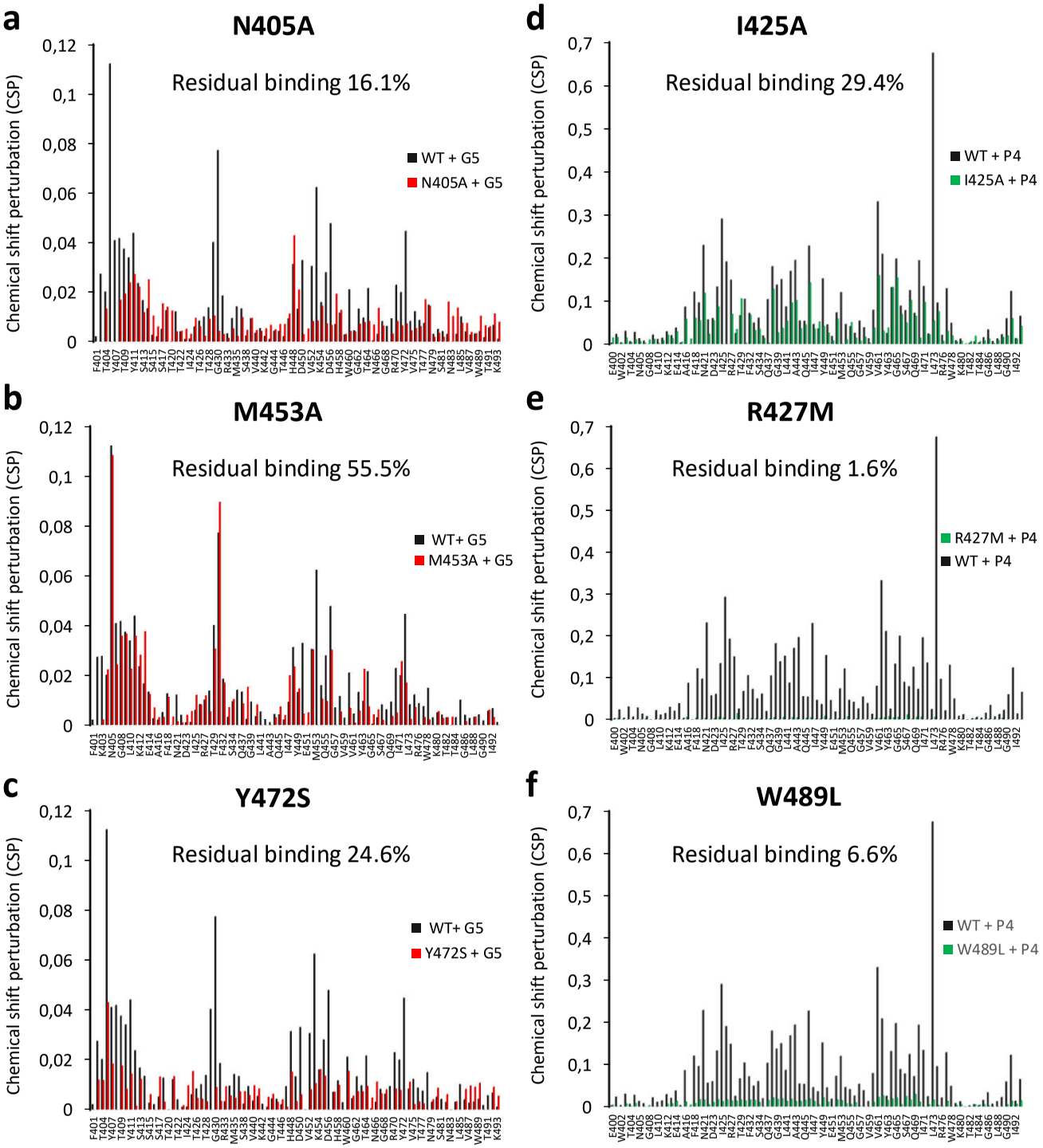


Supplementary Figure 3. Chemical shift perturbation (CSP) analysis of the SH3b protein interactions with a set of six different PG fragments derived from *S. aureus*. Histograms of the observed CSP values calculated as $\Delta\delta=(\Delta\delta H^2+(0.154x\Delta\delta N)^2)$, as a function of the amino acid sequence are shown. The *y*-axis represents the ratio between individual CSPs and the average CSP (taking all residues into account). An arbitrary threshold of 2 was chosen. Residues associated with CSPs above the threshold using G5 as a ligand are in red, those with CSPs above the threshold using P4 as a ligand are in green. Titrations were carried out using 50 µM of protein and G5 (**a**), P4 (**b**), P4-G5 (**c**), P5-G5-P4 (**e**) and GM-P5-G5-GM-P4-G5 (**f**). 0, 0.33, 0.66, 1, 2, 4, 8, 16, 32, and 64 equivalents of ligand were used in (**a**)-(**c**); 16 equivalents in (**d**), and only 4 equivalents in (**e**) as protein aggregation occurred.

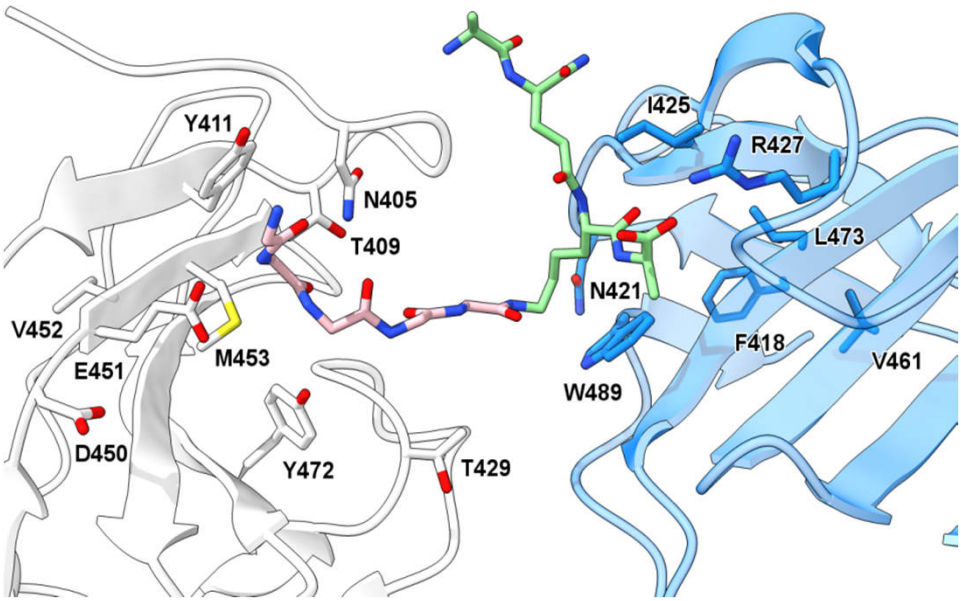


Supplementary Fig 4. Comparison of ligand-binding pocket to other SH3b structures and SH3 superfamily members. **a**, Lysostaphin SH3b domain in complex with the P4-G5 ligand. **b**, Superimposition of the structure shown in A onto the ALE-1 structure (1r77, two symmetry-related monomers orange and yellow). The P4-G5 ligand occupies the same space as an affinity purification tag (helical turn, N-terminus labelled). **c**, Superimposition of the structure shown in A onto the phi7917 structure (5D76, tan). Ligand P3(K)-P4(A) are sterically equivalent to tag residues K269 and V268, respectively. The phi7917 ligand (L-Ala-D-Gln) is positioned with its peptide bond over the P3-P4 peptide. **d,** Sequence alignment of Lysostaphin SH3b (SH3_5 subfamily) with ALE-1 (SH3_5) and the two tandem domains of *Clostridium* phage phiSM101 (4krt, SH3_3 subfamily), G5 and P4-ligating residues annotated with magenta and green block below text, respectively.

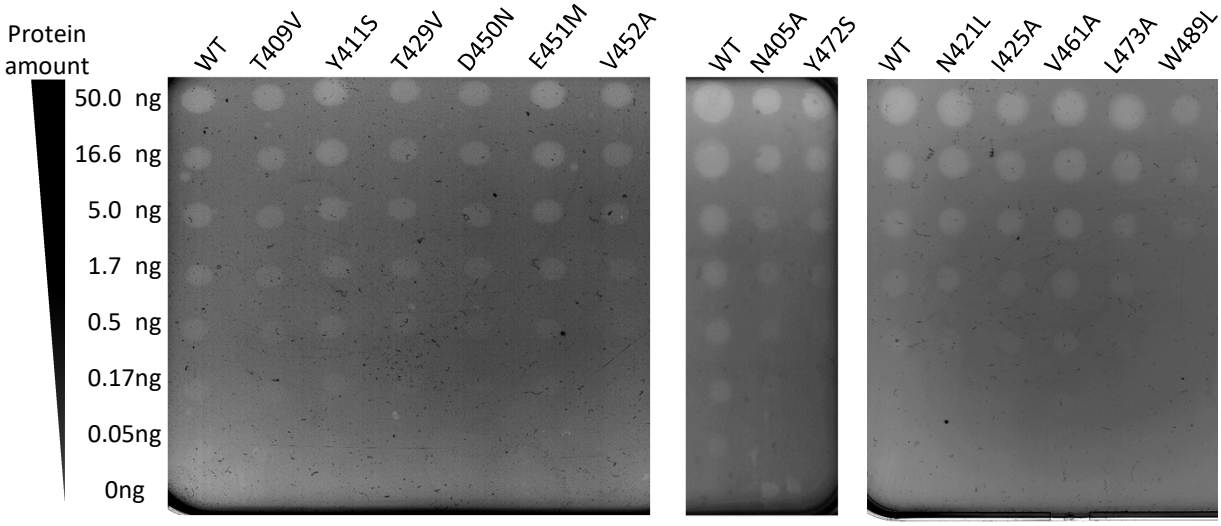
e, Weblogo (Crooks *et al.*, 2004) plot of sequence consensus of SH3_4 subfamily, identifying features with likely equivalence to SH3_3 and SH3_5 alignment: NxR (position 6-8, match lss I425-R427), W (36, match to W460), GxxGW (43-47, match to G468-Y472) and LWG (53-55, match to L488-G490). No structures are currently available for SH3_4 proteins, but structural comparison between Lss/ALE-1 and 4krt confirms conservation of the P-stem D-Ala(4)-carboxylate pocket.



Supplementary Fig 5. Comparison of chemical shift perturbations (CSP) in wild-type and SH3b mutant domains associated with the binding to G5 and P4 ligands. Histograms show individual CSP values from the ^{15}N-HSQC titrations of N405A (**a**), M453A (**b**), and Y472S mutants (**c**) in red compared to the CSP values from the WT protein (in grey) following addition of 32 equivalents of G5. (**c**), (**d**) and (**e**) show CSPs from titrations of I425A, R427M and W489L, respectively (in green) compared to CSP values from the WT protein (in grey) following addition of 32 equivalents of P4. The percentage of residual binding activity deduced from the average CSP values is indicated.



Supplementary Fig 6. Mutagenized residues at the SH3b:P4-G5 interaction interface. The representation is identical to figure 2, but displaying all residues selected for mutation in stick form (to avoid confusion, residues are only displayed once, from the SH3b domain that places them closest to the ligand).



Supplementary Fig 7. Comparison of the enzymatic activity of lysostaphin (Lss) recombinant proteins containing mutations in the SH3b domain. Three independent series of purifications were carried out (left, middle and right panels), each including a wild-type protein as a control. Five µl corresponding to serial dilutions of recombinant Lss proteins were spotted on agar plates containing autoclaved *S. aureus* cells (final OD_{600} of 1) as a substrate. Lytic activities were detected as clearing zones and compared by determining the lowest amount of enzyme giving a detectable digestion of the substrate.

N-terminally His-tagged SH3b (pET-SH3b) for NMR analyses; residues 402-493

MGHHHHHHEFWKTNKYGTLYKSESASFTPNTDIITRTTGPFRSMPQSGVLKAGQTIHYDEVMKQDG
HVWVGYTGNSGQRIYLPVRTWNKSTNTLGVLWGTIK

N-terminally His-tagged SH3b (pET-SH3b-TEV) for X-ray crystallography; residues 402-493

MSGHHHHHHAMGENLYFQG↓SWKTNKYGTLYKSESASFTPNTDIITRTTGPFRSMPQSGVLKAGQTIH
YDEVMKQDGHVWVGYTGNSGQRIYLPVRTWNKSTNTLGVLWGTIKVLWGTIK

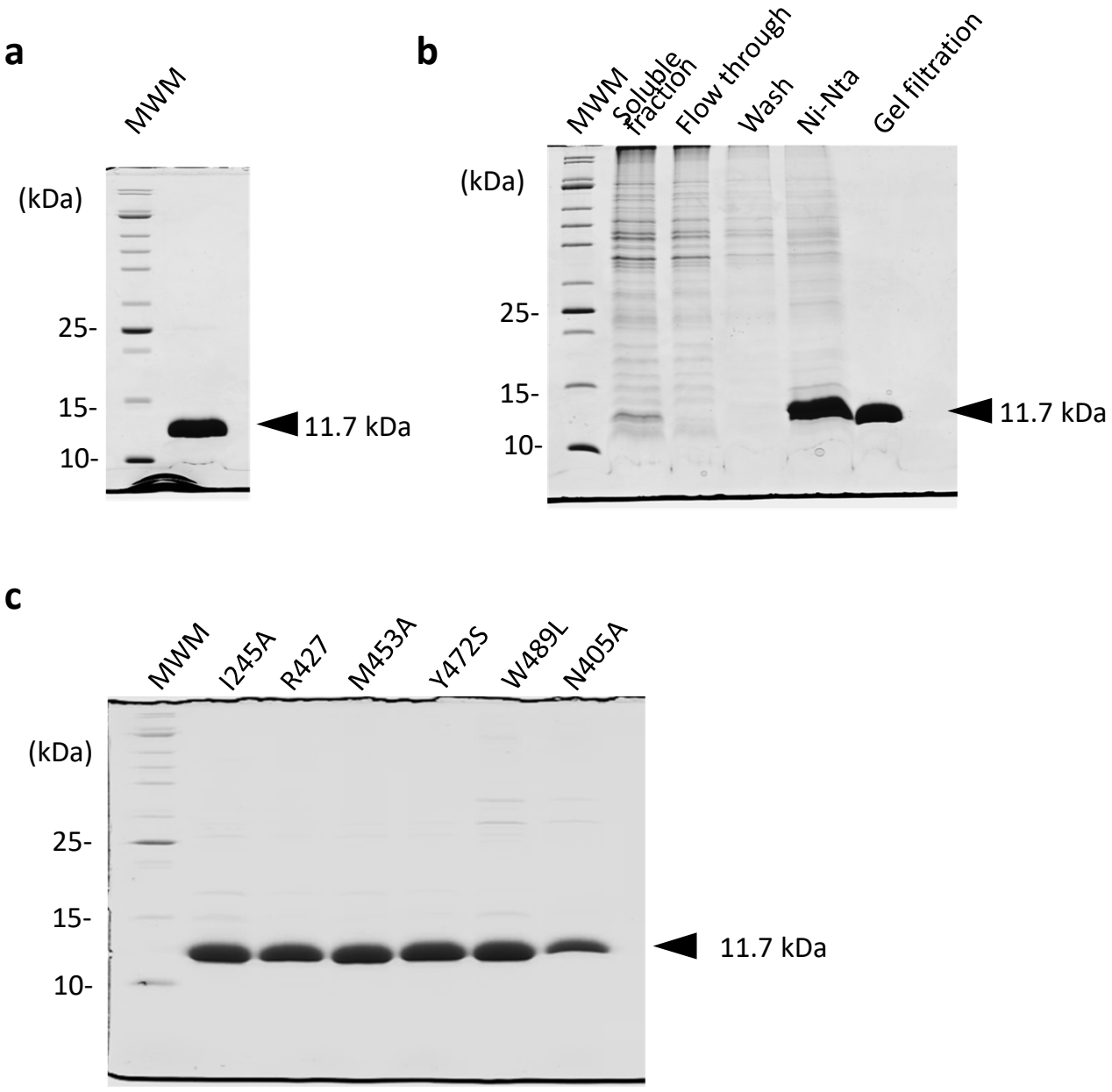
C-terminally his-tagged SH3b-mNeonGreen fusion (pET-SH3b-NG) for binding assays; residues 401-493

MGWKTNKYGTLYKSESASFTPNTDIITRTTGPFRSMPQSGVLKAGQTIHYDEVMKQDGHVWVGYTG
NSGQRIYLPVRTWNKSTNTLGVLWGTIKGSGGSGSGGSNNSGMVSKGEEDNMASLPATHELHIFGSI
NGVDFDMVGQGTGNPNDGYEELNLKSTKGDLQFSPWILVPHIGYGFHQYLPYPDGMSPFQAAMVD
GSGYQVHRTMQFEDGASLTVNYRYTYEGSHIKGEAQVKGTGFPADGPVMTNSLTAADWCRSKKTYPN
DKTIISTFKWSYTTGNGKRYRSTARTTYTFAKPMAANYLKNQPMYVFRKTELKHSKTELNFKEWQKAFT
DVMGMDELYKHHHHHH

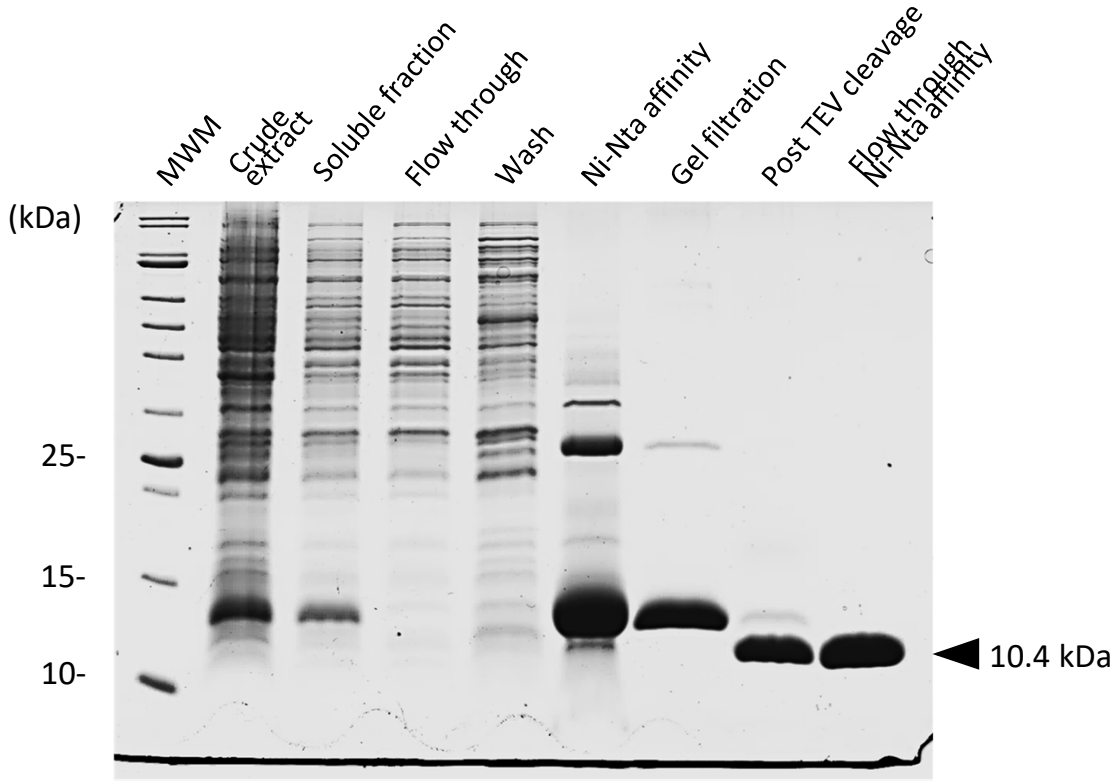
Full length lysostaphin (pET-Lss) for activity assays; residues 248-493

MAATHEHSAQWLNNYKKGYGYGPYPLGINGGMHYGVDFFMNIGTPVKAISSGKIVEAGWSNYGGG
NQIGLIENDGVHRQWYMHLSKYNVKVGDYVKAGQIIGWSGSTGYSTAPHLHFQRMVNSFSNSTAQD
PMPFLKSAGYGKAGGTVTPTPNTGWKTNKYGTLYKSESASFTPNTDIITRTTGPFRSMPQSGVLKAGQ
TIHYDEVMKQDGHVWVGYTGNSGQRIYLPVRTWNKSTNTLGVLWGTIKLEHHHHHH

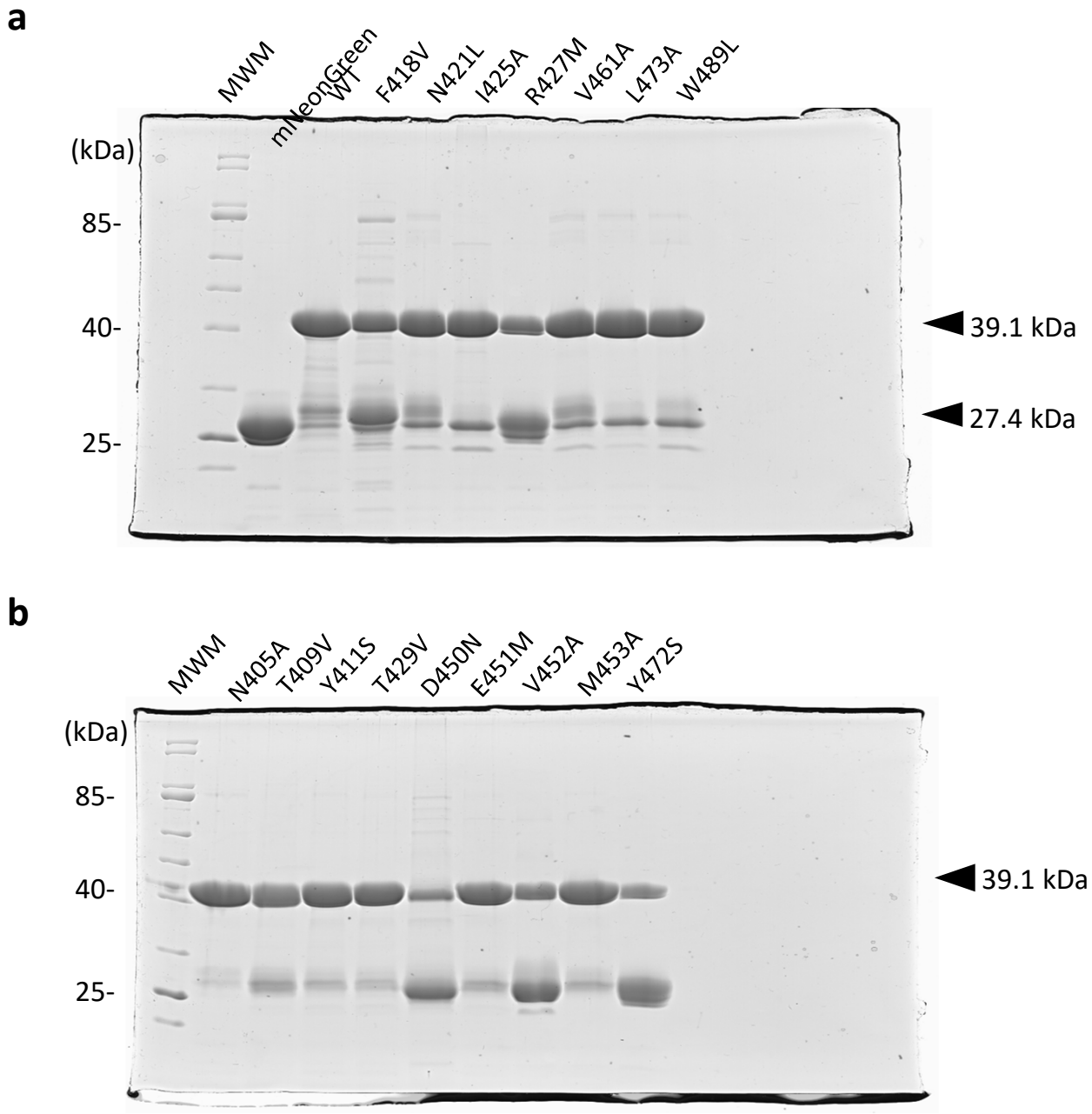
Supplementary Fig 8. Amino acid sequences of the recombinant wild-type proteins used in this study. In each case, both the plasmid encoding the recombinant SH3b protein (in brackets) and the experiment it was used for are indicated. The SH3b domain is in red, amino acids encoded by the expression vector in grey, linker in blue and mNeonGreen in green. The TEV cleavage site is indicated by an arrow.



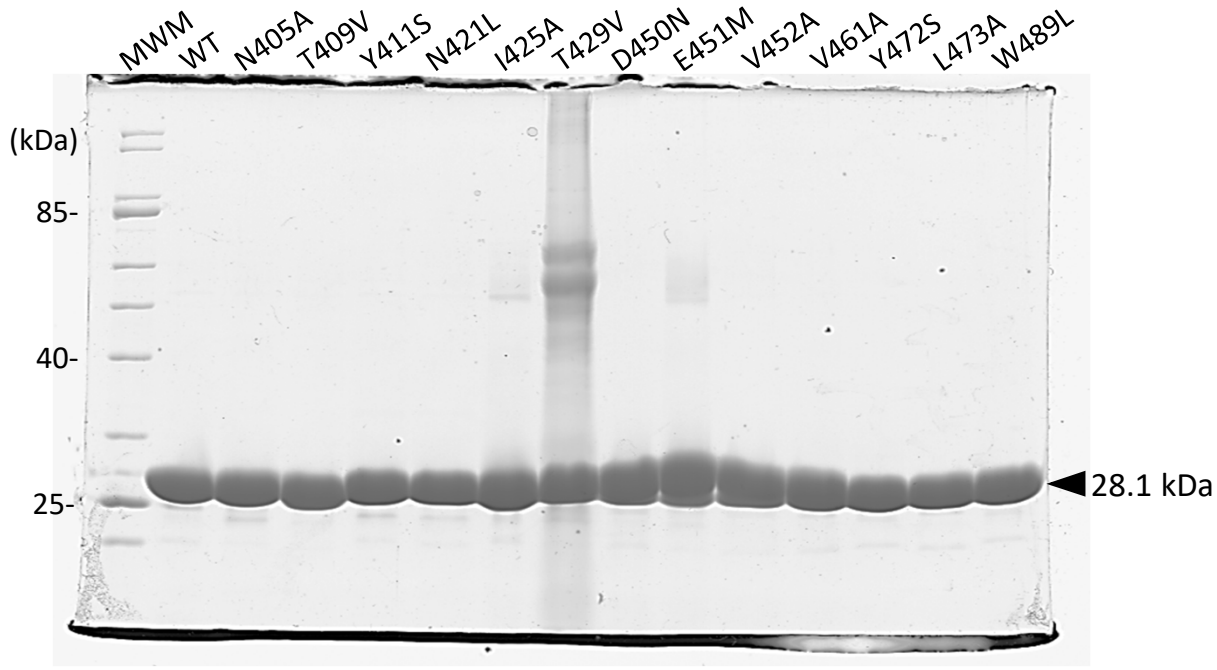
Supplementary Fig 9. SDS-PAGE analysis of recombinant proteins used for NMR studies. **a,** doubly labelled SH3b domain for spectrum assignment described in Supplementary Fig. 2. **b**, singly labelled His-tagged SH3b domain used for NMR titrations with ligands described in Fig. 1 and Supplementary Fig. S3. **c**, singly labelled His-tagged derivatives used for the mutational analysis of the SH3b domain described in Supplementary Fig. 5.



Supplementary Fig 10. SDS-PAGE analysis of recombinant proteins used for X-ray crystallography. The different steps of the purification are described. The final purification product corresponding to the SH3b domain without any His-tag was used for co-crystallisation experiments.



Supplementary Fig 11. SDS-PAGE analysis of recombinant SH3bp-mNeonGreen fusion proteins used for PG binding assays. a, mNeonGreen control and SH3b mutants harbouring mutations in the residues involved in the interaction with G5 ligands. **b**, SH3b mutants harbouring mutations in the residues involved in the interaction with P4 ligands. The amount of protein per binding assay was adjusted using the fluorescent signal intensity of the full-length protein.



Supplementary Fig 12. SDS-PAGE analysis of recombinant SH3bp-mNeonGreen fusion proteins used for PG binding assays. a, mNeonGreen control and SH3b mutants harbouring mutations in the residues involved in the interaction with G5 ligands. **b**, SH3b mutants harbouring mutations in the residues involved in the interaction with P4 ligands. The amount of protein per binding assay was adjusted using the fluorescent signal intensity of the full-length protein.