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Supplementary Materials for

Mechanistic insights into the SNARE complex disassembly

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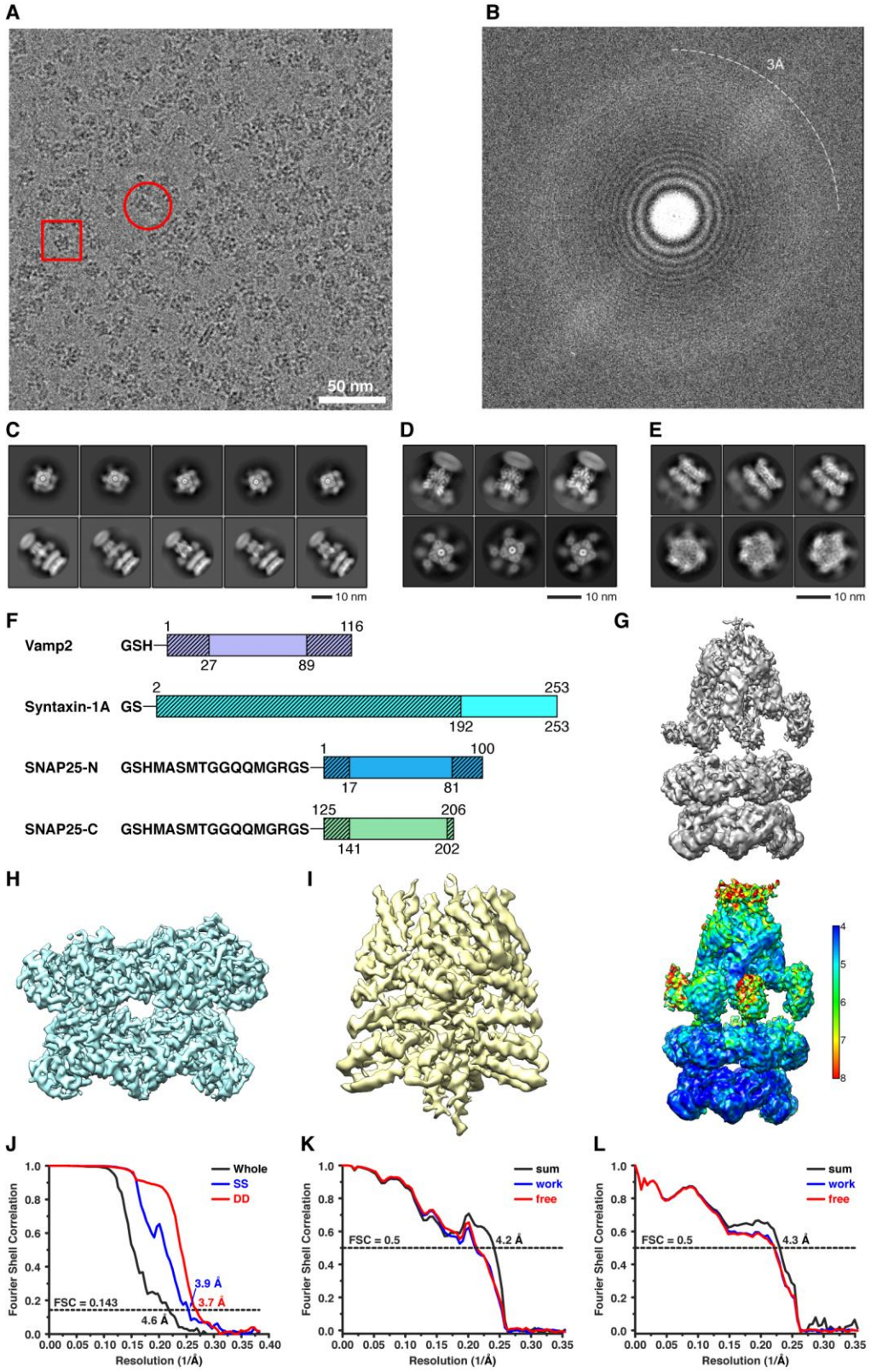

Fig. S1. Cryo-EM analysis of the whole 20S complex, the α-SNAP–SNARE subcomplex, and the NSF-D1D2 part.

(A) A representative motion-corrected electron micrograph of 20S complex with typical particles marked by red circle (side view) and red box (top view). **(B)** Fourier power spectrum of the micrograph showing the Thon ring extending to 3 Å. **(C, D, E)** Typical good reference-free 2-D class averages of the whole 20S complex **(C)**, the α-SNAP-SNARE subcomplex **(D)** and the NSF-D1D2 part **(E)**. **(F)** Schematic of the SNARE proteins used in this study. The lengths of used proteins are indicated above the diagrams. Boundaries of the modeled regions of the SNARE complex are indicated below the diagrams. **(G)** Cryo-EM structure of the whole 20S complex (upper panel) and final density map colored by local resolution (lower panel). **(H)** Cryo-EM structure of the α-SNAP-SNARE subcomplex. **(I)** Cryo-EM structure of the NSF-D1D2 part. **(J)** Gold-standard FSC curves of the final 3D reconstructions of the whole 20S complex (Whole, black), the α-SNAP-SNARE subcomplex (SS, blue) and the NSF-D1D2 part (DD, red). **(K, L)** FSC curves for the cross-validation of the atomic models of the α-SNAP-SNARE subcomplex **(K)** and the NSF-D1D2 part **(L)**. The small difference between work and free FSC curves suggested that the models were not overfitted.

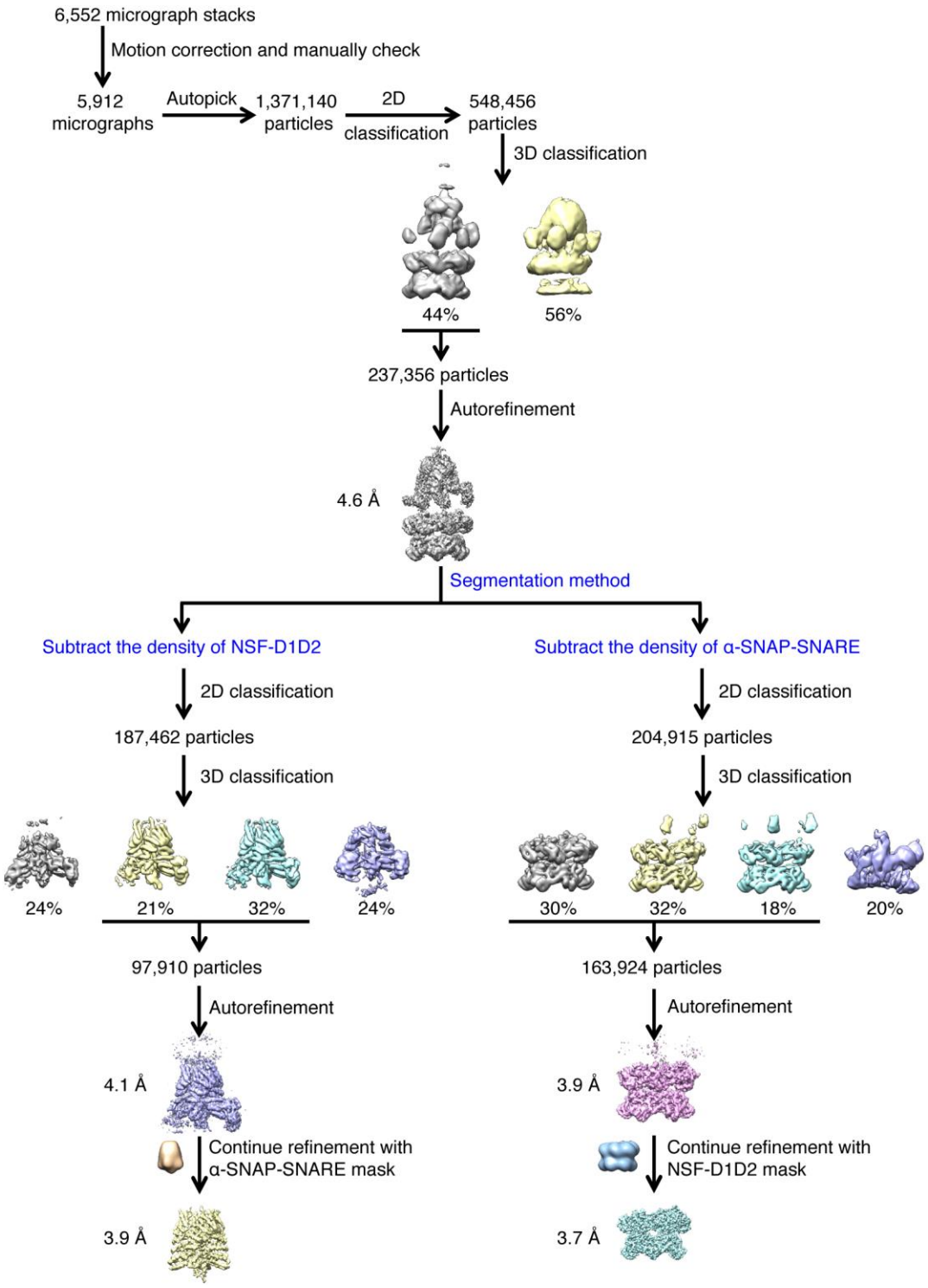

Fig. S2. The flowchart for EM data processing.
Details can be found in Methods.

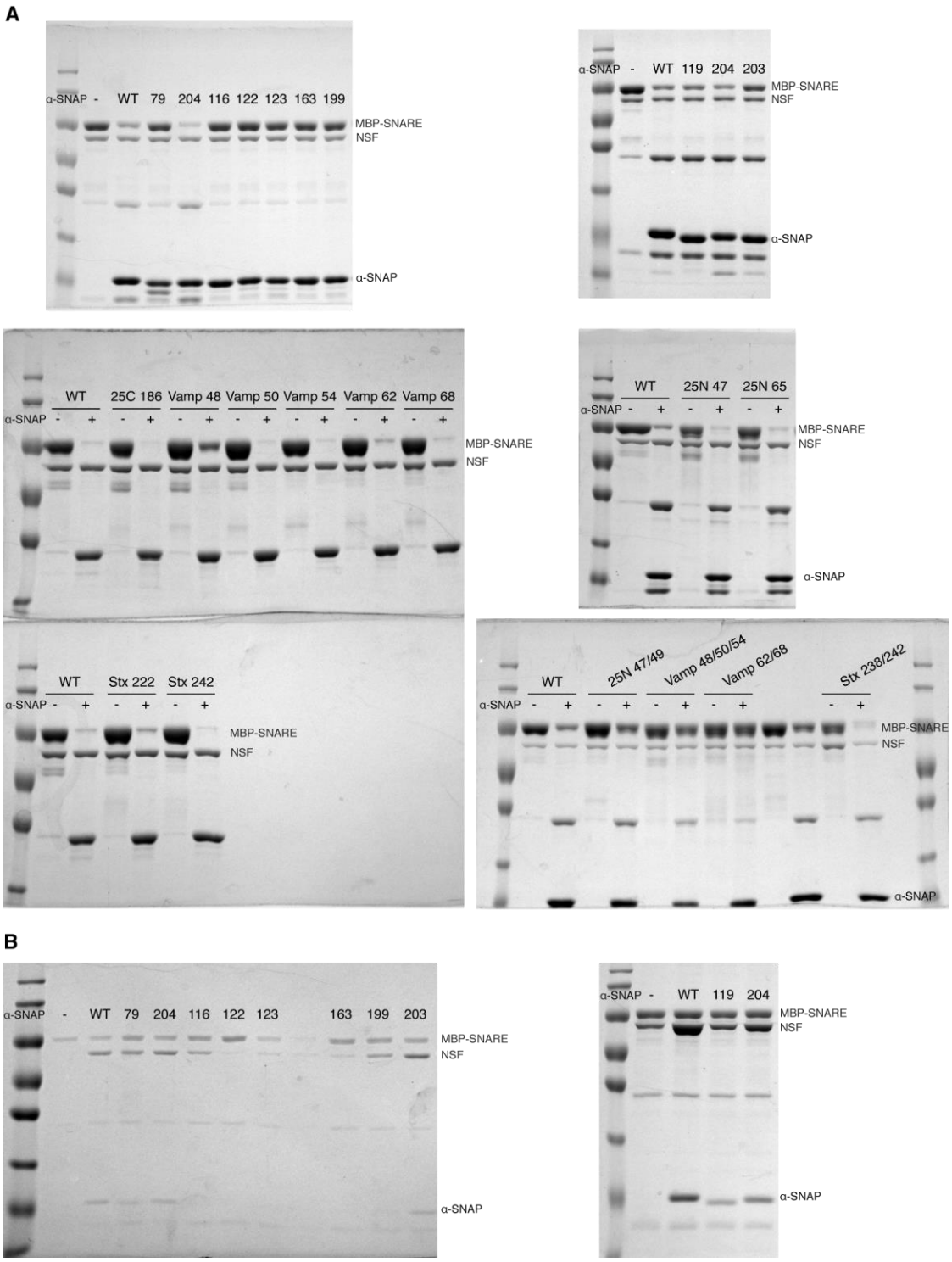

Fig. S3. Representative raw gel images. Representative raw gel images used for both the SNARE complex disassembly (A) and the α-SNAP dependent binding analyses (B), corresponding to Fig. 2A & 2E.
Please note that the data for mutants L197A, L198A and Y200A were from our previous work (30) and so their gel images were not shown here.

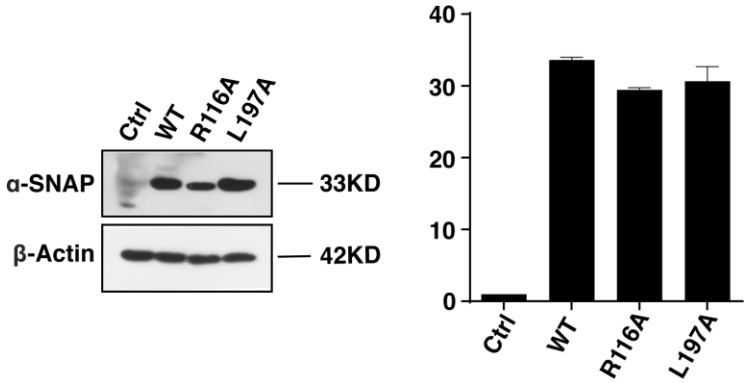

Fig. S4. Analyses of the expression levels of the wild-type α-SNAP and the mutants in the neurons. Left, immunoblot analysis of α-SNAP; n = 3. Right, quantitative reverse transcription PCR (qRT-PCR) analysis. Ctrl, 1.00 ± 0.06; WT, 33.63 ± 0.34; R116A, 29.45 ± 0.31; L197A, 30.66 ± 2.03; n = 3. Error bars, s.e.m.

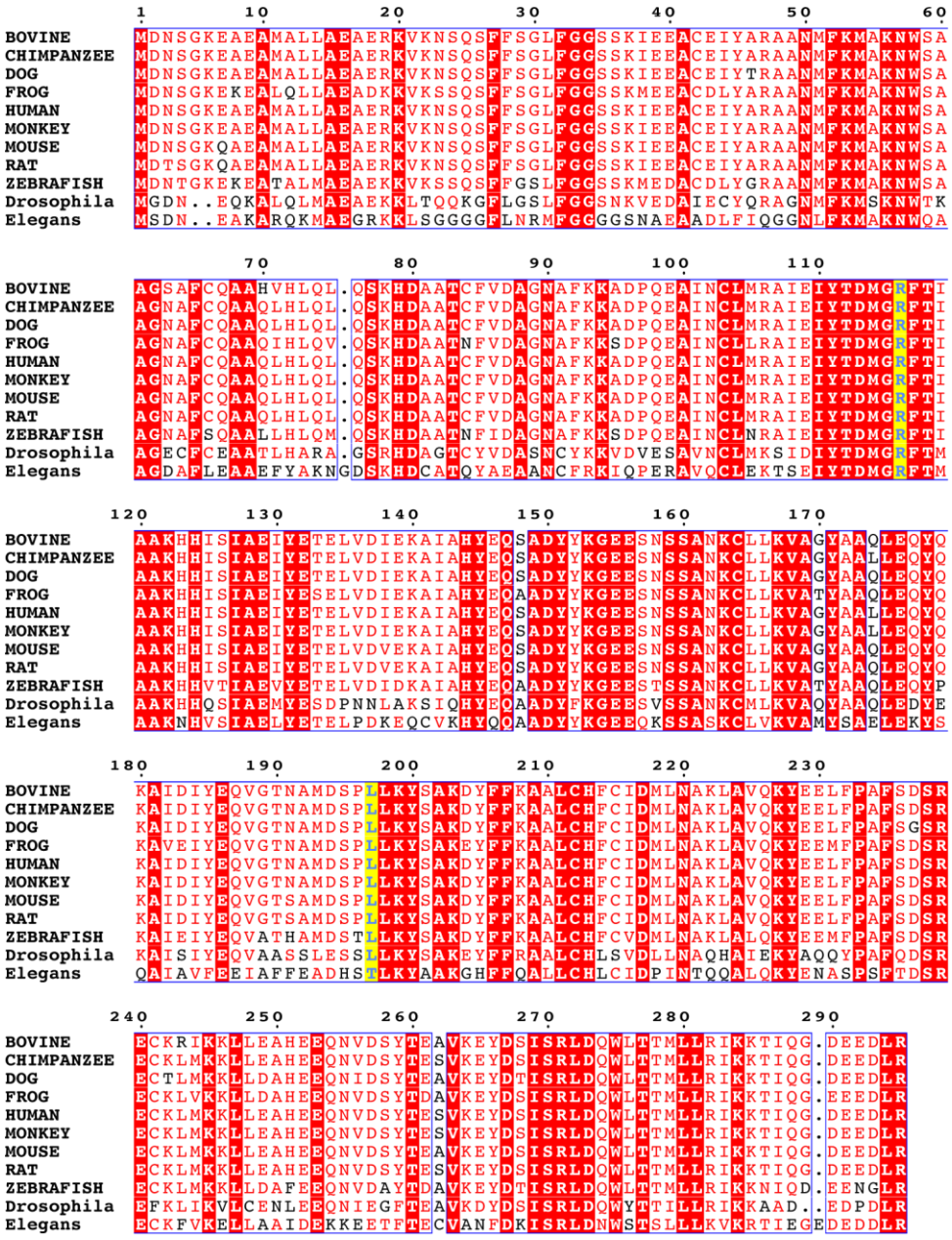

Fig. S5. Sequence alignment of α-SNAP from different species. The conserved residues R116 and L197 are highlighted in yellow. The Uniprot IDs for the aligned sequences are: BOVINE: P81125; CHIMPANZEE: G3RUI1; DOG: E2RQE7; FROG: Q5M8J7; HUMAN: P54920; MONKEY: A0A2K6C5H6; MOUSE: Q9DB05; RAT: P54921; ZEBRAFISH: Q7T2F5; Drosophila: Q23983; Elegans: Q18921.

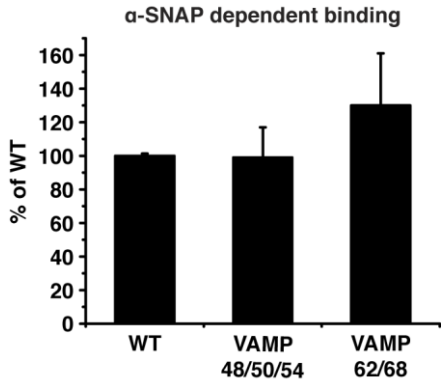

Fig. S6. Effects of multiple point mutations of VAMP on the α-SNAP–dependent binding of NSF to the SNARE complex. The residues of VAMP that interact with α-SNAP's R116 (VAMP 48/50/54) or L197 (VAMP 62/68) were mutated simultaneously. Values are normalized to wild-type SNARE complex (WT) and represent the mean ± SD.

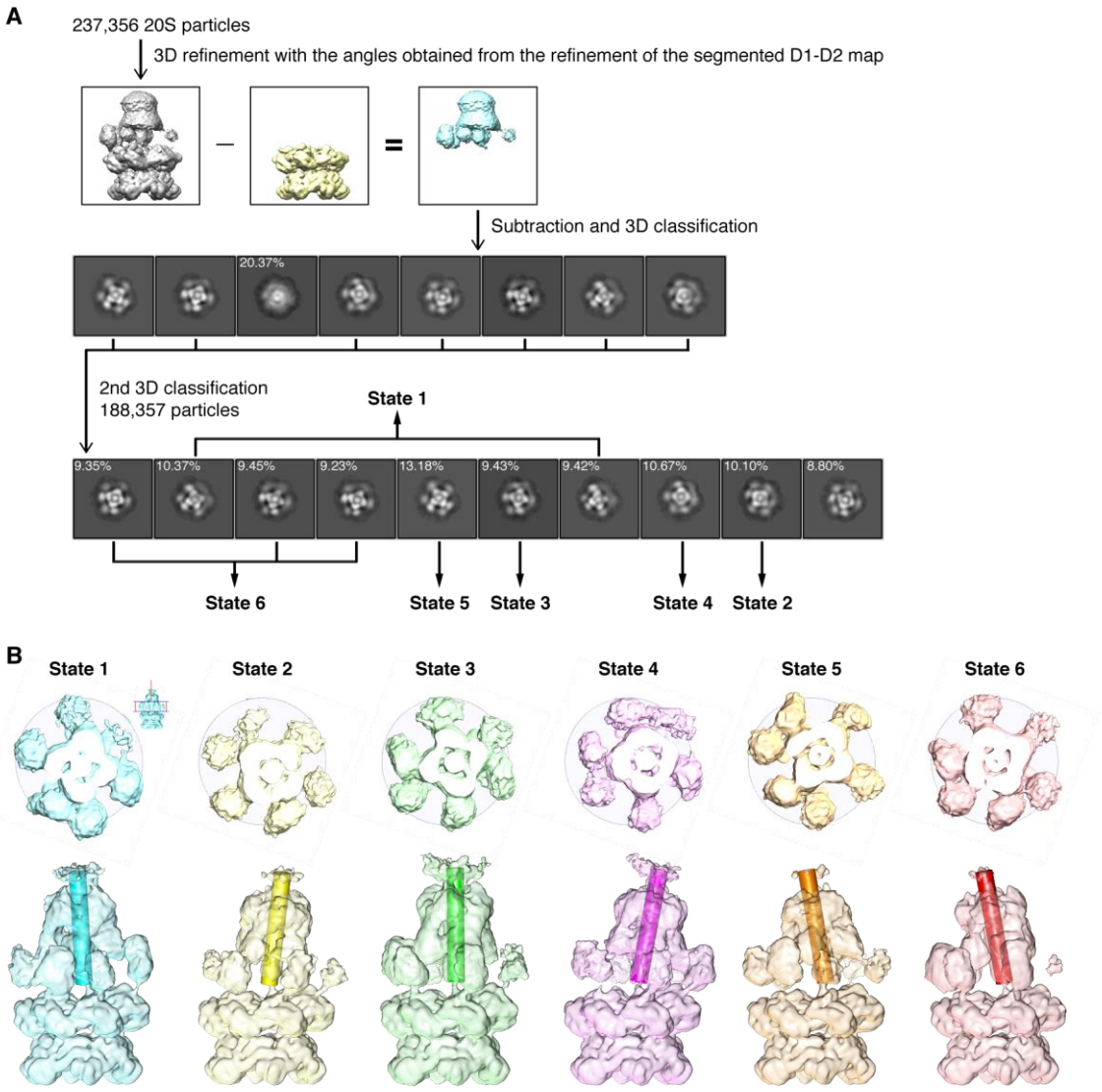

Fig. S7. Focused 3D classification of the α-SNAP–SNARE subcomplex together with the NSF N domain. (A) The flowchart of the focused 3D classification. To better define the flexibility between α-SNAP-SNARE subcomplex and NSF-D1D2 part, NSF-D1D2 signal was subtracted from the raw particle images and alignment-free 3D classification was performed, yielding classes exhibiting distinct patterns of the N-domains relative to the asymmetric D1 ring. **(B)** Upper panel, top view of the reconstruction of six states of 20S complex showing the different pattern in the arrangement of the NSF N-domains relative to the asymmetric D1 ring. Lower panel, side view of the reconstructions of six states of 20S complex showing the varying orientations of α-SNAP-SNARE subcomplex relative to NSF-D1D2. The SNARE complex was represented as a cylinder.

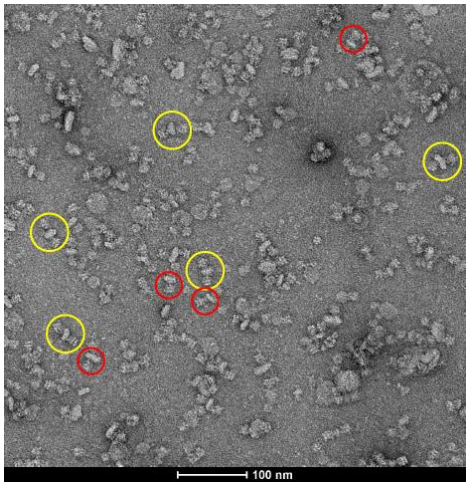

Fig. S8. The N-terminally deleted SNARE proteins can form the 20S complex.
A representative electron micrograph of negatively stained 20S complex formed by the N-terminus deleted SNARE proteins. Typical particles were marked in red (one SNARE complex per nanodisc) and yellow (two SNARE complexes per nanodisc) circles.

Table S1. Cryo-EM data collection and refinement statistics.

	Whole 20S complex (EMDB-9729)	α-SNAP-SNARE subcomplex (EMDB-9697) (PDB 6IP1)	NSF-D1D2 part (EMDB-9698) (PDB 6IP2)
Data collection			
EM equipment		FEI Titan Krios	
Detector		Gatan K2 Summit	
Magnification		22,500	
Voltage (kV)		300	
Electron exposure (e–/Å^2)		~50	
Defocus range (μm)		-1.4 ~ -2.3	
Pixel size (Å)		1.30654	
Number of collected micrographs		6,552	
Number of selected micrographs		5,912	
Reconstruction			
Software		RELION 1.3, RELION 2.0	
Number of initial particles	1,371,140	237,356	237,356
Number of used particles	237,356	97,910	163,942
Symmetry imposed		C1	
Map resolution (Å)	4.6	3.9	3.7
Map sharpening *B* factor (Å^2)	-201.66	-254.53	-170.30
Refinement			
Software		Phenix 1.12rc2-2821	
Model composition			
Non-hydrogen atoms		11246	24666
Protein residues		1420	3120
Ligands		0	12
R.m.s. deviations			
Bond lengths (Å)		0.006	0.007
Bond angles (°)		1.021	1.255
Validation			
MolProbity score		1.90	1.97
Clashscore		7.91	6.66
Poor rotamers (%)		0.34	0.26
Ramachandran plot			
Favored (%)		92.45	87.68
Allowed (%)		7.55	12.19
Disallowed (%)		0	0.13

Table S2. CXMS analysis of the 20S complex formed with either wild-type SNARE proteins or the N-terminally truncated SNARE proteins. Inter-molecular cross-links between SNARE proteins and NSF were obtained with DSS **(A)** or EDC **(B)**. Shown are cross-links identified with confidence. See Methods for filtering criteria.

A

Site 1 - Site 2	#Spec-Total	best E-value	Peptide
25C-D(1)-NSF(304)	9	1.78E-27	GSENLEQVSGIIGNLR(1)-KLFADAEEEQR(1):0
25N(1)-NSF(304)	31	2.91E-25	GSMAEDADMRNELEEMQR(1)-KLFADAEEEQR(1):0
25C(1)-NSF(304)	40	1.05E-20	GSQMAISGGFIR(1)-KLFADAEEEQR(1):0
Stx(84)-NSF(702)	21	6.46E-20	TKEELEELMSDIKK(13)-KVWIGIK(1):0
Stx(95)-NSF(728)	28	1.45E-16	LKSIEQSIEQEEGLNR(2)-KFLALLR(1):0
Stx(95)-NSF(702)	31	1.65E-16	LKSIEQSIEQEEGLNR(2)-KVWIGIK(1):0
Stx-D(1)-NSF(293)	36	3.27E-13	GSSEIIK(1)-VVNGPEILNKYVGESEANIR(10):0
Stx-D(1)-NSF(304)	47	5.21E-13	GSSEIIK(1)-KLFADAEEEQR(1):0
25N(1)-NSF(275)	13	9.21E-13	GSMAEDADMRNELEEMQR(1)-QIGKMLNAR(4):0
Stx(118)-NSF(699)	27	1.25E-12	KTQHSTLSR(1)-TTIAQQVKGK(8):0
25N(1)-NSF(293)	25	5.85E-12	GSMAEDADMRNELEEMQR(1)-VVNGPEILNKYVGESEANIR(10):0
Stx(95)-NSF(699)	12	1.94E-11	LKSIEQSIEQEEGLNR(2)-TTIAQQVKGK(8):0
25N-D(1)-NSF(304)	21	4.35E-11	GSESTRR(1)-KLFADAEEEQRR(1):0
25N(1)-NSF(283)	6	2.08E-09	GSMAEDADMRNELEEMQR(1)-EPKVVNGPEILNK(3):0
Vamp-D(1)-NSF(304)	26	2.12E-05	GSRR(1)-KLFADAEEEQRR(1):0

B

Site 1 - Site 2	#Spec-Total	best E-value	Peptide
Stx(84)-NSF(519)	52	1.42E-31	TKEELEELMSDIKK(13)-VLDDGELLVQQTK(3):0
Stx(84)-NSF(522)	85	8.62E-28	EELEELMSDIKK(11)-VLDDGELLVQQTK(6):0
Stx(84)-NSF(520)	6	3.25E-21	TKEELEELMSDIKK(13)-VLDDGELLVQQTK(4):0
Stx(95)-NSF(744)	26	3.89E-20	SKLKSIEQSIEQEEGLNR(4)-FLALLREEGASPLDFD(16):0
25N(1)-NSF(189)	43	1.28E-19	GSMAEDADMRNELEEMQR(1)-AENSSLNLIGK(2):0
25N(1)-NSF(311)	18	1.31E-19	GSMAEDADMRNELEEMQR(1)-KLFADAEEEQR(8):0
Stx(84)-NSF(744)	58	7.88E-19	TKEELEELMSDIKK(13)-EEGASPLDFD(10):0
Stx(13)-NSF(519)	24	7.23E-18	TAKDSDDDDDVTVTVDRDR(3)-VLDDGELLVQQTK(3):0
25C(1)-NSF(297)	9	6.34E-17	GSQMAISGGFIR(1)-YVGESEANIR(4):0
Stx(95)-NSF(519)	8	7.35E-16	LKSIEQSIEQEEGLNR(2)-VLDDGELLVQQTK(3):0
25C(1)-NSF(189)	13	2.70E-15	GSQMAISGGFIR(1)-AENSSLNLIGK(2):0
Stx(95)-NSF(520)	9	2.76E-15	LKSIEQSIEQEEGLNR(2)-VLDDGELLVQQTK(4):0
25C(1)-NSF(311)	5	2.19E-13	GSQMAISGGFIR(1)-LFADAEEEQR(7):0
Stx(84)-NSF(432)	25	3.61E-12	TKEELEELMSDIKK(13)-ELAVETK(5):0
Vamp(1)-NSF(189)	14	5.65E-12	GSHMSATAATVPPAAPAGEGGPPAPPPNLTSNRR(1)-AENSSLNLIGK(2):0
Stx(84)-NSF(735)	28	2.63E-11	TKEELEELMSDIKK(13)-EEGASPLDFD(1):0
Stx(95)-NSF(735)	7	9.39E-11	LKSIEQSIEQEEGLNR(2)-EEGASPLDFD(1):0
25N(1)-NSF(297)	8	4.84E-10	GSMAEDADMRNELEEMQR(1)-YVGESEANIR(4):0
Stx(118)-NSF(537)	36	5.61E-10	KTQHSTLSR(1)-WGDPVTR(3):0

Table S3. The amino acid sequences of the NSF and SNARE proteins used in this study. Vamp, Stx, 25N and 25C are the wild-type VAMP, Syntaxin, SNAP25-N and SNAP25-C proteins, respectively. Vamp-D, Stx-D, 25N-D and 25C-D are the N-terminally deleted VAMP, Syntaxin, SNAP25-N and SNAP25-C proteins, respectively. The N-terminal sequence before the two amino acids leading up to the layer -7 of each SNARE proteins was deleted in the N-terminally deleted SNARE proteins. Sequences in italics are expressed from the vectors. Amino acids in bold are labeled as 1 in the CXMS analysis. The amino acid of the layer -7 of each SNARE proteins is colored in red.

Name	Amino Acid Sequence
NSF	*MRGSHHHHHHTDPLDVDSEPDVSAK***M**AGRSMQAARCPTDELSLSNCAVVSEKDYQSGQHVI VRTSPNHKYIFTLRTHPSVVPGSVAFSLPQRKWAGLSIGQEIEVALYSFDKAKQCIGTMTI EIDFLQKKNIDSNPYDTDKMAAEFIQQFNNQAFSVGQQLVFSFNDKLFGLLVKDIEAVDPS ILKGEPASGKRQKIEVGLVVGNSQVAFEKAENSSLNLIGKAKTKENRQSIINPDWNFEKMG IGGLDKEFSDIFRRAFASRVFPPEIVEQMGCKHVKGILLYGPPGCGKTLLARQIGKMLNAR EPKVVNGPEILNKYVGESEANIRKLFADAEEEQRRLGANSGLHIIIFDEIDAICKQRGSMA GSTGVHDTVVNQLLSKIDGVEQLNNILVIGMTNRPDLIDEALLRPGRLEVKMEIGLPDEKG RLQILHIHTARMRGHQLLSADVDIKELAVETKNFSGAELEGLVRAAQSTAMNRHIKASTKV EVDMEKAESLQVTRGDFLASLENDIKPAFGTNQEDYASYIMNGIIKWGDPVTRVLDDGELL VQQTKNSDRTPLVSVLLEGPPHSGKTALAAKIAEESNFPFIKICSPDKMIGFSETAKCQAM KKIFDDAYKSQLSCVVVDDIERLLDYVPIGPRFSNLVLQALLVLLKKAPPQGRKLLIIGTT SRKDVLQEMEMLNAFSTTIHVPNIATGEQLLEALELLGNFKDKERTTIAQQVKGKKVWIGI KKLLMLIEMSLQMDPEYRVRKFLALLREEGASPLDFD
Vamp	***GSH***MSATAATVPPAAPAGEGGPPAPPPNLTSNRR**L**QQTQAQVDEVVDIMRVNVDKVLERDQ KLSELDDRADALQAGASQFETSAAKLKRKYWWKNLK
Stx (Syntaxin)	***GS***KDRTQELRTAKDSDDDDDVTVTVDRDRFMDEFFEQVEEIRGFIDKIAENVEEVKRKHSA ILASPNPDEKTKEELEELMSDIKKTANKVRSKLKSIEQSIEQEEGLNRSSADLRIRKTQHS TLSRKFVEVMSEYNATQSDYRERCKGRIQRQLEITGRTTTSEELEDMLESGNPAIFASGII MDSSISKQALSEIETRHSE**I**IKLENSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHAVDY VERAVSDTKK
25N (SNAP25-N)	***GS***MAEDADMRNELEEMQRRADQLADESLES**T**RRMLQLVEESKDAGIRTLVMLDEQGEQLER IEEGMDQINKDMKEAEKNLTDLGKFCGLCVCPCNKLKSSDA
25C (SNAP25-C)	***GS***QMAISGGFIRRVTNDARENEMDEN**L**EQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEK ADSNKTRIDEANQRATKMLGSG
Vamp-D	***GS***RR**L**QQTQAQVDEVVDIMRVNVDKVLERDQKLSELDDRADALQAGASQFETSAAKLKRKY WWKNLK
Stx-D	***GS***SE**I**IKLENSIRELHDMFMDMAMLVESQGEMIDRIEYNVEHAVDYVERAVSDTKK
25N-D	***GS***ES**T**RRMLQLVEESKDAGIRTLVMLDEQGEQLERIEEGMDQINKDMKEAEKNLTDLGKFC GLCVCPCNKLKSSDA
25C-D	***GS***EN**L**EQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKTRIDEANQRATKMLGSG