Supplementary Information for

Structural basis for the trafficking and function mechanism of the vesicular inhibitory amino acid transporter VIAAT

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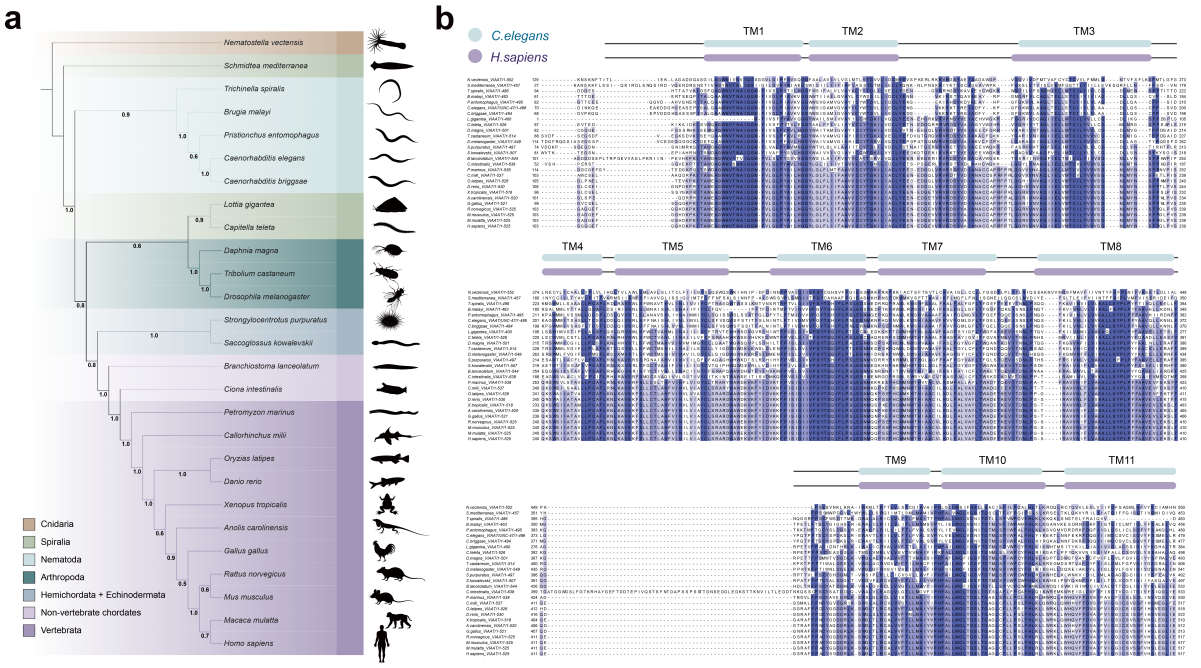
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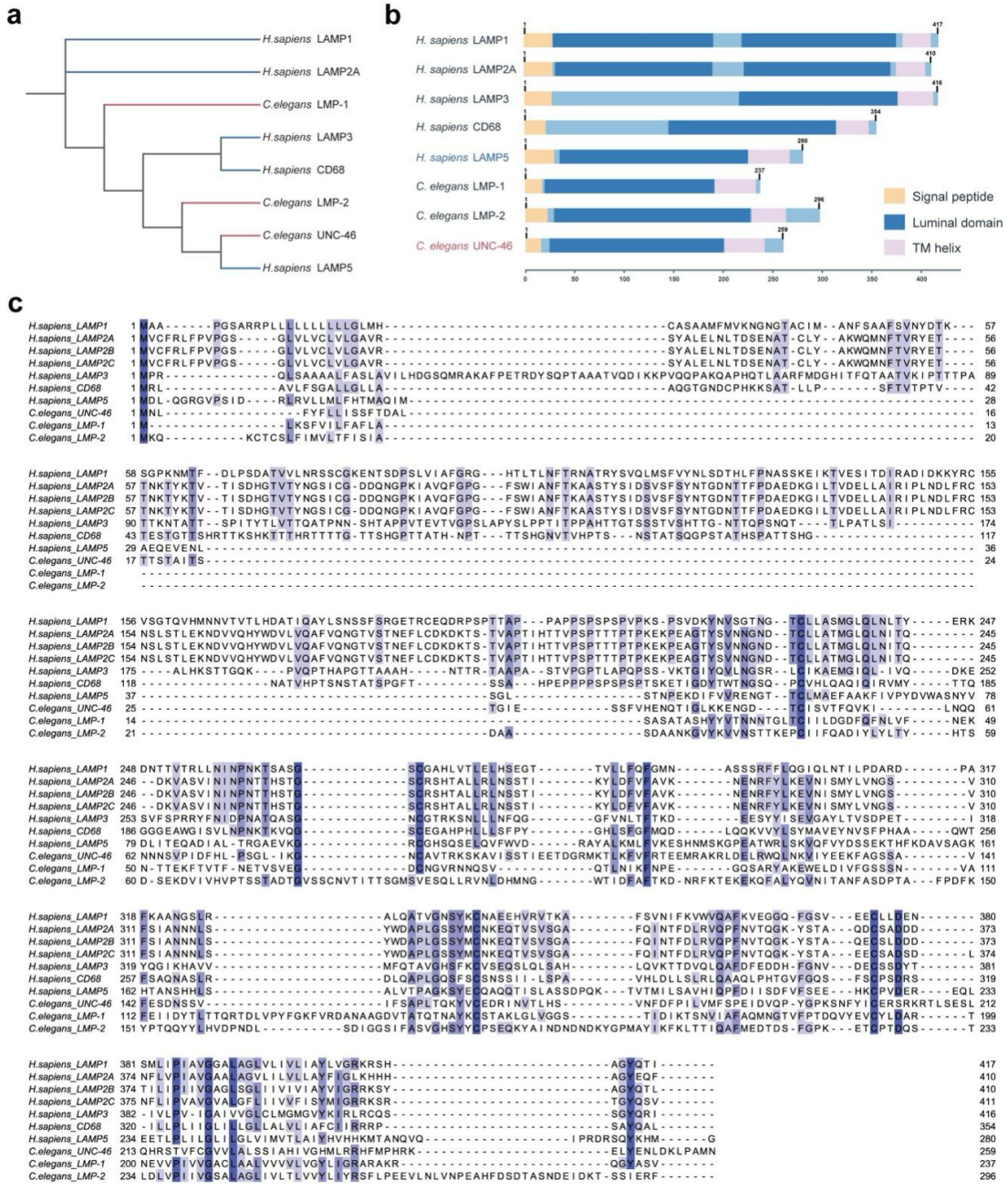
The PDF file includes:

Supplementary Figures S1 to S12

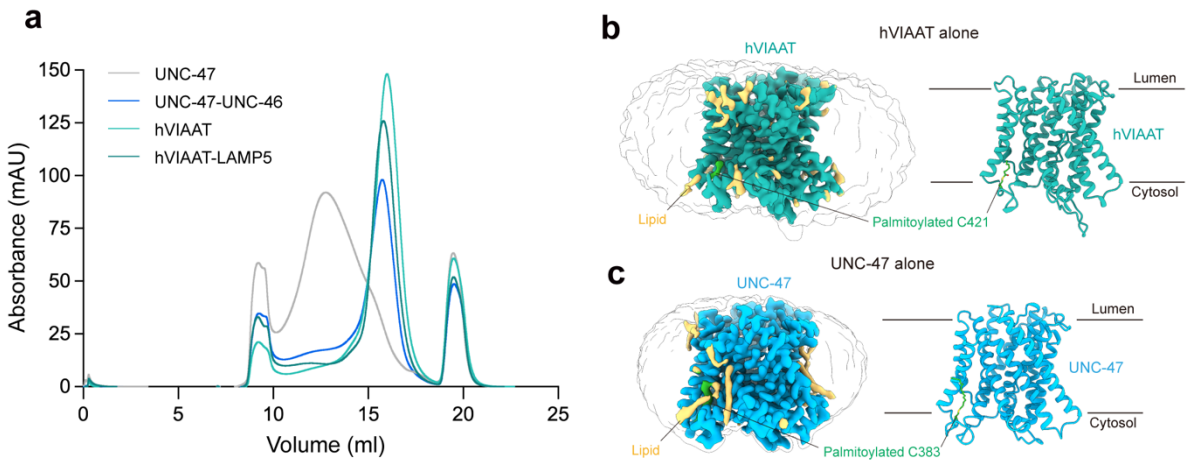
Supplementary Tables S1 to S8



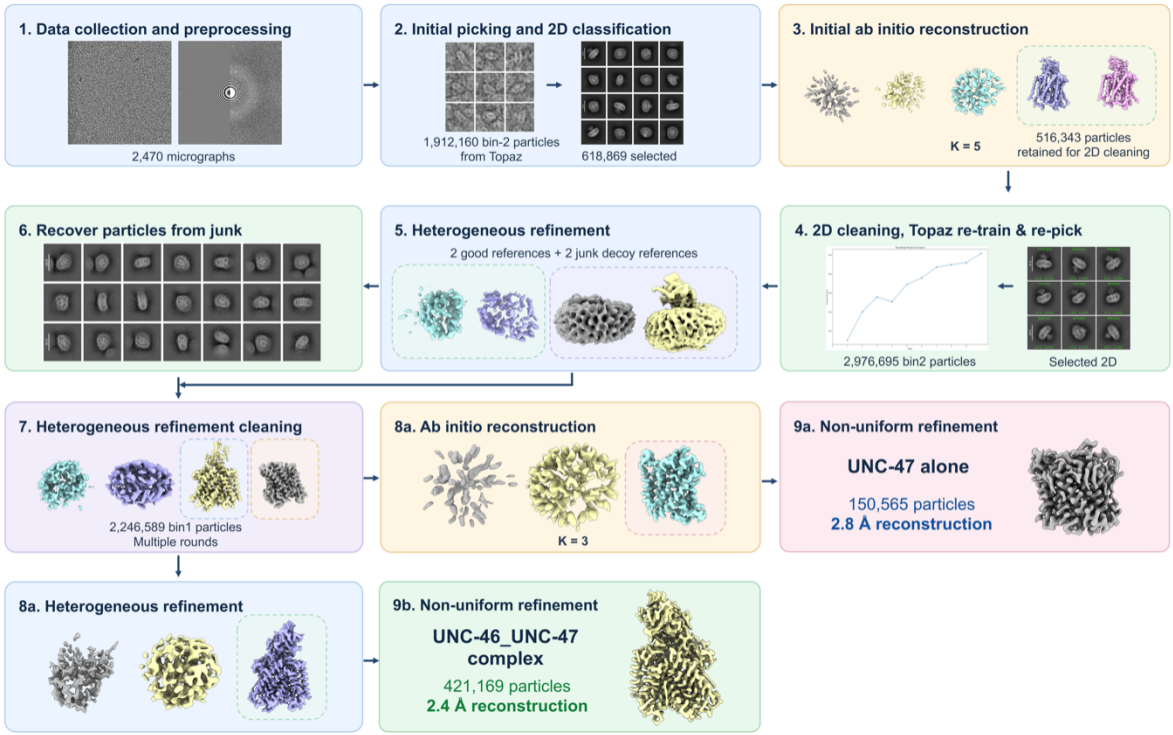
Supplementary Fig. S1 | Phylogenetic relationships and sequence alignment of metazoan VIAAT homologues. a, Maximum-likelihood phylogenetic tree of 27 VIAAT homologues inferred in MEGA using the JTT model. Background colors indicate the taxonomic groups defined in the key, with silhouettes representing the corresponding taxa. Branch lengths are not drawn to scale. **b,** MAFFT alignment of the 27 VIAAT homologues shown in **a**, colored according to the percentage identity scheme in Jalview. Dashes denote alignment gaps, and numbers flanking the sequences indicate residue positions. The transmembrane helices (TM1-TM11) of human VIAAT and *C. elegans* UNC-47, manually assigned based on their experimentally determined structures, are marked above the alignment in purple and light blue, respectively.



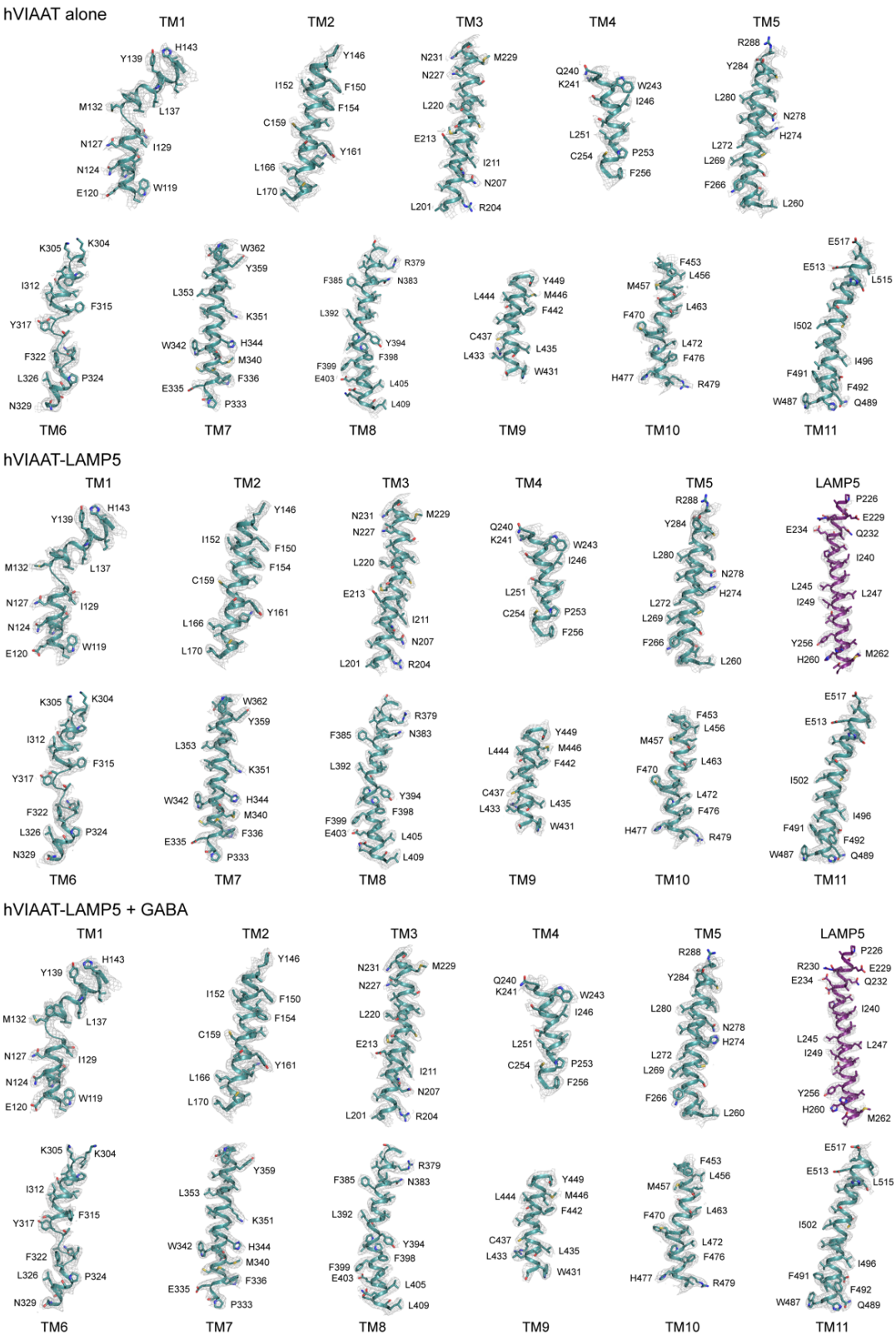
Supplementary Fig. S2 | Comparative phylogenetic and sequence analysis of human and *C. elegans* LAMP-family proteins. a, Maximum-likelihood phylogenetic tree of eight LAMP-family proteins inferred using IQ-TREE, with human LAMP2 represented by its LAMP2A isoform. Branch lengths are not drawn to scale. **b,** Schematic representation of the domain organization of the LAMP-family proteins shown in **a**, based on manual annotation. Signal peptides, luminal domains and transmembrane helices are colored pale orange, blue and pale purple, respectively, whereas other regions are shown in light blue. Bar-end labels indicate protein lengths, and the horizontal axis shows residue positions. **c,** MAFFT alignment of the LAMP-family proteins shown in **a**, together with human LAMP2B and LAMP2C. Residues are colored according to the percentage identity scheme in Jalview. Dashes denote alignment gaps, and flanking numbers indicate residue positions.



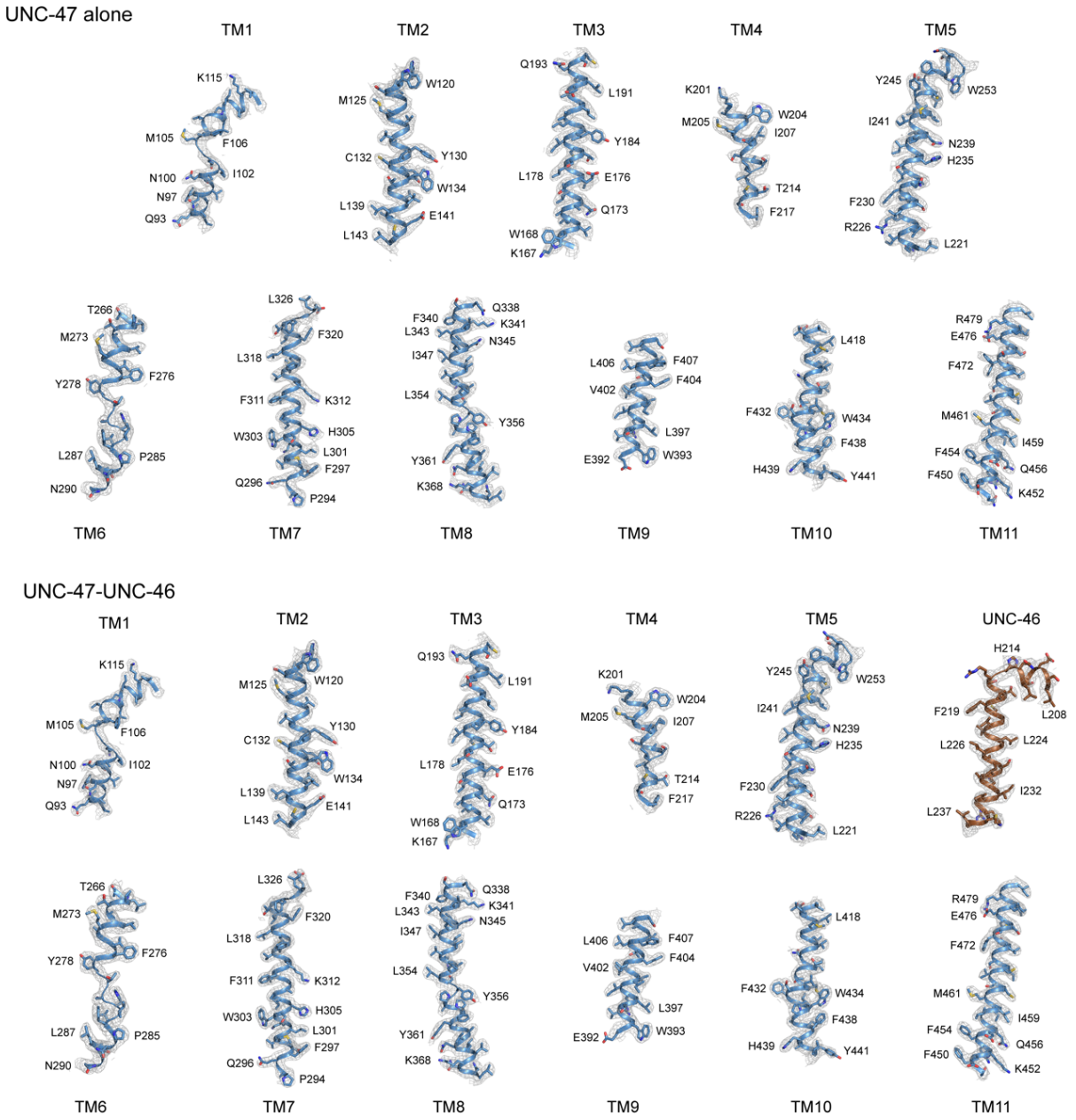
Supplementary Fig. S3 | Purification and cryo-EM structures of apo hVIAAT and UNC-47. a, Size-exclusion chromatography (SEC) profiles of purified UNC-47, UNC-47-UNC-46 complex, hVIAAT and hVIAAT–LAMP5 complex. Cryo-EM density maps (*left*) and corresponding structural models (*right*) of hVIAAT alone (**b**) and UNC-47 alone (**c**), colored cyan and blue, respectively. Bound lipids are shown in yellow, and palmitoylated Cys421 in hVIAAT and Cys383 in UNC-47 are highlighted in green. The luminal and cytosolic sides are indicated.



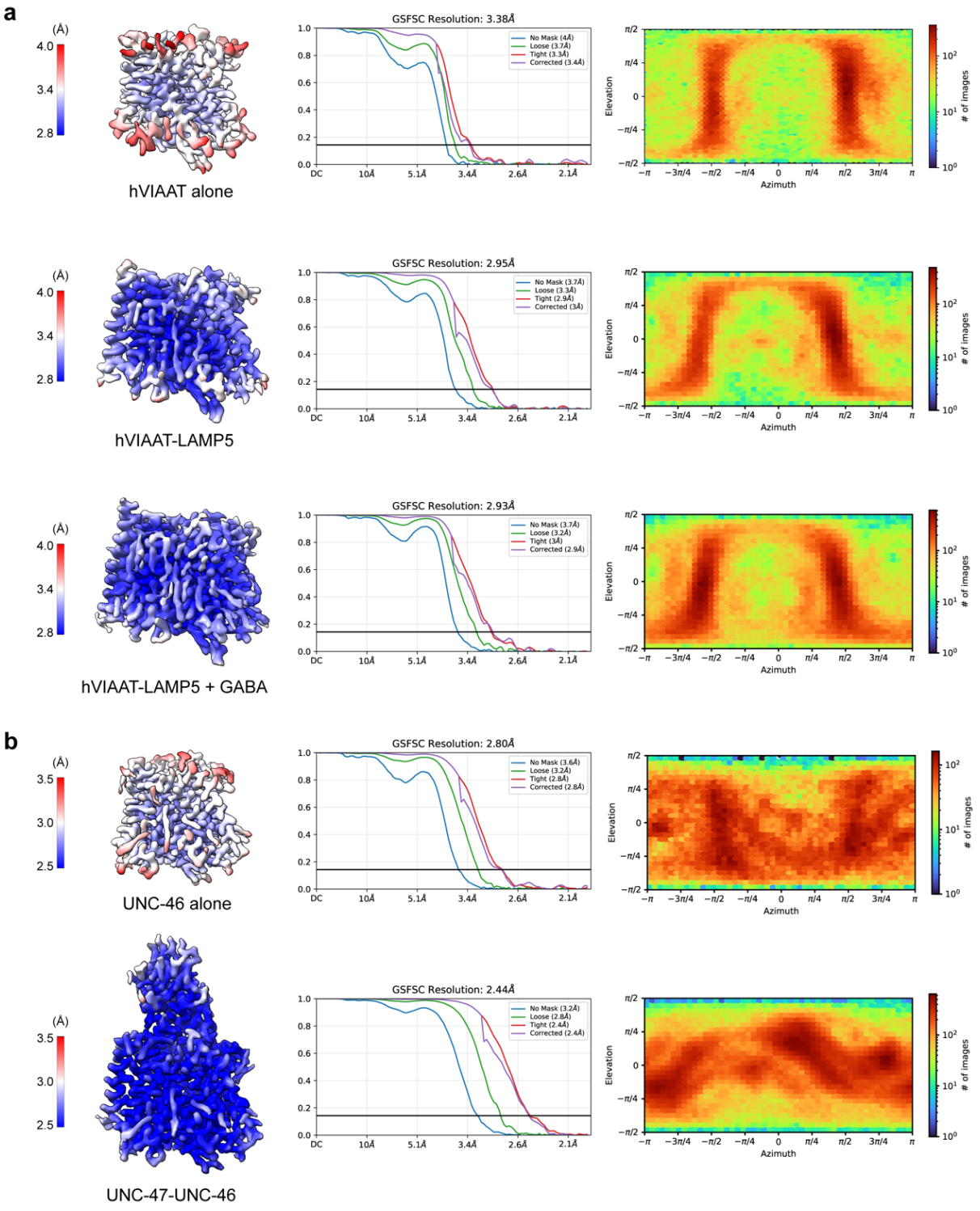
Supplementary Fig. S4 | Representative cryo-EM data processing workflow for the UNC-46–UNC-47 co-expression dataset. Please refer to the Methods section for detailed information.



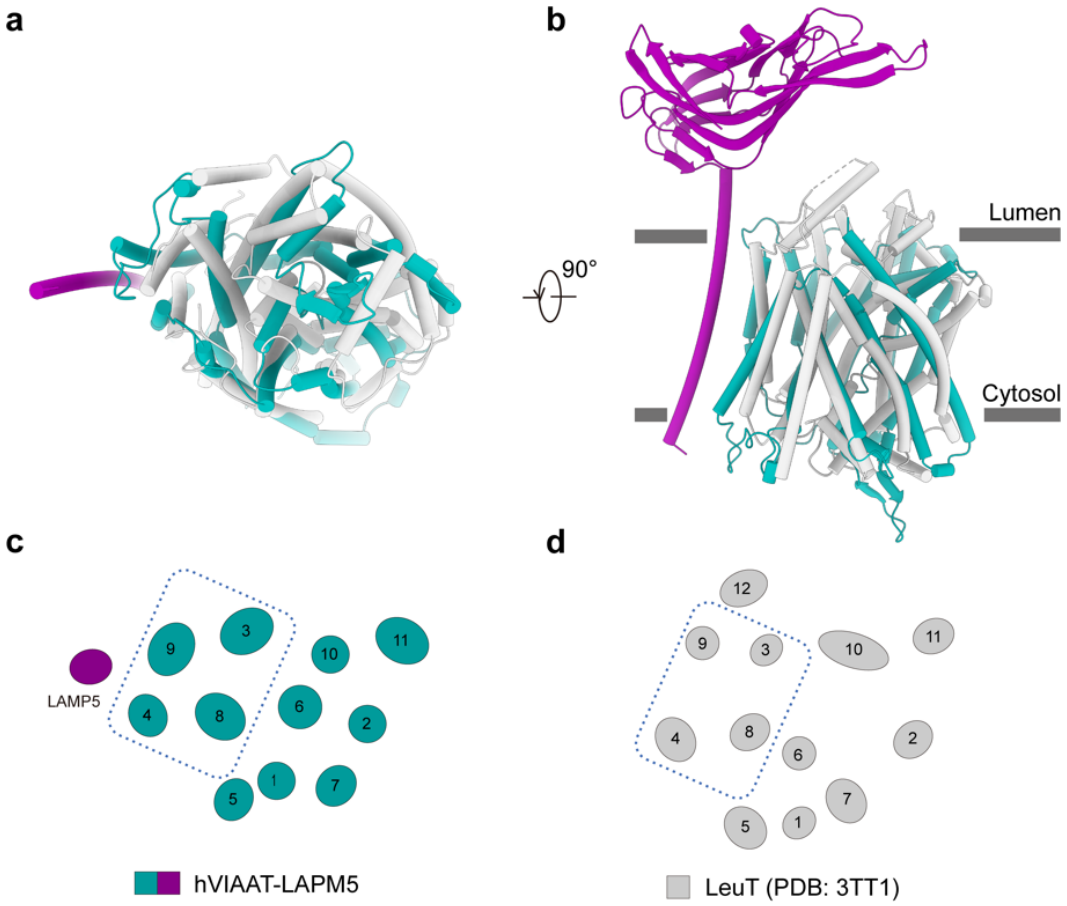
Supplementary Fig. S5 | Segment density of human VIAAT and LAMP5. Local cryo-EM densities corresponding to signature residues across TM1-TM11 helices of human VIAAT and the transmembrane helix of LAMP5. All density maps are displayed at the contour level of 4.5σ.



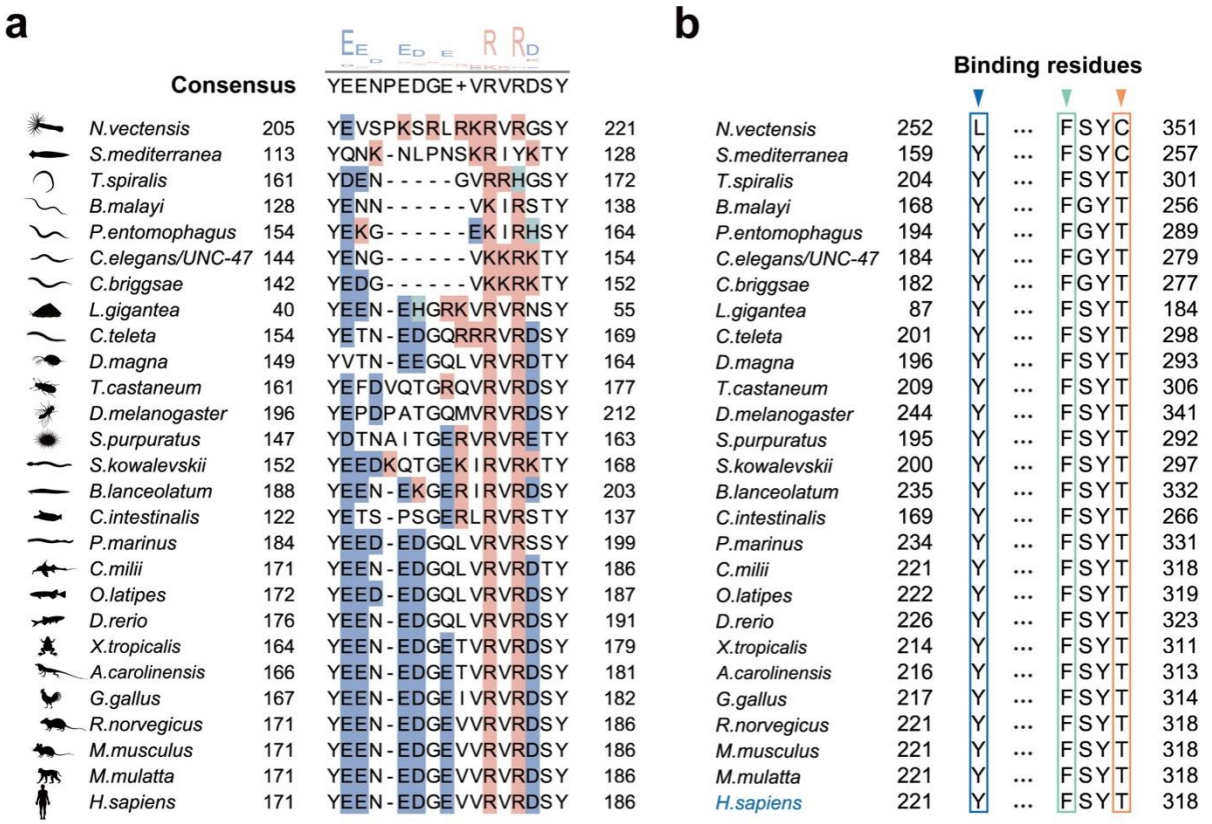
Supplementary Fig. S6 | Segment density of *C. elegans* UNC-47 and UNC-46. Local cryo-EM densities corresponding to signature residues across TM1-TM11 helices of the *C. elegans* UNC-47 and the transmembrane helix of UNC-46. All density maps are displayed at the contour level of 4.5σ.



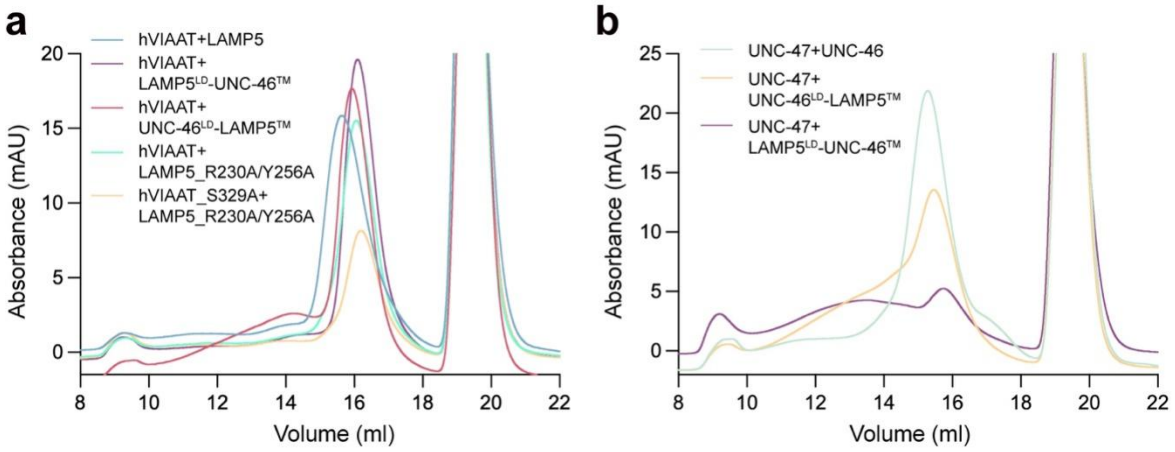
Supplementary Fig. S7 | Cryo-EM map validation of hVIAAT and UNC-47. a-b, Local resolution estimations (*left*), gold-standard Fourier shell correlation (GSFSC) curves (*middle*), and angular distributions (*right*) for (**a**) hVIAAT alone, hVIAAT–LAMP5 complex, and the hVIAAT–LAMP5 complex in the presence of 50mM GABA, and (**b**) UNC47 alone and UNC-47–UNC46 complex.



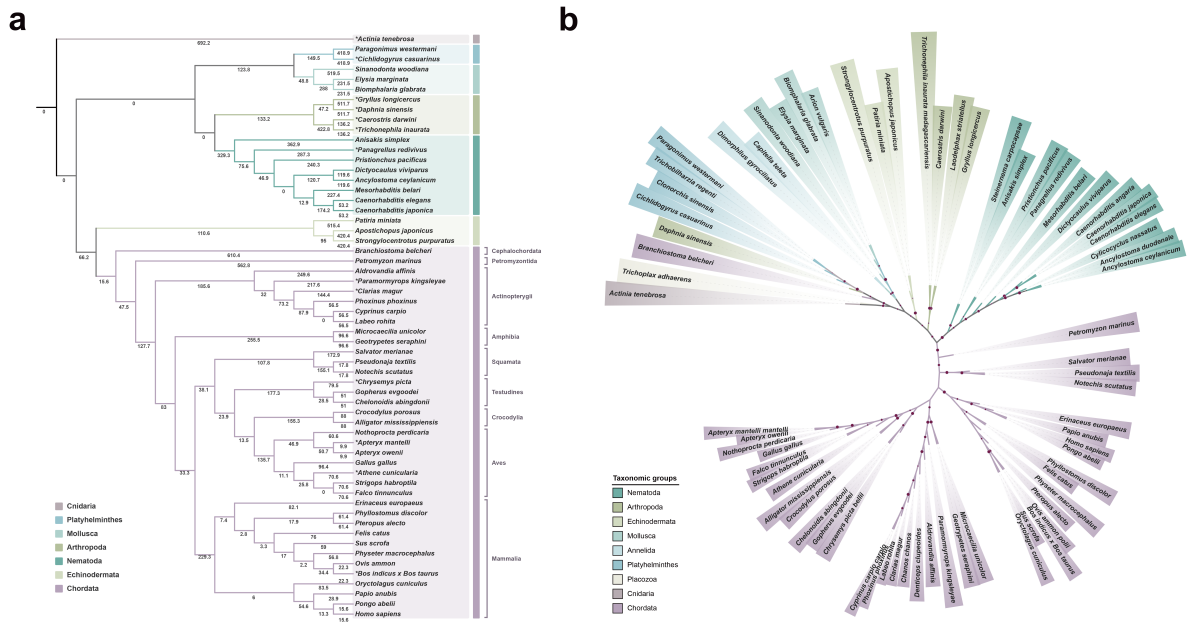
Supplementary Fig. S8 | hVIAAT is a LeuT-like folding transporter. a-b, Superposition of hVIAAT-LAMP5 with LeuT (PDB: 3TT1), shown in two views related by a 90º rotation. hVIAAT, LAMP5 and LeuT are colored cyan, purple and grey, respectively. **c-d,** Schematic cross-sections showing the transmembrane helix arrangement of the hVIAAT-LAMP5 complex (**c**) and LeuT (**d**). Numbers identify individual helices; dashed boxes highlight the scaffold helices TM3, TM4, TM8 and TM9.



Supplementary Fig. S9 | Sequence analysis in the cytosolic and substrate-binding regions across representative VIAAT homologues. Local sequence alignments of 27 VIAAT homologues covering the cytosolic β-sheet region corresponding to residues 171–186 of human VIAAT (**a**) and selected GABA-binding residues (**b**). In **a**, acidic residues (Asp and Glu), basic residues (Lys and Arg) and His are highlighted in blue, salmon and pale cyan, respectively. The sequence logo and consensus sequence are shown above the alignment, with plus sign (+) indicating equally frequent residues at a given position and dashes indicating alignment gaps. In **b**, residues corresponding to Tyr221, Phe315 and Thr318 of human VIAAT are outlined in blue, green and orange, respectively. Ellipses indicate omitted sequence and flanking numbers denote residue positions.

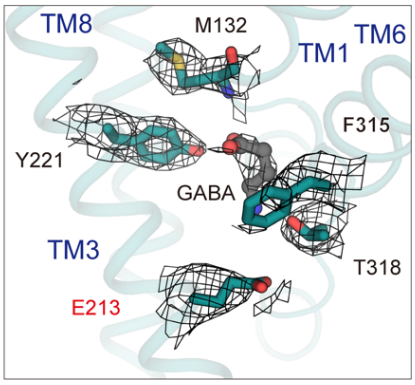


Supplementary Fig. S10 | Size-exclusion chromatography analysis of anti-FLAG pull-down eluates. SEC profiles of anti-FLAG pull-down eluates from hVIAAT and its variants (**a**) or UNC-47 (**b**) co-expressed with the indicated wild-type, mutant or domain-swapped LAMP-family partners. Eluates were analyzed on a Superose 6 Increase 10/300 GL column. Color keys identify the protein combinations. LD, luminal domain; TM, transmembrane region.



Supplementary Fig. S11 | Phylogenetic analysis of LAMP5/UNC-46 homologues. a, TimeTree-derived species phylogeny covering 58 of the 71 sampled taxa across seven animal phyla. Colors denote the corresponding phyla, and brackets highlight selected chordate groups. Branch labels indicate time intervals in millions of years, and branch lengths are not drawn to scale. **b,** Unrooted maximum-likelihood phylogenetic tree of 71 LAMP5/UNC-46 homologues from nine animal phyla, inferred using IQ-TREE. Purple circles indicate support based on 1,000 ultrafast bootstrap replicates, with circle sizes proportional to support values.

a



b

Conformation	Cytosol-open	Luminal-occluded	Luminal-open
Calculated pKa	7.08	7.20	7.51
Cytosol (pH 7.2) Ionization state	GLH 43.1% **GLU⁻ 56.9%**	GLH 50.0% GLU⁻ 50.0%	
Vesicle lumen (pH 5.5) Ionization state		GLH 98.0% GLU⁻ 2.0%	**GLH 99.0%** GLU⁻ 1.0%

Supplementary Fig. S12 | Structural environment and predicted protonation states of Glu213 in different VIAAT conformations. a, Close-up view of the GABA-binding region highlighting Glu213 and its surrounding residues. The corresponding cryo-EM densities are shown as meshes. **b**, Structure-based PROPKA pK_a estimates for Glu213 in the cytosol-open, luminal-occluded and luminal-open conformations, together with the predicted protonation-state populations at cytosolic pH 7.2 and vesicle luminal pH 5.5. The predicted pK_a values are 7.08, 7.20 and 7.51 for the cytosol-open, luminal-occluded and luminal-open conformations, respectively. GLH and GLU^- represent the protonated and deprotonated forms of Glu213, respectively. Protonation-state populations were calculated from the predicted pK_a values at the indicated pH conditions.

Supplementary Table S1 | Summary of the VIAAT sequences from 27 representative metazoan taxa.

No.	Protein	UniProt accession	Species	Length (aa)
1	VIAAT	O35633	*Mus musculus*	525
2	VIAAT	O35458	*Rattus norvegicus*	525
3	VIAAT	Q9H598	*Homo sapiens*	525
4	VIAAT	A1L1T3	*Danio rerio*	530
5	VIAAT	F7E8A5	*Macaca mulatta*	525
6	VIAAT	Q6DIV6	*Xenopus tropicalis*	518
7	VIAAT	A0A1D5PP16	*Gallus gallus*	521
8	VIAAT	G1KPG2	*Anolis carolinensis*	520
9	VIAAT	H2LLZ6	*Oryzias latipes*	526
10	VIAAT	V9KS08	*Callorhinchus milii*	537
11	VIAAT	A0AAJ7T0P0	*Petromyzon marinus*	538
12	VIAAT	A0A8J9ZFP8	*Branchiostoma lanceolatum*	544
13	VIAAT	Q75RZ6	*Ciona intestinalis*	638
14	VIAAT	A0A7M7TH35	*Strongylocentrotus purpuratus*	497
15	VIAAT	A1Z9M6	*Drosophila melanogaster*	549
16	VIAAT	UPI001E1BDD75*	*Daphnia magna*	501
17	UNC-47/VIAAT	P34579	*Caenorhabditis elegans*	486
18	VIAAT	A8X9N7	*Caenorhabditis briggsae*	484
19	VIAAT	A0AAV5T177	*Pristionchus entomophagus*	495
20	VIAAT	A0A0H3YJH0	*Schmidtea mediterranea*	457
21	VIAAT	V4A072	*Lottia gigantea*	400
22	VIAAT	R7TAI2	*Capitella teleta*	526
23	VIAAT	UPI0020770AF4*	*Nematostella vectensis*	552
24	VIAAT	A0ABM0M4R7	*Saccoglossus kowalevskii*	507
25	VIAAT	UPI0001EFD3AF*	*Trichinella spiralis*	486
26	VIAAT	A0A4E9F3D8	*Brugia malayi*	463
27	VIAAT	D6WTB1	*Tribolium castaneum*	514

Supplementary Table S2 | Taxon substitutions and exclusions in the TimeTree phylogeny of representative VIAAT homologues.

Requested taxon	Treatment	TimeTree substitute taxon
Nematostella vectensis	Substituted	*Nematostella*
Strongylocentrotus purpuratus	Substituted	*Strongylocentrotus intermedius*
Pristionchus entomophagus	Substituted	*Pristionchus pacificus*
Schmidtea mediterranea	Substituted	*Archotoplana holotricha*
Saccoglossus kowalevskii	Excluded	—
Capitella teleta	Excluded	—
Trichinella spiralis	Excluded	—

*The final time-calibrated phylogeny comprised 24 terminal taxa, with four substitutions and three exclusions relative to the original set of 27 representative VIAAT homologues.

Supplementary Table S3 | Summary of the human and *C. elegans* LAMP-family protein sequences.

No.	Protein	UniProt accession	Species	Length (aa)
1	LAMP1	P11279-1	*Homo sapiens*	417
2	LAMP2A	P13473-1	*Homo sapiens*	410
3	LAMP3	Q9UQV4	*Homo sapiens*	416
4	CD68	P34810-1	*Homo sapiens*	354
5	LAMP5	Q9UJQ1-1	*Homo sapiens*	280
6	LMP-1	Q11117	*Caenorhabditis elegans*	237
7	LMP-2	Q9GYK0	*Caenorhabditis elegans*	296
8	UNC-46	Q9GZD5	*Caenorhabditis elegans*	259

Supplementary Table S4 | Statistics for cryo-EM data collection and structural refinement.

	UNC-47	UNC-47–UNC-46	hVIAAT	hVIAAT–LAMP5	hVIAAT–LAMP5 + GABA
Data collection and processing					
Magnification	130,000	130,000	130,000	130,000	130,000
Voltage (kV)	300	300	300	300	300
Electron dose (e-/Å^2)	50	50	50	50	50
Defocus range (μm)	-1.2 to -1.6	-1.2 to -1.6	-1.2 to -1.6	-1.2 to -1.6	-1.2 to -1.6
Pixel size (Å)	0.936	0.936	0.936	0.936	0.936
Symmetry	C1	C1	C1	C1	C1
Initial particle images	2,976,695	2,976,695	2,366,997	2,199,315	2,139,359
Final particle images	150,565	421,169	152,254	193,634	297,954
Map resolution (Å)	2.8	2.4	3.4	3.0	2.9
FSC threshold	0.143	0.143	0.143	0.143	0.143
Refinement					
Initial model used	AF3 prediction	AF3 prediction	AF3 prediction	AF3 prediction	AF3 prediction
Map sharpening *B* factor (Å^2)	-108.5	-76.5	-149.1	-120.3	-122.0
Model composition					
Non-hydrogen atoms	3,222	4,984	3,130	4,442	4,450
Protein residues	405	620	400	630	630
Ligands	2	8	0	0	1
B factors (Å^2)					
Protein	112.40	96.29	97.42	128.89	141.47
Ligand	59.34	118.28	/	/	20.00
R.m.s deviations					
Bond lengths (Å)	0.003	0.004	0.003	0.003	0.003
Bond angles (°)	0.527	0.484	0.596	0.621	0.565
Validation					
MolProbity score	1.08	1.00	1.09	0.98	1.14
Clashscore	2.92	2.20	2.99	2.12	3.52
Rotamer outliers (%)	0.85	0.00	0.60	0.79	0.79
CaBLAM outliers (%)	0.25	0.98	0.77	1.13	0.80
Ramachandran plot					
Favored (%)	98.00	98.37	99.24	99.04	99.52
Allowed (%)	2.00	1.63	0.76	0.96	0.48
Disallowed (%)	0.00	0.00	0.00	0.00	0.00

Supplementary Table S5 | Summary of the LAMP-family protein sequences from 71 representative metazoan taxa.

No.	Protein	UniProt accession	Species	Length (aa)
1	LAMP5/UNC-46	A0A016WFN4	*Ancylostoma ceylanicum*	324
2	LAMP5/UNC-46	A0A0C2F5Y6	*Ancylostoma duodenale*	270
3	LAMP5/UNC-46	A0A0M3K7L9	*Anisakis simplex*	267
4	LAMP5/UNC-46	A0A9P1N7Y3	*Caenorhabditis angaria*	247
5	UNC-46	Q9GZD5	*Caenorhabditis elegans*	259
6	LAMP5/UNC-46	A0A8R1E827	*Caenorhabditis japonica*	209
7	LAMP5/UNC-46	A0AA36H482	*Cylicocyclus nassatus*	247
8	LAMP5/UNC-46	A0A0D8YBR3	*Dictyocaulus viviparus*	337
9	LAMP5/UNC-46	A0AAF3F1A5	*Mesorhabditis belari*	258
10	LAMP5/UNC-46	A0A7E4VT93	*Panagrellus redivivus*	278
11	LAMP5/UNC-46	A0A2A6C1T9	*Pristionchus pacificus*	246
12	LAMP5/UNC-46	A0A4U5NWC6	*Steinernema carpocapsae*	256
13	LAMP5/UNC-46	A0AAV4WLM9	*Caerostris darwini*	425
14	LAMP5/UNC-46	A0AAD5LM14	*Daphnia sinensis*	336
15	LAMP5/UNC-46	A0AAN9VYQ2	*Gryllus longicercus*	339
16	LAMP5/UNC-46	A0A482XFG0	*Laodelphax striatellus*	366
17	LAMP5/UNC-46	A0A8X6WMX3	*Trichonephila inaurata madagascariensis*	378
18	LAMP5/UNC-46	A0A0B6Z7D3	*Arion vulgaris*	305
19	LAMP5/UNC-46	A0A9W3A1Q8	*Biomphalaria glabrata*	297
20	LAMP5/UNC-46	A0AAV4FKZ2	*Elysia marginata*	287
21	LAMP5/UNC-46	A0ABD3WM53	*Sinanodonta woodiana*	245
22	LAMP5/UNC-46	R7U686	*Capitella teleta*	260
23	LAMP5/UNC-46	A0A7I8VRE4	*Dimorphilus gyrociliatus*	275
24	LAMP5/UNC-46	A0ABD2PS14	*Cichlidogyrus casuarinus*	337
25	LAMP5/UNC-46	G7YSL1	*Clonorchis sinensis*	244
26	LAMP5/UNC-46	A0A8T0DXC6	*Paragonimus westermani*	262
27	LAMP5/UNC-46	A0AA85JDC3	*Trichobilharzia regenti*	248
28	LAMP5/UNC-46	A0A6P8HIS8	*Actinia tenebrosa*	336
29	LAMP5/UNC-46	A0A2G8LR52	*Apostichopus japonicus*	332
30	LAMP5/UNC-46	A0A914BDY7	*Patiria miniata*	397
31	LAMP5/UNC-46	A0A7M7REJ8	*Strongylocentrotus purpuratus*	310
32	LAMP5/UNC-46	A0A6P4YMC6	*Branchiostoma belcheri*	248
33	LAMP5/UNC-46	A0AAJ7SQZ2	*Petromyzon marinus*	284
34	LAMP5/UNC-46	A0AAD7RGY9	*Aldrovandia affinis*	348

35	LAMP5/UNC-46	A0A6J2VAQ1	*Chanos chanos*	283
36	LAMP5/UNC-46	A0A8J4X8K9	*Clarias magur*	319
37	LAMP5/UNC-46	A0A8C1AQM5	*Cyprinus carpio carpio*	344
38	LAMP5/UNC-46	A0AAY4BZZ4	*Denticeps clupeoides*	409
39	LAMP5/UNC-46	A0A498M3H9	*Labeo rohita*	374
40	LAMP5/UNC-46	A0A3B3RDD2	*Paramormyrops kingsleyae*	295
41	LAMP5/UNC-46	A0AAN9H778	*Phoxinus phoxinus*	311
42	LAMP5/UNC-46	A0A6P8R0R1	*Geotrypetes seraphini*	305
43	LAMP5/UNC-46	A0A6P7XB48	*Microcaecilia unicolor*	360
44	LAMP5/UNC-46	A0A6J1U0A9	*Notechis scutatus*	320
45	LAMP5/UNC-46	A0A670Y6D8	*Pseudonaja textilis*	258
46	LAMP5/UNC-46	A0A8D0ECQ4	*Salvator merianae*	244
47	LAMP5/UNC-46	A0A8C0G547	*Chelonoidis abingdonii*	308
48	LAMP5/UNC-46	A0A8C3IIG0	*Chrysemys picta bellii*	319
49	LAMP5/UNC-46	A0A8C4WJE4	*Gopherus evgoodei*	275
50	LAMP5/UNC-46	A0A151MH96	*Alligator mississippiensis*	307
51	LAMP5/UNC-46	A0A7M4F1E7	*Crocodylus porosus*	324
52	LAMP5/UNC-46	A0A8B7J225	*Apteryx mantelli mantelli*	274
53	LAMP5/UNC-46	A0A8B9SBA1	*Apteryx owenii*	231
54	LAMP5/UNC-46	A0A663N212	*Athene cunicularia*	276
55	LAMP5/UNC-46	A0A8C4TW40	*Falco tinnunculus*	336
56	LAMP5/UNC-46	A0A1L1RQ85	*Gallus gallus*	344
57	LAMP5/UNC-46	A0A8C7EEJ7	*Nothoprocta perdicaria*	287
58	LAMP5/UNC-46	A0A672UTZ3	*Strigops habroptila*	272
59	LAMP5/UNC-46	A0A4W2EJ71	*Bos indicus × Bos taurus*	321
60	LAMP5/UNC-46	A0A5F5Y260	*Felis catus*	310
61	LAMP5/UNC-46	A0A5F9C0U0	*Oryctolagus cuniculus*	333
62	LAMP5/UNC-46	A0AAD4U4Y5	*Ovis ammon polii*	306
63	LAMP5/UNC-46	A0A096NVH7	*Papio anubis*	299
64	LAMP5/UNC-46	A0A834DQA8	*Phyllostomus discolor*	309
65	LAMP5/UNC-46	A0A2Y9EUS1	*Physeter macrocephalus*	374
66	LAMP5/UNC-46	Q5R5V2	*Pongo abelii*	261
67	LAMP5/UNC-46	L5JX04	*Pteropus alecto*	341
68	LAMP5/UNC-46	A0A4X1UR71	*Sus scrofa*	442
69	LAMP5/UNC-46	A0A1S3ARR7	*Erinaceus europaeus*	505
70	LAMP5/UNC-46	B3RKY6	*Trichoplax adhaerens*	293
71	LAMP5	Q9UJQ1	*Homo sapiens*	280

Supplementary Table S6 | Taxon substitutions and exclusions in the TimeTree species phylogenies of representative LAMP5/UNC-46 homologues.

Requested taxon	Treatment	TimeTree substitute taxon
Actinia tenebrosa	Substituted	*Anemonia viridis*
Clarias magur	Substituted	*Clarias gariepinus*
Paramormyrops kingsleyae	Substituted	*Paramormyrops curvifrons*
Bos indicus × *Bos taurus*	Substituted	*Bison bison*
Ovis ammon polii	Substituted	*Ovis ammon*
Apteryx mantelli mantelli	Substituted	*Apteryx mantelli*
Athene cunicularia	Substituted	*Athene brama*
Chrysemys picta bellii	Substituted	*Chrysemys picta*
Patiria miniata	Substituted	*Leptasterias hexactis*
Strongylocentrotus purpuratus	Substituted	*Strongylocentrotus intermedius*
Trichonephila inaurata madagascariensis	Substituted	*Trichonephila inaurata*
Caerostris darwini	Substituted	*Araneus marmoreus*
Daphnia sinensis	Substituted	*Daphnia pulex*
Gryllus longicercus	Substituted	*Gryllus bimaculatus*
Cichlidogyrus casuarinus	Substituted	*Gyrodactylus salaris*
Capitella teleta	Excluded	—
Ancylostoma duodenale	Excluded	—
Clonorchis sinensis	Excluded	—
Caenorhabditis angaria	Excluded	—
Cylicocyclus nassatus	Excluded	—
Steinernema carpocapsae	Excluded	—
Trichobilharzia regenti	Excluded	—
Denticeps clupeoides	Excluded	—
Laodelphax striatellus	Excluded	—
Chanos chanos	Excluded	—
Arion vulgaris	Excluded	—
Trichoplax adhaerens	Excluded	—
Dimorphilus gyrociliatus	Excluded	—

*The final time-calibrated phylogeny comprised 58 terminal taxa, with 15 substitutions and 13 exclusions relative to the original set of 71 representative LAMP5/UNC-46 homologues.

Supplementary Table S7 | Residue ranges and luminal interface areas of predicted VIAAT-LAMP complex across species.

No.	Species	Chain A LAMP luminal core (residues)	Chain B VIAAT (residues)	Mean interface area (Å²)
1	*Ancylostoma ceylanicum*	109-277	80-482	1120.79
2	*Caenorhabditis angaria*	32-197	83-485	1054.27
3	*Caenorhabditis elegans*	39-207	89-486	1021.67
4	*Cylicocyclus nassatus*	32-198	80-477	995.30
5	*Mesorhabditis belari*	45-209	83-480	1052.62
6	*Panagrellus redivivus*	35-223	65-461	1003.77
7	*Pristionchus pacificus*	31-199	97-493	976.07
8	*Steinernema carpocapsae*	32-208	75-472	1121.59
9	*Caerostris darwini*	180-278, 310-373	89-493	376.38
10	*Daphnia sinensis*	106-299	94-499	295.23
11	*Gryllus longicercus*	120-297	148-554	901.05
12	*Laodelphax striatellus*	142-322	107-513	822.13
13	*Trichonephila inaurata madagascariensis*	157-326	88-492	603.35
14	*Biomphalaria glabrata*	69-251	93-497	682.65
15	*Elysia marginata*	60-245	106-544	217.34
16	*Sinanodonta woodiana*	22-203	80-484	192.40
17	*Capitella teleta*	28-222	99-503	736.38
18	*Dimorphilus gyrociliatus*	41-236	56-460	9.67
19	*Cichlidogyrus casuarinus*	114-302	106-528	373.55
20	*Paragonimus westermani*	37-224	172-593	480.39
21	*Patiria miniata*	162-352	103-509	930.37
22	*Strongylocentrotus purpuratus*	83-267	92-497	38.18
23	*Branchiostoma belcheri*	22-200	132-539	86.56
24	*Petromyzon marinus*	44-239	129-538	248.82
25	*Aldrovandia affinis*	109-299	123-530	117.66
26	*Chanos chanos*	42-237	121-530	122.71
27	*Cyprinus carpio carpio*	32-226	117-526	216.15
28	*Denticeps clupeoides*	167-363	117-526	304.03
29	*Labeo rohita*	122-328	122-529	179.10
30	*Paramormyrops kingsleyae*	59-249	120-527	116.01
31	*Phoxinus phoxinus*	70-265	118-525	365.37
32	*Geotrypetes seraphini*	68-258	112-521	119.00
33	*Microcaecilia unicolor*	123-313	112-521	114.51
34	*Notechis scutatus*	82-273	112-521	121.17
35	*Pseudonaja textilis*	43-234	111-520	405.77
36	*Salvator merianae*	8-197	112-521	119.15
37	*Chelonoidis abingdonii*	70-261	112-521	122.66
38	*Chrysemys picta bellii*	40-231	112-521	121.35
39	*Gopherus evgoodei*	44-235	112-521	123.10
40	*Alligator mississippiensis*	69-260	112-521	119.92
41	*Apteryx owenii*	13-184	112-521	121.88
42	*Falco tinnunculus*	98-289	112-521	115.30
43	*Gallus gallus*	106-297	112-521	116.51
44	*Nothoprocta perdicaria*	49-240	112-521	115.65
45	*Strigops habroptila*	34-225	112-521	125.53
46	*Bos indicus* × *Bos taurus*	42-129, 177-274	117-526	110.90
47	*Felis catus*	42-233	117-526	116.75
48	*Oryctolagus cuniculus*	42-233	117-526	167.06
49	*Ovis ammon polii*	58-259	117-526	117.46
50	*Papio anubis*	42-233	116-525	119.15
51	*Phyllostomus discolor*	42-233	117-526	120.51
52	*Physeter macrocephalus*	136-327	117-526	203.90
53	*Pongo abelii*	42-232	116-525	121.57
54	*Pteropus alecto*	102-293	117-526	121.07
55	*Sus scrofa*	42-233	117-526	119.08
56	*Erinaceus europaeus*	267-458	117-526	86.66
57	*Trichoplax adhaerens*	67-257	14-416	1172.90
58	*Homo sapiens*	42-233	116-525	125.45

Supplementary Table S8 | Summary of ΔT_m measurements from the GABA-dependent thermal-shift assay.

hVIAAT variants	WT	Y221A	Y221F	F315A	T318A
independent measrements (°C)	4.3	-1	1.4	2.8	2
	4.3	1.4	0.9	2.8	2.1
	4.2	1.4	1.3	2.8	2.8
	4.8	1.3	1.4	1.64	3.8
	4.34	0.1	2.1	2.7	0.8
	4.42	0.6	1.5	2.35	2.5
	4.4	-1.3		1.9	2.5
	4.9	-0.5		2.6	3
		0.88		2.6	
				3.1	
				2.4	
ΔT_m (°C)	4.46 ± 0.09	0.32 ± 0.35	1.43 ± 0.16	2.52 ± 0.13	2.44 ± 0.31
P	/	< 0.0001	< 0.0001	< 0.0001	< 0.0001
n	8	9	6	11	8

All measurements were performed using the N-FLAG-hVIAAT and N-Strep-LAMP5 constructs or the indicated hVIAAT variants. For each measurement, ΔT_m was calculated from paired measurements as $T_{m,\text{ with GABA}} - T_{m,\text{ without GABA}}$. Data are presented as mean ± s.e.m.; *n* denotes the total number of included independent measurements. Each mutant group was compared with wild-type hVIAAT-LAMP5 using a two-tailed unpaired *t*-test. All four comparisons yielded $P < 0.0001$. Significance thresholds were defined as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.