Supplemental Material

Copper-Transporting P-Type ATPases utilize a Unique Ion-Release Pathway

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Supplementary Figure 1. Comparison of the E2.P_i and E2P states of of LpCopA and SERCA. (a), Domain arrangements in the E2P states of LpCopA and SERCA. LpCopA is shown as in Fig. 1b and SERCA (pdb-id: 3B9B¹³) in green. Superimpositions were made of the intracellular domains using super in PYMOL. TM helices MA and MB, M7-M10 as well as SERCA insertions have been removed for clarity. (b-e), Close-up view of the phosphorylation site of LpCopA and SERCA. The A- and Pdomains (LpCopA and SERCA) are indicated by colors as in Fig. 1a and the catalytic aspartate (Asp426 in LpCopA) and selected residues important for (de)phosphorylation are shown as sticks. (b), The E2P state in complex with BeF₃⁻ (Be in black, F in pink) and the Mg²⁺ ion (green) associated with Asp426. (c), The subsequent (forward reaction) E2.P_i conformation with AlF₄⁻ (Al in brown, F in pink) and the Mg²⁺ ion (green) associated with Asp426 (pdb-id: $3RFU^{18}$). Note the shift in the position of the A-domain relative to the P-domain between the conformations. (d), Equivalent view as in (b) for the E2P state of SERCA (pdb-id: $3B9B^{13}$). (e), Equivalent view as in (c) for the E2.P_i state of SERCA (pdb-id: $3B9R^{13}$).



Supplementary Figure 2. Two E2P crystal forms of LpCopA. (a-d), Crystal packing with the domains are colored as in Fig. 1b. The proteins are arranged as stacked bilayers, held together by hydrophobic interactions between their membrane-spanning regions, typical of the HiLiDe crystallization method⁴¹. (a), View along the membrane bilayer for the C2 (high-resolution) crystal form. (b), Equivalent view for the P2₁2₁2₁ crystal form. Approximate perpendicular views of (a) and (b) are shown in (c) and (d), respectively. Note the more loose packing of the C2 lattice. The unit cell parameters can be found in Table 1. (e), Electron density maps of the low resolution P2₁2₁2₁ form of the E2-BeF₃⁻ complex. The search model is based on the high-resolution structure (C2 space group) and is colored as in Fig. 1b. The 2mFo-DFc (blue, 1 σ contour level) and mFo-DFc (±4 σ contour levels, green and red, respectively) electron density maps were derived upon rigid body refinement and indicate no major deviations from the high-resolution structure



Supplementary Figure 3. MD Simulation Analyses. (a), Evolution of the simulation box cell height. (b), The corresponding relative cell area (X,Y) evolution. (c-d), Backbone RMSD measured over the MD trajectory for states E2.P_i and E2P, and E2.P_i mutants P94A and P710A in the full protein (c) and the TM domain (d). (e), Centers of mass in x, y and z dimensions of the intracellular domains during the E2.P_i simulation. (f-g), Residence times for water molecules within 7 Å of Glu189 associated with the release pathway in the E2.P_i (f) and E2P (g) simulations. Two crystal waters remained associated with the internal water pockets within the release pathway for the entire E2P simulation and hence have residency times of 85 ns (not included in (f)). (h-i), Radii analyses. To determine structural variations of the release pathway within the 10 ns average from the MD simulations presented in Fig. 3d, the last 10 ns of the E2P (h) and E2.P_i (i) simulations were divided into 10 equally spaced 100 ps averages and subjected to Caver analyses.



Supplementary Figure 4. Functional analysis of mutant LpCopA forms. (a), Purity of the assayed constructs used for the *in vitro* assay. SDS-PAGE gels of the purified LpCopA constructs following a first round of scaling using ImageJ. Identical relative amounts of protein were used for the Baginski assay²⁵ for generating the raw data shown in Supplementary Table 1. (**b-c**), The *in vivo* copper susceptibility assay. *E. coli* growth complementation curves from three independent experiments for wild-type LpCopA (WT, red), the inactive Asp426Asn mutant (gray), the high-affinity coordinator mutant Met717Val (yellow) as well as various release pathway mutants are displayed. Experiments 1 and 3 used three replicates each, experiment 2 used 15 replicates. 0 and 3 mM copper ((**b**) and (**c**), respectively) were used in the growth medium. (**d**), Assessment of the reproducibility of the *in vitro* assay. The *in vitro* data shown in Fig. 4c are based on a single experiment (experiment 1, with nine replicates), so wild type, Asp426Asn, Ala714Thr and Met100Glu LpCopA were reproduced in additional experiments (experiment 2, with six replicates). The two independent sets of data are highly consistent (merged data).



Supplementary Figure 5. Support of a proline-dependent opening mechanism. (a-b), Helix dynamics in the E2.P_i simulation with LpCopA colored as in Fig. 1b. (a), Calculated inter-helical distances with spheres indicating C α atoms. Three equally interspersed distances (along the normal to the membrane) in the TM domain were measured for each TM helix pair; gray (intracellular end), red and cyan (extracellular end) dotted lines between helices MA:M6, M1:M4 and M4:M5. (b), The helix-pair distances in (a) plotted as gray (intracellular end), red and cyan (extracellular end) lines. (c-g), Altered hydration patterns for simulations of the Pro94Ala (purple) and Pro710Ala (blue) mutants compared to wild-type (black). (c), Pore radius analyses of average structures from the E2.P_i simulations. (d), Structural representation of the pores predicted by CAVER. (e), Number of water molecules associated with the release pathway. (f), Average representations from the simulations of wild type (solid) and the Pro94Ala mutant (transparent) with the side chains of Pro94, Met717 and Glu189 pinpointed. Water is shown as red (wild type) and green (mutant) iso-density surfaces at 22 % occupancy. (g), Equivalent view as in (f) for wild type (solid, red water) and the Pro710Ala mutant (transparent, blue water).

Figures – Supplementary Figure 6

a _{CopA} ZntA CtpD				MSTPD	N H <mark>G K K</mark> A	PQFAAI	FKPLTT	VQNAN	DCCCDC	GACSSTP
CopA ZntA CtpD	TLSENVS	<mark>g t</mark> r y s w <mark>k v</mark>	SGMD	H <mark>DHHQ</mark>	<mark>GHTHS</mark> G	К G <mark>H А</mark> - С <mark>С</mark> /	AA	S P K T Q	QASSKN	AEGPIVY
CopA ZntA CtpD	T C P M H P E - CARKVE	- I R <mark>Q S</mark> - <mark>A</mark> P N A V R Q L A -	G V N Q V Q Y	VLFAT	EKLVVD	A D <mark>N D I</mark> H	RAQVES	A L Q K A	GY <mark>S</mark> LRI	DEQAAEE M
CopA ZntA CtpD	TALYPAV	EPAPAARP	ARPRSG	PLCGM	A L E P E T	VTVSE	V <mark>S P E Y</mark> <mark>P Q A S</mark> G <mark>W L</mark>	L DMRR RLKEN WTVPS	R F W I A I L P L I T I V R W A A	MLTIPV IVMMAI ALALFL
CopA ZntA CtpD	VILEMGG SWGLEQF TGLAAQL	H <mark>G L K H F I S</mark> N H P L G A P	G N G S S W F Q A	IQLL <mark>L</mark> G <mark>QL</mark> AF VVWTL	A T P V V L I ATT L V Y L A C Y <mark>V</mark>	WG <mark>G</mark> WP G LYPI VGGWEI	F	Q S L K T L I K S - R A L R -	GQLNMI	TLIAMG SYFAIE NRTLDVD
CopA ZntA CtpD	IGVAWIY TLMSVAA LLMIVAA	SMVAVLWP IGALFIGA IGAATIG-	GVFPHA T	FR <mark>SQE</mark>	G <mark>V V A V Y</mark> Q	FEAAA - AEAA VFDGAI	VITTLV AVLLLF LLIVIF	L L G Q V L I C E R A T S G A	LELKAI LEGWA LEDVA	R E Q T G S A S R A R Q G T T R T E R S
CopA ZntA CtpD	I R A L L K L V S A L MAL V R G L L D L	V P E S A H R I K P E T A T R L A P E H A T L L	- KEDG S R KGE GDG S	E E E V S I R E E V A Q R V V A	L D N V A V I N S L R P A A D L R P	GDLLR GDVIE GDVIV	V R P G E K V A A G G R V R P G E R	I PVDG L PADG I SADG	EVQEGI KLLSPI TVIGG	S F V D E S A S F D E S S E V D Q S
CopA ZntA CtpD	MVTGEPI ALTGESI SITGEPL	P V <mark>A K E A</mark> S A P V E R A T G D P A A K D V G D	K V I G A T K V P A G A D V F A G T	INQTG TSVDR VNGSG	S F <mark>V</mark> M K A L V T L E V A L R V <mark>E</mark> V	L H V G S I L S E P G A T R E P S C	OTMLAR ASAIDR QTVVAR	IVQMV ILKLI IVAMV	SDAQRS EEAEEI TEASA	S R A P I Q R R A P I E R F K A T T Q L
CopA ZntA CtpD	L A D T V S G F I D R F S R F I E K I E Q	W F V P A V I L I Y T P A I M A R Y S A G V V V	VAVLSF VALLVT ATLALL	LVPPL TVPL-	L F A A S W M F G A D L	ALLGPC Q R	Q P A L S Y E S	GLIAA WIYKG TLLRA	VSVLI LTLLL MTFMI	I A C P C A L I G C P C A L / A S P C A <mark>V</mark>
CopA ZntA CtpD	G L A T P M S V I S T P A A V L A T M P P	I MVGVGKG I TSGLAAA LLSAIANA	A Q S G V L A R R G A L S R H G V L	IKNAE IKGGA VKSAV	A L E R M E A L E Q L G A M E R L A	K VN T L V R VT Q V D TD V V	V V D K T G A F D K T G V L D K T G	TLTEG TLTVG TLTAG	H P K L T I K P R V - E P V I -	R I <mark>VTDD F</mark>
CopA ZntA CtpD	VEDNA 	I - H PA V T V L I D	TGISES	ELLTL DVLGM	A A A L E H A A A V E Q A A A A E Q	QSEHPI GATHPI FSEHPI	LANAIV LAQAIV LGRAIV	- HAAK RE AA	EKGLSI	GSVEA F
CopA ZntA CtpD	QV-AELA -RGRV	I P T A E S Q R V P E A G D F T	A P T G K G A L V G S G A L P G R G	VVGQV IEAQ- VRAR-	DGHHV- VN- VA	G ER V L G HV V E	IGNA ICA - AG VVS - PA	КН Р А - АҮ <mark>А</mark>	GENAA	DAFTGL 1 - VREHC
CopA ZntA CtpD	- R LMQEH NEL AAI	G G D N A P L F	EKADEL	R <mark>G K</mark> 0 E S A E N D 0	G A S V M F G Q T V V L G G T A V V	M	- AVDGK DV P	TVALL -LG-V -VG-V	V - V E D I I A L Q D T I G L A D I	PIKSSTP FLRADAA RL <mark>RP</mark> DAP
CopA ZntA CtpD	E T I L E L Q T A I S E L N A A V M Q L A	Q S G I - E I - A L G V K G Q L T K H P - P	VMLTGD VILTGD MLLTGD	S <mark>K R</mark> T A I N P R A A N <mark>R R</mark> A A	E A V <mark>A G T</mark> A A I A G E G R L A E E	- L <mark>G I</mark> - H L - G L - H A - G I A I	KKVVAE E - FKAG D - VHAE	IMPED LLPED LLPDG	KSRIV KVKAV KAAAV	5 E L K <mark>D K</mark> G F E L N Q K L Q
CopA ZntA CtpD	L I - QHAP - R DN -	VAMAGD LAMVGD THVLVVGD	GVNDAP GINDAP GVNDAP	AL <mark>AK</mark> A AMKAA AM <mark>AA</mark> A	DIGIAM AIGIAM HTSIAM	GT-GTI GS-GTI GRAGAI	DVAIES DVALET DLTVQT	AGVTL ADAAL ADVVT	LHGDL THNHLI IRDEL	GIAKAR GLVQMI TVPAVI
CopA ZntA CtpD	R L S E S T M E L A R A T H A L A R R A R	S N I R Q N L F A N I R Q N I T R V V I A N L V	FAFIYN IALGLK MAGAAI	VLGVPI GIFLV T <mark>TLVL</mark> V	LA-AGV TTLLGM WDLFGQ	L - Y I T G L W L P L P	PLTGL L	LSPMI L L	AAAAMA AVLAD GV <mark>AG</mark> HI	LSSVSV GATVLV GSTILV
CopA ZntA CtpD	I I NALRL TANALRL ALNGLRL	- K R V L R RR L S NRAW I S	- T L			Poor	,	Conserva	ition	High
d	CtpD/30-202 tr131R194[J1R19/23-195 tr]38GE04[J8GE0/22-192 Lmo0641/5-175 tr[CZD4V0]C2D4V/2-179	30 23 22 5 2 2 2 2 2 2	AALWSVPSVRWAA TRVLALAEARWAA MWQKYHELILAI MKQNWQFITTG • KKLKMTTKLYF	AALALFLTGLA AATVAFLVALF LSGIFILAGWA ISGILIVIGCI FGLILYLLDLV	AQLLGAP LQLTGAP ILTKSEV VGSDVG ILMFSGFN		AV VW AGFW TTAG DFWT SATV	TLYLACYVVG GPLYVLTYVTG IIFYILAYLT AIIFLSAFVIG TSLFLIATVS	GWEPAWVGVRAL GGWEPAWSGLRA GGYVKAKEGITD GGFEQAKEGIQA GGYHVIFEGFEK	RN RT 90 LREKT 82 TI EEKD 64 TI LEESK R 63
<u>م</u> ڤ	tr109A4U91Q9A4U/39-207 tr132VUR0132VUR/17-193 tr1P07VG21F07VG/91-254 tr169WKV31G9WKV/84-247 tr168WS911E8WS9/82-245 L09Ar65-279	39	LAKNAELVLAT RSAARQLTLAMLA RELNAEYQERLIQ RALNEYEDKLVL PETELEOKKRTAL PETELEOKKRTAL	GSGAALAAGWL LGLLGLGLVWF KVILRYGSKLL TVVNRVVNVLF RAGMMLLARP LMLIIPVVIL	L SL RAPG WL APEQ IP	sn	GS5WIQ	LLCFLLAYGL LLLGFASLLV YPVRKAYIVL VPLKRILTGI PVRPILALY LLLATPVVLW	GVFTLKEAIEN ALPVMRSAWYSL KSLKYLWKGFKS KAIPYVLKGMGS SALPYLKKGGAA	L R KRR 97 RY P S
P B-1	A6FW90/108-305 AJSRK6/125-337 AISNK6/199-305 QJB319/77-285 A0L6C8/144-353 A0EC8/1323-595 C1FYP1/464-711 A4AWY2/157-356 B0/TF5/143-372	108 NAT AND 125 SSPA PDRKV 99 DPVAELD 77 - PEERKAGEEK 144 VESRAQTMQ 352 - NAQLESLAKT 464 TIEDRSRAMQL 157 DKGAK 143 FSREQADNM	KAGKELLMRLAMA BEAAHLKRMTILA ARRDVEIARLGRM LKSLRAMKRTVLL QREIRQLQHRLVV REITEWRTAFKTS HERRRLSRLLFT EVNRSLIYKLGVA ATARIAVGVIVGM	GFASMNVMLLS AALTLPVFVLL WRVALVVGLAL VLPPAILMVAV GALLVLVNMAN LSFAIPVFVIS FIAAIPTFFIC GFAFGNVMFLS AVMIOVIVII	MGGHMI MGLMYVPFDLD MLYGMLVPFDLD MHGWLPGVATLI MYFPMLIPFLD IVMMSLVPPTN SFPEYFFEVNEF-	PAFHIWVMS TMD DEG PGSFVVIFPGL EIRKFFYQPIW RTLEYLNOAMA	TIGQUSWSIQ VINPLI Y LGDIIC AGTVSRLEWAL EOYKGVFRWLM SSSGTYFFIVI	AAIALPTIAF FVLTTIVLAW LVVATVTLFW MIIATIILFG GVCMTPVVLW LILTIPVQFG FIITTPVMFY FIFSLPVVFY AVITTVVFFI	GREFFKRAWAN GRDIYQRAWAN GRDIYQRAWAN GRFFKGAWQV IGKRFYVSAYKS GAOVFHVRAFKS GQDVFHVRAFKS GQDVFIRGAYVS	L S 180 L 207 AK 173 V R 153 L 220 MK 438 V R 438 V R 438 V R 232
P ₈₋₂	2mQ119-290 tr[777)8[78]78[74]5-294 tr[X0MSW1]K0MSW1[43-316 tr]32[W1]21[21]W17-354 tr]32[W1]21[21]W17-354 tr]02K6[102K1/85-255 tr]102K6[100K1/85-255 tr]102M87[105K87]56-326 tr]102M87[105K87]56-326 tr]102M87[105K87]56-326 tr]102W87[105K2]56-326 tr]102W87[105K2]51-419	110 - P 116 - P 143 - G 77 - S 72 - G 85 - S 83 - G 156 - S 80 - S 251 - S	CASHERENLPLIT RAKKTWLGAAFVT AAAPARGWRLLAA HGSSFGGATRLVA NEKARRNLYRIII DGREKKTVARLAA EESKAEYYFKSIS IFDYKMEFLKFAA KSNFMLPIFTAVA RMFSFQENARLYL	LIVMMAISWGI LGLLFEFLLTG GGVLAALSEAA ALVLAAGAEAI ATLLVPGLIF GTVLGAAGFLI FIGMFVFFITS ILAALILTVIL SILLFVAGMIT GILLFVAGMIT	CONSTRUCTION		PLHIAD PLHIAD YVAA FWAEY FWAEY FAEW FQF VFKL VAGA SWS	MB	TP I ARQALRLI SI PVVRSGYJSA SI PVVRSGVISA SI DTYQKGLAAL SWPVI RTALRNI SGDI LWKAVRNI SGDI LWKAVRNI SGEVI VSAKNV SYQVFLSGFKAL MR'	K:GSYFAI 182 K:NRS - LDI 186 RNGN - LNI 207 L.RGK - LNI 207 A.RGQFFDE 140 F.RGRVFDE 147 G.KGKFLDE 147 I.KGEVFDE 218 A.RFK - LDE 218 A.RFK - LDE 311

Supplementary Figure 6. Sequence and secondary structure conservation of the P_{IB-1} -, P_{IB-2} - and P_{IB-4} -ATPases. (a), Alignment and sequence conservation of three representative members of the P_{IB-1} -, P_{IB-2} - and P_{IB-4} -ATPases. The sequences used have locus tags lpg1024 (CopA), JW3434 (ZntA) and msmeg_5403 (CtpD). For analysis of conservation level, the ConSurf server was used⁷². Determination of conservation was based on 617 sequences having a sequence identity below 95% for P_{IB-1} -ATPases, 520 sequences below identity=99% for P_{IB-2} -ATPases and 607 sequences below identity=99% for P_{IB-4} -ATPases. Information from (b) was used for adjusting the alignments of helices MA and MB due to poor sequence conservation in this region. The black box indicates the position of Pro94 in LpCopA. (b), Secondary structure based alignment of the N-terminal segment of the ATPase core of P_{IB-1} -, P_{IB-2} - and P_{IB-4} -ATPases. Ten weakly redundant members of each subgroup were selected and aligned based on secondary structure prediction using the PSIPRED server⁷³. Only the region from the start of helix MA to MB' is shown. The UniProtKB entries of the sequences are indicated to the left. Highlighted in bold are first sequences in each subgroup which are also used in (a).

Tables - Supplementary Table 1.

WT	D426N	M717V	P94A	P710A	M711L	A714T	M100L	M100E	E189N	E189Q	
1.483	0.393	0.358	0.615	0.355	0.637	0.492	1.130	0.743	0.564	0.805	
1.466	0.389	0.357	0.603	0.351	0.631	0.498	1.141	0.700	0.568	0.817	
1.308	0.362	0.322	0.582	0.338	0.565	0.474	1.020	0.729	0.533	0.732	
1.479	0.393	0.342	0.615	0.337	0.642	0.503	1.121	0.758	0.533	0.886	
1.395	0.388	0.360	0.596	0.342	0.662	0.507	1.155	0.768	0.540	0.921	
1.588	0.368	0.319	0.632	0.329	0.626	0.501	1.080	0.758	0.519	0.874	
1.649	0.336	0.330	0.588	0.315	0.598	0.481	1.148	0.784	0.564	0.942	
1.659	0.373	0.340	0.588	0.317	0.599	0.496	1.148	0.752	0.557	0.910	
1.708	0.338	0.322	0.534	0.291	0.547	0.480	1.100	0.770	0.511	0.796	
1.500	0.271	0.220	0.505	0.221	Average	es:	1 1 1 (0.751	0.542	0.054	
	0.3/1	0.339	0.595	0.331	0.612	0.492	1.116	0.751	0.543	0.854	
Normalized to WT (100%) and D426N (0%):											
00%	0%	-3%	19%	-4%	21%	11%	64%	33%	15%	42%	
WT	D426N	M717V	P94A	P710A	M711L	A714T	M100L	M100E	E189N	E189Q	
Integrated band intensities:											
7722675	8637304	5480970	6723758	4578525	7638705	6626940	6654735	6480825	6877605	7787190	
Signal scaling factor:											
0.894	1.000	0.635	0.778	0.530	0.884	0.767	0.770	0.750	0.796	0.902	
Raw averages:											
1.526	0.371	0.339	0.595	0.331	0.612	0.492	1.116	0.751	0.543	0.854	
Scaled averages:											
1.707	0.371	0.534	0.764	0.624	0.692	0.642	1.448	1.001	0.682	0.947	

b

a

	Tormanzea to vi i (100 vo) and b izor (0 vo), bused on searce averages											
	100 %	0 %	12 %	29 %	19 %	24 %	20 %	81 %	47 %	23 %	43 %	
Supplementary Table 1. The in vitro data. (a), Raw data from the activity measurements. The												
averaged values have been calculated and, finally, the scaled data where the background, the absorban-												
ce measured for the D426N dead mutant, has been removed and then divided with the average absor-												
bance for the wild type (WT). (b), Process for obtaining the data displayed in Fig. 4c. The LpCopA												
bands from the SDS-PAGE (Supplementary Fig. 4a) were quantified using ImageJ by calculating their												
integrated band intensities. This allowed a scaling factor to be derived, by which the raw absorbance												
signal averages were divided in order to obtain the scaled absorbance averages. The final data were												
computed by removing the background, the absorbance measured for the D426N dead mutant, and then												
divide the resulting background compensated values with the average absorbance for the wild type												
(WT).												

Normalized to WT (100 %) and D426N (0 %), based on scaled averages:

Supplementary References

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