Diverse roles of the metal binding domains and transport mechanism of copper transporting P-type ATPases

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OsHMA4/1-978 LpCopA/1-736 AfCopA/1-804 hATP7B/1-1464 XtATP7B/1-1467 Consensus/241-2513	262 P - KQREA ERHHE - I - RNY RNQ FLWSCLFSVPVFMF - S - MVLPM 71 SEVV - SPEY 91 - G 91 - G 635 NPNAHHLDHKME - I - KQW - KKS - LYVA - AFAGVLL FL - A - H - 636 NPNAHHLDHKME - I - KQW - KKS - FLCSLVFGIPVMAL MIYML 629 AKRD PTAHKLDH - KE - I KQW - KKS - FLCSLVFGIPVMAL MIYML 1321 DEAQADADPAENEELERFEEAIEAELWKRDLRRTRRFISLVTLPLFLLISLUMMGGM	299 103 117 672 665 1380
OsHMA4/1-978 LpCopA/1-736 AfCopA/1-804 hATP7B/1-1464 XtATP7B/1-1467 Consensus/241-2513	300	325 121 131 702 699 1440
OsHMA4/1-978 LpCopA/1-736 AfCopA/1-804 hATP78/1-1464 XtATP78/1-1467 Consensus/241-2513	MB MB' M1 326 CSP VQ FI I GWR FY VGAY H - A L R G - YS - NMDV LVA LGTNAAY FY SVY I V LKA L 122 ATP VV LWGGWP FF K RGWQ S - L - K - T G - Q L - MFT LI AMGI GVAWI Y SMVA VL 132 A LP A I FY SG SS I FK AAF S - A - L - R R R - T L - NMDVMY SMGVGAAF LA SV L	375 168 175 752 744 1500
OsHMA4/1-978 LpCopA/1-736 AfCopA/1-804 hATP78/1-1464 XtATP7B/1-1467 Consensus/241-2513	376 T S E S F E GQD F F E T SAML I S F I L L G K Y L E V V A K G K T S D A L S K L 169 WP G V F P H A - F R S - Q E G V V A Y F E AAAV I T T L V L L G Q V L E L K A R E Q T G S A I R A L 176 - S T A - G V L P R E Y S F Y E T S V L L A F L L L G R T L E A R A K S R T G E A I K K L 733 - A E K A G R S P V T F F D T P P M L F V F I A L G R W L E H L A K S K T S E A L A K L 745 L T V A M V E K A D K S P E T F F D T P P M L F M F I A L G R W L E H I A K S K T S E A L A K L 1501 A P G L T V S W N G L F P A S I F R S G A G G A P H V Y F A A A V I I T L I L L G R Y L E A R A K G R T S F A I R A L A d M R I D	417 219 219 795 792 1560
OsHMA4/1-978 LpCopA/1-736 AfCopA/1-804 hATP7B/1-1464 XtATP7B/1-1467 Consensus/241-2513	418 TE LAPETACLLTLD-KDGNAISETE ISTOLLORNDVIK IVPGEKVPVDGVVI 220 LKLVPESAHRIKED-GSEEVSLDNVAVGDLLRVRPGEKIPVDGVV 220 VGLQAKTAVVIRDGKEIAVPVEEVAVGDIVIVRPGEKIPVDGVV 796 MSLQATEATVVTLG-EDNLIIREEQVPMELVQRGDIVKVVPGGKFPVDGKVL 793 ISLQATEAAVVT-FGANQIILR-EEQVAVELVQRGDIVKVVPGGKFPVDGKVI 1561 LGLOPKTARVVRDDGPIGAAFDDGFGNGSSEEVVVE	468 265 264 846 843 1620
OsHMA4/1-978 LpCopA/1-736 AfCopA/1-804 hATP7B/1-1464 XtATP7B/1-1467 Consensus/241-2513	Adomain 469 K - GQ - SHVNESMITGEARPIAKKPGDKVIGGTVNDNGCIIVKVTHVGSETALSQIVQLVE 266 E - GR - SFVDESMVTGEPIPVAKEASAKVIGATINQTGSFVMKALHVGSDTMLARIVQMVS 265 E - GE - SYVDESMISGEPVPVLKSKGDEVFGATINNTGVLKIRATRVGGETLLAQIVKLVE 847 E - GN - TMADESLITGEAMPVTKKPGSTVIARSINAHGSVLIKATHVGNDTTLAQIVKLVE 844 EG - TSMADESLITGEAMPVTKKPGSMVIAGSINAHGTVLVEATHVGSETTLAQIVKLVE 1621 EGGSASSVDESMLTGESLPVEKKPGDKVIGGTINOTGSLVVRATKVGADTVLAQIIRLVE	526 323 322 904 901 1680
OsHMA4/1-978 LpCopA/1-736 AfCopA/1-804 hATP78/1-1464 XtATP78/1-1467 Consensus/241-2513	M3 527 AAQ LARAP VQK LADR I SRFF V PTV VAAFLTW - LGWF VAGQ F D I Y PR EW 324 DAQR SRAP I Q RLADT V SGWF V PAV I LVAV L SF - I VWALLGP - Q	574 364 363 956 947 1740
OsHMA4/1-978 LpCopA/1-736 AfCopA/1-804 hATP78/1-1464 XtATP7B/1-1467 Consensus/241-2513	M4 575 - I P KAMDS FEL 365 A LQ FG I SV LVVAC P CALG LATPTAVM 366 P LLF 957 PNKH I SQT EVI I R F A FTT LI AV LVVAC P CALG LATPTALT 957 PNKH I SQT EVI I R F A FQT S I TV LC I AC P C S LG LATPTALT 948	610 395 393 996 993 1800
OsHMA4/1-978 LpCopA/1-736 AfCopA/1-804 hATP7B/1-1464 XtATP7B/1-1467 Consensus/241-2513	611 VAT GK GA S Q GV L I K GG NA L E - KA HK VK A I I F D KT GT L T V G K P S V V Q T K V F S K - I 396 V G V G K G A Q S G V L I K NA E A L E RM E K - V NT L V V D K T GT L T E G HP K L T R I V T D D - F	662 446 443 1048 1046 1860
OsHMA4/1-978 LpCopA/1-736 AfCopA/1-804 hATP7B/1-1464 XtATP7B/1-1467 Consensus/241-2513	663 PLLEL CDLAACA EANS EHPLSKAIVEYTKKLREQYGSHSDHIM 447 VEDNALALAAAL EHQSEHPLANAIVHAAKEKGLSL-G 444G	705 482 480 1089 1086 1920

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	N-domain	
OsHMA4/1-978	706 E S K D F E V H P G A G V S A N V E G	726
LpCopA/1-736	483 SVEAFEAPTGKGVVGQVDGGGGG	501
AfCopA/1-804	481 EPEKVEVIAGEGVVA	495
hATP7B/1-1464	1090 YCTDFQAVPGCGIGCK V - SNVEGILAHSERPLSAPASHLNEAGSPAEKD AVPQ	1141
XtATP7B/1-1467	1087 YCTDFQAVPGCGISCKVN-NIESVLVQN	1122
Consensus/241-2513	1921 EVEDFEAIPGKGVSATVDVYDNIEGALRGACNAERIINYENLSVGNPLSNSEFGGGGGKRO	1980
	N-domain	
OsHMA4/1-978	727 - <mark> V L V G N K</mark> <mark>R</mark> L - MQ E - F E V P I S S	746
LpCopA/1-736	502 - HHVAIGNARLMQEH-GGDNAPLFEKADE	528
AfCopA/1-804	496 <u>– D</u> CILVGNK – – R – – L – MEDF – GVA – – <u>– –</u> – – – – – – – – – VS – – – – – – – – <u>– –</u> – – – – – – – – –	514
hATP7B/1-1464	1142 T F S V L I G N R E W - L R R - NG L T I S S	1164
XtATP7B/1-1467	1123 Y R N S L I G T T D S S L I I T P E L L G A Q A P L A H T V L I G N R EWM	1160
Consensus/241-2513	1981 RRRVLLGNRPERLALAMEELNGIDELVPESAEAAATVAEDTASIDAALAAQAEAEVVGWA	2040
	N-domain	
OsHMA4/1-978	747	777
LpCopA/1-736	529 <u></u>	553
AfCopA/1-804	515 NEVE LA LE - K - LEREA KT - AVI - VA RNGRVEGIIAVSD	548
hATP7B/1-1464	1165 S D A - MT D H EMK G Q T - A - I L VA I - D G V L C G M I A I A D	1195
XtATP7B/1-1467	1161 RRNGLHISTDVDEA-MSSHEMKGQTAVLVA-IDGELCGMIAIAD	1202
Consensus/241-2513	2041 KALPLAIEDLEPAALAESDEQAAAEELEAEGATKTEVVYAVAIIDGEVDGRLAGLIAVAD	2100
	B damain	
	r-qonam	
OsHMA4/1-978	778 PLKPHAGRAISYL-SSMGI-SSI-MVTGDNWATAKSIAKEVGIG-TVFAEIDPVGKAEKI	833
LpCopA/1-736	554 PIKSSTPETILELQQ-SGI-EI-VMLTGDSKRTAEAVAGTLGIK-KVVAEIMPEDKSRIV	609
AfCopA/1-804	549 T L K E S A K P A V Q E L K R - MG I - K V - GM I T G D NWR S A E A I S R E L N L D - L V I A E V L P HQ K S E E V	604
hATP7B/1–1464	1196 AVKQEAALAVHT L-QSMGV-DVV-LITGDNRKTARAIATQVGIN-KVFAEVLPSHKVAKV	1251
<i>XtATP7B/1–1467</i>	1203 TVKQEAALAVHTLKS-MGID-V-VLITGDNRKTAKAIATQVGI-KKVFAEVLPSHKVAKV	1258
Consensus/241-2513	2101 PIKPDAAEAIAALKARMGIDRVVVMLTGDNRRTAEAVARQLGIDIEVIAEVLPEDKAAVV	2160
	P-domain	
o		
OsHMA4/1-978	834 KDLQ-MK-GLIVAMVGDGINDSPALAAA-DVGLAIGAGIDVAIEAADIVLMRSSL	885
LpCopA/1-736	610 SEL-KDKGLIVAMAGDGVNDAPALAKAD-IGIAMGTGTDVAIESAGVTLLHGDL	661
AfCopA/1-804	605 KKL-QAKEVVAFVGDGINDAPALAQA-DLGIAVGSGSDVAVESGDIVLIRDDL	655
hATP7B/1–1464	1252 QELQ-N-KGKKVAMVGDGVNDSPALAQA-DMGVAIGTGTDVAIEAADVVLIRNDL	1303
XtATP7B/1-1467	1259 QALQSDNKRVAMVGDGVNDSPALARAD-VGIAIGTGTDVAIEAADIVLIRNDL	1310
Consensus/241-2513	2161 KELOQAESOKGGGKRVVAMVGDGINDAPALAQADDVGIAMGIGIDVAIEAADIILMRGDL	2220
	M5 M6	
0-1111111 079		0.26
$U_{SHWA4} / 1 - 976$		710
LpCOpA/1-750		712
AICOPA/1-804	030 KDVVAATULSKNIMSNIKUNIFWALITNIVILIPAAAGUL-TPIFG-VVKKPEF	1252
NATP7B/1-1404		1333
XIA/P/D/1-140/		1300
Consensus/241-2515	2221 KOVVBAIRLISRAIMKNIKONLITWATITINVLGITLAAGVLLITTEFFFFFNFGGILILLISEMI	2200
	M6	
OsHMA4/1-978		976
LpCopA/1-736	713 AAAAMALSSVSVLINALRLKR-VTL	736
AfCopA/1-804		744
hATP7R/1-1464		1392
XtATP78/1-1467	1361 G SAAMAAS SV SV VI S SLOIK CYRKPDSDRYFARAO-GHMKPI TPSOIS	1407
Consensus/241-2513	2281 AAAAMALSSVSVVTNALRLREFKPPALETREAPALEPAAAPLEAAPTGAANPLIPSTIP	2340
	* *	
OsHMA4/1-978		
LpCopA/1-736		
AfCopA/1-804		
hATP7B/1-1464	1393 P LT - A SQ V S V H I G M DD R W R D S P R A	1415
<i>XtATP7B/1–1467</i>	1408 VH I G - MDD RWRD L P K T K AWDQ I S Y I SQV SR A SQK P K R HG S L V EQQDKWS L L I N E T	1461
Consensus/241-2513	2341 LVOGEMENLSWWSKVSVHSGAPRVSEFLDDLPRDSTSPFPOKHNRPAPGANFWAILRAAR	2400
		-
OsHMA4/1-978	977 LV LV	978
LpCopA/1-736		
AfCopA/1-804	745	751
hATP7B/1–1464	1416 T PWDQVSYVSQVSLSSLTSDKPSRHSAAADDDGDKWSLLLNGRDEEQYI	1464
XtATP7B/1-1467		1467
Consensus/241-2513	2401 A E GA E LWDOR VN F SR SSL SSL LSDRR SL VP LOT V E PGDK E SL LD SDRD E DDD E E Y E GK SY	2460
OcHMA1/1_070		
USTIMA4/1-9/8		
AfConA /1- 201		804
hATP7R/1_1/6/		004
XtATP7R/1_1/67		
Consensus/241-2513	2461 YHCRAHVAQ SYNMEGIAADATELNPAVVF SEGEPTDAWIKTREAAGTEAHLHP	2513

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OsHMA4/1–261 hATP7B/1–634 XtATP7B/1–628	1 – – – – – – – – – – – – – – – – – – –	42 60
OsHMA4/1-261 hATP7B/1-634 XtATP7B/1-628	43 GY EGG L DG L GP S – SQ VAT ST VR I L GMT CQ S C VK S I E DR I SN L K G I I SM K V S L EQ G S A T V K 61 GY EG S P D D L C S L P D D V G S V V A I Q GMT CQ S C VQ S I EGR I S K V S G V V G I N V C L EQ NNA I V N	101 120
OsHMA4/1-261 hATP7B/1-634 XtATP7B/1-628	102 Y V P S V V C L Q Q V C H Q I G D M G F E A S I A E G K A A S W P S R S L P A Q E A V V K L R V E G M T C Q S C V S S I 121 Y L Q T E I T P H K I C E E I E D M G F D A S L S E Q S G M P S S V K S S Y Y G D N V I K I R V E G M T C Q S C V N T I	161 180
OsHMA4/1–261 hATP7B/1–634 XtATP7B/1–628	162 EGKVRKLQGVVRVKVSLSNQEAVITYQPYLIQPEDLRDHVNDMGFEAAIKSKVAPLSLGP 181 EGKIGKIQGVQKIKVSLTGQEAVITYQSHIIQAEDLRKYIEDMGFEASIKNKPDPTKLGT	221 240
OsHMA4/1-261 hATP7B/1-634 XtATP7B/1-628	222 I D I ER LQ ST NP K RP L S SA NQ N F N N S ET L G HQ G S H V V T LQ L R I DGM H C K S C V L N I E E N I G Q 241 I D I ER LQ NS I A E N H	281 285
OsHMA4/1–261 hATP7B/1–634 XtATP7B/1–628	1 282 LLGVQSIQVSLENKTAQVKYDPSCTSPVALQRAIEALPPGNFKVSLPDGAEGSGTDHRSS 286 LAGIQSIRVSLKNKNAVVCLSQGSTSLLSLKESIENLPPGKFKVTLPVGVEKGQSLARNS	21 341 345
OsHMA4/1-261 hATP7B/1-634 XtATP7B/1-628	22 GA SPAGA SPR K ER KTRK VM FNVRG I SCASCAVSI E TVVAG L KGVE SV SV SPLQGQAVV 342 SSH SPG SPPR NQVQGT C STTLIAIAGMT CASCVH SI E GMI SQLEGVQQI SV SLAEGT ATV 346 TH S SHR DQ SM - GG NMAII SI GGMT CQ SSI E NMI SQR KGVLHILVSLDEGNGNI	79 401 400
OsHMA4/1–261 hATP7B/1–634 XtATP7B/1–628	MBD ³ 80 QYRP E EA DART I K EA I EG LNF EV DE LQ	106 460 457
OsHMA4/1–261 hATP7B/1–634 XtATP7B/1–628	MBD ³ 107HT GR L PANHAP DI LAK S PQ STRAVAPQK CFLQIKGMACT SCSESVERALQMVP GV 461 PHT GR L PANHAP DI LAK S PQ STRAVAPQK CFLQIKGMTCASCVSNIERNLQK EAGV 458 G SR DY I L DV L PKK S HP D FANEKY DT AP EKCFLQIT GMTCASCVSNIERNLKK K DG I	139 516 513
OsHMA4/1–261 hATP7B/1–634 XtATP7B/1–628	MBD ² 140 KKAAVGLALEEAKVHFDPNITSRDLIIEAIEDAGFGADLISSGD-DVNKVHLKLEGVSSP 517 LSVLVALMAGKAEIKYDPEVIQPLEIAQFIQDLGFEAAVMEDYAGSDGNIELTITGMTCA 514 VSVLVALMSGKAEVKFYPDRIEPLEIAQLVEDLGFGASVMEDYTASDGNVELIITGMTCA	198 576 573
OsHMA4/1-261 hATP7B/1-634 XtATP7B/1-628	MBD ⁻¹ 199 EDIKLIQSR LESVEGVNNVECDTAGQTIIVAYDPDVTGPRLLIQCIQDAAQPPK 577 SCVHNIESKLTRTNGITYASVALATSKALVKFDPEIIGPRDIIKIIEEIGFHASL 574 SCVHNIESRLMRTPGILQASVALATCKAQVKFDPEIVGPRDIIRIIEGIGFQASL	258 631 628
OsHMA4/1–261 hATP7B/1–634 XtATP7B/1–628	259 Y S P 632 AQ R	261 634

Supplementary figure 1: Conserved sequence features among P_{1B-1} -ATPases. Sequence alignment of copper-transporting P-type ATPases (all containing the CPC motif in M4, DKTGT in the P-domain, YN in M5, and MXXSS in M6 - motifs associated with Cu⁺/Ag⁺ pumps). Alignments were performed using Clustal Omega and visualized using Jalview. Relevant structural features are highlighted above the sequences. **a**, Alignment of *Oryza sativa* HMA4 (Q6H7M3), *Legionella pneumophila* CopA (Q5ZWR1), Archaeoglobus fulgidus CopA (O29777), human ATP7B (P35670) and *Xenopus tropicalis* ATP7B (A0A6I8R0A5) and the consensus of 1713 CopA proteins, spanning from the MA until the C-terminus (the N-termini have been removed for clarity). **b**, Alignment of the N-termini (until MA) of HMA4, hATP7B and XtATP7B.



Supplementary figure 2: *Oryza sativa* HMA4 and human ATP7B sample quality as recovered from *Saccharomyces cerevisiae*. **a**, The size-exclusion chromatography profile following purification of HMA4. **b**, SDS-PAGE analysis of HMA4 following size-exclusion chromatography of HMA4. An unrelated sample is present in the well in-between the markers and OsHMA4. **c**, SDS-PAGE analysis of human ATP7B.



Supplementary figure 3: Data processing of the HMA4^{BeF} structure (E2P state). a, Data processing flowchart. **b**, Representative 2D class averages. The box size was 30 nm. **c**, Gold standard Fourier shell correlation (FSC) curve of the final map. **d**, Particle orientation distributions in the final 3D reconstruction.



Supplementary figure 4: Data processing of the HMA4^{AIF} **structure (E2P state). a**, Data processing flowchart. **b**, Representative 2D class averages. The box size was 30 nm. **c**, Gold standard Fourier shell correlation (FSC) curve of the final map. **d**, Particle orientation distributions in the final 3D reconstruction.

Supplementary figure 5 а Full-frame motion Patch-CTF Blot pick Local motion correction 16,429 movies 1,604, 199 particles 5759,742 particles Ab-initio reconstruction Hetero-refinement 286,161 particles 427,779 particles 237,795 particles 295,660 particles 356,804 particles Ab-initio reconstruction Hetero-refinement 137,100 particles 63,524 particles 79,258 particles 59,950 particles 136,261 particles b 63 258,788 particles map resolution 4.56Å Mutiple runs of Ab-initio d С Hetero-refinement GSFSC Resolution: 3.58Å 1.0 NU-refinement No Mask (4.6Å) Spherical (4.5Å) Loose (4.2Å) Tight (3.6Å) Corrected (3.6Å) 0.8 0.6 104,111 particles 101 0.4 map resolution 3.58Å $-\pi/$ 0.2 n/4 0 Azimuth 0.0 DC 3.4Å 2.8Å 2.3Å 14Å 2Å 6.9Å 4.6Å

Supplementary figure 5: Data processing of the HMA4^{apo} structure (E1 state). a, Data processing flowchart. b, Representative 2D class averages. The box size was 30 nm. c, Gold standard Fourier shell correlation (FSC) curve of the final map. d, Particle orientation distributions in the final 3D reconstruction.





Supplementary figure 6: Data processing of the HMA4^{Cu} **structure (E1-Cu state). a**, Data processing flowchart. **b**, Representative 2D class averages. The box size was 30 nm. **c**, Gold standard Fourier shell correlation (FSC) curve of the final map. **d**, Particle orientation distributions in the final 3D reconstruction.



Supplementary figure 7: Local resolution of the cryo-EM maps of the generated HMA4 structures.



Supplementary figure 8: Cryo-EM map quality of the HMA4^{BeF} structure (E2P state). The protein parts of colored as in Fig. 1. Contour levels are indicated in brackets.



Supplementary figure 9: Cryo-EM map quality of the HMA4^{AIF} structure (E2P state). The protein parts of colored as in Fig. 1. Contour levels are indicated in brackets.



Supplementary figure 10: Cryo-EM map quality of the HMA4^{apo} structure (E1 state). The protein parts of colored as in Fig. 1. Contour levels are indicated in brackets.



Supplementary figure 11: Cryo-EM map quality of the HMA4^{Cu} **structure (E1-Cu state).** The protein parts of colored as in Fig. 1. Contour levels are indicated in brackets.



Supplementary figure 12: The DKTGT-regions in the determined HMA4 structures. Note that the density is shown at high signal-to-noise level where even some secondary structure is missing, and yet support for the phosphate mimics is present in HMA4^{BeF} and HMA4^{AIF}, respectively.



Supplementary figure 13: Structural comparisons of the determined HMA4 structures. Structures were aligned using super in Pymol and using the complete structures. **a**, HMA4^{AlF} versus HMA4^{BeF}. **b**, HMA4^{Cu} versus HMA4^{apo}. **c**, HMA4^{AlF} versus HMA4^{apo}, including a close-view of the CPC-motif showing the CPC-motif is more surface-exposed in HMA4^{apo} than in HMA4^{ALF}.



Supplementary figure 14: Surface analyses of HMA4, with access from the extracellular side to the residues contributing to the high-affinity binding site in the E2P, but not the E1 states. a, HMA4^{BeF}; b, HMA^{AlF}; c, HMA^{apo}; d, HMA^{Cu}.



Supplementary figure 15: Structural comparisons of selected HMA4 structures to SERCA. a, Structural comparison of HMA4^{Cu} and SERCA (PDB 4H1W). **b,** structural comparison of HMA4^{AIF} and SERCA (PDB-ID 3B9B). The alignment was conducted using super in Pymol and using the complete structures. For clarity, the MBD, MA and MB as well as M7-M10 have been removed in HMA4 and SERCA, respectively.



Supplementary figure 16: Comparisons between HMA4^{apo} and AlphaFold structures A, Overview of different P_{IB-I} AlphaFold models, as aligned in PyMol to HMA4^{apo}. Top are overviews of the structures, coloured as in Fig. 1a. Bottom are the same views, but with the core faded out, highlighting the MBDs. The structures shown are HMA4^{apo} (A, including MBDs from the MD simulation in the bottom view), and AlphaFold models of AfCopA (B), hATB7B (C) and HMA4 (D). The MBDs are colored as shown on the left.



Supplementary figure 17: The previously determined structures of human ATP7B, compared to HMA^{apo}. Density maps of the previously determined structure of human ATP7B (PDB-IDs A: 7XUM B: 8IOY), as well as HMA^{apo} (C). Density of the N-domain in red, P-domain in blue, MA-MB in cyan and M1-M6 in gray. The wheat density was previously assigned as MBD⁻¹ while the orange density was left unassigned. We expect the orange density represents MBD⁻¹ (as shown in this manuscript), while the wheat density corresponds to MBD⁻².



Supplementary figure 18: Complementation of Ccc2p deficient Saccharomyces cerevisiae. A: For assessing the function of P_{1B-1} -ATPases, the assay requires low iron levels (-Fe), as Ccc2p is essential for high-affinity iron uptake. Controls grown at normal iron levels (+Fe) are also shown. The indicated yeast strains were spotted at three different densities (from left to right; 5 µL 0.50 OD₄₅₀/mL; 5 µL 0.05 OD₄₅₀/mL; 5 µL 0.005 OD₄₅₀/mL). Plates were imaged after 72 hours. The assessed forms are empty vector (essentially the Ccc2p deficient Saccharomyces cerevisiae strain), hATP7B, WT HMA4 (wild-type HMA4), Δ(N-term)HMA4 (HMA4 lacking the N-terminal tail preceding MBD⁻³), Δ (MBD⁻³)HMA4 (HMA4 lacking MBD⁻³ and preceding amino acids), Δ (MBD⁻³-MBD⁻²)HMA4 (HMA4 lacking MBD⁻² and all preceding parts), Δ (MBD⁻³-MBD⁻¹)HMA4 (HMA4 lacking MBD⁻¹ and all preceding parts) and HMA4 MBD⁻² CXXC - SXXS (HMA4 with the CXXC-motif of MBD⁻² mutated to SXXS). B: Live cell bioimaging of yeast strain PAP6064 expressing C-terminally GFP tagged hATP7B or HMA4 variants. GFP tagged versions of hATP7B, WT HMA4 and HMA4 mutants show similar distinct localization in the $ccc2\Delta$ yeast strain, probably representing the late-Golgi compartment. Localization in the late-Golgi compartment is required for complementation of the high iron requirement of the $ccc2\Delta$ strain. Yeast cultures were inoculated at 30 °C in standard minimal medium with galactose as sole carbon for 24 hours prior to live cell bioimaging. Left side of the figure shows GFP fluorescence while the right side shows the same cells imaged with differential interference contrast. C: GFP fluorescence levels detected in yeast strains expressing hATP7b or HMA4 (WT and different constructs).



Supplementary figure 19: Molecular dynamics simulations. Stability of MBD^{-2} in the starting position. The RMSD traces of MBD^{-2} against starting structures with MBD^{-2} positioned as in AlphaFold model of HMA4 on MB' in simulations of **a**, the E1 and **c**, E2P state, respectively or **e**, with MBD^{-2} positioned as in the XtATP7B structures (in E2P simulations). The average RMSD with standard deviation for the three systems were 0.30 ± 0.082 nm, 0.60 ± 0.10 nm, 0.98 ± 0.81 nm, respectively. **b**,**d**,**f**, shows the final structure of one of the higher RMSD trajectories three separate runs respectively. The M-domain is shown in grey, MA and MB in cyan, P-domain in blue, A-domain in yellow, N-domain in red and MBD^{-2} in orange. The starting position of MBD⁻² is shown in magenta.



Supplementary figure 20: The complex between ATOX1 and a metal binding domain. A. Structure of ATOX1 in complex with MBD⁻⁶ of hATP7A (PDB-ID 2K1R). Side chains of residues located at the interaction interface are shown as lines, the CXXC motifs of each domain, as well as I8 and D64 of the MBD (correspond to L492 and L549 of hATP7B MBD⁻², respectively, are shown as sticks and labelled. **B.** Structural alignment of the ATOX1-MBD complex (PDB-ID 2K1R) and HMA4 (MD simulation of the E1 state). ATOX1-MBD is colored in green-purple, while HMA4 is colored as in Fig. 3c.



Supplementary figure 21: Structural comparisons of the determined HMA4 structures to other structures of P_{1B-1}-type ATPases.





Supplementary figure 22: Densities of the copper binding site A-C, Top are replicates of Fig. 4A-C, with the bottom being the same views including electron density, showing HMA4^{apo} (A), HMA4^{Cu} (B) and HMA4^{AlF} (C). **D.** Closeup of the copper binding site in HMA4^{Cu} (left) and HMA4^{apo} (right), with density shown at two different contour levels in red and black, respectively.

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	HMA4 ^{apo}	HMA4 ^{Cu}	HMA4 ^{AlF}	HMA4 ^{BeF}
	PDB: 8Q73	PDB: 8Q74	PDB: 8Q75	PDB: 8Q76
Data collection				
EM equipment	FEI Titan Krios	FEI Titan Krios	FEI Titan Krios	FEI Titan Krios
Voltage (kV)	300	300	300	300
Detecter	Gatan3	Gatan K3	Falcon 3	Falcon 3
Data collection mode	counting	counting	counting	counting
Pixle size (Å)	0.8617	0.8617	0.832	0.832
Energy filter	/	/	/	/
Electron dose (e ⁻ /Å ²)	50	50	40	40
Defocus range (mm)	-1.2 ~ -2.6	-1.2 ~ -2.6	-1.2 ~ -2.6	$-1.2 \sim -2.6$
Data processing				
Software	cryosparc	cryosparc	cryosparc	cryosparc
Number of final used particles	104111	246045	184645	216398
Symmetry	C1	C1	C1	C1
Map resolution (Å)	3.58	3.68	3.20	3.29
Model refinement				
Total built residues	779	784	774	774
Model-map-fit CC	0.73	0.80	0.84	0.82
R.m.s.d.				
bonds (Å)	0.004	0.004	0.004	0.003
angles (°)	0.916	1.004	0.982	0.739
Molprobity statistics				
Molprobity score	2.23	2.48	2.45	2.32
Ramachandran plot				
Favored (%)	86	83	86	87
Allowed (%)	13	16	12	12
Rotamer outliers (%)	1	1	2	0
Clash score	12	20	22	17
Average B-factor (Å ²)	289.03	291.94	213.03	227.17

Supplementary Table 1. Cryo-EM data collection, data processing and model building statistics.

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SERCA state	PDB ID	HMA4 ^{apo}	HMA4 ^{Cu}	HMA4 ^{AlF}	HMA4 ^{BeF}
		E1-apo	E1-Cu	E2P-AIF	E2P-BeF
E1	4H1W	9.5	8.0	12.1	12.2
[Ca]2 E1	2C9M	10.9	11.8	14.5	14.5
[Ca]2 E1	1SU4	11.2	11.5	13.2	13.0
[Ca]2 E1×ATP	3N8G	10.9	8.2	12.1	12.6
[Ca]2 E1P-ADP	1T5S	11.0	8.3	12.1	12.6
[Ca]2 E1-ADP:AlF4	1T5T	11.0	8.2	12.1	12.6
[Ca]2 E1P:ADP	3BA6	10.9	8.2	11.8	12.3
E2P	3B9B	10.9	13.8	4.8	4.6
E2-P	3N5K	11.2	13.6	3.9	4.0
E2:Pi	3FGO	11.3	13.5	4.1	4.1
E2	3NAL	9.8	10.6	5.5	5.7
P _{1B-1} -ATPases					
LpCopA ^{AlF}	4BYG	8.3	10.1	1.8	1.6
LpCopA ^{BeF}	4BBJ	7.3	8.8	2.9	2.8
AfCopA	7R0I	4.1	3.8	10.8	11.4
XtATP7B	7SI3	7.7	9.7	1.7	1.8
hATP7B	7XUM	4.6	3.8	11.3	11.6
AlphaFold models					
HMA4		3.3	3.4	7.3	7.1
hATP7B		3.5	3.4	7.5	7.5

M-domain	HMA4 ^{apo}	HMA4 ^{Cu}	HMA4 ^{AlF}	HMA4 ^{BeF}
HMA4 ^{apo}		0.9	3.0	3.3
HMA4 ^{Cu}	0.9		3.0	3.1
HMA4 ^{AlF}	3.0	3.0		0.6
HMA4 ^{BeF}	3.3	3.1	0.6	

Supplementary table 2: RMSDs of structural alignments of the determined HMA4 structures to the available structures of relevant P-type ATPases. Alignments were computed using super in Pymol and using the complete structures, except where indicated.

Box System Nr atoms Nr waters Salt, Lipid composition dimensions concentration E1^{apo} x: 120.2 Å 211,256 48,416 KCl: 0.15 M POPC: 383 y: 120.2 Å z: 156.5 Å x: 120.2 Å E2P^{AIF} 218,306 50,648 KCl: 0.15 M POPC: 388 y: 120.2 Å z: 160.3 Å E2P^{Xt} x: 120.1 Å 343,561 92,435 POPC: 387 KCl: 0.15 M y: 120.1 Å z: 171.9 Å

Supplementary table 3

Supplementary table 3: Simulation system overview. Specifications of simulation box dimensions, numbers of atoms, water molecules, salt concentration, and lipid composition of the membrane.

Supplementary table 4

System	Trajectories (samples)	Mean CA RMSD (Å)	Stdev (Å)
E1 ^{apo} MBD ⁻²	10	3.0	0.82
E2P ^{AIF} MBD ⁻²	5	5.9	1.0
E2P ^{Xt} MBD ⁻² all	5	9.8	8.1
E2P ^{Xt} MBD ⁻² stable	3	3.5	0.87

Supplementary table 4: Calculations of MBD⁻² root mean square deviations (RMSD). RMSD for MBD⁻² was calculated compared to the initial position. The mean and standard deviation values presented were calculated from the mean RMSD of each replica. "E2P^{Xt} MBD⁻² stable" refers to the three replicas where MBD⁻² did not dissociate within the simulation time. Uncropped SDS-PAGE gels - Supplementary figure 2 HMA4:



ATP7B:

