Supplementary Information for:

The structure and function of P5A-ATPases

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P5A CtSpf1 ScSpf1 CATP-8 HsATP13A1 P5B CATP-5 HsATP13A2 CtYpk9 ScYpk9	1 1	
CtSpf1 ScSpf1 CATP-8 HsATP13A1 CATP-5 HsATP13A2 CtYpk9 ScYpk9	28 79	EDSHLLGALEDGNH
P5A CtSpf1 ScSpf1 CATP-8 HSATP13A1 P5B CATP-5 HSATP13A2 CtYpk9 ScYpk9	1 1 78 157	MAAAAAVGNAV. MNTSERE AGPVAESVPTSVSAFSHRHGRAESVASFSFYHEQDDQREELEAPPGSLGARL GT.GLSSRRSSIRSFSRASSLS.NAKSYGSFSKRGRSGSRAPQRLGENSDTGFVYHSATHSSSSLSRYTTRERIP
P5A CtSpf1 CATP-8 HSATP13A1 P5B CATP-5 HSATP13A2 CtYpk9 ScYpk9	1 1 12 8 1 139 230	NTD Ma MAPLVDNPQIKSAELLRPLPLYQHAYVWPYVI MTKKSFVSSPVRDSTLLVPKSLIAKPVLPFFV MTKKSFVSSPVRDSTLLVPKSLIAKPVLPFFFI MGVDQLVETIIPYNLRSIATHLYVPFTI PCGARPCGVRPDGQPKGPQPRALLAAGPALIANGDELVAAVWPYRLALLRRLTVLPFAG PLLDTTRNRVYDTTDNPSTKIMKREKDNPK PLLDTTRNRVYDTTDNPSTKIMKREKDNPK. FEEEGLSESEMPEQLDTFGIDLEWGSMNNGYPLIRRSSTHSQFSAHHRLLRRESGVSAAS IELESQTDEILEDESSTHSLES.SDSRRSASENNRGSFSGHDDVHNQHSEYLKPDYHEKFYP
P5A CtSpf1 ScSpf1 CATP-8 HsATP13A1 P5B CATP-5 HsATP13A2 CtYpk9 ScYpk9	33 35 30 73 39 18 199 291	Ma Mb V.WPVFLRVYLTQELYDKYIGAQEWTFVWIISIVTFQTLTWLCTHASVNLNALFTA SIVTFQTLTWLCTHASVNLNALFTA L.YA.TFAQLYFQ.QYDRYIKGPEWTFVYLGTLVSLNILVMLMPAMNVKIKAKFNY TUVSLNILVMLPFCHMMPVVCRIKAKFNY I.TA IMTYVWLNTFGYEEYYELGMLGYAAIFVILALVLFCHMMPVRCFLMC LLYP.AWLGAAAAGCWGWGGSSWVQIPEAALLVLATICLAHALTVLSGHWSVHAHCALTC AAKTISFNQGKLNIGEETCDLYAYKETIGRQILFWL ILTIGTSI.DPLSSSVSSVLSGYCGSPWRVIGYHVVVWMMAGIPLLFRKKPLWGVRLSG GYTGGRSSQKMRLDNDDLTIAISGFITNRIGFAIYIV. GYTGGRSSQKMRLDNDDLTIAISGFITNRIGFAIYIVCCFLTFGLVYL LCVLTGGIAWLFTRMYPKYVKLVG
P5A CtSpf1 CATP-8 HSATP13A1 P5B CATP-5 HSATP13A2 CtYpk9 ScYpk9	88 82 131 99 78 261 353	NTD KKAS
P5A CtSpf1 ScSpf1 CATP-8 HSATP13A1 P5B CATP-5 HSATP13A2 CtYpk9 ScYpk9	120 120 114 163 140 156 316 418	NTD Adomain .VGDNKTNISFLFQKRRFLWYPER.KAFSTLEFDIDAEPKPTLSKFQLSRGIESEDELKRLEQHYGTNTFDIPVP .EAGSLQTFFQCKKRFLWHENE.QVFSPKFLVDESPK.IGDFQKCKGH.SGDLTHLKRLYGENSFDIPIP RD.KQTKLWFEFQRVHYTWDEES.REFQTKTLDTAKPMVFFQKSHGFEVEEHVKDAKYLLGDNKTEMIVP .GEDGLEVLSFEFQKIKYSYDALEKKQFLPVAFPVGNAFSYYQSNRGFQEDSEIRAAEKKFGSNKAEMVVP KQEKVVMRFFTYRKIKYIW2EKOQEWLNPADMDSAAPFNIYQKLTLDVIGLKEQDVIASRKIYNMNALALALT SVGQKRVLRYYLFQQRFYIWIETQQAFYQVSLLDHGRSCDDVHRSRHGLSLQDQMVRKAIYCPNVISIPVK VLRELRSITYCYKFYYHPVLDKFFCCNGW.KDPQWNSMQNARSGLHGDEKAHREAVFGPNSIDVDEQ NVPILITFEYRYIKFIYSPLDDLFKTNNNW.IDPDWVDLSTVSNGLTKGVQEDRELAFGKNQINLRMK
P5A CtSpf1 ScSpf1 CATP-8 HSATP13A1 P5B CATP-5 HSATP13A2 CtTpk9 ScYpk9	193 188 182 233 214 227 383 485	IM1 TM2 .Adomain TFTELFKEHAVAPEFVEOVECVGLWLLDEVMYYSLFTLVMLVVFESTVVWQRQRTLTEFRSMSIKPYPIYYYRLG.KW OFLEMFIERATAPEFVEOVECVGLWCLEDMWYYSLFTLFMLMTEATLVKQQMKNMSEIRNMGNKTYMINVLRGK.KW OFLEMFIERATAPEFVEOVECVGLWCLEDMWYYSLFTLFMLMTFEATLVKQQMKNMSEIRNMGNKTYMINVLRGK.KW DFSELFKERATAPEFVEOVECVGLWCLEDMWYYSLFTLFMLMTFEATLVKQQMKNMSEIRNMGNKTHMIQYYRSR.KW DFSULFKENLGPFYLFOCFSVLWYSDNYAYXASVVIITVGSAAVAVYQMRAQEKRINNVGDTISVIYRGG.HD SYPQLLVDEALNPYYGFQAFSIALWLADHYYWALCIFLISSISICLSLYKTRKQSQTLRDMVKLSMRVCVCRPGGEE SILQLLVSEILTPFYAFOVFSLILWLCDEVYYYAAATHLISAGSIITSLETKETRRIJREMSRFFCEVRVFRGG.FW TTSEILFNEVLHPFYVFOVFSIILWGIDEVYYYAACTFLISVLSIFDSLNEQKKVSRNLAEMSHFHCDVRVLRCVCRPGGEE CAdomain
P5A CtSpf1 ScSpf1 CATP-8 HSATP13A1 P5B CATP-5 HSATP13A2 CtYpk9 ScYpk9	270 265 259 310 291 305 460 562	TEIQSDKLLPGDLVSVTRTKEDSGVACDMIPVEGTAIVNEAMLSGESTPLLKDSIQLRPGDAVLEVDGLDKN VALQTNELLPMDLVSITRTAEESAIPCDLIPLDGSAIVNEAMLSGESTPLLKESIKLRPSDENLQLDGVDKI QKIKIEELVAGDIVSIGRGAEECVPCDLLLLRGPCIVDESMLTGESVPQMKEPIEDVEKDKIFDIETDSRL RPIASDEIVPGDIVSIGRSPQENLVPCDVLLRGRCTDDEAMLTGESVPQMKEPIEDLSPDRVLDLQADSRL ITIDASEIVPMDILIP.S.NTFILPCDLLRGRCTVDEAMLTGESVPVKASLKEADECG.PEIRLSSENR EWVDSSELVPGDCLVLP.Q.EGGLMPCDAALVAGECMVESSLTGESIPVLKTALPE.GLGPYCAETHRR RTFPSSDLVPGDVYEVS.DPSLTQIPADSLLTGDCTWNESMLTGESVAVSKTPATNETLAKLNPAASTFSHDVDK TTISSSELVPGDIYEVS.DPNITILPCD

		A domain	TM3
P5A CtSpf1 ScSpf1 CATP-8 HSATP13A1 P5B CATP-5 HSATP13A2	342 337 331 382 362 372	SLDWGGTKALQITHGTAEEERPKPASGIPPPPDNGAMAVUTKTGFETSQGSUVRTMI AVLHGGTKALQVTPPEHKSDIPPPDNGALAIVTKTGFETSQGSUVRVMI HVIFGGTKIVQHTAPGKAAEGMVKSPDGNCICYVIRTGFNTSQGKULRINH HVIFGGTKVVQHIPPQKATTGL.KPVDSGCVAYVLRTGFNTSQGKULRIINI HTLFSGTTVLQTRNYKGQPVVMARVIRTGFSTLKGQUVRSIM HTLPCGTLILQARAYVGPHVLAVVTRTGFCTAKGGUVSSIM	YSTERVSANNTEALLF LLFLI YSAERVSVDNKEALMPTLFLI YGVKKATANNLETFCF ILFLI YGVKRVTANNLETFIFILFLI YPKPQEKEALKDVMVFILVLO YPRPINFKFYKHSMKFVAALS
CtYpk9 ScYpk9	535 639	HFLYCGTKLIRARQRLADTDEAAAVAL <mark>VVRTGFNTTRGAD</mark> VRSML SFLYN <mark>GT</mark> NIIRARIAPGQTAALAM <mark>VVRTGF</mark> STKGSLVRSMVI	/PKPSKFKFYEDSFRYLKVMG FPKPTGFKFYRDSFK <mark>YI</mark> GF <mark>M</mark> S
P5A CtSpf1 ScSpf1 CATF-8 HsATP13A1 P5B CATF-5 HsATP13A2 CtYpk9 ScYpk9	420 408 403 453 424 434 601 702	TM3 VFALAASWYVWDEGVRK.DRKRSKLILDCILIITSVPPELPMELSLAVNTSLSALAK IFAVIASWYVWVEGVRK.GRQSKLILDCILIITSVPPELPMELTMAVNSSLAALAK IFATAAAAYLWIKGSVDETRSKYKLFLECTLILTSVPPELPELSLAVNTSLSALAK FIALIGFIYTVIEMVSR.GESLKHIIRSLDIITIVVPPALPAAMSVGINANSRLKK VIALLGTIYSIFILYN.RVPLMEIVIRALDLVTVVVPALPAAMTVCTLYAQSRLK CLAGLAFIYSLVNFIRL.KLHWTLILLRALDLITIVVPPALPAAMTVCTLYAQSRLK LIAIFGFCVSCVQFIKL.GLDKKTMILRALDLITIVVPPALPATTIGTSFAVQRLKC VPPELP	P domain FAIFCTDPFRIPFACRIDVA FYVYCTDPFRIPFACRIDVC LGIFCTDPFRIPFACXVDIC LLYMYCTDPFRIPFACXVDC KKIFCTSPTTVNVCCLINVA VGIFCHEDLRINLGCXLDLW KKIFCTSPQRVNVCCXDLM KGIFCISPTRLNISCKIDVM
D54 CtSpf1	497		DGAHTRMVSVHDAGM
ScSpf1 CATE-8 HSATP13A1 P5B CATE-5 HSATP13A2 CYpk9 ScYpk9	485 481 531 501 511 678 779	CFDKTGTLTGEDLVFEGJAGISADSEN. IR CFDKTGTLTDNLVVECVALNNQKEGM. IR CFDKTGTLTSDSLVVEQVAGVAGLRDGK.E. VT CFDKTGTLTDDGLDFNCLKAIRKNEDGKPEFTSEFEELDPVKLS. CFDKTGTLTEDGLDVMGVVP. LKGQAFLPLVPEPRRL CFDKTGTLTEGLDVMGVVP. NKFTELLINVDDLINSCDSVSNGDEVKPAL CFDKTGTLTEDGLDVLGVQISEPNGVRGQKFGELLSDIRQVF. PKFS. DKTGT	HLYSAAEAPE NAEDLPH PVSSIPV AENANL HVDGSSLKKDKTKPLDP.YR LNDCSSPLDFKSR
P5A CtSpf1 ScSpf1 CATP-8	546 524 517	ETTLVLATA <mark>H</mark> ALVKLDEGEI <mark>VGDP</mark> MEKATLNALGNVLGKNDTLT STILVIGAAHALVKLEDGDIV <mark>GDPM</mark> EKATLKAVGWAVERKNSNY. ESLQVLASC <mark>H</mark> SLVRFEE.DLV <mark>GDP</mark> LEKACLSWCGWNLTKGDAVM	
HSATP13A1 P5B CATP-5 HSATP13A2 CtYpk9 ScYpk9	566 551 550 752 838	ETHRALASCHSIMQLDDGTLVGDPLEKAMLTAVDWTLTKDEKVF NIVVAAASCHSITRIDG.TIHGDPLELILVEKSKWIIEEAVNSDEETQDF PLIRALATCHALSRLQD.TPVGDPMDLKMVESTGWVLEEEPAADSAFG AALYVMASCHSIRIVDG.VAVGDPLEVKMFEFTGWSVEEGFIAGEVISTE.G NFFMSLLTCHSIRSVDG.NLLGDPLDFKMFQFTGWSFEEDFQKRAFHSLYEGRHEDDV	PRSIK. DTVQPTVLRPPPEQ. TQVLAVMRPPLWE.P RGDISPSIARPPRYMTS FPENSEIIPAVVHPDSNNRE
P54 CtSpf1	595	N domain	MKML. VIV P EHYERIYKYF
ScSpf1 CATP-8 HSATP13A1 P5B CATP-5 HSATP13A2 CtYpk9 ScYpk9	568 565 615 614 611 819 915	REGTGKLDIIRRFO <mark>FSAL</mark> KRŠASIASHNDALFAAVKGAPETI AKGISGIKIFHRYH P SSAMKRMTVVAGYQSPGTBDTFIVAVKGAPETI TQGLKIHQRFHFASALKRMSVLASYEKLGSTDLCYLAAVKGAPETI ATYHPENNEYSVIKQHPFNSALQRMSVIISTPSEHSAHDMMVFTKGSPEM QLQAMEEPPVVSVLRRFPFSSALQRMSVVIVAWPGATQPEAYVKGSPEDV QEMSIGEAPPAVGVLRAFDPNPLIRRSVINVGNSGGYALVKGSPEDV NTFTDNDPHNFLGVVRSFEFLSEL	RERLSDIPKNYDEIYKSF RNMYADLPSDYDETYTRL HSMFSQCPPDYHHIHTEI ASLCIPDTIPEDYMEVVDEY AGLCNEITVPIDFAQMLQSY PEICRPETIPSDFDZLSYY SEICNKSTLPADFEEVLRCY
		N domain	P domain
P5A CtSpf1 ScSpf1 CATP-8 HsATP13A1 P5B CATP-5 HsATP13A2 CtYpk9 ScYpk9	669 629 632 679 685 681 889 985	N domain TIREGSRVLALAYKQLTTEGELGANKINDLKRESVBADIHFAGFLVLQCPUREDAKQAV TRSGSRVLALASKSLPKMSQSKIDDLNRDDVBSBITFNGFLTHRCPLADALETI TRQGSRVLAMGIRKLGETRVGELRDKKRENFENDIAFAGFVVISCPLKADSAVI SREGARVLALGYKELGHLTHQQAREVKREALESSIKFVGFIVVSCPLKADSAVI AQRGFRLIRVASKAVHLN.FAKALKTPRDIMSSELEFLGLIVMENRLKDVISVI TAACYRVVALASKAVHLN.FAKALKTPRDIMSSELEFLGLIVMENLLKPTTFVI THAGYRVIALASKPLFVS.LEAAQQLTRDIVSGGLDFVGFIIFENKLKPTTTSVI THAGYRVIALASKFLPKTWLYSQKVSREEVBSNIEFLGFIIFQNKLKKETSTI	P domain RMINESSHRVVMITGDNPLT KMINESSHRSIMITGDNPLT REIMDSSHVVAMITGDNPLT REIQNASHVVMITGDNLLT QAIRRTRIRAVMVTGDNLLT QAIRRTRIRAVMVTGDNILT KSLQDANIRTIMCTGDNILT
P5A CtSpf1 ScSpf1 CATP-8 HSATP13A1 P5B CATP-5 HSATP13A2 CtYpk9 ScYpk9	669 629 632 679 685 681 889 985	N domain TRRGSRVLALAYKOLTTEGELGANKINDLKRESVEADTHFAGELVLQCPEREDAKOAV TRSGSRVLALASKSLEKMSQSKIDDLNRDDVESEITENGFDIFHELEKDDAIETI ROGSRVLAMGIRKUGETRVGELRDKRENPSDDJAFAGFVVISCPLKADSKAVI SREGARVLAUGYKELGHLTHQQAREVKREALECSIKEVGFIVVSCPLKADSKAVI AQRGFRLIAVNSKAVHIN.FKRALKTPRDIMSETEFLGLIVMENRLENDVISVT TAAGYRVVALASKPLPTVPSLEAAQQLTRDTVEGDISLLGLIVMENRLENDVISVT THAGYRVIACATKRIPKINLVSVNRMTRDEVEGGISLLGLIVMENRLENPTTPVI THAGYRVIACATKRIPKINLLYSQKVSREEVESNIEELGIIFENKKKFTSTI UHWEKEVETVDEDVILLDADEHSVYGEFSLVMESDVDEVEDDVDEVE DI	P domain RMINESSHRVVMITGDNPLT KMINESSHRSIMITGDNPLT REIMDSSHVVAMITGDNPLT NELSVANIRCVMVTGDNLLT QALRTRIRAVMVTGDNLOT KSLQDANIRTIMCTGDNILT
P5A CtSpf1 ScSpf1 CATP-8 HsATP13A1 P5B CATP-5 HsATP13A2 CtYpk9 ScYpk9 P5A CtSpf1 ScSpf1 CATP-8 HsATP13A1 P5B CATP-5 HsATP13A2 CtYpk9 ScYpk9	669 629 632 679 685 681 889 985 747 704 707 754 757 964 1060	N domain TRRGSRVLDLASKSLEKMSQSKIDDLNRDDVESEITENGFLIFHCLKDDAIETI ROGSRVLDASKSLEKMSQSKIDDLNRDDVESEITENGFLIFHCLKDDAIETI ROGSRVLMGIRKUGETVGELRDKRENFSDDAFAGFVVISCPLKSDTKATM SREGARVLAUGYKELGHLTHQQAREVKREALSCSLKEVGFIVVSCPLKADSKAVI AQRGFRILAVNSKAVHIN.FKRALKFPBDIMSETEFLGLIVMENRLEVDVISVT TAAGYRVVALASKPLPTVPSLEAAQQLTRDTVEGDISLIGLLVMENRLEVPTTPVI THAGYRVIACATKRIPKINLVSVNRMTRDEVSGCLDEVGTIFENKLKFTTISVI THNGYRVIACATKRIPKINLVSVNRMTRDEVSGCLDEVGTIFENKLKFTTISVI THNGYRVIACATKRIPKINLVSVNRMTRDEVSGCLDEVGTIFENKLKFTTISVI THNGYRVIACATKRIPKINLVSVNRMTRDEVSGCLDEVGTIFENKLK P domain AVHVAKEVEIVDRVLIDAPEHSVYGESLVWSNUCHIELGFIIFONKLK KETSETL AVHVAKEVEIVDRVLIDAPEHSVYGESLVWSNUCHIELVDPTKPI ACHVQLHFIKKSLFTLVLDEFADGVDMMKSVDGTIELPLKPETNNK ACHVQLHFIKKSLFTLVLDEFANGCEWRSUGSIVLPLARGSPK. MSVARECGIIRPTKKAFLITHSKTEKDPLGRTKLFKESVSSEND.IDTDNGV AVSVARCGGIIEEHAHCYMPRFIEGNAD.CNAK.LKWESINPFALE.LDPWTLLPMF	P domain RMINESSHRVVMITGDNPLT RMINESSHRSIMITGDNPLT REIMDSSHVVAMITGDNPLT REISVANIRCVMVTGDNLLT OALRRTRIRAVMVTGDNLLT KSLQDANIRTIAVMTGDNILT KSLQDANIRTIMCTGDNILT KSLQDANIRTIMCTGDNILT COMPARIAN FD. HSKLFDRYD ALALEYA SEVRAFDSHE ALALEYA SEVRAFDSHE VPPQTDASLPYDVSNIRNYA LGNNSV.
P5A CtSpf1 ScSpf1 CATP-8 HsATP13A1 P5B CATP-5 HsATP13A2 CtYpk9 ScYpk9 P5A CtSpf1 ScSpf1 CATP-8 HsATP13A2 CtYpk9 ScYpk9 P5B CATP-5 HsATP13A2 CtYpk9 ScYpk9	669 629 632 679 685 681 889 985 747 704 707 754 707 757 964 1060 806 763	N domain TRRGSRVLALAYKOLTTEGELGANKINDLKRESVBADTHFAGELVLQCPDREDAKQAV TRSGSRVLALASKSLPKMSQSKIDDLNDDVSGBTTFNGFLIFHCPRDDALAE TROGSRVLAUGYKELGHLTHQAREVKBEALBCSTKFVGFLVVSCPLKADSKAVI SREGARVLAUGYKELGHLTHQAREVKBEALBCSTKFVGFLVVSCPLKADSKAVI AQRGFRLIAVASKAVHLN.FAKALKTPDIMESBTEFLGLUVMENTLKPQTTVJ THAGYRVVALASKFLPTVPSLEAAQQITRDTVSGDTSLLGLUVMENTLKPQTTVJ THAGYRVVALASKFLPTVFSLEAAQQITRDTVSGTSLGLUVMENTLKFQTTVST THAGYRVVALASKFLPTVFSLEAAQQITRDTVSGTSLGLUVMENTLKFQTTVFJ THAGYRVVALASKFLPTVFSLEAAQQITRDTVSGTSLGLUVVGTITFENKLKPTTTSVI THAGYRVVACATKRIFKINLVSVNRMTRDEVEGGTDFVGTITFENKLKPTTTSVI THAGYRVVACATKRIFKINLVSVNRMTRDEVEGGTDFVGTITFENKLKPTTTSVI THAGYRVIACATKRIFKINLVSVNRMTRDEVEGGTDFVGTITFENKLKPTTSVI THAGYRVIACASKFLPKTWLYSQKVSBEVSSNLEFLGTITFENKLKPTTSVI AVFVAKEVGIVFGETTILDAPEHSDDVQLFRDVETVSIPFDFSKDT. ACHVAQELHFIKAFLINLPPSEKGRQCEWRSUDGSIVLPLARGSPK. AMSVARECGIIRPTKKAFLITHSKTEKDPLGRTKLFIKESVSSEND.IDTD AVTVARGCGUVAPQEHLIVHATHPE.RGQPASLEFLPMESPTAVNVFK AVSVARQCGIIRPTKKAFLITHSKTEKDPLGRTKLFIKESVSSEND.IDTD AVTVARGCGIIRPTKKAFLINDAD.DCNAK.ERWESINPALE.LDPWTLLFMF AISVGREAGLIQCS.RVVVPSIND.TPL.HGEPV.IVWRDVNEPKI.LDTKTLKPVE Pdomain LCVTGYALNAKFKGQVGWKSLLRYTWVYARVSESKOREDILLGLKDMGYYTLMA	P domain RMINESSHRVVMITGDNPLT RMINESSHRVVMITGDNPLT REIMDSSHVAMITGDNPLT REIMDSSHVAMITGDNPLT REIQNASHRVVMITGDNILT QALRRTRIRAVMVTGDNILT KELLSSNIGTVMITGDNILT KSLQDANIRTIMCTGDNILT
P5A CtSpf1 ScSpf1 CATP-8 HsATP13A1 P5B CATP-5 HsATP13A2 CtYpk9 ScYpk9 P5A CtSpf1 ScSpf1 CATP-6 HsATP13A2 CtYpk9 ScYpk9 P5A CtSpf1 ScSpf1 CATP-8 HsATP13A2 CtYpk9 ScYpk9 P5A CtSpf1 ScSpf1 CATP-8 HsATP13A1 P5B CATP-5 HsATP13A2	669 629 632 679 685 889 985 747 704 704 707 754 757 964 1060 806 763 767 809 828 823 1039 1128	N domain TRRGSRVLDLAYKOLTTEGELGANKINDLKRESVBADTHFAGELVLQCPDREDAKQAV TRSGSRVLDLASKSLPKMSQSKIDDLNDDVSBLTFNGFLIFHCLEDDALETI ROGSRVLDMGTRKLGELTVGELDKRENFENDLAFAGFVVISCPLKSDTKATM SREGARVLDMGTKKLGELTVGELDKRENFENDLAFAGFVVISCPLKADSKAVI AQRGFRLIDVASKAVHLN.FAKALKTPDIMESETEFLGLIVMENRLKDVTLSVI THAGYRVNLASKPLFTVSLEAAQQITRDTVSGDISLLGLLVMRNLLKPQTTVJI THAGYRVINCATKRIFKINLVSVNRMTRDEVEGGTDFVGFIIFENKLKPTTTSVI THAGYRVINCATKRIFKINLVSVNRMTRDEVEGGTDFVGFIIFENKLKPTTTSVI THAGYRVINCATKRIFKINLVSVNRMTRDEVEGGTDFVGFIIFENKLKPTTTSVI THAGYRVINCATKRIFKINLVSVNRMTRDEVEGGIDFVGFIIFENKLKPTTSVI THAGYRVINCATKRIFKINLVSVNRMTRDEVEGGIDFVGFIIFENKLK AVTVAKEVGIVFGETLILDAPEHSDUNQLIFRDVETVSIPFDPSKDT. ACHVAQELHFIKKSLFILVLDEPADGVDMMKSVDGTIELPLPETNNK ACHVAQELHFIKKSLFILVLDEPADGVDMMKSVDGTIELPLKPET.NNF ACHVAQELHFIKKSLFILVLDEPANGCPASLEFLDSIDGSIVLPLARGSPK. MSVARECGIIRPTKKAFLITHSKTEKDPLGRTKLFIKESVSSSEND.IDTD AVTVARGCGMVAPQEHLIVHAT.HPE.RGQPASLEFLPMESTAVNGVK AVSVARQCGIIEEHAHCYMPRFIEGNAD.DCNAK.LRWESINNFALE.LDFWTLLFMF ATSVGREAGLIQCS.RVYVPSIND.TPL.HGEPV.IVWRDVNEPDKI.LDTKTLKPVK Pdomain CVTGYALNALEGQVGWKSLENTWVYANVSSKCEFLLNTLKDMGYYTLMA AVSVARQCGILGELAGGVOVVSIND.TPL.HGEPV.IVWRDVNEPDKI.LDTKTLKPVK Pdomain CVTGYALNALEGQVGWKSLENTWVYANVSSKCEFLLNTLKDMGYYTLMA AVSVARQCGILEEHARCYMPRFIEGNAD.DCNAK.LRWESINNFALE.LDFWTLLFMF AISVGREAGLIQCS.RVYVPSIND.TPL.HGEVV.IVWRDVNEPDKI.LDTKTLKPVK Pdomain CVTGYALNALEGQVGWKSLENTWVYANVSSKCEFLLNTLKDMGYYTLMA ANSVARQCGILQCS.RVVVPSIND.TPL.HGEVV.IVWRDVNEPDKI.LDTKTLKPVK PLODELKLLPVVVYANNAPOKKOEFILMELKLSUKVCC CLTSGALHLVHFFRLLPKVLVQGTVFARMAPEOKREVITSLKELGKVTLMC CLTSGULHUKFYPELVDRITAMCDVYARMAPEOKKEFILMELKSLGKVTLMC CLTSGULHUKFYPELVDRITAMCDVYARMAPEOKREVITSLKELGKVTLMC CLTSGULHUKFYPELVDRITAMCDVYARMAPEOKREVITSLKELGKVTLMC CLTSGULHUKFYPELVDRITAMCDVYARMAPEOKREVITSLKELGKVTLMC CLTSGULHUKFYPELVDRITAMCDVYARMAPEOKREVITSLKELGKVTLMC CLTSGULHUKFYPELVDRITAMCDVYARMAPEOKREVITSLKELGKVTLMC CLTSGULHUKFYPELVDRITAMCDVYARMAPEOKREVITSLKELGKVTLMCC	P domain RMLNESSHRVVMITGDNPLT RMLNESSHRVVMITGDNPLT REIMDSSHVAMITGDNPLT REIQNASHVVMITGDNPLT REIQNASHVVMITGDNULT QALRRTRIRAVVMTGDNILT KELLSSNIGTVMITGDNILT KSLQDANIRT MCTGDNILT KSLQDANIRT MCTGDNILT COMPANY KSLQDANIRT AMCTGDNILT COMPANY KSLQDANIRT AMCTGDNILT COMPANY COMPANY COMPANY COMPANY COMPANY COMPANY COMPANY CALKQAHVGIAL GDGTNDVGALKQAHVGIAL GDGTNDVGALKANVGVALL GDGTNDVGALKANVGVALL GDGTNDVGALKANVGISLS GDGANDCAALKAANVGISLS GDGANDCAALKAANVGISLS GDGANDCAALKAANVGISLS
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		TM5	TM6	
P5A CtSpf1 ScSpf1 CATP-8 HsATP13A1 P5B CATP-5 HsATP13A2 CtYpk9 ScYpk9	1034 977 954 973 924 919 1136 1228	I LRQGRCTDVATIQMYKILALNCLISAYSLSVLY I IRQGRCADVNTIQMYKILALNCLISAYSLSVLY VIKQGRCTDVTLQMYKILALNALVSYSLSXIY VIKQGRCTDVTTLQMYKILALNALVSYSLSAX VIKEGRCADVTSYAVSKYMAAYSLNEFLSVMLY VIREGRCADVTSYAVSKYMAAYSLNEFLSVMLY VIREGRASDVTSFSCFKYMSLYSFIQFTSVSFLY VIREGRAALVTSFACFQYMSLYSAIQFITITIL	LEGIKFGDCOITISGML.MSVCFLSISRA MACVKFGDCOATVSGLL.LSVCFLSISRG LDGVKFSDTOATIGGLL.LACCFLFISRS LEGVKFSDTOATLQGLL.LACCFLFISRS NDGTNISDCFLYIDLVITLVALFLGNT TINTNLGDIGFLAIDLVITTVAVLMSRT VSASNLGDTOFLYIDLMIILPIAVEMSWA SRGSNLGDTOFLYIDLLLIVPIAICMSWS	RSVEGISKERPOPNI KPLEKLSKORPOSGI KPLKLISKORPMANI KPLKTLSRERPLPNI EASRKLISGIPPPRL GPALVLGKVRPPGAL GPHLVLCAKRPVSDL KSYEKIDKKRPSANL R
		TM7	. тм	8
P5A CtSpf1 ScSpf1 CATP-8 HsATP13A1 P5B CATP-5 HsATP13A2 CtYpk9 ScYpk9	1111 1054 1031 1050 1002 997 1214 1306	FNFYIIGSILGQFAVHVATLI. YIAQLCDQIEF FNYYIMGSILSQFAVHTATLV. YITTEIYKLEF FNAYTLITVTLQFIVHFSCL. YIVGLAHEAN. FNLYTILTVMLQFFVHFLSLV. YLYREAQARSF ATSAFYFSVFGQMFFNIIQTTGYLLVRGQS LSVPVLSSLLLQMVLVTGVQLGGYFLTLAQF VSRKVLVPLLSHVFVCVMLQALAWVAVRQQF VSRKVLVPLLSVFLVFLFQFIPMIIVQKMS	PRTEVID.LEAEFKPSLINSAVYLLQII REPQVD.LEKEFAPSLLNTGIFIQLV TEKAPVD.LEAKFTPNILNTVYIISMA PEKQEQFVD.LYKEFEPSLVNSTVYIMAMA WYVPNPEELDNTTTMIGTVFFTSCC WFVPLNTVAAPDNLPNYENTVVFSLSSF PWYIPPIVD.TEKSNIENSENTLFFASCF WYIKPIVG.GDD.AVQSSDNTVLFFVSNF	QQISTFAVNYQGRPF QQVSTFAVNYQGEPF LQVCTFAVNYRGRPF MQMATFAINYKGPPF MYLGYAFVYSKGHPY QYLILAAVSKGAPF EYILSGVVLNAGRPF QYILTAIVLSVGPPY
		TM9	TM10	
P5A CtSpf1 ScSpf1 CATP-8 HSATP13A1 P5B CATP-5 HSATP13A2 CtYpk9 ScYpk9	1184 1127 1104 1125 1074 1072 1288 1379	RESLSENKGMFYGIVGVTAIAFACSTEMLPEL.N RENIRSNKGMYYGLLGVTGLALASATEFLPEL.N MESLFENKAMLYSIMFSGGAVFTLASGQATDL.M MESLPENKFLVWSLAVSLLAIIGLLGSSPPF.N RRSVFTNWLLGGIFVIGAINMVMTFTNMGFL.M RRPLYTNVFLVALALLSSVUGUV.LVPGLQG RQSPLETWPFLSAVAVTLIATLLMLLV.PPYWLF REPMSKNFEFIVDITVSIGASLLMTLDTESYLG	NEAMKLVPFNENFKTIMTTV NEAMKFVPMTDDFKIKLTIT. 41QFELVVIPEALRNALLMC. 52GFGLVDIPVEFKLVIAQV NIMGFVYVPSTSMRFILLAISLA FPLALRNTVDTGFKLLLGL. PEFMQLTWMSWIFKITLIAFGFVYFLIAW. SKMLQLTPISNSFTMFIIVW.	MIIDFVACY LLLDFFGSW VTADLVICY LLLDFCLAL GVFLSLLYEHFFVEK VTLNFVGAF TGEHYL. VILNYY
		TM10	C-terminus Helix	
P5A CtSpf1 scSpf1 CATP-8 HSATP13A1 P5B CATP-5 HSATP13A2 CtWpk9	1245 1188 1165 1186 1145 1133	VIEWVLKKLFSD LRARDIAERRPDQLEREF GVEHFFKFFFMDDKPSDISVQQVKIASK IDRGLNFLLGDMF LADRVLQFFLGTPKLKVPS VVAIHFESYLRQ.RRLRNGDPSLSAYEKILAAI MLESVLDQCLPACLRRLRPKRASKKRFKQLERE.	NYRKEKEAREKEEEEERKERERIEAFERRL (GSSPRWFEDEINLSKSIDRKETIESKC LAEQPWPPL.PAGPLR.	EEKRTRLVEAAAQRE
ScYpk9	1438	AQLYIPPSIKGWLKKKKSSKKYKLLIQE.	EMKLKEV	
P5A CtSpf1 ScSpf1 CATP-8 HsATP13A1	1319	00000WAQRR		
P5B CATP-5 HsATP13A2 CtYpk9 ScYpk9		· · · · · · · · · · · · · · · · · · ·		

Supplementary Fig. 1. Structure-based multiple sequence alignment of selected P5A and P5B ATPases. The following sequences are included in the alignment (Uniprot ID in brackets) P5A: CtSpf1 (G0S4Z4), ScSpf1 (P39986), CATP-8 (P90747), HsATP13A1 (Q9HD20); P5B: CATP-5 (Q21286), HsATP13A2 (Q9NQ11), CtYpk9 (G0S7G9), ScYpk9 (Q12697). The alignment is annotated as follows: Purple line, NTD-domain (the soluble part). Yellow line, A-domain. Blue line, P-domain. Red line, N-domain. Gray cylinders, helices Ma and Mb. Orange cylinders, M1 and M2. Wheat cylinders, M3 to M10. Dark gray cylinder, C-terminal helix. Yellow box, conserved dephosphorylation loop (SGE). Blue box, conserved phosphorylation motif (DKTGT). Green box, Plug-domain. Wheat box, P5A conserved motif (PPELPM/IE) of M4. Positively charged residues (circled in blue), may contribute to cargo transport. Sequence alignments were performed using Cluster Omega (online) and visualized using ESPript 3.0 (online).



Supplementary Fig. 2. Functional characterization of CtSpf1. a Localization of CtSpf1 was investigated using fluorescence microscopy, comparing GFP-fluorescence (left), differential interference contrast (DIC) (middle) and merged (right), the scale bar represents 5 µm. Imaging experiments were conducted for three separate clones of each yeast strain. **b** Comparison of the growth on YPdG-agar plates of *S. cerevisiae* cells with wild-type CtSpf1-GFP, inactive CtSpf1-GFP (the D499N mutant), wild-type ScSpf1-GFP and inactive ScSpf1-GFP (the D487N mutant equivalent to the D499N mutant in CtSpf1) in the absence (left) or presence of caffeine (7.5 mM, right). GFP only constructs represent controls using the same plasmid as used for the P5A-ATPase forms. The experiment were performed at 30 °C in wild-type (WT, first row) and ScSpf1-deleted (all rows except for the first) BY4741 yeast strains. The growth assay was performed three times independently with similar results.



Supplementary Fig. 3. Cryo-EM data processing for the E2P state stabilized using BeF₃⁻. **a** Representative motion corrected micrographs (4,777 micrographs were selected for further processing) and processing workflow. **b** Selected 2D classes with different views. **c** Gold standard Fourier shell correlation curves calculated for the reconstructed map based on a FSC 0.143 cut-off. **d** Particle orientation distributions of the final reconstruction. **e** Local resolution map based on a FSC 0.143 cut-off.



Supplementary Fig. 4. Structural comparisons of the E2P (BeF3⁻-stabilized) state of the P5A-ATPase CtSpf1 with the corresponding conformations of ScSpf1 (another P5A-ATPase), a P2-ATPase, a P4-ATPase, and a P5B-ATPase. a Alignment of CtSpf1 (blue) with ScSpf1 (grey, PDB-ID 6XMT) with an overall RMSD of 2.8 Å. The different arrangement of the invariant P5A-motif PPELPM/IE of M4, which adopts a more peripheral location in ScSpf1 is shown in the close-view to the right. b Alignment of CtSpf1 (blue) with the Ca²⁺-ATPase SERCA (grey, PDB-ID 3N5K) with overall RMSD 4.4 Å. **c** Alignment of CtSpf1 (blue) with ATP8A1-Cdc50a (wheat, PDB-ID 6K7L) with overall RMSD 6.0 Å. **d** Alignment of CtSpf1 (blue) with ATP13A2 (salmon, PDB-ID 7VPK) with overall RMSD 3.4 Å. **e** Alignment of CtSpf1 (blue) with CtYpk9 (pink, PDB-ID 7OP3) with overall RMSD 3.9 Å.



Supplementary Fig. 5. Structural comparisons of different states of CtSpf1. a The phosphorylation sites in each state. AMPPNP and ADP are shown as sticks. AlF₄⁻ (grey) and BeF₃⁻ (grey), Mg²⁺ (green) and H₂O (red) are depicted as spheres. Non-protein cryo-EM density is shown in grey. **b** Map and model of the phosphorylation sites in each state as in panel a but with cryo-EM density also for the surrounding region. **c** Structural comparison of the E2P and E2.P_i states, revealing movement of the N-domain while the membrane spanning M-domains are more similar. **d** Structural comparison of the E1 (apo) and E2P states. The close-views display the movement of the transmembrane segments, as aligned on the P-domain and M5-M10. The transport pathway, that is partially lined by M1-M6, deviates substantially between the E1 (with a cytosol-facing cavity) and E2P (the all-through the membrane cleft) state. **e** Comparison of the E1P, E1P-ADP and E1P states.



Supplementary Fig. 6. The P-domain insertion (Plug-domain) in the different structurally determined states. The Plug-domain is highlighted with black arrows. The C-terminal part of the Plug-domain is marked with a red box, as also pinpointed with red arrows. **a** The E1 (apo) state with two reconstructed maps, determined at 3.4 Å (grey) and 3.5 Å (yellow), see Supplementary Fig. 7. **b** The E1-ATP state. **c** Maps of the E1P-ADP state with the feature in the membrane (grey) and without membrane-feature map (yellow), see Supplementary Fig. 11. **d** The E1P state. **e** The E2P state. **f** The E2.P_i state. **g** Modelled CtSpf1 structures (E1, E1P and E2P in cyan) with non-sharpened cryo-EM maps (grey and green) and with the C-termini of the Plug-domain is highlighted in green.



Supplementary Fig. 7. Cryo-EM data processing for the E1P and E2.P_i **states stabilized using AIF**₄**. a** Representative motion corrected micrographs (6,451 micrographs were selected for further processing) and processing workflow. **b** Selected 2D classes with different views. **c** E1P: Gold standard Fourier shell correlation curves calculated for the reconstructed map based on a FSC 0.143 cut-off (left), particle orientation distribution of the final reconstruction (middle), and local resolution map (FSC 0.143) of the reconstructed map based on a FSC 0.143 cut-off (left), particle orientation distribution of the reconstructed map based on a FSC 0.143 cut-off (left), of the reconstructed map (right). **d** E2.P₁^{cargo}: Gold standard Fourier shell correlation curves calculated for the reconstructed map based on a FSC 0.143 cut-off (left), particle orientation distribution of the final reconstruction (middle), and local resolution map (FSC 0.143) of the reconstructed map based on a FSC 0.143 cut-off (left), particle orientation distribution of the final reconstruction (middle), and local resolution for the reconstructed map based on a FSC 0.143 cut-off (left), particle orientation distribution of the final reconstruction (middle), and local resolution map (FSC 0.143) of the reconstructed map (right).



Supplementary Fig. 8. Cryo-EM data processing for E1 (apo). a Representative motion corrected micrographs (4,081) micrographs were selected for further processing) and processing workflow. **b** Selected 2D classes with different views. **c** map1: Gold standard Fourier shell correlation curves calculated for the reconstructed map based on an FSC 0.143 cut-off (left), particle orientation distribution of the final reconstruction (middle), and local resolution map (FSC 0.143) for the reconstructed map based on an FSC 0.143 cut-off (left), particle orientation distribution of the reconstructed map based on an FSC 0.143 cut-off (left), particle orientation distribution of the reconstructed map (right). **d** map2: Gold standard Fourier shell correlation curves calculated for the reconstructed map based on an FSC 0.143 cut-off (left), particle orientation distribution of the final reconstruction (middle), and local resolution map (FSC 0.143) for the reconstructed map (right).



Supplementary Fig. 9. Cryo-EM density and associated model of representative parts of the E1 (apo) state. The transmembrane helices are shown in cartoon with stick sidechains. Soluble domains are shown in cartoon and the densities are show as grey mesh.



Supplementary Fig. 10. Structural comparisons of the E1 (apo) state of the P5A-ATPase CtSpf1 with relevant conformations of a P2-ATPase, a P4-ATPase, another P5A-ATPase (ScSpf1) and a P5B-ATPase. a Alignment of CtSpf1 (blue) with the Ca²⁺-ATPase SERCA (grey, PDB-ID 4H1W). b Alignment of CtSpf1 (blue) with Ca²⁺-ATPase (magenta, PDB-ID 3N8G). c Alignment of CtSpf1 (blue) with ATP8A1-Cdc50a (wheat, PDB-ID 6K7G). d Alignment of CtSpf1 (blue) with ScSpf1-Apo (limon, PDB-ID 6XMP). e Alignment of CtSpf1 (blue) with ATP13A2 (salmon, PDB-ID 7N75).



Supplementary Fig. 11. Cryo-EM data processing for the E1-ATP state stabilized by AMP-PNP. a Representative motion corrected micrographs (3,231 micrographs were selected for further processing) and processing workflow. **b** Selected 2D classes with different views. **c** Gold standard Fourier shell correlation curve calculated for the reconstructed map based on a FSC 0.143 cut-off. **d** Particle orientation distribution of the final reconstruction. **e** Local resolution map based on a FSC 0.143 cut-off.



Supplementary Fig. 12. Cryo-EM data processing for the E1P-ADP and E1P-ADP ADP^{membranous-feature} **states stabilized by AIF**⁴ **and ADP. a** Representative motion corrected micrographs (7,096 micrographs were selected for further processing) and processing workflow. **b** Selected 2D classes with different views. **c** E1P-ADP^{membranous-feature}: Gold standard Fourier shell correlation curve calculated for the reconstructed map based on an FSC 0.143 cutoff (left), particle orientation distributions of the final reconstruction (middle) and local resolution map for the reconstructed map (FSC 0.143) with additional unassigned cryo-EM density (right). **d** E1P-ADP: Gold standard Fourier shell correlation curve calculated for the reconstructed map based on an FSC 0.143 cut-off (left), particle orientation distributions of the final reconstruction (middle) and local resolution map for the reconstructed map (FSC 0.143) without additional unassigned cryo-EM density (right).



Supplementary Fig. 13. Distance difference matrices comparing the positions of TM helices across the different determined CtSpf1 structures. The analysis was conducted as previously reported, using overall alignments ¹⁻³. Each individual matrix shows relative differences in position of helices Ma, Mb, M1, M2, M3, etcetera from left to right and top to bottom. When comparing E1 states with E2P states the helices move as two blocks: Ma-M2 and M4-M10, with M3 remaining relatively stationary.



Supplementary Fig. 14. Identified cryo-EM densities that may represent lipids. Closeviews of complementary non ATPase cryo-EM features. The blue densities close to the cytosol are present in all states. The yellow features are present in all E1, but not in the E2 states recovered here.



Supplementary Fig. 15. Unexplained cryo-EM density (blue) in E2.Pi structure.

Supplementary Table 1

Spf1		PDB ID	6XMP	6XMQ	6XMS	6XMT	6XMU
			Spfl Apo	Spfl (AMP- PCP)	Spfl (AlF ₄ ⁻)	Spfl (BeF ₃ ⁻)	Spf1 (BeF ₃ ⁻) Endogenous substrate bound
Аро		6XMP	0.0	0.7	0.5	6.6	6.7
AMP-PCP bo	und	6XMQ	0.7	0.0	0.6	6.7	6.8
AlF4 ⁻ bound		6XMS	0.5	0.6	0.0	6.5	6.6
BeF ₃ ⁻ bound		6XMT	6.6	6.7	6.5	0.0	0.4
BeF ₃ ⁻ and		6XMU	6.7	6.8	6.6	0.4	0.0
Endogenous s bound	ubstrate						
CtSpf1							
Аро		80P3	1.8	1.9	1.8	7.0	7.1
E1-ATP		80P4	2.1	2.0	1.7	6.8	6.9
E1P:ADP		80P5	1.6	1.4	1.2	6.6	6.7
E1P (AlF ₄ -)		80P6	1.7	1.4	1.4	6.4	6.5
E2-P (BeF ₃ ⁻)		80P7	6.5	6.7	6.6	2.8	2.7
E2:Pi (AlF ₄ ⁻)		80P8	6.2	6.4	6.3	4.2	4.2
						I	
CtSpf1	PDB ID	80P3	80P4	80P5	80P6	80P7	80P8
		Apo	E1-ATP	E1P:ADP	E1P (AlF ₄ -)	E2-P (BeF $_3$ -)	E2:Pi (AlF ₄ -)
Аро	80P3	0.0	2.5	2.0	1.6	6.5	6.5
E1-ATP	80P4	2.5	0.0	1.3	1.6	6.5	6.6
E1P:ADP	80P5	2.0	1.3	0.0	0.6	6.7	6.7
E1P (AlF ₄ ⁻)	80P6	1.6	1.6	0.6	0.0	6.6	6.7
E2-P (BeF ₃ -)	80P7	6.5	6.5	6.7	6.6	0.0	0.7
E2:Pi (AlF ₄ -)	8 O P8	6.5	6.6	6.7	6.7	0.7	0.0

Supplementary Table 1. Overall structural alignments of previously available (of CsSpf1) and here determined (CtSpf1) structures of P5A-ATPases. RMSD (Å) values are indicated. Homologous structures (RMSD < 1 Å) are highlighted in grey and blue, respectively. The most similar structures between CsSpf1 and CtSpf1 are indicated in green.

Supplementary Table 2

	E1 (Apo)	E1-ATP	E1P-	E1Pcytosolic-feature	E2P ^{cargo}	E2.P. cargo
		(AMPPNP)	ADP ^{membranous-}	(AlF_4)	(BeF3 ⁻)	(AlF ₄ ⁻)
	(EMDB-17039)	(EMDB-17040)	feature	(EMDB-17042)	(EMDB-17043)	(EMDB-17044)
	(PDB-8OP3)	(PDB-80P4)	(ADP-AlF4 ⁻)	(PDB-80P6)	(PDB-80P7)	(PDB-8OP8)
			(EMDB-17041)			
			(PDB-80P5)			
Data collection and						
processing						
Magnification	105,000	105,000	105,000	105,000	105,000	105,000
Voltage (kV)	300	300	300	300	300	300
Total exposure (e-/Å2)	50	40	50	50.2	50	50.2
Defocus range (µm)	-1.0 to -2.5	-1.2 to -2.6	-0.7 to -2.7	-0.5 to -2.75	-1.0 to -2.5	-0.5 to -2.75
Pixel size (Å)	1.1	0.832	0.846	0.8464	1.1	0.8464
Symmetry imposed	C1	C1	C1	C1	C1	C1
Initial particle (no.)	648,009	182,187	449,894	495,256	763,922	495,256
Final particle (no.)	196,512	115,476	151,338	107,529	249,499	185,340
Map resolution (Å)	3.5	3.2	3.4	3.7	3.5	3.4
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143
Refinement						
Model resolution (Å)	3.6	3.3	3.6	3.9	3.7	3.6
FSC threshold	0.5	0.5	0.5	0.5	0.5	0.5
Map sharpening B factor (Å ²)	-175.3	-80	-175.3	-130	-120	-130
Model composition						
Non-hydrogen atoms	8,888	8,879	9,007	8,971	9,166	9269
Protein residues	1,127	1,123	1140	1,138	1,162	1186
Ligands		MG: 1; ANP: 1	MG: 2; ALF: 1;	MG: 1; ALF: 1	MG: 1; BEF: 1	MG: 1; ALF: 1;
			ADP: 1			CA: 1
B factors (Å ²)						
Protein	62.35	85.23	92.76	89.54	113.16	131.06
Ligand		71.04	76.44	66.24	85.13	103.81
R.m.s. deviations						
Bond lengths (Å)	0.002	0.003	0.003	0.002	0.003	0.003
Bond angles (°)	0.527	0.726	0.578	0.572	0.605	0.532
Validation						
MolProbity score	1.2	1.36	1.05	1.44	1.44	1.38
Clashscore	2.78	5.02	2.59	4.86	4.27	3.53
Poor rotamers (%)	0	0.21	0.1	0	0.20	0
Ramachandran plot						
Favored (%)	97.3	97.57	97.96	96.89	96.45	96.43
Allowed (%)	2.70	2.43	2.04	3.11	3.55	3.57
Disallowed (%)	0.00	0.00	0.00	0.00	0.00	0.00

Supplementary Table 2. Cryo-EM data collection, refinement and validation statistics

1 Supplementary Table 3

	UniProt	Present in	Protein name	Nr of peptide hits	Unique peptides	Cellular location	Helix type/number of helices	Location of N- terminus	Location of C- terminus	Location of majority of protein
	P16547	More in SpfBef	Mitochondrial outer membrane protein OM45	40	40	Mitochondrial outer membrane	N-terminal helix	Mitochondrial intermembrane space	Cytoplasm	Cytoplasm
	P27614	More in SpfBef	Carboxypeptidase S	28	28	Vacuole	N-terminal helix	Cytoplasm	Lumen	Lumen
	Q08179	SpfBef only	Mitochondrial distribution and morphology protein 38	15	15	Mitochondrial inner membrane	Helix at residues 139- 159	Mitochonrial intermembrane space	Mitochondrial matrix	Mitochondrial matrix
	P40557	SpfBef only	ER-retained PMA1-suppressing protein 1	13	13	ER	C-terminal helix	Lumen	Cytoplasm	Lumen
	P10614	SpfBef only	Lanosterol 14-alpha demethylase CYP51	7	7	ER	N-terminal helix	ER lumen	Cytoplasm	Cytoplasm
TM proteins	P39006	SpfBef only	Phosphatidylserine decarboxylase proenzyme 1	7	7	ER & Mitochondrial inner membrane	Helix at residues 80-98	Mitochondrial matrix	Mitochondrial intermembrane space	Mitochondrial intermembrane space
	P46982	SpfBef only	Alpha-1,2-mannosyltransferase MNN5	7	7	Golgi	N-terminal signal peptide helix	Cytoplasm?	Lumen?	Lumen?
	P29704	SpfBef only	Squalene synthase	5	5	ER & Microsome	C-terminal TA-helix	Cytoplasm	Lumen	Cytoplasm
	P38069	SpfBef only	Alpha-1,2-mannosyltransferase MNN2	5	5	Golgi	N-terminal helix (type II)	Cytoplasm	Lumen	Lumen
-	P38264	SpfBef only	SRP-independent targeting protein 3	5	5	ER & Mitochondria	N-terminal helix	Lumen	Cytoplasm	Cytoplasm
	P37299	SpfBef only	Cytochrome b-c1 complex subunit 10	4	4	Mitochondrial inner membrane	Helix at residues 22-45 out of 77	Mitochondrial matrix	Mitochondrial intermembrane space	Equal
	P38212	SpfBef only	Protein RCR1	4	4	ER	N-terminal helix	Lumen	Cytoplasm	Cytoplasm
	P38736	SpfBef only	Golgi SNAP receptor complex member 1	4	4	Golgi	C-terminal TA-helix	Cytoplasm	Lumen	Cytoplasm
	P47124	SpfBef only	Putative glycosyltransferase HOC1	4	4	Golgi	N-terminal helix (type II)	Cytoplasm	Lumen	Lumen
	P31755	SpfBef only	Initiation-specific alpha-1,6- mannosyltransferase	3	3	ER & Golgi	N-terminal helix (type II)	Cytoplasm	Lumen	Lumen
	P38288	SpfBef only	Protein TOS1	3	3	Secreted	N-terminal signal peptide helix	Cytoplasm	Extracellular space	Extracellular space

	P25297	More in SpfBef	Inorganic phosphate transporter PHO84	24	24	Membrane	12	Cytoplasm	Cytoplasm
	Q03648	More in SpfBef	Uncharacterized protein YMR209C	24	24	Membrane	2	Unknown	Unknown
	P40098	More in SpfBef	Uncharacterized mitochondrial membrane protein FMP10	16	16	Mitochondria	2	Unknown	Unknown
	P32784	SpfBef only	Glycerol-3-phosphate O-acyltransferase 1	10	10	ER	5	Lumen	Cytoplasm
	P38325	More in SpfBef	Mitochondrial outer membrane protein OM14	8	8	Mitochondrial outer membrane	2	Unknown	Unknown
	P39952	SpfBef only	Mitochondrial inner membrane protein OXA1	8	8	Mitochondrial inner membrane	5	Mitochondrial intermembrane space	Mitochondrial matrix
	Q06169	SpfBef only	Peroxisomal membrane protein PEX30	8	8	Peroxisome	4	Unknown	Unknown
eins	P32476	SpfBef only	Squalene monooxygenase	7	7	ER & Microsome	2	Cytoplasm	Cytoplasm
TM prote	Q12029	SpfBef only	Probable mitochondrial transport protein FSF1	7	7	Mitochondria	4	Unknown	Unknown
Multiple	P33310	SpfBef only	ATP-dependent permease MDL1	7	6	Mitochondrial inner membrane	5	Unknown	Unknown
~	P52867	SpfBef only	Dolichyl-phosphate-mannoseprotein mannosyltransferase 5	6	6	ER	10	Lumen	Lumen
	Q99297	SpfBef only	Mitochondrial 2-oxodicarboxylate carrier 2	6	6	Mitochondrial inner membrane	6	Unknown	Unknown
	P47190	SpfBef only	Dolichyl-phosphate-mannoseprotein mannosyltransferase 3	6	4	ER	9	Cytoplasm	Lumen
	P32564	SpfBef only	Protein SCM4	5	5	Membrane	4	Unknown	Unknown
	P47818	SpfBef only	Protein CCC1	5	5	Golgi & Vacuole	5	Cytoplasm	Lumen
	P53337	SpfBef only	ER-derived vesicles protein ERV29	5	5	ER	4	Cytoplasm	Cytoplasm
	Q03713	SpfBef only	Respiratory supercomplex factor 1	5	5	Mitochondria	5	Mitochondrial intermembrane space	Mitochondrial matrix
	P31382	SpfBef only	Dolichyl-phosphate-mannoseprotein mannosyltransferase 2	5	3	ER	9	Cytoplasm	Lumen

P36051	SpfBef only	GPI ethanolamine phosphate transferase 1	4	4	ER, Golgi & Vacuole	15	Cytoplasm	Lumen
Q12144	SpfBef only	Pore and endoplasmic reticulum protein of 33 kDa	4	4	ER & Nucleus	6	Cytoplasm	Cytoplasm
P19145	SpfBef only	General amino-acid permease GAP1	3	3	Cell membrane & ER	12	Cytoplasm	Cytoplasm
P25560	SpfBef only	Protein RER1	3	3	Golgi	2	Cytoplasm	Cytoplasm

2 Supplementary Table 3. List of proteins that were either identified exclusively in CtSpf1/BeF3-, or among the 20 most overrepresented in

3 CtSpf1/BeF₃⁻ relative to the GFP/BeF₃⁻ control, in the MS analysis. The table has been divided into proteins with 1 vs multiple TM helices and

4 sorted by number of peptide hits.

5 Supplementary References

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