

Supporting Information

A Structural Model for Glutathione-Complexed Iron-Sulfur Cluster as a Substrate for ABCB7-Type Transporters

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Experimental Methods

Molecular cloning, expression and purification of recombinant yeast Atm1p.

Yeast Atm1p was cloned into pASK-IBA2 vector, which has an OmpA signal peptide directing the overexpressed protein to the periplasmic space,¹ and a Strep-Tag II sequence (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) on the C-terminus that is used for purification purposes.¹ The pET21b-Atm1p plasmid was used as the template and 100 ng DNA, 100 ng of each primer, 2.5 units of cloned PfuTurbo DNA polymerase, PfuTurbo buffer, and 0.2 mM of each dNTP were used to amplify yeast Atm1p via PCR. Primers were designed as follows: 5' - ATGGTAGGTCTCA↓GGCCTCGCCCGTTATATTTACTGTGTCT-3' and 5'-ATGGTAGGTCTCA↓GCGCTTAGTCTTGCTGGTCTTTTAGTTC A-3', where the red regions denote the BsaI restriction site and the arrows indicate the BsaI cleavage site. The thermocycle used was identical to that described in the PfuTurbo DNA polymerase manual (Stratagene). PCR products were digested with BsaI and ligated to BsaI-digested vector pASK-IBA2 by use of T4 DNA ligase. Cloning into pASK-IBA2 resulted in the production of the gene for C-terminal Strep-tagged yeast Atm1p. Cloning results were confirmed by nucleotide sequencing, and *E. coli* Rosseta(DE3) was used for protein expression. A 10 ml Luria-Bertani (LB) culture with 100 mg/L ampicillin was grown overnight at 37 °C as a starter culture, which was then used to inoculate 1 L LB medium and grown to an OD₅₅₀ of ~ 0.6 prior to induction with 20 μg/L anhydrotetracycline. Cells were pelleted after overnight induction at 30 °C and stored at -80 °C for future use.

The R284E point substituted derivative of Atm1p was obtained by use of standard site-directed mutagenesis protocols. All reagents were purchased from New England BioLabs Inc. In brief, mutation primers were obtained from IDT DNA Inc. and are: TGGAGGACACATTTTGAAAGGGATGCTAACAAGGC, and TTGTTAGCATCCC TTTCAAATGTGTCCTCCA. Experimental conditions such as concentration, volume and PCR time segments are listed in Tables S1 and S2, respectively. Transformation of the resulting mutant plasmid and the subsequent protein purification were performed with the same protocols as described for the wild-type above.

Cloning of yeast Atm1p yields a construct of pASK-IBA2-Atm1p that has an N-terminal OmpA signal peptide and a C-terminal Strep-Tag II sequence (Figure S1). The N-

terminal OmpA peptide is cleaved during periplasmic expression and the Strep-tag is helpful for purification. Successful cloning was confirmed both by restriction enzyme digestion (when digested by NdeI and XmnI, pASK-IBA2-Atm1p results in the 3 predicted fragments), and also by DNA sequencing.

C-terminal Strep-tag Atm1p protein was mostly expressed in the periplasmic space. Cells were suspended in TS buffer (50 mM Tris-HCl pH 8.0, 75 mM NaCl), incubated with 1mg/ml lysozyme and 10 mM EDTA for 30 min on ice, and lysed by sonicating twice for 3 min. Cell debris was removed by centrifugation for 30 min at 3,000 x g. The supernatant was centrifuged again at 150,000 x g to isolate the membrane fraction. The pellet was resuspended in solubilization buffer (20mM MOPS-KOH pH 6.5, 200 mM NaCl, 20% sorbitol), homogenized in a Potter-Elvehjem homogenizer, and brought to a protein concentration of 3 mg/ml. The detergent n-dodecylmaltoside (critical micellar concentration of 0.008%) was added from a 10% stock solution to reach a final concentration of 0.5% (w/v). The suspension was incubated for 30 min on ice and centrifuged at 150,000 x g for 1 h. The supernatant containing the solubilized Atm1p fusion protein was diluted 1:1 with purification buffer (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1mM EDTA, 10% sorbitol, 0.025% n-dodecylmaltoside) and the Atm1p fusion protein was purified on a StrepTactin column. The resin was washed with purification buffer until no more protein eluted. Atm1p was recovered from the resin with purification buffer containing 2.5 mM desthiobiotin.

After purification by StrepTactin column, the purity of Atm1p was confirmed by SDS-PAGE, which shows up as a single band at about 66 kDa (Figure S2). The calculated full length Atm1p has a MW of about 76 kDa, however, the 66 kDa MW is consistent with the reported MW of purified Atm1p protein.²

Liposome preparation and reconstitution of proteoliposomal Atm1p.

Large unilamellar liposomes were prepared by the extrusion method. A lipidic film containing a 1:1:1 molar ratio of DOPC, DOPG and DOPE (Figure S3) was made by evaporation of a chloroform solution under nitrogen flow, and dried under reduced pressure overnight. The film was hydrated with Tris buffer (50 mM Tris, 100 mM NaCl, pH=7.5), and extruded 21 times through a polycarbonate filter with 200 nm pore size. The unilamellarity and size of the liposomes were confirmed by size measurements using dynamic light scattering (Figure S4). The presence of only one peak confirms the unilamellarity of the prepared liposomes and the average diameter is about 246 nm.

The reconstitution of Atm1p into liposomes was conducted following reported protocols.³ Briefly, unilamellar liposomes were resuspended in buffer at 4 mg/mL concentration and exposed to increasing concentration of Triton X-100. Absorbance change was monitored at 550 nm against the concentration of Triton X-100. The optimal concentration of Triton X-100 was then determined from the graph (Figure S5).

Detergent-destabilized liposomes, at the optimal concentration, were then combined with purified Atm1p in a ratio of 100:1(wt/wt) and incubated at room temperature for 15 min with gentle agitation. Bio-Beads SM-2 (Bio-Rad) were used to extract the excess detergent, with 40 mg Bio-Beads added to 1 mL protein-liposome suspension, incubated at room temperature for 30 min, followed by the addition of another 40 mg Bio-Beads

and incubation at 4 °C for 60 min. An additional 40 mg batch of Bio-Beads was added and incubated at 4 °C overnight. After addition of a final batch of 40 mg Bio-Beads and incubation at 4 °C for 120 min, the mixture was filtered to remove the Bio-Beads. The solution was then centrifuged at 4 °C for 20 min at 267,000 x g to collect the proteoliposomes. The proteoliposomes were resuspended in buffer for later use. The presence of Atm1p on the proteoliposomes was confirmed by both SDS-PAGE (Figure S6) and the ATPase activity assay (Figure S7).

ATPase Activity assay.

The ATPase activity of purified Atm1p was monitored by use of the EnzChek Phosphate assay kit (Invitrogen) (Figures S7 and S8). In brief, enzyme were mixed with 200 μM MESG substrate, 1 unit purine nucleoside phosphorylase, 1 mM ATP, 1 mM Mg²⁺ in 1x reaction buffer, and the increase in absorbance at 360 nm due to the formation of ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine was monitored. Initial rates of reaction were measured with increasing concentrations of ATP and a Michaelis-Menten plot of Atm1p ATPase activity of was constructed (Figure S9).

Computational Modeling and Sequence Alignments.

Protein sequence alignments were generated by use of ClustalW (Figure S10).⁴ Model structures for human ABCB7 and yeast Atm1p were generated by SWISS-Model,⁵ and PQR files generated by PDB2PQR from the modeled PDB file by use of the PDB2PQR server (http://nbc-222.ucsd.edu/pdb2pqr_1.8/).⁶ The electrostatic potential maps were then calculated using the generated PQR file and APBS software.^{4,7} The modeled Atm1p and ABCB7 structures (Figure S11) and their electrostatic surface maps (Figure 2, main text) were visualized by use of Chimera.⁸

Table S1: Concentrations and volumes of components for the PCR mutagenesis of wild-type Atm1p to R284E.

	Volume (μL)
10x Reaction buffer	5
Template (0.1 ng/ μL)	10
Forward Primer (125 ng/ μL)	1
Reverse Primer (125 ng/ μL)	1
dNTP mix (10 mM)	1
H ₂ O	32
DNA polymerase (1 unit/ μL)	1
mineral oil	30

Table S2: Reaction times and temperatures for the PCR mutagenesis of wild-type Atm1p to R284E.

Segment	Number of Cycles	Temperature ($^{\circ}\text{C}$)	Time
1	1	95	30 s
2	16	95	30 s
		55	1 min
		68	10 min

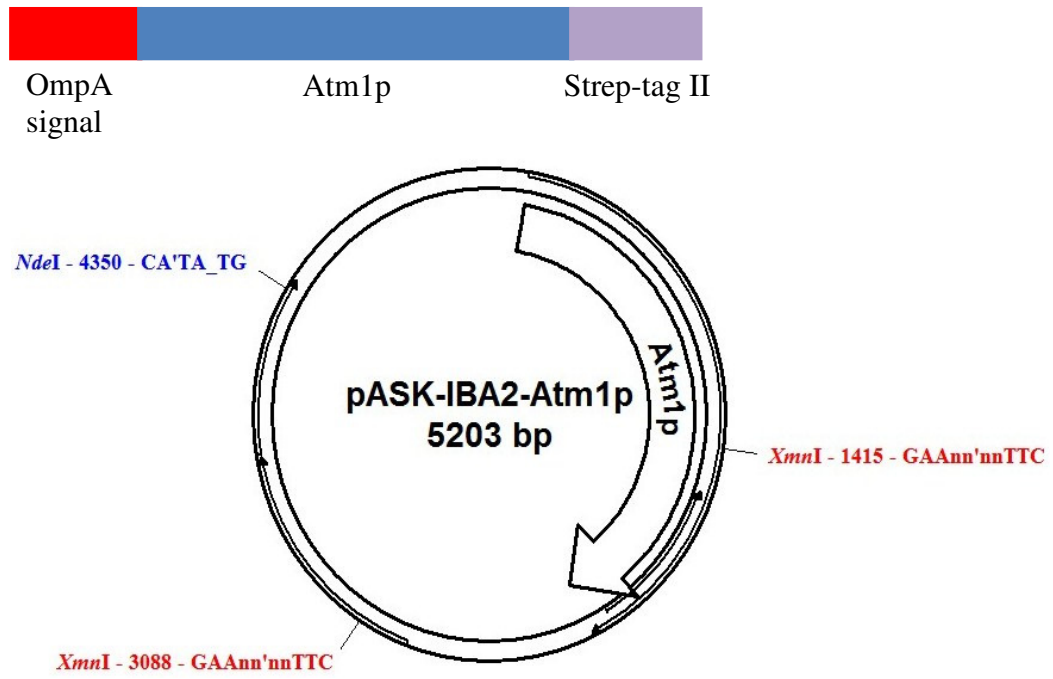


Figure S1. Construct of pASK-IBA2-Atm1p showing the NdeI/XmnI restriction sites.

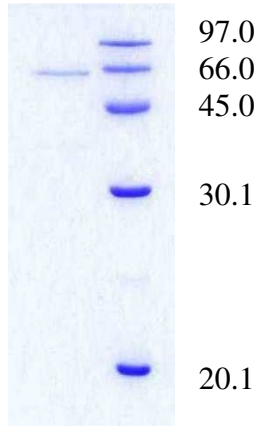


Figure S2. SDS-PAGE for purified Atm1p

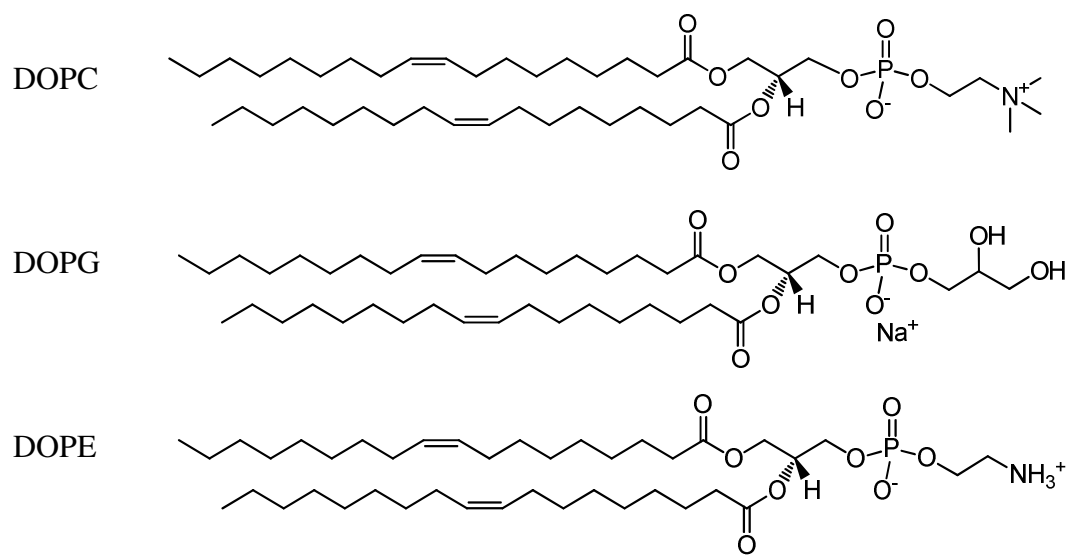


Figure S3. Structures of lipids used to prepare liposomes

Z-Average (d.nm): 293.0
Pdl: 0.117
Intercept: 0.935

	Diam. (nm)	% Number	Width (nm)
Peak 1:	246.8	100.0	89.16
Peak 2:	0.000	0.0	0.000
Peak 3:	0.000	0.0	0.000

Result quality Good

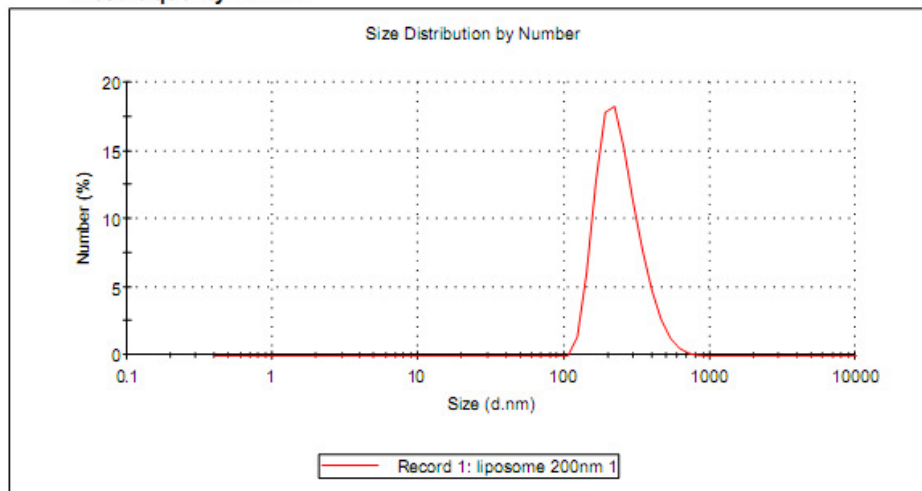


Figure S4. Size measurement for liposomal preparations by use of dynamic light scattering.

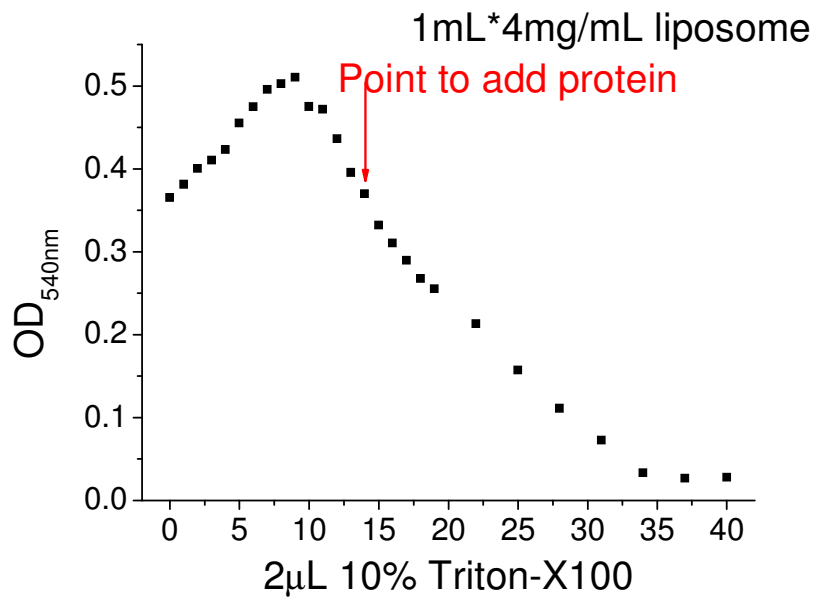


Figure S5. Titration of preformed liposomes with Triton X-100

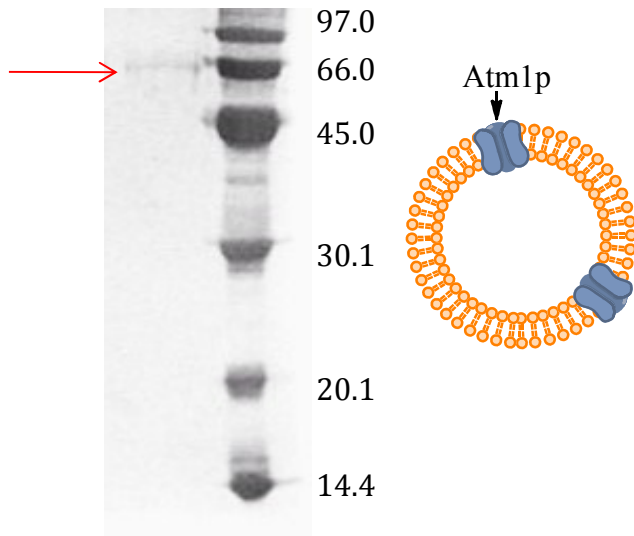


Figure S6. SDS-PAGE confirms the reconstitution of Atm1p into the prepared liposomes

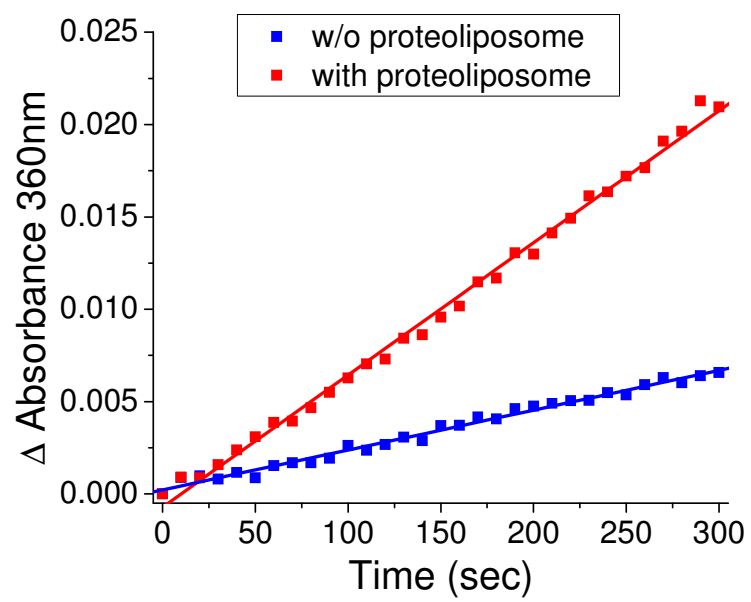


Figure S7. ATPase activity test of reconstituted proteoliposomes.

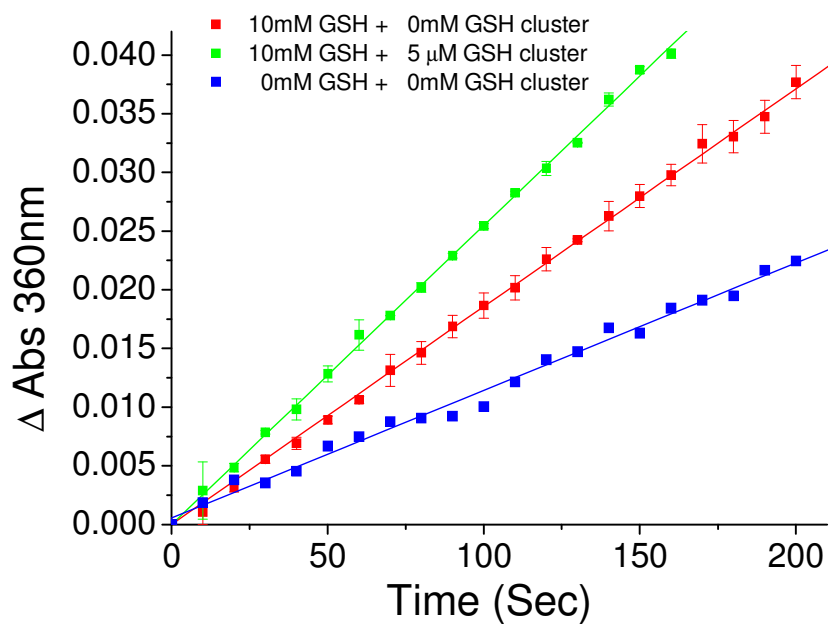
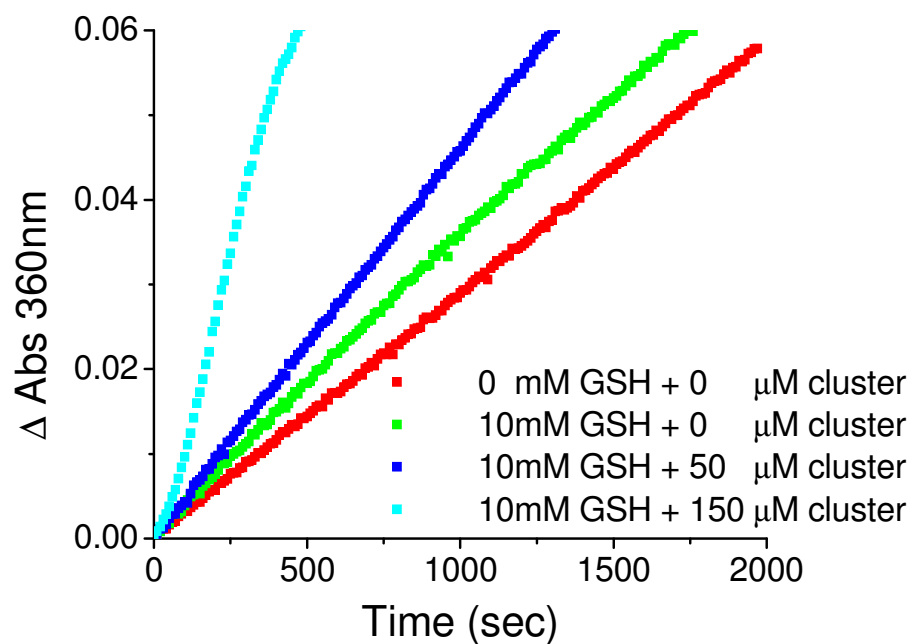


Figure S8. (Top) Solution Stimulation of Atm1p ATPase activity by $[2\text{Fe-2S}](\text{GS})_4$. (Bottom) Stimulation of proteoliposomal ATPase activity by glutathione iron-sulfur cluster complex.

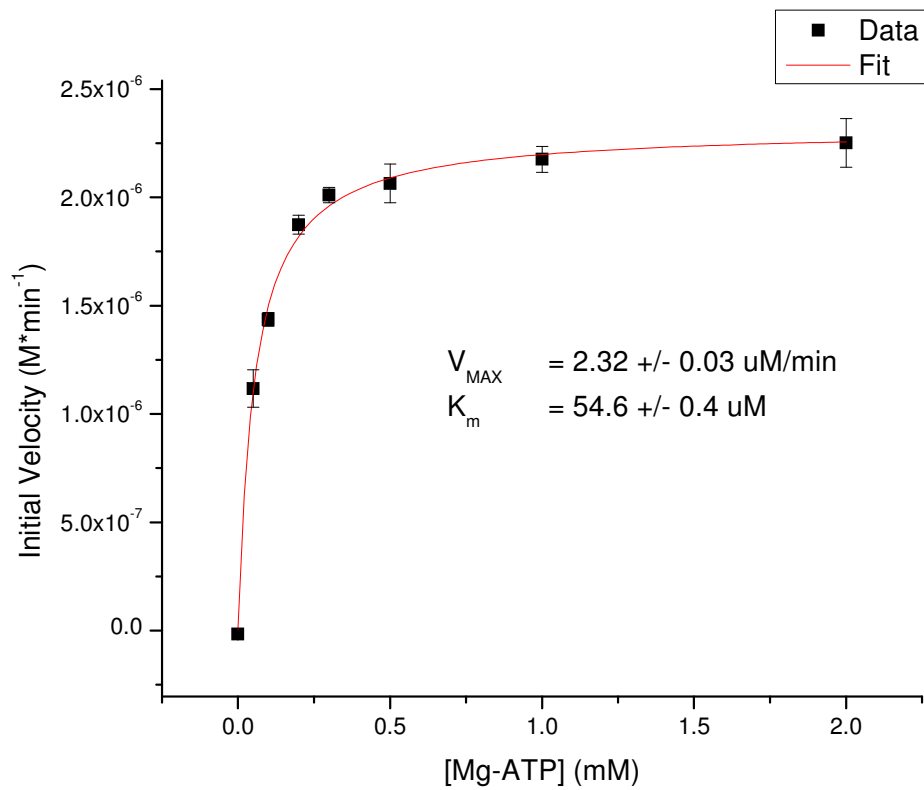


Figure S9. Atm1p and Mg-ATP binding parameters. Data were fit to standard Michaelis-Menten equation to yield V_{max} and K_m values.

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sp|P40416|ATM1_YEAST      MLLLPR----CPVIGRIVRSKFR--SGLIRNHSPVIFTVSKLSTQRP-L 42
sp|O75027|ABCB7_HUMAN    MALLAMHSWRWAAAAAAFEKRRHS--AILIRPLVSVSGSGPQWRPHQLGA 48
sp|Q9NRK6|ABCBA_HUMAN    MRGPPAWPLRLLEPPSPAEPGRLLPVACVWAAASRVPGSLSPFTGLRPAR 50
* . . . : : * : . :

sp|P40416|ATM1_YEAST      LFN SAVNLWNQAQKDI THKKS-----VEQFSSAPKVKTQVKKTSKAP 84
sp|O75027|ABCB7_HUMAN    LGTARAYQIPELKSITWQRLGKGN---SQQFLDAAKALQVWPLIEKRT 94
sp|Q9NRK6|ABCBA_HUMAN    LWGAGPALLWGVGAARRWRSGCRGGGPGASRGVGLGARLLGLWARGPGSC 100
* : . . . : . . . :

sp|P40416|ATM1_YEAST      TLS-----ELKILKDLFRYIWPKGNNK----- 106
sp|O75027|ABCB7_HUMAN    CWHGHAGGGLHTDPKEGLKDVDRKI IKAMLSYVWPKDRPD----- 135
sp|Q9NRK6|ABCBA_HUMAN    RCGAFAGPGAPRLPRARFPGGPAAAAWAGDEAWRRGPAAPPGDGRLRPA 150
.

sp|P40416|ATM1_YEAST      -----VRIRVLIALGLLISAKILNVQVPPFFKQTDIDSMN 140
sp|O75027|ABCB7_HUMAN    -----LRARVAISLGLGGAAMNIVPFMFYAVDSL N 169
sp|Q9NRK6|ABCBA_HUMAN    AAGLPEARKLLGLAYPERRRLAAAVGFLTMSSVISMSAPFFLGKIIDVIY 200
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sp|P40416|ATM1_YEAST      -----IAWDDPTVALPAAIGLTI L CYGVARFGSVLFGELRNAVFAKVAQ 184
sp|O75027|ABCB7_HUMAN    QMSGNMLNLS DAPNTVATMATAVLIGYGVSRAGAAFFNEVRNAVFGKVAQ 219
sp|Q9NRK6|ABCBA_HUMAN    -----TNPTVDYSDNLRRLCLGLSAVFLCGAAANAIRVYLMQTSQG 241
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sp|P40416|ATM1_YEAST      NAIRTVSLQTFQHLMKL DLGWHL SRQTGGLTRAMDRGTKGISQVLTAMVF 234
sp|O75027|ABCB7_HUMAN    NSIRRIAKNVFLHLLHNL DLGFHLSRQTGALS KAIDRGTRGISFVLSALVF 269
sp|Q9NRK6|ABCBA_HUMAN    RIVNRLR TSLFSSILRQEVAF FDKTRT GELINRLSSDTALLGRSVTENLS 291
. . . . . * : . . : . . * * * . . * . . : :

sp|P40416|ATM1_YEAST      HIIPISFEISVVC GILTYQFGASFAAITFSTMLYSIFTIKITAWRTHFR 284
sp|O75027|ABCB7_HUMAN    NLLPIMFEVMLVSGVLYYKCGAQFALVTLGLTGTYTAFTVAVTRWRTRFR 319
sp|Q9NRK6|ABCBA_HUMAN    DGLRAGA QASVGISMMFFVS--PNLATFVLSVVPVPSIIAVIYGRYLRLKT 340
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sp|P40416|ATM1_YEAST      RDANKADNKAASVALD SLINFEAVKYFNNEKYLADKYNGSLMNYRDSQIK 334
sp|O75027|ABCB7_HUMAN    IEMNKADNDAGNAAIDSLN YETVKYFNNEERYEAQR YDGLKTYETASLK 369
sp|Q9NRK6|ABCBA_HUMAN    KVTQDSL AQATQLAEERIGNVRTVRAF GKEMTEIEKYASKVDHVMQLARK 390
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sp|P40416|ATM1_YEAST      VSQSLAFLNSGQNLIFTTALTAMMYMGCTGVIGGNLTVGDLVLI NQLVFQ 384
sp|O75027|ABCB7_HUMAN    STSTLAMLNFGQSAIFSVGLTAIMVLSAQGIVAGTLTVGDLVMVNGLLFQ 419
sp|Q9NRK6|ABCBA_HUMAN    EAFARAGFFGATGLSGNLIVLSVLYKGGLLMGS AHMTV GELSSFLMYAFW 440
. : * : . . . . : : : : . . : : * * * * . *

sp|P40416|ATM1_YEAST      LSVPLNFLG SVYRDLKQSLIDMETL FKLKRNKVEVKIKN--AERPLMLPEN V 432
sp|O75027|ABCB7_HUMAN    LSLPLNFLTGVYRETRQALIDMNTLFTLLKVDQTKDKVMASPLQITPQT 469
sp|Q9NRK6|ABCBA_HUMAN    VGISIGGLSSFYSEL MKGLGAGR L WEL LERE PKLPFN--EGVILNEKSF 488
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sp|P40416|ATM1_YEAST      PYDITFENVTFGY--HPDRKILKNASFTIPAGWKTAIVGSSGSGKSTILK 480
sp|O75027|ABCB7_HUMAN    -ATVAFDNVHFEY--IEGQKVLSGISFEVPAGKVAIVGGSGSGKSTIVR 516
sp|Q9NRK6|ABCBA_HUMAN    QGALEFNKVVHFAYPARPEVPIQDFSLSIPSGSVTALVGPVSGSGKSTVLS 538
. * * * * . : . . * : * * * * * * * * : :

sp|P40416|ATM1_YEAST      LVFRFYDPESGRILINGRDIKEYDIDALRKVIGVVPQDTPLFNDTIWENV 530
sp|O75027|ABCB7_HUMAN    LLFRFYEPQKGS IYLAGQNIQDVSL ES LRAVGVVPQDAVLFHNTIYYNL 566
sp|Q9NRK6|ABCBA_HUMAN    LLLRLYDPASGTISLDGHDIRQLNPVWLR SKIGTVSQEPILFSCSIAENI 588
* : * * * * * * * : : * * * * * * * * * * * * * *

sp|P40416|ATM1_YEAST      KFGR---IDATDEEVITVVEKAQLAPLIKLPQGFDTIVGERGLMISGGE 577
sp|O75027|ABCB7_HUMAN    LYGN---ISASPEEVYAVAKLAGLHDAILRMPHG YDTQVGERGLKLSGGE 613
sp|Q9NRK6|ABCBA_HUMAN    AYGADDPSSVTAE EIQRVAEVANAVAFIRNFPQGFNTVVGKGVLLSGGQ 638
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sp|P40416|ATM1_YEAST      KQRLAIARVLLKNARIMFFDEATSALDTHTEQALLRTIRDNFTSGSRTSV 627
sp|O75027|ABCB7_HUMAN    KQRVAIARAILKDP PVILYDEATSSLD SITEETILGAMKD--VVKHRTSI 661
sp|Q9NRK6|ABCBA_HUMAN    KQRIAIARALLKNPKILLDEATSALDAENYLVQEALDR--LMDGRTVL 686
* * * * * * * * * * * * * * * * * * * * * * * *

sp|P40416|ATM1_YEAST      YIAHRLRTIADADKIIVLDNGRVREEGKHLELLAMPGLYRELWTIQ--- 674
sp|O75027|ABCB7_HUMAN    FIAHRLSTVVDADIIVLDQ GKVAERGTHHGLLANPHSIYSEMWH TQSSR 711
sp|Q9NRK6|ABCBA_HUMAN    VIAHRLSTIKNANMVAVL DQGKITEY GKHELLSKPNGIYRKL MNKQ--- 733
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sp|P40416|ATM1_YEAST      -----EDLDHLENELK DQQL----- 690
sp|O75027|ABCB7_HUMAN    VQNHDNPKWEAKKENISKEEERKQLQEEIVNSVKGCGNCSC 752
sp|Q9NRK6|ABCBA_HUMAN    -----SFISA----- 738
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Figure S10. Sequence alignment of yeast *Atm1p*, human ABCB7 and human ABCB10.

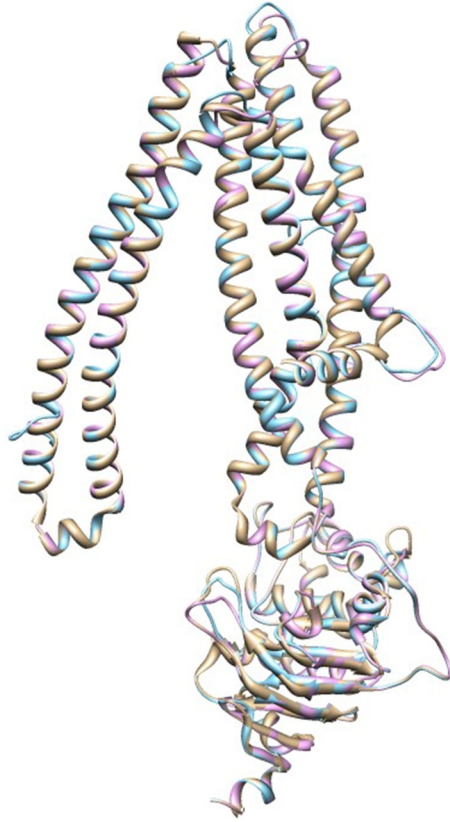


Figure S11. Overlay of the modeled yeast Atm1p structure (pink), modeled human ABCB7 structure (blue), and the template human ABCB10 structure (gold) (PDB:3ZDQ).

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Atmlp_yeast      279 TAWRTHFRRDANK 289
ABCB7_Human     314 TRWRTRFR IEMNK 324
                *  ***  **   **
Atmlp_yeast     385 LSVPLNFLG SVYRDLKQSLI 404
ABCB7_Human     420 LSLPLNFLG TVYRETQALI 443
                **  *  *  *  *  *  *  *  *  *  *

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Figure S12. Sequence alignment of the arginine-rich region of yeast Atmlp and human ABCB7. Positively-charged residues are highlighted in yellow.

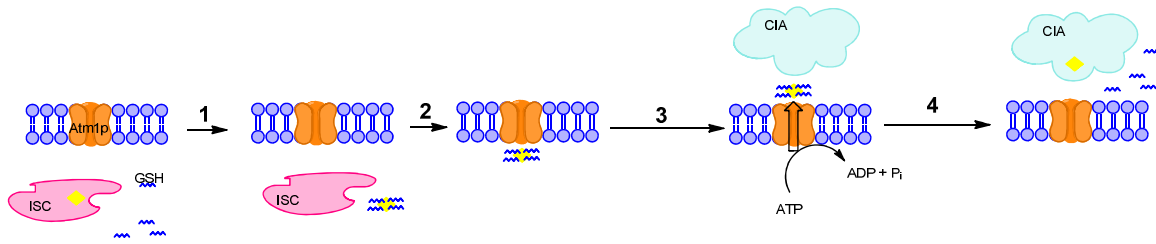


Figure S13. Discrete steps in a proposed mitochondrial Fe-S cluster export pathway, where ISC represents the mitochondrial cluster biosynthesis pathway and CIA represents the cytosolic iron-sulfur cluster handling pathway. The yellow diamond represents the [2Fe-2S] cluster core and the zig-zag line is glutathione (GSH).

References

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