Supplemental Information

Glutathione-Coordinated [2Fe-2S] Cluster. A Viable Physiological Substrate for Mitochondrial ABCB7 Transport

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MATERIALS & METHODS

Synthesis of liposomes

DOPG (Dioleoylphosphatidyl-Glycerol), DOPC (Dioleoylphosphatidyl-Choline), and DOPE (Dioleoylphosphatidyl-Ethanolamine) were purchased from Avanti Polar Lipids, Inc. and dissolved to 15 mM stock concentrations. An equimolar mixture of DOPG, DOPC and DOPE (133 μL, 131 μL and 124 μL, respectively) were vortexed and dried over Argon gas. The resulting residue was suspended in 1 mL 50 mM HEPES, 100 mM NaCl buffered at pH 7.5 and extruded through a 400 nm membrane 21 times. The quality and size of the lipid was checked by Dynamic Light Scattering Spectrometry (Malvern Instruments) and kept at 4 ºC until used. The same liposome synthesis method was followed for fluorescein-encapsulated liposomes, except that the re-suspension HEPES buffer contained 1 mM fluorescein.

Synthesis of Atm1p-incorporated proteoliposomes

The following procedure (illustrated in Figure S6) for proteoliposome synthesis is based on a modified protocol originally established by Poolman et al.[1] The previously synthesized liposome was diluted 2-fold to a total volume of 2 mL. A stock solution of 10% Triton X was titrated into the liposome solution at 2 μL increments until the OD_{550} reached a maximum, and an additional 16 μL was added to reach the "loose" state. It proved to be important to maintain the liposome on ice during the Triton X addition. Subsequently, Atm1p protein (80 μL, 1.2 μM) that had been purified by use of a protocol established by Qi et al.,[2] was added to the loosened liposome and incubated on ice for 15 min. Biobeads were used to remove the Triton X detergent and restore the rigidity of the proteoliposome. Aliquots of 40 mg Biobeads were added at 0, 30, 60 min and overnight. The Biobeads were removed by gentle centrifugation at 3,000 rpm for 1 min and separated from the decantate. The reconstituted proteoliposome was removed from the buffer by ultracentrifugation at 80,000 rpm for 20 min and re-dissolved in 1 mL 50 HEPES, 100 mM NaCl buffered at pH 7.5. The resulting proteoliposome was kept on ice and typically used within 24 hours of synthesis.

Synthesis of fluorescein-labeled glutathione

Oxidized glutathione (GSSG, 25.88 mg, 42 μmol) was dissolved in 0.8 mL 100 mM sodium bicarbonate buffered at pH 8.0. A solution of 5-(and 6-) carboxyfluorescein succinimidyl ester (NHS-Fluorescein, 10 mg, 21 μmol), dissolved in 0.1 mL DMSO, was added drop-wise to the buffered GSSG. The solution was allowed to stir at r.t. for 2 hours in the dark. After reaction, the disulfide bond was reduced by addition of 0.1 mL TCEP (26.4 mg, 0.106 mol) dissolved in bicarbonate buffer, and stirred at r.t. for 10 min. The pure product (Fl-GSH) was purified from

the crude mixture by HPLC (Agilent 1100) equipped with a reversed-phase C-18 column (Gemini 100 x 21.20 mm 5 micron) and eluted with buffers A: nano-pure H_2O, 0.1% TFA, and B: Acetonitrile, 0.1% TFA. The retention time of the product was 37 min with a flow rate of 5 mL/min and gradient of +1% B/min. The resulting fractions were dried over vacuum and stored as a powder until used. The yield of the final fluorescein-GSH conjugate (Fl-GSH) after reduction by TCEP was >90% and characterization by ESI-MS demonstrated a product peak with m/z of 666.1 that corresponded to Fl-GSH ($C_{31}H_{28}N_3O_{12}S^+$).

Synthesis of fluorescein-labeled cluster

Fl-GSH was used to make fluorescein-labeled cluster by combining labeled and unlabeled glutathione in a ratio of Fl-GSH : GSH of 1:20 in order to limit the number of Fl-GSH molecules incorporated per equivalent of cluster to one labeled glutathione molecule. The singly-labeled product appears to be the limiting form produced, most likely reflecting the fact that modification of two GSH yields an unstable cluster, as a result of disruption of stabilizing salt bridges,[3] and so multiply-modified derivatives are not observed.

A solution of glutathione (19.3 mg, 62.9 μmol) was dissolved in 1 mL nano-pure H_2O and purged under argon by 3x freeze-thaw cycles. The pH of this solution was adjusted by addition of stock NaOH solution (5 M, 16.5 μL) to a final pH of 8.6. The mixture of GSH and Fl-GSH was made by mixing 20 μL of the GSH solution with 80 μL of purified Fl-GSH dissolved in H_2O. Solutions of ferric chloride (0.0217 g in 3 mL H_2O) and sodium sulfide (0.0321 g in 3 mL H_2O) were made fresh prior to synthesis and added to the GSH solution (12.5 μL each). The resulting mixture was stirred and purified with the same protocol for $[2Fe\text{-}2S](GS)_4^{2-}$ cluster as previously described. The yield of the final fluorescein-GSH conjugate (Fl-GSH) after reduction

by TCEP was >90% and characterization by ESI-MS demonstrated a product peak with m/z of 1868.6 that corresponded to a cluster with one fluorescein label ($C_{61}H_{70}Fe_2N_{12}Na_5O_{30}S_6^-$).

Flow cytometry analyses of proteoliposomes

Liposomal flow cytometry experiments were conducted on a FACS Calibur Flow Cytometer (BD Biosciences) equipped with 488 nm / 633 nm lasers and dichroic filters at the University Cell Analysis and Sorting Core (UCAS, OSU). A detection filter setting of FL2 (585/42 nm) was used for detection of rhodamine-labeled liposomes and FL1 (530/30 nm) was used to detect fluorescein labeled iron-sulfur cluster. For detection of liposomes, Forward (FSC) and Side (SSC) scattering detector voltages were set at 8.68 V and 5.08 V, respectively. Compensation of the dichroic filters was set at FL1 – 10 % FL2 to remove false positive fluorescence signals. Samples were injected into the instrument at low speed setting and recorded until a final count of 10,000 events. The resulting data was processed on Cell Quest Pro (BD Biosciences) and plots were made as FSC vs. SSC and FL1/2 vs. event count without post-acquisition smoothing.

The fluorescence response from the labeled proteoliposomes was observed to change over the incubation period due to cluster transport into the vesicle (Figure S7). A solution of freshly made proteoliposome (1.0 mL in 50 mM HEPES and 100 mM NaCl at pH 7.5) was mixed with Mg-ATP (200 μL, 75 μM) and cluster (13 mM) in GSH solution (10 μL, 150 mM, pH 8.6) and incubated at 25 ºC for 60 min. To determine the kinetic profile for cluster transport, reactions were started at 10 min intervals up to 50 min. Normalized intensities (average $\pm$ standard deviation) for experiments with cluster and relevant controls were plotted and shown in Figures 1 and S8. Initial slopes were fit to the first 30 min of data for most samples (except for the more rapid cluster transport, which was fit to the initial 10 min) and initial velocities (average

± standard deviations) were plotted in Figure S2. The integrity of the proteoliposome during the experimental conditions described in the main text was checked by use of dynamic light scattering spectroscopy. The measured Z-average and count/s were plotted (Figure S5), and the proteoliposomes were observed to be stable.

For studies of fluorescein-labeled cluster transport, a solution of freshly made proteoliposome (1.0 mL in 50 mM HEPES and 100 mM NaCl at pH 7.5) was mixed with Mg-ATP (200 μL, 75 μM) and fluorescein-cluster (13 mM) in GSH solution (100 μL, 150 mM, pH 8.6) and incubated at 25 ºC for 60 min. Data points were gated with both FSC and SSC > 20 to remove background noise and yield the overall count for the fluorescent proteoliposomes (Figure 2).

Tiron assays

A solution of freshly made proteoliposome (0.2 mL in 50 mM HEPES and 100 mM NaCl at pH 7.5) was mixed with Mg-ATP (40 μL, 75 μM) and cluster (13 mM) in GSH solution (20 μL, 150 mM, pH 8.6) and incubated at 25 ºC for 60 min, and the product mixture was centrifuged at 6,000 rpm for 5 min to remove precipitate formed from cluster hydrolysis. The resulting decantate was removed and centrifuged at 80,000 rpm for 20 min to isolate the proteoliposome. The pellet was resuspended with 100 μL 50 mM HEPES and 100 mM NaCl buffered at pH 7.5. The proteoliposome was denatured by adding concentrated HCl (30 μL, 12 M) to liberate the encapsulated iron. Addition of MES buffer (10 mM, pH 6.5) and NaOH solution (5 M, 692 μL) was used to neutralize the reaction. A stock tiron solution (100 mM, 100 μL in MES buffer) was added to chelate the released ferric ions and incubated at room temperature for 10 min. The absorbance at 550 nm was measured by use of a UV/Vis spectrophotometer (Varian T-50) and

plotted against a standard curve to calculate the iron concentration inside the proteoliposome. The overall scheme for this assay is summarized in Figure S6.

Control experiments with GSH, GSSG and iron were carried out using similar concentrations of these reagents and the results are shown in Figure 3.

Titration of fluorescein-labeled cluster to Atm1p

Labeled cluster from a stock solution (1 mM) was added to a solution of Atm1p (1.2 uM) in HEPES buffer (50 mM HEPES, 2 mM GSH, pH 7.5). The equivalent control experiment without Atm1p was used to calculate the net signal change, and these results are graphed in Figure S3, with a fitted $K_D = 118 \pm 11$ μM.

Titration of GSH to a solution of fluorescein

Addition of GSH quenches the fluorescence response from fluorescein observed in a cuvette measurement. GSH from a stock solution (10 mM) was added to a solution of Fluorescein (10 uM, 1 mL) in HEPES buffer (50 mM HEPES, 100 mM NaCl, pH 7.5). These results are graphed in Figure S9.

Data fitting and Statistical analysis

For the control data shown in Figure S2, the first thirty minutes of the flow cytometry signal was fit to linear functions to obtain the observed initial rate of decrease. Standard p-test was performed for GSH control against experiments with clusters, which yielded $p = 0.0070$ and $z = 2.70$, corresponding to a 99.3% level of confidence.

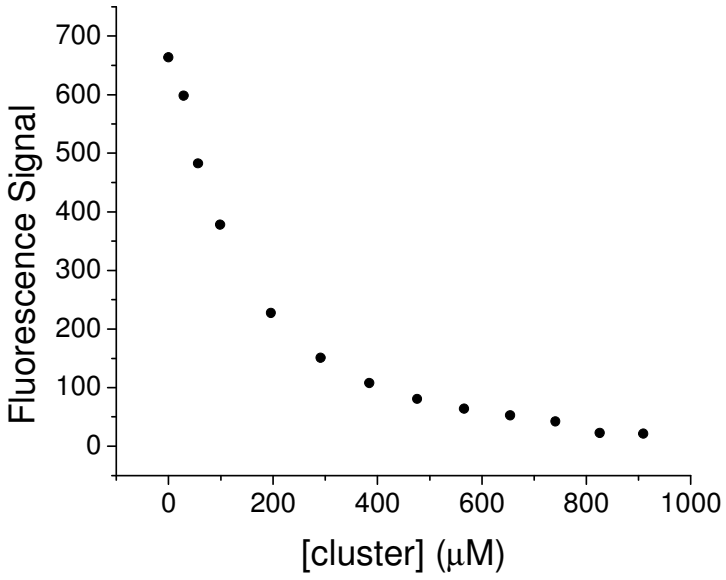

Figure S1. Titration experiment showing cluster quenching the fluorescein fluorescence signal, most likely due to heavy atom and inner filter effects. A solution of glutathione iron-sulfur cluster solution (10 mM) was added in 1uL increments to 100uL 10uM fluorescein solution.

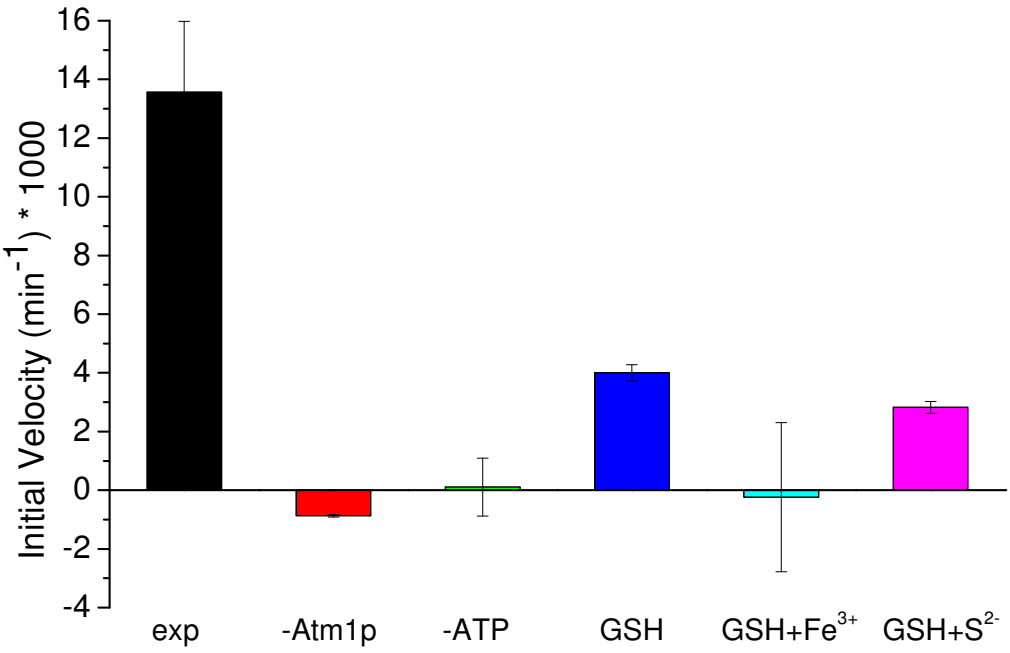

Figure S2. Initial velocity derived from the rate of fluorescein signal quenching following addition of cluster and Mg-ATP (exp). The Atm1p proteoliposome fluorescence signal was measured by flow cytometry at time intervals of 10 min over a period of 50 min. Control experiments in the absence of Atm1p or Mg-ATP demonstrate that cluster transport is absent. A cluster concentration of 13 mM was used, compared to 38 mM of GSH in control experiments.

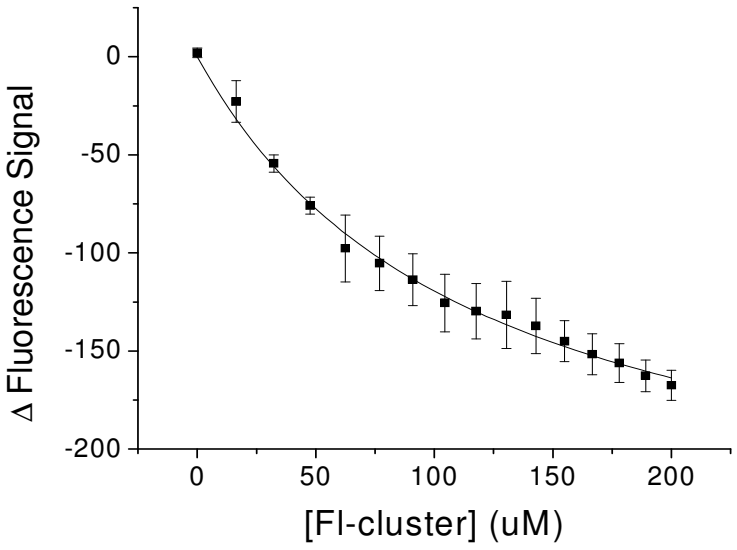

Figure S3. Titration experiment showing the decrease in fluorescence signal intensity as labeled-cluster was added to Atm1p. Labeled cluster from a stock solution (1 mM) was added to a solution of Atm1p (1.2 uM) in HEPES buffer (50 mM HEPES, 2 mM GSH, pH 7.5). The equivalent control experiment lacking Atm1p was used to calculate the net signal change, and these results are graphed and fitted yield a $K_D = 118 \pm 11$ μM.

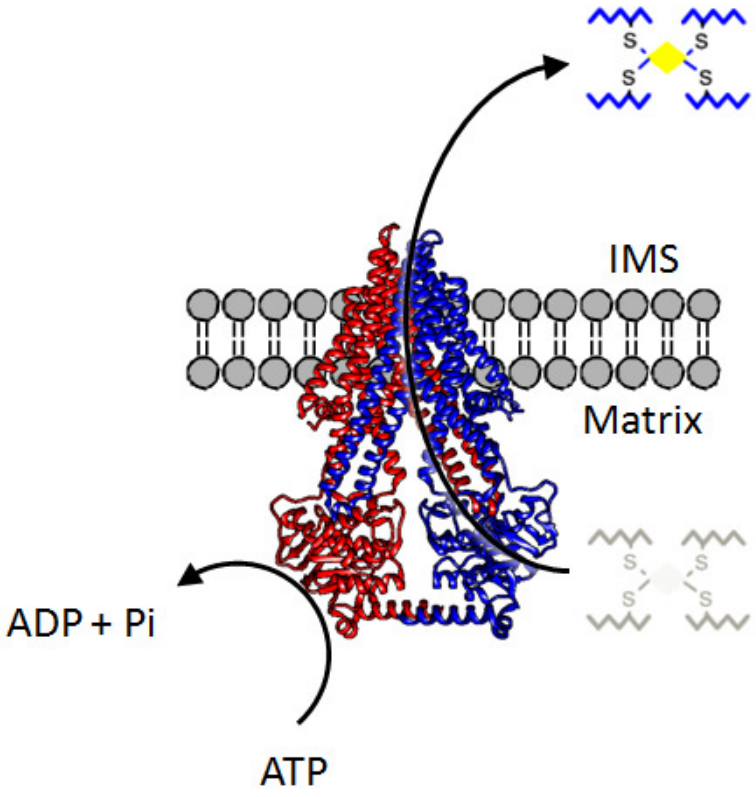

Figure S4. Proposed mechanism for mitochondrial iron-sulfur cluster transport by ABCB7-type export proteins driven by ATP hydrolysis. The transporter shown in the Figure is adapted from the crystal structure of *Novosphingobium aromaticivorans* Atm1p. [4]

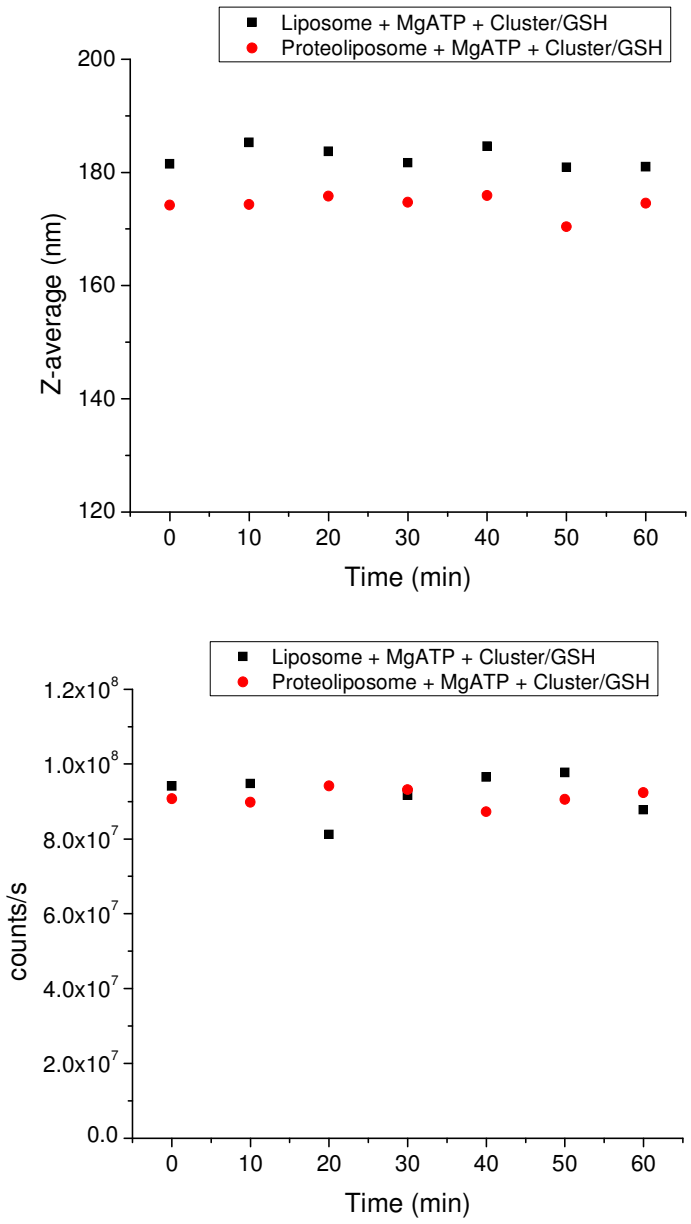

Figure S5. Control experiments to monitor the integrity of the proteoliposomes throughout the transport reaction. Both the size (top) and number (bottom) of the liposomes remained relatively unchanged over the course of the reaction, indicating that the fluorescence change observed during active transport reflects perturbation of the inner content of the proteoliposome. Synthesized proteoliposomes were dissolved in 50 mM HEPES and 100 mM NaCl buffered at pH 7.5. Data were collected by DLS (Malvern Instruments) at room temperature.

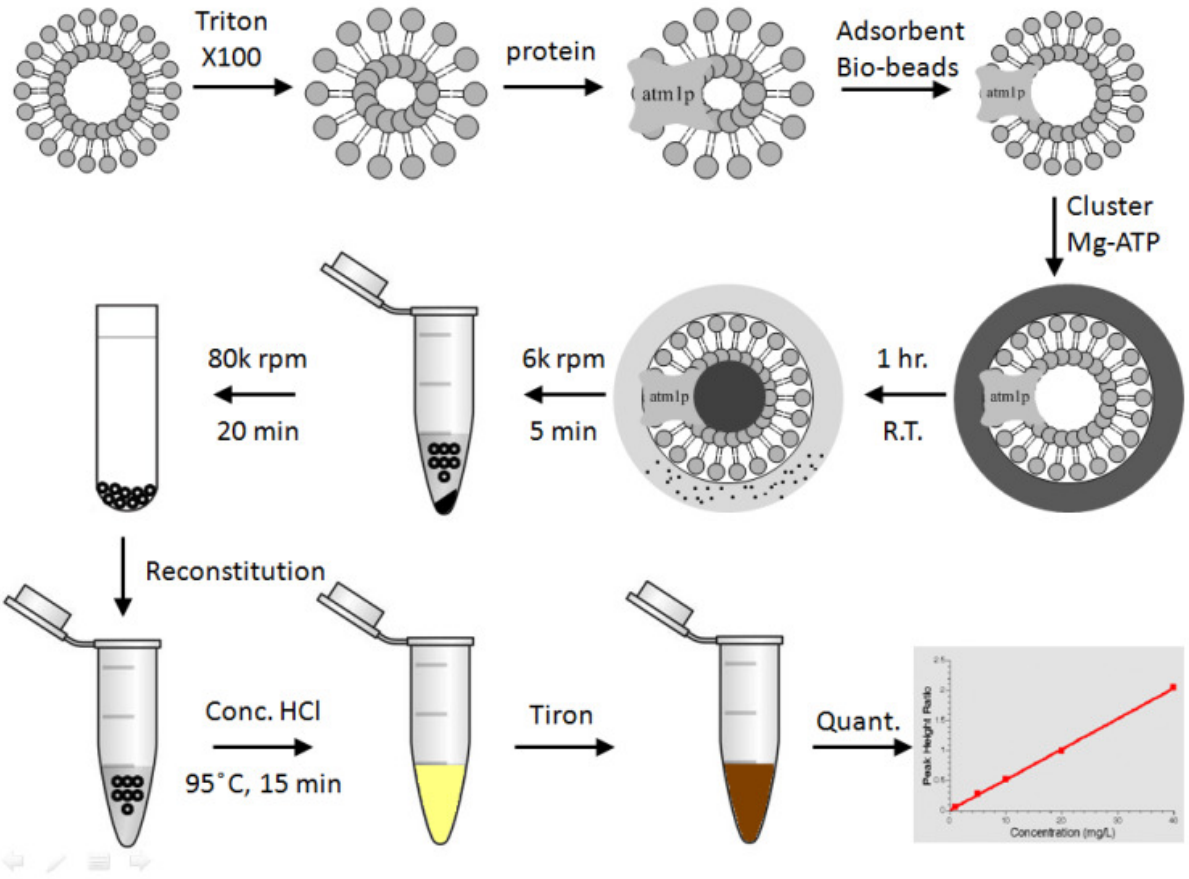

Figure S6. Synthesis of proteoliposome, ATP-driven cluster transport and ferric ion quantitation of product.

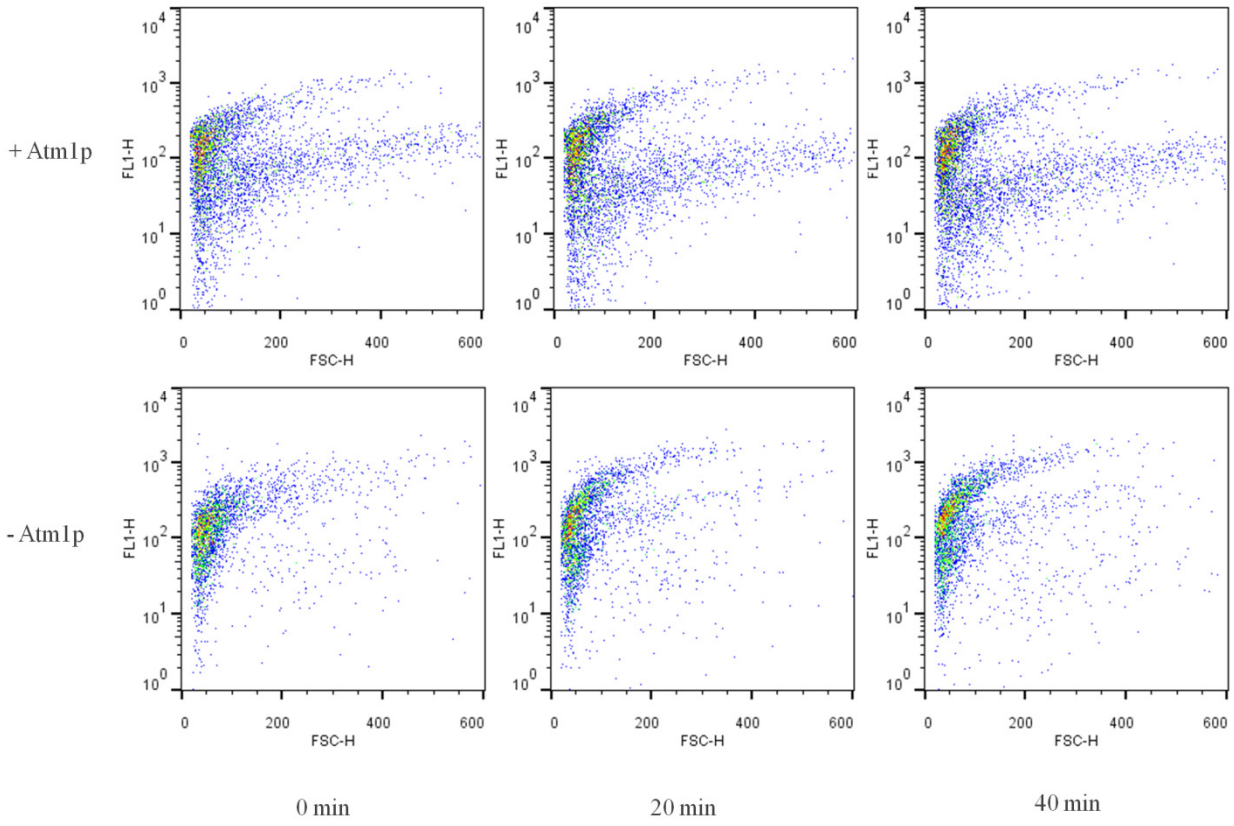

Figure S7. Raw flow cytometry data showing proteoliposomes fluorescent signal changes over time for the experiment and control without Atm1p. FlowJo was used to quantitatively obtain the geometric mean for each experiment.

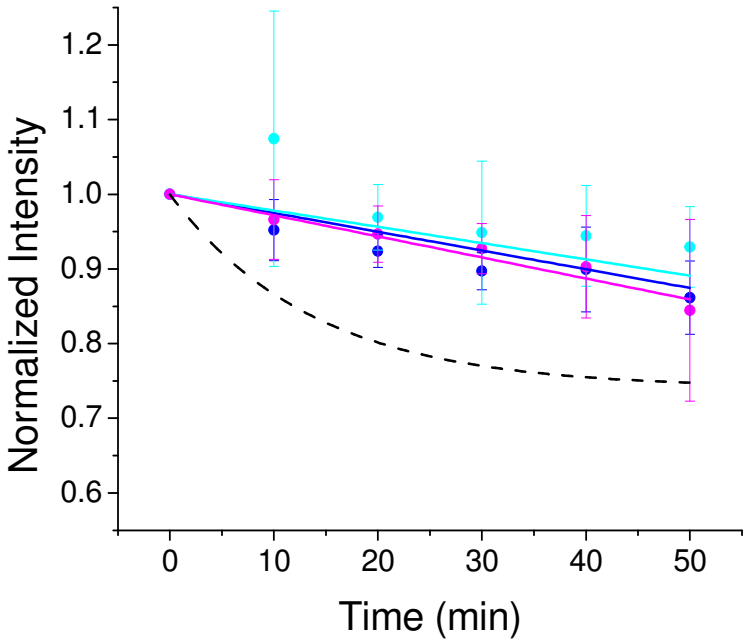

Figure S8. Control experiments for cluster transport into fluorescein-loaded proteoliposome with GSH only (blue), GSH + Fe^{3+} (cyan) and GSH + S^{2-} (magenta). The dashed line corresponds to the observed signal fit from experiments with clusters.

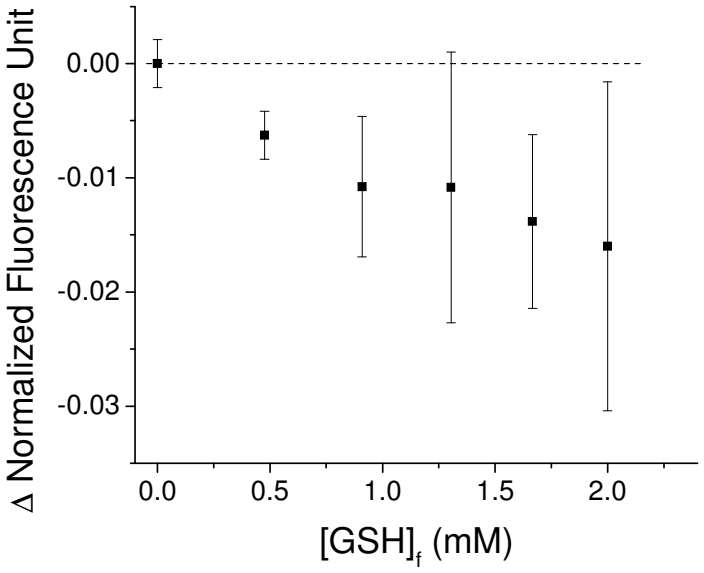

Figure S9. Titration experiment showing GSH quenching the fluorescein fluorescence signal, most likely due to a heavy atom effect. A solution of fluorescein (10 uM , 1 mL) was titrated with a stock solution of glutathione (10 mM) in Hepes buffer (50 mM Hepes, 100 mM NaCl, pH 7.5).

REFERENCES

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