Supporting Information

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SI Materials and Methods

Cloning, Strains, and Plasmids. The gene coding for ABCE1 from *Sulfolobus solfataricus* was cloned in the pSA4 vector for heterologous expression in *Escherichia coli* and in pSVA31 for homologous expression in its natural host as described previously (1, 2). These constructs served as templates for generating the ABCE1 mutant (76–600), lacking the FeS cluster domain by PCR. Mutations of full-length ABCE1^{WT} or ABCE1$^{\Delta FeS}$ were generated by using the Phusion site-directed mutagenesis protocol (Finnzymes). A C-terminal His_6-tag fusion construct of aIF6 from *S. solfataricus* was generated by amplification from genomic DNA via PCR. The amplification product was digested with the NcoI and BamHI restriction enzymes and ligated into pSA4 yielding pDB9. Similarly, the gene of aRF1 was amplified from *S. solfataricus* P2 genomic DNA and cloned into the NcoI and BamHI restriction sites of pSA4 resulting in plasmid pDB10. This cloning strategy allowed for fusion of aRF1 at the C-terminus with a His_6-tag. The identity and integrity of all constructs was verified by sequencing.

Protein Expression and Purification. ***ABCE1.*** Protein expression and purification of ABCE1 from *S. solfataricus* was performed as described previously (1) with following modifications. After metal affinity chromatography, factions containing ABCE1 were dialyzed against A-IEX buffer (20 mM Tris-HCl, pH 8.5) and loaded onto an anion exchange column (HiTrap Q HP; GE Healthcare). Proteins were eluted by a salt gradient (0–1.0 M NaCl). Fractions containing ABCE1 were dialyzed against standard buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT) and stored at −20 °C. Protein concentrations were determined by UV absorbance at 280 nm using extinction coefficients of 58,720 M^{-1} cm^{-1} (full-length ABCE1) and 53,750 M^{-1} cm^{-1} (ABCE1$^{\Delta FeS}$), respectively. A polyclonal antibody was raised against purified ABCE1.

aRF1. *E. coli* BL21(DE3) cells were transformed with the plasmid pDB10 and grown at 37 °C to an OD_{600} of 0.6–0.8. Expression was induced by adding 0.5 mM of isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 3 h at 37 °C. Cell pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM EDTA) and disrupted by sonification using a Branson Sonifier 250 at 60% output in 10 pulses of 1 min on ice. Cell debris and membranes were removed by centrifugation at 100,000 × *g* for 30 min. After heat shock at 70 °C for 10 min, the supernatant was applied to a Ni-NTA affinity column (Qiagen) with a flow rate of 5 mL/ min. aRF1 was purified by washing with 10 column volumes of 40 mM imidazole and eluted at 200 mM of imidazole. Fractions containing aRF1 were pooled and dialyzed against 20 mM Tris-HCl, pH 8.5, 10 mM NaCl, 1 mM DTT and 5 mM $MgCl_2$5 mL/ min and further purified on HiTrap Q sepharose (GE Healthcare). After anion exchange chromatography, fractions containing aRF1 were concentrated and loaded onto a Superdex 75 16/60 prep grade column (GE Healthcare) equilibrated in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT. Fractions corresponding to aRF1 were finally dialyzed in storage buffer (20 mM Tris-HCl, pH 7.5, 40 mM NH_4Cl, 1 mM DTT), concentrated, and snap frozen in liquid nitrogen.

aIF6. The plasmid pDB9 encoding aIF6 was transformed in *E. coli* BL21(DE3) cells and gene expression was performed as described above, except that 0.3 mM IPTG was used for induction. Cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT, 1 mM EDTA) and disrupted by sonification as described above. After centrifugation at 100,000 × *g* for 30 min, a heat shock at 70 °C was applied for 10 min. Denatured proteins were removed by centrifugation at 100,000 × *g* for 30 min and the supernatant was loaded on a HiTrap Chelating column (GE Healthcare) with a flow rate of 2 mL/ min. After two washing steps with 20 and 50 mM imidazole, aIF6 was eluted with 200 mM imidazole. Fractions containing aIF6 were pooled and dialyzed against 20 mM Tris-HCl, pH 8.5, 10 mM NaCl, 1 mM DTT and further purified on HiTrap Q sepharose (GE Healthcare). After anion exchange chromatography, fractions containing aIF6 were concentrated and dialyzed on Amicon concentrators (Millipore) into storage buffer (20 mM Tris-HCl, pH 7.5, 40 mM NH_4Cl, 5 mM $MgCl_2$, 1 mM DTT and 10% (v/v) glycerol), snap frozen in liquid nitrogen, and stored at −20 °C. The protein concentration of aIF6 and aRF1 was determined by using the Coomassie Plus™ Bradford Assay (Pierce) using bovine serum albumin as a standard.

Crystallization and Structure Determination. ABCE1$^{\Delta FeS\text{-}E238/485Q}$ was concentrated to 15 mg/mL (in 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 5% (v/v) glycerol) and incubated at 70 °C with 4 mM of ATP for 5 min. The protein was crystallized by mixing 2 μL protein solution with 2 μL buffer containing 0.1 M NaCacodylate, pH 6.5, 0.2 M $Mg(OAc)_2$, 20% (w/v) PEG 8000. Small rod-shaped crystals of ADP-loaded ABCE1 grew within 2 weeks at 18 °C. Crystals were soaked for 10–20 sec in the reservoir solution supplemented with 15% (v/v) glycerol, mounted in nylon loops (Hampton Research), and flash frozen in liquid nitrogen. The space group P1 datasets were recorded at the SLS beamline (Villingen) and processed with HKL and XDS (3, 4). Phases were calculated by molecular replacement using the program EPMR (5) and coordinates of *Pyrococcus furiosus* ABCE1 (6) as the search model. Refinement of the model was performed with REFMAC5 (7), Phenix (8), and repetitive manual model building with O and Coot (9, 10). Translation Libration Screw (TLS) groups were identified by using the TLSMD server (http://skuld.bmsc.washington.edu/~tlsmd). For cross-validation, 5% of the original reflections were omitted from the refinement and used to calculate the R_{free} factor. Refinement qualities including rotamer and torsion angle analysis were judged by using the MolProbity program (11). Figures were prepared with PyMOL (DeLano Scientific).

Analytical Gel Filtration. ABCE1 (5 μM) was incubated in 50 μL SEC buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT) with 5 mM of ATP, ADP, and AMPPNP, respectively. After 5 min incubation at 80 °C, samples were incubated on ice and subsequently loaded onto a Superdex 200 PC 3.2/30 column (GE Healthcare), preequilibrated in SEC buffer, with a flow rate of 0.05 mL/ min. The absorbance at 254 nm, 280 nm and 410 nm was recorded during the run and fractions of 100 μL were collected. Standard molecular weight markers, including apoferritin (440 kDa), alcohol dehydrogenase (150 kDa), albumin (67 kDa), β-lactalbumin (35 kDa), cytochrome c (12 kDa), vitamin B12 (1.35 kDa) were used for calibration.

For detection of ABCE1/ATP/30S complexes, ABCE1$^{E238/485Q}$ or ABCE1^{WT} (1 μM) were incubated with 500 μM ATP (traced with [γ-^{32}P]-ATP) at 73 °C for 5 min with high-salt washed 30S (1 μM) subunits. After incubation on ice for 2 min, the reaction was loaded onto a TSK4000 SW_{XL} (Tosoh Bioscience) column, preequilibrated in 20 mM triethylamine (TEA) pH 7.2, 10 mM

KCl, 20 mM $MgCl_2$, with a flow rate of 0.4 mL/ min. 500 μL fractions were collected and analyzed by Cerenkov counting.

ATPase Assays. ATP hydrolysis was measured colorimetrically by using the malachite green assay (12). Reactions were performed in 25 μL of ATPase buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$) with various concentrations of ABCE1 (0–10 μM) and ATP (0–5 mM) at 80 °C. ATP hydrolysis was stopped by adding 175 μL of ice-cold H_2SO_4 (20 mM) and placing the samples on ice. 50 μL of malachite green solution (0.28 mM malachite green, 0.17% (v/v) Tween-20, 1.48% (w/v) ammonium molybdate tetrahydrate) was then added and inorganic phosphate release was detected at 620 nm. A K_2HPO_4 solution served as a standard to calculate the amount of P_i released.

ATP hydrolysis was additionally measured by thin layer chromatography (13). Reactions were performed in ATPase buffer supplemented with trace amounts of [γ-^{32}P]-ATP (110 TBq/mmol; Hartmann Analytic). After incubation, reactions were placed on ice and stopped by adding 1% (w/v) SDS and 10 mM EDTA. 1 μL of the reaction was spotted onto polyethyleneimine plates (Merck) and developed in 0.75 M KH_2PO_4 pH 3.4. The dried chromatograms were scanned and quantified in a Phosphor Imager (GE Healthcare). All ATPase measurements were performed in triplicates to calculate the standard deviation.

Stoichiometry of Bound Nucleotides. ABCE1 (5 μM) was incubated in 50 μL ATPase buffer with 500 μM of ATP (traced with [γ-^{32}P]-ATP) for 5 min at 80 °C. Afterward, reactions were immediately applied onto preequilibrated G50-microspin columns (GE Healthcare) and centrifuged for 1 min at 750 × *g* to separate protein bound nucleotides from unbound nucleotides. Protein retained radioactivity was quantified by Cerenkov Counting and corrected for nonspecifically retained radioactivity. The identity of the bound nucleotides was determined by thin layer chromatography as described above.

Preparation of Whole Cell Extracts, Ribosomes, and tRNA. *S. solfataricus* cells were harvested in midlog growth phase and flash frozen as small spherules in ribosome extraction buffer (20 mM Tris-HCl, pH 7.4, 40 mM NH_4Cl, 10 mM MgOAc, 2 mM DTT). Whole cell extracts, ribosomes, crude tRNA and crude translation factors were prepared as described (14, 15). Lysates and ribosomes from *T. celer* were prepared in the same way.

Immunodepletion of ABCE1. 50 μL of Dynabeads® Protein A (Invitrogen) were washed twice in 500 μL of 100 mM Na_2HPO_4 (pH 8.0). Then, 20 μL of anti-ABCE1 or preimmune serum were incubated for 60 min with the magnetic beads at 4 °C. Subsequently, beads were washed three times with 100 mM Na_2HPO_4, pH 7.4, 0.05% (v/v) Tween-20). The beads were washed twice in 1 mL of 200 mM TEA, pH 8.0. Antibodies were coupled to the beads by incubation for 30 min at room temperature in 1 mL of freshly prepared buffer X (200 mM TEA, pH 8.0, 20 mM dimethylpimelidate). The reaction was stopped by adding 500 μL of 50 mM Tris-HCl, pH 7.4. After 15 min at room temperature, beads were washed three times with 100 mM Na_2HPO_4 pH 8.0 containing 0.05% (v/v) Tween-20. 100 μL of *S. solfataricus* P2 cell extract (15 mg/mL) were incubated with the antibody coupled beads for 1 h at 4 °C. The depletion reaction was performed twice to ensure efficient removal of ABCE1 from the lysate.

Coimmunoprecipitation. To probe a direct ABCE1-aRF1 interaction, 50 μL of Dynabeads® Protein A (Invitrogen) were washed three times with 100 mM NaH_2PO_4 pH 8.0 and once with ATPase buffer). Then, 50 μL of the anti-ABCE1 serum were added and incubated at 4 °C for 2 h using an overhead rotator. ABCE1 and aRF1 (2 μM of each) were incubated in 40 μL of CoIP buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.2% Tween-20 and 2 mM nucleotides) for 10 min at 73 °C. After cooling down on ice for 2 min, the reaction was incubated with the washed beads for 3 h at 4 °C under shaking. Beads were washed four times with 1 mL of CoIP buffer and one time with 40 μL of CoIP buffer (wash fraction). The proteins were eluted in 40 μL of SDS-loading buffer. The samples were analyzed by SDS-PAGE (10%) and subsequent immunoblotting using a monoclonal antiHis antibody (Novagen).

To probe the interaction of ABCE1 and aRF1 in the lysate of *S. solfataricus*, 100 μL of whole cell extracts (15 mg/mL) was supplemented with aRF1 and ABCE1 (2 μM of each) in the presence of different nucleotides (5 mM). The reaction volume was increased to 200 μL with ribosome extraction buffer (20 mM Tris-HCl, pH 7.4, 40 mM NH_4Cl, 10 mM MgOAc, 1 mM DTT) and the samples were incubated at 73 °C for 10 min. The immunoprecipitation reaction with anti-ABCE1 coupled Dynabeads® Protein A (Invitrogen) was essentially performed as described above.

Phosphorylation of ABCE1. ABCE1 was phosphorylated with 5 μL of cAMP-dependent protein kinase A (PKA, NEB) in 20 mM Tris-HCl, pH 7.5, 50 mM KCL, 5 mM $MgCl_2$, 1 mM ATP (traced with [γ-^{32}P]-ATP) and in a total volume of 150 μL for 2 h at 30 °C. PKA and unincorporated nucleotides were removed by anion exchange chromatography (HiTrap Q, GE Healthcare), yielding specifically ^{32}P-phosphorylated ABCE1 ($7 * 10^3$ cpm/pmol).

Sucrose Density Gradients. To analyze the colocalization of ABCE1 and ribosomes, 100 μL of WCE (15 mg/mL) from *S. solfataricus* were incubated at 73 °C for 2–4 min with different nucleotides (5 mM), and then loaded on 10–30% sucrose gradients. Centrifugation was performed in a SW41 rotor (Beckman Coulter) at 36.000 rpm for 4 h. Gradients were fractionated from the top (Piston Gradient Fractionator, Biocomp) by detecting the absorbance $A_{254\ nm}$. 500 μL-fractions were mixed with 1 mL of acetone and precipitated at −20 °C. Purified ABCE1 (dialyzed in 20 mM triethanolamine-HCl pH 7.4, 10 mM KCl) was added to WCE or to isolated ribosomes, 30S and 50S (0.5–1 μM) and incubated at 73 °C for 4 min. Fractions were analyzed by immunoblotting using a polyclonal anti-ABCE1 antibody raised in rabbits or an anti-His antibody (Novagen).

Ribosome Sedimentation Assay. Increasing concentrations of ABCE1 (0–10 μM) were mixed with 0.5 μM of 30S or 50S in 40 μL of ribosome extraction buffer in the presence of different nucleotides (5 mM) and incubated for 5 min at 73 °C. After centrifugation at 13,000 × *g* for 10 min to remove aggregates, samples were subsequently loaded on 160 μL of 25% (w/v) sucrose cushion made in ribosome extraction buffer. Sedimentation was performed at 95,000 rpm in the TLA-100 rotor (Beckman Coulter) for 50 min. Ribosomal pellets were washed and resuspended in 40 μL of ribosome extraction buffer. The ribosome concentration was measured at $A_{254\ nm}$. Equimolar amounts were analyzed by SDS-PAGE (15%, Coomassie staining) and immunoblotting.

In Vitro Translation. The vectors pBS800 (28) and pSK_{aIF1} were linearized with AflII or NotI, respectively. 5 μg of DNA was used for run-off in vitro transcription with T7 RNA polymerase (Ambion). Transcribed mRNA-104 and mRNA-aIF1, coding for the small ribosomal protein rpl30 and aIF1, respectively, were purified by phenol-chloroform extraction. In vitro translation reactions were performed in 100 μL buffer T (20 mM Tris-HCl, pH 7.4, 10 mM KCl, 20 mM MgOAc) supplemented with 3.6 mM ATP, 1.8 mM GTP, 4 mM DTT, 10 μg *S. solfataricus* bulk tRNA, 2 μL [^{35}S]-methionine (37 TBq/mmol), 4 μL amino acid mix minus methionine (Promega), 500 μg *S. solfataricus* S30 extract (preincubated at 73 °C for 15 min), and 10 μg mRNA. For in vitro

translation, samples were incubated at 73 °C for up to 60 min. 10 μL of each reaction were withdrawn and analyzed by SDS-PAGE (17.5%). Radioactive bands corresponding to rpl30 were quantified by a PhosphoImager (GE Healthcare). For the stabilization of 70S ribosomes in sucrose gradients, the Tris-buffer was replaced by TEA. After incubation, samples were cross-linked with formaldehyde (1% (v/v) final concentration) for 30 min on ice. Subsequently, the reactions were loaded on sucrose gradients (10–30%) in ribosome extraction buffer and centrifuged for 4 h at 36,000 rpm. For preinitiation complex formation, [^{32}P]-mRNA-104 (30,000 cpm/μg) was added to the in vitro translation reaction and GTP was replaced by GMPPNP (2 mM). SDG fractions (10–20% sucrose) were analyzed by Cerenkov counting.

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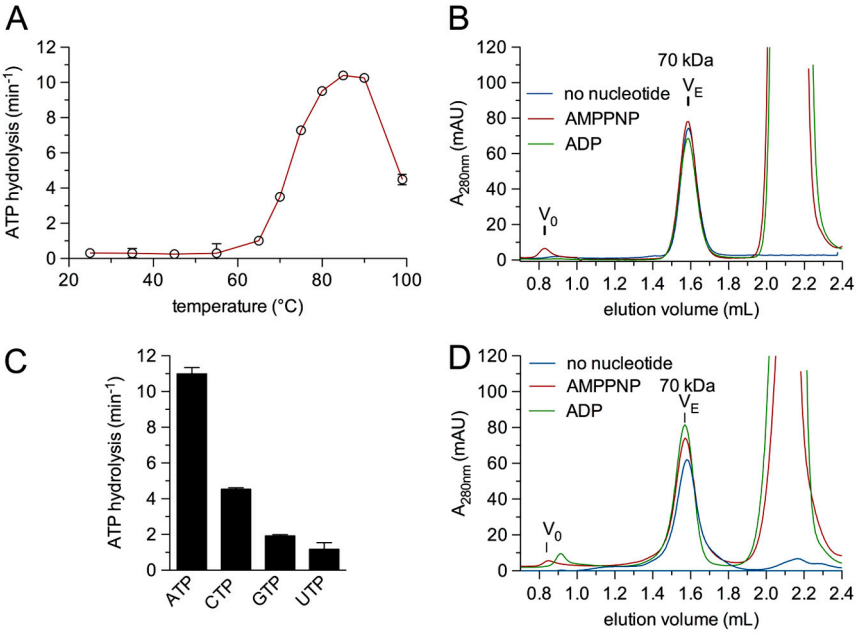

Fig. S1. Temperature dependency of the ATPase activity and monomeric state of ABCE1. (*A*) ABCE1^{WT} (5 μM) was incubated with ATP (5 mM) at different temperatures. P_i-release was measured by the colorimetric Malachite Green assay. Experiments were performed in triplicates and the error bars indicate the standard deviation. (*B*) Analytical gel filtration. ABCE1^{WT} (5 μM) were incubated at 80 °C for 5 min with different nucleotides (5 mM of each). After cooling down on ice for 2 min, 50 μL of the protein samples were loaded on Superdex 200 PC 3.2/30 (GE Healthcare) operating at 4 °C and a flow rate of 0.05 mL/ min. (*C*) ABCE1^{WT} was incubated at 80 °C for 5 min in the presence of different nucleoside triphosphates (2 mM of each). (*D*) Analytical gel filtration of ABCE1^{E485Q} (5 μM) was performed as described in (*B*).

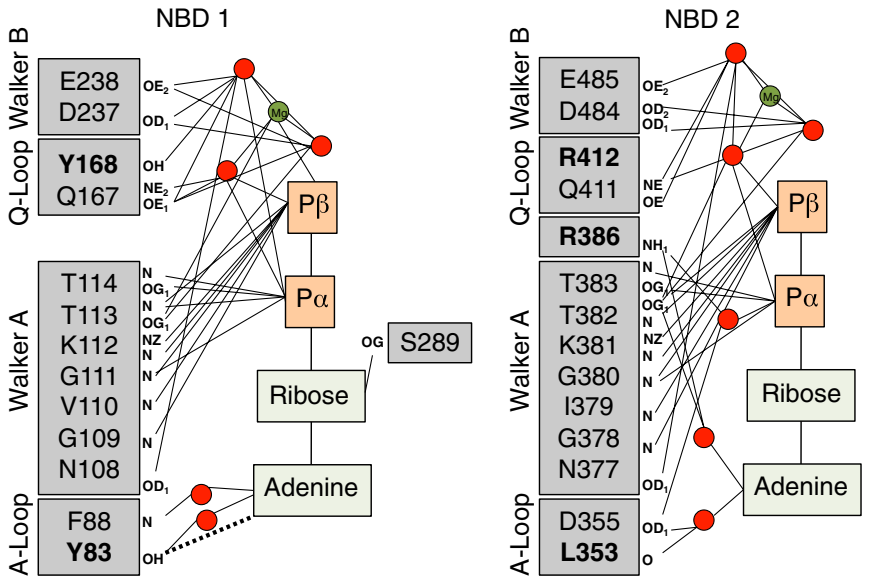

Fig. S2. Contact diagram of the two bound ADP molecules in NBD1 and NBD2 of ABCE1. Residues are labeled based on their location in ABC motifs. Water molecules and magnesium ions are labeled as red and green dots, respectively. Interactions include electrostatic interaction, van der Waals contacts (≤3.6 Å), hydrogen bonds (≤3.3 Å), and π-π electron stacking.

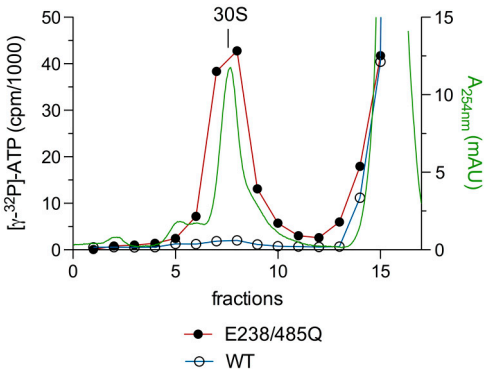

Fig. S3. ABCE1 in its ATP occluded state binds to the 30S ribosome. Isolated 30S subunits (0.5 μM) were incubated at 73 °C for 5 min with different ABCE1 mutants (1 μM) and ATP (2 mM traced with [γ-^{32}P]-ATP) in 20 mM TEA-HCl, pH 7.2, 10 mM KCl, 20 mM $MgCl_2$. After cooling down on ice for 2 min, 200 μL of the reaction volume was loaded onto a TSK4000 SW_{XL} column (Tosoh Bioscience) with a flow rate of 0.4 mL/ min. 500 μL fractions were collected by simultaneous detection of the absorbance at 254 nm and analyzed by Cerenkov counting. A colocalization of [γ-^{32}P]-ATP stably bound to ABCE1 with the 30S ribosomes was observed for ABCE1$^{E238/485Q}$, whereas no [γ-^{32}P]-ATP colocalization above background could be observed in the presence of ABCE1^{WT}.

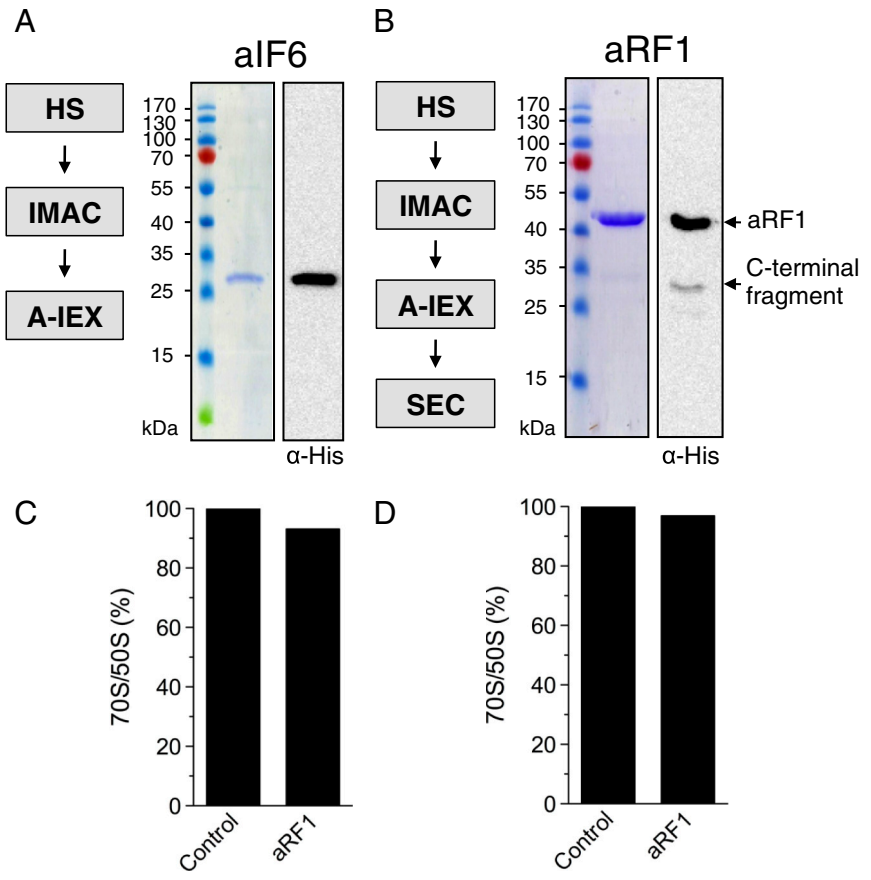

Fig. S4. Isolation of aIF6, aRF1, and effect of aRF1 alone on 70S breakdown. (*A*) Purification strategy for aIF6 expressed in *E. coli*. After a heat shock at 70 °C, the ribosome antiassociation factor aIF6 was isolated via metal chelate affinity (IMAC) and anion exchange chromatography (A-IEX). (*Left*) Purity is judged higher than 95% by SDS-PAGE (12.5%, Coomassie staining). Immunoblotting determined the identity of His-tagged aIF6. (*B*) Purification strategy for aRF1 expressed in *E. coli*. After heat shock, aRF1 is purified by metal affinity, anion exchange, and size exclusion chromatography with a final yield of 1 mg/L cell culture. Purity was judged to 95% by SDS-PAGE (12.5%, Coomassie staining). Immunoblotting determined the identity of aRF1. A C-terminal fragment of aRF1 (23 kDa) was copurified. (*C*) Recombinant aRF1 (1 μM) was added to lysates programmed for translation. After 2 min incubation, samples were cross-linked on ice and analyzed by SDG fractionation. The 70/50S ratio without addition of recombinant aRF1 (control) was set to 100%. (*D*) Isolated 70S ribosomes (1 μM) from *T. celer* were incubated with aRF1 at 73 °C for 4 min. Ribosome reassociation was prevented by addition of aIF6 (5 μM). Data were analyzed by SDG fractionation and calculating the 70S/50S ratio. The reaction in the absence of added aRF1 served as control and the initial 70S/50S ratio was normalized to 100%.

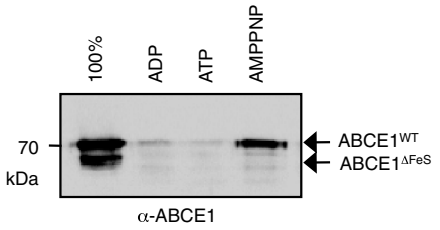

Fig. S5. Nucleotide-dependent binding of ABCE1 to *T. celer* ribosomes. Ribosome pelleting assays of $ABCE1^{WT}$ (5 μM) and isolated 30S subunits (0.5 μM) from *T. celer*. The samples were incubated at 73 °C for 5 min in the presence of various nucleotides (2 mM of each). After ribosome pelleting, bound proteins were analyzed by immunoblotting using a polyclonal anti-ABCE1 antibody. In the control lane (100%), the amount of ABCE1 expected for a 1:1 binding (0.5 μM) to the 30S particle was loaded.

Table S1. Steady-state kinetic parameters of the ATP hydrolysis rate of various ABCE1 mutants

Protein	k_{cat} (min^{-1})*	K_M (mM)*	k_{cat}/K_M (M^{-1} sec^{-1})	Hill coefficient n†
WT	12.5 ± 0.5	0.68 ± 0.05	301	1.03 ± 0.06
ΔFeS	12.3 ± 0.3	0.62 ± 0.11	336	1.20 ± 0.18
C54S	10.3 ± 1.4	0.65 ± 0.17	263	1.12 ± 0.23
C24S	n.d.‡	n.d.	n.d.	n.d.
E238Q	4.7 ± 0.1	0.20 ± 0.06	392	0.97 ± 0.11
E485Q	122.1 ± 5.8	0.76 ± 0.12	2679	1.01 ± 0.05
H269A	n.d.	n.d.	n.d.	n.d.
H518A	124.0 ± 4.4	0.61 ± 0.08	2952	1.24 ± 0.11

*Values were obtained by fitting the experimental data to the Michaelis–Menten equation: $V = V_{max}/(1 + K_M/[S])$.
†The Hill coefficient (n) was determined by using the Hill form of the Michaelis–Menten equation: $V = V_{max}/(1 + K_M/[S])^n$.
‡Not determined.

Table S2. Data collection and refinement statistics

	ADP bound ABCE1$^{\Delta FeS\text{-}E238/485Q}$
Data collection	
Space group	P1
Cell dimensions	
a, *b*, *c* (Å)	58.4, 63.7, 81.8
α, β, γ (°)	89.2, 85.4, 69.8
Resolution (Å)	50.0–2.0 (2.05–2.0)
Completeness (%)	91.3 (92.8)
R_{sym}	4.8 (47.1)
$I/\sigma I$	10.1 (1.7)
Redundancy	1.4 (1.3)
Wavelength	0.979
Refinement	
Number of reflection	59761
Resolution	20.00–2.05
R_{work}/R_{free}	0.186/0.244
Number of atoms	8765
Protein	8245
Ligand/ion	108/4/5
Water	403
B factors	37.91
Rmsd bonds (Å)	0.016
Rmsd angles (°)	1.672
Ramachandran plot statistics	
Favored/allowed/unfavorable (%)	97.5/2.5/0.0

Values in parentheses are for the highest-resolution bin (2.05–2.0 Å) $R_{work} = \Sigma_h||F_{obs}(h) - F_{calc}(h)||/\Sigma_h|F_{obs}(h)|$, calculated over the 95% of the data in the working set. R_{free} is equivalent to R_{work} except that the factor is calculated over 5% of the data assigned to the test set. Geometry statistics were calculated with MOLPROBITY (11).