The extracellular gate shapes the energy profile of an ABC exporter

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ABC exporters harness the energy of ATP to pump substrates across membranes. Extracellular gate opening and closure are key steps of the transport cycle, but the underlying mechanism is poorly understood. Here, we generated a synthetic single domain antibody (sybody) that recognizes the heterodimeric ABC exporter TM287/288 exclusively in the presence of ATP, which was essential to solve a 3.2 Å crystal structure of the outward-facing transporter. The sybody binds to an extracellular wing and strongly inhibits ATPase activity by shifting the transporter’s conformational equilibrium towards the outward-facing state, as shown by double electron-electron resonance (DEER). Mutations that facilitate extracellular gate opening result in a comparable equilibrium shift and strongly reduce ATPase activity and drug transport. Using the sybody as conformational probe, we demonstrate that efficient extracellular gate closure is required to dissociate the NBD dimer after ATP hydrolysis to reset the transporter back to its inward-facing state.
BC exporters are versatile membrane proteins found in all phyla of life. Type I exporters are the best studied class of ABC exporters and minimally consist of two transmembrane domains (TMDs) each comprising six transmembrane helices and two nucleotide binding domains (NBDs) that are universally conserved among all ABC transporters. The NBDs undergo large conformational changes in response to ATP binding and hydrolysis, which are transmitted to the TMDs via coupling helices to assume inward-facing (IF), outward-facing (OF), and outward-occluded (Occ) conformations.1 Alternating access to the TMDs in conjunction with affinity changes towards the transported substrates enable uphill transport across the lipid bilayer.2 Fully closed NBDs are stabilized by two ATP molecules bound at the dimer interface and coincide with TMDs adopting an OF or Occ state.3,4 The transition to the IF state requires the NBDs to separate at least to some degree, a process that is initiated by ATP hydrolysis.5

Many ABC exporters including the entire human ABCCC family exhibit asymmetric ATP binding sites, namely a degenerate site that can bind but not hydrolyze ATP and a consensus site that is hydrolysis-competent.6 Heterodimeric TM287/288 of the thermophilic bacterium Thermotoga maritima was the first structurally analyzed example of an ABC exporter with a degenerate site.7,8 Two closely related IF structures of TM287/288 were solved by X-ray crystallography either containing one AMP-PNP molecule bound to the degenerate site or no nucleotide. In contrast to most other IF structures of ABC exporters, the opened NBDs of TM287/288 are only partially separated due to contacts mediated by the degenerate site D-loop, whereas the consensus site D-loop was found to allosterically couple ATP binding at the degenerate site to ATP hydrolysis at the consensus site.9 The consensus site features distortions in the Walker B motif, which prevents nucleotide binding in the IF transporter.2 DEER studies have revealed that TM287/288 exhibits dynamic IF/OF equilibria in the presence of nucleotides and that nucleotide trapping at the consensus site is required to strongly populate the OF state, whereas in the presence of AMP-PNP the transporter predominantly adopts its IF state.9

Broad distance distributions were found by DEER in the extracellular gate of TM287/288, hinting at conformational flexibility in this external region.9 Similar observations were reported for ABCB1.10 Unbiased Molecular Dynamics (MD) simulations of TM287/288 uncovered spontaneous conformational transitions from the IF state via an Occ intermediate to the OF state.11 Many simulations remained trapped in the Occ state, suggesting that extracellular gate opening represents a major energetic barrier in the conformational cycle. Interestingly, the degree of extracellular gate opening varies greatly among different type I ABC exporters solved in OF states, whereas the gate remains closed in the Occ state.3,4,12 Hence, events occurring at the extracellular gate likely play a key role in substrate transport and must be allosterically coupled to the catalytic cycle of the NBDs. Nevertheless, the underlying molecular mechanism is unknown.

In this work, we generated single domain antibodies that exclusively bind to OF TM287/288 and thereby inhibit the transport cycle. The binders were instrumental to solve a crystal structure of the transporter in its OF state and were used to probe molecular events at the extracellular gate and their allosteric coupling with the NBDs.

**Results**

**Conformational trapping of TM287/288.** Having solved two closely related IF structures of TM287/288, our aim was to obtain an atomic structure of this heterodimeric ABC exporter in its OF state. DEER analyses revealed that TM287/288 carrying the TM288E517Q mutation in the Walker B motif of the consensus site (EtoQ mutation) was almost completely trapped in the OF state in the presence of ATP-Mg or ATPyS-Mg. To further decrease the residual ATPase activity of the EtoQ mutant (turnover of 0.02 min⁻¹) by a factor of 6.5, we instead introduced the EtoA mutation. In addition, we generated single domain antibodies (nanobodies) that exclusively recognize the OF state of TM287/288. To this end, alpacas were immunized with OF TM287/288 containing a cross-linked tetrahelix bundle motif (see Methods). This approach yielded nanobody Nb_TM#1 binding exclusively to TM287/288 in the presence (but not in the absence) of ATP, as shown by surface plasmon resonance (SPR) (Fig. 1d). However, crystals obtained with Nb_TM#1 did not diffract well enough to build a reliable model. Therefore, we selected synthetic nanobodies (sybodies) against TM287/288 (EtoA) in the presence of ATP-Mg completely in vitro.14 Thereby, more than ten OF-specific sybodies were generated and sybody Sb_TM#35 was successfully used to solve the OF structure of TM287/288(EtoA) in the presence of ATPyS-Mg at 3.2 Å resolution (Fig. 1a, Supplementary Table 1).

**Structure of TM287/288-sybody complex.** Sybody Sb_TM#35 binds on top of an extracellular wing of TM287/288 (Fig. 1a) and was crucially involved in establishing crystal contacts (Supplementary Fig. 1). Binding is mediated by aromatic residues of all three complementarity determining regions (CDRs) of the sybody, which are wedged between transmembrane helices (TMs) 1 and 2 of TM287 and TMs 5′ and 6′ of TM288 (Fig. 2a). Since Sb_TM#35 only binds in the presence of ATP (Fig. 1d), we hypothesized that it interferes with the catalytic cycle of the transporter. Indeed, the sybody inhibited the ATPase activity of TM287/288 in detergent (IC50 of 66.1 nM, Fig. 2b), as well as reconstituted in nanodiscs (Supplementary Fig. 2b). Of note, inhibition was less efficient in nanodiscs, presumably due to impaired epitope accessibility of the sybody in the membrane context.

**Two nanobodies addressing epitopes on the NBDs.** Using the high resolution structure of OF TM287/288 for molecular replacement and as template for model building, we solved two additional low resolution structures (3.5–4.2 Å) of the OF transporter determined in complex with alpaca nanobodies Nb_TM#1 and Nb_TM#2 (Fig. 1b, c). Nb_TM#1 specifically recognizes the OF state and binds to the bottom of the closed NBD dimer, occupying an epitope that is shared between NBD287 and NBD288 (Fig. 1d). Akin to Sb_TM#35, state-specific Nb_TM#1 was found to inhibit the transporter’s ATPase activity (Fig. 2b). Nb_TM#2 binds side-ways to NBD288 and exhibits picomolar affinity for the transporter regardless whether ATP is present or not (Fig. 1d, Supplementary Table 2). Nevertheless, this nanobody partially inhibits ATPase activity by around 30% already at the lowest assayed concentration of 20 nM (Fig. 2b). Because the TM287/288 concentration needed to be at least 8 nM to reliably measure ATPase activity, we could not determine the IC50 value for Nb_TM#2. A measurement artifact can be excluded, because an unrelated control sybody did not affect the transporter’s ATPase activity (Fig. 2b).

**IF to OF transition renders TM287/288 more symmetric.** The OF structure of TM287/288 features fully dimerized NBDs that sandwich two ATPyS-Mg molecules at the degenerate and the consensus site (Fig. 3a, Supplementary Fig. 3). An almost identical structure (RMSD of 0.21 Å) was also obtained in the presence of ATP-Mg (Supplementary Fig. 4c, Supplementary Table 1). In contrast to the NBDs of IF TM287/288, which exhibited pronounced asymmetries between degenerate and
consensus site mainly with regard to the D-loops, the closed NBD dimer of the OF transporter is more symmetric (Fig. 3a, Supplementary Fig. 3). Further, the distortions found at the catalytic dyad of the consensus site of the IF structure (E517TM288 and H548TM288) relax during the transition to the OF state and the two key residues adopt a hydrolysis-competent arrangement (Fig. 3b). Interestingly, two tunnels that would allow for release of the cleaved γ-phosphate are present at the consensus site (Fig. 3c). The TMDs consisting of two wings each encompassing six transmembrane helices donated from both protomers are widely opened towards the outside (Supplementary Fig. 4). With an RMSD of 1.73 Å, the structure of TM287/288 most closely resembles the structure of Sav1866. Furthermore, the OF structure is similar to the OF conformation of TM287/288 predicted by MD simulations (Supplementary Fig. 5), although in the MD simulations the protein was embedded in a lipid bilayer instead of the detergent environment used for crystallization. Also the degree of NBD closure and extracellular gate opening is highly similar between TM287/288 and Sav1866 (Supplementary Fig. 6a). The RMSD between TM287 and TM288 decreases from 2.55 Å to 1.98 Å as the transporter is converted from the IF to the OF conformation, indicating that OF TM287/288 is more symmetric (Supplementary Fig. 6b). Whereas a similar degree of symmetry was observed between the half-transporters of OF ABCB1 (PDB: 6C0V, RMSD of 2.07 Å), the equivalent superimpositions exhibit substantial asymmetries in the OF structure of MRP1 (PDB: 6BHU, RMSD of 4.54 Å), mostly owing to asymmetries in the TMDs (Supplementary Fig. 6b). Extracellular gate opening is less pronounced in MRP1 and even less so in ABCB1, and the gate remains almost completely closed in the outward-occluded structure of McjD (Supplementary Fig. 6b). Hence, structures of OF and Occ ABC exporters show their largest structural variation in the extracellular gates.

The sybody acts as a molecular clamp. Interestingly, we did not find steric clashes which would prevent Sb_TM#35 from binding to the IF transporter. Hence, based on structural information

![Diagram](https://example.com/diagram.png)
alone we could not explain why the sybody inhibits ATPase activity. Therefore, we used DEER spectroscopy to unravel the sybody’s impact on the conformational cycle.

The sybody was found to shift the transporter’s equilibrium towards the OF state, as measured in the presence of ATP-EDTA (arrows in Fig. 2c). Pronounced effects were observed in the extracellular region (54 TM287/290 TM288 and 54 TM287/271 TM288), but also when probing distances at the intracellular region of the TMDs (131 TM288/248 TM288) and at the NBDs (460 TM287/363 TM288) (Fig. 2c and Supplementary Fig. 7). Further, we observed a distance increase between two spin labels positioned in the wing underneath the sybody (54 TM287/290 TM288) as a result of sybody binding (dotted vertical lines in Fig. 2c). This suggests that the sybody acts as a wedge at the opened extracellular wing. As expected from the lack of sybody binding to the IF state, we observed negligible effects on the interspin distances when TM287/288 was incubated with the sybody in the absence of nucleotides (apo state) (Supplementary Fig. 7).

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**Fig. 2** The sybody traps TM287/288 in its OF state. a Sybody Sb_TM#35 is shown as cartoon in gray with the CDR1, 2 and 3 highlighted in yellow, orange and red, respectively. Four aromatic residues (Y33, W52, Y59, and W113) that wedge between TMs 1 and 2 of TM287 (teal) and TMs 5′ and 6′ of TM288 (magenta) are highlighted as sticks. b Inhibition of TM287/288’s ATP hydrolysis by Sb_TM#35, Nb_TM#1 and Nb_TM#2. A non-randomized sybody served as control. The data were fitted with a hyperbolic decay function to determine IC50 values, as well as residual activities. The error bars are standard deviations of technical triplicates. c, d DEER analyses of spin-label pairs introduced to probe the extracellular and intracellular TMDs and the NBDs (c), as well as sybody binding to the transporter (d). DEER traces were recorded in the presence of ATP-EDTA with or without unlabeled Sb_TM#35 (c) or in the presence of ATP-EDTA and spin-labeled Sb_TM#35 (d). The graphs show experimental distance distributions, and vertical dotted lines shown in c highlight changes in the mean distances.
To investigate the positioning of the bound sybody relative to the opposite wing, we then focused on the distance between the sybody labeled at position 71 and spin labels introduced either at 54TM287 (the sybody-binding wing) or 27TM287 (opposite wing) of the transporter (Fig. 2d and Supplementary Fig. 8). The main distance peak corresponding to dipolar coupling between 71Sb_TM35 and 54TM287 was very sharp and centered at 3.8 nm, while it was somewhat broader and centered at 3.2 nm between 71Sb_TM35 and 27TM287 placed on the opposite wing. Both distances were visible only in the presence of ATP, and were in close agreement with the simulations based on the OF structure (Supplementary Fig. 8). Both traces also contained a distance peak at around 5.2 nm corresponding to a residual fraction of sybody dimers in solution. In conclusion, the sybody acts as a molecular clamp that keeps the extracellular gate open.

Conserved aspartates seal the extracellular gate. Having shown that the sybody traps the transporter in a fully opened state, we reasoned that mutations facilitating extracellular gate opening would have a similar impact on the transporter’s energy landscape. In IF TM287/288, D41TM287 and D65TM288 placed in TM1 of the respective half-transporter establish hydrogen bonds with backbone amides of the opposite wing (Fig. 4a). Of note, these aspartates are conserved in bacterial ABC exporters (Fig. 4b), but not in eukaryotic members of the family. When the aspartates were substituted with alanines, the ATPase activity of TM287/288 decreased around three-fold for the single mutants and around 10-fold for the double mutant (henceforth called 2xDtoA mutant) (Fig. 4c).

Using again ATP-EDTA to induce the IF to OF transition, DEER analyses revealed an equilibrium shift towards the OF state in the 2xDtoA mutant for all spin-labeled pairs (Fig. 4f and Supplementary Fig. 9). The equilibrium shift was similar to that induced by Sb_TM35. Hence, the aspartates at the extracellular gate constitute an energy barrier that needs to be overcome to switch to the OF state and influence the ATPase cycle in a long-ranged allosteric coupling connecting the extracellular gate with the NBDs.

To probe the atomic details of the conformational dynamics underlying the IF–OF transition, we performed MD simulations of TM287/288 in a POPC lipid bilayer starting from the IF crystal structure (PDB: 4Q4A), after docking a second ATP-Mg molecule into the consensus site11 and introducing the 2xDtoA mutations in the extracellular gate. As in our previous MD simulations of wild-type TM287/28811, we observed spontaneous large-scale conformational transitions from the IF conformation via an Oc state to an OF conformation; this complete transition was observed in 3 out of 20 independent 500 ns simulations (Supplementary Fig. 10). Despite the limited statistics, the transition appears to be slightly more frequent than for the wild-type (6 out of 100 simulations11), in agreement with our experimental results and the notion that the polar contacts of the two aspartate residues increase the energy barrier of extracellular gate opening. Although MD simulations and experimental data are in agreement, we cannot exclude different results if these rather long simulations were conducted in a lipid bilayer containing other lipids such as for example POPE15. To assess the stability of the OF structure reported in this work, 20 independent 400 ns simulations were carried out for both the wild-type and the 2xDtoA mutant. Although the sybody is not present in the simulations, the OF conformation with two bound ATP-Mg molecules is very stable and merely fluctuates around the X-ray structure (Supplementary Fig. 11). Additional control simulations in a POPE (instead of POPC) bilayer confirmed this result (Supplementary Fig. 11), rendering it unlikely that lipid composition (in terms of PC vs. PE head groups) has a large effect on the ATP-Mg-bound OF structure.

Next, we introduced the 2xDtoA mutations into the heterodimeric ABC exporter EfrEF of Enterococcus faecalis (Fig. 4b)16. Ethidium-stimulated ATPase activity profiles of membrane reconstituted EfrEF were found to be strongly affected by the single DtoA mutations, and the ATPase activity of EfrEF containing the 2xDtoA mutations could no longer be stimulated by the drug (Fig. 4d). Supporting this notion, the TM287/288(2xDtoA) mutant reconstituted in nanodiscs exhibited strongly diminished drug stimulation by Hoechst 33342...
Walker B mutant E515QEfrF or the extracellular gate mutants D41AEfrE and D50AEfrF or the corresponding double mutant (2xDtoA).

The single mutants D41AEfrE and D50AEfrF and the corresponding double mutant (2xDtoA) reconstituted into proteoliposomes determined in the absence of ethidium and in the presence of ethidium relative to wild-type TM287/288 determined in detergent.

The error bars are standard deviations of technical triplicates. The basal activity was determined for wild-type transporter and for TM287/288(2xDtoA).

Ethidium fluorescence measurements (Fig.4e). Wild-type EfrEF containing the EtoQ mutation in the NBDs served as negative control exhibiting high ethidium accumulation levels.

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Ethidium fluorescence measurements (Fig.4e). Wild-type EfrEF containing the EtoQ mutation in the NBDs served as negative control exhibiting high ethidium accumulation levels.

Data were normalized to the basal ATPase activity of the respective mutant.

The single mutants D41A {EfrE} and D50A {EfrE} and the corresponding double mutant (2xDtoA) reconstituted into proteoliposomes determined in the absence (basal activity) or in the presence of ethidium at the concentrations indicated. Data were normalized to the basal ATPase activity of the respective mutant.

Wild-type transporter is sufficient expels ethidium from the cell, resulting in a slow increase of ethidium accumulation that reaches a low steady-state level. EfrEF containing the EtoQ mutation in the NBDs served as negative control exhibiting high ethidium accumulation levels. The single DtoA mutants D41A {EfrE} and D50A {EfrE} partially lost their capability of ethidium efflux. Interestingly, the accumulation curve of the 2xDtoA mutant does not reach a steady-state level within the time frame of the experiment. This observation suggests a transporter defect resulting in passive influx of ethidium into the cell mediated by EfrEF carrying the 2xDtoA mutations. In conclusion, the extracellular aspartates are important gate-keeper residues that are allosterically coupled to the NBDs and are required for substrate transport.

Extracellular gate mutant and sybody are synergistic. Because both sybody binding and weakening of the extracellular gate shifted the conformational equilibrium towards the OF state, we reasoned that these effects are additive. Indeed, with an IC_{50} of 18.4 nM (Fig. 4g), inhibition of TM287/288 carrying the 2xDtoA mutations by Sb_TM#35 was found to be more pronounced than the inhibition of the wild-type transporter (IC_{50} = 66.1 nM) (Fig. 2b). In further agreement, the affinity of Sb_TM#35 towards the 2xDtoA mutant (K_{D} = 14 nM) was around eight times higher than towards the wild-type transporter (K_{D} = 110 nM) (Supplementary Fig. 12, Supplementary Table 2). Affinity was as well increased upon introduction of the EtoA or EtoQ mutation into the consensus site of the transporter (K_{D} = 66 nM) which as well exhibit a conformational equilibrium shift towards the OF state and was highest for the combined triple mutant (2xDtoA/EtoA) (K_{D} = 8 nM). An analogous pattern was observed for Nb_TM#1,
which binds to the closed NBD dimer. The $IC_{50}$ was substantially smaller when probing the $2xDtoA$ mutant ($K_D = 16.8 \text{ nM}$) compared to wild-type TM287/288 ($K_D = 460 \text{ nM}$) (Fig. 2b, Fig. 4g). This difference was again reflected by an affinity increase for the $2xDtoA$ mutant (37 nM) vs. the wild-type transporter ($K_D = 184 \text{ nM}$) and the highest affinity was observed for the triple mutant ($K_D = 5 \text{ nM}$) (Supplementary Fig. 12, Supplementary Table 2). In conclusion, Sb_TM#35 and Nb_TM#1 bind to the opposite ends of the transporter but nevertheless exhibit a highly similar biophysical behavior of trapping the OF transporter.

Probing the OF–IF conversion by state-specific binders. We finally tested whether the state-specific nanobodies could be used as probes in SPR to investigate the OF–IF transition of TM287/288. When immobilized TM287/288(EtoA) was charged with ATP-Mg and subsequently washed with buffer devoid of nucleotides, the maximal SPR binding signal for the OF state-unspecific nanobody Nb_TM#2 was used as a control.

Discussion

In this work we unleashed the power of state-specific single domain antibodies obtained from alpacas and entirely in vitro from synthetic libraries to investigate a membrane transporter at the structural and functional level. The strategy of generating state-specific binders against type I ABC exporters has a long history going back to the 90’s of the last century, when the state-specific ABCB1 antibody UIC2 was identified\cite{18}. A recent
nanobodies raised against ABCB1 and PglK, which both sterically prevent extracellular gate opening can be partial while NBD closure is complete. A cyclic peptide raised against CmABCB1 further clamping of the closed extracellular gate was also achieved by the antibody clamps the extracellular loops together, cryo-EM structure of ABCB1 in complex with UIC2 revealed that the antibody clamps the extracellular loops together, thereby preventing extracellular gate opening. Molecular clamping of the closed extracellular gate was also achieved by a cyclic peptide raised against CmABCB1. Further examples of binders that prevent the IF–OF conversion are nanobodies raised against ABCB1 and PglK, which both sterically clash with NBD closure. In contrast, our binders are specific for the OF state and consequently impede the OF–IF conversion.

Sybody binding to an extracellular wing of TM287/288 was essential to solve the OF structure. Both the degenerate and the consensus ATP binding site are fully closed and highly symmetric, but only the consensus site bears the catalytic dyad positioned to catalyze ATP hydrolysis. This suggests that ATP hydrolysis of only one nucleotide is sufficient to initiate dissociation of the NBDs. In further support of this view, the closed NBD dimers feature two possible $P_i$ exit tunnels at the consensus site.

A comparison with other OF and Occ transporter structures revealed major conformational heterogeneity in the degree of extracellular gate opening, which has been discussed to play a potential role in squeezing out substrates or to prevent rebinding of substrates. In this study, we uncover cross-talk between the extracellular gate and the ATPase cycle, a connection that has to the best of our knowledge not yet been investigated at the molecular level (Fig. 6). A sybody stabilizing the opened extracellular wing or mutations weakening the extracellular gate both shifted the conformational equilibrium towards the OF state. Previous experimental and computational studies have uncovered that NBD closure precedes extracellular gate opening during the IF–OF transition, and DEER analyses have revealed that extracellular gate opening can be partial while NBD closure is complete. Hence, there seems to be an inbuilt mechanical principle that the extracellular gate is energetically costly to open. Conversely, using our state-specific nanobodies as conformational probes we were able to show that extracellular gate closure is coupled to the dissociation of the closed NBD dimer after ATP hydrolysis. Our experiments on EfrEF demonstrated that a firmly sealed extracellular gate is in fact crucial for transporter function. Further, the 2xDtoA mutant had a strongly reduced ATPase activity and lost its capacity to be stimulated by drugs. This suggests that extracellular gate closure has become the rate-limiting step of the catalytic cycle of the 2xDtoA mutant. Hence, in this mutant the IF–OF conversion is no longer rate-limiting and consequently cannot be stimulated by drug binding to the inward-oriented high-affinity site (Fig. 6).

The ATP-bound OF state with completely closed NBDs has been referred to as the high-energy state of the transport cycle and in some instances it was proposed that ATP hydrolysis is needed to populate the OF state at all. In contrast, we and others have previously stipulated that ATP binding alone is sufficient for IF–OF conversion and substrate release, in agreement with the ATP-switch model. It should be noted that while the opened extracellular gate indeed represents a high-energy state, the opposite is in fact true for the closed NBD dimer. It adopts a low-energy state and a large energy input is required to dissociate the dimer. This is certainly achieved in part by the hydrolysis of ATP. Importantly, our results suggest that NBD dissociation also involves a mechanical component mediated by extracellular gate closure. A further possibility is the triggering of ATP hydrolysis as a result of extracellular gate closure. Although speculative and neither directly supported nor excluded by our data, such a mechanism would assure that the transporter only reverts to the IF state after substrate release.

Why and when ATP hydrolysis is required to achieve active transport is a recurrent debate in the ABC transporter field. Our data presented here and in previous studies, clearly suggest that ATP binding alone (in case of ATP-EDTA when ATP hydrolysis cannot occur) is sufficient for the IF–OF conversion and presumably the active transport of one substrate molecule. Directionality of transport is then achieved by an affinity switch of the substrate binding site, which inevitably undergoes drastic rearrangements as the TMDs switch from an IF to an OF conformation. Nevertheless, it is unlikely that an ABC exporter can operate efficiently by binding and dissociation of ATP alone, because energy input is lacking and the molecular events would be driven by slow stochastic Brownian motions alone. To strongly populate the OF state under physiological conditions, ATP-Mg needs to be occluded at the consensus site of the closed NBD dimer, a state that can be efficiently mimicked by the Walker B EtoQ or EtoA mutation. As we show here with our binder probes, ATP occlusion firmly traps the transporter in the OF state and prevents transporter cycling. Hence, ATP hydrolysis appears to be strictly required to initiate dissociation of the closed NBD dimer. Once ATP is hydrolyzed, the force exerted by the closed extracellular gate facilitates NBD dissociation. In summary, our results support the notion that ATP hydrolysis is required to drive the transport cycle at the resetting step from the OF to the IF state.

We hope that our results provide a mechanistic framework to further study the functional role of the extracellular gate of type I ABC exporters and to investigate the molecular underpinning of disease-causing mutations found in the extracellular region of medically important ABC exporters such as MRP1 and CFTR.

**Methods**

**Expression and purification.** The genes encoding the heterodimeric ABC transporter TM287/288 were amplified and cloned into pLNT_cat (addgene: Plasmid #46858). The genes were subcloned into pBAD expression vectors by FX cloning.
for crystallization and biochemical analyses into the pBXNH3 expression vector (MWCO) of 50 kDa. Puriﬁed proteins were all analyzed by SEC and did not differ in terms of elution proﬁle and yield from the wild-type transporter.

**Crytalization.** For crystallization of TM287/288(EtoA) or TM287/288(2xDtoA/EtoA) in complex with Sh TM#41, puriﬁed Nb TM#41 (stored at ~80 °C) was added to the transporter puriﬁed in 0.3% (w/v) β-DM at a 1.2-fold molar excess prior to size-exclusion chromatography. After short incubation on ice, the complex was separated from excess nanobodies by size-exclusion chromatography using a Superdex 200 Increase 10/300 GL (GE Healthcare) column equilibrated in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.3% (w/v) β-DM, 3 mM MgCl2, 1.25 mM ATPγS, or 5 mM ATP and 25% (v/v) ethyleneglycol and flash-frozen in liquid nitrogen.

For crystallography of TM287/288(2xDtoA/EtoA) in complex with Nb TM#41, Nb TM#41 was added to the transporter puriﬁed in 0.3% (w/v) β-DM in a 1.2-fold molar excess prior to size-exclusion chromatography. After short incubation on ice, the complex was separated from excess nanobodies by size-exclusion chromatography using a Superdex 200 Increase 10/300 GL (GE Healthcare) column equilibrated in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.3% (w/v) β-DM. The transporter/nanobody complexes were concentrated to 10 mg/mL using an Amicon Ultra-4 concentrator unit (50 kDa MWCO) and incubated with 5 mM ATPγS and 3 mM MgCl2 for 15 min on ice. Crystals were grown by the vapor diffusion method in sitting drops (1:1 protein to reservoir solution) at 20 °C in 0.1 M sodium acetate pH 9.5, 0.225 M NaCl and 21% (v/v) PEG3350. Crystals appeared within 2–3 days and were grown for another 3 weeks. Crystals were cryo-protected in reservoir solution containing 10% (v/v) PEG400 and flash-frozen in liquid nitrogen.

For crystallization of TM287/288(2xDtoA/EtoA) in complex with Nb TM#2, puriﬁed Nb TM#2 (stored at ~80 °C) was added to the transporter puriﬁed in 0.03% (w/v) β-DM in a 1.2-fold molar excess prior to size-exclusion chromatography. After short incubation on ice, the complex was separated from excess nanobodies by size-exclusion chromatography using a Superdex 200 Increase 10/300 GL (GE Healthcare) column equilibrated in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.3% (w/v) β-DM. The transporter/nanobody complexes were concentrated to 10 mg/mL using an Amicon Ultra-4 concentrator unit (50 kDa MWCO) and incubated with 5 mM ATPγS and 3 mM MgCl2 for 15 min on ice. Crystals were grown by the vapor diffusion method in sitting drops (1:1 protein to reservoir solution) at 20 °C in 0.1 M sodium acetate pH 9.5, 0.225 M NaCl and 21% (v/v) PEG3350. Crystals appeared within 2–3 days and were grown for another 3 weeks. Crystals were stored at ~80 °C.

**Data collection and structure determination.** Diffraction data were collected with a wavelength of 1.0 Å at 100 K at the beamlines X06DA and X06SA at the Swiss Light Source (SLS, Villigen, Switzerland). Diffraction data were processed with the program XDS [40] and truncated using the Diffraction Anisotropy Server with default settings [40] due to strong or severe anisotropy, what lead to improved electron density maps (Supplementary Table 1, Supplementary Fig. 13).

The TM278/288(EtoA) – Sh TM#35 – ATPγS-Mg complex structure was solved by molecular replacement in Phaser [41] using a modiﬁed homology model based on Savi1866 (PDB: 2YHD). The crystals belong to the space group P21, containing two TM278/288 heterodimers and two sybodies per asymmetric unit. The structural model was in good agreement with the electron density maps and cross-links retrieved from cross-linking mass spectrometry experiments (www.globalphasing.com), a polyclonal model of a nanobody (PDB: 1ZVH) was manually placed into additional electron density. Multiple iterations of model building in Coot and TLS reﬁnement in Buster resulted in a ﬁnal model with good geometry (Ramachandran favored/outliers: 96.5%/0.08%) (Supplementary Table 1). Chains A (TM278), B (TM288) and E (Sh TM#35) were used for structural analysis and ﬁgures.

In order to determine the TM278/288(2xDtoA/EtoA) – Sh TM#35 – ATPγS-Mg complex structure, the ﬁnal TM278/288(EtoA) – Sh TM#35 – ATPγS-Mg complex structure was used for reﬁnement against the TM278/288(2xDtoA/EtoA) – Sh TM#35 – ATPγS-Mg complex data. The 2xDtoA mutations were introduced and the ATPγS replaced by ATP in Coot. TLS reﬁnement in Buster resulted in a ﬁnal model with good geometry (Ramachandran favored/outliers: 96.58%/0.24%) (Supplementary Table 1). Chains A (TM278) and B (TM288) were used for structural comparison with the TM278/288(EtoA) – Sh TM#35 – ATPγS-Mg complex structure.
The amount of reconstituted EfrEF variants was determined by quantitative SDS-polymerography using a Superdex 200 Increase 10/300 GL (GE Healthcare) column equilibrated in PBS pH 7.4 and 0.03% (w/v) β-DDM at 4 °C and concentrated to 50 µM with an Amicon Ultra-4 concentrator unit with a MWCO of 50 kDa. 10 mM ATP, 3 mM MgCl₂, and 25-fold molar excess of BMOE over transporter were added and the cross-linking mixture incubated for 3 h at 30 °C. The mixture was diluted 5-fold and incubated with 3 C protease (1:10 w/v) overnight at 4 °C. The cross-linked and Tag-free transporter was reloaded on a Ni-NTA gravity chromatography using a Superdex 200 Increase 10/300 GL (GE Healthcare) column equilibrated in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.03% (w/v) β-DDM. Immunizations of alpacas were approved by the Cantonal Veterinary Office in Zurich, Switzerland (animal experiment licence nr. 188/2011). Blood was collected two weeks after the last injection for the preparation of the hyperimmune serum, which was used to generate sera to generate the VHL/nanobody repertoire. Phage libraries were generated and two rounds of phage display were performed against TM287/288 (2xDtoA/EtoA) sOAs. After the final phage display selection round, 91.9-fold enrichment was determined by qPCR using AcrB as background. Two eluted nanobody libraries were cloned into phD plasmid and 94 single clones were analyzed by ELISA in the presence of 1 mM ATP-Mg. The 27 positive ELISA hits were Sanger sequenced and grouped into four families according to their CDRI length and sequence (among them was Nb_TM#1). In another selection cross-linked TM287/288_cl_L200CTM287/S224CTM288 was used for antibody immobilizations resulting in 49 unique ELISA hits, of which 19 were Sanger sequenced and grouped into four binder families (among them was Nb_TM#2). Sotypes were selected against TM287/288 (ES17A) in β-DDM in presence of 1 mM ATP-Mg with our in vitro selection platform14. After the second round of phage display, single clones were analyzed for binding against TM287/288 (E57A) in presence of 100 mM MgCl₂ by ELISA. Sequencing of 48 ELISA positives resulted in 40 unique sotype sequences14.

Spin labeling for DEER. TM287/288 cysteine variants were expressed as described above and purified by Ni-NTA affinity chromatography in presence of 2 mM DTT. For spin labeling, DTT was removed on a PD-10 column (GE Healthcare, 17-0851-01) equilibrated with 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.03% (w/v) β-DDM and MTSL (1-oxyl-2,2,5,5-tetramethyl-pyrroline-3-methyl)methanethiosulfonate (Titan Research, Inc.) was added in a 10-fold molar excess and incubated at 4 °C for 3 h. After overnight free-spin label was removed by size-exclusion chromatography on a Superdex 200 Increase 10/300 GL (GE Healthcare) column equilibrated in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.03% (w/v) β-DDM at 4 °C. The samples were concentrated to 30–50 µM with Amicon Ultra-4 concentrator units with a MWCO of 10 kDa, flash-frozen into liquid nitrogen, and stored at −80 °C ready for DEER experiments. The pairs 131TM287/291TM288 and 460TM287/363TM288 were already used in previous studies15, whereas the extracellular spin-label pairs 54TM287/271TM287 and 54TM287/290TM288 were constructed as part of this study and their APtase activities were determined (Supplementary Table 3).

For site-specific spin labeling of Sb_TM35, a single cysteine was introduced in the framework of the eSI of the sotype at position 71 by site-directed mutagenesis. Sb_TM35_571C was expressed from pBXNPHM1 as described above. Cells were harvested and resuspended in PBS pH 7.4 and 2 mM DTT supplemented with DNase (Sigma) and disrupted with an M-110 Microfluidizer® (Microfluidics). 20 mM imidazole, supplemented with 1 mM ATP, and 20 µM imidazole, supplemented with 20 mM imidazole and a 20 µM imidazole and 2 mM DTT and eluted with 40 column volumes PBS pH 7.4, 300 mM imidazole and 2 mM DTT.

In a next step Sb_TM35_571C fused to His-tagged MBP was incubated with 3 C protease (1:10 w/v), while dialyzing against PBS pH 7.4 and 2 mM DTT overnight at room temperature. Cleaved sotype was reloaded on a Ni-NTA gravity chromatography column equilibrated with 20 column volumes PBS pH 7.4, 40 mM imidazole and 2 mM DTT, followed by size-exclusion chromatography using a Superdex S200 Increase 10/300 GL (GE Healthcare) column equilibrated in PBS pH 7.4 and 0.03% (w/v) β-DDM at 4 °C and concentrated to 50 µM with an Amicon Ultra-4 concentrator unit with a MWCO of 50 kDa. 10 mM ATP, 3 mM MgCl₂, and 25-fold molar excess of BMOE over transporter were added and the cross-linking mixture incubated for 3 h at 30 °C. The mixture was diluted 5-fold and incubated with 3 C protease (1:10 w/v) overnight at 4 °C. The cross-linked and Tag-free transporter was reloaded on a Ni-NTA gravity chromatography column equilibrated with 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 40 mM imidazole and 0.03% (w/v) β-DDM at 4 °C and concentrated to 1 mg/ml with an Amicon Ultra-4 concentrator unit with a MWCO of 50 kDa and immediately used for alpaca immunizations.

**Nanobody and sotype selections.** For the selection of OF state-specific nanobodies, an alpaca was immunized with subcutaneous injections four times in two weeks by single each time with 200 µg purified cross-linked TM287/288 (2xDtoA) cl_L200CTM287/S224CTM288 in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.03% (w/v) β-DDM. Immunizations of alpacas were approved by the Cantonal Veterinary Office in Zurich, Switzerland (animal experiment licence nr. 188/2011). Blood was collected two weeks after the last injection for the preparation of the hyperimmune serum, which was used to generate sera to generate the VHL/nanobody repertoire. Phage libraries were generated and two rounds of phage display were performed against TM287/288 (2xDtoA/EtoA) sOAs. After the final phage display selection round, 91.9-fold enrichment was determined by qPCR using AcrB as background. Two eluted nanobody libraries were cloned into phD plasmid and 94 single clones were analyzed by ELISA in the presence of 1 mM ATP-Mg. The 27 positive ELISA hits were Sanger sequenced and grouped into four families according to their CDRI length and sequence (among them was Nb_TM#1). In another selection cross-linked TM287/288_cl_L200CTM287/S224CTM288 was used for antibody immobilizations resulting in 49 unique ELISA hits, of which 19 were Sanger sequenced and grouped into four binder families (among them was Nb_TM#2). Sotypes were selected against TM287/288 (ES17A) in β-DDM in presence of 1 mM ATP-Mg with our in vitro selection platform14. After the second round of phage display, single clones were analyzed for binding against TM287/288 (E57A) in presence of 100 mM MgCl₂ by ELISA. Sequencing of 48 ELISA positives resulted in 40 unique sotype sequences14.
removed and buffer exchanged using a PD-10 column (GE Healthcare, 17-0851-1) equilibrated with 20 mM Tris-HCl pH 7.5 and 150 mM NaCl. In order to avoid MTSLS, 10% (v/v) D8-water was added to 20 mM Tris-HCl pH 7.5 and 150 mM NaCl. Spin-labeled sybodies were concentrated to the desired concentration with an Amicon Ultra-4 concentrator unit with a 3 kDa MWCO and flash-frozen in liquid nitrogen and stored at −80 °C. Site-specific labeling was confirmed and quantified by mass spectrometry.

DEER measurements. The labeling efficiency of the double cysteine mutants of the transporters solubilized in detergent was measured by comparing the second integral of the spectra detected at 25 °C using an X-band Miniscope 1200 EPR spectrometer (Magnettech by Freiberg Instruments) with that of a standard TEMPO solution in water. The calculated spin labeling efficiencies of the twelve mutants ranged between 80% and 90%. For DEER measurements, 10% (v/v) D$_8$-spectrometer (Magnettech by Freiberg Instruments) with that of a standard transporter solubilized in detergent was measured by comparing the second DEER measurements sinked in 50 mM K-HEPES pH 7.0.

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...with homogeneous dimensions of 1.8 to 3 for the mixture with an Amicon Ultra-4 concentrator unit with a 3 kDa MWCO and concentrated in 50 mM sodium cholate, 100 mM NaCl, and 100 mM mcholate to a final concentration of 38 mg/ml (50 mM), and filtered using a 0.22 µm filter. Lipids ready for nanodisc reconstitutions were stored at −80 °C.

Purified, biotinylated TM287/288 variants solubilized in β-DDM were reconstituted in nanodiscs using a 240:8:1 molar ratio of lipids:MSPI13D1:TM287/288. In order to spare membrane protein, the ideal lipid:MSPI13D1 ratio was determined beforehand by reconstituting empty nanodiscs. Different ratios were tested, ranging from 25:1 to 40:1 (lipid:MSPI13D1). Empty nanodiscs were loaded on a Superdex 200 Increase 10/300 GL (GE Healthcare) column to separate empty nanodiscs from monomeric MSPI13D1 and aggregates. From the elution profile the optimal lipid:MSPI13D1 ratio of 30:1 was determined. For MD simulations, the ATP-bound EfrEF structure was initially recycled by adjusting the Mg-ATP concentration in the incubation buffer to 10 mM MgCl$_2$ and 2.5 mM MgCl$_2$ to maintain Mg-ATP at a 1:1 stoichiometric ratio. Detergent molecules were removed by four rounds of adding and purifying using stretched exponential functions with homogeneous dimensions of 1.8 to 3 for the mixture with an Amicon Ultra-4 concentrator unit with a 3 kDa MWCO and concentrated in 50 mM sodium cholate, 100 mM NaCl, and 100 mM mcholate to a final concentration of 38 mg/ml (50 mM), and filtered using a 0.22 µm filter. Lipids ready for nanodisc reconstitutions were stored at −80 °C.

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Data availability
Data supporting the findings of this manuscript are available from the corresponding author upon reasonable request. A reporting summary for this Article is available as a Supplementary Information file. The source data underlying Figs. 2b, 4c, d, and Supplementary Figs. 2a, b are provided as a Source Data file. The coordinates of the TM28/288 structures have been deposited under accession numbers 6QV0 (Sh_TM35, ATPγS-bound), 6QV1 (Sh_TM28, ATP-bound), 6QV1 (Nb_TM1) and 6QV2 (Nb_TM2). Sbbody Sh_TM35 and nanobodies Nb_TM1 and Nb_TM2 will be distributed for academic research upon reasonable request.

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Author contributions
C.A.J.H., E.B., and M.A.S. conceived the study. C.A.J.H., I.Z., P.E., and S.S. selected nanobodies and sybodies against TM287/288. C.A.J.H. purified and crystallized the protein complexes and solved their structure. L.M.H. conducted the functional and biochemical experiments with EfrEF. C.A.J.H. and L.M.H. conducted all functional experiments with TM287/288. C.A.J.H. cloned, purified, and labeled all samples for DEER analyses. M.H.T. conducted most of the DEER analyses and simulations and discussed the results with E.B. S.K. conducted the sybody-transporter DEER experiments and discussed them with E.B. H.G. carried out MD simulations and analyzed and interpreted them together with M.K. and L.V.S. C.A.J.H., M.H.T., L.M.H., H.G., and M.A.S. created figures. C.A.J.H., L.V.S., E.B., and M.A.S. wrote the manuscript and all authors edited the manuscript.

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