Mutations in ABC subfamily C member 6 (ABCC6) transporter are associated with pseudoxanthoma elasticum (PXE), a disease resulting in ectopic mineralization and affecting multiple tissues. A growing number of mutations have been identified in individuals with PXE. For most of these variants, no mechanistic information is available regarding their role in normal and pathophysiological processes. To assess how PXE-associated mutations alter ABCC6 biosynthesis and structure, we biophysically and biochemically evaluated the N-terminal nucleotide-binding domain. A high-resolution X-ray structure of nucleotide-binding domain 1 (NBD1) of human ABCC6 was obtained at 2.3 Å that provided a template on which to evaluate PXE-causing mutations. Biochemical analysis of mutations in this domain indicated that multiple PXE-causing mutations alter its structural properties. Analyses of the full-length protein revealed a strong correlation between the alterations in NBD properties and the processing and expression of ABCC6. These results suggest that a significant fraction of PXE-associated mutations located in NBD1 causes changes in its structural properties and that these mutation-induced alterations directly affect the maturation of the full-length ABCC6 protein.

Pseudoxanthoma elasticum (PXE)3 is a disease of ectopic calcification of elastic tissues, affecting the vasculature, gastrointestinal tract, skin, and eye (1). The mineralization and subsequent degradation of elastic fibers leads to a progressive loss of tone in the affected tissues, resulting in vascular disease, intestinal bleeding, and loss of vision. Genetic and physiological studies demonstrate that PXE is caused predominantly by loss-of-function mutations in the ABCC6 ATP Binding Cassette (ABC) transporter (2, 3). Recent studies suggest that ABCC6 expression is associated with the regulation of circulating pyrophosphates (4–6). However, models of PXE pathophysiology differ with regards to the exact role of ABCC6 in regulating pyrophosphate and mineralization (4, 7–9). Currently, there are few clinically approved therapies that relieve the symptoms of PXE and/or alter the progression of the disease.

ABCC6 is a member of the C subfamily of ABC transporters, which includes the multidrug resistance-associated proteins (MRPs), sulfonyl urea receptor (SUR) and the cystic fibrosis transmembrane conductance regulator (CFTR) (10). The functional protein is composed of five domains: two nucleotide-binding domains (NBD1 and NBD2) and three transmembrane domains (TMD0, TMD1, and TMD2). The cytosolic NBDs bind and hydrolyze ATP, providing the energy for solute movement. Each of the nucleotide-binding domains contains two ATP-binding “half-sites” comprised of canonical Walker A, Walker B, and LSGGQ signature sequences (11). Nucleotide binding leads to the dimerization of the NBDs, resulting in the formation of two complete functional sites formed by the in trans association of the Walker A and B sequences from one monomer with the LSGGQ sequence from the opposing monomer. The fully formed active sites then hydrolyze ATP and dissociate. The ABC subfamily of transporters, including ABCC6, is partially defined by a degenerate Walker B sequence in the N-terminal NBD (NBD1), a substitution of an aspartate for a glutamate, which potentially influences ATP hydrolysis (Walker B: ABCC, ϕϕϕϕϕDD; consensus: ϕϕϕϕϕDE; ϕ, hydrophobic amino acid).

The ATP-mediated association-dissociation reaction leads to conformational changes in the TMDs that are putatively associated with solute transport through the core TMDs (TMD1 and TMD2). The function of the N-terminal TMD (TMD0) is not known, although it has been implicated in regulating ABCC6 trafficking and protein–protein interactions in other ABC family members (ABCC8/SUR1) (12).

Over 200 different mutations have been identified in abcc6, likely impacting all aspects of gene regulation, protein biosynthesis, stability, trafficking, and function (13, 14). The exact molecular pathologies associated with the majority of these mutations are not known. Recent studies suggest that alterations to the biosynthesis and trafficking of ABCC6 influence a
the ABC subfamily and more broadly across the entire family of ABC proteins (Figs. 1 and 2). As with other members of the ABC subfamily, the extended Walker B sequences in NBD1 of ABCC1/MRP1 and ABCC8/SUR1 also deviate from the canonical \( \phi \phi \phi \phi \phi \phi \phi \) sequence with a substitution of Asp for the ultimate Glu residue (22). These sequences coordinate nucleotide triphosphate, water, and Mg\(^{2+}\) ions for the hydrolytic reaction.

Other conserved sequences are also identified in the ABC family alignment. The A-loop Trp residue, which interacts with the bound adenosine moiety via ring stacking interactions, is invariant in the human ABCC NBD1 sequences. Similarly, the Q-loop Gln residue, which serves as a putative switch to sense nucleotide binding and regulate NBD conformational changes, is also invariant. The H-loop catalytic His residue is also conserved across the ABCC NBD1 proteins with the exception of CFTR NBD1.

Two insertion sites are present in the alignment of ABCC NBD1 sequences. A large insertion is seen near the N terminus of ABCC5, CFTR, ABCC11, and ABCC12. This insertion occurs between the first two \( \beta \)-strands and in CFTR is only partially ordered (19). A second insertion site is present in ABCC8 and ABCC9. This insertion is also not ordered in the available structures (20, 21).

Alignments of existing structures were curated using these conserved elements and used to define the N- and C-terminal boundaries of human ABCC6 NBD1. N- and C-terminal domain boundaries were iteratively refined during protein expression, purification, and crystallization trials to optimize soluble protein production in *Escherichia coli* and crystallization. Purification and crystallization studies identified optimal domain boundaries (positions 623–859) for the generation of soluble and stable protein that produced crystals. Using these domain boundaries, a single crystallization condition was identified for ABCC6 NBD1 that diffracted to 2.3 Å and was used for structure determination (Table 1). Crystals formed in 24–72 h in ammonium sulfate at near neutral pH and appeared as single and joined rods with protein concentrations between 10 and 12 mg/ml. Super stoichiometric ATP and Mg\(^{2+}\) was included in all protein buffers.

The ABCC6 NBD1 structure is composed of three subdomains: a short antiparallel \( \beta \)-sheet, an \( \alpha/\beta \)-core, and an \( \alpha \)-helical subdomain (Figs. 2 and 3). The anti-parallel \( \beta \)-subdomain (\( \beta_1, \beta_2, \beta_4, \) and \( \beta_5 \)) contains the highly conserved A-loop sequence associated with ring stacking with the nucleotide (Fig. 3B). The short linker between the first two strands, the insertion site identified in CFTR, ABCC11, and ABCC12, is well resolved in the ABCC6 NBD1 density. The \( \alpha/\beta \)-core is defined by \( \beta_3, \alpha_1, \beta_6–10, \) and \( \alpha_6–9 \) (Figs. 2 and 3A, violet). This domain contains the canonical Walker A and B sequences as well as the H- and D-loops (Fig. 3B). This core region of the NBD is structurally highly conserved across all the NBD proteins. The \( \alpha \)-helical subdomain, critical for mediating TMD–NBD interactions, via the intracellular coupling helices, and signaling is composed of \( \alpha_2–5 \) (Figs. 2 and 3A, green). This domain is highly similar to those of ABCC1/MRP1 and ABCC8/SUR1 when comparing both local structure and sequence similarity (20, 21). This subdomain contains the LSGGQ signature sequence and the structurally diverse region (Figs. 2 and
Figure 2. ABCC subfamily NBD alignment. A multiple sequence alignment for the human NBD1 ABCC domains is shown. Conserved sequence elements associated with ATP binding and hydrolysis are indicated below the alignment. The secondary structure assignments from the ABCC6 NBD1 structure are shown above the alignment with arrows indicating strands and curling loops indicating helices. The secondary structures symbols are colored by subdomain assignment (β-sheet: yellow; β-hairpin: purple; helical: green). Secondary structure numbering was adapted from the HisP NBD structure (PDB code 1B0U). (44)

The figure was prepared with ESPRIPT and BoxShade. (45)

Structural characterization of ABCC6 NBD1

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3, A and B). The structurally diverse region exhibits the highest B-factors in the ABCC6 NBD1 structure, similar to what has been observed in other NBD structures, suggesting increased mobility in the absence of other protein domains (Fig. 3C). The topology of the ABCC6 NBD is organized similarly to the canonical members of the family with re-entrant features in the β and α/β-core subdomains (Fig. 3D). The α-helical subdomain is the only region formed exclusively by continuous primary sequence.

Nucleotide-binding and NBD1 structure

Although millimolar nucleotide was included in the protein buffer, the ABCC6 NBD1 protein crystallized in the nucleotide-free state. Efforts to crystallize NBD1 in the nucleotide-bound state have been, to date, unsuccessful. However, superposition of ABCC6 NBD1 with the solved structures of ABCC1/MRP1 and ABCC8/SUR1 NBD1 provides a means on which to evaluate the nucleotide-dependent conformational changes in these domains (20, 21, 23). Nucleotide binding to the NBDs has been shown to induce a rigid body movement of the α-helical subdomain in many NBDs. ATP binding, sensed by the Q-loop, causes a rotation of the α-helical subdomain by roughly 15° in prototypical NBD models (24). This conformational change is thought to be coupled to NBD–TMD interactions and to propagate the energy of ATP binding and hydrolysis into the TMDs.

Superposition of the ABCC6 nucleotide-free structure, determined here, with the ABCC1/MRP1 structures was performed by aligning the Walker A and Walker B sequences. By constraining the alignment to the Walker A and B sequences, ATP-dependent changes in the position of the α-helical subdomain can be evaluated. Comparison of the isolated ABCC1/MRP1 NBD1 in ATP bound (PDB code 2CBZ) and nucleotide-free (PDB code 4C3Z) states and the full-length ATP-bound structure (PDB code 6BHJ) showed only minimal changes in protein conformation (Fig. 4A and Table 2) (23, 25). The RMSD was <1.0 Å between all NBD1 structures. No significant rotation of the α-helical domain is observed in the comparison of the ABCC6 and ABCC1/MRP1 structures, regardless of nucleotide state (ATP-bound or nucleotide-free). Similarly, superposition of the ABCC6 NBD1 structure with that of ABCC8/SUR1 NBD1 shows only minimal changes in NBD conformation with an RMSD of 1.39 Å across the NBD structure and not including the ABCC8/ABCC9 unique insertion (Fig. 4B and Table 2) (20, 21). Again, the α-helical subdomain showed no significant rotation between the ATP-bound and nucleotide-free states.

Changes immediately around the nucleotide binding site were also assessed. Although no density for the ATP was identified in the ABCC6 NBD1 crystals, a single SO₄ ion was modeled into electron density tightly coordinated by the Walker A sequence. This position would normally be occupied by the β-phosphate of a bound ATP molecule. The SO₄ ion provided a reference by which Mg²⁺-ATP could be modeled into the structure of NBD1 using the nucleotide conformers from ABCC1/MRP1 (Fig. 4C). Minimal reorientation of the ATP molecule from the ABCC1/MRP1 structure (PDB code 2CBZ) was required to fit the adenosine moiety for a native ring-stacking interaction with conserved A-loop Trp (Trp638) and position the β-phosphate into the SO₄ experimental density, as judged by superposition with other ATP-bound structures (23, 25).

Although large rigid body movements were not observed when evaluating the various ABCC NBD1 proteins, comparison of the various nucleotide-free and ATP-bound structures revealed conformational heterogeneity in the position of the catalytic residues across the ABCC proteins. The catalytic histidine (His812) side chain was readily visible in the electron density map of ABCC6 NBD1 and was oriented toward the active site, despite the lack of bound nucleotide (Fig. 4C). The His side chain was observed in multiple rotamers in the superposition of ABCC1/MRP1 and ABCC8/SUR1 structures (Fig. 4D). In contrast, minimal changes in the positions of the Walker B acidic residues were observed. The canonical Walker B Asp residue (Asp777, φφφφDD) was consistently seen oriented toward the active site and in position to coordinate the Mg²⁺ cofactor. The noncanonical Walker B Asp (Asp778, φφφφDD), associated with the ABCC subfamily NBD1 sequences, was oriented away from the active site in the ABCC structures. The side-chain carboxylate was positioned for hydrogen bonding to the backbone amide of the catalytic histidine (His812).

### ABCC6 PXE mutations and NBD1 structural properties

To better understand how NBD1 structural properties might influence the biosynthesis of full-length ABCC6, PXE-associated variants in ABCC6 were selected for biochemical studies. ABCC6 mutations in the Leiden Open Variation Database (http://www.LOVD.nl/ABCC6) and NCBI ClinVar Database were selected for additional characterization in the isolated NBD1 protein. These protein variants display heterologous

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**Table 1**

**Data collection and refinement statistics**

Statistics for the highest-resolution shell are shown in parentheses.

| Data collection | Resolution range (Å) | Space group | Cell dimensions (Å) | Multiplicity | Completeness (%) | Rmerge (%) | Rfree (%) |
|-----------------|----------------------|-------------|---------------------|--------------|-----------------|----------|---------|
|                 | 40.79–2.3 (2.382–2.3)| P 3 2 1     | a, b, c: 94.20, 94.20, 46.11 | 90.90, 120   | 5.94 (5.94)     | 99.85 (100.00) | 6.0 (2.2) |

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presentation across the PXE patient cohort and show only loose genotype-phenotype correlations (26).

NBD folding was first assessed using an in-cell solubility assay, which has previously been used to characterize the folding and misfolding of multiple NBDs from ABC transporters. This system relies on the structural complementation of β-gal as a reporter for the soluble production of the protein of interest (27). The small α-fragment of β-gal is fused to the C terminus of the NBD protein and complements the coexpressed ω-fragment when the fusion is expressed in a folded, soluble form. Changes in the NBD-α fusion expression, folding, aggregation, degradation, and/or sequestration by chaperones has been shown to alter complementation. This complementation can be readily monitored using fluorescent substrates (fluorescein-di(β-galactose) (FDG). When the NBD-α fusion was coexpressed with the ω-fragment, β-gal activity was detected. This activity was not detected when the NBD-α or ω-fragment proteins were expressed alone or when a stop codon was introduced into the NBD-α upstream of the α-peptide sequence.

The PXE-associated variants tended toward two-state behavior in this folding assay (Fig. 5A). Four mutants showed activity greater than 70% of WT (Q698P, E699D, G755R, and V787I). Thirteen variants produced β-gal signals at roughly

Figure 3. NBD1 structure. The structure and topology of ABCC6 NBD1 is shown. A, a cartoon representation of ABCC6 NBD1 is shown in two orientations rotated by 60°. The subdomains are colored as in Fig. 2 (β-sheet, yellow; α/β-core, purple; α-helical, green). The structurally diverse region, part of the α-helical subdomain, is shown (tan). B, a cartoon representation of ABCC6 NBD1 showing the location of the canonical ATP-binding sequences is shown in two orientations, as in A. The labels of the conserved elements are colored corresponding to the features in the structure. C, a tube diagram of NBD1 is shown colored and scaled by B-factor. D, a topology map of ABCC6 NBD1 is shown colored as in A.
20% or less of WT levels (L677P, R765Q, and A781V). Ten of these variants produced H9252-gal signals 10% of WT (G663C, L673P, L726P, R760W, A766D, D777N, V810M, T811M, A820P, and L826P). These data suggest that multiple PXE-causing variants in NBD1 induce local changes in domain structural properties, which could include gross misfolding, destabilization, or changes in protein solubility.

Positioning the mutants within the structure of NBD1 provides further evidence for their strong influence on domain structural properties (Fig. 5B). The residues associated with the most significant decrease in NBD1 folding show decreased surface accessibility and include multiple proline substitutions into elements of defined secondary structure (Fig. 5, C and D, and Fig. S1). In contrast, those that show little to no effect on
Comparison of ABCC NBD1 structures

| ABCC6 | ABCC1 | ABCC1 ATP | ABCC1* ATP 6BHU | ABCC8* apo 6BAA |
|-------|-------|-----------|-----------------|-----------------|
| SO4 6BZS | SO4 4C3Z | 2CBZ | 0.786 | 1.387 |
| 0.568 | 0.766 | 0.463 | 0.640 | 1.228 |
| — | — | — | 0.718 | 1.240 |
| — | — | — | — | 1.175 |
| — | — | — | — | — |

Figure 5. NBD1 expression. NBD expression was assessed using an enzymatic assay in mammalian cells. A, the effects of PXE mutations are shown on NBD1 expression, normalized to the signal from WT. B, the effects of PXE mutations are shown on NBD1 expression, colored by severity. Two views of the NBD1 are shown rotated by 60°. C, the position of the PXE mutations are shown and colored by fractional surface area. The partially transparent molecular surface is shown as a reference. D, the correlation between fractional surface area and NBD1 expression is shown. Variants showing intermediate or greater expression are labeled for reference. The position of the partially transparent molecular surface is shown as a reference.

Relationship between NBD1 structural properties and ABCC6 trafficking

The biosynthesis of full-length ABCC6 was then assessed using Western blotting to assess changes in its subcellular localization and expression. Proteins were expressed transiently in HEK 293 cells, and differential glycosylation was used as a marker for trafficking into and through the secretory pathway. ABCC6 has a single glycosylation site within the N-terminal extracellular sequence, upstream of TMD0. This sequence is likely cotranslationally modified by ER glycosidases (28). Translational events out of the ER and through the Golgi results in further modification of the core carbohydrates. These differences in glycosylation can be resolved as changes in electrophoretic mobility, with the core glycosylated protein migrating at ~150 kDa and the fully modified protein migrating at ~170 kDa. As previously reported, the WT protein showed both core and complex glycosylation states, consistent with its transit through the ER and Golgi. Both forms of the protein were visible, with the majority of protein being the fully glycosylated species protein that was efficiently exiting the ER and trafficking through the secretory pathway (Fig. 6A).

The effects of the PXE-causing mutation were then assessed by Western blotting (Fig. 6, A and B). The G663C, L673P, L726P, R765Q, A766D, D777N, A820P, and L826P variants most adversely affected ABCC6 trafficking. These mutants showed little to no fully glycosylated species and steady-state expression was reduced to less than 10%, when compared with the WT protein. The reduction in steady-state protein and lack of fully glycosylated species was consistent with ER-associated degradation, as previously described for processing mutants in ABCC6 (15, 28). The R760W, V810M, and T811M mutants produced small amounts of fully glycosylated protein, although steady-state expression was markedly reduced compared with WT. Quantification of these variants suggested total protein levels were 15–20% that of the WT protein. A single variant, L677P, trafficked at roughly 60% that of WT and was the only intermediate variant among the selected NBD1 mutations. In contrast, several mutants, including Q698P, E699D, G755R, L726P, and A781V trafficked at levels similar to WT.

ABCC6 trafficking and function

To better assess how the expression of NBD1 and trafficking of the full-length protein were associated, correlations of the quantified values from the β-gal assay and densitometry of the full-length Western blots were evaluated. Comparison of the NBD expression and full-length trafficking values showed a strong positive correlation (Fig. 7). Mutants that had strong influence on NBD folding similarly had severe impacts on full-length protein trafficking and expression. Those mutants that clustered near WT in the NBD folding assay similarly clustered near WT in the full-length protein.

Two mutations deviated from this apparent two-state clustering: L677P, and A781V. These two variants showed intermediate effects (between 25 and 50% that of WT) on NBD expression. The L677P and A781V mutants deviated furthest from unity in the comparison, appearing to fold and mature more efficiently in the context of the full-length protein than in the isolated NBD.
Discussion

Although many PXE-associated alleles are yet to be characterized, previous studies have implicated protein misfolding as a potential molecular mechanism underlying this disease (15–17, 28). Recent advances in drug development efforts provide evidence that small molecule corrections of these defects can provide therapeutic relief for patients (29). Thus, a more complete view of both the structure and the folding of ABCC6 may prove useful in understanding the molecular defects associated with mutations and disease development, as well as potential mechanisms that could be leveraged for rational therapeutic development.

Using sequence alignments and iterative refinement of domain boundaries, a crystallizable NBD1 protein was identified, and a 2.3 Å structure was determined in the absence of ATP. Although the ABCC6 NBD1 adopts a canonical NBD fold, several key observations arise from this structure and its comparison with other ABCC subfamily members.

First, despite millimolar Mg\textsuperscript{2+}-ATP being included in the protein buffer, the ABCC6 NBD1 protein crystallized in a nucleotide-free state. No electron density for the nucleotide was seen, although a sulfate ion appeared to be well coordinated by the Walker A sequence, occupying the position of the β-phosphate. Nucleotide was required in the protein buffer, and no crystallization conditions have been identified in the absence of ATP to date. This could suggest a direct role for nucleotide during crystallization. Alternatively, the nucleotide may stabilize the free pool of NBD1 and prevent aggregation, as has been demonstrated for other NBDs, thereby indirectly facilitating crystallization (15, 30, 31). The adverse effects of the D777N, reported here, and D778N, previously reported, in the Walker B sequence suggest that Mg-ATP binding may be critical for the folding and/or structural stability of ABCC6 NBD1 (15). Disruption of ATP binding in CFTR NBD1 has similarly been shown to alter NBD1 stability and CFTR folding, suggesting that nucleotide association may be critical for the biosynthesis and/or stability of the ABCC subfamily NBDs (19, 30).

Second, when the ABCC6 NBD1 structure is compared with the structures from ABCC1 and ABCC8, ATP binding does not appear to impact the orientation of the α-helical subdomain. Models of the ABC-transporter mechanochemical cycle suggest that ATP binding to the NBDs induces their dimerization and also induces a rigid body rotation of the α-helical subdomain relative to the α/β core (11, 32). Superposition of the ATP-bound ABCC6 NBD1 structures shows only minimal deviation when compared with either of the ABCC1 NBD1 structures or the ABCC/MRP1 and ABCC8/SUR1 EM structures (Fig. 4 and Table 2) (20, 21, 25). The lack of subdomain movement is also seen in X-ray structures of CFTR NBD1 in apo and ADP- and ATP-bound states and in the ADP- and ATP-bound structures of TAP1 (19, 33–37). As in ABCC6, noncanonical Walker B sequences are found in both of these proteins.

It is possible that the observed conformation represents a ground state that is highly favorable for structural studies. Alternatively, it is possible that the bound SO\textsubscript{4}\textsuperscript{2−} ion is sufficient to induce the conformational movements normally associated

Four mutants clustered closely to the WT (Q698P, E699D, G755R, and V787I), showing minimal changes in both NBD folding and full-length processing. These data suggest that protein biosynthesis and folding are likely not the pathomechanisms associated with PXE for these variants. The Gln\textsuperscript{698} and Glu\textsuperscript{699} (Q-loop) and Gly\textsuperscript{755} (signature sequence) sites are located in canonical ATP binding and hydrolysis sequences (Fig. 3B and Fig. S2). It is not clear how the conservative V787I substitution would influence protein structure or function.

**Figure 6. ABCC6 expression.** PXE-associated mutations in NBD1 were assessed for their impact on the folding and maturation of full-length ABCC6 in cell culture. A, a representative Western blot is shown. Molecular mass markers are indicated on the left (in kDa), and the positions of the core (band B) and complexly glycosylated (band C) protein species are shown on the right. PARP1 is shown as a loading control. A dark exposure was chosen to demonstrate expression of all ABCC6 clones. B, densitometric analysis of Western blotting of ABCC6 expression is shown. The data shown are means ± S.E. from n > 4 experiments. Individual data points used for the analyses are shown for each mutant.

**Figure 7. NBD1 expression and ABCC6 trafficking.** The correlation between NBD expression and full-length trafficking was assessed from data presented in Figs. 5 and 6. NBD1 expression from the β-gal assay is plotted against the steady-state expression of full-length ABCC6. Residues showing intermediate or greater levels of expression are labeled. Residues located in conserved ATP-binding and hydrolysis sequences are shown in green (Q698P, E699D, and G755R). Unity is shown as a dashed line for reference.
with nucleotide binding. Although the canonical interactions with the Q-loop and γ-phosphate are not satisfied by the binding of SO₄ to the Walker A sequence, it is possible that allosteric mechanisms promote this structural transition. Further studies are required to evaluate the apparent conformational constraint seen in these structures.

The structure of NBD1 also provides a template on which to evaluate PXE-causing mutations previously identified in the patient population. The expression of NBD1 variants generally showed a two-state distribution. Many of the PXE-associated variants showed little surface exposure and were buried within the NBD structure (Fig. 5, B and C, and Fig. S1). The reported substitutions at these sites were not well tolerated, impacting both NBD1 and full-length ABCC6 expression (Fig. 7 and Fig. S1). These data suggest that NBD folding is a critical barrier to the maturation of the full-length ABCC6 transporter.

Two mutations deviated most significantly from this apparent bimodal clustering: L677P and A781V. Both of these mutations showed a more severe impact on NBD1 folding than on the maturation of full-length ABCC6. Leu⁶⁷⁷ is located at the C terminus of helix α4, and the substitution for Pro may partially be accommodated in the NBD1 structure without significant disruption of native structure (Fig. S3). The A781V substitution is also located outside of regular secondary structure elements following the catalytic histidine in the H-loop. This substitution, located in the putative NBD heterodimer interface, may alter ATP-dependent dimerization and ABCC6 activity (Fig. S2). Both of the missense mutations showed roughly 4-fold more efficient folding in the full-length protein than in the isolated domain (L677P: 3.8-fold; A781V: 4.5-fold).

Given the location of these two mutants in NBD1, the increased efficiency of full-length folding suggests that domain–domain assembly could provide stabilization of the NBD (Fig. 7). The changes in local structural properties may be partially restored by NBD association with the coupling helices of the transmembrane domains (Fig. S3). Such a case would require the NBD to adopt a near-native, binding competent structure with the domain–domain interaction interface preserved.

Similar domain–domain interactions have been shown to rescue ABC transporter misfolding (15, 30, 38, 39). In ABCC6, stabilization of the NBD–NBD interface has been shown to facilitate partial biosynthetic rescue of multiple misfolding mutations in NBD2 (15). Similarly, stabilization of the NBD1–TMD interface in F508del CFTR results in partial rescue of this misfolding mutation (30, 34). Thus, the increased efficiency of mutant folding in the full-length background may reflect coupled NBD folding and assembly or NBD stabilization via the energy of interactions with other domains (40).

Two PXE variants tested lie within the conserved NBD1–TMD interface and show severe effects on ABCC6 expression. The R765Q and A766D PXE variants, located in the α-helical subdomain, show severe biosynthetic defects in the full-length protein (Figs. 5 and 6 and Fig. S3). These variants likely alter local domain structure and domain–domain association, given their location and the chemical nature of the substitutions (Fig. 3 and Fig. S3). Further studies are necessary to fully understand the energetics of these interactions and the effects of disease-causing mutations on domain–domain dynamics.

Studies of CFTR suggest that domain folding and assembly events may serve as independent checkpoints in the regulation of its biosynthesis and are rescuable genetically, by second site suppressors, or by small molecule correctors. Significant improvements in CFTR biosynthesis have been reported using combinations of suppressors or small molecule correctors, pointing to multiple independent mechanisms of correction. Although exact binding sites and mechanisms of action have yet to be established, correction of multiple steps in the folding pathway may explain the synergy seen with these combinations (34, 41).

Defining the folding pathway and structural defects in ABCC6 provides a framework for our understanding of PXE-causing mutations and their effects on ABCC6 structure and molecular mechanochemistry. Developing this understanding provides insight into the basic defects underlying PXE and the mechanisms by which mutations impact ABCC6 structure and function. Finally, high resolution studies of ABCC6 structure and the molecular defects associated with specific mutations will help facilitate rational and structure-based therapeutic development for the relief of PXE in the patient population.

**Experimental procedures**

**NBD1 expression and purification**

NBD1 was expressed as a Smt3 fusion protein, as previously described (15, 30, 42). The coding region for NBD1, spanning amino acids 623–859, was PCR-amplified from pcDNA-ABCC6 and subcloned as a C-terminal fusion in the pSmt3 vector for expression in *E. coli*. PXE mutations were introduced by PCR-based site directed mutagenesis, and all clones were confirmed by DNA sequencing.

Proteins were expressed in *E. coli* BL21(DE3) cells. Small donor cultures were grown in LB supplemented with 50 mg/ml kanamycin overnight at 37 °C. Expression cultures were inoculated from the donor culture and grown at 37 °C until A₆₀₀ reached ~0.8–1.0. The cultures were then shifted to 15 °C and induced with 1 mM isopropyl-β-D-thiogalactopyranoside for expression overnight (16–18 h). Following expression, the cells were harvested by centrifugation, resuspended in buffer A (50 mM Tris, 150 mM NaCl, 10% glycerol, pH 7.6) supplemented with 2 mM MgATP and 1 mM DTT, and lysed by sonication. The lysates were cleared by centrifugation (40,000× g, 4 °C), and the supernatant was loaded onto a Fast Flow nickel–nitrilotriacetic acid column (GE Lifesciences) pre-equilibrated with 400 mM imidazole. Fractions containing NBD1 were pooled and supplemented with 1 mM DTT and 2 mM MgATP. The fusion protein was then digested overnight with Ulp1 at 4 °C to remove the N-terminal His-Smt3 tag. The digested protein was diluted into HIC buffer supplemented with 1 M ammonium (25 mM Tris-HCl, 100 mM NaCl, and 10% glycerol, pH 7.2) and loaded onto a butyl-Sepharose (GE Life-Sciences) column. The bound protein was washed with HIC buffer containing ammonium sulfate and eluted using a linear
gradient of HIC buffer without ammonium sulfate. The protein-containing fractions were then pooled, concentrated, and loaded onto a Superdex S75 column (GE LifeSciences) pre-equilibrated in 20 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 5 mM DTT, 2 mM ATP, 5 mM MgCl₂, pH 7.2. The protein-containing fractions were pooled, concentrated to 10–12 mg/ml, and flash-frozen in liquid nitrogen prior to storage at −80 °C.

**NBD1 crystallization and structure determination**

The purified NBD1 proteins were screened in parallel using microbatch under oil at the Hauptmann Woodward Institute high-throughput crystallization facility and in-house using sitting-drop vapor-diffusion methods (43). Hits were subsequently confirmed and optimized using sitting-drop vapor diffusion for the production of crystals for diffraction studies. Crystals formed with 10–12 mg/ml protein diluted 1:1 with reservoir solution containing 2.1–2.4 M ammonium sulfate between pH 6.8 and 7.5 at 4 °C. Rod-shaped crystals appeared after 1–3 days. X-ray diffraction was performed in-house using a Rigaku FR-E Superbright rotating anode and Rigaku HTC RAXIS HTC image plate. The data were collected in a nitrogen cryostream at 100 K. Diffraction data were indexed and scaled using d*Trek (Rigaku) with dtdisplay and dtprocess. Phasing and refinement were accomplished in Phenix. Molecular replacement was accomplished using the ABCC1/MRP1 NBD1 structure 2CBZ as an initial search model (23).

**β-gal assay**

NBD1 folding was assessed using the β-gal structural complementation assay, as previously described (27, 28, 30). The NBD1 coding sequence was PCR-amplified and subcloned into pcDNA as an N-terminal fusion to the HA tag and α-fragment from β-gal. Mutations in the NBD1 coding sequence were introduced by PCR mutagenesis and confirmed by DNA sequencing. HEK 293 cells were transfected with the NBD1–HA–α plasmid alone or cotransfected with the α- and ω-fragment plasmids. 48 h post-transfection, the cells were washed twice in PBS (Gibco) and lysed in reporter lysis Buffer (Millipore) on ice. The lysate was clarified by centrifugation (5 min, 21,000 × g) and separated by Tris-glycine SDS–PAGE. After separation, the gels were transferred to polyvinylidene difluoride membrane for Western blotting using M6II-7 or M6II-31 (Santa Cruz) anti-ABCC6 rat monoclonal antibody. Protein was visualized using goat anti-rat horseradish peroxidase–conjugated secondary (Millipore) and Illuminata Classic (Millipore) ECL reagent.

**PDB and structure factor files**

The refined NBD1 model file and structure factor files have been deposited in the Protein Data Bank and assigned the PDB code 6BZS.

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