RECTIFY PHARMA

A structural and mechanistic model for BSEP dysfunction in severe cholestatic disease

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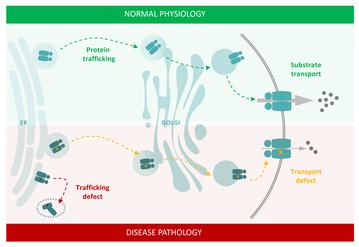
Abstract

ABCB11, (also known as BSEP) transports bile salts across the canalicular membrane of hepatocytes, where they enter the biliary system and are incorporated into the mixed micelles that constitute bile. Biallelic mutations in BSEP result in Progressive Familial Intrahepatic Cholestasis Type 2 (PFIC2), a pediatric disease characterized by hepatic bile acid accumulation leading to hepatotoxicity, resulting in fibrosis, cirrhosis, an increased risk of hepatocellular carcinoma, and ultimately, liver failure. In PFIC2, the extent to which bile acids can be exported from hepatocytes defines the severity and onset of disease; nonsense and frameshift mutations give rise to the most severe and earliest onset forms, while missense mutations, depending on their level of dysfunction, drive a varying degree of severity. BSEP mutations resulting in PFIC2 are most often missense mutations, which exhibit decreased protein expression and impaired trafficking and localization to the canalicular membrane, resulting in significantly reduced BSEP transport function.

To characterize the effects of disease-causing mutations on protein thermodynamic stability, we have carried out in-cell thermal shift (CETSA) measurements for 13 different patient mutations, including the most prevalent BSEP mutations E297G and D482G. Using a novel split luciferase detection method, shifts in aggregation temperature (T_{agg}) could be classified into three groups, a) mutations with no effects on T_{agg}, b) mutations causing mildly destabilizing thermal shifts of -2-4 °C, and c) mutations causing severe destabilization of 9-11° C, including E297G. All highly destabilizing mutations were localized to the NBD2-ICL2 interface of BSEP. Confocal microscopy indicated cytoplasmic versus plasma membrane localization in HEK293 cells, confirming that these mutations result in defective protein trafficking. To determine the structural basis for BSEP protein destabilization, we determined the cryo-EM structure of wild-type BSEP to 3.0 Å resolution. Focusing on mutations in NBD and ICL2, our high-resolution cryo-EM model provides a structural framework for rationalizing the thermal destabilization of these mutants, suggesting a novel NBD2-localized mechanism through which the most severe missense patient mutations drive cholestatic disease.

ABC transporters and link to human disease

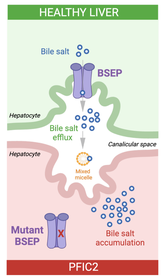
ABC transporter trafficking and transport defects drive human disease states



- ABC transporters are folded in the ER and trafficked to the membrane where they transport biological substrates
- Genetic mutations can cause:
  - Protein trafficking defects
  - Substrate transport defects
- Mutant forms of ABC transporter protein can be etiological causes of rare monogenic diseases

PFIC2 disease

PFIC2 is a form of genetic cholestasis with no disease-modifying therapies



An early onset life-limiting disease

- Progressive familial intrahepatic cholestasis type 2 (PFIC2) is caused by biallelic mutations in the *ABCB11/BSEP* gene
- Pediatric onset of chronic cholestasis and progressive liver failure
- Cholestasis arises due to insufficient efflux of toxic bile salts out of hepatocytes

An unmet need

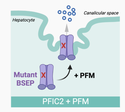
- The epidemiology of PFIC2 is approximately 1/50-100,000 births*
- SoC is limited to symptomatic treatment of pruritis (severe skin itching)
- Correcting BSEP mutations would be disease modifying#

A disease modifying PFM has the potential to transform care of PFIC2

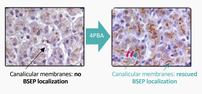
*Orphanet, #Rectify primary research

Proof of concept for discovery of BSEP modulators

Pharmacological rescue of mutant BSEP trafficking → **4-PBA provides clinical proof of concept for BSEP PFM** → **Significant opportunity for PFIC2 disease-modifying therapy**



- Small molecule PFM correctors restore canalicular localization and improved BA efflux



- 5 months 4-PBA therapy (non-selective chaperone)
- Liver biopsy shows rescued canalicular localization of BSEP (PoM)
- Improvement in serum bile acid levels, pruritus, liver function (PoC)

Gonzales et al 2012

Most products are not a cure; there's a huge unmet need for therapies.

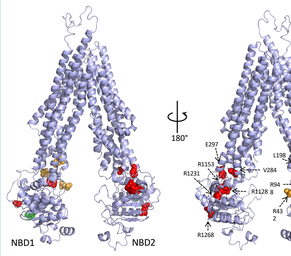
-US PFIC Hepatologist

- Rectify is developing a first-in-class disease modifying therapies for PFIC2 and other cholestatic diseases

- **PFM:** Positive functional modulator, a small molecule that increases transporter function through direct correction of protein trafficking or transport defects

BSEP variants drive PFIC2 disease

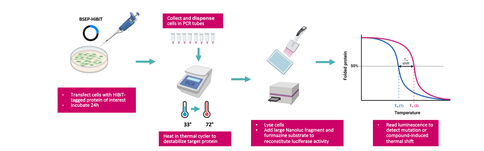
Mutations in ABCB11 (BSEP) have been shown to cause a complete loss of expression, decreased mRNA/protein stability, impaired trafficking, or diminished transport. The most common mutations are missense trafficking mutations, where most newly synthesized BSEP is retained and degraded in the ER. Several mutations in BSEP occur frequently, including the E297G and D482G variants, which are present in 58% of European PFIC2 patients. Reduced cell surface expression of BSEP missense mutants due to defective trafficking is analogous to the most common disease-causing mutations that occur with the cystic fibrosis transmembrane conductance regulator (CFTR). As with CFTR, trafficking defects can be corrected when protein is expressed at low temperature or in the presence of chemical chaperones, and it is hypothesized that trafficking modulators, or correctors, may be a possible approach to partially restore cell surface expression of BSEP. While missense mutations are distributed over both the transmembrane domains and the nucleotide binding domains (NBDs), many prevalent PFIC2 and BRIC mutations, as well as the common polymorph V444A are located in the NBDs. In this study, we have focused on missense mutations in the NBDs and intracellular loops (ICLs).



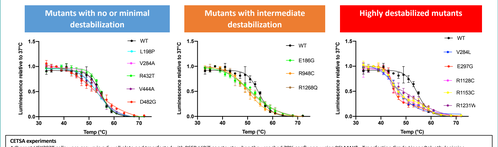
	Mutation	Severity	References
WT			
SNP	V284A		(1,2)
	V444A		(1)
BRIC	E186G		(1,2)
	L198P		(1,2)
	R432T		(1,2)
PFIC2	V284L		(1,2)
	E297G		(3)
	D482G		(3)
	R948C		(1,2,3)
	R1128C		(1,2,3)
	R1153C		(1,2,3)
	R1231W		(1,3)
	R1268Q		(1,3,4)

References
(1) Byrne *et al.* Hepatology (2009)
(2) Kubitz *et al.* Clin Res Hepatol Gastroenterol (2012)
(3) Van Wessel *et al.* J. Hepatol (2020)
(4) Sonika & Boyer Mol. Aspects Med (2014)

In-cell Thermal Shift Assay (CETSA)



Mutants with no or minimal destabilization | **Mutants with intermediate destabilization** | **Highly destabilized mutants**



CETSA experiments

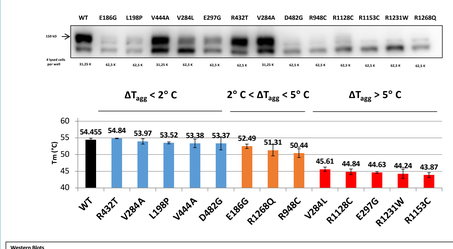
Adherent HEK293T cells were grown in a 6-well plate and transfected with BSEP-HiBiT constructs when they reached 70% confluency using PEI MAX® - Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40,000) (Polysciences) at a 1:3 DNA: PEI mass ratio. Cells were harvested 24h after transfection, counted, washed 1x in DPBS and aliquoted. Cell pellets were stored at -80 °C. Cell pellets were resuspended in lysis buffer (0.2% Triton-X in DPBS supplemented with 1X cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche)), left on ice for 30 minutes and spin down 12 000 rpm at 4 °C for 10 minutes. Supernatant was collected and protein separated by SDS-PAGE on 7.5% gel run at 70V for 30 minutes and 80V for 1 hour, transferred on a PVDF membrane using Trans-Blot Turbo transfer kit (Biorad) that was blocked for 2 hours in 5% milk TBST before being proabed with mouse anti-HiBiT (CS2006A01 clone 30E5) monoclonal antibody overnight at 4 °C (1:4000 in 3%BSA TBST). The next day the membrane was washed in TBST and incubated for 1 hour with HRP-conjugated goat anti-mouse IgG (sc-2055, Santa Cruz biotechnology) (1:5000 in 3%BSA TBST). After a final wash in TBST, bands were revealed with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and the membrane imaged on an Amersham Imager 680.

Melting curve analysis

For each melting curve, two wells per temperature were averaged. Resulting curves were fitted with a non-linear regression model on GraphPad 9, from which an aggregation temperature was extracted.

Destabilization of BSEP variants

Western blots confirm correlation of destabilization with impaired trafficking

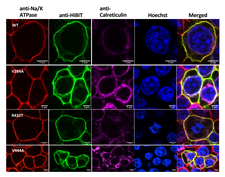


Western Blots

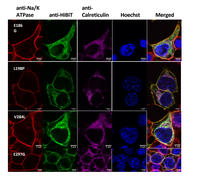
Adherent HEK293T cells were seeded in a 12-well plate and transfected at 70% confluency using PEI MAX® - Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40,000) (Polysciences) at a 1:3 DNA: PEI mass ratio. Cells were harvested 24h after transfection, counted, washed 1x in DPBS and aliquoted. Cell pellets were stored at -80 °C. Cell pellets were resuspended in lysis buffer (0.2% Triton-X in DPBS supplemented with 1X cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche)), left on ice for 30 minutes and spin down 12 000 rpm at 4 °C for 10 minutes. Supernatant was collected and protein separated by SDS-PAGE on 7.5% gel run at 70V for 30 minutes and 80V for 1 hour, transferred on a PVDF membrane using Trans-Blot Turbo transfer kit (Biorad) that was blocked for 2 hours in 5% milk TBST before being proabed with mouse anti-HiBiT (CS2006A01 clone 30E5) monoclonal antibody overnight at 4 °C (1:4000 in 3%BSA TBST). The next day the membrane was washed in TBST and incubated for 1 hour with HRP-conjugated goat anti-mouse IgG (sc-2055, Santa Cruz biotechnology) (1:5000 in 3%BSA TBST). After a final wash in TBST, bands were revealed with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and the membrane imaged on an Amersham Imager 680.

Confocal microscopy and Western blotting - BSEP localization

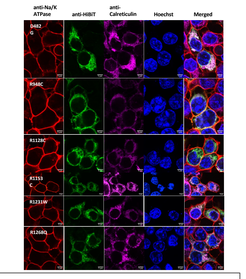
Variants with WT localization

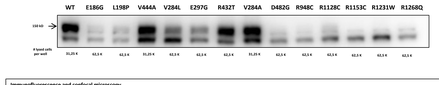


Variants with ER and PM localization



Variants with ER localization

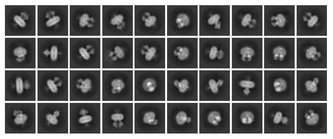


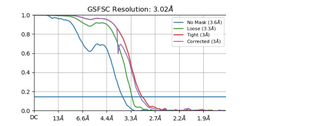


Immunofluorescence and confocal microscopy

HEK293T cells were plated on glass coverslips coated with poly-D-lysine in 24-well plates. The next day cells were transfected with BSEP-HiBiT plasmid with FuGENE® HD Transfection Reagent according to the manufacturer's instructions (Promega). 1-2 days after transfection cells were washed three times with cold DPBS, fixed in 100% methanol for 5 minutes, washed again three times with DPBS, permeabilized with 0.1% Triton-X-100 for 5 minutes and washed one more time in DPBS. Cells were then incubated in blocking buffer (3%BSA in HBSS) for 30 minutes. Primary antibodies were diluted in the same buffer and added to the cells overnight at 4 °C (mouse anti-HiBiT (Promega catalog number CS2006A01 clone 30E5) monoclonal antibody, 1:500; rabbit Alexa Fluor® 647 anti-Calreticulin (abcam catalog number ab196159), 1:400; rat anti Na/K ATPase (abcam catalog number ab283345), 1:400. Cells were washed the next day three times in DPBS and incubated 1h RT with secondary antibodies (rabbit anti-mouse IgG AlexaFluor 488 (abcam catalog number ab150125), 1:500; goat anti-rat IgG Alexa Fluor® 568 (abcam catalog number ab175476), 1:500) and Hoechst stain (Cayman catalog number 15547, 1:1000). After three more washes in DPBS and one wash in water, coverslips were mounted using ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific catalog number P10144) on a glass slide. Images were acquired on an Olympus iXplore spinning disk confocal microscope with Olympus objective 60x (UPLSAPO 52, NA 1. 3, oil) and 100x (UPLSAPO S, NA 1. 35, oil).

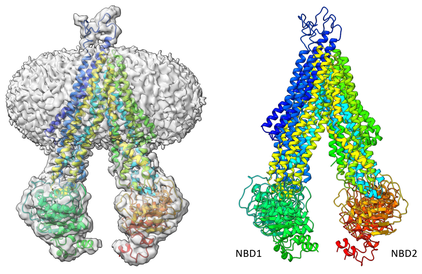
Cryo-EM structure of wild-type BSEP



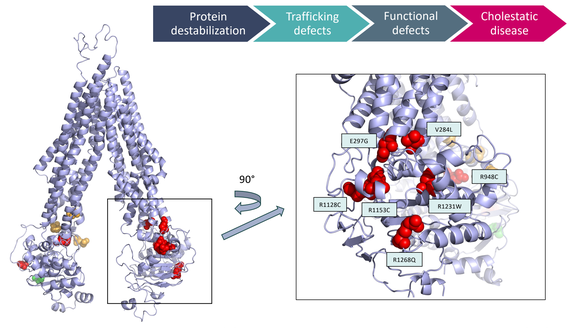


ABCB11 was expressed using BacMam in HEK293 cells. To purify ABCB11, it was extracted using DDM-CHS, followed by Co-NTA metal affinity with an on-column GDN detergent exchange, and finally polished using Superpose 6. Concentrated protein was frozen on Quantifoil UltrAuFoil grids using a Thermofisher Vitrobot. Next, ~3.5k movies were collected on a Thermofisher Titan Krios using a Gatan K3 detector. Data processing was performed using CryoSPARC, and the final model was generated using ~254k particles.

Single-particle cryo-EM structure of BSEP determined at 3.0Å resolution

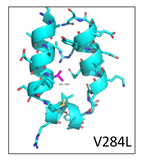


A hotspot for destabilization is localized to NBD2

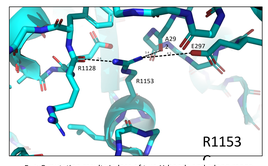


- Missense mutations on NBD/ICL2 cause severe thermal destabilization of BSEP

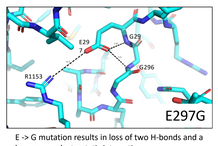
Structural basis of protein destabilization



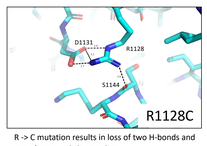
V -> L mutation increases steric bulk and perturbs packing of ICL2 coupling helix



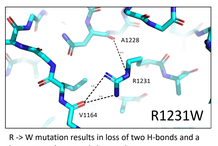
R -> C mutation results in loss of two H-bonds and a long-range electrostatic interaction



E -> G mutation results in loss of two H-bonds and a long-range electrostatic interaction

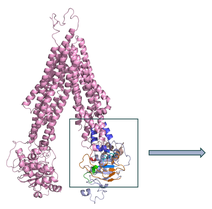


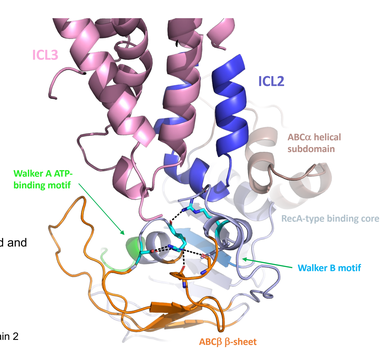
R -> C mutation results in loss of two H-bonds and two electrostatic interactions



R -> W mutation results in loss of two H-bonds and a long-range electrostatic interaction

A network of electrostatic interactions stabilizes the NBD fold

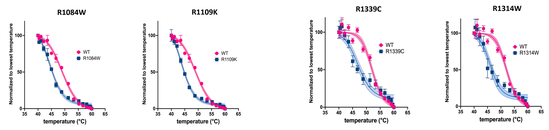




- R1128 and R1153 (cyan) form the core of an electrostatic network that stabilizes the NBD2 fold and NBD2-ICL2 interactions
- Ties together key subdomains of NBD2
  - Walker A ATP binding motif
  - ABCβ subdomain
  - Helix 1 of the RecA type binding core
  - Intracellular loop 2 (ICL2) of transmembrane domain 2 (TMD2)

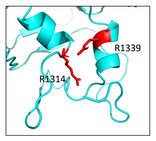
NBD2 destabilization is also observed in B4, C6

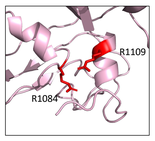
Residues homologous to BSEP R1128, R1153 are also destabilized in ABCB4 and ABCC6



ABCB4 Variant	T_{agg} 95% CI (°C)	ΔT_{agg}
Wild-type	**49.2** +/- 0.4	
R1084W	**44.6** +/- 0.7	**-4.6**
R1109K	**43.9** +/- 0.6	**-5.3**

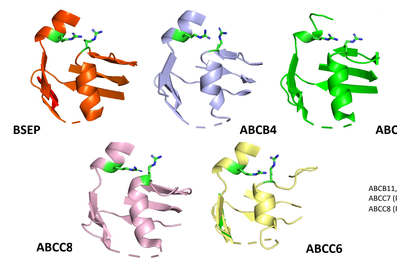
ABCC6 Variant	T_{agg} 95% CI (°C)	ΔT_{agg}
Wild-type	**52.1** +/- 0.4	
R1339C	**47.1** +/- 1.2	**-5.0**
R1314W	**46.1** +/- 0.9	**-6.0**





Pathogenic mutations are structurally conserved

Structural motifs identified in destabilized BSEP variants are structurally conserved in the ABCB and ABCC families



ABCB11, ABCB4, ABCC6 (Rectify)
ABCC7 (PDB 5UAK)
ABCC8 (PDB 6C3O)

Can we design mechanism-based assays to target this hotspot in BSEP and other ABCTs?

Conclusions and future work

- CETSA, Western blotting and confocal microscopy have been used to characterize the stability and cellular localization of key patient variants of BSEP
- All highly destabilizing mutations were localized to the NBD2-ICL2 interface of BSEP
- Western blotting and confocal microscopy demonstrate low protein expression and partial or full ER localization for this set of highly destabilized NBD2 variants
- The cryo-EM structure of wild-type BSEP provides a structural framework for interpreting the thermal destabilization of NBD2-localized mutants, suggesting several NBD2-localized mechanisms through which this subset of severe missense patient mutations may drive severe cholestatic disease
- Destabilizing mutations in BSEP and ABCC6 occur in common NBD2 structural elements and likely possess a similar mechanism of defective protein trafficking
- Mechanism-based assays designed to identify NBD2-specific stabilizing ligands may provide differentiated chemical matter