RESEARCH ARTICLE

Analysis of the Bile Salt Export Pump (ABCB11) Interactome Employing Complementary Approaches

Susanne Przybylla¹, Jan Stindt², Diana Kleinschrodt¹, Jan Schulte am Esch³, Dieter Häussinger², Verena Keitel², Sander H. Smits¹, Lutz Schmitt¹*

¹ Institute of Biochemistry, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany, ² Department of Gastroenterology, Hepatology and Infectious Diseases, University Hospital, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany, ³ Department of General, Visceral and Pediatric Surgery, University Hospital, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

* Lutz.Schmitt@hhu.de

Abstract

The bile salt export pump (BSEP, ABCB11) plays an essential role in the formation of bile. In hepatocytes, BSEP is localized within the apical (canalicular) membrane and a deficiency of canalicular BSEP function is associated with severe forms of cholestasis. Regulation of correct trafficking to the canalicular membrane and of activity is essential to ensure BSEP functionality and thus normal bile flow. However, little is known about the identity of interaction partners regulating function and localization of BSEP. In our study, interaction partners of BSEP were identified in a complementary approach: Firstly, BSEP interaction partners were co-immunoprecipitated from human liver samples and identified by mass spectrometry (MS). Secondly, a membrane yeast two-hybrid (MYTH) assay was used to determine protein interaction partners using a human liver cDNA library. A selection of interaction partners identified both by MYTH and MS were verified by in vitro interaction studies using purified proteins. By these complementary approaches, a set of ten novel BSEP interaction partners was identified. With the exception of radixin, all other interaction partners were integral or membrane-associated proteins including proteins of the early secretory pathway and the bile acyl-CoA synthetase, the second to last, ER-associated enzyme of bile salt synthesis.

Introduction

One of the liver’s major functions is the production of bile. During digestion bile facilitates the absorption of lipids and fat-soluble vitamins. Furthermore, it serves as the main route for excretion of cholesterol and lipophilic waste products.

The bile salt export pump (BSEP, ABCB11) is an ATP binding cassette (ABC)-transporter located in the apical membrane of hepatocytes, which is indispensable for bile flow, as it translocates conjugated bile acids from the cell lumen into the bile canaliculus, driving bile salt-
dependent bile flow [1]. At the canalicular membrane BSEP is accompanied by other ABC-transporters, which transport additional bile components, such as the multidrug resistance protein 3 (MDR3, ABCB4), which is a lipid floppase specific for the phosphatidylcholine family, and the cholesterol transporter ABCG5/8 [2]. These three compounds, the main organic constituents of bile, form mixed micelles in the canaliculus, which mitigate the toxic detergent effect of bile salts on the surrounding hepato- and cholangiocellular membranes and thereby preserve their integrity [3,4].

Dysfunction of BSEP with respect to expression, trafficking or function is often a molecular determinant of cholestasis. Reduced expression of BSEP at the canalicular membrane or impaired activity leads to accumulation of bile salts in the hepatocyte, which is a cause for benign recurrent or progressive familial intrahepatic cholestasis type 2 (BRIC2 and PFIC2, respectively) [5,6]. Due to their detergent character, increased levels of bile salts lead to cellular damage, while their function as signal molecules impinges on cell metabolism at lower concentrations.

Due to its significance in cholestasis development, many aspects of expression as well as the functional regulation of BSEP have been extensively studied [2,7]. In this field, one less studied aspect is the posttranslational regulation of BSEP by protein-protein interaction (PPI), which is, among other functions, a prerequisite for efficient trafficking and localization of BSEP to the canalicular membrane. Some PPIs, which influence the abundance of the ABC-transporter in the apical membrane have already been identified. For example, the adaptor protein complex 2 (AP-2) and HAX-1 take part in the endocytic retrieval from the canalicular membrane and the myosin II regulatory light chain (MLC2) has been shown to influence anterograde trafficking of the transporter to the apical membrane [8–10]. PPI also plays a role in stabilization of membrane proteins by tethering them to the cytoskeleton. One example is the multidrug-resistance-associated protein 2 (MRP2, ABCC2), which depends on scaffolding and membrane-cytoskeletal cross-linking proteins such as the sodium-hydrogen exchanger regulatory factor-1 (NHERF1/EBP50), radixin and ezrin for efficient localization to the apical membrane [11–14]. Additionally, PPI can directly influence the activity of an interaction partner as shown recently for MDR3 (ABCB4). Here, the interaction with a scaffold protein regulates trafficking and cell surface expression [15]. This emphasizes that more detailed information on the interaction network of BSEP is required to understand how its trafficking and activity are regulated.

In this study, full-length, human BSEP was used in a membrane yeast two-hybrid (MYTH) screen [16] using a liver cDNA library to identify previously unknown interaction partners. In parallel, proteins associated with BSEP were identified by tandem mass spectrometry (MS/MS) after co-immunoprecipitation (co-IP) from human liver samples. As a third line of interaction studies, putative interaction partners were verified by in vitro pull-down assays.

Materials and Methods

Yeast strain

The Saccharomyces cerevisiae strain NMY51 was used for the membrane yeast two-hybrid assay (Dualsystems Biotech, Schlieren, Switzerland; MATa his3Δ200 trp1-901 leu2-3,112 ade2 lys2::(lexAop)4 -HIS3 ura3::(lexAop)8–lacZ ade2::(lexAop)8 -ADE2 GAL4). Yeast cells were transformed using the lithium acetate method as described in Gietz et al. [17].

Construction of plasmids for the MYTH screen

For the bait construct full-length human BSEP cDNA [6] was cloned into the pBT3-C vector by homologous recombination. pBT3-C was linearized with the restriction endonucleases NcoI and SfiI. BSEP cDNA was amplified by PCR with the following primer pair 5’ -CAAATACACA
Homologous recombination was performed in the yeast strain NMY51 plated on synthetic defined drop-out medium (SD) lacking leucine. Positive clones were selected by colony PCR. After plasmid isolation and transformation of *E. coli* DH5α the construct was verified by sequencing.

**MYTH screen**

The MYTH assay was carried out as described in the DUALhunter manual (Dualsystems Biotech). Briefly, the yeast strain NMY51 was transformed with the bait construct pBT3-C-BSEP and the functionality of the system with BSEP as bait was assessed employing the recommended controls. Following that, the bait was tested for self-activation with the empty prey vector pPR3-N. To screen for interaction partners NMY51 was transformed with the bait construct and subsequently with 36 μg of a human adult liver NubG-X cDNA library (Dualsystems Biotech; 1.5x10⁵ independent clones) for complete coverage. Clones grown on SD medium lacking leucine, tryptophan and histidine (SD-LWH) were re-plated on SD medium lacking in addition adenine and supplemented with 40 μg/ml X-Gal (SD-LWHAxF). Plasmids of blue colonies were isolated and amplified in *E. coli* DH5α. Yeast cells harboring the pBT3-C-BSEP plasmid were retransformed with the prey plasmids to confirm the interaction. Interaction partners were checked for false positives by a bait dependency test with the SV40 large T antigen fused to an Ost4p membrane anchor as unrelated bait (DUALhunter manual, control plasmid pDHB1-largeT). Remaining candidates were sequenced and identified with the basic local alignment search tool (BLAST) [18]. MYTH controls were performed at least in duplicate.

**Cloning of putative interaction partners for production in *E. coli***

cDNA of potential interaction partners identified in the MYTH screen were cloned into the pET-51b(+) vector (EMD Biosciences, Inc., Darmstadt, Germany) for expression in *E. coli*. cDNA was amplified by PCR with the addition of ZraI and KpnI restriction endonuclease sites and inserted into pET-51b(+) via these restriction sites. All constructs were verified by DNA sequencing.

**Production of putative interaction partners in *E. coli***

Full-length radixin and radixin1-318 were produced in *E. coli* BL21 (DE3), the bile acyl-CoA synthetase (BACS) in *E. coli* Rosetta (DE3) pLysS. LB medium (10 g/l Tryptone/Pepitone from Casein, 5 g/l yeast extract, 5 g/l NaCl) or in the case of radixin and radixin1-318 LBN (10 g/l Trypton/Pepton from Casein, 2 g/l glucose, 29.2 g/l NaCl) was inoculated to an OD₆₀₀ of 0.09 and grown to an OD₆₀₀ of 0.6 at 37°C and 180 rpm. Protein production was induced by addition of 0.5 mM IPTG. The proteins were produced at 18°C for 20 h. After cell harvest (3000 g, 20 min, 4°C) the sediment was suspended in lysis buffer (50 mM sodium phosphate pH 7, 100 mM NaCl, 1 mM EDTA, 20% (w/v) glycerol) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) and lysed by two passages through the Microfluidizer M-110P (Microfluidics, Westwood, MA) at 1.3 kbar. The lysate was cleared by centrifugation at 4000 g, 15 min, 4°C followed by centrifugation at 15000 g, 30 min, 4°C.

**Purification of interaction partners**

Protein interaction partners were purified by affinity chromatography. *E. coli* cell lysate was applied to Strep-Tactin resin (iba GmbH, Göttingen, Germany) by gravity flow and washed...
with buffer (50 mM HEPES pH 7, 150 mM NaCl, 1 mM EDTA). Protein was eluted in elution buffer (50 mM HEPES pH 7, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin) and concentrated with Amicon centrifugal filter units with a molecular weight cut-off of 10 kDa (Merck KGaA, Darmstadt, Germany). Purified protein was flash frozen and stored at -80°C.

**Expression of BSEP**

BSEP was produced in the methylotrophic yeast *Pichia pastoris* (*Komagataella pastoris*) as previously published in Ellinger *et al.* [19]. In brief, *Pichia pastoris* X-33 (Life Technologies, Carlsbad, CA) was transformed with the construct pSGP18-2μ-BSEP. The yeast was fermented according to the Invitrogen Pichia Expression Kit manual in a 15 liter table-top glass fermenter (Applikon Biotechnology, Schiedam, the Netherlands) in 5 l of basal salt medium with addition of 500 ml of 50% (v/v) glycerol. Feeding 500 ml of methanol during 28 h induced protein production. Approximately 800 g of wet cell mass was flash-frozen in liquid nitrogen and stored at -80°C until further use.

**Purification of BSEP**

*Pichia pastoris* cells were suspended in homogenization buffer (50 mM Tris pH 8, 50 mM NaCl, 0.33 M Sucrose, 1 mM EDTA pH 8, 1 mM EGTA pH 8, 0.1 M 6-aminohexanoic acid, 1 mM DTT) supplemented with protease inhibitor cocktail tablets (Roche). Cell disruption was performed with the Microfluidizer M-110P (Microfluidics) in three passes at 2 kbar. Cell debris was sedimented by differential centrifugation (1500 g, 15 min, 4°C; 14000 g, 20 min, 4°C). Crude membranes were obtained by ultracentrifugation at 120000 g for 1 hour at 4°C and suspended in membrane buffer (50 mM Tris pH 8, 50 mM NaCl, 20% glycerol). After a second ultracentrifugation step, the membranes were resuspended in membrane buffer to a protein concentration of 10–20 mg/ml. Membranes equivalent to 30 g of wet cell weight were diluted to a protein concentration of 5 mg/ml as determined by Pierce Coomassie Plus Assay (Thermo Fisher Scientific Inc., Rockford, IL). Fos-choline 16 (Anatrace, Maumee, OH) was added to a concentration of 1% (w/v) and proteins solubilized with rotation at 4°C for 45 min. Aggregates were sedimented by ultracentrifugation at 100000 g, 40 min, 4°C. The supernatant was applied to 1 ml of Calmodulin Affinity Resin (Agilent Technologies, Santa Clara, CA) and incubated with light agitation at 4°C for 30 min. The resin was washed with CBP binding buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM β-mercaptoethanol, 1 mM magnesium acetate, 2 mM calcium chloride, 15% (w/v) glycerol, 0.022% (w/v) DDM). BSEP was eluted with CBP elution buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM β-mercaptoethanol, 2 mM EGTA, 15% (w/v) glycerol, 0.022% (w/v) DDM), stored at 4°C and used within the next 48 h. For removal of the affinity tags 200 μg of BSEP were incubated with 2 units of HRV 3C Protease (Thermo Scientific, Rockford, IL) for 4 h at 4°C. Protease and affinity tags were removed by incubation with 50 μl of Ni-NTA Agarose (Qiagen, Hilden, Germany) for 30 min at 4°C.

**Pull-down assay of BSEP with interactions partners**

For pull-down assays, interaction partners were immobilized on a Strep-Tactin resin. After 30 min incubation at 4°C and washing with pull-down buffer (50 mM sodium phosphate pH 7, 150 mM NaCl, 20% (w/v) glycerol, 0.022% (w/v) DDM) detergent-solubilized, purified BSEP was added for another 30 min at 4°C. Complexes were eluted after 5 times washing and analyzed by immunoblotting. For detection of BSEP, the monoclonal F-6 antibody (Santa Cruz Biotechnology, Dallas, TX, AB_2242103) was used, the Strep-tag II was detected by anti Strep-tag II mouse monoclonal antibody (Merck KGaA, Darmstadt, Germany, AB_10807650).
Horseradish peroxidase-conjugated goat monoclonal secondary antibody was purchased from Dianova (Hamburg, Germany). Pull-down assays were replicated at least three times.

Immunoprecipitation of BSEP from human liver

This study was performed according to the guidelines of the declaration of Helsinki. Written consent was obtained from all patients, and all associated studies on excess material were approved by the local ethics committee (study no. 2852 approved by the local ethics committee of the medical faculty of Heinrich Heine University Düsseldorf). Human liver samples were obtained from the noncancerous resection margin during liver metastasis resection and were immediately processed. All steps were carried out on ice or at 4°C unless stated otherwise. Liver tissue was cut into small pieces and lysed in homogenization buffer (50 mM Tris/HCl pH 7.4, 250 mM sucrose) with eight strokes in a tight-fitting dounce homogenizer. The crude homogenate was centrifuged for 10 min at 1000 g and afterwards for 10 min at 3000 g. 20 μl of the wet pellet were solubilized in 1 ml IP solubilization buffer (1% Triton X-100 or digitonin in 50 mM Hepes pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, Roche protease inhibitor cocktail (EDTA-free)) overnight with slow overhead inversion. Insoluble material was removed by centrifugation at 13000 g for 10 min. For immunoprecipitation the monoclonal BSEP antibody (F-6) was used and naïve mouse IgG2a served as control (Santa Cruz Biotechnology, Dallas, TX). 2 μg of antibody were added to 20 μl of protein A/G+ agarose slurry (Santa Cruz Biotechnology) and the mixture was incubated for 10 min at room temperature with rotation. The preloaded agarose was washed three times with IP solubilization buffer. 1 ml of the solubilization supernatant was added to agarose-bound antibody, samples were incubated for 4 h with slow overhead inversion and centrifuged for 1 min at 1000 g. The resulting pellet was washed three times with IP solubilization buffer. Remaining buffer was removed by aspiration and pellets were stored at -20°C. For further analysis, the agarose pellet was incubated in 64 μl 3% (w/v) SDS for 20 min, followed by addition of 16 μl 5x reducing sample buffer (100 mM Tris/HCl pH 6.8, 3.2% (w/v) SDS, 40% (v/v) glycerol, 0.02% bromophenol blue, 10% β-mercaptoethanol). After incubation at 65°C for 15 min samples were spun down briefly and 40 μl were subjected to SDS-PAGE and immunoblotting. Detection was performed with the following antibodies: polyclonal anti-BSEP K24, which was raised in rabbit against the C-terminal 13 amino acids as described previously [20], mouse monoclonal anti-Radixin (GeneTex, Irvine, CA, AB_10624814), mouse monoclonal anti-Adaptin α (BD Biosciences, Franklin Lakes, NJ, AB_397867) or rabbit polyclonal anti-SLC27A5 (Abcam plc, Cambridge, UK, AB_2190760). These experiments were performed at least twice.

Mass spectrometry

Following detergent removal, a tryptic digest of the co-IP pellet was subjected to shotgun tandem mass spectrometry. The analysis was carried out on an UHPLC-coupled Orbitrap Elite mass spectrometer by the Molecular Proteomics Laboratory at the Biologisch-Medizinisches Forschungszentrum (BMFZ) of Heinrich-Heine-University Düsseldorf as detailed earlier [21].

Immunofluorescence staining of liver cryosections and differentiated HepaRG cells

Cryosections were obtained from human liver tissue used as source material for co-IP experiments. HepaRG cells were cultured and differentiated as described [22]. Briefly, the cells were grown for two weeks without subculturing and culture medium exchanged every two days, followed by two weeks under medium supplemented with 2% (v/v) dimethyl sulfoxide. Differentiated cells were harvested and seeded onto glass coverslips before cultivation for 48h in DMSO-
containing medium. Both cells and liver cryosections were fixed in ice-cold methanol and blocked in Ultra V block (Thermo). BSEP was stained using either mouse F-6 (Santa Cruz, 1:100) or the rabbit serum K24 [20], BACS and MRP2 were stained using rabbit anti-SLC27A5 (Abcam, 1:100) and mouse M2-III6 (1:25; Alexis, AB_2273479), respectively. Goat anti-mouse-IgG2a-AF488 (AB_2535771) and goat anti-rabbit-IgG-AF546 (AB_2534093, both 1:500, Invitrogen) were used as secondary antibodies and cell nuclei stained with Hoechst 34580. All samples were analyzed by confocal laser-scanning microscopy on a Zeiss LSM 510meta confocal laser-scanning microscope.

Results

Three complementary methods identify a novel set of BSEP interaction partners

A comprehensive understanding of the regulation of a protein in regard to function and cellular trafficking requires detailed knowledge of its interactome. Techniques such as yeast two-hybrid approaches, tandem affinity purification or immunoprecipitation, the latter two often combined with mass spectrometry, have been developed and optimized in recent years. However, each of these techniques has its own advantages and disadvantages [23]. For example, in yeast two-hybrid systems the presence of protein tags can prevent interactions or produce false positive results, while mass spectrometry-based approaches lack some sensitivity towards transient interactions. To cope with these limitations and to advance the understanding of BSEP on a molecular level we used three complementary methods to identify interacting proteins of this bile salt transporter.

Firstly, the MYTH assay was used to screen for putative interaction partners that interact directly with BSEP. In contrast to conventional yeast two-hybrid systems [23,24] the MYTH system, which has been established by Stagljar et al., allows for screening of membrane proteins [16,25]. A schematic diagram of the system is provided in Fig 1. The MYTH assay is based on the re-association of two ubiquitin halves. The protein of interest (bait) is produced in yeast as a fusion protein with the C-terminal half of ubiquitin (Cub) and a transcription factor (Lex-A-VP16). The putative interaction partners (prey) are fused to the mutated N-terminal moiety of ubiquitin (NubG). Interaction of bait and prey cause the two ubiquitin halves to reconstitute into the so called split-ubiquitin, which is recognized by an endogenous ubiquitin specific protease. The transcription factor is released and activates three reporter genes, which enable yeast to grow on selective media [16].

For our study the assay was established with full-length, human BSEP as bait. BSEP cDNA was cloned into the pBT3-C bait vector, which adds the required ubiquitin moiety and transcription factor to the C-terminus of the protein for optimal accessibility of the fusion proteins.

Functionality of the MYTH system with BSEP was ascertained by control assays. The first assay activated the system independently of bait interaction and thereby confirmed BSEP expression (Fig 2). The second control assay tested the BSEP construct for self-activation, which is a common complication in applying the MYTH assay. A low bait expression level, which was indicated for this system in the first control assay, is of advantage. BSEP did not show self-activation in our setup (Fig 2).

To screen for interaction partners the BSEP expressing yeast strain was transformed with a human adult liver cDNA library. This library had a complexity of 1.5x10⁶ independent clones and allowed for the screening of liver proteins or protein fragments of up to 450 amino acids.

As in any genetic screening method, the obtained clones contained a number of false positives. To reduce their amount, reporter gene activation was reconfirmed individually for each prey plasmid and subsequently analyzed in a bait dependency test, where the reporter
activation for a prey was compared between BSEP and an unrelated, non-interacting bait. Sequencing and identification of the cDNA fragments by alignment search tools led to elimination of the remaining false positive clones, which occurred due to out of frame readings or gene products from non-relevant compartments. This resulted in the identification of 37 proteins (Table 1).

Secondly, immunoprecipitation of BSEP from human liver and consecutive analysis of the co-precipitated protein complexes by MS/MS allowed for the identification of proteins associated with the transporter. For solubilization of BSEP we tested two commonly used, mild detergents, Triton X-100 and digitonin. To identify BSEP-specific precipitation, protein frequencies from co-IP with BSEP antibody were compared to the frequencies obtained with a control antibody. Using this strategy, 262 proteins were identified.

The adaptor protein complex 2 (AP-2) is a known interaction partner of BSEP [9] and was precipitated in this setup, while it was not identified in the MYTH screen. Subunits of the complex were detected by MS/MS after solubilization of canalicular membranes with Triton X-100, while specific AP-2 signals were absent in samples prepared with digitonin (Fig 3A; compare S9 Fig for an assembly of interaction partners derived from MS data only from all four co-IPs). Immunoblots of the samples however showed that subunits of AP-2 were precipitated with BSEP in both detergents (Fig 3B).

---

Fig 1. Schematic diagram of the membrane yeast two-hybrid system (MYTH) to screen for interaction partners of the ABC-transporter BSEP. The MYTH system is based on a split-ubiquitin approach. The bait, BSEP, is fused to the C-terminus of ubiquitin (Cub) and a transcription factor (LexA-VP16). The preys are soluble or membrane-associated liver proteins, introduced via a cDNA library. They are fused to the N-terminus of ubiquitin (NubG). Upon interaction of BSEP and the liver protein the ubiquitin moieties, which have a low affinity for each other due to a mutation in the N-terminus, come into close proximity. The reassembled ubiquitin is recognized by endogenous ubiquitin specific proteases (UBP). The transcription factor is cleaved off and activates the reporter genes (HIS3, ADE2, lacZ).

doi:10.1371/journal.pone.0159778.g001
Thirdly, in vitro pull-down analyses were used to confirm a subset of identified interaction partners using recombinant, purified proteins. Here, the alpha subunit of the AP-2 complex as a known interaction partner [9] was able to pull down BSEP (Fig 4B). The subcellular localization of the ten identified BSEP interaction partners that were identified in both screens is depicted in Fig 5. In the following section, we will focus on the proteins that were identified with these complementary methods (Table 2).

Factors of the early secretory pathway

Nine out of the ten proteins identified in both screens are membrane proteins of the early secretory pathway. All proteins discussed in the following paragraph co-precipitated specifically with BSEP from human liver and required Triton X-100 for solubilization (see Fig 3, S9 Fig and S1 Table). Also, these proteins showed direct interaction with BSEP as seen in the bait dependency test performed after the MYTH screen (Fig 6).

The translocon associated protein (TRAP) is a complex of four subunits involved in membrane protein topogenesis [26]. The two screens identified the gamma subunit (SSR3) as a direct interaction partner of BSEP, while the alpha and delta subunit (SSR1/4) were found to associate with the transporter in the co-IP / MS/MS screen. While the interaction of TRAP gamma subunit and BSEP displayed a comparatively high background signal in both screens (Fig 6 and S1 Table), the alpha and delta subunits co-precipitated specifically with BSEP (S1 Table, S9 Fig). Taken together, these findings suggest an interaction of the TRAP complex with BSEP.

A second factor connected to the translocation of hydrophobic protein sections is the translocating chain-associated membrane protein 1 (TRAM1). TRAM1 has been found to be
stimulatory or for some targets even necessary for membrane protein translocation. Furthermore, a role for TRAM in dislocation of proteins from the ER has been postulated [27,28].

Another aspect of trafficking is membrane protein sorting and quality control. Bap31 (BCAP31) is an ubiquitous, integral ER membrane protein, which is closely associated with TRAM and involved in protein sorting. Its expression affects a number of membrane proteins,

Table 1. Protein and gene names of identified interaction partners of BSEP derived from the MYTH assay.

| Protein Name                                      | Gene Symbol | GeneID   | GenBank Accession |
|---------------------------------------------------|-------------|----------|-------------------|
| aldolase A, fructose-bisphosphate                 | ALDOA       | 226      | NM_184041.2       |
| asialoglycoprotein receptor 2                     | ASGR2       | 433      | NM_080914.2       |
| B-cell receptor-associated protein 31            | BCAP31      | 10134    | NM_001256447.1    |
| BCL2/adenovirus E1B 19kDa interacting protein 3-like | BNIP3L    | 665      | NM_004331.2       |
| bile acyl-CoA synthetase                          | SLC27A5     | 10998    | NM_012524.2       |
| catechol-O-methyltransferase                      | COMT        | 1312     | NM_000754.3       |
| CD63 molecule                                     | CD63        | 967      | NM_001780.5       |
| CD99 molecule                                     | CD99        | 4267     | NM_0011228898.1   |
| cofilin 1 (non-muscle)                           | CFL1        | 1072     | NM_005507.2       |
| dolichyl-phosphate mannosyltransferase poly peptide 2, regulatory subunit | DPM2       | 8818     | NM_003863.3       |
| ER membrane protein complex subunit 4            | EMC4        | 51234    | NM_016454.2       |
| glutamate receptor, ionotropic, N-methyl-D-aspartate-associated protein 1 | GRINA      | 2907     | NM_001009184.1    |
| heme oxygenase (decycling) 2                     | HMOX2       | 3163     | NM_002134.3       |
| immediate early response 3 interacting protein 1 | IER3IP1     | 51124    | NM_016097.4       |
| interferon induced transmembrane protein 2       | IFITM2      | 10581    | NM_006435.2       |
| interferon induced transmembrane protein 3       | IFITM3      | 10410    | NM_021034.2       |
| mitogen-activated protein kinase binding protein 1 | MAPKBP1    | 23005    | NM_001256511.1    |
| protein disulfide isomerase family A, member 6   | PDIA6       | 10130    | NM_005742.2       |
| radixin                                          | RDX         | 5962     | NM_00260492.1     |
| receptor accessory protein 5                     | RERP5       | 7905     | NM_005669.4       |
| RER1 retention in endoplasmic reticulum 1 homolog (S. cerevisiae) | RER1       | 11079    | NM_007033.4       |
| signal peptidase complex subunit 1 homolog (S. cerevisiae) | SPCS1 | 28972 | NM_014041.3       |
| signal peptidase complex subunit 2 homolog (S. cerevisiae) | SPCS2 | 9789 | NM_014752.2       |
| signal sequence receptor, gamma (translocon-associated protein gamma) | SSR3 | 6747 | NM_001707.3       |
| sphingomyelin phosphodiesterase 4, neutral membrane (neutral sphingomyelinase-3) | SMPD4 | 55627 | NM_017951.4       |
| translocation associated membrane protein 1      | TRAM1       | 23471    | NM_014294.5       |
| transmembrane protein 134                        | TMEM134     | 80194    | NM_025124.2       |
| transmembrane protein 14A                        | TMEM14A     | 28978    | NM_014051.3       |
| transmembrane protein 199                        | TMEM199     | 147007   | NM_015246.1       |
| transmembrane protein 205                        | TMEM205     | 374882   | NM_001145416.1    |
| transmembrane protein 230                        | TMEM230     | 29058    | NM_001009925.1    |
| UDP glucuronosyltransferase 1 family, polypeptide A5 | UGT1A5    | 54579    | NM_019078.1       |
| unconventional SNARE in the ER 1 homolog (S. cerevisiae) | USE1 | 55850 | NM_018467.3       |
| VAMP (vesicle-associated membrane protein)-asso ciated protein B and C | VAPB | 9217 | NR_036633.1       |
| WD repeat domain 83 opposite strand             | WDR83OS     | 51398    | NM_016145.3       |
| Yip1 interacting factor homolog A (S. cerevisiae) | YIP1A      | 10897    | NM_020470.2       |
| ZMYM6 neighbor                                   | ZMYM6NB     | 100506144| NM_001195156.1    |

Highlighted proteins have also been identified in the co-immunoprecipitation / MS/MS screen. Five proteins are human homologs of S. cerevisiae proteins as indicated by the species added in brackets.

doi:10.1371/journal.pone.0159778.001
Some mutations involving BCAP31 could be connected to liver dysfunction and cholestasis [30,31]. The receptor expression-enhancing proteins (REEP) of which proteins 5 and 6 were identified in MYTH and co-IP/MS/MS screen, respectively, are proposed to be ER-shaping proteins which directly interact with cargo proteins to modulate their processing and trafficking [32].

The immediate early response 3 interacting protein 1 (IER3IP1) is a less studied, ER-resident protein [33]. Two mutations have been linked to microcephaly and an abnormal amount of apoptosis [34]. Its homology to the yeast protein Yos1 points towards a function in vesicular transport between Golgi and ER.

Retention in endoplasmic reticulum 1 (RER1) is an early Golgi membrane protein thought to function as a sorting chaperone, which modulates the fate of several membrane proteins [35]. While RER1 came up in both screens, in the MYTH screen only the C-terminal half of the 196 amino acid protein was encoded on the library plasmid. This part codes for the last

---

**Fig 3. co-IP / MS/MS identifies BSEP interaction partners.** (A) Immunoprecipitation of BSEP coupled to complex mass spectrometry reveals new interaction partners of BSEP in human liver. Crude canalicular membrane preparations were solubilized in either digitonin or Triton X-100, and immunoprecipitated samples were subjected to MS/MS. For each detergent, protein frequencies from two co-IPs with BSEP antibody are plotted against the respective negative control with naive mouse IgG. Interaction partners of interest are labeled. For the sake of clarity, proteins found exclusively in either the BSEP or control co-IP are depicted with an MS-score of one instead of zero on the other axis. (B) Immunoblot analysis of co-IPs shown in A.

doi:10.1371/journal.pone.0159778.g003
transmembrane helix and the cytosolic tail of the protein. Studies of the yeast homolog Rer1 show that cargo recognition occurs via transmembrane domain interaction, while the C-terminus has been shown to bind to the coatomer [36].

There is little information on two further interaction partners identified. Transmembrane protein 14A (TMEM14A) was found to localize to or close to ER and mitochondria and ectopic expression resulted in apoptosis suppression [37]. Its molecular function has not yet been elucidated. Transmembrane protein 205 (TMEM205) has been identified in endosomes of the liver and other secretory tissue and its expression was linked to increased cisplatin resistance [38]. Current information would support a role of this small membrane protein in secretion or vesicular trafficking.

All identified proteins are candidates that may directly determine the fate of BSEP at different stages along the secretory pathway.

**BSEP interacts with an ER-anchored enzyme**

In addition to the factors related to protein sorting and quality control, BSEP showed interaction with an ER-anchored enzyme.

The bile acyl-CoA synthetase (BACS, SLC27A5) catalyzes the first step in re-conjugation of bile acids to taurine or glycine [39]. BACS was found to co-precipitate with BSEP as seen in the MS/MS analysis from samples solubilized with Triton X-100 (Fig 3A). With the detergent digitonin corresponding peptides were not found in co-IP samples (S1 Table). Immunoblots of the co-IP samples also showed that BACS is precipitated to a greater degree in the presence of BSEP (Fig 3B). Interestingly, a relative of BACS, the very long-chain acyl-CoA synthetase (VLACS, SLC27A2), was specifically co-precipitated with BSEP in Triton X-100 (Fig 3A). BACS was also identified as a BSEP-interacting protein in the MYTH assay (Fig 4A). Here, the C-terminal 50 amino acids were encoded by the library plasmid, a fragment that belongs to the cytosolic part of the enzyme. This fragment shares 50% identity with the related VLACS.
down analyses, as seen in Fig 4B, confirm the interaction of BSEP with BACS lacking its N-terminal membrane anchor (BACS77-690). This finding might explain the observation of Wlcek et al. [40] who observed a protein dependent influx of taurocholate in the lumen of the rough endoplasmic reticulum.

Since BSEP is localized at the apical, canalicular membrane and BACS is an ER-anchored enzyme, we sought to identify sites of interaction by immunofluorescence analysis of human liver tissue yet found no co-localization of BSEP and BACS (S10A Fig). Strikingly, we find that
in differentiated HepaRG cells, a human hepatoma cell line with many hepatocyte-like characteristics missing in other liver cell lines [22], both BSEP and BACS are found in newly formed pseudo-canaliculuar structures (S10B and S10C Fig). This finding raises the possibility of a canalicular site of interaction between BSEP and one of the enzymes that catalyze the formation of its substrate—amino acid-conjugated bile salts.

**BSEP interacts with the membrane-cytoskeletal cross-linker radixin**

Radixin (RDX) was identified as an interaction partner of BSEP by MYTH, co-IP / MS/MS and specific in vitro pulldown. This protein belongs to the ezrin/radixin/moesin (ERM) family and cross-links membrane proteins to the cytoskeleton [41]. The N-terminal FERM (4.1/ezrin/radixin/moesin) domain interacts with target membrane proteins, for example MRP2, and is only accessible if the protein is activated or the inhibiting C-terminus is removed [11,42]. The C-terminal ERM-associated domain (C-ERMAD) binds the actin cytoskeleton. Alternatively, ERM proteins can bind other scaffolding proteins, such as NHERF1/EBP50 [41]. In the MYTH assay (Fig 4A) a radixin fragment was produced that roughly corresponds to the N-terminal FERM domain. Looking at the co-IP results, radixin showed differential precipitation in the

| Protein Name and Gene Symbol | GenBank Accession |
|------------------------------|------------------|
| B-cell receptor-associated protein 31 (BCAP31) | NM_001256447.1 |
| bile acyl-CoA synthetase (SLC27A5) [SLC27A2] | NM_012254.2 |
| immediate early response 3 interacting protein 1 (IER3IP1) | NM_016097.4 |
| receptor accessory protein 5 (REEP5) [REEP6] | NM_005669.4 |
| retention in endoplasmic reticulum 1 (RER1) | NM_007033.4 |
| radixin (RDX) | NM_001260492.1 |
| signal sequence receptor, gamma (translocon-associated protein gamma) (SSR3) [SSR4] | NM_007107.3 |
| translocation associated membrane protein 1 (TRAM1) | NM_014294.5 |
| transmembrane protein 14A (TMEM14A) | NM_014051.3 |
| transmembrane protein 205 (TMEM205) | NM_001145416.1 |

Gene names of closely related proteins identified in the co-IP screen are added in square brackets.

doi:10.1371/journal.pone.0159778.t002

Fig 6. Bait dependency test with BSEP interaction partners in the early secretory pathway. To confirm the interaction with prey proteins from the initial MYTH screen, reporter gene activation was tested individually for each prey against the bait, BSEP, or a non-interacting control bait, large T antigen. The figure shows one representative result for the positively tested preys, which are denoted by their respective gene name.

doi:10.1371/journal.pone.0159778.g006
two detergents used. While there was no specific co-precipitation with Triton X-100, the complex of BSEP and radixin was stable in the presence of digitonin (Fig 3A). Immunoblot analysis of the co-IPs mirrored the results obtained from MS/MS analysis. With digitonin radixin appears in the presence of BSEP only, while with Triton X-100 it is also present in the control lanes, suggesting nonspecific binding in that detergent (Fig 3B). Pull-down analyses, as seen in Fig 4B, confirmed the interaction of BSEP with the N-terminal half of radixin (radixin1-318). Additionally, we could show that the C-terminal affinity tags used for purification of BSEP do not influence its interaction with radixin. After proteolytic removal of the BSEP affinity tags, pull-down assays show that radixin1-318 can bind BSEP, while full-length radixin does not display a signal as depicted in Fig 4C.

One likely protein interaction partner for BSEP could not be found in our approaches. The scaffolding protein NHERF1 is an interaction partner of several ABC-transporters in the liver, for example CFTR, MRP2 and MRP4 [13,43,44]. NHERF1, like radixin, is predominantly found at the apical membrane of hepatocytes [45] and was tested separately with MYTH, co-IP/ MS/MS and in vitro pull-down. We did not detect interaction of NHERF1 and BSEP in any of the three methods applied here.

**Discussion**

Protein-protein interaction presents the basis of many cellular processes and also plays a pivotal role in the trafficking, localization and activity of membrane proteins. In the case of the bile salt export pump this has been shown for interactions that modulate its cycling at the canalicular membrane [8–10]. In the study presented here, we identified ten novel proteins that interact with BSEP using a three-sided approach.

The first approach was used to screen for proteins that bind directly to BSEP. The MYTH assay probed BSEP interaction with soluble and membrane-associated liver proteins. Of the over 500 initial candidates 37 were verified as interaction partners after applying the appropriate controls (Fig 7). In contrast to currently available methods for human tissues, the MYTH

---

**Fig 7. Venn diagram of potential BSEP interaction partners obtained with MYTH and co-IP / MS/MS.** The numbers represent the potential BSEP interaction partners identified by MYTH and co-IP / MS/MS and the overlapping set of proteins found in both screens.

doi:10.1371/journal.pone.0159778.g007
assay has the advantage of detecting both stable and more transient interactions. The 27 additional BSEP interaction partners detected by MYTH, which were not found by co-IP / MS/MS, may represent these short-lived interactions (Table 1). On the other hand the host organism, the required fusion proteins, and the quality of the cDNA library limit the detection range.

Immunoprecipitation of BSEP from human liver and subsequent identification of interaction partners by MS/MS resulted in more than 200 BSEP-associated proteins (Fig 7). This approach allowed for identification of physiological interaction partners and proteins stably associated with the transporter via mediators. On the downside, the method is not suitable for reliable detection of transient interactions and the required use of detergent can disrupt physiologically relevant interactions. Consequently, we used two different, non-ionic detergents, digitonin and Triton X-100. By binding cholesterol [46] digitonin readily disrupts the cholesterol-rich canalicular membrane and was able to preserve the interaction of BSEP and radixin at the plasma membrane. A somewhat more stringent solubilization by Triton X-100, on the other hand, was required to access interactions in the secretory pathway.

The third method, in vitro pull-down, was used to confirm the interaction of BSEP with soluble proteins or soluble domains of identified interaction partners. This method requires soluble or solubilized proteins and detects stable interactions.

Applying the three presented approaches, which covered both in vitro methods as well as procedures for detecting physiological interactions in human liver, established the validity of the observed interactions. Proteins detected by an individual technique may still be relevant and require a different method for verification. Since methods for detection of PPI directly in human tissue are at present very limited, in vitro and yeast-based approaches help to cover the cases in which PPI cannot yet be screened and visualized in vivo.

The interaction partners identified in the early secretory pathway are likely involved in the topogenesis, sorting and quality control of BSEP. The TRAP complex, of which subunits alpha, gamma and delta have been found to associate with BSEP, assists in membrane protein topogenesis. Interaction of the nascent protein with the TRAP complex may stabilize certain orientations [26]. Influence on the sorting of secretory proteins has been shown for TRAM [27] and Bap31, for the latter in the sorting of CFTR [29]. Members of the REEP family influence the shape of ER membranes and processing of their target proteins [32] and may influence BSEP trafficking by locally altering ER membrane shape. RER1 is highly conserved across species and has been studied in more detail in yeast [47]. In combination with previous studies our findings indicate that Rer1 interacts with BSEP via its C-terminal transmembrane domain. Since several of the interacting proteins in the early secretory pathway are conserved among species, it is possible that yeast enabled an interaction in the MYTH assay with endogenous factors.

The enzymes that were found to interact with BSEP were the acyl-CoA synthetases BACS and VLACS. Their similarities, especially in the C-terminal 50 amino acids that were found to bind to BSEP, may point to a common binding site. BACS and VLACS are both catalyzing steps in the synthesis of BSEP substrates and may be involved in a regulatory feedback loop that modulates trafficking or modification of the bile salt transporter. In light of the results of protein-mediated taurocholate uptake in the ER [40], one might speculate that the proposed transport system is indeed BSEP, which would already in the ER catalyze the transport of bile acids. In contrast, the finding that BACS is localized at pseudocanalicular structures in differentiated HepaRG cells (S10C Fig) supports a canalicular site of interaction between BACS and BSEP. In contrast to the vast majority of existing canaliculi in adult human liver tissue, the pseudocanicular structures in these cells are formed de novo during differentiation, and BACS may play some role in this process. We speculate that in the early stages of canalicular morphogenesis, a canalicular localization of BACS allows for the generation of conjugated bile
salts in direct vicinity of their cognate transporter while later on this process is localized to the cytosolic side of the ER membrane.

Lastly, BSEP interacts with the FERM domain of the cross-linking protein radixin. Radixin has been shown to connect several membrane proteins to the cytoskeleton either directly or via adaptors. In the case of BSEP and MRP2 Wang et al. observed an influence of radixin expression on localization and transport efficiency in rat hepatocytes, where silencing of radixin led to retention of the transporters in subapical compartments [12].

In summary, with the complementary methods of membrane yeast two-hybrid, in vitro pull down assay and co-immunoprecipitation from human liver samples combined with MS/MS we screened for novel interaction partners of BSEP and identified ten proteins. These results define a network of interacting proteins involved in topogenesis, trafficking and functional regulation (Fig 5) and suggest that BSEP is tightly controlled within the cell with respect to localization and function. In addition to the factors in the early secretory pathway, radixin and the bile acyl-CoA synthetase have a possible role in the regulation of the transporter. How these interaction partners regulate BSEP in the physiological context remains to be investigated. Our work provides the foundation for further research on post-translational BSEP regulation by protein-protein interaction.

Supporting Information
S1 Fig. Uncropped immunoblot of Fig 3B anti BSEP.
(PNG)

S2 Fig. Uncropped immunoblot of Fig 3B anti Radixin.
(PNG)

S3 Fig. Uncropped immunoblot of Fig 3B anti Adaptin α.
(PNG)

S4 Fig. Uncropped immunoblot of Fig 3B anti BACS (SLC27A5).
(PNG)

S5 Fig. Uncropped immunoblot of Fig 4B anti BSEP.
(JPG)

S6 Fig. Uncropped immunoblot of Fig 4B anti Strep-tag.
(JPG)

S7 Fig. Uncropped immunoblot of Fig 4C anti BSEP.
(TIF)

S8 Fig. Uncropped immunoblot of Fig 4C anti Strep-tag. Immunoblot is flipped in the final figure.
(TIF)

S9 Fig. MS data of interaction partners from all four co-IP experiments.
(PNG)

S10 Fig. Immunofluorescence staining of human liver cryosections and differentiated HepaRG cells for MRP2, BSEP, and BACS.
(TIFF)

S1 Table. Mass spectrometry data on identified BSEP interaction partners. Data from two independent measurements are shown. See inserted legend for further information.
(XLSX)
Acknowledgments
Vectors for the membrane yeast two-hybrid system were a kind gift from Prof. Dr. Willbold (Düsseldorf). We thank Dr. Poschmann and Prof. Dr. Stühler of the Molecular Proteomics Laboratory at Heinrich-Heine-University Düsseldorf for performing the MS/MS analysis. We are grateful to Iris Fey of the Protein Production Facility for technical assistance.

Author Contributions
Conceived and designed the experiments: SP JS DK VK SHS LS. Performed the experiments: SP JS DK. Analyzed the data: SP JS DK VK SHS LS. Contributed reagents/materials/analysis tools: JSaE DH. Wrote the paper: SP JS VK SHS LS.

References
1. Gerloff T, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, et al. (1998) The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J Biol Chem 273: 10046–10050. PMID:954351
2. Boyer JL (2013) Bile formation and secretion. Compr Physiol 3: 1035–1078. doi:10.1002/cphy.c120027 PMID:23897680
3. de Vree JM, Jacquemin E, Sturm E, Cresteil D, Bosma PJ, Aten J, et al. (1998) Mutations in the MDR3 gene cause progressive familial intrahepatic cholestasis. Proc Natl Acad Sci U S A 95: 282–287. PMID:9419367
4. Small DM (2003) Role of ABC transporters in secretion of cholesterol from liver into bile. Proc Natl Acad Sci U S A 100: 4–6. PMID:12509503
5. Kubitz R, Droge C, Stindt J, Weissenerber K, Haussinger D (2012) The bile salt export pump (BSEP) in health and disease. Clin Res Hepatol Gastroenterol 36: 536–553. doi:10.1016/j.clinre.2012.06.006 PMID:22795478
6. Stindt J, Ellinger P, Weissenerber K, Droge C, Herebian D, Mayatepek E, et al. (2013) A novel mutation within a transmembrane helix of the bile salt export pump (BSEP, ABCB11) with delayed development of cirrhosis. Liver Int 33: 1527–1535. doi:10.1111/liv.12217 PMID:23758865
7. Soroka CJ, Boyer JL (2014) Biosynthesis and trafficking of the bile salt export pump, BSEP: therapeutic implications of BSEP mutations. Mol Aspects Med 37: 3–14. doi:10.1016/j.mam.2013.05.001 PMID:23685087
8. Ortiz DF, Moseley J, Calderon G, Swift AL, Li S, Arias IM (2004) Identification of HAX-1 as a protein that binds bile salt export protein and regulates its abundance in the apical membrane of Madin-Darby canine kidney cells. J Biol Chem 279: 32761–32770. PMID:15159385
9. Hayashi H, Inamura K, Aida K, Naol S, Horikawa R, Nagasaka H, et al. (2012) AP2 adaptor complex mediates bile salt export pump internalization and modulates its hepatocanicular expression and transport function. Hepatology 55: 1889–1900. doi:10.1002/hep.25591 PMID:22262466
10. Chan W, Calderon G, Swift AL, Moseley J, Li S, Hosoya H, et al. (2005) Myosin II regulatory light chain is required for trafficking of bile salt export protein to the apical membrane in Madin-Darby canine kidney cells. J Biol Chem 280: 23741–23747. PMID:15826951
11. Kikuchi S, Hata M, Fukumoto K, Yamane Y, Matsui T, Tamura A, et al. (2002) Radixin deficiency causes conjugated hyperbilirubinemia with loss of Mrp2 from bile canalicular membranes. Nat Genet 31: 320–325. PMID:12068294
12. Wang W, Soroka CJ, Menonne A, Rahner C, Harry K, Pypaert M, et al. (2006) Radixin is required to maintain apical canalicular membrane structure and function in rat hepatocytes. Gastroenterology 131: 878–884. PMID:16952556
13. Li M, Wang W, Soroka CJ, Menonne A, Harry K, Weinman EJ, et al. (2010) NHERF-1 binds to Mrp2 and regulates hepatic Mrp2 expression and function. J Biol Chem 285: 19299–19307. doi:10.1074/jbc.M110.096081 PMID:20404332
14. Chai J, Cai SY, Liu X, Lian W, Chen S, Zhang L, et al. (2015) Canalicular membrane MRP2/ABCC2 internalization is determined by Ezrin Thr567 phosphorylation in human obstructive cholestasis. J Hepatol 63: 1440–1448. doi:10.1016/j.jhep.2015.07.016 PMID:26212029
15. Venot Q, Delaunay JL, Fouassier L, Delaustier D, Falguieres T, Houssset C, et al. (2016) A PDZ-Like Motif in the Biliary Transporter ABCB4 Interacts with the Scaffold Protein EB50 and Regulates ABCB4 Cell Surface Expression. PLoS One 11: e0146962. doi:10.1371/journal.pone.0146962 PMID:26789121
16. Stagljar I, Korostensky C, Johnsson N, te Heesen S (1998) A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. Proc Natl Acad Sci U S A 95: 5187–5192. PMID: 9560251
17. Gietz RD, Schiestl RH (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc 2: 31–34. PMID: 17401334
18. Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J Comput Biol 7: 203–214. PMID: 10890397
19. Ellinger P, Kluth M, Stindt J, Smits SH, Schmitt L (2013) Detergent screening and purification of the human liver ABC transporters BSEP (ABCB11) and MDR3 (ABCB4) expressed in the yeast Pichia pastoris. PLoS One 8: e60620. doi: 10.1371/journal.pone.0060620 PMID: 23593265
20. Noe J, Stieger B, Meier PJ (2002) Functional expression of the canalicular bile salt export pump of human liver. Gastroenterology 123: 1659–1666. PMID: 12404240
21. Poschmann G, Seyfarth K, Besong Agbo D, Klafki HW, Rozman J, Wurst W, et al. (2014) High-fat diet induced isoform changes of the Parkinson’s disease protein DJ-1. J Proteome Res 13: 2339–2351. doi: 10.1021/pr401157k PMID: 24646099
22. Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. (2002) Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci U S A 99: 15655–15660. PMID: 12432097
23. Ngounou Wetie AG, Sokolowska I, Woods AG, Roy U, Deinhardt K, Darie CC (2014) Protein-protein interactions: switch from classical methods to proteomics and bioinformatics-based approaches. Cell Mol Life Sci 71: 205–228. doi: 10.1007/s00018-013-1333-1 PMID: 23579629
24. Fields S, Song O (1989) A novel genetic system to detect protein-protein interactions. Nature 340: 245–246. PMID: 25471163
25. Snider J, Hanif A, Lee ME, Jin K, Yu AR, Graham C, et al. (2013) Mapping the functional yeast ABC transporter interactome. Nat Chem Biol.
26. Sommer N, Junne T, Kales KU, Spiess M, Hartmann E (2013) TRAP assists membrane protein topogenesis at the mammalian ER membrane. Biochim Biophys Acta 1833: 3104–3111. doi: 10.1016/j.bbamcr.2013.08.018 PMID: 24130369
27. Gorlich D, Rapoport TA (1993) Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. Cell 75: 615–630. PMID: 8242736
28. Ng CL, Oresic K, Tortorella D (2010) TRAM1 is involved in disposal of ER membrane degradation substrates. Exp Cell Res 316: 2113–2122. doi: 10.1016/j.yexcr.2010.04.010 PMID: 20430023
29. Wang B, Heath-Engel H, Zhang D, Nguyen N, Thomas DY, Hanrahan JW, et al. (2008) BAP31 interacts with Sec61 translocons and promotes retrotranslocation of CFTRDeltaF508 via the derlin-1 complex. Cell 133: 1080–1092. doi: 10.1016/j.cell.2008.04.042 PMID: 18555793
30. van de Kamp JM, Errami A, Howidi M, Anseel I, Winter S, Phalin-Roque J, et al. (2015) Genotype-phenotype correlation of contiguous gene deletions of SLC6A8, BCAP31 and ABCD1. Clin Genet 87: 5187–5192. doi:10.1111/cge.12355 PMID: 24597975
31. Cacciagli P, Sutera-Sardo J, Borges-Correia A, Roux JC, Dorboz I, Desvignes JP, et al. (2013) Mutations in BCAP31 cause a severe X-linked phenotype with deafness, dystonia, and central hypomyelination and disorganize the Golgi apparatus. Am J Hum Genet 93: 579–586. doi: 10.1016/j.ajhg.2013.07.023 PMID: 24011989
32. Björk S, Hunt CM, Ho VK, Angelotti T (2013) REEPs are membrane shaping adapter proteins that modulate specific g protein-coupled receptor trafficking by affecting ER cargo capacity. PLoS One 8: e76366. doi: 10.1371/journal.pone.0076366 PMID: 24098489
33. Yu WH, Poon JW, Tsui SK, Fung KP, Waiye MM (2004) Cloning and characterization of a novel endoplasmic reticulum localized G-patch domain protein, IER3IP1. Gene 337: 37–44. PMID: 15276200
34. Poulton CJ, Schot R, Kia SK, Jones M, Verheijen FW, Venselaar H, et al. (2011) Microcephaly with simplified gyration, epilepsy, and infantile diabetes linked to inappropriate apoptosis of neural progenitors. Am J Hum Genet 89: 265–276. doi: 10.1016/j.ajhg.2011.07.006 PMID: 21835305
35. Yamasaki A, Hara T, Maejima I, Sato M, Sato K, Sato K (2014) Rer1p regulates the ER retention of immature rhodopsin and modulates its intracellular trafficking. Sci Rep 4: 5973. doi:10.1038/srep05973 PMID: 25096327
36. Snider J, Hanif A, Lee ME, Jin K, Yu AR, Graham C, et al. (2013) Mapping the functional yeast ABC transporter interactome. Nat Chem Biol.
37. Sato K, Sato M, Nakano A (2003) Rer1p, a retrieval receptor for ER membrane proteins, recognizes transmembrane domains in multiple modes. Mol Biol Cell 14: 3605–3616. PMID: 12972550
38. Woo IS, Jin H, Kang ES, Kim HJ, Lee JH, Chang KC, et al. (2011) TMEM14A inhibits N-(4-hydroxyphenyl)retinamide-induced apoptosis through the stabilization of mitochondrial membrane potential. Cancer Lett 309: 190–198. doi: 10.1016/j.canlet.2011.05.031 PMID: 21723035
38. Shen DW, Ma J, Okabe M, Zhang G, Xia D, Gottesman MM (2010) Elevated expression of TMEM205, a hypothetical membrane protein, is associated with cisplatin resistance. J Cell Physiol 225: 822–828. doi: 10.1002/jcp.22287 PMID: 20589834

39. Mihalik SJ, Steinberg SJ, Pei Z, Park J, Kim DG, Heinzer AK, et al. (2002) Participation of two members of the very long-chain acyl-CoA synthetase family in bile acid synthesis and recycling. J Biol Chem 277: 24771–24779. PMID: 11980911

40. Wlcek K, Hofstetter L, Stieger B (2014) Transport of estradiol-17beta-glucuronide, estrone-3-sulfate and taurocholate across the endoplasmic reticulum membrane: evidence for different transport systems. Biochem Pharmacol 88: 106–118. doi: 10.1016/j.bcp.2013.12.026 PMID: 24406246

41. Clucas J, Valderrama F (2014) ERM proteins in cancer progression. J Cell Sci 127: 267–275. doi: 10.1242/jcs.133108 PMID: 24421310

42. Magendantz M, Henry MD, Lander A, Solomon F (1995) Interdomain interactions of radixin in vitro. J Biol Chem 270: 25324–25327. PMID: 7592691

43. Short DB, Trotter KW, Reczek D, Kreda SM, Bretscher A, Boucher RC, et al. (1998) An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. J Biol Chem 273: 19797–19801. PMID: 9677412

44. Hoque MT, Conseil G, Cole SP (2009) Involvement of NHERF1 in apical membrane localization of MRP4 in polarized kidney cells. Biochem Biophys Res Commun 379: 60–64. doi: 10.1016/j.bbrc.2008.12.014 PMID: 19073137

45. Ingraffea J, Reczek D, Bretscher A (2002) Distinct cell type-specific expression of scaffolding proteins EBP50 and E3KARP: EBP50 is generally expressed with ezrin in specific epithelia, whereas E3KARP is not. Eur J Cell Biol 81: 61–68. PMID: 11893083

46. Sperry WM, Webb M (1950) A revision of the Schoenheimer-Sperry method for cholesterol determination. J Biol Chem 187: 97–106. PMID: 14794694

47. Fullekrug J, Boehm J, Rotgger S, Nilsson T, Mieskes G, Schmitt HD (1997) Human Rer1 is localized to the Golgi apparatus and complements the deletion of the homologous Rer1 protein of Saccharomyces cerevisiae. Eur J Cell Biol 74: 31–40. PMID: 9309388