

Identification of Key Amino Acids that Impact Organic Solute Transporter

Alpha/Beta (OST α/β)

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Abbreviations

Alanine (Ala; A); Asparagine (Asn; N); Cystine (Cys; C); Glutamate (Glu; E); Glutamine (Gln; Q); Lysine (Lys; K); Phenylalanine (Phe; F); Serine (Ser; S); Threonine (Thr; T)

ASBT, apical sodium-dependent bile acid transporter; DAPI, 4',6-diamidino-2-phenylindole; ECF, extracellular fluid; FXR, farnesoid X receptor; GCA, glycocholate; HRP, horseradish

peroxidase; MOPS, 4-morpholinepropanesulfonic acid; OST α/β , organic solute transporter alpha/beta; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; sec, second; TCA, taurocholate; TBST, Tris-buffered saline with Tween 20; TMD, transmembrane domain; WT, wild-type

Abstract

Organic solute transporter alpha/beta (OST α/β ; SLC51A/B) is a bidirectional bile acid transporter localized on the basolateral membrane of hepatic, intestinal, and renal epithelial cells. OST α/β plays a critical role in intestinal bile acid reabsorption and is upregulated in hepatic diseases characterized by elevated bile acids, while genetic variants in *SLC51A/B* have been associated with clinical cholestasis. OST α/β also transports and is inhibited by commonly used medications. However, there is currently no high-resolution structure of OST α/β , and structure-function data for OST α , the proposed substrate-binding subunit, are lacking. The present study addressed this knowledge gap and identified amino acids in OST α that are important for bile acid transport. This was accomplished using computational modeling and site-directed mutagenesis of the OST α subunit to generate OST α/β mutant cell lines. Out of the ten OST α/β mutants investigated, four (S228K, T229S, Q269E, Q269K) exhibited decreased [^3H]-taurocholate (TCA) uptake (ratio of geometric means relative to OST α/β WT of 0.76, 0.75, 0.79, 0.13, respectively). Three OST α/β mutants (S228K, Q269K, E305A) had reduced [^3H]-TCA efflux % (ratio of geometric means relative to OST α/β WT of 0.86, 0.65, 0.79, respectively). Additionally, several OST α/β mutants demonstrated altered expression and cellular localization when compared to OST α/β WT. In summary, we identified OST α residues (Ser228, Thr229, Gln269, Glu305) in predicted transmembrane domains that affect expression of OST α/β and may influence OST α/β -mediated bile acid transport. These data advance our understanding of OST α/β structure/function and can inform future studies designed to gain further insight into OST α/β structure, or to identify additional OST α/β substrates and inhibitors.

Significance Statement

OST α/β is a clinically important transporter involved in enterohepatic bile acid recycling with currently no high-resolution protein structure and limited structure-function data. This study identified four OST α amino acids (Ser228, Thr229, Gln269, Glu305) that affect expression of OST α/β and may influence OST α/β -mediated bile acid transport. These data can be utilized to inform future investigation of OST α/β structure and refine molecular modeling approaches to facilitate the identification of substrates and/or inhibitors of OST α/β .

Introduction

The importance of the organic solute transporter alpha/beta (OST α/β ; SLC51A/B) in human physiology and disease is growing in recognition as critical roles for this protein in the transport of bile acids have emerged (Beaudoin et al., 2020a). This bidirectional, heteromeric transport protein is expressed predominantly on the basolateral membrane of intestinal, renal, biliary, and hepatic epithelial cells (Ballatori, 2005; Uhlen et al., 2015). While its expression is low in the healthy human liver (Uhlen et al., 2015), OST α/β protein is significantly upregulated in hepatic diseases associated with altered bile acid homeostasis (*i.e.*, primary biliary cholangitis and nonalcoholic steatohepatitis) (Boyer et al., 2006; Malinen et al., 2018). Disease-associated upregulation of hepatic OST α/β may facilitate basolateral bile acid efflux into sinusoidal blood, thereby protecting hepatocytes from damage due to accumulating toxic bile acids (Boyer et al., 2006; Chai et al., 2015; Malinen et al., 2018). This hypothesis is supported by the finding that OST α/β is inhibited by some xenobiotics associated with cholestatic hepatotoxicity (*e.g.*, troglitazone sulfate, ethinyl estradiol). Also, OST α/β preferentially transports conjugates of relatively hydrophobic, hepatotoxic bile acids such as tauro- and glycochenodeoxycholate compared to more hydrophilic bile acids including taurocholate (TCA) and glycocholate (GCA) (Beaudoin et al., 2020b; Suga et al., 2019). Furthermore, OST α/β expression is tightly regulated by farnesoid X receptor (FXR), a nuclear receptor activated during hepatocellular bile acid accumulation.

OST α/β plays an important role in the enterohepatic circulation of bile acids under non-cholestatic conditions; bile acids in the intestinal lumen are transported into enterocytes by the apical sodium-dependent bile acid transporter (ASBT) and undergo efflux into the mesenteric circulation by OST α/β (Dawson et al., 2005). Case studies in three pediatric patients suffering

from cholestasis, congenital diarrhea, and elevated liver transaminases highlight the importance of OST α/β in hepatobiliary bile acid homeostasis. These patients had rare genetic mutations in *SLC51A* (c.556C>T, p.Q186stop) (Gao et al., 2019) and *SLC51B* (c.79delT, p.F27frameshift) (Sultan et al., 2018) resulting in truncated OST α and OST β protein, respectively. Impaired OST α/β function in hepatocytes, enterocytes, and cholangiocytes likely results in cellular accumulation of bile acids and could explain these clinical phenotypes (Sultan et al., 2018). OST α/β also transports endogenous steroid hormones and several drugs including digoxin, docetaxel, statins, and sulfasalazine (Schwarz, 2012; Seward et al., 2003; Wang et al., 2001). However, the true contribution of this transporter to drug disposition and toxicity in health and disease is unknown and commonly unaccounted for in predictive models utilized during drug development. A better understanding of OST α/β protein structure and function is needed to inform future studies and facilitate accurate predictions of the role of this transporter in drug disposition and drug interactions in healthy and diseased populations.

Twenty years after the initial discovery of this transporter in a hepatic cDNA screen (Wang et al., 2001), OST α/β still lacks a high-resolution 3D structure. Alternative strategies to obtain information on protein structure include evolutionary conservation analysis, prediction of topology, homology modeling, and molecular modeling. Homology-based modeling, in tandem with three-dimensional molecular modeling is a common approach to elucidate protein-substrate interactions and identify key amino acids required for substrate recognition and transport (Schlessinger et al., 2018). The MEMSAT-SVM is a sequence-based platform that predicts pore-lining helices in transmembrane proteins that are essential for substrate interaction (Nugent and Jones, 2012) and can be utilized to guide *in vitro* functional studies. Previous *in vitro* work revealed that OST α is likely the substrate-binding subunit, while co-expression of OST β on the

plasma membrane is required for transport function (Seward et al., 2003). However, more data on OST α/β structure-function pertaining to bile acid transport is needed. Site-directed mutagenesis, informed by computational modeling, offers an attractive opportunity to study the relative contributions of individual amino acids to OST α/β structure and function. This approach has been used previously for other human transporters (Gruetz et al., 2016; Scalise et al., 2018; Zou et al., 2018). The present study employed *in silico* computational tools, *in vitro* site-directed mutagenesis, and *in vitro* functional assays to identify amino acids in OST α that influence bile acid transport.

Materials and Methods

Chemicals and Reagents. Unlabeled TCA and GCA were purchased from Chem-Impex International (Wood Dale, IL) and Sigma-Aldrich (St. Louis, MO), respectively. [^3H]-TCA (6.50–9.74 Ci/mmol, radiochemical purity >97%) and [^{14}C]-GCA, sodium salt (51.58 mCi/mmol, radiochemical purity >97%) were obtained from PerkinElmer Life Sciences (Boston, MA). GibcoTM Dulbecco's Modified Eagle Medium (catalog no. 11960-044), PureLinkTM HiPure Plasmid Miniprep Kit, Corning[®] BioCoatTM 24-well plates (catalog no. 08774124), radioimmunoprecipitation assay lysis and extraction buffer (catalog no. 89900), PierceTM dithiothreitol extraction buffer (catalog no. 20291), PierceTM bicinchoninic acid protein assay kit, NuPAGETM LDS Sample Buffer (catalog no. NP0007), NuPAGE 4-12% Bis-Tris gel (catalog no. NP0322Box), NuPAGE MOPS SDS Running Buffer (catalog no. NP001), NuPAGE Transfer Buffer (catalog no. NP0006), RestoreTM Western Blot Stripping Buffer (catalog no. 21063), LabTek[®] Chamber Slides (catalog no. 177445), fetal bovine serum, L-glutamine, penicillin-streptomycin, and goat-anti-mouse IgG AlexaFluorTM 488 secondary antibody (catalog no. A11001) were all purchased from Thermo Fisher Scientific (Waltham, MA). cCompleteTM ULTRA Tablets, Mini, EDTA-free, EASYpack Protease Inhibitor Cocktail (catalog no. 05892791001), PhosSTOPTM (catalog no. 04906845001), rabbit-anti-OST β antibody (catalog no. HPA008533), mouse-anti- β -actin antibody (catalog no. A1978), and 4',6-diamidino-2-phenylindole (DAPI) (catalog no. D9542) were purchased from Sigma-Aldrich, and rabbit-anti-OST α antibody (catalog no. ab103442) from Abcam (Cambridge, MA). The OST α antibody used for Western blot and immunocytochemistry analysis (ab103442) was a polyclonal mixture with an immunogen corresponding to a region within human OST α C-terminal amino acids (305-335). Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody and HRP-conjugated anti-

mouse antibody were obtained from Jackson ImmunoResearch (West Grove, PA), and Fluoromount-GTM Mounting Medium (catalog no. 0100-01) and 10% normal goat serum from Southern Biotech (Birmingham, AL). The Q5[®] Site-Directed Mutagenesis Kit was obtained from New England Biolabs (Rowley, MA)

Identification of OST α amino acids for mutagenesis. Two approaches were used to select specific OST α amino acids for mutation studies (Fig. 1). The first approach involved evolutionary conservation and topology prediction tools. Evolutionary conservation of amino acids in human OST α (UniProtKB protein identifier: Q86UW1) was evaluated using OST α protein sequences from mouse (Q8R000), rat (D4AC81), bovine (Q3T124), and skate (Q90YM5). In addition, several topology prediction tools (TOPCONS (Tsirigos et al., 2015), CCTOP (Dobson et al., 2015), DAS (Cserzö et al., 1997), PRED-TMR2 (Pasquier et al., 1999), HMMTOP (Tusnády and Simon, 2001), TMHMM (Krogh et al., 2001), SOSUI (Hirokawa et al., 1998) and MEMSAT-SVM (Nugent and Jones, 2012) were used to predict which OST α amino acids belong to the transmembrane domains (TMDs). The majority of these tools predicted a total of seven TMDs in OST α , and at least five TMDs were unanimously predicted. MEMSAT-SVM was used to predict which of the TMDs form the pore-lining region of OST α . Evolutionarily conserved, hydrophilic amino acids (Ser228, Thr229, Gln260, Gln269) in those TMDs were selected for mutagenesis (Fig. 2) in order to study their individual impact on transport function. A number of non-conservative and relatively conservative amino acid substitutions (Bordo and Argos, 1991) were used to generate the following mutants of OST α : S228K, S228T, T229S, Q260K, Q269E, and Q269K.

The second approach involved homology-based models. Since the protein structure of OST α/β is unavailable, the models were constructed based on the assumption that the transmembrane

helical parts of the OST α protein are closely related from a structural standpoint to selected transporter templates even if the exact sequence homology is low. OsSweet, a sugar transporter and a homolog of the human SLC50A1 (Tao et al., 2015), was selected as the template due to its similar seven TMD topology to OST α and the lack of an available seven TMD eukaryotic SLC template. In addition to their similar topology, the OsSweet folding conformation (inside open) is consistent with the physiological function of OST α/β to transport bile acids out of hepatocytes (efflux phase) (Dawson et al., 2010; Guo et al., 2018). Tools such as ClustalW (Larkin et al., 2007), PROMALS3D (Pei et al., 2008), and T-COFFEE (Notredame et al., 2000) were not able to provide reasonable alignments of predicted TMDs. However, locations of the transmembrane helices were successfully predicted with TMHMM (Krogh et al., 2001), Tmpred (Ikeda et al., 2003), and DAS (Cserzö et al., 1997). Aligned human, mouse, bovine, and rat OST α sequences were used for the final TMD prediction. Homology models were constructed based on a manually optimized alignment of transmembrane helices using the standard settings of Discovery Studio (BIOVIA, Dassault Systèmes, San Diego, CA, 2020) from the OsSweet template (PDB: 5CTH) with an inward-open structure that included a co-crystallized additive polyethylene glycol 400 (Supplemental Fig. 1A). The single OST β transmembrane helix was modeled based on helix-helix interactions between subunits of the trimeric OsSweet structure (Supplemental Fig. 1B). Prior to docking studies, the homology models from Discovery Studio were pre-processed and minimized using the Schrödinger Suite 2020-1 protein preparation wizard tool with modules Epik, Impact and Prime (Schrödinger, LLC, New York, NY, 2020). Structure of the TCA substrate was parametrized and minimized using the Ligprep module (Schrödinger, LLC). Molecular docking studies were computed using the induced fit workflow of Schrödinger by employing the SP-setting for the Glide docking module, and side chain

movements of 5 Å were considered for the conformational refinement using the Prime module. Based on the visualized OST α amino acids and their mutual and substrate interactions, this approach led to the selection of four OST α amino acids Cys103, Phe122, Asn298, Glu305 for alanine scanning mutagenesis (Morrison and Weiss, 2001). Finally, to qualitatively study the stability of the homology model of OST α with a docked ligand (TCA) (Fig. 3), a molecular dynamics simulation was performed that included a phospholipid membrane model of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, neutralized with ions and solvated with simple point-charge waters using Desmond (Maestro-Desmond Interoperability Tools, Schrödinger, LLC; Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY, 2021). The structure was first minimized using the protein preparation wizard of Schrodinger with a default heavy atom root-mean-square deviation constraint of 0.3 Å. The Desmond molecular dynamic simulation was unconstrained and run using orthorhombic periodic boundary conditions. In the simulation setup, the position of the membrane bilayer was first placed automatically on helices and the final orientation was adjusted manually. Prior to the molecular dynamics simulation, the system was subjected to a default relaxation protocol of Desmond and heated up to the simulation temperature. A 500-nanosecond simulation was run using the NPT protocol at 300 Kelvin temperature, pressure of 1.01325 bar, Noe-Hoover thermostat and a timestep of 2 femtoseconds. The OST α/β model was inspected using a visualization program Visual Molecular Dynamics (VMD Version 1.9.3, NIH Center for Macromolecular Modeling and Bioinformatics, at the Beckman Institute, University of Illinois at Urbana-Champaign, Champaign, IL). Graphical illustrations were generated using PyMol (The PyMOL Molecular Graphics System, Version 2.0, Schrödinger, LLC).

DNA constructs and mutagenesis. Site-directed mutagenesis and the Flp-In™ cell and vector system were used to incorporate mutations into the OST α subunit. A previously developed pcDNA5.1/FRT expression vector containing human wild-type (WT) *SLC51A* (NM_152672.5, OST α) and *SLC51B* (NM_178859.3, OST β) (Malinen et al., 2018) was used as a template for mutagenesis using the Q5® Site-Directed Mutagenesis Kit, per the manufacturer's instructions. DNA primer pairs for the generation of each *SLC51A* mutant via polymerase chain reaction (PCR) (Supplemental Table 1) were obtained from Integrated DNA Technologies (Coralville, IA). Prior to ligation, the integrity of PCR products was evaluated by agarose gel electrophoresis. Subsequently, plasmids were transformed into high-efficiency New England Biolabs 5-alpha competent *E. coli* cells according to the manufacturer's instructions. Ampicillin-resistant colonies were isolated and expanded overnight in Luria-Bertani medium containing ampicillin (100 μ g/mL) in an orbital shaker (37°C, 160 rpm). The PureLink™ HiPure Plasmid Miniprep Kit was used to isolate plasmids. The concentration and purity of DNA were measured using a NanoDrop spectrophotometer (model ND-1000, Thermo Fisher Scientific). The OST α constructs were then confirmed by Sanger sequencing (Eurofins Genomics LLC, Louisville, KY).

Generation of OST α / β WT and OST α / β mutant overexpressing cell lines. Mock, OST α / β WT, and OST α / β mutant Flp-In™ 293 cell lines were generated using lipofection as described previously (Malinen et al., 2018). Established cell lines stably expressing WT OST α and OST β are referred to hereafter as OST α / β WT cells, and cell lines expressing mutated OST α and WT OST β are referred to hereafter as OST α / β mutants for which the OST α amino acid substitution was specified (*e.g.*, S228K). Cells transfected with empty pcDNA5.1/FRT vector were referred to as Mock cells.

Cell culture/maintenance of Flp-In™ 293 cells. Mock, OST α/β WT, and mutant cells were cultured at 37°C and 5% CO₂ in T-75 cell culture flasks (Sarstedt, Newton, NC) with Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin-100 ug/mL streptomycin. Media was replaced every 3-4 days and cells were subcultured once weekly using 0.25% trypsin-EDTA for detachment. Passages 5-15 were used for all assays. Cell viability was determined by Trypan blue exclusion.

Uptake studies in Mock, OST α/β WT, and mutant Flp-In™ 293 cells. The impact of the selected OST α amino acid substitutions on OST α/β -mediated uptake of [³H]-TCA was assessed at 30 sec, a time point within the linear range of the initial uptake vs. time profile (Beaudoin et al., 2020b; Malinen et al., 2018). A concentration of 5 μ M was selected to study TCA transport as it mimics the physiologic concentration of serum bile acids (4-5 μ M) following a meal (Di Ciaula et al., 2017). Cells were seeded at a density of 5 x 10⁵ cells/well on poly-D-lysine-coated Corning® BioCoat™ 24-well plates. Uptake studies were performed ~48 hr after seeding when cells had formed a confluent monolayer. Medium was aspirated and the cells were briefly washed twice with warm (37°C) sodium-free extracellular fluid (ECF) buffer (125 mM KCl, 25 mM KHCO₃, 0.4 mM K₂HPO₄, 10 mM D-glucose, 1.4 mM CaCl₂, 1.2 mM anhydrous MgSO₄ and 10 mM HEPES; pH 7.4), based on earlier OST α/β uptake studies (Malinen et al., 2018; Malinen et al., 2019). Subsequently, cells were conditioned with 200 μ L/well of 5 μ M [³H]-TCA or 5 μ M [¹⁴C]-GCA dosing solution in warm ECF buffer for 30 seconds (sec) in an incubator (37°C, 5% CO₂). Dosing solution was aspirated, and cells were briefly washed twice with ice-cold (4°C) ECF buffer. Each cell monolayer was solubilized with 400 μ L of lysis buffer (0.5% Triton X-100 and 0.005% antifoam A in phosphate-buffered saline [PBS]) and the contents were mixed vigorously on a VWR VX-2500 Multi Tube Vortexer (Marshall Scientific, Hampton, NH)

for 20 min. To determine the amount of radiolabeled compound in cells, 300 μL aliquots of the cell lysate were added to 10 mL of BioSafe II counting cocktail (RPI, Mt. Prospect, IL) in scintillation vials. Radioactive counting was performed using a liquid scintillation analyzer (Tri-Carb 3100TR, PerkinElmer). Total cellular protein was determined by a Pierce™ bicinchoninic acid protein assay kit using an aliquot (25 μL) of cell lysate. Absorbances were read at 562 nm using a PowerWave XS microplate spectrophotometer (BioTek Instruments). Uptake assays were repeated on three separate days, with three technical replicates on each day, resulting in nine replicate samples for each cell line.

Efflux studies using Mock, OST α/β WT, and mutant Flp-In™ 293 cells. Efflux studies were performed using the same methodology as described for uptake assays, except that the 30-sec substrate conditioning phase was extended to 10 min (preloading phase) to allow for maximal intracellular accumulation of substrate prior to efflux. A 10-min duration for the preload phase was selected to maximize intracellular [^3H]-TCA concentrations based on the finding that OST α/β -mediated cellular uptake of [^3H]-TCA using the same Flp-In™ system plateaued at ~10 min (Malinen et al., 2018). The selected time point for the efflux phase was 5 min based on pilot studies. After this 10-min preloading phase, the dosing solution was aspirated, and cells were briefly washed twice with ice-cold ECF buffer. Efflux of preloaded substrate was initiated by incubating one row of wells with warm ECF buffer (200 μL /well). Plates were placed on a flat external plate warmer at 37°C for 5 min before transferring efflux buffer (*i.e.*, warm ECF buffer) from each well to a 96-deep well plate (Greiner Bio-One, Monroe, NC). Cell lysates after the 10-min preload were collected to evaluate total intracellular substrate content prior to efflux, while cell lysates after the subsequent 5-min efflux were collected separately to determine the residual intracellular substrate content. To determine the amount of radiolabeled compound in cells and

efflux buffer, 10 mL of BioSafe II counting cocktail was added to samples (300 μ L aliquots of cell lysate or 200 μ L of efflux buffer) in scintillation vials. Radioactive counting and measurement of total cellular protein for each cell line was performed as described above. Efflux assays were repeated on three separate days in triplicate samples on each day, resulting in nine replicate samples for each cell line. Efflux assays for homology-informed mutants (C103A, F122A, N298, E305A) were performed on separate occasions (*i.e.*, Group 2) from the other mutants (S228K, S228T, T229S, Q260K, Q269E, Q269K) designated as Group 1, using the same OST α/β WT comparator cell line. Of note, one out of nine replicates for S228K, S228T, Q269E, and Q269K was lost due to a pipetting error.

Western blot analysis. Cells were detached with 5 mM EDTA at 37°C from the T-75 flasks. The resulting cell suspension ($\sim 7 \times 10^6$ cells) was centrifuged at 200g for 5 min. After removing the supernatant, 1 mL of Radioimmunoprecipitation assay Lysis and Extraction buffer, supplemented with cOmplete™ ULTRA Tablets, Mini, EDTA-free, EASYpack Protease Inhibitor Cocktail and PhosSTOP™, was mixed by vortex and whole cell lysates were collected. Total cellular protein for each cell line was quantified using a Pierce™ bicinchoninic acid protein assay kit. Lysate samples containing 20 μ g of total protein were prepared for Western blot analysis in 10% (v/v) of 0.5M Pierce™ dithiothreitol extraction buffer and 25% (v/v) NuPAGE LDS Sample Buffer, and added to separate wells of a NuPAGE 4-12% Bis-Tris gel. After electrophoresis using NuPAGE MOPS SDS Running Buffer, proteins were transferred to a polyvinylidene difluoride membrane by incubating in NuPAGE Transfer Buffer overnight at 4°C. The membrane was blocked in a 5% milk solution for 1 hr before incubation with rabbit-anti-OST α antibody (1:250 dilution) or rabbit-anti-OST β antibody (1:150 dilution) overnight at 4°C in 5% (w/v) bovine serum albumin/Tris-buffered saline with Tween 20 (TBST) buffer. To

establish a loading control, the membrane was incubated in mouse-anti- β -actin antibody (1:5,000 dilution) at room temperature in 5% bovine serum albumin/TBST for 1 hr. The membranes were washed with TBST three times and then incubated with HRP-conjugated anti-rabbit antibody (1:5,000 dilution) for OST α and OST β , and HRP-conjugated anti-mouse antibody (1:10,000 dilution) for β -actin at room temperature in 5% milk/TBST for 1 hr. The imaging signal was produced using chemiluminescence reagents (ECL Select, GE Healthcare Bio-Sciences, Piscataway, NJ). Images were captured and processed using BioRad (ChemiDoc XRS+) imager and Image Lab software (Bio-Rad, Hercules, CA). OST α and OST β were evaluated on the same membrane without stripping, while β -actin loading control was evaluated on the same membrane following stripping with Restore[™] Western Blot Stripping Buffer for 15 min at room temperature (20°C).

Immunocytochemistry. Eight-well LabTek[®] Chamber Slides were coated using a 0.1 mg/mL poly-D-lysine solution. Mock, OST α/β WT, and mutant cells were seeded at a density of 0.4×10^5 cells/well. After growing to ~70% confluence, cells were fixed with 4% paraformaldehyde. Cells were permeabilized for 10 min with 200 μ L of 0.5% saponin in PBS and then blocked with 150 μ L of 10% normal goat serum in 0.1% saponin for 1 hr at room temperature. After removing the blocking solution, cells were incubated with either 150 μ L of rabbit-anti-OST α antibody (1:50 dilution) or rabbit-anti-OST β antibody (1:50 dilution) overnight at 4°C. Cells were washed with PBS three times and incubated with 150 μ L of goat-anti-mouse IgG AlexaFluor[™] 488 secondary antibody (1:200 dilution) for 1 hr at room temperature in the dark. Cells were then washed with PBS and Milli-Q[®] H₂O before incubating in 150 μ L of a DAPI dilution (1:200) for 2 min at room temperature in the dark. Chamber walls were removed before adding 2-3 drops of Fluoromount-G[™] Mounting Medium. A #1.5 glass coverslip (0.17mm) (VWR International,

Radnor, PA) was placed on top of chamber slides before storing at 4°C in the dark for ~24 hr to ensure hardening of the slide. Images were captured using a Nikon ECLIPSE Ti2 microscope (Nikon, Melville, NY) and optimized with Nikon NIS Elements software. All images were taken using a 60X oil immersion objective.

Data analysis. Uptake and efflux data for [³H]-TCA and [¹⁴C]-GCA were normalized to total cellular protein (pmol/mg of protein). OST α/β WT and mutant cell uptake/efflux data were not adjusted for Mock cell function due to negligible (~5%) transport observed in Mock cells. No outliers were detected for uptake or efflux data using Grubbs's test. Percent (%) efflux was calculated by dividing the amount of radiolabeled substrate (pmol) per mg protein in ECF buffer following the 5-min efflux by the amount of radiolabeled substrate in cell lysate (pmol) per mg protein following the 10-min preload. Percent (%) of residual cellular substrate was calculated by dividing the amount of radiolabeled substrate in cell lysate (pmol) per mg protein following the 5-min efflux by the amount of radiolabeled substrate in cell lysate (pmol) per mg protein following the 10-min preload. The ratio of geometric mean uptake and % efflux for each mutant relative to OST α/β WT, and corresponding 95% credible intervals, were calculated using a Bayesian generalized linear mixed model with group-specific terms. Experimental day was included as a random effect group-specific term for Bayesian models of uptake and efflux data. Group number was also included as a random effect group-specific term for the Bayesian model of efflux data (but not uptake data) since, as previously described, two groups of mutants were evaluated on separate occasions for efflux assays only. Protein expression data for OST α/β WT and mutant cells were normalized to loading control (β -actin) and OST α/β expression levels in Mock cells were subtracted from densitometry values from OST α/β WT and each mutant cell line. ImageJ Version 1.53f 25 was used to normalize and quantify band intensity from Western

blots (Schneider et al., 2012). All statistical analyses were performed using R Version 1.4.1103 (R Core Team, 2020) and GraphPad Prism 9 (GraphPad Software, La Jolla, CA). Linear mixed analysis and Bayesian regression modeling were performed using the lme4 (Bates et al., 2015) and rstanarm (Goodrich et al., 2020) R packages, respectively (see Data Supplement 1).

Results

Molecular modeling. The 3D homology model studies indicated that despite the low similarity of residues, OST α helices could be aligned reasonably well with those in the OsSweet template. In docking studies, most of the docking poses for TCA within the OST α cavity were oriented so that the bile acid A-ring was pointing towards the extracellular space. This region of the substrate was consistently in close contact with amino acids Phe122 and Asn298 (Fig. 3). Notably, the corresponding residues in OsSweet were in contact with the respective ligand (Tao et al., 2015). The side chain of TCA was oriented towards the cytoplasm but was more flexible with fewer contacts with the surrounding helices due to the inward-open structure of the template (Fig. 3; Supplemental Fig. 2, Data Supplement 2 and 3). Of residues identified by evolutionary conservation and topology prediction, Thr229 and Gln269 were also oriented towards the predicted cavity of the homology model, Ser228 was facing the neighboring helix and Gln260 was placed away from the cavity (Fig. 3; Supplemental Fig. 2, Data Supplement 2 and 3). The final homology model appeared relatively stable over time, with helices showing the least fluctuation in 500 nanosecond simulations (Supplemental Fig. 3, Data Supplement 4).

OST α / β -mediated Uptake. Out of ten mutants, four showed a decrease in [³H]-TCA uptake (S228K, T229S, Q269E, Q269K) relative to OST α / β WT. Specifically, the respective ratios of geometric mean 30-sec uptake relative to OST α / β WT (95% credible interval) were 0.76 (0.67 – 0.88), 0.75 (0.65 – 0.86), 0.79 (0.68 – 0.90), and 0.13 (0.11 – 0.15) (Fig. 4).

OST α / β -mediated Efflux. [³H]-TCA % efflux (of the preloaded amount) was reduced in three mutant cells, S228K, Q269K and E305A, which showed respective ratios of the geometric mean efflux % relative to OST α / β WT (95% credible interval) of 0.86 (0.78 – 0.95), 0.65 (0.59 – 0.71),

and 0.79 (0.72 – 0.87) (Fig. 4). These mutants also differed from OST α / β WT in other transport parameters. Specifically, S228K and Q269K displayed increased % residual cellular [3 H]-TCA relative to OST α / β WT (Supplemental Table 2). Due to similar [3 H]-TCA and [14 C]-GCA uptake/efflux data for Mock, OST α / β WT, and mutant cell lines, [14 C]-GCA uptake/efflux was only studied for six mutants (S228K, S228T, T229S, Q260K, Q269E, Q269K) (*data not shown*).

OST α and OST β Protein Levels. Protein levels of OST α and OST β in Mock, OST α / β WT, and mutant cells were assessed by densitometry of the Western blots (Supplemental Fig. 4). Mock cells, as expected, had negligible to low expression of both OST α forms and OST β protein. The smaller OST α immature precursor protein is believed to be localized to the endoplasmic reticulum while the mature OST α glycoprotein is expressed on the plasma membrane (Dawson et al., 2005; Soroka et al., 2008). While OST α / β WT cells had a low ratio of immature to mature OST α proteins, three mutants (S228K, Q269E, Q269K) expressed higher absolute levels of immature OST α than mature protein (Fig. 5a; Supplemental Fig. 4f). All mutant cells except Q260K had decreased OST α mature and OST β protein expression compared to OST α / β WT cells (Fig. 5; Supplemental Fig. 4). Mature OST α expression had a strong correlation ($r = 0.99$) with expression of OST β protein for each mutant cell line relative to OST α / β WT (Supplemental Fig. 4g).

OST α and OST β cellular localization. Plasma membrane localization of OST α and OST β was negligible in Mock cells. The localization of OST α and OST β in mutant cell lines with altered [3 H]-TCA uptake and/or efflux (S228K, T229S, Q269E, Q269K, E305A) is shown in Fig. 5, while data for all mutant cells is presented in Supplemental Fig. 5. OST α / β WT, C103A, and N298A cells had apparent plasma membrane localization of OST α and OST β . Based on qualitative/visual inspection of the images (Supplemental Table 2), several mutant cells (F122A,

S228K, S228T, T229S, Q260K, Q269E, Q269K, E305A) appeared to have reduced plasma membrane expression of OST α protein in comparison to OST α/β WT. Five of these mutants (F122A, S228T, T229S, Q260K, E305A) also seemed to have increased intracellular localization of OST α protein relative to OST α/β WT. Two mutants (S228K, Q269K) showed negligible membrane localization of both OST α and OST β protein. Interestingly, F122A, T229S, Q260K, and E305A cells all showed OST β plasma membrane staining similar to OST α/β WT despite a reduction in OST α membrane staining.

Discussion

OST α/β is a clinically relevant transporter involved in enterohepatic recycling of bile acids, particularly in the small intestine, (Dawson et al., 2015; Gao et al., 2019; Sultan et al., 2018) and is upregulated in the human liver in certain diseases (Boyer et al., 2006; Malinen et al., 2018). Some drugs are substrates and/or inhibitors of OST α/β (Beaudoin et al., 2020b; Malinen et al., 2019; Schwarz, 2012; Seward et al., 2003; van de Wiel et al., 2018), which could impact pharmacotherapy. The current lack of a high-resolution OST α/β structure has prevented detailed structure/function studies of this transporter and identification of potential drug substrates and/or inhibitors. Here, we aimed to identify specific OST α residues that are important for OST α/β -mediated bile acid transport through computational modeling and site-directed mutagenesis, followed by analysis of protein expression, localization, and function. Given the bidirectionality of OST α/β transport, both [^3H]-TCA uptake and efflux were evaluated for each OST α/β mutant. Given that OST α is believed to be the substrate-binding subunit (Seward et al., 2003), only OST α was selected for the final molecular dynamic simulation to test stability of the modeled transmembrane helices and to determine whether the TCA ligand remained within the cavity under the conditions tested. While we did consider modeling interactions between OST α and OST β based on the LAT1-4F2hc heterodimer, the homology was insufficient and its physiological function too distant to consider it within the same genetic subfamily; therefore, assessing the location of interaction derived from this complex seemed unlikely. Of the mutants informed by evolutionary conservation and topology prediction tools, both Gln269 mutants Q269E and Q269K displayed reduced uptake, but the Q269K mutant also demonstrated reduced % efflux. Based on the molecular model and docking, Gln269 faces the OST α cavity towards the TCA substrate. The change to glutamate adds a negative charge but this amino acid is similar in

size to glutamine, while lysine adds a positive charge and is considerably larger in volume than both glutamate and glutamine. Therefore, it is reasonable to assume that changes in charge and/or volume of this residue impact electrostatic interactions with the negatively charged TCA and its recognition and/or transport, with Q269K showing more notable effects as expected.

It is currently unknown whether bidirectional transport mediated by OST α/β is truly symmetric or asymmetric (Beaudoin et al., 2020a). While some mutants (i.e., T229S, Q269E, E305A) appear to have a different impact on [³H]-TCA uptake compared with efflux, it is unclear whether this is due to inherent asymmetric OST α/β transport of the substrate, or whether differences in the experimental setup between uptake and efflux studies (e.g., efflux studies required an additional preloading phase) could have contributed, in part, to these observed differences. The S228K mutant (but not S228T) showed decreased uptake and % efflux relative to OST α/β WT. Given the location of the Ser228 residue at the interface between two helices, it is understandable that the small difference between neutral, uncharged hydroxyl-containing serine and threonine residues may not create as drastic a change as the introduction of a basic and larger lysine that might disrupt helix-helix interactions. A relatively conservative mutation at the OST α 229 position (T229S) resulted in reduced uptake but had no effect on efflux. This change could not be easily explained by the model as the residue did not face the OST α cavity. Lastly, the only lysine-substituted mutant (Q260K) that did not show any differences from OST α/β WT in uptake or % efflux could be rationalized by the outward projection of this residue, away from the TCA substrate, in the model. Two mutants (T229K, Q260E) were not investigated due to failed cell line development (*data not shown*). As anticipated, lysine substitution generally had a greater impact on OST α/β -mediated bile acid transport than a more conservative change of the same residue (Bordo and Argos, 1991; Wong and Kwon, 2015). Of

the four OST α residues selected for alanine scanning mutagenesis by using homology models, only the E305A mutant showed alterations in [3 H]-TCA transport, despite orientation of all these amino acids towards the docked TCA substrate. Since OsSweet residues that correspond to OST α Phe122 and Asn298 form a contact with the sugar substrate (Tao et al., 2015), it was somewhat unexpected that F122A and N298A mutations had no effect on [3 H]-TCA transport. Substitutions other than alanine might be more effective, or alternatively, an improved alignment method might indicate neighboring residues that would be more important. Nonetheless, the E305A mutation reduced [3 H]-TCA % efflux. This residue faces the cavity orienting towards TCA's D-ring and side chain (Fig. 3; Supplemental Fig. 2, Data Supplement 2 and 3). Incidentally, the corresponding residue in OsSweet contributes to the intracellular gate that regulates substrate access to the cavity (Tao et al., 2015), suggesting that the OST α residue Glu305 may play a role in efflux of bile acids.

Alterations in OST α and/or OST β membrane localization (Supplemental Fig. 5; Supplemental Table 2) and/or reductions in protein levels (Supplemental Fig. 4) were observed for each mutant. However, several of these mutants (C103A, F122A, S228T, Q260K, N298A) showed similar uptake and % efflux data relative to OST α/β WT (Fig. 4). There are two possible explanations for these observations: First, these mutations in OST α actually increase the efficiency of OST α/β -mediated transport. This phenomenon has been documented in site-directed mutagenesis studies of plant transporters (Fontenot et al., 2015; Wang et al., 2017). Additionally, site-directed mutagenesis of a highly conserved residue in human glutathione S-transferase resulted in increased substrate affinity and metabolic activity of the enzyme (Kalita et al., 2020). Second, OST α/β is a high capacity transporter that is non-saturable at 1 mM [3 H]-TCA (Suga et al., 2019), and limited only by the amount of protein available for transport

(Malinen et al., 2018). In the present study, the level of OST α/β protein available for efficient [^3H]-TCA transport was likely more than sufficient for the majority of mutant cell lines at the studied concentration (5 μM). The Q269K mutant cell line is the most obvious exception, since this mutant exhibited the lowest (i.e., negligible) expression of mature OST α and OST β among all mutant cell lines investigated by Western blot, and had the most prominent effect on transport (both uptake and efflux). The substantial decreases in OST α/β protein expression but modest reductions in [^3H]-TCA transport by mutants S228K, T229S, Q269E and E305A suggest that the observed differences in OST α/β -mediated transport are driven only partially by altered plasma membrane localization, misfolding, or reduced expression. Nevertheless, future studies are needed to investigate the underlying mechanism(s) (e.g., intrinsic activity, protein expression) of altered [^3H]-TCA transport.

In the present study, the uptake and efflux of [^3H]-TCA by OST α/β WT and mutants were investigated only at a single time point and concentration. Kinetic parameters V_{max} and K_m were not assessed here because OST α/β was not saturable at the highest soluble concentration (1 mM) of [^3H]-TCA (Malinen et al., 2018). Therefore, a single time point (i.e., 30-sec uptake, 5-min efflux) and concentration (5 μM) for each assay were selected based on previously published data as described in the Methods section. Another limitation is the adjustment of transport function data using total cellular protein instead of total membrane-associated OST α/β protein. Currently available membrane extraction methods also capture intracellular organelle-bound proteins (Bunger et al., 2009). However, the non-functional, immature OST α associates with intracellular organelles (Dawson et al., 2005; Soroka et al., 2008); therefore, the membrane-bound fraction would also likely capture this nonfunctional transport protein. Additionally, the proper stoichiometry of OST α/β has yet to be definitively determined (Beaudoin et al., 2020a).

Additional studies would be needed to investigate whether the reduction in uptake and/or % efflux observed for select mutants (S228K, T229S, Q269E, Q269K, E305A) was due to decreased OST α/β protein levels and/or plasma membrane localization rather than alterations in intrinsic transport activity.

While previous studies have investigated naturally occurring OST α/β mutations (Schwarz, 2012; Sultan et al., 2018), the present study is the first to investigate synthetic OST α mutations informed by computational modeling to evaluate OST α/β protein structure/function. However, all models with low similarity between the template and the target sequences are sensitive to changes in alignment of the transmembrane helices. A difference of one amino acid in helix assignments may shift one residue that points to the cavity to face the neighboring helix or even the surrounding lipid membrane, thereby altering the quaternary structure of the protein. In summary, our data provide insight that can be utilized to inform future investigations of OST α/β protein structure. With the novel identification of four OST α residues (Ser228, Thr229, Gln269, Glu305) impacting bile acid transport, molecular modeling approaches can be refined for this protein to facilitate identification of additional endogenous and exogenous substrates and/or inhibitors of OST α/β .

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Authorship Contributions

Participated in research design: Murphy, Beaudoin, Malinen, Swaan, Honkakoski, Brouwer.

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Contributed new reagents or analytic tools: Laitinen, Malinen.

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Wrote or contributed to the writing of the manuscript: Murphy, Beaudoin, Laitinen, Sjöstedt, Swaan, Honkakoski, Brouwer.

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Footnotes

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Conflicts of Interest

The authors declare no conflicts of interest.

Figure Legends

Fig. 1. Flow chart of OST α / β mutagenesis and evaluation. Evolutionary conservation and topology prediction tools were used to identify residues for mutagenesis resulting in the following OST α mutants: S228K, S228T, T229S, Q260K, Q269E, and Q269K. Homology models were used to identify additional OST α residues for alanine scanning mutagenesis: C103A, F122A, N298A, and E305A. Following site-directed mutagenesis, stable Flp-In™ 293 cell lines were established for Mock, OST α / β wild-type, and OST α / β mutants. OST α / β -mediated bile acid uptake and efflux, protein localization and expression were evaluated for each stable cell line. Alanine (Ala; A); Asparagine (Asn; N); Cystine (Cys; C); Glutamate (Glu; E); Glutamine (Gln; Q); Lysine (Lys; K); Phenylalanine (Phe; F); Serine (Ser; S); Threonine (Thr; T)

Fig. 2. Predicted OST α transmembrane domains and amino acid residues selected for site-directed mutagenesis. OST α is composed of 340 amino acids, with seven predicted transmembrane domains (The UniProt Consortium, 2021). All amino acid residues selected for mutagenesis (see Fig. 1) are highlighted in red. Every 10th amino acid residue is numbered accordingly. Protter version 1.0 (Omasits et al., 2014) was used to create this figure.

Fig. 3. Homology model of OST α / β with the docked taurocholate (TCA) substrate. The seven OST α helices are shown as barrels with a color gradient from H1 (red) through H4 (purple) to H7 (blue) and the single OST β helix as a golden barrel. All mutated amino acids are displayed with licorice structures and single-letter codes while the TCA backbone is presented as a green ball-and-stick structure with the bile acid A-ring closest to amino acids Phe122 and Asn298. The view on the left is from the cytoplasmic side into the OST α cavity. The side view

on the right shows the extracellular side at the top and cytoplasmic side at the bottom. Made using PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

Fig. 4. Forest plot of OST α / β -mediated [3 H]-TCA 30-sec uptake and [3 H]-TCA % efflux of preloaded amount for OST α / β wild-type (WT) and OST α / β mutant cell lines. Data points represent the ratio of the geometric mean value between OST α / β mutant and OST α / β WT for 30-sec uptake (dark blue) and % efflux (light blue). Error bars represent the 95% credible interval. Ratios of geometric mean values and credible intervals were calculated using a Bayesian generalized linear mixed model adjusted on experimental day for both uptake and efflux data, and group number for efflux data only since a single group was evaluated for uptake assays. Across the three uptake assays, nine independent replicates were collected, in total, for all OST α / β mutant cell lines. Similarly, for the three efflux assays, nine independent replicates were collected for all OST α / β mutant cell lines except S228K, S228T, Q269E, and Q269K, for which eight independent replicates were collected. *95% credible interval does not cross 1.0.

Fig. 5. Western blot and immunocytochemistry of Mock, OST α / β wild-type (WT), and OST α / β mutant cell lines. a) OST α and b) OST β Western blot analysis of whole cell lysate for Mock, OST α / β WT, and OST α / β mutant cell lines. Protein bands for mature (likely plasma membrane-localized) and immature (likely intracellular-localized) OST α are shown slightly above and below 38 kDa, respectively, at ~14 kDa for OST β , and at ~42 kDa for the β -actin loading control. Images of OST α , OST β , and loading control Western blots were cropped from one membrane (see Supplemental Fig. 4 for the full, uncropped membranes). Red frames on the Western blot indicate OST α / β WT and OST α / β mutant cell lines that demonstrated differences in [3 H]-TCA 30-sec uptake and/or efflux %. Immunocytochemistry staining of nuclei using DAPI (4',6-diamidino-2-phenylindole; blue) and c) OST α (green) or d) OST β (green) for OST α / β WT

and for the five OST α/β mutant cell lines that showed differences in uptake and/or efflux.

Qualitative assessment of plasma membrane localization (++; strong), (+; modest), (-; negligible) is provided next to the label for each OST α/β mutant. All qualitative measures of localization are relative to OST α/β WT. Images were taken using a Nikon ECLIPSE Ti2 microscope with a 60X oil immersion objective (field of view \approx 40 μ m wide).

Fig. 1

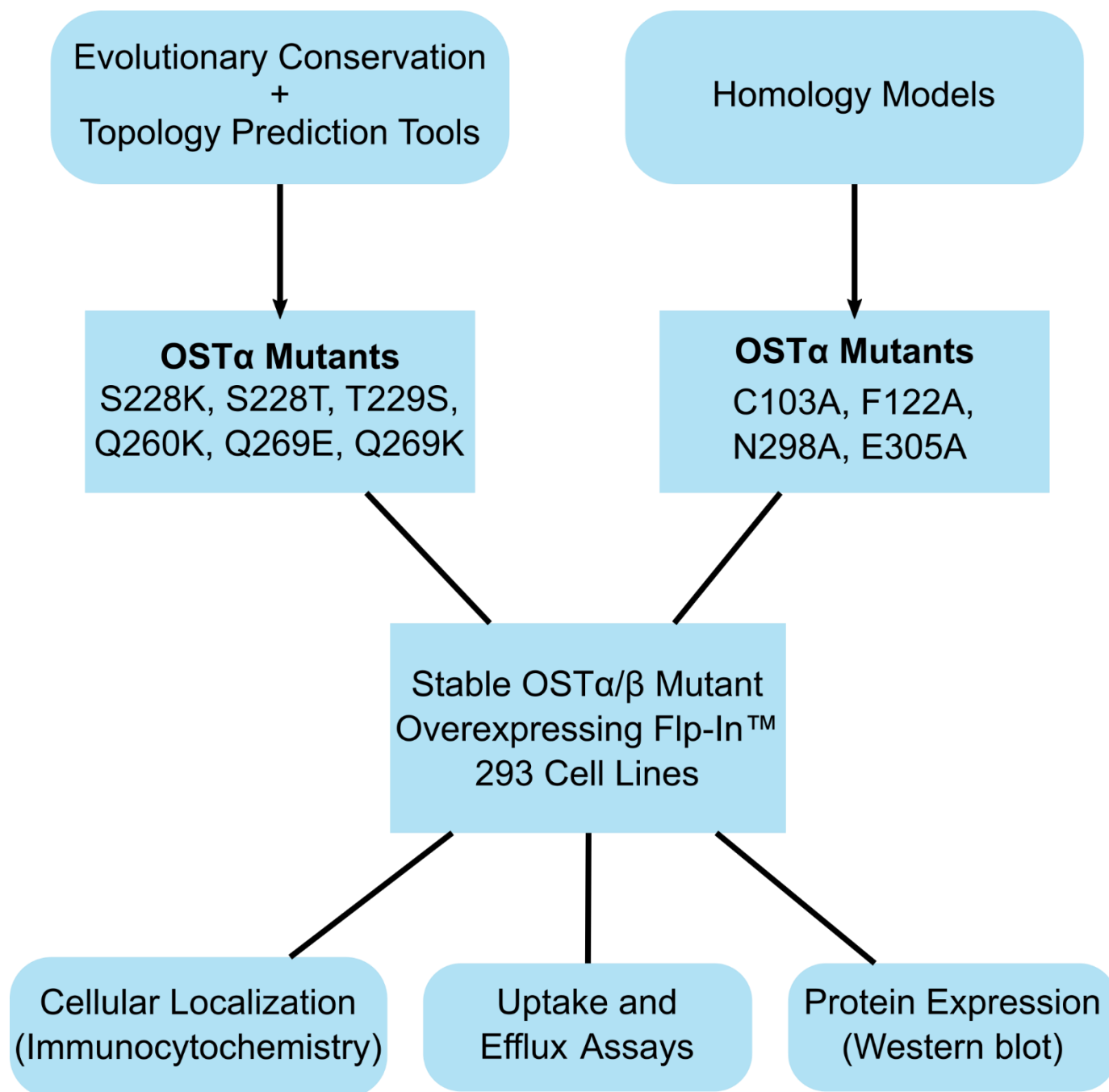


Fig. 3

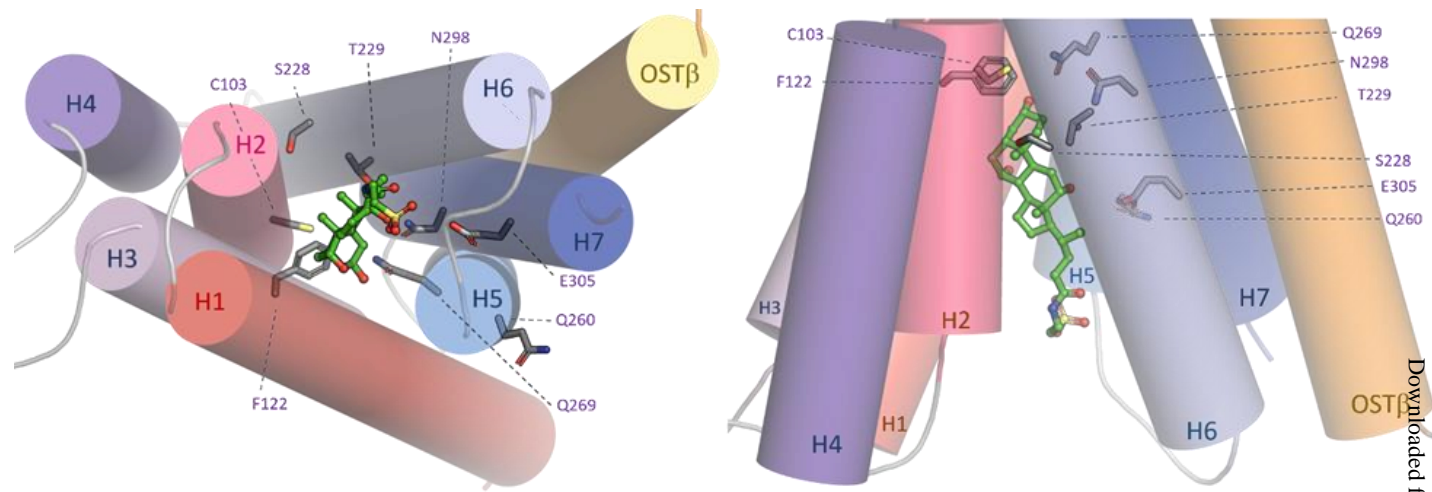


Fig. 4

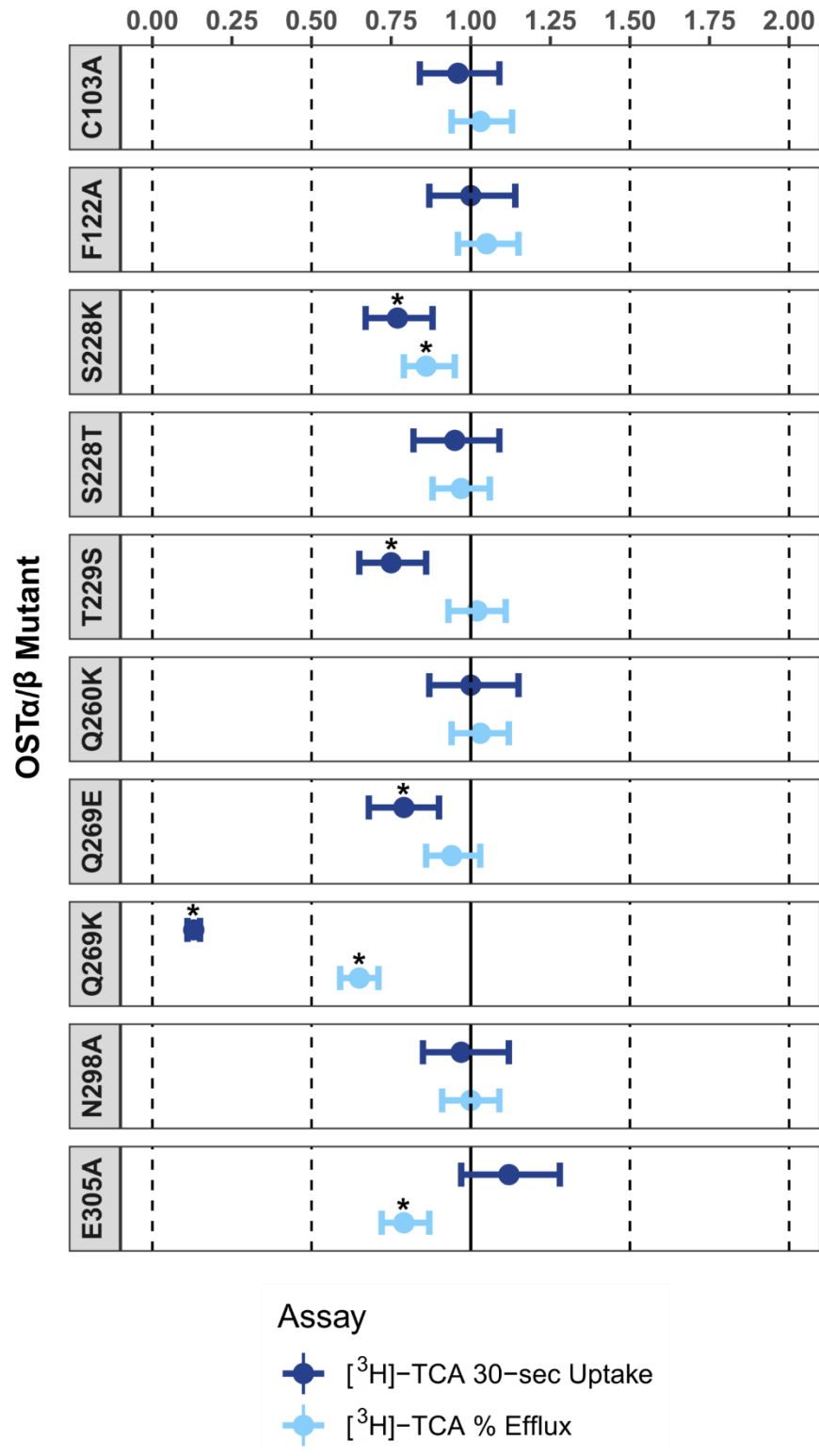
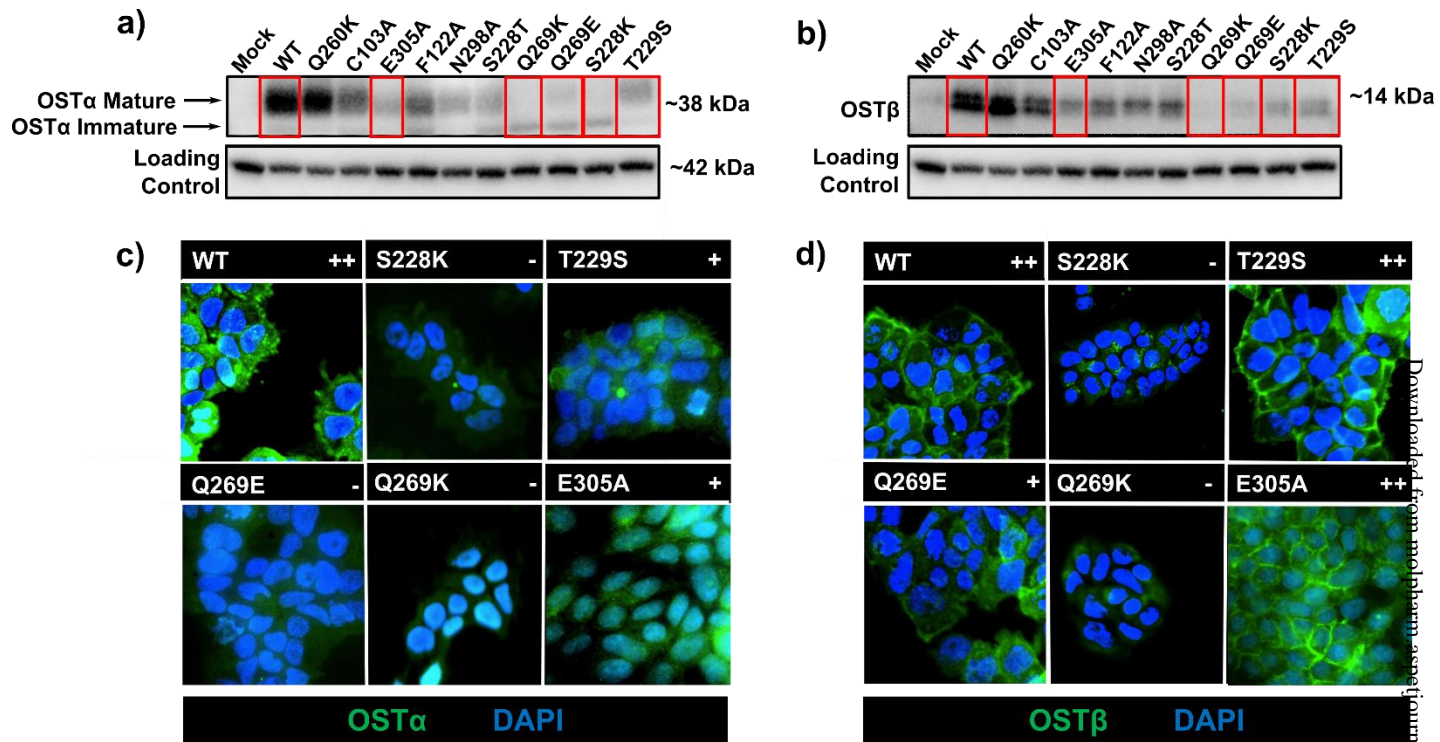


Fig. 5



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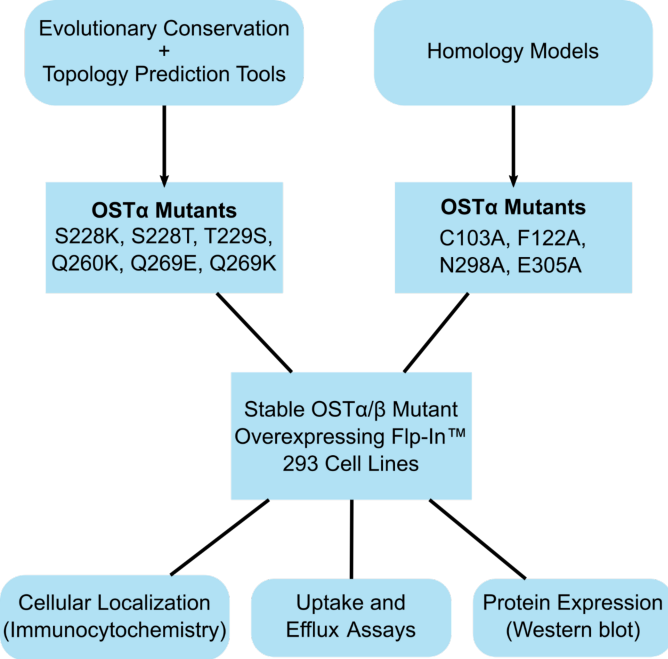


Figure 1

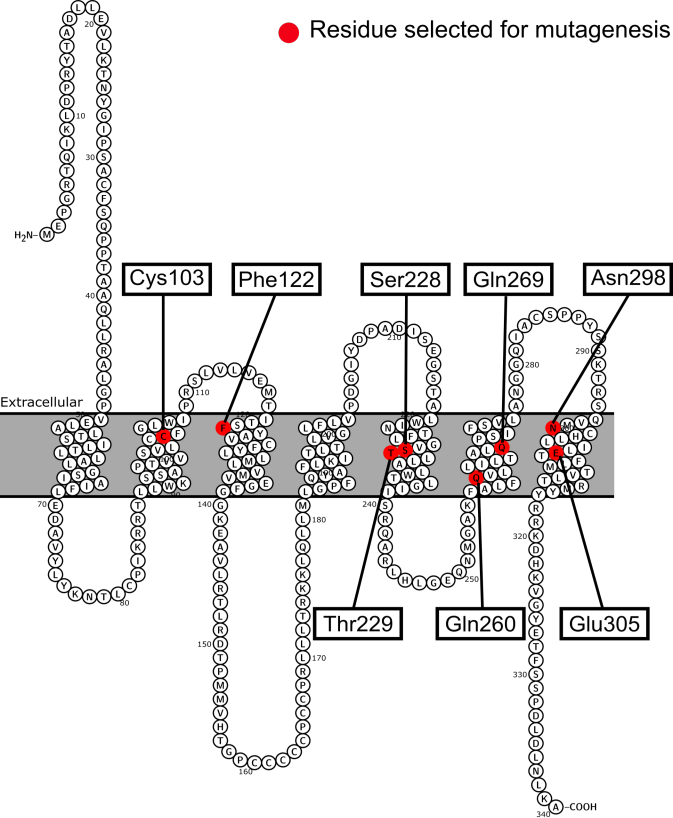


Figure 2

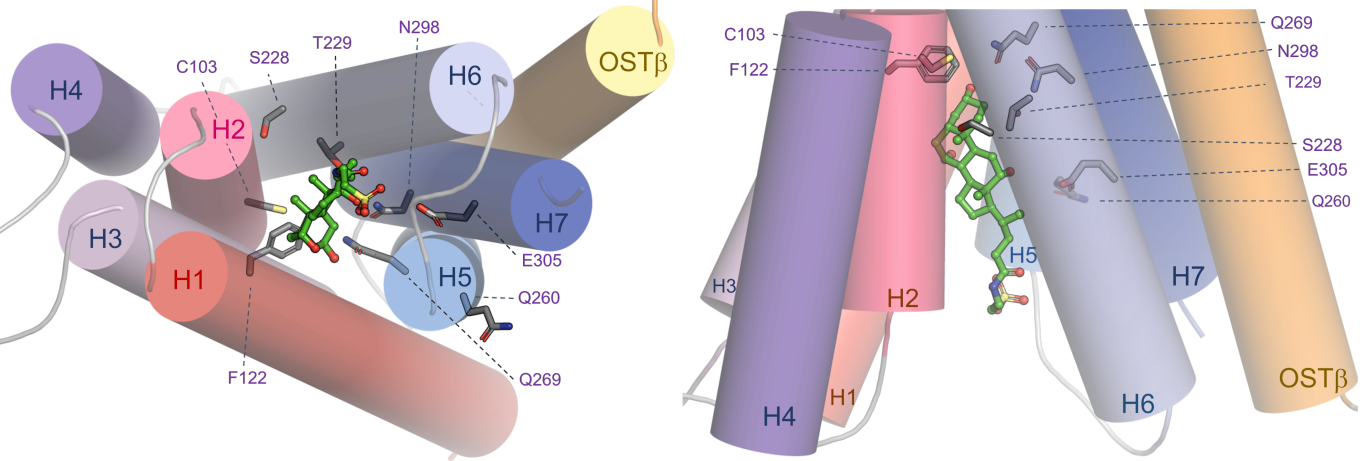
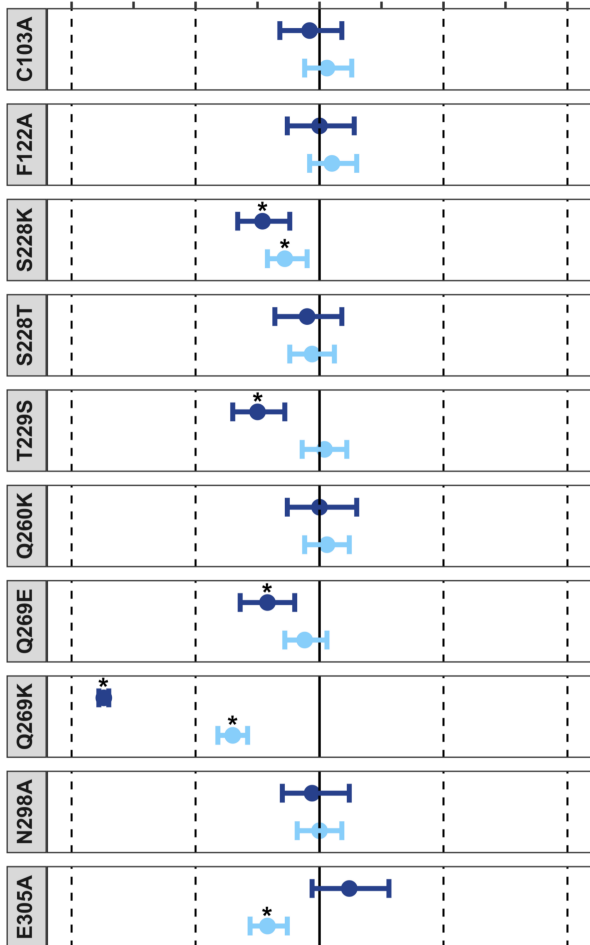


Figure 3

0.00 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00

OST α / β Mutant



Assay



[³H]-TCA 30-sec Uptake



[³H]-TCA % Efflux

Figure 4

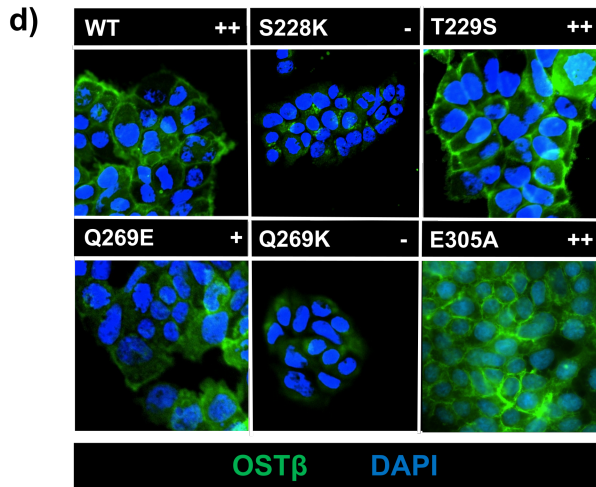
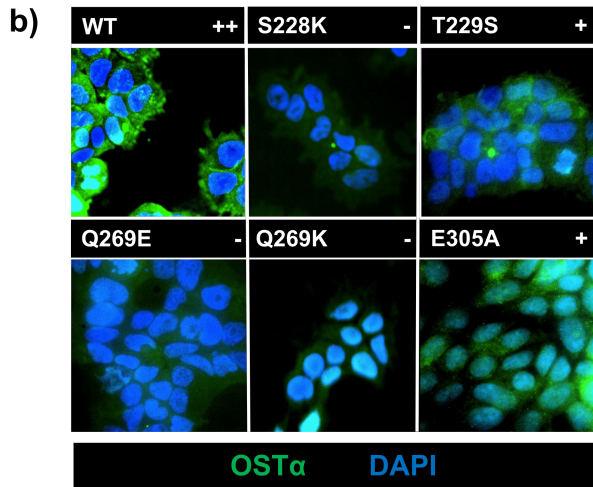
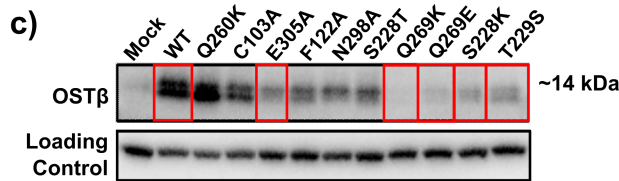
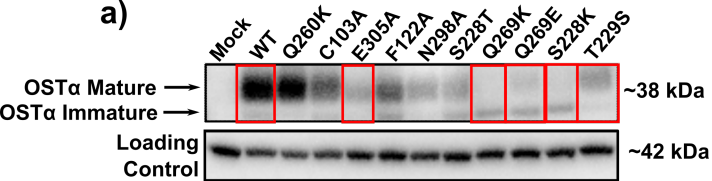


Figure 5

Supplemental Data

Supplemental Table 1. OST α forward primers used to generate OST α / β mutants.

<u>OSTα/β Mutant</u>	<u>Forward Primer</u>
C103A	5'- TGTGCTGTGCG CCTTTGGTCTCTGGATCCC -3'
F122A	5'-CATCACCTCG GCCTATGCCGTGTG -3'
S228K	5'-CCTTGGCGTGA AGACACTGCTGG -3'
S228T	5'-CCTTGGCGTGA CCACACTGCT -3'
T229S	5'-TGGCGTGT CCTCACTGCTGGC -3'
Q260K	5'-TGCTCTGTT CAAGGTTCTCCTC -3'
Q269E	5'-GACTGCCCT AGAGCCCTCCAT -3'
Q269K	5'-GACTGCCCT AAAGCCCTCCAT -3'
N298A	5'-TCAAGTGAT GGCCTGCCACCTCC -3'
E305A	5'- CCTC ACTGGCCACTTTTCTAATGAC -3'

Mutations in OST α DNA are shown in bold italics in respective forward primers. The online NEBaseChanger[®] tool (<https://nebasechanger.neb.com/>) was used to design primers. The 5' ends of the forward primers and the corresponding reverse unmutated primers (*not shown*) were annealed back-to-back to allow for optimal exponential amplification.

NEB, New England Biolabs

Supplemental Table 2. OST α / β wild-type and OST α / β mutant [3 H]-taurocholate (TCA) transport data.

OST α / β Cell Line	[3 H]-TCA 30-sec Uptake ^a (pmol/protein)	[3 H]-TCA Efflux ^b (%)	Residual Cellular [3 H]-TCA ^b (%)	OST α Membrane Localization	OST β Membrane Localization
WT1	54.1 (51.3, 57.0)	64.7 (59.1, 70.3)	25.7 (22.0, 29.4)	++	++
WT2	N/A	55.9 (51.0, 60.8)	23.0 (23.0, 35.0)	++	++
C103A ^{##}	51.8 (48.4, 55.1)	58.7 (52.6, 64.8)	27.7 (22.9, 32.4)	++	++
F122A ^{##}	53.9 (49.8, 58.0)	60.0 (54.3, 65.0)	27.9 (24.5, 31.4)	+	++
S228K [#]	41.3* (38.5, 44.2)	55.4* (50.2, 60.6)	40.3* (36.2, 44.4)	-	-
S228T [#]	51.3 (48.4, 54.1)	62.0 (59.3, 64.7)	28.9 (26.6, 31.2)	+	+
T229S [#]	40.7* (37.4, 44.0)	65.0 (61.0, 68.9)	27.9 (24.5, 31.4)	+	++
Q260K [#]	54.1 (51.8, 56.5)	65.5 (63.5, 67.4)	26.4 (25.4, 27.3)	+	++
Q269E [#]	42.4* (40.2, 44.5)	60.2 (54.3, 66.0)	28.1 (26.4, 29.9)	-	+
Q269K [#]	7.2* (5.7, 8.7)	40.1* (37.6, 42.7)	51.9* (46.2, 57.6)	-	-
N298A ^{##}	52.6 (49.3, 55.8)	56.2 (51.7, 60.7)	25.8 (24.1, 27.4)	++	++
E305A ^{##}	60.3* (58.0, 62.7)	44.3* (39.4, 49.3)	24.2 (22.3, 26.1)	+	++

Data depicted above as arithmetic mean (95% confidence interval).

Values were tested for statistical differences ($p < 0.05$) using analysis of variance (ANOVA) with Dunnett's multiple comparison test.

* $p < 0.05$. All p values were adjusted for multiple (10) comparisons.

[#]WT1 used as comparator (Group 1) in efflux studies.

^{##}WT2 used as comparator (Group 2) in efflux studies.

MOLPHARM 345

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Journal Title: *Molecular Pharmacology*

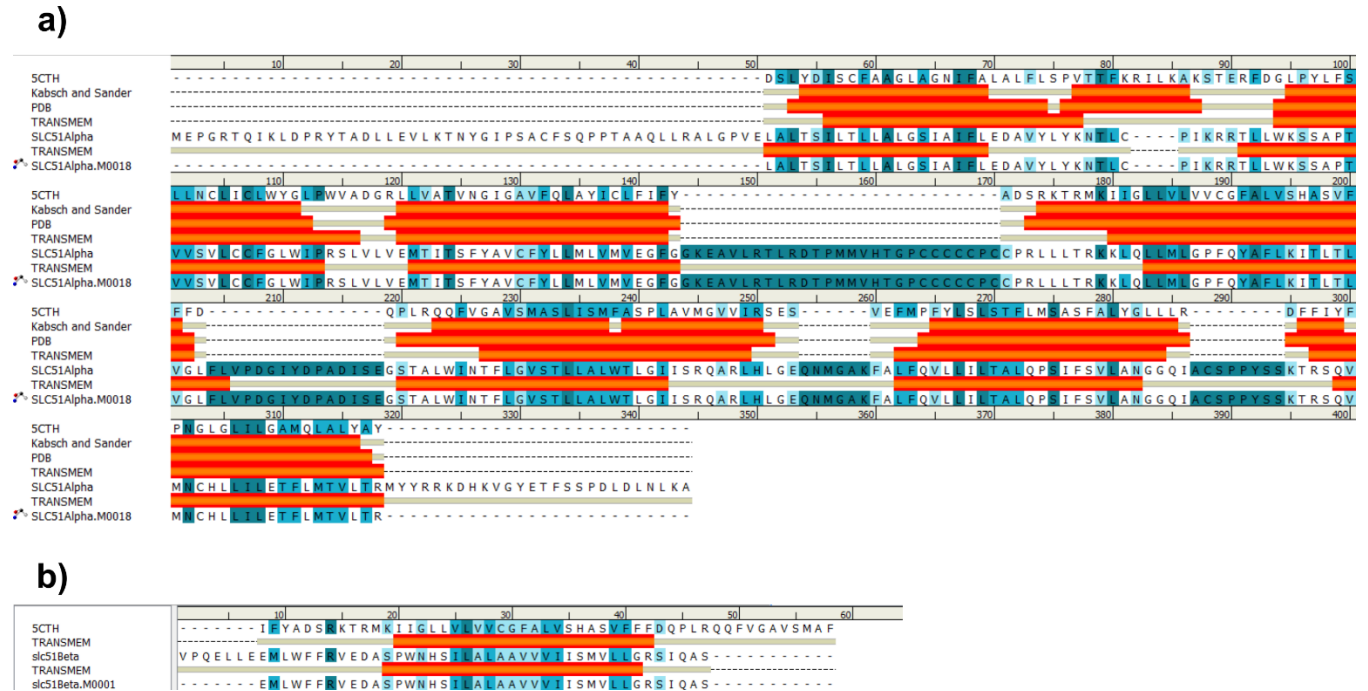
^aData obtained from [³H]-TCA uptake assay.

^bData obtained from [³H]-TCA efflux assay.

Residual Cellular [³H]-TCA (%) = [5-min efflux whole cell lysate (pmol/mg protein)/10-min preload whole cell lysate (pmol/mg protein)].

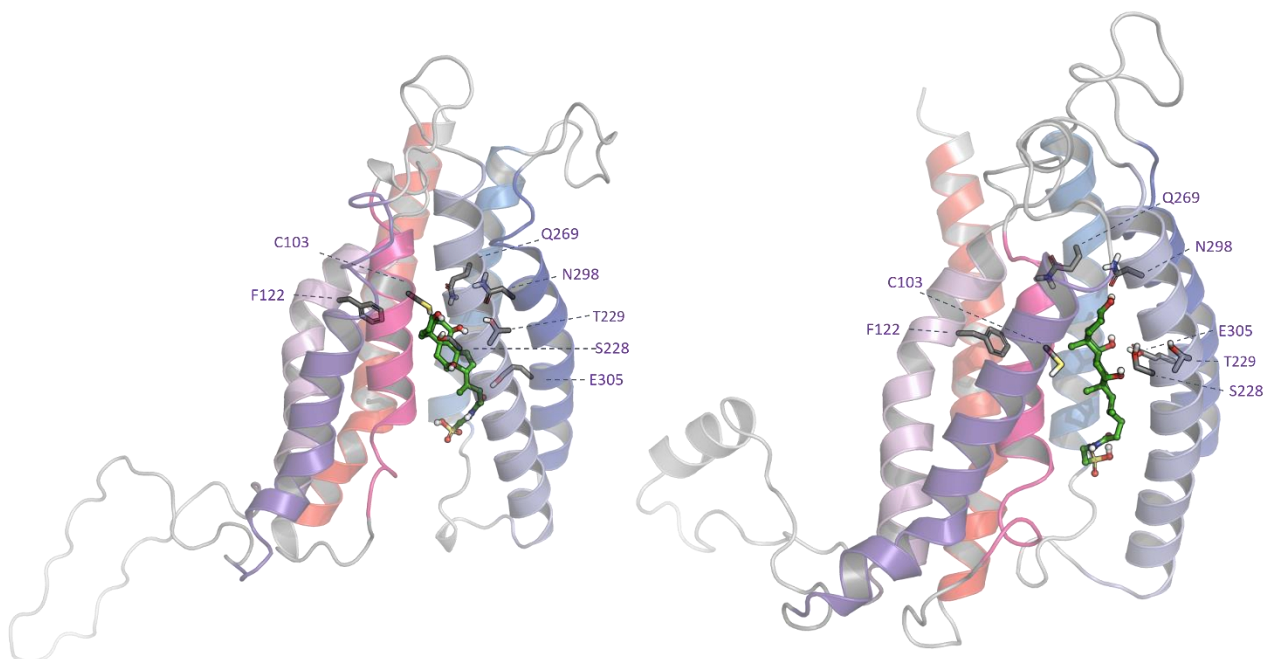
[³H]-TCA Efflux (%) = [5-min efflux ECF buffer (pmol/mg protein)/10-min preload whole cell lysate (pmol/mg protein)].

Membrane Localization = (++; strong), (+; modest), (-; negligible). All measures of localization relative to OST α / β WT (++ for both OST α and OST β).

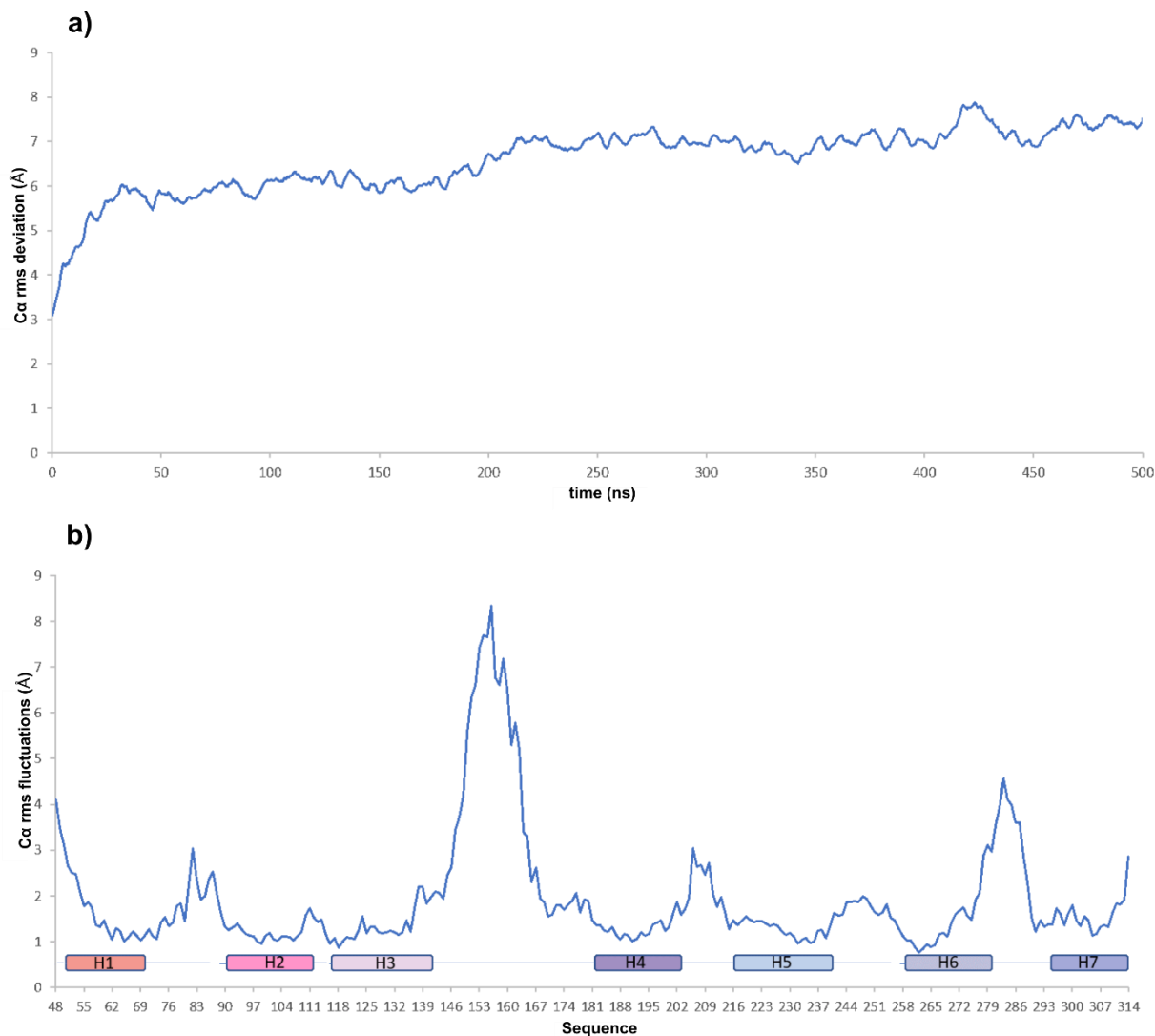


Supplemental Fig. 1. Sequence alignments of OST α and OST β . **a)** Pairwise sequence alignment of OST α (SLC51Alpha) and the A chain of the SWEET/SLC50A1 transporter (PDB ID: 5CTH) used as the template. This comparative modeling approach was based on alignment of transmembrane regions, which hold a sequence identity (*dark teal shading*) of 11.3% and a sequence similarity (*light teal shading*) of 33.3% for the aligned helices, respectively. The overall sequence identity and similarity between the two proteins were 7.6% and 22.5%, respectively. **b)** Pairwise sequence alignment of OST β (SLC51Beta) with helix 4 of chain C of the template (PDB ID: 5CTH).

The row titles indicate the following: **Kabsch and Sander**, secondary structure prediction tool in Discovery Studio based on the DSSP method (Kabsch and Sander, 1983). **PDB**, secondary structure retrieved from the original pdb file. **TRANSMEM**, TM prediction tool in Discovery Studio based on the hidden Markov model (Sonnhammer et al., 1998). SLC51Alpha and slc51Beta indicate the sequences of the targets, while SLC51Alpha.M0018 and slc51Beta.M0001 are the sequences of the top-ranked models based on the PDF Total Energy of the models, respectively.



Supplemental Fig. 2. Snapshot structures of OST α taken from a molecular dynamics (MD) trajectory at the beginning *i.e.*, after relaxation (*left panel*) and after 500 ns of MD simulation (*right panel*). Helices are indicated with a color gradient (H1 = red to H7 = blue), taurocholate substrate with a green ball-and-stick structure with the bile acid A-ring at the top and amino acids C103, F122, S228, T229, Q269, N298 and E305 are shown for orientation purposes. The side view of the protein is shown, with the extracellular side at the top and the intracellular side at the bottom. The lipid membrane, solvent, and ions are not shown for clarity. See Data Supplement 2 for pdb file of MD-trajectory after relaxation (*left panel*) and Data Supplement 3 for pdb file of MD simulation at 500 ns timepoint (*right panel*).

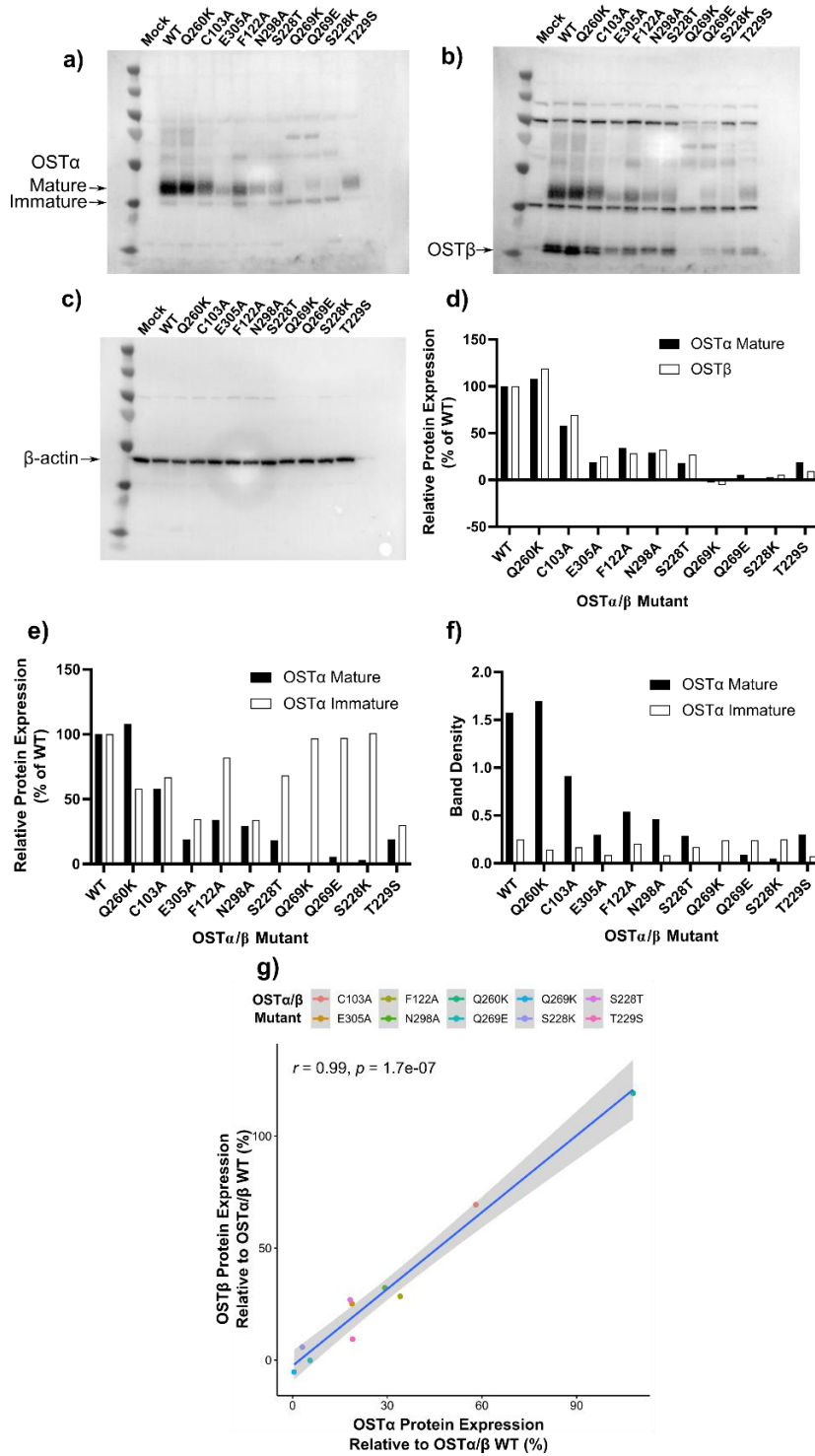


Supplemental Fig. 3. Simulations of the final homology model. **a)** Root-mean-square deviation (\AA) of the main chain $C\alpha$ atoms is shown as the moving average within the 500 nanosecond molecular dynamics (MD) simulation. According to the profile, the backbone of the model has converged after 50 ns and remains rather stable during the rest of the simulation (50-500ns). See Data Supplement 4 for the pdb file of the homology model. **b)** Average fluctuation (\AA) of the main chain $C\alpha$ atoms along the OST α sequence. The fluctuation pattern suggests that helical parts of the model are stable throughout the simulation time. The largest movement was located in the unstructured loop region (residues 140-170), which was quickly rearranged in the beginning of the simulation (panel a, < 50 ns), observed during visualization of the simulation trajectory and shown in Supplemental Fig. 2 (before and after).

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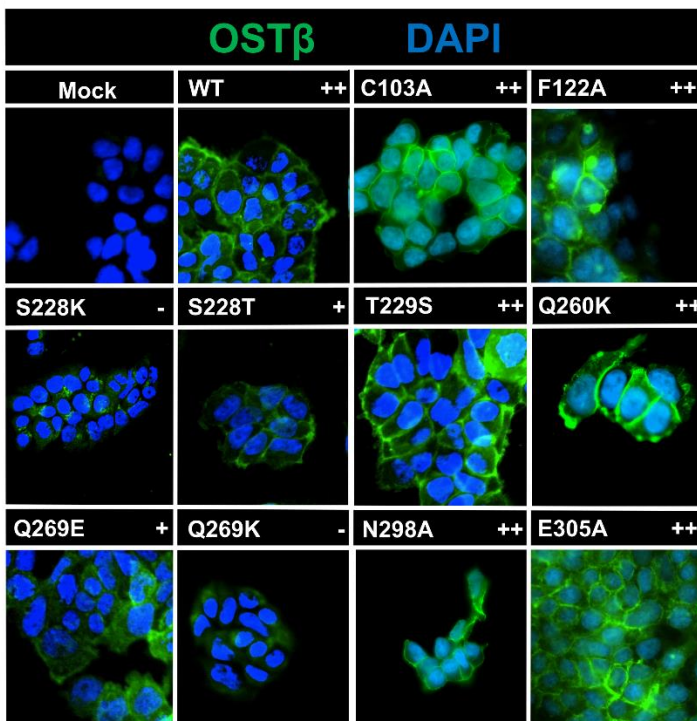
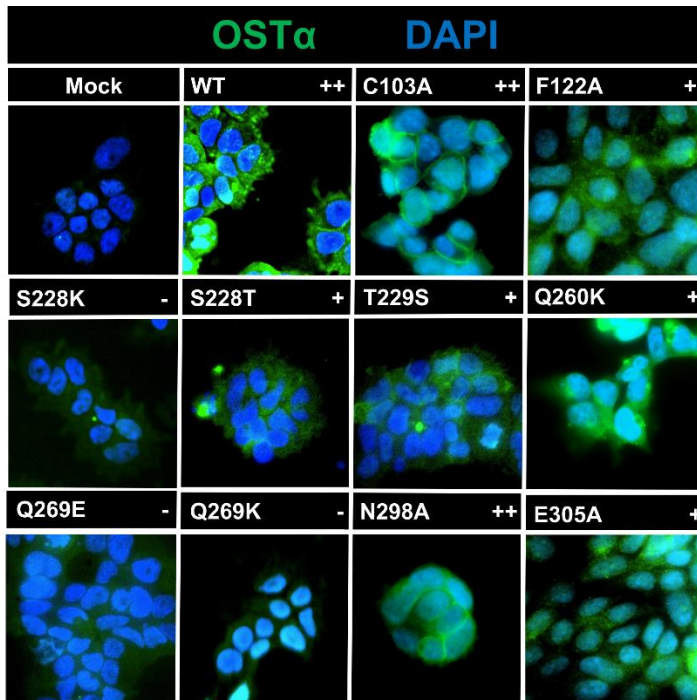
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Supplemental Fig. 4. OST α and OST β Protein Levels. **a)** Full, non-cropped western blot membranes of OST α mature and immature protein (~38 kDa). **b)** Western blot of OST β (~14 kDa) on same polyvinylidene difluoride membrane following OST α primary antibody incubation and visualization. **c)** Western blot of β -actin (loading control; ~42 kDa) after stripping membrane of OST α and OST β protein bands. Page Ruler Plus Pre-Stained ladder (Thermo Fisher Scientific) was used as reference (far left of each blot) to determine approximate protein size in kDa. **d)** Western blot densitometry analysis of OST α and OST β for each OST α / β mutant relative to OST α / β WT (%). **e)** Densitometry analysis of OST α mature and OST α immature protein for each OST α / β mutant relative to OST α / β WT (%). **f)** Absolute band density, determined by densitometry analysis, of OST α mature and OST α immature protein for OST α / β WT and each OST α / β mutant. **g)** Pearson correlation analysis of OST α mature and OST β protein expression relative to OST α / β WT for each OST α / β mutant (gray shading represents 95% confidence interval). Densitometry analysis was performed on the western blot membrane using ImageJ software (Schneider et al., 2012). Band intensity was normalized using the β -actin loading control for each OST α / β mutant and OST α / β WT. OST α mature, OST α immature, and OST β band intensity for Mock cells was subtracted from normalized OST α / β WT and OST α / β mutant band intensity. OST α mature, OST α immature, and OST β bands for OST α / β WT were used as the comparator/reference group for each OST α / β mutant. Pearson correlation analysis was performed using GraphPad software.

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MOLPHARM 345

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Supplemental Fig. 5. Immunocytochemistry of Mock, OST α / β wild-type, and OST α / β mutant cells. Immunocytochemistry staining of DAPI (4',6-diamidino-2-phenylindole; blue) and OST α or OST β (green) for Mock, OST α / β WT and OST α / β mutant cell lines. Qualitative assessment of plasma membrane localization (++; strong), (+; modest), (-; negligible) is provided next to the label for each OST α / β mutant. All qualitative measures of localization are relative to OST α / β WT. Images were taken using a Nikon ECLIPSE Ti2 microscope with a 60X oil immersion objective (field of view \approx 40 μ m wide).

MOLPHARM 345

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Data Supplement File Legends

Data Supplement 1. R script for Bayesian analysis (i.e., generalized linear mixed model with group-specific terms) of OST α / β uptake and efflux data.

Data Supplement 2. Represents the first frame taken from the molecular dynamics (MD)-trajectory presenting structure at the time point zero, after standard relaxation protocol of the Desmond Molecular Dynamics System. Solvent, ions, and plasma membrane lipid bilayer are excluded for clarity.

Data Supplement 3. Represents the OST α structure at the end of the 500 ns molecular dynamics (MD) simulation. Solvent, ions, and plasma membrane lipid bilayer are excluded from the unconstrained periodic simulation for clarity.

Data Supplement 4. The final homology model of OST α , including OST β . The putative interaction with OST β is generated based on interactions detected in the OsSweet template structure, between chains A (OST α) and B (OST β). The OST β structural component is used for graphical illustrations only and should be taken with caution.

MOLPHARM 345

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Schneider CA, Rasband WS and Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**: 671-675.

Sonnhammer EL, von Heijne G and Krogh A (1998) A hidden Markov model for predicting transmembrane helices in protein sequences. *Proc Int Conf Intell Syst Mol Biol* **6**: 175-182.