Supporting Information

Title: Functional transformations of bile acid transporters induced by high-affinity macromolecules

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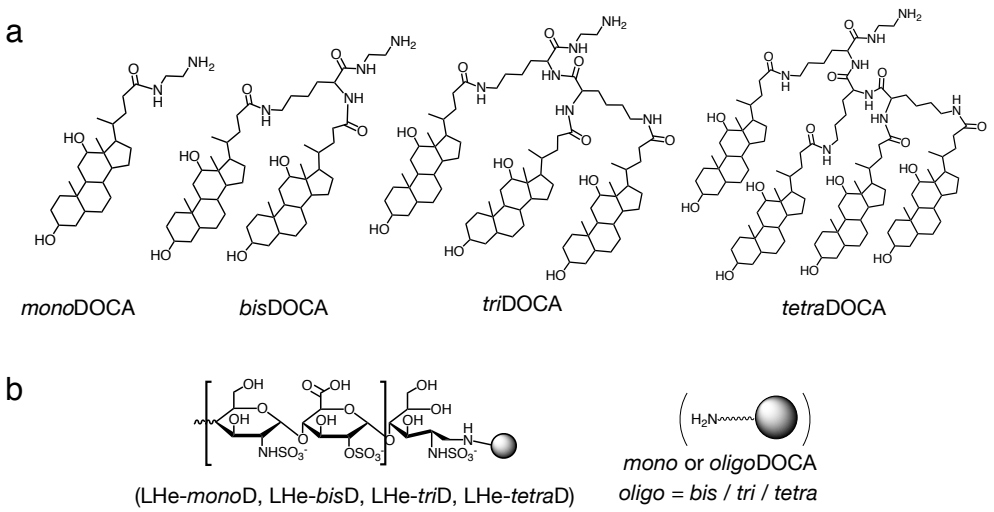
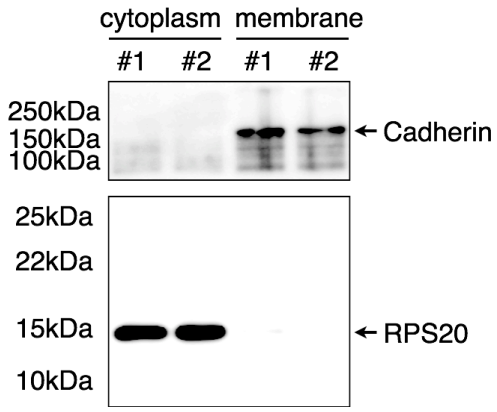
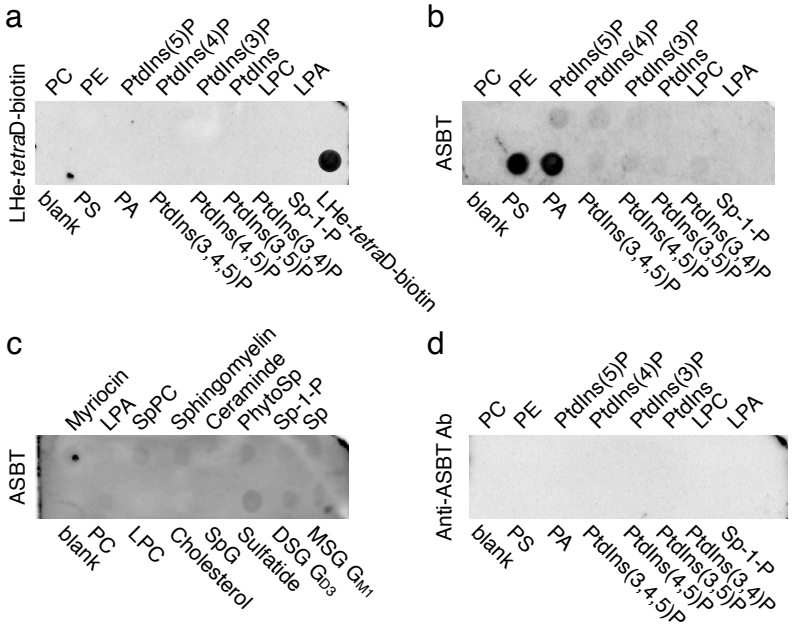


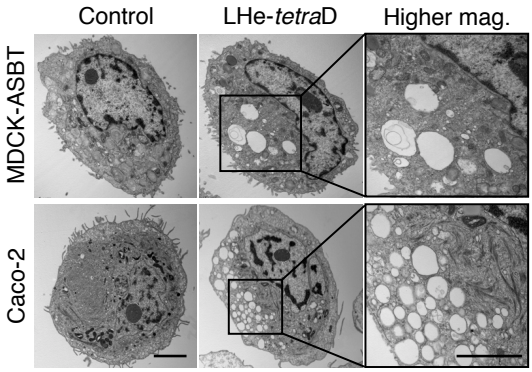
Supplementary Figure S1. (A) Structures of deoxycholic acid (DOCA) derivatives, named as *mono*DOCA, *bis*DOCA, *tri*DOCA, and *tetra*DOCA. (B) The general structure of LMWH-*oligo*DOCA conjugates.



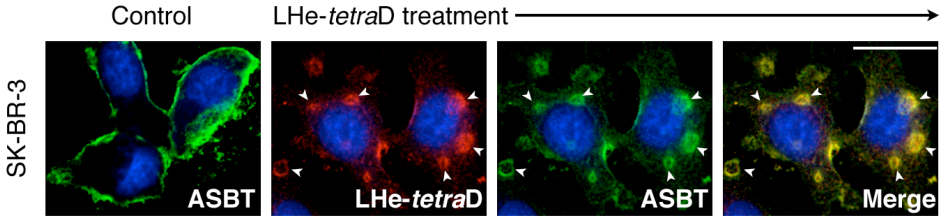
Supplementary Figure S2. Western blot data showing the purity of cytoplasmic and membrane fractions of MDCK-ASBT cells. Antibodies against Cadherin and RPS20 were used as markers for the membrane and cytoplasmic fractions of the cell, respectively. The negative staining of Cadherin in the cytoplasmic fraction and the negative staining of RPS20 in the membrane fraction confirmed the purity of each fraction.



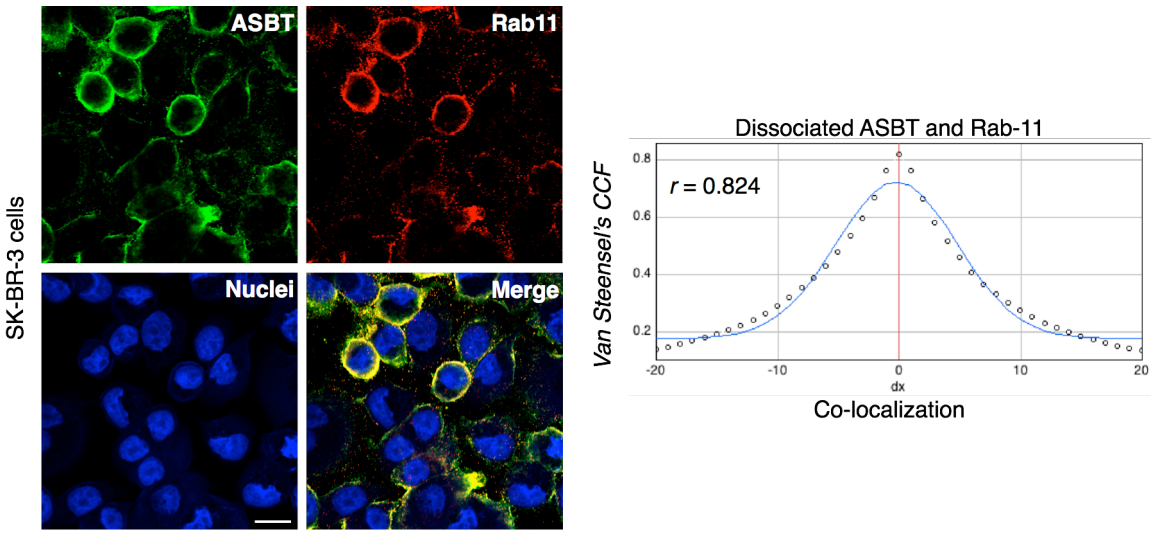
Supplementary Figure S3. The ASBT protein specifically binds to the membrane lipids. (a) LHe-*tetra*D-biotin was first spotted on the nitrocellulose membrane by dot blot method and further incubated to observe the non-specific binding of LHe-*tetra*D-biotin to lipids. Lipid bound or spotted LHe-*tetra*D-biotin was visualized by streptavidin-HRP. LHe-*tetra*D-biotin exhibited no interactions with spotted lipids. Recombinant human ASBT protein was incubated with nitrocellulose strips spotted with various phospholipids (b) and sphingolipids (c). Note that binding of wild-type protein was mostly observed with phosphatidic acid (PA) and phosphatidylserine (PS) lipids. (d) ASBT antibody alone exhibited no interactions. PC; phosphatidylcholine, PE; phosphatidylethanolamine, LPC; lysophosphatidylcholine, LPA; lysophosphatidic acid, PtdIns; phosphatidylinositol, Sp; sphingosine, Sp-1-P; sphingosine 1-phosphate, phytoSp; Phytosphingosine, SpPC; sphingosylphosphocholine, SpG; sphingosylgalactoside, MSG; monosialoganglioside, DSG; disialoganglioside.



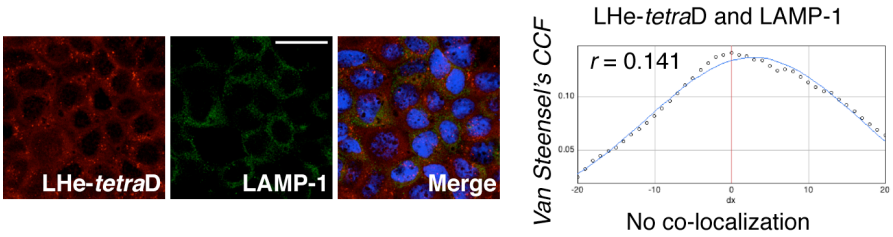
Supplementary Figure S4. LHe-*tetra*D mediated vesicular transport of ASBT. Transmission electron microscope revealed the extent of vesicle formation in the MDCK-ASBT and Caco-2 cells due to LHe-*tetra*D treatment in comparison to non-treated control cells. Scale bars indicate 2 μm; high magnification, 200 nm.



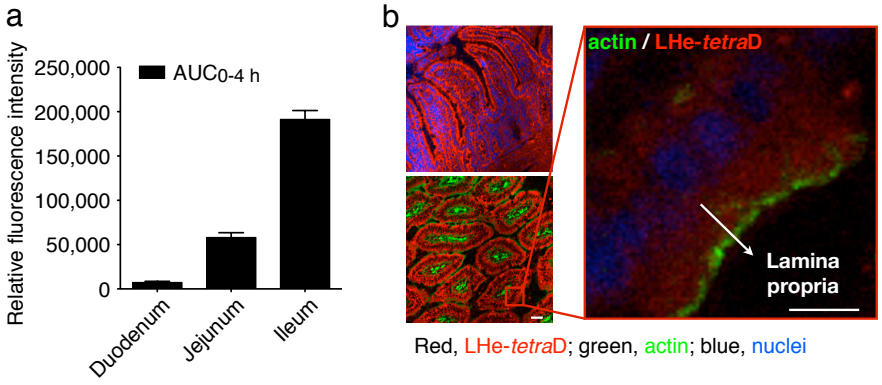
Supplementary Figure S5. Confocal images showing the co-localization of ASBT (green) and LHe-*tetra*D (red) in vesicle-like structures (arrowheads) in the cytoplasm of LHe-*tetra*D treated SK-BR-3 cells, whereas ASBT staining was highly observed in the membrane of control cells. Cells were treated with LHe-*tetra*D-biotin at a concentration of 0.05 mg/mL for 5 min. ASBT and LHe-*tetra*D-biotin was labeled with ASBT (C-terminal) and biotin antibodies, respectively, that were counterstained with Alexa®488 (green) and Alexa®555 (red) labeled secondary antibodies. Nucleus was stained in blue. Scale bar indicates 5 μm.



Supplementary Figure S6. The co-localization of ASBT (green) with recycling endosome marker Rab11 (red) in SK-BR-3 cells. Scale bar indicates 20 μm. Co-localization analysis performed on the original images using Van Steensel's cross-correlation coefficient (CCF) between ASBT and Rab11 using the JACop software. Perfect bell-shaped curves and Pearson coefficient (*r*) ranging from 0.8 to 1 was observed for ASBT with Rab11 (right panel).



Supplementary Figure S7. No co-localization of RITC labeled LHe-*tetra*D with lysosomal marker LAMP-1 (green) in Caco-2 cells suggested the escape of macromolecule from the degradative lysosmal compartment. Nuclei were stained in blue. Scale bar indicates 10 µm. Perfect bell-shaped curve was not observed with LHe-*tetra*D and LMAP-1 (B, right panel).



Supplementary Figure S8. Fluorescent signal in intestinal tissue extracts. (a) Most of the coumarin labeled LHe-*tetra*D was extracted from the ileum of small intestine. (b) The intestinal distribution of LHe-*tetra*D (red) was observed in the epithelium lining and lamina propria of ileum, but not in the tight junctions (green). Nuclei are shown stained in blue. Scale bar indicates 50 μm. (Inset) LHe-*tetra*D (red) was observed to be located in the lamina propria of small intestine. Scale bar indicates 5 μm.

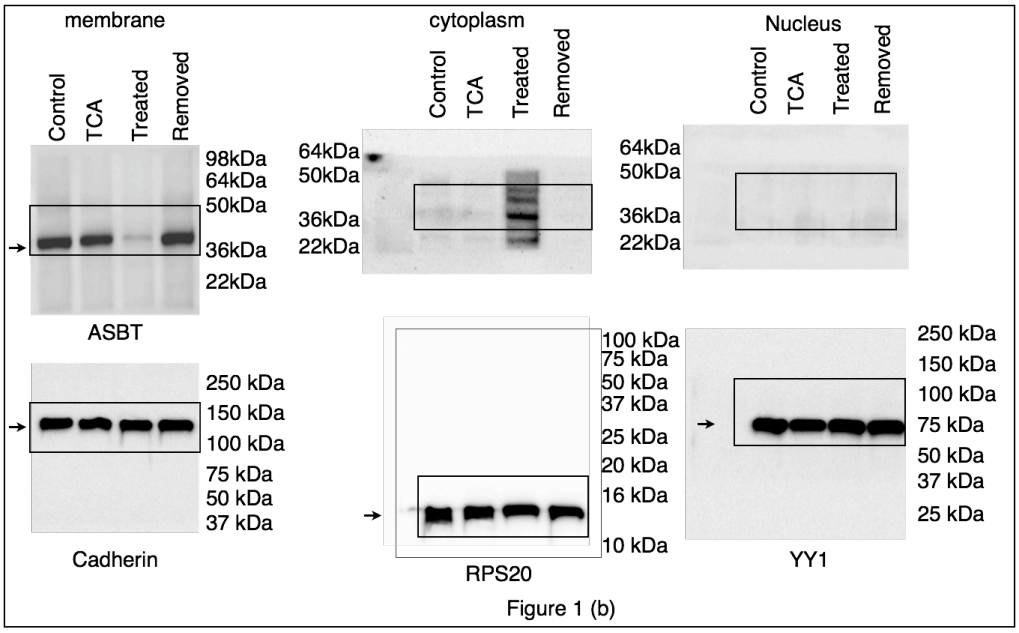


Figure 1 (b)

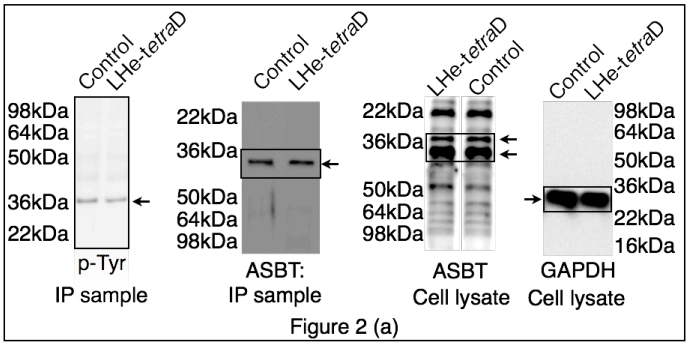


Figure 2 (a)

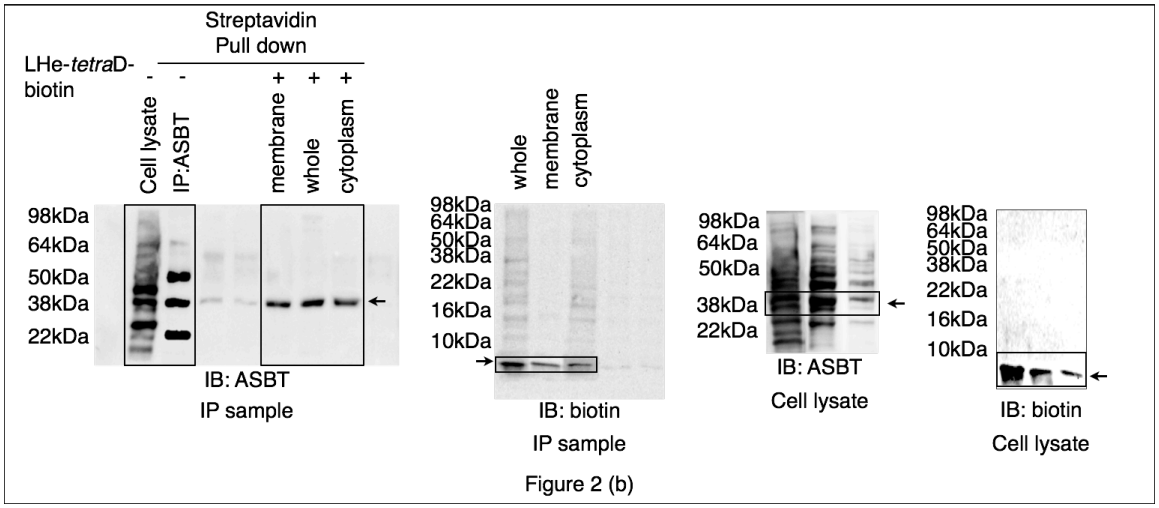
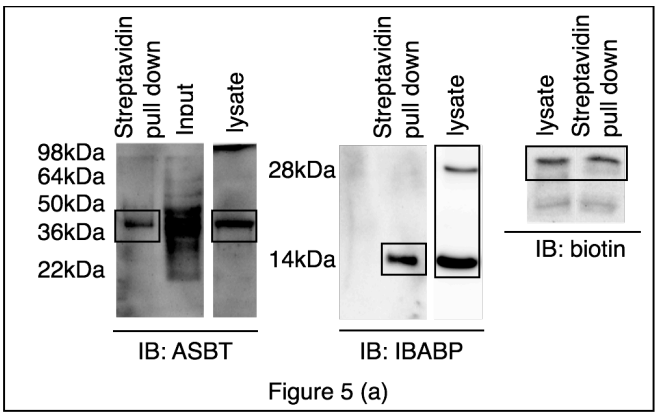
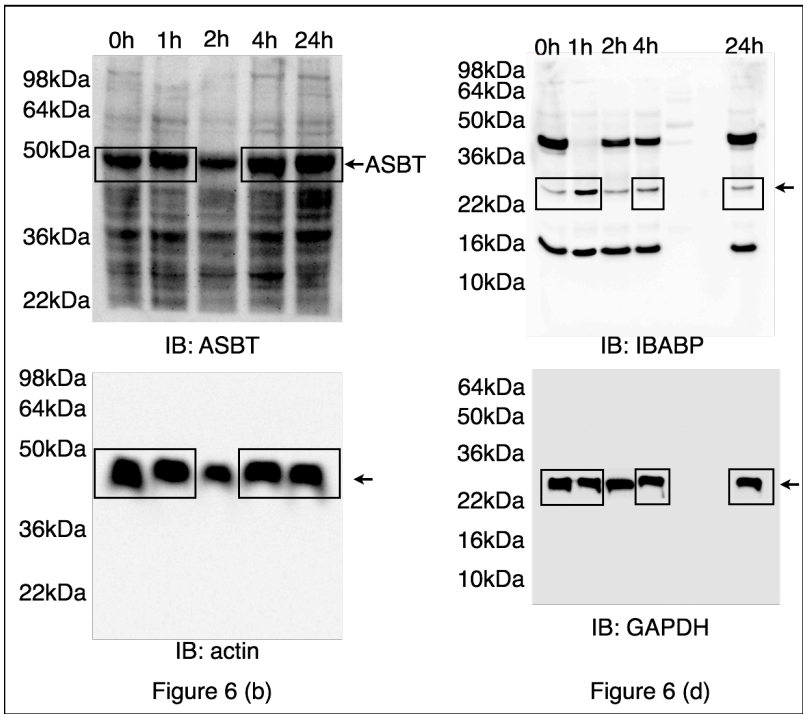


Figure 2 (b)

Supplementary Figure S9. Cropped western blot data.





Supplementary Figure S9 continued. Cropped western blot data.

Supplementary Methods

Materials

ASBT-C-terminal specific antibody (SAB2500947, 1:4000, Sigma, St. Louis, MO), ASBT-N-terminal specific antibody (ARP43749_P050l, 1:4000, Aviva Systems Biology, San Diego, CA), IBABP antibody (HPA012601, 1:2000, Sigma, St. Louis, MO), biotin antibody (ab3780, 1:4000, Abcam, Cambridge, MA), pan cadherin antibody (ab22744, 1:2000, Abcam, Cambridge, MA), RPS20 antibody (HPA003570, 1:1000, Sigma, St. Louis, MO), YY1 antibody (H-10, sc-7341, 1:1000, Santa Cruz, Dallas, Tx), phosphotyrosine antibody, clone 4G10 (05-321X, 1:4000, Millipore, Billerica, MA), EEA1 antibody (2411, 1:100, Cell Signaling Technology, Danvers, MA), Rab11 antibody (5589, 1:100, Cell Signaling Technology, Danvers, MA), LAMP1 antibody (9091, 1:100, Cell Signaling Technology, Danvers, MA), GAPDH antibody (MAB5718, 1:4000, R&D Systems, Minneapolis, MN), actin antibody (CC10028, 1:4000, Cell Applications, San Diego, CA) and horseradish peroxidase (HRP)-conjugated secondary antibody to goat (HAF109, 1:2000, R&D Systems, Minneapolis, MN), HRP-conjugated secondary antibody to mouse (HAF007, 1:2000, R&D Systems, Minneapolis, MN), streptavidin-HRP (3999, 1:1000, Cell Signaling Technology, Danvers, MA), goat TrueBlot® (18-8814, 1:2000, eBioscience, San Diego, CA), and mouse TrueBlot® ULTRA (18-8817, 1:2000, eBioscience, San Diego, CA) were purchased and stored following the manufacturer's guidelines. Recombinant human SLC10A2 protein (H00006555-G01) was purchased from Abnova Corporations (Taipei, Taiwan). Recombinant protein G agarose (15920-010), Dynabeads® streptavidin trial kit (658.01D), Dynabeads® MyOne™ streptavidin C1 (650.01), goat IgG (A10537), Alexa Flour®488-conjugated anti-goat antibody (A11055, 1:200), Alexa Flour®555-conjugated anti-mouse antibody (A21427, 1:200), Alexa Flour®488-conjugated anti-mouse antibody (A11001, 1:200), Cy3-

conjugated anti-rabbit antibody (A10520, 1:200), Alexa Flour®488-conjugated anti-rabbit antibody (A11008, 1:200), and PIP Strips™ (P23751) were purchased from Invitrogen. Protease and phosphatase inhibitor cocktail tablets (S8820) and solutions (P5726) were purchased from Sigma-Aldrich (St. Louis, MO). Rhodamine B isothiocyanate (RITC, 283924), fluorescein isothiocyanate (F7250), 7-(diethylamino)coumarin-3-carbohydrazide (36798), hoechst 33258 (861405), and biotin hydrazide (B7639) was purchased from Sigma-Aldrich (St. Louis, MO). LMWH with an average molecular weight of 4500 Da was obtained from the Nanjing King-Friend Biochemical Pharmaceutical Company Ltd. (Nanjing, China). Other chemicals and solvents were purchased from Sigma unless noted otherwise and were used without further purification.

Nitrocellulose phospholipid binding assays. We used PIP- and Sphingo- strips, which are nitrocellulose membranes spotted with 100 pmol of different lipids per spot. PIP- and Sphingo-strips were blocked for 1 h with 3% fatty acid-free bovine serum albumin in Tris buffer saline (Gentic, Abelbio, Korea) supplemented with 0.1% tween 20 (TBST). Human ASBT protein at a concentration of 0.5 μg/mL was incubated for 2 h at 4 ºC with the nitrocellulose membrane in TBST. After repeated washing, lipid-bound ASBT was detected using anti-ASBT (C-terminal), followed by incubation with HRP-coupled secondary antibody. Membranes were developed using an enhanced chemiluminescence ECL kit following the manufacturer's instructions (Santa Cruz, Dallas, Tx). To check non-specific bindings, ASBT antibody only was incubated with another PIP-Strip and was detected by following the procedure mentioned earlier. Using another PIP-Strip, LHe-*tetra*D (LHe-*tetra*D-biotin; 0.1 μM) labeled with biotin was spotted on the nitrocellulose membrane at sites where lipids were not spotted. The whole membrane was

further incubated with 0.05 mg/mL of LHe-*tetra*D-biotin to allow the interaction with lipids. Lipids bound to or spotted with LHe-*tetra*D-biotin were detected by incubation with streptavidin-HRP.

Immunoprecipitation (IP) and biotin pull-down assays. Whole lysates were collected using IP buffer [20 mM Tris-HCl (pH 7.4), 15% glycerol, 1% triton X-100, 8 mM $MgSO_4$, 150 mM NaCl, 1 mM EDTA, supplemented with β-glycerophosphate, NaF, protease inhibitor, and phosphatase inhibitor] from the treated and non-treated MDCK-ASBT cells. To pull-down ASBT protein, ASBT antibody (2.5 μg) was added to the pre-cleared lysates (total 1 mg protein), which were then incubated overnight at 4ºC with gentle rocking. The final volume was adjusted to 850 μL by adding IP buffer. In another tube, the goat IgG was added as a control. Next, the mixture was poured onto recombinant protein G agarose beads and incubated with gentle rocking for a further 2.5 h at 4 ºC. The beads were centrifuged, washed with IP buffer at least three times, boiled in SDS-sample buffer, separated on a 12% gel, and analyzed by immunoblotting. The membranes were blotted with anti-ASBT (C-terminal) and anti-phosphotyrosine to investigate ASBT phosphorylation, followed by incubation with goat TrueBlot® and mouse TrueBlot® secondary antibodies, respectively.

To check the interactions, cells were treated with biotin-labeled LHe-*tetra*D and the whole lysates or fractions were collected following the procedures explained earlier. At first, the biotin pull-down assay was optimized using the streptavidin trail kit. Among four different Dynabeads® applied here, maximum biotin was pulled down by using MyOne™ streaptavidin C1 beads from the lysate of LHe-*tetra*D-biotin-treated cells.

Next, streptavidin C1 beads were added to the whole-cell lysates (total 2 mg protein) or to the membrane (total 2 mg protein) and cytoplasmic (total 1.2 mg protein) fractions and incubated overnight at 4 ºC with gentle rocking. Streptavidin beads were washed with IP buffer at least three times, heated in deionized water at 70 ºC for 3 min, and subjected to SDS-PAGE analysis. The membranes were separately blotted with ASBT (C-terminal) antibodies against 12% gel and biotin antibodies against 15% gel.

Proximity ligation assay (PLA). For PLA, cells were treated as indicated, immediately fixed in 4% PFA for 20 min, and thereafter subjected to *in situ* PLA using a Duolink Detection kit (Olink Bioscience, Uppsala, Sweden) following the manufacturer's instructions. Briefly, slides were blocked, incubated with anti-ASBT (N-terminal) and anti-biotin, and thereafter incubated with PLA probes, which are secondary antibodies (anti-rabbit and anti-mouse, respectively) conjugated to unique oligonucleotides. Ligation of the oligonucleotides was followed by an amplification step. The products were detected by using a complementary fluorescently labeled probe. Slides were mounted using mounting media and evaluated using the confocal microscope. An ASBT antibody immunogenic to N-terminal region of ASBT was chosen, considering the fact that PLA only allowed the detection of two interacting molecules when it remained in close proximity of less than 40 nm. The use of antibodies that are immunogenic to N-terminal rather than C-terminal region of ASBT minimized the effects associated with the distance between LHe-*tetra*D and antibody binding site in this case.

Interaction with IBABP. The interaction of LHe-*tera*D and IBABP was exclusively studied using the PLA method as described above. Briefly, SK-BR-3 (American type Culture Collection) cells that naturally express ASBT and IBABP were treated with LHe-*tetra*D-biotin for 30 min. Following PLA, ASBT was also stained by the IF method as established above. IBABP antibody was used at a dilution of 1:30 in the diluted blocking buffer overnight. The primary antibodies were stained with Cy3 (red)-labeled secondary antibodies. Nucleus was counterstained by Hoechst 33258 dye. Fluorescence was then observed under a confocal microscope.