Supplementary Materials and methods

RNA isolation and real-time PCR

Cells for expression analysis were lysed and total RNA was prepared from ca. 3 x 10^6 cells using the Qiagen RNeasy Mini kit with QIAShredder and on-column DNaseI treatment, according to the manufacturer's instructions. RNA was reverse-transcribed using M-MLV reverse transcriptase, RNase H^- (Promega) and random hexamer primer (Promega). Gene expression was quantitated in triplicates by real-time PCR using SYBR Green PCR master mix (Roche) and a Roche LightCycler 480 sequence detector. cDNA levels during the linear phase of amplification were normalized against hypoxanthine guanine phosphoribosyl transferase (HPRT) controls and relative quantification was made using the LightCycler 480 Software 1.5.0.

Primer:

huCXCL1, F: GCCCAAACCGAAGTCATAGC, R: CTGGTCAGTTGGATTTGTCACTG; huCXCL2, F: GCCCAAACCGAAGTCATAGC, R: GGTCAGTTGGATTTGCCATTT; huCXCL3, F: GCCCAAACCGAAGTCATAGC, R: TGCAGGAAGTGTCAATGATACG;

huIL-8, F: GGAGAAGTTTTTGAAGAGGGCTGA, R: TGCTTGAAGTTTCACTGGCATCTT; huCCL20, F: GCGAATCAGAAGCAGCAAGC, R: GCCGTGTGAAGCCCACAATA;

huA20, F: AGAAGAGCAACTGAGATCGAGC, R: CTGGTTGGGATGCTGACAC; huPLA2G4C, F: ATGGAGAAGGGCTGTTGCTA, R: CTCACCGCCTTCATCTTCTG; huTNFRSF9, F: TTCAAACAACCATTTATGAGACCAG, R: CCATTTCACAGTTCACATCCTC;

huMAP2K5, F: CATTGTTGATGAGGATTCGC, R: ATTGAACTGCACGATGAACG; mUnc5CL, F: TCTGAGGGTTGGGAGAATGTG, R: GGTTAATGCCGAGCACTCTTC.

Proteinase K protection assays

HEK293T cells stably expressing Unc5CL or control cells were permeabilized for 10' at 4°C with digitonin buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose) containing 0.2 μg/ml digitonin (Sigma, added directly before use). Samples were divided into 3 and left either untreated or were treated with 40 μg/ml PK with or without 1 mM PMSF for 10' at 4°C. Digestion was stopped by addition of 1 mM PMSF. Lysates were centrifuged at 13.000 rpm for 10' at 4°C in a microcentrifuge to obtain a pellet and supernatant fraction and analyzed by western blot using the indicated antibodies.

Confocal microscopy and immunohistochemistry

CaCo-2 cells stably expressing C-terminally FLAG-tagged Unc5CL or Unc5CLΔTM were cultured in Lab-Tek 8-well glass chamber slides (Nunc) until 3 days of post-confluence. Cells were washed with PBS and fixed by incubation with 3.7% formaldehyde in PBS at RT. Cells were washed three times with PBS and permeabilized and blocked by incubation for 30' with blocking solution (10% FCS, 0.3% saponin in PBS). Primary mouse anti-FLAG M2 antibodies (Sigma, 1:500 in blocking solution) were incubated for 1 h at RT. Cells were washed three times with blocking solution and incubated with the secondary antibodies (anti-mouse-Alexa488 H+L, 1:800 in blocking solution, Invitrogen) for 1 h at RT. Cells were washed three times with blocking solution, once with PBS and stained with Hoechst stain in PBS for 10' at RT. Cells were washed twice with PBS and mounted using FluorSafe medium (Calbiochem). Slides were examined using a Zeiss LSM 510 Meta inverted confocal microscope.

For immunohistochemistry, uterine horns were prepared from euthanized female C57BL/6 mice (6-10 weeks of age), flushed with cold PBS, embedded in Tissue-Tek optimum cutting temperature compound (Sakura) and then frozen in an ethanol dry ice bath. Cryostat sections (8 μm in thickness) on Superfrost/Plus glass slides (Fisher Scientific) were air-dried overnight, then fixed for 10 min in ice-cold acetone and rehydrated for 30' in PBS. Slides were incubated for 30' at RT in blocking reagent (0.1% BSA, 1% normal mouse serum, 1% normal donkey serum in PBS), then washed three times with PBS. Primary antibodies were added (rabbit anti-Unc5CL 1:25-1:50 or anti-FLAG 1:500 in 0.1% BSA, 1% normal mouse serum in PBS) and incubated for 1 h at RT. Slides were washed three times with PBS and incubated with the secondary antibodies (anti-rabbit-Alexa488 or anti-Alexa594, Invitrogen, 1:800 in 0.1% BSA, 1% normal mouse serum in PBS) for 45' at RT. Slides were washed three times with PBS, stained with DAPI and mounted on slides using DABCO reagent. Slides were examined using a Zeiss LSM 510 Meta inverted confocal microscope.

For control stainings, mock- or FLAG-tagged Unc5CL-transduced Caco-2 cells were cultured in Lab-Tek 8-well glass chamber slides (Nunc) until 3 days of post-confluence. Cells were washed with PBS and staining was carried out as described above from the acetone fixation step onwards.

Preparation of brush border membrane vesicles (BBMV)

For preparation of BBMVs an adapted protocol from Kessler et al. was used. The murine small intestine was slit open lengthwise and rinsed with ice-cold PBS to remove residual feces. The mucosa was scraped into PBS using a glass slide and centrifuged at 400g for 5' at 4°C. The pellet was resuspended in 15 ml homogenization buffer (50 mM mannitol, 2mM Tris, pH 7.4) containing protease inhibitors (Complete, Roche) and homogenized using a polytron. The homogenate was centrifuged at 3.000g for 15' at 4°C, the pellet was discarded

and the SN was re-centrifuged at 27.000g for 30’ at 4°C. The pellet containing cell membranes was resuspended in 15 ml homogenization buffer containing 10 mM $CaCl_2$ and incubated at 4°C for 20’. The 3.000g and 27.000g centrifugation steps were repeated; the final pellet contained the BBMVs. Selected fractions were analyzed by western blot.