Supplementary Figure S1. The mouse *Ctrb1* gene (from genomic sequence NC_000074.6). Exons are highlighted in yellow, the translation start and stop codons are in green. Exon 4, which is deleted in the *Ctrb1-del* mouse strain, is shown in magenta. Splice sites are underlined and italicized. The polyadenylation signal in the 3' untranslated region is underlined and emboldened. The position of the genotyping primers in exon 2 and intron 4 is marked in bold underlined type.

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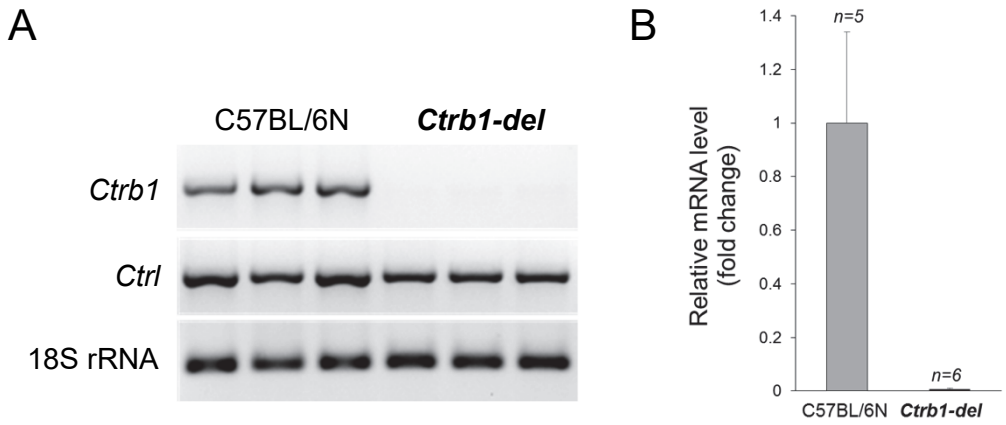
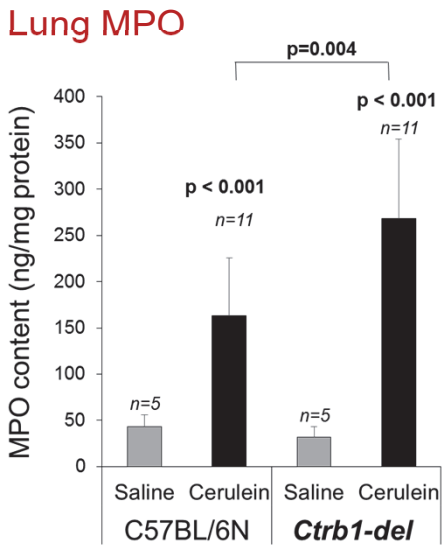
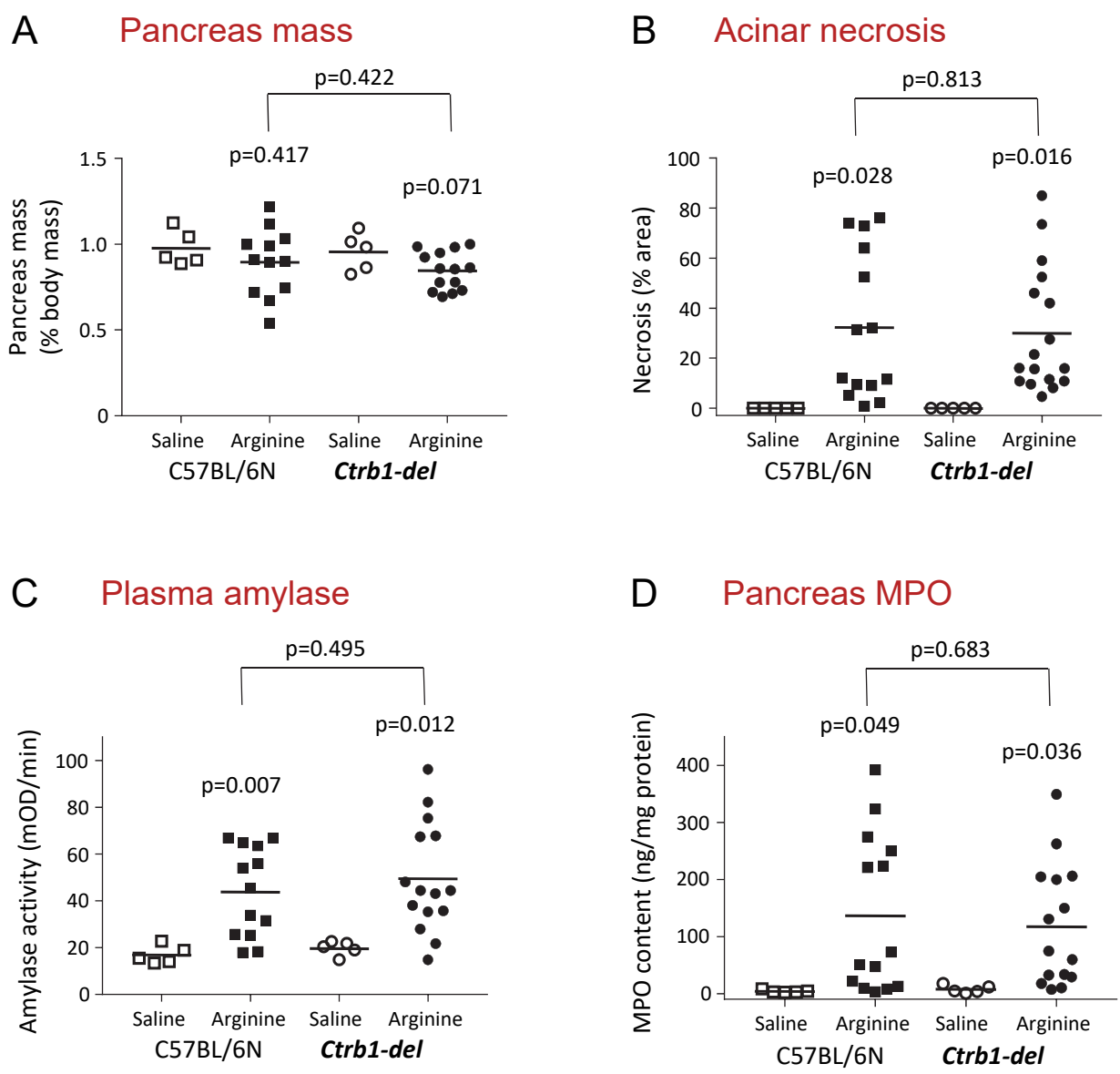
Ctrb1 gene expression



Supplementary Figure S2. Expression of *Ctrb1* mRNA in the pancreas of *Ctrb1-del* and C57BL/6N control mice. **A**, Semi-quantitative reverse-transcription PCR was carried out as described in *Methods*. PCR amplicons were analyzed by agarose gel electrophoresis and ethidium bromide staining. As loading control, 18S rRNA was measured. As an additional control, chymotrypsin-like protease (*Ctrl*) was also amplified. **B**, Quantitative real-time PCR was performed as detailed in *Methods*. Mean values with S.D. are shown. Loss of mRNA expression in the *Ctrb1-del* mice is in all likelihood due to nonsense-mediated mRNA decay, as deletion of exon 4 creates a frame shift with a premature termination codon.



Supplementary Figure S3. Myeloperoxidase (MPO) content in the lung of *Ctrb1-del* and C57BL/6N control mice treated with 10 hourly injections of saline or cerulein, as described in *Methods*. Mean values with S.D. are shown.



Supplementary Figure S4. L-arginine induced pancreatitis in *Ctrb1-del* and C57BL/6N control mice. **A**, Pancreas mass. **B**, Acinar cell necrosis, as judged by visual inspection and scoring of hematoxylin-eosin stained pancreas sections. **C**, Plasma amylase activity. **D**, Myeloperoxidase (MPO) content in the pancreas. Pancreatitis induction and analysis were performed as described in *Methods*. Individual data points were graphed with the mean indicated.

SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Accession numbers. NC_000074.6 (*Mus musculus* strain C57BL/6J chromosome 8, GRCm38.p4 C57BL/6J); NM_025583.2 (*Mus musculus* chymotrypsinogen B1 (Ctrb1), mRNA).

Animals. Animal experiments were performed with the approval and oversight of the Institutional Animal Care and Use Committee (IACUC) of Boston University, including protocol review and post-approval monitoring. The animal care program at Boston University is managed in full compliance with the US Animal Welfare Act, the United States Department of Agriculture Animal Welfare Regulations, the US Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Research Council's Guide for the Care and Use of Laboratory Animals. Boston University has an approved Animal Welfare Assurance statement (A3316-01) on file with the US Public Health Service, National Institutes of Health, Office of Laboratory Animal Welfare, and has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

All mice used in this study were on the C57BL/6N genetic background. The mouse line deficient in CTRB1 (*Ctrb1-del*) was generated by Cyagen, Inc. (Santa Clara, CA) using CRISPR/Cas9-mediated genome engineering. The mouse *Ctrb1* gene is located on chromosome 8; it spans approximately 4.5 kb and contains 7 exons. In the *Ctrb1-del* strain the 79 nucleotides that constitute exon 4 (c.237_315) were deleted (Supplementary Figure S1). *Ctrb1-del* mice were maintained in the homozygous state. C57BL/6N control mice were obtained from Charles River Laboratories (Wilmington, MA) or produced in our breeding facility from the same stock. For the experiments, 10-11-week old mice were used; both males and females (~30%) were studied. Typical weights at this age were 23 g and 18 g, respectively. The number of animals used in each experiment is shown in the figures. This represents a pooled value from three independent experiments using 5-9 animals in the cerulein-treated groups. We used fewer animals (2-4) for saline-treated controls, which typically show no change. Final experimental numbers in the different graphs may vary as not all animals were analyzed for all severity parameters.

Genotyping. To genotype the *Ctrb1-del* mice, we used PCR with primers that annealed to exon 2 and intron 4 and thus flanked the deleted exon 4 (Supplementary Figure S1). The wild-type amplicon size was 570 bp, whereas the deletion allele yielded a 491 bp product. The primer

sequences were as follows. Forward: 5'-TGC CAT CCA ACC CGT GCT GA-3'; Reverse: 5'-AGA ATT CTA AGA CCG GCC CCA GC-3'.

Cerulein-induced pancreatitis. Acute pancreatitis was induced by repeated intraperitoneal injections of the secretagogue peptide cerulein in a supramaximal stimulatory dose. Cerulein (#C9026, Sigma-Aldrich, St. Louis, MO) was dissolved in normal saline and injected hourly 10 times at a dose of 50 μg/kg. Experimental control mice were given normal saline injections. Mice were sacrificed 1 h after the last injection and the pancreas, blood and lung tissue were harvested. For histological analysis, pancreas tissue was fixed in 10% neutral buffered formalin. For myeloperoxidase (MPO) assays, pancreas and lung tissue was flash frozen in liquid nitrogen and stored at -80ºC until use. Blood was collected in heparinized syringes; cells were removed by centrifugation at 2,000g for 15 min and the plasma was stored frozen at -80ºC until use.

L-arginine-induced pancreatitis. A sterile solution of 10% L-arginine monohydrochloride (#11039; Sigma-Aldrich) was prepared in normal saline, and the pH was adjusted to 7.4. Two injections of L-arginine were administered intraperitoneally to mice, one hour apart, at a dose of 3.7 g/kg of body mass each. Contrary to published recommendations [1, 2], we found that two injections of the higher 4.0 g/kg dose or three injections of a lower 3.3 g/kg dose resulted in excessive mortality. This difference likely indicates the higher sensitivity of the C57BL/6N substrain to the unwanted toxic side effects of L-arginine. Controls received injections of normal saline. Mice were returned to their cages and warmed with infrared light during a 3-4 h recovery period with free access to food and water. Animals were sacrificed after 72 hours; blood and pancreas tissue were harvested and processed as described above. In this model, pancreatitis severity was variable and ~20% of treated animals exhibited no histological disease. In our analysis, we used only those animals where histology verified some degree of pancreatic injury.

Intra-pancreatic trypsin and chymotrypsin activation. To avoid the confounding effects of inflammatory cells and damaged acini at later time points, cerulein-induced intra-acinar protease activation was determined at 30 min after a single cerulein injection. The pancreas (~40-50 mg) was homogenized in 1 mL MOPS homogenization buffer (250 mM sucrose, 5 mM MOPS (pH 6.5), 1 mM $MgSO_4$), using a rotor-stator homogenizer (Tissue Master 125, Omni International, Kennesaw, GA). The homogenate was briefly centrifuged (1,000 g, 3 min) and trypsin and chymotrypsin activity in the supernatant was determined using the Boc-Gln-Ala-Arg-AMC and

Suc-Ala-Ala-Pro-Phe-AMC fluorescent substrates (Bachem USA, Torrance CA), respectively. An aliquot (20 uL) of the cleared homogenate was mixed with 30 µL assay buffer (50 mM Tris-HCl (pH 8.1), 150 mM NaCl, 1 mM $CaCl_2$, 0.1 mg/mL BSA) and 150 µL of 200 µM substrate dissolved in assay buffer was added to initiate the reaction. Increase in fluorescence was followed for 5 min in a fluorescent plate reader at 380 nm excitation and 460 nm emission wavelengths. The rate of substrate cleavage was expressed as relative fluorescent units (RFU) per sec and it was normalized to the total protein in the assay mix (RFU/sec/mg protein).

Determination of pancreatic water content. To characterize tissue edema, a 50-100 mg portion of the pancreas was weighed, dried for 72 h in an oven at 65ºC and weighed again. The difference between the wet and dry weights yielded the tissue water content, which was expressed as percent of the wet weight.

Histological analysis. Formalin-fixed pancreas tissue was paraffin-embedded; 5 µm sections were prepared and stained with hematoxylin-eosin (H&E) at the Boston University Experimental Pathology Laboratory Service Core. Arbitrary scoring (scale 0-3) by visual inspection was used to semi-quantitate the extent of tissue edema and inflammatory cell infiltration. Acinar cell necrosis was estimated by visual inspection and expressed as percent of tissue area.

Plasma amylase. Levels of amylase in the blood were measured in a kinetic activity assay using the 2-chloro-*p*-nitrophenyl-α-D-maltotrioside substrate (#A7564-60, Pointe Scientific, Canton MI). An aliquot of plasma (1 µL) was diluted with 9 µL normal saline, mixed with 190 µL substrate and the increase in absorbance due to the release of 2-chloro-nitrophenol was followed in a microplate reader at 405 nm for 2 min. The rate of substrate cleavage was expressed in mOD/min units.

Pancreatic myeloperoxidase (MPO) content. To assess inflammatory cell infiltration, tissue concentration of MPO was determined using an ELISA kit (#HK210-01, Hycult Biotech, Plymouth Meeting, PA) according to the manufacturer's instructions. The ELISA signal measured at 450 nm was converted to ng/mL MPO concentration using a calibration curve; normalized to total protein concentration (0.35 mg/mL in the 100 µL reaction sample) and expressed as ng MPO/mg protein.

Western blot analysis of CTRB1 expression. Pancreas tissue (~30 mg) was homogenized in phosphate buffered saline (pH 7.4) in the presence of Halt protease and phosphatase inhibitor

cocktail (Thermo Scientific) using a rotor-stator homogenizer (Tissue Master 125, Omni International). The homogenate was cleared by centrifugation at 17,000 g for 10 min at 4ºC. An aliquot of the supernatant containing 30 µg total protein was directly mixed with sample buffer and electrophoresed on 15% SDS-PAGE minigels. Proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, MA) at 350 mA for 1 h. CTRB1 was detected with a mouse monoclonal antibody (#sc-398721, Santa Cruz Biotechnology, Dallas, TX) used at a dilution of 1:200 followed by horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG (#HAF007, R&D Systems, Minneapolis, MN) used at 1:2,500 dilution. ERK1/2 was detected with a rabbit monoclonal antibody (#4695, Cell Signaling Technology, Danvers, MA) diluted 1:500 followed by HRP-conjugated goat anti-rabbit IgG (#31460, ThermoFisher Scientific) at a dilution of 1:5,000. Incubations with primary and secondary antibodies were performed at room temperature for 1 h each. HRP was detected using the SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific).

Pancreatic trypsinogen and chymotrypsinogen content. Protease zymogen content of the pancreas was characterized by their enzymatic activity after maximal activation. Pancreas (40 mg) was homogenized in 400 µL 20 mM Na-HEPES (pH 7.4) using a rotor-stator homogenizer (Tissue Master 125, Omni International) and the homogenate was cleared by centrifugation (850 g, 10 min, 4ºC). An aliquot (5 µL) of the supernatant was then treated with 4 µL human enteropeptidase (50x dilution of #1585-SE, R&D Systems) in 100 µL final volume of assay buffer (0.1M Tris-HCl (pH 8.0), 10 mM $CaCl_2$, 0.05% Tween 20) to activate trypsinogen. The endogenous active trypsin served as the activator for chymotrypsinogen. The development of trypsin and chymotrypsin activity was followed every 5 min by withdrawing 2 µL aliquots and mixing it with 48 µL assay buffer and 150 µL *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide and Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide substrates dissolved in assay buffer, respectively. The increase of absorbance at 405 nm was monitored in a microplate reader and rate of substrate cleavage was normalized to the total protein concentration and expressed as percent of the C57BL/6N control value. Under the conditions used, trypsinogen activation followed a relatively slow time course and reached its maximum at 50 min, after which it remained stable. Chymotrypsinogen activation, in contrast, proceeded rapidly and peaked within 5 min followed by a gradual decline.

RNA isolation, reverse transcription, PCR. Total RNA was extracted from mouse pancreas (~30 mg tissue) using the RNeasy Plus Mini Kit (Qiagen, Valencia CA). RNA (2 µg) was

reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (#4368814, ThermoFisher Scientific). Semi-quantitative PCR analysis was carried out using the HotStarTaq DNA Polymerase Kit (Qiagen) and the following primer sets to amplify mouse *Ctrb1* and *Ctrl* cDNAs. Ctrb1 forward primer, 5′-TCA CCA GCA CCA TGG CAT TC-3′; Ctrb1 reverse primer, 5′-GGA CCG TGT ACT TAG TTG GC-3′; Ctrl forward primer, 5′-GTC ACA ATG CTA CTG CTC AGC-3′; Ctrl reverse primer, 5′-GGA ACG GAT CTG TGG ACA G-3′. As loading control, 18S rRNA was measured with forward primer 5′-GAA ACG GCT ACC ACA TCC AAG G-3′ and reverse primer 5′-CCG CTC CCA AGA TCC AAC TAC G-3′. The PCR amplicons were resolved on 1.5% agarose gels and stained with ethidium bromide.

Quantitative real-time PCR was performed with the TaqMan Gene Expression Assay Mm00481616_m1 and TaqMan Universal PCR Mastermix (Life Technologies, Carlsbad, CA). Relative expression levels were estimated using the comparative cycle threshold method (ΔΔCT method). First, CT values for *Ctrb1* were normalized to those of the *Rpl13a* (ribosomal protein L13a) reference gene (ΔCT) and then to the ΔCT value of the C57BL/6N sample (ΔΔCT). Results were expressed as fold change calculated with the formula $2^{-\Delta\Delta CT}$.

Statistical analysis. Results from animal experiments were expressed as mean ± standard deviation. Differences of means between two groups were analyzed by unpaired t-test. $P < 0.05$ was considered statistically significant.

Biochemical Experiments with Recombinant Proteins

Expression and purification of T7 trypsinogen and mouse CTRB1. Mouse cationic trypsinogen (T7) was expressed in *E. coli*, re-folded *in vitro* and purified with ecotin affinity chromatography, as described in [3]. Concentration of purified trypsinogen was calculated from the UV absorbance at 280 nm using the extinction coefficient 39,140 $M^{-1}cm^{-1}$. Mouse CTRB1 carrying a 10His affinity tag was expressed in HEK 293T cells and purified as described in [4]. CTRB1 was activated with trypsin and the active chymotrypsin concentration was determined by titration with ecotin.

Trypsinogen autoactivation. Mouse cationic trypsinogen (T7) was incubated at 2 μM concentration in the absence or presence of 200 nM mouse CTRB1 and 10 nM initial trypsin in 0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$ and 0.05% Tween 20 (final concentrations) at 37ºC. At the indicated times, 1.5 μL aliquots were withdrawn and trypsin activity was measured with 200 μM *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide substrate, as detailed in [3].

Cleavage of trypsinogen by CTRB1. Mouse cationic trypsinogen (T7) was incubated at 2 μM concentration with 200 nM mouse CTRB1 in 0.1 M Tris-HCl (pH 8.0) at 37ºC. No calcium was added to prevent autoactivation during the incubation and to achieve maximal cleavage rates. Reactions (90 μL) were terminated by precipitation with 10% trichloroacetic acid (final concentration) and analyzed by 15% SDS-PAGE and Coomassie Blue staining. Cleavage fragments were transferred to an Immobilon P membrane (Millipore) and subjected to Edman degradation to determine their N-terminal sequences (David McCourt, Midwest Analytical, Inc, St. Louis, MO).

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