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14. ABSTRACT

Inroduction. The most frequent cause of hereditary pancreatitis is the R122H mutation in cationic trypsinogen (PRSS1). This mutation renders trypsinogen resistant to protective degradation by chymotrypsin-dependent mechanisms and thereby results in elevated intra-pancreatic trypsin activity.
Methods. We introduced the p.R123H mutation, which is analogous to human PRSS1 mutation p.R122H, in the mouse T7 trypsinogen gene and characterized acute pancreatitis responses elicited with repetitive cerulein injections.
Results. The *T7R123H* knock-in strain developed no spontaneous pathology in the pancreas or elsewhere. When given cerulein, *T7R123H* mice exhibited higher pancreatic edema and inflammatory cell infiltration, compared with C57BL/6N control mice treated in the same manner.
Conclusion. The observations indicate that the R123H mutation in mouse cationic trypsinogen is insufficient to elicit pancreatitis but it sensitizes the pancreas to harmful stimuli and heighten pathological responses in the secretagouge-induced pancreatitis model in mice.

15. SUBJECT TERMS
None listed

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1. Introduction

The most frequent cause of hereditary pancreatitis is the R122H mutation in cationic trypsinogen (PRSS1). This mutation renders trypsinogen resistant to protective degradation by chymotrypsin-dependent mechanisms and thereby results in elevated intra-pancreatic trypsin activity. High intra-pancreatic trypsin levels drive pancreatitis onset and progression. Although PRSS1-related hereditary pancreatitis is a relatively rare disease, it is one of the best understood forms of human pancreatitis at the mechanistic level and thus it represents a paradigm for this potentially severe inflammatory disorder. Therefore, understanding how PRSS1 mutation R122H initiates pancreatitis should offer essential insight into the pathogenesis of all forms of human chronic pancreatitis. We proposed to generate and characterize a novel mouse model of PRSS1-related hereditary pancreatitis carrying the clinically most relevant R122H mutation. We hypothesize that the mutation should be introduced in the endogenous mouse cationic trypsinogen (isoform T7), because this isoform is highly expressed and its activation properties resemble those of the human paralog. The mutation that corresponds to R122H in the mouse T7 trypsinogen is R123H. We expect that the *T7 R123H* mouse strain will develop either spontaneous pancreatitis or it will respond to secretagogue stimulation with increased pathological responses of pancreatitis. This preclinical mouse model of PRSS1-dependent hereditary pancreatitis will also greatly facilitate drug development and testing.

2. Keywords: pancreas, trypsinogen activation, digestive protease, cerulein, acute pancreatitis, chronic pancreatitis, hereditary pancreatitis, preclinical mouse model

3. Accomplishments

What were the major goals of the project?

Specific Aim 1: Generate new knock-in mouse strain with mutated T7 cationic trypsinogen in the C57BL/6 background, carrying mutation R123H, which correspond to mutation R122H in human cationic trypsinogen (PRSS1).

Milestone: Local IRB/IACUC Approval obtained on February 3, 2020
Milestone: HRPO/ACURO Approval obtained on February 10, 2020
Major Task 1: Generate knock-in strain with mutated T7 trypsinogen gene.
Major Task 2: Trypsinogen expression analyses.
Milestones: Generation and characterization of the newly created mutant *T7 R123H* mouse strain have been completed in July, 2020.

Specific Aim 2: Experiments to assess spontaneous pancreatitis and increased susceptibility to experimental pancreatitis.

Major Task 3: Experiments to assess spontaneous pancreatitis.
Milestones: Development of spontaneous pancreatitis have been followed up to 1 year of age in T7 R123H mice. Task has been completed in February, 2021.
Major Task 4: Investigation of susceptibility to experimental acute pancreatitis.
Milestones: Determination of severity of acute pancreatitis by evaluation of edema, acinar cell necrosis and vacuolization and inflammatory cell infiltration. Task has been completed in October, 2020.
Major Task 5: Investigation of susceptibility to experimental chronic pancreatitis.
Milestones: Determination of chronic pancreatitis severity by evaluation of acinar cell loss, fibrosis, duct dilatations, tubular complexes and inflammatory cell infiltration. Task is in progress (20%).
Major Task 6: Evaluation of intra-acinar trypsin activation.
Milestones: Better understanding the mechanistic basis of the pathogenic effect of the T7 R123H mutation. Task is in progress (50%).

What was accomplished under these goals?

The main hypothesis of this grant is that a mouse strain expressing the R123H mutated form of T7 mouse trypsinogen can be used to model human hereditary pancreatitis associated with the R122H mutation in PRSS1. The objective to be reached is to create *T7 R123H* mice that develop pancreatitis spontaneously or exhibit increased sensitivity to experimentally induced pancreatitis.

Specific Aim 1: Generate new knock-in mouse strain with mutated T7 cationic trypsinogen in the C57BL/6 background, carrying mutation R123H, which correspond to mutation R122H in human cationic trypsinogen (PRSS1).

Major Task 1: Generate knock-in strain with mutated T7 trypsinogen gene.
We were curious whether the mouse model of PRSS1-related hereditary pancreatitis carrying the clinically most relevant R122H mutation will develop either spontaneous pancreatitis or it will respond to secretagogue stimulation with increased pathological responses of pancreatitis. To answer this question, we introduced the R123H mutation into exon 3 of T7 trypsinogen in C57BL/6N mice, as described in *Project Narrative* (Figure 1A). No phenotypic changes were apparent in homozygous *T7R123H* mice, which grew and bred normally.

Major Task 2: Trypsinogen expression analysis.
Expression of T7 trypsinogen was comparable in C57BL/6N and *T7R123H* mice, as judged by by western blotting (Figure 1B) and protein zymogen content (Figure 1C).

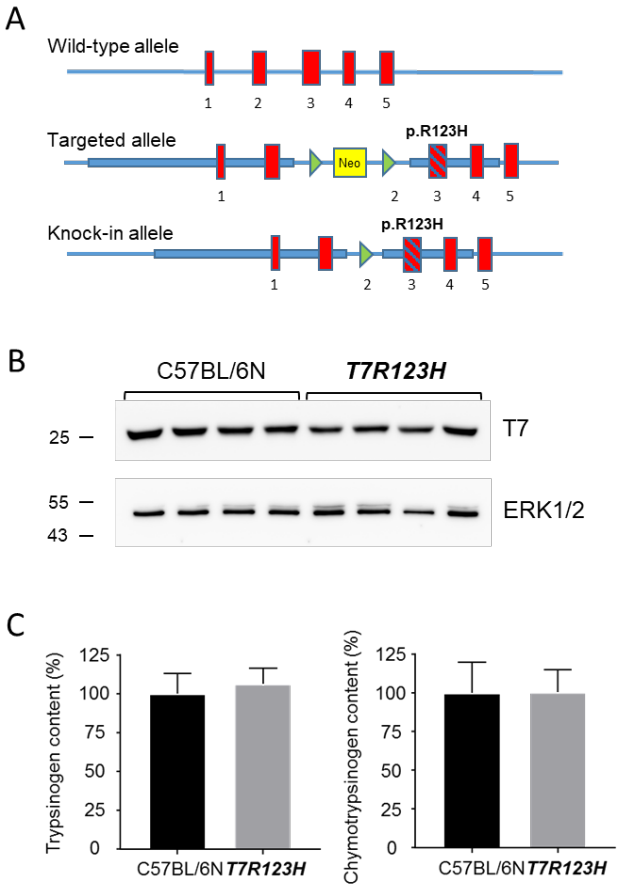

Figure 1. Generation of the *T7R123H* mouse strain and expression of T7 trypsinogen. **A**, Schematic representation of the wild-type T7 trypsinogen gene and the recombined *T7R123H* allele before and after excision of the neomycin cassette by breeding with a Cre-deleter strain. Exons are shown as red boxes. The loxP sites are in green. Thick blue lines indicate the homology arms. **B**, Western blot analysis of T7 trypsinogen protein levels in the pancreas of C57BL/6N and *T7R123H* mice. ERK1/2 was measured as loading control. **C**, Protease zymogen content of the pancreas. Total trypsinogen and (B) chymotrypsinogen content was measured from pancreas homogenates of C57BL/6N and *T7R123H* mice. Trypsinogen and chymotrypsinogen levels in the pancreas were measured enzymatically after maximal activation. Results were expressed as percent of the average C57BL/6N values. Individual data points with mean (horizontal bar) and standard deviation are shown.

Milestones Achieved: Generation and characterization of the newly created mutant T7 R123H mouse strain have been completed.

Major Task 3: Experiments to assess spontaneous pancreatitis.
We speculated that increased autoactivation of the R123H trypsinogen mutant might result in pancreatitis in *T7 R123H* mice or increase their susceptibility to the disease. We observed no spontaneous pancreatitis in *T7 R123H* mice followed up to 1 year of age, as assessed by histology. In 1-year-old *T7 R123H* mice, we occasionally observed patchy acinar cell damage, focal cellular infiltrates and islet hypertrophy (not shown).

Milestones Achieved: No signs of spontaneous pancreatitis have been observed in T7 R123H mice at ages of 1 year.

Major Task 4: Investigation of susceptibility to experimental acute pancreatitis.
We induced experimental acute pancreatitis in C57BL/6N and *T7R123H* mice by 10 hourly intraperitoneal injections of the secretagogue cerulein and sacrificed the mice 1 hour after the last injection. On hematoxylin-

eosin stained pancreas sections from the cerulein-treated mice we observed the characteristic edema, inflammatory cell infiltration and scattered acinar cell necrosis, which were absent in mice given saline (Figure 4A). Histological scoring of multiple sections for edema (Figure 4B), inflammatory cells (Figure 4C) and acinar cell necrosis (Figure 4D) revealed significant infiltration of inflammatory cells in *T7R123H* mice small differences in edema, and necrosis of the pancreas between *T7R123H* mice and C57BL/6N controls. Edema was also evaluated by measuring the pancreas mass and the pancreatic water content (Figure 2). Both parameters increased significantly in response to cerulein treatment in the two strains. Again, a non-significant trend for higher pancreatic mass was evident in cerulein-treated *T7R123H* mice relative to C57BL/6N mice (Figure 2A), but significant differences were seen in pancreatic water content in cerulein-treated *T7R123H* relative to C57BL/6N mice (Figure 2B). We determined changes in plasma amylase levels, and pancreas myeloperoxidase (MPO) content (Figure 3). As expected, marked elevations in plasma amylase activity and pancreatic MPO content were observed in mice given cerulein compared to saline-treated mice. However, we found no significant differences between *T7R123H* and C57BL/6N mice. Pancreatic MPO content was somewhat higher in cerulein-treated *T7R123H* versus C57BL/6N mice (Figure 3B). Taken together, the results indicate that the R123H mutation in T7 trypsinogen does not change the severity of cerulein-induced pancreatitis significantly. The higher pancreatic water content and histological increase in inflammatory cell infiltration in cerulein-treated *T7R123H* mice, however, suggest that this strain is slightly more prone to acinar cell damage than the C57BL/6N parent strain.

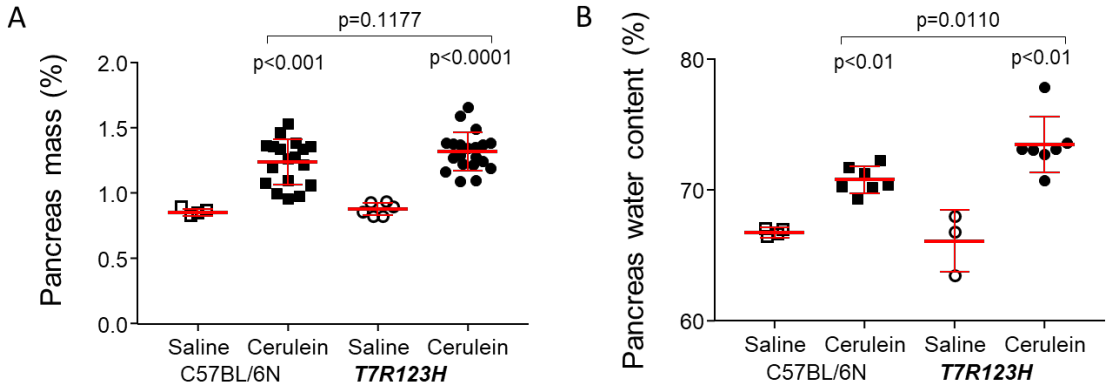

Figure 2. Pancreas mass and pancreatic water content in cerulein-induced pancreatitis. C57BL/6N and *T7R123H* mice were given 10 hourly saline or cerulein injections, as indicated. Mice were sacrificed 1 hour after the last injection. A, Pancreas mass as percent of body mass. B, Pancreas water content expressed as percent of wet pancreas mass. Individual data points were graphed with the mean and SD indicated. The difference of means between two groups was analyzed by two-tailed unpaired t-test.

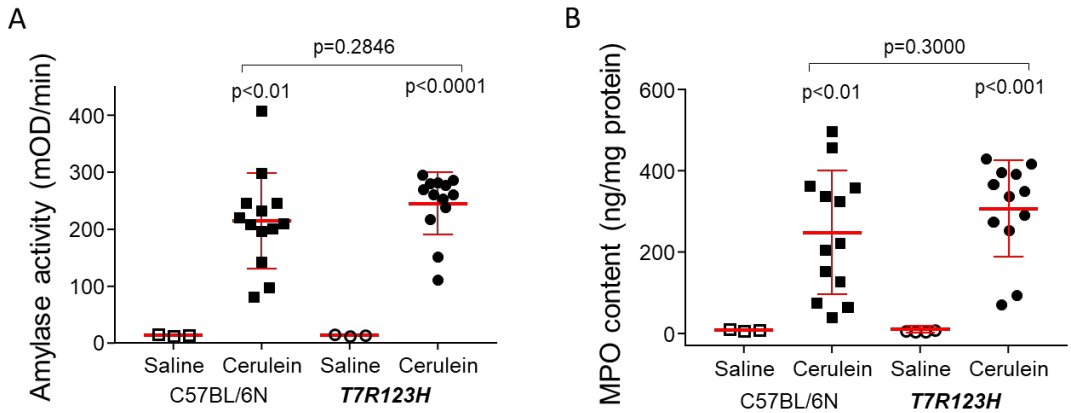

Figure 3. Plasma amylase and pancreas myeloperoxidase (MPO) content in cerulein-induced pancreatitis. C57BL/6N and *T7R123H* mice were given 10 hourly saline or cerulein injections, as indicated. Mice were sacrificed 1 hour after the last injection. **A**, Plasma amylase activity expressed in mOD/min units. **B**, Pancreas MPO content. Individual data points were graphed with the mean and SD indicated. The difference of means between two groups was analyzed by two-tailed unpaired t-test.

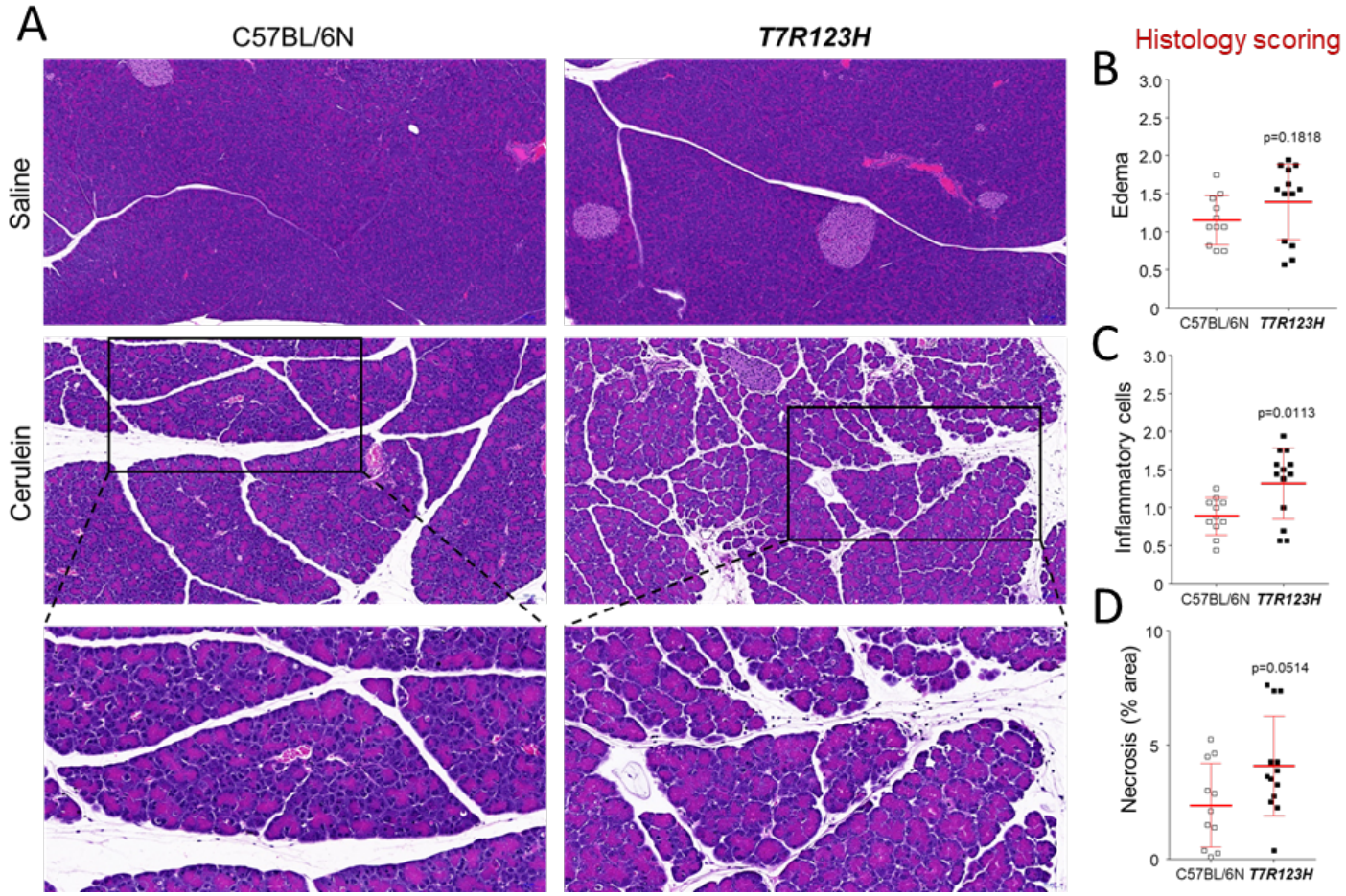

Figure 4. Histology of cerulein-induced acute pancreatitis in *T7R123H* mice. (**A**) Representative hematoxylin-eosin stained histological sections of the pancreas from saline and cerulein-treated C57BL/6N and *Ctrl-KO* mice. (**B**) Histology scoring for edema, inflammatory cell infiltration, and acinar cell necrosis in cerulein-treated mice. Mean values with standard deviation are shown.

Milestone Achieved: Determination of severity of acute pancreatitis by evaluation of edema, acinar cell necrosis and inflammatory cell infiltration have been completed.

Major Task 6: Evaluation of intra-acinar trypsin activation.

To examine whether the increased autoactivation of the R123H mutant translates to higher intrapancreatic protease activation during cerulein-induced pancreatitis, we measured trypsin and chymotrypsin activities from pancreas homogenates 30 min after a single cerulein or saline injection. Previous studies indicated that this is the optimal time point to assess intrapancreatic protease activation as the pancreas is still relatively intact and the acinar tissue is unaffected by necrosis or inflammatory cell infiltration. Both trypsin and chymotrypsin activities were markedly higher in the pancreas of mice given cerulein relative to saline-treated mice (Figure 5). When the cerulein-treated groups were compared, no appreciable differences were observed in trypsin and chymotrypsin activities in *T7R123H* mice relative to C57BL/6N mice. The results indicate that cerulein-induced intrapancreatic protease activation is comparable in the two strains.

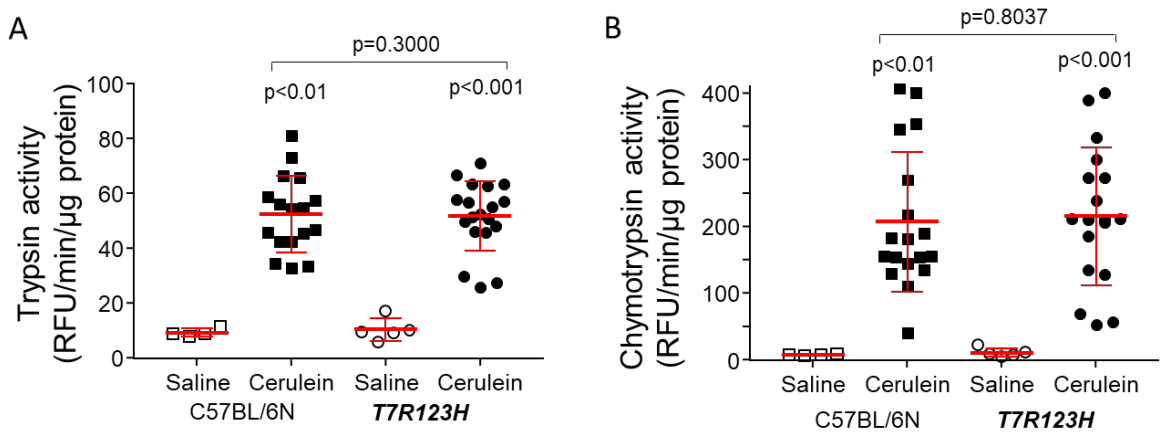

Figure 5. Cerulein-induced intrapancreatic trypsin and chymotrypsin activation. C57BL/6N and *T7R123H* mice were given a single saline or cerulein injection, as indicated, and the mice were sacrificed 30 min later. **A**, Trypsin activity in pancreas homogenates. **B**, Chymotrypsin activity in pancreas homogenates. Individual data points were graphed with the mean and SD indicated. The difference of means between two groups was analyzed by two-tailed unpaired t-test.

Milestone: For better understanding the mechanistic basis of the pathogenic effect of the R123H mutation, we will further study of trypsin activation as well as acinar cell injury on freshly isolated acinar cell preparations in the next reporting period.

What opportunities for training and professional development has the project provided?
Nothing to Report

How were the results disseminated to communities of interest?
Nothing to Report. During the next period of the award, I plan to attend and present my findings at the American Pancreatic Association (APA) annual meeting as well as to publish our data. I will aim to publish high quality paper as first author (or senior author) in high-impact journals.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.
During the next period of the award, I will achieve the last milestone of this project by determine the severity of chronic pancreatitis. In this task I will evaluate the grade of acinar cell loss, fibrosis, duct dilatations, tubular complexes and inflammatory cell infiltration.

Major Task 5: Investigation of susceptibility to experimental chronic pancreatitis. Given the small differences observed in acute pancreatitis severity in *T7R123H* mice, we reasoned that more sustained stimulation with cerulein may amplify the incipient pathology and result in overt disease. Therefore, we treated *T7R123H* and C57BL/6N mice with 8 hourly injections of cerulein on two consecutive days and sacrificed the mice 3 days after the last injection. Preliminary data indicates that cerulein-treated *T7R123H* mice develope severe disease. During the next reporting period we will complete Major task 5 by evaluating changes are characteristic of chronic pancreatitis, such as acinar cell ablation, histological architecture and fibrosis.

The experiments detailed above indicate that the R123H mutation in mouse cationic trypsinogen is insufficient to elicit pancreatitis but it sensitizes the pancreas to harmful stimuli. To obtain additional evidence for this concept, we will cross the *T7R123H* mice with homozygous *Ctrb1-del* mice which are deficient in chymotrypsin B1 (CTRB1), the major mouse chymotrypsin isoform. CTRB1 protects the pancreas against pancreatitis by degrading trypsinogen and deletion of the *Ctrb1* gene increases cerulein-induced trypsin activation and the severity of cerulein-induced pancreatitis. We will assess whether *T7R123H x Ctrb1-del* mice developed spontaneous pancreatitis at ages of 3 months, 6 months and 1 year.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?
We generated a novel mouse model, which will offer conceptual proof for the role of trypsinogen mutations in hereditary pancreatitis. This model will allow the research community to study mechanistic details of the pathogenesis of hereditary pancreatitis. Using the novel mouse strain as preclinical model contributes to the development of novel therapeutic and preventive approaches.

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer? Nothing to Report

What was the impact on society beyond science and technology? Nothing to Report

5. Changes/Problems: Nothing to Report

6. Products: research material such as animal models of hereditary pancreatitis

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	Zsanett Jancso, PhD
Project Role:	PI
Researcher Identifier (ORCID ID):	0000-0002-0572-5452
Nearest person month worked:	3.6
Contribution to Project:	Zsanett Jancso has managed the entire project. She also wrote the necessary protocols. She performed and completed the proposed experiments.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report

What other organizations were involved as partners?
Nothing to Report

8. Special Reporting Requirements: N/A

9. Appendices

Methods:

Generation of the *T7R123H* mouse strain. Mice were on the C57BL/6N genetic background. The gene encoding T7 trypsinogen (2210010C04Rik) is located on chromosome 6; it spans ~3.8 kb and comprises 5 exons. Mutation c. c.368_369GA>AC (p.R123H) was knocked-in using homologous recombination in C57BL/6 embryonic stem (ES) cells (Cyagen, Santa Clara, CA). The targeting vector contained the T7 trypsinogen gene with the p.R123H mutation in exon 3 and a neomycin resistance gene flanked by loxP sites in intron 2 (Figure 1A). Correctly targeted ES cell clones were identified by long-range PCR and confirmed by Southern blot. Mutant ES cells were injected into mouse embryos (blastocysts), which were implanted into pseudopregnant females. The resulting chimeras were bred with wild-type C57BL/6N mice to achieve germline transmission of the mutant allele. To remove the neomycin resistance gene from the mutant allele, mice were bred with a Cre-deleter strain that expresses the Cre recombinase in the early mouse embryo (B6.FVB-Tg(EIIa-cre)C5379Lmgd/J; Jackson Laboratories). The final *T7R123H* knock-in allele contained the p.R123H mutation in exon 3 and a 147 nt residual sequence in intron 2 including a single loxP site. *T7R123H* mice were maintained in the homozygous state. C57BL/6N mice obtained from Charles River Laboratories (Wilmington, MA) or produced in our breeding facility from the same stock were used as experimental controls. The number of animals used in each experiment is shown in the figures.

Both male and female animals were studied. Experimental mice were 11-12-week old and weighed around 25 g (males) and 20 g (females).

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.

Western blotting. T7 trypsinogen was detected using a rabbit polyclonal antibody raised against the peptide sequence LKTAATLNSRVST corresponding to amino-acids 114-126 of mouse T7 pre-trypsinogen. The specificity of the antibody was previously validated on pancreas homogenate from T7-deficient mice. The antibody was used at a final dilution of 1:10,000. Rabbit monoclonal antibody against p44/42 MAPK (ERK1/2) (137F5) was purchased from Cell Signaling Technology (catalog #4695) and used at a final dilution of 1:1,000. The horse-radish peroxidase-conjugated goat anti-rabbit secondary antibody ((#31460, ThermoFisher Scientific)) was used at a dilution of 1:10,000.

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 12 times at a dose of 50 µg/kg. Experimental control received normal saline injections. Mice were sacrificed 1 h after the last injection and the pancreas and blood tissue were harvested. For histological analysis, pancreas tissue was fixed in 10% neutral buffered formalin. For myeloperoxidase (MPO) assays, pancreas 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 at 4ºC and the plasma was stored frozen at -80ºC until use.

Determination of pancreatic water content. To characterize tissue edema, a 50-100 mg portion of the pancreas was weighed, dried for 72 hours in an oven at 65ºC and weighed again. Tissue water content was then expressed as percent of wet mass.

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

Pancreatic myeloperoxidase (MPO) content. To evaluate inflammatory cell infiltration, MPO content of the tissue 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.

Histology and immunohistochemistry. Pancreas tissue was fixed in 10% neutral buffered formalin; paraffin-embedded (FFPE); sectioned and stained with hematoxylin-eosin at The Translational Pathology Core Laboratory (TPCL) at UCLA. Arbitrary scoring (scale 0-3) by visual inspection was used to semi-quantitate the extent of tissue edema and inflammatory cell infiltration. 0 = absent, 1 = minimal (<10% of visual field), 2 = moderate (10% to 50%), and 3 = severe (>50%). Acinar cell necrosis was estimated by visual inspection and expressed as percent of tissue area.

Intra-pancreatic trypsin and chymotrypsin activation. Cerulein-induced intra-pancreatic protease activation was determined at 30 min after a single cerulein (50 µg/kg) injection. The pancreas (~40 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, 2 min, 4°C), and trypsin and chymotrypsin activity in the supernatant was determined using the Z-Gly-Pro-Arg-AMC·HCl and Suc-Ala-Ala-Pro-Phe-AMC fluorescent substrates (Bachem USA, Torrance CA), respectively. Aliquots (5 µL) of the cleared homogenate were mixed with 45 µL assay buffer (0.1M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 0.05% Tween 20) and 150 µL of 200 µM substrate dissolved in assay buffer was added to initiate the reaction. The increase in fluorescence was followed for 5 minutes 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 second and it was normalized to the total protein in the assay mix (RFU/sec/mg protein).

Statistical analysis. Results were plotted as individual data points with the mean and standard deviation indicated. Differences of means between two groups were analyzed by unpaired t-test. $P < 0.05$ was considered statistically significant.