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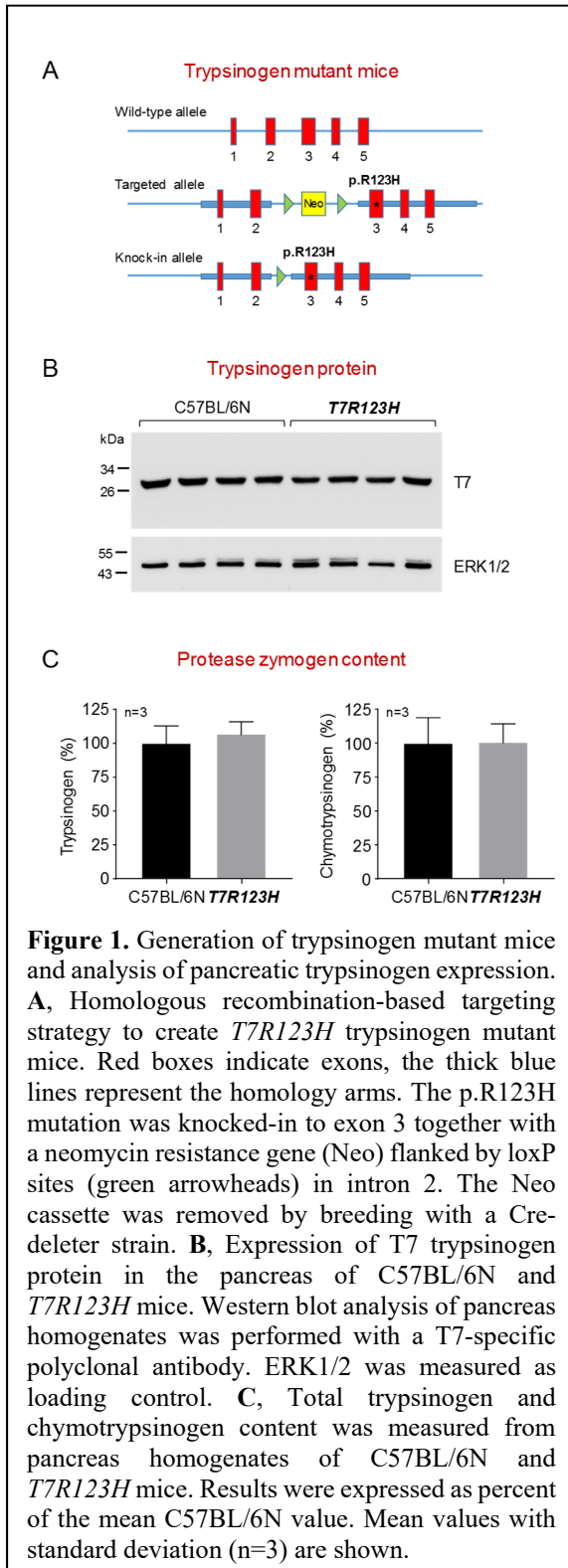
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14. ABSTRACT Inroduction. The most frequent cause of hereditary pancreatitis is the p.R122H mutation in the serine protease 1 (PRSS1) gene, which encodes human cationic trypsinogen. This mutation renders trypsinogen resistant to protective degradation by chymotrypsin and thereby results in elevated intrapancreatic trypsin activity that elicits pancreatitis. Methods. We introduced the p.R123H mutation, which is analogous to human PRSS1 mutation p.R122H, in the mouse cationic trypsinogen (isoform T7) and characterized the severity of experimentally induced pancreatitis in the novel <i>T7R123H</i> mice. Results. The <i>T7R123H</i> knock-in mouse strain developed no spontaneous pathology in the pancreas or elsewhere. When pancreatitis was induced experimentally by cerulein injections, <i>T7R123H</i> mice exhibited similar intrapancreatic trypsin activation and disease severity as C57BL/6N control mice treated in the same manner. Sustained stimulation with cerulein, however, resulted in more severe chronic pancreatitis in <i>T7R123H</i> mice than in C57BL/6N controls. Conclusion. The observations indicate that <i>T7R123H</i> mice exhibit more severe experimental pancreatitis in case of persistent pancreatic injury. The findings are consistent with the proposed pathogenic role of the p.R122H trypsinogen mutation in humans.					
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1. INTRODUCTION

The most frequent cause of hereditary pancreatitis is the p.R122H mutation in cationic trypsinogen (PRSS1). This mutation renders trypsinogen resistant to protective degradation by chymotrypsin and thereby results in elevated intrapancreatic trypsin activity. High intrapancreatic 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 p.R122H initiates pancreatitis should offer essential insight into the pathogenesis of all forms of human chronic pancreatitis. In our work funded by this grant award, we generated a novel mouse model of PRSS1-related hereditary pancreatitis. The *T7R123H* mice carry the p.R123H mutation in mouse cationic trypsinogen (isoform T7). Mutation p.R123H in mouse cationic trypsinogen is analogous to mutation p.R122H in human cationic trypsinogen. The original PI of the grant, Dr. Zsanett Jancso, has left UCLA, and the project was taken over and completed by the new PI, Dr. Miklos Sahin-Toth as of 08/31/2021.



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 p.R123H, which correspond to mutation p.R122H in human cationic trypsinogen (PRSS1).

Major Task 1: Generate knock-in strain with mutated T7 trypsinogen gene.

Major Task 2: Trypsinogen expression analyses.

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

Major Task 3: Experiments to assess spontaneous pancreatitis.

Major Task 4: Investigation of susceptibility to experimental acute pancreatitis.

Major Task 5: Investigation of susceptibility to experimental chronic pancreatitis.

Major Task 6: Evaluation of intrapancreatic trypsin activity.

What was accomplished under these goals?

Both Specific Aims have been completed and the results have been published:

Jancsó Z, Morales Granda NC, Demcsák A, Sahin-Tóth M. Mouse model of *PRSS1* p.R122H-related hereditary pancreatitis highlights context-dependent effect of autolysis-site mutation. *Pancreatology* 2023, 23:131-142.

Specific Aim 1, Major Tasks 1 and 2.

To test the effect of p.R123H *in vivo*, we created a novel knock-in mouse strain carrying the mutation in the native T7 trypsinogen locus of C57BL/6N mice (Figure 1A). Genetic modification was achieved using homologous recombination. Homozygous *T7R123H* mice had no obvious phenotype; they were indistinguishable from wild-type C57BL/6N mice. Macroscopic and microscopic morphology of the pancreas from 1-year-old *T7R123H* mice were normal, with no signs of spontaneous pathology. Western blot analysis of pancreas homogenates indicated comparable expression of T7 trypsinogen in C57BL/6N and *T7R123H* mice (Figure 1B). Measurement of total trypsinogen and chymotrypsinogen content in the pancreas from C57BL/6N and *T7R123H* mice revealed no differences either (Figure 1C).

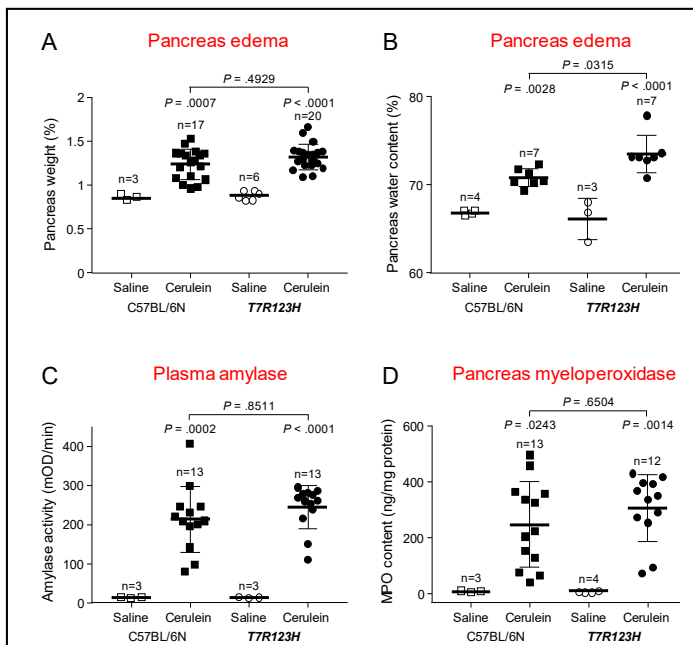


Figure 2. Cerulein-induced acute pancreatitis in *T7R123H* mice. C57BL/6N and *T7R123H* mice were given 10 hourly injections of saline or cerulein. Mice were euthanized 1 h after the last injection. **A**, Pancreas weight normalized to body weight. **B**, Pancreatic water content. **C**, Plasma amylase activity. **D**, Pancreatic myeloperoxidase (MPO) content. Individual data points with mean and standard deviation are shown. The difference of means was analyzed by one-way ANOVA followed by Tukey's post-hoc analysis.

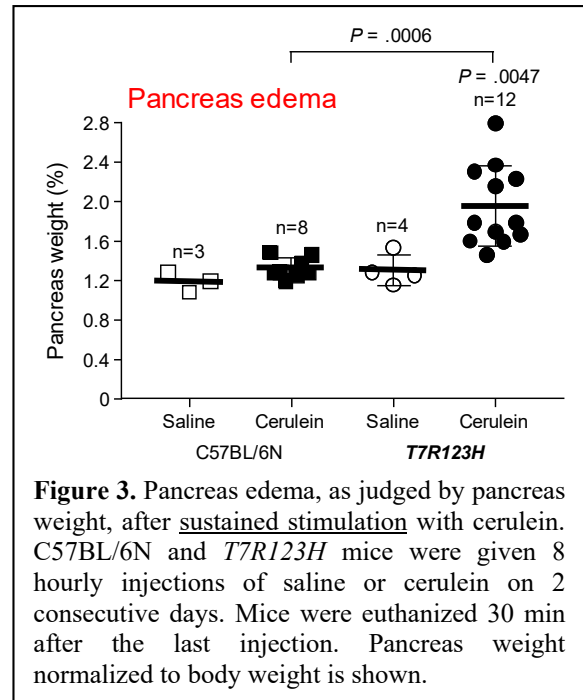
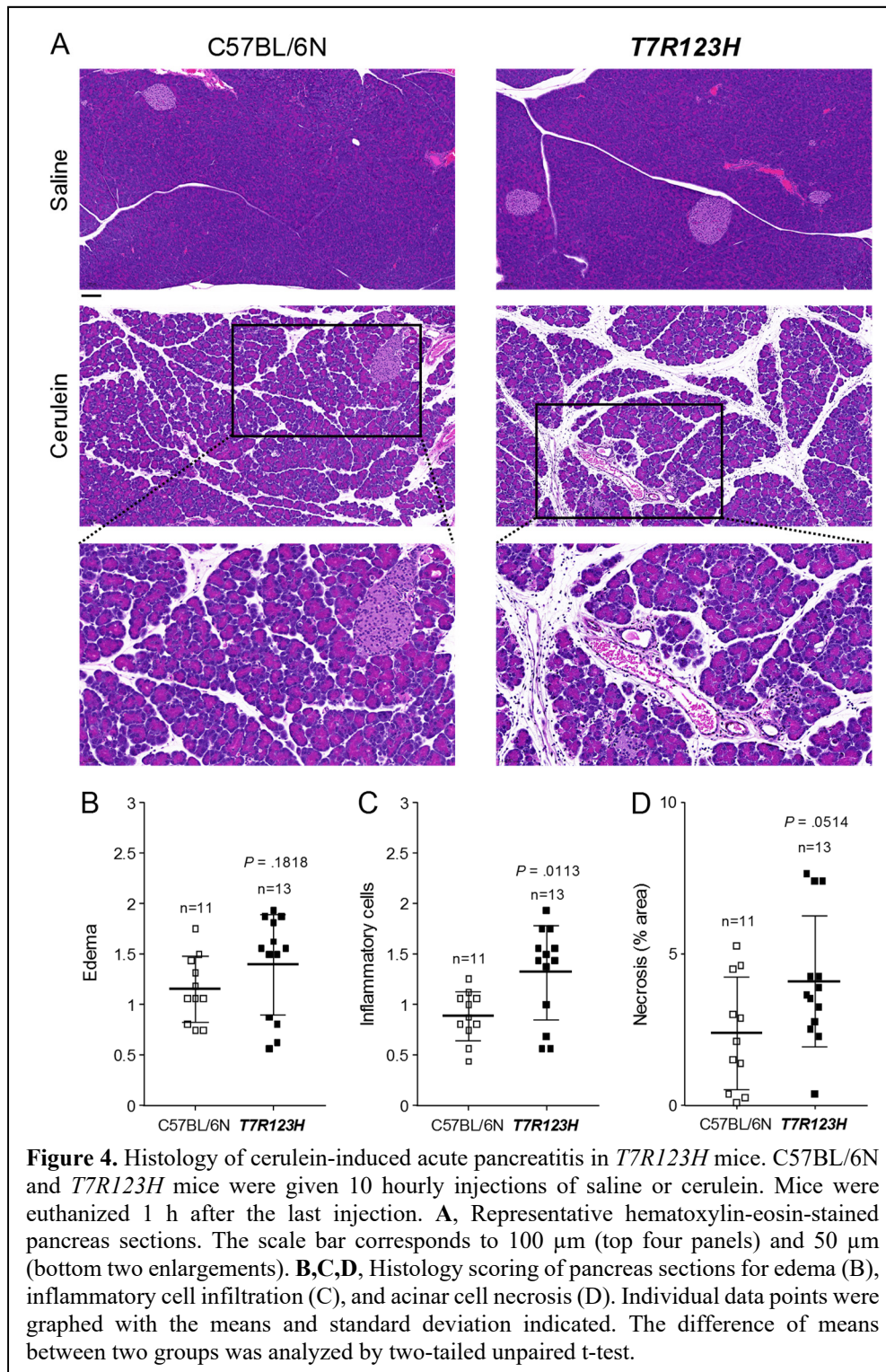


Figure 3. Pancreas edema, as judged by pancreas weight, after sustained stimulation with cerulein. C57BL/6N and *T7R123H* mice were given 8 hourly injections of saline or cerulein on 2 consecutive days. Mice were euthanized 30 min after the last injection. Pancreas weight normalized to body weight is shown.

Specific Aim 2, Major Tasks 3 and 4.

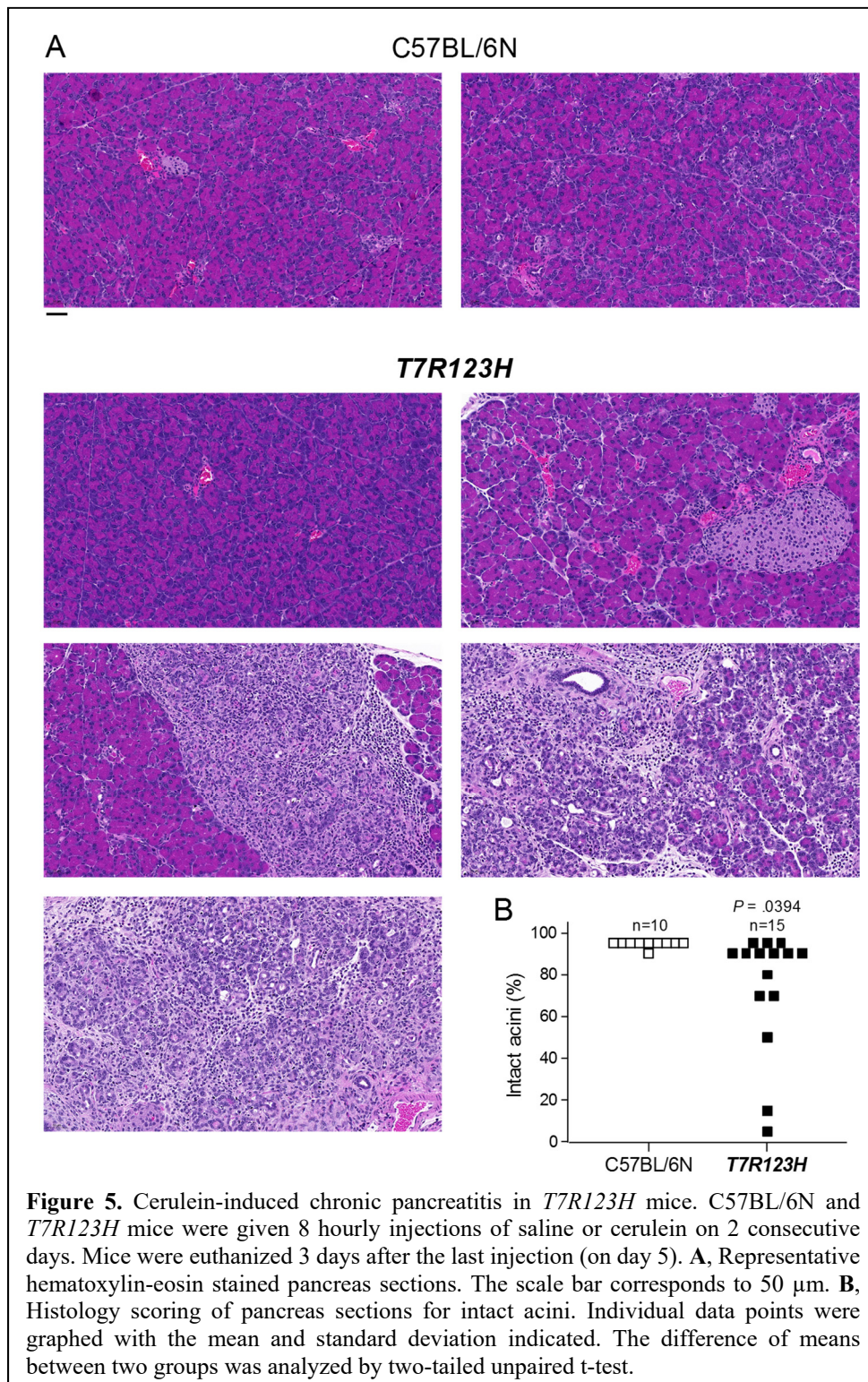
Since the *T7R123H* mice developed no spontaneous pancreas disease, we induced acute pancreatitis in C57BL/6N and *T7R123H* mice by 10 hourly injections of cerulein (50 $\mu\text{g}/\text{kg}$ dose). Mice were euthanized 1 hour after the last injection. We found significant pancreas edema in cerulein-treated mice relative to saline-treated controls, as judged by the pancreas weight and pancreas water content (Figures 2A and B). Similarly, plasma amylase (Figure 2C) and pancreatic myeloperoxidase (MPO) content (Figure 2D) were markedly increased in mice given cerulein versus saline. When the inflammatory response of C57BL/6N and *T7R123H* mice were compared, all parameters were slightly higher in *T7R123H* mice but the difference reached statistical significance only for pancreatic water content. To evaluate the effect of **sustained overstimulation** with cerulein on pancreatic edema, we treated mice with 8 hourly cerulein injections on 2 consecutive days, and euthanized the animals 30 min after the last injection. The pancreas of cerulein-treated *T7R123H* mice was visibly more edematous (not shown) than those of C57BL/6N mice, and this significant difference was also evident when pancreas weights were compared (Figure 3). Histological analysis of pancreas sections of mice given 10 cerulein injections by hematoxylin-eosin staining (Figure 4A) also showed slightly stronger pancreatitis scores in *T7R123H* versus C57BL/6N mice for edema (Figure 4B), inflammatory cells (Figure 4C), and acinar cell necrosis (Figure 4D). The difference in inflammatory cell infiltration was statistically significant. Taken together, the results indicate that *T7R123H* mice develop slightly more severe cerulein-induced AP than the C57BL/6N parent strain.



Specific Aim 2, Major Task 5.

We and others previously found that trypsinogen mutant mice develop progressive pancreatitis after a cerulein-induced acute episode while the pancreas of C57BL/6N mice recovers quickly. Pancreatitis progression in mutant mice is characterized by extensive acinar atrophy, fibrosis, and inflammatory cell infiltration, all features of chronic pancreatitis. Histological recovery is delayed, and often incomplete. To test whether *T7R123H* mice would develop chronic pancreatitis after an acute episode, we treated mice with 8 hourly injections of cerulein on 2 consecutive days and euthanized the animals 3 days after the last injection. Histological analysis of pancreas sections showed essentially normal acinar tissue in C57BL/6N mice, indicating complete recovery from the cerulein-induced AP (Figure 5A). In contrast, pancreas sections from some *T7R123H* mice revealed areas of

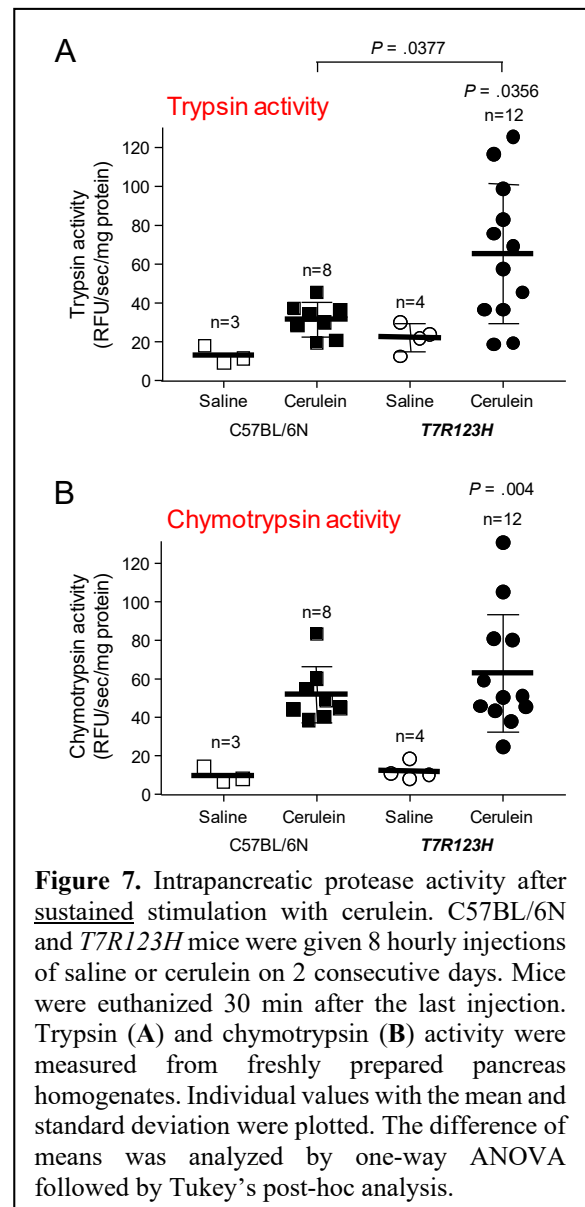
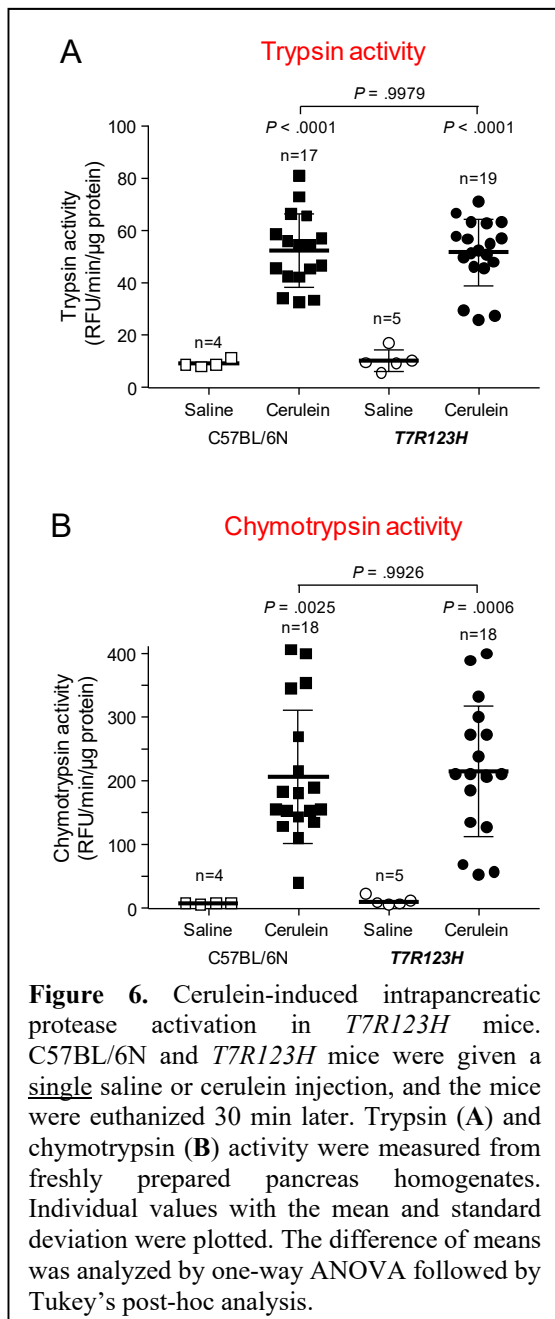
chronic pancreatitis. Scoring of multiple sections for intact acini indicated no significant dropout in C57BL/6N



mice (n=10), whereas 6 of 15 (40%) *T7R123H* mice showed measurable acinar atrophy, with 2 mice exhibiting a complete response (Figure 5B). The results indicate that *T7R123H* mice are somewhat prone to develop progressive chronic pancreatitis after cerulein-induced acute pancreatitis; however, the penetrance is relatively low, not nearly as robust as in the previously published trypsinogen mutant strains.

Specific Aim 2, Major Task 6: Evaluation of intrapancreatic trypsin activation.

We measured trypsin (Figure 6A) and chymotrypsin (Figure 6B) activity from freshly prepared pancreas homogenates 30 min after a single cerulein injection (50 μ g/kg dose). We typically use this early time point to



evaluate intrapancreatic protease activation because the acinar tissue is still relatively intact, unaffected by inflammation. Relative to saline-injected mice, significantly increased trypsin and chymotrypsin activity was observed in the pancreas of cerulein-treated mice, however, no difference was apparent between the C57BL/6N and *T7R123H* strains.

To evaluate the effect of more sustained overstimulation with cerulein on intrapancreatic trypsin and chymotrypsin activity, we treated mice with 8 hourly cerulein injections on 2 consecutive days, and euthanized the animals 30 min after the last injection. Under these conditions, intrapancreatic trypsin activity of cerulein-treated *T7R123H* mice was significantly higher than those of cerulein-treated C57BL/6N controls (Figure 7A), while chymotrypsin activity showed a similar trend without statistical significance (Figure 7B).

Methods

Generation of the *T7R123H* mouse strain. Model generation was performed in the C57BL/6N genetic background (Cyagen, Santa Clara, CA). The final *T7R123H* knock-in allele contained the c.368_369GA>AC (p.R123H) mutation in exon 3 of the mouse cationic trypsinogen gene and a 113 nt residual “scar” sequence in intron 2, between positions c.204-417 and c.204-416. *T7R123H* mice were bred to homozygosity, and were

maintained in this 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. Both male and female mice were studied.

Western blotting. Pancreas tissue (30 mg) was homogenized in 300 μ L phosphate-buffered saline (pH 7.4) containing Halt Protease and Phosphatase Inhibitor Cocktail (from 100 \times stock, catalog number 78440, Thermo Scientific) and 20 μ g total protein of the cleared lysate was loaded per well. Mouse T7 trypsinogen was detected using an in-house rabbit polyclonal antibody (1:10,000 dilution). As loading control, mouse ERK1/2 was measured using a rabbit monoclonal antibody (catalog number 4695, Cell Signaling Technology, Danvers, MA) at a dilution of 1:1,000. The horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody was used at a dilution of 1:10,000 (catalog number 31460, Thermo Scientific).

Measurement of pancreatic protease zymogen content. Pancreas tissue (40 mg) was homogenized in 400 μ L 20 mM Na-HEPES (pH 7.4), and centrifuged at 850g, for 10 min, at 4°C. The supernatant was then used to determine levels of trypsinogen and chymotrypsinogen by measuring their enzyme activity after maximal activation. Protease zymogen content was expressed as percent of the average activity values from C57BL/6N mice.

Cerulein-induced acute pancreatitis. Cerulein (catalog number C9026, Sigma-Aldrich, St. Louis, MO) was dissolved in normal saline, filter-sterilized, and administered in a supramaximal stimulatory dose of 50 μ g/kg. C57BL/6N and homozygous *T7R123H* mice (11-12 weeks of age) were given 10 hourly injections of cerulein, and the animals were euthanized 1 hour after the last injection. Alternatively, where indicated, mice were treated with 8 hourly injections of cerulein on two consecutive days, and euthanasia was performed 30 min after the last injection on the second day. Control mice were treated with saline injections. Blood and pancreas tissue were harvested for analysis.

Cerulein-induced chronic pancreatitis. C57BL/6N and homozygous *T7R123H* mice (11-12 weeks of age) were treated with 8 hourly injections of cerulein on two consecutive days (50 μ g/kg dose). Control mice were given saline injections. Mice were euthanized 3 days after the last injection (on day 5).

Cerulein-induced intrapancreatic protease activity. C57BL/6N and homozygous *T7R123H* mice (11-12 weeks of age) were treated with a single injection of cerulein (50 μ g/kg dose) and euthanized 30 min later. Alternatively, where indicated, mice were treated with 8 hourly injections of cerulein on two consecutive days, and euthanized 30 min after the last injection on the second day. Control mice were given saline injections. Intrapancreatic trypsin and chymotrypsin activity was measured from freshly prepared pancreas homogenates. Activity was expressed as the rate of substrate cleavage in relative fluorescent units (RFU) per second, normalized to the total protein concentration in mg unit.

Pancreatic water content. Tissue edema was estimated by measuring the water content of the pancreas. A portion of the pancreas (50-100 mg) was weighed (wet weight), desiccated for 72 hours at 65°C, and weighed again (dry weight). Water content was calculated as the difference between the dry and wet weights, expressed as percent of the wet weight.

Plasma amylase measurement. Enzyme activity of amylase was determined from 1 μ L blood plasma, using the 2-chloro-p-nitrophenyl- α -D-maltotrioxide substrate. The rate of substrate cleavage was expressed in mOD/min unit.

Pancreas myeloperoxidase (MPO) content. Measurement of MPO levels in pancreas homogenates was carried out with a commercial ELISA kit (catalog number HK210-01, Hycult Biotech, Plymouth Meeting, PA). MPO concentrations were normalized to the total protein concentration, and expressed in ng MPO/mg protein unit.

Histology. Pancreas tissue was fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned, and stained with hematoxylin-eosin.

Statistics. Experimental data were graphed as individual points with the mean and standard deviation values indicated. The difference of means between 2 groups was analyzed by two-tailed unpaired *t*-test. The difference of means between 4 groups was assessed by one-way ANOVA followed by Tukey's post-hoc analysis. $P < .05$ was considered statistically significant.

What opportunities for training and professional development has the project provided?

Nothing to Report.

How were the results disseminated to communities of interest?

Results were published in the journal *Pancreatology* (Jancsó Z, Morales Granda NC, Demcsák A, Sahin-Tóth M. Mouse model of *PRSSI* p.R122H-related hereditary pancreatitis highlights context-dependent effect of autolysis-site mutation. *Pancreatology* 2023, 23:131-142).

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report.

4. IMPACT**What was the impact on the development of the principal discipline(s) of the project?**

This is the first mouse model of *PRSSI* p.R122H-related hereditary pancreatitis that utilized a knock-in approach rather than a transgenic design. We found that T7R123H mice, harboring the analogous p.R123H mutation, exhibited increased intrapancreatic trypsin activity and more severe acute pancreatitis than C57BL/6N mice, after sustained treatment with cerulein. Furthermore, T7R123H mice developed chronic pancreatitis after the acute pancreatitis episode, but with incomplete penetrance. The findings reinforce the pathogenic model of *PRSSI* p.R122H-associated hereditary pancreatitis, where mutation p.R122H blocks chymotrypsin-dependent trypsinogen degradation, and thereby increases trypsinogen autoactivation, and intrapancreatic trypsin activity.

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:

Publication: Jancsó Z, Morales Granda NC, Demcsák A, Sahin-Tóth M. Mouse model of *PRSSI* p.R122H-related hereditary pancreatitis highlights context-dependent effect of autolysis-site mutation. *Pancreatology* 2023, 23:131-142.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**What individuals have worked on the project?**

Name:	Miklos Sahin-Toth, MD, PhD
Project Role:	PI from 08/31/2021
Researcher Identifier (ORCID ID):	0000-0003-4513-9922
Nearest person month worked:	2
Contribution to Project:	PI, Dr. Sahin-Toth replaced Dr. Jancso when she left UCLA.
Funding Support:	N/A

Name:	Alexandra Demcsak, MD, PhD
Project Role:	post-doctoral scholar
Researcher Identifier (ORCID ID):	0000-0002-5391-6931
Nearest person month worked:	1
Contribution to Project:	Dr. Demcsak performed mouse 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.

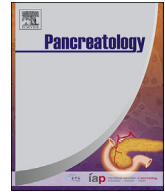
What other organizations were involved as partners?

Nothing to Report.

8. Special Reporting Requirements: N/A.

9. Appendices:

Publication: Jancsó Z, Morales Granda NC, Demcsák A, Sahin-Tóth M. Mouse model of *PRSSI* p.R122H-related hereditary pancreatitis highlights context-dependent effect of autolysis-site mutation. *Pancreatology* 2023, 23:131-142.



Mouse model of *PRSS1* p.R122H-related hereditary pancreatitis highlights context-dependent effect of autolysis-site mutation

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ABSTRACT

Mutation p.R122H in human cationic trypsinogen (*PRSS1*) is the most frequently identified cause of hereditary pancreatitis. The mutation blocks protective degradation of trypsinogen by chymotrypsin C (CTRC), which involves an obligatory trypsin-mediated cleavage at Arg122. Previously, we found that C57BL/6N mice are naturally deficient in CTCRC, and trypsinogen degradation is catalyzed by chymotrypsin B1 (CTRB1). Here, we used biochemical experiments to demonstrate that the cognate p.R123H mutation in mouse cationic trypsinogen (isoform T7) only partially prevented CTRB1-mediated degradation. We generated a novel C57BL/6N mouse strain harboring the p.R123H mutation in the native T7 trypsinogen locus. *T7R123H* mice developed no spontaneous pancreatitis, and severity parameters of cerulein-induced pancreatitis trended only slightly higher than those of C57BL/6N mice. However, when treated with cerulein for 2 days, more edema and higher trypsin activity was seen in the pancreas of *T7R123H* mice compared to C57BL/6N controls. Furthermore, about 40% of *T7R123H* mice progressed to atrophic pancreatitis in 3 days, whereas C57BL/6N animals showed full histological recovery. Taken together, the observations indicate that mutation p.R123H inefficiently blocks chymotrypsin-mediated degradation of mouse cationic trypsinogen, and modestly increases cerulein-induced intrapancreatic trypsin activity and pancreatitis severity. The findings support the notion that the pathogenic effect of the *PRSS1* p.R122H mutation in hereditary pancreatitis is dependent on its ability to defuse chymotrypsin-dependent defenses.

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1. Introduction

Hereditary chronic pancreatitis (CP) is a dominantly inherited inflammatory disorder of the pancreas, typically caused by heterozygous mutations in the serine protease 1 (*PRSS1*) gene that codes for human cationic trypsinogen [1–4]. Mutation p.R122H was the first *PRSS1* variant identified in hereditary pancreatitis families, and it remains the most frequently detected genetic lesion in this disease. To date, 1327 pancreatitis patients carrying a heterozygous p.R122H mutation have been reported in the literature, which accounts for 67% of all missense *PRSS1* mutations found in association with hereditary and idiopathic CP. Mutation p.R122H changes Arg122 to His and thereby eliminates a trypsin-sensitive cleavage site, a so-called autolytic site, on the surface of cationic trypsinogen and trypsin. Trypsin-mediated, autocatalytic cleavage at Arg122

was first observed in preparations of bovine cationic trypsin [5], and later confirmed in the porcine [6], rat [7,8], mouse [9], and human [10–12] orthologs. Whitcomb et al. (1996) hypothesized that the p.R122H mutation exerted its pathogenic effect in hereditary CP by stabilizing human cationic trypsin against autocatalytic degradation [1]. Várallyai et al. (1998) was the first to test this notion by mutating Arg122 in rat anionic trypsin [13]. In their experiments, the p.R122N replacement stabilized trypsin against autocatalytic inactivation, and the authors proposed that cleavage at Arg122 destabilizes trypsin and facilitates extensive proteolysis at multiple cleavage sites, resulting in degradation. Subsequent biochemical studies focused on the role of Arg122 in the degradation of human cationic trypsinogen and trypsin, yielding intriguing results and a plausible mechanism of action for the p.R122H mutation in hereditary CP.

We found that the Arg122-Val123 peptide bond in human cationic trypsinogen and trypsin is thermodynamically stable, and its cleavage leads to an equilibrium mixture of cleaved and intact forms, due to trypsin-mediated re-synthesis of the peptide bond

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[12,14]. The rate of cleavage at Arg122 is much more rapid in trypsinogen than in trypsin [14], suggesting that pancreatic defense mechanisms against high trypsin activity likely work through trypsinogen degradation rather than elimination of active trypsin. Due to the unusual stability of the Arg122 site, trypsin-mediated degradation of human cationic trypsinogen is inefficient, and requires a chymotrypsin C (CTRC)-mediated cleavage at Leu81 [14–17]. Mutation p.R122H blocks CTCRC-mediated trypsinogen degradation primarily by eliminating autolytic cleavage at Arg122 but also by reducing the rate of CTCRC-mediated cleavage at Leu81. When autoactivation of wild-type and p.R122H mutant cationic trypsinogen was compared, the mutant autoactivated slightly (1.5-fold) faster than wild-type, reaching similar final trypsin activity values [16,18]. When the same experiment was performed in the presence of CTCRC, the mutant trypsinogen autoactivated at an increased rate and to much higher trypsin levels than the wild-type did [16]. These observations support a disease model, where the p.R122H mutation causes high intrapancreatic trypsin activity and pancreatitis by blocking protective, chymotrypsin-dependent degradation of human cationic trypsinogen.

Previously, we demonstrated that C57BL/6N mice are naturally deficient in CTCRC [19], and genetic deletion of the major chymotrypsinogen isoform CTRB1 resulted in higher cerulein-induced intrapancreatic trypsin activity, and more severe pancreatitis [20,21]. The results indicated that CTRB1-dependent trypsinogen degradation is protective in murine pancreatitis. Consistently with this interpretation, biochemical experiments showed that CTRB1 cleaved mouse anionic and cationic trypsinogens, and thereby curbed their activation [9,20,21]. In the present study, we knocked in the p.R123H mutation, which corresponds to PRSS1 p.R122H, to the native mouse cationic trypsinogen (isoform T7) locus. We speculated that mutation p.R123H would inhibit CTRB1-mediated degradation, increase intrapancreatic trypsin activity, and cause spontaneous pancreatitis or worsen the severity of experimentally-induced pancreatitis. The results presented below suggest a more nuanced picture, highlighting the context-dependent effect of mutation p.R122H in hereditary CP.

2. Methods

Accession numbers and nomenclature. NC_000072.6, *Mus Musculus* strain C57BL/6J chromosome 6, GRCm38.p4 C57BL/6J; NM_023333.4, *Mus musculus* RIKEN cDNA 2210010C04 gene (2210010C04Rik), mRNA; mouse cationic trypsinogen (isoform T7). Amino-acid residues in trypsinogen were numbered starting from the initiator methionine of the primary translation product. Note that amino-acid numbering of mouse T7 trypsinogen is shifted by 1 relative to human trypsinogens due to an extra Asp residue in the activation peptide. Thus, mutation p.R122H in human PRSS1 corresponds to p.R123H in mouse T7 trypsinogen.

Expression and purification of mouse cationic trypsinogen (isoform T7). Wild-type and p.R123H mutant T7 trypsinogen were expressed as intein fusion proteins, as reported previously [22,23]. Inclusion bodies were isolated, trypsinogen was refolded *in vitro*, and purified by ecotin affinity chromatography using published protocols [22]. The concentration of trypsinogen solutions was calculated from the ultraviolet absorbance at 280 nm using the extinction coefficient $39,140 \text{ M}^{-1} \text{ cm}^{-1}$.

Expression, purification, and activation of mouse chymotrypsinogen B1 (CTRB1). Construction of the pcDNA3.1(–) mouse CTRB1 10His plasmid containing a C-terminal 10His affinity tag was reported recently [21]. Mouse CTRB1 was expressed in HEK 293T cells with transient transfection, and purified from the conditioned medium by nickel-affinity chromatography according to our published protocol [24]. The eluted CTRB1 was dialyzed against 15 mM

Na-HEPES (pH 8.0), 100 mM NaCl, and activated with immobilized TPCK-treated trypsin (catalog number 20230, Thermo Scientific, Waltham, MA). The agarose beads were removed by centrifugation, and the active CTRB1 concentration was determined by titration with ecotin [25].

Measurement of trypsinogen autoactivation. Wild-type and mutant trypsinogens (2 μM) were incubated at 37 °C with 10 nM active T7 trypsin in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂, and 0.05% Tween 20 (final concentrations). Where indicated, autoactivation of trypsinogen was measured in the presence of 25, 100, and 400 nM mouse CTRB1 (final concentrations). At given times, trypsin activity was measured from 2 μL aliquots after the addition of 48 μL assay buffer (0.1 M Tris-HCl (pH 8.0), 1 mM CaCl₂, 0.05% Tween 20), and 150 μL of 200 μM N-CBZ-Gly-Pro-Arg-*p*-nitroanilide substrate (in assay buffer). Alternatively, incubations were performed in the absence of Tween 20, and 100 μL aliquots were precipitated with 10% trichloroacetic acid (final concentration). The precipitate was collected by centrifugation (10 min, 13,200 rpm, 4 °C), and dissolved in 25 μL 2 × Laemmli Sample Buffer (catalog number 1610737, Bio-Rad, Hercules, CA) supplemented with 100 mM dithiothreitol and 150 mM NaOH. The samples were heat-denatured at 95 °C for 5 min, electrophoresed on 15% SDS-polyacrylamide gels, and stained with Brilliant Blue R-250 (Coomassie Blue).

Digestion of trypsinogen with chymotrypsinogen B1 (CTRB1). Wild-type and mutant trypsinogens (2 μM) were incubated at 37 °C with 100 nM mouse CTRB1 in 0.1 M Tris-HCl (pH 8.0) (final concentrations). At the indicated times, 100 μL aliquots were precipitated and analyzed by SDS-PAGE, as described in the previous paragraph. Gels were digitized on a ChemiDoc Touch Imaging System (Bio-Rad) as tif files, and densitometric quantitation of the intensity of the trypsinogen bands was performed with the ImageJ software.

Animal studies approval. Animal experiments were performed at the University of California, Los Angeles with the approval and oversight of the Animal Research Committee, including protocol review and post-approval monitoring. Some of the initial studies were carried out at Boston University with the approval and oversight of the Institutional Animal Care and Use Committee. The animal care programs at these institutions are 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. The University of California Los Angeles and Boston University have approved Animal Welfare Assurance statements (A3196–01 and A3316–01, respectively) on file with the US Public Health Service, National Institutes of Health, Office of Laboratory Animal Welfare. Both institutions are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Generation of the T7R123H mouse strain. Model generation was performed in the C57BL/6N genetic background (Cyagen, Santa Clara, CA), and followed the general strategy and protocols previously reported for the T7D23A, T7K24R, T7G199R, T7D23del, and T7D22N,K24R mouse strains [23,26–29]. The final T7R123H knock-in allele contained the c.368_369GA>AC (p.R123H) mutation in exon 3 of the mouse cationic trypsinogen gene and a 113 nt residual “scar” sequence in intron 2, between positions c.204–417 and c.204–416. T7R123H mice were bred to homozygosity, and were maintained in this 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 the experiments is shown in the figures. Both male and female mice were studied.

Genotyping. To genotype *T7R123H* mice, we used the following primers: forward primer, 5'-CTG TCC TAT AAC ATT GCT CTG CTT -3', reverse primer, 5'-AGA CAC AAG ACA CCT AGT ACC AG -3'. The amplicon sizes for the wild-type and mutant alleles were 681 bp and 794 bp, respectively. The mutant allele yielded a longer product due to the presence of the residual sequence in intron 2.

Western blotting. Pancreas tissue (30 mg) was homogenized in 300 μ L phosphate-buffered saline (pH 7.4) containing Halt Protease and Phosphatase Inhibitor Cocktail (from 100 \times stock, catalog number 78440, Thermo Scientific) and 20 μ g total protein of the cleared lysate was loaded per well. Mouse T7 trypsinogen was detected using a rabbit polyclonal antibody (1:10,000 dilution) raised against a peptide sequence corresponding to amino-acids 114–126 of mouse T7 pre-trypsinogen [23]. As loading control, mouse ERK1/2 was measured using a rabbit monoclonal antibody (catalog number 4695, Cell Signaling Technology, Danvers, MA) at a dilution of 1:1000. The horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody was used at a dilution of 1:10,000 (catalog number 31460, Thermo Scientific).

Measurement of pancreatic protease zymogen content. Pancreas tissue (40 mg) was homogenized in 400 μ L 20 mM Na-HEPES (pH 7.4), and centrifuged at 850 g, for 10 min, at 4 $^{\circ}$ C. The supernatant was then used to determine levels of trypsinogen and chymotrypsinogen by measuring their enzyme activity after maximal activation, as described previously [19]. Protease zymogen content was expressed as percent of the average activity values from C57BL/6N mice. To compare the trypsinogen and chymotrypsinogen levels in the homogenate, we converted the activity values to enzyme concentrations using purified, recombinant mouse CTRB1 and mouse cationic trypsin (isoform T7) as reference standards. The concentration of the recombinant proteases was determined by active-site titration with ecotin.

Cerulein-induced acute pancreatitis. Cerulein (catalog number C9026, Sigma-Aldrich, St. Louis, MO) was dissolved in normal saline, filter-sterilized, and administered in a supramaximal stimulatory dose of 50 μ g/kg. C57BL/6N and homozygous *T7R123H* mice (11–12 weeks of age) were given 10 hourly injections of cerulein, and the animals were euthanized 1 h after the last injection. Alternatively, where indicated, mice were treated with 8 hourly injections of cerulein on two consecutive days, and euthanasia was performed 30 min after the last injection on the second day. Control mice were treated with saline injections. Blood and pancreas tissue were harvested for analysis.

Cerulein-induced chronic pancreatitis. The Jensen protocol [30] was used to induce progressive, atrophic CP in *T7R123H* mice. Briefly, C57BL/6N and homozygous *T7R123H* mice (11–12 weeks of age) were treated with 8 hourly injections of cerulein on two consecutive days (50 μ g/kg dose). Control mice were given saline injections. Mice were euthanized 3 days after the last injection (on day 5).

Cerulein-induced intrapancreatic protease activity. C57BL/6N and homozygous *T7R123H* mice (11–12 weeks of age) were treated with a single injection of cerulein (50 μ g/kg dose) and euthanized 30 min later. Alternatively, where indicated, mice were treated with 8 hourly injections of cerulein on two consecutive days, and euthanized 30 min after the last injection on the second day. Control mice were given saline injections. Intrapancreatic trypsin and chymotrypsin activity was measured from freshly prepared pancreas homogenates using a recently published protocol [31]. Activity was expressed as the rate of substrate cleavage in relative fluorescent units (RFU) per second, normalized to the total protein concentration in μ g unit.

Pancreatic water content. Tissue edema was estimated by measuring the water content of the pancreas. A portion of the pancreas (50–100 mg) was weighed (wet weight), desiccated for

72 h at 65 $^{\circ}$ C, and weighed again (dry weight). Water content was calculated as the difference between the dry and wet weights, expressed as percent of the wet weight.

Plasma amylase measurement. Enzyme activity of amylase was determined from 1 μ L blood plasma, using the 2-chloro-*p*-nitrophenyl- α -D-maltotriose substrate, as reported previously [21]. The rate of substrate cleavage was expressed in mOD/min unit.

Pancreas myeloperoxidase (MPO) content. Measurement of MPO levels in pancreas homogenates was carried out as reported previously [16,26], using a commercial ELISA kit (catalog number HK210-01, Hycult Biotech, Plymouth Meeting, PA). MPO concentrations were normalized to the total protein concentration, and expressed in ng MPO/mg protein unit.

Histology. Pancreas tissue was fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned, and stained with hematoxylin-eosin. Histological assessment (scoring) of cerulein-induced acute pancreatitis (AP) for edema, inflammatory cell infiltration, and acinar cell necrosis was performed as described previously [26]. The extent of acinar cell atrophy in the cerulein-induced CP model was characterized histologically by estimating the number of intact acini as percent of the total tissue area.

Statistics. Experimental data were graphed as individual points with the mean and standard deviation values indicated. The difference of means between 2 groups was analyzed by two-tailed unpaired *t*-test. The difference of means between 4 groups was assessed by one-way ANOVA followed by Tukey's post-hoc analysis. $P < .05$ was considered statistically significant.

3. Results

Effect of mutation p.R123H on mouse cationic trypsinogen.

We produced recombinantly and purified wild-type and p.R123H mutant mouse cationic trypsinogen (isoform T7). Mutation p.R123H in T7 trypsinogen corresponds to p.R122H in human cationic trypsinogen. Autoactivation of wild-type and mutant trypsinogens was nearly indistinguishable at pH 8.0, in 1 mM calcium (Fig. 1A). SDS-PAGE analysis with Coomassie Blue staining confirmed that conversion of the trypsinogen band to trypsin proceeded at the same rate, resulting in similar final trypsin levels (Fig. 1B). N-terminal sequencing of autolytic cleavage fragments identified Arg123 and Lys194 as the sites of autolytic digestion in wild-type T7 trypsinogen, whereas cleavage at Arg123 was absent in the p.R123H mutant (Fig. 1B).

When autoactivation was measured in the presence of mouse CTRB1, final trypsin levels were reduced as a function of the CTRB1 concentration, due to trypsinogen degradation (Fig. 2A). Under the same conditions, mutant p.R123H autoactivated to higher final trypsin levels than wild-type T7 (Fig. 2B). However, degradation of p.R123H trypsinogen was still highly significant. When final trypsin levels generated through autoactivation were plotted as a function of the CTRB1 concentration, the protective effect of mutation p.R123H seemed to diminish at the highest CTRB1 concentration tested (Fig. 2C), which corresponds to a 5:1 trypsinogen-to-chymotrypsin ratio. We measured the trypsinogen and chymotrypsinogen content in pancreas homogenates, as described in *Methods*, and found that their ratio was approximately 1.8. Thus, the concentration of chymotrypsinogen is comparable to or slightly exceeds that of T7 trypsinogen, which constitutes about half of the pancreatic trypsinogen content [9]. These observations indicate that under physiological conditions mutation p.R123H would afford little protection against CTRB1-mediated degradation of T7 trypsinogen.

The CTRB1-mediated cleavages of wild-type and mutant T7 trypsinogens were analyzed by SDS-PAGE and Coomassie Blue staining. To optimize the cleavage reaction, this experiment was

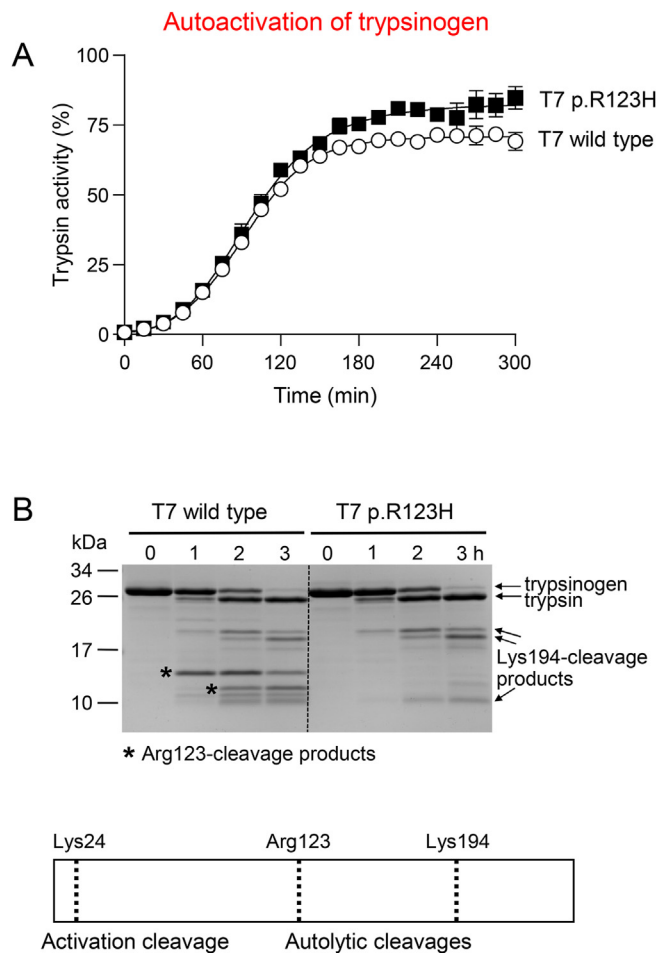


Fig. 1. Effect of mutation p.R123H on the autoactivation of mouse cationic trypsinogen (T7). **A.** Autoactivation of wild-type and p.R123H mutant T7 trypsinogens (2 μ M) was measured at pH 8.0 in 1 mM calcium, as described in *Methods*. Trypsin activity was expressed as percent of potentially attainable activity. Data points represent mean \pm standard error (n = 2). **B.** Autoactivation of wild-type and p.R123H mutant T7 trypsinogen was analyzed by SDS-PAGE and Coomassie Blue staining. A representative gel (n = 2) is shown. Bands generated by trypsin-mediated cleavage (autolysis) of trypsinogen are indicated. Cartoon denotes position of trypsin-sensitive cleavage sites in T7 trypsinogen.

performed in the absence of added calcium. The banding pattern of CTRB1-digested wild-type and mutant trypsinogens was identical (Fig. 3A). N-terminal sequencing identified the primary CTRB1 cleavage site at Leu149, and secondary cleavages at Tyr29 and Leu159. Densitometric evaluation of the cleavage reaction confirmed the comparable degradation kinetics (Fig. 3B). The results indicate that CTRB1 cleavages of T7 trypsinogen are not directly affected by mutation p.R123H. Instead, the mutation protects against CTRB1-mediated degradation by eliminating the autolytic cleavage at the Arg123 site, and thereby delaying the proteolysis-induced conformational disintegration of T7 trypsinogen.

Generation of T7R123H mice and pancreatic expression of trypsinogen. To test the effect of p.R123H *in vivo*, we created a novel knock-in mouse strain carrying the mutation in the native T7 trypsinogen locus of C57BL/6N mice (Fig. 4A). Genetic modification was achieved using homologous recombination, following previously employed protocols [23,26–29]. Homozygous T7R123H mice had no obvious phenotype; they were indistinguishable from wild-type C57BL/6N mice. Macroscopic and microscopic morphology of the pancreas from 1-year-old T7R123H mice were normal, with no signs

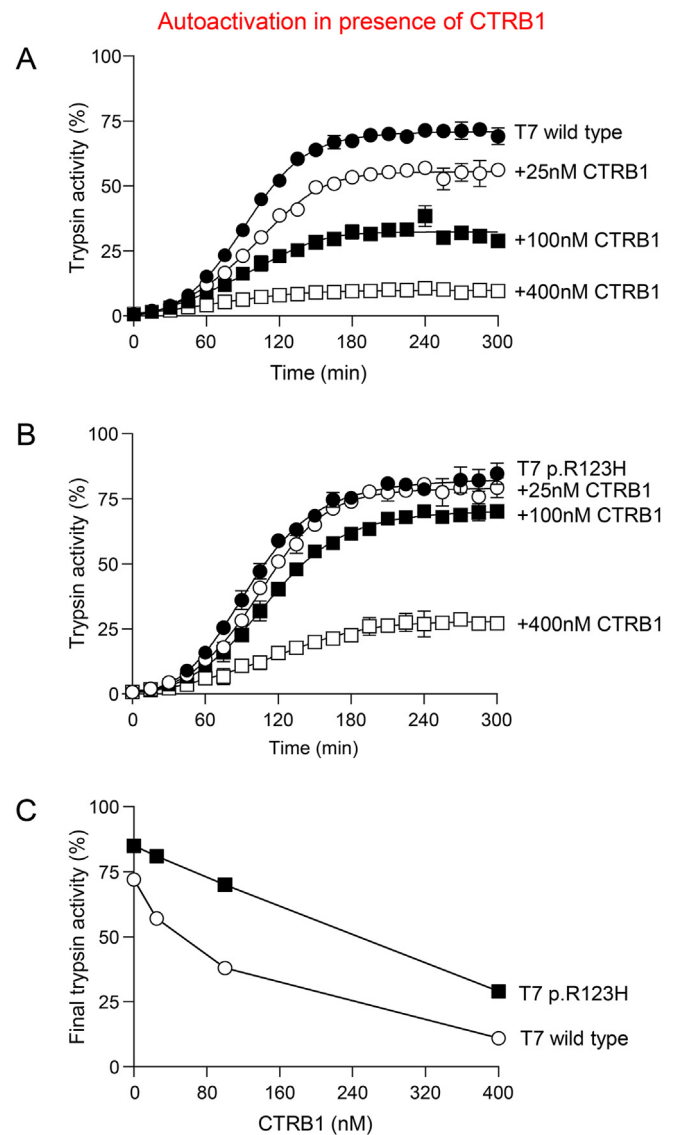


Fig. 2. Effect of mutation p.R123H on the autoactivation of mouse cationic trypsinogen (T7) in the presence of mouse chymotrypsin B1 (CTRB1). Autoactivation was measured in the presence of the indicated CTRB1 concentrations, as described in *Methods*. Trypsin activity was expressed as percent of potentially attainable activity. Data points represent mean \pm standard error (n = 2). **A.** Wild-type T7 trypsinogen. **B.** Mutant p.R123H. **C.** Final trypsin activity as a function of the CTRB1 concentration present in the autoactivation reaction. The plateau trypsin activity values from panels A and B were plotted.

of spontaneous pathology. Western blot analysis of pancreas homogenates indicated comparable expression of T7 trypsinogen in C57BL/6N and T7R123H mice (Fig. 4B). Measurement of total trypsinogen and chymotrypsinogen content in the pancreas from C57BL/6N and T7R123H mice revealed no differences either (Fig. 4C).

Cerulein-induced intrapancreatic protease activity in T7R123H mice. First, we measured trypsin (Fig. 5A) and chymotrypsin (Fig. 5B) activity from freshly prepared pancreas homogenates 30 min after a single cerulein injection (50 μ g/kg dose). We typically use this early time point to evaluate intrapancreatic protease activation because the acinar tissue is still relatively intact, unaffected by inflammation. Relative to saline-injected mice, significantly increased trypsin and chymotrypsin activity was observed in the pancreas of cerulein-treated mice, however, no difference was apparent between the C57BL/6N and T7R123H strains.

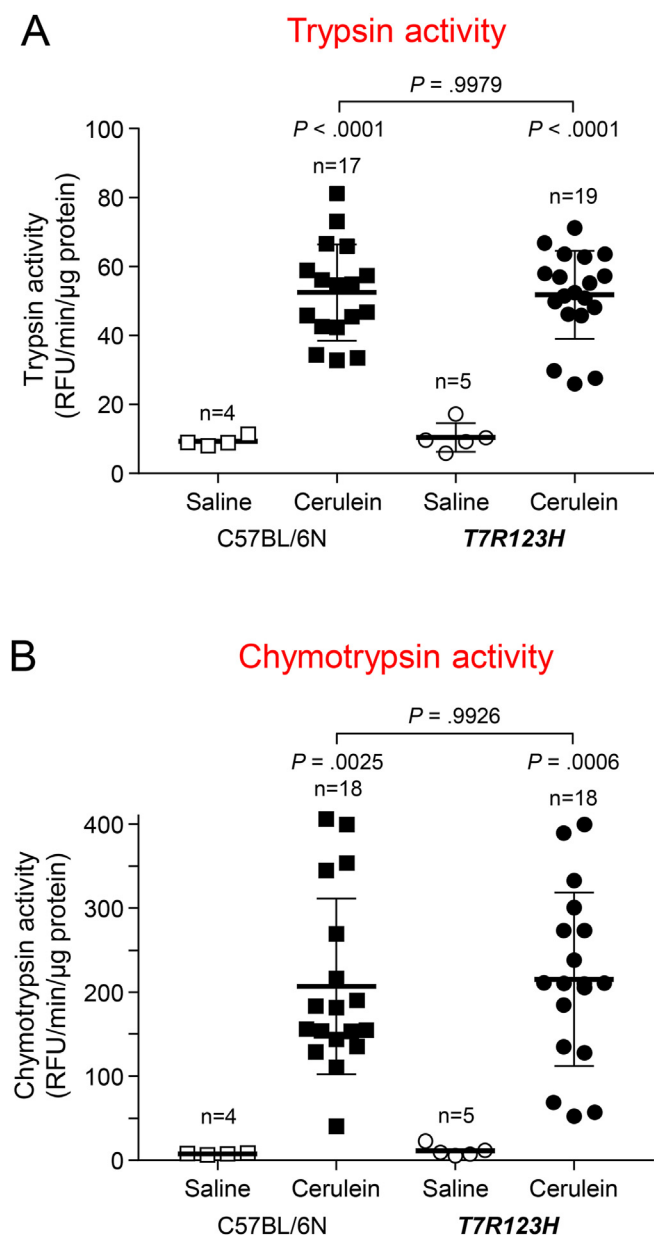


Fig. 5. Cerulein-induced intrapancreatic protease activation in *T7R123H* mice. C57BL/6N and *T7R123H* mice were given a single saline or cerulein injection, and the mice were euthanized 30 min later. Trypsin and chymotrypsin activity were measured from freshly prepared pancreas homogenates, as described in *Methods*. **A**, Trypsin activity. **B**, Chymotrypsin activity. Individual values with the mean and standard deviation were plotted. The difference of means was analyzed by one-way ANOVA followed by Tukey's post-hoc analysis.

eosin staining (Fig. 7A) also showed slightly stronger pancreatitis scores in *T7R123H* versus C57BL/6N mice for edema (Fig. 7B), inflammatory cells (Fig. 7C), and acinar cell necrosis (Fig. 7D). The difference in inflammatory cell infiltration was statistically significant. Taken together, the results indicate that *T7R123H* mice develop slightly more severe cerulein-induced AP than the C57BL/6N parent strain.

To evaluate the effect of more sustained overstimulation with cerulein, we treated mice with 8 hourly cerulein injections on 2 consecutive days, and euthanized the animals 30 min after the last injection. Remarkably, the pancreas of cerulein-treated *T7R123H* mice was visibly more edematous (not shown) than those of C57BL/6N

mice, and this significant difference was also evident when pancreas weights were compared (Fig. 8A). Furthermore, intrapancreatic trypsin activity of cerulein-treated *T7R123H* mice was significantly higher than those of cerulein-treated C57BL/6N controls (Fig. 8B), while chymotrypsin activity showed a similar trend without statistical significance (Fig. 8C).

Cerulein-induced chronic pancreatitis (CP) in *T7R123H* mice.

We and others previously found that trypsinogen mutant mice develop progressive pancreatitis after a cerulein-induced acute episode while the pancreas of C57BL/6N mice recovers quickly [29,32,33]. Pancreatitis progression in mutant mice is characterized by extensive acinar atrophy, fibrosis, and inflammatory cell infiltration, all features of CP. Histological recovery is delayed, and often incomplete. To test whether *T7R123H* mice would develop CP after an acute episode, we treated mice with 8 hourly injections of cerulein on 2 consecutive days and euthanized the animals 3 days after the last injection. This protocol was described by Jensen et al. (2005) to study pancreas regeneration [30]. Histological analysis of pancreas sections showed essentially normal acinar tissue in C57BL/6N mice, indicating complete recovery from the cerulein-induced AP (Fig. 9A). In contrast, pancreas sections from some *T7R123H* mice revealed areas of CP. Scoring of multiple sections for intact acini indicated no significant dropout in C57BL/6N mice ($n = 10$), whereas 6 of 15 (40%) *T7R123H* mice showed measurable acinar atrophy, with 2 mice exhibiting a complete response (Fig. 9B). The results indicate that *T7R123H* mice are somewhat prone to develop progressive CP after cerulein-induced AP; however, the penetrance is relatively low, not nearly as robust as in the previously published trypsinogen mutant strains [23,26,29,33].

4. Discussion

In the present study, we describe the novel *T7R123H* knock-in mouse model harboring the p.R123H mutation (p.R122H in human numbering) in the mouse cationic trypsinogen (isoform T7) locus. When treated with cerulein (10 hourly injections), *T7R123H* mice exhibited unchanged intrapancreatic trypsin activity and slightly increased severity of AP, relative to C57BL/6N controls. However, when cerulein-treatment was extended (8 hourly injections on 2 consecutive days), *T7R123H* mice showed higher intrapancreatic trypsin activity and more pancreatic edema than C57BL/6N animals. Furthermore, after the 2-day acute episode, cerulein-treated *T7R123H* mice developed progressive, CP-like disease with incomplete penetrance, while C57BL/6N mice fully recovered. Previously, we demonstrated that mice deficient in CTRB1 (*Ctrb1-del* strain) showed significantly increased severity of cerulein-induced AP, indicating that CTRB1-mediated trypsinogen degradation is protective in the murine secretagogue-induced disease model [20,21]. Furthermore, when the *Ctrb1-del* allele was crossed with the *T7K24R* mutant trypsinogen allele, mice with the homozygous compound genotype developed severe, early-onset pancreatitis [34]. Since neither the *Ctrb1-del* nor the *T7K24R* allele alone was capable of inducing spontaneous pancreatitis, the striking phenotype of the *Ctrb1-del* × *T7K24R* mice provided further evidence that impairment of the CTRB1-dependent trypsinogen degradation promotes pancreatitis onset and increases severity in mice. Therefore, we expected to see a similar effect in *T7R123H* mice, assuming the p.R123H mutation would protect T7 trypsinogen against CTRB1. Surprisingly, this was not the case. Biochemical analysis revealed that mutation p.R123H inefficiently protected mouse cationic trypsinogen against CTRB1-mediated degradation. This stands in contrast to the robust protective effect of the p.R122H mutation against CTCRC-mediated degradation of human cationic trypsinogen. We believe this biochemical difference explains the relatively modest phenotypic impact of the

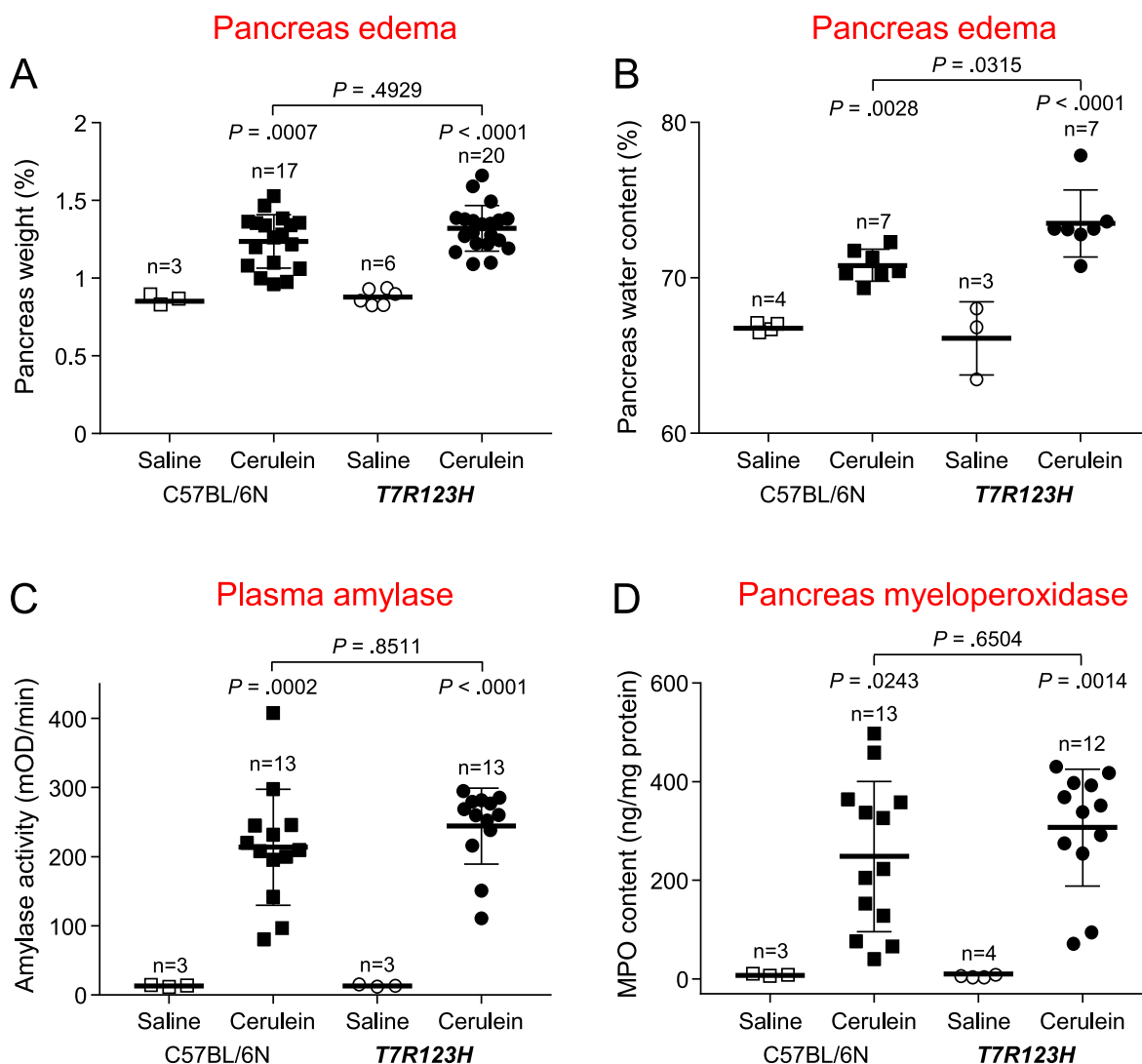


Fig. 6. Cerulein-induced acute pancreatitis in *T7R123H* mice. *C57BL/6N* and *T7R123H* mice were given 10 hourly injections of saline or cerulein, as described in *Methods*. Mice were euthanized 1 h after the last injection. **A**, Pancreas weight normalized to body weight. **B**, Pancreatic water content. **C**, Plasma amylase activity. **D**, Pancreatic myeloperoxidase (MPO) content. Individual data points with mean and standard deviation are shown. The difference of means was analyzed by one-way ANOVA followed by Tukey's post-hoc analysis.

mutation in *T7R123H* mice versus the strong disease-causing effect in hereditary CP.

Previous attempts to model *PRSS1* p.R122H-related hereditary CP in mice utilized various transgenic approaches (Table 1). First, Archer et al. (2006) [35] used a transgenic construct in which the coding DNA for mouse anionic trypsinogen (isoform T8, see Ref. [9]) with the p.R122H mutation was placed under the control of the “short” rat elastase 1 (*Cela1*) promoter [36]. Transgenic mice developed scattered fibro-inflammatory lesions with incomplete penetrance, and exhibited more severe CP than *C57BL/6* controls after 2 weeks of cerulein treatment. The experiments did not include a wild-type transgenic control, and total trypsinogen content of the pancreas of transgenic mice was not measured. Therefore, it remains unclear whether introduction of the extra trypsinogen gene, the p.R122H mutation, or a combination of both were responsible for the observed phenotype.

In the same year, Selig et al. (2006) generated transgenic mice containing the coding DNA for human *PRSS1* with the p.R122H mutation, under the control of the short rat *Cela1* promoter [37]. The authors noted low levels of transgene expression, although no quantitative analysis of pancreatic trypsinogens was performed.

Transgenic mice developed no spontaneous pancreatitis, but showed slightly increased severity of cerulein-induced disease. No wild-type transgenic control was included.

Building on the shortcomings of these pioneering studies, Athwal et al. (2014) generated three transgenic lines harboring the coding DNA for wild-type *PRSS1*, and mutants p.N29I and p.R122H, under the control of the short rat *Cela1* promoter [38]. Approximately 10% of transgenic mice developed spontaneous acinar vacuolization and fibro-inflammatory alterations in the pancreas at or above 9 months of age. Upon treatment with cerulein, transgenic mice exhibited more severe pancreatitis than controls. Strikingly, however, no difference in phenotype was seen among the three lines, indicating that murine expression of human *PRSS1* is sufficient to induce or aggravate pancreatitis. As was the case in prior studies, measurement of pancreatic trypsinogen content was not performed.

More recent mouse models used bacterial artificial chromosome (BAC)-transgene technology to overcome the expression problems seen with the short rat *Cela1* promoter. Huang et al. (2020) described an overly sophisticated transgene design, in which the *PRSS1* coding DNA with or without the p.R122H mutation was

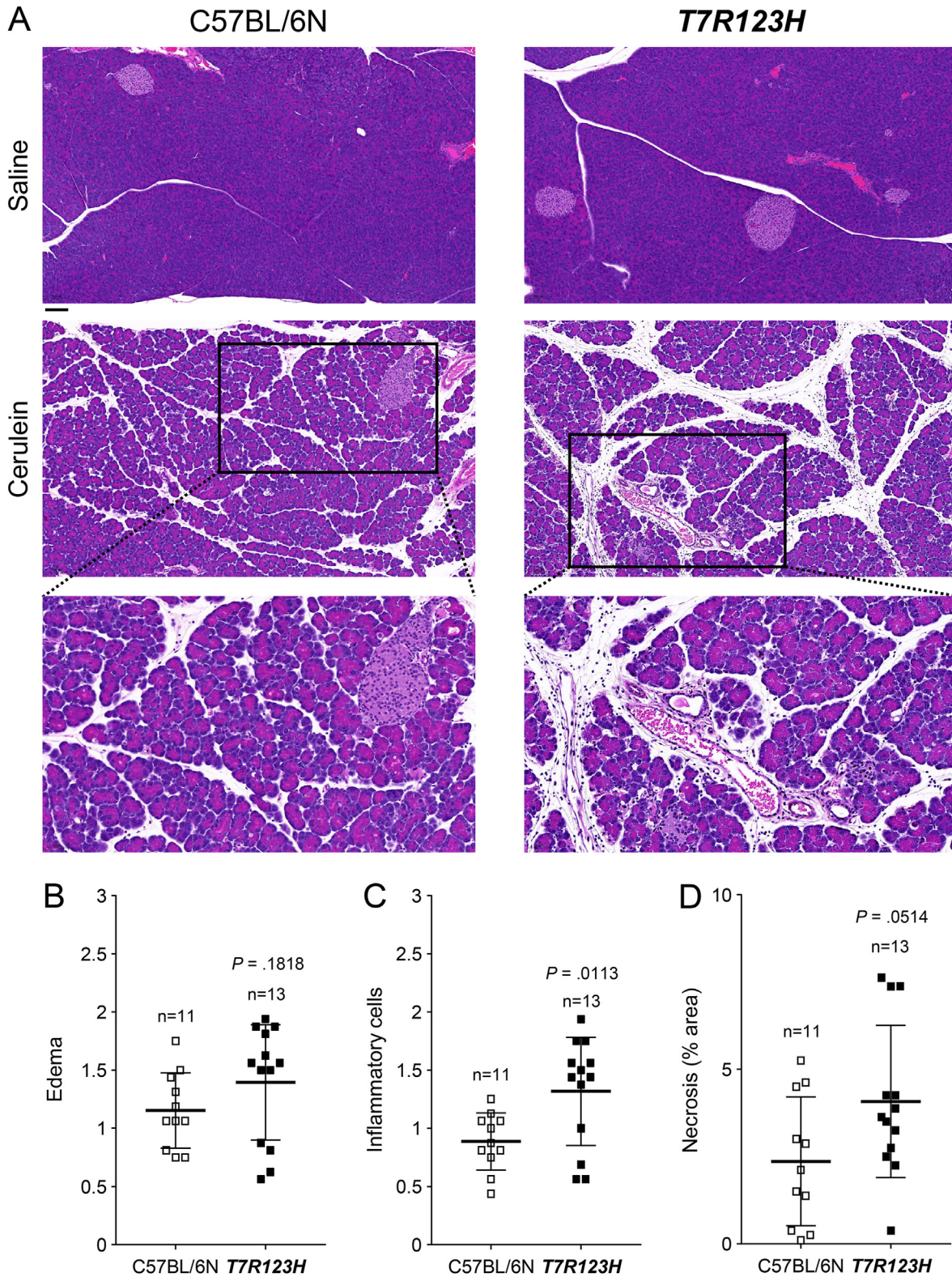


Fig. 7. Histology of cerulein-induced acute pancreatitis in *T7R123H* mice. *C57BL/6N* and *T7R123H* mice were given 10 hourly injections of saline or cerulein, as described in *Methods*. Mice were euthanized 1 h after the last injection. **A**, Representative hematoxylin-eosin-stained pancreas sections. The scale bar corresponds to 100 μm (top four panels) and 50 μm (bottom two enlargements). **B,C,D**, Histology scoring of pancreas sections for edema (B), inflammatory cell infiltration (C), and acinar cell necrosis (D). Individual data points were graphed with the means and standard deviation indicated. The difference of means between two groups was analyzed by two-tailed unpaired *t*-test.

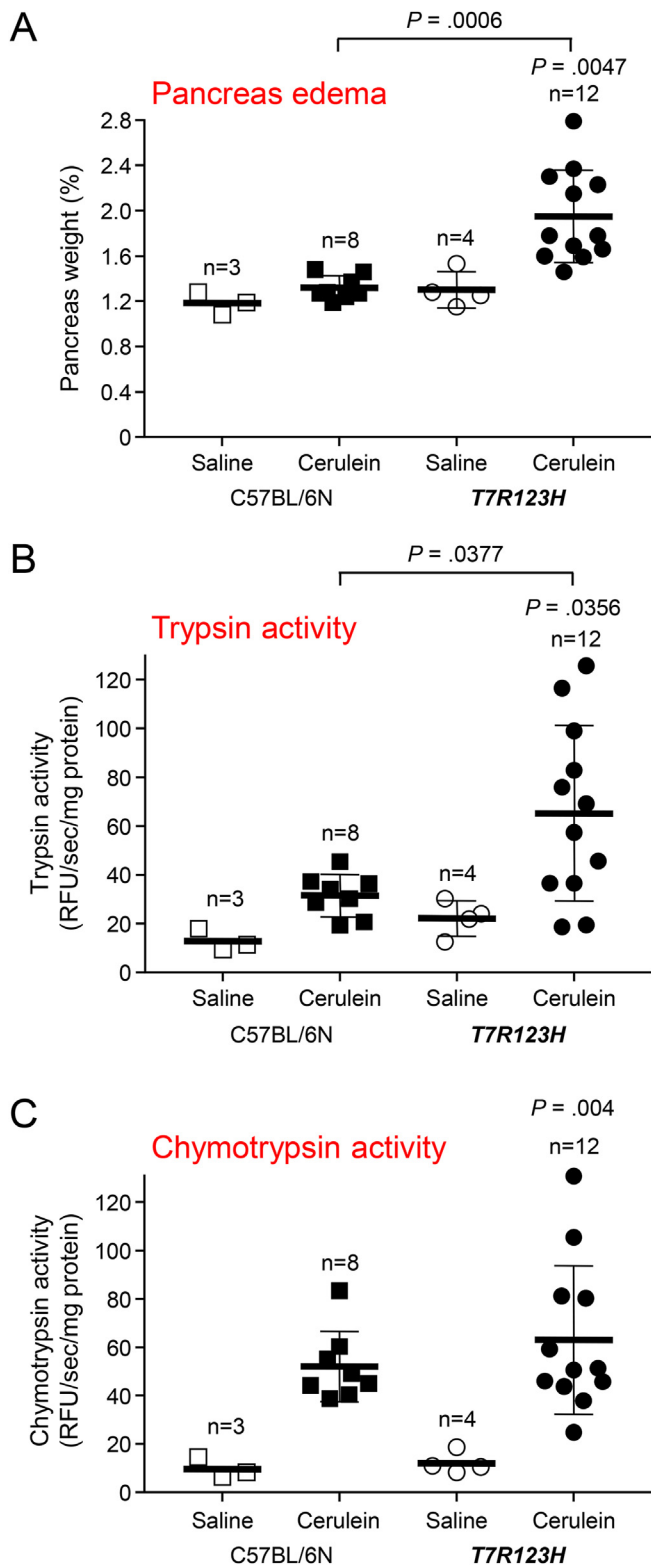


Fig. 8. Pancreas edema and intrapancreatic protease activity after sustained stimulation with cerulein. C57BL/6N and TTR123H mice were given 8 hourly injections of saline or cerulein on 2 consecutive days, as described in *Methods*. Mice were euthanized 30 min after the last injection. Trypsin and chymotrypsin activity were measured from freshly prepared pancreas homogenates, as described in *Methods*. **A**, Pancreas weight normalized to body weight. **B**, Trypsin activity. **C**, Chymotrypsin activity. Individual values with the mean and standard deviation were plotted. The difference of means was analyzed by one-way ANOVA followed by Tukey's post-hoc analysis.

placed downstream of a loxP-GFP-STOP-loxP sequence [39]. This entire cassette was then inserted downstream of the full-length mouse *Cela1* gene within a BAC clone. To remove the floxed STOP cassette, PRSS1-transgenic mice were bred with BAC-*Cela1*-Cre-ERT2 mice [40], and trypsinogen expression was induced by treatment with tamoxifen for 5 days. Transgenic mice with mutation p.R122H developed slightly more focal fibro-inflammatory lesions, and showed more severe cerulein-induced pancreatitis than mice with wild-type PRSS1. Similarly, treatment with lipopolysaccharide, ethanol feeding, or a high-fat diet resulted in more prominent pathological changes in the pancreas of p.R122H mutant mice. Pancreatic trypsinogen content was not measured. Western blot analysis suggested comparable expression of wild-type and p.R122H mutant transgenes whereas immunohistochemistry indicated higher PRSS1 protein expression in the pancreas of p.R122H mutant mice.

Gui et al. (2020) generated transgenic mice using a human BAC clone harboring the genomic sequence for PRSS1 [32]. Mice carrying wild-type PRSS1, p.R122H mutant PRSS1, and a catalytically inactive PRSS1 with the p.R122H,p.S200T double mutation were created. AP induced by 8 hourly injections of cerulein was more severe in p.R122H transgenic mice relative to C57BL/6N controls, and mutant mice developed progressive, CP-like disease after an acute episode whereas C57BL/6N mice recovered rapidly. When the effect of a single cerulein injection was compared in wild-type and p.R122H mutant transgenic mice, mutant mice developed pancreatitis at lower cerulein doses. As expected, the p.R122H,p.S200T double-mutant transgenic mice did not show increased disease severity or sensitivity, indicating that the effect of the p.R122H mutation was strictly dependent on trypsin activity. Unfortunately, in their experiments, the authors did not compare wild-type PRSS1 transgenic mice with C57BL/6N mice, which makes it difficult to separate the effect of the p.R122H mutation from the gene-dosage effect of the extra trypsinogen allele. Total trypsinogen content in the pancreas of the studied strains was not determined either, although Western blot analysis suggested comparable transgene expression in the pancreas of mice with wild-type PRSS1 and mutant transgenes. Wang et al. (2022) published a follow-up study in which the PRSS1 and PRSS1-p.R122H transgenic mice also carried the PRSS2 gene on the same allele [41]. A single low dose of cerulein induced AP in the PRSS1-p.R122H-PRSS2 mice but not in PRSS1-PRSS2 mice. When bred to homozygosity, the increased trypsinogen gene dosage resulted in spontaneous, progressive pancreatitis in PRSS1-p.R122H-PRSS2 mice but not in PRSS1-PRSS2 mice. Similarly, mice with a PRSS2 allele paired with a PRSS1-p.R122H-PRSS2 allele showed spontaneous disease whereas mice harboring the PRSS2 and PRSS1-PRSS2 alleles did not develop pathology. Western blot analysis showed comparable PRSS1 and PRSS2 protein expression in PRSS1-p.R122H-PRSS2 and PRSS1-PRSS2 transgenic mice, indicating that mutation p.R122H was responsible for the observed phenotypic effects.

Taken together, the six studies published to date demonstrate that the presence of the p.R122H mutation increases sensitivity to pancreatitis and is associated with more severe disease. This effect is much more readily observed in later studies utilizing BAC technology with higher trypsinogen expression levels. A general limitation of the published experiments is the lack of information on total pancreatic trypsinogen levels in the various transgenic mice. Some attempts were made to quantify transgene expression, however, these were hardly rigorous. Since transgene incorporation is random, and copy numbers may be variable, it is not readily apparent why trypsinogen levels would be identical among the transgenic lines. Nevertheless, the consistent effect of the p.R122H mutation across all published accounts argues that the observed phenotypes were primarily due to the presence of the mutation and

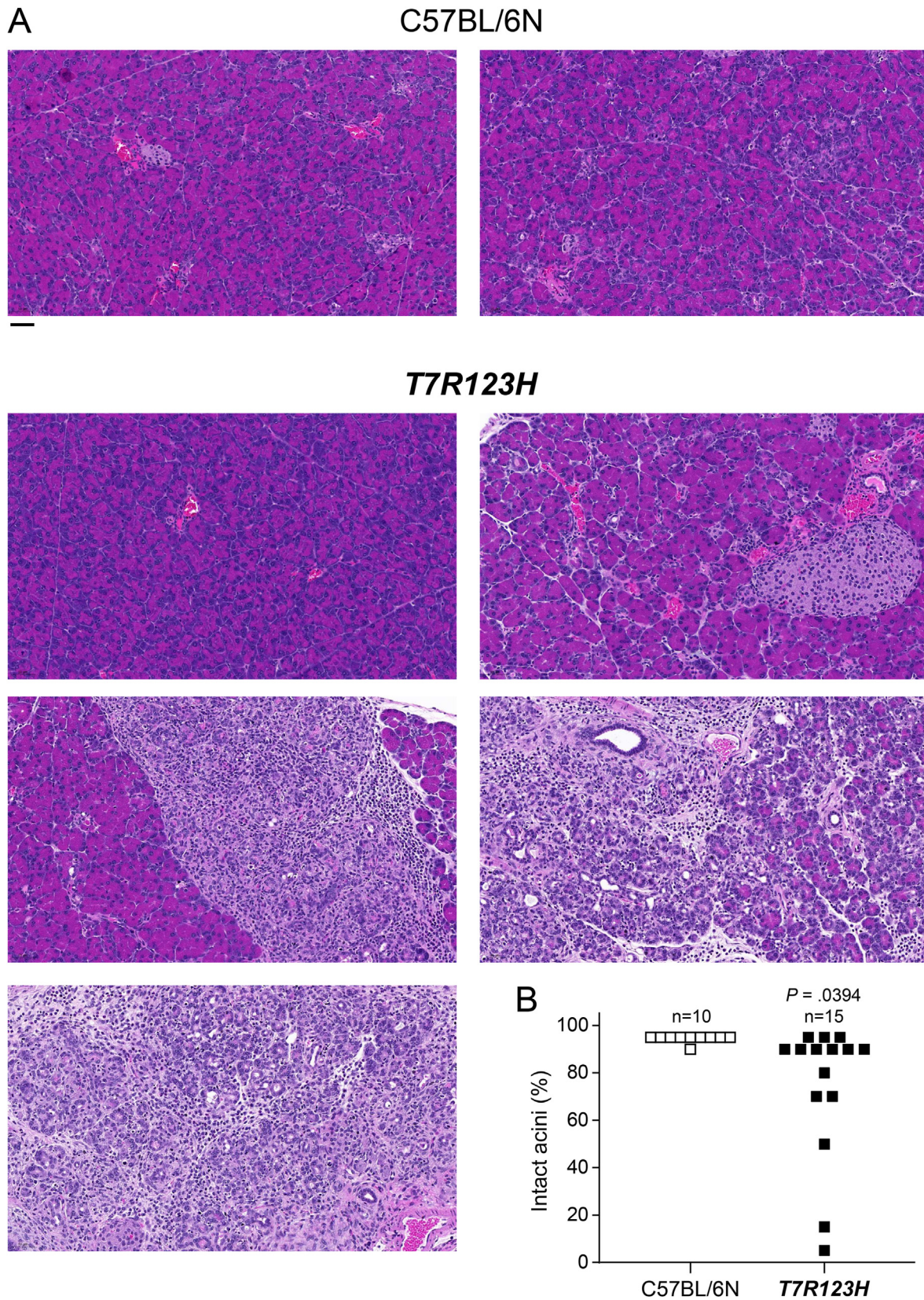


Fig. 9. Cerulein-induced chronic pancreatitis in *T7R123H* mice. *C57BL/6N* and *T7R123H* mice were given 8 hourly injections of saline or cerulein on 2 consecutive days, as described in *Methods*. Mice were euthanized 3 days after the last injection (on day 5). **A**, Representative hematoxylin-eosin stained pancreas sections. The scale bar corresponds to 50 μ m. **B**, Histology scoring of pancreas sections for intact acini. Individual data points were graphed with the mean and standard deviation indicated. The difference of means between two groups was analyzed by two-tailed unpaired *t*-test.

Table 1

Genetically modified mouse models with p.R122H mutant trypsinogen. *Cela1*, chymotrypsin-like elastase 1 gene. BAC, bacterial artificial chromosome. Note that *T7R123H* mice contain the p.R123H mutation, which is analogous to p.R122H in human trypsinogen.

Citation	Method	Promoter	Trypsinogen	Gene
Archer et al., 2006 [35]	transgenic	short rat <i>Cela1</i> promoter	mouse anionic trypsinogen (isoform T8)	coding DNA
Selig et al., 2006 [37]	transgenic	short rat <i>Cela1</i> promoter	human <i>PRSS1</i>	coding DNA
Athwal et al., 2014 [38]	transgenic	short rat <i>Cela1</i> promoter	human <i>PRSS1</i>	coding DNA
Huang et al., 2020 [39]	transgenic	full-length mouse <i>Cela1</i> gene	human <i>PRSS1</i>	coding DNA
Gui et al., 2020 [32]	transgenic	native trypsinogen promoter	human <i>PRSS1</i>	full-length genomic in BAC
Wang et al., 2022 [41]	transgenic	native trypsinogen promoter	human <i>PRSS1</i>	full-length genomic in BAC
This work, <i>T7R123H</i> mice	knock-in	native trypsinogen promoter	mouse cationic trypsinogen (isoform T7)	native genomic locus

not to variability in pancreatic trypsinogen levels. The *T7R123H* mice of the current study represents the first knock-in model that utilizes the native mouse cationic trypsinogen locus, and does not change trypsinogen levels in the pancreas. We performed total trypsinogen measurements and T7-specific Western blot analysis to confirm comparable trypsinogen expression in the pancreas of *T7R123H* and C57BL/6N mice. Therefore, we can conclude that all phenotypic effects seen in *T7R123H* mice were solely due to the p.R123H mutation.

C57BL/6N mice used for most of the published transgenic studies are naturally deficient in mouse CTRC [19], therefore, the effect of the p.R122H mutation cannot be related to protection against CTRC-dependent degradation, as postulated for hereditary CP [3,4]. In biochemical experiments, we found that mouse CTBR1 did not degrade human cationic trypsinogen (unpublished), indicating that regulation of transgenic PRSS1 through mouse CTBR1 is unlikely. It remains unknown whether human cationic trypsinogen can be cleaved by mouse chymotrypsin-like protease (CTRL), although the ineffectiveness of human CTRL [17] would argue against this possibility. It appears, therefore, that the published pathogenic effect of the p.R122H mutation in transgenic mice cannot be readily explained by the inhibition of protective chymotrypsin-dependent trypsinogen degradation. In addition to blocking CTRC-dependent degradation, mutation p.R122H also accelerates autoactivation of human cationic trypsinogen by about 1.5-fold [16,18], and slightly increases secretion levels from transfected cells [42]. These secondary effects of the mutation, amplified by the gene-dosage effect of the transgene, are likely responsible for the increased severity of and/or sensitivity to pancreatitis in the published transgenic models.

In summary, here we present the first knock-in mouse model of the hereditary-pancreatitis associated p.R122H cationic trypsinogen mutation. *T7R123H* mice, harboring the analogous p.R123H mutation, exhibit increased intrapancreatic trypsin activity and more severe AP than C57BL/6N mice, after sustained treatment with cerulein. Furthermore, *T7R123H* mice develop CP after the AP episode, but with incomplete penetrance. The lack of a strong disease phenotype could be explained by the inability of the p.R123H mutation to prevent chymotrypsin-dependent degradation in the context of mouse cationic trypsinogen. The findings indirectly reinforce the pathogenic model of *PRSS1* p.R122H-associated hereditary CP, where mutation p.R122H blocks CTRC-dependent trypsinogen degradation, and thereby increases trypsinogen autoactivation, and intrapancreatic trypsin activity.

Author contributions

MST and ZJ conceived and directed the study. ZJ and MST designed the experiments. ZJ, NCMG, and AD performed the experiments. ZJ, NCMG, AD, and MST analyzed the data. MST wrote the manuscript, ZJ, NCMG, and AD prepared figures. All authors contributed critical revisions and approved the final manuscript.

Declaration of competing interest

The authors have declared that no conflict of interest exists.

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