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*J Clin Invest.* 2019. <https://doi.org/10.1172/JCI129961>.

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## **Elastase 3B mutation links to familial pancreatitis with diabetes and pancreatic adenocarcinoma**

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## **Abstract**

While improvements in genetic analysis have greatly enhanced our understanding of the mechanisms behind pancreatitis, it continues to afflict many families for whom the hereditary factors remain unknown. Recent evaluation of a patient with a strong family history of pancreatitis sparked us to reexamine a large kindred originally reported over 50 years ago with an autosomal dominant inheritance pattern of chronic pancreatitis, diabetes and pancreatic adenocarcinoma. Whole exome sequencing analysis identified a rare missense mutation in the gene encoding pancreas-specific protease Elastase 3B (CELA3B) that cosegregates with disease. Studies of the mutant protein in vitro, in cell lines and in CRISPR-Cas9 engineered mice indicate that this mutation causes translational upregulation of CELA3B, which upon secretion and activation by trypsin leads to uncontrolled proteolysis and recurrent pancreatitis. Although lesions in several other pancreatic proteases have been previously linked to hereditary pancreatitis, this is the first known instance of a mutation in CELA3B and a defect in translational control contributing to this disease.

## **Introduction**

Malfunction or injury to the pancreas, primarily by its own digestive enzymes, results in the inflammation and abdominal pain typical of acute pancreatitis. Repeated injury and inflammation lead to chronic pancreatitis (CP), which is characterized by calcification of the pancreas, scarring and obstruction, and loss of exocrine and endocrine function (1).

This results in a spectrum of clinical manifestations such as malnutrition, increased risks for diabetes and pancreatic cancer, poor quality of life, and reduced survival (2).

There have been several genes linked to hereditary CP, including *PRSS1*, the gene for cationic trypsinogen, which prevents proper auto-inactivation of the enzyme (3). Other causative lesions for inherited CP have been identified in genes that regulate trypsin inhibition/inactivation (*SPINK1*, *CTRC*)(4, 5), and in clearance of trypsin from the pancreas (*CFTR*)(6). Several trypsin-independent genetic causes, such as mutations in carboxypeptidase A1 (*CPA1*), have also been reported (7). Furthermore, alcoholic pancreatitis, the most common cause of sporadic CP, is contingent on genetic and environmental factors (8). Despite these advances, the molecular basis of hereditary pancreatitis remains unknown in at least 30% of affected families (9).

## **Results and Discussion**

In 1968, investigators at UCSF and affiliated hospitals published one of the earliest documented cases of familial pancreatitis in the US, affecting a kindred of 71 people centered in Northern California (10). In affected individuals, abdominal pain manifested between 5-15 years of age with onset of pancreatitis in late childhood to early adulthood that was severe, including several patients requiring surgical intervention. In multiple cases, pancreatitis was confirmed by biopsy. Pancreatitis was accompanied by a high prevalence of diabetes and pancreatic cancer in adulthood, and there was no reported association with smoking or alcohol consumption. The data indicated that the causative

variant was autosomal dominant with an extremely high penetrance. However, without advanced genetic methods, the cause of the disease remained undetermined.

Recently, a patient with a similar history of CP presented at the UCSF Diabetes Clinic, and revealed a connection to the family reported in 1968 (Figure 1A). Like other family members, she had onset of abdominal pain in late childhood and a diagnosis of CP in early adulthood. In addition, she had no history of smoking or alcohol consumption. Sanger sequencing revealed no mutations in *PRSS1* (Figure S1, Supplemental Table 1, Methods), the most common cause of hereditary CP (11). Whole exome sequencing on DNA samples from the patient (Figure 1A, member IV-9), her affected daughter (V-1), and unaffected brother (IV-10) and son (V-2) found no mutations in known pancreatitis-associated genes, but revealed three novel variants that cosegregated with disease and were predicted to be both damaging and rare (Figure S2). Recruitment and Sanger sequencing of an unaffected sister (IV-11) ruled out one variant, leaving two candidate variants. A G→A substitution at c.959 (c.959G>A) resulted in a substitution of arginine for glutamine at codon 320 (p.R320Q) in *FOXM1*, which encodes a transcription factor important for thymic epithelial cells. Homozygous mutations in *FOXM1* cause T-cell immunodeficiency, congenital alopecia, and nail dystrophy (TIDAND)(12). A C→T substitution at c.268 (c.268C>T) resulted in a substitution of arginine to cysteine at codon 90 (p.R90C) in *CELA3B* (Table 1), which encodes chymotrypsin-like elastase family member 3B and is exclusively expressed in the exocrine pancreas (13) (Figure S3). Sanger sequencing confirmed that the *CELA3B* and *FOXM1* variants are present in affected family members and are absent in unaffected family members. As *FOXM1* is not

expressed in the pancreas and heterozygous *FOXN1* mutations are not a cause of pancreatitis (12, 13), we hypothesized that the *CELA3B* p.R90C variant is likely the cause of CP in this family and performed functional studies.

*CELA3B* belongs to a family of six elastases, four of which (2A, 2B, 3A and 3B) are only produced in and secreted by pancreatic acinar cells (13). Structurally, it consists of a signal peptide (residues 1-15), pro-peptide (16-28) and catalytic domain (29-270) (Figure 1B). Upon secretion, *CELA3B* is cleaved at R28 by trypsin, converting it from zymogen to active protease. No pathogenic mutations of *CELA3B* have been reported (14). Sequence alignments of elastases reveal that R90 is highly conserved amongst *CELA3Bs*, while other elastase family members typically have a leucine or isoleucine at this position (Figure 2A and Supplemental Figure 4A), suggesting that position 90 plays a crucial regulatory, structural or functional role.

We explored the functional divergence of WT, R90C (patient mutation) and R90L (evolutionary substitution) variants in 293T cells. Transfection assays revealed that the R90C and R90L substitutions lead to elevated intracellular and secreted *CELA3B* levels (Figure 2B-C) despite equal mRNA transcript levels (Supplemental Figure 4B). Introduction of reverse mutations (L90C and L90R) into *CELA3A*, *CELA3B*'s closest relative, reduced its expression (Supplemental Figure 4C-E), indicating an evolutionarily conserved role for this residue in regulating elastase levels.

Pulse-chase analysis revealed that the R90 substitutions do not appreciably impact CELA3B stability or secretory rates (Supplemental Figure 4F-I), though they significantly increased translation rates in correlation with their steady-state levels (Figure 2D-E). Conversely, CELA3B variants lacking the signal peptide ( $\Delta$ SP) are translated into the cytosol at equal levels (Figure 2F-G), thereby linking differences in expression of the full-length transcripts to co-translational translocation at the endoplasmic reticulum (ER) membrane.

In addition to regulation of expression, mutations in proteases can affect their intrinsic catalytic capabilities and their activation/inactivation by regulatory factors. Michaelis-Menten enzyme kinetics of purified WT, R90C and R90L variants of CELA3B suggest that R90 does not grossly affect the catalytic efficiency of the protease (Supplemental Table 2) (15). No differences in activation efficiency of the R90 variants were detected (P.C.M. and J.P., unpublished observations), though trypsin treatment of unmodified media containing secreted elastases yielded much more rapid activation of the R90C and R90L variants than WT CELA3B (Figure 2H). Additionally, CELA3B binds and impedes premature activation of carboxypeptidases 1 and 2 (CPA1 and CPA2) (16) (17). Therefore, we tested whether mutations in CELA3B might disrupt its binding to CPA1/2 and result in excessive protease activation. However, we detected normal binding of R90C CELA3B and R90L CELA3B to both CPA1 and CPA2, while a known binding-deficient variant (A241G) CELA3B was incapable of interacting with either (Supplemental Figure 5A and B). Taken together, these data suggest that the R90C patient mutation removes a molecular brake to increase translation and secretion of the

CELA3B protease, thereby enhancing its proteolytic activation by trypsin.

To bridge the gap between our molecular and patient data, we employed CRISPR/Cas9-directed genome editing to knock in the homologous mutations (R89C and R89L) at the endogenous *Cela3b* locus in mice. Similar to other familial pancreatitis mouse models, homozygous *Cela3b* mutant mice (both R89C/R89C and R89L/R89L) did not spontaneously develop pancreatitis by 40 weeks of age (18, 19). Current research supports a two-hit theory whereby CP-causing mutations increase the susceptibility of the pancreas to environmental insults, thereby leading to the development of pancreatitis (20). We provided a “second hit” by injecting the cholecystokinin analogue caerulein, a chemical agent used to induce pancreatitis in rodents. Under the dose and regime of caerulein utilized, control mice typically make a full recovery within seven days (21, 22), while pathology is exaggerated in mouse models that are genetically predisposed to pancreatitis (19).

In response to caerulein, homozygous *Cela3b* R89C and R89L animals developed more severe pancreatitis than wildtype control animals, as characterized by increased immune infiltration, acinar de-differentiation, and loss of lobular integrity (Figure 3A-C). Moreover, the R89L animals showed even greater pancreatic injury than the R89C animals, consistent with the effects of these two variants on CELA3B protein expression (Figure 3C).



Although this family was originally described over 50 years ago, the molecular basis underlying their autosomal dominant inherited syndrome of pancreatitis, diabetes, and pancreatic adenocarcinoma had remained a mystery. Using next generation sequencing, we have identified the likely causative lesion in CELA3B: a single amino acid mutation of arginine 90 to cysteine. Intriguingly, this mutation does not alter the catalytic profile of CELA3B, as typically anticipated for a protease, but enhances its rate of translation, thereby increasing the total amount of active enzyme and the risk of pancreatitis in response to pancreatic insult.

Precisely how these mutations increase expression is unclear, but the answer may lie in regulation of translation elongation, a growing field of research with numerous correlations to disease (23). While codon optimization is an obvious candidate, we observed no differences in CELA3B expression when using alternate codons for R90, R90C and R90L (P.C.M., unpublished observations); instead, translation rate appears to depend on amino acid residue. Codon optimization also fails to account for our observation that CELA3B expression is dependent on co-translational translocation at the ER. However, translational elongation can be mediated by stress chaperones such as Hsp70 (23), which raises the possibility that ER-resident chaperones, like BiP, could mediate translation rate. Regardless, this mechanism of action clearly sets it apart from other causative lesions, which typically regulate activation/inactivation of trypsin or lead to ER stress.

Moreover, although the position of CELA3B downstream of the canonical trypsin regulatory mechanism makes it an unexpected candidate to trigger uncontrolled pancreatic proteolysis, our mouse model clearly demonstrates that this elastase is important for propagating pancreatitis and in vitro assays show that excessive expression allows it to be rapidly activated by trypsin. Given the severe pancreatitis phenotype in patients, the possibility remains that mutant CELA3B has undiscovered catalytic properties or targets that enhance its pathogenicity. The severity of this disease also suggests that CELA3B may play a prominent role in other forms of CP. As such, it would be interesting to test if CELA3B knockout animals are resistant to pancreatitis, particularly in the presence of CP-causing trypsin mutations. Support for this notion comes from a recent report that a different variant (c.643-7G>T) in CELA3B is associated with a small protective effect against alcoholic chronic pancreatitis (17). Furthermore, pharmacologic inhibition of elastase family members has shown efficacy in preclinical models of pancreatitis (24).

Importantly, many kindred are still afflicted by hereditary pancreatitis with unknown genetic causes, and future studies will need to determine whether they also carry mutations in CELA3B. As shown here, a coordinated approach using modern human genetics, CRISPR-based mouse models and molecular biology/biochemistry opens new opportunities to strongly link rare variants to disease risk.

## **Methods**

### ***Human Subjects***

The original pedigree was previously described in 1968 (10), which we corroborated and expanded with the help of the proband (Figure 1). The study was approved by the University of California San Francisco (UCSF) Committee on Human Research. All participating family members provided written informed consent to participate in the study.

### ***Animal Study Approvals***

All mouse experiments complied with the Animal Welfare Act and the National Institutes of Health guidelines for the ethical care and use of animals in biomedical research and were performed under the approval of the University of California at San Francisco Institutional Care and Use of Animals Committee (IACUC). Animals were maintained in a specific pathogen-free animal facility on a 12-hr light–dark cycle at an ambient temperature of 21°C. They were given free access to water and food.

### ***Statistics***

All data are expressed as means  $\pm$  SD and significance was defined as  $P < 0.05$ . Data sets with one factor and multiple groups were compared by ordinary or matched (paired) 1-way ANOVA with Tukey's multiple comparisons test; sets with two factors were compared by ordinary or matched (paired) 2-way ANOVA with Tukey's multiple comparisons test. Slopes in Figure 2E were calculated by linear regression and were determined to be different (non-parallel) with  $P = 0.0011$ . Curves in Figure S4G were calculated by exponential decay (one phase decay) with rate constants,  $K$ , determined to differ from the WT control with  $P < 0.0001$ . All statistical analyses were performed in Prism, version 6.0 (Graphpad).

### **Author contributions**

Order of authors: P.C.M. and J.T.C. are co-first authors with P.C.M. listed first due to his major contributions regarding molecular mechanism and manuscript preparation. Conceptualization, P.C.M., J.T.C., M.A. and S.A.O.; Methodology, P.C.M., J.T.C., C.E.C., A.C., J.L.B., M.A. and S.A.O.; Validation, P.C.M., J.T.C. and C.E.C.; Formal Analysis, P.C.M., J.T.C. and C.E.C.; Investigation, P.C.M., J.T.C., C.E.C., A.C., J.L.B. and J.P.; Resources, D.A., A.C.B., Z.Q., A.C., and M.H.C; Writing – Original Draft, P.C.M. and S.A.O.; Writing – Review & Editing, P.C.M., J.T.C., C.E.C., D.A., A.C.B., Z.Q., A.C., M.H.C., M.S.G., M.A. and S.A.O.; Visualization, P.C.M., J.T.C. and C.E.C.; Supervision, M.S.G., M.A. and S.A.O.; Funding Acquisition, M.S.G., M.A. and S.A.O.

### **Acknowledgements**

This work was supported in part by the National Institutes of Health grants: P30DK63720, R01CA219815, R01EY027810, and U01DK108332; the Helmsley Charitable Trust; the Bern Schwartz Family Foundation; and Larry L. Hillblom Foundation grant 2018-D-0006-NET. We would like to specially thank Miklos Sahin-Toth (UCLA) for his generous donation of elastase and carboxypeptidase constructs and for guidance on purification and analysis of these enzymes.

### **Conflict of Interest Disclosure**

M.S.G. holds stock in ViacYTE, Inc. and Encellin, Inc. M.A. holds stock in Medtronic and Merck. S.A.O. is a scientific cofounder, equity holder, and consultant for OptiKira, LLC.

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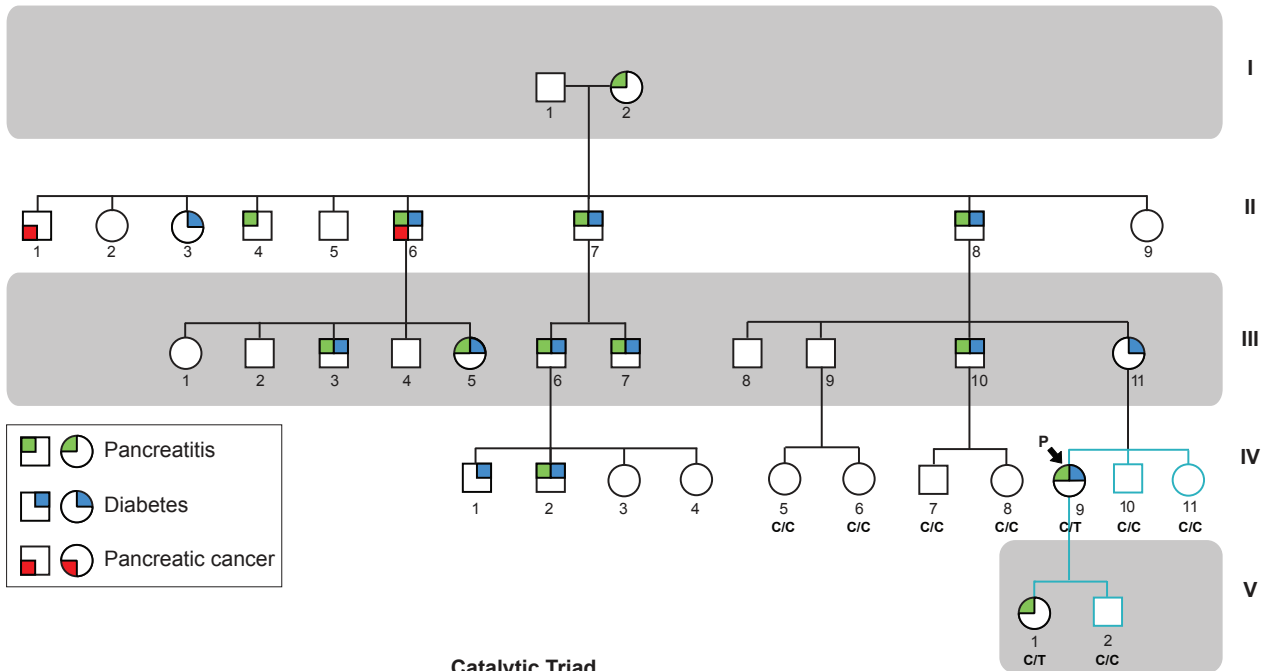
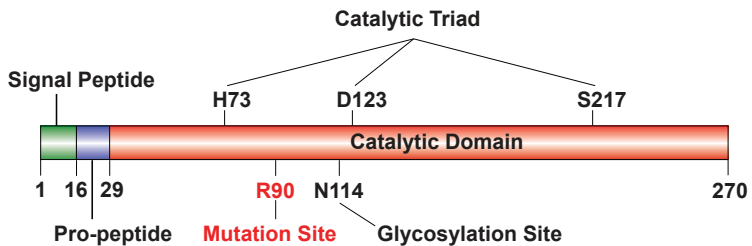
**A****B**

Figure 1

## Figure Legends

**Figure 1: Identification of a pathogenic mutation in family with hereditary pancreatitis. (A and B)** (A) Pedigree of the study family. Squares indicate males while circles indicate females. Shapes are partitioned and colored to indicate conditions (green: pancreatitis, blue: diabetes, red: pancreatic cancer). Generations I-IV were previously reported (Davidson P. et al, 1968) while generation V is newly reported. Teal branches indicate family members that participated in this study to identify the CELA3B p.R90C mutation while black branches indicate previously reported individuals. The proband is indicated by an arrow and labelled with a "P". Mutation status confirmed by sequencing is indicated below shapes and labeled with C/C indicating wild-type individuals or C/T indicating individuals that harbor the CELA3B c.268C>T (p.R90C) variant. (B) Map of human CELA3B, showing known domains and key amino acid residues.



**A**

Elastase 1	50	GTLIRQNWVMTAAHCVDYQ--KTFRVVAGDHNLSQNDGTEQYVSV--QKIVVHPYWNSDN
Neutrophil Elastase	57	ATLIAPNFVMSAAHCVANVNVRAVRVVLGAHNLSRREPTRQVFAV--QRIFENGY--DP
Elastase 2A	60	GSLIANSWVLTAAHCISSS--RTYRVGLGRHNLVVAESGSLAVSV--SKIVVHKDWNNSQ
Elastase 2B	60	GSLIANSWVLTAAHCISSS--GIYRVMLGQHNLYVAESGSLAVSV--SKIVVHKDWNNSQ
Elastase 3A	60	GSLIAPDWVVTAGHCISRD--LTYQVVLGEYNLAVKEGPEQVIPINSEELFVHPLWNRSC
Elastase 3B	60	GSLIAPDWVVTAGHCISSS--RTYQVVLGEYDRAVKEGPEQVIPINSGDLFVHPLWNRSC

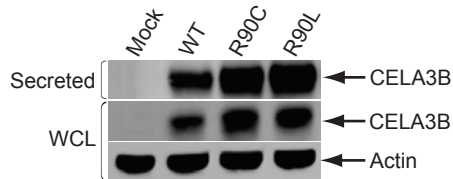
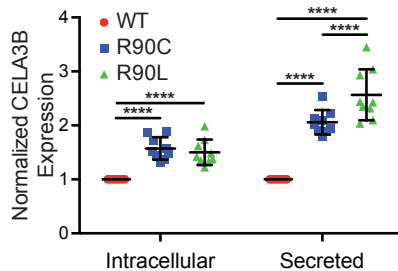
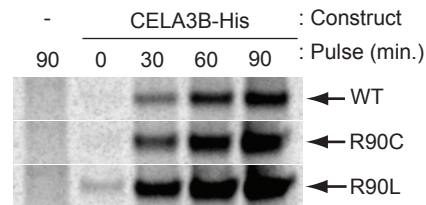
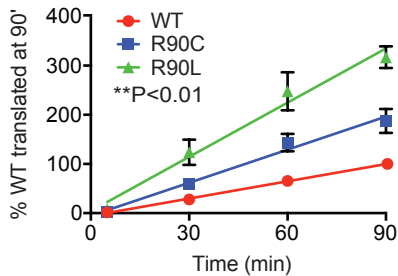
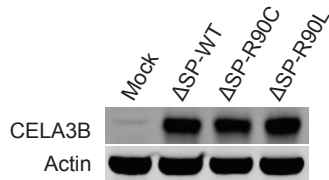
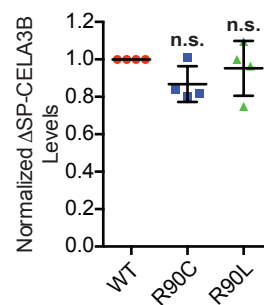
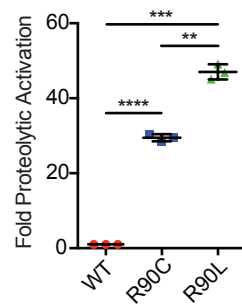
**B****C****D****E****F****G****H**

Figure 2

**Figure 2: Functional characterization of CELA3B mutations at arginine 90. (A-H)** (A) Sequence alignment of the six human elastases centered at position 90 in CELA3B. Arginine (R) residues are labeled in red; leucine (L) residues are labeled in blue. (B) Immunoblots of whole cell lysates (intracellular) and conditioned media (secreted protein) from 293T cells transfected with empty vector (Mock) or the indicated CELA3B variant. (C) Quantification of n=9 experiments as in B. Values are means  $\pm$  SD. (D) Autoradiographs of His-tagged CELA3B variants purified from Brefeldin A-treated 293T cells pulsed with  $^{35}\text{S}$ -labeled methionine and cysteine for the indicated amounts of time. First (control) lane is the same in WT and R90C images. (E) Quantification of n=4 experiments as in D, showing data points normalized to WT levels at 90 minutes and their corresponding lines of best fit (linear regression). Values are means  $\pm$  SD.  $P < 0.01$  between all samples. (F) Western blots of CELA3B variants lacking a signal peptide ( $\Delta\text{SP}$ ). Samples are whole cell lysates from 293T cells transfected with empty vector (Mock) or vector containing  $\Delta\text{SP}$ -CELA3B variants. Blots were probed with the indicated antibodies. (G) Quantification of n=4 experiments as in F, with protein levels normalized to  $\Delta\text{SP}$ -WT CELA3B. Values are means  $\pm$  SD. (H) Efficiency of catalytic activation of CELA3B variants in conditioned media after limited activation with trypsin. Raw catalytic activity was quantified as a change in absorbance due to cleavage of a colorimetric CELA3B substrate; raw values were normalized to total CELA3B protein levels and are provided as means  $\pm$  SD of n=3 experiments. Multiplicity-adjusted P values were determined by matched 2-way ANOVA (panel C) or matched 1-way ANOVA (panels G and H) with Tukey's multiple comparisons test; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  and n.s. = not significant.

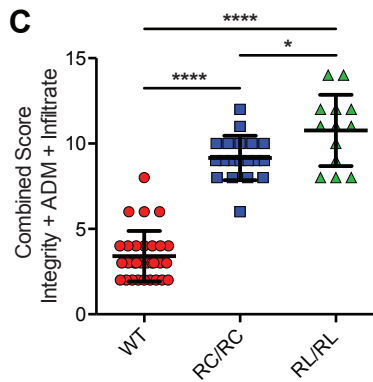
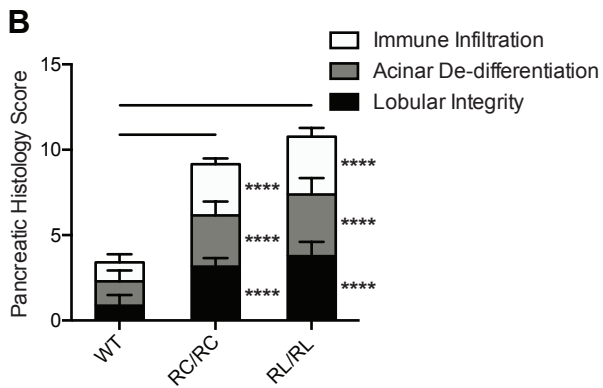
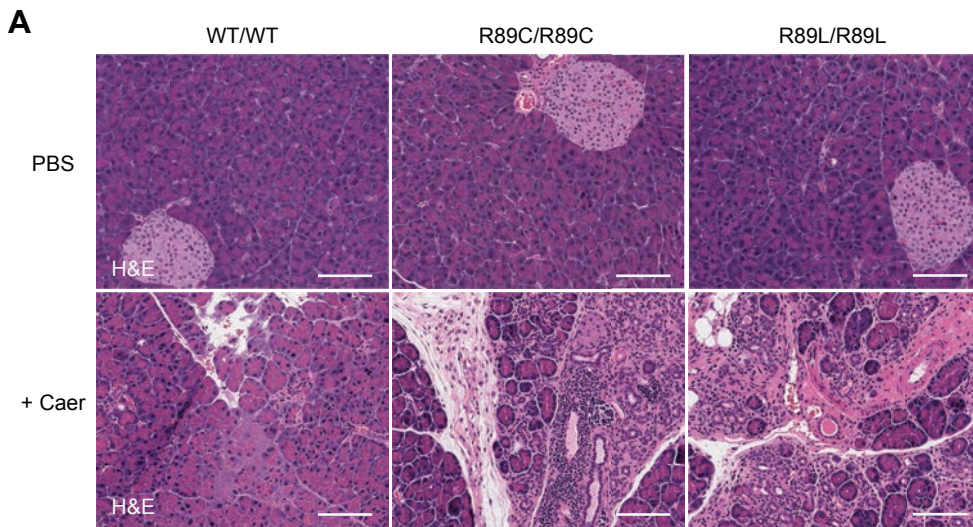


Figure 3

**Figure 3. *Cela3b* mutant mice show increased pancreatic injury following induction of acute pancreatitis. (A-C)** (A) H&E staining of pancreata from WT, *Cela3b* R89C/R89C, and *Cela3b* R89L/R89L C57BL/6 mice 7 days after two consecutive days of 8 hourly intraperitoneal injections with vehicle (PBS) or 75 µg/kg caerulein. Scale bars are 100 µm. (B) Stacked bar graphs classifying pancreatic damage of 13-30 caerulein-treated mice per genotype. Values from 0-5 assigned for immune infiltration (Infiltrate), acinar de-differentiation (ADM) and lobular integrity (Integrity) with means ± SD for each separate classification. (C) Combined scores from B with means ± SD. P values were determined by ordinary 2-way ANOVA (panel B) or ordinary 1-way ANOVA (panel C) with Tukey’s multiple comparisons test; \*P<0.05, and \*\*\*\*P<0.0001.

**Tables**

**Table 1**

<b><i>CELA3B</i> mutation</b>	
Genomic Position	Chr1:22307571
Zygoty	Heterozygous
Transcript variant	c.268C>T
Protein variant	p.R90C
Scaled CADD Score	26
gnomeAD allele frequency	0.0008%