Cathepsin B and D deficiency in the mouse pancreas induces impaired autophagy and chronic pancreatitis

Hideaki Iwama1,2, Sally Mehanna3,7, Mai Imasaka4, Shinsuke Hashidume1, Hiroshi Nishiura4, Ken‑ichi Yamamura3, Chigure Suzuki5, Yasuo Uchiyama6, Etsuro Hatano2 & Masaki Ohmuraya1*

The major lysosomal proteases, Cathepsin B (CTSB), Cathepsin D (CTSD) and Cathepsin L (CTSL), are implicated in autophagic activity. To investigate the role of each cathepsin in the exocrine pancreas, we generated mice in which the pancreas was specifically deficient in Ctsb, Ctsd and Ctsl. Each of these gene knockout (KO) and Ctsb;Ctsl and Ctsd;Ctsl double-knockout (DKO) mice were almost normal. However, we found cytoplasmic degeneration in the pancreatic acinar cells of Ctsb;Ctsd DKO mice, similar to autophagy related 5 (Atg5) KO mice. LC3 and p62 (autophagy markers) showed remarkable accumulation and the numbers of autophagosomes and autolysosomes were increased in the pancreatic acinar cells of Ctsb;Ctsd DKO mice. Moreover, these Ctsb;Ctsd DKO mice also developed chronic pancreatitis (CP). Thus, we conclude that both Ctsb and Ctsd deficiency caused impaired autophagy in the pancreatic acinar cells, and induced CP in mice.

Autophagy is a system of intracellular degradation that involves lysosomal enzymes. The role of autophagy is balancing sources of energy at critical times in development and in response to nutrient stress1. Moreover, autophagy plays a critical role in clearing misfolded or aggregated proteins, removing damaged organelles, such as mitochondria, endoplasmic reticulum and peroxisomes1. Autophagy exerts devastating effects in pancreatic acinar cells by the activation of trypsinogen to trypsin in the early stage of acute pancreatitis2. However, basal autophagy maintains pancreatic acinar cell homeostasis and protein synthesis3 and impaired autophagy induces chronic pancreatitis (CP)4–7.

Cathepsin B (CTSB) and L (CTSL) are cysteine proteases, and Cathepsin D (CTSD) is an aspartic protease8. These cathepsins are major lysosomal proteases and are widely expressed in endosomes and lysosomes. Mice lacking Ctsd in all tissues die one month after birth due to intestinal necrosis9. Mice lacking Ctsb or Ctsl in all tissues grow up almost normally10–12, however, mice lacking both Ctsb and Ctsl in all tissues die 2 weeks after birth due to the massive apoptosis of central neurons13. Thus, the organ-specific roles of these cathepsins are different. Furthermore, Ctsb and Ctsl were reported to be involved in the processing of CtsD14,15, and cathepsins might interact with each other in different ways depending on tissues16,17.

On the other hand, Ctsb and Ctsl play a role in intrapancreatic trypsinogen activation and the onset of acute pancreatitis18–20. Ctsd was reported to regulate Ctsb activation during experimental pancreatitis17, and was implicated in Ctsb and Ctsl degradation, but not in autophagic activity in the pancreas21. Thus, it is not clear which of the cathepsins plays an important role in autophagy of the pancreas.

1Department of Genetics, Hyogo College of Medicine, 1-1, Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan. 2Department of Gastroenterological Surgery, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan. 3Institute of Resource Development and Analysis, Kumamoto University, 2-2-1 Honjo, Chuou-ku, Kumamoto 860-0811, Japan. 4Division of Functional Pathology, Department of Pathology, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan. 5Department of Pharmacology, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. 6Department of Cell Biology and Neuroscience, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. 7Present address: Department of Veterinary Hygiene and Management, Faculty of Veterinary Medicine, Cairo University, Giza 12211, Egypt. *email: ohmuraya@hyo-med.ac.jp
In order to investigate the role of Ctsb, Ctsd and Ctsl in autophagy of the pancreas, we generated mice in which the pancreas was specifically deficient in Ctsb, Ctsd, Ctsb;Ctsd, Ctsb;Ctsl or Ctsb;Ctsd;Ctsl. The purpose of this study was to clarify which of these cathepsins are essential for autophagy in the mouse pancreas.

**Results**

**Generation of pancreas-specific Ctsb-, Ctsd-, and Ctsl-deficient mice.** In order to investigate the role of Ctsb, Ctsd and Ctsl in autophagy of the pancreas, we generated pancreas-specific Ctsb-, Ctsd- or Ctsl-deficient (CtsbΔPan, CtsdΔPan or CtslΔPan) mice using the Cre-loxP system. Pancreas-specific Ctsb, Ctsd and Ctsl double-knockout (DKO) (CtsbΔPan, CtsdΔPan, CtslΔPan) mice were obtained by mating the CtsbΔPan, CtsdΔPan and CtslΔPan mice. Western blotting (WB) of whole tissue lysates of the pancreases of wild-type, CtsbΔPan, CtsdΔPan and CtslΔPan mice confirmed that Ctsb, Ctsd or Ctsl was disrupted in the CtsbΔPan, CtsdΔPan and CtslΔPan pancreases, respectively (Fig. 1A). WB of whole tissue lysates of the pancreases of wild-type Fig. 1, CtsbΔPan, CtsdΔPan, CtslΔPan and CtsbΔPan, CtsdΔPan and CtslΔPan mice confirmed that Ctsb and Ctsd or Ctsd and Ctsl were disrupted in the CtsbΔPan, CtsdΔPan, CtslΔPan and CtsbΔPan, CtsdΔPan and CtslΔPan pancreases, respectively (Fig. 2A). The CtsbΔPan, CtsdΔPan and CtslΔPan mice were all healthy, fertile, and indistinguishable from their wild-type littermates, although the expression of each of the cathepsins was completely deleted.

**The expression of autophagy markers was normal in the pancreas of CtsbΔPan, CtsdΔPan and CtslΔPan mice.** In order to investigate the autophagic activity in the pancreases of CtsbΔPan, CtsdΔPan or CtslΔPan mice, we firstly performed histopathological examinations of the pancreases of CtsbΔPan, CtsdΔPan or CtslΔPan mice. These examinations revealed no abnormalities (Fig. 1Ba–d).

We then investigated the expression of p62/sequestosome 1 (p62), a selective substrate of autophagy, and microtubule-associated protein 1 light chain 3 (LC3), a marker of autophagic vacuole formation, in order to analyze the autophagy function in the pancreases of CtsbΔPan, CtsdΔPan and CtslΔPan mice. As shown in Fig. 1C, no significant difference in the expression of p62 in the pancreases was noted among wild-type, CtsbΔPan, CtsdΔPan and CtslΔPan mice (Fig. 1C). LC3 has two forms. LC3-I is localized in the cytoplasm and is converted into LC3-II. In order to investigate the autophagic activity in the pancreases of CtsbΔPan, CtsdΔPan and CtslΔPan mice, respectively (Fig. 1A). WB of whole tissue lysates of the pancreases of wild-type Fig. 1, CtsbΔPan, CtsdΔPan, CtslΔPan and CtsbΔPan, CtsdΔPan and CtslΔPan mice confirmed that Ctsb and Ctsd or Ctsd and Ctsl were disrupted in the CtsbΔPan, CtsdΔPan, CtslΔPan and CtsbΔPan, CtsdΔPan and CtslΔPan pancreases, respectively (Fig. 2A). The CtsbΔPan, CtsdΔPan and CtslΔPan mice were all healthy, fertile, and indistinguishable from their wild-type littermates, although the expression of each of the cathepsins was completely deleted.

**Impaired autophagy was induced in the pancreas of CtsbΔPan;CtsdΔPan mice.** In order to investigate the autophagic activity in the pancreases of CtsbΔPan;CtsdΔPan or CtslΔPan mice, we performed histopathological examinations of the pancreases of CtsbΔPan;CtsdΔPan or CtslΔPan mice. As shown in Fig. 1E, there was no apparent difference in the expression of p62 in the pancreases of CtsbΔPan;CtsdΔPan mice compared with wild-type, CtsbΔPan;CtsdΔPan and CtslΔPan mice (Fig. 1C). LC3 has two forms. LC3-I is localized in the cytoplasm and is converted into LC3-II. In order to investigate the autophagic activity in the pancreases of CtsbΔPan;CtsdΔPan or CtslΔPan mice, we performed histopathological examinations of the pancreases of CtsbΔPan;CtsdΔPan, CtsbΔPan;CtslΔPan and CtslΔPan mice (Fig. 1F). As shown in Fig. 1F, there was no apparent difference in the expression of p62 in the pancreases of CtsbΔPan;CtsdΔPan mice compared with wild-type, CtsbΔPan;CtsdΔPan and CtslΔPan mice (Fig. 1C). LC3 has two forms. LC3-I is localized in the cytoplasm and is converted into LC3-II. In order to investigate the autophagic activity in the pancreases of CtsbΔPan;CtsdΔPan or CtslΔPan mice, we performed histopathological examinations of the pancreases of CtsbΔPan;CtsdΔPan or CtslΔPan mice. As shown in Fig. 2C, there were no significant differences in the expression of p62 and LC3 among wild-type, CtsbΔPan;CtsdΔPan and CtslΔPan mice. On the other hand, CtsbΔPan;CtsdΔPan or CtslΔPan mice showed a marked increase in the expression of p62 and LC3 in the acinar cells of CtsbΔPan;CtsdΔPan mice (Fig. 2Da, e). Furthermore, we performed immunofluorescent staining of the pancreases of wild-type and CtsbΔPan;CtsdΔPan mice, which demonstrated the accumulation of p62 and LC3 in the acinar cells of CtsbΔPan;CtsdΔPan mice (Fig. 2Da–f).

We also examined the expression of ULK1, Becn1, Lamp2 and Rab7 by WB. The expression of ULK1, Becn1, Lamp2 and Rab7 in wild-type, CtsbΔPan;CtsdΔPan, CtsbΔPan;CtslΔPan and CtslΔPan mice showed no apparent difference (Fig. 2D). These data indicate that autophagy was not impaired in the pancreases of CtsbΔPan, CtsdΔPan or CtslΔPan mice.

**Autolysosomes accumulated in pancreatic acinar cells of CtsbΔPan;CtsdΔPan mice, while autophagosomes were also present in the cells but fewer in number.** In order to clarify which phase of the autophagic pathway was disturbed in the pancreas of CtsbΔPan;CtsdΔPan mice, we performed electron microscopy (EM). A large number of granular structures were observed in the pancreatic acinar cells of CtsbΔPan;CtsdΔPan mice compared with wild-type mice (Fig. 3A,B,E,F). In the cytoplasm of the acinar cells of CtsbΔPan;CtsdΔPan mice, most granular structures that possessed cytoplasmic organelles with degraded or undegraded materials, in particular the granular endoplasmic reticulum, were autolysosome-like structures (Fig. 3A,B). In addition to such autolysosome-like structures, the CtsbΔPan;CtsdΔPan acinar cells had multi-membranous vacuolar structures that corresponded to the autophagosomes often seen in cathepsin D-defective neurons28 (Fig. 3A,B) but were much fewer in number. The CtsbΔPan;CtsdΔPan phagocytic cells that appeared adjacent to
Acinar cells often contained large granules of cellular debris in which zymogen granules and nuclear structures were detected (Fig. 3C). In some cases, inclusion bodies which contained a nucleus with condensed chromatin were detected in acinar cells (Fig. 3D). In contrast, wild-type acinar cells possessed lysosome/autolysosome-like structures in the cytoplasm, but no clear-cut autophagosome-like structures were detected in the cells, in contrast to CtsbΔPan;CtsdΔPan acinar cells (Fig. 3E,F).

Chronic pancreatitis was induced in CtsbΔPan;CtsdΔPan mice. We compared the weight at one, four and eight months old between wild-type mice and CtsbΔPan;CtsdΔPan mice. No significant difference was noted in weight between wild-type mice and CtsbΔPan;CtsdΔPan mice.
Figure 2. Generation of CtsbΔPan;CtsdΔPan, CtsbΔPan;CtslΔPan and CtsdΔPan;CtslΔPan mice. (A) Western blotting of Ctsb, Ctsd and Ctsl in the pancreases of 1-month-old wild-type, CtsbΔPan;CtsdΔPan, CtsbΔPan;CtslΔPan and CtsdΔPan;CtslΔPan mice during normal feeding. The full-length blots/gels and the quantification data of WB are presented in supplementary figure S13–S16. (B) HE staining of pancreas tissue isolated from 2-month-old CtsbΔPan;CtsdΔPan, CtsbΔPan;CtslΔPan, CtsdΔPan;CtslΔPan and Atg5ΔPan mice during normal feeding. Scale bars indicate 50 μm. (C) Western blotting of pancreas extracts with anti-p62 and anti-LC3 antibodies in 1-month-old wild-type, CtsbΔPan;CtsdΔPan, CtsbΔPan;CtslΔPan and CtsdΔPan;CtslΔPan mice during normal feeding. Actin was used as a loading control. The full-length blots/gels and the quantification data of WB are presented in supplementary figure S17–S19. (D) Western blotting of pancreas extracts with anti-ULK1, anti-Becn1, anti-Lamp2 and anti-Rab7 antibodies in 1-month-old wild-type, CtsbΔPan;CtsdΔPan, CtsbΔPan;CtslΔPan and CtsdΔPan;CtslΔPan mice during normal feeding. Akt was used as a loading control. The full-length blots/gels and the quantification data of WB are presented in supplementary figure S20–S24. (E) Immunofluorescent staining of pancreas tissue isolated from 2-month-old wild-type and CtsbΔPan;CtsdΔPan mice during normal feeding. Antibodies against LC3 and p62 were used for immunostaining. Scale bars indicate 100 μm.
Figure 3. Transmission electron microscopy of wild-type and CtsbΔPan;CtsdΔPan mice. Transmission electron microscopy of pancreases from 1-month-old wild-type and CtsbΔPan;CtsdΔPan mice. A large number of granular autolysosome-like structures (AL) are observed in the pancreatic acinar cells of CtsbΔPan;CtsdΔPan mice, and autophagosome-like structures (AV) can also be seen in the cells although fewer in number (A,B) than in the cells of wild-type mice (E,F). Several inclusion bodies with dying acinar cell debris are detected in a phagocytic cell appearing in the vicinity of CtsbΔPan;CtsdΔPan mouse acinar cells as well as CtsbΔPan;CtsdΔPan mouse acinar cells (C,D). All scale bars indicate 20 μm.
between the weights at one and four months old, but the weight at eight months old was significantly lower in CtsbΔPan;CtsdΔPan mice than in wild-type mice (Fig. 4A).

On a histopathologic examination of the pancreas in four-month-old CtsbΔPan;CtsdΔPan mice, numerous vacuoles were observed in the acinar cells, in comparison to wild-type mice (Fig. 4B, a, b, d). The loss of the normal architecture and replacement of acinar cells with fat was observed in eight-month-old CtsbΔPan;CtsdΔPan mice (Fig. 4Bc). Pancreatic fibrosis in the pancreas of four-month-old and eight-month-old CtsbΔPan;CtsdΔPan mice was confirmed by Azan-Mallory staining (Fig. 4Be, f, Ci). Fibrosis and replacement of acinar cells with fat worsened over time in the pancreas of eight-month-old CtsbΔPan;CtsdΔPan mice compared with the pancreas of four-month-old CtsbΔPan;CtsdΔPan mice. Immunostaining of the pancreas in four-month-old CtsbΔPan;CtsdΔPan mice revealed a large number of inflammatory cells, including monocytes (CD11b-positive cells), macrophages (F4/80-positive cells) and plasma cells (CD138-positive cells) infiltrated the pancreas (Fig. 4Ce–h, j–l). No monocyte, macrophage nor plasma cell was detected in the pancreases of four-month-old wild-type mice (Fig. 4Ca–d). Immunofluorescent staining of the pancreas in four-month-old mice showed chronic inflammatory cell infiltration and progressive fibrosis (Fig. 4D).

The expression of mRNAs encoding proinflammatory cytokines, including interleukin 6 (Il6), interleukin 1β (Il1β) and tumor necrosis factor a (Tnfa), was also upregulated in CtsbΔPan;CtsdΔPan mice (Fig. 4E). These data suggest that CP was induced in the CtsbΔPan;CtsdΔPan mouse pancreas.

**Discussion**

The purpose of this study is to clarify the role of cathepsin B, D and L in autophagy of the pancreas. The pancreases of CtsbΔPan;CtsdΔPan mice showed impaired autophagy. On the other hand, autophagy was not impaired in the pancreases of CtsbΔPan and CtsdΔPan mice. These results indicate that Ctsb and Ctsd play an important role and act synergistically in the autophagy of the pancreas.

Ctsd plays a critical role in the autophagy of the intestine9 and the autophagy of central nervous requires Ctsb or Ctsd13. This study proved that the autophagy of pancreatic acinar cells in mice required both Ctsb and Ctsd. In this study, the autophagy in the pancreas was not impaired in CtsbΔPan or CtsdΔPan mice, as reported previously10–12,21. The Ctsb and Ctsl expression was increased in the pancreases of CtsbΔPan mice. The same result was reported previously21. In this study, the expression of LC3-II was not increased in the pancreas of CtsbΔPan mice. A previous report21 evaluated the ratio of LC3-II to LC3-I. LC3-II should be calculated using the ratio of a housekeeping protein, not LC3-I, as LC3-I tends to be less sensitive to detection by certain anti-LC3 antibodies25. This is the reason why we calculated the ratio of LC3-II to actin. In addition, the serine protease inhibitor Kazal type 3 (Spink3), which is a trypsin specific inhibitor in the pancreas, -cre knock-in mice were used in the previous report21, while pancreas transcription factor 1 subunit alpha (Ptf1a) -cre knock-in mice (Ptf1aCre+/−) were used in this study. Spink3 expression was reported in not only the pancreas, but also in the intestine, kidney and epididymis, among other sites26. Ptf1a expression would be more specific to the pancreas than Spink3, so we used Ptf1aCre+/− mice in this study.

The expression of Ctsb in CtsbΔPan;CtsdΔPan mice did not disappeared. However, the expression ratio of pro-Ctsb/Ctsb heavy chain in CtsbΔPan;CtsdΔPan mice was different from in wild-type mice. Ctsd in pancreatic acinar cells was shown to be involved in Ctsb and Ctsl degradation22 and regulated Ctsb activation under conditions of experimental pancreatitis22. Ctsd was shown to be accumulated in the brain of Ctsb/Ctsl DKO mice in all cells13. These results indicate that Ctsb, Ctsd and Ctsl regulate each other in vivo. The CtsbΔPan;CtsdΔPan mice showed a marked increase in the autophagy substrates p62 and LC3. This result indicates that impaired autophagy was induced in the CtsbΔPan;CtsdΔPan mouse pancreas. Because Ctsb and Ctsd are lysosomal enzymes, autophagy in the CtsbΔPan;CtsdΔPan mouse pancreas could be disturbed in the degradation phase in autolysosomes. The accumulation of autolysosomes with a few autophagosomes in the pancreatic acinar cells of CtsbΔPan;CtsdΔPan mice was consistent with this expectation.

In the histopathologic examination, the CtsbΔPan;CtsdΔPan mouse pancreas showed CP, which progressed with time. At least four animal models of CP with impaired autophagy have been previously reported: mice with pancreas-specific disruption of Atg5 that developed a form of CP, mice with pancreas-specific ablation of IκB kinase α, mice lacking Spink3 with a mosaic pattern of SPINK1 expression9, and LAMP-2-deficient mice7. The histological findings of CP, such as inflammation, acinar-to-ductal metaplasia and acinar-cell hypertrophy, have been confirmed in all these models. Additional experiments have been performed, such as qRT-PCR analyses of pancreatic RNA from fibrogenic markers and cytokine and chemokine genes and immune cell markers, assessments of serum levels of pancreatitis markers, evaluations of changes in the body and pancreas weight and immunostaining of the pancreas. On the other hand, impaired autophagy was evidenced by accumulation of p62 in WB, enlarged autophagic vacuoles in HE staining and TEM of pancreatic acinar cells. Immunostaining of the pancreas was also performed as an additional experiment. CtsbΔPan;CtsdΔPan mice may be another animal model of CP with impaired autophagy.

In 1896, Chiari reported that pancreatitis was the result of pancreatic autodigestion27,28. In 1959, Greenbaum reported Ctsb activated trypsinogen to trypsin in vitro29. Steer suggested that Ctsb might be responsible for the intracellular activation of digestive enzymes30–32. It became clear that Ctsb inhibition reduced the severity of pancreatitis33, the release of Ctsb in cytosol caused cell death in acute pancreatitis34, and Ctsb activity initiated experimental pancreatitis35. Ctsb, as described above, is known to be deeply implicated in pancreatitis. The present study also indicated that Ctsb played an important role of autophagy in pancreas. Ctsd has many functions as an aspartic protease36–38, that interacts with other important molecules and influences cell signals39–41. Ctsd is implicated in apoptosis and cancer, but not in the severity of pancreatitis or autophagy activity by itself42. From the above results, both Ctsb and Ctsd play an important synergistic role in the autophagy of the mouse pancreas.
Figure 4. Chronic pancreatitis was induced in *Ctsb<sup>ΔPan</sup>;Ctsd<sup>ΔPan</sup> mice. (A) Body weights of 1-, 4- and 8-month-old wild-type and *Ctsb<sup>ΔPan</sup>;Ctsd<sup>ΔPan</sup> mice (n = 8–15 Wild type, n = 5–8 *Ctsb<sup>ΔPan</sup>;Ctsd<sup>ΔPan</sup>). *P < 0.01. M.O.: month-old. (B) HE staining and Azan-Mallory staining of pancreas tissue isolated from 4-month-old wild-type (a, d) and *Ctsb<sup>ΔPan</sup>;Ctsd<sup>ΔPan</sup> (b, e) mice, and 8-month-old *Ctsb<sup>ΔPan</sup>;Ctsd<sup>ΔPan</sup> (c, f) mice during normal feeding. Scale bars indicate 50 μm. AM: Azan-Mallory. (C) The Immunohistochemical analysis of pancreas tissue isolated from 4-month-old wild-type (a–d) and *Ctsb<sup>ΔPan</sup>;Ctsd<sup>ΔPan</sup> (e–l) mice. Antibodies against CD11b as a monocyte marker, F4/80 as a microphage marker and CD138 as a plasma cell marker were used for immunostaining. Scale bars indicate 50 μm. (D) Immunofluorescent staining of pancreas tissue isolated from 4-month-old *Ctsb<sup>ΔPan</sup>;Ctsd<sup>ΔPan</sup> mice (a–d) during normal feeding. Antibodies against Collagen-I as a marker of fibrosis, and F4/80 and CD138 as a marker of inflammatory cell were used for immunostaining. (E) qRT-PCR of pancreatic RNAs from 2-month-old wild-type and *Ctsb<sup>ΔPan</sup>;Ctsd<sup>ΔPan</sup> mice for cytokine genes. Results are shown as the mean ± SEM. n = 6 mice per condition. *P < 0.05.
In conclusion, both Ctsb and Ctsd deficiency caused impaired autophagy in pancreatic acinar cells and induced CP in mice. A future challenge is to clarify the mechanism underlying the interaction of Ctsb and Ctsd with regard to autophagy.

**Materials and methods**

**Animal protocol and experimental design.** Mice were kept under specific-pathogen-free conditions with ad libitum access to food and water in a 12 h (h) light/dark cycle. C57BL/6N mice were purchased from CREA Japan. All animal experiments were performed with the approval of the Hyogo College of Medicine Institutional Animal Care and Use Committee (18-068), and the ARRIVE guideline. All methods were carried out in accordance with the relevant guidelines of the Hyogo College of Medicine and the ARRIVE guideline, including any relevant details.

**Generation of Ctsb, Ctsd, Ctsl, CtsbΔPan, CtsdΔPan and Ctsd;CtslΔPan deficient mice in the pancreas.** We generated mice where the third exon of Ctsb, the second exon of Ctsd or the third to sixth exon of Ctsl was flanked by loxP sites. Both CtsbΔPan and CtsdΔPan ES cells were purchased from the International Mouse Phenotyping Consortium (IMPC) project. CtsbΔPan are previously described[21]. Mice homozygous for these modifications are denoted by CtsbΔPan or CtsdΔPan. CtsbΔPan or CtsdΔPan mice were crossed with Ptflafl/+ mice[42] and offspring carrying Ptflafl/+ and two copies of the floxed Ctsb, Ctsd or Ctsl allele (CtsbΔPan;Ptflafl/+, CtsdΔPan;Ptflafl/+, CtslΔPan;Ptflafl/+ were used in this study as a homozygous mutant (CtsbΔPan, CtsdΔPan or CtslΔPan) mice. The Ctsb;Ctsd, Ctsb;Ctsl or Ctsd;Ctsl DKO mice were obtained by mating the CtsbΔPan, CtsdΔPan and CtslΔPan mice.

**Antibodies.** Antibodies against cathepsin B (R&D Systems, AF965), cathepsin D (Santa Cruz, sc-6486), cathepsin L (R&B Systems, AF1515), p62 (MBL, PM045), LC3 (Cell Signaling Technology, 2775 and 43566S), UPLK1 (Sigma, A7481), LAMP2 (Sigma, L6688), Rab7 (Sigma, R8779), Becn1 (Santa Cruz, 11427), Akt (Cell Signaling Technology, 9272), Actin (Sigma, A5060), F4/80 (AbD Serotec, MCAP97), CD11b (Abcam, ab133357), CD138 (BD Biosciences, 553712) and Collagen-1 (Abcam, ab34710) were used as primary antibodies in this study. The secondary antibodies used in this study were anti-rabbit (GE Healthcare, NA9340) (Jackson ImmunoResearch, 115-035-146) and anti-goat (CHEMI-CON INTERNATIONAL, AP106P).

**Histological and immunohistochemical analyses.** For the histological analyses, pancreatic tissue was fixed overnight in 15% formalin, embedded in paraffin, sectioned, and subjected to HE and Azan-Mallory staining. Immunohistochemistry was performed using the antibodies listed above.

**Immunofluorescent staining with confocal microscopy.** For multiple immunofluorescent immunostaining, tissue samples were fixed in 15% buffered formalin and embedded in paraffin. In order to investigate fibrosis and inflammatory cells of macrophages and plasma cells, triple fluorescent immunostaining was performed with antibodies against Collagen-I, F4/80 and CD138. Subsequent staining was performed using an Opal 4-color IHC kit (Akoya Biosciences NEL820001KT). Signal amplification and covalent binding of fluorophore was achieved using a tyramide signaling amplification reagent (included in the Opal kit) that is conjugated with a different fluorophore for each cycle. Tissue samples were incubated overnight at 4 °C with the primary antibody, as follows: LC3(1:1500; detected by Opal 570 at 1:300), p62 (1:15:000; detected by Opal 520 at 1:300), Collagen-I (1:1200; detected by Opal 690 at 1:300), F4/80 (1:2000; detected by Opal 570 at 1:300) and CD138 (1:1200; detected by Opal 520 at 1:300). Counterstaining was performed using 4,6-diamidino-2-phenylindole (Akoya Biosciences). Fluorochrome labeling was viewed under a Zeiss LSM780 confocal microscope and documented using the LSM780 software program.

**Western blotting.** Pancreases were disrupted with TissueLyser LT (QIAGEN) and homogenized in RIPA buffer. The homogenates, which included 80 μg proteins, were subjected to SDS-PAGE (ATTO) and transferred onto Immobilon polyvinylidene difluoride membranes (Millipore). Blots were incubated with 5% nonfat dry milk in phosphate-buffered saline with 0.1% Tween 20 at room temperature for 1 h to block nonspecific binding, overnight at 4 °C with primary antibodies in blocking buffer, and finally with horseradish peroxidase conjugated secondary antibodies in blocking buffer at room temperature for 1 h. The membranes were developed with Chemi-Lumi One L and super (Nacalai Tesque), and analyzed in a WSE-6100H LuminoGraph1 (ATTO). Akt and actin were used as loading controls.

**Transmission electron microscopy.** Anesthetized mice were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. Pancreatic tissues were extracted after fixation. Slices of the fixed tissues were postfixed with 2% OsO4, dehydrated in ethanol and embedded in Epon 812 (Okenshoji Co.). Ultrathin sections were cut with an ultramicromotome (ultracut N or UC6: Leica). These sections were stained with uranyl acetate and lead citrate and examined on a Hitachi HT7700 or JEOL JEM-1230 electron microscope.

**Reverse transcription RT-PCR.** Total RNA was isolated using an RNeasy Plus Universal Mini Kit (Qiagen). CDNA was synthesized using a ReverTra Ace qPCR RT Kit (Toyobo). For the detection of Il6 mRNA, Tfα and Il1β, a Thermal Cycler Dice Real Time PCR System III was used with SYBR Premix Ex Taq (Takara).
Statistical analysis. Data in graphs are expressed as the mean ± standard error of the mean (SEM). Results were analyzed by an unpaired Student’s t-test to compare two groups and a one-way analysis of variance (ANOVA) to compare multiple groups using the JMP Pro13 software program (SAS Institute Inc.). P values of <0.05 were considered to indicate statistical significance.
34. Talukdar, R. et al. Release of cathepsin B in cytosol causes cell death in acute pancreatitis. *Gastroenterology* **151**, 747-758 e745. https://doi.org/10.1053/j.gastro.2016.06.042 (2016).
35. Sendler, M. et al. Cathepsin B activity initiates apoptosis via digestive protease activation in pancreatic acinar cells and experimental pancreatitis. *J. Biol. Chem.* **291**, 14717–14731. https://doi.org/10.1074/jbc.M116.718999 (2016).
36. Koike, M. et al. Purkinje cells are more vulnerable to the specific depletion of cathepsin D than to that of Atg7. *Am. J. Pathol.* **187**, 1586–1600. https://doi.org/10.1016/j.ajpath.2017.02.020 (2017).
37. Suzuki, C. et al. Lack of cathepsin D in the renal proximal tubular cells resulted in increased sensitivity against renal ischemia/reperfusion injury. *Int. J. Mol. Sci.* https://doi.org/10.3390/ijms20071711 (2019).
38. Koike, M. et al. Involvement of two different cell death pathways in retinal atrophy of cathepsin D-deficient mice. *Mol. Cell Neurosci.* **22**, 146–161. https://doi.org/10.1016/s1044-7431(03)00035-6 (2003).
39. Liaudet-Coopman, E. et al. Cathepsin D: newly discovered functions of a long-standing aspartic protease in cancer and apoptosis. *Cancer Lett.* **237**, 167–179. https://doi.org/10.1016/j.canlet.2005.06.007 (2006).
40. Zaidi, N., Maurer, A., Nieke, S. & Kalbacher, H. Cathepsin D: a cellular roadmap. *Biochem. Biophys. Res. Commun.* **376**, 5–9. https://doi.org/10.1016/j.bbrc.2008.08.099 (2008).
41. Benes, P., Vetvicka, V. & Fusek, M. Cathepsin D—many functions of one aspartic protease. *Crit. Rev. Oncol. Hematol.* **68**, 12–28. https://doi.org/10.1016/j.critrevonc.2008.02.008 (2008).
42. Taki, K. et al. GNAS(R201H) and Kras(G12D) cooperate to promote murine pancreatic tumorigenesis recapitulating human intraductal papillary mucinous neoplasm. *Oncogene* **35**, 2407–2412. https://doi.org/10.1038/onc.2015.294 (2016).

Acknowledgements
We are grateful to Erika Hayakawa, Ayumu Miwa and Keiko Mitani for their technical assistance. This work was supported by a Grant-in-Aid for Scientific Research (C) (KAKENHI) (19K08405) and a Grant-in-Aid for graduate students, Hyogo college of Medicine.

Author contributions
H.I. wrote the main manuscript text and prepared Figs. 1, 2, 3 and 4. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-85898-9.

Correspondence and requests for materials should be addressed to M.O.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2021