RIP3 attenuates the pancreatic damage induced by deletion of ATG7

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Invalidation of pancreatic autophagy entails pancreatic atrophy, endocrine and exocrine insufficiency and pancreatitis. The aim of this study was to investigate whether depletion of Rip3, which is involved in necroptotic signaling, may attenuate the pancreatic atrophy and pancreatitis resulting from autophagy inhibition. Autophagy and necroptosis signaling were evaluated in mice lacking expression of Rip3 in all organs and Atg7 in the pancreas. Acinar cell death, inflammation and fibrosis were evaluated by using of a compendium of immunofluorescence methods and immunobLOTS. Mice deficient for pancreatic Atg7 developed acute pancreatitis, which progressed to chronic pancreatitis. This phenotype reduces autophagy, increase apoptosis and necroptosis, inflammation and fibrosis, as well as premature death of the animals. Knockout of Rip3 exacerbated the apoptotic death of acinar cells, increased tissue damage, reduced macrophage infiltration and further accelerated the death of the mice with Atg7-deficient pancreas. The pancreatic degeneration induced by autophagy inhibition was exacerbated by Rip3 deletion.

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Autophagy is an evolutionarily conserved process; it is tightly regulated and activated under cellular stress and energy depletion in order to sequester cytoplasmic components for degradation/recycling and generation of substrates for bioenergetic reactions.1,2 Autophagy plays a cytoprotective role in keeping cellular homeostasis. Defective autophagy can lead to accumulation of waste proteins and damaged organelles (such as uncoupled mitochondria), along with ROS production, DNA damage and NFκB activation, culminating in cell death by apoptosis or necrosis and inflammatory responses.3

Inactivation of autophagy can be achieved by deletion of Atg genes, which are essential for this process. Suppression of autophagy in pancreatic cells causes local tissue destruction, leading to a variable degree of insufficiency of the endocrine and exocrine pancreas. Inactivation of floxed Atg5 in the pancreas by means of the p48-Cre recombinase expressed under the control of the Ptf1a promoter (Ptf1a-Cre) caused relatively mild pancreatic injury only in male, not in female mice.4 In contrast, local inactivation of floxed Atg7 using Pdx1-Cre provoked more severe pancreatic damage with no gender difference. Phenotypic alterations included acinar cell degeneration, pancreatic inflammation and extensive fibrosis, leading to pancreatitis, although no effects on life expectancy were reported.5 According to one report, removing floxed Atg7 using Pdx1-Cre in mice expressing a pancreas-specific Kras oncogene improved survival to around 3.6 months and 35% of the remaining mice failed to manifest any PanIN lesions.6

Altogether, it appears that depletion of pancreatic Atg7 or Atg5 induces local cell death, thereby triggering pancreatic atrophy and pancreatitis.5,7,8 Similarly, loss of Atg7 in β-cells causes insulin deficiency and type-1 diabetes due to the rarefaction of β-cells.9

Pancreatic acinar cells are highly efficient in synthesizing and releasing digestive enzymes, meaning that they are continuously exposed to high levels of misfolded or denatured proteins with potentially toxic functions. For this reason, pancreatic acinar cells must quench latent cellular damage by means of autophagy. Disabled autophagy has been linked to multiple distinct pathologies including inflammatory diseases such as pancreatitis.10

Chronic pancreatitis (CP) is initiated by acinar cell necrosis driving subsequent pancreatic fibrogenesis, according to the necrosis–fibrosis hypothesis developed by Kloppel et al.11,12 Importantly, some forms of necrosis may be regulated and executed by specific proteins.13 One version of regulated necrosis, necroptosis, is executed by a kinase, receptor interacting proteins 3 (Rip3), and its downstream phosphorylation target mixed lineage kinase domain-like protein (Mlkl). Phosphorylated Rip3 can recruit and phosphorylate Mlkl, causing its translocation to the plasma membrane culminating in its permeabilization and cell death.14–16 The role of Rip3 in pancreatitis is controversial. One study reported that Rip3-deficient mice are resistant against experimental acute necrotizing pancreatitis,13,17 while another study using identical mouse strains and pancreatitis models failed to detect any...
effect of Rip3. In contrast, Mlkl knockout clearly protected mice from caerulein-induced AP. As a possibility, Mlkl deletion may be more efficient in attenuating necroptosis-associated disease because Rip3 (and its upstream activator Rip1) have additional functions, for instance in the activation of caspase-8 and NFκB. This implies that the inactivation of Rip3 and Rip1 would not only inhibit necroptosis (which is a desired goal) but would also stimulate apoptosis and inflammation (which would be an undesired ‘side effect’).

Here, we characterized a novel system for suppressing pancreatic autophagy, by inactivating floxed Atg7 with the Crep48 system. This model was characterized by a particularly rapid destruction of the pancreas with multiple signs of apoptotic and necrotic cell death, AP and CP that caused premature death of the animals. Depletion of Rip3 furthermore exacerbated this phenotype, creating an interesting precedent in which the inhibition of this necroptosis mediator actually aggravates an organ defect caused by autophagy inhibition.

Results

Loss of pancreatic Atg7 suppresses pancreatic autophagy. We determined whether pancreatic Atg7 deletion is sufficient to trigger CP. For this, Atg7flox/flox mice (Atg7Δ/Δ) were bred with Ptf1a/p48-Cre mice to generate a pancreas-specific Atg7 knockout (Atg7Δpan), which was confirmed by PCR of genomic DNA (Supplementary Figure S1A). Immunoblot of homozygous Atg7Δpan mice indicated that Atg7 was present in the liver, yet was undetectable in the pancreas (Supplementary Figure S1B). Deletion of Atg7 from pancreatic cells was determined by quantitative IF for Atg7Δpan mice compared with heterozygous Atg7Δ/+ mice (Figure 1a). Loss of Atg7 and Atg5 by Atg7Δpan were confirmed by immunoblot image (Figure 1b).

Pancreata from Atg7Δpan mice exhibited a marked reduction in the abundance of LC3-II and hence the LC3-II/LC3-I or LC3-II/Erk1/2 ratios (Figure 1c), as well an accumulation of STQM1/p62, as detected by IF (Figure 1d) and immunoblot (Supplementary Figure S1C). Accumulation of STQM1/p62 in form of cytoplasmic dots has been previously reported following impaired autophagy signaling. Altogether, our data indicate that the autophagic process was disabled in the pancreas of Atg7Δpan mice.

Loss of pancreatic Atg7 causes CP. Hematoxylin–eosin staining and histopathology scoring revealed a remarkable increase in acinar cell vacuolization, inflammatory infiltration, fibrosis and islet damage in Atg7Δpan mice compared with Atg7Δ+/F controls, as detected by IF (Figure 1e) and immunoblot (Supplementary Figure S1C). Lymphocytic infiltration peaked at 8 weeks, followed by a decrease to normal levels at 20 weeks of age (Supplementary Figure S2F). The formation of acinar-to-ductal cell metaplasia (ADM), a reprogramming process that induces acinar cell trans-differentiation into a ductal-like cell type, may represent the precursors for PanIN lesions. Similar to fibrosis, ADM were not detectable at 4 weeks of age, yet increased over time in Atg7Δpan mice (Figure 2e). Altogether, these histopathological analyses indicate that lack of Atg7 expression in pancreatic acinar cells triggers AP that progressively develops into CP with signs of pre-malignancy.

Ablation of Atg7 increases acinar cell apoptosis and necroptosis. We next investigated whether the ablation of Atg7 in pancreatic acinar cells would induce apoptosis and necroptosis. The expression of the proteolytically mature form of caspase-3 (Figure 3a), as well as the enzymatic activity of caspase-3 (Supplementary Figure S3A), were significantly increased in Atg7Δpan compared with Atg7Δ+/F pancreata. Very similar results were obtained for caspase-8 (Figure 3b), caspase-9 (Figure 3c) and Bax (Figure 3d), which all were activated in Atg7Δpan pancreata, suggesting the ignition of both the intrinsic and extrinsic apoptosis pathways. We additionally confirm the expression of cleaved caspase-8 (Supplementary Figure S3B), cleaved caspase-9 (Supplementary Figure S3C) and Bax (Supplementary Figure S3D) by immunoblot experiments.
When compared with Atg7ΔΔ controls, Rip3 protein expression was significantly enhanced in pancreatic tissue from Atg7ΔΔ mice, as determined by quantitative IF (Figure 3e). Mlkl, which was recently described to interact with Rip3 and to participate in TNFα-induced pancreatitis,20 was also significantly elevated in Atg7ΔΔ pancreata (Figure 3f). High mobility group protein B1 (Hmgb1), a chromatin-binding protein that is released from the nuclei of necrotic cells, was reduced in Atg7ΔΔ pancreata (Figure 3g).2,31 We also confirmed the expression of Rip3 (Supplementary Figure S3E), Rip1 (Supplementary Figure S3H), Mlkl (Supplementary Figure S3F) and Hmgb1 (Supplementary Figure S3G) by immunoblot.

Collectively, these results indicate that Atg7 inactivation in pancreatic acinar cells is sufficient to cause substantial cell death by apoptosis and necrosis in the affected organ. Moreover, two necroptosis-relevant proteins, Rip3 and Mlkl, were overexpressed in the context of pancreatitis.

Pancreatic damage induced by Atg7 deletion is exacerbated by removal of Rip3. Since depletion of pancreatic Atg7 resulted into signs of necrosis and upregulation of Rip3, we investigated whether Rip3 depletion would be able to attenuate pancreatitis. For this, we crossed Atg7ΔΔ with Rip3ΔΔ mice. PCR reactions (Figure 4a) and immunoblots of pancreatic extracts from homozygous Atg7ΔΔ–Rip3ΔΔ and Atg7ΔΔ–Rip3ΔΔ mice confirmed that both Atg7 and Rip3 were removed (Figure 4b). Hematoxylin/eosin staining followed by careful scoring of pathological stages failed to reveal any major difference between Atg7ΔΔ–Rip3ΔΔ and Atg7ΔΔ–Rip3ΔΔ pancreata (Figure 4c). The overall pathological severity score of fibrosis, vacuolization, inflammation, edema and islet damage were similar for Atg7ΔΔ–Rip3ΔΔ and Atg7ΔΔ–Rip3ΔΔ mice, although this trend did not reach significance (Figure 4e). However, in the double-deficient Atg7ΔΔ, Rip3ΔΔ mice, pancreatic insulin levels further decreased compared with Atg7ΔΔ–Rip3ΔΔ mice, indicating an aggravation of islet cell dysfunction (Figure 4f). Collagen detection by IF revealed an exacerbated fibrosis in Atg7ΔΔ–Rip3ΔΔ mice compared with Atg7ΔΔ mice (Figure 4g). The aggravation of the endocrine dysfunction induced by removal of Rip3 might explain the shortened lifespan of Atg7ΔΔ–Rip3ΔΔ mice compared with Atg7ΔΔ mice (Figure 2h).
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(a) Histology of Atg7^{fl/fl} and Atg7^{fap} mice at different time points: Atg7^{fl/fl} 12 weeks, 4 weeks, 12 weeks; Atg7^{fap} 12 weeks, 8 weeks, 20 weeks.

(b) Histology Score:
- Fibrosis
- Vasculature
- Inflammation
- Islet Damage

(c) Collagen 1 (% area) comparison:
- Atg7^{fl/fl} n=2
- Atg7^{fap} n=4

(d) Immunofluorescence images of Atg7^{fl/fl} and Atg7^{fap} mice showing different cellular structures.

(e) Time course of fibrosis and ADM in Atg7^{fl/fl} mice.

(f) TGFβ expression levels in Atg7^{fl/fl} and Atg7^{fap} mice. Atg7^{fap} n=5.

(g) Tryptase expression comparison:
- Atg7^{fl/fl} n=4
- Atg7^{fap} n=4

(h) Survival curve of Atg7^{fap} and Atg7^{fap} Rip3^{fap} mice. Atg7^{fap} (n=15), Atg7^{fap} Rip3^{fap} (n=12), Atg7^{fap} Rip3^{fap} (n=7).
Loss of Atg7 and Rip3 exacerbates apoptosis and alters the immune cell infiltrate. The active form of caspase-3 was significantly increased in double-deficient Atg7<sup>pan−Δpan</sup>, Rip3<sup>Δ/Δ</sup> compared with Atg7<sup>Δ/Δ</sup> pancreata (Figure 5a). Very similar results were obtained for Bax (Figure 5b) and caspase-9 (Figure 5c), while the active form of caspase-8 exhibited a tendency to increase (Supplementary Figure S3B), indicating accelerated intrinsic and extrinsic apoptosis activity. Nuclear Hmgb1 staining in pancreatic acinar cells was not different between Atg7<sup>Δ/Δ</sup> and Atg7<sup>Δ/Δ</sup>-Rip3<sup>Δ/Δ</sup> mice (Supplementary Figure S3B). Surprisingly, macrophage infiltration was significantly reduced in the double-deficient Atg7<sup>Δ/Δ</sup>, Rip3<sup>Δ/Δ</sup> compared with Atg7<sup>Δ/Δ</sup> pancreata (Figure 5d). Similarly, removal of both Atg7 and Rip3 led to a nonsignificant reduction in MPO positivity with respect to Atg7<sup>Δ/Δ</sup> pancreata (Figure 5d). In addition, T and B lymphocyte infiltration that may be influenced by Rip3-depleted immune cell reprogramming did not change in the double-deficient Atg7<sup>Δ/Δ</sup>, Rip3<sup>Δ/Δ</sup> compared with Atg7<sup>Δ/Δ</sup> pancreata (Supplementary Figure S3I). Hence, in contrast to our expectations, Rip3 removal did not reduce pancreatic tissue damage, but rather enhanced this damage, though without an increase in local inflammation and even a reduction in macrophage counts.

Discussion

The present study reveals that pancreatic Atg7 deletion causes gender-independent severe pancreatitis with progressive cell death, fibrosis and inflammation. Atg7<sup>Δ/Δ</sup> succumbed to the ever-aggravating exocrine and endocrine dysfunction within a few months. Additional removal of Rip3 further accelerated pancreatic tissue damage and the associated premature mortality. Atg7 deletion alone increased necroptosis, apoptosis and inflammation, and additional RIP3 depletion exacerbated pancreatic apoptosis and reduced local macrophage infiltration, in line with the function of Rip3 not only in necroptosis but also in inflammatory responses.

The observation that Atg7-deleted pancreatic acinar cells progressively degenerate and die, as local tissue damage entails acute and chronic inflammation, emphasizes the importance of basal autophagy in maintaining pancreatic homeostasis. Deletion Atg7 in pancreatic acinar cells was sufficient to induce strong acinar cell death associated with AP that later progressed to CP. Such mice manifested local inactivation of the autophagy machinery tied to accelerated cell death by necroptosis and apoptosis, consequent pancreatic atrophy, inflammation and fibrosis, all of which have been recently described, though with a less severe phenotype, in other knockout models. Atg5 and Atg7 are both essential for autophagy, and lack of either of them close-to-entirely suppresses the autophagic processes. As compared with our results obtained by deletion of Atg7, depletion of pancreatic Atg5 reportedly caused much less severe pancreatic damage with gender-specific pathogenic effects that were more pronounced in males and hardly detectable in females. Although the gender-specific difference in Atg5-deleted mice remain elusive, the function of Atg7 and Atg5 may independent of autophagy processes. Thus it is possible that the impaired autophagy-independent function of Atg7 and Atg5 provide additional pancreatic injury and exacerbate cell death signals. The loss of autophagy-independent function may explain the reported differences of Atg7 and Atg5.

Yet another study in which floxed Atg7 was inactivated by Cre expressed under the control of the Pdx1 promoter (another essential pancreatic embryonic transcription factor frequently used for targeting genes to the pancreas), exhibited endocrine and exocrine damage, though again with less severe damage and only 60% of premature mortality. This is sharp contrast with our findings revealing full (100%) penetrance of the premature mortality phenotype. The exact mechanisms underlying these discrepancies remain elusive. As a possibility, the timing of the Atg7 depletion driven by the two developmental transcription factors p48 and Pdx1 may be important. During pancreatic development, Pdx1 is expressed earlier than p48, and co-expression of these two transcription factors in multipotent progenitor cells is critical for the cellular differentiation of all three main pancreatic cell types (acinar, endocrine and ductal cells). Using either of them as a driver for Cre, recombination is initiated in both endocrine and exocrine cells of the pancreas. After birth, Pdx1 is progressively restricted to endocrine pancreas, while p48 expression is confined to acinar cells. Moreover, p48 is more specific for the pancreas than Pdx1, which is also expressed in the duodenum, antral stomach and bile duct. In the Atg7-p48-Cre or Atg7-Pdx1-Cre mouse models, deletion of Atg7 of pancreas is almost completed before birth, but due to different temporal and spatial expression patterns of p48 and Pdx1, the effectiveness of the Atg7 knockout may be slightly different, hence affecting the severity of pancreatitis and the probability of early death.

Figure 2  
Atg7 depletion induces chronic pancreatitis. (a) Representative H&E staining of pancreatic tissue demonstrated that loss of pancreatic Atg7 increased pancreatic tissue damage in Atg7<sup>Δ/Δ</sup> mice (×20 objective; scale bar = 20 μm). (b) Histopathological evaluation of the pancreatic tissue included: For fibrosis: 0, no fibrosis; 1, mild fibrosis; 2, moderate fibrosis; 3, severe fibrosis. For vacuolization: 0, no vacuoles to 3, maximal vacuoles. For inflammation: 0, absent to 3, high infiltration. For islets damage: 0, no islets damage to 3, severe islets damage, as described previously. (c) Loss of pancreatic Atg7 induces fibrosis. Atg7 quantitation and representative IF microphotographs of Atg7<sup>Δ/Δ</sup> (n = 2) and Atg7<sup>Δ/Δ</sup> pancreata (n = 4) pancreatic tissue stained for DAPI (blue) and Collagen-1 (green) (anti-collagen, ab66043, 1/100, scale bar = 50 μm). (d) Representative transmission electron microscopy image of pancreatic acinar cells from control and Atg7-depleted mice. Arrows indicate collagen fibers indicating tissue fibrosis after loss of pancreatic Atg7. (e) Loss of pancreatic Atg7 increased severity of fibrosis and ADM. Histopathology scores were age dependent plotted for fibrosis (continuous line) and ADM (broken line). (f) Induction of fibrosis was accompanied with an increased level of the pro-fibrotic factor TGFα/TGFβ quantitation and representative IF/scattergram microphotographs of Atg7<sup>Δ/Δ</sup> (n = 4) and Atg7<sup>Δ/Δ</sup> (n = 5) pancreatic tissue stained for DAPI (blue) and TGFβ (green) (anti-TGFβ/ab66043, 1/100, scale bar = 50 μm). (g) Depletion of pancreatic Atg7 increases tissue Tryptosingen/Trypsin expression. Tryptosingen/Trypsin quantitation and representative IF/scattergram microphotographs of Atg7<sup>Δ/Δ</sup> (n = 4) and Atg7<sup>Δ/Δ</sup> (n = 4) pancreatic tissue stained for DAPI (blue) and Tryptosingen/Trypsin (green) (anti-Tryptosingen cs-67388, 1/50, scale bar = 50 μm). (h) Loss of pancreatic Atg7 reduces survival. Survival rate was analyzed by using Kaplan–Meier analysis comparing survival in Atg7<sup>Δ/Δ</sup> (continuous line), Atg7<sup>Δ/Δ</sup> (broken line) and Atg7<sup>Δ/Δ</sup>-Rip3<sup>Δ/Δ</sup> (broken/continuous line). Data are mean ± S.E.M. for the numbers of animals as indicated in the graph, *P < 0.05, **P < 0.01, ***P < 0.0001
Rip1 and Rip3 and activated Mlkl can promote necroptotic cell death in the absence of caspase-8 in multiple disease models. The regulation of necroptosis is complex and blockage of Rip1 or Rip3 suppresses not only necroptosis but also apoptosis and NFκB-dependent inflammation in a context-dependent fashion. However, neither Rip1 nor Rip3 is required for apoptosis execution. As mentioned in the Introduction, the contribution of Rip3 to caerulein-induced pancreatitis is highly controversial as it is in other disease models. In Atg7Δpan mice, removal of Rip3...
accelerated signs of pancreatitis, diabetes and reduced mortality rates. Surprisingly, depletion of Rip3 exacerbated pancreatic apoptosis accompanied with reduced macrophage infiltration. In a recent study, loss of Rip3 has been shown to promote tumorigenic T-cell infiltration in the context of Kras-driven pancreatic oncogenesis. This study showed that loss

Figure 4  Rip3 deletion did not reverse pancreatic tissue damage by the deletion of pancreatic Atg7. (a) Representative PCR agarose gel electrophoreses of mouse tail genotyping. (b) Representative immunoblot of double depleted Atg7 and Rip3 in pancreatic tissue of 12-week-old mouse. (c) Representative H&E staining of pancreatic tissue demonstrating that loss of pancreatic Atg7 and Rip3. Atg7Δpan-Rip3Δd mouse (x20 objective; scale bar = 20 μm). (d) Histopathology was scored for evidence of injury such as fibrosis, vacuolization, inflammation and islets damage in 12-week-old mice. (e) Reduced serum α-Amylase and increased serum glucose level in 12-week-old Atg7Δpan and Atg7Δpan-Rip3Δd mice. (f) Reduced pancreatic insulin level in double-deficient Atg7Δpan-Rip3Δd mice compared with Atg7Δpan mice of 12 weeks of age. Insulin quantitation and representative IF microphotographs of Atg7Δpan (n=7) and Atg7Δpan-Rip3Δd (n=11) pancreatic tissue stained for DAPI (blue) and insulin (red) (anti-insulin B sc-377071, 1/50, scale bar = 50 μm). (g) Loss of pancreatic autophagy and necroptosis (Atg7Δpan-Rip3Δd) exacerbate fibrosis compared with Atg7Δpan mice. Atg7 and Rip3 quantitation and representative IF microphotographs of Atg7Δpan (n=5) and Atg7Δpan-Rip3Δd (n=7) pancreatic tissue stained for DAPI (blue) and collagen-1 (green) (anti-collagen ab6308, 1/50, scale bar = 50 μm). Data are mean ± S.E.M. for the numbers of animals as indicated in the graph, *P<0.05, **P<0.01, ****P<0.0001
of Rip3 resulted into decreased tissue infiltration by macrophages but increased T and B lymphocyte infiltration. Here, Atg7Δpan-Rip3Δ/d mice exhibited reduced pancreatic macrophage infiltration and MPO but no alteration in T and B lymphocyte densities within the pancreatic tissue. The reasons for these discrepancies appear elusive, although the context of the inflammatory responses (cancer versus pancreatitis) may be determinant.

In summary, Atg7Δpan and Atg7Δpan-Rip3Δ/d mice were born healthy, and no pancreatic injury was observed up to 4 weeks of age, followed by minor pancreatic damage at 8 weeks of age. It is only at the age of 8–20 weeks that Atg7Δpan mice exhibited strong destruction of their exocrine and endocrine pancreata, thereby compromising the survival of the animals all of which were dead at the age of 24 weeks. Local inactivation of the autophagic machinery then compromised the survival of pancreatic exocrine acinar cells that activated a diverse array of cell death mechanisms including apoptotic and necroptotic pathways. This process was followed by invasion of the pancreas by inflammatory leukocytes from the myeloid lineage, production of the pro-fibrotic factor TGF-β, and necroptotic pathways. This process was followed by invasion of the pancreas by inflammatory leukocytes from the myeloid lineage, production of the pro-fibrotic factor TGF-β, and necroptotic pathways.

**Figure 5** Exacerbated apoptosis and altered inflammation in double-deficient Atg7Δpan-Rip3Δ/d mice. (a) Depletion of Rip3 and pancreatic Atg7 (Atg7Δpan-Rip3Δ/d) increased pancreatic caspase-3 in 12-week-old mice. Caspase-3 quantitation and representative IF microphotographs of Atg7Δpan (n=5) and Atg7Δpan-Rip3Δ/d (n=5) pancreatic tissue stained for DAPI (blue) and Caspase-3 (red) (anti-active caspase-3 ab2302, 1/50, scale bar = 50 μm). (b) Depletion of Rip3 and pancreatic Atg7 (Atg7Δpan-Rip3Δ/d) increased pancreatic caspase-9 in 12-week-old mice. Caspase-9 quantitation and representative IF microphotographs of Atg7Δpan (n=5) and Atg7Δpan-Rip3Δ/d (n=5) pancreatic tissue stained for DAPI (blue) and Caspase-9 (red) (anti-Bax (sc-526, 1/50)). (c) Depletion of Rip3 and pancreatic Atg7 (Atg7Δpan-Rip3Δ/d) increased pancreatic Bax in 12-week-old mice. Bax quantitation and representative IF microphotographs of Atg7Δpan (n=5) and Atg7Δpan-Rip3Δ/d (n=5) pancreatic tissue stained for DAPI (blue) and Caspase-3 (red) (anti-Bax (sc-526, 1/50)). (d) Depletion of Rip3 and pancreatic Atg7 (Atg7Δpan-Rip3Δ/d) increased pancreatic caspase-9 in 12-week-old mice. Caspase-9 quantitation and representative IF microphotographs of Atg7Δpan (n=5) and Atg7Δpan-Rip3Δ/d (n=5) pancreatic tissue stained for DAPI (blue) and Caspase-9 (red) (anti-caspase-9 NB100-56118, 1/1000). (e) Reduced infiltration inflammation of macrophages (F4/80, NBP2-12506, 1/75) and early T-lymphocytes (MPO, ab9535, 1/50) in double-deficient Atg7Δpan-Rip3Δ/d (n=10) mice compared with pancreatic Atg7Δpan (n=7). Data are mean ± S.E.M. for the numbers of animals as indicated in the graph. *P<0.05, **P<0.01, NS = no significance.
Experimental design. Mice were bred under standard conditions and maintained on a C57BL6 background. Animal breeding was approved by the Institutional Animal Care and Use Committee in accordance with the guidelines of the University of Heidelberg and the Federal Animal Care and Use Board, Karlsruhe, Germany. All mice were sedated with pentobarbital (Nembutal, 60 mg/kg body weight, i.p.) and the whole pancreas was resected, described previously.2,31,40 Parts of the pancreas were immersed in liquid nitrogen and stored at –80 °C or immediately fixed in 4% buffered formalin solution, as described earlier.2 Serum samples were also taken and stored at –80 °C. Serum parameters including α-amylase and lipase were determined by our institutional blood analysis center.

Pancreatic histopathology. FFPE pancreatic tissue were cut into 4-μm thick sections and stained with hematoxylin and eosin. Histopathological evaluation of the pancreatic tissue included the severity of fibrosis, the activity of inflammation, the severity of inflammation, and the severity and activity of the perineural inflammation as described recently.41 Briefly, histopathological evaluation of the pancreatic tissue included: For fibrosis: 0, no fibrosis; 1, mild fibrosis; 2, moderate fibrosis; 3, severe fibrosis. For vacuolization: 0, no vacuoles to 3, maximal vacuoles. For inflammation: 0, absent to 3, high infiltration. For islets damage: 0, no islets damage to 3, severe islets damage, as described previously.5

Genotyping. DNA extraction was accomplished according to the protocol of the Fast Tissue-to-PCR Kit, using mouse tails. Four microtiter DNA extracts were added to a 25 μl PCR reaction system containing Dream Taq PCR Master Mix primers and nuclease-free water (Thermo Fisher Scientific, Waltham, MA, USA). DNA amplification was conducted in a Mastercycler personal PCR machine (Eppendorf, Germany), followed by separation on a 2% agarose gel containing DNA Stain G (SERVA, Heidelberg, Germany) under standard DNA electrophoresis conditions and UV illuminator visibility. The primers used in the PCR reaction are listed: Atg7 forward primer: 5′-TGCGCCTCTCCTG-CATGATGCT-3′; reverse primer: 5′-CAGAGACGAGACCATCAGCTC-3′; p48 cDNA forward primer: 5′-ACC GTCAGTCGATGATCTT-3′; reverse primer: 5′-ACCTGAAGA-TGTTCGCGATTCT-3′; Rip3 primer-1: 5′-CGGTATAGAAGCGCTTACCG3′; Rip3 primer-2: 5′-GCCGCCCATGCAACCTC-3′; Rip3 primer-3: 5′-CCAGAGGCCACCTG-TGA GCC-3′.

Immunofluorescence. IF was conducted using 4 μm thin FFPE pancreatic tissue sections obtained from Atg7 mice and human specimens and processed as described in detail previously.2,31,40 All images were processed using the StrataQuest software (TissueGnostics, Vienna, Austria), allowing the quantitation for inflammation as described recently.41 Briefly, histopathological evaluation of the pancreatic tissue included: For fibrosis: 0, no fibrosis; 1, mild fibrosis; 2, moderate fibrosis; 3, severe fibrosis. For vacuolization: 0, no vacuoles to 3, maximal vacuoles. For infiltration: 0, absent to 3, high infiltration. For islets damage: 0, no islets damage to 3, severe islets damage, as described previously.5

Immunoblot analysis. Immunoblot analysis was performed in order to evaluate variations in the expression of specific proteins involved in apoptosis, autophagy and necrosis signaling. Human frozen tissues were homogenized on ice as described previously.2 Protein loading control was performed with Erk2 (after using a Restore Western Blot Stripping Buffer Plus (Pierce Biotech., Rockford, IL, USA) to ensure equal protein loading, according to the instructions. Protein fragments were processed and analyzed by using computer-assisted software ImageJ (NIH, Bethesda, MD, USA).

EM observation. Freshly isolated pancreatic tissue pieces were fixed in 3% glutaraldehyde and embedded in Epon. Ultra-thin sections were prepared for EM observation.

Caspase-3 activity. The activity of caspase-3 was measured in pancreatic tissue extracts using a modified assay for measuring caspase activity as described previously.42 Results were expressed as the caspase-3 substrate cleavage in release fluorescent units, after subtraction of the nonspecific product formation (substrate plus inhibitor).

Statistical analysis. Statistical analysis was performed using Student’s t-test for each group. For all column data sets, analysis of identified outliers was performed and results were considered significant when P-value < 0.05, indicated with *, using GraphPad Prism 6 software for statistical calculations. (n = x) represent the numbers of animals x, included in this study. All results were reported as mean ± S.E.M. (standard error of the mean) as indicated with the significance score (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001) in the figure legend.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

Study concept, analysis and statistics: XZ, LX, LX, GK and FF, pathology: FB; supervision and funding: MWB, TH and FF; manuscript writing: MWB, GK and TH.
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