Intracellular galectins are carbohydrate-binding proteins capable of sensing and repairing damaged lysosomes. As in the physiological conditions glycosylated moieties are mostly in the lysosomal lumen but not cytosol, it is unclear whether galectins reside in lysosomes, bind to glycosylated proteins, and regulate lysosome functions. Here, we show in gut epithelial cells, galectin-9 is enriched in lysosomes and predominantly binds to lysosome-associated membrane protein 2 (Lamp2) in a Asn(N)-glycan dependent manner. At the steady state, galectin-9 binding to glycosylated Asn\(^{175}\) of Lamp2 is essential for functionality of lysosomes and autophagy. Loss of N-glycan-binding capability of galectin-9 causes its complete depletion from lysosomes and defective autophagy, leading to increased endoplasmic reticulum (ER) stress preferentially in autophagy-active Paneth cells and acinar cells. Unresolved ER stress consequently causes cell degeneration or apoptosis that associates with colitis and pancreatic disorders in mice. Therefore, lysosomal galectins maintain homeostatic function of lysosomes to prevent organ pathogenesis.
Susceptibility to lysosomal membrane permeabilization (LMP) is associated with lysosome dysfunction, cell death, and disease pathogenesis. One consequence of LMP is the leakage of lysosomal hydrolases into the cytosol, posing a risk for lysosomal cell death. Importantly, lysosomal damage or dysfunction has been linked to neurodegeneration or inflammatory bowel disease (IBD) in humans and targeting lysosomes for LMP-induced cell death is considered as a potential strategy for cancer therapy. Therefore, maintenance of lysosomal membrane integrity not only protects cells physically but also facilitates lysosome functionality to prevent intracellular stress and death. The heavily glycosylated Lamp proteins were modeled and shown to constitute an ~8 nm-thick glycoprotein coat called glycocalyx, which associates with the lysosomal membrane and helps build or maintain its physical integrity. Most Asn(N)-linked glycosylation is high-molecular-weight complex poly-N-acetyl-lactosamine (LacNAc) moieties which can protect the membrane from degradation by lysosomal hydrolases. Removal of N-linked glycans from Lamp1/2 causes their rapid proteolysis. As such, lysosome-associated membrane protein 2 (Lamp2) depletion in cells causes increased susceptibility to cell death and impaired lysosome fusion, and is involved in human pancreatitis. In contrast, overexpression of Lamp2 can reverse stress-induced autophagic flux blockade and subsequently alleviate LMP-induced lysosomal cell death. Loss of Lamp2 in humans leads to Danon disease, a lysosome and autophagy disorder linked to neurodegeneration or inflammation. In gut epithelial cells, Lamp2 is also abundantly expressed in colon crypts in healthy human tissues but significantly lower in patients with colorectal cancer (CRC), inflammatory bowel disease (IBD), ulcerative colitis (UC), and CD. Gal-9-mediated lysosome function regulates Paneth cells against infection. In addition to this type of intracellular stress, as autophagy, which can be triggered by ER stress, serves as a protective mechanism to resolve ER stress, we examined whether loss of Gal-9 is associated with increased susceptibility to disease pathogenesis.

**Results**

Gal-9-mediated lysosome function regulates Paneth cells. Lysosome dysfunction, unresolved endoplasmic reticulum (ER) stress, and impaired autophagy are pronounced factors for intestinal pathogenesis especially in autophagy-active Paneth cells. As Gal-9 was previously identified as an autophagy-sensitive factor that affects anti-mycobacterial response and therefore reported as a risk factor for Crohn’s disease (CD), we investigated the expression of Gal-9 in the intestine. Quantitative real-time PCR and immunofluorescence analyses showed Gal-9 is predominantly expressed in gut epithelial cells located at the crypts. Specifically, Gal-9 is weakly expressed in Lgr5+ epithelial stem cells but highly enriched in MMP7+ Paneth cells. Similar to mouse tissues, Gal-9 is also abundantly expressed in colon crypts in healthy human tissues but significantly lower in patients with colorectal cancer (CRC), inflammatory bowel disease (IBD), ulcerative colitis (UC), and CD. Intracellularly, we found in fresh crypts, Gal-9, in intestine-specific long form and common short form, is highly enriched in endolysosomes/lysosomes (Fig. 1a), which are known to contain extensively glycosylated lysosomal membrane proteins such as Lamp1/2, Limp1/2, and NPC1. Under transmission electron microscope (TEM), loss of Gal-9 in mice led to increased accumulation of aberrant degradative lysosomes (Fig. 1b), which was assayed by measuring the reduction of AO intensity. In addition to this type of intracellular stress, as autophagy, which can be triggered by ER stress, serves as a protective mechanism to resolve ER stress, we examined whether loss of Gal-9 is associated with increased susceptibility to disease pathogenesis.

**Intracellular functions for galectins have been documented in the context of their nuclear or cytosolic ligands.** Due to absence of complex glycans in the cytosol, cytosolic galectins were shown, likely only after vesicle rupture, to mediate a host surveillance mechanism for detection, repair, and clearance of damaged vesicles including lysosomes. Such lysosome-mediated lysesophagy is believed to be aided by cytosolic galectins, including galectin-3 (Gal-3), Gal-8, and Gal-9, which can sense sterile lysosomal damage and are rapidly recruited to damaged lysosomes via binding to exposed glycans from the lysosomal lumen. At the molecular level, Gal-3 was shown to cooperate with TRIM16 and ATG16L1 to direct autophagy to defend cells against infection. In addition to this type of intracellular function, endogenous galectins can be secreted and endocytosed to reside in the endocytic or recycling compartments. For example, internalized Gal-3 and Gal-4 are involved in the apical endocytic-recycling of glycoproteins. Similarly, endocytosed Gal-9 has been shown to accumulate in lysosomes and is involved in apical sorting of proteins and lipids, as well as in the regulation of cancer cell death via autophagy or ubiquitination. Intriguingly, while galectins at the steady state via endocytosis have access to endolysosomal compartments which contain abundant luminal glycosylated membrane proteins, it has not been explored that under the physiological conditions whether intracellular or endocytic galectins could physically bind to glycosylated vesicular membrane proteins or whether such luminal interaction affects structure, conformation, maturation or functionality of endocytic compartments. In this study, we reveal that in gut epithelial cells lysosomal Gal-9 preferentially binds to N-glycosylated Lamp2 at the steady state to stabilize lysosomes, maintain lysosomal acidity, and facilitate lysosome-mediated autophagy. More importantly, we show that loss of Gal-9 in mice causes unstable lysosomes in autophagy-active intestinal Paneth cells and pancreatic acinar cells at the steady state, which consequently compromises autophagy functionality leading to increased susceptibility to disease pathogenesis.
in Gal-9−/− organoids. As activation of UPR and accumulation of ER stress might create a tumor-supporting microenvironment for cancer development34, our results therefore support why reduced Gal-9 expression in colon tissues might be associated with CRC development. Taken together, by promoting lysosome-mediated autophagy, Gal-9 targets autophagy-active Paneth cells in the intestine to maintain their homeostasis and help resolve ER stress.

Gal-9 regulates autophagy in Paneth cells to prevent colitis. As Gal-9 is predominantly expressed at the crypt base, we asked whether Gal-9 controls crypt homeostasis, which is mainly governed by the number of epithelial stem cells and Paneth cells located at this position35. Notably, under the physiological conditions, total crypt numbers in the ileum and colon were significantly reduced in Gal-9−/− mice (Supplementary Fig. 2f), suggesting Gal-9 regulates epithelial homeostasis in vivo. As
Fig. 1 Gal-9-mediated lysosome function alleviates Paneth cell ER stress. a Western blot analysis of subcellular fractionation of fresh ileum crypts. PNS: post nuclear supernatant, Cyto: cytosol fraction, Lyso: lysosome fraction. b Transmission electron microscopy (TEM) analysis of ileum Paneth cells. Lysosomes with typical, partial, or aberrant morphology are indicated and quantified. c Flow cytometry analysis of ileum crypts isolated from naïve mice and stained with monodansylcadaverine (MDC) to detect autophagic vacuoles. d Western blot analysis of autophagy markers in fresh ileum crypts. e Freshly isolated crypts were labeled with acridine orange (AO) and then exposed to a 488 nm laser to induce lysosomal damage. Confocal images after laser exposure were taken at the indicated time points. Loss of lysosome stability is determined by the decrease of AO red fluorescence over time which is normalized to cells exposed at 0 s. f TEM analysis of Paneth cells with normal (in wild-type) or degenerating (in Gal-9−/−) electron-dense core secretory vesicles, indicated by arrowheads (left panels). Normal well-developed granular ER with intact lacy strands (in wild-type) or abnormally dilated ER (in Gal-9−/−) around the nucleus of Paneth cells is indicated (middle panels). Normal (in wild-type) or abnormally dilated Golgi complex (in Gal-9−/−) around the ER region of Paneth cells is indicated (right panels). N: nucleus, ER: endoplasmic reticulum, M: mitochondria. g Western blot analysis of ER stress and apoptosis markers in ileum crypts. h Survival curve of tunicamycin-injected mice. i Quantitative real-time PCR analysis of ER stress-associated genes in ileum organoids which were cultured with or without recombinant mouse Gal-9, stimulated with or without tunicamycin to induce ER stress. Each symbol represents organoids derived from one mouse. Data shown are representative or combined (i) results from two independent reproducible experiments. Statistical significance (p value) is indicated (b, c, e, f, Unpaired two-tailed t-test, h: Kaplan–Meier survival curves). Data are presented as mean ± SD. Source data are provided as a Source data file.

unresolved ER stress might cause loss of epithelial stem cells or Paneth cells leading to enteritis22,36, it is possible increased ER stress in Gal-9−/− crypts might compromise the survival and/or function of stem cells and Paneth cells. Indeed, there were fewer Lgr5+ epithelial stem cells or MMP7+ Paneth cells in Gal-9−/− crypts (Fig. 2a). Functionally, the percentage of CD24low Ki67+ proliferating transit-amplifying (TA) cells and in vivo Edu incorporation in Gal-9−/− crypts were both significantly decreased (Supplementary Fig. 2g, h)37, indicative of reduced regeneration capacity. CD24+ Lysozyme-producing Paneth cells were also significantly reduced in Gal-9−/− crypts (Supplementary Fig. 2i)37. Regarding to immunity, we found Paneth-cell defect results in reduced bacteria killing by fresh crypts, which was assayed by anti-microbial contents directly released from stimulated crypts (Fig. 2b)38. Using primary organoids, we confirmed loss of Gal-9 compromised the percentage of Lgr5+ stem cells and Lysozyme+ Paneth cells, while exogenous recombinant Gal-9 effectively rescued the defects of growth and differentiation (Supplementary Fig. 2j). When immunity was tested, we found Gal-9−/− organoids showed less basal and IL-22-induced production of anti-microbial peptides (AMP), which could also be restored by exogenous recombinant Gal-9 (Fig. 2c). Intriguingly, while Gal-9−/− crypts showed reduced regeneration capability, slightly increased apoptosis, and reduced anti-microbial immunity at the steady state, Gal-9+/− mice surprisingly do not develop spontaneous colitis at least as the age of 8 months. To gain more insights whether Gal-9 predominantly targets Paneth cells in vivo, we generated Paneth cell-specific (Defa6-Cre+/Gal-9fl/fl) Gal-9 conditional knockout mice. Defa6-Cre mice drives Cre expression via the α-defensin promoter which is specific to Paneth cells22. We first analyzed and confirmed Gal-9 deletion in Paneth cells by gating on CD24high Lysozyme-producing crypt cells (Fig. 2d)37. Reproducibly, conditional Gal-9 deletion caused colon injury, a decrease in total crypt numbers, and autophagy blockade that likely associate with Paneth cell degeneration (Fig. 2e, f)23. Functionally, while there were fewer CD24high Lysozyme-producing Paneth cells (Fig. 2g, upper panels), CD24low Ki67+ proliferating transit-amplifying or stem cells were also reduced when Gal-9 was conditionally ablated in Paneth cells (Fig. 2g, lower panels). The stem-cell defect was likely due to disrupted niche regulation between Paneth cells and stem cells35,39, where Gal-9−/− Paneth cells might not produce sufficient niche factors to support nearby stem cells. Notably, fresh crypts also showed reduced lysosomal hydrolase activity (Fig. 2h), indicative of lysosome dysfunction in Gal-9−/− Paneth cells. Similar to global knockout mice, Paneth cell-specific Gal-9 conditional knockout mice were more susceptible to dextran sulfate sodium (DSS)-induced colitis, showing increased colon internal bleeding, more body weight loss, higher disease activity index, and enhanced colon injury (Fig. 2i, j). Furthermore, there was increased accumulation of LC3, Lamp2 and p62 in crypts (Fig. 2k), indicative of autophagy blockade in Gal-9−/− Paneth cells. As secretory Paneth cells have high autophagic activity, as a result, they are prone to ER stress that could associate with increased apoptosis22. Indeed, analysis of crypts in DSS-treated mice showed specific loss of Gal-9 in Paneth cells not only caused autophagy block, but also increased ER stress and apoptosis (Fig. 2k). Together these genetic evidence indicate Gal-9-mediated autophagy in Paneth cells is needed to protect mice against colitis.

Gal-9 regulates autophagy flux to prevent ER stress and LMP. To gain more insights about Gal-9-mediated autophagy, we abolished two forms of Gal-9 in colon epithelial cell CMT93 by CRISPR/Cas9 (Supplementary Fig. 3a). Similar to crypts, Gal-9−/− CMT93 cells showed more aberrant lysosomes with partially digested materials5, increased accumulation of LC3 and Lamp2, more MDC+ autophagic vacuoles, and higher lysosomal pH that was associated with reduced lysosomal hydrolase activity (Fig. 3a–d). Furthermore, not only there was increased accumulation of Lamp2+ lysosomes in Gal-9−/− cells, but those lysosomes were mostly peripherally localized in a manner reminiscent of Lamp1−/− cells (Supplementary Fig. 3b, lower left panels)40. This indicates that Gal-9 might regulate autophagosome maturation or lysosome perinuclear mobilization needed for fusion41. To test this hypothesis, we exploited a lysosome rupture model induced by L-Leucyl-L-Leucine methyl ester (LLOMe), a lysosomotropic compound known to induce lysosomal damage and facilitate perinuclear autophagosome formation to sequester damaged lysosomes42,43. By time-course (0–6 h) chasing of maturation and perinuclear formation or mobilization of Rab7+ CD63+ late endosomes or Rab7+ Lamp2+ endolysosomes post LLOMe (Supplementary Fig. 3c, both shown in large yellow perinuclear punctia)28,43, we confirmed that loss of Gal-9 indeed cause impaired perinuclear positioning of Lamp2+ Cathepsin-D+ lysosomes or Lamp2+ LC3+ autophagosomes during lysosomal damage (Supplementary Fig. 3b, d). The absence of perinuclear puncta was specifically caused by Gal-9 deficiency, as addition of exogenous recombinant Gal-9, but not recombinant Gal-3 or presence of other endogenous galectins, effectively restored puncta formation (Supplementary Fig. 3b, d, e). Reproducibly, only exogenous recombinant human Gal-9 can reduce accumulation of MDC+ autophagic vacuoles in human HT-29 Gal-9 knockdown (HT-29^KDf) epithelial cells (Supplementary Fig. 3f).
Next, we asked whether autophagy blockade in the absence of Gal-9 contributes to autophagy flux, which measures autophagic degradation and can be assayed by treating cells with autophagy-inducing rapamycin, with or without fusion-blocking bafilomycin. Notably, there was more basal and rapamycin-induced accumulation of LC3-II in Gal-9−/− cells, addition of bafilomycin further blocked LC3-II degradation (Fig. 3e), suggesting impaired autophagy flux which was also supported by increased p62 accumulation (Fig. 3b). To further confirm this, we analyzed endocytosis of exogenous red fluorochrome-labeled EGF and monitored perinuclear maturation and mobilization of green dye-labeled endosomes or blue dye-labeled lysosomes at the steady...
Fig. 2 Gal-9 regulates lysosomes in Paneth cells to prevent inflammation. a Immunofluorescence analysis of stem-cell marker Lgr5 (with Lgr5-eGFP reporter mice) (left panels) and Paneth cell marker MMP7 (right panels) in ileum crypts. b Freshly isolated ileum crypts were stimulated with carbachol (Cch), muramyl dipeptide (MDP), or IL-22 to induce release of anti-microbial contents. The supernatants were then incubated with live Yersinia and the percentage of bacterial killing was calculated after normalizing to unstimulated crypts. c Quantitative real-time PCR analysis of anti-microbial peptides in ileum organoids which were cultured with recombinant mouse Gal-9, stimulated with IL-22, or both. Each symbol represents organoids derived from one mouse. d Flow cytometry analysis of intracellular Gal-9 levels in the gated Paneth cells in ileum crypts. e Colon length was measured and freshly isolated crypts from naïve mice were counted under phase-contrast microscopy and quantified. f Electron microscopy images of ileum crypts with Paneth cells outlined in yellow (left panels). Vacuoles containing concentric multi-lamellar (finger-print-like) membrane structures, indicative of impaired autophagy, were observed in Defa6-Cre+Gal-9fl/fl mice (lower right panel). g Flow cytometry analysis of CD24+/CI-M6PR+ Paneth cells and CD24low/Ci-M6PR Paneth cells and CD24low/Ci-M6PR+ proliferating cells in ileum crypts from naïve mice. h Lysosomal hydrolase activity of freshly isolated ileum crypts was determined by specific substrates. i DSS-treated mice at day-5 or day-8 were analyzed for colon internal bleeding (indicated by yellow arrowheads) by endoscopy. j Percentage of body weight, disease activity index (combined scores of weight loss, rectal bleeding, and stool consistency), and colon length in DSS-treated mice were recombinant Gal-9. We next asked whether autophagy blockade of Gal-9 localization of Gal-9 (Fig.4a, Supplementary Fig. 4a)20,48. Intriguingly, 2 h after uptake, exogenous Gal-9 was exclusively localized in endolysosomes/lysosomes and almost undetectable in the cytosol. We next asked whether glycan-binding capability of Gal-9 is critical for its endolysosomal/lysosomal localization or regulatory function when Gal-9 is intrinsically expressed. To this end, Gal-9f/f cells were stably reconstituted with either wild-type or glycan-binding-deficient mutants of Gal-9 (Gal-9G64/269A and Gal-9G64/238A, or abbreviated as Gal-9RA2, Supplementary Fig. 4b, c)49. Indeed, we found glycan-binding capability of Gal-9 is required for its endolysosomal/lysosomal localization when Gal-9 is endogenously expressed (Fig.4b). Loss of glycan-binding in Gal-9 also led to increased accumulation of LC3 and Lamp2, more MDC1 autophagic vacuoles, higher lysosomal pH, increased LMP response, elevated ER stress, and increased basal or stress-induced apoptosis (Fig. 4c, Supplementary Fig. 4d–g). These results indicate that Gal-9 binding to glycosylated proteins in the endolysosomal/lysosomal lumen is critical for its lysosomal localization and regulatory function. Next, to identify Gal-9-binding proteins in endolysosomes/lysosomes, mass spectrometry of wild-type or Gal-9-RA2-reconstituted Gal-9−/− CMT93 cells at the steady state was performed by pull-down of Flag-tagged Gal-9. Notably, the volcano plot identified and showed differential Gal-9-binding proteins between glycan-binding sufficient (Gal-9-WT) and deficient (Gal-9-RA2) cells with adjusted p-value significance (Supplementary Fig. 4h). Next, the endolysosomal/lysosomal protein coding genes, based on a database, were selected from the LC-MS/MS result and a heatmap with a high cut-off threshold (score ≥ 300) was generated (Fig. 4d). The map shows the top 25 endolysosomal/lysosomal Gal-9-binding targets in wild-type versus glycan-binding deficient cells. Among the list, Igf2r (Insulin-like growth factor 2 receptor or cation-independent mannose-6-phosphate receptor, CI-M6PR) and Lamp2 were identified as the predominant Gal-9-binding proteins in endolysosomes/lysosomes. N-glycan-dependent Gal-9-M6PR and Gal-9-Lamp2 interactions were further confirmed by co-immunoprecipitation (Fig. 4e).

In the trans-Golgi network, newly synthesized lysosomal hydrolases are tagged with mannose-6-phosphate (M6P) groups exclusively to N-linked glycans in hydrolases. These M6P-tagged hydrolases are recognized by M6P receptor (M6PR) in the lumen of transport vesicles budding from the trans-Golgi network30. In this way M6P-tagged hydrolases are accurately transported to endolysosomal compartments, with the M6PR being trafficking in vesicles between the trans-Golgi network and these specialized compartments (Supplementary Fig. 5a)51. It was proposed that lysosomal enzymes are released from the receptor in the acidic lumen of these specialized M6PR/Lamp-enriched endosomes (so-called late endosomes or prelysosomes) and then subsequently packaged into mature lysosomes51,52. By density gradient ultracentrifugation for subcellular fractionation of CMT93 cells53, we found M6PR is more localized in EE1A1-enriched early endosomes but significantly less in Lamp2-enriched late endosomes/lysosomes (Supplementary Fig. 5b, c). As loss of M6PR has been associated with disrupted endolysosomal transport of hydrolases, decreased lysosome function, and impaired autophagy in cervical cancer cells34, we tested in CMT93 cells and found knockdown of M6PR (M6pRA2) indeed caused reduced activity of lysosomal hydrolases and impaired cargo degradation (Fig. 4f, g, Supplementary Fig. 5d), which were also found in Gal-9−/− cells. However, while Gal-9 co-immunoprecipitates with M6PR,
knockdown of M6PR does not affect lysosomal localization of Gal-9 (Fig. 4h), suggesting Gal-9 might indirectly associate with M6PR and is likely not involved in M6PR-mediated endolysosomal transport and cargo degradation. Due to technical difficulty in performing M6PR (>300 kDa) mutagenesis study in CMT93 cells (Supplementary Fig. 5e), we focused on Lamp2 and asked whether loss of Lamp1 and Lamp2 (Lamp1/2), two most abundant and heavily glycosylated lysosomal membrane proteins, contributes to functionality of lysosome or autophagy. Similar to Gal-9−/− but different from M6prKD cells, knockdown of Lamp1/2 (Lamp1/2KD) caused reduced Gal-9 endolysosomal/lysosomal localization (Fig. 5a, Supplementary Fig. 6a), increased
Lamp1/2 to poly-LacNAc chains confers autophagy functionality. To this also showed increased accumulation of MDC+ or CRC,59,60, we asked whether in CMT93 cells, binding of Gal-9 which also binds to poly-LacNAc (Fig. 6a, Supplementary 3-sialyl-lactose (SiaLac) with high affinity (Fig. 5d). Notably, Lamp2N175D-reconstituted Lamp1/2 prevent ER stress and associated cell apoptosis (summarized in Supplementary Fig. 6f), binding of Gal-9 to Lamp2 was site in Lamp2 did not cause its overall protein instability (Supplementary Fig. 6e). While mutation of each N-glycosylation cell was significantly decreased in the absence of β3Gnt2 or β4GalT1 (Fig. 6f). Together we conclude binding of Gal-9 to poly-LacNAc glycan chains contributes to lysosomal function of Gal-9 and confers lysosome-mediated autophagy and cargo degradation.

Gal-9 regulates lysosome function to prevent pancreatitis. Dysregulation of lysosome function, autophagy, and ER stress in acinar cells has been linked to pancreatic inflammation or fibrosis.10,31,62. As Gal-9 is involved in pancreatic carcinoma and Lamp2 is also associated with pancreatitis,10,26,63, we investigated whether Gal-9 has a function in the homeostasis of pancreatic acinar cells, which, similar to intestinal Paneth cells, are an autophagy-active secretory cell type. By acinar cell fractionation and immunofluorescence (Fig. 7a, Supplementary Fig. 7a), we found under the physiological condition, Gal-9 is weakly expressed in amylase-expressing acini and is detectable in Lamp2-enriched lysosomes but not in syncollin-enriched zymogen granules (ZG). Similar to ileum crypts, Gal-9−/− acinar cells exhibited increased accumulation of LC3, Lamp2, p62, and MDC+ autophagic vacuages, as well as higher lysosomal pH with decreased AO/LysoTracker staining (Fig. 7b, c, Supplementary Fig. 7b), indicative of lysosome dysfunction and autophagy blockade. Impairment of lysosome-associated autophagy in Gal-9−/− acinar cells was further supported by decreased lysosomal hydrolase activity (Fig. 7d) and impaired autophagy flux (Fig. 7e)44. Lysosome dysfunction was also observed under transmission electron microscopy (Supplementary Fig. 7c), showing aberrant accumulation of large autophagic vacules with partially digested materials which is reminiscent of Lamp1−/− mice.65 Notably, levels of amylase and trypsinogen in ZG were not altered in Gal-9−/− acinar cells (Supplementary Fig. 7d), suggesting it was not over-activation of amylase or trypsin that caused pancreas injury. Similar to ileum crypts, Gal-9−/− acinar cells also showed increased ER stress at the steady state and higher LMP response after photo-oxidation challenge (Fig. 7f, g)30, which were likely leading to increased basal or tunicamycin-induced apoptosis (Fig. 7f, g). LMP response in tunicamycin-treated CMT93 cells. Flow cytometry and time-course analysis of active caspase-3 in tunicamycin-treated CMT93 cells. Data shown are representative or combined (d, h) results from two independent reproducible experiments. Statistical significance (p value) is indicated (a, c, d, f, h, i). Unpaired two-tailed t-test. Data are presented as mean ± SD. Source data are provided as a Source data file.

Gal-9 binding to poly-LacNAc moieties regulates autophagy. By X-ray structural analysis, Gal-9 was shown to bind to LacNAc, the biantennary pyridylaminated oligosaccharide (BIPA), and α2-3-sialyl-lactose (SiaLac) with high affinity (Fig. 6a).57,58. As aberrant poly-LacNAc expression is involved in gastric carcinoma or CRC,59,60, we asked whether in CMT93 cells, binding of Gal-9 to poly-LacNAc chains confers autophagy functionality. To this end, β1,3-N-acetyl-glucosaminyl-transferase (β3Gnt2) and β1,4-galactosyl-transferase (β4GalT1)36,61, two key enzymes responsible for the synthesis of poly-LacNAc chains, were knockdowned (KD) by RNA interference (Fig. 6a, b). In β3gnt2KD and β4galT1KD CMT93 cells, binding of tomato lectin (LEA) to LacNAc and binding of Gal-9 to Lamp2 were indeed decreased (Fig. 6c, d)56, which likely led to autophagy blockade, as evidenced by increased accumulation of Lamp2 and MDC+ autophagic vacules (Fig. 6d, e). Notably, while exogenous Gal-9 effectively reduced MDC+ autophagic vacules in Gal-9−/− cells, it failed to do so in β3gnt2KD or β4galT1KD cells (Fig. 6e), suggesting the interaction of Gal-9 with poly-LacNAc chains is functionally linked to autophagy. Similarly, exogenous Gal-9 also failed to rescue defects of autophagy block (measured by LC3, Lamp2), ER stress (measured by GRP78), apoptosis (measured by cleaved caspase-3), and cargo degradation in β3gnt2KD or β4galT1KD CMT93 cells (Fig. 6f, g). Notably, similar to α-lactose pre-treatment, endocytosed recombinant Gal-9 within cells was significantly decreased in the absence of β3Gnt2 or β4GalT1 (Fig. 6f). Together we conclude binding of Gal-9 to poly-LacNAc glycan chains contributes to lysosomal function of Gal-9 and confers lysosome-mediated autophagy and cargo degradation.
required to alleviate ER stress and prevent tissue injury or aberrant induction of fibrosis.

Discussion

Our work here reveal a fundamental and unexplored function of Gal-9 in lysosomal lumen at the steady state in promoting autophagy and protecting cells from ER stress-associated LMP and cell death, especially in autophagy-active secretory cells. At the molecular level, we identified N-glycosylated M6PR and Lamp2 are major lysosomal binding proteins of Gal-9 in gut epithelial cells. It appears that the Gal-9-Lamp2 interaction contributes to endolysosome/lysosome function and cargo degradation. We also reveal that binding of Gal-9 to N-glycosylated Asn175 in Lamp2 is critical for functionality of autophagy under the physiological conditions without endolysosomal/lysosomal damage. We also provide evidence that Gal-9-mediated autophagy is indeed through its binding to poly-LacNAc chains in N-glycosylated targets. At the cellular level, we found Gal-9 preferentially targets intestinal Paneth cells and pancreatic acinar cells, and promotes autophagic degradation to resolve their ER stress and prevent cell death. At the global level in mice, we found lysosome dysfunction, due to loss of Gal-9, could serve as a shared cell-intrinsic defect in the intestine and...
pancreas that renders mice more susceptible to stress or organ pathogenesis. Therefore, highly secretory cells are homeostatically being protected from ER stress and apoptosis via Gal-9-mediated autophagy that otherwise could lead to tissue inflammation or injury. Our study not only provides clues why Gal-9, via targeting and regulating autophagy in Paneth cells, is a contributing risk factor for CD, but also supports that IBD and extraintestinal manifestations (EIMs), such as pancreatitis in this study, are pathologically linked. We therefore propose that lysosome and autophagy are cross-regulated, so patients with IBD or lysosomal/glycogen storage disease (LSD/GSD) are prone to have clinical manifestations simultaneously in multiple tissues or organs.

One key finding in our study is to reveal how a single N-glycosylation site (Asn175) in Lamp2 could have a predominant role in affecting lysosome function, autophagy, and ER stress. Unexpectedly, mutation of N-glycosylation sites in Lamp2 could cause increased, decreased, or unaltered binding of Lamp2 to Gal-9, suggesting in Lamp2 each N-glycosylation chain, either bound or unbound by Gal-9, might have a unique role in maintaining or affecting structural conformation that is consequently associated with protein-protein (Gal-9-Lamp2) interaction or functionality of Lamp2 in lysosome and autophagy. That is, the overall contribution of N-glycosylation to functionality of Lamp2 is the result of delicate and complicated structural positioning of each glycosylated chain, which will also be further affected by Gal-9 binding. As such, loss of one N-glycosylation site, while not affecting the overall binding of galectin, may still cause conformational change leading to protein dysfunction. In the case of Lamp2, Asn175 is close to the linker region (amino acid 189-233) which connects N- and C-terminal subdomains of Lamp2 (Supplementary Fig. 6). It is possible that loss of N-glycosylation in Asn175 affects the hinge function of linker peptides that consequently causes an unfavorable conformational change between two subdomains leading to Lamp2 dysfunction. Likewise, Asn175 in Lamp2 may affect the formation of Cys149-Cys185 disulfide bond, which was shown to stabilize the conserved β-prism fold found in the subdomains of Lamp protein family. Furthermore, it was proposed that not all glycosylation sites in Lamp2 can be accessible to β1,3-N-acetyl-glucosaminyl-transferase for glycan modification. As structural features might determine the addition of poly-LacNAc glycans which then serve as binding targets for Gal-9, it is therefore difficult to distinguish whether it is the poly-LacNAc chain itself or Gal-9-bound poly-LacNAc chain that actually contributes to the functionality of Lamp2. In addition, how many LacNAc moieties will be synthesized and added to a single N-glycosylation site is completely unclear.

However, in our study, we show that poly-LacNAc glycans are indeed required for functionality of Gal-9, as exogenous recombinant Gal-9 fails to rescue autophagy defects in Gal-9−/− cells in the absence of β3GnT2 or β4GalT1 transferase. It is technically challenging to prove that it is solely the Gal-9-Lamp2 interaction that regulates lysosome function, as the binding of Gal-9 to N-glycans in target proteins has no sequence specificity. Correlated to this, it is still unclear why in CMT93 cells other endogenous galectins, whose expression is more abundant than Gal-9, could not functionally rescue those defects in Gal-9−/− cells. However, our mass spectrometry analysis reveals that at least in CMT93 colon epithelial cells, Lamp2 is the predominant regulatory target for Gal-9 with respect to endolysosomal/lysosome function and our results support this conclusion. Phenotypically, similar to Lamp2−/− mice, Gal-9−/− mice also exhibited large autophagic vacuoles with partially digested materials in pancreas, decreased acinar cell homeostasis, and spontaneous pancreatitis. In Gal-9−/− and Lamp2−/− CMT93 cells, endolysosomal/lysosome function and cargo degradation were impaired, indicating a causal effect mediated by the specific interaction of Gal-9 to Lamp2. In humans, while Gal-9 polymorphism is associated with CD25, Lamp2 deficiency causes Danon disease with cardiomyopathy. It is possible that differential expression patterns of Gal-9 and Lamp2 in different cell types offset the requirement of their functions or there are compensatory mechanisms for Lamp2-mediated function when endolysosomal/lysosomal Gal-9 levels are low in certain cells. Interestingly, while intestinal disorders in either spontaneous or experimental models have not been reported in Lamp2−/− mice, impaired phagosome maturation and acidification in Lamp2−/− neutrophils were shown to cause periodontitis in mice, an oral inflammatory EIMs commonly reported in IBD patients. Therefore, phenotypical correlations in Gal-9 and Lamp2 deficiency with respect to IBD, pancreatitis, and EIMs might support Gal-9-Lamp2 axis as a potential therapeutic target for these disorders and therefore is worth further investigation in animal models or human subjects.

Methods

Animals. Galectin-9−/− mice were obtained from Dr. Liu, Fu-Tong, Institute of Biomedical Sciences, Academia Sinica, Taiwan. Galectin-9−/− mice were in C57BL/6 background and we have backcrossed Galectin-9−/− mice to C57BL/6 mice for two more generations before intercrossing them to have littermates for our experiments. Lgr5-eGFP-ires-creERT2 mice were obtained from Jackson Laboratory. Lgr5-eGFP mice were bred with Galectin-9−/− mice to obtain Lgr5−/− mice. Galectin-9 conditional knockout (Galectin-9flox/flox) mice in C57BL/6 background were generated via Transgenic Mouse Core Facility in National Taiwan University by the CRISPR/Cas9. To minimize off-target effects in CRISPR mice, Galectin-9flox/flox mice were first backcrossed to C57BL/6 mice for...
four generations before breeding into Paneth cell-specific Galectin-9 conditional knockout mice (Defa6-Cre; Galectin-9fl/fl). The Defa6-Cre mice were obtained from Dr. Richard S. Blumberg, Division of Gastroenterology, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School. Animals were maintained in a specific-pathogen-free (SPF) facility at a relative humidity 50 ± 10%, 20–26 °C, and in 12 h dark/light cycles (08:00–20:00 light). Experiments were performed on mature animals (age, 8–12 wk) in both male and female mice, unless otherwise indicated. Animal care and experimental protocols (Protocol ID: 15-12-908) have been approved by the Institutional Animal Care and Use Committee (IACUC) at the Institute of Biomedical Sciences, Academia Sinica. Ethical compliance has been observed in the animal study. Dr. John T. Kung is the chair of IACUC and Ethics Committee in Academia Sinica.
Antibodies. The following primary antibodies were used for immunofluorescence, immunohistochemistry or immunoblotting: rabbit anti-Ameylase [a48-511] (LSBio LS-B11116) (IB: 1:500; IF: 1:200), rabbit anti-ATP6V1A [EPR19270] (Abcam #193932) (IB: 1:200), mouse anti-β-actin [C4] (Santa Cruz sc-47778) (IB: 1:1000), rabbit anti-Careticulin [EPR3924] (Abcam #ab92516) (IB: 1:5000; IF: 1:100), rabbit anti-Synclinin [EPR13148] (Abcam #ab178415) (IB: 1:200), rabbit anti-Cathepsin B [EPR21033] (Abcam #ab214428) (IB: 1:1000), rabbit anti-Cathepsin D [EPR3057y] (Abcam #7852) (IB: 1:2000; IF: 1:100), mouse anti-CD63 [NVG-2] (Biologend #143902) (IB: 1:200), rat anti-Galectin-9 [108A2] (BioLegend #137901) (IB: 1:1000; IF: 1:100), rabbit anti-GFP [BGN/06/961] (Abcam #ab36362) (IF: 1:100), rabbit anti-MMP7 (Abcam #3868) (IB: 1:1000; IF: 1:200), mouse anti-Lamab1 [1D4B] (Abcam #ab25245) (IB: 1:1000), rat anti-Lamp1/2 [A93] (Abcam #ab25339) (IB: 1:1000; IF: 1:100), rabbit anti-Lamp2 (Thermo Fisher #PA1-655) (IF: 1:200), rabbit anti-LC3B [D11] (Cell Signaling Technology #3886) (IB: 1:1000; IF: 1:200), mouse anti-Lysozyme [BGN/06/961] (Abcam #ab36362) (IF: 1:100), mouse anti-MMPI7 (Abcam #ab5706) (IF: 1:100), mouse anti-p62 (Abcam #ab56416) (IF: 1:200), rabbit anti-Rab7 [D9F5] (Cell Signaling Technology #9367) (IF: 1:200), rabbit anti-tubulin (Cell Signaling Technology #2144) (IB: 1:1000), mouse anti-Chop [L63F7] (Cell Signaling Technology #2895) (IB: 1:100), rabbit anti-Cleaved Caspase-3 [Asp175] (Santa Cruz #sc-371) (IB: 1:1000), rabbit anti-CD24 [M1/69] APC-eFluor 780 (eBioscience #47-0242-82) (IB: 1:1000), rabbit anti-human galectin-9 (R&D Systems #AF2045) (IB: 1:1000), rabbit anti-human galectin-9 (Sigma #G48566) (IB: 1:1000), rabbit anti-MPR (cution independent) [EPR5990] (Abcam #ab124767) (IB: 1:10,000), and rabbit anti-Niennman Pick C1 antibody [EPR5209] (Abcam #ab134113) (IB: 1:2000), mouse anti-CK19 (Santa Cruz sc-33111) (IB: 1:1000; IF: 1:200), rabbit anti-α-SMA (Abcam #ab32575) (IB: 1:200), rabbit anti-EA1 (C4S810) (Cell Signaling Technology #3288) (IB: 1:1000), rabbit anti-Syntaxin 6 antibody [EP7665] (Abcam #ab140607) (IB: 1:5000), rabbit Anti-Rab5 antibody [EPR21801] (Abcam #ab218624) (IB: 1:1000), Fluorphore-conjugated secondary antibodies (all 1:300 dilution used) for immuonfluorescence were purchased from Cell Signaling Technology unless otherwise mentioned. The following secondary antibodies were used: anti-mouse Alexa Fluor 488 (#4412), anti-rabbit Alexa Fluor 594 (#8899), anti-rabbit Alexa Fluor 488 (#4412), anti-rabbit Alexa Fluor-594 (Thermo Scientific #A-11077). Horseradish peroxidase-conjugated secondary antibodies (all 1:10,000 dilution used) for immunoblotting were purchased from Jackson ImmunoResearch Laboratories, Inc. The following secondary antibodies were used: anti-rabbit mouse-HRP ([#15-035-03-05] and anti-rat-HRP ([#112-035-03-05]). The following antibodies were used for flow cytometry: rabbit anti-CD24 [M1/69] APC-eFluro 780 ( Bioscience #47-0242-82) (1:200), rabbit anti-CD45 [30-F11] Alexa Fluor-700 (Bioscience #56-0451-81) (1:400), rabbit anti-CD326 (EpCam) [Gl8.4] eFluo 450 (Bioscience #48-5791-82) (1:400), rabbit anti-CD44 [IM7] PC5y (BioLegend #130299) (1:200), rabbit anti-active Caspase-3 [C9-205] Alexa Fluor-647 (BD Biosciences #560626) (Sul/test), anti-Ki67 [D0A15] PCy7 (Bioscience #52-5698-82) (1:200), rabbit anti-Lysozyme (EC 1.6.3.1) FITC (1:100, Dako #F307201-1) (1:100), rat anti-mouse Galectin 9 [RG9-35] APC (BioLegend #136110) (1:200), anti-PPAR-γ [1D7] (LifeLearn #147-07) and DyLight 488 Labeled Lymphoprecisor Escentulum (Tomato) Lectin (Vector Laboratories #DL-1174-1) (5ug/ml). Flow cytometry data were collected by BD LSRII with BD FACS DIVA software (v6.1.3) and analyzed by Flowjo (v10.7).
**Fig. 6 Interaction of Gal-9 with poly-LacNAc is critical for functionality of lysosome.**

**a** Schematic illustration of N-glycosylated moieties with the indicated colored carbohydrates. Specific enzymes required for each N-glycan synthesis step are shown in red. Gal-9 binding targets are indicated by shaded green area, while epitopes for natural lectin binding are indicated by dotted rectangle.

**b** Western blot analysis of 4GalT1 proteins in gene-knockdown (KD) CMT93 cells.

**c** Western blot analysis of autophagy, ER stress, and apoptosis markers in the indicated CMT93 cells, cultured with or without exogenous rmGal-9.

**d** Flow cytometry analysis of tomato lectin (LEA) binding efficiency to LacNAc in β3GnT2 and β4GalT1 proteins in gene-knockdown (KD) CMT93 cells.

**e** Flow cytometry analysis of monodansylcadaverine (MDC) fluorescence analysis of the indicated CMT93 cells, cultured with or without exogenous rmGal-9.

**f** Western blot analysis of autophagy, ER stress, and apoptosis markers in the indicated CMT93 cells, cultured with or without exogenous rmGal-9.

**g** Immunofluorescence analysis of the indicated CMT93 cells, cultured with or without exogenous rmGal-9, for degradation of endocytosed fluorochrome-labeled EGF. Cells were pulsed with Alexa Flour 555-conjugated EGF for 5 min and confocal images were captured after 1 h post fixation. Data shown are representative or combined (e) results from two independent reproducible experiments. Statistical significance (p value) is indicated (c, e). Unpaired two-tailed t-test. Data are presented as mean ± SD. Source data are provided as a Source data file.
T7765), diluted in DMEM containing 3% fetal calf serum and 2% penicillin/streptomycin. Survival was monitored over 96 h. To examine ER stress-induced apoptosis, isolated acinar cells, ileum crypts, or tissue sections collected on day 2 after tunicamycin injection were examined by immunofluorescence or flow cytometry. Cell death analysis by TUNEL assay (In Situ Cell Death Detection Kit, TMR red, Roche #12156792910) was performed on tissue sections. In vitro tunicamycin experiments were examined using CMT93 cells. Cells were treated with 1 μg/ml tunicamycin for indicated time and ER stress, LMP, and apoptosis responses were analyzed by Western blotting or flow cytometry.

DSS-induced colitis. For colitis studies, mice were given 2% dextran sodium sulfate (DSS, MP Biologicals, molecular mass 36–40 kDa) in drinking water for the experimental days 1–5 followed by normal drinking water until the end of the experiment (day 8). Colonoscopy images were captured on day 5 and day 8 by using endoscopy system (TESALA AVS, Olympus, Tokyo, Japan).

EGF degradation assay. Epidermal growth factor (EGF), when binding to its receptor (EGFR), sends downstream signals and eventually the ligand-receptor
Fig. 7 Gal-9 regulates lysosomes in acinar cells to prevent pancreatic disorders. a Western blot analysis of subcellular fractions for localization of Gal-9 in fresh acinar cells. Cytosol marker α-tubulin, ZG marker syncoillin, and lysosome marker Lamp2 are indicated. PNS: post nuclear supernatant, Cyto: cytosol fraction, Lyso: lysosome fraction. b Western blot analysis of autophagy markers in fresh acinar cells. c Flow cytometry analysis of fresh acinar cells, stained with monodansylcadaverine (MDC) to detect autophagic vacuoles or with acridine orange (AO) to detect acidic compartments. d Activity of ZG digestive enzymes or lysosomal hydrolases in fresh acinar cells was determined by specific substrates. e Western blot analysis of autophagy flux by the LC3 marker in acinar cells, treated with rapamycin (R), bafloymycin (B), or both (R/B) for 16 h. f Western blot analysis of ER stress markers in fresh acinar cells. g The indicated AO-labeled acinar cells were exposed to a 488 nm laser to induce lysosomal damage. Confocal images after laser exposure were taken at the indicated time points. Loss of lysosome stability was determined by the decrease of AO red fluorescence over time which was normalized to cells exposed at 0 s. h Immunofluorescence of pancreatic tissue sections from naïve or tunicamycin-injected mice at day-2 (TMR-DUTP) analysis. i Serum amylase levels were determined by Fuji Dri-Chem Clinical Chemistry Analyzer FDC 4000i. m m Pancreatic tissues from aged mice were analyzed by H&E (left panels) for acini injury, and by Sirius red (middle panels) and α-SMA staining (right panels) for fibrosis. Data shown are representative or combined (d, i) results from two independent reproducible experiments. Statistical significance (p value) is indicated (c, d, g, i). Unpaired two-tailed t-test. Data are presented as mean ± SD. Source data are provided as a Source data file.

complex is internalized into lysosomes by endocytosis and is digested by lysosomal enzymes. Any defects in lysosomes will result in curtailing the ligand-receptor degradation. To check the EGF degradation in CMT93 cells, cells were grown on sterile silane coated glass slides for 24–48 h with or without rmGal-9 treatment. The cells were kept on ice for 10 min and washed with cold wash solution (Live Cell Imaging Solution containing 20 mM Glucose and 1% BSA) to inhibit endocytosis. Biotinylated EGF-Alexa Fluor 555 streptavidin (Molecular probes #E35350) was loaded on to the cells at a concentration of 2 μg/ml in wash solution and incubated at 37 °C for 5 min. After that cells were washed with warm wash solution and incubated in complete DMEM for 1 h at 37 °C and then fixed with 4% formaldehyde and later imaged. For live imaging, cells were grown on µ-Dish 35 mm, high (ibidi #81156) at 37 °C. Reactions were stopped by addition of 1.0 ml of 0.4 M glycine/NaOH buffer, pH 7.4. Further information on research design is available in the Nature Reporting Summary linked to this article.

Lysosomal stability assay. The isolated pancreatic acinar cells, leafy corals, or cultured CMT93 cells, treated with or without 0.5 µg/ml recombinant mouse galacto-9 for 48 h, were labeled with AO in the growth medium (1:10,000 dilution from the 2% AO stock). The cells were incubated at 37 °C for 20 min and then washed twice with live imaging solution (Invitrogen #A14291D). Live imaging solution was added to cover the cells in a confocal dish and immediately proceeded for microscopic analysis. Groups of cells in the brightfield (eight pre-defined areas of each well) were imaged in the brightfield and lysosomes (visible as black dots) were put in focus very rapidly to avoid any damage to lysosomes since the brightfield also contains blue light. The cells were immediately and continuously exposed to 489-nm light from a 100 mW diode laser while laser scanning micrographs where captured every 484 ms on a ZEISS LSM780 confocal microscope system in two channels defined by secondary bandpass filters for 485–555 nm (green) and LP650 nm (Red) light for 180 s. Loss of lysosome integrity in cells was visualized by the loss of red fluorescence, which is normalized to unexposed cells.

Hydrolase activity assay. β-Hexosaminidase, β-galactosidase, and α-mannosidase activity was assayed by estimation of p-nitrophenol liberation of p-nitrophenol-N-acetylβ-D-glucosaminide (Sigma #N9376), 4-Nitrophenyl-β-D-galactopyranoside (Sigma #N1252), and 4-nitrophenyl-α-D-mannopyranoside (Sigma #N2127), respectively. The assay mixture consisted of 5 mM substrate, 50 mM sodium citrate buffer pH 4.5, 0.1 % Triton X-100, 0.4 % BSA, and enzyme solution in a final volume of 0.2 ml. For β-Hexosaminidase activity, 10 µg cell extract was added to the assay mixture and incubate for 1 h at 37 °C. Reaction was stopped by addition of 1.0 ml of 0.4 M glycine/NaOH buffer, pH 10.4. The absorbance of liberated p-nitrophenol was measured at 405 nm. For β-galactosidase and α-mannosidase activity, 200 µg cell extracts were added to the assay mixture and incubate for 16 h at 37 °C. Reactions were stopped by addition of 1.0 ml of 0.4 M glycine/NaOH buffer, pH 10.4. The absorbance of liberated p-nitrophenol was measured at 515 nm.

Subcellular fractionation of CMT93 cells. A near confluent monolayer of CMT93 cells (3 x 106 cells) were serum starved for 2 h before homogenization to increase the lysosomal contribution. The cells were rinsed twice with PBS and then trypsinized with wash medium (0.25 M sucrose, 10 mM Tris-HCl) at room temperature. The wash medium is replaced with ice-cold Homogenization Media (HM) containing 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, 1x Protease inhibitors at pH 7.4 (p = 1:300 µl/ml), and the cells were scrapped with cell lifter (Coming #3008) into HM. The monolayer was pelleted and homogenized in 1 ml of HM by using 25 strokes of the pestle of a tight-fitting Dounce homogenizer. To ascertain the degree of cell breakage, they were checked under a microscope using Trypan Blue staining solution. After 90% of cell breakage, they were centrifuged at 2600 x g for 10 min at 4 °C to remove large cell debris and nuclei. The supernatant is post-nuclear supernatant (PNS), transferred to a new centrifuge tube and centrifuged at 20,000 x g for 20 min at 4 °C to pellet down the crude lysosomal fraction, and the supernant is cytosolic fraction. The crude lysosomal fraction consisting of mitochondria, lysosomes, endosomes, and peroxo-osomes. The pellet was washed three times with HM, and the cytosolic fraction was cleared by further centrifuging in a Ultra-Clear centrifuge tubes (Beckman Coulter ™ #A14291D) at 150,000 x g for 1 h at 4 °C in Beckman Coulter Optima MAX-XP Ultracentrifuge using TLA 100.3 S/N. 80U 3870 rotor to obtain light membrane fraction (LM) consisting of golgi apparatus and endoplasmatic reticulum. Cells pre-treated with or without rmGal-9 for another 2 h are incubated with rmGal-9 for another 2 h. The fractions are lysed in 1% NP-40 lysis buffer containing 1x Protease Inhibitor Cocktail and analyzed by western blotting.

Pancreatic acinar cell isolation and culture. To isolate pancreatic acinar cells, mice were euthanized and pancreas were collected and immediately washed with ice-cold HBSS twice. Pancreas was transferred to an appendor, well-mixed, and then transferred into a 50 ml tube. Centrifuged for 2 min at 450 x g. The supernatant was discarded to remove cell debris and blood cells. 5 ml of collagenase I solution (HBBS containing 10 mM HEPES, 200 U/ml collagenase IIA (Sigma #C2674), and 0.25 mg/ml of trypsin inhibitor (Sigma #T6522) was added and incubated for 20 min at 37 °C with 200 rpm shaking. The enzymatic reaction was stopped by adding 10 ml of cold HBSS with 5% FBS and then centrifuged for 2 min at 450 x g, 4 °C. The supernatant was aspirated to remove collagenase I solution. Cell pellets were resuspended, washed twice with 10 ml of cold HBSS with 5% FBS and filtered through a 100 µm strainer ( Falcon) to remove non-digested materials. The cells were suspended in Waymouth’s medium and transferred to 6-well plate and placed for 24 h in a 37 °C humidified 5% CO2 incubator. Purity of acinar cells was examined by PNA staining (Vector Laboratories, #FL-1071) via flow cytometry as described. To avoid acinar cells undergoing acinar-to-duodenal metaplasia, fresh acinar cells were used in most of the experiments. If acinar cells were to be treated for 16 h, level of ductal cell marker CK14 was measured.

Statistics. All statistical analyses were performed using GraphPad Prism v7.1 software. The results are expressed as the mean ± S.D. Statistical significance (p value) is indicated. Unpaired two-tailed t-test is performed unless otherwise indicated. The definition of symbols or sample numbers (n) in each figure for statistical analysis is provided in details in the section of “Reproducibility” in Supplementary methods.

Data availability. UniProtKB Mus musculus protein database (https://www.uniprot.org/) was used for mass spectrometry analysis. The heatmap (Fig. 4d) and volcano plot analysis (Supplementary Fig. 4h), based on the online dataset (mouse lysosomal protein coding gene, mLDGv v.1.2, http://lysosome.uniprot.org), were created and uploaded to a public database (Accesson code: MassIVE MIV000085310). Colored Fig. 6a was created and partially adapted from non-colored Fig. 1 (https://doi.org/10.1038/s41598-018-25580-9) by Chung-Geun Lee et al. We acknowledge the original authors’ contribution and indicate that their work is published under a CC BY 4.0 license. All uncorrected Western blots are provided in the Supplementary information. All data presented in this study are available to the public upon request via the corresponding author. Source data are provided with this paper.

Received: 8 August 2019; Accepted: 5 August 2020; Published online: 27 August 2020.
8. Kundra, R. & Kornfeld, S. Asparagine-linked oligosaccharides protect Lamp-1 and Lamp-2 from intracellular proteolysis. J. Biol. Chem. 274, 31039–31046 (1999).

9. Gonzalez-Polo, R. A. et al. The apoptosis/autophagy paradox: autophagic vacuolization before apoptotic death. J. Cell Sci. 118, 3091–3102 (2005).

10. Fortunato, F. et al. Impaired autolysosome formation correlates with Lamp-2 depletion: role of apoptosis, autophagy, and necrosis in pancreatitis. Gastroenterology 137, 360–351 (2009).

11. Cui, L. et al. The lysosomal membrane protein lamp2 alleviates lysosomal cell death by promoting autophagic flux in ischemic cardiomyocytes. Front. Cell Dev. Biol. 8, 31 (2020).

12. Nishino, I. et al. Primary LAMP-2 deficiency causes X-linked vacuolopathy and monocytopenia (Daton disease). Nature 406, 906–910 (2000).

13. Johannes, L., Jacob, R. & Leffler, H. Galectins at a glance. J. Cell Sci. 131, jcs208848 (2018).

14. Papadopoulos, C., Kravic, B. & Meyer, H. Repair or lysophagy: dealing with damaged lysosomes. J. Mol. Biol. 432, 231–239 (2020).

15. Thurston, T. L., Wandel, M. P., von Muhlinen, N., Foeglein, A. & Randow, F. Repair or lysophagy: dealing with damaged lysosomes. J. Mol. Biol. 432, 231–239 (2020).

16. Chauhan, S. et al. TRIMs and galectins globally cooperate and TRIM16 and galectin-3 control autophagy in endoderm-derived mouse homeostasis. Dev. Cell 39, 13–27 (2016).

17. Stechny, L. et al. Galectin-4-regulated delivery of glycoproteins to the brush border membrane of enterocyte-like cells. Traffic 10, 438–450 (2009).

18. Delacour, D. et al. Apical sorting by galectin-3-dependent glycoprotein clustering. Traffic 8, 379–388 (2007).

19. Itoh, A., Nonaka, Y., Ogawa, T., Nakamura, T. & Nishi, N. Galectin-9 induces fatal frustrated autophagy in KRAS mutant colon carcinoma that depends on elevated basal autophagic flux. Autophagy 11, 1408–1424 (2015).

20. Stechny, L. et al. Galectin-4-induced apoptosis vacuolization before apoptotic death. J. Cell Sci. 118, 3091–3102 (2005).

21. Fortunato, F. et al. Impaired autolysosome formation correlates with Lamp-2 depletion: role of apoptosis, autophagy, and necrosis in pancreatitis. Gastroenterology 137, 360–351 (2009).

22. Chauhan, S. et al. TRIMs and galectins globally cooperate and TRIM16 and galectin-3 control autophagy in endoderm-derived mouse homeostasis. Dev. Cell 39, 13–27 (2016).

23. Cadwell, K. et al. Paneth cells as a site of origin for intestinal galectin-9. Nature 503, 272–277 (2013).

24. Kumar, D. et al. Genome-wide analysis of the host intracellular network that regulates survival of Mycobacterium tuberculosis. Cell 140, 731–743 (2010).

25. Justins, L. et al. Host-microbe interactions have shaped the genetic architecture of in nature Commun. 567 (2017).

26. Takeda, T. et al. Upregulation of IGF2R evades lysosomal dysfunction-induced apoptosis of cervical cancer cells via transport of cathepsins. Cell Death Dis. 10, 876 (2019).

27. Biellmann, F., Henion, T. R., Burki, K. & Hennet, T. Impaired behavioral and feeding in male mice deficient for the beta-1-3 N-acetylglucosaminyltransferase-I gene. Mol. Reprod. Dev. 75, 699–706 (2008).

28. Toygayachi, A. et al. BetaGnT2 (B3GNT2), a major polylactosamine synthase: analysis of B3GNT2-deficient mice. Methods Enzymol. 479, 185–204 (2010).

29. De Araujo, M. E., Huber, L. A. & Stasyk, T. Isolation of endocytic organelles by density gradient centrifugation. Methods Mol. Biol. 329, 524–539 (2010).

30. Mellman, I. Endocytosis and molecular sorting. Annu. Rev. Cell Dev. Biol. 12, 575–625 (1996).

31. Griffiths, G., Hoflack, B., Simons, K., Mellman, I. & Kornfeld, S. The mannos-6-phosphate receptor and the biogenesis of lysosomes. Cell 52, 329–341 (1988).

32. de Araujo, M. E., Huber, L. A. & Stasyk, T. Isolation of endocytic organelles by density gradient centrifugation. Methods Mol. Biol. 424, 317–331 (2008).

33. Takeda, T. et al. Upregulation of IGF2R evades lysosomal dysfunction-induced apoptosis of cervical cancer cells via transport of cathepsins. Cell Death Dis. 10, 876 (2019).

34. Biellmann, F., Henion, T. R., Burki, K. & Hennet, T. Impaired behavioral and feeding in male mice deficient for the beta-1-3 N-acetylglucosaminyltransferase-I gene. Mol. Reprod. Dev. 75, 699–706 (2008).

35. Toygayachi, A. et al. BetaGnT2 (B3GNT2), a major polylactosamine synthase: analysis of B3GNT2-deficient mice. Methods Enzymol. 479, 185–204 (2010).

36. De Araujo, M. E., Huber, L. A. & Stasyk, T. Isolation of endocytic organelles by density gradient centrifugation. Methods Mol. Biol. 329, 524–539 (2010).

37. Griffiths, G., Hoflack, B., Simons, K., Mellman, I. & Kornfeld, S. The mannos-6-phosphate receptor and the biogenesis of lysosomes. Cell 52, 329–341 (1988).

38. de Araujo, M. E., Huber, L. A. & Stasyk, T. Isolation of endocytic organelles by density gradient centrifugation. Methods Mol. Biol. 424, 317–331 (2008).

39. Takeda, T. et al. Upregulation of IGF2R evades lysosomal dysfunction-induced apoptosis of cervical cancer cells via transport of cathepsins. Cell Death Dis. 10, 876 (2019).

40. Biellmann, F., Henion, T. R., Burki, K. & Hennet, T. Impaired behavioral and feeding in male mice deficient for the beta-1-3 N-acetylglucosaminyltransferase-I gene. Mol. Reprod. Dev. 75, 699–706 (2008).

41. Toygayachi, A. et al. BetaGnT2 (B3GNT2), a major polylactosamine synthase: analysis of B3GNT2-deficient mice. Methods Enzymol. 479, 185–204 (2010).

42. De Araujo, M. E., Huber, L. A. & Stasyk, T. Isolation of endocytic organelles by density gradient centrifugation. Methods Mol. Biol. 424, 317–331 (2008).

43. Takeda, T. et al. Upregulation of IGF2R evades lysosomal dysfunction-induced apoptosis of cervical cancer cells via transport of cathepsins. Cell Death Dis. 10, 876 (2019).

44. Biellmann, F., Henion, T. R., Burki, K. & Hennet, T. Impaired behavioral and feeding in male mice deficient for the beta-1-3 N-acetylglucosaminyltransferase-I gene. Mol. Reprod. Dev. 75, 699–706 (2008).

45. Toygayachi, A. et al. BetaGnT2 (B3GNT2), a major polylactosamine synthase: analysis of B3GNT2-deficient mice. Methods Enzymol. 479, 185–204 (2010).

46. De Araujo, M. E., Huber, L. A. & Stasyk, T. Isolation of endocytic organelles by density gradient centrifugation. Methods Mol. Biol. 424, 317–331 (2008).

47. Takeda, T. et al. Upregulation of IGF2R evades lysosomal dysfunction-induced apoptosis of cervical cancer cells via transport of cathepsins. Cell Death Dis. 10, 876 (2019).
62. Mareninova, O. A. et al. Impaired autophagic flux mediates acinar cell vacuole formation and trypsinogen activation in rodent models of acute pancreatitis. J. Clin. Invest. 119, 3340–3355 (2009).
63. Mareninova, O. A. et al. Lysoosome-associated membrane proteins maintain pancreatic acinar cell homeostasis: LAMP-2 deficient mice develop pancreatitis. Cell. Mol. Gastroenterol. Hepatol. 1, 678–694 (2015).
64. Gomez-Lazaro, M., Rinn, C., Aroso, M., Amado, F. & Schrader, M. Proteomic analysis of zymogen granules. Expert Rev. Proteom. 7, 735–747 (2010).
65. Tanaka, Y. et al. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. Nature 406, 902–906 (2000).
66. Li, N. et al. Loss of acinar cell IKKalpha triggers spontaneous pancreatitis in mice. J. Clin. Invest. 123, 2231–2243 (2013).
67. Geisz, A. & Sahin-Toth, M. A preclinical model of chronic pancreatitis driven by trypsinogen autoactivation. Nat. Commun. 9, 5033 (2018).
68. Antonini, F., Pizzilli, R., Angelelli, I. & Macarrì, G. Pancreatic disorders in inflammatory bowel disease. World J. Gastrointest. Pathophysiol. 7, 276–282 (2016).
69. Lassen, K. G. & Xavier, R. J. Mechanisms and function of autophagy in intestinal disease. Autophagy 14, 216–220 (2018).
70. Parkinsson-Lawrence, E. J. et al. Lysoosomal storage disease: revealing lysosomal function and physiology. Physiology 25, 102–115 (2010).
71. Visser, G. et al. Neutropenia, neutrophil dysfunction, and inflammatory bowel disease in glycogen storage disease type Ib: results of the European Study on Glycogen Storage Disease type I. J. Pediatr. 137, 187–191 (2000).
72. Terrasawa, K. et al. Lysoosome-associated membrane proteins-1 and -2 (LAMP-1 and LAMP-2) assemble via distinct modes. Biochem. Biophys. Res. Commun. 479, 489–495 (2016).
73. Carlson, S. R. & Fukuda, M. The polylactosaminoglycans of human lysosomal membrane glycoproteins lamp-1 and lamp-2. Localization on the peptide backbone. J. Biol. Chem. 265, 20488–20495 (1990).
74. Beertsen, W. et al. Impaired phagosomal maturation in neutrophils leads to periodontitis in lysosomal-associated membrane protein-2 knockout mice. J. Immunol. 180, 475–482 (2008).
75. Lira-Junior, R. & Figueredo, C. M. Periodontal and inflammatory bowel diseases: Is there evidence of complex pathogenic interactions? World J. Gastroenterol. 22, 7963–7972 (2016).

Acknowledgements

This work was supported by Intramural Grant (#2315-1040738 to J.W.S.) and Career Development Award (#104-CDA-L07 to J.W.S.) from Academia Sinica, Taiwan; and by grants (104-2320-B-001-001-MY2 to J.W.S.) from Ministry of Science and Technology (MOST), Taiwan. We thank Dr. Fu-Tong Liu for providing Defa6-Cre mice for our study. We also thank the Pathology Core (tissue section processing), Flow Cytometry Core, Confocal Microscopy Core facilities at the Institute of Biomedical Sciences, and Imaging Core (transmission electron microscope) Facility at the Institute of Cellular and Organismic Biology, and Institute of Plant and Microbial Biology of Academia Sinica. We thank the DNA Sequencing Core Facility at the Institute of Biomedical Sciences of Academia Sinica for providing DNA sequencing services. We thank the Academia Sinica DNA Sequencing Core Facility (AS-CHIP-108-115), National RNAi Core Facility at the Academia Sinica, for providing shRNA plasmids and related services, and Taiwan Mouse Clinic, at the Academia Sinica, for their technical assistance.

Author contributions

J.N.S. and H.H.L. designed and performed experiments, and contributed to manuscript writing and revision. H.Y.C. performed experiments and contributed to manuscript revision. S.Y.W. and C.N.S. performed experiments and provided experimental assistance. C.S.S and M.J.H. contributed to data collection and interpretation related to structural modeling. Y.M.C. and F.A.L. contributed to data collection and analysis related to mass spectrometry. F.T.L. contributed to manuscript revision. J.W.S. designed experiments and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-18102-7.

Correspondence and requests for materials should be addressed to J.-W.S.

Peer review information Nature Communications thanks Anna Gukovskaya and other anonymous, reviewers for their contributions to the peer review of this work. Peer review reports of this work.

Reprints and permission information is available at http://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) 2020