Autophagy maintains cell and tissue homeostasis through catabolic degradation. To better delineate the in vivo function for autophagy in adaptive responses to tissue injury, we examined the impact of compromised autophagy in mouse submandibular glands (SMGs) subjected to main excretory duct ligation. Blocking outflow from exocrine glands causes glandular atrophy by increased ductal pressure. Atg5−/−;Aqp5-Cre mice with salivary acinar-specific knockout (KO) of autophagy essential gene Atg5 were generated. While duct ligation induced autophagy and the expression of inflammatory mediators, SMGs in Atg5−/−;Aqp5-Cre mice, before ligation, already expressed higher levels of proinflammatory cytokine and Cdkn1a/p21 messages. Extended ligation period resulted in the caspase-3 activation and acinar cell death, which was delayed by Atg5 knockout. Moreover, expression of a set of senescence-associated secretory phenotype (SASP) factors was elevated in the post-ligated glands. Dysregulation of cell-cycle inhibitor Cdkn1a/p21 and activation of senescence-associated β-galactosidase were detected in the stressed SMG duct cells. These senescence markers peaked at day 3 after ligation and partially resolved by day 7 in post-ligated SMGs of wild-type (WT) mice, but not in KO mice. The role of autophagy-related 5 (ATG5)-dependent autophagy in regulating the tempo, duration and magnitude of cellular stress responses in vivo was corroborated by in vitro studies using MEFs lacking ATG5 or autophagy-related 7 (ATG7) and autophagy inhibitors. Collectively, our results highlight the role of ATG5 in the dynamic regulation of ligation-induced cellular senescence and apoptosis, and suggest the involvement of autophagy resolution in salivary repair.

Cell Death and Disease (2014) 5, e1478; doi:10.1038/cddis.2014.428; published online 23 October 2014
autophagy in regulating the injury responses in submandibular glands (SMGs) have not been explored.

To explore how autophagy contributes to salivary (patho)physiology, we established a transgenic mouse model deficient for ATG5 in the salivary acinar cells. Previously, we have identified a role for basal autophagy in salivary homeostatic mechanisms that restrict acinar cell size and the number of secretory granules. Here, we report that ligation of the major SMG excretory duct triggers the glandular atrophy and the induction of autophagy. By comparing the acute and subacute stress responses from autophagy-impaired and -competent SMGs with duct obstruction, we established the intrinsic roles of ATG5-dependent autophagy in modulating salivary inflammatory responses, stress-induced senescence and cell death, which all occur sequentially in response to tissue injury. Our results provide in vivo evidence that stress-induced autophagic response is indispensable for resolving premature senescence in duct cells of the ligated glands, whereas ATG5 deficiency leads to delayed acinar cell death.

Results

Acinar-specific autophagy deficiency. To assess the contribution of autophagy to tissue injury, we impaired Atg5 expression in salivary acinar cells by crossing mice expressing aquaporin 5 (Aqp5)-driven Cre recombinase with mice containing loxP sites that flank the third exon of Atg5 (Figure 1a) and subjected these mice to ductal ligation. Expression of the Aqp5-Cre transgene mirrored endogenous Aqp5 in the salivary glands and was acinar cell specific. Immunohistochemical (IHC) analyses revealed that ATG5 expression was not only abolished in AQP5-expressing acinar cells, but also decreased substantially in AQP5 non-expressors, mainly granular convoluted ducts (GCDs) and other duct cells in the SMGs of Atg5 KO mice (Figure 1b). This is because offspring from Atg5 KO;Aqp5-Cre and Atg5 KO crossbreeds exhibited an ATG5 hypomorphic phenotype in SMGs (Figure 1c, 4 versus F/F) introduced by the flox allele. As expected, higher ATG5-WT ATG12 signals were co-existed with higher abundance of lipidated microtubule-associated protein 1 light chain 3 (MAP1LC3-II) (and lower MAP1LC3-I) in the post-ligated SMGs of Atg5 KO mice at day 1, followed by a decline. Similar patterns of Il1a, Il1b and Ptgs2/Cox-2 mRNAs surges of different magnitudes were observed in ligated SMGs of Atg5 KO mice. To valid induction of proinflammatory cytokines, we measured TNF-α protein level by enzyme-linked immunosorbent assay (ELISA). TNF-α level peaked in L3 SMG from both genotypes at approximately 1.5 ng/mg total protein.

Autophagy alters duct ligation-induced morphological manifestations. Gross examination revealed that SMG size of Atg5 KO mice increased initially at day 1, which could be due to a combination of obstructed saliva outflow and an inflammatory response then leveled off at day 3 and atrophied at day 7 after duct ligation (Supplementary Figure S3). In comparison, significant glandular weight loss was detected in ligated SMGs of Atg5 KO mice at day 3 post ligation (Supplementary Figure S3). On average, we observed 19% decrease in gland weight compared with the contralateral control SMG in the same mouse, and the degree of atrophy in terms of weight loss was indistinguishable between Atg5 KO and Atg5 KO mice at day 7 post ligation (Supplementary Figure S3). However, gland weights of Atg5 KO mice were slightly higher than that of Atg5 KO mice at day 3 post ligation. Microscopically, GCD cells lost many secretory granules and saliva as a result of the outflow obstruction, was particular pronounced in L1 and L3 SMGs of Atg5 KO mice and to a lesser extent in Atg5 KO mice (Figure 2c). The level of duct dilation reduced slowly toward baseline from day 3 to
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Figure 1 Elevated basal expression of proinflammatory cytokines genes in Atg5-knockout SMGs. (a) Schematic diagram of generation of experimental mice. Mice developed a spontaneous heterologous Atg5 deletion after five generations of crossing between Atg5<sup>f/f</sup> and Atg5<sup>f/f</sup>;Aqp5-Cre mice, resulting in Atg5<sup>−/−</sup>:Aqp5-Cre mice. Atg5<sup>−/−</sup>:Aqp5-Cre (Atg5<sup>−/−</sup>) and Atg5<sup>−/−</sup>:Aqp5-Cre (Atg5<sup>−/−</sup>) mice were used in studies herein. (b) Immunohistochemical analyses show decreased ATG5 protein in both SMG granular convoluted ducts (GCDs; labeled D) and acinar cells (labeled A) of Atg5<sup>−/−</sup> mice, compared with that of Atg5<sup>WT</sup> mice. Bar: 100 μm. (c) Correlation of decreased ATG5 expression and impaired MAP1LC3 lipidation in SMGs among different genotypes. Equal amounts of whole SMG lysates from two individual mouse of the indicated genotype were analyzed for ATG5 and MAP1LC3 levels by western blots. ATG5 expression was greatly reduced in the SMGs of Atg5<sup>−/−</sup> than that in Atg5<sup>WT</sup> mice. This hypomorphic phenotype in floxed mouse line has been reported previously in the laxP mouse gene targeting system. In Atg5<sup>−/−</sup> mice, we examined red and green fluorescence patterns through acinar-specific Cre recombinase, whereas GCDs and other non-acinar cells were mostly marked with red fluorescence in the control gland (Figure 3a, Ctrl). Both the size and number of green fluorescent-marked acinar cells in duct-ligated SMGs of mT/mG;Aqp5-Cre mice were notably reduced at day 3 post ligation (Figure 3a). As expected, acinar cells had a distinct pattern of senescence apoptosis and others (Figure 2 and Supplementary Figure S4B). Furthermore, in agreement with the observation of robust inflammatory responses (Supplementary Figure S1), trichrome staining revealed extensive tissue fibrosis in SMGs following prolonged ligation (Figure 2d, Supplementary Figure S4B). Mucin-like material (non-eosinophilic staining) accumulated in acinar cells of L1 SMGs from both genotypes and L3 SMGs from Atg5<sup>−/−</sup> mice only, but not Atg5<sup>WT</sup> mice. Similar to H & E staining, trichrome staining revealed more severe fibrosis in L3 SMGs of Atg5<sup>−/−</sup> mice than the corresponding SMGs of Atg5<sup>−/−</sup> mice. (d) Quantitative RT-PCR analyses show elevated basal expression of selected proinflammatory cytokine genes in SMGs from Atg5<sup>−/−</sup> mice. Results are shown as mean ± S.D.; *P<0.05

day 7 post ligation. In parallel, secretory acinar cells became enlarged and were staining pale from lacking eosinophilic cytoplasmic staining at day 1 in both genotypes (Figure 2c, L1 inset). By day 3, enlarged acinar cells remained in SMGs of Atg5<sup>−/−</sup> mice (Figure 2c, L3, arrowheads); however, they were relatively sparse in the L3 SMGs of Atg5<sup>−/−</sup> mice (Supplementary Figure S4A). By day 7, the atrophied glands from both Atg5<sup>−/−</sup> and Atg5<sup>−/−</sup> mice had residual GCD structures and scattered acinar cells (Figure 2c, Supplementary Figure S4A). Furthermore, in agreement with the observation of robust inflammatory responses (Supplementary Figure S1), trichrome staining revealed extensive tissue fibrosis in SMGs following prolonged ligation (Figure 2d, Supplementary Figure S4B). Mucin-like material (non-eosinophilic staining) accumulated in acinar cells of L1 SMGs from both genotypes and L3 SMGs from Atg5<sup>−/−</sup> mice only, but not Atg5<sup>WT</sup> mice. Similar to H & E staining, trichrome staining revealed more severe fibrosis in L3 SMGs of Atg5<sup>−/−</sup> mice than the corresponding SMGs of Atg5<sup>−/−</sup> mice. (d) Quantitative RT-PCR analyses show elevated basal expression of selected proinflammatory cytokine genes in SMGs from Atg5<sup>−/−</sup> mice. Results are shown as mean ± S.D.; *P<0.05

To confirm the loss of acinar cells in duct-ligated L3 SMGs of Atg5<sup>−/−</sup> mice, we examined red and green fluorescence patterns in duct-ligated SMGs of mT/mG;Aqp5-Cre reporter mice (Figure 3a). As expected, acinar cells had a distinct pattern of green mG fluorescence due to excision of laxP-flanked red mT through acinar-specific Cre recombinase, whereas GCDs and other non-acinar cells were mostly marked with red fluorescence in the control gland (Figure 3a, Ctrl). Both the size and number of green fluorescent-marked acinar cells in duct-ligated SMGs of mT/mG;Aqp5-Cre mice were notably reduced at day 3 post ligation (Figure 3a). Additionally, AQP5 IHC staining revealed reduced number of AQP5-positive acinar cells in L3
Figure 2  Duct ligation-induced autophagy affects morphological manifestations in post-ligated SMGs. (a) Analyses of autophagy-related proteins in post-ligated SMGs from Atg5<sup>WT</sup> and Atg5<sup>KO</sup> mice. Main excretory ducts from right SMG of individual mouse were ligated for 0 day (control; Ctrl), 1 day (L1), 3 days (L3) or 7 days (L7) before tissue harvesting. Equal amounts of whole gland homogenates were analyzed by western blot using the indicated primary antibodies. One representative western blot is shown (N = 3). (b) Immunohistochemical analyses show intense staining of SQSTM1 in acinar cells (arrowhead) of L3 SMGs from Atg5<sup>KO</sup> mice. p62 accumulation was scattered in duct cells (arrow) of ligated glands of Atg5<sup>WT</sup> and control SMGs of Atg5<sup>KO</sup> mice, and became prevalent in both acinar and duct cells of post-ligated L3 SMGs from Atg5<sup>KO</sup> mice. Bar: 100 μm. (c) Autophagy impinges on morphological changes induced by duct ligation. SMGs were dissected from Atg5<sup>WT</sup> and Atg5<sup>KO</sup> mice at day 0, day 1, day 3 and day 7 post ligation. Duct obstruction led to gradually reduced eosinophilic secretory granules (bright pink) from GCDs in SMGs of both genotypes, and GCDs were severely dilated (asterisk), especially in L1 SMGs of Atg5<sup>WT</sup> mice. Acinar cells appeared enlarged in L1 SMGs of both genotypes. The enlarged acinar cells persisted in L3 SMGs of Atg5<sup>KO</sup> (arrowhead), but not Atg5<sup>WT</sup> mice, and very few morphologically typical acinar cells were seen in L7 SMGs of both genotypes. Ctrl: non-operated control SMG. Magnification: ×100, and enlarged view (inset): ×400. Bar: 100 μm. (d) Progression of tissue fibrosis in post-ligated SMGs. FFPE SMG sections were stained with Trichrome. Excess fibrous connective tissues (blue) were present in L3 and L7 SMGs of Atg5<sup>WT</sup> and Atg5<sup>KO</sup> mice. Note the lack of eosinophilic cytoplasmic staining, likely reflecting mucin-like glycoprotein products, in enlarged acinar cells (arrowhead) from L1 glands of both genotypes, and from L3 SMGs of Atg5<sup>KO</sup> mice only. Bar: 100 μm.
SMGs of Atg5<sup>WT</sup> mice compared with L3 SMGs of Atg5<sup>KO</sup> mice (Figure 3b).

**ATG5 KO delays ligation-induced apoptosis in SMG acinar cells.** We next evaluated the functional role of enhanced autophagy in ligation-induced cell death. In situ apoptosis assays revealed that the percentage of ApopTag-positive cells in duct-ligated SMGs of Atg5<sup>WT</sup> mice markedly increased at day 1 to day 3, and then decreased at day 7 (Figure 4a). Conversely, the percentage of ApopTag-positive cells peaked at day 3 and day 7 after ligation in Atg5<sup>KO</sup> mice (Figure 4a). Notably, most ApopTag-positive cells were localized outside the ductal system (Figure 4a). In agreement with in situ apoptosis data, caspase-3 activation was comparable between L3 and L7 SMGs, whereas cleaved caspase-3 levels markedly increased between L1 and L3 SMGs (Figure 4b, Supplementary Figure S5A). In addition, the message abundances of both death executor Casp3 and apoptotic mediators Bcl2l11/Bim and Bmf were significantly upregulated following duct ligation and fold induction of Bmf, compared with control, were higher in Atg5<sup>WT</sup> than in Atg5<sup>KO</sup> mice after 1 day of ligation (Supplementary Figure S2). A decrease in the abundance of acinar cell proline rich, lacrimal 1/mucin-10 (Prol1/Muc-10)
message (Figure 4c), but not MUC-10 protein (Figure 4b, Supplementary Figure S5B), was detected in SMGs from Atg5WT at day 3 and day 7 after duct ligation. In contrast, the abundance of ductal marker kallikrein 1 (Klk1) message was comparable between the two genotypes, except that it significantly increased by approximately 3-fold over the control level in L7 SMGs of Atg5WT mice only (Figure 4c). Notably, the KLK1 protein abundance was higher in SMGs from Atg5WT than that of Atg5KO at day 1 and day 3 after duct ligation (Figure 4b, Supplementary Figure S5C). Since duct
cells contribute to the regeneration of damaged exocrine glands, the increased KLK1 messages in L7 SMGs of Atg5\textsuperscript{wt} mice may reflect the initiation of regeneration.

To validate the participation of ATG5 in cellular injury, H\textsubscript{2}O\textsubscript{2}-induced cell death was examined in Atg5 Tet-Off MEF m5-7 cells\textsuperscript{22} as an in vitro model. ATG5 levels in m5-7 cells were regulated by concentration of doxycycline (Dox; Figure 4d) in culture medium. The decreased ATG5 level clearly compromised cellular autophagic capacity, evident by decreased MAP1LC3-II and increased p62 levels. Notably, an inverse ATG5-dose-dependent correlation was observed between ATG5 protein levels and cell viabilities upon H\textsubscript{2}O\textsubscript{2} treatment (Figure 4d). In addition to Atg5-KO cell model, we also tested sensitivity to H\textsubscript{2}O\textsubscript{2}-induced cell death affected by autophagy inhibition through Atg7-KO and autophagy inhibitors, chloroquine and bafilomycin A1 (BafA1), respectively, in Atg7-KO MEF and salivary Pa-4 cells. Both autophagy-compromised MEFs and salivary Pa-4 cells demonstrated reduced susceptibility to H\textsubscript{2}O\textsubscript{2}-induced cell death (Figure 4d). Taken together, we surmised that autophagy deficiency renders delayed cell death and/or greater apoptotic threshold upon stress.

**Decreased ATG5 primes SMG duct cells for duct ligation-induced senescent phenotypes.** Many upregulated proinflammatory mediator messages in ligated SMGs (Supplementary Figure S2) are also genes encoding SASP factors that are secreted by various cells undergoing premature senescence.\textsuperscript{23} Therefore, we asked whether duct ligation induced cellular senescence. SA-\textbeta-gal activity, which is strongly associated with senescent cells,\textsuperscript{24} was examined in control and ligated SMGs. While SMG SA-\textbeta-gal activity was detected sparingly at day 1 following duct ligation, SMGs from both Atg5\textsuperscript{wt} and Atg5\textsuperscript{KO} mice showed robust SA-\textbeta-gal staining after 3 days of ligation (Figure 5a, L3). Notably, SA-\textbeta-gal activity was mainly detected in duct cells of GCDs of both genotypes. In addition, SA-\textbeta-gal activity was detected in limited duct cells of control SMGs from Atg5\textsuperscript{wt} mice, but not Atg5\textsuperscript{KO} mice. After 7 days of ligation, the SA-\textbeta-gal staining remained intense in the duct cells of post-ligated SMGs of Atg5\textsuperscript{wt} mice, but markedly decreased in the corresponding counterparts of Atg5\textsuperscript{KO} mice (Figure 5a, L7). We postulated that the attenuated autophagy resulting from reduced ATG5 abundance in SMG duct cells of Atg5\textsuperscript{wt} mice (Figure 1b) attributes to the lingering SA-\textbeta-gal activity from day 3 to day 7 in KO mice.

To corroborate the results of SA-\textbeta-gal accumulation, we confirmed that other senescence markers, including cell-cycle inhibitors Cdkn2a/p16ink4a, Cdkn2a/p19Arf, Cdkn2b/p15 and Cdkn1a/p21,\textsuperscript{25} were upregulated following duct ligation (Supplementary Figure S2). Cdkn1a/p21 IHC analyses showed prominent Cdkn1a/p21 staining in GCDs of L3 SMGs (Figure 5b), independently validating duct ligation-induced senescent phenotype in duct cells of ligated SMGs. In that, Cdkn1a/p21 signal was stronger at day 1 post ligation in Atg5\textsuperscript{wt} mice than in Atg5\textsuperscript{KO} mice. Consistent with SA-\textbeta-gal activity (Figure 5a), the majority of GCDs in SMGs of Atg5\textsuperscript{wt} mice at day 7 post ligation stained positive for Cdkn1a/p21, but much less so in L7 SMGs of Atg5\textsuperscript{KO} mice (Figure 5b, Supplementary Figure S6).

We then used a cell-based assay to confirm the role of ATG5 in premature senescence by examining H\textsubscript{2}O\textsubscript{2}-induced senescent phenotype in Atg5 Tet-Off MEF m5-7 and Atg5-KO MEF cells.\textsuperscript{22} Treatment with sublethal concentrations of H\textsubscript{2}O\textsubscript{2} resulted in more SA-\textbeta-gal-positive Atg5-KO MEF cells than in WT MEF cells (Figure 5c). In addition, steady-state abundance of Cdkn1a/p21 protein was substantially increased by sublethal concentrations of H\textsubscript{2}O\textsubscript{2} treatment in Atg5-KO MEF cells (Figure 5d). Time course analysis revealed that p21 level peaked in KO but not in WT cells, 1 day after H\textsubscript{2}O\textsubscript{2} treatment, while p38 was activated immediate (2 h) after the treatment in both cells (Figure 5d). In addition, there is a positive correlation between protein levels of p21 and p62 accumulation (Figure 5d).

**Macrophage activation during duct ligation.** Compare with Atg5\textsuperscript{wt} mice, duct ligation induced sustained senescent phenotypes in SMG duct cells (Figures 5a and b) and delayed apoptosis in acinar cells (Figures 3 and 4) of Atg5\textsuperscript{KO} mice. Macrophages are key components in tissue repair and remodeling during wound healing. To determine whether distinct programs for clearance of damaged cells may have arisen based on cellular autophagy proficiency, we examined recruitment of macrophages following duct ligation. IHC staining for F4/80 revealed that tissue injury induced by duct ligation was accompanied by macrophage recruitment throughout the progression of the tissue injury (Supplementary Figure S7). Notably, F4/80-positive macrophages massively accumulated in injured SMGs at day 3 and day 7 after ligation, however, indistinguishable between WT and KO mice. In addition, expression level of the
chemoattractant chemokine chemokine (C-C motif) ligand 2/monocyte chemoattractant protein-1 (Ccl2/MCP-1), which was highly induced in SMGs following ligation (Supplementary Figure S2), was also comparable between the two genotypes. We concluded that levels of ATG5 did not affect macrophage activation in the duct ligation model.

Discussion

It is now generally recognized that autophagic process has a pivotal role in cellular homeostasis and tissue adaptive responses to stress and injury. Herein, we used an injury model of SMG duct obstruction and conditional Atg5 KO to explore the simultaneous interplay among autophagy, inflammation, stress-induced premature senescence and apoptosis in vivo. Ligation of excretory duct of exocrine glands, such as SMG, pancreas, induces acinar cell injury and death as well as ductal cell proliferation when the passage of secretions is blocked. However, the mechanism dictating the fate of individual cell types, in response to stress, within the same tissue is still unclear. Our report represents the first of its kind using conditional ATG5-KO mice to delineate molecular mechanism of duct cell survival and acinar cell death in atrophied SMGs following duct ligation in vivo. One major finding of this study is that combined effect from reduced ATG5 abundance and disruption of SMG duct ligation-induced...
autophagy causes a phenotype of delayed apoptosis in acinar cells and sustained premature senescence in duct cells of post-ligated SMGs from Atg5 KO mice (Figure 6).

Conceivably, depending on stress intensity, autophagy could regulate cell death pathway with two distinct outcomes: (1) the induction of autophagy in responses to ductal obstruction may be initially beneficial for ligated SMGs by eliminating superfluous proteins accumulated within acinar cells due to blocked secretory outflow. This will allow cells to adapt to the hostile conditions. (2) The sustained autophagy may exacerbate stress condition and induce autophagic cell death (or ‘autosis’) accompanied by ER dilation and nuclear convolution. Alternatively, autophagosomes could serve as platforms for caspase activation, degrading anti-apoptotic factors, and crosstalkling with cell killing mechanisms such as p53 and JNK pathways. Here we showed that acinar cells in post-ligated SMGs underwent programmed cell death after prolonged ligation. Although apoptosis occurred regardless of autophagy status, apoptotic cell death was delayed in ATG5-deficient acinar cells, compared with autophagy-competent cells (Figures 3 and 4a). Furthermore, autophagy-impaired cells were more resistant to H2O2-induce cell lethality (Figure 4d), supporting our notion. ATG5, although an essential protein in autophagosome formation, has additional functions beyond autophagy. For instance, both ATG5-dependent mitotic catastrophe and cell death in cells treated with cytotoxic agents have recently been reported. More importantly, calpain-cleaved ATG5 is known to activate caspases to enhance susceptibility toward apoptotic stimuli, thus ATG5 functions as a molecular link between autophagy and apoptosis. Conceivably, the absence of ATG5-dependent caspase activation could account for the initially delayed apoptosis in day-1 post-ligated SMGs of the Atg5 KO mice. Thus, the delayed Atg5 KO acinar cell death could be either autophagy dependent or ATG5 dependent or their combination.

Senescence is a state of stable cell-cycle arrest. Accumulating evidence from several in vivo senescent mouse models have suggested that the early onset of senescent phenotypes results from the expression, activation or deletion of genes involved in cell-cycle progression. Controversy over the relationship between autophagy and senescence exists because both a direct relationship and an inverse relationship were reported. In agreement with inverse relationships, the emergence of unstrained SA-β-gal activity in duct cells of SMGs was only detected from young (6–8 weeks old) Atg5 KO, but not from Atg5 KO mice (Figure 5a, Ctrl). Moreover, stress-induced senescent phenotypes were activated transiently in duct cells of post-ligated SMGs from Atg5 KO mice, however, persisted through day 7 in Atg5 KO mice (Figures 5a and b). We hypothesize that senescent phenotypes were transient in duct-ligated SMGs from Atg5 KO mice because autophagy removes superfluous proteins resulted from blunted secretion, thus maintains cellular homeostasis. When the clearance mechanism is impaired, damaged organelles and other proteins accumulate, leading to unresolved stress phenotypes in the autophagy-impaired SMGs. These observations of increased p21 protein level and intensified SA-β-gal signals in sublethal H2O2-treated Atg5-compromised MEF cells than the corresponding WT MEF cells (Figures 5c and d) supported the notion that lower ATG5 abundance in duct cells of Atg5 KO mice, compared with Atg5 KO mice (Figure 1b), primed these SMG duct cells susceptibility to persistent senescent-associated phenotypes (Figure 5a).

Extended outflow blockage led to extensive accumulation of p62 protein in SMGs of KO mice (Figures 2a and b). The polyubiquitin-binding protein p62, through interaction with LC3, is involved in delivering cargoes to the autophagy machinery or lysosomes for degradation. In autophagy-deficient Atg5 KO cells, excessive p62, instead, sequesters ubiquitinated proteins from proteasomal degradation, extending their half-life. Conceivably, p21, a short-lived protein, could therefore prevail in day-7 post-ligated SMGs of Atg5 KO mice through reduced turnover (Figure 5b). However, we cannot rule out the possibility that the stressed acinar cells might modulate the fate of duct cells in a paracrine and autophagy-dependent manner. Even though cellular senescence is generally considered as irreversible, escape scenarios, such as p53 pathway inactivation, exist to allow cells to re-enter the cell cycle. Duct ligation clearly leads to a transient senescent phenotype in post-ligated duct cells of Atg5 KO but not Atg5 KO mice. The exact underlying mechanism is still unclear. It is possible that autophagy keeps p62 levels in check, thus promotes resolution of senescent phenotype. Alternatively, autophagy could facilitate the removal of dead cells at the final stage of post-ligation period, minimizing the stress input from local environment.

Collectively, our results explicitly show that ATG5 deficiency primes SMG cells with increased expression of proinflammatory cytokine and Cdkn1a/p21 messages, and the lack of proper duct ligation-induced autophagy dysregulates the tempo and duration of sequential and overlapping elements of tissue injury, including inflammatory responses, stress-induced premature senescence and apoptosis. We therefore conclude that duct ligation-induced autophagy has a dynamic role in preserving the structural integrity of duct and acinar cells in duct-obstructed SMGs. One potentially important mechanism of regulating autophagy in post-ligated SMGs is through the mammalian target of rapamycin (mTOR).
Bozorgi et al.14 have recently reported while that mTOR is switched off in normal salivary glands, it gets switched on during the course of duct ligation. As mTOR inhibition is instrumental for autophagy induction, the activation of mTORC1 appears to be sufficient to suppress autophagy by preventing the formation of ATG complex.47 One possible explanation to reconcile our data with theirs is that mTOR activation could represent a feedback mechanism to escape from autosis or autophagy-induced cell death. Nonetheless, these discordances underlie the complexity of in vivo role ATG5 or autophagy partakes in post-ligated SMGs and hint at their respective interactions with other stress-triggered signaling pathways.

Materials and Methods

Generation of salivary acinar-specific Atg5-deficient mice. Mice with salivary acinar-specific ATG5 deficiency were generated by crossing Atg5lox/lox mice (Atg535) with Cre recombinase under the control of the Acyl promoter. Atg52−/−;Acyl-Cre genotypes arose from breeding Atg52−/− with Atg52lox/lox;Acyl-Cre mice for several generations, most likely through promiscuous Cre recombinase activity during developmental processes, such as gametogenesis.48 These mice were selected for mating with C57BL/6 mice to generate the Atg5−/−;Acyl-Cre mice used in this study. Crossing Atg5−/−;Atg5−/− mice generated Atg5−/−;Atg5−/− (Atg5−/− parental mice) and Atg5−/−;Atg5−/− mice were on a mixed C57BL/6 and 129Sv/EvTac background and appeared normal without gross abnormality. Genotypes were screened using the following PCR primer pairs: Atg5 forward, 5′-AGACCTAGTGCACCACTCATCA-3′, and reverse, 5′-CAGCCTGTAAGTGGTGTT-3′; and for Atg5, 5′-CAGGAGGAAGGTGTTCTCC-3′, and reverse, 5′-GCATCTCAGTAAGGTATTAACCTTCC-3′ to detect deleted the Atg5 allele; and Cre, forward, 5′-GCCCCAGAAGAAGAGGAAGTG-3′, and reverse, 5′-GCCGCTAACCAGTGAACACGAT-3′.

Generation of reporter mice. Reporter mTmG;Atg5-Cre mice were generated by crossing mTmG25 and Atg5-Cre mice.17

Cell line, H2O2 treatment and cell viability assay. WT, Atg5-KO and Atp7b/KO MEF cells (kind gift from Dr. Chengyu Liang, USC) were cultured in DMEM with 10% FBS. Salivary Pa-4 cells were cultured as we described previously.50 The Atp7−/−;Aqp5-Cre reporter (kind gift from Dr. Chengyu Liang, USC) were cultured in DMEM containing 10% FBS with 10% FBS. These cells were treated with up to 1 mM H2O2 alone or in the presence of doxycycline (Dox, 20 ng/ml) to fully suppress Atg5 expression, as we described previously.17 Expression, cells were cultured either in Dox-free media containing different concentrations of doxycycline (0, 1 and 20 ng/ml). At day 4, the cultures were treated with 1 μM BAF and incubated with 100 μM p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO, USA; N4465) in 5 mM in 0.1 M sodium acetate and 0.1% Triton X-100, pH 5.0, for 1 h at 37 °C. The reaction was stopped with the addition of NaOH (10 μl, 1 N) and absorbance measured at 405 nm using a microplate reader. For senescence induction, m7s-5 cells were maintained in growth media containing different concentrations of doxycycline (0, 20 ng/ml). At day 1 after seeding, cells were treated with sublethal dose of H2O2 (75 μM) for 2 h, washed and cultured in growth medium for 5 days. The H2O2 treatment and recovery were repeated once before cell harvesting. WT and Atg5-KO MEF cells were treated with 150 μM H2O2 using the same protocol as for m7s-5 cells, and the cells were analyzed for SA-β-gal activity as below.

Fluorescent microscopy. Fluorescent images of frozen sections from SMG of mTmG;Atg5-Cre mice were acquired using an Olympus AX70 microscope (Olympus, Tokyo, Japan). All images were compiled using Image-Pro (version 6.3, Media Cybernetics, Rockville, MD, USA).

Submandibular duct ligation. Due to gender During morphine in mouse SMGs, male mice (6-8 weeks old) were used exclusively in all ligation studies reported here. Mice were maintained under 12-h light/dark cycles with unlimited access to food and water. Unilateral ligation of the main excretory ducts of right SMGs, for a period from 1 to 7 days, was performed using surgical sutures, according to Turner et al.46 The contralateral left SMGs, which served as ligation controls, from the same mice were also harvested for comparison. Animals were euthanized at indicated times following ligation. Both control and ligated SMGs were removed and either snap-frozen in liquid nitrogen for western and real-time RT-PCR analyses or fixed in 4% parafomaldehyde for Hematoxylin and Eosin (H&E) staining and IHC analyses. City of Hope Institutional Animal Care and Use Committee approved all surgical procedures reported here.

IHC analysis. IHC staining was performed on 5-μm thick tissue sections prepared from formalin fixed, paraffin-embedded (FFPE) tissues. FFPE tissue sections were deparaffinized in xylene then hydrated through graded ethanol and distilled water. Samples were then quenched in 1% hydrogen peroxide and pretreated with steam from citrate buffer (Vector Laboratories, Burlingame, CA, USA; H-3300) to promote antigen retrieval. After antigen retrieval, slides were incubated in Protein Block for 1 h, then incubated with primary antibodies ATG5 (1: 800 dilution, RT, 1 h; Novus Biologicals, Littleton, CO, USA; NB110-53818), SQSTM1/p62 (1:1500 dilution, 4 °C, overnight; Wako Chemicals, Richmond, VA, USA; 018-22141), AQP5 (1:150 dilution, RT, 30 min; Alomone Labs, Jerusalem, Israel; AQP-005), p53 (1:50 dilution, RT, 30 min; Abd Serotec, Raleigh, NC, USA; MCA497R), or CDKN1A/p21 (1:1000 dilution, 4 °C, overnight; Abcam, Cambridge, MA, USA; ab2961), respectively. For ATG5, SQSTM1 and CDKN1A/p21 IHCs, slides were then washed in PBS and incubated with biotinylated secondary antibody (1: 200 dilution, RT, 30 min; Vector Labs, BA-1000) and Vectastain Elite ABC protocol followed (Vector Laboratories, PK-6100). For AQP5 and p80 IHCs, slides were washed in Dako buffer after primary antibody incubation and incubated with EnVision+ (Dako, Carpinteria, CA, USA; K4010) secondary antibody for 30 min. After three more washes in Dako buffer, slides were incubated with chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB), counterstained with hematoxylin, and mounted.

Real-time reverse-transcription PCR. Total RNA was extracted from SMGs with TRizol (Life Technologies, Grand Island, NY, USA), according to the manufacturer’s instructions. Synthesis of cDNA was performed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA; 170-8891). The cDNA was amplified using IQ SYBR Green Supermix and specific primer pairs in a My IQ Real-Time PCR Detection System (Bio-Rad, 170-8882). Relative mRNA expression levels were calculated using the ΔCt method, as previously described,47 against G6c; a message that we found stable throughout the duct ligation process. The primer pairs used are shown in Supplementary Table S1.

Western analyses. Whole tissue lysates were prepared using the Qproteome Mammalian Protein Prep Kit (Qiagen, Valencia, CA, USA; 370401) according to the manufacturer’s protocol, and then supplemented with Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). Equal volume of tissue or cell lysates were separated on SDS-PAGE and then immunoblotted with antibodies that recognize ATG5 (Novus Biologicals, NB110-53818), ATG7 (Cell Signaling, Danvers, MA, USA; 2631), SQSTM1/p62 (American Research Products, Waltham, MA, USA; 03-GP62-C), MAP1LC3 (Boster Biological, Monrovia, CA, USA; MBS422856), KALAEKIN1 (Boster Biological, Monrovia, CA, USA; MBS422856) and IHC analyses. City of Hope Institutional Animal Care and Use Committee approved all surgical procedures reported here.

Quantitation of TNF-α. Tissue lysates were diluted ten-fold with 1X Assay Diluent B and levels of TNF-α were determined by the TNFα Mouse ELISA Kit (Abcam, ab100747) following the manufacturer’s protocol.
SA-β-gal Staining. Control and duct-ligated SMGs were embedded in OCT compound for cryosection and stained for SA-β-gal activity according to the manufacturer’s protocol (Cell Signaling Technology, 9860). Tissue slides were counterstained with hematoxylin before mounting medium was applied. The H2O2-treated m5-7 and MEF cells were fixed in 2% formaldehyde, 0.2% glutaraldehyde in PBS for 10 min at room temperature and stained for SA-β-gal activity as above.

In situ apoptosis detection. Apoptosis was detected in FFPE tissue slides (prepared as described above) using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (EMD Millipore, S7100) following the manufacturer’s instructions. The percentage of apoptotic cells was calculated by dividing the number of ApopTag-positive cells over total number of nuclei enumerated from randomly selected ten microscopic fields (10X) of individual samples using ImagePro.

Statistical analysis. Gene expression data from quantitative RT-PCR were analyzed by using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). The non-parametric Mann-Whitney test was employed to determine the significant differences between the ligated groups and control group of each genotype as well as the significant differences between Atg5/− and Atg5+/+. Statistical significance of in situ apoptosis assays between Atg5/− and Atg5+/+ groups was determined using Student’s t-test. For cell viability assay, data were analyzed with ANOVA followed by Bonferroni-Honest.

Conflict of Interest. The authors declare no conflict of interest.

Acknowledgements. We are sincerely grateful to Drs. Chih-Pin Liu, Mei-Ling Kuo and Peiguo Chu for their valuable reagents, helpful suggestions and critical reading of manuscript. This work was supported in part by National Institute of Health Research Grants R01DE10742 and R01DE14183 (to DKA), RC1DE020335 (to DKA and KHL). We also thank Ms. Sofia Loera of Pathology Core at City of Hope for immunohistochemical analyses, members of Animal Resources at City of Hope, members of Am’s laboratory for helpful discussions, and Dr Margaret Morgan for editing. ZB is the Edgington Chair in Medicine, USC.

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Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)