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AUTOPHAGY SUPPRESSES OXIDATIVE STRESS AND P53 FOR ADULT MICE SURVIVAL AND IS ESSENTIAL FOR TELOCYTES SURVIVAL AND INTESTINAL HOMEOSTASIS

by

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ABSTRACT OF THE DISSERTATION

Autophagy Suppresses Oxidative Stress and p53 for Adult Mice Survival and Is Essential for Telocytes Survival and Intestinal Homeostasis

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Autophagy captures intracellular components and delivers them to lysosomes for degradation and recycling. Conditional autophagy deficiency in adult mice causes liver damage, and shortens lifespan to three months due to neurodegeneration. As autophagy deficiency causes p53 induction and cell death in neurons, we sought to test if p53 mediates the toxic effects of autophagy deficiency. Here we conditionally deleted Trp53 (p53 hereafter) and/or the essential autophagy gene Atg7 throughout adult mice. Compared to Atg7Δ/Δ mice, life span of Atg7Δ/Δp53Δ/Δ mice was extended due to delayed neurodegeneration and resistance to death upon fasting. Atg7 also limited apoptosis induced by the p53 activator Nutlin-3, suggesting that autophagy inhibited p53 activation. To test if increased oxidative stress in Atg7Δ/Δ mice was
responsible for p53 activation, \(\text{Atg7}\) was deleted in the presence or absence of the master regulator of antioxidant defense Nuclear factor erythroid 2-related factor 2 (\(\text{Nrf2}\)). \(\text{Nrf2}^-\text{Atg7}^{\Delta/\Delta}\) mice died rapidly due to small intestine damage, which was not rescued by co-deletion of \(p53\). Thus, autophagy limits p53 activation and p53-mediated neurodegeneration. In turn, NRF2 mitigates lethal intestine degeneration upon autophagy loss. These findings illustrate the tissue-specific roles for autophagy and functional dependencies on the p53 and NRF2 stress response mechanisms.

\(\text{Atg7}\) and \(\text{Atg5}\) are both essential autophagy genes (\(\text{ATG}\)) involved in autophagosome formation and deleting these genes leads to autophagy deficiency. As essential autophagy genes can function differently, we then sought to test whether whole-body conditional \(\text{Atg5}\) deletion in adult mice (\(\text{Atg5}^{\Delta/\Delta}\) mice) would have similar phenotype to that of \(\text{Atg7}\) deletion. In contrast to \(\text{Atg7}^{\Delta/\Delta}\) mice, \(\text{Atg5}^{\Delta/\Delta}\) mice surprisingly lived for less than five days. \(\text{Atg5}^{\Delta/\Delta}\) mice showed selective damage in the ileum part of intestine, with marked epithelial damage and loss of barrier function. In comparison to \(\text{Atg7}^{\Delta/\Delta}\) mice, the ileum of \(\text{Atg5}^{\Delta/\Delta}\) showed evidence of more rapid loss of autophagy, and loss of stem cells and malfunction of Paneth cells. Furthermore, \(\text{Atg5}^{\Delta/\Delta}\) mice had decreased active \(\beta\)-catenin in the ileum, the key transcription factor for Wnt signaling that is essential for intestinal stem cell renewal. \(\text{Atg5}^{\Delta/\Delta}\) mice lost PDGFR\(\alpha^+\) mesenchymal cells (telocytes) in the ileum, which are required to
provide Wnt signals to stem cells. Deletion of Atg5 more gradually overcame
the loss of ileum telocytes and stem cells and resulted in death much later from
neurodegeneration similar to deletion of Atg7 or Atg12. Atg5Δ/Δ telocytes
displayed significantly decreased aspartate and nucleotides, which caused
their loss. These findings reveal a novel function of autophagy in maintenance
of telocytes, Wnt signaling, and thereby stem cells essential for intestinal
homeostasis and the survival of adult mice. As impaired autophagy is
associated with Inflammatory Bowel Diseases, this suggests that failure to
maintain telocyte function is involved in development of this disease.
PREFACE

Part of this research was originally submitted to *Genes&Development* and is being reviewed.

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CHAPTER 1

INTRODUCTION
Autophagy is a dynamic catabolic process by which cells direct their own intracellular proteins, lipids, organelles and pathogens to the lysosomal compartment for degradation (Mizushima, 2010). Autophagy and the ubiquitin-proteosome system (UPS) are the two major cellular quality control systems which is responsible for clearance of proteins and organelles. These two systems formed an interactive network, where decision of pathway is self organized and controlled by different biophysical markers and compartmentalization. Usually, the UPS is in charge of the misfolded soluble proteins and short lived proteins, sending them to the proteosome for degradation; while autophagy deals with larger cytosolic structure and organelles such as damaged mitochondria and ER, long lived protein, protein aggregates and bacteria invading into the cell (Pohl and Dikic, 2019).

There are three types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy (hereafter autophagy) is the major type and is responsible for most of the protein aggregates and organelles degradation. Generally, the autophagy pathway involves the formation of double membrane-bound vesicles called autophagosomes that capture cargos including cytoplasmic proteins, organelles and bacteria. Autophagosomes with cargo then fuse with lysosomes to form autolysosomes where the cargo is degraded (Kaur and Debnath, 2015). The breakdown products are then released into the cytoplasm where they are recycled, and
specifically used as substrates for central carbon metabolism to sustain survival (Guo et al., 2016; Rabinowitz and White, 2010). By doing this, autophagy also serves as the quality control system by eliminating protein aggregates and damaged organelles (Mizushima and Komatsu, 2011).

**Autophagy Signaling Network**

Autophagy is controlled by the autophagy-related proteins (ATG) and other proteins that enable the formation of autophagosomes and recognition and capture of cargos (Mizushima and Komatsu, 2011). Autophagy is usually upregulated by different kinds of stress such as oxidative stress, hypoxia, nutrient deprivation and infection. During nutrient deprivation when cells are low in energy supply, autophagy is activated to recycle substrates to replenish the cellular ATP levels. Low ATP: AMP levels triggers the 5’ AMP activated protein kinase (AMPK), which is the main energy sensor and regulator of cellular metabolism for energy homeostasis, induced autophagy (Kim et al., 2011). In contrast to AMPK, another anabolic metabolic stress sensor, mammalian target of rapamycin multi protein complex 1 (mTORC1), which can be activated by mitogen-activated protein kinase (MAPK) and Phosphoinositide 3-kinase (PI3K)/ Protein Kinase B (AKT) pathway inhibits autophagy. Under nutrient deprivation, AMPK induction by metabolic stress either inhibits or bypasses mTORC1 to initiate autophagy (Jung et al., 2010).
Autophagy machinery is progressed in several steps: autophagosome formation (including initiation, nucleation and elongation), fusion with lysosome and degradation/cargo recycling. These steps are controlled by ATGs and other proteins. The core protein complex for initiation step is the uncoordinated-51-like kinase (ULK) complex, which is composed of ULK1, ATG13, ATG101 and focal adhesion kinase family interacting protein of 200-kDa (FIP200). During sufficient nutrient condition, mTORC1 binds to and phosphorylate the ULK1 at Serine (Ser) 757 and ATG13, prevents their activation and interaction to AMPK (Hosokawa et al., 2009; Kim et al., 2011). Upon starvation, following inhibition of mTORC1, ULK1 is dephosphorylated on the mTORC1 binding site, dissociated from mTORC1, and then undergoes autophosphorylation. The autophosphorylation activates ULK1 followed by phosphorylation of ATG13 and FIP200, thus the full protein complex is activated (Hosokawa et al., 2009; Jung et al., 2010).

Following ULK complex activation, it triggers nucleation of the phagophore by phosphorylating components of the nucleation complex class III PI3K (PI3KC3) complex, which is composed of PI3KC3, vacuolar protein sorting 34 (VPS34), BECN1 (Beclin-1, BCL-2 interacting protein) and general vesicular transport factor (p115), ATG14 for PI3KC3 complex I or UV radiation resistance associated gene protein (UVRAG) in PI3KC3 complex II. The main function for the nucleation complex is to generate local phosphatidylinositol-3-phosphate
(PI3P) at the phagophore assembly site (PAS), which is usually on the endoplasmic reticulum (ER) emanating membrane domains called the omegasome. However, the ER-mitochondria and ER-plasma membrane contact sites, the Golgi complex, plasma membrane and recycling endosomes are also proposed as PAS (Abada and Elazar, 2014). PI3P then recruits the PI3P effector proteins WD repeat domain phosphoinositide interacting proteins 2 (WIPI2) and zinc-finger FYVE domain containing protein 1 (DFCP1) to the omegasome via interaction with their PI3P binding domains (Dikic and Elazar, 2018). Afterwards, ATG9 containing vesicles generated by the cellular secretory pathway were recruited to the PAS, which delivers additional lipids and proteins for the upcoming membrane elongation (Karanasios et al., 2013; Nishimura et al., 2017).

Autophagosome elongation requires two ubiquitin-like systems. The first system is ATG8 family proteins, in mammals including Microtubule-associated proteins 1A/1B light chain 3B (LC3) and γ-aminobutyric acid receptor-associated proteins (GABARAPs). Nascent pro-ATG8 is processed by the cysteine protease ATG4 at its C-terminal to expose a glycine residue essential for the binding of phosphatidylethanolamine (PE). Processed ATG8s are activated by the E1-like ubiquitin activating enzyme ATG7 (for LC3 it is referred to as LC3-I), then conjugated to the PE with the help of ATG3 (for LC3 referred to as LC3-II) (Hamasaki et al., 2013). The other conjugation system is
ATG5-ATG12-ATG16L1 protein complex. ATG12 is activated by ATG7 and then binds to the ATG5 by E2 like ubiquitin conjugating enzyme ATG10 to form ATG12-ATG5 conjugate, which is working as an E3 like ubiquitin ligating enzyme essential for the efficient conjugating of PE to ATG8s (Hamasaki et al., 2013). ATG16L1 directly crosslinks with ATG12-ATG5 conjugate, or forms homooligomers by its coiled-coil domain, then crosslink several ATG12-ATG5 conjugates into a large protein complex, then the ATG16L1-ATG12-ATG5 complex is localized to the PAS by interaction of ATG16L1 with the WIPI2 (Dooley et al., 2014; Kaufmann et al., 2014). Interestingly, besides the processing of C terminal of ATG8 proteins, ATG4 is also capable of deconjugating ATG8s from PE to release them from the ER membrane and prevent phagophore formation (Satoo et al., 2009). Therefore, PE-conjugated ATG8s are protected from ATG4, possibly through inhibition of ATG4 by phosphorylation of ULK1 or mitochondria generated reactive oxygen species (ROS) (Pengo et al., 2017; Scherz-Shouval et al., 2007).

Before sealing the phagophore, one important step is to identify and capture the cargo in the autophagosome. Selective autophagy requires the labeling of cargo with capturing signals (prominently ubiquitin chains), which is recognized by autophagy cargo receptors that link cargo to the ATG8 family proteins autophagosome membrane (Dikic and Elazar, 2018). There are several autophagy cargo receptors identified, including SQSTM1.
(Sequestosome 1, or p62), NIP-like protein X (NIX), FuN14 domain-containing protein 1 (FUNDC1), Bcl2/adenovirus e1B 19 kDa protein-interacting protein 3 (BNIP3), calcium-binding and coiled-coil domain-containing protein 2 (NDP52), Optineurin (OPTN), and neighbor of BRCA1 gene 1 (NBR1) (Dikic and Elazar, 2018; Johansen and Lamark, 2011; Wild et al., 2011). All these cargo receptors have a conserved LC3-interacting region (LIR) essential for cargo interaction with the autophagosome membrane. Besides, p62 and NBR1 contains a ubiquitin associated (UBA) domain, OPTN has a ubiquitin binding in ABIN and NEMO (UBAN) domain and a zinc finger domain, and NDP52 has a zinc finger domain; all the domains are involved in recognition and uptake of these ubiquitinated cargos (Johansen and Lamark, 2011). To degrade protein aggregates, for example, p62 interacts with protein labeled with ubiquitin chains and recognize the LC3 through LIR to enable selective autophagic degradation and is degraded in the lysosome along with cargo (Johansen and Lamark, 2011). Damaged mitochondrion is another important type of cargo. Ubiquitin dependent degradation of mitochondria requires the participation of PTEN-induced putative kinase 1 (PINK1), a mitochondrial kinase; and Parkin, an E3 ubiquitin ligase. When mitochondria are damaged and then depolarized, PINK1 is stabilized and accumulates on the outer membrane of the mitochondria and followed by recruitment of Parkin (Narendra et al., 2010). Parkin ubiquitinates mitochondrial outer membrane proteins, then the damaged mitochondria are recognized by p62, OPTN or NDP52 and brought
to the autophagosome. Mitophagy can also occur in a Ubiquitin independent manner, which utilizes the NIX, FUNC1 or BNIP3 as cargo receptors. These receptors are mitochondria proteins which contain a LIR domain without UBA domain, therefore can directly function as a cargo receptor for mitophagy (Dikic and Elazar, 2018). Some lipids and phospholipids such as cardiolipin and ceramide also serve as cargo receptors for mitophagy. Cardiolipin is located in the inner membrane of mitochondria. However, it is externalized on the mitochondria surface upon mitochondrial damage, where it can be recognized by LC3 and recruited to autophagosome (Chu et al., 2013; Sentelle et al., 2012). Thus, autophagy receptors can recognize damaged organelles and bring them to autophagosome for degradation (Okamoto, 2014). Other autophagy receptors such as OPTN and NDP52 can similarly mediate the degradation of invading bacteria by ubiquitination (Wild et al., 2011).

The next step following elongation, cargo recruitment and sealing of phagophore by LC3-II is the maturation of autophagosome and fusion with lysosome. These steps require the clearance of ATGs on the outer membrane of autophagosome, recruitment of the lysosomal delivery machinery, and the machinery for fusion with the lysosome, which are working in a coordinated manner but the detailed mechanism are poorly characterized (Stolz et al., 2014). ATG8 proteins link autophagosome to kinesins through FYVE and coiled-coil domain containing protein 1 (FYCO1) to drive its maturation (Olsvik
et al., 2015). The fusion machinery that is studied is the SNAREs, which are protein that mediate the fusion of vesicles with target membrane. There are SNARE protein on the vesicle (v-SNARE) and the target membrane (t-SNARE), which combine together to form a trans-SNARE which provides the force for membrane fusion. For autophagosome fusion with the lysosome, Syntaxin 17 (STX17) and synaptosomal- associated protein 29 (SNAP29) are localized on the autophagosome as v-SNARE, while vesicle-associated membrane protein 8 (VAMP8) is on the lysosome as t-SNARE. With the help of homotypic fusion and protein sorting (HOPS) complex, autophagosome and lysosome membrane are tethered together for SNARE- mediated fusion (Diao et al., 2015; Itakura et al., 2012).

Regulation of Autophagy Machinery

Transcriptional Regulation

Autophagy genes are first regulated at the transcriptional level. One evolutionary conserved protein that regulates autophagy is the forkhead box O (FOXO) transcriptional factor. FOXO proteins are activated in response to cellular stress, and regulate genes involved in energy production, oxidative stress, cell viability and proliferation (Dikic and Elazar, 2018). Upon cellular stress, FOXO proteins are phosphorylated by AKT and translocated to the nucleus to upregulate the Atg genes transcriptionally in hepatocytes,
cardiomyocytes, neurons and primary renal proximal tubular cells (Webb and Brunet, 2014; Zhao et al., 2007).

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is another transcription factor that upregulates autophagy, which induces BECN1, BCL2 and p62 expression by binding to their antioxidant response elements (AREs) in the promoter region (Copetti et al., 2009; Jain et al., 2010; Niture and Jaiswal, 2012). Another key regulator for autophagy genes is the transcription factor EB (TFEB). TFEB control the mTORC1 lysosome recruitment for degradation or activity by regulating expression of the mTOR activating Rag GTPase complex component Ras-related GTP binding protein D (RagD) (Di Malta et al., 2017). Thus, TFEB is inhibited by mTORC1 under nutrient sufficient condition, and released upon starvation to trigger the expression level of autophagy related proteins including WIPI, p62 and UVRAG (Settembre et al., 2011).

Recently, the epigenetic reader bromodomain-containing protein 4 (BRD4) were reported as repressors of autophagic genes needed for autophagosome biogenesis at transcriptional level. BRD4 is a member of the bromodomain and extraterminal (BET) family proteins characterized by two N-terminal bromodomains and an extraterminal (ET) domain, which binds to acetylated histones and transcription factors through bromodomains and recruits
transcriptional regulators involved in the activation of genes for cell growth and cell cycle progression. BRD4 suppresses the expression of a series of autophagy genes by binding to the promoter regions under normal growth conditions, and the repression is alleviated when autophagy is upregulated (Sakamaki et al., 2017).

Post-translational Regulation

Autophagy can be triggered by nutrient starvation through inhibition of mTORC1 and activation of AMPK. Low amino acids upregulate autophagy through inhibition of mTORC1 but not mTORC2 (Condon and Sabatini, 2019; Saxton and Sabatini, 2017). In glucose starvation, low cellular energy level and decreased ATP: AMP ratio is sensed by AMPK and serine/threonine- protein kinase STK11 (LKB1). LKB1 activates autophagy through AMPK, which inhibits mTORC1 by activation of mTORC1 inhibitor Tuberous Sclerosis Complex 2 (TSC2) and inhibition of Raptor by phosphorylation, an essential component of the mTORC1 (Tripathi et al., 2013). When LKB1 is deficient, autophagy is activated to maintain metabolic flexibility by modulating lipid metabolism in lung tumors (Bhatt et al., 2019).

ATG proteins are mainly regulated by post-translational modification including phosphorylation, lipidation (i.e. LC3 lipidation of PE, discussed in the previous
section), oxidation and acetylation. During initiation stage, ULK1, ATG13 and FIP200 are inhibited or activated by phosphorylation at different regions as discussed in the previous section. At phagophore nucleation step, BECN1 can be phosphorylated by ULK1 or AMPK. And it can promote autophagy by interacting with the activating molecule in Beclin 1-regulated autophagy protein 1 (AMBRA1). Phosphorylated AMBRA1 by ULK1 is released from microtubules to allow binding of BECN1 and consequent activation of the full nucleation complex PI3KC3-C1 (Di Bartolomeo et al., 2010). At autophagosome elongation, maturation and fusion steps, phosphorylation of LC3 by protein kinase A (PKA) inhibits its activity to negatively regulate autophagy (Cherra et al., 2010). It was recently discovered that phosphorylation of LC3 at Thr50 by the Ste20 Hippo kinase orthologues serine/threonine-protein kinase 3 (STK3) and STK4 is required for fusion of autophagosome with lysosome (Wilkinson et al., 2015). Oxidation also modulates the function of ATGs. During nutrient starvation, increased ROS specifically generates hydrogen peroxide, which activates ATG4 for processing of ATG8 proteins through direct oxidation (Scherz-Shouval et al., 2007). Moreover, ATGs are regulated by acetylation and deacetylation process. ATG5, ATG7 and ATG8s are acetylated in autophagy inactive conditions. During starvation, silent mating type information regulation 2 homolog 1(SIRT1), a deacetylase, directly interacts with these ATGs and deacetylates them to induce autophagosome formation (Lee et al., 2008).
Besides, VPS34 is acetylated by histone acetyltransferase p300, which either diminishes the VPS34 affinity to the PI3KC3-C1 or prevents formation of the nucleation complex. During fasting, p300 inactivates and causes VPS34 deacetylation, coordinates with the activation of autophagy in mouse liver (Su et al., 2017).

**Epigenetic and microRNAs Regulation**

Histone modification of *Atg* genes by histone acetyltransferases (HATs) and histone deacetylases (HDACs) haven’t been experimentally shown yet. However, these histone modifying proteins are related in regulating autophagy. HDAC inhibitors induce autophagy in yeast, mouse embryonic fibroblasts (MEFs), skeletal muscle cells, HeLa cells and glioblastoma cells; but they inhibit autophagy in cardiomyocytes. HAT counterparts the function of HDAC. Esa1, for example, can acetylate ATG3 to induce autophagy in yeast (Fullgrabe et al., 2014).

MicroRNAs (miRNA) are also important regulators of autophagy process from initiation, nucleation and elongation. Currently, the identified miRNA targets are: ULK1 and FIP200 in initiation step; BECN1 and UVRAG in nucleation; ATG3, ATG7, ATG10, ATG5, ATG12, ATG16, p62, LC3 during elongation. There are no miRNAs identified yet to regulate proteins involved in fusion step
Physiological Role of Autophagy

Autophagy maintains organelle function, prevents the accumulation of toxic cellular waste products, and sustains cell metabolism and survival during starvation (Poillet-Perez and White, 2019). Autophagy is required to prevent the accumulation of damaged mitochondria, which is particularly important in the liver, muscle and brain. In fact, the buildup of damaged mitochondria can lead to oxidative stress and perturbation of metabolism (Rabinowitz and White, 2010). Autophagy is also important for removal of damaged proteins, functioning in coordination with proteasome degradation for protein quality control (Pohl and Dikic, 2019). Autophagy defects lead to endoplasmic reticulum (ER) stress and accumulation of chaperone proteins due to loss of the ability to remove the unfolded protein and properly remodel the proteome in response to stress (Mathew et al., 2009; Mathew et al., 2014). Under stress conditions such as nutrient starvation, autophagy is dramatically induced and essential for stress adaptation (Mizushima et al., 2004). Cargo-selective autophagy is also important, for example, to recycle iron from ferritin, which is critical for the iron homeostasis (Mancias et al., 2014).
Autophagy also has a critical role in mouse survival. Constitutively Atg5- or Atg7-deficient mice are born developmentally normal but fail to survive the neonatal starvation period when the trans-placental nutrient supply is interrupted but not yet restored by milk (Komatsu et al., 2005; Kuma et al., 2004). Force feeding only extends survival to neonatal starvation by 24 hours. In contrast to newborn mice, adult mice have a greater tolerance to the loss of autophagy. Conditional whole body ablation of the essential autophagy gene Atg7 in adult mice shortens life span to two to three months due to susceptibility to infection and neurodegeneration (Karsli-Uzunbas et al., 2014). Autophagy also suppresses liver, brain and muscle damage. While adult mice tolerate autophagy deficiency in the short term and in the fed state, fasting is lethal within 16 hours due to hypoglycemia (Karsli-Uzunbas et al., 2014). These findings demonstrate that autophagy is required to maintain systemic metabolism and survival by mitigating metabolic stress during nutrient deprivation (Karsli-Uzunbas et al., 2014). Moreover, there are remarkable tissue-specific dependencies on autophagy with brain, liver, muscle and WAT being particularly autophagy-dependent (Karsli-Uzunbas et al., 2014).

Autophagy also regulates intracellular lipid storage by breaking down triglycerides and cholesterol stored in lipid droplets, which is lipophagy. Impaired autophagy in liver causes accumulation of lipid droplets; in addition to that, neurons, stellate cells and fibroblasts also require autophagy for lipid
breakdown (Liu and Czaja, 2013). Lipophagy activity varies in response to the extracellular supply of nutrients. Free fatty acids and high-fat diet reduces lipophagy through feedback inhibition (Singh et al., 2009). On the other hand, autophagy plays a distinct role in regulating adipose lipid physiology. Autophagy is essential to maintain fat mass and large lipid droplet in adipose tissue instead of breaking down lipids. Adipocyte-specific \( \text{Atg7} \) knockout mice have decreased WAT and increased brown adipose tissue (BAT); and these mice are resistant to high fat diet-induced obesity (Singh et al., 2009). Autophagy is also required to maintain WAT in the whole body \( \text{Atg7} \) knockout mice (Karsli-Uzunbas et al., 2014).

Autophagy plays an important role in maintaining the tissue homeostasis (Karsli-Uzunbas et al., 2014), and dysregulation of autophagy contributes to different types of organ specific diseases. Neurodegenerative diseases are tightly related with autophagy. The first hallmark for neurodegenerative diseases is the aggregates of misfolded or unfolded proteins that accumulate inside neuronal cells including Parkinson’s disease, Alzheimer’s disease and Huntington’s disease (Rubinsztein et al., 2012). In healthy cells, misfolded proteins are labeled with Ub and degraded by the proteasome. However, proteasomal activity is impaired by various internal and external stresses and declines with age. When the capacity of proteasome is overloaded, autophagy is upregulated to compensate for the proteasome function and remove protein
aggregates as well as organelles that are damaged by these non-functional aggregates (Dikic, 2017). Indeed, neurodegeneration accompanied with accumulation of these undegraded ubiquitylated protein aggregates causes death of autophagy deficient mice (Karsli-Uzunbas et al., 2014; Komatsu et al., 2006). Besides, numerous mutated genes identified in neurodegenerative diseases are related to autophagy (Gan-Or et al., 2015) and lysosome function(Moors et al., 2016). p62, as a cargo receptor for autophagy, plays a key role in bringing misfolded protein together for sequestration and then transport them to the autophagosome (Pankiv et al., 2007; Seibenhener et al., 2004). The second hallmark for neurodegenerative disease is the accumulation of dysfunctional mitochondria, which elevates the ROS level that damages the proteome and genome of the cell (Dikic and Elazar, 2018). Damaged mitochondria can be removed by mitophagy using ubiquitin-dependent PINK1-Parkin pathway as described in the previous section, and mutations in PINK1 or Parkin are associated with the early onset of Parkinson’s disease(Walden and Muqit, 2017). NIX functions as an alternative ubiquitin independent cargo for mitophagy. Overexpression of NIX restores mitophagy in Parkin- or PINK1-deficient cell lines derived from patients with Parkinson disease (Koentjoro et al., 2017). Another protein involved in regulating mitophagy in Parkinson’s disease is the TANK- binding kinase 1 (TBK1). TBK1 phosphorylates ubiquitylated autophagy cargo receptors including p62, OPTN, NDP52, enhances their binding affinity to Ub
on the cargo (here damaged mitochondria), LC3 on the autophagosome or both, which contributes to the increased efficiency on recruitment of damaged mitochondria to the autophagosome for degradation (Lazarou et al., 2015; Matsumoto et al., 2015; Richter et al., 2016). Therefore, autophagy is a beneficial machinery that counteracts the neurodegenerative diseases, and its induction is currently being explored as a strategy for prevention or treatment of neurodegenerative disease (Dikic and Elazar, 2018).

Other than neurodegenerative diseases, there’s evidence that autophagy is related to cardiovascular disorders including ischemia, cardiomyopathy, congestive heart failure and Danon’s disease. Autophagosome accumulation was detected in myocardial biopsy samples from cardiovascular patients (Terman and Brunk, 2005). Genetic defects in autophagy exacerbate the propensity of mice to spontaneously develop cardiodegenerative disorders, and alteration in autophagic flux affects disease outcome in rodent models of several cardiovascular disorders including various types of cardiomyopathy, myocardial infarction and atherosclerosis (Bravo-San Pedro et al., 2017). Other organ-specific diseases such as cystic fibrosis muscle and liver disorders are related to altered autophagy, which were mostly based on decreased clearance of abnormal accumulation of defective organelles, proteins and lipids (Schneider and Cuervo, 2014).
Moreover, autophagy regulates mammalian differentiation and development. The very initial autophagic events in development happen in fertilized oocytes, which are ATG5 dependent (Tsukamoto et al., 2008). After fertilization, autophagy is profoundly activated in all the tissues to degrade maternal mRNAs and proteins, except in the brain (Kuma et al., 2004). Autophagy is upregulated during the neonatal period due to starvation, which is consistent with the results that knockouts of Atg3, Atg5, Atg7, Atg9, and Atg16L1 cause neonatal lethality. Interestingly, restoration of ATG5 in the brain is sufficient to rescue Atg5 knockout mice, even though these mice display some abnormalities including hypogonadism and iron-deficiency anemia, suggesting that neuronal loss of Atg5 is the primary cause of death of these mice (Yoshii et al., 2016). Absence of upstream autophagy regulators causes more severe phenotypes. For example, beclin1, Ambra1, and FIP200 deficiency induce embryonic lethality, probably due to autophagy-independent functions (Mizushima and Levine, 2010). In addition to development, autophagy is required for lymphocyte differentiation through NIX-dependent mitochondrial clearance. Autophagy deficiency reduces T and B lymphocyte counts with mitochondrial accumulation, and lymphocytes tend to increase superoxide and sensitivity to apoptosis (Mortensen et al., 2010; Novak et al., 2010). Autophagy is also required for the differentiation and survival of regulatory T cells (Wei et al., 2016).
Autophagy has been demonstrated to involve in immune functions, including inflammation control, inflammatory cytokine secretion, intracellular bacteria removal and antigen presentation (Dikic and Elazar, 2018). Autophagy deficient animals are susceptible to bacterial infection, and autophagy defects are widely displayed in autoimmune diseases, such as diabetes, multiple sclerosis, Crohn’s disease, systemic lupus erythematosus, rheumatoid arthritis, and psoriasis (Karsli-Uzunbas et al., 2014; Rioux et al., 2007; Rockel and Kapoor, 2016). One of the major signaling pathway on inflammation which crosstalks with autophagy is the IκB kinase complex (IKK)-NF-κB pathway. NF-κB induced autophagy by transactivating BECN1, BCL2 and p62 (Copetti et al., 2009; Jain et al., 2010; Niture and Jaiswal, 2012), while IKK induces autophagy in the presence of stress signals (Criollo et al., 2010). However, NF-κB pathway may inhibit autophagy in macrophages infected by E.coli and tumor necrosis factor-α (TNFα)-induced cell death (Djavaheri-Mergny et al., 2006; Schlottmann et al., 2008). On the other hand, autophagy can suppress NF-κB signaling by the autophagic degradation of active IKKβ mediated either by E3 ubiquitin-protein ligase Kelch-like ECH-associated protein 1 (KEAP1) or RO52 (Kim et al., 2010; Niida et al., 2010).

Autophagy can also eliminate a variety of invading bacteria including Mycobacteria, Salmonella, Shigella, Legionella, Listeria and group A streptococcus (Gomes and Dikic, 2014). Once these bacteria invade into the
cytosol, they are labeled with various types of Ub chain and galectin and sequestered by autophagic membranes utilizing the cargo receptors such as p62, NDP52 and OPTN (Gomes and Dikic, 2014). The Ub modifications on the bacteria coat is not only the target of autophagy cargo receptors, it also transforms the bacteria surface into a signaling platform to attract other proteins. The linear Ub chains, for example, activate the NF-κB signaling as well as attracting autophagy cargo receptors to maximize the antibacterial response (Noad et al., 2017; van Wijk et al., 2017). Interestingly, some pathogens develop their strategies to avoid the autophagy machinery by blocking the fusion of autophagosome with lysosome (Nguyen et al., 2005), competing with host LC3-interacting proteins for LC3 binding (Real et al., 2018), or secreting factors to activate host kinases to interfere autophagosome maturation (Neumann et al., 2016). Some bacteria are even able to take use of the autophagy machinery and replicate in the autophagosome-like vesicles (Devenish and Lai, 2015). However, generally autophagy is still considered a valid way to combat bacterial infections.

**p53 and Autophagy**

Many major stress responses are controlled by p53, and there is mounting evidence for a functional interaction between the p53 and the autophagy pathways. p53 is a transcription factor and tumor suppressor that responds to
diverse types of stresses including DNA damage, oncogene activation, oxidative stress and hypoxia (Fischer, 2017). In response to stress p53 can induce apoptosis, senescence, and cell cycle arrest, and alter cell metabolism by regulating multiple p53 target genes (Toledo and Wahl, 2006). It is generally thought that the p53 stress response can provide either protection and facilitate adaptation and recovery (e.g. cell cycle arrest) in the case of mild stress, or can eliminate cells (e.g. apoptosis) with excessive damage in the setting of high levels of stress (Kruiswijk et al., 2015). p53 thereby controls the nature of the stress response and its outcome.

p53 can also regulate autophagy. Under nutrient deprivation, a low ATP: AMP ratio activates AMPK, which then induces p53. Induction of p53 activates the transcription of genes in the AMPK pathway including TSC2 and AMPK itself, and leads to the inhibition of mTOR and activation of autophagy (Feng et al., 2007). Some p53 target genes like BCL2-associated X protein (BAX) and p53-upregulated modulator of apoptosis (PUMA) can directly activate autophagy in MEF cells (Yee et al., 2009). p53 can also directly turn on the expression of essential autophagy genes or induce autophagy via transcriptional activation of Damage-Regulated Autophagy Modulator (DRAM-1) in human and mouse cell lines (Crighton et al., 2006; Kenzelmann Broz et al., 2013; Mah et al., 2012).
CHAPTER 2
MATERIALS AND METHODS
Mouse models

All animal care was carried out in compliance with Rutgers University Institutional Animal Care and Use Committee guidelines. Ubc-Cre<sup>ERT2/+</sup> mice (Ruzankina et al., 2007) (The Jackson Laboratory) and Atg7<sup>flox/flox</sup> mice (Komatsu et al., 2005) (provided by Dr. M. Komatsu, Tokyo Metropolitan Institute of Medical Science) were cross-bred to generate the Ubc-Cre<sup>ERT2/+</sup>; Atg7<sup>flox/flox</sup> mice as previously described (Karsli-Uzunbas et al., 2014). To generate Ubc-Cre<sup>ERT2/+</sup>; Atg7<sup>flox/flox</sup>; p53<sup>flox/flox</sup> mice, p53<sup>flox/flox</sup> mice (Marino et al., 2000) (The Jackson Laboratory) were cross-bred with our previously created Ubc-Cre<sup>ERT2/+</sup>; Atg7<sup>flox/flox</sup> mice. To generate Nrf2<sup>−/−</sup>; Ubc-Cre<sup>ERT2/+</sup> mice and Nrf2<sup>−/−</sup>; Ubc-Cre<sup>ERT2/+</sup>; Atg7<sup>flox/flox</sup> mice, Nrf2<sup>−/−</sup> mice (Chan et al., 1996) (provided by YW Kan, UCSF) were cross-bred with Ubc-Cre<sup>ERT2/+</sup> mice and our previously generated Ubc-Cre<sup>ERT2/+</sup>; Atg7<sup>flox/flox</sup> mice. To generate Ubc-Cre<sup>ERT2/−</sup>; Atg7<sup>flox/flox</sup> mice, Ubc-Cre<sup>ERT2</sup> mice was cross-bred with Atg5<sup>flox/flox</sup> mice (Hara et al., 2006). To generate Ubc-Cre<sup>ERT2/−</sup>; Atg7<sup>flox/flox</sup>; Trp53<sup>flox/flox</sup> mice, Trp53<sup>flox/flox</sup> mice (Marino et al., 2000) (The Jackson Laboratory) were crossbred with previously generated Ubc-Cre<sup>ERT2/−</sup>; Atg5<sup>flox/flox</sup> mice.

Cag-Cre<sup>ERT2/−</sup>; Atg5<sup>flox/flox</sup> mice and Cag-Cre<sup>ERT2/−</sup>; Atg12<sup>flox/flox</sup> mice were provided by Dr. J. Debnath (University of California San Francisco). To generate PDGFRα-Cre<sup>ERT/</sup>; Atg5<sup>flox/flox</sup> mice and PDGFRα-Cre<sup>ERT/</sup>; Atg7<sup>flox/flox</sup> mice, PDGFRα-Cre<sup>ERT/</sup> mice (provided by Dwight E Bergles, Johns Hopkins School of Medicine) (The Jackson Laboratory) was cross-bred with Atg5<sup>flox/flox</sup> mice and
**TAM administration**

For acute deletion of Atg7 and/or p53, detailed rationale and TAM preparation is described as previously published (Karsli-Uzunbas et al., 2014). For TAM delivery to the adult mice, 200 μl of the suspended solution per 20 g of body weight equals (20mg/kg) were injected intraperitoneally (IP) into 8-10 weeks old Ubc-Cre<sup>ERT2/+</sup>; Atg7<sup>flox/flox</sup> mice and Ubc-Cre<sup>ERT2/+</sup>; Atg7<sup>flox/flox</sup>, p53<sup>flox/flox</sup> mice once per day for 5 consecutive days to delete the floxed gene systematically. Additionally, same dosage of TAM were given to Ubc-Cre<sup>ERT2/+</sup> mice and Ubc-Cre<sup>ERT2/+</sup>; p53<sup>flox/flox</sup> mice and these mice were examined as control groups. For Atg5 related experiment, same amount of TAM were injected intraperitoneally (IP) into 8-10 weeks old Ubc-Cre<sup>ERT2/+</sup>; Atg5<sup>flox/flox</sup> mice, Ubc-Cre<sup>ERT2/+</sup>; Atg7<sup>flox/flox</sup> mice, PDGFRα-Cre<sup>ERT/+</sup>; Atg5<sup>flox/flox</sup> mice and PDGFRα-Cre<sup>ERT/+</sup>; Atg7<sup>flox/flox</sup> mice once per day for 5 consecutive days. For slow deletion, same amount of TAM was used once per week for 4 shots to delete the floxed gene systematically.

**Survival**

For mouse Kaplan-Meyer survival curve, mice were monitored daily until they reached the endpoint. Cag-Cre<sup>ERT2</sup>; Atg12<sup>flox/flox</sup> mice were sacrificed 70 days.
after the last TAM injection because they met the criteria for euthanization with a body condition score of 2 and defects in grooming.

**Fasting**

Fasting was conducted as previous described (Karsli-Uzunbas et al., 2014).

**Histology**

Mouse tissues were collected and fixed in 10% formalin solution (Formaldehyde Fresh, Fisher Scientific, SF94-4). Tissues were fixed overnight and then transferred to 70% ethanol for paraffin-embedded sections or 15% sucrose following by 30% sucrose for frozen sections. For Alcian blue staining, paraffin sections were processed with the Alcian blue stain kit (Abcam) following the manufacturer’s protocol. For Bodipy C11 (Thermo Scientific, D3861) stain, 5μM of Bodipy C11 dye were used to stain the frozen sections of intestine for 30 minutes and counterstained with DAPI. For IHC, paraffin sections were stained with antibodies against p53 (Novus Biologicals, NB200-103, 1:400), Active caspase-3 (Cell Signaling, 9661, 1:300), γ-H2AX (Cell Signaling, 9718, 1:480), MDA (Cosmo Bio USA, NOF-N213530-EX, 1:200), OLFM4 (Cell Signaling, 39141, 1:2000), Lysozyme (Agilent, A0099, 1:2000), Active β-Catenin (Cell Signaling, 8814, 1:3000). For quantification of IHC on p53, Active caspase-3 and γ-H2AX, the liver and brain tissue were
analyzed by quantifying at least 10 images at 60X magnification using ImageJ. A minimum of 100 cells in liver and brain were scored for each image. For Immunofluorescence, paraffin sections were stained with antibodies against p62 (Novus 00008878, 1:1000), OLFM4 (cell signaling, 39141, 1:2000), CD34 (Abcam, 81289, 1:150), PDGFRα (R&D systems, AF1062, 1:100) and pictures were taken using confocal microscope.

**Nutlin-3a administration**

TAM were injected via IP into 8-10 weeks old Ubc-Cre<sup>ERT2/+</sup>; Atg<sup>flox/flox</sup> mice and Ubc-Cre<sup>ERT2/+</sup>; Atg<sup>flox/flox</sup>; p53<sup>flox/flox</sup> mice once per day for 5 consecutive days. Additionally, same dosage of TAM were given to Ubc-Cre<sup>ERT2/+</sup> mice and Ubc-Cre<sup>ERT2/+</sup>; p53<sup>flox/flox</sup> mice. After two weeks, 200mg/kg of Nutlin-3 (Cayman Chemicals) resolved in 50% DMSO were delivered to the mice by oral gavage once per day for 7 consecutive days. Mice were sacrificed one day after last administration of Nutlin-3 and tissues were collected for histology and snap-frozen for western blot and real-time PCR.

**Real-time PCR**

Total RNA were isolated from tissue by Trizol (Invitrogen). cDNA were then reverse transcribed from the total RNA by MultiScribe RT kit (Thermo Fisher). Real-time PCR were performed on Applied Biosystems StepOne Plus
machine. *Atg7* and *p53* were performed using SYBR green for deletion detection (*Atg7*: Forward 5' ACTTGACCGGTCTTACCCTG 3'; Reverse 5' TACTCCTGAGCTGTGGTTGC 3'; *p53*: Forward: 5' CGACTACAGTTAGGGGCAC 3'; Reverse: 5' GGAGGAAGTAGTTTCCATAAGCCT 3'; Actin: Forward 5' GAACCCTAAGGCCAACCCTGAAGAGATGAC 3'; Reverse 5' GCAGGATGGCGTGAGGGAGAGCA 3'). Besides that, all the other genes were detected using predesigned commercial Taqman primers for each gene accordingly (*Cdkn1a*: Mm00432448-m1; Actin: Mm00607939-s1; *Uvrag*: Mm00724370-m1; *Ulk1*: Mm00437238-m1; *Ulk2*: Mm03048846-m1; *Vmp1*: Mm00558175-m1; *Atg2b*: Mm00620760-m1; *Atg4a*: Mm04214755-s1; *Atg4c*: Mm00558175-m1; *Atg10*: Mm00470550-m1). Results were calculated using $\Delta\Delta C_T$ method and then normalized to actin. For real time PCR on stem cell markers and Wnt target genes, primers (information kindly from Dr. Michael Verzi, Rutgers University) are:

**OLFM4** (Forward:5' GCCAGATCTTGGCTCTGAAG 3'; Reverse: 5' GCCAGTGGAGCTGAATCACA 3')

**Lgr5** (Forward:5' ACGTCTTGCTGGAAATGCTT 3'; Reverse: 5' CACGTAGCTGATGTGGTTGG 3')

**Axin2** (Forward:5' AGGAGCAGCTCAGCAAAAAG 3'; Reverse: 5' GCTCAGTCCTCCTCCAC 3')

**Sox9** (Forward:5' AGTCGGTGAAAGACGGACAA 3'; Reverse: 5')
Western blot

Different tissues were grounded in a Cryomill machine (Retsch) and then total protein extracts were isolated by Tris lysis buffer (1 mol/L Tris HCl, 1 mol/L NaCl, 0.1 mol/L EDTA, 10% NP40). Separated proteins were probed with antibodies against ATG7 (Sigma; A2856, 1:2000), ATG5 (Abcam, ab108327, 1:1000), LC3 (Novus Biologicals; NB600-1384, 1:1500), p62 (American Research Products, 03-GP62-C, 1:2000), GAPDH (Santa Cruz; sc-365062, 1:1000) and β-actin (Sigma; A1978, 1:5000).

Organoid culture

Organoid culture was performed following standard protocol with modifications (Sato et al., 2009). Generally, crypts from the ileum part of the murine intestine were incubated for 40 min at 4°C in PBS containing 2mM EDTA. Then crypts were pelleted and then plated in 48 well plates with a total of 250 crypts in 25μl of Matrigel (Trevigen, 3433-05). After gel solidified in incubator, 250μl of culture medium (Advanced DMEM/F12 (Thermo Scientific, 12634-010)) containing 50ng/mL of EGF(Peprotech,315-09), 500ng/mL of human...
R-spondin 1 (Peprotech, 120-38) and 100ng/ml of Noggin (Peprotech, 250-38) were added. For in vitro Atg5 and Atg7 deletion, 1μM of 4-hydroxy-TAM (Sigma, H7905) were added to the 48 well plates for 15 hours and then changed back to normal culture medium. For in vitro Wnt ligand supplementation, Wnt3a (Abcam, ab81484) or Wnt2b (R&D systems, 3900-WN-025) were added with a concentration of 100ng/ml.

**In vivo Wnt ligand supplementation**

Mice at the age of 8-10 weeks were first treated with TAM as mentioned before. Wnt3a or Wnt 2b were administered to the mice by intraperitoneal injection with an amount of 50mg/kg per mice, 2 shots per day starting from the last day of TAM injection as previously described (Valenta et al., 2016). Mice were then monitored twice per day for survival curve and sacrificed to collect tissues when they reached the end point.

**Glucose supplementation**

To assess if the glucose can rescue the lethality caused by acute ATG5 deletion, mice were supplemented with 1mg/g of glucose twice per day via intraperitoneal injection starting from the last day of TAM treatment. Blood glucose level was measured daily using blood glucometer (One Touch Ultra 2).
**FITC dextran supplementation**

In order to test the intestinal barrier function, mice were supplemented with FITC-dextran and the blood serum was collected to measure the FITC-dextran concentration using the fluorescence plate reader as previously described (Gupta and Nebreda, 2014).

**Single cell RNA sequencing for PDGFRα⁺ mesenchymal cells**

Single cell RNA sequencing for telocytes were conducted as previously described (Halpern et al., 2019).

**Matrix-assisted laser desorption ionization coupled to imaging mass spectrometry (MALDI-IMS)**

Ileum tissues are snap frozen in liquid nitrogen and stored at -80°C until imaging mass-spectrometry and/or IHC/IF staining. For MALDI sectioned slides are desiccated and coated with a matrix compatible with MALDI-IMS (e.g. 9-AA or DHB) and mass spectrometry data is acquired using a Bruker solariX FTMS at 30-50µM spatial resolution. Conventional procedures for IHC/IF are used and data acquired from both modalities are used to determine metabolite levels using antibodies directed against PDGFRα⁺ mesenchymal
We will confirm the results from imaging spectrometry by alternative techniques, including LC-MS and fluorescence imaging. For LC-MS we will dissect comparable regions as in IMS to perform quantitative analysis of candidate metabolites identified by MALDI and global metabolomics. Qualitative data analysis will be conducted using Bruker FlexImaging software. Relative intensity of individual metabolites of interest will be determined for preliminary studies and standards. For quantitative data analysis approaches, we have developed an analysis pipeline that analyzes IMS data in three stages: 1) Pre-processing, 2) Peak picking, and 3) Post-processing. In the Pre-processing stage, we subtract background noise and matrix peaks, scan for potential adducts of analytes of interest, and perform spectrum alignment. In the peak picking stage, human-assisted automatic integration is performed to quantitate (relative quantification where no standards are run and absolute quantitation where standards are run) the peak area and intensity of the labeled and unlabeled metabolites of interest. All selected peak areas will be corrected for natural isotope abundance. In the post-processing stage, IMS data is overlaid with Hematoxylin and eosin (H&E) or immunohistochemical/immunofluorescence data. In addition, the post processing stage employed image fusion algorithms which infer structural information and metabolite localization from the overlaid images. Finally, we
will use the data generated in the post-processing stage to obtain spatial resolution of metabolic fluxes using metabolites (labeled and unlabeled). Relative quantification of metabolites is performed as part of the normal procedure, and absolute quantification of metabolites will be performed for key metabolites of interest using chemical standards at known concentration dispensed on tissue sections.
CHAPTER 3

AUTOPHAGY PROMOTES MAMMALIAN SURVIVAL BY SUPPRESSING OXIDATIVE STRESS AND P53
Introduction

Autophagy deficiency can cause p53 induction in mouse models of lung and pancreatic and breast cancer, and also neurons, correlating with more apoptosis when p53 is intact, suggesting that autophagy may suppress p53 activation in some but not all cancer settings (Guo et al., 2013; Huo et al., 2013; Rosenfeldt et al., 2013; Strohecker et al., 2013; Yang and Kimmelman, 2014; Yang et al., 2011; Zhang et al., 2009). As autophagy loss promotes p53 activation, and this p53 activation can be damaging, we sought to test the hypothesis that p53 was responsible for degenerative phenotypes induced by conditional autophagy loss *in vivo*.

To address how p53 and autophagy functionally interact *in vivo* and to determine the role that p53 plays in limiting the survival of mice without autophagy, we developed genetically engineered mouse models (GEMMs) to conditionally delete *Atg7* and/or *p53* systemically with tamoxifen (TAM). Whereas conditional, systemic *Atg7* deletion (*Atg7*Δ/Δ) in adult mice limited their survival to two to three months, co-deletion of *p53* and *Atg7* (*Atg7*Δ/Δ *p53*Δ/Δ) remarkably extended lifespan to up to six months. *Atg7*Δ/Δ *p53*Δ/Δ mice showed decreased tissue damage, apoptosis, and DNA damage in liver and brain in comparison to *Atg7*Δ/Δ mice. Activation of p53 by Nutlin-3 was inhibited by autophagy, which prevented liver and brain from p53 hyperactivation and apoptosis, suggesting that autophagy may be a resistance mechanism to
p53-activators. NRF2, in turn, is a resistance mechanism to loss of autophagy as conditional deletion of both *Nrf2* and *Atg7* in adult mice was synthetically lethal. Mice deficient for both *Atg7* and *Nrf2* (*Nrf2−/−Atg7Δ/Δ*) succumbed to damage to the small intestine, which was independent of p53 function. Thus, *Atg7* protects against excessive p53 activation and damage in the liver and brain, whereas NRF-2 protects the intestine from damage upon loss of *Atg7*, demonstrating the functional interdependence and tissue specificity of stress response pathways.
Results

Loss of p53 delays neurodegeneration and prolongs survival of Atg7-deficient mice.

To test whether p53 plays a role in limiting the survival in mice without autophagy, adult mice were engineered with or without floxed alleles of Atg7 (Kuma et al., 2004), p53 (Marino et al., 2000) and a transgene expressing a TAM-regulated Cre recombinase under the control of Ubiquitin C promoter that is ubiquitously expressed in the whole-body (Ubc-CreERT2) (Ruzankina et al., 2007). Injecting TAM activates Cre throughout these mice and the floxed alleles of Atg7 and/or p53 are deleted separately or together (Fig. 3.1A). Mice with systemic loss of Atg7 or p53 or both in all tissues are thereby generated and gene deletion was confirmed by qRT-PCR at two, five and eight weeks following the five consecutive days of TAM administration (Fig. S3.1A). Loss of ATG7 protein expression was also associated with accumulation of unprocessed form of microtubule-associated protein 1A/1B light chain 3 (LC3-I), decreased or absence of the processed (active) form of LC3 (LC3-II), and accumulation of the autophagy substrate protein p62, in both Atg7Δ/Δ and Atg7Δ/Δp53Δ/Δ mice, indicating blockage of autophagy function (Fig. 3.1B). Atg7Δ/Δ mice had a lifespan of about two to three months primarily due to susceptibility to infection early, and to neurodegeneration later, which is consistent with our previous findings (Karsli-Uzunbas et al., 2014). Similar to constitutively deficient p53−/− mice, p53Δ/Δ mice died from lymphoma, which
limited lifespan to up to six months (Fig. 3.1C, D) (Donehower et al., 1995). In contrast to Atg7Δ/Δ mice, a third of the Atg7Δ/Δp53Δ/Δ mice lived longer than three months and up to six months post TAM, while all the Atg7Δ/Δ mice died before three months post TAM (Fig. 3.1C, D). Although Atg7Δ/Δp53Δ/Δ lived longer than Atg7Δ/Δ mice, death was still predominantly from neurodegeneration (Fig. 3.1D). As loss of p53 did not alter survival to Atg7 deficiency early post deletion where death is due to susceptibility to infection (Karsli-Uzunbas et al., 2014), the role of p53 was specific to promoting death due to neurodegeneration (Fig. 3.1C). Therefore, p53 promotes neurodegeneration in mice deleted for Atg7.

**p53 deficiency reduces tissue damage in Atg7Δ/Δ mice**

Histological examination (H&E) of tissues from wild type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice revealed no differences two weeks post TAM (Fig. S3.1B). At five weeks post TAM, Atg7Δ/Δ mice began to show early evidence of loss of hepatocytes in liver, pyramidal neurons in the cerebrum, Purkinje cells in the cerebellum, and depletion of lipid in WAT as reported previously (Karsli-Uzunbas et al., 2014), which was not observed in the p53Δ/Δ or Atg7Δ/Δp53Δ/Δ mice (Fig. S3.1C). Two months post TAM, Atg7Δ/Δ mice showed severe loss of hepatocytes, pyramidal neurons, Purkinje cells, and WAT, and muscle wasting, whereas kidney and lung were not affected (Fig. 3.1E, S3.1D) (Karsli-Uzunbas et al., 2014). In contrast, these tissue damage phenotypes
resulting from \textit{Atg7} deficiency were not observed in wild type, \textit{p53}_{Δ/Δ} and \textit{Atg7}_{Δ/Δ}\textit{p53}_{Δ/Δ} mice (Fig. 3.1E, S3.1D). These results suggest that tissue damage caused by autophagy deficiency is induced by \textit{p53}. \textit{Atg7}_{Δ/Δ}\textit{p53}_{Δ/Δ} mice did display the same phenotype as the \textit{Atg7}_{Δ/Δ} mice at three to six months post depletion (data not shown), indicating that loss of \textit{p53} delays but does not prevent lethal neurodegeneration caused by autophagy deficiency.

\textbf{ATG7 is required for tumorigenesis driven by \textit{p53} deletion}

Constitutive \textit{p53} deficiency leads to the development of lethal thymic lymphomas, which limits lifespan to about six months (Donehower et al., 1995). Here, conditional \textit{p53} deficiency in adult mice (\textit{p53}_{Δ/Δ}) produced the same phenotype as 36 out of 39 mice died of thymic lymphoma (Fig. 3.1D). \textit{Atg7}_{Δ/Δ}\textit{p53}_{Δ/Δ} mice showed similar lifespan limitation as the \textit{p53}_{Δ/Δ} mice, however, the vast majority of the mice died from neurodegeneration without tumor development as 18 out of 21 mice died of neurodegeneration (Fig. 3.1D). Of the three \textit{Atg7}_{Δ/Δ}\textit{p53}_{Δ/Δ} mice that died from cancer, two died from thymic lymphomas and one died from a sarcoma (Fig. 3.1D). Thus, autophagy is required to enable development of lethal thymic lymphoma driven by deletion of \textit{p53}.
p53 deficiency prevents lethality in Atg7Δ/Δ mice during fasting

While Atg7Δ/Δ mice survive in the short term, fasting is immediately lethal due to hypoglycemia (Karsli-Uzunbas et al., 2014). Since p53 deficiency extended the lifespan and attenuated tissue damage in Atg7Δ/Δ mice, we sought to test whether p53 contributes to the death of Atg7Δ/Δ mice during fasting. In contrast to Atg7Δ/Δ mice where fasting was lethal, none of the Atg7Δ/Δ p53Δ/Δ mice died upon fasting, suggesting that p53 was responsible for fasting-induced death of Atg7-deficient mice (Fig. 3.1F).
Fig. 3.1 *Atg7ΔΔ, p53ΔΔ* mice have extended life span, delayed tissue damage and neurodegeneration compared to *Atg7ΔΔ* mice. A.

Experimental design for generation of *Atg7ΔΔ* mice, *p53ΔΔ* mice, and *Atg7ΔΔ p53ΔΔ* mice. Ubc-Cre$^{ERT2/+}$, Ubc-Cre$^{ERT2/+}$; *Atg7flx/flx* mice, Ubc-Cre$^{ERT2/+}$; *p53flx/flx*, Ubc-Cre$^{ERT2/+}$; *p53flx/flx*; *Atg7flx/flx* mice were treated with TAM at 8 to 10 weeks age and analyzed at certain time points afterwards. B. Western blot for ATG7, p62, and LC3 at the indicated time points of the indicated tissues from wild type mice, *Atg7ΔΔ* mice, *p53ΔΔ* mice, and *Atg7ΔΔ p53ΔΔ* mice. β-Actin is used as a loading control. C. Kaplan–Meier survival curve of wild type mice, *Atg7ΔΔ* mice, *p53ΔΔ* mice, and *Atg7ΔΔ p53ΔΔ* mice. * p<0.05, ** p<0.01, **** p<0.0001 (Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test as indicated). D. Percentage distribution for the cause of death of *Atg7ΔΔ*, *p53ΔΔ*, and *Atg7ΔΔ p53ΔΔ* mice. The cause of death was analyzed at 30-90 days post TAM and 90-180 days post TAM. E. Representative histology of liver, muscle, cerebrum, cerebellum, pancreas, white adipose tissue (WAT) and lung by Hematoxylin & Eosin stain (H&E) from wild type, *Atg7ΔΔ*, *p53ΔΔ*, and *Atg7ΔΔ p53ΔΔ* mice at 8 weeks time point. F. Kaplan–Meier survival curve of wild type mice, *Atg7ΔΔ* mice, *p53ΔΔ* mice, and *Atg7ΔΔ p53ΔΔ* mice during starvation at 10 days post TAM. * p<0.05. See also Figure S3.1
ATG7 is required to protect liver and brain from p53-mediated damage

Autophagy deficiency causes p53 induction in neurons and in some cancer models and promotes cell death. Therefore, we tested whether p53 induction occurred in the whole-body after Atg7 deletion. Immunohistochemistry (IHC) for p53 protein revealed that p53 accumulation was detectable at two weeks post TAM administration, and was maintained at five and eight weeks post TAM in the livers and brains of Atg7Δ/Δ mice, while p53 activation was not apparent in wild type, p53Δ/Δ, and Atg7Δ/Δ p53Δ/Δ mice (Fig. 3.2A, S3.2A, S3.2B). qRT-PCR for the p53 target gene cyclin-dependent kinase inhibitor 1A (Cdkn1a, or p21) showed increased Cdkn1a expression in Atg7Δ/Δ mice at two, five and eight weeks post TAM in liver and brain compared to wild type, p53Δ/Δ and Atg7Δ/Δ p53Δ/Δ mice (Fig. 3.2B, C). These data suggest that loss of Atg7 promotes activation of p53.

Whole-body ATG7 deficiency leads to DNA damage and apoptosis in liver and cerebrum (Karsli-Uzunbas et al., 2014), and p53 is known to be activated by different stress signals including DNA damage, oxidative stress and triggers cell cycle arrest and apoptosis (Fischer, 2017). Therefore, we hypothesized that p53 induction in Atg7Δ/Δ mice may promote apoptosis. IHC for the DNA damage response activation marker γ-H2AX revealed accumulation of γ-H2AX in Atg7Δ/Δ liver and cerebrum starting two weeks that was apparent through eight weeks post TAM (Fig. 3.2D, S3.2C, S3.2D). In contrast, γ-H2AX accumulation was not detected in wild type, p53Δ/Δ and Atg7Δ/Δ p53Δ/Δ mice (Fig.
S3.2C, S3.2D). As a likely consequence of p53 activation in \( Atg7^{\Delta/\Delta} \) mice, these mice also showed more apoptosis marked by increased active caspase-3 in liver and brain in comparison to \( Atg7^{\Delta/\Delta}p53^{\Delta/\Delta} \) mice (Fig. 3.2D, S3.2E, S3.2F). These data indicated that p53 induction caused apoptosis in \( Atg7^{\Delta/\Delta} \) mice.

\( Atg7^{\Delta/\Delta} \) mice also displayed increased Malondialdehyde (MDA) in liver by IHC compared to wild type, \( p53^{\Delta/\Delta} \) and \( Atg7^{\Delta/\Delta}p53^{\Delta/\Delta} \) mice, indicating that p53 induction was associated with increased oxidative stress in \( Atg7^{\Delta/\Delta} \) mice (Fig. 3.2E).
**Fig. 3.2. Autophagy is required to protect liver and brain from p53 accumulation, DNA damage response activation and apoptosis.**

A. Representative liver and cerebrum IHC staining of p53 and quantification at indicated time points from wild type and Atg7Δ/Δ mice. 2w, 2 weeks time point. 5w, 5 weeks time point. 8w, 8 weeks time point. B-C. Quantitative real-time PCR of Cdkn1a for liver and brain tissues from wild type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice at indicated time points. D. Representative liver and cerebrum IHC staining for γ-H2AX and active caspase-3 with quantification at indicated time points from wild type and Atg7Δ/Δ mice. 2w, 2 weeks. 5w, 5 weeks. 8w, 8 weeks. *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001 (Unpaired t test) E. Representative liver IHC staining for MDA at indicated time points from wild type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice. See also Figure S3.2.
Regulation of p53 activity relies on the essential p53 antagonist MDM2, which is a direct transcriptional target of p53 and is upregulated when p53 is activated by phosphorylation at specific serine and threonine residues (Bode and Dong, 2004). MDM2 binds to p53, and the ubiquitin E3 ligase of MDM2 ubiquitinylates p53, which decreases its stability by targeting it to the proteasome for degradation (Honda et al., 1997; Kubbutat et al., 1997; Matsumine et al., 1997). Under stress conditions, p53 is released from MDM2, and is thereby stabilized and activated. In this way, p53 and MDM2 form a negative feedback loop resulting from p53-dependent induction of MDM2 and MDM2-dependent suppression of p53 activity, which helps the cell to deal with stress without hyperactivation of p53 (Dotto, 2009; Marine and Lozano, 2010; Montes de Oca Luna et al., 1995). Nutlin-3 works as an MDM2 antagonist and upregulates the cellular p53 level by competing for the binding site of MDM2 for p53, and is being assessed clinically to promote p53 activation for cancer therapy (Vassilev et al., 2004). Since Atg7 limits p53 accumulation and activation we sought to test if Atg7 also limited the ability of Nutlin-3 to activate p53 (Khoury and Domling, 2012) as a potential resistance mechanism.

Following deletion of Atg7 and/or p53, mice were either treated with vehicle or Nutlin-3 (200mg/kg) once per day for one week (Fig. 3.3A). Deletion of Atg7 and p53 were confirmed by qRT-PCR (Fig. S3.3A). Western blot for loss of
ATG7 protein, accumulation of LC3-I and loss of LC3-II, and accumulation of p62 in liver and brain from Atg7\(^{\Delta/\Delta}\) and Atg7\(^{\Delta/\Delta}\)p53\(^{\Delta/\Delta}\) mice indicated blockage of autophagy (Fig. 3.3B). As previously described, liver damage and neuron loss in Atg7\(^{\Delta/\Delta}\) mice were confirmed by H&E, which was not affected by Nutlin-3 (Fig. S3.3B). IHC of liver and brain from Atg7\(^{\Delta/\Delta}\) mice revealed increased p53 compared to wild type mice, which was further increased in Nutlin-3 treated Atg7\(^{\Delta/\Delta}\) mice, suggesting that Nutlin-3 induced p53 in the absence but not in the presence of autophagy. As expected, Nutlin-3 did not affect p53 levels in p53\(^{\Delta/\Delta}\) mice and Atg7\(^{\Delta/\Delta}\)p53\(^{\Delta/\Delta}\) mice (Fig. 3.3C, S3.3C). qRT-PCR for the p53 target gene Cdkn1a showed increased Cdkn1a expression in untreated Atg7\(^{\Delta/\Delta}\) mice, which was further increased in Nutlin-3 treated Atg7\(^{\Delta/\Delta}\) mice in liver and brain compared to vehicle or Nutlin-3 treated wild type mice (Fig. 3.3D). This confirmed that p53 was activated by Nutlin-3 specifically in the absence of autophagy. IHC of liver and brain revealed increased γ-H2AX and active caspase-3 in Atg7\(^{\Delta/\Delta}\) mice compared to wild type mice, and activation of p53 by Nutlin-3 greatly increased γ-H2AX and active caspase-3 levels. Induction of γ-H2AX and active caspase-3 were not observed in p53\(^{\Delta/\Delta}\) and Atg7\(^{\Delta/\Delta}\)p53\(^{\Delta/\Delta}\) mice, suggesting that loss of autophagy induced apoptosis through p53 activation (Fig. 3.3E, Fig. S3.3D, S3.3E). Therefore, autophagy is essential to protect tissues from apoptosis by limiting p53 activation.

Since p53 can induce a series of essential autophagy genes including Atg7 in
MEF cells (Kenzelmann Broz et al., 2013), we hypothesized that upregulation of p53 by Nutlin-3 can turn on essential autophagy genes and protect tissues from damage caused by p53 induction in wild type mice. Real-time PCR on a series of autophagy essential genes indicated no significant difference in the autophagy gene mRNA levels, suggesting that the autophagy transcription program is not detectably induced by p53 at the times the tissues were collected (Supplementary Fig. S3.4).
Fig. 3.3 Activation of p53 by MDM2 antagonist Nutlin-3a in Atg7Δ/Δ mice leads to further increased DNA damage response and apoptosis in liver and brain. A. Experimental design for generation of Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice and Nutlin-3 administration. Nutlin-3 was administered to mice by oral gavage 2 weeks post TAM administration at a dosage of 200mg/kg for one week. B. Western blot for ATG7, p62 and LC3 for liver and brain tissues from wild type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice treated with vehicle or Nutlin-3. β-Actin is used as a loading control. V, treated with Vehicle; N, treated with Nutlin-3. C. Representative liver and cerebrum IHC staining for p53 and quantification at indicated time points from wild type and Atg7Δ/Δ mice treated with vehicle or Nutlin-3. V, Vehicle; N, Nutlin-3. * p<0.05; **** p<0.0001; n.s., not significant (Unpaired t test). D. Quantitative real-time PCR of Cdkn1a for liver and brain tissues from wild type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice treated with vehicle or Nutlin-3. V, Vehicle; N, Nutlin-3. *p<0.05; **p<0.01; n.s., not significant (Unpaired t test). E Representative liver and cerebrum IHC staining for γ-H2AX and active caspase-3 with quantification at indicated time points from wild type and Atg7Δ/Δ mice treated with vehicle or Nutlin-3. V, Vehicle; N, Nutlin-3. * p<0.05; ***p<0.001; ****p<0.0001; n.s., not significant (Unpaired t test). See also Figure S3.3, S3.4
Atg7 deficiency is synthetically lethal in the absence of Nrf2

Autophagy can reduce ROS by removing damaged mitochondria and unfolded protein, and autophagy deficiency leads to increased ROS and accumulation of unfolded protein (Manjithaya et al., 2010; Mizushima, 2010). Since we found induction of oxidative stress markers in Atg7Δ/Δ mice (Fig. 3.2E), we investigated whether the increased oxidative stress was responsible for p53 activation and/or tissue damage. NRF2 is the master regulator of the antioxidant defense and is ubiquitinated by an E3 ubiquitin ligase Kelch-like ECH-associated protein 1 (KEAP1) and degraded by the proteosome pathway under normal conditions (Kensler et al., 2007). With increased ROS, NRF2 is released from KEAP1 and triggers expression of a series of antioxidant genes, and NRF2 is induced by autophagy deficiency (Lau et al., 2010; Levonen et al., 2014). To examine the role of antioxidant defense in mice lacking autophagy, mice with constitutive deficiency in Nrf2 (Chan et al., 1996) were crossed with Ubc-CreERT2/+; Atg7floxflox mice to generate Nrf2-/-; Ubc-CreERT2/+; Atg7floxflox mice (Fig. 3.4A). TAM administration was then used to delete Atg7 in the presence and absence of Nrf2.

In contrast to Atg7Δ/Δ mice that survive for two to three months, Nrf2-/-; Atg7Δ/Δ mice had a lifespan of less than seven days (Fig. 3.4B). Histological examination of tissues by H&E surprisingly showed no damage to liver, brain, pancreas, lung and kidney in Nrf2-/-; Atg7Δ/Δ mice (Fig. S3.5A). The only tissue
with significant damage was the intestine (duodenum, jejunum and ileum), which may be the cause of increased muscle wasting and loss of WAT (Fig. 3.4C, Fig. S3.5B). Bodipy C11 staining of the whole small intestine was significantly increased in Nrf2\(^{-/-}\)Atg7\(^{+/-}\) intestine, indicating increased lipid peroxidation that results from ROS (Fig. 3.4D). IHC for active caspase-3 displayed increased staining in Nrf2\(^{-/-}\)Atg7\(^{+/-}\) intestine in both the crypt and villus, indicating increased apoptosis (Fig. S3.5C-S3.5E). Thus, conditional deletion of the essential autophagy gene Atg7 in adult mice is synthetically lethal in the absence of Nrf2 due to damage to the intestine.

We then investigated which cell type in the intestine was most affected by deficiency in Atg7 in the absence of Nrf2. Alcian blue staining of paraffin sections from intestine tissues was significantly decreased in the Nrf2\(^{-/-}\)Atg7\(^{+/-}\) mouse intestine, suggesting loss of goblet cells (Fig. 3.4E). IHC for the stem cell marker OLFM4 revealed loss of OLFM4 staining in Nrf2\(^{-/-}\)Atg7\(^{+/-}\) but not in wild type, Atg7\(^{+/-}\) and Nrf2\(^{-/-}\) mouse intestine, suggesting loss of stem cells (Fig. 3.4F). IHC for the Paneth cell marker Lysozyme revealed diffuse staining in the Nrf2\(^{-/-}\)Atg7\(^{+/-}\) mouse intestine in comparison to wild type, Atg7\(^{+/-}\) and Nrf2\(^{-/-}\) intestine, which suggests that Paneth cell function is abnormal (Supplementary Fig. S3.5F) (Cadwell et al., 2008). We then investigated whether this deleterious phenotype in the intestine was induced by p53 activation. p53\(^{flox/flox}\) mice were crossed with Nrf2\(^{-/-}\); Ubc-Cre\(^{ERT2/+}\) mice and Nrf2\(^{-/-}\); Ubc-Cre\(^{ERT2/+}\); Atg7\(^{flox/flox}\) mice to generate Nrf2\(^{-/-}\); Ubc-Cre\(^{ERT2/+}\); p53\(^{flox/flox}\) mice and Nrf2\(^{-/-}\); Ubc-Cre\(^{ERT2/+}\); p53\(^{flox/flox}\) mice and Nrf2\(^{-/-}\);
Ubc-Cre\textsuperscript{ERT2+}; p53\textsuperscript{flox/flox}; Atg7\textsuperscript{flox/flox} mice. After TAM administration, we found that Nrf2\textsuperscript{−/−}p53\textsuperscript{Δ/Δ}Atg7\textsuperscript{Δ/Δ} mice did not survive longer than Nrf2\textsuperscript{−/−}Atg7\textsuperscript{Δ/Δ} mice, suggesting that the intestinal damage in the Nrf2\textsuperscript{−/−}Atg7\textsuperscript{Δ/Δ} mice was not caused by p53 (Supplementary Fig. S3.5G).
Fig. 3.4 Atg7 deficiency is synthetically lethal in the absence of Nrf2. A.

Experimental design for generation of Atg7Δ/Δ mice, Nrf2−/− mice, and Nrf2−/− Atg7Δ/Δ mice. B. Kaplan–Meier survival curve of wild type, Atg7Δ/Δ, Nrf2−/−, and Nrf2−/− Atg7Δ/Δ mice. ****p<0.0001 (Log-rank (Mantel-Cox) test). C.

Representative histology of duodenum, jejunum and ileum by H&E at indicated time points from wild type, Atg7Δ/Δ, Nrf2−/−, and Nrf2−/− Atg7Δ/Δ mice. D.

Representative Bodipy C11 stain of duodenum, jejunum and ileum at indicated time points from wild type, Atg7Δ/Δ, Nrf2−/−, and Nrf2−/− Atg7Δ/Δ mice. E.

Representative Alcian blue stain of duodenum, jejunum and ileum at indicated time points from wild type, Atg7Δ/Δ, Nrf2−/−, and Nrf2−/− Atg7Δ/Δ mice. F.

Representative duodenum, jejunum and ileum IHC stain of OLFM4 at indicated time points from wild type, Atg7Δ/Δ, Nrf2−/−, and Nrf2−/− Atg7Δ/Δ mice. See also Figure S3.5.
Figure 5. Mechanism by which autophagy interacts with the p53 and NRF2 stress response mechanisms to protect tissues in a tissue specific manner

See text for explanation.
Discussion

Both autophagy and p53 can protect tissues from stress such as DNA damage, oxidative stress, and hypoxia (Fischer, 2017; Mizushima and Komatsu, 2011), and their overlapping functions have suggested that these two pathways interact. p53 upregulates the expression of essential autophagy genes and autophagy function in vitro (Crighton et al., 2006; Feng et al., 2007; Kenzelmann Broz et al., 2013; Mah et al., 2012; Zhang et al., 2009). In turn, autophagy inhibits p53 in some tumors providing a negative feedback loop (Guo et al., 2013; Rosenfeldt et al., 2013; Strohecker et al., 2013; Yang et al., 2014). Whether autophagy can regulate p53 in normal tissues in vivo, however, was not clear. We found that autophagy suppresses p53 activation in liver and brain, without which hyperactivation of p53 is responsible for damage to these tissues. Thus, autophagy is a tissue-specific negative regulator of p53 and contributes to a negative feedback loop to limit p53 activation in vivo (Fig. 3.5A). Remarkably, eliminating p53 also rescued the survival of Atg7-deficient mice during fasting, suggesting that autophagy restricts p53 activation in response to exogenous as well as endogenous stress (Fig. 3.5). Even when p53 activation is forced by Nutlin-3, autophagy prevents these tissues from p53-mediated damage (Fig. 3.5A, B). These findings also suggest that autophagy may limit the effectiveness of MDM2 antagonists, and this should be tested in the cancer setting.
The NRF2 and autophagy pathways both contribute to antioxidant defense. NRF2 is activated by autophagy deficiency in vitro and in tumors (Lau et al., 2010; Saito et al., 2016; Strohecker et al., 2013). The autophagy substrate p62, which accumulates when autophagy is blocked, interacts with KEAP1, thereby releasing and stabilizing NRF2 and promoting expression of its target genes (Ichimura et al., 2013; Levonen et al., 2014). We found that the protective function of NRF2 is essential for the survival of mice with loss of ATG7, as *Nrf2*−/−; *Atg7Δ/Δ* mice die rapidly, specifically from damage to the small intestine. *Atg5* deficiency in intestine epithelia causes decreased numbers of intestinal stem cells, and these stem cells have a higher ROS level compared to wild type mice, which can be rescued by treating mice with antioxidant N-Acetyl Cysteine (Asano et al., 2017). *Atg16L1* is also required to protect the intestinal epithelium from necroptosis induction in response to virus-induced intestinal bowel disease by maintaining mitochondrial homeostasis (Matsuzawa-Ishimoto et al., 2017). We report here that knockout of NRF2 is synthetically lethal with loss of *Atg7*, as NRF2 is specifically required to protect the survival of intestinal stem cells (Fig. 3.5C). The compensatory protective effect of NRF2 to loss of autophagy may be broad as recent cell-based screens identified NRF2 activation as a resistance mechanism selected for in cancer cells deleted for essential autophagy genes (Towers et al., 2019). In conclusion, autophagy limits p53 activation and damage in liver and brain, while NRF2 limits intestinal stem cells damage due to loss of autophagy by a p53-independent mechanism.
These findings demonstrate the functional interaction and tissue specificity of these stress regulated pathways (Fig. 3.5A-C).
Supplementary Figures

Supplementary Fig. S3.1

A

Liver

Kidney

Muscle

Lung

Brain

Pancreas

B

2 weeks

5 weeks

C

D

2 weeks

5 weeks

8 weeks
Figure S3.1. Related to Figure 3.1. A. Quantitative real-time PCR of Atg7 and p53 for liver, brain, kidney, pancreas, muscle, lung at indicated time points from wild type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice. 2w, 2 weeks time point. 5w, 5 weeks time point. 8w, 8 weeks time point. B-C. Representative histology of liver, muscle, cerebrum, cerebellum, pancreas, WAT and lung by H&E at 2 weeks and 5 weeks time point from wild type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice. D. Representative histology of kidney by H&E at indicated time points from wild type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice. E. Blood glucose level of wild type, Atg7Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice at the end of starvation. *p<0.05; **p<0.01(Unpaired t test)
Figure S3.2. Related to Figure 3.2. A. Representative liver IHC staining of p53 at indicated time points from wild type, \textit{Atg7}\textsuperscript{Δ/Δ}, \textit{p53}\textsuperscript{Δ/Δ}, and \textit{Atg7}\textsuperscript{Δ/Δ}p\textit{53}\textsuperscript{Δ/Δ} mice. 1-4, corresponding to images in Fig. 3.2A. B. Representative cerebrum IHC staining of p53 at indicated time points from wild type, \textit{Atg7}\textsuperscript{Δ/Δ}, \textit{p53}\textsuperscript{Δ/Δ}, and \textit{Atg7}\textsuperscript{Δ/Δ}p\textit{53}\textsuperscript{Δ/Δ} mice. 1-4, corresponding to images in Fig. 3.2A. C. Representative liver IHC staining for γ-H2AX at indicated time points from wild type, \textit{Atg7}\textsuperscript{Δ/Δ}, \textit{p53}\textsuperscript{Δ/Δ}, and \textit{Atg7}\textsuperscript{Δ/Δ}p\textit{53}\textsuperscript{Δ/Δ} mice. 1-4, corresponding to images in Fig. 3.2D. D. Representative cerebrum IHC staining for γ-H2AX at indicated time points from wild type, \textit{Atg7}\textsuperscript{Δ/Δ}, \textit{p53}\textsuperscript{Δ/Δ}, and \textit{Atg7}\textsuperscript{Δ/Δ}p\textit{53}\textsuperscript{Δ/Δ} mice. 1-4, corresponding to images in Fig. 3.2D. E. Representative liver IHC staining for active caspase-3 at indicated time points from wild type, \textit{Atg7}\textsuperscript{Δ/Δ}, \textit{p53}\textsuperscript{Δ/Δ}, and \textit{Atg7}\textsuperscript{Δ/Δ}p\textit{53}\textsuperscript{Δ/Δ} mice. 1-4, corresponding to images in Fig. 3.2D. F. Representative cerebrum IHC staining for active caspase-3 at indicated time points from wild type, \textit{Atg7}\textsuperscript{Δ/Δ}, \textit{p53}\textsuperscript{Δ/Δ}, and \textit{Atg7}\textsuperscript{Δ/Δ}p\textit{53}\textsuperscript{Δ/Δ} mice. 1-4, corresponding to images in Fig. 3.2D.
Figure S3.3. Related to Figure 3.3. A. Quantitative real-time PCR of Atg7 and p53 for liver and brain from wild type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice treated with vehicle or Nutlin-3. B. Representative histology of liver and cerebrum by H&E from wild type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice treated with vehicle or Nutlin-3. C. Representative liver and cerebrum IHC staining for p53 from wild type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice treated with vehicle or Nutlin-3. 1-4, corresponding to images in Fig. 3.3C. D. Representative liver and cerebrum IHC staining for γ-H2AX and quantification from wild type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice treated with vehicle or Nutlin-3. 1-4, corresponding to images in Fig. 3.3E. E. Representative liver and cerebrum IHC staining for active caspase-3 and quantification at indicated time points from wild type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice treated with vehicle or Nutlin-3. 1-4, corresponding to images in Fig. 3.3E.
Figure S3.4. Related to Figure 3.3. Quantitative real-time PCR of *Uvrag*, *Ulk1*, *Ulk2*, *Vmp1*, *Atg2b*, *Atg4a*, *Atg4c* and *Atg10* for liver from wild type, *Atg7Δ/Δ*, *p53Δ/Δ*, and *Atg7Δ/Δ p53Δ/Δ* mice treated with vehicle or Nutlin-3. V, treated with Vehicle; N, treated with Nutlin-3.
Figure S3.5. Related to Figure 3.4. A. Representative histology of liver, cerebrum, cerebellum, pancreas, lung and kidney by H&E at 2 days and 4 days time point from wild type, \( \text{Atg7}^{\Delta/\Delta}, \text{Nrf2}^{-/-} \), and \( \text{Nrf2}^{-/-}\text{Atg7}^{\Delta/\Delta} \) mice. B. Representative histology of muscle and WAT by H&E at 2 days and 4 days time point from wild type, \( \text{Atg7}^{\Delta/\Delta}, \text{Nrf2}^{-/-} \), and \( \text{Nrf2}^{-/-}\text{Atg7}^{\Delta/\Delta} \) mice. C-E. Representative duodenum, jejunum and ileum IHC stain of active caspase-3 at indicated time points from wild type, \( \text{Atg7}^{\Delta/\Delta}, \text{Nrf2}^{-/-} \), and \( \text{Nrf2}^{-/-}\text{Atg7}^{\Delta/\Delta} \) mice. F. Representative duodenum, jejunum and ileum IHC stain of Lysozyme at indicated time points from wild type mice, \( \text{Atg7}^{\Delta/\Delta} \) mice, \( \text{Nrf2}^{-/-} \) mice, and \( \text{Nrf2}^{-/-}\text{Atg7}^{\Delta/\Delta} \) mice. G. Kaplan–Meier survival curve of wild type, \( \text{Atg7}^{\Delta/\Delta}, \text{Nrf2}^{-/-} \), and \( \text{Nrf2}^{-/-}\text{Atg7}^{\Delta/\Delta} \) mice, \( \text{Nrf2}^{-/-}\text{p53}^{\Delta/\Delta} \) and \( \text{Nrf2}^{-/-}\text{p53}^{\Delta/\Delta}\text{Atg7}^{\Delta/\Delta} \) mice.

****p<0.0001. n.s., not significant. (Log-rank (Mantel-Cox) test).
CHAPTER 4

AUTOPHAGY IS ESSENTIAL FOR TELOCYTE SURVIVAL, WNT SIGNALING AND INTESTINAL STEM CELL HOMEOSTASIS
Introduction

Autophagosome formation requires three steps: initiation, nucleation and elongation. During the elongation stage, there are two protein complexes involved: the ATG12-ATG5-ATG16 complex (formed with the help of ATG7 and ATG10) and the LC3 complex (formed with the help of ATG4, ATG7 and ATG3). The ATG12-ATG5-ATG16 complex is recruited to the autophagosome membrane, and facilitates the lipidation of LC3 with (PE), which is required for the elongation and final formation of autophagosomes. Therefore, both Atg5 and Atg7 are essential for the formation of functional autophagosomes and the process of autophagy.

Conditional, whole-body deletion of essential autophagy gene Atg7 in adult mice (Atg7Δ/Δ) leads to liver damage, muscle wasting, and loss of adipose tissue with lifespan decreased to three months due primarily to neurodegeneration (Karsli-Uzunbas et al., 2014). Constitutively Atg5- or Atg7-deficient mice are born normal but unable to survive neonatal starvation when the trans-placental nutrient supply is interrupted but not yet restored by milk (Komatsu et al., 2005; Kuma et al., 2004). Consistent with the role of autophagy in promoting survival in starvation, fasting Atg7Δ/Δ but not wild type mice is lethal (Karsli-Uzunbas et al., 2014). Fasted Atg7Δ/Δ mice rapidly deplete dedicated nutrient stores of glycogen and lipids and fail to maintain circulating glucose levels and die from hypoglycemia (Karsli-Uzunbas et al., 2014).
Since both \textit{Atg7} and \textit{Atg5} are essential autophagy genes, we generated the genetically engineered mouse model (GEMM) for conditional, whole-body \textit{Atg5} deletion to address whether loss of \textit{Atg5} would have similar phenotype as loss of \textit{Atg7}. In contrast to \textit{Atg7}^{\Delta/\Delta} mice which tolerate loss of ATG7 for 2-3 months in the fed state, we found that adult mice with conditional whole-body deletion of \textit{Atg5} (\textit{Atg5}^{\Delta/\Delta}) survive only four days post deletion. \textit{Atg5}^{\Delta/\Delta} mice display severe damage in the ileum part of intestine with loss of barrier function. In contrast to \textit{Atg7}^{\Delta/\Delta} mice, \textit{Atg5}^{\Delta/\Delta} mice showed more rapid loss of autophagy in the ileum with loss of stem cells and also PDGFR\textalpha^{+} mesenchymal cells (telocytes), which provide Wnt ligands to the stem cells for their maintenance. However, a gradual deletion of \textit{Atg5} rescued this phenotype and these mice generated tissue damage and neurodegenerative phenotype similar as whole body loss of \textit{Atg7} and \textit{Atg12}. Loss of telocytes in the \textit{Atg5}^{\Delta/\Delta} ileum caused significantly decreased aspartate and increased nucleotide degradation products. Therefore, \textit{Atg5} is required in maintenance of PDGFR\textalpha^{+} mesenchymal cells which contribute Wnt ligands to the intestinal stem cells for maintenance of intestinal homeostasis.
Results

Conditional, whole-body \textit{Atg5} deletion in adult mice leads to ileum damage and death

As both \textit{Atg7} and \textit{Atg5} function at the same step in the autophagy pathway, we hypothesized that conditional whole-body deletion of either gene will behave similarly. To address this, we generated the inducible \textit{Ubc-Cre}^{ERT2/+}, \textit{Atg5}^{flox/flox} GEMM, treated these adult mice (eight weeks of age) with 5 consecutive daily injections of tamoxifen (TAM) as we described previously to generate \textit{Atg7}^{Δ/Δ} mice (Karsli-Uzunbas et al., 2014) to generate \textit{Atg5}^{Δ/Δ} mice (Fig. 4.1A). To our surprise, \textit{Atg5}^{Δ/Δ} mice showed a significantly decreased lifespan of only about four days post TAM, while \textit{Atg7}^{Δ/Δ} mice have a lifespan of about two to three months (Karsli-Uzunbas et al., 2014) (Fig. 4.1B). In order to confirm the phenotype is specifically caused by loss of \textit{Atg5}, we did the TAM deletion in \textit{Cag-Cre}^{ERT2/+} \textit{Atg12}^{flox/flox} and \textit{Cag-Cre}^{ERT2/+} \textit{Atg5}^{flox/flox} mice, which is another inducible whole body deletion model (Fig. S4.1A), and \textit{Cag-Cre}^{ERT2/+} \textit{Atg12}^{flox/flox} mice was previously published to have similar lifespan and neurodegeneration phenotype as the whole body \textit{Atg7}^{Δ/Δ} mice (Malhotra et al., 2015). We observed similar lifespan of 4 days for the \textit{Atg5}^{Δ/Δ} mice, while \textit{Atg12}^{Δ/Δ} survived as long as 10 weeks post TAM, which is similar as \textit{Atg7}^{Δ/Δ} mice (Fig. S4.1B). To determine why \textit{Atg5}^{Δ/Δ} mice fail to survive immediately post deletion, we compared the histology of wild type, \textit{Atg7}^{Δ/Δ} and \textit{Atg5}^{Δ/Δ} mouse tissues, and the primary damage site is the ileum part of intestine. The
wild type ileum showed normal villous architecture without inflammation or significant apoptosis. The $Atg^{7/Δ}$ ileum maintained the normal villous architecture, but with slightly increased apoptosis in the glandular epithelium. However, $Atg^{5/Δ}$ ileum demonstrated marked epithelial damage exemplified by the shortening of villi, cytoplasmic vacuolation, numerous apoptosis, and sloughing of the surface epithelium especially at Day three post TAM but no significant inflammatory cell infiltration. Duodenum and jejunum didn’t display those damage phenotypes (Fig. 4.1C, S4.1C, S4.1D). Similar damage is observed in Cag-Cre $Atg^{5/Δ}$ ileum but not the duodenum or jejunum (Fig. S4.1E-G).
Fig. 4.1

A. Whole body deletion of Atg7 or Atg5

- Ubc-CreERT2⁺
- Ubc-CreERT2⁺; Atg5⁺⁺
- Ubc-CreERT2⁺; Atg7⁺⁺
- Ubc-CreERT2⁺; Atg7⁺⁺⁻⁻

B. 2-3 months survival Neurodegeneration liver, muscle damage loss of fat tissue

C. H&E stain

D. Blood Glucose

E. TTC staining concentration (OD 492)

F. Western Blot
Figure. 4.1. Conditional, whole-body Atg5 deletion in adult mice leads to ileum damage and death. A. Experimental design for generation of Atg5Δ/Δ mice and Atg7Δ/Δ mice. Ubc-CreERT2/+; Atg5Δ/Δ mice and Ubc-CreERT2/++; Atg7Δ/Δ mice were treated with TAM at 8-10 weeks of age with 5 shots of TAM every consecutive day and analyzed. B. Kaplan-Meier survival curve of TAM-treated wild type, Atg7Δ/Δ and Atg5Δ/Δ mice. **** p<0.0001 (log-rank Mantel-Cox test). C. Representative ileum Hematoxylin&Eosin stained (H&E) histology at indicated time points from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice. D. Blood glucose concentration measured by mg/dL from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice at indicated time points. *p<0.05, ***p<0.001(log-rank Mantel-Cox test) E. FITC-dextran concentration in the blood serum from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice at indicated time points. *p<0.05, **p<0.01, ***p<0.001(log-rank Mantel-Cox test) F. Western blotting for ATG5, ATG7, LC3 at indicated time points from the ileum tissue of wild type, Atg7Δ/Δ and Atg5Δ/Δ mice. β-ACTIN is used as the loading control. See also Figure S4.1, Figure S4.2.
Acute whole body \textit{Atg5} deletion decreases blood glucose and causes loss of intestinal barrier function

As the ileum functions in dietary nutrient absorption, and nutritional deprivation of \textit{Atg7}\textsubscript{Δ/Δ} mice causes lethal hypoglycemia, we sought to test if \textit{Atg5}\textsubscript{Δ/Δ} mice were nutritionally compromised due to loss of ileum function. \textit{Atg5}\textsubscript{Δ/Δ} mice had decreased blood glucose levels starting from day two post TAM which further decreased on day three in comparison to wild type and \textit{Atg7}\textsubscript{Δ/Δ} mice (Fig. 4.1D). Supplementation of glucose by intraperitoneal (IP) injection restored the blood glucose levels but did not rescue the survival of \textit{Atg5}\textsubscript{Δ/Δ} mice, suggesting that this was not solely responsible for death (Fig. S4.2A, S4.2B). Intestinal barrier function is important in defending from bacterial invasion and required for mouse survival. To test the barrier function of \textit{Atg5}\textsubscript{Δ/Δ} mice, we performed FITC-dextran dietary supplementation to wild type, \textit{Atg7}\textsubscript{Δ/Δ} and \textit{Atg5}\textsubscript{Δ/Δ} mice and compared the concentration of FITC-dextran in the circulating blood supply. The FITC-dextran concentration in serum was low with no difference at day one post TAM in wild type control, \textit{Atg7}\textsubscript{Δ/Δ} mice and \textit{Atg5}\textsubscript{Δ/Δ} mice. However, \textit{Atg5}\textsubscript{Δ/Δ} mice began to show increased FITC-dextran in the circulation at day two post TAM, which further increased at day three in comparison to wild type and \textit{Atg7}\textsubscript{Δ/Δ} mice (Fig. 4.1E). These results suggest that \textit{Atg5}\textsubscript{Δ/Δ} mice lost intestinal barrier function and likely also efficient nutritional absorption causing hypoglycemia, which led to death of \textit{Atg5}\textsubscript{Δ/Δ} mice.
Deletion of Atg5 causes more rapid loss of autophagy in the ileum than deletion of Atg7

Deletion of Atg5 in Atg5Δ/Δ mice and deletion of Atg7 in Atg7Δ/Δ mice were detected, but Atg5 deletion occurred more rapidly and thoroughly compared to Atg7 (Fig. S4.2C, S4.2D). Loss of ATG5, and more importantly the ATG5-ATG12 complex, was apparent in the ileum on day one post TAM in Atg5Δ/Δ mice, whereas the ATG5-ATG12 complex persisted in Atg7Δ/Δ mice despite the loss of ATG7 (Fig. 4.1F). This indicates that ATG5-ATG12 complex may be stable and functional longer in the absence of ATG7 but not ATG5. In contrast, accumulation of the unprocessed form of LC3 (LC3-I) and decreased levels of the processed form of LC3 (LC3-II) occurred earlier in the Atg7Δ/Δ ileum compared to the Atg5Δ/Δ ileum, consistent with the direct role of ATG7 in conjugation of PE to LC3 to generate LC3-II (Fig. 4.1F). Similar results were observed in duodenum and jejunum (Fig. S4.2E, S4.2F). These results suggest that whole body deletion of Atg5 and Atg7 can both cause autophagy defect in the intestine, but Atg5Δ/Δ mice lose autophagy more rapidly than Atg7Δ/Δ mice due to more rapid and complete loss of the ATG5-ATG12 complex, which may explain the lethal phenotype of Atg5Δ/Δ mice.
Ileum damage in \textit{Atg5}^{\Delta/\Delta} mice is caused by loss of stem cells

The intestinal epithelium is mainly divided into two compartments: crypt and villus. In the crypt, the crypt base columnar (CBC) cells, or stem cells, are interspersed with Paneth cells at the base region of the crypt. These CBC cells are responsible for the renewal of whole intestine epithelium by continuously generating the proliferating transient amplifying (TA) cells. These TA cells migrate ascendantly towards the base of villi and differentiate into several cell types in villus, mainly enterocytes and goblet cells (Barker, 2014). In order to investigate the cause of ileum damage, we checked which cell type in the ileum was lost in \textit{Atg5}^{\Delta/\Delta} mice. Immunohistochemistry (IHC) of ATG5 and ATG7 protein confirmed the loss of ATG5 and ATG7 protein in the ileum and also duodenum and jejunum in \textit{Atg5}^{\Delta/\Delta} and \textit{Atg7}^{\Delta/\Delta} mice (Fig. 4.2A, Fig. S4.3A-S4.3D). However, the intestine of \textit{Atg5}^{\Delta/\Delta} mice accumulated more p62 aggregates in comparison to \textit{Atg7}^{\Delta/\Delta} mice indicative of a more rapid and efficient loss of autophagy (Fig. 4.2B, Fig. S4.3E, S4.3F). There was more p62 accumulation in Cag-Cre \textit{Atg5}^{\Delta/\Delta} intestine compared to \textit{Atg5}^{\Delta/\Delta} intestine too (Fig. S4.3G-L). Alcian blue staining of the small intestine was decreased in the ileum of \textit{Atg5}^{\Delta/\Delta} mice from day two post TAM and was completely lost on day three, indicating loss of goblet cells in the ileum but not in the duodenum and jejunum of \textit{Atg5}^{\Delta/\Delta} mice, while wild type and \textit{Atg7}^{\Delta/\Delta} mice maintained the goblet cells throughout the intestine (Fig. 4.2C, Fig. S4.4A, S4.4B). Loss of goblet cells was also observed in Cag-Cre \textit{Atg5}^{\Delta/\Delta} ileum but not duodenum or
jejenum (Fig. S4.4C-E). IHC for the stem cell marker OLFM4 revealed loss of OLFM4 staining starting from day two, which was completely lost by day three in $Atg5^{Δ/Δ}$ ileum compared to wild type and $Atg7^{Δ/Δ}$ mice (Fig. 4.2D, Fig. S4.4F, S4.4G). Similar phenotype was observed in Cag-Cre $Atg5^{Δ/Δ}$ ileum but not duodenum or jejunum (Fig. S4.4H-J). Thus, stem cells are lost in the ileum but not in the duodenum and jejunum specifically with systemic deletion of $Atg5$.

IHC for lysozyme showed that staining is maintained in duodenum and jejunum in wild type, $Atg7^{Δ/Δ}$ mice and $Atg5^{Δ/Δ}$ mice (Fig. S4.4K, S4.4L).

However, $Atg5^{Δ/Δ}$ mice displayed a diffuse staining pattern in the ileum at day three in $Atg5^{Δ/Δ}$ mice (Fig. 4.2E). This diffuse stain pattern is also displayed in the Cag-Cre $Atg5^{Δ/Δ}$ ileum but not duodenum or jejunum (Fig. S4.4M-S4.4O).

Immunofluorescence (IF) for OLFM4 and p62 showed that there was increased p62 aggregates colocalized with OLFM4 in $Atg5^{Δ/Δ}$ ileum in comparison to $Atg7^{Δ/Δ}$ ileum at day two but not at day three because of the complete loss of stem cells (Fig. 4.2F). There were increased p62 aggregates in $Atg5^{Δ/Δ}$ duodenum and $Atg5^{Δ/Δ}$ jejunum compared to $Atg7^{Δ/Δ}$ at all time points but not as much as in the ileum (Fig. S4.4P, S4.4Q). Taken together, the ileum of $Atg5^{Δ/Δ}$ mice lost intestinal stem cells and goblet cells and had abnormal Paneth cells, and the autophagy blockage was more severe in the stem cells of $Atg5^{Δ/Δ}$ mice compared to $Atg7^{Δ/Δ}$ mice. These findings suggest that ileum stem cells are acutely sensitive to loss of autophagy that may be more severe upon systemic deletion of $Atg5$ than $Atg7$ in mice.
Figure 4.2. Ileum damage in Atg5Δ/Δ mice is caused by loss of stem cells.

A. Representative ileum Immunohistochemistry (IHC) staining of ATG5 and ATG7 at indicated time points from wild type, Atg7Δ/Δ mice and Atg5Δ/Δ mice. B. Representative ileum IHC staining of p62 at indicated time points from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice. Red arrows indicate p62 aggregates. C. Representative ileum Alcian blue staining at indicated time points from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice. D. Representative ileum IHC staining of OLFM4 at indicated time points from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice. E. Representative ileum IHC staining of Lysozyme at indicated time points from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice. F. Representative ileum Immunofluorescence (IF) costaining of p62 and OLFM4 at indicated time points from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice. Red arrows indicate the p62 aggregates which colocalize with OLFM4. See also Figure S4.3, Figure S4.4.
In vitro deletion of Atg5 lead to growth inhibition and can be rescued by supplementation of Wnt ligands

Since intestinal stem cells are required for the renewal of the other cell types in the intestinal epithelium, we hypothesized that loss of stem cell was the main cause of ileum damage and loss of barrier function. The most important signaling pathway that supports the growth and renewal of the intestinal stem cells is the canonical WNT signaling pathway (Clevers et al., 2014; Fevr et al., 2007). WNT signaling is activated by Wnt ligands Wnt3a or Wnt2b in the crypt region, and β-catenin is the essential signal transducer. To test if conditional deficiency in ATG5 caused loss of ileum stem cells due to failure of WNT signaling, we isolated crypts from the ileum of Atg5^floxed/floxed and Atg7^floxed/floxed mice and plated them in matrigel to culture organoids. Organoids were then treated with TAM to delete either Atg5 or Atg7 genes in vitro (Fig. 4.3A). Atg5^Δ/Δ organoids showed rapid and complete loss of the ATG5-ATG12 complex, while Atg7^Δ/Δ organoids showed loss of ATG7 protein yet maintained some ATG5-ATG12 complex (Fig. 4.3B). Both Atg5^Δ/Δ and Atg7^Δ/Δ organoids accumulated LC3-I and had less LC3-II post TAM (Fig. 4.3B). These findings were consistent with ATG5 loss in ileum organoids causing a more rapid and complete loss of the ATG5-ATG12 complex than loss of ATG7, as was observed in the ileum in vivo. Thus, loss of ATG5 may thereby cause a more profound loss of autophagy function.
To address how loss of ATG5 and ATG7 altered stem cell function, organoids were followed over time. After deletion, the viability and budding number of $\text{Atg5}^{\Delta/\Delta}$ organoids was decreased compared to $\text{Atg7}^{\Delta/\Delta}$ organoids when passaged for three generations, and this phenotype was rescued by supplementation with Wnt3a or Wnt2b (Fig. 4.3C, 4.3D, S4.5A-C, H-J). IF of organoids for OLFM4 and p62 showed significant loss of OLFM4-positive stem cells with more p62 aggregates in $\text{Atg5}^{\Delta/\Delta}$ compared with $\text{Atg7}^{\Delta/\Delta}$ organoids (Fig. 4.3E). Real-time PCR for stem cell maker OLFM4, Lgr5 and Wnt target gene Axin2 and Sox9 revealed decreased transcription level of these genes in $\text{Atg5}^{\Delta/\Delta}$ compared with $\text{Atg5}^\text{Flox/Flox}$ organoids but can be brought back to similar or higher level when supplemented with Wnt3a or Wnt2b (Fig. 4.3F, Fig. S4.5D-G, Fig. S4.5K-N). WB for organoids at passage two and three revealed that ATG7 protein were restored with low LC3-I accumulation and increased LC3-II in $\text{Atg7}^{\Delta/\Delta}$ organoids, whereas in $\text{Atg5}^{\Delta/\Delta}$ organoids ATG5 was still lost with high LC3-I accumulation and no LC3-II, suggesting that the $\text{Atg7}^{\Delta/\Delta}$ organoids were outcompeted by undeleted $\text{Atg7}^{\text{Flox/Flox}}$ organoids and had intact autophagy function (Fig. S4.5O, S4.5P). These data indicated that stem cell depletion in $\text{Atg5}^{\Delta/\Delta}$ organoids is caused by insufficient Wnt signaling.
Figure 4.3. *In vitro* deletion of *Atg5* lead to growth inhibition and can be rescued by supplementation of Wnt ligands. A. PCR of *Atg5* from the untreated *Atg5*\(^{flox/flox}\) organoids, *Atg5\(^{Δ/Δ}\) organoids and *Atg5\(^{Δ/Δ}\) organoids supplemented with Wnt3a or Wnt2b; And PCR of *Atg7* from untreated *Atg7*\(^{flox/flox}\) organoids, *Atg7\(^{Δ/Δ}\) organoids and *Atg7\(^{Δ/Δ}\) organoids supplemented with Wnt3a or Wnt2b. B. Western blotting for ATG5, ATG7, and LC3 from the untreated *Atg5*\(^{flox/flox}\) organoids, *Atg5\(^{Δ/Δ}\) organoids, and *Atg5\(^{Δ/Δ}\) organoids.
supplemented with Wnt3a or Wnt2b and untreated $Atg_{7^{\text{flox/flox}}}$ organoids, $Atg_{7^{\Delta/\Delta}}$ organoids, $Atg_{7^{\Delta/\Delta}}$ organoids supplemented with Wnt3a or Wnt2b at passage 1. β-ACTIN is used as the loading control. C. Representative picture of the untreated $Atg_{5^{\text{flox/flox}}}$ organoids, $Atg_{5^{\Delta/\Delta}}$ organoids, and $Atg_{5^{\Delta/\Delta}}$ organoids supplemented with Wnt3a or Wnt2b and untreated $Atg_{7^{\text{flox/flox}}}$ organoids, $Atg_{7^{\Delta/\Delta}}$ organoids, $Atg_{7^{\Delta/\Delta}}$ organoids supplemented with Wnt3a or Wnt2b at passage 3. D. Alive organoid percentage and average budding numbers of the untreated $Atg_{5^{\text{flox/flox}}}$ organoids, $Atg_{5^{\Delta/\Delta}}$ organoids, $Atg_{5^{\Delta/\Delta}}$ organoids supplemented with Wnt3a or Wnt2b and untreated $Atg_{7^{\text{flox/flox}}}$ organoids, $Atg_{7^{\Delta/\Delta}}$ organoids, $Atg_{7^{\Delta/\Delta}}$ organoids supplemented with Wnt3a or Wnt2b at passage 3. E. Representative organoid IF costaining of p62 and OLFM4 of the untreated $Atg_{5^{\text{flox/flox}}}$ organoids, $Atg_{5^{\Delta/\Delta}}$ organoids, $Atg_{5^{\Delta/\Delta}}$ organoids supplemented with Wnt3a or Wnt2b and untreated $Atg_{7^{\text{flox/flox}}}$ organoids, $Atg_{7^{\Delta/\Delta}}$ organoids, $Atg_{7^{\Delta/\Delta}}$ organoids supplemented with Wnt3a or Wnt2b at passage 3. F. Quantitative real-time PCR of OLFM4, Lgr5, Axin2 and Sox9 of the untreated $Atg_{5^{\text{flox/flox}}}$ organoids, $Atg_{5^{\Delta/\Delta}}$ organoids, $Atg_{5^{\Delta/\Delta}}$ organoids supplemented with Wnt3a or Wnt2b and untreated $Atg_{7^{\text{flox/flox}}}$ organoids, $Atg_{7^{\Delta/\Delta}}$ organoids, $Atg_{7^{\Delta/\Delta}}$ organoids supplemented with Wnt3a or Wnt2b at passage 3. See also Figure S4.5.
**Atg5 is required to maintain PDGFRα⁺ mesenchymal cells.**

Next, we tested whether ATG5-dependent WNT signaling is required for maintenance of stem cells *in vivo*. We observed significant loss of active β-catenin in the ileum crypt in *Atg5*Δ/Δ mice by IHC, and decreased transcription level of Axin2 and Sox9 in *Atg5*Δ/Δ ileum compared with *Atg7*Δ/Δ ileum at Day three post TAM, suggesting failure of Wnt signaling at Day three but not Day one and Day two (Fig. 4.4A, 4.4B, S4.6A, S4.6B). In Cag-Cre *Atg5*Δ/Δ ileum same phenotype was observed (Fig. S4.6C, S4.6D). Wnt ligands can come from Paneth cells in the epithelium (Farin et al., 2012), however, *Atg5* deletion using a epithelium-specific villin-Cre did not cause loss of ileum stem cells (Cadwell et al., 2008), suggesting that ATG5-dependent Wnts were originating outside the epithelium. Recent data demonstrated that PDGFRα⁺ mesenchymal cells or telocytes surrounding the intestinal epithelium are a critical source of Wnt ligands essential for intestinal stem cell maintenance (Greicius et al., 2018; Shoshkes-Carmel et al., 2018; Stzepourginski et al., 2017). Therefore, we tested whether ATG5 was required for the function and survival of PDGFRα⁺ mesenchymal cells, the loss of which in *Atg5*Δ/Δ ileum was responsible for ileum stem cell loss *in vivo*. IF for PDGFRα and CD34 indicated that PDGFRα and CD34 positive cells were present at day one and two post TAM in the *Atg5*Δ/Δ and *Atg7*Δ/Δ ileum (Fig. S4.6E, S4.6F). However, there was nearly complete loss of PDGFRα and CD34 positive cells on day three post TAM specifically in the *Atg5*Δ/Δ ileum but not in the in *Atg7*Δ/Δ ileum.
In Cag-Cre Atg5Δ/Δ ileum, PDGFRα and CD34 positive cells were present at day one, and almost complete loss of these cells on day two but not in the Atg12Δ/Δ ileum (Fig. S4.6G, S4.6H). Thus, deletion of Atg5 caused loss of telocytes in Atg5Δ/Δ but not Atg7Δ/Δ or Atg12Δ/Δ ileum. We also observed increased p62 in telocytes on day one and two in Atg5Δ/Δ compared to Atg7Δ/Δ ileum, whereas p62 staining was lost on day three in Atg5Δ/Δ ileum due to loss of telocytes (Fig. S4.7A, 4.4C, S4.7B). Similar phenotype of p62 accumulation was also displayed in Cag-Cre Atg5Δ/Δ ileum on day one but not as much in Atg12Δ/Δ ileum (Fig. S4.7C, S4.7D). Greater p62 accumulation in Atg5Δ/Δ ileum is constant with more rapid and complete loss of autophagy function. We then tested whether Wnt ligand supplementation could compensate for the loss of PDGFRα+ mesenchymal cells in the Atg5Δ/Δ ileum. Atg5Δ/Δ mice supplemented with Wnt ligands Wnt3a or Wnt2b showed a twenty four hours extension of their lifespan (Fig. 4.4D). In addition, there was an increase of OLFM4 IHC signal in the ileum of Atg5Δ/Δ mice supplemented with Wnt3a and Wnt2b compared to untreated Atg5Δ/Δ mice, but not to the extent as seen in wild type mice (Fig. 4.4E). These data indicated that PDGFRα+ mesenchymal cells require autophagy to survive and are essential in maintaining intestinal stem cells by providing them with Wnt ligands.
Figure 4.4. *Atg5* is required to maintain PDGFRα⁺ mesenchymal cells.  

A. Representative ileum IHC staining of Active β-catenin at Day 3 post TAM from wild type, *Atg7Δ/Δ* and *Atg5Δ/Δ* mice.  
B. Representative ileum IF costaining of CD34 and PDGFRα at Day 3 post TAM from wild type, *Atg7Δ/Δ* and *Atg5Δ/Δ* mice.  
C. Representative ileum IF costaining of p62 and PDGFRα at Day 2 from wild type, *Atg7Δ/Δ* and *Atg5Δ/Δ* mice. White arrows indicate colocalization of p62 with PDGFRα.  
D. Kaplan-Meier survival curve of *Atg5Δ/Δ* mice, *Atg5Δ/Δ* mice supplemented with Wnt3a and *Atg5Δ/Δ* mice supplemented with Wnt2b. *p*<0.05, **, *p*<0.01 (log-rank Mantel-Cox test)  
E. Representative ileum IHC of OLFM4 at Day 3 on the duodenum, jejunum and ileum from *Atg5Δ/Δ* mice, *Atg5Δ/Δ* mice supplemented with Wnt3a and *Atg5Δ/Δ* mice supplemented with Wnt2b. See also Figure S4.6, S4.7.
Telocyte specific deletion of \textit{Atg5} causes similar ileum damage phenotype.

We then sought to investigate whether the ileum damage phenotype is specifically caused by loss of PDGFRα+ mesenchymal cells in the whole body \textit{Atg5}\textsuperscript{Δ/Δ} mice. To test this, we crossed the PDGFRα-Cre\textsuperscript{ERT/+} mice with \textit{Atg7}\textsuperscript{flox/flox} and \textit{Atg5}\textsuperscript{flox/flox} mice to generate the PDGFRα-Cre\textsuperscript{ERT/+}; \textit{Atg7}\textsuperscript{flox/flox} and the PDGFRα-Cre\textsuperscript{ERT/+}; \textit{Atg5}\textsuperscript{flox/flox} mice and then administered with TAM to delete \textit{Atg7} or \textit{Atg5} specifically in the PDGFRα+ mesenchymal cells (Fig. 4.5A). Similar to whole body \textit{Atg5}\textsuperscript{Δ/Δ} mice, almost seventy percent of the PDGFRα-Cre \textit{Atg5}\textsuperscript{Δ/Δ} mice died within six days, while wild type and PDGFRα-Cre \textit{Atg7}\textsuperscript{Δ/Δ} mice survived (Fig. 4.5B). There’s moderate p62 aggregates in PDGFRα+ mesenchymal cells in both PDGFRα-Cre \textit{Atg7}\textsuperscript{Δ/Δ} and \textit{Atg5}\textsuperscript{Δ/Δ} mice at Day two post TAM, suggesting that autophagy function was still intact at this time point in PDGFRα+ mesenchymal cells (Fig. S4.8A). We observed increased p62 aggregates in PDGFRα+ mesenchymal cells from PDGFRα-Cre \textit{Atg5}\textsuperscript{Δ/Δ} mice at Day three, following the complete loss of these cells at Day four, indicating blockage of autophagy caused loss of PDGFRα+ mesenchymal cells in PDGFRα-Cre \textit{Atg5}\textsuperscript{Δ/Δ} ileum (Fig. 4.5C, 4.5D). Significant loss of active β-catenin and OLFM4 in the ileum crypt of PDGFRα-Cre \textit{Atg5}\textsuperscript{Δ/Δ} mice by IHC at Day four were observed, suggesting loss of Wnt signaling and stem cells (Fig. 4.5E, 4.5F). Alcian blue staining of the PDGFRα-Cre \textit{Atg5}\textsuperscript{Δ/Δ} ileum was largely decreased from day three and was completely lost on day
four, indicating loss of goblet cells (Fig. S4.8B). IHC for Lysozyme revealed that staining is maintained and no diffused pattern detected in PDGFRα-Cre $Atg5^{Δ/Δ}$ ileum, suggesting that Paneth cells were functioning normally (Fig. S4.8C). Similar to whole body $Atg5^{Δ/Δ}$ ileum, PDGFRα-Cre $Atg5^{Δ/Δ}$ ileum also demonstrated epithelial damage exemplified by shortening of villi, cytoplasmic vacuolation, numerous apoptosis, and sloughing of the surface epithelium at Day four post TAM but no significant inflammatory cell infiltration, which is not found in duodenum and jejunum (Fig. 4.5G, S4.8D, S4.8E). For the PDGFRα-Cre $Atg5^{Δ/Δ}$ mice that survived longer than six days, PDGFRα$^+$ mesenchymal cells were maintained with moderate p62 aggregates, and the small intestine was intact, which indicated that $Atg5$ might be undeleted in the PDGFRα$^+$ mesenchymal cells of these mice (Fig. S4.8F, S4.8G). These data indicated that the ileum damage phenotype we observed in the whole body $Atg5^{Δ/Δ}$ mice was specifically caused by loss of PDGFRα$^+$ mesenchymal cells.
Figure 4.5. Telocyte specific deletion of \textit{Atg5} causes similar ileum damage phenotype. A. Experimental design for generation of PDGFRα-Cre\textsuperscript{ERT/+} \textit{Atg5}\textsuperscript{Δ/Δ} and \textit{Atg7}\textsuperscript{Δ/Δ} mice. B. Kaplan-Meier survival curve of TAM-treated wild type, PDGFRα-Cre \textit{Atg7}\textsuperscript{Δ/Δ} and PDGFRα-Cre \textit{Atg5}\textsuperscript{Δ/Δ} mice. * p<0.05 **** p<0.0001 (log-rank Mantel-Cox test) C and D. Representative ileum IF costaining of p62 and PDGFRα at Day 3 and Day 4 from wild type, PDGFRα-Cre \textit{Atg7}\textsuperscript{Δ/Δ} and PDGFRα-Cre \textit{Atg5}\textsuperscript{Δ/Δ} mice. White arrows indicate colocalization of p62 with PDGFRα. E. Representative ileum H&E histology at indicated time points from wild type, PDGFRα-Cre \textit{Atg7}\textsuperscript{Δ/Δ} and PDGFRα-Cre \textit{Atg5}\textsuperscript{Δ/Δ} mice. F. Representative ileum IHC staining of Active β-catenin at Day 4 post TAM from wild type, PDGFRα-Cre \textit{Atg7}\textsuperscript{Δ/Δ} and PDGFRα-Cre \textit{Atg5}\textsuperscript{Δ/Δ} mice. G. Representative ileum IHC staining of OLFM4 at indicated time points from wild type, PDGFRα-Cre \textit{Atg7}\textsuperscript{Δ/Δ} and PDGFRα-Cre \textit{Atg5}\textsuperscript{Δ/Δ} mice. See also Figure S4.8.
Deleting *Atg5* more gradually rescues intestinal ileum function.

Systemic deletion of *Atg5* causes more rapid loss of the ATG5-ATG12 complex and greater p62 accumulation in the ileum that systemic deletion of *Atg7*, suggesting a more rapid and complete loss of autophagy. This raised the possibility that gradual loss of autophagy may allow adaptation and ileum survival. We then tested whether giving *Atg5*\(^{\text{flox/flox}}\) mice TAM once per week for 4 weeks (slow deletion) instead of on 5 consecutive days (fast deletion) to delete *Atg5* more gradually could allow adaptation and rescue the lethal phenotype of *Atg5*\(^{\Delta/\Delta}\) mice (Fig. 4.6A). In stark contrast to *Atg5*\(^{\Delta/\Delta}\) mice generated by fast deletion that died in four days, *Atg5*\(^{\Delta/\Delta}\) mice generated by slow deletion survived three months post TAM, more comparable to *Atg7*\(^{\Delta/\Delta}\) mice generated by slow (or fast) deletion (Fig. 4.1B, 4.6B). Importantly, both *Atg5*\(^{\Delta/\Delta}\) and *Atg7*\(^{\Delta/\Delta}\) mice generated by slow deletion died of neurodegeneration similar to *Atg7*\(^{\Delta/\Delta}\) mice generated by fast deletion (Karsli-Uzunbas et al., 2014). Tissues in *Atg5*\(^{\Delta/\Delta}\) and *Atg7*\(^{\Delta/\Delta}\) mice by slow deletion maintained intact at Day three, and started to show loss of Purkinje cells in cerebellum (Fig. S4.9A, S4.9B). At Week 5, there were more loss of Purkinje cells compared to Week 2 in *Atg5*\(^{\Delta/\Delta}\) mice, but less severe compared to *Atg7*\(^{\Delta/\Delta}\) mice at this time point, along with severe loss of hepatocytes in liver, muscle wasting, loss of white adipose tissue and massive loss of Purkinje cells (Fig. S4.9C). At Week 12, *Atg5*\(^{\Delta/\Delta}\) mice also developed massive loss of Purkinje cells, loss of hepatocytes, loss of white adipose tissue and muscle wasting which were similar as *Atg7*\(^{\Delta/\Delta}\)
mice at Week 5 (Fig. S4.9D). We observed normal villous architecture without inflammation or significant apoptosis in \(\text{Atg}^{\Delta/\Delta}\) ileum at day three with slow deletion, whereas fast deletion in these mice caused severe ileum damage (Fig. 4.6C, Fig. 4.1E), with no damage at duodenum and jejunum (Fig. S4.9E).

Deletion of \(\text{Atg}5\) in \(\text{Atg}^{\Delta/\Delta}\) mice and deletion of \(\text{Atg}7\) in \(\text{Atg}^{\Delta/\Delta}\) mice were confirmed (Fig. S4.9F). Loss of ATG5-ATG12 complex was displayed in the ileum on day three post TAM in \(\text{Atg}^{\Delta/\Delta}\) mice by slow deletion, whereas the ATG5-ATG12 complex still maintained in \(\text{Atg}^{\Delta/\Delta}\) mice which was similar as observed in whole body knockout mice generated by acute deletion (Fig. 4.6D, 4.1F). Accumulation of the unprocessed form of LC3 (LC3-I) and decreased levels of the processed form of LC3 (LC3-II) was observed in the \(\text{Atg}^{\Delta/\Delta}\) ileum compared to the \(\text{Atg}^{\Delta/\Delta}\) ileum, suggesting higher autophagy blockage in \(\text{Atg}^{\Delta/\Delta}\) ileum (Fig. 4.6D). Similar results were observed in duodenum and jejunum (Fig. S4.9G). We also observed loss of ATG5 in duodenum, jejunum and ileum in \(\text{Atg}^{\Delta/\Delta}\) mice and loss of ATG7 in \(\text{Atg}^{\Delta/\Delta}\) mice, suggesting these proteins are lost in intestine after TAM (Fig. 4.6E). There were increased p62 aggregates observed in duodenum, jejunum and mostly in ileum part of \(\text{Atg}^{\Delta/\Delta}\) mice compared to \(\text{Atg}^{\Delta/\Delta}\) mice (Fig. 4.6F, S4.9H). In contrast to \(\text{Atg}^{\Delta/\Delta}\) mice generated by fast deletion, OLFM4-positive ileum stem cells were retained in \(\text{Atg}^{\Delta/\Delta}\) mice generated by slow deletion, as were the PDGFR\(\alpha\) and CD34 positive telocytes (Fig. 4.6G, 4.6H). There were no decrease in the relative transcriptional level of Axin2 and less than two fold change of Sox9,
suggesting Wnt signaling is still functioning (Fig. S4.9I). Moderate p62 accumulation in PDGFRα+ mesenchymal cells in both Atg5Δ/Δ ileum and Atg7Δ/Δ ileum were observed, suggesting some PDGFRα+ mesenchymal cells lost function but most of them maintained intact (Fig. 4.6I). These data suggested that Atg5Δ/Δ mice by slow deletion overcame the deleterious phenotype and behave similarly as the Atg7Δ/Δ mice.
Figure 4.6. Deleting Atg5 more gradually rescues intestinal ileum function. A. Experimental design for generation of Atg5Δ/Δ and Atg7Δ/Δ mice by slow deletion. B. Kaplan-Meier survival curve of TAM-treated wild type, Atg7Δ/Δ and Atg5Δ/Δ mice. * p<0.05 **** p<0.0001 (log-rank Mantel-Cox test) C. Representative ileum H&E histology from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice by slow deletion at indicated time points. D. Western blotting for ATG5, ATG7, LC3 at Day 3 from ileum of wild type, Atg7Δ/Δ and Atg5Δ/Δ mice by slow deletion. β-ACTIN is used as the loading control. E. Representative duodenum, jejunum and ileum Immunohistochemistry (IHC) staining of ATG5 and ATG7 at indicated time points from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice by slow deletion. F. Representative ileum IHC staining of p62 at indicated time points from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice by slow deletion. Red arrows indicate p62 aggregates. G. Representative duodenum, jejunum and ileum IHC staining of OLFM4 at indicated time points from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice by slow deletion. H. Representative ileum IF costaining of CD34 and PDGFRα at Day 3 from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice by slow deletion. I. Representative ileum IF costaining of p62 and PDGFRα at Day 3 on the ileum from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice by slow deletion. White arrows indicate colocalization of p62 with PDGFRα. See also Figure S4.9.
Autophagy is required to maintain mitochondria function, TCA cycle and nucleotide metabolism in PDGFRα+ mesenchymal cells

We then sought to test why autophagy is required for the maintenance of PDGFRα+ mesenchymal cells. It was discovered that autophagy deficiency will trigger p53 to induce downstream cell apoptosis in lung cancer, breast cancer and pancreatic cancer, and deletion of p53 will attenuate the tumor growth (Guo et al., 2013; Huo et al., 2013; Rosenfeldt et al., 2013; Strohecker et al., 2013; Yang and Kimmelman, 2014; Yang et al., 2014; Yang et al., 2011). Thus, we crossed the Trp53<sup>flox/flox</sup> (p53 hereafter) with our Ubc-Cre<sup>ERT2/+</sup>; Atg5<sup>flox/flox</sup> mouse to generate Ubc-Cre<sup>ERT2/+</sup>; Atg5<sup>flox/flox</sup>; Trp53<sup>flox/flox</sup> mice. TAM was injected to these mice to generate Atg5<sup>Δ/Δ</sup>p53<sup>Δ/Δ</sup> mice and compared with Atg5<sup>Δ/Δ</sup> mice (Fig. S4.10A). Whole body codeletion of p53 together with Atg5 did not rescue the survival of Atg5<sup>Δ/Δ</sup> mice, suggesting that Atg5 deletion induced death cannot be rescued by p53 deficiency (Fig. S4.10B).

Autophagy is also required for maintenance of cellular metabolism and mitochondria function (Guo et al., 2013; Guo et al., 2016; Karsli-Uzunbas et al., 2014). We then sought to investigate whether autophagy is required to maintain normal cellular metabolism and mitochondria function in telocytes. Single cell RNA sequencing data from the PDGFRα+ mesenchymal cells revealed a higher expression level of several essential autophagy genes in the crypt and villus telocytes compared to fibroblasts, especially the genes involved in the autophagosome assembly including Gabarap and Gabarap2,
Map1lc3a (LC3-I), Sqstm1 (p62), suggesting higher autophagy activity in telocytes (Fig. 4.7A). We then performed Matrix-assisted laser desorption ionization coupled to imaging mass spectrometry (MALDI-IMS) to identify the level of metabolites on wild type and Atg5Δ/Δ ileum. Fourteen metabolites showed significant difference between wild type and Atg5Δ/Δ PDGFRα+ mesenchymal cells. These metabolites including TCA cycle intermediates aconitate and citrate/isocitrate, which is accumulating in Atg5Δ/Δ PDGFRα+ mesenchymal cells compared to wild type, suggesting malfunction of TCA cycle in Atg5Δ/Δ PDGFRα+ mesenchymal cells (Fig. 4.7B). Atg5Δ/Δ PDGFRα+ mesenchymal cells had more glucose-1 phosphate but less glycerol-phosphate, suggesting more usage of glycogen but less lipids (Fig. 4.7B). Another group of metabolites were TCA cycle derived amino acids: aspartate, glutamate and glutamine. Atg5Δ/Δ PDGFRα+ mesenchymal cells displayed decreased aspartate and glutamate but accumulation of glutamine, suggesting dysfunction of electron transport chain (ETC) in mitochondria as previously published (Guo et al., 2016; Jain et al., 2019) (Fig. 4.7B). The other metabolites were related to nucleotide synthesis and degradation. Atg5Δ/Δ PDGFRα+ mesenchymal cells showed increased ribose phosphate, AMP, inosine, hypoxanthine, xanthine and uric acid and greatly decreased cytidine compared to wild type, indicating increased purine synthesis, purine and pyrimidine degradation (Fig. 4.7B). These data suggested that autophagy deficient PDGFRα+ mesenchymal cells altered their metabolism with blockage
of TCA cycle and increased nucleotide degradation; both related to mitochondria dysfunction caused by autophagy deficiency (Guo et al., 2016) Thus, we tested the mitochondria numbers in PDGFRα+ mesenchymal cells. There’s moderate TOM20 in PDGFRα+ mesenchymal cell at Day one, which was increased in these cells on day two in Atg5Δ/Δ compared to Atg7Δ/Δ and wild type ileum, suggesting increased abnormal mitochondria numbers as we discovered before in autophagy deficient tumors (Guo et al., 2013; Joshi et al., 2015; Karsli-Uzunbas et al., 2014), indicating the altered metabolism in Atg5Δ/Δ PDGFRα+ mesenchymal cells was likely to be caused by mitochondria dysfunction. (Fig. 4.7C, S4.10C).
Fig. 4.7. Autophagy is required to maintain mitochondria function, TCA cycle and nucleotide metabolism in PDGFRα+ mesenchymal cells. A. single cell RNA sequencing data of essential autophagy genes. Y axis, in units of fraction of cellular mRNA (averaged over all the sequenced cells from the indicated cluster). B. MALDI-IMS data from wild type and Atg5Δ/Δ ileum at Day 1 and Day 2 post TAM. The scale bar on the left of each image set represents the intensity range of ions detected for each metabolite. The white pixels on the telocyte image panel represent the PDGFRα+ mesenchymal cells. C. Representative ileum IF costaining of TOMM20 and PDGFRα at Day 2 from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice. See also Figure S4.10.
Fig. 4.8. Mechanism by which autophagy is required for telocytes survival to maintain intestinal homeostasis.

See text for explanation.
Discussion

Previous work has suggested that mice with Atg5, Atg16L1 or Atg7 deficiency in the intestine epithelium layer (under the control of Villin-Cre) had a granule abnormality in Paneth cells but the general intestine structure is maintained and mice can survive (Cadwell et al., 2008; Wittkopf et al., 2012). These findings indicate that autophagy in the epithelial compartment is not essential for ileum function, in contrast to the loss of ileum function with the whole body conditional Atg5 deletion shown here. It remains possible that adaptation to the loss of autophagy occurs in these models as we observed with slow deletion of Atg5. Similarly, Atg5−/− mice with transgenic Atg5 expression in neurons to rescue neonatal lethality showed intestinal morphological changes with shorter and wider villi and deeper crypts that did not affect mouse survival (Yoshii et al., 2016). Adaptation to loss of autophagy may also occur in this setting. Autophagy in the intestinal epithelium layer is also required to maintain enough number of intestinal stem cells and prevent irradiation induced damage and necroptosis (Asano et al., 2017; Matsuzawa-Ishimoto et al., 2017). All these phenotypes caused by intestine specific autophagy deficiency were not lethal to the mice. However, in our model we observed a lethal phenotype caused by acute loss of Atg5 in the whole body with a lifespan of only 4 days. Even though the major damage site is only in the ileum part of intestine, the damage is severe with complete loss of stem cells villus cells besides the granules abnormality in Paneth cells. The key reason for the
difference is that in previous study the essential autophagy gene was deleted only in the epithelium layer of intestine by using the Villin-Cre recombinase but we deleted the target gene in the whole body. Therefore, we are blocking autophagy function in more cell populations, especially in the PDGFRα+ mesenchymal cells which are not affected in the Villin-Cre models. Thus, in Villin-Cre models, the intestinal stem cells still receive decent amount of Wnt ligands to maintain their renewal and proliferation and mice can survive unless damage was induced by other factors like irradiation (Asano et al., 2017). However, in our case, we blocked autophagy function in these PDGFRα+ mesenchymal cells and we found that rapid loss of autophagy caused depletion of these cells. Therefore, the intestinal stem cells cannot get enough Wnt ligands to turn on Wnt signaling properly (Fig. 4.8). Thus, the intestinal homeostasis cannot be maintained and cause the mice to die of hypoglycemia and loss of intestinal barrier function.

Autophagy is important in maintaining cellular metabolism and the pool size of TCA cycle intermediates, amino acids and nucleotides (Guo et al., 2016; Karsli-Uzunbas et al., 2014; Poillet-Perez et al., 2018). In our MALDI-IMS experiment, the fourteen metabolites which showed significant differences all belonged to these groups, and aspartate is probably a key metabolite among all of them. Aspartate is a TCA cycle derived non-essential amino acid, and its synthesis needs proper activation of ETC in mitochondria, which is required for
cell proliferation and growth (Birsoy et al., 2015; Sullivan et al., 2015). Here in our data, accumulation of glutamine and TOMM20 in $Atg^{5Δ/Δ}$ PDGFRα+ mesenchymal cells suggesting dysfunction of ETC and mitochondria, which can explain the decreased aspartate in these cells (Jain et al., 2019).

Aspartate also serves as an indispensable component for purine synthesis by donating its amino group to IMP to make AMP and also directly contributes in forming the pyrimidine ring. However, in $Atg^{5Δ/Δ}$ PDGFRα+ mesenchymal cells, aspartate cannot be synthesized properly and support nucleotide synthesis due to loss of ETC function caused by autophagy deficiency; At the same time, short of energy supply caused nucleotide degradation marked by increased xanthine and uric acid. These caused decreased nucleotide pool similar as in the tumor derived cell lines, which is very likely to be the cause of death for $Atg^{5Δ/Δ}$ PDGFRα+ mesenchymal cells (Guo et al., 2016).

Inflammatory bowel disease (IBD), including Crohn’s disease (CD) and ulcerative colitis (UC), are complex diseases which start with chronic relapsing and remitting intestinal inflammation which finally leads to tissue fibrosis. The driver of IBD is usually a combination of genetic, environmental and microbiota factors in the intestine or colon (Rieder and Fiocchi, 2008). Several genetic variants have been identified by GWAS to be related to autophagic defect and linked to IBD onset. The most outstanding one is $Atg16L1$, which is an essential autophagy gene directly involved in autophagosome formation. In
patients with CD the essential autophagy gene *Atg16L1* is usually a risk allele with a polymorphism of T300A, and intestine of the patient displays abnormality in Paneth cells granule secretion as seen in the Villin-Cre *Atg16L1* KO mice (Cadwell et al., 2008). This *Atg16L1* T300A polymorphism also causes morphological defect in goblet cells and increased production of cytokine IL-1β (Lassen et al., 2014). Interestingly, *Atg16L1* polymorphism in intestine causes failure of sensing protective signals from the microbiome, which is a critical gene-environment etiology for development of IBD (Chu et al., 2016). Another IBD susceptible gene related to autophagy is NOD2, which is a pattern recognition receptor that is involved in the homeostasis of intestinal immunity and works together with *Atg16L1*. Under normal condition, NOD2 will be activated by invading bacteria and recruits ATG16L1 to the plasma membrane at the bacteria invading site and triggers autophagosome formation to wrap the bacteria for clearance; Mutant NOD2 fails to recruit ATG16L1 to the plasma membrane and bacteria clearance by autophagy will be impaired (Cooney et al., 2010; Homer et al., 2010; Travassos et al., 2010). Another gene identified is ULK1, which can directly phosphorylate wild type Atg16L1 in response to infection and promotes xenophagy, but will destabilize the mutant Atg16L1 with CD polymorphism (Alsaadi et al., 2019).

It has been shown that in both types of IBD, the PDGFRα+ mesenchymal cells were significantly decreased as the disease progressed to advanced stage
In our study, we unraveled an important role of autophagy in maintaining the survival of PDGFRα+ mesenchymal cells, which is essential for maintaining intestinal homeostasis. Therefore, the decreased number of PDGFRα+ mesenchymal cells in IBD patient is very likely caused by gradual mutation or loss of essential autophagy genes in these cells as the disease gets more advanced. Thus, our study presents a possible mechanism for development of IBD, and upregulation of autophagy function in those PDGFRα+ mesenchymal cells or supplementing Wnt ligands in early IBD patients could potentially serve as a therapy to deal with this disease.
Supplemental Figures

Supplementary Fig. S4.1

A. Whole body deletion of Alg12 or Alg5

Cag-CreERT2<sup>+</sup> → Algin<sup>−</sup>
Cag-CreERT2<sup>−</sup> (Wild Type)

Cag-CreERT2<sup>−</sup>: Alg12<sup>−/−</sup> → Cag-CreERT2<sup>−</sup>: Alg12<sup>+/−</sup>

Cag-CreERT2<sup>−</sup>: Alg5<sup>−/−</sup> → Cag-CreERT2<sup>−</sup>: Alg5<sup>+/−</sup>

2-3 months survival
Neurodegeneration
Liver, muscle damage
Loss of fat tissue

B. Graph showing survival rates of different genotypes

Wild Type (n=45)
Cag-Cre Alg12<sup>−/−</sup> (n=29)
Cag-Cre Alg5<sup>−/−</sup> (n=18)

Days post TAM

C. H&E duodenum

D. H&E jejunum

E. H&E ileum

F. H&E duodenum

G. H&E jejunum
**Figure S4.1. Related to Figure 4.1.** A. Kaplan-Meier survival curve of TAM-treated wild type, Cag-Cre *Atg12Δ/Δ* and Cag-Cre *Atg5Δ/Δ* mice. **** p<0.0001 (log-rank Mantel-Cox test). B. Representative duodenum Hematoxyin&Eosin stained (H&E) histology at indicated time points from wild type, *Atg7Δ/Δ* and *Atg5Δ/Δ* mice. C. Representative jejunum H&E histology at indicated time points from wild type, *Atg7Δ/Δ* mice and *Atg5Δ/Δ* mice. D. Representative ileum H&E histology at indicated time points from wild type, Cag-Cre *Atg12Δ/Δ* mice and Cag-Cre *Atg5Δ/Δ* mice. E. Representative duodenum H&E histology at indicated time points from wild type, Cag-Cre *Atg12Δ/Δ* mice and Cag-Cre *Atg5Δ/Δ* mice. F. Representative jejunum H&E histology at indicated time points from wild type, Cag-Cre *Atg12Δ/Δ* mice and Cag-Cre *Atg5Δ/Δ* mice.
Figure S4.2. Related to Figure 4.1. A. Blood glucose concentration measured by mg/dL from wild type and Atg5Δ/Δ mice supplemented with/without glucose solution by IP injection at indicated time points. n.s, not significant (log-rank Mantel-Cox test). B. Kaplan Meyer survival curve of wild type and Atg5Δ/Δ mice supplemented with/without glucose solution by IP injection at indicated time points. n.s, not significant (log-rank Mantel-Cox test).

C. Representative PCR of Atg5 from the Atg5Δ/Δ mice at indicated time points.

D. Representative PCR of Atg7 from the Atg7Δ/Δ mice at indicated time points.

E. Western blotting for ATG5, ATG7, LC3 at indicated time points from the duodenum of wild type, Atg7Δ/Δ mice and Atg5Δ/Δ mice by slow deletion. β-ACTIN is used as the loading control.

F. Western blotting for ATG5, ATG7, LC3 at indicated time points from the jejunum of wild type, Atg7Δ/Δ mice and Atg5Δ/Δ mice by slow deletion. β-ACTIN is used as the loading control.
Figure S4.3. Related to Figure 4.2. A-B. Representative duodenum and jejunum IHC staining of ATG5 at indicated time points from wild type, Atg7Δ/Δ mice and Atg5Δ/Δ mice. C-D. Representative duodenum and jejunum IHC staining of ATG7 at indicated time points from wild type, Atg7Δ/Δ mice and Atg5Δ/Δ mice. E-F. Representative duodenum and jejunum IHC staining of p62 at indicated time points from wild type, Atg7Δ/Δ mice and Atg5Δ/Δ mice. G-H. Representative ileum p62 staining at indicated time points from wild type, Cag-Cre Atg12Δ/Δ mice and Cag-Cre Atg5Δ/Δ mice. I-J. Representative duodenum p62 staining at indicated time points from wild type, Cag-Cre Atg12Δ/Δ mice and Cag-Cre Atg5Δ/Δ mice. K-L. Representative jejunum p62 staining at indicated time points from wild type, Cag-Cre Atg12Δ/Δ mice and Cag-Cre Atg5Δ/Δ mice.
**Figure S4.4. Related to Figure 4.2.** A-B. Representative duodenum and jejunum Alcian staining at indicated time points from wild type, *Atg*7Δ/Δ mice and *Atg*5Δ/Δ mice. C. Representative ileum Alcian staining at indicated time points from wild type, Cag-Cre *Atg*12Δ/Δ mice and Cag-Cre *Atg*5Δ/Δ mice. D-E. Representative duodenum and jejunum Alcian staining at indicated time points from Cag-Cre *Atg*12Δ/Δ mice and Cag-Cre *Atg*5Δ/Δ mice. F-G. Representative duodenum and jejunum IHC staining of OLFM4 at indicated time points from wild type, *Atg*7Δ/Δ mice and *Atg*5Δ/Δ mice. H. Representative ileum IHC staining of OLFM4 at indicated time points from wild type, Cag-Cre *Atg*12Δ/Δ mice and Cag-Cre *Atg*5Δ/Δ mice. I-J. Representative duodenum and jejunum IHC staining of OLFM4 at indicated time points from wild type, Cag-Cre *Atg*12Δ/Δ mice and Cag-Cre *Atg*5Δ/Δ mice. K-L. Representative duodenum and jejunum IHC staining of Lysozyme at indicated time points from wild type, *Atg*7Δ/Δ mice and *Atg*5Δ/Δ mice. M. Representative ileum IHC staining of Lysozyme at indicated time points from wild type, Cag-Cre *Atg*12Δ/Δ mice and Cag-Cre *Atg*5Δ/Δ mice. N-O. Representative ileum IHC staining of Lysozyme at indicated time points from wild type, Cag-Cre *Atg*12Δ/Δ mice and Cag-Cre *Atg*5Δ/Δ mice. P-Q. Representative duodenum and jejunum IF co staining of p62 and OLFM4 at indicated time points from wild type, *Atg*7Δ/Δ mice and *Atg*5Δ/Δ mice.
Figure S4.5. Related to Figure 4.3. A. Representative picture of the untreated \( Atg^{5\Delta/\Delta} \) organoids, \( Atg^{5\Delta/\Delta} \) organoids, and \( Atg^{5\Delta/\Delta} \) organoids supplemented with Wnt3a or Wnt2b and untreated \( Atg^{7\Delta/\Delta} \) organoids, \( Atg^{7\Delta/\Delta} \) organoids, \( Atg^{7\Delta/\Delta} \) organoids supplemented with Wnt3a or Wnt2b at passage 1. B-C. Alive organoid percentage and average budding numbers of the untreated \( Atg^{5\Delta/\Delta} \) organoids, \( Atg^{5\Delta/\Delta} \) organoids, \( Atg^{5\Delta/\Delta} \) organoids supplemented with Wnt3a or Wnt2b and untreated \( Atg^{7\Delta/\Delta} \) organoids, \( Atg^{7\Delta/\Delta} \) organoids, \( Atg^{7\Delta/\Delta} \) organoids supplemented with Wnt3a or Wnt2b at passage 1. D-G. Quantitative real-time PCR of OLFM4, Lgr5, Axin2 and Sox9 of the untreated \( Atg^{5\Delta/\Delta} \) organoids, \( Atg^{5\Delta/\Delta} \) organoids, \( Atg^{5\Delta/\Delta} \) organoids supplemented with Wnt3a or Wnt2b and untreated \( Atg^{7\Delta/\Delta} \) organoids, \( Atg^{7\Delta/\Delta} \) organoids, \( Atg^{7\Delta/\Delta} \) organoids supplemented with Wnt3a or Wnt2b at passage 1. H. Representative picture of the untreated \( Atg^{5\Delta/\Delta} \) organoids, \( Atg^{5\Delta/\Delta} \) organoids, and \( Atg^{5\Delta/\Delta} \) organoids supplemented with Wnt3a or Wnt2b and untreated \( Atg^{7\Delta/\Delta} \) organoids, \( Atg^{7\Delta/\Delta} \) organoids, \( Atg^{7\Delta/\Delta} \) organoids supplemented with Wnt3a or Wnt2b at passage 2. I-J. Alive organoid percentage and average budding numbers of the untreated \( Atg^{5\Delta/\Delta} \) organoids, \( Atg^{5\Delta/\Delta} \) organoids, \( Atg^{5\Delta/\Delta} \) organoids supplemented with Wnt3a or Wnt2b and untreated \( Atg^{7\Delta/\Delta} \) organoids, \( Atg^{7\Delta/\Delta} \) organoids, \( Atg^{7\Delta/\Delta} \) organoids supplemented with Wnt3a or Wnt2b at passage 2. K-N. Quantitative real-time PCR of OLFM4, Lgr5, Axin2 and Sox9 of the untreated \( Atg^{5\Delta/\Delta} \) organoids, \( Atg^{5\Delta/\Delta} \) organoids, \( Atg^{5\Delta/\Delta} \) organoids supplemented with Wnt3a or Wnt2b and untreated \( Atg^{7\Delta/\Delta} \) organoids, \( Atg^{7\Delta/\Delta} \) organoids, \( Atg^{7\Delta/\Delta} \) organoids supplemented with Wnt3a or Wnt2b at passage 2.
Wnt2b and untreated Atg7^{flox/flox} organoids, Atg7^{Δ/Δ} organoids, Atg7^{Δ/Δ} organoids supplemented with Wnt3a or Wnt2b at passage 2. O-P. Western blotting for ATG5, ATG7, and LC3 from the untreated Atg5^{flox/flox} organoids, Atg5^{Δ/Δ} organoids, and Atg5^{Δ/Δ} organoids supplemented with Wnt3a or Wnt2b and untreated Atg7^{flox/flox} organoids, Atg7^{Δ/Δ} organoids, Atg7^{Δ/Δ} organoids supplemented with Wnt3a or Wnt2b at passage 2 and passage 3. β-ACTIN is used as the loading control.
**Figure S4.6. Related to Figure 4.4.** A-B. Representative ileum IHC staining of Active β-catenin at Day 1 and Day 2 post TAM from wild type, *Atg7Δ/Δ* mice and *Atg5Δ/Δ* mice. C-D. Representative ileum IHC staining of Active β-catenin at Day 1 and Day 2 post TAM from wild type, Cag-Cre *Atg12Δ/Δ* mice and Cag-Cre *Atg5Δ/Δ* mice. E-F. Representative ileum IF costaining of CD34 and PDGFRα at Day 1 and Day 2 post TAM from wild type, *Atg7Δ/Δ* mice and *Atg5Δ/Δ* mice. G-H. Representative ileum IF costaining of CD34 and PDGFRα at Day 1 and Day 2 post TAM from wild type, Cag-Cre *Atg12Δ/Δ* mice and Cag-Cre *Atg5Δ/Δ* mice.
Figure S4.7. Related to Figure 4.4. A-B. Representative ileum IF costaining of p62 and PDGFRα at Day 1 and Day 3 post TAM from wild type, $Atg^{7\Delta/\Delta}$ mice and $Atg^{5\Delta/\Delta}$ mice. White arrows indicate colocalization of p62 with PDGFRα. C-D. Representative ileum IF costaining of p62 and PDGFRα at Day 1 and Day 2 post TAM from wild type, Cag-Cre $Atg^{12\Delta/\Delta}$ mice and Cag-Cre $Atg^{5\Delta/\Delta}$ mice. White arrows indicate colocalization of p62 with PDGFRα.
Figure S4.8. Related to Figure 4.5. A. Representative ileum IF costaining of p62 and PDGFRα at Day 2 from wild type, PDGFRα-Cre Atg7Δ/Δ and PDGFRα-Cre Atg5Δ/Δ mice. White arrows indicate colocalization of p62 with PDGFRα. B. Representative ileum Alcian blue staining at indicated time points from wild type, PDGFRα-Cre Atg7Δ/Δ and PDGFRα-Cre Atg5Δ/Δ mice. C. Representative ileum IHC staining of Lysozyme at Day 4 post TAM from wild type, PDGFRα-Cre Atg7Δ/Δ and PDGFRα-Cre Atg5Δ/Δ mice. D-E. Representative duodenum and jejunum H&E histology at indicated time points from wild type, PDGFRα-Cre Atg7Δ/Δ and PDGFRα-Cre Atg5Δ/Δ mice. F. Representative ileum IF costaining of p62 and PDGFRα at Day 8 from wild type, PDGFRα-Cre Atg7Δ/Δ and PDGFRα-Cre Atg5Δ/Δ mice. White arrows indicate colocalization of p62 with PDGFRα. G. Representative duodenum, jejunum and ileum H&E histology at Day 8 from wild type, PDGFRα-Cre Atg7Δ/Δ and PDGFRα-Cre Atg5Δ/Δ mice.
**Figure S4.9. Related to Figure 4.6.** A-C. Representative liver, muscle, cerebrum, cerebellum, pancreas, White adipose tissue (WAT) and lung H&E histology at Day 3, Week 2 and Week 5 from wild type, \(\textit{Atg}^{7\Delta/\Delta}\) mice and \(\textit{Atg}^{5\Delta/\Delta}\) mice by slow deletion. D. Representative liver, muscle, cerebrum, cerebellum, pancreas, WAT and lung H&E histology at Week twelve from wild type and \(\textit{Atg}^{5\Delta/\Delta}\) mice by slow deletion. E. Representative duodenum and jejunum H&E histology from wild type, \(\textit{Atg}^{7\Delta/\Delta}\) mice and \(\textit{Atg}^{5\Delta/\Delta}\) mice by slow deletion at indicated time points. F. Representative PCR of \(\textit{Atg}5\) from the \(\textit{Atg}^{5\Delta/\Delta}\) mice and \(\textit{Atg}7\) from the \(\textit{Atg}^{7\Delta/\Delta}\) mice at Day 3 post TAM. G. Western blotting for ATG5, ATG7, LC3 at Day 3 post TAM from the duodenum and jejunum of wild type, \(\textit{Atg}^{7\Delta/\Delta}\) mice and \(\textit{Atg}^{5\Delta/\Delta}\) mice by slow deletion. \(\beta\)-ACTIN is used as the loading control. H. Representative duodenum and jejunum IHC staining of p62 at indicated time points from wild type, \(\textit{Atg}^{7\Delta/\Delta}\) mice and \(\textit{Atg}^{5\Delta/\Delta}\) mice by slow deletion.
Figure S4.10. Related to Figure 4.7. A. Experimental design for generation of Atg5Δ/Δ and Atg5Δ/Δp53Δ/Δ mice. B. Kaplan-Meier survival curve of TAM-treated wild type, Atg5Δ/Δ and Atg5Δ/Δp53Δ/Δ mice. n.s. not significant (log-rank Mantel-Cox test) C. Representative ileum IF costaining of TOMM20 and PDGFRα at Day 1 from wild type, Atg7Δ/Δ and Atg5Δ/Δ mice.
CHAPTER 5

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