A Novel Atg5-shRNA Mouse Model Enables Temporal Control of Autophagy in vivo

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Abstract: Autophagy is an evolutionary conserved catabolic pathway whose modulation has been linked to diverse disease states, including age-associated disorders. Conventional and conditional whole body knockout mouse models of key autophagy genes display perinatal death and lethal neurotoxicity, respectively, limiting their applications for in vivo studies. Here, we have developed an inducible shRNA mouse model targeting Atg5, allowing us to dynamically inhibit autophagy in vivo, termed Atg5i mice. The lack of brain-associated shRNA expression in this model circumvents the lethal phenotypes associated with complete autophagy knockouts. We show that Atg5i mice recapitulate many of the previously described phenotypes of tissue-specific knockouts. While restoration of autophagy in the liver rescues hepatomegaly and other pathologies associated with autophagy deficiency, this coincided with the development of hepatic fibrosis. These results highlight the need to consider the potential side effects of systemic anti-autophagy therapies.

Key Words: Autophagy, Atg5, Genetically engineered mouse model, Liver, Fibrosis, shRNA
Introduction

Macroautophagy (herein referred to as autophagy) is an evolutionary conserved, bulk cellular degradation system required to maintain cellular and energy homeostasis. Through autophagy, cytoplasmic components such as lipids, proteins and entire organelles are isolated in double-membrane vesicles (autophagosomes) and subsequently delivered to the lysosome to facilitate degradation and recycling of their respective constitutive components. Deregulation of autophagic flux, the rate at which autophagosomes form, fuse with lysosomes, and breakdown constitutive components, is believed to play a key role in the development of age-associated disorders, neurodegenerative conditions, and cancer{White:2015bv, Kroemer:2015im, GarciaPrat:2016bu, Komatsu:2006iq, Takamura:2011kv, Rosenfeldt:2013em, Menzies:2015hg}.

Conventional knockout mouse models of essential autophagy genes, such as Atg5 and Atg7, display perinatal lethal phenotypes within the first day after birth. This coincides with a period of metabolic stress wherein neonates must adapt and engage the autophagic machinery to mobilize their own food stores{Kuma:2004hq, Komatsu:2005jc}. Additionally, conditional whole-body knockout of Atg7 in adult mice has provided further evidence for the requirement of autophagy to survive acute metabolic stress. These mice also experience premature death due to neurotoxicity in metabolically unstressed states{KarsliUzunbas:2014kg}.

Due to these limitations there has been a reliance on tissue-specific knockouts to provide insight into the role of basal autophagy in tissue development and homeostasis{Mizushima:2010hx}. However, in all such classical approaches there is
a dependence on the complete and irreversible abrogation of key autophagy genes, a situation not generally associated with the etiology of human disease or therapeutic modulation of autophagy. These approaches preclude the ability to perform reversal experiments and restore autophagy as a mechanism to reverse or modulate the disease state.

With this in mind, we have generated an inducible Atg5-shRNA mouse model, which enables us to inhibit and restore Atg5-dependent autophagy in vivo. The model enables temporal control of Atg5 levels, which can be made ubiquitous or cell type-specific through breeding with appropriate Cre-recombinase expressing strains. Herein we have chosen to focus on the characterization of systemic Atg5 down-regulation and provide evidence that these mice display key phenotypes analogous to those described in knockout models (e.g. hepatomegaly, reduced adipose tissue, and pancreatic degeneration), indicating a high degree of Atg5 knockdown and autophagy inhibition in these tissues. Importantly we further utilize the system to ascertain how reversible these pathological states are, and provide evidence that autophagy inhibition and subsequent restoration may have pathological consequences.

Results

To investigate the effects that a reduction in autophagy would have on organismal homeostasis, and the degree to which these effects are reversible, we have developed a mouse model incorporating a doxycycline (dox)-inducible shRNA system (Fig. 1A){Dow:2012iw, Premsrirut:2011bm}. Using a sensor-based screening system{Fellmann:2011ee}, we first obtained a panel of shRNAs against Atg5, an essential component for autophagosome formation, and then selected an shRNA with
the greatest knockdown in vitro, Atg5_1065 (see Fig. S1, and Methods). Subsequently mice were generated carrying a single copy of Atg5_1065 under the control of doxycycline (dox). Briefly, the TRE (Tetracycline-Responsive Element)-regulated Atg5-shRNA is downstream of the Collal locus and is GFP-linked providing a non-invasive reporter system of activation. Its transcription is driven in mice by rtTA3 in the presence of dox, of which spatial expression is restricted courtesy of a loxp-stop-loxp (LSL) cassette (this model is termed LSL-Atg5i mice). To generate a second version of this mouse model, wherein Atg5 can be ubiquitously knocked-down, we crossed the LSL-Atg5i mouse to a PGK-Cre expressing strain\{Lallemand:1998wn\}. This resulted in germ-line excision of the LSL ‘STOP cassette’, which was passed on to subsequent generations while the PGK-Cre was rapidly bred out (Atg5i mice).

Atg5i mouse embryonic fibroblasts (MEFs) show a reduction in Atg5 levels and the conversion of soluble LC3-I to membrane bound LC3-II, an Atg5-dependent process, by western blot analysis upon administration of dox (Fig. 1B).

Using a tamoxifen-inducible Cre (Cre-ERT2) system, Atg7 was recently knocked out systemically in adult mice\{KarsliUzunbas:2014kg\}. To test the extent to which Atg5i mice recapitulate gross phenotypes of the whole-body somatic Atg7 knockout (KO) mice, eight-week old Atg5i mice were placed on a dox-containing diet for 6 weeks. Similar to the systemic Atg7 KO mice, Atg5i mice on dox appear smaller in size with smaller weight gain in both genders (Fig. 1C). Anatomical inspection revealed, as in the whole-body somatic Atg7 KO mice and/or Atg5/7 KO mice, a reduction of fat\{Singh:2009bk\} and muscle tissues (Fig. 1D and E)\{Masiero:2009bq\}, with the presence of hepatomegaly\{Komatsu:2005jc\}, splenomegaly, and seminal vesicle atrophy (Fig. 1F, G and H)\{Yoshii:2016gc\}.
We next validated Atg5 knockdown in tissues from 6-week dox-treated Atg5i mice. Western blotting displayed a strong reduction in Atg5 levels as well as a reduction in the conversion of soluble LC3-I to membrane bound LC3-II (Fig. 2A). This was reflected in immunohistochemistry (IHC) analyses by the accumulation of p62 (also known as SQSTM1), an autophagy substrate, forming aggregates to various degrees depending on tissue/cell type (Fig. 2B and Fig. S2). As reported before, the increase of poly-ubiquitinated proteins was evident particularly in muscle and heart (Fig. S2B)\cite{Kuma:2017fq}. In addition, pathological features of autophagy deficiency were also reproduced: e.g. in liver, hepatocytes were enlarged with intracellular proteinaceous aggregates, and in the pancreas, acinar and islet degeneration was noted in the dox-treated Atg5i mice (Fig. 2C). These results indicate that the single copy integration of *sh-Atg5* in the genome is sufficient for robust down-regulation of Atg5 and, as a result, of autophagy activity *in vivo*.

One notable exception, however, was brain tissue, where we failed to detect any alterations in Atg5 and poly-ubiquitinated protein levels (Fig. 2A and S2B). This is consistent with the reported inefficient expression of shRNA in the brain using this system\cite{Dow:2012iw}. Additionally, in contrast to whole body somatic *Atg7* KO mice, which develop lethal neurodegeneration\cite{KarsliUzunbas:2014kg}, Atg5i mice displayed no evidence of overt neurological or motor phenotypes, and presented with normal limb clasping reflexes and brain histology when treated with dox for up to 8 months (Fig. S3). Despite this exception, Atg5i mice recapitulate many of the major phenotypes associated with autophagy deficiency at cellular, organ, and organismal levels.
To test the resilience of Atg5i mice to survive the perinatal starvation period, *sh-Atg5* homozygous mice were crossed with *rtTA3* heterozygous mice and fed a dox-containing diet (Fig. 3A). Only resultant offspring inheriting both components of the system are able to induce *sh-Atg5* expression. Consistent with embryonic *Atg5* KO mice, Atg5i mice were born at close to expected Mendelian ratios (Observed 40%; Expected 50%){Kuma:2004hq}. However, unlike *Atg5* KO mice, all embryonic Atg5i mice were able to survive the neonatal starvation period. Similar results were recently described wherein restoration of *Atg5* expression, ectopically driven from a rat neuron-specific enolase promoter (*NSE*), was sufficient to rescue neonatal lethality of conventional *Atg5* KO mice, suggesting that the neonatal lethality of embryonic *Atg5* KO mice is primarily due to neurological dysfunction including a suckling defect{Kuma:2004hq, Yoshii:2016gc}. Although it is formally possible that this lack of neonatal death phenotype in the Atg5i model is due to hypomorphism of autophagy deficiency, IHC analyses showed strong accumulation of p62 aggregation in neonatal liver (Fig. 3B). In addition, and congruent with previous publications, while Atg5i neonates appeared indistinguishable at birth to their littermate counterparts, their postnatal development of body size and weight was severely impaired (Fig. 3C-D). These data reinforce the developmental role of autophagy in neuronal tissues, which is essential for survival during periods of neonatal metabolic stress{Yoshii:2016gc}.

Autophagy has a critical role in maintaining energy homeostasis during periods of starvation-induced stress. Eighty percent of whole-body somatic *Atg7* KO mice die with lethal hypoglycemia during a 24hr fasting period{KarsliUzunbas:2014kg}. To test whether the Atg5i mice also recapitulate this phenotype, eight-week old Atg5i
mice were treated with dox for a period of two-weeks and then fasted for 24 hours with free access to water, to replicate the same experimental design as previously reported{KarsliUzunbas:2014kg}. At this time point, diminished expression of Atg5 in comparison to control mice was associated with p62 aggregation in the liver (Fig. 4A and B). Unlike whole-body somatic Atg7 KO mice{KarsliUzunbas:2014kg}, Atg5i mice displayed no evidence of fasting-induced death (Fig. 4C) and maintained blood glucose levels similar to that of control mice, despite continued suppression of Atg5 at least in the liver (Fig. 4D). These results indicate that both embryonic and somatic Atg5i mice are highly robust under metabolic stress conditions, although it remains to be elucidated whether or not the unaltered autophagy activity in the brain of Atg5i mice is also responsible for rescuing starvation-induced death in the somatic model. Furthermore, these results reinforce the unique nature of the Atg5i model, which provides an opportunity for longer-term experiments involving autophagy defective adults.

Next, we examined the effects of restoring autophagy in the Atg5i mice by taking advantage of the regulatable nature of the system. As in Fig. 2, 8-week old Atg5i and control mice were fed a dox-infused diet for 6-weeks to induce whole-body Atg5 deficiency. At this point, mice were then switched to a standard diet (absent from dox) for a further 6 weeks. Within this time window at least, the extent to which the Atg5 knockdown-associated phenotypes recover upon Atg5 restoration varied depending on the tissue type (Fig. S4 and S5). Strikingly, during necropsy, livers from these Atg5-restored mice were found to display no evidence of hepatomegaly (Fig. 5A). The complete reversibility of hepatomegaly was confirmed through a time-course analysis using MRI imaging (Fig. 5B, C and S5B). Re-expression of Atg5 was
confirmed at the protein level by western blot analysis and was associated with a normalization of LC3 levels (Fig. 5D), suggesting that autophagic flux had been re-established.

Additionally, the histopathological alterations associated with autophagy deficiency, such as swollen hepatocytes and increased proliferation and apoptosis, were absent in the Atg5 reconstituted livers (Fig. 6A and Fig. S6A and B). Evidence of liver damage and impaired liver function in Atg5i mice 6 weeks on dox, as measured by elevated serum alanine aminotransferase (ALT) and reduced serum albumin levels, also reverted to control levels upon dox withdrawal (Fig. S6C and D).

Interestingly, we found that autophagy restoration in the liver was also associated with the induction of hepatic fibrosis as determined by picrosirius red (Fig. 6B and C). This effect was not seen in Atg5i mice fed dox continuously for 6 or 12 weeks, suggesting that the increased fibrosis is not an outcome of autophagy deficiency per se but likely to be a secondary effect of autophagy restoration. This phenotype was also recapitulated in a short-term setting, where autophagy was restored after dox addition for 3 weeks (around which point hepatomegaly became evident as shown in Fig. 5C), although the induction of fibrosis following 3 weeks off dox was modest, showing only a 2.5-fold increase in collagen staining (Fig. S7), in comparison to a 6.96-fold increase in the 6-week on/off regimen (Fig. 6C). We next stained for α-SMA, a marker of activated hepatic stellate cells (HSCs), mediators of liver fibrosis, and found a substantial increase of α-SMA after 6-weeks on dox in Atg5i mouse
livers (Fig. 6D and E). However, consistent with the picrosirius red staining (Fig. 6C),
Col1α1, a major component of liver fibrosis, did not show any increase in these same
livers, but was instead upregulated only after autophagy restoration (Fig. 6B-D). Note
the major source of Col1α1 in the liver is activated HSCs, thus our data suggest that
while HSCs can be activated in the absence of autophagy, they are not fibrogenic.
Interestingly, it has been shown that inhibition of autophagy, either genetically or
pharmacologically, in HSCs can prevent Col1α1 expression and fibrosis during liver
injury{HernandezGea:2012hn}{Thoen:2011bh}. Thus, it is possible that in the Atg5i
mice, systemic autophagy deficiency triggers hepatocyte damage, which activates
HSCs, but the activated HSCs are not fully functional and that the restoration of
autophagy enables the primed HSCs to perform their functional roles, including the
deposition of collagen. Thus, although pathological features of autophagy deficiency
in the liver are largely reversible, transient autophagy inhibition may confer
unforeseen adverse effects.

In the pancreas, the removal of dox was also associated with the re-expression of
Atg5 and near complete normalization of LC3 levels as evidenced by whole tissue
western blot analysis (Fig. 7A). However, while p62 levels were elevated in both
acinar and islet compartments during dox administration in Atg5i mice, only the
acinar cells of the pancreas displayed a normalization of p62 levels upon dox removal
(Fig. 7C). Consistently, while the acinar portion of the pancreas histologically
recovered, the islets still appeared degenerative with areas of vacuolization apparent
(Fig. 7B and C). Thus, the data suggest that, similar to the liver, autophagy
deficiency-associated phenotypes of the pancreatic acinar are also reversible.
However, in contrast to the liver, the pancreas displayed no evidence of fibrosis after
Atg5 restoration (Fig. 7C). The reason for the observed irreversibility of islet phenotype is unclear. Of note, the acinar was found to display evidence of increased proliferation that was not seen in the islets and may reflect the natural abilities of these cellular populations to recover upon stress (Fig. S8).

Discussion

Here we report the first temporally-regulatable mouse model of autophagy, enabling both down-regulation, and subsequent re-expression of endogenous Atg5 in vivo. Although this model can be both systemic and tissue-specific, in this study, we focused on the systemic model to evaluate the overall effects of the system. As previously described with this system{Dow:2012iw}, Atg5i mice exhibited no apparent Atg5 knockdown in brain. Interestingly, although further detailed analyses are required to determine the Atg5 knockdown efficiency in entire tissues/cell types, the lack of Atg5 knockdown in brain has created a unique situation analogous to the recently developed Atg5-/-;NSE-Atg5 mice. In these mice, Atg5 is ectopically expressed under a neuron specific promoter in the conventional Atg5 KO background{Yoshii:2016gc}. In contrast to Atg5-/-;NSE-Atg5 mice, which is an embryonic system, in Atg5i mice the shRNA can be induced either embryonically or somatically, the latter is particularly useful to separate developmental phenotypes from the role of autophagy in tissue homeostasis. Thus, together with its dynamic nature, the Atg5i mouse model offers unique and complementary resource for autophagy studies. However, it is important to note any possibility of off-target effects of the RNAi in this system. While our model exploits a recently developed inducible shRNA system, wherein the shRNA is expressed in a miR-E cassette to allow physiological processing and reduced off-target effects{Fellmann:2013ji},
currently we only use one targeting sequence. As such it will be important to develop
further RNAi models targeting alternative sequences of Atg5, or other key autophagy
genes, for further validation of newly described phenotypes.

We report that Atg5i mice appeared refractory to metabolic stress unlike conventional
and conditional autophagy knockout mice. Analogous to the Atg5-/-;NSE-Atg5 model,
dox-treated Atg5i neonates (where dox was administered throughout embryogenesis)
did not display the characteristic rapid perinatal lethality. Moreover, 24 hours food
withdrawal in adult Atg5i mice was not associated with a lethal hypoglycemic
response, in contrast to somatic Atg7 KO mice, which develop extensive brain
damage. Although, in our Atg5i model, it is not possible to study direct effects of
dynamic autophagy modulation in brain, data from this model raise an interesting
question; whether basal autophagy in the adult brain plays a critical role in systemic
metabolic homeostasis under starvation conditions.

We leveraged the dynamic nature of our system and show a tissue- or cell type-
specific difference in the reversibility of any alterations-associated with autophagy
deficiency, at least during the time range tested. Further studies will be required to
determine the exact source of this heterogeneity and whether longer restoration times
are required for some tissues. Nevertheless, our model showed near compete
reversibility in the liver and pancreatic acinar. Importantly, in the liver, despite the
full reversibility of pathologies derived from Atg5 knockdown, autophagy restoration
enhanced fibrosis. This does not appear to affect liver function (Fig S6C) but may
alter the long-term fate of the tissue microenvironment. Hepatocyte cell death due to
autophagy inhibition has been previously reported to lead to the activation of HSCs
and drive fibrosis\cite{Ni:2014bu}. It was also shown that Atg5-deficient macrophages facilitate chemically-induced liver fibrosis through stimulating myofibroblasts (likely to be activated HSCs\cite{Iwaisako:2014jk} to express fibrogenic genes)\cite{Lodder:2015jk}. In our Atg5i mice, however, despite hepatocyte cell death, HSC activation and Atg5 knockdown in immune cells/macrophages (Fig. S9), the livers of Atg5i mice on dox failed to exhibit collagen deposition and fibrosis which only developed after autophagy restoration (Fig. 6), reinforcing the critical role of autophagy within activated HSCs for the fibrogenic activity of these cells\cite{HernandezGea:2012hn, Thoen:2011bh}.

In the clinical setting, autophagy-modulating therapies have garnered interest for life-and health-span modulation, as well as in the field of oncology \cite{Levine:2015ds, Rubinsztein:2012hm, Kroemer:2015im}. Particularly for the latter, inhibition of autophagy (considered as cytoprotective program) has generally been suggested for use in conjunction with standard chemotherapy. As such the temporal modulation of autophagy is considered a rational goal to achieve clinical benefit. However, regimens to-date that modulate autophagic flux do not act specifically on the autophagy machinery. Instead they often target other components of the cellular system to alter autophagy and as such distinguishing autophagy specific effects is often difficult. Our data suggest that the systemic Atg5i mice may be utilized to model specific anti-autophagy therapies. Additionally, there is very little understanding of the potential adverse effects of switching systemically from an autophagy-low state to an autophagy-high or restored state, which, as highlighted here, may be associated with further complications. As shorter regimens of autophagy inhibition appear to result in reduced fibrosis in this system, we speculate that the timing of dosing, as well as the
degree of autophagy inhibition, may be a critical determinant in the generation of pathological effects.

Methods

Antibodies

For Western: Anti-Atg5 (Abcam ab108327, 1/1000), Anti-LC3 (Nanotools Clone 5F10, 1/1000), Anti-β Actin (Sigma), Anti-Actin (Santa Cruz I-19, 1/5000), Anti-α SMA (Abcam ab5694, 1/1000), Anti-Col1a1 (Abcam ab34710, 1/2000), Anti-poly Ubiquitin (Enzo Clone FK1, 1:5000), Anti-turboGFP (Pierce PA5-22688, 1/2000), Anti-Rabbit HRP and Anti-Mouse HRP (1/5000)

For Immunohistochemistry (IHC): Anti-p62 (Enzo, BML-PW9860, 1/750), Anti-Ki67 (Abcam, ab16667, 1/1000), Anti-Cleaved Caspase 3 (Cell Signalling, #9664, 1/200), Anti-α SMA (Abcam ab5694, 1/500), Anti-Col1a1 (Abcam ab34710, 1/1000).

Western Blot Analysis

Western blot analysis was performed as previously{Young:2009ew}. Cells and tissues were lysed in laemmli buffer; tissues samples were homogenized with the Precellys 24 tissue homogenizer in laemmeli buffer. Samples were run on 12.5% or 15% gels and transferred to PVDF membranes (Immobilon, Millipore). The membrane was blocked for 1hr at room temperature (5% milk solution in TBS-Tween 0.1%) before incubating with primary antibody at 4°C overnight. Subsequently an appropriate HRP-conjugated secondary antibody was incubated at room temperature for 1hr. Western blots were visualized with chemiluminescence reagents (Sigma, RPN2106).
IHC

Formalin-fixed paraffin-embedded samples were de-waxed and rehydrated before antigen unmasking with citrate buffer (pH 6) in a pressure cooker for 5 minutes at 120°C. Remaining steps were according to the Dako Envision+ Rabbit kit instructions.

Picrosirius Red Staining and Quantification

Briefly FFPE slides were de-waxed and rehydrated before being stained with Weigert's Haematoxylin (8 mins), washed in running water (10 mins), immersed in picrosirius red solution (1hr; Sigma) and washed in acidified water (0.5% acetic acid) for 2 changes. Slides were then dehydrated and cover slipped. Once stained images were taken at random using a Nikon T-2000 inverted microscope and DSFi-1 camera. The specimen was illuminated with circularly polarized light by setting the de Senarmont compensator of the Nikon microscope with the polarizer at 45 degrees to the fast/slow axes of the quarter-wave plate. A circular polarizer was placed in the light path from the objective lens, producing a dark field as its circular polarization was opposite to that of the illuminating beam. In this condition, the birefringent specimen is essentially placed between crossed circular polarizers and remains bright at all azimuthal positions. If an area was found to overlap with a large vessel it was discounted and images not taken for analysis, in all conditions at least 10 images were used for analysis. Subsequently, images were analyzed in Fiji by generating a threshold in a control sample and using this across all samples. The remaining area count provided ‘positive fibrotic area’. Each sample was then normalized to the mean value of all the controls.
Blood Glucose Homeostasis During Starvation

Blood glucose measurements were taken prior to, and after 24 hours of, food withdrawal using the ACCU-CHEK Aviva blood glucose monitor. As doxycycline is provided in the diet of mice, mice also received doxycycline IP (20ml/kg of a 4mg/ml solution) to ensure continued expression of $Atg5_{1065}$.

Ki67 Counting

For livers, automated counting of Ki-67 across the entire liver section was performed using ImageScope™ (Leica Biosystems) customized for the liver and reported as a percentage of nuclei that stained positively. For the pancreas acinar and islets were counted separately using HALO™ software (Indicalab) and reported as a percentage of nuclei that stained positively.

Cleaved Caspase 3 Analysis

For livers, automated counting of stain positivity across the entire liver section was performed using ImageScope™ (Leica Biosystems) customized for the liver and reported as a percentage of pixels in the image that stained positively, known as a positive pixel count.

Islet Degeneration

Each pancreas had all islets available in the histological section imaged and counted for the presence or absence of islet degeneration. The number of degenerative islets was calculated as a percentage of the total number of islets visible on one section.
from a single mouse. This was calculated for control and Atg5i mice and a mean and standard deviation was calculated for each group and displayed graphically.

**MRI**

MRI scans were acquired on a 9.4T Agilent MRI scanner running VnmrJ 3.1A and equipped with gradients of maximum strength 40 G/cm and inner diameter 120mm. A quadrature millipede coil of inner diameter 40mm was used for all imaging studies. Coronal fast spin-echo images were acquired with and without fat suppression employing a chemical-shift-selective sinc-profile RF pulse. Slice thickness was 1mm, field of view 100mm x 50mm, 512x256 points providing in-plane resolution of 200µm. Up to 18 slices were acquired to cover the full body of the mouse. The effective echo time was 44ms, echo train length 8, and four averages were acquired to improve SNR in the liver, which is hypointense with these parameters. Nominal TR was 3 seconds, but in practice this was determined by the respiratory gating employed to minimise motion artifacts in the upper abdomen. This sequence gives good contrast between different soft tissues. Organ volumes were measured by drawing ROIs on the fat-suppressed images. Fat volume was assessed by subtracting the unsuppressed images from the suppressed images and thresholding the resulting image.

**Generation and Maintenance of Atg5i Mice**

A panel of shRNAs in a Mir-E design{Fellmann:2013ji} targeting Atg5 was obtained from Mirimus Inc. through a sensor-based screening system {Fellmann:2011ee} in a pLPE backbone and was used to generate retroviral supernatant. We tested knockdown efficiency of those shRNAs in NIH3T3 cells and an shRNA showing the strongest knockdown even with the highest dilution (1% v/v) of viral supernatant was
taken forward for mouse generation in collaboration with Mirimus Inc (Fig. S1). The shRNA (Atg5_1065; Guide sequence: TATGAAGAAAGTTATCTGGGTA) was inserted downstream of the Collal locus via recombinase-mediated cassette exchange which enables efficient targeting of a transgene to a specific genomic site 500bp downstream of the 3’ UTR in D34 ES cells expressing CAGS-rtTA3 knocked into the Rosa26 locus{Beard:2006hz}{Dow:2014be} (Fig. S1C). Mice were maintained on a mixed C57Bl/6 X 129 background and littermate controls were used in all experiments. All experimental mice were maintained as heterozygous for both the Atg5_1065 and CAGS-rtTA3 alleles, while control littermates were absent for one of the alleles. Mice were maintained in a specific pathogen-free environment under a 12 hr light/dark cycle, having free access to food and water unless otherwise stated. Mice were fed either a laboratory diet (PicoLab Mouse Diet 20; 5R58) or the same diet containing doxycycline at 200PPM (PicoLab Mouse Diet; 5A5X). All experiments were performed in accordance with national and institutional guidelines, and the study was approved by the ethical review committee of the University of Cambridge. Imaging of neonates for tGFP expression was conducted using excitation lamp (460-494nm) and emission filter (500-515nm) (BLS, FHS/LS-1B) optimized for fluorescent proteins in the green wavelength.

Liver Immune Cell Isolation: Dissected livers were homogenized (Miltenyi Liver Dissociation Kit) and passed through a 70μm filter. After centrifugation, red blood cells were lysed with RBC Lysis Buffer (eBioscience) for 10 minutes. After centrifugation samples were washed twice in PEB buffer (PBS, EDTA 5μM, BSA 0.5%). Immune cells were initially isolated using an Optiprep gradient (Sigma) and the F4/80+ population isolated by incubating the immune cell population with F4/80
MicroBeads (Miltenyi) and passing the mixture through two MACs columns (Miltenyi) sequentially. Macrophages purified by MACS were stained with Fixable Viability Dye eFluor™ 780 (Thermo Fisher Scientific) to distinguish live cells from dead cells. Subsequently, cells were blocked with TruStain fcX™ (anti-mouse CD16/32) antibodies (Biolegend) and then stained with fluorochrome-conjugated antibodies against CD45 (clone 30-F11, Biolegend) and F4/80 (clone BM8, Biolegend). Stained cells were analyzed using FACS LSR II (BD) and acquired results were analyzed by the use of FlowJo software (v10.4, FlowJo, LLC).

RNA Analysis: RNA was isolated using Qiagen RNEasy Micro Kit and cDNA generated using Superscript™ III Reverse Transcriptase (Invitrogen) and random hexamers. Primer sequences as follows:

**Atg5**: Forward 5’-GCCGAACCCCTTTGCTCAATG-3’
Reverse 5’-TGGTCACCTTAGAAATACCCAC-3’

**β-Actin**: Forward 5’-CAAGAGAGGTATCCTGACCCTGAAG-3’
Reverse 5’-CATTGTAGAAGGTGTGGTGCCAG-3’

Disclosure of Interest
The authors report no conflict of interest.

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**Contributions**

L.D.C. and M.N. designed the experiments and wrote the manuscript. A.R.J.Y and P.A.P-M. generated the mouse model. L.D.C and A.R.J.Y performed all experiments except for shRNA screening in vitro (performed by P.A.P-M.) brain isolation, fixation and histopathology analysis (performed by B.N., T.R., R.J.G., D.R) and MRI capture (D.J.O.M). Macrophage isolation was performed by L.D.C, H.C.C. and M.D.L.R. H-C.C performed flow cytometry on enriched samples. A.J. and S.P. analyzed seminal vesicles. A.J and A.R.J.Y performed RNA isolation and qPCR on isolated immune cells. All additional tissue histopathology was analyzed blinded by a trained pathologist (R.B). All authors commented on the manuscript.
References

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**Figure Legends**

**Figure 1.** Generation of Atg5i Mice

(A) Graphical illustration of doxycycline (dox) inducible Atg5-RNAi (Atg5i) system. Only through crossing with the appropriate Cre-expressing strain is the loxP-STOP-loxP (LSL) cassette excised and rtTA3 protein produced. In the presence of dox, rtTA3 is able to bind to the tet-responsive element (TRE) and drive the expression of Atg5-shRNA in a Mir-E backbone. (B) Western blots for indicated proteins in MEFs isolated from Atg5i mice and littermate control in the presence or absence of dox (3 days). Control littermates lack either the rtTA or sh-Atg5 cassette. (C-G) Eight-week old Atg5i mice fed on a dox containing diet for 6-weeks display a decrease in weight (Males, \( P=0.0017 \) n=16 control and Atg5i; Females, \( P=0.0239 \) n=16 control and 9 Atg5i mice) in C, reduction in inguinal fat weight (\( P=0.0286, \) n=4 males per condition) in D, reduction in muscle weight (\( P=0.0286, \) n=4 males per condition) in E, hepatomegaly (\( P=0.0006, \) n=7; 3 females and 4 males per condition) in F, splenomegaly (\( P=0.0286 \) n=4 males per condition) in G, and seminal vesicle atrophy in H (\( P=0.0022 \) n=6 males per condition). All pairwise comparisons determined using Mann-Whitney test (\(*P<0.05, \) **\( P<0.01 \) and ***\( P<0.001 \)). Error bars represent s.d. around the means.

**Figure 2.** In vivo validation of autophagy inhibition upon Atg5 down-regulation

(A) Atg5 shows a down-regulation across a range of tissues in adult mice treated with dox for 6-weeks by western blot, except the brain, which displays no alterations in Atg5 in whole tissue extracts. \( \beta \)-Actin serves as a loading control in all tissues except for heart and muscle for which total Actin was used instead. (B) Atg5i mice display an increase in p62 in the indicated tissues via IHC. (C) Atg5 down-regulation is
associated with the development of large proteinaceous aggregates in the liver (yellow arrows). Additionally, cellular degeneration of the exocrine and endocrine pancreas is visible by H&E analysis (yellow asterisk). Scale bars= 100µm in B and C.

**Figure 3. Perinatal survival of Atg5i neonates**

(A) Breeding strategy for the generation of Atg5i neonates. A doxycycline containing diet was fed to the parents who each have one component of the two-component system, thus are unable to induce Atg5-shRNA. Any embryos with both components however will induce the system. Cont, control. (B) IHC analysis of 14-day-old neonates highlights the presence of p62 aggregates in the livers of Atg5i mice in comparison to control. Scale bars= 100µm. (C) Atg5i neonates are born at, and survive at, expected mendelian ratios. They appear indistinguishable from their littermate controls except for their positivity for tGFP (bottom). (D) While initially indistinguishable, Atg5i do not show the same growth kinetics (n= 6 males in both conditions).

**Figure 4. Adult Atg5i mice survive starvation-induced stress and maintain glucose homeostasis**

(A) Atg5i mice administered dox for 2 weeks and food withdrawal for 24 hours prior to sacrifice display down-regulation of Atg5 and a reduction of LC3-I to LC3-II conversion in the liver. (B) These same livers also show the formation of p62 aggregates as seen through immunohistochemical analysis. Scale bars = 100µm. (C) These mice show no evidence of starvation induced mortality when food is removed for 24 hours (Control n=6, Atg5i n=4). (D) Blood glucose levels before and after food
withdrawal show no significant difference between control and experimental (free feeding P=0.66; Starved P=0.37, Mann Whitney). N.S., not significant.

Figure 5. Restoration of Atg5 is associated with reversal of hepatomegaly

(A) Consistent with the results in Figure 1F, adult mice treated with dox for 6-weeks develop hepatomegaly in comparison to control mice (P= 0.0035). However, the restoration of Atg5 levels in Atg5i mice is associated with a significant reduction in liver size (P=0.007) to a weight similar to control mice on the same feeding regime. (n= 8-10 mice per group; Kruskal-Wallis with Dunn’s post test, **P<0.01, N.S., not significant) (B) Example images of an MRI scan from Atg5i mice at the 6-week on dox time point, as well as the 6-week on dox -> 6-week off dox time point displaying hepatomegaly and reversal to normal size, respectively. Yellow dotted lines encircle livers. (C) Time series analyses of liver size after dox addition, followed by dox withdrawal using MRI (n=2 mice per condition, average value is shown; see Figure S5B for individual data). (D) Adult Atg5i fed on a dox-containing diet for 6-weeks display a down-regulation of Atg5 and an increase in LC3-I by western blot analysis. When switched back to a normal diet for 6-weeks mice show a recovery in Atg5 levels and LC3-I, similar to control mice.

Figure 6. Restoration of Atg5 is associated with the induction of hepatic fibrosis

(A) Representative histology and IHC from the livers of control and Atg5i mice. Yellow arrows highlight large intracellular inclusions only found in Atg5i mice on dox. Scale bars= 100μm. (B) Representative images of Picrosirius Red staining in sections of control and Atg5i mice in the indicated conditions. Scale bars= 100μm. (C) Only Atg5i mice treated with dox for 6-weeks and off dox for 6-weeks showed the presence of fibrosis (P=0.0468 Welch’s t-test, error bars represent s.d. around the
means). **P<0.01; N.S., not significant. (D) Immunohistochemical analysis of livers from each time point highlights that α-SMA positive activated stellate cells are only present during the 6-week on dox time point, with Col1a1 staining positivity only present in the 6-week on dox->6-week off dox time point. (E) Whole tissue protein extracts display a similar trend with Col1a1 present only in the Atg5i mice at the 6-week on dox -> 6-week off dox time point.

**Fig 7. Atg5 restoration in the pancreas leads to partial phenotypic rescue**

(A) Adult Atg5i fed on a dox-containing diet for 6-weeks display a down-regulation of Atg5 and an increase in LC3-I by western blot analysis. When returned to a normal diet for 6-weeks mice show a recovery in Atg5 and LC3-I levels, similar to those seen in control mice. (B) Dox treated adult mice display evidence of islet degeneration that is not reversed upon dox withdrawal. Mann-Whitney test, ***P<0.001. (C) Representative histology, IHC, and picrosirius red staining (marker of fibrosis) from the pancreas of control and Atg5i mice. While p62 levels are increased in both the islets and acinar of Atg5i mice on dox, only the acinar display a reversal once dox is removed. Islets do not show a reversal of the degeneration phenotype as viewed by H&E. Scale bars= 100μm.

**Figure S1. In vitro screening for potent shRNA targeting Atg5**

(A) A panel of shRNAs was obtained from Mirimus Inc. in a pLPE backbone. Retrovirus for each shRNA was generated and knockdown efficiency tested in NIH3T3 cells. shAtg5#1 (Atg5_1065) provided the greatest knockdown efficiency by western blot analysis during infection. (B) This occurs at the lowest dilutions of retrovirus tested with a near complete abrogation of LC3-II conversion. Dilutions are
shown as percent volume of the retroviral soup directly derived from packaging cell culture and diluted into the media of NIH3T3. (C) Schematic illustrates the relation between Col1a1 gene and shRNA cassette. Targeting of the shRNA was achieved using recombinase-mediated cassette exchange at a specific site 500bp downstream of the Col1a1 3’UTR in D34 ES Cells{Dow:2014be}{Beard:2006hz}. (D) Homozygous targeting of the shRNA does not alter Col1a1 expression in MEFs in comparison to littermate wild-type controls.

**Figure S2. Extended Characterisation of Atg5i Mice**

(A) An increase in the staining for the autophagy adaptor protein p62 can be seen in muscle and heart tissue. Spleen tissue provides a heterogeneous staining even in the control tissue with an increase in intensity in the Atg5i mice. (B) Western blotting for poly-ubiquitin highlights an increase particularly in heart and muscle. Brain tissue shows no alterations. (C) A single gel containing all four tissues (5μg loaded per lane) highlights the differences in the expression of tGFP, a marker of system activation. As expected brain tissue displays a much reduced expression due to the bioavailability of dox.

**Figure S3. Atg5i mice display no evidence of neurotoxicity**

(A) Adult mice fed on dox-based diet for 6-weeks display normal limb claspers in both control and Atg5i cohorts. Note the limbs are extended in both control and Atg5i mice, unlike limb claspers towards the body as seen in some models of neurodegeneration. (B) Age-matched brain histology from mice treated with dox for 5-6 months (n=3 Atg5i and control mice). Sagittal sections (10 μm) were assessed blinded across various brain regions with particular emphasis on the cortex and
hippocampus. Representative images are given for regions spanning cortical layers and hippocampal pyramidal CA2/3 region layers. Haematoxylin and Eosin (H&E) staining showed no consistent differences between groups with a staining pattern reflective of aged mice, such as observable vacuolization. No above baseline TUNEL positive staining was observed between groups. Scale bars= 20μm.

**Figure S4. Reversibility of phenotype induced by Atg5 knockdown is tissue type dependent**

(A) Inguinal fat weights are significantly reduced in the Atg5i cohorts during dox administration and do not display a recovery in weight during the 6-week on dox -> 6-week off dox time point. (B) Similarly, splenic weights are significantly different while on dox, and also do not appear to significantly recover at the 6-week on dox -> 6-week off dox time point. (C) Muscle weight was significantly different between control and Atg5i mice while on dox, however this significance was lost during the 6-week on dox -> 6-week off dox time point. Mann-Whitney test *P<0.05, **P<0.01, ***P<0.001; N.S., not significant. (D) Time series analyses of total body fat content after dox addition, followed by dox withdrawal using MRI (n=2 mice per condition, average value is shown; see Figure S5A for individual data).

**Figure S5. Dynamics of adipose tissue and liver during doxycycline administration**

Individual data points from MRI studies of fat (Fig. S4D) and liver (Fig. 5C) in adult Atg5i fed on a dox-containing diet for 6-weeks, before being moved to a dox-free diet for 6-weeks. (A) Relative fat content and (B) Relative liver volume (n=2 mice per condition).
Figure S6. Liver functionality reflects Atg5 levels in vivo

(A) Proliferation, as measured through the presence of Ki-67 positive cells in IHC, was increased after 6-weeks of dox in the Atg5i group in comparison to controls (P<0.001) and displayed a significant decrease upon Atg5 restoration (P<0.001) to levels comparable to control mice. One-way ANOVA, n= 5-6 mice per condition. (B) Apoptosis, as measured by cleaved-caspase 3 (CC3) in IHC, is higher in Atg5i mouse livers during dox administration compared to control mice (P<0.001). Restoration of Atg5 is associated with a reduction in CC3 positivity (P<0.001) and a return to basal levels. One-way ANOVA, n= 3-7 mice per condition. All error bars represent s.d. around the means. ***P<0.001; N.S, not significant. (C) Serum albumin levels in Atg5i mice are suppressed in the presence of dox for 6 and 12 weeks in comparison to controls, P= 0.0460 and P=0.0116 respectively. Serum albumin levels recover to normal levels once dox is removed from the diet and Atg5 levels are restored. Kruskal-Wallis Test. n= 4-12 mice per condition with individual values displayed. (D) Serum ALT levels are significantly increased during a 6-week (P<0.0025, Mann-Whitney) or 12-week period on dox in Atg5i mice (P<0.0001, Mann-Whitney) which is not present in the 6 weeks on- 6 weeks off dox cohort. *P<0.05, **P<0.01, ***P<0.001; N.S., not significant.

Figure S7. Short-term inhibition and restoration of Atg5 is also associated with liver fibrosis

(A) Atg5i mice treated with dox for 3 weeks and subsequently moved to a dox-free diet for 3 weeks show a similar fibrosis phenotype to the 6-week on dox->6-week off dox time point. (B) Quantification of sirius red staining at the end of this time course.
(C-D) Atg5i mice displayed a larger liver after 3 weeks of dox, which reduced in size to normal levels after dox had been removed as seen by MRI (C) and tissue weight upon death (D). Values are means of two mice.

Figure S8. Proliferative index in the pancreas of Atg5i mice

(A) The pancreatic acinar of Atg5i mice displays a higher level of proliferation, as measured through the presence of Ki-67 positive cells in IHC, in comparison to controls both during dox administration and even after dox was removed from the diet, although only the 6-weeks on dox and 6-weeks off dox regimen reaches significance. (B) Conversely, the pancreatic islets did not show an increase in proliferation. Pairwise comparisons based on Mann-Whitney test.

Figure S9. Immune cells residing in the liver of Atg5i mice display evidence of Atg5 knockdown

(A-B) qPCR for Atg5 mRNA (relative to β-actin) in total immune cells and macrophages (F4/80 positive cells) isolated from the liver of control (Cont) and Atg5i mice on dox for 6 weeks. (C) Example flow cytometry confirming a high level of enrichment of F4/80 cells after MACs column enrichment with F4/80 microbeads (control or Atg5i?). (D) Isolated cells displayed both a high level of viability and a high level of F4/80 enrichment.