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

Liam D. Cassidy[1,5], Andrew RJ. Young[1,5], Pedro A. Pérez-Mancera[1,5†], Birgit Nimmervoll[1], Adil Jaulim[1], Hung-Chang Chen[1], Dominick J. O. McIntyre[1], Rebecca Brais[2], Thomas Ricketts[3], Simon Pacey[4], Maike de la Roche[1], Richard J. Gilbertson[1], David Rubinsztein[3], Masashi Narita[1*]

[1]Cancer Research UK Cambridge Institute, University of Cambridge, Cambridge, United Kingdom

[2]Department of Histopathology, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK

[3]Cambridge Institute for Medical Research, Department of Medical Genetics, Wellcome Trust/MRC Building, Cambridge CB2 0XY, UK

[4] Department of Oncology, University of Cambridge

[5] These authors contributed equally to this work

[†]Present Address: Department of Molecular and Clinical Cancer Medicine, University of Liverpool, Liverpool, United Kingdom

*Correspondence to Masashi.Narita@cruk.cam.ac.uk

23   **Abstract:** Autophagy is an evolutionary conserved catabolic pathway whose

24   modulation has been linked to diverse disease states, including age-associated

25   disorders. Conventional and conditional whole body knockout mouse models of key

26   autophagy genes display perinatal death and lethal neurotoxicity, respectively,

27   limiting their applications for *in vivo* studies. Here, we have developed an inducible

28   shRNA mouse model targeting Atg5, allowing us to dynamically inhibit autophagy *in*

29   *vivo*, termed Atg5i mice. The lack of brain-associated shRNA expression in this

30   model circumvents the lethal phenotypes associated with complete autophagy

31   knockouts. We show that Atg5i mice recapitulate many of the previously described

32   phenotypes of tissue-specific knockouts. While restoration of autophagy in the liver

33   rescues hepatomegaly and other pathologies associated with autophagy deficiency,

34   this coincided with the development of hepatic fibrosis. These results highlight the

35   need to consider the potential side effects of systemic anti-autophagy therapies.

36

37   **Key Words:** Autophagy, Atg5, Genetically engineered mouse model, Liver, Fibrosis,

38   shRNA

39
40

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

67   a dependence on the complete and irreversible abrogation of key autophagy genes, a

68   situation not generally associated with the etiology of human disease or therapeutic

69   modulation of autophagy. These approaches preclude the ability to perform reversal

70   experiments and restore autophagy as a mechanism to reverse or modulate the disease

71   state.

72

73   With this in mind, we have generated an inducible *Atg5-shRNA* mouse model, which

74   enables us to inhibit and restore Atg5-dependent autophagy *in vivo*. The model

75   enables temporal control of Atg5 levels, which can be made ubiquitous or cell type-

76   specific through breeding with appropriate Cre-recombinase expressing strains.

77   Herein we have chosen to focus on the characterization of systemic Atg5 down-

78   regulation and provide evidence that these mice display key phenotypes analogous to

79   those described in knockout models (e.g. hepatomegaly, reduced adipose tissue, and

80   pancreatic degeneration), indicating a high degree of Atg5 knockdown and autophagy

81   inhibition in these tissues. Importantly we further utilize the system to ascertain how

82   reversible these pathological states are, and provide evidence that autophagy

83   inhibition and subsequent restoration may have pathological consequences.

84

85   **Results**

86   To investigate the effects that a reduction in autophagy would have on organismal

87   homeostasis, and the degree to which these effects are reversible, we have developed

88   a mouse model incorporating a doxycycline (dox)-inducible shRNA system (Fig.

89   1A){Dow:2012iw, Premsrirut:2011bm}. Using a sensor-based screening

90   system{Fellmann:2011ee}, we first obtained a panel of shRNAs against *Atg5*, an

91   essential component for autophagosome formation, and then selected an shRNA with

92   the greatest knockdown *in vitro*, *Atg5_1065* (see Fig. S1, and Methods). Subsequently

93   mice were generated carrying a single copy of *Atg5_1065* under the control of

94   doxycycline (dox). Briefly, the TRE (Tetracycline-Responsive Element)-regulated

95   Atg5-shRNA is downstream of the *Col1a1* locus and is GFP-linked providing a non-

96   invasive reporter system of activation. Its transcription is driven in mice by rtTA3 in

97   the presence of dox, of which spatial expression is restricted courtesy of a loxp-stop-

98   loxp (LSL) cassette (this model is termed LSL-Atg5i mice). To generate a second

99   version of this mouse model, wherein Atg5 can be ubiquitously knocked-down, we

100 crossed the LSL-Atg5i mouse to a PGK-Cre expressing strain{Lallemand:1998wn}.

101 This resulted in germ-line excision of the LSL 'STOP cassette', which was passed on

102 to subsequent generations while the PGK-Cre was rapidly bred out (Atg5i mice).

103 Atg5i mouse embryonic fibroblasts (MEFs) show a reduction in Atg5 levels and the

104 conversion of soluble LC3-I to membrane bound LC3-II, an Atg5-dependent process,

105 by western blot analysis upon administration of dox (Fig. 1B).

106

107 Using a tamoxifen-inducible Cre (Cre-ERT2) system, *Atg7* was recently knocked out

108 systemically in adult mice{KarsliUzunbas:2014kg}. To test the extent to which Atg5i

109 mice recapitulate gross phenotypes of the whole-body somatic *Atg7* knockout (KO)

110 mice, eight-week old Atg5i mice were placed on a dox-containing diet for 6 weeks.

111 Similar to the systemic *Atg7* KO mice, Atg5i mice on dox appear smaller in size with

112 smaller weight gain in both genders (Fig. 1C). Anatomical inspection revealed, as in

113 the whole-body somatic *Atg7* KO mice and/or *Atg5/7* KO mice, a reduction of

114 fat{Singh:2009bk} and muscle tissues (Fig. 1D and E){Masiero:2009bq}, with the

115 presence of hepatomegaly{Komatsu:2005jc}, splenomegaly, and seminal vesicle

116 atrophy (Fig. 1F, G and H){Yoshii:2016gc}.

117

118 We next validated Atg5 knockdown in tissues from 6-week dox-treated Atg5i mice.

119 Western blotting displayed a strong reduction in Atg5 levels as well as a reduction in

120 the conversion of soluble LC3-I to membrane bound LC3-II (Fig. 2A). This was

121 reflected in immunohistochemistry (IHC) analyses by the accumulation of p62 (also

122 known as SQSTM1), an autophagy substrate, forming aggregates to various degrees

123 depending on tissue/cell type (Fig. 2B and Fig. S2). As reported before, the increase

124 of poly-ubiquitinated proteins was evident particularly in muscle and heart (Fig.

125 S2B){Kuma:2017fq}. In addition, pathological features of autophagy deficiency were

126 also reproduced: e.g. in liver, hepatocytes were enlarged with intracellular

127 proteinaceous aggregates, and in the pancreas, acinar and islet degeneration was noted

128 in the dox-treated Atg5i mice (Fig. 2C). These results indicate that the single copy

129 integration of *sh-Atg5* in the genome is sufficient for robust down-regulation of Atg5

130 and, as a result, of autophagy activity *in vivo*.

131

132 One notable exception, however, was brain tissue, where we failed to detect any

133 alterations in Atg5 and poly-ubiquitinated protein levels (Fig. 2A and S2B). This is

134 consistent with the reported inefficient expression of shRNA in the brain using this

135 system{Dow:2012iw}. Additionally, in contrast to whole body somatic *Atg7* KO

136 mice, which develop lethal neurodegeneration{KarsliUzunbas:2014kg}, Atg5i mice

137 displayed no evidence of overt neurological or motor phenotypes, and presented with

138 normal limb clasping reflexes and brain histology when treated with dox for up to 8

139 months (Fig. S3). Despite this exception, Atg5i mice recapitulate many of the major

140 phenotypes associated with autophagy deficiency at cellular, organ, and organismal

141 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{Takamura:2011kv}, 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 determine 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

216 livers (Fig. 6D and E). However, consistent with the picrosirius red staining (Fig. 6C),

217 Col1a1, a major component of liver fibrosis, did not show any increase in these same

218 livers, but was instead upregulated only after autophagy restoration (Fig. 6B-D). Note

219 the major source of Col1a1 in the liver is activated HSCs, thus our data suggest that

220 while HSCs can be activated in the absence of autophagy, they are not fibrogenic.

221 Interestingly, it has been shown that inhibition of autophagy, either genetically or

222 pharmacologically, in HSCs can prevent Col1a1 expression and fibrosis during liver

223 injury{HernandezGea:2012hn}{Thoen:2011bh}. Thus, it is possible that in the Atg5i

224 mice, systemic autophagy deficiency triggers hepatocyte damage, which activates

225 HSCs, but the activated HSCs are not fully functional and that the restoration of

226 autophagy enables the primed HSCs to perform their functional roles, including the

227 deposition of collagen. Thus, although pathological features of autophagy deficiency

228 in the liver are largely reversible, transient autophagy inhibition may confer

229 unforeseen adverse effects.

230

231 In the pancreas, the removal of dox was also associated with the re-expression of

232 Atg5 and near complete normalization of LC3 levels as evidenced by whole tissue

233 western blot analysis (Fig. 7A). However, while p62 levels were elevated in both

234 acinar and islet compartments during dox administration in Atg5i mice, only the

235 acinar cells of the pancreas displayed a normalization of p62 levels upon dox removal

236 (Fig. 7C). Consistently, while the acinar portion of the pancreas histologically

237 recovered, the islets still appeared degenerative with areas of vacuolization apparent

238 (Fig. 7B and C). Thus, the data suggest that, similar to the liver, autophagy

239 deficiency-associated phenotypes of the pancreatic acinar are also reversible.

240 However, in contrast to the liver, the pancreas displayed no evidence of fibrosis after

241 Atg5 restoration (Fig. 7C). The reason for the observed irreversibility of islet

242 phenotype is unclear. Of note, the acinar was found to display evidence of increased

243 proliferation that was not seen in the islets and may reflect the natural abilities of

244 these cellular populations to recover upon stress (Fig. S8).

245

246 **Discussion**

247 Here we report the first temporally-regulatable mouse model of autophagy, enabling

248 both down-regulation, and subsequent re-expression of endogenous Atg5 *in vivo*.

249 Although this model can be both systemic and tissue-specific, in this study, we

250 focused on the systemic model to evaluate the overall effects of the system. As

251 previously described with this system{Dow:2012iw}, Atg5i mice exhibited no

252 apparent Atg5 knockdown in brain. Interestingly, although further detailed analyses

253 are required to determine the Atg5 knockdown efficiency in entire tissues/cell types,

254 the lack of Atg5 knockdown in brain has created a unique situation analogous to the

255 recently developed *Atg5-/-;NSE-Atg5* mice. In these mice, *Atg5* is ectopically

256 expressed under a neuron specific promoter in the conventional *Atg5* KO

257 background{Yoshii:2016gc}. In contrast to *Atg5-/-;NSE-Atg5* mice, which is an

258 embryonic system, in Atg5i mice the shRNA can be induced either embryonically or

259 somatically, the latter is particularly useful to separate developmental phenotypes

260 from the role of autophagy in tissue homeostasis. Thus, together with its dynamic

261 nature, the Atg5i mouse model offers unique and complementary resource for

262 autophagy studies. However, it is important to note any possibility of off-target

263 effects of the RNAi in this system. While our model exploits a recently developed

264 inducible shRNA system, wherein the shRNA is expressed in a miR-E cassette to

265 allow physiological processing and reduced off-target effects{Fellmann:2013ji},

266   currently we only use one targeting sequence. As such it will be important to develop

267   further RNAi models targeting alternative sequences of *Atg5*, or other key autophagy

268   genes, for further validation of newly described phenotypes.

269

270   We report that Atg5i mice appeared refractory to metabolic stress unlike conventional

271   and conditional autophagy knockout mice. Analogous to the *Atg5-/-;NSE-Atg5* model,

272   dox-treated Atg5i neonates (where dox was administered throughout embryogenesis)

273   did not display the characteristic rapid perinatal lethality. Moreover, 24 hours food

274   withdrawal in adult Atg5i mice was not associated with a lethal hypoglycemic

275   response, in contrast to somatic Atg7 KO mice, which develop extensive brain

276   damage. Although, in our Atg5i model, it is not possible to study direct effects of

277   dynamic autophagy modulation in brain, data from this model raise an interesting

278   question; whether basal autophagy in the adult brain plays a critical role in systemic

279   metabolic homeostasis under starvation conditions.

280

281   We leveraged the dynamic nature of our system and show a tissue- or cell type-

282   specific difference in the reversibility of any alterations-associated with autophagy

283   deficiency, at least during the time range tested. Further studies will be required to

284   determine the exact source of this heterogeneity and whether longer restoration times

285   are required for some tissues. Nevertheless, our model showed near compete

286   reversibility in the liver and pancreatic acinar. Importantly, in the liver, despite the

287   full reversibility of pathologies derived from Atg5 knockdown, autophagy restoration

288   enhanced fibrosis. This does not appear to affect liver function (Fig S6C) but may

289   alter the long-term fate of the tissue microenvironment. Hepatocyte cell death due to

290   autophagy inhibition has been previously reported to lead to the activation of HSCs

291 and drive fibrosis{Ni:2014bu}. It was also shown that Atg5-deficient macrophages

292 facilitate chemically-induced liver fibrosis through stimulating myofibroblasts (likely

293 to be activated HSCs{Iwaisako:2014jk} to express fibrogenic

294 genes){Lodder:2015jk}. In our Atg5i mice, however, despite hepatocyte cell death,

295 HSC activation and Atg5 knockdown in immune cells/macrophages (Fig. S9), the

296 livers of Atg5i mice on dox failed to exhibit collagen deposition and fibrosis which

297 only developed after autophagy restoration (Fig. 6), reinforcing the critical role of

298 autophagy within activated HSCs for the fibrogenic activity of these

299 cells{HernandezGea:2012hn}{Thoen:2011bh}.

300

301 In the clinical setting, autophagy-modulating therapies have garnered interest for life-

302 and health-span modulation, as well as in the field of oncology {Levine:2015ds,

303 Rubinsztein:2012hm, Kroemer:2015im}. Particularly for the latter, inhibition of

304 autophagy (considered as cytoprotective program) has generally been suggested for

305 use in conjunction with standard chemotherapy. As such the temporal modulation of

306 autophagy is considered a rational goal to achieve clinical benefit. However, regimens

307 to-date that modulate autophagic flux do not act specifically on the autophagy

308 machinery. Instead they often target other components of the cellular system to alter

309 autophagy and as such distinguishing autophagy specific effects is often difficult. Our

310 data suggest that the systemic Atg5i mice may be utilized to model specific anti-

311 autophagy therapies. Additionally, there is very little understanding of the potential

312 adverse effects of switching systemically from an autophagy-low state to an

313 autophagy-high or restored state, which, as highlighted here, may be associated with

314 further complications. As shorter regimens of autophagy inhibition appear to result in

315 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 4ºC overnight. Subsequently an appropriate HRP-conjugated secondary antibody was incubated at room temperature for 1hr. Western blots were visualized with chemiluminsence reagents (Sigma, RPN2106).

341

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.

347

Picrosirius Red Staining and Quantification

Briefly FFPE slides were de-waxed and rehydrated before being stained with Weigers 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.

366

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

390 from a single mouse. This was calculated for control and Atg5i mice and a mean and

391 standard deviation was calculated for each group and displayed graphically.

392

393 **MRI**

394 MRI scans were acquired on a 9.4T Agilent MRI scanner running VnmrJ 3.1A and

395 equipped with gradients of maximum strength 40 G/cm and inner diameter 120mm. A

396 quadrature millipede coil of inner diameter 40mm was used for all imaging studies.

397 Coronal fast spin-echo images were acquired with and without fat suppression

398 employing a chemical-shift-selective sinc-profile RF pulse. Slice thickness was 1mm,

399 field of view 100mm x 50mm, 512x256 points providing in-plane resolution of

400 200μm. Up to 18 slices were acquired to cover the full body of the mouse. The

401 effective echo time was 44ms, echo train length 8, and four averages were acquired to

402 improve SNR in the liver, which is hypointense with these parameters. Nominal TR

403 was 3 seconds, but in practice this was determined by the respiratory gating employed

404 to minimise motion artifacts in the upper abdomen. This sequence gives good contrast

405 between different soft tissues. Organ volumes were measured by drawing ROIs on the

406 fat-suppressed images. Fat volume was assessed by subtracting the unsuppressed

407 images from the suppressed images and thresholding the resulting image.

408

409 **Generation and Maintenance of Atg5i Mice**

410 A panel of shRNAs in a Mir-E design{Fellmann:2013ji} targeting Atg5 was obtained

411 from Mirimus Inc. through a sensor-based screening system {Fellmann:2011ee} in a

412 pLPE backbone and was used to generate retroviral supernatant. We tested

413 knockdown efficiency of those shRNAs in NIH3T3 cells and an shRNA showing the

414 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 *Col1a1* 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 fluorchrome-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'-GCCGAACCCTTTGCTCAATG-3'*

       Reverse 5'-TGGTCACCTTAGGAAATACCCAC-3'

β-**Actin:** *Forward 5'-CAAGAGAGGTATCCTGACCCTGAAG-3'*

       Reverse 5'-CATTGTAGAAGGTGTGGTGCCAG-3'

Disclosure of Interest

The authors report no conflict of interest.

465 Light Microscopy (S. Reichelt, J. Pike, L. Berry for Picrosirius imaging and analysis),

466 and BRU (M. Clayton and H. Jones for animal husbandry).

467

478

479 **Contributions**

480 L.D.C. and M.N. designed the experiments and wrote the manuscript. A.R.J.Y and

481 P.A.P-M. generated the mouse model. L.D.C and A.R.J.Y performed all experiments

482 except for shRNA screening in vitro (performed by P.A.P-M.) brain isolation, fixation

483 and histopathology analysis (performed by B.N., T.R., R.J.G., D.R) and MRI capture

484 (D.J.O.M). Macrophage isolation was performed by L.D.C, H-C.C. and M.D.L.R. H-

485 C.C performed flow cytometry on enriched samples. A.J. and S.P. analyzed seminal

486 vesicles. A.J and A.R.J.Y performed RNA isolation and qPCR on isolated immune

487 cells. All additional tissue histopathology was analyzed blinded by a trained

488 pathologist (R.B). All authors commented on the manuscript.

489

490

491

References

{papers2_bibliography}

Figure Legends

496   **Figure 1. Generation of Atg5i Mice**

497   (**A**) Graphical illustration of doxycycline (dox) inducible Atg5-RNAi (Atg5i) system.

498   Only through crossing with the appropriate Cre-expressing strain is the loxP-STOP-

499   loxP (LSL) cassette excised and rtTA3 protein produced. In the presence of dox,

500   rtTA3 is able to bind to the tet-responsive element (TRE) and drive the expression of

501   Atg5-shRNA in a Mir-E backbone. (**B**) Western blots for indicated proteins in MEFs

502   isolated from Atg5i mice and littermate control in the presence or absence of dox (3

503   days). Control littermates lack either the rtTA or sh-Atg5 cassette. (**C-G**) Eight-week

504   old Atg5i mice fed on a dox containing diet for 6-weeks display a decrease in weight

505   (Males, P=0.0017 n=16 control and Atg5i; Females, P=0.0239 n=16 control and 9

506   Atg5i mice) in **C**, reduction in inguinal fat weight (P=0.0286, n=4 males per

507   condition) in **D**, reduction in muscle weight (P=0.0286, n=4 males per condition) in

508   **E**, hepatomegaly (P=0.0006, n=7; 3 females and 4 males per condition) in **F**,

509   splenomegaly (P=0.0286 n=4 males per condition) in **G,** and seminal vesicle atrophy

510   in H (P=0.0022 n=6 males per condition). All pairwise comparisons determined using

511   Mann-Whitney test (*P<0.05, **P<0.01 and ***P<0.001). Error bars represent s.d.

512   around the means.

513

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

515   (**A**) Atg5 shows a down-regulation across a range of tissues in adult mice treated with

516   dox for 6-weeks by western blot, except the brain, which displays no alterations in

517   Atg5 in whole tissue extracts. β-Actin serves as a loading control in all tissues except

518   for heart and muscle for which total Actin was used instead. (**B**) Atg5i mice display

519   an increase in p62 in the indicated tissues via IHC. (**C**) Atg5 down-regulation is

520   associated with the development of large proteinaceous aggregates in the liver (yellow

521   arrows). Additionally, cellular degeneration of the exocrine and endocrine pancreas is

522   visible by H&E analysis (yellow asterisk). Scale bars= 100μm in **B** and **C**.

523

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

525   (**A**) Breeding strategy for the generation of Atg5i neonates. A doxcycline containing

526   diet was fed to the parents who each have one component of the two-component

527   system, thus are unable to induce Atg5-shRNA. Any embryos with both components

528   however will induce the system. Cont, control. (**B**) IHC analysis of 14-day-old

529   neonates highlights the presence of p62 aggregates in the livers of Atg5i mice in

530   comparison to control. Scale bars= 100μm. (**C**) Atg5i neonates are born at, and

531   survive at, expected mendelian ratios. They appear indistinguishable from their

532   littermate controls except for their positivity for tGFP (bottom). (**D**) While initially

533   indistinguishable, Atg5i do not show the same growth kinetics (n= 6 males in both

534   conditions).

535

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

537   **glucose homeostasis**

538   (**A**) Atg5i mice administered dox for 2 weeks and food withdrawal for 24 hours prior

539   to sacrifice display down-regulation of Atg5 and a reduction of LC3-I to LC3-II

540   conversion in the liver. (**B**) These same livers also show the formation of p62

541   aggregates as seen through immunohistochemical analysis. Scale bars = 100μm. (**C**)

542   These mice show no evidence of starvation induced mortality when food is removed

543   for 24 hours (Control n=6, Atg5i n=4). (**D**) Blood glucose levels before and after food

544   withdrawal show no significant difference between control and experimental (free

545   feeding P=0.66; Starved P=0.37, Mann Whitney). N.S., not significant.

546

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

548   (**A**) Consistent with the results in Figure 1F, adult mice treated with dox for 6-weeks

549   develop hepatomegaly in comparison to control mice (P= 0.0035). However, the

550   restoration of Atg5 levels in Atg5i mice is associated with a significant reduction in

551   liver size (P=0.007) to a weight similar to control mice on the same feeding regime.

552   (n= 8-10 mice per group; Kruskal-Wallis with Dunn's post test, **P<0.01, N.S., not

553   significant) (**B**) Example images of an MRI scan from Atg5i mice at the 6-week on

554   dox time point, as well as the 6-week on dox -> 6-week off dox time point displaying

555   hepatomegaly and reversal to normal size, respectively. Yellow dotted lines encircle

556   livers. (**C**) Time series analyses of liver size after dox addition, followed by dox

557   withdrawal using MRI (n=2 mice per condition, average value is shown; see Figure

558   S5B for individual data). (**D**) Adult Atg5i fed on a dox-containing diet for 6-weeks

559   display a down-regulation of Atg5 and an increase in LC3-I by western blot analysis.

560   When switched back to a normal diet for 6-weeks mice show a recovery in Atg5

561   levels and LC3-I, similar to control mice.

562

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

564   (**A**) Representative histology and IHC from the livers of control and Atg5i mice.

565   Yellow arrows highlight large intracellular inclusions only found in Atg5i mice on

566   dox. Scale bars= 100μm. (**B**) Representative images of Picrosirius Red staining in

567   sections of control and Atg5i mice in the indicated conditions. Scale bars= 100μm.

568   (**C**) Only Atg5i mice treated with dox for 6-weeks and off dox for 6-weeks showed

569   the presence of fibrosis (P=0.0468 Welch's t-test, error bars represent s.d. around the

570   means). **P<0.01; N.S., not significant. (**D**) Immunohistochemical analysis of livers

571   from each time point highlights that α-SMA positive activated stellate cells are only

572   present during the 6-week on dox time point, with Col1a1 staining positivity only

573   present in the 6-week on dox->6-week off dox time point. (**E**) Whole tissue protein

574   extracts display a similar trend with Col1a1 present only in the Atg5i mice at the 6-

575   week on dox -> 6-week off dox time point.

576

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

578   (**A**) Adult Atg5i fed on a dox-containing diet for 6-weeks display a down-regulation

579   of Atg5 and an increase in LC3-I by western blot analysis. When returned to a normal

580   diet for 6-weeks mice show a recovery in Atg5 and LC3-I levels, similar to those seen

581   in control mice. (**B**) Dox treated adult mice display evidence of islet degeneration that

582   is not reversed upon dox withdrawal. Mann-Whitney test, ***P<0.001. (**C**)

583   Representative histology, IHC, and picrosirius red staining (marker of fibrosis) from

584   the pancreas of control and Atg5i mice. While p62 levels are increased in both the

585   islets and acinar of Atg5i mice on dox, only the acinar display a reversal once dox is

586   removed. Islets do not show a reversal of the degeneration phenotype as viewed by

587   H&E. Scale bars= 100μm.

588

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

590   (**A**) A panel of shRNAs was obtained from Mirimus Inc. in a pLPE backbone.

591   Retrovirus for each shRNA was generated and knockdown efficiency tested in

592   NIH3T3 cells. shAtg5#1 (Atg5_1065) provided the greatest knockdown efficiency by

593   western blot analysis during infection. (**B**) This occurs at the lowest dilutions of

594   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-ubiqutin 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 bioavailibilty 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 clasping reflexes in both control and Atg5i cohorts. Note the limbs are extended in both control and Atg5i mice, unlike limb clasping 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 leveler 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.