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Temporal inhibition of autophagy reveals segmental reversal of ageing with increased cancer risk

Liam D. Cassidy[1], Andrew R.J. Young[1], Christopher N.J. Young[2], Elizabeth J. Soilleux[3], Edward Fielder[4], Bettina M. Weigand[4,5,6], Anthony Lagnado[5,6], Rebecca Brais[7], Nicholas T. Ktistakis[8], Kimberley A. Wiggins[9], Katerina Pyrillou[9], Murray C.H. Clarke[9], Diana Jurk[5,6], Joao F. Passos[4,5,6] & Masashi Narita[1,10*]

Autophagy is an important cellular degradation pathway with a central role in metabolism as well as basic quality control, two processes inextricably linked to ageing. A decrease in autophagy is associated with increasing age, yet it is unknown if this is causal in the ageing process, and whether autophagy restoration can counteract these ageing effects. Here we demonstrate that systemic autophagy inhibition induces the premature acquisition of age-associated phenotypes and pathologies in mammals. Remarkably, autophagy restoration provides a near complete recovery of morbidity and a significant extension of lifespan; however, at the molecular level this rescue appears incomplete. Importantly autophagy-restored mice still succumb earlier due to an increase in spontaneous tumour formation. Thus, our data suggest that chronic autophagy inhibition confers an irreversible increase in cancer risk and uncovers a biphasic role of autophagy in cancer development being both tumour suppressive and oncogenic, sequentially.

[1] University of Cambridge, Cancer Research UK Cambridge Institute, Robinson Way, Cambridge CB2 0RE, UK. [2] Leicester School of Allied Health Sciences, Faculty of Health & Life Sciences, De Montfort University, Leicester LE1 5RR, UK. [3] Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK. [4] Institute for Cell and Molecular Biosciences, Newcastle University Institute for Ageing, Newcastle University, Newcastle upon Tyne, UK. [5] Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN, USA. [6] Robert and Arlene Kogod Center on Aging, Mayo Clinic, Rochester, MN, USA. [7] Department of Histopathology, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK. [8] Signalling [illegible]

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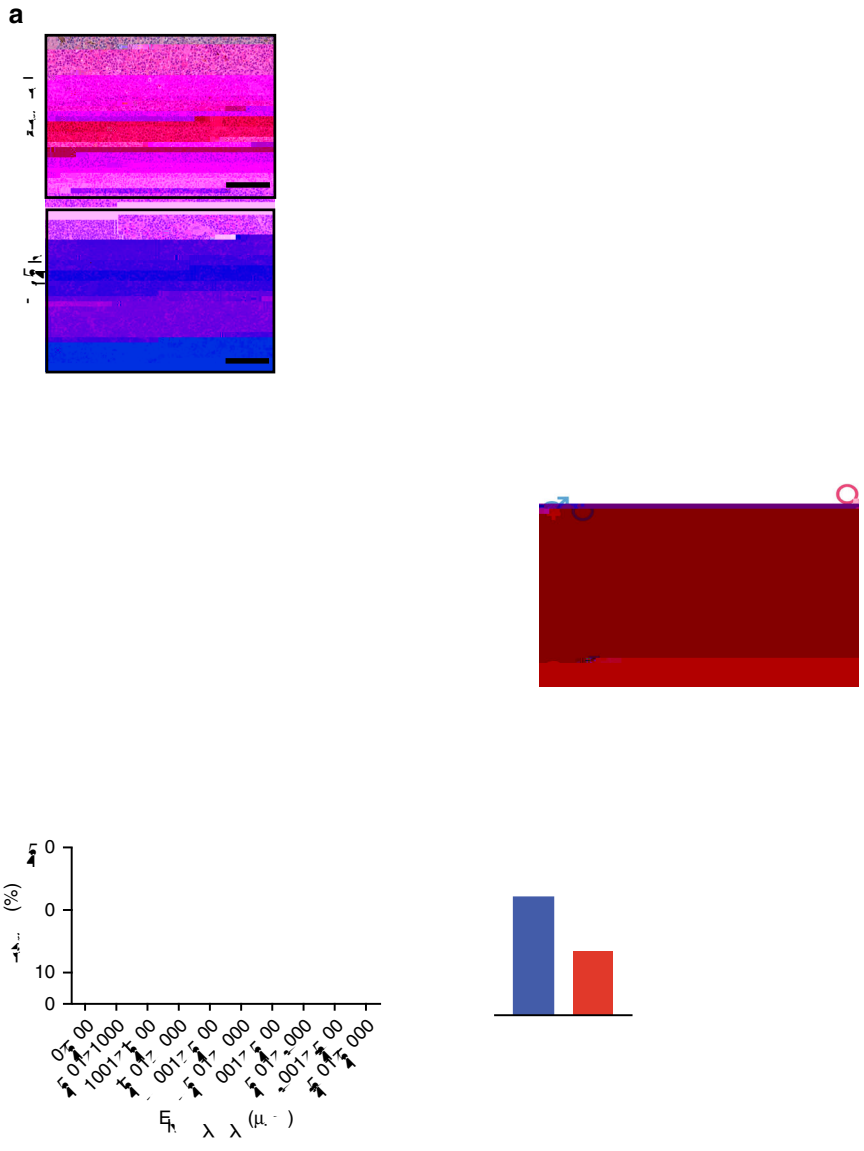
displayed evidence of premature greying to varying degrees (Fig. 1h). Furthermore, LT-Atg5i mice displayed evidence of extramedullary hematopoiesis (Fig. 2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of Pax7 positive satellite cells, and an increase in central nucleation in comparison with age-matched littermate control mice (Fig. 2f–i, Supplementary Fig. 6a, b). Central nucleation represents muscle fibre regeneration after acute muscle injury but an increase in basal frequency of centrally nucleated myofibres is also a sign of sarcopenia at geriatric age both in mice and human[22]. In addition, LT-Atg5i muscle fibres displayed increased staining positivity for the mitochondrial marker Tom20 indicative of increased mitochondrial mass and a reduction in autophagy mediated turnover (Fig. 2j).

The accumulation of senescent cells is considered a key marker of chronological ageing. Autophagy has been reported to have context dependent and sometimes opposing roles during cellular senescence: typically basal autophagy is considered to promote fitness and its loss may promote senescence, whereas in oncogene-induced senescence, autophagy may be important for the establishment of senescent phenotypes[23–26]. To determine if the systemic loss of basal autophagy is sufficient to drive the establishment of cellular senescence in vivo, we performed western blotting across a number of tissues from 4-month dox treated LT-Atg5i mice and found an increased staining pattern for key senescence markers (i.e. p16, p21, and p53) (Fig. 3a–c and

Atg5 knockout mice, including hepatomegaly and splenomegaly (Supplementary Fig. 7d–f). These findings in particular are important as they establish that the reduction in longevity and presence of ageing phenotypes is not dependent on the hepatomegaly and splenomegaly phenotypes encountered in the original LT-Atg5i mouse strain with the highest degree of autophagy inhibition.

Combined these data support a role for basal autophagy in maintaining tissue and organismal homoeostasis and provide evidence that causally links autophagy inhibition to the induction of ageing-like phenotypes in mammals.

Autophagy restoration partially reverses ageing phenotypes. We next sought to determine whether autophagy restoration alone is able to reverse the ageing-like phenotypes by removing dox from the diet. 8-week old Atg5i and control mice treated with dox for 4 months, the point at which they universally presented with kyphosis, were switched back to a diet absent of dox leading to a restoration in Atg5 levels and autophagy (termed R-Atg5i cohort) (Fig. 4a, b and Supplementary Fig. 8a)[13]. Interestingly, while p16 levels reduced in the livers R-Atg5i mice, they still appeared elevated in comparison with age-matched control mice 4-months post dox removal (Fig. 4b). This is in contrast to the kidney that exhibited only a mild increase in p16 that was mostly reversed upon autophagy restoration. While further systematic analyses would be required, the data suggest a differential susceptibility to autophagy inhibition across organs.

An increase in chronological age is generally associated with the deviations in multiple health parameters that when measured can be combined into a clinical 'frailty-score'[30]. As expected, R-Atg5i mice displayed an initial increase in their frailty scores during autophagy inhibition in comparison with littermate controls, yet once mice have been switched back to a diet absent of dox, the frailty scores displayed a significant decrease over the next 4 months (Fig. 4c, Supplementary Movie 1). In contrast, LT-Atg5i mice treated on dox for 6 months (median survival is around ~6 months on dox) continued to display a significant

homoeostatic effects that cannot be distinguished in ageing models based on constitutive or in utero genetic modifications.

In addition, it should be noted that whilst the LT-Atg5i model

particular mitochondrial dysfunction[35,36], however it remains to be seen whether mitochondrial function is altered in this setting. In addition, we cannot rule out synergistic effects of doxycycline side-effects with autophagy inhibition, as such comparison with other inducible models would be required to exclude this possibility.

Several health and life-span extending regimens in mammals, such as calorie restriction or pharmacological modulation, have been posited to exert their effects through the regulation of autophagy[7,37]. However, these effects are also pleiotropic in nature and alter a multitude of cellular processes, making it impossible to deconvolute and ascribe the role of autophagy in these settings. Whilst recent genetic models that promote autophagic flux continuously throughout life have demonstrated an extension of health- and life-span in mammalian systems[11,12], it is unclear if the damage established by a loss of autophagy is sufficient for age acceleration and can be reversed. If therapeutic regimens in humans are to be established later in life, once

time-point to restore autophagy as this provided a clear and ubiquitous distinction between control and autophagy inhibited mice, shorter time points or intermittent dosing regimens may display further heterogeneity in damage and recovery phenotypes.

Our unexpected finding, that the temporal inhibition of autophagy predisposes to increased tumour development, provides a potential genetic explanation for the context-dependent role of autophagy in tumorigenesis:[38,39] i.e. autophagy can be a tumour suppressor[33,40,41] or a tumour promoter[42–44]. The irreversible damage induced by autophagy inhibition (e.g. genomic instability), might confer tumour susceptibility, while autophagy activity is perhaps required for actual malignant transformation. The clinical implication of our data is not limited to the advanced age state. As some pathophysiological states, such as obesity, are associated with an insufficient level of autophagy[45], it would be interesting to determine if obese individuals retain an increased risk of tumour development even upon weight loss, in comparison with never obese populations.

Methods

Atg5i mouse maintenance and aging. The generation and initial characterisation of the Atg5i transgenic line have previously been described in detail[13]. Mice were maintained on a mixed C57Bl/6 × 129 background with littermate controls used in all experiments. All experimental mice were maintained as heterozygous for both the shRNA allele and CAG-rtTA3 alleles, whereas control littermates were lacking one of the alleles. Guide sequences were as follows: Atg5i (Atg5_1065) TATGAAG AAAGTTATCTGGGTA[13]; Atg5i_2 (Atg5_1654) TTATTTAAAAATCTCTCACT GT. Atg5_1654 was chosen after an initial screen for shRNA knockdown efficiency wherein it displayed the second highest efficiency of knockdown[13]. The shRNA guides in a miR-E design were inserted downstream of the *Col1a1* locus via recombinase-mediate cassette exchange which enables efficient targeting of a transgene to a specific genomic site 500 base pairs downstream of the 3′UTR in D34 ES cells. Mice were maintained in a specific pathogen-free environment under a 12-h light/dark cycle, having free access to food and water. These mice were fed either a laboratory diet (PicoLab Mouse Diet 20, 5R58) or the same diet containing doxycycline at 200 ppm (PicoLab Mouse Diet, 5A5X). For this study mice were aged for 2 months before doxycycline administration in the diet. Mice were enroled either to time-point study groups or long-term longevity cohorts (LT- and R-groups). Experienced animal technicians checked mice daily in a blinded fashion, and additionally mice were weighed and hand-checked on a weekly basis. Mice found to be of deteriorating health were culled under the advice of senior animal technicians if displaying end of life criteria. These signs include a combination of (1) hunched body position with matted fur, (2) piloerection, (3) poor body condition (BC) score (BC1 to 2), (4) failure to eat or drink, (5) cold to touch, and or (6) reduced mobility, including severe balance disturbances and ataxia. In accordance with UK home office regulations any mice suffering a 15% loss of body weight were also considered to be at an end-point. Note that for LT- longevity cohorts a portion of control mice were culled to generate age-matched littermate control tissue. These mice are marked as censored events on the survival curve. For analysis, mice were treated as alive up to the point of their removal from the study where they are considered lost to follow-up and are not included in the calculations of median

or both), vision loss, menace reflex, nasal discharge, malocclusions, rectal prolapse, prolapse (vaginal, uterine, or penile), diarrhoea, altered respiratory rate, alterations to mouse grimace, piloerection, body temperature and weight.

Doxycycline serum measurements. An LC-MS/MS assay was developed for the analysis of doxycycline in mouse plasma with demeclocycline as an internal standard.

0.45 mm filtered paraformaldehyde solution in 1 × PBS for 15 min at 4 °C. PFA

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