Autophagy Is Required for Glucose Homeostasis and Lung Tumor Maintenance

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INTRODUCTION

Autophagy is a process that captures cytoplasmic proteins and organelles in vesicles called autophagosomes, which then fuse with lysosomes and are degraded. Autophagy functions at a low basal level to remove damaged cellular components to maintain protein and organelle quality, thereby preventing the gradual toxic accumulation of intracellular waste material (1). Autophagy is dramatically upregulated by starvation where the catabolism and recycling of cellular components sustain energy homeostasis essential for cells and newborn mice to survive without nutrients (2–4). These functions of autophagy are conserved from yeast through mammals and mice to survive without nutrients (2–4). These functions of autophagy are conserved from yeast through mammals and mice.

Mice constitutively deficient in Atg5 or Atg7 are born developmentally normal but fail to survive the neonatal starvation period due to metabolic insufficiency, illustrating the importance of autophagy to supply metabolic substrates to bridge gaps in nutrient availability (2, 3). Neuronal-specific deficiency in Atg5 or Atg7 results in the accumulation of autophagy substrates such as aggregated and ubiquitinated proteins and damaged organelles, motor and behavioral defects, neurodegeneration, and lethality between 1 and 6 months after birth (5, 6). These findings suggest that autophagy is critical for preventing the toxic accumulation of damaged proteins and organelles in postmitotic tissues, although there is a potential additional contribution of autophagy to brain energy metabolism. Mosaic or liver-specific deletion of Atg5 or liver-specific deficiency in Atg7 also causes accumulation of autophagy substrates, steatosis, and eventual hepatico development, suggesting that autophagy prevents liver damage and limits liver cancer initiation (2, 7, 8). Additional tissue-specific knockout studies underscore the importance of autophagy in tissue homeostasis, metabolism, and stem cell maintenance (1).

Autophagy has a context-dependent role in cancer (9). It is upregulated and required for the survival of tumor cells in hypoxic tumor regions (9). Oncogenic RAS transformation upregulates basal autophagy required for maintenance of mitochondrial metabolism and progression of tumorigenesis (10–12). Moreover, studies knocking out essential autophagy genes in genetically engineered mouse models (GEMM) for cancer have demonstrated a protumorigenic role for autophagy (13). Deletion of Atg7 in KrasG12D and BrafV600E non–small cell lung cancer (NSCLC) in adult mice causes tumors to accumulate defective mitochondria and become metabolically impaired. Without ATG7, tumor cell proliferation is suppressed and tumors progress to benign oncocytomas rather than adenomas and carcinomas, extending the mice’s lifespan (14–16). In some but not all contexts,
autophagy promotes tumorigenesis by mitigating Trp53 activity (14–19). Although these studies point to a protumorigenic role for autophagy, they involved Atg gene deletion specifically in tumor cells, and thus do not demonstrate that autophagy deficiency is selectively detrimental to tumor tissue. Furthermore, as Atg gene deletion occurred concurrently with activation of oncogenic mutations that initiate tumorigenesis, these prior studies do not model acute, systemic autophagy ablation as would occur during autophagy inhibition for cancer therapy. To address the tumor selectivity of autophagy ablation in cancer, we engineered the mice to conditionally [tamoxifen (TAM)-inducible] and systemically delete Atg7 throughout adult mice. We found that adult mice with Atg7 acute whole-body deletion (Atg7Δ/Δ mice) manifested systemically blocked autophagy without extensive organ damage at 5 weeks after deletion, although a small fraction of mice succumbed to Streptococcus infection. In contrast, by 6 to 12 weeks after deletion, extensive liver and muscle damage was evident and neurodegeneration limited survival to 2 to 3 months. Atg7Δ/Δ mice suffered lethality upon fasting, where mice displayed extreme muscle wasting and died of hypoglycemia, indicating that autophagy is required for glucose homeostasis. This reveals a new role for autophagy in the management of systemic energy balance. In the setting of cancer, 5 weeks of acute autophagy ablation converted established lung adenocarcinomas to oncocytes and blocked mTOR and MAP kinase signaling, and cell proliferation and survival. Compared with tumor-specific autophagy deficiency produced concurrently with tumor initiation, acute systemic autophagy ablation was more destructive to tumors. Thus, established tumors have greater autophagy dependency than both newly developing tumors and most normal tissues. This suggests that, with proper control of the extent and/or timing of autophagy inhibition, there is likely to be a therapeutic window to suppress tumorigenesis while mostly sparing normal tissue.

RESULTS

Acute Atg7 Deletion in Adult Mice Produces Systemic Autophagy Defect

Adult mice were engineered with floxed alleles of Atg7 (2) and a transgene expressing the TAM-regulated Cre recombinase fusion protein under the control of the ubiquitously expressed ubiquitin C (Ubc) promoter (20). Eight- to 10-week-old adult mice retain intact Atg7 (wild-type mice or mice with Atg7 floxed alleles with or without the Ubc-CreERT2 allele) and express ATG7 protein (Supplementary Fig. S1A), but when provided TAM, Cre is activated only in mice with Atg7 floxed and Ubc-CreERT2 alleles, deleting the Lox-P sites and Atg7, producing a near-complete and sustained loss of ATG7 protein (Atg7Δ/Δ mice) in all tissues examined by 2 weeks following the last day of the 5-day course of TAM administration (Fig. 1A and B and Supplementary Fig. S1B). Atg7 deletion throughout mouse tissues was also confirmed by PCR (data not shown).

The loss of ATG7 correlated with accumulation of the autophagy substrate p62 and the unprocessed form of microtubule-associated protein 1A/1B-light chain 3 (LC3-I) and the absence of the cleaved, lipidated, and active (autophagosome-associated) form LC3-II (Fig. 1B and Supplementary Fig. S1). These findings are consistent with efficient, conditional ablation of ATG7 expression and loss of autophagy in adult mouse tissues, allowing the assessment of the role of autophagy in adult mice for the first time.

Atg7 Deficiency Causes Depletion of White Adipose Tissue and Damage to Liver and Muscle

In comparison with Atg7 wild-type mice, Atg7Δ/Δ mice at 2 months after TAM were smaller in size and had reduced body weight (Fig. 1C and D), despite similar feeding behavior and energy expenditure. Liver weight was increased in Atg7Δ/Δ mice (Supplementary Fig. S1C), consistent with steatosis that also results from liver-specific Atg7 or Atg5 deletion (2, 7, 8, 21). Echo magnetic resonance imaging (EchoMRI) analysis of body composition showed less absolute fat and lean body mass in Atg7Δ/Δ mice compared with control mice at 2 months after TAM (Fig. 1E and F), explaining the decreased body size of Atg7Δ/Δ mice. Histologic examination of tissues from the Atg7Δ/Δ mice revealed limited tissue damage at 5 weeks after TAM that included the beginning of liver abnormalities, splenic enlargement, testicular degeneration, and depletion of the lipid content of white adipose tissue (WAT; Fig. 1G and Supplementary Fig. S1D and S1I). Spleens of Atg7Δ/Δ mice were enlarged with accumulation of megakaryocytes (Fig. 1G), although bone marrow seemed normal (data not shown). We observed testicular degeneration in Atg7Δ/Δ mice, which was partially due to Cre toxicity; testes were smaller in Atg7Δ/Δ mice with elevated p62 aggregates mainly in Leydig cells (Fig. 1B and Supplementary Fig. S1I). Autophagy is essential for adipocyte differentiation and formation of WAT (22, 23). Here, acute autophagy ablation rapidly depleted lipid stores and reduced established WAT by converting WAT to brown adipose tissue (BAT) or by blocking BAT to WAT transdifferentiation. In contrast, marked liver, brain, muscle, and pancreas damage and further depletion of WAT were apparent in Atg7Δ/Δ mice at 2 months after TAM (Fig. 1G). Thus, systemic Atg7 deficiency for 5 weeks produces limited toxicities but this is not the case at 2 to 3 months.

Tissue damage resulting from acute, systemic Atg7 deficiency in adult mice for 2 months included increased liver enlargement where hepatocytes accumulated p62 and LC3-I aggregates, mitochondria (as indicated by the mitochondrial marker TOM20), and lipid droplets (Oil red O staining; Fig. 1B and G and Supplementary Fig. S1E–S1G). These mice also had a decreased number of large pyramidal neurons and Purkinje cells (Fig. 1G). At the same time, we observed degenerative changes in muscle with centrally nucleated, small myofibers (Fig. 1G). These findings are consistent with a time-dependent destructive effect to normal tissues upon loss of autophagy as reported with tissue-specific knockout of Atg5 or Atg7 (5, 6, 21–26). We also observed intra-acinar vacuolization in the pancreas (Fig. 1G). Consistent with the role of autophagy in lipid metabolism, kidney and lung tissues accumulated lipids (Supplementary Fig. S1G). Other tissues were not visibly affected by acute Atg7 ablation. Interestingly, constitutive autophagy deficiency impairs lung function in neonates (27), but as this did not occur in Atg7Δ/Δ adult mice, this suggests that developing tissues can be more reliant on autophagy than adult ones.
Figure 1. Conditional whole-body deletion of Atg7 abrogates autophagy and impairs long-term survival. **A**, experimental design for generation of Atg7Δ/Δ mice. Atg7fl/fl or Ubc-CreERT2/+Atg7fl/fl mice were treated with TAM at 8 to 10 weeks of age by intraperitoneal injection and analyzed at times thereafter. **B**, Western blotting for ATG7, p62, and LC3 at the indicated times (w, weeks; m, months) of the indicated tissues from TAM-treated Atg7fl/fl or Atg7Δ/Δ adult mice. Atg7fl/fl control tissues are from a 5 weeks post–TAM-treated mouse. Tumor derived cell lines (TDCL) from Atg7- intact and Atg7-deficient tumors (16) were used as controls for ATG7, LC3, and p62 protein expression. β-Actin serves as a protein loading control. **C**, representative pictures of 2 months post–TAM-treated Atg7fl/fl or Atg7Δ/Δ female and male mice (n = 3 for each genotype). **D**, body weight differences of TAM-treated Atg7fl/fl or Atg7Δ/Δ adult mice at the indicated times (w, weeks; m, months). Error bar, SEM; *, P < 0.05; ***, P < 0.01 (two-way ANOVA with the Bonferroni posttest). **E**, body fat composition as determined by EchoMRI of 10 days post–TAM-treated Atg7fl/fl or Atg7Δ/Δ adult mice. Error bar, SEM; *, P < 0.05 (two-way ANOVA with the Bonferroni posttest). **F**, lean muscle mass as determined by EchoMRI of 10 days post–TAM-treated Atg7fl/fl or Atg7Δ/Δ adult mice. Error bar, SEM; ***, P < 0.001 (two-way ANOVA with the Bonferroni posttest). **G**, representative liver, cerebral cortex, cerebellum, muscle, pancreas, spleen, and adipose histology (hematoxylin and eosin (H&E) stained) of TAM-treated Atg7fl/fl or Atg7Δ/Δ adult mice at indicated times. Arrowheads in cerebellum point to Purkinje cells; arrowheads in muscle point to centrally nucleated myofibers; arrowheads in pancreas point to intra-acinar vacuolization; arrows in liver point to macrovesicular fatty acid accumulation; arrowheads in spleen point to intra-acinar vacuolization of adipocytes; arrows in adipose tissue point to intracellular vacuolization of adipocytes. **H**, the Kaplan-Meier survival curve of TAM-treated Atg7fl/fl or Atg7Δ/Δ adult mice (w, weeks; m, months). *, mice that died of infection; P < 0.0001 (log-rank Mantel-Cox test).
ATG7 Extends Adult Lifespan by Suppressing Susceptibility to Infection and Neurodegeneration

A cohort of wild-type and Atg7Δ/Δ mice was followed to assess the consequence to overall survival. Lifespan of Atg7Δ/Δ mice was shortened to 2 to 3 months, demonstrating that retention of Atg7 for functional autophagy is required for the viability of adult mice (Fig. 1H). Death of Atg7Δ/Δ mice resulted from two distinct causes. Several mice (12%) succumbed to *Streptococcus* infection shortly after Atg7 deletion (Fig. 1H, asterisks, and Supplementary Fig. S1G), consistent with the finding that autophagy is required for eliminating intracellular *Streptococcus* in cells *in vitro* (28). The vast majority of the Atg7Δ/Δ mice, however, died between 2 to 3 months due to neurodegeneration (Fig. 1H), similar to findings with neuronal-specific deficiency in Atg5 or Atg7 (5, 6). With conditional whole-body deletion of Atg7, we observed progressive motor and behavioral deficits such as tremors and ataxic walking and limb-clasping reflexes when held by their tails, whereas control animals tend to extend their limbs (Supplementary Movie S1). Atg7Δ/Δ mice also displayed very severely impaired balance and bradykinesia (abnormally slow movements) shortly before death, collectively indicating that death results from neurologic defects. Thus, the predominant toxicity of autophagy deficiency is neurodegeneration.

Autophagy Is Required for Adult Mice to Survive Fasting

The above analysis focused on unstressed mice. A major function of autophagy conserved from yeast to mammals is stress survival. This is evident in the requirement for autophagy to survive the stress of the neonatal starvation period (2, 3). Similarly, autophagy is robustly induced in adult mouse tissues in response to starvation (4). To test if autophagy is functionally important for adult mice to survive fasting, Atg7 was deleted in 8- to 10-week-old mice by TAM administration (mice with Atg7 floxed alleles plus the Ubc-CreERT2 allele; Atg7Δ/Δ) or left intact (mice with Atg7 floxed alleles without the Ubc-CreERT2 allele; Atg7-intact). Ten days following the last day of a 5-day schedule of TAM administration when ATG7 expression was extinguished (Fig. 2A), mice were subjected to fasting (free access to water without food) for 24 hours. We chose this short-term autophagy inactivation to establish the acute and direct requirement for autophagy. All fed mice (Atg7-intact or Atg7Δ/Δ) and all fasted mice with Atg7 intact survived for 24 hours as expected (Fig. 2B). In contrast, the majority of Atg7Δ/Δ mice failed to survive fasting for 24 hours (Fig. 2B). Thus, similar to neonates, adult mice also require autophagy to tolerate starvation. Serum insulin and leptin levels were downregulated by fasting independent of autophagy status (Supplementary Fig. S2), suggesting that the hormonal response to starvation was normal and thus not the cause of the fasting lethality of Atg7-deficient mice.

Autophagy Maintains Fat Stores and the Mobilization of Lipids during Fasting

The physiology and metabolic requirements of neonates are distinct from those of adult mice, raising the question of whether the role of autophagy in supporting survival during fasting also differed. Neonates require autophagy to maintain serum amino acid but not glucose levels (3). To address the mechanism by which autophagy contributes to the survival of adult mice during fasting, tissues and serum were examined during fed and fasted conditions in the presence and absence of ATG7.

There was a striking depletion of WAT, apparent with short-term Atg7 deletion under fed conditions (Fig. 2C). Histologic examination revealed marked reduction of epididymal adipose mass due to a reduction in the size of fat droplets and accumulation of mitochondria within adipocytes in Atg7Δ/Δ mice in the short term (Fig. 2C and Supplementary Fig. S1F). Thus, the presence of Atg7 favors WAT, whereas Atg7 deficiency favors BAT. To assess absolute total body fat mass, live Atg7-intact or Atg7Δ/Δ mice were subjected to EchoMRI. Fed Atg7Δ/Δ mice displayed reduced absolute fat mass compared with mice with Atg7 intact, indicating that acute autophagy deficiency causes depletion of fat stores (Fig. 2D). Atg7 deficiency promotes adipose differentiation favoring BAT formation, possibly due to a decreased capacity for lipid storage and increased lipid metabolism through β-oxidation. This suggests that the reduction in dedicated lipid stores may compromise survival during fasting. Indeed, fat mass was more severely depleted in fasted Atg7Δ/Δ mice than in those with Atg7 intact (Fig. 2C). Mobilization of free fatty acids (FFA) in serum during fasting was also defective without Atg7, revealing a metabolic deficiency that can contribute to lethality (Fig. 2E).

Glycogen Stores Are Depleted in Fasted Atg7-Deficient Mice

Glycogen store mobilization, particularly in the liver, provides an important source of glucose in fasted conditions that contributes to serum glucose homeostasis. Fasting reduced liver size to a greater extent in Atg7Δ/Δ mice (17% with Atg7 intact, compared with a 29% in Atg7Δ/Δ mice), and these mice had higher serum levels of the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) when fasted, indicative of some liver damage. Indeed, these mice had increased apoptosis coincident with DNA damage response activation (γH2AX–positive hepatocytes), although healthy liver tissue was still present (Fig. 2F and G). Importantly, in contrast to fasted Atg7-intact mice, fasted Atg7Δ/Δ mice had complete depletion of liver glycogen stores (Fig. 2G). Thus, autophagy is critical to prevent depletion of glycogen stores during fasting. Because acute deficiency in Atg7 produced accelerated depletion of lipid and glycogen stores, this indicates a systemic requirement for autophagy that may contribute to their failure to tolerate fasting.

ATG7 Prevents Muscle Wasting during Fasting

Degradation of muscle protein sustains homeostasis during fasting (29). Gastrocnemius muscle mass was slightly reduced in Atg7Δ/Δ mice with short-term deletion under fed conditions in comparison with that from Atg7-intact mice (Fig. 2H), as was total muscle mass, as revealed by EchoMRI (Fig. 2I). Fasting, however, induced severe muscle wasting with DNA damage response activation (γH2AX–positive myofibers) in Atg7Δ/Δ mice compared with Atg7-intact mice (Fig. 2H). Thus, without autophagy, fasting induces severe muscle wasting. Ultimately, metabolic failure can lead to brain damage...
Figure 2. Autophagy is required for adult mice to survive fasting. A, ATG7 Western blot of tissue lysates from Atg7flox/flox or Atg7Δ/Δ adult mice at 10 days post-TAM-treated, fed, and fasted. β-Actin serves a protein loading control. B, the Kaplan–Meier survival curve of Atg7flox/flox or Atg7Δ/Δ adult mice at 10 days post-TAM-treated, fed, and fasted. P = 0.0072 (log-rank Mantel–Cox test). C, representative male pictures (left), tissue weight (middle), and histology (H&E; right) of epididymal adipose tissues of Atg7flox/flox or Atg7Δ/Δ adult mice at 10 days post–TAM-treated, fed, and fasted (n = 4, for each). Error bar, SEM; *, P < 0.05 (two-way ANOVA with the Bonferroni posttest). D, body fat composition as determined by EchoMRI of Atg7flox/flox or Atg7Δ/Δ adult mice at 10 days post–TAM-treated. E, quantification of serum FFA levels of Atg7flox/flox or Atg7Δ/Δ adult mice at 10 days post–TAM-treated, fed, and fasted. Error bar, SEM; *, P < 0.05 (two-way ANOVA with the Bonferroni posttest). F, liver weight (left) and serum level of liver enzymes AST (middle) and ALT (right) from Atg7flox/flox or Atg7Δ/Δ adult mice at 10 days post–TAM-treated, fed, and fasted. Error bar, SEM; **, P < 0.01 (two-way ANOVA with the Bonferroni posttest). G, representative liver histology (H&E) and liver glycogen levels (periodic acid–Schiff staining) of Atg7flox/flox or Atg7Δ/Δ adult mice at 10 days post–TAM-treated, fed, and fasted. Arrowheads point to glycogen. IHC staining for active caspase-3 shows apoptosis induction, and IHC staining for γ-H2AX shows DNA damage response activation in Atg7-deficient liver tissue when fasted. H, representative gross image of calf muscle (left) and weight of gastrocnemius muscle and representative muscle histology (H&E) from Atg7flox/flox or Atg7Δ/Δ adult mice at 10 days post–TAM-treated, fed, and fasted. IHC staining for γ-H2AX shows DNA damage response activation in Atg7-deficient muscle tissue when fasted. Error bar, SEM; **, P < 0.01 (two-way ANOVA with the Bonferroni posttest). I, lean muscle mass as determined by EchoMRI of Atg7flox/flox or Atg7Δ/Δ adult mice at 10 days post–TAM-treated. Error bar, SEM; ***, P < 0.001 (two-way ANOVA with the Bonferroni posttest). J, representative brain histology (H&E) of Atg7flox/flox or Atg7Δ/Δ adult mice at 10 days post–TAM-treated, fed, and fasted. IHC staining for γ-H2AX shows DNA damage response activation in Atg7-deficient brain tissue when fasted.
and death. In contrast to fed and fasted mice with Atg7 intact, fasted Atg7/Δ mice showed loss of motor control indicative of neurologic impairment, which was consistent with the occurrence of extensive brain damage (Fig. 2J).

**Fasted Atg7-Deficient Adult Mice Die from Hypoglycemia**

To address the mechanism by which autophagy sustains mouse survival during fasting, we first examined levels of serum amino acids by LC/MS in fed and fasting conditions, in Atg7-intact and Atg7/Δ mice. In contrast to Atg7-deficient neonates undergoing neonatal starvation, adult Atg7/Δ mice with short-term Atg7 deletion sustained serum amino acid levels with the exception of arginine (Fig. 3A). Liver amino acids were also similar in fed and fasted mice (data not shown), suggesting that this was not responsible for the survival defect. Serum β-hydroxybutyrate levels were elevated in fasting in both Atg7-intact and Atg7/Δ mice, suggesting that serum amino acids were sufficient to induce ketogenesis (Fig. 3B). Extensive muscle wasting in fasted Atg7/Δ mice may sustain serum amino acids and ketogenesis, partly compensating for depleted fat and glycogen stores.

We next examined serum glucose levels and found that the majority of fasted Atg7/Δ mice were severely hypoglycemic consistent with loss of viability (Fig. 3C). Indeed, glucose supplementation was sufficient to rescue serum glucose levels, muscle wasting, and survival of fasted Atg7/Δ mice (Fig. 3D). Thus, autophagy in adult mice is required to maintain fasting serum glucose levels, preventing hypoglycemia and death, revealing different metabolic roles for autophagy in the systemic metabolism of neonatal and adult mice.

**Atg7 Deletion Alters Gene Expression Responses to Fasting**

Functional analysis of differentially expressed genes revealed that molecular functions related to inflammatory response were highly enriched in livers from Atg7/Δ mice (Fig. 3E, Supplementary Fig. S3A, and Supplementary Tables S1 and S3). This suggests that liver function is altered by Atg7 deficiency under fed conditions, although this was not the case for muscle, consistent with normal histology (Fig. 1G). Fasting Atg7-intact mice repressed gene expression for lipid metabolic processes and insulin signaling and elevated those for fatty acid oxidation in liver (Fig. 3F and Supplementary Tables S1 and S3), but induced few changes in muscle gene expression (Fig. 3F, Supplementary Fig. S3B, and Supplementary Tables S2 and S4). Fasting Atg7/Δ mice reduced signatures for liver immune response–related genes; however, there was not a significant change in lipid metabolism–related gene expression as opposed to Atg7-intact mice (Fig. 3G, Supplementary Fig. S3A, and Supplementary Tables S1 and S3). Gluconeogenesis predominantly occurs in the liver, producing glucose that supports metabolism in the brain and other tissues. Genes associated with gluconeogenesis were induced by fasting in the liver in Atg7/Δ mice (e.g., Pparg1a, Atpf, Amt1, Pik3k, Got1, Lepr, Supplementary Fig. S3A and S3C and Supplementary Table S3), suggesting that it may be defective because of insufficient substrates. For example, depletion of FFAs may cause more use of amino acids for ketogenesis instead of gluconeogenesis. Depletion of WAT would limit the availability of glycerol for use as a substrate for gluconeogenesis. Fasting caused activation of genes involved in catabolic processes for autophagy and atrophy (e.g., Ulk1, Bnip3, Foxo1, Foxo3, Mti, and Mtd, Fig. 3G, Supplementary Fig. S3B, S3D, and S3E, and Supplementary Tables S2 and S4). The muscle atrophy signature in the fasted Atg7/Δ mice is consistent with extensive muscle wasting.

In summary, healthy adult mice have lipid and glycogen stores that are mobilized during fasting, reducing the demand for muscle catabolism to supply amino acids for gluconeogenesis. Liver and muscle autophagy may also supply substrates for gluconeogenesis. Lipid and glycogen stores in Atg7/Δ mice were depleted even under fed conditions, and fasting accelerated glycerol elimination and caused excessive muscle wasting (Fig. 4 and Supplementary Fig. S4). Although serum amino acid levels and ketogenesis were sustained in Atg7/Δ mice, they nonetheless died from hypoglycemia. Therefore, this suggests that adult mice require autophagy to provide substrates for glucose homeostasis to survive fasting.

**Atg7 Deficiency Does Not Alter Lung Tumor Initiation**

To test if autophagy influences tumor initiation, mice were engineered with Frt alleles of both KrasG12D and Trp53 without and with the conditional floxed system for generating Atg7/Δ mice. Mice then had Atg7 ablated by TAM administration and deletion of the Lox-P sites in the Atg7 alleles by Cre recombinase, followed later by initiation of lung tumorigenesis by intranasal administration of adenosine expressing Flp recombinase (Ad-FLPo) and simultaneous activation of KRAS and deletion of Trp53 by excision of Frt sites (Fig. 5A).

When Atg7 was deleted and lung tumorigenesis was initiated by RAS activation and Trp53 deletion, the number and size of tumors were monitored 4 and 8 weeks after TAM (3 and 7 weeks after Ad-FLPo). There was no difference in tumor frequency or burden 3 weeks after Ad-FLPo regardless of Atg7 status, indicating that the functional status of autophagy does not alter the ability of KrasG12D activation and Trp53 deficiency to induce formation of lung tumors (Fig. 5B–D). At 7 weeks after FLPo, however, there were fewer tumors and a reduction in tumor burden in Atg7/Δ mice (Fig. 5D and Supplementary Fig. S5), coincident with emergence of oncocyteomas rather than adenocarcinomas (Fig. 5B). This suggests that loss of autophagy does not affect the ability of activated RAS and Trp53 deficiency to initiate tumorigenesis but rather it diminishes tumor growth over time.

**Acute Atg7 Ablation Alters Tumor Fate and Compromises Maintenance**

To perform the converse experiment, lung tumors were generated, and at 12 weeks after Ad-FLPo administration, TAM administration produced acute autophagy deficiency in both established tumors and normal tissues (Fig. 6A). Tumorigenesis was then assessed 5 weeks after TAM (17 weeks after Ad-FLPo), at which point there was little effect of autophagy ablation in normal tissues (Fig. 1G). Lung tumor burden was similar among all mice at the time of TAM administration as determined by micro-CT (Fig. 6B). At 17 weeks after FLPo and 5 weeks after TAM, as expected, lung tumor burden, with KRAS activation and Trp53 deficiency, produced large adenocarcinomas, encompassing most of the
Acute Autophagy Ablation Selectively Impairs NSCLC

Figure 3. Autophagy sustains glucose homeostasis required to survive fasting. A, quantification of serum amino acid levels in Atg7Δflox/flox or Atg7ΔΔ adult mice at 10 days post-TAM-treated, fed, and fasted. BCAA, branched chain amino acid. Error bar, SEM; *, P < 0.05; **, P < 0.01 (two-way ANOVA with the Bonferroni posttest). B, quantification of serum β-hydroxybutyrate levels in Atg7Δflox/flox or Atg7ΔΔ adult mice at 10 days post-TAM-treated, fed, and fasted. Error bar, SEM; *, P < 0.05 (two-way ANOVA with the Bonferroni posttest). C, quantification of blood glucose levels of Atg7Δflox/flox or Atg7ΔΔ adult mice at 10 days post-TAM-treated, fed, and fasted. Error bar, SEM; *, P < 0.05 (two-way ANOVA with the Bonferroni posttest). D, the Kaplan–Meier survival curve (left), serum glucose levels (middle), and weight of gastrocnemius muscle (right) from Atg7ΔΔ adult mice at 10 days post-TAM-treated, fasted, without or with glucose supplemented, P = 0.0022 (log-rank Mantel–Cox test). Error bar, SEM; *, P < 0.05; **, P < 0.01 (two-way ANOVA with the Bonferroni posttest). E to G, analysis of differentially expressed genes from liver and muscle tissue in Atg7Δflox/flox or Atg7ΔΔ adult mice at 10 days post–TAM-treated, fed, and fasted, showing upregulated (blue) or downregulated (pink) genes for significant biologic processes as determined by GO term DAVID Analysis. Number scale, number of upregulated (positive number) and downregulated (negative number) genes in the indicated biologic processes.
**Figure 4.** Mechanism by which autophagy supports survival of adult mice during fasting. See text for explanation.

Airspace in the lobes of the lungs with intact *Atg7* (Fig. 6B and Supplementary Fig. S6A). In contrast, in *Atg7Δ/Δ* mice at 17 weeks after FLPo and 5 weeks after TAM, tumors were strikingly smaller, had poorly-defined margins, and were composed of oncocytes and dead tumor cells (Fig. 6B and Supplementary Fig. S6A). There was no detectable difference in normal adjacent lung tissue in tumor-bearing *Atg7Δ/Δ* mice (Fig 6B). The difference in tumor burden in *Atg7Δ/Δ* mice with 5 weeks of *Atg7* deletion was apparent by examination of wet lung weight and by determining tumor burden in scanned lung sections, with tumor area reduced by one third (Fig. 6C and Supplementary Fig. S6A). In contrast, in *Atg7Δ/Δ* mice with 5 weeks of *Atg7* deletion was apparent by examination of wet lung weight and by determining tumor burden in scanned lung sections, with tumor area reduced by one third (Fig. 6C and Supplementary Fig. S6A).

Loss of ATG7 protein expression and autophagy ablation in tumors in the *Atg7Δ/Δ* mice were confirmed by Western blotting and IHC. These tumors specifically accumulated p62 and LC3 aggregates, and abnormal mitochondria as detected by TOM20 IHC and electron microscopy (EM; Fig. 6D and E and Supplementary Fig. S6B). Tumors in *Atg7Δ/Δ* mice also displayed lipid accumulation (Supplementary Fig. S6C) consistent with defective mitochondrial fatty acid oxidation as reported previously with tumor-specific *Atg7* deficiency (16). The impaired tumorigenesis in *Atg7Δ/Δ* mice was attributed to increased apoptosis (active caspase-3; Fig. 6F) and ablation of proliferation, mTOR and MAP kinase signaling [Ki67, loss of phosphorylated S6 (P-S6), 4E-BP1 (P-4E-BP1), and ERK (P-ERK) IHC; Fig. 6G and H and Supplementary Fig. S6D]. Serum glucose levels were similar in wild-type and *Atg7Δ/Δ* tumor-bearing mice, indicating that hypoglycemia was not a factor contributing to defective tumor growth and survival (data not shown). Although tumor-specific deletion of *Atg7* concurrently with tumor initiation by KRAS activation and Tpr53 deletion also reduces tumor burden, generates oncocytomas, suppresses proliferation, and induces tumor cell death progressively beyond 6 weeks after deletion (16), the kinetics and magnitude of these events are greatly accelerated by acute ablation of *Atg7* throughout the mouse (Fig. 6I).

**DISCUSSION**

Acute autophagy ablation using conditional whole-body deletion of *Atg7* in adult mice demonstrated the essential, systemic role of autophagy in host defense against *Streptococcus* infection and in preventing neurodegeneration, which together limit lifespan to no more than 3 months. This contrasts with constitutive autophagy-deficient mice, where neonates fail to survive much more than 24 hours after birth even when force-fed (2, 3), suggesting that autophagy is more critical in newborn than adult mice.
Acute Autophagy Ablation Selectively Impairs NSCLC

Figure 5. Acute, systemic autophagy ablation does not alter the efficiency of tumor initiation. A, experimental design to induce conditional whole-body Atg7 deletion before tumor induction. Ubc-CreERT2+/−;KrasG12D-frt/+,Trp53frt/frt,Atg7+/+ or Ubc-CreERT2+/−;KrasG12D-frt/+,Trp53frt/frt,Atg7fl ox/fl ox mice were treated with TAM at 8 to 10 weeks of age by intraperitoneal injection to delete Atg7 throughout the mouse, then these mice were infected with Ad-FLPo at 1 week after TAM to activate RAS and delete p53 and were analyzed at times thereafter. B, representative lung tumor histology (H&E) at 3 weeks after Ad-FLPo, 4 weeks after TAM (left), and 7 weeks after Ad-FLPo, 8 weeks after TAM (right). Arrows point to oncocytes. C, representative lung lobes at 7 weeks after Ad-FLPo and 8 weeks after TAM in the indicated genotypes. D, quantification of tumor numbers (left) and tumor burden (right) at the indicated times. Error bar, SEM, *, P < 0.05 (two-way ANOVA with the Bonferroni posttest).
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Figure 6. Acute, systemic Atg7 deficiency compromises tumorigenesis. A, experimental design to induce lung tumors before conditional whole-body Atg7 deletion. Ubc-CreERT2;KrasG12D-frt/+;Trp53frt/frt;Atg7+/+ and Ubc-CreERT2;KrasG12D-frt/+;Trp53frt/frt;Atg7fl ox/fl ox mice were infected with Ad-FLPo at 6 to 8 weeks of age to activate RAS and delete Trp53, then these mice were treated with TAM at 12 weeks after Ad-FLPo by intraperitoneal injection to create Atg7Δ/Δ mice. Tumor progression was analyzed at various times thereafter. B, representative micro-CT three-dimensional reconstruction and quantification of lung volume showing healthy air space before TAM treatment and equivalent tumor burden at the time of TAM treatment of mice with the indicated genotypes. Kras+/+;Trp53+/+;Atg7+/+ mice were used as a control for normal lung airspace. Tumor histology and normal adjacent lung tissue (H&E images) were analyzed at 5 weeks after TAM (last three plots). Arrows point to dead cells. C, quantification of wet lung weight (top) and tumor burden (bottom). Error bar, SEM; P values are calculated using two-way ANOVA with the Bonferroni posttest. D, representative Western blotting for ATG7, p62, S6, P-S6, and LC3 of Atg7+/+ or Atg7Δ/Δ tumor tissues. β-Actin serves as a protein loading control. E, representative IHC for ATG7, p62, TOM20, and EM images of tumor tissue. Arrows in second plot point to p62 aggregates, and arrows in the third plot point to TOM20 accumulation in tumors from Atg7-deficient mice. N, nucleus; M, mitochondria; Ly, lysosome; AP, autophagosome; L, lipid. F, representative IHC images for active caspase-3 (left) with quantification (right). Error bar, SEM; P values are calculated using two-way ANOVA with the Bonferroni posttest. G, representative IHC images for Ki67 (left) with quantification (right). Error bar, SEM; P values are calculated using two-way ANOVA with the Bonferroni posttest. H, representative IHC images for S6, P-S6, 4E-BP1, P-4E-BP1, ERK, and P-ERK. I, a model for the destructive effect of autophagy on established tumors.
Loss of autophagy in adult mice rapidly decreases the mass of WAT, suggesting that autophagy deficiency creates systemic metabolic deficiency even under fed conditions. Autophagy deficiency may necessitate the catabolism of dedicated fat stores in WAT by cytosolic lipases, or may cause browning of adipose tissue that increases β-oxidation and lipid consumption, which is consistent with mitochondrial accumulation (30). This defective adipose homeostasis and mobilization of FFAs in starved Atg7-deficient mice can contribute to systemic metabolic impairment.

One property common to adult and neonatal mice is the requirement for autophagy to survive starvation, although the underlying mechanisms seem distinct. In contrast to wild-type adult mice, we found that those with acute autophagy ablation are intolerant to fasting and die of hypoglycemia. As WAT is depleted in autophagy-deficient mice, fasting results in accelerated depletion of glycogen stores and excessive muscle catabolism in comparison with wild-type mice. The autophagy-deficient adult mice sustain serum amino acid levels and ketogenesis, but not FFA and glucose levels, indicating that gluconeogenesis in the liver is insufficient for survival. Moreover, despite excessive muscle wasting and maintenance of serum amino acid levels, this is apparently insufficient for gluconeogenesis. This suggests that liver autophagy and catabolism of lipid stores may be critical either directly or indirectly for gluconeogenesis and glucose homeostasis during fasting. The fasted autophagy-deficient mice display rapid liver, muscle, and brain damage, demonstrating the essential requirement for systemic autophagy to survive fasting. Thus, autophagy is required by both neonates and adults to survive starvation, but for different reasons. Neonates need autophagy to generate amino acids to sustain metabolic homeostasis, in contrast to adult mice that need autophagy to prevent fatal hypoglycemia. Fasted adult mice deficient for autophagy have depleted lipid stores and compensate for metabolic impairment by extensive muscle wasting, which is ultimately insufficient for viability. Liver-specific deficiency in autophagy is not fatal to fasted mice (2, 31), suggesting that autophagy in other tissues, most likely including WAT (which is lost in the absence of autophagy), is essential for survival and glucose homeostasis. RAS-driven cancers often upregulate autophagy and are dependent on autophagy for survival, although the point at which autophagy functions during tumorigenesis was not known (13). To address this, we ablated Atg7 and then initiated tumorigenesis by activating RAS and deleting Trp53 in the lung. Autophagy seemed dispensable for tumor initiation in this setting, although over time the suppressed proliferation and emergence of oncocytomas as seen previously in the lung tumor-specific Atg7 knockouts were apparent. This suggests that functional autophagy status does not alter the efficiency by which KrasG12D activation and Trp53 loss initiate lung tumor formation, but rather it is instead critical for tumor maintenance.

Acute knockdown of essential autophagy genes in many RAS-mutant human cancer cell lines can rapidly suppress growth and survival, whereas knockout of essential autophagy genes in GEMMs at the time of cancer initiation suppresses tumor growth gradually over time (10, 11, 16, 18, 19). This indicates that acute autophagy ablation may be more detrimental to tumorigenesis than constitutive ablation. To test this hypothesis, Atg7 was acutely ablated in mice with established KrasG12D-activated and Trp53-deficient NSCLC. Five weeks of systemic autophagy deficiency was dramatically destructive to these preexisting NSCLCs, which underwent loss of mTOR and MAP kinase signaling, proliferative arrest, apoptosis, and conversion of adenocarcinomas to oncocytomas. Branched chain amino acids and arginine are mTOR inducers, and there were low levels of serum valine and arginine in response to autophagy inhibition, which may contribute to loss of mTOR signaling along with defective mitochondrial metabolism (Fig. 3A).

Acute autophagy ablation more severely compromises tumorigenesis in comparison with tumors that evolve with deficiency in essential autophagy genes. Established tumors may be more autophagy-dependent, systemic autophagy deficiency may be more destructive to tumors than tumor-specific deficiency, or tumors evolving without autophagy may have some capacity to adapt, diminishing the impact of autophagy loss. In support of systemic autophagy deficiency being more detrimental to tumorigenesis, it also causes the elimination of WAT, perhaps creating a systemic environment metabolically unsupportive of tumor growth.

The key to determining if autophagy inhibition will be potentially effective to diminish tumorigenesis is establishing if tumors are more susceptible to acute autophagy inactivation than normal tissues, therefore providing a therapeutic window. Our data demonstrate that complete systemic autophagy inhibition for prolonged durations is not likely to be tolerable from a safety perspective. That said, the most serious observed effects on normal health were bacterial infection and neurodegeneration. Infection can presumably be managed through prophylactic antibiotics, and central nervous system toxicities should be considered in the design of pharmacologic agents. Note that genetic ablation as we have done here is complete and irreversible inhibition of autophagy, not likely achievable with small-molecule inhibitors, and so such represents an extreme situation. It would, however, be interesting to compare genetic and pharmacologic inhibition of autophagy (with chloroquine, for example) to help establish how effective autophagy inhibition needs to be to impair tumor growth. To what extent the detrimental effects of acute autophagy ablation are reversible in normal and tumor tissue also remains to be addressed. Finally, it will be interesting to test systemic deletion of other autophagy-related genes in this model to resolve any potential autophagy-independent functions associated with loss of specific ATG genes. With these issues addressed, our observation that 5 weeks of acute Atg7 ablation was sufficient to shut off mTOR and MAP kinase signaling and cause widespread tumor proliferative arrest and death while largely sparing normal tissues raises great hopes for antitumor efficacy.

**METHODS**

**Mice**

All animal care and treatments were carried out in compliance with Rutgers University Institutional Animal Care and Use Committee guidelines. Ubc-CreERT2 mice (ref. 20; The Jackson Laboratory) and Atg7flx/flx mice (ref. 2; provided by Dr. M. Komatsu, Tokyo Metropolitan Institute of Medical Science) were cross-bred to generate...
Ubc-CreERT2/\textsuperscript{+}/Atg7\textsuperscript{fl ox/fl ox} mice. Details of the TAM treatment to induce Atg7 deletion can be found in Supplementary Methods.

To assess the consequence of acute Atg7 deletion in response to fasting, 10 days following the last day of 5-day TAM administration, mice were fasted for 24 hours with free access to water but no food. At 20 to 24 hours after fasting, plasma was collected for hormonal and biochemical analysis, and tissues were harvested for histologic examination. For glucose supplementation, mice were provided drinking water containing 10% glucose throughout fasting.

To assess the consequence of acute Atg7 deletion to tumorigenesis, compound Ubc-CreERT2/\textsuperscript{+}/Atg7\textsuperscript{+/−} mice were bred to FSF-Kras\textsuperscript{G12D} mice (ref. 32; The Jackson Laboratory) and Tpr53\textsuperscript{frt/frt} mice (ref. 33; provided by Dr. L. Johnson, Genentech) to generate Ubc-CreERT2/\textsuperscript{+}/Kras\textsuperscript{G12D+/−}/Tpr53\textsuperscript{frt/frt}/Atg7\textsuperscript{+/−} and Ubc-CreERT2/\textsuperscript{+}/Kras\textsuperscript{G12D+/−}/Tpr53\textsuperscript{frt/frt}/Atg7\textsuperscript{fl ox/fl ox} mice. To activate RAS and delete Tpr53 in the lung and produce tumors, mice were infected intranasally with recombinant, replication-deficient Ad-FloxO (University of Iowa Adenoviral Core) at 1.2 × 10\textsuperscript{8} pfu/mouse at 6 to 8 weeks of age as described previously (16).

Gene Expression Analysis

Liver and muscle tissues were snap-frozen for RNA extraction. Extracted RNAs were processed, labeled, and hybridized to the Affymetrix GeneChip Mouse Genome 430A 2.0 array. The raw Affymetrix CEL files for liver and muscle samples were analyzed with Agilent GeneSpring GX11 software. See Supplementary Methods for details of gene expression analysis.

Serum Assays

Blood glucose was measured with a True2Go glucose meter (Nipro Diagnostics). Serum insulin and leptin levels were determined with ultrasensitive mouse insulin (Crystal Chem Inc.; 90030) and leptin (Crystal Chem Inc.; 90030) ELISA kits. Serum FFAs and β-hydroxybutyrate were measured using commercial kits from Cayman Chemical (700310 and 700190, respectively). Serum AST and ALT levels were determined using commercial kits from Idexx Bioresearch.

Histology

Histology was performed at the Cancer Institute of New Jersey (CINJ) Shared Resources and White laboratory members for histology quantification, R. Patel for EM, and Cancer Institute of New Jersey (CINJ) Shared Resources and White laboratory members for helpfull comments.

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