Autophagy promotes mammalian survival by suppressing oxidative stress and p53

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Autophagy captures intracellular components and delivers them to lysosomes for degradation and recycling. Conditional autophagy deficiency in adult mice causes liver damage, shortens life span to 3 mo due to neurodegeneration, and is lethal upon fasting. As autophagy deficiency causes p53 induction and cell death in neurons, we sought to test whether p53 mediates the lethal consequences of autophagy deficiency. Here, we conditionally deleted Trp53 (p53 hereafter) and/or the essential autophagy gene Atg7 throughout adult mice. Compared with Atg7Δ/Δ mice, the life span of Atg7Δ/Δ p53Δ/Δ mice was extended due to delayed neurodegeneration and resistance to death upon fasting. Atg7 also suppressed apoptosis induced by p53 activator Nutlin-3, suggesting that autophagy inhibited p53 activation. To test whether increased oxidative stress in Atg7Δ/Δ mice was responsible for p53 activation, Atg7 was deleted in the presence or absence of the master regulator of antioxidant defense nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2−/− Atg7Δ/Δ mice died rapidly due to small intestine damage, which was not rescued by p53 codeletion. Thus, Atg7 limits p53 activation and p53-mediated neurodegeneration. In turn, NRF2 mitigates lethal intestine degeneration upon autophagy loss. These findings illustrate the tissue-specific roles for autophagy and functional dependencies on the p53 and NRF2 stress response mechanisms.

[Keywords: autophagy; ATG7; p53; DNA damage; apoptosis; NRF2; oxidative stress]

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Autophagy is the process by which cells direct their own intracellular proteins, lipids, and organelles to the lysosomal compartment for degradation [Mizushima 2010]. Generally, the autophagy pathway involves the formation of double membrane-bound vesicles called autophagosomes that capture cargo such as cytoplasmic proteins, organelles, and bacteria. Autophagosomes with cargo then fuse with lysosomes to form autolysosomes where the cargo is degraded [Kaur and Debnath 2015]. The breakdown products are then released into the cytoplasm where they are recycled and specifically used as substrates for central carbon metabolism to sustain survival [Rabinowitz and White 2010]. Cargo-selective mutations in the autophagy-related genes (Atg) and other proteins that enable the formation of autophagosomes and recognition and capture of cargos [Mizushima and Komatsu 2011].

Autophagy maintains organelle function, prevents the accumulation of toxic cellular waste products, and sustains cell metabolism and survival during starvation [Poil-
from ferritin, which is critical for the iron homeostasis (Mancias et al. 2014).

Autophagy also has a critical role in mouse survival. Constitutively, Atg5- or Atg7-deficient mice are born developmentally normal but fail to survive the neonatal starvation period when the transplacental nutrient supply is interrupted but not yet restored by milk (Kuma et al. 2004; Komatsu et al. 2005). Force feeding only extends survival of autophagy-deficient newborn mice to neonatal starvation by 24 h. In contrast to newborn mice, adult mice have a greater tolerance to the loss of autophagy. Conditional whole-body ablation of the essential autophagy gene Atg7 in adult mice shortens life span to 2 to 3 mo due to susceptibility to infection and neurodegeneration (Karsli-Uzunbas et al. 2014). Autophagy also suppresses liver, brain, and muscle damage and prevents depletion of white adipose tissue (WAT). While adult mice tolerate autophagy deficiency in the short term in the fed state, fasting is lethal within 16 h due to hypoglycemia (Karsli-Uzunbas et al. 2014). These findings demonstrate that autophagy is required to maintain systemic mammalian metabolism and survival by mitigating metabolic stress during nutrient deprivation (Karsli-Uzunbas et al. 2014). Moreover, there are remarkable tissue-specific dependencies on autophagy, with brain, liver, muscle, and WAT being particularly autophagy-dependent (Karsli-Uzunbas et al. 2014).

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

p53 can also regulate autophagy. Under nutrient deprivation, a low ATP/AMP ratio activates 5′ AMP-activated protein kinase (AMPK), which then induces p53. Induction of p53 activates the transcription of genes in the AMPK pathway including tuberous sclerosis complex 2 (TSC2) and AMPK itself, and leads to the inhibition of mTOR and activation of autophagy (Feng et al. 2007). Some p53 target genes like BCL2-associated X protein (BAX) and p53-up-regulated modulator of apoptosis (PUMA) can directly activate autophagy in MEF cells (Yee et al. 2009). p53 can also directly turn on the expression of essential autophagy genes or induce autophagy via transcriptional activation of damage-regulated autophagy modulator (DRAM-1) in human and mouse cell lines (Crighton et al. 2006; Mah et al. 2012; Kenzelmann Broz et al. 2013). Autophagy deficiency can cause p53 induction in mouse models of lung, pancreatic, and breast cancer, and also in neurons, correlating with more apoptosis when p53 is intact, suggesting that autophagy may suppress p53 activation in some settings (Zhang et al. 2009; Yang et al. 2011; Guo et al. 2013; Huo et al. 2013; Rosenfeldt et al. 2013; Strohecker et al. 2013; Yang and Kimmelman 2014). As autophagy loss promotes p53 activation, and this p53 activation can be damaging, we sought to test the hypothesis that p53 was responsible for degenerative phenotypes induced by conditional autophagy loss in vivo.

To address how p53 and autophagy functionally interact in vivo and to determine the role that p53 plays in limiting the survival of mice without autophagy, we developed genetically engineered mouse models (GEMMs) to conditionally delete Atg7 and/or p53 systemically with tamoxifen (TAM). Whereas conditional, systemic Atg7 deletion (Atg7Δ/Δ) in adult mice limited their survival to 2–3 mo, codeletion of p53 and Atg7 (Atg7Δ/Δ p53Δ/Δ) remarkably extended life span to up to 6 mo and sustained survival during fasting. Atg7Δ/Δ p53Δ/Δ mice showed decreased tissue damage, apoptosis, and DNA damage in the liver and brain in comparison with Atg7Δ/Δ mice. Activation of p53 by Nutlin-3 was inhibited by autophagy, which protected liver and brain from p53 hyperactivation and apoptosis, suggesting that autophagy may be a resistance mechanism to p53 activators. NRF2, in turn, is a resistance mechanism to loss of autophagy as conditional deletion of both Nrf2 and Atg7 in adult mice was synthetically lethal. Mice deficient for both Atg7 and Nrf2 (Nrf2−/− Atg7Δ/Δ) succumbed to damage to the small intestine, which was independent of p53 function. Thus, Atg7 protects against excessive p53 activation and damage in the liver and brain, whereas NRF-2 protects the intestine from damage upon loss of Atg7, demonstrating the functional interdependence and tissue specificity of stress response pathways.

Results

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

To test whether p53 plays a role in limiting the survival in mice without autophagy, adult mice were engineered with or without floxed alleles of Atg7 (Kuma et al. 2004), p53 (Marino et al. 2000), and a transgene expressing a TAM-regulated Cre recombinase under the control of ubiquitin C promoter that is ubiquitously expressed in the whole body (Ubc-CreERT2) (Ruzankina et al. 2007). Injecting TAM activates Cre throughout these mice and the floxed alleles of Atg7 and/or p53 are deleted separately or together (Fig. 1A). Mice with systemic loss of Atg7 or p53 or both in all tissues are thereby generated and gene deletion was confirmed by qRT-PCR at 2, 5, and 8 wk following the five consecutive days of TAM administration [Supplemental Fig. S1A]. Loss of ATG7 protein expression was also associated with accumulation of an unprocessed form of microtubule-associated protein 1A/1B light chain 3 (LC3-I), decrease in or absence of the processed (active) form of LC3 (LC3-II), and accumulation of the autophagy suppresses oxidative stress, p53 for survival
Figure 1. Atg7Δ/Δ, p53Δ/Δ mice have extended life span, delayed tissue damage and neurodegeneration compared with Atg7Δ/Δ mice. (A) Experimental design for generation of Atg7Δ/Δ, p53Δ/Δ mice, and Atg7Δ/Δ p53Δ/Δ mice. Ubc-CreERT2/+; Atg7flox/flox mice were treated with TAM at 8–10 wk of age and analyzed at certain time points afterward. (B) Western blot for ATG7, p62, and LC3 at the indicated times of the indicated tissues from wild-type mice, Atg7Δ/Δ mice, p53Δ/Δ mice, and Atg7Δ/Δ p53Δ/Δ mice. β-Actin was used as a loading control. (C) Kaplan-Meier survival curve of wild-type mice, Atg7Δ/Δ mice, p53Δ/Δ mice, and Atg7Δ/Δ p53Δ/Δ mice. Dotted line indicates 109 d, when the first lymphoma was identified in p53Δ/Δ mice. (n.s.) Not significant; (∗) P < 0.05; (∗∗) P < 0.01; (∗∗∗) P < 0.0001 (log-rank test and Gehan-Breslow-Wilcoxon test as indicated). (D) Percentage distribution for the cause of death of Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δ p53Δ/Δ mice. The cause of death was analyzed at 30–90 d after TAM and 109–180 d after TAM. (E) Representative histology of liver, muscle, cerebrum, cerebellum, pancreas, white adipose tissue (WAT), and lung by hematoxylin and eosin stain (H&E) from wild-type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δ p53Δ/Δ mice at the 8-wk time point. Black arrows indicate the damage site for these tissues. (F) Kaplan-Meier survival curve of wild-type mice, p53Δ/Δ mice, and Atg7Δ/Δ p53Δ/Δ mice that died after 109 d. Black dots on the survival curve indicate the censoring times that mice died of no tumor development. (∗∗∗∗) P < 0.0001 (log-rank test). (G) Kaplan-Meier survival curve of wild-type mice, Atg7Δ/Δ mice, p53Δ/Δ mice, and Atg7Δ/Δ p53Δ/Δ mice during starvation at 10 d after TAM. ∗ P < 0.05 (log-rank test). See also Supplemental Figure S1.
constitutively deficient due to susceptibility to infection early, and to neurodegeneration later, which is consistent with our previous findings [Fig. 1C,D; Karsli-Uzunbas et al. 2014]. Similar to constitutively deficient p53/−/− mice, p53ΔΔ mice died from lymphoma, which limited life span to up to 6 mo [Fig. 1C,D; Donehower et al. 1995]. In contrast to Atg7ΔΔ mice, one-third of the Atg7ΔΔ/p53ΔΔ mice lived >3 mo and up to 6 mo after TAM, while all of the Atg7ΔΔ mice died before 3 mo after TAM [Fig. 1C,D]. Although Atg7ΔΔ/p53ΔΔ mice showed severe loss of hepatocytes in the liver, pyramidal neurons, Purkinje cells, and depletion of lipid in WAT as reported previously (Kar- sli-Uzunbas et al. 2014), which was not observed in the p53ΔΔ mice that died from cancer, none of the Atg7ΔΔ/p53ΔΔ mice died after 109 d revealed that death probability from lymphoma in Atg7ΔΔ/p53ΔΔ mice is much lower compared with p53ΔΔ mice [P-value<0.0001, Log-rank test] [Fig. 1F]. Thus, Atg7 promotes development of lethal lymphomas driven by deletion of p53, consistent with the tumor-promoting role for autophagy reported in other settings [Poillet-Perez and White 2019].

p53 deficiency prevents fasting lethality in Atg7ΔΔ mice
While Atg7ΔΔ mice survive in the short term, in contrast to wild-type mice, fasting is lethal within 16 h due to hypoglycemia [Karsli-Uzunbas et al. 2014]. Since p53 deficiency extended the life span and attenuated tissue damage in Atg7ΔΔ mice, we sought to test whether p53 contributes to the death of Atg7ΔΔ mice during fasting. In contrast to Atg7ΔΔ mice where fasting was lethal, none of the Atg7ΔΔ/p53ΔΔ mice died upon fasting, suggesting that p53 was responsible for fasting-induced death of Atg7-deficient mice [Fig. 1F].

ATG7 is required to protect the liver and brain from p53-mediated damage
Autophagy deficiency causes p53 induction in neurons and in some cancer models and promotes cell death. Therefore, we tested whether p53 induction occurred in the whole body after Atg7 deletion. Immunohistochemistry [IHC] for p53 protein revealed that p53 accumulation was detectable at 2 wk after TAM administration, and was maintained at 5 and 8 wk after TAM in the livers and brains of Atg7ΔΔ mice, while p53 activation was not apparent in wild-type, p53ΔΔ, and Atg7ΔΔ/p53ΔΔ mice [Fig. 2A; Supplemental Fig. S2A,B]. qRT-PCR for the p53 target genes cyclin-dependent kinase inhibitor 1A [Cdkn1a, or p21], BCL2-associated X [Bax], and BCL2-binding component 3 [Bbc3, or Puma] showed increased Cdkn1a, Bax, and Bbc3 expression in Atg7ΔΔ mice at 2, 5, and 8 wk after TAM in the liver and brain compared with wild-type, p53ΔΔ, and Atg7ΔΔ/p53ΔΔ mice [Fig. 2B, C]. These data suggest that loss of Atg7 promotes activation of p53.

Whole-body ATG7 deficiency leads to DNA damage and apoptosis in the liver and cerebrum [Karsli-Uzunbas et al. 2014] and p53 is known to be activated by different stress signals including DNA damage and oxidative stress, and triggers cell cycle arrest and apoptosis [Fischer 2017]. Therefore, we hypothesized that p53 induction in Atg7ΔΔ mice may promote apoptosis. IHC for the DNA damage response activation marker γ-H2AX revealed accumulation of γ-H2AX in Atg7ΔΔ liver hepatocytes, neurons, and nonneuronal cells in cerebrum starting at 2 wk that
Figure 2. Autophagy is required to protect liver and brain from p53 accumulation, DNA damage response activation, and apoptosis. (A) Representative liver and cerebrum IHC staining of p53 and quantification at the indicated times from wild-type and Atg7Δ/Δ mice. Black arrows indicate p53-positive cells. (2w) 2-wk time point; (5w) 5-wk time point; (8w) 8-wk time point. (B, C) Quantitative real-time PCR of Cdkn1a, Bax, and Bbc3 for liver and brain tissues from wild-type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice at the indicated times. (*) P < 0.01; (**) P < 0.001; (***) P < 0.0001 (unpaired t-test). (D) Representative liver and cerebrum IHC staining for γ-H2AX and active caspase-3 with quantification at the indicated times from wild-type and Atg7Δ/Δ mice. Black arrows indicate γ-H2AX or active caspase-3-positive cells. (2w) 2 wk; (5w) 5 wk; (8w) 8 wk. [*] P < 0.05; [**] P < 0.01; [***] P < 0.001 [****] P < 0.0001 (unpaired t-test). (E) Representative liver IHC staining for MDA at the indicated times from wild-type, Atg7Δ/Δ, p53Δ/Δ, and Atg7Δ/Δp53Δ/Δ mice. See also Supplemental Figure S2.
was apparent through 8 wk after TAM (Fig. 2D; Supplemental Fig. S2,C,D). In contrast, γ-H2AX accumulation was not detected in wild-type, p53Δ/Δ, and Atg7Δ/Δ p53Δ/Δ mice [Supplemental Fig. S2,C,D]. As a likely consequence of p53 activation in Atg7Δ/Δ mice, they also showed more apoptosis marked by increased active caspase-3 in liver hepatocytes, neurons, and nonneuronal cells in cerebrum in comparison with Atg7Δ/Δ/p53Δ/Δ mice [Fig. 2D; Supplemental Fig. S2,E,F]. These data indicated that p53 induction caused apoptosis in Atg7Δ/Δ mice. Atg7Δ/Δ mice also displayed increased malondialdehyde [MDA] in the liver by IHC compared with wild-type, p53Δ/Δ, and Atg7Δ/Δ/p53Δ/Δ mice, indicating that p53 induction was associated with increased oxidative stress in Atg7Δ/Δ mice [Fig. 2E]. As the phenotype of the Atg7Δ/Δ/p53Δ/Δ mice was similar to the Atg7Δ/Δ mice, just delayed, this suggests that other responses similar to p53 act later when p53 is absent. It is intriguing to speculate that other p53 family members, such as p63 and p73, may be responsible for the neurodegeneration in the Atg7Δ/Δ/p53Δ/Δ mice.

**ATG7 limits p53 activation by Nutlin-3a**

Regulation of p53 activity relies on the essential p53 antagonist MDM2, which is a direct transcriptional target of p53 and is up-regulated when p53 is activated by phosphorylation at specific serine and threonine residues [Bode and Dong 2004]. MDM2 binds to p53, and the ubiquitin E3 ligase of MDM2 ubiquitylates p53, which decreases its stability by targeting it to the proteasome for degradation [Honda et al. 1997; Kubb tat et al. 1997; Matsumine et al. 1997]. Under stress conditions, p53 is phosphorylated at its transactivation domain [Ser15, Ser20, and Thr18], which disrupts its binding of MDM2, and is thereby stabilized and activated [Craig et al. 1999]. In this way, p53 and MDM2 form a negative feedback loop resulting from p53-dependent induction of MDM2 and MDM2-dependent suppression of p53 activity, which helps the cell to deal with stress without hyperactivation of p53 [Montes de Oca Luna et al. 1995; Dotto 2009; Marine and Lozano 2010]. Nutlin-3 works as an MDM2 antagonist and up-regulates the cellular p53 level by competing for the p53-binding site on MDM2, and is being assessed clinically to promote p53 activation for cancer therapy [Vassilev et al. 2004; Drost et al. 2015; Yee-Lin et al. 2018; Forte et al. 2019]. Since Atg7 limits p53 accumulation and activation, we sought to test whether Atg7 also limited the ability of Nutlin-3 to activate p53 [Khoury and Domling 2012] as a potential resistance mechanism in normal tissues.

Following deletion of Atg7 and/or p53, mice were treated with either vehicle or Nutlin-3 (200 mg/kg) once per day for 1 wk [Fig. 3A]. Deletion of Atg7 and p53 was confirmed by qRT-PCR [Supplemental Fig. S3A]. Western blot for loss of ATG7 protein, accumulation of LC3-I and loss of LC3-II, and accumulation of p62 in the livers and brains from Atg7Δ/Δ and Atg7Δ/Δ/p53Δ/Δ mice indicated blockage of autophagy [Fig. 3B]. As described previously, liver damage and neuron loss in Atg7Δ/Δ mice were confirmed by H&E, which was not affected by Nutlin-3 [Supplemental Fig. S3B]. IHC of the livers and brains from Atg7Δ/Δ mice revealed increased p53 compared with wild-type mice, which was further increased in Nutlin-3-treated Atg7Δ/Δ mice, suggesting that Nutlin-3-induced p53 in the absence but not in the presence of autophagy. As expected, Nutlin-3 did not affect p53 levels in p53Δ/Δ mice and Atg7Δ/Δ/p53Δ/Δ mice [Fig. 3C; Supplemental Fig. S3C]. qRT-PCR for the p53 target genes Cdkn1a, Bax, and Bbc3 showed increased Cdkn1a, Bax, and Bbc3 expression in untreated Atg7Δ/Δ mice, which was further increased in Nutlin-3-treated Atg7Δ/Δ mice in the liver and brain compared with vehicle or Nutlin-3-treated wild-type mice [Fig. 3D]. This suggested that p53 was activated by Nutlin-3 only in the absence of Atg7 in normal tissues such as liver and brain. IHC of liver and brain revealed increased γ-H2AX and active caspase-3 in Atg7Δ/Δ mice compared with wild-type mice, and activation of p53 by Nutlin-3 greatly increased γ-H2AX and active caspase-3 levels. Induction of γ-H2AX and active caspase-3 were not observed in p53Δ/Δ and Atg7Δ/Δ/p53Δ/Δ mice, suggesting that loss of autophagy induced apoptosis through p53 activation [Fig. 3E; Supplemental Fig. S3D,E]. Therefore, autophagy is essential to protect tissues from apoptosis by limiting p53 activation.

Since p53 can induce a series of essential autophagy genes including Atg7 in MEF cells [Kenzelmann Broz et al. 2013], we hypothesized that up-regulation of p53 by Nutlin-3 can turn on essential autophagy genes and protect tissues from damage caused by p53 induction in wild-type mice. Real-time PCR on a series of autophagy essential genes indicated no significant difference in the autophagy gene mRNA levels, suggesting that the autophagy transcription program is not detectably induced by p53 at the times the tissues were collected [Supplemental Fig. S4].

**Atg7 deficiency is synthetically lethal in the absence of Nrf2**

Autophagy can reduce reactive oxygen species [ROS] by removing damaged mitochondria and unfolded protein, and autophagy deficiency leads to increased ROS and accumulation of unfolded protein [Manjithaya et al. 2010; Mizushima 2010]. Since we found induction of oxidative stress markers in Atg7Δ/Δ mice [Fig. 2E], we investigated whether the increased oxidative stress was responsible for tissue damage caused by p53 activation. Nrf2 is the master regulator of the antioxidant defense and is ubiquinated by an E3 ubiquitin ligase Kelch-like ECH-associated protein 1 [KEAP1] and degraded by the proteasome pathway under normal conditions [Kensler et al. 2007]. With increased ROS, Nrf2 is released from KEAP1 and triggers expression of a series of antioxidant genes, and NRF2 is induced by autophagy deficiency [Komatsu et al. 2010; Lau et al. 2010; Levonen et al. 2014]. To examine the role of antioxidant defense in mice lacking autophagy, mice with constitutive deficiency in Nrf2 [Chan et al. 1996] were crossed with Ubc-CreERT2fl; Atg7fl mice to generate Nrf2−/−; Ubc-CreERT2fl; Atg7fl mice [Fig.
Figure 3. Activation of p53 by MDM2 antagonist Nutlin-3a in Atg7ΔΔ mice leads to further increased DNA damage response and apoptosis in the liver and brain. [A] Experimental design for generation of Atg7ΔΔ, p53ΔΔ, and Atg7ΔΔ/p53ΔΔ mice and Nutlin-3 administration. Nutlin-3 was administered to mice by oral gavage 2 wk after TAM administration at a dosage of 200 mg/kg for 1 wk. [B] Western blot for ATG7, p62, and LC3 for liver and brain tissues from wild-type, Atg7ΔΔ, p53ΔΔ, and Atg7ΔΔ/p53ΔΔ mice treated with vehicle or Nutlin-3. β-Actin was used as a loading control. [V] Treated with vehicle; [N] treated with Nutlin-3. [C] Representative liver and cerebrum IHC staining for p53 and quantification from wild-type and Atg7ΔΔ mice treated with vehicle or Nutlin-3. Black arrows indicate p53-positive cells. [V] Vehicle; [N] Nutlin-3. (•) P < 0.05; (••••) P < 0.0001; [n.s.] not significant (unpaired t-test). [D] Quantitative real-time PCR of Cdkn1a, Bax, and Bbc3 for liver and brain tissues from wild-type, Atg7ΔΔ, p53ΔΔ, and Atg7ΔΔ/p53ΔΔ mice treated with vehicle or Nutlin-3. [V] Vehicle; [N] Nutlin-3. (•) P < 0.05; (••) P < 0.01; (•••) P < 0.001; [n.s.] not significant (unpaired t-test). [E] Representative liver and cerebrum IHC staining for γ-H2AX and active caspase-3 with quantification from wild-type and Atg7ΔΔ mice treated with vehicle or Nutlin-3. Black arrows indicate γ-H2AX or active caspase-3-positive cells. [V] Vehicle; [N] Nutlin-3. (•) P < 0.05; (•••) P < 0.001; (••••) P < 0.0001; [n.s.] not significant (unpaired t-test).
Figure 4. Atg7 deficiency is synthetically lethal in the absence of Nrf2. (A) Experimental design for generation of Atg7 Δ/Δ mice, Nrf2−/− mice, and Nrf2−/−/Atg7 Δ/Δ mice. (B) Kaplan-Meier survival curve of wild-type, Atg7 Δ/Δ, Nrf2−/−, and Nrf2−/−/Atg7 Δ/Δ mice. (***P < 0.0001 [log-rank [Mantel-Cox] test]). (C) Representative histology of duodenum, jejunum, and ileum by H&E at the indicated times from wild-type, Atg7 Δ/Δ, Nrf2−/−, and Nrf2−/−/Atg7 Δ/Δ mice. (D) Representative Bodipy C11 stain of duodenum, jejunum, and ileum at the indicated times from wild-type, Atg7 Δ/Δ, Nrf2−/−, and Nrf2−/−/Atg7 Δ/Δ mice. (E) Representative Alcian blue stain of duodenum, jejunum, and ileum at the indicated times from wild-type, Atg7 Δ/Δ, Nrf2−/−, and Nrf2−/−/Atg7 Δ/Δ mice. (F) Representative duodenum, jejunum, and ileum IHC stain of OLFM4 at the indicated times from wild-type, Atg7 Δ/Δ, Nrf2−/−, and Nrf2−/−/Atg7 Δ/Δ mice.
In contrast to Atg7Δ/Δ and Nrf2−/− mice, most of which survive, Nrf2−/−Atg7Δ/Δ mice had a life span of <7 d (Fig. 4B). Histological examination of tissues by H&E surprisingly showed no damage to the liver, brain, pancreas, lungs, and kidneys in Nrf2−/−Atg7Δ/Δ mice (Supplemental Fig. S5A). The only tissue with significant damage was the intestine (duodenum, jejunum, and ileum), which may be the cause of increased muscle wasting and loss of WAT (Fig. 4C; Supplemental Fig. S5B). The only tissue with significant loss of OLFM4 staining in Nrf2−/−Atg7Δ/Δ intestine, indicating increased apoptosis (Supplemental Fig. S5C–E). Thus, conditional deletion of the essential autophagy gene Atg7 in adult mice is synthetically lethal in the absence of Nrf2 due to damage to the intestine.

We then investigated which cell type in the intestine was most affected by deficiency in Atg7 in the absence of Nrf2. Alcian blue staining of paraffin sections from intestine tissues was significantly decreased in Nrf2−/−Atg7Δ/Δ mouse intestine, suggesting loss of goblet cells (Fig. 4E). IHC for the stem cell marker Lysozyme revealed diffuse staining in the Nrf2−/−Atg7Δ/Δ intestine, indicating decreased lipid peroxidation that results from ROS (Fig. 4D). IHC for active caspase-3 displayed increased staining in Nrf2−/−Atg7Δ/Δ intestine in both the crypt and villus, indicating increased apoptosis (Supplemental Fig. S5F; Cadwell et al. 2008). We then investigated whether Paneth cell function is abnormal (Supplemental Fig. S5G). TAM administration was then used to delete p53 in the intestine was confirmed by qRT-PCR, and we found that Nrf2−/−p53Δ/ΔAtg7Δ/Δ mice did not survive longer than Nrf2−/−Atg7Δ/Δ mice, suggesting that the intestinal damage in the Nrf2−/−Atg7Δ/Δ mice was not caused by p53 (Supplemental Fig. S5G,H).

**Discussion**

Both autophagy and p53 can protect tissues from stress such as DNA damage, oxidative stress, and hypoxia (Mizushima and Komatsu 2011; Fischer 2017), and their overlapping functions have suggested that these two pathways interact. p53 up-regulates the expression of essential autophagy genes and autophagy function in vitro (Crighton et al. 2006; Feng et al. 2007; Zhang et al. 2009; Mah et al. 2012; Kenzelmann Broz et al. 2013). In turn, autophagy inhibits p53 in some tumors providing a negative feedback loop (Guo et al. 2013; Rosenfeldt et al. 2013; Strohecker et al. 2013; Yang et al. 2014). Whether autophagy can regulate p53 in normal tissues in vivo, however, was not clear. We found that Atg7 suppresses p53 activation in the liver and brain, without which hyperactivation of p53 is responsible for damage to these tissues. Thus, essential autophagy gene Atg7 is a tissue-specific negative regulator of p53 and contributes to a negative feedback loop to limit p53 activation in vivo (Fig. 5A). Remarkably, eliminating p53 also rescued the survival of Atg7-deficient mice during fasting, suggesting that Atg7 restricts p53 activation in response to exogenous as well as endogenous stress (Fig. 5). Even when p53 activation is forced by Nutlin-3, Atg7 prevents these tissues from p53-mediated damage (Fig. 5A,B). However, how p53 is activated remains unclear, which could either be a direct effect of loss of Atg7, or an indirect effect caused by changes in the cellular microenvironment after Atg7 deletion. These findings are consistent with the observation that Nutlin-3 does not activate p53 in normal tissues unless under stress conditions caused by Atg7 deficiency, but can efficiently activate p53 in different types of cancer cells without side effects on normal tissues (Vassilev et al. 2004; Drost et al. 2015; Yee-Lin et al. 2018; Forte et al. 2019). Therefore, autophagy may limit the effectiveness of MDM2 antagonists, and this should be tested in the cancer setting.

The Nrf2 and autophagy pathways both contribute to antioxidative defense. NRF2 is activated by autophagy...
deficiency in vitro and in tumors [Lau et al. 2010; Strohecker et al. 2013; Saito et al. 2016]. The autophagy substrate p62, which accumulates when autophagy is blocked, interacts with KEAP1, thereby releasing and stabilizing NRF2 and promoting expression of its target genes [Komatsu et al. 2010; Lau et al. 2010; Ichimura et al. 2013; Levonen et al. 2014]. We found that the protective function of NRF2 is essential for the survival of mice with loss of ATG7, as in stark contrast to Atg7/A mice, Nrf2/-/-; Atg7/A mice die rapidly, specifically from damage to the small intestine. Atg5 deficiency in intestine epithelia causes decreased numbers of intestinal stem cells, and these stem cells have a higher ROS level compared with wild-type mice, which can be rescued by treating mice with antioxidant N-acetyl cysteine [Asano et al. 2017]. Atg16L1 is also required to protect the intestinal epithelium from necroptosis induction in response to virus-induced intestinal bowel disease by maintaining mitochondrial homeostasis [Matsuzawa-Ishimoto et al. 2017]. We report here that knockout of NRF2 is synthetically lethal with loss of Atg7, which causes death within 1 wk before liver and brain damage can be observed, as NRF2 is specifically required to protect the survival of intestinal stem cells [Fig. 5C]. The compensatory protective effect of NRF2 to loss of autophagy may be broad as recent cell-based screens identified NRF2 activation as a resistance mechanism selected for in cancer cells deleted for essential autophagy genes [Towers et al. 2019]. In conclusion, autophagy limits p53 activation and damage in the liver and brain, while NRF2 limits intestinal stem cells damage due to loss of autophagy by a p53-independent mechanism. These findings demonstrate the functional interaction and tissue specificity of these stress regulated pathways [Fig. 5A–C].

ATG7 is an essential autophagy protein in the canonical autophagy pathway where it functions as an E1-like ubiquitin activating enzyme [Mizushima and Klionsky 2007]. ATG7, however, also contributes to the other noncanonical LC3-dependent pathways LC3-associated phagocytosis (LAP) and LC3-associated endocytosis (LANDO). Macrophages use LAP to engulf and degrade particles and pathogens, which is immune-suppressive [Sanjuan et al. 2007; Florey et al. 2011; Martinez et al. 2011, 2015; Kim et al. 2013]. LANDO is also immune suppressive and functions in microglia to remove the β-amyloid aggregates and protect the brain from neurodegeneration [Heckmann et al. 2019]. Canonical autophagy, LAP, and LANDO all require autophagy machinery proteins including Beclin1, vacuolar protein sorting 34 (VPS34), ATG7, ATG5, and LC3, but activation of LAP or LANDO does not require some proteins in the autophagy initiation complexes such as RB1-inducible coiled-coil protein 1 [FIP200] [Martinez et al. 2011, 2015; Heckmann et al. 2019]. Although LAP and LANDO have not been identified in hepatocytes, neurons, or intestinal stem cells as examined here, it would be interesting to test whether Fip200 deletion activates the p53 response in the liver and brain, or causes dependence on NRF2 in the intestine to distinguish roles of canonical and noncanonical autophagy pathways.

Material and methods

Mouse models

All animal care was carried out in compliance with Rutgers University Institutional Animal Care and Use Committee guidelines. Ubc-CreERT2/+, mice (The Jackson Laboratory) (Ruzankina et al. 2007) and Atg7fl/fl mice (provided by Dr. M. Komatsu, Tokyo Metropolitan Institute of Medical Science) [Komatsu et al. 2005] were cross-bred to generate the Ubc-CreERT2/+, Atg7fl/fl mice as described previously [Karsli-Uzunbas et al. 2014]. To generate Ubc-CreERT2/+, Atg7fl/fl; p53fl/fl mice, p53fl/fl mice [The Jackson Laboratory] (Marino et al. 2000) were cross-bred with our previously created Ubc-CreERT2/+, Atg7fl/fl mice. To generate Nrf2-/-; Ubc-CreERT2/+, and Nrf2-/-; Ubc-CreERT2/+, Atg7fl/fl mice, Nrf2-/- mice [provided by YW Kan, University of California at San Francisco] (Chan et al. 1996) were cross-bred with Ubc-CreERT2/+ mice and our previously generated Ubc-CreERT2/+, Atg7fl/fl mice.

TAM administration

For acute deletion of Atg7 and/or p53, detailed rationale and TAM preparation is described as published previously [Karsli-Uzunbas et al. 2014]. For TAM delivery to the adult mice, 200 μL of the suspended solution per 20 g of body weight was injected intraperitoneally (IP) into 8- to 10-wk-old Ubc-CreERT2/+, Atg7fl/fl mice and Ubc-CreERT2/+, Atg7fl/fl; p53fl/fl mice once per day for five consecutive days to delete the floxed gene systematically. Additionally, the same dosages of TAM were given to Ubc-CreERT2/+ mice and Ubc-CreERT2/+, p53fl/fl mice, and these mice were examined as control groups.

Statistical methods

Survival curves were estimated using the Kaplan-Meier method. Comparisons of survival curves were made using the log-rank test and Gehan-Breslow-Wilcoxon test [Cox and Oakes 1984].

Fasting

Fasting was conducted as described previously [Karsli-Uzunbas et al. 2014].

Histology

Mouse tissues were collected and fixed in 10% formalin solution [Formaldehyde Fresh, Fisher Scientific SF94-4]. Tissues were fixed overnight and then transferred to 70% ethanol for paraffin-embedded sections or 15% sucrose following by 30% sucrose for frozen sections. For acute deletion of Atg7 and/or p53, detailed rationale and TAM preparation is described as published previously [Karsli-Uzunbas et al. 2014]. For TAM delivery to the adult mice, 200 μL of the suspended solution per 20 g of body weight was injected intraperitoneally (IP) into 8- to 10-wk-old Ubc-CreERT2/+, Atg7fl/fl mice and Ubc-CreERT2/+, Atg7fl/fl; p53fl/fl mice once per day for five consecutive days to delete the floxed gene systematically. Additionally, the same dosages of TAM were given to Ubc-CreERT2/+ mice and Ubc-CreERT2/+, p53fl/fl mice, and these mice were examined as control groups.

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Histology

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Nutlin-3a administration

TAM were injected via IP into 8- to 10-wk-old Ubc-CreERT2/\textsuperscript{fl}, Atg7\textsuperscript{-/}, Atg5\textsuperscript{-/}, and p53\textsuperscript{-/-} mice once per day for five consecutive days. Additionally, same dosages of TAM were given to Ubc-CreERT2/\textsuperscript{fl}, p53\textsuperscript{-/-} mice. After 2 wk, 200 mg/kg Nutlin-3 (Cayman Chemicals) resolved in 50% DMSO was delivered to the mice by oral gavage once per day for seven consecutive days. Mice were sacrificed 1 d after the last administration of Nutlin-3 and tissues were collected for histology and snap-frozen for Western blot and real-time PCR.

Real-time PCR

Total RNA were isolated from tissue by Trizol [Invitrogen]. cDNA were then reverse-transcribed from the total RNA by MultiScribe RT kit [Thermo Fisher]. Real-time PCR were performed on Applied Biosystems StepOne Plus machine. Atg7 and p53 were performed using SYBR Green for deletion detection [Atg7: forward 5′-ACTTGACGCCCTTACCTGCG-3′; reverse 5′-TAC TCTTGACGGTCGTTGC-3′; p53: forward 5′-CGACTACATT TAGGGGGCAC-3′; reverse 5′-GGACGAATGTGGTTC TACACAAGCCT-3′; Actin: forward 5′-GAACCTTAAGGCCAAC CTTGAAAGATGAC-3′; reverse 5′-GCAAGATGCGTGAGGGA GACCA-3′]. Besides that, all of the other genes were detected using predesigned commercial TaqMan primers for each gene accordingly (Cdkn1a: Mm00432448-m1; Bax: Mm00432050; Bbc3: Mm00519268; Actin: Mm0067939-s1; Uvrag: Mm00724370-m1; Ulk1: Mm0437238-m1; Ulk2: Mm03048846-m1; Vmp1: Mm00774656-m1; Atg2b: Mm00062076-m1; Atg4a: Mm04214755-s1; Atg4c: Mm00558175-m1; and Atg10: Mm00470550-m1). Results were calculated using the \( \Delta\Delta C_T \) method and then normalized to actin.

Western blot

Different tissues were ground in a Cryomill machine [Retsch] and then total protein extracts were isolated by Tris lysis buffer (1 mol/L Tris HCl, 1 mol/L NaCl, 0.1 mol/L EDTA, 10% NP40). Separated proteins were probed with antibodies against ATG7 (1:1000; Santa Cruz Biotechnology sc-365062), and GAPDH (1:1000; BioVision 40-8200) and ATG4A, p62 (1:2000; American Research Products 03-GP62-C), Bcl-3, Bax (1:1000; Cell Signaling Technologies), Bcl-2 (1:1000; Cell Signaling Technologies), and Bcl-w (1:1000; Cell Signaling Technologies). Resulting blots were visualized on Applied Biosystems StepOne Plus machine.


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Author contributions: Y.Y. performed the majority of the experimental work and wrote the manuscript. G.K.-U. performed some of the survival experiments and the fasting experiment and assisted with IHC tissue preparation from wild-type, Atg7\textsuperscript{+/+}, p53\textsuperscript{+/+} and Atg7\textsuperscript{+/}p53\textsuperscript{+/} mice. L.P.-P. and A.S. assisted with the Western blot and quantification of IHC. Z.S.H. assisted with the manuscript. G.K.-U. performed IHC tissue preparation from wild-type, some of the survival experiments and the fasting experiment and wrote the manuscript. D.M. assisted with the statistics. E.W. was the leading principal investigator who conceived and supervised on the project and edited the paper.
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