Autophagy Sustains Mitochondrial Glutamine Metabolism and Growth of \textit{Braf}^{\text{V600E}}-Driven Lung Tumors

Anne M. Strohecker\textsuperscript{1,2}, Jessie Yanxiang Guo\textsuperscript{1,2}, Gizem Karsli-Uzunbas\textsuperscript{1,2}, Sandy M. Price\textsuperscript{1,2}, Guanghua Jim Chen\textsuperscript{1,2}, Robin Mathew\textsuperscript{1,2}, Martin McMahon\textsuperscript{3}, and Eileen White\textsuperscript{1,2}
Autophagic elimination of defective mitochondria suppresses oxidative stress and preserves mitochondrial function. Here, the essential autophagy gene Atg7 was deleted in a mouse model of \( \text{Braf}^{V600E} \)-induced lung cancer in the presence or absence of the tumor suppressor \( \text{Trp53} \). Atg7 deletion initially induced oxidative stress and accelerated tumor cell proliferation in a manner indistinguishable from \( \text{Nrf2} \) ablation. Compound deletion of \( \text{Atg7} \) and \( \text{Nrf2} \) had no additive effect, suggesting that both genes modulate tumorigenesis by regulating oxidative stress and revealing a potential mechanism of autophagy-mediated tumor suppression. At later stages of tumorigenesis, \( \text{Atg7} \) deficiency resulted in an accumulation of defective mitochondria, proliferative defects, reduced tumor burden, conversion of adenomas and adenocarcinomas to oncocytomas, and increased mouse life span. Autophagy-defective tumor-derived cell lines were impaired in their ability to respire and survive starvation and were glutamine-dependent, suggesting that autophagy-supplied substrates from protein degradation sustains \( \text{Braf}^{V600E} \) tumor growth and metabolism.

**SIGNIFICANCE:** The essential autophagy gene \( \text{Atg7} \) functions to promote \( \text{Braf}^{V600E} \)-driven lung tumorigenesis by preserving mitochondrial glutamine metabolism. This suggests that inhibiting autophagy is a novel approach to treating \( \text{Braf}^{V600E} \)-driven cancers.

See related commentary by Chen and Guan, p. 1225.

---

**INTRODUCTION**

Autophagy is a catabolic process activated by metabolic stress whereby cellular proteins and organelles are engulfed and targeted to lysosomes for degradation. This process sustains metabolism by recycling intracellular components for use in macromolecular synthesis and prevents the accumulation of damaged proteins and organelles that can be toxic. The role of autophagy in cancer is complex and still poorly understood (1). Mice with allelic loss of the essential autophagy gene \( \text{Beclin1} \) are tumor prone, and mosaic or liver-specific deletion of \( \text{Atg5} \) or \( \text{Atg7} \) produces benign hepatomas, suggesting a role for autophagy in tumor suppression (2–4). However, autophagy localizes to metabolically stressed, hypoxic regions of solid tumors, where it enables tumor cell survival (5–7). Indeed, \( \text{Kras}^{G12D} \) -driven tumors upregulate autophagy that is required for maintenance of functional mitochondria, survival during starvation, and tumor growth (8, 9). In the absence of autophagy, defective mitochondria accumulate, triggering a reduction in mitochondrial respiration that is ultimately incompatible with proliferation. This compromises stress tolerance, prompting us to label these tumors “autophagy-addicted” (8–10). Autophagy addiction was recently confirmed in genetically engineered mouse models of \( \text{Kras}^{G12D} \)-driven non–small cell lung carcinoma (NSCLC), where autophagy deletion contributed to development of adenomas and adenocarcinomas to oncocytomas, a benign tumor type associated with an accumulation of defective mitochondria (11–14). Thus, the emerging picture is that the role of autophagy in cancer is context-dependent. In some settings, autophagy may limit tumorigenesis by preserving protein and organelle quality control (preventing oxidative stress and tissue damage), whereas in other contexts autophagy promotes metabolism, adaptation to stress, and tumor growth. A comprehensive understanding of the role of the pathway in different tissue types as well as the spatial and temporal variations in autophagy dependence in tumors driven by specific oncogenic events will be required before modulation of autophagy can become an effective therapeutic strategy for cancer (15–17).

Because \( \text{Kras}^{G12D} \)-driven tumors are exquisitely sensitive to autophagy inhibition, we turned our attention to its downstream effector \( \text{BRAF} \). Mutations in \( \text{Braf} \) are found in many human tumors, including melanoma (65%), lung cancer (3%), papillary thyroid cancer (40%), ovarian cancer (4%), and colorectal cancer (18%, refs. 18–20), with the most common alteration being a valine to glutamic acid substitution at residue 600 (\( \text{Braf}^{V600E} \)) leading to constitutive activation of the kinase and RAS independence. To examine the role of autophagy in \( \text{Braf}^{V600E} \)-driven lung cancers, mice with Cre-activatable \( \text{Braf}^{V600E} \) with and without conditional alleles of the essential autophagy gene \( \text{Atg7} \) were generated, lung tumorigenesis was induced via intranasal administration of adenoviral Cre recombinase, and the resulting tumors were followed over time. In addition, conditional alleles of the tumor suppressor \( \text{Trp53} \) were bred into the \( \text{Braf} \) and \( \text{Atg7} \) compound mutant mice to assess the consequences of \( \text{Trp53} \) loss on the need for autophagy to sustain metabolism and tumorigenesis in the mouse lung.

We report here that autophagy is required for growth of \( \text{Braf}^{V600E} \)-driven lung tumors and identify the metabolic alterations underlying autophagy addiction. Moreover, we provide direct evidence that the tumor-suppressive function of autophagy can be explained by reactive oxygen species.
(ROS) mitigation early in tumorigenesis. Most importantly, the dominant tumor-promoting function of autophagy can be explained by preservation of mitochondrial function and the supply of metabolic substrates that confer the stress tolerance likely necessary for sustained tumorigenesis. Thus, autophagy prevents metabolic dysfunction and ensures the health of *Braf*<sup>V600E</sup>-driven lung tumors.

**RESULTS**

**Deletion of Atg7 Blocks Autophagy in *Braf*<sup>V600E</sup>-Driven Lung Cancer**

To examine the role of autophagy in tumor establishment and progression, mice homozygous for a Cre-activatable *Braf<sup>CA</sup>* allele (21) were mated to *Atg7<sup>fl/fl</sup>* mice (22), generating animals in which intranasal administration of adenoviral Cre recombinase simultaneously enabled expression of the oncogenic *Braf*<sup>V600E</sup> variant while deleting the floxed allele of *Atg7* in the mouse lung. The *Braf*<sup>V600E</sup> model was chosen both for its rapid progression (hyperplasia in 2–4 weeks with discrete adenoma formation by 6–8 weeks) and for the capacity to give rise to a spectrum of malignancies when mated to loss- and gain-of-function tumor models. The *Braf*<sup>V600E</sup> mouse lung tumor model yields benign adenomas unless crossed to mice with deletions of the known tumor suppressors Ink4A/Arf or Trp53, at which point the lesions progress to adenocarcinomas (21). Thus, the *Braf*<sup>V600E</sup> mouse lung tumor model enables direct testing of the hypothesis that autophagy functions as a regulator of lung tumorigenesis with the potential to reveal both promotion and inhibition of tumor growth by autophagy.

In this genetically engineered mouse model of lung cancer, all tumors are initiated by the constitutively active *Braf*<sup>V600E</sup> protein (21). *Atg7* deletion and loss of ATG7 protein expression was confirmed by PCR (data not shown) and at early (5 and 7 weeks) and late (20 and 30 weeks) stages of tumorigenesis in the *Braf*<sup>V600E</sup>-derived tumors by immunohistochemistry (IHC; Fig. 1A and Supplementary Fig. S1). *Atg7* deletion was detected in tumor and not normal lung tissues, as adenoviral-Cre infects only a small fraction of lung cells, and only those with activated BRAF<sup>V600E</sup> form tumors and expand the population of *Atg7* wild-type or deleted cells. Thus, assessment of autophagy function in normal lung cells is not addressable in this setting. *Atg7* deletion alone by adenoviral-Cre did not...
cause tumors (Supplementary Fig. S2; ref. 13). All tumors were positive for surfactant protein c, a marker of type II alveolar epithelial cells, confirming tumor initiation in the lung (Fig. 1A). Tumor lysates from BrafV600E+/−; Atg7−/− mice failed to convert LC3-I to LC3-II and accumulated the autophagy substrate p62 (Fig. 1B). Similarly, LC3 puncta were visible in the tumor tissue of BrafV600E+/−; Atg7−/− mice, whereas only diffuse cytoplasmic staining was detected in tumor tissues from the BrafV600E+/−; Atg7−/+ mice (Fig. 1C). Taken together, these data show that Atg7 deletion is sufficient to block autophagy in BrafV600E-driven lung tumors.

**Autophagy Ablation Causes Robust Early Tumor Establishment in the Lung**

Next, we conducted time-course analyses to assess tumor burden over time in mice bearing BrafV600E+/− and either Atg7+/+ or Atg7−/− lung tumors. A marked difference in tumor burden was revealed upon examination of the lung histology. At 5 weeks post-Cre, Atg7-deficient BrafV600E+/− tumors increased tumor burden by nearly 40% in comparison with Atg7+/+ tumors (Fig. 2A–C). This increased early tumorigenesis in the BrafV600E+/−; Atg7−/− mice was also observed in micro-computed tomography (CT) analysis of the mice at 3 and 5 weeks post-Cre (data not shown). Importantly, the BrafV600E+/−; Atg7−/− tumors increased tumor burden by nearly 40% in comparison with BrafV600E−/−, driven lung tumors (P = 0.0017; Fig. 2G). Mice with BrafV600E+/−; Atg7+/+ tumors were the first to succumb, exhibiting a median survival of 16 weeks post-Cre. In comparison, BrafV600E+/−; Atg7−/− tumor-bearing mice exhibited a median survival of 31 weeks. Tumor-specific allelic loss of Atg7 or whole-body allelic loss of the essential autophagy gene Beclin1, which significantly impairs but does not ablate autophagy (23), did not alter survival of mice bearing BrafV600E lung tumors (Fig. 2G and H), suggesting that complete and not partial ablation of autophagy is necessary to suppress tumor growth and increase mouse survival (23). Of note, the median survival of the BrafV600E+/− lung tumor–bearing mice (15.1 weeks) was nearly identical to that of mice bearing BrafV600E−/− tumors (15.8 weeks). Importantly, the accumulation of the autophagy substrate p62 was accelerated in BrafV600E+/−; Atg7−/− tumors in comparison with BrafV600E+/−; Beclin1−/− tumors, consistent with a more profound autophagy defect induced by Atg7 deficiency (data not shown).

**Autophagy Deficiency Alters BrafV600E Tumor Fate from Adenomas to Oncocytomas**

Examination of the histology revealed that BrafV600E+/−; Atg7−/+ tumors were adenomas, as expected, whereas BrafV600E+/−; Atg7−/− tumors were composed of tumor cells with an enlarged, granular cytoplasm characteristic of oncocytomas (Fig. 2D). Oncocytomas are a rare but predominantly benign tumor type characterized by an accumulation of defective mitochondria (12). Indeed, Atg7 deficiency similarly altered progression of KrasG12D-driven NSCLCs to oncocytomas (13).

Consistent with Atg7 deficiency altering tumor fate from adenomas to oncocytomas, the autophagy-deficient tumors accumulated significantly more mitochondria than their Atg7−/-expressing counterparts as measured by IHC for TOM20 (Fig. 2E). Electron microscopy revealed that the abundant mitochondria in the BrafV600E+/−; Atg7−/− tumors were swollen and misshapen in contrast to the normal mitochondria observed in the BrafV600E+/−; Atg7−/+ tumors. Moreover, mitochondria in the BrafV600E+/−; Atg7−/− tumors were poorly functional, measured by markedly decreased enzymatic activity of cytochrome c oxidase, an essential component of the electron transport chain, compared with their Atg7−/+ expressing counterparts (Fig. 2E). Reduced expression of Cox IV was observed in tumor lysates from Atg7-deficient tumors compared with those expressing Atg7, indicating that autophagy ablation results in a functional defect in the mitochondria of these tumors (Figs. 2E and F). Thus, autophagy deficiency is sufficient to alter tumor fate from adenomas to oncocytomas in BrafV600E lung tumors, similarly to those with KrasG12D mutations.

**Atg7 Deficiency Extends Life Span of Mice with BrafV600E-Driven Lung Tumors**

Aggressive tumors, such as those driven by oncogenic HRAS or KRAS, are dependent on autophagy for survival (8, 9, 13, 15). In keeping with these findings and consistent with the conversion of Atg7-deficient tumors to a more benign fate, we observed that complete blockade of autophagy was sufficient to extend and nearly double the survival of mice with BrafV600E, driven lung tumors (P = 0.017; Fig. 2G). Mice with BrafV600E+/−; Atg7+/+ tumors were the first to succumb, exhibiting a median survival of 16 weeks post-Cre. In comparison, BrafV600E+/−; Atg7−/− tumor-bearing mice exhibited a median survival of 31 weeks. Tumor-specific allelic loss of Atg7 or whole-body allelic loss of the essential autophagy gene Beclin1, which significantly impairs but does not ablate autophagy (23), did not alter survival of mice bearing BrafV600E lung tumors (Fig. 2G and H), suggesting that complete and not partial ablation of autophagy is necessary to suppress tumor growth and increase mouse survival (23). Of note, the median survival of the BrafV600E+/− lung tumor–bearing mice (15.1 weeks) was nearly identical to that of mice bearing BrafV600E−/− tumors (15.8 weeks). Importantly, the accumulation of the autophagy substrate p62 was accelerated in BrafV600E+/−; Atg7−/− tumors in comparison with BrafV600E+/−; Beclin1−/− tumors, consistent with a more profound autophagy defect induced by Atg7 deficiency (data not shown).

Increased survival of mice bearing Atg7−/+ compared with Atg7−/−; BrafV600E+/− tumors was accompanied by reduced tumor cell proliferation assessed by nuclear Ki67 staining, and increased p53, p21, and γH2AX levels at 14 weeks post-Cre administration (Fig. 2I and Supplementary Figs. S4 and S5). Comparable numbers of cells in wild-type and Atg7-deficient tumors were positive for senescence-associated β-galactosidase (Supplementary Fig. S6A), eliminating senescence as the mechanism for growth arrest and reduced tumor burden with Atg7 deficiency at 14 weeks. Similarly, no significant changes in active caspase-3 were detected 14 weeks post-Cre administration (Supplementary Fig. S6B).

**p53 Induction in Atg7-Deficient BrafV600E Lung Tumors Limits Tumor Growth**

The induction of p53 and p21 in Atg7-deficient tumors led us to question whether the mechanism by which autophagy deficiency suppressed tumor proliferation was through induction of a p53 response. To test this hypothesis, we deleted Trp53 in BrafV600E lung tumors with and without Atg7 and followed tumorigenesis in the resulting mice.

Deletion of Atg7 in Trp53-deficient, BrafV600E lung tumors caused a loss of ATG7 protein expression (residual ATG7 protein is due to the stromal component of the tumor that is not deleted for Atg7), the accumulation of LC3-I, and the autophagy substrate p62, indicative of autophagy inactivation (Fig. 3A). Ablation of Trp53 did not alter the increased
Figure 2. Atg7 deficiency has distinct consequences for tumor establishment and maintenance in BrafV600E-driven lung tumors and is associated with the emergence of oncocytomas and failure of mitochondrial function. A, representative hematoxylin and eosin (H&E) staining of lung lobes at the indicated times post-Cre. Scale bar, 3mm. Scanned H&E slides of the full time course are provided in Supplementary Fig. S2. B, quantification of tumor burden (2 mice/group, all lobes analyzed) from H&E slides across the indicated time points via MATLAB. Error bars indicate SEM. Scanned H&E slides of the full time course are provided in Supplementary Fig. S2. C, representative low-power images of H&E slides 5, 7, and 14 weeks post-Cre. Scale bar, 100 μm. Higher-magnification images are provided in the inset. Scale bar, 50 μm. D, representative high-magnification images of H&E staining of tumor for the indicated time course. Note the increasing large cytoplasm in the BrafV600E/−Atg7+/− tumors indicative of oncocytoma. Scale bar, 10 μm. E, BrafV600E/−; Atg7−/− tumors have an accumulation of defective mitochondria. Representative images of TOM2O IHC, electron micrographs, and the enzymatic cytochrome c oxidase assay at the indicated time post-Cre. For TOM2O, scale bar, 10 μm. For electron microscopy (EM): N, nucleus; MT, mitochondria. Scale bar, 500 nm. Arrows point to swollen mitochondria in BrafV600E/−; Atg7−/− tumors. For cytochrome c oxidase, arrows point to tumor regions, indicated by dashed line, with defective mitochondria in the BrafV600E/−; Atg7−/− tumors. Scale bar, 100 μm. F, Western blot analysis of Cox IV in tumor lysates of BrafV600E/−; Atg7−/− and BrafV600E/−; Atg7+/− mice 10 weeks post-Cre. Numbers refer to individual mice. A blank lane was intentionally included between the normal lung and tumor lysates. An additional Atg7−/−null tumor lysate was spliced out for aesthetic reasons. G, Kaplan-Meier analysis of overall survival of BrafV600E/−; Atg7−/− and BrafV600E/−; Atg7+/− mice post-Cre. Numbers of animals per group and median survival are indicated. H, Kaplan-Meier analysis of overall survival of BrafV600E/−; Beclin1+/− and BrafV600E/−; Beclin1−/− mice post-Cre. Numbers of animals per group and median survival are indicated. I, representative IHC of Ki67, p53, and p21 from BrafV600E/−; Atg7−/− and BrafV600E/−; Atg7+/− mice 14 weeks post-Cre. Scale bar, 10 μm. Quantification of IHC at 5, 7, and 14 weeks post-Cre is shown below. Images for entire time course are provided in Supplementary Fig. S4.
**Figure 3.** Loss of p53 does not ablate the increased early tumorigenesis or life span extension of mice carrying BrafV600E; Atg7−/− tumors. **A,** Western blot analysis of Atg7 expression, LC3-I to LC3-II conversion, and accumulation of p62 in tumor lysates from Trp53−/−; BrafV600E+/+, Atg7−/−, BrafV600E+/+, Trp53−/−; BrafV600E+/+, Atg7+/−, Trp53−/−; BrafV600E+/+, Atg7+/+, Trp53+/+; BrafV600E+/+, Atg7+/+, and Trp53+/+; BrafV600E+/+, Atg7+/+, tumors at 3 and 5 weeks post-Cre. Numbers refer to individual mice. **B,** representative micro-CT images and 3D reconstructions of Trp53−/−; BrafV600E+/+, Atg7+/+, and Trp53−/−; BrafV600E+/+, Atg7−/− mice at early time points. Numbers refer to individual mice. Quantification of normal lung volume is shown at right. **C,** representative micro-CT images and 3D reconstructions of Trp53−/−; BrafV600E+/+, Atg7+/+, and Trp53−/−; BrafV600E+/+, Atg7+/+, tumors at 7 and 10 weeks post-Cre showing reduced tumor in the Trp53−/−; BrafV600E+/+, Atg7−/− mice at late time points. Numbers refer to individual mice. Quantification of normal lung volume is shown at right. **D,** representative images of Ki67 staining (hematoxylin and eosin [H&E], TOM20 IHC, electron microscopy). **E,** Kaplan-Meier analysis of overall survival of Trp53+/+ and Trp53−/−; BrafV600E+/+, Atg7+/+ and BrafV600E+/+, Atg7−/− mice post-Cre. Numbers of mice per group and median survival are indicated on graph. Note: this study was ended at 22 weeks once the P value was highly significant (P = 0.0003). **F,** representative images of histology [hematoxylin and eosin (H&E), TOM20 IHC, electron micrographs, and the enzymatic cytochrome c oxidase assay at the indicated time post-Cre. For H&E, scale bar, 10 μm. For TOM20, scale bar, 2 μm. For electron microscopy: N, nucleus; MT, mitochondria. Scale bar, 500 nm. Arrows point to swollen mitochondria in Trp53−/−; BrafV600E+/+, Atg7+/+ tumors. For cytochrome c oxidase, arrows point to tumor regions, indicated by dashed lines, with defective mitochondria in the Trp53−/−; BrafV600E+/+, Atg7+/+ tumors. Scale bar, 100 μm.
early tumorigenesis of the Atg7-deficient tumors, shown as decreased normal/healthy lung volume in the quantitation of the micro-CT analysis at 3 and 5 weeks (Fig. 3B), or the eventual reduction in tumor burden, shown as increased normal/healthy lung volume at 10 weeks, compared with Atg7-wild-type tumors (Fig. 3C). As observed in mice bearing Trp53-intact, BrafV600E-activated tumors (Fig. 2I), this phenotype was associated with a proliferative defect shown via reduction in nuclear Ki67 staining (Fig. 3D). Kaplan–Meier analysis showed that autophagy deficiency extended the life span of tumor-bearing mice independent of p53 status. However, the kinetics are different; mice with Trp53-deficient tumors succumb to their tumors more rapidly than those with intact Trp53 (Fig. 3E). Thus, loss of Atg7 causes premature p53 induction in BrafV600E lung tumors that suppresses proliferation and tumor growth. Atg7 deficiency suppresses growth of Trp53-deficient tumors in a statistically significant manner (P = 0.04), revealing a second, Trp53-independent, growth-suppressive mechanism in response to tumor-specific loss of Atg7. However, we note that Trp53 has a major role in suppressing tumor growth and promoting overall survival of mice bearing Atg7-deleted tumors.

Consistent with the emergence of oncocytomas upon Atg7 deletion in the Trp53+/−; BrafV600E/+ lung tumors, these tumors were characterized by the accumulation of defective mitochondria (indicated by increased staining with the mitochondrial marker TOM20), ultrastructural mitochondrial abnormalities, and loss of enzymatic activity for cytochrome c oxidase (Fig. 3F). The fact that Trp53 deficiency did not influence the conversion of BrafV600E-driven adenocarcinomas to oncocytomas induced by loss of Atg7 suggests that autophagy is critical in preventing the accumulation of defective mitochondria and maintaining adenocarcinoma fate. Importantly, despite the early growth advantage provided by Atg7 deficiency, tumor growth was ultimately impaired, indicating that the requirement for autophagy to promote tumorigenesis is dominant. Therefore, autophagy inhibition may be an important therapeutic tool to alter lung tumor progression to a more benign fate. **Atg7 Deficiency Induces Nrf2 Accumulation in BrafV600E Lung Tumors**

Having established opposing tumor growth-suppressing and -promoting roles for autophagy in BrafV600E-driven lung tumors, we turned our attention to identifying the underlying mechanisms. One explanation for the early enhancement of tumor growth upon loss of Atg7 was that increased ROS generated by abnormal mitochondria was responsible. To test this hypothesis, we first examined the levels of the master regulator of antioxidant defense, NRF2, in BrafV600E/wt, Atg7+/− and BrafV600E/+, Atg7−/− tumors. NRF2 is normally ubiquitinated and degraded via the proteasome through its interaction with KEAP1 (24). Autophagy deficiency results in the accumulation of the autophagy substrate p62, which competes with NRF2 for binding sites on KEAP1. Thus, under autophagy-deficient conditions, NRF2 accumulates and translocates to the nucleus where it activates the cellular antioxidant defense program (25–29). Indeed, induction of NRF2 was observed in Atg7-deficient tumors both by IHC examining levels of nuclear NRF2 and by Western blotting of tumor lysates (Fig. 4A and B). Induction of nuclear NRF2 correlated with p62 accumulation in Atg7-deficient tumors as expected (Fig. 4A). However, it was unclear whether this induction was caused by Atg7 deletion as a consequence of the accumulation of p62, or rather resulted from increased ROS production due to failure of mitochondrial quality control that also induces NRF2 (26). To resolve this question, we examined the functional consequences of Nrf2 loss in BrafV600E-driven lung tumorigenesis in the presence or absence of Atg7.

**Nrf2 Deficiency Enhances Early and Impedes Late BrafV600E Lung Tumor Growth**

Mice with Cre-activatable BrafV600E with and without conditional alleles of Atg7 were mated to Nrf2 knockout mice, tumorigenesis was induced in the lung via intranasal administration of adenoviral-Cre recombinase, and the resulting tumors were followed over time. Surprisingly, Nrf2 ablation caused a profound yet comparable increase in early tumor growth independent of autophagy status, indicating that the early tumor growth is driven by loss of antioxidant defense (Fig. 4C). Compound deficiency in both Nrf2 and Atg7 produced no additional increase in early tumor growth compared with loss of either gene alone (Fig. 4C). Stimulation of early tumorigenesis induced by Nrf2 deficiency was associated with activation of the DNA damage response (γ-H2AX positivity) consistent with elevated ROS production and oxidative stress (Fig. 4D). Compound loss of both Nrf2 and Atg7 failed to produce significant increases in the levels of phosphorylated γ-H2AX compared with loss of either alone, suggesting that they function in the same pathway (Fig. 4D). These findings are consistent with increased positivity for the ROS-specific DNA adduct 8-oxo-2′-deoxyguanosine (8-oxo-dG) in tumor sections upon loss of either or both Atg7 and Nrf2 but not in Atg7−/− and Nrf2−/-wild-type tumors at 17 weeks post-Cre administration (Fig. 4D and E).

**Deficiency in Nrf2 Phenocopies Atg7 Loss in Survival Analyses**

Despite the marked promotion of early tumorigenesis, loss of Nrf2 extended survival of mice in a similar manner to what was observed with Atg7 deletion previously: median survival of Nrf2 intact mice was 17.4 weeks compared with 21.6 weeks for the Nrf2-deleted mice (P = 0.003; Figs. 2G and 4F). Critically, there was no further increase in survival when both Atg7 and Nrf2 were deleted (P = 0.61), indicating that Nrf2 ablation phenocopies Atg7 loss and that the two genes are epistatic to one another. Further strengthening this argument is the comparable survival of Nrf2+/−; BrafV600E/wt, Atg7+/− (26.4 weeks) and Nrf2−/−; BrafV600E/wt, Atg7−/− (21.6 weeks) mice (P = 0.55). Thus, loss of either Nrf2 or Atg7 or loss of both genes reduces tumor growth and increases survival as previously reported for Nrf2 deletion in BrafV600E-induced lung tumors (30). Taken together, this suggests that although oxidative stress through loss of antioxidant defense or defective autophagy, or both, stimulates early tumor growth, this early promotion of tumor cell proliferation is not sustained and eventually results in a tumor growth defect.

**Loss of Atg7 Impairs Mitochondrial Metabolism and Survival during Starvation**

We hypothesized that the autophagy-deficient tumors eventually fail to thrive because they evolve into oncocytomas...
Autophagy Promotes BrafV600E-Driven Lung Tumor Growth

**Figure 4.** Loss of Nrf2 phenocopies Atg7 deletion and is associated with increased lifespan. **A**, representative immunohistochemical staining for p62 and Nrf2 in lung tumors of BrafV600E/+; Atg7−/− and BrafV600E/++; Atg7−/− mice at the indicated time post-Cre. Scale bar, 10 μm. Quantification is shown below. Error bars are SEM. **B**, Western blot analysis of Nrf2 accumulation in lysates of tumors harvested from BrafV600E/++; Atg7−/− and BrafV600E/++; Atg7−/− mice 10 weeks post-Cre. Numbers refer to individual animals. A blank lane was intentionally included between the normal lung and tumor lysates. An additional Atg7−/− null tumor lysate was spliced out for aesthetic reasons. **C**, hematoxylin and eosin (H&E) staining of single lung lobes showing dramatic increase in size and number of tumors in Nrf2−/− mice independent of autophagy status 4 weeks post-Cre. Scale bar, 3 mm. Quantification of tumor burden is shown at right. The Nrf2−/− and Nrf2−/− graph is the aggregate of autophagy competent and deficient Braf tumors. These data are plotted individually in the next graph. The difference in tumor burden between the Nrf2−/−; BrafV600E/++; Atg7−/− and Nrf2−/−; BrafV600E/++; Atg7−/− mice is not statistically significant (P = 0.63). The difference in tumor burden between the Nrf2−/−; BrafV600E/++; Atg7−/− and Nrf2−/−; BrafV600E/++; Atg7−/− mice is not statistically significant in this experiment (P = 0.49). Please refer to similar experiments in Figs. 2A-C and 3B and Supplementary Fig. 5. **D**, representative images of H&E and immunohistochemical analysis of γ-H2AX 4 weeks post-Cre. Scale bar, 10 μm. **E**, representative images of 8-oxo-dG 17 weeks post-Cre. Scale bar, 10 μm. **F**, Kaplan-Meier analysis of overall survival of the Nrf2−/−; Braf; Atg7 compound mutant mice. The number of mice per group and median survival are indicated. Note that the increased survival between the Nrf2−/−; BrafV600E/++; Atg7−/− and Nrf2−/−; BrafV600E/++; Atg7−/− mice just misses statistical significance (P = 0.06).
that are metabolically compromised. To test this hypothesis, we generated \textit{Atg7}−/−-wild-type and -deficient \textit{Trp53}−/−; \textit{Braf} \textit{V600E}+/− tumor-derived cell lines (TDCL) and directed our efforts toward the identification of the metabolic defects responsible for the blunted tumorigenesis observed in the autophagy-deficient setting. Western blot analysis confirmed deficiency in \textit{ATG7} in the \textit{Atg7}−/− TDCLs but not in those derived from \textit{Atg7}−/− tumors (Fig. 5A). During starvation in Hanks’ balanced salt solution (HBSS), the autophagy block in the \textit{Atg7}−/− TDCLs manifested as a persistent accumulation of LC3-I that was not processed to LC3-II (Fig. 5B), confirming that \textit{Atg7}−/− TDCLs were autophagy deficient (Fig. 5B).

Deficiency in \textit{Atg7} dramatically impaired both starvation survival and tumor growth in nude mice compared with their \textit{Atg7}−/−-wild-type counterparts (Fig. 5C and D), recapitulating the findings from the genetically engineered mice (Fig. 2G). Consistent with the mitochondrial defects observed with \textit{Atg7} deficiency in tumors in the genetically engineered mice, the autophagy-deficient TDCLs possessed greater numbers of mitochondria and yet had a smaller fraction of functional mitochondria than their wild-type counterparts (Fig. 5E).

Next, we examined the oxygen consumption rates (OCR) of autophagy-competent and -deficient TDCLs maintained in normal growth media (RPMI) or following short-term starvation with HBSS (4 hours) as a first approximation of their metabolic fitness. Basal and starvation-induced OCR levels of the autophagy-deficient TDCLs were significantly lower than the rates measured with the autophagy-competent TDCLs (Fig. 5F). Importantly, the mitochondrial reserve capacity (OCR postinjection of the uncoupler trifluorocarbonylcyanide phenylhydrazone, FCCP) of the autophagy-deficient TDCLs was nearly absent following starvation, lending further support to the hypothesis that autophagy supplies metabolites, which maintain mitochondrial function (Fig. 5F).

We next identified the metabolic component supplied by autophagy that enabled survival during starvation. The addition of exogenous glutamine or to a lesser extent sodium pyruvate, but not glucose, or the ROS scavenger N-acetyl-cysteine (NAC), was sufficient to rescue survival of the \textit{Atg7}−/−-deficient TDCLs in response to starvation (HBSS) in a clonogenic assay (Fig. 5G and Supplementary Fig. S7). This suggests that autophagy deficiency renders TDCLs glutamine-dependent. The inability of NAC to protect \textit{Atg7}−/− TDCLs from starvation-induced death suggests that loss of viability does not result from excess ROS production but rather resulted from metabolic insufficiency (Supplementary Fig. S7). OCR analysis revealed that the autophagy-deficient TDCLs were capable of using glutamine to turn the tricarboxylic acid (TCA) cycle, resulting in an increased OCR compared with glutamine-starved conditions, although they were not as efficient at doing so as their autophagy-competent counterparts (Fig. 5H). Taken together, these data suggest that the failure of the late-stage \textit{Atg7}−/−-deficient tumors to thrive is due to both an impairment of overall mitochondrial function and substrate limitation (particularly glutamine) of the remaining functional mitochondria, which together lead to metabolic crisis that is incompatible with continued tumor growth.

**DISCUSSION**

The role of autophagy in cancer is complex; it can either suppress or promote tumorigenesis depending on cellular context (1,15). Whether these differential effects of autophagy are due to the artificiality of the models examined or occur in human cancer remains to be determined. Factors with the potential to influence the role of autophagy in cancer include tissue type, nature of the driver mutations, the metabolic demands imposed by growth and proliferation, level of inherent metabolic stress, as well as the particular metabolic pathways engaged. It is clear that physiologic, genetic, and in \textit{vivo} approaches are needed to resolve these issues.

To begin to address the context-dependent role of autophagy in cancer, we ablated \textit{Atg7} in the Cre-activatable mouse model of \textit{Braf} \textit{V600E}-driven lung tumorigenesis that normally results in benign tumors (21). We showed that defective autophagy through loss of \textit{Atg7} and the concomitant loss of mitochondrial quality control that increases oxidative stress promotes early tumorigenesis, revealing a potential mechanism by which autophagy can limit tumor growth. Persistent mitochondrial dysfunction, however, eventually overshadows this initial tumor growth advantage, resulting in metabolic impairment, reduction in tumor burden, the emergence of oncocytomas, and an increase in the overall mouse survival.

Several important points emerge from these studies. First, \textit{Atg7} deficiency in the context of \textit{Braf} \textit{V600E}-driven lung tumorigenesis promotes early tumor growth. This initial stimulation of tumor cell proliferation was associated with increased oxidative stress, suggesting that it may be a ROS-mediated event. Similarly, loss of the master regulator of antioxidant defense, \textit{Nrf2}, initially promoted proliferation of \textit{Braf} \textit{V600E}-driven lung tumors, and loss of both \textit{Atg7} and \textit{Nrf2} provided no additional growth advantage. This suggests that oxidative stress from loss of either autophagy or antioxidant defense may stimulate early \textit{Braf} \textit{V600E} lung tumor growth and that \textit{Atg7} and \textit{Nrf2} are genetically epistatic.

It is well established that ROS induces genomic damage and mutations that can promote cancer, and that autophagy deficiency increases ROS levels, mutation rates, and genome instability (6,7). However, these consequences of ROS production are unlikely to be playing a role here, as the growth promotion induced by \textit{Atg7} or \textit{Nrf2} loss is temporary. ROS activates oncogenic signaling pathways, including HIF, \textit{Wnt/β-catenin}, Notch, and extracellular signal-regulated kinase (ERK), and it is more likely that these signaling events are responsible for the transient growth stimulation observed in the \textit{Braf} \textit{V600E} lung tumors deficient for \textit{Atg7} or \textit{Nrf2}. It remains to be seen whether the eventual diminution of tumor burden observed with \textit{Atg7} or \textit{Nrf2} loss corresponds to the progressive deterioration of mitochondrial function and loss of ROS production. We propose that autophagy deficiency results in the accumulation of malfunctioning mitochondria, giving rise to oxidative stress (as is the case with loss of \textit{Nrf2}-mediated antioxidant defense) that initially stimulates the growth of \textit{Braf} \textit{V600E}-driven lung tumors by activating growth-promoting signaling pathways. The potential signaling events activated in this setting remain to be determined, although no difference was observed in the levels of phospho-ERK in
Figure 5. Loss of Atg7 impairs mitochondrial metabolism and survival during starvation. A, Western blot analysis of Atg7 expression in TDCLs isolated 9 weeks post-Cre from Trp53+/−; BrafV600E/+, Atg7+/− or Trp53+/−; BrafV600E/+, Atg7−/− mice. Numbers refer to individual clones. An additional Atg7−/− null tumor lysate was spliced out for aesthetic reasons. B, Western blot analysis of conversion of LC3-I to LC3-II during a 3-day HBSS starvation time course. Numbers refer to individual clones. Two pairs of Atg7−/-wild type and Atg7−/-null derived TDCLs are shown. C, clonogenic assay of autophagy-competent and -deficient TDCLs are shown. D, alligraft growth of Trp53+/−; BrafV600E/+, Atg7−/− and Trp53+/−; BrafV600E/+, Atg7−/− TDCLs. n = 8 tumors from each genotype were monitored. E, FACS analysis of total mitochondrial mass and relative mitochondrial membrane potential of TDCLs. F, Seahorse measurement of basal and reserve OCR of autophagy-competent and -deficient TDCLs under normal growth conditions or following 4 hours of HBSS starvation. G, clonogenic survival of TDCLs incubated with RPMI, HBSS, or HBSS supplemented with exogenous glutamine (2 mmol/L), sodium pyruvate (1 mmol/L), or glucose (4.5 g/L) during 3-day HBSS starvation and 4-day recovery in normal growth media. H, Seahorse measurement of OCR in the TDCLs incubated with HBSS before and after the addition of 2 mmol/L glutamine (Q). Injection timing is indicated by arrow.
Atg7-wild-type and deficient BrafV600E lung tumors (data not shown). Alternatively, insufficient levels of autophagy-supplied substrates, such as glutamine from protein degradation, may impair mitochondrial metabolism, ROS production, and metabolic fitness (Fig. 6).

Second, autophagy is required for sustaining the growth of BrafV600E-driven lung tumors and for dictating their fate. Specifically, autophagy ablation shifts tumor progression from adenomas (Trp53-intact tumors) and adenocarcinomas (Trp53-null tumors) to oncocytomas. These data suggest that altering tumor fate to benign oncocytomas by inhibiting autophagy could be a novel therapeutic approach to BrafV600E-driven lung cancer and may extend to other tumors driven by mitogen-activated protein kinase (MAPK) signaling. Importantly, deficiency in either of the essential autophagy genes Atg7 or Atg5 in RAS-transformed cancer cell lines impairs tumor growth and causes the accumulation of defective mitochondria (8, 9), as seen here for BrafV600E-induced tumors. Thus, the oncocytoma phenotype and defective tumor growth are a consequence of defective autophagy and not related to any autophagy-independent function of Atg7. Although Atg7 deficiency also alters the fate of KrasG12D-driven adenomas and carcinomas to oncocytomas (13), in BrafV600E lung tumors this occurs more rapidly and confers a greater survival advantage, suggesting that MAPK signaling may be a key driver for autophagy dependence. It will be of great interest to test the role of autophagy in other BrafV600E-driven malignancies. It will also be very important to determine whether acute autophagy ablation is sufficient to convert established tumors to oncocytomas.

Production of oncocytic tumors by autophagy ablation was associated with reduced proliferation, indicating that cell-cycle inhibition was the main cause of reduced tumor burden. Enhanced p53 induction caused by Atg7 deficiency contributed to but was not essential for the proliferative arrest in response to Atg7 deficiency, revealing both p53-dependent and -independent mechanisms by which autophagy promotes tumor cell proliferation, as is the case with KrasG12D-driven lung tumors (13). Autophagy deficiency (either ablation or allelic loss) activates the DNA damage response (6, 23). Recently, allelic loss of Beclin1 was shown to activate a p53-mediated block to mammary tumorigenesis (31). Tumors apparently activate autophagy to suppress the p53 response as part of a tumor-promotion mechanism. These findings are consistent with autophagy being of central importance for maintenance of mitochondrial metabolism in RAS-driven tumors (8–10, 13) and extend the generalizability of the phenomenon to include BrafV600E-driven lung tumors and possibly those driven by other oncogenic events (32).

Third, we identify a potential mechanism to explain the eventual growth defect in Atg7-deficient BrafV600E-driven lung tumors. Autophagy was originally identified in yeast as a pathway required for survival to nitrogen starvation (33). Mammals also require autophagy to survive starvation

Figure 6. Role of Atg7 in the growth of BrafV600E-driven lung tumors. See text for details.
Autophagy Promotes BrafV600E-Driven Lung Tumor Growth

**METHODS**

**Animal Husbandry and Infection**

All experiments were carried out in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines. BrafCA/CA mice were bred to Atg7fl/fl (provided by Dr. M. Komatsu, Tokyo Metropolitan Institute of Medical Science) or Beclin1+/− (provided by Dr. Z. Yue, Mount Sinai) to generate BrafCA/CA; Atg7fl/fl or BrafCA/CA; Beclin1+/− mice. Compound Braf Atg7 mice were bred to Trp53fl/fl (The Jackson Laboratory) or Nrf2−/− (Y.W. Kan, University of California San Francisco), generating triple-mutant mice. For adenoviral Cre infection, mice 6 to 8 weeks of age were anesthetized with isoflurane, and high-titer replication defective adenoviral-Cre (4 × 10⁶ pfu/mouse; University of Iowa Gene Vector Core) was administered intranasally as a calcium precipitate. Mice were monitored until fully recovered from the anesthetic before returning to their housing. For allograft studies of the TDCLs, 10° cells were injected bilaterally into the flank of 6-week-old nude mice (Taconic). Tumor size was measured every 2 days. Mice were sacrificed at 14 days postinjection. Tumors were fixed for histology as described below.

**Micro-CT Analysis**

Respiratory-gated, low-resolution CT images of maximally ventilated mice were obtained using a Siemens Inveon PET/CT and the INVEON Acquisition Workplace software. Using INVEON Research Workplace software, reconstructed data were processed through a Gaussian filter and tissue determined to be pulmonary was segmented and its volume was calculated. Three-dimensional (3D) reconstructions of normal lung tissue and transverse sections were generated using Osiris software.

**Antibodies**

The following antibodies were used for Western blotting or immunohistochemical analysis: ATG7 (Sigma; Cat#: A2856), Surfactant C protein (Seven Hills Bioreagents; Cat#: WARAB-SPC), LC3 (Nano Tools; Cat#: LC3-5F10), p62 (antiscera raised against MBP full-length p62 (8) or purchased from Enzo Life Sciences P9480-0100), Ki67 (Abcam; Cat#: ab-15580), p53 (Leica; product code: NCL-p53- CM5P), p21 (BD Biosciences; Cat#: 56431), active caspase-3 (Cell Signaling Technology; Cat#: 9661), γH2AX (Cell Signaling Technology; Cat#: 2577), TOM20 (Santa Cruz Biotechnology; Cat# sc-11415), Cox-IV (Molecular Probes), 8-oxo-G (clone 2E2; Trevigen), phospho-ERK (Cell Signaling Technology; Cat# 4376), NFR2 (Epitomics; Cat #2178), and β-actin (Calbiochem; Cat #C01).
Bodipy Staining

A total of 0.6 \times 10^6 cells were plated on coverslips in 6-well plates and starved for 24 hours in HBSS. After starvation, cells were rinsed twice with PBS before incubation with Bodipy (1 mg/mL) for 15 minutes and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Slides were rinsed in water, mounted with Prolong Antifade Gold, and imaged.

Western Blotting

Tumor pieces were snap-frozen, then ground in liquid nitrogen with mortar and pestle. Material was lysed in radioimmunoprecipitation assay buffer, assessed for protein concentration with the Bio-Rad BCA reagent, and analyzed via SDS-PAGE. For TDCLs, whole-cell lysates were prepared as described previously (5).

Mitochondrial Mass and Potential Measurements

Live TDCLs were colabeled with 25 nmol/L MitoTracker-Red CMXRos (Molecular Probes/Invitrogen) to assess mitochondrial membrane potential (MMP) and 25 nmol/L MitoTracker-Green FM (Molecular Probes/Invitrogen) to measure mitochondrial mass for 30 minutes under normal growth conditions. After labeling, samples were washed twice in growth medium, trypsinized and analyzed by flow cytometry (BD Influx Cell Sorter; BD Biosciences). The mean mitochondrial mass fluorescence is shown. Relative MMP was calculated by dividing the mean MMP fluorescence (red) with the mean of mitochondrial mass fluorescence (green).

OCR Measurement

OCR rates were measured with the Seahorse Biosciences extracellular flux analyzer (XF24) as described previously (8). A total of 5 \times 10^5 cells were seeded per well in normal growth media (RPMI with 10% FBS) and allowed to attach 24 to 30 hours at 38.5°C and 8.5% CO₂ before study. Basal measurements were collected following 4 hours of incubation with either RPMI or HBSS. SRC (maximal respiratory capacity-based respiration rate) and total reserve capacity were measured by injecting the mitochondrial uncoupler FCCP (1.5 μmol/L) and Complex III inhibitor Antimycin (20 μmol/L) from the XF24 ports as indicated. For glutamine utilization experiments, cells were plated as above, incubated with HBSS for 30 minutes, and transferred to the Seahorse for analysis where l-glutamine (2 mmol/L) was injected via the XF24 port. Measurements were collected pre-/post-glutamine addition as indicated.

Electron Microscopy

Tumors were fixed in 2.5% glutaraldehyde/4% paraformaldehyde/8 mmol/L calcium chloride in 0.1 mol/L cacodylate buffer pH 7.4. Samples were stored at 4°C overnight before delivery for further processing. They were postfixed in buffered 1% osmium tetroxide and subsequently dehydrated in a graded series of acetone before embedding in epon resin. Thin (90 nm) sections were cut on a Leica electron microscopy UC6 ultra-microtome. Sectioned grids were stained with saturated solution of uranyl acetate and lead citrate. Images were captured with an AMT XR41 digital camera at 80 kV on a JEOL 1200EX transmission electron microscope as described previously (5).

Clonogenic Survival Assays

A total of 0.07 \times 10^5 TDCLs were seeded in 12-well plates in normal growth media and allowed to attach overnight. The cells were washed twice with PBS before incubation with either normal growth media, HBSS, or HBSS supplemented with 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 4.5 g/L glucose, or 4 mmol/L NAC as indicated for 3 days. Following starvation, cells were allowed to recover in normal growth media for 4 days before methanol fixation and Giemsa staining.

Statistical Analysis

Statistical analyses were conducted with GraphPad Prism version 5.0. Statistical significance was calculated by two-way ANOVA with Bonferroni posttest. Significance in the Kaplan–Meier analyses was calculated using the log-rank test. For quantification of tumor burden from immunohistochemical slides, specimens were digitized with a Trestle MedScan whole slide scanner. An automated image processing protocol was built on MATLAB 2011b. Analysis was conducted on a whole slide map of each specimen to produce tumor area and total tissue area measurements. Tumor and whole tissue masks were created for each slide. The segmentation masks were used for generation of ratios of tumor burden. For quantitation of IHC, a minimum of 200 cells were scored from multiple images for each genotype.

Disclosure of Potential Conflicts of Interest

M. McMahon has received commercial research support from Novartis, Plexxicon, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: A.M. Strohecker, J.Y. Guo, E. White
Development of methodology: A.M. Strohecker, J.Y. Guo, M. McMahon
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Strohecker, R. Mathew, M. McMahon
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.M. Strohecker, G. Karsli-Uzunbas, R. Mathew
Writing, review, and/or revision of the manuscript: A.M. Strohecker, J.Y. Guo, G. Karsli-Uzunbas, M. McMahon, E. White
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.M. Price
IHC of tissue sections: G.J. Chen

Acknowledgments

The authors thank M. Komatsu for the conditional Atg7 mice, Z. Xue for the Bedmi10 mice, and Y.W. Kan for the Nrf2 knockout mice. The authors also thank R. Patel for electron microscopy, N. Campbell for micro-CT, W. Chen for histology quantification, A. Roberts for fluorescence-activated cell sorting (FACS) analysis, the Cancer Institute of New Jersey (CINJ) Tissue Analytic Services staff, P. Chin for technical assistance, and members of the White laboratory for helpful discussions.

Grant Support

This work was supported by grants from the NIH (R37 CA53370, RO1 CA130893, RO1 CA163591, and RCI CA147961 to E. White; and CA131261 to M. McMahon) and a postdoctoral fellowship from New Jersey Commission on Cancer Research (NJCCR) to A.M. Strohecker (09-2406-CRR-EO).

Received July 17, 2013; revised August 16, 2013; accepted August 20, 2013; published OnlineFirst August 21, 2013.

REFERENCES

Autophagy Promotes BrafV600E-Driven Lung Tumor Growth


Autophagy Sustains Mitochondrial Glutamine Metabolism and Growth of \textit{Braf}^{V600E}–Driven Lung Tumors

Anne M. Strohecker, Jessie Yanxiang Guo, Gizem Karsli-Uzunbas, et al.