Atg7 Overcomes Senescence and Promotes Growth of Braf$^{V600E}$-Driven Melanoma

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**ABSTRACT**

Macroautophagy (autophagy hereafter) may promote survival and growth of spontaneous tumors, including melanoma. We utilized a genetically engineered mouse model of melanoma driven by oncogenic BrofV600E and deficiency in the Pten tumor suppressor gene in melanocytes to test the functional consequences of loss of the essential autophagy gene autophagy-related-7, Atg7. Atg7 deficiency prevented melanoma development by BrofV600E and allelic Pten loss, indicating that autophagy is essential for melanomagenesis. Moreover, BrofV600E-mutant, Pten-null, Atg7-deficient melanomas displayed accumulation of autophagy substrates and growth defects, which extended animal survival. Atg7-deleted tumors showed increased oxidative stress and senescence, a known barrier to melanomagenesis. Treatment with the BRAF inhibitor dabrafenib decreased tumor growth and induced senescence that was more pronounced in tumors with Atg7 deficiency. Thus, Atg7 promotes melanoma by limiting oxidative stress and overcoming senescence, and autophagy inhibition may be of therapeutic value by augmenting the antitumor activity of BRAF inhibitors.

**SIGNIFICANCE:** The essential autophagy gene Atg7 promotes development of BrofV600E-mutant, Pten-null melanomas by overcoming senescence, and deleting Atg7 facilitated senescence induction and antitumor activity of BRAF inhibition. This suggests that combinatorial BROFV600E and autophagy inhibition may improve therapeutic outcomes in patients whose tumors have BROFV600E/K mutations, an approach currently being explored in clinical trials. Cancer Discov; 5(4); 1–14. © 2015 AACR. See related commentary by Thorburn and Morgan, p. 353.

**INTRODUCTION**

Malignant melanoma is the most dangerous cancer of the skin and extremely difficult to treat when disease recurs. In 2014, approximately 10,000 melanoma-related deaths were expected to occur in the United States, with many thousands more in other nations (1). Recent advances have improved the treatment landscape of this disease (2–4), the use of which may allow 5-year survival to approach 30% to 40% in the near future. Nonetheless, the majority of patients will die from their disease, making it imperative to build on these recent advances to develop more effective therapeutic strategies.

One of the most important events in the field has been the development of highly potent and specific inhibitors of the MAPK pathway, as nearly half of melanomas harbor a BRAFV600E or BRAFV600K mutation that leads to hyperactivation of the MAPK pathway, which promotes tumor growth and survival (5). The selective BRAFV600E/K inhibitors vemurafenib and dabrafenib lead to significant improvements in clinical response rate, progression-free survival (PFS), and overall survival, compared with chemotherapy in BRAFV600E/K-mutant patients (3, 6). However, clinical resistance develops in nearly all patients, and durable disease remissions are uncommon. Thus, elucidation of critical tumor survival mechanisms that can be coordinately targeted along with BRAF inhibition remains an important area of investigation, with the goal of improving the initial antitumor activity of BRAF inhibitors to prevent the emergence of resistance or to effectively treat disease that is progressing.

Autophagy is one such mechanism that may be abrogated for therapeutic gain in patients with melanoma. Autophagy captures and degrades intracellular proteins and organelles to control their quality and to recycle their components to sustain metabolism in starvation (7). Levels of autophagy are normally low but are profoundly induced by starvation and other stressors. Although autophagy may be important for tumor suppression in some settings, the mechanism remains to be defined. Autophagy suppresses p53 activation and p53-mediated tumor suppression, but the mechanism is not yet known (8). Autophagy also promotes tumorigenesis in the absence of p53. Autophagy is upregulated in and required for the survival of tumor cells in hypoxic tumor regions (9), and autophagy defects cause tumor cells to be greatly sensitized to metabolic stress in vitro independent of p53 status (10, 11). This suggests that the accumulated defective mitochondria and the inability to generate metabolic substrates from recycling create a metabolic liability that compromises tumorigenesis. Neither the role of defective mitochondria nor the critical substrates provided by autophagy are known. Nonetheless, this suggests that autophagy inhibition may be a valuable strategy for cancer therapy. This may be particularly relevant in melanoma, where indications of high basal levels of autophagy in malignant tumors have been reported (12–14) and have been found to predict inferior response to treatment and shorter survival (13). Indeed, concomitant inhibition of autophagy and either MAPK or PI3K/AKT/mTOR signaling has led to promising preclinical results, with increased cell death in both BRAFV600E-driven and BRAF–wild-type melanoma tumors (14–16). However, it is important to examine the loss of functional autophagy...
deficiency is particularly profound in antitumor activity of autophagy will have therapeutic value by promoting senescence to promote growth of melanomas and that inhibiting this suggests that autophagy overcomes the senescence barrier also enhanced in the setting of targeted BRAF inhibition. The mechanism of tumor promotion by autophagy is suppression of senescence that correlated with reduction (21). The mechanism of tumor suppression problematic. Moreover, Some cancers such as those driven by oncogenic KRAS and BRAF upregulate and require autophagy for their growth and survival. It is possible that these tumors, including melanoma, are more aggressive in nature and more dependent on alleviation of metabolic stress for their growth, and, as such, may be most sensitive to disruption of autophagy. Deletion of the essential autophagy genes Atg7 or Atg5 in GEMMs for <italic>Kras<sup>G12D</sup></italic>, and <italic>Braf<sup>V600E</sup></italic>-driven lung and pancreatic cancers causes accumulation of defective mitochondria and protein aggregates, tumor cell growth arrest and cell death, and progression to more benign disease (10, 11, 17–19). The antitumor activity of Atg7 deficiency is particularly profound in <italic>Braf<sup>V600E</sup></italic>-driven lung tumors, where it produces a dramatic lifespan extension accompanying the reduction in tumor burden (11).

Whether autophagy is ubiquitously required for other <italic>Braf<sup>V600E</sup></italic>-driven cancers is not yet clear, although evidence suggests it is important to melanoma tumorigenesis. Deprivation of the amino acid leucine and blockade of adaptive autophagy activation in melanoma xenografts with activated RAS–MEK signaling increase caspase-dependent apoptotic cell death and reduce tumor growth (20). Autophagy is also an important mechanism of the development of resistance to treatment in melanomas that harbor <italic>Braf<sup>V600E</sup></italic> mutations. Patients whose melanoma showed higher levels of autophagosomes after BRAF or BRAF/MEK inhibition experienced lower response rates and shorter PFS, and this autophagy activation in preclinical models was dependent on MAPK-driven expansion of the endoplasmic reticulum (ER) stress response that can be abrogated with combined BRAF and autophagy inhibition (15).

We report here using GEMMs that deletion of Atg7 suppresses the growth and extends survival of mice bearing <italic>Braf<sup>V600E</sup></italic> and <italic>Pten</italic>-null melanomas. This suggests that the antitumor activity of Atg7 deficiency extends to settings of <italic>Pten</italic> deficiency, a common scenario in human melanoma (21). The mechanism of tumor promotion by autophagy is suppression of senescence that correlated with reduction of oxidative stress, a known senescence trigger, which was also enhanced in the setting of targeted BRAF inhibition. This suggests that autophagy overcomes the senescence barrier to promote growth of melanomas and that inhibiting autophagy will have therapeutic value by promoting senescence in these tumors.

RESULTS

Tumor-Specific Atg7 Deficiency Prevents Development of <italic>Braf<sup>V600E</sup></italic>-Driven Melanomas in the Context of <italic>Pten</italic> Heterozygosity

To test the hypothesis that autophagy is required for the growth of spontaneous, <italic>Braf<sup>V600E</sup></italic>-driven melanomas, we employed a GEMM where oncogenic <italic>Braf</italic> is activated by the removal of Lox-P sites with Cre recombinase (22). Expression of the Cre transgene (Tyr-Cre/ERT2) is restricted to melanocytes by both the melanocyte-specific tyrosinase promoter and by the fusion of Cre to the tamoxifen-responsive element that is activated upon topical administration of 4-hydroxytamoxifen (4-HT). Because <italic>Braf<sup>V600E</sup></italic> activation alone is insufficient to cause melanoma in this model, a single floxed allele of the <italic>Pten</italic> tumor suppressor gene was introduced that is coordinately deleted in melanocytes with activation of <italic>Braf<sup>V600E</sup></italic>. Upon topical administration of 4-HT, Cre activates the <italic>Braf<sup>V600E</sup></italic> and deletes the <italic>Pten</italic> alleles specifically in melanocytes, initiating melanomagenesis that gradually progresses over 10 months to melanoma, presumably with spontaneous loss of heterozygosity of the remaining <italic>Pten</italic> allele (ref. 23; Fig. 1). To address the role of autophagy in this setting, floxed alleles of the essential autophagy gene Atg7 (24) were introduced that can be coordinately deleted with <italic>Braf<sup>V600E</sup></italic> activation and heterozygous <italic>Pten</italic> deletion specifically in melanocytes. Adult mice with the genotypes of <italic>Tg[Tyr-Cre/ERT2]<sup>FLOX/FLOX</sup>; Lsl-Braf<sup>V600E/+</sup>; Pten<sup>FLOX/+</sup>; Atg7<sup>FLOX/FLOX</sup>; or Atg7<sup>FLOX/FLOX</sup>; or Atg7<sup>FLOX/FLOX</sup> were administered 4-HT to the skin on the lower back to initiate melanomagenesis in the presence and absence of Atg7, and the mice were monitored for 40 weeks.

As expected in the setting of functional autophagy (<italic>Atg7<sup>+/−</sup></italic>), a subset of the mice gradually developed pigmented lesions, some of which progressed to melanoma tumors, resulting in lethality in 4 of 11 mice (Fig. 1A and B). Heterozygosity for Atg7 (<italic>Atg7<sup>+/−</sup></italic>) had no significant effect on tumorigenesis, consistent with previous results that retention of a single allele of Atg7 is sufficient for functional autophagy (11, 24). In contrast, there were fewer pigmented lesions with no tumors developing in the setting of Atg7 deficiency (<italic>Atg7<sup>−/−</sup></italic>), and mouse survival was identical to wild-type mice (Fig. 1A and B). Thus, Atg7 is required for development of melanomas driven by <italic>Braf<sup>V600E</sup></italic> and <italic>Pten</italic> loss-of-heterozygosity (<italic>Pten<sup>+/−</sup></italic>). This is important as it suggests that autophagy inactivation blocks both initiation of, and gradual progression to, malignant melanoma in a physiologic setting. The near-complete absence of premalignant tissue with Atg7 deficiency made determining the mechanism of tumor suppression problematic. Moreover, the consequence of Atg7 ablation in the context of more aggressive disease was not known.

<italic>Atg7</italic> Facilitates Growth and Lethality of <italic>Braf<sup>V600E</sup></italic>-Driven, <italic>Pten</italic>-Deficient Melanomas

To determine if Atg7 deficiency impedes aggressive melanoma growth, we tested the consequence of Atg7 ablation for the development and progression of <italic>Braf<sup>V600E</sup></italic>-driven, <italic>Pten</italic>-deficient melanomas. The 4-HT was administered to the skin on the lower back of adult mice with the genotypes of <italic>Tg[Tyr-Cre/ERT2]<sup>FLOX/FLOX</sup>; Lsl-Braf<sup>V600E/+</sup>; Pten<sup>FLOX/FLOX</sup>; Atg7<sup>FLOX/FLOX</sup>; or Atg7<sup>FLOX/FLOX</sup> to initiate melanomagenesis in the presence and absence of Atg7, and the mice were monitored for 6 months. As expected, melanomagenesis was greatly accelerated by complete deficiency in <italic>Pten</italic> (<italic>Pten<sup>−/−</sup></italic>) in the context of activation of oncogenic <italic>Braf<sup>V600E</sup></italic> (Fig. 2A–D), which limited survival to less than 4 months (Fig. 2E; ref. 23). The absence of a single allele of Atg7 caused a slight reduction in tumor volume (Fig. 2C), but had no significant effect on overall survival (Fig. 2E),
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**Figure 1.** Tumor-specific Atg7 deletion in a BrafV600E-driven and Pten heterozygous melanoma mouse model slows melanoma development. A, the table shows numbers of 4-HT-induced mice and the breakdown of lesion type by genotype. The bar graph shows the percentage of mice with development of either pigmented lesions or melanoma by genotype during the 10-month postinduction period. Representative photographs of induced pigmented lesions and melanoma tumors on the lower back skin of the mice are shown. B, Kaplan–Meier survival curve of mice with the indicated tumor genotypes. Number of mice per group and P values (log-rank Mantel–Cox test) are indicated.

A

<table>
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<th>Mice with tumors</th>
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<td>2</td>
<td>0</td>
</tr>
<tr>
<td>TyrCre/BrafV600E/+/Atg7/+/Δ</td>
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<td>0</td>
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</tr>
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B

Consistent with partial deficiency in Atg7 retaining autophagy function. Loss of both Atg7 alleles, however, profoundly suppressed tumor growth (Fig. 2A–D). Indeed, Atg7 deficiency in melanomas extended mouse lifespan to a median survival of 14.5 weeks, compared with 10.5 weeks for mice bearing melanomas with Atg7 intact (Fig. 2E). Nonetheless, and in contrast with the setting above with tumors initiated with heterozygous deficiency in Pten (Fig. 1B), Atg7 deficiency did not completely suppress melanomagenesis, and mice ultimately died from their tumors (Fig. 2E). In summary, these findings demonstrate for the first time that tumor-specific ablation of an essential autophagy gene suppresses growth of spontaneous melanomas driven by active BRAF and Pten deficiency, significantly extending mouse survival.

Recent evidence suggests that systemic rather than tumor-specific Atg7 ablation in mice has more potent antitumor...
Figure 2. Tumor-specific Atg7 ablation in BrafV600E-driven and Pten-deficient melanomas impedes tumor growth and extends survival. A, representative photographs of the lower back skin of the mice 6 weeks after 4-HT induction. Mice with TyrCre/PtenΔ/ΔBrav600E+/Atg7Δ/Δ melanomas have greater tumor burden compared with those with Atg7 deletion. The control mice with the genotypes of TyrCre and TyrCre/Atg7Δ/Δ show no tumor burden. B, tumor growth curves of mice with TyrCre/PtenΔ/ΔBrav600E+/Atg7Δ/Δ and TyrCre/PtenΔ/ΔBrav600E+/Atg7Δ/Δ melanomas. Tumors with Atg7 deletion show slower growth. The number of mice per group and P value (two-way ANOVA, Bonferroni posttests) are indicated. C, tumor size comparison. Tumor volumes were measured at week 6. D, tumor weight comparison. In an independent experiment, 8 weeks after 4-HT induction, mice were euthanized and tumor samples were collected for weight measurement. E, Kaplan-Meier survival curve of mice with the indicated genotypes. The number of mice per group and P values (log-rank Mantel-Cox test) are indicated. F, melanomas were induced with 4-HT to generate TyrCre/PtenΔ/ΔBrav600E+/Atg7Δ/Δ tumors. When tumor volumes reached the size of approximately 100 mm³, HCQ (130 mg/kg daily) or vehicle control was administered i.p. Tumor sizes were measured every 4 days. The tumor growth curve is indicated in the left plot with P values (two-way ANOVA, Bonferroni posttests), and the weights of tumor samples collected the day after the last HCQ injection are indicated in the right plot with P value (t-test, unpaired two-tailed).
activity in Kras-driven lung cancers (17). To explore potential antimelanoma activity of systemic autophagy deficiency, pharmacologic inhibition (as opposed to genetic ablation) of autophagy was tested by administration of the lysosomal autophagy inhibitor hydroxychloroquine (HCQ). Administration of HCQ (130 mg/kg daily for 24 days) to the GEMMs with established melanomas driven by active BRAF and Pten deficiency with Atg7 intact profoundly suppressed tumor growth (Fig. 2F). These data indicate that autophagy promotes the growth of aggressive melanomas by a tumor cell-autonomous mechanism and that systemic pharmacologic autophagy inhibition with HCQ has antimelanoma activity without apparent toxicity.

**Atg7-Deficient Melanomas Manifest Autophagy Defects**

To address the functional consequences of Atg7 deficiency and loss of protein and organelle quality control to melanomas, tumors were examined histologically at 6, 9, and 12 weeks after melanoma induction. As the findings were similar throughout tumorigenesis, tumors 9 weeks after induction that are representative of all tumors examined are shown in Fig. 3. In comparison with Atg7 wild-type tumors, Atg7-null tumors contained melanocytes with a greatly expanded cytoplasm and the striking accumulation of the autophagy substrates p62 and LC3 (Fig. 3A). Cells with accumulated p62 and LC3 contained melanin in melanosomes, indicating that they were tumor cells and not stromal in origin (Fig. 3A). Note that the accumulated LC3 in the Atg7-deficient tumors was diffusely localized, consistent with a block of autophagy, and not found in discrete puncta associated with LC3-I to LC3-II conversion and initiation of autophagosome formation (Fig. 3A). As expected, ATG7 protein expression was lost in Atg7-null tumor cells (Fig. 3A). Consistent with these findings, Western blot analysis of tumor tissue demonstrated reduction in ATG7 and accumulation of LC3-I and p62 in Atg7-deleted tumors (Fig. 3B). Note that tumors ablated for Atg7 contain a considerable stromal component that is not deleted for Atg7, explaining the presence of some ATG7 and LC3-II (Fig. 3B). In addition to accumulating autophagy protein substrates, electron microscopy revealed the dramatic accumulation of swollen, abnormal mitochondria that were also apparent in tumor sections stained for the mitochondrial outer membrane marker protein TOM20 (Fig. 3C). Thus, melanomas with Atg7 deletion display failure of both protein and organelle quality control similar to other cancer GEMMs, where accumulation of defective mitochondria and reduced tumor burden have been observed in the setting of Atg7 deficiency (10, 11, 17).

**Atg7 Promotes Melanoma Proliferation and Suppresses Senescence and Fibrosis**

To address the mechanism by which ATG7 promoted melanoma growth, levels of proliferation and senescence were examined in matched wild-type and deficient tumors. There were fewer Ki67-positive melanoma cells in the Atg7-deficient tumors compared with the wild-type throughout the time course, with the representative 9 weeks after melanoma induction data shown (Fig. 4A). None of the autophagy-deficient tumor cells were Ki67 positive as revealed by coimmunofluorescence staining with p62 (Fig. 4B). Thus, loss of autophagy suppresses proliferation of melanoma cells, which likely contributes to a reduction in tumor burden and increased mouse survival.

Reduced proliferation in melanoma is often associated with activation of senescence; therefore, the matched Atg7 wild-type and deficient tumors were assessed for the activity of senescence-associated β-galactosidase (SA-β-gal; ref. 25). Atg7 deficiency increased the level of SA-β-gal activity in tumor tissue, resulting in more SA-β-gal-positive cells in larger patches with a greater level of activity compared with matched wild-type tumors (Fig. 4C–E). Importantly, the pigmented melanosome-containing tumor cells were commonly positive for SA-β-gal in the tumors (Fig. 4D). Increased induction of the senescence-responsive CDK inhibitors p21 and p16 was also apparent in the Atg7-deficient compared with the wild-type tumors (Fig. 4F and G), consistent with inhibition of proliferation and induction of senescence. Thus, ATG7 expression is important for suppressing senescence in spontaneous BrafV600E-driven and Pten-deficient melanomas, explaining why Atg7 deficiency compromises tumorigenesis.

An important consequence of increased senescence can be increased fibrosis (25). We noticed that Atg7-deficient tumors appeared to contain not only greatly enlarged tumor cells but also more extracellular matrix material and possibly nontumor cells indicative of fibrosis. To test if part of the altered histology of Atg7-deficient tumors was due to increased fibrosis, Atg7-deficient and wild-type tumors were stained with Masson’s trichrome stain that visualizes excessive collagen fibers or fibrin-like deposition associated with fibrosis (25). This revealed a dramatic increase in collagen fibers or fibrin-like connective tissue (blue indicator) in the Atg7-deficient tumors compared with the wild-type (Fig. 4H). Thus, BrafV600E-driven and Pten-deficient melanomas require ATG7 to suppress senescence that would otherwise limit tumor growth.

**Atg7 Suppresses Oxidative Stress**

Senescence is triggered by many factors, and given that autophagy deficiency produces elevated oxidative stress and DNA damage (26, 27), which are known triggers of senescence, in other settings, we assessed whether this was occurring at an increased level in Atg7-deficient compared with wild-type melanomas. We first examined levels of the marker for DNA double-strand breaks, γ-H2AX, in tumor tissue. Atg7-deficient melanomas have greatly increased numbers of γ-H2AX–positive tumor cells in comparison with wild-type melanomas (Fig. 5A). Elevated γ-H2AX levels in Atg7-deficient tumors were also apparent when wild-type and deficient tumors were examined by Western blotting (Fig. 5A). Atg7-deficient tumors also had higher levels of active caspase-3, indicative of elevated apoptosis (Fig. 5A). Accumulation of damaged mitochondria in autophagy-deficient cells can result in elevated production of ROS that we hypothesized was the underlying basis for the increased senescence. Although direct measurement of ROS in tumor tissue is problematic, the free radical–induced oxidative lesion marker, 8-Oxo-2′-deoxyguanosine (8-oxo-dG), can be readily measured. There was a striking increase in 8-oxo-dG in Atg7-deficient melanomas in comparison with wild-type melanomas, indicative of elevated oxidative damage and consistent with elevated ROS production (Fig. 5B).
ATG7 Suppresses BRAF Inhibitor–Induced Senescence

With the knowledge that ATG7 combats senescence and promotes growth of BrfV600E-driven melanoma, we hypothesized that ATG7 also opposes senescence induced by pharmacologic BrfV600E inhibition. To test this, we examined matched wild-type and Atg7-deficient tumors growth treated with the BRAFV600E inhibitor dabrafenib and its vehicle control. Melanoma tumors were induced with 4-HT. When tumor volumes reached 300 to 750 mm³, dabrafenib (20 mg/kg daily) and its vehicle control were administered by oral gavage. As expected, BrfV600E inhibition resulted in dramatic tumor volume reduction in both Atg7-null and wild-type tumors. However, tumor reduction was more profound in Atg7-null tumors compared with wild-type tumors (Fig. 6A). This more pronounced reduction in tumor growth was associated with a significantly greater induction of senescence as measured by SA-β-gal in Atg7-null tumors (Fig. 6B). Indeed, BRAF inhibitor–treated mice with Atg7-null tumors additionally demonstrated
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Figure 4. Atg7 promotes melanoma proliferation and suppresses senescence and fibrosis. All histology is from 9 weeks after melanoma induction. A, representative images of Ki67 immunofluorescence staining and quantification. B, representative images of communofluorescence staining of Ki67 and p62 with quantification. p62-positive cells show no staining of Ki67. DAPI, 4,6-diamidino-2-phenylindole. C, representative images of different types of positive SA-β-gal staining. Type I, single-cell–positive stain. Type II, small groups (small patches) of cells show positive stain. Type III, large groups (large patches) of cells show positive stain. Type IV, large groups of cells show strong positive stain (large patches, strong stain). D, image at high magnification shows positive SA-β-gal staining overlapping with melanin, indicating the cells are melanoma cells. E, table shows the percentage of different types of positive SA-β-gal staining in all TyrCre/PtenΔ/ΔBrafV600E/Atg7+/+ and TyrCre/PtenΔ/ΔBrafV600E/Atg7−/− tumor tissue samples at 6, 9, and 12 weeks after melanoma induction. F, representative images of p21 immunofluorescence staining and quantification show augmented positive staining with Atg7−/− deficient melanoma samples. G, representative images of p16 immunofluorescence staining and quantification show increased positive staining with Atg7−/− deficient melanoma samples. H, representative images of Masson’s trichrome stain show more blue connective tissue and tumor cells with larger cytoplasm in Atg7−/− deficient melanoma samples than those that are wild-type. The induced skin sample obtained from TyrCre mouse shows no tumor and serves as a negative control.
Figure 5. Functional autophagy suppresses oxidative stress and DNA damage. A, representative images of γH2AX immunofluorescence staining (top); Western blot of γH2AX and active caspase-3 of Atg7 wild-type and deficient mouse melanoma samples 6, 9, 12, and 15 weeks after induction; Actin serves as a protein loading control (middle). The densitometric ratios from the Western blots of γH2AX and active caspase-3 versus actin with the \( P \) value (t test, unpaired two-tailed) at the indicated times are shown (bottom). The images show more DNA damage response activation and apoptosis induction when Atg7 is deleted. B, representative images of immunofluorescence staining for 8-oxo-dG (left) with quantification (right), and positivity for 8-oxo-dG colocalizes with melanin (brown), indicating that these cells are melanoma cells.

further evidence of increased senescence through induction of p21 and p16 (Fig. 6C). In addition, there was a significant increase in apoptosis as measured by active caspase-3. Although suppression of proliferation was similar to the wild-type as measured by Ki67, and DNA damage response activation as measured by γH2AX was not significantly increased (Fig. 6C), this is possibly due to the timing of the analysis. Taken together, these findings demonstrate that senescence induced by treatment with BRAF inhibition in spontaneous melanomas is further increased by Atg7 deficiency, supporting the hypothesis that ATG7 overcomes senescence. It also must be considered that elimination of senescent cells by the immune system and promotion of apoptosis may also play a role in Atg7-null tumor shrinkage.
**Figure 6.** BRAF inhibition and autophagy gene deletion cooperate to impede melanoma tumor growth. **A,** melanomas were induced with 4-HT to generate TyrCre/PtenΔ/Δ/BrafV600E/Atg7Δ/Δ and TyrCre/PtenΔ/Δ/BrafV600E/Atg7Δ/Δ tumors. When tumors reached the size of 300 to 750 mm³, dabrafenib (20 mg/kg daily) and vehicle control were administered via oral gavage. Tumor sizes were measured and fold changes in tumor volumes were calculated. The tumor growth curve is indicated in the left plot with P values [two-way ANOVA, Bonferroni posttests], and the fold change at the last time point is indicated in the right plot with P value (t test, unpaired two-tailed). Each data point represents the mean tumor volume changes from 10 to 13 mice. 

**B,** representative images of SA-β-gal staining with tumor samples from mice treated with vehicle control or dabrafenib, respectively, for 4 days with samples collected at day 6 (left). Quantification of the images (right) revealed significantly more SA-β-gal staining in Atg7-null tumors treated with dabrafenib compared with vehicle control–treated samples, or with the Atg7 wild-type samples with dabrafenib or vehicle control treatment. P values were calculated by unpaired two-tailed t test. Quantification was based on 5 samples of each group and at least of 3 views of each sample. **C,** representative images of immunofluorescence staining for p21, p16, Ki67, γ-H2AX, and active caspase-3 with quantification and their P values as indicated (right).
DISCUSSION

It is known that autophagy plays a complex role in cancer and can both suppress and promote tumorigenesis (28). In general, it is thought that autophagy is used by cancers to promote tumor survival, with several lines of evidence supporting the role of deregulated autophagy in melanoma (29). Importantly, adult mice with systemic ablation of the Atg7 gene showed destruction of tumor tissues before normal tissues, underscoring the potential therapeutic utility of autophagy inhibition (17). We sought to determine the effects of the genetic ablation of Atg7 in a BrafV600E-driven genetically engineered melanoma model that shares salient features of human disease to determine the role of ATG7 in promoting melanoma survival (23).

Our results demonstrate that Atg7 deficiency impeded the development of BrafV600E-driven and Pten heterozygous melanomas, which extended mouse survival. We also found that Atg7 deficiency in more aggressive BrafV600E-driven and Pten-deficient melanomas produced slower growing and more senescent melanomas in comparison with those that were wild-type for Atg7. Importantly, melanoma-specific deficiency in Atg7 dramatically increased the survival of these mice. These findings provide the proof of concept that ATG7 provides a melanoma-promoting function in the context of a spontaneously arising tumor with its normal microenvironment and in the presence of an intact immune system. It is also significant that this is the first GEMM where defects of autophagy in tumors with TP53 loss, as opposed to p53 loss, result in impaired tumor growth. This is an important observation due to the common frequency of TP53 loss or PI3K pathway activation has been shown to contribute to melanomagenesis in BrafV600E-driven melanoma models (34). Increased activation of this endogenous senescence program was noted in Atg7-deficient melanomas described here. This is consistent with the findings of others examining the deletion of Atg7 in melanocytes using the Cre-loxP system (35). Atg7 deletion suppressed lipodystrophy of LC3 in melanocytes, which then entered into senescent premature growth arrest and accumulated ROS damage, ubiquitinated proteins, and the polyubiquitin binding protein p62. The authors suggested that Atg7-dependent autophagy is dispensable for melanogenesis but necessary for achieving the full proliferative capacity of melanocytes, also consistent with our observation of delayed growth of Atg7-deficient melanomas.

Understanding the role of autophagy in the development of resistance to treatment, as opposed to simply in the process of tumorigenesis, is also critical in the development of novel therapeutics that may target this process. Ma and colleagues (13) reported that patients whose tumors developed resistance to BRAF inhibitors displayed higher levels of autophagosomes, and that treatment of multiple melanoma cell lines with BRAF inhibitors induced autophagy. Coordinate BRAF and autophagy inhibition with chloroquine-related compounds that interfere with lysosome function promotes tumor regression in BRAF inhibitor-resistant xenografts, underscoring that autophagy is a likely mechanism of resistance to BRAF inhibition (15). Thus, autophagy is important for melanoma survival that should be coordinately circumvented in targeted therapy to activated BRAF. The authors reported that this cytoprotective autophagy was activated by the binding of mutant BRAF to the ER stress gatekeeper GRP78, rapidly expanding the ER, followed by GRP78–PERK dissociation and a PERK-dependent ER stress response. These findings represent a different context compared with our examination of Atg7 in tumorigenesis, as they focus on a resistant model. However, these tumorigenic and resistance pathways are likely to be linked. Significant cross-talk is known to exist between the ER response, generation of ROS, and the unfolded protein response that is critical for the pathogenesis of many diseases (36). Expansion of the ER stress response is a recognized consequence of autophagy inhibition (37); however, activation of an uncontrolled ER stress response can also contribute to cell death (38) through increased generation of ROS. Our group and others have reported ROS-dependent cell death in the setting of autophagy inhibition in melanoma and kidney cancer models (14, 16, 39), and ROS is also a potent trigger of senescence. In both developing and resistant tumors, resulting activation of autophagy is potentially linked to a protein and organelle quality control response that is blunted when autophagy is inhibited or ablated, resulting in decreased tumor growth.
The clinical benefit of autophagy inhibition in human patients with cancer is an area of active investigation (40). Recent reports suggest that, indeed, inhibition of autophagy combined with proteasome inhibitors or inhibitors of histone deacetylases or mTOR produced clinical benefit in multiple tumor types (41–43). An intriguing case report documents an improved clinical outcome in a patient with a BRAFV600E-mutant ganglioglioma (44). The patient initially responded to treatment with the BRAFV600E inhibitor vemurafenib, but subsequently developed resistance; chemosensitivity was restored with the addition of the autophagy inhibitor HCQ. BRAF-mutant central nervous system tumor cell lines were found to be autophagy dependent, and coordinate BRAF and autophagy inhibition enhanced tumor cell death. These findings support our conclusions that autophagy, specifically ATG7, plays an important role in tumorigenesis, and also support the conclusions of others that suggest that autophagy inhibition may circumvent an important mechanism of resistance (15).

Whether up-front coordinate BRAF and autophagy inhibition could improve the initial activity of BRAFV600E inhibitors is an important clinical question. In this highly relevant melanoma model with an intact immune system, HCQ had potent antitumor activity as a single agent. This underscores that systemic autophagy inhibition with a currently available pharmacologic agent actively suppresses melanoma in the same setting where genetic ablation of autophagy compromises melanoma growth. It is noteworthy that this approach may have the highest clinical yield in patients whose tumors are most inherently reliant on autophagy (13, 15). This may be particularly applicable in patients whose tumors have PTEN aberrations, given the link between PTEN deficiency, resulting PI3K activation, and increased autophagy in human tumors (45, 46). Because PTEN and PI3K pathway aberrations have been implicated in the early development of resistance to BRAF inhibitors (47–49), it is perhaps this population of patients that would best respond to concomitant BRAF and autophagy inhibition. This question will be explored in current clinical trials, and will be relevant to the design of future trials including newer, more potent autophagy inhibitors that have been reported (50).

In summary, Atg7 promotes the growth of BRAFV600E melanoma and inhibits activation of a senescence program, likely through the elimination of defective mitochondria and limiting oxidative stress. Tumors from melanoma GEMMs with Atg7 deficiency demonstrated defects in autophagy and mitochondrial quality control, consistent with findings in other cancer GEMMs, which indicate that this impaired mitochondrial function may be key to impairing tumor growth. Our observations provide genetic proof-of-concept evidence that autophagy is thus an important program in the survival and proliferation of BRAFV600E-mutant, Pten-deficient melanoma. Abrogation of this pathway in autophagy-dependent tumors for therapeutic gain remains a promising clinical approach.

METHODS

Mice

All animal care and experiments were carried out in compliance with Rutgers University Institutional Animal Care and Use Committee guidelines. Animals were obtained as follows: TyrCreER mice (The Jackson Laboratory; stock #: 013590); Atg7Flox/Flox mice (provided by Dr. M. Komatsu, Tokyo Metropolitan Institute of Medical Science); PtenFlox/Flox mice (The Jackson Laboratory; stock #: 004597); and Brafca/− mice (provided by Dr. Martin McMahon, University of California, San Francisco; ref. 22). Mice were crossbred to generate the following mouse lines: (A), TyrCrePtenFlox/Flox/Braf+/−Atg7ca/ca; (B), PtenFlox/Flox/Braf+/−Atg7ca/ca; (C), TyrCrePtenFlox/Flox/Braf+/−Atg7Flox/Flox; (D), PtenFlox/Flox/Braf−/−Atg7Flox/Flox. Crossbreeding (A) and (B) produces TyrCre/PtenFlox/Flox/Braf−/−Atg7Flox/Flox mice; crossbreeding (A) and (D) produces TyrCre/PtenFlox/Flox/Braf+/−Atg7Flox/Flox mice; and crossbreeding (C) and (D) produces TyrCre/PtenFlox/Flox/Braf−/−Atg7Flox/Flox mice.

When mice reached 6 to 8 weeks of age, depliation was performed by shaving a small region of the back with an electric shaver to produce a 2-cm diameter region above the base of animal tails. A small, defined volume (3 μL) of 4-HT (10 mmol/L, 70% Z-isomer; Sigma) dissolved in ethanol (99%) was placed in the center spot of the shaved skin for 3 consecutive days. Melanoma tumor volumes were measured and calculated with the following formula: volume = π/6 × L × W × H. For tumors that begin as multiple islands, individual volumes for those islands were added together for measurements before the eventual merging of these islands as a single tumor lesion.

Western Blotting

Collected tumor samples were ground in liquid nitrogen, lysed, and probed with antibodies against Atg7 (Sigma-Aldrich; A2856), LC3 (Novus Biologicals; NB600-1384), p62, γH2AX (Cell Signaling Technology; 2577), active caspase-3 (Cell Signaling Technology; 9661), and β-actin (Sigma; A1978).

Immunofluorescence

Freshly collected tumor samples were fixed overnight in 10% formalin solution (Formalde-Fresh; Fisher Scientific) before transfer to 70% ethanol and sectioned. The tumor paraffin slides were deparaffinized, hydrated, and incubated with primary antibody against Atg7 (Sigma-Aldrich; A2856), LC3 (Novus Biologicals; NB300-1384), p62, γH2AX (Cell Signaling Technology; 2577), active caspase-3 (Cell Signaling Technology; 9661), and β-actin (Sigma; A1978).

Electron Microscopy

Small pieces of excised tumor tissue were immersed in fixation buffer (2.5% glutaraldehyde, 4% paraformaldehyde, 8 mmol/L CaCl2, 0.1 mol/L cacodylate, pH 7.4) for 24 hours at 4°C before handover for further processing, and analyzed by a JEOL 1200EX electron microscope.

Senescence-Associated β-Galactosidase Assay

Tumor tissue samples were fixed with 10% formalin solution overnight before transfer to 15% sucrose for 4 hours and finally to 30% sucrose. Frozen tissue sections (6 μm thick) were prepared according to the standard procedures. Senescence β-Galactosidase assay was performed following the manufacturer’s instructions (Senescence β-Galactosidase Staining Kit #9860).

Masson’s Trichrome Stain

Tumor paraffin slides were prepared as mentioned above and subjected to Masson’s trichrome stain according to the manufacturer’s instructions (Trichrome Stain Masson Kit; Sigma-Aldrich; HT15).
Drug Treatment
Mice at the age of 6 to 8 weeks with genotypes of TyrCre/Pten^FloX/FloX/Beaf^+/+Atg7^−/− and TyrCre/Pten^FloX/FloX/Beaf^+/+Atg7^+/+ were induced with 4HT at the small region of the low back, randomized into four groups (two groups per genotype), and placed on respective treatment regimens. Dabrafenib (Chemie Tek) and vehicle control were administered via oral gavage (20 mg/kg daily). Tumor sizes were measured every 3 days. HCQ (Acros Organics) and vehicle control were administered i.p. (130 mg/kg). Tumor sizes were measured every 4 days. The tumor volumes were calculated by the following formula: volume = π/6 × L × W × H. Mice were weighed once per week.

Disclosure of Potential Conflict of Interest
E. White is a consultant/advisory board member for Forma Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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