Autophagy Is Critical for Pancreatic Tumor Growth and Progression in Tumors with p53 Alterations

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ABSTRACT
Pancreatic ductal adenocarcinoma is refractory to available therapies. We have previously shown that these tumors have elevated autophagy and that inhibition of autophagy leads to decreased tumor growth. Using an autochthonous model of pancreatic cancer driven by oncogenic Kras and the stochastic LOH of Trp53, we demonstrate that although genetic ablation of autophagy in the pancreas leads to increased tumor initiation, these premalignant lesions are impaired in their ability to progress to invasive cancer, leading to prolonged survival. In addition, mouse pancreatic cancer cell lines with differing p53 status are all sensitive to pharmacologic and genetic inhibition of autophagy. Finally, a mouse preclinical trial using cohorts of genetically characterized patient-derived xenografts treated with hydroxychloroquine showed responses across the collection of tumors. Together, our data support the critical role of autophagy in pancreatic cancer and show that inhibition of autophagy may have clinical utility in the treatment of these cancers, independent of p53 status.

SIGNIFICANCE: Recently, a mouse model with embryonic homozygous Trp53 deletion showed paradoxical effects of autophagy inhibition. We used a mouse model with Trp53 LOH (similar to human tumors), tumor cell lines, and patient-derived xenografts to show that p53 status does not affect response to autophagy inhibition. These findings have important implications on ongoing clinical trials.
responses in human and mouse PDAC cell lines and tumor models (2). Because of its extensive use in human patients for other indications, multiple clinical trials have opened using the CQ derivative hydroxychloroquine (HCQ) to inhibit autophagy in patients with pancreatic cancer (www.clinicaltrials.gov).

Autophagy is a catabolic mechanism that recycles cellular components, and the level is dynamically controlled to maintain cell function. When cells are under stress such as starvation, autophagy is elevated to redistribute energy to sustain cell survival; however, it could also lead to cell death when the attempts to sustain viability have failed (3). The dual role of autophagy applies to tumorigenesis as it can both serve as a barrier to cancer initiation and promote cancer cell survival; however, it could also lead to cell death when the attempts to sustain viability have failed (3). The dual role of autophagy is elevated to redistribute energy to sustain cell function. When cells are under stress such as starvation, autophagy is elevated to redistribute energy to sustain cell survival; however, it could also lead to cell death when the attempts to sustain viability have failed (3). The dual role of autophagy may play a particularly important role in cancers of tumors possess Atg7 (4). Previous data have also shown that autophagy serves as a barrier to cancer initiation and promotes cancer growth by providing energy for advanced malignancies (4). This dynamic role of autophagy in cancer has been supported using various mouse models. Mosaic deletion of Atg5 predisposes mice to benign liver adenomas that do not progress to malignant tumors (5). Previous data have also shown that autophagy may play a particularly important role in cancers driven by oncogenic Kras (2, 6, 7). Indeed, mice engineered to express oncogenic Kras in the lung with concurrent Atg5 or Atg7 loss develop increased benign lesions (adenomas), but fail to progress to malignancy (8, 9).

Sequence analysis of PDAC has shown that more than 90% of tumors possess KRAS mutations (10), and mouse models have validated the critical role of this oncogene in driving PDAC formation (11, 12) and in tumor maintenance (13). In addition, PDACs demonstrate a mixture of tumor-suppressor genes in all of human cancer, and it is found mutated or lost to constrain tumor progression, including TP53, CDKN2A, and SMAD4 (14). TP53 is one of the most commonly altered genes in all of human cancer, and it is found mutated or lost in the majority (~75%) of PDAC (15). In most cases, these genetic mutations, including in the tumor suppressor gene TP53, are acquired via LOH during tumor development (15). As a result, a conditional Trp53 deletion allele or a Trp53 mutant allele is often crossed to an activated Kras allele to accelerate tumorigenesis in PDAC genetically engineered mouse models (GEMM). This is typically done as a single copy, as mice with homozygous Trp53 deletion have rapidly progressive disease that is nonmetastatic (16), and for these reasons most treatment studies using PDAC GEMMs have focused on heterozygous Trp53 models (17). The heterozygous Trp53 mice develop tumors dependent on the stochastic LOH at the Trp53 locus, analogous to human PDAC (18).

Recently, the role of autophagy in PDAC progression was explored using a GEMM (19). Although loss of Atg5 or Atg7 in the pancreas with an activating Kras mutation completely inhibited progression of premalignant lesions to invasive cancer, in the context of an embryonic homozygous Trp53 deletion in the pancreas, autophagy loss seemed to accelerate the progression to invasive cancer (19). However, because of the nature of the Trp53 homozygous model used, the conclusion generated from this model might not be fully representative of human tumors where Trp53 alterations occur by LOH. In this study, we used a Trp53 heterozygous PDAC GEMM to study how loss of autophagy affects tumor development in a situation where p53 is present before tumor initiation and lost in fully developed tumors, mimicking human PDAC development. We also explored how tumor cell lines with TP53 deletions or mutations responded to autophagy inhibition. Finally, we determined how human patient-derived xenografts responded to autophagy inhibition using HCQ treatment to assess the clinical significance of TP53 genotypes on human tumor response.

RESULTS
To study the function of autophagy in pancreatic cancer development, we generated pancreatic conditional Atg5 knockout mice using a Pdx1 promoter–driven Cre recombinase (Fig. 1A; ref. 20). We first assessed the impact of homozygous deletion of Atg5 (Atg5<sup>L/L</sup>) in the pancreas. Pancreatic deletion of Atg5 was not embryonic lethal and did not cause signs of malignant transformation; however, it caused a cellular disruption in endocrine tissues starting at 12 weeks, leading to a reduction in insulin-producing β-cells, as had been shown previously with Atg7 deletion in that compartment (ref. 21; Supplementary Fig. S1A and S1B). Thus, Atg5<sup>L/L</sup> mice had a reduced long-term survival that was consistent with previous reports (Fig. 1B; ref. 19). Given the relatively restricted phenotype of the Atg5<sup>L/L</sup> mice and the long survival of this cohort, we crossed Atg5<sup>L/L</sup> mice into the PDAC GEMM (Pdx1Cre<sup>+</sup>; lidsKras<sup>G12D</sup>/lslKras<sup>G12D</sup>; Trp53<sup>−/−</sup>) to assess the role of autophagy in PDAC development. To this end, we generated three cohorts with differing Atg5 allelic status: Atg5<sup>−/−</sup>, Atg5<sup>L/L</sup>, and Atg5<sup>L/L</sup>. This GEMM recapitulates the human condition beginning from premalignant pancreatic intraepithelial neoplasia (PanIN) to invasive and malignant PDAC (16, 18). We first compared the overall survival between the groups and found that the Atg5<sup>L/L</sup> cohort had the longest median survival (Fig. 1C). Indeed, there were significantly more long-term survivors (>30 weeks of age) in this group compared with either the Atg5<sup>L/L</sup> or Atg5<sup>L/L</sup> cohorts (P < 0.0005). Interestingly, we observed that approximately 20% of the Atg5<sup>L/L</sup> mice died before 6 weeks of age, which was not seen in the Atg5<sup>L/L</sup> cohort (0%) and infrequently (4%) in the Atg5<sup>L/L</sup> group. Analysis of this small population of Atg5<sup>L/L</sup> mice revealed that they did not die of PDAC but due to acinar destruction as shown by massive acinar to ductal metaplasia, which led to the disruption of most of the exocrine pancreas and left on average less than 10% normal pancreas (Supplementary Fig. S2A and S2B). The majority of the Atg5<sup>L/L</sup> cohort survived past this early insult and when death due to PDAC was assessed, the Atg5<sup>L/L</sup> mice had a significantly prolonged survival compared with the Atg5<sup>L/L</sup> and Atg5<sup>L/L</sup> cohorts (P = 0.0032; Fig. 1D). To investigate the mechanism behind the prolonged survival in the Atg5<sup>L/L</sup> mice, we performed a detailed histologic assessment of pancreata at an early time point (6 weeks) and a later time point (15 weeks) to determine how Atg5 loss affects both tumor initiation and progression. At 6 weeks, there was a marked difference in formation of noninvasive precursor lesions, PanIN, between the cohorts, with the Atg5<sup>L/L</sup> group having on average more than 50 PanINs per mouse compared with the Atg5<sup>L/L</sup> group, which showed only 2 to 5 PanINs per mouse (Fig. 1E). The difference in PanIN formation was further quantified by comparing the fraction of normal pancreas remaining with that containing PanIN and surrounding inflammation (22). The Atg5<sup>L/L</sup> cohort showed 41.7% normal
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**Figure 1.** Loss of autophagy extends PDAC survival. A, pancreatic sections stained by IHC for ATG5 expression, showing Pdx1-driven Cre-mediated deletion of ATG5. Scale bar, 50 μm. B, Kaplan–Meier analysis comparing overall survival of Pdx1-Cre ATG5+/+ (blue) and ATG5−/− (red) mice. Representative sections stained with hematoxylin and eosin (H&E), illustrating the β-cell disruption in the ATG5−/− mice. ATG5+/+ mice are shown as controls. Scale bar, 100 μm. C, Kaplan–Meier analysis comparing overall survival of Trp53fl/fl KrasG12D/+Pdx1Cre−/− ATG5−/− mice with ATG5+/+ (black), ATG5+/− (blue), and ATG5−/− (red). There are significantly more ATG5−/− mice with long-term survival (>30 weeks) than in the ATG5+/− or ATG5+/+ cohorts (P < 0.0005, Fisher exact test). H&E staining of representative Trp53fl/fl KrasG12D/+Pdx1Cre−/− ATG5+/+ mice that died before 6 weeks of each due to pancreatic disruption. Scale bar, 100 μM. D, Kaplan–Meier analysis comparing PDAC-specific survival of ATG5+/+ mice with ATG5−/− (black), ATG5+/− (blue), and ATG5−/− (red) mice with Trp53fl/fl KrasG12D/+Pdx1Cre−/−. The survival of the ATG5+/− cohort was significantly longer than that of the ATG5+/+ cohort (*, P = 0.003, log-rank test). E, quantification of the normal pancreas area of ATG5+/+ and ATG5−/− mice at 6 weeks. H&E staining of representative ATG5+/+ and ATG5−/− pancreata at 6 and 15 weeks. Proportion of mice that developed PDAC (vs. only PanIN) at 15 weeks in ATG5+/+ (n = 10) and ATG5−/− (n = 8) mice. Scale bar, 100 μM. F, pancreatic tumors from indicated genotypes stained with cleaved caspase-3, γH2AX, and Ki67 antibody. The number of cleaved caspase-3 or γH2AX-positive cells per field was counted from five random fields per mouse. Scale bar, 50 μM. G, growth curve comparing proliferation of ATG5+/+ (black, n = 4) and ATG5−/− (red, n = 4) tumor cell lines at both high- and low-serum conditions. Error bars, SD of the results from four cell lines of each genotype. *, P < 0.05 by t test.

Pancreatic parenchyma versus 98.6% in the ATG5+/+ group (P = 0.0015; Fig. 1E). At 15 weeks, the normal pancreatic parenchyma in the ATG5−/− group further decreased to 31.7%, but interestingly only 25% of the mice (2 of 8) had invasive PDAC, whereas 60% of the ATG5+/+ mice (6 of 10) developed PDAC (Fig. 1E). Together, these data indicate that although autophagy deficiency increased PanIN development (tumor initiation), it inhibits PanIN progression to PDAC. IHC for ATG5 and LC3 confirmed that all tumors developed in ATG5−/− mice lacked ATG5 expression and had absent autophagy.

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(Supplementary Fig. S3A and S3B). Interestingly, cell lines derived from two of the deleted tumors reestablished Atg5 expression after a few passages, indicating that rare subclones escaped Pdx1-cre-mediated excision and can grow out in a small fraction of the tumors (Supplementary Fig. S4A). As expected, tumors from all three genotypes show high LOH of the remaining Trp53 allele and did not express p53 protein (Supplementary Fig. S4B). We also assessed tumors from the three groups for the expression of cleaved caspase-3, γH2AX, and Ki67 (Fig. 1F) to measure apoptosis, DNA damage, and proliferation, respectively, and found that there was elevation of both apoptosis and DNA damage as well as a decrease in proliferation in the Atg5L/L group compared with the Atg5+/− or Atg5−/− cohorts (Fig. 1F). Therefore, in the tumors that were able to form in the setting of autophagy loss, there was increased DNA damage and cell death. This is consistent with the fact that cell lines derived from these autophagy-incompetent tumors proliferate at a significantly slower rate than Atg5−/− tumors under both high-serum and low-serum conditions (Fig. 1G).

Together, our data suggest that autophagy is required for proper progression of premalignant lesions to invasive PDAC, and those tumors that do progress are less robust.

Previously, we reported that human PDAC cell lines and tumors have elevated basal autophagy, and CQ treatment, as well as suppression of autophagy using RNAi against critical autophagy genes, could inhibit cell growth in vitro as well as in xenografts. In addition, CQ treatment significantly prolonged survival in the lslKrasG12D; Trp53−/− PDAC GEMM (2). A recent study using a Kras-driven PDAC GEMM with an embryonic Trp53 homozygous deletion showed that either Atg5 or Atg7 deletion promoted tumor progression, thus reducing mouse survival. In their model, CQ treatment seemed to have a modest reduction in lifespan as well. Given the differences between our prior and current data using the Trp53−/− PDAC GEMM, and that reported by Rosenfeldt and colleagues (19) using the Trp53−/− GEMM, we examined the impact of autophagy inhibition in a panel of murine PDAC cell lines with varying Trp53 genotypes (Trp53−/−, Trp53+/-, and p53R172H/+). As the majority of the human clinical trials are using CQ or its derivative, HCQ, we focused on response to CQ given its clinical relevance. As has been reported in the past (18), the wild-type (WT) allele of Trp53 was lost in Trp53−/−, Trp53+/-, and the Trp53R172H/+ lines, as confirmed by PCR (Supplementary Fig. S5A). Western blot analysis showed loss of p53 expression in the Trp53−/− and Trp53+/- lines, whereas Trp53 mutations were detected in the Trp53R172H/+ lines (DNA point mutation confirmed by sequencing; Supplementary Fig. S5B and S5C). All PDAC lines, independent of the Trp53 genotype, showed a significant, dose-dependent reduction in clonogenic growth when treated with CQ (Fig. 2A). We had previously shown that one of the consequences of autophagy inhibition in human PDAC cells was a decrease in oxidative phosphorylation (measured by oxygen consumption; ref. 2). Consistent with these findings, all cell lines, independent of TP53 status, had a significantly reduced baseline oxygen consumption rate (OCR; Fig. 2B). To validate the CQ data, we repeated the clonogenic assays using shRNAs to either ATG5 or ATG7. Suppression of expression of both ATG genes and autophagy inhibition was confirmed by Western blot analysis (Supplementary Fig. S6). Similar to the CQ data, suppression of autophagy via RNAi significantly attenuated clonogenic growth independent of the TP53 genotype (Fig. 2C).

Finally, to model the therapeutic situation that is occurring in ongoing human clinical trials, we performed efficacy trials using 12 individual human patient-derived pancreatic cancer xenografts (PDX) treated with HCQ or saline control. Mice with established pancreatic tumors were treated with HCQ, and tumor growth was compared with the vehicle-treated mice. The overwhelming majority of the PDX lines showed a reduction in tumor volume compared with controls (P < 0.05), with a third of the PDX lines showing more than 20% inhibition of tumor growth compared with the tumors in the vehicle-treated mice (Fig. 3B). All tumors had KRAS mutations (except P410) and TP53 mutations (except JH024; Supplementary Table S1). Interestingly, consistent with previous findings of the role of autophagy in KRAS-mutant cancers (2, 8), the KRAS WT tumor did not seem to have elevated autophagy by transmission electron microscopy (TEM) and did not respond to HCQ treatment (Fig. 3A and B). IHC for LC3 in the treated tumors showed that HCQ increased the LC3 punctate staining in the HCQ-treated samples, consistent with effective autophagy inhibition. In line with the TEM data, the KRAS WT tumor had the lowest amount of basal puncta that did show an increase upon HCQ treatment (Fig. 3C). In addition, the fact that all the TP53-mutant tumors showed varying degrees of response further supports the fact that disruption of the p53 axis does not affect response to antiautophagy therapies. Ki67 and cleaved caspase-3 staining of the three best responders versus the KRAS WT nonresponder was consistent with its effect on tumor volume: HCQ treatment significantly inhibited tumor cell proliferation and increased apoptosis in the responders but had minimal impact on the nonresponder (Fig. 3D and E).

**DISCUSSION**

In this study, we have used multiple orthogonal approaches (autonomous models, cell lines, and human tumor xenografts) to demonstrate that disruption of the p53 axis (a finding observed in 75% of PDAC) has no impact on the efficacy of autophagy inhibition. We used a PDAC GEMM (Pdx1Cre; lslKrasG12D/+; Trp53−/−) in which Trp53 is lost by stochastic LOH as seen in human tumors and determined the role of autophagy in PDAC progression using a conditional Atg5 allele. We found that deletion of Atg5 predisposed mice to premalignant pancreatic lesions as evidenced by the increased occurrence of PanINs. On the other hand, mice with Atg5 deletion were significantly less likely to develop PDAC and therefore had improved survival.

The role of autophagy in tumorigenesis is controversial because there are studies supporting both its being a suppressor and a promoter. Evidence to support autophagy as a tumor suppressor comes from studies in which autophagy genes were deleted in mice. With the exception of Beclin1, in which the heterozygote was used and autophagy was only partially attenuated, these studies have shown that loss of autophagy predisposes mice to benign tumors (4, 23, 24). In contrast, evidence to support autophagy as a tumor promoter comes from studies of advanced tumors, in which blocking autophagy inhibits tumor growth and can synergize with
Figure 2. Autophagy inhibition reduces colony formation and reduces baseline OCR independent of p53 status. A, CQ reduces clonogenic growth (blank bar, 0 μmol/L CQ; 10% dotted bar, 7.5 μmol/L CQ; 25% dotted bar, 15 μmol/L CQ). Error bars, SD of a representative experiment performed in triplicate. *, P < 0.005 by t test. B, CQ reduces baseline OCR in tumor cell lines. A representative OCR plot is shown for one cell line of each genotype. Error bars, SD of triplicate wells. *, P < 0.05 by t test. Bottom, quantification of the reduction in baseline OCRs from cell lines of each indicated Trp53 genotype. Error bars, SD from three experiments. C, clonogenic assay shows that knocking down autophagy reduces colony formation independent of Trp53 genotype. Blank bar, shGFP; 10% dotted bar, shAtg5; 25% dotted bar, shAtg7. Error bars, SD of a representative experiment performed in triplicate. *, P < 0.05 by t test.

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A dual role for autophagy in PDAC development, whereby autophagy loss increases the initiation of tumors, but abrogates the efficient progression to invasive cancer.

Several prior studies have shown that autophagy deletion attenuates malignant tumor formation in lslKrasG12D/+ tumor models (8, 9). However, the role of p53 in this process is complex, with studies reporting results that differ in terms of how tumorigenesis affects autophagy loss with concurrent Trp53 deletion (9, 19). A recent study using a PDAC GEMM showed that the impact of autophagy inhibition differed depending on whether Trp53 was concurrently deleted or not (19). Some of these differences could be due to the models used, or, alternatively, that particular ATG genes may have nonoverlapping...
Figure 3. HCQ treatment selectively suppressed tumor growth and proliferation in PDXs with active baseline autophagy. A, representative TEM photomicrographs of autophagy in PDXs. Arrows, autophagosomes. B, efficacy of HCQ in a panel of 12 individual patient-derived PDXs (n = 8–10 tumors per PDX). C, LC3 staining of PDX tumors treated with vehicle or HCQ. Small black squared area is enlarged in the left corner. Scale bar, 20 μm. Numbers of LC3 puncta per field were quantified (*, P < 0.05, t test). D, representative photomicrographs of Ki67-stained tumor sections in PDXs sensitive or resistant to HCQ treatment. The number of Ki67+ cells was quantified for each line. Five fields were counted from two tumors for each PDX (*, P < 0.05; **, P < 0.01, t test). Scale bar, 50 μm. E, cleaved caspase-3 staining of PDX tumors and positive cells per field were quantified (*, P < 0.05). Scale bar, 50 μm.

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functions that are independent of autophagy. In addition, there are likely differences regarding whether Trp53 is deleted homozygously in the germline, or if one copy is lost by somatic LOH (mimicking the cognate human phenomenon). Our data are consistent with prior reports showing that loss of autophagy can promote the initiation of tumorigenesis in the pancreas, and prevent the progression to cancer in the setting of oncogenic Kras mutations (19). However, unlike the data from Rosenfeldt and colleagues (19), our work shows that Atg5 deletion impairs the progression of premalignant PanIN to invasive PDAC in the setting of Trp53 loss. We believe that this difference stems from the fact that in their study, Trp53 was homozygously deleted during embryogenesis. Therefore, in the physiologic setting of Trp53 loss during
tumor progression, autophagy seems to be required for optimal PDAC development. The intricate and complex relationship of autophagy and p53 is of great importance and awaits further study (26).

Perhaps most relevant to cancer treatment, we showed that acute inhibition of autophagy by CQ treatment or RNAi inhibited growth of murine PDAC cell lines with various Trp53 alterations, and is consistent with our prior data using human PDAC cell lines, which almost all harbor TP53 mutations (2). Finally, we used a large panel of patient-derived PDAC xenografts and performed treatment studies using HCQ. HCQ treatment attenuated the growth of the majority of primary patient-derived PDAC xenografts that harbor TP53 mutations.

Together, our data continue to support the integration of autophagy therapies into the treatment of PDAC. Ongoing human clinical trials in PDAC will determine whether this approach is feasible and effective in patients.

METHODS

Genetically Engineered Mice

Atg5<sup>L/L</sup> mice were kindly provided by Dr. Noboru Mizushima (The University of Tokyo, Tokyo, Japan; ref. 27). Trp53<sup>R172H</sup> mice were obtained from Anton Berns (Netherlands Cancer Institute, Amsterdam, The Netherlands; ref. 28). Pdx1-Cre was obtained from Doug Melton (Harvard University, Boston, MA; ref. 29). All animal experiments were approved by the Institutional Animal Care and Use Committee under protocol 10-055 at the Dana-Farber Cancer Institute (Boston, MA). Mice were maintained on a mixed background. Survival was determined by humane endpoints as specified by the protocol, including showing signs of being moribund, significant weight loss, skin ulceration, or in rare cases being found dead. All mice with PDAC-specific death were histologically confirmed.

Histology

All tissues were fixed in 10% formalin overnight and embedded in paraffin. For IHC, tumors were deparaffinized and rehydrated. After antigen retrieval in citrate buffer (pH 6.0), tumors were labeled with primary antibody overnight and then detected using the VECTASTAIN Elite ABC Kit (pk-6100; Vector Labs) and DAB (sk-4100; Vector labs). Antibodies used for immunohistochemistry are ATG5 (1:200; NB110-53818; Novus Biologicals), cleaved caspase-3 (1:200; D175; Cell Signaling Technology), γH2AX (1:100; clone JBW301; Millipore), and LC3 (1:100, NB1-19167; Novus Biologicals). Ki67 staining was performed using an anti–Ki67 antibody (Ventana Medical Systems; clone K2; 1:100 dilutions) as previously described (29). Sections from two tumors per treatment group were examined microscopically, and more than five representative fields from each slide were photographed under &times;200 magnifications (except LC3, which was taken at &times;1,000 magnification; ref. 30).

Cell Culture

All cell lines were derived from mouse primary tumors and grown in DMEM (11965; Invitrogen) with 10% FBS and 1% Penstrep. The Trp53<sup>R172H</sup> and Trp53<sup>L/L</sup> lines were obtained from Dr. H. Ying (The University of Texas MD Anderson Cancer Center, Houston, TX) and Dr. D. Tuveson (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). Cells were regenotyped to verify p53 status, and Western blot analyses were used to confirm p53 protein expression. Trp53-mutant cell lines were sequenced to verify the presence of the mutation. Atg5<sup>L/L</sup> lines were harvested from an approximately 5-mm<sup>2</sup> chunk of tumor, minced, and digested in 4% collagenase/dispace for 1 hour. Cell lines were routinely tested and all were negative for mycoplasma infection.

Clonogenic Assay

Cells were plated in 6-cm dishes at 500 cells per dish in growth medium with 10% FBS and treated with CQ the day after seeding. After 7 days, cells were fixed in 80% methanol and stained with 0.2% crystal violet, and colonies were counted. The surviving fraction was calculated using the plating efficiency.

Growth Curves

Cells were plated in 24-well plates at 3,000 cells per well in 1 mL of media. Media were not changed throughout the course of the experiment. At the indicated time points, cells were fixed in 10% formalin and stained with 0.1% crystal violet. Dye was extracted with 10% acetic acid, and the relative proliferation was determined by attenuation (D) at 595 nm.

OCR

Oxygen consumption measurements: 1.5 × 10<sup>5</sup> cells were seeded in a 96-well Seahorse plate, and OCRs were measured using the Seahorse XF96 Instrument (Seahorse Biosciences). Basal mitochondrial respiration (3 mmol/L glucose) and ATP production (2 mmol/L oligomycin) were measured. Maximal respiration was obtained by carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 0.5 mmol/L), and non-mitochondrial OCRs were obtained by adding 2 mmol/L antimycin A. Values were normalized by protein concentration to account for cell number.

Western Blot Analysis

Proteins were extracted by RIPA buffer and separated on 4% to 12% stacking SDS–PAGE gel. Proteins were then transferred to polyvinylidenefluoride membrane (Bio-Rad). Membranes were blocked with 5% nonfat dry milk and then incubated with the primary antibody overnight at 4°C. Following Tris Buffered Saline with Tween 20 (TBST) washing, membranes were incubated with peroxidase-conjugated secondary antibody for 1 hour and exposed on film using the Enhanced Chemiluminescence (ECL) Detection System (Thermo Scientific). Antibodies used were as follows: ATG5 (1:500; NB110-53818; Novus Biologicals), ATG7 (1:300; A2856; Sigma), LC3B (1:500; NB600-1384; Novus Biologicals), p53 (1:1,000; FL-393; Santa Cruz Biotechnology), and β-actin (1:3,000; A2066; Sigma).

Lentivirus-Mediated shRNAs

All shRNA vectors were obtained from the RNA Interference Screening Facility of Dana-Farber Cancer Institute. Atg5 (TRCN0000037819) and Atg7 (TRCN0000092163) shGFP: forward 5', CCGGCCGCAAGCTGACTCTAGGATGAACTTCA GGTCAGCTTCTCGTTT, reverse 5', AATTTAAAGCAAGCTTGAGC CCGTGAAGTTCATCTCTTGAATGAACTCAGGGTCAGCTTGCG (31). Lentivirus was produced using 293T cells, as previously described (2).

Electron Microscopy

Freshly harvested subcutaneous tumors from mice were fixed immediately with 3% paraformaldehyde, 1.5% glutaraldehyde, and 2.5% sucrose in 0.1 mol/L sodium cacodylate and cut into 1-mm<sup>3</sup> squares. After postfixation in 1% osmium tetroxide for 1 hour on ice and dehydration, the samples were embedded in a mixture of Epon–araldite. Thin sections from four blocks were collected on uncoated grids, stained with uranyl and lead citrate. Samples were sectioned and examined using an FEI Tecnai 12 Transmission Electron Microscope equipped with a 16-bit 2K × 2K FEI Eagle bottom-mount camera and an SIS MegaView III wide-angle camera. Images were captured at &times;12,000 magnification (32).
In Vivo Efficacy of HCQ in Human PDXs

Animal experiments were conducted following approval and in accordance with the Institutional Animal Care and Use Committee guidelines of the Johns Hopkins University under protocol M006M335. A total of 12 human PDXs established from the primary tumors, resected from patients with pancreatic cancer at the Johns Hopkins Hospital, were used for the study (33). The mutational status of these tumors was previously reported (14) and is shown as Supplementary Table S1. Fresh tumors resected from mice were cut into cubes of 2 mm^3, and were subcutaneously implanted on both flanks of 6-week-old female nude/nu athymic mice (Harlan). When cohorts of tumors reached approximately 150 mm^3, animals (5 mice per group, each group with 8–10 tumors) were randomly assigned to receive vehicle or HCQ (60 mg/kg, i.p., once daily for 4 weeks) treatments (2). Tumors were measured twice per week, and tumor volumes were calculated using the following formula: \( V = \frac{a \times b^2}{2} \), where \( a \) is the largest dimension and \( b \) the smallest. Tumor growth in HCQ-treated animals was compared with that in vehicle-treated mice.

**Statistical Analysis**

Overall survival events included death as defined by the protocol with censoring for alive at last follow-up. Events for PDAC-specific survival included deaths attributable to PDAC with uninformative censoring for deaths related to other causes or at last follow-up. Survival plots were generated using the Kaplan–Meier method. The log-rank test was used to compare survival distributions between groups. The proportion of mice alive at 30 weeks or longer in the Atg5-/- group was compared with the other two groups using a Fisher exact test. Statistical analyses were performed using R version 3.0.2 (34).

**Disclosure of Potential Conflicts of Interest**

A.C. Kimmelman has received honoraria from the speakers’ bureau of US Oncology and is a consultant/advisory board member for Forma Therapeutics and Gilead. No potential conflicts of interest were disclosed by the other authors.

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