Autophagy is Essential to Suppress Cell Stress and to Allow BCR-Abl-Mediated Leukemogenesis

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Abstract

Hematopoietic cells normally require cell extrinsic signals to maintain metabolism and survival. In contrast, cancer cells can express constitutively active oncogenic kinases such as BCR-Abl that promote these processes independent of extrinsic growth factors. When cells receive insufficient growth signals or when oncogenic kinases are inhibited, glucose metabolism decreases and the self-digestive process of autophagy is elevated to degrade bulk cytoplasm and organelles. While autophagy has been proposed to provide a cell-intrinsic nutrient supply for mitochondrial oxidative metabolism and to maintain cellular homeostasis through degradation of damaged organelles or protein aggregates, its acute role in growth factor deprivation or inhibition of oncogenic kinases remains poorly understood. We therefore developed a growth factor-dependent hematopoietic cell culture model in which autophagy can be acutely disrupted through conditional Cre-mediated excision of the autophagy-essential gene Atg3. Treated cells rapidly lost their ability to perform autophagy and underwent cell cycle arrest and apoptosis. While Atg3 was essential for optimal upregulation of mitochondrial oxidative pathways in growth factor withdrawal, this metabolic contribution of autophagy did not appear critical for cell survival, as provision of exogenous pyruvate or lipids could not completely rescue Atg3-deficiency. Instead, autophagy suppressed a stress response that otherwise led to p53 phosphorylation and upregulation of p21 and the pro-apoptotic Bcl-2 family protein Puma. Importantly, BCR-Abl-expressing cells had low basal levels of autophagy but were highly dependent on this process, and rapidly underwent apoptosis upon disruption of autophagy through Atg3 deletion or treatment with chemical autophagy inhibitors. This dependence on autophagy extended *in vivo,* as Atg3 deletion also

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Online Supplemental Material

Online supplemental material includes supplemental figures S1-S9, accompanying figure legends, and supplemental methods on A3C cell transduction and treatment, immunoblots, and mouse studies.

Competing Interests

The authors declare no competing financial interests.

prevented BCR-Abl-mediated leukemogenesis in a cell transfer model. Together these data demonstrate a critical role for autophagy to mitigate cell stress, and that cells expressing the oncogenic kinase BCR-Abl appear particularly dependent on autophagy for cell survival and leukemogenesis.

Keywords

Autophagy; BCR-Abl; Puma; apoptosis; metabolism; p53

INTRODUCTION

Normal metazoan cells require growth factors to sustain metabolism, maintain cellular homeostasis, and prevent cell death (Raff 1992, Rathmell et al 2000). In contrast, cancer cells can express oncogenic kinases to mimic growth factors and render cells independent of extrinsic signals (DeBerardinis et al 2008, Hanahan and Weinberg 2000). Insufficient access to or inhibition of these signals can lead cellular atrophy with decreased nutrient uptake and activation of the self-digestive process of autophagy (Altman and Rathmell 2009, Lum et al 2005a, Plas et al 2001, Rathmell et al 2000, Wieman et al 2007). Autophagy likely plays a key role in this stress response (Altman et al 2009, Colell et al 2007), but the acute role of autophagy in cells deprived of growth signals and how inhibition of autophagy may influence targeted cancer therapies, however, remains largely uncertain.

Autophagy may influence cells deprived growth signals through several pathways. Autophagosomes may specifically target damaged mitochondria (Colell et al 2007, Kim et al 2007), peroxisomes (Iwata et al 2006), or protein aggregates (Pankiv et al 2007) to maintain intracellular homeostasis of organelles and proteins. Indeed, protein aggregates can lead to DNA damage and genomic instability (Mathew et al 2007, Mathew et al 2009), and accumulation of damaged mitochondria can promote apoptosis (Mathew et al 2009). Autophagy may also provide nutrients through lysosomal degradation of intracellular components for mitochondrial oxidation (Altman et al 2009, Lum et al 2005b, Mathew et al 2007, Singh et al 2009). Under cases of prolonged growth factor deprivation when apoptosis is suppressed, this metabolic contribution appears critical to sustain cell viability (Lum et al 2005a).

Given its potential function in metabolism and cell survival, manipulation of autophagy may provide a critical means to eliminate cancer cells. Heterozygosity for the autophagy-essential gene *Beclin-1* led to greatly increased rates of tumorigenesis (Liang et al 1999, Yue et al 2003), possibly due to genomic instability when autophagy was reduced (Mathew et al 2007). Conversely, autophagy has also been shown to promote survival of cancer cells following stress or nutrient deprivation (Degenhardt et al 2006, Jin et al 2007, Karantza-Wadsworth et al 2007, Lum et al 2005a). A promising setting for inhibition of autophagy may be in treatment of cancers driven by oncogenic kinases that mimic growth signals, such as BCR-Abl, where inhibition of autophagy may enhance kinase inhibitor-induced apoptosis (Bellodi et al 2009, Carew et al 2007, Kamitsuji et al 2008). Nevertheless, the precise role

for autophagy and how inhibition of this process may impact BCR-Abl[+] leukemia is uncertain.

To directly address the role of autophagy in control of cell metabolism and viability, we have generated a model for acute genetic inhibition of the autophagy pathway using conditional deletion of the autophagy-essential gene Atg3. Cre-mediated Atg3 excision led to disruption of autophagy, cell cycle arrest, and eventual apoptotic cell death, both in the presence and absence of growth factor. We observed specific metabolic contributions from autophagy, but these nutrients did not appear critical for cell survival. Rather, disruption of autophagy led to p53 pathway activation with cell cycle arrest, Puma induction, and apoptosis. Interestingly, cells expressing oncogenic BCR-Abl had low levels of basal autophagy but were highly sensitive to autophagy disruption and failed to generate leukemia upon Atg3 deletion *in vivo*. Together, these data directly demonstrate that inhibition of autophagy can enhance cell stress to promote cell death and prevent BCR-Abl-driven leukemogenesis.

RESULTS

Generation of a hematopoietic cell model for conditional Atg3 deletion

We developed a model for conditional deletion of the autophagy-essential gene Atg3 to examine the role of autophagy. Hematopoietic precursor cells from Atg3[F/F] Cre-ER (A3C) mice were isolated and infected with Hox11-expressing virus to suppress cell differentiation (Figure 1A) (Zinkel et al 2005). This mixed population of hematopoietic progenitor cells could be expanded in IL3 and Atg3 was readily excised when treated with 4-hydroxytamoxifen (4OHT) to activate Cre recombinase (Figure 1B). 4OHT did not cause Atg3 loss (Supp. Figure S1A) or cytotoxicity in cells lacking Cre-ER (Supp. Figure S1B), and activation of Cre by 4OHT in cells with wild-type Atg3 did not result in loss of viability or growth (Supp. Figure S1C-D). To ensure Cre-mediated DNA excision did not elicit a DNA damage response, bone marrow from control and IL7R[F/F] Cre-ER mice (Jacobs et al 2010) was harvested and treated with either 4OHT to delete IL7R, a gene not expressed in hematopoietic precursor cells, or etoposide to directly cause DNA damage. γH2A.X, a marker of DNA double-strand breaks (Kurz and Lees-Miller 2004), was observed after etoposide treatment, but not 4OHT treatment (Supp. Figure S1E).

To examine the role of autophagy in cell metabolism and apoptosis, A3C cells were transduced to stably overexpress the glucose transporter Glut1, the anti-apoptotic protein Bcl-2, or a truncated and non-functional human nerve growth factor receptor (NGF) marker as a control (Supp. Figure S2A). Cell surface Glut1 was observed by flow cytometric staining (Supp. Figure S2B) (Wieman et al 2007). In each cell population, 4OHT treatment resulted in decreased Atg3 expression (Supp. Figure S2C). Functionally, Atg3 deletion disrupted autophagy, as 4OHT treatment impaired accumulation of the autophagy markers and Atg3 targets LC3-II, GABARAP, and Gate-16, and increased accumulation of p62, a protein normally degraded by autophagy (Bjorkoy et al 2005) (Figure 1C). Because autophagy leads to lysosomal degradation of autophagosomes and total levels of LC3-II, GABARAP, Gate-16, and p62 reflect both processing and degradation, cells were treated with chloroquine to block lysosomal proteolysis (Figure 1D). In each case, Atg3 excision

prevented protein processing. A3C cells were also analyzed in the presence or absence of 4OHT by high throughput image analysis to quantitate GFP-LC3 punctae (Figure 1E). 4OHT-treated cells had fewer GFP-LC3 punctae, indicating that Atg3-deletion disrupted basal levels of autophagy. Together, these data show that 4OHT and Cre recombination are not intrinsically cytotoxic and that Atg3 can be efficiently excised in the A3C cell population leading to acute genetic blockade of autophagy.

Atg3 deletion leads to cell cycle arrest and enhanced death

The establishment of A3C cells allowed direct examination of the role of autophagy on cell growth and survival. Atg3 was essential for cell accumulation over time in culture with IL3 (Figure 2A). This was due in part to cell cycle arrest, as expression of Bcl-2 to suppress apoptosis did not prevent cell cycle arrest and Bcl-2-expressing A3C cells did not efficiently incorporate BrdU (Figure 2B) and arrested in G1 or G2 of the cell cycle (Figure 2C) after Atg3-deletion. Growth factor deprivation leads to atrophy and decreased cell metabolism through increased proteolysis and reduced nutrient uptake (Edinger and Thompson 2002, Rathmell et al 2000, Wieman et al 2007), and Atg3 deletion partially maintained cell size of apoptosis-resistant Bcl-2 expressing A3C cells deprived IL3 (Figure 3A). Importantly, deletion of Atg3 led to increased cell death in both the presence and absence of IL3, but this death was partially inhibited by expression of Bcl-2, which allowed extended survival in the absence of both Atg3 and IL3 (Figure 3B). These data suggested a role for apoptosis and Bcl-2 family proteins in the death of IL3 deprived Atg3-deficient cells. Indeed, IL3 withdrawal led to loss of Mcl-1 and induction of the pro-apoptotic proteins Puma and Bim, and this was enhanced in Atg3-deficient cells (Figure 3C). Further supporting apoptosis as the mechanism of cell death, deletion of Atg3 also resulted in accumulation of cells with activated Bax and sub-diploid DNA content (Supp. Figure S3A-B).

Autophagy supports but does not appear essential for metabolism of acutely growth factor-deprived cells

Pharmacologic inhibition or chronic genetic deletion experiments have shown that lipid oxidation of nutrients made available through autophagic degradation of intracellular components (Altman and Rathmell 2009, Singh et al 2009) can support the survival of metabolically stressed cells (Altman et al 2009, Degenhardt et al 2006). Indeed, Atg3-deficient cells were more sensitive to cell death upon treatment with the glycolytic inhibitor 2-deoxyglucose (2DG) than autophagy-competent cells (Supp. Figure S4).

Autophagy may also support cell metabolism upon decreased nutrient uptake following growth factor deprivation (Lum et al 2005a). IL3 deprivation decreased glycolytic flux (Figure 4A), demonstrating that growth factors mediate control of glycolytic metabolism in A3C cells. To directly establish the contribution of autophagy to cell metabolism in this isogenic system, we undertook mass spectrometry-based metabolomic analyses of A3C cells that were either treated or untreated with 4OHT to delete Atg3 and cultured in the presence or absence of IL3. Consistent with growth factor regulation of glycolytic rate, IL3 deprivation reduced levels of intracellular lactate and organic acids derived from glucose metabolism and the tri-carboxylic acid (TCA) cycle regardless of Atg3 status (Figure 4B and Supp. Figure S5A). Acyl-carnitines, which are indicative of fuels available for

mitochondrial oxidation and are derived from fatty acids that may be produced from the breakdown of membranes, were increased upon IL3 deprivation in an Atg3-dependent manner (Altman et al 2009, An et al 2004) (Figure 4C). Intracellular levels of some amino acids also increased in IL3 deprived cells that expressed Atg3 but not in Atg3-deficient cells (Supp. Figure S5B-D).

Provision of nutrients may mediate the survival benefits of autophagy in growth factor deprivation, and addition of exogenous nutrients may replace autophagy to support the viability of growth factor-deprived cells. Control and Bcl-2 expressing cells were cultured in the presence or absence of 4OHT, withdrawn from IL3, and provided methyl-pyruvate, a cell permeable form of the end-glycolytic product pyruvate, a 1:1 mixture of the long-chain fatty acids oleate and palmitate (OP), or a combination of these nutrients (Figure 4D,E). No condition rescued Atg3-deficient cells from enhanced death after growth factor withdrawal. Neither methyl-pyruvate nor OP significantly altered cellular ATP levels (Supp. Figure 6A), suggesting that autophagy deficient cells were not under strong metabolic stress or were unable to efficiently uptake and utilize these nutrients. To ensure nutrient access, the glucose transporter Glut1 was expressed to enhance and maintain glucose uptake even after removal of IL3 (Zhao et al 2007). While Glut1 expression itself did not affect cell death of Atg3-deleted cells, provision of both methyl-pyruvate and OP modestly increased survival –IL3 in both control and 4OHT treated Glut1-expressing cells (Figure 4F). The inability to fully rescue survival suggested a lack of metabolic stress; similarly, the metabolic stress protein phospho-AMP kinase (AMPK), its downstream target phospho-acetyl-CoA carboxylase (ACC), as well as phospho-S6 were unchanged following Atg3 deletion (Supp. Figure S6B, C). Together, these data suggest that autophagy-derived nutrients are essential in response to direct metabolic stress, but not in the initial days after growth signal deprivation.

Disruption of autophagy leads to induction of pro-apoptotic proteins and p53 activation

In addition to metabolism, autophagy has been implicated in the homeostasis of many pathways and organelles, including the mitochondria and endoplasmic reticulum (Bernales et al 2006, Hoyer-Hansen and Jaattela 2007, Kim et al 2007). Acute disruption of autophagy led to a small increase in induction of the ER stress marker Chop, though this was modest compared to treatment with the ER-stress-inducing compound Tunicamycin, and Atg3 deletion led to no increase in the markers BiP or Calnexin (Figure 5A). Autophagy disruption may also lead to mitochondrial dysfunction and ROS stress (Mathew et al 2009) to activate the p53 pathway (Karawajew et al 2005, Liu et al 2008, Niizuma et al 2009). Indeed, p53 was strongly phosphorylated on serine 18 (equivalent of human S15), and total p53 protein accumulated after Atg3 deletion (Figure 5B). In addition to induction of Puma (Figure 3C), the p53 target gene p21 was induced after Atg3 deletion. Interestingly, γH2A.X, a marker of DNA double-strand breaks that may be upstream of p53 activation (Kurz and Lees-Miller 2004), was also increased by disruption of autophagy, particularly after grown factor withdrawal.

While appearance of γH2A.X suggested DNA damage, it was unclear why this followed p53 phosphorylation and induction of the cell-cycle arrest protein p21 (Figure 5B). Temporal differences in detection of γH2A.X and markers of the DNA damage response may account

for this discrepancy, but γH2A.X may also be induced independent of DNA damage. Consistent with this latter possibility, IL3 withdrawal of the IL3-dependent cell line FL5.12 cells led to accumulation of γH2A.X but not p21 (Figure 5C), indicating that γH2A.X may be a general marker of cell stress or apoptosis. These data suggest that disruption of autophagy in A3C cells may cause growth arrest and eventual death through p53 phosphorylation, activation, and induction of pro-apoptotic target genes including Puma.

Autophagy is critical to inhibit p53 in p185 BCR-Abl-transformed A3C cells

Oncogenic kinases such as BCR-Abl mimic growth factor signals and are the targets of selective kinase inhibitors in cancer therapy (Bellodi et al 2009, Kamitsuji et al 2008). To examine the role of autophagy in cells expressing an oncogenic kinase, p185 BCR-Abl was stably expressed in A3C cells alone or with Glut1 (Supp. Figure S7A,B). Efficient Atg3 excision was observed in the BCR-Abl and BCR-Abl/Glut1-expressing cells (Supp. Figure S7C) and autophagy was suppressed, indicated by accumulation of p62 (Supp. Figure S7C) and decreased LC3-II (Supp. Figure S7D). Interestingly, BCR-Abl-expressing cells had less basal LC3-II than control cells (Supp. Figure S7D), suggesting lower levels of basal autophagy. Consistent with a survival role for autophagy in BCR-Abl-expressing cells, imatinib treatment blocked BCR-Abl kinase activity and signaling (Supp. Figure S7E) (Kharas and Fruman 2005, Modi et al 2007) and led to cell death that was augmented by Atg3 deletion (Figure 6A).

Given the enhancement of death with simultaneous disruption of autophagy and BCR-Abl inhibition, we next sought to determine how basal autophagy impacted cell growth and survival in BCR-Abl-expressing cells. BCR-Abl-expressing cells were capable of growth independent of IL3 (Figures 6B,C), but Atg3 excision in either the presence or absence of IL3 led to cell cycle arrest and rapid cell death similar to control cells (Figures 6B,C). Notably, Glut1 expression did not confer protection from this death (Supp. Figures S8A,B), and there was no activation of the downstream AMPK target phospho-ACC (Supp. Figure 6C), suggesting cell death was not likely due to metabolic stress. Rather, Atg3-deletion led to greater p53 phosphorylation and accumulation, as well as increased induction of p21, Puma, and γH2A.X (Figure 6D). Some cells persisted, but this was likely the consequence of inefficient Atg3 deletion and outgrowth of autophagy-competent Atg3-expressing cells, as Atg3 and processed LC3-II were readily detected in surviving cells at later time points (Supp. Figure S9).

The loss of cell proliferation and viability upon Atg3 deletion may have been due to general inhibition of autophagy or potential Atg3-specific effects. We therefore chemically inhibited autophagy by culturing A3C control and BCR-Abl-expressing cells in either chloroquine, which can inhibit the final lysosomal degradation step of autophagic digestion (Solomon and Lee 2009), or 3-methyladenine (3MA), which inhibits a PI3K upstream of autophaghy (Seglen and Gordon 1982). Chloroquine had a minor effect on growth and survival of control A3C cells cultured in IL3, but strongly inhibited both growth and survival of BCR-Abl-expressing cells (Figures 7A,B). Likewise, 3MA treatment inhibited cell accumulation and led to death in control and BCR-Abl-expressing cells (Figures 7C,D), though BCR-Abl expressing cells were somewhat more resistant to 3MA. Together, these data support the

notion that BCR-Abl-expressing A3C cells are reliant on autophagy rather than an Atg3-specific function.

Cell cycle arrest and apoptosis upon Atg3 deletion may have been due to activation of p53. To examine the importance of the p53 pathway in the death of Atg3-deficient Bcr-Abl-expressing cells, cells were transduced to stably express a p53 shRNAi (Mason et al 2010). This partial p53 knockdown reduced induction of both p21 and Puma in 4OHT-treated cells (Figure 8A, quantitation for p21, Puma, and γH2A.X provided), and allowed continued cell accumulation (Figure 8B) and a modest reduction in death (Figure 8C) even after Atg3 deletion. The tumor suppressor p53, therefore, is essential for cell cycle arrest and may contribute to apoptosis in BCR-Abl expressing cells following deletion of Atg3.

Inhibition of autophagy prevents formation of BCR-Abl⁺ leukemia

The sensitivity of BCR-Abl-expressing cells to disruption of autophagy suggested that autophagy may play an important role to support BCR-Abl-driven leukemia *in vivo*. To directly examine the role of autophagy in leukemia, control or BCR-Abl-expressing A3C cells were intravenously injected into immuno-deficient recipients. Mice were then control-treated or were treated with tamoxifen for three days to activate Cre-ER and promote *in vivo* deletion of Atg3 (Figure 9A). At no point did animals that received control A3C cells develop disease. In contrast, mice that received BCR-Abl-expressing A3C cells and were control-treated all developed leukemias (Figure 9A) that were characterized by splenomegaly (Figures 9B) and disruption of splenic architecture (Figure 9C). None of the animals that received BCR-Abl cells and were treated with tamoxifen to delete Atg3, however, showed signs of leukemia or significant alteration of spleen size or architecture in this period. In a separate experiment, mice injected with BCR-Abl expressing cells and then treated with tamoxifen immediately or two weeks after injection did not develop enlarged spleens compared to control-treated mice (Figure 9D), suggesting that disruption of autophagy can affect the course of a more established cancer. Thus, acute deletion of Atg3 and disruption of autophagy was sufficient to prevent progression of BCR-Abl-dependent leukemia *in vivo*.

DISCUSSION

Autophagy can play a critical role to promote survival, so we generated a growth-factor dependent cell model in which the autophagy-essential gene Atg3 could be inducibly excised by activation of Cre-ER. This acute genetic approach to disrupt autophagy avoided non-specific or partial effects from pharmacologic or RNAi-mediated inhibition of autophagy (Altman et al 2009) as well as developmental compensation from germline knockouts. Cells lacking Atg3 underwent more rapid cell death, had reduced levels of lipid metabolism, and showed a mild ER stress and robust activation of the p53 pathway that was essential for efficient p21 and Puma induction as well cell cycle arrest. Importantly, BCR-Abl-expressing cells relied on autophagy, as Atg3 excision or pharmacologic inhibition of autophagy led to cell death and prevented BCR-Abl-dependent leukemia. The acute genetic disruption of autophagy demonstrates the critical importance of this pathway in control of cell stress and survival.

Deletion of Atg3 appears to have strongly suppressed autophagy. Atg3 is a highly conserved E2-like enzyme responsible for the lipidation of several downstream targets critical in autophagy, including LC3, GABARAP, and Gate-16 (Weidberg et al 2010), which each showed decreased processing upon Atg3 excision. Protection of p62 and decreased GFP-LC3 punctae formation in Atg3-deficient cells further suggested autophagic disruption. Although complete blockade of autophagy was not observed in the entire cell population at timepoints considered, this may have been due to incomplete deletion of Atg3 or degradation of Atg3 protein after gene excision. Additionally, while Atg3 targets are important in autophagy, GABARAP and Gate-16 have been shown to be critical in multiple membrane trafficking events unrelated to autophagy (Lainez et al 2010, Legesse-Miller et al 2000, Sagiv et al 2000). Altered lipidation of these proteins may, therefore, also affect other cellular processes independent of autophagy. Similar findings using pharmacologic inhibitors 3-MA and chloroquine, however, suggest that autophagy itself was essential to growth and survival.

It remains unclear why autophagy was necessary for cell survival. While growth factors are essential to promote glucose uptake and glycolysis, autophagy can derive metabolic precursors from lysosomal digestion of organelles and other materials for metabolism in the absence of growth factor input (Altman et al 2009, Lum et al 2005a, Singh et al 2009). Here we show that autophagy was essential to increase mitochondrial oxidation of long chain fatty acids in growth factor-deprived cells. This autophagy-dependent contribution to cell metabolism was critical when glycolysis was directly disrupted, but did not appear essential in growth factor withdrawal. This finding contrasts with that of Lum et al, in which chronically growth factor deprived cells deficient in apoptosis relied on autophagy to support basal cell metabolism (Lum et al 2005a). It is likely that in apoptosis-competent cells, autophagy provides nutrients, yet is essential to alleviate other acute cell stresses. Autophagy can also contribute to apoptosis by promoting induction of the pro-apoptotic protein Bim (Altman et al 2009, Kiyono et al 2009). In these cases, RNAi partially suppressed autophagy rather than the more complete blockade caused by Atg3 deletion. Autophagy may affect cell stress and apoptotic pathways through multiple means, therefore, that depend in part on the overall levels of autophagic flux.

Autophagy can degrade damaged organelles such as mitochondria (Colell et al 2007) and endoplasmic reticulum (Bernales et al 2006), mitigate ROS stress, degrade protein aggregates, and prevent DNA damage (Mathew et al 2007, Mathew et al 2009, Pankiv et al 2007). Two distinct stress pathways were induced downstream of autophagy disruption that may contribute to the cellular need for autophagy. We observed an apparent mild ER stress that was characterized by a modest induction of the pro-apoptotic transcription factor Chop/GADD153. The endoplasmic reticulum can be regulated by autophagy (Bernales et al 2006, Hoyer-Hansen and Jaattela 2007), and Chop is important in the transcription of the pro-apoptotic proteins Bim and Puma (Altman et al 2009, Ishihara et al 2007, Puthalakath et al 2007), which were also transcribed downstream of autophagy disruption. Previously we had shown using RNAi that partial inhibition of autophagy led to increased Chop induction relative to cells with normal levels of autophagy (Altman et al 2009). Partial changes in levels of autophagy may, therefore, have distinct effects of cellular outcome. Alternatively, cell-type specific effects of autophagy-deficiency may also lead to distinct effects on Chop.

Loss of autophagy led to a robust stress response that included activation of p53 and accumulation of the double-strand DNA break marker γH2A.X (Kurz and Lees-Miller 2004). p53 was strongly phosphorylated and accumulated after autophagy disruption, and we observed induction of p53 targets including the cell cycle inhibitor p21 and the pro-apoptotic Bcl-2 family protein Puma. While p53 can be upstream of autophagy (Crighton et al 2006, Feng et al 2005, Wan et al 2008), this induction of the p53 pathway downstream of autophagy disruption may reflect a novel mechanism by which autophagy can suppress cell stress. Indeed, p53 was critical in cell cycle arrest and death after Atg3 deletion. γH2A.X accumulation suggested DNA damage, but this marker was also induced upon growth factor withdrawal in the absence of p21 accumulation. It is not clear, therefore, if the discrepancy between γH2A.X and phospho-p53 and p21 accumulation is due to a low level of DNA damage or an alternate cell stress pathway to activate p53. Similar findings of cell stress upon inhibition of autophagy have been reported in other systems and may be due to reactive oxygen species or protein aggregates (Mathew et al 2007, Mathew et al 2009). While the partial effects on cell cycle and death by p53 RNAi may be due to remaining p53 protein or through an alternate pathway, these data indicate that p53 contributes to cell fate when autophagy is suppressed.

There has been significant interest in determining if manipulation of autophagy may provide alternative approaches to cancer therapy. Indeed, cancer cells that resist apoptosis have been shown to require autophagy to survive nutrient stress (Degenhardt et al 2006, Jin and White 2007, Karantza-Wadsworth et al 2007) and clear protein aggregates (Mathew et al 2009, Pankiv et al 2007). Importantly, we found that while autophagy can serve as a source of nutrients, this did not appear to represent the critical function for autophagy to support cell viability in acute settings when growth signals were suppressed or in cells with active BCR-Abl. Rather, a key function of autophagy was to alleviate cell stress and p53 activation. Autophagy is constitutively activated at low levels in p185 BCR-Abl-expressing cells and is cytoprotective both under normal conditions and after treatment with the kinase inhibitor imatinib. Given the reliance of BCR-Abl expressing cells on autophagy and that autophagy can be activated by many cancer treatments (Bellodi et al 2009, Carew et al 2007, Kamitsuji et al 2008, Lomonaco et al 2009, Ren et al 2010), cancer cells may have a "non-oncogene addiction" to autophagy that could be exploited even in nutrient replete conditions (Luo et al 2009). Consistent with this notion, the autophagy and lysosome-inhibiting drug chloroquine has shown efficacy in enhancing cell death following treatment with other chemotherapeutics (Amaravadi et al 2007, Kamitsuji et al 2008, Solomon and Lee 2009). It will be important in future studies to further examine and understand how autophagy impacts cell stress and the p53 pathway in cancer cells that express oncogenic kinases so that inhibition of autophagy may be effectively combined with targeted therapies to treat cancer.

METHODS AND MATERIALS

Generation and Culture of A3C cells

Murine hematopoietic progenitor cells were isolated from Atg3$^{F/F}$ Rosa26-Cre-ER or Ub-Cre-ER bone marrow with a Progenitor Enrichment Kit (Stemcell Technologies, Vancouver,

BC) and co-cultured with Hox-11 retrovirus producing NIH-3T3 cells (generously provided by S. Zinkel, Vanderbilt University) and cytokines as previously described (Zinkel et al 2005). Non-adherent cells were recovered and cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 10% FBS (Gemini Bioproducts, West Sacramento, CA), 100 u/mL Penicillin/Streptomycin (Invitrogen, Chicago, IL), 2 mM L-Glutamine (Invitrogen) and 0.5 ng/mL IL3 (Ebioscience, San Diego, CA). The resulting cell population, A3C cells, were treated with 4-hydroxytamoxifen (4OHT, 0.5 μM in ethanol; Sigma) (Jacobs et al 2010) to excise Atg3. CrePos cells, from Ub-Cre-ER mice, were cultured as previously described (Zinkel et al 2005). FL5.12 cells were cultured as previously described (Altman et al 2009).

Amnis Multispectral Imaging Flow Cytometry

GFP-LC3-expressing cells were stained with the nuclear stain DRAQ5 (Cell Signaling), fixed in 2% PFA in PBS and analysed on an ImageStreamX (Amnis Corporation, Seattle, WA). Images were collected and analyzed using the manufacturer's software on GFP+, focused, single non-apoptotic cells using the spot counting feature as directed by the manufacturer (at least 1300 cells per condition).

Cell Accumulation and Death Analysis

Cell accumulation was assessed using a Coulter Z2 Particle Counter (Beckman Coulter, Brea, CA). Cell death was by flow cytometry analysis (FACScan, Becton Dickinson) using propidium iodide exclusion (PI, 1 μg / ml, Invitrogen) and analysis with Flowjo software (Treestar Inc., Ashland, OR). Active Bax and sub-diploid DNA were analyzed as previously described (Altman et al 2009).

BrdU and Cell Cycle Analysis

Cells were cultured in bromo-2-deoxyuridine (10 μM BrdU, Sigma) for eight hours and stained with Alexa Fluor 488 anti-Brdu (Invitrogen) for BRDU incorporation and 10 μg/ml PI (Invitrogen) for cell cycle analysis by flow cytometry as described (Darzynkiewicz and Juan 2001).

Glycolysis and ATP Assay

Glycolytic rate was measured as previously described (Vander Heiden et al 2001). ATP was measured using the ATP Bioluminescence Assay Kit CLS II (Roche Applied Science, Indianapolis, IN).

Mass Spectrometry Metabolic Analysis

Samples were prepared as previously described for intracellular organic acid analysis (Zhao et al 2007) and acyl-carnitine analyses (Altman et al 2009, An et al 2004).

Mouse Leukemia Assays

Fox Chase SCID Beige Mice (Charles River Laboratories, Wilmington, MA) were injected intravenously with 10^6 A3C NGF or BCR-Abl-expressing cells and injected with tamoxifen or vehicle control corn oil by intraperitoneal injection for three days after cell injection, or for three consecutive days two weeks after cell injection. Mice were sacrificed on onset of

overt moderate illness according to approved protocols. Histological sections were prepared and stained by the Duke University Pathology Laboratory.

Microscopy

Histologic images were acquired with a Zeiss Axio microscope (Carl Zeiss) using a 40X Zeiss Plan Apochromat objective and analyzed using MetaMorph 7.5 software (Molecular Devices).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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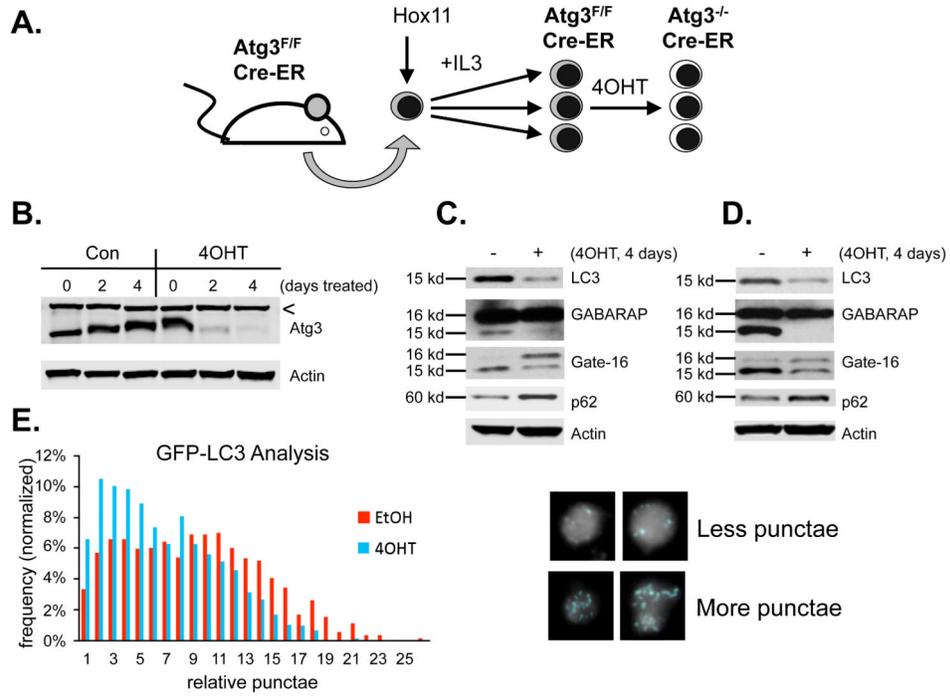
Figure 1. Autophagy can be acutely disrupted by inducible excision of Atg3 in the hematopoietic A3C cell population

A. Generation and model of immortalization, culture conditions, and Atg3 excision from Atg3$^{F/F}$ Rosa26-Cre-ER mouse of A3C cells. **B.** A3C cells were cultured with ethanol or 0.5 μM 4-hydroxytamoxifen (4OHT) for the indicated times, and loss of Atg3 was observed by immunoblot. A non-specific band is indicated by <. **C.,D.** Bcl-2-expressing A3C cells were cultured with ethanol or 4OHT for 4 days, then cultured in the absence (**C**) or presence (**D**) of 40 μM chloroquine (CQ) for 10 hours, and LC3-II, GABARAP, Gate-16, and p62 protein accumulation were assessed by immunoblot. LC3 antibody used preferentially recognized LC3-II. **E.** A3C GFP-LC3-expressing cells were cultured with ethanol or 4OHT for two days and subjected to analysis on the Amnis ImagestreamX Multispectral Imaging Flow Cytometer for LC3-puncta. Left panel shows histogram of the number of LC3-puncta in individual cells, right panel shows examples of more punctate vs less punctate patterns. Data shown are representative of three or more experiments.
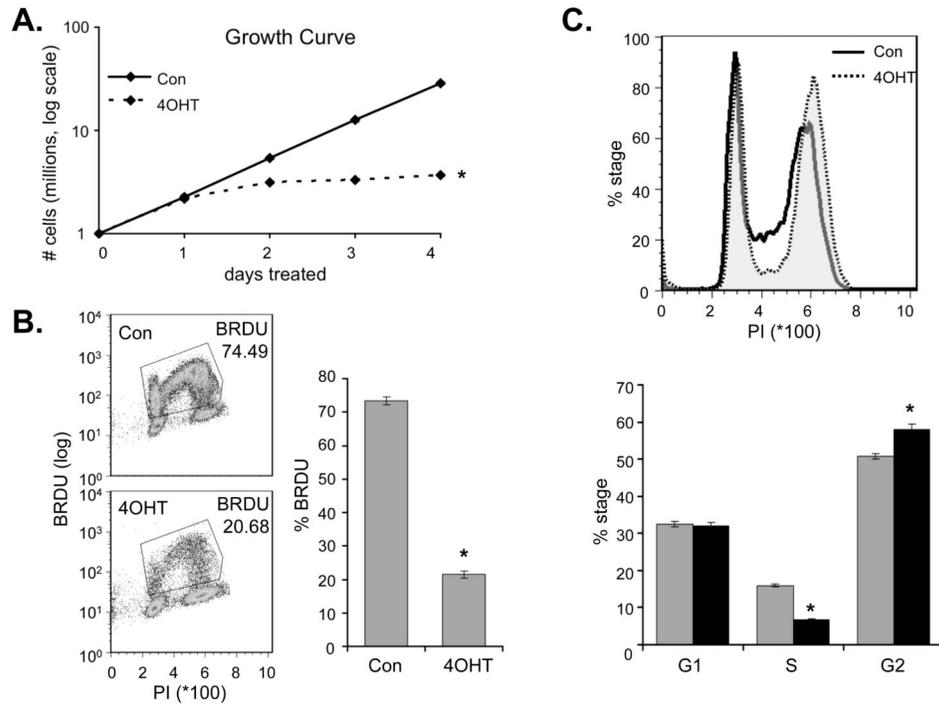
Figure 2. Disruption of autophagy causes cell cycle arrest in culture
A. NGF-expressing A3C control cells were cultured in the presence or absence of 4OHT, and cell number was assessed daily using a Coulter Z2 Particle Counter. **B,C.** Bcl-2-expressing A3C cells were cultured with ethanol or 4OHT for three days, with media being changed and new drug being added on day two, then cultured with Bromo-2-deoxyuridine (BrdU) and BrdU incorporation and PI staining (to indicate DNA content) were assessed by flow cytometry for (**B**) BrdU incorporation and (**C**) PI cell-cycle analysis. Means and standard deviations of triplicate samples are shown. Asterisks denote $p < 0.05$ by Student's t-Test of 4OHT-treated samples compared to control-treated samples.
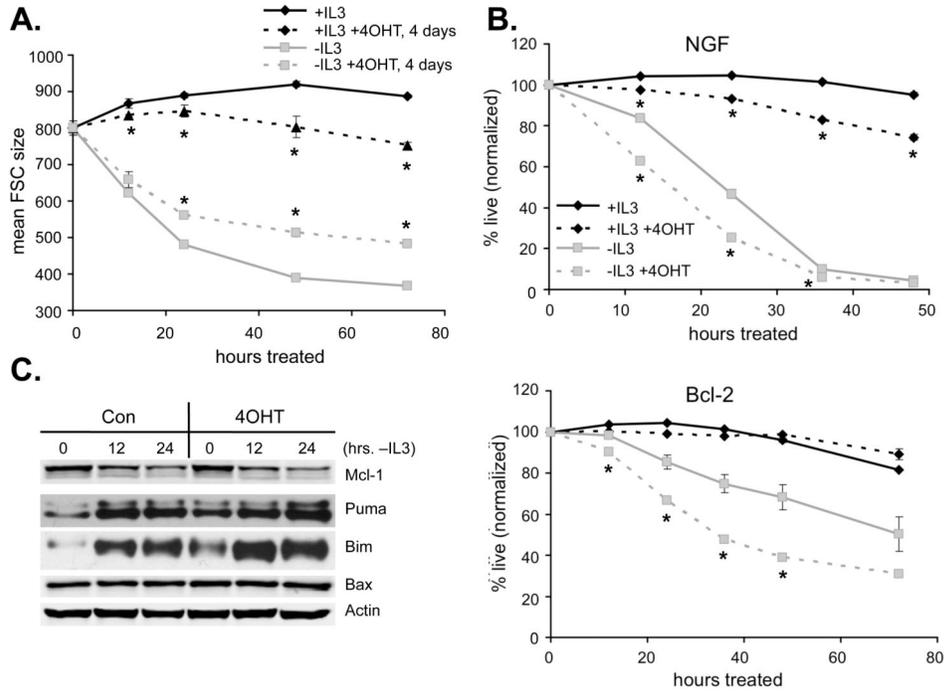
Figure 3. Autophagy disruption delays atrophy and enhances cell death in both the presence and absence of growth factor

A. Bcl-2-expressing cells were cultured for four days in the presence or absence of 4OHT to delete Atg3, then cultured in the presence or absence of both 4OHT and IL3. Cell size of Bcl-2-expressing cells was assessed by forward scan flow cytometry. **B.** Control NGF or Bcl-2-expressing A3C cells were cultured for two days with ethanol or 4OHT, then cultured in the presence or absence of both 4OHT and IL3, and survival of control NGF and Bcl-2-expressing cells was assessed by propidium iodide exclusion flow cytometry. *Note different time scales*. **C**. Bcl-2-expressing A3C cells were cultured for two days with ethanol or 4OHT to prevent autophagy, and withdrawn from IL3 for indicated times. Mcl-1, Puma, Bim, and Bax, were analyzed by immunoblot. Means and standard deviations of triplicate samples are shown. Data shown are representative of three or more experiments. Asterisks denote $p < 0.05$ by Student's t-Test of 4OHT-treated samples compared to control-treated samples.
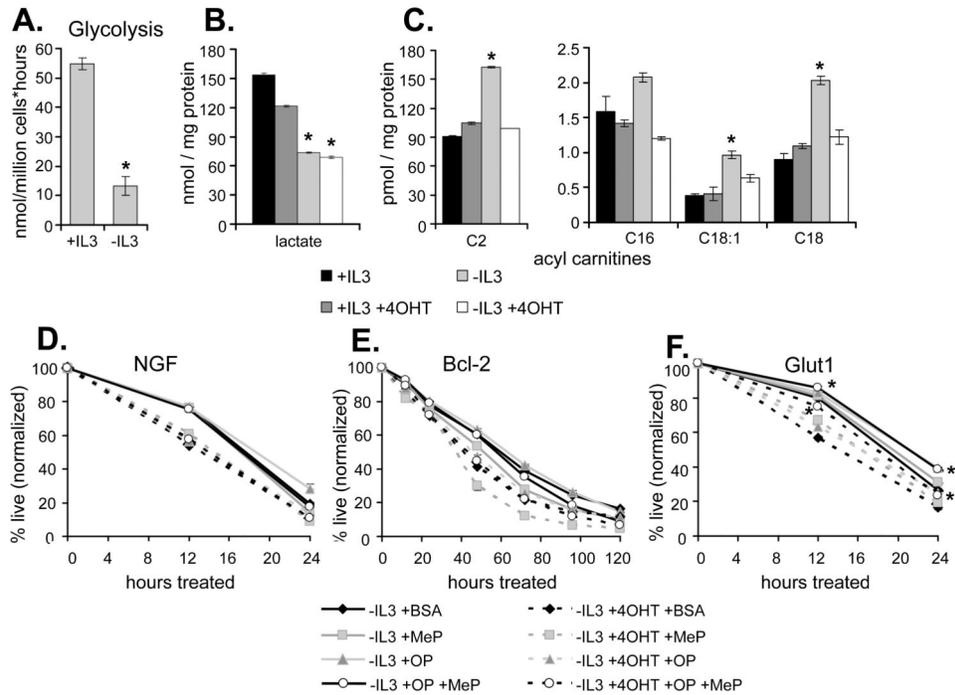
Figure 4. Atg3 deletion affects cell metabolism, but these changes do not appear critical for survival early in growth factor withdrawal

A-C. Bcl-2-expressing A3C cells were cultured with IL3 or withdrawn from IL3 for 24 hours and (**A**) glycolysis, (**B**) intracellular lactate, and (**C**) short-chain C2 (acetyl) and the long-chain acyl carnitines C16 (palmitate), C18:1 (oleate) and C18 (stearate) were measured. **D-F**. Control NGF (**D**), Bcl-2-expressing (**E**), or Glut1-expressing (**F**) were cultured for two days with either ethanol or 4OHT to inhibit autophagy, then cultured in the presence or absence of IL3 along with a 1:1 mixture of 5 mM oleate and palmitate (OP), 10 mM methyl-pyruvate (MeP), OP + MeP, or BSA as a control, and cell viability was assessed. *Note different time scales*. Means and standard deviations of triplicate samples are shown, standard errors for mass spectrometry experiments. Data shown are representative of three or more experiments. Asterisks denote p < 0.05 by Student's t-Test of IL3-withdrawn samples compared to control samples for **B,C**, 4OHT-treated samples compared to control-treated samples for **F**.
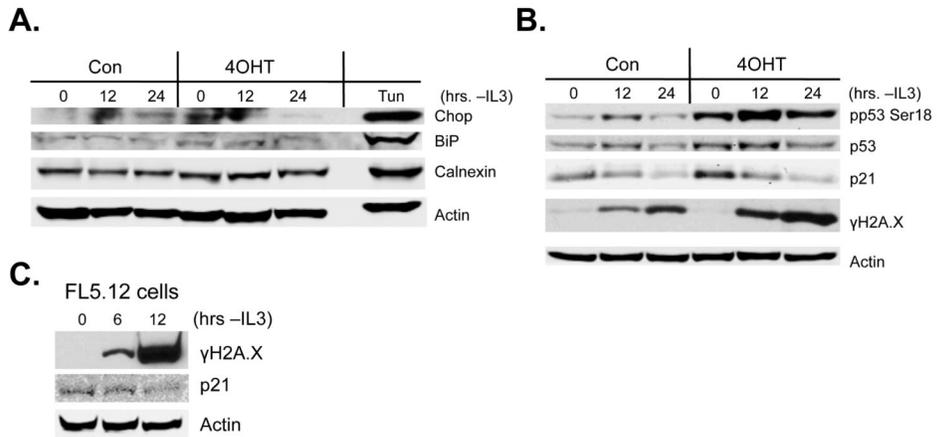
A.

B.

C. FL5.12 cells

Figure 5. Atg3 deletion leads to cell stress and p53 activation
A,B. Bcl-2-expressing A3C cells were cultured in the presence or absence of 4OHT for two days followed by withdrawal from IL3 for indicated times. (**A**) The ER stress markers Chop, BiP, and Calnexin were analyzed by immunoblot. Some cells were treated with 2 μg/mL tunicamycin (Tun) for 12 hours. (**B**) Phospho-p53 serine 18, total p53, p21, and the DNA damage response protein γH2A.X (phospho histone H2A.X serine 139) were analyzed by immunoblot. **C.** FL5.12 cells were withdrawn from IL3 for the indicated times, and γH2A.X and p21 were analyzed by immunoblot. Data shown are representative of three or more experiments.
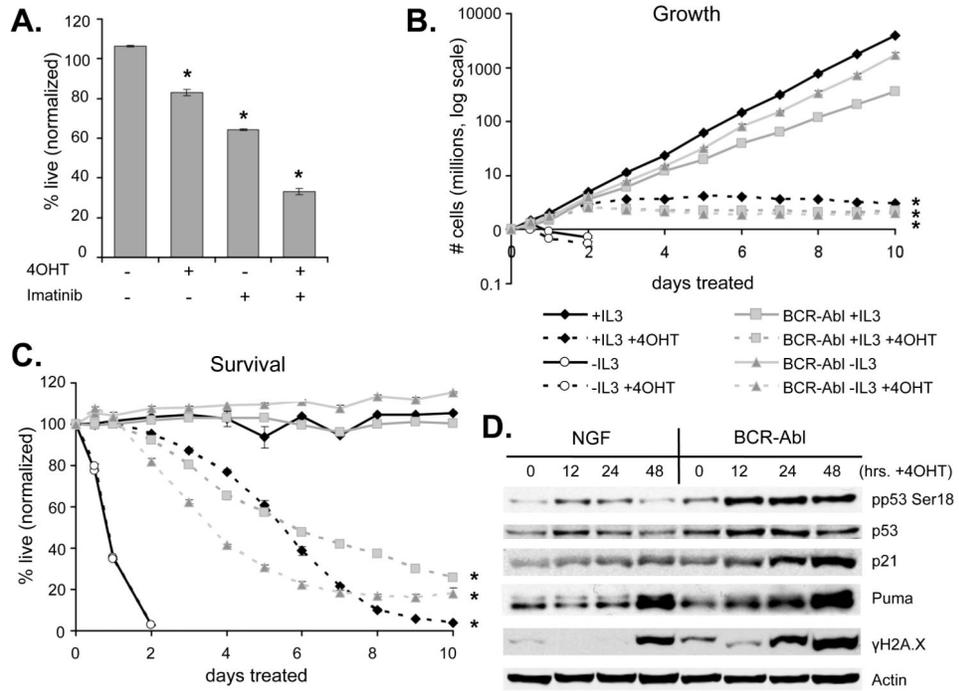
Figure 6. BCR-Abl-transformed A3C cells are sensitive to loss of autophagy
A. BCR-Abl-expressing A3C cells were cultured for two days in the presence or absence of 4OHT and 0.1 μM imatinib, and survival was assessed. **B,C**. Control NGF and BCR-Abl-expressing cells were cultured in the presence or absence of both IL3 and 4OHT for ten days, with media being changed and new 4OHT added on day two, and growing cells being split every two days. (**B**) Cell accumulation over time was measured by quantitation on a Coulter Z2 Particle Counter, and (**C**) death was measured by flow cytometry. **D.** Control NGF and BCR-Abl-expressing cells were treated with 4OHT for the times indicated, and phospho-p53 serine 18, total p53, p21, Puma, and γH2A.X were analyzed by immunoblot. Means and standard deviations of triplicate samples are shown. Data shown are representative of three or more experiments. Asterisks denote p < 0.05 by Student's t-Test of 4OHT-treated samples compared to control-treated samples.
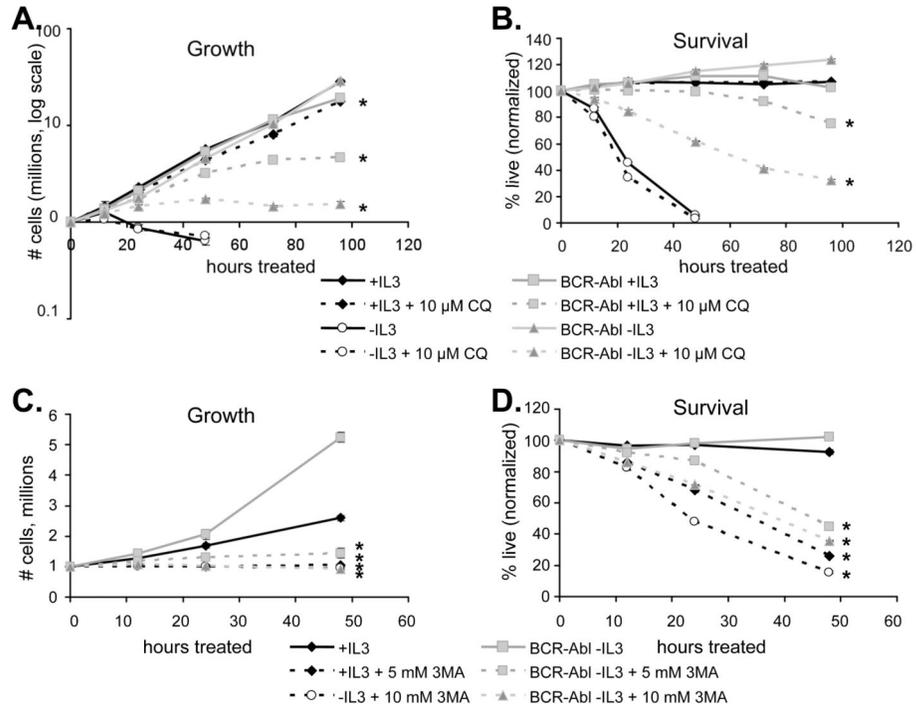
Figure 7. Pharmacologic inhibition of autophagy promotes death of BCR-Abl-transformed A3C cells

A,B. Control NGF and BCR-Abl-expressing cells were cultured in the presence or absence of both IL3 and 10 μM chloroquine (CQ), with media being changed, new 4OHT being added, and growing cells being split on day two. (**A**) Cell accumulation over time was measured by quantitation on a Coulter Z2 Particle Counter, and (**B**) death was measured by flow cytometry. **C,D.** Control NGF and BCR-Abl-expressing cells were cultured in the presence of IL3 or absence of IL3 for BCR-Abl-expressing cells, and with 0, 5, or 10 mM 3-methyladenine (3MA). (**A**) Cell accumulation over time was measured by quantitation on a Coulter Z2 Particle Counter, and (**B**) death was measured by flow cytometry Means and standard deviations of triplicate samples are shown. Data shown are representative of three or more experiments. Asterisks denote $p < 0.05$ by Student's t-Test of 4OHT-treated samples compared to control-treated samples.
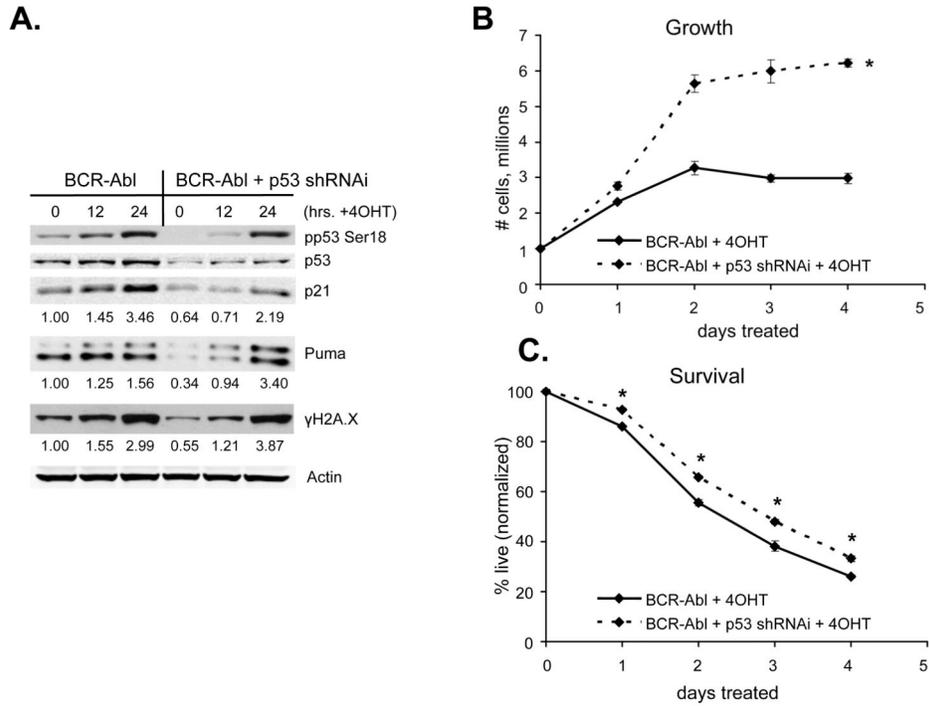
Figure 8. shRNAi targeting of p53 alleviates some effects of Atg3 excision
A. BCR-Abl-expressing cells stably transduced with a control vector or p53 shRNAi were treated with 4OHT for the indicated times, and phospho-p53 serine 18, total p53, p21, Puma, and γH2A.X were analyzed by immunoblot. Quantitations are provided for p21, Puma, and γH2A.X, normalized to relative background and to actin. **B,C.** BCR-Abl-expressing cells with or without p53 shRNAi were treated with 4OHT for the indicated times, with media being changed, new 4OHT added, and growing cells being split on day two. (**B**) Cell accumulation over time was measured by quantitation on a Coulter Z2 Particle Counter, and (**C**) death was measured by flow cytometry. Data shown are representative of three or more experiments. Asterisks denote p < 0.05 by Student's t-Test of 4OHT-treated samples compared to control-treated samples.
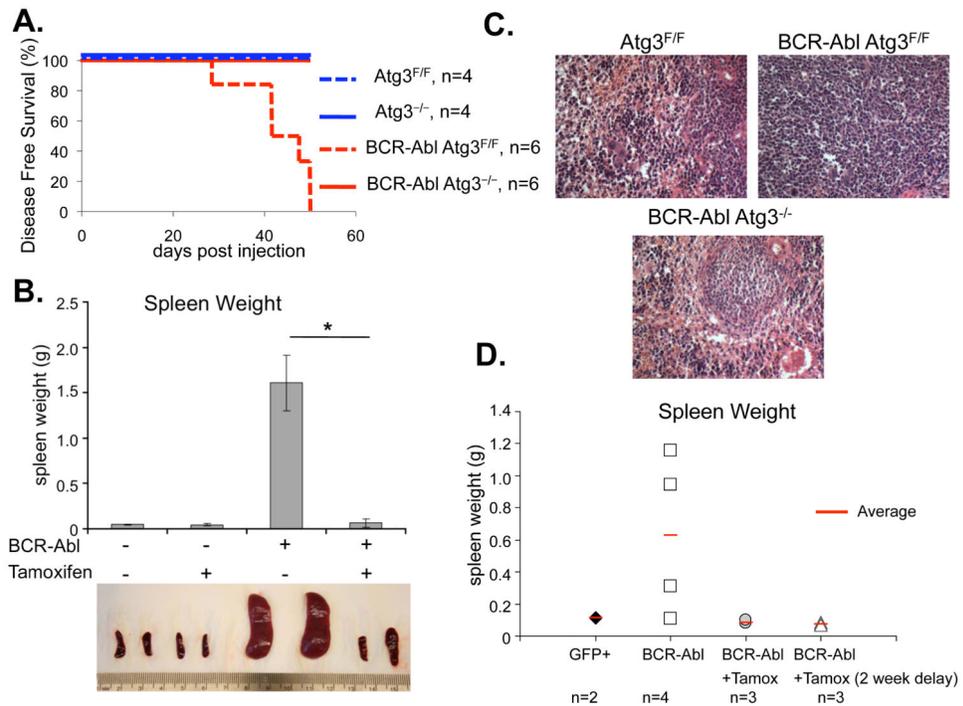
Figure 9. Loss of autophagy prevents formation of BCR-Abl+ cancer in an allograft model of leukemia

A. Immunocompromised mice were injected with control or BCR-Abl-expressing A3C cells and mice received intraperitoneal injections vehicle or tamoxifen for three days and time of disease-free survival was measured. **B-C.** Spleens were removed from each animal and (**B**) weighed, with representative spleens are shown in the bottom panel and (**C**) subject to histological examination with hematoxylin and eosin. Means and standard deviations are from six mice each for injections with BCR-Abl-expressing cells, and four mice each for injections with NGF-expressing cells. Asterisks denote p < 0.05 by Student's t-Test. **D.** Immunocompromised mice were injected with GRP+ or BCR-Abl A3C cells and mice received intraperitoneal injections vehicle or tamoxifen for three days either immediately after cell injection, or three consecutive days two weeks after cell injection, as indicated. Mice were sacrificed at 50 days after cell injection, and individual spleens were weighed.