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# Growth Factor Regulation of Autophagy and Cell Survival in the Absence of Apoptosis

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## Summary

**In animals, cells are dependent on extracellular signals to prevent apoptosis. However, using growth factor-dependent cells from Bax/Bak-deficient mice, we demonstrate that apoptosis is not essential to limit cell autonomous survival. Following growth factor withdrawal, $Bax^{-/-}Bak^{-/-}$ cells activate autophagy, undergo progressive atrophy, and ultimately succumb to death. These effects result from loss of the ability to take up sufficient nutrients to maintain cellular bioenergetics. Despite abundant extracellular nutrients, growth factor-deprived cells maintain ATP production from catabolism of intracellular substrates through autophagy. Autophagy is essential for maintaining cell survival following growth factor withdrawal and can sustain viability for several weeks. During this time, cells respond to growth factor readdition by rapid restoration of the ability to take up and metabolize glucose and by subsequent recovery of their original size and proliferative potential. Thus, growth factor signal transduction is required to direct the utilization of sufficient exogenous nutrients to maintain cell viability.**

## Introduction

Animal cells depend on extrinsic factors to provide signals for growth and proliferation. When these signals are lost, both growth and division cease, and programmed cell death is initiated through the intrinsic mitochondrial pathway (Danial et al., 2003; Green and Reed, 1998). An additional consequence of growth factor limitation is a rapid decline in the surface expression of nutrient transporters including the major glucose transporter GLUT1, the LDL receptor, amino acid transporters and receptors for iron uptake (Edinger and Thompson, 2002; Kan et al., 1994). This decrease in nutrient transporter expression has been proposed to perturb mitochondrial physiology resulting in the induction of apoptotic cell death (Rathmell et al., 2000). However, an alternative explanation is that the decline in surface expression of nutrient transporters simply reflects a secondary response to the decreased metabolic demand on the cell following the cessation of growth and the withdrawal from the cell cycle (Conlon and Raff, 1999). In this model, perturbations in mitochondrial physiology result from the activities of apoptotic regulatory factors.

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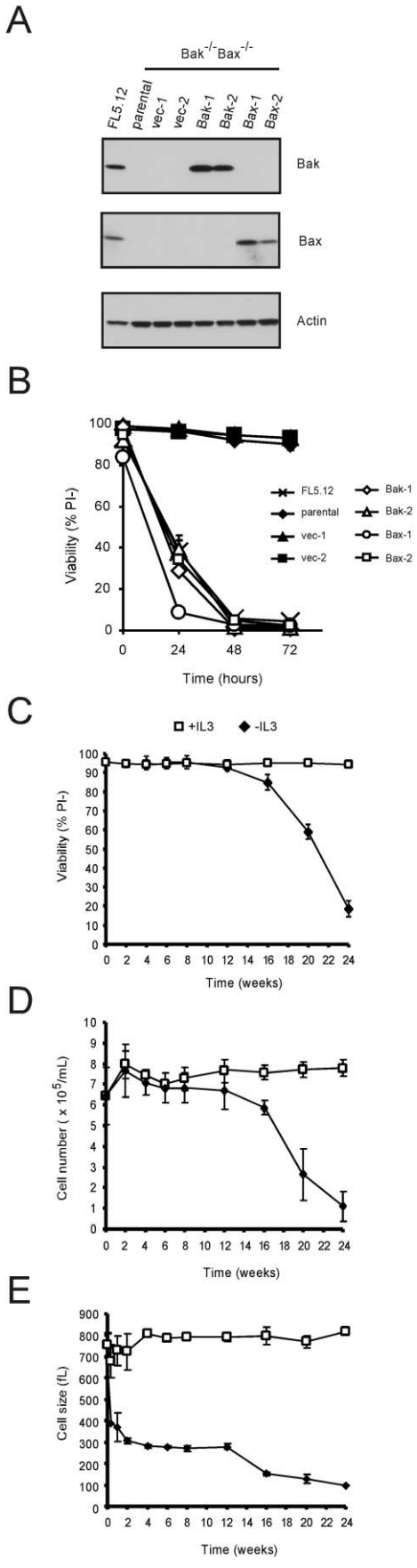
In yeast, which lack the central components of the mitochondrial apoptotic pathway, nutrient deprivation results in compromised bioenergetics and activation of a cell survival response termed macroautophagy (Klionsky and Emr, 2000; Takeshige et al., 1992; Thumm et al., 1994). During macroautophagy, regions of the cytosol become sequestered in double membrane vesicles known as autophagosomes (Baba et al., 1994). Upon fusion with the vacuole, the contents of autophagosomes are degraded, and the resulting degradation products are then either reutilized to maintain basal macromolecular synthesis or oxidized in the mitochondria to maintain bioenergetics. Yeast can survive for several weeks in the absence of extracellular nutrients through macroautophagy. A role for autophagy in organismal survival during starvation has been observed in plants, worms, flies, and mice (Hanaoka et al., 2002; Melendez et al., 2003; Scott et al., 2004; Kuma et al., 2004). In addition, autophagy has been reported to initiate cell death in response to intracellular damage caused by hypoxia, chemotherapeutic agents, virus infection, or toxins (Daido et al., 2004; Kanzawa et al., 2003, 2004; Paglin et al., 2001; Talloczy et al., 2002; Nelson and White, 2004; Qu et al., 2003; Yu et al., 2004; Yue et al., 2003). Autophagy may also contribute to disease pathology, as it has been observed in a variety of neurodegenerative diseases (reviewed in Shintani and Klionsky [2004]). Macroautophagy initiated as a response to intracellular damage may provide a multicellular organism with a mechanism to eliminate damaged cells even if the ability of the cells to respond by apoptosis becomes impaired.

Recently, Bax and Bak have been demonstrated to be required for cells to initiate apoptosis through the intrinsic mitochondrial pathway (Cheng et al., 2001; Wei et al., 2001). Cells from $Bax^{-/-}Bak^{-/-}$ animals fail to undergo apoptosis in response to serum deprivation, loss of attachment, and growth factor withdrawal (Wei et al., 2001). Thus, Bax and Bak are essential and redundant regulators of apoptosis and extracellular signals are no longer necessary to suppress mitochondrial initiation of apoptosis in $Bax^{-/-}Bak^{-/-}$ cells. To determine if metabolic changes that result from growth factor withdrawal result from a primary effect of growth factor signal transduction or as a consequence of Bax and Bak activity, we examined the effects of growth factor withdrawal on IL-3-dependent $Bax^{-/-}Bak^{-/-}$ cells. In addition to withdrawing from the cell cycle, cells cultured in the absence of growth factor underwent progressive atrophy. This atrophy correlated with the inability to utilize extracellular glucose and the induction of macroautophagy. Despite the abundance of oxidizable nutrients in the extracellular media, growth factor-deprived cells became dependent on the autophagic degradation of intracellular contents to maintain ATP production. Prevention of autophagy by RNAi-mediated suppression of autophagy genes or chemical inhibitors of autophagosome function led to rapid cell death. These data provide a demonstration that autophagy is critical for maintaining cell survival following growth factor withdrawal.




While, by its very nature, macroautophagy is a self-limited survival strategy, it was able to promote growth factor-independent survival for several weeks. During this period, readdition of growth factor led to stimulation of glycolysis and complete cell recovery. Together, these data suggest that growth factor signal transduction is required to maintain a sufficient level of nutrient utilization to support the survival of mammalian cells.

## Results

### Growth Factor Withdrawal Results in Progressive Atrophy of $Bax^{-/-}Bak^{-/-}$ Cells

To study the effects of growth factor withdrawal on cells lacking the intrinsic apoptotic pathway, immortalized interleukin-3- (IL-3) dependent cell lines were generated from the bone marrow of $Bax^{-/-}Bak^{-/-}$ mice. These cells failed to undergo apoptosis following growth factor withdrawal (Figure 1) but remained dependent on IL-3 for proliferation in culture. Transfection of either Bax or Bak fully restored apoptosis in these cells in response to IL-3 withdrawal with comparable kinetics to that observed in wild-type IL-3-dependent cells (Figures 1A and 1B). Following IL-3 withdrawal, the $Bax^{-/-}Bak^{-/-}$ cells exited from the cell cycle and the cell number in the culture did not change during the first several weeks (Figure 1D). Although the initial decline in cell size that occurs in the first two days after growth factor withdrawal results from the arrest of the cells in the $G_0/G_1$ phase of the cell cycle, cell size continued to decline at subsequent time points, and no stable cell size was achieved as measured by either cell size or protein content (Figure 1E and data not shown). Beginning at approximately 12 weeks, cell number and viability also began to decline and >95% of cells were dead by 24 weeks of culture (Figures 1C and 1D).

Figure 1. $Bax^{-/-}Bak^{-/-}$ Cells Undergo Atrophy and Maintain Prolonged Survival Following Withdrawal of Growth Factor

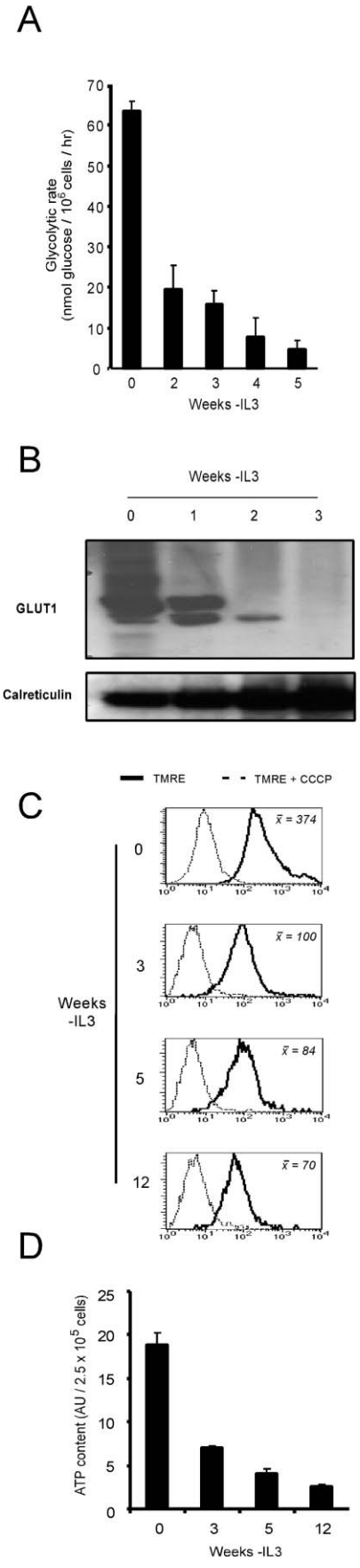
(A) Two independent clones of $Bax^{-/-}Bak^{-/-}$ IL-3-dependent cells (parental) stably transfected with either Bax, Bak, or empty vector (vec) were generated, and expression levels were assessed by Western blot. The IL-3-dependent $Bax^{+/+}Bak^{+/+}$ cell line FL5.12 is shown for comparison.

(B) Kinetics of cell death in Bax- or Bak-reconstituted cells following IL-3 withdrawal. Viability was measured by propidium iodide exclusion. Data are averages of three experiments ± standard deviation (SD).

(C) Cell viability of $Bax^{-/-}Bak^{-/-}$ cells in the presence or absence of IL-3. Cells were washed and cultured in the presence (open squares) or absence (closed diamonds) of IL-3. At the indicated time points, cells were collected and viability was assessed. Cells grown in the presence of IL-3 were passaged every 2–3 days to restore a cell concentration of $7.5 \times 10^5$ cells/ml. The medium in IL-3-deprived cultures was replaced with an identical volume of fresh complete medium without IL-3 every 10 days. Data are averages of three independent experiments ± SD.

(D) Cell numbers of cultures that were grown in the presence or absence of IL-3 and were cultured as in (C). Data are averages of three independent experiments ± SD.

(E) Cell size of cultures that were grown in the presence or absence of IL-3 and were cultured as in (C). Data are averages of three independent experiments ± SD.




## Autophagosome Formation Is Induced by Growth Factor Withdrawal

Cells grown in the presence of IL-3 were highly glycolytic (Figure 2A). In contrast, glycolysis declined rapidly following IL-3 withdrawal, and there was a time-dependent loss of GLUT1, the major glucose transporter expressed on these cells (Figure 2B). Coincident with the decline in glycolysis, there was a decline in mitochondrial membrane potential (Figure 2C). Cellular ATP levels also fell, but the decline in glucose transporter expression was greater than that expected based on the ATP decline (Figure 2D), suggesting that cells were utilizing alternative substrates to maintain their bioenergetics. Furthermore, the continued decline in cell size of the $G_0/G_1$ arrested cells following growth factor withdrawal suggested the possibility that cells were utilizing macroautophagy to catabolize intracellular substrates to maintain their survival. These observations prompted a characterization of the cells during growth factor withdrawal by electron microscopy. By 48 hr after growth factor withdrawal, early autophagosomes were visible in the cytosol of the cells (Figures 3Aa–3Ac). The presence of autophagosomes when quantitated by electron micrographs was significantly increased in the IL-3-deprived cells in comparison to cells maintained in IL-3 (Figure 3Ad; $p < 0.001$). To confirm the extent and specificity of this autophagosome induction, the cells were stained with an antibody specific for the mammalian homolog of the yeast Atg8 protein, microtubule-associated protein-1 light chain-3 (LC3). LC3 becomes physically associated with forming autophagic vesicles and is a well-characterized marker for autophagosome formation (Asanuma et al., 2003; Kabeya et al., 2000). Using confocal microscopy, we observed a redistribution of LC3 from diffuse cytoplasmic staining in cells grown in the presence of IL-3 (Figure 3Ba) to discrete vesicular structures following IL-3 withdrawal (Figure 3Bb). This redistribution was confirmed biochemically by Western blot analysis. The intracellular LC3 underwent a conversion from the LC3-I isoform to the LC3-II isoform that is specific for autophagosomes (Figure 3C, lane 1 versus lane 4).

Figure 2. Metabolic Effects of IL-3 Withdrawal on $Bax^{-/-}Bak^{-/-}$ Cells

(A) Glycolytic rate of cells grown in the absence of IL-3 as measured by the conversion of 5-$^3$H-glucose to $^3H_2O$ at the indicated time points. The data presented at week 0 represent values of control cells growing in IL-3 throughout the time course of the experiment. Data are averages of three experiments ± SD.

(B) Western blot analysis of GLUT1 expression in cells cultured in the absence of IL-3. The GLUT1 expression at week 0 is representative of GLUT1 expression of cells grown in IL-3.

(C) Mitochondrial membrane potential as measured by TMRE staining in cells grown without IL-3 (solid histogram) at the indicated time point. Baseline TMRE was determined by using cells treated with the uncoupler CCCP (dotted histogram). The numbers in the top right corner indicate the average mean fluorescence intensity of three independent experiments. The week 0 time point indicates the mean fluorescence intensity of cells growing in IL-3 and is representative of the values obtained for such cells over the time course of the experiment.

(D) ATP levels in cells grown without IL-3 and expressed as arbitrary units (AU). ATP levels for IL-3-grown cells did not decline significantly over the time course of the experiment (data not shown). Data are averages of three independent experiments ± SD.

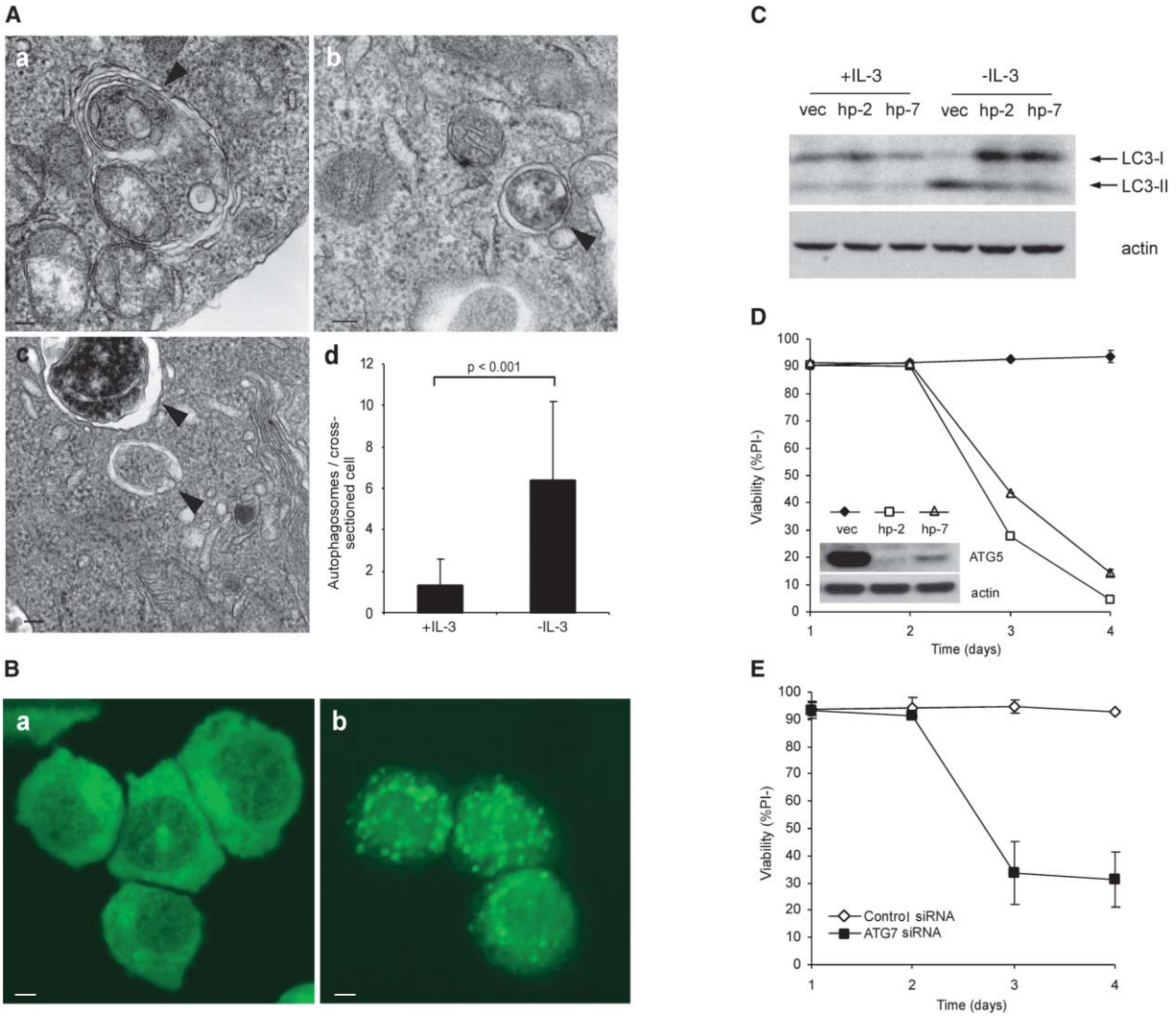



Figure 3. Growth Factor Withdrawal Induced Autophagosome Formation Is Required for Survival

(A) Electron microscopy of cells grown in the absence of IL-3 for 48 hr (a–c) showing the presence of autophagosomes. Arrowheads depict representative autophagosomes quantitated in (d). Scale bar, 100 nm. (d) Quantitation of the number of autophagosomes per cross-sectioned cell cultured in the presence or absence of IL-3 for 48 hr. Error bar represents ±SD. Statistical significance determined by Student's t test.

(B) Immunofluoresence with anti-LC3 antibody on cells grown in the presence (a) or absence (b) of IL-3 for 48 hr. Scale bar, 5 μm.

(C) Immunoblot analysis of LC3-I processing into LC3-II in cells transfected with control or two independent shRNA constructs against *ATG5* (hp-2 and hp-7) followed by culture in the presence or absence of IL-3 for 48 hr. Actin was used as a loading control.

(D) Time course of cell viability following IL-3 withdrawal in cells with inactivation of *ATG5*. Data are averages of three experiments ± SD. Western blot analysis of *ATG5* protein expression in cells transfected with vector control, hp-2, or hp-7 shRNA is shown as a representative experiment. Actin was used as loading control.

(E) Time course of cell viability following IL-3 withdrawal in cells transfected with FITC tagged-siRNA for *ATG7* (Yu et al., 2004) or a control siRNA. Cells which had incorporated the siRNA for *ATG7* or control were purified by FACS sorting based on FITC-positive cells, and viability was assessed at the indicated time points. Data are averages of three experiments ± SD.

### Inhibition of Autophagy Leads to Cell Death

The ability of cells to initiate autophagosome formation is dependent on the ATG12-ATG5 complex. To test whether macroautophagy plays a role in maintaining growth factor-independent cell survival, shRNA against *ATG5* were introduced into the IL-3 dependent cells. Cells transfected with two independent shRNA constructs against *ATG5* (hp-2 and hp-7) or a control had no effect on the size or viability of cells grown in the presence of IL-3 (data not shown). In contrast, if cells transfected with *ATG5* hairpins were withdrawn from IL-3, their viability began to decline at 48 hr after withdrawal, and virtually all cells were dead by 96 hr (Figure 3D). The onset and rapidity of decline in cell viability correlated with the extent of ATG5 protein suppression by shRNA (Figure 3D), with the absence of autophagic processing of LC3 in growth factor-deprived cells in which *ATG5* is suppressed (Figure 3C, lane 4 versus lanes 5 and 6) and a statistically significant reduction in autophagosomes observed in electron micrographs at

48 hr after IL-3 withdrawal (data not shown). Similar results on cell survival were obtained when autophagy was suppressed with siRNA against *ATG7* (Figure 3E).

While macroautophagy in yeast and plant cells is required to promote cell survival in the absence of nutrients, the macroautophagy observed following IL-3 deprivation occurred in the presence of abundant extracellular nutrients. The IL-3-deprived cells were maintained in complete RPMI medium supplemented with 10% serum, and the medium was replaced every 10 days. The medium removed from these cultures was not nutrient deficient since it supported proliferative expansion of the parental $Bax^{-/-}Bak^{-/-}$ cells when supplemented with IL-3 (data not shown). Therefore, macroautophagy in $Bax^{-/-}Bak^{-/-}$ cells was induced by growth factor withdrawal and not by a lack of nutrients in the extracellular environment.

### Macroautophagy Is an Ongoing Process in Surviving Growth Factor-Deprived Cells

To determine if macroautophagy persisted during the weeks the cells survived growth factor deprivation, electron micrographs were obtained from later time points following IL-3 withdrawal (Figure 4). In the weeks following IL-3 deprivation, the cytoplasm of the cells became progressively replaced by vesicular structures, some of which contained residual remnants of degraded organelles and others the characteristics of lysosomes (Figures 4Aa–4Ad). High-power transmission electron microscopy images demonstrate continued presence of autophagosomes and late autophagosomes fusing with lysosomes (Figures 4Ae and 4Af). These structures were quantitatively increased in IL-3-deprived cells even after 6 weeks in culture (Figure 4B; $p < 0.001$). The continued presence of autophagosomes was confirmed by the persistent vesicular staining pattern of LC3 (Figure 4Ca versus 4Cb). This persistent formation of autophagosomes correlated with a progressive reduction of definitive intracellular organelles. By six weeks, ribosomes were difficult to find and the Golgi/ER network was not observed in any of the sections examined. The few mitochondria that remained were perinuclear in distribution and highly condensed. The nucleus displayed a reduced number of nuclear pores, reduced nucleolar size, and a more organized heterochromatin.

### The TCA-Substrate Methylpyruvate Maintains the Survival of Growth Factor-Deprived Cells Treated with Inhibitors of Autophagosome/Lysosome Function

We next tested whether the continued degradation of metabolic substrates within the autophagosome/lysosome system was required to maintain cell viability at these late time points after growth factor withdrawal. Existing shRNA transfection methods proved ineffective in cells that had undergone prolonged growth factor withdrawal, therefore we used two independent and widely used inhibitors of macroautophagy, 3-methyladenine (3-MA) and chloroquine (CQ) (Mizushima et al., 2002; Petiot et al., 2000) to block autophagy. Treatment with either 5 mM 3-MA or 10 μM CQ had no significant effect on survival of cells grown in the presence of IL-3 (Figure 5). However, when cells deprived of growth factor for 6 weeks were cultured with 3-MA or CQ, cell viability began to decline within 18 hr. By 48 hr, treatment with 3-MA or CQ had resulted in the death of 72% and 82% of the cells in the cultures, respectively. These effects of 3-MA or CQ were dose dependent (data not shown). Cell death induced by 3-MA and CQ correlated with a reduction in the number of autophagosomes as evidenced by the redistribution of LC3 from highly localized punctate staining to diffuse cytoplasmic staining (Figure 5Bb versus Figure 5Bd and Figure 5Bb versus Figure 5Bf). This death did not appear to be apoptotic in nature. DNA extracted from the dying cells lacked oligonucleosomal length fragments characteristic of apoptosis (Figure 5C). In addition, the dying cells lost their capacity to exclude propidium iodide prior to becoming annexin V positive (Figure 5D), and caspase inhibitors failed to prevent this death (data not shown).

Since autophagy is required in yeast to provide mitochondria with substrates to maintain oxidative phosphorylation during nutrient deprivation (Levine and Klionsky, 2004), we tested whether the cell death observed following 3-MA and CQ treatment could be reversed by supplying the cell with an alternative metabolic substrate. A cell-permeable form of pyruvate, methylpyruvate (MP), was added to the cultures at the time of 3-MA or CQ treatment. Once internalized, this substrate can be oxidized in the tricarboxylic acid cycle to produce NADH to fuel electron transport and ATP production. The addition of methylpyruvate suppressed the death observed in response to 3-MA and CQ in a time- and dose-dependent fashion (Figure 5E and data not shown).

To confirm that suppression of autophagy led to compromised cellular bioenergetics that are restored by methylpyruvate, ATP levels were measured in growth factor-deprived cells treated with 3-MA or CQ. After 8 hr of addition of either drug, there was no observable cell death in the culture. Despite this, the IL-3-deprived cells treated with 3-MA (Figure 5F) or CQ (data not shown) experienced a dramatic decline in ATP levels. The ATP decline could be suppressed by supplying a cell-permeant bioenergetic substrate, methylpyruvate. Despite an abundance of oxidizable substrates in the medium including serum lipids, amino acids, and glucose, the IL-3-withdrawn cells were unable to utilize them to maintain ATP production.

### Growth Factor Regulates Autophagy in Primary $Bax^{-/-}Bak^{-/-}$ Bone Marrow Cells

The properties described above were reproduced in two independently-derived IL-3-dependent $Bax^{-/-}Bak^{-/-}$ cell lines. To determine whether these results also applied to primary cells, we isolated $Bax^{-/-}Bak^{-/-}$ bone marrow cells from mice and cultured the cells in the presence or absence of IL-3 for 14 days immediately following isolation. Consistent with the data from the immortalized IL-3-dependent cells, bone marrow cells grown in the absence of IL-3 were smaller, displayed LC3-positive autophagosomes in their cytoplasm, and were dependent on autophagy to support cell survival and ATP production (Figures 6A and 6B). Many cells in the culture retained the ability to grow and proliferate when IL-3 was restored.

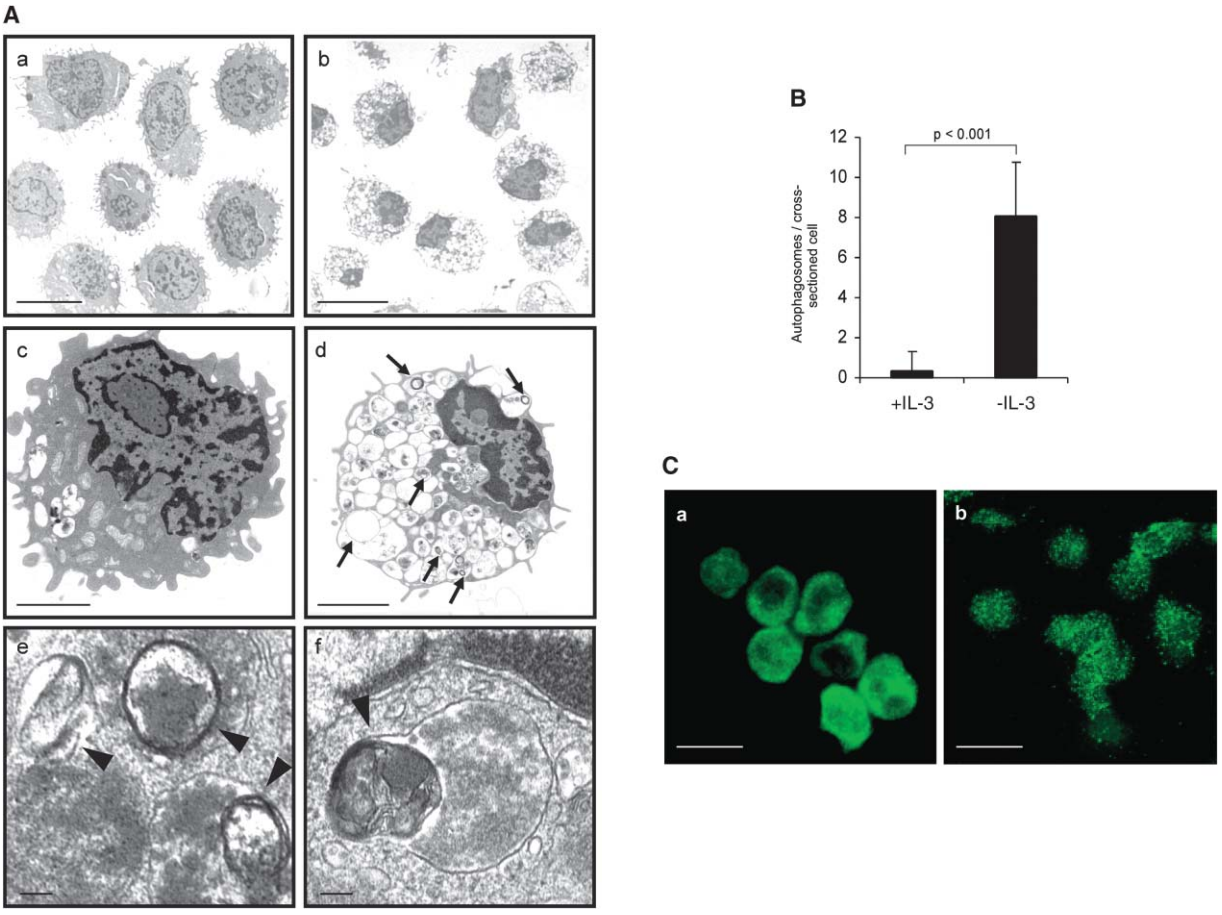



Figure 4. Persistent Autophagy in Long-Term Growth Factor-Withdrawn Cells

(A) Electron microscopy of cells grown in the presence (a) or absence (b) of IL-3 for 6 weeks. Scale bar, 8.5 μm. Magnification image of a cell grown in the presence (c) or absence (d) of IL-3 showing autophagosomes (arrows). Scale bar, 2.3 μm. Higher magnification of cells grown in the absence of IL-3 (e and f). Arrowheads depict autophagosomes in cells containing recognizable cellular material (e) or a late autophagosome fusing with a lysosome (f). Arrowheads depict representative autophagosomes quantitated in (B). Scale bar, 100 nm.

(B) Quantitation of the number of autophagosomes per cross-sectioned cells cultured in the presence or absence of IL-3 for 6 weeks. Error bar represents ±SD. Statistical significance was determined by Student's t test.

(C) Immunofluorescence of cells grown in the presence (a) or absence (b) of IL-3 for 6 weeks probed with anti-LC3 antibodies to detect autophagosomes. Scale bar, 12 μm.

### Growth Factor Readdition Restores Glycolysis and Cell Growth/Proliferation

In unicellular organisms, an important feature of autophagic maintenance of cell survival is the ability of the cells to recover and proliferate if nutrients reappear (Levine and Klionsky, 2004). Despite the loss of cell surface nutrient transporters, the absence of an observable Golgi/ER, and a profound decline in total protein content, the cells cultured in the absence of IL-3 had higher levels of surface IL-3 receptor (Figure 7A) than cells grown in the presence of IL-3. In addition, the IL-3 receptor-activated transcription factor STAT3, a known regulator of GLUT1 expression, was still expressed (data not shown). Therefore, we determined whether the ability of IL-3 to regulate glucose uptake and metabolism was intact. Within 4 hr of IL-3 readdition the glycolytic rate of the cells increased almost 5-fold and by 24 hr increased to levels comparable to those of cells grown in the presence of IL-3 (Figure 7B).

Although glycolysis recovered rapidly following IL-3 readdition, cells did not regain their ability to grow and proliferate immediately. The recovery time for cell size and proliferation varied depending on the length of time the cells had been deprived of IL-3. Virtually all cells deprived of IL-3 for 2 or 6 weeks were ultimately able to recover as measured by cell growth and proliferation when placed in IL-3-containing medium (Figures 7C and 7D). However, the kinetics of recovery were dramatically different depending on the length of time the cells were deprived of IL-3. All of the cells in both cultures began to grow in size in response to IL-3 (Figure 7E). After only 3 days of IL-3, the average cell in the 2 week-deprived cultures had grown from 276 fL to 439 fL. In contrast, even after 11 days of IL-3, the average cell in the 6 week-deprived cultures had only grown from 241 fL to 353 fL (Figure 7E). In comparison to the cells in the 2 week-deprived cultures, it took over a week longer for cells in the 6 week-deprived cultures to begin to divide and

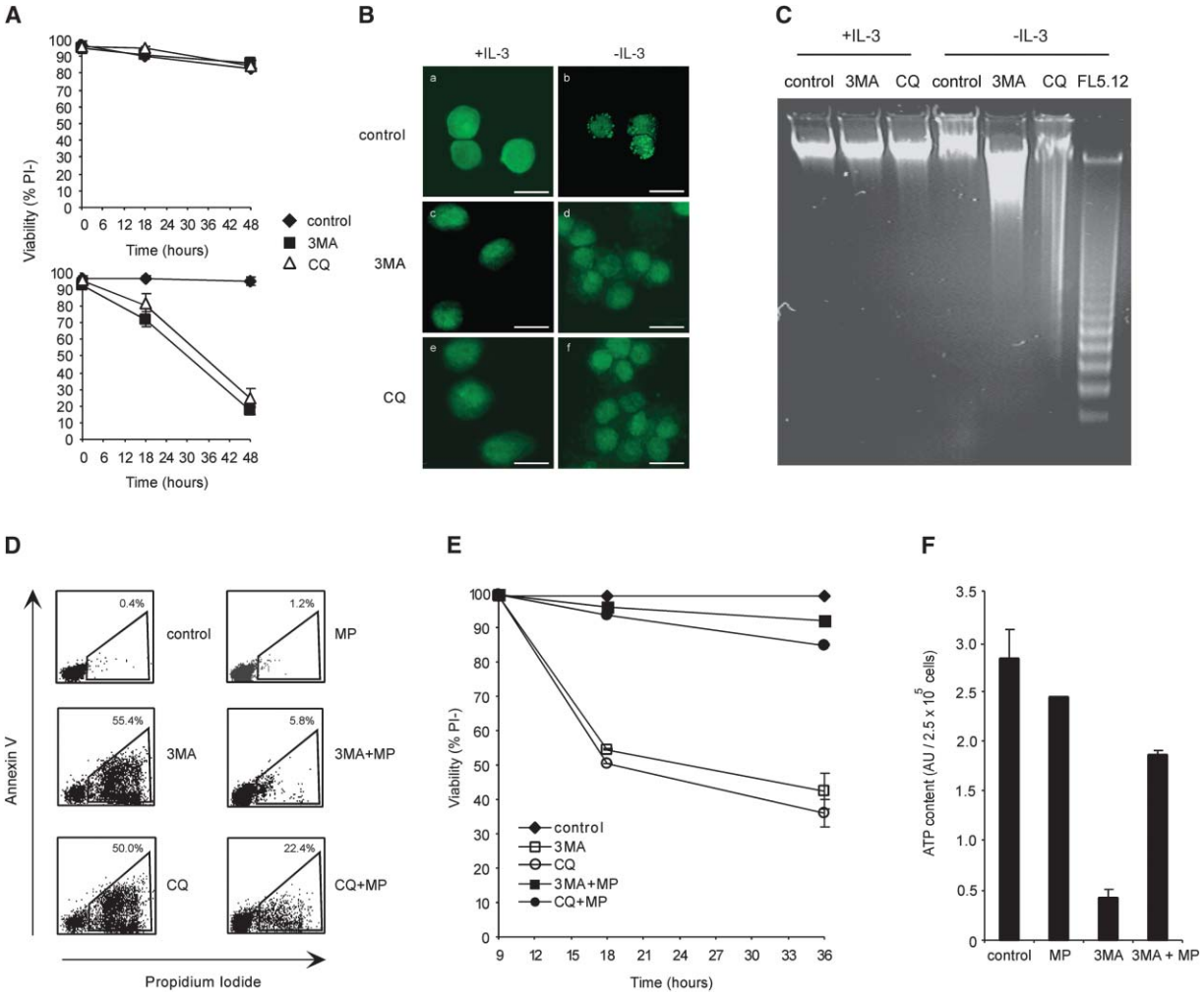



Figure 5. Cell Death Following Inhibition of Autophagy Is Reversed by Methylpyruvate

(A) Viability of cells grown in the presence (top panel) or absence (bottom panel) of IL-3 for 6 weeks treated with 5 mM 3-MA (closed squares) or 10 μM CQ (open triangles). PBS was used as a vehicle control (closed diamonds). Data represent averages of three experiments ± SD.

(B) Immunofluorescence staining of LC3 in cells grown in the presence (a) or absence (b) of IL-3 for 6 weeks. Cells grown in the presence or absence of IL-3 were treated for 18 hr with 5 mM 3-MA (c and d) or 10 μM CQ (e and f) followed by LC3 staining. PBS was used as a vehicle control. Scale bar, 10 μm.

(C) DNA fragmentation assay was performed on $Bax^{-/-}Bak^{-/-}$ cells grown in the presence or absence of IL-3 for 6 weeks and treated for 36 hr with 5 mM 3-MA, 10 μM CQ, or PBS as a vehicle control. IL-3-dependent $Bax^{+/+}Bak^{+/+}$ FL5.12 cells grown in the absence of IL-3 for 36 hr were used as a positive control for DNA laddering.

(D) Viability of cells grown in the absence of IL-3 for 6 weeks after 18 hr of treatment with 5 mM 3-MA or 10 μM CQ in the presence or absence of 10 mM MP.

(E) Viability of cells deprived of IL-3 for 6 weeks that were treated with 5 mM 3-MA or 10 μM CQ (open symbols) in the presence (closed squares and circles) or absence of 10 mM MP. Control cells were left untreated (closed diamonds). Data represent averages of three experiments ± SD.

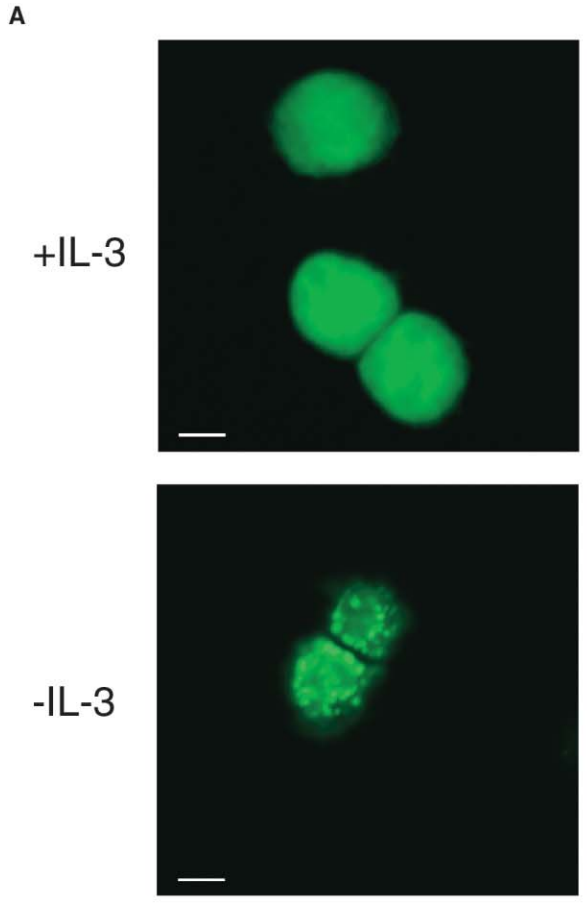
(F) ATP levels of cells grown in the absence of IL-3. Cells withdrawn from IL-3 for 6 weeks were treated with 10 mM MP or 5 mM 3-MA alone or together for 8 hr. ATP levels expressed as AU. Data represent average of three independent experiments ± SD.

accumulate. When fully recovered, both populations had a size distribution and doubling time indistinguishable from the starting population.

## Discussion

### Growth Factors Regulate Exogenous Nutrient Utilization and Survival

The above results suggest that in addition to regulating apoptosis, growth factors promote cell survival by maintaining the ability of cells to take up sufficient nutrients to maintain ATP production and to support self-sustaining macromolecular biosynthesis (anapleurosis). Previous work has suggested that extracellular ligands primarily regulate anabolic processes, hence the collective term growth factors. Anapleurotic processes that are self maintaining have been thought to be intrinsically controlled by cells because the extracellular environment of a healthy animal has an abundant supply of extracellular nutrients. However, the present work suggests that he-



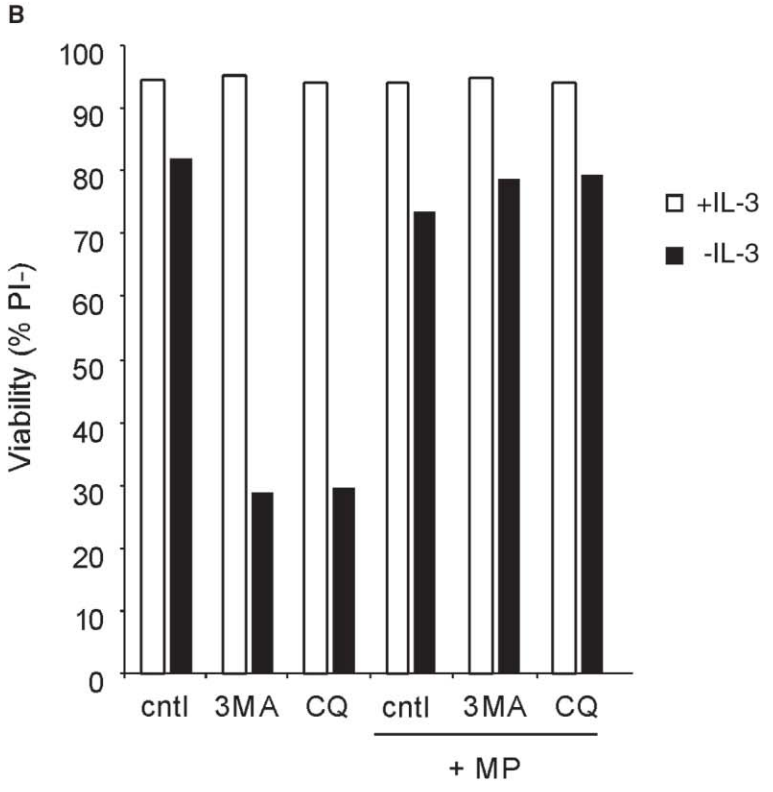




Figure 6. Cell Survival in Primary Bone Marrow $Bax^{-/-}Bak^{-/-}$ Cells Is Controlled by Macroautophagy and Growth Factor Availability

(A) Immunofluorescence staining with anti-LC3 antibodies of cells cultured in the presence or absence of IL-3 for 14 days. Scale bar, 5 μm.

(B) $Bax^{-/-}Bak^{-/-}$ bone marrow cells were cultured in the presence (open bars) or absence (solid bars) of IL-3 for 14 days. On day 14, cells were treated with 5 mM 3-MA or 10 μM CQ in the presence or absence of 10 mM MP. Cell viability was assessed by propidium iodide exclusion 36 hr later.

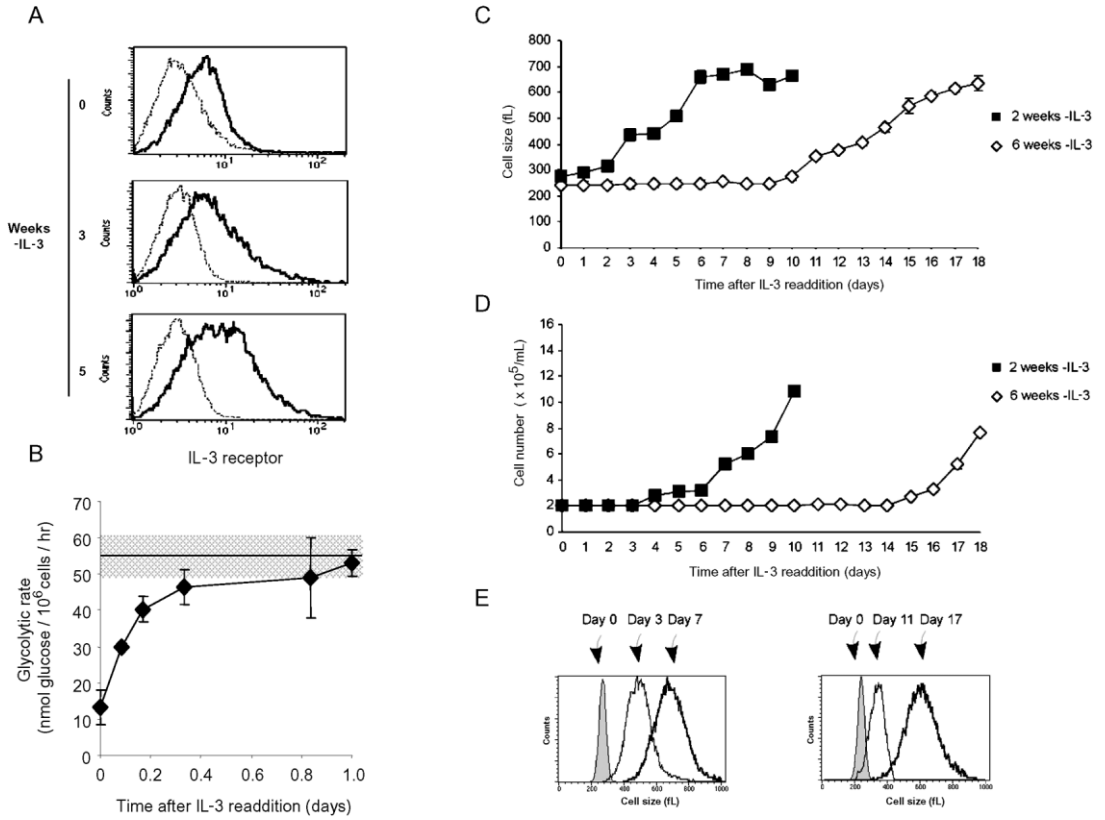



Figure 7. IL-3 Restimulates Glycolysis and Growth/Proliferation in Growth Factor-Deprived Cells

(A) Cell surface staining of IL-3 receptor $\alpha$ chain. Dotted histogram represents isotype control and solid histogram represents IL-3 receptor expression. Representative of three independent experiments.

(B) Glycolytic rate of cells following readdition of IL-3. IL-3 was readded to cells that were cultured in the absence of IL-3 for 4 weeks and collected at the indicated time points for measurement of glycolytic rate. Solid line indicates average glycolytic rate of cells grown in the presence of IL-3 over the time course of the experiment. The hatched area represents $\pm$1 SD. Representative of three independent experiments.

(C and D) Cell size and cell number of cultures cultured without IL-3 for 2 (closed squares) or 6 (open triangles) weeks followed by readdition of IL-3. Data represent average of three experiments $\pm$ SD.

(E) Cell size recovery following IL-3 readdition is dependent on the duration of deprivation. Histogram of mean cell size (fL) in cells restimulated with IL-3 for the indicated number of days following 2 (left panel) or 6 (right panel) weeks of growth factor withdrawal.

matopoietic cells depend on extracellular signals like IL-3 for self-maintenance even when cultured in otherwise complete medium. In the absence of signal transduction by the lineage-specific factor IL-3, receptor-expressing cells undergo progressive atrophy and must use macroautophagy to support a sufficient level of ATP production to maintain viability. Such cellular catabolism can promote cell survival for a number of weeks in the absence of extracellular signals, but this mechanism of promoting cell autonomous survival is necessarily self limited and ultimately results in death unless growth factor is resupplied. Thus, growth factor signal transduction is absolutely required to maintain hematopoietic cell survival.

### Macroautophagy Is a Conserved but Self-Limited Survival Mechanism

Based on the results, macroautophagy appears to be an evolutionarily conserved survival strategy. Macroautophagy can support growth factor-independent cell survival of hematopoietic cells for several weeks. Readdition of growth factor during this period leads to cell recovery. Similarly, in both plants and yeast, survival in response to nutrient deprivation is dependent on macroautophagy (Aubert et al., 1996; Baba et al., 1994; Takeshige et al., 1992; Tsukada and Ohsumi, 1993). Macroautophagy can support survival for several weeks, during which time nutrient readdition supports recovery. Thus, it appears eukaryotic cells share a common survival pathway that promotes cell-autonomous survival in the face of starvation and/or neglect. Animal cells may have evolved an apoptotic response in part to limit this form of cell-autonomous survival. Nevertheless, as previously demonstrated in unicellular organisms, macroautophagy is a self limited survival strategy and ultimately will result in cell death if not reversed.

The catabolic effects of macroautophagy do have significant consequences for the cell. Although cells retain the ability to rapidly respond to growth factor stimulation by upregulating glycolysis, their ability to proliferate in response to growth factor stimulation becomes impaired. For example, cells deprived of IL-3 for 6 weeks take 14 days following IL-3 readdition to reenter S phase. During this time, they must reverse the catabolic effects

of macroautophagy by resynthesizing cellular organelles and cell cycle regulatory proteins.

In contrast to the role of autophagy in promoting cell survival either during nutrient (Aubert et al., 1996; Baba et al., 1994; Melendez et al., 2003; Scott et al., 2004; Takeshige et al., 1992; Tsukada and Ohsumi, 1993) or growth factor deprivation as described here, cell death associated with autophagy has been observed in response to viral infection, ER stress, toxins, and chemotherapy drugs (Daido et al., 2004; Kanzawa et al., 2004; Paglin et al., 2001; Talloczy et al., 2002). In some of these cases, inhibition of autophagy prevents cells from undergoing nonapoptotic death (Yu et al., 2004). These results do not argue against a protective role for autophagy during cellular stress since autophagy may be a strategy to limit cell death by clearance of damaged organelles which can activate apoptosis. However, this compensatory mechanism if overactivated may compromise the ability of a cell to ultimately recover if autophagy-mediated clearance results in complete elimination of an essential organelle.

The concept of nutrient starvation-induced autophagy and its essential role in survival originated from studies in yeast and has begun to be extended to multicellular organisms. In response to starvation, *C. elegans* larvae enter dauer, a latent developmental state. Inactivation of *ATG* homologs disrupts normal dauer formation (Melendez et al., 2003). Recent results suggest that fruit flies also require autophagy to adapt to organismal nutrient starvation (Rusten et al., 2004; Scott et al., 2004). In plants with mutant autophagy genes, nitrogen starvation induces development defects including accelerated senescence and enhanced chlorosis (Hanaoka et al., 2002). These effects on survival are observed in *D. discoideum* where defective fruiting bodies are formed in autophagy mutants in response to nutrient starvation (Otto et al., 2003, 2004). Autophagy is also critical to maintain the survival of neonatal mice during the period between birth and the establishment of their ability to be nursed effectively by their mothers (Kuma et al., 2004). However, while mammalian cells normally have ample nutrient resources in their extracellular environment in the fed state, the present data demonstrate that growth factor withdrawal results in the loss of the cellular ability to utilize extracellular nutrients to maintain themselves. When conserved components of the autophagic program are inactivated, cells succumb to cell death despite abundant extracellular nutrients.

## Implications for the Regulation of Cell Death during Development

Although apoptosis is not absolutely required for growth factor regulation of cell survival, these data may also help explain why apoptosis is so important in the development of animals. Because macroautophagy can maintain cell survival for a number of weeks following growth factor withdrawal, the timescale of growth factor withdrawal-induced death in the absence of apoptosis is too slow to permit effective culling of extraneous cells produced during embryonic patterning. The lack of rapid culling of extraneous cells is most clearly seen during the embryonic development of mice with genetic defects in core apoptotic genes such as $Bax^{-/-}Bak^{-/-}$, APAF-$1^{-/-}$, caspase $3^{-/-}$, or caspase $9^{-/-}$ mice. These deficiencies are associated with perinatal death resulting from the failure to eliminate the excess neurons produced during the development of the central nervous system (CNS) (Lindsten et al., 2000; Woo et al., 1998; Yoshida et al., 1998).

Together, these data suggest that apoptosis is not the only mechanism by which animals can limit the survival of unwanted cells. Elimination of extracellular factors on which cells depend to maintain anapleurotic reactions and bioenergetics will be as effective at eliminating them, albeit more slowly. These results may explain how other multicellular organisms such as plants can limit the survival of cells that accumulate in excess despite the apparent lack of homologs of the central apoptotic control genes in their genomes (Lam, 2004; Pennell and Lamb, 1997; Yu et al., 2002).

## Experimental Procedures

### Cell Culture, Reagents, and Inhibition Assays

Immortalized IL-3-dependent cells were obtained from the bone marrow of two independent $Bax^{-/-}Bak^{-/-}$ mice using previously established protocols (Dexter et al., 1980). Subsequent experiments using these cells were performed in complete media consisting of RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Gemini), 10 units/ml penicillin/streptomycin, and 2 mM L-glutamine (Invitrogen), 50 μm β-mercaptoethanol (Sigma) and 10 mM HEPES (Invitrogen). For cells grown in the presence of IL-3, the complete medium was supplemented with 3.5 ng/ml of murine recombinant IL-3 (BD Pharmingen). For IL-3 withdrawal experiments, cells were washed three times in medium without IL-3 and serum. After the final wash, cells were resuspended in complete medium without IL-3. Medium for cells cultured in the absence of IL-3 was replaced with fresh complete medium without IL-3 every 10 days. To inhibit autophagy, cells cultured in the absence of IL-3 were pelleted and resuspended in IL-3-deficient medium containing either 5 mM 3-methyladenine (Sigma) or 10 μM chloroquine (Sigma) in the presence or absence of 10 mM methylpyruvate (Sigma). Cell size and number were assessed by using a Coulter Z2 particle analyzer. For readdition experiments, cells grown in the absence of IL-3 at the indicated time points were pelleted by centrifugation and resuspended in complete media containing 3.5 ng/ml IL-3. The FL5.12 cell line was maintained in complete media supplemented with 0.35 ng/ml IL-3.

### Primary Bone Marrow Cultures

Primary cell cultures were prepared from $Bax^{-/-}Bak^{-/-}$ bone marrow cells and were cultured in the presence or absence of 3.5 ng/ml recombinant IL-3 in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Gemini), 10 units/ml penicillin/streptomycin and 2 mM L-glutamine (Invitrogen), 50 μm β-mercaptoethanol (Sigma), and 10 mM HEPES (Invitrogen). Nonadherent cells were collected every two days and resuspended in fresh media with or without IL-3.

### Constructs, Retroviral Infections, and RNAi

Bax and Bak were subcloned into pBabe-IRES GFP containing retroviral vectors and transfected into the Phoenix packaging cell line. Viral supernants were used to infect $Bax^{-/-}Bak^{-/-}$ cells in the presence of 10 ng/ml polybrene and 3.5 ng/ml IL-3. Ten days postinfection, single GFP-positive cells were FACS sorted into 96 well plates and expanded as required. Short hairpin RNA constructs were generated against *ATG5* using previously established protocols (Edinger et al., 2003). Briefly, hairpin specific primers were used in a PCR reaction using pEF6-hU6 as a template. The PCR products were subcloned into TOPO-TA, digested with BamHI and EcoRV, and ligated into pKD-GFP. The *ATG5* sense primers were: hp-2 5′ GGC ATT ATC CAA TTG GTT TA, hp-7 5′ GCA GAA CCA TAC TAT TTG CT. Eight micrograms of DNA were introduced into $Bax^{-/-}Bak^{-/-}$

cells by Nucleofector transfection (Amaxa) using program T20. Twenty-four to forty-eight hours posttransfection, GFP-positive cells were FACS sorted, and the resulting population was placed in complete medium supplemented with or without IL-3. Both an oligoribonucleotide for *ATG7* (Yu et al., 2004) and a control oligoribonucleotide were synthesized with an N-terminally-conjugated fluorescein-5-isothiocyanate (FITC) tag (Invitrogen). Each ribonucleotide (0.5 nmol) was introduced into $Bax^{-/-}Bak^{-/-}$ cells by Nucleofector transfection (Amaxa) using program T20. Twenty-four to forty-eight hours posttransfection, FITC-positive cells were FACS sorted and the resulting population was placed in complete medium supplemented with or without IL-3.

### Membrane Potential and Cell Death Assays

For measurement of mitochondrial membrane potential, cells were incubated for 30 min with 50 nM of tetramethyl rhodamine ethyl ester (TMRE; Molecular Probes) in the presence or absence of 50 μM CCCP (Sigma). Viability was performed by incubating cells with annexin V conjugated fluorescein isothiocyanate (BD Pharmingen) in buffer containing 1 μg/ml propidium iodide (Molecular Probes) followed by FACS analysis. DNA fragmentation assay was performed as previously described (Zong et al., 1997).

### Electron Microscopy

Cells were fixed with 2.5% glutaraldehyde/2% formaldehyde with 0.1 M sodium cacodylate and stored at 4°C until embedding. Cells were postfixed with 2% osmium tetroxide followed by an increasing gradient dehydration step using ethanol and propylene oxide. Cells were then embedded in LX-112 medium (Ladd) and sections were cut ultrathin (90 nm), placed on uncoated copper grids, and stained with 0.2% lead citrate and 1% uranyl acetate. Images were examined with a JEOL-1010 electron microscope (JEOL) at 80 kV. For quantitation of autophagosomes, the data obtained from a minimum of 50 independent cells was averaged (mean ± SD)

### Immunoblotting, Immunofluorescence, and Surface Staining

Cells were lysed in RIPA buffer (Zong et al., 2004), and proteins were subjected to SDS-PAGE on 4%–12% NuPAGE gels (Invitrogen) (Zong et al., 2004). Antibodies (all 1:1000 dilution) used were GLUT1 (Research Diagnostic Inc.), calreticulin (StressGen), STAT3 (Cell Signaling), actin (Sigma), LC3 (gift from T. Yoshimori), ATG5 (gift from N. Mizushima), Bax (Santa Cruz), and Bak (Upstate Technologies). For immunofluorescence staining, cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, washed three times (PBS containing 0.01% Triton X-100 and 10% FBS), followed by incubation with anti-rabbit LC3. A 1:100 dilution of Alexa 488 (Molecular Probes) secondary antibody was used. Images were captured using a Zeiss 510 confocal microscope. For surface analysis, cells were fixed in 4% paraformaldehyde, stained with 1:100 biotin-anti-IL-3 α chain receptor (BD Pharmingen) and a 1:100 dilution of streptavidin conjugated fluorescein isothiocyanate (BD Pharmingen) followed by FACS analysis.

### Measurement of Glycolysis and ATP

The conversion of 5-$^3$H-glucose to $^3H_2O$ was used to measure the glycolytic rate (Vander Heiden et al., 2001). The level of ATP was measured as described previously (Vander Heiden et al., 1999).

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