In the absence of apoptosis, myeloid cells arrest when deprived of growth factor, but remain viable without ATG5-dependent autophagy or uptake of glucose or glutamine.

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Summary

Withdrawal of the growth factor interleukin 3 from IL3-dependent myeloid cells causes them to undergo Bax/Bak1-dependent apoptosis, whereas factor-deprived *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells remain viable, but arrest. It was previously reported that withdrawal of IL3 from *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells caused decreased expression of the glucose transporter Glut1, leading to reduced glucose uptake, so that arrested cells required Atg5-dependent autophagy for long-term survival. In other cell types, a decrease in Glut1 is mediated by thioredoxin-interacting protein Txnip, which is induced in IL3-dependent myeloid cells when growth factor is removed. To confirm whether the sustained viability of IL3-deprived *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells required Atg5-dependent autophagy, and to determine whether Txnip was necessary for the reduction in Glut1, we mutated *Atg5* and *Txnip* by CRISPR/Cas9. We found that Atg5-dependent autophagy was not necessary the long-term viability of cycling or arrested *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells, and that Txnip was not required for the decrease in Glut1 expression in response to withdrawal of growth factor. Surprisingly, in glucose-free medium, both wild-type and *Bax*/*Bak1* double mutant cells not only survived, but continued to cycle when IL3 was present. In contrast, *Bax*$^{+/+}$*Bak1*$^{+/+}$ cells died by apoptosis when deprived of glutamine, and while *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells deprived of glutamine survived, they arrested even when IL3 was present. Thus, glutamine, but neither Atg5-dependent autophagy nor glucose uptake, is necessary for cell division, and none are needed for arrested *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells to survive for several weeks.

Introduction

Mammalian cells import a variety of extracellular nutrients to support their survival and growth. *In vitro*, cells are typically propagated in medium supplemented with glucose, glutamine and fetal bovine serum. To generate chemical energy, glucose can be metabolised by oxidative phosphorylation in the mitochondria or by anaerobic glycolysis. Glutamine can be used as an energy source through glutaminolysis, and can also be used, together with a multitude of factors present in serum, to allow the synthesis of essential macromolecules.

Factor dependent myeloid (FDM) progenitor cell lines (Perkins and Cory, 1993; (Ekert et al., 2004; Perkins and Cory, 1993) require the haematopoietic growth factor interleukin 3 (IL3) to survive and multiply in culture. When IL3 is not provided, FDM cells undergo Bax/Bak1-dependent apoptosis. FDM cells derived from *Bax*$^{-/-}$*Bak1*$^{-/-}$ mice survive in the absence of IL3, but they undergo cell cycle arrest, similar to those over-expressing Bcl2 (Lum et al., 2005; Vaux et al., 1988). Previously it was reported that to survive longer than 4 days, IL3-deprived *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells required Atg5-dependent autophagy (Lum et al., 2005), an intracellular catabolic process that can support the survival of starved or arrested cells (Mizushima and Komatsu, 2011).

Cells import glucose when it is available and growth factors can stimulate the amount of glucose that is consumed. *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells arrested by IL3 withdrawal decrease expression of the glucose transporter Glut1, which was proposed to reduce glucose uptake and metabolism (Lum et al., 2005). Several mechanisms have been put forward to explain the regulation of expression and plasma membrane translocation of Glut1. Activation of autophagy was shown to be required for the efficient surface

expression of Glut1 in mouse embryonic fibroblasts cultured under hypoxic conditions (Roy et al., 2017). In human hepatocytes subjected to glucose starvation, a mechanism involving proteasomal degradation of the thioredoxin-interacting protein Txnip was required for Glut1 stabilization (Wu et al., 2013). Moreover, it was shown that Txnip directly binds to Glut1 and promotes its internalization, thus reducing Glut1 on the plasma membrane, and the ability of cells to take up glucose (Wu et al., 2013).

In this study, we employed *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells to genetically test whether Atg5-dependent autophagy is required for the sustained survival of arrested cells deprived of growth factor. We also asked whether autophagy and/or Txnip are required for the reduction in Glut1 expression and glucose uptake by arrested myeloid cells, and whether cells required glucose uptake in order to cycle.

Results

Bax$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells shrink and arrest when deprived of IL3, but survive.*

Factor dependent myeloid (FDM) cells require interleukin 3 (IL3) to survive and proliferate (Dexter et al., 1980; Ekert et al., 2004; Lum et al., 2005). When deprived of IL3 for 4 days, >80 % of wild-type *Bax*$^{+/+}$*Bak1*$^{+/+}$ FDM cells died, as indicated by loss of plasma membrane integrity and uptake of propidium iodide (Figs. 1A and Supplementary S1A). By contrast, and consistent with the findings of Lum *et al.*, (Lum et al., 2005) *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells survived in the absence of IL3 for more than 14 days. After 48 h IL3 withdrawal, these cells shrank in size and arrested (Fig. 1B and C). However, this quiescent state was reversible, because when IL3 was returned, the cells increased in size and resumed cycling. Thus, in the absence of

Bax/Bak1-mediated apoptosis, FDM cells can survive for weeks without IL3, but they shrink and arrest.

$Bax^{-/-}Bak1^{-/-}$ myeloid cells arrested by deprivation of IL3 do not require autophagy to survive.

During macroautophagy, LC3B-I is lipidated by a multiprotein complex containing Atg5 to form LC3B-II, which can incorporate into the growing autophagosome, and is a common marker of autophagy. Lysates of $Bax^{-/-}Bak1^{-/-}$ FDM cells contained similar amounts LC3B-II, whether they were cultured in the presence or absence of IL3 (Fig. 2A, lanes 1 and 2). This suggests autophagy is occurring in both cycling and arrested cells. To determine if autophagy was needed to support the sustained survival of arrested IL3-starved cells, we used CRISPR/Cas9 to delete *Atg5* in $Bax^{-/-}Bak1^{-/-}$ cells, generating $Bax^{-/-}Bak1^{-/-}\Delta Atg5$ triple knockout FDM cell clones. Western blotting confirmed the three $Bax^{-/-}Bak1^{-/-}\Delta Atg5$ clones had much less LC3B-II than the $Bax^{-/-}Bak1^{-/-}$ cells, whether they were cultured with IL3 or not (Fig. 2A). After 14 days culture without IL3, all $Bax^{-/-}Bak1^{-/-}\Delta Atg5$ clones remained ~80% viable (Figs. 2B and Supplementary S1B). Similar to $Bax^{-/-}Bak1^{-/-}$ FDMs, $Bax^{-/-}Bak1^{-/-}\Delta Atg5$ cells arrested and shrank in size within 48 h of growth factor withdrawal (Figs. 2C and D). These results confirm that IL3 receptor signals are needed for $Bax^{-/-}Bak1^{-/-}$ FDM cells to maintain their normal size, but show that Atg5-dependent autophagy is not required for their survival, whether they are cycling or are arrested by IL3 withdrawal. Furthermore, because culturing without IL3 caused a similar decrease in size of the arrested $Bax^{-/-}Bak1^{-/-}$ as of the $Bax^{-/-}Bak1^{-/-}\Delta Atg5$ cells, this shrinkage is not due to Atg5-dependent autophagy.

Bax$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells arrested by IL3 deprivation survive in the absence of glucose.*

Lum *et al*. reported that when growth factor was removed, *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells decreased expression of the glucose transporter Glut1 (Lum et al., 2005). A possible mechanism for this was proposed by Wu et al., who found that thioredoxin-interacting protein (Txnip) could not only bind to Glut1 and induce its internalization through clathrin-coated pits, thus removing it from the plasma membrane, but could also reduce the level of Glut1 messenger mRNA (Wu et al., 2013). Consistent with Lum *et al*. and Wu *et al*., Txnip was increased, and Glut1 was decreased, in lysates of factor-starved *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells 48 h following removal of IL3 (Fig. 3A, lanes 1 and 2).

To determine whether absence of Txnip caused or correlated with changes in Glut1 levels, we deleted *Txnip* in *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells using CRISPR/Cas9. Western blotting confirmed the absence of Txnip protein in three *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Txnip* clones (Fig. 3A). We found that Glut1 levels were unaffected by deletion of *Txnip*, whether IL3 was added or not, indicating that the increase in Txnip was not required for the decrease in Glut1 when cells were cultured without IL3. Furthermore, withdrawal of IL3 caused G1 arrest in *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Txnip* cells as rapidly as in *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells (Fig. 1A and Supplementary Fig. S2A). Because induced over-expression of FLAG-Txnip in *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells had no impact on levels of Glut1 protein or cell cycle progression (Fig. 3B and Supplementary Fig. S2B), an increase in Txnip is not sufficient to reduce Glut1. Therefore, in FDM cells, the abundance of Txnip has no effect on levels of Glut1, whether they are treated with IL3 or not.

Levels of Glut1 were similar among proliferating *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells of all genotypes, including *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clones, and the decrease in Glut1 expression following IL3 withdrawal in *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells was unaffected (Supplementary Fig. S2C). Together, these results indicate that although IL3 can promote expression of Glut1, neither Txnip nor autophagy is required for the decrease in Glut1 when growth factor is removed from myeloid cells, and neither absence nor overexpression of Txnip affects the levels of Glut1.

Because Glut1 levels are reduced in IL3-starved *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells, we asked whether glucose utilization was reduced. The rate of glucose metabolism in *Bax*$^{-/-}$ *Bak1*$^{-/-}$ FDMs was reduced after IL3 withdrawal, but they nevertheless still responded to the addition of glucose, as indicated by the increased extracellular acidification rate (ECAR, Fig. 3C). In the absence of IL3, *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM lines that also lacked Txnip or Atg5 had similar rates of glucose metabolism to the parental Bax/Bak1-null cells (Fig. 3C and Supplementary Fig. S2D). Thus, arrested FDM cells retain the capacity to import and metabolise glucose, albeit at a reduced level.

Because the arrested IL3-starved cells were able to take up glucose, we hypothesised that this was sufficient to support their sustained survival despite the decrease in Glut1. To test their requirement for glucose, the cells were cultured in medium that lacked glucose. Surprisingly, ≥ 80% of IL3-starved *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$ Δ*Txnip* cells remained viable at 24 days and beyond without glucose (Fig. 3D). Furthermore, when IL3 was present, these cells were able to proliferate (Supplementary Fig. S2E). These results indicate that FDM cells will import glucose

whether IL3 is present or not, and that they can still proliferate even in the absence of glucose.

$Bax^{-/-}Bak1^{-/-}$ myeloid cells required glutamine to proliferate, but could survive in its absence for weeks.

When glucose is limiting, lymphoid cells can metabolise glutamine to survive and proliferate (Le et al., 2012). We therefore asked whether FDM cells exhibited similar requirements for glutamine when cultured in the absence of glucose. In medium that lacked both glucose and glutamine, with or without IL3, >95% of wild-type $Bax^{+/+}Bak1^{+/+}$ FDM cells died as indicated by uptake of PI by day 4 (Supplementary Fig. S3A), and consistent with their death being due to apoptosis, the nuclei of the cells had sub-2n DNA (Fig. 4A). Apoptosis was Bax/Bak1 dependent, because Bax/Bak-null cells did not die when cultured in medium lacking glucose and glutamine, but they arrested instead (Fig. 4A and Supplementary Fig. S3B). When IL3 was present, they arrested as large cells, whereas when cultured without IL3, they arrested as small cells (Fig. 4B and Supplementary Fig. S3C).

In medium without glucose, glutamine, or IL3, the viability of $Bax^{-/-}Bak1^{-/-}$ FDM cells only started to decline at day 20 (Fig. 4C). By day 28, ~15% of cells remained PI negative. Interestingly, in the presence of IL3, ~75% of cells cultured with neither glucose nor glutamine continued to survive beyond 28 days. $Bax^{-/-}Bak1^{-/-}\Delta Atg5$ clones cultured in medium lacking glucose and glutamine also maintained ~80% viability at day 16, with or without IL3, and their decline in viability beyond day 20 was similar to that observed for $Bax^{-/-}Bak1^{-/-}$ cells (Supplementary Fig. S3D; data not shown).

When *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells were starved of glutamine, most arrested in G_1 with 2n DNA, but some arrested in G_2/M with 4n DNA (Fig. 4A, D). When glutamine was added back to *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells that had been cultured for 7 days without glucose or glutamine, the arrested cells resumed cycling within 48 h in the presence of IL3, whereas adding back glucose had no effect (Fig. 4D). These results indicate that IL3-treated *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells require glutamine to proliferate, but can survive up to 3 weeks or more in its absence. Because *Bax*$^{-/-}$*Bak1*$^{-/-}$ Δ*Atg5* FDMs behaved similarly to *Bax*$^{-/-}$*Bak1*$^{-/-}$ DKO cells, Atg5-dependent autophagy is not required for cell survival, even in the absence of glucose and glutamine.

Withdrawal of IL3 or glutamine caused reduced expression of Mcl-1 and key regulators of cell cycle progression.

To elucidate how growth factor and nutrient deprivation impacted FDM cell survival and proliferation, we performed Western blotting to determine protein levels of apoptotic and cell cycle regulators. We found that levels of the pro-survival protein Mcl-1 were markedly reduced in *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells after 3 days culture without glucose and glutamine (Fig. 5A), suggesting that the decrease in normal levels of Mcl-1 allowed activation of Bax/Bak1 and the apoptosis of wild-type FDM cells observed in Fig. 4A. Consistent with this notion, addition of a specific Mcl-1 inhibitor S63845 (Kotschy et al., 2016) to *Bax*$^{+/+}$*Bak1*$^{+/+}$ cells was sufficient to cause apoptosis within 24 h, even when the cells were cultured in medium containing glutamine and IL3 (Fig. 5B).

As well as causing arrest, IL3 withdrawal reduced c-Myc protein to barely detectable levels in both *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells (Fig. 5A). Cdk4 and cyclin D3

are key drivers of cell cycle progression in haematopoietic cell lines (Dolezal et al., 2017; Sawai et al., 2012), and these proteins were also markedly reduced in the IL3-starved, Bax/Bak1-deficient myeloid cells (Fig. 5A), suggesting a mechanism for their arrest (Figs. 1C and 2C). Withdrawal of glutamine from these cells also caused cell cycle arrest, even in the presence of IL3 (Fig. 4A, Supplementary Fig. S3B). Under these conditions, IL3 was still able to stimulate the expression of c-Myc and Cdk4, but the levels of these proteins were significantly lower than in the cells cultured with both glutamine and IL3 (Fig. 5A). The transcription factor Atf4 accumulates in response to a variety of cellular stresses including amino acid depletion (Harding et al., 2003). In glutamine-starved *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells, Atf4 levels increase strongly, but intriguingly only in the presence of IL3. These results indicate that the signal transduction pathway activated by ligation of the IL3 receptors remains largely intact in cells lacking glutamine, and can stimulate the cells to remain large, but that glutamine is essential for high levels of c-Myc and Cdk4/cyclin D3 expression, and for the cells to divide.

Bax$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells require serum factors to survive long-term in the absence of glutamine.*

The cells cultured without glutamine or glucose would need a source of energy to survive, so we tested whether they were obtaining it from fetal bovine serum (FBS) in the medium. When cultured in 2% FBS, all *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells died after 4 days, whereas at the same time ~20% of cells cultured with 4% FBS were viable (Fig. 6A). More than 80% of cells remained viable for 16 days and beyond when cultured with 6%, 8% or 10% FBS. Similar results were observed in *Bax*$^{-/-}$*Bak1*$^{-/-}$ Δ*Atg5* cells (Fig. 6B). These data suggest that *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells survive for several weeks

without glucose and glutamine by consuming nutrients in the FBS, rather than by using autophagy.

Discussion

IL3-dependent myeloid cell lines have provided many insights into the regulation of fundamental processes such as cell death, cell division, and autophagy, as well as the signaling pathways that regulate them. For example, it was experiments on an IL3-dependent myeloid cell line that first revealed that the function of Bcl-2 was to inhibit cell death (Vaux et al., 1988). When genes for Bax and Bak1 are deleted in factor-dependent myeloid (FDM) cells, they fail to undergo apoptosis when IL3 is removed, but nevertheless will die if starved of nutrients needed to maintain their metabolism. When *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDMs are cultured without IL3, they decrease expression of the glucose transporter Glut1, shrink, and arrest, but do not die (Lum et al., 2005). This led to the proposal that the cells could not take up glucose, so they needed autophagy to maintain their metabolism. Consistent with this idea, Lum et al. found that inhibition of autophagy either by 3-methyladenine or shRNA knockdown of Atg5 caused the cells to die. However, we also used shRNA to reduce Atg5 levels and found that Atg5-dependent autophagy was not required for the survival *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells deprived of IL3 (Lindqvist et al., 2014). In this report, we have genetically eliminated *Atg5* expression in IL3-dependent *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells using CRISPR/Cas9. Unlike Lum et al., who concluded that autophagy was necessary to sustain the survival of IL3 deprived FDM cells beyond 4 days, our results demonstrate that canonical Atg5-dependent autophagy pathways are not required to support the sustained viability of IL3-deprived *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells (Fig. 2).

We were able to confirm the observations of Lum *et al*. that culture of FDM cells without IL3 reduced the levels of the glucose transporter Glut1 (Lum et al., 2005) (Fig. 3A), and wondered if the mechanism for reduction of Glut1 would be similar to that described by Wu *et al*. who found that thioredoxin interacting protein (Txnip) could reduce the levels of Glut1 in hepatocytes (Wu et al., 2013). Although we found that Txnip increased in cells when IL3 was removed, levels of Glut1 decreased by the same amount when IL3 was removed from Txnip-null cells, and over-expression of exogenous Txnip did not reduce the levels of Glut1 (Figs. 3A and B). Therefore, although we confirmed the reported inverse correlation between levels of Txnip and Glut1, Txnip is neither required nor sufficient for IL3 to regulate levels of Glut1 in FDMs.

Canonical autophagy was shown to be required for the efficient surface expression of Glut1 (Roy et al., 2017). Our Atg5-deficient *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells had similar levels of Glut1 to parental *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells, and they decreased Glut1 to a similar extent when cultured without IL3. Furthermore, the *Bax*$^{-/-}$*Bak1*$^{-/-}$*Atg5*$^{-/-}$ cells showed similar levels of glucose uptake to *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells both when cultured with IL3 and without. Therefore, in FDM cells, Atg5-dependent autophagy is not needed to maintain normal levels of Glut1, and does not affect the ability of cells to take up and metabolize glucose (Figs. S2C and D).

To prove whether reduced uptake of glucose was responsible for the arrest of *Bax*$^{-/-}$ *Bak1*$^{-/-}$ FDM cells when IL3 was removed, we cultured the cells in glucose-free media. We were surprised to find that *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$*Atg5*$^{-/-}$ FDM cells not only remained viable in the absence of glucose when arrested due to culture

without IL3, but that *Bax*$^{-/-}$*Bak1*$^{-/-}$, *Bax*$^{-/-}$*Bak1*$^{-/-}$*Atg5*$^{-/-}$, and even wild-type (*Bax*$^{+/+}$*Bak1*$^{+/+}$) FDM cells not only survived, but also proliferated in the absence of added glucose when IL3 was present (Figs. 3D, S2E; data not shown). Therefore, even though FDM cells will take up and metabolise glucose when it is available, even if they are arrested and their Glut1 levels are decreased, they have no obligatory requirement for glucose, either to survive or to proliferate.

Given that both arrested and proliferating *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells could survive for weeks without glucose or Atg5-dependent autophagy, we wondered if glutamine in the media provided the energy needed for survival and the source of amino acids needed for growth. When cultured with IL3, but in media lacking glutamine, wild-type (*Bax*$^{+/+}$*Bak1*$^{+/+}$) FDM cells died by apoptosis, but *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells remained viable, but they arrested. When IL3 was not added, the *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells cultured without glutamine also arrested and remained viable, but these cells, unlike those cultured with IL3, shank in size (Fig. 4). These experiments show that glutamine is needed for the cells to proliferate, but that they can maintain enough metabolic activity to survive even in its absence.

Several pathways have been proposed to explain how engagement of IL3 receptors prevents activation of Bax/Bak1. For example, it was proposed that IL3 receptors signaled via Akt to phosphorylate Bad, preventing it from binding to Bcl-x, to thereby free Bcl-x to inhibit apoptosis (Datta et al., 1997; Zha et al., 1997). However, experiments in which genes for Bad or Akt have been mutated have shown that unlike Bax/Bak1, they play relatively minor roles in the regulation of apoptosis by IL3 (Ekert et al., 2006; Green et al., 2013). These experiments, and those where genes for

all the Bcl-2 family member were deleted (O'Neill et al., 2016) suggest that Bax/Bak1 dependent apoptosis is not always signaled via BH3-only proteins, but more often regulated by controlling the abundance of anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-x, and Mcl-1 (Goodall et al., 2016). The results presented here are consistent with this, and show that apoptosis of FDMs following the removal of IL3, or culture in the absence of glutamine, is due to the decrease in abundance in Mcl-1 (Fig. 5A), rather than through increased activity of a BH3-only protein. Cytokines including IL3 keeps cells alive by driving *Mcl-1* transcription, most likely via Jak2 and Stat5 (Callus and Mathey-Prevot, 1998; Chao et al., 1998; Moulding et al., 1998), and glutamine prevents default apoptosis by maintaining production of Mcl-1. Consistent with a requirement for Mcl-1 to prevent spontaneous activation of Bax/Bak1, a specific inhibitor of Mcl-1 was able to induce apoptosis of wild-type $Bax^{+/+}Bak1^{+/+}$ FDM cells cultured with IL3 and glutamine (Fig. 5B).

In the absence of glutamine, pathways activated by IL3 could still converge to induce the expression of c-Myc in $Bax^{-/-}Bak1^{-/-}$ FDM cells, and although this was response was blunted, the arrested cells maintained their large size (Figs. 4B and 5A). We found that the transcription factor Atf4 was strongly induced by IL3 in glutamine-starved cells (Fig. 5A), but at present, it is unclear how or why signaling from the IL3 receptor leads to increased Atf4 production. Given that IL3 can stimulate the expression of ER chaperones in FDM cells (Brewer et al., 1997) and that Atf4 translation increased in cells starved of amino acids or exposed to stimuli that induced ER stress (Harding et al., 2003), it is plausible that IL3 activated the unfolded protein response in the glutamine-deprived FDMs. Atf4 is also known to regulate the expression of several autophagy genes (B'Chir et al., 2013), although we found that its

accumulation did not induce LC3B lipidation in the glutamine-starved FDM cells (data not shown). Furthermore, IL3 also induced Atf4 expression in *Bax*$^{-/-}$*Bak1*$^{-/-}$*Atg5*$^{-/-}$ cells cultured in glutamine-free media (Fig. 5A), suggesting that Atf4 does not promote canonical autophagy to maintain cell viability under these conditions. The importance of Atf4 for the sustained survival of *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells arrested by glutamine withdrawal will be determined when we genetically delete *Atf4* in these cells.

In the absence of Atg5-dependent autophagy, glucose, and glutamine, *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells remained viable for weeks, and their sensitivity to low concentrations of fetal bovine serum (Fig. 6) are consistent with the hypothesis that they took up and used components present in serum to maintain enough metabolism in order to survive. Serum factors are likely scavenged by endocytic processes such as macropinocytosis, which can be stimulated by growth factors (Lim et al., 2015). Accordingly, the observation that *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells deprived of glutamine survived longer when growth factor was provided (>4 weeks; Fig. 4C) suggests the possibility that increased macropinocytic uptake of serum proteins prolonged the viability of these cells. We have begun to test whether arrested FDM cells catabolize the ingested serum proteins through lysosomal and/or proteasomal degradation. Preliminary data suggests that, in the absence of growth factor, glucose, glutamine and Atg5-dependent autophagy, arrested *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells survive by utilizing amino acids derived from intracellular proteolysis of ingested serum proteins.

We found that *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells cultured in the absence of glutamine express low levels of the cell cycle regulators Cdk4 and cyclin D3 (Fig. 5A). Reduced levels

of these proteins, combined with possibly altered levels of other cell cycle regulators, could explain why glutamine-starved cells arrested, even in the presence of IL3 that stimulated them to maintain a large size (Fig. 7). Future studies will determine whether glutamine-starved myeloid cells consume serum proteins and how they use these to generate the energy they need to stay viable under conditions of limited nutrients.

Materials & Methods

Cell culture

Wild-type *Bax*$^{+/+}$*Bak1*$^{+/+}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$ IL3-dependent mouse myeloid progenitor cell lines (FDM) have been described (Ekert et al., 2006). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher Scientific Cat# 11885-084; containing 1 g/L glucose and 4 mM glutamine) supplemented with 10% fetal bovine serum (FBS, ThermoFisher Scientific Cat# 16000-044) and 0.25 ng/ml recombinant murine interleukin 3 (IL3, PeproTech, Cat# 213-13) in a 37ºC humidified incubator with 10% CO_2. Cells were deprived of IL3, glucose, or glucose and glutamine by washing 3 times with medium (+10% FBS) that lacked those nutrients, then resuspended in the same medium for analysis at the indicated time points. DMEM lacking D-Glucose and sodium pyruvate (Cat# 11966-025) and DMEM lacking D-Glucose, sodium pyruvate, L-Glutamine and phenol red (Cat# A14430-01) were purchased from ThermoFisher Scientific. D-Glucose was purchased from Sigma (Cat# G7528).

Lentiviral constructs for CRISPR/Cas9 and inducible overexpression

An inducible lentiviral CRISPR/Cas9 system (Aubrey et al., 2015) was used to mutate *Txnip* in *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells. Guide RNA oligos targeting mouse *Txnip* (guide 1: CCA GCA GGT GAG AAC GAG A; guide 2: TAT GGT TGC GTA GAC TAC T) were cloned into the *Bsm*BI site of the pFgH1tUTG GFP lentiviral vector. Guide RNA oligo targeting mouse *Atg5* (AAG ATG TGC TTC GAG ATG TG) was cloned into the *Bsm*BI site of the lentiviral vector lentiCRISPR v2 (Addgene #52961; (Sanjana et al., 2014). For overexpression studies, the coding sequence of mouse *Txnip* was PCR amplified from pEGFP-C1-Txnip (Addgene #18758; Wang et al., 2006) with the incorporation of an N-terminal FLAG-tag. The PCR product was cloned into the *Eco*RI/*Nhe*I sites of a doxycycline inducible pFTREtight MCS rtTAadvanced GFP lentiviral vector (Brumatti et al., 2013).

Antibodies and compounds

Antibodies: LC3B (D11, Cell Signaling Technology #3868), Atg5 (D5F5U, Cell Signaling Technology #12994); β-actin (AC-15, Sigma #A1978), Glut1 (Abcam ab15730), Txnip (D5F3E, Cell Signaling Technology #14715S), Glut4 (1F8, Cell Signaling Technology #2213), Mcl-1 (Rockland 600-401-394S), c-Myc (Abcam ab32072), Cdk4 (Santa Cruz Biotechnology sc-260), Cyclin D3 (Cell Signaling Technology #2936), Atf4 (D4B8, Cell Signaling Technology #11815). Compounds: Mcl-1 inhibitor S63845 was synthesized according to Kotschy et al. (Kotschy et al., 2016) by SYNthesis Med Chem Pty Ltd (Parkville, Australia); DMSO (dimethyl sulfoxide) was purchased from Sigma (Cat# 472301).

Cell death and cell cycle assays

Cell death and cell cycle were assayed using propidium iodide (PI, Sigma) and flow cytometry. For death assays, cells were harvested and resuspended in PBS containing 1 μg/ml PI. For cell cycle analysis, cells were harvested and resuspended in PBS containing 0.1% Triton X-100, 5 μg/ml PI and 10 μg/ml RNaseA (Thermo Scientific), then incubated at 37 °C for 15 min. Samples were processed on FACSCalibur or LSRFortessa X-20 flow cytometers (BD Biosciences) and the acquired data analysed with FlowJo software.

Glucose uptake assays

Extracellular acidification (ECAR) rates were measured in live cells using a Seahorse Bioscience XFe96 Analyzer (Agilent Technologies). In brief, cells were resuspended in non-buffered DMEM (Seahorse Biosciences) and 200 x 10^3 cells were seeded per well in Seahorse Bioscience 96-well plates. The cellular ECAR was analyzed in non-buffered DMEM containing 2 mM glutamine with the following compounds: 5 mM glucose, 1 μM oligomycin and 50 mM 2-deoxy-glucose (2DG). For each assay cycle, measurement time points of 2 min mix, 2 min wait and 5 min measure were collected. Data shown for each cell line are representative of at least 2 independent experiments performed in triplicate.

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Figure Legends

Figure 1. *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells shrink and arrest but remain viable when cultured without IL3.

A. *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells survive long-term in the absence of IL3. *Bax*$^{+/+}$*Bak1*$^{+/+}$ wild-type (black circles) and *Bax*$^{-/-}$*Bak1*$^{-/-}$ (green squares) FDM cells were cultured without IL3. At the indicated times, cells were harvested, resuspended in PBS containing propidium iodide (PI) and analysed by flow cytometry.

B. IL3 withdrawal caused cells to shrink. PI negative populations of *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells cultured for 48 h in the presence (light gray) or absence (dark gray) of IL3, and the latter that had IL3 returned for 72 h (green), were compared by forward scatter as in indicator of cell size.

C. IL3 withdrawal caused cell cycle arrest, which was reversed by re-addition of IL3. Nuclei from *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells cultured as described in (B) were stained with PI to indicate DNA content by flow cytometry.

Figure 2. *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* FDM cells shrink and arrest but remain viable when IL3 is withdrawn.

A. Whole cell lysates from *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells and 3 *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* FDM clonal lines cultured for 48 h in the presence or absence of IL3 were subjected to Western blotting. The Atg5 immunoblot demonstrates successful CRISPR/Cas9-mediated deletion of *Atg5*. The LC3B immunoblot indicates greatly reduced LC3B lipidation (isoform II) in Δ*Atg5* cell clones.

B. *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* FDM cells survive long-term in the absence of IL3. The indicated *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clones were cultured without IL3 and viability was determined by PI uptake at the indicated times.

C. Cells from *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clone 1 were cultured for 48 h in the presence or absence of IL3 then processed in PI buffer to measure DNA content by flow cytometry.

D. *Bax*$^{-/-}$*Bak1*$^{-/-}$ Δ*Atg5* clone 1 cells were cultured for 48 h in the presence or absence of IL3 and PI negative populations were compared by forward scatter to indicate differences in cell size.

Figure 3. *Bax$^{-/-}$Bak1$^{-/-}$ FDM cells arrested by* IL3 *deprivation survive in the absence of glucose.*

A. Txnip is not required for the decrease in Glut1 following IL3 withdrawal. Whole cell lysates from *Bax$^{-/-}$Bak1$^{-/-}$* and 3 independent *Bax$^{-/-}$Bak1$^{-/-}$*Δ*Txnip* FDM cell clones cultured for 48 h in the presence or absence of IL3 were analysed by Western blotting to detect Glut1 and Txnip protein.

B. Forced expression of Txnip is not sufficient to decrease Glut1 protein levels. Independent *Bax$^{-/-}$Bak1$^{-/-}$* FDM clones bearing a doxycycline (dox)-inducible FLAG-Txnip construct or empty vector control were cultured for 72 h in the absence or presence of dox. Whole cell lysates were prepared and analysed by Western blotting to detect Glut1 and Txnip protein.

C. *Bax$^{-/-}$Bak1$^{-/-}$* and *Bax$^{-/-}$Bak1$^{-/-}$*Δ*Txnip* cells can take up and metabolise glucose. Cells cultured for 72 h in the presence or absence of IL3 were analysed on the Seahorse bioanalyzer to measure the extracellular acidification rate (ECAR) as an indicator of glucose metabolism. The increased ECAR following addition of glucose signifies increased glycolytic flux in cells. Oligomycin inhibits mitochondrial ATP synthase and the change in ECAR upon its addition represents the glycolytic reserve capacity of cells. Glycolysis is inhibited by 2-deoxyglucose (2-DG) so upon its addition, the ECAR values are produced from non-glycolytic sources. Equal numbers of cells were seeded for analysis. To correct for the reduced size of IL3-starved cells, protein concentration was assayed and the ECAR values were normalized to 1 μg total protein per sample. Error bars: ±SD, n=3 replicate wells.

D. *Bax$^{-/-}$Bak1$^{-/-}$* and *Bax$^{-/-}$Bak1$^{-/-}$*Δ*Txnip* cells deprived of IL3 survive long-term in the absence of glucose. *Bax$^{-/-}$Bak1$^{-/-}$* cells were cultured without IL3 in complete medium or glucose-free medium and analysed for PI uptake at the indicated times.

Figure 4. When starved of glutamine, *Bax*$^{+/+}$*Bak1*$^{+/+}$ FDM cells undergo apoptosis, whereas *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells arrest, but can survive for weeks.

A. *Bax*$^{+/+}$*Bak1*$^{+/+}$ cells undergo apoptosis in the absence of glutamine (upper panels), whereas *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells survive in the absence of glutamine, but arrest even when IL3 is present (lower panels). Cells were cultured with or without IL3 in glucose- and glutamine-free medium for 4 days then processed in PI buffer to assess DNA content by flow cytometry. Note the absence of cells in S phase when *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells were cultured without glutamine.

B. PI negative populations of *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells cultured with or without IL3 in medium without glucose or glutamine for 4 days were analysed by flow cytometry.

C. *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells deprived of IL3 survive long-term in the absence of glutamine. Cells were cultured with or without IL3 in glucose- and glutamine-free medium. At the indicated times, cells were assayed for viability by PI uptake.

D. *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells require glutamine to divide. Cells were cultured with or without IL3 for 7 days in glucose- and glutamine-free medium. They were then split in three, and resuspended in medium without glucose and glutamine, or containing only glucose (1 g/L), or only glutamine (4 mM), for a further 2 days. Cells were harvested and resuspended in PI buffer to assess DNA content by flow cytometry.

Figure 5. Withdrawal of IL3 or glutamine reduced the expression of Mcl-1 and several key regulators of cell cycle progression.

A. *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells cultured for 72 h with or without IL3 in complete or glucose- and glutamine-free medium. Whole cell lysates were prepared

and analysed by Western blotting to detect the indicated proteins. Immunoblots from 3 independent gels are shown with β-actin loading control blots for each gel.

B. *Bax*$^{+/+}$*Bak1*$^{+/+}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells cultured in complete medium with IL3 were treated with DMSO (vehicle control) or the 10 μM of the Mcl-1 inhibitor S63845 for 24 h. Cells were harvested and viability determined by PI uptake and flow cytometry. Bars depict the mean of 3 replicate cultures represented by the solid symbols.

Figure 6. *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells require serum and proteasome activity to survive in the absence of glutamine.

A. Even though they were incapable of undergoing Bax/Bak1-dependent apoptosis, *Bax*$^{-/-}$*Bak*$^{-/-}$ FDM cells starved of glucose and glutamine died rapidly when cultured in medium with less than 4% fetal bovine serum. *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells were cultured with the indicated concentrations of FBS in medium with IL3 but without supplemented glucose and glutamine. Viability was determined by failure to take up PI at the indicated time points as assessed by flow cytometry.

B. *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* FDM cells starved of glucose and glutamine died rapidly when cultured in medium with less than 4% fetal bovine serum. *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells were cultured and analyzed for viability as described above in (A).

Figure 7.

The growth factor IL3 stimulates the expression of Mcl-1 that inhibits activation of Bax/Bak1, and stimulates expression of c-Myc, Cdk4 and cyclin D3 to promote cell growth and proliferation. Glutamine is required to maintain levels of these proteins needed to prevent activation of Bax/Bak1 (Mcl-1), as well as for cell proliferation (c-Myc, Cdk4, cyclin D3 and possibly others). In the absence of growth factor, glutamine, glucose, and Atg5-dependent autophagy, we hypothesise that *Bax*$^{-/-}$*Bak1*$^{-/-}$

myeloid cells survive by consuming proteins present in fetal bovine serum, and degrading these in lysosomes and proteasomes to maintain a sufficient pool of amino acids for energy production.

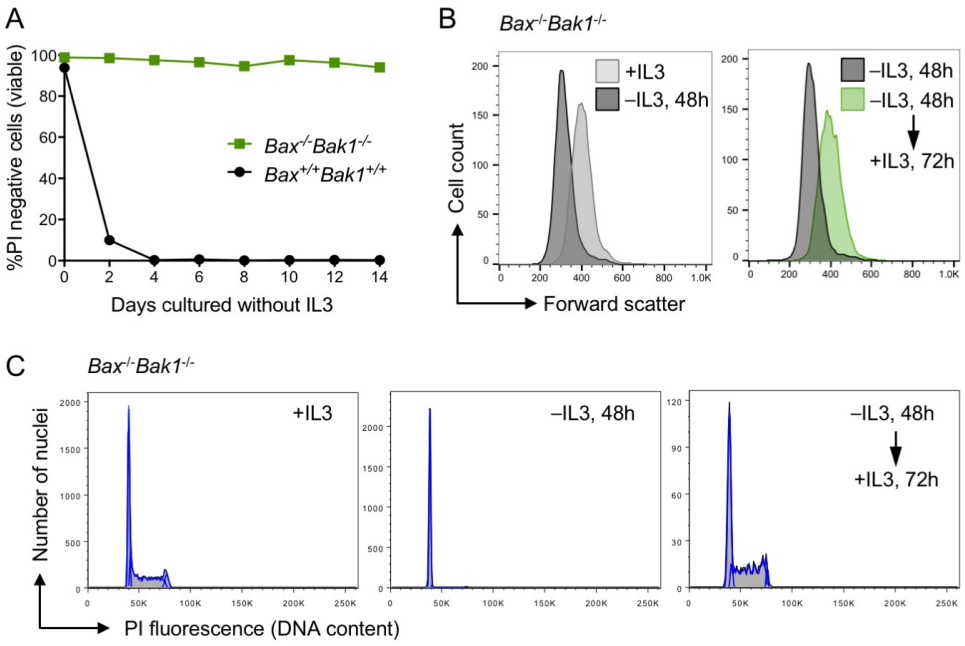
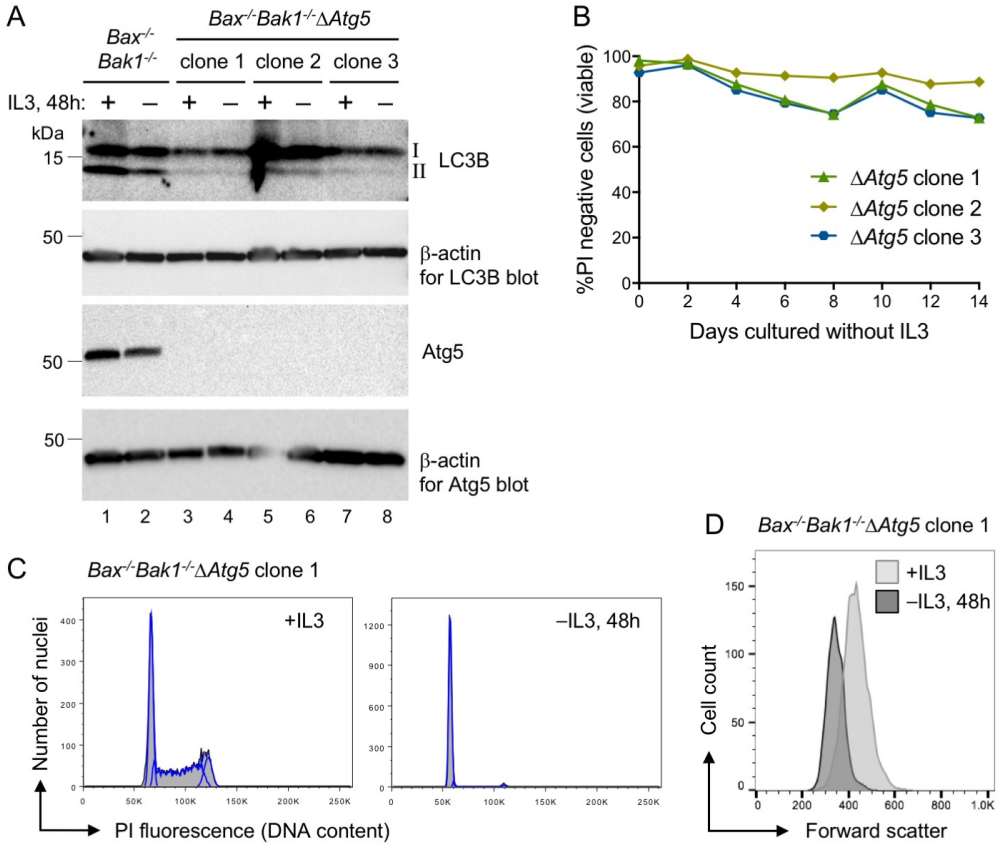
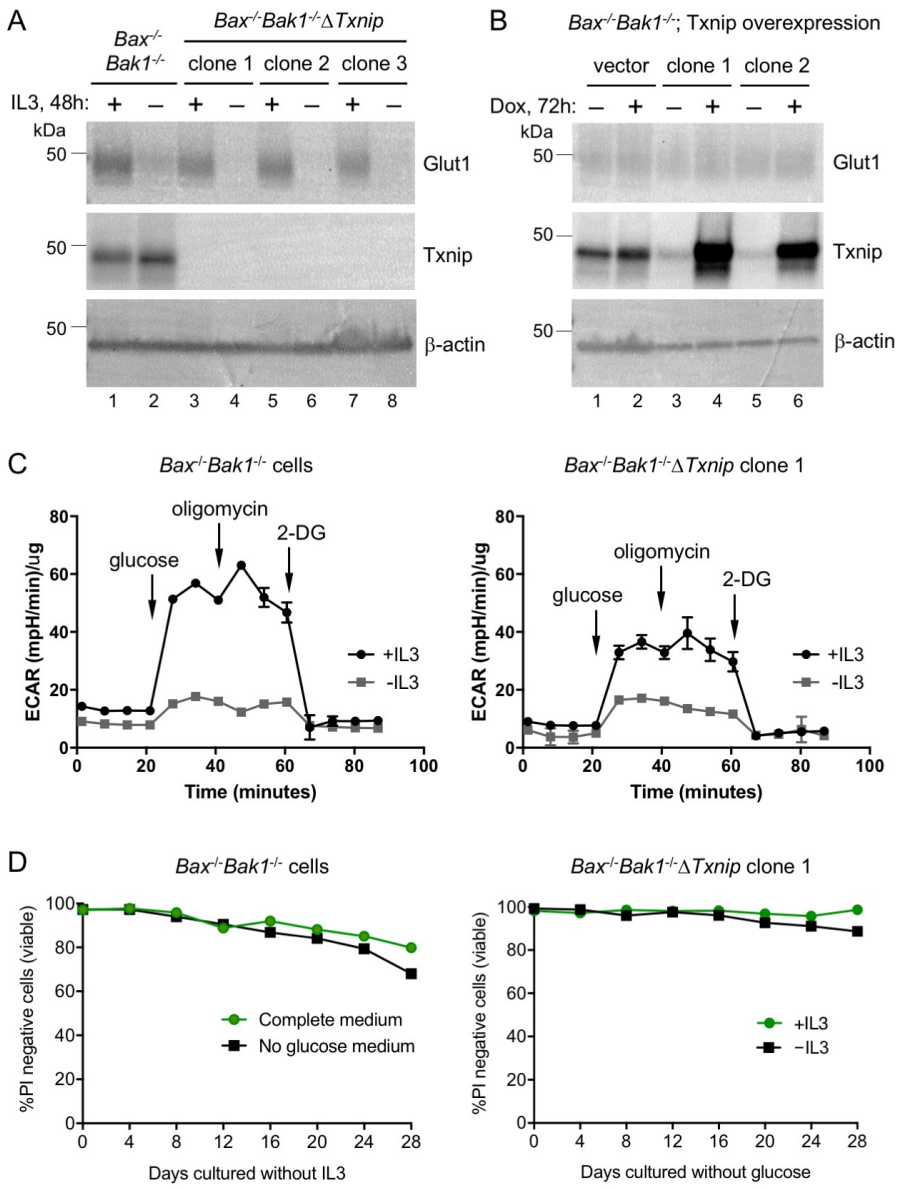
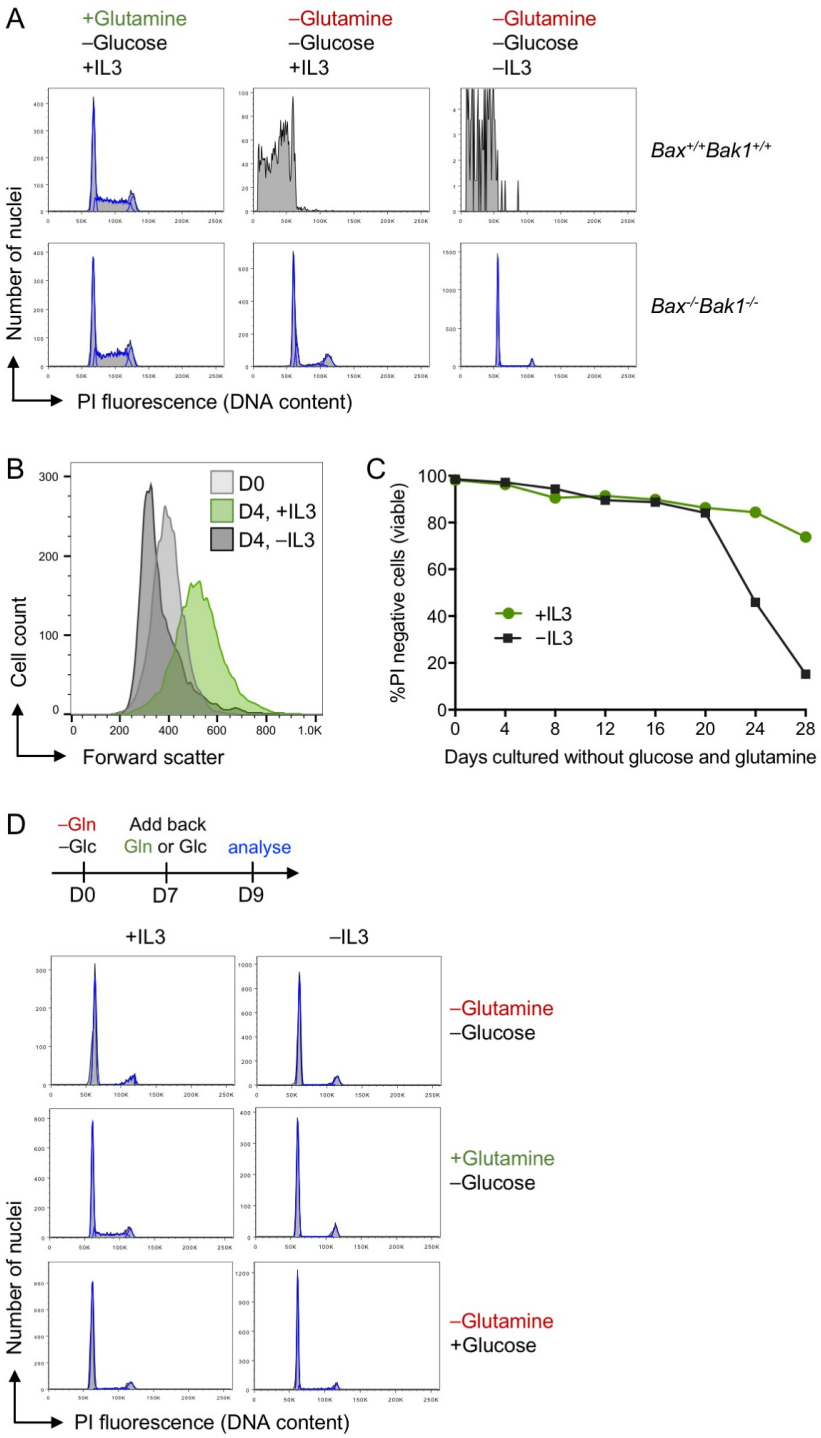
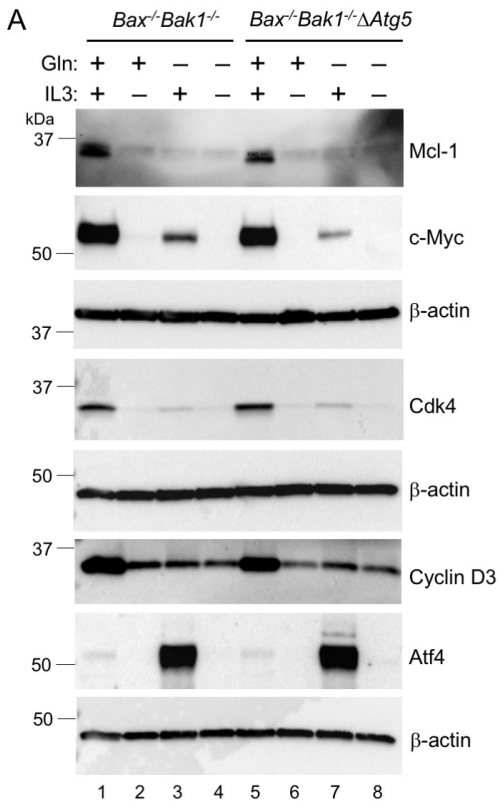

A
%PI negative cells (viable)
Days cultured without IL3
Bax-/-Bak1-/-
Bax+/+Bak1+/+
B
Bax-/-Bak1-/-
Cell count
Forward scatter
+IL3
–IL3, 48h
–IL3, 48h
+IL3, 72h
C
Bax-/-Bak1-/-
Number of nuclei
PI fluorescence (DNA content)
+IL3
–IL3, 48h
–IL3, 48h
+IL3, 72h

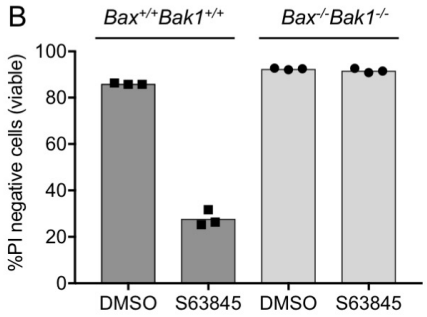
FIGURE 2 Dong et al.

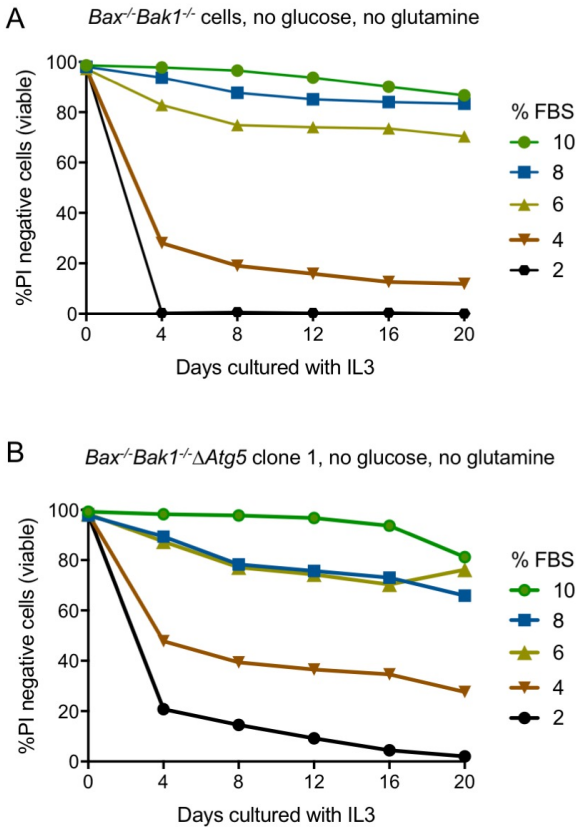


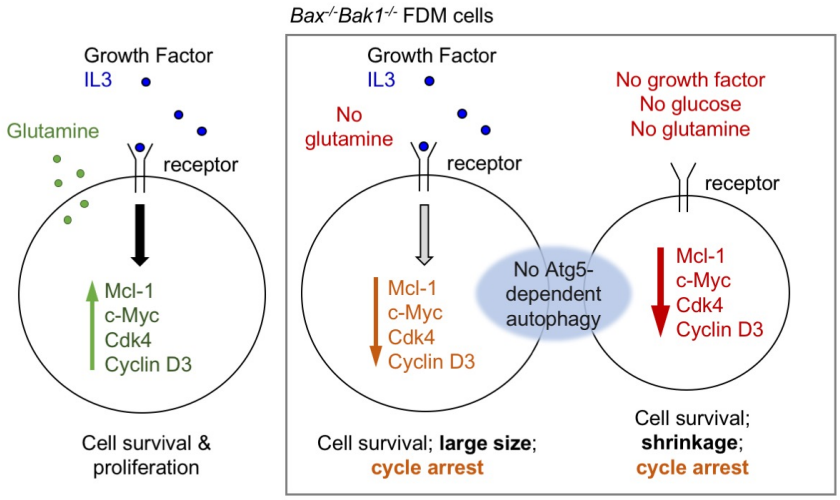

A
Bax-/- Bak1-/-
Bax-/-Bak1-/-ΔTxnip
clone 1 clone 2 clone 3
IL3, 48h: + – + – + – + –
kDa
50
Glut1
Txnip
β-actin
1 2 3 4 5 6 7 8
B
Bax-/-Bak1-/-; Txnip overexpression
vector clone 1 clone 2
Dox, 72h: – + – + – +
1 2 3 4 5 6
C
Bax-/-Bak1-/- cells
Bax-/-Bak1-/-ΔTxnip clone 1
glucose
oligomycin
2-DG
ECAR (mpH/min)/ug
Time (minutes)
+IL3
-IL3
D
Bax-/-Bak1-/- cells
Bax-/-Bak1-/-ΔTxnip clone 1
%PI negative cells (viable)
Complete medium
No glucose medium
Days cultured without IL3
+IL3
−IL3
Days cultured without glucose


A
+Glutamine
–Glucose
+IL3
–Glutamine
–Glucose
+IL3
–Glutamine
–Glucose
–IL3
Bax+/+Bak1+/+
Bax-/-Bak1-/-
Number of nuclei
PI fluorescence (DNA content)
B
D0
D4, +IL3
D4, –IL3
Cell count
Forward scatter
C
%PI negative cells (viable)
+IL3
–IL3
Days cultured without glucose and glutamine
D
–Gln
–Glc
Add back
Gln or Glc
analyse
D0
D7
D9
+IL3
–IL3
–Glutamine
–Glucose
+Glutamine
–Glucose
–Glutamine
+Glucose
Number of nuclei
PI fluorescence (DNA content)


A
Bax-/-Bak1-/-
Bax-/-Bak1-/-ΔAtg5
Gln: + + – – + + – –
IL3: + – + – + – + –
kDa
37
Mcl-1
50
c-Myc
37
β-actin
37
Cdk4
50
β-actin
37
Cyclin D3
50
Atf4
50
β-actin
1 2 3 4 5 6 7 8


B
Bax+/+Bak1+/+
Bax-/-Bak1-/-
%PI negative cells (viable)
100
80
60
40
20
0
DMSO
S63845
DMSO
S63845


A
Bax-/-Bak1-/- cells, no glucose, no glutamine
%PI negative cells (viable)
100
80
60
40
20
0
0
4
8
12
16
20
Days cultured with IL3
% FBS
10
8
6
4
2
B
Bax-/-Bak1-/-ΔAtg5 clone 1, no glucose, no glutamine
%PI negative cells (viable)
100
80
60
40
20
0
0
4
8
12
16
20
Days cultured with IL3
% FBS
10
8
6
4
2


Bax-/-Bak1-/- FDM cells
Growth Factor
IL3
Glutamine
receptor
Mcl-1
c-Myc
Cdk4
Cyclin D3
Cell survival &
proliferation
Growth Factor
IL3
No
glutamine
receptor
Mcl-1
c-Myc
Cdk4
Cyclin D3
Cell survival; large size;
cycle arrest
No Atg5-
dependent
autophagy
No growth factor
No glucose
No glutamine
receptor
Mcl-1
c-Myc
Cdk4
Cyclin D3
Cell survival;
shrinkage;
cycle arrest