In the Absence of Apoptosis, Myeloid Cells Arrest When Deprived of Growth Factor, But Remain Viable by Consuming Extracellular Glucose

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Running Head: Cell Survival Without ATG5-dependent Autophagy

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Abstract

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 and shrink. It was 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 the thioredoxin-interacting protein Txnip, which is induced in IL3-dependent myeloid cells when growth factor is removed. We mutated *Atg5* and *Txnip* by CRISPR/Cas9 and found that Atg5-dependent autophagy was not necessary for 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 IL3 withdrawal. Surprisingly, Atg5-deficient *Bax*/*Bak1* double mutant cells survived for several weeks in medium supplemented with 10% fetal bovine serum (FBS), without high concentrations of added glucose or glutamine. When serum was withdrawn, the provision of an equivalent amount of glucose present in 10% FBS (~0.5 mM) was sufficient to support cell survival for more than a week, in the presence or absence of IL3. Thus, *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells deprived of growth factor consume extracellular glucose to maintain long-term viability, without a requirement for Atg5-dependent autophagy.

51 **Introduction**

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

59

60 Factor dependent myeloid (FDM) progenitor cell lines[1, 2] require the hematopoietic
61 growth factor interleukin 3 (IL3) to survive and multiply in culture. When IL3 is not
62 provided, FDM cells undergo Bax/Bak1-dependent apoptosis. FDM cells derived
63 from *Bax*$^{-/-}$*Bak1*$^{-/-}$ mice survive in the absence of IL3, but they undergo cell cycle
64 arrest, similar to those over-expressing Bcl-2[3, 4]. It has been reported that to survive
65 longer than 4 days, IL3-deprived *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells required Atg5-dependent
66 autophagy[4], an intracellular catabolic process that can support the survival of starved
67 or arrested cells[5].

68

69 Cells import glucose when it is available and growth factors can stimulate the amount
70 of glucose that is consumed. *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells arrested by IL3 withdrawal
71 decrease expression of the glucose transporter Glut1, which was proposed to reduce
72 glucose uptake and metabolism[4]. Several mechanisms have been put forward to
73 explain the regulation of expression and plasma membrane translocation of Glut1.
74 Activation of autophagy was shown to be required for the efficient surface expression
75 of Glut1 in mouse embryonic fibroblasts cultured under hypoxic conditions[6]. In

76 human hepatocytes subjected to glucose starvation, a mechanism involving
77 proteasomal degradation of the thioredoxin-interacting protein Txnip was required for
78 Glut1 stabilization[7]. Moreover, it was shown that Txnip directly binds to Glut1 and
79 promotes its internalization, thus reducing Glut1 on the plasma membrane and the
80 ability of cells to take up glucose[7].
81
82 In this study, we employed *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells to genetically test whether Atg5-
83 dependent autophagy is required for the sustained survival of arrested cells deprived
84 of growth factor. We also asked whether autophagy and/or Txnip are required for the
85 reduction in Glut1 expression and glucose uptake by arrested myeloid cells.
86
87 **Results**
88 *Bax*$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells shrink and arrest when deprived of IL3, but survive.*
89 Factor dependent myeloid (FDM) cells require interleukin 3 (IL3) to survive and
90 proliferate[2, 4, 8]. When deprived of IL3 for 4 days, >80% of wild-type *Bax*$^{+/+}$*Bak1*$^{+/+}$
91 FDM cells died, as indicated by loss of plasma membrane integrity and uptake of
92 propidium iodide (Figs. 1A and Supplementary S1A). By contrast, and consistent with
93 the findings of Lum *et al*.[4], *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells survived in the absence of IL3 for
94 more than 14 days. After 48 h IL3 withdrawal, these cells shrank in size and arrested
95 (Fig. 1, B and C). This quiescent state was reversible, because when IL3 was returned,
96 the cells increased in size and resumed cycling. Thus, in the absence of Bax/Bak1-
97 mediated apoptosis, FDM cells can survive for weeks without IL3, but they shrink
98 and arrest.
99

100 *Bax*$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells arrested by deprivation of IL3 do not require Atg5-*
101 *dependent autophagy to survive.*
102 During macroautophagy, LC3B-I is lipidated by a multiprotein complex containing
103 Atg5 to form LC3B-II, which is incorporated into the growing autophagosome, and is
104 commonly used as a marker of autophagy[9]. Lysates of *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells,
105 whether they were cultured in the presence or absence of IL3, contained similar
106 amounts LC3B-II, suggesting that autophagy is occurring in both cycling and arrested
107 cells (Fig. 2A; lanes 1, 3 and 4). Notably, the low levels of both LC3B isoforms I and
108 II in *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells cultured in serum, amino acid and glucose-free Hank's
109 balanced salt solution (HBSS; Fig. 2A, lane 2) is indicative of a strong increase in
110 autophagic flux, which causes the coincident degradation of LC3B proteins after
111 autophagosomes fuse with lysosomes[9].
112
113 To determine if Atg5-dependent autophagy was needed to support the sustained
114 survival of IL3-starved cells, we used CRISPR/Cas9 to delete *Atg5* in *Bax*$^{-/-}$*Bak1*$^{-/-}$
115 cells, generating *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* triple knockout FDM cell clones. Western
116 blotting confirmed in three *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clones the absence of the LC3B-II
117 isoform, whether they were cultured with or without IL3, or in HBSS (Fig. 2A).
118 Moreover, levels of LC3B-I in *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clones remained similar across the
119 different culture conditions. These results indicate that Atg5-dependent autophagy and
120 autophagic flux are blocked in the *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* lines. After 14 days cultured
121 without IL3, all *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clones remained ~80% viable (Fig. 2B). Similar to
122 *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDMs, *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells decreased in size and arrested within 48 h
123 of growth factor withdrawal (Fig. 2, C and D).
124

125 These results confirm that IL3 receptor signals are needed for *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells
126 to maintain their normal size and divide, but show that Atg5-dependent autophagy is
127 not required for their survival, whether they are cycling or are arrested by IL3
128 withdrawal. Furthermore, because culturing without IL3 caused a similar decrease in
129 size of the arrested *Bax*$^{-/-}$*Bak1*$^{-/-}$ to that of the *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells, this shrinkage is
130 not due to Atg5-dependent autophagy.
131
132 *Bax*$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells arrested by IL3 deprivation downregulate Glut1*
133 *independently of Txnip or Atg5 and survive in the absence of added glucose.*
134 Lum *et al*. reported that when IL3 growth factor was removed, *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM
135 cells decreased expression of the glucose transporter Glut1[4]. A possible mechanism
136 for this was proposed by Wu *et al*., who found that thioredoxin-interacting protein
137 (Txnip) could not only bind to Glut1 and induce its internalization through clathrin-
138 coated pits, thus removing it from the plasma membrane, but could also reduce the
139 level of Glut1 messenger mRNA[7]. Consistent with Lum *et al*. and Wu *et al*., levels of
140 Txnip increased, and those of Glut1 decreased, in lysates of *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells 48 h
141 following removal of IL3 (Fig. 3A, lane 2).
142
143 To determine whether expression of Txnip caused or correlated with changes in Glut1
144 levels, we deleted *Txnip* in *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells using CRISPR/Cas9. Western
145 blotting confirmed the absence of Txnip protein in three *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Txnip* clonal
146 lines (Fig. 3A). We found that Glut1 levels were unaffected by deletion of *Txnip*,
147 whether IL3 was added or not, indicating that the increase in Txnip was not required
148 for the decrease in Glut1 when cells were cultured without IL3. Furthermore, similar
149 to *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells, withdrawal of IL3 caused G_1 arrest in *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Txnip* cells

150 within 48 h (Supplementary Fig. S1A). Because induced over-expression of FLAG-
151 Txnip in *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells had no impact on levels of Glut1 protein or cell cycle
152 progression (Figs. 3B and Supplementary S1B), an increase in Txnip is not sufficient
153 to reduce Glut1. Therefore, in FDM cells, the abundance of Txnip has no effect on
154 levels of Glut1, whether they are treated with IL3 or not. Of note, IL3 deprivation
155 caused specific downregulation of Glut1, but not Glut4, protein in *Bax*$^{-/-}$*Bak1*$^{-/-}$ and
156 *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clones; and levels of Glut1 were similar among proliferating
157 Bax/Bak1-null cells of all genotypes (Figs. 3A, B and Supplementary Fig. S1C).
158 Together, these results indicate that although IL3 can promote the expression of
159 Glut1, neither Txnip nor Atg5-dependent autophagy is required for the decrease in
160 Glut1 when growth factor is removed from myeloid cells, and neither absence nor
161 overexpression of Txnip affects the levels of Glut1.
162
163 Because Glut1 levels are reduced in IL3-starved *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells, we asked
164 whether glucose utilization was reduced. Proliferating *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells in the
165 presence of IL3 responded strongly to the addition of glucose, as indicated by the
166 increased extracellular acidification rate (ECAR, Fig. 3C), signifying that they readily
167 consumed and metabolized the added glucose. Following 3 days cultured without IL3,
168 the rate of glucose metabolism in *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells was reduced, but they
169 nevertheless still responded to the addition of glucose. With or without IL3, the rates
170 of glucose metabolism in *Bax*$^{-/-}$*Bak1*$^{-/-}$ lines that also lacked Txnip or Atg5 were
171 similar to the rates of the parental Bax/Bak1-null cells (Figs. 3C and Supplementary
172 S1D). When cells were treated with oligomycin to block oxidative phosphorylation
173 shortly after they reached their maximal glycolytic rate, the steady decline in ECAR
174 continued relatively unchanged until glycolysis was inhibited with 2-deoxyglucose (2-

175 DG), causing ECAR values to fall rapidly to baseline. These results indicate that
176 FDM cells rely predominantly on glycolysis for energy production, and when
177 deprived of IL3, they retain the capacity to import and metabolise glucose, albeit at a
178 reduced level.
179
180 Because IL3-starved *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDMs took up and metabolized glucose, we
181 hypothesised that this was sufficient to support their sustained survival despite the
182 decrease in Glut1. For FDM cell culture, we used DMEM medium supplemented with
183 4 mM glutamine, 5.5 mM glucose and 10% fetal bovine serum (FBS), estimated to
184 contain ~0.5 mM glucose (Supplementary Fig. S1E). To test their requirement for
185 glucose, the cells were cultured in medium supplemented with 4 mM glutamine and
186 10% FBS, but lacking the added 5.5 mM glucose. Surprisingly, > 80% of IL3-starved
187 *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Txnip* cells remained viable for 3 weeks in culture
188 without added glucose (Fig. 3D). Furthermore, when IL3 was present, these cells were
189 able to proliferate (Supplementary Fig. S1F). These results suggest that FDM cells do
190 not require high concentrations of glucose to maintain long-term viability, whether
191 IL3 is present or not, and that they consume the glucose (and other nutrients) present
192 in FBS and/or the added glutamine to support survival or to multiply when stimulated
193 with growth factor.
194
195 *Bax*$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells require glutamine to proliferate but can survive in its*
196 *absence for weeks.*
197 When glucose is limiting, lymphoid cells metabolise glutamine to survive and
198 proliferate[10]. We therefore asked whether IL3-dependent myeloid cells exhibited
199 similar requirements for glutamine. In medium supplemented with 10% FBS but

200 without added glucose or glutamine, with or without IL3, >95% of wild-type
201 *Bax*$^{+/+}$*Bak1*$^{+/+}$ FDM cells died as indicated by uptake of PI by day 4 (Supplementary
202 Fig. S2A), and consistent with their death being due to apoptosis, the nuclei of the
203 cells had sub-2n DNA (Fig. 4A). Apoptosis was Bax/Bak1-dependent, because
204 Bax/Bak1-null cells, including clones also lacking Atg5, did not die when cultured in
205 medium without added glucose and glutamine, but they arrested instead, even when
206 IL3 was provided (Fig. 4A). When cultured with neither added glucose nor glutamine,
207 in the presence or absence of IL3, >80% of the arrested *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$
208 Δ*Atg5* cells survived for more than 12 days (Figs. 4B and Supplementary S2B).
209
210 When *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells were starved of glucose and glutamine, most arrested in G_1
211 with 2n DNA, but some arrested in G_2/M with 4n DNA (Fig. 4A). When glutamine
212 was added back to *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells that had been cultured for 7 days in glucose- and
213 glutamine-free medium, the arrested cells resumed cycling within 48 h in the presence
214 of IL3, whereas adding back glucose had no effect (Supplementary Fig. S2C). These
215 results indicate that IL3-treated *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells require glutamine to
216 proliferate, but can survive for weeks in its absence. Because *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells
217 behaved similarly to *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells, Atg5-dependent autophagy is not required for
218 the sustained survival of growth factor-deprived *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDMs, even in cultures
219 without high levels of glucose and glutamine.
220
221 *Bax*$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells arrested by glutamine deprivation shrink in a manner*
222 *independent of Atg5-mediated autophagy.*
223 Because *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells arrested by IL3 withdrawal
224 decreased in size (Figs. 1B and 2C), we asked whether cells arrested by glutamine

225 withdrawal also shrink. In medium containing 10% FBS but lacking added glucose
226 and glutamine, IL3-treated *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells showed a gradual
227 decrease in size to almost 1/3 of their original volume over a 20-day period (Fig. 5).
228 Without IL3, the shrinkage was more acute and cells reached ~1/3 of their original
229 volume within 7 days. These results suggest that IL3 provided a signal to *Bax*$^{-/-}$*Bak1*$^{-/-}$
230 myeloid cells arrested by glutamine deprivation to maintain a large size, and confirm
231 that the shrinkage of these cells upon IL3 and/or nutrient withdrawal do not require
232 Atg5-dependent autophagy.
233
234 *Withdrawal of IL3 or glutamine caused reduced expression of Mcl-1 and key*
235 *regulators of cell cycle progression.*
236 To elucidate how growth factor and nutrient deprivation impacted FDM cell survival
237 and proliferation, we performed Western blotting to determine protein levels of
238 apoptotic and cell cycle regulators. We found that levels of the pro-survival protein
239 Mcl-1 were markedly reduced in *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells after 3 days
240 in culture without added glucose and glutamine (Fig. 6A), suggesting that the
241 decrease in normal levels of Mcl-1 allowed activation of Bax/Bak1 and the apoptosis
242 of wild-type FDM cells observed in Fig. 4A. Consistent with this notion, addition of a
243 specific Mcl-1 inhibitor S63845[11] to *Bax*$^{+/+}$*Bak1*$^{+/+}$ cells was sufficient to cause
244 significant apoptosis within 24 h, even when the cells were cultured with IL3 in
245 complete medium containing 10% FBS and high concentrations of glucose and
246 glutamine (Fig. 6B).
247
248 As well as causing arrest, IL3 withdrawal reduced c-Myc protein to barely detectable
249 levels in both *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells (Fig. 6A). Cdk4 and cyclin D3

250 are key drivers of cell cycle progression in haematopoietic cell lines[12, 13], and these
251 proteins were also markedly reduced in the IL3-starved, Bax/Bak1-deficient myeloid
252 cells, suggesting a mechanism for their arrest (Figs. 1C and 2D). Withdrawal of
253 glutamine from these cells also caused cell cycle arrest, even in the presence of IL3
254 (Fig. 4A). Under these conditions, IL3 was able to stimulate the expression of c-Myc
255 and Cdk4, but the levels of these proteins were significantly lower than in the cells
256 cultured with both glutamine and IL3 (Fig. 6A). The transcription factor Atf4
257 accumulates in response to a variety of cellular stresses including amino acid
258 starvation[14]. In glutamine-starved *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells, Atf4
259 levels increase strongly, but intriguingly only in the presence of IL3. These results
260 indicate that the signal transduction pathway activated by ligation of the IL3 receptors
261 remains largely intact in cells cultured without supplemented glutamine, and can
262 stimulate the cells to remain large, but that glutamine is essential for high levels of c-
263 Myc and Cdk4/cyclin D3 expression, and for the cells to divide.
264
265 *Bax*$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells consume extracellular glucose to maintain long-term*
266 *viability.*
267 The Bax/Bak1-null cells cultured without added glucose and glutamine would need a
268 source of energy to survive (Figs. 4B and S2B), so we tested whether they were
269 obtaining it from the supplemented FBS. In IL3-containing medium lacking added
270 glucose, glutamine and FBS, all *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* died after 2 days
271 (Fig. 7A). In the absence of serum, we asked if adding back glutamine or glucose
272 alone was sufficient to support cell survival. While <40% of cells cultured in medium
273 supplemented with 4mM glutamine remained viable after 2 days (and all died within
274 4 days, Supplementary Fig. S3A), >90% of cells cultured with 5.5 mM glucose

275 survived (for more than 6 days, Supplementary Fig. S3B). In IL3-starved cultures, we
276 observed a similar dependence on glucose, but not glutamine, for cell viability (Fig.
277 7B). Strikingly, we found that 0.5 mM glucose only (an equivalent amount present in
278 10% FBS) was sufficient to support the survival of >80% of IL3-deprived *Bax*$^{-/-}$*Bak1*$^{-}$
279 $^{/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells (Fig. 7B) for at least 1 week (Fig. 7C). Collectively,
280 these results suggest that in the absence of growth factor, *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells
281 consume extracellular glucose to maintain long-term viability, without the need to
282 invoke Atg5-dependent autophagy.
283

284 **Discussion**

285 IL3-dependent myeloid cell lines have provided many insights into the regulation of
286 fundamental processes such as cell death, cell division, and autophagy, as well as the
287 signaling pathways that regulate them. For example, it was experiments on an IL3-
288 dependent myeloid cell line that first revealed that the function of Bcl-2 was to inhibit
289 cell death[3]. When genes for Bax and Bak1 are deleted in factor-dependent myeloid
290 (FDM) cells, they fail to undergo apoptosis when IL3 is removed, but nevertheless
291 will die if starved of nutrients needed to maintain their metabolism. When *Bax*$^{-/-}$*Bak1*$^{-}$
292 $^{/-}$ FDMs are cultured without IL3, they decrease expression of the glucose transporter
293 Glut1, shrink and arrest, but do not die[4]. This led to the proposal that the cells could
294 not take up glucose, so they needed autophagy to maintain their metabolism to
295 survive. Consistent with this idea, Lum *et al.* found that inhibition of autophagy either
296 by 3-methyladenine, chloroquine or shRNA knockdown of Atg5 caused the cells to
297 die[4]. However, we also used chloroquine to inhibit autophagy and shRNA to reduce
298 Atg5 levels, and found that Atg5-dependent autophagy was not required for the
299 survival *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells deprived of IL3[15]. It is conceivable that the two

300 Atg5-targeting shRNA sequences used by Lum *et al*., which is different to our
301 shRNA, had off-target effects (such as induction of interferons) that sensitized their
302 cells to autophagy inhibition. Notwithstanding, it is unlikely that off-target effects of
303 chloroquine caused such contrasting survival outcomes in similar IL3-dependent
304 lines.
305
306 In this report, we have genetically eliminated *Atg5* expression in IL3-dependent *Bax*$^{-/-}$
307 *Bak1*$^{-/-}$ cells using CRISPR/Cas9. Unlike Lum *et al*., who concluded that autophagy
308 was necessary to sustain the survival of IL3-deprived *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells beyond
309 4 days[4], our results demonstrate that canonical Atg5-dependent autophagy pathways
310 are not required to support the long-term viability of these cells (Fig. 2). We generated
311 IL3-dependent FDM lines by co-culturing E14.5 fetal liver single-cell suspensions
312 from *Bax*$^{-/-}$*Bak1*$^{-/-}$ embryos with fibroblasts expressing a *HoxB8* retrovirus (to
313 immortalize the cells) in the presence of IL3[16]. Lum *et al*. obtained IL3-dependent
314 myeloid progenitors from the bone marrow of adult *Bax*$^{-/-}$*Bak1*$^{-/-}$ mice, but did not
315 specify how these cells were immortalized[4]. It is plausible that differences in the age,
316 organ of origin, or immortalization protocols used to generate IL3-dependent myeloid
317 progenitors caused the discrepancy in results between our study and those reported by
318 Lum and colleagues. An experiment to rule out these differences would be to mutate
319 *Bax*, *Bak1* and *Atg5* genes by CRISPR/Cas9 in IL3-dependent myeloid lines isolated
320 from the fetal livers and bone marrow of wild-type mice, then determine whether
321 these cells survive long-term in the absence of IL3.
322
323 We were able to confirm the observation that culture of FDM cells without IL3
324 reduced the levels of the glucose transporter Glut1[4], and wondered if the mechanism

325 for reduction of Glut1 would be similar to that described by Wu *et al*. who found that
326 thioredoxin interacting protein (Txnip) could reduce the levels of Glut1 in human
327 hepatocytes[7]. Although we found that Txnip increased in FDM cells when IL3 was
328 removed, levels of Glut1 decreased by the same amount when IL3 was removed from
329 Txnip-null cells, and over-expression of exogenous Txnip did not reduce the levels of
330 Glut1 (Fig. 3). Although we confirmed the reported inverse correlation between levels
331 of Txnip and Glut1, Txnip is neither required nor sufficient for IL3 to regulate levels
332 of Glut1 in FDMs. It remains possible that the Txnip-mediated downregulation of
333 Glut1 is a mechanism specific to hepatocytes and other cell types in response to
334 glucose availability[7].
335
336 Canonical autophagy was shown to be required for the efficient surface expression of
337 Glut1[6]. Our Atg5-deficient *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells had similar levels of Glut1 to
338 parental *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells, and they decreased Glut1 to a similar extent when cultured
339 without IL3. Furthermore, the *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells showed similar levels of
340 glucose uptake to *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells when cultured with IL3 and without. Therefore, in
341 FDM cells, Atg5-dependent autophagy is not needed to maintain normal levels of
342 Glut1, and does not affect the ability of IL3-starved cells to take up and metabolize
343 glucose (Figs. 3C and S1D).
344
345 Consistent with the requirement of glutamine for lymphoid cell survival[10], we found
346 that wild-type factor dependent myeloid cells died following glutamine deprivation
347 (Fig. 4). Surprisingly, a high concentration of glutamine was not required to sustain
348 the survival of Bax/Bak1-null FDM cells in serum-containing cultures not
349 supplemented with extra glucose, and that these glutamine-deprived cells maintained

350 a large size in the presence of IL3, but arrested. This suggests that glutamine provided
351 the source of amino acids needed for cell division, but that in its absence, these cells
352 import the glucose present in serum to maintain enough metabolic activity to survive
353 (Fig. 7). Hecht *et al.*[17] reported that the acute shrinkage of B an T lymphocytes
354 following growth factor withdrawal occurred before the induction of autophagy.
355 Consistent with Hecht *et al.*, the shrinkage of FDM cells following growth factor or
356 glutamine withdrawal also occurred independently of Atg5-mediated autophagy (Fig.
357 5).
358
359 In the absence of high levels of glutamine, pathways activated by IL3 could still
360 converge to induce the expression of c-Myc in Bax/Bak1-null FDM cells, and
361 although this was response was blunted, the arrested cells maintained a relatively
362 large size for at least 1 week (Fig. 5). We found that the transcription factor Atf4 was
363 strongly induced by IL3 in glutamine-starved cells, but at present, it is unclear how or
364 why signaling from the IL3 receptor leads to increased Atf4 production. Although
365 Atf4 was shown to activate the expression of several autophagy genes to promote cell
366 survival[18], we have not been able to confirm that its accumulation induced LC3B
367 lipidation in the glutamine-starved FDM cells. That IL3 also induced Atf4 expression
368 in Atg5-deficient *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells following glutamine deprivation (Fig. 6), suggests
369 that Atf4 does not promote canonical autophagy to maintain cell viability under these
370 conditions. Moreover, cells starved of both IL3 and glutamine showed no detectable
371 expression of Atf4, yet survived for weeks.
372
373 Several pathways have been proposed to explain how engagement of IL3 receptors
374 prevents activation of Bax and Bak1. For example, it was proposed that IL3 receptors

375 signaled via Akt to phosphorylate Bad, preventing it from binding to Bcl-x, to thereby
376 free Bcl-x to inhibit apoptosis[19, 20]. However, experiments in which genes for Bad or
377 Akt have been mutated have shown that unlike Bax/Bak1, they play relatively minor
378 roles in the regulation of apoptosis by IL3[16, 21]. These experiments, and those where
379 genes for all the Bcl-2 family member were deleted[22] suggest that Bax/Bak1
380 dependent apoptosis is not always signaled via BH3-only proteins, but more often
381 regulated by controlling the abundance of anti-apoptotic Bcl-2 family members, such
382 as Bcl-2, Bcl-x, and Mcl-1[23]. The results presented here are consistent with this, and
383 show that apoptosis of FDMs following the removal of IL3, or culture in the absence
384 of glutamine, is due to the decrease in abundance of Mcl-1. Cytokines including IL3
385 keeps cells alive by driving *Mcl-1* transcription, most likely via Jak2 and Stat5[24, 25, 26],
386 and glutamine is required to maintain the production of Mcl-1 to prevent default
387 apoptosis. Consistent with a requirement for Mcl-1 to prevent spontaneous activation
388 of Bax/Bak1, a specific inhibitor of Mcl-1 was able to induce apoptosis of wild-type
389 $Bax^{+/+}Bak1^{+/+}$ FDM cells cultured with IL3 and glutamine (Fig. 6).
390
391 In the absence of growth factor and high concentrations of added glucose and
392 glutamine, Atg5-deficient $Bax^{-/-}Bak1^{-/-}$ cells remained viable for weeks in serum-
393 containing medium, and their rapid death in glucose-free cultures was consistent with
394 the hypothesis that they took up and used the glucose present in serum to survive (Fig.
395 7). That these cells predominantly generate chemical energy through glycolysis (Fig.
396 3) was also consistent with their ability to consume only diluted concentrations of
397 glucose to remain viable for at least a week. Serum-starved Bax/Bak1-null cells
398 supplemented with high concentrations of glucose and glutamine survived for several
399 weeks, but arrested, with or without IL3 (data not shown). Collectively, our work

400 suggests that in the absence of growth factor, *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells, including
401 mutant lines lacking Atg5-dependent autophagy, import extracellular glucose and
402 amino acids to maintain long-term viability. Their metabolic requirement for glucose
403 and amino acids is a mechanism to support survival even in cultures where levels of
404 these nutrients are not optimal for cell proliferation, and the reduced levels of c-Myc,
405 Cdk4 and cyclin D3, combined with possibly altered levels of other cell cycle
406 regulators, could explain why growth factor and/or nutrient-starved *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells
407 arrested (Fig. 8)
408

409 **Methods & Materials**

410 *Cell culture*

411 Wild-type *Bax*$^{+/+}$*Bak1*$^{+/+}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$ IL3-dependent mouse myeloid progenitor
412 (FDM) cell lines have been described[16]. We routinely test our cells for mycoplasma
413 contamination. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM,
414 ThermoFisher Scientific Cat# 11885-084; containing 5.55 mM D-glucose and 4 mM
415 L-glutamine) supplemented with 10% fetal bovine serum (FBS, Sigma Cat# F9423)
416 and 0.25 ng/mL recombinant murine interleukin 3 (IL3, PeproTech, Cat# 213-13) in a
417 37ºC humidified incubator with 10% CO_2. Cells were deprived of IL3, glucose,
418 glucose and glutamine, or varying concentrations of FBS, by washing 3 times with
419 medium that lacked those nutrients, then resuspended in the same medium for
420 analysis at the indicated time points. Unless indicated otherwise, 1 x 10^5 cells were
421 seeded in 1 mL of the specified medium per well of a 24-well tissue culture plate
422 (Corning, Cat# 353047), and one well was analysed for each timepoint. For
423 experiments without FBS, plastic tubes and tissue culture dishes were pre-coated with
424 20 μg/mL bovine serum albumin (Sigma Cat# A1470) and rinsed with serum-free

425 medium before cell seeding. DMEM lacking D-Glucose and sodium pyruvate but
426 containing 4 mM L-Glutamine (Cat# 11966-025) and DMEM lacking D-Glucose,
427 sodium pyruvate, L-Glutamine and phenol red (Cat# A14430-01) were purchased
428 from ThermoFisher Scientific. D-Glucose was purchased from Sigma (Cat# G7528).
429 The glucose concentration in FBS was determined using an Accu-Chek Performa
430 glucose meter, according to the manufacturer's instructions.
431
432 *Lentiviral constructs for CRISPR/Cas9 and inducible overexpression*
433 An inducible lentiviral CRISPR/Cas9 system[27] was used to mutate *Txnip* in *Bax*$^{-/-}$
434 *Bak1*$^{-/-}$ FDM cells. Guide RNA oligos targeting mouse *Txnip* (guide 1: CCA GCA
435 GGT GAG AAC GAG A; guide 2: TAT GGT TGC GTA GAC TAC T) were cloned
436 into the *Bsm*BI site of the pFgH1tUTG GFP lentiviral vector. Guide RNA oligo
437 targeting mouse *Atg5* (AAG ATG TGC TTC GAG ATG TG) was cloned into the
438 *Bsm*BI site of the lentiviral vector lentiCRISPR v2 (Addgene #52961[28]). For
439 overexpression studies, the coding sequence of mouse *Txnip* was PCR amplified from
440 pEGFP-C1-Txnip (Addgene #18758[29]) with the incorporation of an N-terminal
441 FLAG-tag. The PCR product was cloned into the *Eco*RI/*Nhe*I sites of a doxycycline
442 inducible pFTREtight MCS rtTAadvanced GFP lentiviral vector[21].
443
444 *Antibodies and small molecules*
445 Antibodies: LC3B (D11, Cell Signaling Technology #3868), Atg5 (D5F5U, Cell
446 Signaling Technology #12994); β-actin (AC-15, Sigma #A1978), Glut1 (Abcam
447 ab15730), Txnip (D5F3E, Cell Signaling Technology #14715S), Glut4 (1F8, Cell
448 Signaling Technology #2213), Mcl-1 (Rockland 600-401-394S), c-Myc (Abcam
449 ab32072), Cdk4 (Santa Cruz Biotechnology sc-260), Cyclin D3 (Cell Signaling

450 Technology #2936), Atf4 (D4B8, Cell Signaling Technology #11815). Small
451 molecules: Mcl-1 inhibitor S63845 was synthesized according to Kotschy *et al.*[11] by
452 SYNthesis Med Chem Pty Ltd (Parkville, Australia) or by Active Biochem (Catalog#
453 A-6044 – a kind gift from David Huang); DMSO (dimethyl sulfoxide; Cat# 472301)
454 and propidium iodide (Cat# P4864) were purchased from Sigma.
455
456 *Measurements of cell death, cell cycle and cell size*
457 Cell death and cell cycle were assayed using propidium iodide (PI) and flow
458 cytometry. For death assays, cells were harvested and resuspended in PBS containing
459 1 μg/mL PI. For cell cycle analysis, cells were harvested and resuspended in PBS
460 containing 0.1% Triton X-100, 5 μg/mL PI and 10 μg/mL RNaseA (Thermo
461 Scientific), then incubated at 37 °C for 15 min. Samples were processed on
462 FACSCalibur or LSRFortessa X-20 flow cytometers (BD Biosciences) and the
463 acquired data analyzed with FlowJo software. Cell volume in Figure 5 was measured
464 on a CASY instrument (Roche Innovatis AG), a Coulter-based cell counter operating
465 on the Electrical Current Exclusion principle. In brief, 200 μL aliquots of cells
466 cultured under the specified conditions were mixed with 5 mL of CASY electrolyte
467 buffer and analyzed in a 60 μm CASY capillary. Multiple parameters including the
468 peak cell volume were collected.
469
470 *Glucose uptake assays*
471 Extracellular acidification (ECAR) rates were measured in live cells using a Seahorse
472 Bioscience XFe96 Analyzer (Agilent Technologies). In brief, cells were resuspended
473 in non-buffered DMEM (Seahorse Biosciences) and 200 x 10^3 cells were seeded per
474 well in Seahorse Bioscience 96-well plates. The cellular ECAR was analyzed in non-

475 buffered DMEM containing 2 mM glutamine with the following compounds: 5 mM
476 glucose, 1 μM oligomycin and 50 mM 2-deoxy-glucose (2DG). For each assay cycle,
477 measurement time points of 2 min mix, 2 min wait and 5 min measure were collected.
478
479 *Data reproducibility*
480 Unless indicated otherwise, all experiments shown have been reproduced at least 3
481 independent times with similar results. While not all repeats were performed wholly
482 or in exact detail, we ensured that our experimental design allowed us to interpret and
483 draw conclusions from multiple independent experiments. This is aided by the fact
484 that our results are unambiguous: the majority of cells are either alive or dead, cycling
485 or arrested, large or small; and immunoblots show clear changes in protein levels or
486 modification. We have endeavored to perform experiments that provide consistent
487 evidence in mechanistically independent ways, rather than repeating the same
488 experiment many times until statistical significance is reached. The experimental
489 triangulation of our evidence[30] is best exemplified by multiple independent assays
490 confirming that autophagy-deficient FDM cells survived for weeks without growth
491 factor under a variety of different culture conditions (Figs. 2B, 4B, 5, 7C, S3B).
492
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638 **Figure Legends**

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

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

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

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

652

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

655 **A.** Whole cell lysates from *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells and 3 *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clonal lines
656 cultured in the presence of IL3, in Hank's Buffered Saline Solution (HBSS) for 2 h, or
657 in the absence of IL3 for 2 or 48 h, were subjected to Western blotting. The Atg5
658 immunoblot demonstrates successful CRISPR/Cas9-mediated deletion of *Atg5*. The
659 LC3B immunoblot demonstrates lack of LC3B lipidation (isoform II) and autophagic
660 flux (HBSS treatment) in Δ*Atg5* cell clones.

661 **B.** *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* FDM cells survive long-term in the absence of IL3. The
662 indicated *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clones were cultured without IL3 and viability was
663 determined by PI exclusion and flow cytometry at the indicated times.
664 **C.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ Δ*Atg5* cells (clone 1) were cultured for 48 h in the presence or absence
665 of IL3 and PI negative populations were compared by forward scatter to indicate
666 differences in cell size.
667 **D.** Cells from *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clone 1 were cultured for 48 h in the presence or
668 absence of IL3 then processed in PI buffer to measure DNA content by flow
669 cytometry.
670
671 **Figure 3.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells arrested by IL3 deprivation survive in the absence
672 of added glucose.
673 **A.** Txnip is not required for the decrease in Glut1 following IL3 withdrawal. Whole
674 cell lysates from *Bax*$^{-/-}$*Bak1*$^{-/-}$ and 3 independent *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Txnip* FDM cell clones
675 cultured for 48 h in the presence or absence of IL3 were analyzed by Western blotting
676 to detect Glut1 and Txnip protein.
677 **B.** Forced expression of Txnip is not sufficient to decrease Glut1 protein levels.
678 Independent *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM clones bearing a doxycycline (dox)-inducible FLAG-
679 Txnip construct or empty vector control were cultured for 72 h in the absence or
680 presence of dox. Whole cell lysates were prepared and analyzed by Western blotting
681 to detect Glut1 and Txnip protein.
682 **C.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Txnip* cells can take up and metabolise glucose.
683 Cells cultured for 72 h in the presence or absence of IL3 were analyzed on the
684 Seahorse bioanalyzer to measure the extracellular acidification rate (ECAR) as an
685 indicator of glucose metabolism. The increased ECAR following addition of glucose

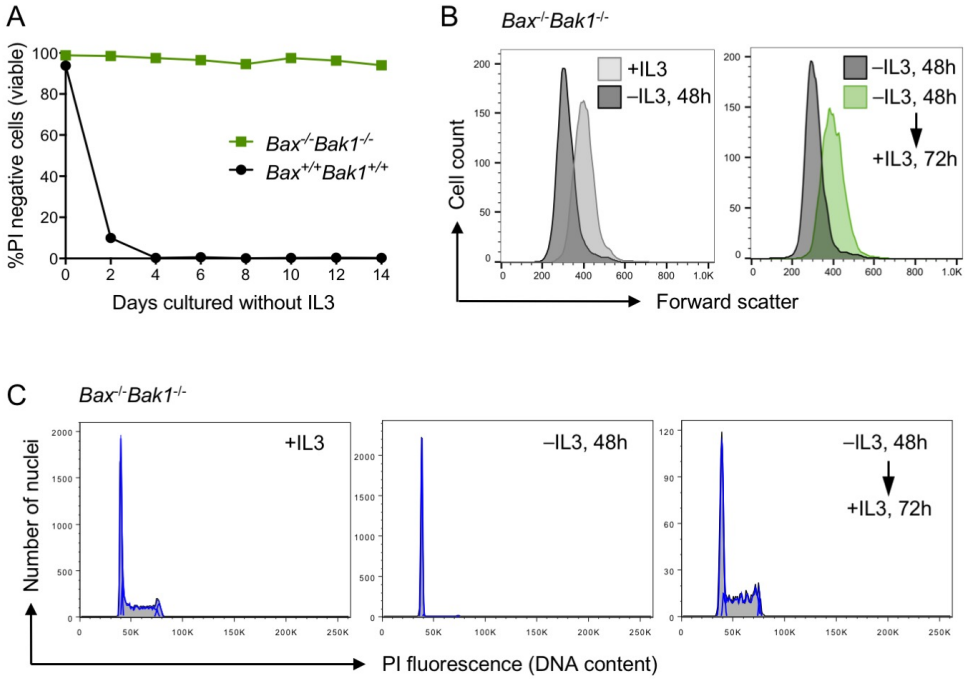
686 signifies increased glycolytic flux in cells. Oligomycin inhibits mitochondrial ATP
687 synthase and the change in ECAR upon its addition represents the glycolytic reserve
688 capacity of cells. Glycolysis is inhibited by 2-deoxyglucose (2-DG) so upon its
689 addition, the ECAR values are produced from non-glycolytic sources. Equal numbers
690 of cells were seeded for analysis. To correct for the reduced size of IL3-starved cells,
691 protein concentration was assayed and the ECAR values were normalized to 1 μg
692 total protein per sample. Curves represent the mean of 3 replicate samples depicted as
693 solid symbols for each time point.
694 **D.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Txnip* cells deprived of IL3 survive long-term in the
695 absence of added glucose. Cells were cultured without or with IL3 in medium
696 supplemented with 10% FBS, 4 mM glutamine and 5.5 mM glucose (medium with
697 added glucose) or in medium supplemented with 10% FBS and 4 mM glutamine
698 (medium without added glucose). Cell viability was monitored by PI exclusion and
699 flow cytometry at the indicated times.
700
701 **Figure 4.** When starved of glutamine, *Bax*$^{+/+}$*Bak1*$^{+/+}$ FDM cells undergo apoptosis,
702 whereas *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells arrest, but can survive for weeks.
703 **A.** *Bax*$^{+/+}$*Bak1*$^{+/+}$ cells undergo apoptosis in the absence of glutamine (upper panels),
704 whereas *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells survive in the absence of glutamine,
705 but arrest even when IL3 is present (lower panels). Cells were cultured in medium
706 supplemented with 10% FBS, with or without IL3 and 4 mM glutamine, in the
707 absence of added glucose, for 4 days. Cells were processed in PI buffer to assess
708 DNA content by flow cytometry. Note the absence of cells in S phase when
709 Bax/Bak1-null cells were cultured without added glutamine.

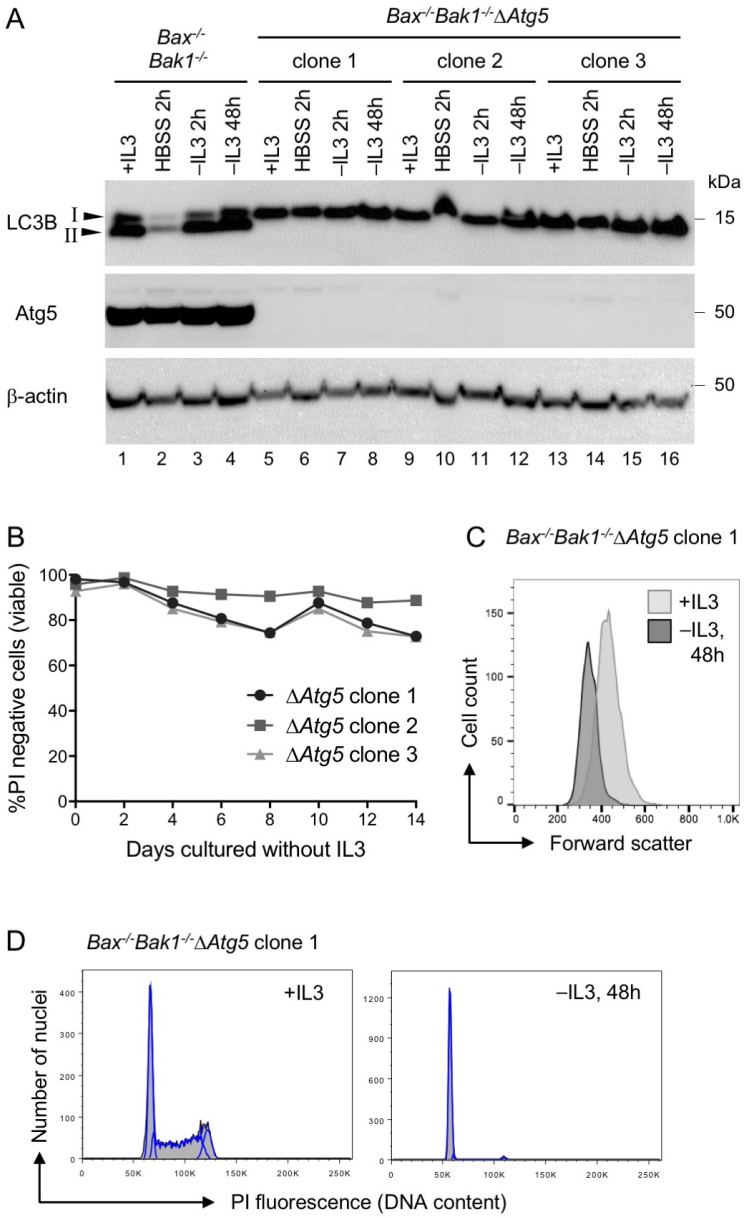
710 **B.** *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells survive for weeks in the absence of glutamine. Three
711 independent *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clones were cultured with or without IL3, in medium
712 supplemented with 10% FBS, without added glucose or glutamine. At the indicated
713 times, cells were assayed for viability by PI exclusion and flow cytometry.
714
715 **Figure 5.** Withdrawal of IL3 or glutamine caused cell shrinkage independent of Atg5-
716 mediated autophagy.
717 *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells were cultured in medium supplemented with
718 10% FBS, with or without IL3, in the absence of added glucose and glutamine. At the
719 indicated times, cell volume was measured in a Coulter-based CASY cell counter.
720
721 **Figure 6.** Withdrawal of IL3 or glutamine reduced the expression of Mcl-1 and
722 several key regulators of cell cycle progression.
723 **A.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clone 1 were cultured for 72 h in medium
724 supplemented with (+) or without (–) added glucose (Glc) and glutamine (Q), as
725 indicated, in the presence or absence of IL3 (+/–). Whole cell lysates were prepared
726 and analyzed by Western blotting to detect the indicated proteins. Three independent
727 membranes were used for immunoblotting and β-actin loading control blots for each
728 are shown. Membrane #1: anti-Mcl-1 and anti-c-Myc; membrane #2: anti-Cdk4;
729 membrane #3: anti-cyclin D3 and anti-Atf4.
730 **B.** *Bax*$^{+/+}$*Bak1*$^{+/+}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells cultured in complete medium with IL3 were
731 treated with DMSO (vehicle control) or 10 μM of the Mcl-1 inhibitor S63845 for 24
732 h. Cells were harvested and viability determined by PI exclusion and flow cytometry.
733 Bars depict the mean of 3 independent experiments represented by the 3 different
734 symbols, performed in triplicate. The experiment represented by the solid circles was

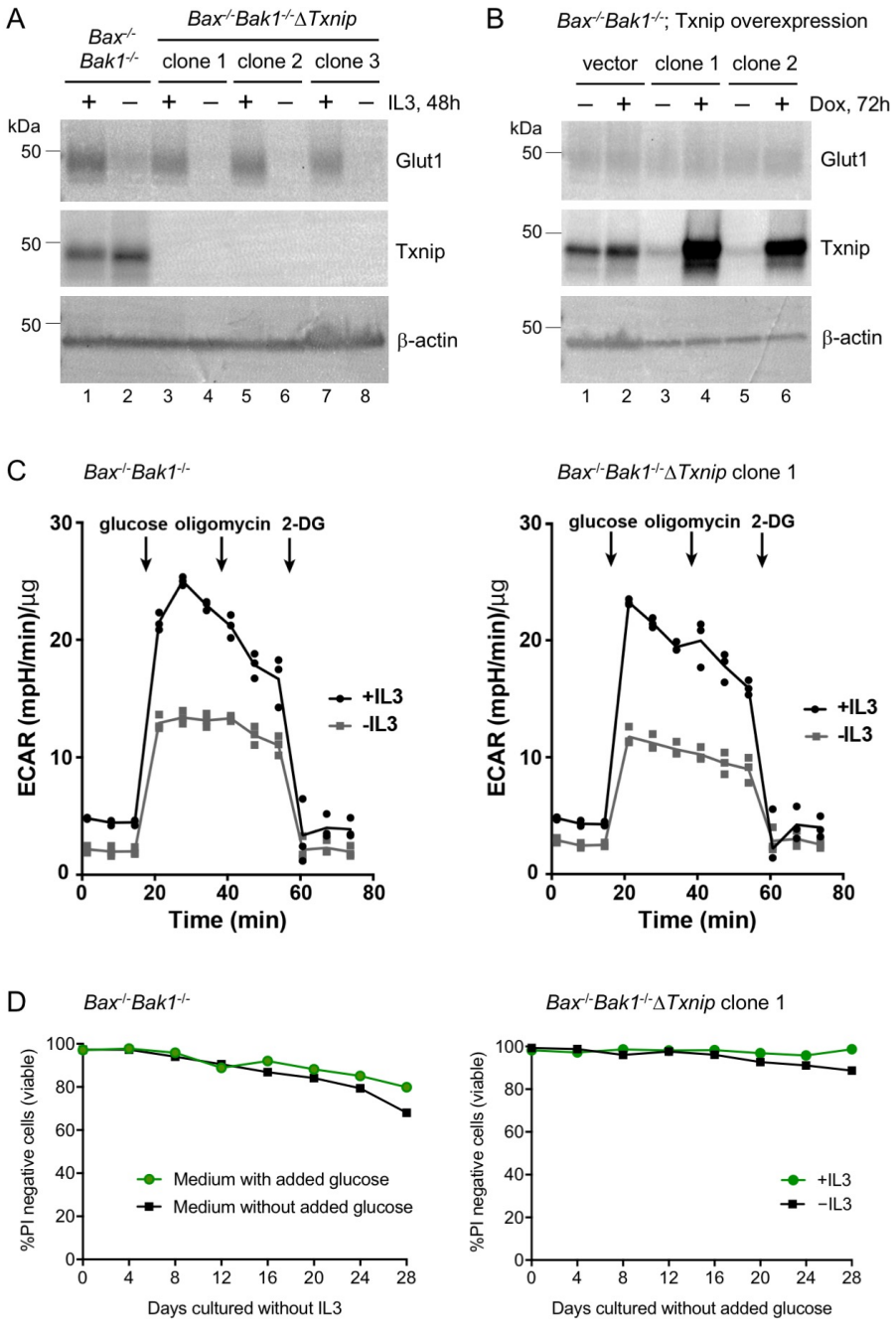
735 performed with S63845 synthesized by SYNthesis Med Chem. Experiments
736 represented by the solid triangles and open hexagons were performed with S63845
737 synthesized by Active Biochem (see Materials and Methods).
738
739 **Figure 7.** In the absence of Atg5-dependent autophagy, IL3-deprived *Bax*$^{-/-}$*Bak1*$^{-/-}$
740 myeloid cells consume extracellular glucose to maintain long-term viability.
741 **A.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clone 1 were cultured in the absence of
742 FBS for 48 h in medium without or with the specified concentration of glutamine
743 and/or glucose, with IL3. Cells were assayed for viability by PI exclusion and flow
744 cytometry. Bars represent the average of 3 independent experiments (depicted by the
745 3 different symbols).
746 **B.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clone 1 were cultured in the absence of
747 FBS for 48 h in medium with the specified concentration of glutamine or glucose,
748 without IL3. Cells were assayed for viability by PI exclusion and flow cytometry.
749 Bars represent the average of 2 independent experiments (depicted by the 2 different
750 symbols) performed with technical replicates as indicated.
751 **C.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells were cultured in medium supplemented
752 with 0.5 mM glucose; without IL3, glutamine or FBS. At the indicated times, cells
753 were assayed for viability by PI exclusion and flow cytometry. The curves connect the
754 average values of data points derived from 2 independent experiments (depicted by
755 the 2 different symbols) performed with technical replicates or without (dotted
756 symbols) as indicated.
757
758
759

760 **Figure 8.**

761 The growth factor IL3 stimulates the expression of Mcl-1 that inhibits activation of
762 Bax/Bak1, and stimulates expression of c-Myc, Cdk4 and cyclin D3 to promote cell
763 growth and division. Optimal concentrations of glucose and glutamine are required to
764 maintain levels of these proteins needed to prevent activation of Bax/Bak1 (Mcl-1), as
765 well as for cell proliferation (c-Myc, Cdk4, cyclin D3 and possibly others). In the
766 absence of growth factor, or when nutrient levels are low, sufficient levels of glucose
767 transporters (Glut1, Glut4 and possibly others) remain to allow *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid
768 cells to consume extracellular glucose to survive, even in cells lacking Atg5-
769 dependent autophagy.


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


A
Bax-/- Bak1-/-
Bax-/-Bak1-/-ΔAtg5
clone 1
clone 2
clone 3
+IL3
HBSS 2h
–IL3 2h
–IL3 48h
kDa
LC3B
I
II
15
Atg5
50
β-actin
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
B
%PI negative cells (viable)
Days cultured without IL3
ΔAtg5 clone 1
ΔAtg5 clone 2
ΔAtg5 clone 3
C Bax-/-Bak1-/-ΔAtg5 clone 1
+IL3
–IL3, 48h
Cell count
Forward scatter
D Bax-/-Bak1-/-ΔAtg5 clone 1
+IL3
–IL3, 48h
Number of nuclei
PI fluorescence (DNA content)


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-/-
Bax-/-Bak1-/-ΔTxnip clone 1
glucose oligomycin 2-DG
ECAR (mpH/min)/µg
Time (min)
+IL3
-IL3
D
Bax-/-Bak1-/-
Bax-/-Bak1-/-ΔTxnip clone 1
%PI negative cells (viable)
Medium with added glucose
Medium without added glucose
Days cultured without IL3
+IL3
−IL3
Days cultured without added glucose

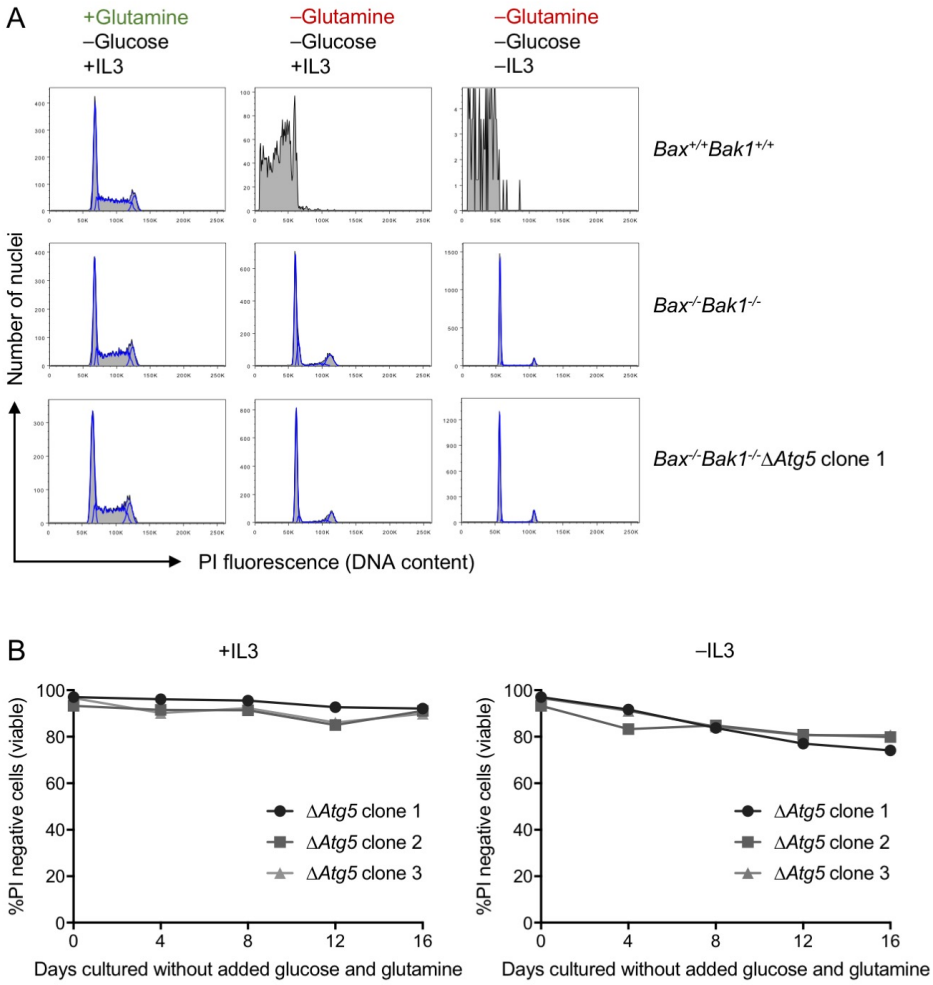
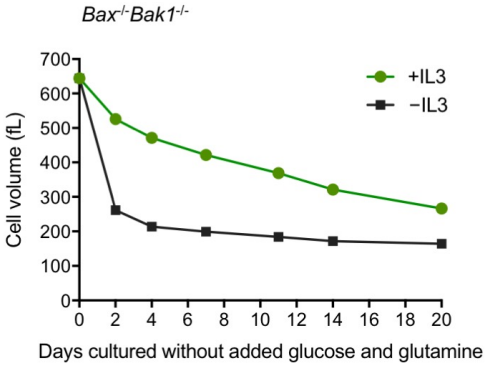
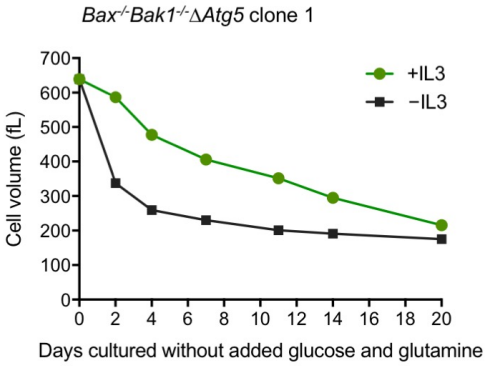
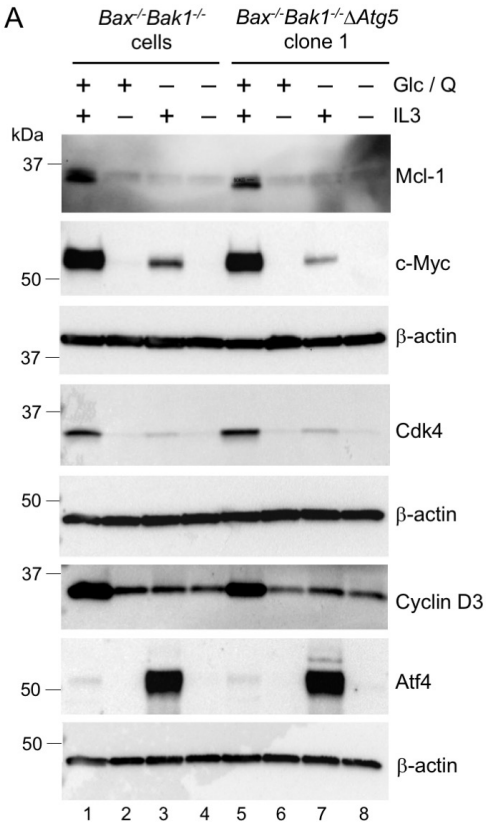
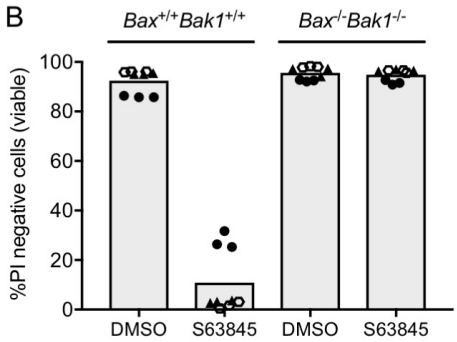

A
+Glutamine
–Glucose
+IL3
–Glutamine
–Glucose
+IL3
–Glutamine
–Glucose
–IL3
Bax+/+Bak1+/+
Bax-/-Bak1-/-
Bax-/-Bak1-/-ΔAtg5 clone 1
Number of nuclei
PI fluorescence (DNA content)
B
+IL3
–IL3
%PI negative cells (viable)
ΔAtg5 clone 1
ΔAtg5 clone 2
ΔAtg5 clone 3
Days cultured without added glucose and glutamine

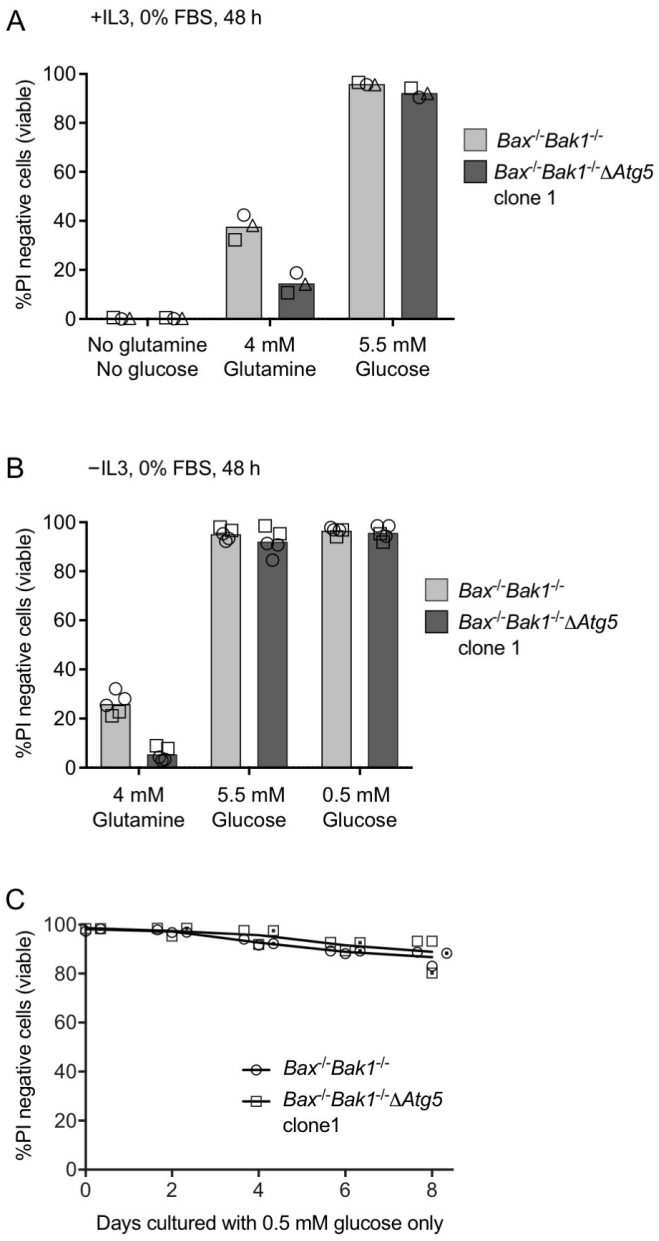
FIGURE 5 Dong et al.

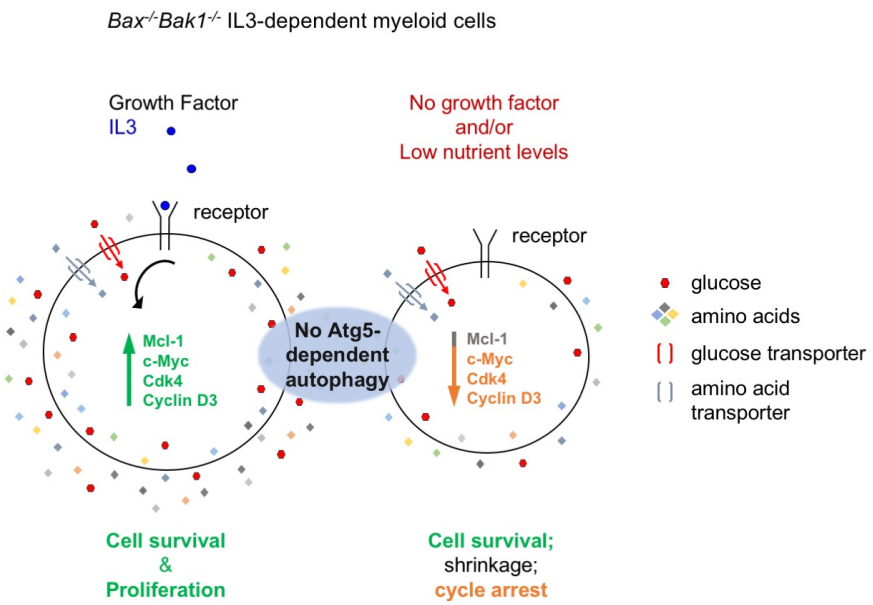





A
Bax-/-Bak1-/- cells
Bax-/-Bak1-/-ΔAtg5 clone 1
+ + – – + + – – Glc / Q
+ – + – + – + – IL3
kDa
37
Mcl-1
50
c-Myc
37
β-actin
37
Cdk4
50
β-actin
37
Cyclin D3
50
Atf4
50
β-actin
1 2 3 4 5 6 7 8


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


A
+IL3, 0% FBS, 48 h
%PI negative cells (viable)
100
80
60
40
20
0
No glutamine
No glucose
4 mM
Glutamine
5.5 mM
Glucose
Bax-/-Bak1-/-
Bax-/-Bak1-/-ΔAtg5
clone 1
B
−IL3, 0% FBS, 48 h
%PI negative cells (viable)
100
80
60
40
20
0
4 mM
Glutamine
5.5 mM
Glucose
0.5 mM
Glucose
Bax-/-Bak1-/-
Bax-/-Bak1-/-ΔAtg5
clone 1
C
%PI negative cells (viable)
100
80
60
40
20
0
0
2
4
6
8
Days cultured with 0.5 mM glucose only
Bax-/-Bak1-/-
Bax-/-Bak1-/-ΔAtg5
clone1


Bax-/-*Bak1*-/- IL3-dependent myeloid cells
Growth Factor
IL3
receptor
Mcl-1
c-Myc
Cdk4
Cyclin D3
No Atg5-
dependent
autophagy
No growth factor
and/or
Low nutrient levels
receptor
Mcl-1
c-Myc
Cdk4
Cyclin D3
glucose
amino acids
glucose transporter
amino acid
transporter
Cell survival
&
Proliferation
Cell survival;
shrinkage;
cycle arrest