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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Running Head: Cell Survival Without Glucose, Glutamine, or Autophagy

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Abstract

Withdrawal of 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 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. 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, in glucose-free medium, 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.

51 **Introduction**

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

59

60 Factor dependent myeloid (FDM) progenitor cell lines (1, 2) require the
61 haematopoietic growth factor interleukin 3 (IL3) to survive and multiply in culture.
62 When IL3 is not provided, FDM cells undergo Bax/Bak1-dependent apoptosis. FDM
63 cells derived from *Bax*$^{-/-}$*Bak1*$^{-/-}$ mice survive in the absence of IL3, but they undergo
64 cell cycle arrest, similar to those over-expressing Bcl2 (3, 4). Previously it was
65 reported that to survive longer than 4 days, IL3-deprived *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells required
66 Atg5-dependent autophagy (4), an intracellular catabolic process that can support the
67 survival of starved 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, and
86 whether cells required glucose uptake in order to cycle.
87
88 **Results**
89 *Bax*$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells shrink and arrest when deprived of IL3, but survive.*
90 Factor dependent myeloid (FDM) cells require interleukin 3 (IL3) to survive and
91 proliferate (2, 4, 8). When deprived of IL3 for 4 days, >80 % of wild-type
92 *Bax*$^{+/+}$*Bak1*$^{+/+}$ FDM cells died, as indicated by loss of plasma membrane integrity and
93 uptake of propidium iodide (Figs. 1A and Supplemental S1A). By contrast, and
94 consistent with the findings of Lum *et al*. (4), *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells survived in the
95 absence of IL3 for more than 14 days. After 48 h IL3 withdrawal, these cells shrank in
96 size and arrested (Fig. 1, B and C). This quiescent state was reversible, because when
97 IL3 was returned, the cells increased in size and resumed cycling. Thus, in the
98 absence of Bax/Bak1-mediated apoptosis, FDM cells can survive for weeks without
99 IL3, but they shrink and arrest.
100

101 *Bax*$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells arrested by deprivation of IL3 do not require autophagy to*
102 *survive.*
103 During macroautophagy, LC3B-I is lipidated by a multiprotein complex containing
104 Atg5 to form LC3B-II, which can incorporate into the growing autophagosome, and is
105 a common marker of autophagy. Lysates of *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells contained similar
106 amounts LC3B-II, whether they were cultured in the presence or absence of IL3 (Fig.
107 2A, lanes 1 and 2). This suggests autophagy is occurring in both cycling and arrested
108 cells. To determine if autophagy was needed to support the sustained survival of
109 arrested IL3-starved cells, we used CRISPR/Cas9 to delete *Atg5* in *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells,
110 generating *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* triple knockout FDM cell clones. Western blotting
111 confirmed the three *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clones had much less LC3B-II than the *Bax*$^{-/-}$
112 *Bak1*$^{-/-}$ cells, whether they were cultured with IL3 or not (Fig. 2A). After 14 days in
113 culture without IL3, all *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clones remained ~80% viable (Figs. 2B
114 and Supplemental S1B). Similar to *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDMs, *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells
115 arrested and shrank in size within 48 h of growth factor withdrawal (Fig. 2, C and D).
116 These results confirm that IL3 receptor signals are needed for *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells
117 to maintain their normal size, but show that Atg5-dependent autophagy is not required
118 for their survival, whether they are cycling or are arrested by IL3 withdrawal.
119 Furthermore, because culturing without IL3 caused a similar decrease in size of the
120 arrested *Bax*$^{-/-}$*Bak1*$^{-/-}$ as of the *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells, this shrinkage is not due to
121 Atg5-dependent autophagy.
122
123 *Bax*$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells arrested by IL3 deprivation survive in the absence of*
124 *glucose.*

125 Lum *et al*. reported that when growth factor was removed, *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells
126 decreased expression of the glucose transporter Glut1 (4). A possible mechanism for
127 this was proposed by Wu *et al*., who found that thioredoxin-interacting protein
128 (Txnip) could not only bind to Glut1 and induce its internalization through clathrin-
129 coated pits, thus removing it from the plasma membrane, but could also reduce the
130 level of Glut1 messenger mRNA (7). Consistent with Lum *et al*. and Wu *et al*., Txnip
131 was increased, and Glut1 was decreased, in lysates of factor-starved *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells
132 48 h following removal of IL3 (Fig. 3A, lanes 1 and 2).
133
134 To determine whether expression of Txnip caused or correlated with changes in Glut1
135 levels, we deleted *Txnip* in *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells using CRISPR/Cas9. Western
136 blotting confirmed the absence of Txnip protein in three *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Txnip* clones
137 (Fig. 3A). We found that Glut1 levels were unaffected by deletion of *Txnip*, whether
138 IL3 was added or not, indicating that the increase in Txnip was not required for the
139 decrease in Glut1 when cells were cultured without IL3. Furthermore, withdrawal of
140 IL3 caused G1 arrest in *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Txnip* cells within 48 h, similar to *Bax*$^{-/-}$*Bak1*$^{-/-}$
141 cells (Figs. 1A and Supplemental S2A). Because induced over-expression of FLAG-
142 Txnip in *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells had no impact on levels of Glut1 protein or cell cycle
143 progression (Figs. 3B and Supplemental S2B), an increase in Txnip is not sufficient to
144 reduce Glut1. Therefore, in FDM cells, the abundance of Txnip has no effect on levels
145 of Glut1, whether they are treated with IL3 or not.
146
147 Levels of Glut1 were similar among proliferating Bax/Bak1-null cells of all genotypes
148 and the decrease in Glut1 expression following IL3 withdrawal in *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5*
149 clones compared to *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells was unaffected (Supplemental Fig. S2C).

150 Together, these results indicate that although IL3 can promote expression of Glut1,
151 neither Txnip nor Atg5-dependent autophagy is required for the decrease in Glut1
152 when growth factor is removed from myeloid cells, and neither absence nor
153 overexpression of Txnip affects the levels of Glut1.
154
155 Because Glut1 levels are reduced in IL3-starved *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells, we asked
156 whether glucose utilization was reduced. The rate of glucose metabolism in *Bax*$^{-/-}$
157 *Bak1*$^{-/-}$ FDMs was reduced after IL3 withdrawal, but they nevertheless still responded
158 to the addition of glucose, as indicated by the increased extracellular acidification rate
159 (ECAR, Fig. 3C). In the absence of IL3, *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM lines that also lacked
160 Txnip or Atg5 had similar rates of glucose metabolism to the parental Bax/Bak1-null
161 cells (Figs. 3C and Supplemental S2D). Thus, arrested FDM cells retain the capacity
162 to import and metabolise glucose, albeit at a reduced level.
163
164 Because the arrested IL3-starved cells were able to take up glucose, we hypothesised
165 that this was sufficient to support their sustained survival despite the decrease in
166 Glut1. To test their requirement for glucose, the cells were cultured in medium that
167 lacked glucose. Surprisingly, ≥ 80% of IL3-starved *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$
168 Δ*Txnip* cells remained viable at 24 days without glucose (Fig. 3D). Furthermore,
169 when IL3 was present, these cells were able to proliferate (Supplemental Fig. S2E).
170 These results indicate that FDM cells will import glucose whether IL3 is present or
171 not, and that they can still proliferate even in the absence of glucose.
172
173 *Bax*$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells require glutamine to proliferate, but can survive in its*
174 *absence for weeks.*

175 When glucose is limiting, lymphoid cells can metabolise glutamine to survive and
176 proliferate (9). We therefore asked whether FDM cells exhibited similar requirements
177 for glutamine when cultured in the absence of glucose. In medium that lacked both
178 glucose and glutamine, with or without IL3, >95% of wild-type *Bax*$^{+/+}$*Bak1*$^{+/+}$ FDM
179 cells died as indicated by uptake of PI by day 4 (Supplemental Fig. S3A), and
180 consistent with their death being due to apoptosis, the nuclei of the cells had sub-2n
181 DNA (Fig. 4A). Apoptosis was Bax/Bak1 dependent, because Bax/Bak-null cells did
182 not die when cultured in medium lacking glucose and glutamine, but they arrested
183 instead (Figs. 4A and Supplemental S3B). When IL3 was present, they arrested as
184 large cells, whereas when cultured without IL3, they arrested as small cells (Figs. 4B
185 and Supplemental S3C).
186
187 In medium without glucose, glutamine, or IL3, *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells exhibited a
188 slight decline in viability at day 16 and beyond compared to cultures with IL3 (Fig.
189 4C). Interestingly, in the presence of IL3, >90% of cells cultured with neither glucose
190 nor glutamine continued to survive beyond 28 days. *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clones
191 cultured in medium lacking glucose and glutamine also maintained ~80% or greater
192 viability at day 16, with or without IL3 (Supplemental Fig. S3D).
193
194 When *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells were starved of glucose and glutamine, most arrested in
195 G_1 with 2n DNA, but some arrested in G_2/M with 4n DNA (Fig. 4, A and D). When
196 glutamine was added back to *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells that had been cultured for 7 days
197 without glucose or glutamine, the arrested cells resumed cycling within 48 h in the
198 presence of IL3, whereas adding back glucose had no effect (Fig. 4D). These results
199 indicate that IL3-treated *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells require glutamine to proliferate,

200 but can survive up to 4 weeks or more in its absence. Because *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5*
201 FDMs behaved similarly to *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells (Supplemental Fig. S3B), Atg5-
202 dependent autophagy is not required for sustained cell survival, even in the absence of
203 glucose and glutamine.
204
205 *Withdrawal of IL3 or glutamine caused reduced expression of Mcl-1 and key*
206 *regulators of cell cycle progression.*
207 To elucidate how growth factor and nutrient deprivation impacted FDM cell survival
208 and proliferation, we performed Western blotting to determine protein levels of
209 apoptotic and cell cycle regulators. We found that levels of the pro-survival protein
210 Mcl-1 were markedly reduced in *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells after 3 days
211 in culture without glucose and glutamine (Fig. 5A), suggesting that the decrease in
212 normal levels of Mcl-1 allowed activation of Bax/Bak1 and the apoptosis of wild-type
213 FDM cells observed in Fig. 4A. Consistent with this notion, addition of a specific
214 Mcl-1 inhibitor S63845 (10) to *Bax*$^{+/+}$*Bak1*$^{+/+}$ cells was sufficient to cause significant
215 apoptosis within 24 h, even when the cells were cultured in complete medium
216 containing glutamine and IL3 (Fig. 5B).
217
218 As well as causing arrest, IL3 withdrawal reduced c-Myc protein to barely detectable
219 levels in both *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells (Fig. 5A). Cdk4 and cyclin D3
220 are key drivers of cell cycle progression in haematopoietic cell lines (11, 12), and
221 these proteins were also markedly reduced in the IL3-starved, Bax/Bak1-deficient
222 myeloid cells (Fig. 5A), suggesting a mechanism for their arrest (Figs. 1C and 2C).
223 Withdrawal of glutamine from these cells also caused cell cycle arrest, even in the
224 presence of IL3 (Figs. 4A and Supplemental S3B). Under these conditions, IL3 was

225 able to stimulate the expression of c-Myc and Cdk4, but the levels of these proteins
226 were significantly lower than in the cells cultured with both glutamine and IL3 (Fig.
227 5A). The transcription factor Atf4 accumulates in response to a variety of cellular
228 stresses including amino acid depletion (13). In glutamine-starved *Bax*$^{-/-}$*Bak1*$^{-/-}$ and
229 *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells, Atf4 levels increase strongly, but intriguingly only in the
230 presence of IL3. These results indicate that the signal transduction pathway activated
231 by ligation of the IL3 receptors remains largely intact in cells lacking glutamine, and
232 can stimulate the cells to remain large, but that glutamine is essential for high levels
233 of c-Myc and Cdk4/cyclin D3 expression, and for the cells to divide.
234
235 *Bax*$^{-/-}$*Bak1*$^{-/-}$ *myeloid cells require serum factors to survive long-term in the absence*
236 *of glutamine.*
237 The cells cultured without glutamine or glucose would need a source of energy to
238 survive, so we tested whether they were obtaining it from fetal bovine serum (FBS) in
239 the medium. When cultured in 2% FBS, all *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells died after 4 days,
240 whereas at the same time ~20% of cells cultured with 4% FBS were viable (Fig. 6A).
241 More than 80% of cells remained viable for 16 days and beyond when cultured with
242 6%, 8% or 10% FBS. Similar findings were observed for *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells
243 (Fig. 6B). These results suggest that *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells survive for several
244 weeks without glucose and glutamine by consuming nutrients in the FBS, rather than
245 by using autophagy.
246
247 **Discussion**
248 IL3-dependent myeloid cell lines have provided many insights into the regulation of
249 fundamental processes such as cell death, cell division, and autophagy, as well as the

250 signaling pathways that regulate them. For example, it was experiments on an IL3-
251 dependent myeloid cell line that first revealed that the function of Bcl-2 was to inhibit
252 cell death (3). When genes for Bax and Bak1 are deleted in factor-dependent myeloid
253 (FDM) cells, they fail to undergo apoptosis when IL3 is removed, but nevertheless
254 will die if starved of nutrients needed to maintain their metabolism. When *Bax*$^{-/-}$*Bak1*$^{-}$
255 $^{/-}$ FDMs are cultured without IL3, they decrease expression of the glucose transporter
256 Glut1, shrink and arrest, but do not die (4). This led to the proposal that the cells could
257 not take up glucose, so they needed autophagy to maintain their metabolism to
258 survive. Consistent with this idea, Lum *et al*. found that inhibition of autophagy either
259 by 3-methyladenine or shRNA knockdown of Atg5 caused the cells to die (4).
260 However, we also used shRNA to reduce Atg5 levels and found that Atg5-dependent
261 autophagy was not required for the survival *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells deprived of
262 IL3 (14). In this report, we have genetically eliminated *Atg5* expression in IL3-
263 dependent *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells using CRISPR/Cas9. Unlike Lum *et al*., who concluded
264 that autophagy was necessary to sustain the survival of IL3-deprived *Bax*$^{-/-}$*Bak1*$^{-/-}$
265 FDM cells beyond 4 days (4), our results demonstrate that canonical Atg5-dependent
266 autophagy pathways are not required to support the long-term viability of these cells
267 (Fig. 2).
268
269 We were able to confirm the observation that culture of FDM cells without IL3
270 reduced the levels of the glucose transporter Glut1 (4) (Fig. 3A), and wondered if the
271 mechanism for reduction of Glut1 would be similar to that described by Wu *et al*. who
272 found that thioredoxin interacting protein (Txnip) could reduce the levels of Glut1 in
273 human hepatocytes (7). Although we found that Txnip increased in FDM cells when
274 IL3 was removed, levels of Glut1 decreased by the same amount when IL3 was

275 removed from Txnip-null cells, and over-expression of exogenous Txnip did not
276 reduce the levels of Glut1 (Fig. 3, A and B). Although we confirmed the reported
277 inverse correlation between levels of Txnip and Glut1, Txnip is neither required nor
278 sufficient for IL3 to regulate levels of Glut1 in FDMs.
279
280 Canonical autophagy was shown to be required for the efficient surface expression of
281 Glut1 (6). Our Atg5-deficient *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells had similar levels of Glut1 to
282 parental *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells, and they decreased Glut1 to a similar extent when cultured
283 without IL3. Furthermore, the *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells showed similar levels of
284 glucose uptake to *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells when cultured with IL3 and without. Therefore, in
285 FDM cells, Atg5-dependent autophagy is not needed to maintain normal levels of
286 Glut1, and does not affect the ability of cells to take up and metabolize glucose (Fig.
287 S2, C and D).
288
289 We were surprised to find that Bax/Bak1-null FDM cells of all genotypes, when
290 cultured in the absence of glucose and arrested due to withdrawal of IL3, not only
291 survived, but that these cells, and even wild-type *Bax*$^{+/+}$*Bak1*$^{+/+}$ cells, proliferated in
292 the absence of glucose when IL3 was present (Figs. 3D and S2E; data not shown).
293 Therefore, even though FDM cells will take up and metabolise glucose when it is
294 available, even if they are arrested and their Glut1 levels are decreased, they have no
295 obligatory requirement for glucose, either to survive or to proliferate. Our work
296 demonstrates that reduced uptake of glucose is not responsible for the arrest of *Bax*$^{-/-}$
297 *Bak1*$^{-/-}$ FDM cells upon IL3 withdrawal.
298

299 Consistent with the requirement of glutamine for lymphoid cell survival (9), we found
300 that wild-type factor dependent myeloid cells died following glutamine deprivation
301 (Fig. 4). It was nevertheless surprising that glutamine was not required to sustain the
302 survival of *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells in the absence of glucose or Atg5-dependent
303 autophagy, and that the glutamine-deprived cells maintained a large size in the
304 presence of IL3, but arrested. This suggests that glutamine provided the source of
305 amino acids needed for cell division, but that these cells can maintain enough
306 metabolic activity to survive even in its absence.
307
308 Several pathways have been proposed to explain how engagement of IL3 receptors
309 prevents activation of Bax and Bak1. For example, it was proposed that IL3 receptors
310 signaled via Akt to phosphorylate Bad, preventing it from binding to Bcl-x, to thereby
311 free Bcl-x to inhibit apoptosis (15, 16). However, experiments in which genes for Bad
312 or Akt have been mutated have shown that unlike Bax/Bak1, they play relatively
313 minor roles in the regulation of apoptosis by IL3 (17, 18). These experiments, and
314 those where genes for all the Bcl-2 family member were deleted (19) suggest that
315 Bax/Bak1 dependent apoptosis is not always signaled via BH3-only proteins, but
316 more often regulated by controlling the abundance of anti-apoptotic Bcl-2 family
317 members, such as Bcl-2, Bcl-x, and Mcl-1 (20). The results presented here are
318 consistent with this, and show that apoptosis of FDMs following the removal of IL3,
319 or culture in the absence of glutamine, is due to the decrease in abundance of Mcl-1
320 (Fig. 5A). Cytokines including IL3 keeps cells alive by driving *Mcl-1* transcription,
321 most likely via Jak2 and Stat5 (21-23), and glutamine is required to maintain the
322 production of Mcl-1 to prevent default apoptosis. Consistent with a requirement for
323 Mcl-1 to prevent spontaneous activation of Bax/Bak1, a specific inhibitor of Mcl-1

324 was able to induce apoptosis of wild-type *Bax*$^{+/+}$*Bak1*$^{+/+}$ FDM cells cultured with IL3
325 and glutamine (Fig. 5B).
326
327 In the absence of glutamine, pathways activated by IL3 could still converge to induce
328 the expression of c-Myc in *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells, and although this was response
329 was blunted, the arrested cells maintained their large size (Figs. 4B and 5A). We
330 found that the transcription factor Atf4 was strongly induced by IL3 in glutamine-
331 starved cells (Fig. 5A), but at present, it is unclear how or why signaling from the IL3
332 receptor leads to increased Atf4 production. Given that IL3 can stimulate the
333 expression of ER chaperones in FDM cells (24) and that Atf4 translation increased in
334 cells starved of amino acids or exposed to stimuli that induced ER stress (13), it is
335 plausible that IL3 activated the unfolded protein response in the glutamine-deprived
336 FDMs. Atf4 has also been shown to promote the expression of several autophagy
337 genes (25), but we have not been able to confirm that its accumulation induced LC3B
338 lipidation in the glutamine-starved FDM cells. Nevertheless, IL3 also induced Atf4
339 expression in Atg5-deficient *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells cultured in glutamine-free media (Fig.
340 5A), suggesting that Atf4 does not promote canonical autophagy to maintain cell
341 viability under these conditions. The importance of Atf4 for the sustained survival of
342 *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells arrested by glutamine withdrawal will be determined when
343 we genetically delete *Atf4* in these cells.
344
345 In the absence of glucose, glutamine and Atg5-dependent autophagy, *Bax*$^{-/-}$*Bak1*$^{-/-}$
346 FDM cells remained viable for weeks, and their sensitivity to low concentrations of
347 fetal bovine serum (Fig. 6) are consistent with the hypothesis that they took up and
348 used components present in serum to maintain enough metabolism in order to survive.

349 Serum factors are likely scavenged by endocytic processes such as macropinocytosis,
350 which can be stimulated by growth factors (26). Increased endocytic uptake of serum
351 proteins could account for the observation that at ~2 weeks and beyond in culture
352 without glutamine, more Bax/Bak1-null cells survived when growth factor was
353 provided (Figs. 4C and S3E).
354
355 *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells cultured in the absence of glutamine express low levels of the
356 cell cycle regulators Cdk4 and cyclin D3 (Fig. 5A). Reduced levels of these proteins,
357 combined with possibly altered levels of other cell cycle regulators, could explain
358 why glutamine-starved cells arrested, even in the presence of IL3 that stimulated them
359 to maintain a large size (Fig. 7). In the absence of growth factor, glucose, glutamine
360 and Atg5-dependent autophagy, we hypothesise that *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid cells
361 survive by consuming proteins present in bovine serum, and degrading these in
362 lysosomes and/or proteasomes to maintain a sufficient pool of amino acids. Future
363 studies will address this hypothesis and determine how cells generate the energy they
364 need to stay viable under conditions of limited nutrients.
365
366 **Materials & Methods**
367 *Cell culture*
368 Wild-type *Bax*$^{+/+}$*Bak1*$^{+/+}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$ IL3-dependent mouse myeloid progenitor
369 cell lines (FDM) have been described (18). Cells were cultured in Dulbecco's
370 Modified Eagle Medium (DMEM, ThermoFisher Scientific Cat# 11885-084;
371 containing 1 g/L glucose and 4 mM glutamine) supplemented with 10% fetal bovine
372 serum (FBS, ThermoFisher Scientific Cat# 16000-044) and 0.25 ng/ml recombinant
373 murine interleukin 3 (IL3, PeproTech, Cat# 213-13) in a 37ºC humidified incubator

374 with 10% CO_2. Cells were deprived of IL3, glucose, or glucose and glutamine by
375 washing 3 times with medium (+10% FBS) that lacked those nutrients, then
376 resuspended in the same medium for analysis at the indicated time points. DMEM
377 lacking D-Glucose and sodium pyruvate (Cat# 11966-025) and DMEM lacking D-
378 Glucose, sodium pyruvate, L-Glutamine and phenol red (Cat# A14430-01) were
379 purchased from ThermoFisher Scientific. D-Glucose was purchased from Sigma
380 (Cat# G7528).
381
382 *Lentiviral constructs for CRISPR/Cas9 and inducible overexpression*
383 An inducible lentiviral CRISPR/Cas9 system (27) was used to mutate *Txnip* in *Bax*$^{-/-}$
384 *Bak1*$^{-/-}$ FDM cells. Guide RNA oligos targeting mouse *Txnip* (guide 1: CCA GCA
385 GGT GAG AAC GAG A; guide 2: TAT GGT TGC GTA GAC TAC T) were cloned
386 into the *Bsm*BI site of the pFgH1tUTG GFP lentiviral vector. Guide RNA oligo
387 targeting mouse *Atg5* (AAG ATG TGC TTC GAG ATG TG) was cloned into the
388 *Bsm*BI site of the lentiviral vector lentiCRISPR v2 (Addgene #52961; (28)). For
389 overexpression studies, the coding sequence of mouse *Txnip* was PCR amplified from
390 pEGFP-C1-Txnip (Addgene #18758; (29)) with the incorporation of an N-terminal
391 FLAG-tag. The PCR product was cloned into the *Eco*RI/*Nhe*I sites of a doxycycline
392 inducible pFTREtight MCS rtTAadvanced GFP lentiviral vector (30).
393
394 *Antibodies and compounds*
395 Antibodies: LC3B (D11, Cell Signaling Technology #3868), Atg5 (D5F5U, Cell
396 Signaling Technology #12994); β-actin (AC-15, Sigma #A1978), Glut1 (Abcam
397 ab15730), Txnip (D5F3E, Cell Signaling Technology #14715S), Glut4 (1F8, Cell
398 Signaling Technology #2213), Mcl-1 (Rockland 600-401-394S), c-Myc (Abcam

399 ab32072), Cdk4 (Santa Cruz Biotechnology sc-260), Cyclin D3 (Cell Signaling
400 Technology #2936), Atf4 (D4B8, Cell Signaling Technology #11815). Compounds:
401 Mcl-1 inhibitor S63845 was synthesized according to Kotschy et al. (10) by
402 SYNthesis Med Chem Pty Ltd (Parkville, Australia); DMSO (dimethyl sulfoxide) was
403 purchased from Sigma (Cat# 472301).
404
405 *Cell death and cell cycle assays*
406 Cell death and cell cycle were assayed using propidium iodide (PI, Sigma) and flow
407 cytometry. For death assays, cells were harvested and resuspended in PBS containing
408 1 μg/ml PI. For cell cycle analysis, cells were harvested and resuspended in PBS
409 containing 0.1% Triton X-100, 5 μg/ml PI and 10 μg/ml RNaseA (Thermo Scientific),
410 then incubated at 37 $^{\circ}$C for 15 min. Samples were processed on FACSCalibur or
411 LSRFortessa X-20 flow cytometers (BD Biosciences) and the acquired data analysed
412 with FlowJo software.
413
414 *Glucose uptake assays*
415 Extracellular acidification (ECAR) rates were measured in live cells using a Seahorse
416 Bioscience XFe96 Analyzer (Agilent Technologies). In brief, cells were resuspended
417 in non-buffered DMEM (Seahorse Biosciences) and 200 x 10^3 cells were seeded per
418 well in Seahorse Bioscience 96-well plates. The cellular ECAR was analyzed in non-
419 buffered DMEM containing 2 mM glutamine with the following compounds: 5 mM
420 glucose, 1 μM oligomycin and 50 mM 2-deoxy-glucose (2DG). For each assay cycle,
421 measurement time points of 2 min mix, 2 min wait and 5 min measure were collected.
422 Data shown for each cell line are representative of at least 2 independent experiments
423 performed in triplicate.

424 **Acknowledgments**

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428 operational infrastructure grants through the Australian Government Independent
429 Research Institutes Infrastructure Support (IRISS) and the Victorian State
430 Government OIS.

431

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539

540 **Figure Legends**

541 **Figure 1.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells shrink and arrest but remain viable when cultured
542 without IL3.
543 **A.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells survive long-term in the absence of IL3. *Bax*$^{+/+}$*Bak1*$^{+/+}$
544 wild-type (black circles) and *Bax*$^{-/-}$*Bak1*$^{-/-}$ (green squares) FDM cells were cultured
545 without IL3. At the indicated times, cells were harvested, resuspended in PBS

546 containing propidium iodide (PI) and analysed by flow cytometry. See Supplemental
547 Fig. S1A for a representative independent repeat of this experiment.
548 **B.** IL3 withdrawal caused cells to shrink. PI negative populations of *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells
549 cultured for 48 h in the presence (light gray) or absence (dark gray) of IL3, and the
550 latter that had IL3 returned for 72 h (green), were compared by forward scatter as an
551 indicator of cell size. Data shown are representative of 2 independent experiments.
552 **C.** IL3 withdrawal caused cell cycle arrest, which was reversed by re-addition of IL3.
553 Nuclei from *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells cultured as described in (B) were stained with PI to
554 indicate DNA content by flow cytometry. Data shown are representative of 2
555 independent experiments.
556
557 **Figure 2.** *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* FDM cells shrink and arrest but remain viable when IL3
558 is withdrawn.
559 **A.** Whole cell lysates from *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells and 3 *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* FDM clonal
560 lines cultured for 48 h in the presence or absence of IL3 were subjected to Western
561 blotting. The Atg5 immunoblot demonstrates successful CRISPR/Cas9-mediated
562 deletion of *Atg5*. The LC3B immunoblot indicates greatly reduced LC3B lipidation
563 (isoform II) in Δ*Atg5* cell clones. Data shown are representative of 2 independent
564 experiments.
565 **B.** *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* FDM cells survive long-term in the absence of IL3. The
566 indicated *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clones were cultured without IL3 and viability was
567 determined by PI uptake at the indicated times. See Supplemental Fig. S1B for a
568 representative independent repeat of this experiment.

569 **C.** Cells from *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clone 1 were cultured for 48 h in the presence or
570 absence of IL3 then processed in PI buffer to measure DNA content by flow
571 cytometry. Data shown are representative of 2 independent experiments.
572 **D.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ Δ*Atg5* clone 1 cells were cultured for 48 h in the presence or absence
573 of IL3 and PI negative populations were compared by forward scatter to indicate
574 differences in cell size. Data shown are representative of 2 independent experiments.
575
576 **Figure 3.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM cells arrested by IL3 deprivation survive in the absence
577 of glucose.
578 **A.** Txnip is not required for the decrease in Glut1 following IL3 withdrawal. Whole
579 cell lysates from *Bax*$^{-/-}$*Bak1*$^{-/-}$ and 3 independent *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Txnip* FDM cell clones
580 cultured for 48 h in the presence or absence of IL3 were analysed by Western blotting
581 to detect Glut1 and Txnip protein. Data shown are representative of 2 independent
582 experiments.
583 **B.** Forced expression of Txnip is not sufficient to decrease Glut1 protein levels.
584 Independent *Bax*$^{-/-}$*Bak1*$^{-/-}$ FDM clones bearing a doxycycline (dox)-inducible FLAG-
585 Txnip construct or empty vector control were cultured for 72 h in the absence or
586 presence of dox. Whole cell lysates were prepared and analysed by Western blotting
587 to detect Glut1 and Txnip protein. The data shown represent 1 experiment.
588 **C.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Txnip* cells can take up and metabolise glucose.
589 Cells cultured for 72 h in the presence or absence of IL3 were analysed on the
590 Seahorse bioanalyzer to measure the extracellular acidification rate (ECAR) as an
591 indicator of glucose metabolism. The increased ECAR following addition of glucose
592 signifies increased glycolytic flux in cells. Oligomycin inhibits mitochondrial ATP
593 synthase and the change in ECAR upon its addition represents the glycolytic reserve

594 capacity of cells. Glycolysis is inhibited by 2-deoxyglucose (2-DG) so upon its
595 addition, the ECAR values are produced from non-glycolytic sources. Equal numbers
596 of cells were seeded for analysis. To correct for the reduced size of IL3-starved cells,
597 protein concentration was assayed and the ECAR values were normalized to 1 μg
598 total protein per sample. Curves represent the mean of 3 replicate samples depicted as
599 solid symbols for each time point. Data shown are representative of 2 independent
600 experiments.

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

604

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

607 **A.** *Bax*$^{+/+}$*Bak1*$^{+/+}$ cells undergo apoptosis in the absence of glutamine (upper panels),
608 whereas *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells survive in the absence of glutamine, but arrest even when
609 IL3 is present (lower panels). Cells were cultured with or without IL3 in glucose- and
610 glutamine-free medium for 4 days then processed in PI buffer to assess DNA content
611 by flow cytometry. Note the absence of cells in S phase when *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells were
612 cultured without glutamine. Data shown are representative of 2 independent
613 experiments.

614 **B.** PI negative populations of *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells cultured with or without IL3 in
615 medium lacking glucose or glutamine for 4 days (D4) were compared to cells at day 0
616 (D0) by forward scatter as an indicator of cell size. Data shown are representative of 2
617 independent experiments.

618 **C.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells survive long-term in the absence of glutamine. Cells were
619 cultured with or without IL3 in glucose- and glutamine-free medium. At the indicated
620 times, cells were assayed for viability by PI uptake. See Supplemental Fig. S3E for an
621 independent repeat of this experiment.
622 **D.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells require glutamine to divide. Cells were cultured with or without
623 IL3 for 7 days in glucose- and glutamine-free medium. They were then split in three,
624 and resuspended in medium without glucose and glutamine, or containing only
625 glucose (1 g/L), or only glutamine (4 mM), for a further 2 days. Cells were harvested
626 and resuspended in PI buffer to assess DNA content by flow cytometry. Data shown
627 are representative of 2 independent experiments.
628
629 **Figure 5.** Withdrawal of IL3 or glutamine reduced the expression of Mcl-1 and
630 several key regulators of cell cycle progression.
631 **A.** *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells and *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* clone 1 were cultured for 72 h with or
632 without IL3 in complete or glucose (Glc)- and glutamine (Gln)-free medium. Whole
633 cell lysates were prepared and analysed by Western blotting to detect the indicated
634 proteins. Three independent membranes were used for immunoblotting and β-actin
635 loading control blots for each are shown. Membrane #1: anti-Mcl-1 and anti-c-Myc;
636 membrane #2: anti-Cdk4; membrane #3: anti-cyclin D3 and anti-Atf4. Data shown are
637 representative of 2 independent experiments.
638 **B.** *Bax*$^{+/+}$*Bak1*$^{+/+}$ and *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells cultured in complete medium with IL3 were
639 treated with DMSO (vehicle control) or the 10 μM of the Mcl-1 inhibitor S63845 for
640 24 h. Cells were harvested and viability determined by PI uptake and flow cytometry.
641 Bars depict the mean of 3 replicate cultures represented by the solid symbols. The
642 data shown represent 1 experiment.

643

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

646 **A.** Even though they were incapable of undergoing Bax/Bak1-dependent apoptosis,
647 *Bax*$^{-/-}$*Bak*$^{-/-}$ FDM cells starved of glucose and glutamine died rapidly when cultured in
648 medium with less than 4% fetal bovine serum. *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells were cultured with
649 the indicated concentrations of FBS in medium with IL3 but without supplemented
650 glucose and glutamine. Viability was determined by failure to take up PI at the
651 indicated time points as assessed by flow cytometry. The data shown represent 1
652 experiment.

653 **B.** *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* FDM cells starved of glucose and glutamine died rapidly when
654 cultured in medium with less than 4% fetal bovine serum. *Bax*$^{-/-}$*Bak1*$^{-/-}$Δ*Atg5* cells
655 were cultured and analyzed for viability as described above in (A). The data shown
656 represent 1 experiment.

657

658 **Figure 7.**

659 The growth factor IL3 stimulates the expression of Mcl-1 that inhibits activation of
660 Bax/Bak1, and stimulates expression of c-Myc, Cdk4 and cyclin D3 to promote cell
661 growth and proliferation. Glutamine is required to maintain levels of these proteins
662 needed to prevent activation of Bax/Bak1 (Mcl-1), as well as for cell proliferation (c-
663 Myc, Cdk4, cyclin D3 and possibly others). In the absence of growth factor, glucose
664 glutamine and Atg5-dependent autophagy, we hypothesise that *Bax*$^{-/-}$*Bak1*$^{-/-}$ myeloid
665 cells survive by consuming proteins present in fetal bovine serum, and degrading
666 these in lysosomes and proteasomes to maintain a sufficient pool of amino acids for
667 energy production.

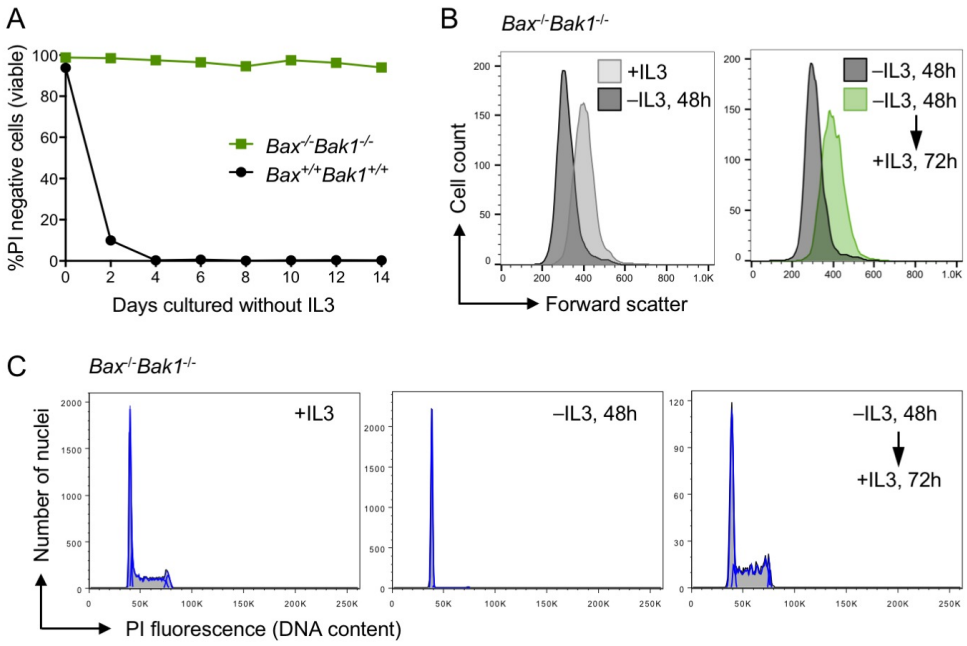
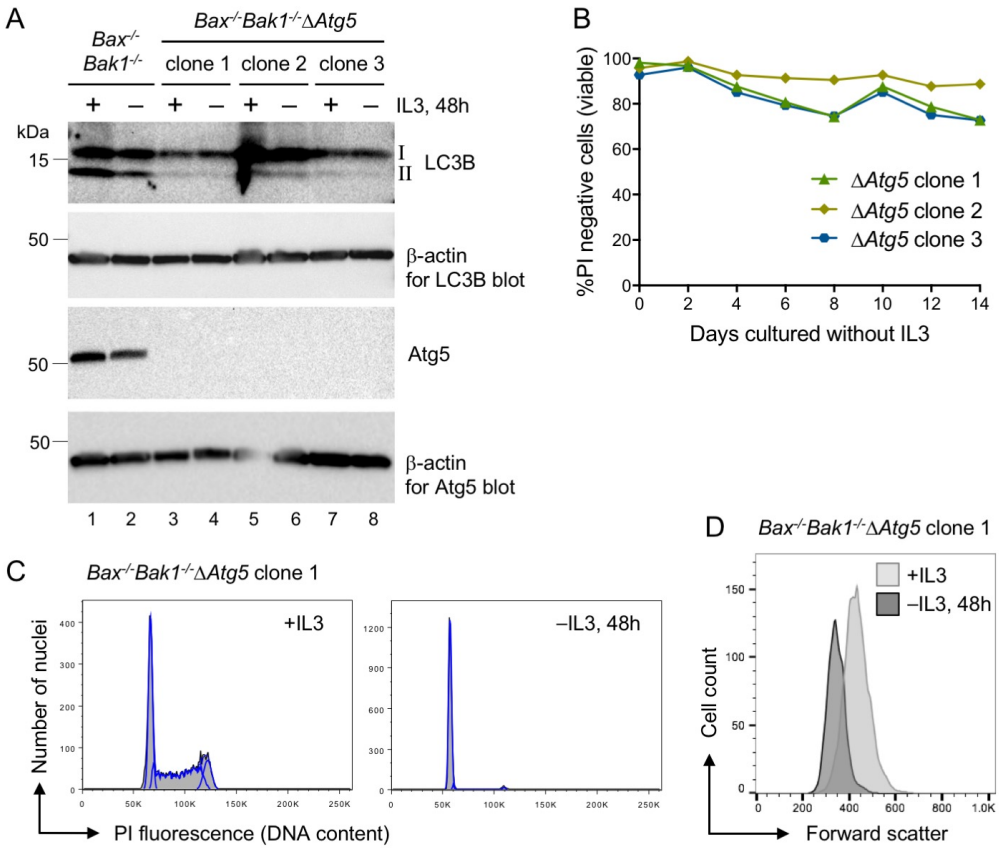
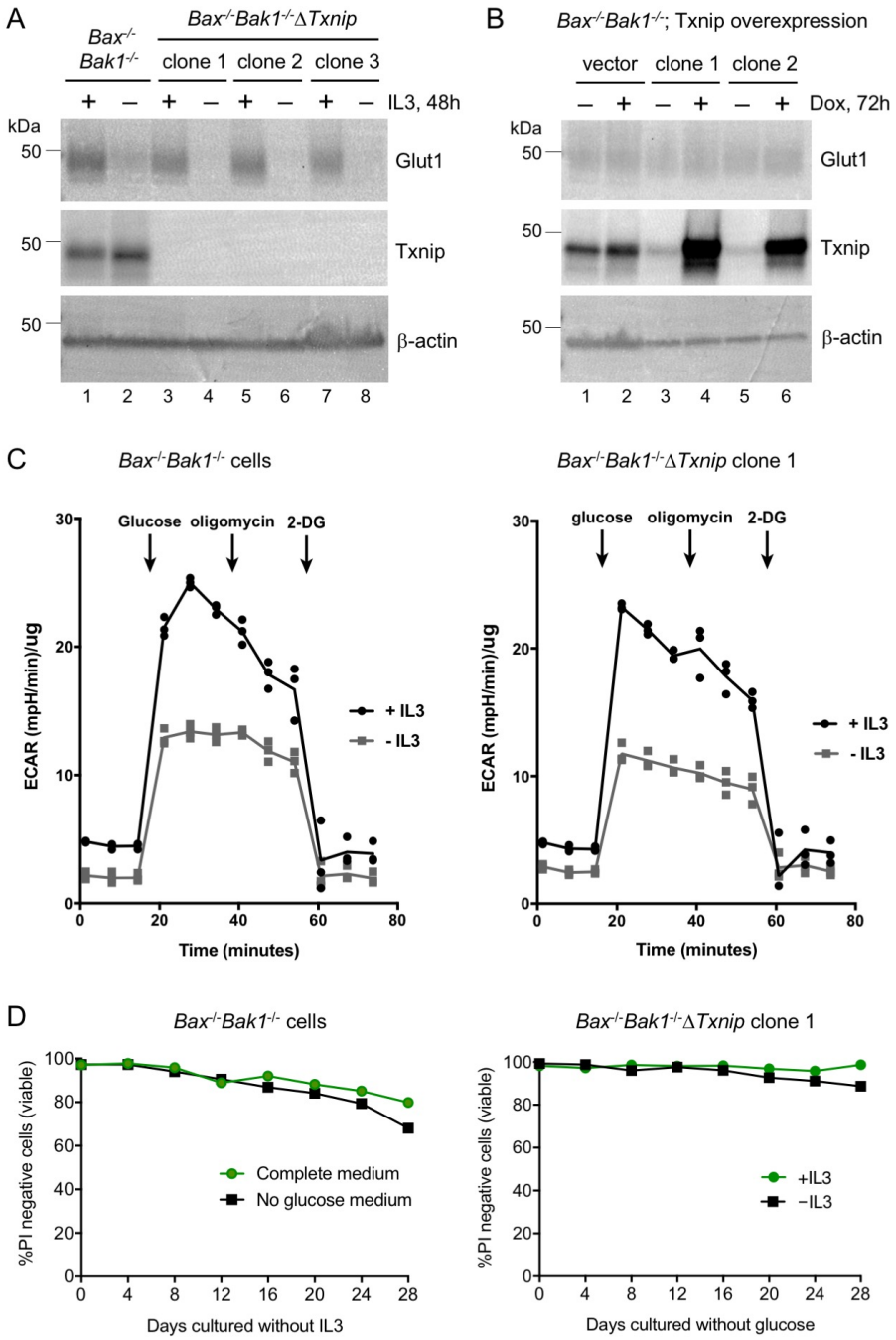
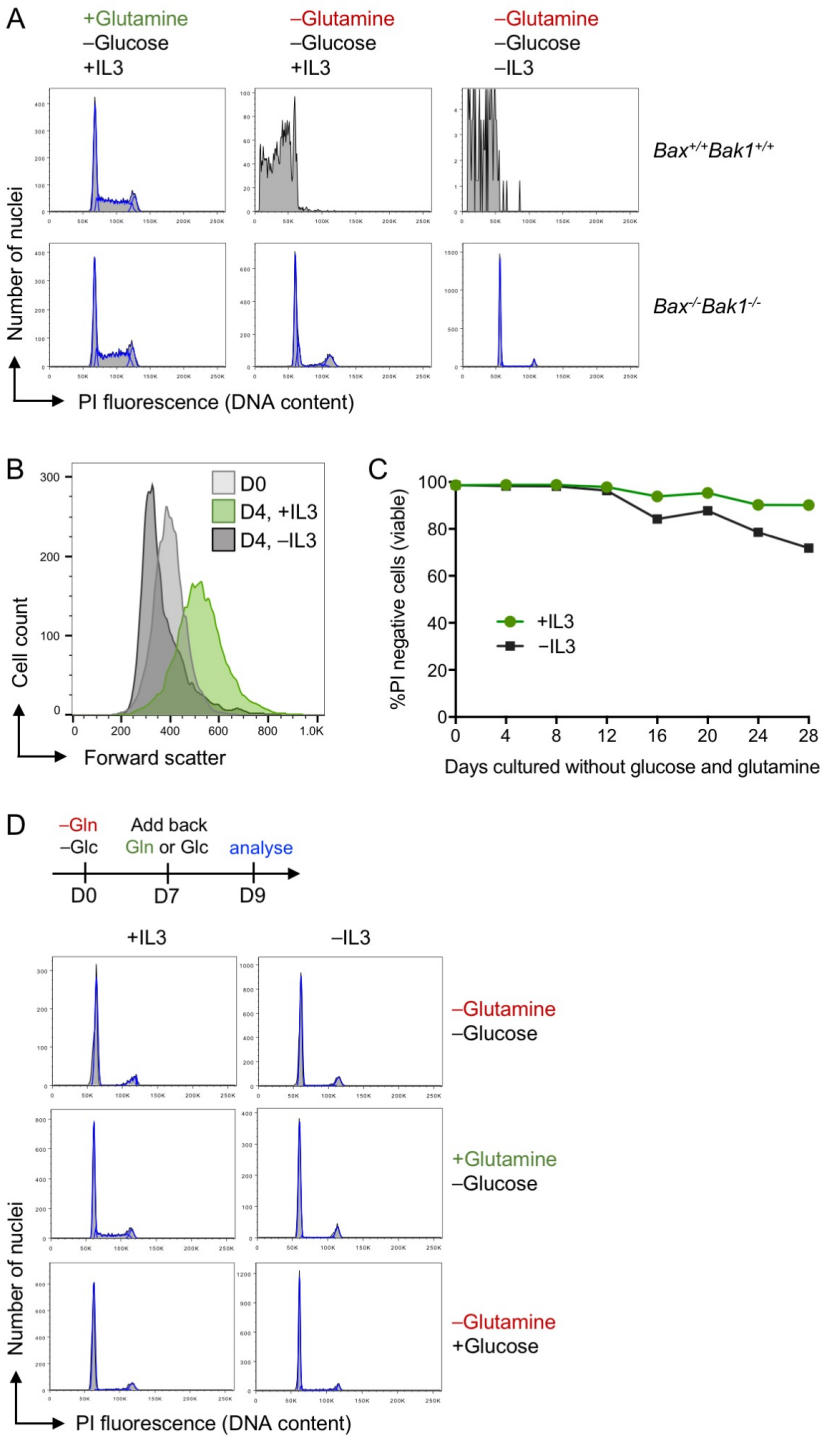
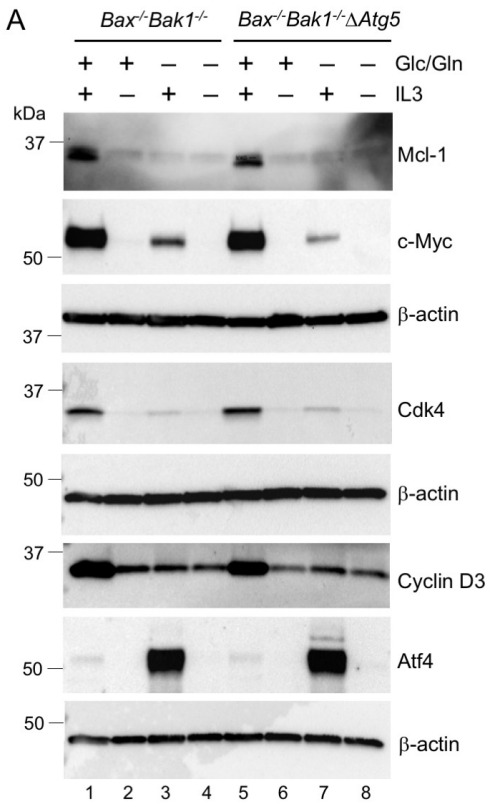

A
%PI negative cells (viable)
Days cultured without IL3
Bax-/-Bak1-/-
Bax+/+Bak1+/+
B
Bax-/-Bak1-/-
Cell count
Forward scatter
+IL3
–IL3, 48h
–IL3, 48h
+IL3, 72h
C
Bax-/-Bak1-/-
Number of nuclei
PI fluorescence (DNA content)
+IL3
–IL3, 48h
–IL3, 48h
+IL3, 72h

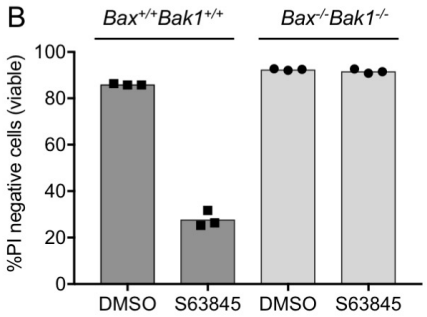
FIGURE 2 Dong et al.

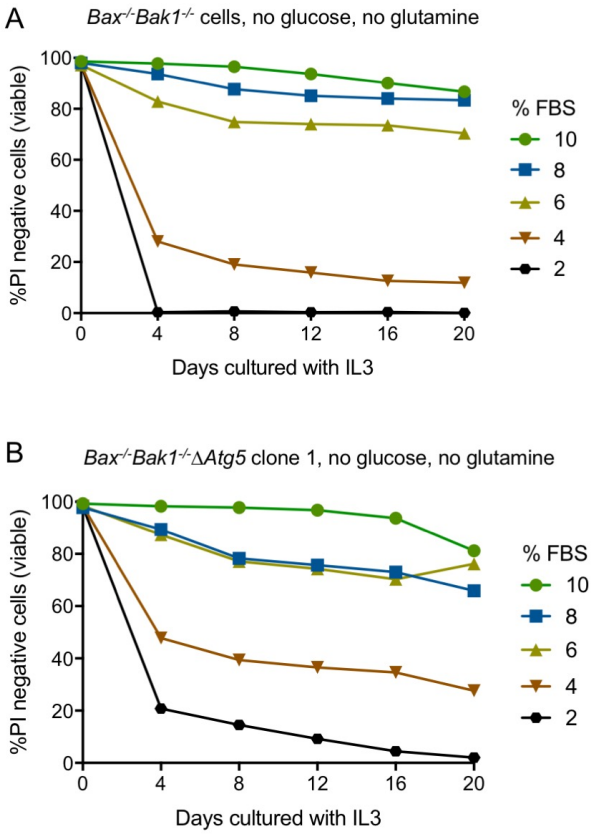



A
Bax-/- Bak1-/-
Bax-/-Bak1-/-ΔTxnip
clone 1 clone 2 clone 3
+ − + − + − + − IL3, 48h
kDa
50
Glut1
Txnip
β-actin
1 2 3 4 5 6 7 8
B
Bax-/-Bak1-/-; Txnip overexpression
vector clone 1 clone 2
− + − + − + Dox, 72h
1 2 3 4 5 6
C
Bax-/-Bak1-/- cells
Bax-/-Bak1-/-ΔTxnip clone 1
Glucose oligomycin 2-DG
glucose 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
+ + – – + + – – Glc/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

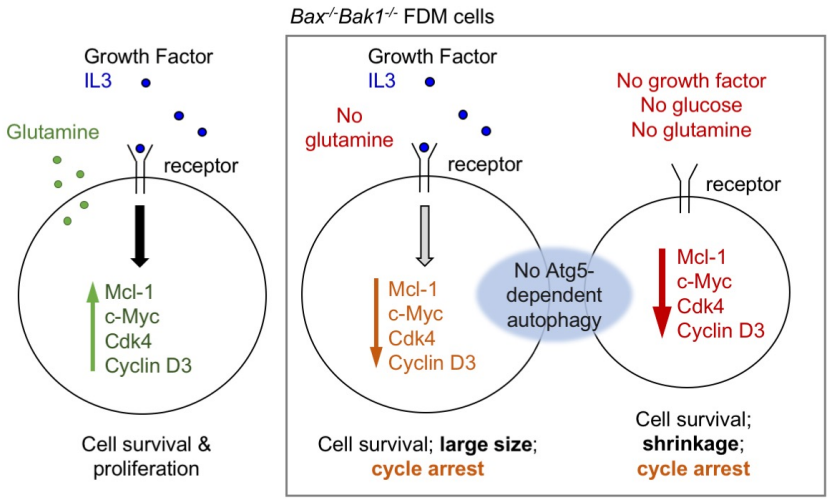



FIGURE 7 Dong et al.