

Tumor suppressors BTG1 and BTG2 regulate early mouse B-cell development

Transcription factors that regulate B-cell differentiation are frequently targeted by genetic aberrations in B-cell malignancies. The B-cell translocation gene 1 (*BTG1*), which encodes a transcription coregulator,^{1,2} is recurrently affected by gene deletions in B-cell precursor acute lymphoblastic leukemia (BCP-ALL),³⁻⁵ and targeted by missense mutations in diffuse large B-cell lymphomas (DLBCL).^{6,7} Similarly, the closely related *BTG2* gene is subject to mutations in DLBCL, Burkitt lymphoma and follicular lymphoma.⁶⁻⁹ To establish the role of BTG1 and BTG2 in B lymphopoiesis, we studied the composition of the hematopoietic compartment in mice deficient for

either *Btg1* or *Btg2*, as well as in *Btg1*;*Btg2*-double knock-out animals. We found that *Btg1* and *Btg2* are both required at multiple stages of early B-cell development, but also display specific functions in progenitor B-cells. Our data demonstrate that transcriptional cofactors BTG1 and BTG2 act as important regulators of B-cell differentiation, providing an important link to their role as tumor suppressors in B-cell malignancies.

To determine the function of *Btg1* and *Btg2* during normal hematopoiesis we performed flow cytometric analysis of the bone marrow (BM) compartment and spleen. The fraction of B220⁺ progenitor B-cells was significantly reduced in *Btg1*^{-/-} and *Btg2*^{-/-} mice, ranging from a 10% decrease in the *Btg2*^{-/-} mice, 20% in *Btg1*^{-/-} mice, to 40% in the *Btg1*^{-/-};*Btg2*^{-/-} double knockout animals relative to control mice (Figure 1A). Similarly, B-cell counts in the

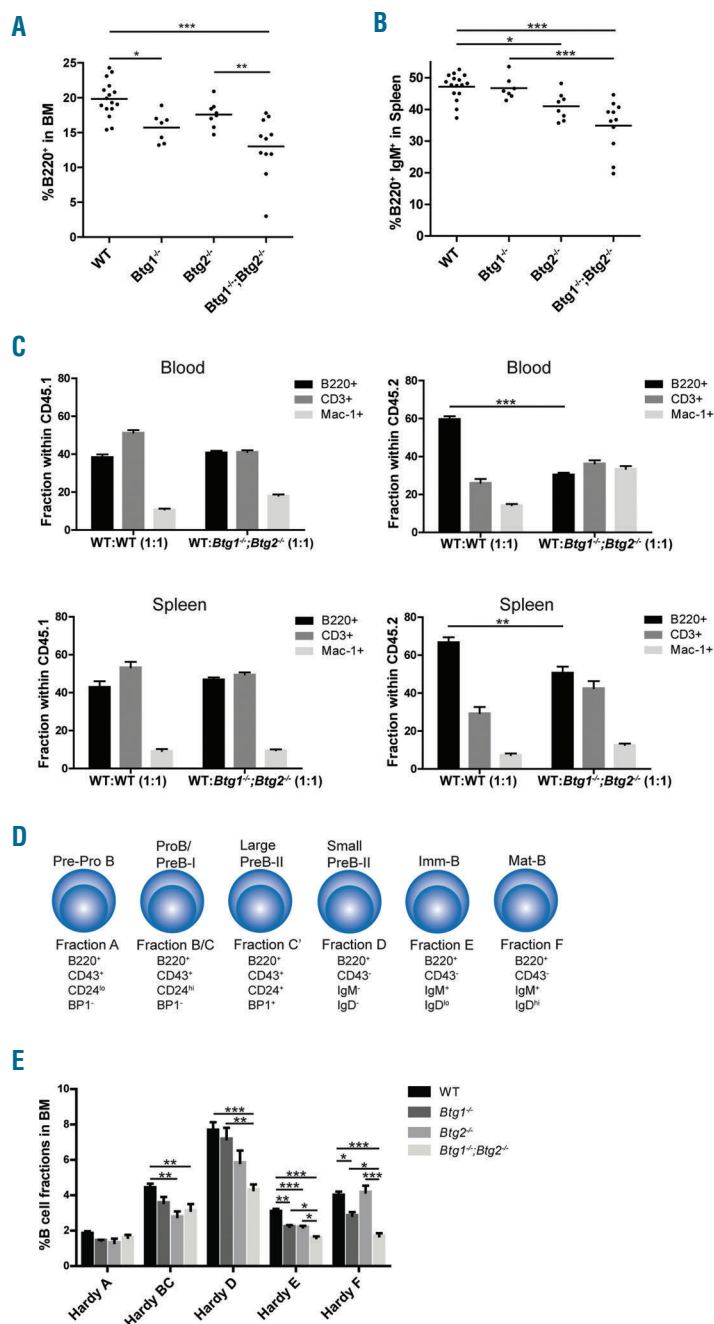


Figure 1. Decreased B-cell numbers in mice deficient for *Btg1* and *Btg2*. (A-B) Flow cytometric analysis was performed on mononuclear cells isolated from bone marrow (BM) and spleen of wild-type (WT) control (n=16), *Btg1*^{-/-} (n=7), *Btg2*^{-/-} (n=8) and *Btg1*^{-/-};*Btg2*^{-/-} mice (n=11). The fractions of B220⁺ BM cells (A) and B220⁺IgM⁺ splenocytes (B) are indicated. Each dot represents an individual sample and the horizontal line indicates the mean. (C) Competitive repopulation assay of wild-type and *Btg1*^{-/-};*Btg2*^{-/-} donor cells (CD45.2) derived of BM mixed 1:1 with CD45.1 wild-type cells which were transplanted into 9Gy (Gray) irradiated C57BL6/J mice (CD45.1). Flow cytometric analysis was performed on blood and spleen. The fraction of B220, CD3 and Mac-1 of WT:WT (CD45.1:CD45.1) (n=4) and WT:*Btg1*^{-/-};*Btg2*^{-/-} (CD45.1:CD45.2) (n=6) cells are indicated. (D) Schematic representation of the different B-cell developmental stages according to Hardy nomenclature. Cell surface markers that distinguish specific Hardy fractions are indicated. (E) The percentage for the different Hardy B-cell fractions within BM is indicated as determined in WT control (n=16), *Btg1*^{-/-} (n=7), *Btg2*^{-/-} (n=8), and *Btg1*^{-/-};*Btg2*^{-/-} (n=11) mice. Each bar represents the mean of each group of mice \pm the standard error of the mean (SEM). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

spleen were most significantly reduced in *Btg1*^{-/-}; *Btg2*^{-/-} mice, with about 30% fewer B220⁺IgM⁺ B-cells (Figure 1B). In contrast, the fraction of myeloid progenitor cells in BM was not altered in the *Btg1*^{-/-} and *Btg2*^{-/-} deficient animals (Online Supplementary Figure S1). In addition, we did not observe any specific defects in T-cell development in mice lacking *Btg1* and *Btg2* expression (Online Supplementary Figure S2).

To assess whether the defect in B lymphopoiesis was not related to defective support from the BM microenvironment in the *Btg1* and *Btg2* knockout animals, competitive repopulation assays were performed. BM cells derived from either wild-type (CD45.2) or *Btg1*^{-/-}; *Btg2*^{-/-} mice (CD45.2) were mixed with competitor wild-type cells (CD45.1) and transplanted into lethally irradiated C57BL/6J mice (CD45.1). FACS analysis on peripheral blood and spleen demonstrated that contribution to the B-cell lineage was significantly reduced using *Btg1*^{-/-}; *Btg2*^{-/-} donor cells when compared to wild-type donor cells (Figure 1C), arguing that the observed defect in B-cell development is cell autonomous.

To establish at what stage of early B-cell development *Btg1*^{-/-} and *Btg2*^{-/-} deficiency affect B-cell differentiation, we investigated the relative frequencies of the different B-cell

subsets within the BM compartment. Analysis of the common lymphoid progenitor (CLP) fraction, defined as lineage negative (Lin⁻) Sca-1⁻c-Kit⁺Flt3⁺Il-7Rα⁺ cells, revealed no significant differences in *Btg1*^{-/-}, *Btg2*^{-/-} and *Btg1*^{-/-}; *Btg2*^{-/-} mice compared to wild-type controls (Online Supplementary Figure S3). Next, we employed the cell surface markers B220, CD43, CD24, IgD and IgM, to distinguish different B-cell precursor populations, generally known as Hardy fractions (Figure 1D). When we examined the single knockout animals, the *Btg2*^{-/-} mice displayed decreased B-cell fractions at both the early and later stages of B-cell differentiation, while *Btg1*^{-/-} deficiency resulted only in reduced B-cell fractions beyond the pre-B cell stage, including the immature B-cells (Hardy fractions E and F) (Figure 1E). In the *Btg1*^{-/-}; *Btg2*^{-/-} mice, we observed a similar phenotype as the *Btg2*^{-/-} mice at the early stages of B-lineage differentiation, with a moderate decrease in the pool of pro-B cells (Hardy BC) compared to control mice. However, from the pre-B cell stage onwards, the *Btg1*^{-/-}; *Btg2*^{-/-} mice displayed a more pronounced phenotype in comparison to the *Btg1*^{-/-} and *Btg2*^{-/-} single knockout animals, showing a 2-fold reduction in the B220⁺CD43⁻ cell fraction relative to control mice (Hardy D, E and F) (Online Supplementary Figure S4).

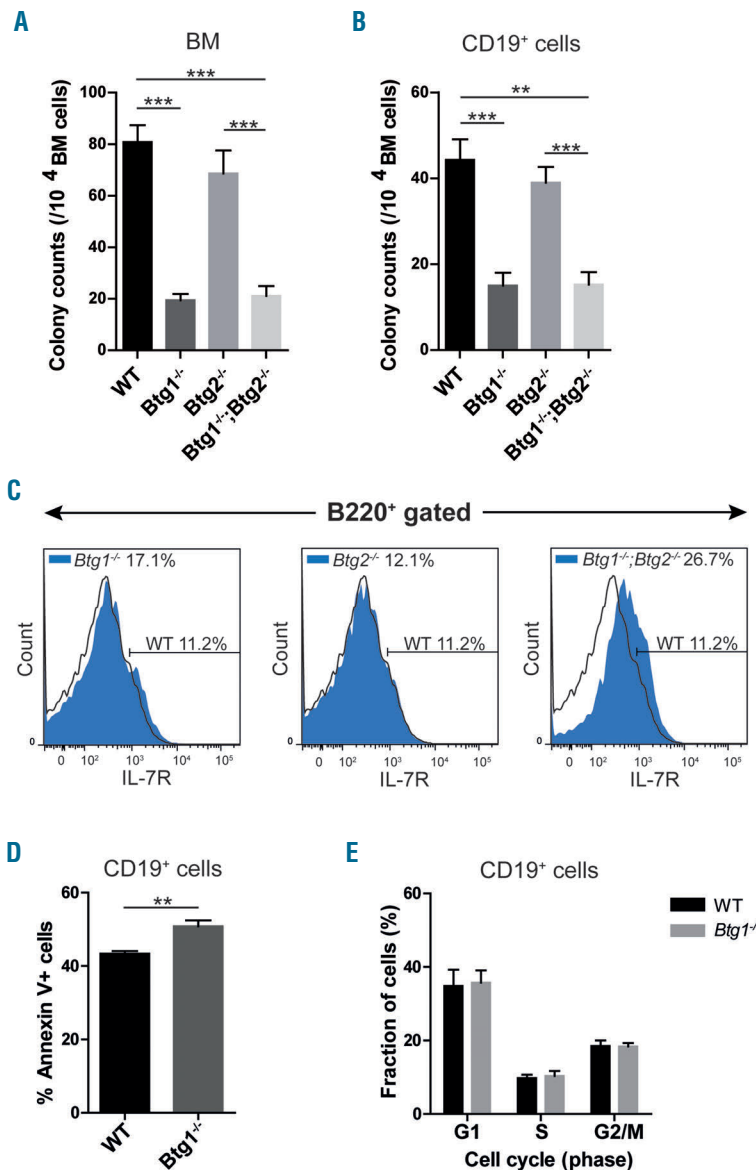


Figure 2. *Btg1* stimulates B-cell progenitor outgrowth in response to IL-7. (A) Bone marrow (BM) mononuclear cells (1×10^5 cells) were isolated from wild-type (WT) control ($n=18$), *Btg1*^{-/-} ($n=7$), *Btg2*^{-/-} ($n=8$) and *Btg1*^{-/-}; *Btg2*^{-/-} mice ($n=11$) and added in methylcellulose containing 10 ng/mL IL-7. (B) BM mononuclear cells were isolated from WT control ($n=10$), *Btg1*^{-/-} ($n=7$), *Btg2*^{-/-} ($n=4$) and *Btg1*^{-/-}; *Btg2*^{-/-} mice ($n=4$) and added after CD19 isolation (2×10^4 CD19⁺ cells) in methylcellulose containing 10ng/ml IL-7. (A, B) Mean colony counts (and SEM) were determined (>30 cells/colony) after seven days of culture. Data are representative of at least two independent experiments. $^{***}P<0.01$, $^{****}P<0.001$. (C) BM cells from WT, *Btg1*^{-/-}, *Btg2*^{-/-}, and *Btg1*^{-/-}; *Btg2*^{-/-} mice (all $n=2$) were stained with B220 and IL-7Rα/CD127 antibodies and the percentage of IL-7Rα-positive cells within the B220⁺ fraction was determined. The black line indicates IL-7Rα expression in the WT cells, while the blue peak shows the IL-7Rα expression in the *Btg1*^{-/-}, *Btg2*^{-/-} and *Btg1*^{-/-}; *Btg2*^{-/-} double knockout cells. (D) Apoptosis assay of CD19⁺ BM cells isolated from WT ($n=4$) and *Btg1*^{-/-} ($n=7$) cultured for 4 days in B-cell specific medium, by measuring Annexin V using flow cytometry. The percentage of Annexin V-positive cells in CD19⁺ BM cells is indicated. (E) Proliferation assay of CD19⁺ BM cells isolated from WT ($n=3$) and *Btg1*^{-/-} ($n=3$) cultured for 4 days in B-cell specific medium, measuring Ki-67 by flow cytometry. The cell cycle distribution according to Ki-67 staining in CD19⁺ BM cells is indicated. Each bar represents the mean of each group of mice \pm the standard error of the mean (SEM). $^{**}P<0.01$, $^{***}P<0.001$.

We conclude from these results that *Btg1* and *Btg2* are both required for mouse B-cell development, while deficiency for each of the genes shows a different outcome on the relative abundance of the specific Hardy fractions.

To more specifically assess the function of *Btg1* and *Btg2* in early B lymphopoiesis, we investigated whether interleukin-7 (IL-7) dependent outgrowth of B-lymphoid progenitor cells was affected in the absence of *Btg1* and *Btg2* expression. BM cells derived from *Btg1*^{-/-}, *Btg2*^{-/-} single knockout and *Btg1*^{-/-};*Btg2*^{-/-} double knockout mice were plated in methylcellulose and cultured for one week in the presence of cytokine IL-7. The number of colonies in methylcellulose was about 4-fold lower in cultures derived from *Btg1*^{-/-} and *Btg1*^{-/-};*Btg2*^{-/-} BM cells relative to

those derived from control BM cells (Figure 2A). Moreover, colony size was also reduced in these two knockout genotypes (*data not shown*). In contrast, *Btg2*-deficiency had no significant impact on the outgrowth of lymphoid progenitor cells in the IL-7-dependent methylcellulose colony formation assay. Given the fact that the number of B220⁺ B-cell progenitors was decreased in the BM of the different *Btg1* and *Btg2* knockout lines, we also analyzed the colony outgrowth of similar amounts of purified CD19⁺ progenitor B-cells under the same conditions. Again, colony formation was significantly impaired using CD19⁺ cells derived from *Btg1*^{-/-} and *Btg1*^{-/-};*Btg2*^{-/-} mice, but not with *Btg2*^{-/-} progenitor B-cells, which indicates that the loss of *Btg1* function is

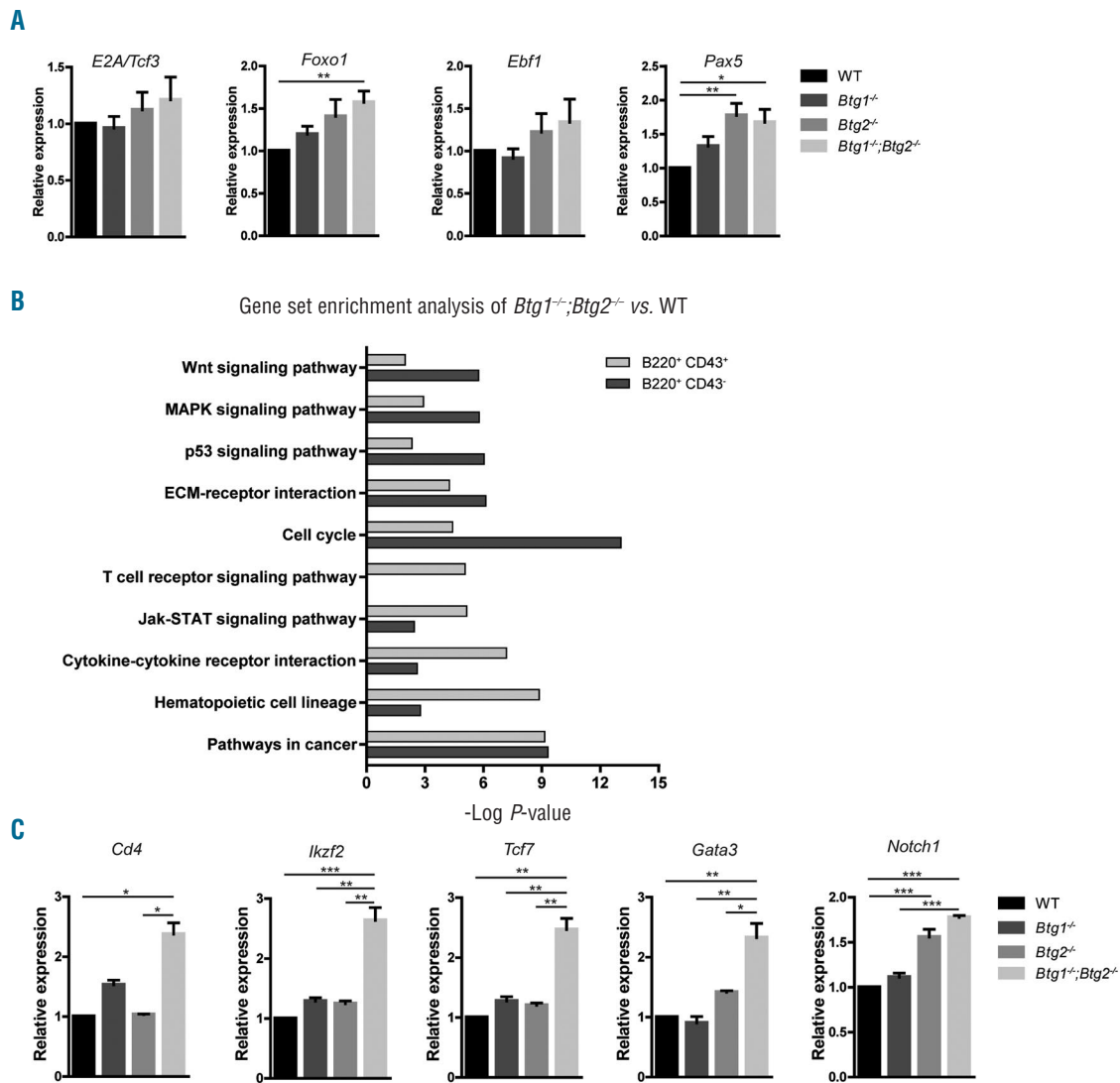


Figure 3. Aberrant T-lineage gene expression in progenitor B-cells deficient for *Btg1* and *Btg2*. (A) Relative expression levels of B-cell transcription factors *E2A* (*Tcf3*), *Foxo1*, *Ebf1* and *Pax5* was determined using cDNA generated from B220⁺ bone marrow (BM) cells of wild type control (WT), *Btg1*^{-/-}, *Btg2*^{-/-} and *Btg1*^{-/-};*Btg2*^{-/-} mice by qRT-PCR and normalized to the expression of the housekeeping gene *TBP*. (B) RNA was isolated from B220⁺CD43⁺ and B220⁺CD43⁻ cells derived from wild type control and *Btg1*^{-/-};*Btg2*^{-/-} mice (n=4) and analyzed by microarray using the Illumina BeadArray platform. The data obtained were RMA normalized and relative gene expression differences of >1.6-fold was determined. KEGG pathway analysis was performed using the online Gene Set Enrichment Analysis (GSEA) tool and relevant pathways with highest *P*-value that are differentially expressed between WT and *Btg1*^{-/-};*Btg2*^{-/-} cells are indicated. (C) Relative expression levels of *Cd4*, *Ikzf2*, *Tcf7*, *Gata3* and *Notch1* were determined on cDNA generated from B220⁺ BM cells of the different genotypes by real-time PCR and normalized to *TBP* expression. (A and C) Data represent the mean and SEM of three independent experiments containing cDNA derived from 2 different biological samples. **P*<0.05, ***P*<0.01, ****P*<0.001.

largely responsible for the defective outgrowth of B-lymphoid progenitors (Figure 2B).

To assess whether differences in colony formation could be attributed to reduced expression levels of the IL-7 receptor alpha (IL-7R α /CD127), CD127 expression was measured on B220⁺ BM cells by flow cytometry. We observed that IL-7R α expression was not decreased in *Btg1*^{-/-} progenitor B-cells, but was even upregulated in *Btg1*^{-/-};*Btg2*^{-/-} cells (Figure 2C), arguing that the low number of colonies due to *Btg1*-deficiency was not related to reduced IL-7R α expression levels. Next, we examined whether *Btg1*-deficient CD19⁺ cells displayed intrinsic differences in the level of apoptosis and proliferation. We observed a moderate but significant increase in the fraction of Annexin V-positive cells derived from *Btg1*^{-/-} mice (Figure 2D; *Online Supplementary Figure S5*), while the cell cycle distribution, as determined by Ki-67 staining, was not affected (Figure 2E). Thus, *Btg1* appears to be required for optimal IL-7 dependent B-lymphoid colony formation, in part by promoting cell survival in early progenitor B-cells.

To elucidate how *Btg1* and *Btg2* regulate B-cell development *in vivo*, we examined the expression of key transcription factors involved in early B-cell differentiation, including *E2a* (*Tcf3*), *Foxo1*, *Ebf1*, and *Pax5*.^{10,11} Quantitative reverse transcription polymerase reaction (qRT-PCR) on RNA isolated from B220⁺ sorted BM cells of *Btg1*^{-/-}, *Btg2*^{-/-} and *Btg1*^{-/-};*Btg2*^{-/-} mice demonstrated that the expression of these master regulators of B-cell differentiation was not decreased (Figure 3A). On the contrary, mRNA expression levels of these transcription factors were even somewhat elevated in the absence of *Btg1* and *Btg2*. Hence, the partial block in B-cell differentiation observed in the *Btg1*^{-/-};*Btg2*^{-/-} animals cannot be explained by diminished expression levels of these B-lineage transcription factors.

Next, we performed microarray based gene expression profiling using early (B220⁺CD43⁺) and late progenitor B-cells (B220⁺CD43⁻) isolated from control and *Btg1*^{-/-};*Btg2*^{-/-} mice and performed Gene Set Enrichment Analyses (GSEA) using the Molecular Signatures Database (MSigDB) to define KEGG pathways that were specifically affected (Figure 3B). Interestingly, several signaling pathways were identified, including the presence of a T-cell receptor signaling signature in early B-cells of *Btg1*^{-/-};*Btg2*^{-/-} mice showing upregulation of *Cd3d* (*Cd3delta*) and *Cd3g* (*Cd3gamma*) (*Online Supplementary Table S1*). Therefore, we aimed to assess whether *Btg1*;*Btg2*-deficient progenitor B-cells displayed aberrant expression of other T-lineage specific genes, including *Cd4*, *Irf2*, *Tcf7*, *Gata3* and *Notch1*. Indeed, qRT-PCR revealed that *Btg1*^{-/-};*Btg2*^{-/-} B220⁺ BM cells showed significantly increased expression levels of these T-lineage marker genes (Figure 3C), while we also observed higher expression of the CD3, CD4 and CD8 cell surface markers on *Btg1*^{-/-};*Btg2*^{-/-} committed B-cells (*Online Supplementary Figure S6*). These findings suggest that *Btg1* and *Btg2* are implicated in regulating B-lineage commitment by suppressing the expression of lineage-inappropriate genes.

In summary, using genetic mouse models, we demonstrate that both *Btg1* and *Btg2* promote mouse B-cell differentiation at multiple stages of development, and mostly display overlapping functions during B lymphopoiesis, which corresponds with an additive phenotype in the *Btg1*^{-/-};*Btg2*^{-/-} mice. On the other hand, *Btg1* fulfills a more prominent role in promoting the proliferative expansion of lymphoid progenitors by inhibiting apoptosis. This is in agreement with the finding that *Btg1* ablation in the

mouse brain decreases the pool of proliferating stem and progenitor cells, which is accompanied by increased apoptosis.¹² Importantly, *Btg1* and *Btg2* are both required to repress T-lineage gene expression in progenitor B-cells. These findings are partially overlapping with the phenotype observed in *Ebf1* and *Pax5* knockout mice, where it has been shown that EBF1 and PAX5 are critical regulators of B-lineage specification and commitment by repressing alternative non-B-cell fates.¹³⁻¹⁵ As the expression of *Ebf1* and *Pax5* itself is not inhibited in *Btg1*^{-/-};*Btg2*^{-/-} progenitor B-cells, the BTG1 and BTG2 transcriptional coregulators may affect B-lineage commitment by modifying the function of these B-cell specific transcription factors or by epigenetic regulation of their target genes. Notably, *PAX5* and *EBF1*, similar to *BTG1*, are also subject to gene deletions in BCP-ALL, and combinations of these deletions often co-occur in the same leukemia.⁴ Together our findings suggest that the block in differentiation observed in BCP-ALL may result from gene alterations affecting multiple master regulators of B-cell differentiation.

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