1 **Tumor suppressor BTG1 limits activation of BCL6 expression downstream of ETV6-**

2 **RUNX1**

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Highlights

- Loss of BTG1 potentiates ETV6-RUNX1-mediated self-renewal capacity

- Tumor suppressor BTG1 limits activation of BCL6 by ETV6-RUNX1

- BCL6 enhances the clonogenicity of primitive B lymphoid progenitors

Abstract

Translocation t(12;21) (p13;q22), giving rise to the *ETV6-RUNX1* fusion gene, is the most common genetic abnormality in childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL). This translocation usually arises *in utero*, but its expression is insufficient to induce leukemia and requires other cooperating genetic lesions for BCP-ALL to develop. Deletions affecting the transcriptional coregulator BTG1 are frequently observed in *ETV6-RUNX1*-positive leukemia. Here we show that *Btg1* deficiency enhances the self-renewal capacity of ETV6-RUNX1 positive mouse fetal liver derived hematopoietic progenitors (FL-HPCs). Combined expression of the fusion protein and a loss of BTG1 drives upregulation of the proto-oncogene *Bcl6* and downregulation of BCL6 target genes, such as *p19Arf* and *Tp53*. Similarly, ectopic expression of BCL6 promotes the self-renewal and clonogenic replating capacity of FL-HPCs, by suppressing the expression of *p19Arf* and *Tp53*.

Together these results identify BCL6 as a potential driver of ETV6-RUNX1 mediated leukemogenesis, which could involve loss of BTG1 dependent suppression of ETV6-RUNX1 function.

Introduction

B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common malignancy in children and characterized by the presence of distinct genetic abnormalities. The t(12;21) (p13;q22) chromosomal translocation arises *in utero,* giving rise to ETV6-RUNX1 fusion protein, generating a preleukemic clone, which requires additional cooperating events before

49 overt leukemia develops [1, 2]. Genomic profiling of *ETV6-RUNX1*-positive BCP-ALL has

50 identified recurrent copy number aberrations (CNAs), including deletions affecting *B-cell*

51 *translocation gene 1 (BTG1)* [3, 4]. We and others have shown that monoallelic *BTG1*

52 deletions in *ETV6-RUNX1*-positive BCP-ALL are the result of genomic rearrangements

53 mediated by aberrant RAG recombinase activity [5, 6]. Focal deletions affecting *BTG1* are

54 present in 9% of all BCP-ALL cases, but are enriched to about 20% in *ETV6-RUNX1*-positive

55 leukemia, without affecting patient outcome [3, 4, 6, 7]. BTG1 functions as a transcriptional

56 cofactor that acts by recruiting effector molecules, such as the protein arginine

57 methyltransferase I (PRMT1) to specific transcription factors, thus affecting proliferation and

58 differentiation [8, 9]. In this paper, we investigated if loss of BTG1 affects the self-renewal

59 capacity of ETV6-RUNX1 in mouse fetal liver hematopoietic progenitor cells (FL-HPCs) by

60 performing clonogenic B cell progenitor assays and gene expression analysis.

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62

63 **Methods**

64 **Mice**

65 The generation of C57BL/6J *Btg1* knockout (KO) mice has been previously described by

66 Farioli-Vecchiolo et al. [10]. The animals were maintained under specific pathogen-free

67 conditions at our animal facility. All animal experiments were approved by the Animal

68 Experimental Committee of the Radboud university medical center and were performed in

69 accordance with institutional and national guidelines. For the retroviral transduction

70 experiments, fetal liver cells derived from day 13.5 of wild-type and $Btg1^{-/-}$ embryos were

71 used.

72

73 **Retroviral transduction of fetal liver-derived progenitor cells and adult bone marrow**

74 Primitive fetal liver hematopoietic progenitor cells (FL-HPCs) were purified as c-Kit$^+$Ter119$^-$

75 cells from fetal livers of embryonic day 13.5 (E13.5) of C57Bl6/J wild-type and $Btg1^{-/-}$ mice by

76 MACS sort using Ter119 and cKit (CD117) beads (Miltenyi Biotec). FL-HPCs were expanded

in Iscoves modified Dulbecco medium (IMDM) (Gibco) supplemented with 10% fetal-bovine

serum (FBS) (Greiner Bio-One), 1% Penicillin-Streptomycin (Invitrogen) and 55μM 2-ME

(Gibco) in the presence of 10ng/ml *fms*-like tyrosine kinase-3 ligand (Flt-3L), 20ng/ml

Interleukin 7 (IL-7) (and 100ng/ml stem cell factor (SCF) (all R&D) for 24 hours. Cells were

transduced with retrovirus by spinoculation for 45 min at 700xg (25°C) in the presence of

5μg/ml polybrene and cultured with the same growth factors for 48 hours, as has been

described by Morrow et al. [11]. CD19^{+} bone marrow cells were purified from 10 weeks old

wild-type and *Btg1^{-/-}* mice by MACS sorting using CD19 beads (Miltenyi Biotec). CD19^{+} cells

were cultured in improved minimal essential medium (IMEM) (Gibco) supplemented with 2%

FBS (Greiner Bio-One), 1% Penicillin-Streptomycin (Invitrogen), 0.03% primatone and 55μM

2-ME   in the presence of 20ng/ml IL-7 (R&D) on OP9 stroma for 48 hours. Cells were

transduced by spinoculation (45 min at 700xg, 25°C) in the presence of 10μg/ml protamine

sulphate and cultured in the same pro-B cell medium for 24 hours. Transduction efficiency

was determined by analyzing EGFP expression using flow cytometry.

B-cell colony-forming assays

Transduced FL-HPCs (1x10^{4} cells/plate) and CD19^{+} bone marrow cells (1x10^{5} cells/plate)

derived of wild-type and *Btg1^{-/-}* animals, were cultured in duplicate methylcellulose cultures

using 35mm plates (M3630, STEMCELL Technologies), 48 hours after viral transduction.

The FL-HPC methylcellulose cultures were supplemented with 10ng/ml FLT3L, 20ng/ml IL-7

and 100ng/ml SCF . After 6-10 days, colonies (>30 cells) were scored, pooled and counted.

Replating of 1x10^{4} cells/plate was performed under identical conditions.

Results and discussion

To examine contribution of BTG1 loss to ETV6-RUNX1 positive ALL, we compared *in vitro*

self-renewal capacity of wild-type and *Btg1*-deficient FL-HPCs. Therefore, FL-HPCs isolated

from C57Bl/6J wild-type and *Btg1^{-/-}* embryos at 13.5 days post coitum (dpc) were transduced

105 with either control or ETV6-RUNX1 retrovirus and propagated in B-lineage promoting culture

106 conditions. Transduction was observed in 40%-70% of both wild-type and $Btg1^{-/-}$ FL-HPCs by

107 flow cytometry and confirmed by western blot analysis (Supplementary Figure E1). Wild-type

108 and $Btg1^{-/-}$ FL-HPCs transduced with either control or ETV6-RUNX1 virus were cultured in

109 methylcellulose containing cytokines to facilitate expansion and differentiation of early B cell

110 progenitors. Subsequently, cells were analyzed for their ability to maintain self-renewal

111 capacity by serial replating assays. We observed that introduction of the ETV6-RUNX1

112 fusion protein enhanced self-renewal capacity, particularly in a $Btg1$-deficient background

113 (Fig. 1A). However, transformation to a full leukemia in $vivo$ by transplantation of ETV6-

114 RUNX1 transduced wild-type and $Btg1^{-/-}$ HPCs was not observed (results not shown). These

115 data suggest that BTG1 function limits the ETV6-RUNX1-mediated self-renewal capacity of

116 colony-forming fetal progenitor B-cells, but that loss of $Btg1$ is not sufficient to drive

117 leukemogenesis in this experimental model.

118   To determine whether this phenotype could also be observed in adult-type B cell

119 progenitors, we examined the effect of ETV6-RUNX1 expression in CD19^{+}-purified adult

120 bone marrow cells, derived from wild-type or $Btg1^{-/-}$ mice. Consistent with previous reports

121 [12], ETV6-RUNX1 suppressed colony formation of adult progenitor B-cells, which was

122 further enhanced in the absence of BTG1 (Supplementary Figure E2). These findings are

123 consistent with the notion that ETV6-RUNX1-mediated leukemic transformation occurs

124 primarily in the developing embryo and not in adult progenitor B-cells.

125   To elucidate the molecular mechanism by which $Btg1$ deficiency enhances ETV6-

126 RUNX1-induced self-renewal capacity of FL-derived progenitor B-cells, we performed gene

127 expression analysis of FL-HPCs cultured in our standard cytokine-containing medium. Gene

128 Set Enrichment Analysis (GSEA) was performed to define KEGG pathways that were

129 specifically affected by ETV6-RUNX1 expression in the two different genetic backgrounds

130 (Fig. 1B and Supplementary Table E1). Interestingly, we identified a p53 signature in both

131 wild-type and $Btg1^{-/-}$ FL-HPCs in the presence of ETV6-RUNX1 expression, which involved

132 downregulation of p53 target genes $Cdkn2a,$ $Gadd45g$ and $CcnD2$ expression

133 (Supplementary Table E1). As we observed reduced expression of *Tp53* in ETV6-

134 RUNX1;*Btg1*$^{-/-}$ fetal progenitor B-cells, we hypothesized that an upstream regulator of the

135 p53 pathway might be affected in these cells. Notably, we observed a 4-fold upregulation of

136 *Bcl6* mRNA levels in ETV6-RUNX1-positive *Btg1*-deficient fetal progenitor B-cells relative to

137 control cells. By qRT-PCR we validated that *Bcl6* expression was induced in ETV6-

138 RUNX1;*Btg1*$^{-/-}$ pro-B cells, accompanied by repression of known BCL6 target genes such as

139 *p19Arf, Tp53, CcnD2, Bcl2 and c-Myc* (Fig. 1C). However, due to poor quality of the

140 available antibodies, we were unable to detect native BCL6 protein in these fetal liver cells.

141   As p53 is subject to regulation of the E3 ubiquitin-protein ligase MDM2, and ETV6-

142 RUNX1 has been shown to activate MDM2 expression [13], we examined *Mdm2* mRNA

143 expression in these FL-HPCs. However, no significant differences in expression of *Mdm2*

144 were found (Fig. 1C). By western blot analysis we confirmed that p53 protein levels were

145 reduced in ETV6-RUNX1-positive fetal progenitor B-cells relative to control and *Btg1*$^{-/-}$ cells,

146 while expression was even further reduced in ETV6-RUNX1;*Btg1*$^{-/-}$ pro-B-cells (Fig. 1D).

147 These data demonstrate that BTG1 loss synergizes with ETV6-RUNX1 in activating BCL6

148 expression and repression of its downstream targets such as *Tp53* and *p19Arf.*

149   To study whether increased BCL6 expression itself was responsible for the enhanced

150 self-renewal capacity observed in ETV6-RUNX1-positive *Btg1*$^{-/-}$ FL-HPCs, we transduced

151 wild-type FL-derived progenitor B-cells with either control or BCL6 retrovirus. Flow cytometric

152 analysis showed a transduction efficiency of 30%-50% GFP-positive cells (data not shown).

153 Indeed, forced expression of BCL6 efficiently promoted the clonogenic replating capacity of

154 FL-HPCs to a similar extent as observed in ETV6-RUNX1;*Btg1*$^{-/-}$ cells (Fig. 2A). Gene

155 expression analyses by qRT-PCR revealed a significant reduction in *p19Arf* and *Tp53*

156 expression, which mirrored the phenotype observed in ETV6-RUNX1;*Btg1*$^{-/-}$ fetal progenitor

157 B-cells (Fig. 2B). Western blot analysis confirmed that p53 protein levels were decreased in

158 BCL6 transduced fetal pro-B-cells (Fig. 2C). These results indicate that BCL6 enhances the

159 clonogenicity of primitive B lymphoid progenitors, which is in sharp contrast to its effects on

160 adult definitive precursor B-cells [14].

161   Previous studies have shown that RUNX1 is methylated by the arginine

162 methyltransferase PRMT1 [15-17] and that BTG1 acts by recruiting and stimulating the

163 enzymatic activity of PRMT1 [8, 9, 18]. Consistent with these findings, we could demonstrate

164 that ETV6-RUNX1 associates with both BTG1 and PRMT1 when co-expressed in HEK293

165 cells (Fig. 3A-B). In addition, by performing PRMT1 co-immunoprecipitation in control and

166 *Btg1*$^{-/-}$ FL-HPCs protein lysates, we observed that BTG1 is required for the association

167 between ETV6-RUNX1 and PRMT1 (Fig. 3C). However, how modulation of ETV6-RUNX1

168 function by the BTG1-PRMT1 complex affects expression of BCL6 remains to be explored.

169   BCL6 has been identified as a critical determinant of leukemic stem cell survival in

170 chronic myeloid leukemia (CML) and *BCR-ABL1*-positive ALL, involving repression of

171 p14*ARF* and *TP53* [14, 19]. Previous reports have shown that the RUNX1 fusion protein

172 AML1-ETO, an important driver of acute myeloid leukemia, represses transcription of

173 p14ARF [20]. Our study shows that downstream targets of BCL6, including *p19Arf* and *Tp53,*

174 are downregulated in ETV6-RUNX1-positive fetal progenitor B-cells, particularly in the

175 absence of BTG1 function. Furthermore, ectopic expression of BCL6 in primitive progenitor

176 B-cells induced a similar proliferative expansion, accompanied by downregulation of *TP53*

177 *and p19Arf* expression. These data indicate that BCL6 promotes proliferation of primitive B

178 lymphoid progenitors by suppressing the p53 pathway. Future studies will need to establish

179 whether inhibition of BCL6 could be effective in the eradication of leukemia-initiating cells in

180 *ETV6-RUNX1*-positive ALL.

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185 **Potential conflict of interest**: The authors declare no competing financial interests.

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251   **Figures**

252   **Figure 1.** BTG1 loss enhances self-renewal of ETV6-RUNX1-positive fetal progenitor B-cells

253   by activation of BCL6 pathway. (**A-D**) Fetal liver-derived hematopoietic progenitor cells (FL-

254   HPCs) were isolated from wild-type (WT) and *Btg1$^{-/-}$* embryos at day 13.5dpc and transduced

255   with control and ETV6-RUNX1 retrovirus. (**A**) Control and ETV6-RUNX1 transduced FL-

256   HPCs (1x10^4 cells) were seeded 48 hours after transduction in duplicate in B cell specific

257   methylcellulose supplemented with FLT3L, IL-7 and SCF. Serial replating was performed

258   under identical conditions. Mean colony counts (and SEM) were determined (>30

259   cells/colony) after 6-10 days of culture. Data set is representative of 2 independent

260   experiments. *, $P< 0.05$, **, $P< 0.01$. (**B**) Gene set enrichment analyses on gene expression

261   data of WT, ETV6-RUNX1, *Btg1$^{-/-}$* and ETV6-RUNX1;*Btg1$^{-/-}$* fetal progenitor B-cells. Total

262   RNA was extracted from FL-HPCs, which were expanded for 12 days in liquid medium

263   supplemented with FLT-3L, IL-7 and SCF. The data were RMA normalized and relative gene

264   expression differences of >1.6-fold were included. KEGG pathway analysis was performed

265   using the online Gene Set Enrichment Analysis (GSEA) tool and relevant pathways that are

266   differentially expressed between wild-type and ETV6-RUNX1 as well ETV6-RUNX1;*Btg1$^{-/-}$*

267   FL cells are indicated. (**C**) Relative expression levels of *Bcl6, Tp53, p19Arf, Mdm2, CcnD1,*

268   *CcnD2, Bcl2* and *c-Myc* were determined by quantitative reverse-transcription PCR (qRT-

269   PCR) and normalized to the expression of the housekeeping gene TATA box binding protein

270   (TBP). Data represent the mean and SEM of three independent experiments. *, $P< 0.05$, **,

271   $P< 0.01$, ***, $P< 0.001$. (**D**) Protein lysates were generated from FL-HPCs that were

272   expanded for 12 days in liquid medium supplemented with FLT-3L, IL-7 and SCF. The

273   immunoblot demonstrates expression of p53, while β-actin serves as a loading control.

274   Quantitation of relative expression levels is indicated.

275

276   **Figure 2.** BCL6 stimulates self-renewal of early B cell progenitors. (**A-C**) Fetal liver-derived

277   hematopoietic progenitor cells (FL-HPCs) were isolated from wild-type embryos at day

278   13.5dpc and transduced with control or BCL6 retrovirus. (**A**) Control and BCL6 transduced

279   FL-HPCs (1×10^4 cells) were cultured 48 hours after transduction in B cell specific

280   methylcellulose supplemented with FLT-3L, IL-7 and SCF. Serial replating was performed

281   under identical conditions. Mean colony counts (and SEM) were determined (>30

282   cells/colony) after 6-10 days of culture. Data set is representative of 2 independent

283   experiments. **, $P< 0.01$. (**B**) RNA was isolated from FL-HPCs that were expanded for 10

284   days in liquid cultures in the presence of cytokines FLT-3L, IL-7 and SCF. Relative

285   expression levels of *Tp53*, *p19Arf*, *Mdm2, CcnD1*, *CcnD2*, *Bcl2* and *c-Myc* were determined

286   on cDNA generated from transduced wild-type FL progenitor cells by real-time PCR and

287   normalized to TBP. Data represent the mean and SEM of three independent experiments. *,

288    $P< 0.05$, **, $P< 0.01$, ***, $P< 0.001$. (**C**) Protein lysates were generated from FL-HPCs that

289   were expanded for 13 days in liquid cultures in the presence of cytokines FLT-3L, IL-7 and

290   SCF. The immunoblot shows expression of p53 and the loading control β-actin. Quantitation

291   of relative expression levels is indicated.

292

293   **Figure 3.** ETV6-RUNX1 interacts with PRMT1 and its cofactor BTG1. (**A**) HEK293 cells were

294   transfected with expression plasmids encoding HA-ETV6-RUNX1 and FLAG-BTG1 and

295   treated for 16 hours with 5µM of the proteasome inhibitor MG132. Protein lysates were

296   generated and subjected to immunoprecipitation (IP) by incubation with FLAG antibody (Ab).

297   Immunoblot demonstrates the expression of BTG1 using a FLAG-Ab and ETV6-RUNX1 with

298   a HA-Ab in whole cell extracts (WCE) and IP samples. (**B**) HEK293 cells were transfected

299   with expression plasmids encoding HA-ETV6-RUNX1 and PRMT1 and treated for 16 hours

300   with 5µM of the proteasome inhibitor MG132. Protein lysates were subjected to IP using HA-

301   Ab. The immunblot shows expression of PRMT1 using a PRMT1-Ab and ETV6-RUNX1 with

302   a HA-Ab in WCE and IP. (**C**) FL-HPCs (cKit$^+$Ter119$^-$) were isolated from wild-type and *Btg1$^{-/-}$*

303   embryos at day 13.5dpc and transduced with control and ETV6-RUNX1 virus. Protein lysates

304   were generated from FL-HPCs that were expanded for 12 days in liquid cultures in the

305   presence of cytokines FLT-3L, IL-7 and SCF and subjected to IP using PRMT1 Ab. The

306   immunoblot shows expression of PRMT1 using a PRMT1-Ab and HA-ETV6-RUNX1 using an

307   HA-Ab in WCE and IP from wild-type FL cells.

308