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Role of STAT5 in controlling cell survival and immunoglobin gene recombination during pro-B cell development

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Abstract

STAT5 and IL-7 signaling are thought to control B-lymphopoiesis by regulating key transcription factor genes and activating V_H gene segments at the *Igh* locus. Using conditional mutagenesis, we demonstrate that transgenic *Bcl2* expression rescued the development of *Stat5*-deleted pro-B cells by compensating for the loss of Mcl-1. *Ebf1* and *Pax5* expression as well as V_H gene recombination were normal in Bcl2-rescued pro-B cells lacking STAT5 or IL-7Rα. In agreement with this finding, STAT5-expressing pro-B cells contained little or no active chromatin at most V_H genes. In contrast, *Igk* rearrangements were increased in STAT5-or IL-7Rα-deficient pro-B cells. Hence, STAT5 and IL-7 signaling control cell survival and the developmental ordering of immunoglobulin gene rearrangements by suppressing premature *Igk* recombination in pro-B cells.

Keywords

STAT5; IL-7 signaling; cell survival; *Pax5*; *Ebf1*; immunoglobulin gene rearrangements; chromatin activation

Introduction

Interleukin-7 (IL-7) is a nonredundant cytokine for T and B cell development[1] and transmits its signal through the IL-7 receptor (IL-7R) consisting of the common γ chain (γc) chain and the IL-7Rα subunit (encoded by the *Il7r* gene). T-lymphopoiesis is strongly reduced in the thymus, and B-cell development is stringently arrested at the uncommitted pre-pro-B cell stage in the bone marrow of *Il7r*$^{-/-}$ mice[2, 3]. Transgenic expression of the anti-apoptotic Bcl-2 protein efficiently rescues T-lymphopoiesis[4, 5], but not B-cell development in *Il7r*$^{-/-}$ mice[5,

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AUTHOR CONTRIBUTIONS S. Malin performed most experiments; S. McManus contributed the ChIP-chip analyses; C.C. generated and characterized the *Ikzf1*^{Ebf1} transgenic mouse; A.D. carried out the histological analysis; M.N. did the bioinformatical analysis of the *Igh* locus; P.B. and A.S. provided the *Mcl1*$^{fl/fl}$ mouse; and M.B. and S. Malin planned the project, designed the experiments and wrote the manuscript.

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[6], which indicates an important role of IL-7R signaling in controlling T-cell survival and early B-lymphopoiesis.

IL-7R signaling stimulates the Jak-STAT pathway leading to activation of the transcription factor STAT5[7]. STAT5 consists of two highly related isoforms, STAT5A and STAT5B, which are encoded by separate genes and fulfill largely redundant roles within the lymphoid system[7]. Different strategies have been used for simultaneous inactivation of the two closely linked *Stat5a* and *Stat5b* genes. Teglund et al.[8] disrupted the first protein-coding exons of *Stat5a* and *Stat5b*, which still results in expression of N-terminally truncated and partially functional STAT5 proteins ($Stat5^{\Delta N}$ allele)[9, 10]. Consequently, T and B cell development is minimally affected in $Stat5^{\Delta N/\Delta N}$ mice[11, 12]. The subsequent generation of a conditional *Stat5* allele ($Stat5^{fl}$) containing *Stat5a* and *Stat5b* between *loxP* sites facilitated Cre-mediated deletion of both genes ($Stat5^{-}$ allele)[13]. Complete *Stat5* inactivation causes perinatal lethality, severely reduced development of B and αβ T cells as well as the absence of γδ T-lymphocytes in $Stat5^{-/-}$ mice[9, 10]. Similar to $Il7r^{-/-}$ mice[3], B-cell development is arrested at the uncommitted pre-pro-B cell stage in the bone marrow of $Stat5^{-/-}$ mice[9, 10].

An instructive role of STAT5 in B-lymphopoiesis was suggested based on the partial rescue of B-cell development in $Il7r^{-/-}$ mice by a transgene expressing a constitutively active form of STAT5B[14]. STAT5 has furthermore been implicated in the regulation of the B-cell commitment gene *Pax5* by binding to its promoter region[15, 16]. STAT5-mediated IL-7 signaling is also thought to activate expression of the B-cell specification factor EBF1 in common lymphoid progenitors (CLPs) and pre-pro-B cells[3, 17, 18]. Finally, IL-7 is sufficient to promote *in vitro* differentiation of wild-type CLPs to committed pro-B cells[2]. These data together with the inability of transgenic Bcl-2 expression to rescue B-lymphopoiesis in $Il7r^{-/-}$ mice[5, 6] suggest that IL-7 signaling fulfills an instructive role in early B-cell development by STAT5-mediated activation of the B-cell regulatory genes *Ebf1* and *Pax5*.

STAT5 and IL-7R signaling have also been implicated in controlling the chromatin accessibility of the immunoglobulin heavy-chain (*Igh*) gene in pro-B cells. The *Igh* locus consists of a 2.5 megabase (Mb) cluster of 195 variable (V) gene segments and a proximal 0.27 Mb region containing the diversity (D), joining (J) and constant (C) gene segments[19]. At the onset of B-cell development, the *Igh* locus is thought to undergo stepwise chromatin activation, whereby IL-7 signaling is reportedly responsible for activation of the large distal V_HJ558 gene family[20, 21]. Consistent with this model, germline transcription and recombination of distal V_H genes are strongly impaired, whereas proximal V_H genes undergo normal germline transcription and rearrangements in B-cell progenitors of $Il7r^{-/-}$ and $Stat5^{\Delta N/\Delta N}$ mice[22, 23]. Hence, these data further support an instructive role for STAT5 and IL-7R signaling in early B-cell development.

Here we have investigated the cell intrinsic role of STAT5 in B-lymphopoiesis by conditional mutagenesis in adult mice. The B-cell developmental effect of STAT5 deficiency was previously studied in the fetal liver of $Stat5^{-/-}$ embryos or bone marrow of the rare growth-retarded $Stat5^{-/-}$ survivors[9, 10], whose hematopoietic stem cells (HSCs) were severely impaired in their function[9]. As the *Rag1* gene is not expressed in HSCs, but is subsequently activated in early lymphoid progenitors (ELPs)[24], we used a $Rag1^{cre}$ knock-in allele[25] for Cre-mediated deletion of *Stat5* specifically at the onset of lymphopoiesis. Contrary to previous reports, our conditional mutagenesis data demonstrate that STAT5 and IL-7R signaling are not involved in the regulation of *Ebf1* and *Pax5* or in the chromatin activation and V_H-DJ_H recombination of distal V_HJ558 genes. Instead, the primary role of IL-7-mediated STAT5 activation during pro-B cell development is to maintain cell survival by activating the anti-apoptotic gene *Mcl1* and to prevent premature *Igk* gene rearrangements by binding to the intronic iEκ enhancer.

RESULTS

STAT5 promotes pro-B cell development by controlling cell survival

To determine the activity of the *Rag1*^{cre} allele in early hematopoiesis, we used a Cre reporter gene[26] to show that Rag1-Cre-mediated deletion was initiated in 18% of multipotent progenitors (LSK cells) and was completed in all bone marrow pro-B cells and thymic pro-T (DN1/2) cells (Supplementary Fig. 1 online). As the *Rag1*^{cre} allele deletes the floxed *Stat5* allele not only in B-cells but also in all T-cells, *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice became autoimmune within 2 weeks after birth and died at the age of 2-3 months due to autoimmune pathologies that are likely caused by the dramatic reduction of Treg cell numbers[27] (Supplementary Fig. 2). Consequently, we first examined the B-cell phenotype caused by conditional *Stat5* inactivation under non-autoimmune conditions. Fetal liver pro-B cells (c-Kit^{+}CD19^{+}IgM^{-}) and pre-B cells (CD2^{+}CD19^{+}IgM^{-}) were reduced 10-15 fold in *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ compared to *Stat5*$^{fl/fl}$ embryos at day 18.5 (Supplementary Fig. 3a). Moreover, the bone marrow of T-cell-deficient *Rag1*$^{cre/cre}$ *Stat5*$^{fl/fl}$ mice contained similar amounts of CLPs, but 7-fold reduced numbers of pro-B cells compared to *Stat5*$^{fl/fl}$ mice, indicating that the loss of STAT5 interferes with the differentiation of CLPs to committed pro-B cells (Supplementary Fig. 3b).

Pro-B, pre-B and total B cell numbers were similarly reduced in the bone marrow of *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice compared to *Stat5*$^{fl/fl}$ mice (Fig. 1a-c). Deletion of the floxed *Stat5* allele in the few residual pro-B cells of *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice indicated that STAT5 is not absolutely required for pro-B cell development (Fig. 1d). Importantly, transgenic Bcl-2 expression efficiently rescued pro-B cell development (Fig. 1a,b) despite complete *Stat5* deletion and STAT5 protein loss (Fig. 1d,e) in *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice. Hence, the pro-survival protein Bcl-2 can compensate for STAT5 deficiency in pro-B cell development. In contrast, pre-B cells and total B-cells were only minimally increased in *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice (Fig. 1a,b). Interestingly, no cell expansion was observed during the development of pro-B to pre-B cells in *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice (Fig. 1c) consistent with a decrease of larger, blasting pro-B cells (Fig. 1e). Hence, STAT5 is an essential mediator of the collaboration between IL-7 and pre-BCR signaling[28] during this developmental transition.

The Bcl-2-mediated rescue of STAT5-deficient pro-B cells begged the question whether the STAT5 protein was already lost at the preceding pre-pro-B cell stage in *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice. Indeed, the floxed *Stat5* allele was efficiently deleted, and the *Stat5a* mRNA was ~20-fold reduced in sorted pre-pro-B cells (Kit^{+}B220^{+}CD19^{-}DX5^{-}Ly6C^{-}) of *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ compared to control mice (Supplementary Fig. 4). Moreover, intracellular staining of STAT5 protein revealed the same background fluorescence for pre-pro-B and pro-B cells of *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice, indicating that the pre-pro-B cells contained little or no STAT5 protein similar to the pro-B cells of these mice (Supplementary Fig. 5).

We next investigated whether the transcription factor EBF1 could rescue the STAT5 deficiency, as suggested by published data[3, 17, 18]. An *Ikzf1*^{Ebf1} transgene, that expresses *Ebf1* under the control of the pan-hematopoietic *Ikzf1* (Ikaros) locus (Supplementary Fig. 6a-d), rescued pro-B cell development in *Ebf1*$^{-/-}$ mice (Supplementary Fig. 6e), but could only partially restore the generation of *Stat5*-deficient pro-B cells in *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ *Ikzf1*^{Ebf1} mice (Supplementary Fig. 7). Together, these data demonstrate that STAT5 fulfills a permissive rather than instructive role in pro-B cell development.

Normal development of mature B-cell types in the absence of STAT5

STAT5 was shown by gain-of-function experiments to control the differentiation and self-renewal potential of human memory B-cells[29]. To verify these findings by loss-of-function

analysis in the mouse, we used the *Cd23-cre* and *Aicda-cre* lines[26] to inactivate the floxed *Stat5* allele in splenic B cells. Despite efficient *Stat5* deletion, the mature ($IgD^{hi}IgM^{lo}CD19^{+}$), follicular (FO; $CD21^{int}CD23^{hi}$) and marginal zone (MZ; $CD21^{hi}CD23^{lo}$) B-cells were equally abundant in *Cd23-cre Stat5*$^{fl/fl}$ compared to control *Stat5*$^{fl/fl}$ mice, (Fig. 2a,e). Likewise, similar numbers of germinal center (GC) B-cells ($B220^{+}Fas^{+}$) were generated in the spleen of immunized *Aicda-cre Stat5*$^{fl/fl}$ and control *Stat5*$^{fl/fl}$ mice (Fig. 2b,c). NP-specific $IgG1^{+}$ memory B-cells ($Lin^{-}CD38^{hi}B220^{+}CD19^{+}IgG1^{+}NP^{+}$) with complete *Stat5* inactivation were also found in the spleen of *Cd23-cre Stat5*$^{fl/fl}$ mice eight weeks after immunization with NP-KLH (Fig. 2d,e and Supplementary Fig. 8). Finally, similar titers of anti-NP-IgG1 antibodies were detected in the sera of *Cd23-cre Stat5*$^{fl/fl}$ and control *Stat5*$^{fl/fl}$ mice 2 and 4 weeks after NP-KLH immunization as well as 2 weeks after NP-KLH boosting of the memory B-cell response (Fig. 2f). Together, these data indicate that STAT5 is dispensable for late B-lymphopoiesis, memory B-cell formation and plasma cell differentiation.

Development of committed pro-B cells in the absence of IL-7 signaling

As STAT5 is a downstream mediator of IL-7 signaling[7], we next determined the role of the IL-7R in controlling pro-B cell development. Consistent with published reports[2, 3], the pro-B, pre-B and total B cells were drastically reduced in the bone marrow of adult *Il7r*$^{-/-}$ mice (Supplementary Fig. 9a). Importantly, the bone marrow from the two hind legs (femur and tibia) of 4-6-week-old *Il7r*$^{-/-}$ mice contained on average 5,000 committed c-$Kit^{+}CD19^{+}IgM^{-}$ pro-B cells, indicating that IL-7R signaling is not strictly required for the formation of committed pro-B cells (Supplementary Fig. 9a). Transgenic Bcl-2 expression resulted in a 5-10-fold increase of pro-B, pre-B and total B cells in *Vav-bcl2 Il7r*$^{-/-}$ mice (Supplementary Fig. 9a). Moreover, the majority of the Bcl-2-rescued *Il7r*$^{-/-}$ pro-B cells were small in size and thus failed to proliferate (Supplementary Fig. 9b). Hence, the development of committed pro-B cells was only partially rescued by transgenic Bcl-2 expression in *Vav-bcl2 Il7r*$^{-/-}$ mice in contrast to its efficient rescue in *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice.

STAT5- and IL-7R-dependent gene regulation in pro-B cells

The Bcl-2-mediated rescue of STAT5- and IL-7Rα-deficient pro-B cells provided the unique opportunity to FACS-sort sufficient pro-B cells (Supplementary Fig. 10) for investigating the transcriptional role of STAT5 and IL-7R signaling in early B-cell development. Semiquantitative RT-PCR analysis demonstrated that the transcription factor genes *Pax5*, *Ebf1* and *Tcfe2a* (E2A) were similarly expressed in sorted bone marrow pro-B cells of *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ and control *Vav-bcl2 Stat5*$^{fl/fl}$ mice (Fig. 3a,c). Similar results were obtained with bone marrow pro-B cells of *Vav-bcl2 Il7r*$^{-/-}$ and *Vav-bcl2 Il7r*$^{+/+}$ mice (Fig. 3b) as well as with the rare pro-B cells isolated from the fetal liver of *Stat5*$^{-/-}$embryos at day 18.5 (Supplementary Fig. 11). As all three transcription factors are essential for early B-lymphopoiesis, their normal expression in *Stat5* and *Il7r* mutant pro-B cells further supports a permissive rather than instructive role for STAT5 and IL-7R signaling in pro-B cell development.

Expression of the pro-survival gene *Bcl2l1* (encoding Bcl-xL) was unaffected by the loss of STAT5 or IL-7Rα (Fig. 3a,b). In contrast, transcripts of the *Mcl1* gene were 8-fold reduced and the Mcl-1 protein was undetectable in pro-B cells of *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice compared to *Vav-bcl2 Stat5*$^{fl/fl}$ mice (Fig. 3c,d), suggesting that STAT5 mediates the survival of pro-B cells by activating the *Mcl1* gene. As pro-B cells are still generated in the bone marrow of *Cd19-cre Mcl1*$^{fl/fl}$ mouse[30], we examine the role of *Mcl1* in *Rag1*$^{cre/+}$ *Mcl1*$^{fl/fl}$ mice. These mice entirely failed to produce pro-B, pre-B and B cells in their bone marrow (Fig. 3e and Supplementary Fig. 12). In contrast, pro-B cells were normally generated in *Rag1*$^{cre/+}$ *Bcl2l1*$^{fl/fl}$ mice despite efficient *Bcl2l1* deletion, whereas pre-B cells were strongly reduced,

thus indicating a critical function for Bcl-xL in pre-B cells and later stages of B-cell development (Fig. 3e and Supplementary Fig. 12). Together these data demonstrate that STAT5 controls the survival of pro-B cells by regulating the *Mcl1* but not the *Bcl2l1* gene.

Normal *Igh* recombination in pro-B cells lacking STAT5 or IL-7Rα

We next investigated the role of STAT5 and IL-7R signaling in controlling distal V_H-DJ_H recombination of the *Igh* locus by semiquantitative PCR detection and quantification of the different *Igh* rearrangements in FACS-sorted pro-B cells (c-Kit$^+$CD19$^+$IgM$^-$) from *Vav-bcl2 Rag1$^{cre/+}$ Stat5$^{fl/fl}$*, *Vav-bcl2 Il7r$^{-/-}$*, control wild-type and *Vav-bcl2* mice (Fig. 4a,b and Supplementary Fig. 13). As expected[20, 22, 23], we observed no or only a minor (~2-fold) difference in the frequency of D_H-J_H and proximal V_H7183-DJ_H rearrangements in *Vav-bcl2 Rag1$^{cre/+}$ Stat5$^{fl/fl}$* and *Vav-bcl2 Il7r$^{-/-}$* pro-B cells compared to wild-type and *Vav-bcl2* pro-B cells (Fig. 4a,b and Supplementary Fig. 13). Surprisingly however, we could not detect a significant difference in the frequency of distal V_HJ558-DJ_H rearrangements between STAT5-deficient, *Il7r* mutant and control pro-B cells (Fig. 4a,b and Supplementary Fig. 13). RT-PCR analysis confirmed this result as rearranged V_H7183-DJC_μ and V_HJ558-DJ_μ transcripts were expressed at similar levels in *Vav-bcl2 Rag1$^{cre/+}$ Stat5$^{fl/fl}$* and *Vav-bcl2 Il7r$^{-/-}$* pro-B cells compared to control *Vav-bcl2* pro-B cells (Fig. 4c). Moreover, germline transcripts (GLT) of the distal V_HJ558 genes were also expressed in pro-B cells of the different genotypes (Fig. 4c). In contrast, Bertolino et al.[23] reported a 5-fold reduction of distal V_HJ558-DJ_H rearrangements in B220$^+$IgM$^-$ bone marrow B-lymphocytes (pro-B and pre-B cells) of *Stat5$^{\Delta N/\Delta N}$* mice[8] and a 10-fold reduction of V_HJ558 germline transcripts in cultured *Stat5$^{\Delta N/\Delta N}$* pro-B cells compared to wild-type control cells. However, our own analysis of *ex vivo* sorted pro-B cells from the bone marrow of *Stat5$^{\Delta N/\Delta N}$* mice failed to demonstrate any effect of the N-terminally truncated (ΔN) STAT5A and STAT5 proteins on V_HJ558 germline transcription, V_HJ558-DJ_H recombination and expression of rearranged V_HJ558-DJ_μ transcripts (Supplementary Fig. 14; see legend for detailed explanation of the discrepancy). We therefore conclude that neither STAT5 nor IL-7R signaling play a critically important role in controlling V_H-DJ_H recombination at the *Igh* locus.

Absence of detectable active chromatin at most V_H genes in *Rag2$^{-/-}$* pro-B cells

STAT5 and IL-7 signaling have previously been implicated in controlling the accessibility of distal V_H genes to the recombination machinery by promoting histone acetylation[20, 21, 23]. We therefore studied this aspect of STAT5 and IL-7 signaling by determining whether the distal V_H genes contain active chromatin in IL-7-treated pro-B cells. As chromatin activation of the distal V_H genes is thought to precede V(D)J recombination[20, 21], we used *Rag2$^{-/-}$* pro-B cells for high-resolution ChIP-chip mapping of active histone modifications along the *Igh* locus. To this end, bone marrow pro-B cells from *Rag2$^{-/-}$* mice were expanded for only 5 days in the presence of IL-7 and OP9 stromal cells prior to chromatin immunoprecipitation (ChIP) with antibodies that recognized acetylated lysine 9 (H3K9ac), dimethylated lysine 4 (H3K4me2) or trimethylated lysine 4 (H3K4me3) on histone H3. The precipitated DNA was subjected to either T7-based linear amplification[31] or whole genome amplification (WGA)[32] prior to fluorescent labeling and hybridization together with the input DNA probe onto a high-density 50-mer oligonucleotide array containing the non-repetitive sequences of the *Igh* locus (Fig. 5a). Probes prepared with the two different protocols gave rise to similar results as shown in Fig. 5 (linear amplification) and Supplementary Fig. 15 (WGA amplification). The three active histone marks H3K9ac, H3K4me2 and H3K4me3 were strongly enriched in a region centered on the intronic Eμ enhancer and extending from the DQ52 element to the C_μ gene (Fig. 5b and Supplementary Fig. 15b). Active histone modifications were absent throughout the central D_H region and at all proximal V_H7183 and V_HQ52 genes in *Rag2$^{-/-}$* pro-B cells (Fig. 5b,c and Supplementary Fig. 15b,c), as published[20, 21, 33]. Surprisingly however, we detected no or only very low enrichment of active histone marks at distal V_HJ558 genes despite the fact that

the *Rag2*$^{-/-}$ pro-B cells had been stimulated with IL-7 for 5 days (Fig. 5d and Supplementary Fig. 15d). In contrast, the distal V_H3609 genes, which are interspersed with the V_HJ558 genes, carried relatively high levels of active H3K9ac and H3K4me2 marks (Fig. 5e and Supplementary Fig. 15e), although the H3K4me3 modification was barely detectable in contrast to its abundance at promoters of other expressed B-cell-specific genes[31] such as the *Ikzf1* gene (Fig. 5f and Supplementary Fig. 15f).

Systematic peak-finder analysis demonstrated that *Rag2*$^{-/-}$ pro-B cells carried active H3K9ac marks at 10 V_H3609 genes and H3K4me2 modifications at all 12 V_H3609 genes present on the microarray hybridized with linearly amplified probes (Fig. 6a). Except for 2 V_HSM7 genes, no significant peaks of active histone modifications could be detected at all other V_H genes including the large V_HJ558 gene family (Fig. 6a). A similar picture was obtained with the microarray hybridized with WGA-amplified probes. The H3K4me2 marks were present at 14 of 16 V_H3609 genes, all 4 V_HSM7 genes, 3 V_HGAM genes and only at 10 of 86 V_HJ558 genes, whereas significant peaks of H3K9ac could be detected only at 4 V_H3609 genes (Fig. 6b). In summary, we conclude that IL-7-stimulated *Rag2*$^{-/-}$ pro-B cells lack active histone modifications at most V_H genes including the large V_HJ558 gene family with the notable exception of the V_H3609 and V_HSM7 genes. Importantly, ChIP-chip analysis also revealed a similar chromatin profile of the *Igh* locus in wild-type pro-B cells undergoing *Igh* rearrangements (Supplementary Fig. 16). Hence, the V_H gene cluster of the rearranging *Igh* locus is characterized by a distinct chromatin structure that differs from that of other expressed B-cell-specific genes.

Increased *Igk* recombination in pro-B cells lacking STAT5 or IL-7Rα

As pro-B cells undergo differentiation and *Igk* recombination upon IL-7 withdrawal[34, 35], we tested the hypothesis that STAT5 and IL-7R signaling may repress *Igk* gene rearrangements at the pro-B cell stage. PCR analysis and quantification of *Igk* rearrangements revealed that V_κ-J_κ recombination involving the downstream J_κ4 and J_κ5 elements was 5-6-fold increased in FACS-sorted pro-B cells from *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice compared to wild-type mice (Fig. 7a). Increased *Igk* recombination likely resulted in secondary rearrangements, which could explain the preferential usage of downstream J_κ elements in Bcl-2-rescued STAT5-deficient pro-B cells (Fig. 7a). Consistent with enhanced *Igk* recombination, RT-PCR analysis revealed a ~10-fold increase of rearranged V_κ-JC_κ transcripts in sorted pro-B cells of *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice compared to control *Vav-bcl2* pro-B cells (Fig. 7b). The proximal and distal κ^o germline transcripts (GLT) were also 5- to 20-fold induced in STAT5-deficient pro-B cells (Fig. 7b) and reached the expression level of wild-type pre-B cells (Fig. 7c). In addition to the V_κ-JC_κ transcripts, rearranged V_λ1-JC_λ mRNA were strongly increased in pro-B cells of *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ as well as *Vav-bcl2 Il7r*$^{-/-}$ mice (Fig. 7b), indicating that *Igk* and *Igl* rearrangements or transcription were similarly activated in Bcl-2-rescued pro-B cells lacking STAT5 or IL-7Rα. Importantly, the pro-B cells of *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice expressed the 'pro-B cell-specific' genes *Vpreb1, Igll1* (λ5), *Dntt* (TdT) and *Lef1* (Fig. 7c) and exhibited the same cell surface phenotype (c-Kit$^+$CD19$^+$CD25$^-$CD2$^-$MHCII$^-$) as wild-type pro-B cells (Supplementary Fig. 17). Hence, the STAT5-deficient pro-B cells did not acquire pre-B cell characteristics other than increased *IgL* gene recombination and expression, indicating that STAT5 deficiency alone does not promote the progression from pro-B to pre-B cells. Moreover, ChIP analysis of IL-7-stimulated wild-type pro-B cells demonstrated that STAT5 specifically interacted with the 3' region of the intronic iEκ enhancer among all *Igk* regulatory elements tested (Supplementary Fig. 18) in agreement with recently published data obtained with *Irf4*$^{-/-}$ *Irf8*$^{-/-}$ pre-B cells[36]. In summary, our loss-of-function data identified a critical role for STAT5 and IL-7R signaling in suppressing premature *Igk* and *Igl* recombination in pro-B cells.

DISCUSSION

STAT5 and IL-7 signaling have been considered to control early B-cell development in an instructive manner by regulating the B-cell-specific transcription factor genes *Ebf1* and *Pax5* (ref. [3, 16, 17]) and by controlling distal V_H-DJ_H recombination at the *Igh* locus[20-23]. Here we have reached a different conclusion, as conditional mutagenesis and transgenic *Bcl-2* rescue experiments revealed a critical role for STAT5 and IL-7 signaling in controlling cell survival and repressing premature *Igk* recombination during pro-B cell development.

Cell survival control is known to be a primary function of IL-7R signaling in T-cell development[4, 5]. In contrast, the development of pro-B cells (defined as $CD43^+HSA^+B220^+IgM^-$ cells) is apparently not rescued by transgenic Bcl-2 expression in *Il7r*$^{-/-}$ mice[6], contributing to the notion of instructive IL-7R signaling in pro-B cell development. We observed, however, a partial rescue of committed pro-B cells (defined as c-$Kit^+CD19^+IgM^-$ cells) in *Vav-bcl2 Il7r*$^{-/-}$ mice, indicating that Bcl-2 expression can substitute for the survival, but not the proliferation function of IL-7R signaling in early B-lymphopoiesis. Moreover, transgenic Bcl-2 expression efficiently rescued pro-B cell development in *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice, demonstrating that STAT5 mediates the survival function of IL-7 signaling at the onset of B-lymphopoiesis.

The pro-B to pre-B cell transition requires pre-BCR signaling, which also modulates IL-7 responsiveness via an ERK/MAP kinase-dependent pathway to facilitate selective cell expansion under low IL-7 conditions[28, 37]. The absence of this cell expansion in *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice suggests that IL-7R signaling critically depends on STAT5 for its collaboration with the pre-BCR. STAT5 may thereby synergize with pre-BCR signaling by promoting the proliferation and/or survival of pre-BCR^+ cells. STAT5 has also been implicated in late B-cell development, as retroviral expression of a constitutive active form of STAT5B induces the proliferation and differentiation of human tonsillar B-cells to memory B-cells[29]. Contrary to these *in vitro* gain-of-function studies, our conditional *Stat5* inactivation experiments demonstrate that STAT5 is not essential for the *in vivo* generation of functional memory B-cells or other mature B cell types.

STAT5 directly regulates the pro-survival gene *Bcl2l1* (Bcl-xL) in erythroid cells in response to erythropoietin signaling[38] and in myeloid cells upon GM-CSF stimulation[39]. *Bcl2l1* is, however, not an activated STAT5 target gene in early B-cell development, as it is normally expressed in Bcl-2-rescued STAT5- and IL-7Rα-deficient pro-B cells. Notably, the Bcl-xL protein is more highly expressed in pre-B cells than in pro-B cells[40]. Accordingly, pre-B cells but not pro-B cells were lost upon conditional inactivation of *Bcl2l1*, which demonstrates a critical survival function for Bcl-xL in pre-B cells as suggested by previous analysis of *Bcl2l1*$^{-/-}$ mice[41]. In contrast, the pro-survival protein Mcl-1 was no longer expressed in Bcl-2-rescued STAT5-deficient pro-B cells, and its conditional loss entirely abrogates pro-B cell development, indicating that STAT5 mediates its survival function by activating the *Mcl1* gene in pro-B cells.

STAT5 and IL-7 signaling have also been implicated in the regulation of the B-cell-specific transcription factor genes *Ebf1* and *Pax5*. *Ebf1* can be induced in *Il7*$^{-/-}$ pre-pro-B cells by IL-7 stimulation[3] and in BaF/3 progenitor cells by expression of constitutive active STAT5A[18], suggesting that IL-7 signaling induces B-cell specification in pre-pro-B cells by activating the *Ebf1* gene. However, retroviral expression of *Ebf1* in *Il7r*$^{-/-}$ HSCs leads to a minimal rescue of B-cell development[3], and STAT5 regulates only indirectly the activity of the *Ebf1* promoter[18]. Our Bcl-2 rescue experiments demonstrate, however, that neither IL-7R signaling nor STAT5 are essential for normal *Ebf1* transcription in bone marrow pro-B cells. STAT5 is also thought to regulate *Pax5* by binding to sequences upstream of its distal promoter[15, 16].

However like *Ebf1*, *Pax5* was also normally expressed in *Stat5*$^{-/-}$ fetal liver pro-B cells as well as in Bcl-2-rescued bone marrow pro-B cells lacking STAT5 or IL-7Rα, consistent with the fact that we could not detect STAT5 binding to any regulatory element of the *Pax5* locus[42].

STAT5 and IL-7 signaling have also been associated with V(D)J recombination of the *Tcrg* and *Igh* loci. *Il7r*$^{-/-}$ mice and *Stat5*$^{-/-}$ embryos are unable to generate γδ T cells due to a severe defect in V_γ-J_γ recombination[9, 10], as STAT5 induces chromatin accessibility and germline transcription at the *Tcrg* locus by direct binding to the J_γ promoters[43]. A similar role has been suggested for STAT5 and IL-7R signaling in controlling the chromatin accessibility and rearrangements of distal V_H genes at the *Igh* locus[20, 22, 23]. In contrast to these published data, we could not detect any significant difference in distal V_HJ558-DJ_H rearrangements in Bcl-2-rescued *Il7r*$^{-/-}$ and *Stat5*$^{\Delta/\Delta}$ pro-B cells compared to control cells. In an attempt to explain these discrepancies, we note that Corcoran et al.[22] detected similar numbers of pro-B cells in the bone marrow of wild-type and *Il7r*$^{-/-}$ mice in marked contrast to our study and other reports[2, 3]. The CD43$^+$B220$^+$ 'pro-B cells' analyzed by Corcoran et al.[22] were likely a mixture of pro-B cells, recirculating CD43$^+$ B1 cells, NK cell progenitors and plasmacytoid dendritic cells[44, 45], which could explain the apparent decrease of distal V_HJ558-DJ_H recombination in this impure cell population isolated from *Il7r*$^{-/-}$ mice. As Bertolino et al.[23] also observed decreased germline transcripts and rearrangements of distal V_HJ558 genes in B220$^+$IgM$^-$ B-lymphocytes (pro-B and pre-B cells) of *Stat5*$^{\Delta N/\Delta N}$ mice, we repeated these experiments to realize that V_HJ558 germline transcription and recombination is normal in *Stat5*$^{\Delta N/\Delta N}$ pro-B cells similar to Bcl-2-rescued STAT5-deficient pro-B cells. Finally, Chowdhury and Sen[20] described a role for IL-7 signaling in controlling the chromatin accessibility of distal V_H genes in *Rag2*$^{-/-}$ pro-B cells. Our extensive ChIP-chip analyses revealed, however, no or only very low levels of the active histone modifications H3K4me2 and H3K9ac at most V_H genes in IL-7-stimulated *Rag2*$^{-/-}$ pro-B cells. Active chromatin was, however, consistently present at V_H3609 and V_HSM7 genes. As wild-type pro-B cells exhibited a similar H3K4me2 and H3K9ac modification pattern throughout the V_H gene cluster as *Rag2*$^{-/-}$ pro-B cells, we conclude that germline transcription and V_H-DJ_H recombination can proceed in the absence of overt chromatin activation of V_H genes. As active chromatin at V_H genes can currently only be analyzed at the cell population level, our data are also compatible with the remote possibility that each individual pro-B cell is able to induce active chromatin only at a small random subset of V_H genes, which would escape detection in the entire pro-B cell population. In contrast to the V_H genes, the three active marks H3K9ac, H3K4me2 and H3K4me3 accumulate to high levels at the intronic Eμ enhancer and J_H elements, which suggests the following sequence of epigenetic events during *Igh* recombination. The V(D)J recombinase consisting of RAG1 and RAG2 is likely tethered to the J_H elements through specific binding of H3K4me3 by the C-terminal PHD finger of RAG2 (ref. [46]) and may act from this proximal location to undergo synapse formation with D_H and V_H elements. Following D_H-J_H recombination in B-lymphoid progenitors, the rearranged D_H element is incorporated into the active chromatin domain at the J_H-Eμ region, which facilitates subsequent V_H-DJ_H recombination upon juxtaposition of V_H genes by Pax5-dependent contraction of the *Igh* locus in committed pro-B cells[47]. This model could explain why active chromatin is asymmetrically distributed at the proximal J_H region but not throughout the V_H gene cluster in pro-B cells.

Under physiological conditions, the expression of IL-7Rα is down-regulated in response to pre-BCR signaling, which renders small pre-B cells unresponsive to IL-7 signals, leading to the loss of STAT5 activation[37, 48]. Moreover, pro-B cells upon IL-7 withdrawal initiate pre-B cell differentiation and *Igk* rearrangements[34, 35]. Here we have shown that the loss of STAT5 and IL-7 signaling is sufficient to increase *Igk* germline transcription and rearrangements in Bcl-2-rescued pro-B cells in the absence of further differentiation. As *Igk* rearrangements can be detected in only 15% of wild-type pro-B cells[49], the observed 5-6 fold increase in recombination frequency predicts that almost all Bcl-2-rescued pro-B cells undergo *Igk*

recombination in the absence of STAT5. Hence, an important function of STAT5 and IL-7 signaling is the suppression of premature *Igk* rearrangements in pro-B cells. STAT5 directly binds to the intronic iEκ enhancer in wild-type pro-B cells (this study) and *Irf4*$^{-/-}$ *Irf8*$^{-/-}$ pre-B cells[36], where it likely represses the recombination-activating function of the iEκ enhancer[50]. Importantly, our genetic *in vivo* analysis demonstrates a key role for STAT5 and IL-7 signaling in suppressing *Igk* germline transcription as well as recombination in pro-B cells and thus provides strong complementary evidence to a recently published *in vitro* study[36], which describes an inverse correlation between STAT5 activation and *Igk* germline transcription during the developmental transition of *Irf4*$^{-/-}$ *Irf8*$^{-/-}$ large pre-B cells to small pre-B cells.

Constitutive activation of STAT5 is associated with human leukemias and myeloproliferative disorders[7]. In particular, the presence of activated STAT5 is a characteristic feature of B-cell acute lymphophastic leukemia (B-ALL) carrying the *BCR-ABL1* chromosomal translocation[7]. A critical role of STAT5 in the generation of this B-ALL subset is indicated by the fact that retroviral BCR-ABL (p185) expression in *Stat5*$^{-/-}$ hematopoietic progenitors fails to induce leukemia in the mouse[10]. Our study has now provided a molecular explanation suggesting that the oncogenic function of STAT5 in B-ALL is likely to control the survival of leukemic B-lymphocytes by activating the *Mcl1* gene.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. von Freeden-Jeffry U, et al. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med 1995;181:1519–1526. [PubMed: 7699333]
2. Miller JP, et al. The earliest step in B lineage differentiation from common lymphoid progenitors is critically dependent upon interleukin 7. J Exp Med 2002;196:705–711. [PubMed: 12208884]
3. Kikuchi K, Lai AY, Hsu C-L, Kondo M. IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF. J Exp Med 2005;201:1197–1203. [PubMed: 15837809]
4. Maraskovsky E, et al. Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant *rag-1*$^{-/-}$ mice. Cell 1997;89:1011–1019. [PubMed: 9215624]
5. Akashi K, Kondo M, von Freeden-Jeffry U, Murray R, Weissman IL. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. Cell 1997;89:1033–1041. [PubMed: 9215626]
6. Maraskovsky E, Peschon JJ, McKenna H, Teepe M, Strasser A. Overexpression of Bcl-2 does not rescue impaired B lymphopoiesis in IL-7 receptor-deficient mice but can enhance survival of mature B cells. Int Immunol 1998;10:1367–1375. [PubMed: 9786436]

7. Hennighausen L, Robinson GW. Interpretation of cytokine signaling through the transcription factors STAT5A and STAT5B. Genes Dev 2008;22:711–721. [PubMed: 18347089]
8. Teglund S, et al. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 1998;93:841–850. [PubMed: 9630227]
9. Yao Z, et al. Stat5a/b are essential for normal lymphoid development and differentiation. Proc Natl Acad Sci USA 2006;103:1000–1005. [PubMed: 16418296]
10. Hoelbl A, et al. Clarifying the role of Stat5 in lymphoid development and Abelson-induced transformation. Blood 2006;107:4898–4906. [PubMed: 16493008]
11. Moriggl R, et al. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. Immunity 1999;10:249–259. [PubMed: 10072077]
12. Sexl V, et al. Stat5a/b contribute to interleukin 7-induced B-cell precursor expansion, but *abl*- and *bcr/abl*-induced transformation are independent of Stat5. Blood 2000;96:2277–2283. [PubMed: 10979977]
13. Cui Y, et al. Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. Mol Cell Biol 2004;24:8037–8047. [PubMed: 15340066]
14. Goetz CA, Harmon IR, O'Neil JJ, Burchill MA, Farrar MA. STAT5 activation underlies IL7 receptor-dependent B cell development. J Immunol 2004;172:4770–4778. [PubMed: 15067053]
15. Hirokawa S, Sato H, Kato I, Kudo A. EBF-regulating Pax5 transcription is enhanced by STAT5 in the early stage of B cells. Eur J Immunol 2003;33:1824–1829. [PubMed: 12811842]
16. Goetz CA, et al. Restricted STAT5 activation dictates appropriate thymic B versus T cell lineage commitment. J Immunol 2005;174:7753–7763. [PubMed: 15944278]
17. Dias S, Silva H Jr, Cumano A, Vieira P. Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors. J Exp Med 2005;201:971–979. [PubMed: 15767371]
18. Roessler S, et al. Distinct promoters mediate the regulation of *Ebf1* gene expression by IL-7 and Pax5. Mol Cell Biol 2007;27:579–594. [PubMed: 17101802]
19. Johnston CM, Wood AL, Bolland DJ, Corcoran AE. Complete sequence assembly and characterization of the C57BL/6 mouse Ig heavy chain V region. J Immunol 2006;176:4221–4234. [PubMed: 16547259]
20. Chowdhury D, Sen R. Stepwise activation of the immunoglobulin μ heavy chain gene locus. EMBO J 2001;20:6394–6403. [PubMed: 11707410]
21. Johnson K, Angelin-Duclos C, Park S, Calame KL. Changes in histone acetylation are associated with differences in accessibility of V_H gene segments to V-DJ recombination during B-cell ontogeny and development. Mol Cell Biol 2003;23:2438–2450. [PubMed: 12640127]
22. Corcoran AE, Riddell A, Krooshoop D, Venkitaraman AR. Impaired immunoglobulin gene rearrangement in mice lacking the IL-7 receptor. Nature 1998;391:904–907. [PubMed: 9495344]
23. Bertolino E, et al. Regulation of interleukin 7-dependent immunoglobulin heavy-chain variable gene rearrangements by transcription factor STAT5. Nat Immunol 2005;6:836–843. [PubMed: 16025120]
24. Igarashi H, Gregory SC, Yokota T, Sakaguchi N, Kincade PW. Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. Immunity 2002;17:117–130. [PubMed: 12196284]
25. McCormack MP, Forster A, Drynan L, Pannell R, Rabbitts TH. The LMO2 T-cell oncogene is activated via chromosomal translocations or retroviral insertion during gene therapy but has no mandatory role in normal T-cell development. Mol Cell Biol 2003;23:9003–9013. [PubMed: 14645513]
26. Kwon K, et al. Instructive role of the transcription factor E2A in early B lymphopoiesis and germinal center B cell development. Immunity 2008;28:751–762. [PubMed: 18538592]
27. Yao Z, et al. Nonredundant roles for Stat5a/b in directly regulating *Foxp3*. Blood 2007;109:4368–4375. [PubMed: 17227828]
28. Fleming HE, Paige CJ. Pre-B cell receptor signaling mediates selective response to IL-7 at the pro-B to pre-B cell transition via an ERK/MAP kinase-dependent pathway. Immunity 2001;15:521–531. [PubMed: 11672535]

29. Scheeren FA, et al. STAT5 regulates the self-renewal capacity and differentiation of human memory B cells and controls Bcl-6 expression. Nat Immunol 2005;6:303–313. [PubMed: 15711548]
30. Opferman JT, et al. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. Nature 2003;426:671–676. [PubMed: 14668867]
31. Schebesta A, et al. Transcription factor Pax5 activates the chromatin of key genes involved in B cell signaling, adhesion, migration and immune function. Immunity 2007;27:49–63. [PubMed: 17658281]
32. O'Geen H, Nicolet CM, Blahnik K, Green R, Farnham PJ. Comparison of sample preparation methods for ChIP-chip assays. BioTechniques 2006;41:577–580. [PubMed: 17140114]
33. Chakraborty T, et al. Repeat organization and epigenetic regulation of the D_H-Cμ domain of the immunoglobulin heavy-chain gene locus. Mol Cell 2007;27:842–850. [PubMed: 17803947]
34. Grawunder U, Haasner D, Melchers F, Rolink A. Rearrangement and expression of κ light chain genes can occur without μ heavy chain expression during differentiation of pre-B cells. Int Immunol 1993;5:1609–1618. [PubMed: 8312230]
35. Johnson K, et al. Regulation of immunoglobulin light-chain recombination by the transcription factor IRF-4 and the attenuation of interleukin-7 signaling. Immunity 2008;28:335–345. [PubMed: 18280186]
36. Mandal M, et al. Ras orchestrates exit from the cell cycle and light-chain recombination during early B cell development. Nat Immunol 2009;10:1110–1117. [PubMed: 19734904]
37. Marshall AJ, Fleming HF, Wu GE, Paige CJ. Modulation of the IL-7 dose-response threshold during pro-B cell differentiation is dependent on pre-B cell receptor expression. J Immunol 1998;161:6038–6045. [PubMed: 9834086]
38. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF. Fetal anemia and apoptosis of red cell progenitors in Stat5a$^{-/-}$5b$^{-/-}$ mice: a direct role for Stat5 in Bcl-X_L induction. Cell 1999;98:181–191. [PubMed: 10428030]
39. Kieslinger M, et al. Antiapoptotic activity of *Stat5* required during terminal stages of myeloid differentiation. Genes Dev 2000;14:232–244. [PubMed: 10652277]
40. Grillot DAM, et al. *bcl-x* exhibits regulated expression during B cell development and activation and modulates lymphocyte survival in transgenic mice. J Exp Med 1996;183:381–391. [PubMed: 8627151]
41. Motoyama N, et al. Massive cell death of immature hematopoietic cells and neurons in bcl-x-deficient mice. Science 1995;267:1506–1510. [PubMed: 7878471]
42. Decker T, et al. Stepwise activation of enhancer and promoter regions of the B cell commitment gene *Pax5* in early lymphopoiesis. Immunity 2009;30:508–520. [PubMed: 19345119]
43. Ye S-K, et al. The IL-7 receptor controls the accessibility of the TCR_γ locus by Stat5 and histone acetylation. Immunity 2001;15:813–823. [PubMed: 11728342]
44. Rolink A, et al. A subpopulation of B220$^+$ cells in murine bone marrow does not express CD19 and contains natural killer cell progenitors. J Exp Med 1996;183:187–194. [PubMed: 8551222]
45. Tudor KS, Payne KJ, Yamashita Y, Kincade PW. Functional assessment of precursors from murine bone marrow suggests a sequence of early B lineage differentiation events. Immunity 2000;12:335–345. [PubMed: 10755620]
46. Matthews AG, et al. RAG2 PHD finger couples histone H3 lysine 4 trimethylation with V(D)J recombination. Nature 2007;450:1106–1110. [PubMed: 18033247]
47. Fuxa M, et al. Pax5 induces *V*-to-*DJ* rearrangements and locus contraction of the *immunoglobulin heavy-chain* gene. Genes Dev 2004;18:411–422. [PubMed: 15004008]
48. Schebesta M, Pfeffer PL, Busslinger M. Control of pre-BCR signaling by Pax5-dependent activation of the *BLNK* gene. Immunity 2002;17:473–485. [PubMed: 12387741]
49. Novobrantseva TI, et al. Rearrangement and expression of immunoglobulin light chain genes can precede heavy chain expression during normal B cell development in mice. J Exp Med 1999;189:75–87. [PubMed: 9874565]
50. Xu Y, Davidson L, Alt FW, Baltimore D. Deletion of the Igκ light chain intronic enhancer/matrix attachment region impairs but does not abolish VκJκ rearrangement. Immunity 1996;4:377–385. [PubMed: 8612132]

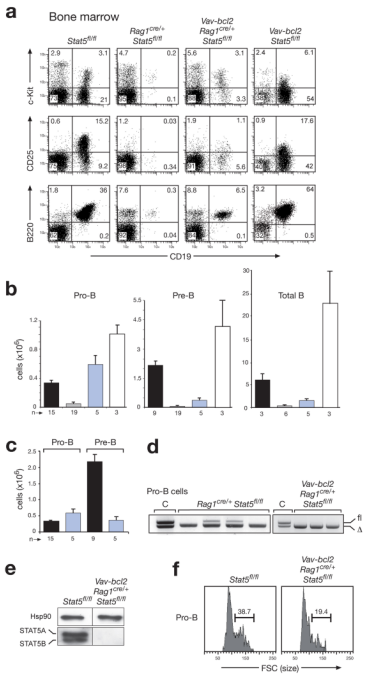

Figure 1. Rescue of STAT5-deficient pro-B cells by transgenic Bcl-2 expression

(**a-c**) Restoration of pro-B cell development by compensation of the STAT5 deficiency with transgenic Bcl-2 expression. Flow cytometric analysis (**a**) and cell counting were used to determine the absolute cell numbers (**b,c**) of pro-B (c-Kit$^+$CD19$^+$IgM$^-$), pre-B (CD25$^+$CD19$^+$IgM$^-$) and total B cells (B220$^+$CD19$^+$) in the bone marrow of *Rag1$^{cre/+}$ Stat5$^{fl/fl}$* (grey bar) and *Vav-bcl2 Rag1$^{cre/+}$ Stat5$^{fl/fl}$* mice (blue bar) compared to control *Stat5$^{fl/fl}$* (black bar) and *Vav-bcl2 Stat5$^{fl/fl}$* mice (white bar) at the age of 4-6 weeks. Bone marrow was isolated from the femur and tibia of the two hind legs. n, number of mice analyzed. (**c**) Reanalysis of the absolute numbers of pro-B and pre-B cells (**b**) revealed the absence of proliferative cell expansion during the pro-B to pre-B cell transition in *Vav-bcl2 Rag1$^{cre/+}$ Stat5$^{fl/fl}$* mice compared to control *Stat5$^{fl/fl}$* mice. (**d**) PCR genotyping of the floxed (fl) and deleted (Δ) *Stat5* alleles in sorted pro-B cells from the bone marrow of 4 *Rag1$^{cre/+}$ Stat5$^{fl/fl}$* and 3 *Vav-bcl2 Rag1$^{cre/+}$ Stat5$^{fl/fl}$* mice. Genomic *Stat5$^{fl/Δ}$* DNA was used as control (C). (**e**) Immunoblot analysis of sorted CD19$^+$ bone marrow B cells of the indicated genotypes with a mixture of STAT5A and STAT5B antibodies or a control Hsp90 antibody. (**f**) Size distribution of pro-B cells of the indicated genotypes, as determined by their forward scatter (FSC) profile.

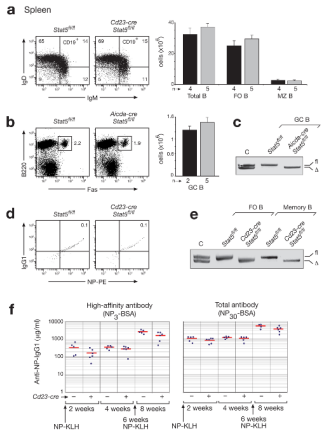

Figure 2. Normal development and immune responses of mature B cells in the absence of STAT5

(**a**) Normal development of mature ($IgD^{hi}IgM^{lo}CD19^{+}$), follicular (FO; $CD21^{int}CD23^{hi}$) and marginal zone (MZ; $CD21^{hi}CD23^{lo}$) B cells in the spleen of nonimmunized *Cd23-cre Stat5*$^{fl/fl}$ mice (grey bar) compared to control *Stat5*$^{fl/fl}$ mice (black bar) at the age of 8-10 weeks. Note that the *Cd23-cre* line 5 initiates Cre-mediated gene deletion in immature B cells and results in complete deletion in MZ and FO B cells of the spleen[26]. Absolute numbers of the different B cell types and total B cells ($CD19^{+}B220^{+}$) are shown to the right together with the number (n) of mice analyzed. (**b**) Normal GC B cell formation in *Aicda-cre Stat5*$^{fl/fl}$ mice. The abundance of GC B cells ($Fas^{+}B220^{+}$) in control *Stat5*$^{fl/fl}$ (back bar) and *Aicda-cre Stat5*$^{fl/fl}$ (grey bar) mice was determined by flow cytometric analysis 15 days after immunization with sheep red blood cells. n, number of mice analyzed. (**c**) Efficient deletion of *Stat5* in sorted GC B cells of *Aicda-cre Stat5*$^{fl/fl}$ mice 15 days after immunization. Genomic *Stat5*$^{fl/\Delta}$ DNA was used as control (C). (**d**) Presence of memory B cells in the spleen of *Cd23-cre Stat5*$^{fl/fl}$ 8 weeks after the initial immunization with NP-KLH (100 μg in Alum) and 2 weeks after boosting with NP-KLH (10 μg in PBS). Memory B cells were identified as $Lin^{-}CD38^{hi}B220^{+}CD19^{+}IgG1^{+}NP^{+}$ cells as shown in Supplementary Fig. 8. (**e**) PCR genotyping of the floxed (fl) and deleted (Δ) *Stat5* alleles in sorted FO and memory B cells from mice of the indicated genotypes. (**f**) NP-specific immune responses. Control *Stat5*$^{fl/fl}$ (−) and *Cd23-cre Stat5*$^{fl/fl}$ (+) mice were immunized with NP-KLH (100 μg in Alum) and boosted with NP-KLH (10 μg in PBS) 6 weeks later. After 2, 4 and 8 weeks, the serum titers of NP-specific antibodies were determined by ELISA using NP_3-BSA- or NP_{30}-BSA-coated plates for detecting high-affinity or total IgG1 antibodies, respectively. NP-specific IgG1 concentrations (μg/ml) were determined relative to a standard NP-binding IgG1 antibody.

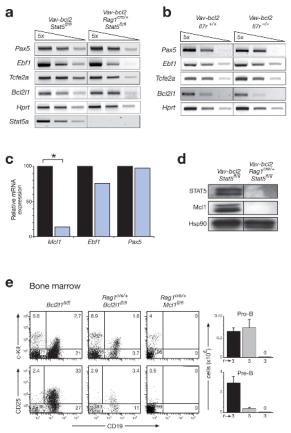

Figure 3. STAT5 controls the survival of pro-B cells by activating the *Mcl1* gene

(**a,b**) Normal expression of B cell transcription factor genes in the absence of STAT5 (**a**) or IL-7R signaling (**b**). Expression of the indicated genes was determined by semiquantitative RT-PCR analysis using 5-fold serial dilutions of cDNA prepared from FACS-sorted pro-B cells (Supplementary Fig. 10) of 4-6-week-old mice of the indicated genotypes. *Tcfe2a* (E2A); *Bcl2l1* (Bcl-x). (**c**) Reduced *Mcl1* expression in STAT5-deficient pro-B cells. Transcripts of the indicated genes were analyzed by real-time RT-PCR of cDNA prepared from sorted pro-B cells of control *Vav-bcl2 Stat5*$^{fl/fl}$ (black bar) and *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ (blue bar) mice. The transcript levels of control pro-B cells were set to 100%. Statistical significance was determined by the student's *t* test (*, $p < 0.05$). (**d**) Loss of Mcl-1 expression in the absence of STAT5. Whole cell extracts of sorted CD19^{+} bone marrow cells from mice of the indicated genotypes were analyzed by immunoblot analysis with STAT5, Mcl-1 and Hsp90 antibodies. (**e**) Distinct functions of *Mcl1* and *Bcl2l1* in early B cell development. Pro-B and pre-B cells were analyzed by flow cytometric analysis in the bone marrow of control *Bcl2l1*$^{fl/fl}$ (black bar), *Rag1*$^{cre/+}$ *Bcl2l1*$^{fl/fl}$ (grey bar) and *Rag1*$^{cre/+}$ *Mcl1*$^{fl/fl}$ mice (denoted by 0). Absolute cell numbers are shown to the right together with the number (n) of mice analyzed. A more comprehensive FACS analysis is shown in Supplementary Fig. 12.

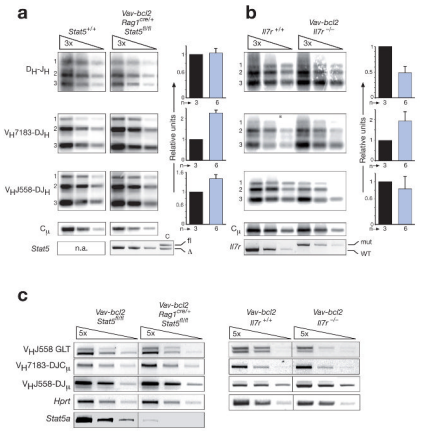

Figure 4. Normal *Igh* gene recombination in pro-B cells lacking STAT5 or IL-7Rα

(**a**) PCR analysis of D_H-J_H, V_H7183-DJ_H and V_HJ558-DJ_H rearrangements in c-Kit$^+$CD19$^+$IgM$^-$ pro-B cells, which were FACS-sorted from the bone marrow of control *Stat5*$^{+/+}$ (black bar) and *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice (blue bar) at the age of 4-6 weeks as described in Supplementary Fig. 10. Input DNA was normalized by PCR amplification of an *Igh* Cμ fragment. Threefold serial DNA dilutions were analyzed by semiquantitative PCR and Southern blotting. The radioactive signals of each rearrangement type were quantified for each DNA dilution by phosphorimager analysis and normalized relative to the corresponding value of the Cμ PCR fragment. For each experiment, the average of the normalized signals obtained with the three DNA dilutions was determined, and the average value of all experiments is displayed as a bar graph for each rearrangement type to the right. The average values obtained with wild-type (*Stat5*$^{+/+}$) pro-B cells were set to 1, and the number (n) of independent experiments is shown. Numbers along the left margin indicate rearrangements to the J_H1, J_H2 and J_H3 gene segments. n.a., not applicable. (**b**) PCR analysis of *Igh* rearrangements in sorted pro-B cells from 4-6-week-old *Il7r*$^{+/+}$ (black bar) and *Vav-bcl2 Il7r*$^{-/-}$ mice (blue bar) was performed and evaluated as described in (**a**). (**c**) RT-PCR analysis. Expression of the V_HJ558 germline transcripts (GLT) as well as the rearranged V_H7183-DJC_μ and V_HJ558-DJ_μ transcripts was analyzed by semiquantitative RT-PCR analysis using 5-fold serial dilutions of cDNA prepared from FACS-sorted pro-B cells of the indicated genotypes.

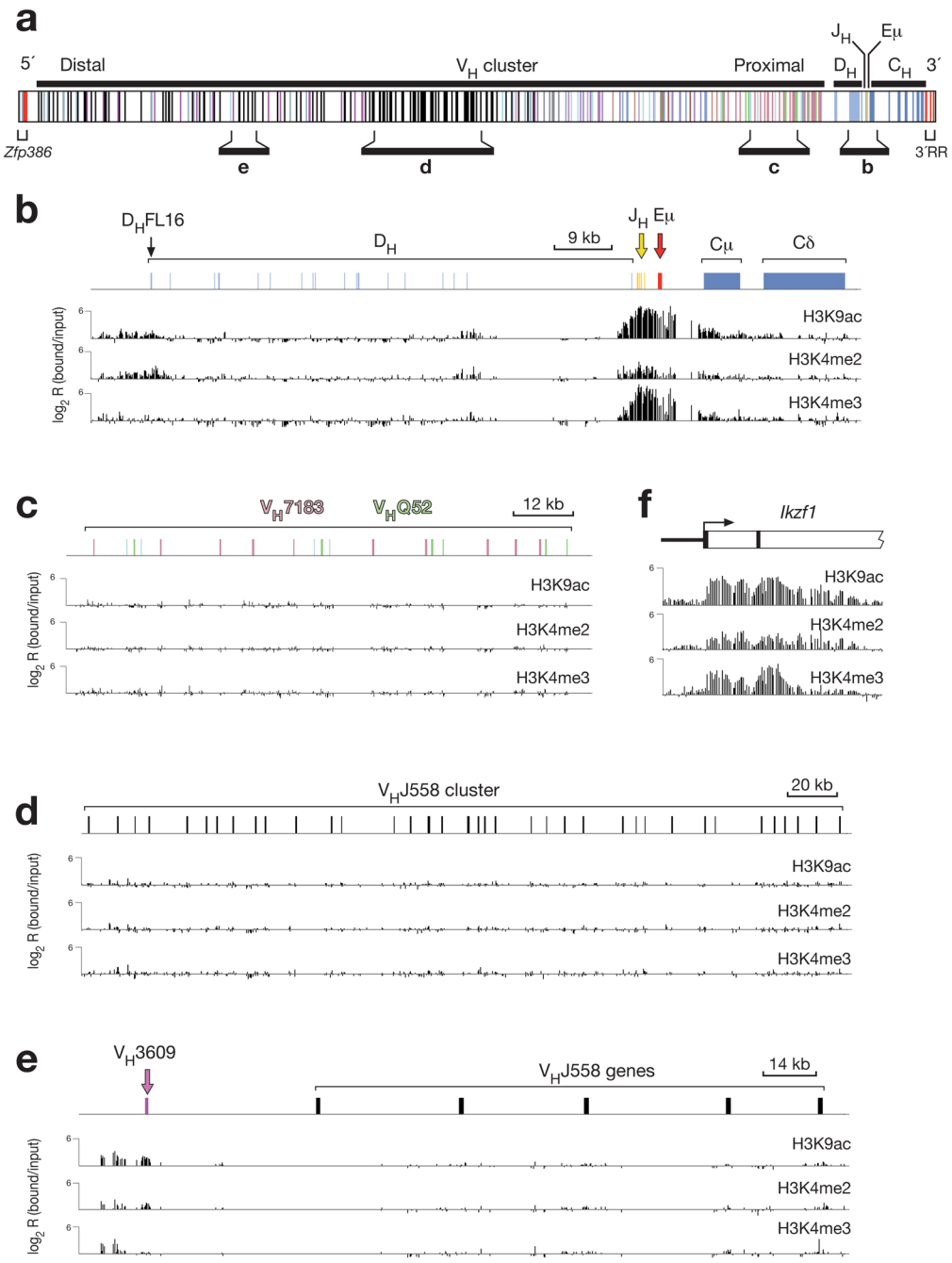

Figure 5. Mapping of active histone modifications along the *Igh* locus in *Rag2*$^{-/-}$ pro-B cells

(**a**) Structure of the *Igh* locus indicating the location of variable (V_H), diversity (D_H), joining (J_H) and constant (C_H) gene segments together with the intronic (Eμ) enhancer, the 5' DNase I hypersensitive sites (red) and 3' regulatory region (3'RR; red). Different colors indicate the members of distinct V_H gene families, of which the V_HJ558 (black), V_H3609 (pink), V_HQ52 (green) and V_H7183 (rosa) genes are relevant for this study. The diagram of the *Igh* locus is based on the mm8 genome sequence and the published V_H gene assembly[19] of the C57BL/6 mouse. (**b-f**) Mapping of active chromatin marks in the D_H-C_μ domain (**b**) and representative proximal (**c**) and distal (**d,e**) V_H gene regions of the *Igh* locus as well as at the 5' end of the *Ikzf1* (Ikaros) gene (**f**) in *Rag2*$^{-/-}$ pro-B cells. Antibodies recognizing acetylated lysine 9

(H3K9ac), dimethylated lysine 4 (H3K4me2) or trimethylated lysine 4 (H3K4me3) on histone H3 were used for ChIP-chip analysis[31] of *Rag2*$^{-/-}$ bone marrow pro-B cells, which were expanded *in vitro* for 5 days in the presence of IL-7 and OP9 stromal cells. The ChIP-precipitated DNA was amplified with the T7-based linear amplification protocol[31] prior to fluorescent labeling and co-hybridization with the input DNA probe onto a high-density 50-mer oligonucleotide array containing the mouse *Igh* locus at 100-bp resolution (produced by NimbleGen Systems). The logarithmic ratio ($\log_2$) of the hybridization intensities between antibody-precipitated and input DNA (bound/input) is shown as a bar for each oligonucleotide on the microarray. The results shown are representative of two ChIP-chip experiments, which were performed with independently prepared DNA probes from *Rag2*$^{-/-}$ pro-B cells. A scale bar is shown in kb. Supplementary Fig. 15 shows the result of a similar ChIP-chip analysis, which was performed with DNA probes amplified with the WGA method[32]. The mm8 sequence coordinates for the displayed regions of the *Igh* locus on chromosome 12 are 116,520,000-113,586,000 (**a**), 113,938,000-113,852,500 (**b**), 114,234,000-114,086,000 (**c**), 115,441,500-115,087,000 (**d**) and 115,859,000-115,780,000 (**e**).

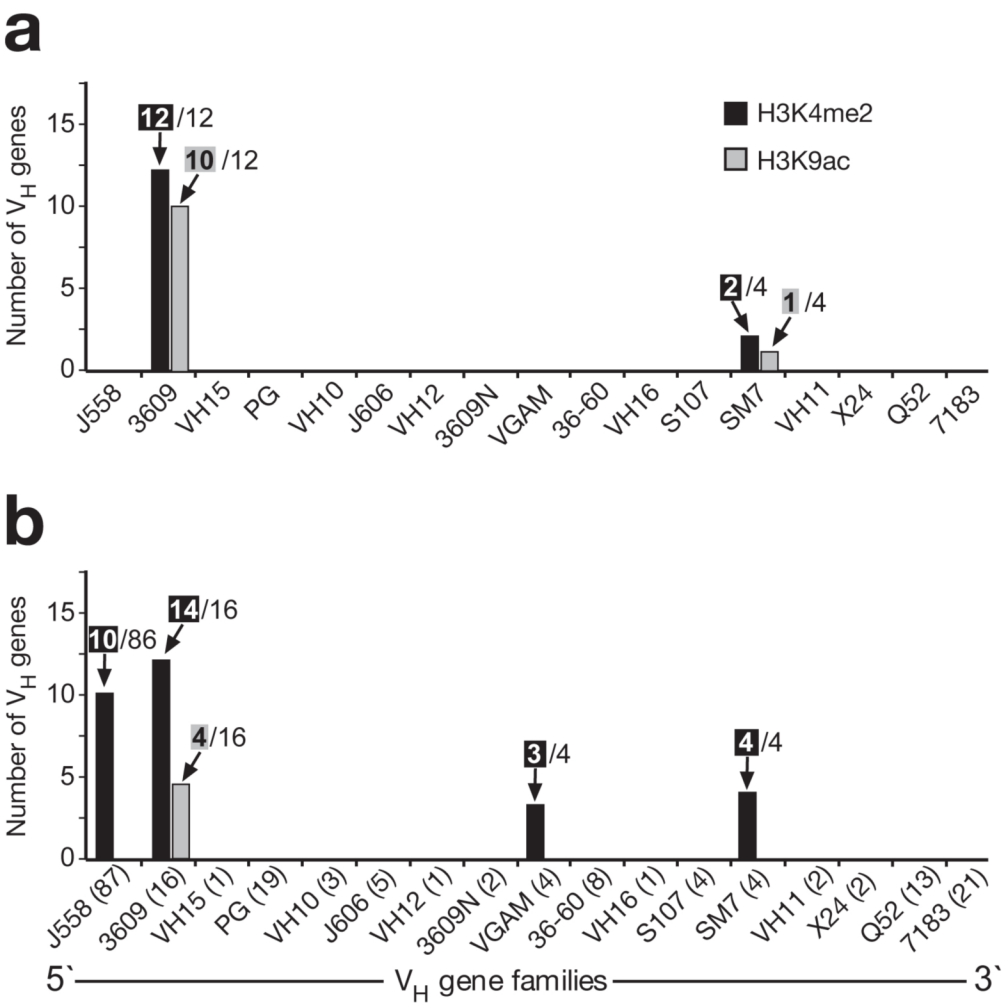

Figure 6. Distribution of active histone marks along the V_H gene cluster in *Rag2*$^{-/-}$ pro-B cells

Peak-finder analysis was used to systematically evaluate the ChIP-chip data, which were obtained with linearly amplified (**a**) or WGA-amplified (**b**) probes as shown in Fig. 5 and Supplementary Fig. 15, respectively. The number of V_H genes, which contained a peak of H3K4me2 (black bar) or H3K9ac (gray bar) above the threshold of the peak-finder analysis, are shown together with the number of all genes of each V_H gene family that were present on the microarray. The V_H gene families are arranged according to their position in the *Igh* locus. The number of all annotated genes is shown in brackets (**b**) for each V_H gene family of the C57BL/6 mouse[19].

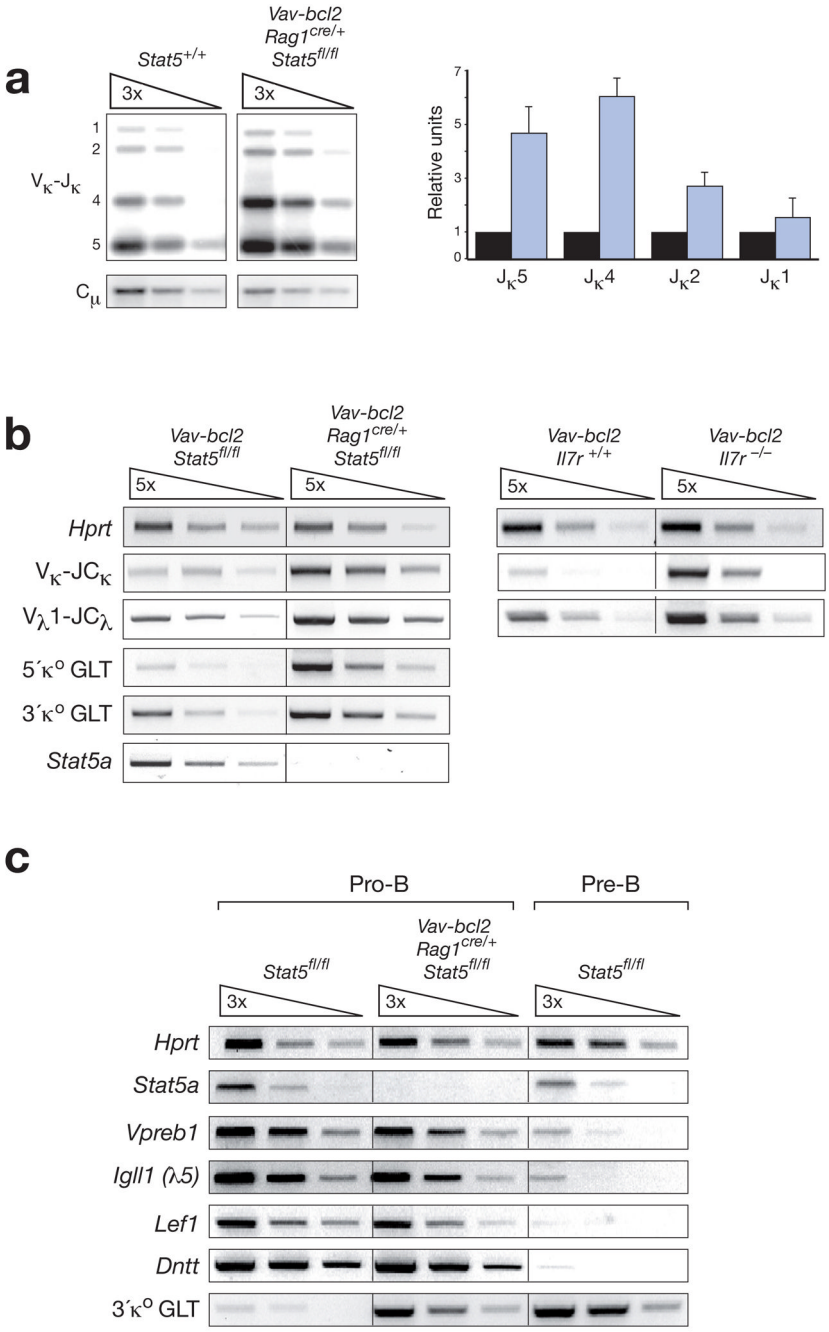

Figure 7. STAT5 and IL-7 signaling repress *Igk* and *Igl* gene rearrangements in pro-B cells

(**a**) Increased *Igk* gene rearrangements in pro-B cells sorted from *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice (blue bar) compared to wild-type mice (black bar). V_κ gene rearrangements to the $J_\kappa1$ to $J_\kappa5$ gene segments (indicated by numbers along the left margin) were analyzed by semiquantitative PCR of 3-fold serially diluted DNA followed by Southern blot detection. The radioactive signals corresponding to these rearrangements were quantified by phosphorimager analysis, normalized to the value of the control Cμ PCR fragment and displayed in the bar graph to the right. The normalized value obtained for each V_κ-J_κ rearrangement in wild-type pro-B cells was set to 1. The quantification of 3 independent experiments is shown. (**b**) RT-PCR analysis of *Igk* transcripts. The expression of rearranged V_κ-JC_κ and $V_\lambda1$-JC_λ transcripts

as well as the two κ^o germline transcripts (GLT) were analyzed by semiquantitative RT-PCR using 5-fold serial dilutions of cDNA prepared from FACS-sorted pro-B cells of the indicated genotypes. The 5' and 3' κ^o GLTs are transcribed from distinct promoters (see Supplementary Fig. 18a) and give rise to 1.1-kb and 0.8-kb spliced transcripts, respectively. (**c**) Expression of pro-B cell-specific genes in Bcl-2-rescued STAT5-deficient pro-B cells. FACS-sorted pro-B cells (c-Kit$^+$CD19$^+$CD25$^-$IgM$^-$) and pre-B cells (CD19$^+$CD25$^+$c-Kit$^-$IgM$^-$) of control *Stat5*$^{+/+}$ or *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice were analyzed by semiquantitative RT-PCR for expression of the 'pro-B cell-specific' genes *Vpreb1*, *Igll1* (λ5), *Lef1* and *Dntt* (TdT)[48] and the 'pre-B cell-specific' 3' κ^o GLT.