Function of BCL6 in pre-B cells and Philadelphia chromosome-positive acute lymphoblastic leukemia

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To those who never give up and are still able to see the world with open and imaginative eyes

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Related Articles

This thesis is based on the following papers, which will be referred to in the results section by their numbers:

1. **Duy C**, Yu JJ, Nahar R, Swaminathan S, Kweon SM, Polo JM, Valls E, Klemm L, Shojaee S, Cerchietti L, Schuh W, Jack HM, Hurtz C, Ramezani-Rad P, Jäck HM, Herzog S, Jumaa H, Koeffler HP, de Alborán IM, Melnick AM, Ye BH & Müschen M. BCL6 is critical for the development of a diverse primary B cell repertoire. *J Exp Med.* 2010; 207:1209-1221

2. **Duy C**, Hurtz C, Shojaee S, Cerchietti L, Geng H, Swaminathan S, Klemm L, Kweon SM, Nahar R, Braig M, Park E, Kim YM, Hofmann W-K, Herzog S, Jumaa H, Koeffler PH, Yu JJ, Heisterkamp N, Graeber TG, Wu H, Ye BH, Melnick A & Müschen M. BCL6 enables Ph^+ acute lymphoblastic leukaemia cells to survive *BCR–ABL1* kinase inhibition. *Nature.* 2011; in press

3. Klemm L, **Duy C**, Iacobucci I, Kuchen S, von Levetzow G, Feldhahn N, Henke N, Li Z, Hoffmann TK, Kim YM, Hofmann WK, Jumaa H, Groffen J, Heisterkamp N, Martinelli G, Lieber MR, Casellas R & Müschen M. The B cell mutator AID promotes B lymphoid blast crisis and drug resistance in chronic myeloid leukemia. *Cancer Cell*. 2009; 16:232-45.

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1 Introduction

1.1 B cells

B cells represent a subset of lymphocytes that are involved in the humoral immune response by producing antibodies (also called immunoglobulins) against pathogens. Specific B cell receptors enable the recognition of pathogens based on their foreign antigens. Since there is an immense variety of pathogens, nature developed a mechanism, termed V(D)J (variable-diversity-joining) recombination, to ensure that B cells recognize a broad spectrum of antigens (Brack et al., 1978; Weigert et al., 1978).

1.1.1 V(D)J recombination

V(D)J recombination (also referred to as somatic recombination) leads to diversification of the B cell antigen receptor (BCR) by random rearrangement of gene segments at the immunoglobulin (Ig) gene loci (Figure 1). Activated B cells (plasma cells) can secrete a soluble form of their BCR as antibodies composed of two heavy and two light chains, which are encoded by Ig genes at three loci. The immunoglobulin heavy chain (*IGH*) locus is located at chromosome 14q32, the Ig kappa and lambda light chain loci at chromosome 2p12 (*IGK*) and 22q11 (*IGL*) (Croce et al., 1979; Erikson et al., 1981; Hobart et al., 1981; Kirsch et al., 1982; Malcolm et al., 1982; McBride et al., 1982). A cluster of *V* (variable), *D* (diversity), and *J* (joining) gene segments encodes the variable region while a cluster of *C* (constant) genes expresses the constant region of an immunoglobulin heavy chain (IgH; Figure 1) (Early et al., 1980; Tonegawa 1983; Matsuda et al., 1998). The variable region of Ig light chain is generated by only joining *V*

and *J* gene segments, either at the κ or λ locus.

V, D, and J gene segments are flanked by recognition motifs (referred to as RSS, recombination signal sequence), which mark the segments as blocks and serve as substrates for V(D)J recombination (Sakano et al., 1979). During recombination, lymphoid-specific recombination-activating genes 1 and 2 (RAG1 & RAG2) form an endonuclease complex generating double-stranded DNA breaks between the gene segments and their adjacent RSSs (Schatz et al., 1989; Oettinger et al., 1990).

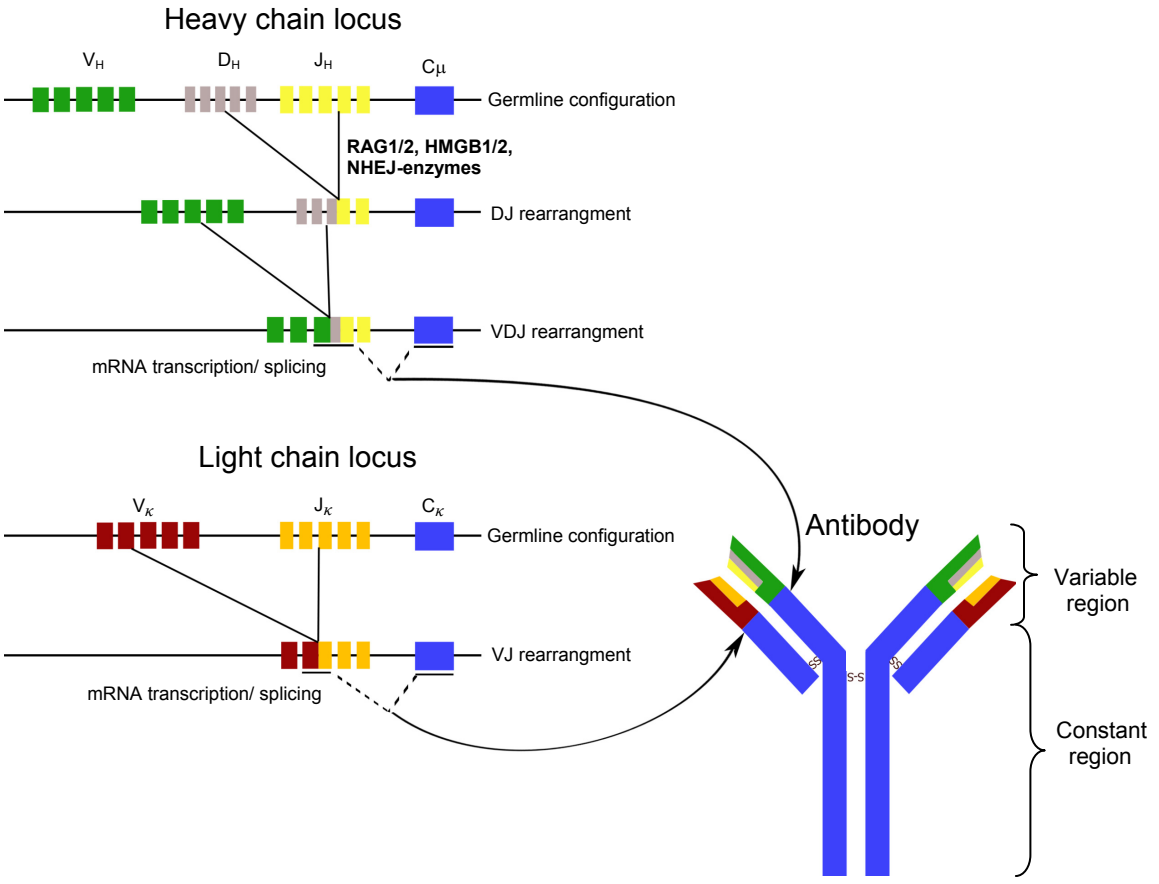


Figure 1: *Schematic representation of V(D)J recombination and an antibody molecule*

Rearrangements of V, D, and J segments are shown at the Ig gene loci. The membrane-bound form of an antibody is part of the B cell receptor. Abbreviations: C, constant; D, diversity; H, heavy; J, joining; κ, kappa; V, variable; SS, disulfide bridge. See text for details.

A RSS motif consists of a strongly conserved DNA heptamer sequence (CACAGTG) and a less well-conserved nonamer sequence (ACAAAAACC) that are separated by a poorly conserved spacer whose length is either about 1 or 2 helical turns (12 or 23 base pairs) of the DNA helix. Efficient V(D)J recombination occurs between a 12-bp spacer RSS and a 23-bp spacer signal sequence (Akamatsu et al., 1994). High mobility group B proteins 1 and 2 (HMGB1 & HMGB2) interact with RAG proteins to facilitate the binding and recombination process (Shirakata et al., 1991; Aidinis et al., 1999; Bergeron et al., 2005).

RAG1/2-generated double-strand breaks (DSBs) are processed and rejoined by the non-homologous DNA end-joining (NHEJ) pathway containing a number of proteins such as Ku70, Ku86, DNA-PKcs, Artemis, DNA polymerase μ (Pol μ), DNA polymerase λ (Pol λ), XLF (Cernunnos), XRCC4, and DNA ligase IV (Rooney et al., 2004; Lieber 2010). These ubiquitously expressed components of a DNA repair process utilize enzymes that capture and repair DSBs without sequence homology in a synaptic DNA-protein complex. During the rejoining process, non-template nucleotides (N-nucleotides) are occasionally added through random integration by the lymphoid-specific terminal deoxynucleotidyl-transferase (TdT) to increase the diversity of VDJ coding sequences of IgH (Desiderio et al., 1984).

V(D)J recombination at both the heavy chain and the light chain locus is subject to allelic exclusion, in which a functional rearrangement on one allele prevents further rearrangement at the other allele (Pernis et al., 1965; Alt et al., 1980; Alt et al., 1984; Mostoslavsky et al., 2004). In addition, functional rearrangements at the Ig light chain loci of *IGK* and *IGL* are mutually exclusive, a process referred to as isotypic exclusion.

Allelic and isotypic exclusion ensure that B cells express only an antigen receptor with a single binding specificity.

Lack of RAG function or fundamental defects in repairing RAG-mediated DNA breaks by the NHEJ-pathway will cause a severe combined immunodeficiency (SCID), a disease that is also known as the "bubble boy" syndrome (Mombaerts et al., 1992; Nowak 1993; Schwarz et al., 1996; Moshous et al., 2001; O'Driscoll et al., 2001; Buckley 2004; de Villartay 2009). Although the benefit of somatic recombination is necessary for an intact immune system, there are also risks creating genetic aberrations that could induce cancer. In order to minimize the potential risks, the complete V(D)J recombination process is strongly regulated and temporally ordered. B cells express RAG1/2 only at specific developmental stages, and the recombination is preferentially restricted to the G0/G1 phases of the cell cycle (Lin and Desiderio 1994). Furthermore, the accessibility of Ig loci is controlled in part by a combination of epigenetic modifications of histones, Ig locus contraction, and changes in intranuclear localization (Kwon et al., 1998; Kosak et al., 2002; Liu et al., 2007b; Matthews et al., 2007; Jhunjhunwala et al., 2008).

1.1.2 B cell development

Conventional B cells (B-2 cells) develop postnatally from self-renewing hematopoietic stem cells (HSCs) in the bone marrow (Kondo et al., 2003; Murre 2009). B cell lymphopoiesis is regulated by a network of factors that control cell fate and stepwise differentiation. Distinct stages of B cells are characterized by the presence or absence of specific cell surface molecules, intracellular proteins, and rearrangements of Ig genes (Figure 2) (Rolink et al., 1995; Hardy and Hayakawa 2001; van Zelm et al., 2005).

Studies in mice demonstrated that the B cell lineage starts after differentiation of a common lymphoid progenitor (CLP) (Kondo et al., 1997; Miller et al., 2002). The generation of CLPs depends on the activity of two key transcription factors IKAROS (*Ikzf1*) and PU.1 (*Sfpi1*) (Allman et al., 2003; Dakic et al., 2005; Iwasaki et al., 2005; Thompson et al., 2007). *Ikaros* encodes multiple transcription factors of *Krüppel*-type zinc finger proteins that can function both as an activator and as a repressor of gene expression (Molnar and Georgopoulos 1994; Ng et al., 2007). *Ikzf1*$^{-/-}$ mice lack B cells from the earliest detectable stage (Figure 2), in part because the progenitor cells do not express the important FMS-like tyrosine kinase 3 (FLT3) cytokine receptor (Georgopoulos et al., 1994; Wang et al., 1996; Yoshida et al., 2006).

PU.1 is an E-twenty six (Ets) family transcription factor that is involved in the regulation of the interleukin-7 receptor, an additional important cytokine receptor for B cell differentiation. PU.1-deficient hematopoietic progenitors fail to express the interleukin-7 receptor alpha gene (*IL-7Rα)* and are unable to differentiate into CLP cells (DeKoter et al., 2002). The myocyte enhancer factor 2c (*Mef2c*) was also identified as a transcriptional target of PU.1 (Stehling-Sun et al., 2009). Lack of MEF2c in mice is

associated with profound defects in the production of lymphoid cells. These results demonstrate that PU.1 controls the early B cell development in part by regulating IL-7Rα and MEF2c. During the CLP stage, PU.1 and interleukin-7 signaling induce the differentiation of pre-pro B cells through upregulation of the early B cell factor 1 (EBF1) and E2A (*Tcf3*) (Dias et al., 2005; Kikuchi et al., 2008). EBF1 and E2A, together with activation of the paired box protein 5 (*Pax5* as known as *Bsap*), regulate many genes required for B cell differentiation: *cd79a* (Ig-α), *cd79b* (Ig-β), *λ5*, *VpreB*, *Rag1/2,* etc. (Busslinger 2004; Lin et al., 2010). E2A proteins also bind to enhancer regions of the Ig-μ heavy-chain locus and activate germ-line transcription for VDJ recombination. In addition, the leukemia/lymphoma-related factor (*Lrf* also described as *Pokemon* or *Fbi-1*), B cell lymphoma gene 11a (*Bcl11a*), and forkhead box O1 (*Foxo1*) were also found to play a regulatory role in the B cell lineage commitment. Absence of one of these transcription factors results in an arrest in early B cell development at the CLP (*Ebf1*$^{-/-}$, *Tcf3*$^{-/-}$, *Bcl11a*$^{-/-}$), pre-pro-B (*Lrf*$^{-/-}$), and pro-B cell stages (*Bsap*$^{-/-}$, *Foxo1*$^{-/-}$) (Bain et al., 1994; Zhuang et al., 1994; Urbanek et al., 1994; Lin and Grosschedl 1995; Nutt et al., 1999; Maeda et al., 2007; Dengler et al., 2008; Amin and Schlissel 2008).

Besides initiating differentiation, transcriptional regulators such as PAX5 are also required for maintaining B cell commitment through activation of B cell lineage-associated molecules and repression of essential factors for other lineage pathways (Mikkola et al., 2002; Holmes et al., 2006; Cobaleda et al., 2007; Pridans et al., 2008).

V(D)J recombination of the *IgH* locus begins in the CLP population with D_HJ_H rearrangements and proceed with V_H to DJ_H rearrangements in the pro-B cell stage (Borghesi et al., 2004; Rumfelt et al., 2006). The early step of V(D)J rearrangement is

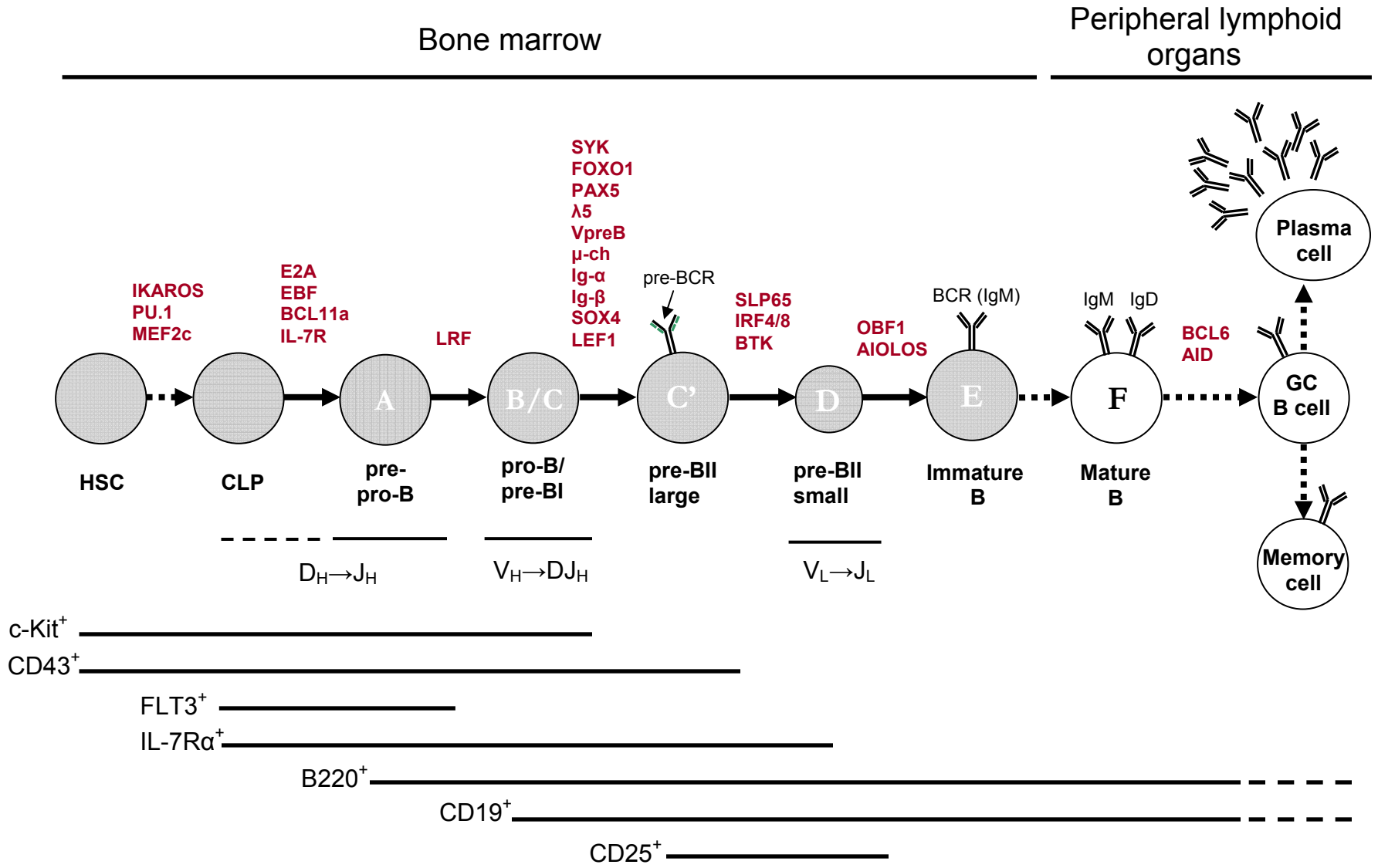


Figure 2: *B cell development*

Stages of B cells are shown according to Hardy fractions (capital letters) and the Fritz/Melchers-defined B cell classification (Osmond et al., 1998; Hardy and Hayakawa 2001). Shown are V(D)J rearrangements and selected surface markers (without relative expression levels) at specific B cell stages. Developmental blockages of selected knockouts are shown in red and the surrogate light chain in green. HSC, hematopoietic stem cell; BCR, B cell receptor (IgM); CLP, common lymphoid progenitor; D, diversity; GC, germinal center; H, heavy; J, joining; L, light; V, variable. See text for details.

initiated by relocation of the *IgH* locus from the nuclear periphery to more central positions within the nucleus (Kosak et al., 2002). This subnuclear repositioning facilitates chromatin opening and large-scale contraction of the *IgH* locus. During locus contraction of *IgH*, the DNA forms chromosomal loops that juxtapose distal V_H gene segments next to the rearranged proximal DJ_H cluster, thus promoting distal V_H-DJ_H rearrangements (Sayegh et al., 2005; Jhunjhunwala et al., 2008). Contraction of the *IgH* locus is in part regulated by STAT5, PAX5, IKAROS, the zinc finger transcription factor YY1 (yin yang 1), and histone methyltransferase EZH2 (enhancer of zeste homolog 2) (Su et al., 2003; Fuxa et al., 2004; Roldan et al., 2005; Liu et al., 2007a; Reynaud et al., 2008). Rearranging Ig gene segments are enriched with trimethylated histones (H3 at lysine-4; H3K4me3), increasing the stable association of the RAG complexes with their targets (Perkins et al., 2004; Liu et al., 2007b; Matthews et al., 2007).

Successful rearrangement of *IgH* leads to the formation of the pre-B cell receptor (pre-BCR) and characterizes the large pre-BII cell stage (Figure 2). The pre-BCR is a complex of the productively VDJ-rearranged Ig-μ heavy chain associated with a surrogate light chain (SLC; consisting of VpreB and λ5) and the subunits Ig-*α*/Ig-β. Large pre-BII cells require expression of the pre-BCR for their expansion and selection (Hess et al., 2001). The absence of a single component of the pre-BCR complex causes predominantly an arrest at the pro-B cell stage (Figure 2) (Hendriks and Middendorp 2004).

SLP65, a linker molecule, plays a critical role in pre-BCR-mediated differentiation (Fu et al., 1998; Gangi-Peterson et al., 1998; Goitsuka et al., 1998; Wienands et al., 1998). Expression of the pre-BCR initiates ligand-independent signaling,

which, in addition to phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) of Ig-*α,* leads to phosphorylation of tyrosine 204 in a non-ITAM, which is the main ligand for SLP65 (Cambier 1995; Engels et al., 2001; Kabak et al., 2002; Patterson et al., 2006). After binding to Ig-*α*, SLP65 is phosphorylated by the spleen tyrosine kinase (SYK) and enables the interaction of Bruton's tyrosine kinase (BTK) with phospholipase C (PLC)-γ2, generating subsequently a Ca^{2+} signal that regulates several processes including proliferation of B cells (Dolmetsch et al., 1997; Healy et al., 1997; Su et al., 1999; Ishiai et al., 1999; Kurosaki and Tsukada 2000). Absence of SLP65 or BTK was shown to result in an enhanced proliferation rate and a partial arrest of B cells at large pre-BII cell stage (Middendorp et al., 2002; Flemming et al., 2003).

Pre-BCR-mediated signaling induces downregulation of the IL-7 receptor; after several cell divisions, large pre-BII cells transit into small pre-BII cells and arrest in the G1 cell cycle phase (Rolink et al., 2000). Transition into small pre-BII cells is associated with reactivation of the recombination machinery for immunoglobulin light chain rearrangements. Some transcription factors such as E2A and PAX5, needed for IgH recombination, are again required for recombination at the Igκ locus, whereas other factors like STAT5 are inhibitory for Igκ rearrangements and need to be inactivated (Sato et al., 2004; Lazorchak et al., 2006; Malin et al., 2010). Interferon regulatory factor (IRF) family members IRF4 and IRF8 are uniquely required for Ig light chain recombination (Lu et al., 2003; Ma et al., 2006; Johnson et al., 2008). IRF4/8 orchestrate the transition from large to small pre-BII cells by inducing expression of the IKAROS family members IKAROS and AIOLOS, which in turn downregulate the pre-BCR by suppressing the expression of surrogate light chain (Thompson et al., 2007; Ma et al., 2008; Karnowski et

al., 2008). IRF4 also induces the chemokine receptor CXCR-4, which promotes migration of pre-B cells away from IL-7-producing bone marrow stroma (Johnson et al., 2008). Deficiency of IRF4/8 results in an accumulation of large pre-BII cells that fail to undergo light-chain recombination (Figure 2).

Once the Ig light chain is successfully rearranged, it will replace the SLC and, together with Ig μ-heavy chain, will form the fully functional B cell receptor (BCR; also referred to as IgM). This stage defines the immature B cell that is sensitive to antigen binding. The majority of newly developed immature B cells are self-reactive, and will recognize a self-antigen that is normally present in the host (Levine et al., 2000; Wardemann et al., 2003). To avoid auto-reactive immune responses, B cells undergo negative selection via several processes such as clonal deletion, receptor editing, clonal anergy, and immunological ignorance (Nossal 1983; Goodnow et al., 1988; Nemazee and Burki 1989; Hartley et al., 1991; Russell et al., 1991; Gay et al., 1993; Tiegs et al., 1993). Immature B cells migrate to the spleen and lymph nodes to differentiate further into mature naïve B cells. Mature B cells that have not bound a self-antigen concomitantly express membrane-bound IgD and IgM via differential mRNA splicing of a single VDJCμ- Cδ transcript.

The peripheral maturation of B cells involves an ordered series of events that is regulated by various factors, including some from the early B cell stages in the bone marrow (Loder et al., 1999; Schubart et al., 2001; Johnson and Calame 2003; Heyzer-Williams and Heyzer-Williams 2005; Matthias and Rolink 2005; Cattoretti et al., 2006).

1.1.3 B cell activation

B cells in peripheral lymphoid organs (spleen, lymph nodes, tonsils, Peyer's patches) are located in a compartment known as the lymphoid follicle (LF) that serves as an antigen filter (MacLennan 1994). Engagement of the BCR with an antigen initiates B cell activation. For the majority of protein antigens, activation of a B cell requires the interaction with its cognate T cell, a helper T cell that is primed by the same antigen. B cells that have bound antigen internalize and process it into peptides, in order to present them to T cells on major histocompatibility complex (MHC) class II molecules, where they are recognized by the T cell receptor (Allen et al., 2007; Goodnow et al., 2010). Following a T cell-dependent antibody response, activated B cells differentiate either along the extrafollicular pathway into short-lived plasma cells that produce low affinity antibodies, or form secondary follicles referred to as germinal centers (GCs) (Jacob et al., 1991; Liu et al., 1991). Stronger antigen binding preferentially induces an extrafollicular pathway, whereas responding clones with weaker antigen reactivity require an improvement and are directed to GC (Paus et al., 2006).

Once B cells enter the GC reaction, they become rapidly proliferating centroblasts and form the GC dark zone compartment. Here, centroblasts undergo somatic hypermutation (SHM) of their Ig variable-region genes to increase diversity and affinity of the BCR (Bross et al., 2000; Papavasiliou and Schatz 2000). Centroblasts differentiate into centrocytes and migrate to the GC light zone, where the modified BCR is selected with help from T follicular helper (T_{FH}) cells and follicular dendritic cells (FDCs) for improved binding to the immunizing antigen. Depending on the T cell interaction and cytokine exposure, centrocytes undergo class switch recombination (CSR), a process that

changes the Cμ constant region to another constant region gene (Cγ, Cα, or Cε) to alter Fc-dependent effector functions (Honjo et al., 1981; Heyzer-Williams and Heyzer-Williams 2005; Stavnezer et al., 2008). Selected B cells exit the GC as long-lived plasma cells or memory B cells, while the remaining B cells either undergo apoptosis or re-enter the cell cycle for further rounds of SHM.

During the early stages of GC differentiation, B cells upregulate various factors; among them, PAX5, IRF8, PU.1, the activation-induced cytidine deaminase (AID), and B cell lymphoma 6 (BCL6) (Cattoretti et al., 2006; Klein and Dalla-Favera 2008; Schmidlin et al., 2009). PAX5 is a suppressor of plasma cell differentiation and is downregulated along with PU.1 in plasma cells (Horcher et al., 2001; Nagy et al., 2002; Nera et al., 2006; Delogu et al., 2006). IRF8 induces AID and BCL6 to promote the development of GC B cells (Dent et al., 1997; Ye et al., 1997; Lee et al., 2006). Since BCL6 arrests plasma cell differentiation by repressing B-lymphocyte-induced maturation protein-1 (BLIMP-1, also called PRDM1), inactivation of BCL6 is required for further B cell differentiation (Reljic et al., 2000; Tunyaplin et al., 2004; Kuo et al., 2007).

AID promotes somatic hypermutation and class switch recombination of Ig genes in GC B cells (Muramatsu et al., 2000; Revy et al., 2000). Both processes are initiated by AID through deamination of Ig gene cytidines into uracils, thereby creating DNA mismatches that are ultimately processed into mutations or DNA breaks (Petersen-Mahrt et al., 2002; Chaudhuri et al., 2003; Dickerson et al., 2003). While AID primarily acts on Ig variable and switch regions, non-Ig genes including *BCL6, CD79A, CD79B, CD83,* and *PAX5* are occasionally targeted by hypermutation in GC B cells (Shen et al., 1998; Liu et al., 2008). AID off-target activity is implicated in malignant transformation of GC-

derived B cell lymphomas and plasmacytomas (Ramiro et al., 2006; Chesi et al., 2008; Pasqualucci et al., 2008; Robbiani et al., 2008; Takizawa et al., 2008). Additional studies suggest that AID together with RAG1/2 enzymes may also contribute to chromosomal translocations at early stages of B cell development (Tsai et al., 2008).

Under physiological circumstances, AID gene expression is induced in mature germinal center or activated B cells by crosslinking of the BCR, CD40, IL-4R, and Toll-like receptors (TLR) (Muramatsu et al., 1999; Crouch et al., 2007). Downstream activation of NF-κB, along with B cell-specific transcription factors PAX5 and E2A, are required for transcriptional activation of AID (Gonda et al., 2003; Sayegh et al., 2003; Dedeoglu et al., 2004; Gourzi et al., 2007)

1.1.4 Malignant transformation

During B cell development, genetic and epigenetic aberrations can result in the selection of B cells that have acquired mutations allowing uncontrolled proliferation and survival. These transformed B cells will eventually go on to cause B cell malignancies. One outcome of B cell transformation is leukemia, a clonal disease that originates from abnormal growth of a single cell. This uncontrolled proliferation leads to the progressive accumulation of malignant cells resulting in suppression of normal haematopoiesis and infiltration of a variety of extramedullary sites. Depending on the developmental stage in which the molecular malfunction manifests (Figure 3), leukemia can be divided into a chronic form that typically takes months to years to progress, and an acute form that is highly aggressive due to rapid expansion of leukemic cells. Without treatment, patients with acute leukemia die within weeks or a few months, mainly because of severe

infections or bleeding complications (Hersh et al., 1965). Malignant transformation of other B cell stages can cause, besides leukemia, lymphoma or plasmacytoma/myeloma (Figure 3) (Cobaleda and Sanchez-Garcia 2009).

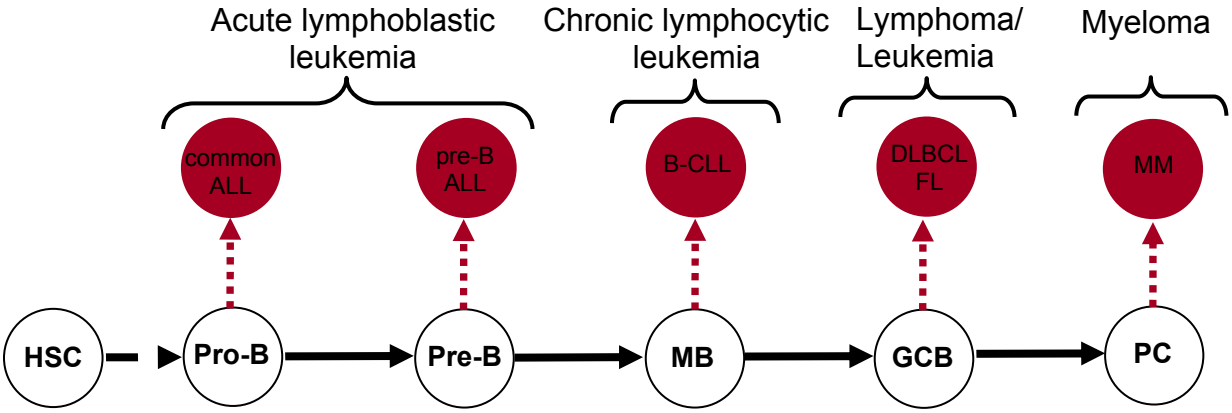


Figure 3: *Partial overview of B cell malignancies*

Selected types of transformed stage-specific cells are indicated during the B cell development. Abbreviations: ALL, acute lymphoblastic leukemia; B-CLL, B cell chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma; FL, follicular cell lymphoma; GCB, germinal center B cell; HSC, hematopoietic stem cell; MB, mature B cell; MM, multiple myeloma; Pre-B, precursor-B cell; Pro-B, progenitor-B cell; PC, plasma cell.

Leukemia is the most common cancer found in children, comprising about one-third of all childhood cancers in the industrialized countries with an annual incidence rate of 3-5 per 10^5 children (variations occur in ethnic and socioeconomic groups) (Stiller 2004). Acute lymphoblastic leukemia (ALL) represents the most frequent form of acute leukemia in children, accounting for about 80% of childhood leukemias and ~20% of acute leukemias in adults. B cell ALL is the most dominant form of lymphoblastic leukemia in both children and adults (Pui et al., 2004). B cell ALL cases are further

classified into pro-B ALL ($CD10^-$ and cytoplasmic Ig^-), common precursor B ALL ($CD10^+$ and cytoplasmic Ig^-), and pre-B ALL ($CD10^+$ and cytoplasmic Ig^+).

ALL originates from a variety of genetic and molecular mutations that contribute to malignant transformation by maintaining or enhancing an unlimited self-renewal capacity, suppressing the controls of normal proliferation, blocking differentiation, and promoting resistance to apoptosis (Pui et al., 2004; Armstrong and Look 2005). However, the initiation and cascade of genetic events, as well as their individual contributions, that finally results in ALL remain incompletely understood (Greaves 2006). Only a small percentage of leukemia cases (<5%) are linked with inherited, predisposing genomic instability syndromes such as Bloom-, Down-, and Nijmegen breakage syndrome, as well as ataxia-telangiectasia (Pui et al., 2008; Teitell and Pandolfi 2009). Environmental factors including ionizing radiation, infections, and exposure to specific chemotherapeutic agents are also associated with leukemogenesis (Eden 2010). Some cases of pediatric ALL implicate an *in utero*–initiating first mutation, followed by postnatal genetic events that act as secondary contributors (Greaves and Wiemels 2003; Hong et al., 2008).

Since ALL is a biologically heterogeneous disease, most studies of ALL cases take into account specific underlying genetic abnormalities. Genetic alterations include the aberrant expression of tumor suppressor genes and proto-oncogenes, as well as structural chromosome changes and aneuploidy. Gene-chip analyses demonstrate that specific chromosomal translocations define distinct biological and clinical subtypes of ALL (Armstrong et al., 2002; Yeoh et al., 2002; Mullighan et al., 2007; Harvey et al., 2010).

Chromosomal translocations result in either the transcriptional deregulation of the translocated gene by promoter substitution or the creation of a chimeric gene through fusion of two genes. Fusion genes often encode proteins that act as constitutively active tyrosine kinases or as novel transcription factors. While chromosomal translocations in the majority of B cell lymphomas lead to a transcriptional deregulation, translocations in B cell leukemias mostly result in the expression of chimeric proteins (Look 1997; Küppers 2005).

The most frequent translocation with a prevalence of around 25% in pediatric ALL involves chromosomes 12 and 21 (t(12;21)(p13;q22)) (Golub et al., 1995; Loh et al., 1998). The resulting chimeric protein TEL-AML1 contains the basic helix-loop-helix (bHLH) domain of TEL (*ETV6*) fused to the DNA-binding and transactivation domains of AML1 (*RUNX1*). Nowadays, TEL-AML1-positive ALL belongs to the low-risk leukemia category.

Among adult ALL patients, the most common chromosomal aberration results from a reciprocal translocation between the long arms of chromosomes 9 and 22 (t(9;22)(q34;q11)) creating the so-called Philadelphia (Ph) chromosome (Figure 4) (Nowell and Hungerford 1960; Rowley 1973). The Ph chromosome is present only in ~3% of pediatric ALL patients, but it increases in adolescents and reaches frequencies between 40 to 44% in adult ALL patients older than 45 years (Secker-Walker et al., 1991; Schlieben et al., 1996; Burmeister et al., 2008).

1.2 Philadelphia (Ph) chromosome

The Ph chromosome results from a chromosomal translocation, which fuses the breakpoint cluster region (*BCR*) gene on chromosome 22 to the *ABL1* gene (non-receptor tyrosine kinase) on chromosome 9, encoding the BCR-ABL1 fusion protein (Figure 4) (de Klein et al., 1982; Groffen et al., 1984; Heisterkamp et al., 1985). While the normal function of c-ABL1 has been extensively studied, the primary function of the ubiquitously expressed BCR protein is still unknown. BCR contains domains of an N-terminal serine-threonine kinase, SH2 binding, GTP/GDP exchange, and a C-terminal GTPase-activating domain (Maru and Witte 1991; Boguski and McCormick 1993). It was shown that BCR is a GTPase-activating protein for RAC1 and CDC42, and it interacts with Mint3 and PDZK1 to control vesicular trafficking in cells (Diekmann et al., 1991; Malmberg et al., 2004; Olabisi et al., 2006).

The ubiquitously expressed non-receptor tyrosine kinase c-ABL1 is the human homologue of the *v-abl* oncogene carried by the Abelson murine leukemia virus (A-MuLV). C-ABL1 is localized both in the nucleus and the cytoplasm, and is involved in a variety of cellular functions. Nuclear localization of c-ABL1 modulates the cellular response induced by DNA damage and leads to cell cycle arrest and apoptosis (Gong et al., 1999; Preyer et al., 2007). In contrast, cytoplasmic c-ABL1 is activated by various extracellular stimuli and promotes mitogenesis. Furthermore, cytoplasmic c-ABL1 is associated with morphogenesis, receptor trafficking and F-actin dynamics (Plattner et al., 1999; Woodring et al., 2002; Furstoss et al., 2002; Sini et al., 2004; Tanos and Pendergast 2006; Huang et al., 2008; Yogalingam and Pendergast 2008).

Under normal conditions, the enzymatic activity of c-ABL1 is regulated through several intra-molecular interactions that maintain the kinase domain in a closed inactive conformation (Barila and Superti-Furga 1998; Pluk et al., 2002; Hantschel et al., 2003; Nagar et al., 2003; Hantschel et al., 2005; Nagar et al., 2006). Fusion of ABL1 with BCR disrupts this autoinhibition and results in a form of ABL1 that has constitutive kinase activity and is predominantly localized in the cytoplasm.

BCR-ABL1 activates various signaling pathways such as PI3K, Hedgehog, RAS, NF-κB, and STAT5, which lead to oncogenesis. (Konopka et al., 1984; Puil et al., 1994; Cortez et al., 1996; Skorski et al., 1997; Reuther et al., 1998; Nieborowska-Skorska et al., 1999; Deininger et al., 2000; Ren 2005). Additionally, BCR-ABL1 is able to induce AID and endogenous reactive oxygen species (ROS) causing DNA damage and mutagenesis (Koptyra et al., 2006; Feldhahn et al., 2007). This genomic instability in combination with aberrant regulation of DNA repair pathways can generate mutations that confer drug resistance and progression of leukemia.

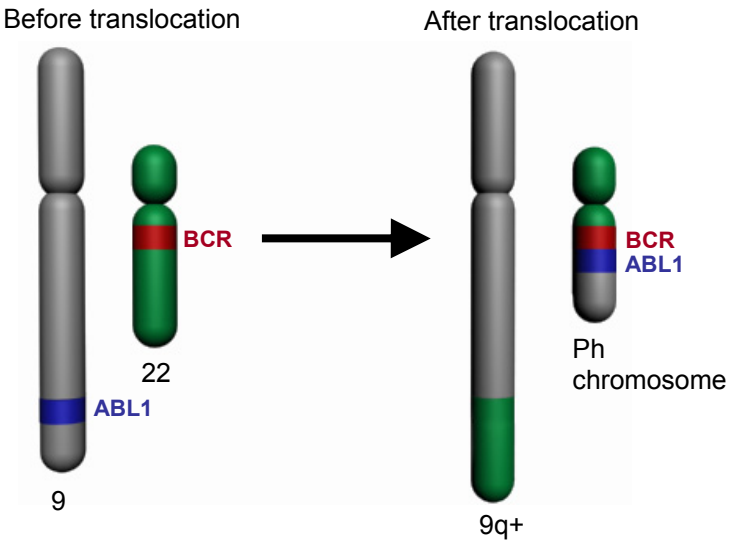


Figure 4: *The Philadelphia (Ph) chromosome*

Shown is the reciprocal translocation between chromosome 9 and 22, creating the Philadelphia chromosome (Ph). The Ph chromosome encodes the oncogenic BCR-ABL1 fusion protein.

1.2.1 BCR-ABL1-positive leukemia

BCR-ABL1 is found in almost every case of chronic myelogenous leukemia (CML), a chronic type of leukemia affecting myeloid lineage cells. CML develops from a hematopoietic stem cell and consequently displays multilineage differentiation potential (Calabretta and Perrotti 2004). If not efficiently treated, CML follows a triphasic clinical course with an initial indolent chronic phase (CML-CP; 5-15 years) followed by an intermediate accelerated phase and eventually a blast crisis of the myeloid (CML-MBC; ~60% incidence), B lymphoid (CML-LBC; 30% incidence) or biphenotypic myeloid/lymphoid (~10% incidence) lineage (Calabretta and Perrotti 2004). The molecular basis of chronic phase to blast crisis transformation is still largely unknown; however, the majority of patients in blast crisis acquire secondary mutations or deletions at *ARF, MYC, RB1, AML1, TP53,* and *RAS* genes, which are believed to accelerate disease progression (Melo and Barnes 2007).

Apart from CML, BCR-ABL1 is also present in a subset of B cell ALL with particularly poor prognosis. In the majority of adult ALL patients, BCR-ABL1 induces a common ALL phenotype (~78%) followed by a manifestation of pre-B (~20%) and pro-B (~2%) ALL (Burmeister et al., 2008).

Although both Ph^+ ALL and CML are caused by BCR-ABL1, these leukemias differ at the genetic and molecular level. Mouse models have shown that activated Src-family kinases such as LYN, HCK, and FGR are required for Ph^+ ALL pathogenesis but not for CML-CP (Hu et al., 2004). Presence of additional genetic abnormalities in Ph^+ ALL such as mutations and deletions involving *IKZF1*, *JAK*, or *CDKN2A* may contribute to the relatively poor prognosis of Ph^+ ALL (Mullighan et al., 2009a; Mullighan et al.,

2009b). Also involved are different forms of BCR-ABL1 that usually contain the same portion of c-ABL but different lengths of the BCR sequence. Three main variants of BCR-ABL1 were identified: p185/p190$^{BCR-ABL1}$ (minor-BCR), p210$^{BCR-ABL1}$ (major-BCR), and p230$^{BCR-ABL1}$ (micro-BCR) (Groffen et al., 1984; Fainstein et al., 1987; Saglio et al., 1990). The intrinsic tyrosine kinase activity decreases with the size of BCR-ABL1, and each form of BCR-ABL1 is associated with a distinct type of human leukemia (Melo 1996; Laurent et al., 2001; Lugo et al., 1990; Wong and Witte 2004). p190$^{BCR-ABL1}$, with the highest kinase activity among the three forms, is mainly found in Ph$^+$ ALL but rarely observed in CML. In contrast, p210$^{BCR-ABL1}$ is present in the majority of CML cases, and is additionally found in acute lymphoid and myeloid leukemias. The lowest intrinsic tyrosine kinase activity is observed in p230$^{BCR-ABL1}$, which is commonly linked to milder myeloproliferative disorders.

1.2.2 Drug treatment and resistance

In order to target the oncogenic kinase activity of BCR-ABL1, small molecule tyrosine kinase inhibitors (TKI) were rationally designed. The small-molecule inhibitor Imatinib mesylate (known as STI571 or Gleevec®) inhibits the kinase activity by blocking adenosine triphosphate (ATP) from entering into the nucleotide-binding pocket of BCR-ABL1 (Figure 4a) (Schindler et al., 2000). Inhibition of ABL1 kinase activity suppresses the proliferation of BCR-ABL1-driven leukemia cells and induces apoptosis (Druker et al, 1996; Kantarjian et al, 2002; Kurzrock et al, 2003). While CML-CP can be effectively treated with Imatinib for many years (5-year overall survival ~ 95%), CML-LBC and Ph$^+$

ALL are invariably multidrug-resistant and fatal within weeks or months (Druker et al., 2001; Druker et al., 2006).

Resistance to Imatinib is associated with mutations in the ABL1 kinase domain, overexpression of BCR-ABL1, increased drug-efflux activity, and activation of BCR-ABL1-independent survival pathways. In the majority of resistant or relapsed cases, BCR-ABL1 acquired predominantly mutations affecting T315I (threonine is substituted by isoleucine at amino acid position 315) and E255K (glutamic acid is substituted by lysine), which dramatically increases the IC_{50} value resulting in a virtual insensitivity to Imatinib (Shah et al., 2002) (Figure 5b). Second generation inhibitors of BCR-ABL1 (Dasatinib, Nilotinib, Bosutinib and Bafetinib) are more potent than Imatinib and capable of inhibiting most of the known BCR-ABL1 mutations, with the exception of the clinically relevant T315I mutation (Puttini et al., 2006; Talpaz et al., 2006; Redaelli et al., 2009; Kantarjian et al., 2010). The isoleucine substitution at residue 315 is called ‘‘gatekeeper’’ because it creates steric hindrance that precludes the binding of all currently approved TKIs and therefore prevents therapeutic inhibition. AP24534 (Ponatinib), a third-generation BCR-ABL1 inhibitor, is currently being tested to determine whether it has the potential to overcome the T315I mutation (O'Hare et al., 2009). It has been further shown that a combined strategy of TKI with an allosteric BCR-ABL1 inhibitor is able to suppress the gatekeeper mutation (Zhang et al., 2010b). Moreover, promising therapeutic strategies have been designed where TKIs are combined with inhibitors that target either BCR-ABL1 stabilizing factors or other molecules of the oncogenic signaling. These different inhibitors target: Aurora kinases, farnesyl transferases, HDAC, SMO, NFAT, MAPK, Rac GTP, PI3K, JAK, HSP90, and mTOR

(Gorre et al., 2002; Hoover et al., 2002; Carter et al., 2005; Nimmanapalli et al., 2003; Thomas et al., 2007; Kharas et al., 2008; Zhao et al., 2009; Gregory et al., 2010; Samanta et al., 2010; Zhang et al., 2010a).

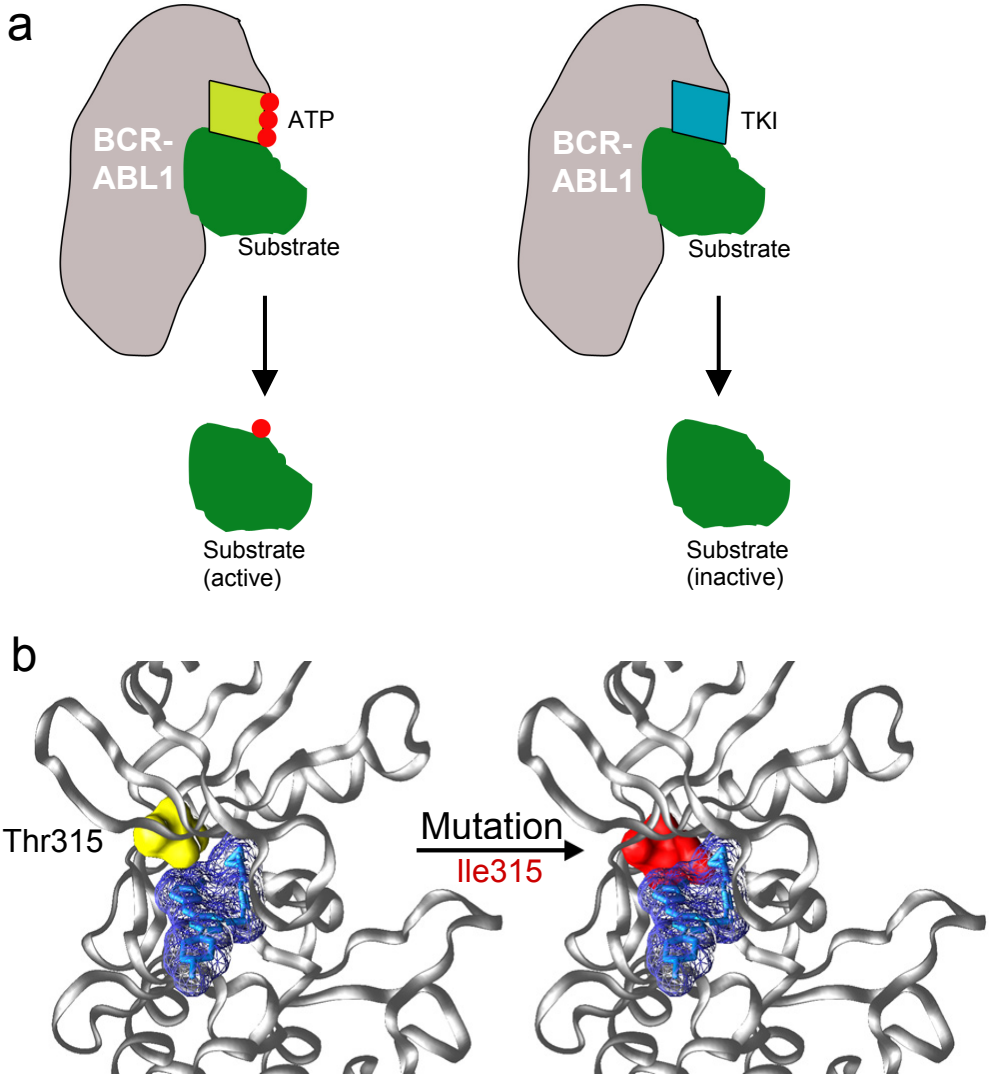


Figure 5: *TKI binding to BCR-ABL1*

a) ATP binding allows BCR-ABL1 to activate substrates by tyrosine phosphorylation (left layer). Binding of tyrosine kinase inhibitor (TKI) prevents the essential phosphorylation of substrates (right layer) and interrupts oncogenic signaling. **b)** Shown is the crystal structure of the ATP binding pocket of ABL1 (grey) and the surface projection of a BCR-ABL1 kinase inhibitor (blue). The kinase inhibitor fits into its binding pocket (left layer) with a Thr315-residue (yellow), whereas

the gatekeeper residue Ile315 (red, right layer) creates steric hindrance and prevents the binding of the conventional TKIs. (Used Protein Data Bank (PDB) accessions: 2F4J, 2V7A)

1.2.3 Quiescent leukemia cells

Despite significant advances in the treatment of leukemia over the past four decades, the rate of long-term survival has recently reached a plateau where large numbers of leukemia patients continue to die because of relapse and drug-resistance (Arico et al., 2000; Pui and Evans 2006; Mullighan et al., 2009a). TKIs are widely used to treat patients with leukemia driven by oncogenic tyrosine kinases. Although TKI treatment achieves complete cytogenetic remission (CCR) in the majority of CML-CP patients, detectable BCR-ABL1 transcripts in almost every patient indicate the persistence of minimal residual disease. This drug-resistance and relapse of leukemia is recently attributed to a subpopulation of quiescent leukemia cells, described as quiescent leukemia-initiating cells (LICs) or leukemia stem cells (LSCs).

Conventional treatment with TKIs target leukemia primarily through suppression of abnormally increased cell proliferation and induce cell death typically in rapidly dividing leukemia cells. In contrast, non-dividing or rarely dividing leukemia cells (quiescent leukemia cells) are resistant to TKI-induced cell death. LSCs also often express transporter proteins (e.g. ABCG2) for extracellular export of cytotoxic drugs (Abbott et al., 2002; Jordanides et al., 2006). In addition, cellular quiescence represents a defining feature of LSCs – as opposed to the bulk of proliferating leukemia cells with a progenitor/transient amplifying phenotype (Holyoake et al., 1999; Holyoake et al., 2001; Graham et al., 2002; Holtz et al., 2005; Michor et al., 2005). Since traditional cytotoxic drug-therapy approaches mainly target rapidly proliferating cells, LSCs would typically evade these therapies and subsequently give rise to relapse. Besides stem cell quiescence,

LSCs are distinguished from bulk leukemia cells by their longevity and extensive self-renewal potential and, hence, their ability to initiate leukemia even starting from very small cell numbers (Lapidot et al., 1994; Bonnet and Dick 1997). Since oncogenes driving leukemogenesis also induce senescence in bulk leukemia cells through an Arf/p53-dependent pathway, self-renewal capacity and longevity of LSCs require a mechanism to negatively regulate oncogene-induced senescence (Schmitt et al., 2002; Wang et al., 2008). For instance, the presence or absence of Arf determines the susceptibility of B cell ALL to oncogene-induced senescence (Wang et al., 2008).

Stem cell populations in myeloid leukemias (acute myeloid leukemia, AML; chronic myeloid leukemia, CML) have been classified by surface markers resembling normal hematopoietic stem cells (e.g. Lin^- $CD34^+$ $CD38^-$ $CD48^-$ $CD150^+$) (Lapidot et al., 1994; Bonnet and Dick 1997; Jamieson et al., 2004; Krivtsov et al., 2006). The definition of stem cells in AML and CML is mainly based on a developmental hierarchy with a rare stem cell population at its apex (Bonnet and Dick 1997; Hope et al., 2004; Jamieson et al., 2004; Perez-Caro et al., 2009).

While it was previously suggested that B cell ALL develops hierarchically from a phenotypically distinct leukemia stem cell, a number of recent studies provide evidence against a hierarchical model for B cell ALL (Cox et al., 2004; Castor et al., 2005; Kelly et al., 2007; Hong et al., 2008; Kong et al., 2008; le Viseur et al., 2008; Heidenreich and Vormoor 2009; Morisot et al., 2010). In a Myc-transgenic mouse model, Kelly et al. (2007) demonstrated that Myc-transgenic leukemia cells were able to initiate the disease in congenic transplant recipients regardless of their phenotype at very low numbers. The authors argued that differential LIC potential observed between phenotypically distinct

leukemia subpopulations in a NOD/SCID xenotransplant model would rather reflect variations in the ability of leukemia cells to cope with a xenoenvironment rather than differences in self-renewal capacity (Kelly et al., 2007). Other studies observed that the pool of leukemia stem cells or initiating cells is much larger in B cell ALL than in myeloid leukemias (Hong et al., 2008; Kong et al., 2008; le Viseur et al., 2008; Heidenreich and Vormoor 2009; Morisot et al., 2010).

Central signaling pathways of LSCs have been identified that are required for maintenance in AML and CML. Aberrant activation of HOXA9/MEIS1/MEF2c factors were identified as a requirement for LSC maintenance in AML and aberrant activation of the WNT/β-catenin signaling pathway for LSC self-renewal in CML cells (Jamieson et al., 2004; Krivtsov et al., 2006; Somervaille et al., 2009; Zhao et al., 2009). These and other "Achilles heel" screens for leukemia stem cell pathways have subsequently led to the development of drugs for specific eradication of malignant stem cells both in AML and CML (Guzman et al., 2007; Zhao et al., 2009).

In contrast to myeloid leukemias, the distinction between LSC and bulk leukemia at the committed progenitor or transient amplifying cell level is blurred in B cell ALL: in contrast to lineage antigen-negative leukemia stem cells in AML and CML, leukemia stem/initiating cells in ALL typically express the B lineage antigen CD19 (Hong et al., 2008; Kong et al., 2008; le Viseur et al., 2008; Heidenreich and Vormoor 2009; Morisot et al., 2010).

Since quiescent leukemia cells represent a source of disease maintenance, targeting and elimination of these cells is required for the achievement of long-term remission in a greater proportion of patients.

1.3 BCL6

BCL6 (B cell lymphoma/leukemia gene 6) was first discovered on the basis of its frequent translocations involving the chromosomal region 3q27 in B cell non-Hodgkin's lymphoma (B-NHL) (Baron et al., 1993; Deweindt et al., 1993; Kerckaert et al., 1993; Ye et al., 1993). Chromosomal *BCL6* rearrangements are observed in approximately one-third of diffuse large B cell lymphoma (DLBCL) cases and up to 14% of follicular lymphoma (FL) cases (Ye et al., 1993; Bastard et al., 1994; Lo et al., 1994; Miki et al., 1994; Ohno et al., 1994; Otsuki et al., 1995; Akasaka et al., 2003).

BCL6 translocations usually juxtapose the coding region of *BCL6* with the promoter of a different active gene in B cells, such as an immunoglobulin gene or several non-*IG* genes (e.g., *PIM1, RHOH, IL21R, IKAROS, HSP90β,* etc.) (Ye et al., 1995; Akasaka et al., 2000; Ohno 2006). This promoter substitution leads to the deregulated expression of *BCL6* rather than altering the protein function. However, small deletions and somatic point mutations are also present in the *BCL6* coding region (Pasqualucci et al., 2001; Liu et al., 2008). It is thought that mutations in the 5' region of *BCL6* contribute to the progression of FL to DLBCL (Lossos and Levy 2003; Jardin et al., 2005). Frequent mutations induced by AID are found in *BCL6* translocations, indicating the possibility that somatic hypermutation destabilizes the *BCL6* gene structure and makes the promoter region particularly susceptible to translocation events (Pasqualucci et al., 2001; Liu et al., 2008).

1.3.1 Structure and regulation

BCL6 encodes a transcription factor of the POK (Poxviruses zinc finger and *Krüppel*) family that is comprised of an N-terminal POZ/BTB (Broad Complex, Tramtrack, Bric à brac) domain and several *Krüppel*-type zinc fingers at the C-terminus (Figure 6) (Rosenberg et al., 1986; Zollman et al., 1994; Bardwell and Treisman 1994; Chang et al., 1996). The structure of BCL6 is similar to several developmental regulators of Drosophila, and it is highly conserved among vertebrate species (Allman et al., 1996). Amino acid sequences between murine and human BCL6 are 94% identical in overall protein composition, containing the highest degree of identity at the POZ (98%) and zinc finger domain (100%) (Allman et al., 1996).

BCL6 was identified as a transcriptional repressor of gene expression by in vitro studies of BCL6 binding to DNA promoter sequences in reporter assays (Kawamata et al., 1994; Chang et al., 1996; Seyfert et al., 1996; Baron et al., 1997). The mechanism of BCL6-mediated gene repression requires the physical interaction of co-repressor proteins such as B-CoR (BCL6-interacting corepressor), N-CoR (nuclear receptor corepressor) or SMRT (silencing mediator of retinoid acid and thyroid hormone receptor)/mSIN3A (mammalian SIN3A) with BCL6 at target gene loci (Dhordain et al., 1997; Huynh and Bardwell 1998; Dhordain et al., 1998; Huynh et al., 2000). Homo-dimerization of the BTB/POZ domain of BCL6 forms a lateral groove that is bound by SMRT, BCoR, and NCoR in a mutually exclusive manner using a 17-aa-long binding sequence (Ahmad et al., 2003; Ghetu et al., 2008). The BCL6 co-repressor complex can recruit histone deacetylase (HDAC) proteins, indicating at least that BCL6 suppresses gene expression

by modulating the chromatin structure (Dhordain et al., 1998; Burke and Baniahmad 2000; Miles et al., 2005; Karagianni and Wong 2007).

The central region of BCL6 contains a second repression domain (RDII) and PEST motifs, which regulate the degradation and activity of BCL6, respectively. PEST sequences contain MAPK phosphorylation sites that are involved in the degradation of BCL6 by the ubiquitin-proteasome pathway (Moriyama et al., 1997; Niu et al., 1998). Additionally, within the PEST-rich region, degradation can be induced via acetylation of a KKYK motif, which disrupts the ability of BCL6 to recruit HDACs and blocks BCL6-mediated gene repression (Bereshchenko et al., 2002). In contrast, the RDII domain facilitates the transcriptional repression potential of BCL6 by recruiting the NuRD (nucleosome remodeling and deacetylase) complex through direct binding to the MTA3 (metastasis associated 3) subunit (Fujita et al., 2004).

Figure 6: *Structure of BCL6*

The diagram shows the full-length BCL6 protein (out of different splicing forms of the human BCL6 gene). Domains of BCL6 mediate protein-protein interactions, transcriptional repression, post-translational regulation and specific DNA-binding. Abbreviations: DBD, DNA binding domain; ZF, zinc finger; PEST, proline(P)- glutamic acid(E)- serine(S)- threonine(T) motif; RDII, second repression domain. BCL6 synonyms: BCL5, LAZ3, ZBTB27, and ZNF51. See text for details.

The DNA binding domain of BCL6 not only recognizes and binds with its *Krüppel*-type zinc fingers to specific DNA sequences but also interacts with other factors such as JUN and ETO (eight-twenty-one) (Vasanwala et al., 2002; Chevallier et al., 2004). ETO binds

to the fourth zinc finger of BCL6, which forms a complex on the promoter, and enhances BCL6-mediated gene repression in an HDAC-dependent manner.

The DNA binding consensus sequence for the BCL6 zinc fingers was initially identified using random oligonucleotide selection. High affinity sites were found to contain a core sequence of 9 and 14 nucleotides: TTCCT(A/C)GAA and (T/A)NCTTTC NAGG(A/G)AT (Kawamata et al., 1994; Chang et al., 1996; Seyfert et al., 1996). Recently, however, studies using chromatin immunoprecipitation and computational approaches have identified more degenerate recognition sequences of BCL6 (Ci et al., 2009; Basso et al., 2010). While BCL6 can directly bind to promoter sequences to mediate target gene repression, recruitment to some other genes (e.g. *BCL2* and *CDKN1A*) involves indirect binding via an interaction with the transcription factor MIZ-1 (Phan et al., 2005; Saito et al., 2009). In addition, BCL6 can also regulate gene expression via PU.1-mediated recruitment to promoter regions containing PU.1 DNA binding sites (Wei et al., 2009).

An integrative approach that combines ChIP-on-chip data with gene expression profiles showed that BCL6 recruitment to gene control regions does not always lead to gene repression, as only a fraction of the bound genes are repressed (Basso et al., 2010). Furthermore, a mass spectrometry analysis of BCL6 interacting proteins from a lymphoma cell line identified over 60 proteins bound to BCL6, suggesting a broader functional spectrum for BCL6 besides transcriptional repression (Miles et al., 2005). Therefore, BCL6-mediated gene regulation is complex and controlled by multiple mechanisms.

1.3.2 Biological relevance

BCL6 transcript levels are high in resting B and T cells but are downregulated after mitogenic activation *in vitro,* whereas activated B cells in GC maintain high levels of BCL6 (Allman et al., 1996). Through somatic hypermutation and DNA double-strand break (DSB) events resulting from class-switch recombination in GCs, combined with replication errors owing to a high proliferation rate, GC B cells are exposed to a high level of cellular stress and DNA damage (Schlissel et al., 2006; Liu et al., 2008). In GC cells, BCL6 allows these physiological processes to occur by repressing the expression of DNA damage response and checkpoint genes (CHEK1, ARF, p21, p27, p53) as well as the DNA damage sensor ATR, thus enabling GC B cell proliferation and survival even in the presence of genotoxic stress (Shaffer et al., 2000; Shvarts et al., 2002; Phan and Dalla-Favera 2004; Phan et al., 2005; Ranuncolo et al., 2007). In the absence of BCL6, GC formation is abrogated and, as a consequence, high-affinity antibodies are lacking in these mice (Dent et al., 1997; Ye et al., 1997). This is partly attributed to the effector function of BCL6 in T follicular helper cells (T_{FH}), which are required for GC formation, and to the negative regulatory effect of BCL6 on DNA damage response genes in GC B cells (Phan and Dalla-Favera 2004; Ranuncolo et al., 2007; Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009).

BCL6 in GC B cells is regulated by several mechanisms involving BCR-, TLR-, DNA damage-, cytokine-, and CD40-mediated signaling. Antigenic stimulation of BCR activates MAPK-dependent phosphorylation of BCL6 and triggers the proteolytic degradation of BCL6 (Niu et al., 1998). Both BCR and CD40 receptor signaling were reported to activate NF-κB and, subsequently, *IRF4* expression, which in turn

downregulates *BCL6* at the transcriptional level (Saito et al., 2007; Ranuncolo et al., 2007; Teng et al., 2007). On the other hand, it was shown that BCL6 can also directly repress the expression of NF-κB, thereby inhibiting the NF-κB-mediated regulation (Li et al., 2005; Perez-Rosado et al., 2008). Another regulatory mechanism suppressing BCL6 involves the inactivation of BCL6-inducing factors such as FOXO proteins (Tang et al., 2002; Fernandez de Mattos et al., 2004). Cytokine signaling (e.g. IL-2 and IL-5) can also suppress *BCL6* by activation of STAT5 binding to promoter sites of *BCL6* (Walker et al., 2007). Additionally, BCL6 is downregulated by microRNA (miR)-127 and probably by miR-155, which is induced by BCR and TLR signaling (Saito et al., 2006; Dorsett et al., 2008; Teng et al., 2008). Besides several other factors that suppress BCL6 gene expression, BCL6 is also negatively autoregulated (Kikuchi et al., 2000; Wang et al., 2002). Although BCL6 represses DNA damage response genes and allows GC B cells to tolerate some DNA damage, accumulated genotoxic stress can subsequently lead to ubiquitin-dependent degradation of BCL6 (Ranuncolo et al., 2007; Phan et al., 2007). Thus, various signaling pathways lead to downregulation of BCL6 and ensure that damaged B cells undergo cell death.

Besides B cells, BCL6 is also found expressed in a variety of different cell types including erythrocytes, keratinocytes, macrophages, olfactory sensory neurons, pancreatic beta-cells, spermatocytes, myocytes, and T cells (Yoshida et al., 1996; Yoshida et al., 1999; Toney et al., 2000; Kojima et al., 2001; Ichii et al., 2002; Asari et al., 2005; Yu et al., 2005; Yoshida et al., 2006a; Ichii et al., 2007; Glauser and Schlegel 2009; Otaki et al., 2010).

1.3.3 Aberrant expression

Since unchecked DNA damage can result in malignant transformation, GC B cells must tightly regulate the expression and activity of BCL6 during the GC reaction, in which genotoxic stress is tolerated. Deregulation of BCL6 is primarily reported in the pathogenesis of B cell lymphoma, but it is also associated with a number of other cancer types such as bladder, breast, colon, gastric and skin cancer (Kanazawa et al., 1997; Bos et al., 2003; Logarajah et al., 2003; Saito et al., 2006; Barros et al., 2009; Pinto et al., 2009; Tran et al., 2010). However, BCL6 appears to play an ambivalent role in developing malignancies (Albagli-Curiel 2003). Moreover, the function of BCL6 depends on a combination of the presence of interacting factors and the absence of its inhibitors.

Constitutive expression of *BCL6* is required for survival of lymphoma cell lines and induces formation of DLBCL in transgenic mice (Baron et al., 2004; Phan and Dalla-Favera 2004; Polo et al., 2004; Cattoretti et al., 2005; Cerchietti et al., 2010). On the other hand, some studies suggest that *BCL6* translocations correlate with a more favorable clinical prognosis or at least with a similar clinical outcome to lymphoma patients carrying germline *BCL6* sequences (Bastard et al., 1994; Offit et al., 1995; Pescarmona et al., 1997; Takeshita et al., 2000; Braaten et al., 2003; Seegmiller et al., 2010).

Although BCL6 can directly repress cell cycle inhibitors, *in vitro* experiments have shown that BCL6 induces growth arrest and senescence through a p53-dependent pathway when BCL6 is ectopically expressed (Ranuncolo et al., 2008). Also, BCL6 suppresses known oncogenes such as *MYC* and *CCND2* and induces apoptosis by

downregulation of the anti-apoptotic factors *BCL-2* and *BCL-X_L* (Albagli et al., 1999; Yamochi et al., 1999; Shaffer et al., 2000; Tang et al., 2002). Therefore, BCL6 can also act as a tumor suppressor under certain circumstances, demonstrating its variable role in carcinogenesis.

1.4 Aims

The first part of this work will focus on survival factors during early B cell development. V(D)J recombination-induced double-strand breaks (DSBs) activate a multi-functional expression program including pro-apoptotic pathways that can promote cell death unless survival pathways are concomitantly activated. It is currently unclear as to which mechanisms protect pre-B cells from extensive DNA damage stress caused by DSB events during V(D)J recombination.

The aims of this part are:

- Identification of factors that protect pre-B cells from DNA damage-induced apoptosis.
- Analysis of the consequences of loss of the identified protective factor in a loss-of-function model.

The second part of this work will focus on drug-resistance mechanisms against TKI in BCR-ABL1-driven leukemia. Although treatment of leukemia has significantly improved, many patients still die because of drug-resistant relapse. The mechanisms responsible for TKI resistance in BCR-ABL1-driven leukemia remain largely unresolved.

The aims of this part are:

- Identification of factors that are involved in TKI-resistance in BCR-ABL1-driven leukemia cells;
- Elucidating the mechanisms and target genes contributing to drug-resistance and self-renewal signaling;

- Validation of pharmacological inhibition of the identified factor as a therapy strategy.

2 Materials and Methods

2.1 Patient samples, cell lines and human bone marrow cells

Normal human pro-B cells ($CD19^+$ $CD34^+$ $VpreB^-$), pre-B cells ($CD19^+$ $CD34^-$ $VpreB^+$) and naive B cells ($CD19^+$ $CD27^-$ IgD^+) were sorted from bone marrow (from healthy donors; Cambrex, Verviers, Belgium) by flow cytometry using antibodies from BD Biosciences (San Jose, CA) and a FACSVantage SE cell sorter (BD Biosciences). Human germinal center B cells ($CD77^{high}$ $CD38^+$ IgD^-) were isolated from tonsillar resectates (TKH) and sorted by FACS.

Patient samples (Table S1 & S2) were provided from the Departments of Hematology and Oncology, University Hospital Benjamin Franklin, Berlin, Germany (WKH) and the USC Norris Comprehensive Cancer Center in compliance with Institutional Review Board regulations (approval from the Ethik-Kommission of the Charité, Campus Benjamin Franklin and the IRB of the University of Southern California Health Sciences Campus). Primary leukemia cells, isolated from established NOD/SCID xenografts, were cultured on OP9 stroma cells in Alpha Minimum Essential medium (Alpha-MEM, Invitrogen, Carlsbad, CA) without ribonucleotides and deoxyribonucleotides, supplemented with 20% fetal bovine serum, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 100 IU/ml penicillin and 100 µg/ml streptomycin.

The human ALL cell lines (BV173, NALM-1, SUP-B15, TOM1), CML cell lines (EM2, JK1, JURL-MK1, K562, KCL22, KYO, LAMA84, MEG1) and B cell lymphoma cell lines (Karpas-422, MN60, DB) were obtained from DMSZ, Braunschweig, Germany. Human cell lines were maintained in Roswell Park Memorial Institute medium (RPMI-

1640, Invitrogen) with GlutaMAX containing 20% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cell cultures were kept at 37°C in a humidified incubator under a 5% CO_2 atmosphere.

2.2 Mouse models

Mouse model	Provided by	Literature
AID$^{-/-}$	Dr. Nussenzweig's lab	(Muramatsu et al., 2000)
AID-GFP	Dr. Casellas's lab	(Crouch et al., 2007)
BCL6$^{-/-}$	Dr. Dalla-Favera's lab	(Ye et al., 1997)
CDKN1A$^{-/-}$ *(p21)*	The Jackson laboratory	(Brugarolas et al., 1995)
CDKN1B$^{-/-}$ *(p27)*	The Jackson laboratory	(Fero et al., 1996)
CDKN2A$^{-/-}$ *(Arf)*	NCI Frederick	(Serrano et al., 1996)
MYC$^{fl/fl}$ *x mx-Cre*	Dr. Moreno de Alboráno's lab	(Baena et al., 2005)
RAG1$^{-/-}$	The Jackson laboratory	(Mombaerts et al., 1992)
RAG2$^{-/-}$ *tTA/µ-chain*	Dr. Jäck's lab	(Hess et al., 2001)
p53$^{-/-}$	The Jackson laboratory	(Jacks et al., 1994)
PTEN$^{fl/fl}$	Dr. Wu's lab	(Lesche et al., 2002)
SLP65$^{-/-}$	Dr. Jumaa's lab	(Jumaa et al., 1999)
STAT5$^{fl/fl}$	Dr. Henninghausen's lab	(Cui et al., 2004)

2.3 Extraction and culturing of bone marrow cells from mice

To avoid inflammation-related effects in BCL6$^{-/-}$ mice (Dent et al., 1997), bone marrow cells were extracted from young age-matched BCL6$^{+/+}$ and BCL6$^{-/-}$ mice (<6 weeks of age) without signs of inflammation. Bone marrow cells were obtained by flushing cavities of femur and tibia with PBS. After filtration through a 70-µm filter and depletion of erythrocytes using a lysis buffer (BD PharmLyse, BD Biosciences, San Jose, CA), washed cells were either frozen for storage or subjected to further experiments.

Bone marrow cells from knockout and respective wildtype mice were retrovirally transformed by BCR-ABL1 (Pear et al., 1998) in the presence of 10 ng IL-7/ml in RetroNectin- (Takara, Madison, WI) coated petri dishes as described below.

Deletion of Myc from bone marrow B cell precursors of *Myc*$^{fl/fl}$ x *mx-Cre* and *Myc*$^{fl/fl}$ mice (Baena et al., 2005) was induced by IFNβ-mediated (2,500 U/ml; PBL Interferon Source, NJ) induction of mx-Cre activation.

Rag2$^{-/-}$ tTA/μ-chain-transgenic mice are unable to express an endogenous μ-chain because of lack of Rag2-dependent V(D)J recombination, but carry a functionally pre-rearranged μ-chain under control of tetracycline operator (tetO) sequences in the germline (Hess et al., 2001). In addition, *Rag2*$^{-/-}$ tTA/μ-chain-transgenic mice express a tetracycline-controlled transactivator (tTA) under control of endogenous μ-chain regulatory elements.

All pre-B cells derived from bone marrow of mice were maintained in Iscove's modified Dulbecco's medium (IMDM, Invitrogen, Carlsbad, CA) with GlutaMAX containing 20% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol and 10 ng/ml recombinant mouse IL7 (Peprotech, Rocky Hill, NJ) at 37°C in a humidified incubator with 5% CO_2.

2.4 In vivo model for BCR-ABL1-transformed ALL and bioluminescence imaging

After cytokine-independent proliferation, BCR–ABL1-transformed ALL cells were labelled with a lentiviral vector encoding firefly luciferase with a neomycin selection marker. After selection with 0.5–2 mg ml^{-1} G418 for 10 days, luciferase-labelled ALL cells were injected into sublethally irradiated (250 cGy) NOD/SCID mice. Human primary leukaemia cells were transduced with a lentiviral firefly luciferase carrying a

GFP marker. After expansion of sorted GFP^+ cells, 1×10^5 cells were injected through the tail vein into sublethally irradiated NOD/SCID mice. Bioimaging of leukaemia progression in mice was performed at different time points using an *in vivo* IVIS 100 bioluminescence/optical imaging system (Xenogen). D-Luciferin (Promega) dissolved in PBS was injected intraperitoneally at a dose of 2.5 mg per mouse 15 min before measuring the luminescence signal. General anaesthesia was induced with 5% isoflurane and continued during the procedure with 2% isoflurane introduced through a nose cone. All mouse experiments were subject to institutional approval by the Children's Hospital Los Angeles Institutional Animal Care and Use Committee.

2.5 Retroviral transduction

Transfection of retroviral constructs encoding BCR-ABL1-IRES-GFP, BCR-ABL1-IRES-Neo, BCL6-IRES-GFP, MYC-IRES-GFP, SLP65-IRES-GFP, STAT5-CA, AID-IRES-GFP, PAX5-IRES-GFP, CD44S Puro, FOXO4 Puro, BCL6-ER^{T2}-GFP, DN-BCL6-ER^{T2}-GFP, ER^{T2}-GFP, Cre-ER^{T2} Puro, Cre-IRES-GFP, Puro-, Neo- and GFP-empty vector controls were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with Opti-MEM media (Invitrogen) (Onishi et al., 1998; Pear et al., 1998; Shaffer et al., 2000; Godar et al., 2008; Kumar et al., 2009). Retroviral supernatant was produced by co-transfecting HEK 293FT cells with the plasmids pHIT60 (gag-pol), pHIT123 (ecotropic env), pHIT456 (amphotropic env) or VSV-G (pseudotyped env), respectively (Emi et al., 1991; Soneoka et al., 1995). Cultivation of 293FT cells was performed in high glucose Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with GlutaMAX containing 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 25 mmol/l HEPES, 1 mmol/l sodium pyruvate and 0.1 mmol/l non-

essential amino acids. Regular media was replaced after 16 hours by growth media containing 10 mmol/l sodium butyrate. After 8 hours of incubation, the media was changed back to regular growth media. 24 hours later, the virus supernatant was harvested, filtered through a 0.45 μm filter and loaded twice by centrifugation (2,000 x g, 90 min at 32°C) on 50 μg/ml RetroNectin (Takara, Madison, WI) coated non-tissue six-well plates. 2-3 x 10^6 cells per well were transduced in the six-well plate by centrifugation at 500 x g for 30 minutes and maintained overnight at 37°C with 5% CO_2 before transferring into culture flasks. Transduced cells with oestrogen receptor fusion proteins were induced with 4-hydroxytamoxifen (500 nM).

2.6 BCR-ABL1 Tyrosine Kinase Inhibitors (TKI)

Imatinib (STI571) and Nilotinib (AMN107) were obtained from Novartis Pharmaceuticals (Basel, Switzerland) and LC Laboratories (Woburn, MA). Stock solutions of Imatinib were prepared in sterile distilled water at 10 mmol/l and stored at -20ºC. Nilotinib were either dissolved in DMSO (Dimethyl sulfoxide) or NMP (N-Methyl-2-pyrrolidone) just prior to administration. Nilotinib dissolved in DMSO were vortexed with 4 volumes of peanut butter till a homogeneous mixture was formed. Nilotinib (free base) solubilized in NMP was diluted with PEG 300 (Polyethylene glycol 300) in a 10/90 (vol/vol) ratio. Cohorts of mice were treated with oral administration of vehicle or Nilotinib (25 mg/kg or 50 mg/kg) once daily at indicated time points.

2.7 Flow cytometry

Antibodies against mouse CD19 (1D3), B220 (RA3-6B2), c-kit (2B8), CD25 (7D4), CD3 (17A2), CD43 (S7), CD45.1 (A20), CD45.2 (104), CD43 (S7), CD44 (IM7), IgM or μ-

chain (R6-60.2 or II/41 (for intracellular staining)), IgD (11-26) and κ light chains (187.1) as well as respective isotype controls were purchased from BD Biosciences, San Jose, California. Anti-mouse Sca-1 antibody (clone 177228) was obtained from R&D Systems (Minneapolis, MN) and anti-mouse IL-7Rα (A7R34) from eBioscience (San Diego, CA). Anti-mouse λ light chain (JC-5-1) antibody was purchased from Southern Biotechnology (Birmingham, AL). Antibodies against human CD19, CD13, CD34, CD38, CD44, IgD, VpreB, CD79B (Ig-β), IL-7Ra, and μ-chain and respective isotype controls were purchased from BD Biosciences. Annexin V, propidium iodide, and 7-AAD were used for apoptosis and cell viability analyses.

2.8 In vitro pre-B cell differentiation assays

Polyclonal pre-B cells were either propagated in the presence of 10 ng/ml IL-7 (Peprotech, Rocky Hill, NJ) or transformed using an MSCV retrovirus encoding *BCR-ABL1*-IRES-GFP or *BCR-ABL1*-IRES-Neo. Induction of differentiation was either induced by withdrawal of IL-7 or inhibition of BCR-ABL1 kinase activity using 2 μmol/l STI571. After three days of IL-7 withdrawal or STI571-treatment, successful induction of differentiation was verified by flow cytometry analysis of κ light chain surface expression. Alternatively, 10-20 μg/ml of neutralizing anti-mouse IL-7 antibody (R&D, Minneapolis, MN) was used to induce differentiation of IL-7-dependend pre-B cells.

2.9 V(D)J recombination reporter assay

For construction of the recombination reporter plasmid, a PCR fragment including both 5′ and 3′ recombination signal sequences (RSS) amplified from the vector pJH288 was ligated into the retroviral expression vector pMOWS containing a puromycin resistance

gene for selection (Wossning et al., 2006). GFP cDNA was ligated in between the RSS in reverse orientation. *BCL6*$^{-/-}$ and wildtype BCR-ABL1^{+} pre-B cells were transduced with the RSS-GFP reporter retrovirus. Three days after transduction, transduced cells were selected in the presence of 1 μg/ml puromycin for five days. At this time, no viable cells were detected in a non-transduced parallel culture.

2.10 Clonality analysis and spectratyping of B cell populations

V_H-DJ_H gene rearrangements from B cell populations were amplified using PCR primers specific for the J558 V_H region gene together with a primer specific for the Cμ constant region gene. Using a FAM-conjugated Cμ constant region or J_H gene-specific primer in a run-off reaction, PCR products were labeled and subsequently analyzed on an ABI3100 capillary sequencer by fragment length analysis. Sequences of primers used are given in Table S4.

2.11 Retro-Inverso BCL6 Peptide Inhibitor (RI-BPI)

Homo-dimerization of the N-terminal BTB (Broad Complex, Tramtrack, Bric à brac) domain of BCL6 forms a lateral groove motif, which is required to recruit co-repressor proteins such as BCoR (BCL6-corepressor), N-CoR (nuclear receptor corepressor), and SMRT (silencing mediator of retinoid and thyroid receptors). BCoR, NCoR and SMRT interact in a mutually exclusive manner with an 18-aminoacid long motif in the lateral groove of the BCL6 BTB domain to form a BCL6 repression complex (Ahmad et al., 2003; Ghetu et al., 2008). A recombinant peptide containing the SMRT BBD (BCL6 binding domain) along with a cell-penetrating TAT domain was able to inhibit the transcriptional repressor activity of BCL6 (Polo et al., 2004). Based on this initial work,

the peptidomimetic molecule RI-BPI (retro-inverso BCL6 peptide inhibitor) with superior potency and stability was developed (Cerchietti et al., 2009b) and used for BCL6-inhibition. RI-BPI represents a retro-inverso TAT-BBD-Fu (fusogenic) peptide (Cerchietti et al., 2009b) that was synthesized by Biosynthesis, Inc (Lewisville, TX) and stored lyophilized at -20°C until reconstituted with sterile, distilled, degassed water immediately before use. The purity determined by HPLC-MS was 95% or higher. RI-BPI was injected intraperitoneally into mice.

2.12 Quantitative RT-PCR

Total RNA from cells was extracted using RNeasy isolation kit from Qiagen (Valencia, CA). cDNA was generated using a poly(dT) oligonucleotide and the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed with the SYBRGreenER mix (Invitrogen) and the ABI7900HT real-time PCR system (Applied Biosystems, Foster City, CA) according to standard PCR conditions. Primers for quantitative RT-PCR are listed in Table S4.

2.13 Western blotting

Cells were lysed in CelLytic buffer (Sigma, St. Louis, MO) supplemented with 1% protease inhibitor cocktail (Pierce, Rockford, IL). Protein samples were separated on NuPAGE (Invitrogen, Carlsbad, CA) 4-12% Bis-Tris gradient gels and transferred on PVDF membranes (Immobilion, Millipore, Temecula, CA). For the detection of mouse and human proteins by Western blot, primary antibodies were used together with the WesternBreeze immunodetection system (Invitrogen). The following antibodies were used: Human BCL6 (Clones D8 and N3, Santa Cruz Biotechnology, Santa Cruz, CA),

mouse BCL6 (rabbit polyclonal, Cell Signaling Technology, Beverly, MA), ARF (4C6/4, Cell Signaling Technology), p53 (1C12, Cell Signaling Technology), PTEN (A2B1, Santa Cruz), global STAT5 (3H7, Cell Signaling Technology), AID (L7E7; Cell Signaling Technology), c-Myc (N-262 and 9E10, Santa Cruz), phospho-Y694 STAT5 (14H2, Cell Signaling technology), and β-Actin (C4, Santa Cruz).

2.14 Affymetrix GeneChip analysis

Total RNA from cells used for microarray was isolated by RNeasy (Qiagen, Valencia, CA) purification. RNA quality was first assessed using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the 28S:18S rRNA ratios of all samples. cDNA was generated from 5 μg of total RNA using a poly(dT) oligonucleotide that contains a T7 RNA polymerase initiation site and the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Biotinylated cRNA was generated and fragmented according to the Affymetrix protocol and hybridized to U133A 2.0 human or 430 mouse microarrays (Affymetrix, High Wycombe, UK). After scanning (GeneChip Scanner 3000 7G; Affymetrix) of the GeneChip arrays, the generated CEL files were imported to BRB Array Tool (http://linus.nci.nih.gov/BRB-ArrayTools.html) and processed using the RMA algorithm (Robust Multi-array Average) for normalization and summarization. Relative signal intensities of probesets were determined by comparing the signal intensity from TKI-treated and untreated cells to the average signal value of the respective cell line or a group of cell lines. The calculated signal ratios of probesets were visualized as a heatmap with Java Treeview.

2.15 Chromatin immunoprecipitation, ChIP-on-chip

OCI-Ly1 cells were fixed, washed and chromatin was sheared to an average length of 600 bp. BCL6-DNA complexes were immunoprecipitated using an anti-BCL6 polyclonal antibody (N3, Santa Cruz, CA). Enrichment of known target genes was validated by quantitative real time PCR. ChIP products and their respective input genomic fragments were amplified by ligation-mediated PCR as previously reported (Ranuncolo et al., 2007). QChIP was performed again at this stage for selected positive control loci to verify that the enrichment ratios were retained. The genomic products of two biological ChIP replicates were labeled with Cy5 (for ChIP products) and Cy3 (for input) and cohybridized on custom-designed genomic tiling arrays generated by NimbleGen Systems Inc. These high-density tiling arrays contain 50-residue oligonucleotides with an average overlap of 25 bases, omitting repetitive elements. Included in the arrays were the genomic region of the *CDKN2A* locus (Chr9:21950629- 22003329) according to the human genome May 2004 assembly. After hybridization, the relative enrichment for each probe was calculated as the signal ratio of ChIP to input. Peaks of enrichment for BCL6 relative to input were captured with a five-probe sliding window, and the results were uploaded as custom tracks into the University of California Santa Cruz genome browser and graphically represented as histograms. The cutoff threshold is defined as 2.5 times the standard deviation above the average relative enrichment on the entire array. Peaks involving five or more oligonucleotide probes above this threshold were considered positive hits.

2.16 Quantitative chromatin immunoprecipitation (QChIP)

3 x 10^7 cells were used for chromatin immunoprecipitation. Cells were double cross-

linked with 1.5 mmol/l EGS (Pierce, Rockford, IL) for 30 minutes, followed by 1% Formaldehyde (Sigma, St. Louis, MO) for 10 minutes at room temperature. They were lysed (1% Triton-x-100, 150 mmol/l NaCl, 20 mmol/l Tris pH 8. 0 and 1 mmol/l EDTA) and sonicated using Sonicator (BRANSON Digital Sonifier 450) for 40 minutes. Cells were immunoprecipitated with rabbit polyclonal antibody to BCL6 (N-3, Santa Cruz Biotechnologies, Santa Cruz, CA) and rabbit polyclonal antibody to β-actin (H-196, Santa Cruz Biotechnologies) as a control. Eluted DNA was quantified by PCR (Opticon Engine 2; MJ Research) with the Qiagen SYBR Green Kit (Qiagen, Carlsbad, CA). Primers to confirm binding of BCL6 to the Arf (*CDKN2A*) promoter region are given in Table S4. Serial dilutions of input DNA were performed in order to assess the relative amount of product that was enriched after IP. Percentage of input is represented on the graph.

2.17 Target validation of RI-BPI in human Ph$^+$ ALL cells

Ph$^+$ ALL cell lines (BV-173, NALM-1 and TOM-1) were treated with vehicle (control), 10 μmol/l Imatinib or Imatinib + 20 μmol/l RI-BPI for 24 h and maintained in Allprotect (Qiagen, Valencia, CA) at -80 °C until RNA isolation using RNeasy Plus kit (Qiagen). RNA integrity was determined using the RNA 6000 Nano LabChip Kit on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Two independent samples were analyzed for each condition. RNA (1 μg) was hybridized to Agilent 60-mer Whole Human Genome Microarrays (part number G4112A) according to the manufacturer's recommendations. After hybridization, the processed microarrays were scanned with the Agilent DNA microarray scanner (part number G2505C) and extracted with Agilent Feature Extraction software v8.5 (GE1-v5_10_Apr08). For computational analysis of

signal we used the dye-normalized signal after surrogate algorithm (gProcessedSignal) extracted from the .txt files and process for each array and for all the probes. This value was subjected to $\log_2$ transformation and median array normalization. The fold change of Imatinib vs. Control and (Imatinib + RI-BPI) vs. Imatinib were calculated for each cell line and for each gene. A dataset containing previously identified BCL6 target genes (obtained from NimbleGen arrays) was mapped into the Agilent probesets using the Agilent and NimbleGen array annotation files. To determine if two datasets differ significantly we compared the fold change in BCL6 target genes versus the fold change in BCL6 non-target genes for each dataset (Imatinib vs. Control and (Imatinib+RI-BPI) vs. Imatinib) by means of the Kolmogorov-Smirnov test (Chakravarti and Roy 1967). The Kolmogorov-Smirnov test (KS-test) determines if two data sets (gene expression values for BCL6 target genes and Non-target genes) differ significantly. Heat maps and other analysis were obtained using the R statistical software (http://www.r-project.org).

2.18 ChIP-on-chip analysis

Chromatin immunoprecipitations were performed with modifications as described (Ci et al., 2009). Briefly, 2.5 x 10^7 Ph^+ ALL cells (BV-173, NALM-1 and TOM-1) were treated with/without 10 μmol/l Imatinib for 24 h. Then the cells were double cross-linked with 2 mmol/l EGS cross linker and 1% formaldehyde. After sonication, immunoprecipitations were performed using 5 μg BCL6 (N3; Santa Cruz Biotechnology, Santa Cruz, CA) or control IgG antibody (Sigma-Aldrich, St. Louis, MO) from the chromatin fragments of 2.5 x 10^7 Ph^+ ALL cells. After validation of enrichment by Q-ChIP, BCL6 or control IgG ChIP products and their respective input genomic fragments were amplified by ligation-mediated PCR (LM-PCR). The products were co-hybridized with the respective input

samples to NimbleGen promoter arrays (human genome v. 35, May 2004) (NimbleGen Systems, Madison, WI). QChIP was performed again at this stage for selected positive control loci to verify that the enrichment ratios were retained. The genomic products of two biological ChIP replicates were labeled with Cy5 (for ChIP products) and Cy3 (for input) and cohybridized on custom-designed genomic tiling arrays generated by NimbleGen Systems Inc. These high-density tiling arrays contain 50-residue oligonucleotides with an average overlap of 25 bases, omitting repetitive elements. After hybridization, the relative enrichment for each probe was calculated as the signal ratio of ChIP to input. Peaks of enrichment for BCL6 relative to input were captured with a five-probe sliding window, and the results were uploaded as custom tracks into the University of California Santa Cruz genome browser and graphically represented as histograms. Two replicates were performed with each condition.

2.19 Data analysis of ChIP-on-chip experiments

To identify target genes of BCL6 in these experiments, we compute the log-ratio between the probe intensities of the ChIP product and input and take moving averages of log-ratio of 3 neighboring probes and determine maximum value for each gene promoter and also the random permutation probes as background control (Polo et al., 2007). The cut-off for each array is established as higher than 99th percentile of the 24,175 log-ratio values generated from random permutation probes. A locus with maximum moving average above cut-offs in two replicates is considered a potential binding site. Since this high stringent-overlapping approach can produce a high false negative rate, we also computed the correlations among peaks between the replicates as a way to rescue promoters that did not pass cut-off in one replicate. We calculated the Pearson correlation coefficient of the

probes signal of the promoter between replicates, and promoters with a correlation higher that 0.8 were rescued and included in our final set of BCL6 targets. In addition, all peaks were mapped back to the genome using BLAT (The BLAST-like Alignment Tool, http://genome.ucsc.edu) to identify genes on opposite strands that could be regulated from the same bidirectional promoter. Two genes were considered to be bidirectional partners when they were located on the opposite strands in a "head-to-head" orientation and their transcription start sites were separated by less than 0.5kb.

2.20 Comparative Genomic Hybridization (CGH)

After 4 month of cell culturing BCR-ABL1-transformed $BCL6^{+/+}$ and $BCL6^{-/-}$ ALL cells, genomic DNA was extracted using the PureLink genomic DNA kit (Invitrogen). Three samples of each ALL type were co-hybridized with genomic DNA extracted from normal untransformed mouse cells to NimbleGen mouse 720k Whole-Genome Tiling arrays (NimbleGen Systems, Madison, WI) in accordance with the manufacturer's recommendations. Copy number variations were analyzed using the FASST-segmentation algorithm in Nexus software (BioDiscovery, El Segundo, CA). Copy-number analysis was performed using a significance threshold of $1x10^{-7}$ and a $\log_2$ ratio cut-off at ±0.2 for regions sized 1000 kbp.

2.21 Cell viability assay

5×10^4 BCR-ABL1 transformed ALL cells per well were seeded in a volume of 100 µl B cell medium on Optilux 96-well plate (BD Biosciences, San Jose, CA). Imatinib was diluted in medium and added at the indicated concentration in a total culture volume of 150 µl. After 3 days culturing, 15 µl of Resazurin (R&D, Minneapolis, MN) was added

on each well and incubated for 4 hours at 37 °C. The fluorescent signal was monitored using 535 nm excitation wavelength and 590 nm emission wavelength. Fold changes were calculated using baseline values of untreated cells as a reference (set to 100 percent).

2.22 Colony forming assay

The methylcellulose colony-forming assays were performed with 10,000 BCR-ABL1-transformed mouse $BCL6^{-/-}$ or $BCL6^{+/+}$ cells or 10,000 human Ph^{+} ALL cells. Cells were resuspended in murine MethoCult medium (StemCell Technologies, Vancouver, BC, Canada) and cultured on dishes (3 cm diameter) with an extra water supply dish to prevent evaporation. After 7 to 14 days, colony numbers were counted.

2.23 Cell cycle analysis

For cell cycle analysis in BCR-ABL1 ALL cells, the BrdU flow cytometry kit for cell cycle analysis (BD Biosciences, San Jose, CA) was used according to manufacturer's instructions. BrdU incorporation (APC-labeled anti-BrdU antibodies) was measured together with DNA content (7-amino-actinomycin-D) in fixed and permeabilized cells. The analysis was gated on viable cells that were identified based on scatter morphology (Trageser et al., 2009).

2.24 Senescence-associated β-galactosidase assay

Senescence-associated β-galactosidase activity was performed on cytospin preparations as described (Braig et al., 2005). Briefly, a fixative solution (0.25% glutaraldehyde, 2% paraformaldehyde in PBS, pH 5.5 for mouse cells and pH 6.0 for human cells) was freshly generated. To this end, 1 g paraformaldehyde is dissolved in 50 ml PBS at pH5.5

by heating followed by addition of 250 µl of a 50% stock glutaraldehyde solution. A 1 x X-gal staining solution was prepared as follows (10 ml): 9.3 ml PBS/$MgCl_2$, 0.5 ml 20 x KC solution (i.e. 820 mg $K_3Fe(CN)_6$ and 1,050 mg $K_4Fe(CN)_6$ x $3H_2O$ in 25 ml PBS) and 0.25 ml 40 x X-gal (i.e. 40 mg 5-bromo-4-chloro-3-indolyl β-D-galactoside per ml of N,N-dimethylformamide) solution were mixed. For *BCR-ABL1*-transformed ALL cells, 100,000 cells/cytospin were used (700 rpm, 8 minutes). The fixative solution was pipetted on the cytospins and incubate for 10 minutes at room temperature, then washed twice for 5 minutes in PBS/$MgCl_2$. Cytospin preparations were submerged in 1 x X-gal solution, incubated over night at 37°C in a humidified chamber, washed twice in PBS. Slides were mounted before they dried.

2.25 MicroRNA-155 expression in mouse and human CML-LBC

Mouse $CD43^-$ naive B cells were activated ex vivo in the presence of LPS/IL-4 for 72 h. Human germinal center B cells ($CD77^{high}$ $CD38^+$ IgD^-) were isolated from tonsil samples by cell sorting. Total RNA was isolated from 10^6 cells, and microRNA was processed for Illumina's deep sequencing using the manufacturer's protocol (Illumina, San Diego, CA). Mature microRNA sequences (tags) were quantified on the basis of 1 million tags aligned to the mouse and human microRNome as defined by miRBase (http://microrna.sanger.ac.uk/sequences/search.shtml).

2.26 Mutation analysis and ligation-mediated PCR

For mutation analysis of *ARF*, *BCL6*, and *MYC* genes, genomic fragments were amplified and sequenced using *Pyrococcus furiosus* DNA polymerase (New England Biolabs, Ipswich, MA). For each PCR product, both DNA strands were sequenced, and mutations

were counted only if they were found both in the forward and reverse sequence. PCR primers used for amplification of *ARF*, *BCL6*, and *MYC* fragments are listed in Table S4. The ABL1 kinase portion of *BCR-ABL1* transcripts was amplified in two rounds of PCR: To prevent coamplification of normal *ABL1* transcripts, the first round of PCR used *BCR*-specific (exon 13) and *ABL1*-specific (exon 9) primers in 10 cycles of amplification. The second round of amplification (35 cycles) focused on the ABL1 kinase domain (exons 4–6). The rate of *Pfu* DNA polymerase errors was calculated at 4×10^{-5}/ PCR cycle using genomic DNA from MACS-sorted $CD3^+$ human T cells (immunomagnetic beads from Miltenyi Biotech).

2.27 Single-cell RT-PCR analysis

AID/GFP- or GFP-transduced CML cells were FACS-sorted into 0.5 µl reaction tubes containing 5 µl of 2 x ribonuclease inhibitor mix (2 µl RNasin [40 u/µl] plus 18 µl, 0.15 mol/l NaCl, 10 mmol/l Tris-HCl [pH 8.0], and 5 mM DTT). The cells were then quickly frozen on dry ice and stored at -80°C. After one freezing and thawing step, which leads to cell lysis, 10 µl of cell lysates was added to 50 µl RT-PCR using the Access RT-PCR System (Promega, Madison, WI). RT-PCR primers for *BCR-ABL1*, *ABL1*, or *COX6B* (Table S4) were used (50 pmoles each per reaction). To prevent coamplification of normal ABL1 transcripts, the first round of PCR used *BCR*-specific (exon 13) and *ABL1*-specific (exon 9) primers for specific amplification of *BCR-ABL1* fusion transcripts. The second round of amplification focused on the (BCR-) ABL1 kinase domain (exons 4–6). The rate of *Taq* DNA polymerase errors was calculated at 2×10^{-4}/ PCR cycle, and PCR products were directly sequenced.

2.28 Single nucleotide polymorphism mapping assay and comparative genomic hybridization

Genomic DNA was extracted from AID^{high} and AID^{low} Ph^{+} ALL samples and was subjected to 250K NspI SNP array analysis. For comparative genomic hybridization, bone marrow cells from $AID^{-/-}$ and $AID^{+/+}$ mice were transduced with *BCR-ABL1*. After 9 weeks in cell culture, genomic DNA was isolated, and amplifications and deletions were detected using the Mouse Genome CGH Microarray Kit 244A (Agilent Technologies, Inc., Santa Clara).

3 Results

3.1 BCL6 is critical for the development of a diverse primary B cell repertoire

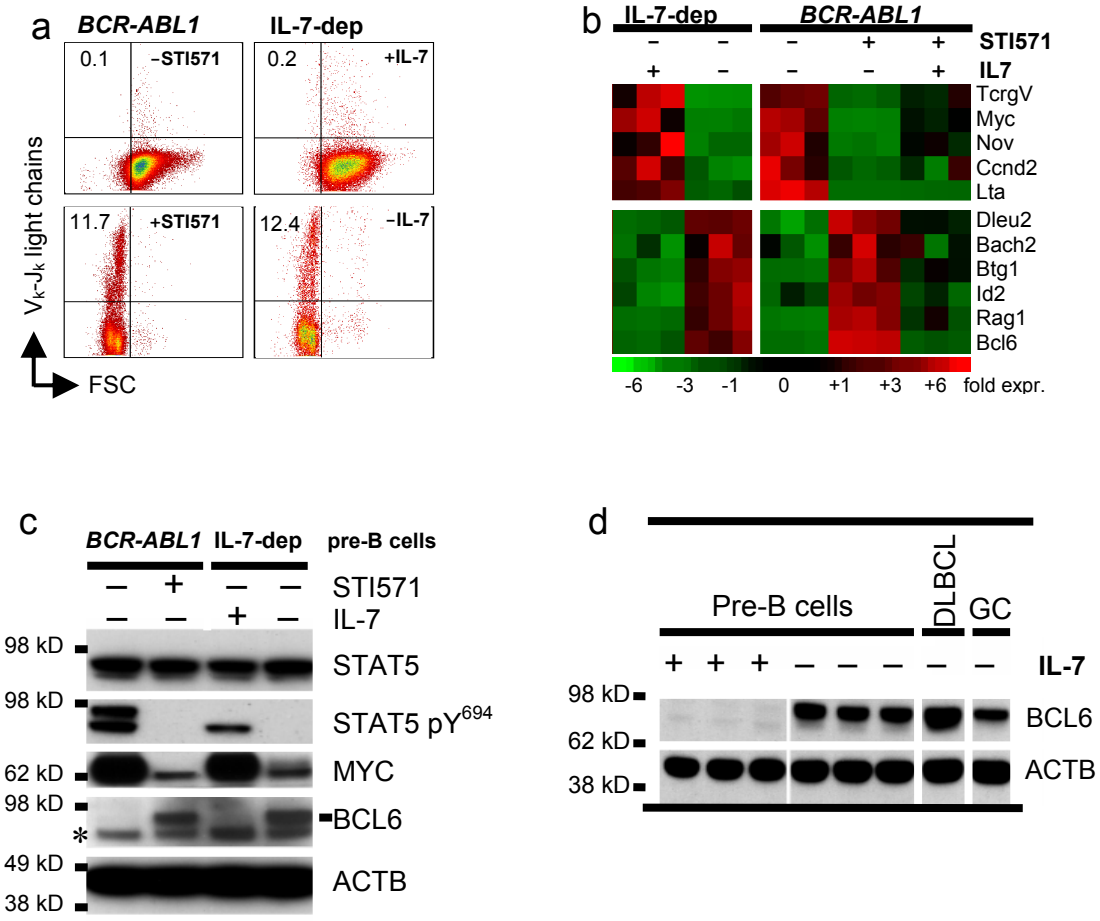
BCL6 is upregulated during Ig light chain gene rearrangement

To initiate Vκ-Jκ and Vλ–Jλ gene rearrangements in pre-B cells and to study factors that protect pre-B cells against DSB-mediated DNA damage stress during these recombination events, we used two cell-culture systems that were previously described by us and others (Rolink et al., 1993; Muljo and Schlissel 2003; Klein et al., 2005). Although a recent study demonstrated that IL-7-STAT5 signaling prevents premature Ig light chain rearrangement in pro-B cells (Malin et al., 2010), withdrawal of IL-7 from pre-B cell cultures strongly induces differentiation and Ig light chain gene rearrangement (Rolink et al., 1993). More recently, pharmacological inhibition of v-ABL1 and BCR-ABL1 in transformed pre-B cells was demonstrated to have the same effect (Muljo and Schlissel 2003; Klein et al., 2005). In this study, we confirmed that withdrawal of IL-7 from IL-7-dependent mouse pre-B cells and inhibition of BCR-ABL1 kinase activity in BCR-ABL1-transformed pre-B cells induced the transition from large cycling pre-B cells to small resting pre-B cells with subsequent expression of κ light chains on the cell surface (Figure 7a). Inhibition of ABL1 kinase activity in transformed pre-B cells recapitulates the pre-B to immature B cell transition including sequential initiation of DSB events at the Vκ, KDE and Vλ gene segments (Muljo and Schlissel 2003; Klein et al., 2005). These experiments were performed using IL-7–dependent and BCR-ABL1–

transformed pre–B cells from SLP65-deficient mice. In the presence of IL-7 or upon transformation by BCR-ABL1, SLP65-deficient B cell precursors give rise to large cycling pre-BII cells, whereas IL-7–dependent and BCR-ABL1–transformed SLP65 wildtype cells are arrested at the pro-B/pre-BI cell stage of development. To search for factors that are up-regulated in response to induced Ig light chain gene rearrangement in pre–B cells as potential protective factors against DSB-induced DNA damage stress, we performed Affymetrix GeneChip analyses of IL-7–dependent and BCR-ABL1–transformed pre–B cells before and after inhibition of IL-7 and BCR-ABL1 kinase signaling (Figure 7b). IL-7 withdrawal and BCR-ABL1 kinase inhibition by STI571 (10 μmol/l STI571 for 16 hours) resulted in very similar gene expression changes (Figure 7b). Some of these gene expression changes can be attributed to dephosphorylation of STAT5-Y694 (Walker et al., 2007), at which both the IL-7 and (BCR-) ABL1 signaling pathways converge (Banerjee and Rothman, 1998; Figure 7c). When genes were sorted based on fold increase after induced differentiation, *BCL6* ranked first in the analysis (Figure 7b). Of note, the *MYC* proto-oncogene was among the genes on the opposite extreme of this analysis. Silencing of *MYC* and *de novo* expression of *BCL6* upon inhibition of IL-7- or BCR-ABL1-signaling was confirmed at the protein level by Western blot analysis and correlated with STAT5 dephosphorylation at Y694 (Figure 7c). BCL6 is expressed at very high levels in GC B cells and serves a critical role in GC B cell survival (Dent et al., 1997; Ye et al., 1997; Phan and Dalla-Favera 2004). Likewise, BCL6 functions as a proto-oncogene in DLBCL cells, where it is often expressed at very high levels owing to the *BCL6-IGH* translocation (t(3;14)(q27;q32); Ye et al., 1993). For these reasons, we studied BCL6 protein levels in pre-B cells upon IL-7 withdrawal as

compared with GC B cells DLBCLs by Western blotting (Figure 7d). Of note, withdrawal of IL-7 resulted in dramatic upregulation of BCL6 protein expression, which reached levels comparable to both DLBCLs and GC B cells.

Figure 7: *Regulation of BCL6 during inducible pre-B cell differentiation*



(**a**) IL-7–dependent and BCR-ABL1–transformed pre–B cells were induced to differentiate by withdrawal of 10 ng/ml IL-7 and ABL1 kinase inhibition (2 µmol/liter STI571), respectively. Cell size (FSC) and κ light chain surface expression were monitored by flow cytometry (*n* = 5). Numbers indicate percentages. (**b**) To identify genes that are differentially regulated during induced pre–B cell differentiation, we studied pre–B cells stimulated to differentiate in a microarray analysis. Genes were sorted based on the ratio of gene expression values observed upon withdrawal of IL-7 from IL-7–dependent pre–B cells. (**c**) Likewise, protein lysates from pre–B cells in the presence or absence of induced differentiation (treatment with 10 µmol/liter STI571 or withdrawal

of IL-7 for 24 h) were analyzed by Western blotting using antibodies against STAT5, phosphorylated STAT5 at Y694, BCL6 (clone N3), MYC, and an ACTB antibody as loading control (*n* = 6). The asterisk denotes a nonspecific band that is consistently observed with the N3 BCL6 antibody. Of note, BCR-ABL1 kinase signaling results in stronger STAT5 tyrosine phosphorylation at Y694 and detection of two phosphoproteins compared with IL-7–dependent STAT5 phosphorylation, where only one band is detected (see d). (**d**) To directly compare BCL6 protein expression levels in pre–B cells upon IL-7 withdrawal and DLBCL cells and tonsillar GC B cells, Western blot analyses were repeated with cell lysates from these cell populations (cell lysates from three IL-7 withdrawal experiments). FSC, forward scatter.

The balance between MYC and BCL6 regulates Vκ-Jκ light chain gene recombination

To investigate whether these *in vitro* observations are relevant for mechanisms of pre-B cell differentiation *in vivo*, we measured the mRNA levels of *BCL6* and *MYC* at various stages of B lymphopoiesis isolated from primary mouse bone marrow (Figure S31.1). To this end, hematopoietic progenitor cells (HPCs, c-kit$^+$ Sca-1$^+$), pro-B cells (c-kit$^+$ B220$^+$; fractions B and C), large pre-BII cells (CD25$^+$ B220^{low}, fraction C') and small pre-BII cells (fraction D) as well as immature B cells (B220^{low} IgM$^+$; fraction E) were isolated and studied by quantitative RT-PCR for *BCL6* and *MYC* mRNA levels (Figure 8a). In most subsets of early B lymphopoiesis, *MYC* mRNA levels were significantly higher when compared with *BCL6*. In particular, the ratio of *BCL6*/*MYC* mRNA levels was very low in large cycling pre-BII cells (fraction C'; ratio = 0.1). Strikingly, however, this ratio increased by >150-fold in small resting pre-BII cells (fraction D; ratio = 18). Given the role of MYC in cell cycle regulation, lower mRNA levels of *MYC* in small resting compared with large cycling pre-BII cells were expected. The finding of a 20-fold up-regulation of *BCL6* in small resting compared with large cycling pre-BII cells is novel albeit consistent with the cytokine context of pre-B cells at this differentiation stage: Pre-BII cells transitioning from the large cycling to the small resting stage downregulate expres-

sion of the IL-7 receptor (Hardy and Hayakawa 2001; Johnson et al., 2008). Withdrawal of IL-7 receptor signaling results in strong *de novo* expression of *BCL6* as demonstrated in Figure 7, which correlates with dephosphorylation of STAT5 and downregulation of MYC (Figure 7c). The inverse relationship of BCL6 and MYC expression is consistent with our finding in BCR-ABL1-transformed pre-B cells that BCL6 is recruited and directly binds to the MYC promoter in BCR-ABL1–transformed pre–B cells (Figure 8b), suggesting that BCL6 functions as a transcriptional repressor of MYC. In addition to BCL6, expression levels of MYC are also negatively regulated by STI571 itself, i.e., via dephosphorylation of STAT5.

To study the functional significance of concomitant upregulation of *BCL6* and downregulation of *MYC* during induced pre-B cell differentiation and Ig light chain gene recombination, we performed gain- and loss-of-function experiments (Figure 8c-e). In one set of experiments, IL-7– and BCR-ABL1–driven pre-B cells were transduced with retroviral BCL6 and MYC vectors or a GFP empty vector control. Upon induced pre-B cell differentiation, the pool of κ light chain expressing cells was significantly increased by *BCL6* overexpression (Figure 8c, d). It should be noted that overexpression of BCL6 likely results in a competitive disadvantage of the BCL6-GFP–transduced cells. As shown here in transformed pre-B cells (Figure 8b) and previously, in DLBCLs (Ci et al., 2009), BCL6 functions as a transcriptional repressor of MYC and may, hence, contribute to the quiescent phenotype of small resting pre-BII cells ($BCL6^{high}$ MYC^{low}) as opposed to large cycling pre-BII cells ($BCL6^{low}$ MYC^{high}; Figure 8a).

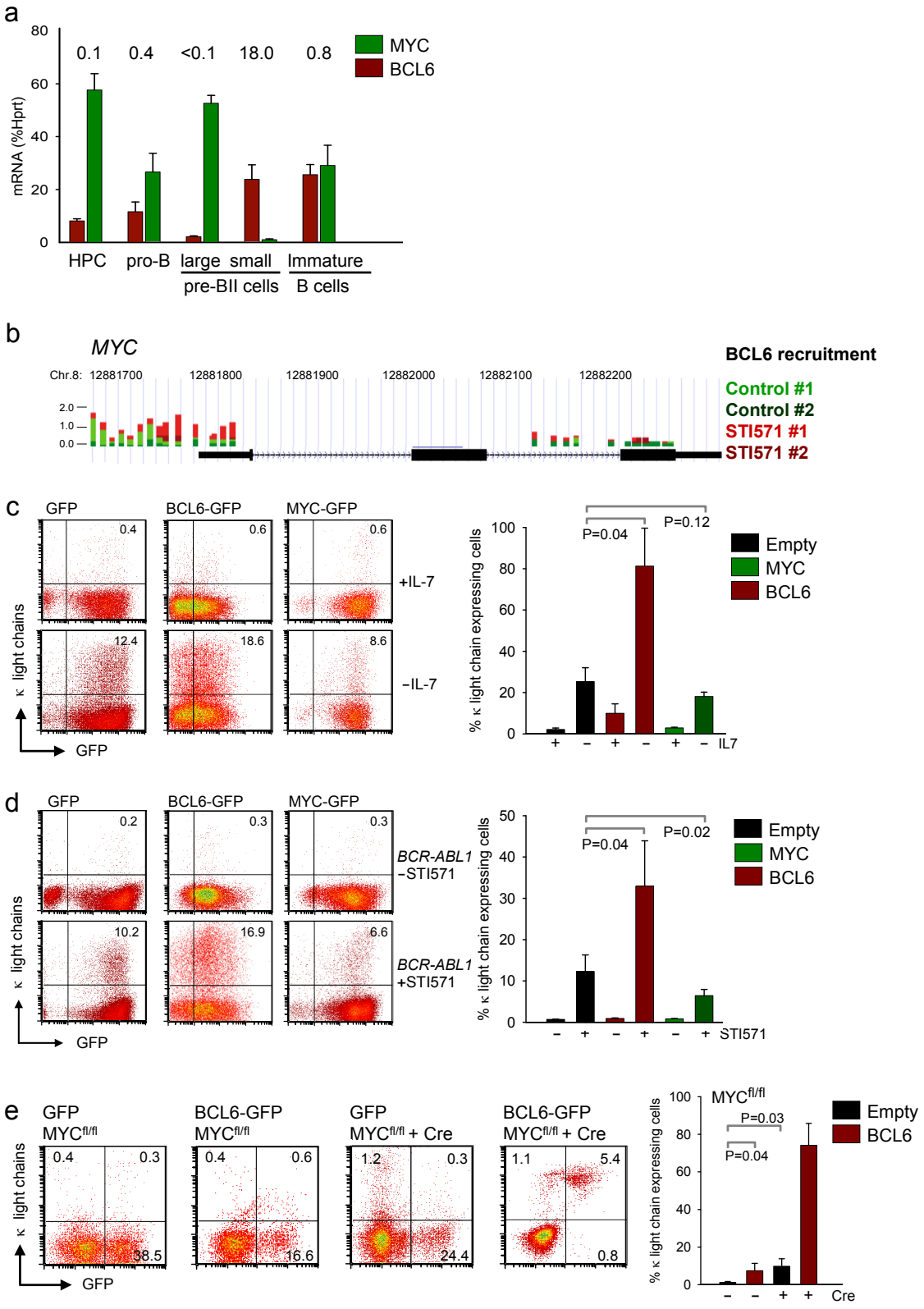
In contrast, overexpression of *MYC* reduced the frequency of κ light $chain^{+}$ cells as compared with GFP empty vector controls in BCR-ABL1–transformed pre–B cells

(treatment with STI571; Figure 8d). Also, IL-7–dependent pre–B cells showed a trend toward MYC-induced reduction of κ light chain^{+} cells, which, however, was not statistically significant. Collectively, these findings suggest that BCL6 and MYC have opposite effects on the regulation of pre–B cell differentiation. To test this possibility in a genetic experiment, we combined retroviral overexpression of *BCL6* with conditional deletion of *MYC* in IL-7–dependent pre–B cells from MYC$^{fl/fl}$ mice (Figure 8e). Overexpression of *BCL6* alone and conditional deletion of *MYC* alone already had a statistically significant, albeit subtle, effect on MYC$^{fl/fl}$ pre–B cells in the presence of IL-7. Strikingly, however, combined overexpression of *BCL6* with Cre-mediated deletion of *MYC* strongly promoted differentiation of pre–B cells into κ light chain–producing immature B cells, even in the presence of IL-7 (Figure 8e). The results demonstrate that the balance between BCL6 and MYC determines the large cycling (fraction C') versus small resting (fraction D) phenotypes of pre–BII cells. We conclude that the genetically induced inversion of the BCL6/MYC ratio in pre–BII cells in this experiment is required and sufficient to recapitulate the effect of IL-7 withdrawal on pre–B cell differentiation.

Pre-B cell receptor-activation induces expression of BCL6 via downregulation of IL7-responsiveness

In Figure 7c, we observed that up-regulation of BCL6 correlated with dephosphorylation of STAT5 Y694 upon withdrawal of IL-7 receptor signaling. This finding suggests that active STAT5 (phosphorylated at Y694) functions as a negative regulator of BCL6 expression downstream of the IL-7 receptor. To mechanistically address this possibility, IL-7–dependent pre–B cells were transduced with a retroviral vector encoding a constitutively active STAT5 mutant (Onishi et al., 1998) or an empty vector control.

Figure 8: *The balance between MYC and BCL6 regulates Vκ-Jκ light chain gene recombination*



(**a**) HPCs, pro–B cells (fractions B and C), large cycling pre–BII cells (fraction C'), small resting pre–BII cells (fraction D), and immature B cells (fraction E) were sorted from normal mouse bone marrow (*n* = 3) and subjected to quantitative RT-PCR to measure mRNA levels of BCL6 and MYC relative to Hprt. Mean values ± SD of three experiments are given. Numbers in the bar chart denote the ratios of BCL6 versus MYC mRNA levels. (**b**) Recruitment of BCL6 to the *MYC* promoter was identified by ChIP-on-chip analysis and confirmed by single locus QChIP analysis. ChIP-on-chip analysis was performed for three BCR-ABL1–transformed pre–B cell lines (each two replicates) under control conditions or after treatment with 10 μmol/liter STI571 for 24 h. Two replicate experiments for one BCR-ABL1–transformed pre–B ALL cell line are shown. (c and d) IL-7–dependent (**c**) and BCR-ABL1–transformed (**d**) pre–B cells were induced to differentiate by IL-7 withdrawal or inhibition of BCR-ABL1 kinase activity. To test the function of MYC and BCL6 during induced pre–B cell differentiation, pre–B cells were transduced with retroviral vectors encoding BCL6-IRES-GFP, MYC-IRES-GFP, or an IRES-GFP empty vector control. Percentages of κ light chain$^+$ GFP$^+$ cells are indicated (mean values of three experiments ± SD). (**e**) IL-7–dependent bone marrow pre–B cells from MYC$^{fl/fl}$ and MYC$^{fl/fl}$ × *mx-Cre* mice were transduced with retroviral vectors encoding BCL6-IRES-GFP or an IRES-GFP (empty vector control) and cultured in the presence of 10 ng/ml IL-7. 2,500 U/ml IFN-β was added to all cultures to induce expression of Cre and MYC deletion in *Mx*-Cre × MYC$^{fl/fl}$ but not MYC$^{fl/fl}$ pre–B cells (mean values ± SD; *n* = 3).

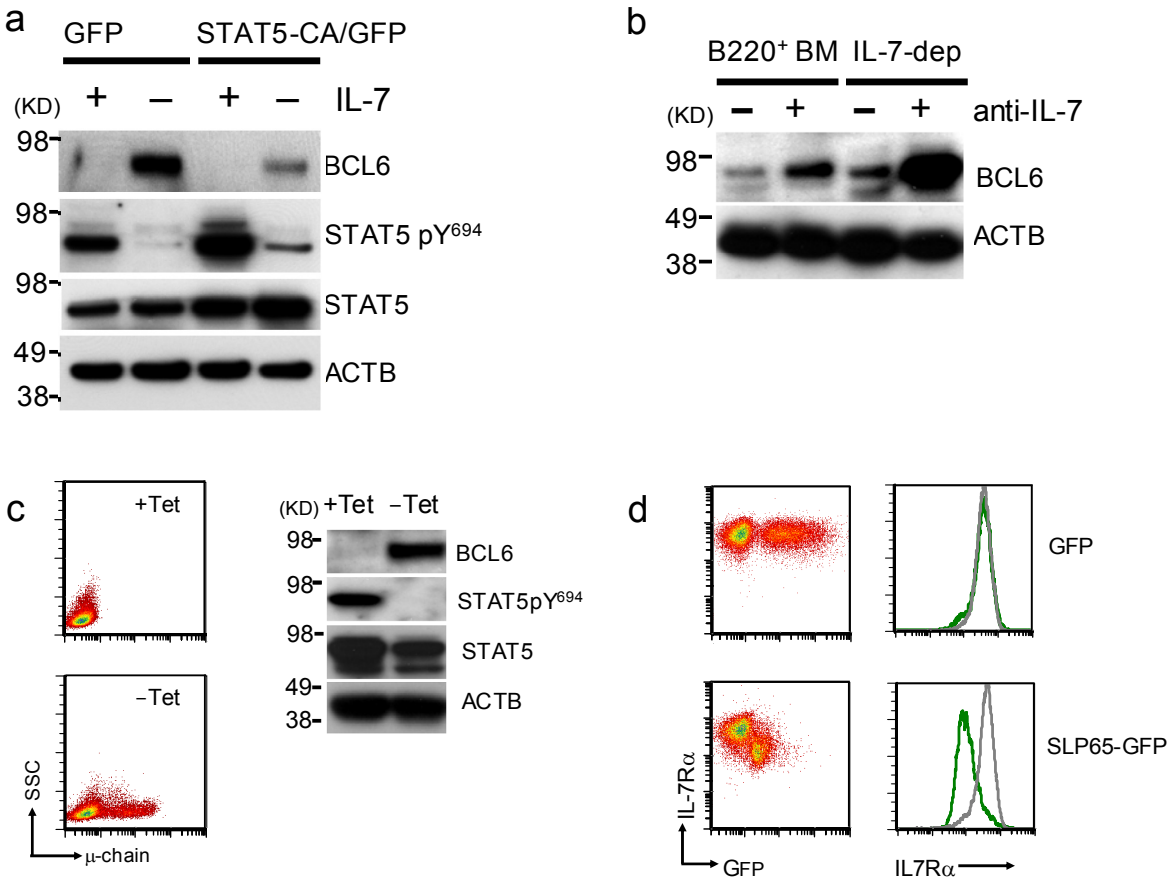
Withdrawal of IL-7 resulted in both dephosphorylation of STAT5 and up-regulation of BCL6 in pre–B cells that were transduced with an empty vector. In contrast, pre–B cells transduced with the constitutively active STAT5 mutant maintained some STAT5 phosphorylation even after IL-7 withdrawal, and up-regulation of BCL6 was greatly attenuated (Figure 9a). This finding demonstrates that active STAT5 functions as a negative regulator downstream of the IL-7 receptor. To verify that withdrawal of IL-7 not only results in BCL6 up-regulation in long-term IL-7–dependent pre–B cell cultures, we used a neutralizing anti–IL-7 antibody to inactivate IL-7 receptor signaling in freshly ex vivo–isolated (B220$^+$ MACS) bone marrow B cell precursors (Figure 9b). Although both populations strongly up-regulated BCL6 in response to anti–IL-7 antibody treatment, the antibody had a stronger effect in long-term pre–B cell cultures than in freshly isolated *ex*

vivo $B220^+$ bone marrow cells. This difference likely reflects a higher degree of enrichment for IL-7–responsive cells in long-term IL-7 pre–B cell cultures than in $B220^+$ bone marrow cells (Figure 9b).

Although these findings establish that IL-7Rα–STAT5 signaling negatively regulates BCL6 expression in pre–BII cells, it is not clear how BCL6 expression can be activated in these cells. To address this question, we used a model in which inducible *de novo* synthesis of the pre–B cell receptor recapitulates the transition of pro–B cells (fractions B and C) into large cycling pre–BII cells (after 2 d; fraction C') and then small resting pre–BII cells (after 4 d; fraction D): pro–B cells from $RAG2^{-/-}$ tetracycline-controlled transactivator (tTA)/μ chain–transgenic mice (Hess et al., 2001) are unable to express an endogenous μ chain because of a lack of RAG2-dependent V(D)J recombination. Although these pro–B cells carry their endogenous Ig heavy chain loci in germline configuration, they also harbor a functionally prerearranged μ chain under the control of tetracycline operator (tetO) sequences. In addition, $RAG2^{-/-}$ tTA/μ chain–transgenic mice express a tTA under control of endogenous μ chain regulatory elements. We verified induction of μ chain expression in $RAG2^{-/-}$ tTA/μ chain–transgenic IL-7–dependent pro–B cell cultures by flow cytometry (Figure 9c). In agreement with previously published data (Schuh et al., 2008), we observed that induction of μ chain expression in $RAG2^{-/-}$ tTA/μ chain–transgenic pro–B cells results in down-regulation of CD43 and up-regulation of CD25, which is consistent with a pro–B to pre–B cell transition (Hardy and Hayakawa 2001). 2 d after induction of μ chain expression, the cells had assumed a large cycling phenotype (fraction C'), followed by cell-cycle exit and reduction of cell size (small resting pre–BII cell; fraction D; (Trageser et al., 2009)). A

Western blot analysis performed with cells that were harvested after 4 d of μ chain induction showed strong up-regulation of BCL6 together with near complete dephosphorylation of STAT5 (Figure 9c).

Figure 9: *Pre-B cell receptor activation induces expression of BCL6 via downregulation of IL-7 responsiveness*



IL-7–dependent pre–B cells were transduced with a retroviral vector encoding a constitutively active STAT5 mutant (STAT5-CA-GFP; Onishi et al., 1998) or an empty vector control (GFP). (**a**) GFP-expressing transduced cells were sorted, subjected to IL-7 withdrawal, and analyzed by Western blotting for expression of BCL6 and tyrosine phosphorylation of STAT5 using β-actin as a loading control (*n* = 3). (**b**) Mouse B cell precursors were isolated by $B220^+$ MACS from freshly harvested bone marrow cells. Freshly isolated bone marrow B cell precursors and IL-7–dependent pre–B cell cultures were treated with 10 μg/ml of a neutralizing anti–IL-7 antibody overnight and were subjected to Western blot analysis (three experiments were performed). (**c**) Bone marrow B cell precursors from $RAG2^{-/-}$ tTA/μ chain–transgenic mice are unable to express

an endogenous μ chain but carry a functionally prerearranged μ chain under control of tetO sequences (Hess et al., 2001). These mice express a tTA under control of endogenous μ chain regulatory elements, and withdrawal of tetracycline results in activation of μ chain expression (routinely performed quality control). The effect of tetracycline-inducible activation of μ chain expression on BCL6 expression and STAT5 tyrosine phosphorylation was determined by Western blotting (*n* = 3; right). (**d**) IL-7–dependent pre–B cells lacking the pre–B cell receptor–related linker molecule SLP65 were transduced with retroviral expression vectors encoding either SLP65-GFP or GFP alone. Surface expression levels of IL-7Rα chain were measured by flow cytometry (the experiment was performed twice). The histogram shows the IL-7Rα levels in transduced GFP^+ (green) and untransduced (gray) cells.

These findings demonstrate that the transition from IL-7Rα–STAT5 signaling in large cycling pre–BII cells (fraction C') to BCL6 expression in small cycling pre–BII cells (fraction D) is initiated by the pre–B cell receptor. The finding of pre–B cell receptor–mediated down-regulation of IL-7Rα–STAT5 signaling is consistent with a recent study, which demonstrated that IL-7Rα–STAT5 signaling prevents premature Ig light chain gene recombination in pro–B cells (Malin et al., 2010). Experiments to delineate the details of pre–B cell receptor–dependent activation of BCL6 are currently under way. In this context it is noteworthy that reconstitution of the pre–B cell receptor linker molecule SLP65 in $SLP65^{-/-}$ pre–B cells results in down-regulation of IL-7Rα surface expression (Figure 9d) and reduced IL-7 responsiveness (Schebesta et al., 2002). In addition, a recent study showed that pre–B cell receptor–dependent activation of SLP65 leads to dephosphorylation of STAT5 via inhibition of JAK3 (Nakayama et al., 2009). We conclude that BCL6 activation in B cell precursors is naturally confined to the pre–B cell compartment and is induced after de novo synthesis of the pre–B cell receptor based on productively rearranged V_H-DJ_H gene segments. We propose that down-regulation of IL-7 responsiveness represents an important aspect through which pre–B cell receptor

signaling induces expression of BCL6 in small resting pre–BII cells.

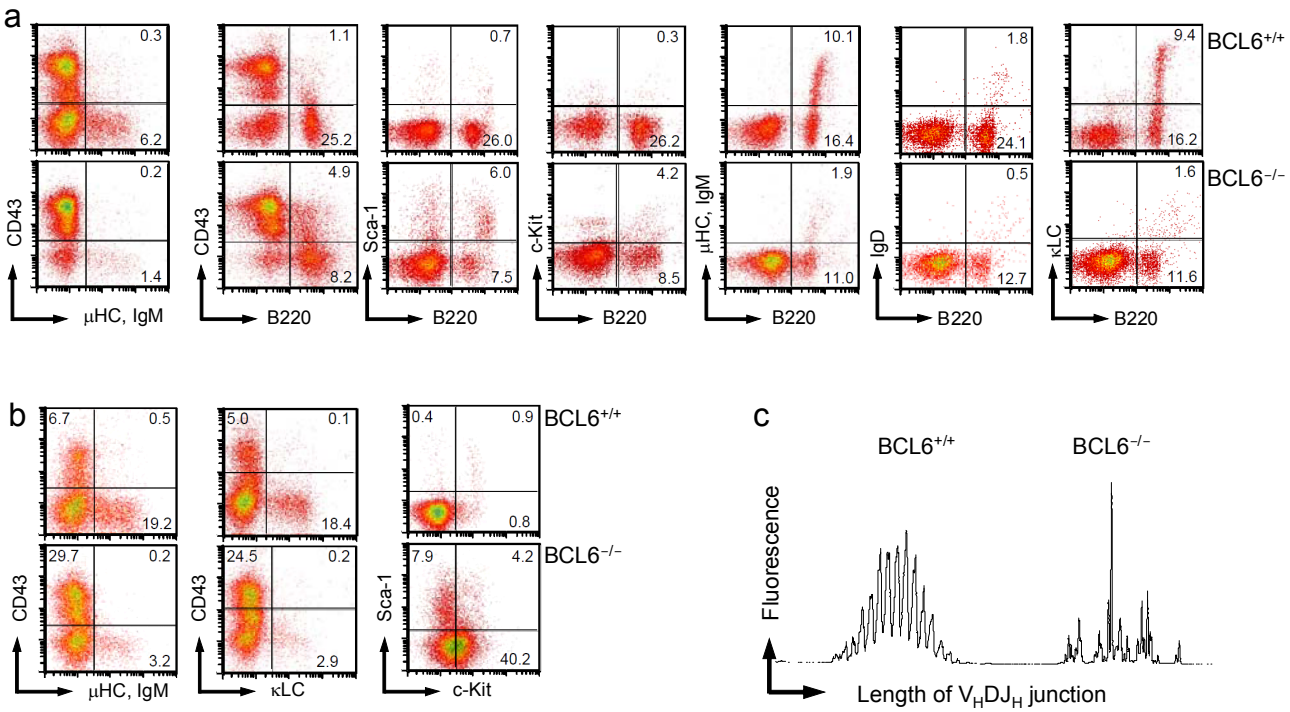
BCL6 is required for normal polyclonal B lymphopoiesis

To investigate the physiological relevance of BCL6 during early B cell development, we performed a detailed flow cytometry analysis of bone marrow samples from age-matched $BCL6^{-/-}$ and $BCL6^{+/+}$ mice (n = 4). Although the total number of $B220^+$ B cell lineage cells was only slightly reduced in $BCL6^{-/-}$ compared with wild-type bone marrow, the frequency of IgM^+ and κ light $chain^+$ immature B cells was significantly diminished (Figure 10a, b; statistical analysis in Table S31.1). Compared with wild-type cells, c-kit^+ $B220^+$ (fraction B) and $CD43^+$ $B220^+$ (fraction C) pro–B cell populations were slightly expanded in $BCL6^{-/-}$ bone marrow. In agreement with previous studies (Dent et al., 1997; Ye et al., 1997), κ light chain–expressing immature B cells (fraction E) were detected in $BCL6^{-/-}$ bone marrow, but their frequency was approximately fivefold lower than in wild-type mice (P = 0.03; Table S31.1).

To study the clonal composition of IgM^+ κ^+ bone marrow immature B cells (fraction E) that developed in the absence of BCL6, we sorted 8,000 $B220^+$ IgD^- κ^+ bone marrow cells from both *BCL6*$^{-/-}$ and $BCL6^{+/+}$ bone marrow and analyzed them by spectratype analysis. Although wild-type $B220^+$ IgD^- κ^+ bone marrow immature B cells exhibit a broad polyclonal repertoire, the profile of $BCL6^{-/-}$ bone marrow immature B cells was oligoclonal (Figure 10c). These findings are based on sorted immature B cells from four pairs of $BCL6^{+/+}$ and $BCL6^{-/-}$ mice, and suggest that BCL6 is required for normal polyclonal B cell production. To verify the clonal composition of the primary B cell repertoire in $BCL6^{+/+}$ and $BCL6^{-/-}$ mice, we performed a detailed sequence analysis for two pairs of mice where immature B cells from bone marrow and mature B cells from

spleens were available for cell sorting from the same animals (Figure 11). Interestingly, in two BCL6$^{-/-}$ mice, spectratyping identified clonal expansions with the same peak size (i.e., the same length of the V_H-DJ_H junction; Figure 11a) that were amplified from both the bone marrow and the spleen of these mice. This finding raises the question of whether the clonal expansions among bone marrow immature B cells and mature splenic B cells in BCL6$^{-/-}$ mice are derived from the same dominant clone.

Figure 10: *Normal polyclonal B lymphopoiesis requires BCL6 survival signaling in late pre-B cells*



Bone marrow mononuclear cells from BCL6$^{+/+}$ and BCL6$^{-/-}$ mice were analyzed by flow cytometry using the indicated antibody combinations (numbers indicated percentages). (**a**) Bone marrow cells were analyzed using a FSC/SSC gate together with propidium iodide exclusion (viable lymphocytes). (**b**) Cells were analyzed using a B220$^+$ gate. Four mice were studied in each group, and a detailed statistical analysis including absolute cell numbers is presented in Table S31.1. (**c**) To examine the clonal composition of κ$^+$ immature B cells (fraction E), B220$^+$ IgD$^-$ κ$^+$ cells were sorted and analyzed by Ig spectratyping. Spectratyping analysis separates Ig gene

rearrangements based on the length of their V_H-DJ_H junction, and the height (fluorescence intensity) of each size peak indicates its relative representation within the B cell population analyzed. Individual size peaks are typically separated by 3 bp, reflecting the reading frame in functional V_H-DJ_H gene rearrangements. In total, four pairs of $BCL6^{+/+}$ and $BCL6^{-/-}$ bone marrow–derived immature B cell samples were analyzed by spectratyping. FSC, forward scatter; SSC, side scatter.

To test this possibility, we performed a comprehensive sequence analysis of V_H-DJ_H junctions in bone marrow immature B cells and splenic mature B cells from $BCL6^{+/+}$ and $BCL6^{-/-}$ mice, respectively (Figure 11b and Table S31.2). Consistent with the finding of a restricted B cell repertoire in $BCL6^{-/-}$ mice (Figure 10c and Figure 11a), we amplified multiple clonally related sequences from $BCL6^{-/-}$ but not from $BCL6^{+/+}$ mice (available from GenBank/EMBL/DDBJ under accession nos. FN652762, FN652763, FN652764, FN652765, FN652766, FN652767, FN652768, FN652769, FN652770, FN652771, FN652772, FN652773, FN652774, FN652775, FN652776, FN652777, and FN652778; Table S31.2). In addition, clonal expansions within the bone marrow immature B cell and mature splenic B cell pool in $BCL6^{-/-}$ mice carried the same V_H-DJ_H junction and, hence, share the same clonal origin (Figure 11b). Two large clones were amplified from the bone marrow and spleen of $BCL6^{-/-}$ mouse #I, and one clonal expansion in the bone marrow of $BCL6^{-/-}$ mouse #II was also recovered from the spleen. These findings further illustrate that even though the size of a peripheral B cell pool may be normal in $BCL6^{-/-}$ mice (Dent et al., 1997; Ye et al., 1997), the primary B cell repertoire in $BCL6^{-/-}$ mice is restricted to a small number of dominant clones.

A more detailed analysis of V_H-DJ_H junctions revealed that many other seemingly unique V_H-DJ_H rearrangements amplified from $BCL6^{-/-}$ mature splenic B cells in fact harbor the same D-J_H junction (gray shading in Table S31.2) but have rearranged

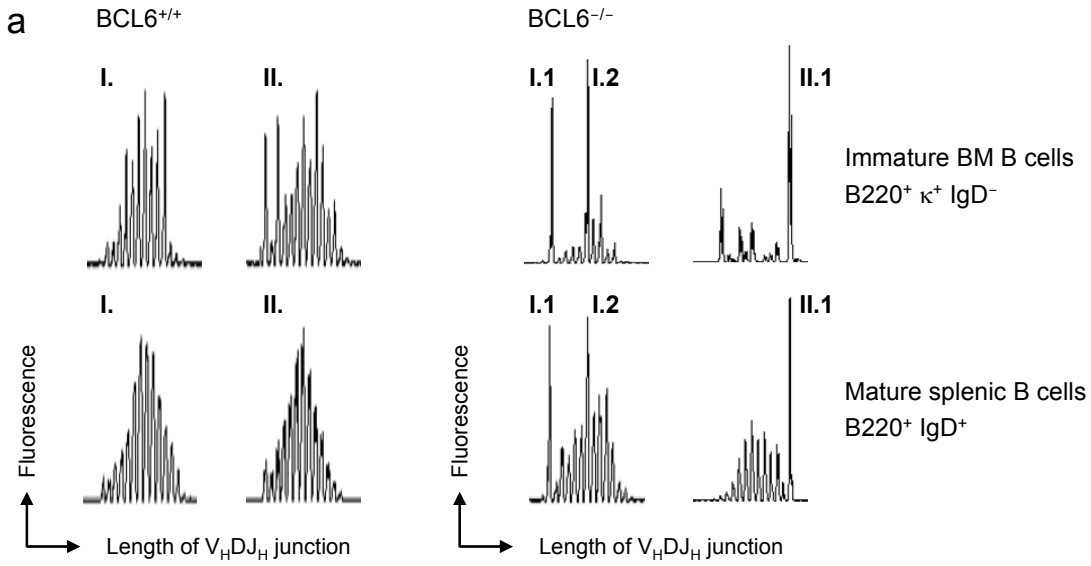
different V_H gene segments. These clones are derived from one parental bone marrow immature B cell clone but subsequently diversified in the spleen by V_H replacement, i.e., by substitution of the initially rearranged V_H segment by a V_H segment located upstream. V_H replacement (Reth et al., 1986) involves the recombination signal sequence (RSS) of a nonrearranged V_H gene and a cryptic RSS (i.e., an isolated heptamer) within the preexisting V_HDJ_H joint. The cryptic RSS (highlighted in red in Figure 11b) is located at the 3′ end of the majority of V_H gene segments in mice and humans (Radic and Zouali 1996). The spectratyping profiles shown in Figure 11a seem to suggest that the extreme oligoclonality observed among BCL6$^{-/-}$ bone marrow immature B cells is alleviated to some degree within the pool of BCL6$^{-/-}$ mature splenic B cells. It is likely, however, that many of these additional clones have diversified by V_H gene replacement, share the same D-J_H junction, and are, hence, derived from a very small number of immature B cells that have left the bone marrow. We conclude that the ability of small resting pre–BII cells to up-regulate BCL6 is critical for the development of a diverse primary B cell repertoire.

BCL6 is required for self-renewal signaling at the pre-B cell stage

Previous work demonstrated that a fraction of BCL6$^{-/-}$ mice suffers from chronic inflammation (Dent et al., 1997; Ye et al., 1997), which may affect the composition of the B cell progenitor populations within the bone marrow. In the animals we analyzed in this study, no signs of inflammatory disease were noted. In addition, we tested the ability of BCL6$^{-/-}$ pre–B cells to differentiate under cell-culture conditions, which excludes potential side effects from inflammation (Figure S31.2). We first incubated bone marrow samples from BCL6$^{+/+}$ and BCL6$^{-/-}$ mice in the presence of 10 ng/ml IL-7, which selectively induces proliferation of bone marrow pre–B cells. In the presence of high IL-7

concentrations, the majority of the bone marrow HPCs die, whereas B cell lineage cells vigorously proliferate (Figure S31.2a).

Figure 11: *BCL6 is required for the development of a diverse primary B cell repertoire*



b

BCL6$^{-/-}$	Clone	3'V_H	N/P1	D_H	N/P2	5'J_H
Bone marrow	I.1 V_H1-15	ATCTGAAGACTCTGCGGTCTATTACTGTGCAAGA	..GG	.....AGGG	.....GG	.....TACTGGGGCCAAGGCACCACT
Spleen	I.1 V_H1-15	ATCTGAAGACTCTGCGGTCTATTACTGTGCAAGA	..GG	.....AGGG	.....GG	.....TACTGGGGCCAAGGCACCACT
Spleen	I.1 V_H1-12	ATCTGAAGACTCTGCGGTCTATTTTCTGTGCAA	C	.....↓GGG	.....GG	.....TACTGGGGCCAAGGCACCACT
Bone marrow	I.2 V_H1-11	ATCTGAGGACCCTGCTGTCTATTACTGTGGAAG	.....G	...AGACAGACCGGG	.....GT	....ACTACTTTGACTACTGGGGCCAAGGCACCACT
Spleen	I.2 V_H1-11	ATCTGAGGACCCTGCTGTCTATTACTGTGGAAG	.....G	...AGACAGACCGGG	.....GT	....ACTACTTTGACTACTGGGGCCAAGGCACCACT
Spleen	I.2 V_H1-12	ATCTGAAGACTCTGCGGTCTATTT	.....	↓ACCGGG	.....GT	....ACTACTTTGACTACTGGGGCCAAGGCACCACT
Spleen	I.2 V_H1-5	AAATGAGGACTCTGCGGTCT	.....	↓CAGACCGGG	.....GT	....ACTACTTTGACTACTGGGGCCAAGGCACCACT
Spleen	I.2 V_H1-11	ATCTGAGGACCCTGCTGTCTATTAC	.....	CG↓G..AGACAGACCGGG	.....GT	....ACTACTTTGACTACTGGGGCCAAGGCACCACT
Spleen	I.2 V_H1-15	ATCTGAGGACTCTGCCGTCTATTACT	.....	GCCAG↓G..AGACAGACCGGG	.....GT	....ACTACTTTGACTACTGGGGCCAAGGCACCACT
Bone marrow	II.1 V_H1-34	ATCTGAGGACTCTGCAGTCTATTACTGTGCAAGA	..GATCCCT	..CTACGGTAGT	..CCCCTTTT	..ACTACTTTGACTACTGGGGCCAAGGCACCACT
Spleen	II.1 V_H1-34	ATCTGAGGACTCTGCAGTCTATTACTGTGCAAGA	..GATCCCT	..CTACGGTAGT	..CCCCTTTT	..ACTACTTTGACTACTGGGGCCAAGGCACCACT
Spleen	II.1 V_H1-5	AAATGAGGACTCTGCGGTCTAT	.....	↓GGTAGT	..CCCCTTTT	..ACTACTTTGACTACTGGGGCCAAGGCACCACT
Spleen	II.1 V_H1-12	ATCTGAAGACTCTGCGGTCTATTTTCTG	.....	↓TAGT	..CCCCTTTT	..ACTACTTTGACTACTGGGGCCAAGGCACCACT

(**a**) For two pairs of BCL6$^{+/+}$ and BCL6$^{-/-}$ mice (I and II), in addition to bone marrow samples spleen tissue was also available. Immature B cells (fraction E) from bone marrow and mature B cells from spleens (fraction F) were sorted and subjected to spectratyping analysis to study length diversity of V_H-DJ_H junctions. In two BCL6$^{-/-}$ mice, clonal expansions were identified that occurred both in the bone marrow and in the spleen with the same peak size, i.e., the same length of the V_H-DJ_H junction. Two such clonal expansions were found in BCL6$^{-/-}$ mouse I (I.1 and I.2) and one expansion was found in BCL6$^{-/-}$ mouse II (II.1). (**b**) To test whether these expansions with the same length of the V_H-DJ_H junction indeed belong to one clone, we performed a detailed

sequence analysis of these V_H-DJ_H junctions. Alignments of V_H, D, and J_H gene segments are shown as well as N/P nucleotides in the junctional regions. Internal heptamers are highlighted in red, and sites at which the junctional homology with the parental V_H-DJ_H rearrangement ends are indicated by arrows.

We verified that the IL-7–dependent and BCR-ABL1–transformed cells used are indeed pre–B cells based on positive intracellular staining for μ chain and CD25 expression (Figure S31.2b). IL-7–dependent pre–B cell proliferation was robustly induced in bone marrow samples from $BCL6^{+/+}$ mice, and pre–B cell cultures can be expanded for up to 2 mo until pre–B cell cultures become senescent (unpublished data). In striking contrast, $BCL6^{-/-}$ pre–B cells consistently failed to propagate ex vivo even in the presence of 10 ng/ml IL-7. Although wild-type pre–B cells start to vigorously expand on day 3, no viable B cell precursors can be detected in IL-7 cultures from $BCL6^{-/-}$ bone marrow (Figure S31.2a). Because $BCL6^{-/-}$ pre–B cells failed to differentiate and died upon IL-7 withdrawal even after short-term culture (Figure S31.2a), we studied differentiation in BCR-ABL1–transformed pre–B cells, as described in Figure 7a. Compared with wild-type pre–B cells, transformation of fresh $BCL6^{-/-}$ pre–B cells was significantly delayed, cells proliferated at a reduced rate, and in some instances transformation failed entirely (unpublished data). However, we found after successful BCR-ABL1 transformation of $BCL6^{+/+}$ and $BCL6^{-/-}$ cells that inhibition of BCR-ABL1 kinase activity induced differentiation into κ^+ immature B cells in 3% of wild-type pre–B cells within 3 d, whereas no significant differentiation but markedly enhanced cell death was observed in $BCL6^{-/-}$ pre–B cells (Figure S31.3a).

BCL6 protects pre-B cells against apoptosis during Vκ-Jκ recombination

We next compared V(D)J recombinase activity in BCL6$^{-/-}$ and wild-type pre–B cells before and after induced differentiation. To this end, we transduced *BCR-ABL1*–transformed pre–B cells with a retrovirus carrying an inverted GFP cassette flanked by RSS as in Vκ and Jκ gene segments. Upon inversion-mediated recombination of the RSS-flanked GFP, the GFP cassette will be in the correct orientation and expressed from the retroviral long terminal repeat (Figure S31.3b). Equal numbers of BCL6$^{-/-}$ and wild-type pre–B cells were transduced and subjected to selection for antibiotic resistance encoded by the RSS-GFP reporter construct (Wossning et al., 2006). We detected spontaneous V(D)J recombinase activity in both untreated *BCR-ABL1*–transformed BCL6$^{+/+}$ and BCL6$^{-/-}$ pre–B cells, and the frequency of cells with recombinase activity was higher in the wild-type cells (~20% compared with ~2%; $P < 0.01$; Figure S31.3b). When *BCR-ABL1*–transformed pre–B cells were stimulated to differentiate by BCR-ABL1 kinase inhibition, the frequency of cells undergoing RSS-dependent recombination events increased in both BCL6$^{-/-}$ and wild-type pre–B cells. RSS-targeted recombination events were followed by de novo expression of κ light chains in wild-type pre–B cells. Strikingly, however, the frequency of κ^+ cells did not significantly increase in STI571-treated BCL6$^{-/-}$ pre–B cells despite recombination activity in these cells (Figure S31.3b). This difference between BCL6$^{-/-}$ and BCL6$^{+/+}$ pre–B cells could indicate that BCL6 is required for targeting of the V(D)J recombinase to the Ig κ light chain locus. Alternatively, BCL6 may have a role in the intracellular processing of κ light chains and their pairing with Ig heavy chains. Finally, Vκ-Jκ rearranging pre–B cells might undergo apoptosis in the absence of BCL6 before κ light chains can be expressed on the cell surface. To directly test the latter hypothesis, we measured the percentage of apoptotic

cells (annexin V^+) in $BCL6^{+/+}$ and $BCL6^{-/-}$ pre–B cells with ($RSS\text{-}GFP^+$) and without ($RSS\text{-}GFP^-$) indication of V(D)J recombinase activity (Figure S31.3c). If lack of κ light chain expression on $BCL6^{-/-}$ pre–B cells was indeed linked to apoptosis during Vκ-Jκ rearrangement, one would expect that $RSS\text{-}GFP^+$ cells were preferentially apoptotic in the case of $BCL6^{-/-}$ but not $BCL6^{+/+}$ cells. Induction of differentiation by STI571 treatment resulted in a strong increase of V(D)J recombinase activity, both in $BCL6^{+/+}$ and $BCL6^{-/-}$ pre–B cells (Figure S31.3c). Among $BCL6^{+/+}$ cells, only a minority of V(D)J recombining pre–B cells ($RSS\text{-}GFP^+$) was apoptotic in the presence (24% of all $RSS\text{-}GFP^+$ cells) or absence (13% of $RSS\text{-}GFP^+$ cells) of STI571-treatment. In contrast, however, no enrichment of viable cells was observed among $BCL6^{-/-}$ pre–B cells that underwent V(D)J recombination: approximately one half of $BCL6^{-/-}$ V(D)J recombining pre–B cells ($RSS\text{-}GFP^+$) was apoptotic in the presence and absence of STI571 treatment (Figure S31.3c). In summary, STI571 treatment induced increased V(D)J recombinase activity and increased cell death both in $BCL6^{+/+}$ and $BCL6^{-/-}$ cells. However, the outcome of STI571-induced V(D)J recombination activity differs between $BCL6^{+/+}$ and $BCL6^{-/-}$ cells: in $BCL6^{+/+}$ cells, STI571-induced recombinase activity results in higher output of κ^+ cells. In $BCL6^{-/-}$ cells, STI571-induced recombination results in increased cell death (Figure S31.3b & c).

These findings suggest that DNA DSB events during Vκ-Jκ gene rearrangement are directly linked to apoptosis in $BCL6^{-/-}$ pre–B cells. To test this possibility, we induced differentiation in IL-7–dependent B cell precursors carrying a homozygous deletion of the RAG1 gene, which is required for V(D)J recombination. Upon withdrawal of IL-7, $RAG1^{-/-}$ B cell precursors undergo phenotypic changes consistent with

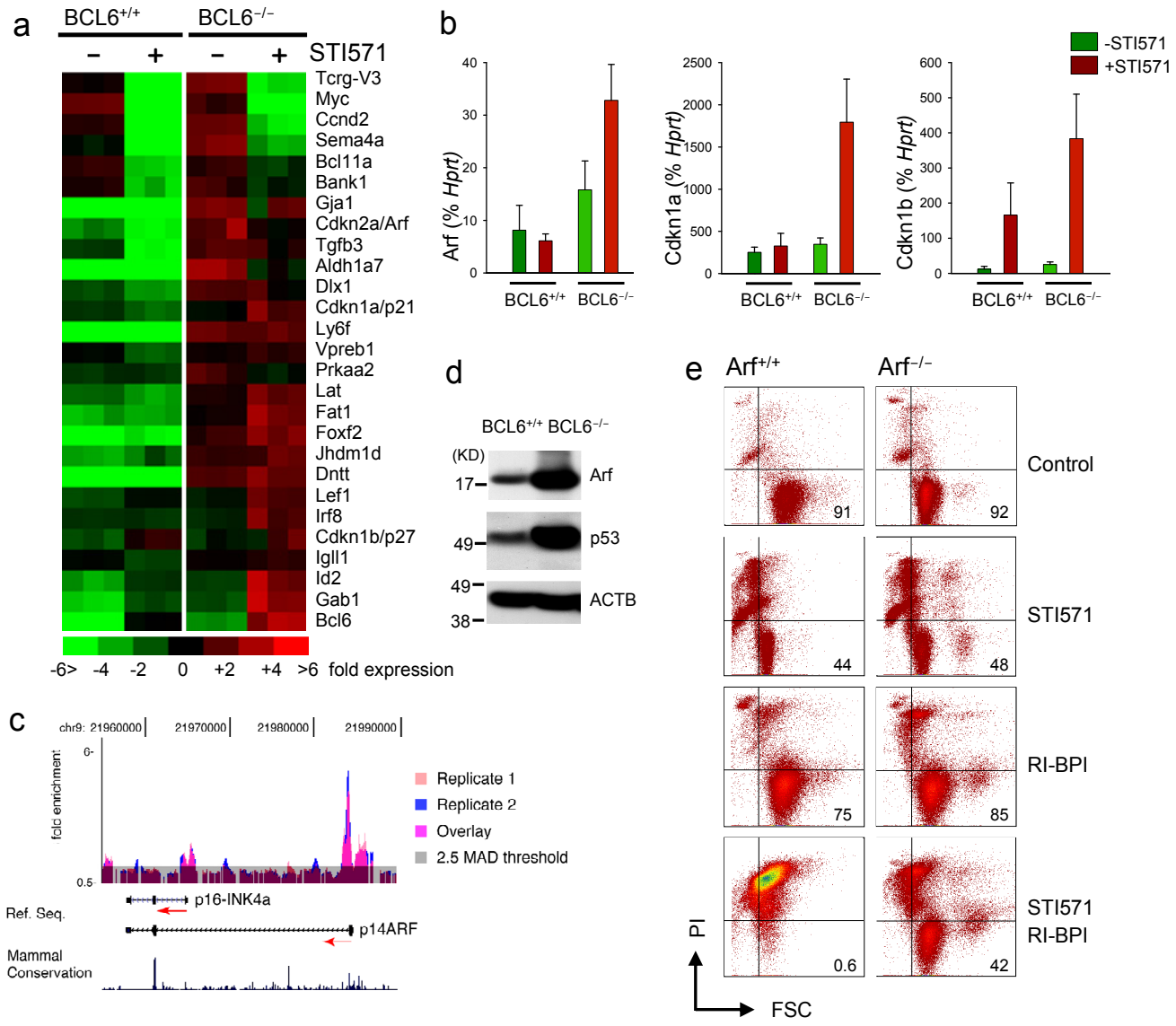
differentiation, as in wild-type pre–B cells (Figure S31.4), but fail to introduce DNA DSBs at Vκ- and Jκ genes. To test the role of BCL6 in this system, we used a peptidomimetic inhibitor of BCL6 (retro-inverso BCL6 peptide inhibitor [RI-BPI]; (Cerchietti et al., 2009b)), which was recently developed for the treatment of GC-derived B cell lymphoma (Polo et al., 2004). Withdrawal of IL-7 had a similar effect in both $RAG1^{-/-}$ and wild-type B cell precursors, and induced phenotypic differentiation (down-regulation of CD43 and up-regulation of CD25) in most cells but apoptosis only in a small fraction (Figure S31.4). Concomitant withdrawal of IL-7 and peptidomimetic inhibition of BCL6 increased the fraction of apoptotic cells in both *Rag1*$^{-/-}$ and wild-type B cell precursors. Although >50% of $RAG1^{-/-}$ B cell precursors survived simultaneous inhibition of IL-7 and BCL6 signaling, almost all cells in which DNA DSBs could be introduced during induced Vκ-Jκ gene rearrangement underwent apoptosis (annexin V^+; Figure S31.4). Likewise, concomitant inhibition of IL-7 and BCL6 compromised the survival of differentiating ($CD25^+$ $CD43^-$) subclones, unless introduction of DNA DSBs during V(D)J recombinase activity was prevented in $RAG1^{-/-}$ B cell precursors (Figure S31.4). These striking differences indicate that BCL6 function in differentiating pre–B cells is mainly needed to counteract the apoptotic DNA damage response induced by DNA DSBs during Ig light chain gene rearrangement.

BCL6 is required for transcriptional repression of DNA damage response genes during Ig light chain recombination

To elucidate the mechanism of BCL6-mediated pre–B cell survival signaling, we next investigated the gene expression pattern in $BCL6^{-/-}$ and $BCL6^{+/+}$ pre–B cells (Figure 12a). Importantly, well-known cell-cycle checkpoint regulators (CDKN1A/p21,

CDKN1B/p27, and CDKN2A/Arf) and inducers of cellular senescence (CDKN2A/Arf) are among the targets of BCL6-mediated transcriptional repression (Figure 12a, b), which directly supports a central role of BCL6 in pre–B cell self-renewal signaling and protection against DNA damage–induced apoptosis (e.g., during Vκ-Jκ gene rearrangement). These differences could be confirmed by quantitative RT-PCR for ARF, p21, and p27 (highlighted in Figure 12a, b). In the absence of BCL6, p21, p27, and ARF showed excessive up-regulation after STI571 treatment, and Arf mRNA levels were increased in BCL6-deficient pre–B cells even in the absence of induced differentiation. Although p21 and p27 were previously identified as target genes of BCL6-mediated transcriptional repression (Phan and Dalla-Favera 2004; Phan et al., 2005; Shaffer et al., 2000), transcriptional silencing of ARF by BCL6 represents a novel finding. To determine whether BCL6 could bind to the *ARF* locus, we performed BCL6 chromatin immunoprecipitation (ChIP)–on-chip using a genomic microarray tiling across the entire *CDKN2A* locus (Figure 12c) in the human B cell lymphoma cell line OCI-Ly1. The *CDKN2A* gene contains two start exons (1α and 1β) that give rise to two alternative transcripts, $p14^{ARF}$ and $p16^{INK4A}$. The ChIP-on-chip result demonstrated that BCL6 was strongly bound to sequences proximal to the $p14^{ARF}$ transcriptional start site (Figure 12c). ChIP-on-chip also showed that BCL6 also bound, albeit weakly, to sequences related to $p16^{INK4a}$ (Figure 12c). BCL6 binding to the p14ARF promoter was confirmed by performing additional quantitative ChIP (QChIP) assays using primers to amplify this site. BCL6 single-locus QChIP for *CDKN2A*/$p14^{ARF}$ showed 0.74 ± 0.2% of input for BCL6 compared with 0.03 ± 0.01% of input using a β-actin antibody as negative control ($n = 3$; $P = 0.02$).

***Figure 12**: BCL6 promotes pre-B cell survival by negative regulation of ARF*



(**a**) BCR-ABL1–transformed BCL6$^{+/+}$ and BCL6$^{-/-}$ pre–B cells were induced to differentiate (10 µmol/liter STI571) and studied by microarray analysis. Genes were sorted based on the ratio of gene expression values in STI571-treated BCL6$^{+/+}$ compared with BCL6$^{-/-}$ pre–B cells. (**b**) Differences in mRNA levels of checkpoint genes (ARF, p21, and p27) were verified by quantitative RT-PCR using HPRT as a reference (mean values ± SD; *n* = 3). (**c**) Recruitment of BCL6 to the genomic region of the *CDKN2A* locus (*ARF*) was verified by ChIP-on-chip analysis and confirmed by single locus QChIP analysis (*n* = 3). BCL6–DNA complexes were immunoprecipitated using an anti-BCL6 polyclonal antibody. ChIP products and their respective input genomic fragments were amplified by ligation-mediated PCR. QChIP was performed again at this stage for selected positive control loci to verify that the enrichment ratios were retained

(not depicted). The genomic products of two biological ChIP replicates were labeled with Cy5 (for ChIP products) and Cy3 (for input) and cohybridized on genomic tiling arrays including the genomic region of the *CDKN2A* locus (Chr9:21,950629–22,003329). After hybridization, the relative enrichment for each probe was calculated as the signal ratio of ChIP to input. Peaks of enrichment for BCL6 relative to input were uploaded as custom tracks into the University of California, Santa Cruz genome browser and graphically represented as histograms. Peaks involving five or more oligonucleotide probes above threshold (2.5-fold SD above average enrichment) were considered positive. (**d**) To determine whether recruitment of BCL6 to the Arf (*CDKN2A*) promoter affects expression levels of proteins in the Arf–p53 pathway, BCR-ABL1–transformed $BCL6^{+/+}$ and $BCL6^{-/-}$ pre–B cells were analyzed by Western blotting. (**e**) BCR-ABL1–transformed pre–B cells from $ARF^{+/+}$ and $ARF^{-/-}$ mice were induced to differentiate (+STI571) or cultured under control conditions in the presence or absence of 5 µmol/liter of a peptidomimetic BCL6 inhibitor (RI-BPI; Cerchietti et al., 2009). Cell size (FSC) and viability (propidium iodide uptake) were measured by flow cytometry after 1 and 3 d (n = 3). Percentages of viable cells are indicated for each condition. FSC, forward scatter; PI, propidium iodide.

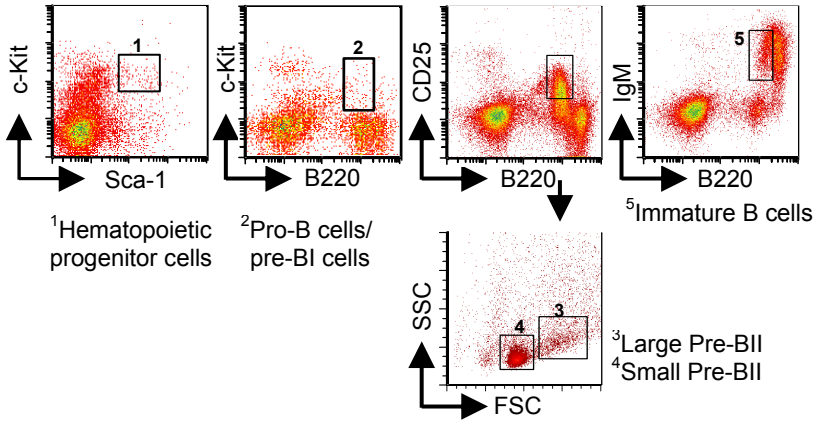
BCL6 promotes pre-B cell survival by negative regulation of ARF

Consistent with the identification of *CDKN2A* (ARF) as a direct transcriptional target of BCL6, we found that protein levels of both Arf and its downstream effector p53 are excessively increased in BCR-ABL1–transformed $BCL6^{-/-}$ pre–B cells (Figure 12d). To investigate the functional significance of transcriptional repression of ARF by BCL6, we studied the role of ARF in a genetic loss-of-function model. We tested whether pre–B cell apoptosis resulting from inhibition of BCL6 is in part mediated by excessive activation of Arf. To this end, we induced differentiation in wild-type and Arf-deficient BCR-ABL1 pre–B cells by treatment with STI571 in the presence or absence of concomitant BCL6 inhibition (RI-BPI; Figure 12e). Induced differentiation (STI571) or inhibition of BCL6 (RI-BPI) alone had little effect on the viability of both wild-type and $ARF^{-/-}$ pre–B cells. Simultaneous induction of differentiation and inhibition of BCL6, however, induced apoptosis in the vast majority of wild-type pre–B cells (viability < 1%). In contrast, a significant portion of $ARF^{-/-}$ pre–B cells survived concomitant induction of

differentiation and inhibition of BCL6 (viability > 40%; Figure 12e). We conclude that de novo expression of BCL6 at the transition from IL-7–dependent to –independent stages of B cell development is required to promote pre–B cell survival by negative regulation of Arf.

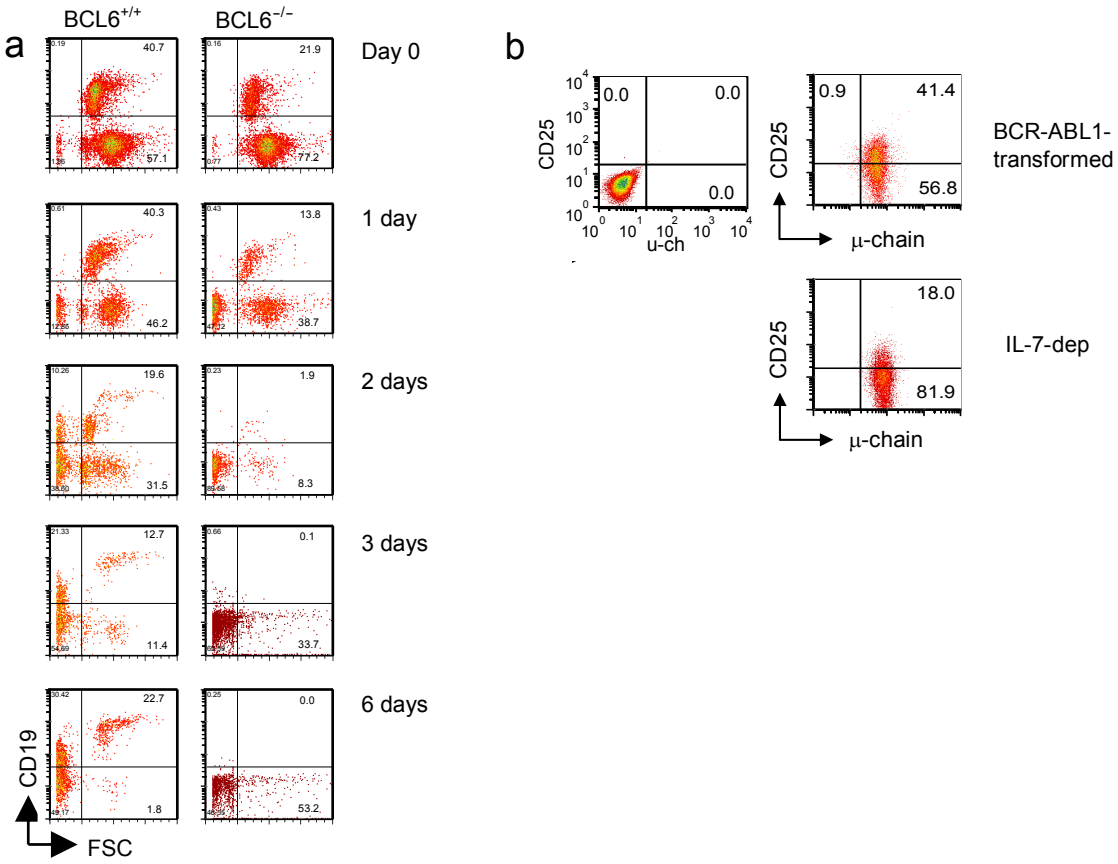
Supplemental figures in 3.1:

Figure S31.1: *Sorted subsets from mouse bone marrow*



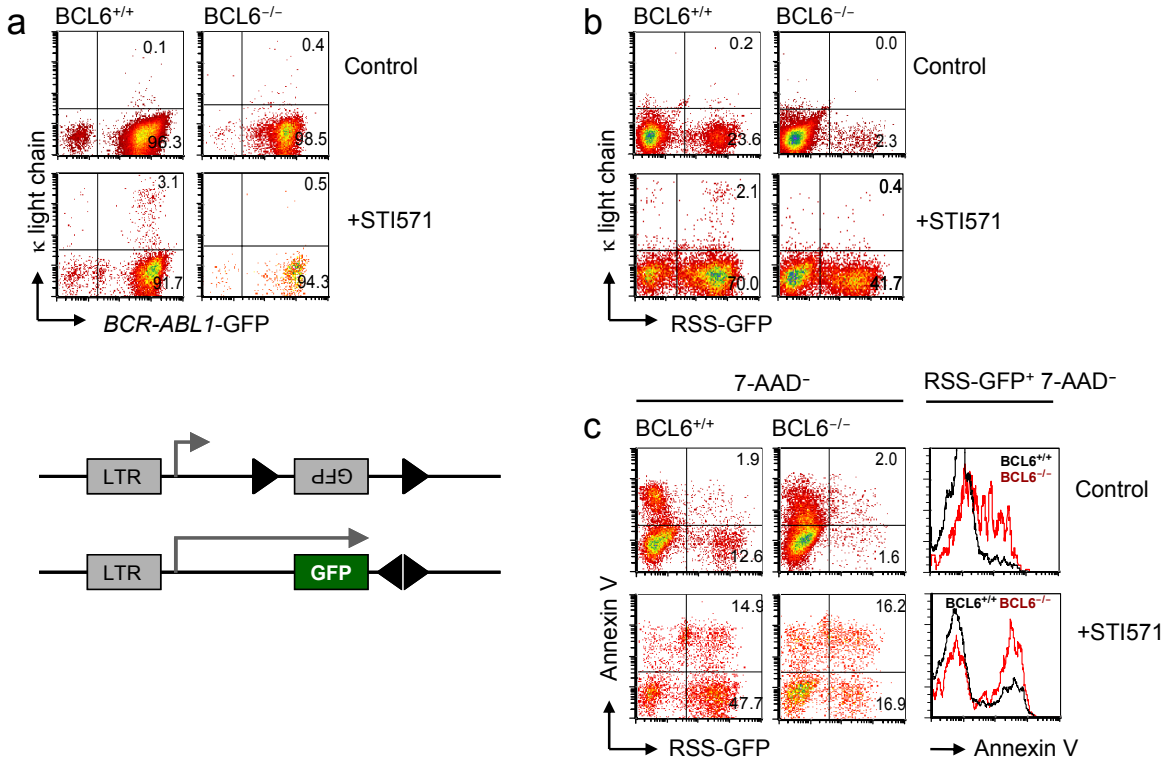
Legend: Hematopoietic progenitor cells, pro-B cells (Fraction B and C), large cycling pre-BII cells (Fraction C'), small resisting pre-BII cells (Fraction D) and immature B cells (Fraction E) were sorted from normal mouse bone marrow.

Figure S31.2: *BCL6 is required for pre-B cell self-renewal in vitro*



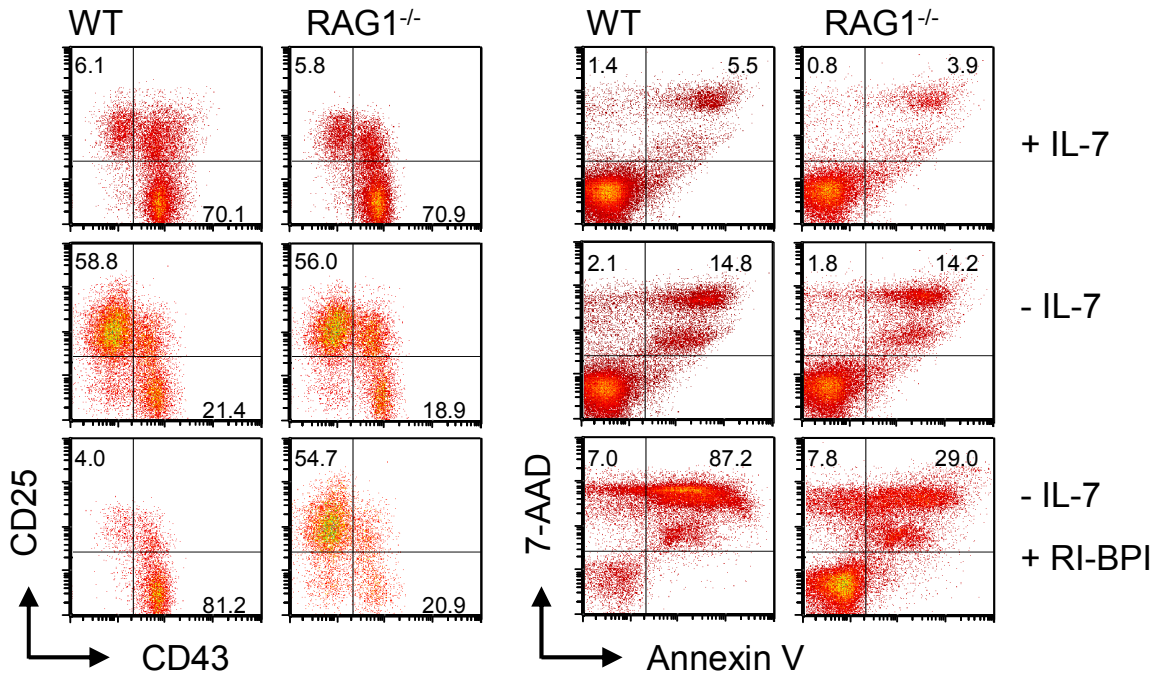
Legend: Bone marrow pre-B cells from BCL6$^{-/-}$ mice and wildtype littermates were cultured in the presence of 10 ng/ml IL-7 for the times indicated. After 1, 2, 3 and 6 days, cells were stained with antibodies against CD19 and viability was determined by propidium iodide exclusion (**a**). Numbers indicate percentages of viable CD19^{+} and CD19^{-} cells in cell culture. BCR-ABL1-transformed B cell precursors and IL-7-dependent long-term cultures (SLP65$^{-/-}$) were stained for surface expression of CD25 and intracellular expression of μ-chain (**b**). It should be noted that BCL6$^{-/-}$ pre-B cells cannot be propagated as IL7-dependent cell cultures.

Figure S31.3: *BCL6 protects pre-B cells against apoptosis during Vκ-Jκ recombination*



Legend: Bone marrow pre-B cells from BCL6$^{-/-}$ and wildtype littermates were transformed by BCR-ABL1/GFP and induced to differentiate by inhibition of BCR-ABL1 kinase activity (2 µmol/l STI571). The percentages of κ light chain expressing cells were measured after three days of treatment with and without STI571 (a; n=3). In a different set of experiments, BCL6$^{-/-}$ and BCL6$^{+/+}$ pre-B cells were transformed with a BCR-ABL1-Neo retrovirus and subsequently transduced with a recombination reporter plasmid carrying an inverted GFP gene flanked by recombination signal sequences (RSS) and a puromycin resistance gene (b and c; n=3). Transduced cells were subjected to puromycin selection and then induced to differentiate as shown in (**a**). κ light chain expression was measured in V(D)J recombining (RSS-GFP+) and non-recombining (RSS-GFP^{-}) BCL6$^{-/-}$ and wildtype pre-B cells after three days of STI571-treatment (2 µmol/l; **b**). In addition, apoptosis (Annexin V^{+}) was measured in V(D)J recombining (RSS-GFP^{+}) and non-recombining (RSS-GFP^{-}) BCL6$^{-/-}$ and BCL6$^{+/+}$ pre-B cells after three days of induced differentiation (STI571) or incubation under control conditions (**c**). The right panel in (**c**) shows a histogram overlay of Annexin V staining gated on Vκ -Jκ rearranging RSS-GFP^{+} pre-B cells from BCL6$^{-/-}$ (red histograms) and BCL6$^{+/+}$ (black histograms) mice.

Figure S31.4: *RAG1-dependent Vκ-Jκ recombination activity causes apoptotic propensity of BCL6-deficient pre-B cells*



Legend: Bone marrow pre-B cells from RAG1$^{-/-}$ mice and wildtype littermates were cultured in the presence of 10 ng/ml IL-7 (+IL-7), or IL-7 was withdrawn (-IL-7). In one set of experiments, 5 µmol/l of the peptidomimetic BCL6 inhibitor RI-BPI was added upon withdrawal of IL-7 (-IL-7 + RI-BPI). After three days, induction of differentiation was measured by flow cytometry (upregulation of CD25, downregulation of CD43) as well as induction of apoptosis (measurement of 7-AAD/Annexin V).

Note: Unlike other experiments, where *de novo* expression of κ light chains was measured as indicator for pre-B cell differentiation, this measurement was impossible here because RAG1$^{-/-}$ pre-B cells cannot rearrange Vκ- and Jκ- gene segments and therefore cannot express κ light chains. For this reason, CD25 and CD43 were chosen as positive and negative markers of differentiation, respectively.

Table S31.1: *Phenotypic characterization of bone marrow B cell precursors in BCL6$^{-/-}$ mice*

Phenotype group)	Gate	Number of cells		% Gated		% All		N (mice per	
		BCL6$^{+/+}$	BCL6$^{-/-}$	BCL6$^{+/+}$	BCL6$^{-/-}$	BCL6$^{+/+}$	BCL6$^{-/-}$	BCL6$^{+/+}$	BCL6$^{-/-}$
μ-chain^{+} CD43^{-}	PI^{-}, FSC	1,379 ± 413 p=0.013	419 ± 173	7.4 ± 2.8 p=0.109	2.3 ± 0.6	2.8 ± 0.8 p=0.013	0.8 ± 0.4	4	4
μ-chain^{+} B220^{+}	PI^{-}, FSC	1,687 ± 451 p=0.079	579 ± 168	9.1 ± 3.3 p=0.044	3.3 ± 0.6	3.4 ± 0.9 p=0.076	1.2 ± 0.3	4	4
μ-chain^{-} B220^{+}	PI^{-}, FSC	2,669 ± 641 p=0.014	1,359 ± 471	14.6 ± 4.7 p=0.039	6.8 ± 1.0	5.4 ± 1.3 p=0.024	2.6 ± 0.7	4	4
CD43^{-} B220^{+}	PI^{-}, FSC	3,218 ± 1,060 p=0.021	942 ± 525	17.4 ± 7.1 p=0.159	5.5 ± 2.9	6.5 ± 2.1 p=0.022	1.9 ± 1.1	4	4
IgD^{+} B220^{+}	PI^{-}, FSC	128 ± 49 p=0.099	65 ± 43	2.1 ± 0.3 p=0.001	0.7 ± 0.3	0.3 ± 0.1 p=0.207	0.2 ± 0.1	4	4
Igκ^{+} B220^{+}	PI^{-}, FSC	933 ± 264 p=0.024	146 ± 51	15.1 ± 3.5 p=0.033	2.1 ± 1.1	1.8 ± 0.6 p=0.016	0.4 ± 0.3	4	4

Notes: PI, propidium iodide; FSC, forward scatter (lymphocytes); mean values ± S.D. are indicated. P-values were calculated using double-sided T-test.

Table S31.2: *Clonally restricted repertoire of bone marrow and splenic B cells in BCL6$^{-/-}$ mice*

V_H	3'V_H	N1	D_H	N2	5'J_H	J_H	
BCL6$^{+/+}$ Bone marrow			B220$^+$ κ$^+$ IgD$^-$				
V1-11	tggggaa	a	ttattagcacggtagtagc	c	ctgtgactactgg	J2	
V1-11	tgtggaag	c	ttattactacggtagtagct	ttttt	actattttgactactgg	J2	
V1-11	tgtggaag	cc	ggga	tttt	actattttgactactgg	J2	
V1-11	tgtgggaga	ct	cagaccggg	gtttt	ctactttgactactgg	J2	
V1-15	tgtac	ga	tatagtaact	c	acttcgatgtctgg	J1	
V1-15	tgtacaag	g	aggct	ttt	ctactgg	J2	
V1-18	tgtgcaaga	tttaacgg	tgattacgac	caggaattt	tttgactactgg	J2	
V1-18	tgtgcaaga	ttaag	ctggg	caaatt	actactttgactactgg	J2	
V1-23	tgtacaaga	tcc	tctttgatggtt		actactttgactactgg	J2	
V1-23	tgtacaag	tg	gaccgg	cc	tactttgactactgg	J2	
V1-34	tgtgcaaga	g	tctacta	t	ttgactactgg	J2	
V1-5	tgtacaaga	c	gggattag	tttc	actttgactactgg	J2	
V1-5	tgtacaaga	at	acggtagta	a	ctactttgactactgg	J2	
V1-82	tgtgcaaga	cgg	actacggtagtagc	cc	actttgactactgg	J2	
V1-9	tgtgcaa		gggattag	ca	actggtacttcgatgtctgg	J1	
V1-9	tgtgcaa		actggg		actttgactactgg	J2	
V1-9	tgtgcaa		gtaactacggtt	cc	ggtacttcgatgtctgg	J1	
BCL6$^{+/+}$ Spleen			B220$^+$ κ$^+$ IgD$^+$				
V1-11	gaag	tca	tctacta	cc	gtacttcgatgtctgg	J1	
V1-11	tgtggaag	cccc	tctttgatggtt	caaatttt	actattttgactactgg	J2	
V1-11	g	tc	cagaccggg	cccctact	gtacttcgatgtctgg	J1	
V1-11	tgtgggaga	cg	ctacggtagtagct	gc	actttgactactgg	J2	
V1-12	tgtgcaaga		ctatgattacg	g	tgaccactgg	J2	
V1-15	tgtacaag	gc	ttattactacggtagtagct	tttt	tactttgactactgg	J2	
V1-34	tgtgcaaga	ggg	attactacggtagtagctac	gc	ctactggtacttcgatgtctgg	J1	
V1-5	tgtacaa	aa	agaatgggggctac	g	gactactgg	J2	
V1-82	tgtgcaaga	gcg	actggg	gc	tgactactgg	J2	
V1-82	tgtgcaaga		tacggtagtag	catt	gactactgg	J2	
V1-9	tgtgcaaga	tggggc	actacggtagtagc	agcccctctaagggtt	tgg	J2	
V1-9	tgtgcaaga	agggaggtga	tctactatggtaa	aggggg	ctactgg	J2	
V1-9	tgtgcaag	g	tactacggtagtagct	cccct	tactttgactactgg	J2	
V1-9	tgtgcaaga	ggcc	ctggg	g	ttgactactgg	J2	
V1-9	tgtgcaaga	ag	actacggtagtagc	c	ggtacttcgatgtctgg	J1	
BCL6$^{-/-}$ Bone marrow			B220$^+$ κ$^+$ IgD$^-$				
V1-11	**tgtggaag**	**g**	**agacagaccggg**	**gt**	**actactttgactactgg**	J2	**BCL6$^{-/-}$ Bone marrow #1**
V1-11	**tgtggaag**	**g**	**agacagaccggg**	**gt**	**actactttgactactgg**	J2	
V1-11	**tgtggaag**	**g**	**agacagaccggg**	**gt**	**actactttgactactgg**	J2	
V1-9	tgtgcaaga	c	gtaactacggtt	ttgtt	tggtacttcgatgtctgg	J1	
V1-15	**tgtacaaga**	**gg**	**aggg**	**gg**	**tactgg**	J2	
V1-15	**tgtacaaga**	**gg**	**aggg**	**gg**	**tactgg**	J2	
V1-15	**tgtacaaga**	**gg**	**aggg**	**gg**	**tactgg**	J2	
V1-15	**tgtacaaga**	**gg**	**aggg**	**gg**	**tactgg**	J2	
V1-82	tgtgcaaga	tcc	ggtgattac	acg	tttgactactgg	J2	
V1-11	tgtggaag	ggagggga	tttattactacggtag	ag	ggaacttcgatgtctgg	J1	
V1-11	tgtggaag	ggagggga	tttattactacggtag	ag	ggaacttcgatgtctgg	J1	
V1-15	tgtacaaga		gatggttact	ctc	ttgactactgg	J2	
V1-18	tgtgcaaga	ttaag	ctttgatggt	aatt	tgaccactgg	J2	
V1-9	attactgtgc	tgc	acggtagta	aagg	cttcgatgtctgg	J2	
V1-18	tattactgtgc	tt	actggg	caat	tactttgactactgg	J2	
V1-34	**tgtgcaaga**	**gatccct**	**ctacggtagt**	**ccccttttt**	**actactttgactactgg**	J2	**BCL6$^{-/-}$ Bone marrow #2**
V1-34	**tgtgcaaga**	**gatccct**	**ctacggtagt**	**ccccttttt**	**actactttgactactgg**	J2	
V1-34	**tgtgcaaga**	**gatccct**	**ctacggtagt**	**ccccttttt**	**actactttgactactgg**	J2	
V1-34	**tgtgcaaga**	**gatccct**	**ctacggtagt**	**ccccttttt**	**actactttgactactgg**	J2	
V1-82	tgtgcaaga	gaaa	tctatgatggttac	cct	tactttgactactgg	J2	
V1-9	tgtgcaaga	tcta	tctatgatggttac	ctgt	actactttgactactgg	J2	
V1-9	tgtgcaaga	tcta	tctatgatggttac	ctgt	actactttgactactgg	J2	
V1-11	tgtggaag	ct	ctacggtagt	ttcccc	tgactactgg	J2	
V1-5	tgtacaaga		tactactacggtagtagcta	tcctttct	actactttgactactgg	J2	
V1-5	tgtacaaga		tactactacggtagtagcta	tcctttct	actactttgactactgg	J2	
V1-9	tgtgcaaga		ggga	tt	actactttgactactgg	J2	
V1-11	tgtggaag	taga	agtaactac	ttgcg	ctactgg	J2	
V1-11	tgtggaag	taga	agtaactac	ttgcg	ctactgg	J2	
V1-11	tgtggaag	taga	agtaactac	ttgcg	ctactgg	J2	
V1-23	tgtacaaga	tc	ctggg	ag	ctactttgactactgg	J2	
V1-82	tgtgcaaga	gcgtcc	tctact	g	tgactactgg	J2	

BCL6$^{-/-}$ Spleen B220^{+} κ^{+} IgD^{+}

V1-11	**tgtggaag**	**g**	**agacagaccggg**	**gt**	**actactttgactactgg**	J2	
V1-12	ctgcggtctattt		**accggg**	**gt**	**actactttgactactgg**	J2	
V1-12	ctgcggtctattt		**accggg**	**gt**	**actactttgactactgg**	J2	
V1-5	actctgcggtct		**cagaccggg**	**gt**	**actactttgactactgg**	J2	
V1-15	tctattact	gccagg	**agacagaccggg**	**gt**	**actactttgactactgg**	J2	
V1-11	tctattac	cgg	**agacagaccggg**	**gt**	**actactttgactactgg**	J2	
V1-11	tgtggaagagg		tactacgggggg	gg	ctactgg	J2	
V1-15	**tgtacaaga**	**gg**	**aggg**	**gg**	**tactgg**	J2	**BCL6$^{-/-}$**
V1-12	tgtgcaa	c	**ggg**	**gg**	**tactgg**	J2	Spleen
V1-15	tgtacaaga	gg	aggtgggg	g	ctttgactactgg	J2	#1
V1-11	tgtgg		ggggg	gggg	ctttgactacggg	J2	
V1-11	tgtgg		ggggg	gggg	ctttgactactgg	J2	
V1-11	tgtggaaaagg		ctgggac	gggg	ggtacttcgatgtctgg	J1	
V1-15	tgtacaaga	gatc	atgatggttac	gggtgg	tttgactactgg	J2	
V1-11	tgtggaag	gacgga	gattacgac	gtgtgggg	actactttgactactgg	J2	
V1-15	tgta	gggggg	taactggg	t	ctttgactactgg	J2	
V1-34	**tgtgcaaga**	**gatccct**	**ctacggtagt**	**cccctttt**	**actactttgactactgg**	J2	
V1-34	**tgtgcaaga**	**gatccct**	**ctacggtagt**	**cccctttt**	**actactttgactactgg**	J2	
V1-5	tctgcggtctat	ac	**....ggtagt**	**cccctttt**	**actactttgactactgg**	J2	
V1-12	gtctatttctg	gg	**......tagt**	**cccctttt**	**actactttgactactgg**	J2	
V1-82	tgtgcaaga	gtagaggt	ctactatagta	t	ctttgactaccgg	J2	
V1-34	tgtgcaaga	ggga	actatagta	tccg	ctttgactactgg	J2	**BCL6$^{-/-}$**
V1-34	tgtgcaaga	ggga	actatagta	tccg	ctttgactactgg	J2	Spleen
V1-82	tgtgcaaga	gtagaggt	ctactatagta	t	ctttgactaccgg	J2	#2
V1-11	tgtggaa		actatgattacgac	gc	gactactgg	J2	
V1-9	tgtgcaaga	agagg	gtatggtaactac	gggaccagcc	ttgactactgg	J2	
V1-15	tgtacaaga	ggttgg	aactgg	gt	tactttgactactgg	J2	
V1-34	tgtgcaaga	tccct	ctacggtagt	tt	tttgactactgg	J2	
V1-11	tgtggaagagg	tac	ttactacggtagtagcta	tgc	ctactgg	J2	

Notes: Light shades denote immunoglobulin rearrangements that were found more than once on one sample (either bone marrow or spleen). Dark shades denote immunoglobulin gene rearrangements that were found both in the bone marrow and the spleen from the same mouse. Complete sequence data are available from EMBL/GenBank under accession numbers FN652762-FN652778.

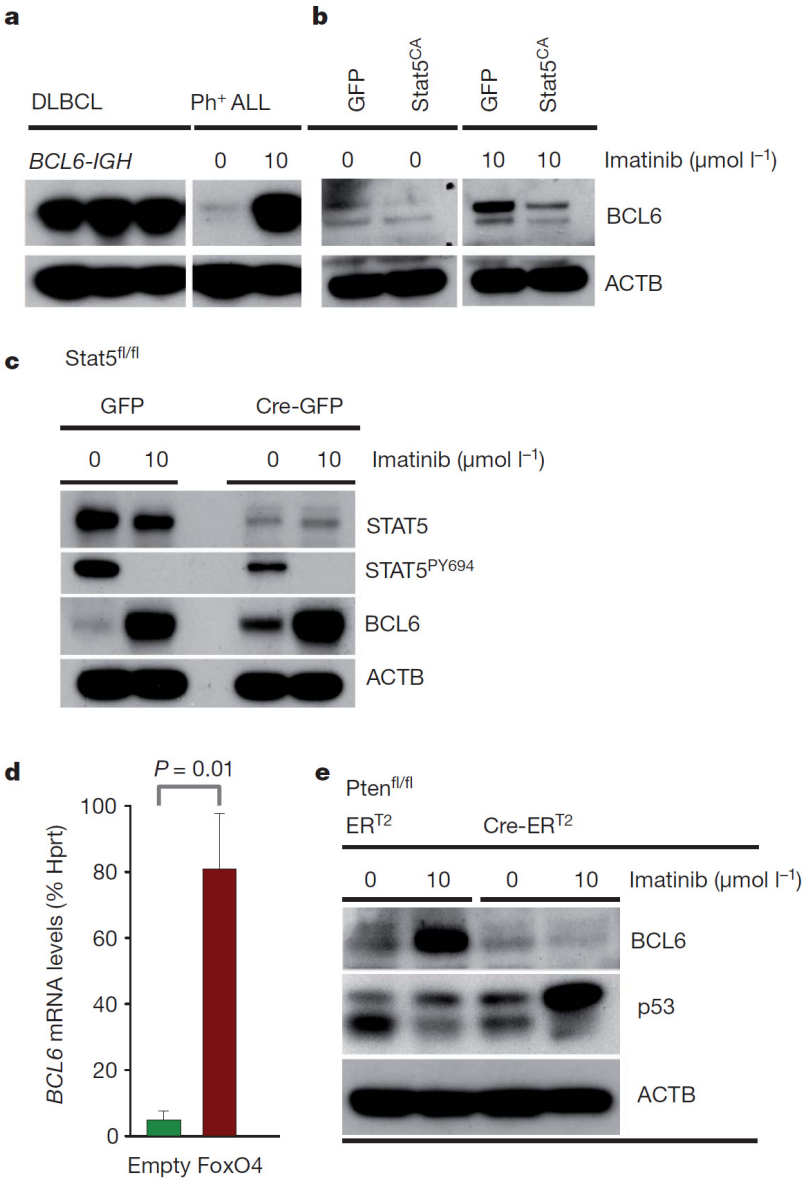
3.2 BCL6 enables Ph+ acute lymphoblastic leukaemia cells to survive BCR–ABL1 kinase inhibition

In response to TKI treatment, *BCR–ABL1* acute lymphoblastic leukaemia (ALL) cells upregulate BCL6 protein levels by approximately 90-fold: that is, to similar levels as in DLBCL (Figure 13a). To elucidate mechanisms of TKI resistance in tyrosine-kinase-driven leukaemia, we performed a gene expression analysis including our and published data of TKI-treated leukaemia (Geo dataset 2010). We identified *BCL6* as a top-ranking gene in a set of recurrent gene expression changes, some of which are shared with mitogen-activated protein-kinase kinase (MEK) inhibition in $BRAF^{V600E}$ mutant solid tumour cells (Pratilas et al., 2009) (Figure S32.1 and S32.2). TKI-induced upregulation of BCL6 mRNA levels was confirmed in multiple leukaemia subtypes carrying oncogenic tyrosine kinases (Figure S32.1). The BCR–ABL1 kinase, encoded by the Philadelphia chromosome (Ph), represents the most frequent genetic lesion in adult ALL, defines the subtype with a particularly poor prognosis (Druker et al., 2001; Shah et al., 2004; O'Hare et al., 2009) and was therefore chosen as focus for this study.

To elucidate the regulation of BCL6 in Ph^+ ALL, we investigated the JAK2/STAT5 (Choudhary et al., 2009) and PI3K/AKT (Janes et al., 2010) pathways downstream of BCR–ABL1. We and others have shown that STAT5 suppresses BCL6 in B cells (Fernandez de Mattos et al., 2004; Walker et al., 2007; Duy et al., 2010). TKI-mediated upregulation of BCL6 was diminished by constitutively active STAT5 (Figure 13b) and deletion of *STAT5* was sufficient to upregulate BCL6, even in the absence of TKI treatment (Figure 13c). In agreement with previous work (Fernandez de Mattos et al., 2004), overexpression of FoxO4 induced BCL6 (Figure 13d). In Ph^+ ALL cells, FoxO

factors are inactivated by PI3K/AKT (Janes et al., 2010) signalling, which is reversed by Pten (Figure S32.3). Deletion of *Pten*, hence, abrogated the ability of the leukaemia cells to upregulate BCL6 in response to TKI treatment (Figure 13e).

Figure 13: *Regulation of BCL6 expression in BCR–ABL1 ALL cells.*



(**a**), Ph+ ALL cells were treated with and without imatinib (10 μmol/l) for 24 h. Upregulation of BCL6 was compared with expression levels in DLBCL by western blot. (**b**), *BCR–ABL1*-transformed mouse ALL cells were transduced with a constitutively active Stat5 mutant ($STAT5^{CA}$) or a control vector (green fluorescent protein, GFP) and treated either with or without imatinib. BCL6 western blot was performed using β-actin as loading control. (**c**), BCL6 expression upon imatinib treatment was studied by western blot in the presence or absence of Cre-mediated deletion of *Stat5* in BCR–ABL1-transformed $Stat5^{fl/fl}$ mouse ALL. (**d**), Mouse *BCR–ABL1* ALL cells were transduced with FoxO4-puromycin or a puromycin control vector and subjected to antibiotic selection. Cells were collected and BCL6 mRNA levels were measured by qRT–PCR relative to Hprt. (**e**), Imatinib-induced BCL6 expression was studied

by western blot in the presence or absence of Cre-mediated deletion of *Pten* in *BCR–ABL1*-transformed $Pten^{fl/fl}$ mouse ALL cells.

In DLBCL, BCL6 is frequently translocated and suppresses p53-mediated apoptosis (Phan and Dalla-Favera 2004; Saito et al., 2007). Although TKI treatment is less effective in $p53^{-/-}$ Ph^+ ALL (Wendel et al., 2006), recent studies showed that TKI paradoxically prevents the upregulation of p53 in response to DNA damage in Ph^+ ALL and chronic myelogenous leukaemia (Goldberg et al., 2004; Skorta et al., 2009). A comparative gene expression analysis of $BCL6^{-/-}$ and $BCL6^{+/+}$ ALL cells (Figure S32.4) identified Cdkn2a (Arf), Cdkn1a (p21), p53 and p53bp1 as potential BCL6 target genes (Figure S32.5). Arf and p53 protein levels were indeed unrestrained in $BCL6^{-/-}$ ALL (Figure 14a). TKI treatment of $BCL6^{+/+}$ ALL resulted in strong upregulation of BCL6 with low levels of p53, whereas $BCL6^{-/-}$ ALL cells failed to curb p53 expression levels (Figure S32.6). Likewise, TKI treatment increased excessively p53 levels when *Pten*-deficient ALL cells failed to upregulate BCL6 (Figure 13e).

Identifying direct targets of BCL6 by chromatin immunoprecipitation (ChIP) in Ph^+ ALL (Figure S32.7–10), p53, p21 and p27 were among the genes with the strongest recruitment of BCL6 in TKI-treated ALL (Figure 14b and Figure S32.8–10). Given that cell-cycle arrest and senescence-associated genes were among the BCL6 targets, we studied the cell-cycle profile of leukaemia cells. $BCL6^{-/-}$ ALL cells divided at a slightly reduced rate compared with $BCL6^{+/+}$ ALL cells (Figure 14c). Treatment with adriamycin (0.05 μg ml^{-1}) had no significant effect on $BCL6^{+/+}$ ALL cells in a senescence-associated β-galactosidase assay (Kamijo et al., 1997; Braig et al., 2005) but revealed that most $BCL6^{-/-}$ leukaemia cells were poised to undergo cellular senescence (Figure 14d). These

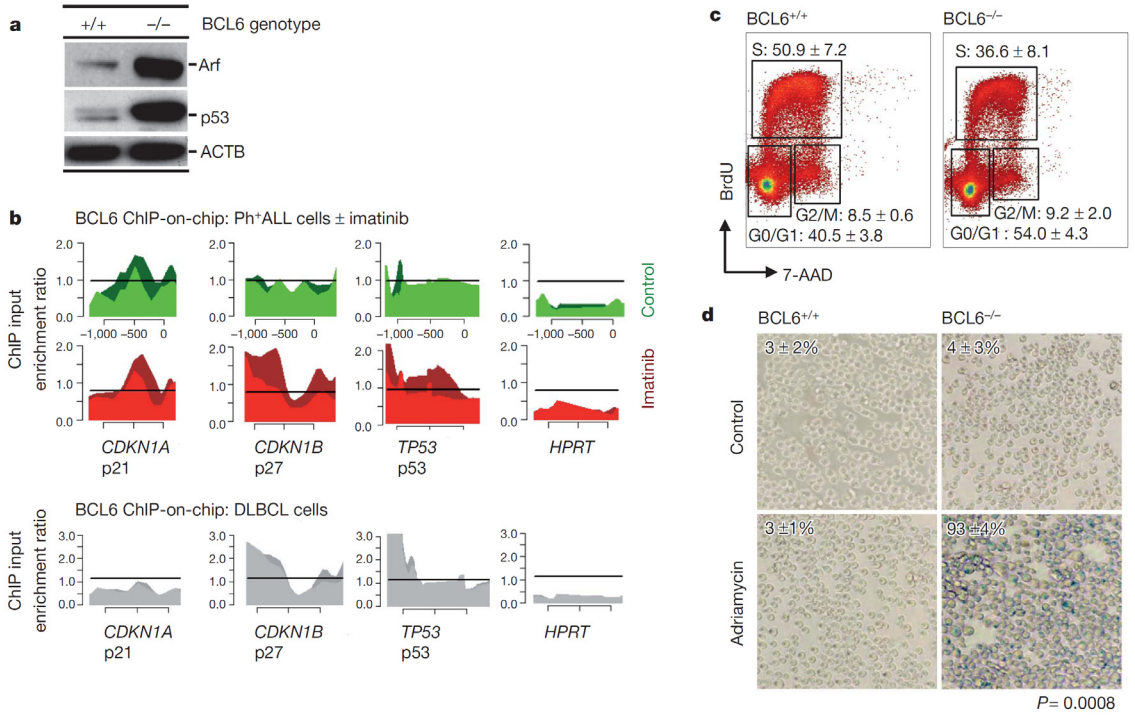
findings demonstrate that even low levels of BCL6 in the absence of TKI treatment are critical to downregulate Arf/p53.

Clonal evolution of leukaemia involves acquisition of genetic lesions through DNA damage (Mullighan et al., 2008). Interestingly, a comparative genomic hybridization analysis revealed that genetic lesions were less frequent in BCL6 deficient ALL (Figure S32.11), suggesting that increased sensitivity to DNA damage limits clonal evolution in the absence of BCL6. Because Arf and p53 are critical negative regulators of self-renewal (Williams et al., 2006), we performed colony-forming assays. The colony frequencies of $BCL6^{-/-}$ ALL cells were reduced by approximately 20-fold compared with $BCL6^{+/+}$ ALL (Figure 15a). To study self-renewal *in vivo*, we measured the ability of $BCL6^{+/+}$ and $BCL6^{-/-}$ ALL cells to initiate leukaemia in transplant recipients (Figure 15b). Using luciferase bioimaging, leukaemia engraftment was observed in both groups after 8 days. $BCL6^{+/+}$ ALL cells rapidly expanded and initiated fatal leukaemia, whereas $BCL6^{-/-}$ ALL cells failed to expand from the initial engraftment foci (Figure 15c). Some mice that received $BCL6^{-/-}$ ALL cells ultimately succumbed to leukaemia (Figure 15b). Flow cytometry, however, revealed that the leukaemias in the $BCL6^{-/-}$ group were in fact derived from endogenous $CD45.1^+$ cells of the irradiated recipients and not from the injected $CD45.2^+$ donor ALL cells (Figure S32.12 and asterisks in Figure 15b).

Defective leukaemia initiation may be a consequence of impaired homing to the bone marrow niche. Indeed, $BCL6^{-/-}$ ALL cells lack expression of CD44 (Figure S32.13), which is critical for homing of *BCR–ABL1* LICs to the bone marrow microenvironment (Krause et al., 2006). Retroviral reconstitution of CD44 markedly

increased homing of BCL6$^{-/-}$ ALL cells to the bone marrow niche, but failed to rescue defective leukaemia initiation (Figure S32.13).

Figure 14: *BCL6 is required for transcriptional inactivation of the Arf/p53 pathway in BCR-ABL1$^+$ ALL*



(**a**), Western blot analysis of CDKN2A (Arf) and p53 expression in BCL6$^{-/-}$ and BCL6$^{+/+}$ *BCR–ABL1* ALL cells. (**b**), Human Ph$^+$ ALL cells (Tom1) were treated with and without imatinib (10 µmol/l) for 24 h and were subjected to ChIP-on-chip analysis using a BCL6-specific antibody. The y axis indicates enrichment versus input, the x axis the location of probes within the respective loci relative to the transcriptional start site. The dark and light green (control) or red (imatinib) tracings depict two replicates. Recruitment to *CDKN1A*, *CDKN1B*, *TP53* and *HPRT* (negative control) is shown in Ph$^+$ ALL cells and one DLBCL cell line (OCI-Ly7). (**c**), Cell-cycle analysis (BrdU/7-AAD staining). (**d**), Staining for senescence-associated β-galactosidase (SA-β-gal). ALL cells were treated with or without 0.05 µg/ml adriamycin for 48 h to induce a low level of DNA damage. Percentages of SA-β-gal$^+$ cells are indicated (means ± SD; n=3).

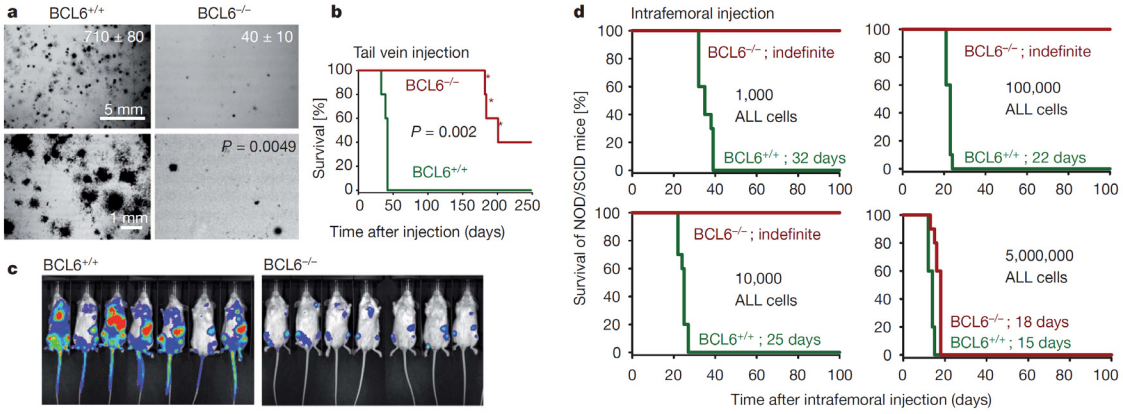
Using intrafemoral injection to circumvent homing defects, a limiting dilution experiment (Figure 15d) showed that 5 million $BCL6^{-/-}$ ALL cells compared with only 10^3 $BCL6^{+/+}$ ALL cells were needed to initiate fatal leukaemia. These findings suggest that the frequency of LIC in $BCL6^{-/-}$ ALL (fewer than 1 in 100,000) is reduced by more than 100-fold compared with $BCL6^{+/+}$ ALL (at least 1 in 1,000). An alternative interpretation would be that LICs occur at a similar frequency in $BCL6^{-/-}$ ALL but with reduced self-renewal activity. To address potential 'exhaustion' of LICs, we performed a serial transplantation with ALL cells that gave rise to disease in primary recipients after injection of 5 million ALL cells. From the bone marrow, we isolated $CD19^+$ ALL cells for secondary intrafemoral injection. $BCL6^{-/-}$ leukaemia was not transplantable in secondary recipients (Figure S32.14). Although these findings do not exclude the possibility that the LIC frequencies are reduced in $BCL6^{-/-}$ ALL, they support the notion of LIC 'exhaustion' after secondary transplantation.

To explore the therapeutic usefulness of pharmacological inhibition of BCL6, we tested a BCL6 inhibitor (*retro-inverso* BCL6 peptide-inhibitor (RI-BPI)), which blocks the repressor activity of BCL6 (Cerchietti et al., 2009b). Gene expression analysis confirmed that RI-BPI is a selective and potent inhibitor of BCL6 (Figure S32.15). We investigated the effect of RI-BPI on the self-renewal capacity of primary Ph^+ ALL and the initiation of leukaemia in a mouse xenograft model. Treatment with RI-BPI resulted in a reduction of colony formation and delayed progression of leukaemia. Likewise, treatment of Ph^+ ALL with RI-BPI induced cellular senescence (Figure S32.16).

We next examined how gene dosage of BCL6 affects responses to TKI. For instance, $Pten^{-/-}$ ALL cells lack the ability to upregulate the p53-repressor BCL6 and are more sensitive to imatinib (Figure 13e and Figure S32.17). Dose–response studies in

BCL6$^{+/+}$, BCL6$^{+/-}$ and BCL6$^{-/-}$ ALL (Figure 16a) showed that sensitivity to imatinib was significantly increased in BCL6$^{-/-}$ (half-maximal effective concentration (EC_{50}) 0.17 µmol l^{-1}) and even in BCL6$^{+/-}$ ALL cells (EC_{50} 0.67 µmol l^{-1}) compared with BCL6$^{+/+}$ ALL cells (EC_{50} 1.10 µmol l^{-1}; Figure 16a). These findings indicate that maximum levels of BCL6 expression are required to prevent TKI-induced cell death. Indeed, inducible activation of BCL6-ER^{T2} constructs (Shaffer et al., 2000) in BCL6$^{-/-}$ ALL cells conferred a strong survival advantage in the presence of imatinib (Figure 16b). Activation of BCL6 in BCL6$^{+/+}$ ALL cells induced cell-cycle exit (not shown) and no additional survival advantage, because these cells already achieved maximal upregulation of endogenous BCL6 (Figure 13a).

Figure 15: *BCL6 is required for leukemia-initiation in BCR-ABL1$^+$ ALL*



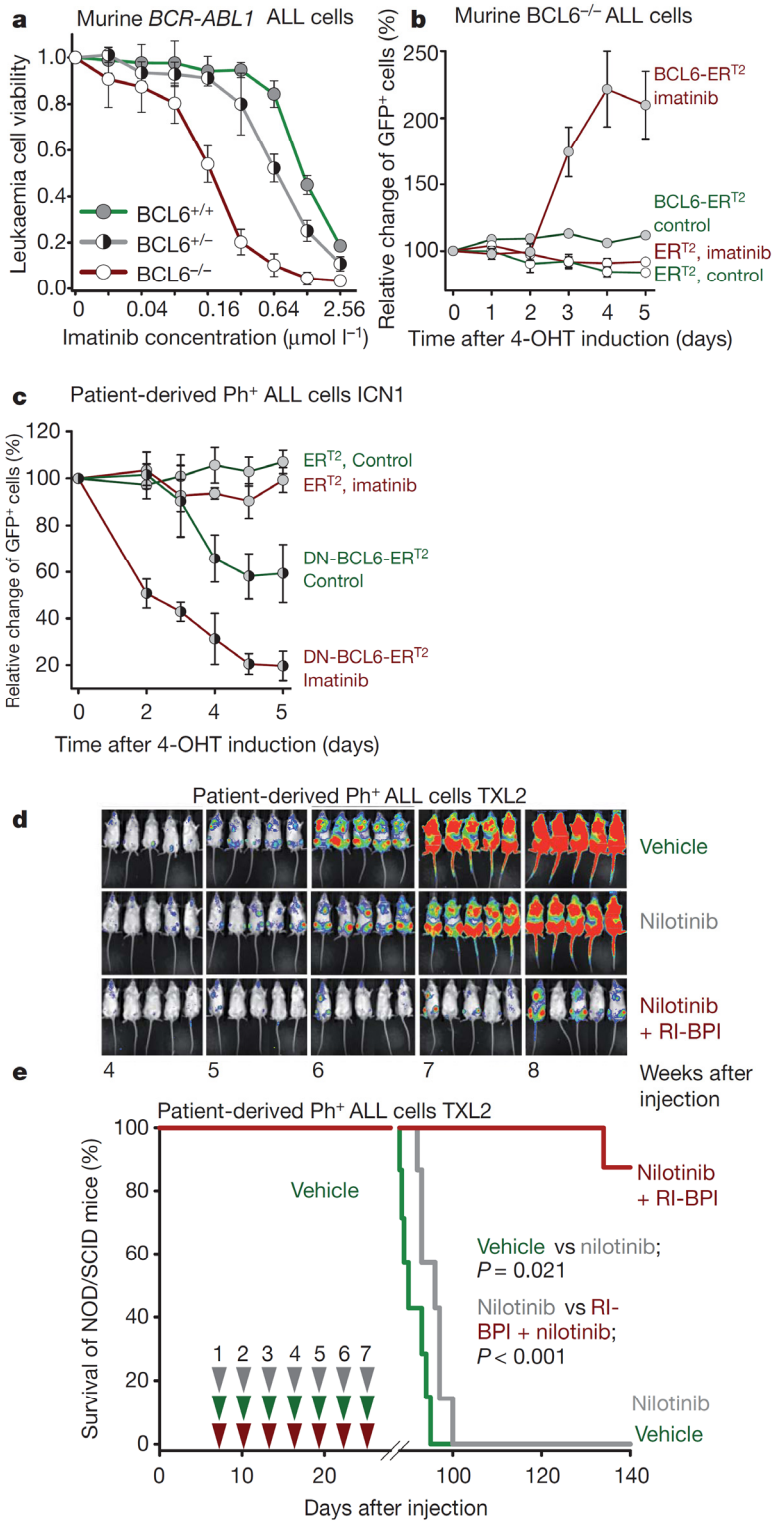
(**a**), Ten thousand BCL6$^{-/-}$ or BCL6$^{+/+}$ *BCR–ABL1* ALL cells were plated in semisolid agar, and colonies were counted after 10 days (numbers denote means ± SD, n=3). (**b**), Overall survival of mice injected with 100,000 BCL6$^{-/-}$ and BCL6$^{+/+}$ *BCR–ABL1* ALL cells was compared by Kaplan–Meier analysis. Mice that developed CD45.1$^+$ endogenous leukaemia instead of leukaemia from injected CD45.2$^+$ cells are indicated by asterisks (see Figure S32.12). (**c**), For an SCID LIC (SL-IC) experiment, BCL6$^{-/-}$ and BCL6$^{+/+}$ *BCR–ABL1* ALL cells were labelled with firefly luciferase and intravenously injected into sublethally irradiated NOD/SCID mice. (**d**), The SL-IC assay was repeated as a limiting dilution experiment (10^3, 10^4, 10^5, 5 million cells) and leukaemia cells were directly injected into the femoral bone marrow to circumvent potential engraftment defects.

To address the role of BCL6-mediated repression of p53 in TKI-resistance, $p53^{-/-}$ and $p53^{+/+}$ ALL cells were treated with RI-BPI. The synergistic effect between TKI treatment and RI-BPI is indeed partly p53 dependent (Figure S32.18). In $p53^{-/-}$ ALL cells, the effect of RI-BPI was significantly diminished compared with $p53^{+/+}$ ALL.

To confirm that BCL6 has a similar function in patient-derived Ph^+ ALL, primary ALL cells were transduced with a dominant-negative BCL6 mutant (DN-BCL6-ER^{T2}) (Shaffer et al., 2000), which resulted in a marked competitive disadvantage of Ph^+ ALL cells, that was further enhanced by imatinib treatment (Figure 16c). Similar observations in mouse ALL and in an established Ph^+ ALL cell line demonstrated that BCL6 promotes survival of TKI-treated Ph^+ ALL (Figure S32.19).

To test the effect of BCL6 inhibition on TKI resistance, we cultured four primary Ph^+ ALL in the presence or absence of imatinib, RI-BPI or a combination of both (Figure S32.20). Initially, all four Ph^+ ALL cases responded to imatinib treatment, but subsequently rebounded and were no longer sensitive to imatinib (10 μmol l^{-1}). RI-BPI alone showed only slight effects, whereas the combination of RI-BPI and imatinib rapidly induced cell death and effectively prevented a rebound in all four cases (Figure S32.20). These findings suggest that prolonged treatment with a combination of imatinib/RI-BPI prevents acquisition of TKI-resistance. We next examined the effect of imatinib/RI-BPI combinations on primary TKI-resistance in Ph^+ ALL. To this end, four human Ph^+ ALL cell lines that lacked *BCR–ABL1* kinase mutations (Table S1) but which were highly refractory to imatinib (10 μmol 1^{-l}) were treated with or without imatinib, RI-BPI or a combination of both.

Figure 16: *BCL6 promotes survival of TKI-treated BCR-ABL1+ ALL cells*



(**a**), Imatinib sensitivity of BCL6$^{-/-}$, BCL6$^{+/-}$ and BCL6$^{+/+}$ ALL cells was measured in a resazurin viability assay. (**b**), BCL6$^{-/-}$ ALL cells were transduced with BCL6-ER^{T2} or ER^{T2} vectors (tagged with GFP). ALL cells were treated with or without 1 µmol/l imatinib, and BCL6-ER^{T2} or ER^{T2} were induced by 4-hydroxytamoxifen. Relative changes of GFP^{+} cells after induction are indicated. (**c**), Patient-derived Ph^{+} ALL cells (ICN1) were transduced with inducible dominant-negative BCL6 (DN-BCL6-ER^{T2}) or ER^{T2} control vectors. ALL cells were treated with or without 10 µmol/l imatinib and DN-BCL6-ER^{T2} or ER^{T2} were induced by 4-hydroxy-tamoxifen.Relative changes of GFP^{+} cells after induction are indicated. (**d**), Patient-derived Ph^{+} ALL cells (TXL2) were labelled with luciferase and 100,000 cells were injected. Mice were treated seven times with either vehicle (green), nilotinib (25mg/kg; grey) or a combination of nilotinib and RI-BPI (25 mg/kg; red). Treated mice are shown in (**e**), a Kaplan–Meier survival analysis. Treatment days are indicated by arrowheads.

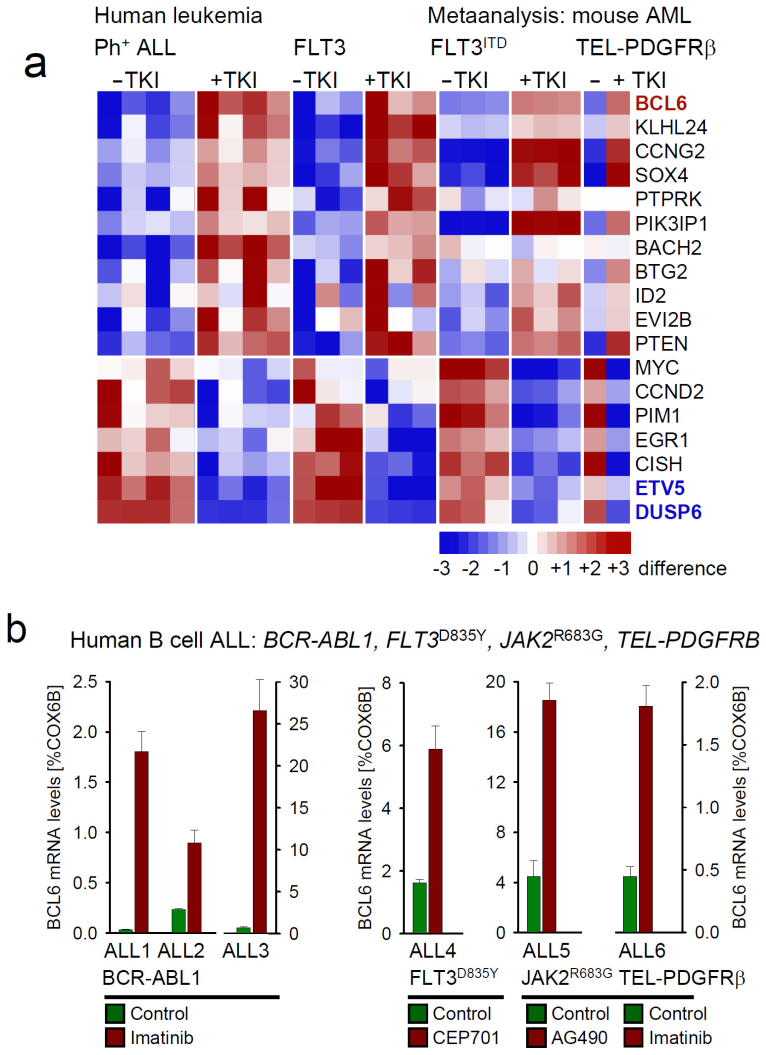
Imatinib alone did not achieve a therapeutic response, whereas the combination with RI-BPI potentiated the effect of imatinib on the refractory ALL cells (Figure S32.21).

To study the efficacy of combined tyrosine kinase and BCL6 inhibition *in vivo*, primary Ph+ ALL cells were labelled with luciferase and xenografted into mice. Recipient mice were treated with either vehicle, nilotinib or a combination of nilotinib and RI-BPI. Nilotinib is more potent than imatinib, which only has marginal effects in mice (Williams et al., 2007; Gruber et al., 2010). Bioimaging demonstrated that seven to ten injections of RI-BPI significantly enhanced the effect of nilotinib (Figure 16d,e and Figure S32.22). Whereas all mice treated with nilotinib alone succumbed to leukaemia within 99 days after injection, seven of eight mice treated with RI-BPI/nilotinib combination were still alive after 140 days (Figure 16d,e). Also, in a model for full-blown mouse leukaemia, TKI/RI-BPI combinations proved effective and significantly prolonged survival (Figure S32.23). Establishing a potential therapeutic window of nilotinib/RI-BPI combinations, we found no evidence of relevant toxicity (Figure S32.24 and S32.25 and Table S3).

Although transcription factors have been considered intractable therapeutic targets, the recent development of a small molecule inhibitor against BCL6 (Cerchietti et al., 2010) holds promise for effectively targeting TKI-resistance in patients with Ph+ ALL. Because TKI-resistance develops in virtually all cases of Ph+ ALL, it appears particularly important to target this novel pathway of TKI-resistance.

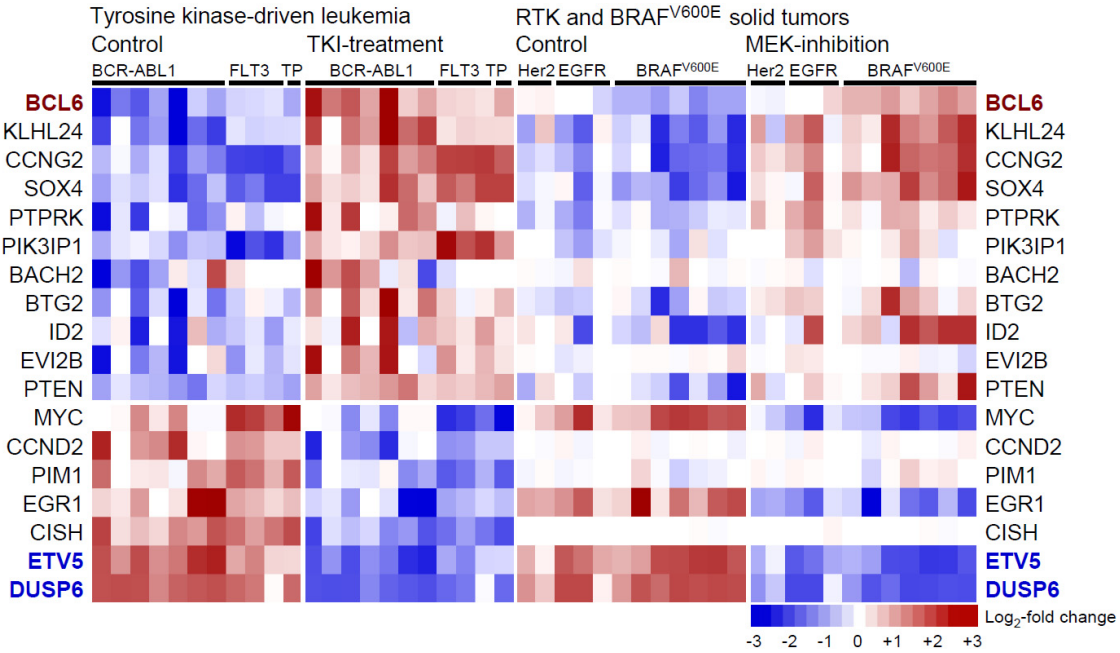
Supplemental figures in 3.2:

Figure S32.1*: Tyrosine kinase-driven leukemia cells respond to oncogene withdrawal by upregulation of BCL6*



(**a**) Seven human leukemia cell lines carrying the BCR-ABL1 kinase (4 Ph$^+$ ALL, 3 CML) were treated with or without Imatinib (10 µmol/l) for 16 hours. In addition, a meta-analysis of gene expression data of mouse myeloid leukemia cells carrying FLT3^{ITD} and TEL-PDGFRβ kinases that were treated with MLN518 (0.5 µmol/l, 4 hours) or Imatinib (5 µmol/l, 4 hours) is shown. Gene expression data were sorted based on ratio [log_2] of gene expression values in treated vs untreated leukemia cells. Microarray data used in this analysis are available from GEO under accession numbers GSE23743, GSE11794 and GSE24493. (**b**) TKI-induced upregulation of BCL6 was confirmed by quantitative RT-PCR in six cases of tyrosine kinase-driven leukemia (3 Ph$^+$ ALL, 1 *FLT3*^{D835Y} ALL, 1 *JAK2*^{R683G} ALL, 1 *TEL-PDGFR*β ALL; means of triplicate measurements ± SD).

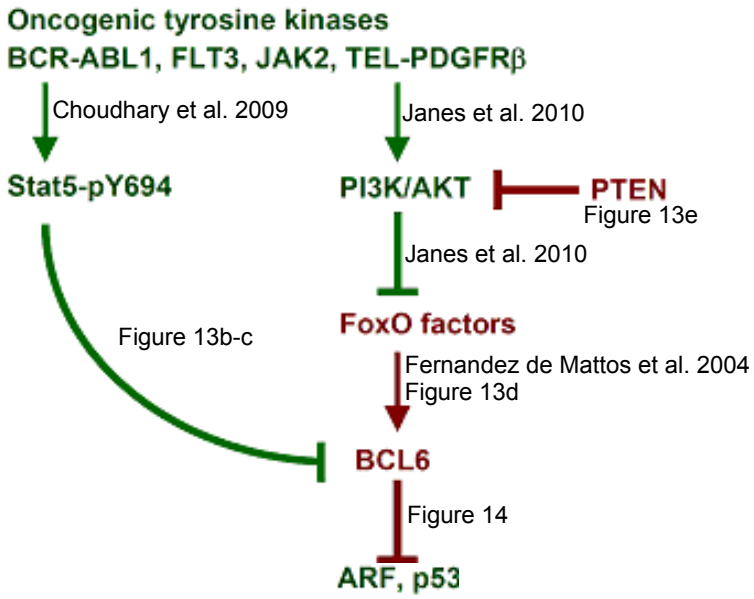
Figure S32.2: *Similarities between TKI-induced gene expression changes in leukemia cells and MEK inhibition in BRAF^{V600E} mutant solid tumors*



A set of genes with a similar pattern of regulation by TKI across multiple oncogenic tyrosine kinases was identified (see Figure S32.1a). Gene expression values in BCR-ABL1 (7 Ph$^+$ ALL), FLT3^{ITD} AML (FLT3; n=3) and TEL-PDGFRβ AML (TP; n=1) before and after TKI-treatment were plotted against gene expression values in solid tumor cells expressing oncogenic receptor-type tyrosine kinases (RTK) or mutant BRAF^{V600E} (GEO accession number GSE10086). Oncogenic RTK include Her2-overexpressing breast cancer (n=2; BT474, SKBR3), EGFR-mutant bronchioalveolar adenocarcinoma (n=1; H1650) and EGFR-overexpressing epidermoid and breast cancer (n=2; A431, MDA468) cell lines. EGFR-mutant and –overexpressing cell lines are collectively denoted as “EGFR”.

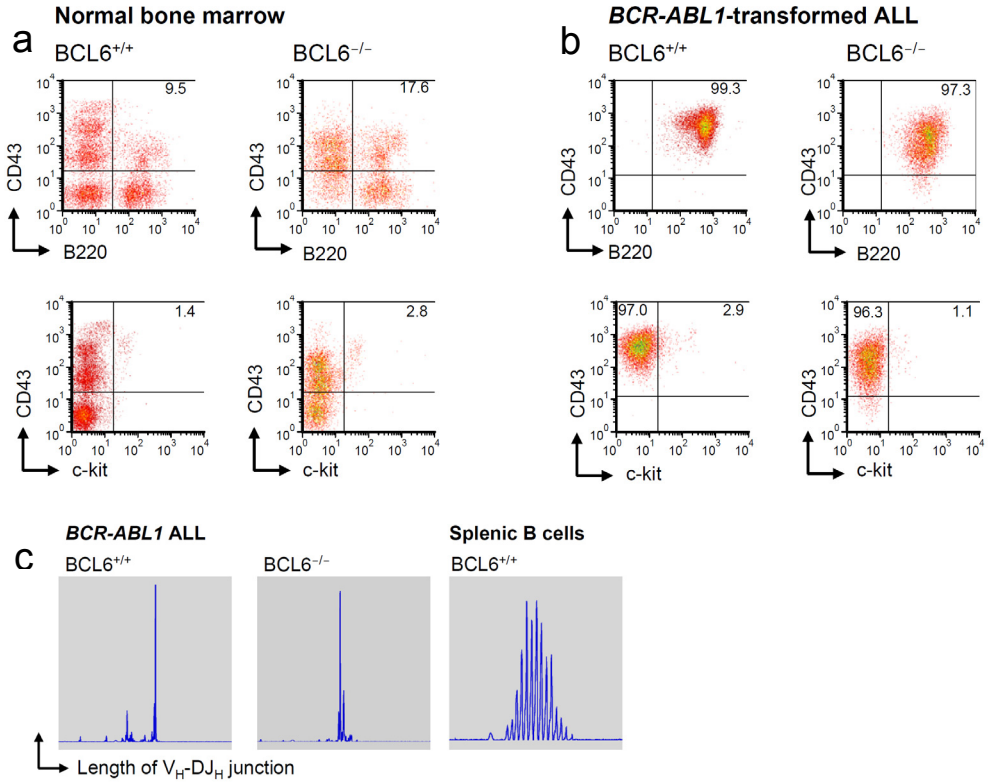
Human leukemia cell lines carrying the BCR-ABL1 kinase were treated with Imatinib (10 µmol/l) for 16 hours. Mouse leukemia cells carrying FLT3^{ITD} and TEL-PDGFRβ kinases were treated with MLN518 (0.5 µmol/l, 4 hours) or Imatinib (5 µmol/l, 4 hours). MEK-inhibition was performed with 50 nmol/l PD0325901 for 8 hours.

Figure S32.3: *Regulation of BCL6 expression in tyrosine kinase-driven leukemias*



Oncogenic tyrosine kinases negatively regulate BCL6 expression via Stat5 (direct repression of BCL6 transcription (Walker et al. 2007 and Figure 13b-c) and PI3K/AKT (inactivation of FoxO factors; Janes et al. 2010). PTEN antagonizes PI3K/AKT function and thereby favors activation of FoxO factors and hence, transcriptional activation of BCL6. Here we show that PTEN is required for transcriptional activation of BCL6 (Figure 13e) and, hence, BCL6-mediated repression of ARF and p53 (Figure 14).

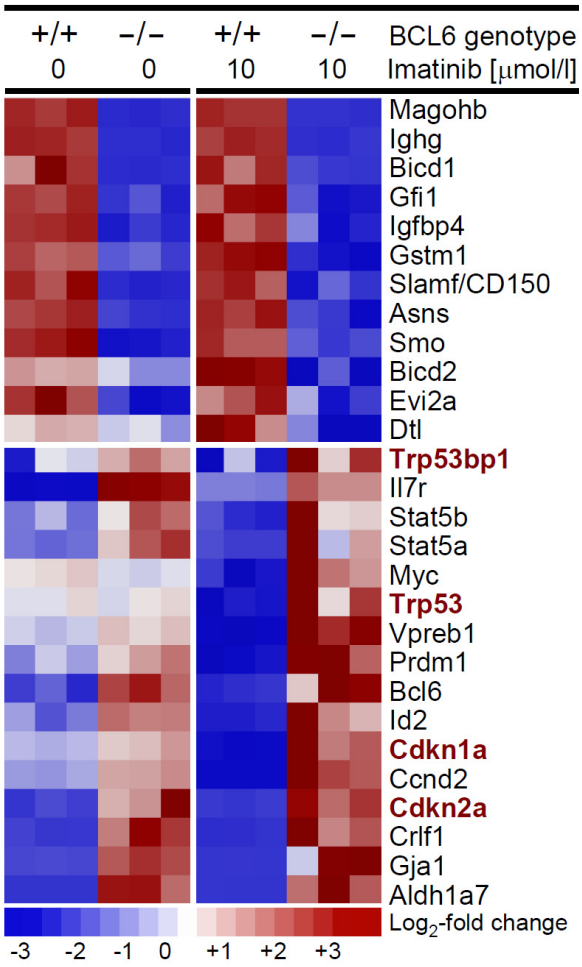
Figure S32.4: *BCR-ABL1 transforms comparable B cell precursor subsets in BCL6$^{+/+}$ and BCL6$^{-/-}$ mice*



Rationale: This experiment addresses the question of whether the same cell type is transformed by *BCR-ABL1* in BCL6$^{+/+}$ and BCL6$^{-/-}$ bone marrow. While late pre-BII cells are diminished in BCL6$^{-/-}$ mice (Duy et al., 2010), the pro- and pre-BI subsets, which represent the target of *BCR-ABL1*-mediated transformation, are slightly expanded in the bone marrow of BCL6$^{-/-}$ mice (**a**).

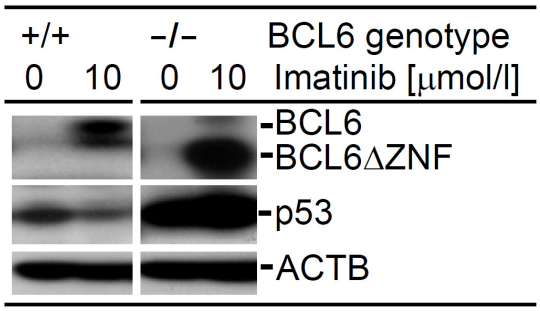
The CD43^{+} B220^{+} c-Kit$^{low/-}$ pro-/pre-BI cell population represents the target of *BCR-ABL1*-mediated transformation and is present both in BCL6$^{+/+}$ and BCL6$^{-/-}$ bone marrow (**a**). *BCR-ABL1*-transformed ALL cells from BCL6$^{+/+}$ and BCL6$^{-/-}$ bone marrow have a similar pro-/pre-BI cell phenotype and express high levels of CD43 and B220 and low levels of the pro-B cell antigen c-Kit (**b**). As determined by immunoglobulin heavy chain spectratyping, both BCL6$^{+/+}$ and BCL6$^{-/-}$ BCR-ABL1 ALL cells carry a clonal V_H-DJ_H rearrangement. Splenic B cells were used as positive control to depict a polyclonal repertoire of V_H-DJ_H junctions (**c**).

Figure S32.5: *BCL6-dependent gene expression changes in BCR-ABL1+ ALL cells*



Gene expression changes in response to Imatinib-treatment were studied in $BCL6^{-/-}$ and $BCL6^{+/+}$ *BCR-ABL1* ALL cells by Affymetrix GeneChip analysis and data were sorted based on the ratio of gene expression values in $BCL6^{-/-}$ and $BCL6^{+/+}$ *BCR-ABL1* ALL cells in the presence of Imatinib. Microarray data used in this analysis are available from GEO under accession number GSE20987.

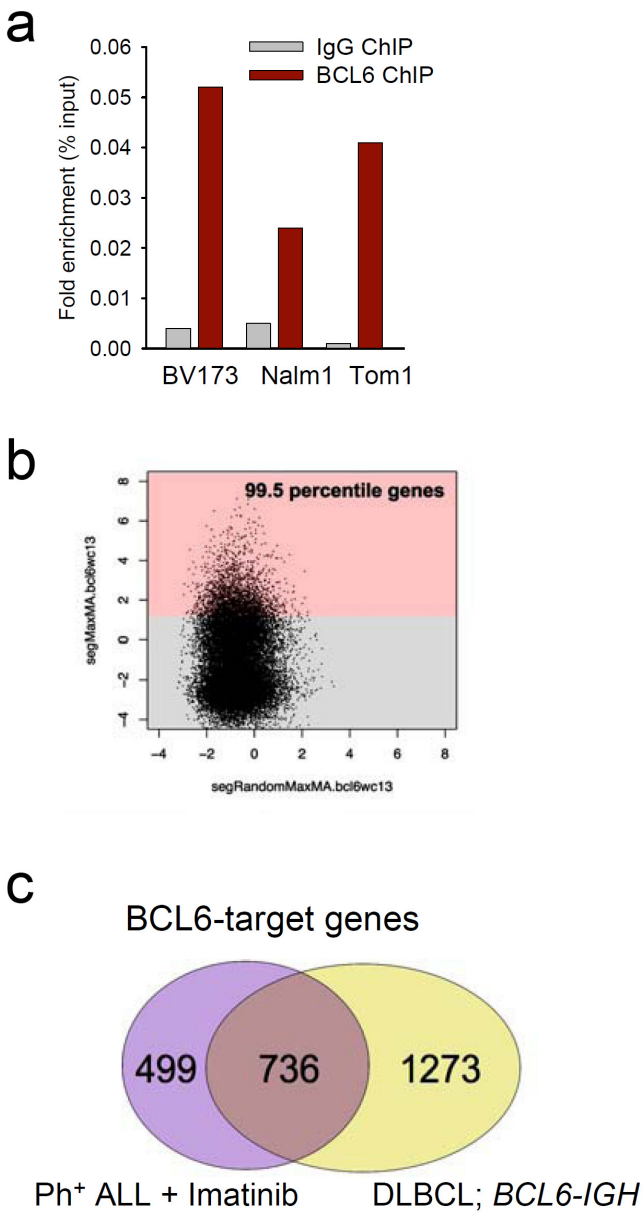
Figure S32.6: *TKI-treatment results in BCL6-mediated downregulation of p53*



Previous studies showed that treatment of BCR-ABL1 leukemia cells with Imatinib paradoxically results in inhibition of p53 accumulation in response to DNA damage. Likewise, treatment of BCR-ABL1 ALL cells with 10 µmol/l Imatinib resulted in downregulation of p53 in parallel with BCL6 upregulation (left panel). In $BCL6^{-/-}$ BCR-ABL1 ALL, p53 levels are constitutively high and further increased by Imatinib-treatment (right panel). Hence, BCL6 is required for Imatinib-induced downregulation of p53 in Ph^+ ALL.

For Western blotting, antibodies against BCL6 and p53 were used and β-actin as loading control. BCL6ΔZNF denotes a truncated protein that is still expressed in "$BCL6^{-/-}$" mice, in which critical zinc finger domains of BCL6 are deleted (Ye et al., 1997).

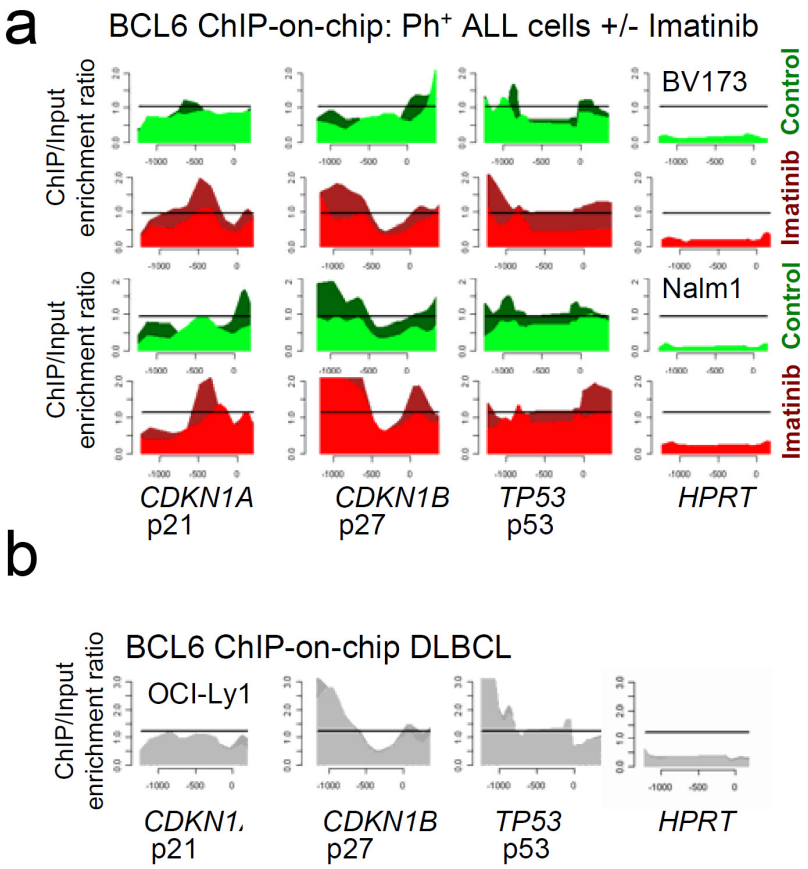
Figure S32.7: *BCL6 ChIP-on-chip analysis of Ph+ ALL cell lines*



(**a**) ChIP was performed with BCL6 antibodies followed by QPCR to detect the BCL6 binding site in exon 1 of the *BCL6* locus in TOM-1, NALM-1 and BV-173 ALL cell lines treated with Imatinib. Treatment with Imatinib induces BCL6 expression. Red bars denote BCL6- ChIP and gray bars denote control IgG. (**b**) A random permutation analysis was performed using a sliding window of three oligos to identify BCL6 binding peaks in the Ph+ ALL cell lines (the panel shows one of the Nalm1 replicates as an example). The graph shows the non-zero centered comparison between BCL6 peaks (y-axis) vs. a random permutation of the oligos (x-axis). The red field at the top

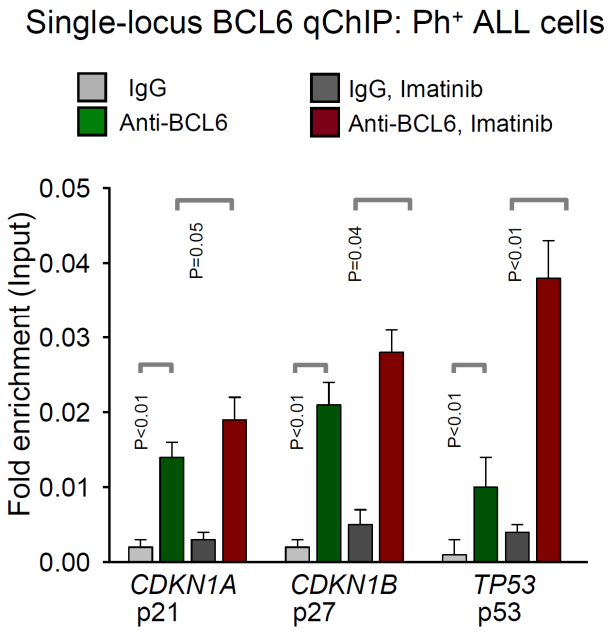
denotes those peaks scoring > than 99.5 percentile, which captured ~1,300 genes as being true positive BCL6 targets in Nalm1 cells. Single locus validation confirmed > 95% accuracy.

Figure S32.8: *Specific recruitment of BCL6 to CDKN1A, CDKN1B and TP53 promoters in Ph+ ALL cells*



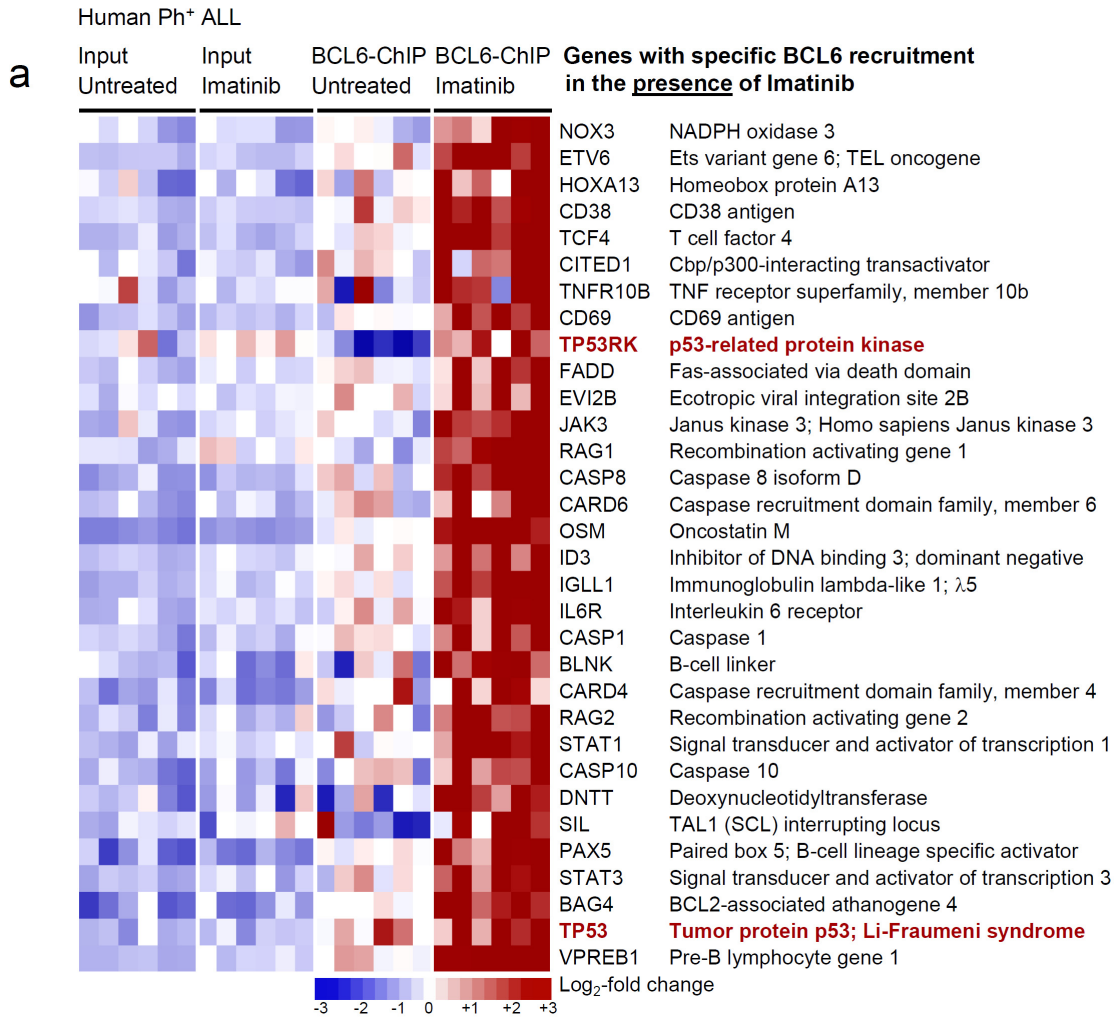
Three Ph+ ALL cell lines (BV173, Nalm1, Tom1) were treated in the presence or absence of Imatinib (10 µmol/l) and subjected to BCL6 ChIP-on-chip analysis. BV173 and Nalm1 are shown here, ChIP profiles for Tom1 are shown in Figure 14. The y-axis indicates enrichment versus input and the x-axis the location of probes within the respective loci relative to the transcriptional start site. The dark and light green (Control) or red (Imatinib) tracings depict two replicates. Recruitment to *CDKN1A*, *CDKN1B*, *TP53* and *HPRT* (negative control) is shown in (**a**) Ph+ ALL cell lines BV173 and Nalm1 and (**b**) one DLBCL cell line (OCI-Ly1).

Figure S32.9: *Single-locus quantitative ChIP verification of BCL6 recruitment to CDKN1A, CDKN1B and p53 promoters*



Single locus quantitative ChIP shows recruitment of BCL6 to *CDKN1A* (p21), *CDKN1B* (p27) and *TP53* promoters. Means of fold-enrichment (duplicate measurements for three cell lines, n=3; means ± SD) are indicated. The cell lines used are BV173, Nalm1 and TOM1.

Figure S32.10: *BCL6 target genes with specific recruitment of BCL6 in Imatinib-treated Ph+ ALL cells*

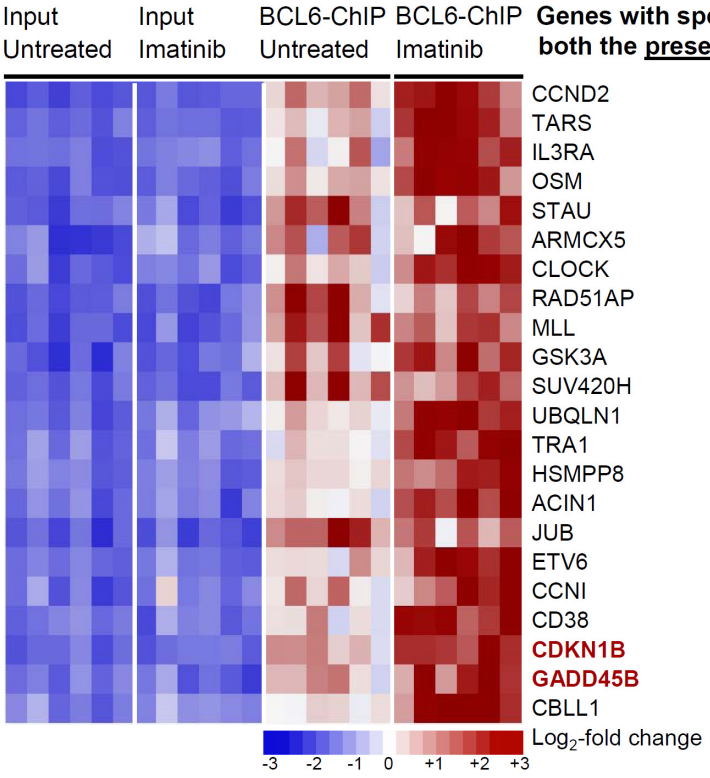


Three Ph+ ALL cell lines (BV-173, NALM-1, TOM-1) were treated in the presence or absence of Imatinib (10 µmol/l) and subjected to BCL6 ChIP-on-chip analysis. Most of the BCL6 target genes show BCL6 recruitment only in the presence of Imatinib-treatment. The heatmap presented in (**a**) depicts a list of genes that are specifically targeted by BCL6 in the presence of Imatinib (1,024 target genes). For instance, *TP53* only shows significant enrichment of BCL6 to its promoter when Ph+ ALL were treated with Imatinib (highlighted in red). The heatmap presented in (**b**) shows gene loci with significant recruitment of BCL6 also when Ph+ ALL were not treated with Imatinib. For instance, the *CDKN1B* (p27) and *GADD45B* loci show significant binding of BCL6 even in the absence of Imatinib-treatment (highlighted in red). 211 genes are in this group. Table (**c**) summarizes how Imatinib-treatment impacts on BCL6-recruitment in Ph+ ALL cells. Only 5 loci show specific recruitment of BCL6 in the absence of Imatinib. Recruitment of BCL6 to these loci, however, needs independent verification by single locus quantitative ChIP as we performed here

for *CDKN1A* (p21), *CDKN1B* (p27), *GADD45B* and *TP53*. ChIP-on-chip data are available from GEO under accession number GSE24426.

Figure S31.10 (continued): *BCL6 target genes with specific recruitment of BCL6 in Imatinib-treated Ph+ ALL cells*

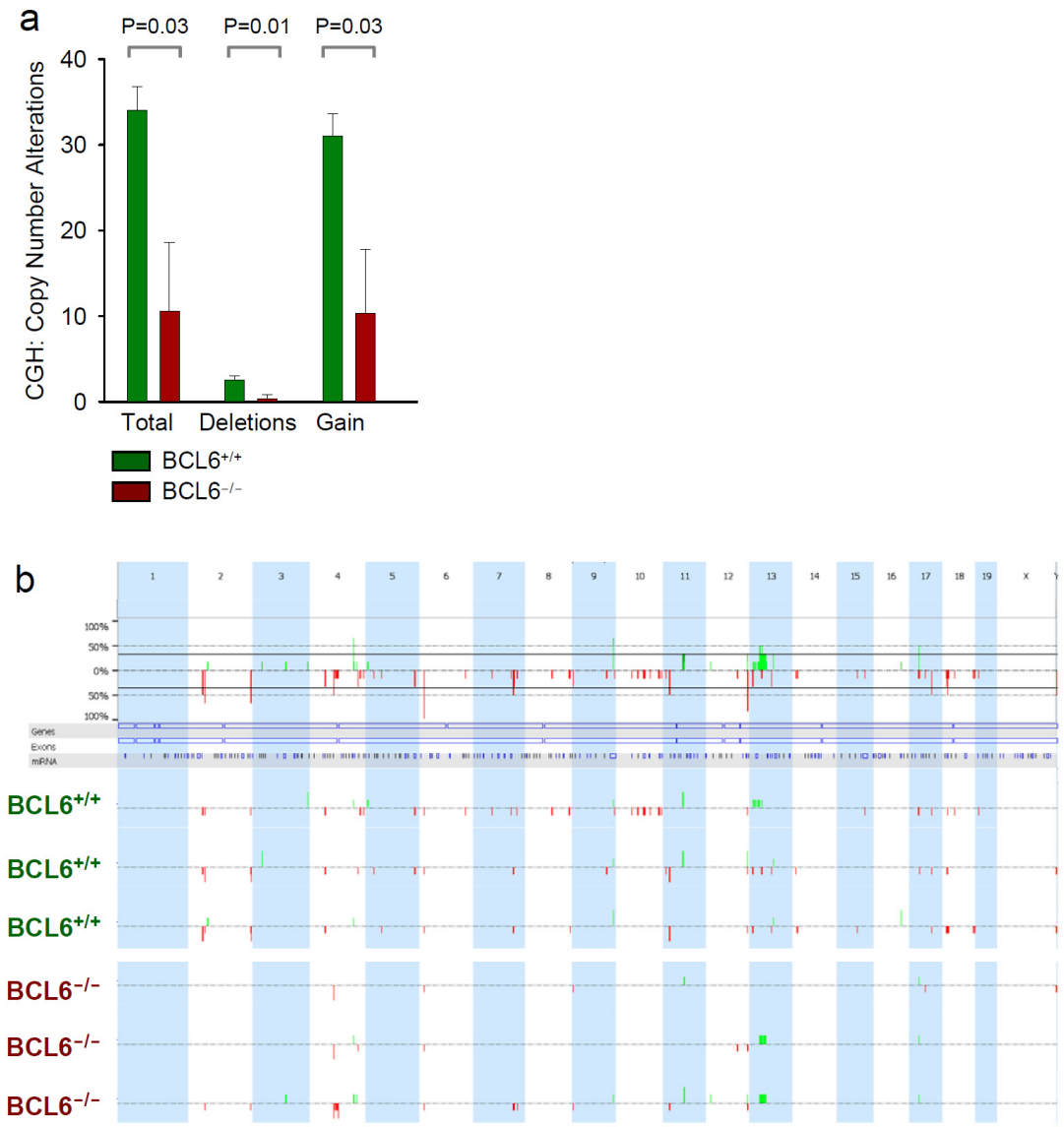
b



c

BCL6 recruitment	
1,235	In the presence of Imatinib
1,024	Only in the presence of Imatinib
211	Also in the absence of Imatinib
5	Only in the absence of Imatinib
1,240	Total BCL6 target genes in Ph+ ALL

Figure S32.11: *Analysis of genetic instability in BCL6$^{+/+}$ and BCL6$^{-/-}$ BCR-ABL1-transformed ALL cells*



Comparative genomic hybridization (CGH) analysis (NimbleGen, 720k Whole Genome Tiling arrays) of three transformed BCL6$^{+/+}$ and BCL6$^{-/-}$ BCR-ABL1 leukemias was performed. (**a**) The frequencies of copy number alterations in BCR-ABL1-transformed BCL6$^{+/+}$ and BCL6$^{-/-}$ leukemias were determined (mean values ± SD) and depicted for all events, deletions and gains/amplifications. In (**b**), genetic lesions that were specifically acquired either in BCL6$^{+/+}$ or BCL6$^{-/-}$ leukemia cells are depicted. In (**c**), a distribution of all copy number alterations over the mouse chromosomes is depicted. CGH array data are available from GEO under accession number GSE24400.

Figure S32.11 (continued): *Analysis of genetic instability in BCL6$^{+/+}$ and BCL6$^{-/-}$ BCR-ABL1-transformed ALL cells*

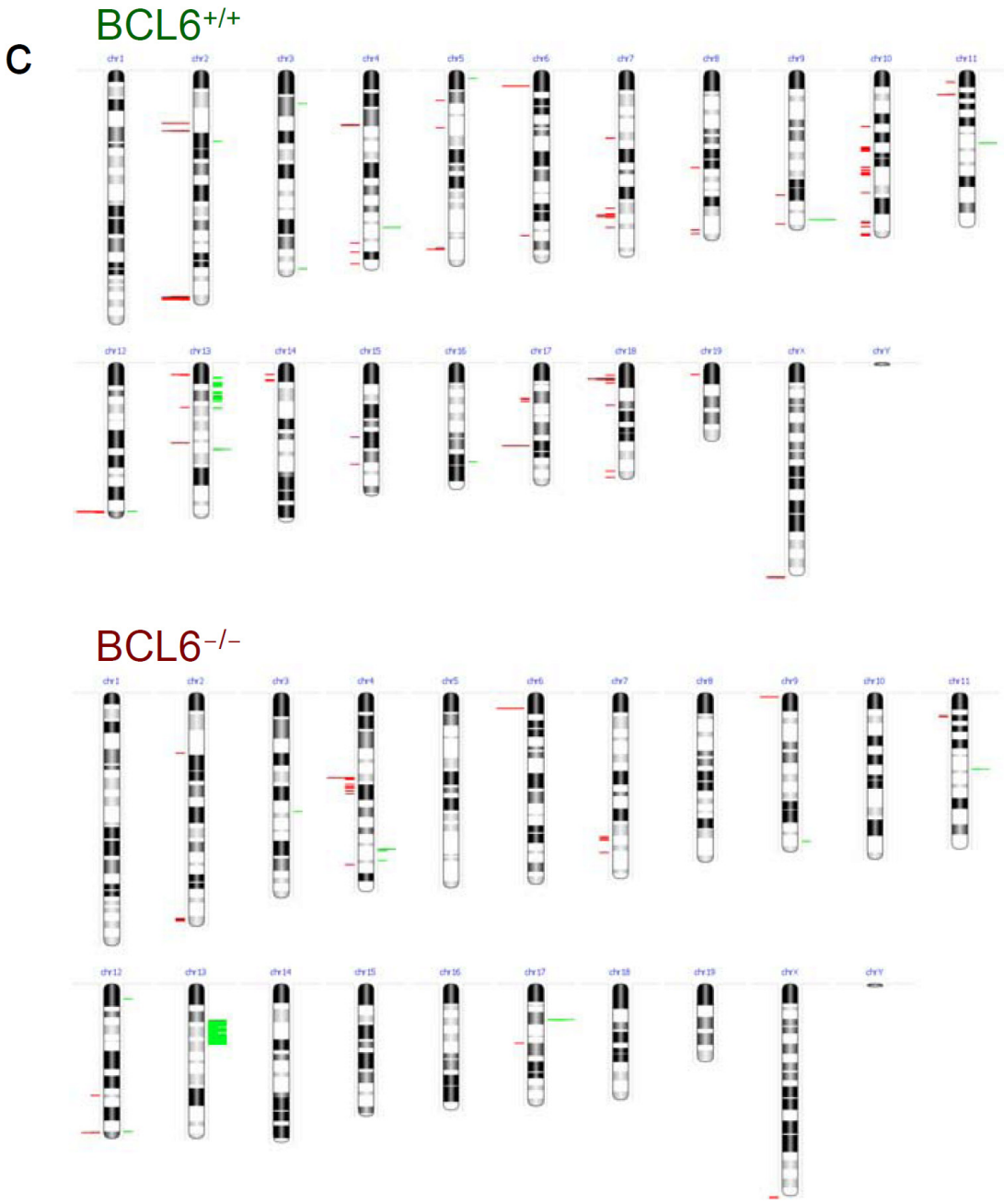
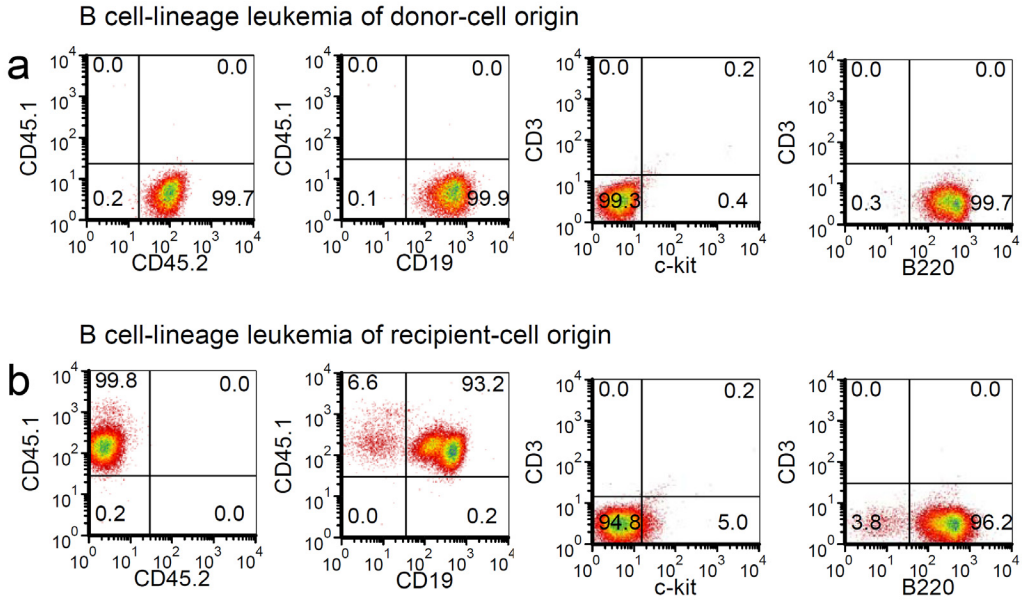
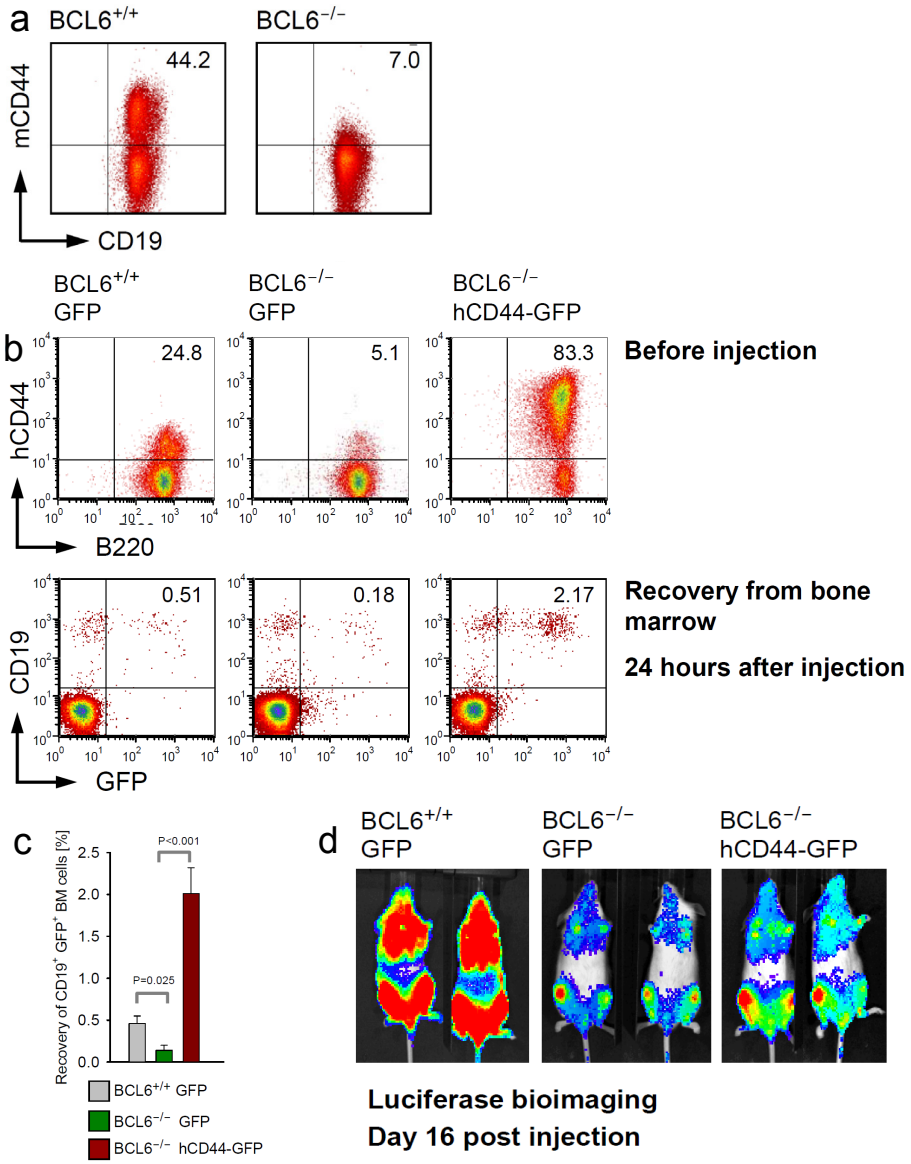


Figure S32.12: *Phenotypic analysis of donor- or recipient-origin of leukemia developing in irradiated NOD/SCID mice*



BCR-ABL1-transduced ALL cells from BCL6$^{+/+}$ or BCL6$^{-/-}$ mice express CD45.2 but not CD45.1. These leukemia cells can be identified as CD45.2^{+} cells after injection into CD45.1^{+} NOD/SCID recipient mice (**a**). In some cases, irradiated NOD/SCID recipient mice develop endogenous leukemia, in particular in older mice (see Figure 15b, where mice were monitored for 250 days). Endogenous leukemia cells can be identified as CD45.1^{+} cells of recipient origin (**b**). For further characterization, leukemia cells were stained for expression of the B cell antigens CD19 and B220, the T cell antigen CD3 and c-kit to identify particularly immature cells.

Figure S32.13: *Reconstitution of CD44 in BCL6$^{-/-}$ leukemia cells rescues engraftment but not leukemia initiation*

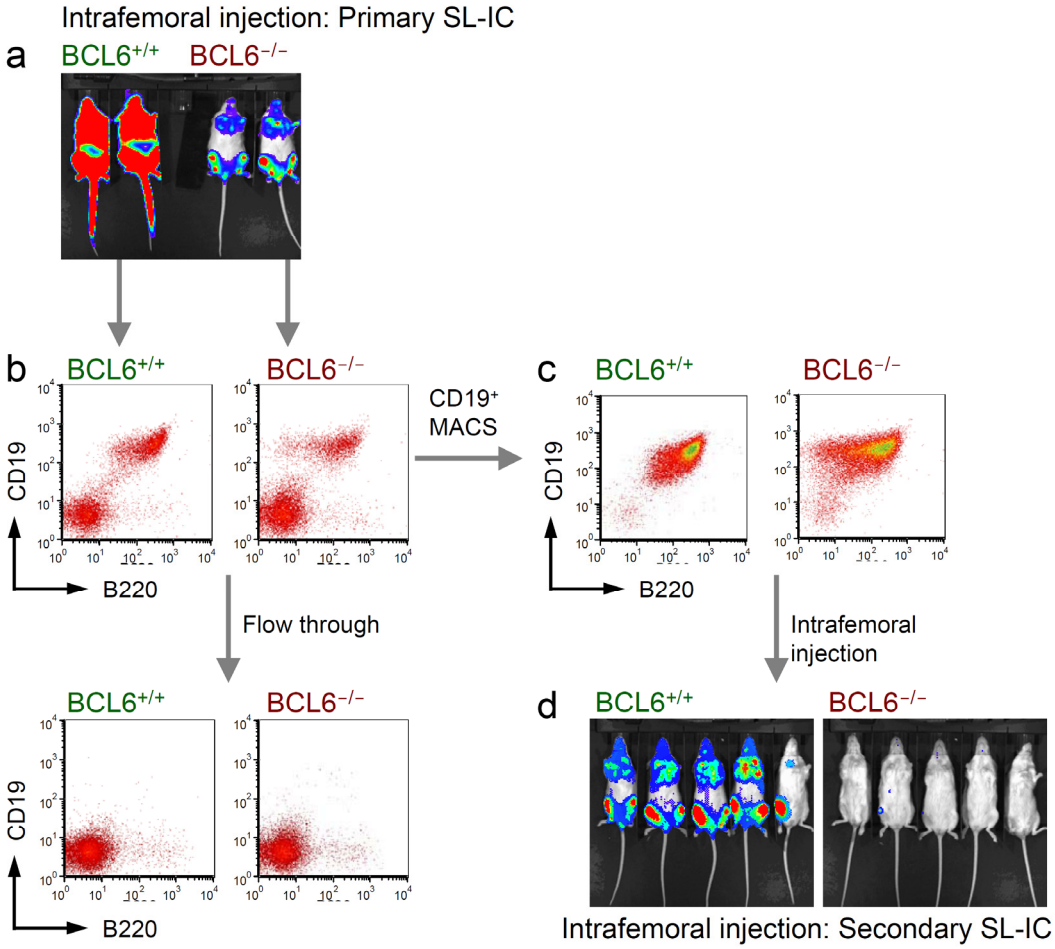


BCL6$^{-/-}$ *BCR-ABL1* ALL cells lack expression of the homing receptor CD44 (**a**), which is critical for homing of leukemia-initiating cells to the bone marrow microenvironment (Krause et al., 2006). *BCR-ABL1* ALL cells were transduced with human CD44 (hCD44-GFP) or an empty vector control (**b**). To directly address the role of CD44, we reconstituted CD44 expression in BCL6$^{-/-}$ *BCR-ABL1* ALL cells using a retroviral expression vector and determined how CD44 reconstitution affects engraftment and leukemia-initiation from BCL6$^{-/-}$ *BCR-ABL1* ALL cells. 2 x 10^6 cells were injected via tail vein. Three mice in each group were sacrificed after 24 hours and engraftment of GFP$^+$ leukemia cells in the bone marrow was measured by flow cytometry.

Consistent with lack of CD44 surface expression, recovery of BCL6$^{-/-}$ ALL cells from the bone marrow of transplant recipients was significantly reduced compared to BCL6$^{+/+}$ ALL cells 24 hours after tail vein injection (**b** and **c**; p=0.025). Retroviral reconstitution of CD44 expression increased the recovery of injected cells from the bone marrow by more than 10-fold (**b-c**; p<0.001). However, while overexpression of CD44 rescued homing of BCL6$^{-/-}$ *BCR-ABL1* ALL cells to the bone marrow, CD44 reconstitution failed to restore leukemia-initiation in BCL6$^{-/-}$ ALL cells (**d**). Hence, the failure of BCL6$^{-/-}$ ALL cells to initiate leukemia is not primarily owing to a CD44-dependent defect in homing to the bone marrow niche.

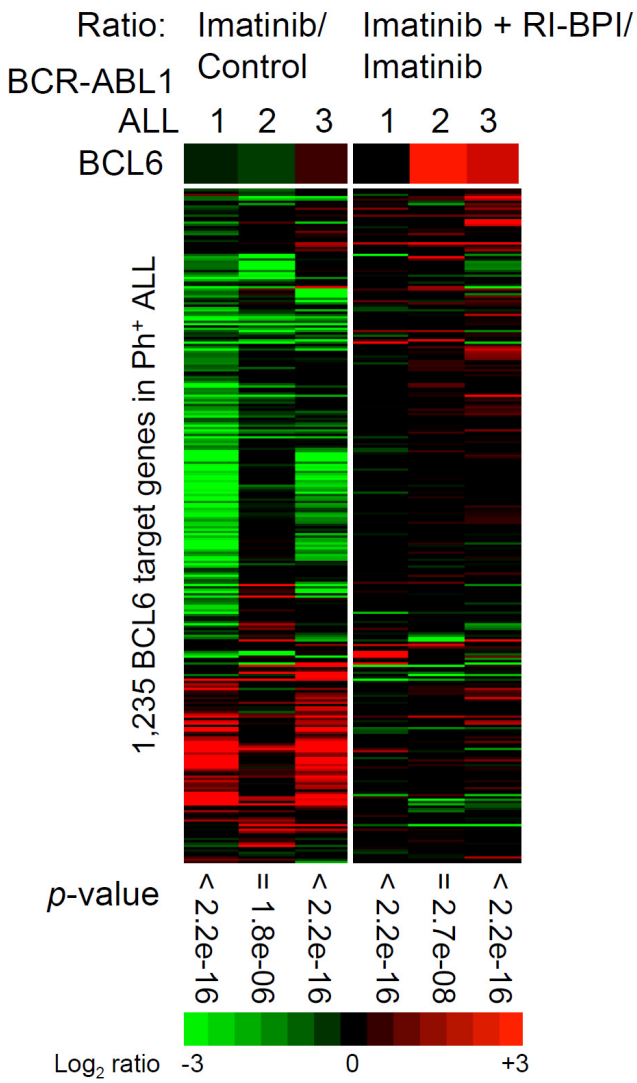
In (**c**), the quantitative analysis of recovery of GFP$^+$ cells from the bone marrow of engrafted leukemia cells is summarized (n=3; means ± SD). Leukemia cells from BCL6$^{+/+}$ and BCL6$^{-/-}$ mice were labeled with firefly luciferase to track leukemia-initiation after initial engraftment of leukemia cells. Leukemia-initiation was measured 16 days after injection by luciferase bioimaging in the remaining mice (**d**).

Figure S32.14: *BCL6$^{-/-}$ leukemia is not transplantable in serial transplant recipients*



5,000,000 BCL6$^{+/+}$ and BCL6$^{-/-}$ BCR-ABL1-transformed ALL cells were intrafemorally injected into sublethally irradiated NOD/SCID mice (see Figure 15d). Intrafemoral injection of large numbers of leukemia cells bypasses the leukemia-initiation step: as shown by luciferase bioimaging, the mice develop leukemia soon after injection of 5,000,000 leukemia cells (**a**; luciferase bioimaging on day 10 after injection). Leukemia cells were harvested from ill mice and stained for expression of CD19 and B220 (**b**). Using CD19 MACS beads, B lymphoid leukemia cells were enriched (Example; **c**). 5,000,000 BCL6$^{+/+}$ and BCL6$^{-/-}$ leukemia cells were intrafemorally injected into secondary recipients. Despite the large number of intrafemorally injected cells, BCL6$^{-/-}$ leukemia was not transplantable into secondary recipients (**d**; luciferase bioimaging on day 8 after injection).

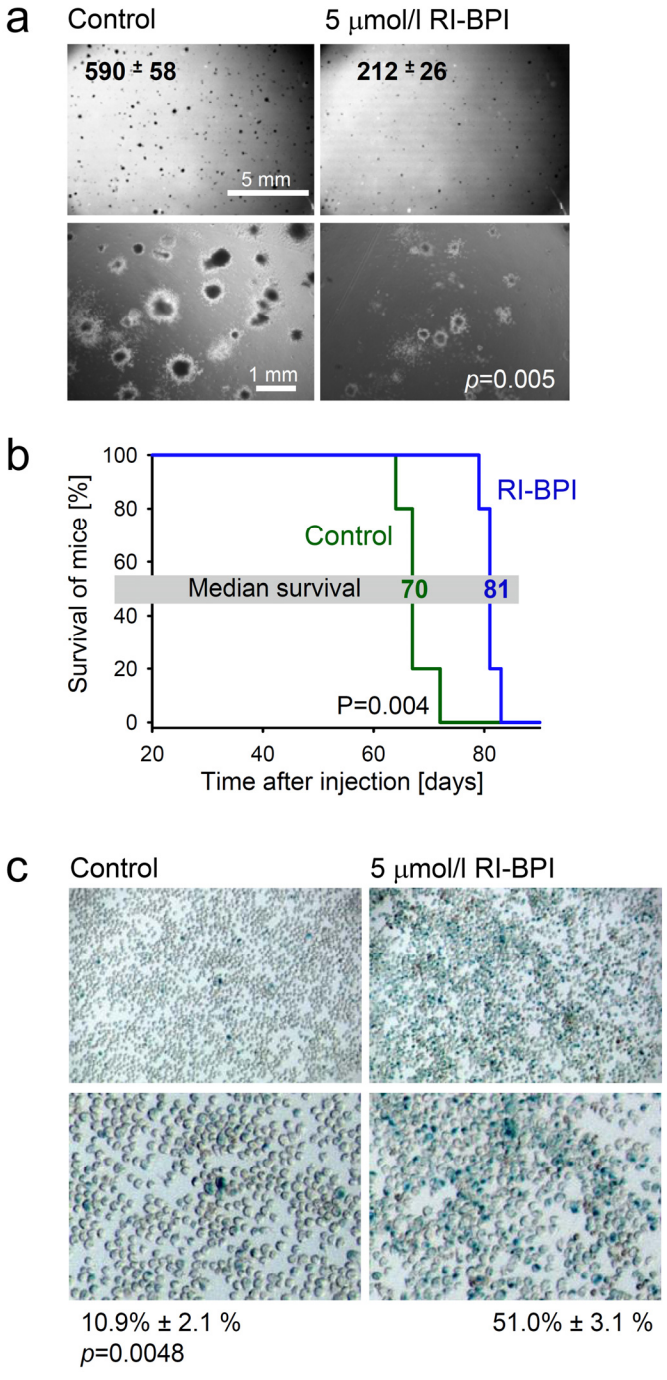
Figure S32.15: *RI-BPI reverses BCL6-dependent gene expression changes in human Ph$^+$ ALL cells*



Three human Ph$^+$ ALL cell lines were treated in the presence or absence of 10 µmol/l Imatinib or in the presence of both 10 µmol/l Imatinib and 20 µmol/l RI-BPI for 24 hours. Gene expression changes upon treatment with Imatinib or Imatinib + RI-BPI were analyzed using Agilent gene expression arrays. Kolmogorov-Smirnov test (KS-test) to determine if two data sets differ significantly was performed. 1,235 known BCL6 target genes (covered by 2,956 probe sets) and Non-target genes (covered by 38,137 probe sets) were compared using Agilent gene expression arrays. For Imatinib vs. Control (left), we tested if BCL6 target genes have lower values ($\log_2$ fold change) than Non-target genes. Lower gene expression values would be consistent with BCL6-mediated repression. For Imatinib + RI-BPI vs. Imatinib (right), we tested if BCL6 target genes have higher values ($\log_2$ fold

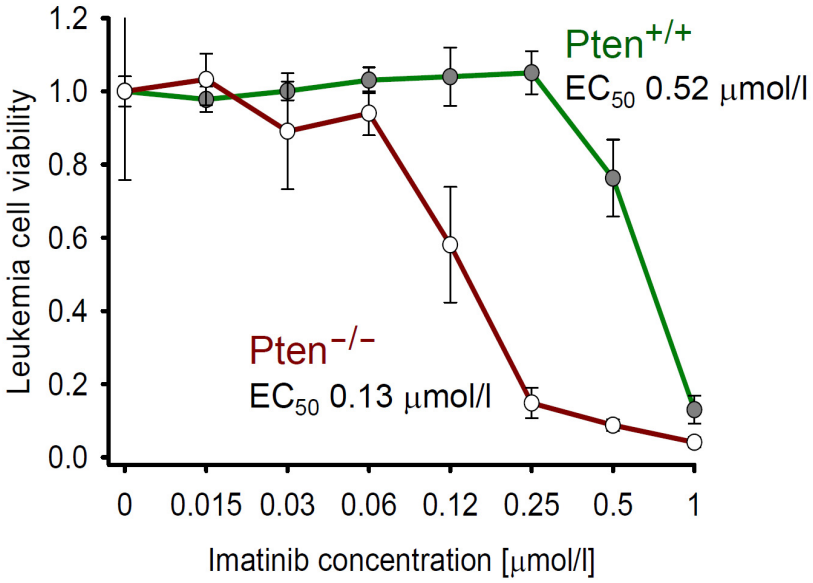
change) than Non-target genes. Higher gene expression values would be consistent with RI-BPI-mediated de-repression of BCL6-target genes. As one example, negative autoregulation of BCL6 is shown in the top row: Treatment with RI-BPI leads to transcriptional derepression of BCL6. The bottom panel indicates *p*-values for the comparison of gene expression values for known BCL6 target genes and Non-target genes as determined by KS-test. Data are available from GEO superseries GSE24426 including GEO accession numbers GSE24381 and GSE24404.

Figure S32.16: *RI-BPI compromises self-renewal and induces senescence in human Ph+ ALL cells*



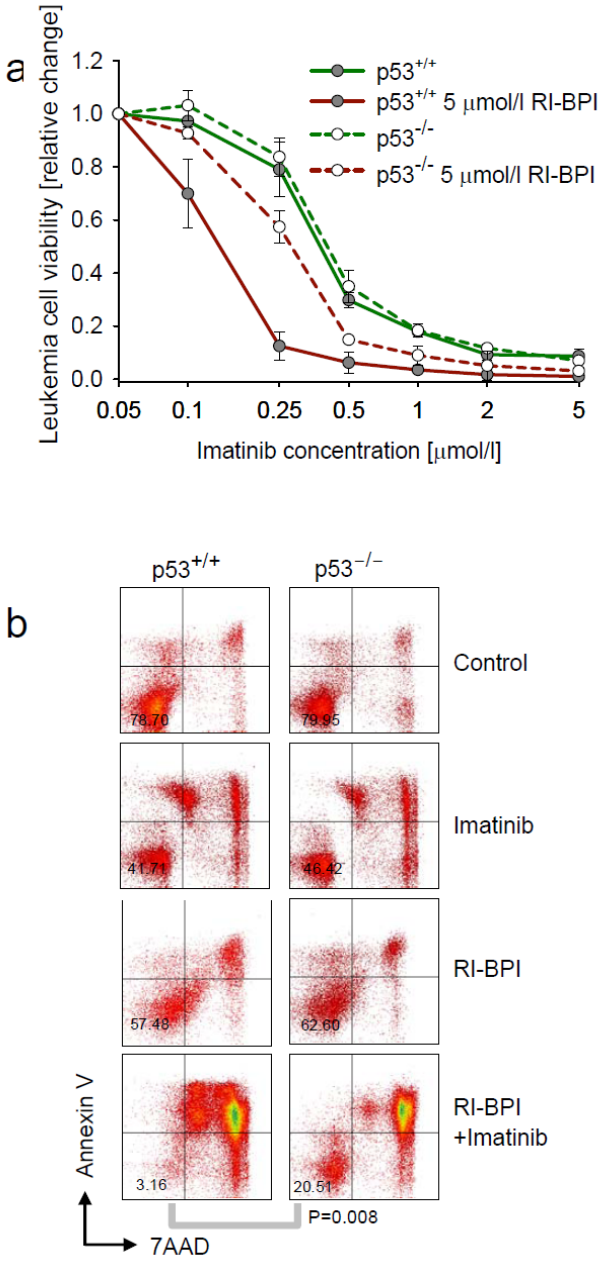
(**a**) Human Ph+ ALL cells (10,000) were plated in semisolid agar in either the presence or absence of 5 µmol/l *retro-inverso* BCL6 peptide-inhibitor (RI-BPI). Colonies were counted after ten days (numbers denote means ± SD, n=3). (**b**), Patient-derived Ph+ ALL cells (BLQ5; Table S1) were incubated *ex vivo* for two days in the presence of RI-BPI (5 µmol/l) and injected into sublethally irradiated NOD/SCID mice. A Kaplan-Meier analysis of survival is shown (5 mice per group, p=0.004). (**c**), Human Ph+ ALL cells were treated with or without 5 µmol/l *retro-inverso* BCL6 peptide-inhibitor (RI-BPI) for three days. Senescence-associated β-galactosidase (SA-β-gal) activity as an indicator of cellular senescence was measured on cytospin preparations. Percentages reflect SA-β-gal+ cells (means ± SD, n=3).

Figure S32.17: *Deletion of Pten sensitizes BCR-ABL1-transformed ALL cells to TKI-treatment*



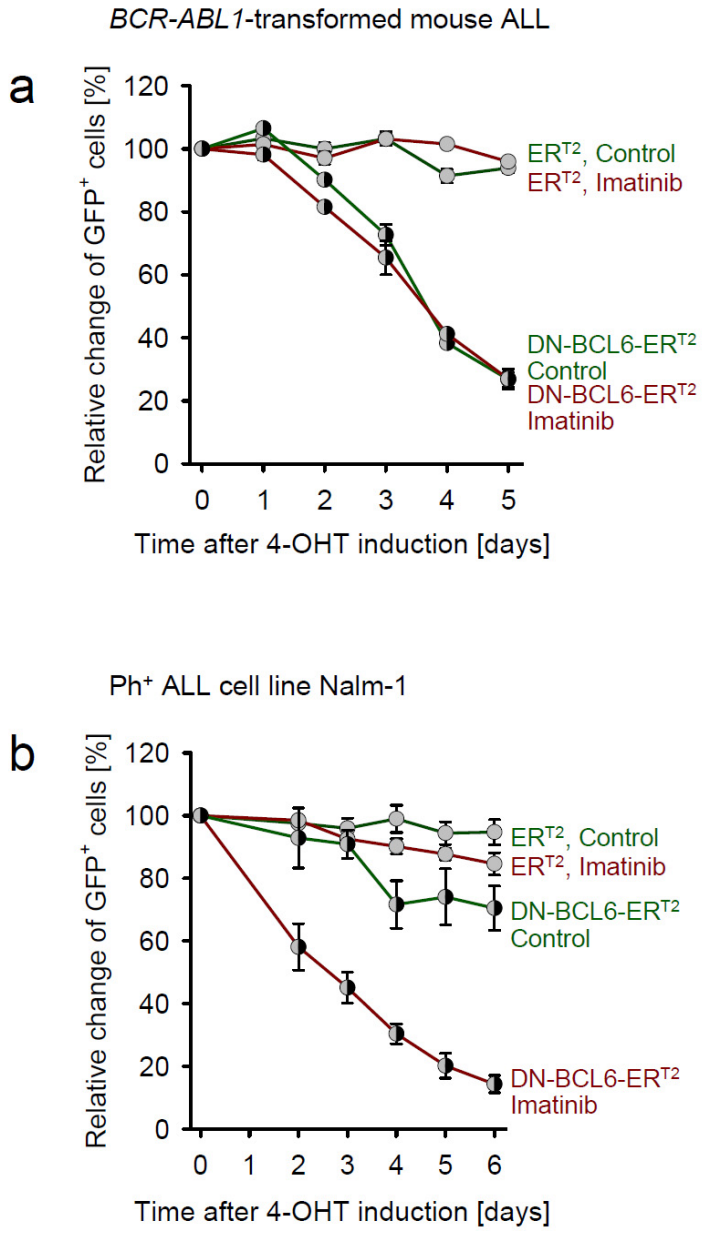
Upon inducible deletion of *Pten*, *BCR-ABL1*-transformed ALL cells lose the ability to upregulate BCL6 and Imatinib-treatment results in very high levels of p53 (see Figure 13e). Consistent with impaired ability to upregulate BCL6, inducible deletion of *Pten* results in increased sensitivity of leukemia cells to Imatinib treatment (p=0.002). Curves depict means (± SD) of triplicate measurements.

Figure S32.18: *p53 is suppressed by BCL6 and contributes to Imatinib-mediated apoptosis in BCR-ABL1+ ALL*



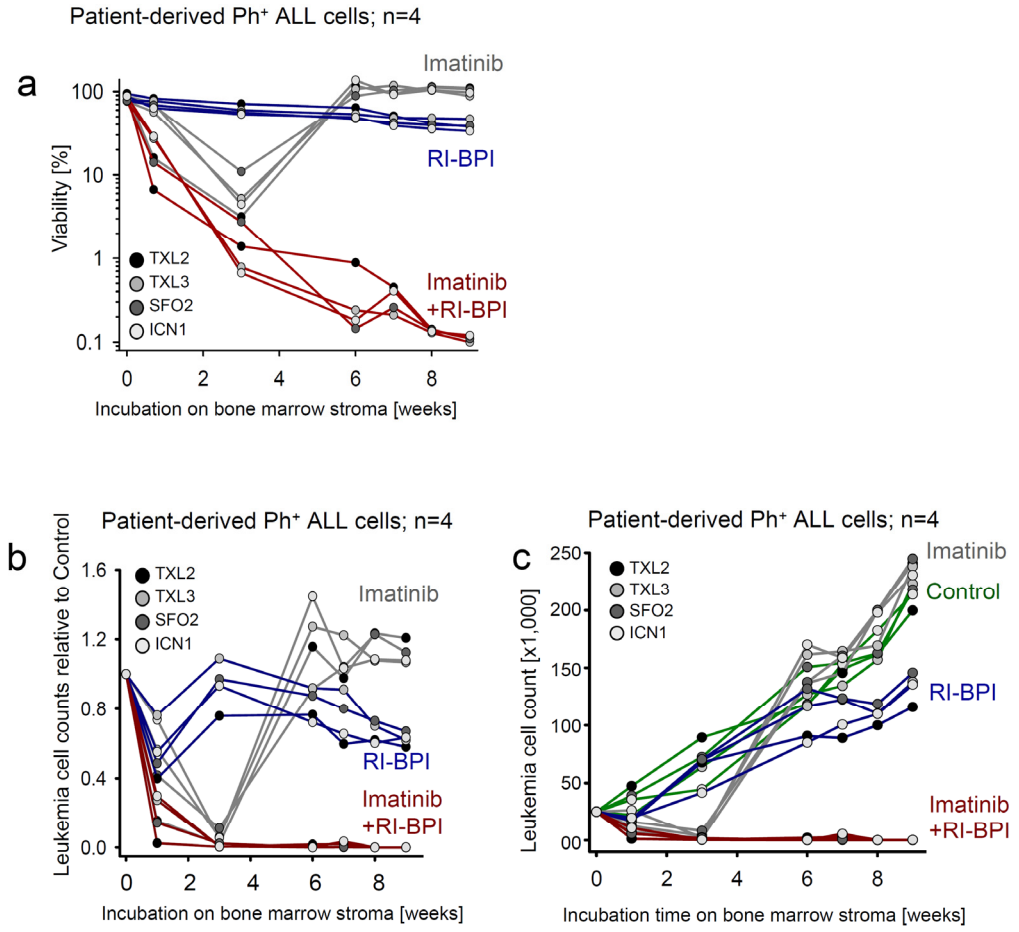
B cell precursors from $p53^{+/+}$ and $p53^{-/-}$ mice were transformed by *BCR-ABL1*. After full transformation, leukemia cells were treated with or without Imatinib or a combination of Imatinib and RI-BPI (5 µmol/l). In (**a**), drug-responses to different doses of Imatinib are measured to test whether RI-BPI sensitizes $p53^{+/+}$ and $p53^{-/-}$ leukemia cells to Imatinib (Resazurin assay). While RI-BPI had a significant effect on $p53^{+/+}$ ALL cells, the effect of RIBPI was reduced on $p53^{-/-}$ ALL cells. Likewise, a combination of Imatinib and RI-BPI was strongly synergistic in $p53^{+/+}$ ALL cells whereas the effect of RI-BPI was reduced on $p53^{-/-}$ ALL cells as measured by flow cytometry (**b**).

Figure S32.19: *Inducible activation of a dominant-negative BCL6 mutant in BCR-ABL1-transformed ALL cells*



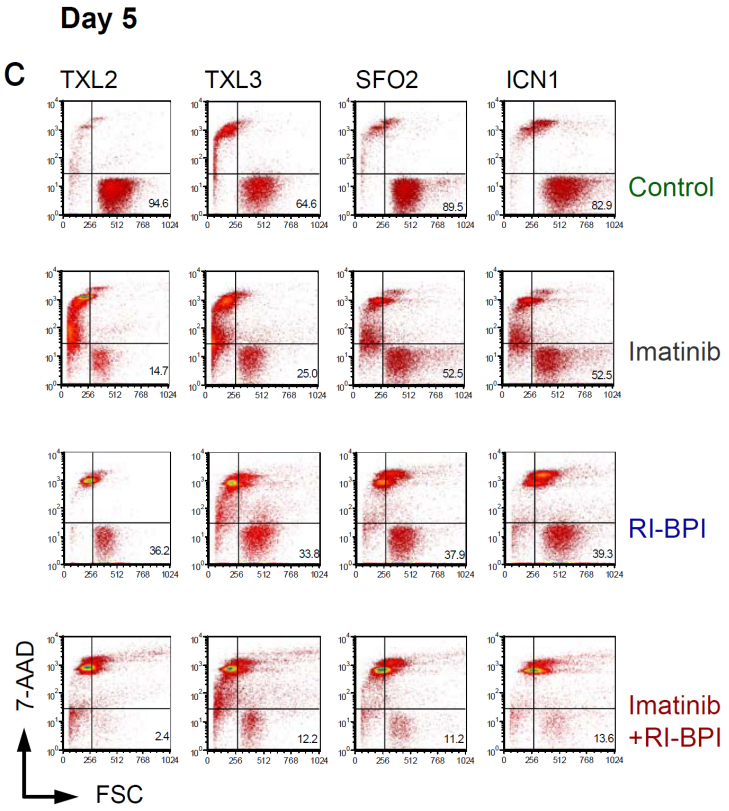
BCR-ABL1-transformed mouse ALL cells (pro-/pre-BI phenotype) were transduced with 4-OHT inducible dominant-negative BCL6 (DN-BCL6-ER^{T2}) or ER^{T2} empty vectors (Shaffer et al., 2000), which are tagged with GFP (**a**). In a parallel experiment, a human Ph$^+$ ALL cell line (NALM1) was transduced with the same constructs (**b**). Mouse and human leukemia cells were then treated with or without 1 µmol/l Imatinib (mouse) or 10 µmol/Imatinib (human cell line) and DN-BCL6-ERT2 or ER^{T2} were induced by addition of 4-OHT. The relative changes of GFP$^+$ cells after 4-OHT induction are indicated. Curves denote mean values ± SD; n=3.

Figure S32.20: *RI-BPI-mediated inhibition of BCL6 prevents outgrowth of Imatinib-resistant subclones in patient-derived Ph+ ALL*



Primary patient-derived Ph+ ALL cells from bone marrow biopsies of 4 patients (TXL2, TXL3, SFO2 and ICN1; see Table S1) were isolated and incubated on OP9 stroma cells. In all 4 cases, samples were obtained at the time of diagnosis and none of the patients was previously treated with Imatinib. Sequence analysis revealed that the BCR-ABL1 kinase domain in patient-derived ALL cells was unmutated in all 4 cases. BCR-ABL1 ALL cells were treated in the presence or absence of Imatinib (10 µmol/l) and/or RI-BPI (10 µmol/l). After 5 days, 3 weeks, 6 weeks, 7 weeks, 8 weeks and 9 weeks, aliquots from cell cultures were analyzed by flow cytometry for cell viability (Annexin V, 7-AAD and forward scatter) and cell number. (**a**) Shows a synopsis of viability measurements (log scale). (**b**) Shows changes of cell number relative to untreated control (set as 1.0). Individual flow cytometry measurements after 5 days (**c-d**) and 7 weeks of incubation (**e-f**) are shown.

Figure S32.20 (continued): *RI-BPI-mediated inhibition of BCL6 prevents outgrowth of Imatinib-resistant subclones in patient-derived Ph+ ALL cells*



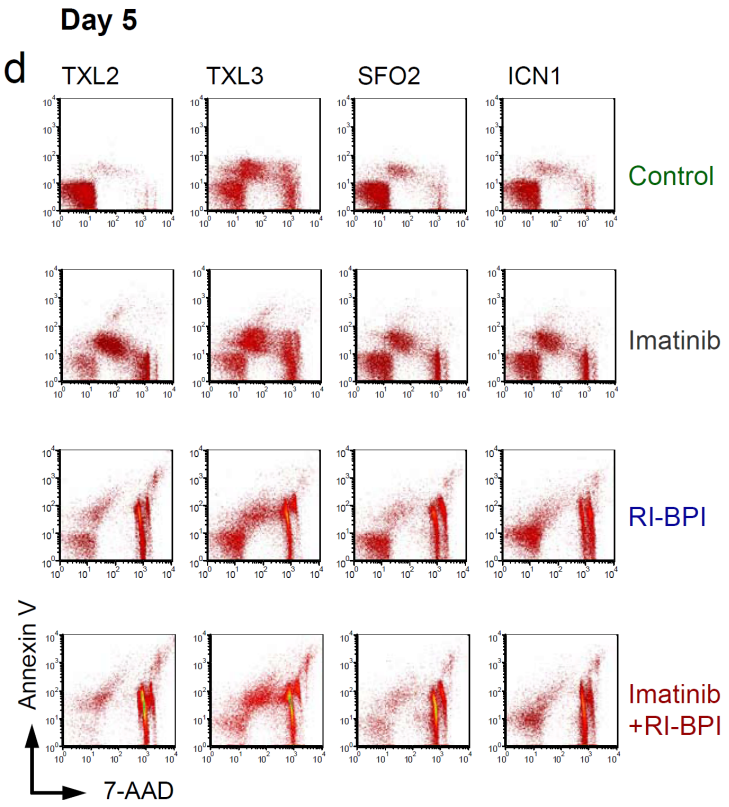


Figure S32.20 (continued): *RI-BPI-mediated inhibition of BCL6 prevents outgrowth of Imatinib-resistant subclones in patient-derived Ph+ ALL cells*

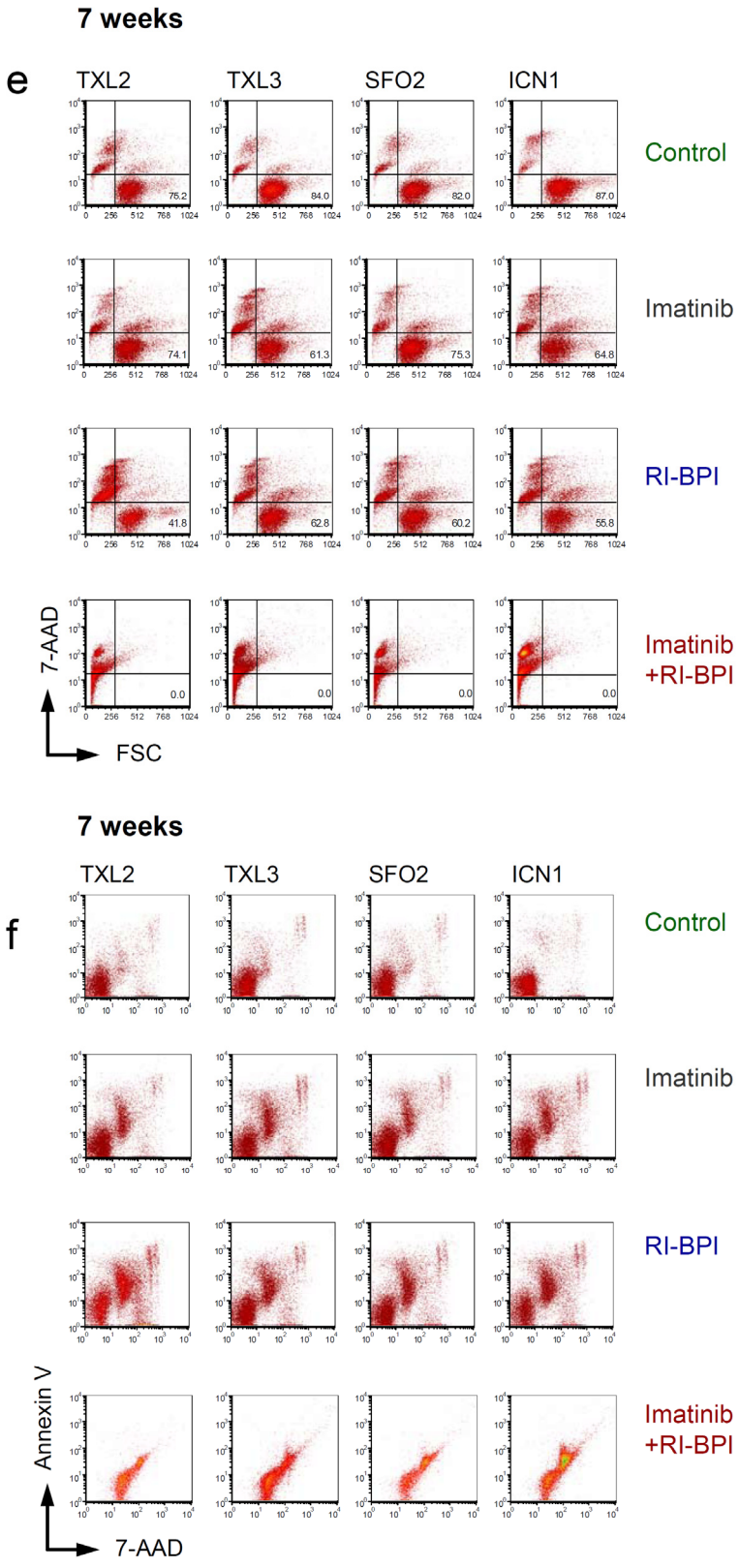
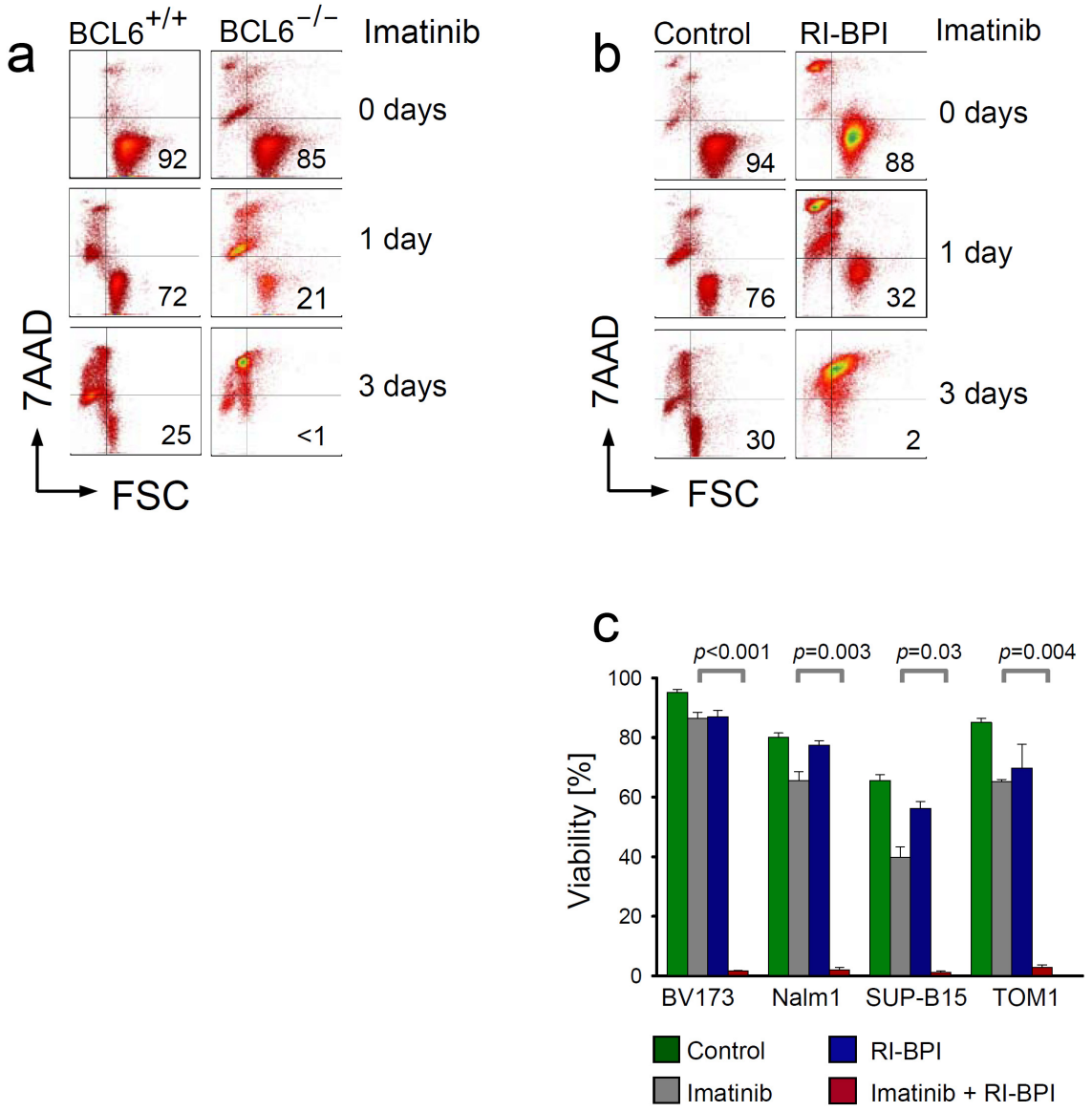
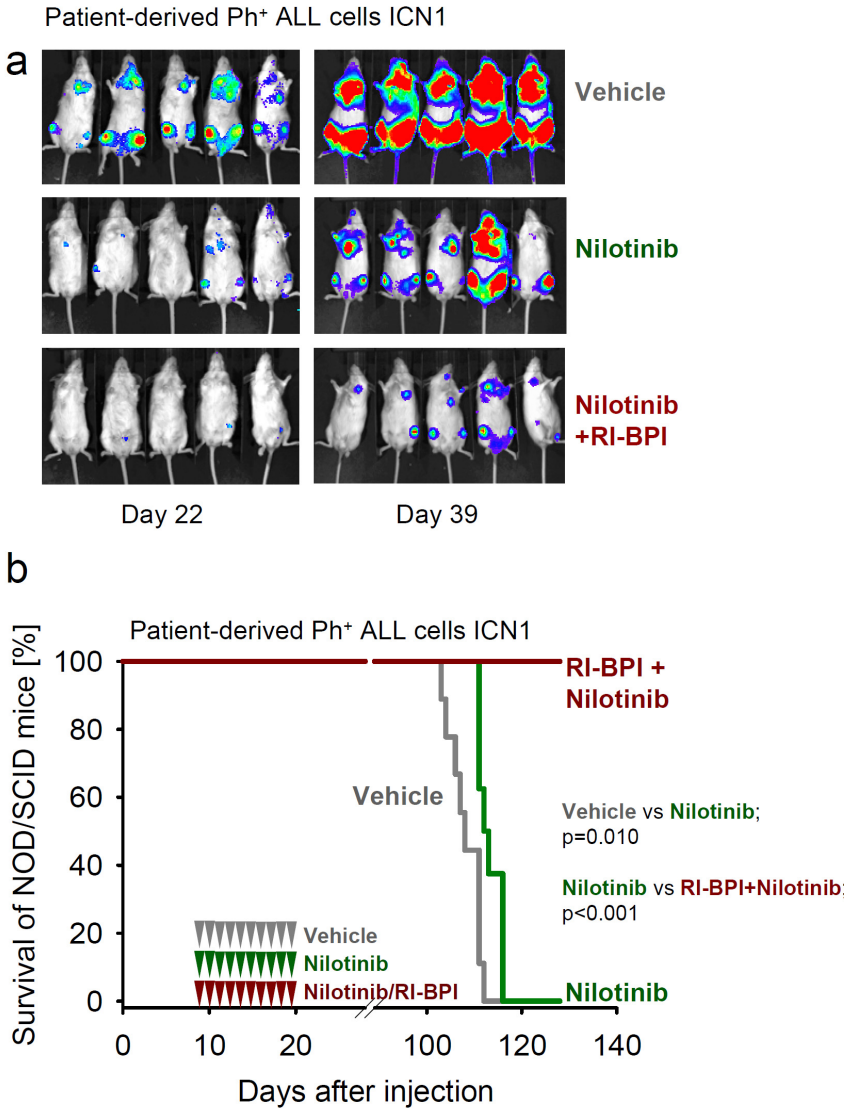


Figure S32.21 *RI-BPI-mediated inhibition of BCL6 induces a similar degree of TKI-sensitivity as in BCL6$^{-/-}$ leukemia cells*



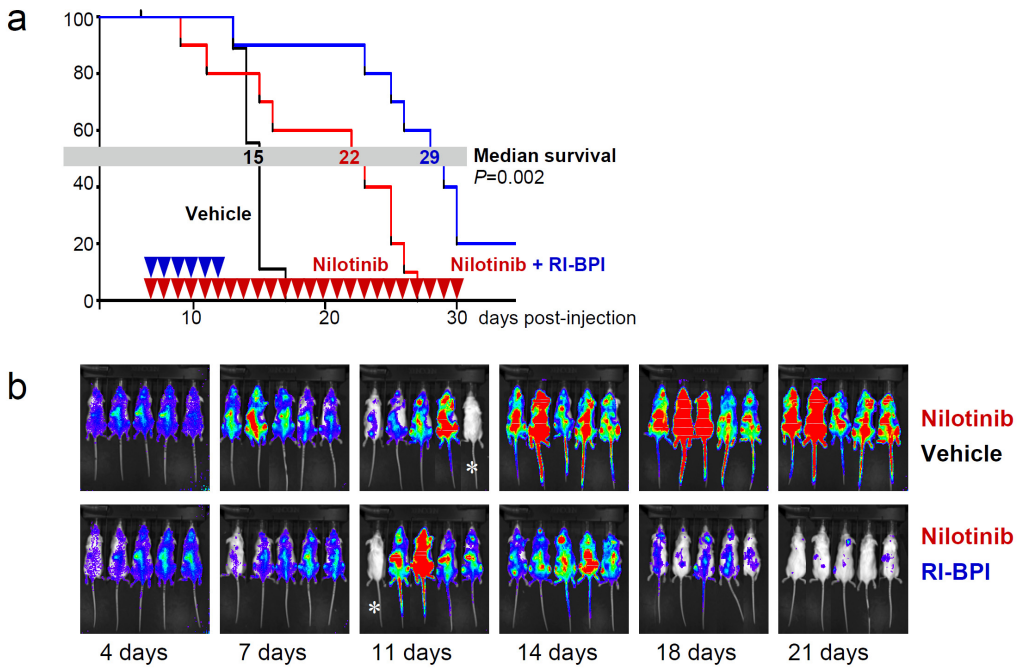
B cell precursors from BCL6$^{+/+}$ and BCL6$^{-/-}$ mice were transformed by *BCR-ABL1*. After full transformation, leukemia cells were treated with or without Imatinib (**a**) or a combination of Imatinib and 5 µmol/l RI-BPI (**b**) and viability was analyzed by flow cytometry using 7-AAD and forward scatter (FSC) parameters. Likewise, four human Ph$^+$ ALL cell lines (BV173, Nalm1, SUP-B15 and TOM1) were treated with or without 10 µmol/l Imatinib or with or without RI-BPI (20 µmol/l) and viability was measured after three days of treatment (**c**). Means (± SD) of triplicate measurements are indicated for each cell line. *p*-values were calculated for the differences between Imatinib only and Imatinib + RI-BPI combinations (double-sided t-test).

Figure S32.22: *BCL6 peptide inhibition sensitizes patient-derived Ph+ ALL to TKI-treatment in vivo*



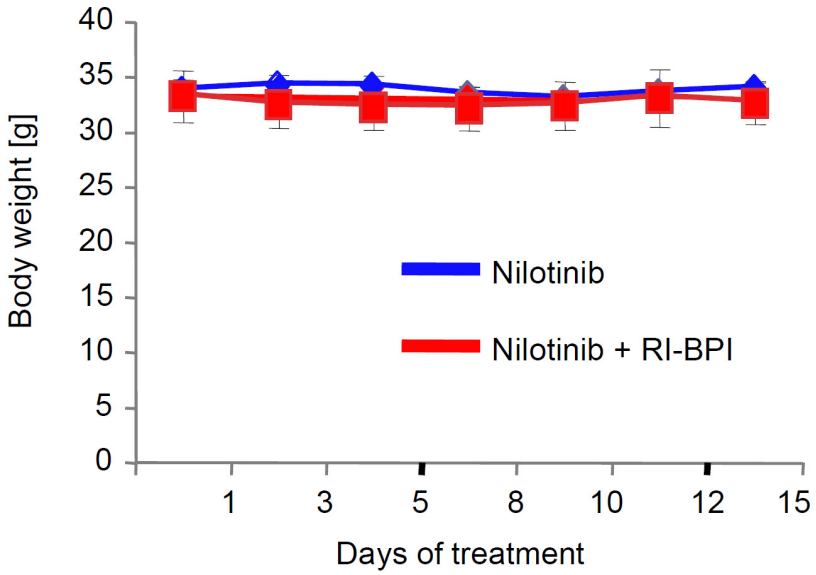
(**a**) Leukemia cells from a bone marrow biopsy of a patient with Ph+ ALL (ICN1, Table S1) were xenografted into sublethally irradiated NOD/SCID mice via tail vein injection. After first passage in mice, leukemia cells were harvested and transduced with lentiviral firefly luciferase and 100,000 transduced leukemia cells were injected into sublethally irradiated secondary NOD/SCID recipients. The mice were treated with daily injections of vehicle, Nilotinib (50 mg/kg) alone or Nilotinib (50 mg/kg) and RI-BPI (25 mg/kg) over a period of 10 days (days 8 to 17). Luciferase bioimaging on days 22 and 39 showed that co-treatment with RI-BPI delayed leukemia formation. (**b**) A Kaplan Meier-analysis of overall survival of mice in the three groups is shown. Arrowheads depict times of treatment.

Figure S32.23: *RI-BPI plus Nilotinib combination reduces disease burden in mice with full-blown leukemia*



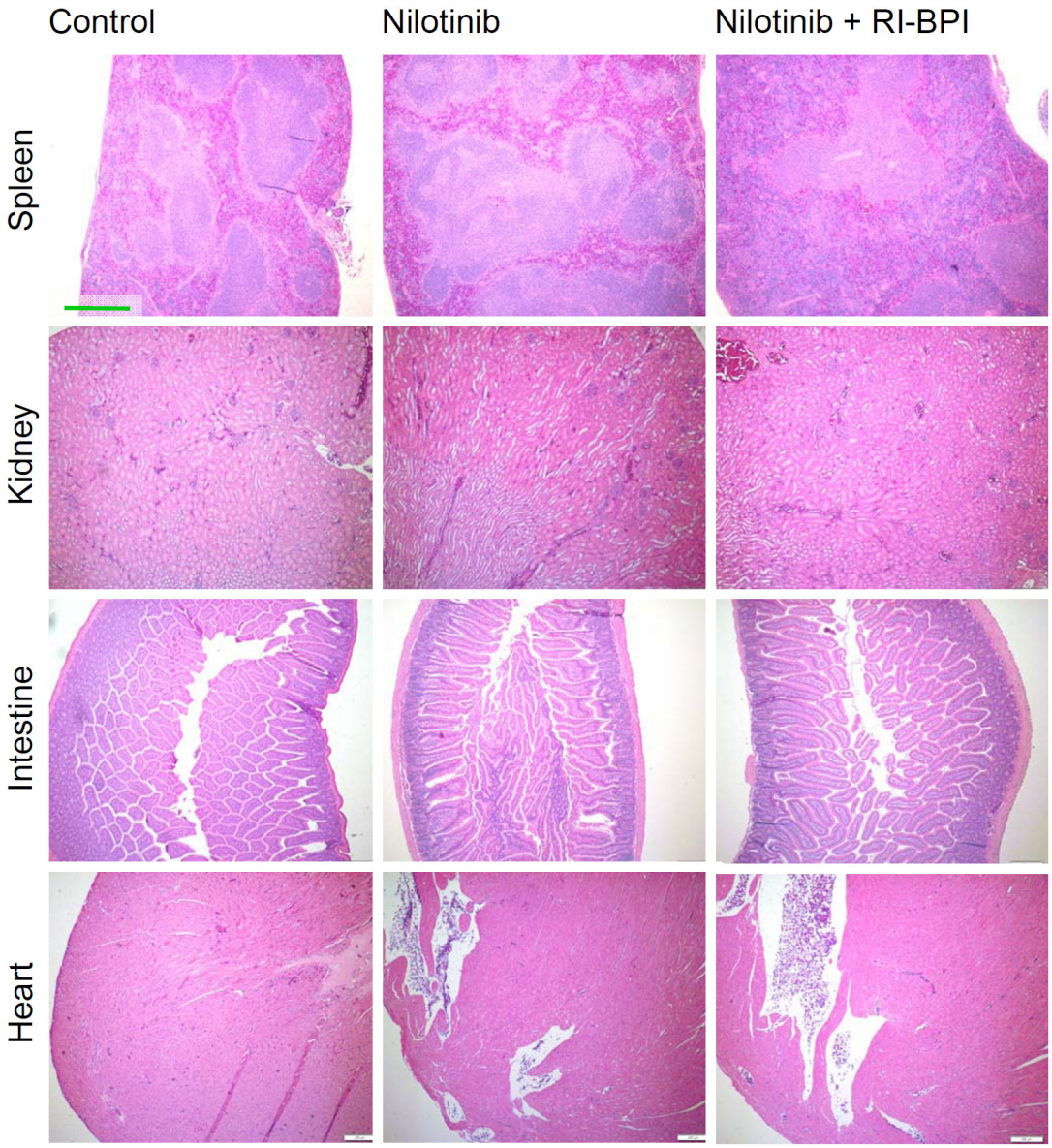
1 x 10^6 mouse *BCR-ABL1* ALL cells were labeled with lentiviral firefly luciferase and injected into NOD/SCID mice. 4 days after injection, the first signs of engraftment were observed by luciferase bioimaging. Mice were treated six times starting on day 8 after leukemia cell injection with either the second generation TKI Nilotinib alone (25 mg/kg; red arrow heads) or a combination of Nilotinib and RI-BPI (20 mg/kg; blue arrow heads). After day 13, treatment with RI-BPI was discontinued and mice were treated daily with Nilotinib only. A Kaplan-Meier analysis of survival of mice treated with vehicle, Nilotinib alone or a combination of Nilotinib and RI-BPI (days 8-13) is shown (**a**). The kinetics of leukemia engraftment, progression and reduction of leukemia cell burden in case of Nilotinib + RI-BPI-treatment are shown by luciferase bioimaging (**b**). Asterisks denote mice with misinjections of D-luciferin.

Figure S32.24: *In vivo toxicology studies for Nilotinib/RI-BPI combinations -Body weight*



Ten adult C57BL/6 mice were exposed to intraperitoneal administration of RI-BPI 20 mg/kg of body weight 3 times a week (mean values ± SD; n=5) or RI-BPI 20 mg/kg of body weight 3 times a week plus Nilotinib 25 mg/kg of body weight 3 times a week by oral gavage (mean values ± SD; n=5). Five additional mice were treated with vehicle for control. One mouse in the combination group was excluded for analysis due to a severe bite wound with infection that required additional treatment. No toxic effect, such as lethargy, weight loss (see above), failure to thrive or any other indicator of sickness or organ damage, was noted with either treatment schedule. Complete blood counts showed no alteration of leukocyte, platelet and erythrocyte numbers. One mouse in the combination group presented a low hematocrit value due to intraperitoneal bleeding as consequence of the IP injection. Bone marrow examination (smear) and peripheral blood examination showed normal cell morphology. There were no alterations in the biochemistry parameters for liver function, kidney function and electrolytes (see Table S3).

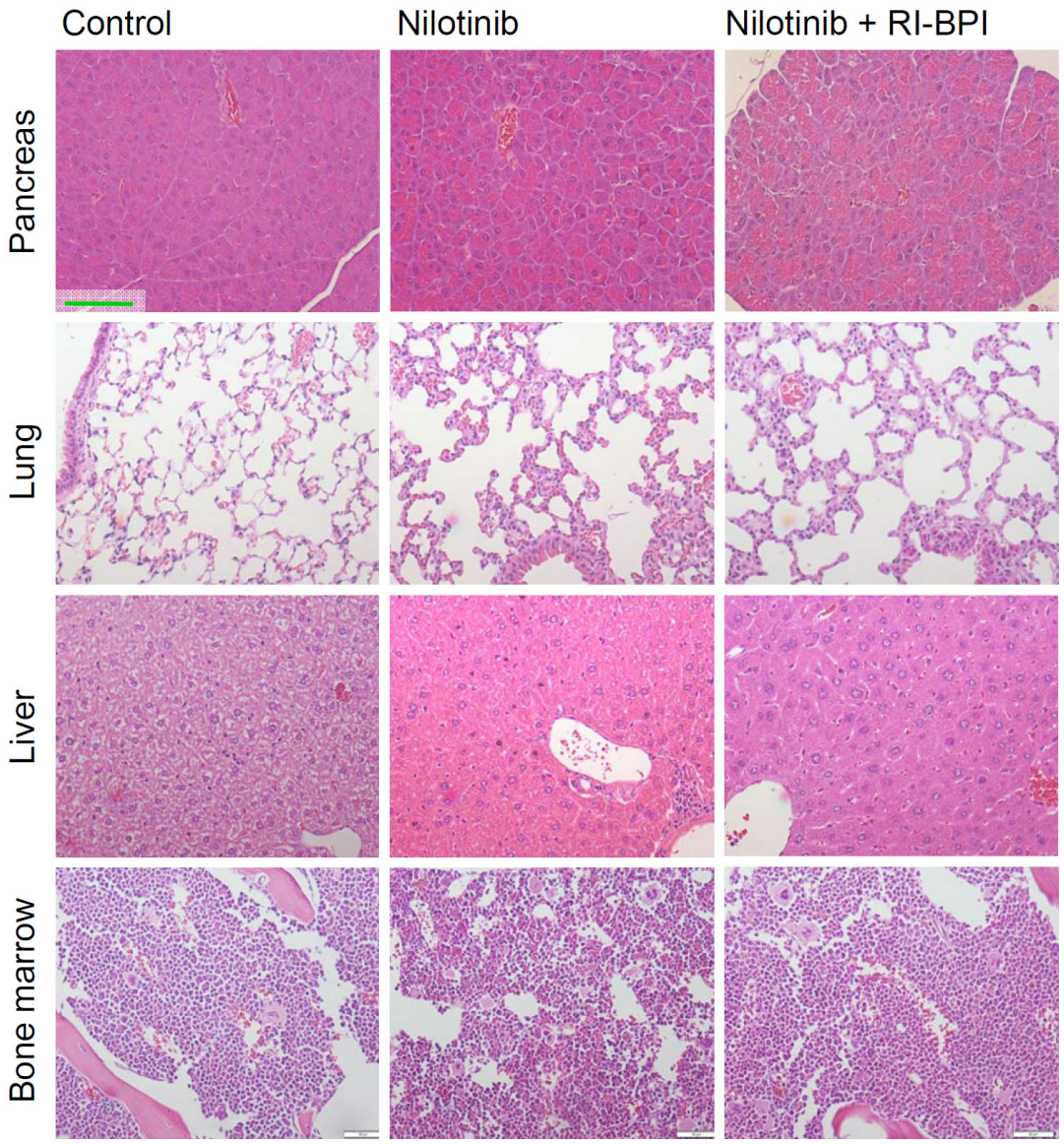
Figure S32.25: *In vivo toxicology studies for Nilotinib/RI-BPI combinations - Histology*



Note: Line represents 400 µm

Representative microphotographs from the several tissues (spleen, kidney, intestine, heart, pancreas, lung, liver and bone marrow) harvested from vehicle, Nilotinib and Nilotinib+RI-BPI treated animals for toxicity assessment. Pictures were taken using a digital camera (Olympus DP72, Olympus Corp, Tokyo, Japan) attached to a light microscope (Axioskop, Carl Zeiss Crop., Maple Grove, MN) with 4x and 20x Plan Neofluar objectives (Carl Zeiss Crop., Maple Grove, MN).

Figure S32.25 (continued): *In vivo toxicology studies for Nilotinib/RI-BPI combinations - Histology*



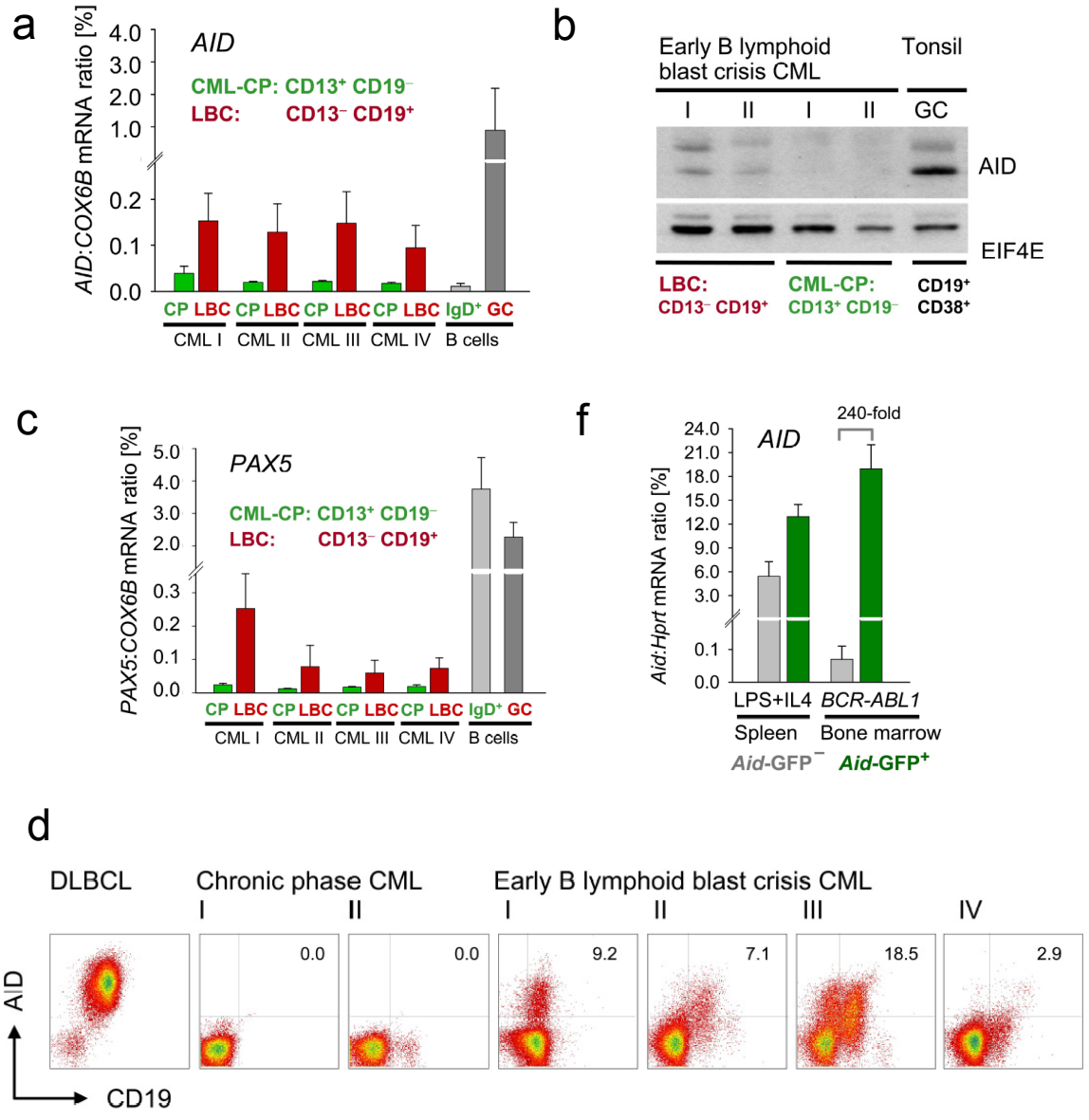
Note: Line represents 100 µm

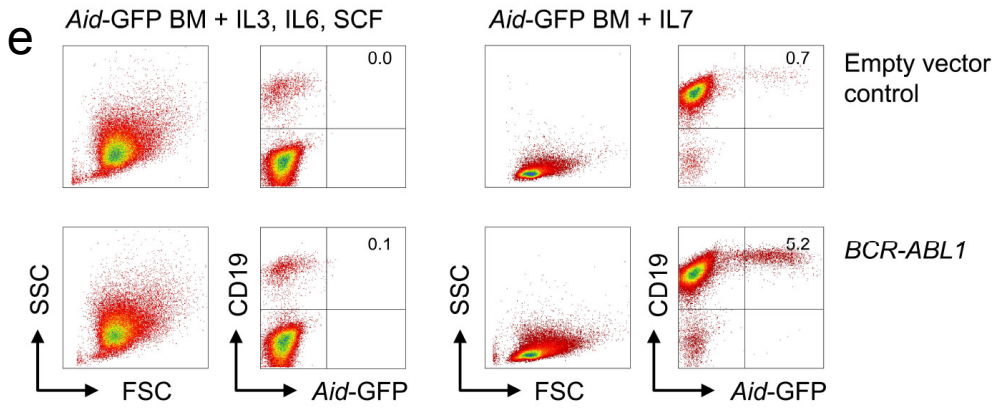
3.3 The B cell mutator AID promotes B lymphoid blast crisis and drug-resistance in chronic myeloid leukemia

To determine whether AID is implicated in CML progression into fatal blast crisis, we examined AID expression in patients with co-existing chronic phase CML (CML-CP) and B lymphoid blast crisis CML (CML-LBC). Leukemia cells of either phenotype were clearly distinguished in bone marrow samples from these patients based on myeloid (CP; $CD13^+CD19^-CD34^+$) and B lymphoid (LBC; $CD13^-CD19^+CD34^+$) markers. Real-time RT-PCR showed significant expression of AID mRNA in CML-LBC blasts, but little or no expression in CML-CP cells (6-10 fold difference, $P = 0.013$, Figure 17a). By comparison, AID expression in primary LBC blasts was 5-10 fold lower relative to germinal center (GC) B cells isolated from human tonsils (Figure 17a). Western blot analysis also showed AID protein present in LBC but absent in CML-CP cells (Figure 17b). Significantly, AID was in all cases coexpressed with the B cell lineage-inducer PAX5 (Nutt et al., 1999), which has been shown to promote AID gene transcription (Gonda et al., 2003; Sayegh et al., 2003) (Figure 17c). To measure AID protein expression on a per cell basis, we performed intracellular staining with an AID-specific antibody. Compared to GC-derived diffuse-large B cell lymphoma cells (DLBCL) that express high levels of AID (Lenz et al., 2007), AID protein was undetectable in bone marrow samples from patients with myeloid CML-CP (Figure 17d). In contrast, patients with early CML-LBC harbored cells expressing AID at varying levels (3-18%) along with the B cell antigen CD19 (Figure 17d). Surface expression of the hematopoietic progenitor cell antigen CD34 on these cells excluded the possibility of sample contamination with mature, AID^+ activated B cells. We conclude that progression of

CML-CP into CML-LBC is characterized by aberrant expression of PAX5 and its transcriptional target AID.

Figure 17: *B cell lineage-specific activation of AID in BCR-ABL1-transformed leukemia cells*





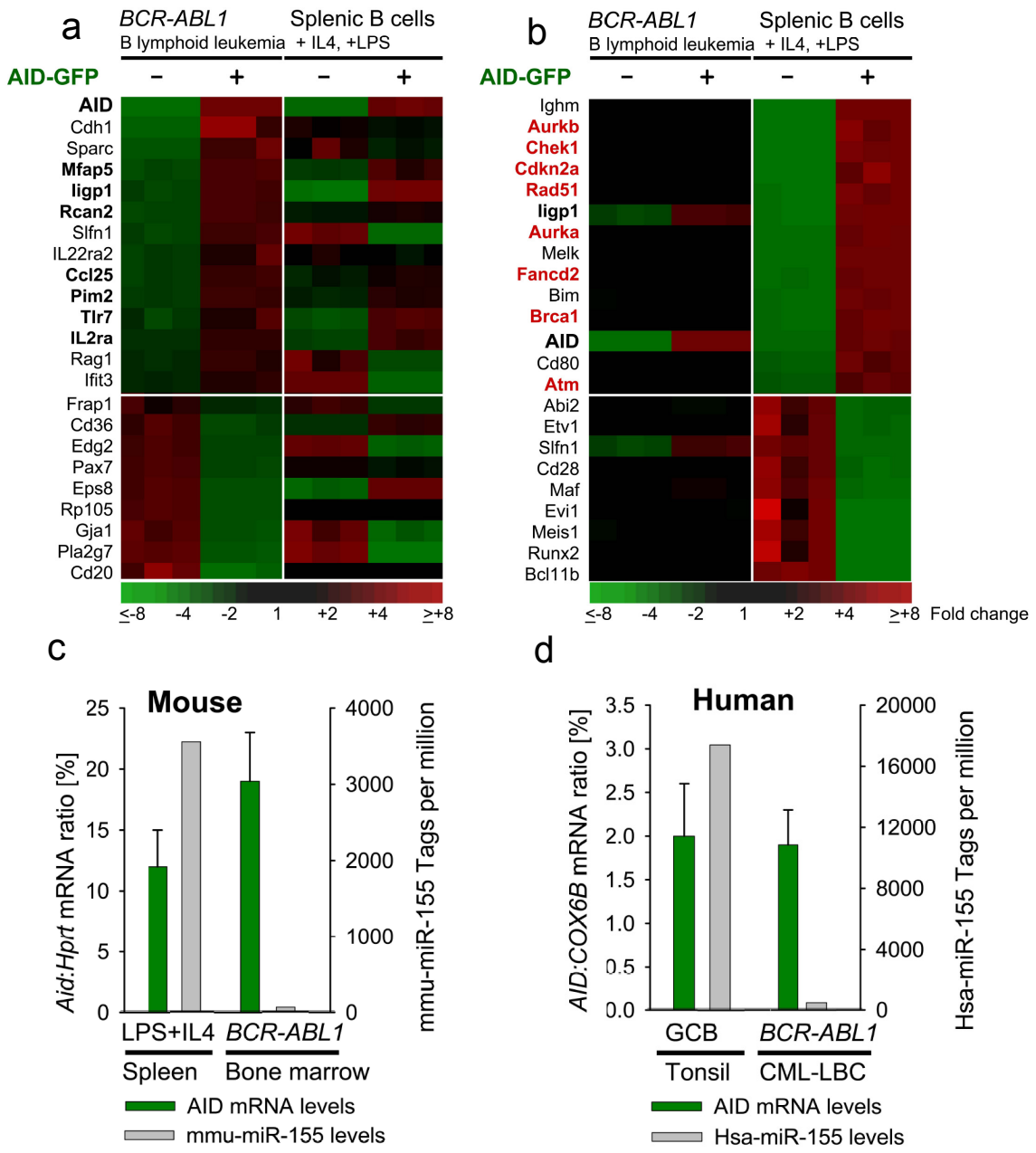
(**a**) Quantitative RT-PCR analysis of AID mRNA expression in $CD13^-$ $CD19^+$ B lymphoid (CML-LBC) and $CD13^+$ $CD19^-$ myeloid (CML-CP) bone marrow cells sorted from four patients (I-IV) with early B lymphoid blast crisis CML. Peripheral blood naïve (IgD^+) and germinal center (GC) B cells were used as negative and positive controls, respectively. AID mRNA levels (means ± SD) were normalized based on *COX6B* transcripts. (**b**) AID protein levels in CML-LBC and CML-CP cells from two patients (I-II) were compared to those of tonsillar GC B cells by Western blotting using EIF4E as loading control. (**c**) PAX5 mRNA levels in sorted CML-CP and CML-LBC cells were measured by quantitative RT-PCR (means ± SD). (**d**) AID protein expression in chronic phase and lymphoid blast crisis CML cells (I-IV) was measured by flow cytometry (intracellular staining) using diffuse large B cell lymphoma (DLBCL) cells as control. (**e**) Bone marrow cells from AID-GFP reporter transgenic mice were transduced with *BCR-ABL1* or empty vector control and cultured under myeloid (IL3, IL6, SCF) or B lymphoid (IL7) growth conditions. (**f**) AID mRNA levels (means ± SD) in sorted GFP^- (gray) and GFP^+ (green) cell populations from LPS/IL4-stimulated splenic B cells or *BCR-ABL1*-transformed leukemia cells from AID-GFP transgenic mice were measured by quantitative RT-PCR

We next examined *BCR-ABL1*-mediated transformation of bone marrow cells from mice carrying an *AID*-GFP reporter transgene (Crouch et al., 2007). Isolated bone marrow cells from *AID*-GFP animals were transduced with a retrovirus expressing human *BCR-ABL1* (Pear et al., 1998), or a retroviral empty vector control (MSCV). Following transduction, cells were cultured on bone marrow stroma in the presence of either myeloid (IL3, IL6 and SCF) or B lymphoid (IL7) growth factors following classical models of myeloid CML and B lymphoid LBC/Ph^+ ALL respectively (Li et al., 1999). Under myeloid culture conditions, neither normal nor *BCR-ABL1*-transformed myeloid progenitor cells

displayed *AID*-GFP reporter expression (Figure 17e). In contrast, ~5% of the *BCR-ABL1*-transformed B lymphoid progenitors exhibited strong *AID*-GFP (Figure 17e). In agreement with previous findings (Gourzi et al., 2006), retroviral infection with empty vector control also induced *AID*-GFP fluorescence in a small number of B lymphoid progenitor cells (0.7%, Figure 17e). Of note, AID expression in *BCR-ABL1* AID-GFP$^+$ leukemia cells was commensurate to splenic B cells activated with LPS and IL4, and about 240-fold higher than in AID-GFP$^-$ counterparts (Figure 17f).

To further characterize AID$^+$ B lymphoid leukemias, we analyzed AID-GFP$^+$ and AID-GFP$^-$ cells among *BCR-ABL1*-transformed B lymphoid leukemia and LPS/IL4-activated splenic B cells with Affymetrix 430 GeneChips (Figure 18). A number of genes typically coexpressed with AID in activated B cells were also upregulated in *BCR-ABL1* leukemias (*Mfap5, Iigp1, Rcan2*, *Ccl25, Pim2, Tlr7, Ilr2a*, Figure 18a). Conversely, a subset of DNA repair genes were strongly upregulated with AID in activated splenic B cells but absent in *BCR-ABL1* leukemia cells (Figure 18b). Among these, *Brca1*, *FancD2* and *Rad51* (Longerich et al., 2008), *Atm* and *Cdkn2a* (Ramiro et al., 2006), and *Chek1* (Gourzi et al., 2006) have been previously implicated either in the repair of AID-induced DNA lesions or negative regulation of AID-induced chromosomal translocation events. Thus, *BCR-ABL1*-transformed B lymphoid leukemia cells express AID in the absence of the DNA repair mechanisms that typically safeguard genome integrity during normal B cell activation in GCs.

Figure 18: *BCR-ABL1-transformed B lymphoid leukemia cells express AID in the absence of protective mechanisms to maintain genome integrity*



Bone marrow cells and splenic B cells were isolated from Aid-GFP reporter transgenic mice. Bone marrow B cell precursors were transformed with retroviral *BCR-ABL1,* splenic B cells were activated by LPS and IL4. From *BCR-ABL1*-transformed leukemia cells and LPS/IL4-activated B cells, Aid-GFP$^+$ and Aid-GFP$^-$ cells were sorted and subjected to RNA isolation and microarray analysis using the 430 GeneChip platform (GEO accession number GSE13611). For both cell types, genes that were differentially expressed between Aid-GFP$^+$ and Aid-GFP$^-$ populations

were identified and sorted according to the ratio of gene expression values in Aid-GFP$^+$ and Aid-GFP$^-$ populations from *BCR-ABL1*-transformed leukemia cells (**a**) and LPS/IL4-activated B cells (**b**). Genes that are concordantly upregulated with AID expression both in BCR-ABL1 leukemia (**a**) and activated splenic B cells (**b**) are highlighted in boldface. Genes related to DNA damage and repair and chromosomal stability are highlighted in red. In (**c**) and (**d**), AID mRNA levels (% of reference gene (means $\pm$ SD) as determined by quantitative RT-PCR) were plotted against expression levels of miR-155 (microRNA tags per million, as determined by Illumina deep sequencing). To this end, Aid mRNA and mmu-miR-155 levels were measured in murine LPS/IL4-activated splenic B cells and mouse bone marrow B cell precursors that were transformed by *BCR-ABL1* (**c**). Likewise, AID mRNA and hsa-miR-155 levels were measured in sorted human tonsillar germinal center B cells (GCB; CD77^{high}CD38$^+$IgD$^-$) and human B lymphoid CML-LBC cells (**d**).

In addition to DNA repair molecules, the micro RNA miR-155 acts as a tumor suppressor by protecting B cells from AID-induced chromosomal translocations (Dorsett et al., 2008). This mechanism relies on the ability of mir-155 to negatively regulate AID protein levels and to curb excessive AID activity during B cell activation in GCs (Teng et al., 2008). Deep sequencing analysis of human GC B cells and mouse activated B cells confirmed that expression of AID in these cells is matched by high levels of miR-155 expression (Figure 18c, d). In contrast, human B lymphoid CML-LBC and mouse B lymphoid *BCR-ABL1* leukemia cells expressed high levels of AID in the absence of miR-155 (Figure 18c, d). Hence, while AID is readily expressed in B lymphoid CML-LBC, some of the mechanisms that normally constrain AID-mediated genetic instability are not concurrently induced.

To determine whether AID is functional in human CML-LBC, we next assessed somatic hypermutation of immunoglobulin heavy chain (*IGHM*), *BCL6,* and *MYC* genes, which are targeted by AID in germinal center B cells ((Liu et al., 2008; Shen et al., 1998) for a schematic of regions selected for sequence analysis, see Figure S33.1a-c). In contrast to AID-negative CML-CP cells, AID$^+$ CML-LBC cells carried mutations in

IGHM, *BCL6*, and *MYC* at frequencies comparable to those previously reported for GC B cell-derived lymphoma ((Pasqualucci et al., 2001); Figure 19a). To determine whether AID promotes overall genetic instability in *BCR-ABL1* tumors, we measured gene copy number alterations (i.e. deletions or amplifications) in 23 primary cases of Ph^+ ALL using a 250K NspI SNP array (Figure 19b). Based on AID mRNA levels, Ph^+ ALL samples were classified as either AID^{high} (16 cases) or AID^{low} (7 cases). SNP analysis revealed a higher frequency of gene copy number alterations in the AID^{high} as compared to the AID^{low} group (median 14 [range 6-50] vs. median 5 [range 2-8]; $P = 0.02$; Figure 19b). Notably, deletion frequencies at the tumor suppressor genes *ARF* (*CDKN2A*) and *INK4B* (*CDKN2B*) at 9p21 were considerably higher in the presence of AID ($P = 0.04$; Figure 19b).

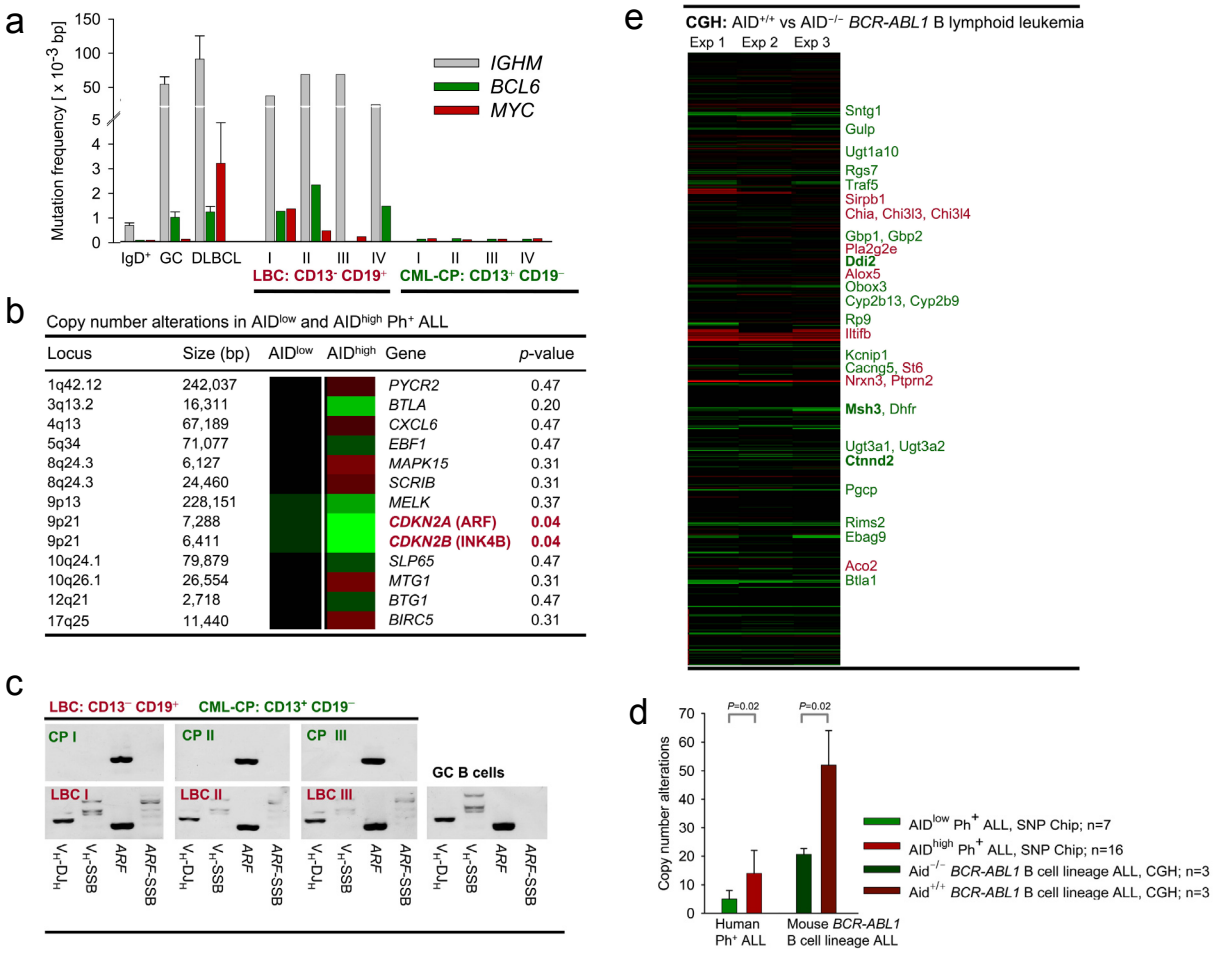
Deletion of *ARF* has previously been implicated in the progression of myeloid CML-CP into B lymphoid CML-LBC (Calabretta and Perrotti 2004). To determine whether AID directly targets *ARF* in LBC cells, we assayed this locus by ligation-mediated PCR for the presence of ssDNA breaks, which are intermediates of AID-mediated hypermutation (Unniraman and Schatz 2007). We found evidence of ssDNA breaks at the *ARF* locus of sorted AID^+ LBC cells, whereas these lesions were absent in AID^- CML-CP cells isolated from the same patients (Figure 19c), indicating *ARF* is a potential AID target in LBC cells. In support of this view, the *CDKN2A* (ARF) gene promoter carries five E box motifs (CAGGTG). E box motifs are tightly associated with AID hypermutation activity at Ig and non-Ig genes alike (Michael et al., 2003). In addition, analysis of a database of published *ARF* mutations in B lymphoid Ph^+ ALL and CML-LBC revealed a significant preference for mutations at RGYW or WRCY DNA motifs (13 of 60 mutations (21.7%), $P<0.05$), which are hotspots for AID-induced

hypermutation (Dorner et al., 1997). No such preference was observed in non-lymphoid tumors (24 of 461 mutations (5.2%), $P>0.22$; Figure S33.2). These observations suggest that AID promotes genetic instability in CML-LBC via widespread somatic hypermutation. To further explore this idea, we transformed bone marrow B cell precursors from $AID^{-/-}$ and $AID^{+/+}$ mice with *BCR-ABL1*-expressing retroviruses. After 9 weeks in cell culture, we assessed genomic deletions and amplifications by comparative genomic hybridization (CGH). The frequency of copy number alterations was significantly higher in $AID^{+/+}$ B lymphoid leukemia cells (52 ± 12) as compared to their $AID^{-/-}$ counterparts (20.6 ± 2.1; $P=0.02$; Figure 19d). Of note, 22 genes were consistently deleted in the presence but not in the absence of AID (Figure 19e). Among these, we found examples of genes implicated in the DNA repair and/or DNA damage response: Deletions of the *Msh3*, *Ddi2* and *Ctnnd2* genes were recurrently found in $AID^{+/+}$ but not $AID^{-/-}$ B lymphoid BCR-ABL1 leukemias (Figure 19e), suggesting that AID promotes genetic instability in *BCR-ABL1* lymphoid leukemias in part via hypermutation of DNA repair and DNA damage response genes.

Acquisition of *BCR-ABL1* kinase domain mutations represents a critical event in the progression of CML-CP into fatal CML-LBC, as they often confer resistance to Imatinib treatment (Shah et al., 2002). To investigate a potential role of AID in this process, we amplified and sequenced the *BCR-ABL1* kinase domain from single AID-negative myeloid and AID^{+} B lymphoid CML cells isolated from four patients with early CML-LBC under Imatinib-treatment. Single-cell PCR analysis and direct sequencing revealed the presence of L248V and T315I mutations in a significant fraction of B lymphoid but not myeloid CML cells in three of the patients (Figure 20a). The fourth patient showed positive PCR products in only a few sorted B lymphoid CML cells.

However, the E255K mutation was repeatedly amplified from bulk CML-LBC cells whereas myeloid cells from the same patient were unmutated (not shown). Since these findings show a straight correlation between AID expression and the acquisition of clinically relevant *BCR-ABL1* mutations in CML-LBC, we investigated whether enforced AID expression can induce *BCR-ABL1* mutations in CML.

Figure 19: *Evidence of aberrant AID activity in B lymphoid BCR-ABL1 leukemias*



(**a**) CML-LBC ($CD13^-$ $CD19^+$ $CD34^+$) and CML-CP ($CD13^+CD19^-$ $CD34^+$) cells were sorted from four patients and analyzed for somatic mutations within rearranged *IGHM* loci, as well as *BCL6* and *MYC* genes (means of mutation frequencies ± SD). Mutation frequencies in naïve (IgD^+) and germinal center (GC) B cells, as well as DLBCL cells were studied as a reference. CML-CP cells carry *IGHM* loci in germline configuration, which precludes mutation analysis. (**b**) SNP mapping

analysis was performed for 23 bone marrow samples from patients with B lymphoid Ph^+ ALL (GEO accession number GSE13612). Samples were selected based on particularly high (n=16; AID^{high}) or low (n=7; AID^{low}) AID mRNA levels. Deletions or duplications of gene loci are represented in green or red respectively, with color intensities reflecting the frequency of these lesions based on the total number of samples analyzed. (**c**) Short-lived DNA single-strand break (SSB) intermediates were determined at the *ARF* and *IGHM* loci by ligation-mediated PCR (LM-PCR) in LBC and CML-CP, as well as in germinal center B cells (GC). The presence of *ARF* and rearranged V_H-DJ_H genes was verified by genomic PCR in each sample. (**d**) Gene copy number alterations (CNA) were studied in *BCR-ABL1*-transformed bone marrow B cell precursors from $AID^{-/-}$ and $AID^{+/+}$ mice by Comparative Genomic Hybridization (means of CNA frequencies $\pm$ SD; GEO accession number GSE15093) using genomic DNA from normal splenic B cells as a reference. AID-specific lesions were identified in three CGH experiments comparing $AID^{+/+}$ *BCR-ABL1* leukemia cells against $AID^{-/-}$ *BCR-ABL1* leukemia (**e**). Data for all genes/loci where a copy number change between $AID^{-/-}$ and $AID^{+/+}$ *BCR-ABL1* leukemia was found in at least one experiment (sorted by chromosomal localization, chromosomes 1 through 19; recurrent deletions (green) or amplifications (red) are highlighted; **e**).

To this end, we transduced eight AID-negative myeloid CML cell lines with a retroviral vector encoding AID/GFP or GFP alone (Figure S33.3). Transduced cells were cultured in the presence or absence of Imatinib for three weeks and the relative enrichment or depletion of GFP^+ cells was monitored by flow cytometry. While GFP^+/GFP^- ratios were unchanged in untreated cultures, we observed a preferential expansion of AID-GFP transduced CML cells in the presence of Imatinib (Figure 20b). Importantly, single-cell PCR analysis consistently showed the presence of clinically relevant *BCR-ABL1* kinase mutations in Imatinib-treated AID/GFP^+ but not GFP^+ CML cells (Table 1). These results were confirmed in a complementary assay based on enzymatic digestion of *BCR-ABL1* kinase amplification products (Figure S33.4). We conclude that enforced AID expression can promote mutagenesis of the *BCR-ABL1* oncogene in CML cells. To examine whether this activity leads to Imatinib-resistance *in vivo*, we then labeled GFP^+ and AID/GFP^+ transduced CML cells with lentiviral firefly luciferase and injected them intrafemorally

into sublethally irradiated NOD/SCID mice. Leukemia engraftment was first observed 12 days post-injection (Figure 20c), as determined by bioluminescence imaging.

Table 1: *Single-cell RT-PCR analysis of BCR-ABL1 kinase domain mutations*

Cell Type	*ABL1* exons 4-6 Sequence analysis	Cells	Cytidine-Deamination	Amino acid change	IC_{50} [µmol/l Imatir
EM2-GFP	Wildtype	12/12			
EM2-AID/GFP	742 CTG→GTG	3/10	No	L248V	7.0
	944 ACT→ATT	5/10	Yes	T315I	16
	Wildtype	3/10			0.6
JK1-GFP	Wildtype	11/11			0.6
JK1-AID/GFP	742 CTG→GTG	3/10	No	L248V	7.0
	835 GAG→GTG	1/10	No	Silent	0.6
	944 ACT→ATT	2/10	Yes	T315I	16
	Wildtype	4/10			0.6
JURL-GFP	Wildtype	12/12			0.6
JURL-AID/GFP	742 CTG→GTG	3/10	No	L248V	7.0
	764 GAG→AAG	1/10	Yes	E255K	12.1
	944 ACT→ATT	3/10	Yes	T315I	16
	Wildtype	3/10			0.6
K562-GFP	742 CTG→GTG	3/10	Yes	L248V	7.0
	944 ACT→ATT	3/10	Yes	T315I	16
	Wildtype	5/10			0.6
K562-AID/GFP	742 CTG→GTG	3/10	No	L248V	7.0
	764 GAG→AAG	1/10	Yes	E255K	12.1
	819 TTG→TTA	1/10	Yes	Silent	0.6
	944 ACT→ATT	3/10	Yes	T315I	16
	Wildtype	3/10			0.6
KYO-GFP	Wildtype	11/11			
KYO-AID/GFP	742 CTG→GTG	1/10	No	L248V	7.0
	764 GAG→AAG	1/10	Yes	E255K	12.1
	819 TTG→TTA	1/10	Yes	Silent	0.6
	944 ACT→ATT	4/10	Yes	T315I	16
	Wildtype	3/10			0.6
LAMA84-GFP	Wildtype	9/9			0.6
LAMA84-AID/GFP	742 CTG→GTG	9/10	No	L248V	7.0
	764 GAG→AAG	1/10	Yes	E255K	12.1
	837 GAG→GTG	1/10	No	E279V	9.9
	944 ACT→ATT	4/10	Yes	T315I	16
	Wildtype	2/10			0.6
MEG1-GFP	Wildtype	11/11			0.6
MEG1-AID/GFP	742 CTG→GTG	3/11	No	L248V	7.0
	764 GAG→AAG	1/11	Yes	E255K	12.1
	819 TTG→TTA	1/11	Yes	Silent	0.6
	944 ACT→ATT	4/11	Yes	T315I	16
	Wildtype	2/11			0.6

The BCR-ABL1 kinase domain (exons 4–6 of ABL1) was subjected to sequence analysis. To prevent coamplification of normal ABL1 transcripts from nonrearranged alleles, the first round of

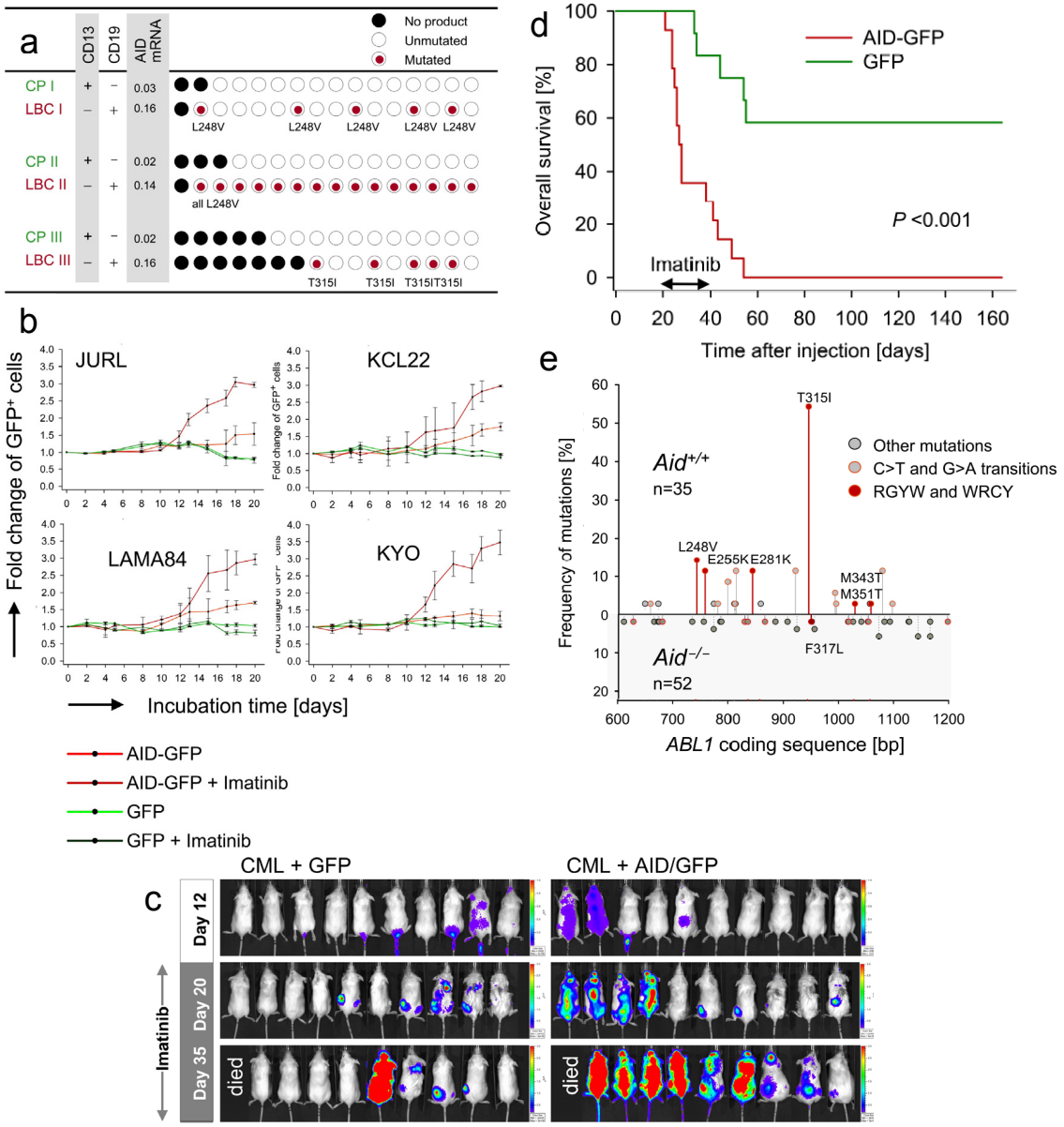
PCR used a BCR-forward and an ABL1-reverse primer. The second round of amplification used ABL1 gene-specific primers (exons 4–6). The rate of Taq DNA polymerase errors was calculated at 8×10^{5} /PCR cycle in the first round of PCR amplification. The CML cell lines K562 carries preexisting BCR-ABL1 kinasemutations (L248V and T315I), which is consistentwith Imatinib resistance of these cells in cell culture (data not shown). IC_{50} values are derived from publications referenced in Table S33.2.

When focal expansion of leukemic growth became evident (day 20), treatment with Imatinib (100 mg/kg twice daily) was started. By day 35, all 14 mice injected with AID/GFP-transduced CML cells had developed progressive disease as opposed to 5 of 12 GFP control mice ($P < 0.02$, Figure 20c). Correspondingly, all mice injected with AID/GFP^{+} CML cells were dead at day 54, whereas about half of the mice injected with GFP^{+} CML cells were still alive 162 days post-injection (Figure 20d).

Our transduction studies clearly show that while not absolutely required, AID can accelerate the acquisition of *BCR-ABL1* kinase domain mutations in human CML. To test AID-dependence of these mutations in a genetic experiment, we transduced bone marrow B cell precursors from $AID^{+/+}$ and $AID^{-/-}$ mice with *BCR-ABL1* to generate $AID^{+/+}$ and $AID^{-/-}$ B lymphoid *BCR-ABL1* leukemias. $AID^{+/+}$ and $AID^{-/-}$ B cell precursors transformed by *BCR-ABL1* at a similar efficiency, showed similar leukemic growth kinetics, viability, and colony formation potential in a methyl-cellulose semi-solid culture system (Figure S33.5). Six weeks following transformation, $AID^{+/+}$ and $AID^{-/-}$ leukemia cells were treated with increasing concentrations of Imatinib (from 0.1 up to 1.75 μmol/l) over a period of two weeks. Under these conditions, we observed a reproducible survival advantage of $AID^{+/+}$ relative to $AID^{-/-}$ leukemia cells (not shown). Importantly, sequence analysis of the *BCR-ABL1* kinase domain revealed multiple $AID^{+/+}$ clones carrying *BCR-ABL1* kinase mutations commonly observed in Imatinib-resistant LBC ((Shah et al.,

2002); Table S33.2; Figure 20e). Conversely, the *BCR-ABL1* mutation frequency in AID$^{-/-}$ leukemias was within the range of *Pfu* DNA polymerase error rate (P = 0.09; Figure 20e). A significant number of mutations in AID$^{+/+}$ cells were C→T (e.g. T315I) or G→A (e.g. E255K) transitions, suggesting that AID could directly target the *BCR-ABL1* fusion gene.

Figure 20: *AID expression in CML cells induces Imatinib-resistance in vitro and in vivo*



(**a**) Single CML-CP and LBC cells from three patients (I-III) with early lymphoid blast crisis under Imatinib-treatment were sorted into individual PCR reaction tubes and studied by single-cell PCR. AID mRNA levels (percentage of *COX6B* mRNA) for each cell population is indicated. (**b**) Four AID-negative human myeloid CML cell lines were transduced with retroviral vectors encoding AID-GFP or GFP alone and cultured for three weeks in the presence or absence of 2 µmol/l Imatinib. Relative outgrowth or depletion of GFP^+ cells was monitored by flow cytometry (means $\pm$ SD). (**c**) Firefly luciferase-labeled AID-GFP^+ and GFP^+ CML cells were intrafemorally injected into sublethally irradiated NOD/SCID recipient mice. Leukemia cell growth was monitored by bioluminescence imaging at the times indicated. (**d**) Overall survival of NOD/SCID mice injected with either AID-GFP^+ or GFP^+ CML cells and treated with 100 mg/kg/day Imatinib (day 20-40) is depicted by a Kaplan-Meier analysis. (**e**) Bone marrow B cell precursor cells from $AID^{+/+}$ and $AID^{-/-}$ mice were transformed by *BCR-ABL1* and cultured for 6 weeks under B lymphoid growth conditions. Subsequently, $AID^{+/+}$ and $AID^{-/-}$ *BCR-ABL1* B lymphoid leukemia cells were treated with gradually increasing concentrations of Imatinib (0.1 to 1.75 µmol/l) for two weeks. Surviving cells were subjected to sequence analysis for BCR-ABL1 kinase mutations. Red circles: *ABL1* mutations conferring clinical Imatinib-resistance, orange circles: C→T or G→A transitions, gray circles: other mutations.

The transcription factor PAX5 regulates both AID-gene expression (Table S33.1; (Gonda et al., 2003; Sayegh et al., 2003)) and B cell lineage commitment of hematopoietic progenitor cells (Nutt et al., 1999). To investigate whether ectopic expression of PAX5 in myeloid CML cells was sufficient to induce AID transcription and B cell lineage conversion, we transduced PAX5-negative CML cells with a retroviral vector expressing PAX5/GFP or GFP alone. Enforced expression of PAX5 resulted in significant upregulation of known B cell-specific PAX5-target genes including *AID*, *SLP65* (BLNK), and *CD79A* (Ig-α; Figure 21a). This was accompanied by surface expression of CD19 in a small subset of PAX5/GFP^+ but not in GFP^+ CML cells (Figure 21b). Upon prolonged treatment with Imatinib, $CD19^+$ CML subclones were positively selected to more than 16% after 6 weeks (Figure 21b). Furthermore, immunoglobulin spectratyping analysis of PAX5/GFP-transduced CML cells showed evidence of PAX5-induced *de novo* immunoglobulin V_H-DJ_H rearrangement (Figure 21c), which is

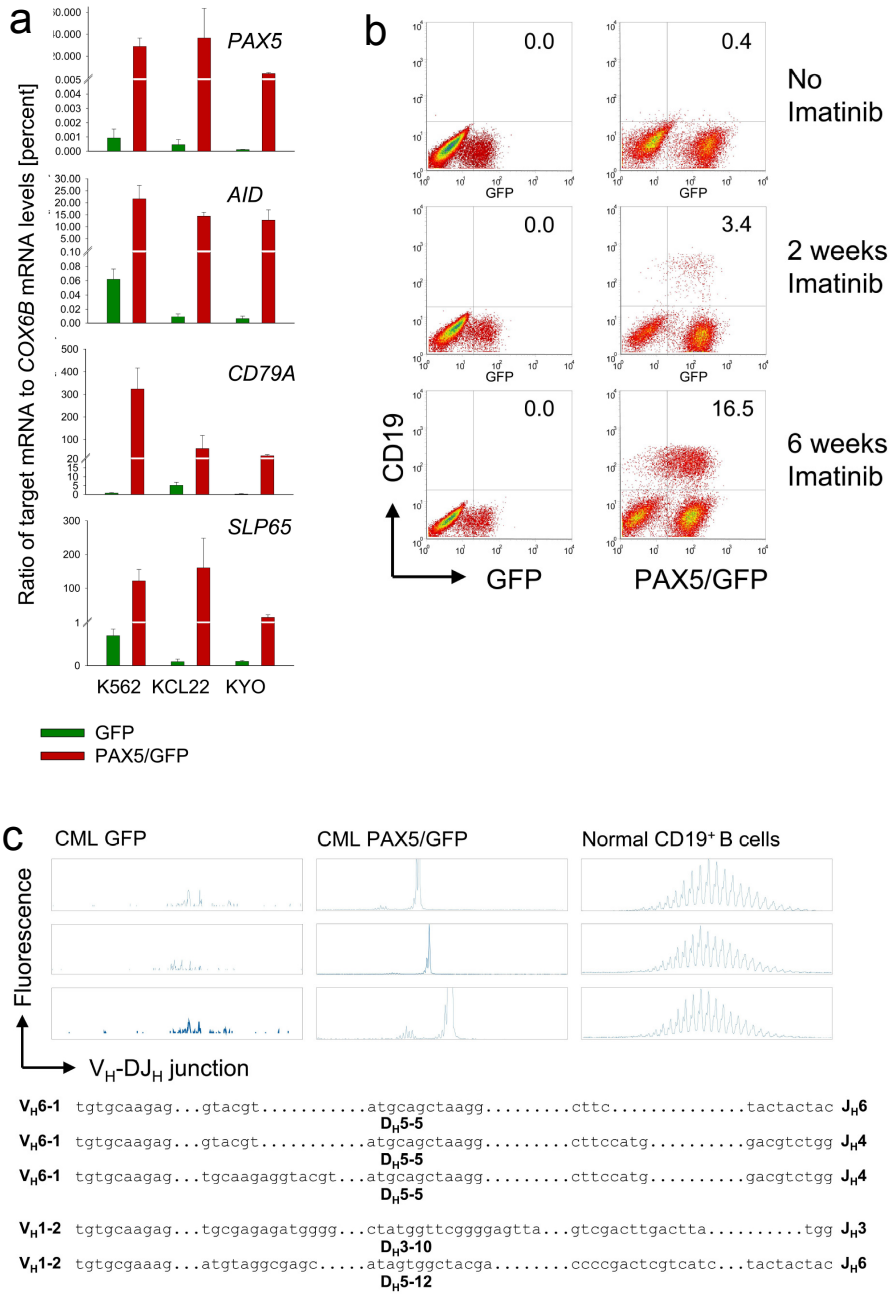
consistent with the role of PAX5 at promoting V_H-DJ_H recombination (Zhang et al., 2006). Sequence analysis of immunoglobulin gene rearrangements in PAX5/GFP-transduced CML cells revealed a striking preference for the most proximal located V_H gene segments: among 37 informative clones, only V_H6-1 and V_H1-2 gene segments (#1 and #2 most proximal located among 123 V_H segments) were found rearranged. *PAX5*-induced V(D)J recombination was also aberrant: instead of an initial D_H-J_H joint rearranging to different V_H-segments, a pre-existing V_H-D_H joint (V_H6-1-D_H5-5) was rearranged to various J_H segments (J_H4 and J_H6; Figure 21c). We conclude that ectopic expression of PAX5 in myeloid CML cells induces partial B cell lineage conversion and AID expression.

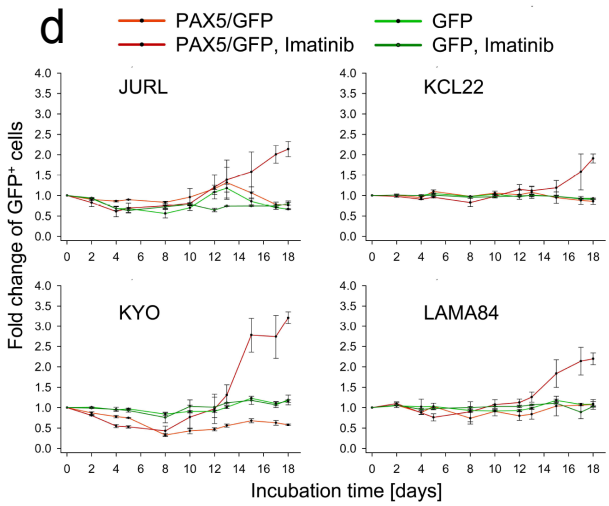
To determine whether *PAX5* can promote Imatinib-resistance in CML, PAX5/GFP$^+$ and GFP$^+$ human leukemia cells were cultured in the presence of increasing concentrations of Imatinib (0.1 μmol/L to 1.75 μmol/L). In analogy to results obtained with AID/GFP (Figure 20b), we observed a time-dependent outgrowth of PAX5/GFP$^+$ CML cells in the presence of Imatinib (Figure 21d). Sequence analysis confirmed the presence of mutations within the *BCR-ABL1* kinase domain of PAX5/GFP but not in GFP-transduced CML cells (Table 2). We conclude that enforced *PAX5* expression in myeloid CML cells can lead to partial B cell lineage conversion, AID expression, and Imatinib-resistance via *BCR-ABL1* mutation.

AID-mediated hypermutation preferentially targets RGYW and WRCY hotspot motifs (Dorner et al., 1997) and displays a biased towards C→T and G→A transitions over transversion mutations (Di and Neuberger 2002; Faili et al., 2002). To explore whether *BCR-ABL1* is a direct target of AID, we assembled and analyzed a database of 700 published *BCR-ABL1* kinase domain mutations (572 from CML-CP and 128 from

CML-LBC/Ph$^+$ ALL; Figure S33.6; Table S33.2). In support of direct AID-mediated hypermutation of BCR-ABL1, we found the frequency of C→T and G→A transitions to be notably higher in B lymphoid CML-LBC/Ph$^+$ ALL (66.4%) relative to CML-CP (19.9%; P<0.01; Figure 22b; Table S33.2).

Figure 21: *Ectopic expression of PAX5 in CML cells induces partial B cell lineage conversion, AID expression and Imatinib-resistance*





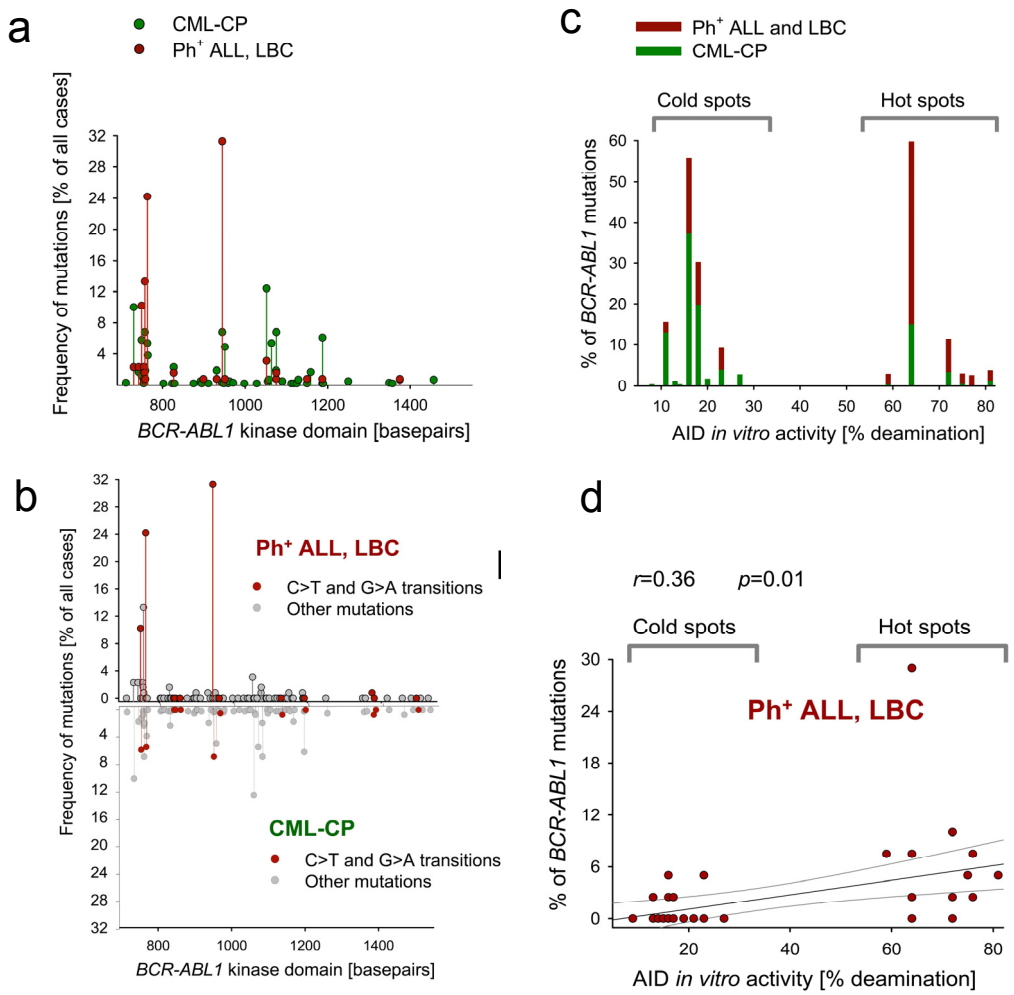
(**a**) CML cell lines were transduced with retroviral vectors encoding GFP (green) or *PAX5*/GFP (red) and expression of B cell lineage specific genes *PAX5*, *AID*, *CD79A* (Igα) and *SLP65* (BLNK) was monitored by quantitative RT-PCR (means ± SD). (**b**) FACS analysis of $CD19^+$ subclones emerging from GFP or *PAX5*/GFP-transduced CML cells cultured in the presence or absence of Imatinib. (**c**). Spectratyping analysis of rearranged immunoglobulin heavy chain genes from GFP-transduced (left), *PAX5*/GFP-transduced CML cells (middle), or primary $CD19^+$ peripheral blood B cells pooled from four healthy donors (right). The bottom panel shows the sequence analysis of oligoclonal *IGHM* gene rearrangements amplified from PAX5/GFP-transduced CML cells. (**d**) Four different CML cell lines were transduced with GFP or *PAX5*/GFP and grown in the presence or absence of rising concentrations of Imatinib (from 0.1 µmol/l to 1.75 µmol/l) for 16 days. The percentage of GFP^+ cells (means ± SD) was monitored over time by flow cytometry.

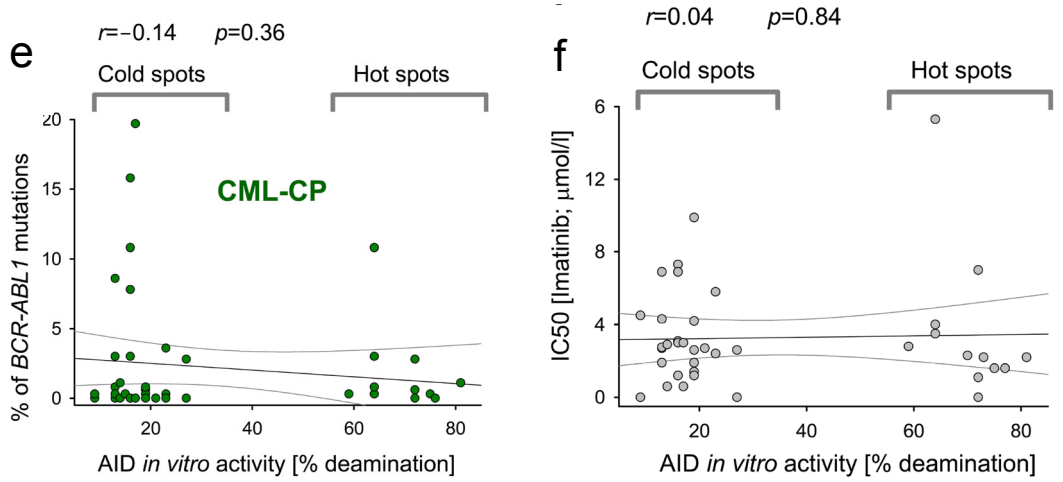
***Table 2:** Mutation analysis of BCR-ABL1 kinase domain mutations in PAX5-transduced CML cells*

Cell Type	$CD19^+$ clones	AID mRNA [% *COX6B*]	*ABL1* exons 4-6 Sequence analysis	Clones	Amino acid change	IC_{50} [µmol/l Imatinib]
KYO-GFP	< 0.01%	<0.01	764 GAG→AAG	1/35	E255K	12.1
			Wildtype and silent	34/35		0.6
			Imatinib-resistant	1/35 (2.9%)		
KYO-PAX5/GFP	~0.5%	4.24 ± 0.95	742 CTG→GTG	4/48	L248V	7.0
			764 GAG→AAG	5/48	E255K	12.1
			944 ACT→ATT	16/48	T315I	16
			1078 ATC→GTC	2/48	I360V	unknown
			1187 CAT→CGT	2/48	H396R	1.8
			Wildtype and silent	21/48		0.6
			Imatinib-resistant	27/48 (56%)		

This difference was exemplified by the 763G→A transition leading to E255K, and the 764A→T transversion leading to E255V. Both mutations target the same codon and are associated with a similar degree of Imatinib-resistance (IC_{50} [Imatinib] at 12.1 µmol/l and 17 µmol/l, respectively). In contrast to E255V, the E255K G→A transition occurs 5 times more frequently in CML-LBC/Ph^+ ALL than in CML-CP. The E255V A→T transversion however, was found in 22 cases of CML-CP (3.8%), but not in a single case of CML-LBC/Ph^+ ALL (Table S33.2).

Figure 22: *Characteristics of BCR-ABL1 kinase domain mutations in myeloid chronic phase CML and B lymphoid Ph^+ ALL/CML lymphoid blast crisis*





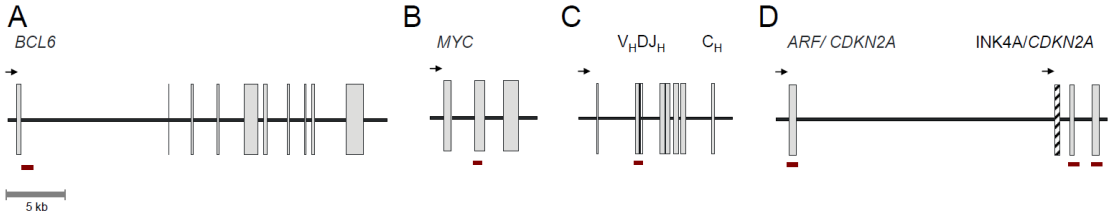
In (**a**), the distribution of *BCR-ABL1* kinase mutations in CML-CP (n=572; green) as compared to Ph^+ ALL/LBC (n=128; red) is compared. The x-axis denotes the nucleotide position of mutations. In (**b**), the frequencies of *BCR-ABL1* mutations in B lymphoid Ph^+ALL/LBC (top) and myeloid CML-CP (bottom) are compared with respect to C→T and G→A transitions (red). As a quantitative measure of AID-specific mutability, data from a comprehensive AID *in vitro* deamination assay based on 68 C-based motifs (Yu et al., 2004) were used. Motifs with AID *in vitro* activity below 27% and above 59% were considered coldspots and hotspots, respectively. In (**c-e**), the distribution of *BCR-ABL1* kinase mutations in B lymphoid Ph^+ALL/LBC (red) and myeloid CML-CP (green) are compared with respect to AID *in vitro* activity at the site of mutation (% deamination; based on data from (Yu et al., 2004)). In (**c**), the frequency of mutations at AID hotspot and AID coldspot motifs are compared in Ph^+ALL/LBC (red) and myeloid CML-CP (green). A potential correlation between the frequency of BCR-ABL1 mutations and AID-specific mutability at these sites (% deamination) was tested separately for Ph^+ALL/LBC (red; **d**) and CML-CP (green; **e**). In (**f**) the possibility of a potential bias introduced into our analysis is tested: Mutations with a high IC50 for Imatinib confer a high degree of drug-resistance and, hence, a strong selective survival advantage, which may skew the pattern of mutations (e.g. frequency of AID hotspot targeting). For 41 unique mutations, information on both IC_{50} (Imatinib) as well as AID *in vitro* activity (% deamination; (Yu et al., 2004)) was available (**f**). Correlation coefficients (r) and p-values were calculated based on Pearson product moment correlation. Linear regressions and 95% confidence intervals are indicated by black and gray lines, respectively.

We next investigated whether *BCR-ABL1* kinase mutations occurred preferentially at RGYW/WRCY motifs based on a previous cytosine deamination quantitative assay, which determined the ability of 68 cytosine-based DNA motifs to serve as a substrate for AID (Yu et al., 2004). We found a significant correlation between potential AID activity

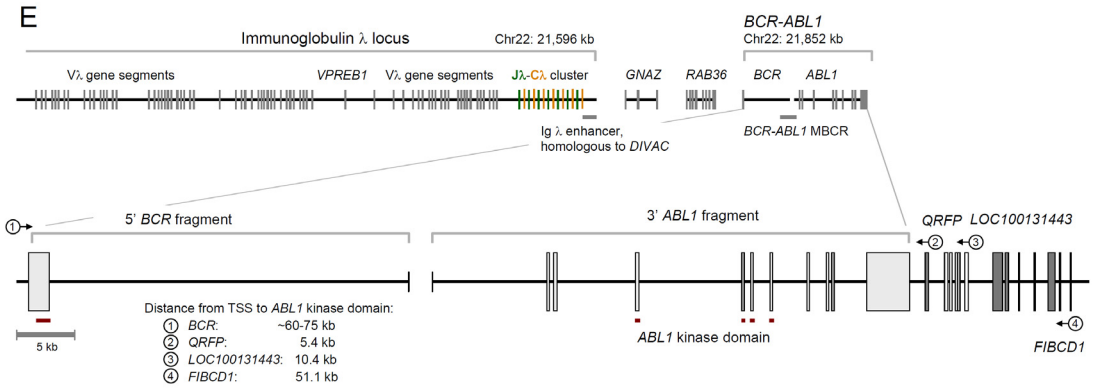
(percentage *in vitro* deamination) and *BCR-ABL1* mutation frequency in CML-LBC/Ph$^+$ ALL (r=0.36; p=0.01; Figure 22c, d; Figure S33.6). In contrast, no such correlation was observed for CML-CP (r=-0.14; p=0.36; Figure 22e). Importantly, statistical analyses showed no significant correlation between IC_{50} values and AID targeting (r=0.04; p=0.84), a feature that argues against the possibility that our results are biased by selective advantage of individual mutations (see Figure 22f and S33.6 for detailed explanation).

Supplemental data in 3.3:

Figure S33.1: *Genomic organization of potential target genes of AID-mediated hypermutation in BCR-ABL1-driven B cell lineage leukemia*



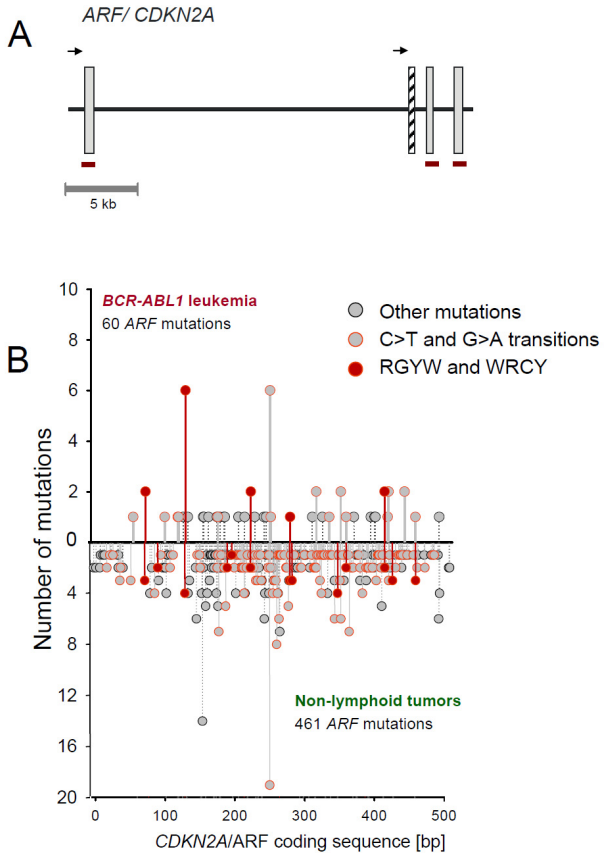
Sequence analysis was performed for the *BCL6* (A), *MYC* (B), *IGHM* (rearranged locus; C) and *CDKN2A* (ARF; D) genes. Gray boxes denote exons, arrows indicate the transcription start sites (TSS) and red bars indicate the region that was selected for sequence analysis.



The upper panel depicts the regional context of the *BCR-ABL1* fusion gene at 22q11 relative to the immunoglubulin λ locus, *VPREB1* and homology regions to chicken *DIVAC* at the Ig λ

enhancer (cis-acting element, Diversification Activator, induces AID-dependent hypermutation of Ig and Non-Ig genes; (Blagodatski et al., 2009)). The MBCR (major breakpoint cluster region) is typically observed in CML and B lymphoid blast crisis of CML and encodes the p210 form of the BCR-ABL1 fusion protein (210 kD). The genomic organization of the *BCR-ABL1* fusion gene is depicted in the lower panel. Gray boxes denote exons, arrows indicate the transcription start sites (TSS) and red bars indicate the regions that were selected for sequence analysis. The distances from TSS of the *BCR*, *QRFP*, *LOC100131443* and *FIBCD1* genes relative to the *BCR-ABL1* kinase domain are indicated.

Figure S33.2: *Mutation analysis of the ARF/CDKN2A gene*



Legend:

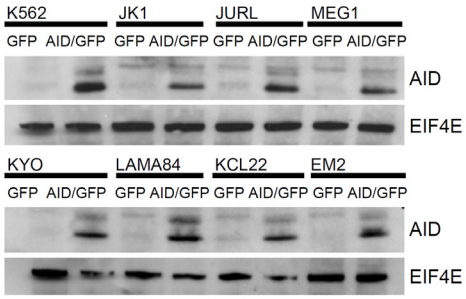
The *CDKN2A* locus with two transcription start sites (arrows) is depicted in (A). Red bars denote the coding exons of ARF that were analyzed. Sequence analysis of the *ARF* coding sequence (471 bps) in *BCR-ABL1* driven B cell lineage leukemias (Ph^+ ALL and LBC, top) and non-lymphoid tumors (bottom) based on publicly available databases (see below). Mutations were classified as targeting RGYW or WRCY hotspot motifs (red circles), C>T and G>A transitions (orange circles), or others (gray circles).

Mutation data were obtained from:

1. https://biodesktop.uvm.edu/perl/p16

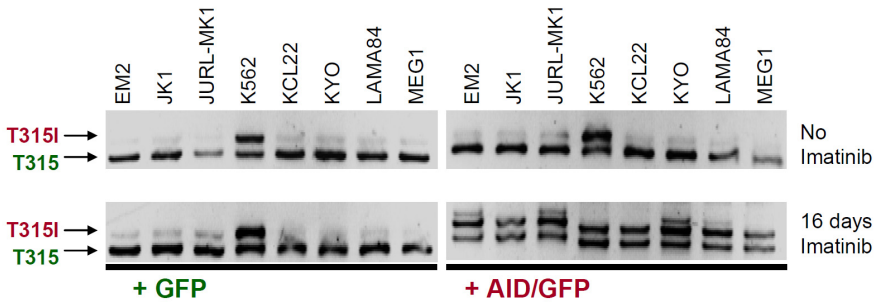
2.http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=bygene&ln=CDKN2A

Figure S33.3: *Ectopic expression of AID in CML cells*



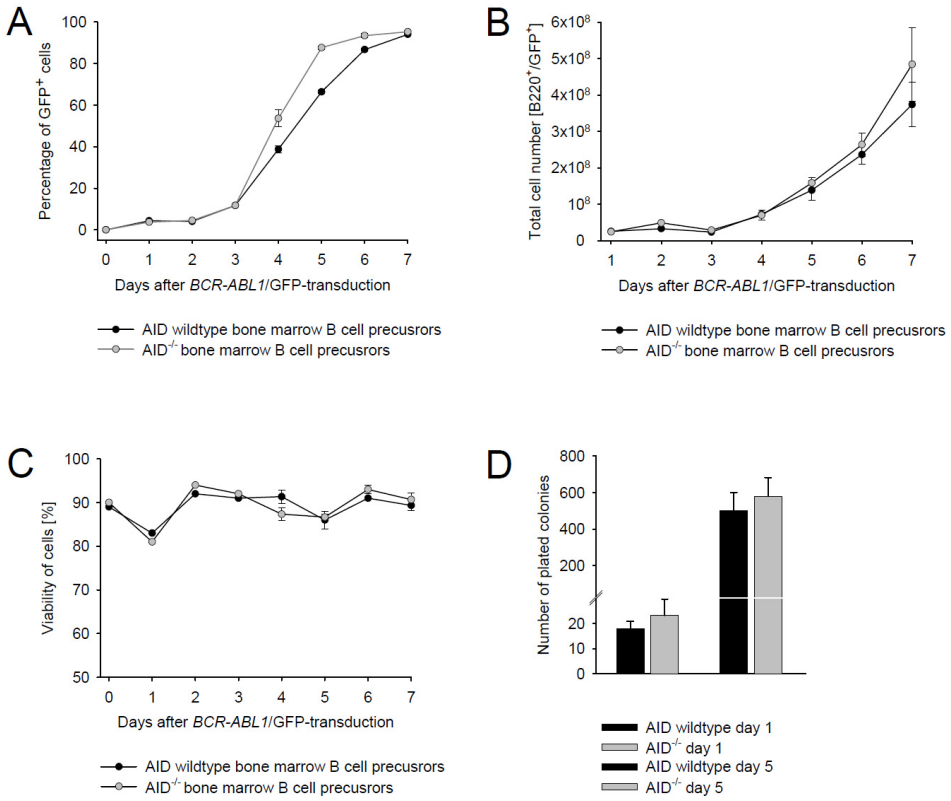
Legend. Eight AID$^-$ CML cell lines were transduced with a retroviral (MSCV) vector encoding AID and GFP or GFP alone. Transduction efficiency was monitored by flow cytometry (GFP$^+$ cells, not shown) and ectopic expression of AID was verified by Western blot using EIF4E as a loading control.

Figure S33.4: *Ectopic expression of AID in CML cells induces Imatinib-resistance in vitro*



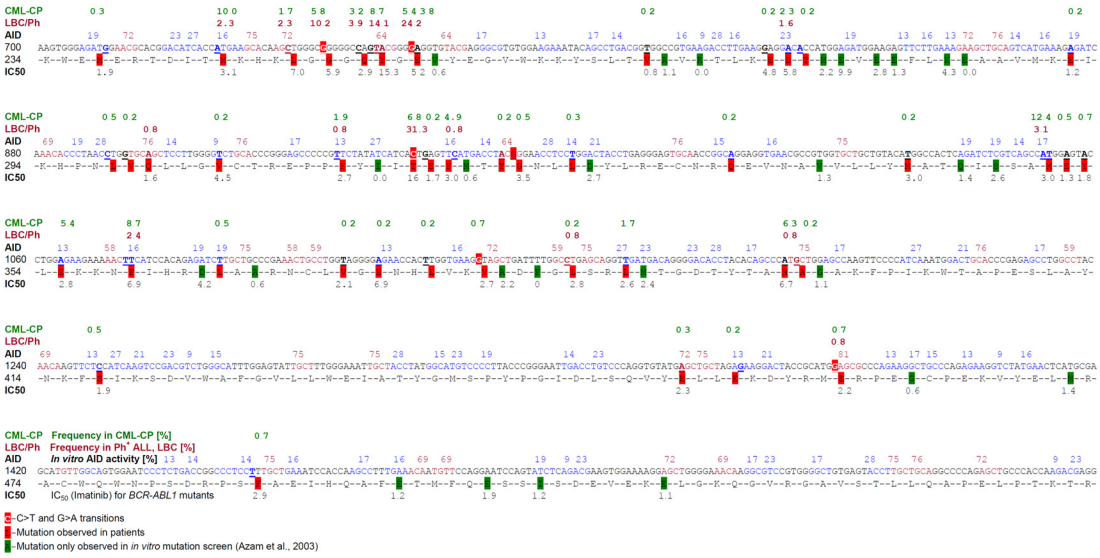
On the first and last day of treatment with Imatinib, aliquots were taken from *AID*/GFP$^+$ and GFP$^+$ CML cells. *ABL1* exon 6 from *BCR-ABL1* fusions was amplified by PCR and subjected to *Dde*I restriction digest, which distinguishes between wildtype (bottom) and T315I mutant (top) sequences. The PCR product is cleaved in the absence but not in the presence of the T315I mutation. Note that an uncleavable PCR product was obtained from K562 cells even prior to Gleevec-treatment and regardless of AID/GFP$^-$ or GFP-transduction, which is consistent with the finding of a pre-existing T315I mutation in these cells (see Table 1).

Figure S33.5: *Transformation potential of AID$^{-/-}$ and AID$^{+/+}$ bone marrow B cell precursors by BCR-ABL1*



Bone marrow cells from BALB/CJ wildtype and AID-deficient mice were cultured for 24 h in IMDM with 20% FBS, 50 µM 2-mercaptoethanol and 10 ng/ml IL-7. 2.5 x 10^6 cells per well were infected with *BCR-ABL1*-GFP Virus using RetroNectin reagent (Takara Bio, Madison) coated six-well plates. Each well was loaded with *BCR-ABL1*-GFP virus supernatant and the plate was centrifuged at 2000 g for 90 minutes. After the supernatant was removed, we centrifuged the cells at 400 g for 30 minutes on top of the virus loaded plates. Viability (C) and cell count (B; means ± SD) was determined using a Vi-CELL (Beckman Coulter, Fullerton). The transduction efficiency and the outgrowth of the *BCR-ABL1*-GFP positive cells were measured by FACS (A). The percentage of the total amount of GFP^+ cells and the amount of GFP^+B220^+ cells was determined on a daily base. On day 1 and day 5, a colony-forming cell assay was performed (D; means of colony numbers ± SD). We prepared a 10X concentrated cell suspension of 2 x 10^5 cells per ml in IMDM. 0.4 ml of cell suspension were added to 4 ml of MethoCult (STEMCELL Technologies, Vancouver). After vortexing, we allowed the bubbles to dissipate before dispensing 1.1 ml of cells + MethoCult mixture using a 3 ml syringe attached to a 16 gauge bluntend needle to each of three 35 mm dishes. 4 ml of sterile water was added to 2 extra uncovered 35 mm dish. Macroscopic colonies of transformed B cell precursors were counted.

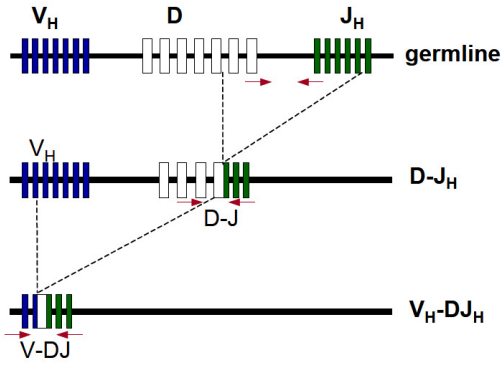
Figure S33.6: *Sequence analysis of the BCR-ABL1 kinase domain in chronic phase CML and B lymphoid CML blast crisis/Ph$^+$ALL*



Control for potential bias resulting from selection effects of individual *BCR-ABL1* kinase mutations: *BCR-ABL1* kinase mutations are subject to strong positive selection, because they provide a selective survival advantage to the leukemia cells in the event of Imatinib-treatment. However, the degree of Imatinib-resistance conferred by individual *BCR-ABL1* kinase mutations is highly variable, which may introduce a bias into our analysis. The possibility of bias was tested based on the following rationale: The degree of Imatinib-resistance conferred by an individual mutation is quantitatively reflected by its IC_{50} value for Imatinib. Mutations with a particularly strong selective advantage should have a high IC_{50} value for Imatinib, reflecting a higher degree of drug-resistance. A bias would occur in our analysis, if by chance mutations that represent AID-hotspots also have a high IC_{50} value. A quantitative measure of AID hotspot targeting was derived from an *in vitro* cytosine deamination assay of AID enzymatic activity using 68 C- or G-based motifs as DNA substrates ((Yu et al., 2004); % in vitro deamination indicated in this Figure). For each individual substrate motif, the percentage of AID-induced cytosine deamination provides a quantitative measure of AID hotspot (highlighted in red) or coldspot (highlighted in blue) targeting. To test the possibility of a potential bias in our analysis, we plotted AID-specific mutability (% deamination values indicated here) against IC_{50} values for all mutations (indicated below amino acide sequence) where both *in vitro* cytosine deamination and IC_{50} (Imatinib) data were available. The statistical analysis showed that there is no significant correlation between IC_{50} values and AID targeting (r=0.04; p=0.84; Figure 22f), which argues against the possibility that our analysis is biased by selective advantage of individual mutations.

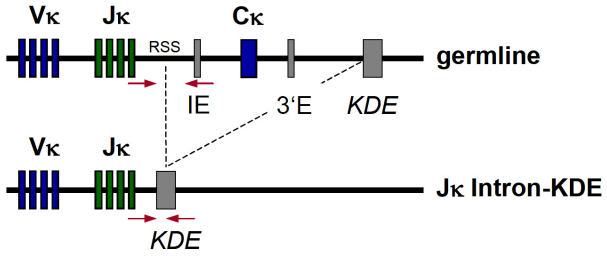
Figure S33.7: *B lymphoid-specific gene rearrangements in B lymphoid and myeloid CML blast crisis*

A.) Analysis of the configuration of the *IGHM* locus including mutation analysis of V region genes



Sample	*IGHM*	Mutations [x10^{-3}bp]	Phenotype
Blank Control	None		None
Normal T cells	Germline		T cell lineage
CML-CP1	Germline		Chronic phase CML
CML-CP2	Germline		Chronic phase CML
CML-CP3	Germline		Chronic phase CML
CML-CP4	Germline		Chronic phase CML
CML-CP5	Germline		Chronic phase CML
CML-CP6	Germline		Chronic phase CML
CML-CP7	Germline		Chronic phase CML
NALM1	V_H1-8-J_H2 V_H3-9-D2-21-J_H5	37 22	B lymphoid blast crisis
BV173	V_H3-21-D2-15-J_H3 Germline	22	B lymphoid blast crisis
LBC1	V_H1-18-D2-2-J_H6 Germline	26	B lymphoid blast crisis
LBC2	V_H1-3-D1-14-J_H4 V_H3-7-D2-2-J_H6	7 15	B lymphoid blast crisis
LBC3	V_H1-8-D5-18-J_H6 Germline	0	B lymphoid blast crisis
LBC4	V_H3-30-D2-15-J_H6	63	B lymphoid blast crisis
LBC5	V_H2-5-J_H5	7	B lymphoid blast crisis
CML-T1	V_H4-34-D2-8-J_H2 Germline	22	T cell/myeloid blast crisis
K562	V_H6-1-D3-9-J_H6	22	Myeloid blast crisis
JURL	Germline		Myeloid blast crisis
EM2	V_H6-1-D3-9-J_H4 V_H1-2-D7-27-J_H4 Germline	3 3	Myeloid blast crisis
LAMA84	V_H6-1-D5-5-J_H3 V_H6-1-D5-18-J_H4 Germline	19 10	Myeloid blast crisis
KYO	Germline		Myeloid blast crisis
KCL22	Germline		Myeloid blast crisis
JK1	V_H6-1-D5-5-J_H4 Germline	21	Myeloid blast crisis
MBC1	V_H3-48-D3-22-J_H4 Germline	48	Myeloid blast crisis
MBC2	V_H3-9-D3-3-J_H3 Germline	37	Myeloid blast crisis
MBC3	Germline		Myeloid blast crisis
MBC4	V_H2-5-D3-16-J_H6 Germline	51	Myeloid blast crisis

B.) Analysis of the configuration of the κ-deleting element (*KDE*) locus including junction analysis



Sample	*KDE*	Phenotype
Blank Control	None	None
T cells	Germline	T cell lineage
CML-CP1	Germline	Chronic phase CML
CML-CP2	Germline	Chronic phase CML
CML-CP3	Germline	Chronic phase CML
CML-CP4	Germline	Chronic phase CML
CML-CP5	Germline	Chronic phase CML
CML-CP6	Germline	Chronic phase CML
CML-CP7	Germline	Chronic phase CML
NALM1	Jκ Intron-KDE	B lymphoid blast crisis
BV173	Jκ Intron-KDE; germline	B lymphoid blast crisis
LBC1	Germline	B lymphoid blast crisis
LBC2	Jκ Intron-KDE; germline	B lymphoid blast crisis
LBC3	Germline	B lymphoid blast crisis
LBC4	Jκ Intron-KDE; germline	B lymphoid blast crisis
LBC5	Jκ Intron-KDE; germline	B lymphoid blast crisis
CML-T1	n.d.	T cell/myeloid blast crisis
K562	Jκ Intron-KDE; germline	Myeloid blast crisis
JURL	Jκ Intron-KDE; germline	Myeloid blast crisis
EM2	Jκ Intron-KDE; germline	Myeloid blast crisis
LAMA84	Jκ Intron-KDE; germline	Myeloid blast crisis
KYO	Germline	Myeloid blast crisis
KCL22	Jκ Intron-KDE; germline	Myeloid blast crisis
JK1	n.d.	Myeloid blast crisis
MBC1	Jκ Intron-KDE; germline	Myeloid blast crisis
MBC2	Jκ Intron-KDE; germline	Myeloid blast crisis
MBC3	Germline	Myeloid blast crisis
MBC4	Jκ Intron-KDE; germline	Myeloid blast crisis

Sequence analysis of *KDE* rearrangements

	Jκ-intron RSS		KDE-RSS
Germline	ATGCTGCCGTAGCCAGCTTTCCTGATG**CACAGTG**		**CACTGTG**GGAGCCCTAGTGGCAGCCCAGGGCGACTCC
NALM1	ATGCTGCCGTAGCCAGC	CCTTCGGG	AGTGGCAGCCCAGGGCGACTCC
BV173	ATGCTGCCGTAGCCAGCTTTCCT	CGTC	GCCCTAGTGGCAGCCCAGGGCGACTCC
LBC2	ATGCTGCCGTAGCCAGCTTTCCTGAT	CC	GAGCCCTAGTGGCAGCCCAGGGCGACTCC
LBC4	ATGCTGCCGTAGCCAGCTTTCC		CCCTAGTGGCAGCCCAGGGCGACTCC
LBC5	ATGCTGCCGTAGCCAGCT	TC	CCTAGTGGCAGCCCAGGGCGACTCC
K562	ATGCTGCCGTAGCCAGCTTTCCTGA		CCCTAGTGGCAGCCCAGGGCGACTCC
JURL	ATGCTGCCGTAGCCAGCTTTCCT	C	GGAGCCCTAGTGGCAGCCCAGGGCGACTCC
EM2	ATGCTGCCGTAGCCAGCTTTCCTGAT	CCGC	TGGGAGCCCTAGTGGCAGCCCAGGGCGACTCC
LAMA84	ATGCTGCCGTAGCCAGCTTTCCTGATG	CT	TGGCAGCCCAGGGCGACTCC
KCL22	ATGCTGCCGTAGCCAGCTTTCCTGA		CTAGTGGCAGCCCAGGGCGACTCC
MBC1	ATGCTGCCGTAGCCAGCTTTCCT	GC	GCCCTAGTGGCAGCCCAGGGCGACTCC
MBC2	ATGCTGCCGTAGCCAGCTTTCCTGATG	CGC	GTGGGAGCCCTAGTGGCAGCCCAGGGCGACTCC
MBC4	ATGCTGCCGTAGCCAGCTTTCC	CT	TGGGAGCCCTAGTGGCAGCCCAGGGCGACTCC

Table S33.1: *Requirements for AID expression and features of chronic phase and B lymphoid blast crisis CML/Ph$^+$ ALL*

Feature	IgD$^+$	GCB	DLBCL	CML-CP	LBC/Ph$^+$ALL	Reference
BCR-ABL1	-	-	-	+	+	(Heisterkamp et al., 1985)
NF-κB activation	-	+	+	+	+	(Reuther et al., 1998) (Gourzi et al., 2007) (Dedeoglu et al., 2004)
PAX5, E2A	+	+	+	-	+	(Gonda et al., 2003) (Sayegh et al., 2003) Figure 17c, Figure 21
AID	-	+	+	-	+	Figure 17
ARF deletions	n/a	n/a	+	-	50%	(Perrotti and Calabretta 2004) Figure 19, Figure S33.2
Aberrant hypermutation *IGHM, BCL6, MYC*	-	-	+	-	+	(Pasqualucci et al., 2001) Figure 19a
BCR-ABL1 mutations	n/a	n/a	n/a	14%	83%	(Soverini et al., 2006) Figure 20, Table 1
DFS (5 years)	n/a	n/a	n/a	87%	4%	(Kantarjian et al., 2002) (Druker et al., 2006)

Notes: IgD$^+$, naïve B cell; GCB, germinal center B cell, DLBCL, Diffuse large B cell lymphoma (GC-derived); CML-CP, chronic phase CML; LBC, lymphoid blast crisis; DFS, disease-free survival

Table S33.2: Metaanalysis of BCR-ABL1 kinase mutations in Ph+ ALL/CML-LBC and CML-CP

BCR-ABL1 kinase mutations						Ph+ ALL and CML-LBC			CML-CP			References for Ph+ ALL and CML-LBC													References for CML-CP																									
bp	aa	DNA	IC50	AID activity	C>T; G>A	Cases	%	C>T; G>A	Cases	%	C>T; G>A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	1	2	3	15	16	17	18	19	20	21	22	23	24	25	26	27	10	28	29	30	31	32	11	12	33
711	M237I	711G>T	1.9	19		0	0.0		2	0.3																			2																					
730	M244V	730A>G	3.1	16		3	2.3		57	10.0						1						2					1				6	5		1	7	1	6		5	9	1	1	1	1		1	2	9		
742	L248V	742C>G	7	72		3	2.3		10	1.7												1		2			1				2	3					1							2				1		
749	G250E	749G>A	5.9		Y	13	10.2	10.2	33	5.8	5.8	1								5		6		1			2					9				2	10		3	2			1				2	2		
749.5	G250A	749G>C	1.4			0	0.0		7	1.2																1														5						1				
754	Q252E	754C>G	2.9			0	0.0		1	0.2																																			1					
754.5	Q252R	754C>A				3	2.3		4	0.7											1	2																		3								1		
756	Q252H	756G>C				2	1.6		13	2.3						1				1							6				1			1	3		1			1										
757	Y253H	757T>C	15.3	64		17	13.3		39	6.8		1				4				2		7	2	1	1	1				3	5	6		4	4	1	4		4				1		1	1	2			1
758	Y253F	758A>T	3.5	64		1	0.8		11	1.9											1						2			1	2			1												1	2	2		
763	E255K	763G>A	5.2		Y	31	24.2	24.2	31	5.4	5.4	1				1		4	6	4	2	9	3	1	1	2	4				5	6		2	2	1				4		1				1	1	1		
764	E255V	764A>T	18			0	0.0		22	3.8																	1			1	2	2	1	3	3		2			1							2	2	1	1
767	V256A	767T>G	0.6			0	0.0		0	0.0																																								
802	V268A	802T>G	0.8			0	0.0		1	0.2																												1												
805	A269V	805G>T	1.1			0	0.0		0	0.0																																								
812	K271N	812G>C	0	9		0	0.0		0	0.0																																								
823	E275Q	823G>C	4.8			0	0.0		1	0.2																														1										
827	D276G	827A>G	5.8	23		2	1.6		13	2.3										1		1							3		1						2	2		3								1		1
829	T277A	829A>IG		23		0	0.0		1	0.2																					1																			
832	M278L	832A>T	2.2			0	0.0		0	0.0																																								
838	E279K	838G>A	9.9	19	Y	0	0.0	0.0	0	0.0	0.0																																							
841	E281K	841G>A	2.8		Y	0	0.0	0.0	0	0.0	0.0																																							
844	E282D	844G>T	1.3			0	0.0		0	0.0																																								
855	K285N	855A>C	4.3	13		0	0.0		0	0.0																																								
856	E286K	856G>A	0	72	Y	0	0.0	0.0	0	0.0	0.0																																							
875	E292V	875A>T	1.2	19		0	0.0		1	0.2																											1													
892	L298V	892C>G				0	0.0		3	0.5																											1			1						1				
895	V299L	895G>T				0	0.0		1	0.2																											1													
899	Q300R	899A>G	1.6	76		1	0.8		0	0.0					1																																			
900	Q300H	900G>C	1.6	76		0	0.0		0	0.0																																								
911	V304G	911T>G	4.5	9		0	0.0		1	0.2																			1																					
931	F311L	931T>C	2.7	13		1	0.8		11	1.9										1											1						2			1	2					2	1	2		
937	I313V	937A>G	0	27		0	0.0		0	0.0																																								
944	T315N	944C>A				0	0.0		2	0.3															1													1												
944.5	T315I	944C>T	16		Y	40	31.3	31.3	39	6.8	6.8	2	1	1		6	6	3	1	4	4	7	2	3			1				4	5		4	3	1	2		2	9	3	2	1					2		
948	E316D	948G>T	1.7			0	0.0		1	0.2																												1												
951	F317L	951C>G	3	16		1	0.8		28	4.9										1						1	2				1	4					7		3	5		3					1	1		
954	M318L	954A>T	0.6	14		0	0.0		0	0.0																																								
959	Y320C	959A>G		64		0	0.0		1	0.2																														1										
962	G321E	962G>A	3.5	64	Y	0	0.0	0.0	3	0.5	0.5																		3																					
971	L324Q	971T>A				0	0.0		2	0.3																					1									1										
973	D325H	973G>C	2.7	21		0	0.0		0	0.0																																								
998	Q333R	998G>A		15		0	0.0		1	0.2																																		1						
1013	V338G	1013T>G	1.3			0	0.0		0	0.0																																								
1028	M343T	1028T>C	3			0	0.0		1	0.2																												1												
1038	Q346H	1038G>C	1.4	19		0	0.0		0	0.0																																								
1043	S348L	1043T>G	2.6	19		0	0.0		0	0.0																																								
1052	M351T	1052T>C	3	17		4	3.1		71	12.4												4			2	3	8	1	1	3	7	6		4		2	3	1	5	9	2	4			2		1	7		
1055	E352G	1055A>G	1.3			0	0.0		3	0.5																			3																					
1057	Y353H	1057T>C	1.8			0	0.0		4	0.7																			2									2												
1064	E355G	1064A>G	2.75	13		0	0.0		31	5.4																	3				2	4		1			7			6			1			1	1	5		
1075	F359I	1075T>A	7.3	16		1	0.8		11	1.9												1			1					1		2				2	2	1		1										1
1075.5	F359V	1075T>G	6.9	16		2	1.6		39	6.8						1						1			1		2			2	1	7			2	1	1			5		2	1			1	1	11		1
1088	D363G	1088A>G	4.2	19		0	0.0		0	0.0																																								
1090	L364I	1090T>A		19		0	0.0		3	0.5																											2			1										
1097	A366D	1097C>A	0.6			0	0.0		0	0.0																																								
1112	V371A	1112T>C	2.1			0	0.0		1	0.2																			1																					
1118	E373G	1118A>G	6.9	13		0	0.0		1	0.2																			1																					
1125	L376V	1125T>G				0	0.0		1	0.2																														1										
1129	V379I	1129G>A	2.7		Y	0	0.0	0.0	4	0.7	0.7																					4										0								
1138	A380S	1138G>T	2.2	72		0	0.0		0	0.0																																								
1146	F382L	1146T>A	0			0	0.0		0	0.0																																								
1150	L384M	1150C>A	2.8	59		1	0.8		1	0.2										1																														1
1159	L387M	1159T>A	2.6	27		0	0.0		10	1.7																					3					1	2			1		1						1		1
1162	M388L	1162A>C	2.4	23		0	0.0		0	0.0																																								
1187	H396P	1187A>C	6.7			1	0.8		1	0.2													1														1													
1187.5	H396R	1187A>G	1.8			0	0.0		35	6.1															1		1			1	5	2		1	10		3			3		1				1	2	4		
1189	A397P	1189G>C		75		0	0.0		1	0.2																																								1
1192	G398R	1192G>A	1.1		Y	0	0.0	0.0	0	0.0	0.0																																							
1250	S417Y	1250C>A	1.9	13		0	0.0		3	0.5																	1			1																		1		
1349	E450G	1349A>G	2.3	72		0	0.0		2	0.3																					2																			
1357	E453Q	1357G>C		13		0	0.0		1	0.2																											1													
1375	E459K	1375G>A	2.2	81	Y	1	0.8	0.8	4	0.7	0.7		1														1												3											
1381	G463D	1381G>A	0.6	17	Y	0	0.0	0.0	0	0.0	0.0																																							
1416	M472I	1416G>C	1.4			0	0.0		0	0.0																																								
1457	F486S	1457T>C	2.9	14		0	0.0		4	0.7																	1										2											1		
1481	E494A	1481G>C	1.2	16		0	0.0		0	0.0																																								
1495	E499K	1495G>A	1.9		Y	0	0.0	0.0	0	0.0	0.0																																							
1506	I502M	1506C>G	1.2	19		0	0.0		0	0.0																																								
1527	E509D	1527G>C	1.1	72		0	0.0		0	0.0																																								
80 unique mutations						**128 cases**		**66.4**	**572 cases**		**19.9**													**128 cases**																										**572 cases**

Footnotes: bp, base pair; aa, amino acid; DNA, nucleotide position

References: 1 (Branford et al., 2002); 2 (Branford et al., 2003); 3 (Chien et al., 2008); 4 (Grammatico et al., 2009); 5 (Jones et al., 2008); 6 (Gorre et al., 2001); 7 (Gorre et al., 2001); 8 (Hofmann et al., 2002); 9 (Pfeifer et al., 2007); 10 (Shah et al., 2002); 11 (Soverini et al., 2006); 12 (von et al., 2002); 13 This study; 14 (Al-Ali et al., 2004); 15 (Chu et al., 2005); 16 (Deininger et al., 2004); 17 (Ernst et al., 2008); 18 (Guilhot et al., 2007); 19 (Hochhaus et al., 2001); 20 (Hochhaus et al., 2002); 21 (Hochhaus et al., 2007); 22 (Irving et al., 2004); 23 (Jabbour et al., 2006); 24 (Jiang et al., 2007); 25 (le Coutre et al., 2008); 26 (Nicolini et al., 2006); 27 (Roche-Lestienne et al., 2002); 28 (Sherbenou et al., 2007); 29 (Sherbenou et al., 2008); 30 (Sorel et al., 2004); 31 (Soverini et al., 2004); 32 (Soverini et al., 2005); 33 (Wang et al., 2006).

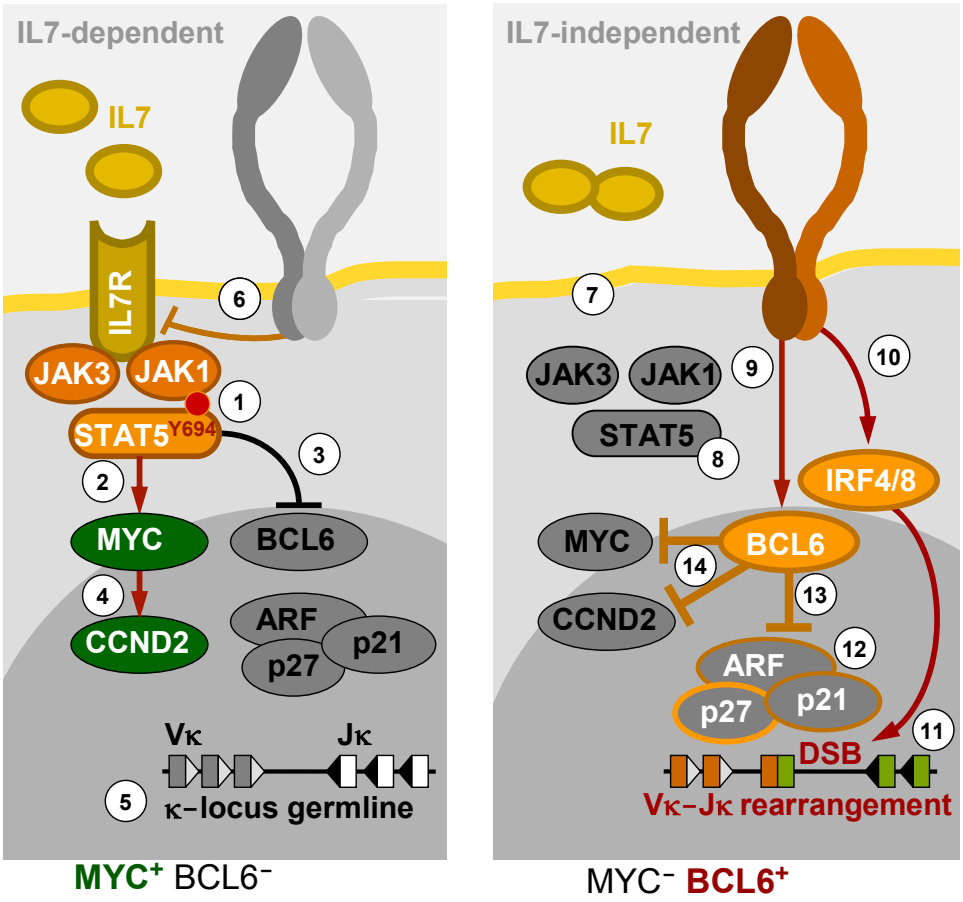
4 Discussion

BCL6 is critical for the development of a diverse primary B cell repertoire

Based on our observations, we propose the following scenario of BCL6-mediated survival signaling at the transition from IL-7–dependent to IL-7–independent stages of B cell development (Figure 23): Large cycling pre–BII cells express high levels of the IL-7 receptor (Hardy and Hayakawa 2001; Johnson et al., 2008), ligation of which induces tyrosine phosphorylation of JAK1, JAK3 and STAT5 (Banerjee and Rothman 1998; Walker et al., 2007). STAT5 phosphorylation at Y694 leads to transcriptional suppression of BCL6 (Walker et al., 2007) and transcriptional activation of MYC, which in turn promotes cell cycle progression via CCND2 (Bouchard et al., 2001). A recent study demonstrated that IL-7-Stat5 signaling in these cells actively prevents Ig light chain gene recombination (Malin et al., 2010). The Ig light chain loci IL-7–STAT5–dependent pro– and large cycling pre–BII cells are in germline configuration and the level of DNA damage (e.g. DSB) is low (Figure 23, left). Activation of pre–B cell receptor signaling in large cycling pre-BII cells, however, induces downregulation of the IL-7 receptor (Schebesta et al., 2002; Johnson et al., 2008) and ultimately dephosphorylation of STAT5 (Figure 9c). Dephosphorylation of STAT5 may either result from reduced IL-7 responsiveness (Schebesta et al., 2002; Johnson et al., 2008) or interference of the pre–B cell receptor signaling molecule SLP65 with JAK3–STAT5 signal transduction (Nakayama et al., 2009). Termination of IL-7Rα–JAK1–STAT5 signaling leads to down-regulation of MYC-CCND2 and, hence, loss of the “large cycling” phenotype of pre–BII cells. At the same time, loss of IL-7Rα–JAK1–STAT5 signaling allows transcriptional

activation of BCL6, which leads to further transcriptional repression of MYC-CCND2 (Figure 8b; (Ci et al., 2009)). The identification of MYC and CCND2 as transcriptional targets of BCL6 can explain that expression of MYC and BCL6 are mutually exclusive at the transition from large cycling pre–BII cells (MYC^+ $BCL6^-$) to small resting pre–BII cells (MYC^- $BCL6^+$).

Figure 23: *Scenario of BCL6-mediated survival signaling at the transition from IL-7–dependent to IL-7–independent stages of B cell development*



Legend: (1) Binding of IL-7 to the IL-7 receptor (composed of the IL-7Rα chain and common γ chain) activates JAK3 and JAK1, which leads to STAT5 phosphorylation (Figure 7c; Palmer et al., 2008). (2) Phosphorylated STAT5 activates MYC (Figure 7c) and (3) represses BCL6 expression (Figure 7c, Figure 3a; Walker et al., 2007). (4) The IL-7–STAT5 signaling mediates the upregulation of CCND2 (Figure 7b; Bouchard et al., 2001) and (5) arrests the κ locus in germline

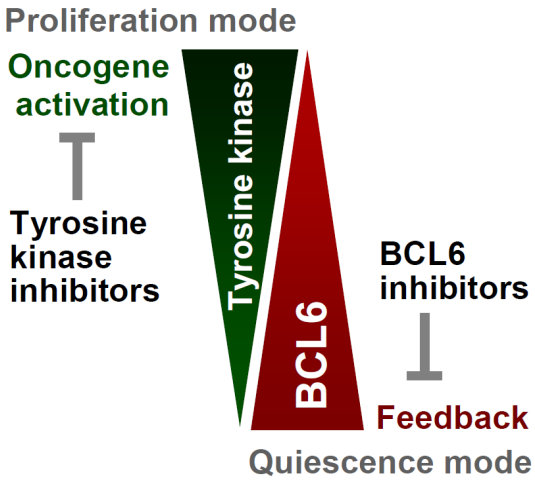
configuration (Malin et al., 2010). (6) Expression of the pre-B cell receptor induces the downregulation of IL-7Rα expression (Figure 9d). (7) Withdrawal of the IL-7Rα–STAT5 signaling (Figure 9c, d) results in (8) BCL6 upregulation (Figure 7c-d, Figure 9a-b). (9) Pre-B cell receptor mediates the upregulation of BCL6 (Figure 9c) and (10) activates IRF4/8 (Johnson et al., 2008). (11) IRF4 induces Vκ-Jκ rearrangement (Johnson et al., 2008), resulting in (12) extensive DNA damage and DNA DSB (Klein et al., 2005). (13) BCL6 suppresses DNA damage response and checkpoint genes (Figure 12a-d; (Phan et al., 2005; Ranuncolo et al., 2007)) as well as (14) CCND2 (Fernandez de Mattos et al., 2004) and MYC (Figure 8d).

After productive V_H-DJ_H recombination, pre–B cell receptor signaling induces upregulation of IRF4/8 and other factors needed for immunoglobulin light chain gene rearrangement (Schebesta et al., 2002; Johnson et al., 2008). In consequence, *de novo* expression of BCL6 upon IL-7 withdrawal coincides with initiation of immunoglobulin light chain gene rearrangement. Recombination events at Ig light chain loci (Vκ-Jκ, *KDE*-Jκ Intron and *KDE*-Vκ at 2p12; Vλ–Jλ at 22q11) involve multiple DNA-DSBs and induce activation of DNA damage response genes and checkpoint genes including ARF, p21 and p27, which are all negatively regulated by BCL6 (Figure 12a-d). In the absence of BCL6, Ig light chain gene rearrangement leads to excessive activation of DNA damage response and checkpoint genes (ARF, p53, p21, and p27; Figure 12) and only a few small resting pre–BII cells survive the transition from IL-7–dependent to IL-7–independent B cell development. Based on the timing of its expression and its ability to curb an excessive DNA damage response during light chain gene recombination, BCL6 plays a central role in the generation of a diverse primary B cell repertoire.

BCL6 enables leukemia cells to survive inhibition of oncogenic tyrosine kinases

Tyrosine kinase inhibitors (TKI) are widely used to treat patients with leukemia driven by *BCR-ABL1* (Druker et al., 2001) and other oncogenic tyrosine kinases (Meydan et al., 1996; Armstrong et al., 2003). Recent efforts focused on the development of more potent TKI that also inhibit mutant tyrosine kinases (Shah et al., 2004; O'Hare et al., 2009). However, even effective TKI typically fail to eradicate leukemia-initiating cells (Graham et al., 2002; Oravecz-Wilson et al., 2009; Naka et al., 2010), which often cause recurrence of leukemia after initially successful treatment. We identified BCL6 as a central component of this drug-resistance pathway and demonstrate that targeted inhibition of BCL6 leads to eradication of drug-resistant and leukemia-initiating subclones (Figure 24). We propose that dual targeting of tyrosine kinase signaling (TKI, "proliferation mode") and BCL6-dependent protective feedback (BCL6 inhibitors, "quiescence mode") represents a novel strategy to eradicate drug-resistant and leukemia-initiating subclones in tyrosine kinase-driven leukemia.

Figure 24: *Scenario - Dual targeting of oncogenic tyrosine kinase signaling and BCL6-dependent feedback in leukemia*



Proposed concept of dual targeting of oncogenic tyrosine kinase signaling (e.g. BCR-ABL1, FLT3, JAK2, PDGFR) and BCL6-dependent feedback signaling in tyrosine kinase-driven leukemia. While effective TKI induce cell death in the bulk of the rapidly dividing, tyrosine kinase-dependent leukemia population ("proliferation mode"), TKI typically fail to eradicate quiescent leukemia-initiating cells ("quiescence mode"). BCL6 provides protection in response to TKI-treatment of leukemia cells by suppression of ARF/p53-dependent apoptosis and by preventing cellular senescence. In this situation, the cells are highly drug-resistant and in "quiescence mode". Currently, a peptide inhibitor (RI-BPI; (Cerchietti et al., 2009b)) and a small molecule inhibitor (79-6; (Cerchietti et al., 2010)) are available to block the ability of BCL6 to recruit its co-repressors (NCoR, SMRT, BCoR). Upon cessation of TKI-treatment, oncogenic tyrosine kinase activity resumes and the leukemia cells revert into "proliferation mode", which represents the onset of leukemia recurrence after initially successful treatment.

AID-induced blast crisis progression in CML

We have shown here that the transition from chronic phase CML into B lymphoid blast crisis is accompanied by expression of PAX5 and its transcriptional target, the B cell-specific mutator AID. The presence of hypermutation at *MYC*, *ARF*, *BCL6*, and the *IGHM* loci clearly demonstrates the enzymatic activity of AID in CML-LBC. In like manner, a subset of *BCR-ABL1*-induced mouse leukemia cells express very high levels of AID and carry deletions and amplifications at an increased frequency relative to *BCR-ABL1*-induced leukemia cells from $AID^{-/-}$ mice. The first hint to AID tumorigenic activity was given by studies of transgenic mice expressing AID constitutively and ubiquitously under the chicken β-actin promoter (Okazaki et al., 2003). These animals rapidly succumbed to T cell lymphomas, lung microadenomas, and to a lesser extent sarcomas, hepatocellular carcinomas and melanomas. Surprisingly, B cell ontogeny was not directly affected by AID overexpression. Most importantly, B cell malignancies were not detected in these mice, suggesting that B lymphocytes might have evolved B cell-specific mechanisms to suppress AID tumorigenic activity (Casellas et al., 2009). Error-

free base excision and mismatch repair pathways for instance, correct the vast majority of AID-mediated mutations outside the immunoglobulin loci in germinal center B cells (Liu et al., 2008). Likewise, DNA repair proteins, cell cycle regulators, and microRNA mir155 prevent AID-induced DNA double-strand breaks from developing into chromosomal translocations (Dorsett et al., 2008). In stark contrast to activated or GC B cells, B lymphoid AID^+ CML-LBC cells fail to upregulate the full repertoire of protective mechanisms that normally accompany AID activity. This feature, which is likely to be a recurrent theme in AID^+ malignancies, would exacerbate AID-mediated hypermutation and/or deletion of tumor suppressor genes. Based on these observations, we propose that aberrant activity of AID and the lack of mechanisms restricting its ability to introduce deletions and chromosomal translocations combine to accelerate the progression from chronic phase into fatal B lymphoid blast crisis in CML patients.

In addition to genomic instability, our data uncovered a correlation between AID expression, the acquisition of *BCR-ABL1* kinase domain mutations and Imatinib resistance in CML-LBC. This correlation is underscored by the observation that *BCR-ABL1* kinase mutations are at least six times more frequent in B lymphoid CML-LBC than in myeloid CML-CP (Table S33.1; (Soverini et al., 2006)). Three sets of experiments argue for a causative role of AID in the acquisition of *BCR-ABL1* kinase mutations: i) forced expression of AID induces *BCR-ABL1* kinase mutations and Imatinib-resistance in human CML cells; ii) forced expression of PAX5 in CML cells leads to AID gene transcription and the acquisition of *BCR-ABL1* kinase mutations and iii) $AID^{+/+}$ but not $AID^{-/-}$ mouse BCR-ABL1 leukemias acquire *BCR-ABL1* mutations and Imatinib-resistance within a short period of time. While *BCR-ABL1* mutations do

obviously occur in the absence of AID (e.g. as in some cases of CML-CP), our findings demonstrate that AID dramatically accelerates the acquisition of such mutations and, hence, increases their overall frequency (e.g. as in CML-LBC; Table S33.1).

In this context, *BCR-ABL1* mutations could arise either *directly* by way of direct AID-targeted hypermutation, or *indirectly* as a result of AID-mediated genetic instability (e.g. by targeting of genes that are involved in DNA repair). Below we explore these arguments in detail.

In germinal center B cells, somatic hypermutation occurs mostly within a 2 kb window downstream of a gene's transcription start site (TSS), and loses its activity exponentially with increasing distance from this window (Rada and Milstein 2001). Depending on the precise location of the breakpoints within the *BCR* and *ABL1* genes, the *BCR-ABL1* kinase domain is situated approximately 60 to 75 kb downstream of the *BCR* TSS (Figure S33.1E), a feature that argues against the direct targeting hypothesis. On the other hand, hypermutation of immunoglobulin switch domains is known to occur up to 10 kb downstream of sterile promoters during isotype switching. Hence, if recruited to an unknown transcription start site downstream of the *BCR* promoter, AID could in principle exhibit enough residual activity to target the *BCR-ABL1* kinase domain. Since many *BCR-ABL1* kinase mutations confer drug-resistance, such lesions will be eventually selected from a heterogeneous leukemia cell population even if introduced at extremely low frequencies. Attempts to uncover hypermutation near the *BCR* promoter in CML-LBC cells have been inconclusive so far (unpublished observation). A negative result, however, does not necessarily rule out direct targeting of *BCR-ABL1* by AID, as hypermutation of a large number of genes in germinal center B cells is only evident in the

absence of error-free repair proteins Msh2 and UNG1 (Liu et al., 2008). Alternatively, AID hypermutation could be recruited by the *QRFP*, *LOC100131443* or *FIBCD1* genes, which are actively transcribed at 5.4 kb, 10.4 kb and 51.1 kb downstream of and in opposite orientation vis-à-vis *BCR-ABL1* (Figure S33.1E).

Of note, the *BCR-ABL1* fusion gene is located at chromosome 22q11 in close proximity to the human immunoglobulin λ locus (Figure S33.1E), which strongly recruits AID. The ability of the immunoglobulin λ locus to recruit AID was further underscored by the recent identification of a cis-acting Diversification Activator (*DIVAC*) element, which induces AID-dependent hypermutation of both immunoglobulin and non-immunoglobulin genes in DT40 chicken B cells (Figure S33.1E; (Blagodatski et al., 2009; Kothapalli et al., 2008)). *DIVAC* encompasses the immunoglobulin light chain (*IgL*) enhancer in chicken, a region that is highly homologous to the human immunoglobulin λ locus (*IGLV*), which is located in close proximity to the *BCR-ABL1* fusion gene (Figure S33.1E). It is conceivable that enhanced transcriptional activity following the chromosomal break event at 22q11 in the regional context of both the immunoglobulin λ locus and *DIVAC*-like elements may lead to recruitment of AID and aberrant hypermutation of the *BCR-ABL1* fusion gene.

Although based on correlative observations, the pattern of BCR-ABL1 kinase mutations in CML-LBC/Ph^+ ALL indeed suggests direct targeting by AID at a low frequency (Figure 22). Our data however do not rule out that mechanisms downstream of or independent from AID are also at play. Genomic instability resulting from AID-mediated deletion of DNA repair genes would be an example of the former, the acquisition of random mutations as byproducts of cellular oxidative stress or DNA

replication errors would exemplify the latter. For instance, a recent mathematical model for drug-resistance in CML proposes that DNA replication errors lead to the acquisition of BCR-ABL1 kinase mutations at a rate of 4×10^{-7} per cell division (Michor et al., 2005). This model explains the high frequency of BCR-ABL1 kinase mutations in CML blast crisis on the basis of the particularly high proliferation rate of these cells.

CML develops from a hematopoietic stem cell and consequently displays multilineage differentiation potential. During the chronic phase of the disease, the vast majority (>95%) of tumor cells differentiate into committed myeloid progenitors, while a small fraction (<5%) develops into the B lymphoid lineage likely as a result of PAX5 upregulation (Takahashi et al., 1998; Verstegen et al., 1999). In this context, we have shown that enforced expression of PAX5 promotes B cell lineage conversion in CML cells and induces aberrant expression of AID. We propose that this is a key step in the progression from CML-CP to fatal CML-LBC.

The development of Imatinib resistance in CML-MBC, which are $PAX5^{-}$ AID^{-} (unpublished observation), argues against a role of AID in the acquisition of BCR-ABL1 mutations in myeloid blast crisis. However, CML typically exhibits lineage infidelity and in ~10% of cases, B/myeloid biphenotypic leukemia cells give rise to CML blast crisis. By oscillating between myeloid and B lymphoid phenotypes (Takahashi et al., 1998; Verstegen et al., 1999), Imatinib-resistant MBC could in principle originate from mutated B lymphoid CML cells that have subsequently lost their B cell identity. There are several precedents supporting this view: *MYC*-driven hematopoietic progenitor cell leukemias are known to alternate between B lymphoid and

myeloid lineages through spontaneous silencing and reactivation of PAX5-dependent B cell lineage-induction (Yu et al., 2003).

Furthermore, Hodgkin's disease represents a prominent example of a human B cell-derived malignancy that has lost its B cell identity through aberrant expression of ID2, an inhibitor of PAX5/E2A (Mathas et al., 2006). Interestingly, we have found that in nearly all cases of CML-MBC analyzed, *IGHM* V_H-DJ_H and the Ig κ deleting element (KDE) are rearranged (Figure S33.7). Rearrangement of these loci is considered as a permanent imprint of B cell identity that would be retained even after subsequent myeloid lineage conversion. Importantly, V_H-DJ_H genes in CML-MBC show evidence of somatic hypermutation (Figure S33.7), whereas the *IGHM* and *KDE* loci are unmutated and in germline configuration in CML-CP. These findings support a B lymphoid origin for at least some cases of CML-MBC, and raise the intriguing possibility that AID activity may promote blast crisis transformation also in the case of CML-MBC.

5 Appendix

5.1 Abbreviations

4-OHT	4-hydroxytamoxifen
aa	amino acid
ABL1	Abelson leukemia viral oncogene homolog 1
ACTB	beta-actin
ARF	alternative reading frame
ALL	acute lymphoblastic leukemia
AID	activation-induced cytidine deaminase
BCL6	B cell lymphoma 6
BCR	B cell receptor or the breakpoint cluster region gene
bHLH	basic helix-loop-helix
BM	bone marrow
bp	base pair
BPI	BCL6 peptide inhibitor
BTB	broad complex–tramtrack–bric à brac
BTK	Bruton's tyrosine kinase
C	constant
CCND2	cyclin D2
CD	cluster of differentiation
CDKN1A	cyclin-dependent kinase inhibitor 1A
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
Chr	chromosome
CLL	chronic lymphocytic leukemia
CLP	common lymphoid progenitor
CML	chronic myelogenous leukemia
CML-BC	blast crisis CML
CML-CP	chronic phase CML
CML-LBC	CML in lymphoid blast crisis
CML-MBC	CML in myeloid blast crisis
CRE	Cre recombinase
COX6B	cytochrome c oxidase subunit 6B
CSR	class-switch recombination
cRSS	cryptic RSS
D	diversity
DLBCL	diffuse large B cell lymphoma
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dox	doxycycline
DSB	double-strand break
ER^{T2}	mutated estrogen receptor

fl	flanked loxP recognition sites
g	gravity
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	germinal center
GFP	green fluorescent protein
GL	germline
HDAC	histone deacetylase
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HMGB	high mobility group B
HSC	hematopoietic stem cell
Ig	immunoglobulin
IgH	immunoglobulin μ heavy-chain
IgK	immunoglobulin κ light chain
IgL	immunoglobulin λ light chain
IFN-β	interferon beta
IL	interleukin
IP	immunoprecipitation
ITAM	immunoreceptor tyrosine-based activatory motif
IRES	internal ribosome entry site
J	joining
JAK	Janus kinase
kDa	kilodalton
KDE	κ deleting element
KO ($^{-/-}$)	knockout
K-S	Kolmogorov-Smirnov
LM-PCR	ligation-mediated PCR
LIC	leukemia initiating cell
LSC	leukemia stem cell
Luc	firefly luciferase
MACS	magnetic cell separation
MAPK	mitogen activated protein kinase
miRNA	microRNA
MLL	mixed lineage leukemia
MM	multiple myeloma
Mx	myxovirus-resistance protein promoter
Myc	myelocytomatosis oncogene
Neo	neomycin
NHEJ	non-homologous end joining
NMP	N-methyl-2-pyrrolidone
NOD	non-obese diabetic
PAX5	paired box gene 5
PEG-300	polyethylene glycol-300
PCR	polymerase chain reaction
PLCγ	Phospholipase-C gamma
Ph	Philadelphia chromosome
PI3K	phosphatidylinositol-3 kinase
PTK	protein tyrosine-kinase

PTP	protein tyrosine-phosphatase
Puro	puromycin
Q-ChIP	quantitative chromatin immunoprecipitation
QLC	quiescent leukemia cell
qPCR	quantitative-PCR
RAG	recombination-activating genes
RI-BPI	retro-inverso BCL6 peptide inhibitor
RNA	ribonucleic acid
RSS	recombination signal sequence
SCID	severe combined immunodeficiency
SD	standard deviation
SEM	standard error of the mean
SH2	SRC-homology domain 2
SH3	SRC-homology domain 3
SHM	somatic hypermutation
SLC	surrogate light chain
SLP65	SH2 containing leukocyte-specific phosphoprotein of 65 kDa
Spl	spleen
SRC	cellular homolog of Rous sarcoma virus
STAT5	signal transducer and activator of transcription 5
TdT	desoxynucleotidyl transferase
Tet	tetracycline
TKI	tyrosine kinase inhibitor
TLR	toll-like receptor
Tyr	tyrosine
V	variable
WT ($^{+/+}$)	wildtype

5.2 Supplementary Information

Table S1: *Overview over patient-derived samples of Ph^+ ALL and Ph^+ ALL cell lines*

Case	Cytogenetics	*BCR-ABL1*	Sequence analysis	Clinical course	Gender/Age
Primary cases					
LAX2	t(9;22)(q34;q11)	p210	T315I	Relapse (Imatinib)	m/38
LAX9	t(9;22)(q34;q11) del(12)(p12;p13) del(11)(q23)	p190	unmutated	at diganosis	m
SFO2	t(9;22)(q34;q11)	p210	unmutated	at diagnosis and Relapse (Nilotinib)	m/7 m/8
BLQ1	FISH der(9), der(22)	p210	T315I	Relapse (Imatinib)	
BLQ4	FISH der(9), der(22)	p210	unmutated-	Relapse (Imatinib)	f
BLQ5	FISH der(9), der(22)	p190	T315I	Relapse (Imatinib)	f
BLQ6	FISH der(9), der(22)	n.d.	n.d.	Relapse (Imatinib)	m
BLQ11	FISH der(9), der(22)	p210	T315I	Relapse (Imatinib)	m
TXL1	t(9;22)(q34;q11)	n.d.	unmutated	at diagnosis	m/19
TXL2	t(9;22)(q34;q11)	p210	unmutated	at diagnosis	
TXL3	t(9;22)(q34;q11)	p210	unmutated	at diagnosis	
TXL4	t(9;22)(q34;q11)	n.d.	unmutated	at diagnosis	f/56
ICN1	t(9;22)(q34;q11)	p210	unmutated	at diagnosis	
ICN10	der(9)(q10)t(9;22)(q34;q11) der(22) t(9;22)(q34;q11)	n.d.	n.d.	at diagnosis	f
Cell lines					
BV173	add(1)(q42), add(8)(p23), der(22)t(9;22)(q34;q11)	p210	unmutated	B lymphoid blast crisis	m/45
Nalm1	der(7)t(7;9;15)(q10;?;q15), t(9;9)(p24;q33-q34) t(9;22) (q34;q11), der(15)t(7;9;15)	p210	unmutated	B lymphoid blast crisis	f/3
SUP-B15	t(1;1)(p11;q31), add(3)(q27), der(4)t(1;4)(p11;q35), add(10)(q25), t(9;22)(q34;q11)	p190	unmutated	Relapse	m/9
TOM1	del(7)(p14),der(9)del(9)(q13q34) der(22)t(9;22)(q34;q11)	p190	unmutated	Refractory	f/54

Notes: All samples are bone marrow biopsies, blast content >80%; LAX, Los Angeles; BLQ, Bologna; TXL, Berlin; SFO, San Francisco; ICN, Seoul; n.d., not done; f, female; m, male

***Table S2**: Immunophenotype of patient derived CML samples*

Patient	Age, sex	Blasts [%]	CD10 [%]	CD19	CD24	TdT	CD13 [%]	CD15	CD33	CD54	CD64	CD65	MPO	CD34 [%]
Early B lymphoid blast crisis														
I	62, female	22	44	19	91	22	35	37	32	26	39	43	n.d.	8
II	72, male	28	24	21	n.d.	11	15	10	4	n.d.	n.d.	12	4	18
III	male	25	32	14	29	19	64	29	59	78	n.d.	63	n.d.	19
IV	female	27	31	18	42	12	50	38	44	76	n.d.	48	23	24
Late B lymphoid blast crisis														
V	51, male	70	78	73	59	58	33	2	22	55	2	12	n.d.	76
VI	28, female	84	83	80	91	73	22	20	11	49	11	10	n.d.	43
VII	79, male	87	93	85	94	78	21	10	10	65	10	10	n.d.	70
VIII	24, female	81	84	79	87	74	22	n.d.	24	73	n.d.	11	n.d.	13
Chronic phase CML														
IX	male	4	2	1	15	1	45	34	30	83	55	67	n.d.	9
X	female	8	1	1	23	0	56	41	42	78	60	63	n.d.	12

Notes: TdT, terminal deoxynucleotidyl transferase; MPO, myeloperoxidase; FACS analyses of surface antigens, TdT and MPO are gated on leukemic blasts, percentages refer to positive cells among leukemic blasts. $CD34^+$ $CD13^-$ $CD19^+$ B lymphoid or $CD34^+$ $CD13^+$ $CD19^-$ myeloid leukemic blasts were sorted from bone marrow samples, using a FACStar 440.

The definition of “Early B lymphoid blast crisis” is based on the WHO criteria for staging of CML (>20% blasts within the bone marrow; (Cortes et al., 2006)). The traditional classification (Sokal et al., 1988) uses 30% bone marrow blasts as cut off. Therefore, the four samples studied here (22% to 28% leukemic blasts) are referred to as “early B lymphoid blast crisis”. Samples from these particular patients were chosen for analysis because they included chronic phase CML cells and also a subpopulation of B lymphoid blasts, which indicate the onset of lymphoid blast crisis

***Table S3**: In vivo toxicology studies for Nilotinib/RI-BPI combinations, biochemistry parameters for liver, kidney function and electrolytes*

	Nilotinib		**Nilotinib + RI-BPI**			
Parameter	*Mean (n = 5)*	*SEM*	*Mean (n = 4)*	*SEM*	*Reference values*	
White blood cells	5.34	0.77	6.89	0.62	3.5-12	K/μL
Red blood cells	8.53	0.26	7.06	0.44	8.2-10.4	M/μL
Platelets	974.40	103.25	1112.75	194.84	799-1300	K/μL
WBC morphology	normal		normal			
RBC morphology	normal		normal			
Platelets morphology	normal		normal			
ALP	39.80	1.62	50.50	13.28	23-181	IU/L
GGT	0.20	0.20	0.50	0.50	0-2	IU/L
Albumin	2.54	0.07	2.28	0.05	2.5-3.9	g/dL
Total Protein	4.46	0.11	4.13	0.20	4.1-6.4	g/dL
Globulin	1.92	0.09	1.85	0.16	1.3-2.8	g/dL
Total Bilirubin	0.36	0.02	0.25	0.03	0-0.3	mg/dL
Blood Urea Nitrogen	19.60	2.18	28.00	5.37	14-32	mg/dL
Creatinine	0.14	0.02	0.10	0.00	0.1-0.6	mg/dL
Cholesterol	77.20	1.85	64.50	4.11	74-190	mg/dL
Glucose	293.00	28.18	190.25	46.97	76-222	mg/dL
Calcium	8.48	0.04	8.58	0.09	7.6-10.7	mg/dL
Phosphorus	6.78	0.47	9.03	0.99	4.6-9.3	mg/dL
Bicarbonate	21.00	1.22	19.75	1.65	11-18	mEq/L
Chloride	110.40	0.51	113.75	1.18	103-115	mEq/L
Potassium	4.92	0.27	4.93	0.34	3.4-5.5	mEq/L
Sodium	148.80	0.97	151.50	0.96	146-155	mEq/L
Osmolality	309.20	2.06	311.50	1.31	300-330	mOsm/kg

Note: Single-agent treatment with 20 mg/kg RI-BPI was recently evaluated over a period of one year (weekly injections over 52 weeks) and found to be non-toxic in mice (Cerchietti et al., 2005).

***Table S4:** List of Primers*

Sequences of oligonucleotide primers used Clonality and spectratyping analysis (mouse)

V_H1_F	5'-AAGGCCACACTGACTGTAGAC-3'
J_H2_R	5'-GAGGAGACTGTGAGAGTGGTG-3'
Cμ_R	5'-TGGCCACCAGATTCTTATCAG-3'
J_H1-FAM_R	5'-GACGGTGACCGTGGTCCCTGT-3'
J_H2-FAM_R	5'-GACTGTGAGAGTGGTGCCTTG-3'
J_H3-FAM_R	5'-GACAGTGACCAGAGTCCCTTG-3'

J_H4-FAM_R	5'-GACGGTGACTGAGGTTTCTTG-3'
Cμ-FAM_R	5'-AGACGAGGGGGAAGACATTTG-3'

Notes: V_H1_F binds to rearranged V_H gene segments of the J558 family.
FAM denotes dye-labeled oligonucleotide. The label is attached to the 5' end.

Human IGHM spectratyping primers

V_HFRIIICONS	5'-ACACGGCYSTGTATTACTGT-3'
Cμ	5'-TCAGGACTGATGGGAAGC-3'
Cμ Run off	5'-FAM-GCTGCTGATGTCAGAGTTGT-3'

Human IGHM

V_H1	5'-CAGTCTGGGGCTGAGGTGAAGA-3'
V_H2	5'-GTCCTRCGCTGGTGAAACCCACACA-3'
V_H3	5'-GGGGTCCCTGAGACTCTCCTGTGCAG-3'
V_H4	5'-GACCCTGTCCCTCACCTGCRCTGTC-3'
V_H5	5'-AAAAAGCCCGGGGAGTCTCTGARGA-3'
V_H6	5'-ACCTGTGCCATCTCCGGGGACAGTG-3'
Cμ	5'-AGACGAGGGGGAAAAGGGTT-3'
J_H1.2.4.5	5'-ACCTGAGGAGACGGTGACCAGGGT-3'
J_H3	5'-ACCTGAAGAGACGGTGACCATTGT-3'
J_H6	5'-ACCTGAGGAGACGGTGACCGTGGT-3'

Human KDE rearrangements

KDE germline	5'-CTCACTGAGCCTCCCTTGAATAGTCC-3'
Jκ-Cκ intron	5'-CCGCGGTTCTTTCTCGATTGAGTGG-3'
KDE_R1	5'-CTTCATAGACCCTTCAGGCACATGC-3'
KDE_R2	5'-AGACAGGTCCTCAGAGGTCAGAGC-3'

Human genomic DNA mutation analysis

MYC 1 F	5'-CACCGGCCCTTTATAATGCG-3'
MYC 1 R	5'-CGATTCCAGGAGAATCGGAC-3'

MYC 2 F	5’-CTTTGTGTGCCCCGCTCCAG-3’
MYC 2 R	5’-GCGCTCAGATCCTGCAGGTA-3’
BCL6 F	5’-ATGCTTTGGCTCCAAGTT-3’
BCL6 R	5’-CACGATACTTCATCTCATC-3’

BCR-ABL1 Single-cell RT-PCR and mutation analysis

BCR exon 13	5’-TTCAGAAGCTTCTCCCTGACAT-3’
ABL1 exon 9	5’-CTTCGTCTGAGATACTGGATTCCT-3’
ABL1 exon 4 F	5’-CGAGTTGGTTCATCATCATTC-3’
ABL1 exon 7 R	5’-CTTGATGGAGAACTTGTTGTAGG-3’
ABL1 exon 4 F	5’-GTGGAAGAAATACAGCCTGAC-3’
ABL1 exon 6 R	5’-CTCAGGTAGTCCAGGAGGTTC-3’

BCR gene hypermutation analysis

*BCR*_F1	5’-AAGGTCAACGACAAAGAGGTGT-3’
*BCR*_R1	5’-GTCGATCAGGTTGTCCTTCAG-3’
*BCR*_F2	5’-AAGGTCAACGACAAAGAGGTGT-3’
*BCR*_R2	5’-AACTCGGCGTCCTCGTAGT-3’

Human RT-PCR primers

*GAPDH*_F	5’-TTAGCACCCCTGGCCAAGG-3’
*GAPDH*_R	5’-CTTACTCCTTGGAGGCCA-3’
*BCR-ABL1*_F	5’-ACCTCACCTCCAGCGAGGAGGACTT-3’
*BCR-ABL1*_R	5’-TCCACTGGCCACAAAATCATACAGT-3’

LM-PCR primers

*ARF*_F	5’-CCAGGAATAAAATAAGGGGAATA-3’
*ARF*_F2	5’-GGAATAAAATAAGGGGAATAGGG-3’
*ARF*_R	5’-CTTTCCTACCTGGTCTTCTAGG-3’
V3-21_R	5’-CTCTCGCACAGTAATACACAGC-3’
V1-2_R	5’-CTCTCGCACAGTAATACACGAC-3’

V1-69_R	5’-TCTCTCGCACAGTAATACACG-3’
V3-73_R	5’-GGTTTTCAGGCTGTTCATTT-3’
V3-53_R	5’-CACCTTTTAAAATAGCAACAAGG-3’
V3-30_R	5’-AGCATAGCTACTGAAGGTGAAT-3’
Linker_F1	5’-CTGCTCGAATTCAAGCTTCT-3’
Linker_F2	5’-GCTTCTAACGATGTACGGGG-3’
Linker_R1	5’-GTACATCGTTAGAAGCTTGAA-3’
Linker_R2	5’-GTTAGAAGCTTGAATTCGAGC-3’

Human primers for quantitative RT-PCR

*SLP65*_F	5’-AAAGTCAAAGCACCTCCAAG-3’
*SLP65*_R	5’-TGTCATCAGCGTTCTCCTC-3’
*CD79A*_F	5’-AAGAACCGAATCATCACAGC-3’
*CD79A*_R	5’-CTGCCCACATCCTGGTAG-3’
*PAX5*_F	5’-AACTTTTCCCTGTCCATTCC-3’
*PAX5*_R	5’-GTAGTCCGCCAGAGGATAG-3’
*COX6B*_F	5’-AACTACAAGACCGCCCCTTT-3’
*COX6B*_R	5’-GCAGCCAGTTCAGATCTTCC-3’
*AID*_F	5’-TCCTTTTCACTGGACTTTGG-3’
*AID*_R	5’-GACTGAGGTTGGGGTTCC-3’

Quantitative RT-PCR (mouse)

*Aid*_F	5’-AAATGTCCGCTGGGCCAA-3’
*Aid*_R	5’-CATCGACTTCGTACAAGGG-3’
*Bcl6*_F	5’-CCTGCAACTGGAAGAAGTATAAG-3’
*Bcl6*_R	5’-AGTATGGAGGCACATCTCTGTAT-3’
*Cdkn2a*_F	5’-GGACCAGGTGATGATGATG-3’
Cdkn2a _R	5’-ATCGCACGATGTCTTGATG-3’
Cdkn1a _F	5’-ACAAGAGGCCCAGTACTTC-3’
*Cdkn1a*_R	5’-CTTGCAGAAGACCAATCTG-3’

Cdkn1b _F	5’-GTGTCCAGGGATGAGGAAG-3’
*Cdkn1b*_R	5’-CGGAGCTGTTTACGTCTGG-3’
*Hprt*_F	5’-GGGGGCTATAAGTTCTTTGC-3’
*Hprt*_R	5’-TCCAACACTTCGAGAGGTCC-3’
*Myc*_F	5’-ATCATCCAGGACTGTATGTGGAG-3’
*Myc*_R	5’-TTCTTGCTCTTCTTCAGAGTCG-3’
*Trp53*_F	5’-TCCTTACCATCATCACACTGG-3’
*Trp53*_R	5’-CGGATCTTGAGGGTGAAATAC-3’

Quantitative chromatin immunoprecipitation (QChIP)

*CDKN2A*_F	5’-GCGTGCAGCGGTTTAGTTTA-3’
*CDKN2A*_R	5’-TCAGGAGGCTGAATGTCAGTT-3

5.3 Contribution to Publication

In this doctoral thesis, the experimental and scientific work has been supported and supervised by Prof. Dr. Markus Müschen.

The published results presented in chapter 3 involved besides my own experimental data also the contributions from my colleagues.

In the following, the contributions are outlined for each publication separately.

3.1: BCL6 is critical for the development of a diverse primary B cell repertoire.

Duy C, Yu JJ, Nahar R, Swaminathan S, Kweon SM, Polo JM, Valls E, Klemm L, Shojaee S, Cerchietti L, Schuh W, Jack HM, Hurtz C, Ramezani-Rad P, Jäck HM, Herzog S, Jumaa H, Koeffler HP, de Alborán IM, Melnick AM, Ye BH & Müschen M.; *J Exp Med.* 2010; 207:1209-1221

Cihangir Duy:

-Generating and analyzing gene expression profiles of microarray experiments

-Real-time PCR analysis of various samples using several designed primer sets

-Production of various retroviral particles and viral transduction of cells

-Generation of BCR-ABL1-transformed pre-B ALL cells

-Cell culturing and treatment of BCR-ABL1-transformed and IL-7-dependent cells used in the experiments

-Extraction of bone marrow and separation of cell populations using MACS

-Analyzing cell lines and bone marrow populations by flow cytometry using various antibodies

-Western blot analysis for the expression of several proteins

-Spectratyping analysis of VDJ rearrangements

-Planning experimental design

-Discussion and work on the manuscript together with Markus Müschen

J. Jessica Yu, Rahul Nahar, Srividya Swaminathan, Soo-Mi Kweon, Jose M. Polo, Ester Valls, Ester Valls, Lars Klemm, Seyedmehdi Shojaee, Leandro Cerchietti, Wolfgang Schuh, Hans-Martin Jäck, Christian Hurtz, Parham Ramezani-Rad, Sebastian Herzog, Hassan Jumaa, H. Phillip Koeffler, Ignacio Moreno de Alborán, Ari M. Melnick, B. Hilda Ye & Markus Müschen:

-Providing various mouse models, plasmids and RI-BPI

-Generating and analyzing ChIP-on-chip profiles

-Western blot analysis for the expression of several proteins

-Flow cytrometry analysis and Western blot of *Rag2*$^{-/-}$ tTA/μ-chain-cells

-Discussion on the manuscript

3.2: BCL6 enables leukemia cells to survive inhibition of oncogenic tyrosine kinases

Duy C, Hurtz C, Shojaee S, Cerchietti L, Geng H, Swaminathan S, Klemm L, Kweon SM, Nahar R, Braig M, Park E, Kim YM, Hofmann W-K, Herzog S, Jumaa H, Koeffler PH, Yu JJ, Heisterkamp N, Graeber TG, Wu H, Ye BH, Melnick A & Müschen M.; *Nature.* 2010: in press

Cihangir Duy:

-Generating and analyzing gene expression profiles from Affymetrix microarrays

-Cell culturing and treatment of cell lines and patient-derived ALL used in the experiments

-Generating and analyzing CGH chip experiments

-Production of various retroviral particles and viral transduction of cells

-Generation of BCR-ABL1-transformed ALL cells

-Treatment and analysis of long-term survival of primary ALL cases

-Western blot analysis for the expression of BCL6

-Analyzing cell lines and bone marrow population by flow cytometry using various antibodies

-Bioimaging and treatment of mice as well as creating survival curves

-Colony forming assays of primary ALL cases and cell lines

-Extraction and analysis of bone marrow populations

-Analyzing survival of drug-treated and transduced cells by flow cytometry

-Planning experimental design

-Discussion and work on the manuscript together with Markus Müschen

Christian Hurtz, Seyedmehdi Shojaee, Leandro Cerchietti, Huimin Geng, Srividya Swaminathan, Lars Klemm, Soo-mi Kweon, Rahul Nahar, Melanie Braig, Eugene Park, Yong-mi Kim, Wolf-Karsten Hofmann, Sebastian Herzog, Hassan Jumaa, H Phillip Koeffler, J. Jessica Yu, Nora Heisterkamp, Hong Wu, B. Hilda Ye, Ari Melnick & Markus Müschen:

-Providing various mouse models, primary Ph^+ ALL cases and plasmids

-Spectratype-analysis of VDJ rearrangements

-Real-time PCR analysis of various samples for expression of BCL6

-Generating and analyzing ChIP-on-chip profiles and QChIP data

-Generating and analyzing gene expression profiles from Agilent microarrays

-Western blot analysis for the expression of several proteins

-Analyzing cell cycle and senescence

-Colony forming assays of several ALL cells

-Injection and treatment of mice

-Discussion on the manuscript

3.3: The B cell mutator AID promotes B lymphoid blast crisis and drug resistance in chronic myeloid leukemia.

Klemm L, **Duy C**, Iacobucci I, Kuchen S, von Levetzow G, Feldhahn N, Henke N, Li Z, Hoffmann TK, Kim YM, Hofmann WK, Jumaa H, Groffen J, Heisterkamp N, Martinelli G, Lieber MR, Casellas R & Müschen M.; *Cancer Cell*. 2009;16:232-45.

Cihangir Duy:

-Colony PCR of BCR-ABL1 samples

-Sequencing of BCR-ABL1 samples

-Extraction of bone marrow cells

-Discussing experimental design

Lars Klemm, Ilaria Iacobucci, Stefan Kuchen, Gregor von Levetzow, Niklas Feldhahn, Nadine Henke, Zhiyu Li, Thomas K. Hoffmann, Yong-mi Kim, Wolf-Karsten Hofmann, Hassan Jumaa, John Groffen, Nora Heisterkamp, Giovanni Martinelli, Michael R. Lieber, Rafael Casellas & Markus Müschen:

-Providing several mouse models and plasmids

-Generating and analyzing gene expression profiles of microarray experiments

-Spectratype-analysis of VDJ rearrangements

-Real-time PCR analysis of various samples for expression of several genes

-LM-PCR amplifying of short-lived DNA single-strand breaks at the *ARF* and *IGM* loci

-Generating and analyzing expression levels of miR-155

-Analyzing cell lines and bone marrow population by flow cytometry using various antibodies

-Generating and analyzing CGH chip experiments

-Western blot analysis for the expression of AID

-Injection and treatment of mice

-Bioimaging and treatment of mice as well as creating survival curves

-Analyzing survival of drug-treated and transduced cells by flow cytometry

-Colony forming assays of several ALL cells

-Mutation-analysis of BCR-ABL1

-Generating and analysis of SNP data

-Discussion and work on the manuscript

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7 Summary

The oncogenic BCR-ABL1 tyrosine kinase is encoded by the Philadelphia chromosome (Ph), which is present in virtually all cases of chronic myeloid leukemia (CML) and represents the most frequent genetic lesion in adult acute lymphoblastic leukemia (ALL). Tyrosine kinase inhibitors (TKIs) are widely used to treat patients with BCR-ABL1-driven leukemia. Although treatment of leukemia has significantly improved, many patients still die because of drug-resistant leukemia relapses. Recent efforts focused on the development of more potent TKIs that also inhibit mutant tyrosine kinase forms. However, even effective TKI typically targets only rapidly dividing leukemia cells, while non-dividing leukemia cells (quiescent leukemia cells "QLC") are resistant to TKI-induced cell death.

In our studies, we have discovered the transcriptional repressor BCL6 as a central component of a fundamentally novel pathway of TKI-resistance and QLC-maintenance: BCL6 is strongly upregulated in response to TKI treatment and prevents QLC depletion through negative regulation of the Arf/p53/p21 pathway in BCR-ABL1-positive ALL. In the BCL6-activated situation, the cells are highly drug-resistant and in "quiescence mode". Upon cessation of TKI-treatment, oncogenic tyrosine kinase activity resumes and the leukemia cells revert into "proliferation mode", which represents the onset of leukemia recurrence after initially successful treatment. According to this scenario, BCR-ABL-driven ALL cells can switch between a BCL6-dependent "static/stem cell" mode and an oncogenic kinase-dependent "dynamic/progenitor cell" mode.

We found that TKI-treatment of patient-derived BCR-ABL1$^+$ ALL cells increase BCL6 expression levels to similar levels as in diffuse large B cell lymphoma (DLBCL),

where BCL6 is frequently translocated. Even though expressed at low levels in untreated leukemia cells, BCL6 is still critical to suppress Arf/p53/p21, to promote leukemia cell engraftment and enable self-renewal capacity. Compared to leukemias from $BCL6^{-/-}$ mice, BCL6 is required for the development of drug-resistance, leukemia cell colony formation and leukemia-initiation in serially transplanted NOD/SCID mice. In absence of BCL6, BCR-$ABL1^{+}$ ALL cells are poised to undergo cellular senescence and are extremely sensitive to TKI-treatment. Pharmacological inhibition of BCL6 using a novel *retro-inverso* BCL6 peptide inhibitor (RI-BPI) strongly synergizes with TKI in the treatment of BCR-$ABL1^{+}$ ALL cells *in vitro* and *in vivo*.

We demonstrated that dual targeting of tyrosine kinase signaling (TKI, "proliferation mode") and BCL6-dependent protective feedback (BCL6 inhibitors, "quiescence mode") represents a novel strategy to eradicate drug-resistant and leukemia-initiating subclones in BCR-ABL1-driven ALL cells.

Furthermore, we found that BCL6 is critical in the early B cell development for pre-B cell survival at the transition from IL-7-dependent to -independent stages. At this transistion, BCL6 protects pre-B cells from DNA damage-induced apoptosis during immunoglobulin (Ig) light chain gene recombination. In the absence of BCL6, DNA breaks during Ig light chain gene rearrangements lead to excessive up-regulation of Arf and p53. As a consequence, the pool of new bone marrow immature B cells is markedly reduced in size and clonal diversity. We showed that negative regulation of Arf by BCL6 is required for pre-B cell self-renewal and the formation of a diverse polyclonal B cell repertoire.

8 Zusammenfassung

Die krebsverursachende BCR-ABL1-Tyrosinkinase, die durch das Philadelphia Chromosom (Ph) exprimiert wird, ist in nahezu allen Fällen von chronischer myeloischer Leukämie (CML) vorhanden und stellt daneben die häufigste genetische Veränderung bei erwachsenen Patienten mit akuter lymphoblastischer Leukämie (ALL) dar. Zur medikamentösen Behandlung von Patienten mit BCR-ABL1-positiven Leukämien werden häufig Tyrosinkinase-Inhibitoren (TKI) eingesetzt. Trotz einer deutlich verbesserten Behandlung von Leukämien sterben viele Patienten aufgrund von Leukämie-Rückfällen durch TKI-resistente Leukämiezellen. Die derzeitigen Forschungsansätze konzentrieren sich vor allem auf die Entwicklung verbesserter TKI, die in der Lage sind, resistente Tyrosinkinase-Formen wirkungsvoll zu inaktivieren. Jedoch wirken selbst die effektivsten TKI in erster Linie auf sich schnell teilende Leukämiezellen, wohingegen nicht teilungsaktive Leukämiezellen (quieszente Leukämiezellen, QLC) weitestgehend unangetastet bleiben.

In unseren Studien haben wir den transkriptionellen Repressor BCL6 als einen zentralen Bestandteil eines neuen Signalwegs für Resistenzen gegen TKI und die Aufrechthaltung der QLC identifiziert. BCL6 wird entscheidend durch die TKI-Behandlung der BCR-ABL1-positiven ALL-Zellen hochreguliert und verhindert die Eliminierung der QLC durch Unterdrückung der Arf/p53/p21-Faktoren des Apoptose- und Seneszenz-Programms. Im BCL6-aktivierten Zustand befinden sich die Leukämiezellen im „Quieszenz-Modus“ und sind gegenüber TKI resistent. Nach Beendigung der Behandlung mit TKI erlangt die BCR-ABL1-Tyrosinkinase wieder ihre volle Aktivität, wodurch die Leukämiezellen in den aktiven „Proliferationsmodus“

zurückkehren und nach einem anfänglichen Behandlungserfolg einen neuen Ausbruch der Leukämie hervorrufen. Unserem Modell entsprechend können BCR-ABL1-positive ALL-Zellen zwischen einem BCL6-abhängigen „statischen bzw. Stammzellen-Zustand“ und dem krebsverursachenden Tyrosinkinase-abhängigen „dynamischen bzw. Vorläuferzellen-Zustand“ wechseln.

Wir konnten feststellen, dass eine TKI-Behandlung der von Patienten stammenden BCR-ABL1-positiven ALL-Zellen eine BCL6-Hochregulation induziert, die vergleichbar mit den Expressionsmengen von diffus großzelligen B-Zell-Lymphomen (DLBCL) ist, die eine BCL6-Translokationen aufweisen. Selbst die in unbehandelten Leukämiezellen gering vorliegenden BCL6-Expressionsmengen sind wichtig, um Arf/p53/p21-Faktoren soweit zu unterdrücken, dass sowohl das Engraftment als auch die Selbsterneuerungsfähigkeit der Leukämiezellen gefördert werden. Verglichen mit BCL6-defizienten Leukämiezellen ist ersichtlich, dass BCL6 für die Bildung von Resistenzen, Zellkolonien sowie von Leukämien in seriellen Transplantationsexperimenten mit NOD/SCID-Mäusen notwendig ist. In Abwesenheit von BCL6 sind BCR-ABL1-positive ALL-Zellen gezwungen, die zelluläre Seneszenz einzuleiten, wodurch die Leukämiezellen anfällig gegenüber der Behandlung mit TKI sind. Die pharmakologische Inhibition von BCL6 mittels eines neuen „*retro-inverso*-BCL6-Peptid-Inhibitors“ (RI-BPI) zeigt in Kombination mit TKI einen starken synergistischen Effekt *in vitro* sowie *in vivo*.

Wir konnten somit demonstrieren, dass ein dualer Angriff des Tyrosinkinase-Signalwegs (TKI, „Proliferationsmodus“) und des BCL6-abhängigen Schutzmechanismus (BCL6 Inhibitoren, „Quieszenz-Modus“) eine neuartige Strategie

zur Bekämpfung von resistenten und Leukämie-initiierenden Zellen in BCR-ABL1-positiver ALL darstellt.

Des Weiteren haben wir gezeigt, dass während der frühen B-Zell-Entwicklung BCL6 kritisch für das Überleben der prä-B-Zelle im Übergang vom IL-7-abhängigen zum IL-7-unabhängigen Stadium ist. In diesem Übergang „beschützt“ BCL6 die prä-B-Zellen vor DNA-Doppelstrangbruch-induzierter Apoptose, die durch Genrekombination der leichten Immunglobulin-(Ig-)Kette verursacht wird. In Abwesenheit von BCL6 führen DNA-Brüche der Ig-Leichtketten-Rearrangements zu einer exzessiven Hochregulation von Arf und p53. Als Konsequenz ist der Pool an neuen unreifen B-Zellen im Knochenmark in Größe und in klonaler Diversität deutlich reduziert. Somit konnten wir feststellen, dass die BCL6-negative Regulation von Arf für die Selbsterneuerung der prä-B-Zelle und die Formierung eines polyklonalen B-Zell-Repertoires erforderlich ist.

9 Danksagung

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10 Curriculum vitae

Education & Position

1990-1996	FORQ, Volkspark-Hauptschule, Dinslaken, Germany
1996-1999	Abitur, Theodor-Heuss-Gymnasium, Dinslaken, Germany
1999-2000	Civilian service, Duisburg, Germany
2000-2005	Diploma (B.Sc. and M.Sc.) in Biology, Heinrich-Heine-Universität Düsseldorf, Germany
2004-2005	Diploma thesis in Biology and Biophysics, Forschungszentrum Jülich, Germany
2006-2010	PhD (Dr. rer. nat.) student, Heinrich-Heine-Universität Düsseldorf, Germany
2006-2010	PhD study, University of Southern California/Childrens Hospital Los Angeles, CA, USA

Conference abstracts

Oral sessions

12/2008 50th American Society of Hematology (ASH), San Francisco, CA. Oral presentation: "Aberrant splicing of the SLP65 SH2 domain enables pre-B cell transformation and compromises the leukemia-suppressive function of the pre-B cell receptor"

12/2008 50th American Society of Hematology (ASH), San Francisco, CA. Oral presentation: "BCL6-Mediated Survival Signaling Promotes Drug-Resistance in BCR-ABL1-Driven Acute Lymphoblastic Leukemia"

12/2009 51st American Society of Hematology (ASH), New Orleans, LA. Oral presentation: "BCL6 Is Critical for the Development of a Diverse Primary B Cell Repertoire"

12/2009 51st American Society of Hematology (ASH), New Orleans, LA. Oral presentation: "Inactivation of Pre-B Cell Receptor-Mediated Tumor Suppression by Aberrant Splicing in Ph^+ Acute Lymphoblastic Leukemia"

12/2009 51st American Society of Hematology (ASH), New Orleans, LA. Oral presentation: "BCL6-Dependent Negative Regulation of Cell Cycle Checkpoint Regulators Enables Drug-Resistance in Ph^+ Acute Lymphoblastic Leukemia"

12/2010 52nd American Society of Hematology (ASH), Orlando, FL. Oral presentation: "Mechanisms of pre-B Cell Receptor-Inactivation in Acute Lymphoblastic Leukemia"

Plenary session at the 2010 EURO Cancer conference in Paris

6/2010 EURO Cancer 2010, Paris, France. "Mechanisms of drug-resistance in Chronic Myeloid Leukemia".

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12 Erklärung

Hiermit erkläre ich, dass die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe von mir angefertigt wurde. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht.

Düsseldorf, den 31. Januar 2011

Cihangir Duy