Mimicry of a constitutively active pre-B cell receptor in BCR-ABL1-transformed pre-B leukemia cells

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Chapter 1

Introduction

1.1 Commitment to the B cell lineage

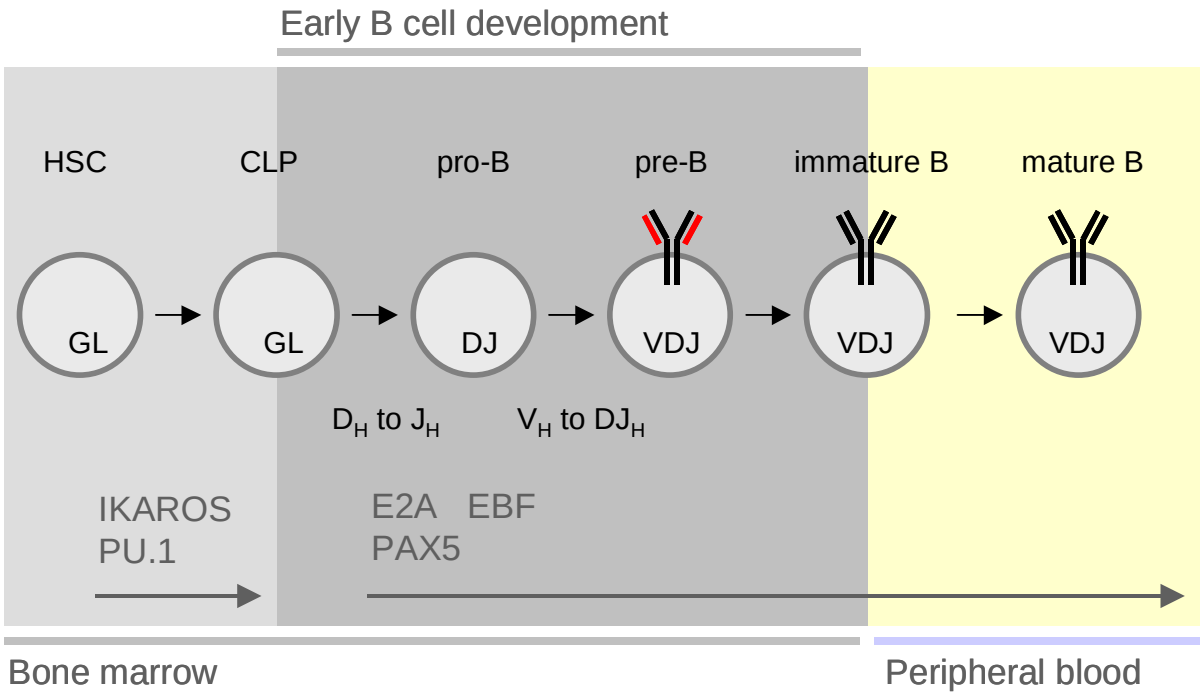
The development of B cells in the human originates from pluripotent hematopoietic stem cells (HSCs) in the bone marrow or the fetal liver. HSCs exhibit extensive self-renewal capacity and regenerate all hematopoietic lineages throughout life. Development from the HSC towards the lymphoid lineages is accompanied with the loss of the self-renewal capacity of HSCs (Adolfsson et al., 2001) and guided by several transcription factors that initiate or maintain gene expression specific for lineage and stage of differentiation. Early B cell development is characterized by the opening and rearrangement of the immunoglobulin heavy chain (*IGH*) locus, which is mediated by the *recombination activating genes* 1 and 2 (RAG1/2). Somatic rearrangement of gene segments within the *IGH* locus encoding the variable region of the immunoglobulin heavy chain occurs at the transition to the pro-B cell and pre-B cell stages of development, respectively (Figure 1). Functional rearrangement of *IGH* V (variable) region gene segments is a prerequisite for the expression of the pre-B cell receptor and further differentiation to mature B cells (Kitamura et al., 1991).

Commitment to the B- or T-lymphoid lineages is dependent on the activity of two key transcription factors, IKAROS and PU.1, thereby promoting the differentiation of HSCs into common lymphoid progenitor (CLP) cells: Deficiency of IKAROS in mice prevents the development of all lymphoid cells (Georgopoulos et al., 1994). Likewise, the level of PU.1 expression controls B versus myeloid differentiation as shown by retroviral reconstitution experiments with PU.1 deficient progenitor cells (DeKoter and Singh; 2000). Notably, IKAROS is an unusual transcription factor as it may either repress or activate target genes by closing or opening their loci through the induction of chromatin remodeling (Kim et al., 1999).

The differentiation from the CLP to the pro-B cell stage, which represents the commitment to the B cell lineage, critically depends on the three transcription factors E2A, EBF and PAX5. Deficiency of any of these transcription factors results in a B cell differentiation arrest at earliest stages and in the absence of *IGH* V region gene rearrangements (Bain et al., 1994; Lin and Grosschedl, 1995; Urbanek et al., 1994). In agreement with the phenotype of E2A- and EBF-deficient mice, it has been shown that E2A and EBF act in concert to regulate the expression of RAG1 and RAG2 and germline transcription of the *IGH* locus (Romanow et al., 2000), which are both essential for recombination processes within the *IGH* locus. E2A and EBF also promote the transition from the pro-B cell to the pre-B cell stage as they induce the transcription of several crucial components of the pre-B cell receptor complex, like the elements of the surrogate light chain

VpreB and λ5 (Sigvardsson et al., 1997; Kee and Murre, 1998). Deficiency of these components leads to a developmental arrest at the pro-B cell stage (Papavasiliou et al., 1996). Despite already advanced differentiation, B cell lineage commitment is still reversible at this stage of development and critically depends on the sustained expression of PAX5. Conditional deletion of PAX5 in mice is sufficient to reactivate in early B cells a broad developmental potential (Mikkola et al., 2002). Even in mature B cells, the conditional inactivation of PAX5 leads to the loss of B cell identity (Horcher et al., 2001), demonstrating the crucial role of continuous PAX5 expression for the maintenance of the B cell identity.

Figure 1: *Early B cell development is guided by distinct transcription factors*



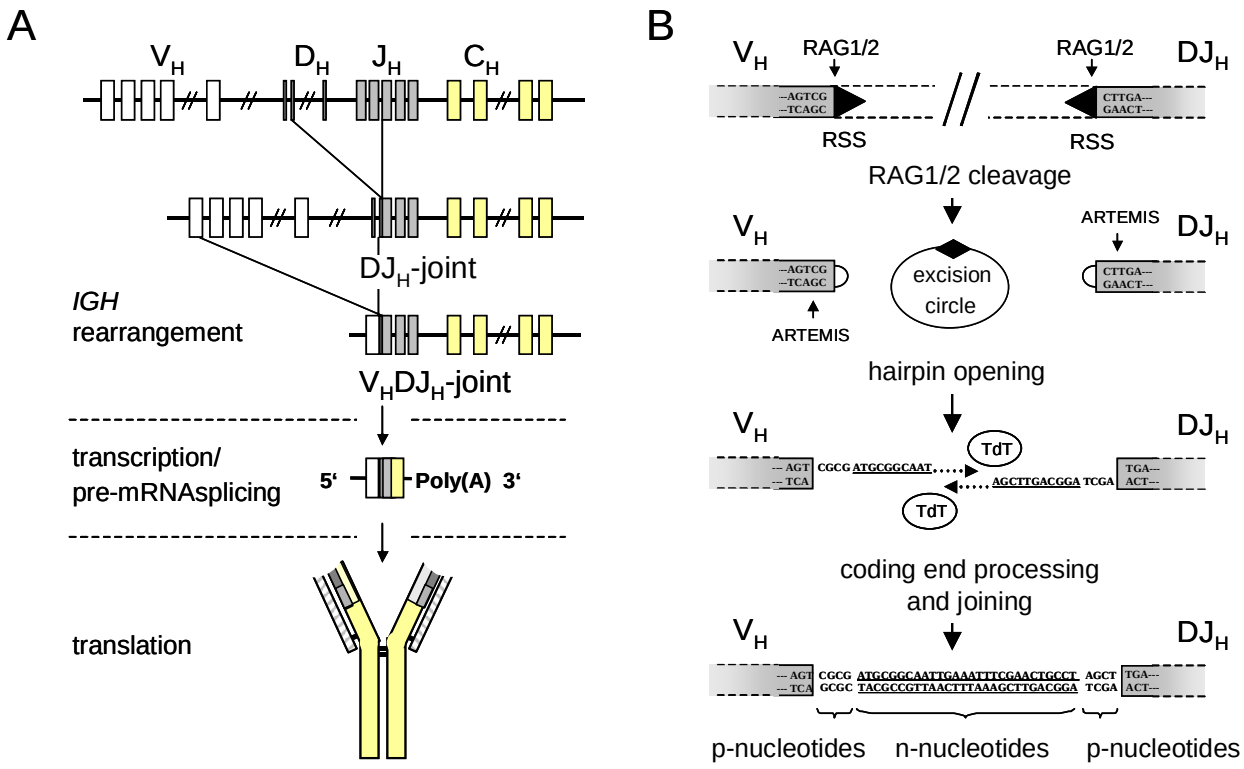
Schematic diagram of early B cell development. Differentiation of a hematopoietic stem cell (HSC) into the lymphoid lineage (CLP, common lymphoid progenitor) is guided by the transcription factors IKAROS and PU.1. Early B cell development is defined by a sequence of somatic rearrangements within the immunoglobulin heavy chain locus (*IGH*): The rearrangement of a D_H to J_H gene segment occurs during the transition from the CLP to the pro-B cell stage followed by a rearrangement of a V_H gene segment to the DJ_H joint during the transition from the pro-B to the pre-B cell stage. Early B cell development is guided by the transcription factors E2A, EBF and PAX5. Pre-B cells express a the μ-heavy chain (black) in combination with the surrogate light chain (red) on the surface. Selected pre-B cells downregulate their pre-B cell receptor, initiate the rearrangement of the immunoglobulin light chain loci and further differentiate into immature and mature B cells, which express a B cell receptor on their surface.

1.2 V(D)J recombination

Early B cell differentiation is characterized by a sequence of somatic rearrangements within the immunoglobulin heavy chain (*IGH*) locus. If the rearrangement was functional, pre-B cells are able to express the gene product of the rearrangement, the µ-heavy chain, as part of the pre-B cell receptor on their surface and are selected to undergo further differentiation (Kitamura et al., 1991; Rajewsky, 1996).

The *IGH* locus contains clusters of V (variable), D (diversity) and J (joining) gene segments for the generation of a variable region and a cluster of C (constant) genes encoding the different constant regions of an immunoglobulin heavy chain (Ravetch et al., 1981; Matsuda et al., 1988; Matsuda et al., 1998; Figure 2a).

Figure 2: *V(D)J recombination is a prerequisite for the expression of a µ-heavy chain*

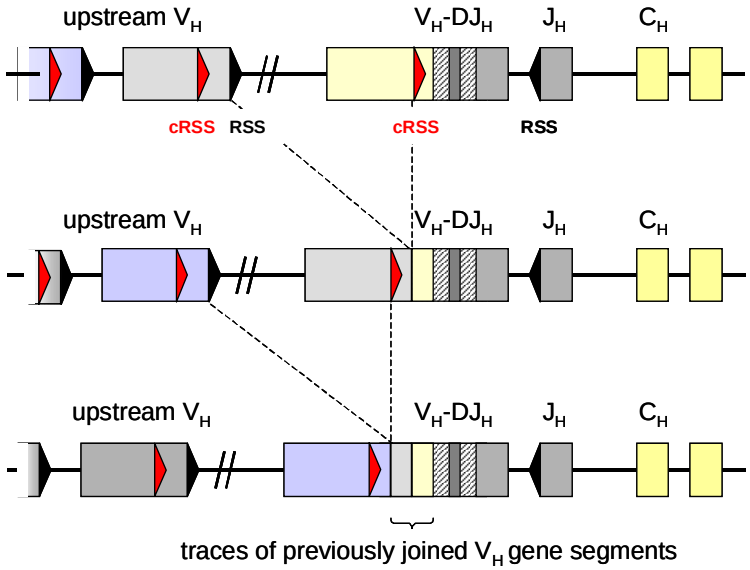


(A) The rearrangement of a D_H segment to a J_H segment followed by the rearrangement of a V_H segment to the DJ_H-joint during early B cell development is a prerequisite for the expression of the µ-heavy chain within the pre-B cell receptor. The V_HDJ_H-joint encodes the variable region and is expressed with a gene segment encoding the constant region µ. The newly formed heavy chain expressed in combination with the surrogate light chain (stripes) forms the pre-B cell receptor. (B) V(D)J-recombination is initiated by RAG1/2 proteins, which recognize RSS motifs and induce DNA double-strand breaks (DSBs). The coding ends are sealed by hairpin formation and the signal ends are sealed resulting in an excision circle. The activity of ARTEMIS, TdT and components of the NHEJ pathway mediate coding end processing and end joining to generate a unique junction.

Prior to expression of a functional µ-chain, one V, D and J gene segment has to be recombined by a process termed V(D)J recombination, thereby generating the variable region (Ravetch et al., 1981). The somatic recombination process occurs in two steps: a rearrangement of a D_H to a J_H gene segment (DJ_H-joint) at the transition to the pro-B cell stage of development is followed by the rearrangement of a V_H segment to the DJ_H-joint (V_HDJ_H-joint) at the transition to the pre-B cell stage of development (Figure1 and 2a). At the transcriptional level, the variable region is linked to the constant region by pre-mRNA splicing. Each V_H, D_H and J_H gene segment carries recombination signal sequences (RSS), immediately flanking V_H and J_H segments at their 3' and 5' border, respectively, or on both sides for D_H segments (Sakano et al., 1981). RSS sequences are the target sequences for the V(D)J recombinase encoded by the *recombination-activating genes* 1 and 2 (RAG1/2). The RSS motif consists of a conserved heptamer and nonamer sequence separated by a spacer of a conserved length of either 12 or 23 bp. Efficient V(D)J recombination requires participitation of two RSS motifs, one with a spacer of 12 bp and one with a spacer of 23 bp in length (12/23 rule; Akamatsu et al., 1994). Before the onset of recombination, the *IGH* locus has to be rendered accessible for germline transcription. The introduction of DNA double-strand breaks (DSBs) between the RSS site and the flanking gene segment results in two asymmetric DNA ends: the coding end is covalently sealed into a hairpin while the signal end is blunt ended, 5' phosphorylated (Schlissel et al., 1993) and subsequently ligated to form an episomal excision circle (Figure 2b). The hairpins represent the substrate for ARTEMIS, which opens the hairpins by single-strand cleavage at random position (Ma et al., 2002; see Figure 2b). Subsequently, unfolding of the hairpin results in a palindromic sequence (P-nucleotides), which can be further extended by the addition of non-templated nucleotides (N-nucleotides) through enzymatic activity of the *terminal deoxynucleotidyl transferase* (TdT; Alt and Baltimore, 1982; see Figure 2b). Joining of the ends of the two coding joints involves the components of the non-homologous end joining (NHEJ) pathway (Grawunder et al., 1998). Statistically, two thirds of all V(D)J recombination events result in an out-of-frame rearrangement lacking coding capacity for a µ-heavy chain. As ARTEMIS and TdT activity is randomly, the frequency of functional rearrangements is further decreased by the possible generation of stop-codons within the junction. If V(D)J recombination resulted in a productively rearranged *IGH* V region gene, the pre-B cell is capable of expressing a µ heavy chain, which in combination with the surrogate light chain forms the pre-B cell receptor. If the initial rearrangement was non-functional, the pre-B cell can initiate V(D)J recombination on the second allele. Furthermore, the already rearranged V_HDJ_H-joint can undergo another

recombination process termed V_H replacement (Figure 3). In this case, the V_H gene segment of the V_HDJ_H-joint is replaced by an upstream located V_H gene segment. Within the previously rearranged V_H segment, RAG1/2 proteins recognize a cryptic RSS motif (cRSS; see Figure 3). Such an cRSS motif is present within the extreme 3' region of 40 out of 44 functional V_H gene segments. However, V_H replacement is relatively rare as only 5 % of normal immature B cells show traces of V_H replacement (Zhang et al., 2003).

Figure 3: *V_H-DJ_H-joints can undergo a further rearrangement by V_H-replacement*



An already rearranged V_HDJ_H-joint can undergo further rearrangements by V_H-replacement. While the original RSS site is lost during the initial V(D)J-recombination, rearrangement is initiated at the cRSS motifs present in the 3' end of the coding region of 40 out of 44 functional V_H gene segments. Replacement of a V_H gene segment usually leaves a footprint of the previously used V_H within the newly formed junction which represent traces of the V_H-replacement process.

1.3 Selection for the expression of a pre-B cell receptor

The pre-B cell receptor is assembled by the µ-heavy chain, the two signaling chains Igα and Igβ, and the surrogate light chain (SL), which consists of the two proteins VpreB and λ5 (Tsubata et al., 1990; Karasuyama et al., 1990; Lassoued et al., 1996). A functional rearrangement of the µ-chain by V(D)J recombination is a prerequisite for the expression of a pre-B cell receptor. Remarkably, only the half of all newly rearranged functional µ-chains are capable to pair with the SL to form a pre-B cell receptor in mice (ten Boekel et al., 1997). However, if the configuration of the µ-heavy chain allows its expression within the pre-B cell receptor on the surface, pre-B cell receptor signaling is initiated. Whether this signaling is ligand-dependent or autonomous, remains controversial: Galectin-1 (GAL1), anchored to glycosylated counterreceptors on stromal cells, was proposed to represent a ligand for the pre-B cell receptor selection in the human, as GAL1 can induce pre-B cell receptor signaling through the interaction with the non-Ig portion of the λ5 protein (Gauthier et al., 2002).

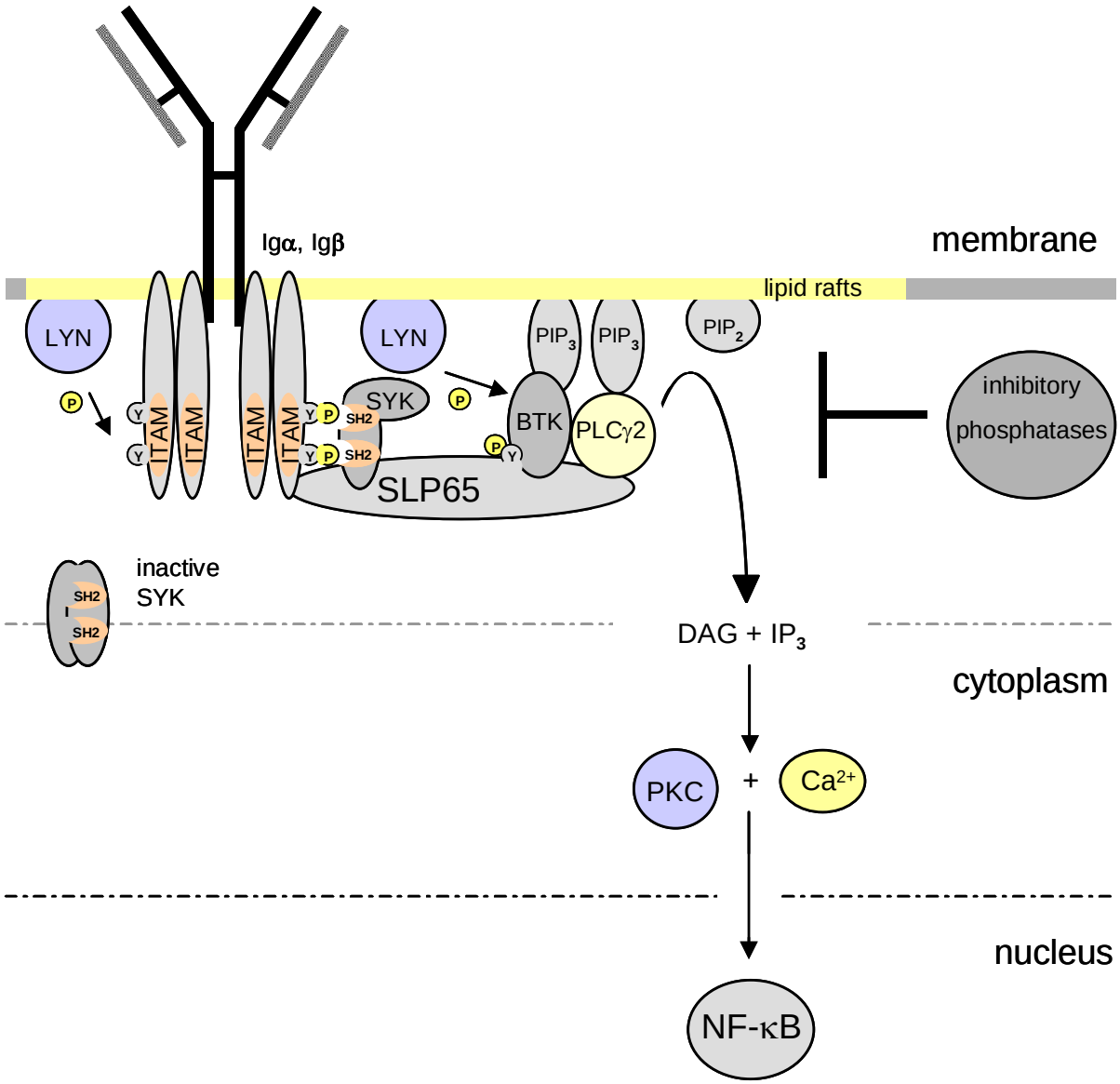
However, it was shown that GAL1 does not exhibit this function in mice, instead heparan sulfate was proposed to act as a pre-B cell receptor ligand in mice (Bradl et al., 2003). In striking contrast with this notion, *ex vivo* isolated µ-chain expressing pre-B cells undergo further cell divisions and differentiation in the absence stromal cells (Rolink et al., 2000). Autonomous pre-B cell receptor signaling can be explained by crosslinking of pre-B cell receptor molecules through the non-Ig portion of λ5 (Ohnishi and Melchers, 2003).

The expression of a pre-B cell receptor on the cell surface is necessary for cell cycle progression of pre-B cells (Hess et al., 2001; Flemming et al., 2003). To limit proliferation and induce further differentiation, pre-B cell receptor signals initiate a negative feedback signal: The expression of the pre-B cell receptor downregulates the expression of VpreB and λ5 (Lu et al., 2003; Parker et al., 2005) and the pre-B cell receptor molecules present on the cell surface are presumably downregulated by proliferation-mediated dilution. The pre-B cell receptor-dependent change in gene expression is mediated by transcriptional activation of IRF4 and IRF8 (Lu et al., 2003). These transcription factors do not only terminate pre-B cell receptor signaling but also promote further differentiation by the initiation of the rearrangement of *Igκ* and *Igλ* light chain genes leading to the expression of a B cell receptor (Lu et al., 2003). Therefore, the expression of a pre-B cell receptor on the surface of pre-B cells and subsequent pre-B cell receptor signaling represents a positive selection mechanism, which controls expansion and further differentiation of pre-B cells.

1.4 Pre-B cell receptor signaling

Signal transduction of the pre-B cell receptor is essential for the survival, proliferation and differentiation of pre-B cells (Hess et al., 2001; Tarlinton et al; 1997). Transmission of pre-B cell receptor signals is mediated by three types of protein tyrosine kinases (PTKs): the SRC (cellular homolog of *Rous sarcoma virus*) family kinases (Aoki et al., 1994; Saijo et al., 2003), the SYK (spleen tyrosine kinase) family kinases (Schweighoffer et al., 2003) and the TEC family kinases (Ellmeier et al., 2000). The correct assembly of the pre-B cell receptor signalosome also requires the presence of adapter molecules, which stabilize the interaction of signaling molecules (Su et al., 1999; Flemming et al., 2003; Ishiai et al., 1999; Figure 4). Signaling activity of these molecules is regulated through autoinhibition (Shiue et al., 1995; Rolli et al., 2002; Tolar et al., 2005), regulation of membrane-localisation (Bolland et al., 1998) and the activity of inhibitory protein tyrosine phosphatases (PTPs; Ravetch and Lanier, 2000). The need for this tight regulation is underscored by the finding that molecules

Figure 4: *Schematic diagram of pre-B cell receptor-induced signal transduction*



Signal transduction by the pre-B cell receptor is mediated by members of three major tyrosine kinase families: the SRC-family kinases (e.g. LYN), the SYK-family kinases (e.g. SYK) and the TEC-family kinases (e.g. BTK). For the accurate assembly of the pre-B cell receptor signalosome, the presence of adapter molecules like SLP65 is of critical importance. Activation of this pathway leads to PLCγ2-mediated generation of the second messengers diacylglycerol (DAG) and Ca^{2+}. Subsequent activation of transcription factors like NF-κB results in survival and differentiation of the pre-B cell. The (pre-)B cell receptor signal underlies a general inhibition by inhibitory phosphatases. Inactivation of these phosphatases is of high importance for the initiation of efficient signaling and is mediated by the generation of reactive oxygen species (ROS) by the (pre-)B cell receptor.

implicated in pre-B cell receptor signaling have initially been identified as oncogenes (Rodrigues and Park, 1994).

Crosslinking of the pre-B cell receptor leads to the accumulation of signaling molecules within cell surface microdomains of high activation, the glycolipid-enriched membrane domains (GEMs) or so-called lipid rafts (Guo et al., 2000). The assembly of this microdomains is induced by pre-B cell receptor activation and essential for effective signaling (Saeki eta al., 2003). Recruitment of the pre-B cell receptor to the lipid raft fraction allows the phosphorylation of the pre-B cell receptor associated signal chains Igα and Igβ by the SRC kinase LYN, which is constitutively present in these microdomains (Cheng et al., 1999). Interestingly, localisation within the lipid raft fraction itself maintains LYN in an active state (Young et al., 2003). In parallel to its function in pre-B cell activation, LYN also initiates the termination of pre-B cell receptor signaling by the activation of inhibitory phosphatases through the phosphorylation of inhibitory receptors (Smith et al., 1998). In agreement with this, B cells from LYN-deficient mice exhibit enhanced signaling in response to B cell receptor stimulation (Chan et al., 1997). LYN positively transmits the initial signal by phosphorylation of one tyrosine within an *immunoreceptor tyrosine-based activation motif* (ITAM; Reth, 1989) of Igα and Igβ (Flaswinkel et al., 1994; Rolli et al., 2002; see Figure 4). Phosphorylated ITAMs allow binding of the tyrosine kinase SYK, which is thereby released from autoinhibition (Rolli et al., 2002). The autoinhibition of SYK is mediated through binding one of the two N-terminal SH2 domains to the own kinase domain (Wossning and Reth, 2004) and relieved through binding of the SH2 domains to the tyrosine-phosphorylated ITAMs (Rolli et al., 2002). In addition to the activation of downstream targets, SYK initiates a positive feedback loop by tyrosine-phosphorylation of ITAMs of further Igα and Igβ molecules as well as by tyrosine-phosphorylation of further SYK molecules (Rolli et al., 2002; Keshvara et al., 1998). As a consequence, the amount of initially activated SYK molecules is largely amplified. SYK activity is highly regulated by dephosphorylation of its binding partners Igα and Igβ by the inhibitory phosphatase SHP1 (Adachi et al., 2001). In addition, attenuation of SYK activity is mediated by ubiquitin ligase CBL, which targets SYK for degradation (Sohn et al., 2003). The important role of SYK family kinases is demonstrated by the complete block at the pro-B cell stage in SYK family kinases deficient mice (Schweighoffer et al., 2003). Activated SYK phosphorylates SLP65, an adapter molecule that stabilizes the pre-B cell receptor signalosome through binding to phosphorylated Igα and downstream targets including GRB2, VAV, NCK, BTK and PLCγ2 (Fu et al., 1998; see Figure 4). SYK also induces the recruitment of the TEC family kinase BTK (Bruton's

tyrosine kinase) and PLCγ2 to the pre-B cell receptor signalosome at the plasma membrane by the activation of phosphatidylinositol-3-kinase (PI3K; Beitz et al., 1999). PI3K activity generates the membrane bound phospho-lipid *phosphatidylinositol-3,4,5-triphosphat* (PIP_3), which serves as a membrane-anchor for the PH-domains of BTK and PLCγ2 (Saito et al., 2003). The activity of both enzymes is negatively regulated by their membrane-delocalisation through PIP_3-degradation mediated by the inhibitory phosphatidyl-inositol phosphatases SHIP (Bolland et al., 1998) and PTEN (Satterthwaite et al., 2000). Binding of membrane-localized BTK to the phosphorylated adapter molecule SLP65 brings BTK in close proximity to LYN, which in turn activates BTK through phosphorylation (Rawlings et al., 1996). BTK and SLP65 are essential for further transmission of pre-B cell receptor-induced differentiation signals, as either BTK- or SLP65-deficiency results in a severe B cell differentiation arrest in the human (Noordzij et al., 2002; Minegishi et al., 1999). However, proliferation signals can also be transduced in a SLP65- and BTK-independent pathway in mice, documented by the rapid expansion of pre-B cells in tumors in the absence of SLP65 and BTK (Kersseboom et al., 2003). Binding of BTK to SLP65 brings BTK also in close proximity to his target PLCγ2. Activated PLCγ2 generates diacylglycerol (DAG) and inositol-3-phosphate (IP_3) through the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), which in turn results in the release of Ca^{2+} from intracellular stores by triggering IP_3 receptors on the endoplasmic reticulum (ER). The second messengers DAG and Ca^{2+} promote differentiation and survival by induction of the NF-κB pathway.

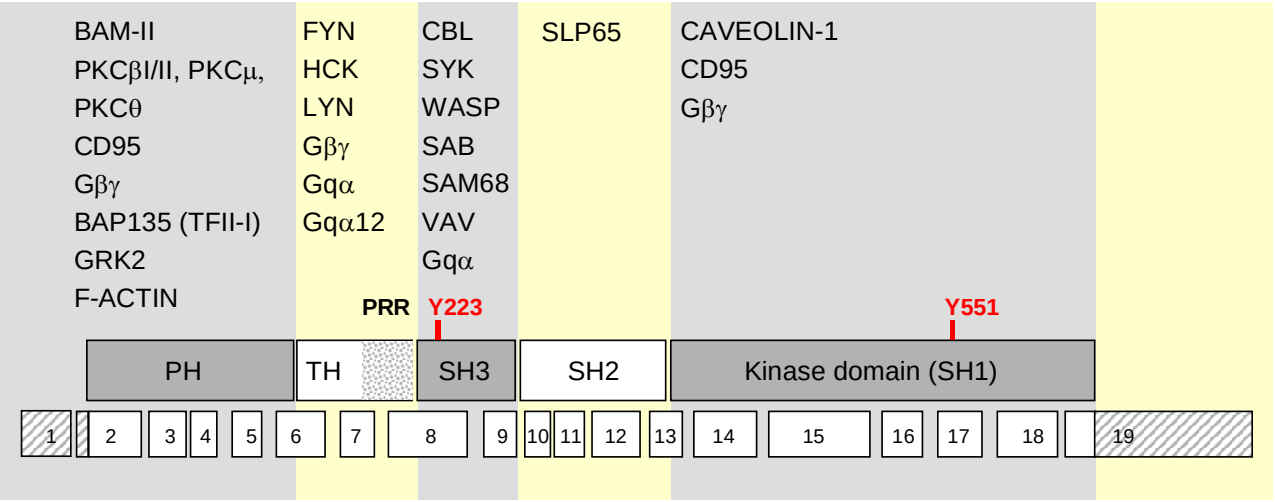
The pre-B cell receptor-mediated activation of a network of tyrosine kinases (PTKs) allows rapid amplification of the initial stimulus. While the tight regulation of their activity by autoinhibition and delocalisation is necessary for the specificity of the pre-B cell receptor signal, their inhibition by inhibitory PTPs is superior to the activating signal: PTPs have a 10- to 100-fold higher turnover rate than PTKs (Honjo T, *Molecular Biology of B cells*, 2004, S.162). Efficient pre-B cell receptor signaling therefore requires both, kinase activation and phosphatase inhibition. Recently, the oxidation of a cystein within the catalytic center of PTPs has been identified as a mechanism for the reversible inactivation of PTPs (Meng et al., 2002). The oxidation is mediated by H_2O_2, which is generated by the membrane-bound NADPH-oxidase complex in response to (pre-) B cell receptor stimulation (Singh et al., 2005). The stimulation of the pre-B cell receptor therefore induces a highly specific signal in a limited time frame of activation resulting in survival, proliferation and differentiation of pre-B cells.

1.5 Bruton's tyrosine kinase

In 1993, mutations of a gene encoding a novel cytoplasmic tyrosine kinase, termed Bruton's tyrosine kinase (BTK), were decribed as the cause of the human immunodeficiency *X-linked agammaglobulinemia* (XLA; Vetrie et al., 1993; Tsukada et al., 1993). Defective expression of this tyrosine kinase leads to a severe differentiation arrest at the pre-B cell stage in the bone marrow and the absence of mature B cells in the periphery of XLA patients (de Weers et al., 1993; Noordzij et al., 2002). Expression of BTK in healthy humans appears from the early pro-B cell till the mature memory B cell stage, but is downregulated in plasma cells (de Weers et al., 1993). While BTK has a crucial function in normal B cells, BTK is also expressed in myeloid cells but does not play an essential role, since myeloid differentiation in XLA patients does not seem to be affected (de Weers et al., 1993).

BTK belongs to the SRC-related family of TEC protein tyrosine kinases (Desiderio and Siliciano, 1994; Ohta et al., 1994), which share the following structural domains: The amino-terminal Pleckstrin-homology (PH) domain, the TEC-homology (TH) domain, the SRC homology 3 (SH3) domain, the SH2 domain and the carboxy-terminal kinase domain (Figure 5). With the exception of one member, termed TXK (Ohta et al., 1996), all TEC family kinases carry a PH domain, which confers membrane-localisation of BTK depending on PIP_3 and brings BTK into close proximity with its interaction partners within the (pre-) B cell receptor signalosome. Each domain of BTK is required for a specific interaction with specific molecules of the (pre-)B cell receptor, thereby also acting as a linker in addition to its kinase function (Figure 5): The PH and kinase domain of BTK together allow binding to the cytoplasmic domain of CD95 (Vassilev et al., 1999). The TH domain of BTK contains a proline-rich region which binds the SH3 domain of other signaling molecules (Hansson et al., 2001). Those intermolecular interactions can be formed between BTK and the SRC kinases LYN, FYN and HCK (Cheng et al., 1994; Yang et al., 1995), the G protein Gα (Ma et al., 1998) as well as in self-association of BTK molecules (Laederach et al., 2002). The SH3 and SH2 domains also confer intermolecular interactions by binding to the proto-oncogene CBL (Patel et al., 1997), the spleen tyrosine kinase (SYK; Morrogh et al., 1999), the WAS protein (WASP; Morrogh et al., 1999), the guanine exchange factor VAV, the RNA-binding protein SAM68 (Guinamard et al., 1997) and the adapter molecule SLP65 (Su et al., 1999). In agreement with this, recent work demonstrated that kinase-inactive BTK, acting as a linker, can partially restore the differentiation arrest in BTK-deficient mice (Middendorp et al., 2003).

Figure 5: *BTK confers several intermolecular interactions*



The intermolecular interactions of BTK with several proteins occur at distinct domains: The Pleckstrin-homology (PH), TEC-homology (TH) including the proline-rich region (PRR), SH3, SH2 and Kinase domain (SH1). The critical tyrosines 223 and 551 are shown in red. The exon organisation is shown at the bottom.

While activation of BTK can occur through distinct cell surface receptors (Jeffries et al., 2003; Wahl et al., 1997; Brunner et al 2002), the most prominent activation of BTK is induced through stimulation of the (pre-)B cell receptor: Following stimulation, BTK is recruited to the membrane through binding to PIP_3. Membrane-localisation is a prerequisite for BTK function in normal B cells (Bolland et al., 1998). Likewise, the expression of the E41K *BTK*-transgene, which confers constitutive membrane-localisation caused by a mutation within the PH domain, leads to hyperresponsive (pre-)B cell receptor signaling (Dingjan et al., 1998; Maas et al., 1999). Recruitment of BTK to the plasma membrane is positively regulated through the generation of PIP_3 by PI3K (Saito et al., 2003) and negatively regulated by PIP_3 degradation through the activity SHIP (Bolland et al., 1998) and PTEN (Satterthwaite et al., 2000; Figure 6). Once at the plasma membrane, BTK itself enhances the function of PI3K and PLCγ2 by recruitment of PIP5K, which generates the substrate, PIP_2, for both molecules (Saito et al., 2003). While SHIP- and PTEN-activity is regulated by inhibitory receptors (Okada et al.1998; Brown et al., 2004; Ravetch and Lanier, 2000), BTK itself regulates its membrane recruitment by a PKCβ-mediated negative feedback loop (Kang et al., 2001). Within the (pre-)B cell receptor signalosome, BTK is phosphorylated by the SRC family kinase LYN at the key tyrosine 551 (Y551) within the activation loop (Rawlings et al., 1996; Figure 6). Phosphorylation of Y551 is essential for the catalytic activity of BTK (Rawlings et al., 1996). In a second step, another important tyrosine within the SH3 domain, tyrosine 223 (Y223), is autophosphorylated (Rawlings et al., 1996; Park et al., 1996; Nisitani et al., 1999).

The relevance of Y223 phosphorylation for BTK function remains controversial: Phosphorylation of Y223 enhances binding properties of signaling molecules like SYK to the SH3 domain of BTK (Morrogh et al., 1999), suggesting a positive role of Y223. In contrast, an inhibitory function of Y223 was proposed, as it was shown that mutation of Y223 potentiates the capacity of a gain-of-function mutant to transform fibroblasts (Park et al., 1996). Moreover, it was shown that the expression of a *BTK*-transgene with an Y233F mutation could reconstitute B cell development in *BTK*-deficient mice (Middendorp et al., 2003).

Activated BTK is essential for cell cycle progression and survival in response to (pre-) B cell receptor stimulation (Petro et al., 2000). Following activation, BTK phosphorylates PLCγ2 facilitated by the interaction with the adapter molecules CBL-B and SLP65 (Yasuda et al., 2002). PLCγ2-mediated release of the second messengers diacylglycerol (DAG) and Ca^{2+} into the cytoplasm induces the activation of PKCβ (Saijo et al., 2002). The subsequent activation of NF-κB and CYCLIN D2 allows entry into the S phase of the cell cycle and cell growth (Petro et al., 2001; Glassford et al., 2003). However, while BTK-dependent activation of PLCγ2 and the subsequent Ca^{2+} release is mediated by tyrosine-phosphorylation (Watanabe et al., 2001), it has recently been shown, that also a *BTK*-transgene lacking kinase activity could partially restore B cell development in *BTK*-deficient mice (Middendorp et al., 2003). In parallel to cell cycle progression, BTK provides survival signals through NF-κB associated expression of the anti-apoptotic protein $BCLX_L$ (Suzuki et al., 2003). In addition, BTK activity leads to the activation of STAT5 (Mahajan et al., 2001), which also results in the upregulation of $BCLX_L$ (Nosaka et al., 1999). Contrary to the function in survival signaling by prevention of CD95-mediated apoptosis (Vassilev et al., 1999) and upregulation of $BCLX_L$, BTK can also act as a mediator of apoptosis: BTK kinase activity is responsible for radiation-induced apoptosis in the chicken lymphoma cell line DT40 (Uckun et al., 1996) and forced expression induces apoptosis in the human epithelial carcinoma cell line HELA (Islam et al., 2000; Islam and Smith, 2000).

There may be additional functions of BTK in regulating gene expression. BTK binds to the two transcription factors BAP135/TFII-I (Yang and Desiderio, 1997) and BRIGHT (Rajaiya et al., 2005) and can translocate to the nucleus, which may be critical during B cell development and differentiation (Mohamed et al., 2000).

In summary, it has been shown that Bruton's tyrosine kinase (BTK) represents a crucial component of the (pre-) B cell receptor signal transduction cascade (Fluckiger et al., 1998). Deficiency of BTK leads to a differentiation arrest at the pre-B cell stage (Noordzij et

al., 2002) and reduced survival and proliferation, as in the bone marrow of XLA patients the amount of B cells is decreased compared to bone marrow healthy humans (Genevier and Callard, 1997). Surprisingly, in cooperation with the linker molecule SLP65, BTK was recently described as a tumor suppressor in mice (Kersseboom et al., 2003). This underlines the important role of BTK in different signaling pathways leading to survival, proliferation or differentiation of pre-B cells.

1.6 X-linked agammaglobulinemia

The severe human immunodeficiency *X-linked agammaglobulinemia* (XLA) was firstly characterized as human *agammaglobulinemia* in 1952 by Ogden C. Bruton, who reported the complete absence of the immunoglobulin γ-fraction in the serum of an eight year old boy (Bruton, 1952). The immune defect was further characterized by the X-linked pattern of inheritance (Janeway et al., 1953) and renamed as XLA. The disease is, at a prevalence of 1 out of 200.000 individuals (Sideras and Smith, 1995), rare and characterized by an increased susceptibility to extracellular bacterial and enteroviral infections (Lederman and Winkelstein, 1985). While in XLA patients the myeloid cell and T cell populations are unaffected, the B cell population exhibits a differentiation arrest between the pro-B and pre-B cell stage resulting in a near complete loss of mature B cells (Campana et al., 1990; Noordzij et al., 2002). The gene defective in XLA was mapped to the Xq21.3-22 region (Guioli et al., 1989) and afterwards described as the gene encoding a cytoplasmic tyrosine termed ATK (agammablobilinemia tyrosine kinase; Vetrie et al., 1993) or BPK (B cell progenitor kinase; Tsukada et al., 1993), later renamed as Bruton's tyrosine kinase (BTK). BTK is currrently characterized as a crucial component of the (pre-) B cell receptor signalosome (Fluckiger et al., 1998) promoting the developmental progression of pre-B cells (Middendorp et al., 2003). Mutation of the mouse homolog causes a less severe phenotype termed *X-linked immunodeficiency* (Xid; Rawlings et al., 1993; Thomas et al., 1993), which is owing to the presence of BTK-redundant TEC family kinases that may serve as a backup in the absence of BTK (Ellmeier et al., 2000). In contrast to XLA, Xid mice still have about 50 % of the normal number of B cells and are able to generate an antibody response against T cell dependent antigens (Klaus et al., 1997; Wicker and Scher, 1986). In the human, about 85 % of defects in early B cell development are caused by a mutation in BTK, while 5-10 % are caused by defects in other components of the (pre-) B cell receptor signalosome (Conley et al., 2005). The so far described mutations within the *BTK* gene are collected in the BTK database BTKbase (http://bioinf.uta.fi/BTKbase/), which represents a public XLA-mutation registry

established in 1994 (Vihinen et al., 1996). Currently, about 561 unique mutations within the *BTK* gene from 839 families are known, of which 185 are missense mutations. While frameshift and nonsense mutations are distributed randomly over the whole gene, missense mutations are predominantly present within the carboxyterminal kinase domain and the aminoterminal PH domain of BTK (Lindvall et al., 2005). Notably, only the SH3 domain of BTK is completely devoid of missense mutations in XLA patients. From *in vitro* kinase assays of BTK mutants derived from *BTK*-deficient X-linked agammaglobulinemia (XLA) patients, it is known that in all cases of XLA, BTK kinase activity is abolished or severely reduced (http://bioinf.uta.fi/BTKbase/BTKbasebrowser.html). Even replacement mutations in the distal portion of the kinase domain often result in a complete loss of BTK kinase activity (Holinski-Feder et al., 1998).

1.7 Chromosomal translocations as a cause of B cell precursor leukemia

Acute lymphoblastic leukemia (ALL) with a pre-B cell immunophenotype is characterized by deregulated cell growth, differentiation and survival signaling. In many cases, leukemogenic deregulation is owing to translocation events. In 52% of cases of all ALLs in children and 68% of cases of all ALLs in adults, chromosomal aberrations were detected in the leukemia cells (Pui et al., 2004). As a general phenomenon of cancer, translocations typically result in transcriptional deregulation of the target gene or lead to the generation of a fusion gene if the breakpoint involves coding exons on both chromosomes. In this regard it is noteworthy that recurrent chromosomal translocations in mature B cell lymphoma cells, in almost all instances, result in transcriptional deregulation. In contrast, most if not all recurrent chromosomal rearrangements in the leukemia cells result in the expression of chimeric proteins (Look , 1997).

The most frequent genetic aberration in children (22% of all ALLs) involves a rearrangement of the oligomerisation domain of the *TEL* gene on chromosome 12 to the entire coding region of the *AML1* gene on chromosome 21 (Pui et al., 2004). While TEL belongs to the ETS family of transcriptional repressors and *AML1* encodes the RUNT-domain of the transcription factor termed core-binding factor (CBF), the *TEL-AML1* gene product oligomerizes and represses genes normally activated by AML1 (Hiebert et al., 1996). In contrast to the normal AML1 proteins, which recruit other co-activators like histone-acetyl-transferases (HATs) to the target gene, the oncogenic TEL-AML1 protein reverses the initial function by recruiting histone-deacetylases (HDACs) that close the chromatin structure by

methylation and inhibit transcription (Hiebert et al., 1996). In addition to the reversal of AML1 function, the translocation event also prevents normal function of TEL.

Another example for an oncogenic trancription factor chimera generated by a translocation event in B cell precursor leukemia represents the fusion protein E2A-PBX1. Fusion of the transcriptional activation domains of the *E2A* gene on chromosome 19 to the DNA-binding domain of the *PBX1* gene on chromosome 1 results in a new transcription factor that deregulates PBX1-target genes. This deregulation alone is sufficient to promote proliferation and survival of leukemia cells as transgenic expression of *E2A-PBX1* leads to leukemia in mice (Dedera et al., 1993).

The most frequent type of lymphoid leukemia in adults (25%) is the pre-B cell leukemia carrying a translocation of the *BCR* gene on chromosome 22 to the *ABL1* gene on chromosome 9, the so-called Philadelphia (Ph) chromosome (Piu et al., 2004; Rowley, 1973). With identification of the *BCR-ABL1* translocation, it was for the first time that a chromosomal aberration could be linked to a specific cancer (Nowell and Hungerford, 1960). The *BCR-ABL1* rearrangement is present in B cell progenitor leukemia, but also drives malignant transformation in >95% if not all cases of chronic myeloid leukemia (CML; Shepherd et al., 1995). While in CML, leukemia cells express predominantly a BCR-ABL1 onco-protein of 210 kd (BCR-ABL1^{p210}), pre-B cell leukemias with a *BCR-ABL1* gene rearrangement mainly express the form of 190 kd size (BCR-ABL1^{p190}; Clark et al., 1988). Notably, *BCR-ABL1*–positive CML can be cured in many cases with the BCR-ABL1 kinase inhibitor STI571 (also termed Imatinib or Gleevec), but the same treatment leads to a relapse in all patients with *BCR-ABL1*–positive pre-B cell leukemia within a median of 4 months (Druker et al., 2001). However, the reasons for this striking difference have yet remained unclear. The translocation of the *BCR* to the *ABL1* gene deletes the autoinhibition domain of the ABL1 tyrosine kinase resulting in a constitutively active BCR-ABL1 tyrosine kinase. The expression of *BCR-ABL1* alone is sufficient for malignant transformation of pre-B cells (Klucher et al., 1998). The transformation capacity is dependent on the kinase activity of BCR-ABL1 as kinase-inhibitors against BCR-ABL1 induce apoptosis in the leukemia cells (Druker et al., 1996). BCR-ABL1 kinase activity leads to the deregulation of cellular signaling pathways and is of critically importance for the promotion of survival of the leukemia cells (Huettner et al., 2000).

1.8 Signaling activity of the oncogenic BCR-ABL1 kinase

The activity of the normal c-ABL1 tyrosine kinase is regulated by an autoinhibition mechanism (Pluk et al., 2002). This autoinhibition is mediated through binding of the N-terminal myristoyl modification of c-ABL1 to the kinase domain, thereby stabilizing the inactive conformation of c-ABL1 (Nagar et al., 2003). The translocation resulting in the *BCR-ABL1* fusion gene deletes the N-terminal part of c-ABL1 required for autoinhibition. To the contrary, the *Abelson murine leukemia virus* gene product v-Abl, which also exhibits constitutive ABL1 kinase activity, still contains the complete N-terminal domain but carries a deletion within the C-terminus also resulting in the relief of autoinhibition (Shore et al., 1990). The rearrangement of *BCR* and *ABL1* results in fusion proteins of different sizes depending on the breakpoint within the *BCR* gene. Comparison of the kinase activity of the two major forms of BCR-ABL1, p190 and p210, demonstrated that the p190 isoform exhibits increased kinase activity compared to p210 (Lugo et al., 1990). BCR-ABL1^{p190} also induces lymphoid leukemia with a shorter latency than p210 in mice (Li et al., 1999). However, both forms are present in *BCR-ABL1*-positive pre-B cell leukemia and a significant difference in the response to treatment has not been observed (Gleissner et al., 2002).

For the activation of c-ABL1, the phosphorylation of tyrosine 412 is of critical importance (Hantschel et al., 2003). Relief of the autoinhibition of c-ABL1 results in the recruitment to specific membrane domains like lipid rafts, where c-ABL1 is activated by phosphorylation. In contrast, BCR-ABL1 is not membrane-associated but requires oligomerization to homotetramers for full activation (Maru et al., 1996). Oligomerization is dependent on a coiled-coiled domain within the BCR gene which is present in all BCR-ABL1 forms (Tauchi et al., 1997). The oligomerisation status of BCR-ABL1 proteins within the cell directly determines their autophosphorylation and transformation capacity (Maru et al., 1996).

The activated BCR-ABL1 kinase is sufficient to modulate distinct cellular signaling pathways promoting cell growth and survival: Several molecules that promote cell-cycle progression and growth like CYCLIN D2 (Parada et al., 2001), MYC (Skorski et al., 1997) and the RAS pathway (Cortez et al., 1997) have been identified to be regulated by BCR-ABL1 in leukemia cells. In addition, BCR-ABL1 prevents apoptosis by upregulation of the anti-apoptotic protein $BCLX_L$ through phosphorylation of STAT5 (Gesbert and Griffin, 2000) and activation of the anti-apoptoic protein BCL2 through the PI3K/AKT pathway (Skorski et al., 1997). Also molecules involved in cytoskeletal modulation (including PAXILLIN and CBL), DNA repair (including RAD51 and DNA-PK) and signal transduction (including RAS and JNK) have been described to be also regulated by BCR-ABL1 (Wong and Witte, 2004).

As several of BCR-ABL1-regulated molecules are also implicated in the signal transduction of the pre-B cell receptor (e.g. PI3K, AKT, SHP1, CBL, GRB2 and VAV), potential interference of BCR-ABL1-induced oncogenic signaling with pre-B cell receptor signaling in the pre-B leukemia cells was systematically investigated in this thesis.

1.9 Aims of the thesis

During early B cell development, B cell precursor cells undergo a sequence of somatic rearrangements within the *IGH* locus, which are required for the expression of a µ-heavy chain within the pre-B cell receptor complex on the surface of pre-B cells. Expression of a functional pre-B cell receptor and subsequent signal transduction serves as an early checkpoint of B cell development, thereby initiating the expansion, survival and further differentiation of pre-B cells into immature B cells (Hess et al., 2001; Tarlinton et al., 1997).

B cell lineage leukemia cells expressing the oncogenic BCR-ABL1 tyrosine kinase typically exhibit a differentiation block at the pre-B cell stage (Young and Witte, 1988). Oncogenic signaling initiated by the BCR-ABL1 kinase is sufficient for malignant transformation of pre-B cells (Huettner et al., 2000).

Based on these findings, the following questions should be addressed within this thesis:

i) Is the differentiation block of BCR-ABL1-transformed pre-B cells induced by the oncogenic BCR-ABL1 kinase and how is this arrest maintained in the leukemia cells?

ii) BCR-ABL1-transformed pre-B leukemia cells exhibit enhanced proliferation, which could also be induced by pre-B cell receptor signaling. Does the pre-B cell receptor function as an oncogene within the leukemia cells?

iii) Do the oncogenic BCR-ABL1 signaling pathway and the pre-B cell receptor signal transduction pathway coincide within the leukemia cell and if so, do they interfere with each other?

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Chapter 2

Results and discussion

2.1 The BCR-ABL1 kinase bypasses the selection for the expression of a pre-B cell receptor in transformed pre-B cells

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The expression of a pre-B cell receptor on the cell surface and its signaling capacity represents a critical checkpoint during early B cell development and confers survival and proliferation (Rajewsky, 1996; Hess et al., 2001).

In order to investigate if *BCR-ABL1*-positive leukemia cells are selected for the expression of a pre-B cell receptor, the configuration of the *IGH* locus of twelve leukemia cases was analyzed for the capacity to encode a functional µ-heavy chain. Surprisingly, only three out of twelve cases harbored a functionally rearranged *IGH* allele, which was compatible with the expression of a pre-B cell receptor. Within the non-functional *IGH* gene rearrangements, traces of secondary V_H rearrangements were found, which may have rendered an initially productive rearrangement non-functional (see 1.2). Notably, the usuage of this mechanism in normal B cells is rare as only 5 % of normal immature B cells show traces of secondary V_H rearrangements (Zhang et al., 2003).

Comparing genome-wide gene expression profiles of pre-B leukemia cells harboring a *BCR-ABL1* rearrangement and human pre-B cells by the *serial analysis of gene expression* (SAGE) method (Velculescu et al., 1995), the leukemia cells exhibit specific silencing of many genes involved in pre-B cell receptor signaling. The presence of non-functional *IGH* V region gene rearrangements in the majority of cases and the severely reduced expression of pre-B cell receptor signaling molecules indicates the absence of selection pressure for the expression of a functional pre-B cell receptor in the leukemia cells.

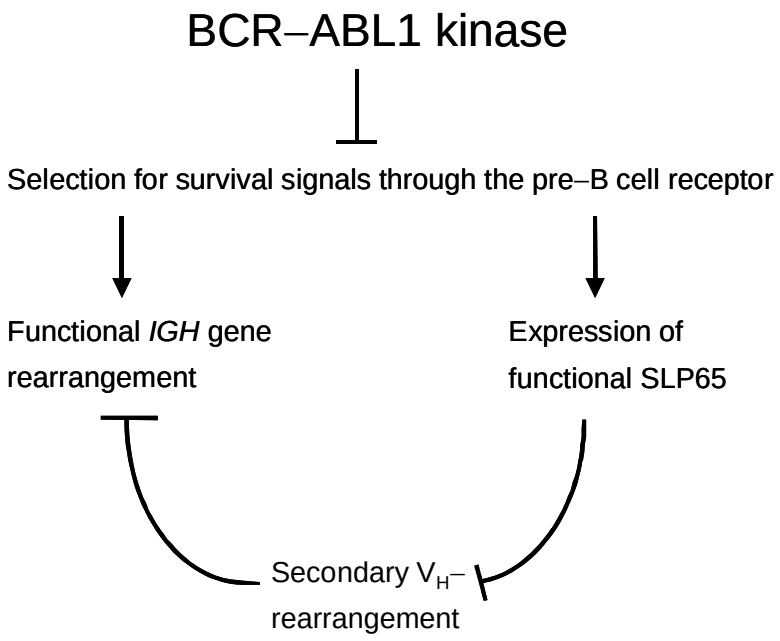
Secondary V_H rearrangement may result from an ongoing recombination process in the leukemia cells. To investigate possible reasons for ongoing recombination, the expression of the adapter molecule SLP65, which stabilizes the correct assembly of the pre-B cell receptor signaling cascade, was examined. SLP65 functions as a sensor for the expression of a productive *IGH* V region gene rearrangement within the pre-B cell receptor and halts the recombination machinery to prevent further, potentially inactivating, rearrangement at the *IGH* locus (Schebesta et al., 2002). Indeed, BCR-ABL1 kinase activity was linked to the expression of a truncated non-functional isoform of SLP65 (SLP65ΔC). In addition to ongoing rearrangement within the *IGH* locus resulting from defective SLP65 expression (Sprangers et al., 2005), truncated SLP65 splice variants may also interfere with normal

SLP65 function in pre-B cell receptor signal transduction by competition for upstream binding partners like the SYK kinase. Inhibition of BCR-ABL1 kinase activity resulted in apoptosis in the majority of leukemia cells, but leads to the outgrowth of a small subset of leukemia cells, which survived the inhibition of BCR-ABL1. In the absence of BCR-ABL1 kinase activity, the surviving cells expressed a pre-B cell receptor on their surface and showed normal expression of SLP65 and other molecules related to pre-B cell receptor signaling. Specific outgrowth of pre-B cell receptor-responsive leukemia cells surviving inhibition of BCR-ABL1 indicates that selection for the capacity to initiate pre-B cell receptor signaling is reconstituted upon inhibition of BCR-ABL1 (Figure 1).

As three out of twelve leukemia cases tested are able to express a pre-B cell receptor on their surface, the signaling capacity of the pre-B cell receptor was analyzed in these cells. Stimulation of the pre-B cell receptor did not lead to a Ca^{2+} response in any of the three cases tested. Instead, the leukemia cells exhibit an autonomous oscillatory Ca^{2+} signaling, which was dependent on BCR-ABL1 kinase activity.

In summary, these results demonstrate that the BCR-ABL1 kinase bypasses the selection for the expression of a pre-B cell receptor. In addition, BCR-ABL1 also actively interferes with pre-B cell receptor signal transduction in the leukemia cells by the expression of defective SLP65 and the induction of an autonomous Ca^{2+} signal.

Figure 1: *BCR-ABL1 bypasses the selection for the expression of a pre-B cell receptor*



2.2. BCR-ABL1 interferes with differentiation by the induction of a dominant-negative isoform of the transcription factor IKAROS

Published in *Oncogene* (5.2 in chapter 5).

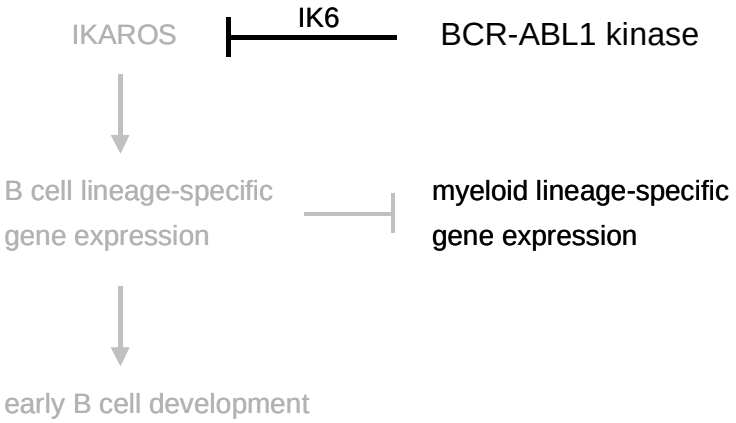
Early B cell development in the human is guided by several transcription factors that initiate differentiation and maintain the identity of B cell precursors (Busslinger et al., 2004). B lymphoid cells transformed by the BCR-ABL1 kinase typically exhibit an a differentiation block at the pre-B cell stage (Young and Witte, 1988).

Comparison of genome-wide gene expression profiles of leukemia cells carrying a *BCR-ABL1* rearrangement and normal human pre-B cells by SAGE, revealed that multiple components of the pre-B cell receptor signaling cascade and molecules maintaining B cell identity were transcriptionally silenced in the leukemia cells (see 2.1). In addition, myeloid lineage-specific molecules were upregulated, thereby promoting a mixed-lineage phenotype of the transformed pre-B cells. The usage of a BCR-ABL1-specific kinase inhibitor, termed STI571, and a murine cell line carrying an inducible *BCR-ABL1* transgene showed that this mixed lineage phenotype was dependent on BCR-ABL1 kinase activity. Furthermore, induced expression of BCR-ABL1 resulted in aberrant splicing of IKAROS, a transcription factor essential for the commitment to the lymphoid lineages (Georgopoulos et al., 1994). Previous studies have shown that deficiency of IKAROS prevents early B cell development (Georgopoulos et al., 1994) and even prevents normal function of mature B cells (Kirstetter et al., 2002). In transformed pre-B cells, BCR-ABL1 induces the expression of a dominant-negative IKAROS isoform, termed IK6, which has the capacity to block B lymphoid differentiation of HSCs (Tonnelle et al., 2001). Unlike full-length IKAROS, alternatively-spliced IK6 is lacking the IKAROS DNA-binding domain (Sun et al., 1996; Sun et al., 1999). As a consequence, IK6 is localized to the cytoplasm and also prevents nuclear localisation of full-length IKAROS molecules through formation of heterodimers (Sun et al., 1996). To investigate the specific contribution of *IK6* expression to the mixed-lineage phenotype of *BCR-ABL1*-positive B cell precursor leukemia, *IK6* transcripts were specifically silenced in the leukemia cells using RNA interference. Upon inhibition of IK6 expression, the leukemia cells partially restored their B lineage identity while the expression of myeloid lineage-specific molecules was downregulated.

In summary, it has been shown that BCR-ABL1 kinase activity promotes a loss of B cell identity by the relief of selection pressure for the expression of a pre-B cell receptor (see

2.1). In addition, the BCR-ABL1 kinase directly interferes with differentiation by the induction of the expression of a dominant-negative isoform of IKAROS, IK6, which results in the downregulation of B cell-specific genes and upregulation of myeloid-specific genes (Figure 2).

Figure 2: *The BCR-ABL1 kinase interferes with IKAROS-dependent B lymphoid differentiation by the induction of the dominant-negative isoform IK6*



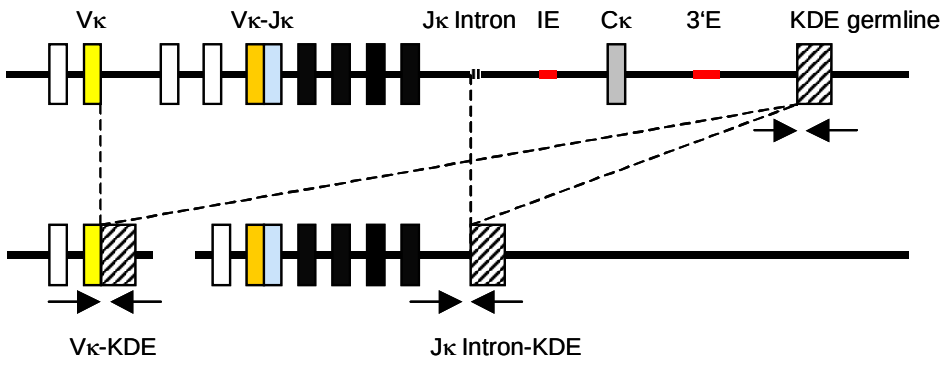
2.3 Inhibition of BCR-ABL1 kinase activity relieves the differentiation block of transformed pre-B cells and indicates that the *Igκ* and *Igλ* light chain loci rearrange sequentially during early B cell development

Published in *the Journal of Immunology* (5.3 in chapter 5).

BCR-ABL1-transformed B cell progenitor cells exhibit a differentiation block at the pre-B cell stage of development. While normal pre-B cells are selected for the expression of a pre-B cell receptor and further differentiate into immature B cells, this selection process is impaired in the leukemia cells (see 2.1). However, inhibition of BCR-ABL1 kinase activity by STI571, induces apoptosis in the majority of the leukemia cells, but also partially relieves the differentiation block mediated by BCR-ABL1: A small subset of pre-B cell receptor expressing leukemia cells selectively survives, by their capacity to generate survival signals

through the pre-B cell receptor. These cells further differentiate to the immature B cell stage demonstrated by the expression of λ-light chains within the B cell receptor complex on their surface. Likewise, treatment of patients suffering from *BCR-ABL1*-positive pre-B cell leukemia with STI571 induces the expression of the transcription factors IRF4 and SPIB in the surviving leukemia cells, both of which are neccessary for efficient immunoglobulin light chain (*IGL*) rearrangement (Lu et al., 2003; Muljo and Schlissel, 2003). To investigate the cause for the selective expression of λ-light chains in the surviving leukemia cells, the specific targeting of the recombination machinery within the *IGL* loci was monitored by the amplification of DNA double-strand break (DSB) intermediates, reflecting an active recombination process: Surprisingly, DSBs within the Igκ light chain locus were already detectable after one hour of STI571 treatment. After six hours, initially rearranged VκJκ joints were deleted by the rearrangement of the κ-deleting element (KDE, see Figure 3A) within the Igκ light chain locus followed by rearrangment of the Igλ light chain locus after 12 hours.

Figure 3A: *Initially rearranged Vκ-Jκ joints can be deleted by the rearrangement of the κ-deleting element (KDE)*

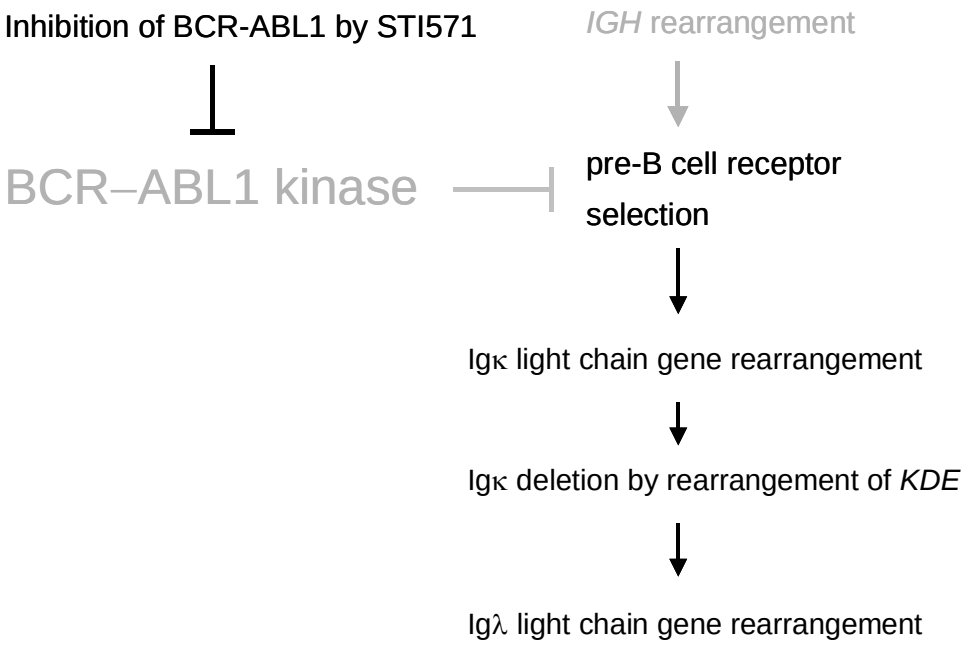


A preformed Vκ-Jκ-joint (top) can be inactivated by two types of rearrangement of the κ-deleting element (KDE). Using an upstream Vκ-RSS of an unrearranged Vκ-gene segment, rearrangement of the KDE results in a large deletion within the κ locus including downstream unrearranged Vκ-gene segments, the pre-existing Vκ-Jκ-joint, unrearranged Jκ-gene segments and the Cκ-gene (left). In alternative, the KDE can rearrange to an intronic RSS between the cluster of Jκ-gene segments and the Cκ gene. In this case, KDE-rearrangement results in a deletion of the Cκ-gene and both κ enhancers (right).

Therefore, the selective expression of Igλ-light chains by the surviving leukemia cells is explained as a result of continuous activity of the recombination machinery within the Igκ and Igλ light chain loci. This likely reflects the absence of a negative feedback regulation of the recombinase in the differentiating leukemia cells.

In summary, inhibition of BCR-ABL1 kinase activity demonstrates that BCR-ABL1 continuously maintains the differentiation block in the leukemia cells. Furthermore, inhibition of BCR-ABL1 kinase activity directly shows that the Igκ light chain locus, KDE and the Igλ light chain locus are rearranged in a sequential order (Figure 3B).

Figure 3B: *Inhibition of BCR-ABL1 kinase activity relieves the differentiation block and induces the pre-B to immature B cell transition*



2.4 Mimicry of a constitutively active pre-B cell receptor: Aberrant splicing links Bruton's tyrosine kinase to BCR-ABL1 in pre-B lymphoblastic leukemia cells

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BCR-ABL1-transformed pre-B cells have lost their capacity to express a functional pre-B cell receptor, which is consistent with lack of selection pressure for the expression of an active pre-B cell receptor (see 2.1). While pre-B cell receptor signals are critical for the survival and proliferation of normal human pre-B cells, BCR-ABL1 induces an autonomous oscillatory Ca^{2+} signal that interferes with signals initiated by the pre-B cell receptor (see 2.1).

In order to identify the mechanism by which BCR-ABL1 kinase activity interferes with pre-B cell receptor signaling, the activation of the proximal signaling cascade in response to stimulation of the pre-B cell receptor was analyzed. As expected, the key molecules SYK, SLP65 and *Bruton's tyrosine kinase* (BTK) could not be activated in response to pre-B cell receptor engagement. Instead, BTK was constitutively phosphorylated in the leukemia cells. The analysis of BTK in B cell precursor leukemia cells harboring other chromosomal aberrations demonstrated, that constitutively activated BTK is a specific feature of *BCR-ABL1*-positive leukemia cells. Usage of the BCR-ABL1-specific kinase inhibitor STI571 and a murine pro-B cell line carrying an inducible *BCR-ABL1*-transgene revealed that BTK is specifically tyrosine-phosphorylated by BCR-ABL1.

Further investigations showed that BTK is "captured" by the oncogenic BCR-ABL1 kinase: BTK was identified as a crucial component of the BCR-ABL1 signalosome responsible for the initiation of survival signals through STAT5-mediated upregulation of the anti-apoptotic protein $BCLX_L$. Inhibition of BTK kinase activity induced apoptosis in the leukemia cells at comparable levels as inhibition of the BCR-ABL1 kinase activity itself. The initially observed autonomous oscillatory Ca^{2+} signal initiated by BCR-ABL1 (see 2.1) was also dependent on BTK kinase activity and mediated by activation of PLCγ1. Notably, even inhibition of PLCγ1 expression decreased survival of the leukemia cells significantly.

However, the interaction between BCR-ABL1 and BTK is difficult to explain, because previous studies have shown that BCR-ABL1 cannot directly bind to BTK. Instead, BCR-ABL1 kinase activity induces the expression of a splice variant of BTK that has the capacity to bind to BCR-ABL1 as well as to full-length BTK molecules. This BTK isoform, termed BTK^{p52} (referring to its molecular weight of 52 kD), is C-terminally truncated and

lacks kinase activity. Notably, BTK^{p52} can act as a linker between the two kinases thereby facilitating the activation of BTK by BCR-ABL1. Indeed, specific inhibition of BTK^{p52} expression by RNA interference abolished the oncogenic activation of full-length BTK and downstream targets like STAT5 and PLCγ1 and subsequently induced apoptosis in the leukemia cells. In a co-transfection assay, the SH3 domain of BTK^{p52} was sufficient to induce BCR-ABL1- and BTK-dependent phosphorylation of STAT5 and PLCγ1.

BCR-ABL1-induced aberrant splicing of BTK interferes with normal membrane-associated pre-B cell receptor signaling, because the kinase-inactive BTK isoforms are exluded from BTK-dependent phophorylation events and may also compete with full-length BTK molecules for binding to upstream interaction partners. In contrast, truncated BTK isoforms are essential for the transmission of the oncogenic signal initiated by BCR-ABL1 providing survival of the leukemia cells.

In conclusion, BTK function in the leukemia cells exemplifies that components of the pre-B cell receptor signal cascade are essential for the autonomous survival signal in *BCR-ABL1*-positive B cell precursor leukemia cells. Instead of being dependent on pre-B cell receptor signals, which can also initiate apoptosis, the BCR-ABL1 kinase mimics an aberrant pre-B cell receptor signal by activation of BTK, which leads to proliferation and survival of the leukemia cells (Figure 4).

Legend to Figure 4*:* *Mimicry of a constitutively active pre-B cell receptor in pre-B cell leukemia cells by the oncogenic BCR-ABL1 kinase*

In normal pre-B cells (left), signals initiated by the pre-B cell receptor result in the activation of the tyrosine kinases SYK and BTK and the subsequent initiation of PLCγ2-mediated Ca^{2+} release. The adapter molecule SLP65 stabilizes the accurate assembly of the pre-B cell receptor signalosome. As a result, the trancription factor NF-κB translocates into the nucleus and initiates gene expression leading to survival and differentiation. In BCR-ABL1-transformed pre-B cells (right), the pre-B cell receptor is not active. Instead, the oncogenic BCR-ABL1 kinase activates BTK and induces an autonomous survival signal mediated by PLCγ1 and STAT5. For the interaction between the two kinases, BCR-ABL1 and BTK, the presence of a BCR-ABL1-induced isoform of BTK, BTK^{p52}, is essential, which lacks kinase activity. Acting as a linker, BTK^{p52} facilitates BCR-ABL1-mediated activation of full-length BTK and downstream signaling molecules.

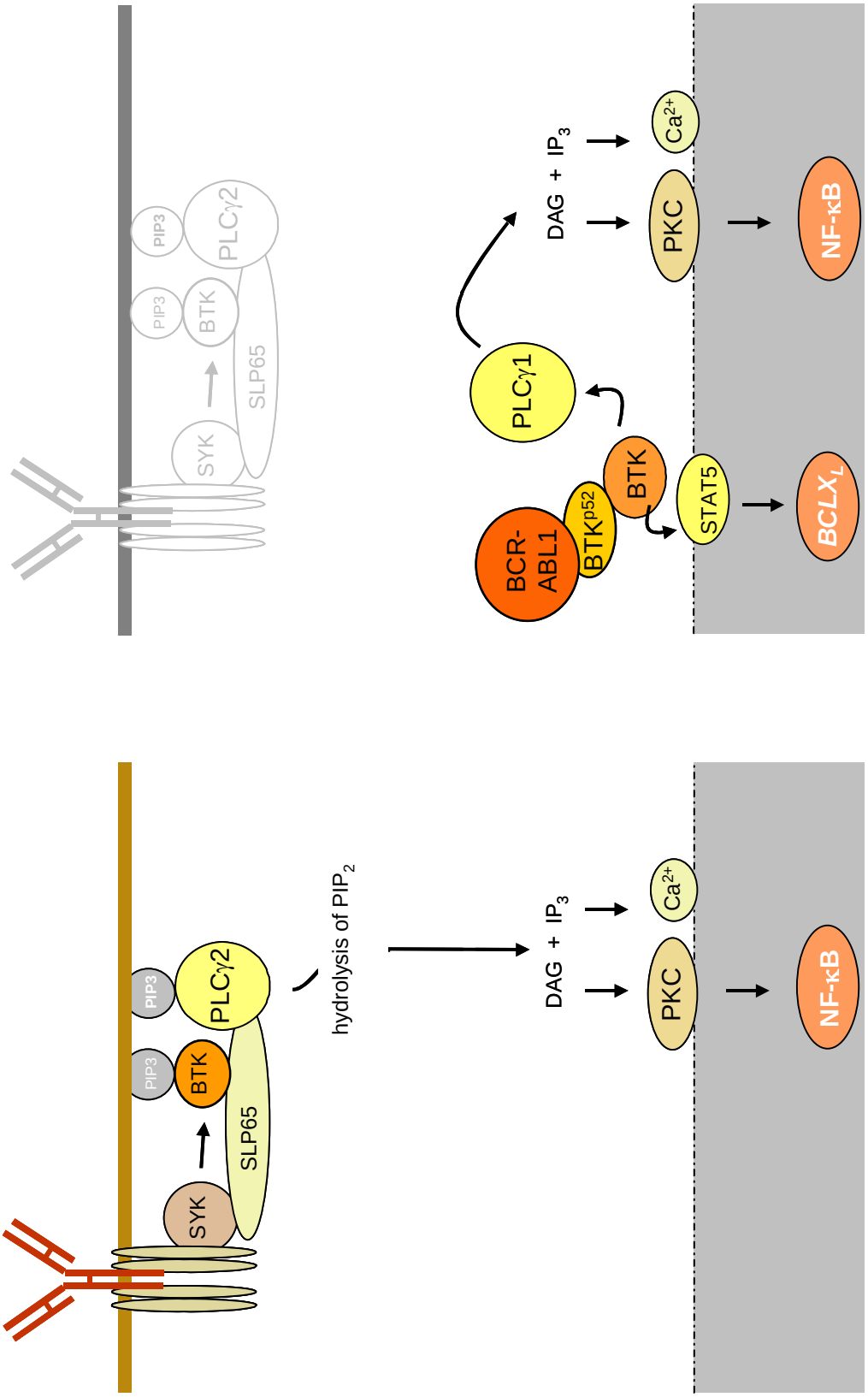

Figure 4: *Mimicry of a constitutively active pre-B cell receptor in pre-B leukemia cells by the oncogenic BCR-ABL1 kinase*

2.5 Activation-induced cytidine deaminase acts as a mutator in pre-B cell leukemia cells carrying a *BCR-ABL1* gene rearrangement

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Pre-B cell leukemia carrying a *BCR-ABL1* fusion gene defines a subset of acute lymphoblastic leukemia (ALL) with a particular unfavorable prognosis (Druker et al., 2001). Since malignant transformation by the oncogenic BCR-ABL1 tyrosine kinase has been extensively studied, a specific inhibitor of BCR-ABL1 kinase activity was identified, termed STI571, which is used in the therapy of *BCR-ABL1*-positive leukemia (Wong and Witte, 2004). However, while *BCR-ABL1*-positive chronic myeloid leukemia (CML) can be treated efficiently by STI571, *BCR-ABL1*-positive pre-B cell leukemia cells usually become resistant to STI571 in most if not all cases after a median of four months (Druker et al., 2001). A frequent cause leading to resistance is the pre-existence of point mutations within the kinase domain of BCR-ABL1, which prevent binding of STI571 (von Bubnoff et al., 2002). The origin of these mutations is to date not known.

Within the germinal center reaction, normal mature B cells modify the affinity and effector function of their B cell receptor by somatic hypermutation (SHM) and class switch recombination (CSR), respectively. Both events require the activity of the *activation-induced cytidine deaminase* (AID), which introduces DNA-single strand breaks (SSB) into the immunoglobulin (Ig) variable (V) region or constant (C) region genes, thereby resulting in SHM or the initiation of CSR. At low frequency, AID can also target non-Ig genes in germinal center B cells (Pasqualucci et al., 1998; Shen et al., 1998; Müschen et al., 2000) and may even act as a genome-wide mutator (Wang et al., 2004).

To examine the potential contribution of AID to leukemia development, B cell precursor leukemia cells were analyzed for *AID* expression. Neither normal pre-B cells nor pre-B cell leukemia cells express *AID*. Surprisingly, *AID* expression was specifically found in leukemia cells with a *BCR-ABL1* gene rearrangement. *AID* expression was directly dependent on BCR-ABL1 kinase activity *in vitro* and *in vivo*, demonstrated by retroviral delivery of *BCR-ABL1* expression in a human *BCR-ABL1*-negative cell line and the usage of matched patient samples, respectively. Consistent with aberrant *AID* expression, immunoglobulin V region genes were mutated in 28 out of 32 cases of *BCR-ABL1*-positive pre-B cell leukemia analyzed, but unmutated in 44 out of 48 cases among *BCR-ABL1*-negative pre-B cell leukemia. In 5 out of 21 cases of *BCR-ABL1*-positive leukemia, also class-switched Ig

transcripts could be detected. To further investigate the capacity of AID to act as a mutator in the leukemia cells, Ig and non-Ig genes were analyzed for the presence of DNA-SSBs. In both, imunoglobulin V region genes and the non-Ig gene *CDKN2B*, DNA-SSBs could be detected in *BCR-ABL1*-positive cells. These DNA-SSBs were dependent on AID activity, as demonstrated by specific silencing of *AID* expression by RNA interference.

In summary, AID could be identified as a mutator of Ig and and non-Ig genes in pre-B cell leukemia cells harboring a *BCR-ABL1* gene rearrangement. AID activity induced by the BCR-ABL1 kinase might lead to an accumulation of somatic mutations that favor the development of resistance against chemotherapy and treatment with STI571.

2.6 Deficiency of Bruton's tyrosine kinase in B cell precursor leukemia cells without a *BCR-ABL1* rearrangement

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Pre-B cell receptor-induced signal transduction is essential for the initiation of survival, proliferation and differentiation of human pre-B cells. In normal B cells, defective expression of components of the pre-B cell receptor complex or the pre-B cell receptor signaling cascade usually results in a developmental block at the pre-B cell stage. Likewise, inherited deficiency of BTK results in a differentiation block at the pre-B cell stage leading to the severe immunodeficiency XLA in the human and in the milder form XID in mice (Campana et al., 1990; Rawlings et al., 1993).

As shown in chapter 2.4 and 5.4 of this thesis, oncogenic activation of BTK is essential for BCR-ABL1-induced transformation. Since BCR-ABL1-induced oncogenic activation of BTK provides survival and proliferation-related signaling pathways but does not promote BTK-induced differentiation, the leukemia cells typically exhibit a pre-B cell differentiation block. A pre-B cell differentiation block is also a common feature of many other types of B cell lineage leukemia besides BCR-ABL1-induced leukemogenesis (Schiff et al., 1991; LeBien, 2000). To investigate a potential contribution of BTK to the differentiation block of *BCR-ABL1*-negative B cell precursor leukemia cells, BTK expression was analyzed in leukemia cells harboring an *E2A-PBX1*, *MLL-AF4*, *TEL-AML1* or *TEL-PDGFRB*

rearrangement. In contrast to *BCR-ABL1*-positive pre-B cell leukemia cells, where the BTK kinase has a unique role within the transmission of oncogenic signaling, in none of the *BCR-ABL1*-negative leukemias constitutive activation of BTK by tyrosine-phosphorylation could be detected. However, all leukemia types tested, expressed aberrant splice variants of BTK, which had in common that they lack kinase activity. Unlike *BCR-ABL1*-positive pre-B cell leukemia (see 2.4), the expression of a specific splice variant was not linked to a specific leukemia type. While in four out of eight cases of *MLL-AF4*-positive B cell progenitor leukemia analyzed, expression of full-length BTK was missing, the majority of leukemia cases typically coexpressed BTK splice variants with full-length BTK.

To investigate if the expression of kinase-inactive BTK splice variants could interfere with pre-B cell receptor-mediated differentiation and therefore could contribute to the differentiation block of the leukemia cells, we transfected a pre-B cell line, which exhibited a normal pre-B cell receptor signal and only expression of wildtype BTK, with a retroviral expression vector encoding kinase-inactive BTK. While in the presence of only wildtype BTK, the engagement of the pre-B cell receptor resulted in a typical Ca^{2+} response, this signal was completely blocked upon coexpression of kinase-inactive BTK. In another assay, in which the differentiation from the pre-B to immature B cell stage could be induced, the presence of kinase-deficient BTK splice variants prevented this differentiation process. Therefore, kinase-deficient BTK isoforms, frequently expressed in leukemia cells, can act as dominant-negative BTK in human pre-B cells regarding pre-B cell receptor-induced differentiation.

Surprisingly, reconstitution of B cell precursor leukemia cells expressing kinase-deficient BTK splice variants with full-length BTK rendered the cells sensitive to apoptosis, In order to examine if the expression of dominant-negative BTK splice variants contributes to prevention of BTK mediated apoptosis, B cell precursor leukemia cells were γ-irradiated and BTK isoform expression was monitored in the surviving and in pre-apoptotic cells. Consistent with a role of wildtype BTK as apoptosis-sentisizer, kinase-deficient BTK splice variants in the leukemia cells were predominantly expressed by the non-apoptotic leukemia cells.

The specific contribution of the expression of dominant-negative BTK isoforms to the differentiation arrest and apoptosis prevention in *BCR-ABL1*-negative pre-B cell leukemia cells remains unclear as full-length BTK is still expressed in the leukemia cells. Though, these findings show that the expression of dominant-negative splice variants of BTK i) can inhibit differentiation beyond the pre-B cell stage and ii) can interfere with BTK-mediated apoptosis signals initiated by the pre-B cell receptor.

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Chapter 3

Concluding remarks

3.1 *Splicing for survival* – selection for aberrant splice variants in *BCR-ABL1*-transformed pre-B cell leukemia cells

Accurate splice site targeting is mandatory for efficient protein synthesis. Previous studies (Jumaa et al., 2003) and the results described here (see 2.1, 2.2, 2.4 and 2.6) demonstrate that inaccurate splice site targeting is a frequent event in B cell progenitor leukemia cells. Aberrant splice events in leukemia cells may reflect advantageous changes in protein function, which were positively selected during leukemogenesis. As shown for BTK, IKAROS and SLP65, deranged function as a result of aberrant splicing promotes tumor progression and survival of the leukemia cells (see 2.1, 2.2, 2.4 and 2.6). While many types of B cell progenitor leukemia exhibit aberrant splicing of B cell-specific proteins (Jumaa et al., 2003 and chapter 2.6), a direct relation between aberrant splicing and a specific leukemia type could only be established for pre-B cell leukemia cells harboring a *BCR-ABL1* gene rearrangement.

As shown here, aberrant splicing of BTK, IKAROS and SLP65 is induced by BCR-ABL1 kinase activity. Previous studies indicate that these processes are enabled by the increased expression of splice factors including hnRNPA2/B1, TLS, RNA-helicase II and SRPK1 in *BCR-ABL1*-positive cells (Salesse et al., 2004; Perrotti et al., 1998). Increased expression of these proteins is directly dependent on BCR-ABL1 kinase activity (Salesse et al., 2004) and in the case of hnRNPA1 and TLS mediated by increased protein stability owing to reduced ubiquitin/proteasome-dependent degradation (Iervolino et al., 2002; Perrotti et al., 2000). Interference with one of these splice factors resulted in the reduction of the tumorigenic potential of the leukemia cells in either case (Perrotti et al., 1998; Iervolino et al., 2002).

While it remains unclear whether the expression of aberrant splice variants of SLP65, IKAROS and BTK in *BCR-ABL1*-positive pre-B cell leukemia is a result of loss of stringency in splice site selection or represents specific targeting of particular pre-mRNAs, it is evident that these aberrant splice events contribute to survival signaling by BCR-ABL1: SLP65-deficiency caused by aberrant splicing in the leukemia cells results in ongoing recombination within the *IGH* locus (Sprangers et al., 2005), thereby perturbing pre-B cell receptor expression at the plasma membrane. SLP65-mediated absence of pre-B cell receptor expression favors the maintenance of BCR-ABL1-induced leukemia in the human (described in 2.1). SLP65-deficiency was also described to promote uncontrolled pre-B cell expansion in mice (Flemming et al., 2003). Deranged expression of IKAROS contibutes to the

differentiation block in *BCR-ABL1*-positive pre-B cell leukemia (see 2.2). Interference with differentiation maintains the leukemia cell in an autonomous state independent from regulation by a specific developmental program. Moreover, deranged expression of IKAROS may also contribute to the malignant transformation itself as reduced expression of IKAROS in *IKAROS* $^{+/-}$ mice leads to lymphoproliferation and rapid development of T cell leukemia (Winandy et al., 1995) and is associated with disease progression of CML (Nakayama et al., 1999). The most striking example of aberrant splicing in BCR-ABL1-induced leukemogenesis involves BTK: The survival of the leukemia cells strictly relies on the presence of an aberrant BTK splice variant, the isoform BTK^{p52}. Acting as a linker, BTK^{p52} facilitates the activation of full-length BTK by BCR-ABL1 and subsequently the activation of downstream targets of BTK (see 2.4). Constitutive activation of BTK mimics a constitutively active pre-B cell receptor thereby providing survival signals for the leukemia cells.

3.2 The pre-B cell receptor pathway is a target of malignant transformation of pre-B cells by BCR-ABL1

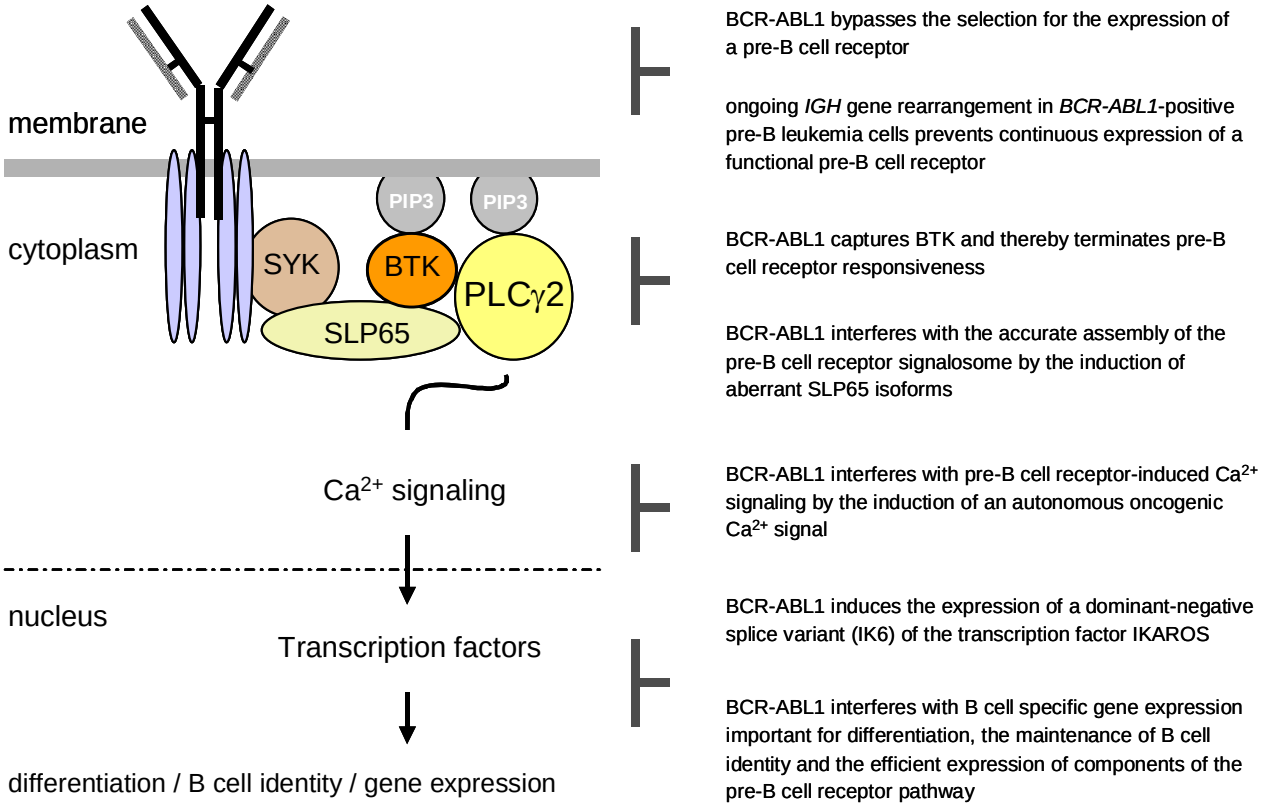
In normal pre-B cells, signal transduction through the pre-B cell receptor initiates the transition from the pre-B to immature B cell stage by a combination of signals leading to survival, proliferation and differentiation (Hess et al., 2001). In contrast, *BCR-ABL1*-positive leukemia cells typically exhibit an enhanced capacity to proliferate and to maintain survival in the absence of developmental progression.

The findings presented in this thesis identify the pre-B cell receptor pathway as a target for malignant transformation of *BCR-ABL1*-positive pre-B cell leukemia cells: Interference with the normal pre-B cell receptor signal and oncogenic activation of essential components of the pre-B cell receptor signal transduction cascade results in independence from regulation by the pre-B cell receptor. BCR-ABL1 interferes with pre-B cell receptor signal transduction at nearly all levels of the signaling cascade (Figure 1): At the cell surface, the expression of the pre-B cell receptor itself is prevented, as ongoing recombination activity within the *IGH* locus renders previously productive V_H-DJ_H-joints non-functional. The relief of selection pressure for the expression of a functional pre-B cell receptor (see 2.1) enables the leukemia cells to survive even in the absence of a functional µ-heavy chain. Within the proximal signaling cascade, uncoupling of BTK from the pre-B cell receptor complex by BCR-ABL1 interferes with pre-B cell receptor signaling by the disruption of the signaling

cascade (see 2.4). Interestingly, also the SRC-kinases LYN, HCK and FGR were collectively identified as a target of BCR-ABL1 (Hu et al., 2004). BCR-ABL1-induced expression of non-functional isoforms of the adapter molecule SLP65 also interferes with an efficient assembly of the pre-B cell receptor signalosome (see 2.1). Aberrant expression of SLP65 also leads to ongoing *IGH* V region gene rearrangement (Sprangers et al., 2005), which may render a previously productively rearranged V_HDJ_H-joint non-functional. Within the nucleus, BCR-ABL1-induces deranged expression of the transcription factor IKAROS (see 2.2), which is consistent with global silencing of genes important for the maintenance of B cell identity. As a result, the transformed pre-B cells exhibit a developmental arrest and become independent from regulation through the pre-B cell receptor. Oncogenic activation of BTK independently from the pre-B cell receptor generates a signal which is comparable to constitutive pre-B cell receptor signaling but results selectively in autonomous proliferation and survival of the leukemia cells.

In summary, aberrant splicing in BCR-ABL1-induced leukemia may interfere with membrane-associated pre-B cell receptor signaling and allow BCR-ABL1-induced oncogenic activation of components of the pre-B cell receptor signal transduction cascade (Figure 1).

Figure 1: *Targeting at various levels of the pre-B cell receptor pathway by the BCR-ABL1 kinase*



3.3 Tumor suppressor or oncogenic function of the pre-B cell receptor pathway

Recently, an important component of the pre-B cell receptor cascade, SLP65, was described to function as a tumor suppressor in mice to limit proliferation of murine pre-B cells and suppress leukemogenesis (Flemming et al., 2003). Remarkably, $SLP65^{-/-}BTK^{-/-}$ double-deficient mice exhibit a dramatic increase of leukemia-development: While about 6% of $SLP65^{-/-}$ single-deficient mice develop leukemia, this is the case in about 75% of $SLP65^{-/-}$ $BTK^{-/-}$ double-deficient mice (Kersseboom et al., 2003). In both mice strains, the proliferating cells exhibited a differentiation arrest at the pre-B cell receptor-positive stage. The proliferation capacity of the tumor cells was dependent on the expression of a pre-B cell receptor and linked to deranged assembly of the pre-B cell receptor signalosome. Of note, both molecules, SLP65 and BTK, exhibit linker function and, as demonstrated for BTK, loss of linker function is the pivotal cause for the development of tumors in SLP65/BTK-double deficient mice (Middendorp et al., 2005). However, as the majority of SLP65-deficient mice did not develop tumors and even in SLP65/BTK-double deficient mice, leukemia did not arise in all instances (75% of mice developed tumors; Kersseboom et al., 2003), other linker molecules likely have residual capacity to prevent tumor development. These could be adapter molecules like LAT and SLP76, which, indeed in the case of LAT in SLP65/LAT double-deficient mice, also exhibit the capacity to limit uncontrolled pre-B cell expansion (Su et al., 2004). Therefore, it seems that uncontrolled pre-B cell receptor signaling caused by deficiency of adapter proteins, may lead to enhanced pre-B cell expansion and the development of leukemia. Likewise, oncogenic activation of BTK by BCR-ABL1 represents an equivalent scenario of uncontrolled constitutive pre-B cell receptor signaling independent of the activity of adapter molecules. Moreover, constitutively active variants of BLK and SYK, which result in an deregulated pre-B cell receptor signal, enhance proliferation of early B cells which overrides developmental regulation: Expression of active BLK in mice lacking a functional pre-BCR induced proliferation and relieved the selection for productive Ig heavy chain rearrangement (Tretter et al., 2003). In addition, a constitutively active TEL-SYK fusion gene has the capacity transform the IL-3-dependent murine pro-B cell line BAF3 to be growth factor independent (Kanie et al., 2004).

However, a comprehensive analysis of the configuration of the *IGH* locus in 317 cases of B cell precursor leukemia revealed that in only 21% of cases the leukemia cells carried a productive *IGH* V region gene rearrangement compatible with the expression of a pre-B cell

receptor (Li et al., 2004). This is even below the statistical probability of 33% to generate a functional rearrangement in the complete absence of selection. Loss of pre-B cell receptor expression on the majority of leukemia cells suggests that the expression of a pre-B cell receptor as such, despite its oncogenic potential, does not confer a survival advantage in leukemia cells. Furthermore, loss of the pre-B cell receptor may provide independence of regulation by the pre-B cell receptor pathway, which may also induce apoptosis or differentiation rather than proliferation and apoptosis-resistance. Indeed, the normal pre-B cell receptor signal can also act as a tumor suppressor, as reconstitution of the deranged pre-B cell receptor signal in SLP65-single or SLP65/BTK-double deficient mice can limit uncontrolled proliferation of pre-B cells (Flemming et al., 2003; Middendorp et al., 2005) and in the majority of B lineage leukemia cells pre-B cell receptor expression and signal transduction is impeded. However, abrogation of pre-B cell receptor signaling, for example by deletion of the µ-heavy chain, does not lead to enhanced proliferation or tumor development (Hess et al., 2001).

The pre-B cell receptor pathway therefore appears ambiguous with respect to leukemogenesis: The pre-B cell receptor limits uncontrolled pre-B cell expansion thereby acting as a tumor suppressor. In contrast, the pre-B cell receptor also induces pre-B cell proliferation thereby acting as an oncogene.

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Chapter 4

Summary

4.1 Summary

The expression of a pre-B cell receptor on the surface of pre-B cells represents a critical checkpoint during early B cell development. While pre-B cells expressing a functional pre-B cell receptor are selected to proliferate and initiate further differentiation, they undergo apoptosis in the absence of pre-B cell receptor-mediated survival signals. The regulation by the pre-B cell receptor is of critical importance to selectively induce the proliferation of further differentiating cells but also to prevent uncontrolled pre-B cell expansion. Bruton's tyrosine kinase (BTK) is an essential component of the proximal signal transduction cascade of the pre-B cell receptor.

BCR-ABL1 represents an oncogenic tyrosine kinase sufficient to transform pre-B cells by the induction of an oncogenic survival and proliferation signal. In order to investigate if *BCR-ABL1*-transformed pre-B cell leukemia cells are still selected for the expression of a pre-B cell receptor, the capacity of the leukemia cells to express a functional pre-B cell receptor was examined. Surprisingly, in the vast majority of cases, the leukemia cells were unable to express a pre-B cell receptor, which would otherwise be a requirement for survival of normal pre-B cells. Studying how the *BCR-ABL1*-mediated oncogenic signal helps the pre-B leukemia cells to escape apoptosis, BCR-ABL1-dependent signal transduction was investigated. The leukemia cells were identified to capture and constitutively activate BTK by the oncogenic BCR-ABL1 kinase. Uncoupling of BTK from the pre-B cell receptor thereby prevents the capacity of the pre-B cell receptor to limit pre-B cell expansion, but induces an aberrant and oncogenic mimicry of the pre-B cell receptor signal leading to uncontrolled proliferation and survival of the leukemia cells. In addition, the oncogenic signaling by the BCR-ABL1 kinase interferes with differentiation of the leukemia cells, which are typically arrested at the pre-B cell stage of development. Interference with differentiation further maintains the autonomous proliferation capacity of the leukemia cells.

The oncogenic pre-B cell receptor signal in the leukemia cells also induces the expression of a B cell-specific mutator enzyme, termed AID, which targets Ig and non-Ig genes in the leukemia cells. The accumulation of mutations might contribute to the frequent development of therapy-resistance, which notoriously develops in BCR-ABL1-induced pre-B cell leukemia.

In summary, the BCR-ABL1 kinase induces an oncogenic mimicry of a constitutively active pre-B cell receptor, which drives uncontrolled pre-B cell expansion and maintains the leukemia cells in an undifferentiated state. Furthermore, the induction of a mutator phenotype in the leukemia cells may lead to the development of therapy-resistance.

4.2 Zusammenfassung

Die Expression des Prä-B Zell Rezeptors auf der Oberfläche von Prä-B Zellen stellt einen zentralen Kontrollpunkt während der frühen B Zell Entwicklung im Menschen dar. Während die Expression des Prä-B Zell Rezeptors in Prä-B Zellen Proliferation und Differenzierung auslöst, erleiden Prä-B Zellen bei Abwesenheit von Prä-B Zell Rezeptor-abhängigen Überlebenssignalen Apoptose. Die Regulation über den Prä-B Zell Rezeptor garantiert daher die gezielte Expansion von differenzierenden Prä-B Zellen und verhindert unkontrollierte Proliferation. Bruton's Tyrosin Kinase (BTK) ist ein essentieller Bestandteil der proximalen Signaltransduktion des Prä-B Zell Rezeptors.

BCR-ABL1 ist eine onkogene Tyrosin-Kinase, die für die Entstehung eines autonomen Überlebens- und Proliferationssignals in Prä-B Leukämiezellen notwendig und ausreichend ist. Um zu klären, ob BCR-ABL1-transformierte Prä-B Zellen auf die Expression eines Prä-B Zell Rezeptors angewiesen sind, wurde untersucht, ob die Leukämiezellen die Fähigkeit besitzen, einen Prä-B Zell Rezeptor zu exprimieren. Überraschender Weise war die Mehrheit der getesteten Leukämien nicht in der Lage, einen Prä-B Zell Rezeptor zu exprimieren, obwohl dieser für das Überleben von normalen Prä-B Zellen notwendig ist. Um zu klären, wie BCR-ABL1-transformierte Prä-B Zellen Apoptose bei Abwesenheit von Prä-B Zell Rezeptor-vermittelten Überlebenssignalen verhindern, wurden die von BCR-ABL1 ausgehenden onkogenen Signale untersucht. Es konnte festgestellt werden, daß in den Leukämiezellen BTK gezielt von BCR-ABL1 aktiviert wird. Die Vereinnahmung von BTK durch BCR-ABL1 verhindert die Kontrolle der Prä-B Zell Expansion durch den Prä-B Zell Rezeptor und induziert zudem ein Mimikry des Prä-B Zell Rezeptor Signals, welches die Proliferation und das Überleben der Leukämiezellen ermöglicht. Dieses von BCR-ABL1-ausgehende onkogene Signal verhindert die weitere Differenzierung der transformierten Prä-B Zellen und sichert damit die autonome Proliferation der Leukämiezellen.

Über das onkogene Prä-B Zell Rezeptor-ähnliche Signal wird außerdem die Expression des B Zell-spezifischen Mutator-Enzyms AID induziert. AID Aktivität innerhalb und außerhalb von Immunglobulingenen führt zu einer Ansammlung von Mutationen, die vermutlich zur Entwicklung von Therapieresistenz beitragen, die typischerweise bei der Behandlung von BCR-ABL1-induzierten Prä-B Zell Leukämien auftritt.

Es konnte gezeigt werden, daß die BCR-ABL1 Kinase das onkogene Mimikry eines konstitutiv-aktiven Prä-B Zell Rezeptors induziert. Dieses Signal führt zur unkontrollierten Vermehrung der Prä-B Leukämiezellen und sichert deren Überleben und Unabhängigkeit von Differenzierungsvorgängen. Die Expression von AID verursacht die Ansammlung von Mutationen und unterstützt somit vermutlich die Entwicklung von Therapieresistenz.

Chapter 5

Published results

5.1 The BCR-ABL1 kinase bypasses selection for the expression of a pre-B cell receptor in pre-B acute lymphoblastic leukemia cells

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Summary

The BCR-ABL1 kinase expressed in acute lymphoblastic leukemia (ALL) drives malignant transformation of human pre-B cells. Comparing genome-wide gene expression profiles of *BCR-ABL1*$^+$ pre-B ALL and normal bone marrow pre-B cells by SAGE, many genes involved in pre-B cell receptor signaling are silenced in the leukemia cells. While normal pre-B cells are selected for the expression of a functional pre-B cell receptor, *BCR-ABL1*$^+$ ALL cells mostly do not harbor a productively rearranged *IGH* allele. In these cases, we identified traces of secondary V_H gene rearrangements, which may have rendered an initially productive V_H region gene nonfunctional. Even *BCR-ABL1*$^+$ ALL cells harboring a functional V_H region gene are unresponsive to pre-B cell receptor engagement and exhibit autonomous oscillatory Ca^{2+} signaling activity. Conversely, leukemia subclones surviving inhibition of BCR-ABL1 by STI571 restore responsiveness to antigen receptor engagement and differentiate into immature B cells expressing Ig light chains. BCR-ABL1 kinase activity is linked to defective pre-B cell receptor signaling and the expression of a truncated isoform of the pre-B cell receptor-associated linker molecule *SLP65*. Also in primary leukemia cells, truncated *SLP65* is expressed before but not after treatment of the patients with STI571. We conclude that inhibition of BCR-ABL1 reconstitutes selection for leukemia cells expressing a functional (pre-) B cell receptor.

Introduction

A number of transcription factors, including *PAX5*, *E2A* and *EBF*, guide commitment of hematopoietic stem cells to the B cell lineage (1). Committed B cell precursors then undergo a sequence of immunoglobulin (Ig) gene rearrangements defining distinct stages of early B cell development (2). During their early development within the bone marrow, B cell precursors have to pass checkpoints, at which only cells carrying functional Ig gene rearrangements are selected for further development along the B cell lineage (3). For instance, the presence of a productive *IGH* gene rearrangement is a prerequisite for the expression of the Ig μ heavy chain as a component of the pre-B cell receptor on the surface of a pre-B cell (4). If these cells initially fail to express a functional pre-B cell receptor on their surface because of a nonproductive *IGH* gene rearrangement on one allele, they can continue to rearrange Ig V region genes on the second *IGH* allele or undergo secondary V_H gene recombination on the nonproductively rearranged allele (5). Pre-B cells are destined to die by apoptosis unless they are rescued by survival signals through their pre-B cell receptor. The pre-B cell receptor complex including the Ig μ heavy chain, the surrogate light chain composed of λ5 and VpreB, CD19 and the Igα and Igβ signal chains does not only convey survival signals but also terminates the rearrangement process within the *IGH* locus. A critical component in the pre-B cell receptor signaling cascade is the adapter molecule SLP65, which links SYK to downstream effector pathways including PLCγ2, BTK and VAV (6). While somatic *SLP65*-deficiency is a frequent aberration in pre-B ALL in humans (7), *SLP65*$^{-/-}$ mutant mice exhibit a differentiation block at the pre-B cell stage (8), autonomous proliferation, ongoing rearrangement of *IGH* V region genes and development of leukemia (9).

In most cases of acute lymphoblastic leukemia (ALL), pre-B cells represent the normal counterpart of the malignant clone, which in many cases carries specific oncogenic gene rearrangements defining both biological and clinical subentities (10). Among these translocation events, the t(9;22) (q34;q11) results in a fusion of the *BCR* and *ABL1* genes (11), which codes for a potent tyrosine kinase and represents the most frequent recurrent genetic aberration leading to ALL in adults (12). Comparing pre-B ALL carrying a *BCR-ABL1* gene rearrangement to pre-B ALL harboring other translocations, recent studies using the cDNA microarray technique identified differentially expressed genes predicting a *BCR-ABL1* fusion. Aiming at molecular classification of leukemia, these studies searched for differentially expressed genes discriminating between various subentities of ALL (13, 14). In an alternative approach using the SAGE technique, we compared genome-wide gene expression profiles of normal hematopoietic bone marrow populations including pre-B cells,

hematopoietic progenitor cells, myeloid progenitor cells, T lymphoid precursors and *BCR-ABL1*$^+$ pre-B ALL, which are thought to represent the malignant outgrowth of pre-B cells (12). The leukemia cells were also compared to mature B cell populations including naïve B cells, germinal center B cells, memory B cells and plasma cells. These comparisons were meant i.) to identify novel target genes of BCR-ABL1-mediated transformation and ii.) to elucidate how the oncogenic BCR-ABL1 kinase may interfere with normal pre-B cell receptor signaling.

Results and Discussion

Pre-B cell receptor-associated signaling molecules are silenced in BCR-ABL1$^+$ *pre-B ALL cells*

To identify target genes of the oncogenic BCR-ABL1 kinase, we compared genome-wide gene expression profiles of two cases of *BCR-ABL1*$^+$ pre-B ALL and normal hematopoietic populations using the serial analysis of gene expression (SAGE) technique. For the SAGE-analysis of normal hematopoietic populations, a total of 592,000 SAGE-tags have been collected, including CD10$^+$ CD19$^+$ human bone marrow pre-B cells as the normal precursors of pre-B ALL, CD7$^+$ CD10$^+$ T lymphoid progenitor cells (TLP), CD15$^+$ common myeloid progenitor cells (CMP), CD34$^+$ CD38^{low} hematopoietic progenitor cells (HSC), CD19$^+$ CD27$^-$ naïve (NBC), CD20$^+$ CD77$^+$ germinal center (GCB) and CD19$^+$ CD27$^+$ memory B cells (MBC) and CD19$^+$ CD138$^+$ plasma cells (PC; Figure 1). Comparing the gene expression pattern in *BCR-ABL1*$^+$ pre-B ALL and normal human pre-B cells, many genes conferring B cell lineage commitment and signal transduction through the pre-B cell receptor were transcriptionally silenced in the leukemic cells (Figures 1 and 2). Loss of pre-B cell receptor-related molecules in the pre-B ALL cells involves nuclear transcription factors (*OBF1, PAX5, E2A, OCT2, EBF* and *IRF4*; see Figure 2), cytoplasmic kinases and linker molecules (*LYN, BLK, BTK, BRAG, SLP65, SYK, BAP37, Igα BP1, BRDG1, PLCγ2, VAV1-3, HPK1, LCK, FYN, BAM32, AKT, SHC1, SAP, p62DOK, CIN85, NIK* and *IKK*; Figure 1) and membrane associated receptor molecules (*CD19, IGHμ, VpreB, Igβ, Igα*; Figure 1). Conversely, transcription factors related to primitive hematopoiesis including *AML1* and *GATA1* are upregulated in the leukemia cells as compared to their normal counterpart (see Figure 2). Also, signaling molecules related to *NF-κB, JAK-STAT, GAB2* and *GRB2* pathways are expressed in the leukemia cells at similar or higher levels than in pre-B cells (see bottom part of Figure 1).

Figure 1: *Silencing of pre-B cell receptor-related molecules in BCR-ABL1^{+} pre-B ALL*

HSC	CMP	TLP	ALL II	ALL IX	pre-B	NBC	GCB	MBC	PC	Name	Proposed function	Reference
1		1	304	408	3299	184	497	628	324	*CD19*	co-ligation enhances BCR signals	Carter & Fearon, 1992
7	10	8	48	144	865	144	334	451	425	*IGHCμ*	IGH μ chain	Raff et al., 1976
		1	4	12	278					*VpreB*	surrogate light chain	Kudo & Melchers, 1987
1		19	4		125					*RAG2*	V(D)J recombination	Schatz et al., 1989
4	22	23	4	8	99	51	21	13	41	*LYN*	attentuates BCR signaling	Yamanashi et al., 1991
1		1			101		17	13	16	*BLK*	recruits GRB2	Aoki et al., 1994
3	17	109	14	31	125	8	27	17	12	*NFAT*	activated by Vav2 upon BCR crosslinking	Doody et al., 2002
			8	4	62	8	73	58	68	*Igα*	ITAM	Flaswinkel et al., 1995
5		16	4	1	83					*RAG1*	V(D)J recombination	Menetski & Gellert, 1990
1		38	49	44	155	4	3			*TdT*	generation of junctional diversity	Kung et al., 1975
3	1	1	4		71	4	17	27	8	*BTK*	PLCγ activation upon BCR crosslinking	Cheng et al., 1994
1			8		77		11	13	4	*BRAG*	enhances survival signals through BCR	Verkoczy et al., 2000
1	1		4	8	73	4	17	33	8	*SLP65*	links Syk to PLCγ2, GRB2, Vav and NCK	Fu et al., 1998
3	22	13	4	8	65	8	41	44	4	*SYK*	promotes BCR signaling	Kurosaki et al., 1994
2	74	51	8	8	45	8	115	76	36	*CD45*	antagonizes SHP1, CSK	Pani et al., 1997
3	1		118	81	175	46	166	121	81	*Igβ*	ITAM	Cassard et al., 1996
21	2	4	16	16	76		11	21	16	*BAP37*	IgM specific transmembrane signaling	Terashima et al., 1994
2	15	42	31	12	79	4	11	21	32	*RHO p115*	actin polymerisation upon BCR ligation	Moores et al., 2000
1	8	1	19	8	61		11	37	4	*IgαBP1*	signal transduction through Igα	Grupp et al., 1995
1				4	38		7	7		*BRDG1*	BCR-dependent activation of CREBP	Ohya et al., 1999
3	7		12		33	4	41	23	12	*PLCγ2*	part of downstream BCR signaling complex	Okazaki et al., 2001
	3				15	12	12	21	44	*VAV2*	BCR signals synergistic with CD19	Doody et al., 2000
2	4	16	4		15	12	34	53	8	*HPK1*	augments BCR signaling through BLNK	Tsuji et al., 2001
	2	2			16		7	17	7	*PI3K p110*	activates BTK	Okkenhaug et al., 2002
1	2	72			13		21	11	10	*LCK*	activated upon BCR ligation	Gold et al., 1994
1	7			8	22		11	11	4	*FYN*	reduction of BCR signaling in FYN$^{-/-}$ mice	Appleby et al., 1995
1	5		4	4	12	4	34	21	16	*WIP*	cytosceleton-associated activation signals	Anton et al., 2000
1	2	9			9		11	17	12	*BAM32*	higher Ca^{2+} upon BCR crosslinking	Niiro et al., 2002
1					12	4	7	7	4	*AKT*	promotes PI3K signals upon BCR ligation	Astoul et al., 1999
2	1				11		5	9	4	*SHC1*	Ras-activation upon BCR-crosslinking	Saxton et al., 1994
7	15	181	164	104	231	8	3	37	16	*NF-κB2*	reduction of BCR-signal threshold	Claudio et al., 2002
	1				6		7	13	8	*SAP*	recruitment of SHIP or SHP2 to SLAM	Shlapatska et al., 2001
7	3	6			4	16			4	*p62DOK*	mediates ITIM-dependent BCR-inhibition	Tamir et al., 2000
2	4						17	13	4	*CIN85*	Inhibition of CBL	Beckwith et al., 1996
	6		4		6		3	7	4	*VAV1*	B cell activation through BCR	Moores et al., 2000
13	74	25	19	12	11	31	24	24	56	*IκBα*	inhibits NF-κB activation upon BCR ligation	Petro et al., 2000
1	1	1					11	11		*SHP2*	links BCR to GRB2, activates RAS	Nakamura et al., 1998
6	1	1				18				*CBL*	recruits CBL	Wakioka et al., 1999
	25	32				16				*SIRPα1*	SHP1 substrate	Cambier, 1997
1	1	1					13			*SIT*	inhibition of BCR by ITIM-dependent signals	Marie-Cardine et al., 1999
1	1	19	4	4	6			7	8	*SLP76*	Igα-downstream signaling in early B cells	Nagata et al., 1997
1	1	1					3		4	*NIK*	activates NF-κB by IκBα degradation	Claudio et al., 2002
			4	4	4		3	7	8	*VAV3*	activation of PI3K, increased Ca^{2+} flux	Inabe et al., 2002
	13	61	34	16	33		17	17	30	*IKK*	degradates IκBα upon activation by BTK	Petro et al., 2000
2	4		4	4			7	13		*GAB1*	amplifies BCR signaling via PI3 kinase	Ingham et al., 2001
			12	12	12	4	7	21	8	*REL*	protects from BCR-mediated apoptosis	Chen et al., 2001
10	23	26	27	16	19	58	7	7	24	*SHIP*	ITIM-dependent inhibition of BCR-signals	Ono et al., 1997
15			8	4		19	3			*APS*	recruits CBL	Wakioka et al., 1999
1	27	45	31	44	24		47	33	32	*GRB2*	interacts with SHC, activates Ras	Smit et al., 1994
9	9	11	22	40	7	70	3	9	52	*CSK*	inhibits BCR-signals together with p62DOK	Vuica et al., 1997
8	31	15	42	16	7	36	11	7	25	*SHP1*	antagonizes CD45	Pani et al., 1997
6	6		33	51	23	4	28	44	4	*NF-κB*	BCR-induced proliferation	Petro et al., 2000
3	2		32	48	23		3	13		*GAB2*	substrate of SHP2	Nishida et al., 1999
1			38	61	18		3	17		*JAK3*	required for pre-BCR signal transduction	Thomis et al., 1995

Legend to Figure 1:

Using the SAGE method, ten genome-wide gene expression profiles have been generated for $CD34^+$ $CD38^{low}$ hematopoietic progenitor cells (HSC), $CD15^+$ common myeloid progenitor cells (CMP), $CD7^+$ $CD10^+$ T cell lineage precursors (TLP), $CD10^+$ $CD19^+$ $Ig\mu^+$ pre-B cells (pre-B), $CD19^+$ $CD27^-$ naïve B cells (NBC), $CD20^+$ $CD77^+$ germinal center B cells (GCB), $CD19^+$ $CD27^+$ memory B cells (MBC), $CD19^+$ $CD138^+$ plasma cells (PC) and compared with two cases of pre-B ALL carrying a *BCR-ABL1* gene rearrangement (ALL, case II and IX). SAGE-tag counts are given as numbers and depicted in colors, with red indicating high and green low levels or no expression. SAGE data were sorted according to the ratio of SAGE-tag counts for pre-B cells and SAGE-tag counts in *BCR-ABL1*$^+$ ALL cases. In addition to gene names, annotations of proposed gene functions (including a reference) are given.

However, NF-κB activation (23) and GAB2/ GRB2 phosphorylation (24, 25) together with expression of JAK and STAT proteins (26) reflect oncogenic BCR-ABL1 kinase activity or requirements for transformation by BCR-ABL1 rather than pre-B cell receptor signaling in the leukemia cells.

BCR-ABL1$^+$ *pre-B ALL cells carry nonproductive* V_H *gene rearrangements in most cases*

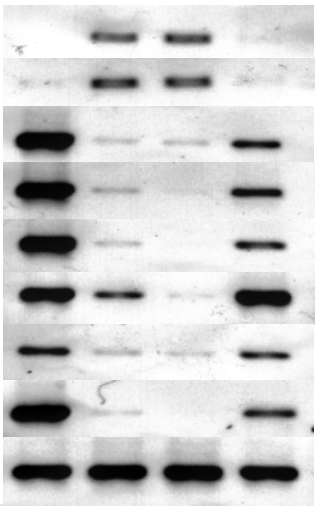
These findings suggest that *BCR-ABL1*$^+$ pre-B ALL cells as opposed to normal bone marrow pre-B cells do not express an active pre-B cell receptor. Therefore, we investigated whether the genomic configuration of the *IGH* loci in *BCR-ABL1*$^+$ pre-B ALL cells is compatible with the expression of a functional pre-B cell receptor. Only three out of 12 *BCR-ABL1*$^+$ pre-B ALL cases harbored a potentially functional *IGH* gene rearrangement (Table II). In the remaining nine cases, the coding capacity of *IGH* alleles was compromised by loss of the reading frame or generation of stop codons within the junction between V, D and J segments during the rearrangement process. To formally exclude that in these cases a productive V_H-D_HJ_H rearrangement on the second allele might have been missed during the PCR amplification, we also amplified the second *IGH* allele using primers specific for *IGH* germline configuration or D_H-J_H rearrangements (Table II). In one case (case IV, Table II), two *IGH VDJ* rearrangements were amplified, both of which were nonfunctional.

Hence, the majority of pre-B ALL clones carrying the oncogenic *BCR-ABL1* gene rearrangement can survive and even clonally expand in the absence of pre-B cell receptor survival signals. We conclude that *BCR-ABL1*$^+$ leukemia cells can bypass selection for the expression of a pre-B cell receptor.

However, since clonal V_H gene rearrangements were amplified in a single round of PCR amplification at a relatively low sensitivity (detection limit of about 10^4 clonal cells in 5 x 10^6 total cells), we cannot exclude that small subclones carrying a productive *IGH* allele were among the leukemia cells. Of note, the two leukemia cases analyzed by SAGE also

differred with respect to the coding capacity of their *IGH* alleles. However, irrespective of whether the leukemia cells harbored a potentially productive *IGH* allele or not, mRNA expression of pre-B cell receptor-related signaling molecules was invariably downregulated or missing in both cases (Figure 1).

Figure 2: *Inhibition of BCR-ABL1 in pre-B ALL cells can restore expression of B cell-specific transcription factors*

Pre-B	*BCR-ABL1*$^+$ pre-B ALL				SAGE-tag counts (per 10^5 total tags)			
–	–	+	+	STI571				
CD10$^+$ CD19$^+$	–	μ^-	μ^+	MACS	pre-B	ALL II	ALL IX	UniGene
				*AML1*	6	57	40	129914
				GATA1	1	8	11	765
				E2A	164	12	12	101047
				EBF	178			185708
				OBF1	118	8	4	2407
				OCT2	48			1101
				IRF4	18		4	82132
				PAX5	66	4	4	22030
				GAPDH	311	503	541	169476

SAGE-tag counts of hematopoietic stem cell-related transcription factors AML1 and GATA1 and B cell-specific transcription factors E2A, EBF, OBF1, OCT2, IRF4 and PAX5 are compared between normal pre-B cells and two cases of *BCR-ABL1*$^+$ pre-B ALL (cases II and IX). Differential expression of these transcription factors in pre-B as compared to leukemia cells was verified by semiquantitative RT-PCR using cDNAs from normal pre-B cells and *BCR-ABL1*$^+$ SUP-B15 cells. Inhibition of BCR-ABL1 by STI571 could restore a pre-B cell-like expression pattern of these transcription factors in Igμ$^+$ but not in Igμ$^-$ leukemia cells. Igμ$^+$ and Igμ$^-$ leukemia cells were separated by MACS.

Inhibition of BCR-ABL1 kinase activity reconstitutes selection of pre-B ALL cells expressing Ig μ-chains at high levels

To determine whether the BCR-ABL1 kinase has an effect on the expression of the pre-B cell receptor, we measured expression levels of surface Ig μ heavy chain on three *BCR-ABL1*$^+$ pre-B ALL cell lines (SUP-B15, BV173 and NALM1) in the presence and absence of STI571, a specific inhibitor of BCR-ABL1 kinase activity. CD19$^+$ bone marrow B lineage cells exhibit surface Ig μ heavy chain expression at variable levels (Figure 3A). In contrast, Ig μ heavy

chain protein was detectable only at very low levels on the surface of SUP-B15 cells (Figure 3B) and NALM1 cells (not shown). On BV173 cells, Ig μ heavy chains were detected neither on the cell membrane nor in the cytoplasm (not shown), which is consistent with the absence of a productive *IGH* allele in these cells (case XI, Table II). However, after 19 hours of STI571-treatment, a small subset of about 3% of the leukemia cells expressed surface Ig μ heavy chain at high levels (Figure 3C). Only few ALL cells survived continued STI571-treatment, which further increased the fraction of Ig μ^{high} cells to 23% after four days (Figure 3F) and 48% after six days (Figure 3D). In STI571-treated leukemia cells, expression of Ig μ heavy chain surface correlated with viability of the cells (i.e. lack of both annexin V membrane expression and uptake of propidium iodide; Figure 3F). Conversely, almost no Ig μ heavy chain protein could be detected on the surface of cells undergoing apoptosis induced by STI571 (Figure 3F). During six days of STI571-treatment more than 95% of all leukemia cells died by apoptosis. Thus, the relative gain of Ig μ^{high} cells can be fully attributed to selective deletion of Ig μ^{-} leukemia cells. We conclude that ablation of BCR-ABL1 kinase activity by STI571 induces selection of leukemia subclones expressing high levels of μ-chains on their surface.

Table I: *Clinical data on BCR-ABL1^{+} pre-B ALL cases studied*

Case	Age	Sex	*BCR-ABL1* Fusion	Clinical course
I	34	m	p190	Complete remission after BMT
II	32	m	p190	Died after BMT
III	76	m	p190	Chemotherapy, died three weeks after onset of therapy
IV	30	m	p190	Complete remission after BMT, currently GVHD
V	66	f	p190	Complete remission after chemotherapy
VI	10	f	p190	Died after BMT
VII	56	m	p190	Chemotherapy, currently in remission
VIII	10	f	p190	Died after BMT, GVHD
IX	16	m	p210	Complete remission after BMT
X	9	m	p190	Died after relapse (SUP-B15 cell line)
XI	45	m	p210	Died after relapse (BV173 cell line)
XII	3	f	p210	Died after relapse (NALM1 cell line)
XIII	39	m	p210	Relapse after STI571 treatment
XIV	54	f	p190	Relapse after STI571 treatment
XV	61	f	p190	Relapse after STI571 treatment
XVI	51	m	p190	Relapse after STI571 treatment
XVII	N.N.	N.N.	p190	Relapse after STI571 treatment
XVIII	65	m	p190	Relapse after STI571 treatment
XIX	47	f	p190	Relapse after STI571 treatment

Notes:
m, male; f, female; BMT, bone marrow transplantation; N.N., not known; GVHD, graft versus host disease; Sequencing analysis for *IGH* alleles: cases I to XII; SAGE analysis: cases II and IX; Comparison of *SLP65* isoform expression before and after STI571 treatment: cases XIII to XIX

In STI571-treated patients, expression of μ-chains on few leukemia subclones may confer a survival advantage and, hence, represent an escape mechanism, through which some leukemic cells can temporarily evade STI571-induced apoptosis.

In the absence of BCR-ABL1 kinase activity, the malignant cells would undergo apoptosis unless they successfully reactivate other survival signals by default, e.g. through the (pre-) B cell receptor. After extended STI571-treatment, Ig μ^{high} cells were not only seen in SUP-B15 cells and NALM1 cells, which both carry a functional *IGH* allele (cases X and XII, Table II). Unexpectedly, also BV173 cells, from which we amplified two non-productive *IGH* alleles, gave rise to Ig μ^{high} cells after prolonged exposure to STI571. This could be explained by selective outgrowth of few Ig μhigh subclones of the BV173 cell line that were already existing before STI571-treatment, e.g. as a result of spontaneous secondary *IGH* gene rearrangement. Alternatively, secondary *IGH* gene rearrangements might be induced by STI571-mediated inhibition of BCR-ABL1. Remedy of compromised coding capacity for a functional (pre-) B cell receptor by BCR-ABL1-inhibition in the case of BV173 cells therefore exemplifies that survival signals through the (pre-) B cell receptor would indeed allow leukemia cells to temporarily evade STI571-mediated apoptosis.

BCR-ABL1 kinase activity overrides (pre-) B cell receptor signaling in leukemia cells

To elucidate whether the BCR-ABL1 kinase activity in leukemic pre-B cells can not only bypass selection for but also interfere with (pre-) B cell receptor signaling, we compared antigen receptor responsiveness in human bone marrow pre-B cells ($VpreB^+$) and immature B cells ($Ig\kappa^+$ or $Ig\lambda^+$) and three *BCR-ABL1*$^+$ leukemia cell lines. Two of the three cell lines (SUP-B15 and NALM1, cases X and XII, Table II) indeed carry a productive *IGH* allele and express surface Ig μ-heavy chain at low levels together with VpreB (Figure 4). To this end, we measured Ca^{2+} release in response to antigen receptor engagement by anti-VpreB, anti-κ, anti-λ and anti-μ antibodies in normal pre-B and immature B cells and *BCR-ABL1*$^+$ leukemia cells at the single-cell level. Pre-B cells and immature B cells showed the typical increase of cytoplasmic Ca^{2+} reaching a plateau about one minute after pre-B cell receptor crosslinking (Figure 5A). Comparing engagement of pre-B cell receptors by anti-VpreB and crosslinking of B cell receptors by anti-κ or anti-λ, the Ca^{2+} signal initiated by the pre-B cell receptor had a lower amplitude and was slightly delayed (Figure 5A). In contrast, *BCR-ABL1*$^+$ SUP-B15 leukemia cells did not respond to anti-Ig μ trigger at all even if 5-fold higher antibody concentrations were used. Instead, the leukemia cells displayed a peculiar autonomous oscillatory Ca^{2+} signal activity (Figure 5B).

Table II: Sequence analysis of *IGH* alleles in *BCR-ABL1*$^+$ pre-B ALL cases

Case	Allele	Configuration of *IGH* loci	Coding capacity		Indication for secondary V_H-gene rearrangement
I	#1	V_H4.34-D_H6.13-J_H5	-	out-of-frame	+ (previously rearranged: V_H1.8)
	#2	D_H5-J_H			
II	#1	V_H4.61-D_H2.2-J_H4	-	out-of-frame, 2 stop codons in junction	+ (previously rearranged: V_H1.58)
	#2	D_H2.4-J_H4			
III	#1	V_H3.33-D_H3.22-J_H4	-	out-of-frame	-
	#2	D_H7.27-J_H; germline			
IV	#1	V_H5.51-D_H3.22-J_H4	-	in frame; stop codon in junction	-
	#2	V_H5.51-D_H6.19-J_H3	-	out-of-frame	-
V	#1	V_H5.51-D_H1.26-J_H1	-	in frame; stop codon in junction	+ (previously rearranged: V_H2.5)
	#2	D_H7.27-J_H; germline			
VI	#1	V_H3.30-D_H3.22-J_H3	-	out-of-frame	+ (previously rearranged: V_H1.2)
	#2	D_H5-J_H			
VII	#1	V_H1.46-D_H6.13-J_H4	-	out-of-frame	+ (previously rearranged: V_H2.5)
	#2	D_H5-J_H			
VIII	#1	V_H2.5-D_H3.22-J_H4	-	in frame; 2 stop codons in junction	+ (previously rearranged: V_H2.70)
	#2	D_H7.27-J_H; germline			
IX	#1	V_H1.46-D_H3.10-J_H5	+	in frame	+ (previously rearranged: V_H1.24)
	#2	D_H3.9-J_H6			
X[a]	#1	V_H3.53-D_H2.8-J_H6	+	in frame	+ (previously rearranged: V_H3.38)
	#2	n.d.			
XI[a]	#1	V_H3.48-D_H2.15-J_H3	-	out-of-frame, stop codon in junction	+ (previously rearranged: V_H3.38)
	#2	D_H2-J_H			
XII[a]	#1	V_H3.9-D_H2.21-J_H3	+	in frame	-
	#2	D_H4-J_H			

Note: a: cases X, XI and XII correspond to *BCR-ABL1*$^+$ pre-B ALL cell lines SUP-B15, BV173 and NALM1, respectively.

Autonomous Ca^{2+} oscillations were not only seen in SUP-B15 cells (Figure 5B) but also in *BCR-ABL1*$^{+}$ BV173 and NALM1 leukemia cells (not shown). To test whether autonomous undulatory Ca^{2+} signals are causally linked to BCR-ABL1 kinase activity, we treated *BCR-ABL1*$^{+}$ SUP-B15, BV173 and NALM1 cells with STI571. In the presence of STI571, the leukemia cells did not exhibit any autonomous Ca^{2+} signal activity. Instead, antigen receptor responsiveness was partially restored in STI571-treated leukemia cells. Upon pre-B or B cell receptor engagement by anti-μ chain antibodies, some of the STI571-treated leukemia cells responded by release of Ca^{2+} from cytoplasmic stores while many other leukemia cells did not respond to antigen receptor crosslinking (not shown).

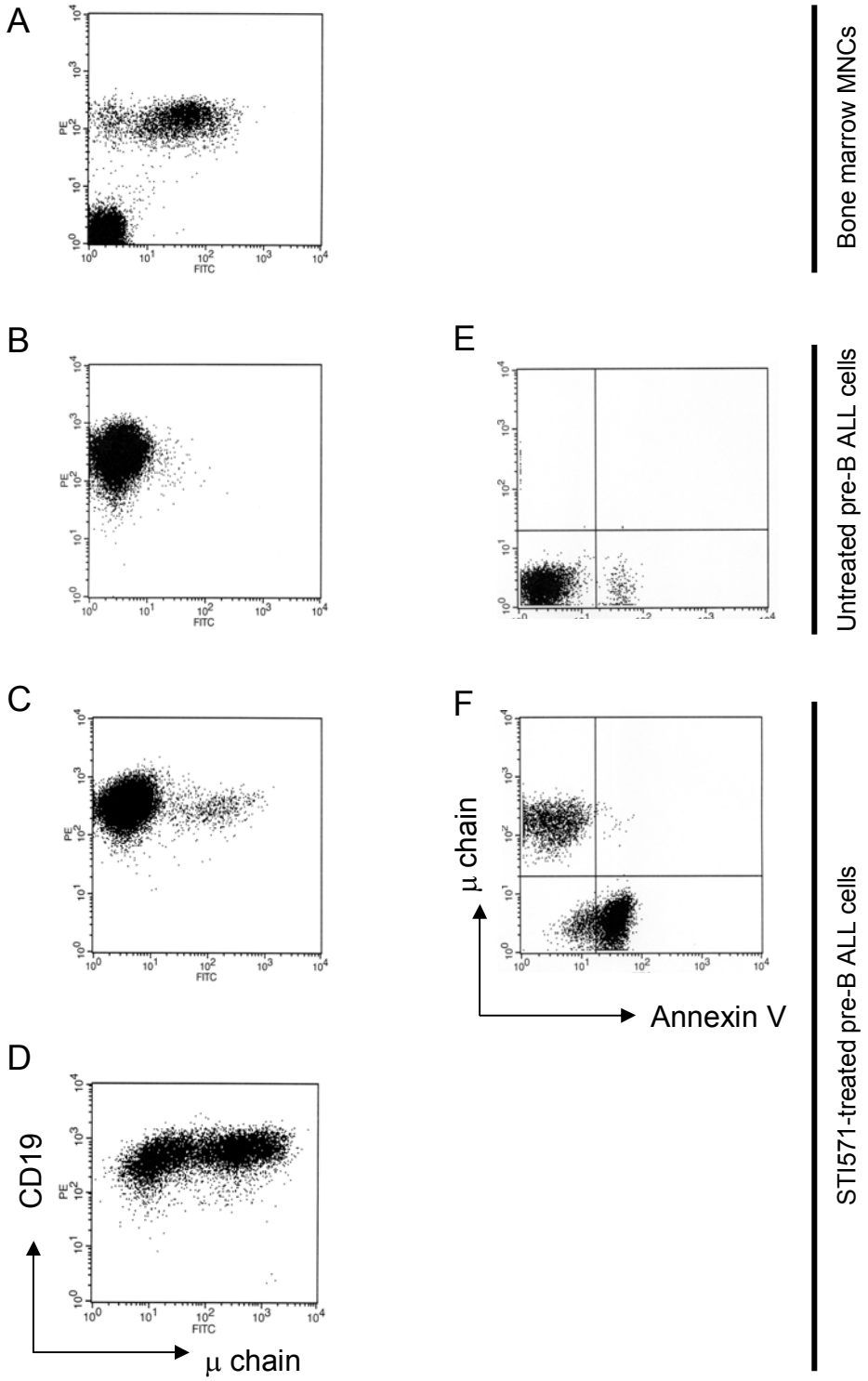
In order to determine whether regained responsiveness of (pre-) B cell receptors was restricted to differentiating Ig μ^{high} cells among the STI571-treated leukemia cells, Ig μ^{high} and Ig μ^{-} cells were separated by MACS after prolonged STI571-treatment. While 48 of 56 Ig μ^{high} STI571-treated cells responded by an oscillatory Ca^{2+} signal, all 45 Ig μ^{-} STI571-treated cells remained silent upon (pre-) B cell receptor engagement (Figure 5C).

We conclude that the BCR-ABL1 kinase establishes an unusual autonomous signaling activity, which overrides signal transduction initiated by the (pre-) B cell receptor. While the kinetics of the Ca^{2+} signal in STI571-treated leukemia cells still clearly differs from the signaling pattern in normal pre-B and immature B cells, inhibition of BCR-ABL1 kinase activity restores responsiveness of the (pre-) B cell receptor in these leukemia cells.

BCR-ABL1^{+} *leukemia cells surviving STI571-treatment differentiate into immature B cells*

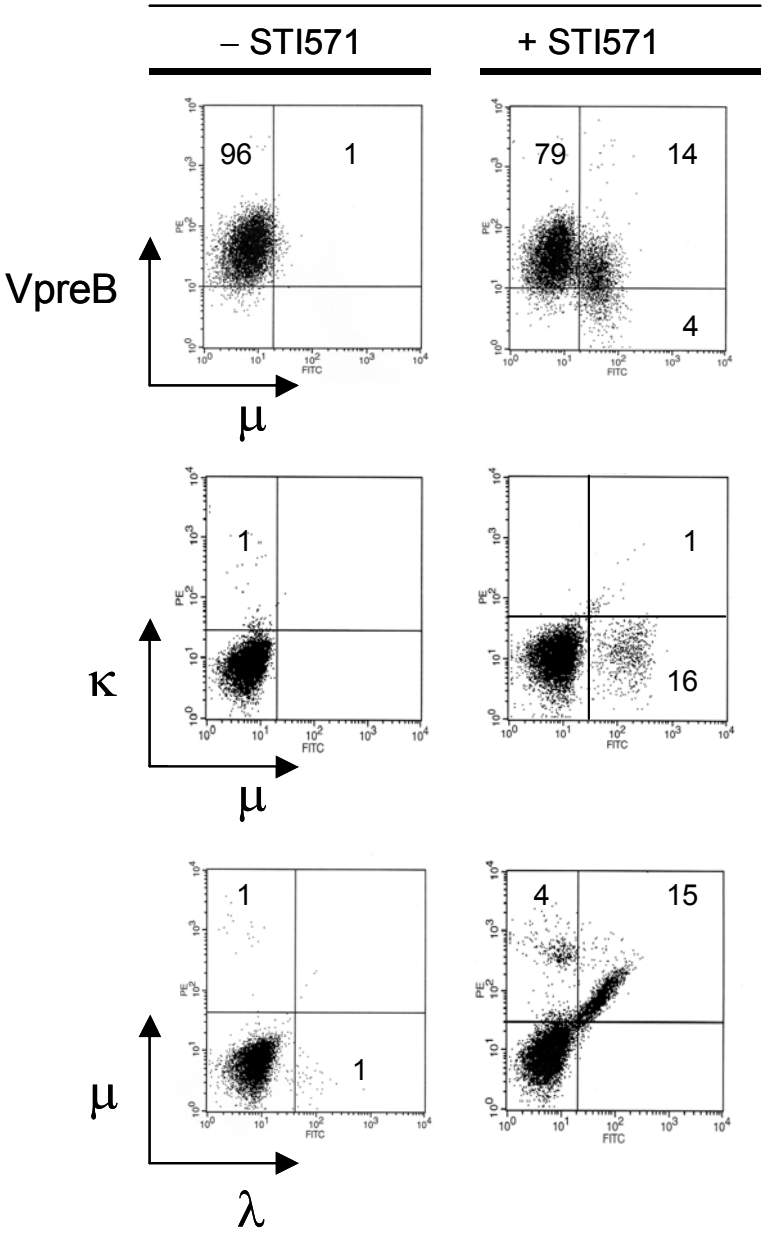
Assuming a pre-B cell origin of the three *BCR-ABL1*$^{+}$ leukemia cell lines analyzed, Ig μ heavy chains on the surface of leukemia cells surviving extended STI571-treatment were expressed at surprisingly high levels (Figure 3D, E), which are characteristic for μ-chain expression in the context of a B cell receptor rather than a pre-B cell receptor (12). We therefore tested whether cells surviving prolonged STI571-treatment were in fact differentiating into immature B cells, which express high levels of surface μ-chain as part of a B cell receptor. As shown in Figure 4, Ig μ^{high} leukemia cells downregulated VpreB as compared to Ig μ^{low} cells (top). Indeed, surrogate light chains on Ig μ^{high} leukemia cells were replaced with conventional light chains (Figure 4, middle and bottom). We conclude that the three *BCR-ABL1*$^{+}$ leukemia cell lines treated with STI571 exhibit preferential outgrowth of subclones differentiating into immature B cells. These findings are in agreement with a recent study on the effect of STI571 on v-abl-mediates differentiation arrest in murine pre-B cells (27).

Figure 3: *Inhibition of BCR-ABL1 induces selection for μ–chain⁺ leukemia cells*



Normal bone marrow mononuclear cells (MNCs) include B lineage cells expressing Ig μ heavy chains at variable levels, depending on their differentiation stage as pro-B cells ($Igμ^-$) pre-B cells ($Igμ^{low}$) or immature B cells ($Igμ^{high}$; A). *BCR-ABL1*⁺ SUP-B15 (B-F), BV173 (not shown) and NALM1 (not shown) pre-B ALL cells were incubated in the absence (B, E) or presence of 10 μmol/l STI571 (C, D, F) for 19 hours (B, C), two (E, F) or six days (D) and measured for surface expression of CD19 (A-D), Annexin V (E, F) and Ig μ chains (A-F). Comparing *BCR-ABL1*⁺ SUP-B15 pre-B ALL cells to BV173 and NALM1 cells, similar results were obtained (not shown).

Figure 4: *Inhibition of BCR-ABL1 in pre-B ALL cells results in selection for subclones differentiating to immature B cells with preferential expression of Ig λ light chains*



BCR-ABL1^{+} SUP-B15, BV173 and NALM1 pre-B ALL cells were cultured in the presence or absence of 10 μmol/l STI571 for two days and surviving cells were analyzed for surface expression of Ig μ heavy chains, Vpre B surrogate light chains (top), Igκ light chains (middle) and Igλ light chains (bottom). Inhibition of BCR-ABL1 by STI571 results in preferential outgrowth of Igμ^{high} cells, which downregulate VpreB as compared to Igμ^{low} cells and express conventional, predominantly λ, light chains.

However, in the three *BCR-ABL1*$^+$ leukemia cell lines tested, the pattern of light chain expression was heavily biased in favor of λ-light chains (Figure 4, bottom) and only few κ-expressing cells could be detected (Figure 4, middle) while Igκ$^+$ cells in control stainings can easily be identified by the antibody we used (not shown).

Extensive κ-deleting element rearrangement results in preferential λ-light chain expression on STI571-surviving leukemia cells

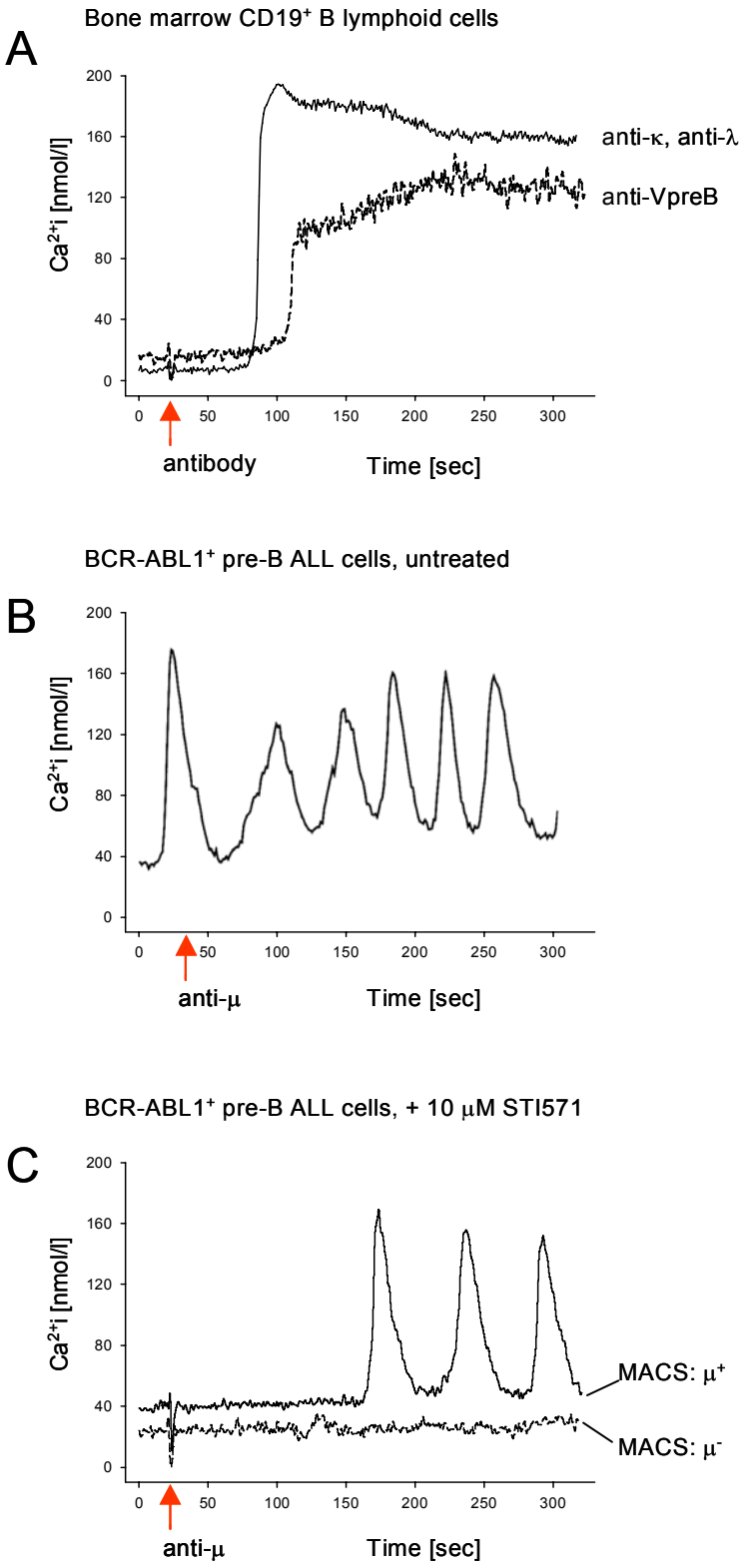
Searching for a potential explanation for predominant λ-light chain expression on differentiating leukemia cells surviving STI571-treatment, we investigated whether rearrangement of the so-called κ deleting element (KDE) may contribute to reduced κ light chain usage in STI571-treated leukemia cells. KDE rearrangement may lead to inactivation of productively recombined Vκ-Jκ joints and hence represents another level at which Ig light chain expression can be regulated (28).

We amplified specific DNA fragments for KDE-germline configuration, KDE rearrangement to an RSS within the Jκ intron and KDE rearrangement to RSS-sites flanking Vκ gene segments from three *BCR-ABL1*$^+$ leukemia cells in the absence or presence of STI571. While KDE was found in germline configuration in untreated leukemia cells, STI571-mediated inhibition of BCR-ABL1 was associated with rearrangement of KDE to Jκ intron RSS sites and –to lesser extent- to Vκ RSS sites (not shown). Thus, predominant expression of λ light chains is consistent with extensive KDE rearrangement observed here. In the absence of BCR-ABL1 kinase activity, differentiating leukemia cells seem to undergo multiple rounds of rearrangement targeting both κ and λ light chain loci, which ultimately leads to preferential λ light chain expression expression due to KDE-mediated deletion of potentially productive Vκ-Jκ joints.

Ig μ^{high} leukemia cells surviving STI571-treatment restore expression of B cell-specific transcription factors

Propensity of STI571-treated leukemia cells to differentiate into light chain-expressing immature B cells suggests that these cells exhibit a more mature phenotype as compared to untreated leukemia cells. Therefore, we tested whether STI571-treated differentiating leukemia cells can restore expression of B cell-specific transcription factors, which were downregulated in the untreated leukemia cells as compared to normal pre-B cells (SAGE data; Figure 2). *BCR-ABL1*$^+$ SUP-B15 pre-B ALL cells were cultured either in the presence

Figure 5: *Inhibition of BCR-ABL1 restores (pre-) B cell receptor responsiveness in pre-B ALL cells*



$CD19^+$ B lineage cells were purified from bone marrow mononuclear cells using immunomagnetic beads. Release of Ca^{2+} from cytoplasmic stores in these cells in response to pre-B or B cell receptor engagement by anti-VpreB or a mixture of anti-κand anti-λ chain antibodies (arrows) was measured using a laser scanning microscope (A). Untreated *BCR-ABL1*$^+$ SUP-B15 (B), BV173 and NALM1 (not shown) pre-B ALL cells and *BCR-ABL1*$^+$ pre-B ALL cells incubated with 10μmol/l STI571 for 24 hours (C) were stimulated using an anti-μ chain antibody, which can crosslink both pre-B and B cell receptors. Among STI571-treated leukemia cells, $Ig\mu^+$ cells were separated from $Ig\mu^-$ cells and analyzed independently (C). In (B), 5-fold higher concentrations of the anti-μ antibody than in (A) and (C) were used. The data shown here refer to SUP-B15 cells. Analyzing BV173 and NALM1 cells, similar results were obtained (not shown).

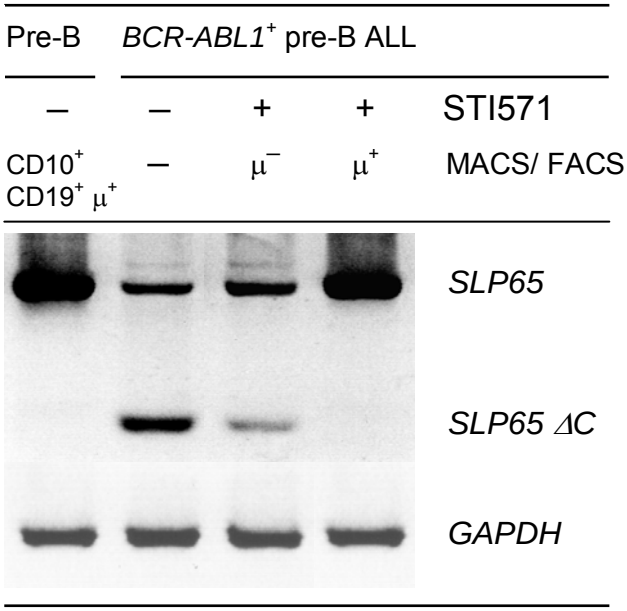
or absence of STI571. Among STI571-treated leukemia cells, Ig μ^{high} and Ig μ^{-} cells were separated by MACS using immunmagnetic beads. From normal pre-B cells, untreated ALL cells, STI571-treated Ig μ^{high} and STI571-treated Ig μ^{-} cells, cDNAs were synthesized and subjected to semiquantitative RT-PCR analysis for mRNA levels of B cell-specific transcription factors (E2A, EBF, OBF1, OCT2, IRF4 and PAX5) and the stem cell-related transcription factors AML1 and GATA1 (Figure 2). The expression patterns of these transcription factors in STI571-treated differentiating Ig μ^{high} and normal pre-B cells were similar indicating that STI571-treated Ig μ^{high} leukemia cells indeed reversed the de-differentiated stem cell-like phenotype seen in untreated leukemia cells and STI571-treated Ig μ^{-} leukemia cells (Figure 2).

Ablation of BCR-ABL1 kinase activity corrects expression of truncated SLP65 in pre-B ALL cells from STI571-treated patients

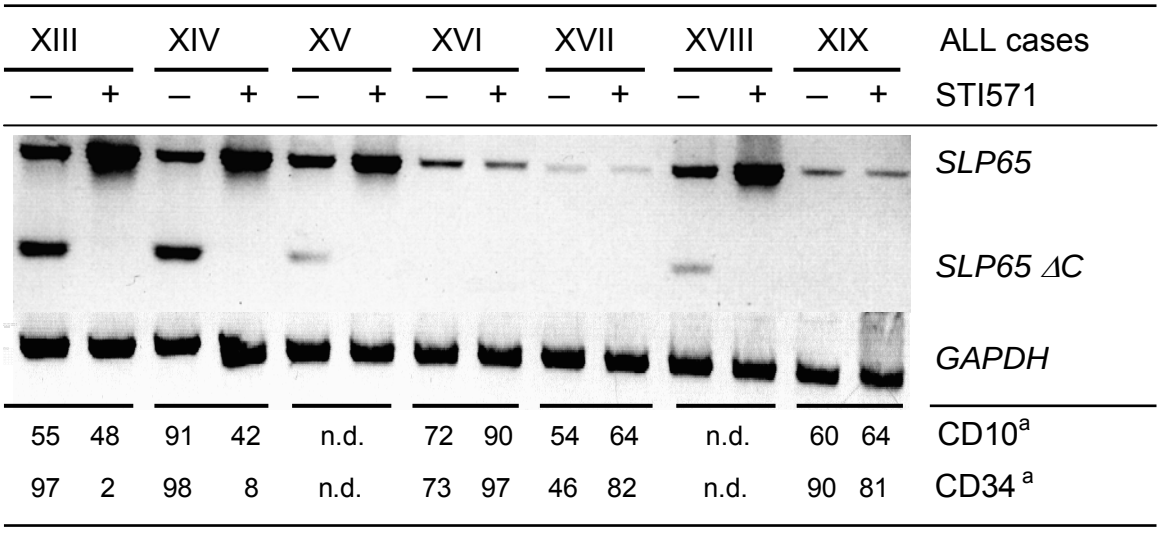
An earlier study demonstrated that in the human, functional *SLP65* is required for the expression of a pre-B cell receptor: B cell precursors in a patient carrying a deleterious mutation of the *SLP65* gene are arrested at the pro-B cell stage of development (29). In mice, *SLP65*-deficiency does not prevent expression of a pre-B cell receptor but results in a partial differentiation block at the pre-B cell stage (7). Of note, in about 50 percent of childhood pre-B ALL cases, *SLP65* expression is defective, which results in compromised pre-B cell receptor signaling (8). In these cases, either no *SLP65* was expressed or an aberrant splice variant including one or two additional exons inserted between *SLP65* exons III and IV (8). Inclusion of additional exons, termed IIIa and IIIb introduced a premature translation stop resulting in loss of the C-terminus of SLP65 (SLP65 ΔC). The C-terminus of SLP65 encompasses tyrosine 96, whose phosphorylation is critical for SLP65 activity in mice (8). Therefore, we tested whether *SLP65* expression is deranged in *BCR-ABL1*$^{+}$ pre-B ALL cells and whether *SLP65* deficiency is linked to the BCR-ABL1 kinase activity. To this end, we analyzed *SLP65* isoform expression in primary pre-B cells from the bone marrow of four healthy donors and STI571-treated and untreated *BCR-ABL1*$^{+}$ pre-B ALL cells (Figure 6A). While normal pre-B cells expressed high levels of the canonical *SLP65* isoform and only very little alternatively spliced *SLP65* ΔC, this pattern was almost inverted in *BCR-ABL1*$^{+}$ pre-B ALL cells.

Figure 6: *Expression of SLP65 isoforms in BCR-ABL1$^+$ pre-B ALL cells*

A



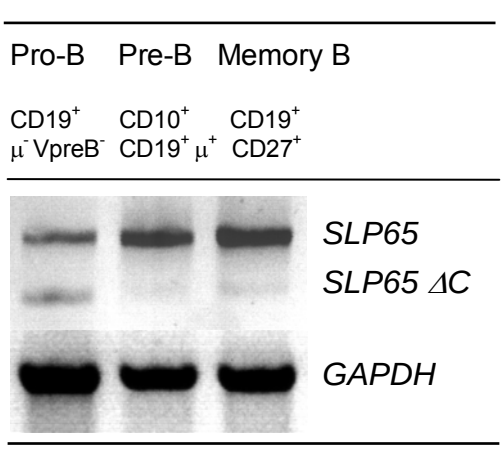
B



	XIII		XIV		XV		XVI		XVII		XVIII		XIX		ALL cases
STI571	–	+	–	+	–	+	–	+	–	+	–	+	–	+	
CD10[a]	55	48	91	42	n.d.		72	90	54	64	n.d.		60	64	
CD34[a]	97	2	98	8	n.d.		73	97	46	82	n.d.		90	81	

Note:
a. Percentage of CD19$^+$ leukemia cells coexpressing CD10 or CD34

C



Legend to Figure 6:

SLP65 cDNA fragments were amplified from normal human $CD19^+$ $CD34^+$ $Ig\mu^-$ pro-B, $CD10^+$ $CD19^+$ $Ig\mu^{low}$ pre-B and $CD10^-$ $CD19^+$ $CD27^+$ $Ig\mu^{high}$ memory B cells (C) and *BCR-ABL1*$^+$ ALL cells, including a *BCR-ABL1*$^+$ cell line in the presence or absence of STI571 (A) and enriched ALL cells from seven leukemia patients (cases XIII to XIX, Table I) before and after treatment with STI571 (B). cDNA amounts were normalized using *GAPDH* as a standard (A-C). The primers used amplified full-length SLP65 and an isoform encoding truncated SLP65 (SLP65 ΔC). For leukemia cases XIII, XIV, XVI, XVII and XIX described in (B), FACS data on coexpression of CD10 and/ or CD34 on leukemic $CD19^+$ cells was available before and after STI571-treatment (B, lower panel).

Also, when *BCR-ABL1*$^+$ pre-B ALL cells were incubated in the presence of STI571 for 48 hours, the leukemia cells expressed the *SLP65* ΔC transcript, although at lower levels. Given that short-term treatment with STI571 partially restored (pre-) B cell receptor responsiveness (Figure 5C) and induced selection for differentiating leukemia cells expressing high levels of Ig μ heavy chains (Figure 3), we sorted Ig μ^+ leukemia cells. STI571-treated leukemia cells, which have recovered (pre-) B cell receptor expression also regained normal *SLP65* expression (Figure 6A) in parallel with restored pre-B cell receptor responsiveness (Figure 5C). In humans, deleterious mutation of the *SLP65* gene prevents expression and function of the pre-B cell receptor on mutant B cell precursors (29). However, inactivating mutations within pre-B cell receptor-related genes (including *IGHCμ*, *Igα*, *Igβ*, *VpreB* and *λ5*) do not interfere with *SLP65* expression (29). Therefore, deranged *SLP65* expression is likely the cause rather than the consequence of compromised pre-B cell receptor signaling in *BCR-ABL1*$^+$ pre-B ALL cells.

To determine whether deranged *SLP65* expression is linked to BCR-ABL1 kinase activity also in primary *BCR-ABL1*$^+$ pre-B ALL leukemia cells, we analyzed the pattern of *SLP65* isoform expression in enriched leukemia cells from seven patients before and after treatment with STI571. Comparing matched pairs of leukemia samples, we identified three patients, in which the leukemia cells expressed *SLP65* only at low levels, if at all (cases XVI, XVII and XIX; Figure 6B). In these cases, treatment by STI571 had no effect on *SLP65* isoform expression. Given that the leukemia samples had a purity of about 80 percent, weak *SLP65* expression in these cases can be attributed to few contaminating normal bystander B cells. In four cases, expression of the canonical *SLP65* isoform encoding the full-length protein was accompanied by the expression of *SLP65* ΔC. Treatment with STI571 not only increased mRNA levels of full-length *SLP65* but also abolished expression of the truncated splice variant (Figure 6B).

Given that STI571-treatment of the *BCR-ABL1*$^+$ ALL cell lines BV173, NALM1 and SUP-B15 resulted in the preferential outgrowth of more differentiated subclones (Figures 2

and 3), which further differentiate into Ig μ^{high} light chain-expressing immature B cells (Figure 4), we wondered whether also in patients the *BCR-ABL1*$^+$ leukemia cells surviving STI571-treatment tend to exhibit a more mature phenotype. Therefore, we analyzed primary B lymphoid bone marrow cells from seven leukemia patients before and after STI571-treatment by flow cytometry and calculated the percentage of $CD19^+$ cells, which coexpress CD34 and/ or CD10. In the human, $CD19^+$ $CD34^+$ cells are considered as pro-B cells, $CD19^+$ $CD10^+$ $CD34^-$ cells include pre-B cells and immature B cells, whereas mature B cell subsets in human bone marrow and the peripheral blood typically express CD19 but neither CD10 nor CD34 (12). Based on this rough classification, between 46 and 98 percent of *BCR-ABL1*$^+$ leukemia cells coexpress CD34 and thus exhibit a pro-B cell-like phenotype (Figure 6B). After STI571-treatment, the percentage of cells displaying a pro-B cell-like phenotype remains high in three cases (XVI, XVII and XIX). In two other cases, however, the fraction of pro-B cell-like leukemia cells drops to 2 and 8 percent (cases XIII and XIV; Figure 6B, lower panel). The remaining cells show a more mature phenotype resembling pre-B or immature B cells. Of note, STI571-induced conversion of primary leukemia cases from a pro-B cell-like to a pre-B cell-/ immature B cell-like phenotype correlates with therapy-related correction of *SLP65* isoform expression in these cases (Figure 6B). Three cases, in which STI571-treatment had no effect on SLP65 mRNA expression, also did not convert to a more differentiated phenotype in response to STI571-therapy (cases XVI, XVII and XIX; Figure 6B). From two cases, no flow cytometry data was available. *SLP65 ΔC* is nonfunctional and by competing with full-length SLP65 for binding to upstream kinases (e.g. SYK), it may have an inhibitory effect on signal transduction initiated from the pre-B cell receptor in pre-B ALL cells. While the negative regulatory effect of truncated SLP65 is not fully elucidated, our results indicate that its expression is linked to BCR-ABL1 kinase activity.

A potential link between SLP65 deficiency, secondary V_H gene rearrangement and loss of coding capacity for a μ heavy chain in BCR-ABL1$^+$ *pre-B ALL cells*

SLP65 also functions as a sensor for the expression of a productive V_H region gene within a pre-B cell receptor complex and then halts the recombination machinery to prevent further, potentially inactivating, rearrangement of *IGH* gene segments (1). In the absence of functional SLP65, the recombination machinery remains active and generates secondary V_H region gene rearrangements even if V, D and J segments have been productively rearranged (9). Assuming that *SLP65* deficiency is a common feature of *BCR-ABL1*$^+$ ALL, one would predict that in most if not all cases the leukemia clone has undergone one or more rounds of secondary V_H gene rearrangement. Such secondary rearrangements can occur by replacement of an already

rearranged V_H gene segment by another (in most cases upstream) V_H gene segment (22). V_H gene replacement typically uses a cryptic recombination signal sequence (cRSS), which is present in the 3' part of 40 out of 44 functional V_H gene segments (22). A hallmark for V_H gene replacement are short sequences (5 to 7 bp) within the V_H-D_H junction that are foreign to the actually rearranged V_H gene segment but are matching exactly the 3' end adjacent to the cRSS of another previously rearranged V_H gene. It is very unlikely that 5 to 7 bp stretches matching the extreme 3' part of another V_H gene within the V_H-D_H joint can be randomly generated as a byproduct of junctional diversification (p=0.009; reference 22). Therefore, these short sequences are considered as vestiges of a previously rearranged V_H gene segment. Analysing the *IGH* VDJ-rearrangements for footprints of secondary rearrangements, we found indication of V_H-gene replacement in 9 of 12 leukemia cases (Table III). In eight of nine cases, the potential secondary rearrangement uses a donor V_H segment located upstream from the initially rearranged (recipient) V_H-gene segment (Table III). One potential V_H-replacement might reflect an inversion or transrecombination event as described earlier (22). A comprehensive analysis of *IGH* VDJ-rearrangements amplified from normal human immature B cells identified traces of V_H-gene replacement in 16 out of 343 sequences (5%; reference 22). Hence, the frequency of V_H-gene replacement in *BCR-ABL1*$^+$ ALL cases is, at about 75%, surprisingly high.

Therefore, defective *SLP65* expression (Figure 6) is associated with and potentially the reason for secondary, mostly nonproductive, V_H gene rearrangements in 9 of the 12 leukemia cases we analyzed (Table II). In agreement with a pre-B cell origin of *BCR-ABL1*$^+$ ALL one might envision that the precursor of the leukemia clone underwent malignant transformation after having passed the pre-B cell receptor checkpoint but subsequently lost coding capacity of an initially productive Ig V_H region gene by a deleterious secondary V_H gene rearrangement.

Assuming that BCR-ABL1 can relieve the selection pressure for survival signals through the pre-B cell receptor, we next tested whether truncated *SLP65* is also expressed in normal pro-B cells, in which selection for the expression of a functional pre-B cell receptor is not operative. While CD19$^+$ Ig μ chain$^-$ VpreB$^-$ pro-B cells express full-length *SLP65* at similar levels as in CD19$^+$ Ig μ chain$^+$ Vpre-B$^+$ pre-B cells and CD19$^+$ CD27$^+$ memory B cells, *SLP65 ΔC* is only expressed in pro-B cells (Figure 6C). As *SLP65* isoform expression is similar in *BCR-ABL1*$^+$ pre-B ALL and normal human CD19$^+$ Igμ$^-$ VpreB$^-$ pro-B cells, we conclude that *SLP65 ΔC* is not a marker for B lineage leukemia cells but rather for B lymphoid cells that are not selected for the expression of an active (pre-) B cell receptor.

Table III: Indication of potential V_H-gene replacement in V_H region genes of *BCR-ABL1*$^+$ pre-B ALL clones

Case		3' part of recipient V_H		3' part of donor V_H	V_H-D_H	D_H	
I	V_H1.8	CGTGTATT**TACTGTG**CGAGAGG	V_H4.34	GTGTATT**TACTGTG**CG	AGAGGGGGGTA	GGGAATAGCA	D_H6.13-J_H5
II	V_H1.58	CGTGTATT**TACTGTG**CGGCAGA	V_H4.61	GTGTATT**TACTGTG**CGAGAGA	CACGGCAGATG	TATTGTAGTAGTACCCCC	D_H2.2-J_H4
V	V_H2.5	CACATATT**TACTGTG**CACACAGACC	V_H5.51	ATGTATT**TACTGTG**CG	AGACACACAGT	GAGAGAAACCAGCC	D_H1.26-J_H1
VI	V_H1.2[a]	CGTGTATT**TACTGTG**CGAGAGA	V_H3.30	GTGTATT**TACTGTG**CG	CGAGAGATCCCCCTTC	TATTACTATGA	D_H3.22-J_H3
VII	V_H2.5	CACATATT**TACTGTG**CACACAGACC	V_H1.46	GTGTATT**TACTGTG**CGAG	AGACCGCT	GGGTATAGCAGCAGCTG	D_H6.13-J_H4
VIII	V_H2.70[b]	CACATATT**TACTGTG**CACACAGAC	V_H2.5	ACATATT**TACTGTG**CA	CACAGCCCCGGGATCCC	CGGGATAGTAGTG	D_H3.22-J_H4
IX	V_H1.24	CGTGTATT**TACTGTG**CAACAGA	V_H1.46	GTGTATT**TACTGTG**CGAGAGA	CCAACAG	ACCGTGGTTCGGGGA	D_H3.10-J_H5
X	V_H3.38	CGTGTATT**TACTGTG**CCAGATATA[c]	V_H3.53	GTGTATT**TACTGTG**CGAGA	GTTGCCAGGGGG	TGGTGTATGCTATACC	D_H2.8-J_H6
XI	V_H3.38	CGTGTATT**TACTGTG**CCAGATATA	V_H3.48	GTGTATT**TACTGTG**GCGA	GCCAGATATTGT	AGTGGTGGTAGCT	D_H2.15-J_H3

Notes: a: The footprint of this potential V_H-replacement may be also derived from V_H1.3, V_H1.18, V_H3.7, V_H3.11, V_H3.20, V_H3.21 and V_H4.4 gene segments
b: Likely generated by inversion or transrecombination events
c: The footprint of V_H-replacement in this case also involves the last two nucleotides of the cRSS motif
bold: cRSS motifs; underlined: footprints of recipient V_H-gene segments

Perspective

A broad range of pre-B cell receptor-related signaling molecules and transcription factors is transcriptionally silenced in *BCR-ABL1*$^+$ pre-B ALL as compared to human pre-B cells. Loss of (pre-) B cell receptor-related molecules as observed by SAGE (Figures 1 and 2) may reflect that the BCR-ABL1 kinase may have relieved the selection pressure for the expression of these molecules. In the presence of autonomous BCR-ABL1 kinase activity, pre-B ALL cells become nonresponsive to (pre-) B cell receptor engagement (Figure 5). As a consequence, molecules needed for transduction of survival signals through the pre-B cell receptor, disappear from the gene expression program of the leukemic cells (SAGE, Figure 1). Conversely, inhibition of BCR-ABL1 reconstitutes dependence on survival signals initiated from surface receptors including the (pre-) B cell receptor (Figure 3). It is even conceivable that default signaling through the (pre-) B cell receptor represents a mechanism to acquire drug resistance that is available to *BCR-ABL1*$^+$ pre-B ALL cells but not to other malignant cells carrying a *BCR-ABL1* gene rearrangement, e.g. chronic myeloid leukemia (CML) cells. In this regard, it is interesting to note that treatment failure of STI571 is frequent in pre-B ALL but not in CML harboring a *BCR-ABL1* fusion gene (30). Moreover, STI571-resistant as opposed to STI571-sensitive *BCR-ABL1*$^+$ pre-B ALL cells exhibit high expression levels of Bruton's tyrosine kinase (BTK), which represents a critical component of (pre-) B cell receptor signal transduction cascade (15). Therefore, resumption of (pre-) B cell receptor signaling may represent a mechanism to escape immediate apoptosis induced by STI571 and open a time frame, during which secondary transforming events that confer permanent STI571-resistance (e.g. mutations within the ATP-binding site of the ABL1 kinase domain) can occur.

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Materials and Methods

Patient samples and cell purification

In total, 19 cases of *BCR-ABL1*$^+$ pre-B ALL were studied. For cases I to IX (Table I), *BCR-ABL1*$^+$ pre-B ALL cells obtained from bone marrow samples of untreated patients had a purity of >95% as assessed by bone marrow morphology. Cases X, XI and XII (Table I) correspond to *BCR-ABL1*$^+$ pre-B ALL cell lines, termed SUP-B15, BV173 and NALM1 (DSMZ, Braunschweig, Germany), respectively. Purification of ALL cells from cases XIII to XIX (Table I) was described earlier (15). In these cases, *SLP65* isoform expression before and after treatment with the BCR-ABL1 inhibitor STI571 and expression of CD10, CD19 and CD34 was studied (Figure 4; cases XIII to XIX; Table I). For nine primary cases (including two cases analyzed by SAGE, cases II and IX) and three cell lines, the configuration of *IGH* loci was examined (cases I to XII in Table II). For all cases, breakpoints within the major or minor breakpoint cluster region of the *BCR* gene, leading to p210 and p190 fusion molecules, respectively, were detected by PCR using primers and PCR conditions as previously described (16). Clinical data for all 19 cases are given in Table I. Normal hematopoietic cell populations including CD34$^+$ CD38^{low} CD133$^+$ Lin$^-$ hematopoietic progenitor cells (HSC), CD15$^+$ myeloid progenitor cells (CMP), CD7$^+$ CD10$^+$ T lymphoid precursors (TLP), CD19$^+$ VpreB$^-$ Igμ chain$^-$ pro-B cells (no SAGE-profile available) and CD10$^+$ CD19$^+$ Igμ chain$^+$ pre-B cells (pre-B) were enriched from human bone marrow by depletion of unwanted cells and enrichment of the populations of interest using immunomagnetic beads and cell sorting. Mature B cell populations including CD19$^+$ CD27$^-$ naïve B cells (NBC), CD19$^+$ CD27$^+$ memory B cells (MBC) and CD19$^+$ CD138$^+$ plasma cells (PC) were from pooled peripheral blood samples of 12 healthy donors. CD20$^+$ CD77$^+$ germinal center B cells (GCB) were isolated from human tonsillectomy resectates. HSC, CMP, TLP, pre-B cells, naïve B cells, memory B cells and plasma cells were subjected to SAGE-analysis as described earlier (17-19). SAGE data on germinal center B cells were kindly provided by Ines Schwering and Ralf Küppers (University of Cologne, Cologne, Germany and University of Essen, Essen, Germany).

SAGE analysis

cDNA-synthesis, SAGE analysis, cloning and sequencing of SAGE concatemers was carried out as previously described (17-19). The UniGene reference data base (March 2001) was obtained at http://www.sagenet.org/SAGEDatabases/unigene.htm. A total of 592,000 SAGE tags were collected for 10 SAGE profiles. 106,000 tags were analyzed from the HSC library, 99,000 for common myeloid progenitor cells, 110,000 for pre-B cells, 96,500 for T lymphoid

precursors and each about 30,000 tags for two cases of bone marrow-derived pre-B ALL carrying a *BCR-ABL1* gene rearrangement, naïve B cells, germinal center B cells, memory B cells and plasma cells. All SAGE libraries were normalized to 100,000 tags. SAGE data were graphically visualized using the Cluster and Treeview software (http://rana.lbl.gov/) and sorted according to the ratio between SAGE-tag counts in pre-B cells and in *BCR-ABL1*$^+$ ALL cases. In a comprehensive search for pre-B cell receptor components and pre-B cell receptor-related signaling molecules in PubMed, UniGene (http://www.ncbi.nlm.nih.gov/UniGene/) and OMIM (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM), we identified 54 genes in at least one of the SAGE-libraries analyzed, for which a role in the assembly or regulation of the pre-B cell receptor was shown.

Semiquantitative RT-PCR analysis and verification of quantitative accuracy of SAGE data

In order to corroborate quantitative differences in gene expression as determined by SAGE, semiquantitative RT-PCR analysis was performed for a set of 45 selected genes differentially expressed between HSC and pre-B cells and 41 genes differentially expressed between naïve and memory B cells as previously described (17, 18).

To verify differential gene expression observed by SAGE-analysis of pre-B cells and the *BCR-ABL1*$^+$ pre-B ALL cell line SUP-B15, we performed semiquantitative RT-PCR using 5'-TTAGCACCCCTGGCCAAG-3' and 5'-CTTACTCCTTGGAGGCCATG-3' for *GAPDH* (for standardization), 5'-AATGATGAAAACTACTCGGCT-3' and 5'-TTGGTCTGATCATCTAGTTTC-3' for *AML1*, 5'-AGTCTTTCAGGTGTACCCAT-3' and 5'-AAAGAAGGTACTGGAAAAGTC-3' for *GATA1*, 5'-AAGGAAAAAGAAGCCAACAG-3' and 5'-GGACAAACATGTTATCAGGA-3' for *EBF*, 5'-CTGAAAGCAAGCAACAAAAC-3' and 5'-CACGGGGTCTTTTTAATACA-3' for *E2A*, 5'-CCATGTAAATACCTTCTTGC-3' and 5'-ATTCTGCTTCGGAAAAGTAG-3' for *PAX5*, 5'-CAAAATAAGACCTCCCCATT-3' and 5'-TGAGGTAGCTGGAATAGATT-3' for *OCT2*, 5'-GGCTTCAAAGAGAAAAGGCA-3' and 5'-TCTGTCGTGACATTGGTGAT-3' for *OBF1* and 5'-CAAGAGCAATGACTTTGAGG-3' and 5'-TGGGACATTGGTACGGGAT-3' for *IRF4*. The same primer sets were also used for RT-PCR analysis of STI571-treated SUP-B15 ALL cells after MACS-enrichment of μ^+ and μ^- subclones. In addition, quantitative differences between the SAGE-libraries for pre-B cells and *BCR-ABL1*$^+$ pre-B ALL cases were verified at the protein level for ten surface molecules by flow cytometry using antibodies against CD19, IL7Rα, CD10, CD38, CD40, CD150/SLAM, CD72, VpreB, Igα and CD34 (BD Biosciences, Heidelberg, Germany; data not shown).

Sequence analysis of IGH loci

In order to characterize the configuration of the *IGH* loci in nine primary *BCR-ABL1*$^+$ pre-B ALL cases (cases I to IX, Table II) and three cell lines (SUP-B15, BV173 and NALM1; cases X to XII, Table II), three primer sets were used to amplify germline configuration, *D-J* and *V-DJ* gene rearrangements from both alleles of the *IGH* locus. Clonal V_H gene rearrangements were amplified in a single round of PCR amplification using 35 PCR cycles at a detection limit of about 10^4 clonal cells in 5 x 10^6 total cells. PCR primers and conditions were used as previously described (20) and amplification products were directly sequenced and aligned according to Matsuda et al. (21) using the IMGT software at http://www.dnaplot.de/input/human_v.html. To identify footprints from formerly rearranged V_H gene segments within potential secondary VDJ-rearrangements, a list of V_H and D_H germline gene segments was obtained at http://imgt.cines.fr and cryptic recombination signal sequences (cRSS) were identified as previously described (22).

Measurement of pre-B cell receptor responsiveness

Primary human CD19$^+$ bone marrow mononuclear cells were enriched from bone marrow samples of four healthy donors using immunomagnetic MACS beads as previously described (17). SUP-B15, BV173 and NALM1 pre-B ALL cells (DMSZ, Braunschweig, Germany) carrying a *BCR-ABL1* gene rearrangement (Table II, cases X, XI and XII) were cultured with 20% fetal calf serum in RPMI 1640 medium in the presence or absence of 10 μM STI571 (Novartis, Basel, Switzerland) for the times indicated. Among STI571-treated leukemia cells, Igμ$^+$ and Igμ$^-$ cells were separated using a non-stimulating, biotin-conjugated anti-μ antibody (BD Biosciences) together with streptavidin-microbeads (Miltenyi Biotech). After MACS-enrichment or pre-incubation, cells were washed and stained with Fluo-3 dye (Calbiochem, Bad Soden, Germany) for 30 minutes. Changes of cytosolic Ca^{2+} were measured by laser scans using confocal microscopy (18, 19). After 10 to 30 seconds of measurement, antibodies against human μ chains (Jackson ImmunoResearch), VpreB (BD Biosciences) or Igκ- together with Igλ-light chains (BD Biosciences) were added to CD19$^+$ bone marrow mononuclear cells or preincubated pre-B ALL cells (in the presence or absence of STI571; cases X to XII). Cytosolic Ca^{2+} concentrations were determined as previously described (18). As a negative control, cells were also treated with an antibody against CD3 (BD Bioscience), which induces Ca^{2+} mobilization in T but not B lineage cells.

Flow cytometry

Surface expression of VpreB surrogate- or Igκ- or Igλ-light chains, Ig μ-chains and CD19 on BV173, NALM1, and SUP-B15 pre-B ALL cells in the presence or absence of 10 μM STI571

was monitored using antibodies against VpreB, Igκ- or Igλ-light chains, the Ig μ heavy chain and CD19 (BD Biosciences, Heidelberg, Germany) after the incubation times indicated. Apoptotic or dead cells were identified by staining with anti-Annexin V antibodies and propidium iodide (BD Biosciences).

RT-PCR analysis of SLP65 isoform expression

cDNA amounts were normalized by OD measurements and amplification of a specific fragment of the *GAPDH* gene using 5'-TTAGCACCCCTGGCCAAGG-3' as forward and 5'-CTTACTCCTTGGAGGCCATG-3' as reverse primers. For amplification of *SLP65* isoforms, forward primers specific for exon I (5'-TGGACAGTTATTCGTGTCTCTT-3'), and additional exon IIIb (5'-AGAGTGTGTTGACCTTGGTG-3') were used together with a reverse primer specific for additional exon IIIb (5'-TTGGCTTAGAGGGTTTTGG-3') and exon VII (5'-GTGAACTGCTTTCTGTGGGA-3') as described earlier (8). For amplification of another additional exon IIIa, the forward primer 5'-TTTAATCTCTCCTGGAATGCAG-3' was used. However, a splice variant including exon IIIa (8) could not be amplified with this primer (not shown). cDNAs derived from human pre-B cells, *BCR-ABL1*$^+$ pre-B ALL cells in the presence or absence of STI571 and MACS-sorted μ-chain$^+$ and μ-chain$^-$ *BCR-ABL1*$^+$ pre-B ALL cells after treatment with 10 μM STI571, were used as RT-PCR templates.

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5.2 BCR-ABL1 induces aberrant splicing of *IKAROS* and lineage infidelity in pre-B lymphoblastic leukemia cells

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Summary

Pre-B lymphoblastic leukemia cells carrying a *BCR-ABL1* gene rearrangement exhibit an undifferentiated phenotype. Comparing genome-wide gene expression profiles of normal B cell subsets and *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells by SAGE, the leukemia cells show loss of B lymphoid identity and aberrant expression of myeloid lineage-specific molecules. Consistent with this, *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells exhibit defective expression of *IKAROS*, a transcription factor needed for early lymphoid lineage commitment. As shown by inducible expression of BCR-ABL1 in human and murine B cell precursor cell lines, BCR-ABL1 induces the expression of a dominant-negative *IKAROS* splice variant, termed IK6. Comparing matched leukemia sample pairs from patients before and during therapy with the BCR-ABL1 kinase inhibitor STI571 (Imatinib), inhibition of BCR-ABL1 partially corrected aberrant expression of IK6 and lineage-infidelity of the leukemia cells. To elucidate the contribution of IK6 to lineage infidelity in *BCR-ABL1*$^+$ cell lines, IK6-expression was silenced by RNA interference. Upon inhibition of IK6, *BCR-ABL1*$^+$ leukemia cells partially restored B lymphoid lineage commitment. Therefore, we propose that BCR-ABL1 induces aberrant splicing of *IKAROS*, which interferes with lineage identity and differentiation of pre-B lymphoblastic leukemia cells.

Introduction

The *BCR-ABL1* gene rearrangement resulting from the t(9;22)(q34;q11) translocation represents the most frequent recurrent genetic aberration in B lymphoid leukemia in adults (Look, 1997). *BCR-ABL1* fusion genes encode constitutively active tyrosine kinase molecules mostly of a molecular weight of either 190 or 210 kD (p190 or p210, Laurent et al. 2001). BCR-ABL1 kinase activity is required and sufficient to drive malignant transformation of B cell precursors in mice (Huettner et al., 1998). Pre-B lymphoblastic leukemia cells carrying a *BCR-ABL1* gene rearrangement typically exhibit a differentiation block at the pre-B cell stage of development (Klein et al., 2004; Klein et al., 2005).

Early B cell development is guided by a number of transcription factors including PAX5, E2A, EBF and IKAROS (Busslinger, 2004). In order to search for downstream targets of BCR-ABL1 that contribute to a differentiation block in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells, genome-wide gene expression profiles of this leukemia type were compared with normal hematopoietic progenitor populations by serial analysis of gene expression (SAGE; Velculescu et al., 1995).

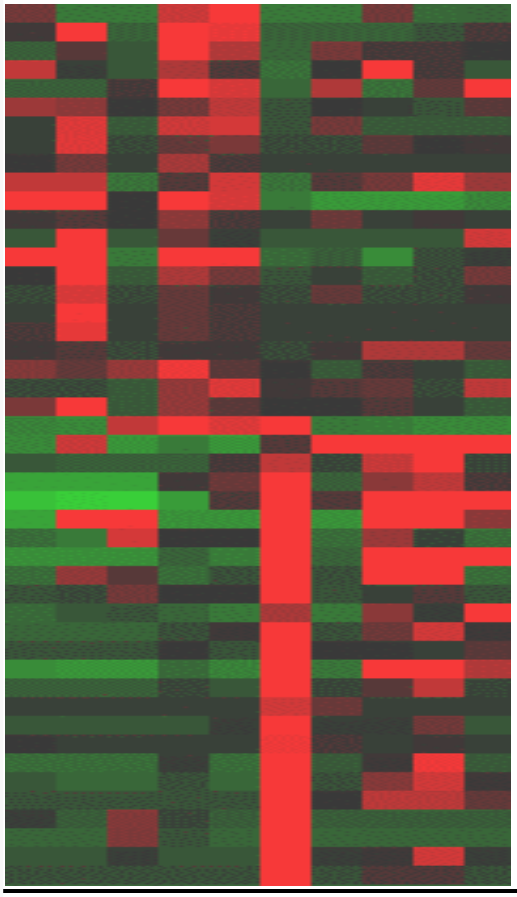
Results and Diskussion

BCR-ABL1 interferes with B lymphoid differentiation in BCR-ABL1$^+$ *pre-B lymphoblastic leukemia cells*

Comparing genome-wide gene expression profiles of leukemia cells from two leukemia cases of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia with normal pre-B cells by SAGE, a number of myeloid lineage-specific genes including transcription factors (*MLF2, MZF1, AML1, GATA1*), surface receptors (*CSF3R, CSF1R, CD14, CD11A*) and signaling molecules (*IRAK1, MYD88*) were upregulated in the pre-B lymphoblastic leukemia cells as in normal myeloid progenitor cells (Figure 1). Hence, the pattern of gene expression in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells as compared to their normal pre-B cell counterpart is skewed to the myeloid lineage. Consistent with this, transcription factors and signaling molecules involved in early lymphoid differentiation (*IKAROS, E2A, IL7Rα, RAG1, RAG2, TdT*) and B lymphoid lineage commitment (*EBF, PAX5*) were silenced in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells (Figure 1). These findings lead to the hypothesis that lymphoid lineage commitment is impaired in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells.

We next studied whether derangement of lymphoid lineage commitment and B cell differentiation in the *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells are indeed owing to BCR-ABL1 kinase activity. To this end, we induced BCR-ABL1 expression in a murine B

Figure 1: *SAGE-Analysis of differentially expressed genes in BCR-ABL1+ B cell precursor leukemia cells and normal pre-B cells*



UniGene	Gene name	Proposed function	Reference
81994	*GYPC*	erythroid/ myeloid differentiation	Robinson et al., 1981
2175	*CSF3R*	GCSF-receptor	Dong et al., 1996
181002	*MSF1*	related to myeloid leukemia	Osaka et al., 1999
85289	*CD133*	hematopoietic stem cell antigen	Yin et al., 1997
182018	*IRAK1*	IL1 signaling in myeloid cells	Cao et al., 1996
79026	*MLF2*	myeloid leukemia factor 2	Kuefer et al., 1996
169832	*MZF1*	myeloid zinc finger gene 1	Hromas et al., 1991
112255	*NUP98*	related to myeloid leukemia	Ahuja et al., 1999
174142	*CSF1R*	colony-stimulating factor 1 receptor	Gisselbrecht et al., 1987
460463	*IL3Rα*	Shared subunit with GM-CSFR	Kitamura et al., 1991
1817	*MPO*	myeloperoxidase	Weil et al., 1988
89633	*PML*	related to myeloid leukemia	Cleary, 1996
1239	*CD13*	myeloid differentiation antigen	Look et al., 1986
129914	*AML1*	implicated in myelopoiesis	Tanaka et al., 1995
153837	*MNDA*	myeloid nuclear differentiation antigen	Briggs et al., 1994
83731	*CD33*	myeloid differentiation	Peiper et al., 1988
31551	*CBFA2T2*	related to myeloid leukemia	Kitabayashi et al., 1998
765	*GATA1*	myeloid differentiation	Qian et al., 2002
174103	*CD11A*	macrophage differentiation	Springer et al., 1985
196352	*NCF4*	oxidative burst in myeloid cells	Zhan et al., 1996
279751	*SIGLEC8*	myeloid inhibitory receptor	Kikly et al., 2000
82116	*MYD88*	IL1 signaling in myeloid differentiation	Adachi et al., 1998
272537	*TdT*	generation of junctional diversity	Kung et al., 1975
89414	*CXCR4*	SDF1 receptor, pre-B cells	Nagasawa et al., 1996
25648	*CD40*	Proliferation of B cell precursors	Hasbold et al., 1994
96023	*CD19*	B cell co-receptor	Carter & Fearon, 1992
285823	*IGHCM*	IGH μ chain	Raff et al., 1976
170121	*CD45*	Antagonizes SHP1, CSK	Pani et al., 1997
101047	*E2A*	Initiation of *IGH* gene rearrangement	Bain et al., 1994
79630	*Igα*	ITAM signaling chain	Flaswinkel et al., 1995
74101	*SYK*	B cell receptor signaling	Kurosaki et al., 1994
237868	*IL7Rα*	Lymphoid differentiation	Uckun et al., 1991
66052	*CD38*	Ligation causes tyrosine phosphorylation	Kitanaka et al., 1999
167746	*BLNK*	Linker in B cell receptor signaling	Fu et al., 1998
82132	*IRF4*	*IGK* and *IGL* gene rearrangement	Lu et al., 2003
2407	*OBF1*	*IGK* gene transcription	Casellas et al., 2002
22030	*PAX5*	Required for pro- to pre-B cell transition	Nutt et al., 1999
158341	*TACI*	Growth control of early B cells	Yan et al., 2001
54452	*IKAROS*	Critical for early B cell development	Kirstetter et al., 2002
1521	*IgMBP2*	Signal transduction through Igα	Grupp et al., 1995
3631	*IgαBP1*	Involvement in B cell receptor signaling	Fukita et al., 1993
159494	*BTK*	Critical for (pre-) B cell receptor signaling	Cheng et al., 1994
192861	*SPIB*	*IGK* and *IGL* gene rearrangement	Su et al., 1996
73958	*RAG1*	V(D)J recombination	Menetski & Gellert, 1990
159376	*RAG2*	V(D)J recombination	Schatz et al., 1989
1101	*OCT2*	Regulates *IGH* gene transcription	Staudt et al., 1988
192824	*EBF*	Required for B lymphopoiesis	Lin & Grosschedl, 1995

cDNA-synthesis, SAGE analysis, cloning and sequencing of SAGE concatemers was carried out as previously described (Müschen et al., 2001; Feldhahn et al., 2001; Klein et al., 2003). A total of 592,000 SAGE tags were collected for 10 SAGE profiles. All SAGE libraries were normalized to 100,000 tags. Leukemia cells from two cases of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells (p190 and p210) and normal bone marrow populations and mature B cell subsets were compared by SAGE. Upregulated genes are depicted in red, downregulated genes in green. For each gene, a proposed function along with a reference is given. SAGE extracts a 14-bp fragment of any transcript in the analyzed cell population as a unique identifier (SAGE-tag) of an expressed gene using the reference database UniGene. By counting the number of SAGE-tags per 100,000, the representation of a transcript within the transcriptome can be quantified. Normal bone marrow populations analyzed include CD34$^+$ hematopoietic progenitor cells (HSC), CD15$^+$ myeloid progenitor cells (CMP), CD7$^+$ CD10$^+$ T lymphoid progenitor cells (TLP) and CD10$^+$ CD19$^+$ pre-B cells (pre-B). In addition, CD19$^+$ CD27$^-$ naïve B cells (NBC), CD20$^+$ CD77$^+$ germinal center B cells (GCB), CD19$^+$ CD27$^+$ memory B cells (MBC) and CD19$^+$ CD138$^+$ plasma cells (PC) were analysed. In both cases, the leukemia cells harbor an *IGH VDJ* gene rearrangement on one allele (V_H4.61-D_H2.2-J_H4 and V_H3.38-D_H3.10-J_H5) and an *IGH DJ* gene rearrangement on the second allele (D_H2.4-J_H4 and D_H3.9-J_H6). However, *IGK* and *IGL* loci were in germline configuration in both cases (not shown). An *IGH VDJ*-gene rearrangement in the absence of *IGK* or *IGL* gene rearrangement defines a pre-B cell stage of development. We therefore sorted SAGE-data based on the ratio of SAGE-tag frequencies in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells and normal pre-B cells. SAGE data were graphically visualized using the Cluster and Treeview software (http://rana.lbl.gov/) and sorted according to the ratio between SAGE-tag counts in pre-B cells and in *BCR-ABL1*$^+$ ALL cases.

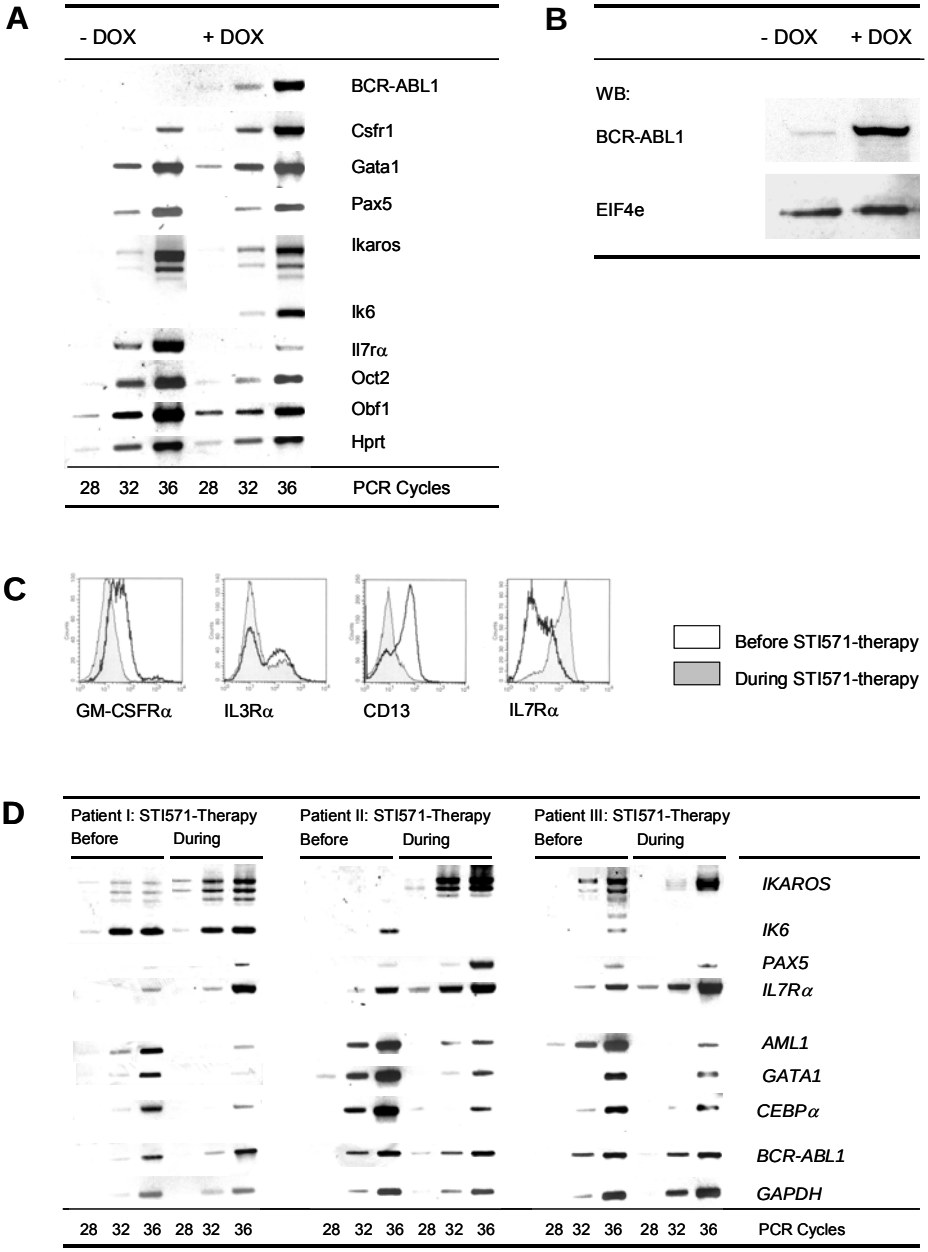
lymphoid cell line carrying an inducible *BCR-ABL1*-transgene (Klucher et al., 1998, Figure 2A, B). BCR-ABL1 expression was induced by addition of doxycycline as monitored by measurement of BCR-ABL1 mRNA expression and Western blot analysis (Figure 2B). Induced expression of BCR-ABL1 resulted in upregulation of mRNA levels for the myeloid lineage-specific genes *Csfr1* and *Gata1* and downregulation of mRNA levels for *Il7rα*, which is critical for early lymphoid development. Of note, inducible BCR-ABL1 expression also resulted in aberrant splicing of *IKAROS*, leading to the expression of a dominant-negative form of Ikaros (IK6; Figure 2A). These findings indicate that BCR-ABL1 can promote lineage infidelity in the murine B cell precursor line. However, mRNA levels for *Oct2*, *Obf1* and *Pax5* remained unchanged.

Attenuation of lymphoid lineage commitment was also linked to BCR-ABL1 kinase activity in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells directly isolated from leukemia patients (Figure 2C, D). From three leukemia patients, matched sample pairs of leukemia cells were available before and during treatment with the BCR-ABL1 kinase-inhibitor STI571 (Imatinib). Comparing leukemia cell samples before and during STI571-therapy by flow cytometry, inhibition of BCR-ABL1 kinase activity resulted in downregulation of the myeloid antigens GM-CSFRα, IL3Rα and CD13 (Figure 2C). Conversely, STI571-treated leukemia cells upregulated IL7Rα surface expression. In agreement with this, we found that inhibition of BCR-ABL1 kinase activity during STI571-therapy results in increased mRNA levels of *IL7Rα* while mRNA levels of myeloid transcription factors *AML1*, *GATA1* and *CEBPα* were downregulated (Figure 2D).

BCR-ABL1 induces defective expression of IKAROS

Previous studies showed that loss of functional *IKAROS* prevents normal B cell development (Kirstetter et al. 2002, Georgopoulos et al. 1994). Since the expression of a dominant negative splice variant IK6 can inhibit B cell differentiation (Tonnelle et al. 2001), lineage infidelity in human pre-B lymphoblastic leukemia cells may result from BCR-ABL1-induced derangement of *IKAROS* pre-mRNA splicing leading to aberrant expression of IK6. Furthermore, the expression of dominant negative *IKAROS* splice variants lacking the DNA-binding domain was recently reported in both childhood and adult B cell lineage acute lymphoblastic leukemia (Sun et al., 1999; Nakase et al., 2000). Studying *IKAROS* isoform expression in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia, expression of dominant-negative IK6 was detected in 6 of 7 primary cases (Cases XIII to XIX in Klein et al., 2004) and two of three cell lines (in BV173 and SUP-B15 but not in Nalm1 cells).

Figure 2: *BCR-ABL1 kinase activity results in lineage infidelity and differentially regulates the expression of lymphoid versus myeloid-specific genes*



(A, B) A lymphoid derivative of the murine cell line TONB210 (Klucher et al., 1998) carrying an inducible BCR-ABL1 transgene under the control of a doxycycline-dependent promoter was used for inducible expression of a BCR-ABL1 transgene. The cells were cultured in the presence or absence of 1 µg/ml doxycycline (DOX). In the absence of BCR-ABL1 expression the cells remain viable in the presence of 2 ng/ml IL-3. BCR-ABL1 expression was induced by addition of 1 µg/ml doxycycline and verified by Western blot analysis (B). EIF4e was used as a loading control. mRNA levels for human *BCR-ABL1* and murine *Csfr1, Gata1, Pax5, Ikaros* splice variants, *Il7rα, Oct2, Obf1* and *Hprt* were monitored (A). From three leukemia patients, matched leukemia sample pairs (Patients I to III correspond to cases XIII,

Legend to Figure 2 (continued):

XIV and XVIII in Klein et al., 2004) before (white histograms) and during (grey histograms) therapy with the BCR-ABL1 kinase inhibitor STI571 were analyzed by flow cytometry for surface expression of GM-CSFRα, IL3Rα,CD13 and IL7Rα (C). One representative case out of three is shown. These patient samples were also subjected to RT-PCR analysis for mRNA expression of *IKAROS* splice variants, *PAX5, IL7Rα* and the myeloid transcription factors *AML1*, *GATA1, CEBPα* and normalized for cDNA amounts and leukemia cell content by amplification of *BCR-ABL1* and *GAPDH* cDNA fragments (D). Specificity of the inhibitory effect of STI571 on BCR-ABL1 with respect to B lymphoid versus myeloid lineage markers was tested using three *MLL-AF4*$^+$ leukemia cell lines (BEL1, RS4;11 and SEM) that did not carry a *BCR-ABL1* gene rearrangement yet exhibited a mixed lineage (lymphoid/ myeloid) phenotype. These three cell lines were cultured in the presence or absence of 10 µmol/l STI571 for 48 hours. STI571 had no effect on expression levels (carried out by FACS analysis) of myeloid-lineage related surface molecules GM-CSFRα, IL3Rα and CD13 and the lymphoid lineage marker IL7Rα (not shown). Primers used for semiquantitative RT-PCR analysis are listed in supplementary Table 1.

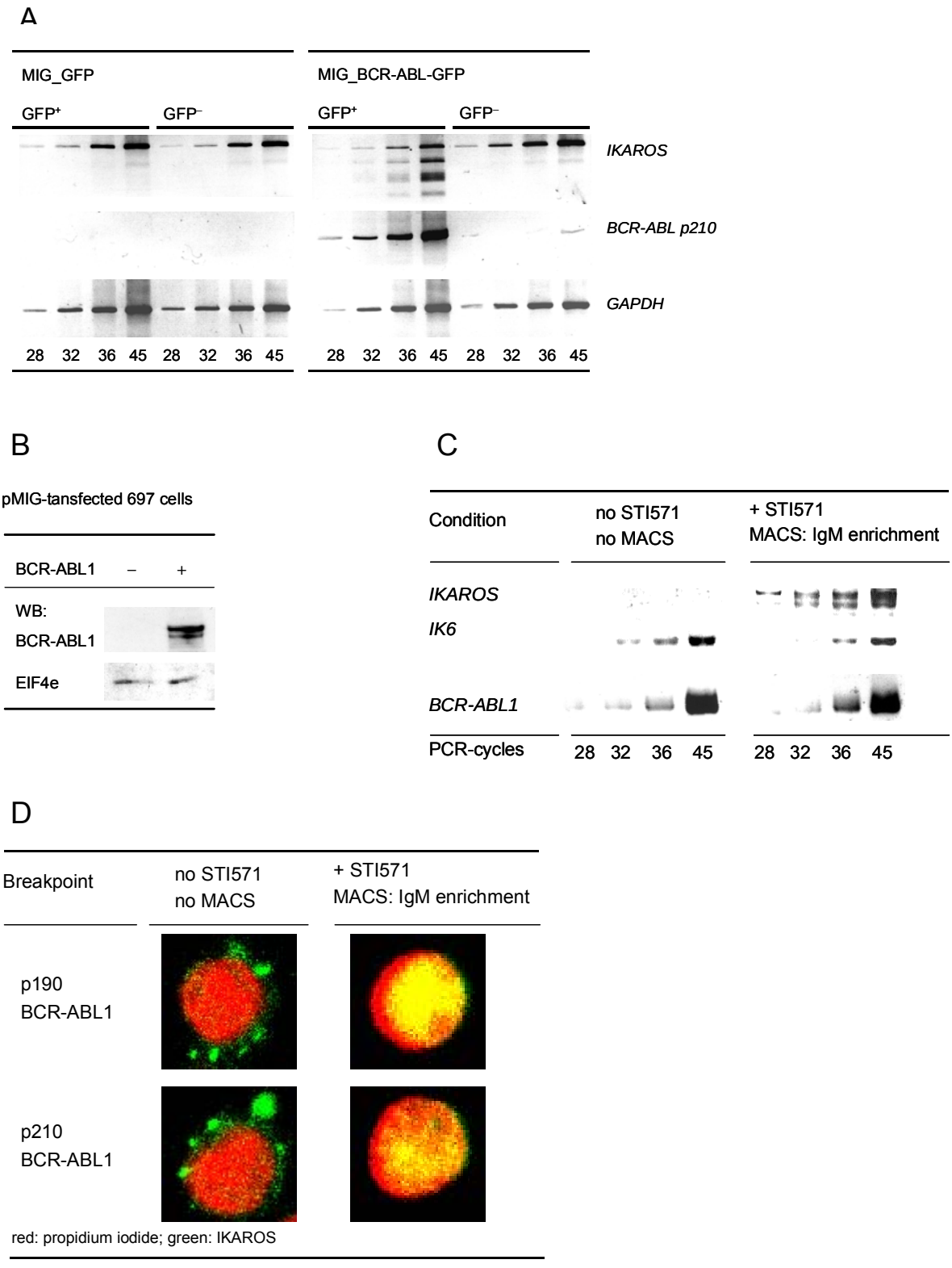
As shown by us and others, BCR-ABL1 can induce aberrant splicing of various genes (Perrotti and Calabretta, 2001) including *SLP65* (Jumaa et al., 2003; Klein et al., 2004), *PYK2* (Salesse et al., 2004) and BTK (Feldhahn et al., 2005). Consistent with BCR-ABL1-induced derangement of *IKAROS* pre-mRNA splicing, patient-derived leukemia cells express dominant-negative IKAROS (IK6), before but not during extended therapy with the BCR-ABL1 kinase inhibitor STI571 (Figure 2D).

To directly analyze the effect of BCR-ABL1 expression on *IKAROS* pre-mRNA splicing in human pre-B lymphoblastic leukemia cells, 697 cells that carry an *E2A-PBX1* but not a *BCR-ABL1* gene rearrangement and only exhibit expression of full-length *IKAROS*, were transiently transfected with expression vectors encoding either GFP only or BCR-ABL1 and GFP (Figure 3A). For both transfections, GFP$^+$ and GFP$^-$ cells were sorted and separately analyzed for the expression of *IKAROS* splice variants. Expression of the BCR-ABL1 kinase in transfected cells was verified by Western blot analysis (Figure 3B). As shown in Figure 3A, induced expression of BCR-ABL1 results in aberrant splicing of *IKAROS* leading to the expression of IK6.

Inhibition of BCR-ABL1 kinase corrects splicing and nuclear localization of IKAROS in pre-B lymphoblastic leukemia cells

Treatment of the leukemia cells with STI571 for four days induced selective outgrowth of differentiating subclones that downregulate IL3Rα expression and exhibit surface IgM expression (not shown), which indicates the pre-B to immature B cell transition (Klein et al., 2005). We studied mRNA expression of *IKAROS* in undifferentiated leukemia cells and in IgM$^+$ MACS-enriched differentiating subclones. Whereas undifferentiated leukemia cells

Figure 3: *BCR-ABL1 induces aberrant splicing of IKAROS in human pre-B lymphoblastic leukemia cells*



Legend to Figure 3:

(A) 697 pre-B lymphoblastic leukemia cells carrying an *E2A-PBX1* gene rearrangement were transiently transfected through electroporation with an expression vector encoding only GFP (pMIG_GFP) or BCR-ABL1 and GFP (pMIG_BCR-ABL1/GFP). Expression of the BCR-ABL1 kinase was identified by Western blot analysis (B). EIF4e was used as loading control. 24 hours after electroporation, for each transfection, GFP^+ and GFP^- cells were sorted, subjected to RNA isolation, cDNA synthesis and analyzed for the expression of *IKAROS* splice variants (A). cDNA amounts were normalized for *COX6B* mRNA levels.

BCR-ABL1$^+$ pre-B lymphoblastic leukemia cells (BV173) were cultured in the presence or absence of 10 µmol/l STI571 for four days. Differentiating IgM^+ subclones were enriched by MACS as previously described (Klein et al., 2004). Undifferentiated *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells (IgM^-) and differentiating subclones (IgM^+) were subjected to RT-PCR analysis for *IKAROS* isoform expression (C). *IK6* expression (*IKAROS* splice variant lacking exons 3 to 6) was identified by sequence analysis. (Sequence data is available from EMBL/GenBank under accession number, AM085310). cDNA amounts were normalized by amplification of *BCR-ABL1* fusion transcripts. Localization of IKAROS (D) was studied by confocal laser microscopy in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells (BV173 and SUP-B15) expressing either a p210 or p190 BCR-ABL1 fusion molecule, respectively. For localization of IKAROS, undifferentiated and MACS-enriched IgM^+ differentiating subclones were stained with propidium iodide (nucleus) and with anti-IKAROS antibodies. Nuclear or cytoplasmic localization of IKAROS was analyzed using primary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) together with anti-rabbit IgG-Cy2 (Jackson Immunoresearch). Cells were fixed with 0.4% para-formaldehyde and incubated for 10 minutes in 90% methanol on ice and subjected to confocal laser-scanning microscopy as previously described (Klein et al., 2003).

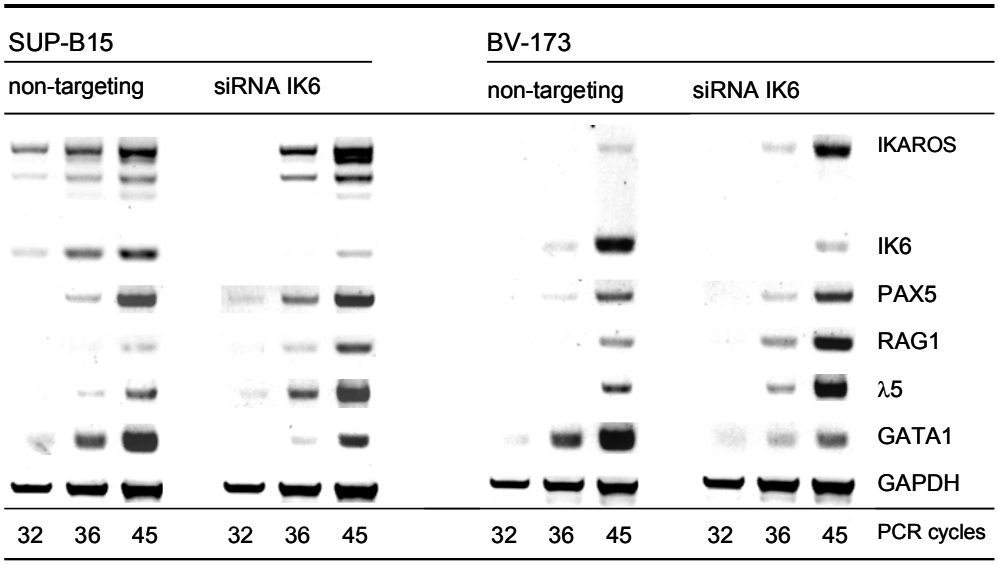
predominantly express dominant negative IK6, expression of functional IKAROS transcripts was largely restored in IgM^+ differentiating subclones (Figure 3C).

While nuclear localization of IKAROS is critical for its function as a transcription factor, in two untreated leukemia cell lines expressing either p190 or p210 BCR-ABL1 proteins, IKAROS protein is localized in the cytoplasm, which is consistent with the expression of non-DNA binding IK6 (Figure 3D). However, differentiating subclones that were MACS-enriched for IgM-expression, exhibit a nuclear pattern of staining for IKAROS, which is consistent with expression of DNA-binding forms of IKAROS (Figure 3D).

Silencing of dominant negative IK6 partially restores B lymphoid lineage commitment

Expression of dominant-negative IK6 can effectively block B cell differentiation of hematopoietic progenitor cells from umbilical cord blood (Tonnelle et al., 2001). In the absence of functional IKAROS, hematopoietic stem cells cannot give rise to lymphoid cells and are exclusively diverted into the myeloid lineages (Georgopoulos et al., 1994). In order to provide evidence for a possible link between the expression of IK6 and lineage infidelity in pre-B lymphoblatic leukaemia cells, we silenced IK6 expression in two *BCR-ABL1*$^+$ pre-B

Figure 4: *Specific silencing of IK6 partially restores lineage determination in BCR-ABL1$^+$ pre-B leukemia cells.*



BCR-ABL1$^+$ pre-B lymphoblastic leukemia cell lines (SUP-B15 and BV173) were transfected either with a pool of siRNA duplicies targeting IK6 or a non-targeting siRNA duplex serving as a negative control. Three different IK6 siRNAs were designed and synthesized (MWG Biotech, Ebersberg, Germany) to target the junction of exon2 and exon7, which defines the *IKAROS* splice variant IK6 (sequence data available from EMBL/GenBank under accession number, pending). The control non-targeting siRNA duplex did not match a known mRNA sequence. The IK6-specific siRNA duplexes were designed according to the guidelines described by Dr. Thomas Tuschl at http://www.rockefeller.edu/labheads/tuschl/sirna.html. All siRNA duplices were labeled with Cy2 using an siRNA labeling kit (Ambion, Austin, TX) according to the manufacturer's protocol. siRNAs were applied to the cells with a final concentration of 100 nmol/l for each siRNA, using Oligofectamine (Invitrogen) in Opti-MEM1 medium (Invitrogen) according to the manufacturer's instructions. After 24 hours, leukemia cells were re-transfected with labeled siRNAs and subsequently incubated for further 24 hours. The silencing effect of siRNAs for IKAROS isoform IK6 was controlled by RT-PCR analysis of IK6 in Cy2$^+$ sorted cells. siRNA transfected cells were subjected to RT-PCR analysis as previously described and investigated for the expression of *IKAROS, IK6, PAX5, RAG1, 5, GATA1* and *GAPDH*. Oligonucleotides used for semiquantitative RT-PCR analysis and siRNA oligonucleotides are listed in supplementary Table 1.

lymphoblastic leukemia cell lines (SUP-B15 and BV173). Three different siRNA duplices against IK6 and one non-targeting siRNA duplex were used in transfection experiments. All siRNA duplices were labeled with a Cy2 fluorochrome to identify transfected cells. Cy2$^+$ cells carrying siRNAs were sorted by FACS and subjected to RT-PCR analysis. In both cell lines, *IK6* expression was significantly diminished (Figure 4). Using cells transfected with a non-targeting siRNA duplex as a reference, mRNA levels for the V(D)J-recombinase molecule

RAG1 and the B cell specific component of the surrogate light chain *λ5* where increased, while mRNA levels of the myeloid transcription factor *GATA1* were reduced in parallel with *IK6*. However, no differences were detected for the mRNA expression levels of *PAX5* (Figure 4). Specificity of the IK6-targeting siRNA duplices were controlled by co-amplification of *GAPDH*, mRNA levels of which remained stable (Figure 4). We conclude that BCR-ABL1-induced expression of IK6 contributes to lineage infidelity observed in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells. However, we cannot exclude that also other molecules than IK6 also contribute BCR-ABL1-induced loss of lineage determination.

Taken together, these findings show that BCR-ABL1 kinase activity i.) induces lineage infidelity in pre-B lymphoblastic leukemia cells ii.) interferes with IKAROS pre-mRNA splicing and iii.) that aberrant expression of IK6 contributes to lineage infidelity. Therefore, we propose that defective lineage commitment and B cell differentiation in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells can occur as a consequence of BCR-ABL1-induced derangement of *IKAROS*-expression.

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5.3 Tracing the pre-B to immature B cell transition in human leukemia cells reveals a coordinated sequence of primary and secondary *IGK* gene rearrangement, *IGK* deletion and *IGL* gene rearrangement

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Summary

The BCR-ABL1 kinase expressed in acute lymphoblastic leukemia (ALL) drives malignant transformation of pre-B cells and prevents further development. We studied whether inhibition of BCR-ABL1 kinase activity using STI571 can relieve this differentiation block. STI571 treatment of leukemia patients induced expression of the Ig light chain-associated transcription factors *IRF4* and *SPIB*, upregulation of RAG1 and RAG2, Cκ and Cλ germline transcription and rearrangement of *IGK* and *IGL* genes. However, STI571-treated pre-B ALL cells expressed λ- but almost no κ-light chains. This could be explained by STI571-induced rearrangement of the κ-deleting element (KDE), which can delete productively rearranged Vκ-Jκ joints. Amplifying double-strand breaks at recombination signal sequences within the *IGK*, *KDE* and *IGL* loci revealed a coordinated sequence of rearrangement events induced by STI571: Recombination of *IGK* gene segments is already initiated within one hour after STI571-treatment, followed by KDE-mediated deletion of Vκ-Jκ-joints six hours later and, ultimately, *IGL* gene rearrangement after 12 hours. Consistently, upregulation of Cκ and Cλ germline transcripts, indicating opening of *IGK* and *IGL* loci, was detected after one and six hours for *IGK* and *IGL*, respectively. Continued activity of the recombination machinery induced secondary *IGK* gene rearrangements, which shifted preferential usage of upstream located Jκ- to downstream Jκ-

gene segments. Thus, inhibition of BCR-ABL1 in pre-B ALL cells i.) recapitulates early B cell development, ii.) directly shows that *IGK*, *KDE* and *IGL* genes are rearranged in sequential order and iii.) provides a model for Ig light chain gene regulation in the human.

Introduction

B cell precursors within the bone marrow undergo a sequence of immunoglobulin (Ig) gene rearrangements defining distinct stages of early B cell differentiation (1). During their early development, B cell precursors have to pass checkpoints at which only cells carrying functional Ig gene rearrangements are selected for further development along the B cell lineage (2). For instance, the presence of a productive *IGH* gene rearrangement is a prerequisite for the expression of the Ig μ heavy chain as a component of the pre-B cell receptor on the surface of a pre-B cell. As soon as a B cell precursor successfully rearranges V_H, D_H and J_H gene segments on one allele, the recombination machinery is halted, preventing further rearrangement of the second allele, which constitutes allelic exclusion at the *IGH* locus (3). Termination of the rearrangement process at the *IGH* locus is followed by the initiation of Ig light chain gene recombination at the *IGK* and *IGL* loci, which defines the pre-B to immature B cell transition. Human B cells express more frequently κ- than λ-light chains, at a κ/λ ratio of about 1.4 (4). To explain the relative overrepresentation of *IGK* gene rearrangements, which is even more striking in mice, two models have been proposed: While the 'stochastic model' (5, 6) postulates that *IGK* and *IGL* loci are independently accessible to recombinase activity and that cis-acting regulatory elements (e.g. the 3' κ-enhancer; references 7, 8) determine the ratio of κ- and λ-light chain expressing cells. This model was supported by the finding that λ-expressing B cells could arise from mice carrying inactivated *IGK* loci on both alleles (9, 10). On the contrary, the 'ordered model' proposes that *IGK* genes, as a rule, rearrange prior to *IGL* gene recombination (11, 12). Consistent with an ordered model of *IGK* and *IGL* gene rearrangement, recent work showed that single human λ-expressing B cells (13) or B cell precursor leukemia clones (14) harbored inactivated Vκ-Jκ gene rearrangements almost in all instances in addition to productive Vλ-Jλ joints but not vice versa: only few κ-expressing B cells or leukemic B cell clones also carried inactive Vλ-Jλ joints in addition to productive Vκ-Jκ gene rearrangements. These findings argue for sequential rearrangement of *IGK* and *IGL* genes. However, these studies do not formally demonstrate that rearrangement of *IGK* genes in λ-expressing B cells do necessarily precede successful Vλ-Jλ gene recombination. In λ-expressing B cells, for instance, *IGL* genes may

well be rearranged first, followed by needless Vκ-Jκ gene rearrangements, which then would subsequently be inactivated by rearrangement of the κ-deleting element (KDE). The KDE may rearrange to recombination signal sequences (RSS) flanking germline Vκ gene segments or an RSS within the intron between the Jκ cluster and the Cκ gene, which leads to inactivation of a preexisting Vκ-Jκ joint in either case (15). Also the existence of B cells expressing κ- together with λ-light chains (16) suggests that recombination events in the *IGK* and *IGL* loci are not mutually exclusive in all instances. Instead of κ- or λ-light chains, human pre-B cells express surrogate light chains composed of VpreB and λ5.

In the vast majority of acute lymphoblastic leukemia (ALL), the malignant clone represents the outgrowth of a transformed pre-B cell. In many cases, pre-B ALL clones carry specific oncogenic gene rearrangements defining both biological and clinical subentities (17). Among these translocation events, the t(9;22)(q34;q11) results in a fusion of the *BCR* and *ABL1* genes, which codes for a constitutive active variant of the ABL1 tyrosine kinase (18) and represents the most frequent recurrent aberration leading to ALL in adults (19). *ABL1* represents the human homologue of the transforming gene of the Abelson murine leukemia virus, v-abl. While v-abl has been used for many years to transform murine pre-B cells, recent work showed that v-abl also confers a differentiation block at the pre-B cell stage of development and, hence, prevents rearrangement of *IGK* or *IGL* light chain genes (20, 21). Recently, a specifically designed inhibitor of BCR-ABL1, termed STI571, has become available and is now widely used as anti-leukemia drug for BCR-ABL1$^+$ leukemias (22). We, therefore, investigated whether and in which way inhibition of BCR-ABL1 kinase activity by STI571 might induce differentiation including *IGK* and *IGL* gene rearrangement in pre-B ALL cells.

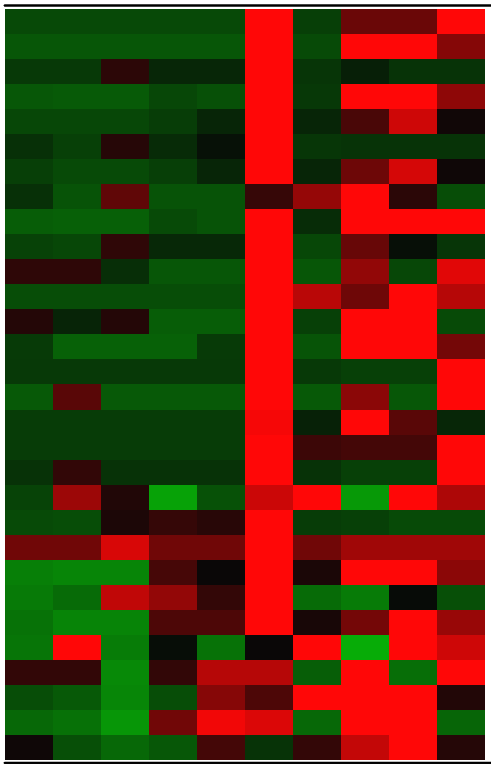
Results and Discussion

Regulation of genes implicated in Ig-light chain expression by BCR-ABL1

Comparing genome-wide gene expression profiles from normal bone marrow pre-B cells with two cases of *BCR-ABL1*$^+$ pre-B ALL generated by the serial analysis of gene expression (SAGE) technique, we identified 16,786 individual transcripts (unique tags) in pre-B cells (24) and each about 9,200 in the pre-B ALL cases respectively. In order to elucidate transcriptional regulation during the pre-B to immature B cell transition, we identified 182 genes, for which a specific role in early B cell development is known (24, 25, 30). Among these genes, transcription factors, such as *PAX5, E2A* and *EBF* and genes coding for (pre-) B

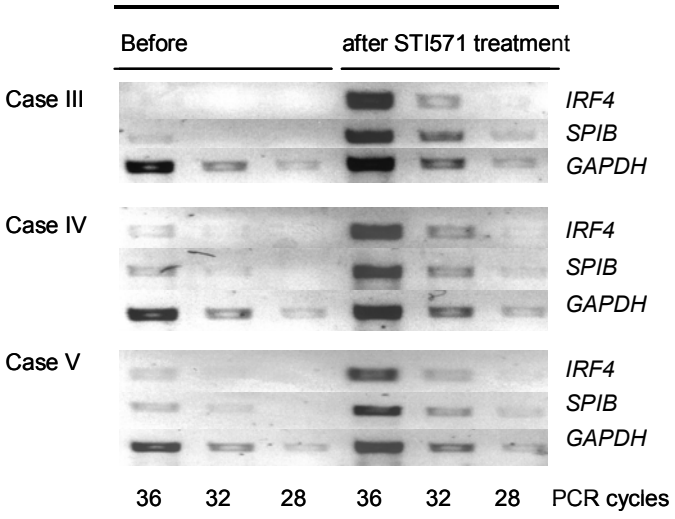
Figure 1: *mRNA levels of Ig light chain gene-associated genes in BCR-ABL1⁺ pre-B ALL cells*

A



Gene	Proposed function in light chain regulation	Reference
IRF4	required for *IGK* and *IGL* gene rearrangement;	Eisenbeis *et al.*, 1995
SPIB	activates *IGK* and *IGL* transcription;	Su *et al.*, 1996
RAG2	recombination activating gene;	Schatz *et al.*, 1989
OBF1	required for recombination of some *IGK* genes;	Casellas *et al.*, 2002
BLNK	increases IGK light chain production;	Xu and Lam, 2002
RAG1	recombination activating gene;	Schatz *et al.*, 1989
BTK	increases *IGL* gene rearrangement;	Dingjan *et al.*, 2001
IRF8	required for *IGK* and *IGL* gene rearrangement;	Lu *et al.*, 2003
Igα	opens *IGK* and *IGL* loci for rearrangement;	Maki *et al.*, 2000
E2A	activates *IGK* and *IGL* gene transcription;	Kee and Murre, *1998*
RASA1	induces *IGK* and *IGL* gene rearrangement;	Shaw *et al.*, 1999
AP4	activates *IGK* gene transcription;	Aranburu *et al.*, 2001
KRAS2	induces *IGK* and *IGL* gene rearrangement;	Shaw *et al.*, 1999
PU.1	activates *IGK* and *IGL* gene transcription;	Schwarzenbach *et al.*, 1995
MEF2B	activates *IGK* and *IGL* gene transcription;	Satyaraj and Storb, 1998
CREM	activates *IGK* and *IGL* gene transcription;	Pongubala and Atchison, 1995
HRAS	induces *IGK* and *IGL* gene rearrangement;	Shaw *et al.*, 1999
MEF2A	activates *IGK* and *IGL* gene transcription;	Satyaraj and Storb, 1998
ATF1	activates *IGK* and *IGL* gene transcription;	Pongubala and Atchison, 1995
JUN	activates *IGK* and *IGL* gene transcription;	Schanke *et al.*,1994
TdT	N-nucleotide addition	Landau *et al.*, 1987
NRAS	induces *IGK* and *IGL* gene rearrangement;	Shaw *et al.*, 1999
Igβ	opens *IGK* and *IGL* loci for rearrangement;	Maki *et al.*, 2000
NF-κB2	activates *IGK (*and *IGL)* gene transcription;	Sen and Baltimore, 1986
REL-B	activates *IGK (*and *IGL)* gene transcription;	Sen and Baltimore, 1986
FOS	activates *IGK* and *IGL* gene transcription;	Schanke *et al.*,1994
REL-A	activates *IGK (*and *IGL)* gene transcription;	Sen and Baltimore, 1986
POLμ	required for *IGK* and *IGL* gene rearrangement;	Satyaraj and Storb, 1998
NF-κB1	activates *IGK (*and *IGL)* gene transcription;	Sen and Baltimore, 1986
YY1	represses *IGK* gene transcription;	Park and Atchinson, 1991

B



(A) Focusing on thirty genes implicated in the regulation of Ig light chain expression, genome-wide SAGE-profiles of two *BCR-ABL1*⁺ pre-B ALL cases (ALL1 and ALL2) were compared to SAGE-profiles of normal CD34⁺ CD38^{low} hematopoietic progenitor cells (HSC), CD15⁺ common myeloid progenitor cells (CMP), CD7⁺ CD10⁺ T lymphoid progenitor cells (TLP), CD10⁺ CD19⁺ pre-B cells (PBC), CD19⁺ CD27⁻ naïve B cells (NBC), CD20⁺ CD77⁺ germinal center B cells (GCB), CD19⁺ CD27⁺ memory B cells (MBC) and CD19^{low} CD138⁺ plasma cells (PC). SAGE-tag counts are visualized by colors, red indicating high and green depicting low levels or no expression, respectively. SAGE data were sorted according to the ratio of SAGE-tag counts in normal B cell subsets to leukemia samples. (B) From three patients with *BCR-ABL1*⁺ pre-B

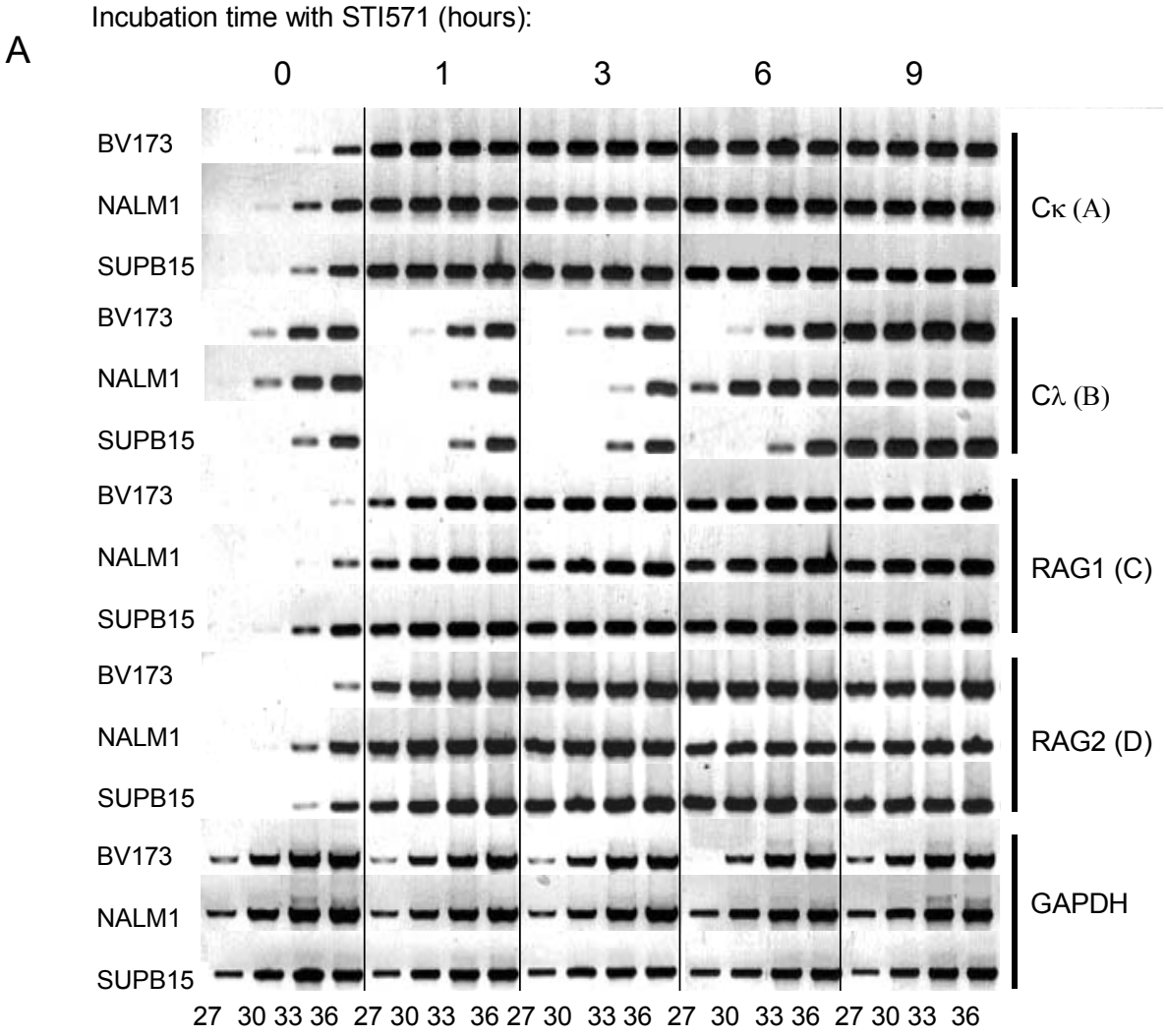
Legend to Figure 1 (continued):

ALL, leukemia samples were available before and after treatment with the BCR-ABL1 kinase inhibitor STI571. In these matched leukemia sample pairs, mRNA levels of *IRF4* and *SPIB* were compared by semiquantitative RT-PCR. cDNA amounts were normalized by OD measurements and amplification of a specific fragement of the housekeeping gene *GAPDH*.

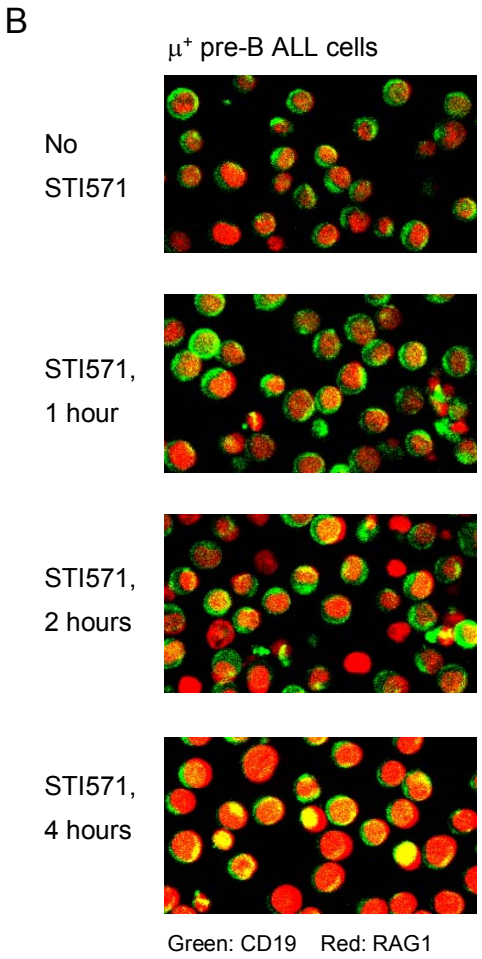
cell receptor signaling molecules (e.g. *LYN, BTK, SYK, FYN)* are silenced in the leukemia cells (30). Furthermore, we focused on a subset of 30 genes, which are specifically implicated in Ig light chain regulation (Figure 1). Here, transcription factors that positively regulate Ig light chain gene transcription (*IRF4*, *SPIB*, *OBF1*, *IRF8*, *E2A*, *AP4*, *PU.1*, *CREM*, *MEF2*, *ATF1*, *JUN*) are downregulated in the leukemia cells compared to normal pre-B cells, which argues for a differentiation block at the pre-B cell to immature B cell transition. However, besides genes implicated in Ig light chain gene rearrangement (pre-B to immature B cell transition), also genes coding for components of the pre-B cell receptor (μ-chain: 565 tags in pre-B cells and 48 and 148 tags in leukemia cells (two cases in Figure 1); VpreB: 278 tags in pre-B cells and 4 and 12 tags in leukemia cells; λ5: 1,932 tags in pre-B cells and 12 and 20 tags in leukemia cells) at the pro-B to pre-B cell transition are downregulated in the leukemia cells. This may suggest that in the leukemia cells, expression and function of the pre-B cell receptor is impaired in general, including its specific function at the pre-B cell to immature B cell checkpoint (2). We next investigated whether downregulation of the Ig light chain-associated transcription factors *IRF4* and *SPIB* is indeed linked to BCR-ABL1 kinase activity. To this end, we analysed primary *BCR-ABL1*$^+$ leukemia samples, which were derived from three patients before and after treatment with the BCR-ABL1-inhibitor STI571 (cases III, IV, V, reference 30, Figure 1B). Comparing matched pairs of patient-derived leukemia cells, *IRF4* and *SPIB* mRNA levels were increased when BCR-ABL1 kinase activity was blocked by STI571.

Before rearrangement of Ig light chain genes can be initiated, *IGK* and *IGL* loci have to be opened to become accessible to the recombination machinery. Therefore, Cκ and Cλ germline transcription indicating transcriptional activation of Ig light chain loci was analyzed by RT-PCR in *BCR-ABL1*$^+$ cell lines treated with or without STI571 (Figure 2A). Upon inhibition of BCR-ABL1, germline transcription of Cκ was upregulated already after one hour, followed by Cλ germline transcription after six hours. However, low germline transcription activity at both loci could also be detected in the absence of STI571. These data suggest that *IGK* and *IGL* light chain loci become in sequential order accessible to Ig-specific transcription factors and the recombination machinery.

Figure 2: *Inhibition of BCR-ABL1 by STI571 results in C κ and C λ germline transcription after 1 and 6 - 9 hours respectively (A) and rapid upregulation of RAG1/2 (A,B)*



Three *BCR-ABL1*$^+$ pre-B ALL cell lines were incubated in the presence or absence of 10 µmol/L STI571 for the times indicated and analyzed for the expression of Cκ and Cλ germline transcription, *RAG1*, *RAG2* and *GAPDH* (control) by RT-PCR (A). Nuclear RAG expression in individual leukemia cells was measured by staining for CD19 (green) and intracellular RAG1 expression (red; B).



As constituents of the recombination machinery, the expression of recombination activation genes, *RAG1* and *RAG2*, was analyzed by RT-PCR (Figure 2A). mRNA levels for both *RAG* genes were significantly upregulated after one hour of STI571-treatment. In order to investigate RAG1 expression in individual cells, RAG1 protein was stained by immunofluorescence in a leukemia cell line (SUP-B15), from which we previously amplified a productive $V_H3.53$-$D_H2.8$-J_H6 Ig heavy chain gene rearrangement (29), in the presence or absence of the BCR-ABL1-inhibitor STI571. While some cells already exhibit nuclear RAG1 expression before STI571-treatment, RAG1 protein levels were visibly upregulated after two hours in some and after four hours in almost all cells (Figure 2B).

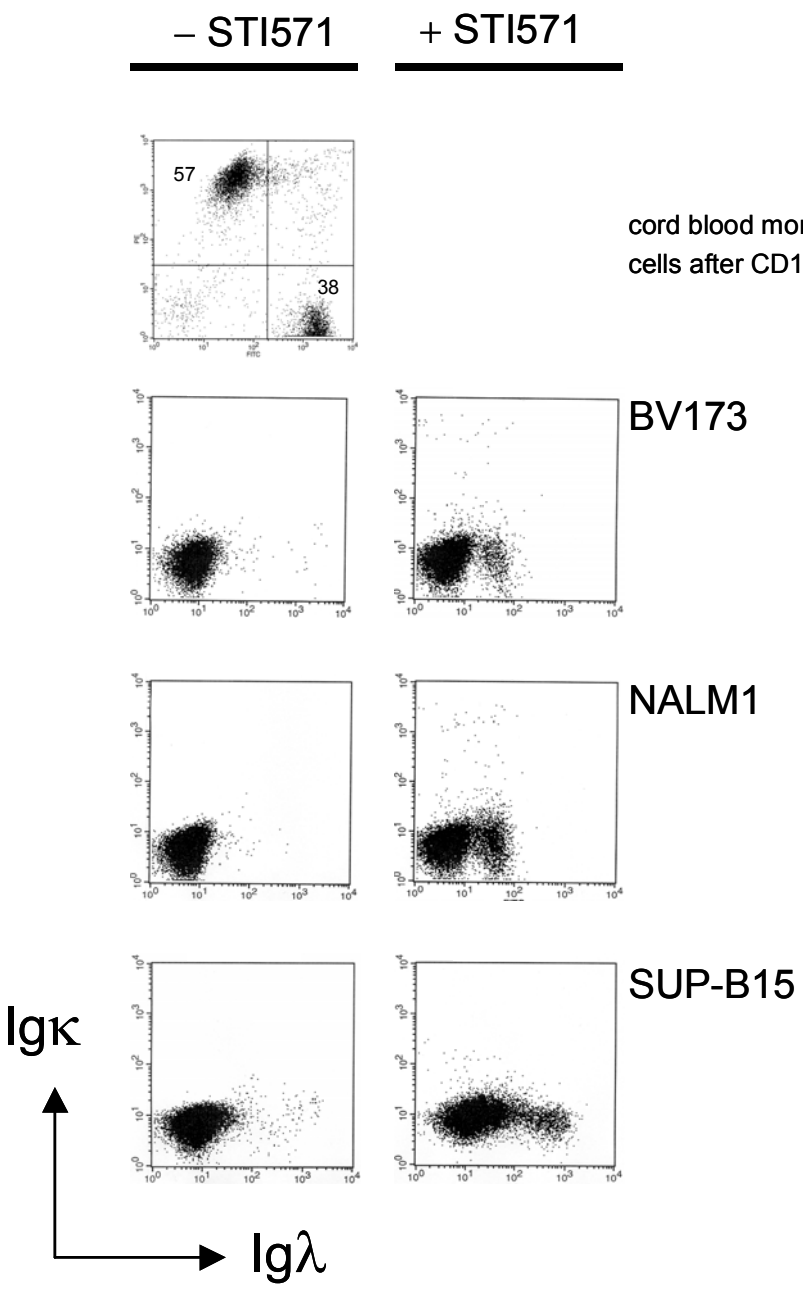
To determine whether upregulation of Ig-light chain-associated transcription factors *IRF4* and *SPIB*, increased expression of RAG1 and RAG2 and Cκ and Cλ germline transcription is followed by the expression of conventional light chains, we analyzed three *BCR-ABL1*$^+$ pre-B ALL cell lines for κ and λ light chain expression in the presence or absence of STI571 by flow cytometry (Figure 3). After 48 hours of STI571 treatment, more than 50 percent of the cells had already undergone apoptosis and were excluded from the

analysis. Apoptotic or dead cells were identified by annexin V membrane expression and uptake of propidium iodide. Among the surviving cells, about 10 percent exhibit *de novo* Ig light chain expression (Figure 3). Unexpectedly, BCR-ABL1-inhibition resulted in a pattern of light chain expression heavily biased for λ-light chains (Figure 3). Only a few κ-light chain-producing leukemia cells were detectable, while the anti-κ antibody used clearly identified normal κ-expressing B cells (Figure 3). For control stainings, B cells from umbilical cord blood were used that were enriched by MACS for CD19 expression (Figure 3).

Extensive κ-deleting element rearrangement results in preferential λ-light chain expression on STI571-surviving leukemia cells

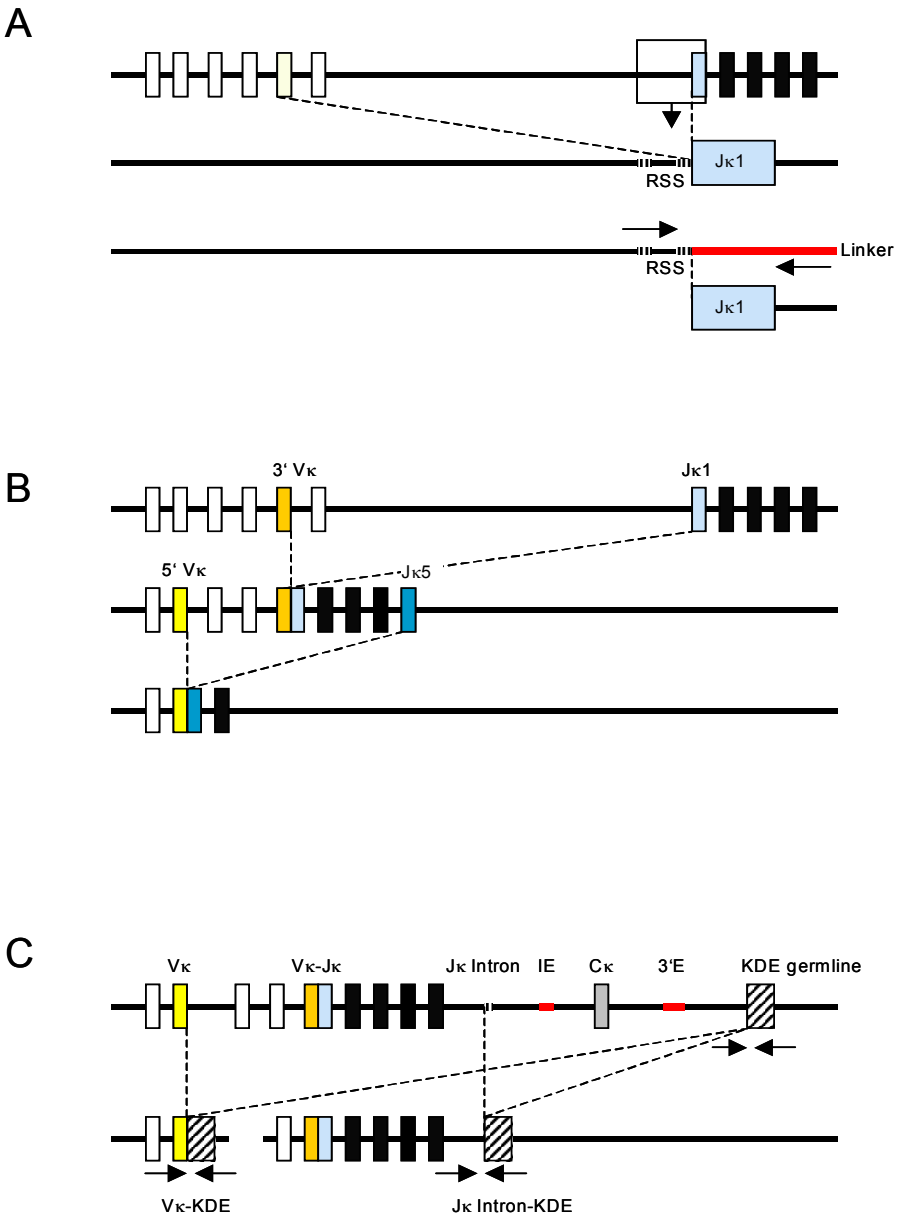
Using three different PCR strategies (Figure 4), we investigated at which level the expression of κ light chains may have been impaired. By ligation-mediated PCR (LM-PCR; Figure 4A), we first showed that inhibition of BCR-ABL1 kinase activity induces DNA double-strand breaks at recombination signal sequences (RSS) within the *IGK* locus after eight hours (Figure 5A). For validation of the LM-PCR assay, PCR products were cloned and five randomly chosen clones were sequenced. Sequence analysis confirmed Jκ1 RSS-linker ligation products (not shown). Such RSS-specific DNA strand breaks represent an initial step within the recombination process of *IGK* genes and demonstrate that the *IGK* locus is indeed targeted by the recombination machinery upon treatment with STI571. Since Jκ-RSS-specific DNA strand breaks represent a pre-condition for Vκ-Jκ gene rearrangements, we next amplified Vκ-Jκ rearrangements from genomic DNA of B cell precursor leukemia cells treated with or without STI571 (Figure 4B). While sporadic Vκ-Jκ and Vλ-Jλ joints were already detectable under control conditions, V-J rearrangements at both *IGK* and *IGL* loci were clearly inducible by STI571 (Figure 5B). As a specificity control, genomic DNA of *BCR-ABL1*$^{+}$ chronic myeloid leukemia cells was also subjected to amplification of Vκ-Jκ and Vλ-Jλ rearrangements but no product was obtained. Integrity of DNA isolated from chronic myeloid leukemia cells was verified by amplification of a Cβ1 germline fragment of the TCRβ locus (Figure 5B). In agreement with a previous study on the effect of STI571 on v-abl-transformed murine pre-B cell lines (21), we observed a polyclonal pattern of Vκ and Vλ gene rearrangement upon STI571-treatment. STI571-induced V-J gene rearrangements involved all six *IGK* and all nine *IGL* V gene families. Whether *IGK* and *IGL* genes were also rearranged at similar frequencies in response to STI571-treatment, however, remains unclear, because the PCR approach used here was not quantitative

Figure 3: *Ig light chain expression resulting from BCR-ABL1 inhibition is heavily biased for λ light chains*



BCR-ABL1$^+$ BV173 cells, NALM1 cells and SUP-B15 cells were cultured in the presence or absence of 10 µmol/l STI571 for two days and surviving cells were analyzed for surface expression of κ and λ light chains by flow cytometry. B lymphocytes from umbilical cord blood were enriched by MACS and used as a positive control for κ and λ staining.

Figure 4: *Molecular analysis of recombination events within the IGK locus*



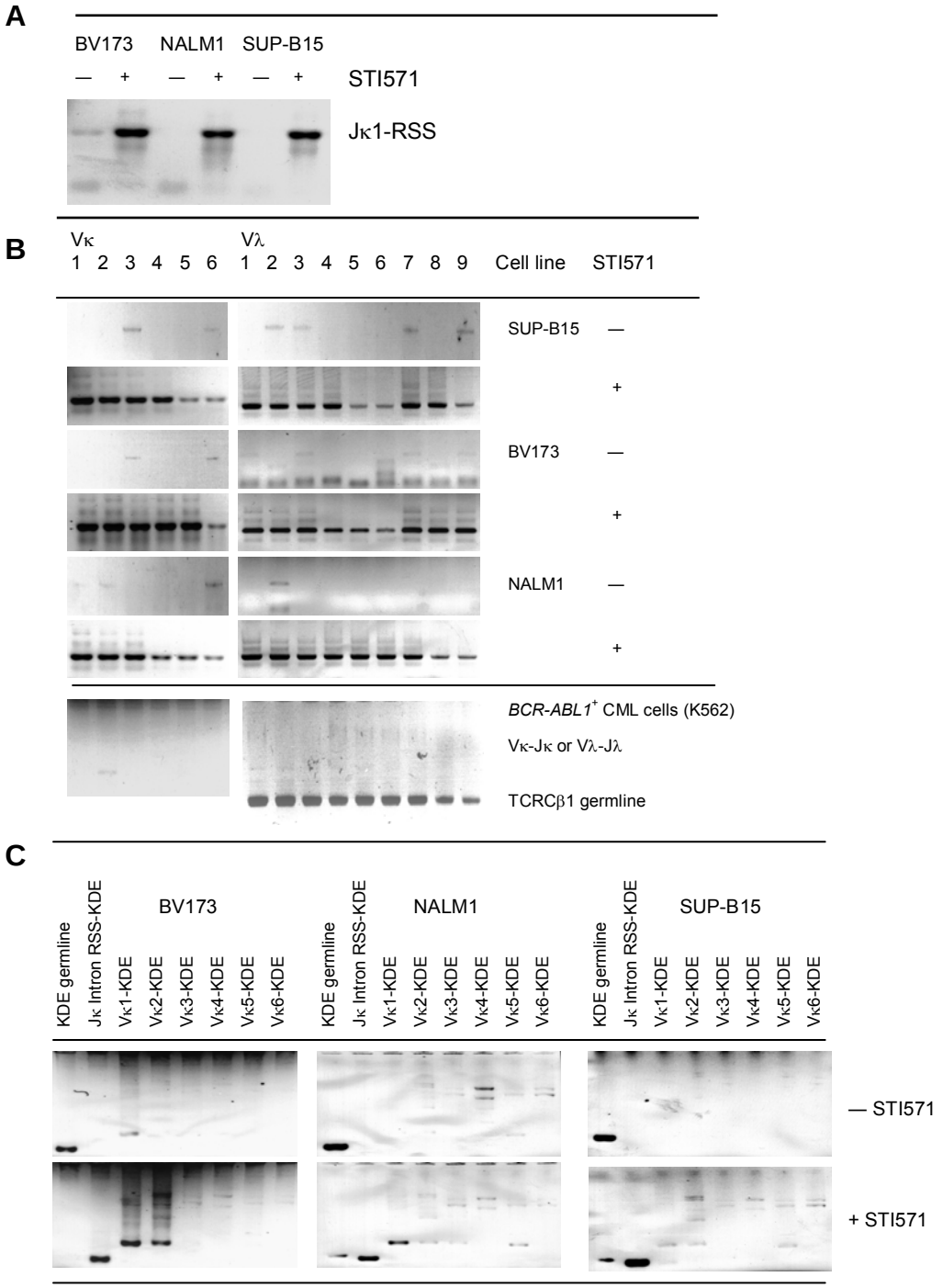
(A) During primary recombination events, a Vκ gene segment rearranges to a (mostly upstream located) Jκ gene segment. In secondary rearrangements, pre-existing Vκ–Jκ joints are deleted by juxtaposition of more upstream V to a more downstream J element. (B) The rearrangement of Vκ to Jκ segments is initiated by DNA double-strand breaks at the heptamer RSS sequence (RSS) immediately flanking the Jκ segment. DNA double-strand breaks result in hairpin-formation at the Jκ-break and 5’ phosphorylated blunt ends at the RSS-break, which can be ligated to a blunt-ended linker molecule (Linker). Using PCR primers specific for sequences upstream of the RSS and the ligated linker sequence, broken-ended Jκ-RSS DNA intermediates can be amplified. (C) A preformed Vκ-Jκ-joint (top) can be inactivated by two types of rearrangement of the κ-deleting element (KDE). Using an upstream Vκ-RSS of an unrearranged Vκ-gene segment, rearrangement of the KDE results in a large deletion within the κ locus including downstream unrearranged Vκ-gene segments, the pre-existing Vκ-Jκ-joint, unrearranged Jκ-gene segments and the Cκ-gene (bottom, left). In alternative, the KDE can rearrange to an intronic RSS between the cluster of Jκ-gene segments and the Cκ gene. In this case, KDE-rearrangement results in a deletion of the Cκ-gene and both κ enhancers (bottom, right).

Rearrangement of the κ-deleting element (KDE) may lead to inactivation of productively recombined Vκ-Jκ joints and hence represents another level at which Ig light chain expression may be impaired (15). To assess whether pre-existing functional Vκ-Jκ joints may have been inactivated by rearrangement of the KDE, we analysed both types of KDE-recombination within the *IGK* locus (Figure 4C). The KDE can rearrange either to an RSS site within the intron region between the cluster of Jκ gene segments and the κ intronic enhancer or to an RSS site immediately flanking a Vκ gene segment (Figure 4C). KDE rearrangement leads to deletion of both κ enhancers and the Cκ gene in the former (Jκ intron RSS-KDE) or to deletion of a Vκ-Jκ joint in the latter case (Vκ RSS-KDE). To investigate whether KDE rearrangement may contribute to reduced κ light chain usage in STI571-treated leukemia cells, we amplified specific DNA fragments for KDE-germline configuration, Jκ intron RSS-KDE rearrangement and Vκ RSS-KDE rearrangement from three *BCR-ABL1*$^+$ leukemia cell lines (BV173, NALM1, SUP-B15) in the absence or presence of STI571 (Figure 5C). While KDE was found in germline configuration in untreated leukemia cells, STI571-mediated inhibition of BCR-ABL1 induced deletion of *IGK* alleles by rearrangement of the *KDE* to Jκ intron RSS sites and to Vκ RSS sites. Targeting of the *IGK* locus by the recombination machinery and generation of Vκ-Jκ joints appears to be normal, but overrepresentation of λ light chains can be attributed to extensive KDE rearrangement observed here (Figure 5C). In the absence of BCR-ABL1 kinase activity, differentiating leukemia cells seem to undergo multiple rounds of rearrangement targeting both κ and λ light chain loci, which ultimately leads to preferential λ light chain expression expression due to KDE-mediated deletion of potentially productive Vκ-Jκ joints.

Rearrangements at the IGK and IGL loci occur in sequential order

For this reason, we considered STI571-induced Ig light chain gene rearrangement as a model for the pre-B to immature B cell transition in normal B cell development and investigated whether *IGK*, *KDE* and *IGL* gene rearrangements follow a defined sequence of events. Sequential rearrangement of *IGK*, *KDE* and *IGL* genes would be predicted by the 'ordered model' of light chain gene recombination. To the contrary, according to the 'stochastic model' of light chain gene rearrangement, *IGK*, *KDE* and *IGL* genes would be recombined independently at each locus. Therefore, we incubated the three *BCR-ABL1*$^+$ pre-B ALL cell lines in the presence or absence of STI571 and analyzed genomic DNA for RSS-specific double-strand breaks within the *IGK*, *KDE* and *IGL* loci at different time points (Figure 6).

Figure 5: *Inhibition of BCR-ABL1 causes extensive rearrangement of κ and λ light chain genes including the κ-deleting element*



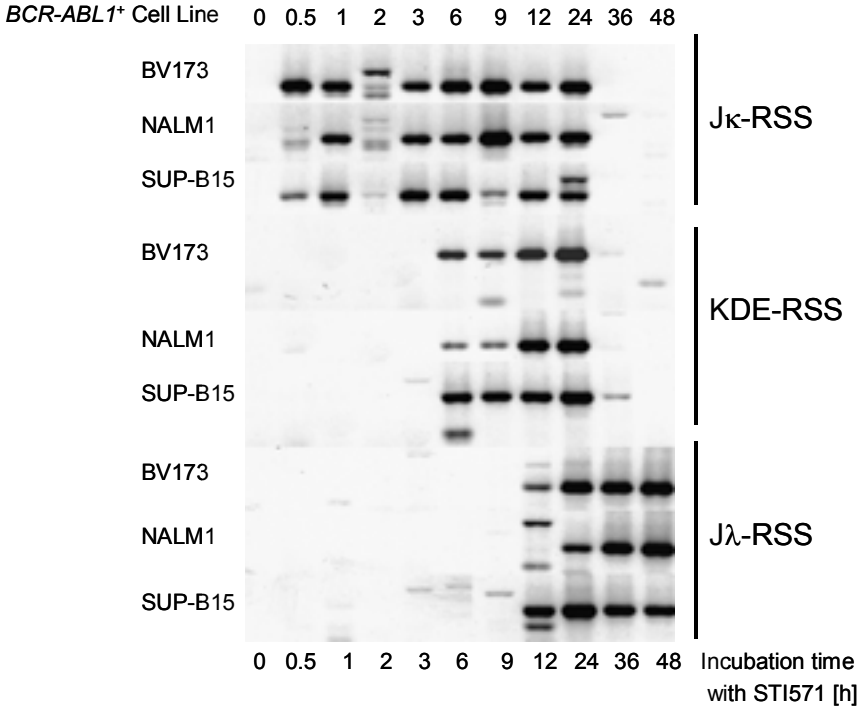
Using LM-PCR, we could amplify shortlived Jκ1-RSS DNA-double strand breaks predominantly in STI571-treated cells, which demonstrates active recombinase activity within the *IGK* loci (A). We next investigated Vκ-Jκ and Vλ-Jλ gene rearrangements in STI571-treated and -untreated leukemia cells. Comparing *IGK* and *IGL* loci, there was no indication of preferential Vλ-Jλ gene rearrangement (B). Amplifying specific fragments of KDE germline configuration, KDE rearrangement to an RSS within the Jκ intron and KDE rearrangement to an Vκ-associated RSS, we observed that the KDE was mainly in germline configuration in untreated leukemia cells, whereas STI571-treatment induced KDE rearrangement predominantly to the Jκ intron RSS (C).

In the three cell lines, RSS-specific DNA double-strand breaks were already detectable at the *IGK* locus within one hour after STI571-treatment. RSS-breaks flanking the *KDE* followed after six hours of STI571-treatment and RSS-DNA-breaks were found at the *IGL* locus only after 12 hours (Figure 6). These findings are consistent with a sequential order of rearrangement events: Ablation of BCR-ABL1 kinase activity almost immediately initiates *IGK* gene rearrangements, many of which are subsequently deleted by *KDE*- and ultimately replaced by *IGL* gene rearrangements (Figure 6). Of note, upregulation of Cλ germline transcripts did not precede the onset of KDE RSS-breaks (Figure 2A and 6). Unlike normal B cell development, only very few STI571-treated leukemia cells seem to stay at a κ^+ stage, while the vast majority of cells continue to rearrange, first *KDE* and ultimately *IGL* genes. It should be noted that between 12 and 24 hours of STI571-incubation, RSS-breaks could be detected concomitantly in *IGK*, *KDE* and *IGL* loci (Figure 6). Therefore, we cannot exclude that during this period of time, *IGL* gene rearrangements may have occurred before recombination within the *IGK* locus. Stability of RSS-strand break intermediates is cell-cycle dependent and mainly found in G_1 and G_0 phase (27). Indeed, STI571-treatment induces cell cycle arrest in *BCR-ABL1*$^+$ B cell precursor leukemia cells (30). Thus, the detection of Jκ-RSS breaks for an extended period of time (one to 24 hours), partially overlapping with KDE-RSS and even Jλ-RSS breaks, can be explained by cell cycle arrest and increased stability of RSS-breaks induced by STI571.

Indication for secondary IGK gene rearrangements

On the other hand, the relatively wide time frame during which *IGK* gene rearrangements can occur might reflect that the leukemia cells are not all synchronized with respect to STI571-induced *IGK* gene rearrangement. This is consistent with a rather heterogenous pattern of of RAG1 upregulation following treatment with STI571 (Figure 2). Moreover, RSS-specific DNA-strand breaks within the *IGK* locus do not necessarily reflect a single definitive Vκ-Jκ gene rearrangement and would also arise from any secondary recombination event during light chain revision (Figure 4B). To determine whether ongoing κ light chain editing may extend the time window for RSS-specific DNA-strand breaks, we searched for traces of secondary rearrangements within the *IGK* locus. A pre-existing Vκ-Jκ joint can only be replaced by a rearrangement which uses an upstream-located Vκ- together with a downstream-located Jκ segment (Figure 4B). Individual Vκ segments are not arranged in the order of Vκ gene families within the *IGK* locus. We, therefore, only compared the usage of upstream Jκ1 and Jκ2 segments to downstream Jκ5 elements (see Figure 4B) at different time points after STI571-induced initiation of light chain gene rearrangement. Consistent with a

Figure 6: *Sequentially ordered recombination events within the IGK, KDE and IGL loci*



BCR-ABL1$^+$ BV173, NALM1 and SUP-B15 cells were were cultured in the presence or absence of 10 μmol/l STI571 for the times indicated. Genomic DNA was isolated from 5 x 10^6 cells. Broken-ended DNA strand breaks were ligated to linker molecules and subjected to two rounds of PCR-amplification using primers specific for breaks at Jκ-RSS, KDE-RSS and Jλ-RSS sites.

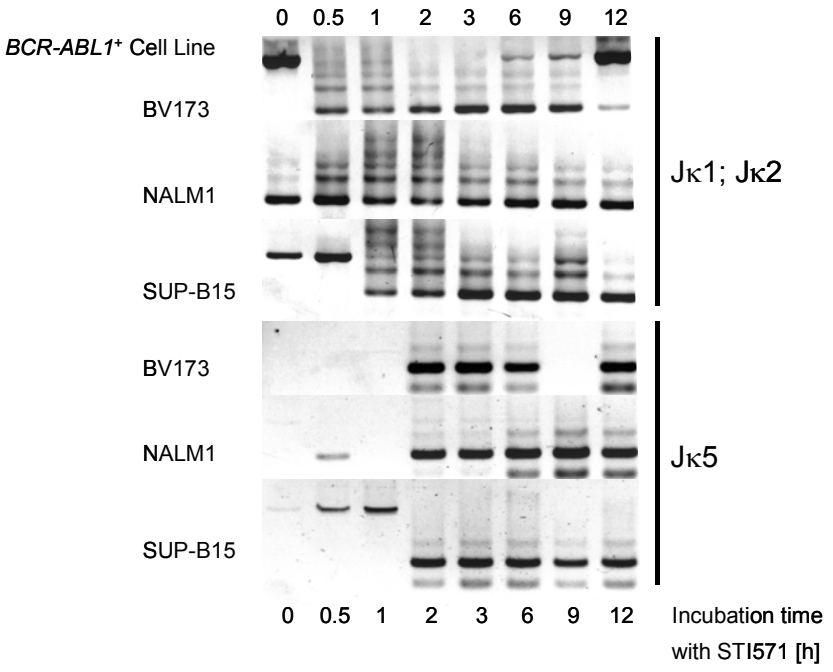
shift from upstream to downstream Jκ elements, Jκ1 and Jκ2 gene rearrangements were detected slightly earlier than rearrangements involving the Jκ5 gene segment (Figure 7). Given that the two round PCR approach used here for analysis of Vκ-Jκ gene rearrangements is not quantitative, the relative amount of PCR products does not necessarily reflect the usage of individual Jκ segments.

Concluding remarks

We conclude that inhibition of BCR-ABL1 initiates a coordinated sequence of Ig light chain gene rearrangement events: Almost immediately after ablation of BCR-ABL1 kinase activity by STI571, the *IGK* locus opens and is targeted by the recombination machinery. The continuous generation of new Jκ RSS-specific DNA strand breaks even after 24 hours together with a shift from upstream Jκ1 and Jκ2 elements to downstream Jκ5 segments suggests that initial Vκ-Jκ rearrangements are followed by at least one, perhaps multiple

rounds of secondary rearrangements. A possible initiation signal for subsequent *KDE* rearrangement could be exhaustion of the recombination potential of *IGK* alleles in multiple rounds of Vκ-Jκ rearrangement. Recombination of the *KDE* deletes Vκ-Jκ rearrangements from the chromosome and may initiate Vλ-Jλ gene rearrangements, which can first be detected after 12 hours. According to these observations, it is not surprising that the vast majority of leukemia subclones that have been primed to differentiate, ultimately express λ light chains on their surface (Figure 3). Obviously, the leukemia cells, unlike normal pre-B cells, are lacking a feedback mechanism which prompts them to halt the recombination machinery in the presence of a productively rearranged *IGK* allele. Upon inhibition of BCR-ABL1, the leukemia cells seem to enter a pre-determined ("ordered") sequence of rearrangement events, in which κ-expressing cells would only exist as a transition stage, inevitably leading to the expression of λ light chains.

Figure 7: *Shifting the preferential Jκ usage from upstream to downstream located Jκ gene segments indicates secondary IGK gene rearrangements*



Vκ1 gene rearrangements were amplified from three *BCR-ABL1*$^+$ pre-B ALL cell lines using Vκ1 leader- and Vκ1 framework region I-specific primers together with either primers matching to Jκ1 and Jκ2 or primers matching to Jκ5.

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Materials and Methods

Patient samples, primary cells and cell lines

BCR-ABL1$^+$ pre-B ALL cells from five patients were analysed. From three of these patients, leukemia cells were available before and after treatment with STI571 (Cases III, IV and V, reference 23). Samples of two other cases (Cases I and II) were used for SAGE analysis. Normal human CD10$^+$ VpreB$^+$ pre-B cells, CD34$^+$ CD38^{low} CD133$^+$ hematopoietic progenitor cells, CD15$^+$ CD34$^-$ myeloid progenitor cells, CD7$^+$ CD10$^+$ T lymphoid pogenitor cells and mature B cell subsets including CD19$^+$ CD27$^-$ naïve B cells, CD20$^+$ CD77$^+$ germinal center B cells, CD19$^+$ CD27$^+$ memory B cells and CD19^{low} CD138$^+$ plasma cells were isolated by MACS and FACS from bone marrow, umbilical cord blood, peripheral blood or tonsils from healthy donors as previously described (24-26). For cell culture experiments, the *BCR-ABL1*$^+$ pre-B ALL cell lines BV173, NALM1 and SUP-B15 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Heidelberg, Germany) were used. Cell lines were treated with 10µmol/l STI571 (Novartis, Basel, Switzerland) for 0 to 48 hours.

SAGE analysis

To identify differentially expressed genes between BCR-ABL1$^+$ pre-B ALL cells and normal pre-B cells with respect to Ig light chain expression, we analysed expression profiles generated by SAGE for mRNA expression levels of genes that are implicated in the regulation of Ig light chain expression. Genome-wide SAGE profiles for two primary cases of BCR-ABL1$^+$ pre-B ALL (cases I and II) and the normal hematopoietic populations mentioned above were generated and compared as described previously (24-26). In these SAGE-profiles, 30 genes were identified which have been implicated in the regulation of Ig-light chain expression. These genes were searched in the literature, as well as the UniGene (http://www.ncbi.nlm.nih.gov/UniGene) and OMIM (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM) databases.

RT-PCR analysis of IRF4, SPIB, RAG1/2 expression and germline κ and λ light chain transcripts

mRNA levels of the Ig light chain-associated transcription factors *IRF4* and *SPIB* in *BCR-ABL1*$^+$ were measured in leukemia samples from three patients (cases III, IV and V) before and after treatment with STI571 (Figure 1). From patient samples, total RNA was isolated and

transcribed into cDNA as previously described (24-26). Amounts of cDNA were normalized by OD measurement and semiquantitative RT-PCR using 5'-TTAGCACCCCTGGCCAAG-3' and 5'-CTTACTCCTTGGAGGCCATG-3' for amplification of a cDNA fragment of *GAPDH.* For semiquantitative RT-PCR analysis of *IRF4* the oligonucleotides 5'-CAAGAGCAATGACTTTGAGG-3' and 5'-TGGGACATTGGTACGGGAT-3' were used and for *SPIB* 5'-AAGACTTACCGTTGGACAGC-3' and 5'-CTTGGAGGAGAACTGGAAGA-3'.

RNA was isolated from BV173, NALM1 and SUP-B15 cell lines with or without STI571incubation (10μmol/l), transcribed into cDNA, and normalized as described above. In order to determine light-chain locus germline transcription by RT-PCR, the primers for constant regions in each locus were used. For κ constant region transcription, 5'-TTCAACAGGGGAGAGTGTTAGAG-3' and 5'- ATGCGCCTTAGGATGACTACATA-3' were used, and 5'- TGTCTGATCAGTGACTTCTACCC-3' and 5'-CTGTAGCTTCTGTGGGACTTC-3' were used for λ constant region transcription. RT-PCR on RAG1 and RAG2 was carried out using the oligonucleotides 5'-ATAGAAGAAAGCAACACAAAAGC -3' and 5'- ATACTGAGTTCAATCCCTGAAGA -3' for RAG1 and 5'-ATAGCAAGAGCTCTACACACTCC-3' and 5'-AAAAATCAGATCAGAAATCCTCA-3' for RAG2 (Figure 2A).

Analysis of nuclear RAG1 protein expression

BCR-ABL1$^+$ pre-B ALL cells (BV173 and SUP-B15) were cultured in the presence or absence of 10 μmol/l STI571 for 1, 2 or 4 hours and subsequently stained with a Cy5-labeled antibody against CD19. After treatment with methanol and 4% paraformaladelhyde, cells were stained with a mouse anti-human RAG1 IgG1 (BD Biosciences, Heidelberg, Germany) together with a Cy2-labeled goat anti-mouse IgG antibody. Expression of CD19 and RAG1 was visualized by immunofluorescence (Figure 2).

Flow cytometry

Surface expression of Igκ- or Igλ-light chains on BV173, NALM1, and SUP-B15 pre-B ALL cells in the presence or absence of 10 μmol/l STI571 was monitored using antibodies against Igκ- or Igλ-light chains (BD Biosciences, Heidelberg, Germany) after the incubation times indicated (Figure 3). Apoptotic or dead cells were identified by staining with FITC-labelled Annexin V and propidium iodide (BD Biosciences) and removed with the gate.

Amplification of double-strand recombination signal sequence breaks by ligation-mediated PCR

From about 2.5 x 10^6 untreated BV173, NALM1 and SUP-B15 cells or after treatment with 10 µmol/l STI571 after the times indicated, genomic DNA was isolated and ligated to a blunt-end linker using T4 DNA ligase (Invitrogen, Karlsruhe, Germany) at 14°C overnight. The linker was constructed by annealing the oligonucleotides 5'-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACATG 3' and 3' amino (C7)-GACGAGCTTAAGTTCGAAGATTGCTACATGCCCCT -5' and protruding 3' overhangs were removed by 3'→ 5' exonuclease activity of the Klenow fragment of E. *coli* DNA polymerase I (Invitrogen, Karlsruhe, Germany). Ligation-mediated PCR (LM-PCR; 27) was carried out with modifications as previously described (26). In two semi-nested rounds of amplification (35 and 45 PCR cycles at an annealing temperature of 59°C), RSS-intermediates with a DNA double-strand break at the 5' heptamer of Jκ gene segments were amplified (see Figure 4, 5A) using 5'-GTAATTAACATTCAGTCTACTTTC-3' as external forward and 5'-TAACATTCAGTCTACTTTCTAAAA-3' as internal forward primers together with 5'-TCCCCGTACATCGTTAGAAG–3' as reverse primer specific for DNA-ligated linker molecules. To amplify RSS-intermediates with a DNA double-strand break at the 5' heptamer of Jλ gene segments, 5'-TTCTCACTTCTTCCATGGTGAC-3' and 5'-ACTTCTTCCATGGTGACAGTCT-3' were used in two rounds of PCR amplification as described above (Figure 6). Accordingly, 5'-TCCTCCTCACTGAGCCTCCCTTGAAT-3' and 5'-CTCACTGAGCCTCCCTTGAATAGTCC-3' were used to amplify RSS-intermediates with a DNA double-strand break at the 5' heptamer of the κ-deleting element (KDE).

Amplification of Vκ-Jκ and Vλ-Jλ gene rearrangements

Genomic DNA from 5 x 10^6 STI571-treated (24 hours) or untreated BV173, NALM1 and SUP-B15 cells was isolated and subjected to two rounds of seminested PCR (35 and 45 cycles) using six Vκ- and nine Vλ-family-specific primers together with Jκ- and Jλ-specific primers, respectively. Primers and PCR conditions were used as previously described (28).

Analysis of the genomic configuration of the κ deleting element (KDE)

Three primers (KDE germline: 5'-CTCACTGAGCCTCCCTTGAATAGTCC-3'; JκCκ Intron : 5'-CCGCGGTTCTTTCTCGATTGAGTGG-3'; KDE external reverse: 5'-CTTCATAGACCCTTCAGGCACATGC-3') were added to Vκ-family-specific primers for amplification of Vκ-KDE rearrangements (Vκ primers and KDE external reverse primer), JκCκ intron RSS-KDE rearrangements (JκCκ Intron and KDE external reverse primer), and KDE in germline configuration (KDE germline and KDE external reverse primer). 1 µl-

aliquots of the first rounds were used in separate second rounds for the six family-specific Vκ primers together with 5' Jκ primers and the internal reverse KDE primer (5'-AGACAGGTCCTCAGAGGTCAGAGC-3'), and one second round with JκCκ Intron and KDE internal reverse and another with KDE germline and internal reverse KDE (see Figure 5). Vκ-family-specific primers used are 5'-GACATCCRGWTGACCCAGTCTCCWTC-3' for Vκ1, 5'- CAGWCTCCACTCTCCCTGYCCGTCA-3' for Vκ2, 5'-TTGTGWTGACRCAGTCTCCAGSCACC-3' for Vκ3, 5'-AGACTCCCTGGCTGTGTCTCTGGGC-3' for Vκ4, 5'-CAGTCTCCAGCATTCATGTCAGCGA-3' for Vκ5 and 5'-TTTCAGTCTGTGACTCCAAAGGAGAA-3' for Vκ6. Jκ-specific primers used are 5'-TTGATYTCCASCTTGGTCCCYTGGC-3' for Jκ1 and Jκ2, 5'-TTGATATCCACTTTGGTCCCAGGGC-3' for Jκ3, 5'-TTGATCTCCACCTTGGTCCCTCCGC-3' for Jκ4 and 5'-TTAATCTCCAGTCGTGTCCCTTGGC-3' for Jκ5.

Analysis of secondary IGK gene rearrangement

To analyse potential secondary *IGK* recombination events, we tested whether rearrangement of individual Jκ-gene segments occurs sequentially. Genomic DNA isolated from untreated *BCR-ABL1*$^{+}$ pre-B ALL cells or treated with STI571 (10μmol/l) for the times indicated and subjected to two rounds of seminested PCR. 5'-GCTCAGCTCCTGGGGCTCCTGC-3' and 5'-GACATCCRGWTGACCCAGTCTCCWTC-3' were used as primers specific for the leader and framework region I, respectively, of Vκ1 gene segments. 5'-TTGATYTCCASCTTGGTCCCYTGGC-3' was used for amplification of Jκ1 and Jκ2 gene rearrangements and 5'- TTAATCTCCAGTCGTGTCCCTTGGC-3' for Jκ5.

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5.4 Mimicry of a constitutively active pre-B cell receptor: Aberrant splicing links Bruton's tyrosine kinase to BCR-ABL1 in pre-B lymphoblastic leukemia

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Summary

Pre-B cells undergo apoptosis unless they are rescued by pre-B cell receptor-dependent survival signals. We previously showed that the BCR-ABL1 kinase expressed in pre-B lymphoblastic leukemia bypasses selection for pre-B cell receptor-dependent survival signals. Investigating possible interference of BCR-ABL1 with pre-B cell receptor signaling, we found that neither SYK nor SLP65 can be phosphorylated in response to pre-B cell receptor engagement. Instead, Bruton's tyrosine kinase (BTK) is constitutively phosphorylated by BCR-ABL1. Activated BTK is essential for survival signals that would otherwise arise from the pre-B cell receptor, including activation of PLCγ1, autonomous Ca^{2+} signaling, STAT5-phosphorylation and upregulation of *BCLX$_L$*. Inhibition of BTK kinase activity specifically induces apoptosis in *BCR-ABL1*$^+$ leukemia cells to a similar extent as inhibition of BCR-ABL1 kinase activity itself. However, BCR-ABL1 cannot directly bind to full-length BTK. Instead, BCR-ABL1 induces the expression of a truncated splice variant of BTK acting as a linker between the two kinases. As opposed to full-length BTK, truncated BTK is lacking kinase activity yet can bind to BCR-ABL1 through its SH3 domain. Acting as a linker, truncated BTK enables BCR-ABL1-dependent activation of full-length BTK, which initiates downstream survival signals mimicking a constitutively active pre-B cell receptor.

Introduction

The *BCR-ABL1* gene rearrangement encodes a constitutively active tyrosine kinase and drives malignant transformation of pre-B cells in humans (1). In a B cell-specific mouse model, expression of *BCR-ABL1* is sufficient to induce pre-B lymphoblastic leukemia and required for maintenance of leukemic transformation (2).

During their development, human B cells have to pass multiple checkpoints at which they are selected for the expression of a functional pre-B or B cell receptor (3). However, signals from the pre-B cell receptor may also initiate apoptosis (4). Therefore, the expression of a functional pre-B cell receptor constitutes an effective means to tightly regulate survival or elimination of individual B cell clones. In this regard, it is not surprising that in a number of B cell-derived malignancies, pre-B or B cell receptor function is compromised (5). For instance, Hodgkin- and Reed-Sternberg cells in classical Hodgkin's disease carry "crippled" immunoglobulin (Ig) variable (V) region gene rearrangements due to the acquisition of deleterious somatic mutations (6). Likewise, transformation of human B cells by the Epstein-Barr virus (EBV) not only mimicks antigen receptor-dependent survival signals through the viral oncoprotein LMP2A but also prevents the B cell receptor from signaling by excluding it from glycolipid-enriched membrane domains (7). Autonomous growth and survival of transformed B cells seem to favor a situation in which the B cell receptor is not active. In accordance with this notion, we recently observed that pre-B lymphoblastic leukemia clones harboring a *BCR-ABL1* fusion gene carry only non-productively rearranged immunoglobulin (Ig) variable region genes in 9 of 12 cases (8). Even the few leukemia clones that do express a pre-B cell receptor were nonetheless not responsive to pre-B cell receptor engagement (8). In analogy to the EBV-encoded LMP2A protein, the expression of the oncogenic BCR-ABL1 kinase may not only confer autonomous survival signals but also interfere with antigen receptor signaling.

Among others, Bruton's tyrosine kinase (BTK) represents a crucial component of the (pre-) B cell receptor signaling chain and links the (pre-) B cell receptor to Ca^{2+} signals in B lymphoid cells (9). In humans, deficiency of BTK leads to X-linked agammaglobulinemia (XLA) resulting from a differentiation block at the pre-B cell stage (10). Of note, BTK not only promotes developmental progression of pre-B cells (11) but also acts as a tumor suppressor to prevent pre-B lymphoblastic leukemia in mice (12, 13). In order to elucidate how BCR-ABL1 can bypass selection for pre-B cell receptor-dependent survival signals, the current study investigates possible interference of BCR-ABL1 with pre-B cell receptor-related signaling molecules including SYK, SLP65, PLCγ2 and BTK.

Results

BCR-ABL1 kinase activity induces tyrosine-phosphorylation of BTK

Normal pre-B cells can only survive in human bone marrow if they receive survival signals through their pre-B cell receptor (14). In a previous study, we observed that in the majority of cases of pre-B lymphoblastic leukemia expressing the oncogenic BCR-ABL1 kinase, the leukemia cells do not harbor a functionally rearranged *IGH* allele and, hence, cannot express a pre-B cell receptor on their surface (8). Therefore, we wondered how a pre-B cell-derived leukemia cell can survive and even clonally expand in the absence of pre-B cell receptor-dependent survival signals.

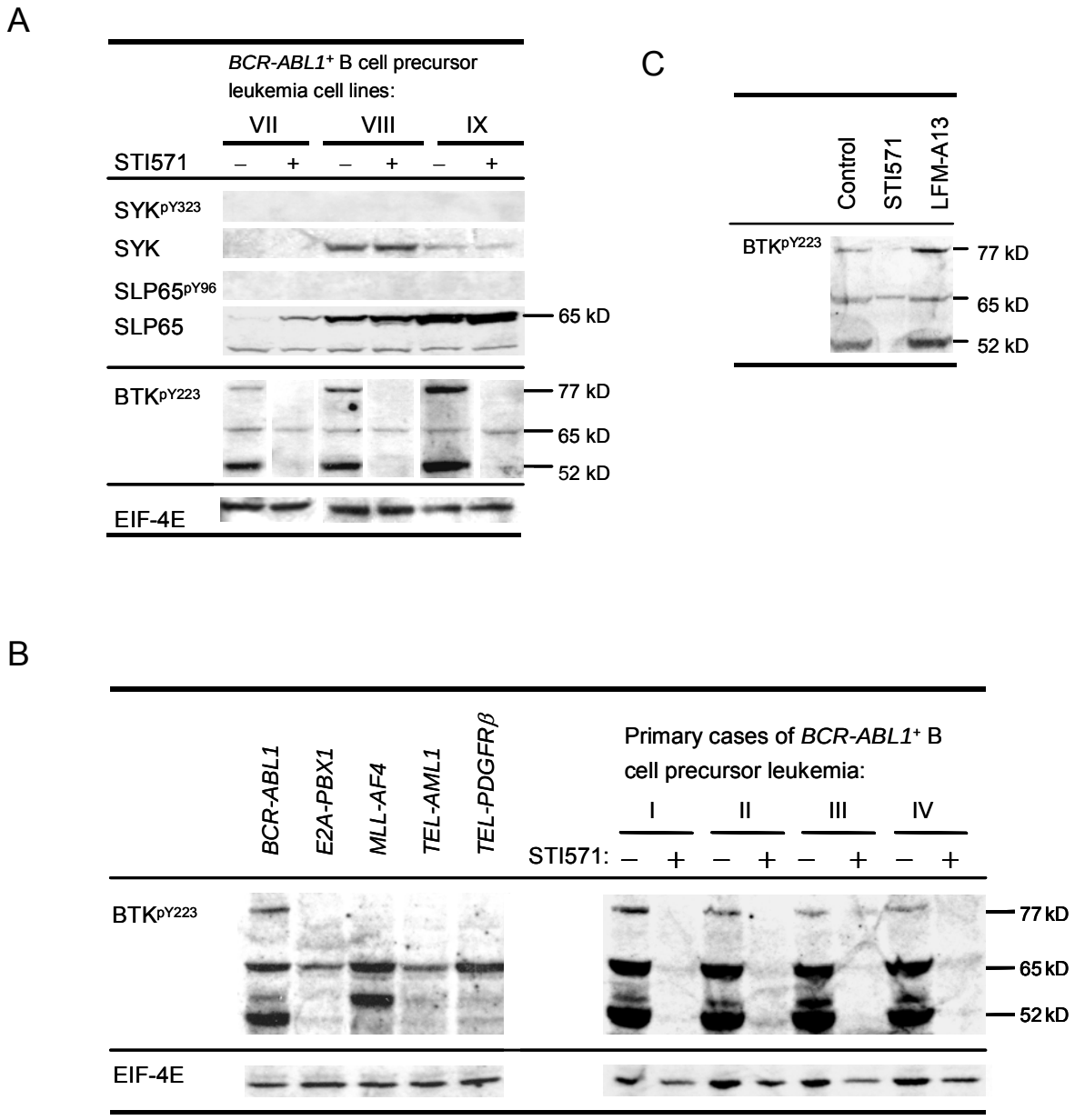
To determine whether BCR-ABL1 interferes with signal transduction of the pre-B cell receptor, we examined whether the pre-B cell receptor-related signaling molecules SYK, SLP65 and BTK are expressed and whether they can be phosphorylated upon pre-B cell receptor engagement in the leukemia cells.

Western blot using antibodies against phosphorylated SYK^{Y323}, $SLP65^{Y96}$ and BTK^{Y223} showed that neither SYK nor SLP65 were phosphorylated in response to crosslinking of the pre-B cell receptor (not shown). However, BTK was constitutively phosphorylated in the leukemia cells (at 77kD; Figure 1A).

Using a specific inhibitor of BCR-ABL1, termed STI571 (15), we found that phosphorylation of BTK was sensitive to inhibition of BCR-ABL1 kinase activity (77kD; Figure 1A). To determine whether constitutive phosphorylation of 77kD BTK is specific for leukemia cells carrying a *BCR-ABL1* gene rearrangement, BTK-phosphorylation was also studied in pre-B lymphoblastic leukemias harboring an *E2A-PBX1*, *MLL-AF4*, *TEL-AML1* or *TEL-PDGFRβ* gene rearrangement (Figure 1B, Table I). While the BTK^{Y223}-specific antibody detected smaller tyrosine-phosphorylated proteins also in other leukemias, constitutive Y223-phosphorylation of BTK (77 kD) was specific for *BCR-ABL1*$^+$ pre-B cell lymphoblastic leukemia cell lines (Figure 1B, left panel).

To confirm that BTK-phosphorylation is indeed depending on BCR-ABL1 kinase activity, we induced BCR-ABL1 expression in a murine B lymphoid cell line TONB210 (16) carrying an inducible *BCR-ABL1* transgene. As opposed to *BCR-ABL1*$^+$ leukemia cells, this cell line grows independently from BCR-ABL1 in the presence of IL-3. In the presence but not in the absence of doxycycline-induced BCR-ABL1 expression, BTK was heavily phosphorylated (see Figure 5C). We conclude that BTK is constitutively phosphorylated by BCR-ABL1 but not by the pre-B cell receptor in the leukemia cells.

Figure 1: *BCR-ABL1 induces phosphorylation of BTK in pre-B lymphoblastic leukemia cells*



Protein expression and tyrosine-phosphorylation of the pre-B cell receptor downstream signaling molecules SYK, SLP65 and BTK was studied by Western blot in three *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cell lines (VII, VIII and IX in Table I) in the presence or absence of STI571. The BTK^{pY223}-antibody detected full-length BTK (77kD) as well as two additional tyrosine-phosphorylated proteins of 65 and 52 kD size (A-C). To confirm that constitutive phosphorylation of 77 kD full-length BTK is specific for BCR-ABL1 kinase activity, BTK^{Y223}-phosphorylation was studied in four BCR-ABL1-negative B cell precursor cell lines (B, left panel; see XI, XII, XIII and XV in Table I). Dependence of BTK on BCR-ABL1 kinase activity was also confirmed in four primary cases of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia, from which matched sample pairs before and during therapy with STI571 were available (B, right panel; cases I to IV in Table I). EIF-4E was used as a loading control. To determine whether Y223-phosphorylation of BTK is mediated by transphosphorylation through BCR-ABL1 or autophosphorylation, *BCR-ABL1*$^+$ leukemia cells (IX; Table I) were treated for 24 hours with the BCR-ABL1 kinase inhibitor STI571 or the BTK kinase inhibitor LFM-A13 (C).

Table I: *Description of leukemia and lymphoma cases and cell lines studied*

Case	Age	Sex	Translocation	IgH	Igκ/λ	*IGH* configuration		Clinical course
			Fusion gene		or SLC	Alleles	Coding capacity	
I[a]	39	m	*BCR-ABL1*; p210	–	–	#1 V_H4.34-D_H6.13-J_H5 #2 D_H5-J_H	no; out-of-frame	ALL, relapse after STI571 treatment
II[a]	54	f	*BCR-ABL1*; p190	–	–	#1 V_H4.61-D_H2.2-J_H4 #2 D_H2.4-J_H4	no; out-of-frame, stop in junction	ALL, relapse after STI571 treatment
III[a]	61	f	*BCR-ABL1*; p190	n.d.	n.d.	n.d.		ALL, relapse after STI571 treatment
IV[a]	47	f	*BCR-ABL1*; p190	n.d.	n.d.	n.d.		ALL, relapse after STI571 treatment
V[a]	34	m	*BCR-ABL1*; p190	–	–	#1 V_H4.34-D_H6.13-J_H5 #2 D_H5-J_H	out-of-frame	ALL, complete remission after BMT
VI[a]	32	m	*BCR-ABL1*; p190	–	–	#1 V_H4.4-D_H2.2-J_H4 #2 D_H2.4-J_H4	out-of-frame, stop codons in junction	ALL, died after BMT
VII[a]	45	m	*BCR-ABL1*; p210	–	–	#1 V_H3.48-D_H2.15-J_H3 #2 D_H2-J_H	no; out-of-frame, stop in junction	ALL, died after relapse (BV173 cell line)
VIII[a]	3	f	*BCR-ABL1*; p210	μ chain	SLC	#1 D_H4-J_H #2 V_H3.9-D_H2.21-J_H3	no yes	ALL, died after relapse (NALM1 cell line)
IX[a]	9	m	*BCR-ABL1*; p190	μ chain	SLC	#1 V_H3.53-D_H2.8-J_H6	yes[b]	ALL, died after relapse (SUP-B15 cell line)
X	32	f	*MLL-AF4*	–	–	#1 V_H3.20-D_H2.8-J_H5 #2 V_H6.1-D_H1.7-J_H4	no; out-of-frame no, stop in junction	ALL, relapse after chemotherapy (RS4;11 cell line)
XI	5	f	*MLL-AF4*	–	–	#1 D_H3-J_H #2 *IGH* in germline	no	ALL, relapse after chemotherapy (SEM cell line)
XII	15	f	*TEL-AML1*	–	–	#1 V_H3.15-D_H3.10-J_H6 #2 *IGH* in germline	no; out-of-frame	ALL, relapse after chemotherapy (REH cell line)
XIII	12	m	*E2A-PBX1*	μ chain	SLC	#1 V_H4.34-D_H3.22-J_H2 #2 V_H2.26-D_H2.2.-J_H4	no; out-of-frame, stop in junction yes	ALL, relapse after chemotherapy (697 cell line)
XIV	15	m	*E2A-PBX1*	μ chain	SLC	#1 V_H3.7-D_H3.10-J_H4	yes[b]	ALL, cell line (KASUMI-2) established at diagnosis
XV	19	m	*TEL-PDGFRβ*	μ chain	SLC	#1 V_H1.69-D_H3.10-J_H6	yes[b]	ALL, relapse after chemotherapy (Nalm6 cell line)
XVI	5	m	*IGH-MYC*	IgM	Igλ	#1 V_H3.53-D_H3.3-J_H6 #2 V_H4.34-D_H2.15-J_H5	no, stop in junction yes	Burkitt's lymphoma, cell line (MHH-PREB1) established at diagnosis
XVII	73	f	*IGH-BCL2*	IgG	Igκ	#1 V_H4.39-D_H3.10-J_H6[d] #2 V_H3.9-D_H3.22-J_H6[d] #3 V_H3.23-D_H6.13-J_H5[d]	yes no, out-of-frame no, out-of-frame	Diffuse large B cell lymphoma, cell line (Karpas-422) established at diagnosis

Notes:

a. These cases have already been described in Klein et al., 2004 (reference 8)
b. The second *IGH* allele was not subjected to sequence analysis if a potentially functional allele was already amplified
c. n.d., not done; ALL, acute lymphoblastic leukemia; SLC, surrogate light chain
d. Three *IGH VDJ*-gene rearrangements are compatible with three chromosomes 14 in these B cell lymphoma cells. Alternatively, the cell line may comprise subclones with different *IGH VDJ* gene rearrangement(s).

BTK-phosphorylation depends on BCR-ABL1 kinase activity in vivo

To confirm that phosphorylation of BTK depends on BCR-ABL1 kinase activity also in primary leukemia cells, expression of tyrosine-phosphorylated BTK was compared in matched leukemia sample pairs from four patients before and during continued therapy with STI571 (cases I to IV, Figure 1B, right panel). The content of *BCR-ABL1*$^+$ leukemia cells was similar in the samples taken before and during STI571-treatment as assessed by semiquantitative amplification of *BCR-ABL1* fusion transcripts (see Figure 2A). As with inhibition of BCR-ABL1 kinase by STI571 *in vitro*, in all four patients *in vivo* ablation of BCR-ABL1 kinase activity resulted in loss of BTK-phosphorylation (Figure 1B, right panel).

BTK^{Y223} can be transphosphorylated by BCR-ABL1

To further clarify BCR-ABL1-dependent phosphorylation of BTK^{Y223}, we investigated whether phosphorylation at Y223 depends only on BCR-ABL1 or also on endogenous BTK kinase activity. In normal B cells, B cell receptor engagement leads first to LYN-mediated transphosphorylation of C-terminal BTK^{Y551} (17). BTK^{Y223}-phosphorylation occurs as a secondary event by autophosphorylation through endogenous BTK kinase activity (17). Therefore, the effect of the BTK kinase inhibitor LFM-A13 on BTK^{Y223}-phosphorylation in *BCR-ABL1*$^+$ pre-B cell lymphoblastic leukemia cells was examined. BTK^{Y223} phosphorylation was sensitive to BCR-ABL1 kinase inhibition by STI571 but not to BTK kinase inhibition by LFM-A13 (Figure 1C). We conclude that BTK^{Y223} is accessible to transphosphorylation by BCR-ABL1 in the absence of endogenous BTK kinase activity.

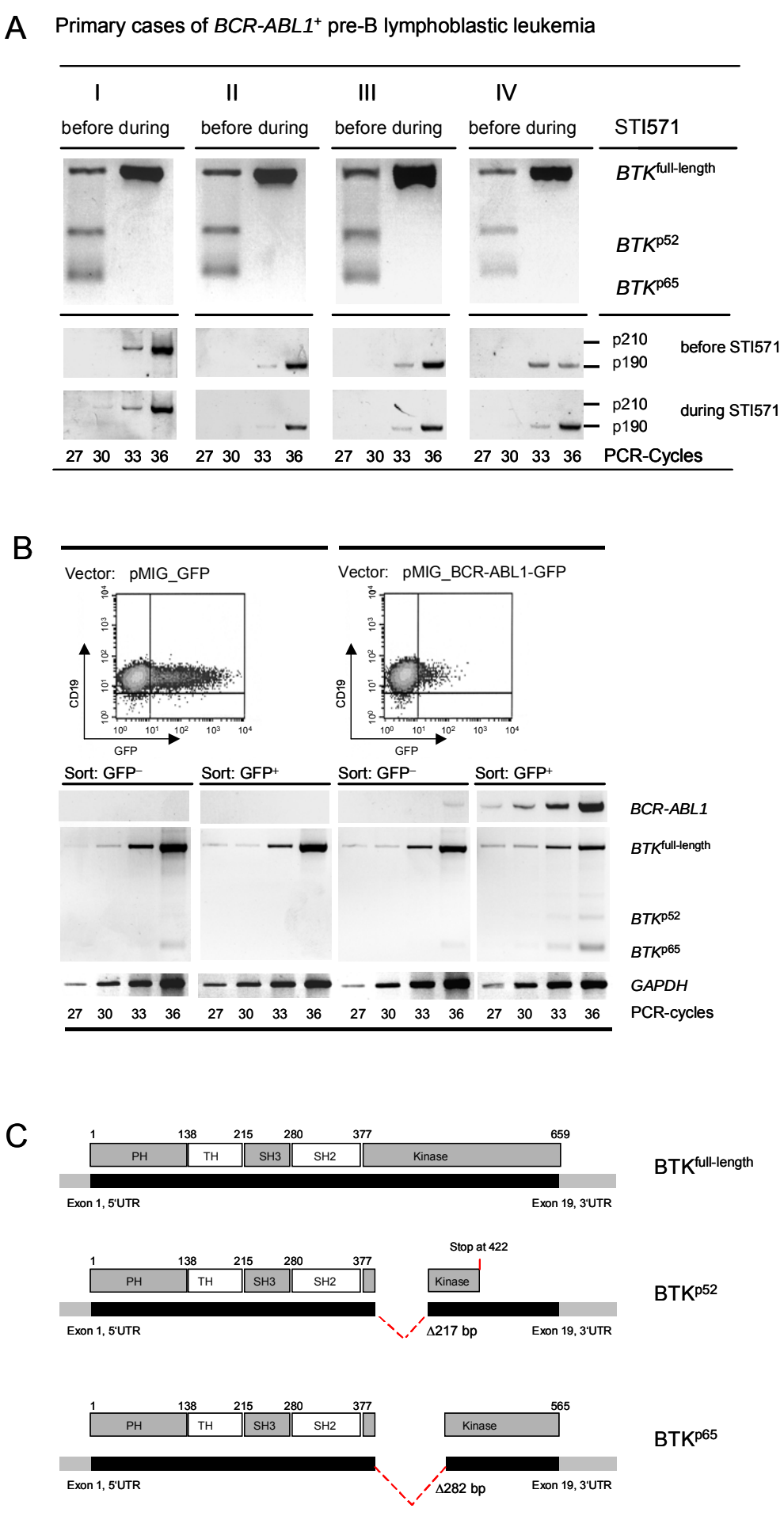
Expression of truncated BTK splice variants in BCR-ABL1$^+$ *pre-B lymphoblastic leukemia*

In the BTK Western blot analysis of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cell lines, we observed that the antibody recognizing phosphorylated BTK^{Y223} at the expected size of 77kD also detected two smaller tyrosine-phosphorylated proteins of about 65 kD and 52 kD in size (Figure 1A-C). This prompted us to investigate splice variants of BTK at the mRNA level.

Amplifying specific BTK cDNA fragments covering the entire coding region of BTK, we detected shorter PCR products in all three *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cell lines (not shown) and also in six primary cases of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia (Cases I to VI, Table I and Figure 2A).

In total, sequence analysis revealed six different BTK isoforms in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells (Supplementary Table II; sequence data available from EMBL/GenBank under accession numbers AJ888376 to AJ888381). All these splice variants have in common that they carry large deletions within the kinase domain (Supplementary Table II).

Figure 2: *BCR-ABL1 induces expression of kinase-deficient BTK isoforms*



Legend to Figure 2:

Comparing matched leukemia sample pairs taken before and during continued therapy with the BCR-ABL1 kinase-inhibitor STI571, BTK transcripts including splice variants ($BTK^{p52}$and BTK^{p65}) were amplified (A, top). cDNA samples were normalized for equal content of leukemia cells by amplification of BCR-ABL1 (A, bottom). To determine whether aberrant splicing of BTK is induced by BCR-ABL1, pre-B lymphoblastic leukemia cells carrying an *E2A-PBX1* but no *BCR-ABL1* rearrangement were transiently transfected with a retroviral expression vector encoding either BCR-ABL1 and GFP, or GFP alone (B). GFP^- and GFP^+ cells were sorted after 24 hours (B, top) and subjected to RT-PCR analysis. cDNA amounts were normalized by amplification of a *GAPDH* fragment (B, bottom).

In addition to full-length BTK, sequence analysis of BTK transcripts revealed six isoforms (see supplementary Table II). Among these isoforms, two were recurrently amplified: BTK^{p52} and BTK^{p65} were detected in all nine cases of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia studied (C). Both isoforms carry a deletion within their kinase domain. Skipping of exon 15 (BTK^{p52}) leads to the loss of the reading frame and C-terminal truncation due to a premature translation stop at codon 442 (C, middle). BTK^{p65} lacks exon 15 and 16 leading to an in-frame deletion of 282 bp (BTK^{p65}).

Four of these isoforms were unique, were generated by usage of cryptic splice sites and were only amplified from individual cases of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia. However, two BTK isoforms were recurrently identified:

In addition to a previously described BTK isoform lacking exons 15 and 16 (reference 18; BTK^{p65} in Figure 2A), another truncated splice variant lacking only exon 15 was amplified from all nine cases of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia (Supplementary Table II). Joint skipping of exons 15 (217 basepairs) and 16 (65 basepairs) leads to an in-frame 282 basepair deletion (BTK^{p65}; Figure 2C). The predicted size of the protein encoded by BTK^{p65} matches the 65 kD protein recognized by the BTK^{Y223} antibody (Figure 1A-C). Skipping of exon 15 alone leads to loss of the reading frame and generates a pre-mature stop codon resulting in the expression of a truncated protein of only 442 instead of 659 amino acids (BTK^{p52}; Figure 2C). The predicted truncated BTK^{p52} (442 aa) matches exactly the size of the heavily phosphorylated 52 kD protein we detected with the phospho-specific anti-BTK^{Y223} antibody (Figure 1A-C).

Comparing matched sample pairs of leukemia cells before and during continued therapy with the BCR-ABL1 kinase-inhibitor STI571, we amplified BTK^{p52} (lacking exon 15 alone) or BTK^{p65} (lacking exons 15 and 16) only in the presence but not in the absence of BCR-ABL1 kinase activity (Figure 2A). These findings suggest that aberrant splicing of BTK may be linked to BCR-ABL1 kinase activity.

BCR-ABL1 was previously implicated in altered splice-site selection and exon-skipping through upregulation of RNA-binding proteins including FUS, hnRNP A1 and hnRNP E2 (19). In addition, a recent study shows that BCR-ABL1 induces aberrant splicing

of PYK2 pre-mRNA in human hematopoietic progenitor cells, which can be reverted by STI571 (20). These findings exemplify that BCR-ABL1 frequently interferes with normal regulation of pre-mRNA splicing e.g. by reduction of stringency of splice-site selection.

To directly assess whether BCR-ABL1 can interfere with splicing of BTK pre-mRNA, we transiently transfected pre-B lymphoblastic leukemia cells carrying an *E2A-PBX1* but no *BCR-ABL1* gene rearrangement with an expression vector either encoding GFP only or GFP and BCR-ABL1. For both transfections, GFP^+ and GFP^- cells were sorted and separately analyzed for expression of BTK splice variants (Figure 2B). While *E2A-PBX1*$^+$ pre-B lymphoblastic leukemia cells under control conditions only express BTK^{p65} at low levels, induced expression of BCR-ABL1 leads to the expression of multiple BTK splice variants (Supplementary Table II) including BTK^{p52} (Figure 2B). We conclude that BCR-ABL1 induces aberrant splicing of BTK and expression of BTK^{p52}.

Comparing 19 genome-wide gene expression profiles by serial analysis of gene expression (SAGE), we identified three SAGE-tags that distinguish between full-length BTK, BTK^{p65} and BTK^{p52} (supplementary Table III). A SAGE-tag represents a 14-bp cDNA sequence immediately flanking the extreme 3' restriction site (CATG) of the tagging enzyme (NlaIII) that is used as unique identifier of any processed transcript. While full-length BTK is widely expressed in normal B cell populations and BTK^{p65} is expressed also in non-*BCR-ABL1*-B lymphoid malignancies, BTK^{p52} appears to be specifically expressed in *BCR-ABL1*$^+$ leukemia cells (supplementary Table III).

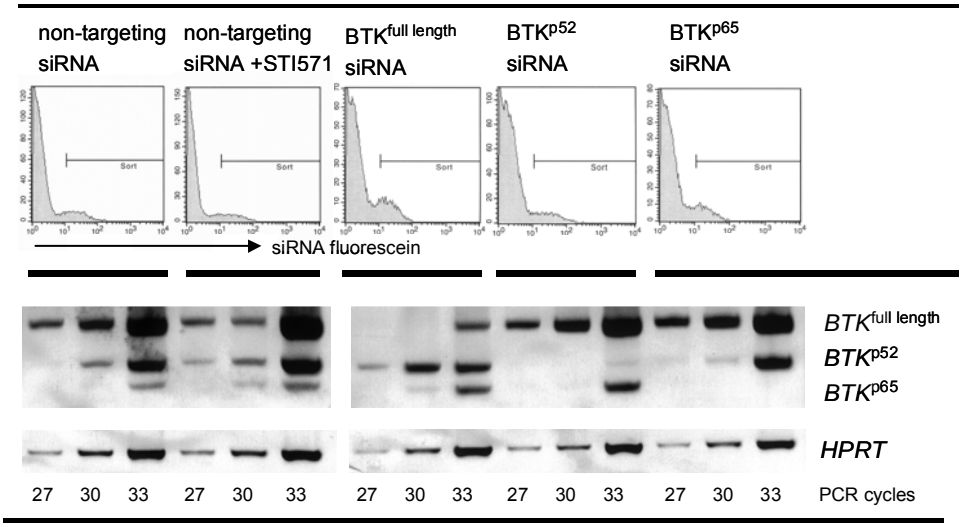
Specific silencing of BTK splice variants by RNA interference

To investigate the function of full-length BTK and its splice variants in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells, we established an RNA interference approach to specifically silence full-length BTK and BTK isoforms.

Among the six BTK splice variants (Supplementary Table II), only BTK^{p52} and BTK^{p65} were recurrently amplified. We therefore focused our analysis on BTK^{p52} and BTK^{p65}. To clarify the specific function of BTK^{p52} (lack of exon 15, loss of reading frame) and BTK^{p65} (in-frame deletion of exons 15 and 16), we designed three siRNA duplices against the junction of BTK exons 14 and 16 (corresponding to BTK^{p52}) and the junction of BTK exons 14 and 17 (corresponding to BTK^{p65}). For each target, the three siRNA duplices were mixed and fluorescein-labeled. Also for full-length BTK, three siRNA duplices were synthesized (specifically binding to BTK exon 15), mixed and fluorescein-labeled. As a control, a fluorescein-labeled non-targeting siRNA duplex was used. After two transfections, the proportion of siRNA-carrying cells ranged between 3 and 5 percent (Figure 3). For each

condition, 1 x 10^5 fluorescein$^+$ cells were sorted and subjected to RT-PCR analysis for BTK splice variants. mRNA levels of the reference genes *HPRT* and *COX6B* remained unchanged (Figure 3 and 8). Sorted cells exhibit specific silencing of either full-length BTK, BTK^{p52} or BTK^{p65} and no cross-inhibition of BTK isoform expression was observed (Figure 3). As lymphoid cells are typically difficult to transfect, we conclude that double-transfection of a mixture of three siRNA duplices and cell sorting of siRNA-carrying cells represents an effective strategy to specifically silence target mRNA sequences in B lymphoid leukemia cells.

Figure 3: *Specific silencing of full-length BTK or BTK splice variants by RNA interference*



BCR-ABL1$^+$ pre-B lymphoblastic leukemia cells were tranfected with a non-targeting siRNA duplex in the presence or absence of STI571 (for 12 hours), as well as with a mixture of siRNAs against full-length BTK, BTK^{p52} or BTK^{p65}, respectively. All siRNA duplices were labeled with fluorescein prior to transfection. Transfection was repeated after 24 hours and 1 x 10^5 cells carrying fluorescein-labeled siRNAs (between 3 and 5 percent of all viable cells) were sorted by FACS 48 hours after the first transfection (upper panel). Total RNA was isolated and subjected to semiquantitative RT-PCR analysis of BTK isoform expression (lower panel). mRNA levels for *HPRT* and *COX6B* (see Figure 8C) were stable under all conditions.

From *in vitro* kinase assays of BTK mutants derived from *BTK*-deficient X-linked agammaglobulinemia (XLA) patients, it is known that even replacement mutations in the distal portion of the kinase domain result in a complete loss of BTK kinase activity (http://bioinf.uta.fi/BTKbase/BTKbasebrowser.html). Hence, only full-length BTK but none of the BTK splice variants identified here should exhibit BTK kinase activity. We therefore

investigated the relevance of BTK kinase activity for BCR-ABL1-mediated survival signaling in pre-B lymphoblastic leukemia cells.

Inhibition of BTK kinase activity induces apoptosis specifically in human BCR-ABL1$^+$ pre-B lymphoblastic leukemia cells

To clarify the role of full-length BTK as compared to kinase-deficient truncated BTK in pre-B lymphoblastic leukemia cells, we analyzed the effect of BTK kinase inhibition on the survival of *BCR-ABL1*$^+$ leukemia cells in comparison to inhibition of BCR-ABL1 by STI571. As a control, we used several B lymphoid cell lines that do not carry a *BCR-ABL1* gene rearrangement. Neither inhibition of endogenous c-ABL1 (by STI571) nor BTK (by LFM-A13) kinase activity alone significantly reduced viability of *BCR-ABL1*-negative B lymphoid cells (Figure 4A, B). Conversely, inhibition of BCR-ABL1 rendered almost all *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells apoptotic within five days (Figure 4A). In both *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cell lines tested but not in *BCR-ABL1*-negative cell lines, inhibition of BTK had a very similar effect as inhibition of BCR-ABL1 and also induced apoptosis in about 90 percent of the leukemia cells within five days (Figure 4A, B). These findings are in agreement with an RNA interference experiment showing that silencing of full-length BTK expression (Figure 3) induces apoptosis in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells (see Figure 8A). Thus, BTK kinase activity is required for sustained growth and survival of BCR-ABL1-transformed human pre-B lymphoblastic leukemia cells.

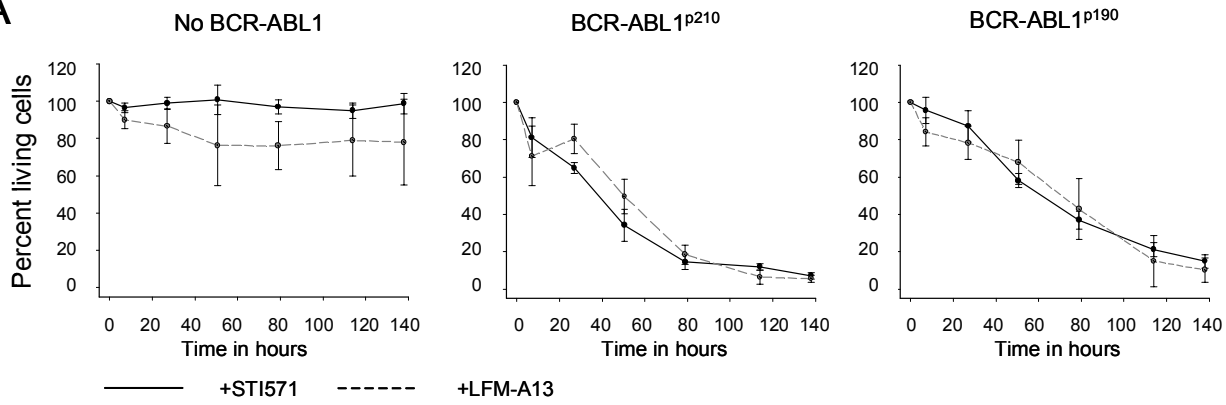
BCR-ABL1-induced tyrosine-phosphorylation and nuclear localization of STAT5 requires BTK kinase activity

Besides other survival signals, STAT5-activation is critical for BCR-ABL1-mediated transformation (21-22). Given that BTK kinase activity can activate STAT5 through phosphorylation at tyrosine 694 (23), we investigated the impact of BCR-ABL1- or BTK kinase-inhibition on STAT5^{Y694}-phosphorylation in three *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cell lines by intracellular staining and flow cytometry. STAT5^{Y694} is constitutively phosphorylated in all three leukemia cell lines and exhibits nuclear localization (Figure 4C), which is required for STAT5 activity (21-22). However, both ablation of BCR-ABL1 kinase activity by STI571 and BTK-inhibition by LFM-A13 largely reduced phosphorylation and nuclear localization of STAT5^{Y694} (Figure 4C).

In addition, three siRNA duplices against full-length BTK were mixed, fluorescein-labelled and transfected into *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells (IX in Table I) using fluorescein-labelled non-targeting siRNAs as a control.

Figure 4: *Inhibition of BTK induces apoptosis specifically in BCR-ABL1$^+$ pre-B lymphoblastic leukemia cells*

A

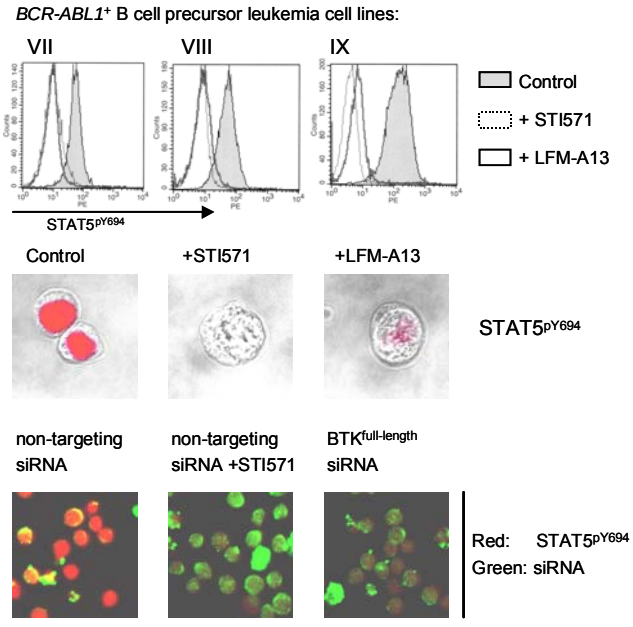


B

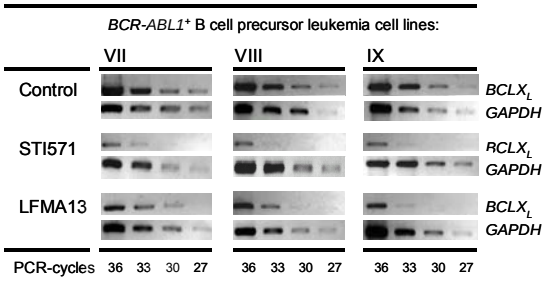
Survival [%]	Cell line	Cell type	Translocation	Genes involved
93.6 ± 0.6	RS4;11	pro-B cell leukemia	t(4;11)(q21;q23)	*MLL-AF4*
97 ± 5.1	SEM	pro-B cell leukemia	t(4;11)(q21;q23)	*MLL-AF4*
49.4 ± 7.6	REH	pre-B cell leukemia	t(12;21)(p12;q22)	*TEL-AML1*
66.8 ± 8.4	697	pre-B cell leukemia	t(1;19)(q23;p13)	*E2A-PBX1*
88.4 ± 3.4	KASUMI-2	pre-B cell leukemia	t(1;19)(q23;p13)	*E2A-PBX1*
89.2 ± 1.3	LCL	polyclonal EBV$^+$ mature B cells	no chromosomal aberration	
85.2 ± 9.6	MHH-PREB-1	Burkitt's Lymphoma	t(8;14)(q24;q32)	*MYC-IGH*
76.2 ± 7.0	KARPAS-422	Diffuse Large B Cell Lymphoma	t(14;18)(q32;q21)	*BCL2-IGH*
7.3 ± 4.1	BV173	pre-B cell leukemia	t(9;22)(q34;q11)	*BCR-ABL1*
19.3 ± 15.5	SUP-B15	pre-B cell leukemia	t(9;22)(q34;q11)	*BCR-ABL1*

Relative survival [%] average: 80.7 ± 5.4 in non-*BCR-ABL1*$^+$ B cell lineage cells
13.3 ± 9.8 in *BCR-ABL1*$^+$ pre-B cell leukemia

C



D



Legend to Figure 4:

BCR-ABL1-negative B lymphoid leukemia and lymphoma cell lines (A, left panel) and two *BCR-ABL*$^+$ leukemia cell lines (A, middle and right panel) were incubated for the times indicated under control conditions, with STI571 (solid line) or with the BTK-inhibitor LFM-A13 (dashed line; A). Relative viability (percent living cells) was calculated as the ratio of viability in the presence of STI571 or LFM-A13 and viability in untreated cells. Two *BCR-ABL1*$^+$ and eight *BCR-ABL1*-negative B lymphoid cell lines were incubated in the presence of LFM-A13 or under control conditions for 114 hours (B). Relative survival of the treated cells as compared to control conditions is given as the mean of three independent experiments $\pm$ S.D.

To analyze BCR-ABL-mediated and BTK-dependent tyrosine-phosphorylation of STAT5 (C), three *BCR-ABL1*$^+$ B cell precursor cell lines (VII, VIII and IX in Table I) were treated with the BCR-ABL1 kinase-inhibitor STI571 (dotted line), with the BTK-inhibitor LFM-A13 (grey solid line) or incubated under control conditions (shaded histograms; C). Nuclear localization of activated STAT5 (red) in treated and untreated leukemia cells was visualized by confocal laser microscopy (C; red stain, middle panel). *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells were transfected with a mixture of fluorescein-labeled siRNAs against full-length BTK as shown in Figure 3. Fluorescein$^+$ cells carrying siRNA molecules (green) were stained for tyrosine-phosphorylated STAT5 (red) and analyzed by confocal laser microscopy (C, lower panel). As a negative control, non-targeting siRNA-duplices were used. Next, mRNA levels of *BCLX$_L$* were studied in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells in the presence or absence of STI571 or LFM-A13. Total RNA was isolated from the leukemia cells and subjected to semiquantitative RT-PCR analysis for mRNA levels of *BCLX$_L$* (D). cDNA amounts were normalized by amplification of a *GAPDH* cDNA fragment.

After two transfections, fluorescein$^+$ leukemia cells were sorted and analyzed for STAT5-phosphorylation (Figure 4C, lower panel). Efficiency and specificity of BTK full-length silencing was controlled by RT-PCR (Figure 3). In agreement with BTK-inhibition by LFM-A13, silencing of full-length BTK by RNA interference largely attenuated STAT5^{Y694}-phosphorylation as shown by confocal lasermicroscopy (Figure 4C) and intracellular FACS-staining (Figure 8B).

BTK-mediated transcriptional activation of BCLX$_L$ in BCR-ABL1$^+$ *pre-B lymphoblastic leukemia cells*

In *BCR-ABL1*$^+$ leukemia cells, STAT5 mainly accomplishes its anti-apoptotic effect by transcriptional activation of *BCLX$_L$* (24). Comparing the frequency of SAGE-tags that distinguish between anti-apoptotic BCLX$_L$ and its pro-apoptotic isoform BCLX$_S$ in 19 genome-wide SAGE-libraries, SAGE-tags specific for BCLX$_L$ were particularly frequent in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells (supplementary Table III).

Analyzing mRNA levels for *BCLX$_L$* in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells in the presence or absence of STI571 or LFM-A13, we found that inhibition of BCR-ABL1 or BTK leads to a considerable reduction of *BCLX$_L$* expression in the leukemia cells

(Figure 4D). This is consistent with the effect of siRNA-mediated silencing of full-length BTK on $BCLX_L$-mRNA levels (Figure 8C).

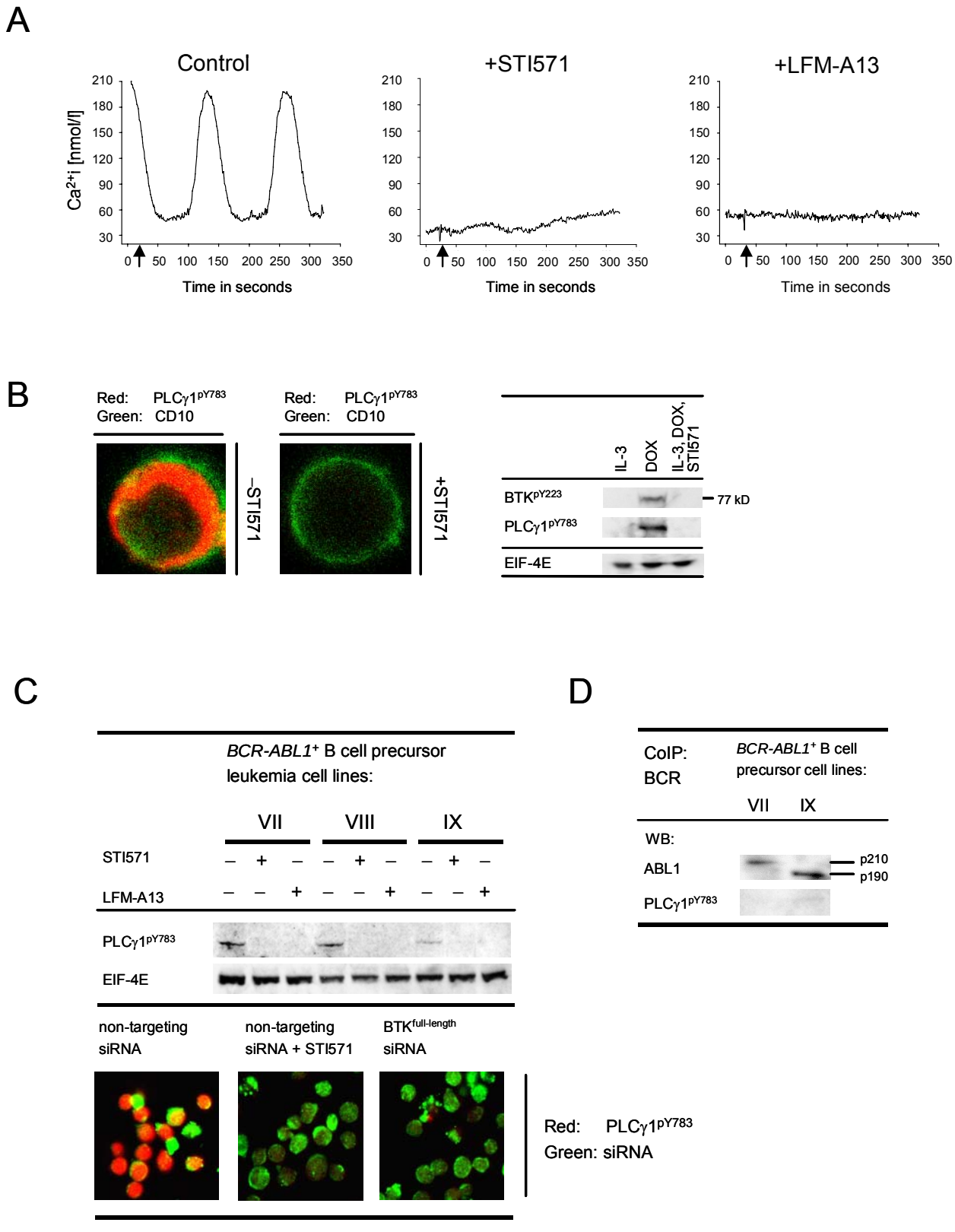
A recent study identified SRC-kinase activity as a requirement for BCR-ABL1-mediated transformation of murine pre-B cells (25). The findings presented here indicate that also BTK kinase activity is specifically required for the BCR-ABL1-dependent survival-signals and may thus represent an important target for the therapy of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia.

BTK kinase activity is required for autonomous Ca^{2+} signal activity in BCR-ABL1$^+$ pre-B lymphoblastic leukemia cells

In normal pre-B cells, BTK kinase activity induces Ca^{2+}-release upon pre-B cell receptor-engagement through BTK-dependent phosphorylation of PLCγ2. Constitutive phosphorylation of BTK by BCR-ABL1 and specific requirement of BTK kinase activity for the survival of *BCR-ABL1*$^+$ leukemia cells suggests that BCR-ABL1 can also trigger BTK-dependent Ca^{2+} release from cytoplasmic stores. In *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells expressing a pre-B cell receptor on the surface (reference 8; Table I), BCR-ABL1 kinase activity was linked to an autonomous oscillatory Ca^{2+} signal, which was not changed by pre-B cell receptor-engagement (arrows; Figure 5A, left panel). Analysis of Ca^{2+} release in multiple pre-B lymphoblastic leukemia cells at the single-cell level showed that inhibition of BCR-ABL1 terminated the autonomous Ca^{2+} signaling activity (Figure 5A, middle panel). Autonomous Ca^{2+} signals did not only require BCR-ABL1 kinase activity but were also abrogated by inhibition of BTK kinase activity through treatment with LFM-A13 (Figure 5A, right panel). Comparing sorted fluorescein$^+$ leukemia cells either transfected with full-length BTK–specific or non-targeting siRNAs, autonomous oscillatory Ca^{2+}-signals were greatly diminished in the leukemia cells carrying full-length BTK–specific siRNAs (see Figure 9B).

Pre-B cell receptor-dependent Ca^{2+} signals are typically initiated by BTK-mediated phosphorylation of PLCγ2 (9). Assuming that BCR-ABL1 targets downstream molecules of the pre-B cell receptor signal cascade in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells, we investigated whether BCR-ABL1-dependent autonomous Ca^{2+} signals are also transduced by BTK and PLCγ2. Unexpectedly, PLCγ1 but not PLCγ2 (not shown) is phosphorylated by BCR-ABL1 in human leukemia cells (Figure 5B, C) and in mouse B lymphoid cells carrying an inducible BCR-ABL1 transgene (Figure 5B). As shown by co-immunoprecipitation, BCR-ABL1 does not directly bind to PLCγ1 (Figure 5D).

Figure 5: *BTK links BCR-ABL1 to PLCγ1-mediated Ca^{2+} signals in pre-B lymphoblastic leukemia cells*



Legend to Figure 5:

Cytoplasmic Ca^{2+} levels were measured in single leukemia cells by confocal laser-microscopy. Before Ca^{2+} measurement, cells were either kept under control conditions or treated for 24 hours with STI571 or LFM-A13 (A). Pre-B cell receptor engagement by addition of µ-heavy chain-specific antibodies (arrows) did not change cytoplasmic Ca^{2+} concentrations. While PLCγ1^{Y783} is phosphorylated in the presence but not in the absence (+ STI571) of BCR-ABL1 kinase activity (B, left panels), PLCγ2 was not tyrosine-phosphorylated by BCR-ABL1 (not shown). As a control for non-specific side-effects of STI571, murine B lymphoid cells carrying a doxycycline-inducible *BCR-ABL1* transgene were analyzed in the presence or absence of 1 µg/ml doxycycline (DOX). These cells remain viable in the absence of BCR-ABL1 expression if the cell culture fluid is supplemented with 2 ng/ml murine recombinant IL-3. Protein extracts from these cells were subjected to Western blot using human- and mouse-reactive antibodies (B, right panel). Tyrosine-phosphorylation of PLCγ1 was studied in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells in the presence or absence of STI571 or LFM-A13. EIF-4E was used as a loading control in both Western blots (B, C). Expression of full-length BTK was silenced by RNA interference in a *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cell line (IX in Table I). Sorted cells carrying fluorescein-labeled siRNAs (green) were permeabilized, stained for PLCγ1^{pY783} (red) and analyzed by confocal laser microscopy (C, lower panel). As a negative control non-targeting siRNA duplices were used. BCR-ABL1-binding proteins were co-immunoprecipitated using an antibody against BCR. The immunoprecipitation was controlled by a Western blot using an ABL1-specific antibody (D).

Phosphorylation of PLCγ1 was sensitive to inhibition of BTK by LFM-A13 (Figure 5C) indicating that PLCγ1 is activated through BTK as in normal lymphocytes and not directly by BCR-ABL1. Silencing of full-length BTK by RNA interference using fluorescein-labeled full-length BTK-specific siRNAs also results in a marked reduction of PLCγ1^{Y783}-phosphorylation as assessed by confocal lasermicroscopy (Figure 5C) and intracellular FACS-staining (Figure 9A).

PLCγ1 contributes to survival signals in BCR-ABL1$^+$ *pre-B lymphoblastic leukemia cells*

Whereas the role of STAT5 in BCR-ABL1-mediated survival signaling is well established (21, 22, 24), a possible role of PLCγ1 to promote survival of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells was not investigated so far.

To determine whether BCR-ABL1- and BTK-dependent activation of PLCγ1 and PLCγ1-mediated Ca^{2+} release contributes to BCR-ABL1-mediated survival signals, we silenced PLCγ1 mRNA expression by RNA interference in three pre-B lymphoblastic leukemia cell lines carrying a *BCR-ABL1* gene rearrangement (VII to IX, Table I) and four cell lines carrying other chromosomal translocations (*MLL-AF4*, X; *TEL-AML1*, XII; *E2A-PBX1*, XIII; *TEL-PDGFRB*, XV; Table I). As a control, non-targeting siRNAs were used. Cells were transfected twice with fluorescein-labeled siRNAs. To asses viability, fluorescein$^+$ cells were analyzed for propidium iodide uptake and Annexin V-staining after 48 hours.

Among the three *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cell lines, viability (mean of three experiments $\pm$ S.D.) was 90.3 $\pm$ 2.7 percent with non-targeting siRNAs and 18.6 $\pm$ 13.7 percent with PLCγ1-specific siRNAs. Among four leukemia cell lines carrying other gene rearrangements, viability was 84.3 $\pm$ 12.2 percent with non-targeting siRNAs and 67.6 $\pm$ 16.9 percent with PLCγ1-specific siRNAs. Unlike *BCR-ABL1*-negative leukemia cells, silencing of PLCγ1 in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells results in a 5-fold reduction of viability. Thus, PLCγ1 contributes to survival signaling specifically in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cell lines.

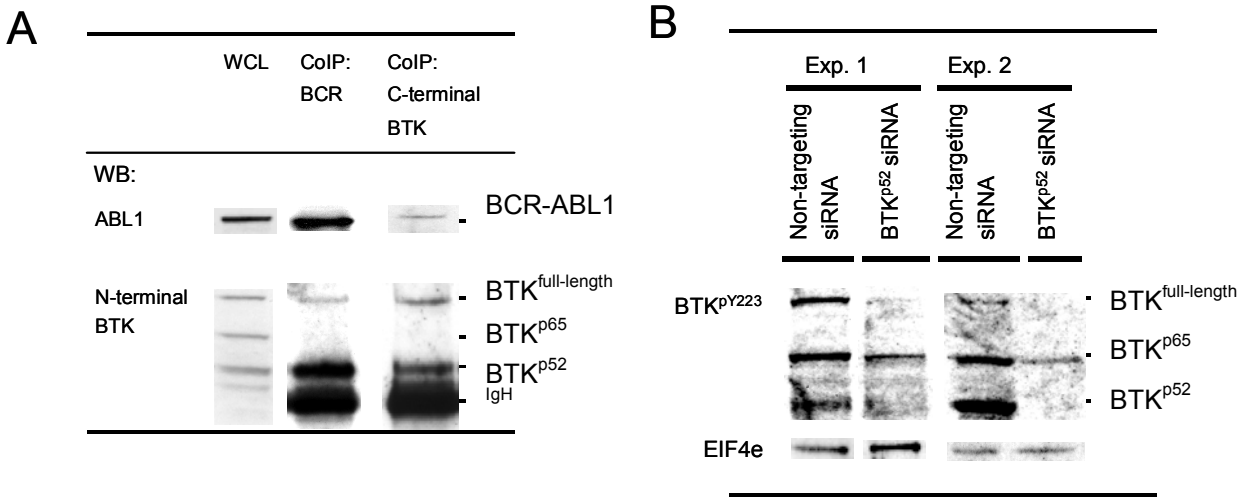
C-terminally truncated BTK functions as a linker between full-length BTK and BCR-ABL1

BCR-ABL1-dependent phosphorylation of BTK suggests that BTK represents a substrate of the BCR-ABL1 kinase. However, previous work demonstrated that BCR-ABL1 cannot directly phosphorylate full-length BTK but the N-terminal BTK-SH3 domain fused to GST (26).

To determine how BCR-ABL1 can induce phosphorylation of full-length BTK, we studied whether full-length BTK or BTK splice variants are part of the BCR-ABL1-signalosome. Using protein extracts of two *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cell lines, BCR-ABL1 signaling complexes were immunoprecipitated with a BCR-specific antibody. Immunoprecipitation was controlled using an anti-ABL1 antibody. Western blot showed that low amounts of full-length BTK indeed co-immunoprecipitates with BCR-ABL1 (Figure 6A). As a control for quantitative distribution of full-length BTK, BTK^{p65} and BTK^{p52} before co-immunoprecipitation with BCR-ABL1, whole cell lysates (WCL) from two *BCR-ABL1*$^+$ leukemia cell lines were subjected to Western blot (Figure 6A). While full-length BTK, BTK^{p65} and BTK^{p52} are expressed at similar levels in the leukemia cells (WCL, Figure 6A), the amount of full-length BTK co-immunoprecipitating with BCR-ABL1 was approximately 20-fold less than the amount of BTK^{p52} co-immunoprecipitating with BCR-ABL1. While BTK^{p65} is clearly expressed in the leukemia cells (WCL, Figure 6A), co-immunoprecipitation of BTK^{p65} with BCR-ABL1 could not be detected (Figure 6A).

Preferential binding of BCR-ABL1 to BTK^{p52} lacking the C-terminus but retaining the N-terminal BTK SH3-domain is consistent with previous findings demonstrating that BCR-ABL1 cannot directly phosphorylate full-length BTK but a BTK-SH3 domain-GST fusion molecule (26). However, binding of BTK^{p52} to BCR-ABL1 may also be indirect in this case. That the N-terminal BTK SH3-domain but not full-length BTK can be readily phosphorylated by BCR-ABL1 suggests that the C-terminus of BTK may interfere with binding of BCR-ABL1 to BTK.

Figure 6: *C-terminally truncated BTK functions as a linker between full-length BTK and BCR-ABL1*



Proteins binding to BCR-ABL1 were co-immunoprecipitated with an anti-BCR antibody (A). Immunoprecipitation was controlled by an anti-ABL1-specific Western blot showing the characteristic BCR-ABL1 fusion protein expressed by the leukemia cell line. Proteins binding to full-length BTK were co-immunoprecipitated with an antibody against C-terminal BTK. Immunoprecipitation was controlled by Western blot using an antibody against N-terminal BTK. Full-length BTK and BTK^{p52} proteins co-immunoprecipitating with either BCR-ABL1 or full-length BTK were visualized by Western blot using an antibody against N-terminal BTK (A). As a control for quantitative distribution of full-length BTK and BTK^{p52} before co-immunoprecipitation with BCR-ABL1 or full-length BTK, whole cell lysates (WCL) were used (A). To analyze the effect of BTK^{p52} on BCR-ABL1-dependent phosphorylation of full-length BTK, BTK^{p52} expression was inhibited by RNA interference as described in Figure 3. As a control, non-targeting siRNA duplices were used. siRNAs were fluorescein-labeled and $fluorescein^{+}$ cells were sorted and subjected to Western blot using antibodies specific for tyrosine-phosphorylated BTK^{Y223} (B). As patterns of tyrosine-phosphorylation varied between replicate experiments, Western blots of two representative experiments (Exp. 1 and 2) are shown. EIF4e was used as a loading control.

Likewise, BTK^{p65} molecules that harbor an in-frame deletion but retain the BTK C-terminus do not immunoprecipitate with BCR-ABL1. In this regard, the expression of truncated BTK splice variants lacking the C-terminus of BTK may represent a mechanism to facilitate phosphorylation of full-length BTK by BCR-ABL1. As BTK molecules can also self-associate through N-terminal SH3 domains (27), BTK^{p52} may bind to BCR-ABL1 as well as to full-length BTK acting as linker between the two kinases.

We next investigated whether full-length BTK can bind to BTK^{p52}. Using an antibody specific for C-terminal BTK (only recognizing full-length BTK) for immunoprecipitation and an N-terminal BTK-antibody (recognizing both full-length BTK and BTK^{p52}) for subsequent Western blotting, we observed that BTK^{p52} co-immunoprecipitates with full-length BTK

(Figure 6A). These findings suggest a role for BTK^{p52} as a linker between BCR-ABL1 and full-length BTK.

In this case, BTK^{p52} would also facilitate tyrosine phosphorylation of full-length BTK by BCR-ABL1. Therefore, we tested whether silencing of BTK^{p52} in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells results in decreased tyrosine-phosphorylation of full-length BTK. To this end, *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells were transfected with either non-targeting or BTK^{p52}-specific fluorescein-labeled siRNAs (Figure 3). Fluorescein$^+$ cells were sorted and analyzed for expression of tyrosine-phosphorylated BTK by Western blot (Figure 6B). Silencing of BTK^{p52} mRNA expression by siRNAs did not interfere with mRNA expression of full-length BTK (Figure 3), yet tyrosine-phosphorylation of full-length BTK was decreased by more than 90 percent (Figure 6B).

Given that i.) BCR-ABL1 and full-length BTK cannot directly bind to each other, ii.) BTK^{p52} can bind to both BCR-ABL1 and full-length BTK and iii.) expression of BTK^{p52} is required for efficient tyrosine-phosphorylation of full-length BTK, we conclude that BTK^{p52} can act as a linker between BCR-ABL1 and full-length BTK. This is in agreement with previous work suggesting that kinase-deficient BTK may act as a linker molecule between upstream kinases (e.g. SYK) and downstream effector molecules (e.g. PLCγ2; reference 11).

C-terminally truncated BTK can link full-length BTK to BCR-ABL1 through its SH3 domain

Based on this assumption, one would predict that the coexpression of BTK^{p52} with full-length BTK can facilitate the interaction between BTK and BCR-ABL1 and, hence, the phosphorylation of downstream signaling molecules. To test this possibility, BCR-ABL1, BTK and its truncated variant BTK^{p52} were expressed in 293T embryonic kidney cells either alone, in various combinations or in the presence of BTK- (LFM-A13) or BCR-ABL1- (STI571) kinase inhibitors. As a readout of this experiment, tyrosine phosphorylation of PLCγ1 and STAT5 was measured. According to a previous study, 293T cells do not express BTK but both PLCγ1 and STAT5 (28).

The expression of either full-length BTK, BTK^{p52} or BCR-ABL1 alone had no effect on tyrosine-phosphorylation of downstream molecules in general. However, to some extent, single transfection with full-length BTK induced activation of PLCγ1. Likewise, BCR-ABL1 alone induced to some extent tyrosine phosphorylation of STAT5 (Figure 7AB), indicating that BCR-ABL1 can partially activate STAT5 in the absence of BTK. Co-expression of BTK^{p52} lacking a functional kinase domain together with BCR-ABL1 did not increase tyrosine-phosphorylation as compared to expression of BCR-ABL1 alone. However, co-expression of BTK^{p52} with full-length BTK and BCR-ABL1 drastically upregulated both

PLCγ1 and STAT5-phosphorylation (Figure 7A). As assessed by intracellular staining, phosphorylated PLCγ1 and STAT5 was distributed in the cytoplasm and in the nucleus, respectively (Figure 7B).

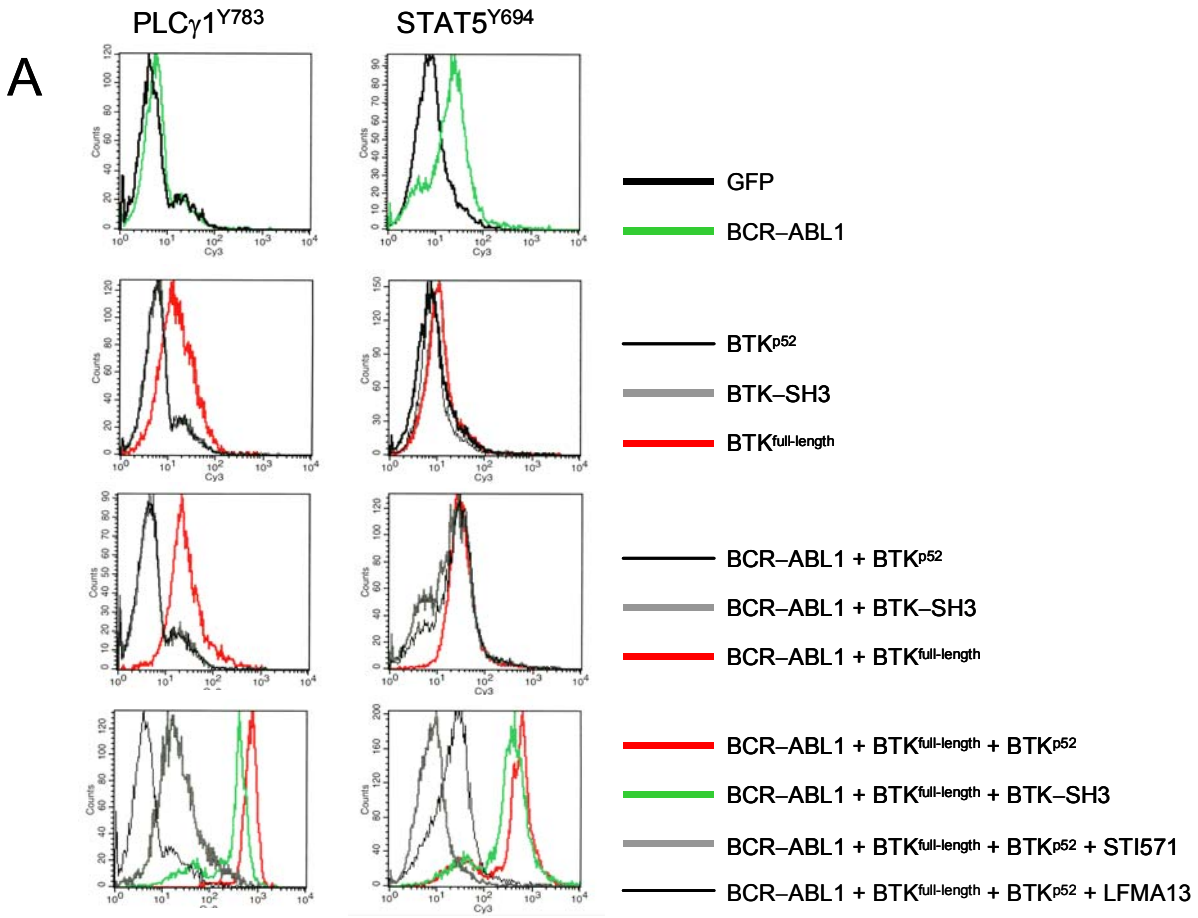
Activation of PLCγ1 and STAT5 upon triple-transfection with full-length BTK, BTKp52 and BCR-ABL1 was largely sensitive to BTK- (LFM-A13) or BCR-ABL1- (STI571) kinase inhibition. Hence, enzymatic activity of both kinases is required for activation of PLCγ1 and STAT5. As a control, the enzymatic activity of BTK expressed in transfected 293T cells was confirmed in an *in vitro* kinase assay as described in the Materials and Methods section. The kinase activity of BTK immunoprecipitated from 1 x 10^5 transfected 293T cells was roughly comparable to that of 25 ng (0.008 U) recombinant BTK (Figure 7C).

As BTK-SH3 domains can directly bind to BCR-ABL1 (26) and also bind to proline-rich regions of other BTK molecules (27), we tested whether the BTK-SH3 domain is sufficient to link full-length BTK to BCR-ABL1. In fact, transfection of 293T cells with a vector encoding only the BTK-SH3 domain had a similar effect compared to transfection with BTK^{p52} comprising PH, TH, SH3, SH2 and a truncated kinase domain (Figure 2C). Indeed, the SH3 domain was sufficient to enable BCR-ABL1-driven activation of PLCγ1 and STAT5 in the presence of active full-length BTK (Figure 7A). We conclude that BTK^{p52}, mainly through its SH3 domain, can act as a linker between full-length BTK and BCR-ABL1.

BTK^{p52} but not BTK^{p65} cooperates with full-length BTK to transduce BCR-ABL1-dependent survival signals

To clarify the specific contribution of BTK^{p52} and BTK^{p65} to BCR-ABL1-downstream survival signaling, Annexin V expression, STAT5-phosphorylation and $BCLX_L$ mRNA levels were measured in leukemia cells after transfection with fluorescein-labelled siRNAs against BTK^{p52} and BTK^{p65} and non-targeting siRNAs (Figure 8 A-C). Transfection of leukemia cells with non-targeting siRNAs or siRNAs against BTK^{p65} had no effect on viability as assessed by Annexin V-staining (Figure 8A), tyrosine-phosphorylation of STAT5 (Figure 8B) and $BCLX_L$ mRNA levels (Figure 8C). In contrast, siRNA-mediated silencing of BTK^{p52} induced apoptosis in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells to a similar extent as full-length BTK (Figure 8A). Likewise, silencing of either full-length BTK or BTK^{p52} decreased tyrosine-phosphorylation of STAT5 (Figure 8B) and reduced mRNA levels of $BCLX_L$ in the leukemia cells (Figure 8C). These findings collectively indicate, that survival signals in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells not only require BTK kinase activity but also BTK^{p52} acting as a linker between full-length BTK and BCR-ABL1.

Figure 7: *BTK^{p52} facilitates BTK- and BCR-ABL1-dependent activation of PLCγ1 and STAT5 through its SH3-domain*



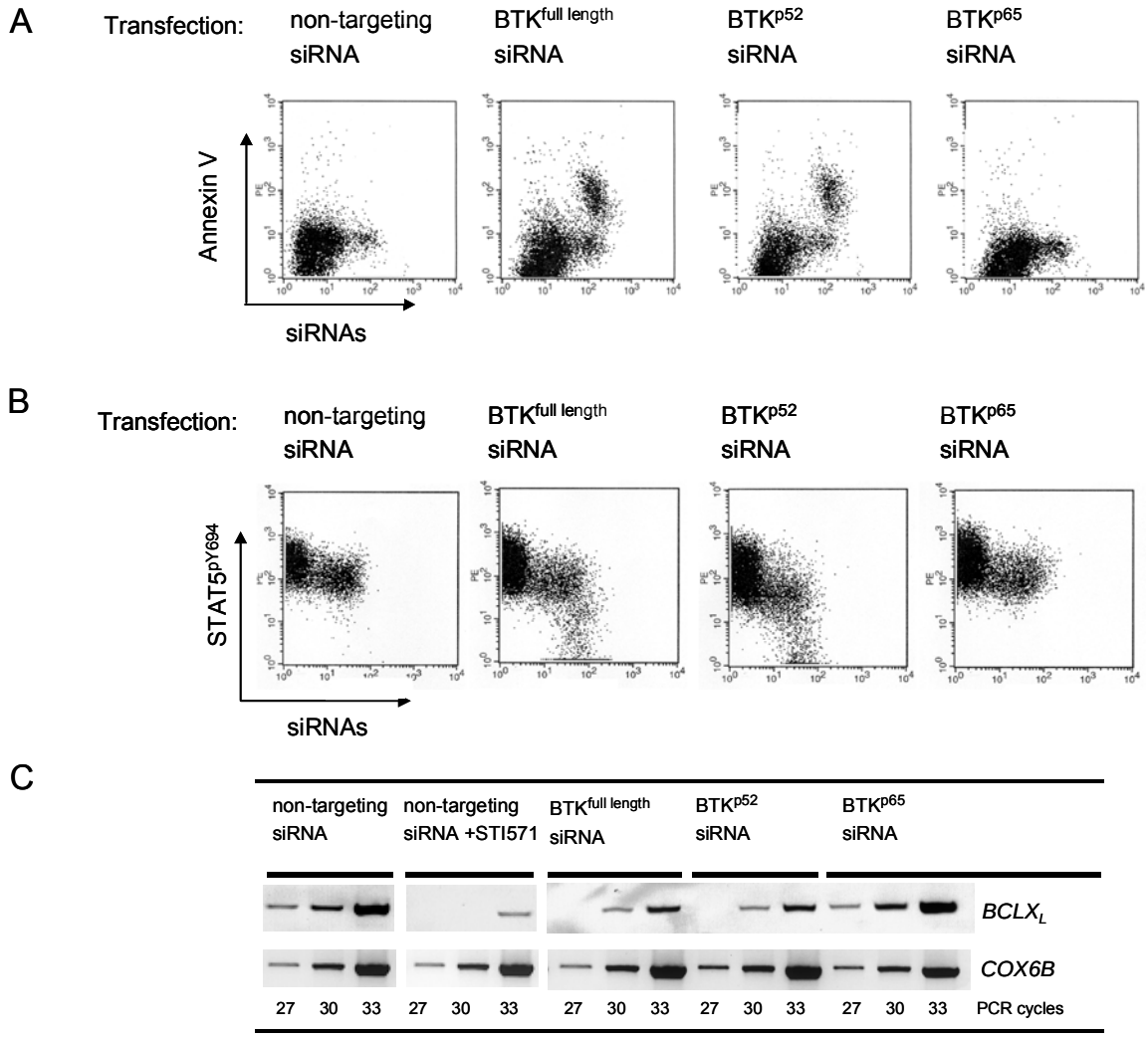
Legend to Figure 7:

BCR-ABL1, full-length BTK, BTK^{p52} and the SH3-domain of BTK were expressed in 293T embryonic kidney cells either alone or in various combinations in the presence or absence of STI571 or LFM-A13 as indicated. As a readout, cells were harvested, subjected to intracellular staining for tyrosine-phosphorylated PLCγ1 or STAT5 and analyzed by flow cytometry (A). To visualize cytoplasmic localization of tyrosine phosphorylated PLCγ1 and nuclear localization of activated STAT5, the stained cells were also subjected to analysis by confocal laser-microscopy (B). 1 x 10^6 293T cells were transiently transfected with full-length BTK for 24 hours and subjected to immunoprecipitation of full-length BTK. Immunoprecipitation was controlled by an BTK-specific Western blot (C). Kinase activity of immunoprecipitated BTK was analyzed in an *in vitro* kinase assay using 150 ng of a PLCγ1 fragment (amino acids 530 to 850) as substrate. In parallel, 25 ng of recombinant active BTK and 100 ng of kinase-deficient SH3-domain of BTK were used in kinase assays as positive and negative controls, respectively.

Requirement of BTK^{p52} for BCR-ABL1-driven autonomous Ca^{2+} signaling activity

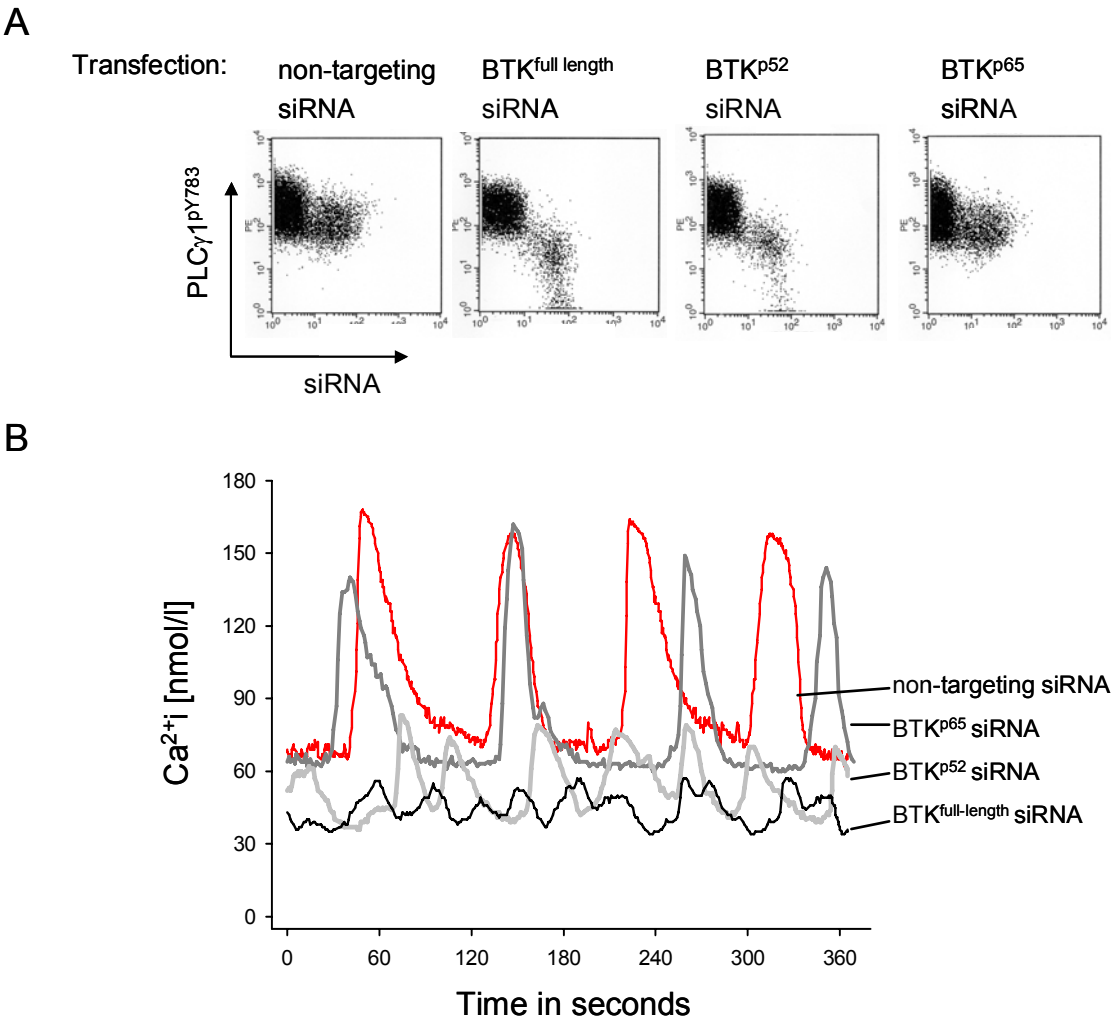
Autonomous oscillatory Ca^{2+} signals in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells require kinase activity of both BCR-ABL1 and BTK (Figure 5A) as well as PLCγ activity (9). Therefore, we investigated the impact of specific silencing of full-length BTK, BTK^{p52} and BTK^{p65} on PLCγ1 activation (Figure 9A). siRNA-mediated knockdown of full-length BTK- and BTK^{p52}-expression similarly reduced tyrosine-phosphorylation of PLCγ1, whereas siRNAs against BTK^{p65} had no effect (Figure 9A). In agreement with this, siRNA-mediated inhibition of either full-length BTK or BTK^{p52} expression but not expression of BTK^{p65} largely reduced the amplitude of autonomous Ca^{2+} oscillations in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells (Figure 9B). We could not identify a specific function of BTK^{p65} with respect to BCR-ABL1-mediated transformation in pre-B lymphoblastic leukemia cells.

Figure 8: *BTK^{p52} but not BTK^{p65} promotes cell survival, tyrosine-phosphorylation of STAT5 and upregulation of $BCLX_L$*



BCR-ABL1$^+$ pre-B lymphoblastic leukemia cells (IX, Table 1) were transfected with fluorescein-labeled siRNA duplices against full-length BTK, BTK^{p52} or BTK^{p65} as described above (see Figure 3) and analyzed by flow cytometry for Annexin V expression (A). As a negative control, non-targeting siRNA duplices were used. Likewise, siRNA-treated leukemia cells were subjected to intracellular staining for tyrosine-phosphorylated STAT5 and analyzed by flow cytometry (B). For the analysis of mRNA levels of $BCLX_L$, *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells were transfected with a non-targeting siRNA duplex in the presence or absence of STI571 (for 12 hours), as well as with siRNAs against full-length BTK, BTK^{p52} or BTK^{p65}. 1 x 10^5 leukemia cells carrying fluorescein-labeled siRNAs sorted by FACS and subjected to semiquantitative RT-PCR analysis for mRNA levels of $BCLX_L$ (C). mRNA levels for *COX6B* (C) and *HPRT* (see Figure 3) remained stable in all experiments.

Figure 9: *Specific silencing of BTK$^{full\text{-}length}$ or BTK^{p52} reduces PLCγ1 phosphorylation and autonomous Ca^{2+} oscillations in BCR-ABL^{+} pre-B lymphoblastic leukemia cells*



BCR-ABL1^{+} pre-B lymphoblastic leukemia cells were transfected with non-targeting siRNAs or siRNAs against full-length BTK, BTK^{p52} or BTK^{p65} (A), then subjected to intracellular staining for tyrosine-phophorylated PLCγ1 and analyzed by flow-cytometry (A). Cytoplasmic Ca^{2+} levels [nmol/l] were measured in single siRNA-containing cells by confocal laser-scanning microscopy by continuous scanning for six minutes (B). Prior to analysis of Ca^{2+} levels, leukemia cells carrying fluorescein-labeled siRNAs were sorted by FACS. For each condition, approximately 50 individual cells were recorded. As a control, cytoplasmic Ca^{2+} levels were measured in cells with non-targeting siRNAs (red line).

Discussion

The leukemogenic BCR-ABL1 kinase mimicks a constitutively active pre-B cell receptor in pre-B lymphoblastic leukemia cells. While the leukemia cells frequently carry only non-functional *IGH*-alleles (8), pre-B cell receptor signaling is even compromised in the few cases, in which the leukemia cells express a pre-B cell receptor on their surface: Important components of the pre-B cell receptor signaling cascade including SYK, SLP65 and BTK are not phosphorylated in response to pre-B cell receptor-engagement, and BTK is constitutively phosphorylated by BCR-ABL1. BCR-ABL1-dependent activation of BTK is critical for autonomous survival signals that would otherwise arise from the pre-B cell receptor. We conclude that BTK kinase activity is no longer responsive to pre-B cell receptor-dependent signals but contributes to multiple aspects of BCR-ABL1-driven survival signaling in pre-B lymphoblastic leukemia cells.

While BCR-ABL1 cannot directly interact with full-length BTK (26), however, BCR-ABL1-induced aberrant splicing of BTK pre-mRNA leads to the expression of a truncated splice variant of BTK acting as a linker molecule between the two kinases. Acting as a linker, truncated BTK^{p52} can not only facilitate BCR-ABL1-dependent activation of full-length BTK but also of downstream molecules including PLCγ1, STAT5 and $BCLX_L$.

The interaction between BCR-ABL1 and BTK is critical because not only inhibition of BCR-ABL1 by STI571 but also interference with BTK kinase activity specifically induces apoptosis in *BCR-ABL1*$^+$ B lymphoid leukemia cells. As resistance to STI571 is frequent in the therapy of this leukemia entity (15), inhibition of BTK or its truncated splice variant potentially represents a therapeutic approach to circumvent STI571-resistance of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells.

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Materials and Methods

Patient samples and cell lines

Clinical data on patient samples and cytogenetic data on cell lines used are summarized in Table I and are described in detail in supplementary information. All studies on human materials were performed on samples provided in compliance with Institutional Review Board regulations (Department of Hematology, University of Frankfurt, Frankfurt/Main, Germany).

Sequence analysis of BTK and semiquantitative RT-PCR

In a search for BTK isoforms, fragments of the BTK cDNA were amplified covering the entire coding region using the PCR primer pairs listed in supplementary Table I. *BTK* amplification products were sequenced as previously described (29). Primers used for semiquantitative RT-PCR analysis of human *BCR-ABL1*, $BCLX_L$, *GAPDH*, *HPRT* and *COX6B* transcripts are listed in supplementary Table I.

Transient expression of BCR-ABL1 in pre-B lymphoblastic leukemia cells

pre-B lymphoblastic leukemia cells (697 cell line) carrying an *E2A-PBX1* but no *BCR-ABL1* gene rearrangement were transiently transfected by electroporation (250 V and 950 μF) with pMIG-GFP or pMIG-GFP_BCR-ABL1 vectors encoding either GFP only or GFP and BCR-ABL1 as previously described (30). For both transfections, GFP^+ and GFP^- cells were sorted after 24 hours and subjected to mRNA isolation.

RNA interference with BTK and PLCγ1 expression

For each target, three different siRNA duplices were synthesized (MWG Biotech, Ebersberg, Germany). Sequences of siRNA duplices are listed in supplementary Table I.

For knockdown of PLCγ1 mRNA expression, a pool of three validated siRNAs targeting different PLCγ1 exons was used (Upstate, Lake Placid, NY). As a control, a non-targeting siRNA duplex was used that does not match a known mRNA sequence. All siRNA duplices were labeled with fluorescein using an siRNA labeling kit (Ambion, Austin, TX) according to the manufacturer's protocol. *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells were transfected with a mixture of three fluorescein-labeled siRNAs for each target sequence at a concentration of 100 nmol/l using Oligofectamine in Opti-MEM1 medium (Invitrogen). After 24 hours, leukemia cells were re-transfected with labeled siRNAs and subsequently incubated for further 24 hours. Transfection efficiency was controlled by fluorescence microscopy and by FACS. The silencing effect of siRNAs for specific BTK isoforms was controlled by RT-PCR analysis of BTK splice variants in sorted of fluorescein$^+$ cells. siRNA-containing fluorescein$^+$ cells were sorted using a FACStar 440 cell sorter. For each condition, 5 x 10^5 cells were sorted and subjected to RNA isolation and cDNA synthesis.

Western blotting

For the detection of signaling molecules by Western blot, antibodies against BTK, PLCγ1, PLCγ2, SLP65 and SYK (Cell Signaling Technology, Beverly, MA), as well as phosphotyrosine-specific antibodies against BTK^{Y223}, $PLC\gamma1^{Y783}$, $PLC\gamma2^{Y1217}$, $SLP65^{Y96}$ and SYK^{Y323}(Cell Signaling Technology) were used together with the WesternBreeze immunodetection system (Invitrogen). Mouse monoclonal antibodies against tyrosine-phosphorylated BTK^{Y223} and BTK^{Y551} were kindly provided by Dr. Owen N. Witte, Los Angeles, CA.

Co-immunoprecipitation

For immunoprecipitation of BCR-ABL1 or BTK, 1.7 mg protein lysate in a volume of 800 µl was incubated with 30 µl rProtein G Agarose beads (Invitrogen) for two hours at 4°C on a shaker. The supernatant was separated from the agarose beads and incubated with 6 µg of an antibody against BCR (Santa Cruz Biotechnology) or with 15 µg of an antibody against C-terminal BTK (kindly provided by Dr. Owen N. Witte, Los Angeles, CA). After 8 hours, 30 µl agarose beads were added and incubation was continued overnight. Afterwards, the agarose beads were washed twice with lysis buffer, resuspended in 35 µl of lysis buffer and subjected to Western blotting.

Flow cytometry

For FACS analysis of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells, antibodies against tyrosine-phosphorylated BTK^{Y223}, tyrosine-phosphorylated $PLC\gamma1^{Y783}$ and tyrosine-phosphorylated $STAT5^{Y694}$ (Cell Signaling Technologies) were used. Apoptotic or dead cells were identified by propidium iodide or PE-labelled Annexin V (BD Biosciences). As secondary antibody, anti-rabbit or anti-mouse IgG-Cy3 (Jackson Immunoresearch) was used.

Immunofluorescence and confocal lasermicroscopy

Nuclear, cytoplasmic or membrane localization of phosphorylated BTK^{Y223}, $PLC\gamma1^{Y783}$ and $STAT5^{Y694}$ was analyzed using primary antibodies from Cell Signaling Technology together with anti-rabbit IgG-Cy5 (Jackson Immunoresearch) as secondary antibody, respectively. Cells were fixed with 0.4% para-formaldehyde and incubated for 10 minutes in 90% methanol on ice and subjected to confocal laser-scanning microscopy as previously described (14).

Expression of BCR-ABL1, full-length BTK, BTK^{p52} and the BTK-SH3 domain in 293T cells

1 x 10^6 293T embryonic kidney cells were plated on a 24-well plate 24 hours prior to transfection. Different combinations of expression vectors were prepared as indicated and 5 µg of each vector was incubated with 8 µl of FuGENE6 (Roche) and 50 µl of serum-and antibiotic-free RPMI medium for 15 minutes at room temperature. The expression vector

pMIG-bcr/abl for *BCR-ABL1*^{p210} (kindly provided by Dr. David Baltimore, La Jolla, CA) was used as previously described (30). The expression vector *pMIG-flagBtk* for human full-length *BTK* was generated by cloning of the human BTK cDNA into the pMIG-R vector (30). For expression of *BTK*^{p52} (EMBL/GenBank accession number AJ888378), the expression vector pcDNA3.1 was used together with the directional TOPO Expression Kit (Invitrogen).

For expression of the BTK-SH3 domain, the pEBG BTK-SH3 vector (kind gift from Dr. Magnus Bäckesjø, Karolinska Institute, Huddinge, Sweden) was used as previously described (26).

Measurement of Ca^{2+} signals in BCR-ABL1$^+$ pre-B lymphoblastic leukemia cells

Pre-B lymphoblastic leukemia cells carrying a *BCR-ABL1* gene rearrangement were cultured in the presence or absence of the BCR-ABL1-inhibitor STI571 (from Novartis, Basel, Switzerland) or the BTK-inhibitor LFM-A13 (Calbiochem, Darmstadt, Germany) for the times indicated. In a different set of experiments, cells were transfected with fluorescein-labeled siRNA duplices and fluorescein$^+$ cells were sorted and conditioned in RPMI-medium at 37°C before measurement of cytoplasmic Ca^{2+} concentrations. Cells were washed and stained with Fluo-3 dye (Calbiochem) for 30 minutes. Changes of cytosolic Ca^{2+} were measured by confocal laser microscopy (31).

For each condition, Ca^{2+}-release of approximately 50 individual cells was recorded.

In vitro kinase assay

BTK immunoprecipitated from transfected 293T cells was used in a kinase assay including a Mg^{2+}/ATP cocktail (100 µmol/l) and a 61 kD fragment of PLCγ1 (Santa Cruz Biotechnology) as a substrate of BTK. Equal amounts of PLCγ1 peptide (150 ng) and ATP were incubated for 10 minutes at 30°C with BTK-immunoprecipitates from 1 x 10^5 transfected 293T cells, 25 ng active human recombinant BTK (Upstate) as a positive control or 100 ng of a peptide corresponding to the BTK-SH3 domain (Labvision, Westinghouse, CA; lacking kinase activity) as a negative control. The kinase activity of BTK immunoprecipitated from 5 x 10^5 transfected 293T cells was roughly comparable to that of 25 ng (0.008 U) recombinant BTK (Figure 7C).

Online supplemental material

A detailed description of patient samples and cell lines used in this paper is available as Supplementary Information. Supplementary Table I, lists all PCR-primers and siRNA duplices used. Supplementary Table II describes all aberrant splice variants of BTK amplified from *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells. Supplementary Table III depicts SAGE-data on mRNA levels of BCLX and BTK isoforms in normal B cell subsets and *BCR-*

ABL1^{+} leukemia cells. Supplementary information and Tables I to III are available at http://www.jem.org/cgi/content/full/jem/(URL pending).

Supplementary Table I: *PCR primers used for BTK sequence analysis, semiquantitative RT-PCR and RNA interference*

PCR-primers	
BTK exon 2 forward	5'-ATCCCAACAGAAAAAGAAAACAT-3'
BTK exon 8 reverse	5'-GTTGCTTTCCTCCAAGATAAAAT-3'
BTK exon 8 forward	5'-ATCTTGAAAAAGCCACTACCG-3'
BTK exon 12 forward	5'-TGATACGTCATTATGTTGTGTGTT-3'
BTK exon 13 forward	5'-ACTCATATCCAGGCTCAAATATC-3'
BTK exon 14 reverse	5'-ATCATGACTTTGGCTTCTTCAAT-3'
BTK exon 14 forward	5'-AATTGATCCAAAGGACCTGAC-3'
BTK exon 15 forward	5'-GAGAAGCTGGTGCAGTTGTAT-3'
BTK exon 16 reverse .1	5'-GGCCGAAATCAGATACTTTAAC-3'
BTK exon 16 reverse.2	5'-CTTTAACAACTCCTTGATCGTTT-3'
BTK exon 17 reverse	5'-ATTTGCTGCTGAACTTGCTATAC-3'
BTK exon 18 reverse	5'-TATACCTTCTCTGAAGCCAGATG-3'
BTK exon 19 reverse.1	5'-AAATTTCAGGCACAATAATTTCT-3'
BTK exon 19 reverse.2	5'-GGATTCTTCATCCATGACATCTA-3'
BTK^{p52} forward	5'CTTCCTCTCTGGACTGTAAGAAT-3'
BTK^{p52} reverse	5'-ATACAACTGCACCAGCTTCTC-3'
BCR-ABL1 forward	5'-ACCTCACCTCCAGCGAGGAGGACTT-3'
BCR-ABL1 reverse	5'-TCCACTGGCCACAAAATCATACAGT-3'
BCLX$_L$ forward	5'-AATCTTATCTTGGCTTTGGAT-3'
BCLX$_L$ reverse	5'-GTTCTCTTCCACATCACTAAA-3'
COX6B forward	5'-AACTACAAGACCGCCCCTTT-3'
COX6B reverse	5'-GCAGCCAGTTCAGATCTTCC-3'
GAPDH forward	5'-TTAGCACCCCTGGCCAAG-3'
GAPDH reverse	5'-CTTACTCCTTGGAGGCCATG-3'
HPRT forward	5'-CCTGCTGGATTACATCAAAGCACTG-3'
HPRT reverse	5'-CACCAGCAAGCTTGCGACC-3'
siRNA duplices	
Non-targeting siRNA	5'-UUGUACCUAAUUUCGUCCCAC-3' 3'-CAUGGAUUAAAGCAGGGUGUU-5'
BTK$^{full-length}$ siRNA.1	5'-UUGCUGGUGCAGUUGUAUGGC-3' 3'-CGACCACGUCAACAUACCGUU-5'
BTK$^{full-length}$ siRNA.2	5'-UUGUUGUAUGGCGUCUGCACC-3' 3'-CAACAUACCGCAGACGUGGUU-5'
BTK$^{full-length}$ siRNA.3	5'-UUGUACAUGGCCAAUGGCUGC-3' 3'-CAUGUACCGGUUACCGACGUU-5'
BTK^{p65} siRNA.1	5'-UUUCAUGAUGUAUGUCCUGGA-3' 3'-AGUACUACAUACAGGACCUUU-5'
BTK^{p65} siRNA.2	5'-UUAAGUCAUGAUGUAUGUCCU-3' 3-'UUCAGUACUACAUACAGGAUU-5'
BTK^{p65} siRNA.3	5'-UUCCAAAGUCAUGAUGUAUGU-3' 3'-GGUUUCAGUACUACAUACAUU-5'

BTK^{p52} siRNA.1	5’-UUUCAUGAUGCAGCUCGAAAC-3’
	3’-AGUACUACGUCGAGCUUUGUU-5’
BTK^{p52} siRNA.2	5’-UUAGUCAUGAUGCAGCUCGAA-3’
	3’-UCAGUACUACGUCGAGCUUUU-5’
BTK^{p52} siRNA.3	5’-UUCCAAAGUCAUGAUGCAGCU-3’
	3’-GGUUUCAGUACUACGUCGAUU-5’

Supplementary Table II: *Sequence analysis of aberrant splice variants of BTK in BCR-ABL1$^+$ pre-B lymphoblastic leukemia*[a]

Exons deleted	Splice sites	Transcript	Translation	Western blot	Kinase activity[c]	C-terminus	Frequency
Exon 14	Cryptic	ΔExon 14, Δ62 bp exon 13, Δ133 bp exon 15	Truncated SH2 and kinase domain	n.d.	–	–	unique (1/9)
Exon 15	Regular	ΔExon 15	Truncated kinase domain	52 kD protein	–	–	recurrent (9/9)
Exons 14 and 15	Cryptic	ΔExon 15, Δ41 bp exon 14	Truncated kinase domain	n.d.	–	–	unique (1/9)
Exons 15 and 16[b]	Regular	ΔExons 15, 16	In-frame deletion in kinase domain	65 kD protein	–	+	recurrent (9/9)
Exons 15 to 18	Cryptic Slippage	ΔExons 15-18, Δ79 bp exon 14, Ins 3 bp, Δ24 bp exon 19	Truncated kinase domain	n.d.	–	–	unique (1/9)
Exons 13 to 17	Cryptic	Δexons 13-17, Δ8 bp exon 12, Δ95 bp exon 18	Truncated SH2 and kinase domain	n.d.	–	–	unique (1/9)

Notes: a.: Sequence data are available from EMBL/GenBank under accession numbers AJ888376, AJ888377, AJ888378, AJ888379, AJ888380, AJ888381

b.: This splice variant was previously described by Goodman et al., 2003 (see reference 18)

c.: Kinase activity predicted based on phenotype of XLA patients (http://bioinf.uta.fi/BTKbase/BTKbasebrowser.html)

SH2: SRC homology domain 2; Ins: Insertion; Δ: Deletion

Supplementary Table III: *Frequency of BTK and BCLX splice variants in normal bone marrow progenitors and B cell subsets, and B lymphoid leukemia and lymphoma cases*

	$BTK^{full\text{-}length}$	BTK^{p65}	BTK^{p52}	$BCLX_L$	$BCLX_S$
SAGE-Tag	GATGAAGAAT	ATGTATGTCC	ATGCAGCTCG	CCTGTCCAGC	ACTGTGGCCG
Cell Type					
$CD34^+$ Hematopoietic progenitors	3			7	2
$CD15^+$ Myeloid progenitors				5	2
$CD7^+$ $CD10^+$ T cell progenitors				4	3
MLL-AF4 pro-B ALL case I		4		4	
MLL-AF4 pro-B ALL case II				4	
MLL-AF4 pro-B ALL case III		4		2	
MLL-AF4 pro-B ALL case IV				3	
TEL-AML1 pre-B ALL case I		4		3	
TEL-AML1 pre-B ALL case II				3	
BCR-ABL1 pre-B ALL case I	4	4	11	15	
BCR-ABL1 pre-B ALL case II		4	16	12	
BCL1-IGH MCL case I		4		4	
BCL1-IGH MCL case II				6	
$CD10^+$ $CD19^+$ pre-B cells	71			3	6
$CD5^+$ $CD19^+$ B1 cells	7			4	
$CD19^+$ $CD27^-$ Naive B cells	4			4	
$CD20^+$ $CD77^+$ Germinal center	17			3	3
$CD19^+$ $CD27^+$ Memory B cells	27			11	
$CD19^+$ $CD138^+$ Plasma cells	8			4	

Notes:
All SAGE-tag counts are normalized to 100,000 total SAGE-tags.
BTK^{p51} was not expressed in the two cases of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cases studied by SAGE
ALL, acute lymphoblastic leukemia; MCL, mantle zone B cell lymphoma
MLL-AF4, *TEL-AML1*, *BCR-ABL1* and *BCL1-IGH* denote gene rearrangements resulting from chromosomal t(4;11)(q21;q23), t(12;21)(p13;q22), t(9;22)(q34;q11) and t(11;14)(q13;q32) translocations, respectively

SAGE analysis

To identify differentially expressed genes between *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells and normal pre-B cells that play a role in malignant transformation of pre-B cells by BCR-ABL1, we analyzed gene expression profiles generated by the serial analysis of gene expression (SAGE) method. A total of 887,000 SAGE tags were collected for 19 SAGE profiles. $CD34^+$ hematopoietic progenitor cells, $CD15^+$ common myeloid progenitor cells, T cell progenitors, $CD10^+$ $CD19^+$ pre-B cells, $CD5^+$ $CD19^+$ B1 cells, $CD19^+$ $CD27^-$ naïve B cells, $CD20^+$ $CD77^+$ germinal center B cells, $CD19^+$ $CD27^+$ memory B cells, $CD19^+$ $CD138^+$ plasma cells, pre-B lymphoblastic leukemia cells carrying a *BCR-ABL1, MLL-AF4* or *TEL-AML1* gene rearrangement and *CCND1-IGH*$^+$ mantle zone B cell lymphoma cells were analyzed by SAGE (supplementary Table II). In order to identify isoforms of *BTK* and *BCLX*, SAGE-tags were analyzed that distinguish between full-length *BTK*, BTK^{p65} and BTK^{p52} as well as between $BCLX_L$ and $BCLX_S$. Based on SAGE-tag counts/100,000 SAGE-tags, the relative frequency of splice variants of BTK and BCLX was analyzed.

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5.5 Activation-induced cytidine deaminase acts as a mutator in BCR-ABL1-induced pre-B lymphoblastic leukemia and lymphoid blast crisis of chronic myeloid leukemia

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Summary

The Philadelphia chromsome (Ph) encoding the oncogenic BCR-ABL1 kinase defines a subset of pre-B acute lymphoblastic leukemia (ALL) with a particularly unfavorable prognosis. Here we show that oncogenic BCR-ABL1 kinase activity induces aberrant somatic hypermutation in Ph-positive ALL cells. In mature germinal center B cells, activation induced cytidine deaminase (AID) introduces somatic mutations into immunoglobulin variable (V) region genes. Comparing Ph-positive and Ph-negative ALL cells, AID expression was found in 23 of 26 Ph-positive but only 2 of 74 Ph-negative ALLs. Consistent with aberrant AID expression in Ph-positive ALL, immunoglobulin V region genes were mutated in most Ph-positive but unmutated in Ph-negative cases. Forced expression of *BCR-ABL1* in Ph-negative pre-B cells and usage of the BCR-ABL1-kinase inhibitor STI571 revealed that BCR-ABL1 kinase activity causes aberrant expression of AID.

Of note, aberrantly expressed AID in Ph-positive ALL also targets non-immunoglobulin genes. For instance, AID introduced DNA-single-strand breaks within the tumor suppressor gene *CDKN2B* in Ph-positive ALL cells,

which was sensitive to both inhibition of BCR-ABL1 kinase activity and silencing of AID expression by RNA interference.

These findings identify AID as a BCR-ABL1-induced mutator in B lymphoid leukemia cells.

Introduction

Somatic hypermutation (SHM) and class-switch recombination (CSR) represent physiological processes that modify variable (V) and constant (C) regions of immunoglobulin genes in mature germinal center B cells (1). Both SHM and CSR critically depend on expression of activation-induced cytidine deaminase (AID), which introduces single-strand breaks into target DNA (2). AID-mediated DNA single-strand breaks leading to SHM and CSR are specifically introduced into V or C regions of immunoglobulin genes, respectively. At much lower frequency, however, AID can also target non-immunoglobulin genes in germinal center B cells (3) and may even act as a genome-wide mutator (4). Such targeting errors carry the risk of oncogenic mutation leading to the transformation of a germinal center B cell, which may give rise to B cell lymphoma. For instance, aberrant SHM or CSR may lead to chromosomal translocation of proto-oncogenes including *MYC*, *BCL2*, *BCL6* and *CCND1* and cause various types of B cell lymphoma (3). Therefore, tight regulation of AID expression in germinal center B cells and control of DNA strand breaks related to SHM and CSR is critical to prevent B cell malignancy.

Results

Aberrant AID expression correlates with the Philadelphia chromosome in pre-B acute lymphoblastic leukemia

Pre-B cells represent the normal precursor of pre-B acute lymphoblastic leukemia (ALL), do not express AID (Table 1; Figure 1A) and carry immunoglobulin genes that have neither undergone SHM nor CSR (5). Therefore, it was unexpected that AID is expressed in a subset of ALL (Table 1, Figure 1B). Interestingly, AID expression correlates with the presence of t(9;22)(q34;q11), the so-called Philadelphia chromosome (Ph). Ph encodes the oncogenic BCR-ABL1 kinase and defines a subgroup of ALL with a particularly unfavorable prognosis (6). Studying AID mRNA expression in 100 cases of pre-B ALL, AID mRNA was detected in 23 of 26 cases of Ph-positive ALL but only in 2 of 74 cases of Ph-negative ALL (Supplementary Figure 1). Compared to normal germinal center B cells, mRNA levels for AID are lower in some but similar in other cases of Ph-positive ALL (Figure 1B).

Table 1: *Somatic hypermutation and class-switch recombination in Ph-positive pre-B acute lymphoblastic leukemia cells*

Cell type	Phenotype	Mutated[a] V_H	Mutation frequency [x 10^{-3} bp]		C_H regions	AID
			all V_H	mutated[a] V_H	Class switch[b]	mRNA expression[c]
Bone marrow pre-B cells	$CD19^+$ $VpreB^+$	1/ 36 clones	4.4 ± 4	11	No; Cμ only	No[d]
Peripheral blood naive B cells	IgD^+ $CD27^-$	0/ 12 clones	3.1 ± 1	not applicable	No; Cμ and Cδ	No[d]
Tonsillar germinal center B cells	$CD19^+$ $CD38^+$	12/ 14 clones	40.2 ± 12	46.5 ± 8	Yes; Cγ1, Cγ2, Cγ3, Cε, Cα	Yes[d]
Peripheral blood memory B cells	$CD19^+$ $CD27^+$	52/ 54 clones	45.2 ± 9	46.8 ± 8	Yes; Cγ1, Cγ2, Cγ3, Cε, Cα	No[d]
Ph-negative pre-B ALL	$CD10^+$ $CD19^+$	4/ 48 cases	4.3 ± 5	29.1 ± 7	0/ 10 cases; Cμ only	2/ 74 cases
Ph-positive pre-B ALL	$CD10^+$ $CD19^+$	28/ 32 cases	36.1 ± 29	40.1 ± 8	5/ 21 cases; Cγ1, Cγ2, Cγ3, Cα	23/ 26 cases

Notes:

a.: All V_H sequences carrying more than 10 x 10^{-3} bp mismatches to germline V_H genes are considered mutated.
b.: For normal B cell subsets, rearranged V_H and C_H regions were amplified from bulk populations using V_H- together with Cμ, Cδ, Cγ1, Cγ2, Cγ3, Cγ4, Cε and Cα-specific primers in 45 cycles. For leukemia cells, individual cases were analysed. All RT-PCR products were confirmed by sequence analysis.
c.: AID transcripts were amplified from normal B cell populations and leukemia cells in 45 cycles of RT-PCR. For each population, cDNA amounts corresponding to 10^5 cells were used.
d.: For RT-PCR analysis of AID expression in normal B cell subsets, see Figure 1A.

Table 2A: *V_H region genes in human Ph-negative pre-B lymphoblastic leukemia cells*

Case	V_H	D_H	J_H	Number of mutations [x 10^{-3} bp]	Cytogenetics	Genes involved
Philadelphia chromosome-negative pre-B lymphoblastic leukemia						
1	V6-1	D2-2	J4	0	Hyperdiploid	N.N.
2	V4-61	none	J4	7 (26)	t(9;15)(p2;q1)	N.N.
3	V3-13	D2-15	J5	0	Hyperdiploid	N.N.
4	V3-66	none	J4	18 (67)	Hyperdiploid ; del(6)(q2)	N.N.
5	V4-34	none	J6	0	Hyperdiploid	N.N.
6	V4-34	D3-22	J4	8 (30)	del(6)(q2q5)	N.N.
7	V4-39	D3-22	J6	1 (4)	Hyperdiploid, trisomy 14	N.N.
8	V4-59	D3-10	J6	0	Hyperdiploid	N.N.
9	V3-64	none	J5	5 (19)	t(17;22)(q25;q11)	*BCR* locus
	V7-4	D6-19	J2	6 (22)		
	V1-18	D3-3	J6	3 (11)		
10	V3-33	D2-2	J6	0	t(3;12)(p11;p13)	*TEL* rearranged
	V4-4	none	J1	1 (4)		
11	V4-34	D1-20	J4	0	Hyperdiploid	N.N.
12	V1-3	D6-6	J5	0	Hyperdiploid	N.N.
13	V6-1	D2-8	J6	1 (4)	Hyperdiploid	N.N.
14	V5-a	D2-15	J6	1 (4)	t(4;11)(q21;q23)	*MLL-AF4*
15	V3-74	D2-2	J4	0	t(1;12)(p32;p12),t(7;7)(q22;q36)	*TEL* rearranged
16	V3-33	none	J6	0	no gross abnormality	N.N.
17	V6-1	D2-21	J6	0	t(2;14)(q23;q32); t(12;21)(p13;q22)	*IGH* locus; *TEL-AML1*
18	V6-1	D3-10	J6	0	Hyperdiploid	N.N.
19	V4-34	D3-16	J4	1 (4)	Hyperdiploid	N.N.
20	V1-3	none	J4	1 (4)	del1(q?)	N.N.
21	V2-5	D5-12	J5	2 (7); 69-bp deletion in V_H	Hyperdiploid	N.N.
22	V2-70	D3-10	J4	1 (4)	der(9)t(9;22)(p11;q11)	*BCR* locus
23	V3-9	D3-3	J6	2 (7)	Hyperdiploid	N.N.
24	V1-58	none	J6	1 (4), 70-bp deletion in V_H	Hyperdiploid	N.N.
25	V2-5	D2-15	J4	0	Hyperdiploid	N.N.
26	V2-5	D3-10	J6	2 (7)	t(11;19)(q23;p13.3)	*MLL-ENL*
27	V3-23	D2-2	J6	1 (4)	Hyperdiploid	N.N.
28	V2-5	D3-10	J6	0	Hyperdiploid	N.N.
29	V4-34	D6-19	J4	1 (4)	Hyperdiploid	N.N.
30	V3-53	D2-21	J4	1 (4)	Hyperdiploid	N.N.
31	V6-1	D2-2	J6	0	Hyperdiploid	N.N.
32	V4-34	D3-9	J6	0	no gross abnormality	N.N.
33	V1-8	D3-3	J4	1 (4)	t(1;19)(q23;p13)	*E2A-PBX1*
	V3-66	D2-2	J4	0		
34	V2-70	D3-10	J6	0	der(12)t(12;14)(p12;q11)	*TEL* rearranged
35	V6-1	D2-2	J6	0	del(10)(p11)	N.N.
36	V3-21	none	J6	0	del(9)(p11),del(17)(p11)	N.N.
37	V3-9	D2-15	J4	0	Hyperdiploid	N.N.
38	V3-30	D3-10	J6	0	Hyperdiploid	N.N.
39	V2-5	D2-2	J3	0	t(12;21)(p13;q22)	*TEL-AML1*
	V3-15	D3-19	J6	1 (4)		
40	V4-34	D2-15	J6	1 (4)	t(12;21)(p13;q22)	*TEL-AML1*
	V4-39	none	J6	0		
41	V3-13	D3-22	J6	2 (7)	t(4;11)(q21;q23)	*MLL-AF4*
	V2-5	D2-2	J6	0		
42	V3-20	D2-8	J5	0	t(4;11)(q21;q23)	*MLL-AF4*
	V6-1	D1-7	J4	0		
43	V3-15	D3-10	J6	1 (4)	t(12;21)(p13;q22), t(16;21)(q24 ;q22)	*TEL-AML1*
44	V4-34	D3-22	J2	0	t(1;19)(q23;p13), del(6)(q21)	*E2A-PBX1*
	V2-26	D2-2	J4	1 (4)		
45	V3-7	D3-10	J4	1 (4)	t(1;19)(q23;p13), t(3;14)(q27;q24)	*E2A-PBX1*
46	V3-15	D3-16	J5	0	t(1;19)(q23;p13); t(9;9)(q21;q11)	*E2A-PBX1*
47	V1-69	D3-10	J6	2 (7)	t(5 ;12)(q33 ;p13)	*TEL-PDGFRB*
48	V6-1	D5-5	J6	0	Hyperdiploid	N.N.
	V4-59	D2-8	J6	0		
Mean:				**1.2 ± 2 (4.3 ± 5)**		

Notes:

Cases 1-9 were previously described in Height et al., 1996 (reference 7). Sequence data were re-analysed based on the full sequence of human germline immunoglobulin heavy chain V genes (reference 8). Cases 1-40 are primary cases of Ph-negative leukemia. Cases 41-48 represent leukemia cell lines BEL1 (41), RS4;11 (42), REH (43), 697 (44), Kasumi-2 (45), MHH-CALL3 (46), Nalm-6 (47) and HPB-Null (48). Deletions were counted as one mutation.

Table 2B: *V_H region genes in human Ph-positive pre-B lymphoblastic leukemia cells*

Case	V_H	D_H	J_H	Number of mutations [x 10^{-3} bp]	Cytogenetics	Genes involved
Philadelphia chromosome-positive pre-B lymphoblastic leukemia						
49	V3-66	D3-3	J4	12 (44)	t(9;22)(q34;q11)	*BCR-ABL1*
50	V3-49	D2-21	J4	21 (78)	t(9;22)(q34;q11)	*BCR-ABL1*
	V3-48	D6-13	J4	49 (181)		
51	V4-30	D4-23	J6	7 (26)	t(9;22)(q34;q11)	*BCR-ABL1*
52	V2-70	D2-2	J6	8 (30); 87-bp deletion in V_H	t(9;22)(q34;q11)	*BCR-ABL1*
53	V4-59	D3-9	J4	0	t(9;22)(q34;q11)	*BCR-ABL1*
54	V1-18	D3-3	J3	4 (15)	t(9;22)(q34;q11)	*BCR-ABL1*
	V3-74	D4-17	J4	3 (11)		
	V3-7	D6-13	J4	10 (37)		
	V3-53	D3-22	J6	16 (59)		
55	V4-31	D2-2	J4	13 (48)	t(9;22)(q34;q11)	*BCR-ABL1*
56	V4-34	D5-12	J2	17 (63)	t(9;22)(q34;q11)	*BCR-ABL1*
	V4-31	D2-2	J4	13 (48)		
57	V3-7	D6-13	J5	4 (15)	t(9;22)(q34;q11)	*BCR-ABL1*
	V1-2	none	J5	4 (15)		
	V3-74	D3-3	J5	13 (48)		
58	V3-49	none	J5	21 (78)	t(9;22)(q34;q11)	*BCR-ABL1*
	V1-46	D5-5	J4	6 (22)		
59	V3-49	D7-27	J6	22 (81)	t(9;22)(q34;q11)	*BCR-ABL1*
60	V4-34	D6-13	J5	4 (15)	t(9;22)(q34;q11)	*BCR-ABL1*
61	V4-61	D2-2	J4	0	t(9;22)(q34;q11)	*BCR-ABL1*
62	V3-33	D3-22	J4	0	t(9;22)(q34;q11)	*BCR-ABL1*
63	V5-51	D3-22	J4	4 (15)	t(9;22)(q34;q11)	*BCR-ABL1*
	V5-51	D6-19	J3	3 (11)	t(9;22)(q34;q11)	*BCR-ABL1*
64	V5-51	D1-26	J1	5 (19)	t(9;22)(q34;q11)	*BCR-ABL1*
65	V3-30	D3-22	J3	4 (15)	t(9;22)(q34;q11)	*BCR-ABL1*
66	V1-46	D6-13	J4	0	t(9;22)(q34;q11)	*BCR-ABL1*
	V1-2	D3-10	J5	4 (15)	t(9;22)(q34;q11)	*BCR-ABL1*
67	V2-5	D3-22	J3	3 (11)	t(9;22)(q34;q11)	*BCR-ABL1*
68	V1-46	D3-10	J5	6 (22)	t(9;22)(q34;q11)	*BCR-ABL1*
69	V1-18	D2-2	J6	7 (26); intraclonal diversity	t(9;22)(q34;q11)	*BCR-ABL1*
	V3-21	D6-25	J6	4 (15)		
70	V1-3	D5-18	J6	0	t(9;22)(q34;q11)	*BCR-ABL1*
	V3-7	D2-2	J6	4 (15); intraclonal diversity		
	V3-30	D5-5	J4	11 (41)		
71	V1-8	D5-18	J6	0	t(9;22)(q34;q11)	*BCR-ABL1*
	V1-46	D2-21	J5	36 (133); intraclonal diversity		
72	V3-33	D2-2	J4	24 (89); intraclonal diversity	t(9;22)(q34;q11)	*BCR-ABL1*
	V3-30	D6-6	J4	21 (78); intraclonal diversity		
	V3-23	D2-2	J4	14 (52)		
	V3-30	D2-15	J6	17 (63); intraclonal diversity		
73	V2-5	none	J5	2 (7)	t(9;22)(q34;q11)	*BCR-ABL1*
74	V3-21	D2-15	J3	6 (22); intraclonal diversity	t(9;22)(q34;q11)	*BCR-ABL1*
75	V4-34	D2-8	J2	6 (22)	t(9;22)(q34;q11)	*BCR-ABL1*
	V3-74	none	J5	37 (137); intraclonal diversity		
76	V3-43	none	J4	3 (11)	t(9;22)(q34;q11)	*BCR-ABL1*
	V6-1	D3-9	J6	6 (22)		
77	V1-8	none	J2	10 (37); intraclonal diversity	t(9;22)(q34;q11)	*BCR-ABL1*
	V2-5	D3-16	J4	3 (11)		
	V2-70	D3-16	J4	7 (26); intraclonal diversity		
	V3-9	D2-21	J5	6 (22); intraclonal diversity		
	V4-31	D3-16	J4	7 (26); intraclonal diversity		
	V4-59	D3-16	J4	6 (22); intraclonal diversity		
78	V1-46	none	J4	6 (22)	t(9;22)(q34;q11)	*BCR-ABL1*
	V3-30	none	J5	12 (44)		
	V3-30	D6-13	J3	11 (41); intraclonal diversity		
	V4-34	none	J5	14 (52)		
79	V1-2	D2-2	J6	19 (70); intraclonal diversity	t(9;22)(q34;q11)	*BCR-ABL1*
	V3-53	D2-8	J6	3 (11)		
	V4-4	none	J6	12 (44)		
80	V1-69	D3-22	J6	4 (15)	t(9;22)(q34;q11)	*BCR-ABL1*
	V4-55	D3-22	J6	2 (7)		
Mean:				**9.7 ± 8 (36.1 ± 29)**		

Notes to Table 2B:

Cases 49-58 were previously described in Height et al., 1996 (reference 7). Sequence data were re-analysed based on the full sequence of human germline immunoglobulin heavy chain V genes (reference 8). Cases 49-73 are primary cases of Ph-positive leukemia. Cases 69-73 represent lymphoid blast crisis of chronic myeloid leukemia. Cases 74-80 represent cell lines BV173 (74), CMLT1 (75), K562 (76), Nalm-1 (77), SD1 (78), SUP-B15 (79) and TOM1 (80). Intraclonal diversity denotes the presence of multiple sequences for one V_H gene rearrangements, which share common mutations but differ in diversifying mutations. Deletions were counted as one mutation.

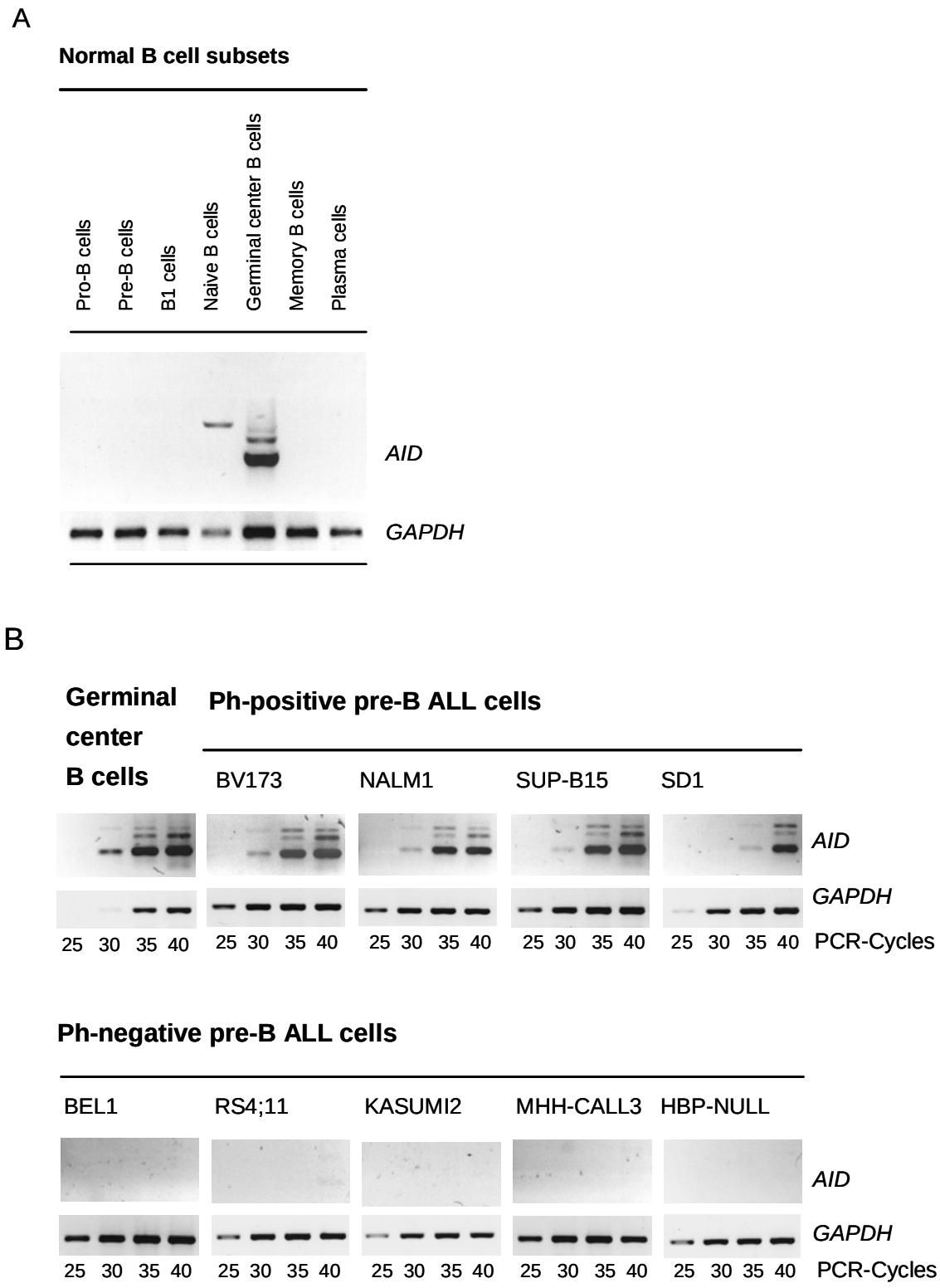
Somatic hypermutation and class-switch recombination in Ph-positive pre-B ALL

This raises the possibility that immunoglobulin V region and C region genes are subject to SHM and CSR in Ph-positive ALL, respectively. Therefore, we analysed the sequence of V_H region genes in 48 Ph-negative and 32 Ph-positive cases of ALL. Among Ph-positive ALL, 28 of 32 cases carry somatically mutated V_H region genes (Table 1, Table 2). In contrast, only 4 of 48 Ph-negative pre-B ALL cases harbor mutated V_H gene rearrangements. Counting only Ph-positive and Ph-negative leukemia cases, for which information on both AID expression and V_H region sequence was available, this correlation was even more conspicuous: 16 of 18 Ph-positive leukemia cases, all expressing AID, also carried mutated V_H region genes, whereas 10 Ph-negative leukemia cases, all lacking AID expression, also all carried unmutated V_H gene rearrangements. The average mutation frequency was 36.1 $\pm$ 29 mutations/ 10^3 bp in Ph-positive and 4.3 $\pm$ 5 mutations/ 10^3 bp in Ph-negative ALL among all sequences ($p<0.05$). Counting only mutated sequences, the mutation frequency observed in Ph-positive ALL came close to mutation frequencies in germinal center B cells and memory B cells (Table 1). Also among the 4 of 48 Ph-negative cases carrying mutated V_H region genes, the mutation frequency was high, suggesting that in a small subset of pre-B ALL, aberrant SHM may be also induced in the absence of BCR-ABL1.

Consistent with an active somatic hypermutation mechanism, we detected traces of ongoing subclonal diversification of V_H gene segments in 15 of 32 cases of Ph-positive ALL (Table 2B). Reflecting their clonal evolution, many Ph-positive leukemia clones exhibit common mutations shared between multiple subclones, which differ from each other by individual diversifying mutations. As an example, genealogic trees for two Ph-positive pre-B ALL cell lines are shown in Figure 2.

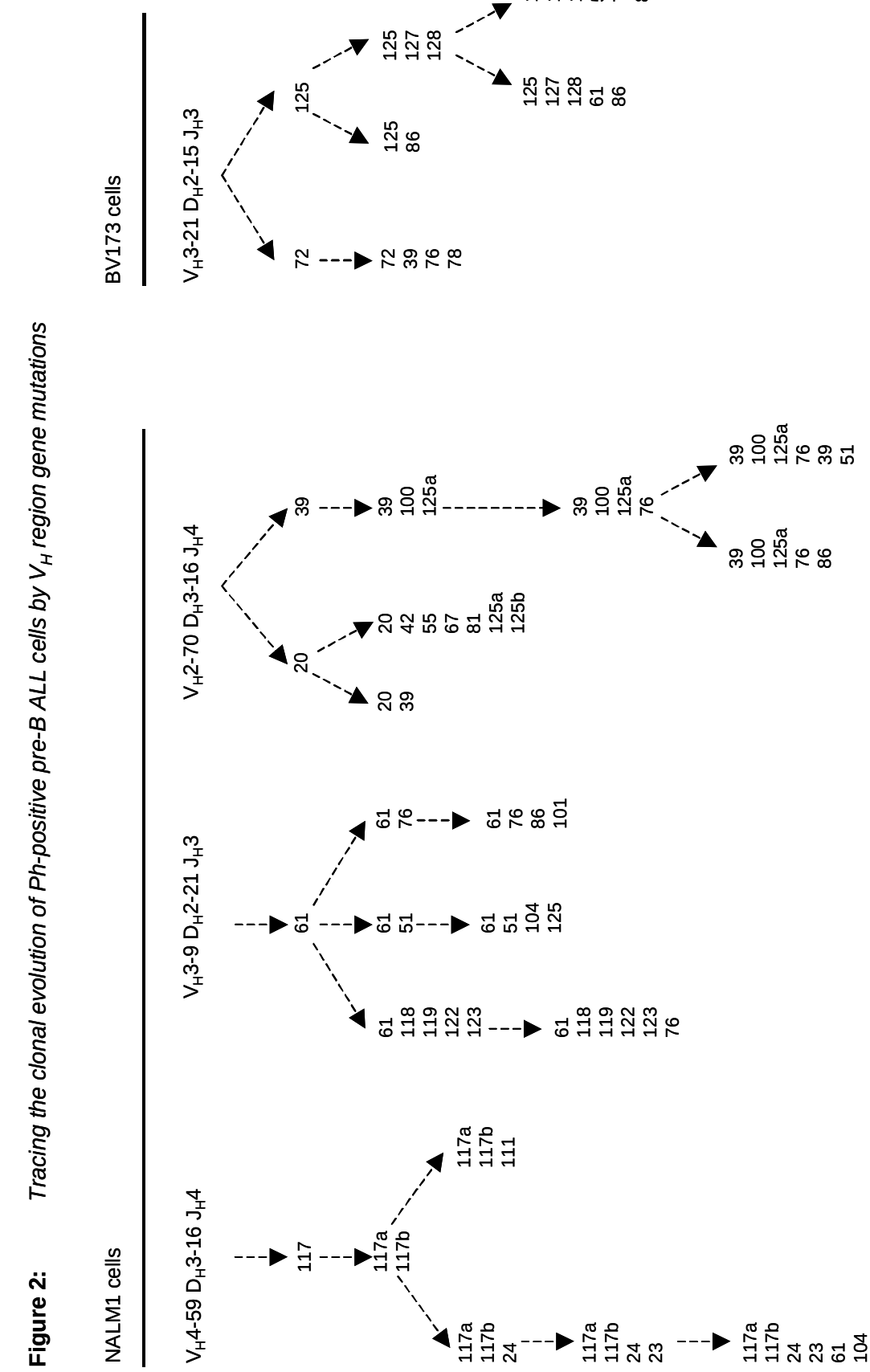
Analysing CSR of immunoglobulin C_H region genes Ph-positive and Ph-negative ALL, we identified switched $C\gamma1$, $C\gamma2$, $C\gamma3$ and $C\alpha_2$ transcripts in 5 of 21 Ph-positive (Table 1 and Table 3) but not in any of 10 Ph-negative ALL cases (Table 1). We conclude that immunoglobulin genes in Ph-positive ALLs are frequently targeted by SHM and occasionally by CSR, which is consistent with specific expression of AID in Ph-positive ALL.

Figure 1: *AID expression in Ph-positive pre-B ALL cells*



mRNA expression of *AID* was measured in normal human pro-B, pre-B, B1, naïve, germinal center and memory B cells as well as plasma cells by RT-PCR (A). In a semiquantitative RT-PCR, *AID* mRNA expression in Ph-positive ALL cells was compared to germinal center B cells and Ph-negative ALL cells (B). *GAPDH* was used for normalization of cDNA amounts (A and B).

Figure 2: *Tracing the clonal evolution of Ph-positive pre-B ALL cells by V_H region gene mutations*



A genealogic tree of ongoing somatic hypermutation of V_H gene segments in three subclones of the Ph-positive pre-B ALL cell line NALM1 and in the Ph-positive pre-B ALL cell line BV173 is shown. Numbers indicate the mutated codons within the rearranged variable region. Each group of codon numbers represents one V_H sequence, a and b denote distinct mutations within the same codon.

BCR-ABL1-induced AID expression in Ph-positive pre-B ALL

We next investigated whether the Ph-encoded BCR-ABL1 kinase contributes to aberrant AID expression in Ph-positive ALL. As shown in Figure 3, inhibition of BCR-ABL1 kinase activity by STI571 downregulates AID expression in Ph-positive ALL at the mRNA (Figure 3A) and protein level (Figure 3B). Conversely, activation of transgenic expression of *BCR-ABL1* in a murine pro-B cell line (Figure 3C) and forced retroviral expression of *BCR-ABL1* in a Ph-negative ALL (Figure 3D) induces *de novo* expression of AID. To validate the causative link between BCR-ABL1 kinase activity and aberrant AID expression in patients suffering from Ph-positive ALL, we compared matched samples pairs of Ph-positive ALL before the onset and during continued therapy with the BCR-ABL1 kinase inhibitor STI571. Confirming *in vitro* observations, primary patient-derived Ph-positive ALL cells downregulate AID in the presence but not in the absence of the BCR-ABL1 kinase inhibitor STI571 (Figure 3E). We conclude that BCR-ABL1 kinase activity is required for aberrant AID expression in Ph-positive ALL cells.

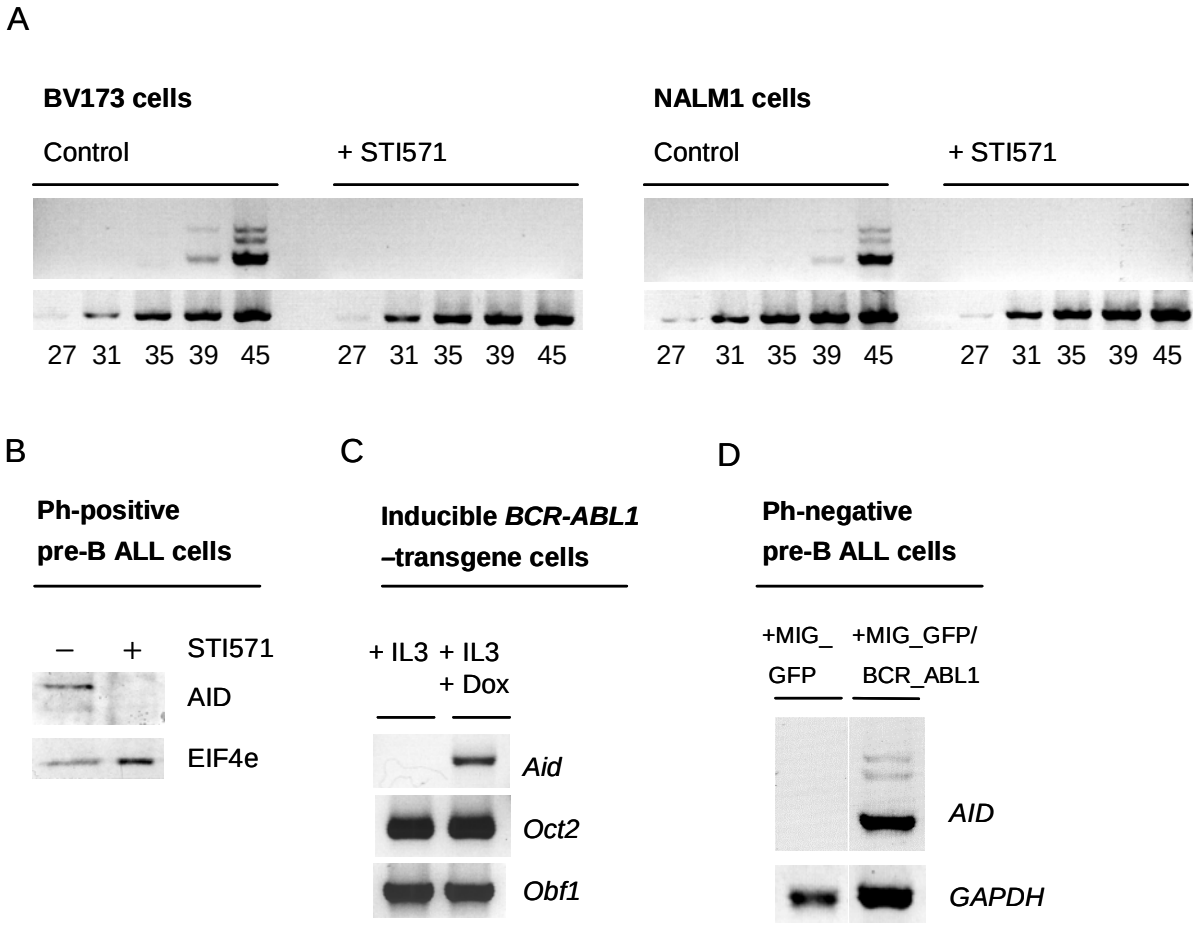
BCR-ABL1 represses ID2, a negative regulator of AID

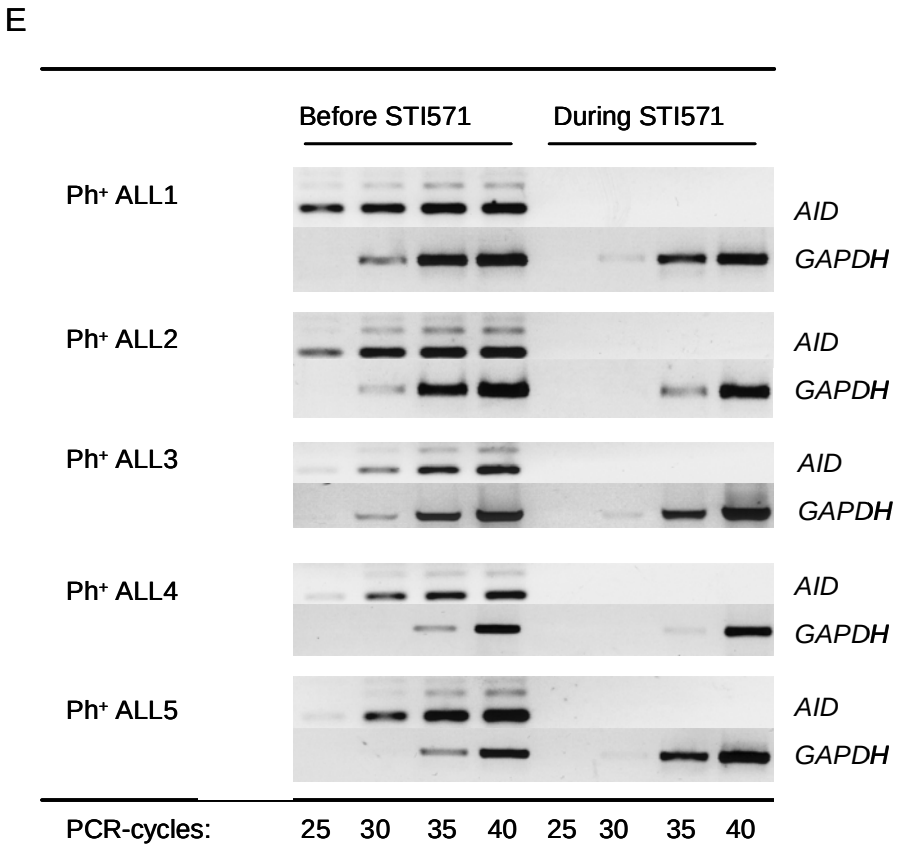
Previous studies demonstrated that AID expression is tightly regulated by the transcription factor E2A and its inhibitors ID2 and ID3 (9, 10). Interestingly, previous work showed that ID2 is among the genes that are transcriptionally silenced by v-abl in murine Abelson-virus-transformed pre-B cells (11). We therefore investigated the relationship between BCR-ABL1 kinase activity, ID2 and AID expression in four Ph-positive ALL cell lines. In untreated Ph-positive ALL cells, ID2 was repressed whereas AID was clearly detectable at the protein level (Supplementary Figure 2). Inhibition of BCR-ABL1 kinase by STI571, however, relieved repression of ID2 while AID expression decreased below the detection limit (Supplementary Figure 2). Consistent with the previously described role of ID2 as a repressor of AID, these findings identify an inverse relationship between ID2 and AID, which depends on BCR-ABL1 kinase activity.

Lineage-specific regulation of AID in Ph-positive leukemia

Given that Ph-induced leukemia not only comprises ALL but also chronic myeloid leukemia (CML), which derives from myeloid lineage progenitors, we investigated whether BCR-ABL1 kinase activity drives aberrant expression of AID irrespective of lineage derivation in both Ph-positive ALL and CML. To this end we sorted B cell, T cell and myeloid lineage subclones from one multilineage CML cell line (CML-T1) and five primary cases of CML in multilineage blast crisis and analyzed B- and T lymphoid as well as myeloid subclones for AID expression (Figure 4).

Figure 3: *AID expression in Ph–positive pre-B ALL cells depends on BCR-ABL1 kinase activity*





Legend to Figure 3:

Ph-positive ALL cell lines (BV173 and Nalm1) were treated with or without 10 µmol/l STI571 for 24 hours and subjected to semiquantitative RT-PCR analysis for AID and *GAPDH* mRNA expression (A). Protein lysates from the same cells were used for Western blotting (B) together with antibodies against AID and EIF4E (used as a loading control). IL3-dependent murine pro-B cells carrying a doxycycline (Dox)-inducible BCR-ABL1 transgene were incubated with or without 1 µg/ ml doxycycline for 24 hours and subjected to RT-PCR analysis of aid, oct2 and obf1 mRNA expression (C). Ph-negative pre-B ALL cells were transfected with a MIG GFP_IRES_BCR-ABL1 and a MIG_GFP vector as a control. After 24 hours, GFP-expressing cells were sorted and subjected to RT-PCR analysis for AID expression (D). Matched sample pairs from five patients with Ph-positive ALL before the onset and during continued treatment with the BCR-ABL1 kinase inhibitor STI571 were analyzed for *AID* mRNA levels by semiquantitative RT-PCR (E). The content of Ph-positive ALL cells in all samples was normalized by semiquantitative RT-PCR analysis of BCR-ABL1 fusion transcripts (not shown). As a loading control for cDNA amounts, a cDNA fragment of GAPDH was amplified.

AID was highly expressed in B lymphoid subclones, detectable at low levels in T lineage cells and absent in myeloid subclones. These findings are consistent with a key role of the (B-) lymphoid transcription factor E2A in the regulation of AID expression (9), which is not active in myeloid lineage cells (12).

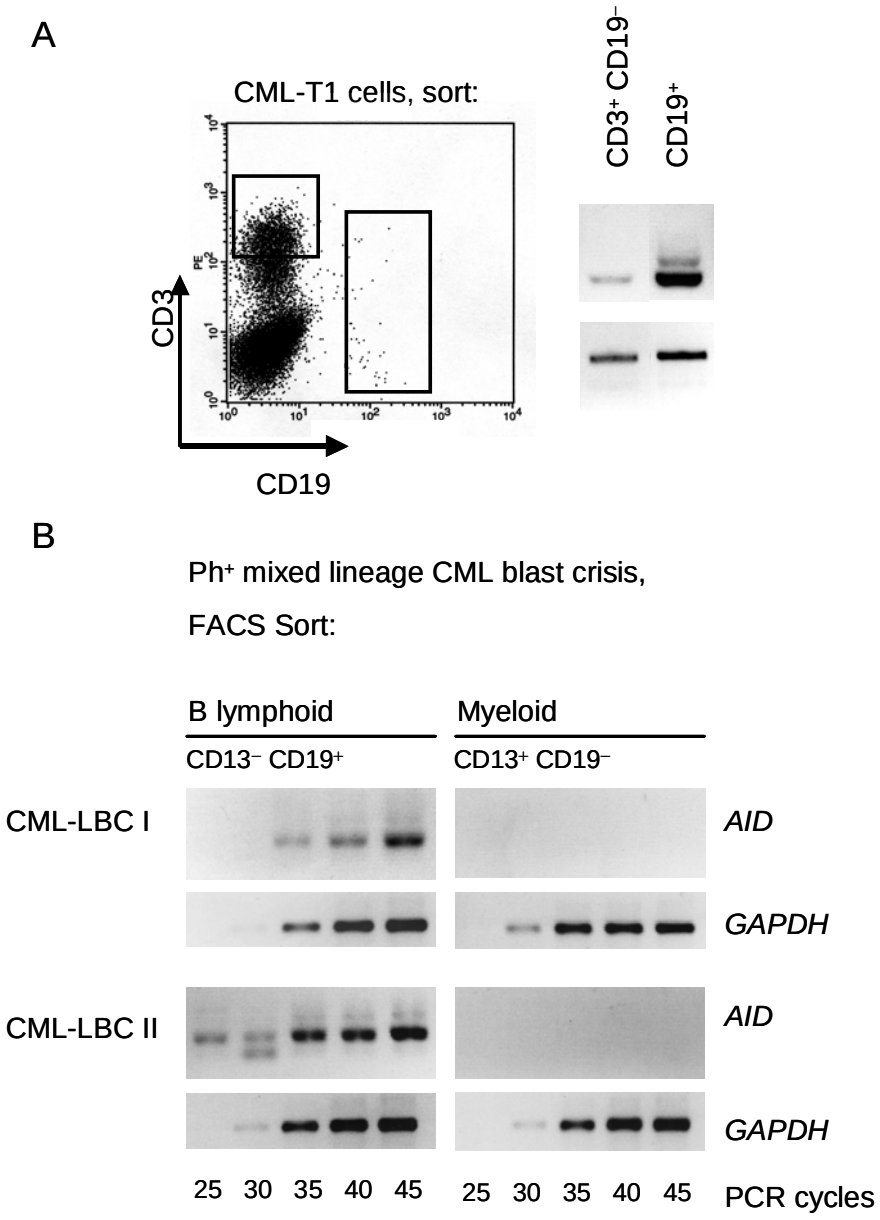
While CML typically develops as a myeloid lineage leukemia in a chronic phase for up to 15 years, transformation of chronic phase CML into B lymphoid blast crisis dramatically changes the kinetics of the disease and defines the terminal stage of CML (13). Therefore, we studied whether AID may contribute to clonal evolution and tumor progression in Ph-positive ALL and B lymphoid subclones of lymphoid blast crisis CML.

AID-induced DNA-single-strand breaks in Ph-positive leukemia cells

First, we investigated whether transgenic AID expression is sufficient to pre-maturely induce SHM in bone marrow-derived pre-B cells, which represent the normal precursor of Ph-positive ALL. To this end, we sorted pre-B cells from the bone marrow of *Aid*-transgenic mice and wildtype littermates and analyzed genomic DNA from the pre-B cell populations for DNA single-strand breaks (SSB) within rearranged *Vh* gene segments and the *myc* gene. DNA-SSB were detected in both *Vh* and *myc* loci of *Aid*-transgenic but not wildtype pre-B cells (Figure 5A).

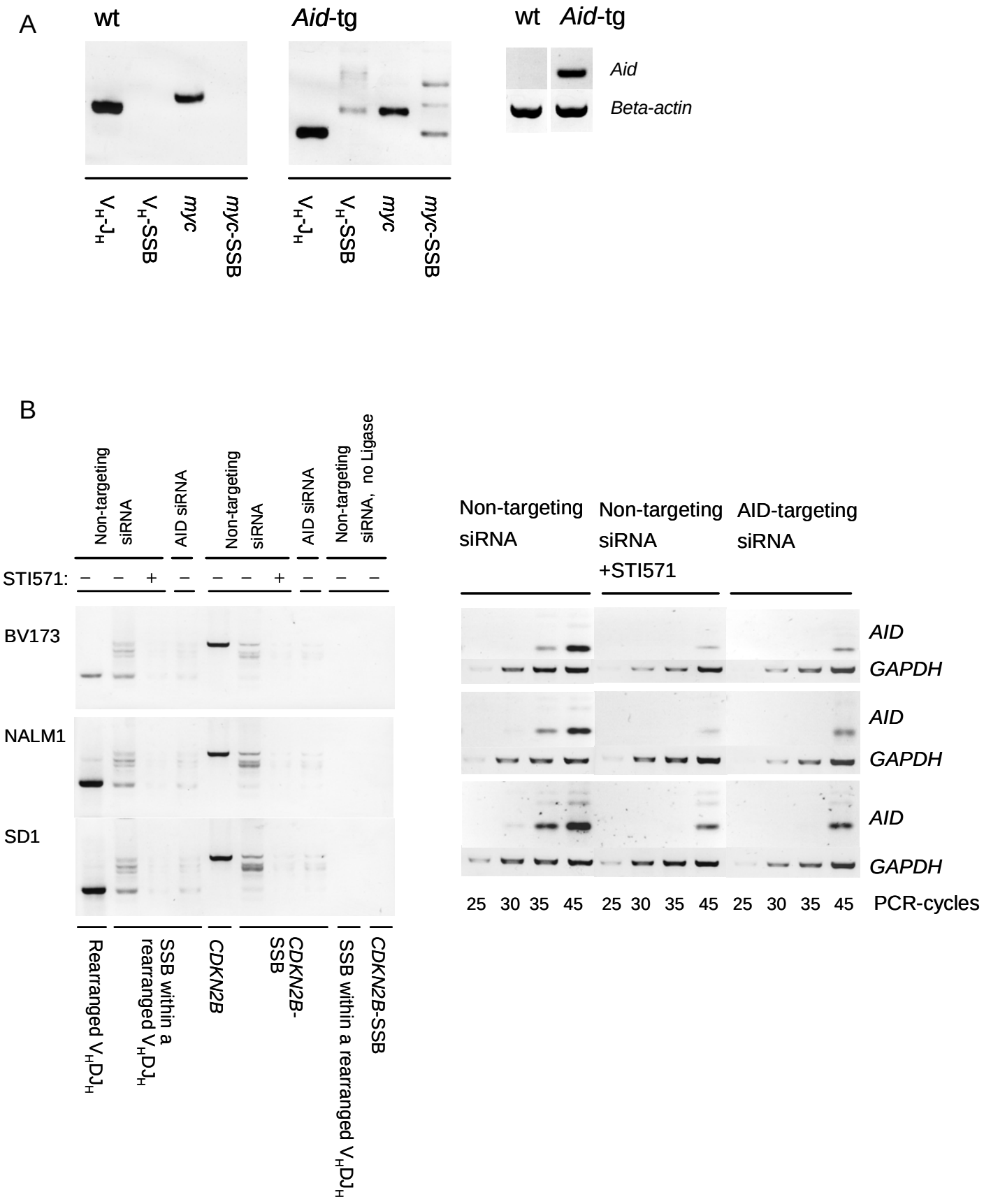
To test whether AID expression and introduction of DNA-SSB are causally linked in Ph-positive ALL, we silenced AID expression in three Ph-positive ALL cell lines by RNA interference (Figure 5B; left). AID-targeting and non-targeting siRNA duplices were fluorochrome-labeled and repeatedly transfected into Ph-positive ALL cells. Fluorochrome-positive cells were sorted by flow cytometry and subjected to further analysis.

Figure 4: *AID expression in Ph-positive multi-lineage blast crisis cells is restricted to lymphoid cells*

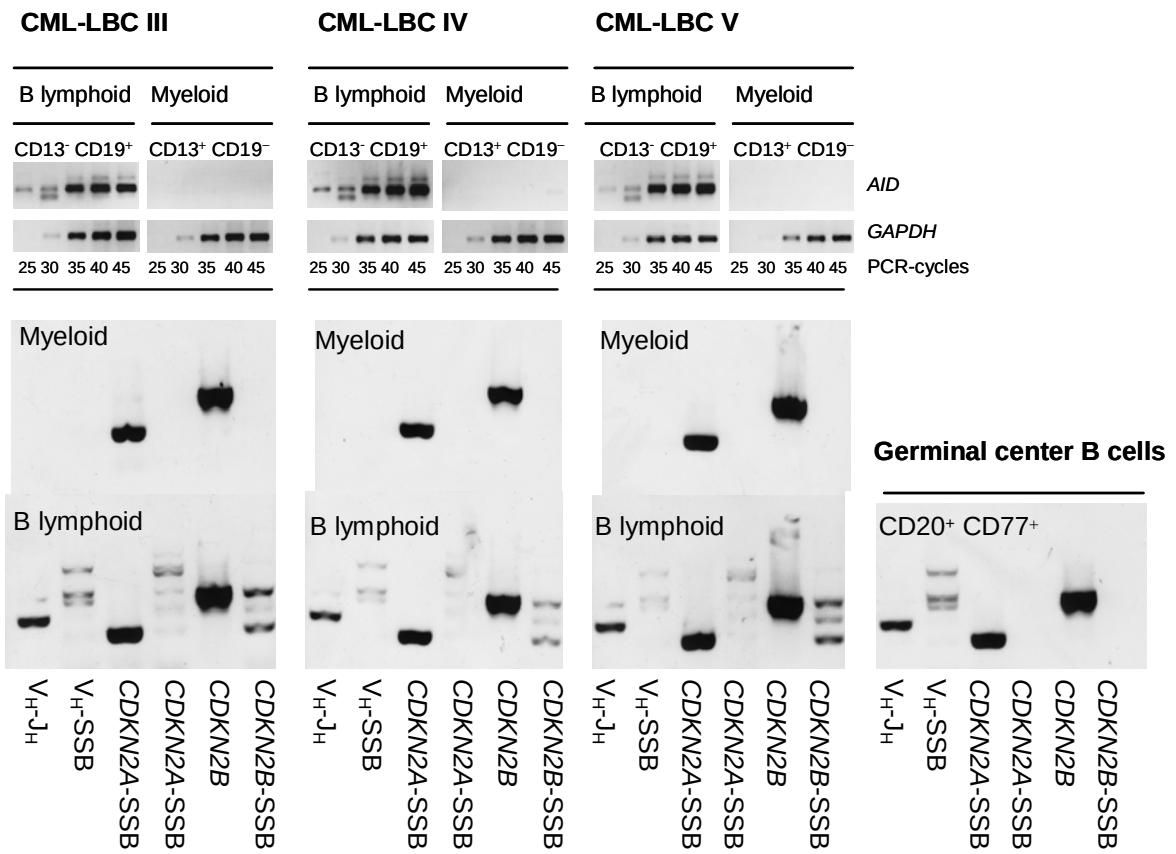


$CD3^+$ $CD19^-$ T lymphoid and $CD19^+$ B lymphoid subpopulations from the multi-lineage CML blast crisis cell line CMLT1 were sorted by FACS (A) and subjected to cDNA synthesis. Thereafter, *AID* mRNA expression of the sorted subsets was analyzed by RT-PCR (A; right). FACS-sorted B lymphoid and myeloid subclones from two primary cases of CML in multilineage blast crisis were analyzed for *AID* mRNA expression by semiquantitative RT-PCR with the cycle numbers indicated (B). As a loading control, a cDNA fragment of *GAPDH* was amplified (A; B).

Figure 5: *AID induces single-strand breaks (SSB) in immunoglobulin and non-immunoglobulin genes*



C



Pre-B cells were sorted from the bone marrow of mice carrying a B cell-specific *Aid*-transgene (*Aid*-tg) and from wildtype littermates (wt). Specific expression of *Aid* in *Aid*-transgenic pre-B cells but not pre-B cells from wt-littermates was confirmed by RT-PCR (A, right). Genomic DNA was isolated from *Aid*-tg and wt pre-B cells and analyzed for DNA single-strand breaks (SSB) within rearranged *Vh* gene segments and the *myc* locus by LM-PCR. As a loading control, genomic fragments of rearranged *Vh* region genes and of the *myc* gene were amplified (A, left).

To establish a causative link between AID function and the occurrence of DNA-SSB, *AID* mRNA expression was silenced in three Ph-positive ALL cell lines by RNA interference using fluorochrome-labeled siRNAs against AID or non-targeting siRNA duplices as a control. Fluorochrome-labeled cells were sorted and analysed for silencing efficiency and specificity by RT-PCR (B, right panel), and for DNA-SSB within rearranged V_H gene segments and the *CDKN2B* gene by LM-PCR (B, left panel). For BV173, Nalm1 and SD1 cells, DNA-SSB intermediates in rearranged V_H3-21, V_H3-9 and V_H3-30 gene segments were amplified, respectively. As a loading control for genomic DNA, V_H gene rearrangements and a genomic fragment of the *CDKN2B* gene were amplified (B). From three patients with multilineage blast crisis of chronic myeloid leukemia (CML-BC), myeloid ($CD13^+$ $CD19^–$) and B lymphoid ($CD13^–$ $CD19^+$) subclones were sorted and subjected to LM-PCR for DNA-SSB within rearranged V_H gene segments and *CDKN2A* and *CDKN2B* genes (C, middle and bottom panel). In these CML-BC cases, only B lymphoid but not myeloid subclones express AID (C, top panel). As a loading control for genomic DNA, V_H gene rearrangements and genomic fragments of the *CDKN2A* and *CDKN2B* genes were used. Reflecting their lineage identity, from myeloid subclones of CML-BC no V_H gene rearrangements were amplified. Two additional cases of CML-BC (not shown) were not informative for DNA-SSB within *CDKN2A* and *CDKN2B*. In these cases, no *CDKN2A* or *CDKN2B* fragments could be amplified owing to large bi-allelic deletions at these loci. As a positive control, genomic DNA from normal germinal center B cells was used.

RNA interference substantially reduced AID mRNA levels in the three Ph-positive ALL cell lines (Figure 5B; left). Comparing Ph-positive ALL cells carrying non-targeting siRNAs with Ph-positive cells carrying AID-specific siRNA duplices, DNA-SSB were detected within rearranged V_H gene segments and also within the *CDKN2B* gene in the former but not in the latter case (Figure 5B; right). Likewise, inhibition of BCR-ABL1 kinase activity by STI571 largely reduced the frequency of DNA-SSB within V_H and *CDKN2B* genes (Figure 5B; right). *CDKN2B* encodes the p15/INK4B tumor suppressor in ALL and CML and is frequently deleted in lymphoid but not myeloid lineage blast crisis of CML (14, 15). We also studied the immediately adjacent *CDKN2A* locus, which we found already deleted in BV173 and Nalm1 cells and therefore not informative for analysis of DNA-SSB (not shown).

These findings identify AID as a lineage-specific mutator within but also outside the immunoglobulin gene loci in Ph-positive ALL cells.

To test whether such a mutation mechanism is also active in primary patient-derived CML multi-lineage blast crisis, we studied DNA-SSB in rearranged V_H gene segments, the *CDKN2A* and the *CDKN2B* loci in myeloid and B cell lineage subclones sorted from five cases of multilineage CML blast crisis (Figure 5C). As a control, tonsillar germinal center B cells were used. Consistent with differential expression of AID in myeloid and B cell lineage subclones of CML-blast crisis in these patients (Figure 5C, top), DNA-SSB within rearranged V_H, *CDKN2A* and *CDKN2B* were exclusively detected in the B lymphoid but not the myeloid compartment (Figure 5C). Reflecting their lineage identity, we could not amplify rearranged V_H gene segments from myeloid subclones of CML blast crisis, thus lacking the target for DNA-SSB at this locus. In two of five cases, the *CDKN2A* locus or both *CDKN2A* and *CDKN2B* loci were already deleted (not shown). We conclude that an AID-induced, lineage specific mutation mechanism is indeed active in primary CML blast crisis.

Discussion

In summary, these findings identify AID as a mutator in Ph-positive ALL and lymphoid blast crisis CML. Aberrant expression of AID leading to somatic hypermutation inside and outside the immunglobulin loci is driven by oncogenic BCR-ABL1 kinase activity and likely contributes to the particularly unfavorable prognosis of Ph-positive leukemia (6). Ph-positive leukemias typically develop resistance to chemotherapy and inhibition of BCR-ABL1 kinase activity within a short period of time (6). Further studies that address a specific role of AID in the acquisition of mutations leading to drug resistance in Ph-positive ALL are currently under way.

Of note, a small fraction of cases of Ph-negative pre-B ALL carry mutated V_H region genes (and presumably express AID) in the absence of BCR-ABL1. This indicates that other genetic aberrations besides the Philadelphia chromosome may likewise induce aberrant somatic hypermutation in pre-B ALL cells. If BCR-ABL1-induced upregulation of AID indeed mainly results from repression of ID2 and indirect activation of E2A, one would envision that other factors that influence the balance between ID2 and E2A may have a similar effect in Ph-negative leukemia cells.

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Materials and Methods

Patient samples and cell lines

Normal pro-B cells, pre-B cells, B1 cells, naïve, germinal center and memory B cells as well as plasma cells were sorted from human bone marrow, umbilical cord blood, peripheral blood and tonsils of multiple healthy donors as previously described (16).

In total, 100 cases of acute lymphoblastic leukemia (ALL) were analysed for AID expression. 26 cases of Ph-positive leukemia including seven cell lines (BV173, CMLT1, K562, NALM1, SD1, SUP-B15 and TOM1 from DSMZ, Braunschweig, Germany) and 74 cases of Ph-negative ALL including eight cell lines (BEL1, HBP-NULL, KASUMI2, MHH-CALL3, NALM6, REH, RS4;11and 697; DSMZ) were analyzed by RT-PCR (see below). V_H gene rearrangements were amplified and sequenced from 80 cases of ALL, including 32 Ph-positive and 48 Ph-negative cases. For 28 cases (18 Ph-positive, 10 Ph-negative), information on both, AID expression and V_H region gene sequence was available.

A detailed description of the patient samples and cell lines is given in Table 2. Patient samples were provided from the Department of Hematology and Oncology, Universität Frankfurt (WKH) and the Department of Medical Biosciences, Pathology, Umea University, Umea, Sweden (AL) in compliance with Institutional Review Board regulations.

Induced expression of BCR-ABL1

A murine IL3-dependent pro-B cell line TONB210, which carries an inducible *BCR-ABL1* transgene under the control of a doxycycline-dependent promoter and Ph-negative pre-B ALL cells transiently transfected with pMIG-GFP or pMIG-GFP_BCR-ABL1 vectors were studied in cell culture experiments as previously described (17). pMIG-GFP or pMIG-GFP_BCR-ABL1 vectors encode either GFP only or GFP and BCR-ABL1 and were transfected by electroporation (250 V and 950 μF). For both transfections, GFP^+ and GFP^- cells were sorted after 24 hours and subjected to further analysis.

Aid-transgenic mice

A murine *Aid* transgene is expressed under the control of the B cell-specific Vλ2 promotor/Vλ2-4 enhancer minigene in FVB/N mice ensuring ectopic expression of aid in murine B cell precursors already from the $CD43^+$ $CD25^-$ late pro-B cell stage. $B220^+$ $AA4.1^+$ pre-B cells from murine bone marrow of transgenic and wildtype mice were isolated by cell sorting und subjected to cDNA synthesis. Transgenic expression of *Aid* was verified by RT-PCR (Figure 5A). As controls, pre-B cells from wildtype littermates were used.

Cell sorting and flow cytometry

$CD19^+$ $CD13^-$ B lymphoid and $CD13^+$ $CD19^-$ myeloid lineage subpopulations from five primary cases of CML in multilineage blast crisis as well as $CD3^+$ $CD19^-$ T lymphoid and $CD19^+$ B lymphoid subpopulations from one multilineage CML cell line (CMLT1) were stained using antibodies against CD19, CD3 and CD13 (BD Biosciences) and sorted using a FACStar 440 cell sorter.

Sequence analysis of V_H and C_H region genes and semiquantitative RT-PCR

In order to characterize the configuration of V_H and C_H region genes, two primer sets were used amplifying the variable (V) region alone or the variable (V) region together with the constant (C) region of rearranged immunoglobulin heavy chain genes as previously described (18). Mutation frequencies of V_H region genes of 10 /10^3 bp or higher were considered significantly ($p< 0.01$) above the frequency of mutations introduced by Taq DNA polymerase error, which was experimentally determined as 2.5 /10^3 bp. Primers used for semiquantitative RT-PCR analysis of human *AID* and *GAPDH*, and for RT-PCR analysis of murine *Aid*, *Oct2* and *Obf1* transcripts are listed in supplementary Table 1.

Western blotting and immunofluorescence

For the detection of AID by Western blot, an antibody against human AID (L7E7; Cell Signaling Technology, Beverly, MA), was used together with the WesternBreeze immunodetection system (Invitrogen, Karlsruhe, Germany). Detection of EIF4e was used as a loading control (Santa Cruz Biotechnology, Heidelberg, Germany). ID2 and AID expression were analyzed by immunofluorescence using primary antibodies against human ID2 (Santa Cruz Biotechnology) and AID (L7E7; Cell Signaling Technology) together with anti-rabbit IgG-Cy2 and anti-mouse IgG-Cy3 (Jackson Immunoresearch) as secondary antibodies, respectively. Prior to staining, cells were fixed with 0.4% para-formaldehyde and incubated for 10 minutes in 90% methanol on ice.

Silencing of AID mRNA expression by RNA interference

For silencing of AID mRNA expression one previously validated siRNA (19) and a non-targeting siRNA duplex was used. All siRNA duplices (for sequences, see Supplementary Table 3) were labeled with fluorescein using an siRNA labeling kit (Ambion, Austin, TX) according to the manufacturer's protocol. Transfected fluorescein-positive cells were sorted by FACS as previously described (17). RNA interference was verified by RT-PCR.

Ligation-mediated PCR for detection of DNA single-strand breaks

Genomic DNA from 2.5 x 10^6 cells containing a nick on the lower strand is denatured for 10 minutes at 95°C, and a gene-specific primer (Supplementary Table 3) is hybridized and

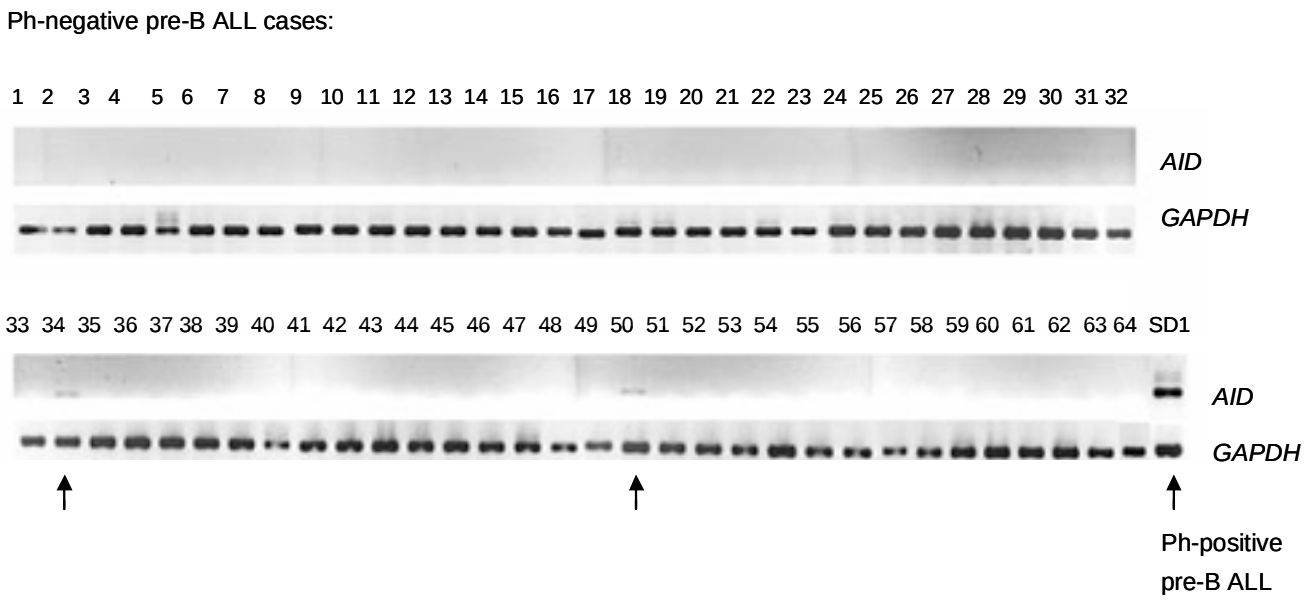
extended to the position of the nick as previously described (20) (first strand extension), leaving a blunt end using Vent DNA polymerase (New England Biolabs, Beverly, MA). Next, a double-stranded linker is ligated to the newly created blunt end using T4 DNA ligase (Invitrogen) at 14°C overnight. The linker was constructed by annealing of the oligonucleotides 5'-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACATG 3' and 3' amino (C7)- GACGAGCTTAAGTTCGAAGATTGCTACATGCCCCT -5' and protruding 3' overhangs were removed by 3'→ 5' exonuclease activity of the Klenow fragment of E. *coli* DNA polymerase I (Invitrogen). Ligation-mediated PCR (reference 21; LM-PCR) was carried out with modifications as previously described (22). In two semi-nested rounds of amplification at an annealing temperature of 59°C, linker-ligated intermediates of DNA-single-strand breaks within various genes were amplified using gene specific primers together with two linker specific primers (Supplementary Table 1).

Table 3: *Class-switch recombination in Ph-positive ALL cells*

Case[a]	V_H	D_H	J_H rearrangement	C_H region
70 (FACS-sort: CD19$^+$ CD34$^+$) Lymphoid blast crisis CML	TATGTATTATTGTACGAAAGA -G-------C---G------- V_H3-30	**GGGTTCAGCTATGG** --A-A--------- D_H5-5	CCGACCTGAACTTCTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAG**CCTCCACCAAGGGCCCAT** A-T--T-TG---A---------- J_H4	$C\gamma_2$
71 (FACS-sort: CD19$^+$ CD34$^+$) Lymphoid blast crisis CML	GCCTTCTATTATTGTGCGCGA ---G-G-----C------A-- V_H1-46	**GGTGATACC** ACAGTCAGCGGGA ------TG- D_H2-21	CCAACTGGCTCGCCCCCTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCTG**CCTCCACCAAGGGCCCAT** A-------T---A----------A- J_H5	$C\gamma_2$
72 (FACS-sort: CD19$^+$ CD34$^+$) Lymphoid blast crisis CML	TCTGTATTACTGTGTGAAAGG A -G-----------C-----A V_H3-30	**GAGT** GGGC ---- D_H6-6	CATTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAG**CCTCCACCAAGGGCCCAT** -T---------- J_H4	$C\gamma_1$
	GCCGTATATTACTGTGCGAAA TGGTA ---------- V_H3-23	**CGGACATTG** CAGTAGTACCACCTGCACGAC T---T---- D_H2-2	CCTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAG**CCTCCACCAAGGGCCCAT** -T---------- J_H4	$C\gamma_1$
	CGGCCATATATTACTGTGCGA CCACCGA -----G-G---------- V_H3-53	**GGGGATAGCAGTGGCTGGTAC** TTCGT ---T---------- D_H6-19	CTGGGGCCAAGGGACCACGGTCATCGTCTCCTCCG**CCTCCACCAAGGGCCCAT** ----------A---CT-----C---------A- J_H5	$C\gamma_1$
	CCGTTTATTACTGTGCGAAA TTGGAACG ----A---------- V_H3-23	**TATGGTGACTACC** GGTCGGC --C---------- D_H4-17	GGCCTTCCCCTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAG**CATCCCCGACCAGCCCCA** -TT-GA---------- J_H5	$C\alpha_2$
	TGTGTATTACTGTGCGAGAGA ---------- V_H3-33	**TCGGGGGCTGGCAG** CTACTC G-A-T------T-C D_H2-2	ACTTTGACTCCTGGGGCCAGGGAACCCTGGTCACCGTCTCTTCAG**CATCCCCGACCAGCCCCA** ---------A----------C---- J_H4	$C\alpha_2$
	TGTTTATTACTGTGCGAAAGA TGCAG ---G---------- V_H3-30	**AAAATGAGGCAGTGA** CTGGTAGGGG -T-T--T--TG---- D_H2-15	CTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAG**CATCCCCGACCAGCCCCA** ---------- J_H6	$C\alpha_2$
78 Ph$^+$ cell line SD1	GCTGTCTATTATTGTGTGAAA ---G-----C----C---- V_H3-30	**CCGATGGGAC** CCTACCGCGAG -A-C---T-- D_H6-13	GCTTTTGATATCTGGGGCCAAGGGACAGTGGTCACCGTCTCTTCAG**CCTCCACCAAGGGCCCAT** ----------A---------- J_H3	$C\gamma_1$
77 Ph$^+$ cell line Nalm-1	AGTCTGAGAGCTGAGGACACC TGGGGG ---------A---------- V_H3-43	**TACTTTGACT** ACTGGGGCC --T------- D_H3-9	AGGGAACCCTGGTCACCGTCTCCTCAG**CTTCCACCAAGGGCCCAT** ---------- J_H5	$C\gamma_3$

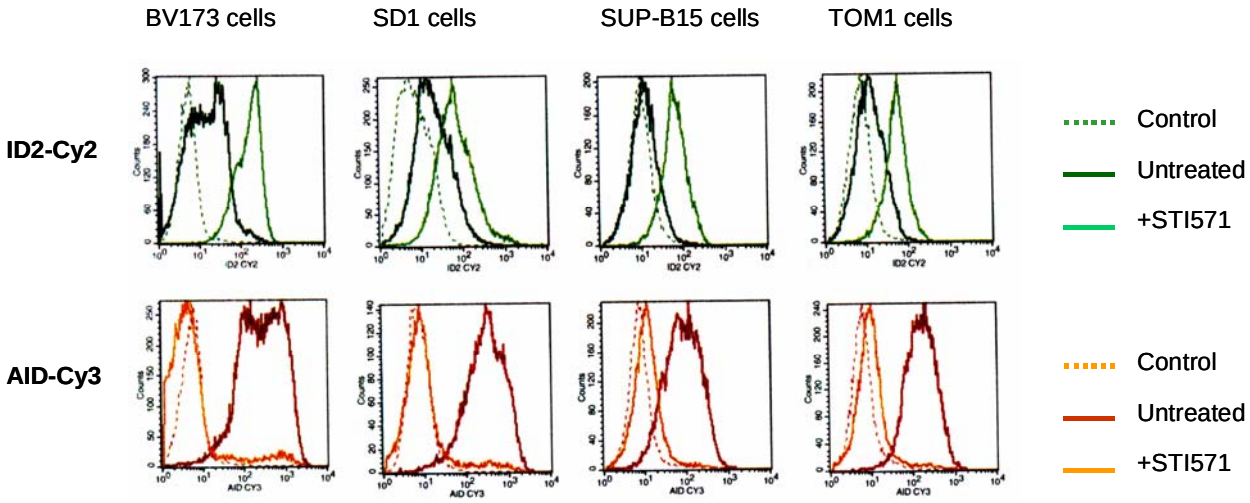
Note: a. Numbers for cases refer to Table 2B

Supplementary Figure 1: *Lack of AID expression in Ph-negative pre-B ALL cells*



cDNA of 64 cases of Ph-negative and of one case of Ph-positive pre-B ALL was subjected to RT-PCR for the analysis of *AID* mRNA expression. As a loading control, a cDNA fragment of *GAPDH* was amplified. Arrows indicate pre-B ALL cases expressing *AID*.

Supplementary Figure 2: *BCR-ABL1 kinase activity represses ID2, a negative regulator of AID*



The expression of AID and ID2 in the presence (+STI571) or absence (Untreated) of 10 μmol/l of the BCR-ABL1 kinase inhibitor STI571 was analyzed by flow cytometry. In all experiments, a fluorochrome-labeled isotype-control antibody was used (Control).

Supplementary Table 1: *Summary of oligonucleotides used for RT-PCR, RNA interference and ligation-mediated PCR*

PCR primers	
GAPDH forward	5'-TTAGCACCCCTGGCCAAG-3'
GAPDH reverse	5'-CTTACTCCTTGGAGGCCATG-3'
BCR-ABL1 forward	5'-ACCTCACCTCCAGCGAGGAGGACTT-3'
BCR-ABL1 reverse	5'-TCCACTGGCCACAAAATCATACAGT-3'
mAid forward	5'- AAATGTCCGCTGGGCCAA-3'
mAid reverse	5'- CATCGACTTCGTACAAGGG-3'
AID forward	5'-TGCTCTTCCTCCGCTACATC-3'
AID reverse	5'-CCTCATACAGGGGCAAAAGG-3'
mObf1 forward	5'-AGCTCCCTGACCATTGAC-3'
mObf1 reverse	5'-CTGTCCCATCCCCCTGTAA-3'
mOct2 forward	5'-ATCGAGACGAATGTCCGCTT-3'
mOct2 reverse	5'-GTAGCTGGTCGGCTTTCC-3'
Cμ_R	5'-AGACGAGGGGGAAAAGGGTT-3'
Cγ1_R	5'-GAGTTTTGTCACAAGATTTGGGCT-3'
Cγ2_R	5'-GCACTCGACACAACATTTGCG-3'
Cγ3_R	5'-TCACCAAGTGGGGTTTTGAGC-3'
Cγ4_R	5'-ATGGGCATGGGGGACCATTT-3'
Cα2_R	5'-TGTTGGCGGTTAGTGGGGTC-3'
Cε_R	5'-GAGGTGGCATTGGAGGGAAT-3'
Cδ_R	5'-AGAGCTGGCTGCTTGTCATG-3'
mJ558 forward	5'-GCAAGGCTTCTGGCTACACA-3'
$mJ_H2/3$ reverse	5'-CCAGTAAGCAAACCAGGCACA-3'
siRNA duplices	
Non-targeting siRNA	5'-UUGUACCUAAUUUCGUCCCAC-3' 3'-CAUGGAUUAAAGCAGGGUGUU-5'
AID siRNA	5'-UUGCUCUUCCUCCGCUACAUU-3' 3'-CGAGAAGGAGGCGAUGUAAUU-3'
LM-PCR primers	
CDKN2A_F1	5'-CCAGGAATAAAATAAGGGGAATA-3'
CDKN2A_F2	5'-GGAATAAAATAAGGGGAATAGGG-3'
CDKN2A_R	5'-CTTTCCTACCTGGTCTTCTAGG-3'
CDKN2B_F1	5'-GTGAACATTCCCAAAATATTAGC-3'
CDKN2B_F2	5'-AAAATATTAGCCTTGGCTTTACTG-3'
CDKN2B_R	5'-AGACTCCTGTACAAATCTACATCG-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'
Mmyc_F1	5'-ATCCTGAGTCGCAGTATAAAAGA-3'
Mmyc_F2	5'-TTTTCTGACTCGCTGTAGTAATTC-3'
Mmyc_R1	5'-CTCAGAGACTGGTAAGTCAGAAG-3'

Linker_F1	5'-CTGCTCGAATTCAAGCTTCT-3'
Linker_F2	5'-GCTTCTAACGATGTACGGGG-3'
Linker_R1	5'-GTACATCGTTAGAAGCTTGAA-3'
Linker_R2	5'-GTTAGAAGCTTGAATTCGAGC-3'

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5.6 Deficiency of Bruton's tyrosine kinase in B cell precursor leukemia cells

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Summary

Bruton's tyrosine kinase (BTK)-deficiency results in a differentiation block at the pre-B cell stage. Likewise, acute lymphoblastic leukemia cells are typically arrested at early stages of B cell development. We therefore investigated BTK function in B cell precursor leukemia cells carrying a *BCR-ABL1*, *E2A-PBX1*, *MLL-AF4*, *TEL-AML1* or *TEL-PDGFRB* gene rearrangement. While somatic mutations of the *BTK* gene are rare in B cell precursor leukemia cells, we identified kinase-deficient splice variants of BTK throughout all leukemia subtypes. Unlike infant leukemia cells carrying an *MLL-AF4* gene rearrangement, where expression of full-length BTK was only detectable in four out of eight primary cases, in leukemia cells harboring other fusion genes full-length BTK was typically coexpressed with kinase-deficient variants. As shown by overexpression experiments, kinase-deficient splice variants can act as a dominant-negative BTK in that they suppress BTK-dependent differentiation and pre-B cell receptor responsiveness of the leukemia cells. On the other hand, induced expression of full-length BTK rendered the leukemia cells particularly sensitive to apoptosis. Comparing BTK expression in surviving or pre-apoptotic leukemia cells after 10 Gy γ-radiation, we observed

selective survival of leukemia cells that exhibit expression of dominant-negative BTK forms. These findings indicate that lack of BTK expression or expression of dominant-negative splice variants in B cell precursor leukemia cells i.) can inhibit differentiation beyond the pre-B cell stage and ii.) protect from radiation-induced apoptosis.

Introduction

Recent findings by us and others suggested a role for the pre-B cell receptor and related signaling molecules as a tumor suppressor to prevent the development or limit the proliferation of leukemic cells (1, 2). *BCR-ABL1*$^{+}$ pre-B lymphoblastic leukemia cells frequently exhibit defective expression of the pre-B cell receptor related signaling molecule SLP65 (1) and acquire independence from pre-B cell receptor-dependent survival signals (2). The analysis of mouse mutants of the pre-B cell receptor-related signaling molecules SLP65 and Bruton's tyrosine kinase (BTK) demonstrated that SLP65 and BTK cooperate to suppress leukemic transformation (3).

Pre-B lymphoblastic leukemia cells typically exhibit a differentiation block at the pre-B cell stage of development (2); likewise *BTK*-deficiency in humans leading to X-linked agammaglobulinemia (XLA) results in a breakdown of pre-B cell receptor signals and a differentiation block at the pre-B cell stage (4). To elucidate a possible role for BTK in leukemic transformation of human B cell precursors, we investigated BTK function in pre-B acute lymphoblastic leukemia cells.

Results and Discussion

Rare occurrence of somatic mutations of the BTK gene in B cell precursor leukemia cells

Pre-B lymphoblastic leukemia cells frequently exhibit defective expression of the pre-B cell receptor-related linker molecules SLP65 (1). Comparing single slp65$^{-/-}$ and slp65$^{-/-}$/btk$^{-/-}$ double mutant mice, Btk was identified as a critical co-factor in preventing leukemic transformation of murine B cell precursors (3). We therefore searched for somatic mutations of the *BTK* gene in 12 cases of B cell precursor leukemia including four cases with *MLL-AF4*, three cases with *E2A-PBX1*, four cases with *BCR-ABL1* and one case with a *TEL-AML1* gene rearrangement. Because the majority of the *BTK* gene mutations in the germline leading to X-linked agammaglobulinemia (XLA), have been found in the BTK kinase domain (see: http://bioinf.uta.fi/BTKbase/BTKbasebrowser.html), we focused our analysis on this region

Table 1: *Sequence analysis of aberrant splice variants of BTK in pre-B acute lymphoblastic leukemia*[a]

Recurrent BTK splice variants

Gene rearrangement	Cases [d]	Exons involved	Splice site	Transcript	Translation	Western Blot	Frequency
MLL-AF4 **BCR-ABL1** *E2A-PBX1* *TEL-AML1* *TEL-PDGFRB*	SEM, RS411, BEL1, ***IX, X, XI, XIV,*** BV173, NALM1, SUP-B15, SD1, KASUMI2, MHH-CALL3, NALM6	Exons 15 and 16 [b]	Regular	ΔExons 15, 16	In-frame deletion in KD	65 kD protein	14/29
BCR-ABL1	IX, X, XI, XIV, BV173, NALM1, SUP-B15, SD1	Exon 15 [c]	Regular	ΔExon 15	Truncated KD	52 kD protein	8/29
MLL-AF4 *BCR-ABL1*	VII and IX	Exons 14 and 15	Cryptic	ΔExon 15, Δ41 bp exon 14	Truncated KD	n.d.	2/29

Unique BTK splice variants

Gene rearrangement	Case [d]	Exons involved	Splice site	Transcript	Translation	Western Blot
MLL-AF4	V	Exon 18	Cryptic	Δ33 bp	In frame deletion in KD	n.d.
	SEM	Exons 16 and 17	Cryptic	ΔExons 16, 17, Δ119 bp exon 15, Δ136 bp exon 18	Truncated KD	n.d.
BCR-ABL1	X	Exons 16 [b]	Regular	ΔExon 16	Truncated KD	n.d.
	XI	Exons 15 to 18	Cryptic, Slippage	ΔExons 15-18, Δ79 bp exon 14, Ins 3 bp, Δ24 bp exon 19	Truncated KD	n.d.
	XV	Exon 14	Cryptic	ΔExon 14, Δ62 bp exon 13, Δ133 bp exon 15	Truncated SH2 and KD	n.d.
TEL-AML1	XVIII	Exons 13 to 18	Cryptic, Slippage	ΔExons 14-18, Δ31 bp exon 12, Δ22 bp exon 13, 212 bp in exon 19 and 3'UTR	Truncated SH2 and KD	n.d.
		Exons 14 to 16	Regular	ΔExons 14-16	Truncated KD	47 kD protein
	XIX	Exons 13 to 18	Cryptic, Slippage	ΔExons 13-18, Ins of a 106 bp fragment of Intron18	Truncated SH2 and KD	n.d.
		Exons 14 to 17	Regular	Loss of exons 14-17	In-frame deletion in KD	56 kD protein
E2A-PBX1	KASUMI2	Exons 15 to 17	Cryptic, Slippage	ΔExons 15-17, Δ18 bp exon 14, Ins 1 bp, Δ33 bp exon 18	Truncated KD	n.d.
	MHH-CALL3	Exons 13 to 17	Cryptic	ΔExons 13-17, Δ13 bp exon 12, Δ92 bp exon 18	In-frame deletion in KD	n.d.

Notes:
a.: Sequence data are available from EMBL/GenBank under AM051275-AM051286 numbers (pending)
b.: These splice variants were previously described by Goodman et al., 2003 (reference 15)
c.: The specific function of this splice variant in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia was investigated by Feldhahn et al., 2005 (reference 6)

using PCR primers for BTK exons 12 to 19. Deleterious somatic mutations of the *BTK* gene were found in one out of 12 cases. In this case, the leukemia cells in a female patient carry an *MLL-AF4* gene rearrangement and deleterious mutations of the *BTK* gene on both alleles (both X chromsomes). One *BTK* allele is inactivated by a mutation Lys→Stop at codon 420 (AAA→TAA), while the second allele harbors a frameshift mutation due to a 1-bp deletion in codon 386. In the 11 other cases studied, no clonal replacement mutations of the *BTK* gene were detected. This analysis does not exclude deleterious mutations in other regions of the BTK gene, yet indicates that inactivation of the *BTK* gene by somatic mutation is rare in B cell precursor leukemia cells.

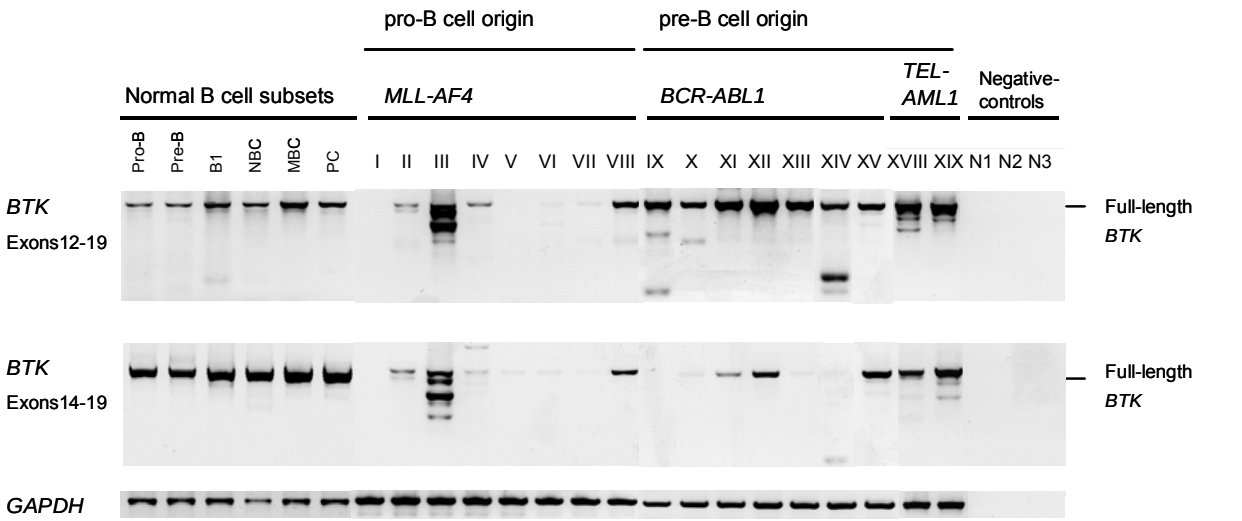
Expression of kinase-deficient splice variants of BTK in pre-B lymphoblastic leukemia cells

We next examined BTK mRNA expression in 29 human leukemias including 12 cell lines and 17 primary cases. In a PCR strategy covering the entire coding region of BTK, we identified 14 aberrant mRNA splice variants (Table 1). As a control, BTK cDNA fragments were amplified from normal human pro- and pre-B cells (isolated from bone marrow) and mature B cell subsets isolated from peripheral blood (Figure 1). At least one aberrant splice variant was co-amplified with full-length BTK in 21 of 29 cases (Table 1). In only four out of 29 cases, full-length BTK was exclusively expressed. Of note, absence of BTK expression was found in four out of 29 cases which all harbor an *MLL-AF4* rearrangement. In the remaining cases of primary B lymphoid leukemia carrying an *MLL-AF4* gene rearrangement, three exhibit very low expression of full-length BTK or high expression of aberrant splice variants while one shows exclusive expression of full-length BTK (Figure 1). In contrast, full-length BTK could be detected (not shown) in all three leukemia cell lines with an *MLL-AF4* fusion gene (BEL1, RS4;11, SEM).

The aberrant BTK splice variants all have in common that they encode a truncated BTK kinase domain (Table 1, supplementary Figure 1). From *in vitro* kinase assays of BTK mutants derived from BTK-deficient XLA patients, it is known that truncation of the BTK kinase domain at amino acid 520 and even replacement mutations in the distal portion of the kinase domain result in a complete loss of BTK kinase activity (4). Hence, only full-length BTK but none of the truncated BTK splice variants identified here should exhibit BTK kinase activity (Table 1, supplementary Figure 1). We conclude that BTK function is compromised at least in the B cell precursor leukemias that either express only kinase-deficient BTK or no BTK at all. In all these cases, the leukemia cells harbor an *MLL-AF4* gene rearrangement.

In many leukemia cases with other gene rearrangements, however, concomitant expression of full-length BTK was also detected. Among the 14 splice variants, three were recurrently amplified and two previously described variants of *BTK* (17) were also detected in this

Figure 1: *BTK mRNA expression in primary B cell precursor leukemia cells*



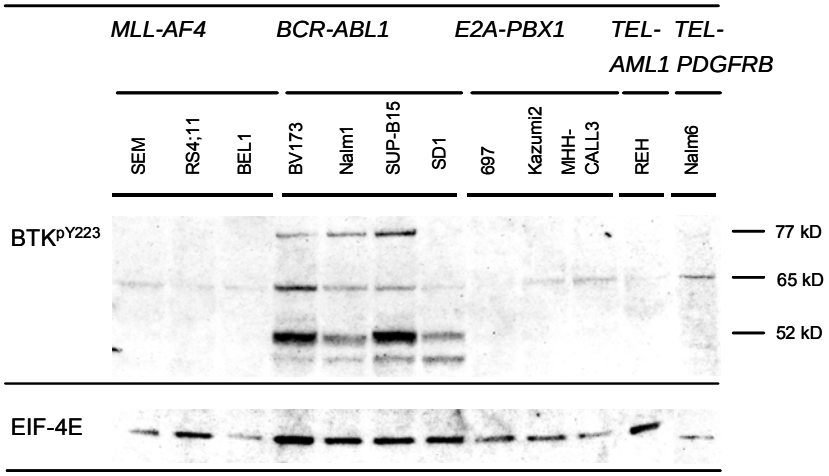
Using PCR-primers for exons 12, 14 and 19, two regions of the *BTK* mRNA were analyzed by RT-PCR in normal human pro-B cells, pre-B cells, B1 cells, naïve B cells (NBC), memory B cells (MBC) and plasma cells (PC) as well as in eight primary cases of B cell precursor leukemia with *MLL-AF4* gene rearrangement, seven primary cases with *BCR-ABL1* and two primary cases with *TEL-AML1* fusion genes. As a loading control for all samples, a *GAPDH* cDNA fragment was amplified.

analysis. While a splice variant lacking exon 15 was consistently and specifically amplified from leukemia cells carrying a *BCR-ABL1* fusion gene (Table 1), the expression of other recurrent splice variants was not linked to a specific chromosomal rearrangement (Table 1). Of note, aberrant splicing of BTK in the leukemia cells not only leads to the expression of defective BTK transcripts. The splice mechanism itself also seems to be deranged as cryptic splice sites were frequently used (9 of 29 cases) and splice site slippage leading to small nucleotide insertions or deletions was observed in four of 29 cases (Table 1).

As BTK activity is mainly regulated by tyrosine-phosphorylation, we investigated the expression of tyrosine-phosphorylated BTK protein in B cell precursor leukemia cell lines carrying *MLL-AF4*, *E2A-PBX1*, *BCR-ABL1*, *TEL-AML1* and *TEL-PDGFRB* gene rearrangements. Constitutive tyrosine-phosphorylation of full-length BTK was only observed in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells (three of the four cell lines tested; Figure 2). Whereas BTK lacking exon 15 alone (52 kD) was heavily phosphorylated specifically in

BCR-ABL1^{+} pre-B lymphoblastic leukemia cells, another recurrent BTK variant lacking exons 15 and 16 (65 kD) was also tyrosine-phosphorylated in other leukemias (Figure 2).

Figure 2: *Tyrosine-phosphorylation of BTK and kinase-deficient splice variants in pre-B lypmphoblastic leukemia cells*



Using an antibody against phosphorylated BTK^{Y233}, tyrosine-phosphorylation of BTK (77 kD) and BTK isoforms was analyzed by Western blot in 12 B cell precursor leukemia cell lines. The defining gene rearrangements of the B cell precursor leukemia cases analyzed are indicated. Western analysis of EIF4E expression was used as a loading control.

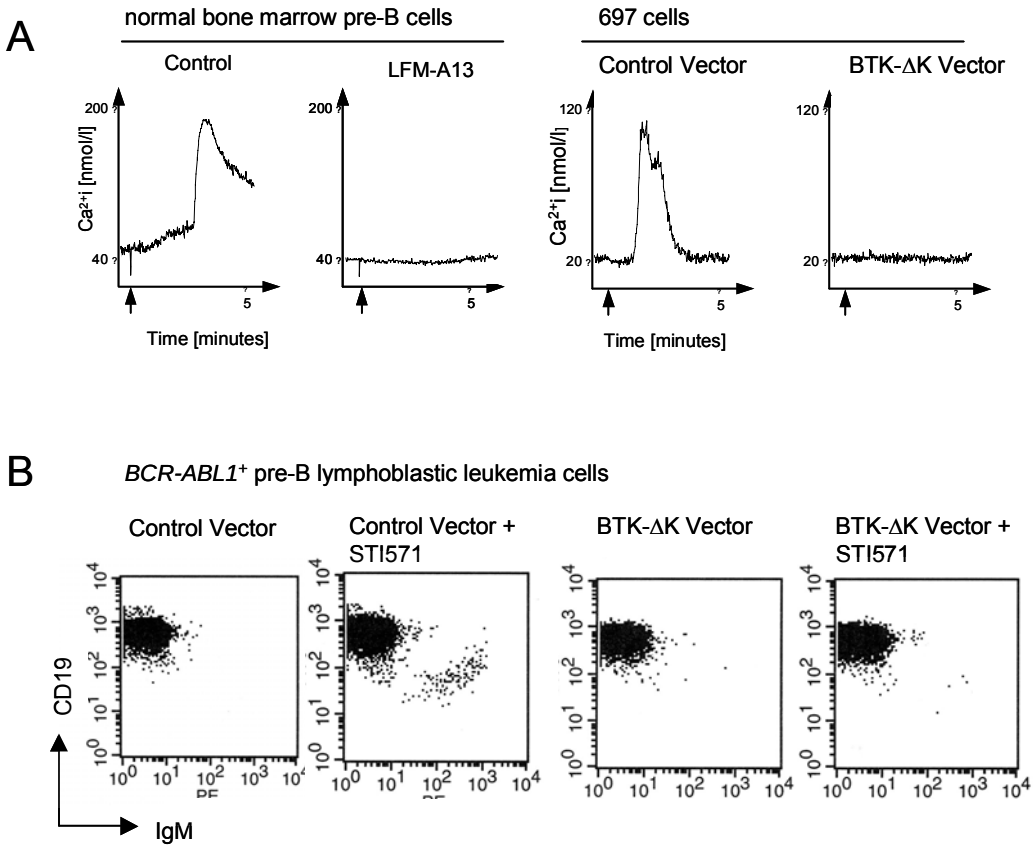
Expression of kinase-deficient BTK prevents pre-B cell receptor-driven differentiation of B cell precursor leukemia cells

BTK kinase-deficiency in humans leading to X-linked agammaglobulinemia (XLA) results in a breakdown of pre-B cell receptor signals and a differentiation block at the pre-B cell stage (4). In normal pre-B cells, engagement of the pre-B cell receptor using a μ-chain-specific antibody (arrows, Figure 3A, left) induces a strong Ca^{2+}-signal, which is sensitive to BTK kinase inhibition by LFM-A13 (Figure 3A, left).

As BTK kinase activity is required for the transduction of pre-B cell receptor-dependent Ca^{2+} signals, the kinase-deficient *BTK* splice variants (BTK-ΔK) expressed in pre-B lymphoblastic leukemia cells likely cannot contribute to pre-B cell receptor-dependent Ca^{2+} signals. However, kinase-deficient forms of BTK may also act as a linker in the signal transduction of B lymphocytes (6, 18). To investigate the function of BTK-ΔK, we expressed kinase-deficient BTK using a retroviral expression system in 697 cells that exhibit active pre-

B cell receptor signaling (Figure 3A, right). 697 cells transduced with a control vector responded by vigorous Ca^{2+} release upon pre-B cell receptor engagement. However, 697 cells carrying the BTK-ΔK expression vector entirely lost pre-B cell receptor responsiveness (Figure 3A, right). We conclude that expression of BTK-ΔK suppresses Ca^{2+} signals in response to pre-B cell receptor-stimulation.

Figure 3: *Expression of kinase-deficient BTK suppresses pre-B cell receptor-dependent Ca2+ signals and differentiation*



Normal pre-B cells were sorted from bone marrow of four healthy donors using antibodies against CD19 and VpreB. Sorted pre-B cells were incubated in cell culture medium in the presence or absence of the BTK kinase inhibitor LFM-A13 for 12 hours (A, left). The pre-B lymphoblastic leukemia cell line 697 transduced with a retroviral expression vector for kinase-deficient BTK (BTK-ΔK) or a control vector (A, right). Using an anti-μ chain antibody (arrows), Ca^{2+} release in response to pre-B cell receptor engagement was measured by laser scanning microscopy (A).

To elucidate the function of kinase-deficient BTK splice variants (BTK-ΔK), *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia SUP-B15 cells were transduced with either a control vector or a BTK-ΔK expression vector. In both cases, SUP-B15 cells were induced to differentiate by inhibition of BCR-ABL1 kinase activity through 10 μmol/l STI571 for four days. Thereafter, leukemia cells were stained using antibodies against CD19 and IgM (B). To clarify the effect of BTK-ΔK expression on STI571-induced differentiation, outgrowth of differentiating subclones in the presence or abscence of BTK-ΔK expression was analyzed by flow cytometry (B). Dead cells were identified and excluded from analysis by propidium iodide uptake.

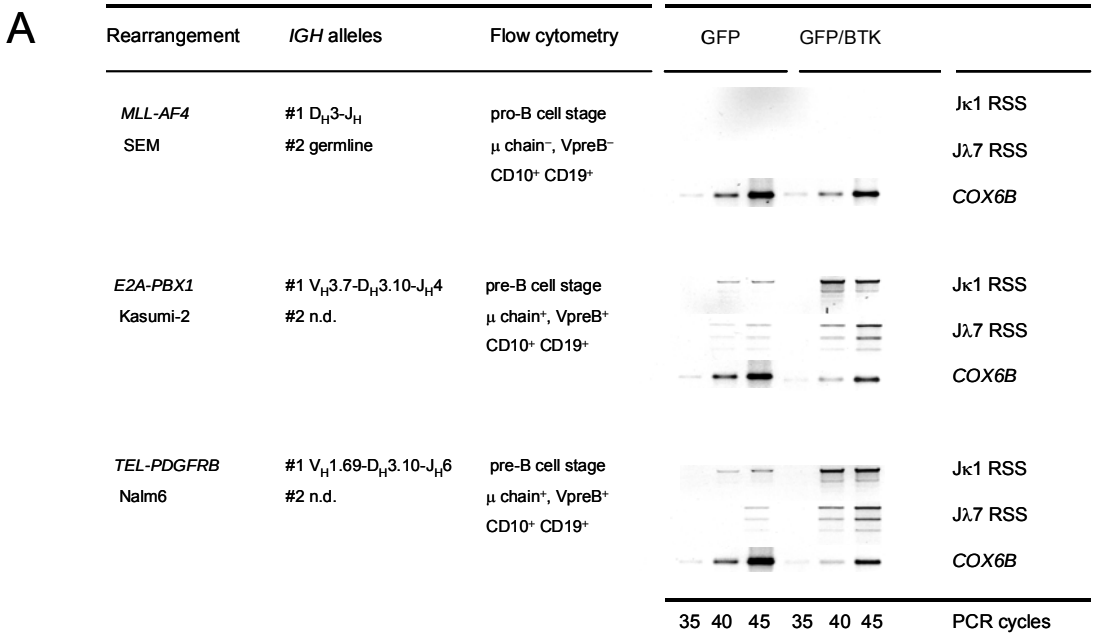
We next tested whether BTK-ΔK also interferes with pre-B cell receptor-driven differentiation. As shown by us and others (16, 19), inhibition of oncogenic kinase activity in *BCR-ABL1*$^+$ leukemia cells by STI571 induces differentiation in a minor population of cells that survive in the absence of BCR-ABL1 kinase activity. Therefore, STI571-induced differentiation in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells was used as a model to study the effect of kinase-deficient BTK on differentiation. To this end, *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells were transduced either with a retroviral control vector or a retroviral expression vector for BTK-ΔK. Inhibition of BCR-ABL1 kinase activity in *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells carrying a control vector induced the outgrowth of a small fraction of differentiating IgM$^+$ subclones (5 percent after two days; Figure 3B). However, upon forced expression of BTK-ΔK, less than 0.5 precent of *BCR-ABL1*$^+$ pre-B lymphoblastic leukemia cells differentiated into IgM$^+$ subclones (Figure 3B).

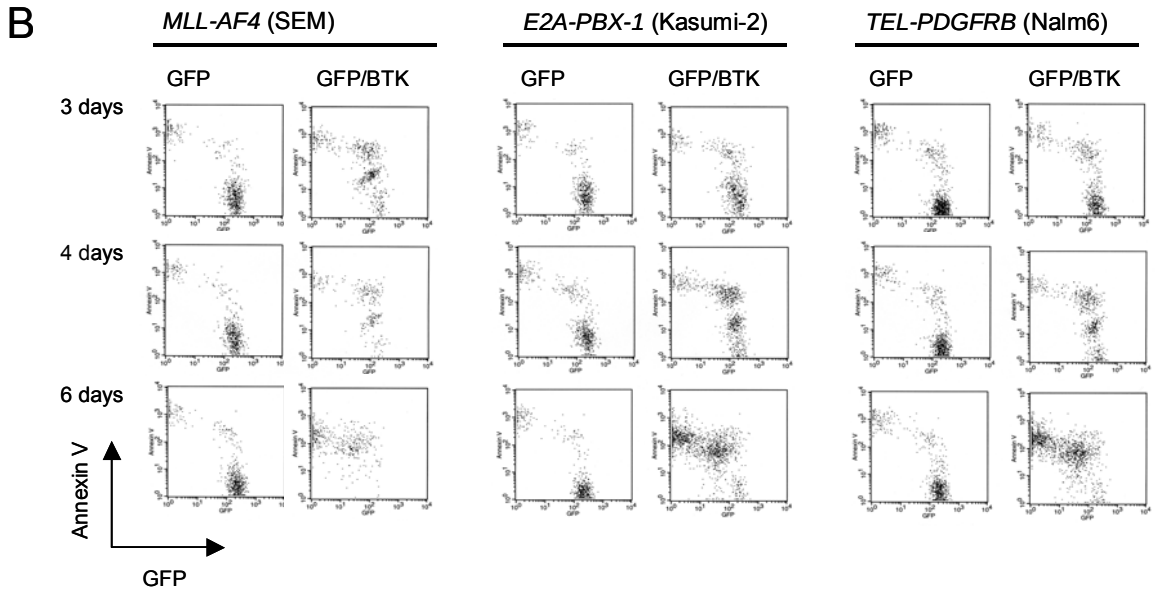
We conclude that BTK-ΔK can act as a dominant negative form of BTK with respect to i.) Ca^{2+} release in response to pre-B cell receptor engagement and ii.) to pre-B cell receptor-driven differentiation.

Reconstitution of functional BTK expression induces differentiation and confers propensity to apoptosis in B cell precursor leukemia cells

Given that kinase-deficient BTK splice variants can act in a dominant-negative way, we next investigated whether induced expression of functional BTK can rescue normal B cell differentiation in B cell precursor leukemia cells. To this end, B cell precursor leukemia cells carrying an *MLL-AF4*, *E2A-PBX1* or *TEL-PGFRB* gene rearrangement were transduced with a retroviral vector expressing functional BTK together with GFP or GFP alone as a control. GFP-expressing cells carrying either the control or the BTK expression vector were sorted and analyzed separately. We tested whether BTK-transduced pre-B cell leukemia cells initiated immunoglobulin light chain gene rearrangement. Rearrangement of immunoglobulin κ and λ light chain genes and subsequent replacement of surrogate light chains with conventional κ or λ light chains represent a hallmark of pre-B cell differentiation into mature B cells. Genomic DNA was isolated from GFP$^+$ or GFP$^+$/BTK$^+$ leukemia cells and subjected to ligation-mediated PCR for DNA double-strand break intermediates at recombination signal sequences (RSS) within the *IGK* or *IGL* loci (Supplementary Figure 2). Locus-specific DNA double-strand break intermediates were amplified at RSS flanking Jκ1 and Jλ7 gene segments, which would indicate ongoing Vκ-Jκ1 or Vλ-Jλ7 gene rearrangement, respectively. While Jκ1-RSS and Jλ7-RSS breaks can be detected at low level in two pre-B lymphoblastic leukemia cell lines transduced with the control vector, the frequency of Jκ1-RSS and Jλ7-RSS

Figure 4: *Reconstitution of BTK expression in acute lymphoblastic leukemia cells initiates differentiation and induces propensity to apoptosis*





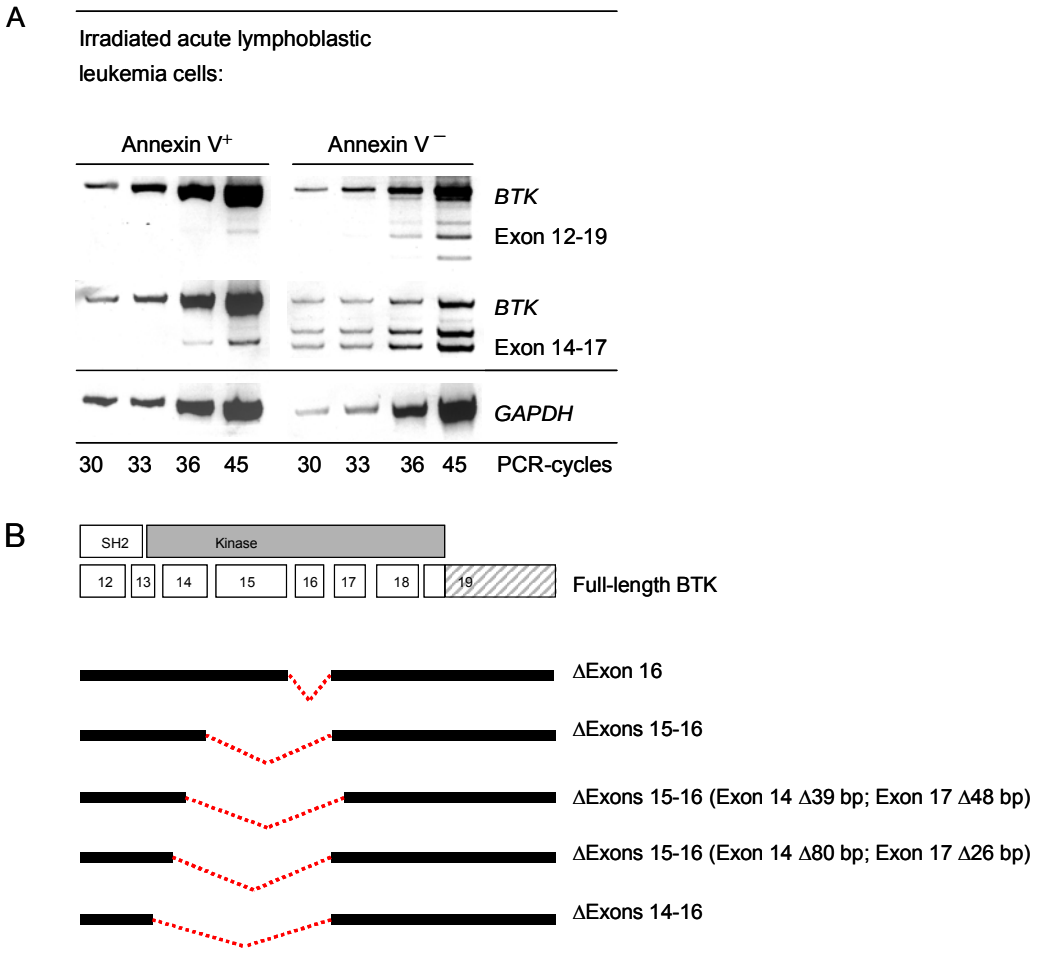
Acute lymphoblastic leukemia cell lines carrying either an *MLL-AF4* (SEM), *E2A-PBX1* (Kasumi-2) or *TEL - PDGFRB* (Nalm6) gene rearrangement were characterized by sequence analysis of their *IGH* loci and flow cytometry. The leukemia cells were transduced either with a retroviral MIG_GFP or a MIG_BTK-IRES-GFP expression vector. GFP-expressing cells were sorted and genomic DNA was extracted from the sorted cells. Amounts of genomic DNA were normalized by amplification of a genomic fragment of the *COX6B* gene. Genomic DNA from cells transduced with the control MIG_GFP-vector or MIG_GFP-IRES-BTK was subjected to ligation with a double-stranded DNA-linker of known sequence. To detect DNA-double strand breaks within the *IGK* and *IGL* loci, linker-ligated DNA was subjected to ligation-mediated PCR using linker-specific primers together with primers binding to recombination signal sequences flanking the Jκ1- (Jκ1 RSS) or the Jλ7- gene segment (Jλ7 RSS). Sorted GFP-expressing leukemia cells were also kept under cell culture conditions for three, four and six days and pre-apoptotic cells were labelled with Annexin V. After six days, almost all BTK-transduced leukemia cells underwent apoptosis whereas viability of leukemia cells transduced with the GFP-control vector remained unchanged (B).

immunoglobulin light chain gene rearrangement not only requires expression of functional BTK but also the expression of a functional pre-B cell receptor, which is present in pre-B lymphoblastic leukemia but missing in pro-B lymphoblastic leukemia cells carrying an *MLL-AF4* gene rearrangement (Figure 4A). While reconstitution of BTK expression expression initiated immunoglobulin light chain gene rearrangement in three of four cell lines tested, we could not detect κ or λ light chains on the surface of BTK-GFP-transduced leukemia cells (not shown). Attempting to establish permanent lines from the control-GFP- or BTK-GFP-transduced leukemia cells, we noted that reconstitution of expression of functional BTK substantially increases sensitivity of the leukemia cells to apoptosis. Comparing acute lymphoblastic leukemia cells carrying control-GFP or BTK-GFP vectors, the BTK-transduced cells underwent apoptosis spontaneously after six days in cell culture (Figure 4B).

Dominant-negative BTK splice variants can protect pre-B lymphoblastic leukemia cells against radiation-induced apoptosis

BTK-mediated propensity to apoptosis was observed in *MLL-AF4*$^+$, *E2A-PBX1*$^+$ and *TEL-PDFRB*$^+$ acute lymphoblastic leukemia cells irrespective of pre-B cell receptor expression (Figure 4). An earlier study invoked BTK kinase activity as a sensitizer to γ-radiation-induced apoptosis in chicken DT-40 lymphoma cells (20). Therefore, kinase-deficient dominant-negative BTK splice variants may have a protective effect against γ-radiation-induced apoptosis in human acute lymphoblastic leukemia cells. To test this hypothesis, we γ-irradiated *E2A-PBX1*$^+$ acute lymphoblastic leukemia cells with 10 Gy. 24 hours after irradiation, more than 90 percent of the cells had already undergone apoptosis as assessed by propidium iodide uptake. Among the remaining cells, we sorted Annexin V$^+$ pre-apoptotic cells and Annexin V$^-$ viable cells and compared BTK splice variant expression between pre-apoptotic and surviving leukemia cells. In two different PCR approaches, the entire kinase domain (using primers for exons 12 to 19) and at a higher level of resolution, a hotspot-region of aberrant splicing comprising exons 14 to 17 was amplified. Consistent with a protective effect against γ-radiation-induced apoptosis, surviving leukemia cells exhibit preferential expression of kinase-deficient dominant-negative BTK splice variants (Figure 5A). Sequence analysis of these splice variants revealed that some of the BTK splice variants identified here in the context of resistance to γ radiation-induced apoptosis were recurrently amplified from various leukemia subentities (see Table 1). However, novel BTK splice variants were also amplified (Figure 5B), which again all have in common their lack of a functional kinase domain.

Figure 5: *Acute lymphoblastic leukemia cells surviving γ-radiation are selected for the expression of dominant-negative BTK isoforms*



The pre-B lymphoblastic leukemia cell line Kasumi-2 carrying a *E2A-PBX1* gene rearrangement were γ-irradiated with 10 Gy. After 24 hours, more than 90 percent of the leukemia cells were apoptotic. Among the remaining cells, AnnexinV$^+$ pre-apoptotic cells and AnnexinV$^-$ surviving cells were sorted separately. Sorted cells were subjected to RT-PCR analysis for BTK splice variant expression and cDNA amounts were normalized by amplification of a GAPDH cDNA fragment. Splice variants were detected by amplification of the entire BTK kinase domain using primers for exons 12 and 19 (low resolution; A, top) or for exons 14 and 17 (high resolution, A, bottom). PCR products of this amplification were subjected to sequence analysis (B).

Concluding remarks

Taking these findings together, we conclude that somatic mutation of the *BTK* gene is rare in B cell precursor leukemia cells. However, in the majority of cases, the leukemia cells exhibit deranged expression of BTK. While in four out of eight primary cases with an *MLL-AF4* gene rearrangement BTK expression is missing, B cell precursor leukemia cells typically exhibit co-expression of full-length BTK with kinase-deficient, dominant-negative BTK splice

variants. These dominant-negative BTK splice variants can interfere with pre-B cell receptor-dependent signal transduction, induce a differentiation block and prevent immunoglobulin κ or λ light chain gene rearrangement. While overexpression of full-length BTK in B lymphoid leukemia cells induces propensity to apoptosis, expression of dominant-negative BTK splice variants can protect against radiation-induced apoptosis.

Acknowledgements:

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Materials and Methods

Patient samples, cell lines and cell purification

Normal $CD19^+$ μ-$chain^-$ pro-B cells and $CD19^+$ $VpreB^+$ pre-B cells were sorted from human bone marrow from four healthy donors (purchased from Cambrex, Baltimore, MD) using immunomagnetic beads against CD19 (Miltenyi Biotech, Bergisch Gladbach, Germany) and cell sorting using antibodies against CD19, VpreB (BD Biosciences, Heidelberg, Germany) and the μ-chain (Jackson Immunoresearch, West Grove, PA). Similarly, $CD5^+$ $CD19^+$ B1 cells, IgD^+ $CD27^-$ naïve B cells, $CD19^+$ $CD27^+$ memory B cells and $CD19^+$ $CD138^+$ plasma cells were sorted from peripheral blood of four healthy donors using antibodies against CD5, CD19, CD27, CD138 and IgD (BD Biosciences). In total, 29 B cell precursor leukemias including 12 cell lines and 17 primary cases were studied. 11 cases of B cell precursor leukemia with *MLL-AF4* gene rearrangement [t(4;11)(q21;q23)] including eight primary cases (I to VIII, Table 1) and three cell lines (BEL1, RS4;11 and SEM) were analyzed. 11 samples carrying a *BCR-ABL1* gene rearrangement [t(9;22)(q34;q11)] including seven primary cases (IX to XV, Table 1) and four cell lines (BV173, Nalm1, SD1 and SUP-B15) were studied. In addition, three leukemia cell lines carrying an *E2A-PBX1* gene rearrangement [t(1;19)(q23;p13); 697, Kasumi2 and MHH-CALL3], three cases of pre-B lymphoblastic

leukemia with *TEL-AML1* fusion gene [t(12;21)(p12;q22)] including two primary cases (XVIII and XIX, Table 1) and the cell line REH and one pre-B lymphoblastic leukemia cell line harboring a *TEL-PDGFRB* gene rearrangement [Nalm6; t(5;12)(q33.2;p13.2)] were studied. For all cases, fusion transcripts resulting from oncogenic gene rearrangements, were detected by PCR as previously described (5). Clinical data for all primary cases were described previously (2).

Western blotting

For the detection of tyrosine-phosphorylated BTK by Western blot, a phosphotyrosine-specific antibody against BTK^{Y223} and EIF4E (Cell Signaling Technology, Beverly, MA) were used. Western blot experiments were carried out as previously described (6).

Inhibitors of BCR-ABL1 and BTK

For inhibition of BCR-ABL1 kinase activity, the anti-leukemic drug STI571 (Novartis, Basel, Switzerland) was used at a concentration of 10 µmol/l. For inhibition of BTK, cells were incubated with alpha-cyano-beta-hydroxy-beta-methyl-N-(2,5-dibromophenyl) propenamide (LFM-A13; Calbiochem, Darmstadt, Germany) at a concentration of 50 µg/ml for the times indicated.

Measurement of pre-B cell receptor responsiveness

Primary human bone marrow pre-B cells were enriched using immunomagnetic MACS beads as previously described (7). Pre-B lymphoblastic leukemia cells were cultured with 10% fetal calf serum in RPMI 1640 medium for the times and under the conditions indicated. After pre-incubation, cells were washed and stained with Fluo-3 dye (Calbiochem, Bad Soden, Germany) for 30 minutes. Changes of cytosolic Ca^{2+} were measured by laser scans using confocal microscopy (8, 9). After 10 to 30 seconds of measurement, antibodies against human µ-chains (Jackson ImmunoResearch) were added to bone marrow pre-B cells or preincubated pre-B lymphoblastic leukemia cells (in the presence or absence of LFM-A13). Cytosolic Ca^{2+} concentrations were determined as previously described (8).

Flow cytometry

Surface expression of IgM and CD19 on pre-B lymphoblastic leukemia cell lines and primary leukemia cells was monitored using antibodies from BD Biosciences, Heidelberg, Germany, after the incubation times indicated. Pre-apoptotic or dead cells were identified by staining with Annexin V and propidium iodide (BD Biosciences).

Sequence analysis of BTK and semiquantitative RT-PCR

In a search for BTK isoforms and somatic mutations of the BTK gene, BTK cDNA fragments covering the entire coding region or genomic DNA fragments of the *BTK* gene were amplified

using 5'-ATCCCAACAGAAAAAGAAAACAT-3' (BTK exon 2 forward), 5'-GTTGCTTTCCTCCAAGATAAAAT-3' (BTK exon 8 reverse), 5'-ATCTTGAAAAAGCCACTACCG-3' (BTK exon 8 forward), 5'-TGATACGTCATTATGTTGTGTGTT-3' (BTK exon 12 forward), 5'-TGATACGTCATTATGTTGTGTGTT-3' (BTK exon 13 forward), 5'-ATCATGACTTTGGCTTCTTCAAT-3' (BTK exon 14 reverse), 5'-CTCAAATATCCAGTGTCTCAACA-3' (BTK exon 14 forward), 5'-CTTTAACAACTCCTTGATCGTTT-3' (BTK exon 17 reverse) and 5'-GGATTCTTCATCCATGACATCTA-3' (BTK exon 19 reverse) were used. For normalization of cDNA and genomic DNA amounts 5'-TTAGCACCCCTGGCCAAG-3' (GAPDH forward) and 5'-CTTACTCCTTGGAGGCCATG-3' (GAPDH reverse) and 5'-AACTACAAGACCGCCCCTTT-3' (COX6B forward) and 5'-GCAGCCAGTTCAGATCTTCC-3' (COX6B reverse) were used.

BTK amplification products were sequenced as previously described (6). Sequences of novel BTK isoforms are available from Genbank/EMBL (AM051275-AM051286).

Retroviral expression of a kinase-deficient BTK splice variant

All aberrant BTK splice variants identified lack a functional kinase domain (see supplementary Figure 1). Therefore, we generated a cDNA fragment of human BTK comprising the entire coding region but lacking the C-terminal portion of the kinase domain (exons 15 to 19; BTK-ΔK). Following digestion with *Not*I (New England Biolabs, Frankfurt am Main, Germany), the PCR-product was ligated into the retroviral S11IN expression vector (10). This vector is based on the retroviral plasmid SFβ11 (kindly provided by Dr. Christopher Baum, Hannover, Germany) in which a multicloning site with *Not*I, *Eco*RI and *Bam*HI restriction sites was introduced, followed by an IRES NEO cassette.

293T cells were cultured in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Invitrogen), 2 mM L-glutamine (Invitrogen), penicillin G (100 units/ml) and streptomycin (100 µg/ml) (Invitrogen). 293T cells were cotransfected with 10 µg of the helper plasmid pHIT60, 10 µg of pczVSV-G envelope (11) and 10 µg of S11IN (as a control vector) or S11-BTK-ΔK-IN using Fugene 6 (Roche, Basel, Switzerland) following manufacturer's instructions. Both vectors are based on SF11 (10) with the 3'LTR of the spleen focus-forming virus (SFFVp) and internal ribosome entry site (IRES). Alternatively, MIG-GFP (12) und MIG-BTK-IRES-GFP vectors were used for retroviral delivery of full-length BTK. For construction of the MIG-BTK-IRES-GFP vector, the cDNA encoding human full-length BTK was cloned into the MIG vector (12). 24h after transfection, the

medium was changed for IMDM (Iscove's modified Medium). 48h after the transfection, supernatants were filtered through a 0.45 μm filter and used to infect pre-B acute lymphoblastic leukemia cell lines on plates coated with 2 $\mu g/cm^2$ of retronectin (Takara Shuzo, Otsu, Japan; reference 13). Plates were pre-loaded three times with fresh supernatant (14) and subsequently 2,5 x 10^5 cells were added to each well. 48 h after infection, cells expressing the S11IN vector were selected using G418 to a final concentration of 0.5 mg/ml.

Cell sorting

GFP-expressing cells or Annexin V^+ and Annexin V^- cells were sorted using a FACStar 440 cell sorter. Cells were sorted under sterile conditions and either kept under cell culture conditions or subjected to RNA isolation and RT-PCR analysis.

Amplification of double-strand recombination signal sequence breaks by ligation-mediated PCR

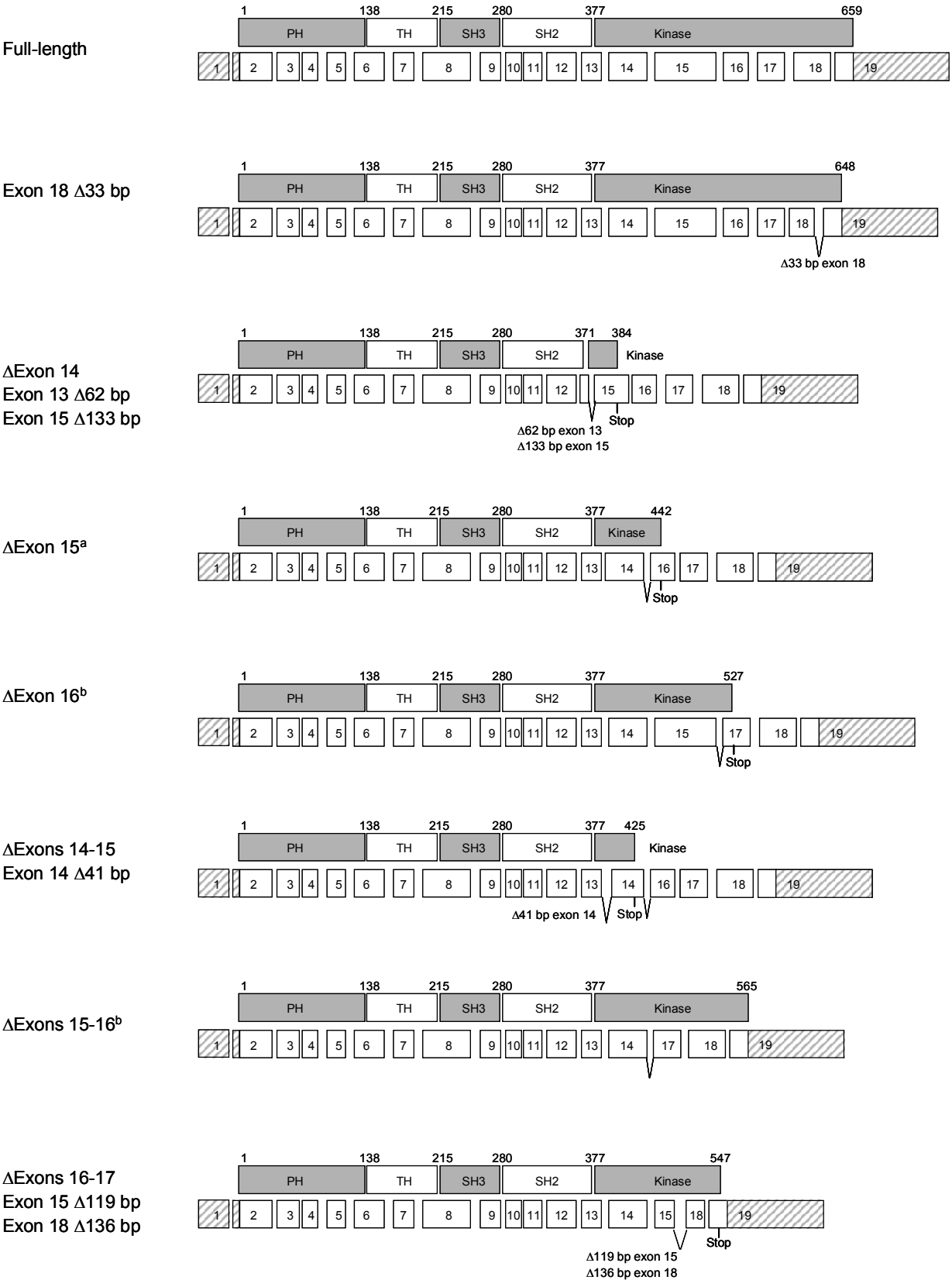
Genomic DNA was isolated from about 2.5 x 10^6 leukemia cells carrying retroviral expression vectors for either GFP alone or GFP and BTK and ligated to a blunt-end linker using T4 DNA ligase (Invitrogen, Karlsruhe, Germany) at 14°C overnight. The linker was constructed by annealing the oligonucleotides 5'-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACATG 3' and 3' amino (C7)-GACGAGCTTAAGTTCGAAGATTGCTACATGCCCCT -5' and protruding 3' overhangs were removed by 3'→ 5' exonuclease activity of the Klenow fragment of E. *coli* DNA polymerase I (Invitrogen, Karlsruhe, Germany). Ligation-mediated PCR (LM-PCR; reference 15) was carried out with modifications as previously described (16). In two semi-nested rounds of amplification at an annealing temperature of 59°C, RSS-intermediates with a DNA double-strand break at the 5' heptamer of Jκ1 gene segments were amplified (see supplementary Figure 2) using 5'-GTAATTAACATTCAGTCTACTTTC-3' as external forward and 5'-TAACATTCAGTCTACTTTCTAAAA-3' as internal forward primers together with 5'-TCCCCGTACATCGTTAGAAG–3' as reverse primer specific for DNA-ligated linker molecules. To amplify RSS-intermediates with a DNA double-strand break at the 5' heptamer of Jλ7 gene segments, 5'-TTCTCACTTCTTCCATGGTGAC-3' and 5'-ACTTCTTCCATGGTGACAGTCT-3' were used in two rounds of PCR amplification as described above.

γ-radiation

Pre-B lymphoblastic leukemia cells were irradiated with 10 Gy γ-radiation at the Clinic for Radiotherapy and Radiological Oncology, Heinrich-Heine-Universität Düsseldorf,

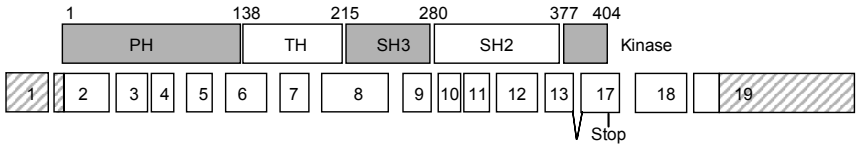
Düsseldorf, Germany. 24 hours after radiation, leukemia cells were stained with Annexin V and propidium iodide to separately sort pre-apoptotic or surviving cells.

Supplementary Figure 1a: *BTK splice variants in B cell precursor leukemia*

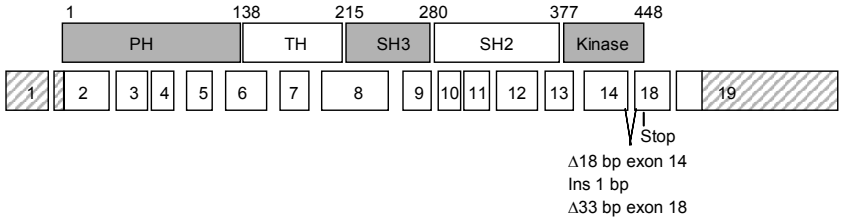


Supplementary Figure 1b: *BTK splice variants in B cell precursor leukemia*

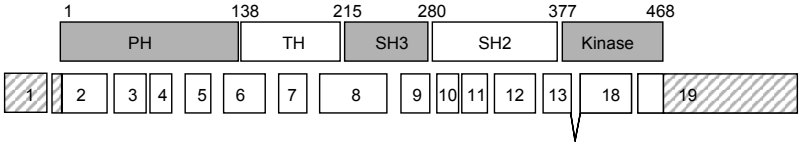
ΔExons 14-16



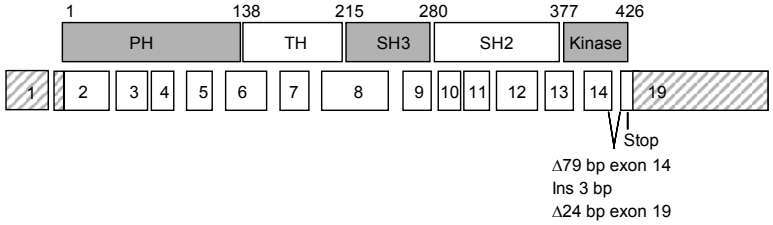
ΔExons 15-17
Exon 14 Δ18 bp
Ins 1 bp
Exon 18 Δ33 bp



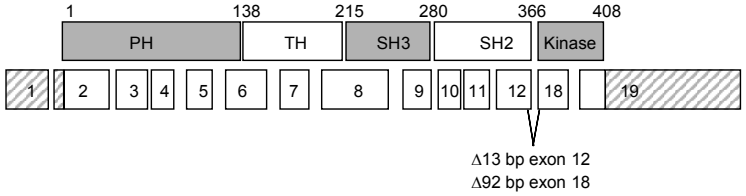
ΔExons 14-17



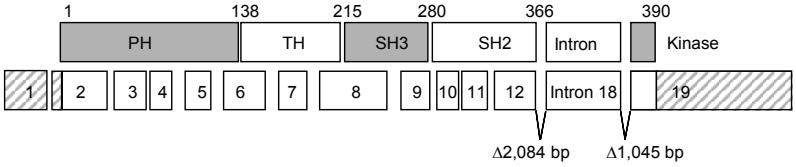
ΔExons 15-18
Exon 14 Δ79 bp
Ins 3 bp
Exon 19 Δ24 bp



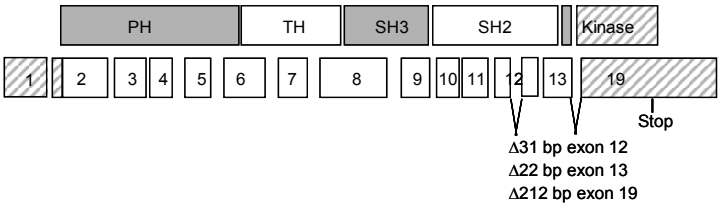
ΔExons 13-17
Exon 12 Δ13 bp
Exon 18 Δ92 bp



ΔExons 13-18
Ins 106 bp Intron 18



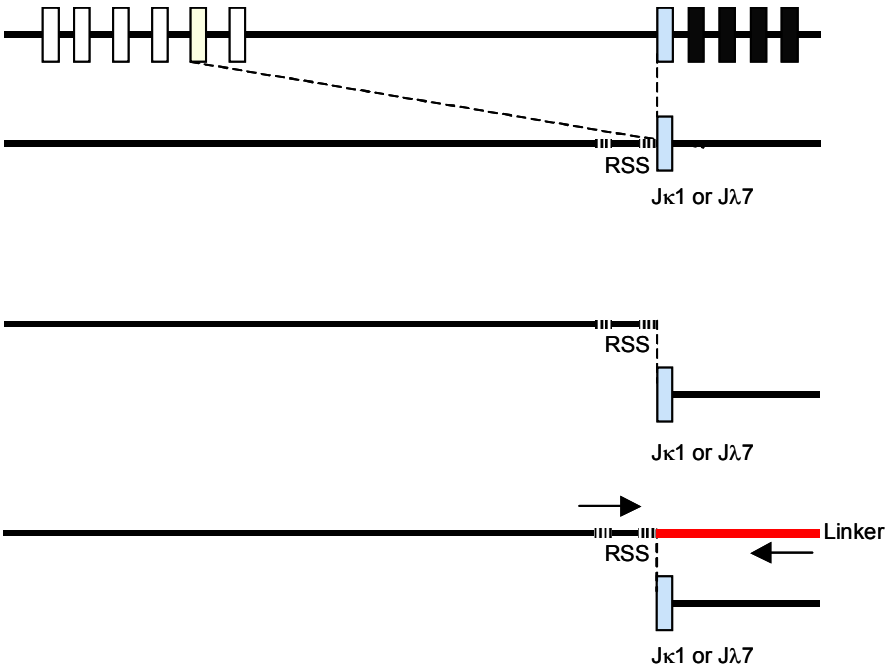
ΔExons 14-18
Exon 12 Δ31 bp
Exon 13 Δ22 bp
Exon 19 Δ212 bp



Legend to supplementary Figure 1: *BTK splice variants in B cell precursor leukemia*

BTK isoform expression was analyzed by RT-PCR in 29 cases of B cell precursor leukemia carrying *BCR-ABL1*, *E2A-PBX1*, *MLL-AF4*, *TEL-AML1* or *TEL-PDGFRB* gene rearrangements. 14 aberrant BTK isoforms could be identified in addition to full-length BTK. None of these BTK isoforms carry a functional kinase domain resulting from the usage of regular or cryptic splice sites leading to large deletion and/or the generation of pre-terminal translation-stops. Protein domains (top) and mRNA exons (bottom) are depicted. Untranslated regions are indicated by hatched boxes. Sequence data of all splice variants is available from EMBL/GenBank under accession numbers (AM051275-AM051286).

Supplementary Figure 2: *LM-PCR amplification of DNA-double strand break intermediates at Jκ1 and Jλ7 recombination signal sequences*



Immunoglobulin light chain gene rearrangement involves the introduction of DNA double strand breaks at recombination signal sequences (RSS) flanking V and J segments. DNA double strand breaks at RSS can be ligated to double stranded DNA linker molecules. In order to detect DNA double-strand breaks preceding V-J recombination at the *IGK* or *IGL* loci, linker-ligated DNA can be amplified using Jk-RSS or Jl-RSS-specific primers together with a linker-specific primer.

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Chapter 6

Appendix

6.1 Supplementary information

PCR primers

GAPDH forward	5'-TTAGCACCCCTGGCCAAG-3'
GAPDH reverse	5'-CTTACTCCTTGGAGGCCATG-3'
BCR-ABL1 forward	5'-ACCTCACCTCCAGCGAGGAGGACTT-3'
BCR-ABL1 reverse	5'-TCCACTGGCCACAAAATCATACAGT-3'
mAid forward	5'- AAATGTCCGCTGGGCCAA-3'
mAid reverse	5'- CATCGACTTCGTACAAGGG-3'
AID forward	5'-TGCTCTTCCTCCGCTACATC-3'
AID reverse	5'-CCTCATACAGGGGCAAAAGG-3'
mObf1 forward	5'-AGCTCCCTGACCATTGAC-3'
mObf1 reverse	5'-CTGTCCCATCCCCCTGTAA-3'
mOct2 forward	5'-ATCGAGACGAATGTCCGCTT-3'
mOct2 reverse	5'-GTAGCTGGTCGGCTTTCC-3'
Cμ_R	5'-AGACGAGGGGGAAAAGGGTT-3'
Cγ1_R	5'-GAGTTTTGTCACAAGATTTGGGCT-3'
Cγ2_R	5'-GCACTCGACACAACATTTGCG-3'
Cγ3_R	5'-TCACCAAGTGGGGTTTTGAGC-3'
Cγ4_R	5'-ATGGGCATGGGGGACCATTT-3'
Cα2_R	5'-TGTTGGCGGTTAGTGGGGTC-3'
Cε_R	5'-GAGGTGGCATTGGAGGGAAT-3'
Cδ_R	5'-AGAGCTGGCTGCTTGTCATG-3'
mJ558 forward	5'-GCAAGGCTTCTGGCTACACA-3'
$mJ_H2/3$ reverse	5'-CCAGTAAGCAAACCAGGCACA-3'
BTK exon 2 forward	5'-ATCCCAACAGAAAAAGAAAACAT-3'
BTK exon 8 reverse	5'-GTTGCTTTCCTCCAAGATAAAAT-3'
BTK exon 8 forward	5'-ATCTTGAAAAAGCCACTACCG-3'
BTK exon 12 forward	5'-TGATACGTCATTATGTTGTGTGTT-3'
BTK exon 13 forward	5'-ACTCATATCCAGGCTCAAATATC-3'
BTK exon 14 reverse	5'-ATCATGACTTTGGCTTCTTCAAT-3'
BTK exon 14 forward	5'-AATTGATCCAAAGGACCTGAC-3'
BTK exon 15 forward	5'-GAGAAGCTGGTGCAGTTGTAT-3'
BTK exon 16 reverse .1	5'-GGCCGAAATCAGATACTTTAAC-3'
BTK exon 16 reverse.2	5'-CTTTAACAACTCCTTGATCGTTT-3'
BTK exon 17 reverse	5'-ATTTGCTGCTGAACTTGCTATAC-3'
BTK exon 18 reverse	5'-TATACCTTCTCTGAAGCCAGATG-3'
BTK exon 19 reverse.1	5'-AAATTTCAGGCACAATAATTTCT-3'
BTK exon 19 reverse.2	5'-GGATTCTTCATCCATGACATCTA-3'
BTK^{p52} forward	5'CTTCCTCTCTGGACTGTAAGAAT-3'
BTK^{p52} reverse	5'-ATACAACTGCACCAGCTTCTC-3'
$BCLX_L$ forward	5'-AATCTTATCTTGGCTTTGGAT-3'
$BCLX_L$ reverse	5'-GTTCTCTTCCACATCACTAAA-3'
COX6B forward	5'-AACTACAAGACCGCCCCTTT-3'
COX6B reverse	5'-GCAGCCAGTTCAGATCTTCC-3'
HPRT forward	5'-CCTGCTGGATTACATCAAAGCACTG-3'
HPRT reverse	5'-CACCAGCAAGCTTGCGACC-3'

LM-PCR primers

CDKN2A_F1	5’-CCAGGAATAAAATAAGGGGAATA-3’
CDKN2A_F2	5’-GGAATAAAATAAGGGGAATAGGG-3’
CDKN2A_R	5’-CTTTCCTACCTGGTCTTCTAGG-3’
CDKN2B_F1	5’-GTGAACATTCCCAAAATATTAGC-3’
CDKN2B_F2	5’-AAAATATTAGCCTTGGCTTTACTG-3’
CDKN2B_R	5’-AGACTCCTGTACAAATCTACATCG-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’
Mmyc_F1	5’-ATCCTGAGTCGCAGTATAAAAGA-3’
Mmyc_F2	5’-TTTTCTGACTCGCTGTAGTAATTC-3’
Mmyc_R1	5’-CTCAGAGACTGGTAAGTCAGAAG-3’
Linker_F1	5’-CTGCTCGAATTCAAGCTTCT-3’
Linker_F2	5’-GCTTCTAACGATGTACGGGG-3’
Linker_R1	5’-GTACATCGTTAGAAGCTTGAA-3’
Linker_R2	5’-GTTAGAAGCTTGAATTCGAGC-3’

siRNA duplices

Non-targeting siRNA	5’-UUGUACCUAAUUUCGUCCCAC-3’ 3’-CAUGGAUUAAAGCAGGGUGUU-5’
AID siRNA	5'-UUGCUCUUCCUCCGCUACAUU-3' 3'-CGAGAAGGAGGCGAUGUAAUU-3'
$BTK^{full\text{-}length}$ siRNA.1	5’-UUGCUGGUGCAGUUGUAUGGC-3’ 3’-CGACCACGUCAACAUACCGUU-5’
$BTK^{full\text{-}length}$ siRNA.2	5’-UUGUUGUAUGGCGUCUGCACC-3’ 3’-CAACAUACCGCAGACGUGGUU-5’
$BTK^{full\text{-}length}$ siRNA.3	5’-UUGUACAUGGCCAAUGGCUGC-3’ 3’-CAUGUACCGGUUACCGACGUU-5’
BTK^{p65} siRNA.1	5’-UUUCAUGAUGUAUGUCCUGGA-3’ 3’-AGUACUACAUACAGGACCUUU-5’
BTK^{p65} siRNA.2	5’-UUAAGUCAUGAUGUAUGUCCU-3’ 3-‘UUCAGUACUACAUACAGGAUU-5’
BTK^{p65} siRNA.3	5’-UUCCAAAGUCAUGAUGUAUGU-3’ 3’-GGUUUCAGUACUACAUACAUU-5’
BTK^{p52} siRNA.1	5’-UUUCAUGAUGCAGCUCGAAAC-3’ 3’-AGUACUACGUCGAGCUUUGUU-5’
BTK^{p52} siRNA.2	5’-UUAGUCAUGAUGCAGCUCGAA-3’ 3’-UCAGUACUACGUCGAGCUUUU-5’
BTK^{p52} siRNA.3	5’-UUCCAAAGUCAUGAUGCAGCU-3’ *3’-GGUUUCAGUACUACGUCGAUU-5’*

Accession numbers:

BTK^{p52}	AJ888378
BTK isoforms in *BCR-ABL1*-positive pre-B cell leukemia	AJ888376-AJ888381
BTK isoforms in *BCR-ABL1*-negative pre-B cell leukemia	AM051275-AM051286
IK6	AM085310

Sequence data available from GenBank/EMBL/DDBJ under the accession numbers given in the table.

6.2 Abbreviations

ALL, acute lymphoblastic leukaemia
AID, activation-induced cytidine deaminase
BTK, Bruton's tyrosine kinase
C, constant;
CLP, common lymphoid progenitor
CML, chronic myeloid leukemia
CSR, class-switch recombination
cRSS, cryptic RSS
D, diversity
DAG, diacylglycerol
GL, germline
HSC, hematopoietic stem cell
IGH, immunoglobulin heavy-chain
IGK, immunoglobulin κ light chain

IGL, immunoglobulin λ light chain

ITAM, immunoreceptor tyrosine-based activatory motif

ITIM, immunoreceptor tyrosine-based inhibitory motif

J, joining

KDE, κ deleting element

LM-PCR, ligation-mediated PCR

NHEJ, non-homologous end joining

Ph, Philadelphia chromosome

PH, Pleckstrin-homology

PIP_2, phosphatidylinositol-4,5-bisphosphate

PIP_3, phosphatidylinositol-3,4,5-trisphosphate

PTK, protein tyrosine-kinase

PTP, protein tyrosine-phosphatase

RSS, recombination signal sequence

SAGE, serial analysis of gene expression

siRNA, small interfering RNA

SH2, SRC homology domain 2

SH3, SRC-homology domain 3

SHM, somatic hypermutation

SRC, cellular homolog of *Rous sarcoma virus*

TH, TEC-homology

V, variable

XLA, X-linked agammaglobulinemia

Xid, X-linked immunodeficiency

6.3 Curriculum vitae

Niklas Feldhahn

15.10.1976	Born in Bad Oldesloe (Germany)
1983-1987	Primary school (Lübeck/Germany)
1987-1996	Gymnasium (Lübeck/Germany)
01.06.1996	Abitur disposal (Lübeck/Germany)
1996-1997	Community service
1997-1999	Basic study period of biology (University of Osnabrück/Germany)
27.08.1999	Intermediate diploma
1999-2002	Main study period of biology (University of Cologne/Germany)
2001	Assistant at the *Science Factory* (company for bioinformatics/Cologne/Germany)
2001-2002	Diploma thesis in the laboratory of PD Dr. Müschen (Universität zu Köln/Germany)
19.09.2002	Diploma disposal graded with *sehr gut* (1.0): "*Signale des Antigenrezeptors humaner B Zell Populationen: eine funktionelle Genomanalyse*"
2003-2006	Thesis in the laboratory of Prof. Müschen (Heinrich-Heine Universität Düsseldorf/Germany)
21.02.2006	Disposal of the *Leukemia Clinical Research Award 2006* by the DGHO to Markus Müschen and Niklas Feldhahn

Invited talks

2003	First annual B cell meeting (Freiburg/Germany)
2004	Second annual B cell meeting (Berlin/Germany)
2004	Joint Annual Meeting of the German (DGfI) and Dutch Society (NVvI) of Immunology (Maastricht/Netherlands)
2005	Joint Annual Meeting of the German (DGfI) and Scandinavian Society (SSI) of Immunology (Kiel/Germany)

6.4 List of publications

2002

Müschen M, Lee S, Zhou G, **Feldhahn N**, Barath VS, Chen J, Moers C, Krönke M, Rowley JD, Wang SM. Molecular portraits of B cell lineage commitment. *Proc Natl Acad Sci U S A.* 2002; 99: 10014-10019

Feldhahn N, Schwering I, Lee S, Wartenberg M, Klein F, Wang H, Zhou G, Wang SM, Rowley JD, Hescheler J, Krönke M, Rajewsky K, Kuppers R, Müschen M. Silencing of B cell receptor signals in human naive B cells. *J Exp Med.* 2002; 196: 1291-1305

2003

Klein F, **Feldhahn N**, Lee S, Wang H, Ciuffi F, von Elstermann M, Toribio ML, Sauer H, Wartenberg M, Barath VS, Krönke M, Wernet P, Rowley JD, Müschen M. T lymphoid differentiation in human bone marrow. *Proc Natl Acad Sci U S A.* 2003; 100: 6747-6752

2004

Klein F, **Feldhahn N**, Harder L, Wang H, Wartenberg M, Hofmann WK, Wernet P, Siebert R, Müschen M. The BCR-ABL1 kinase bypasses selection for the expression of a pre-B cell receptor in pre-B acute lymphoblastic leukemia cells. *J Exp Med.* 2004; 199: 673-685

Klein F, **Feldhahn N**, Müschen M. Interference of BCR-ABL1 kinase activity with antigen receptor signaling in B cell precursor leukemia cells. *Cell Cycle.* 2004; 3: 858-860

Kögler G, Sensken S, Airey JA, Trapp T, Müschen M, **Feldhahn N**, Liedtke S, Sorg RV, Fischer J, Rosenbaum C, Greschat S, Knipper A, Bender J, Degistirici O, Gao J, Caplan AI, Colletti EJ, Almeida-Porada G, Muller HW, Zanjani E, Wernet P. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J Exp Med*. 2004; 200: 123-35

Su YW, Herzog S, Lotz M, **Feldhahn N**, Müschen M, Jumaa H. The molecular requirements for LAT-mediated differentiation and the role of LAT in limiting pre-B cell expansion. *Eur J Immunol*. 2004; 34: 3614-3622

2005

Klein F, **Feldhahn N**, Mooster JL, Sprangers M, Hofmann WK, Wernet P, Wartenberg M, Müschen M. Tracing the pre-B to immature B cell transition in human leukemia cells reveals a coordinated sequence of primary and secondary IGK gene rearrangement, *IGK* deletion, and *IGL* gene rearrangement. *J Immunol.* 2005; 174: 367-375

Feldhahn N, Klein F, Mooster JL, Hadweh P, Sprangers M, Wartenberg M, Bekhite MM, Hofmann WK, Herzog S, Jumaa H, Rowley JD, Müschen M. Mimicry of a constitutively active pre-B cell receptor in acute lymphoblastic leukemia cells. *J Exp Med*. 2005; 201: 1837-1852

Feldhahn N, Río P, Ndikung Bejeng Soh B, Liedtke S, Sprangers M, Klein F, Wernet P, Jumaa H, Hofmann WK, Hanenberg H, Rowley JD, Müschen M. Deficiency of Bruton's tyrosine kinase in B cell precursor leukemia cells. *Proc Natl Acad Sci USA.* 2005; 102: 13266-13271

Soh BN, Klein F, **Feldhahn N**, Müschen M. B-lymphoid or myeloid lineage identity of cell lines derived from chronic myeloid leukemia blast crisis. *Cancer Genet Cytogenet.* 2005; 161: 187-188

2006

Klein F, **Feldhahn N,** Herzog S, Sprangers M, Mooster JL, Jumaa H, Müschen M. BCR-ABL1 induces aberrant splicing of *IKAROS* and lineage infidelity in pre-B lymphoblastic leukemia cells. *Oncogene*. 2006; 25: 1118-24

Sprangers M, **Feldhahn N**, Herzog S, Hansmann ML, Reppel M, Hescheler J, Jumaa H, Siebert R, Müschen M. The SRC family kinase LYN redirects B cell receptor signaling in human SLP65-deficient B cell lymphoma cells. *Oncogene*. 2006 Mar 27;

Sprangers M, **Feldhahn N**, Liedtke S, Jumaa H, Siebert S, Müschen M. *SLP65*-deficiency results in perpetual V(D)J-recombinase activity in pre-B lymphoblastic leukemia and B cell lymphoma cells. *Oncogene.* 2006;

6.5 Specific contribution of Niklas Feldhahn to the publications shown in chapter 5.1-5.2

In this doctoral thesis, a number of aspects related to BCR-ABL1-mediated survival signals in pre-B lymphoblastic leukemia cells are described. The published results are presented in detail in chapter 3.1-3.6 and 5.1-5.6. Besides my own experimental data, this work also involves the contributions from colleagues and members of the laboratory of Markus Müschen.
In the following, my own experimental contribution to the specific publications is outlined:

Chapter 5.1: **The BCR-ABL1 kinase bypasses selection for the expression of a pre-B cell receptor in pre-B acute lymphoblastic leukemia cells**

By Klein F, **Feldhahn N**, Harder L, Wang H, Wartenberg M, Hofmann WK, Wernet P, Siebert R, Müschen M.; *J Exp Med.* 2004; 199: 673-685

- Generation of gene-expression profiles for naïve and memory B cells using the SAGE-method for the comparison of BCR-ABL1-positive pre-B leukemia cells with normal B cell subsets
- Analysis of the configuration of the *IGH* locus of both alleles of 12 BCR-ABL1-positive pre-B leukemia cases
- Identification of footprints of potential V_H-replacement in 9 out of 12 BCR-ABL1-positive pre-B leukemia cases

Chapter 5.2: **BCR-ABL1 induces aberrant splicing of *IKAROS* and lineage infidelity in pre-B lymphoblastic leukemia cells.**

By Klein F, **Feldhahn N,** Herzog S, Sprangers M, Mooster JL, Jumaa H, Müschen M. *Oncogene*. 2005; 24: 1-7

- Western blot for the doxycylin-induced expression of the *BCR-ABL1* transgene in the murine pro-B cell line TON-B210

- FACS analysis of the expression of B lineage- and myeloid lineage-specific surface receptors before and during treatment with STI571
- Western blot for the expression of the *BCR-ABL1* transgene in pMIG-BCR-ABL1-IRES-GFP- and pMIG-GFP-transfected 697 cells
- Participation in the RNA-interference-mediated silencing of the expression of the IKAROS isoform IK6 in BCR-ABL1-positive pre-B leukemia cells

Chaper 5.3: **Tracing the pre-B to immature B cell transition in human leukemia cells reveals a coordinated sequence of primary and secondary IGK gene rearrangement, *IGK* deletion, and *IGL* gene rearrangement**

By Klein F, **Feldhahn N**, Mooster JL, Sprangers M, Hofmann WK, Wernet P, Wartenberg M, Müschen M. *J Immunol.* 2005; 174: 367-375

- Analysis of RAG1 protein expression in BCR-ABL1-positive pre-B leukemia cells following treatment with STI571
- FACS analysis of immunoglobulin light-chain expression on the surface of BCR-ABL1-positive pre-B leukemia cells after treatment with STI571

Chapter 5.4: **Mimicry of a constitutively active pre-B cell receptor in acute lymphoblastic leukemia cells**

By **Feldhahn N**, Klein F, Mooster JL, Hadweh P, Sprangers M, Wartenberg M, Bekhite MM, Hofmann WK, Herzog S, Jumaa H, Rowley JD, Müschen M *J Exp Med.* 2005; 201: 1837-1852

- Western blot analysis of protein expression and tyrosine-phosphorylation of the signaling molecules SYK, SLP65 and BTK in BCR-ABL1-positive and -negative pre-B leukemia cell lines and primary cases

- RT-PCR analysis of *BTK* expression in BCR-ABL1-positive pre-B leukemia cells and subsequent sequence analysis
- Silencing of *BTK* isoform expression by RNA-interference
- Survival assay of BCR-ABL1-positive and –negative pre-B leukemia cell lines in the presence or absence of the BCR-ABL1 kinase inhibitor STI571 and/or the BTK kinase inhibitor LFM-A13
- Analysis of Ca^{2+} signals in BCR-ABL1-positive pre-B cell leukemia cells in the presence or absence of STI571 and/or LFM-A13 by confocal laser scan microscopy
- Analysis of the phosphorylation status of STAT5 and PLCγ1 in BCR-ABL1-positive pre-B leukemia cell lines in the presence or absence of STI571 and/or LFM-A13 by confocal laser scan microscopy
- Analysis of PLCγ1- and PLCγ2-phosphorylation in three human BCR-ABL1-positive pre-B leukemia cell lines and the murine pro-B cell line TON-B210 by western blot
- Co-immunoprecipitation of BCR-ABL1 and BTK
- Generation of a retroviral expression-vector encoding BTK^{p52}
- Co-transfection of the embryonic kidney cell line 293T with retroviral expression vectors encoding GFP, BCR-ABL1, full-length BTK, BTK^{p52} or BTK-SH3
- Analysis of the phosphorylation status of PLCγ1 and STAT5 in differentially co-transfected 293T cells

Chapter 5.5: **Deficiency of Bruton's tyrosine kinase in B cell precursor leukemia cells**

By **Feldhahn N**, Río P, Ndikung Bejeng Soh B, Liedtke S, Sprangers M, Klein F, Wernet P, Jumaa H, Hofmann WK, Hanenberg H, Rowley JD, Müschen M. *Proc Natl Acad Sci USA.* 2005; 102: 13266-13271

- RT-PCR analysis of BTK expression in BCR-ABL1-positive and –negative pre-B leukemia cells
- Detection of BTK tyrosine-phosphorylation in 13 pre-B leukemia cell lines

- Ca^{2+} flux analysis of a BCR-ABL1-negative pre-B cell line transfected with a retroviral expression vector encoding GFP or BTK-ΔK
- FACS-analysis of STI571-induced differentiation of a BCR-ABL1-positive pre-B leukemia cell line expressing GFP or BTK-ΔK
- Reconstitution of BTK expression in three BTK-deficient pre-B cell lines
- γ-irradiation of a BTK-deficient pre-B cell leukemia cell line and subsequent sorting of pre-apoptotic and non-apoptotic cells using FACS
- Analysis of BTK isoform expression by RT-PCR and sequence analysis in the γ-irradiated BTK-deficient pre-B cell leukemia cell line

Chapter 5.6: **Activation-induced cytidine deaminase acts as a mutator in BCR-ABL1-induced pre-B lymphoblastic leukemia and lymphoid blast crisis of chronic myeloid leukemia**

By **Feldhahn N**, Henke N, Soh BNB, Klein F, Mooster JL, Sprangers M, Jumaa H, Hofmann W, Greeve J, Li A, Rowley JD, Müschen M. submitted for publication 2005

- Sequence and mutation analysis of the rearranged IGH V region genes of BCR-ABL1-positive pre-B leukemia cell lines
- Analysis of *AID* mRNA expression by RT-PCR in FACS-sorted normal human B cell subsets
- Western blot of AID and EIF4e in BCR-ABL1-positive pre-B leukemia cells
- FACS-sort of B lymphoid and T lymphoid cells within the CML cell line CML-T1
- Silencing of AID in three BCR-ABL1-positive pre-B leukemia cell lines using RNA-interference

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Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe keine erfolglosen Promotionsversuche unternommen.

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