The role of SLP65 in malignant transformation of human B cells

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Chapter 1

Introduction

B cells in immune responses

Host organisms are protected against diseases caused by pathogens like bacteria and viruses by the immune system. Many different cell types are involved in eliminating these pathogens. A primary defence line against microorganisms is the non-specific, innate, immune system. It consists of external barriers against infection like the skin and mucous membranes. But foreign invaders are also destroyed by natural killer cells, macrophages, granulocytes and the complement system (Metchnikoff, 1893). The specific, acquired, immune system has two additional features. First, it can distinguish different antigens, which is mediated by membrane receptors that have an extremely large variability of structures of antigen-binding sites (Burnet, 1959). Its second feature is its immunological memory. An increasingly effective immune response upon several encounters with the same pathogen is the result. In addition, the cells that are needed are directed to the sites of foreign antigen entry (Burnet, 1959).

The specific arm of the immune system consists of T and B lymphocytes. T lymphocytes mature in the thymus. They recognize foreign organisms in an indirect manner by recognizing short peptide fragments that are derived from proteins degraded inside antigen presenting cells. A major histocompatibility complex presents these antigens at the cell surface. B lymphocytes mature in the bone marrow and are activated by binding of antigens to their antigen receptors, called immunoglobulin (Ig) molecules or B cell receptors (BCR) (Ehrlich, 1892). After activation it receives signals to differentiate into a plasma cell and synthesize the secreted form of its BCR, the antibody molecule (Jerne, 1955). Antigens derived from the foreign organisms will specifically bind these antibodies and are eliminated.

The B cell receptor

The antibody molecule is made out of two identical heavy and two identical light chains bound together by interchain disulfide bonds. Both chains have a constant (C) and a variable region (V). The C region is involved in membrane expression, complement binding and binding to other cells in the immune system. The V region recognizes and binds antigens. Three hypervariable complementary determining regions (CDR) are present in the V region and contribute to the antibody specificity. A large repertoire of specificities is important as one specific antibody can only recognize one specific antigenic structure. The V region consists of several V (variable), D (diversity) and J (joining) regions (Ravetch et al., 1981, Matsuda et al., 1998). Different recombinations of these V, D and J segments are made to

produce different sequences and achieve a broad variation. Further diversity results from the generation of palindromic sequences (P-elements) and the insertion of nucleotides at the N region between the V, D and J segments (Alt and Baltimore, 1982).

V(D)J recombination

Clusters of genes on three different chromosomes code for heavy chains (chromosome 14), κ light chains (chromosome 2) and λ light chains (chromosome 22). For the variable region of the heavy chain the V, D and J gene segments are joined. For the variable region of the light chain V and J gene segments are joined. These gene rearrangements occur in an ordered fashion by a process called V(D)J rearrangement (Ravetch et al., 1981). First, a D_H segment is rearranged to a J_H segment. This is followed by a V_H to DJ_H joining, which can either be in frame (i.e. in the correct reading frame encoding the antibody sequences) or out of frame. If the rearrangement was not successful, a second attempt can be made on the other heavy chain allele. After a successful rearrangement of the heavy chain the cell can proceed with light chain gene rearrangements (Grawunder et al., 1995).

The V(D)J recombination is initiated by the V(D)J recombinase, composed of RAG1 and RAG2 (Oettinger et al., 1990). RAG1 and RAG2 together are both necessary to catalyze the cleavage of the gene segments (Fugmann et al., 2000; McBlane et al., 1995). They recognize and bind a pair of complementary recombination signal sequences (RSSs), which are each composed of conserved heptamer and nonamer sequences separated by a spacer region of 12 bp or 23 bp (Figure 1). This ensures recombination of the correct coding sequences as the V and J segments have RSS with a 23-bp spacer and the D segments have 12 bp spacers on both ends: V(D)J joining takes place by the 12/23 spacer rule in which joining only occurs between segments with RSS of different spacer lengths (Tonegawa, 1983). RAG1 and RAG2 cleave exactly between the RSS and the coding sequences of the two rearranging gene segments, leaving two blunt RSS signal ends and two covalently sealed hairpin coding ends (Ramsden et al., 1996; Figure 1). The signal ends are precisely joined, releasing the intervening DNA as a DNA excision circle. The hairpin structures of the coding ends are opened by Artemis and the two ends are joined by nonhomologous end-joining (NHEJ) factors (Bassing et al., 2002). During opening of the hairpins palindromic (P) nucleotides may be added but it also often nucleotides are removed from the coding ends by exonucleolytic digestion. Before joining usually non-germline nucleotides (N-sequences) are added by the lymphocyte specific terminal nucleotidyltransferase (TdT), further increasing the diversity of V region genes (Alt and Baltimore, 1982). In addition to the RAG proteins, other proteins are

involved in non-homologous end joining (Bassing et al., 2002), including DNA-PK (which is composed of the DNA-PK catalytic subunit and the Ku70 and Ku80 proteins), DNA ligase IV and XRCC4 (Fugmann et al, 2000).

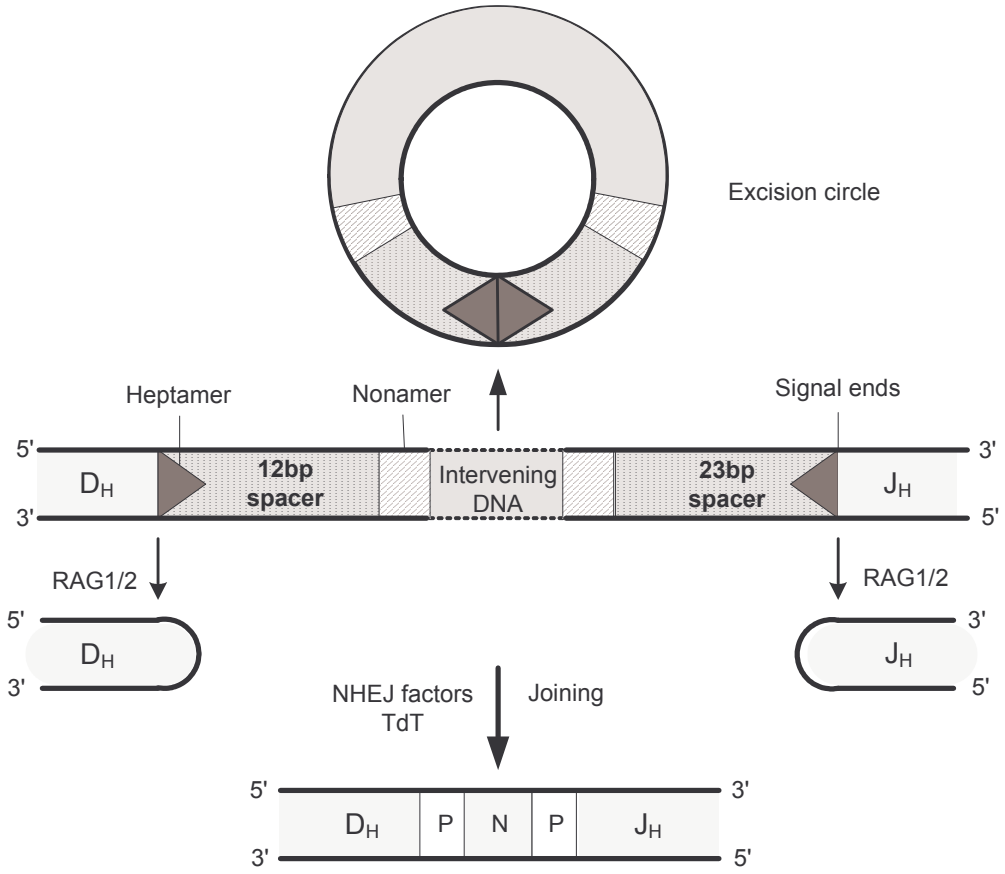

Figure 1: *Joining of V, D and J gene segments*
The RAG 1 and RAG2 enzymes cleave the DNA at the coding ends of the recombination signal sequences (RSS), consisting of a heptamer, nonamer and a spacer, flanking the rearranging gene segments (here a D_H and J_H segment). At the coding joints phosphorylated hairpin structures are generated, whereas the signal joints are blunt end ligated. Upon opening of the hairpins, nucleotides may be removed from the DNA ends, or non-germline encoded nucleotides (N) may be added before the gene segments are joined. The intervening DNA is released as a episomal excision circle. (Based on Roitt and Delves, 2001).

V_H replacement

To increase the specificity of a rearranged immunoglobulin gene or to prevent two non-functional rearrangements, a B cell may undergo a secondary rearrangement on the same chromosome (Nemazee et al., 2003). Most rearranged light-chain genes carry both unrearranged V_L gene segments upstream and unrearranged J_L segments downstream of a V_L-J_L rearrangement. So for light chains it is possible to form a new V_LJ_L rearrangement, thereby deleting the original rearrangement. This is different at the heavy chain locus. This locus

contains only one rearranged D_H segment and all unused D_H segments are deleted during the D_H-J_H and V_H-DJ_H rearrangements. However, initial V(D)J_H-rearrangements can be changed by replacing the previous V_H segment for a new V_H segment. This was reported first in a mouse pre-B cell-line carrying non-functional IgH rearrangements which achieved a functional IgH gene by secondary rearrangement (Reth et al., 1986). Because the D_H segment of the rearranged VDJ_H exon no longer has its own 5' RSS, a cryptic RSS within the V_H segment has to be used (Kleinfield et al., 1989). The consensus sequence of the heptamer on the 5' end of the D_H segment is CACTGTG. Close to the 3' end of the V_H segment is the sequence TACTGTG which only differs with 1 bp from the consensus heptamer and can act as a surrogate for the original RSS on the 5' of the D_H segment (Covey et al., 1990). Recombination takes place after cleavage at the conventional RSS of a new (upstream) V_H segment and at the 3' cryptic RSS in the V_H part of the recombined V(D)J_H. This leaves a small footprint of the previously rearranged V_H segment into the newly rearranged V(D)J_H (Figure 2).

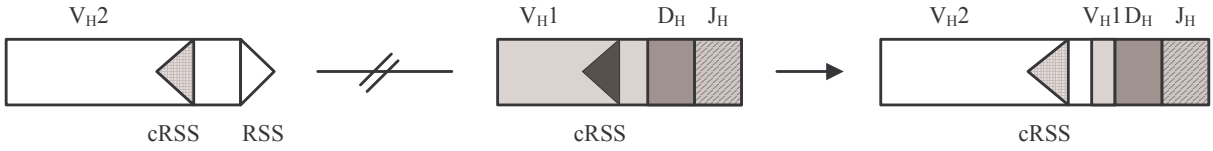

Figure 2: *V_H replacement*
Recombination of the RSS of the new V_H gene with the cryptic RSS (cRSS) of the rearranged V_H gene leaving a footprint of V_H1 (red) in the new V(D)J_H rearrangement.

B cell development

The development of B cells takes place in the bone marrow and in secondary lymphoid organs. The early B cell development in the bone marrow can be divided into several stages according to the sequential rearrangements of the immunoglobulin gene loci and the differential expression of various transcription factors (Figure 3; Miosge et al., 2005). After production of a functional BCR on their surface, the B cells migrate to the secondary lymphoid organs (Rolink et al., 1998). As fully differentiated memory B cells, they circulate through the body until they encounter antigens they can respond to. After recognizing and binding to antigen, B cells may differentiate into antibody producing plasma cells or into memory B cells (Smith et al., 1996).

B cell development represents an ongoing process throughout life. The percentage of B cell precursors in the total lymphoid cell pool is much higher in fetal bone marrow than in adult bone marrow (Brashem et al., 1982). Adult bone marrow also differs from fetal bone marrow in that there are recirculating mature B cells present (Nuñez et al., 1996). RT-PCR shows that there are similar levels of the recombinase activating genes RAG1 and RAG2 and terminal deoxynucleotidyl transferase TdT (genes important for the rearrangement of the immunoglobulin gene) present in pro-B cells from 18-week old fetal bone marrow and 62-year old adult bone marrow (Nuñez et al., 1996).

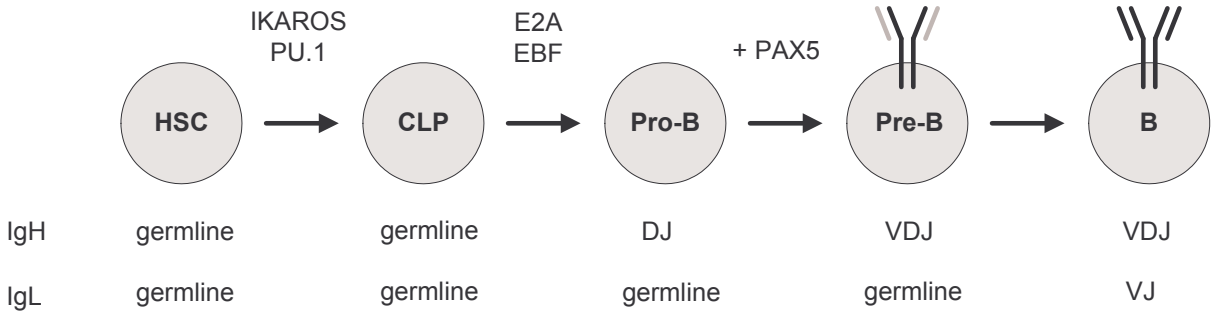

Figure 3: *Early B cell development*

Schematic overview of early B cell development. Hematopoietic stem cells (HSC) can differentiate into a common lymphoid progenitor (CLP) under the influence of IKAROS and PU.1. Further differentiation into the B lymphoid lineage requires the E2A transcription factor together with the early B cell factor EBF. For the transition to the pre-B cell stage and further stages of differentiation, PAX5 is needed as well. During early B cell development, a series of somatic rearrangements at the Ig loci take place. At the transition to the pre-B cell stage, the rearrangement of the D_H to the J_H gene segment take place. This is followed by further rearrangement of the V_H to the DJ_H gene segment at the pre-B cell stage. The Ig μ heavy chain of pre-B cells is expressed together with a surrogate light chain (grey). From the immature B cell stage on, the cells express a conventional κ or λ light chain in combination with their heavy chain.

Transcription factors

Various transcription factors have been shown to be important during early B-cell development including PU.1 (deKoter et al., 2000), IKAROS (Liberg et al., 2003), E2A (O'Riordan et al., 1999), EBF (O'Riordan et al., 1999) and Pax5 (Nutt et al., 1999) (Figure 1). At the beginning of B cell development, the transcription factors E2A and EBF activate the B cell specific gene expression program (O'Riordan et al., 1999). Subsequently, PAX5 activation commits B cell progenitors to the B cell lineage by repressing the transcription of lineage-inappropriate genes and activating B cell lineage-specific genes (Nutt et al., 1999).

Many functions in the B cell lineage have been established by phenotypic analysis of mice with targeted null alleles of genes encoding these transcription factors. Mice fully

defective in the *PU.1* gene exhibit a general defect in the development of myeloid and lymphoid cells, but do develop erythrocytes, megakaryocytes and platelets (Singh, 1996). Low PU.1 expression specifically promotes B lymphoid development, whereas high PU.1 expression promotes the formation of macrophages, members of the myeloid cell lineage (de Koter et al., 2000). This could indicate that PU.1 expression critically controls the decision between myelopoiesis and lymphopoiesis.

The decision to enter T and B lymphoid development appears to be controlled by the *IKAROS* gene (Georgopoulos et al., 1994). Its N-terminal domain is involved in the DNA binding of the protein, while the C-terminus is essential for its dimerization. Several isoforms of IKAROS are expressed due to alternative splicing. Long isoforms with at least three zinc fingers can efficiently bind DNA, whereas short isoforms with less than three zinc fingers dimerize with each other and act as dominant-negative isoforms (Sun et al., 1996). IKAROS is expressed in all hemopoietic progenitors and highest levels were found in mature thymocytes, T, B and NK cells (Georgopoulos et al., 1997). The role of IKAROS in lymphoid development is shown by a complete lack of lymphoid cells following *IKAROS* gene inactivation in mice (Georgopoulos et al., 1994). In contrast, *IKAROS* null mice generate T cells a few weeks after birth but B cells remained absent throughout life and myeloid development was also perturbed (Wang et al., 1996).

The decision to enter the B lymphoid pathway is controlled by two transcription factors: the basic helix-loop-helix protein E2A and the early B cell factor (EBF). In the absence of one of them, B cell development is arrested before the start of D_H-J_H rearrangement of the Ig heavy chain (Bain et al., 1994 and Lin et al., 1995)

The transcription factors E2A and EBF are not sufficient to drive the B cells into the pre-BCR and BCR stages of differentiation. In the absence of PAX5, B cell development is arrested at the pro-B cell stage at which the cells carry a DJ_H gene rearrangement but are blocked from V_H to DJ_H rearrangements (Urbanek et al., 1994) showing that PAX5 is needed for progression beyond the pro-B cell stage. After PAX5 activation it stays expressed in all subsequent stages of B cell differentiation, from pre-B to mature B cells but not to plasma cells (Urbanek et al., 1994).

Common lymphoid progenitors

Lymphoid lineages within the bone marrow and the thymus develop from hematopoietic stem cells. The common lymphoid progenitor (CLP) is defined as a progenitor which can develop into T, B, natural killer (NK) or lymphoid dendritic cells (DC), but it can

not develop into nonlymphoid lineages like myeloid and erythroid cells (Galy *et al*., 1995). CLP have best been characterized as murine bone marrow cells which express interleukin 7 receptor α chain (IL7Rα), and the stem cell markers $Sca1^{lo}$ and $c\text{-}Kit^{lo}$ on the surface, while Thy 1 and markers of mature blood cell lineages (Lin) are absent (Kondo et al., 1997). Survival of lymphoid stem cells and their subsequent development into B lymphocytes is dependent on attachment to and secretion of growth factors by bone marrow stromal cells and is characterized by the expression of certain transcription factors (Rolink et al., 2000).

Pro-B cells

Human pro-B cells are a well-characterized population expressing CD10, CD34 and CD19 (Loken et al., 1987). CD19 is the earliest B cell surface marker and is expressed until the plasma cell stage of development (Poe et al., 2001). VDJ recombination starts in pro-B cells with the rearrangement of the heavy chain gene by joining of the D_H and J_H gene segments (Ravetch et al., 1981). Early pro-B cells typically undergo DJ_H rearrangement before beginning V_H-DJ_H rearrangements. After the initial DJ_H rearrangement in a pro-B cell, pro-B cells proliferate with the potential to rearrange different V_H gene segments to the original DJ_H (Li et al., 1993). If a V_H-DJ_H rearrangement on one allele is productive (correct reading frame), the resulting μ heavy chain is expressed on the cell surface in complex with the surrogate light chain and the Ig-α/ CD79a and Ig-β/ CD79b signaling chains to form the pre-BCR complex (Martensson et al., 2002). Functional V_HDJ_H rearrangement is essential for normal pro-B cell differentiation into the pre-B compartment. If the rearrangement on the first allele is not functional, V_H-DJ_H rearrangement is attempted on the second allele. If this rearrangement is not productive either, the pro-B cell dies (Lewis, 1994).

Pre-B cells

Differentiation into pre-B cells is characterized by loss of CD34 expression and surface expression of μ heavy chains along with non-rearranging λ5 and V_{preB} surrogate light chain components and the Igα/Igβ signal transducing heterodimer to form the pre-BCR. As soon as the pre-BCR is expressed, the expression of λ5 and V_{preB} is turned off (Grawunder et al., 1995). The surrogate light chain functions as a chaperone to facilitate pre-BCR assembly and expression (LeBien, 2000). Signaling initiated via the pre-BCR induces clonal expansion of $Ig\mu^+$ pre-B cells where after VpreB and λ5 transcription is silenced to limit this expansion (Parker et al., 2005). In these cells, the recombination-activating genes *RAG1* and *RAG2* and

TdT are temporarily down-regulated to terminate further Ig heavy chain rearrangements, assuring allelic exclusion (Grawunder et al., 1995). This rearrangement machinery will be reactivated in the immature B cells for rearrangements at the κ and λ Ig light chain loci. After a limited number of cell divisions, the pre-B cells stop cycling and the light chain genes attempt V_L-J_L rearrangements (Lu et al., 2003). Functional light chain rearrangement in pre-B cells leads to the expression of the BCR and the transition to immature B cells.

Immature B cells

Differentiation into IgM^+ immature B cells is dependent on a productive rearrangement of the Ig light chain at the Ig kappa (*IGK*) and Ig lambda (*IGL*) loci. The recombination machinery is activated again and the B cell progenitor rearranges κ or λ genes where *IGK* rearrangement takes place first (Klein et al., 2005). When the rearrangement is successful and the light chain can pair with the μ heavy chain and the resulting BCR is not autoreactive, the cells are transferred to the immature B cell compartment. If a productive light chain is not created in these initial attempts, the cell may be rescued by attempting further rearrangements of one or more κ or λ V region genes, a process termed receptor editing (Radic et al., 1996). If the cell fails to generate a functional light chain by these secondary rearrangements, the cell dies by apoptosis (Rolink et al., 1999).

Between 10 and 20% of the immature B cells produced in the bone marrow, migrate to the spleen (Rolink et al., 1998). Immature B cells, which fail to enter lymphoid follicles have a half-life of about 3 days and probably die by apoptosis (Rolink et al., 1998). Immature B cells which successfully enter follicles mature into naïve B cells and typically express IgM and IgD on the surface.

B1 cells

The B1 phenotype in mice is characterized by high expression levels of surface IgM, low surface IgD and B220 (Abrahao et al., 2003). Many B1 cells can also express CD5 (Berland and Wortis, 2002). Although B1 cells can shift to a B2 phenotype, and possibly vice versa, there is a minimal conversion between the two lineages under normal circumstances (Stall et al., 1996). B1 cells are produced in the fetus in the bone marrow, the liver and other gut-associated regions. They can maintain their populations by self-renewal and limit their *de novo* production from progenitors by feedback regulation (Herzenberg, 2000). B1 cells express the BCR and often react with a variety of autoantigens as well as with foreign

antigens like bacteria and parasites (Hayakawa et al., 1999). Furthermore, B1 cells are in a lowly activated state in which they do not divide in response to foreign antigens, as proliferating B cells do in germinal centers. In this way they can escape from the short half-life of a previously immature B cell (Potter and Melchers, 2000). This unresponsiveness to BCR-mediated signaling is regulated by negative signals from CD5, CD22 and CD72 co-receptors (Ochi and Watanabe, 2000). A major factor influencing self-renewal could be the constitutive production of IL10 promoted by CD5 (Gary-Gouy et al., 2002).

Mature B cells

In the secondary lymphoid organs (spleen, lymph nodes, tonsils, and Peyer's patches) the mature naïve B cells will be activated by antigen-specific binding. If the naïve B cell is activated by antigen and it receives appropriate T_H cell help, the B cell becomes a lymphoblast which divides and secretes IgM. The lymphoblasts' progeny undergoes class-switching and IgG, IgE or IgA secretion. Most of these cells become plasma cells which secrete large amounts of IgG, IgE or IgA and are short-lived (Smith et al., 1996). A few cells become long-lived memory B cells which have IgG, IgE or IgA as receptors on their surface (Manz et al., 1997).

Newly formed B lymphocytes enter the spleen via the blood. There they migrate to the B cell area in the follicle or the marginal zone (MZ) in the white pulp (Brelinska et al., 1982). The MZ contains a unique population of resting marginal zone B cells which do not circulate. The peripheral lymphoid tissues are organized with different T and B lymphocyte compartments. Marginal zone B cells that manage to migrate into follicles become part of the long-lived mature B cell pool (Manz et al., 1997). They migrate from the follicles into the lymphatic vessels and so return to the circulation. The lymphatic vessels also carry antigens from the site of infection to the lymph nodes. Within the lymph nodes, the B cells are located in the outer cortex and the T cells separately in the paracortical area. In the outer cortex, secondary follicles are present and some of them contain areas called germinal centers (Camacho et al., 1998). After secondary antigen challenge, naïve B-cells enter the dark zone of the germinal center as centroblasts which rapidly divide. They divide in response to strong stimuli from complexes on follicular dendritic cells and from cytokines (IL4) released by T-cells. Clonal expansion, isotype switching and somatic hypermutation take place in the dark zone centroblasts (Liu et al., 1997). Thereafter, the centroblasts transform in the nondividing centrocytes, which are vulnerable and die unless rescued by association with antigen on a follicular dendritic cell (Mac Lennan, 1998). Cells either migrate to the sites of plasma cell

activity (lymph node medulla) (Smith et al., 1996) or expand the memory B cell pool depending upon expressed cytokines (Manz et al., 1997). CD40 engagement by CD40 ligand expressed by T cells also leads the B cell into the memory compartment (Arpin et al., 1995). Memory B cells can be found in the marginal zone, tonsils and Payer's Patches (Laichalk et al., 2002).

(Pre-) B cell receptor signaling

One important checkpoint of B cell development is the expression of a pre-BCR, which is essential for the selection and expansion of pre-B cells (Hess et al., 2001). The pre-BCR is transiently expressed on the cell surface when a productively rearranged Ig heavy V region gene generates a membrane-bound μ chain, which can associate with a (surrogate) light chain. Engagement of the pre-BCR or BCR results in the activation of three different families of protein tyrosine kinases (PTKs) for its initial signaling. These are the SRC (cellular homolog of the transforming gene of the Rous sarcoma virus) family kinases, the SYK (spleen tyrosine kinase) family kinases and the TEC family kinases (DeFranco, 1997). Deficiency in one of these proteins results in defective B cell function and development (Campbell, 1999). The phosphorylation events that these PTKs perform, change catalytic activity of effector molecules and mediate protein-protein interactions that bring together the signal transduction molecules. Another group of cellular proteins, the adaptor proteins, play a role in coordinating the signaling events (Flemming et al., 2003; Ishiai et al., 1999). These proteins lack enzymatic activity but possess structural domains that mediate intermolecular protein-protein and protein-lipid interactions (Kurosaki, 2002). After activation of the BCR, all these signaling molecules accumulate in the glycolipid-enriched membrane domains (GEMs), the so-called lipid rafts (Katagiri et al., 2001).

The BCR is non-covalently associated with the disulfide-linked heterodimer of Igα and Igβ. Igα and Igβ each contain a single immunoreceptor tyrosine-based activation motif (ITAM) within their cytoplasmic tail that initiates signal transduction following BCR aggregation (Flaswinkel and Reth, 1994). The ITAM is a conserved motif composed of two precisely spaced tyrosine residues with a consensus sequence that has specific binding sites for SH2 domain-containing effector molecules (Reth, 1989). The BCR moves into the lipid rafts upon BCR-activation, where the ITAM tyrosine residues are phosphorylated (Cheng et al., 1999) by src-family kinases, like Lyn, Fyn, Blk or Lck (Johnson et al., 1995). Although Lyn is not essential for the initiation of BCR signaling, it is the primary src-kinase used in

BCR-signaling (Gauld and Cambier, 2004). The doubly phosphorylation of Igα/ Igβ results in the binding and the activation of the kinase Syk. Syk-deficient B cells have a defect in BCR-mediated activation of downstream signaling pathways (Takata et al., 1994). Thus, Syk is essential to couple the BCR to distal signaling molecules. This coupling takes place via phosphorylation and interaction with the adaptor molecule SLP65 (also known as BLNK or BASH) (Goitsuka et al., 1998; Wienands et al., 1998). SLP65 is rapidly phosphorylated by Syk and serves as a primary docking site for PLCγ2, as well as other effector and adaptor molecules. So has SLP65 been shown to associate with the SH2 domain of BTK, a TEC family protein tyrosine kinase. Dual phosphorylation of PLCγ2 by Syk and BTK is required for optimal activation of the lipase (Hashimoto et al., 1999; Ishiai et al., 1999). Activated PLCγ2 cleaves membrane-associated phosphoinositide PI(4,5)P2 into the second messengers I(1,4,5)P3 and DAG. I(1,4,5)P3 generation causes the mobilization of Ca^{2+} from intra and extracellular stores. Elevated Ca^{2+} levels are required for the activation of transcription factors such as NF-κB and NF-AT by atypical PKCs and Ca^{2+}-calmodulin, respectively (Dolmetsch et al., 1997; Saijo et al., 2002). DAG represents a classical activator of protein kinase C isoforms which regulate the MAPK family.

Although the mature BCR initiates signaling through antigen stimulation, it is not completely clear how the pre-BCR starts its signaling cascade. There are reports that show that interaction with a stroma cell ligands (heparin sulfate and galectin-1) triggers pre-BCR activity (Bradl et al., 2001; Gauthier et al., 2002). However, other experiments indicate that pre-BCR signaling does not need a ligand but depends on the presence of the λ5 protein. Two models were proposed: either the pre-BCR serves as its own ligand, whereby neighbouring pre-BCRs directly interact with each other; or pre-B cells express a molecule on their cell surface that has binding sites for the non-Ig-like unique region of λ5 (Ohnishi et al., 2003).

The adaptor protein SLP65

The adaptor proteins regulate the interaction of effector molecules with the BCR and their targets (Leo et al., 2002). One of these adaptor proteins is SLP65. It is a 65 kDa protein consisting of a N-terminal tyrosine-rich-domain, a proline-rich domain and a C-terminal SH2 domain (Figure 4). A highly conserved leucine zipper in its N terminus is responsible for its membrane association (Köhler et al., 2005). With its SH2 domain, it binds the phosphorylated Igα tyrosine 204, which is located outside of the ITAM motif (Engels et al., 2001; Kabak et al., 2002). Recently HPK1 (hematopoietic progenitor kinase 1) has been identified as a

molecule that binds the SLP65 SH2-domain (Sauer et al., 2001; Tsuji et al., 2001). HPK1 was found to be activated upon antigen receptor stimulation (Liou et al., 2000) and its activation results in NK-kB activation (Arnold et al., 2001). BNAS2 (BASH N terminal associated protein 2) has been found to bind the SLP65 N-terminus (Imamura et al., 2004). BNAS2 has also been found to co-immunoprecipitate with BTK and ERK2 and may facilitate ERK activation by signaling from cell-surface receptors (Imamura et al., 2004). Furthermore, SLP65 has at its N-terminal binding domains for the SH2 domain of other signaling molecules including BTK (Y96), PLCγ2 (Y84, Y178, Y189), Vav and Nck (Y72), and the SH3 domain of Grb2 binds at the proline-rich domain (Chiu et al., 2002). In normal B cells, two SLP65 isoforms are found: full-length and a variant lacking exon 8 coding for a part of the proline-rich domain and previously designated as BLNK-S (Fu et al., 1998).

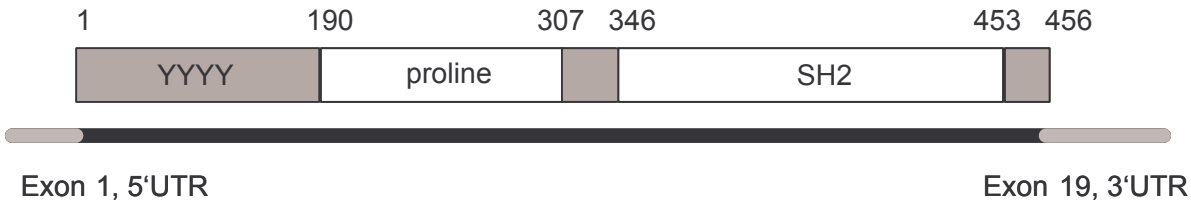

Figure 4: *SLP65 structure*
SLP65 consists of a tyrosine-rich domain (YY) from amino acid 1 to 190 at the N terminal followed by a proline-rich domain from amino acid 190 to 307. At the C-terminal SLP65 consists of an SH2 domain from amino acid 346 to 453.

SLP65-deficient mice show a block at the pro-B to the pre-B cell stage of development (Jumaa et al., 1999; Pappu et al., 1999). Evaluating a patient without mature B cells, defective splicing of SLP65 was identified. Normal numbers of pro-B cells but no pre-B or mature B cells were detected in this patient, indicating that SLP65 plays a role in the pro-B to pre-B cell transition (Minegishi et al., 1999). That the block in early B cell development in SLP65 deficient mice is incomplete, suggested that other signaling molecules could partly compensate for SLP65-deficiency in B cells (Jumaa et al., 1999). LAT and SLP76 are two analogues of SLP65 in T cells. As SLP76 expression was also reported in pro-B cells, it could be possible that SLP76 was involved in compensating SLP65-deficiency (Nagata et al., 1997). SLP76, however, requires co-expression of LAT to reconstitute BCR function in $SLP65^{-/-}$ DT40 chicken B cells (Wong et al., 2000) and in $SLP65^{-/-}$ mice (Su et al., 2003). However, when SLP65 was expressed in SLP76-deficient Jurkat T cells, SLP65 could not rescue TCR-mediated signaling (Wong et al., 2000). It also was suggested that CD19 could partly compensate SLP65 deficiency as CD19/SLP65 double mutant mice showed a complete block

in B cell development at the pre-B cell stage (Hayashi et al., 2003). Other potential candidates are the adaptor molecules LAB (Brdicka et al., 2002; Janssen et al., 2003) and Bam32 (Niiro et al., 2002), but their exact role in BCR signaling is not clear yet.

Importantly, SLP65 is known as a tumor suppressor (Flemming et al., 2003). Unlike wild-type B cells, SLP65-deficient BM-derived B cells show high pre-BCR expression and enhanced proliferation *in vitro*, which correlates with the development of pre-B cell lymphomas *in vivo* (Flemming et al., 2003). Reconstitution of SLP65 expression in a SLP65-deficient pre-B cell line led to enhanced differentiation *in vitro* and prevented the development of pre-B cell leukemia in immune-deficient mice (Jumaa et al., 2003). The murine SLP65-deficient pre-B cell leukemia resembles human childhood pre-B ALL. Indeed many leukemia patients show a loss or drastic reduction of SLP65 expression (Jumaa et al., 2003), although another report shows that SLP65 deficiency is a rare occurrence in childhood B-lineage ALL (Imai et al., 2004). In *BCR-ABL1* positive patients, truncated forms of SLP65 are found which disappear after treatment with the ABL1-kinase inhibitor STI571 (Klein et al., 2004).

Src-family kinases

The Src-family protein tyrosine kinases (SFKs: Lyn, Fyn, Blk, Hck, Fgr and Lck) are about 60 kDa big and have a common domain structure. Every src-kinase consists of six functional domains (Tatosyan et al., 2000): an N-terminal SH4 domain (with acylation sites) followed by a 'unique' domain, an SH3, SH2, a catalytic (or SH1) domain, followed by a C-terminal region. The SH4 domain plays a role in the localization to the cell membrane. The SH3 and SH2 domains play key roles in regulating the catalytic activity of the SFKs. The SH3 domain binds proline-rich domains necessary for binding to intracellular substrates. The SH2 domain also binds intracellular substrates but has a preference for certain phosphotyrosine containing motifs including the ITAM motifs of Igα/β (Tatosyan et al., 2000). SFK activation is regulated by the intramolecular associations between SH3 and SH2 domains (Xu et al., 1999). In its resting, inactive state, the SFK molecules are kept in a closed conformation by binding of the C-terminal phospho-Y527 to the SH2 domain. Y527 is phosphorylated by the protein Csk which promotes its inactivity (Okada et al., 1991). Dephosphorylation of this tyrosine residue is needed for activation of SFKs and is believed to be performed by the protein tyrosine phosphatase CD45. Following dephosphorylation, the SFK is unfolded and Y416 is exposed. Phosphorylation of Y416 is required for full activity (Cahir McFarland et al., 1993).

Activation of the BCR induces various signaling cascades. An important step in this process is the phosphorylation of the ITAM motifs of the cytoplasmic tails of Igα/β. This phosphorylation is secondary to the activation of SFKs like Lyn, the major SFK in B cell signaling. In resting B cells, there is a weak association of Lyn with the BCR by an interaction between the N-terminus of Lyn and the nonphosphorylated ITAM of Igα (Yamanashi et al., 1991). A continuously changing balance between inactive and partially active states of Lyn in resting B cells is due to opposing effects of Csk (phosphorylation) and CD45 (dephosphorylation) on the C-terminal Y508. Although Lyn is known to phosphorylate directly both Syk and Btk, studies show that Lyn-deficient B cells are hyperresponsive to BCR stimulation (Chan et al., 1997). This can be explained by the role Lyn plays in the phosphorylation of both CD19 and CD22. Lyn is known to be an important component in the phosphorylation of inhibitory, ITIM-containing co-receptors. One of these inhibitory BCR coreceptors is CD22 (Nishizumi et al., 1998).CD22 recruits the phosphatase SHP1 which activation leads to negative signaling (Otipoby et al., 2001). The activation of Lyn can amplify BCR signal strength by the phosphorylation of tyrosine residues on the cytoplasmic tail of CD19 (Roifman et al., 1993). It has been suggested that Lyn and CD19 are involved in a 'progressive amplification loop' in which Lyn is essential for CD19 phosphorylation and CD19 then acts as a site for further Lyn recruitment and activation (Fujimoto et al., 2001). It is thought that CD19 and CD22 oppose each other in the regulation of signal strength.

B cell leukemia and lymphoma

Human leukemias are malignancies derived from hematopoietic progenitors developing in the lymphoid or myeloid pathway. Both groups can be divided into acute and chronic leukemia. A frequent mechanism in leukemic transformation involves chromosomal rearrangements that place a proto-oncogene next to an immunoglobulin locus which results in the deregulated expression of the proto-oncogene. Another mechanism of oncogene activation are chromosomal rearrangements that fuse the coding sequences of two different genes. These fusion genes often encode chimeric oncoproteins that act as constitutively active tyrosine kinases or as novel transcription factors to activate transcriptional programs that are oncogenic (Look, 1997). Of note, chromosomal translocations in mature B cell lymphoma cells, result in transcriptional deregulation in almost all cases. In contrast, most chromosomal rearrangements in the leukemia cells result in the expression of chimeric proteins (Look, 1997).

The most common translocation found in childhood acute lymphoblastic leukemia (ALL) is t(12;21)(p13;q22). This rearrangement leads to the fusion of the oligomerization domain of *TEL* on chromosome 12 to the entire coding region of *AML1* (Golub et al., 1995). *TEL* is a member of the ETS family of sequence-specific transcriptional repressors (Lopez et al., 1999) and is fused with different partners. *AML1* is a transcription factor needed for hematopoiesis in mammals and is also involved in the pathogenesis of myeloid leukemias (Roumier et al., 2003). The exact role of TEL-AML1 remains unclear but TEL-AML1 forms dimers with itself and with normal TEL protein (McLean et al., 1996).

The most common translocation found in adult leukemia is t(9;22)(q34;q11) leading to a *BCR-ABL1* fusion gene, encodes a chimeric tyrosine kinase oncoprotein (Gordon, 1999). The 5' part of the *BCR* gene on chromosome 22 is fused with the 3' part of the *ABL1* gene on chromosome 9, also known as the Philadelphia (Ph) chromosome (Pui et al., 2004). The *BCR-ABL1* rearrangement is found in almost all cases of chronic myeloid leukemia (CML) but also in about 5% of children and 30% of adults with B-precursor ALL (Shepherd et al., 1995; Devaraj et al., 1995). The breakpoints of the chromosome 22 *BCR* gene differ in CML and ALL. This results in a larger transcript encoding a 210 kDa protein expressed in 95% of CML and 40% of ALL and a smaller transcript of 190 kDa is expressed in 60% of ALL (Clark et al., 1988).

Aims of the thesis

B cells and their precursors critically depend on the expression and survival signaling capacity of a functional (pre-) B cell receptor. Therefore it is noteworthy that B cell lineage leukemia and lymphoma cells frequently exhibit compromised antigen receptor signal transduction. For example, pre-B acute lymphoblastic leukemia cells carrying a *BCR-ABL1* gene rearrangement are not responsive to pre-B cell receptor engagement (Klein et al., 2004). Likewise, the Epstein-Barr virus (EBV)-encoded oncoprotein LMP2A can mimic B cell receptor signals (Dykstra et al., 2001), thus allowing EBV-infected lymphoma cells to survive in the absence of functional immunoglobulin V region gene rearrangements (Bräuninger et al., 2001). B cell receptor signaling can also be compromised by defects of one or more components of the B cell receptor signaling cascade. In mice, the (pre-) B cell receptor-related linker proteins SLP65 and BTK cooperate as tumor suppressors and limit B cell lymphoproliferation (Kersseboom et al., 2003). Recent work demonstrated that deficiency of SLP65 is a frequent feature in acute lymphoblastic leukemia cells (Jumaa et al., 2003) and that alternative isoforms are present in patients carrying the *BCR-ABL1* translocation (Klein et al., 2004).

Based on these data, the following questions should be addressed:

- What are the consequences of SLP65 deficiency on (pre-) B cell receptor signal transduction in B cell leukemia and lymphoma?
- What is the effect of SLP65-deficiency on differentiation and proliferation capacity?
- What is the function of alternative isoforms of SLP65 in B cell development?
- Is SLP65 involved in the negative feedback regulation of V(D)J-recombination?

References

Abrahao, T.B., Freymuller, E., Mortara, R.A., Lopes, J.D., Mariano, M. (2003) Morphological characterization of mouse B-1 cells. *Immunobiol.* **208**, 401-411

Alt, F.W., Baltimore, D. (1982) Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. *Proc. Natl. Acad. Sci. USA.* **79**, 4118-4122

Arnold, R., Liou, J., Drexler, H.C.A., Weiss, A., Kiefer, F. (2001) Caspase-mediated cleavage of hematopoietic progenitor kinase 1 (HPK1) converts an activator of NFκB into an inhibitor of NFκB. *J. Biol. Chem.* **276**, 14675-14684

Arpin, C., Dechanet, J., Van, K.C., Merville, P., Grouard, G., Briere, F., Bancherau, J., Liu, Y.J. (1995) Generation of memory B cells and plasma cells in vitro. *Science* **268**, 720–722

Bain, G., Maandag, E.C., Izon, D.J., Amsen, D., Kruisbeek, A.M., Weintraub, B.C., Krop, I., Schlissel, M.S., Feeney, A.J., van-Roon, M., van der Valk, M., te Riele, H.P.J., Berns, A., Murre, C. (1994) E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell*. **79**, 885-892

Bassing, C.H., Swat, W., Alt, F.W. (2002) The mechanism and regulation of chromosomal V(D)J recombination. *Cell* **109**, S45-S55

Berland, R., Wortis, H.H. (2002) Origins and functions of B-1 cells with notes on the role of CD5. *Annu. Rev. Immunol.* **20**, 253-262

Bradl, H., Jack, H.M. (2001) Surrogate light chain-mediated interaction of a soluble pre-B cell receptor with adherent cell lines. *J.Immunol.* **167**, 6403-6407

Brashem, C.J., Kersey, J.H., Bollum, F.J., LeBien, T.W. (1982) Ontogenic studies of lymphoid progenitor cells in human bone marrow. *Exp. Hematol.* **10**, 886-892

Bräuninger, A., Spieker, T., Willenbrock, K., Gaulard, P., Wacker, H.H., Rajewsky, K., Hansmann, M.L., Küppers, R. (2001) Survival and clonal expansion of mutating "forbidden" (immunoglobulin receptor-deficient) epstein-barr virus-infected B cells in angioimmunoblastic T cell lymphoma. *J. Exp. Med.* **194**, 927-940

Brdicka, T., Imrich, M., Angelisova, P., Brdickova, N., Horvath, O., Spicka, J., Hilgert, I., Luskova, P., Draber, P., Novak, P., Engels, N., Wienands, J., Simeoni, L., Österreicher, J., Aguado, E., Malissen, M., Schraven, B., Horejsi, V. (2002) Non-T cell avtivation linker (NTAL): a transmembrane adaptor protein involved in immunoreceptor signaling. *J. Exp. Med.* **196**, 1617-1626

Brelinska, R., Pilgrim, C. (1982) The significance of the subcompartments of the marginal zone for directing lymphocyte traffic within the splenic pulp of the rat. *Cell. Tissue. Res.* **226**, 155-165

Burnet, D. (1959) The clonal selection theory of acquired immunity. *Nashville, TN: Vanderbilt Uniniversity Press.*

Cahir McFarland, E.D., Hurley, T.R., Pingel, J.T., Sefton, B.M., Shaw, A., Thomas, M.L. (1993) Correlation between Src family member regulation by the protein-tyrosine-phosphatase CD45 and transmembrane signaling through the T-cell receptor. *Proc. Natl. Acad. Sci. USA.* **90**, 1402-1406

Camacho, S.A., Kosco-Vilbois, M.H., Berek, C. (1998) The dynamic structure of the germinal center. *Immunol. Today.* **19**, 511-514

Campbell, K.S., Signal transduction from the B cell antigen-receptor. *Curr. Opin. Immunol.* **11**, 256-264

Chan, V.W., Meng, F., Soriano, P., DeFranco, A.L. (1997) Characterization of the B lymphocyte populations in Lyn-deficient mice and the role of Lyn in signal initiation and down-regulation. *Immunity.* **7**, 69-81

Cheng, P.C., Dykstra, M.L., Mitchell, R.N., Pierce, S.K. (1999) A role for lipid rafts in B-cell antigen receptor signaling and antigen targeting. *J. Exp. Med.* **190**, 1549-1560

Chiu, C.W., Dalton, M., Ishiai, M., Kurosaki, T., Chan, A. (2002) BLNK: molecular scaffolding through 'cis'-mediated organization of signaling proteins. *EMBO J.* **21**, 6461-6472

Clark, S.S., Mclaughlin, J., Timmons, M., Pendergast, A.M., Ben-Neriah, Y., Dow, L.W., Crist, W., Rovera, G., Smith, S.D., Witte, O.N. (1988) Expression of a distinctive BCR-ABL oncogene in Ph1-positive acute lymphoblastc leukemia (ALL). *Science* **239,** 775-777

Covey, L.R., Ferrier, P., Alt, F.W. (1990) VH to VHDJH rearrangement is mediated by the internal VH heptamer. *Int. Immunol.* **2**, 579-583

DeFranco, A.L. (1997) The complexity of signaling pathways activated by the BCR. *Curr. Opin. Immunol.* **9**, 296-308

DeKoter, R.P., Singh, H. (2000) Regulation of B lymphocyte and macrophage development by graded expression of PU.1 *Science* **288**, 1439-1441

Devaraj, P.E., Foroni, L., Kitra-Roussos, V., Secker-Walker, L.M. (1995) Detection of BCR-ABL and E2A-PBX1 fusion genes by RT-PCR in acute lymphoblastic leukaemia with failed or normal cytogenetics. *Br. J. Haematol.* **89**, 349-355

Dolmetsch, R.E., Lewis, R.S., Goodnow, C.C., Healy, J.I. (1997) Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature* **386**, 855-858

Dykstra, M.L., Longnecker, R., Pierce, S.K. (2001) Epstein-Barr virus coopts lipid rafts to block the signaling and antigen transport functions of the BCR. *Immunity.* **14**, 57-67

Ehrlich, P.Z. (1892) *Hyg. Infektkr.* **12**, 183-203. English translation in *Collected papers of Paul Ehrlich.* **2**, 31-44 (Pergamon, London, 1957)

Engels, N., Wollscheid, B., Wienands, J. (2001) Association of SLP65/BLNK with the B cell antigena receptor through a non-ITAM tyrosine of Ig-α. *Eur. J. Immunol.* **31**, 2126-2134

Flaswinkel, H., Reth, M. (1994) Dual role of the tyrosine activation motif of the Ig-α protein during signal transduction via the B cell antigen receptor. *EMBO* **13(1)**, 83-89

Flemming, A., Brummer, T., Reth, M., Jumaa, H. (2003) The adaptor protein SLP-65 acts as a tumor suppressor that limits pre-B cell expansion. *Nat. Immunol.* **4**, 38-43

Fu, C., Turck, C.W., Kurosaki, A.C., Chan, A.C. (1998) BLNK: a central linker protein in B cell activation. *Immunity.* **9**, 93-103

Fugmann, S.D., Lee, A.I., Shockett, P.E., Villey, I.J., Schatz, D.G. (2000) The RAG proteins and V(D)J recombination: complexes, ends, and transposition. *Annu. Rev. Immunol.* **18**, 495-527

Fujimoto, M., Poe, J.C., Hasegawa, M., Tedder, T.F. (2001) CD19 amplification of B lymphocyte Ca2+ responses: a role for Lyn sequestration in extinguishing negative regulation. *J. Biol. Chem.* **276**, 44820-44827

Galy, A., Travis, M., Cen, D., Chen, B. (1995) Human T, B, natural killer and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity*, **3**, 459-473

Gary-Gouy, H., Harriague, J., Bismuth, G., Platzer, C., Schmitt, C., Dalloul, A.H. (2002) Human CD5 promotes B-cell survival through stimulation of autocrine IL-10 production. *Blood.* **100**, 4537-4543

Gauld, S.B., Cambier, J.C. (2004) Src-family kinases in B-cell development and signaling. *Oncogene* **23**, 8001-8006

Gauthier, L., Rossi, B., Roux, F., Termine, E., Schiff, C. (2002) Galectin-1 is a stromal cell ligand of the pre-B cell receptor (BCR) implicated in synapse formation between pre-B and stromal cells and in pre-BCR triggering. *Proc. Natl. Acad. Sci. USA* **99**, 13014-13019

Georgeopoulos, K., Bigby, M., Wang, J. (1994) The Ikaros gene is required for the development of all lymphoid lineages. *Cell* **79**, 143-156

Georgopoulos, K., Winandy, S., Avitahl, N. (1997) The role of the Ikaros gene in lymphocyte development and homeostasis. *Annu. Rev. Immunol.* **15**, 155-176

Goitsuka, R., Fujimura, Y., Mamada, H., Umeda, A., Morimura, T., Uetsuka, K., Doi, K., Tsuji, S., Kitamura, D. (1998) BASH, a novel signaling molecule preferentially expressed in B cells of the bursa of Fabricius. *J.Immunol.* **161**, 5804-5808

Golub, T.R., Barker, G.F., Bohlander, S.K., Hiebert, S.W., Ward, D.C., Bray-Ward, P., Morgan, E., Raimondi, S.C., Rowley, J.D., Gilliland, D.G. (1995) Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A.* **92**, 4917-4921

Gordon, M.Y. (1999) Biological consequences of the BCR/ABL fusion gene in humans and mice. *J Clin Pathol*. 52, 719-22.

Grawunder, U., Leu, T.M.J., Schatz, D.G., Werner, A., Rolink, A.G., Melchers, F., Winkler, T.H. (1995) Down-regulation of RAG1 and RAG2 gene expression in pre-B cells after functional immunoglobulin heavy chain rearrangement. *Immunity*. **3**, 601-608

Hashimoto, S., Iwamatsu, A., Ishiai, M., Okawa, K., Yamadori, T., Matsushita, M., Baba, Y., Kishimoto, T., Tsukada, S. (1999) Identification of the SH2 domain binding protein of Bruton's tyrosine kinase as BLNK-Functional significance of BTK-SH2 domain in B-cel antigen receptor-coupled calcium signaling. *Blood.* **7**, 2357-2364

Hayakawa, K., Asano, M., Shinton, S.A., Gui, M., Allman, D., Stewart, C.L., silver, J., Hardy, R.R. (1999) Positive selection of natural autoreactive B cells. *Science.* **285**, 113-116

Hayashi, K., Yamamoto, M., Nojima, T., Goitsuka, R., Kitamura, D. (2003) Distinct signaling requirements for Dm selection, IgH allelic exclusion, pre-B cell transition, and tumor suppression in B cell progenitors. *Immunity.* **18**, 825-836

Herzenberg, L.A. (2000) B1 cells, the lineage question revisited. *Immunol. Reviews*, **175**, 9

Hess, J., Werner, A., Wirth, T., Melchers, F., Jack, H.M., winkler, T.H. (2001) Induction of pre-B cell proliferation after de novo synthesis of the pre-B cell receptor. *Proc. Natl. Acad. Sci. UAS.* **98**, 1745-1750

Imai, C., Ross, G., Coustan-Smith, E., Schultz, K.R., Pui, C.H., Downing, J.R., Campana, D. (2004) Expression of the adaptor protein BLNK/SLP-65 in childhood acute lymphoblastic leukemia. *Leukemia.* **18**, 922-925

Imamura, Y., Katahira, T., Kitamura, D. (2004) Identification and characterization of a novel BASH N terminus-associated protein, BNAS2. *J Biol. Chem.* **279**, 26425-26432

Ishiai, M., Kurosaki, M., Pappu, R., Okawa, K., Ronko, I., Fu, C., Shibata, M., Iwamatsu, A., Chan, A.C., Kurosaki, T. (1999) BLNK required for coupling Syk to PLCgamma2 and Rac1-JNK in B cells. *Immunity.* **10**, 117-125

Janssen, E., Zhu, M., Zhang, W., Koonpaew, S., Zhang, W. (2003) LAB: a new membrane-associated adaptor molecule in B cell activation. *Nat. Immunol.* **4**, 117-123

Jerne, N.K. (1955) The natural selection theory of antibody formation. *Proc. Natl. Acad. Sci. USA*. **41**, 849-857

Johnson, S., Pleiman, C.M., Pao, L., Schneringer, J., Hippen, K., Cambier, J.C. (1995) Phosphorylated immunoreceptor signaling motifs (ITAMs) exhibit unique abilities to bind and activate Lyn and Syk tyrosine kinases. *J. Immunol.* **155**, 4596-4603

Jumaa, H., Wollscheid, B., Mitterer, M., Wienands, J., Reth, M., Nielsen, P.J. (1999) Abnormal development and function of B lymphocytes in mice deficient for the signaling adaptor protein SLP-65. *Immunity.* **11**, 547-554

Jumaa, H., Bossaller, L., Portugal, K., Storch, B., Lotz, M., Flemming, A., Schrappe, M., Postila, V., Riikonen, P., Pelkonen, J., Niemeyer, C.M., Reth, M. (2003) Deficiency of the adaptor SLP-65 in pre-B-cell acute lymphoblastic leukemia. *Nature.* **423**, 452-456

Kabak, S., Skaggs, B.J., Gold, M.R., Affolter, M., West, K.L., Foster, M.S., Siemasko, K., Chan, A.C., Aebersold, R., Clark, M.R. (2002) The direct recruitment of BLNK to immunoglobulin α couples the B-cell antigen receptor to distal signaling pathways. *Mol. Cell. Biol.* **22**, 2524-2535

Katagiri, Y.U., Kiyokawa, N., Fujimoto, J. (2001) A role for lipid rafts in immune cell signaling. *Microbiol. Immunol.* **45**, 1-8

Kersseboom, R., Middendorp, S., Dingjan, G.M., Dahlenborg, K., Reth, M., Jumaa, H., Hendriks, R.W. (2003) Bruton's tyrosine kinase cooperates with the B cell linker protein SLP65 as a tumor suppressor in pre-B cells. *J. Exp. Med.* **198**, 91-98

Klein, F., Feldhahn, N., Harder, H., Wang, H., Wartenberg, M., Hofmann, P., Wernet, P., Siebert, R., Müschen, M. (2004) 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.* **199**, 673-685

Klein, F., Feldhahn, N., Mooster, J.L., Sprangers, M., Hofmann, W., Wernet, P., Wartenberg, M., Müschen, M. (2005) 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.* **174**, 367-375

Kleinfield, R., Weigert, M. (1989) Analysis of V_H gene replacement events in a B-cell lymphoma. *J. Imunol.* **142**, 4475-4481

Kondo, M., Weissman, I.L., Akashi, K. (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell*. **91**, 661-672

Köhler, F., Storch, B., Kaluthu, Y., Herzog, S., Kuppig, S., Reth, M., Jumaa, H. (2005) A leucine zipper in the N terminus confers membrane association to SLP65. *Nat. Immunol.* **6**, 204-210

Kurosaki, T. (2002) Regulation of B-cell signal transduction by adaptor proteins. *Nat Rev Immunol.* **2**, 354-363

Laichalk, , L.L., Hochberg, D., babcock, G.J., Freeman, R.B., Thorley-Lawson, D.A. (2002) The dispersal of mucosal memory B cells: evidence from persistent EBV infection. *Immunity.* **16**, 745-754

LeBien, T.W. (2000) Fates of human B-cell precursors. *Blood.* **96**, 9-23

Leo, A., Wienands, J., Baier, G., Horejsi, V., Schraven, B. (2002) Adapters in lymphocyte signaling. *J.Clin.Invest.* **109**, 301-309

Lewis, S.M. (1994) The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses. *Adv. Immunol.* **56**, 27-150

Li, Y.S., Hayakawa, K., Hardy, R.R. (1993) The regulated expression of B lineage-associated genes during B cell differentiation in bone marrow and fetal liver. *J. Exp. Med.* **178**, 951-960

Liberg, D., Smale, S.T., Merkenschlager, M. (2003) Upstream of Ikaros. *TRENDS in Immunol.* **24**, 567-570

Lin, H., Grosschedl, R. (1995) Failure of B-cell differentiation in mice lacking the transcription factor EBF. *Nature*. **376**, 263-267

Liou, J., Kiefer, F., Dang, A., Hashimoto, A., Cobb, M.H., Kurosaki, T., Weiss, A. (2000) HPK1 is activated by lymphocyte antigen receptors and negatively regulates AP-1. *Immunity.* **12**, 399-408

Liu, Y.J., Arpin, C. (1997) Germinal center development. *Immunol. Rev.* **156**, 111-126

Loken, M.R., Shah, V.O., Dattilio, K.L., Civin, C. (1987) Flow cytometric analyss of human bone marrow, II: normal B lymphocyte development. *Blood*. **70**, 1316-1324

Look, A.T. (1997) Oncogenic transcription factors in the human acute leukemias. *Science* **178**, 1059-1064

Lopez, R.G., Carron, C., Oury, C., Gardellin, P., Bernard, O. (1999) TEL is a sequence-specific transcriptional repressor. *J Biol Chem.* **274**, 30132-30138

Lu, R., Medina, K.L., Lancki, D.W., Singh, H. (2003) IRF-4,8 orchestrate the pre-B-to-B transition in lymphocyte development. *Genes Dev.* **17**, 1703-1708

MacLennan, I.C., (1998) B-cell receptor regulation of peripheral B cells. *Curr. Opin. Immunol.* **10**, 220–225.

Manz, R.A., Thiel, A., Radbruch, A. (1997) Lifetime of plasma cells in the bone marrow. *Nature.* **10**, 133-134

Martensson, I.L., Rolink, A., Melchers, F., Mundt, C., Licence, S., Shimizu, T. (2002) The pre-B cell receptor and its role in proliferation and Ig heavy chain allelic exclusion. *Semin. Immunol.* **14**, 335-342

Matsuda, F., Ishii, K., Bourvagnet, P., Kuma, K., Hayashida, H., Miyata, T., Honjo, T. (1998) The complete nucleotide sequence of the human immunoglobulin heavy-chain variable region locus. *J. Exp. Med.* **188**, 2151-2162

McBlane, J.F., van Gent, D.C., Ramsden, D.A., Romeo, C., Cuomo, C.A., Gellert, M., Oettinger, M.A. (1995) Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occur in two steps. *Cell.* **83**, 387-395

McLean, T.W., Ringhold, S., Neuberg, D., Stegmaier, K., Tantravahi, R., Ritz, J., Koeffler, H.P., Takeuchi, S., Janssen, J.W., Seriu, T., Bartram, C.R., Sallan, S.E., Gilliland, D.G., Golub, T.R. (1996) TEL/AML-1 dimerizes and is associated with a favorable outcome in childhood acute lymphoblastic leukemia. *Blood.* **88**, 4252-4258

Metchnikoff, E. (1968, reprint of the 1893 english translation) Lectures on the comparative pathology of inflammation. p. xvii

Minegishi, Y., Rohrer, J., Coustan-Smith, E., Lederman, H.M., Pappu, R., Campana, D., Chan, A.C., Conley, M.E. (1999) An essential role for BLNK in human B cell development. *Science* **286**, 1954-1957

Miosge, L.A., Goodnow, C.C. (2005) Genes, pathways and checkpoints in lymphocyte development and homeostasis. *Imm. Cell. Biol.* **83**, 318-335

Nagata, K., Nakamura, T., Kitamura, F., Kuramochi, S., Karasuyama, H. (1997) The Igα/Igβ heterodimer on μ-negative proB cells is competent for transducing signals to induce early B cell differentiation. *Immunity.* **7**, 559-570

Nemazee, D., Hogquist, K.A. (2003) Antigen receptor selection by editing or downregulation of V(D)J recombination. *Curr. Opin. Immunol.* **15**, 182-189

Niiro, H., Maeda, A., Kurosaki, T., Clark, E.A. (2002) The B lymphocyte adaptor molecule of 32 kD (Bam32) regulates B cell antigen receptor signaling and cell survival. *J. Exp. Med.* **195**, 143-149

Nishizumi, H., Horikawa, K., Mlinaric-Rascan, I., Yamamoto, T. (1998) A double-edged kinase Lyn: a positive and negative regulator for antigen receptor-mediated signals. *J. Exp. Med*. **187**, 1343-1348

Nuñez, C., Nishimoto, N., Gartland, G.L. (1996) B cells are generated throughout life in humans. *J. Immunol.,***156**, 866-872

Nutt, S.L., Heavey, B., Rolink, A.G., Busslinger, M. (1999) Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature.* **401**, 556-562

Ochi, H., Watanabe, T. (2000) Negative regulation of B cell receptor-mediated signaling in B-1 cells through CD5 and Ly49 co-receptors via Lyn kinase activity. *Int. Immunol.* **12**, 1417-1423

Oettinger, M.A., Schatz, D.G., Gorka, C., Baltimore, D. (1990) RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science.* **248**, 1517-1523

Ohnishi, K., Melchers, F. (2003) The nonimmunoglobulin portion of l5 mediates cell-autonomous pre-B cell receptor signaling. *Nat.Immunol.* **4**, 849-856

Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T., Nakagawa, H. (1991) CSK: a protein-tyrosine kinase involved in regulation of src family kinases. *J. Biol. Chem.* **266**, 24249-24252

O'Riordan, M., Grosschedl, R. (1999) Coordinate regulation of B cell differentiation by the transcription factors EBF and E2A. *Imunity.* **11**, 21-31

Otipoby, K.L., Draves, K.E., Clark, E.A. (2001) CD22 regulates B cell receptor-mediated signals via two domains that independently recruit Grb2 and SHP-1. *J. Biol. Chem.* **276**, 44315-44322

Pappu, R., Cheng, A.M., Li, B., Gong, Q., Chiu, C., Griffin, N., White, M., Sleckman, B.P., Chan, A.C. (1999) Requirement for B cell linker protein (BLNK) in B cell development. *Science* **286**, 1949-1954

Parker, M.J., Licence, S., Erlandsson, L., Galler, G.R., Chakalova, L., Osborne, C.S., Morgan, G., Fraser, P., Jumaa, H., Winkler, T.H., Skok, J., Mertensson, I.L. (2005) The pre-B-cell receptor induces silencing of VpreB and lambda5 transcription. *EMBO J.* **24**, 3895-3905

Pui, C.H., Relling, M.V., Downing, J.R. Acute lymphoblastic leukemia. *N. Engl. J. Med.* **350**, 1535-1548

Poe, J.C., Hasegawa, M., Tedder, T.F. (2001) CD19, CD21 and CD22: Multifaceted response regulators of B lymphocyte signal transduction. *Int. Rev. Immunol.* **20**, 739-762

Potter, M., Melchers, F. (2000) Opinions on the nature of B-1 cells and their relationship to B cell neoplasia. *Curr. Top. Microbiol. Immunol.* **252**, 307-324

Radic, M.Z., Zouali, M. (1996) Receptor editing, immune diversification, and self-tolerance. *Immunity.* **5**, 505-511

Ramsden, D.A., McBlane, J.F., van Gent, D.C., Gellert, M. (1996) Distinct DNA sequence and structure requirements for the two steps of V(D)J recombination signal cleavage. *EMBO J.* **15**, 3197-3206

Ravetch, J.V., Siebenlist, U., Korsmeyer, S., Waldmann, T., Leder, P. (1981) Structure of the human immunoglobulin mu locus: characterization of embryonic and rearranged J and D genes. *Cell.* **27**, 583-591

Reth, M., Gehrmann, P., Petrac, E., Wiese, P. (1986) A novel VH to VHDJH joining mechanism in heavy-chain-negative (null) pre-B cells results in heavy-chain production. *Nature* **322**, 840-842

Reth, M. (1989) Antigen receptor tail clue. *Nature.* **338**, 383-384

Roifman, C.M., Ke, S. (1993) CD19 is a substrate of the antigen receptor-associated protein tyrosine kinase in human B cells. *Biochem. Biophys. Res. Commun.* **194**, 222-225

Roitt, I.M., Delves, P.J. (Tenth edition) Roitt's essential Immunology. Chapter 3: Antibodies.

Rolink, A.G., Andersson, J., Melchers, F. (1998) Characterization of immature B cells by a novel monoclonal antibody, by turnover and by mitogen reactivity. *Eur. J. Immunol.* **28**, 3738-3748

Rolink, A.G., ten Boekel, E., Yamagami, T., Ceredig, R., Andersson, J., Melchers, F. (1999) B cell development in the mouse from early progenitors to mature B cells. *Immunol. Lett.* **68**, 89-93

Rolink, A.G., Schaniel, C., Busslinger, M., Nutt, S.L., Melchers, F. (2000) Fidelity and infidelity in commitment to B-lymphocyte lineage development. *Immunol. Rev.* **175**, 104-111

Roumier, C., Fenaux, P., Lafage, M., Imbert, M., Eclache, V., Preudhomme, C. (2003) New mechanisms of AML1 gene alteration in hematological malignancies. *Leukemia.* **17**, 9-16

Saijo, K., Mecklenbräuker, I., Santana, A., Leitger, M., Schmedt, C., Tarakhovsky, A. (2002) Protein kinase C β controls nuclear factor κB activation in B cells through selective regulation of the IκB kinase α. *J. Exp. Med.* **195**, 1647-1652

Sauer, K., Liou, J., Singh, S.B., Yablonski, D., Weiss, A., Perlmutter, R.M. (2001) Hematopoietic progenitor kinase 1 associates physically and functionally with the adaptor proteins B cell linker protein and SLP-76 in lymphocytes. *J. Biol. Chem.* **276**, 45207-45216

Shepherd, P., Suffolk, R., Halsey, J., Allan, N. (1995) Analysis of molecular breakpoint and m-RNA transcripts in a prospective randomized trial of interferon in chronic myeloid leukemia: no correlation with clinical features, cytogenetic response, duration of chronic phase, or survival. *Br. J. Haematol.* **89**, 546-554

Singh, H. (1996) Gene targeting reveals a hierarchy of transcription factors regulating specification of lymphoid cell fates. *Curr. Opin. Immunol.* **8**, 160-165

Stall, A.M., Well, S.M., Lam, K.P (1996) B-1 cells: Unique origins and functions. *Semin. Immunol.* **8**, 45-59

Su, Y., Jumaa, H. (2003) LAT links the pre-BCR to calcium signaling. *Immunity.* **19**, 295-305

Sun, L., Georgopoulos, K. (1996) Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development. *EMBO J.* **15**, 5358-5369

Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H., Kurosaki, T. (1994) Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca^{2+} mobilization through distinct pathways. *EMBO J.* **13**, 1341-1349

Tatosyan, A.G., Mizenina, O.A. (2000) Kinases of the Src family: structure and functions. *Biochemistry (Mosc.)* **65**, 49-58

Tonegawa, S. (1983) Somatic generation of antibody diversity. *Nature.* **302**, 575-581

Tsuji, S., Okamoto, M., Yamada, K., Okamoto, N., Goitsuka, R., Arnold, R., Kiefer, F., Kitamura, D. (2001) B cell adaptor containing Src homology 2 domain (BASH) links the B cell receptor signaling to the activation of hematopoietic progenitor kinase 1. *J. Exp. Med.* **194**, 529-539

Urbanek, P., Wang, Z.Q., Fetka, I., Wagner, E.F., Busslinger, M. (1994) Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking PAX5/BSAP. *Cell.* **79**, 901-912

Wang, H., Nichogiannopoulou, A., Wu, L. (1996) Selective defects in the development of the fetal and adult lymphoid system in mice with Ikaros null mutation. *Immunity* **5**, 537-549

Wienands, J., Schweikert, J., Wollscheid, B., Jumaa, H., Nielsen, P.J., Reth, M. (1998) SLP65: a new signaling component in B lymphocytes which requires expression of the antigen receptor for phosphorylation. *J. Exp. Med.* **188,** 791-795

Wong, J., Ishiai, M., Kurosaki, T., Chan, A. (2000) Functional complementation of BLNK by SLP-76 and LAT linker proteins. *J. Biol. Chem.* **42**, 33116-33122

Xu, W., Doshi, A., Lei, M., Eck, M.J., Harrison, S.C. (1999) Crystal structures of c-Src reveal features of its autoinhibitory mechanism. *Mol. Cell.* **3**, 629-638

Yamanashi, Y., Kakiuchi, T., Mizuguchi, J., Yamamoto, T., Toyoshima, K. (1991) Association of B cell antigen receptor with protein tyrosine kinase Lyn. *Science,* **251**, 192-194

Chapter 2

SLP65-deficiency results in perpetual V(D)J-recombinase activity in pre-B lymphoblastic leukemia and B cell lymphoma cells

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Summary

Perpetual V(D)J-recombinase activity involving multiple DNA double strand-break events in B cell lineage leukemia and lymphoma cells may introduce secondary genetic aberrations leading towards malignant progression. Here we investigated defective negative feedback signaling through the (pre-) B cell receptor as a possible reason for deregulated V(D)J-recombinase activity in B cell malignancy. Studying 28 cases of pre-B lymphoblastic leukemia and 27 B cell lymphomas, expression of the (pre-) B cell receptor-related linker molecule SLP65 was defective in 7 and 5 cases, respectively. SLP65-deficiency correlates with RAG1/2 expression and unremitting V_H-gene rearrangement activity. Reconstitution of SLP65-expression in SLP65-deficient leukemia and lymphoma cells results in downregulation of RAG1/2 expression and prevents both *de novo* V_H-DJ_H rearrangements and secondary V_H replacement. We conclude that iterative V_H gene rearrangement represents a frequent feature in B lymphoid malignancy, which can be attributed to SLP65-deficiency in many cases.

Introduction

Perpetual V(D)J recombinase activity continuously generates DNA double-strand breaks and may give rise to secondary transforming events during the malignant progression of early leukemia and lymphoma cells (Khanna et al., 2001). In B cell precursors, V(D)J-recombination is regulated through a negative feedback signal: upon successful rearrangement, a μ-heavy chain encoded by a productively rearranged V_H region gene signals termination of recombination activity at the *IGHV* locus (Grawunder et al., 1995). How this negative feedback signal is deranged in leukemia and lymphoma cells is not yet resolved.

Recent work demonstrated that deficiency of SLP65 is a frequent feature in acute lymphoblastic leukemia cells (Jumaa et al., 2003; Klein et al., 2004). While a recent report questioned these findings (Imai et al., 2004), this study shows that defective SLP65-expression is not only frequent in human pre-B lymphoblastic leukemia but also occurs in a fraction of mature B cell lymphoma cases. Identifying three leukemia and one lymphoma cell line lacking expression of functional SLP65, we studied the contribution of SLP65 to the control of the V(D)J recombinase activity in B cell lineage leukemia and lymphoma cells.

Perpetual V(D)J-recombinase activity in B cell lineage leukemia and lymphoma cells

In order to investigate ongoing V(D)J recombinase activity in B cell precursor leukemia and B cell lymphoma cells, we first analyzed the configuration of immunoglobulin (Ig) gene loci in leukemia and lymphoma cell lines. Among 22 clonal pre-B lymphoblastic leukemia and B cell lymphoma cell lines, 5 of 12 pre-B lymphoblastic leukemia and 2 of 10 B cell lymphoma cell lines express RAG1 and RAG2 and carry more than two productively rearranged Ig heavy chain V region genes, indicating that negative feedback signaling of the (pre-) B cell receptor to V(D)J recombinase activity was impaired in these cells (Table 1). In (pre-) B lymphoblastic cell lines harboring only one productively rearranged *IGHV* allele, expression of RAG1 and RAG2 does not necessarily indicate defective negative feedback signaling of the (pre-) B cell receptor and may also reflect active rearrangement of *IGKV* and *IGLV* light chain genes. In addition, ongoing V(D)J recombinase activity represents a typical feature of pre-B lymphoblastic leukemia cells carrying a *BCR-ABL1* gene rearrangement as previously shown by us and others (Height et al., 1996; Klein et al., 2004), suggesting that BCR-ABL1 kinase activity interferes with negative feedback signaling of the pre-B cell receptor (Klein et al., 2004).

Results and discussion

SLP65-deficiency in B cell precursor leukemia and B cell lymphoma cells

In murine B cells, the (pre-) B cell receptor-associated linker molecule SLP65 is required to downregulate V(D)J recombinase activity (Hayashi et al., 2003) and acts as a tumor suppressor in pre-B lymphoblastic leukemia cells (Jumaa et al., 2003). Studying SLP65 expression in B cell precursor leukemia and B cell lymphoma by Western blot, we found that expression of SLP65 protein was defective in 7 of 28 leukemia cases (4 of 16 primary cases and 3 of 12 cell lines; Figure 1 A) and 5 of 27 lymphomas (4 of 17 primary cases and 1 of 10 cell lines; Figure 1 A). Sequence analysis revealed that SLP65 transcripts frequently lost their coding capacity for full-length SLP65 protein due to aberrant splicing with exon skipping and usage of cryptic splice sites and splice site slippage (Table 1). In one case of diffuse large B cell lymphoma (DLBCL; Karpas-422) aberrant splicing was the result of a genomic deletion of the 3' splice site of exon 3 of the *SLP65* gene (Figure 1 B). Due to a 28-bp deletion of the 3' part of exon 3 and the 5' part of intron 3-4, full-length SLP65 can no longer be expressed from this allele. Of note, the second *SLP65* allele in these DLBCL cells was lost due to a large deletion at 10q23 (R. S., unpublished). Chromosomal deletion and loss of heterozygosity by somatic mutation is consistent with a role of *SLP65* as a tumor suppressor gene in these DLBCL cells.

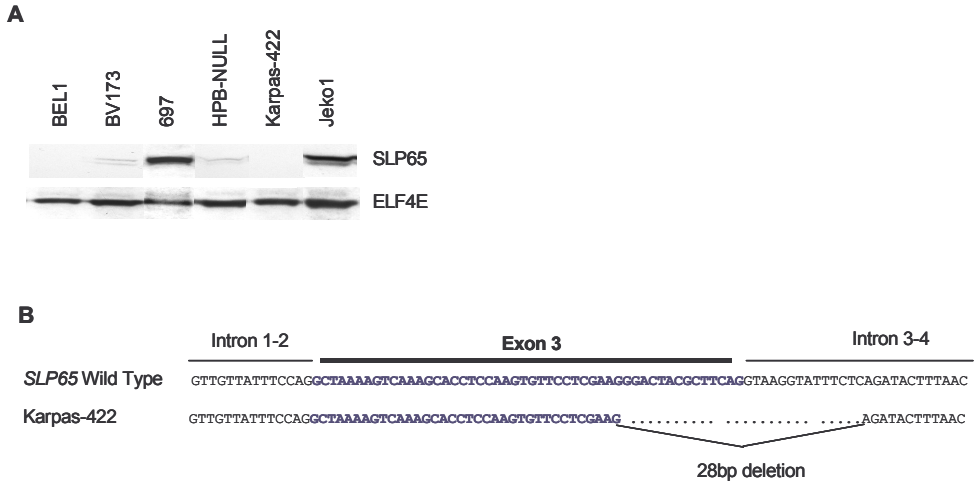


Figure 1: *SLP65-deficiency in B cell precursor leukemia and B cell lymphoma cells*
Western blot analysis shows defective SLP65 expression in BEL1, BV173, HPB-NULL and Karpas-422 cells. EIF4E was used as a loading control (A). Sequence analysis of one allele of *SLP65* in Karpas-422 diffuse large B cell lymphoma cells reveals a 28 bp deletion of the 3' part of exon 3 and the 5' part of intron 3-4 (B). Amplification and sequencing of *SLP65* was done as previously described (Feldhahn et al., 2005) using the primer pairs listed in Supplementary Table 1. The second *SLP65* allele is missing in a classical loss of heterozygosity situation due to a large chromosomal deletion at 10q23. A detailed description of the cell lines used is given in Table 1 and in the supporting information.

Table 1A: *Pre-B cell receptor configuration, V(D)J recombinase activity and SLP65 expression in pre-B acute lymphoblastic leukemia cells*

Case	Entity	Chromosomal rearrangement	*IGHV*	CC	Recombinase activity RAG expression	Active V_H rearrangement	SLP65 WT	*SLP65* mutations	SLP65 Splice variants *
BEL1	pre-B ALL	*MLL-AF4*	V1-2 D3-22 J6 V2-5 D2-2 J6 V3-13 D2-2 J6 V4-31 D2-2 J6 V7-4 D2-2 J6 V3-7 D3-22 J6 V3-13 D3-22 J6 V3-23 D3-22 J6 V3-30 D3-22 J6 V6-1 D3-22 J6 Germline	+ + + – – – – + – – 	RAG1, RAG2	*De novo* V_H-DJ_H Secondary V_H-Replacement	No	N27S	ΔPRD (Exons 5 to 8)
RS4;11	pre-B ALL	*MLL-AF4*	V3-20 D2-8 J5 V6-1 D1-7 J4	– –	RAG1	None	Yes	N.D.	N.D.
SEM	pro-B ALL	*MLL-AF4*	D3-10 J5 Germline		None	None	Yes	None	None
REH	pre-B ALL	*TEL-AML1*	V3-15 D3-10 J6 Germline	–	RAG1	None	Yes	N.D.	N.D.
BV173	pre-B ALL	*BCR-ABL1*	V3-48 D2-2 J3 V3-38 D2-2 J3 V3-21 D2-15 J3 D2-15 J3	– – –	RAG1, RAG2	*De novo* V_H-DJ_H Secondary V_H-Replacement	No	L39P E82K S436F	ΔPRD, SH2 (Exons 6 to 17) ΔPRD, SH2 (Exons 5 to 17) INS Intron 3-4
SUP-B15	pre-B ALL	*BCR-ABL1*	V3-53 D2-8 J6-2 V1-2 D2-2 J6-2 V4-4 J6-2 V6-1 D5-5 J6-2 Germline	+ – – +	RAG1, RAG2	*De novo* V_H-DJ_H Secondary V_H-Replacement	Yes	G30S	ΔPRD, SH2 (Exons 8 to 17) ΔPRD, SH2 (Exons 6 to 16) INS Intron 3-4
Nalm1	pre-B ALL	*BCR-ABL1*	V1-8 J2 V1-8 J4 V2-5 D3-16 J4 V2-70 D3-16 J4 V3-9 J6 V4-31 D5-24 J4 V4-34 D3-16 J4 V4-59 D3-16 J4 2. V4-61 D3-16 J4 V5-51 D3-16 J4 V6-1 D3-16 J4	– – – – + + – – + + +	RAG1, RAG2	*De novo* V_H-DJ_H Secondary V_H-Replacement	Yes	N.D.	ΔPRD, SH2 (Exons 8 to 17 INS Intron 3-4
Nalm6	pre-B ALL	*TEL-PDGFRB*	V1-69 D3-10 J6	+	RAG1	None	Yes	N.D.	N.D.
Kasumi-2	pre-B ALL	*E2A-PBX1*	V3-7 D3-10 J4	+	RAG1	None	Yes	N.D.	N.D.
MHH-CALL3	pre-B ALL	*E2A-PBX1*	V3-15 D3-16 J5	+	RAG1	None	Yes	N.D.	N.D.
697	pre-B ALL	*E2A-PBX1*	V2-26 D2-2 J4	+	RAG1	None	Yes	N.D.	N.D.
HPB-NULL	pre-B ALL	hyperdiploid	V3-9 D3-22 J6 V4-59 D2-8 J6 V6-1 D5-5 J6 V6-1 D6-25 J6 Germline	– + + –	RAG1, RAG2	*De novo* V_H-DJ_H Secondary V_H-Replacement	No	P165S W232R T314A	ΔPRD, SH2 (Exons 5 and 6) ΔPRD, SH2 (Exons 4 to 6)

Table 1B: *B cell receptor configuration, V(D)J recombinase activity and SLP65 expression in B cell lymphoma cells*

Case	Entity	Chromosomal rearrangement	*IGHV*	CC	Recombinase activity RAG expression	Active V_H rearrangement	SLP65 WT	*SLP65* mutations	SLP65 Splice variants *
MEC1	B-CLL	hyperdiploid	V4-59 D3-3 J4 V4-59 D2-21 J4 V2-70 D3-22 J3 V2-70 D3-22 J4	+ + – +	None	None	Yes	Deletion at 10q23	ΔPRD, SH2 (Exons 4 to 6) ΔPRD, SH2 (Exons 8 and 9) ΔPRD, SH2 (Exon 6)
Granta-519	MCL	*CCND1-IGH*	V4-59 D5-5 J4	+	None	None	Yes	None	None
Jeko-1	MCL	*CCND1-IGH*	V2-70 D3-3 J4	+	RAG1, RAG2	None	Yes	N.D.	N.D.
HBL-2	MCL	*CCND1-IGH*	V3-11 D3-22 J1	+	None	None	Yes	None	None
JVM-2	MCL	*CCND1-IGH*	V3-9 D6-19 J4	+	None	None	Yes	N.D.	N.D.
SP49	MCL	*CCND1-IGH*	V4-34 D3-22 J2	+	None	None	Yes	None	None
NCEB-1	MCL	*CCND1-IGH*	V3-53 D2-21 J6	+	None	None	Yes	N.D.	N.D..
MHH-PREB	Burkitt's	*MYC-IGH*	V4-34 D2-15 J5 V3-53 D3-3 J6	+ –	RAG1, RAG2	None	Yes	N.D.	N.D.
MC-116	Burkitt's	*MYC-IGH*	V1-2 D1-26 J4	+	None	None	Yes	None	None
Karpas-422	DLCBL	*BCL2-IGH*	V1-3 D3-3 J6 V1-18 D1-26 J4 V1-18 D2-2 J6 V2-70 D3-22 J3 V3-7 D5-12 J4 V3-9 D3-22 J6 V3-33 D6-13 J4 V6-1 D3-22 J4 V3-73 D2-15 J4 V4-39 D3-10 J6 V4-39 J6 D3-22 J6 Germline	+ + + + + – – + + + +	RAG1, RAG2	*De novo* V_H-DJ_H Secondary V_H-Replacement	No	Deletion at 10q23 LOH: Δ28bp in Exon 3and Intron 3-4	ΔPRD, SH2 (Exons 3 and 7 to 16) ΔPRD, SH2 (Exons 3 and 4) ΔPRD, SH2 (Exons 3 to 5) ΔPRD, SH2 (Exons 3 and 8 to 9)
Normal B cells	None	None	Polyclonal	+/–	None	None	Yes	None	None

Notes:

CC, coding capacity; SH2, SRC-homology domaín 2; PRD, proline-rich domain; N.D., not determined; WT, wild type

De novo V_H-DJ_H rearrangement: rearrangement of a pre-existing DJ_H joint to a V_H gene segment

Secondary V_H replacement: Replacement of a previously rearranged V_H segment within a V_HDJ_H joint by rearrangement of an upstream V_H segment to a cryptic RSS within the 3'part of the previously rearranged V_H.

From MEC1 cells, amplification of RAG1 transcripts yielded a weak band in one experiment, which could not be reproduced in three repeat-experiments.

* Sequence data is a vailable from EMBL/ GenBanl under accession numbers AM180327 to AM180347

Sequence analysis of the coding region of *SLP65* and intronic splice sites revealed a number of other somatic mutations leading to amino acid changes or loss of the reading frame (Table 1). Somatic mutations of the *SLP65* gene were amplified from BEL1, BV173, SUP-B15 and HPB-NULL cells (Table 1A). Non-functional SLP65 mRNA splice variants were amplified from all cases of B cell lineage leukemia and lymphoma lacking negative feedback signaling through the (pre-) B cell receptor (ongoing RAG expression together with multiple V_H gene rearrangements; Table 1). These findings suggest that SLP65 is required to halt the recombination machinery upon successful VDJ-rearrangement at the *IGHV* locus.

V_H replacement in SLP65-deficient leukemia and lymphoma cells

As previously shown by us and others (Klein et al., 2004; Zhang et al., 2003), perpetual V(D)J recombinase activity may involve *de novo* V_H to DJ_H rearrangements or secondary rearrangements by replacement of a previously rearranged V_H gene segment by a yet unrearranged upstream V_H gene segment. In this case, a previously rearranged V_H gene segment is cleaved at a cryptic RSS in its 3' part with only 5 to 7 basepairs remaining as a relict of the initially rearranged V_H gene segment. Such footprints could indeed be detected in the *IGH* VDJ-rearrangements of five pre-B lymphoblastic leukemia cell lines (BEL1, BV173, SUP-B15, Nalm1 and HPB-NULL) and one B cell lymphoma cell line (Karpas-422; Table 2). In two of these five cell lines, the leukemia cells exhibit expression of SLP65. Ongoing V(D)J-recombinase activity in these two cases (SUP-B15 and Nalm1), despite expression of SLP65, reflects that these leukemia cells express the oncogenic BCR-ABL1 kinase, which interferes with negative feedback signaling of the pre-B cell receptor (Klein et al., 2004).

To test if *de novo* rearrangement and V_H replacement are caused by SLP65 deficiency, we reconstituted SLP65 expression in SLP65-deficient pre-B lymphoblastic leukemia cells (BEL1) and diffuse large B cell lymphoma cells (DLBCL; Karpas-422) by nucleofection. After two days, SLP65-reconstituted cells were sorted and analysed for expression of RAG1 and RAG2 and the presence of short-lived DNA double-strand break intermediates at recombination signal sequences (RSS) flanking V_H and J_H gene segments.

RSS-DNA double-strand break intermediates specific for yet unrearranged J_H5 gene segments were amplified to detect *de novo* D_H to J_H5 rearrangements (Supplementary Figure 1). For detection of secondary rearrangements by V_H replacement, we amplified DNA double-strand break intermediates at the cryptic RSS of an already rearranged V_H gene

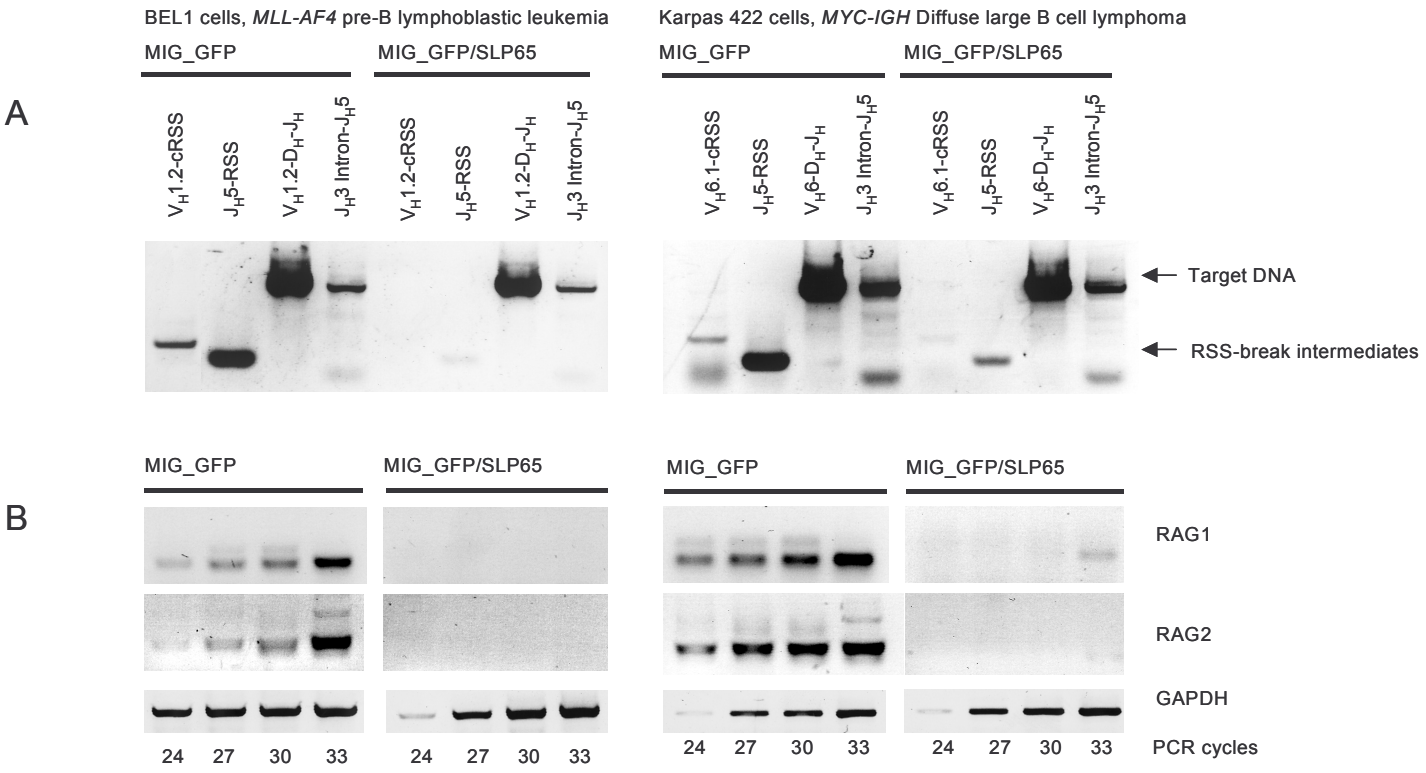


Figure 2: *SLP65-deficiency results in perpetual V(D)J-recombinase activity in pre-B acute lymphoblastic leukemia and B cell lymphoma cells*.

SLP65-deficient pre-B lymphoblastic leukemia (BEL1) and lymphoma (Karpas-422) cells were reconstituted with SLP65 by nucleofection according to the manufacturers' protocol (Amaxa Biosystems, Cologne, Germany) using MIG_GFP_IRES_SLP65 and a MIG_GFP vector as a control. The cells were cultured for 24 hours and nucleofection was repeated. After two days, 5 x 10^4 GFP-expressing cells were sorted using a FACStar 440 cell sorter and kept under cell culture conditions or subjected to DNA or RNA isolation for ligation-mediated PCR or RT-PCR analysis, respectively. Short lived RSS-DNA double-strand break intermediates were determined by ligation-mediated PCR. Target DNA for potential recombination events was amplified as loading control (A). Ligation-mediated PCR (LM-PCR) was carried out as previously described (Klein et al., 2005) and as outlined in supplementary figure 1. In two rounds of semi-nested amplification, DNA-intermediates with a double-strand break at the cryptic recombination signal sequence (cRSS) of rearranged V_H gene segments were amplified using the primers listed in Supplementary Table 1. V_H cRSS-specific primers were used together with a primer specific for DNA-ligated linker molecules. To amplify RSS-intermediates with a DNA double-strand break at the 5' heptamer of unrearranged J_H5 gene segments, nested forward primers flanking the J_H5 RSS were used in two rounds of PCR amplification together with a linker-specific primer. To ensure that equivalent amounts of target DNA for potential DNA double-strand breaks by V_H-replacement or by *de novo* VDJ-rearrangement were present in all LM-PCR reactions, pre-existing V_H1-2 and V_H6-1 gene rearrangements and a genomic region containing the non-rearranged J_H5 gene segment were amplified in one round of PCR.

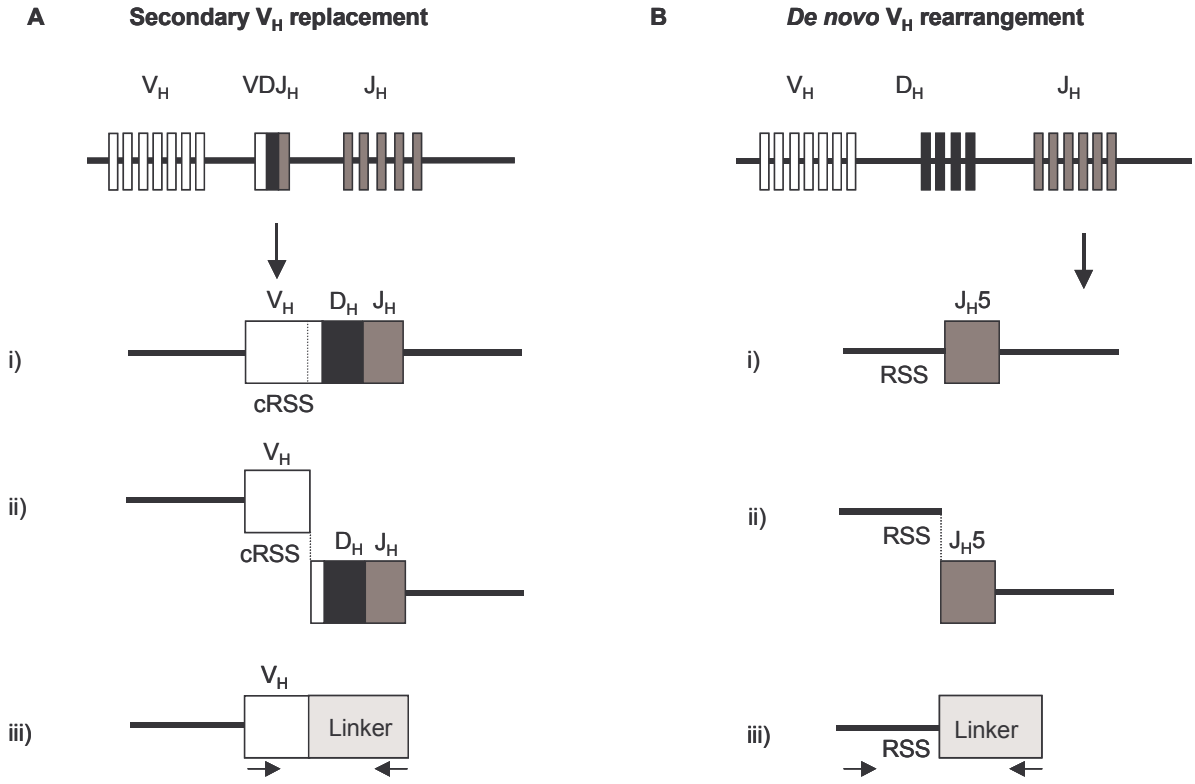
RAG1 and RAG2 expression was analysed by semiquantitative RT-PCR as previously described (Feldhahn et al., 2005) using the primer pairs listed in Supplementary Table 1 (B). cDNA amounts were normalized by amplification of GAPDH transcripts using the PCR cycle numbers indicated.

Table 2: *Analysis of de novo V_H to DJ_H rearrangement and secondary V_H gene replacement in V_H region genes of pre-B lymphoblastic leukemia and B cell lymphoma cells*

Case	3' of recipient V_H-D_H junction		3' of donor V_H		V_H-D_H junction	D_H–J_H junction	
BEL1, allele 1	V_H4-31	CGTGTAT**TACTGTG**CGAGA-GAGGGGTTTGAGCGGG	V_H3-13	TGTGTAT**TACTGTG**CAAGAGA	GTTTGAGCGGG	TATTGTAGTAGTACCAGCTGC	D_H2-2 J_H6
	V_H4-31	CGTGTAT**TACTGTG**CGAGA-GAGGGGTTTGAGCGGG	V_H2-5	CACATAT**TACTGTG**CACAC	CCCCCCGGGGGGGGTTTGAGCGGG	TATTGTAGTAGTACCAGCTGC	D_H2-2 J_H6
	V_H4-31	CGTGTAT**TACTGTG**CGAGA-GAGGGGTTTGAGCGGG	V_H7-4	CGTGTAT**TACTGTG**CGAGA	GGGTTTGAGCGGG	TATTGTAGTAGTACCAGCTGC	D_H2-2 J_H6
BEL1, allele 2 (*de novo* V_H to DJ_H rearrangement)							
	–		V_H3-30	TGTGTAT**TACTGTG**CGAGAGA	TA	ATTACTATGATAGTAGTGG	D_H3-22 J_H6
	–		V_H3-23	CGTATAT**TACTGTG**CGA	TCCTCG	AGTGG	D_H3-22 J_H6
	–		V_H3-13	TGTGTAT**TACTGTG**CAAGAGA		TACTATGATAGTAGTGG	D_H3-22 J_H6
	–		V_H3-7	TGTGTAT**TACTGTG**CGAGAGA	C	GTATTACTATGATAGTAGTGG	D_H3-22 J_H6
	–		V_H1-2	CGTGTAT**TACTGTG**CGAGAGA		TATTACTATGATAGTAGTGG	D_H3-22 J_H6
	–		V_H6-1	TGTGTAT**TACTGTG**CAAGAGA	CCGTATAGCAGTGGCTG	GTATTACTATGATAGTAGTGG	D_H3-22 J_H6
BEL1, allele 3 (germline)							
NALM1, allele 1	V_H1-45	CATGTAT**TACTGTG**CAAGATA	V_H1-69	GTGTAT**TACTGTG**CGAGAG	GTCAA	GATATTGTAGTGGTGGTAGCTGCT	D_H2-15 J_H 3
NALM1, allele 2 (*de novo* V_H to DJ_H rearrangement)							
	–		V_H2-5	ACATAT**TACTGTG**CACACAGATCG		ACACAGATCGGGGGGTACTTTG	D_H3-16 J_H4
	–		V_H4-34	GTGTAT**TACTGTG**CGAGAGG		TCCCCTCGGGGGGGTACTTTT	D_H3-16 J_H4
	–		V_H6-1	GTGTAT**TACTGTG**CAAGAGATTGTGCA		AGAGATATGGGGGGGGTACTTTT	D_H3-16 J_H4
	–		V_H4-61	GTGTAT**TACTGTG**C		AGAGAGATGGGGGGGGGTACTTTT	D_H3-16 J_H4
	–		V_H2-70	ACGTAT**TACTGTG**GCACGGATGTGTGCA		CGGATGGGGGACTACGGGGGGTACTTTG	D_H3-16 J_H4
	–		V_H4-31	GTGTAT**TACTGTG**TGAGAGAA		AGAGAAGGCTACGGGGGGTACTTTT	D_H3-16 J_H4
	–		V_H4-59	GTGTAT**TACTGTG**GCGAGGATAAA		AGGGACTACGGGGGGTACTTTG	D_H3-16 J_H4
	–		V_H5-51	ATGTAT**TACTGTG**GCGAGC		GAGCCTCTACGGGGGGTACTTTT	D_H3-16 J_H4
BV173[a]	V_H3-38	CGTGTAT**TACTGTG**CCAGATATA	V_H3-48	GTGTAT**TACTGTG**GCGA	GCCAGATATTGT	AGTGGTGGTAGCT	D_H2-2 J_H3
SUP-B15[a]	V_H3-38	CGTGTAT**TACTGTG**CCAGATATA	V_H3-53	GTGTAT**TACTGTG**CGAGA	GTTGCCAGGGGG	TGGTGTATGCTATACC	D_H2-8 J_H6
HPB-NULL[b]	V_H3-13	CGTGTAT**TACTGTG**CAAGAGA	V_H4-59	GTGTAT**TACTGTG**CGAGA	CTAAGAGATGG	–	J_H6[c]
KARPAS-422[d]	V_H1-58	TGTGTAT**TACTGTG**CGGCAGA	V_H6-1	GTGTAT**TACTGTG**CAAGAG	TGGGCAGCT	CGTCAAGGGGAGGT	D_H3-22 J_H4

Notes:
BEL1 and NALM1 pre-B lymphoblastic leukemia cells exhibit ongoing V_H replacement on one allele (allele 1) and *de novo* V_H to DJ_H rearrangement on the other allele (allele 2). From BEL1 cells, also an *IGHV* germline allele was amplified (referred to as allele 3).
Likely donor-recipient relationships between multiple V_H-DJ_H gene rearrangements were depicted based on the localisation of V_H gene segments in the *IGH* locus.
cRSS motifs (bold), footprints of recipient V_H gene segments (red color, underlined),
a V_H gene replacements were already described in Klein et al., 2004.
b The footprint of this potential V_H replacement may be also derived from a V_H3-74 or V_H6-1 gene segment.
c The D_H gene segment could not be identified.
d Likely generated by inversion or transrecombination events.

segment (V_H1-2 in BEL1 cells and V_H6-1 in Karpas-422 cells; Figure 2A; Supplementary Figure 1). To ensure that the amount of target DNA for double-strand breaks was equal, a germline DNA fragment including the J_H5 RSS (*de novo* rearrangements) and the pre-existing VDJ-rearrangements (V_H1-2 D_H3-22 J_H6 in BEL1 cells; V_H6-1 D_H3-22 J_H4 in Karpas-422 cells) were amplified. In the case of BEL1 cells, we amplified one germline allele of the *IGHV* locus in addition to two rearranged alleles (Table 1, Table 2), suggesting that this cell line comprises subclones that carry at least one germline allele.



Supplementary Figure 1: *Analysis of DNA double-strand breaks by ligation mediated PCR*

While DNA double-strand breaks involved in both *de novo* and secondary rearrangements were clearly detectable in SLP65-deficient leukemia and lymphoma cells carrying a GFP-control vector, reconstitution of SLP65 expression in these cells resulted in a dramatic decrease of the frequency of DNA double-strand breaks (Figure 2A). Likewise, SLP65-deficient leukemia and lymphoma cells carrying only the GFP-control vector express both RAG1 and RAG2, which was sensitive to SLP65-reconstitution in these cells (Figure 2B). We conclude that re-expression of SLP65 in pre-B lymphoblastic leukemia and

lymphoma cells was sufficient to terminate aberrant VDJ-recombinase activity. This function of SLP65 may have important implications for the clonal evolution of a SLP65-deficient leukemia or lymphoma because perpetual expression and activity of RAG1 and RAG2 carries the risk of continuous DNA double-strand breaks and the accumulation of secondary transforming events in the leukemia and lymphoma cells. These findings establish a causative link between perpetual VDJ-recombinase activity and SLP65-deficiency not only in pre-B lymphoblastic leukemia but also in B cell lymphoma cells.

References

Feldhahn. N., F. Klein, J.L. Mooster, P. Hadweh, M. Sprangers, M. Wartenberg, M.M. Bekhite, W.K. Hofmann, S. Herzog, H. Jumaa, J.D. Rowley, M. Müschen. Mimicry of a constitutively active pre-B cell receptorin acute lymphoblastic leukemia cells. *J Exp Med*. 201: 2005, 1837-1852

Grawunder, U., T.M. Leu, D.G. Schatz, A. Werner, A.G. Rolink, F. Melchers, and T.H. Winkler. Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. *Immunity*. 1995, 3: 601–608

Hayashi, K., M. Yamamoto, T. Nojima, R. Goitsuka, D. Kitamura. Distinct signaling requirements for Dμ selection, IgH allelic exclusion, pre-B cell transition, and tumor suppression in B cell progenitors. *Immunity*. 2003, 18: 825-36.

Height, S.E., G.J. Swansbury, E. Matutes, J.G. Treleaven, D. Catovsky, M.J.S. Dyer. Analysis of clonal rearrangements of the Ig heavy chain locus in acute leukemia. *Blood*. 1996, 87: 5242-5250

Imai, C., M.E. Ross, G. Reid, E. Coustan-Smith, K.R. Schultz, C.H. Pui, J.R. Downing, D. Campana. Expression of the adaptor protein BLNK/SLP-65 in childhood acute lymphoblastic leukemia. *Leukemia.* 2004, 18: 922-925.

Jumaa H, Bossaller L, Portugal K, Storch B, Lotz M, Flemming A, Schrappe M, Postila V, Riikonen P, Pelkonen J, Niemeyer CM, Reth M. Deficiency of the adaptor SLP-65 in pre-B-cell acute lymphoblastic leukemia. *Nature*. 2003, 423: 452-6.

Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet.* 2001, 27: 247-54.

Klein, F., N. Feldhahn, L. Harder, H. Wang, M. Wartenberg, W.K. Hofmann, P. Wernet, R. Siebert, M. Müschen. 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., N. Feldhahn, J.L. Mooster, M. Sprangers, W.K. Hofmann, P. Wernet, M. Wartenberg and M. Müschen. 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.

Zhang, Z., M. Zemlin, Y.H. Wang, D. Munfus, L.E. Huye, H.W. Findley, S.L. Bridges, D.B. Roth, P.D. Burrows, M.D. Cooper. Contribution of V_H gene replacement to the primary B cell repertoire. *Immunity.* 2003, 19: 21-31.

Chapter 3

The SRC family kinase LYN redirects B cell receptor signaling in human SLP65-deficient B cell lymphoma cells

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Summary

SLP65 represents a critical component in (pre-) B cell receptor signal transduction but is compromised in a subset of pre-B cell-derived acute lymphoblastic leukemia. Based on these findings, we investigated i.) whether SLP65-deficiency also occurs in mature B cell-derived lymphoma and ii.) whether SLP65-deficient B cell lymphoma cells use an alternative B cell receptor signaling pathway in the absence of SLP65.

Indeed, expression of SLP65 protein was also missing in a fraction of B cell lymphoma cases. While SLP65 is essential for B cell receptor-induced Ca^{2+} mobilization in normal B cells, B cell receptor engagement in SLP65-deficient as compared to SLP65-reconstituted B cell lymphoma cells resulted in an accelerated yet shortlived Ca^{2+}-signal. B cell receptor engagement of SLP65-deficient lymphoma cells involves SRC kinase activation, which is critical for B cell receptor-dependent Ca^{2+}-mobilisation in the absence but not in the presence of SLP65.

As shown by RNA interference, the SRC kinase LYN is required for B cell receptor-induced Ca^{2+} release in SLP65-deficient B cell lymphoma cells but dispensable after SLP65-reconstitution. B cell receptor engagement in SLP65-deficient B cell lymphoma cells also resulted in tyrosine-phosphorylation of the proliferation- and survival-related MAPK1 and STAT5 molecules, which was sensitive to silencing of the SRC kinase LYN. Inhibition of SRC kinase activity resulted in growth arrest and cell death specifically in SLP65-deficient lymphoma cells.

Introduction

The linker molecule SLP65 (also known as BLNK (Fu et al., 1998), BASH (Hayashi et al., 2000) represents a critical component of the (pre-) B cell receptor signaling cascade (Wienands et al., 1998). Through a number of phosphorylated tyrosine residues, SLP65 can connect with upstream and downstream signaling molecules including SYK, BTK, NCK, VAV and PLCγ2 (Middendorp et al., 2003). These interactions are essential for the assembly of the signaling cascade ultimately leading to the release of Ca^{2+} from the endoplasmic reticulum (Fu et al., 1998). In the absence of SLP65, B cell development is arrested at the pro-B to pre-B cell transition (Pappu et al., 1999; Minegishi et al., 1999), which parallels complete breakdown of (pre-) B cell receptor signaling in SLP65-deficient B cells (Pappu et al., 1999; Ishiai et al., 1999; Hayashi et al., 2003). While one study reported normal expression of SLP65 in childhood acute lymphoblastic leukemia (Imai et al., 2004), recent work by us and others (Flemming et al., 2002; Jumaa et al., 2003; Kersseboom et al., 2003; Hayashi et al., 2003; Klein et al., 2004) indicated a role of SLP65 as a tumor suppressor in human and murine leukemia derived from pre-B cells. Based on these findings, we investigated i.) whether SLP65-deficiency also occurs in mature B cell lymphoma and ii.) whether SLP65-deficient B cell lymphoma cells use an alternative B cell receptor signaling pathway in the absence of SLP65.

Results and Discussion

SLP65-deficiency in B cell lymphoma cells

Studying SLP65 expression in Non-Hodgkin's B cell lymphomas including small lymphocytic lymphoma/ B-CLL, mantle cell lymphoma, follicular lymphoma, Burkitt's lymphoma, diffuse large B cell lymphoma (DLBCL) and plasmablastic B cell lymphoma (PBBCL) by RT-PCR and Western blot, we found normal expression of SLP65 mRNA and protein in the majority of B cell lymphoma cases analysed (Figure 1).

Full-length SLP65 transcripts were detected in seven of eight B cell lymphoma cell lines. From one diffuse large B cell lymphoma (DLBCL; Karpas-422) cell line, only shorter SLP65 mRNAs were amplified and in one Burkitt's lymphoma-derived cell line (MC116), truncated SLP65 mRNAs were co-amplified with full-length transcripts (Figure 1A). Sequence analysis revealed that truncated SLP65 transcripts amplified from MC116 and Karpas-422 do not encode functional SLP65 proteins because of introduction of pre-terminal translation stops and or deletion of tyrosine residues that are important for SLP65 function (Supplementary Table 1; sequence data available from EMBL/GenBank under accession

numbers pending). Two other non-functional SLP65 mRNAs lacking exons 8 or 16 were amplified from various B cell lymphoma samples. SLP65 mRNA lacking exon 8 was previously described and termed BLNK-S (Fu et al., 1998). However, these SLP65 splice variants cannot be attributed to malignant transformation because they were also amplified from $CD19^+$ peripheral blood B cells purified from four healthy donors (Supplementary Table 1, Figure 1).

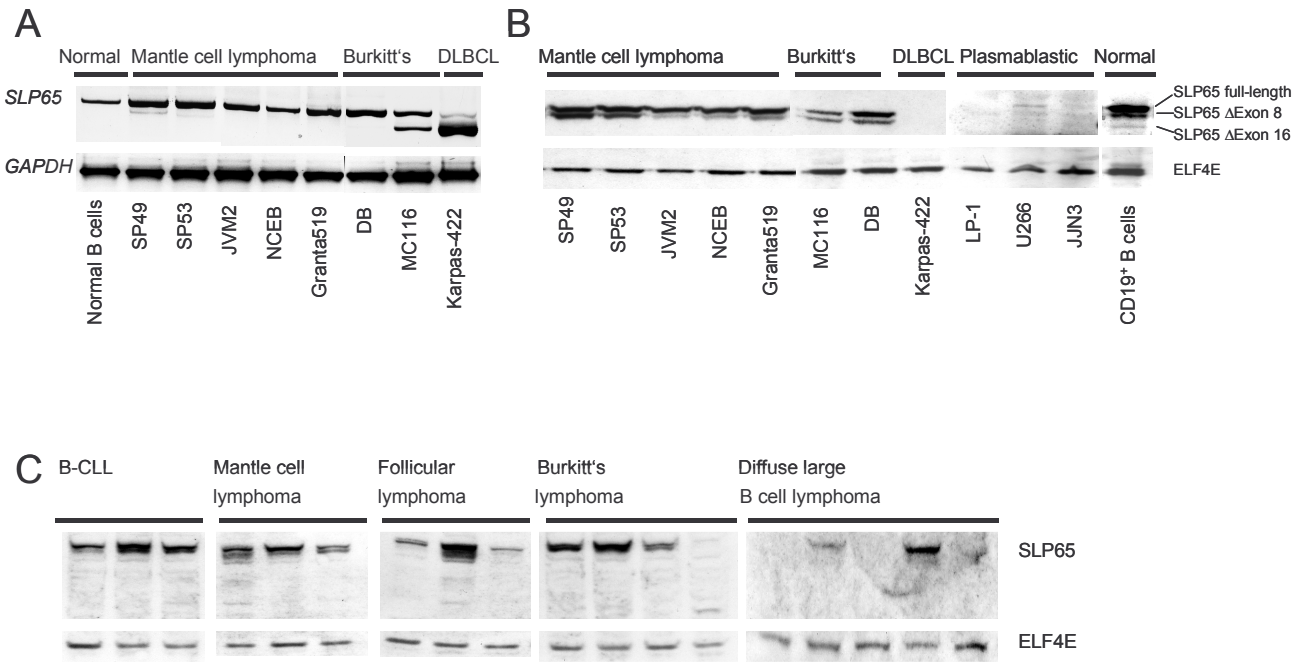

Figure 1: *Analysis of SLP65 expression in B cell lymphoma.*
SLP65 mRNA (A) and protein (B) expression in normal B cells and B cell lymphoma cells were analysed by RT-PCR and Western blot. *SLP65* and *GAPDH* transcripts were amplified as previously described (Klein et al., 2004). $CD19^+$ B cells were purified from peripheral blood of four healthy donors using immunomagnetic beads against CD19 (Miltenyi Biotech, Bergisch Gladbach, Germany). For Western blot analysis of SLP65 expression, total cell lysates of B cell lymphoma cell lines and normal B cells (B) and primary cases of B cell lymphoma (C) were used together with antibodies against SLP65 and ELF4E (Cell Signaling Technology, Beverly, MA). ELF4E was used as a loading control.

Protein expression of SLP65 was missing in 3 of 11 cell lines (Figure 1B) and 4 of 18 primary cases of B cell lymphoma (Figure 1C). In conclusion, SLP65-deficiency does not only occur in B cell precursor leukemia but also lymphoma derived from mature B cells.

In one case (DLBCL cell line, Karpas-422), aberrant splicing of SLP65 was the result of a deletion of the 3' splice site of exon 3 of the *SLP65* gene. Due to a 28-bp deletion of the 3' part of exon 3 and the 5' part of intron 3-4, full-length SLP65 can no longer be expressed from this allele (Figure 1B; sequence data available from EMBL/GenBank under accession number, pending). Of note, the second *SLP65* allele in these DLBCL cells was lost due to a

large deletion at 10q23 (R. S., unpublished FISH data). Chromosomal deletion and loss of heterozygosity by somatic mutation is consistent with a role of *SLP65* as a tumor suppressor gene in these DLBCL cells. In two plasmablastic B cell lymphoma (PBBCL) cell lines (LP-1 and JJN3), SLP65 protein expression was missing (Figure 1B) despite expression of regularly spliced SLP65 transcripts. Studying genomic DNA of these cell lines, no mutations or deletions within the SLP65 coding region and splice sites were found (not shown).

Because SLP65 is essential for B cell receptor signal transduction in normal B cells, we investigated the consequences of SLP65-deficiency on B cell receptor signal transduction in two B cell lymphoma cell lines (Karpas-422 and LP-1).

LYN-dependent B cell receptor signaling in SLP65-deficient B cell lymphoma cells

Unexpectedly, B cell receptor-stimulation in two SLP65-deficient B cell lymphoma cell lines (DLBCL, Karpas-422; PBBCL, LP-1) excited a very vigorous yet shortlived Ca^{2+}-signal despite SLP65-deficiency of these cells (Figure 2A, D, E). In comparison to normal B cells, Ca^{2+}-release in response to B cell receptor engagement in SLP65-deficient lymphoma cells occurred after a substantially reduced lag-phase (26 ± 7 seconds in DLBCL and 29 ± 6 seconds in PBBCL as compared to 94 ± 8 seconds in normal B cells; n = 4). Also the signal amplitude was higher in SLP65-deficient lymphoma cells as compared to normal B cells (Figure 2A, D, E), but the signal was very unstable in the SLP65-deficient lymphoma cells and returned to baseline levels within 90 seconds whereas elevated cytoplasmic Ca^{2+}-levels were maintained in normal B cells for more than six minutes (Figure 2A). These findings suggest that an alternative SLP65-independent pathway may link the B cell receptor to Ca^{2+} mobilization in diffuse large B cell lymphoma cells. Given that the SLP65-deficient lymphoma cell lines express the SRC kinase LYN, FYN and BLK (not shown) and exhibit activating tyrosine-phosphorylation of SRC-kinases in response to B cell receptor engagement (Figure 2B), we tested whether SRC-kinase activity contributed to B cell receptor-dependent Ca^{2+}-release in these cells. While global inhibition of SRC-kinase activity by PP2 had little effect in normal B cells, B cell receptor-dependent Ca^{2+}-release was completely abolished by PP2 in SLP65-deficient lymphoma cells (Figure 2A, D, E). Only few PP2-treated lymphoma cells responded to B cell receptor engagement at all and with a delay of more than five minutes (Figure 2A).

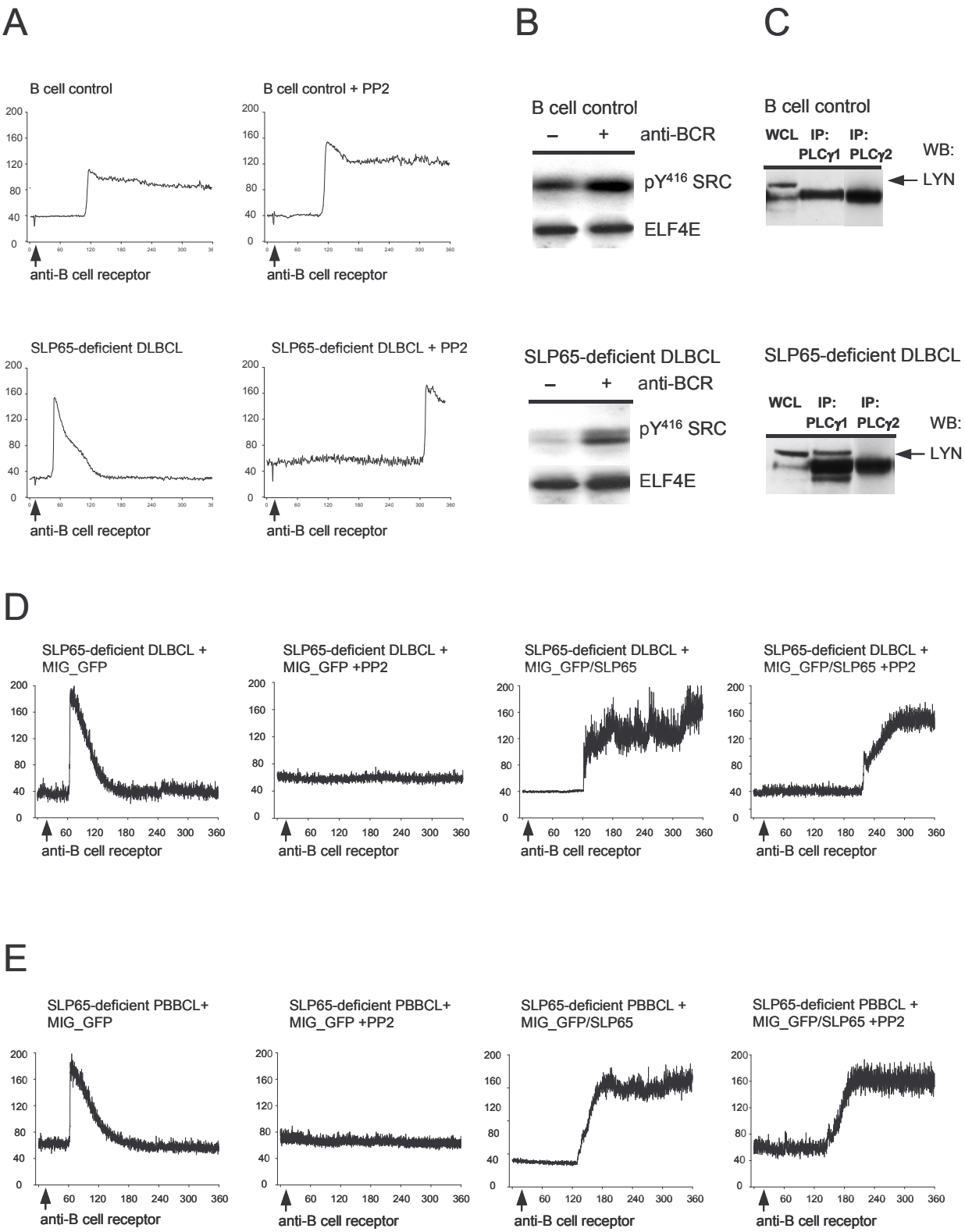

Figure 2: *SRC kinase-dependent B cell receptor signaling in SLP65-deficient B cell lymphoma cells.*

Figure legend to Figure 2: *SRC kinase-dependent B cell receptor signalling in SLP65-deficient B cell lymphoma cells*
Normal B cells or SLP65-deficient diffuse large B cell lymphoma cells (DLBCL; Karpas-422) were incubated in the presence or absence of 10 μmol/l of the SRC-kinase inhibitor PP2 (Calbiochem, Bad Soden, Germany) for 24 hours prior to Ca^{2+}-measurement (A). After pre-incubation, cells were washed and stained with Fluo-3 dye (Calbiochem) for 30 minutes. Changes of cytosolic Ca^{2+} upon B cell receptor engagement were measured by laser scans using confocal microscopy as previously described (Klein et al., 2004). After 30 seconds of measurement, antibodies against the B cell receptor (Jackson Immunoresearch) were added. To measure changes of tyrosine-phosphorylation upon B cell receptor (BCR)-stimulation at a common activation-motif of SRC-kinases (Y416), cell lysates of normal B cells and SLP65-deficient DLBCL cells were analysed by Western blot using phosphotyrosine-specific antibodies against Y416 SRC-kinases (B; Cell Signaling Technology). To measure potential interactions between the SRC kinase LYN and tyrosine-phosphorylated PLCγ1 and PLCγ2 in SLP65-deficient and SLP65-expressing B cell lymphoma cells (C), whole cell lysates (WCL) were generated and subjected to immunoprecipitation of phosphorylated $PLC\gamma1^{Y783}$ (IP: PLCγ1) or $PLC\gamma2^{Y1217}$ (IP: PLCγ2) as previously described (Feldhahn et al., 2005). Thereafter, immunoprecipitated proteins were analysed for the presence of LYN by Western blotting. As a control, normal B cells were used. To analyse the relationship between SRC kinase- and SLP65-dependent signaling pathways, SLP65-deficient DLBCL cells and SLP65-deficient plasmablastic B cell lymphoma cells (PBBCL, LP-1) were transfected with a MIG-GFP or a MIG-GFP/SLP65 vector and incubated in the presence or absence of 10 μmol/l of the SRC-kinase inhibitor PP2. Cells were stimulated with an anti-B cell receptor antibody (arrows) and cytoplasmic Ca^{2+} levels were measured as previously described (Reppel et al., 2005).

Consistent with an alternative SRC kinase-dependent pathway of Ca^{2+} mobilization in SLP65-deficient B cell lymphoma cells, we identified the SRC kinase LYN co-immunoprecipitating with tyrosine-phosphorylated PLCγ1, which generates IP3 acting as a ligand for Ca^{2+} channels of the endoplasmic reticulum. Surprisingly, the B cell-specific PLCγ isozyme PLCγ2, which binds to SLP65 in normal B cells, does not interact with LYN (Figure 2C). Conversely, an interaction between LYN and PLCγ1 was not found in normal B cells (Figure 2C), suggesting that this interaction is specific for SLP65-deficient lymphoma cells. However, in a larger panel of SLP65-expressing B cell lymphomas and pre-B cell leukemias, we also identified LYN-PLCγ1 interactions in some SLP65-expressing pre-B cell leukemia cell lines (not shown). These findings indicate that SRC kinase (LYN)- and SLP65-dependent signaling pathways are not mutually exclusive.

Therefore, we tested the relationship between SRC kinase- and SLP65-dependent B cell receptor signaling in SLP65-deficient B cell lymphoma in more detail: Two SLP65-deficient B cell lymphoma cell lines were transfected with a control vector or a vector encoding wildtype SLP65 (Figure 2D, E). Control- and SLP65-transfectants were cultured in the presence or absence of the SRC kinase inhibitor PP2 and B cell receptor responsiveness was measured as Ca^{2+} release after stimulation with anti-B cell receptor antibodies. In the absence of SLP65-expression, both lymphoma cell lines exhibited an atypical Ca^{2+} signal in

response to B cell receptor engagement, which was entirely abolished by SRC-kinase inhibition through PP2. Upon reconstitution of SLP65 in the lymphoma cell lines, however, several aspects of normal B cell receptor signaling were restored: as opposed to SLP65-deficient lymphoma cells, the signal was retarded but stable for more than four minutes and no longer sensitive to SRC-kinase inhibition by PP2 (Figure 2 D, E).

As PP2 blocks activity of all SRC family kinases, we investigated the specific contribution of the SRC-family kinase LYN, which we found in complex with PLCγ1 (Figure 2C). To this end, normal B cells and SLP65-deficient diffuse large B cell lymphoma cells were transfected with fluorochrome-labeled non-targeting siRNAs and a mixture of three fluorochrome-labeled LYN-targeting siRNA duplices by nucleofection. After repeated nucleofection, the successfully transfected cells were sorted based on uptake of fluorochrome-labeled siRNAs. Efficiency of RNA interference-mediated silencing of LYN was verified by semiquantitative RT-PCR (Figure 3A).

Measurement of Ca^{2+}-release in response to B cell receptor engagement among the transfected cells showed that RNA interference with LYN expression greatly diminished Ca^{2+}-release in SLP65-deficient B cell lymphoma but not in normal B cells (Figure 3B). To test whether SLP65- and LYN-dependent signals are components of mutually exclusive signaling pathways, B cell receptor-dependent Ca^{2+}-release was also measured in SLP65-reconstituted B cell lymphoma cells that were transfected with non-targeting siRNA or siRNA duplices specific for LYN. Reconstitution of SLP65-expression in the B cell lymphoma cells did not only restore the Ca^{2+}-signal kinetics observed in normal B cells (i.e. a sustained signal at a lower amplitude and a longer lag-phase; Figure 3B). Re-expression of SLP65 also rendered B cell receptor-dependent Ca^{2+}-release in the B cell lymphoma insensitive to LYN-inhibition by siRNAs as observed in normal B cells (Figure 3B). These findings suggest that the B cell receptor in SLP65-deficient DLBCL cells signals by default through a pathway dependent on the SRC-kinase LYN.

Taken together, we propose that SLP65-deficient B cell lymphoma cells use an alternative, likely aberrant, B cell receptor signaling pathway that short-circuits SLP65 and potentially other SLP65-binding molecules like BTK and PLCγ2. This alternative B cell receptor signaling pathway is dependent on the SRC kinase LYN and involves tyrosine phosphorylation of PLCγ1. In the absence of SLP65, LYN together with PLCγ1 can maintain the capacity of the B cell receptor to mobilize Ca^{2+} from cytoplasmic stores.

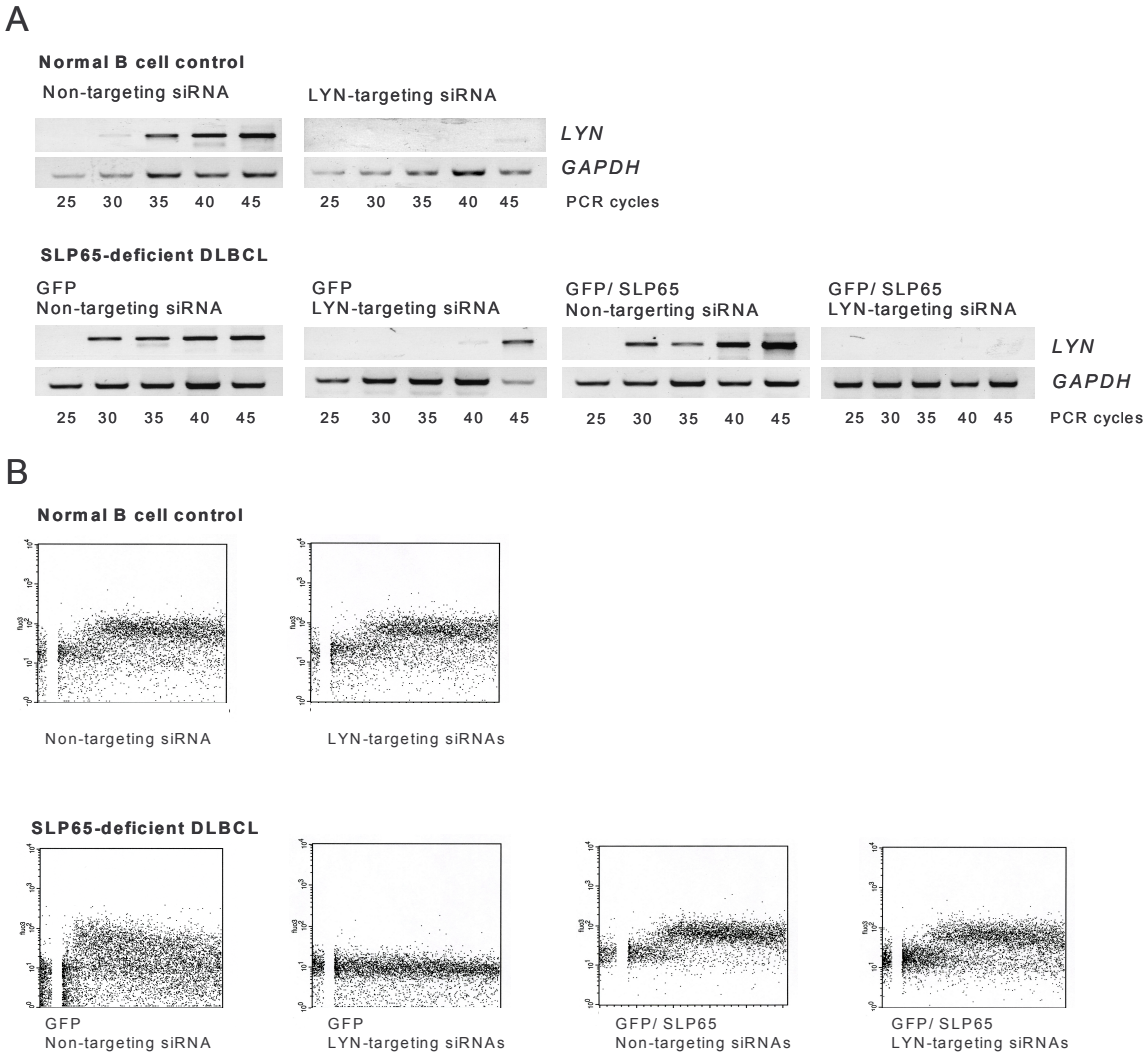

Figure 3: *B cell receptor signaling in DLBCL cells in the presence or absence of LYN and SLP65.*

Normal B cells, SLP65-deficient DLCBL cells and SLP65-reconstituted DLCBL cells were transfected with LYN-targeting siRNAs by nucleofection (Amaxa). Three siRNA duplices were synthesized for RNA interference with LYN expression (MWG Biotech, Ebersberg, Germany; Supplementary Table 2). As a control, a non-targeting siRNA duplex was used that does not match a known mRNA sequence (Supplementary Table 2). All siRNA duplices were labeled with CY3 using a siRNA labeling kit (Ambion, Austin, TX) according to the manufacturer's protocol. DLBCL or normal B cells were transfected with a mixture of the three CY3-labeled siRNAs or the non-targeting siRNA at a concentration of 50 nmol/l each by nucleofection (Amaxa Biosystems): Briefly, for each cell line 5 x 10^6 cells were resuspended in 100 μl nucleofector-solution (Amaxa Biosystems) and 5 μg of plasmid DNA were subsequently added for electroporation. The cells were cultured for 24 hours and nucleofection was repeated. After 24 hours, CY3-positive cells were sorted under sterile conditions using a FACStar 440 cell sorter and either kept under cell culture conditions or subjected to RNA isolation for RT-PCR analysis. Transfection efficiency was controlled by FACS. The silencing effect of siRNAs for LYN was controlled by RT-PCR analysis of LYN mRNA levels (A) using the primers listed in supplementary Table 1. SLP65-deficient B cell lymphoma cells were transfected with MIG-GFP/SLP65 and a MIG-GFP vector as a control using a nucleofection system according to the manufacturers' protocol (Amaxa Biosystems, Cologne, Germany). GFP-positive cells were sorted under sterile conditions using a FACStar 440 cell sorter. Changes of cytosolic Ca^{2+} were measured by flow-cytometry. After 20 seconds of measurement, antibodies against the B cell receptor were added (B).

LYN is required for B cell receptor-induced tyrosine phosphorylation of STAT5 and MAPK1 in SLP65-deficient B cell lymphoma cells

We next investigated, whether LYN-dependent B cell receptor signaling in SLP65-deficient lymphoma cells can promote survival and proliferation. In normal B cells, B cell receptor engagement results in activation of STAT5 (Karras et al., 1996) and mitogen activated protein kinases (MAPK; Richards et al., 2001), which induce survival and proliferation, respectively. Likewise, B cell receptor engagement induced tyrosine phosphorylation of STAT5 and MAPK1 in two SLP65-deficient and one SLP65-expressing B cell lymphoma cell lines (Figure 4). To determine whether LYN is required for B cell receptor-induced STAT5- and MAPK1-activation in the absence of SLP65, SLP65-deficient and SLP65-expressing B cell lymphoma cells were transfected either with non-targeting fluorochrome-labeled siRNAs or siRNA duplices against LYN.

Comparing transfected and non-transfected cells, non-targeting siRNAs had no effect. However, inhibition of LYN significantly diminished both STAT5- and MAPK1-activation in response to B cell receptor engagement in two SLP65-deficient B cell lymphoma cell lines but had no significant effect on SLP65-expressing B cell lymphoma cells (Figure 4). Of note, these B cell lymphoma cells express also other SRC kinases besides LYN including FYN and BLK (not shown). Therefore, these findings indicate that among SRC kinases, LYN has an important contribution to survival and proliferation signaling in SLP65-deficient B cell lymphoma cells.

SRC-kinase activity contributes to survival and proliferation signals in SLP65-deficient B cell lymphoma cells

To directly compare the contribution of SRC-kinases to survival signaling in SLP65-deficient versus SLP65-expressing B cell lymphoma cells, we incubated two SLP65-deficient and three SLP65-expressing B cell lymphoma cell lines in the presence or absence of the SRC kinase inhibitor PP2 for ten days. In one set of experiments, proliferation of SLP65-deficient and SLP65-expressing lymphoma cells was measured by CSFE-labeling and counting of viable cells in the cell cultures in the presence or absence of PP2. SRC-kinase inhibition had only a mild and transient effect on the number of cell divisions (CSFE labeling) and the growth of viable cells (counting; Supplementary Figure A and B) in SLP65-expressing lymphoma cells. In contrast, SRC kinase inhibition resulted in growth arrest in two SLP65-deficient B cell lymphoma cell lines (Supplementary Figure A, B).

Figure 4: *LYN is required for tyrosine-phosphorylation of STAT5 and MAPK1 in SLP65-deficient B cell lymphoma cells*

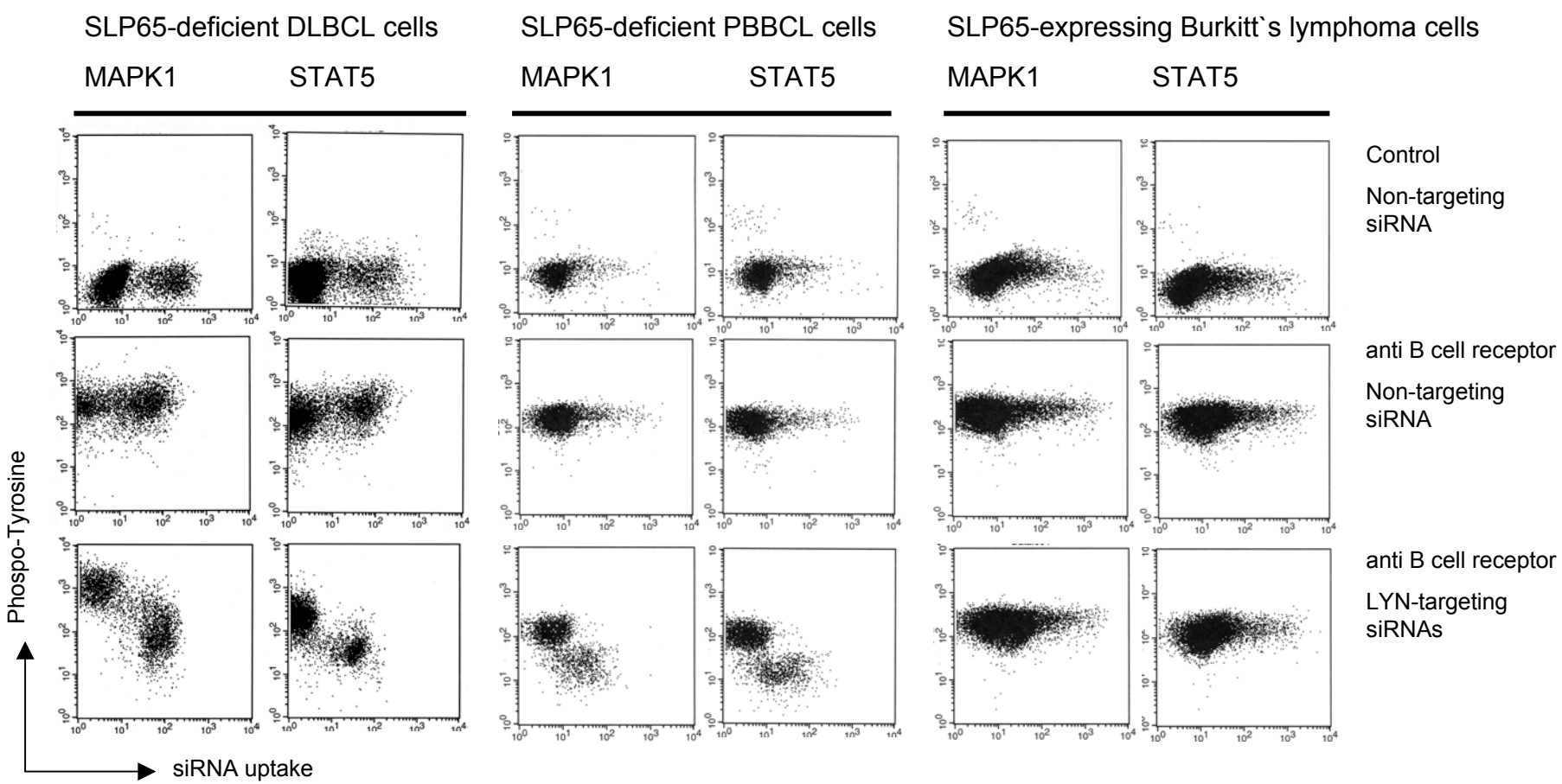

Figure legend to Figure 4: *LYN is required for tyrosine-phosphorylation of STAT5 and MAPK1 in SLP65-deficient B cell lymphoma cells.*
SLP65-deficient diffuse large B cell lymphoma cells (DLBCL; Karpas-422) , SLP65-deficient plasmablastic lymphoma cells (PBBCL, LP-1) and SLP65-expressing Burkitt's lymphoma cells (BL, MHH-PREB) were transfected either with non-targeting fluorochrome-labeled siRNAs or siRNAs against LYN. The cells were analysed for uptake of CY3-labeled siRNAs and STAT5- and MAPK1-tyrosine phosphorylation in response to B cell receptor engagement. Cytoplasmic staining of phosphorylated $STAT5^{Y694}$ and $MAPK1^{Y204}$ was performed using primary antibodies against these phospho-tyrosines (Cell Signaling Technology together with anti-mouse IgG-CY2 and anti-rabbit IgG-CY2 (Jackson Immunoresearch) as secondary antibodies, respectively. Cells were fixed with 0.4% para-formaldehyde and incubated for 10 minutes in 90% methanol on ice and analyzed by flow-cytometry.

Likewise, contribution of SRC-kinase activity to viability of SLP65-deficient versus SLP65-expressing lymphoma cells was compared. Viability of the cells (as defined by exclusion of apoptotic or dead cells by staining for annexin V and propidium iodide uptake) was measured as the ratio of the precentage of living cells at specific incubation times and the percentage of living cells at the outset (Supplementary Figure, C). While SRC-kinase inhibition had no significant effect on SLP65-expressing lymphoma cells, more than 50 percent of cells among the two SLP65-deficient lymphoma cell lines were apoptotic after one week of incubation in the presence of PP2 (Supplementary Figure, C).

SRC-kinase-dependent activation of survival and proliferation signals in B cell lymphoma is in agreement with previous studies that implicate the SRC-kinase LYN in anti-apoptotic signaling in B cell chronic lymhocytic leukemia (Contri et al., 2005) and Philadelphia chromosome-positive acute lymphoblastic leukemia (Ptasznik et al., 2004; Hu et al., 2004). Also LYN-dependent activation of the proliferation-related MAPK1 in SLP65-deficient lymphoma cells is in accordance with two previous studies showing that LYN is essential for proliferation of lymphoma cells either driven by the Karposi sarcoma-associated herpesvirus K1 protein (Prakash et al., 2004) or by interleukin 6 in multiple myeloma (Li et al., 2005).
Consistent with an emerging role of LYN in malignant B cell lymphoproliferation (Prakash et al., 2005; Ptaznik et al., 2004; Hu et al., 2004; Contri et al., 2005; Li et al., 2005), these findings identify the SRC-kinase LYN as an important component of a novel aberrant signaling pathway in SLP65-deficient B cell lymphoma cells.

Supplementary Figure: *Inhibition of SRC kinase activity reduces viability and proliferation of SLP65-deficient B cell lymphoma cells*

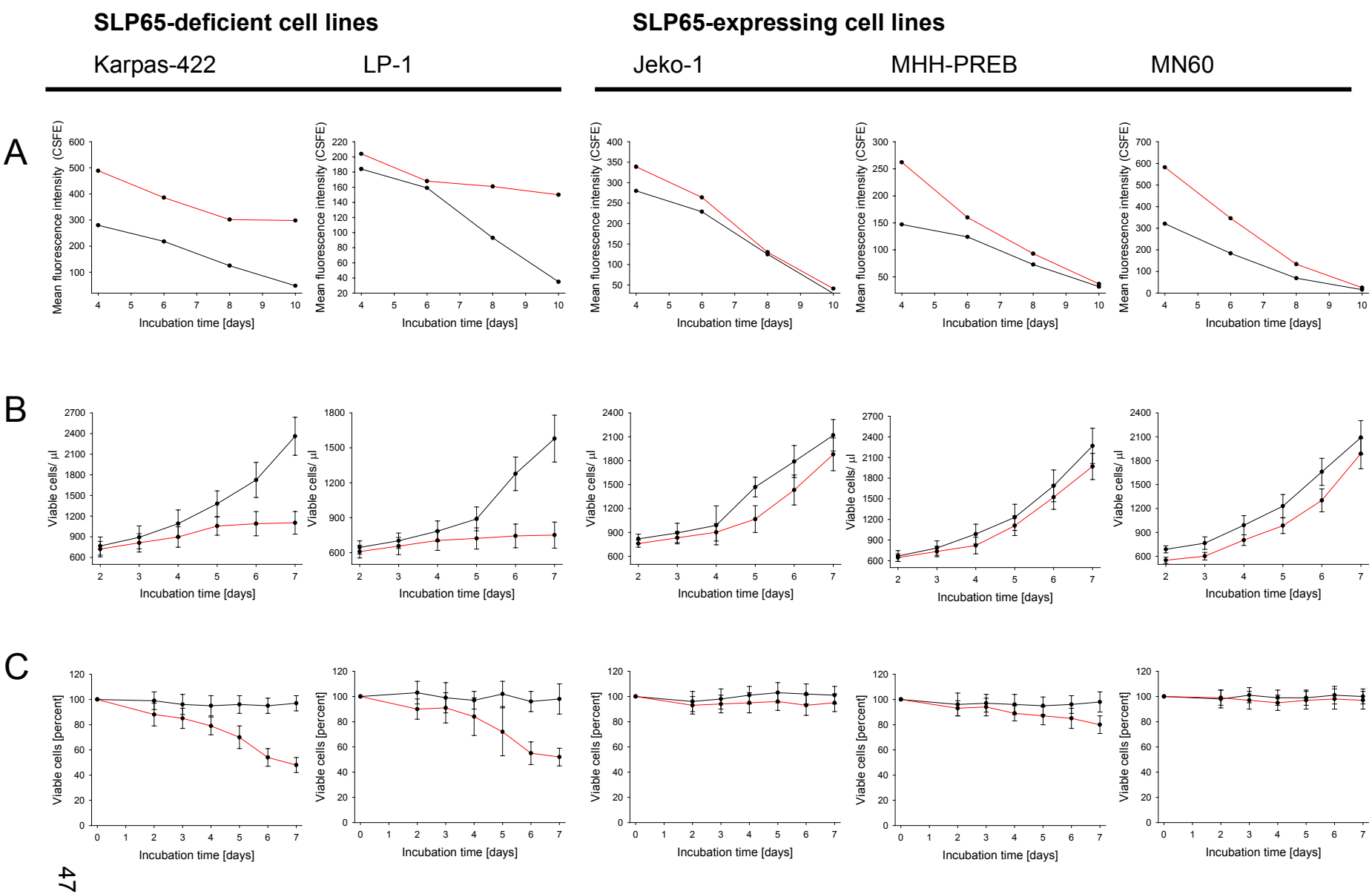

Figure Legend to Supplementary Figure: *Inhibition of SRC-kinase activity reduces viability and proliferation of SLP65-deficient B cell lymphoma cells*.
For measurement of cell divisions, SLP65-deficient (Karpas-422 and LP-1) and SLP65-expressing (Jeko-1, MHH-PREB and MN60) B cell lymphoma cells were labeled with the fluorescent dye carboxyfluorescein diacetate succinimyl ester (CFSE, Calbiochem) as previously described (Cooperman et al., 2004) and incubated in the presence (red lines in A-C) or absence (black lines in A-C) of 10 μmol/l of the SRC kinase-inhibitor PP2 (A). The mean fluorescence intensity was measured as an indicator of cell division-linked dilution of the fluorescent dye. In addition, aliquots of these cell cultures were taken and cells were counted at the times indicated (B). For measurement of viability, cells were incubated in presence (red line) or absence (black line) of PP2 and analysed for staining with antibodies against Annexin V and propidium iodide uptake (C). Viability at the beginning of the incubation time was set as 100 percent.

Supplementary Table 1: *Sequence analysis of aberrant SLP65-splice variants in B cell lymphoma cell lines*

Cell type	Entity	Chromosomal rearrangement	WT SLP65	Somatic mutations	Splice variants Exons involved	Splice sites	Transcript	Predicted Protein
Normal B cells	Not applicable	None	Yes	None	Exon 8	regular	Δ69 bp (in frame)	ΔPRD
					Exon 16	regular	Δ156 bp (in frame)	ΔSH2
MC116	Burkitt's	*MYC-IGH*	Yes	Deletion at 10q23	Exon 3	cryptic	INS 5 bp of Intron 3-4, splice site slippage	
					Exons 5 and 6	regular	Δ321 bp (in frame)	ΔY72, Y84, Y96
					Exon 6	regular	Δ164 bp (STOP)	ΔPRD, SH2
					Exons 4 to 6	regular	Δ362 bp (STOP)	ΔY72, Y84, Y96, PRD, SH2
					Exons 8 and 9	regular	Δ139 bp (STOP)	ΔPRD, SH2
					Exon 8 and 16	regular	Δ225 bp (STOP)	ΔPRD, SH2
Karpas-422	DLBCL	*BCL2-IGH*	No	Deletion at 10q23 LOH: Δ28 bp[a] in Exon 3 and Intron 3-4[a]	Exon 3	regular	Δ50 bp (STOP)	ΔY72,84,96,178,189 ,PRD ,SH2
					Exons 3 and 7 to 16	regular	Δ776 bp (STOP)	ΔY72,84,96,178,189 ,PRD ,SH2
					Exons 3 and 4	regular	Δ91 bp (STOP)	ΔY72,84,96,178,189 ,PRD ,SH2
					Exons 3 to 5	cryptic	Δ12 bp in exon 5	
					Exons 3, 8 and 9	regular	Δ189 bp (in frame)	ΔY72,84,96,178,189 ,PRD ,SH2
					Exon 16	regular	Δ156 bp (in frame)	ΔSH2

Note: a.: Sequence data available from EMBL/GenBank under accession number (pending)
Binding sites for PLCγ2: Y84, Y178, Y189
Binding site for Nck and Vav: Y72
Binding site for BTK: Y96;
PRD: proline-rich-domain; INS: insertion; Δ, deletion; SH2, SRC-homology domain 2

References

Contri, A., A.M. Brunati, L. Trentin, A. Cabrelle, M. Miorin, L. Cesaro, L.A. Pinna, R. Zambello, G. Semenzato, A. Donella-Deana. 2005. Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis. *J Clin Invest*. 115:369-78.

Cooperman J, Neely R, Teachey DT, Grupp S, Choi JK. Cell division rates of primary human precursor B cells in culture reflect in vivo rates. *Stem Cells.* 2004; 22: 1111-11120.

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

Flemming, A., T. Brummer, M. Reth, H. Jumaa. 2003. The adaptor protein SLP-65 acts as a tumor suppressor that limits pre-B cell expansion. *Nat Immunol.* 4: 38-43.

Fu, C., C.W. Turck, T. Kurosaki, A.C. Chan. 1998. BLNK: a central linker protein in B cell activation. *Immunity* 9: 93-103.

Hayashi K, Nittono R, Okamoto N, Tsuji S, Hara Y, Goitsuka R, Kitamura D. The B cell-restricted adaptor BASH is required for normal development and antigen receptor-mediated activation of B cells. *Proc Natl Acad Sci U S A*. 2000; 97: 2755-60.

Hayashi K, Yamamoto M, Nojima T, Goitsuka R, Kitamura D. Distinct signaling requirements for Dμ selection, IgH allelic exclusion, pre-B cell transition, and tumor suppression in B cell progenitors. *Immunity.* 2003;18: 825-36.

Hu, Y., Y. Liu, S. Pelletier, E. Buchdunger, M. Warmuth, D. Fabbro, M. Hallek, R.A. Van Etten, S. Li. 2004. Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. *Nat Genet*. 36: 453-61.

Imai, C., M.E. Ross, G. Reid, E. Coustan-Smith, K.R. Schultz, C.H. Pui, J.R. Downing, D. Campana. 2004. Expression of the adaptor protein BLNK/SLP-65 in childhood acute lymphoblastic leukemia. *Leukemia.* 18: 922-925.

Ishiai M, Sugawara H, Kurosaki M, Kurosaki T. Cutting edge: association of phospholipase C-γ2 Src homology 2 domains with BLNK is critical for B cell antigen receptor signaling. *J Immunol.* 1999; 163:1746-1749.

Jumaa H, Bossaller L, Portugal K, Storch B, Lotz M, Flemming A, Schrappe M, Postila V, Riikonen P, Pelkonen J, Niemeyer CM, Reth M. Deficiency of the adaptor SLP-65 in pre-B-cell acute lymphoblastic leukaemia. *Nature*. 2003; 423: 452-6.

Karras, J.G., Z. Wang, S.J. Coniglio, D.A. Frank, T..L Rothstein. 1996. Antigen-receptor engagement in B cells induces nuclear expression of STAT5 and STAT6 proteins that bind and transactivate an IFN-γ activation site. *J Immunol*. 157: 39-47.

Kersseboom, R., S. Middendorp, G.M. Dingjan, K. Dahlenborg, M. Reth, H. Jumaa, R.W. Hendriks. 2003. Bruton's tyrosine kinase cooperates with the B cell linker protein SLP-65 as a tumor suppressor in pre-B cells. *J Exp Med*. 198: 91-98.

Klein, F., N. Feldhahn, L. Harder, H. Wang, M. Wartenberg, W.K. Hofmann, P. Wernet, R. Siebert, M. Müschen. 2004. 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*. 199: 673-685.

Li FJ, Tsuyama N, Ishikawa H, Obata M, Abroun S, Liu S, Otsuyama K, Zheng X, Ma Z, Maki Y, Kawano MM. A rapid translocation of CD45RO but not CD45RA to lipid rafts in IL-6-induced proliferation in myeloma. *Blood.* 2005; 105: 3295-302.

Middendorp S, Dingjan GM, Maas A, Dahlenborg K, Hendriks RW. Function of Bruton's tyrosine kinase during B cell development is partially independent of its catalytic activity. *J Immunol*. 2003;171: 5988-5996.

Minegishi Y, Rohrer J, Coustan-Smith E, Lederman HM, Pappu R, Campana D, Chan AC, Conley ME. An essential role for BLNK in human B cell development. *Science.* 1999; 286:1954-1957.

Pappu R, Cheng AM, Li B, Gong Q, Chiu C, Griffin N, White M, Sleckman BP, Chan AC. Requirement for B cell linker protein (BLNK) in B cell development. *Science.* 1999; 286:1949-1954.

Prakash, O., O.R. Swamy, X. Peng, Z.Y. Tang, L. Li, J.E. Larson, J.C. Cohen, J. Gill, G. Farr, S. Wang, F. Samaniego. 2005. Activation of Src kinase Lyn by the Kaposi sarcoma-associated herpesvirus K1 protein: implications for lymphomagenesis. *Blood.* 105: 3987-94.

Ptasznik, A., Y. Nakata, A. Kalota, S.G. Emerson, A.M. Gewirtz. 2004. Short interfering RNA (siRNA) targeting the Lyn kinase induces apoptosis in primary, and drug-resistant, BCR-ABL1(+) leukemia cells. *Nat Med*. 10: 1187-9

Reppel M, Sasse P, Piekorz R, Tang M, Roell W, Duan Y, Kletke A, Hescheler J, Nürnberg B, Fleischmann BK. S100A1 enhances the L-type Ca^{2+} current in embryonic mouse and neonatal rat ventricular cardiomyocytes. *J Biol Chem.* 2005; 280: 36019-36028.

Richards, J.D., S.H. Dave, C.H. Chou, A.A. Mamchak, A.L. DeFranco. 2001. Inhibition of the MEK/ERK signaling pathway blocks a subset of B cell responses to antigen. *J Immunol*. 166:3855-64.

Wienands J, Schweikert J, Wollscheid B, Jumaa H, Nielsen PJ, Reth M. SLP-65: a new signaling component in B lymphocytes which requires expression of the antigen receptor for phosphorylation. *J Exp Med.* 1998; 188: 791-795.

Chapter 4

An alternative SLP65 isoform lacking exon 16 in normal B lymphocytes

Summary

SLP65 is an important adaptor protein in (pre-) B cell receptor signaling. We identified an alternatively spliced isoform lacking exon 16 (SLP65ΔE16) in normal B lymphocytes. Exon 16 is a major part of the SH2 domain which is known to bind Igα (Engels et al., 2001; Kabak et al., 2002). While SLP65 is essential for B cell receptor-induced Ca^{2+} signaling, SLP65ΔE16 induces a weaker Ca^{2+} signal upon BCR stimulation. Expression analysis shows that SLP65ΔE16 is expressed in all normal B cell subsets with particularly high expression in B1 cells, and also in most pre-B cell leukemia and B cell lymphoma cells. As B1 cells are frequently autoreactive (Herzenberg, 2000), we investigated the role of SLP65ΔE16 in autoimmunity. Overstimulation of the B cell receptor cells indeed caused upregulation of SLP65ΔE16. Ca^{2+} measurement of SLP65-expressing cells transfected with a vector encoding SLP65ΔE16 showed a diminished Ca^{2+} signal indicating that SLP65ΔE16 is a dominant-negative isoform of SLP65. Phosphorylation studies show that SLP65ΔE16 is not phosphorylated upon BCR-stimulation by itself but is forming a complex with other phosphorylated signaling proteins. A major process by which autoreactive B cells are deleted is BCR-induced apoptosis (Rolink et al., 2001). We conclude that SLP65ΔE16 may play an important role in the protection of autoreactive B cells against BCR-induced apoptosis.

Introduction

SLP65 (also known as BLNK or BASH) is known as an important adaptor protein in (pre-) B cell receptor (BCR) signaling (Hayashi et al., 2000; Wienands et al., 1998). After phosphorylation by the kinase Syk, SLP65 couples the kinase BTK to PLCγ2, resulting in the phosphorylation and activation of the latter (Kurosaki and Tsukada, 2000). Activated PLCγ2 cleaves membrane-associated phosphoinositide PI(4,5)P2 into the second messengers I(1,4,5)P3 and DAG. I(1,4,5)P3 generation leads to the release of Ca^{2+} from the endoplasmic reticulum (Fu et al. 1998). SLP65 has binding domains for BTK, PLCγ2 and other signaling molecules at its N terminus (Chiu et al., 2002). The N-terminal basic domain of SLP65 is responsible for its membrane association (Köhler et al., 2005). The Src homology 2 (SH2) domain of SLP65 interacts with a phosphorylated non-immunoreceptor tyrosine-based activation motif (non-ITAM) tyrosine in the (pre-)BCR signal transduction subunit immunoglobulin α (Igα) (Engels et al., 2001; Kabak et al., 2002).

An alternatively spliced isoform lacking exon 8 (BLNK-S) has been found in normal B lymphocytes (Fu et al., 1998). We characterised another SLP65 isoform in normal B lymphocytes lacking exon 16, a part of the SH2 domain.

Results and discussion

Characterisation of SLP65ΔE16

Sequence analysis of SLP65 in normal B lymphocytes resulted in the characterisation of the full-length transcript and three different isoforms. Among these isoforms was the already characterised BLNK-S lacking exon 8, a part of the proline-rich domain (Fu et al., 1998). However, we also identified a SLP65-isoform lacking amino acids 366 to 417 which is an in-frame deletion of exon 16, a part of the SH2 domain (Figure1). The third isoform was lacking both exon 8 and exon 16.

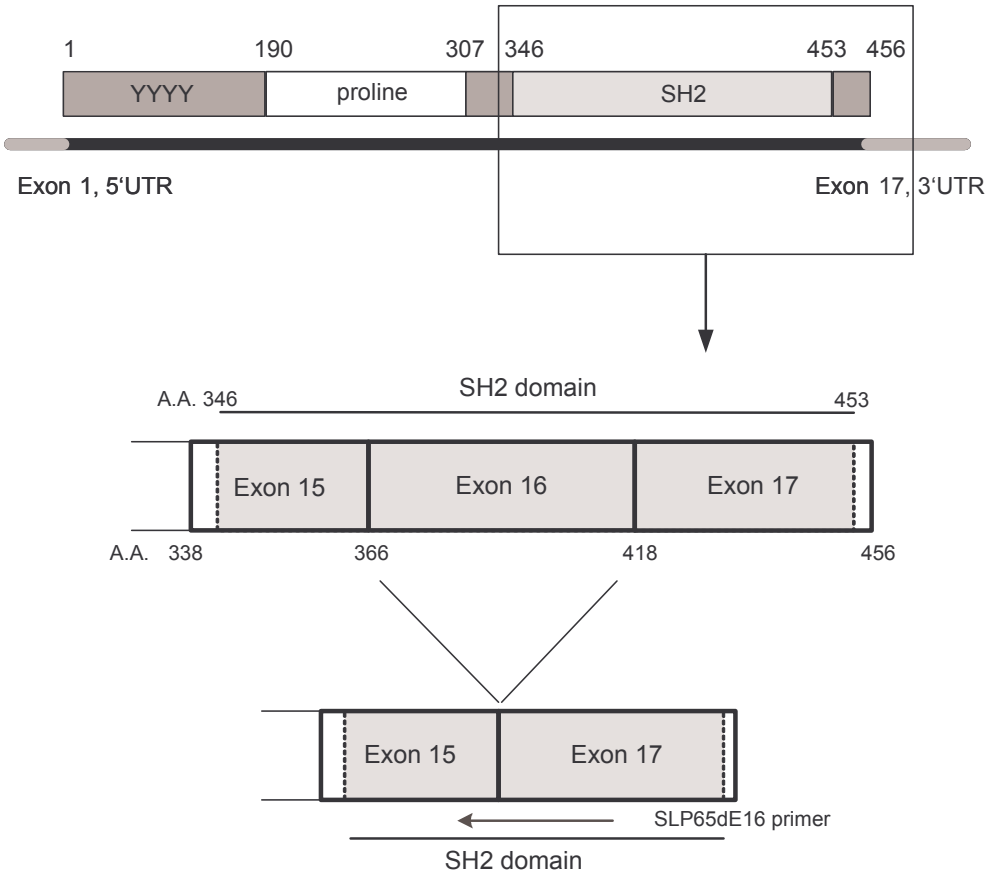

Figure 1: *SLP65 structure*
Overview of the SLP65 domains indicating the N terminus containing many tyrosine binding domains for the SH2 domains of BTK, PLCγ2, Vav and Nck, the proline-rich domain binding the SH3 domain of Grb2; and the SH2 domain (blue) before and after splicing of exon 16. A part of exon 15 (amino acids 338 to 365), exon 16 (amino acids 366 to 417) and a part of exon 17 (amino acids 418 to 456) form the SH2 domain (amino acids 346 to 453). Characterisation of the presence of SLP65ΔE16 is performed by RT-PCR using a reverse primer spanning exons 15 and 17 as indicated with the arrow in the lower figure.

By skipping of exon 16, a major part of the SLP65 SH2 domain is deleted. As the SH2 domain of SLP65 binds directly to Y204 of the non-ITAM of Igα, which is required for SLP65-dependent signaling (Kabak et al., 2002), SLP65ΔE16 functionality would be expected to be disturbed. Therefore Ca^{2+} -release was measured in murine $SLP65^{-/-}$ pre-B cells reconstituted with SLP65 full-length, SLP65ΔE16 or with SLP65ΔSH2. Transfection with the vector encoding full-length SLP65 induced Ca^{2+} release upon stimulation with anti-BCR antibodies (Figure 2). Upon transfection with the vector encoding SLP65ΔSH2, no Ca^{2+}signal could be determined after BCR stimulation (data not shown). However, cells reconstituted with SLP65ΔE16 showed a weaker Ca^{2+} signal (Figure 2) indicating that parts of the SH2 domain not encoded by exon 16 also substantially contribute to SLP65 function. Ca^{2+}-signaling leads to differentiation which is indicated by downregulation of the pre-BCR

and κ gene rearrangements (Flemming et al., 2003). According with Ca^{2+} signalling in the SLP65-expressing cells, the pre-BCR is downregulated and κ light chains were expressed. In the SLP65ΔE16 reconstituted cells, pre-BCR downregulation was diminished and κ rearrangement was less as compared to the SLP65-wild type expressing cells (Figure 2).

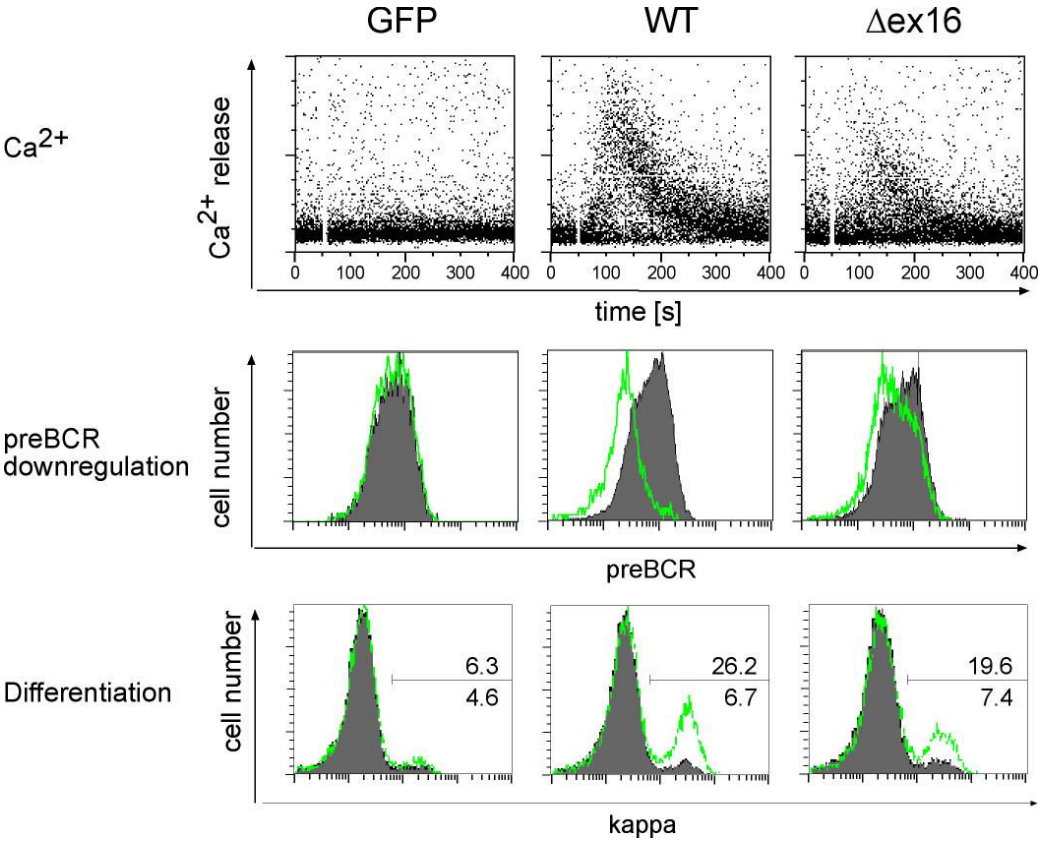

Figure 2: *Ca^{2+} response of SLP65 and SLP65ΔE16*

Murine SLP65-deficient pre-B cells are reconstituted with SLP65 full-length (wt), SLP65ΔE16 (delta-ex16) and as a control a GFP-transgene. Upon BCR-stimulation with an anti-μ antibody, a Ca^{2+} signal is induced in cells expressing full-length SLP65. This Ca^{2+}-signal is reduced in cells expressing SLP65ΔE16. As Ca^{2+} signalling leads to differentiation, two differentiation markers are measured: pre-BCR downregulation and kappa light chain expression. Grey histogram-plots show the non-transfected cells, green plots the transfected pre-B cell line. The percentage of kappa-rearranging cells are indicated in the plots: lower numbers indicate spontaneous differentiation in the non-transfected cells, upper numbers differentiation the transfected cells.

Expression analysis of SLP65ΔE16

SLP65 isoforms were characterised in mature peripheral blood $CD19^+$ B cells. To determine its expression pattern in different B cell subsets and B cell leukemia and lymphoma cells, RT-PCR was performed using a reverse primer overlapping exon 17 to 15 (as indicated in Figure 1) which amplifies when exon 16 is missing. Figure 3A shows that SLP65ΔE16 is present in 9 out of 10 B cell acute lymphoblastic leukemia cell lines (ALL), 4 out of 4 mantle cell

lymphomas (MCL), 3 out of 3 Burkitt's lymphomas (BL), 3 out of 3 diffuse large B cell lymphomas (DLBCL), 2 out of 4 Hodgkin lymphomas (HL), 1 out of 1 plasma cell leukemia (PC) and is not expressed in multiple myeloma (MM). SLP65ΔE16 has not been found in non-B cells (including 293, Hela, K562, Jurl-22, Ku-182, Lama-84, MEG, JK1, $CD3^+$, Jurkat). As these cDNAs are not normalised and RT-PCR is performed for 45 cycles nothing can be said about the relative amounts of expression. Figure 3B shows that SLP65ΔE16 is expressed in all B cell subsets including pre-B cells, B1 cells, naïve B cells, centrocytes, centroblasts, memory cells and plasma cells with the highest level of expression in B1 cells. This result is confirmed by Western blot comparing normalised amounts of naïve B cells, B1 cells and memory B cells (Figure 3C).

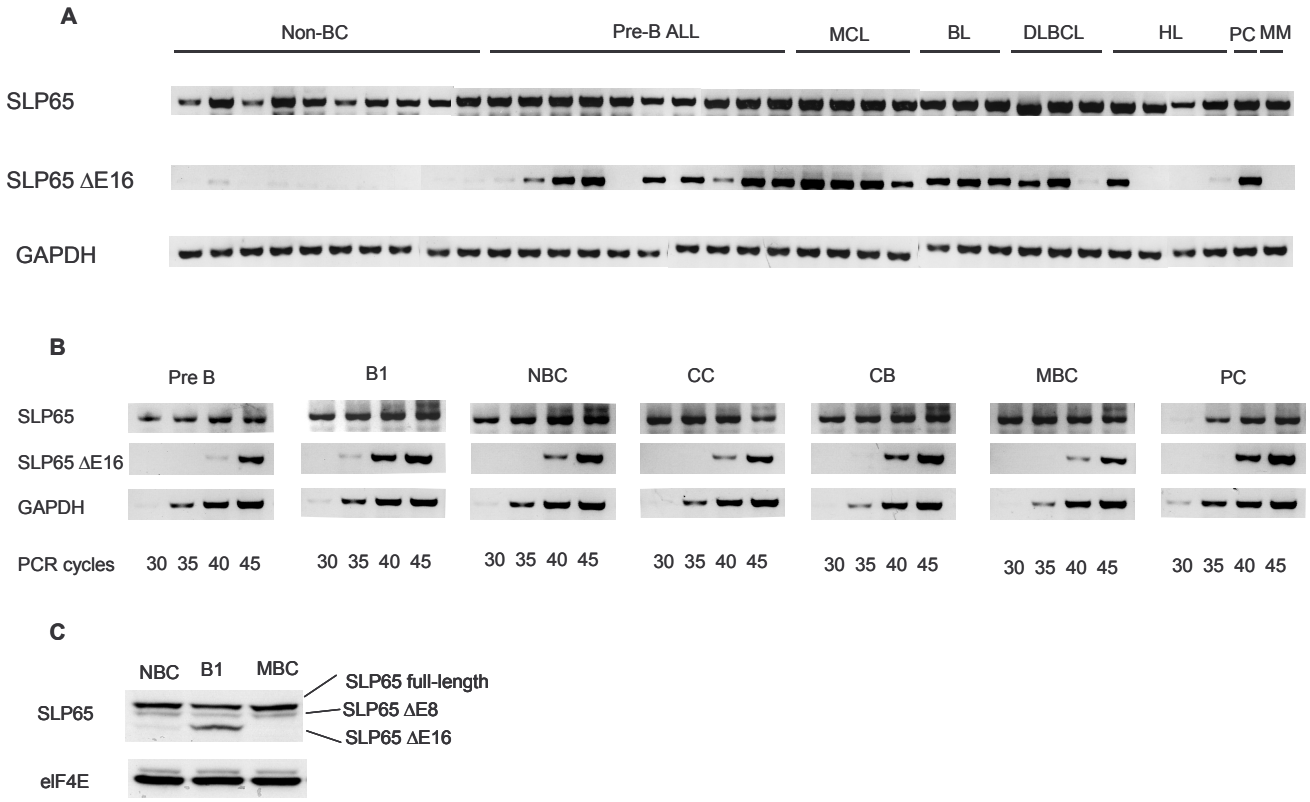

Figure 3: *Expression pattern of SLP65 and SLP65ΔE16*

SLP65 and SLP65ΔE16 mRNA (A and B) and protein (C) expression has been characterised by RT-PCR and Western blot. *SLP65* and *GAPDH* transcripts were amplified as previously described (Klein et al., 2004). Different non-B lymphocytes (Non-BC: 293, Hela, K562, Jurl-22, Ku-182, Lama-84, MEG, JK1, $CD3^+$, Jurkat), pre-B cell acute lymphoblastic leukemia (pre-B ALL), mantle cell lymphoma (MCL), Burkitt's lymphoma (BL), diffuse large B cell lymphoma (DLBCL), Hodgkin's lymphoma (HL), plasma cell leukemia (PC) and multiple myeloma (MM) were amplified. cDNA amounts were normalised for equal expression of GAPDH as a reference gene (A). A semi-quantitative RT-PCR shows expression of SLP65 and SLP65ΔE16 in the different B cell subsets: pre-B cells (Pre B), B1 cells, naïve B cells (NBC), centrocytes (CC), centroblasts (CB), memory B cells (MBC) and plasma cells (PC). cDNA amounts are normalised by GAPDH expression (B). For Western blot expression of

full-length SLP65, SLP65ΔE8 and SLP65ΔE16 and EIF4E (Cell Signaling Technology, Beverly, MA), total cell lysates of naïve B cells (NBC), B1 cells and memory B cells (MBC) were used. As a loading control an eIF4E antibody was used (C).

SLP65ΔE16 involvement in autoreactivity

Expression analysis showed a high expression of SLP65ΔE16 in B1 cells. B1 cells are a minor population of B lineage cells and are distinguished by their expression of CD5 (Hardy et al., 1994). B1 cells are a major contributor of IgM to the serum, but generate a restricted antibody repertoire dominated by a specific set of V genes, many of which encode autoreactive antibodies (Bath et al., 1992; Herzenberg, 2000; Kasaian and Casali, 1993; Murakami and Honjo, 1995; Shirai et al., 1991; Sidman et al., 1986). Therefore we investigated the role of SLP65ΔE16 in autoimmunity.

$CD19^+$ B cells were incubated with the caspase inhibitor ZVAD to inhibit direct apoptosis and cultured for 18 hours in the presence or absence of an anti- μ antibody. The extended BCR-overstimulation creates the atmosphere of autoimmunity. Indeed, both RT-PCR as Western blot analysis showed an upregulation of SLP65ΔE16 after anti-μ treatment (Figure 4A and B).

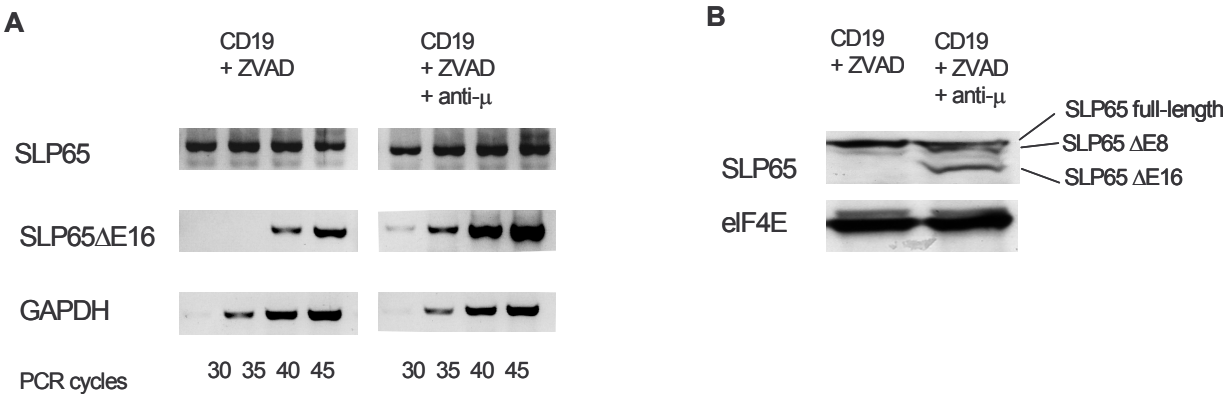

Figure 4: *SLP65ΔE16 upregulation under autoreactive circumstances*
Autoreactivity is created upon extended BCR-overstimulation of $CD19^+$ cells with an anti-μ antibody (Jackson ImmunoResearch, Cambridgeshire, UK). To inhibit direct apoptosis cell are treated with ZVAD (Calbiochem, Schwalbach, Germany). $CD19^+$ B cells were purified from peripheral blood of four healthy donors using immunomagnetic beads against CD19 (Miltenyi Biotech, Bergisch Gladbach, Germany). Upregulation of SLP65ΔE6 upon μ-treatment is shown by RT-PCR (A) and Western blot (B). As controls GAPDH was amplifies and an eIF4E antibody in Western blot was used.

These findings suggest that SLP65ΔE16 might play a negative role in BCR-signaling in autoreactive B cells. As full-length SLP65 expression does not seem affected, SLP65ΔE16 could play a dominant-negative role. A dominant-negative role of alternatively spliced genes

in the regulation of BCR-signaling has been detected in leukemic B lymphocytes, e.g. IKAROS (Sun et al., 1996), BTK (Feldhahn et al., 2005). Therefore we transfected two SLP65-expressing cell lines (MHH-preB and Jeko-1) with a control vector expressing GFP and a vector encoding SLP65ΔE16. Western blot analysis shows that expression levels of SLP65ΔE16 in these transfected cells are similar as SLP65ΔE16 expression levels of normal B1 cells (Figure 5A). The cells transfected with the control vector show a normal Ca^{2+} response upon receptor stimulation. However the Ca^{2+} signal in the Jeko-1 cells transfected with the vector encoding SLP65ΔE16 was downregulated (Figure 5B). The effect on the Ca^{2+} signal in the MHH-preB cells transfected with the vector encoding SLP65ΔE16 was not significant.

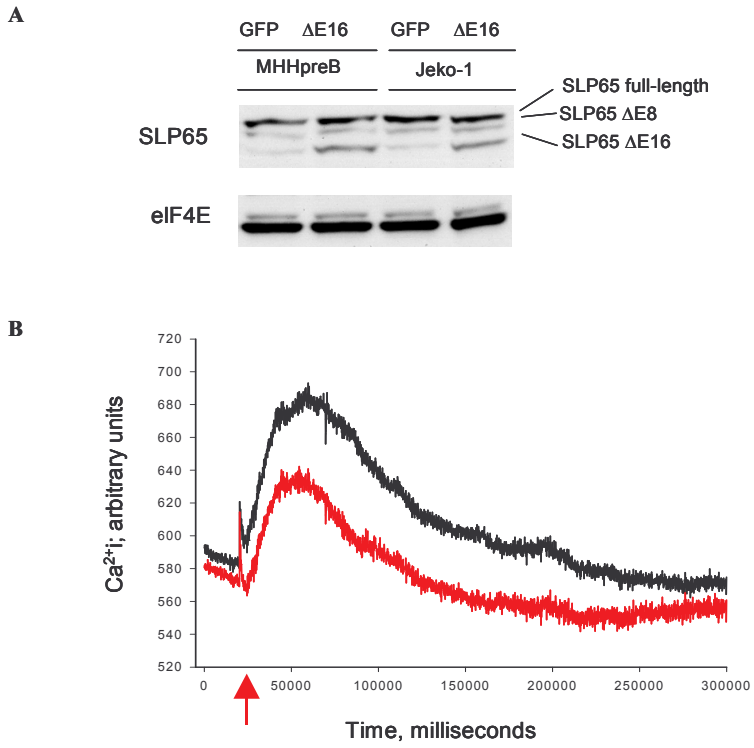

Figure 5: *Effect of SLP65ΔE16 expression on the Ca^{2+} response in normal B cells*
Two SLP65-expressing cell lines (MHH-preB and Jeko-1) are transfected with the vector expressing SLP65ΔE16. As a control, the cells are transfected with a vector expressing GFP. Western blot analysis shows the expression-level of SLP65 in the transfected cells (A). The Ca^{2+} response for Jeko-1 is measured upon antibody stimulation (B). The black line shows the control cells transfected with a vector expressing GFP. The red line shows the effect of the transfection with the vector expressing SLP65ΔE16.

This dominant-negative effect of SLP65ΔE16 on normal B cell signaling could be another mechanism of negative regulation of BCR-signaling. Known mechanisms of negative BCR-signaling include the signaling through immunoreceptor tyrosine-based inhibitory

motifs (ITIMs) that recruit either SHIP or SHP-1 and inhibit Ca^{2+} signals (D'Ambrosio et al., 1996; Doody et al., 1995). Another example includes the release of Lyn into the cytosol following caspase-dependent cleavage which acts as a negative regulator of BCR-induced apoptosis (Luciano et al., 2003).

Mechanism of negative BCR-regulation through SLP65ΔE16

As SLP65ΔE16 can not bind Igα, is not able to introduce a Ca^{2+}-response and even inhibits Ca^{2+} signaling in SLP65 expressing cells, we determined if SLP65ΔE16 could still be phosphorylated and if it was still able to bind other phosphorylated signaling proteins. Murine $SLP65^{-/-}$ pre-B cells were transfected with a control vector encoding GFP and a vector encoding SLP65ΔE16. These cells were stimulated with an anti-μ antibody and whole cell lysates were made from stimulated and non-stimulated cells. As a control, whole cell lysates were made from the SLP65-expressing cell line MHH-preB. To test whether SLP65ΔE16 could still bind other signaling proteins, a co-immunoprecipitation was performed with a 4G10 antibody which globally binds phosphorylated tyrosine residues. Western blot analysis with an antibody detecting SLP65 shows a band with SLP65ΔE16 transfected cells which means that SLP65ΔE16 forms a complex with phosphorylated signaling proteins or is tyrosine-phosphorylated by itself (Figure 6). However, Western blot analysis with an antibody detecting phosphorylation of SLP65 at tyrosine residue 96, shows no band in the SLP65ΔE16-transfected cells (Figure 6), indicating that SLP65ΔE16 is not phosphorylated at this residue.

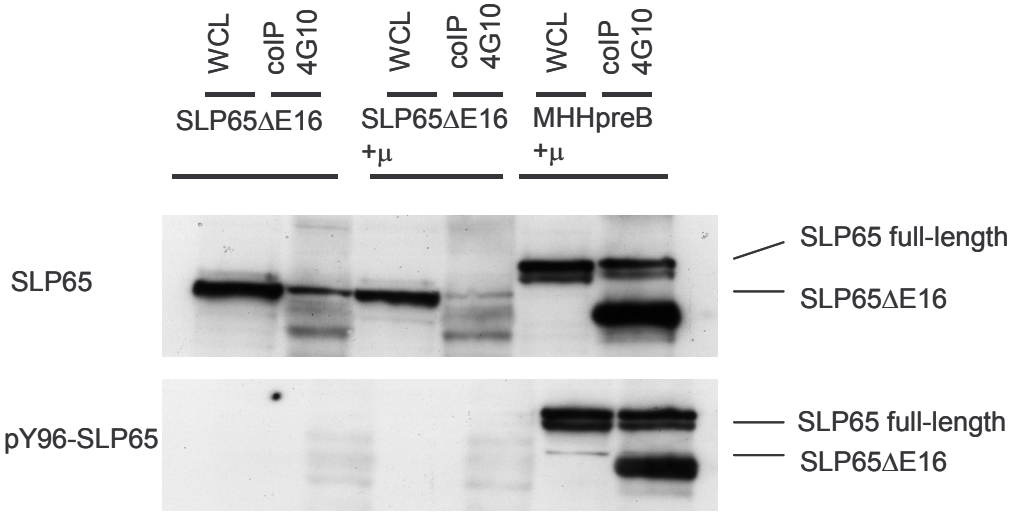

Figure 6: *SLP65ΔE16 phosphorylation and complex formation*
To test the phosphorylation status of SLP65ΔE16 and its interaction with phosphorylated proteins, whole cell lysates (WCL) or co-immunoprecipitates (coIP) with the 4G10 antibody (Cell signaling) were loaded for Western blotting. Co-immunoprecipitation has been performed as previously described (Feldhahn et al., 2005). Western blot analysis is performed with a SLP65 antibody (Cell Signaling) or a antibody detecting phosphorylated SLP65 at Y96 (Cell Signaling). Samples are SLP65$^{-/-}$ pre B cells transfected with a vector encoding SLP65ΔE16 and as a control MHHpreB cells expressing full-length SLP65. Cells are stimulated with an anti-μ antibody.

Discussion

We showed that an alternatively spliced isoform of SLP65 lacking exon 16 (SLP65ΔE16) is expressed in all B cell subsets and is upregulated in B1 cells. As many B1 cells are known to be autoreactive we show that SLP65ΔE16 may play a role in negative BCR-signaling. Autoreactive immature B cells are eliminated by negative selection (BCR-induced apoptosis) or inactivated (anergy) if their BCR-specificity is not revised by receptor editing (Niro and Clark, 2002). SLP65ΔE16 acts in a dominant-negative way inhibiting Ca^{2+} signaling in the presence of full-length SLP65. We show that SLP65ΔE16 is not phosphorylated by itself but forms a complex with other tyrosine phosphorylated signaling proteins. In this way, it might protect autoreactive B cells against BCR-induced apoptosis and be the molecular basis of so-called “anergy”.

With its truncated SH2-domain, SLP65ΔE16 might not only be unable to bind Igα (Engels et al., 2001; Kabak et al., 2002). Also hematopoietic progenitor kinase 1 (HPK1) has been determined to bind the SH2 domain of SLP65 (Sauer et al., 2001). This serine/threonine kinase is activated upon BCR-engagement (Liou et al., 2000). In SLP65$^{-/-}$

DT40 cells and in SLP65ΔSH2 expressing DT40 cells, HPK1 activation was reduced but not completely abolished (Tsuji et al., 2001). HPK1 activation results in the activation of the IKK complex and NF-κB translocation to the nucleus (Arnold et al., 2001). Also BCR-signaling finally leads to the activation of the transcription-factor NF-κB (Saijo et al., 2000) (Figure 7A). This indicates that SLP65ΔE16 could negatively regulate two independent pathways downstream of the BCR, which are BTK-mediated PLCγ2 activation and HPK1 activation, both responsible for NF-κB activation.

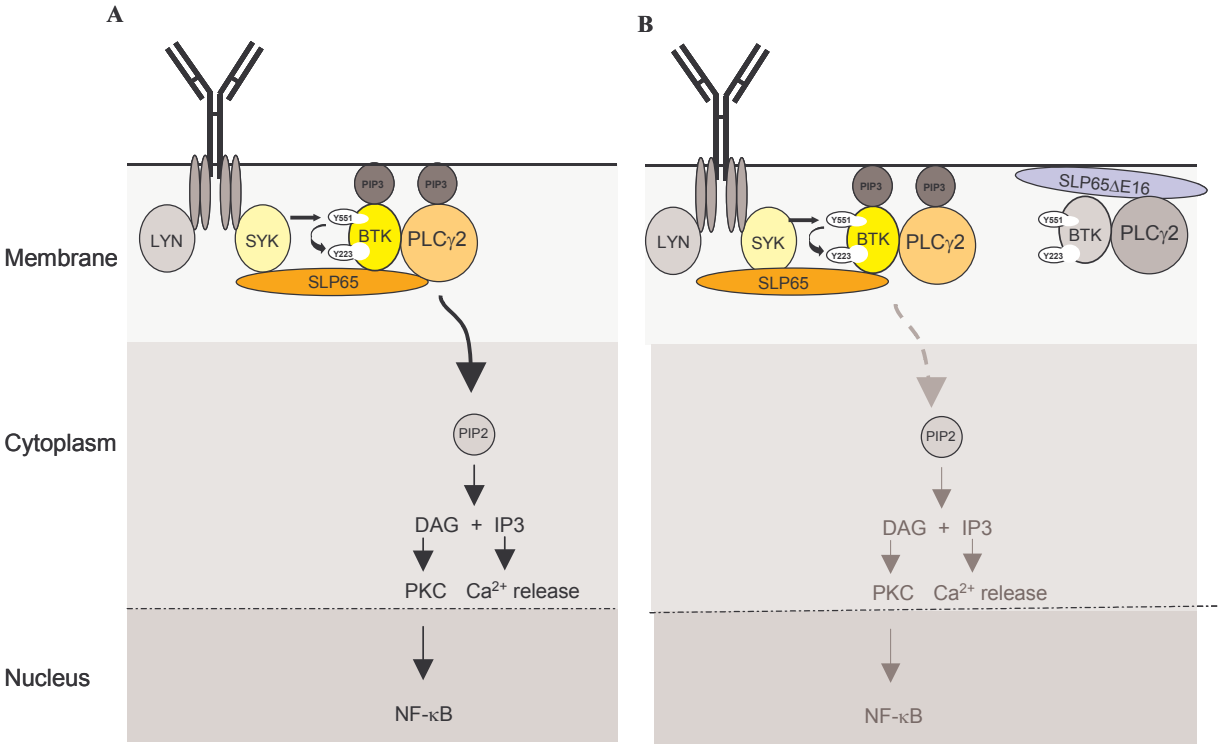

Figure 7: *BCR-dependent signaling in normal B cells (A) and in cells expressing SLP65ΔE16 (B)*

References:

Arnold, R., Liou, J., Drexler, H.C., Weiss, A., Kiefer, F. (2001) Caspase-mediated cleavage of hematopoietic progenitor kinase 1 (HPK1) converts an activator of NFkappaB into an inhibitor of NFkappaB. *J. Biol. Chem.* **276**, 14675-14684

Bhat, N.M., Kantor, A.B., Bieber, M.M., Stall, A.M., Herzenber, LA., Teng, N.N. (1992) The ontogeny and functional characteristics of human B-1 (CD5+ B) cells. *Int. Immunol.* **4**, 243-252

Chiu, C.W., Dalton, M., Ishiai, M., Kurosaki, T., Chan, A. (2002) BLNK: molecular scaffolding through 'cis'-mediated organization of signaling proteins. *EMBO J.* **21**, 6461-6472

D'Ambrosio, D., Fong, D.C., Cambier, J.C. (1996) The SHIP phosphatase becomes associated with Fc gammaRIIB1 and is tyrosine phosphorylated during 'negative' signaling. *Imunol. Lett.* **54**, 77-82

Doody,G.M., Justement, L.B., Delibrias, C.C., Matthews, R.J., Lin, J., Thomas, M.L., Fearon, D.T. (1995) A role in B cell activation for CD22 and the protein tyrosine phosphatase SHP. *Science.* **269**, 242-244

Engels, N., Wollscheid, B., Wienands, J. (2001) Association of SLP65/BLNK with the B cell antigena receptor through a non-ITAM tyrosine of Ig-α. *Eur. J. Immunol.* **31**, 2126-2134

Feldhahn, N., Klein, F., Mooster, J.L., Hadweh, P., Sprangers, M., Wartenberg M.M., Bekhite, W.K., Hofmann, S., Herzog, H., Jumaa, H., Rowley, J.D., Müschen, M. (2005) Mimicry of a contitutively active pre-B cell receptor in acute lymphoblastic leukemia cells. *J. Exp. Med.* **201**, 1837-1852

Feldhahn, N., Rio, P., Ndikung Bejeng Soh, B., Liedtke, S., Sprangers, M., Klein, F., Wernet, P., Jumaa, H., Hofmann, W., Hanenberg, H., Rowley, J.D., Müschen, M. (2005) Deficiency of Bruton's tyrosine kinase in B cell precursor leukemia. *Proc. Natl. Acad. Sci. USA.* **102**, 13266-13271

Flemming, A., Brummer, T., Reth, M., Jumaa, H. (2003) The adaptor protein SLP65 acts as a tumoursuppressor that limits pre-B cell expansion. *Nature Immunol.* **4**, 38-43

Fu, C., Turck, C.W., Kurosaki, T., Chan, A.C. (1998) BLNK-A central linker protein in B cell activation. *Immunity.* **9**, 93-103

Hardy, R.R., Carmack, C.E., Li, S., Hayakawa, K. (1994) Distinctive developmental origins and specificities of murine CD5+ B cells. *Immunol. Rev.* **137**, 91-98

Hayashi, K., Nittono, R., Okamoto, N., tsuji, S., Hara, Y., Goitsuka, R., Kitamura, D. (2000) The B cell-restricted adaptor BASH is required for normal development and antigen receptor-mediated activation of B cells. *Proc. Natl. Acad. Sci USA.* **97**, 2755-2760

Herzenberg, L.A. (2000) B-1 cells: the lineage question revisited. *Immunol. Rev.* **175**, 9-22

Kabak, S., Skaggs, B.J., Gold, M.R., Affolter, M., West, K.L., Foster, M.S., Siemasko, K., Chan, A.C., Aebersold, R., Clark, M.R. (2002) The direct recruitment of BLNK to immunoglobulin α couples the B-cell antigen receptor to distal signaling pathways. *Mol. Cell. Biol.* **22**, 2524-2535

Kasaian, M.T., Casali, P. (1993) Autoimmunity-prone B-1 (CD5 B) cells, natural antibodies and self recognition. *Autoimmunity.* **15**, 315-329

Klein, F., Feldhahn, N., Harder, L., Wang, H., Wartenberg, M., Hofmann, W.K., Wernet, P., Siebert, R., Müschen, M. (2004) 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.* **199**, 673-685

Köhler, F., Storch, B., Kulathu, Y., Herzog, S., Kuppig, S., Reth, M., Jumaa, H. (2005) A leucine zipper in the N terminus congers membrane association to SLP65. *Nat. Immunol.* **6**, 204-210

Kurosaki, T., Tsukada, S. (2000) BLNK: connecting Syk and BTK to calcium signals. *Immunity.* **12**, 1-5

Liou, J., Kiefer, F., Dang, A., Hashimoto, A., Cobb, M.H., Kurosaki, T., Weiss, A. (2000) HPK1 is activated by lymphocyte antigen receptors and negatively regulates AP-1. *Immunity.* **12**, 399-408

Luciano, F., Herrant, M., Jacquel, A., Ricci, J., Auberger, P. (2003) The P54-cleaved form of the tyrosine kinase Lyn generated by caspases during BCR-induced cell death in B lymphoma acts as a negative regulator of apoptosis. *FASEB J.* **17**, 711-735

Murakami, M., Honjo, T. (1995) Involvement of B-1 cells in mucosal immunity and autoimmunity. *Immunol. Today.* **16**, 534-539

Rolink, A.G., Schaniel, C., Andersson, J., Melchers, F. (2001) Selection events operating at various stages in B cell development. *Curr. Opin. Imunol.* **13**, 202-207

Saijo, K., Mecklenbräuker, I., Santana, A., Leitger, M., Schmedt, C., Tarakhovsky, A. (2002) Protein kinase C β controls nuclear factor κB activation in B cells through selective regulation of the IκB kinase α. *J.Exp.Med.* **195**, 1647-1652

Sauer, K., Liou, J., Singh, S.B., Yablonski, D., Weiss, A., Perlmutters, R.M. (2001) Hematopoietic progenitor kinase 1 associates physically and functionally with the adaptor proteins B cell linker protein and SLP76 in lymphocytes. *J. Biol. Chem.* **276**, 45207-45216

Shirai, T., Hirose, S., Okada, T., Nishimura, H. (1991) CD5+ B cells in autoimmune disease and lymphoid malignancy. *Clin. Immunol. Immunopathol.* **59**, 173-186

Sidman, C.L., Shultz, L.D., Hardy, R.R., Hayakawa, K., Herzenberg, L.A. (1986) Production of immunoglobulin isotypes by Ly-1+ B cells in viable motheaten and normal mice. *Science.* **232**, 1423-1425

Sun, L., Georgopoulos, K. (1996) Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development. *EMBO J.* **15**, 5358-5369

Tsuji, S., Okamoto, M., Yamada, K., Okamoto, N., Goitsuka, R., Arnold, R., Kiefer, F., Kitamura, D. (2001) B cell adaptor containing src homology 2 domain (BASH) links B cell receptor signaling to the activation of hematopoietic progenitor kinase 1. *J. Exp. Med.* **194**, 529-539

Wienands J., Schweikert, J., Wollscheid, B., Jumaa, H., Nielsen, P.J., Reth, M. (1998) SLP-65: a new component in B lymphocytes whih requires expression of the antigen receptor for phosphorylation. *J. Exp. Med.* **188**, 791-795

Chapter 5

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 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

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, □41 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.
1. 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, Δ24bp exon 19	***Truncated KD***	***n.d.***
	XV	Exon 14	Cryptic	ΔExon 14, Δ62 bp exon 13, ***2. 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, Δ33bp exon 18	Truncated KD	n.d.
	MHH-CALL3	Exons 13 to 17	Cryptic	ΔExons 13-17, Δ13 bp exon 12, Δ92bp 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)
d.: Roman numerals indicate primary cases of B cell precursor leukemia, cell lines are indicated by their names

SH2: SRC homology domain 2; KD: kinase domain; Ins: Insertion; n.d., not done

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 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).

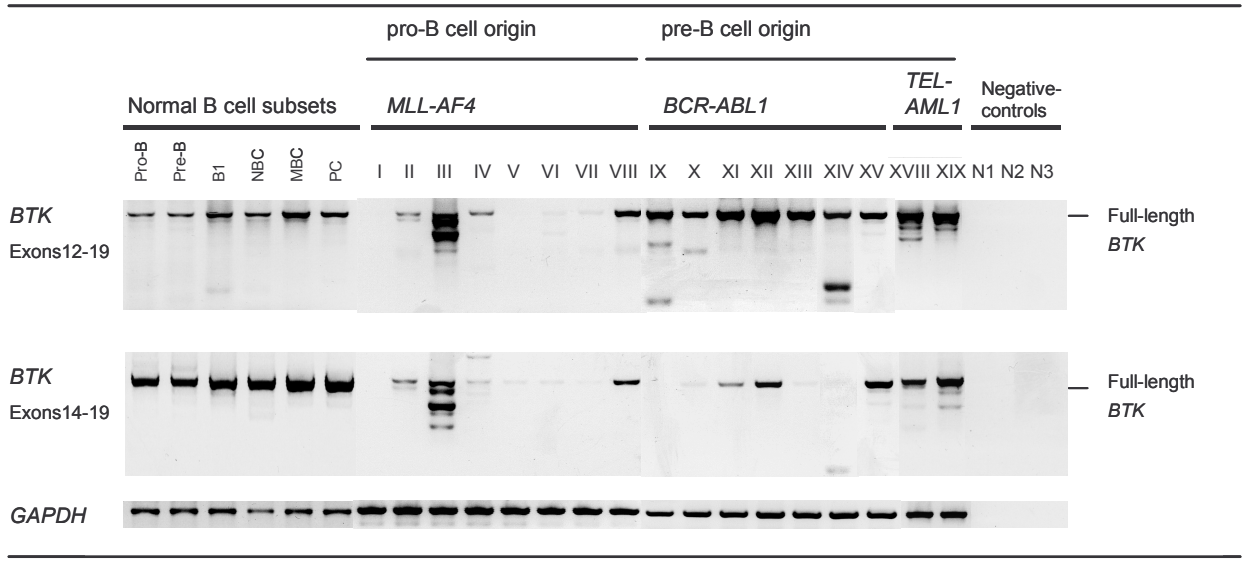


Figure 1: *BTK mRNA expression in primary B cell precursor leukemia cells*
Using PCR-primers for exons 12, 14 and 19, two regions of the *BTK* mRNA were analyzed by RT-PCR in normal human pro-B cells, pre-B cells, B1 cells, naïve B cells (NBC), memory B cells (MBC) and plasma cells (PC) as well as in 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.

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).

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

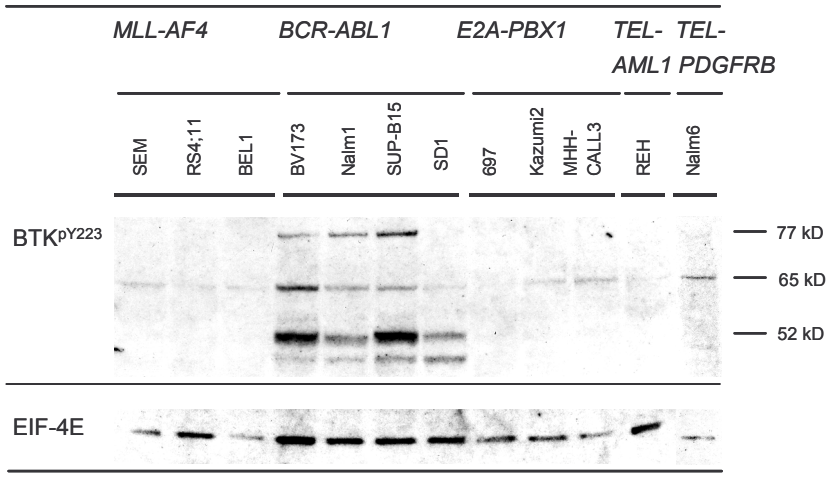


Figure 2: *Tyrosine-phosphorylation of BTK and kinase-deficient splice variants in pre-B lympphoblastic leukemia cells.*
Using an antibody against phosphorylated BTK^{Y233}, tyrosine-phosphorylation of BTK (77 kD) and BTK isoforms was analyzed by Western blot in 12 B cell precursor leukemia cell lines. 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.

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.

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).

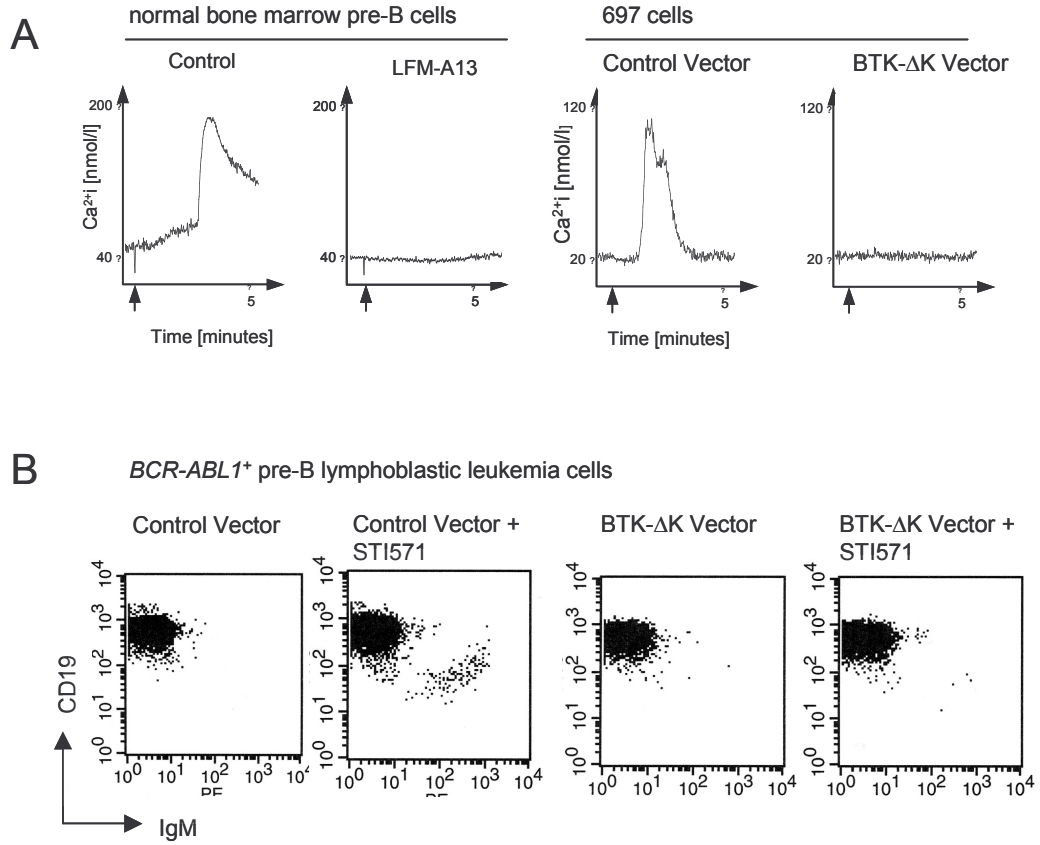


Figure 3: *Expression of kinase-deficient BTK suppresses pre-B cell receptor-dependent Ca^{2+} signals and differentiation.*

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 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 DNA double-strand breaks was increased in leukaemia cells transduced with the BTK-IRES-GFP expression vector (Fig. 4A). This indicates that overexpression of functional BTK can partially relieve the differentiation block of acute lymphoblastic leukemia cells that were arrested at the pre-B cell stage. However, *MLL-AF4*$^+$ acute lymphoblastic leukemia cells, which are arrested at the pro-B cell stage do not initiate immunoglobulin light chain gene rearrangement in response to BTK overexpression. This might indicate that the initiation 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

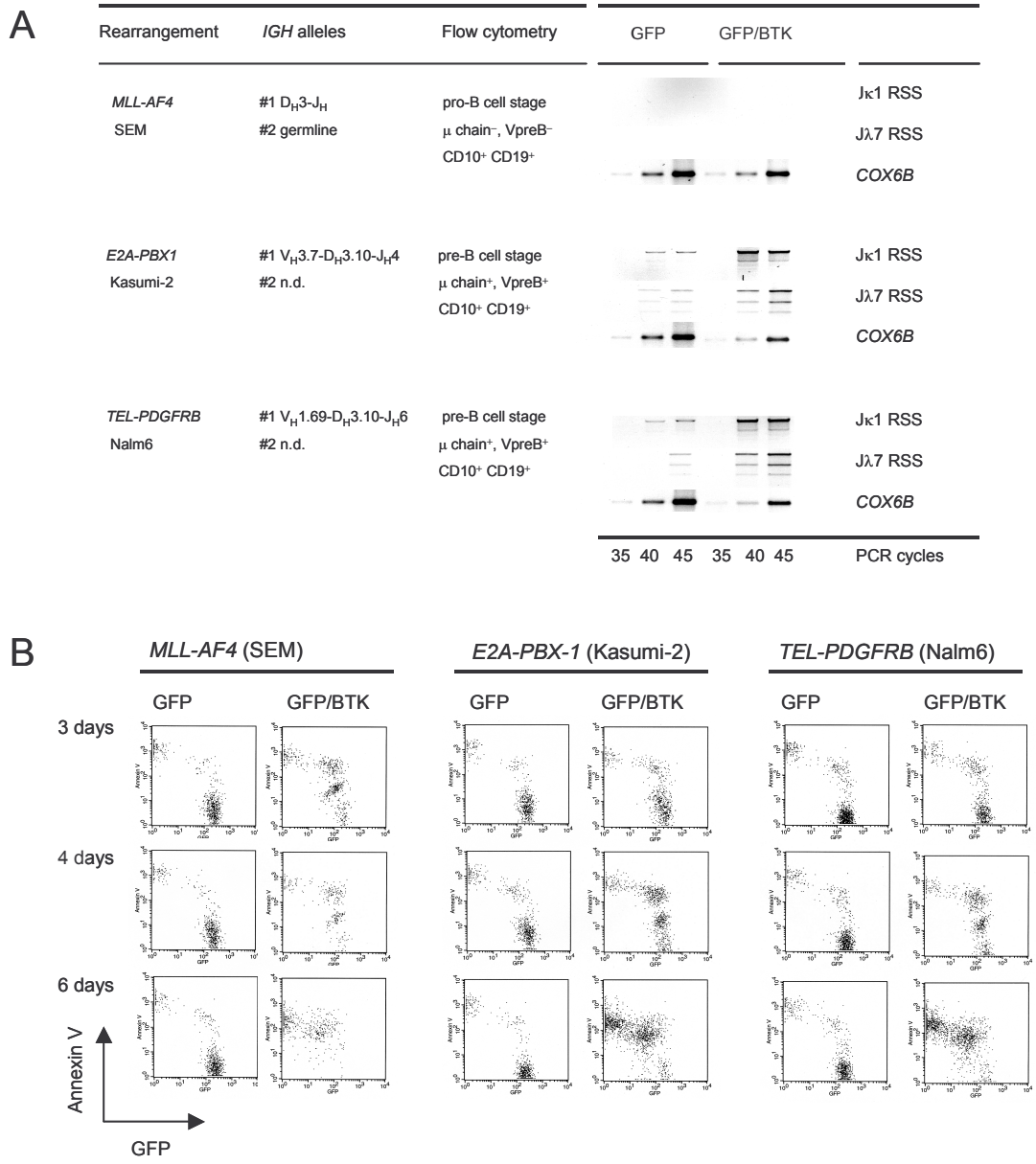


Figure 4: *Reconstitution of BTK expression in acute lymphoblastic leukaemia cells initiates differentiation and induces propensity to apoptosis.*
Acute lymphoblastic leukemia cell lines carrying either an *MLL-AF4* (SEM), *E2A-PBX1* (Kasumi-2) or *TEL -PDGFRB* (Nalm6) gene rearrangement were characterized 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).

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.

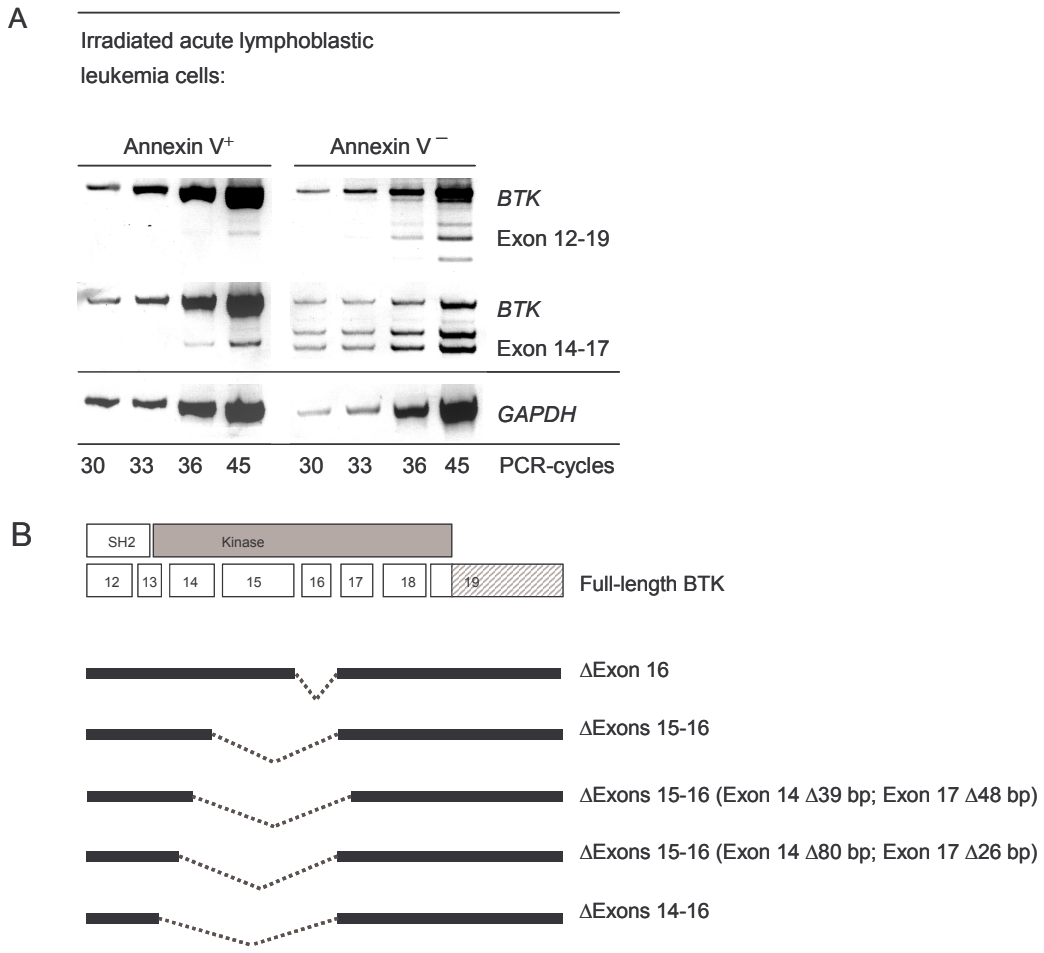


Figure 5: *Acute lymphoblastic leukemia cells surviving γ-radiation are selected for the expression of dominant-negative BTK isoforms.*
The pre-B lymphoblastic leukemia cell line Kasumi-2 carrying a *E2A-PBX1* gene rearrangement were γ-irradiated with 10 Gy. After 24 hours, more than 90 percent of the 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).

Conclusion

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.

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.

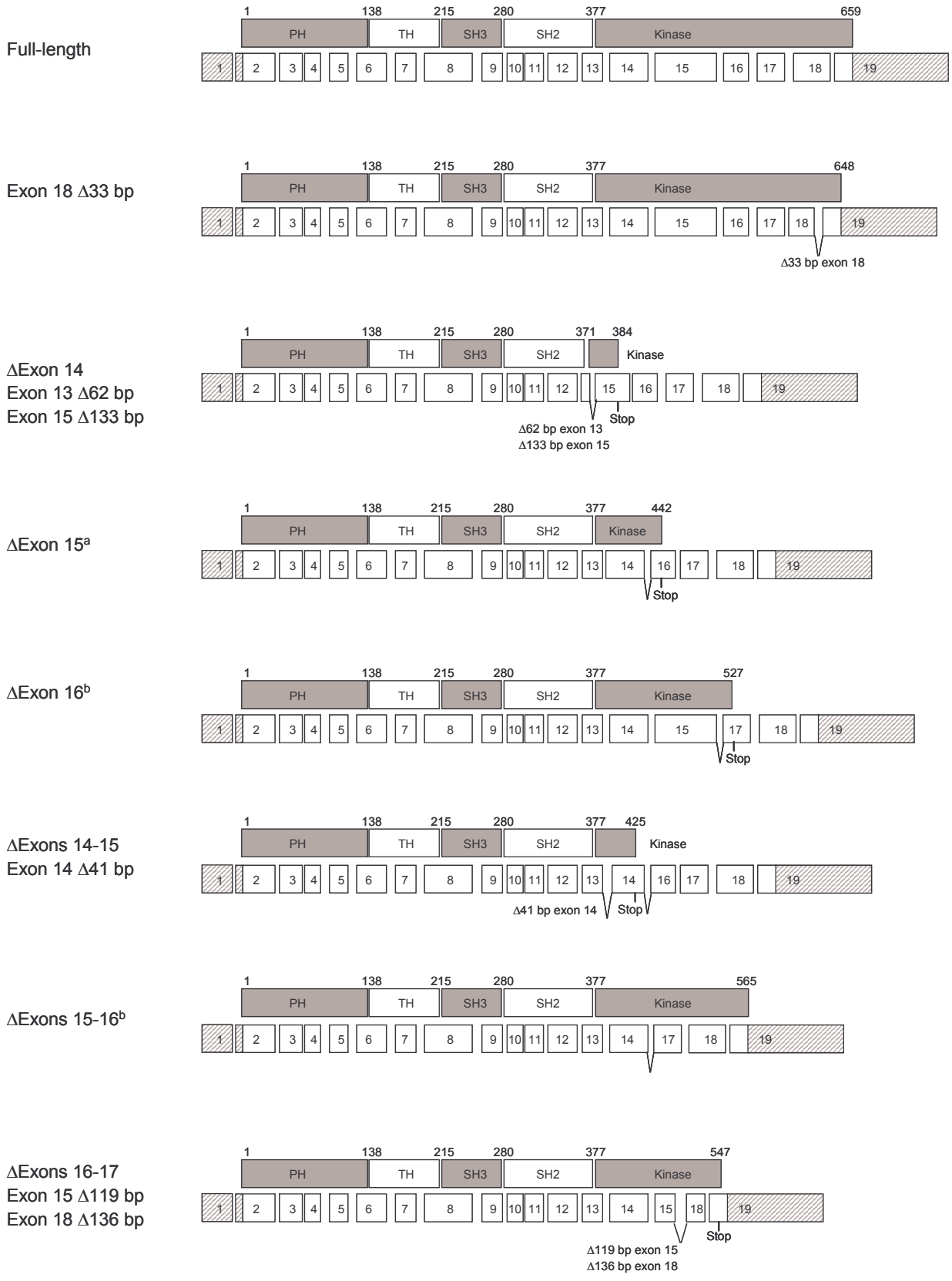
Amplification of double-strand recombination signal sequence breaks by ligation-mediated PCR

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

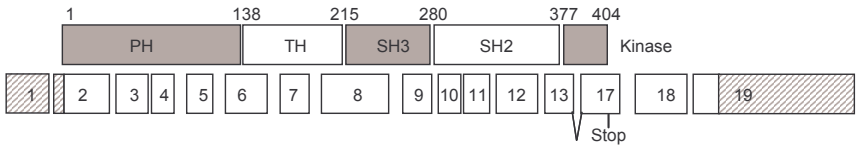
Pre-B lymphoblastic leukemia cells were irradiated with 10 Gy γ-radiation at the Clinic for Radiotherapy and Radiological Oncology, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany. 24 hours after radiation, leukemia cells were stained with Annexin V and propidium iodide to separately sort pre-apoptotic or surviving cells.

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

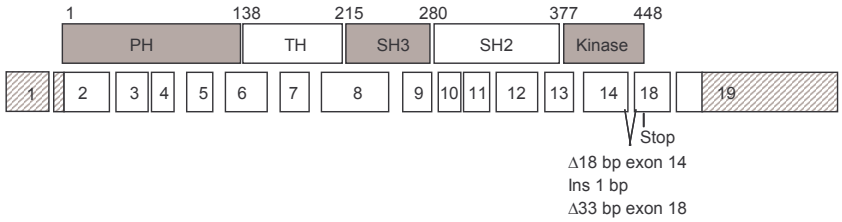


Supplementary Figure 1b: *BTK splice variants in B cell precursor leukaemia*

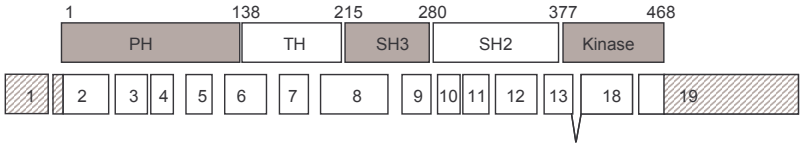
ΔExons 14-16



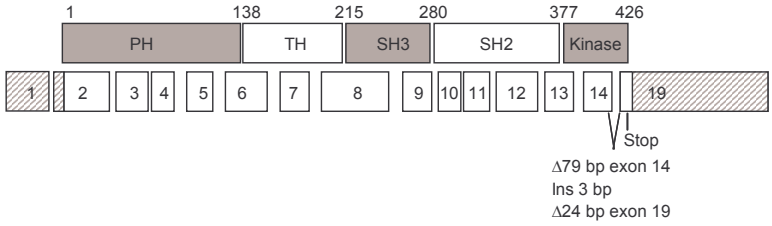
ΔExons 15-17
Exon 14 Δ18 bp
Ins 1 bp
Exon 18 Δ33 bp



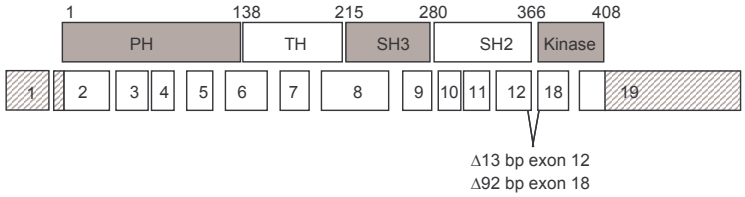
ΔExons 14-17



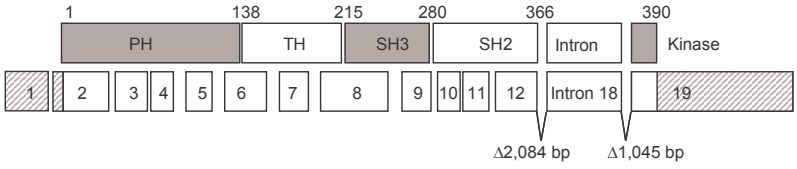
ΔExons 15-18
Exon 14 Δ79 bp
Ins 3 bp
Exon 19 Δ24 bp



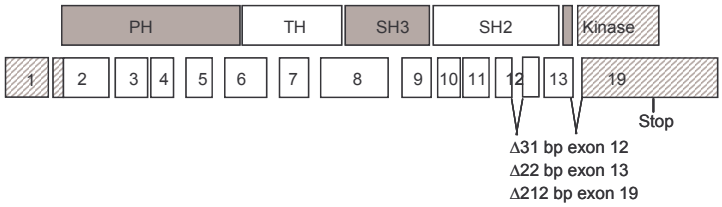
ΔExons 13-17
Exon 12 Δ13 bp
Exon 18 Δ92 bp



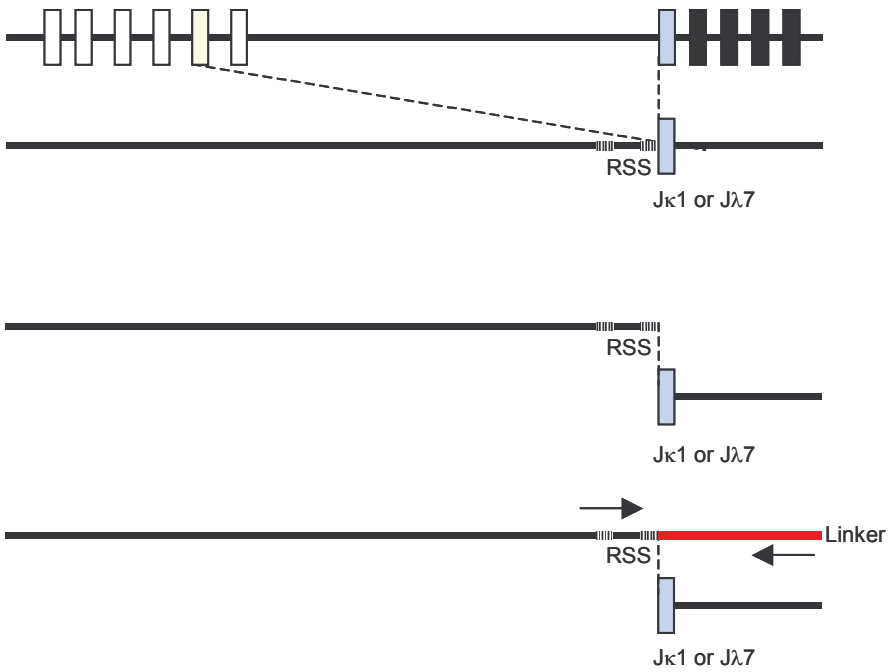
ΔExons 13-18
Ins 106 bp Intron 18



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.

References

1. Jumaa H, Bossaller L, Portugal K, Storch B, Lotz M, Flemming A, Schrappe M, Postila V, Riikonen P, Pelkonen J, Niemeyer CM, Reth M. Deficiency of the adaptor SLP-65 in pre-B-cell acute lymphoblastic leukaemia. *Nature*. 2003; 423: 452-6.
2. 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; 199:673-85.
3. Kersseboom R, Middendorp S, Dingjan GM, Dahlenborg K, Reth M, Jumaa H, Hendriks RW. Bruton's tyrosine kinase cooperates with the B cell linker protein SLP-65 as a tumor suppressor in Pre-B cells. *J Exp Med*. 2003; 198: 91-98.
4. Holinski-Feder E, Weiss M, Brandau O, Jedele KB, Nore B, Backesjo CM, Vihinen M, Hubbard SR, Belohradsky BH, Smith CI, Meindl A. Mutation screening of the BTK gene in 56 families with X-linked agammaglobulinemia (XLA): 47 unique mutations without correlation to clinical course. *Pediatrics*. 1998; 101: 276-284.
5. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, Delabesse E, Davi F, Schuuring E, Garcia-Sanz R, van Krieken JH, Droese J, Gonzalez D, Bastard C, White HE, Spaargaren M, Gonzalez M, Parreira A, Smith JL, Morgan GJ, Kneba M, Macintyre EA. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations. *Leukemia*. 2003;17:2257-317.
6. 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: Aberrant splicing links Bruton's tyrosine kinase to BCR-ABL1 in pre-B lymphoblastic leukemia. *J Exp Med*, 201: in press (2005)
7. 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: 10014-9.
8. Feldhahn N, Schwering I, Lee S, Wartenberg M, Klein F, Wang H, Zhou G, Wang SM, Rowley JD, Hescheler J, Krönke M, Rajewsky K, Küppers R, Müschen M. Silencing of B cell receptor signals in human naive B cells. *J Exp Med*. 2002; 196:1291-305.
9. 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: 6747-52.
10. Hildinger M, Abel KL, Ostertag W, Baum C. Design of 5' untranslated sequences in retroviral vectors developed for medical use. *J Virol* 1999; 73: 4083-4089.
11. Pietschmann T, Heinkelein M, Heldmann M, Zentgraf H, Rethwilm A, Lindemann D. Foamy virus capsids require the cognate envelope protein for particle export. *J Virol* 1999; 73: 2613-2621.
12. Pear WS, Miller JP, Xu L, Pui JC, Soffer B, Quackenbush RC, Pendergast AM, Bronson R, Aster JC, Scott ML, Baltimore D. Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood* 1998; 92: 3780-3792.

13. Hanenberg H, Xiao XL, Dilloo D, Hashino K, Kato I, Williams DA. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat Med.* 1996; 2: 876-882.
14. Hanenberg H, Hashino K, Konishi H, Hock RA, Kato I, Williams DA. Optimization of fibronectin-assisted retroviral gene transfer into human CD34+ hematopoietic cells. *Hum Gene Ther.* 1997; 8: 2193-2206.
15. Schlissel M, Constantinescu A, Morrow T, Baxter M, Peng A. Double-strand signal sequence breaks in V(D)J recombination are blunt, 5'-phosphorylated, RAG-dependent, and cell cycle regulated. *Genes Dev* 1993; 7: 2520-2532.
16. Klein F, Feldhahn N, Mooster JL, Sprangers M, Hofmann WK, Wernet P, Wartenberg M and 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
17. Middendorp S, Dingjan GM, Maas A, Dahlenborg K, Hendriks RW. Function of Bruton's tyrosine kinase during B cell development is partially independent of its catalytic activity. *J Immunol* 2003; 171: 5988-5996.
18. Goodman PA, Wood CM, Vassilev AO, Mao C, Uckun FM. Defective expression of Bruton's tyrosine kinase in acute lymphoblastic leukemia. Leuk Lymphoma 2003; 44: 1011-1008.
19. Muljo SA, Schlissel MS. A small molecule Abl kinase inhibitor induces differentiation of Abelson virus-transformed pre-B cell lines. *Nat Immunol* 2003; 4: 31-7
20. Uckun FM, Waddick KG, Mahajan S, Jun X, Takata M, Bolen J, Kurosaki T. BTK as a mediator of radiation-induced apoptosis in DT-40 lymphoma B cells. *Science* 1996; 273:1096-100.

Chapter 6

Discussion

Influence of SLP65 in negative feedback signaling during immunoglobulin V region gene rearrangement

In pre-B cells, V(D)J recombination is regulated through a negative feedback signal: a functional rearrangement at the *IGHV* locus leads to expression of the μ protein as part of the pre-B cell receptor which functions as an important checkpoint to establish allelic exclusion on the second, unrearranged allele (Kitamura and Rajewsky, 1992). This inhibition of V(D)J recombination on the second allele (referred to as allelic exclusion) was initially noted in mice (Nussenzweig et al., 1987). After μ signalling, the expression of RAG1 and RAG2 is downregulated thereby preventing further rearrangements at the other allele (Grawunder et al., 1995). Besides RAG1 and RAG2 regulation, allelic exclusion is controlled by the accessibility of the different gene loci and segments to the V(D)J recombinase (Stanhope-Baker et al., 1996). This accessibility is organised at multiple levels including subnuclear relocation (Kosak et al., 2002) and chromatin remodelling (Maes et al., 2001). By histone deacetylation the accessibility of the V_H genes are reduced after μ expression on the B cell surface (Chowdhury and Sen, 2003). It is not known yet, how this negative feedback signalling leading to allelic exclusion is exactly organised, however we speculated that SLP65 plays an important role.

We found that all SLP65-deficient pre-B lymphoblastic leukemia and B cell lymphoma cell lines carry more than two productively rearranged Ig heavy chain V region genes consistent with continuous expression of RAG1 and RAG2. The presence of several functionally rearranged heavy chains together with RAG expression indicates that there was a defect in the negative feedback signaling in these cells. From all cases of B cell leukemia and lymphoma, non-functional SLP65 mRNA splice variants were amplified. This suggested that SLP65 plays as role in ending V(D)J recombination upon a successful rearrangement at the *IGHV* gene. Indeed, RAG1 and RAG2 expression was downregulated upon reconstitution of SLP65 in these SLP65-deficient B cell leukemia and lymphoma cells.

Two recent reports (Hayashi et al., 2003; Xu et al., 2000) show that allelic exclusion is intact in $SLP65^{-/-}$ mice. These data, however, are based on SLP65 mutant mice. As these mice are healthy besides SLP65-deficiency, their locus control is intact. This intact locus-control in combination with SLP65-deficiency shows that SLP65-deficiency alone does not compromise allelic exclusion. Our data, however, are based on SLP65-deficient pre-B leukemia and B cell lymphoma cell lines. As B cell leukemia and lymphoma cells exhibit a differentiation block at their specific differentiation stage (Klein et al., 2004; Barr, 1998), no locus control is present

in these cells. As allelic exclusion is based on both recombinase activity and on the accessibility of the locus (Yancopoulos et al., 1986) this could be an explanation why there is ongoing V(D)J recombination in SLP65-deficient leukemia and lymphoma but not in SLP65-deficient B cells (Figure 1).

	Normal B cells	SLP65$^{-/-}$ B cells	SLP65$^{+/+}$ leukemia	SLP65$^{-/-}$ leukemia
Controlled RAG 1/ 2 expression	+	-	+	-
Locus control	+	+	-	-
Allelic exclusion?	Yes	Yes	Yes	No

Figure 1: *Principle of allelic exclusion*
Productive V(D)J rearrangement at one *IgH* allele inhibits further rearrangement on the other *IgH* allele; a negative feedback mechanism known as allelic exclusion. Allelic exclusion is based on controlled RAG1 and RAG2 expression and on the accessibility of the locus for the recombinase (locus control). Failure of either controlled RAG expression or of locus control does not have an effect on allelic exclusion. Assuming that SLP65 inhibits RAG expression this scheme explains why allelic exclusion is inhibited in SLP65-deficient leukemia cells but not in SLP65-deficient B cells.

The continuous expression of RAG1 and RAG2 is of great significance for leukemia and lymphoma as it might increase the risk of other genetic aberrations. There is indication that the V(D)J recombination machinery may target non-Ig genes by mistake. V(D)J recombinase activity probably was invloved in large deletions affecting the *hprt* locus in T cells (Fuscoe et al., 1991), the transcription factor *TAL1* (Aplan et al., 1990) and the *MTS1* gene (Cayuela et al., 1997). In each of the three genes, heptamer-like sequences were detected near the breakpoints of the deletions, and the breakpoints show deletion of nucleotides as well as addition of non-templates nucleotides. These features support the idea that the deletions are mediated by V(D)J recombinase activity. Although each of these examples has been identified in T cells there is no reason why similar deletion events could not occur in B cells. A different type of V(D)J recombinase activity was found in follicular lymphoma where the *BCL2* gene was inserted into the IgH locus between a D_H and J_H gene segment (Vaandrager et al., 2000)., cryptic RSS sites were identified at the breakpoints of the *BCL2* gene and each breakpoint junction showed nucleotide insertions. This suggests that the insertion of *BCL2* was RAG-mediated and happened during D_HJ_H rearrangement.

Src-kinases and malignant transformation in B cell lymphoma cells

BCR engagement leads to the phosphorylation of the ITAMs located in the cytoplasmic tails of Igα/Igβ by the Src-kinase Lyn. This phosphorylation creates the docking sites for the recruitment and activation of the Syk tyrosine kinase subsequently triggering downstream signaling leading to PLCγ2 phosphorylation and Ca^{2+} mobilization (Figure 2A). We found that in the absence of SLP65, the major Src-kinase Lyn (Gauld and Cambier, 2004), binds PLCγ1 directly leading to a Ca^{2+} signal (Figure 2B). We show that this SLP65-independent signaling pathway results in the tyrosine-phosphorylation of MAP kinase 1 and STAT5, which induce proliferation and survival respectively (Richards et al., 2001; Karras et al, 1996).

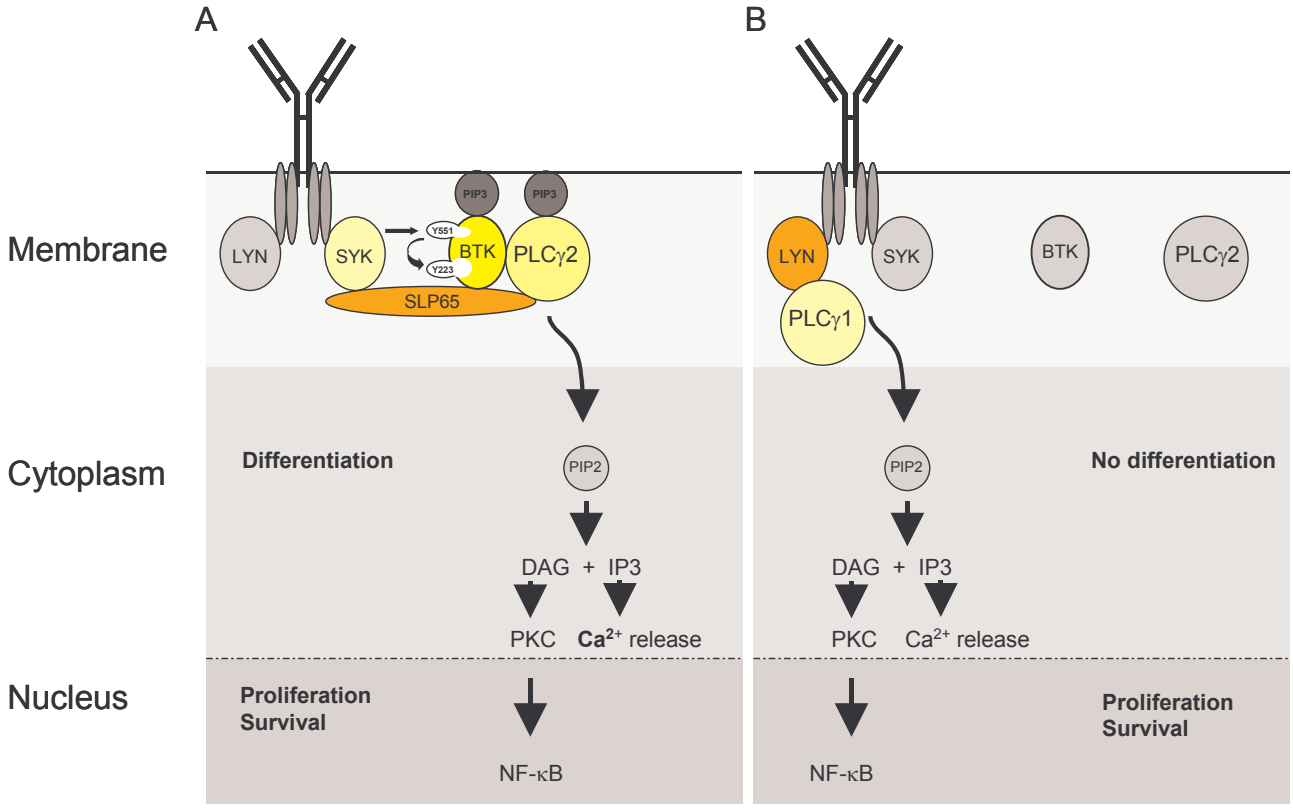


Figure 2: *BCR-signaling in the presence (A) and in the absence (B) of SLP65.*
In the presence of SLP65 (A) the BCR signals via Syk, SLP65, BTK and PLCg2 resulting in differentiation, proliferation and survival. In SLP65-deficient cells (B) the signal is transduced via Lyn to PLCγ1 and leads to proliferation and survival but not to differentiation.

Src is the first proto-oncogene characterized (Bishop, 1985). In 1911, Francis Peyton Rous discovered that healthy chickens injected with a cell-free extract of the tumor of a sick chicken also became cancer. The virus that caused this sarcoma was named the Rous Sarcoma

Virus (RSV). RSV has only 4 genes and one is *src* (v-*src*), encoding a tyrosine kinase and responsible for the virus' oncogenic character. In 1976 Bishop reported that normal avian cells have a gene that is very similar to v-*src*, subsequently named c-*src* (cellular-*src*) a proto-oncogene, normally involved in the control of cell growth. This c-*src* has become an oncogene in RSV after being mutated.

A number of recent studies suggested a role for aberrant Lyn kinase activity in the origin or malignant progression of human B cell lineage leukemia and lymphoma. In chronic lymphocytic leukemia B cells, it was shown that Lyn is remarkably overexpressed at the protein level as compared with normal B lymphocytes (Contri et al., 2005). Moreover, Lyn is constitutively activity and was also detected in the cytosol of the malignant B cells. The release of Lyn into the cytosol following caspase-dependent cleavage of the tyrosine kinase at its N-terminus has been described as a general mechanism in hematopoietic cells during BCR-induced apoptosis (Luciano et al., 2001). The soluble form of Lyn acts as an inhibitor of B lymphocyte death likely by changing c–Myc expression (Luciano et al., 2003). Treatment of *BCR-ABL1*$^{+}$ chronic myelogenous leukemia cells with Lyn-siRNAs, induced apoptosis in 80-95% of the cells whereas normal hematopoietic cells remained viable (Hu et al., 2004), indicating a great dependence of leukemia cells on Lyn.

However, a role of Lyn in aberrant activation of B lymphoid precursor cells of leukemia or lymphoma is unexpected as B cell receptor-dependent B cell activation is typically increased in Lyn$^{-/-}$ as compared to wild type murine B cells (Chan et al., 1998; Nishizumi et al., 1998). Indeed, normal B cell receptor signaling is negatively regulated by Lyn through activation of the inhibitory protein tyrosine kinase SHP1 (Otipoby et al, 2001) and through the activation of CBL which induces degradation of Syk (Ota and Samelson, 1997). However, the classical signaling pathway (Figure 2A) is not active in SLP65-deficient pre-B leukemia and B cell lymphoma cells. In this alternative signaling pathway (Figure 2B) the stimulatory effect of Lyn on proliferation through phosphorylation of CD19, PI3K and MAPK1 is no longer overshadowed by its inhibitory effect on the classical B cell receptor signal transduction (Gauld and Cambier, 2004; O'Laughlin-Bunner et al., 2001; Nishizumi et al., 1995).

We therefore propose that the Src-kinase Lyn redirects (pre-) B cell receptor signaling in SLP65-deficient leukemia and lymphoma cells to proliferation signals through PLCγ1-mediated Ca^{2+}-mobilization and activation of the MAP kinase pathway. Although BCR-signaling normally takes place via PLCγ2 (Kang et al., 1996), PLCγ1 has been detected in BCR-signaling in B cell leukemia before (Feldhahn et al., 2005).

Aberrant splicing of (pre-) B cell receptor-related signaling molecules in leukemia and lymphoma cells

Exons contain splice-site sequences at the intron/ exon borders. These splice-site sequences are almost always consensus sequences including almost invariant dinucleotides at each end of the intron: GT at the 5' end of the intron and AG at the 3' end of the intron. The spliceosome, a multicomponent splicing complex, binds to this consensus-sequences. Two primary functions of splicing are performed by the spliceosome: i) recognition of the intron/ exon borders and ii) catalysis of the cut-and-paste reactions whereby introns are removed and exons are joined (Faustino and Cooper, 2003). The short consensus sequences are not long enough for splice-site recognition as they have to be distinguished from pseudo splice-site sequences that look like the consensus splice-sites but are never used. Therefore, exonic and intronic splicing enhancers and silencers, the so-called *cis*-elements, also play a role in the recognition of the exons (Lim et al., 2001).

Alternative splicing is the joining of different 5' and 3' splice sites. In this way individual genes are able to express multiple mRNAs that encode proteins with diverse and even antagonistic functions (Modrek and Lee, 2002). This is the case with SLP65 in many B cell leukemias and lymphomas. By alternative splicing, variation of mRNA structure can occur by insertion or removal of amino acids, shifting of the reading frame, or by introducing a termination codon. In many cases of alternative splicing the cell undergoes specific regulation depending on cell type, developmental stage, gender, or external stimuli. This shows that splicing is a complex mechanism whereby defects easily can occur. This defect can be caused by a mutation in the *cis*-element leading to aberrant splicing of a single gene (expression of unnatural mRNAs) or a mutation can occur that disrupts a component of the splicing machinery and thereby has an effect on several genes (Faustino et al., 2003).

Most of the mutations that disrupt splicing are single nucleotide changes within the consensus splice sites. But also new splice sites can be introduced by mutations. It was shown that 15% of point mutations result in human genetic disease by a failture of splicing (Krawczak et al., 1992). In fact, the primary mechanism of disease-causing exonic mutations do not have an effect on the coding potential but lead to a splicing abnormality (Cartegni et al., 2002).

In this thesis many alternatively spliced isoforms have been described for several pre-B cell leukemia and B cell lymphoma cells. As for SLP65 no point mutations in the consensus

sequence have been found and this splicing recognition site is still intact, it is very unlikely that mutations outside this consensus sequence had an effect on splicing. It is more likely to believe that there is a failure in the splicing machinery in many, if not all, leukemia and lymphoma cases. Besides alternative SLP65 splicing, many other genes have been aberrantly spliced in leukemia and lymphoma. This confirms the hypothesis that in leukemia and lymphoma a general failure in the splicing machinery is causing alternatively spliced genes.

BTK has been identified as a critical cofactor of SLP65 in preventing leukemic transformation of murine B cell precursors (Kersseboom et al., 2003). As described in chapter 5 of this thesis, several BTK splice-variants are found in pre-B leukemia cells. Also here the splice mechanism itself seems to be deranged as cryptic splice sites were frequently used and splice site slippage leading to small nucleotide insertions or deletions were also observed. The splice-variants affected the BTK kinase domain leading to a complete loss of BTK kinase activity. This BTK-ΔK acts as a dominant-negative form of BTK by inhibiting Ca^{2+} release in response to pre-BCR stimulation and by inhibiting pre-BCR driven differentiation. Thereby it was shown that these dominant-negative BTK splice variants can protect against radiation-induced apoptosis. Another BTK splice-variant, BTK^{p52}, functions as a linker molecule by activation of full-length BTK by BCR-ABL1 (Feldhahn et al., 2005).

The transcription factor *IKAROS* is another well-known example of alternative splicing in leukemia. With its N-terminal zinc-finger domains IKAROS is involved in the DNA binding, while its C-terminal is essential for dimerization with other IKAROS isoforms or other family members (Morgan et al., 1997). Several isoforms of IKAROS are expressed due to alternative splicing. Long isoforms with at least three zinc fingers can efficiently bind DNA, whereas short isoforms with less than three zinc fingers dimerize with each other and act as dominant-negative isoforms (Sun et al., 1996). We have shown that *BCR-ABL1* induces aberrant splicing of *IKAROS* by expressing the dominant-negative isoform Ik6 (Klein et al., 2005). By inhibition of the BCR-ABL1 kinase activity normal IKAROS expression is restored. A role of Ik6 in pre-B leukemia has been described by other groups as well (Nakase et al., 2000; Tonnelle et al., 2003) but also other IKAROS splice-forms (Ik4 to Ik8) which cannot bind DNA, are involved in leukemia (Olivero et al., 2000; Sun et al., 1999).

References

Aplan, P.D., Lombardi, D.P., Ginsberg, A.M., Cossman, J., Bertness, V.L., Kirsch, I.R. (1990) Disruption of the human SCL locus by "illegitimate" V-(D)-J recombinase activity. *Science.* **250**, 1426-1429

Barr, F.G. (1998) Translocations, cancer and the puzzle of specificity. *Nat. Genet.* **19**, 121-124

Bishop, J. (1985) Viral oncogenes. *Cell.* **42**, 23-28

Cartegni, L., Chew, S.L., Krainer, A.R. (2002) Listening to silence and understanding nonsence: Exonic mutations that affect splicing. *Nat. Rev. Genet.* **3**, 285-298.

Cayuela, J.M., Gardie, B., Sigaux, F. (1997) Disruption of the multiple tumor suppressor gene MTS1/p16(INK4a)/CDKN2 by illegitimate V(D)J recombinase activity in T-cell acute lymphoblastic leukemias. *Blood.* **90**, 3720-3726

Chan, V.W., Mecklenbrauker, I., Su, I., Texido, G., Leitges, M., Carsetti, R., Lowell, C.A., Rajewsky, K., Miyake, K., Tarakhovsky, A. (1998) The molecular mechanism of B cell activation by toll-like receptor protein RP-105. *J. Exp. Med.* **188**, 93-101

Chowdhury, D., Sen, R. (2003) Transient IL-7/IL-7R signaling provides a mechanism for feedback inhibition of immunoglobulin heavy chain gene rearrangements. *Immunity.* **18**, 229-241

Contri, A., Brunati, A.M., Trentin, L., Cabrelle, A., Miorin, M., Cesaro, L., Pinna, L.A., Zambello, R., Semenzato, G., Donella-Deana, A. (2005) Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis. *J. Clin. Invest.* **115**, 369-378

Faustino, N.A., Cooper, T.A. (2003) Pre-nRNA splicing and human disease. *Genes and Dev.* **17**, 419-437

Feldhahn, N., Klein, F., Mooster, J.L., Hadweh, P., Sprangers, M., Wartenberg, M., Bekhite, M.M., Hofmann, W., Herzog, S., Jumaa, H., Rowley, J.D., Müschen, M. (2005) Mimicry of a constitutively active pre-B cell receptor:. *J. Exp. Med.* **201**, 1837-1852

Fuscoe, J.C., Zimmerman, L.J., Lippert, M.J., Nicklas, J.A., O'Neill, J.P., Albertini, R.J. (1991) V(D)J recombinase-like activity mediates hprt gene deletion in human fetal T-lymphocytes. *Cancer res.* **51**, 6001-6005

Gauld, S.B., Cambier, J.C. (2004) Src-family kinases in B-cell developmet and signalling. *Oncogene.* **23**, 8001-8006

Grawunder, U., Leu, T.M., Schatz, D.G., Werner, A., Rolink, A.G., Melchers, F., Winkler, T.H. (1995) Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement. *Immunity.* **3**, 601-608

Hayashi, K., Yamamoto, M., Nojima, T., Goitsuka, R., Kitamura, D. (2003) Distinct signaling requirements for Dm selection, IgH allelic exclusion, pre-B cell transition, and tumor suppression in B cell progenitors. *Immunity.* **18**, 825-836

Hu, Y., Liu, Y., Pelletier, S., Buchdunger, E., Warmuth, M., Fabbro, D., Hallek, M., van Etten, R.A., Li, S. (2004) Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukaemia but not chronic myeloid leukemia. *Nat. genet.* **36**, 453-461

Karras, J.G., Wang, Z., Coniglio, S.J., Frank, D.A., Rothstein, T.L. (1996) Antigen-receptor engagement in B cells induces nuclear expression of STAT5 and STAT6 proteins that bind and transactivate an IFNg activation site. *J. Immunol.* **157**, 39-47

Kang, J.S., Kohlhuber, F., Hug, H., Marme, D., Eick, D., Ueffing, M. (1996) Cloning and functional analysis of the hematopoietic cell-specific phospholipase C(gamma)2 promoter. *FEBS Lett.* **399**, 14-20

Kersseboom, R., Middendorp, S., Dingjan, G.M., Dahlenborg, K., Reth, M., Jumaa, H., Hendriks, R.W. (2003) Bruton's tyrosine kinase cooperates with the B cell linker protein SLP65 as a tumor suppressor in pre-B cells. *J. Exp. Med.* **198**, 91-98

Kitamura, D., Rajewsky, K. (1992) Targeted disruption of m chain membrane exon causes loss of heavy-chain allelic exclusion. *Nature.* **356**, 154-156

Klein, F., Feldhahn, N., Harder, L., Wang, H., Wartenberg, M., Hofmann, W.K., Wernet, P., Siebert, R., Müschen, M. (2004) 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.* **199**, 673-685

Kosak, S.T., Skok, J.A., Medina, K.L., Riblet, R., Le Beau, M.M., Fisher, A.G., Singh, H. (2002) Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development. *Science.* **296**, 158-162

Krawczak, M., Reiss, J., Cooper, D.N. (1992) The mutational spectrum of single base-pair sunstitutions in messenger RNA splice junctions of human genes – Causes and consequences. *Hum. Genet.* **90**, 41-54

Lim, L.P., Burge, C.B. (2001) A computational analysis of sequence features involved in recognition of short introns. *Proc. natl. Acad. Sci.* **98**, 11193-11198

Luciano, F., Ricci, J.F., Auberger, P. (2001) Cleavage of Fyn and Lyn in their N-terminal unique regions during induction of apoptosis: a new mechanism for Src kinase regulation. *Oncogene.* **16**, 4935-4941

Luciano, F., Herrant, M., Jacquel, A., Ricci, J., Auberger, P. (2003) The P54-cleaved form of the tyrosine kinase Lyn generated by caspases during BCR-induced cell death in B lymphoma acts as a negative regulator of apoptosis. *FASEB J.* **17**, 711-735

Maes, J., O'Neill, L.P., Cavelier, P., Turner, B.M., Rougeon, F., Goodhardt, M. (2001) Chromatin remodelling at the Ig loci prior to V(D)J recombination. *J. Immunol.* **167**, 866-874

Modrek, B., Lee, C. (2002) A genomic view of alternative splicing. *Nat. Genet.* **30**, 13-19

Morgan, B., Sun, L., Avitahl, N., Andrikopoulos, K., Ikeda, T., Gonzales, E., Wu, P., Neben, S., Georgopoulos, K. (1997) Aiolos, a lymphoid restricted transcription factor that interacts with Ikaros to regulate lymphocyte differentiation. *EMBO J.* **16**, 2004-2013

Nakase, K., Ishimaru, F., Avitahl, N., Dansako, H., Matsuo, K., Fujii, K., Sezaki, N., Nakayama, H., Yano, T., Fukuda, S., Imajoh, K., Takeuchi, M., Miyata, A., Hara, M., Yasukawa, M., Takahashi, I., Taguchi, H., Matsue, K., Nakao, S., Niho, Y., Takenaka, K., Shinagawa, K., Ikeda, K., Niiya, K., Harada M. (2000) Dominant negative isoform of the Ikaros gene in patients with adult B-cell acute lymphoblastic leukemia. *Cancer Research*. **60**, 4062-4065

Nishizumi, H., Taniuchi, I., Yamanashi, Y., Kitamura, D., Ilic, D., Mori, S., Watanabe, T., Yamamoto, T. (1995) Impaired proliferation of peripheral B cells and indication of autoimmune disease in lyn-deficient mice. *Immunity.* **3**, 549-560

Nishizumi, H., Horikawa, K., Mlinaric-Rascan, I., Yamamoto, T. (1998) A double-edged kinase Lyn: a positive and negative regulator for antigen receptor-mediated signals. *J. Exp. Med.* **187**, 1343-1348

Nussenzweig, M.C., Shaw, A.C., Sinn, F., Danner, D.B., Holmes, K.L., Morse, H.C., Leder, P. (1987) Allelic exclusion in transgenic mice that express the membrane form of immunoglobulin mu. *Science.* **236**, 816-819

O'Laughlin-Bunner, B., Radosevic, N., Taylor, M.L., Shivakrupa, C., DeBerry, C., Metcalfe, D.D., Zhou, M., Lowell, D., Linnekin, D. (2001) Lyn is required for normal stem cell factor-induced proliferation and chemotaxis of primary hematopoietic cells. *Blood.* **98**, 343-350

Olivero, S., Maroc, C., Beillard, E., Gabert, J., Nietfeld, W., Chabannon, C., Tonnelle, C (2000) Detection of different Ikaros isoforms in human leukaemias using real-time quantitative polymerase chain reaction. *Br. J. Haematol.* **110**, 826-830

Ota, Y., Samelson, L.E. (1997) The product of the proto-oncogene c-cbl: A negative regulator of the Syk tyrosine kinase. *Science.* **276**, 418-420

Otipoby, K.L., Draves, K.E., Clark, E.A. (2001) CD22 regulates B cell receptor-mediated signals via two domains that independently recruit Grb2 and SHP-1. *J. Biol. Chem.* **276**, 44315-44322

Richards, J.D., Dave, S.H., Chou, C.H., Mamchak, A.A., DeFranco, A.L. (2001) Inhibition of the MEK/ERK signaling pathway blocks a subset of B cell responses to antigen. *J. Immunol.* **166**, 3855-3864

Stanhope-Baker, P., Hudson, K.M., Shaffer, A.L., Constantinescu, A., Schlissel, M.S. (1996) Cell type-specific chromatine structure determines the targeting of V(D)J recombinase activity *in vitro*. *Cell* **85**, 887-897

Sun, L., Heerema, N., Crotty, L., Wu, X., Navara, C., Vassilev, A., Sensel, M., Reaman, H., Uckun, F.M. (1999) Expression of dominant-negative and mutant isoforms of the antileukemic transcription factor Ikaros in infant acute lymphoblastic leukemia. *Proc. Natl., Acad., Sci. USA.* **96**, 680-685

Tonnelle, C., Imbert, M.C., Sainty, D., Granjeaux, S., N'Guyen, C., Chabannon, C. (2003) Overexpression of dominant-negative Ikaros 6 protein is restricted to a subset of B common adult acute lymphoblastic leukemias that express high levels of the CD34 antigen. *Hematol. J.* **4**, 104-109

Vaandrager, J.W., Schuuring, E., Philippo, K., Kluin, P.M. (2000) V(D)J recombinase-mediated transposition of the BCL2 gene to the IGH locus in follicular lymphoma. *Blood.* **96**, 1947-1952

Xu, S., Wong, S., Lam., K. (2000) B cell linker protein is dispensable for the allelic exclusion of immunoglobulin heavy chain locus but required for the persistence of $CD5^+$ B cells. *J. Immunol.* **165**, 4153-4157

Yanopoulos, G.D., Alt, F.W. (1986) Regulation of the assembly and expression of variable-region genes. *Annu. Rev. Immunol.* **4**, 339-368

Summary

B cell development is characterized by recombination of immunoglobulin heavy and light chain genes. Pro-B cells start rearranging their immunoglobulin V heavy chain genes. Human pre-B cells undergo apoptosis, unless they are rescued through survival signals of the pre-B cell receptor, which is expressed as a result of successful V_H region gene rearrangement. After (pre-) B cell receptor activation, a number of signaling proteins are activated finally leading to a Ca^{2+} signal. One of the proteins involved in this cascade is the linker molecule SLP65. It is known that SLP65 expression is defective in a substantial fraction of cases of acute lymphoblastic leukemia. In addition, we also found defective expression of SLP65 in a fraction of cases of B cell lymphoma. Therefore, we investigated the consequences of SLP65-deficiency on (pre-) B cell receptor signal transduction in B cell lymphoproliferative diseases.

We found that SLP65-deficient leukemia and lymphoma cells carry multiple *IGHV* region genes and also exhibit RAG1 and RAG2 expression together with ongoing V(D)J-recombinase activity. Reconstitution of SLP65-expression in SLP65-deficient leukemia and lymphoma cells results in downregulation of RAG1/2 expression and prevents both *de novo* V_H-DJ_H rearrangements and secondary V_H replacement. While SLP65 is essential for B cell receptor-induced Ca^{2+} mobilization in normal cells, B cell receptor engagement in SLP65-deficient as compared to SLP65-reconstituted B cell lymphoma cells resulted in an accelerated and enhanced yet short-lived Ca^{2+}-signal. In a search for components of a SLP65-independent B cell receptor signaling pathway, we identified a critical interaction between the *src*-kinase LYN and PLCγ1. As shown by RNA interference, LYN is required for B cell receptor-induced Ca^{2+} release in SLP65-deficient but not in SLP65-reconstituted B cell lymphoma cells. LYN also transduced B cell receptor-dependent survival and proliferation signals including tyrosine-phosphorylation of STAT5 and MAPK1 in SLP65-deficient B cell lymphoma cells.

We conclude that ongoing V_H gene rearrangements are a frequent feature in B lymphoid malignancy which can be attributed to SLP65 in many cases. This function of SLP65 may have important implications for the clonal evolution of a SLP65-deficient leukemia or lymphoma because perpetual expression and activity of RAG proteins carries the risk of continuous DNA double-strand breaks and the accumulation of secondary transforming events in the leukemia and lymphoma cells. The aberrant signaling pathway in SLP65-deficient B cell lymphoma cells indicates that the src-kinase LYN can short-circuit B cell receptor signaling in SLP65-deficient B cell lymphoproliferation and thereby promote activation of survival and proliferation-related molecules.

Supporting information

Oligonucleotides for RT-PCR analysis:

RAG1	5’-TGCAGACATCTCAACACTTTGGCCAG-3’
	5’-TTTCAAAGGATCTCACCCGGAACAGC-3’
RAG2	5’-AGCAGCCCCTCTGGCCTTCAG-3’
	5’-CATGGTTATGCTTTACATCCAGATG-3’
SLP65_Exon 2F	5’-GGCAGCTTCAAAAGATGGTC-3’
SLP65_Exon 17R	5’-TTTCCCCCTTTATGAAACTTTA-3’
GAPDH	5’- TTAGCACCCCTGGCCAAGG3’
	5’- CTTACTCCTTGGAGGCCATG-3’
rearranged V_H1-2	5’-TTCTGGATACACCTTCACCGGCT-3’
	5’-GATACACCTTCACCGGCTACTAT-3’
rearranged V_H6-1	5’-CTCGCAGACCCTCTCACTCAC-3’
	5’-CAGACCCTCTCACTCACCTGT-3’
germline J_H5	5’-CTTTAACTCTGAAGGGTTTTGCT-3’
	5’-AACTCTGAAGGGTTTTGCTGCAT-3’
	5’-CCCTAAGTGGACTCAGAGAGG-3’
Linker	5’-TCCCCGTACATCGTTAGAAG-3’
	5’-GTACATCGTTAGAAGCTTGA-3’
GAPDH	5’-TTAGCACCCCTGGCCAAGG-3’
	5’-CTTACTCCTTGGAGGCCATG-3’
LYN	5’-GACGATGGAGTAGATTTGAAGAC-3’
	5’-TTCCTGGTTATATCCTTGAAAAA-3’
SLP65_Exon1F	5’-TGGACAGTTATTCGTGTCTCTT-3’
SLP65_Exon7R	5’-GTGAACTGCTTTCTGTGGGA-3’
SLP65_Exon2F	5’-GGCAGCTTCAAAAGATGGTC-3’
SLP65_Exon17R	5’-TTTCCCCCTTTATGAAACTTTA-3’
SLP65_dExon16R	5’-CAACACTTCCAAAGTACTTGT-3’

Oligonucleotidesfor linker construction:

5’-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACATG-3’

3’ amino (C7)-GACGAGCTTAAGTTCGAAGATTGCTACATGCCCCT-5’

Oligonucleotides for sequence analysis of a genomic deletion in Karpas-422 DLBCL cells:

SLP65_Intron2-3F 5'-CATCAAATCTGTCCATTGATTG-3'

SLP65_Intron3-4R 5'-ACCTTTTGTTTTCAAATGAGGA-3'

Oligonucleotides for RNA interference:

5'-AGAUUGGAGAAGGCUUGUAUU-3'

5'-AAUACAAGCCUUCUCCAAUCU-3' (LYN1 siRNA)

5'-CCAUCACUGGUUGCACUUAUU-3'

5'-AAUAAGUGCAACCAGUGAUGG-3' (LYN2 siRNA)

5'-AGUAUUCUGUACUCUUAGAUU-3'

5'-AAUCUAAGAGUACAGAAUACU-3' (LYN3 siRNA).

5'-UUGUACCUAAUUUCGUCCCAC-3'

5'-GUGGGACGAAAUUAGGUACAA-3' (Non-targeting siRNA).

Patient samples and cell lines

28 B cell precursor leukemia cases including 16 primary cases of B cell precursor leukemia and 12 B cell precursor leukemia cell lines were studied (Table 1A). In addition, 27 cases of B cell lymphoma including 17 primary cases and 10 cell lines were investigated (Table 1B). The collection of B cell lymphomas included B-CLL, mantle cell lymphoma, follicular lymphoma, Burkitt's lymphoma and diffuse large B cell lymphoma cases. BEL1, HPB-NULL, HBL-2 and SP49 cells were a kind gift from Dr. Ruoping Tang (Paris, France), Dr. Yoshinobu Matsuo (Okayama, Japan), Dr. Wolfram Klapper (Kiel, Germany) and Dr. Masanori Daibata (Kochi, Japan). Primary leukemia samples were provided by R.S. (Kiel, Germany) and Dr. Wolf-Karsten Hofmann (Berlin, Germany). Primary lymphoma samples were provided by Dr. Wolfram Klapper and Dr. Reza Parwaresch (Kiel, Germany) and Dr. Martin-Leo Hansmann (Frankfurt, Germany).

Abbreviations

ALL	acute lymphoblastic leukemia
B-CLL	B cell chronic lymphocytic leukaemia
BCR	B cell receptor
BTK	Bruton's tyrosine kinase
BTK-ΔK	kinase-deficient BTK isoform
C	constant
CLP	common lymphoid progenitor
cRSS	cryptic RSS
D	diversity
DLBCL	diffuse large B cell lymphoma
IGH	immunoglobulin heavy-chain
IGK	immunoglobulin κ light chain
IGL	immunoglobulin λ light chain
IRES	internal ribosome entry side
ITAM	immunoreceptor tyrosine-based activatory motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
J	joining
LM-PCR	ligation-mediated PCR
NHEJ	non-homologous end joining
PTK	protein tyrosine kinase
RSS	Recombination signal sequence
SH2	SRC homology domain 2
siRNA	short interfering RNA
SH2	SRC homology domain 2
SH3	SRC homology domain 3
SLP65	SH2 domain-containing leukocyte protein of 65 kD
SLP65ΔE16	SLP65 without exon 16
SRC	cellular homolog of *Rous sarcoma virus*
V	variable

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Curriculum Vitae

Mieke Sprangers was born on the 23rd of April 1976 in Heerlen, the Netherlands. After she had finished in 1994 the HAVO at the Laurens College in Rotterdam she started the HLO (higher laboratory education) in Delft. During this education she specialized in biochemistry. For this study she did an internship at the University of Oslo, Norway. Under the supervision of professor Kristian Prydz research was done on proteoglycans involved in endocytosis. She finished this part of her education in 1998 and continued by studying biology at the University of Leiden. In this 2-year program she spend half a year at the Molecular Plant Institute in Leiden under supervision of Dr. Bert van der Zaal in the department of professor Hooykaas and studied root-formation in *Arabidopsis*. She rounded off this study in 2000 with an internship at the University of Sydney, Australia, where she wrote a thesis about the Alzheimer disease.

From 2001 till 2003 she worked at Crucell Holland BV within the immunology group as a research technician. She started in 2003 her PhD-study at the Heinrich-Heine-Universität in Düsseldorf in the group of Professor Markus Müschen. Here she studied the malignant development of pre-B cell leukemia and B cell lymphoma cells as described in this thesis.
Since January 2006 she is an employee of Qiagen in Hilden, Germany, in the function of technical service specialist.

List of publications

Klein F, Feldhahn N, Mooster JL, **Sprangers M**, Hofmann WK, Wernet P, Wartenberg M, Müschen M. (2005) 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.*; **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. (2005) Mimicry of a constitutively active pre-B cell receptor in acute lymphoblastic leukemia cells. *J Exp Med*. **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. (2005) Deficiency of Bruton's tyrosine kinase in B cell precursor leukemia cells. *Proc Natl Acad Sci USA*. **102**, 13266-13271

Klein F, Feldhahn N**,** Herzog S, **Sprangers M**, Mooster JL, Jumaa H, Müschen M. (2005) BCR-ABL1 induces aberrant splicing of *IKAROS* and lineage infidelity in pre-B lymphoblastic leukemia cells. *Oncogene*. **24**, 1-7

Sprangers M, Feldhahn N, Liedtke S, Jumaa H, Siebert S, Müschen M. (2005) *SLP65*-deficiency results in perpetual V(D)J-recombinase activity in pre-B lymphoblastic leukemia and B cell lymphoma cells. *Oncogene.* In press

Sprangers M, Feldhahn N, Herzog S, Hansmann M, Reppel M, Jumaa H, Siebert R, Müschen M. (2006) The SRC family kinase LYN redirects B cell receptor signaling in human SLP65-deficient B cell lymphoma cells. *Oncogene.* In press

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 bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 03.02.2006

Mieke Sprangers