DISSECTING THE ROLES OF THE DIFFERENT ISOFORMS OF THE LYMPHOID-SPECIFIC TRANSCRIPTIONAL COACTIVATOR OBF-1

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Abstract

Abstract

B cell differentiation depends on a highly regulated transcriptional machinery. The octamer sequence (ATGCAAAT) is a conserved DNA motif that confers transcriptional B cell specificity. This sequence is mainly found in immunoglobulin promoters, but also in some B cell specific genes. OBF-1, also known as Bob.1 or OCA-B, is a B lymphocyte-specific transcription factor which coactivates Oct1 and Oct2 on such octamer sites. The main function of OBF-1 was identified in late stage B cells, as *OBF-1* deficient mice exhibit a severe reduction of T cell-dependent immune response and a lack of germinal center formation in the spleen. However little is known about the role of OBF-1 in early B cells.

The most relevant biological finding of this thesis illustrates the fact that OBF-1 expression level has to be tightly regulated to allow early B cell development. Most OBF-1 target genes were identified in late B cells. This thesis proposes for the first time three direct OBF-1 target genes in early B cells, namely *Id2*, *Id3*, and *EBF1*. Indeed OBF-1 can bind their respective promoters. Aberrant OBF-1 expression led to increased levels of Id2 and Id3 transcripts, which resulted in differentiation blocks before and after B cell commitment. In fact Id2 and Id3 are known to be negative regulators of B cell differentiation probably by antagonizing E2A DNA binding. EBF1 is a transcription factor involved in B cell commitment and was reported to induce OBF-1 expression. EBF1 was upregulated in mice overexpressing OBF-1, therefore the activation of *EBF1* promoter by OBF-1 constitutes a positive feedback loop.

The *OBF-1* gene contains two active start codons giving rise to nuclear and myristoylated cytoplasmic isoforms. The second part of this thesis describes the investigation of the physiological role of each isoform. BAC transgenic mice expressing only one isoform were generated to decipher this issue. The nuclear isoform was found to be the main player among the isoforms, as the corresponding transgenic mice did not have any OBF-1 associated phenotype and most of the OBF-1 regulated genes were under its control. IL-7 dependent cultures of preBI cells from mice deficient for *OBF-1* were reported to proliferate faster. In fact the cytoplasmic OBF-1 isoform might have a role under these culture conditions, as the nuclear OBF-1 isoform could only partially rescue this hyperproliferation.

1 Introduction

1.1 The lymphoid system

Living organisms have different ways to protect themselves against pathogens. Vertebrates have a common immune system which uses the generation of specific immunoglobulins as a main weapon against harmful entities. There are two types of immune responses to defend the host organism against pathogens, the innate and adaptive immune responses. The adaptive response needs previous exposure to pathogen on the contrary to the innate response. The immune system comprises lymphoid, myeloid and Drendritic cells. These cell types are orchestrating the innate and adaptive immune responses which are intrically linked. This introduction will focus on the lymphoid system and later will emphasize the B cell ontogeny.
The lymphoid system is constituted of B, T and NK cells. The B and T cells are responsible for the adaptive immunity, whereas the NK cells are involved in the innate immunity. All blood cells arise from a type of cell called the hematopoietic stem cell (HSC) that reside mainly in the bone marrow of adult mammals. Commitment to the different hematopoietic lineages and differentiation of HSCs into the different effector types of cells of the immune response proceeds in a cascade of well defined steps. Early in hematopoiesis, a multipotent progenitor (MPP) differentiates along one of two pathways, giving rise to either a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP) (Fig. 1). During the development of the lymphoid and myeloid lineages, stem cells differentiate into progenitor cells, which have lost the capacity for self-renewal and are committed to a particular cell lineage. Common lymphoid progenitor cells give rise to B, T, and NK (natural killer) cells. Myeloid stem cells generate progenitors of red blood cells (neutrophils, eosinophils, basophils, monocytes, mast cells), and platelets (Fig. 1).
The development of B lymphocytes (B lymphopoiesis) from a hematopoietic stem cell proceeds along a highly ordered, but flexible, pathway. The genetic dissection of this pathway is quite advanced and transcription factors have been found to be essential at many steps of B cell development. Three different nomenclatures for B cell stages are commonly used for historical reasons. Two of these nomenclatures were introduced by Hardy and Hayakama (Philadelphia nomenclature (Hardy and Hayakawa, 2001; Hardy et al., 1991) and a slightly different convention by Rolink and Melchers (Basel nomenclature (Osmond et al., 1998)). For the sake of simplicity the Basel nomenclature will be used in this thesis. The development of the lymphoid cells is depicted in Figure 1.

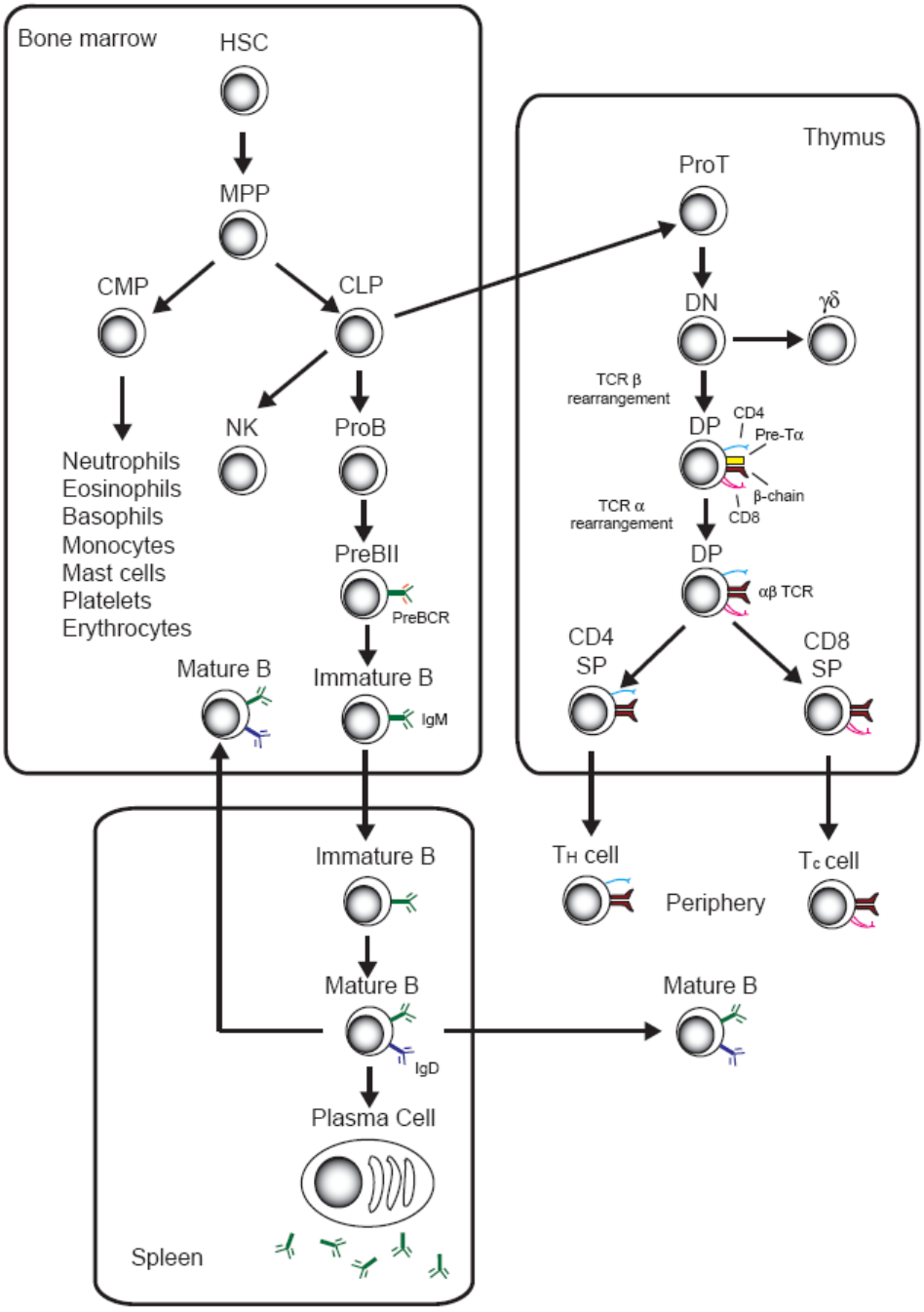


Figure 1: Lymphoid system
This scheme represents the B and T cell differentiation in the bone marrow, thymus and spleen. The myeloid differentiation in the bone marrow is not illustrated in detail as it is not part of the lymphoid system.

1.1.1 B cell development

Bone marrow

B lymphocytes arise from a common lymphoid progenitor (CLP) detectable early in embryonic development in the fetal liver and, during adulthood, in the bone marrow. These CLP then differentiate into Progenitor B cells (Pro B cells) which are characterized by surface expression of the CD45 phosphatase isoform B220 and to a

lesser extend c-Kit. The ProB cell population is heterogeneous as they contain Early Progenitors with Myeloid and Lymphoid potential (EPLM) and Precursor B cells (preBI cells). The EPLM is only a quick transient stage between the CLP and the preBI cells which are multipotent and B cell committed respectively. The EPLM cells have Ig loci still in germline configuration and the transition to preBI cells is monitored by CD19 expression.
The preBI cells proliferate within the bone marrow and differentiate into preBII cells after complete heavy chain gene rearrangement (see 1.1.1.1) and express a membrane immunoglobulin consisting of the heavy chain and surrogate light chains, Vpre-B and $\lambda 5$ forming the the preBCR (preB cell receptor).
The preBII cells, which are characterized by B220 and CD25 surface expression, enter the cell cycle and undergo a proliferative burst, leading to amplification (clonal expansion) of the few cells that underwent productive rearrangement. The preBII cells continue to develop into immature B cell when a productive light-chain gene rearrangement is performed and replaces the surrogate light chain to give rise to mIgM (membrane IgM) on the cell surface.
Therefore the immature B cells are characterized by B220 and IgM surface expression. The immature B cells undergo negative selection in the bone marrow. Autoreactive B cells have to be removed or inactivated and accordingly, encounter of immature B cells with antigen leads to one of three results (Hardy and Hayakawa, 2001): (a) elimination of the respective cell (clonal deletion), this is believed to happen in case of high-affinity antigen binding, (b) cells become nonresponsive (anergy), and (c) cells revise their BCR in a process called receptor editing, involving secondary rearrangements (Radic and Zouali, 1996; Nussenzweig, 1998). (b) and (c) are the options probably chosen in case of low-affinity antigen binding. The detailed development process of early B cells is described in the chapter 1.2.

Secondary lymphoid organs

The immature B cells then leave the bone marrow to enter secondary lymphoid organs such as lymph nodes or spleen. The spleen is the best characterized secondary organ. In the spleen the immature B cells emigrate initially to the red pulp and can be identified by the IgM^{high} $IgD^{low}CD21^{low}CD23^{-}$ surface markers (Cariappa and Pillai, 2002). These transitional B cells express also the 493 antigen.
After a short period, some of these cells colonize the B cell follicles with concomitant expression of high IgD and CD23 levels giving rise to mature B cells. Some of these cells reduce their CD21 and IgM expression level and recirculate to peripheral lymphoid organs and to the bone marrow (Fig. 1). The mature B cells can be activated in a similar way in the lymph nodes or in the spleen upon stimulation by antigen presenting cells.
In the spleen the initial activation of the B cells occurs in the periarteriolar lymphoid sheeth (PALS) which is heavily populated with T cells and contains Interdigitating Dendritic Cells (IDC). After B cell activation, small foci of proliferating B cells form at the edges of the PALS. The B cells within these foci differentiate into short-lived plasma cells secreting IgM and IgG isotypes. A few activated B cells then migrate from the foci to primary follicles in the marginal zone which will develop into secondary follicles.
The marginal zone is heavily populated with long-lived mature B cells which have high CD21 expression level ($IgM^{high}IgD^{-}CD23^{-}CD21^{high}$) and this region is the first line of defence against a pathogen (Cariappa and Pillai, 2002) (Fig. 2a).

The activated B cells migrate towards the center of the secondary follicle, forming the germinal center (GC). The Germinal Center is reviewed in Hess et al., 1998, Tarlinton, 1998, and van Eijk et al., 2001. The proliferating activated B cells (centroblasts) then move to one edge of the follicle thus forming the dark zone where they perform somatic hypermutation and give rise to small centrocytes. Then they move into the light zone of the germinal center containing numerous follicular dendritic cells (FDC) which select high-affinity centrocytes by binding to antigen-antibody complexes. Within the light zone the B cells perform class switching (see 1.1.1.5) and differentiate into small memory B cells and large plasmablasts. The plasmablasts develop into long-lived plasma cells and begin to secrete antibodies (Fig. 2b).

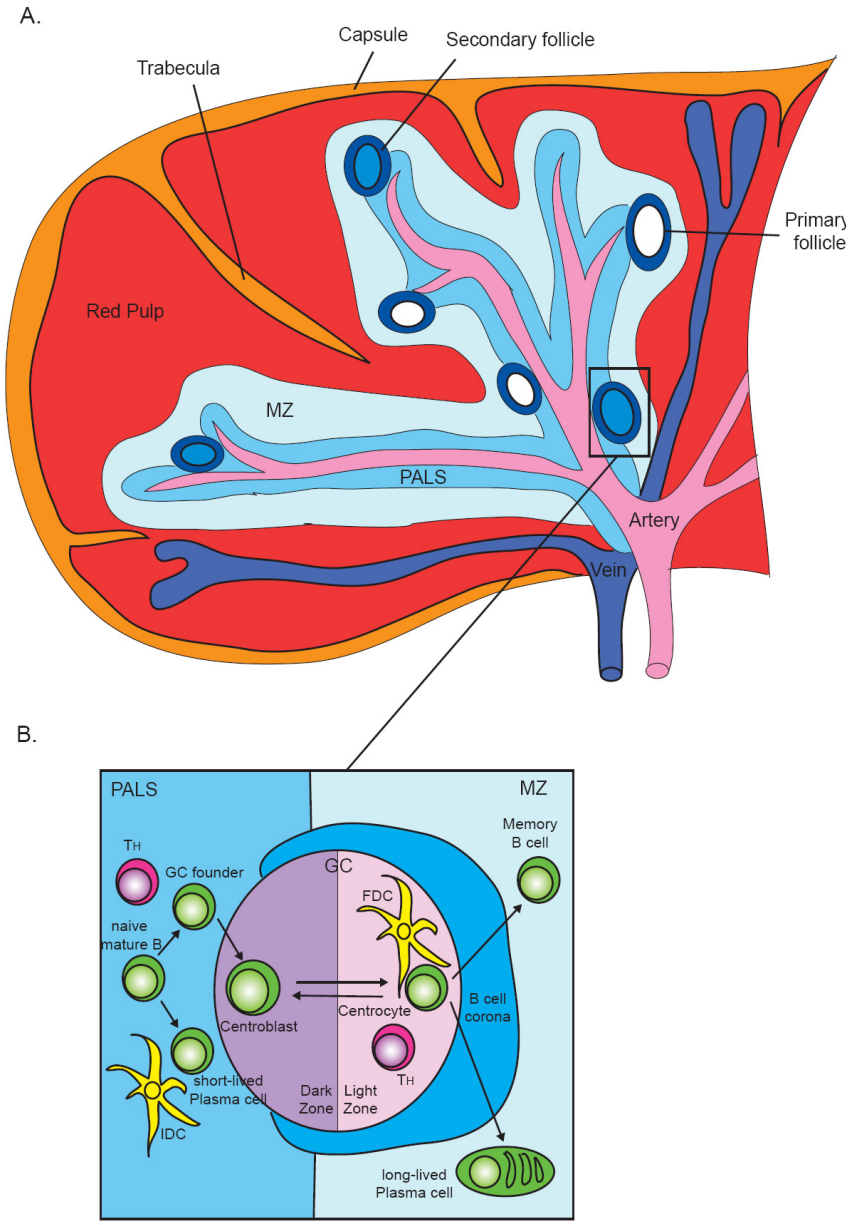


Figure 2: Spleen architecture.
(A) Structure of the spleen. The blood born pathogens are filtrated in the Red Pulp so that the B cells in the PALS are activated and form primary and secondary follicles. (B) The structure of secondary follicles containing the Germinal Center is depicted. The activated B cells enter the Dark Zone of the GC and undergo affinity maturation. The Centrocytes that have a high affinity for the antigen survive and differentiate into Plasma cells and Memory B cells. PALS: Periarteriolar Lymphoid Sheath; IDC: Interdigitating Dendritic Cells; GC: Germinal Center; FDC: Follicular Dendritic Cells; MZ: Marginal Zone.

1.1.1.1 Heavy chain rearrangement

An immunoglobulin molecule (antibody) is constituted of a pair of identical Ig heavy chains and a pair of identical Ig light chains. The N-terminal part of the heavy chain and the light chain has an amino acid sequence called variable region which is involved in specific antigen binding. The C-terminal part is more conserved and therefore is called the constant region. It prescribes the class and effector function of an antibody. The variable regions are assembled from germline *V*, *D*, *J* gene segments through a site-specific recombination reaction known as V(D)J recombination (Jung and Alt, 2004; Bassing et al., 2002).
The murine *IgH* locus spans about 3 Mb (Jung et al., 2006). About 150 functional V_H gene segments, each with their own promoter and leader sequence (for protein sorting), comprise 15 V_H segment families upstream of 12-13 D_H gene segments. Four J_H gene segments are located downstream of the D_H segments, and 8 constant region exons span about 200kb downstream of the J_H segments (Fig. 3). The V(D)J

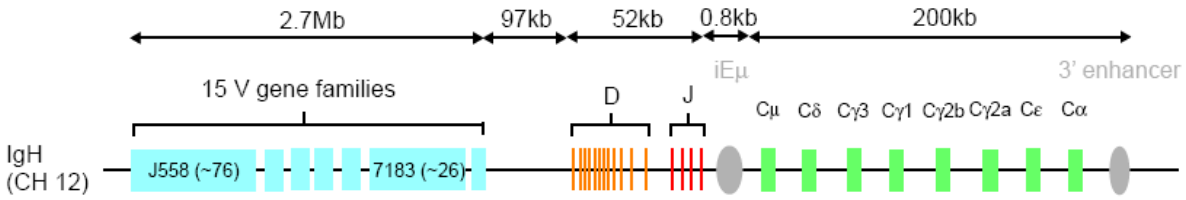
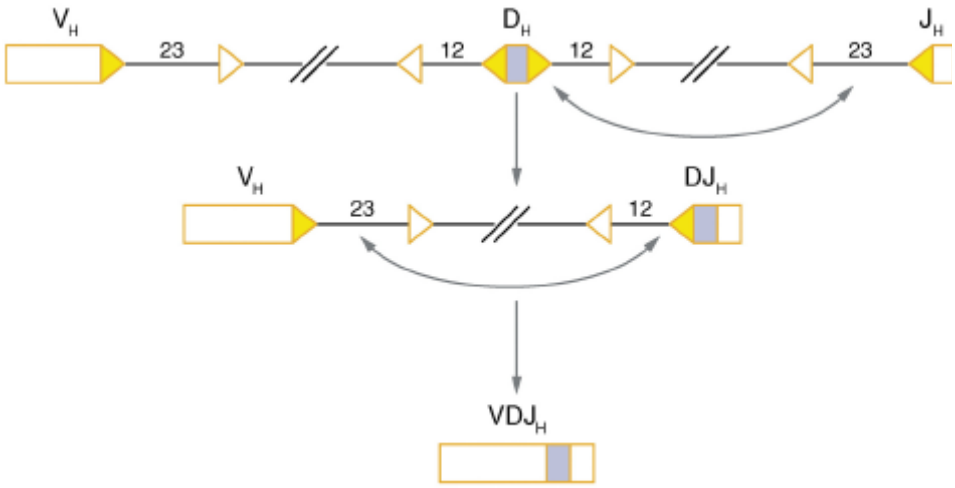


Figure 3: Organization and control elements of the murine immunoglobulin heavy (*IgH*) gene locus (not drawn to scale).
The variable (V), diversity (D), joining (J) and constant (C) gene segments are represented as boxes. The enhancers are shown in grey. Distances shown are from the murine 129 strain and vary between strains.

recombination is generated by RAG mediated double strand breaks (DSBs) followed by religation at the borders between two coding segments and their flanking recombination signal sequences (RSS) bringing first the *D* and *J* regions, and then the *V* to *DJ* regions together. The RSS (nonamer-12/23bp-heptamer) are present 3' of each *V* region, 5' and 3' of *D* regions and 5' of each *J* region. The V-D-J order of recombination is ensured by the rule that a RSS with a 12 bp spacer can only join with a RSS with a 23 bp spacer (Fig. 4a). The V(D)J recombination results in placing the germline V_H promoter in close proximity to a strong enhancer element ($iE\mu$) that lies in the intron between the J_H and $C\mu$ exons (Fig. 4b). Furthermore there is a large enhancer region 3' of Cα (*3' enhancer*) which also regulate the expression of the *IgH* gene. By default, IgH polypeptide utilizes the μ constant regions or –in mature B cells- the Cδ region, which can replace Cμ by alternative splicing mechanism. The diversity of *Ig* genes during V(D)J recombination is increased by the terminal deoxynucleotidyl transferase (TdT), which randomly adds N-nucleotides at the rearrangement joints. After one allele is productively rearranged, the recombination machinery is quickly downregulated so that generally, in any one cell only one successfully rearranged *IgH* and *IgL* locus is found (allelic exclusion). In immature B cells the yield of unproductive rearrangement is increased by a mechanism called receptor editing, which drives further recombination of regions that lie outside of the already recombined regions.

A.



B.

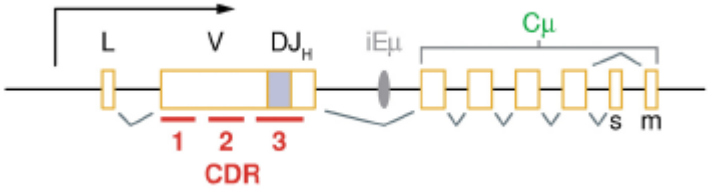


Figure 4: Assembly and expression of IgH genes.
(A) Variable (V_H), diversity (D_H), and joining (J_H) gene segments are shown, along with their flanking recombination signal sequences (RSS). RS heptamers are depicted as yellow triangles, whereas RS nonamers are depicted as white triangles. Spacer lengths are indicated above the various RSS. (B) Location of the three complementarity determining (antigen contact) regions (CDRs) on the assembled V_HDJ_H exon is shown. Transcription initiates upstream of the assembled V_HDJ_H exon and proceeds through the four $C\mu$ exons and the membrane (m) and secreted (s) exons. Possible splicing events are indicated. (from Jung et al., 2006)

1.1.1.2 Light chain rearrangement

The light chain is encoded by the κ and the λ loci that are alternatively used. The κ light chain locus lacks the *D* regions and there is only one *C* region. Enhancers are found on similar positions as the *IgH* gene. The λ light chain locus has multiple sequential pairs of *J-C* and has no intronic enhancer (Fig. 5). The recombination machinery is identical to the heavy chain, the κ light chain is generally rearranged first, and in case of unproductive rearrangement the λ light chain is used.

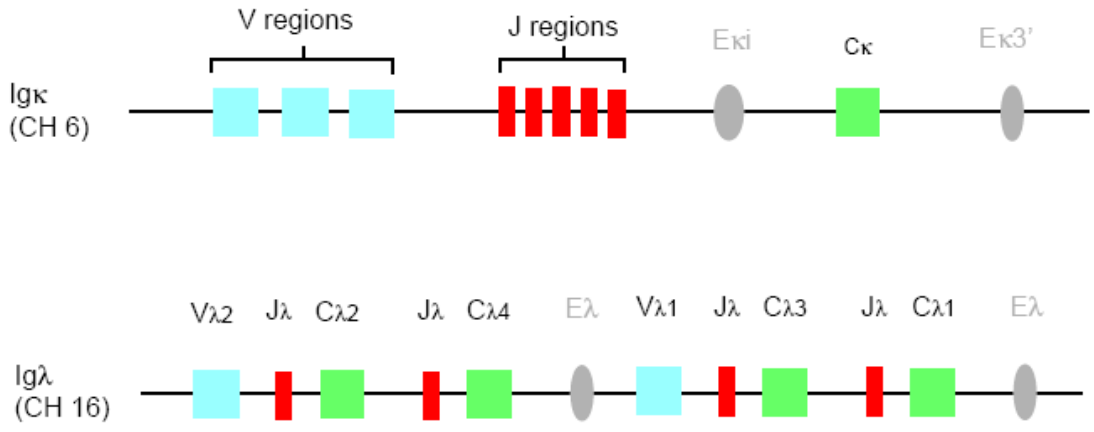


Figure 5: Organization and control elements of the murine immunoglobulin light chain (*Igκ*, *Igλ*) gene loci.
The variable (*V*), joining (*J*) and constant (*C*) gene segments are represented as boxes. The enhancers are shown in grey.

1.1.1.3 Immunoglobulin affinity maturation

The germinal center is the place where B cells are activated in a T-dependent manner. The GC drives the generation of high affinity antibodies through repeated rounds of selection and somatic hypermutation of the BCR (collectively called affinity maturation). The heavy- and light-chain variable region of the immunoglobulin genes are hypermutated when centroblasts proliferate in the dark zone of the germinal center. The germinal center B cells undergo several rounds of cell division in the dark zone and accumulate mutations, then leave and experience selection in the light zone. The Follicular Dendritic Cells (FDCs) are coated with antigen and give a survival signal to high affinity centrocytes. The centrocytes must also receive signals generated by interaction with T_H cells to survive. Some of the B cells return to the dark zone for additional rounds of division and mutation (Fig. 2b). Subsequent selection of these cells in the light zone allows the emergence of cells producing antibodies of higher affinity.

1.1.1.4 Generation of plasma cells and memory B cells

The centrocytes, which have undergone affinity maturation, differentiate into Plasma cells and memory B cells in the light zone. The centrocytes need proliferation cytokines like IL-2, IL-4 and IL-5 and differentiation cytokines like IL-2, IL-4, IL-5, IFN-γ and TGF-β in order to perform class switching (see 1.1.1.5) and differentiate into Plasma cells. Plasma cells are highly specialized in the secretion of large amounts of antibodies and have a life span of about one month. They lack detectable membrane-bound immunoglobulin and synthesize high levels of secreted antibody. Memory B cells are latent cells that are the basis for an enhanced and more rapid response to repeated antigen challenge and are very similar to naive B cells, but they express additional immunoglobulin isotypes, including IgG, IgA, and IgE and may be long-lived.

1.1.1.5 Class switch recombination (CSR)

Class switching is the process by which the heavy chain of the immunoglobulin gene is rearranged to produce antibodies with different effector functions, but same antigen binding. For example IgM is secreted during a primary immune response and is able to activate the complement system. IgG isotypes constituted of IgG1, IgG2a, IgG2b and IgG3 in mice are secreted during a secondary immune response and are involved in antigen neutralisation, opsonisation, sensitization for killing by NK cells and activation of the complement system. IgE is responsible for the sensitization of mast cells, and IgA can neutralize pathogens in the digestive tract. CSR is a deletional process that places a constant region (*C*) gene other than $C\mu$ or $C\delta$ directly downstream of the rearranged *VDJ* segments, leading to the expression of other, secondary Ig isotypes encoded by downstream *C* genes (Stavnezer, 1996). This enables antibody specificity to remain constant while the biological effector activities of the molecule vary. The cytokine combination determines what Ig class is chosen when an IgM-bearing cell undergoes class switching.

1.1.2 T cell development

T lymphocytes play an essential role in the immune system as they can stimulate B cells to set up an antigen specific response (Th cells) and they can induce the destruction of virus infected cells directly (Tc cells). This thesis is focusing mainly in B lymphocytes, therefore only a short overview of T cell development is described.
In the bone marrow the Common Lymphoid Progenitors give rise to ProT cells, which migrate to the thymus. They enter the outer cortex and start to proliferate. The cells then express c-Kit, CD44 and CD25 and continue proliferating, but do not rearrange the TCR genes yet. The TCR is a membrane receptor that recognizes antigens associated with MHC class I and II. The MHC I is ubiquitously expressed on all the cells and the MHC II is expressed on Antigen Presenting Cells (APC) such as Dendritic Cells, Macrophages or activated B cells.
Then the thymocytes stop expressing c-Kit and downregulate CD44. The thymocytes that make productive rearrangements of both the γ- and δ-chain genes develop into double-negative $CD3^+$ $\gamma\delta$ T cells (<5% of thymocytes). As soon as they upregulate the pre-TCRα gene (pTα) they stop proliferating and start rearranging the *TCR β-chain* genes. When the *TCR β* gene rearrangement is productive the cells start to express CD4 and CD8 and become small non-proliferating DP thymocytes. Then the thymocytes rearrange the *TCR α-chain* genes.
The cells undergo positive and negative selection to ensure the mature T cells that are exported from the thymus are functional (self-MHC restricted) and self-tolerant respectively. Actually about 98% of all thymocytes do not mature, but die by apoptosis within the thymus either because they fail to make a productive *TCR*-gene rearrangement or because they fail to survive thymic selection. The positive and negative selections take place in the cortex and in the medulla of the thymus respectively. The cells that pass the negative and positive selection processes have a mature TCR that respond with low affinity and express either CD4 or CD8.
The CD4 SP and CD8 SP thymocytes give rise to T-helper cells (Th) and cytotoxic T lymphocytes (Tc) respectively. The Th cells and the Tc cells recognize MHC class II and class I respectively on target cells. The development of the T cells is depicted in Figure 1.

1.2 Review paper

Intrinsic and extrinsic factors in early B cell development

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B cell ontogeny in the bone marrow is a complex and well regulated process which is essential for efficient immune responses in secondary lymphoid organs. B cell development is governed by multiple levels of regulation. Intrinsic factors regulating B cell ontogeny are constituted mainly of transcription factors, but also comprise intracellular molecules involved in signalling pathways. The cytokines and chemokines are part of the extrinsic factors. This review summarizes our current understanding of the intrinsic and extrinsic factors guiding early B cell development. Furthermore it also describes the role of the redox environment, which appears to play a major role in B cell ontogeny.

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1. Introduction

B cell ontogeny is a tightly regulated process where intrinsic (transcription factors, signalling molecules) and extrinsic factors (cytokines, chemokines) work together to establish and maintain the right B cell differentiation programme. Early B cells in the bone marrow originate from multipotent stem cells which are able to generate all the precursor cells to establish the whole immune system. As these stem cells differentiate they gradually loose their multipotency. The hinge of B cell ontogeny is at the preBI cell stage, the point at which the cells become fully committed to the B cell lineage: at this stage the B cell transcription programme is turned on and the cells cannot differentiate anymore into other lineages. B cell differentiation in the bone marrow consists in a sequential rearrangement of the immunoglobulin (*Ig*) genes to obtain B cells expressing antibodies with many different antigen affinities and to select those B cells that are not autoreactive. The immunoglobulin gene rearrangement mechanism will not be described in this review, however a recent overview can be found in Cobb et al., 2006 (Cobb, Oestreich et al. 2006). The final output in B cell ontogeny occurs in secondary lymphoid organs such as the lymph nodes and the spleen, where the B cells have the potential to become activated by an antigen and to induce an immune response by secreting antigen-specific antibodies.
The transition from a cell stage to the next depends on the activity of specific transcription factors which are turned on and off in a temporally regulated manner (Medina and Singh 2005; Pelayo, Welner et al. 2005; Hagman and Lukin 2006;

Pelayo, Welner et al. 2006). A dozen transcription factors have been identified by knock out studies to play a role in B cell development. The four most important transcription factors for early B cell development are E2A, early B-cell factor (EBF), Pax5, and Sox-4, since the knock out mice have a strong differentiation block before B cell commitment.
B cell development in the bone marrow also requires extrinsic factors such as chemokines and cytokines. The establishment of bone marrow derived stromal cell lines resulted in the discovery of soluble proteins involved in efficient early B cell proliferation and homing. The deletion of these cytokines and their receptors allowed understanding the *in vivo* function of cytokines in B cell responses.
The redox regulation in B cell development is a new field of research, as H_2O_2 was reported to be a second messenger in BCR signalling only recently (Reth 2002). Evidence supports the notion that conformational changes of transcription factors mediated by oxidizing or reducing agents can modulate their DNA binding and transactivation activity.
In this review we will discuss about the different steps of B cell ontogeny with a focus on populations downstream of the common lymphoid progenitor (CLP) stage where the cells become specified to the B cell lineage. The initiation and the sequential transcriptional programme with the microenvironmental cues of early B cell development will also be described in detail. Finally the redox issues influencing B cell development will be discussed.

2. Stages of B lymphopoiesis in the bone marrow

This section describes the developmental pathway leading to B cell precursors (section 2.1) followed by the B cell ontogeny in the bone marrow (section 2.2). Fig. (**1**) gives an overview of the different B cell developmental stages leading to immature B cells in relation to the expression of stage-specific cell surface markers and extrinsic factors.

2.1 Stem cells and multipotent progenitors

The hematopoietic stem cells (HSCs) are the stem cells of the hematopoietic lineage in the bone marrow from which all blood cells ultimately derive. These cells express c-Kit and Sca-1, but do not yet express markers associated with definitive differentiated lineages (e.g. B cells, T cells, etc…), so-called lineage markers. The HSCs can be divided into two subpopulations: the long-term HSCs, which are able to repopulate all hematopoietic cells for the life of the recipient, and the short-term HSCs, which can repopulate only transiently the hematopoietic compartment (Morrison and Weissman 1994). The HSCs then differentiate into multipotent progenitors (MPPs) which express the tyrosine kinase receptor Flt3. Section 4.2 describes the role of Flt3 just before B cell commitment. The MPPs give rise to the common myeloid progenitors (CMPs) (Akashi, Traver et al. 2000) and to the early lymphoid progenitors (ELPs) (Tudor, Payne et al. 2000). ELPs express an intermediate level of CD127 (IL-7R). IL-7 plays mainly a role in preB cell development (section 4.4). Some ELPs express the recombinase-activating genes (*RAG*) *RAG1* and *RAG2*, which are essential for *Ig* gene recombination. Therefore some ELPs have immunoglobulin D_H-J_H Immunoglobulin Heavy Chain (*IgHC*) gene rearrangements (Igarashi, Gregory et al. 2002), and start to set up the genetic machinery to give rise to B cell precursors. ELPs are also precursors of the

intrathymic early T lymphocyte lineage progenitor (ETP) (Allman, Sambandam et al. 2003). In fact recombined D_H-J_H can be detected in thymocytes and other hematopoietic cells (Kurosawa, von Boehmer et al. 1981). ELPs then differentiate into common lymphoid progenitors (CLPs), which express low levels of c-Kit and Sca-1, but express well CD127 and CD93 (aka AA4.1) (Kondo, Weissman et al. 1997). At this stage the *IgHC* gene starts beeing rearranged. CLPs are referred to as B lineage specified rather than B lineage committed progenitors, because the non-B lineage developmental programs have not been completely turned off and these cells still have the potential to generate T (Kondo, Weissman et al. 1997; Allman, Sambandam et al. 2003; Petrie 2007), natural killer (NK) (Kouro, Kumar et al. 2002), and dendritic cells (DC) (Kondo, Weissman et al. 1997; Bjorck and Kincade 1998; Kouro, Kumar et al. 2002; Karsunky, Merad et al. 2003; Martin, Aifantis et al. 2003; Shigematsu, Reizis et al. 2004; Pelayo, Welner et al. 2006). CLPs then differentiate into early progenitors with lymphoid and myeloid development potential (EPLMs) which express CD45R, Notch1 and CD43. EPLM have still the capacity to give rise to T and myeloid cells, when cultured *in vitro* or when they are used to reconstitute γ-irradiated recipient mice (Rolink, Massa et al. 2006). At this stage the *IgHC* gene rearrangement is still undergoing. *In vivo* these cells are only a transient stage and differentiate fast into committed preBI cells by upregulating CD19.

2.2 B cell lineage

PreBI cells:
In the literature the ProB cells are defined as $B220^+cKit^+$ cells. However this population is heterogeneous and comprises the EPLM and preBI cells among others. In this review we will make this distinction. At the preBI cell stage the cells are fully committed to the B lymphocyte lineage. The trigger is the expression of the transcription factor Pax5 which induces the expression of CD19. The condition to differentiate into preBII cells is the successful rearrangement of one of the two *IgHC* gene alleles. First the heavy-chain D_H to J_H gene rearrangement occurs, then the V_H to D_HJ_H rearrangement proceeds. If the first rearrangement is not productive, it will continue on the other chromosome. It has been estimated that only one-third of the preBI cells undergo productive *IgHC* recombination and matures into preBII cells (Alt, Blackwell et al. 1987).

PreBII cells:
The preBII cells can be divided into two subpopulations, the large and the small preBII cells. The preBII cells stop expressing c-Kit and begin to express CD25, which is the α chain of the IL-2 receptor. CD43 is downregulated within the large preBII cell stage and remains turned off for the rest of B cell ontogeny. The preBII cells have rearranged the heavy chain genes but not the light chain yet. Therefore the membrane μ chain is associated with a surrogate light chain. This complex consists of a V-like sequence called Vpre-B and a C-like sequence called λ5, which associate noncovalently to form a light-chain-like structure. The μ heavy chain and the surrogate light chain associate with the Ig-α/Ig-β heterodimer to form the pre-B-cell receptor (preBCR) (Melchers, Karasuyama et al. 1993; Karasuyama, Rolink et al. 1996). The physiological role of the preBCR is not clear; it might recognize an unidentified ligand on the stromal cell surface, thus transmitting a signal that prevents V_H to D_HJ_H rearrangement of the other heavy-chain allele leading to allelic exclusion. The preBCR is also critical for the survival of the cells, since impaired preBCR results

in developmental arrest and cell death (Lam, Kuhn et al. 1997; Meffre, Casellas et al. 2000; Gauld, Dal Porto et al. 2002; Meffre and Nussenzweig 2002; Kraus, Alimzhanov et al. 2004). Once the preBCR is formed the cells divide to produce 32 to 64 descendants (5-6 divisions) giving rise to small preBII cells. After the preBII cell stage the successful rearrangement of the light-chain gene is the condition to differentiate into immature B cells.

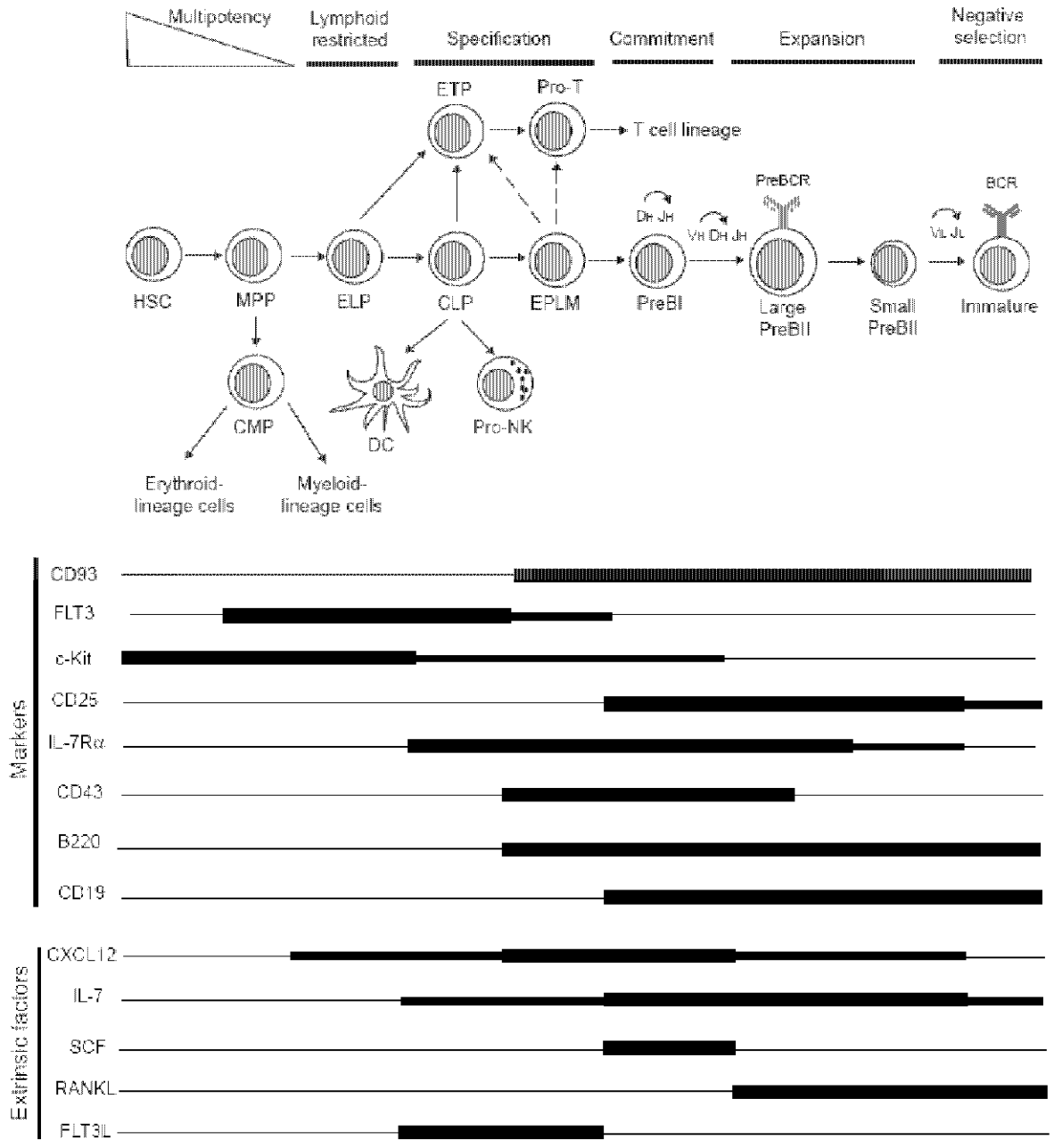

Figure 1: Early B cell development
Discrete stages of B cell development have been defined based on expression of cell surface markers, cell size, the status of Ig heavy (H) and light (L) chain rearrangement, and of the preBCR and BCR complex (IgH chain, (surrogate) IgL chain, Igα and Igβ chains). Extrinsic factors and their expression profile are also depicted.

Immature B cells:
Immature B cells have rearranged Heavy and Light chain genes and start to express mIgM (membrane IgM) on the cell surface. They loose the preBCR and stop expressing CD25. Immature B cells undergo negative selection in the bone marrow: cells that express self-reactive mIgM and bind self-antigens in the bone marrow die by apoptosis. However negative selection of immature B cells does not always result in their elimination. Self-reactive B cells are able to edit their B cell receptor (BCR). When this scenario occurs the maturation is arrested and the cells upregulate RAG-1 and RAG-2 and begin rearranging again their Light Chain genes. For example they can replace the κ light chain of the self-antigen reactive antibody with a λ chain. The cells are then not self-reactive anymore and can leave the bone marrow.

3. Intrinsic factors

This section summarizes the roles of the intrinsic factors in the different bone marrow populations up to the immature B cell stage. The overview of the intrinsic factors in every cell stage is depicted in Fig. (**2**).

Table 1: Intrinsic factors in early B cell development.

Intrinsic factors (Gene Symbol)	Ectopic Expression Phenotype	Knock out phenotype with respect to B cell lineage	Direct target genes	Refs.
PU.1 (*Sfpi1*)	In *PU.1*$^{-/-}$ fetal-liver cells graded amounts of PU.1 rescue B cell and macrophage development.	Lack of fetal B cells and CLPs.	*Flt3*, *IL7-R*, *EBF*, *B220*, *c-rel*	(Scott, Simon et al. 1994; McKercher, Torbett et al. 1996; Hu, Rao et al. 2001; Medina, Pongubala et al. 2004; Iwasaki, Somoza et al. 2005; Nutt and Kee 2007)
Ikaros (*Ikzf1*)	N.D.	Lacks all stages of B cell differentiation.	*Flt3*, *RAG1/2*, *λ5*	(Georgopoulos, Bigby et al. 1994; Wang, Nichogiannopoulou et al. 1996; Nichogiannopoulou, Trevisan et al. 1999; Sabbattini, Lundgren et al. 2001; Lopez, Schoetz et al. 2002)
E2A (*Tcfe2a*)	Induces cell-cycle arrest and apoptosis in T or B cell lines, in the 70Z/3 macrophage line induces B cell-lineage conversion.	Block at the CLP stage.	*EBF*, *IgHC* gene enhancer regions, *mb-1* (*Igα*), *B29* (*Igβ*), *λ5*, *VpreB*, *RAG1/2*, *TdT*	(Bain, Maandag et al. 1994; von Freeden-Jeffry, Vieira et al. 1995; Greenbaum and Zhuang 2002; Nutt and Kee 2007)
Early B cell factor, EBF1 (*Ebf1*)	Induces B cell differentiation in multipotent progenitors, rescues B lymphopoiesis in *PU.1*$^{-/-}$, *E2A*$^{-/-}$ or *IL-7*$^{-/-}$ progenitors.	Block at the CLP stage, no cells with *Igh* gene rearrangement.	*Igα*, *Igβ*, *λ5*, *VpreB*, *Bcl11a*, *Pax5*, *Blk*	(Lin and Grosschedl 1995; Akerblad, Rosberg et al. 1999; Akerblad and Sigvardsson 1999; Sigvardsson 2000; Nutt and Kee 2007; Fields, Ternyak et al. 2008)

Pax5 (*Pax5*)	Impairs T cell development and promotes T cell-lymphoma formation. Variably affects myeloid and erythroid differentiation.	Fetal liver lacks B lineage cells. Adult bone marrow block at EPLM stage but have *D-J$_H$* and only a few proximal *V-DJ$_H$* rearrangements.	Activated: *CD19*, *mb-1*, *Blnk* (*SLP65*), CD72, *EBF1*, CD23, *Lef-1*, *Irf4*, *Irf8*, *SpiB*, *Aiolos*, *RAG-2* Repressed: *Flt3*, *M-CSFR*, *J-chain*, *Notch1*	(Urbanek, Wang et al. 1994; Nutt, Heavey et al. 1999; Horcher, Souabni et al. 2001; Mikkola, Heavey et al. 2002; Cobaleda, Schebesta et al. 2007; Nutt and Kee 2007)
Aiolos (*Ikzf3*)	N.D.	Spontaneous germinal center formation with high IgG and IgE serum level. Decreased number of peritoneal B cells, MZ, and recirculating bone marrow B cells.	*λ5*	(Wang, Avitahl et al. 1998; Sabbattini, Lundgren et al. 2001; Kioussis 2007)
Sox4 (*Sox4*)	N.D.	Lethal at e13.5, proB cells fail to expand in IL-7 and few proB cells after fetal-liver transfer into irradiated adults.	N.D.	(Nutt and Kee 2007)
Lymphoid enhancer factor, Lef1 (*Lef1*)	N.D.	Decreased proB cells in fetal liver and neonatal bone marrow, proB cells respond to IL-7 but not Wnt3a.	*RAG-2*	(Nutt and Kee 2007)
Bcl11a (*Evi9*)	N.D.	Block after CLP.	*Notch* or *Bcl6* ?	(Durum 2003; Liu, Keller et al. 2003)
RAG1 (*Rag1*)	N.D.	Lack of B and T cells associated with inability to perform *V(D)J* recombination.	Immunoglobulin Heavy and Light Chain genes	(Mombaerts, Iacomini et al. 1992)
RAG2 (*Rag2*)	N.D.	Lack of B and T cells associated with inability to perform *V(D)J* recombination.	Immunoglobulin Heavy and Light Chain genes	(Shinkai, Rathbun et al. 1992)
TdT (*Dntt*)	Forced expression of *TdT* in fetal thymus results in a decrease in gammadelta T cells and random dissemination of Vγ3Vδ1 T cells in skin of newborn but not adult mice.	Impaired junctional diversity in *V(D)J* recombination due to lack of N-nucleotides.	Immunoglobulin Heavy Chain genes	(Gilfillan, Dierich et al. 1993; Komori, Okada et al. 1993; Aono, Enomoto et al. 2000)
Spi-B (*Spib*)	N.D.	T-dependent immunodeficiency due to impaired BCR mediated responses and defecting germinal	*c-rel*, *IgLC*, *Grap2*	(Eisenbeis, Singh et al. 1995; Su, Chen et al. 1997; Brass, Zhu et al. 1999; Hu, Rao et al. 2001; Escalante, Brass et al. 2002; Garrett-

		center formation.		Sinha, Hou et al. 2005)
IRF-4 (*Irf4*)	N.D.	Block after the small preBII stage.	*Ikaros*, *Aiolos*, *IgLC*	(Eisenbeis, Singh et al. 1995; Brass, Zhu et al. 1999; Escalante, Brass et al. 2002; Lu, Medina et al. 2003; Ma, Pathak et al. 2008)
IRF-8 (*Irf8*)	N.D.	Block after the small preBII stage.	*Ikaros*, *Aiolos*	(Lu, Medina et al. 2003; Ma, Pathak et al. 2008)
c-rel (*Rel*)	N.D.	Normal B cell development, however immunodeficiency due to reduced survival upon B cell activation.	*Bcl-x*, *E2F3a*	(Kontgen, Grumont et al. 1995; Tumang, Owyang et al. 1998; Chen, Edelstein et al. 2000; Cheng, Hsia et al. 2003)
Foxp1 (*Foxp1*)	N.D.	Block after preBI.	*RAG1/2*	(Hu, Wang et al. 2006)
Ets-1 (*Ets1*)	N.D.	Reduced number of T cells and splenic NK cells. Impaired proB to preB cell transition. Increased number of IgM-secreting plasma cells.	*EBF*, *IgHC*	(Nelsen, Tian et al. 1993; Bories, Willerford et al. 1995; Barton, Muthusamy et al. 1998; Eyquem, Chemin et al. 2004; Roessler, Gyory et al. 2007)
c-Myb (*Myb*)	N.D.	Embryonic lethality due to impaired adult-type erythropoiesis in the fetal liver.	*RAG-2*	(Mucenski, McLain et al. 1991; Jin, Kishi et al. 2002; Kishi, Jin et al. 2002)

3.1 The HSC to MPP transition

The Ikaros proteins belong to the family of zinc finger transcription factors. They can function as transcriptional activators or repressors (Georgopoulos 2002; Cobb and Smale 2005; Ng, Yoshida et al. 2007) and are expressed in all hematopoietic lineages. *PU.1* is a member of the Ets transcription factor family and is expressed in HSCs, MPPs and all differentiating cells except the erythroid, megakaryocytic, and T lineages (Scott, Fisher et al. 1997). The transition from HSCs to MPPs is dependent on the expression of the transcription factors *Ikaros* and *PU.1*, which regulate the expression of the cytokine tyrosine kinase receptor *Flt3* (Nichogiannopoulou, Trevisan et al. 1999; Adolfsson, Borge et al. 2001; DeKoter, Lee et al. 2002). The expression of *Flt3* is associated with a loss of self-renewal capacity but sustained lymphoid-restricted reconstitution potential (Adolfsson, Borge et al. 2001).

3.2 The MPP to ELP transition

The MPP stage is a developmental hinge between the lymphoid and the myeloid lineage. It is not clear what drives the lymphoid versus the myeloid development as both *Ikaros* and *PU.1* are expressed in the MPP. Retroviral transduction experiments suggested that low level of PU.1 expression favours the lymphoid development

(DeKoter and Singh 2000). GATA family members are transcription factors that contain a two-zinc finger domain. GATA-1 was reported to specify the MPP into the myeloid lineage (Arinobu, Mizuno et al. 2007). In fact GATA-1 is able to repress PU.1-dependent transcription by direct interaction (Nerlov, Querfurth et al. 2000).

3.3 The ELP to CLP transition

Lymphoid specification occurs already in some ELPs. The ELP can give rise to CLP or ETP cells which follow the B lymphocyte and T lymphocyte specifications respectively. Notch1 is a membrane bound transcription factor, which is activated upon proteolytic cleavage and translocates into the nucleus to induce gene expression. Notch signalling is necessary for ETP differentiation (Sambandam, Maillard et al. 2005; Tan, Visan et al. 2005).
The B cell lineage specification comprises expression of the *RAG* and the Terminal deoxynulceotidyl transferase (*TdT*) genes. These genes initiate the *IgHC* D_H-J_H recombination and induce N-nucleotides insertion into junctions between Immunoglobulin variable region genes respectively (Medina, Garrett et al. 2001; Igarashi, Gregory et al. 2002). The proper transition to CLP depends on the expression of the *IL-7R* (CD127), which is regulated by PU.1 (DeKoter, Lee et al. 2002; Medina, Pongubala et al. 2004) and by signalling through the Flt3 receptor (Borge, Adolfsson et al. 1999). Ikaros is not essential for ELP development, but may play a role in further specification into the lymphoid pathway as expression of lymphoid genes like *IL-7R* and *RAG1* are reduced in *Ikaros*$^{-/-}$ mice (Yoshida, Ng et al. 2006). This may be due to impaired expression of IL-7R and Flt3 suggesting redundant functions of Ikaros and PU.1 in early lymphoid specification. The transcription factor E2A is also implicated in CLP generation as no B cell progenitors develop in the knock out mice (Bain, Robanus Maandag et al. 1997; Borghesi, Aites et al. 2005). However the mechanism is not understood well as the known E2A target genes are not involved in CLP development. Furthermore one should note that a subset of ELP which expresses CD62L is also T lineage specified and can give rise to the early T-lineage progenitor (ETP) (Perry, Welner et al. 2006).

3.4 The CLP to preBI transition

CLPs are B lineage-specified and still have the potential to give rise to T, NK and dendritic cells. A short overview of the non-B cell pathways will be followed by a detail description of the CLP to preBI cell transition.

<u>T, NK and dendritic cells development:</u>
Notch1 promotes T cell differentiation from bone marrow progenitors and inhibits B cell development (Pui, Allman et al. 1999) by inducing the expression of the transcriptional repressor Hes-1 (Sasai, Kageyama et al. 1992; Jarriault, Brou et al. 1995). *Notch1* can be inhibited by the modifiers Lunatic Fringe (Koch, Lacombe et al. 2001) and Deltex1 (Izon, Aster et al. 2002).
The development of the NK cells depends on transcription factors like Ets (Barton, Muthusamy et al. 1998) and Id2 (Yokota, Mansouri et al. 1999), which inhibits B cell development.

Ikaros and PU.1 are critical transcription factors for the development of dendritic cells (DC) (Wu, Nichogiannopoulou et al. 1997; Anderson, Perkin et al. 2000; Guerriero, Langmuir et al. 2000; Allman, Dalod et al. 2006). Id2 is a negative regulator of *E2A*, which is involved in DC differentiation (Spits, Couwenberg et al. 2000; Schotte, Nagasawa et al. 2004). The Flt3 signalling mediated by STAT3 is also necessary for the development of DCs (Laouar, Welte et al. 2003). Furthermore the transcriptional repressor *Gfi1* has been shown to control DC development through regulating STAT3 activation (Rathinam, Geffers et al. 2005).

PreBI cell differentiation:

The B cell lineage transcriptional programme is triggered by the sequential expression of the transcription factors E2A, EBF1 and Pax5. The key role of these transcription factors for B lineage commitment was highlighted by knock out mice studies; indeed $E2A^{-/-}$ and $EBF^{-/-}$ mice have a strong differentiation block at the CLP stage before D_H-J_H rearrangement (Zhuang, Soriano et al. 1994; Lin and Grosschedl 1995). $Pax5^{-/-}$ mice have a B cell lineage block after the EPLM cell stage at the *V* to *DJ* recombination (Nutt, Urbanek et al. 1997; Nutt, Heavey et al. 1999; Schaniel, Bruno et al. 2002).

The *E2A* gene produces two proteins of the basic-helix-loop-helix (bHLH) family, E47 and E12, through alternative splicing (Engel and Murre 2001). They bind to control regions of many B cell specific genes such as *IgHC* gene enhancers, *mb-1*, *λ5*, *RAG1* and *RAG2*, and *VpreB*, either as homodimers or as heterodimers with other bHLH proteins. E12 and E47 are both not expressed in a B cell-restricted manner, however E47 homodimers are exclusively found in B cells. E2A proteins are subject to different kinds of post-translational modifications such as intermolecular disulfide bond formation and phosphorylation which may have the potential to alter E2A stability or activity (Benezra 1994; Sloan, Shen et al. 1996; Markus and Benezra 1999). Id proteins are negative regulators of bHLH transcription factors such as E2A through direct interaction (Benezra, Davis et al. 1990; Ellis, Spann et al. 1990; Christy, Sanders et al. 1991; Sun, Copeland et al. 1991; Riechmann, van Cruchten et al. 1994). *In vivo* E2A was shown to be inhibited by dimerizing with Id proteins (Deed, Jasiok et al. 1998) and Hes-1 (Sasai, Kageyama et al. 1992; Jarriault, Brou et al. 1995). PreBI cell differentiation is promoted when Id proteins are downregulated and E2A upregulated (Murre, McCaw et al. 1989; Sun and Baltimore 1991; Bain, Maandag et al. 1994). Once expressed at sufficient levels, E2A is thought to take over the repressive effect of Id and Hes-1 and activates the transcription of *EBF*. Therefore regulation of E2A protein level and activity is critical to drive B cell ontogeny.

Early B lymphocyte factor (EBF) has a novel zinc-binding motif and a bHLH-like domain for homodimerization (Hagman, Gutch et al. 1995). E2A and EBF control the initial D_H-J_H rearrangement step by activating the expression of *Rag* genes and promoting the accessibility of the D_H-J_H region within the *IgH* locus (Romanow, Langerak et al. 2000; Goebel, Janney et al. 2001). *EBF* expression is regulated by E2A, PU.1 (Hagman and Lukin 2006) and signalling through the IL-7R (Medina, Pongubala et al. 2004; Seet, Brumbaugh et al. 2004). IL-7R signalling is thought to occur via Stat5, as constitutively active Stat5 restores CLP block and *EBF* expression in $IL\text{-}7R^{-/-}$ mice (Goetz, Harmon et al. 2004). Furthermore $Stat5^{-/-}$ mice have a developmental block after the CLP stage associated with reduced EBF expression (Yao, Cui et al. 2006). B220 is a B cell lineage marker and starts to be expressed in the EPLM cell stage. In fact B220 was reported to be a likely PU.1 target gene in B

lineage progenitors (Medina, Pongubala et al. 2004). It was also reported that distinct promoters mediate the regulation of *Ebf1* gene expression by IL-7 and Pax5 (Roessler, Gyory et al. 2007). The proximal promoter is activated by Ets1, PU.1 and Pax5. The distal promoter is activated by IL-7 signalling, E2A and EBF1. Pax5 potentiates the activation of the distal promoter by inhibiting *Notch1*, which is a negative regulator of *EBF1*. Thus EBF1 regulates its own expression when *Pax5* is upregulated. EBF1 in turn then upregulates *λ5*, *VpreB* and *mb1*. It was shown that EBF promotes demethylation of critical B lineage genes like *Bcl11a* and *Pax5*, thus potentiating their expression (Liu, Keller et al. 2003). OBF-1 is a transcription factor that coactivates Oct1 and Oct2 on octamer sites in B cell specific gene promoters such as the *3'-IgH* enhancer (Tang and Sharp 1999; Stevens, Ong et al. 2000). Little is known about the function of OBF-1 in preB cells. OBF-1 was reported to interact and regulate the stability of the tyrosine kinase Syk to control the proliferation of preB cells (Siegel, Kim et al. 2006). EBF1 has been very recently identified as a potentially direct regulator of *OBF-1* expression (Zandi, Mansson et al. 2008).
The *paired box* homeodomain transcription factor Pax5 represses non-B cell lineage genes involved in cell signalling, cell adhesion, cell migration, transcription, and cellular metabolism (Chiang and Monroe 1999; Nutt, Heavey et al. 1999; Chiang and Monroe 2001; Busslinger 2004; Delogu, Schebesta et al. 2006). It also blocks *Notch1* gene expression, which is critical for T cell differentiation. A recent microarray screen identified more than 100 target genes for Pax5 repression (Delogu, Schebesta et al. 2006). On the other hand Pax5 activates the expression of B cell lineage genes like *mb-1* (*Igα*), *N-myc*, *LEF-1* and *CD19* (Nutt, Morrison et al. 1998). Pax5 needs specific partners to repress or activate its target genes. To repress genes Pax5 cooperates with PU.1 and recruits corepressors of the Groucho protein family (Busslinger 2004). Pax5 and Ets proteins bind and activate cooperatively the *mb-1* promoter (Maier, Ostraat et al. 2004). Furthermore Pax5 induces large-scale contraction of the *IgHC* locus which is crucial for the second stage of V_H-DJ_H recombination (Fuxa, Skok et al. 2004). *Pax5* is turned on following *EBF* expression and sets the transcriptional programme to drive the cells irreversibly to the B cell lineage. Therefore *Pax5* is the most important player for B cell commitment.
The transcription factor *LEF1* is a Pax5 target and the downstream effector of WNT signalling, which is involved in ProB cell proliferation (section 4.7). However the LEF1 target genes in the ProB cells are not identified yet. *LEF1*$^{-/-}$ mice have impaired preBI cell differentiation (van Genderen, Okamura et al. 1994).
The transcription factor *Sox4* is another factor crucial for early B cell development, as reconstituted mice with *Sox4*$^{-/-}$ fetal liver cells have a severe block at the ProB cell stage (Schilham, Oosterwegel et al. 1996). However no target genes have been identified yet for Sox4.

3.5 The preBI to preBII transition

V_H-D_HJ_H recombination:
During the preBI and preBII cell stages the recombinase enzymes *RAG-1* and *RAG-2* are expressed to catalyze heavy chain gene rearrangement between the *V* and *D* regions. Ikaros and C/EBP were reported to cooperatively bind and activate the *Rag* enhancer (Wei, Dohkan et al. 2005). The forkhead transcription factor Foxp1 was shown to be essential for the *V(D)J* recombination activity (Hu, Wang et al. 2006). In fact *Rag2*$^{-/-}$ mice reconstituted with *Foxp1*$^{-/-}$ fetal liver cells have a differentiation

defect after the preBI cell stage combined with impaired V_H-DJ_H recombination. Transcription profile analysis suggests that Foxp1 together with E2A bind the *Erag* enhancer which activates the *RAG* locus and promotes *V(D)J* recombination (Hsu, Lauring et al. 2003).
Recombination of the V_H genes in preBI cells depends on several factors. IL-7R signalling promotes the chromatin accessibility of *DJ*-distal V_H genes, such as those of the V_HJ558 family, via the Stat5 transcription factor (Chowdhury and Sen 2001; Chowdhury and Sen 2003; Bertolino, Reddy et al. 2005). Pax5 promotes locus contraction and removal of histone H3-K9 methylation at the V_H loci (Johnson, Pflugh et al. 2004). Histone H3-K9 methylation is associated with inactive chromatin, and prevents *V(D)J* recombination in non-B lineage cells. Yin Yang 1 (YY1) is a ubiquitous transcription factor and might cooperate with Pax5 in *IgHC* locus contraction (Calame and Atchison 2007). Furthermore the Polycomb group protein Ezh2 was reported to promote histone H3-K27 methylation at the V_HJ558 segments which may facilitate DNA recombination (Su, Basavaraj et al. 2003).
The enzyme terminal deoxyribonucleotidyl transferase (*TdT*) is expressed during the preBI cell stage to insert N-nucleotides at the V_H-D_H-J_H coding joints and is dowregulated in a preBCR dependent manner (Wasserman, Li et al. 1997) early in the preBII cell stage. Therefore N-nucleotides are not found in the rearranged light chain genes, since the *TdT* is turned off after the preBII cell stage when the rearrangement occurs. In fact ectopic expression of E2A identified *TdT* as a potential target gene (Greenbaum and Zhuang 2002).

PreBCR signalling:
The preBCR consists of a pair of Immunoglobulin Heavy chains both associated with a Surrogate Light chain made of λ5 and VpreB. The preBCR is a marker for preBII cells, and is expressed when a productive V_H to DJ_H recombination occurs. The preBCR is necessary for the survival and further differentiation of preB cells. Data suggest that *λ5* and *VpreB* are potential target genes of E2A and EBF (Sigvardsson, O'Riordan et al. 1997; Sigvardsson 2000). The preBCR can signal into the cell through the association with Igα and Igβ, which have immunoreceptor tyrosine-based activation motifs (ITAMs). Many components of the preBCR and BCR are positively regulated Pax5 target genes such as *CD19*, *Blnk*, *CD79a* (*Igα*), and *λ5* (Cobaleda, Schebesta et al. 2007).
The ITAM of the preBCR is believed to be phosphorylated by Src-family protein tyrosine kinases like Fyn, Lyn, Blk and Lck (Dal Porto, Burke et al. 2004; Gauld and Cambier 2004). Phosphorylated ITAMs become docking sites for Syk-family protein tyrosine kinases like Syk and ZAP-70 (Futterer, Wong et al. 1998; Rolli, Gallwitz et al. 2002). The activated Syk then phosphorylates SLP-65, which activates downstream pathways (Ishiai, Kurosaki et al. 1999). Other adaptor proteins like Linker of Activated T cells (LAT) (Su and Jumaa 2003) and Bruton's tyrosine kinase (Btk) (Middendorp, Zijlstra et al. 2005) are able to mediate some of the signalling functions of the preBCR. Interestingly preBI cultures of *OBF-1*$^{-/-}$ mice are hyperproliferative and it was suggested that OBF-1 might regulate preBCR signalling by modulating Syk stability (Siegel, Kim et al. 2006).
Results suggest that the Ras-MAP kinase pathway plays a role downstream of Src, Syk, SLP-65 and Btk (Iritani, Forbush et al. 1997; Shaw, Swat et al. 1999; Shaw, Swat et al. 1999; Nagaoka, Takahashi et al. 2000). Erk is a component of the Ras-MAP kinase pathway and was reported to link the signals between preBCR and IL-7R. PreB cells have a better response to IL-7 when the preBCR is expressed

(Marshall, Fleming et al. 1998); furthermore Erk is upregulated upon preBCR expression and IL-7R signalling (Fleming and Paige 2001). It is thought that Erk has a threshold effect in the integration of the signals from the preBCR and IL-7R and modulates preB cell proliferation (Ohnishi and Melchers 2003; Fuentes-Panana, Bannish et al. 2005; Melchers 2005; Monroe 2006). The transcription factor early growth response 1 (Egr-1) contains three tandem zinc finger motifs that target genes by binding to GC-rich DNA elements in their promoters (Gashler and Sukhatme 1995). Egr-1 is usually associated with cell proliferation and differentiation in B lymphocytes (Sukhatme, Cao et al. 1988; Muthukkumar, Han et al. 1997; Ke, Gururajan et al. 2006). Egr-1 was reported to facilitate the developmental progression of preBI cells. Analysis of changes in the expression pattern of potential Egr-1 target genes revealed that Egr-1 enhances the expression of the aminopeptidase *BP-1/6C3* in preB cells (Dinkel, Warnatz et al. 1998). *Egr-1* was identified as an immediate target gene downstream of the Ras/Erk pathway in B lymphocytes (McMahon and Monroe 1995).

3.6 The preBII to immature B cell transition

Large preBII cells are highly proliferating cells which express the preBCR. Signalling through the preBCR is critical, since this shuts down recombination at the *IgHC* locus (allelic exclusion), facilitates positive selection past the preBI / preBII checkpoint and integrates IL-7R signals to facilitate clonal expansion. Furthermore it initiates the recombination of the *IgLC* locus (Reth, Petrac et al. 1987; Kitamura and Rajewsky 1992; Grawunder, Leu et al. 1995). On the other hand the small preBII cells are resting cells and have downregulated the preBCR so that the light chain gene recombination can occur in order to generate a functional B cell receptor (BCR). This section will describe these mechanisms.

Termination of Immunoglobulin Heavy Chain gene rearrangement:
The inhibition of the recombination machinery is critical for allelic exclusion of the heavy chain genes, the mechanism whereby B cells express only one immunoglobulin. To this end, the IL-7R, the RAG proteins and TdT are downregulated in a preBCR mediated manner (Wasserman, Li et al. 1997; Chowdhury and Sen 2003; Chowdhury and Sen 2004).

PreBCR downregulation:
Recent data shows that Irf4 and 8 orchestrate the transition from large to small preB cells by inducing the expression of *Ikaros* and *Aiolos* to downregulate the preBCR and by promoting cell-cycle arrest (Ma, Pathak et al. 2008). *IRF-4/8* knock out mice are unable to downregulate the preBCR and have an impaired *IgLC* transcription and recombination (Lu, Medina et al. 2003). It was also reported that Spi-B and PU.1 regulate the expression of *c-rel* which promotes the survival of early B cells (Hu, Rao et al. 2001). Furthermore *SpiB* and *IRF-4* knock out mice have a block after the preBII cell stage (Hu, Rao et al. 2001; Lu, Medina et al. 2003) supporting the notion that they play an important role in preBCR-dependent B cell development. Data shows that Pax5 represses about as many genes as it activates (Nutt and Kee 2007). The transcription factors *SpiB*, *Aiolos*, *Irf4* and *Irf8* belong to the activated target genes, suggesting that Pax5 reinforces B cell commitment and promotes subsequent B cell differentiation.

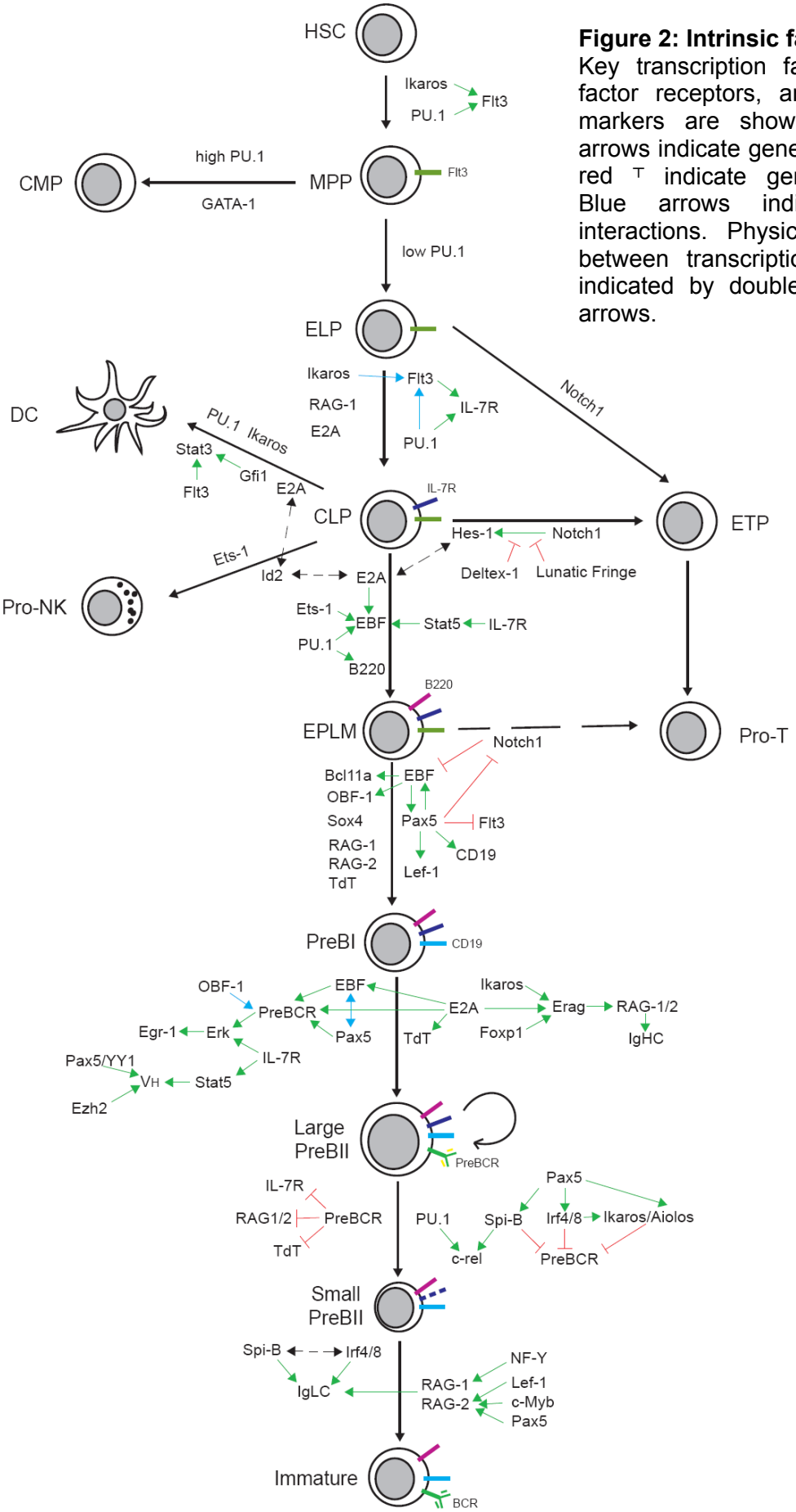

Figure 2: Intrinsic factors
Key transcription factors, growth-factor receptors, and cell-surface markers are shown. The green arrows indicate gene activation, the red ⊤ indicate gene repression. Blue arrows indicate positive interactions. Physical interactions between transcription factors are indicated by double-pointed black arrows.

Light Chain gene recombination:
The Ras/Erk/Egr-1 pathway is triggered by the preBCR to regulate PreB cell differentiation; however Immunoglobulin Light Chain (*IgLC*) gene recombination is probably regulated by additional signalling pathways. Muljo and Schliessel reported that inhibiting the Abelson protein tyrosine kinase (Abl) induces RAG1/2 and initiates *IgLC* recombination (Muljo and Schlissel 2003). *Spi-B* and *IRF-4* were identified as Abl-suppressed target genes by microarray analysis. Interestingly Spi-B and IRF-4 were shown to bind the *IgLC* enhancer (Eisenbeis, Singh et al. 1995; Brass, Zhu et al. 1999; Escalante, Brass et al. 2002). Therefore the downregulation of Abl activity induces the expression of SpiB and IRF-4 which leads to RAG1,2 induction and initiation of *IgLC* recombination and expression (Monroe and Dorshkind 2007). Furthermore the *RAG1* promoter was reported to be potentially activated by NF-Y (Brown, Miranda et al. 1997). The *RAG2* promoter is activated by LEF-1, c-Myb and Pax5 in immature B cells (Jin, Kishi et al. 2002; Kishi, Jin et al. 2002).

4. Extrinsic factors

This section describes the roles of the extrinsic factors (cytokines and chemokines with their respective receptors) in the different bone marrow populations up to the immature B cell stage. The expression profile of these extrinsic factors is depicted in Fig. (**1**).

4.1 Regulators of HSCs

Hematopoietic stem cells (HSCs) have to maintain their self-renewal potency, preserving the stem cell pool, in addition they have also to constantly produce progeny that differentiate into different hematopoietic lineage cells (Ogawa 1993). Different cytokines are able to regulate the proliferation and differentiation of the HSCs. We first discuss about the bone morphogenetic proteins (BMP) and then about other cytokines regulating HSCs.
Bone morphogenetic proteins (BMPs) are members of secreted signalling proteins that belong to the transforming growth factor-β superfamily. BMPs have an important role in specification of HSCs during development (Larsson and Karlsson 2005). For example, BMP4 was shown to positively regulate the proliferation and survival of adult HSCs (Bhatia, Bonnet et al. 1999) and BMP4 signalling is also important in mediating Sonic Hedgehog-induced proliferation of primitive human progenitors (Bhardwaj, Murdoch et al. 2001).
BMPs influence also osteoblasts in the stem cell niche as the deletion of the BMP type I receptor results in increased numbers of HSC-supporting osteoblastic cells lining the bone surface, which leads to an increased number of HSCs (Zhang, Niu et al. 2003). Therefore BMPs illustrate the notion that the microenvironment is crucial for HSCs development.
A cross-talk between BMP receptor 2 (Bmpr2) and c-kit signalling involving Smad proteins, Erk and p38 kinases was reported (Hassel, Yakymovych et al. 2006). It was shown that Bmpr2 and c-kit form a complex and can cooperate upon BMP2 and SCF stimulation in osteoblastic differentiation.
Low levels of Id proteins are necessary to allow E2A driving B cell commitment (Deed, Jasiok et al. 1998; Ji, Li et al. 2008). Indeed Id proteins were reported to be

negative regulators of B cell development (Sun 1994; Kee, Rivera et al. 2001). Although BMP6 is mainly involved in bone formation (Solloway, Dudley et al. 1998; Kugimiya, Kawaguchi et al. 2005), BMP6 was reported to inhibit the proliferation of preBI cells by upregulating Id1 and Id3 (Kersten, Dosen et al. 2006).
Thrombopoietin (Tpo) and its receptor c-mpl are expressed on HSCs and were shown to promote the survival of primitive progenitors *in vitro* (Borge, Ramsfjell et al. 1996). *In vivo* Tpo was reported to greatly promote the self-renewal and expansion of HSCs following bone marrow transplantation (Fox, Priestley et al. 2002).
The Notch pathway plays an important role by modulating self-renewal and lineage fate determination mainly during lymphopoiesis (Ohishi, Katayama et al. 2003). Retroviral expression experiments with a constitutively active Notch suggested that Notch signalling could modulate self-renewal to maintain stem cell numbers and could drive lymphoid differentiation of the progeny (Varnum-Finney, Xu et al. 2000; Stier, Cheng et al. 2002). Furthermore ectopic expression of Notch ligands such as Delta-1, Jagged-1, or Jagged-2 was shown to increase HSCs proliferation (Jones, May et al. 1998; Varnum-Finney, Purton et al. 1998; Han, Ye et al. 2000; Tsai, Fero et al. 2000).
Little is known about negative regulators of HSCs, however TGF-β, TNF-α and IFN-γ were shown to be associated with negative regulation of hematopoietic progenitors (Jacobsen, Ruscetti et al. 1992; Maciejewski, Selleri et al. 1995; Zhang, Harada et al. 1995; Yang, Dybedal et al. 2005).

4.2 FLT3L and EPLM development

FLT3 is already expressed at the surface of MPP cells and is downregulated at the transition between EPLM and preBI cells. Its ligand FLT3L is expressed during the CLP and EPLM cell stage. The role of FLT3 is localized mainly at the beginning of B cell commitment, since the numbers of EPLM and preBI cells are reduced about twofold in *FLT3*$^{-/-}$ mice. However the cells can recover from the lack of FLT3 as the numbers of preBII, immature and mature B cells are relatively normal in these mice (Mackarehtschian, Hardin et al. 1995).
In *FLT3L*$^{-/-}$ mice the CLP precursors are not impaired (Ikuta and Weissman 1992; Adolfsson, Mansson et al. 2005), however the downstream populations like CLPs and ETPs are strongly reduced suggesting that FLT3L is crucial for the generation of CLPs (Sitnicka, Bryder et al. 2002; Sambandam, Maillard et al. 2005). It is also possible that FLT3L acts specifically on ELP and ETPs. Furthermore *FLT3L*$^{-/-}$ mice have a dramatic reduction of EPLM cells, slightly less preBI and preBII cells, and normal numbers of immature B cells. These observations indicate that FLT3L is an essential extrinsic factor in EPLM development (Sitnicka, Bryder et al. 2002; Sitnicka, Brakebusch et al. 2003). *In vitro* FLT3L was shown to stimulate the growth of EPLM cells synergistically with IL-7 (Hunte, Hudak et al. 1996).
It is not clear why the deficiency of FLT3L leads to more dramatic consequences than its receptor FLT3. It is possible that FLT3L has another unknown receptor. It was also reported that the numbers of dendritic cells (McKenna, Stocking et al. 2000; Brawand, Fitzpatrick et al. 2002) and NK cells (McKenna, Stocking et al. 2000), which arise from CLP (Rolink, ten Boekel et al. 1996; Diao, Winter et al. 2004; Pelayo, Hirose et al. 2005; Pelayo, Welner et al. 2005), are also reduced in *FLT3L*$^{-/-}$ mice suggesting also a role for the development of these lineages.

4.3 CXCL12 and pro B cell development

The chemokine CXCL12 (also known as SDF1 and PBSF) and its receptor CXCR4 play a role at the early stage of B cell development since the lack of one of them results in a severe block of B cell lymphopoiesis (Ma, Jones et al. 1998; Zou, Kottmann et al. 1998). Indeed *CXCL12*$^{-/-}$ embryos and chimera mice reconstituted with *CXCR4*$^{-/-}$ fetal cells have a strong decrease of EPLM cells numbers.
CXCR4 expression is highest on preB cells, decreased in immature B cells, and increases again upon transition to mature B cells. Beside its role in early B cell development CXCR4 was also reported to be involved in the retention of hematopoietic cells in lymphoid organs (Ma, Jones et al. 1999). In chimera mice reconstituted with *CXCR4*$^{-/-}$ fetal cells the number of preB cells is increased in the peripheral blood suggesting that CXCR4 is necessary for retaining these cells in the bone marrow (Ma, Jones et al. 1999; Nie, Waite et al. 2004). Furthermore *CXCR4* deficient plasma cells are unable to home to the bone marrow (Hargreaves, Hyman et al. 2001; Tokoyoda, Egawa et al. 2004). These observations suggest that CXCR4 transmits a retention signal to preB cells and mature B cells, but not to immature B cells (Honczarenko, Douglas et al. 1999).
In line with the CXCR4 observations, CXCL12 was reported to play also a role in the bone marrow colonization of hematopoietic stem cells (Nagasawa, Hirota et al. 1996; Ma, Jones et al. 1998; Tachibana, Hirota et al. 1998; Ara, Tokoyoda et al. 2003). These observations indicate that CXCL12 and its receptor are essential for early B cell development (Egawa, Kawabata et al. 2001) and homing of end-stage B cells, potentially by attracting and retaining CXCR4 expressing cells in specific niches.

4.4 IL-7 and preB cell development

The IL-7 receptor (IL-7 R) is a heterodimer constituted of a developmentally regulated α chain and a ubiquitously expressed common γ chain (γc). The α chain expression begins at the CLP stage when the cells have seeded on the stromal cells and starts to be downregulated at the preB cell stage.
IL-7 is a member of the cytokine family that activates common γ chain receptors and was shown to promote the survival of developing progenitors *in vitro* (Boise, Minn et al. 1995; Lu, Lejtenyi et al. 1999). IL-7 is produced by reticular stromal cells (Namen, Lupton et al. 1988) and its release may require contact with B cell precursors (Stephan, Reilly et al. 1998). preBI cells require the microenvironment provided by the stromal cells to proliferate and differentiate into preBII cells. The role of the stromal cells is to interact directly with preB cells and support their development by secreting various cytokines such as IL-7. The interaction of the progenitor cells with the stromal cells is mediated by several cell-adhesion molecules like VLA-4 on the progenitors and its ligand VCAM-1 on the stromal cells. When the progenitor cells are settled on the stroma, the tyrosine kinase receptor c-Kit interacts with stem cell factor (SCF) on the stromal cells. This interaction activates c-Kit driving proliferation and differentiation into the B cell lineage. Large preBII cells then downregulate the adhesion molecules so that they can detach from the stromal cells. At this stage the cells do not need a direct contact with the stroma anymore but still require IL-7 to proliferate and differentiate.
The following paragraphs describe the role of IL-7 in immunoglobulin gene rearrangement, in instructing B cell lineage and in selecting preB cells that have successfully generated a preBCR.

IL-7 plays an important role in *IgHC* gene rearrangements by regulating the expression of RAG proteins (Muegge, Vila et al. 1993) and promoting the recombination of variable (*V*)-region genes with a rearranged D_H-J_H locus (Corcoran, Riddell et al. 1998). *IL-7*$^{-/-}$ mice have a developmental block at the preBI-preBII transition (von Freeden-Jeffry, Vieira et al. 1995) although *VDJ* recombination is not impaired, indicating that other factors may partially rescue IL-7 function in this aspect (Carsetti 2000). In absence of IL-7 signals the CLPs are not able to induce properly the expression of the transcription factors necessary for the initiation of the B lineage differentiation programme (E2A, EBF, Pax5), which results in impaired B cell differentiation (Akashi, Kondo et al. 1998; Miller, Izon et al. 2002). However they are still able to differentiate into T- and NK-cell progenitors. This observation indicates that IL-7 not only modulates the proliferation and survival of B cells, but also can instruct cell lineage decisions.
The transcription factor EBF1 alone is able to rescue the B cell potential of the *IL-7*$^{-/-}$ mice indicating that IL-7 regulates EBF1 expression already at the uncommitted lymphoid progenitor stage (Dias, Silva et al. 2005). Knock out mice lacking the *IL-7R*α *chain* (Peschon, Morrissey et al. 1994) or the γ *chain* (DiSanto, Muller et al. 1995) have a strong and a milder block at the ProB cell stage, respectively. Furthermore *IL-7R*$\alpha^{-/-}$ mice have a V_H to D_H-J_H recombination defect (Corcoran, Riddell et al. 1998) illustrating the fact that the IL-7R chains are also components of other cytokine receptor complexes and that deleting a cytokine or its receptor does not have always the same physiological impact.
Areas of high IL-7 concentration support the expansion of EPLM and preBI cells prior to and during *IgHC* recombination. An adjacent area of limited IL-7 availability selects for those cells which express a preBCR (large preBII) and restricts the preBI population to the permissive, high IL-7 compartment. In fact preBCR expression results in a reduced threshold of IL-7 responsiveness and provides a mechanism by which preBCR expressing cells can be selected (Fleming and Paige 2002). This mechanism is due to the fact that phosphorylated Erk1,2 integrates the preBCR and IL-7R signalling (see 3.5). In compartments that contain high concentrations of IL-7, sufficient ERK1,2 is phosphorylated to reach the threshold for proliferation whether or not a preBCR signal is present. In areas of low IL-7 availability, only cells that express the preBCR reach the ERK1,2 phosphorylation threshold needed to proliferate (Fleming and Paige 2002). Furthermore, IL-7 induced proliferation *in vivo* is not as strong for all the cells within a population, as preBCR containing distal *IgHC* V_H regions expand better (ten Boekel, Melchers et al. 1997). This observation might be due to better pairing of distal *IgHC* V_H regions with the Surrogate Light Chain (Martensson, Rolink et al. 2002). A more stable preBCR might generate a stronger signal for continued developmental progression and survival. This prediction is supported by the observation that mutated heavy chains which pair poorly to the Surrogate Light Chain are poorly represented in peripheral B lymphocytes (Wang, Ye et al. 2001; Wang and Clarke 2007).

4.5 SCF and the development of preBI and preBII cells

The membrane-bound and secreted form of stem cell factor (SCF) is the ligand for the c-Kit receptor. The SCF-cKit axis appears to be redundant in fetal, neonatal and young mice, as B cell development is not impaired in absence of *SCF* or *c-Kit* (Takeda, Shimizu et al. 1997). However SCF is crucial for B cell ontogeny in adult mice as the naturally occurring mutant mice that lack SCF (*Sl/Sl* mice) have reduced

number of CLP and have a block after the EPLM stage (Takeda, Shimizu et al. 1997; Waskow, Paul et al. 2002). Investigation of mutant mice lacking the transmembrane domain of SCF, demonstrated that a membrane-bound isoform of SCF might have a role in B cell development (McCulloch, Siminovitch et al. 1965). Therefore it is postulated that cells expressing membrane-bound SCF might function as a niche for preBI and preBII cells (Driessen, Johnston et al. 2003).

4.6 RANK Ligand and B cell development

RANKL is a transmembrane protein highly expressed on bone-marrow-derived primary stromal cells and osteoblasts (Yasuda, Shima et al. 1998), however little is known about lymphoid expression. RANK is the receptor for RANKL and is expressed by dendritic cells, T cells and osteoclast precursors (Anderson, Maraskovsky et al. 1997; Dougall, Glaccum et al. 1999). In fact *Rag1*$^{-/-}$ mice reconstituted with *Rankl*$^{-/-}$ fetal liver cells have reduced numbers of preBII and immature B cells. However *Rankl*$^{-/-}$ mice reconstituted with wild-type bone marrow cells have a normal B cell development (Dougall, Glaccum et al. 1999; Kong, Yoshida et al. 1999). This reconstitution studies showed that RANKL expression by lymphoid cells is important for B cell ontogeny.

4.7 Wnt signalling

There are 19 *WNT* genes in the human and mouse genome encoding lipid-modified secreted glycoproteins (van Noort and Clevers 2002). WNT signalling results from the binding of a WNT protein to the cysteine-rich domain of a receptor of the frizzled (FZ) family and a co-receptor of the low-density-lipoprotein-receptor-related-protein family (LRP5 or LRP6) (Tamai, Semenov et al. 2000; Mao, Wu et al. 2001; Mao, Wang et al. 2001). There are ten different *FZ*-family members in the human and mouse genomes (Hsieh 2004). WNT signals by inhibiting a multiprotein destruction complex via the protein dishevelled (DVL), which protects β-catenin from proteasome mediated degradation. β-catenin then translocates into the nucleus and activates *Lef-1* by replacing the bound Groucho co-repressors (Staal and Clevers 2005). Relatively little is known about the role of WNT signalling in B cell ontogeny. However WNT signalling was shown to be implicated in the self-renewal capacity of hematopoietic stem cells (Reya, Duncan et al. 2003; Willert, Brown et al. 2003) and to stimulate the proliferation of ProB cells (Reya, O'Riordan et al. 2000).

4.8 C-Fos and the bone microstructure

The c-Fos protein is a major component of the activator protein 1 (AP-1) transcription factor complex (Distel and Spiegelman 1990; Angel and Karin 1991). *c-Fos*$^{-/-}$ mice exhibit an altered B cell differentiation and develop severe osteopetrosis, characterized by foreshortening of the long bones, ossification of the marrow space, and absence of tooth eruption. However reconstitution experiments demonstrated that the hematopoietic stem cells lacking *c-Fos* have full developmental potential. Therefore the B cell development defect was probably due to the impaired bone marrow environment as a consequence of osteopetrosis (Okada, Wang et al. 1994). This observation shows that the bone integrity is also an important factor for B cell ontogeny.

5. Redox environment might influence B cell development

Eukaryotes undergo a constant oxidative stress due to the oxygen they breathe and because of their metabolism which generates oxidants. The oxidative stress appears to be involved in increasing numbers of diseases such as cancer, diabetes mellitus or atherosclerosis (Droge 2002). The cells have developed mechanisms to counteract this oxidative stress, and increasing numbers of observations support the notion that the cellular reduction oxidation (redox) status also plays a role in B cell development. Indeed it appeared that redox-regulation of receptors and transcription factors are important for lymphocyte activation (Roth and Droge 1987), differentiation and apoptosis (Buttke and Sandstrom 1995). Therefore the redox environment can be considered as an intrinsic and extrinsic factor regulating B cell development. However this field of research is only beginning and we do not have yet a clear understanding of the mechanisms regulating the redox level in B cell ontogeny.
The deleterious effect of an imbalanced redox level on B cell development can be illustrated in aging. Aged mice have decreased number of bone marrow early B cells and compromised *V(D)J* recombinase activity. These defects appear to be due to changes in bone marrow microenvironment (Labrie, Borghesi et al. 2005). For example production and/or release of IL-7 have been shown to decrease in the bone marrow of aged mice (Stephan, Reilly et al. 1998). Aging is generally accompanied by accumulation of oxidants (Droge 2002). Furthermore the DNA binding activity of Pax5, which depends on its oxidative state, is decreased in B lymphocytes of aged mice (Anspach, Poulsen et al. 2001). Therefore the modification in the bone marrow microenvironment might be partly due to an imbalanced redox status in old mice. This section gives an overview of what is known about the redox system in early B cells.

Reducing agents:
Thioredoxin (Trx) is an oxidoreductase that is induced in lymphocytes by hydrogen peroxide, ultraviolet (UV) irradiation, and other oxidative stress (Sachi, Hirota et al. 1995; Matsui, Oshima et al. 1996; Taniguchi, Taniguchi-Ueda et al. 1996). Trx is a positive regulator of Bcl-2 family proteins and is thought to prevent apoptosis and promote differentiation in B cells upon oxidative stress. In fact Trx and Bcl-2 are highly expressed in early B cell stages (Pro-B and Pre-B) and downregulated in small resting B cells, where negative selection occurs (Nilsson, Soderberg et al. 2004).
Protein disulfide isomerase (PDI) is expressed in bone marrow B cell populations, but the expression peak occurs in plasma cells which encounter stress due to the high immunoglobulin production. In fact PDI has an important role in the immunoglobulin assembly-machinery by promoting disulfide-bond isomerization (Nilsson, Soderberg et al. 2004).
Therefore Trx and PDI are key redox-regulating proteins and oxidative stress sensors in B cell ontogeny (Nilsson, Soderberg et al. 2004).
The apurinic/apyrimidinic endonuclease 1 APE1 (also known as Ref-1, HAP1 and Apex1) is an enzyme that nicks the DNA backbone at abasic sites to create DNA single-stranded (SS) breaks (SSBs) (Christmann, Tomicic et al. 2003) following DNA lesions generated by spontaneous hydrolysis or by exposure to reactive oxygen radicals (Doetsch and Cunningham 1990). Ref-1 is essential for early embryonic development (Fung and Demple 2005) and is involved in the redox regulation of DNA binding of transcription factors such as p53 (Jayaraman, Murthy et al. 1997; Gaiddon, Moorthy et al. 1999), AP-1 (Ordway, Eberhart et al. 2003), Egr-1 (Huang and

Adamson 1993), Pax5 (Tell, Zecca et al. 2000) and NF-κB (Xanthoudakis, Miao et al. 1992).

Oxidizing agents:
In lymphocytes most of the reactive oxygen species (ROS) are generated by T cells to kill pathogens (Droge 2002). However H_2O_2, which is a strong oxidant, can act as a second messenger in BCR signalling (Reth 2002). Upon antigen binding the BCR localizes close to a ROS-producing NADPH oxidase (NOX). The H_2O_2 produced by NOX generates an oxidizing environment that inhibits protein tyrosine phosphatase (PTP) and promotes Syk activation. Syk can further activate NOX resulting in increased H_2O_2 production (Reth 2002). The signalling machinery of the preBCR is very similar to that of the BCR, therefore we can speculate that the same effect occurs in large preBII cells Fig. (**3b**). Oxidation by H_2O_2 has been reported to prevent the activity of some transcription factors like Egr-1, c-Myb and Ets1, or stimulate the activity of others like the p50 subunit of NF-κB (Sun and Oberley 1996).

Redox mediated regulation of B cell transcription factors:
E2A is a helix-loop-helix transcription factor that was shown to form homodimers by intermolecular disulfide cross-link in order to bind DNA (Benezra 1994). Two isoforms of protein disulfide isomerase (PDI) were reported to regulate the formation of the disulfide cross-link and the dimerization state of E2A. PDI-mediated reduction of the disulfide cross-link appeared to dissociate E2A homodimers and favours heterodimer formation with other basic helix-loop-helix proteins (Markus and Benezra 1999). It is suggested that PDI may play an important role in the regulation of E2A dimerization and B cell ontogeny.
The transcription factor NF-κB provides a preBCR mediated survival signal and initiates Immunoglobulin Light Chain gene recombination, thus facilitating early B cell development (Siebenlist, Brown et al. 2005). The DNA binding activity of NF-κB was reported to be stimulated by reducing proteins such as Trx and Ref-1 acting on the p50 subunit (Toledano and Leonard 1991). On the other hand the oxidant H_2O_2 can stimulate NF-κB nuclear translocation by inhibiting the dephosphorylation of IκB and promoting its release from NF-κB Fig. (**3b**) (Sun and Oberley 1996).
The transcriptional activity of Pax5 depends on the function of the paired (Prd) domain that is able to recognize specific DNA sequences. The Pax5 structure and DNA binding activity was shown to be controlled by the redox potential (Tell, Scaloni et al. 1998). The protein Ref-1 was shown to contribute to the redox regulation of Prd DNA binding activity of Pax5. It is postulated that this control mechanism might be involved in switching among different DNA sequences and therefore different target genes. Furthermore exposure of B cells to oxidative stress resulted in rapid nuclear translocation of Ref-1 associated with an increase in Pax-5 binding activity (Tell, Zecca et al. 2000). Alternative splicing of Pax5 leads to different isoforms with specific DNA binding and transactivation properties Fig. (**3a**). *Pax-5a* is the full length isoform and can be spliced into the *Pax-5d* and *Pax-5e* isoforms. Pax-5d has an intact DNA binding domain, but lacks the transactivation domain and can compete with Pax5-a. Pax-5e lacks also the transactivation domain, but has an impaired DNA binding domain. Pax5e was reported to be structurally complexed to Trx (Lowen, Scott et al. 2001) and has a positive effect on Pax5-a activity. In fact B cell proliferation correlates with increased levels of Pax5e/thioredoxin (Lowen, Scott et al. 2001), so this mechanism may occur in the highly proliferating large preBII cells Fig. (**3b**). In contrast, Pax-5d was reported to be expressed in small resting B cells

(Lowen, Scott et al. 2001) suggesting that it could negatively regulate Pax-5a in these cell types Figure (**3c**).

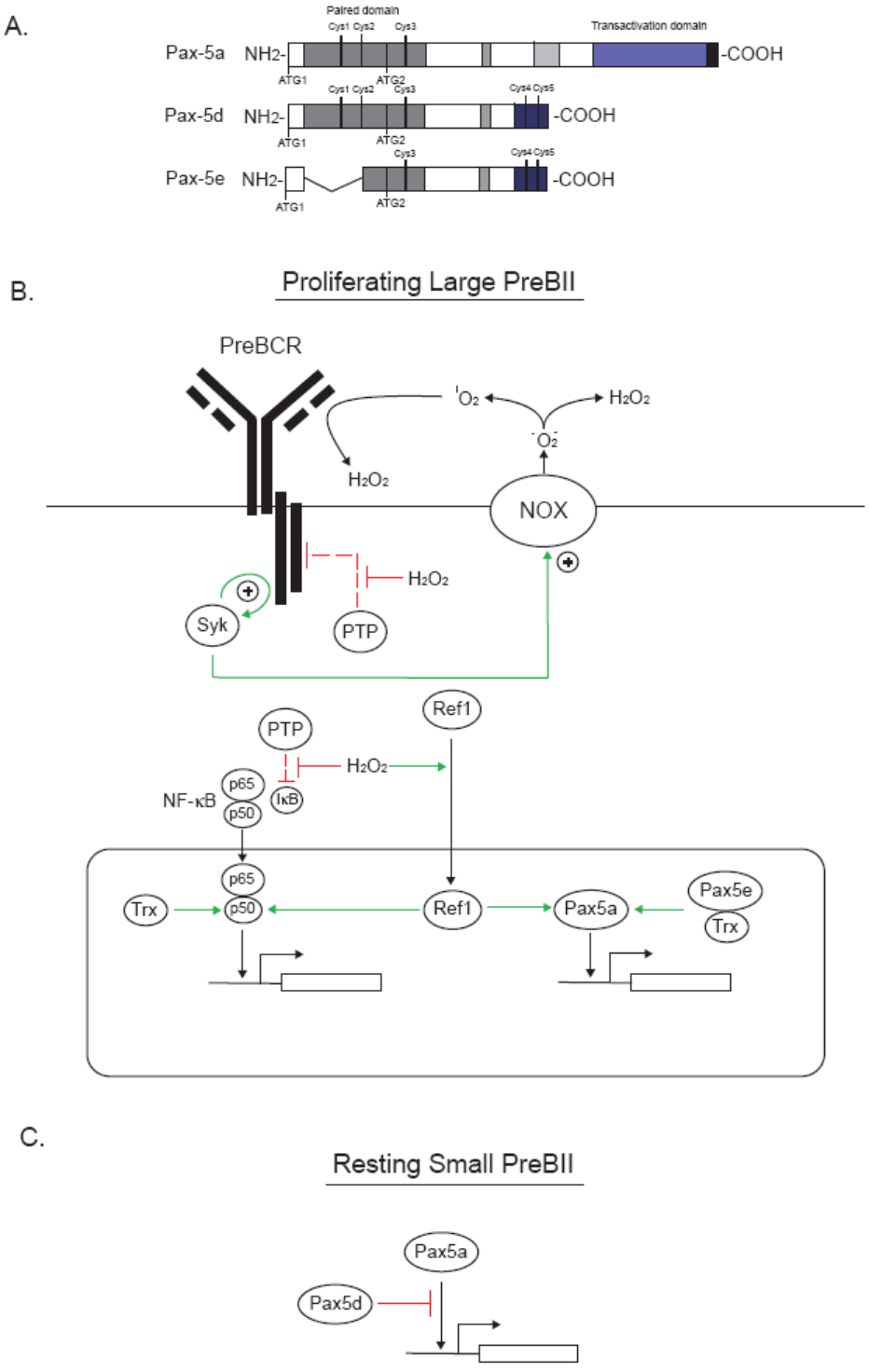

Figure 3: Model for redox regulation of NF-κB and Pax5
(A) *Pax-5a* is the full length isoform containing 10 exons. Pax5 is structured as the following from the N- to the C-terminus: Paired domain, octamer sequence, homeobox homology region, transactivation domain, and repression domain. *Pax-5d* lacks exons 6-10 and *Pax-5e* lacks exons 2 and 6-10. Pax-5d and -5e have a unique C-terminal sequence. All Pax-5 proteins contain two in-frame translation start codons (ATG1 and 2). The cysteine residues

(Cys) that are subject to redox regulation are depicted. (B) Putative model for Pax5 and NF-κB (p50) redox regulation in large preBII cells. During the conversion of $\cdot O_2^-$ into H_2O_2, singlet oxygen ($^1O_2$) is produced that is reduced by the catalytic activity of immunoglobulin into H_2O_2. (C) Putative model for Pax5 regulation in small preBII cells.

6. Conclusions and perspectives

As described here the regulation of early B cell development involves a combination of many factors that have to be set up in a specific spatial and temporal way. The transcription factors belong to the executive entities that integrate all the signalling pathways induced by the extrinsic factors and mediate the lineage specific programming. However, yet another level of regulation is the modulation of locus accessibility through modifications such as histone acetylation and demethylation of CpG islands or the regulation of global changes in the condensation of chromatin, silencers and locus control regions (Ernst and Smale 1995). Histone acetylation and DNA methylation are associated with gene activation and repression respectively. Other histone modifications such as phosphorylation, methylation or ubiquitinylation can modulate locus accessibility and gene expression. These chromatin remodelling events come from epigenetic modifications, which were only marginally discussed in this review. The enzymes (e.g. histone acetyl transferase, histone deacetylase, histone methyl transferase, histone demethylase, DNA methyl transferase, etc...) that regulate these epigenetic marks can be considered also as intrinsic factors in early B cell development.

It is not clear yet what is the mechanism for allelic exclusion. In fact DNA demethylation associated to specific subnuclear localization of the locus is thought to be involved in allelic exclusion (Skok, Brown et al. 2001; Goldmit, Schlissel et al. 2002; Kosak, Skok et al. 2002). Furthermore preBCR signalling was reported to induce rapid decontraction of successfully recombined immunoglobulin loci and IL-7 signalling attenuation leads to repositioning of one *IgHC* allele to repressive centromeric domains (Roldan, Fuxa et al. 2005). However Will et al. argued that indeed allelic exclusion does not originate from IL-7 receptor signalling attenuation (Will, Aaker et al. 2006). Therefore this attenuation mechanism may be necessary, but not essential for allelic exclusion. In fact we do not understand yet how the remodelling factors are recruited to the right recombination sites at the right developmental stage.

Increasing evidence suggests that the oxidation level plays a crucial role in B cell ontogeny. So far a few transcription factors were shown to be regulated by the redox status. However we can speculate that many aspects in the developmental machinery depend on this issue as the redox modulating protein thioredoxin is a crucial player in B cell development. Furthermore the role of the oxidative stress generated with aging is so far poorly understood.

In brief, many aspects of the recombination process and allelic exclusion at the DNA and histone level are still challenging issues. The identification of the chromatin remodelling factors that allow opening and closing of recombination sites will be key achievements to answer these issues. In addition the spatial organization of the immunoglobulin loci before, during and after recombination in such a way that each variable region has an equal probability to rearrange will help to understand this complex phenomenon. Furthermore the understanding of the epigenetic regulation of

transcription factor expression associated with their recruitment to specific loci at the right time is also challenging. Finally the role of the oxidative status together with the aging process will shed light on the fine tuning of the B cell ontogeny.

Abbreviations: HSC, hematopoietic stem cell; MPP: multipotent progenitor; CMP: common myeloid progenitor; ELP: early lymphoid progenitor; ETP: early T lymphocyte lineage progenitor; CLP: common lymphoid progenitor; EPLM, early progenitor with lymphoid and myeloid potential; Ig, immunoglobulin; *IgHC*: immunoglobulin heavy chain; *IgLC*: immunoglobulin light chain; BCR, B cell receptor; DC, dendritic cell; NK cell, natural killer cell; TdT, terminal deoxynucleotidyl transferase; bHLH, (basic) helix-loop-helix; ITAM: immunoreceptor tyrosin-based activation motif; SLC: surrogate light chain; BMP: bone morphogenetic protein; Tpo: thrombopoietin; TGF-β: tumor growth factor β; TNF-α: tumor necrosis factor α; IFN-γ, interferon-γ; FZ: frizzled; Trx: thioredoxin; PDI: protein disulfide isomerase; SS: single stranded; SSBs: single strand breaks; ROS: reactive oxygen species; NOX: NADPH oxidase; Prd: paired; PTP: protein tyrosine phosphatase; HDAC, histone deacetylase; HAT, histone acetyl transferase.

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1.3 OBF-1 characteristics

1.3.1 OBF-1 is a B cell specific coactivator of Oct 1 and Oct 2 on octamer sites

Gene transcription has to be tightly regulated for the proper B cell development. The proteins Oct1 and Oct2 and the coactivator OBF-1 (also known as OCA-B or Bob1) are part of the transcription factors essential for the transcriptional regulation of genes implicated in B lymphopoiesis. Oct1 and Oct2 are members of the POU family of transcription factors which share a conserved bipartite DNA-binding domain called the POU domain. Members of this family bind to specific DNA sequences such as the octamer motif (ATGCAAAT). Oct1 is expressed in almost all tissues and regulate a broad spectrum of target genes. Oct2 on the other hand has a restricted number of target genes and is mainly found in lymphoid cells. The octamer motif is essential for B cell-specific transcription of various genes. This motif can be found in almost all Immunoglobulin (*Ig*) promoters and enhancers (Staudt *et al.*, 1991). Although Oct2 is B-cell-specific it is not the major tissue-specific regulator of *Ig* transcription (Luo *et al.*, 1992; Feldhaus *et al.*, 1993; Corcoran *et al.*, 1993; Sauter and Matthias, 1998).
OBF-1 is also expressed in cells of the lymphoid system and coactivates Oct1 and Oct2 monomers and dimers by clamping their POU subdomains on the octamer motif and inducing gene transcription by its transactivation domain (Luo *et al.*, 1992; Gstaiger *et al.*, 1995; Luo and Roeder, 1995; Strubin *et al.*, 1995; Sauter and Matthias, 1998; Tomilin *et al.*, 2000). However B cells from *OBF-1* knock out mice continue transcribing *Ig* genes, indicating that it is also not the major tissue-specific regulator of *Ig* transcription (Schubart *et al.*, 1996a, 2000, 2001). OBF-1 was reported to activate the transcription of octamer sites in proximal positions of B cell specific promoters (Gstaiger et al., 1995; Luo and Roeder, 1995; Pfisterer et al., 1995; Strubin et al., 1995). On the other hand the activation of transcription from distal positions depends on Oct2 and an unknown coactivator (Schubart et al., 1996a; Pfisterer et al., 1995; Pfisterer et al., 1994; Annweiler et al., 1992). However it was reported that OBF-1 and Oct2 can functionally interact with the *3'-IgH* enhancer element (Tang and Sharp, 1999; Stevens et al., 2000a).
The N-terminus of OBF-1 (amino acids 26 to 32) is crucial for interacting with the POU domain of Oct1 and Oct2. The transactivation domain of OBF-1 was localized in its C-terminus (Pfisterer et al., 1995; Gstaiger et al., 1996; Luo et al., 1998). In the octamer sequence, the position 5 (ATGC**A**AAT) has to be an Adenosine to allow OBF-1 binding (Gstaiger et al., 1996; Cepek et al., 1996). The crystal structure of an OBF-1 peptide with Oct1 and an octamer sequence is depicted in figure 6 to illustrate the importance of the Adenosine in the position 5 (Fig. 6).

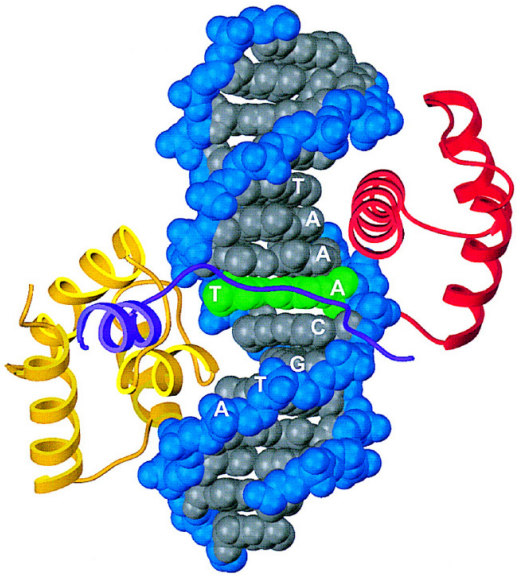
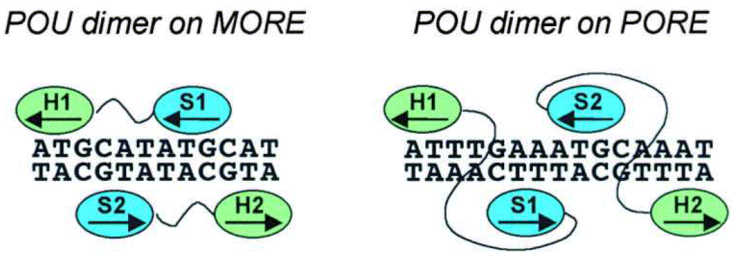


Figure 6: Crystal structure of a ternary complex containing an OBF-1 peptide, the POU domain of Oct1 and an octamer site.
The OBF-1 peptide is shown in purple; the POU_S domain and the POU_H are represented in yellow and red, respectively. The OBF-1 peptide traverses the octamer site at position 5, as highlighted in green (from Chasman et al., 1999)

Distinct octamer sequences were suggested to favor or disfavor recruitment of OBF-1. On some *Ig* heavy chain promoters, Oct-1 was reported to bind to the octamer site in a dimeric conformation (MORE) that prevents recruitment of OBF-1 to the DNA (Tomilin et al., 2000). On the other hand some genes such as *osteopontin* contain a permissive dimeric binding site (PORE) for Oct factors that recruits and is stabilized by OBF-1 (Lins et al., 2003). These octamer configurations may define a structural basis for OBF-1-regulated genes (Tomilin et al., 2000; Remenyi et al., 2001). The MORE and PORE concept is depicted in Figure 7.

Figure 7: Model of an Oct-1 POU domain bound either to a *MORE* or a *PORE* sequence
In each case, the POU_S and POU_H subdomains of one molecule are labeled S1 and H1 or S2 and H2. The flexible linker characteristic of the POU domain, is depicted as well (from Tomilin et al., 2000)



1.3.2 *In vivo* function of OBF-1 in B cells

1.3.2.1 OBF-1 expression in B cells

OBF-1 expression peaks in cycling, low-density splenic B cells (including Germinal Center centroblasts) and can also be induced to high levels by stimulation that mimics T-cell help or bacterial Toll-like receptor 4 (TLR4) responses to lipopolysaccharide (LPS) (Qin *et al.*, 1998; Stevens *et al.*, 2000b; Greiner *et al.*, 2000; Schubart *et al.*, 2001). On the other hand OBF-1 is not detected in resting, high-density splenic B cells (naïve B cells, memory B cells and GC centrocytes). In addition the mRNA levels of *OBF-1* are high and in similar amounts in all B cell populations suggesting that there is a posttranslational regulation of OBF-1 protein expression. Indeed it was reported that the Siah RING finger proteins could interact with OBF-1 and mediate its degradation by the ubiquitin-proteasome pathway (Tiedt *et al.*, 2001; Boehm et al., 2001).

Little is known about the transcription factors that drive *OBF-1* expression. OBF-1 is expressed in bone marrow B cells and in splenic B cells. However OBF-1 is upregulated in late B cell populations, suggesting the involvement of a different transcriptional machinery in early and late B cells. The transcription factor EBF1 has been recently identified as a potential direct regulator of *OBF-1* expression in ProB cells (Zandi et al., 2008). The level of IgG_1 produced by a B cell activated by CD40L and IL4 is increased following CD86 and β_2-adrenergic receptor (β_2AR) stimulation (Kasprowicz et al., 2000; Podojil et al., 2004; Suvas et al., 2002; Podojil and Sanders, 2003). CD86 and β_2AR signaling pathways were reported to increase Oct2 and OBF-1 expression respectively followed by an increased binding to the *3'-IgH enhancer*, which is known to control *IgG_1* expression (Podojil et al., 2004). Therefore the coordinated upregulation of Oct2 and OBF-1 expression by CD86 and β_2AR stimulation, respectively, might explain why the increase in IgG_1 is additive when both receptors are stimulated (Podojil and Sanders, 2005). Stimulation of the β_2AR is associated with an increase in the level of intracellular cAMP (adenosine 3',5'-cyclic monophosphate) activation of protein kinase A (PKA) and an increase in the level of phosphorylated CREB (Sanders et al., 2001). Indeed the *OBF-1* promoter contains a CREB binding site (Stevens et al., 2000b), which explains its sensitivity to β_2AR stimulation. OBF-1 is also upregulated during Plasma cell differentiation (Corcoran et al., 2005). XBP-1 is required for terminal Plasma cell differentiation (Reimold et al., 2001; Iwakoshi et al., 2003) and is activated by the unfolded protein response (UPR), which is triggered by massive immunoglobulin synthesis in the endoplasmatic reticulum (Yoshida et al., 2001; Calfon et al., 2002). Recently Shen and Hendershot reported that *OBF-1* was a direct target of XBP-1 during Plasma cell differentiation (Shen and Hendershot, 2007). The XBP-1 binding occurs through a UPR element conserved in both murine and human OBF-1 promoters.

1.3.2.2 OBF-1 controls transitional B cell survival and chemotaxis

It has been reported that total splenic B cells are reduced to two- to four-fold in *OBF-1* knock out mice (Nielsen *et al.*, 1996; Schubart *et al.*, 1996a). This observation is partly due to the notion that the transitional B cells are more apoptotic and are associated with a reduced splenic seeding (Schubart *et al.*, 2000; Hess *et al.*, 2001). Crossing *OBF-1*$^{-/-}$ mice with transgenic mice overexpressing *Bcl2* could rescue the apoptotic transitional B cells suggesting that Bcl2 might be involved in this phenotype (Brunner et al., 2003b). In addition the chemokine receptor *BLR-1*, which is required for proper homing of B cells to splenic follicles, was reported to be an OBF-1 target gene (Wolf et al., 1998).

1.3.2.3 The roles of OBF-1 in the spleen

The number of Marginal Zone B (MZB) cells was also found to be sharply reduced in *OBF-1* null mice, but this phenotype was strain specific (Qin *et al.*, 1998; Nielsen *et al.*, 1996; Schubart *et al.*, 1996a; Kim *et al.*, 1996; Samardzic *et al.*, 2002b). *OBF-1*$^{-/-}$ B cells have a reduction in the receptor for B-lymphocyte chemoattractant (BLC) called Burkitt lymphoma receptor 1 (BLR1), which could compromise B cell movement in the Marginal Zone (Schubart et al., 2001; Samardzic et al., 2002b; Wolf et al., 1998). Furthermore the mRNA levels of two BAFF receptors, *BAFFR* and *BCMA* (B-cell maturation factor), are reduced in MZB of *OBF-1*$^{-/-}$ mice (Samardzic et al., 2002b). The deletion of the BAFF-BAFFR axis results in impaired MZB

development (Schiemann et al., 2001). Therefore the MZB-cell deficit in some *OBF-1*$^{-/-}$ strains might be due to impaired BLC-BLR1 and/or BAFF-BAFFR or BCMA signaling cascades and perturbations in cell trafficking and sub-compartmentalization (Forster et al., 1996).
In addition to the decrease in the number of mature splenic B cells in *OBF-1* knock out mice it has been observed that the primary follicular B cells were not able to form germinal centers or produce isotype-switched secondary Ig (Nielsen *et al.*, 1996; Schubart *et al.*, 1996a; Kim *et al.*, 1996). The deficiency in the production of secondary Ig isotypes is not due to a failure of the isotype switching process per se, but may be due to decreased transcription of normally switched immunoglobulin heavy-chain loci (Kim *et al.*, 1996). This impaired transcription appears to be due to deficiencies in the function of the *3'-IgH* enhancer elements in class switched Ig in OBF-1 knock out mice (Tang et al., 1999; Stevens et al., 2000a).
OBF-1$^{-/-}$ mice perform normal antigen-independent B cell maturation. However, antigen-dependent maturation of B cells is greatly affected, because the proliferative response to BCR stimulation is impaired (Kim *et al.*, 1996). Furthermore reconstitution experiments showed that the T-dependent immunodeficiency of *OBF-1*$^{-/-}$ mice is B cell-intrinsic (Schubart et al., 1996a). *Spi-B* was identified to be one possible target gene explaining the impaired capacity of *OBF-1*$^{-/-}$ B cells to be activated (Bartholdy *et al.*, 2006). Spi-B is required for normal BCR signalling (Garrett-Sinha *et al.*, 1999) and appears to initiate the production of germinal centers within splenic primary B cell follicles (Su *et al.*, 1997).
In addition to the role of OBF-1 in BCR signaling, it was reported that in mature B cell culture OBF-1 is critical for the final stages of antibody-secreting cell differentiation. In the absence of *OBF-1*, the repressor protein Blimp1/PRDM1 fails to be induced, and downstream targets, such as *Pax-5* or *Bcl6*, are not down-regulated (Corcoran et al., 2005).
The decreased number of splenic B cells observed in *OBF-1* knock out mice (Nielsen *et al.*, 1996; Schubart *et al.*, 1996a) can be explained by the impaired proliferative response to antigen, deficiency in Transitional B cell seeding, decreased number of MZB cells and lack of germinal center formation. These deficiencies in several stages of B cell development leads to an increased sensitivity of *OBF-1*$^{-/-}$ mice to both T-independent Vesicular stomatitis virus (VSV) and T-dependent lymphocytic choriomeningitis virus (LCMV) infection (Fehr et al., 2000). So far OBF-1 seems to have little effect on early B cell development, however specific *Igκ* V(D)J rearrangements and expression of certain recombined *IgVκ* are decreased in *OBF-1* null mice (Casellas *et al.*, 2002) which is not the case for the heavy chain loci (Schubart *et al.*, 2000). These results would mean that octamer motifs in Ig promoters and enhancers need to be flanked by co-stimulatory sequences for proper Ig expression and that some are altered in specific V*k* promoter regions as structural studies suggested (Sauter *et al.*, 1998; Chasman *et al.*, 1999; Cepek *et al.*, 1996; Chang *et al.*, 1999; Tomilin *et al.*, 2000; Lins *et al.*, 2003). As a result of these B cell deficiencies *OBF-1* knock out mice have an immunodeficiency as reported either for T-independent or T-dependent humoral immunity (Fehr *et al.*, 2000).

1.3.2.4 Potential roles of OBF-1 in B cell malignancy

OBF-1 is probably important, alone or in conjunction with other factors such as Aiolos, at several distinct stages of B cell development. The deletion of OBF-1 reverts the phenotype of *Aiolos* knock out mice (Sun *et al.*, 2003), which is B-cell

hyperproliferation and spontaneous germinal center formation in the absence of inciting antigens leading to a systemic lupus erythematous like autoimmune disease (Wang *et al.*, 1998). In line with Aiolos, the loss of OBF-1 was also reported to prevent the development of autoantibodies in the autoimmune disease mouse model MRL-*lpr* (Zuo et al., 2007). However the rescuing properties of the *OBF-1* deletion are different in *Aiolos*$^{-/-}$ and MRL-*lpr* mice. OBF-1 has a role in the transition between preB and immature B cells in *Aiolos*$^{-/-}$ mice (Sun et al., 2003; Matthias and Rolink, 2005). On the other hand the lack of *OBF-1* in MRL-*lpr* mice prevents the generation of antibody/autoantibody-secreting cells. This observation correlates with the notion that OBF-1 is required for plasma cell differentiation *in vitro* (Corcoran et al., 2005).
Surveys of human non-Hodgkin B-cell lymphomas (B-NHLs) show strong OBF-1 protein expression in GC-derived malignancies and no expression in pre- and post-GC derived cases (Greiner et al., 2000; Pileri et al., 2003). In contrast to B-NHL, OBF-1 is aberrantly silenced in the malignant Reed-Sternberg subtype (RS) of classical Hodgkin disease (cHD), which derives from GC B cells and is characterized by impaired immunoglobulin gene transcription (Saez et al., 2002; Re et al., 2001; Jundt et al., 2002; Hertel et al., 2002; Theil et al., 2001; Stein et al., 2001).

1.3.2.5 OBF-1 target genes

Yet, only a few target genes have been identified that can explain the phenotypes of the *OBF-1* knock out mice. Gene profiling and genetic systems were used to understand the molecular function of OBF-1. Several genes were identified to be regulated by OBF-1 and some of these genes were shown to be direct target genes. The table 1 was adapted from a review (Teitell, 2003) and gives an overview of these OBF-1 dependent genes. The classification of these genes suggests that they affect various aspects of B cell physiology such as signal transduction (*CD22*, *Ms4a11*, *B29*, *Kcnn4*, *Lck*, *Spi-B*, *Btk*), cell adhesion (*CD22*, *Osteopontin*), differentiation (*B4galt1*, *BAFFR*, *BCMA*, *Ahd2-like*), proliferation (*Bcl2*, *CDC37*, *CyclinD3*, *Kcnn4*, *S100a10*), chemotaxis (*Osteopontin*, *Blr1*, *CCR-5*) and cytokine secretion (*PU.1*, *IFNγ*, *IL2*).

Table 1: Non-Ig gene and/or protein expression affected in *OBF-1* deficient mice and OBF-1 direct target genes[a]

Affected gene[b]	Function	Cell type	Methods of assessment	Refs
B4galt1	Glycosylation	splenic B cells	RNA (microarray, NB)	Kim et al., 2003
BAFFR	Surface receptor (MZB cell development)	peripheral B-1 and B-2 cells	RNA (RT-PCR)	Samardzic et al., 2002
Bcl2	Cell survival	Pre-B cells	RNA (RPA); protein (WB, Impox)	Brunner et al., 2003
BCMA	Surface receptor (MZB cell development)	peripheral B-1 and B-2 cells	RNA (RT-PCR)	Samardzic et al., 2002b
CD22	BCR antagonist, cell adhesion	Pre-B and immature B cells	Protein (WB, Impox)	Samardzic et al., 2002a
CD36	Scavenger receptor	splenic B cells	RNA (NB)	Schubart et al., 2001

CDC37	Cell cycle	splenic B cells	RNA (microarray, NB, QPCR)	Kim et al., 2003
Cyclin D3	Cell cycle	splenic B cells	RNA (microarray, QPCR)	Kim et al., 2003
Ms4a11	Transmembrane protein (signal transduction)	splenic B cells	RNA (microarray, NB)	Kim et al., 2003
Osteopontin	Cell migration and adhesion	splenocytes	RNA (NB)	Lins et al., 2003
S100a10	Calcium binding protein (BCR-induced expansion)	splenic B cells	RNA (microarray, NB, QPCR)	Kim et al., 2003
Blr1	Chemokine receptor	lymph node B cells	Protein (flow cytometry)	Wolf et al., 1998
Blr1	Chemokine receptor	splenic B cells (no effect)	RNA (NB)	Schubart et al., 2001
CCR-5	Chemokine receptor	T cells	RNA (RT-PCR), protein (flow cytometry, Luc)	Moriuchi and Moriuchi, 2001
B29 (Igβ)	Signal transduction	B cells and plasma cells	Protein (EMSA, Luc)	Malone and Wall, 2002
Kcnn4	Ion transport (BCR-induced expansion)	splenic B cells	RNA (microarray, NB, QPCR); protein (ChIP)	Kim et al., 2003
Lck	Signal transduction	splenic B cells	RNA (microarray, NB, QPCR); protein (ChIP)	Kim et al., 2003
Ahd2-like	Aldehyde dehydrogenase (NAD) activity, oxidoreductase activity (RA synthesis for hematopoiesis)	Pre-B cells and T cells	RNA (microarray, NB, RT-PCR); protein (EMSA, ChIP, Luc)	Brunner et al., 2003a
Spi-B	Transcription factor, BCR signaling	Pre-B cells, splenic B cells and T cells	RNA (microarray, NB, QPCR, RPA); protein (EMSA, ChIP, Luc, IHC)	Bartholdy et al., 2006
Btk	Signal transduction	Pre-B cells, splenic B cells and B cells	RNA (NB); protein (WB, EMSA, ChIP, Luc)	Brunner et al., 2006
PU.1	Transcription factor inhibiting TH2 cytokine expression	T cells	RNA (QPCR); protein (WB, EMSA, ChIP, Luc)	Brunner et al., 2007
IFNγ	TH1 Cytokine	T cells	RNA (RT-PCR, QPCR); protein (EMSA, ChIP, Luc)	Brunner et al., 2007
IL2	TH1 Cytokine	T cells	Protein (EMSA, Luc)	Brunner et al., 2007

[a]Abbreviations: *BAFF*, B-cell activating factor; MZ, Marginal Zone; *BCMA*, B-cell maturation factor; BCR, B-cell receptor; *Blr1*, Burtkitt lymphoma receptor 1; RA, retinoic acid; ChIP, chromatin immunoprecipitation; Impox, immunoperoxidase tissue staining; NB, northern blot; QPCR, semi-quantitative real-time RT-PCR; RPA, RNA protection assay; WB, western blot; Luc, Luciferase assay; EMSA, electromobility shift assay; IHC, immunohistochemistry.

[b]All analyses resulted in a decrease in expression of the listed RNA and/or protein except for *CD22*, which was elevated compared to wild type controls. A discordant result for *Blr1* is noted by a double listing and is potentially explained by post-translational processing, as for OBF-1 in resting versus proliferating splenic B cells (Tiedt et al., 2001). The first part of the gene list corresponds to genes affected by *OBF-1* deletion and the second bold part corresponds to confirmed OBF-1 direct target genes.

1.3.3 *In vivo* function of OBF-1 in T cells

OBF-1 was originally considered as a B cell specific transcription factor. However OBF-1 expression can be induced in T cells after stimulation with αCD3 + αCD28 antibodies, or after co-stimulation with phorbol ester and ionomycin (Sauter and Matthias, 1997; Zwilling et al., 1997; Moriuchi and Moriuchi, 2001). Furthermore the transactivation activity of OBF-1 is also modulated by co-stimulation (Zwilling et al., 1997). OBF-1 expression was also observed in human T cell lymphomas (Marafioti et al., 2003).
Barthodly identified *Spi-B* as an OBF-1 direct target gene by overexpressing *OBF-1* in T cells (Bartholdy et al., 2006). However Spi-B had a more relevant biological function in B cells than in T cells by regulating BCR activity.
Brunner et al. analyzed in detail the T cell development and function in *OBF-1*$^{-/-}$ mice (Brunner et al., 2007). They showed that OBF-1 was critical for T helper cell function by controlling the balance of TH1 and TH2 immune responses. OBF-1 promotes TH1 cytokine expression by activating the *IFNγ* and *IL2* promoters. On the other hand OBF-1 inhibits indirectly TH2 cytokine expression by regulating the expression of the transcription factor *PU.1*, which interferes with GATA-3 binding to promoter DNA (Chang et al., 2005). Therefore the imbalanced immune responses in *OBF-1*$^{-/-}$ mice result in increased susceptibility to *Leishmania major* infection, which depends on the release of TH1-derived IFNγ (Brunner et al., 2007).

1.3.4 OBF-1 has different isoforms

Yu et al. reported the presence of a novel OBF-1 isoform (p40) that results from utilization of an upstream alternative translation initiation codon (Yu *et al.*, 2001). Indeed the *OBF-1* gene contains two active start codons (CTG start codon followed by an ATG start codon). The CTG start codon gives rise to a 40 kDa isoform, which is quickly processed into a 35 kDa isoform (Fig. 8). This isoform gets myristoylated at its N-terminal Glycine and localizes to the cell membrane where its function is not clearly understood. The ATG start codon generates a 34 kDa isoform which transclocates into the nucleus and coactivates gene transcription with Oct proteins (Yu *et al.*, 2001). Both p34 and p35 isoforms are expressed at a similar level from bone marrow preBI cells till splenic mature B cells (Qin et al., 1998; Andersson et al., 2000).
In fact most of the studies on OBF-1 have been performed only with the 34 kDa isoform, therefore the biological functions of the other isoforms are not well understood yet. So far two partners of p35 have been identified, which both are involved in BCR regulation. The nuclear and the cytoplasmatic OBF-1 isoforms, p34 and p35 respectively, were found to interact and regulate the stability of Syk, a key tyrosine kinase in pre-BCR and BCR signalling (Siegel *et al.*, 2006). The second partner of p35 that was identified was Galectin-1, which is a negative regulator of B cell proliferation and tyrosine phosphorylation upon BCR stimulation. OBF-1 was shown to modulate the stability of Galectin-1 thus regulating BCR signalling (Yu *et al.*, 2006).

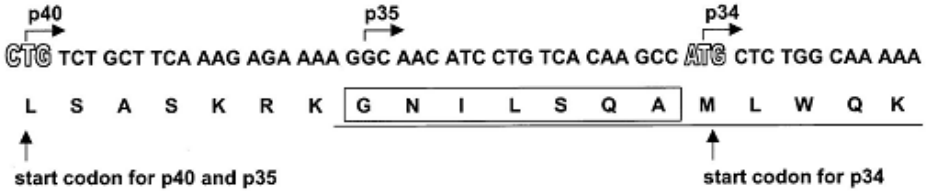


Figure 8: N-terminal sequence of p40
Two translational start codons (CTG and ATG, respectively) are shown in bold face. A conserved myristoylation motif is boxed (from Yu et al., 2001).

1.3.5 p40 might be cleaved into the nucleus

In vitro experiments were performed to understand the machinery leading to p35 generation. These experiments are described in the Appendix. Briefly it is proposed that p40 translocates into the nucleus where it may be processed into p35 potentially after a specific signal. p35 is probably exported into the cytoplasm by interacting with the nuclear exportin CRM1, then it gets myristoylated and anchors into the membrane.
p35 was reported to stabilize Syk, potentially having a positive effect on preBCR signalling. p35 was also reported to destabilize Galectin1 (GAL1). Galectin1 is secreted by B cells and is a negative regulator of the CD45 phosphatase. CD45 is a positive regulator of the BCR signalling. Thus p35 might modulate BCR signalling by regulating Galectin1 level. Furthermore given that p40 is nuclear, one can postulate that, in addition to be a transcriptionally active protein, it represents a "storage" for the membrane bound p35 isoform. Together a general model can be depicted (Fig. 9).

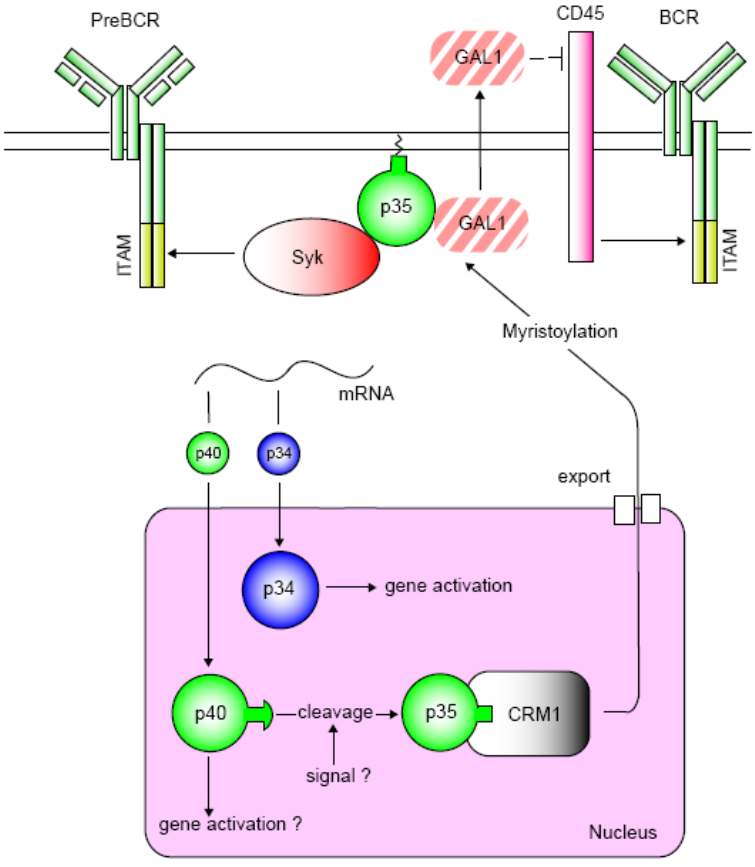


Figure 9: Proposed OBF-1 model
The model was designed according to the experiments in the Appendix, and to the following papers: Yu et al., 2001; Siegel et al., 2006; Yu et al., 2006. p40 might be cleaved into p35 in the nucleus. p35 then interacts with CRM1 and is exported into the cytoplasm where it is myristoylated. At the Plasma membrane p35 stabilizes Syk and destabilizes Galectin1 to modulate preBCR and BCR activity respectively.

1.4 Objective of the research projects

This section gives a short overview of the different projects that were carried out during the PhD thesis. The studies using transgenic mice are described in the "Results" part in the form of publications. Initially the main project was the establishment of BAC (Bacterial Artificial Chromosome) transgenic mice to study the OBF-1 isoforms, however investigating transgenic mice overexpressing OBF-1 in B cells lead to more biological relevant results. Some *in vitro* experiments are depicted in the Appendix.

1.4.1 Identifying the role of OBF-1 in early B cells

This project was set up to identify new OBF-1 target genes by overexpressing OBF-1 in B cells. It turned out that enforced expression of this transcription factor is deleterious for early B cell differentiation. This transgenic mouse led to the discovery of potential new direct target genes by microarray and quantitative PCR analysis.

1.4.2 Dissecting the physiological role of the different OBF-1 isoforms

The generation of BAC transgenic mice was employed to dissect the function of the OBF-1 isoforms *in vivo*. The main approach was to establish BAC transgenic mice that can express only one of the isoforms by mutating either the CTG or the ATG start codons of the *OBF-1* gene. A BAC transgenic mouse containing the wild type *OBF-1* gene was also generated to have a positive control. In addition a *GFP* reporter mouse was established as a negative control. Investigating BAC transgenic mice in an *OBF-1*$^{-/-}$ background allowed understanding what the physiological role of the respective isoforms is. The use of BACs was chosen, because they offer several advantages for functional genomics. They can carry sufficient DNA to encompass most eukaryotic genes, including all *cis*-acting regulatory elements, as well as many eukaryotic gene clusters in a single molecule. However, conventional cloning methods rely on the use of restriction enzymes and *in vitro* purification steps, which preclude engineering of large molecules. To alleviate this limitation the BAC containing the mouse *OBF-1* gene was modified by a recent method based on homologous recombination *in vivo* in E.coli called "ET cloning" (Gene Bridges©). This procedure allows a wide range of modifications at any chosen position. The genomic part of the BAC that was used for ET cloning and for the establishment of transgenic mice is depicted in figure 10.

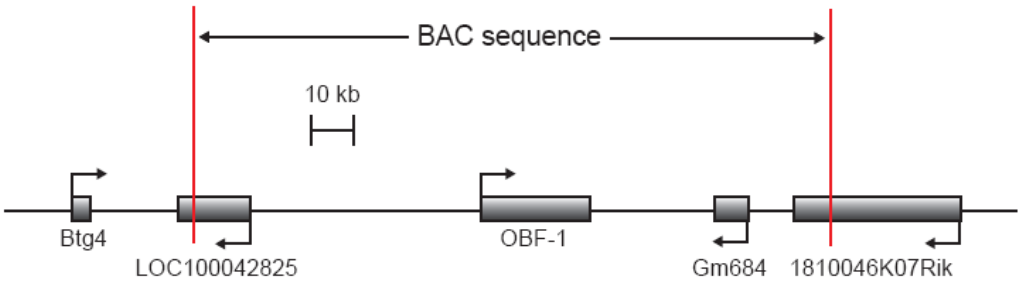


Figure 10: Genomic sequence of the BAC
The BAC is constituted of a 154 kb mouse genomic part containing the *OBF-1* gene. LOC100042825, Gm684 and 1810046K07Rik are putative genes derived by automated computational analysis.

1.4.3 Understanding the machinery for the p35 isoform generation

Yu et al. showed by *in vitro* transcription and translation assays that the p40 isoform is the precursor to p35 and that is quickly processed after translation. In addition Yu et al. demonstrated that p35 and p34 isoforms represent ultimate translational products from alternative start codons (Yu et al., 2001). However the machinery for the generation of the myristoylated p35 isoform was not understood well. Transient transfection experiments suggested that p35 might have a specific function at the plasma membrane, as data supported the idea that p40 is cleaved into p35 in the nucleus and then is exported into the cytoplasm where it is myristoylated (Appendix). Furthermore the observation that BCR stimulation seems to be defective in *OBF-1*$^{-/-}$ mice (Kim *et al.*, 1996) suggests that OBF-1 might interact with components of signal transduction complexes.

2 Results

2.1 Research Publication (submitted to PlosOne)

Enforced Expression of the Transcriptional Coactivator Factor OBF1 Impairs B cell Differentiation at the Earliest Stage of Development

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OBF1, also known as Bob.1 or OCA-B, is a B lymphocyte-specific transcription factor which coactivates Oct1 and Oct2 on B cell specific promoters. So far, the function of OBF1 has been mainly identified in late stage B cell populations. The central defect of OBF1 deficient mice is a severely reduced immune response to T cell-dependent antigens and a lack of germinal center formation in the spleen. Relatively little is known about a potential function of OBF1 in developing B cells. Here we have generated transgenic mice overexpressing OBF1 in B cells under the control of the immunoglobulin heavy chain promoter and enhancer. Surprisingly, these mice have greatly reduced numbers of follicular B cells in the periphery and have a compromised immune response. Furthermore, B cell differentiation is impaired at an early stage in the bone marrow: a first block is observed during B cell commitment and a second differentiation block is seen at the large preB2 cell stage. The cells that succeed to escape the block and to differentiate into mature B cells have post-translationally downregulated the expression of transgene, indicating that expression of OBF1 beyond the normal level early in B cell development is deleterious. Transcriptome analysis identified genes deregulated in these mice and *ID2* and *ID3*, known negative regulators of B cell differentiation, were found to be upregulated in the EPLM and preB cells of the transgenic mice. Furthermore, the *ID2* and *ID3* promoters contain octamer-like sites, which can bind to OBF1. These results provide evidence that tight regulation of OBF1 expression in early B cells is essential to allow efficient B lymphocyte differentiation.

Running title: Role of OBF1 in early B cell development
Keywords: Transcription factors, B cells, B cell development, Gene Regulation

Introduction

The development of B lymphocytes is under precise control by a large number of transcription factors acting at distinct stages to promote cellular differentiation, survival or proliferation. Critical factors for early B cell differentiation and commitment are E2A, early B cell factor 1 (EBF1) and Pax5 and other factors play important roles at later stages (reviewed in (Matthias and Rolink 2005; Fuxa and Skok 2007; Monroe and Dorshkind 2007; Nutt and Kee 2007)). OBF1 is a B cell-restricted transcriptional coactivator, although it can be expressed in activated T cells (Sauter and Matthias 1997; Zwilling, Dieckmann et al. 1997; Moriuchi and Moriuchi 2001). OBF-1 forms a ternary complex with the POU domain transcription factors Oct1 and/or Oct2 and the DNA on conserved octamer motifs (ATGCAAAT) of immunoglobulin (Ig) and other target genes. While it was initially thought that OBF1 is an essential factor for Ig gene transcription (Luo, Fujii et al. 1992), analysis of OBF1 deficient mice revealed that in B cells of these mice the level of unswitched Ig mu gene expression is normal (Kim, Qin et al. 1996; Schubart, Rolink et al. 1996; Schubart, Massa et al. 2001), therefore suggesting that this factor must have other target genes. Work from several laboratories has shown that OBF1 has an important function in late B cell development. Ablation of OBF1 leads to reduced splenic seeding by transitional B cells and to lower numbers of recirculating B cells in the bone marrow (Schubart, Rolink et al. 2000; Hess, Nielsen et al. 2001). Furthermore, OBF1 mutant mice have a severely impaired T cell dependent (TD) humoral immune response with low levels of isotype-switched secondary immunoglobulins (IgGs) and *OBF1*$^{-/-}$ follicular B cells fail to form germinal centers (GCs) (Kim, Qin et al. 1996; Nielsen, Georgiev et al. 1996; Schubart, Rolink et al. 1996; Qin, Reichlin et al. 1998). This absence of GCs may be due in part to the impaired expression of the Ets factor SpiB, which we found to be a direct target of OBF1 in B cells (Bartholdy, Du Roure et al. 2006) and is itself important for GC formation (Su, Chen et al. 1997). In a pure *C57BL/6* genetic background OBF1 is also crucial for marginal zone (MZ) B cells (Samardzic, Marinkovic et al. 2002).
Although the first identified functions of OBF1 are found in the periphery, increasing evidence suggests that this factor also plays a significant role at early stages of B cell ontogeny. In the bone marrow OBF1 promotes the survival of transitional B cells (Schubart, Rolink et al. 2000; Hess, Nielsen et al. 2001), and it is also critical for V(D)J recombination and transcription of a subset of IgVκ genes (Casellas, Jankovic et al. 2002) and thereby has an impact on the IgVκ repertoire (Jankovic and Nussenzweig 2003). In addition, when the OBF1 mutation is combined with a mutation in the zinc finger transcription factor Aiolos, a severe reduction of the immature B cell pool in the bone marrow is observed that defines a crucial function for OBF1 at the preB2 to immature B cell transition (Sun, Matthias et al. 2003; Karnowski et al, submitted). Intriguingly, a recent study has demonstrated that the cytoplasmic isoform of OBF1 interacts with the tyrosine kinase Syk, thus contributing to regulation of preBCR signaling and preB cell proliferation (Siegel, Kim et al. 2006).

Here we have generated transgenic mice expressing OBF1 in B cells under the control of the Ig heavy chain promoter and μ intron enhancer and characterized their phenotype. Surprisingly, we observed that these mice have strongly reduced numbers of follicular B cells in the periphery as well as of preB cells in the bone marrow. In addition, these mice show defects in the immune response elicited by follicular B cells, but have a normal MZ B cell response. We present evidence that these defects are due to the premature expression of OBF1 in early progenitors with lymphoid and myeloid potential, the so-called EPLM cells, which normally do not yet express this factor. Furthermore, we identified a number of genes which are deregulated in the transgenic cells, among which the negative regulators *ID2* and *ID3*. Thus, strict control of the level of OBF1 expression during the earliest stage of B cell development is critical for the formation of a functional B cell compartment.

Materials and Methods

Mouse strains and transgenic mice generation

The *Eμ-V_H-OBF1* construct contains a N-terminally HA epitope-tagged human *OBF1* cDNA under the control of the murine VDJ enhancer (Eμ) coupled to the V_H promoter. Transgenic mouse lines were obtained and bred in *B6CF1* x *C57BL/6* after which they were intercrossed. All the presented analyses were done with littermates of the different genotypes (WT or BCS). Animal experimentation was carried out according to regulations effective in the Kanton of Basel-Stadt, Switzerland as well as in accordance with the FMI internal regulations under supervision of the FMI Animal Committee. The mice were housed in groups of one to six animals at 25°C with a 12:12 h light-dark cycle. They were fed a standard laboratory diet containing 0.8% phosphorus and 1.1% calcium (NAFAG 890, Kliba, Basel, Switzerland). Food and water was provided *ad libitum*.

Splenic B cell purification

The splenic B cells were positively separated with CD19 microbeads following the manufacturer's protocol (Miltenyi Biotec).

Immunizations

To induce a T-independent antibody response, mice were injected intravenously with 100 μg NIP-Ficoll. Sera were collected from tail bleeding prior to and 10 days after immunization and stored at -20°C.
To induce a T-dependent antibody response, mice were injected subcutaneously with 50 μg alum-precipitated NIP-ovalbumin or DNP-KLH. Sera were obtained from tail bleeding prior to and 14 days after immunization and stored at -20°C.

ELISA

96-well microplates were coated over night at 4°C with DNP-BSA or NIP-BSA (5μg/ml in PBS). After extensive washing with PBS the microplates were blocked 1 hour with ELISA buffer (4% BSA, 0.2% Tween20 in PBS). After extensive washing 3 times serial dilutions of serum samples in ELISA buffer were incubated for 2 hours at room temperature. The serum was removed by extensive washing and alkaline phosphatase-labeled anti-IgM or anti-IgG antibodies (1:2000, at room temperature for 2 hours) were used as developing reagents. After washing, substrate buffer (100 mg/ml nitrophenylphosphate, 0.1 g/l $MgCl_2x6H_2O$, 10% diethanolamine pH 9.8) was

used to reveal bound antibodies. The plates were analyzed on an ELISA reader at 405 nm. All antibodies were from Southern Biotech Associates (Birmingham, AL). The antibody titers were determined by taking the dilutions which correspond to three times the value of the background, considering that it is in the linear phase.

Real-Time PCR

RNA was purified with the RNeasy Microkit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized with the Thermoscript Reverse Transcriptase Kit (Invitrogen). Quantitative real-time PCR (qPCR) was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using a SybrGreen-based kit from Eurogene. Normalization was done by amplification of RNA polymerase II (RPII) transcripts.

Primer sequences for qPCR:

OBF1-HA: 5'-CAC TCT CTC TGT GGA AGG CTT TG-3' and 5'-TTC TCA GCT CTA GAC GGC GTA GT-3'

mOBF1: 5'-CAC GCC CAG TCA CAT TAA AGA A-3' and 5'-TGT GGA TTT TTG CCA GAG CAT-3'

RPII: 5'-TGC GCA CCA CGT CCA ATG ATA-3' and 5'-AGG AGC GCC AAA TGC CGA TAA-3'

E2A: 5'-GCA TGA TGT TCC CGC TAC CTG T-3' and 5'-ACC TTC GCT GTA TGT CCG GCT A-3'

EBF1: 5'-AGA TTG AGA GGA CGG CCT TTG T-3' and 5'-TCT GTC CGT ATC CCA TTG CTG-3'

PAX5: 5'-AAT CGC TGA GTA CAA ACG CCA A-3' and 5'-TCC GAA TGA TCC TGT TGA TGG A-3'

ID2: 5'-TCT CCT CCT ACG AGC AGC AT-3' and 5'-CCA GTT CCT TGA GCT TGG AG-3'

ID3: 5'-ACG ACA TGA ACC ACT GCT ACT CG-3' and 5'-AGT GAG CTC AGC TGT CTG GAT C-3'

Syndecan1: 5'-GCG GCA CTT CTG TCA TCA AAG-3' and 5'-GCT GTG TTC TCC CCA GAT GTT T-3'

Immunofluorescent staining and flow cytometry (FACS) analysis

FACS analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA). Cell sorting was performed on a MoFlo (DakoCytomation) or on a FACS Aria (BD Biosciences).

FITC-, PE-, APC-, or biotin-conjugated monoclonal antibodies (mAb) specific for B220, CD3, CD4, CD5, CD8, CD11b, CD19, CD21, CD23, CD25, CD43, CD45.2, CD117, IgM, and NK1.1 were purchased from Pharmingen (BD Biosciences), San Diego, CA. Anti-CD117-APC was purchased from e-Bioscience (San Diego, CA). Anti-CD93 (PB493/AA4.1), anti-IgM and anti-IgD antibodies were purified from the hybridoma supernatant and labeled with biotin in our laboratory by standard methods.

For EPLM cell sorting, erythrocyte-depleted bone marrow cells were stained in IMDM 2% FBS with saturating concentrations of anti-B220-FITC, anti-CD19-PE + anti-NK1.1-PE, anti-CD117-APC and biotinylated anti-CD93 antibodies. After 30 min incubation at 4°C, the cells were washed and resuspended in PBS containing streptavidin-PE/Cy7. After a further 30 min at 4°C, the cells were washed, filtered and resuspended at ~2 x 10^7 cells/ml in PBS 2% FBS before sorting.

Intracellular FACS
After immunostaining of the surface markers, the cells were fixed 10 min with 3% formaldehyde in PBS. The cells were then permeabilized for 10 min with 0.1% Saponin in PBS. After washing with 0.1% Saponin, the cells were incubated 30 min on ice with FITC-coupled anti-HA antibody (Roche). FACS analysis was performed after extensive washing with 0.1% Saponin.

EPLM cell culture
The OP9 mouse stromal cell line was maintained and expanded in IMDM supplemented with 50μM β-mercaptoethanol, 1 mM glutamine, 0.03% w/v primatone (Quest, Naarden, The Netherlands), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2% FBS, as described before (Balciunaite, Ceredig et al. 2005; Ceredig, Rauch et al. 2006). OP9 stromal cells were plated 2 days before the addition of sorted EPLMs and were γ-irradiated with 3000 rad at semi-confluency. The culture medium was then replaced by fresh medium supplemented with 100 U/ml IL-7.

Limiting Dilution Assay
Sorted EPLMs from bone marrow of 3 mice were pooled and plated on semi-confluent γ-irradiated OP9 cells in flat-bottom 96-well plates. Then fresh medium containing ~100 U/ml IL-7 was added, and 48 replicates with increasing numbers of sorted EPLMs were included. At days 10-14 of culture, all wells were inspected using an inverted microscope. Wells containing colonies of more than 50 cells were scored as positive. The frequency of proliferation was calculated with the L-Calc software. The horizontal line was set at 37% and the vertical lines give the inverse of the frequency as the Poisson law.

Chimeric mice
5 *C57BL/6* mice were irradiated with 9.5 Gy and $5x10^6$ bone marrow cells (50% from *C57BL/6* mice and 50% from BCS mice) were injected intravenously. After one month, organ cell suspensions were prepared by mechanical disruption, stained, and subsequently analyzed by flow cytometry.

RNA preparation and hybridization to Affymetrix Microarrays
Cells were FACS sorted and RNA was purified with RNeasy Microkit from Qiagen. Three individual mice per genotype were used for the EPLM cell sorting. Three and four individual mice from WT and BCS mice respectively were used for the large preB cell sorting. Total RNA (~50 ng) from each replicate was reverse transcribed and labeled using the Affymetrix 2-cycles labeling kit according to manufacturer's instructions. Biotinylated cRNA was fragmented by heating with magnesium (as per the Affymetrix instructions) and this fragmented cRNA was hybridized to Mouse 430v2 GeneChips (Affymetrix, Santa Clara, Calif.). Data were analyzed using Expressionist (Genedata AG). The normalized data were subjected to a Student *t*-test ($P < 0.01$) and were required to have a median fold change of at least 2. The microarray data has been deposited in Gene Expression Omnibus (GEO) system under the accession number GSE12421.

Chromatin immunoprecipitation (ChIP)
ChIP was performed with 4.5×10^7 Abelson B cells as described (Bertolino, Reddy et al. 2005). Immunoprecipitation was performed with 5 μg of monoclonal OBF-1 antibody C-20 (SC-955 X; Santa Cruz). As a negative control, the chromatin was

immunoprecipitated with rabbit IgG (Sigma). The samples were amplified using Taq DNA polymerase using the following primers:
ID2: 5'-TGA CAA AGA GCT TCC CAA GAG-3' and 5'-CAC GAC AGG TTT AGC GTG AA-3'
ID3: 5'-AGC ACT AGG GAG GCA GAT CA-3' and 5'-AAA ATC ATG GCC TTC AGT GC-3'
EBF1: 5'-GCC ACA AGG AGA AGG ATG TT-3' and 5'-GGA GAA AGA GAG GGG TGG AC-3'

Results

Mice overexpressing OBF1 have reduced numbers of follicular B cells

OBF1 expression is largely B cell-restricted, and is modulated during B cell development, with a first peak of expression in the bone marrow at the preB stage and a second peak in germinal center cells of immunized mice (Schubart, Sauter et al. 1996; Qin, Reichlin et al. 1998; Greiner, Muller et al. 2000). To define whether tightly regulated expression of OBF1 is critical for B cell development and/or function we generated transgenic mice expressing an HA epitope-tagged OBF1 cDNA under the control of an immunoglobulin variable heavy chain (Ig V_H) promoter and μheavy chain enhancer (Eμ, Fig. 1A). This promoter/enhancer combination has been widely used to express transgenes at high level in B cells, with expression starting already very early in B cell ontogenesis (Banerji, Olson et al. 1983; Grosschedl and Baltimore 1985; Mason, Williams et al. 1985; Dreyfus, Doyen et al. 1987). The transgene was designed in such a way that only the p34 isoform of OBF1 is expressed (the hOBF1 cDNA was inserted at the ATG start codon). Three independent transgenic lines were obtained, hereafter called BCS mice, which all exhibited the phenotype described below.
We first examined the peripheral B cell compartment by analyzing splenic B cells with flow cytometry, using combinations of specific antibodies. In the spleen, the newly formed, so-called transitional, B cells are $CD93^+$, whereas the mature B cells are $CD93^-$ (Rolink, Andersson et al. 2004). The mature B cell gate can be further subdivided into the sessile MZ B cells ($CD23^{low}$ $CD21^{high}$) and the follicular B cells ($CD23^{high}$ $CD21^{low}$). Unexpectedly, the BCS transgenic mice showed a strong reduction in the number of splenic transitional and follicular B cells (Fig. 1B, left). The increased relative percentage in the MZ gate by FACS analysis is due to the reduction of the follicular B cell compartment and not to an increase of MZ B cell number (Fig. 1B, right). In line with this, total splenic cellularity is reduced about five fold in BCS mice, with the numbers of B cells and T cells being reduced about 7 fold and 2 fold, respectively (data not shown).
We then measured the level of secreted Igs in the serum of BCS and wild type mice; as shown in Figure 1C, BCS mice have a slightly but significantly, elevated total IgM level, while total IgG levels are not altered. Furthermore, when specific IgG isotypes were examined, no significant difference was observed between BCS and WT mice.

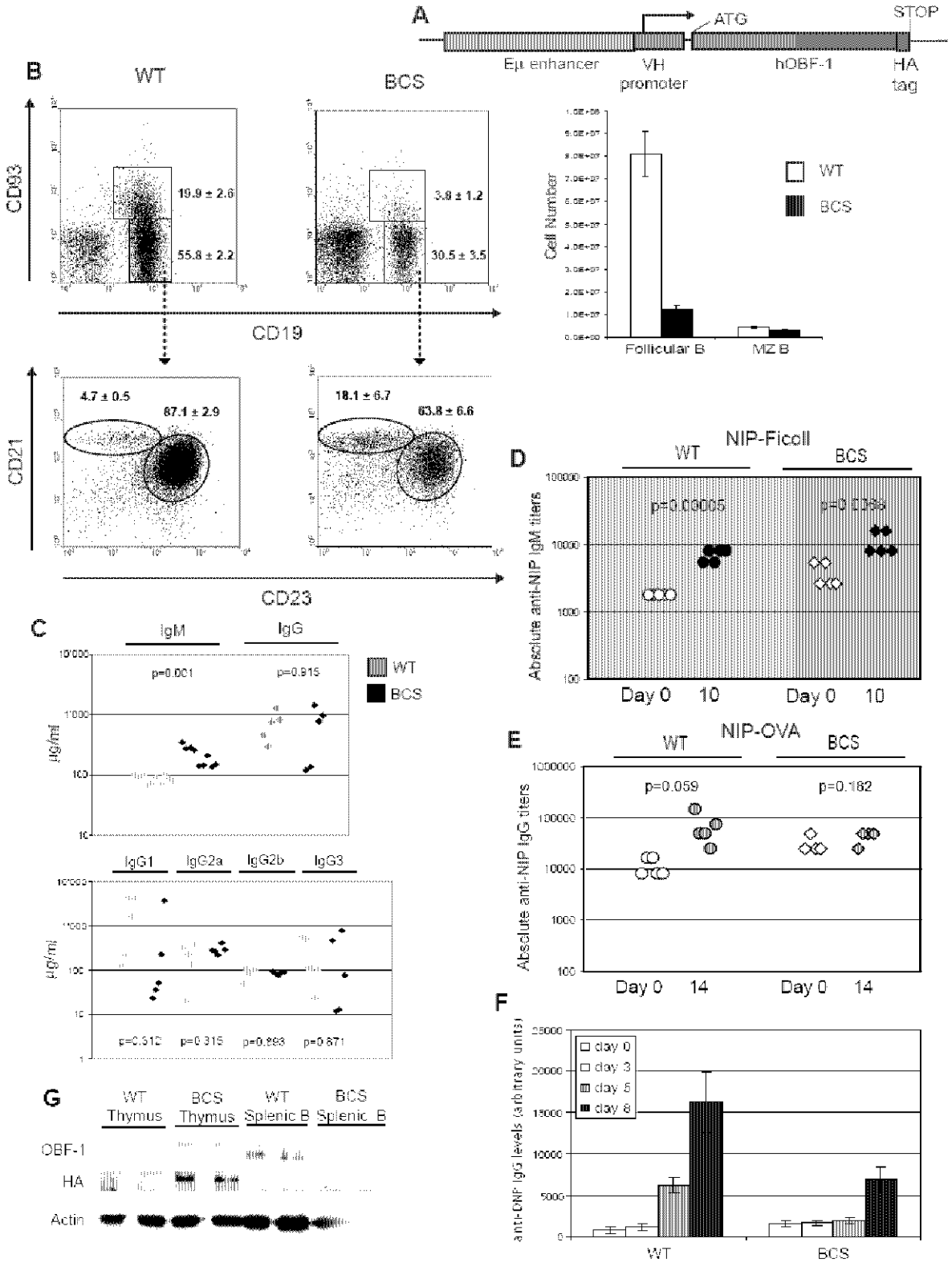

Figure 1: Transgenic *Eμ-V$_H$-OBF1* mice are immunodeficient due to decreased numbers of follicular B cells

(A) Schematic of the transgene consisting of an HA-tagged *OBF1* cDNA under the control of the murine *Eμ* enhancer/*V$_H$* promoter. (B) FACS analysis of splenocytes. Single cell suspensions were stained with antibodies against the indicated markers and representative dot plots are presented. Biotinylated anti-CD93 in combination with streptavidin-PE-Cy7, anti-CD19-APC, anti-CD21-FITC and anti-CD23-PE antibodies were used. The transitional B

cells are CD19$^+$ CD93$^+$, the mature B cells are CD19$^+$ CD93$^-$; within the mature cells, the MZ B cells are CD21$^+$ CD23^{low} and the follicular B cells are CD21^{low} CD23$^+$ (left). The absolute number of follicular and MZ B cells is presented (right). Shown values are the mean percentage and absolute number, ± SE, of four individual mice. (C) Immunoglobulin level in unimmunized mice. The level of total IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3 were measured in unimmunized WT and BCS mice. (D) T-independent immune response. The mice were immunized with NIP-Ficoll *i.v.* and serum antigen-specific IgM titers were analyzed by ELISA after 10 days; for each genotype, five mice were analyzed. (E) T-dependent immune response. The mice were immunized with NIP-OVA and serum antigen-specific IgG titers were analyzed after 14 days. Five control and four BCS mice were used. (F) T-dependent immune response. The mice were immunized with DNP-KLH and serum antigen-specific IgG levels were measured at day 0, 3, 5 and 8 after immunization. The histograms represent the mean ± SE of three mice per genotype. (G) OBF-1 and transgene protein level in thymocytes and splenic B cells. OBF-1, HA and Actin were detected by Western blot from the thymus and splenic B cells of two mice per genotype.

We next monitored the immune response of MZ B cells in BCS mice by injecting them with NIP-Ficoll and measuring the anti-NIP IgM serum titers after 10 days. Indeed, this T-independent immune response was robust in the BCS mice (Fig. 1D), although the basal level of anti-NIP IgM was slightly higher than in the control mice. The immune response of follicular B cells was also investigated by injecting NIP-OVA subcutaneously and measuring the NIP-specific anti-IgG serum titers 14 days later. In this case, this TD immune response was found to be significantly weaker in the BCS than in the control mice (Fig 1E). The impaired T-dependent immune response was further confirmed by immunizing mice with DNP-KLH, another TD antigen, and examining specific IgG serum titers at different time points (Fig. 1F); in this case, a delayed and reduced response in the BCS mice was also observed, in good agreement with the observations presented above.
Western blot was performed to investigate the expression of the transgene in splenic B cells and in the thymus, since the promoter used to construct the transgene can also be active in T cells (Fig. 1G). As expected the HA-tagged transgene is expressed in thymocytes. However the transgene is not detected in splenic B cells suggesting that the impaired splenic B cell populations and the T dependent immunodeficiency have originated from an earlier developmental stage.

The decrease of splenic B cell populations is due to impaired early B cell differentiation

To identify the cause of the reduced splenic B cell compartment in BCS mice, the bone marrow B cell populations were investigated by FACS analysis using a number of antibodies allowing to define the early stages of B cell development. The cellularity of the total bone marrow is reduced by about 10 % in the BCS mice. Among the B220$^+$ cells, the IgM negative and positive gates contain the preB/proB cells and the immature/mature B cells, respectively. Furthermore, within the IgM negative cells, expression of c-kit and CD25 can be used to distinguish the proB and the preB cells; in WT mice, the vast majority of CD25$^+$ preB cells are small and quiescent and derive from large cycling cells (Zhang, Srivastava et al. 2004). As shown in Figure 2A, BCS mice show a strong decrease in the number of CD25$^+$ preB cells, and a relative increase in the proportion of the large preB cells. Furthermore, this latter population has predominantly a high CD43 staining, indicating that the impaired differentiation occurs within the large preB cell stage, at the transition between CD43$^+$ and CD43$^-$

(Fig. 2A, right panel). It results that all the downstream populations, immature and mature recirculating B cells (B220$^+$ IgM$^+$), are strongly reduced in these mice.

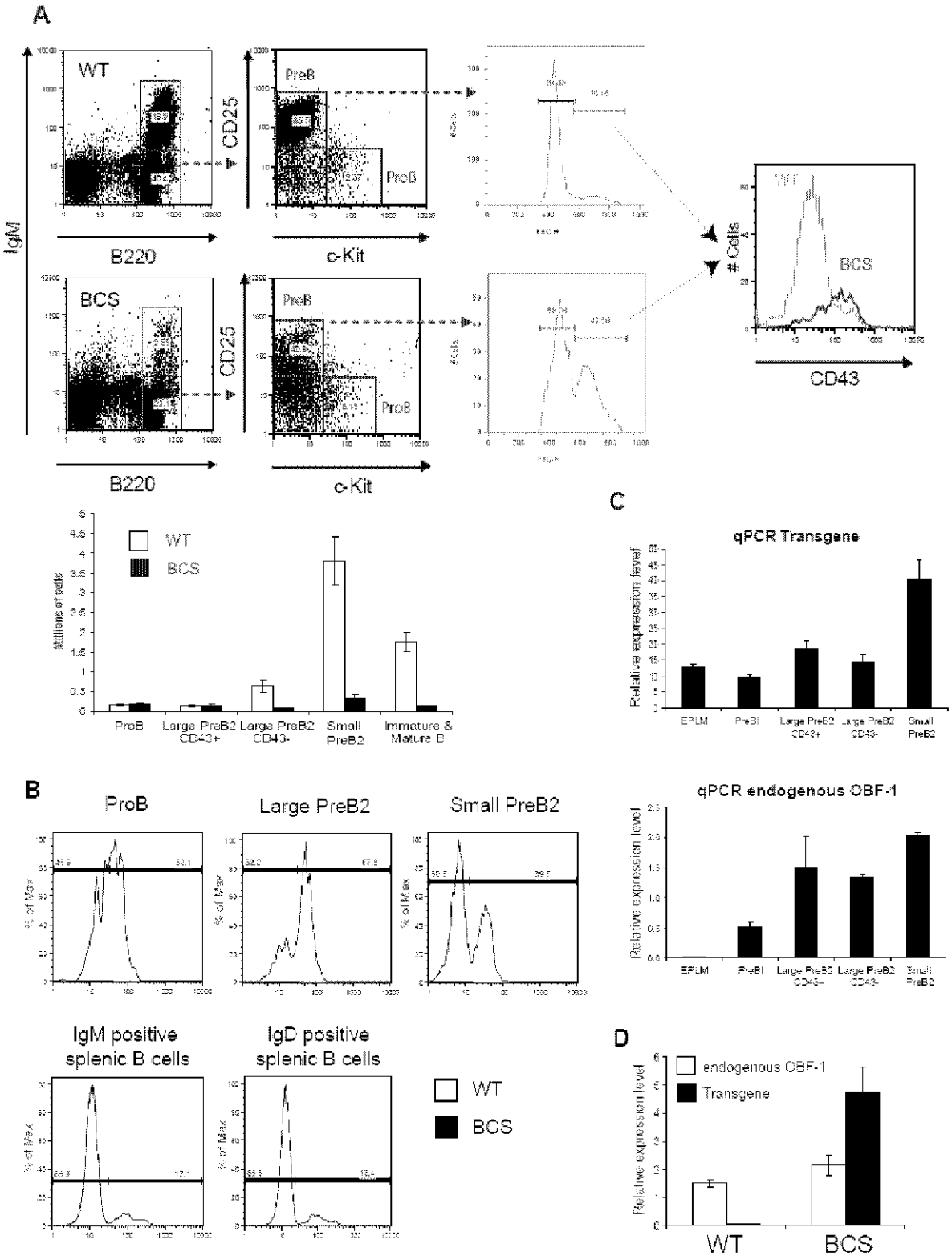

Figure 2: Enforced expression of OBF1 impairs B cell differentiation at the earliest stage

(A) FACS analysis of bone marrow cells. B cells were labeled with an anti-B220-APC antibody and the stages of differentiation were identified with anti-c-kit-PE, biotinylated anti-CD25 in combination with streptavidin-PE-Cy5.5, anti-CD43-PE, and anti-IgM-FITC. The

CD43 expression profile is shown specifically for the large preB2 cells (upper right). Representative dot plots are shown and the histogram with the cell numbers presents the mean ± SE in the different fractions based on three individual mice of each genotype. (B) Intracellular expression of the transgenic OBF1 protein was detected with an anti-HA-FITC antibody in combination with various B cell stage-specific antibodies: In the bone marrow proB cells were detected with anti-B220-APC and anti-c-kit-PE, preB2 cells were labeled with anti-B220-APC together with biotinylated anti-CD25 combined with streptavidin-PE-Cy5.5 and further discriminated for size; splenocytes were labeled with anti-B220-APC, together with either biotinylated anti-IgM or anti-IgD in combination with streptavidin-PE-Cy5.5. (C) qPCR of endogenous and transgenic OBF1 RNA from the indicated bone marrow cell populations; the EPLM and preB1 populations were identified and isolated as described in Figure 3. The histograms represent the mean ± SE of three individual mice for the EPLM and preB1 cells and two individual mice for the large and small preB2 cells. (D) qPCR of endogenous and transgenic OBF1 RNA from splenic B cells. The histogram represents the mean ± SE of two individual mice.

Intracellular FACS analysis with an α-HA antibody was performed to investigate the expression of the transgene during B cell development. As shown in Figure 2B, transgenic OBF1 protein is well expressed until the large preB cell stage and is gradually downregulated in cells that have passed this developmental stage, resulting in a dramatic loss of expression in mature splenic B cells. Unlike the protein, the transgene RNA is expressed from the earliest stage examined (EPLM, see below) and its expression remains relatively constant throughout B cell differentiation (Fig. 2C), including in splenic B cells (Fig. 2D), indicating that the downregulation of OBF1 protein takes place at the post-transcriptional level. In contrast, in WT mice endogenous OBF1 RNA is not detectable in EPLMs and shows a low level of expression in preB1 cells, followed by higher expression starting at the large preB2 cell stage (Fig. 2C).

The EPLMs have a strong B cell commitment deficiency in BCS mice

At a first glance the proB cell population ($B220^+$ $c\text{-}kit^+$) is normal in the BCS mice. However proB cells form an heterogeneous population, which in majority contains already committed B cells (preB1: $CD93^+$ $CD19^+$), but also uncommitted progenitors of several kinds, including NK1.1 positive cells and others. Within these uncommitted progenitors a significant faction of the cells are "early precursors with lymphoid and myeloid potential", so-called EPLMs ($CD93^+$ $CD19^-$ $NK1.1^-$). These cells, while not committed yet to the B cell lineage, under normal conditions preferentially become B cells *in vivo*, are able to generate T cells under transplantation conditions and also have the capacity to differentiate *in vitro* along the myeloid pathway (Balciunaite, Ceredig et al. 2005).

We therefore examined these populations and surprisingly observed that the EPLMs are strongly increased in percentage and number while the preB1 cell numbers are reduced in the BCS mice, indicating an initial differentiation block at this stage already (Fig. 3A). We then sorted EPLMs from WT and BCS mice and tested their capacity to expand and differentiate *in vitro* under culture conditions promoting B cell growth. As shown in Figure 3B, the BCS EPLMs expand very slowly and their differentiation, monitored by the appearance of CD19 expression, is significantly impaired. Intracellular FACS analysis of the EPLM cultures indicated that the cells that succeed to upregulate CD19 also downregulate the OBF1 transgene (Fig. 3C), much like what had been observed in early B cells progressing through

developmental stages *in vivo*. Next, limiting dilution assays (LDA) of EPLMs on OP9 feeders were performed to compare, in EPLM cell populations of WT or BCS origin, the frequency of precursors capable of establishing a colony (Ceredig, Rauch et al. 2006). In this assay, the WT cells showed a normal frequency (1/8), while the BCS cells had a dramatically lower frequency (1/346; Fig. 3C). Together these results demonstrate that the BCS cells are impaired in their B cell commitment potential.

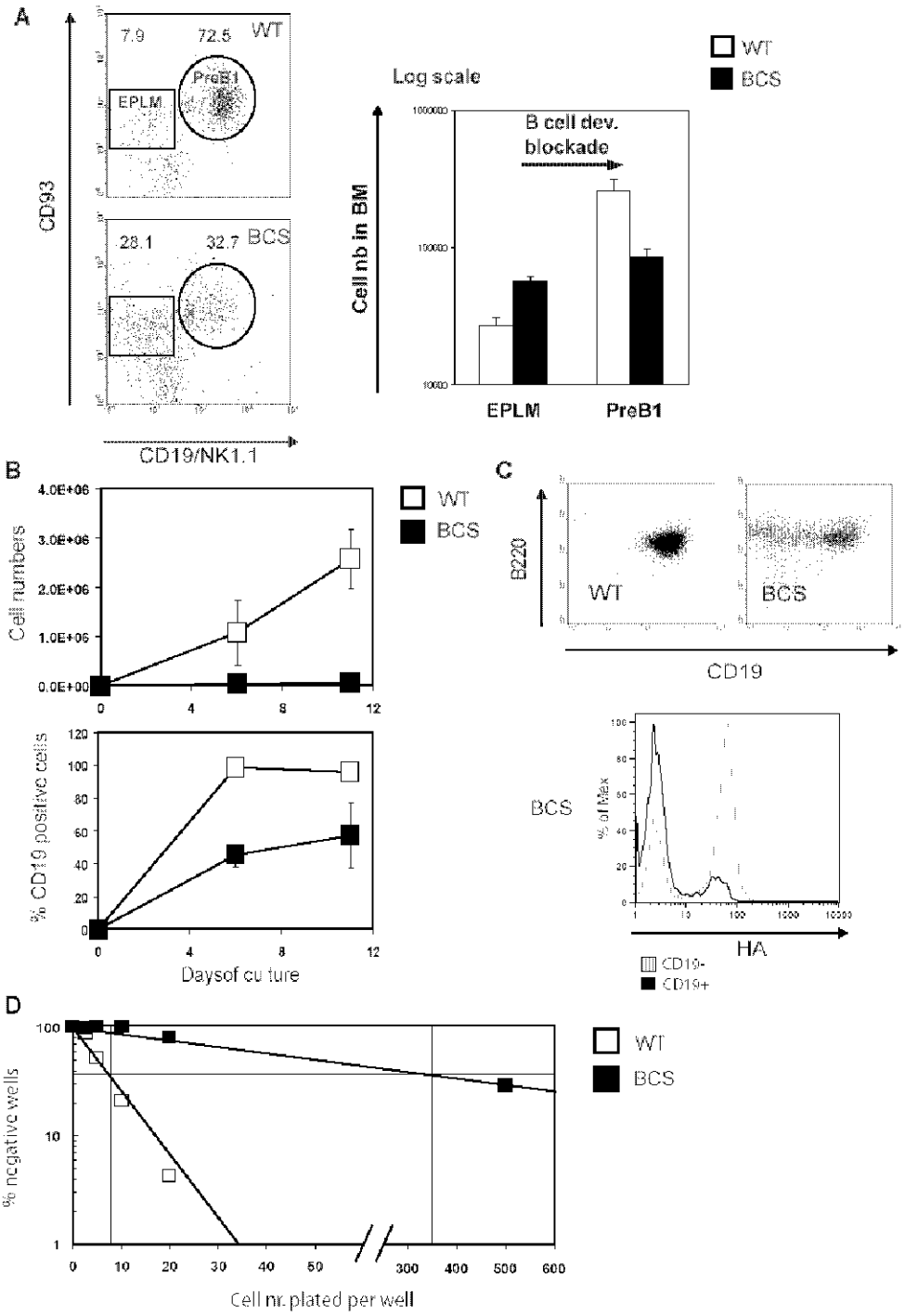

Figure 3: Differential block after the EPLM cell stage and impaired B cell commitment
(A) Detailed FACS analysis of the proB cell compartment in the bone marrow. ProB cells were detected by labeling bone marrow cells with anti-B220-FITC and anti-c-kit-APC. The proB cell gate was then subdivided into EPLM and preB1 cells on the basis of staining with

anti-CD19-PE/anti-NK1.1-PE and biotinylated anti-CD93 in combination with streptavidin-PE-Cy7 (left). The cell number of the different bone marrow populations is presented in the histogram with the mean ± SE of three individual mice (right). (B) *In vitro* culture of EPLMs on OP9 feeders. 10'000 EPLMs from pooled mice (n=3) were plated on OP9 feeders in a 24 well microplate. Cell number and percentage of cells positive for CD19 expression were determined at the indicated times. The graphs represent the mean ± SD from 2 independent samples per time point. (C) Expression of CD19 in cultured EPLMs was determined at day 11 by staining with anti-B220-APC and anti-CD19-PE; expression of the transgene was examined by additional intracellular staining with anti-HA-FITC (lower part). (D) LDA for B cell commitment of EPLMs cultured on OP9 feeders. WT or BCS EPLMs were cultured at increasing dilutions and the number of positive wells was determined under an inverted microscope after 11 to 14 days.

The differentiation block is intrinsic to B cells

The experiments presented so far demonstrate that enforced OBF1 expression in EPLMs impairs their differentiation potential and leads to a developmental block: only cells that successfully downregulate the transgene can differentiate normally along the B cell pathway. To investigate whether the differentiation defect is intrinsic to B cells, competitive chimera mice were generated. For this, the bone marrow of BCS mice (CD45.2, aka Ly5.2) and "competitor" bone marrow from *C57BL/6* mice (CD45.1, aka Ly5.1) were mixed at a 50:50 ratio and used to inject into γ-irradiated mice having the same haplotype as the competitor (CD45.1; Fig. 4A). The reconstituted mice were then sacrified one month post-injection and analyzed. The developing T cell compartment of the chimera mice was not affected, as seen by examining the expression of CD4 and CD8 on thymocytes: for all the developmental stages examined ca. 30% of the thymocytes were BCS-derived. Likewise, in the spleen about 25% of the T cells were of BCS origin. In contrast, the B cell compartment of the BCS haplotype (CD45.2) was strongly impaired in the bone marrow and also in the spleen, as had been initially observed in the BCS mice (Fig. 4B). Indeed, the bone marrow of reconstituted mice showed a clear block at the EPLM-preB1 transition, and the chimerism percentage was found to be inverted just between these two stages: 80% of the EPLMs, but only 10% of the preB1 cells, were BCS-derived (Fig. 4C). These results demonstrate that the differentiation deficiency is intrinsic to the B cells and not due to the environment in the stroma.

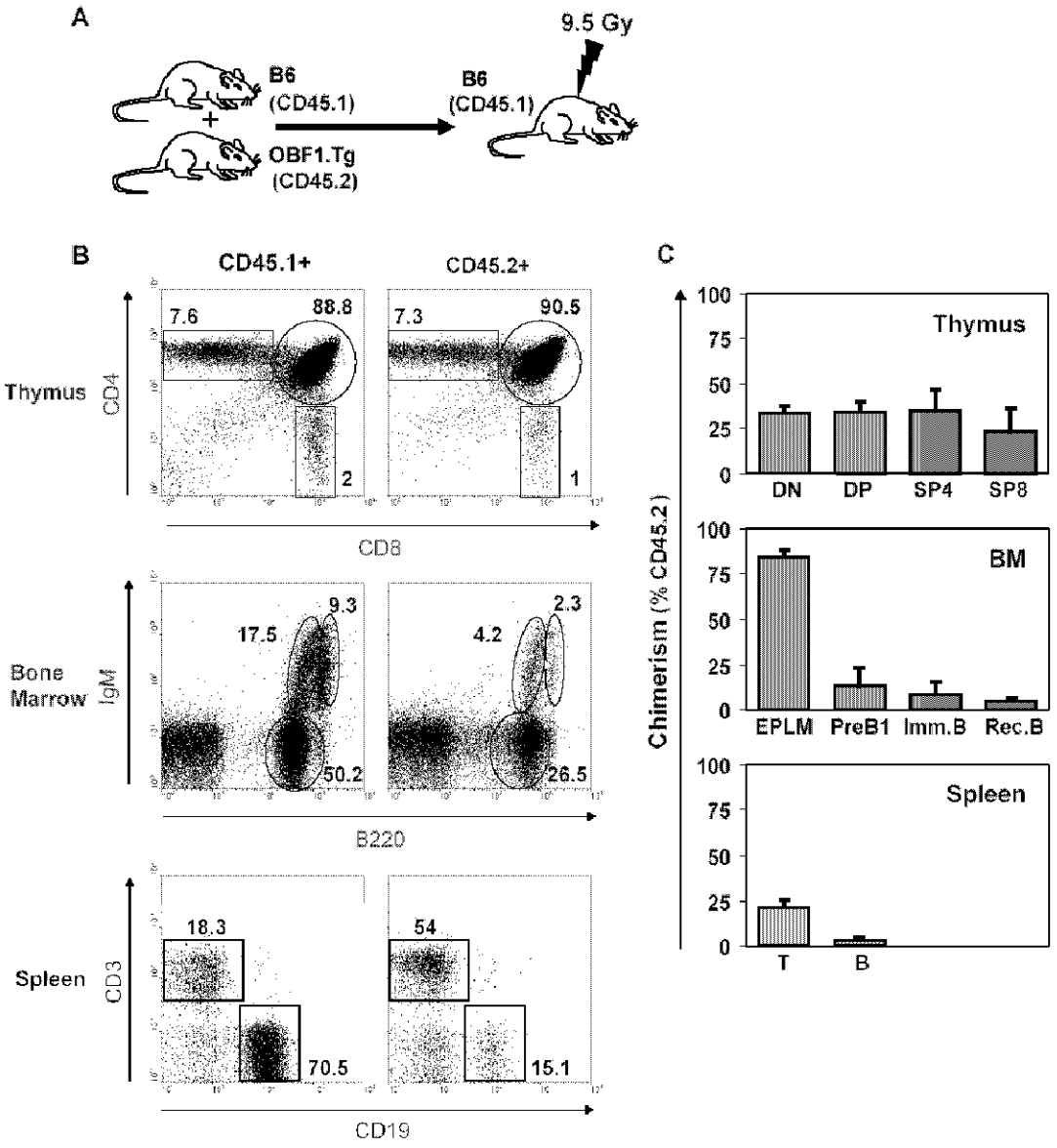

Figure 4: The differentiation block is intrinsic to B cells
(A) The experimental strategy for the mixed bone marrow chimera is depicted. (B) FACS analysis of thymus, bone marrow and spleen of reconstituted mice. Thymocytes were stained with anti-CD4-FITC and anti-CD8-PE. Bone marrow B cells were stained with anti-B220-PE and anti-IgM-APC. Splenocytes were stained with anti-CD3-PE and anti-CD19-APC. In addition, cells of BCS origin were stained with an anti-CD45.2 (Ly5.2) conjugated to either APC or FITC. (C) Percentage of chimerism in the thymus, the bone marrow and the spleen. The EPLM and preB1 cells were analyzed using anti-B220-FITC, anti-CD19-PE, anti-NK1.1-PE, anti-cKit-APC and biotinylated anti-CD45.2 combined with streptavidin-PE-Cy7 antibodies. BM, bone marrow. DN, double negative cells; DP, double positive; SP4, single positive $CD4^+$; SP8, single positive $CD8^+$. Imm. B, immature B cells; Rec. B, recirculating B cells.

The negative regulators *ID2* and *ID3* are OBF1 direct target genes

To get an insight in the molecular origin of the differentiation blocks caused by OBF1 overexpression, the transcriptome of EPLM and large preB2 cells of each genotype was determined by microarray analysis. The scheme for microarray analysis is

depicted in Fig. 5A. We considered genes misregulated at least 2 fold with a stringent p-value of 1%; with these criteria, 569 genes were deregulated in EPLMs and 287 in large preB2 cells, with 40 genes overlapping between the two populations (Fig. 5B). The genes common to EPLMs and large preB cells are presented in Table 1.

Table 1:

Accession number	Name, description	Cluster
1427670_a_at	Transcription factor 12	a
1445093_at	Transcription factor 12	a
1417168_a_at	ubiquitin specific peptidase 2	a
1417336_a_at	synaptotagmin-like 4	a
1417460_at	interferon induced transmembrane protein 2	a
1417976_at	adenosine deaminase	a
1418294_at	erythrocyte protein band 4.1-like 4b	a
1418406_at	phosphodiesterase 8A	a
1418507_s_at	suppressor of cytokine signaling 2	a
1419028_at	cyclic AMP-regulated phosphoprotein, 21	a
1425553_s_at	huntingtin interacting protein 1 related	a
1426755_at	cytoskeleton-associated protein 4	a
1448390_a_at	dehydrogenase/reductase (SDR family) member 3	a
1449109_at	suppressor of cytokine signaling 2	a
1460651_at	linker for activation of T cells	a
1432886_at	RIKEN cDNA 5730488B01 gene	a
1459847_x_at	glial cell line derived neurotrophic factor family receptor alpha 2	a
1452985_at	uveal autoantigen with coiled-coil domains and ankyrin repeats	a
1456772_at	neutrophil cytosolic factor 1	b
1434248_at	protein kinase C, eta	c
1439494_at	solute carrier family 5 (sodium/glucose cotransporter), member 9	d
1445169_at	gb:BM232503 /DB_XREF=gi:17867773 /DB_XREF=K0324B04-3	d
1446294_at	Transcribed locus	d
1421908_a_at	transcription factor 12	d
1439619_at	transcription factor 12	d
1449455_at	hemopoietic cell kinase	d
1429001_at	pirin	d
1458802_at	human immunodeficiency virus type I enhancer binding protein 3	e
1434572_at	histone deacetylase 9	f
1423104_at	insulin receptor substrate 1	h
1416762_at	S100 calcium binding protein A10 (calpactin)	h
1456642_x_at	S100 calcium binding protein A10 (calpactin)	h
1418102_at	hairy and enhancer of split 1 (Drosophila)	h
1419481_at	selectin, lymphocyte	i
1433741_at	CD38 antigen	i
1452679_at	tubulin, beta 2b	i
1447807_s_at	pleckstrin homology domain containing, family H (with MyTH4 domain) member 1	i
1455646_at	RIKEN cDNA 2010004M13 gene	i
1415943_at	syndecan 1	i
1437279_x_at	syndecan 1	i

Table 1:
List of the genes that are deregulated both in EPLMs and preB1 cells of BCS origin; the cluster corresponding to their expression pattern (Fig. 5B) is indicated.

All deregulated genes were then clustered in 9 expression pattern families with the Expressionist program (Fig. 5C). The cluster “a” is possibly the most interesting group, as these genes are upregulated both in EPLM and large preB2 cells of BCS mice, and are therefore putative OBF1 direct target genes. The top upregulated genes in this cluster are presented in Figure 5D; based on the gene ontology (GO) classification, the genes in cluster “a” are mainly involved in lymphocyte development

and activation (Fig. 5E). Furthermore, p53 signaling is also affected, as evidenced by the deregulation of the *Cyclin D2* and *Gadd45β* genes (Fig. 5E).

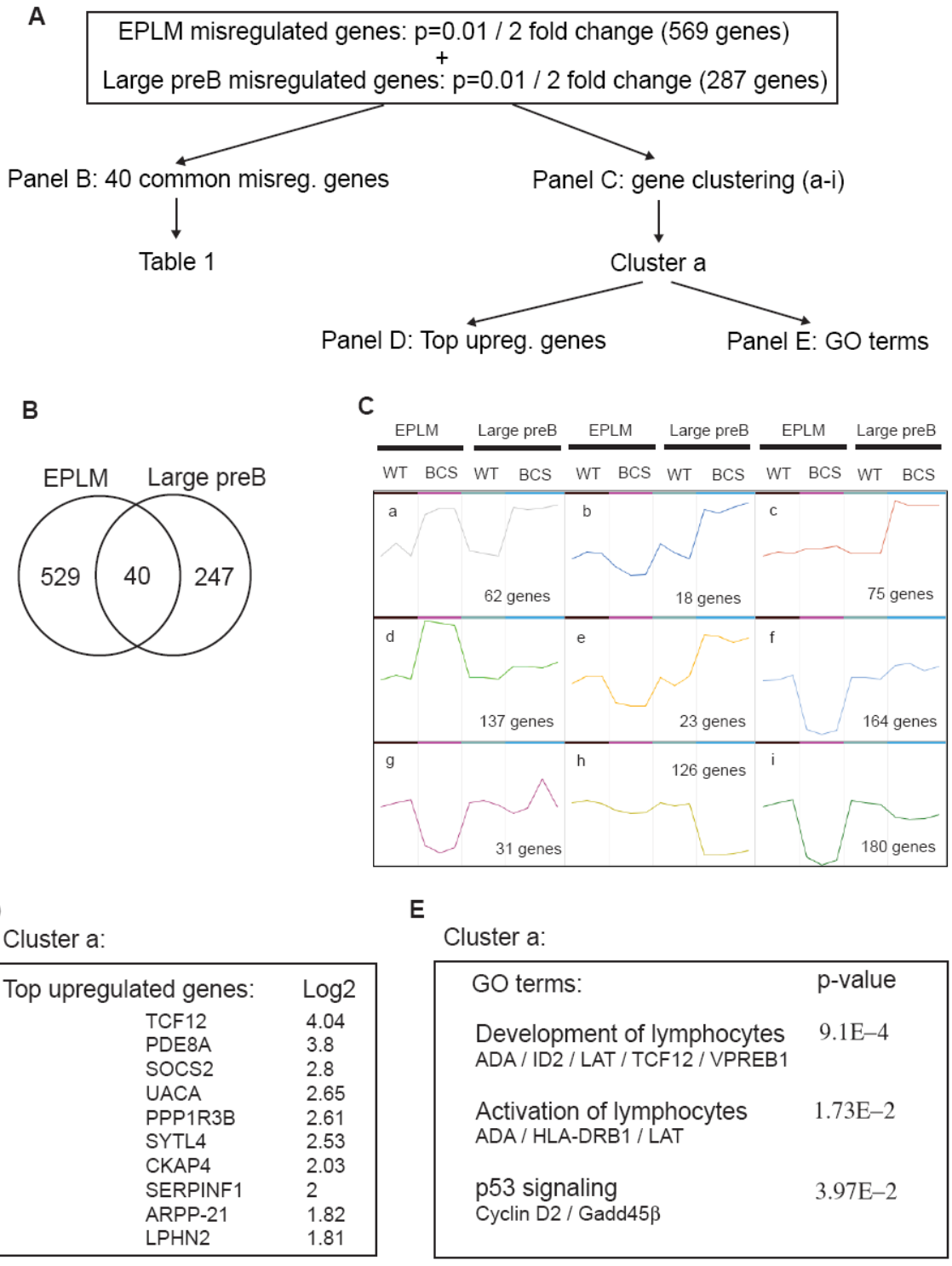

Figure 5: Microarray analysis of EPLM and large preB2 cells
(A) Scheme for the microarray analysis. (B) Venn diagram representing the genes that are misregulated at least 2 fold with a p value of 0.01 in EPLM and large preB2 cells. (C) Gene clustering. The deregulated genes were clustered in 9 families according to their expression patterns. (D) Top upregulated genes from the cluster “a” with the upregulation level monitored in EPLM cells. (E) Gene Ontology (GO) terms in the cluster “a”.

In addition, cluster "a" also contains the *ID2* gene, which encodes an inhibitor of the basic helix-loop-helix (bHLH) protein E2A. Cluster "d", which corresponds to genes specifically deregulated in EPLMs, is also particularly interesting, as it contains another ID gene, *ID3*. Thus, *ID2* and *ID3* are both upregulated in EPLMs of BCS mice and *ID2* is also upregulated in large preB2 cells of BCS mice.
A same gene can appear in different clusters (e.g. *TCF12* in clusters a and d), because different probes recognize different forms of the gene transcript. Often the meaning of these different transcripts is not understood well.
We next examined the microarray data for the main transcription factors critical for early development, such as *E2A*, *Pax-5* or *EBF-1*; while *E2A* expression was similar in BCS and wild type cells, expression of *Pax-5* and *EBF-1* was significantly elevated in BCS EPLMs (cluster "d"). Finally, we found in cluster "i" the *Syndecan1* gene, which is downregulated both in EPLM and large preB2 cells (Fig. 5C). This gene, whose upregulation is often used as a marker for plasma cell differentiation, has been reported earlier to show higher expression at the surface of *OBF1*$^{-/-}$ B cells (Corcoran, Hasbold et al. 2005).
To validate these observations we set up quantitative reverse transcriptase PCR reactions with RNA isolated from cells of different developmental stages: EPLMs, preB1 cells, CD43 positive or negative large preB2 cells and also small preB2 cells. As shown in Figure 6, most of the microarray results could be verified in these experiments. *ID2* and *ID3* were found overexpressed in EPLMs expressing OBF1, and also to a lesser extend in large preB2 CD43$^-$ cells, the two stages where developmental blocks had been identified. *Pax-5* is upregulated in transgenic EPLMs and also slightly downregulated in large preB2 CD43$^-$ cells. Furthermore, BCS EPLMs show a ca. 5 fold upregulation of *EBF-1* and also a robust upregulation of endogenous *OBF1* expression; the latter could be caused by the elevated EBF-1 expression, as it has recently been shown that this factor directly regulates OBF1 expression in progenitors (Zandi, Mansson et al. 2008). Furthermore, *Syndecan1* is downregulated in all the early B cell populations of the BCS mice (Fig. 6), further confirming that it is negatively regulated by OBF1.

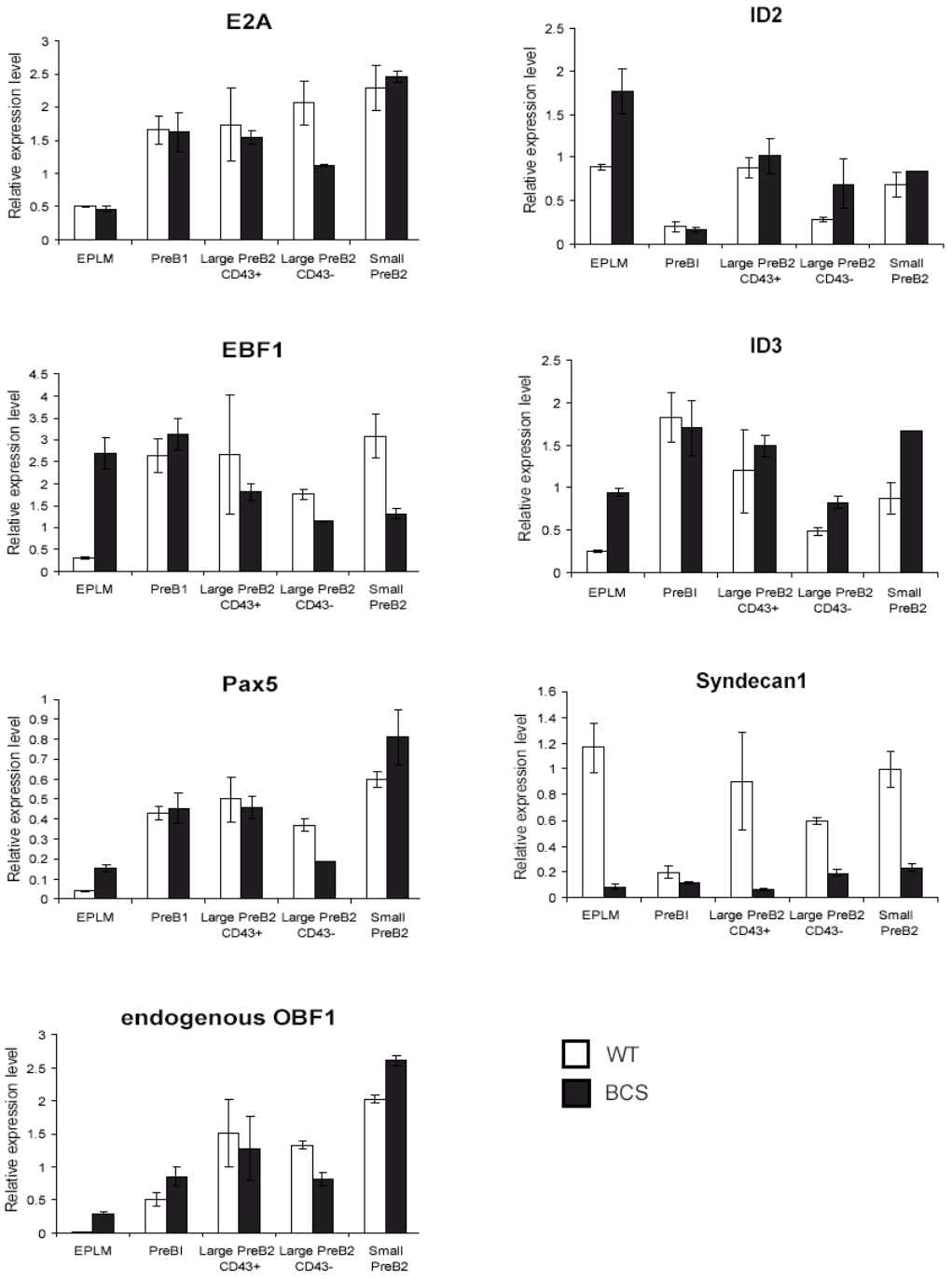

Figure 6: qPCR analysis of early B cell populations
Quantitative RT-PCR analysis of E2A, EBF1, Pax5, endogenous OBF1, ID2, ID3 and Syndecan1 expression in the indicated cell populations. The histograms represent the mean ± SE of three individual mice for the EPLM and preB1 cells and two individual mice for the large and small preB2 cells.

Since *ID2* and *ID3* genes are in gene clusters corresponding to putative OBF1 direct targets (Fig. 5C) and EBF1 is strongly upregulated in EPLM cells (Fig. 6) we searched for potential binding sites in their regulatory region, using the Transcription Element Search System (TESS, http://www.cbil.upenn.edu/cgi-bin/tess/tess). As presented in Figure 7B, the human and the mouse *ID2* and *3* genes contain several elements with homology to the conserved octamer motif found in *Ig* promoters.

Furthermore, one of these elements is conserved in sequence and location between the human and mouse *ID2* promoter. The human and the mouse *EBF1* gene contains also octamer sites. The first sequence is conserved between the human and mouse promoters and the second element in the human promoter is a perfect octamer site. Abelson cell lines were used to investigate the interaction between OBF1 and the respective octamer sites in the *ID2*, *ID3* and *EBF1* promoters. As expected the Abelson cell line from BCS mice express strongly the transgene (Fig. 7A). Chromatin immunoprecipitation was performed with Abelson cell lines from BCS, WT and OBF1$^{-/-}$ mice (Fig. 7C). Immunoprecipitation was performed with anti-OBF1 antibody.and the chromatin regions that were amplified are depicted in red boxes in Fig. 7B. OBF1 interacts with *ID2* and *ID3* promoters in BCS and WT cells. OBF1 interacts with *EBF1* promoter only in BCS cells (Fig. 7C). One should note that EBF1 was not clustered in Fig. 5B, because its p value (0.04) was higher than the cut off of 0.01, although the fold change in EPLM cells was 2.2.

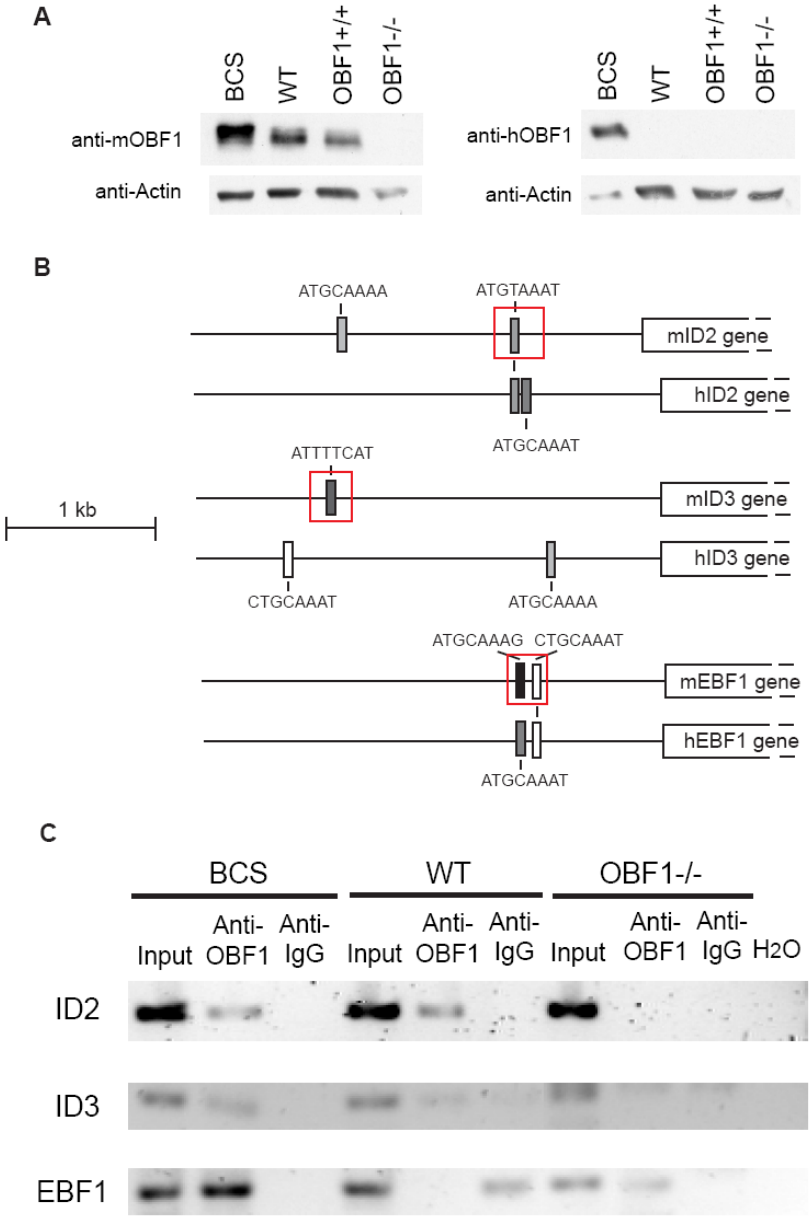

Figure 7: ChIP
(A) OBF1 protein level in Abelson cell lines. Murine OBF1, human OBF1 and Actin were detected by Western blot from pre-B cell cultures established from fetal livers by transformation with the Abelson (Abl) murine leukaemia virus. (B) Octamer-like sites in the mouse ID2, ID3 and EBF1 promoter. The relative locations of the motifs with respect to the translation start codon are as follows: mouse ID2: -835, -2068; human ID2: -1048, -1067; mouse ID3: -2200; human ID3: -760, -2518; mouse EBF1: -822, -933; human EBF1: -808, -919. (C) ChIP of *ID2*, *ID3* and *EBF1* promoters. ChIP using anti-OBF1 antibody was performed from the BCS, WT and OBF1$^{-/-}$ Abl cell lines.

Discussion

Here we present evidence that overexpressing OBF1 at a very early stage of B cell ontogeny is deleterious for B cell development. This misregulated expression pattern of OBF1 ultimately has a dramatic impact on mature B cells in the spleen, as the mice have an impaired T-dependent immune response accompanied with a strong reduction of follicular B cells (Fig.1B and E, F). This immunodeficiency is likely due to the reduced number of follicular B cells, since the T-independent immune response is not impaired (Fig. 1C). Surprisingly, the number of marginal zone B cells is not affected despite the B cell developmental block in the bone marrow and the decrease in splenic immature B cells. One explanation might be that the transitional B cells entering the spleen first repopulate the MZ compartment and then the follicles, and are in sufficient number to fill the MZ. This hypothesis is supported by the earlier observation that the MZ compartment is normal in several other lymphopenic mutant mice such as *IL-7*$^{-/-}$ mice (von Freeden-Jeffry, Vieira et al. 1995) or *Lambda5*$^{-/-}$ mice (Harfst, Andersson et al. 2005). Furthermore, the response to immunization with NIP-Ficoll, which is known to be dependent on the MZ B compartment (Zandvoort and Timens 2002), is also normal in BCS mice. Thus, by these two criteria the follicular and MZ B cell compartment are differentially affected by the presence of the transgene. The total IgM level in unimmunized BCS mice was higher than in the WT mice suggesting that it could be the cause for the high NIP specific IgM background (Fig. 1C). In fact the number of B1b B cells is increased in the peritoneal cavity of BCS mice (Supplementary figure), which could explain the higher IgM level in these mice. On the other hand the total IgG level, as well as specific IgG isotypes, were not altered in unimmunized BCS mice (Fig. 1C). Surprisingly the transgene on the protein level is expressed in the thymus, but not in the splenic B cells, which suggested that the cause for decreased number of immature and follicular B cells is localized at an earlier B cell developmental stage (Fig. 1G). OBF1 was reported to function in determining TH cell polarity (Brunner et al., 2007). Therefore the expression of the transgene in thymocytes could in turn influence TD antibody responses; however T cell development and the numbers of CD4 and CD8 T cells in the thymus are not affected by the transgene (data not shown).

Investigation of the bone marrow, which is the site of early B cell development, allowed to identify the cause of the reduced splenic B cell numbers. A first block was detected between the EPLM and the preB1 cell stage. In the normal situation, EPLMs are mostly committed to the B cell lineage (Balciunaite, Ceredig et al. 2005) and do not yet express OBF1 (Fig. 2C). However, enforcing expression of OBF1 in this population induces an accumulation of EPLMs with a strong B cell commitment deficiency (Fig. 3). Indeed, the cells that succeed to pass this developmental block downregulate the transgene post-translationally, indicating that the level of OBF1 has to be low at this stage for proper B cell differentiation. OBF1 is known to be regulated at the protein level in mature B cells, potentially through interaction with the Ring finger protein SIAH (Boehm, He et al. 2001; Tiedt, Bartholdy et al. 2001). Our results suggest that the OBF1 protein level may also be modulated in early B cells, at least in the case of the transgenic mice described here. Whether this modulation of OBF1 protein levels is mediated by SIAH, or by other mechanisms, is not known. Remarkably, *in vitro* cultures of EPLMs showed that premature expression of OBF1 in this cell compartment severely impairs their proliferation and differentiation potential (Fig. 3). This is in stark contrast to the effect observed in OBF1 deficient IL-7 dependent pro-preB cells: in this case, cellular proliferation is markedly improved in

comparison to WT cells (Siegel, Kim et al. 2006) and data not shown). Thus, in very early B cells OBF1 appears to antagonize cell proliferation and fine regulation of its expression level may be used to set a regulatory threshold. A second differentiation block was also observed after the large preB2 ($CD43^+$) cell stage. It is not clear whether this is directly due to the increased expression of OBF1 in the preB cells, or whether this is a secondary effect whose origin is in the EPLMs. Generation of mice overexpressing OBF1 starting at the preB1 or preB2 stage might allow to address this point.

Mixed bone marrow chimera mice could fully recapitulate the initial phenotype and confirmed that the differentiation defect is intrinsic to the BCS B cell precursors (Fig. 4). When a 1:1 mix of WT and BCS bone marrow was injected into irradiated mice, we observed that about 40% of the thymic developing T cells were of BCS origin and in the spleen the proportion was still about 25%. In striking contrast, the BCS-derived B cell compartment was underrepresented and contained only a few percent of mature B cells in the spleen. Furthermore, a strong developmental block was evident in the bone marrow with a dramatic accumulation of EPLMs accompanied with a deficit to progress to the preB1 stage.

How the deregulated expression of OBF1 in the early EPLM compartment leads to the defects described here is not understood yet. As a first attempt to address this question, we have analyzed the transcriptome of EPLM and large preB cells in WT or BCS mice (Fig. 5). We found significantly more genes misregulated at the EPLM stage than at the large preB cell stage and relatively little overlap between the two sets of genes. However, several of the main transcription factors and known regulatory molecules of early B cell differentiation were either not affected or rather expressed at a slightly higher level in the BCS-derived EPLMs. For example, the helix-loop-helix factor *E2A* is expressed at a normal level, while *EBF1* and *Pax5* are both upregulated (Fig. 6). Generally our results point to the critical importance of maintaining proper regulation of OBF1 expression during early B cell differentiation. So far, relatively little is known about how the *OBF1* gene is regulated and the DNA sequences controlling its cell-specific and temporal expression have not been delineated yet. A functionally important cAMP response element (CRE) binding site has been identified in the proximal OBF1 promoter (Stevens, Wang et al. 2000) but it can not explain the regulated B cell-specific expression of this gene and in transfection experiments the *OBF1* promoter does not appear to be clearly B cell-specific (Massa, Junker et al. 2003). Interestingly, EBF1 has been very recently identified as a potentially direct regulator of OBF1 expression in progenitors (Zandi, Mansson et al. 2008). As shown here, OBF1 is co-expressed from the preB1 stage onwards together with transcription factors like EBF1 that drive B cell commitment (Fig. 2C and Fig. 6). However, in the BCS mice the OBF1 transgene is expressed already before EBF1 and this altered sequence of expression compromises the development of the proB cells. In fact, the elevated level of endogenous OBF1 expression in EPLMs of BCS mice may be a direct consequence of the EBF1 upregulation (Fig. 6), in agreement with the findings of Zandi et al. (2008). However, although EBF1 and Pax5 are misregulated in BCS-derived EPLMs, this does not explain the observed B cell commitment defect, as enforced expression of these genes favours B cell differentiation (Cotta, Zhang et al. 2003; Zhang, Cotta et al. 2003). Interestingly EBF1 showed the same pattern of expression as Id2 and Id3 in EPLM cells from BCS mice. The *EBF1* promoter contains conserved octamer sequences, which can be targeted by OBF1 (Fig. 7). Therefore the EBF1-OBF1 axis constitutes a positive feedback loop, as OBF1 is an EBF1 target gene. The

upregulation of EBF1 in BCS mice can also be explained by the upregulation of Pax5 in EPLM cells, which was reported to activate the proximal promoter of *EBF1* (Roessler, Gyory et al. 2007).
The *ID2* and *ID3* genes were both found deregulated in the microarray as well as in the qPCR validation experiments (Figs. 5 and 6); in particular, elevated expression was found in EPLMs and also in large preB2 cells, which are just the stages were the developmental blocks have been observed in BCS mice *in vivo*. Expression of ID3 has been reported to be repressed by OBF1 in preB cell line expressing inducible OBF1-ER fusion protein (Brunner, Laumen et al. 2003), however this opposite effect might be due to the fusion of ER to OBF1. The identification of several motifs with homology to the octamer site in the *ID2* and *ID3* promoters suggests that these genes could be direct OBF1 targets. Chromatin immunoprecipitation showed that indeed OBF1 can bind to these octamer sites (Fig. 7). Generally ID proteins have been found to antagonize the activity of bHLH proteins, and in particular of E2A. Several previous studies have shown that low levels of ID proteins are necessary to allow E2A to drive B cell commitment (Deed, Jasiok et al. 1998; Ji, Li et al. 2008). In line with this, constitutive expression of ID proteins downstream of the preB1 stage was found to impair B cell development, indicating that ID downregulation is critical for B cell ontogeny (Sun 1994). Furthermore, it was also reported that ID3 inhibits the growth and survival of B lymphocyte progenitors (Kee, Rivera et al. 2001). These observations therefore suggest that elevated expression of ID2 and ID3 could result in a differentiation block at the EPLM and large preB2 cell stage, as seen in the BCS mice.
Finally, syndecan1 is a plasma cell marker whose *in vivo* function is not clear yet. However, syndecan1 was reported previously to be upregulated on the surface of *OBF1*$^{-/-}$ splenic B cells (Corcoran, Hasbold et al. 2005) and our microarray analysis of *OBF1*$^{-/-}$ mice showed that syndecan1 is upregulated also at the mRNA level (data not shown). Interestingly, we found here that syndecan1 expression was strongly downregulated in all the early B cell populations of the BCS mice (Fig. 6), indicating that there is a negative correlation between OBF1 and syndecan1 expression. Thus, *syndecan1* represents a novel OBF1 target gene.

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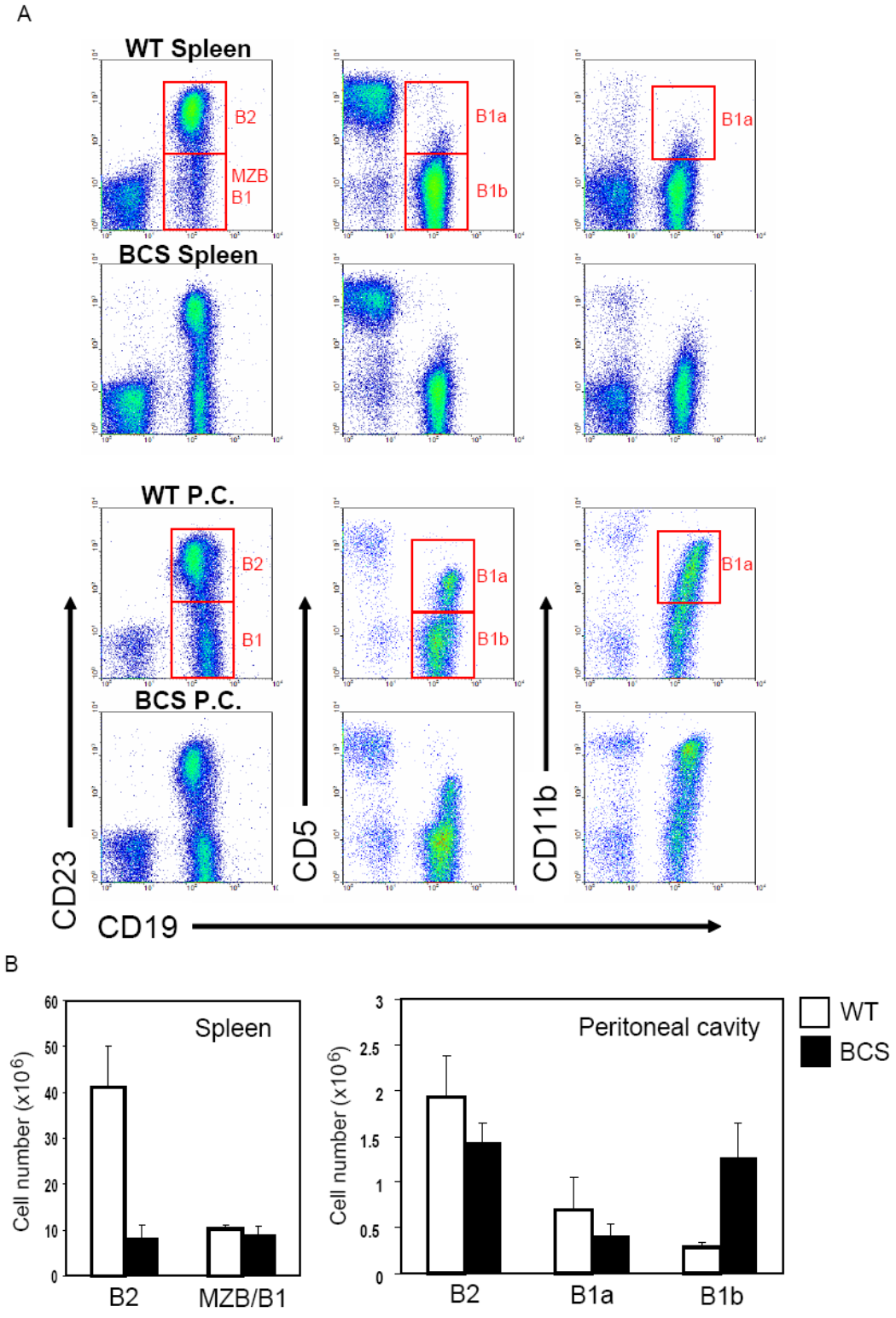
Author Contributions
Conceived and designed the experiments: AB, BB, NB, CDR, PM. Performed the experiments: AB, BB, NB, CDR, HK, GM. Analyzed the data: AB, BB, NB, CDR, GM, AGR, PM. Contributed reagents/materials/analysis tools: AB, BB, NB, AGR, GM. Wrote the paper: AB, PM.



Supplementary figure

(A) FACS analysis of the spleen and peritoneal cavity. B cells were labeled with an anti-CD19-PE antibody. The B2 cells were stained with anti-CD23-FITC. The B1a cells were stained with anti-CD5-FITC antibody or with anti-CD11b-FITC antibody. (B) B1, B1a, B1b and B2 B cell populations in the spleen and peritoneal cavity. The B2 and MZB/B1 B cells are $CD19^{+}CD23^{+}$ and $CD19^{+}CD23^{-}$ populations respectively. The B1a and B1b B cells are $CD19^{+}CD5^{+}$ and $CD19^{+}CD23^{-}CD5^{-}$ populations respectively. The histograms represent the mean ± SD of three individual mice per genotype.

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2.2 Research Publication

Dissecting the functions of the B cell specific transcription factor OBF-1

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The B cell specific transcription factor OBF-1 comprises a nuclear and a cytoplasmic isoform that are generated from two active start codons. The cytoplasmic isoform is myristoylated at its N-terminus and localizes to intracellular membranes where its function is not clear yet. *OBF1*$^{-/-}$ mice have a severe B-cell immunodeficiency and lack germinal centers. The phenotype of these mice has been usually attributed to the nuclear isoform, as the function of OBF-1 outside the nucleus is not clear. BAC transgenic mice expressing each isoform were generated to investigate their capacity in rescuing the *OBF-1*$^{-/-}$ phenotype. Indeed phenotypically the transgenic mice expressing the nuclear isoform looked like wild type mice indicating that it is the main player among the isoforms. However the *in vitro* hyperproliferation of ProB cells from *OBF-1*$^{-/-}$ mice was not rescued by the nuclear isoform. As expected, the comparison of microarray data from *OBF-1*$^{-/-}$ mice and BAC transgenic mice expressing only the nuclear isoform showed that the majority of OBF-1 regulated genes are under the control of the nuclear OBF-1 isoform. These observations suggest that the cytoplasmic isoform might play a role in regulating proliferation of early B cells *in vitro* and confirm the notion that the nuclear OBF-1 isoform controls most of the genes misregulated in *OBF-1*$^{-/-}$ mice.

Introduction

OBF-1 (also known as OCA-B or Bob1) is expressed in cells of the lymphoid system and coactivates Oct1 and Oct2 monomers and dimers by clamping their POU subdomains on the conserved octamer motif (ATGCAAAT) and inducing gene transcription by its transactivation domain (Luo *et al.*, 1992; Gstaiger *et al.*, 1995; Luo and Roeder, 1995; Strubin *et al.*, 1995; Sauter and Matthias, 1998; Tomilin *et al.*, 2000). Oct1 is expressed in almost all tissues and regulates a broad spectrum of target genes. On the opposite Oct2 has a restricted number of target genes and is mainly found in lymphoid cells. The octamer motif is essential for B cell-specific transcription of various genes. This motif can be found in almost all Immunoglobulin (*Ig*) promoters and enhancers (Staudt *et al.*, 1991) and is critical for *Ig* gene

transcription (Bergman et al., 1984; Mason et al., 1985; Dreyfus et al., 1987; Wirth et al., 1987; Jenuwein and Grosschedl 1991; Matthias 1998). Although Oct2 is specifically expressed in lymphocytes and can bind octamer sites on *Ig* promoters, it is not the major tissue-specific regulator of *Ig* transcription (Luo et al., 1992; Feldhaus et al., 1993; Corcoran et al., 1993; Sauter and Matthias, 1998). *In vitro*, OBF-1 was shown to coactivate octamer-containing promoters such as the one from the immunoglobulin κ light chain gene (Luo and Roeder 1995; Strubin et al., 1995; Schubart et al., 1996). Furthermore Oct2 and OBF-1 can functionally interact with the *3' IgH* enhancer element (Tang and Sharp, 1999; Stevens et al., 2000). However B cells from *OBF-1* knock out mice continue transcribing *Ig* genes, indicating that *in vivo* it is not required for initial transcription of *Ig* genes (Schubart et al., 1996; Schubart et al., 2001).

The main phenotype of *OBF-1*$^{-/-}$ mice is a T-dependent immunodeficiency due to lack of germinal centers (Nielsen et al., 1996; Schubart et al., 1996; Kim et al., 1996). *OBF-1*$^{-/-}$ mature B cells are still able to perform class switching, however the transcription of normally switched immunoglobulin heavy-chain loci is impaired (Kim et al., 1996). Furthermore the antigen-dependent maturation of *OBF-1*$^{-/-}$ B cells is also affected, presumably because of impaired BCR-mediated proliferation (Kim et al., 1996). In one *OBF-1*$^{-/-}$ mouse strain the number of Marginal Zone B (MZB) cells was also reported to be reduced (Qin et al., 1998; Nielsen et al., 1996; Schubart et al., 1996; Kim et al., 1996; Samardzic et al., 2002). The bone marrow Transitional B cells of *OBF-1*$^{-/-}$ mice are also more apoptotic and have a reduced splenic seeding, which leads to a decrease of total splenic B cells (Schubart et al., 2000; Hess et al., 2001; Nielsen et al., 1996; Schubart et al., 1996). Crossing *OBF-1*$^{-/-}$ mice with transgenic mice overexpressing *Bcl2* could rescue the apoptotic Transitional B cells suggesting that Bcl2 might be involved in this phenotype (Brunner et al., 2003). In the bone marrow OBF-1 appears to have additional effects on the V(D)J recombination and transcription of a subset of IgVκ genes (Casellas et al., 2002). Furthermore OBF-1 was suggested to cooperate with the zinc finger transcription factor Aiolos to promote the preB2 to immature B cell transition (Sun et al., 2003; Karnowski et al., in press).

To date, a few OBF-1 direct target genes have been identified, which could explain the phenotypes of the *OBF-1* knock out mice, although their physiological relevance for GC formation and TD immune responses remains unclear. The chemokine receptor *BLR1* is required for proper homing of B cells to splenic follicles, and was reported to be cooperatively regulated by NF-κB, OBF-1 and Oct2 in B cells (Wolf et al., 1998), which could partly explain the impaired Transitional B cell seeding in the spleen of *OBF-1*$^{-/-}$ mice. The *Kcnn4*, *Lck*, and *Btk* promoters, with their corresponding genes involved in signal transduction, were reported to be directly bound and activated by OBF-1 (Kim et al., 2003; Brunner et al., 2006). Recently the Ets factor *Spi-B*, which is required for normal BCR signaling (Garrett-Sinha et al., 1999) and appears to initiate the production of germinal centers (Su et al., 1997), was identified as a direct critical target of OBF-1 (Bartholdy et al., 2006). The downregulation of *Spi-B* might be one cause for the impaired capacity of *OBF-1*$^{-/-}$ B cells to be activated. In addition to the role of OBF-1 in BCR signaling, it has been reported that in mature B cell culture OBF-1 is critical for the final stages of antibody-secreting cell differentiation. In absence of *OBF-1*, the repressor protein Blimp1/PRDM1 fails to be induced, and downstream targets, such as *Pax5* or *Bcl6*, are not downregulated (Corcoran et al., 2005).

The *OBF-1* gene contains two active start codons (CTG start codon followed by an ATG start codon). The CTG start codon gives rise to a 40 kDa isoform, which is quickly processed into a 35 kDa isoform. This isoform gets myristoylated at its N-terminus and localizes to the cell membrane where its function is still unclear. The ATG start codon generates a 34 kDa isoform which translocates into the nucleus and coactivates gene transcription with Oct proteins (Yu *et al.*, 2001). In fact most of the studies on OBF-1 have been performed only with the 34 kDa isoform, therefore the biological function of the other isoforms are not well understood yet. So far two partners of p35 were identified, and these proteins are both involved in BCR regulation. The nuclear and the cytoplasmatic OBF-1 isoforms, p34 and p35 respectively, were found to interact and regulate the stability of Syk, a key tyrosine kinase in pre-BCR and BCR signalling (Siegel *et al.*, 2006). The second partner of p35 is Galectin-1, a negative regulator of B cell proliferation and tyrosine phosphorylation upon BCR stimulation. OBF-1 was shown to modulate the stability of Galectin-1, thus regulating BCR signalling (Yu *et al.*, 2006).

Materials and methods

Mouse strains and transgenic mice generation

The Bacterial Artificial Chromosomes were modified with by ET cloning (Muyrers et al., 1999) as per the manufacturer's protocol (Gene Bridges©). In a two-step approach a counter-selection cassette was first introduced at the location to be modified and in the second step replaced by non-selectable DNA containing the desired mutation. The counter-selection cassette contains the *rpsL* and the *neomycin* genes which confer Streptomycin sensitivity and Kanamycin resistance respectively. The first selection was performed with Kanamycin and the counter-selection for non-selectable DNA replacement was done with Streptomycin. The modified BACs were then used to generate transgenic founders in a *B6CF1* x *C57BL/6* background. The mice were then crossed with *OBF1*$^{-/-}$ mice which were in a *129SV-C57BL/6* background.

Western blot and Odyssey detection

For Western blotting, the cells were lysed by repeated cycles of freeze and thaw in 20 mM HEPES pH7.9, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 400 mM KCl and Complete Protease Inhibitor cocktail (Roche). Protein concentrations were determined with Bradford reagent (BioRad). The protein extracts were resolved by SDS-PAGE with subsequent transfer onto Protran Nitrocellulose membrane (Whatman). The proteins were detected (ECL system from Amersham) with rabbit anti-OBF1, mouse anti-Actin, mouse anti-GFP, and rabbit anti-Syk.
For protein quantification the Odyssey detection (LI-COR Biosciences) was used as the manufacturer's protocol.

ELISA

96-well microplates were coated over night at 4°C with DNP-BSA (5µg/ml in PBS). After extensive washing with PBS the microplate was blocked 1 hour with ELISA buffer (4% BSA, 0.2% Tween20 in PBS). After extensive washing 10% serial dilutions of serum samples in ELISA buffer were incubated for 2 hours at room temperature.

The serum was removed by extensive washing and alkaline phosphatase-labeled anti-m-IgM, anti-m-IgG or anti-m-IgG1,2a,2b,3 antibodies (1:2000, at room temperature for 2 hours) were used as developing reagents. After extensive washing, substrate buffer (100 mg/ml nitrophenylphosphate, 0.1 g/l $MgCl_2x6H_2O$, 10% diethanolamine pH 9.8) was used to reveal bound antibodies. The plates were analyzed on an ELISA reader at 405 nm. All antibodies were from Southern Biotech Associates (Birmingham, AL).

Immunocytohistochemistry

Spleen cryosections were prepared from immunized mice. Germinal Center formation was detected by double staining with PE-coupled anti-B220 and biotinylated lectin PNA that was revealed with streptavidin-Alexa-488.

***In vitro* proliferation assay of mature B cells**

The naïve mature B cells from the spleen were negatively separated with CD43 microbeads following the manufacturer's protocol (Miltenyi Biotec). The cells were then stained with 5μM CFSE for 10 min. in warm serum free RPMI medium. The reaction was stopped by adding one volume of FCS. The cells were then washed twice with RPMI medium supplemented with FCS. The cells were stimulated for 5 days with anti-CD40 2μg/ml and IL4 10ng/ml.

Immunofluorescent staining and flow cytometry (FACS) analysis

FACS analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA). Cell sorting was performed on a MoFlo (DakoCytomation).
Biotinylated peanut agglutinin (PNA-biotin, Vector Lab) was used for immunohistochemistry.
FITC-, PE-, APC-, or biotin-conjugated monoclonal antibodies (mAb) specific for B220, CD19, CD22, IgM, and NK1.1 were purchased from Pharmingen (BD Biosciences), San Diego, CA. Anti-CD117-APC was purchased from e-Bioscience (San Diego, CA). Anti-CD93 (PB493/AA4.1), anti-IgM and anti-IgD antibodies were purified from the hybridoma supernatant and labeled with biotin in our laboratory by standard methods.
For EPLM cell sorting erythrocyte-depleted bone marrow cells were stained in IMDM 2% FBS with saturating concentrations of anti-B220-PECy7, anti-CD19-PE and anti-CD117-APC antibodies. After 30 min. incubation at 4°C, the cells were washed, filtered and resuspended at ~20 x 10^6 cells/ml in PBS 2% FBS before sorting. For cell sorting, a FACS Aria (BD Biosciences) was used.

EPLM cell culture

OP9 mouse stromal cell line was maintained and expanded as described before in IMDM supplemented with 50μM β-mercaptoethanol, 1 mM glutamine, 0.03% w/v primatone (Quest, Naarden, The Netherlands), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2% FBS (Balciunaite *et al.*, 2005; Ceredig *et al.*, 2006). OP9 stromal cells were plated 2 days before the addition of sorted EPLM cells. OP9 cells were γ-irradiated with 3000 rad at semi-confluency. Then culture medium was replaced by fresh medium supplemented with 100 U/ml IL-7.

Cell cycle assay

The preBI cell cultures were pulsed with 1 mM BrdU for 45 min. Then the cells were stained with anti-BrdU-FITC and 7AAD as the manufacturer's protocol (BD Pharmingen).

RNA preparation and hybridization to Affimetrix Microarrays

Cells were FACS sorted and RNA was purified with RNeasy Microkit from Qiagen. Total RNA (~50 ng) from each replicate was reverse transcribed and labeled using the Affymetrix 2-cycles labeling kit according to manufacturer's instructions. Biotinylated cRNA was fragmented by heating with magnesium (as per the Affymetrix instructions) and this fragmented cRNA was hybridized to Mouse 430v2 GeneChips (Affymetrix, Santa Clara, Calif.). Data were analyzed using Expressionist (Genedata AG). The normalized data were subjected to a Student *t*-test ($P < 0.01$) and were required to have a median fold change of at least 1.5.

Results

Generation of BAC transgenic mice expressing single OBF-1 isoforms

The main approach to dissect the function of the OBF-1 isoforms *in vivo* was to establish BAC (Bacterial Artificial Chromosome) transgenic mice that can express only one of the isoforms. The use of BACs was chosen, because they offer several advantages for functional genomics. They can carry sufficient DNA to encompass most eukaryotic genes, including their *cis*-acting regulatory elements, as well as many eukaryotic gene clusters in a single molecule. The BAC containing the mouse *OBF-1* gene was modified by "ET cloning" (Muyrers et al., 1999; Zhang et al., 2000; Muyrers et al., 2004) following the Gene Bridges© protocol.
We used a WT BAC, which expresses all OBF-1 isoforms, as well as a BAC where the CTG start codon has been mutated so that only the p34 isoform is expressed from it. The ATG start codon was also mutated in another BAC to restrict the OBF-1 expression to the p35 isoform. In addition a GFP reporter BAC was constructed to follow the expression of the *OBF-1* gene: *EGFP* was inserted just after the ATG start codon so that the *OBF-1* gene is inactivated and GFP is transcribed in the same way as OBF-1 (Fig. 1a). The mouse founders containing the different BACs were crossed with *OBF-1*$^{-/-}$ mice and the progeny was intercrossed to obtain BAC transgenic mice in an *OBF-1*$^{-/-}$ background. The resulting transgenic mice containing the WT BAC, the CTG mutated BAC, the ATG mutated BAC and the GFP BAC in the KO background were called "*BAC WT*", "*BAC p34*", "*BAC p35*" and "*BAC GFP*" respectively. For all practical purposes of our analyses *BAC GFP* mice are equivalent to *OBF-1*$^{-/-}$ mice.
Western blot of splenocytes from the different BAC transgenic mouse lines was performed to make sure the cells have the expected OBF-1 expression profile (Fig. 1b). In the *BAC WT* mice the two main bands correspond to the p34 and p35 isoforms. In the *BAC p34* mice the CTG start codon was effectively inactivated, since only the p34 band was visible. On the other hand OBF-1 was very weakly expressed in the *BAC p35* mice, therefore this BAC mouse line was not investigated in detail. The *BAC GFP* mouse line expressed GFP only and lacked OBF-1 protein expression, as expected. Total splenic B cells were purified with CD19 microbeads to quantify the OBF-1 expression (Fig. 1c).

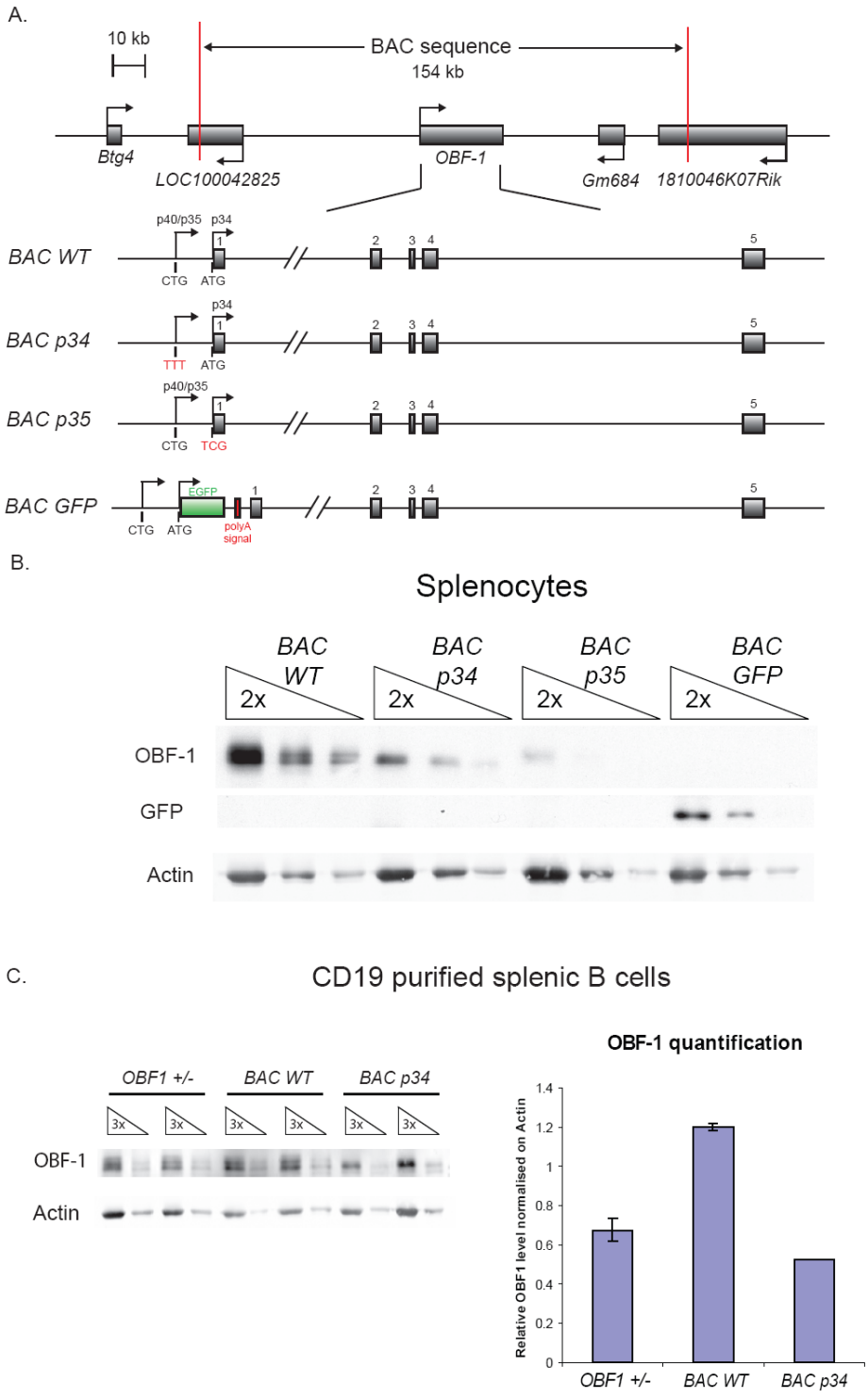

Figure 1: The *BAC WT*, *p34* and *GFP* transgenic mice have the expected expression pattern

(A) The BAC is constituted of a 154 kb mouse genomic part containing the *OBF-1* gene. *LOC100042825*, *Gm684* and *1810046K07Rik* are putative genes derived by automated computational analysis. A schematic representation of the mutations and insertions in the BAC transgenic mice is depicted. The 5 exons in the *OBF-1* gene are numbered. (B) Western blot of the splenocytes from *BAC WT*, *BAC p34*, *BAC p35* and *BAC GFP* transgenic mice. (C) Western blot of purified naïve mature B cells from *OBF-1*$^{+/-}$, *BAC WT* and *BAC p34* mice. Quantification of the OBF-1 level was determined with Odyssey system. OBF-1 and Actin were detected with Alexa 680 and 800 respectively. Shown values are the mean relative OBF-1 protein level ± SE of two individual mice of each genotype.

The B cells from *BAC WT* mice expressed about 2 times more OBF-1 than *OBF-1*$^{+/-}$ and *BAC p34* mice. Therefore the *BAC WT* mice expressed OBF-1 like *OBF-1*$^{+/+}$ mice. In the bone marrow OBF-1 was detected in none of the mouse lines, as the OBF-1 expression was below the detection level (data not shown).

The germinal centers and the immune response are normal in the *BAC p34* mice

The lack of germinal centers (GCs) and impaired immune response constitute the most striking phenotype of *OBF-1* knock out mice (Schubart et al., 1996; Kim et al., 1996). DNP-KLH induces a T-dependent immune response associated with the formation of GCs. BAC transgenic mice were immunized with DNP-KLH/Alum *i.p.* to investigate whether mice expressing only the nuclear OBF-1 isoform form GCs normally 10 days later. As shown in Fig. 2a, in the *BAC p34* mice germinal centers were absolutely normal in terms of number and size. As expected, the *BAC GFP* mice were not able to generate germinal centers (Fig. 2a).

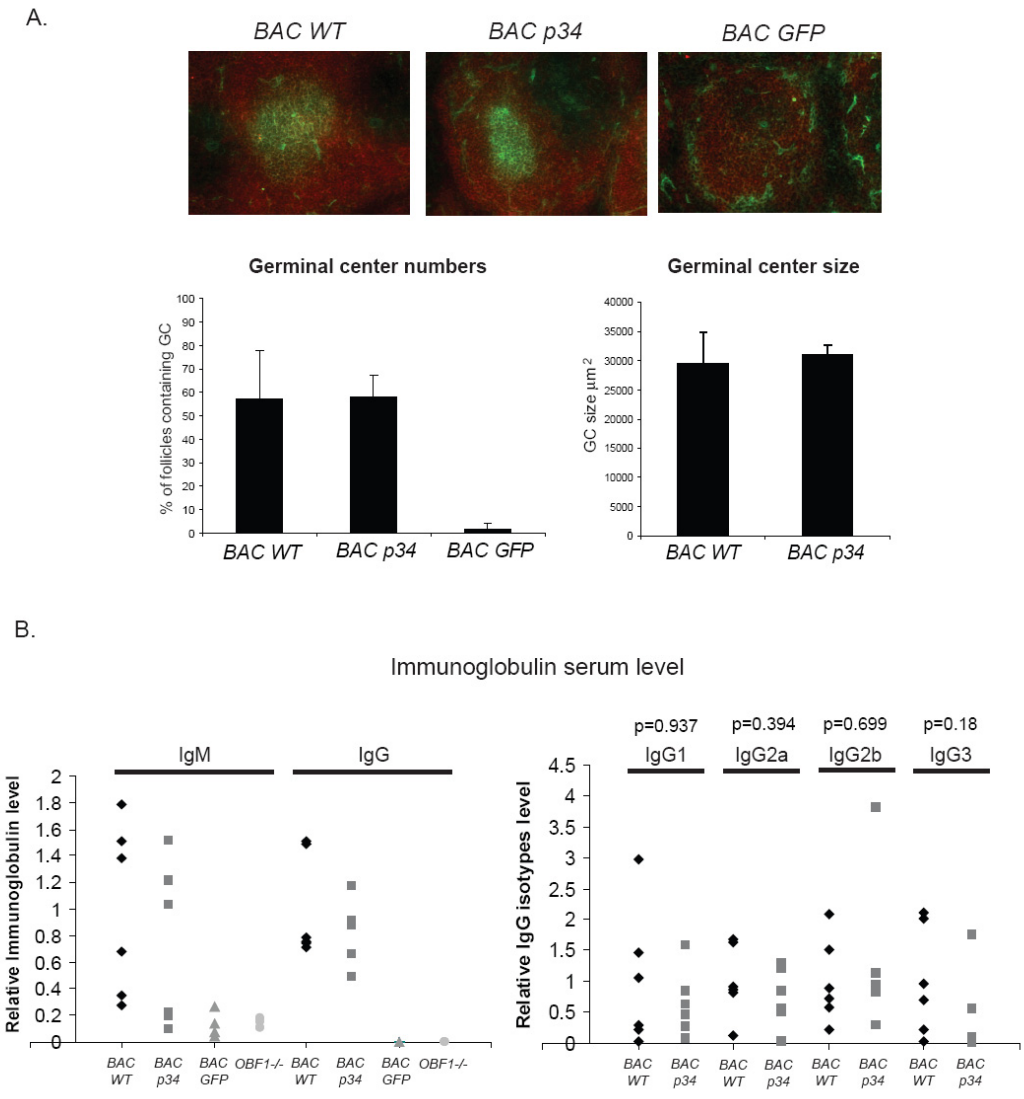

Figure 2: p34 is sufficient to rescue the germinal centers and immune response
(A) Germinal center immunostaining. *BAC WT*, *BAC p34* and *BAC GFP* transgenic mice were immunized with DNP-KLH/Alum and the spleens were processed after 10 days. Spleen

cryosections were stained with PNA-biotin/SA-Alexa466 (green) and with IgM-PE (red). The percentage of Follicles containing Germinal Centers was calculated. The size of the Germinal Centers of *BAC WT* and *BAC p34* mice was measured with ImagePro software. Shown values are the mean percentage and absolute number ± SE of three and five individual mice respectively. (B) T-dependent immune response. *BAC WT*, *BAC p34*, *BAC GFP* and *OBF-1*$^{-/-}$ mice were immunized with DNP-KLH/Alum and serum antigen-specific IgM and IgG titers were analyzed after 10 days. Six mice from each genotype were used. Serum antigen-specific IgG isotypes titers were analyzed for the *BAC WT* and *BAC p34* mice. The p values were calculated using the Wilcoxon rank sum test.

The antigen specific IgM and IgG serum levels were compared with immunized *OBF-1*$^{-/-}$ mice to investigate whether the antibody secretion was impaired. The immunoglobulin levels for the BAC p34 mice were the same as the BAC WT mice and the immune response of the BAC GFP mice was impaired to the same extend as the *OBF-1*$^{-/-}$ mice (Fig. 2b). Although the IgG level was not impaired in the BAC p34 mice, there was still a possibility that the level of some IgG isotypes could be affected. Therefore the different antigen specific isotypes levels were measured, however no difference was detected between the BAC WT and the BAC p34 sera (Fig. 2c).

Transitional B cells and Syndecan1 expression level are normal in the spleen of *BAC p34* mice

Another aspect of the *OBF-1* knock out phenotype in the spleen is a decreased Transitional B cell number (Schubart et al., 2000; Hess et al., 2001). This Transitional B cell defect might be one cause for the lack of germinal centers in knock out mice. The Transitional B cells (immature B cells) and the mature B cells are CD93 positive and negative respectively. Downregulation of the Transitional B cells was also observed in the *BAC GFP* mice, and the *BAC p34* mice had a largely normal Transitional B cell population (Fig. 3).
Syndecan1 is usually used as a marker for Plasma cell differentiation (Kopper and Sebestyen, 2000), but is also expressed at various levels in developing B cells (Sanderson et al., 1989). Expression of Syndecan1 was recently reported to be upregulated in *OBF-1*$^{-/-}$ mice (Corcoran et al., 2005). As expected, the Syndecan1 upregulation on the mature B cells of the *BAC GFP* mice was clearly visible. The percentage of Syndecan1 positive cells was the same between the *OBF-1*$^{+/-}$ and *BAC p34* mice, however there were moderately fewer cells in the *BAC WT* mice (Fig. 3).
Interestingly, in the *BAC GFP* mice, GFP expression was low in Transitional B cells and increased in mature B cells with a maximum in Syndecan1 positive cells (Fig. 3). This GFP expression profile was identical in *BAC GFP* transgenic mice in an *OBF-1*$^{+/+}$ or *OBF-1*$^{-/-}$ background indicating that it is not influenced by the presence of OBF-1 (data not shown).
The decreased number of immature B cells is due to increased apoptosis and impaired splenic seeding of these cells (Schubart et al., 2000; Hess et al., 2001). To verify that the B cell ontogeny was not impaired in the *BAC p34* mice we performed *in vivo* BrdU labeling following the procedure of Hess et al., (Hess et al., 2001). As expected, the *BAC GFP* as well as *OBF-1*$^{-/-}$ mice had significantly less BrdU positive splenic mature B cells than the *BAC WT* and *BAC p34* transgenic mice (data not

shown). Therefore the *BAC p34* mice have a normal rate of appearance of B cells in the periphery.

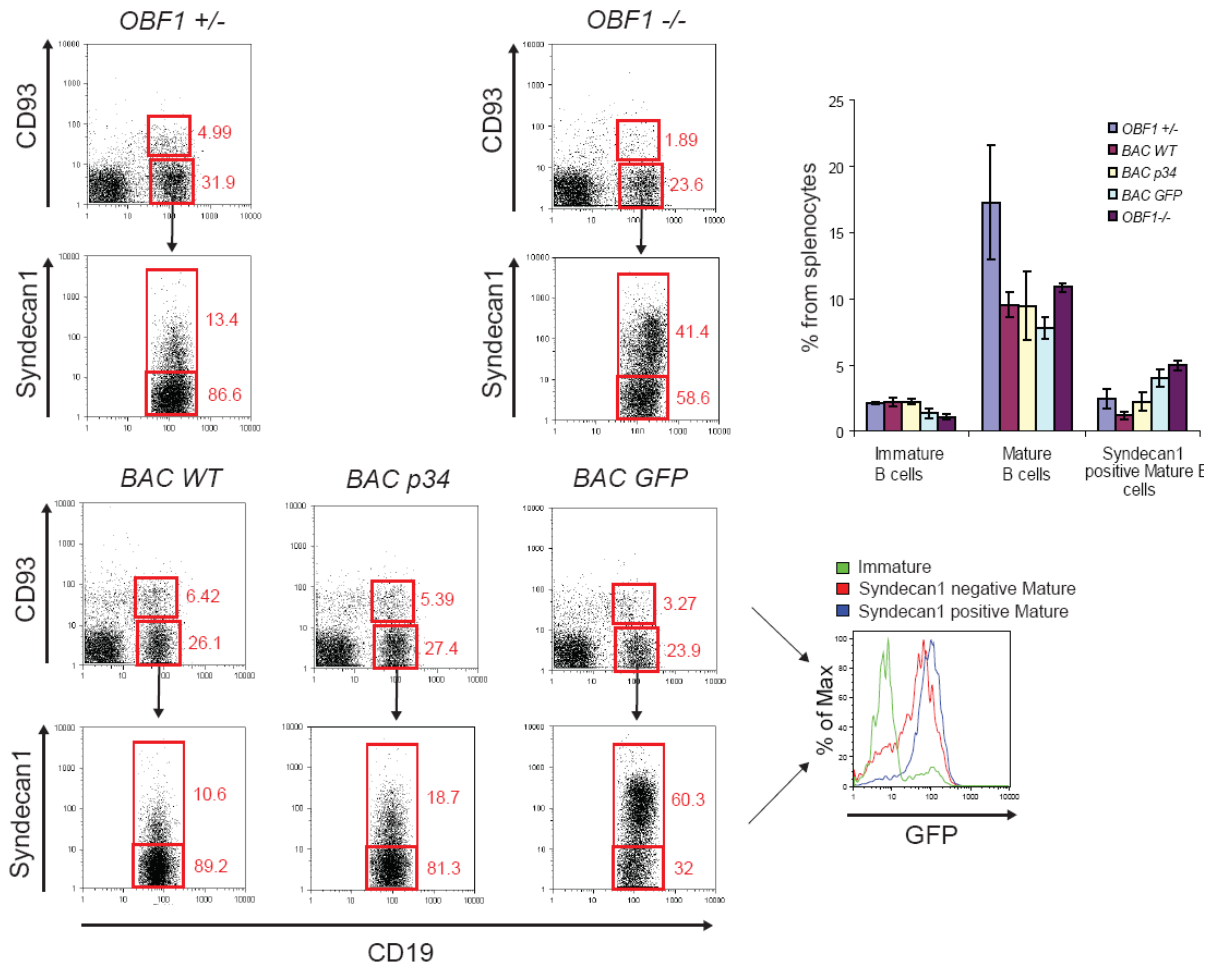

Figure 3: p34 can rescue the splenic immature B cells and the misregulated Syndecan1 profile
FACS analysis of splenocytes. The percentage of immature, mature and Syndecan1 positive mature B cells from total splenocytes is presented. Shown values are the mean percentage and absolute number, ± SE, of three individual mice. Single cell suspensions were stained with antibodies against the indicated markers and representative dot plots are presented. Biotinylated anti-CD93 in combination with streptavidin-PE-Cy5.5, anti-CD19-APC, and anti-Syndecan1-PE antibodies were used. The immature B cells are $CD19^+$ $CD93^+$, the mature B cells are $CD19^+$ $CD93^-$. Syndecan1 profile from the mature B cell gate is depicted. For the *BAC GFP* mice, a GFP expression histogram of immature, mature and Syndecan1 positive mature B cells is illustrated.

The *in vitro* differentiation of mature B cells from *BAC p34* mice is normal

As neither the immune response (Fig.2) nor the composition of the splenic B cell populations (Fig. 3) were impaired in the *BAC p34* mice we investigated whether the *in vitro* Plasma cell differentiation was affected. It has been reported previously that *in vitro* B cell differentiation of *OBF-1*$^{-/-}$ mice is impaired upon α-CD40 and IL-4 stimulation (Corcoran et al., 2005). Under these culture conditions the cells accumulated intermediate level of Syndecan1 due to a block after this differentiation stage. Indeed the *OBF-1*$^{-/-}$ cells are unable to initiate the genetic program for plasma

cell differentiation: *Blimp-1/prdm1* induction fails, and *bcl-6*, *Pax5*, and *AID* are not properly repressed.
In vitro plasma cell differentiation experiments were therefore performed to test whether p34 is sufficient to drive complete Plasma cell differentiation. Naïve mature B cells from the different transgenic mice were purified with CD43 microbeads. The purified cells from *BAC GFP* and *OBF-1*$^{-/-}$ mice maintained the Syndedan1 overexpression phenotype observed *ex vivo* and all the cells from BAC GFP mice were positive for GFP expression (Fig. 4a).

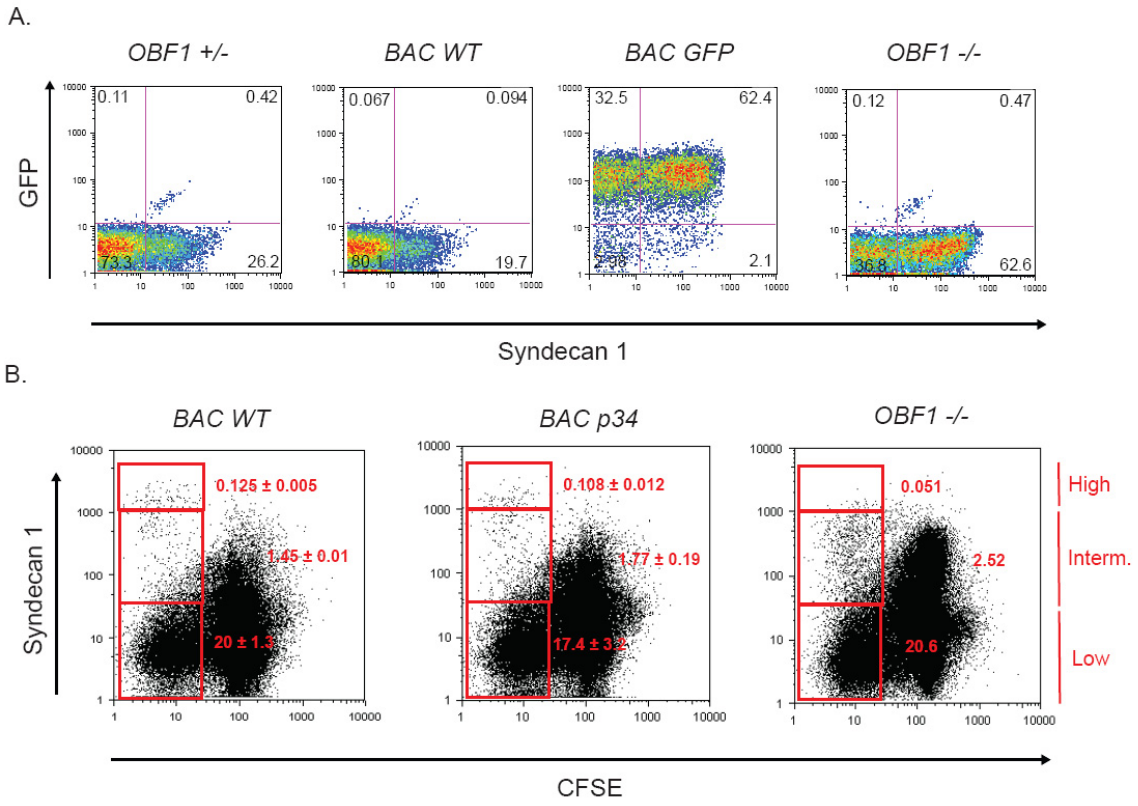

Figure 4: p34 fully rescues *in vitro* plasma cell differentiation
(A) FACS analysis of naïve mature B cells. Naïve mature B cells were negatively purified with CD43 microbeads from total spleen. The purified cells were stained with α-Syndecan1-PE antibody. (B) 5 days stimulation. The purified mature B cells were CFSE stained and cultured for 5 days with α-CD40 and IL-4. The cells were then stained with α-Syndecan1-APC antibody. The gates represent the different levels of Syndecan1 expression in proliferating cells. Shown values are mean percentage ± SE, of living cells from two individual mice per genotype.

The cells were then stained with the cell division-tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE) and stimulated for 5 days with α-CD40 and IL-4. This culture system allows monitoring the rate of proliferation and Plasma cell differentiation. The cells that did not divide much (high CFSE signal) displayed the same Syndecan1 profile as day 0 respectively: normal Syndecan1 level on cells from *BAC WT* and *BAC p34* mice, and increased Syndecan1 expression on cells from *OBF-1*$^{-/-}$ mice. Conversely, the *OBF-1*$^{-/-}$ phenotype described by Corcoran et al. was clearly visible in the cells that proliferated strongly (low CFSE). Indeed the cultures from *BAC WT* and *BAC p34* mice yielded significant numbers of Syndecan1^{high} cells, and there was a clear deficit of Syndecan1^{high} cells in the culture from *OBF-1*$^{-/-}$ mice

that marked the Plasma cell population. Furthermore, the number of cells from *OBF-1*$^{-/-}$ mice expressing an intermediate Syndecan1 level was significantly increased (Fig. 4b). Therefore, expression of only p34 in these cells is sufficient to fully rescue the differentiation defect under these culture conditions.

The B cell populations are normal in the bone marrow of *BAC p34* mice

The bone marrow B cell populations can be subdivided into pro and preB cells ($B220^+$, IgM^-), immature B cells ($B220^{low}$, IgM^+), Transitional B cells ($B220^+$, IgM^{high}), and recirculating mature B cells ($B220^{high}$, IgM^+). The numbers of transitional and mature B cells have been previously reported to be reduced in *OBF-1* knock out mice (Schubart et al., 2000; Hess et al., 2001).
The BAC transgenic mice were compared to the *OBF-1*$^{+/-}$ and *OBF-1*$^{-/-}$ mice to make sure that the Transitional B cells from the *BAC p34* mice were effectively normal (Fig. 5a). Indeed the bone marrow B cell populations from *BAC WT* and *BAC p34* mice were similar to the *OBF-1*$^{+/-}$ mice, and the numbers of transitional and mature B cells from the bone marrow of *BAC GFP* mice were decreased like those of the *OBF-1*$^{-/-}$ mice (Fig. 5a). The results show that p34 can fully rescue the decreased number of transitional and mature B cells in *OBF-1*$^{-/-}$ mice (Fig. 5b).
In line with GFP expression in splenic Transitional B cells of *BAC GFP* mice (Fig. 3), the bone marrow B cell populations are partially expressing GFP. During B cell ontogeny GFP was slightly upregulated starting with 70% GFP positive pro and preB cells up to 90% GFP positive Transitional B cells (data not shown). Therefore GFP was significantly expressed in Transitional B cells in the bone marrow, and then expressed at a lower level in splenic Transitional B cells. When the cells differentiated into mature B cells in the spleen, GFP was expressed again. Obviously GFP was transiently downregulated when the Transitional B cells entered the spleen (Fig. 3).
CD22 is a B lineage adhesion receptor and undergoes a steady increase of expression during early B cell development. CD22 is known as a negative regulator of B cell receptor signaling (Nitschke et al., 1999). In fact CD22 was reported to be upregulated in the Transitional B cells of *OBF-1* knock out mice suggesting that it might impair BCR signaling, which results in decreased number of Transitional B cells (Samardzic et al., 2002). Therefore the Mean Fluorescence Intensity (MFI) of CD22 in each bone marrow B cell populations was measured to confirm the notion that p34 can rescue the decreased number of bone marrow Transitional B cells in mice deficient for OBF-1. In agreement with Samardzic et al. the *OBF-1*$^{-/-}$ mice had a moderately increased surface expression of CD22 on the Transitional B cells. As expected, the Transitional B cells from *BAC WT* and *BAC p34* mice had a normal CD22 expression (Fig. 5c).

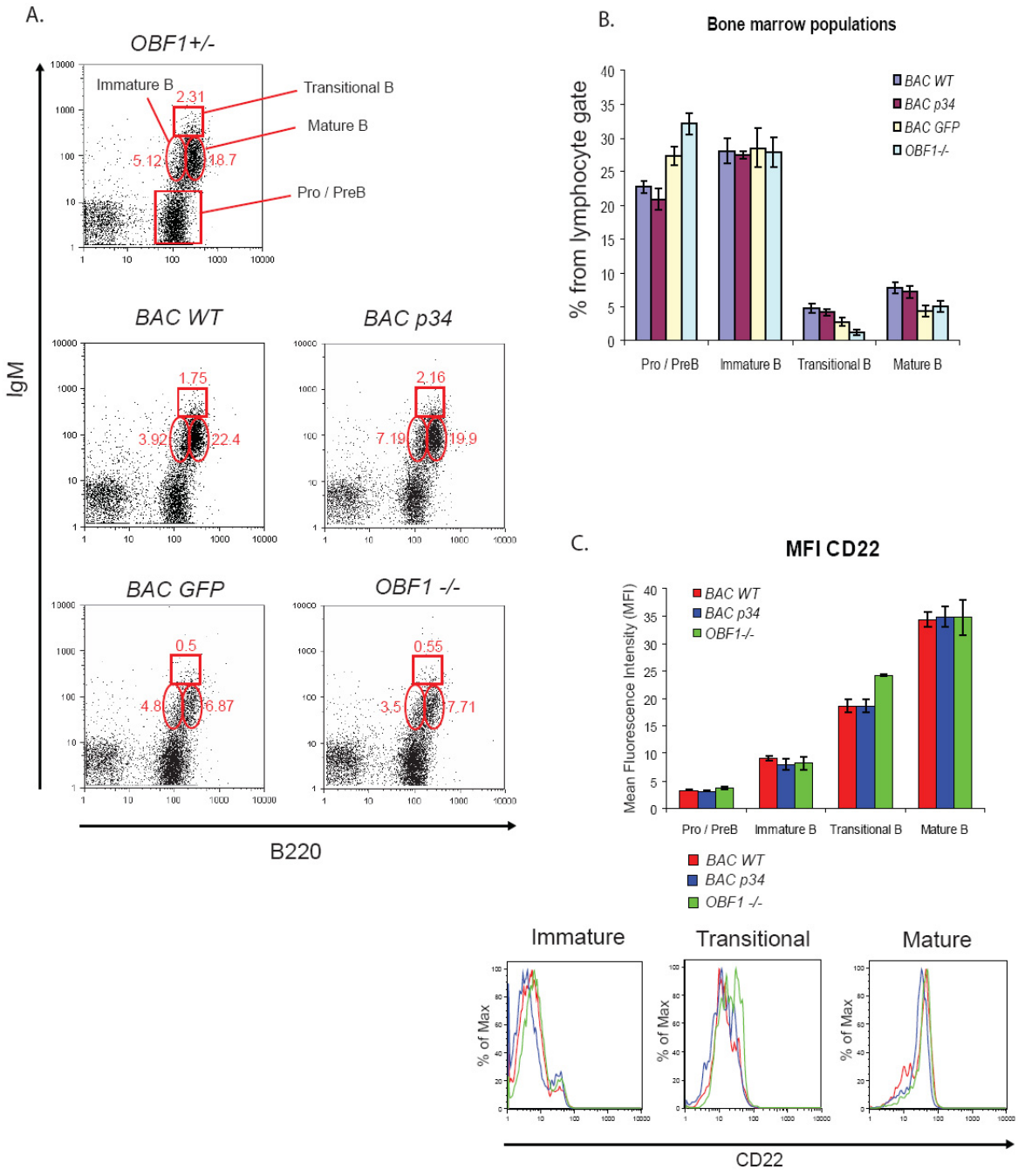

Figure 5: p34 can rescue the bone marrow transitional B cells
(A) FACS analysis of bone marrow cells. Single cell suspensions of bone marrow were stained with anti-B220-APC and anti-IgM-PE antibodies and representative dot plots are presented. (B) The percentage of pro and preB, immature, transitional and recirculating mature B cells is presented. Shown values are the mean percentage from the lymphocyte gate ± SE, of three individual mice. (C) CD22 expression profile. The diagram shows Mean Fluorescence Intensity (MFI) of CD22 for each of the bone marrow populations. Shown values are average MFI ± SE of three individual mice (upper panel). Representative histograms for CD22 intensity are illustrated (lower panels).

The early B cell populations of the *BAC p34* mice are impaired *in vitro*

We wanted to know at which stage the cells start to express GFP. The ProB cell compartment ($B220^+$, c-Kit^+) are mostly CD19 positive, which is a marker for commited B cells (Rolink et al., 2000). However a fraction of this population contains cells that are CD19 negative and were reported to be early progenitors with lymphoid and myeloid potential (so-called EPLM cells) (Rolink et al., 2006). Interestingly GFP started to be expressed in the preBI cells when CD19 was upregulated (Fig. 6a). At this stage only about 50% of the cells expressed GFP. Then GFP is upregulated when the cells differentiate into preBII cells, as about 70% of preB cells were GFP positive (data not shown).
The ProB cells of *OBF-1* knock out mice were reported to proliferate better *in vitro*, potentially due to a decreased Syk level (Siegel et al., 2006). In IL-7 cultures the EPLM cells express very rapidly CD19 (Fig. 6b) and pro/preB cell surface markers (Balciunaite et al., 2005). *In vitro* cultures of EPLM and preBI cells were performed to investigate the contribution of the p34 isoform in rescuing the hyperproliferation of the cells lacking OBF-1. Syk expression level was monitored by Western blot of EPLM cell cultures derived from the different BAC transgenic mice. Syk level was decreased in cells from *BAC p34*, *BAC GFP* and *OBF-1*$^{-/-}$ mice (Fig. 6c). As expected, EPLM cultures from *BAC WT* or *BAC p34* mice expressed OBF-1 by Western blot (data not shown) in line with the notion that the *OBF-1* promoter (monitored by GFP expression *in vivo*) is activated when the cells express CD19 (Fig. 6a).
The primary $CD19^+$ ProB cells (called preBI cells) were plated on OP9 feeders to investigate the proliferation potential of each genotype *in vitro*. The cells from the *OBF-1*$^{+/-}$ and *BAC WT* mice proliferated normally, however the cells from the *BAC p34*, *BAC GFP* and *OBF-1*$^{-/-}$ mice had a stronger proliferation (data not shown), which correlated with the observations of Siegel et al. (Siegel et al., 2006). To confirm these proliferation differences, the preBI culture cells were labeled for 45 minutes with BrdU. The cell cycle of each genotype was monitored with anti-BrdU and 7AAD. As expected, the number of cells in S phase from the *BAC GFP* and *OBF-1*$^{-/-}$ mice was higher confirming their hyperproliferation. Interestingly the number of cells in S phase from the *BAC p34* mice was intermediate between the cells deficient for OBF-1 and the cells expressing both OBF-1 isoforms (Fig. 6d), suggesting that p34 may not be able to fully correct the hyperproliferation. As observed *ex vivo*, the preBI culture cells expressed GFP (Fig. 6e). One can also notice that CD19 expression is relatively stronger in preBI than in EPLM culture cells (Fig. 6b and 6e).

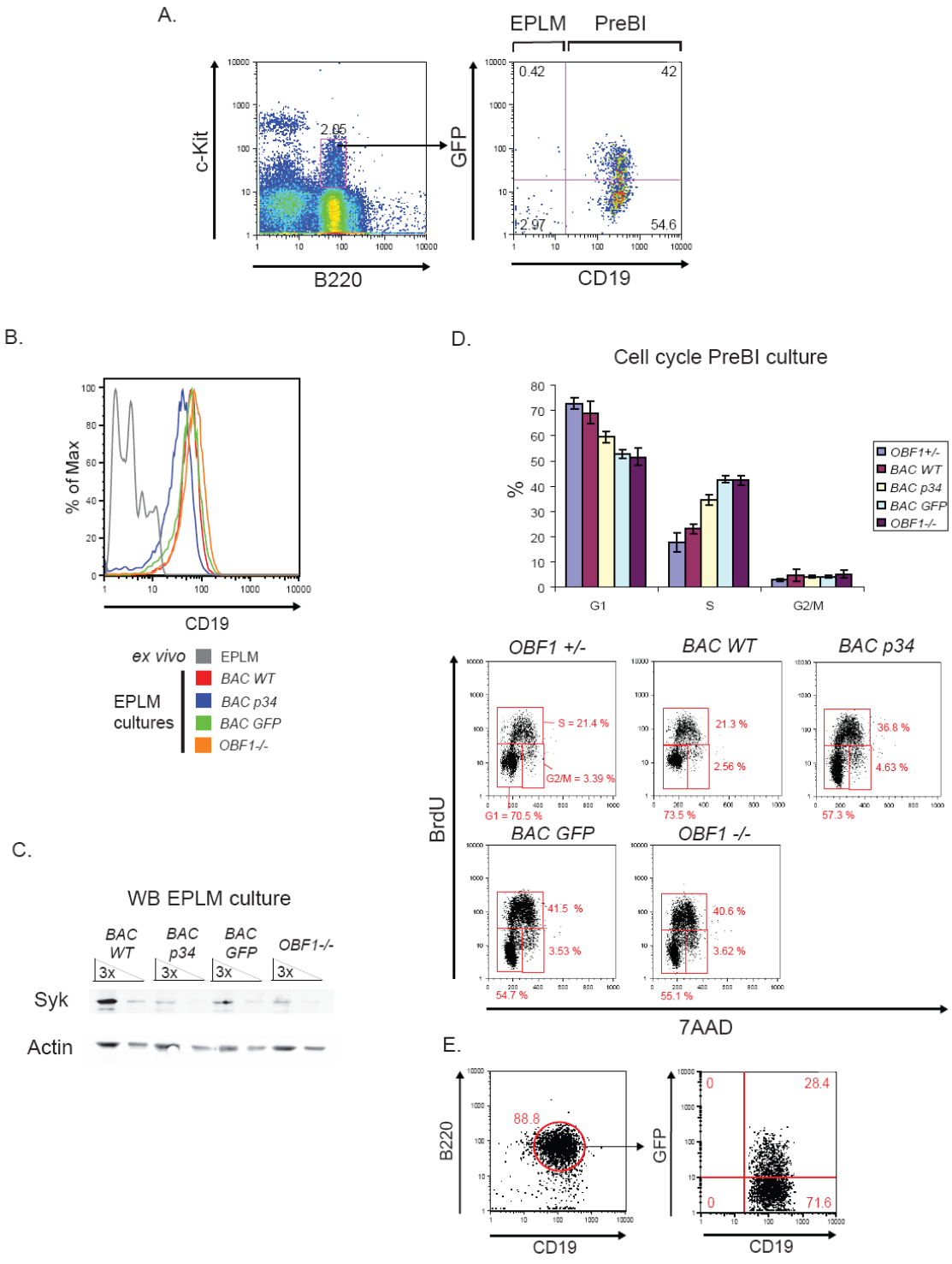

Figure 6: p34 cannot fully rescue the *in vitro* proliferation of preBI cells
(A) FACS analysis of ProB cells. Single cell suspension of bone marrow from *BAC GFP* mice was stained with anti-B220-biotin/SA-PE-Cy5.5, anti-cKit-APC, and anti-CD19-PE antibodies. The ProB cell population is B220/cKit positive. Within this gate the EPLM and preBI cells are $CD19^-$ and $CD19^+$ respectively. (B) *In vitro* differentiation assay of cells from BAC transgenic mice. EPLM cells were cultured with IL-7 on OP9 feeder cells. The cells were stained with anti-CD19-PE antibody. The *ex vivo* EPLM cells were compared with the cells cultured under IL-7 condition. (C) WB of Syk and Actin expression in EPLM cultures. (D) Cell cycle assay of preBI cells. After 8 days culture the preBI cells were pulsed with BrdU for 45 min. and stained with anti-BrdU-FITC antibody and 7AAD. The G1, S and G2/M phases were calculated from

the BrdU/7AAD profile. Shown values represent mean ± SE of 2 samples per genotype. Representative dot plots for each genotype are presented. (E) FACS analysis of preBI cells from *BAC GFP* mice after 8 days culture. The cells were stained with anti-CD19-PE and anti-B220-APC antibodies.

Most of the OBF-1 regulated genes are under the control of the p34 isoform

Phenotypically the *BAC p34* mice appear very similar to the *BAC WT* mice. A number of genes have been previously identified as being OBF-1 dependent such as *Kcnn4*, *Lck* and *Spi-B* (Teitell, 2003; Kim et al., 2003; Bartholdy et al., 2006). However it is clear that the identified OBF-1 regulated genes do not explain all the different aspects of the *OBF-1* knock out phenotype.
Two independent microarray experiments with different mouse lines were performed to identify which genes are dependent on OBF-1. First 380 misregulated genes in total splenic B cells between *BAC WT* and *BAC GFP* mice were identified. Then we identified 352 misregulated genes in splenic mature B cells between *OBF-1*$^{+/+}$ and *OBF-1*$^{-/-}$ mice. The misregulated genes in the first experiment were compared with those of the second experiment (Fig. 7a). We identified 88 common genes between both experiments and called them OBF-1 dependent genes. Using the hypergeometric distribution, a p value of 9.027e-72 was calculated for the group containing these 88 OBF-1 dependent genes. We conclude that the gene lists from the respective experiments are strongly associated, even though there are differences in their design and experimental conditions. Therefore the 88 common genes are the ones most likely to be misregulated due to the deletion of OBF-1 (Fig. 7a).
We have then analysed the microarray data of total splenic B cells from *BAC p34* mice to investigate whether the p34 isoform could rescue all these misregulated genes. The p34 dependent genes were identified by comparing the OBF-1 dependent genes with the misregulated genes in total splenic B cells between *BAC p34* and *BAC GFP* mice (Fig. 7b).
The genetic difference between the *BAC p34* and *BAC WT* mice is the expression of the p35 isoform. Therefore the p35 dependent genes were identified by comparing the OBF-1 dependent genes with the misregulated genes in the total splenic B cells between *BAC WT* and *BAC p34* mice (Fig. 7b).
Within the 88 OBF-1 dependent genes, 39 and 33 genes can be considered as fully and partially rescued by the p34 isoform respectively and were called p34 specific genes. On the other hand only 7 genes were not rescued by p34 and thus can be considered as p35 specific (Fig. 7b).
The expression values of the OBF-1 dependent genes from the data sets considered previously were compared with expression values of bone marrow mature B cells from BAC transgenic mice (Fig. 7c). The p34 and p35 specific genes were clustered in a heat map with their respective color code (blue and orange).

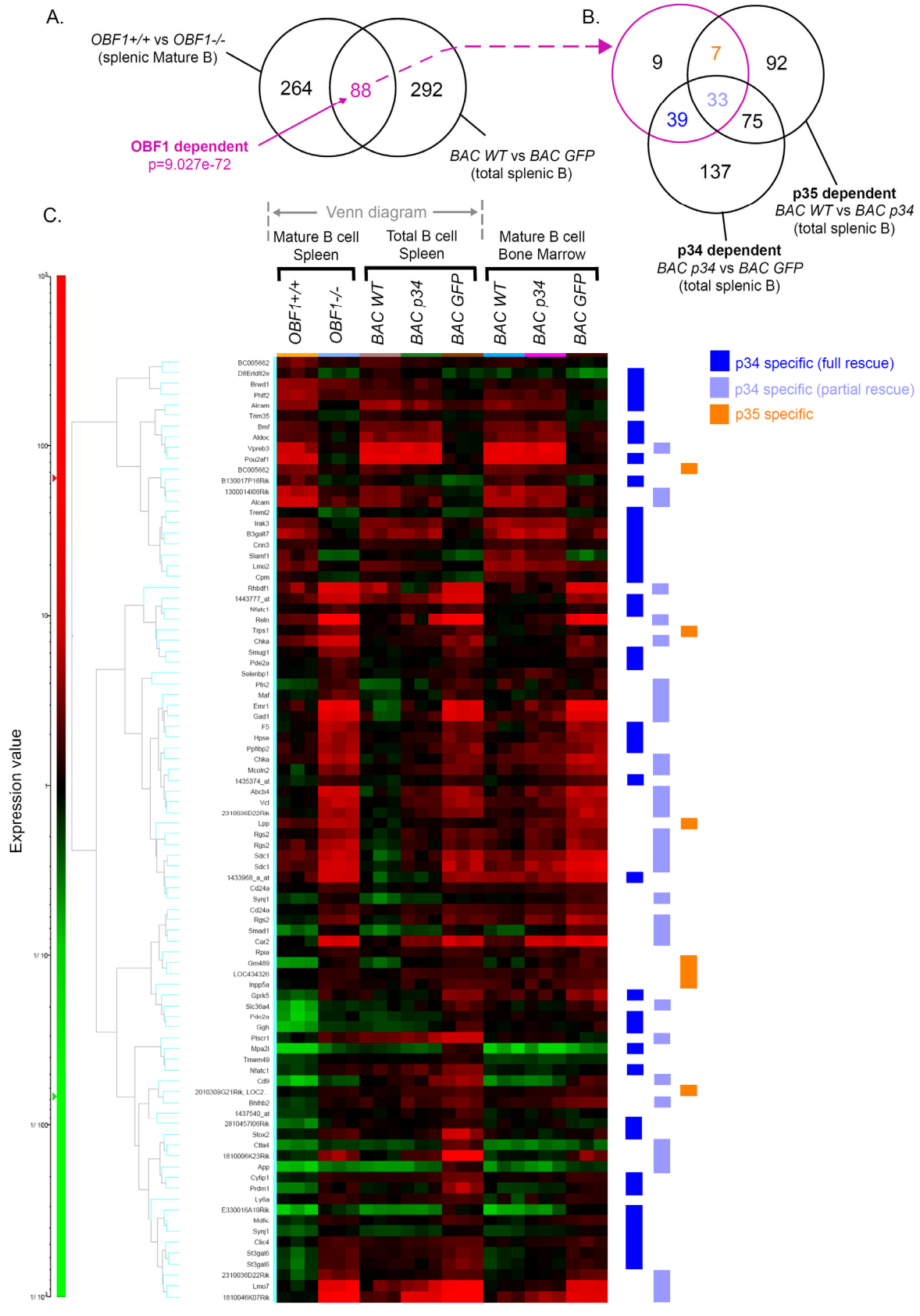

Figure 7: Microarray analysis of *OBF-1*$^{+/+}$, *OBF-1*$^{-/-}$ and BAC transgenic mice
The splenic mature B cells were FACS sorted with anti-B220-APC, anti-IgM-FITC and anti-IgD-biotin antibodies, followed by Streptavidin-PE. The total splenic B cells were FACS sorted with anti-B220-APC antibody. The bone marrow mature B cells were FACS sorted

with anti-B220-APC and anti-IgM-biotin antibodies, followed by Streptavidin-Cy5.5. For every cell population three individual mice were used. The microarray data were analyzed with the Expressionist program; genes with a fold median change of at least 1.5 and a p value of at least 0.01 were considered misregulated. (A) Identification of OBF-1 dependent genes. The splenic mature B cells from *OBF-1*$^{+/+}$ and *OBF-1*$^{-/-}$ mice and the total splenic B cells from *BAC WT* and *BAC GFP* mice were FACS sorted to perform microarray analysis. The respective misregulated genes were compared with a Venn diagram. The p value of the group containing the OBF-1 dependent genes was calculated using the hypergeometric distribution. (B) Identification of the p34 and p35 specific genes. Total splenic B cells from *BAC p34* mice were FACS sorted. The microarray data from this population were compared with the microarray data of *BAC GFP* (p34 dependent genes) and *BAC WT* total splenic B cells (p35 dependent genes). The intersections with the group containing the OBF-1 dependent genes identify the p34 and p35 specific genes respectively. (C) Clustering of the OBF-1 dependent genes. The expression values of the OBF-1 dependent genes from *OBF-1*$^{+/+}$, *OBF-1*$^{-/-}$ and BAC transgenic mice were compared. The p34 and p35 specific genes are in blue and orange respectively.

The list of the p35 specific genes is presented in Table 1.

Table 1:

Accession number	Name, description	Gene Symbol
1451359_at	cDNA sequence BC005662	BC005662
1428719_at	RIKEN cDNA 2010309G21 gene, hypothetical protein LOC207685, similar to Ig lambda-2 chain	2010309G21Rik, LOC207685, LOC547243
1449530_at	trichorhinophalangeal syndrome I (human)	Trps1
1433605_at	inositol polyphosphate-5-phosphatase A	Inpp5a
1454899_at	LIM domain containing preferred translocation partner in lipoma	Lpp
1457707_at	gene model 489, (NCBI)	Gm489
1457728_at	similar to B-cell novel protein isoform 1; BCNP1	LOC434326

Table 1: p35 specific genes
List of the genes that are OBF-1 dependent and are not rescued by p34.

Discussion

Here we present results indicating that the nuclear isoform is responsible for the major roles of OBF-1 *in vivo*. Indeed the nuclear isoform is able to rescue most of the known defects of the *OBF-1*$^{-/-}$ mice and the majority of the OBF-1 dependent genes are under its control. However by looking at the very early stage of B cell ontogeny in the bone marrow, the cytoplasmic OBF-1 isoform appeared to be potentially involved in *in vitro* B cell proliferation.
Point mutations of the respective start codons in the *OBF-1* gene confirmed that the p34 isoform is translated from the ATG start codon and that the p35 isoform is derived from CTG mediated translation (Fig. 1b). The *BAC p35* mice had an impaired OBF-1 expression compared to *BAC p34* mice (Fig. 1b). The efficiency of non-ATG codon initiation is very variable and mutating the ATG start codon might be deleterious for its proper activity as the use of alternative start codons depends on the sequence context and the secondary structure surrounding the codon (Touriol et al., 2003). The *BAC GFP* mice did not express OBF-1 at all; therefore the mouse line

could be used as a negative control (Fig. 1b). In an *OBF-1*$^{+/-}$ mouse the level of p34 and p35 expression is equivalent (Yu et al., 2001). The *BAC p34* mice expressed the same OBF-1 level like *OBF-1*$^{+/-}$ mice suggesting that the CTG mutation did not interfere with the ATG mediated translation. On the other hand the *BAC WT* mice expressed about 2 times more OBF-1 than *OBF-1*$^{+/-}$ mice suggesting that potentially two copies of *BACs* were incorporated in the mouse genome (Fig. 1c).
The main *OBF-1* knock out phenotype is a B-cell immunodeficiency due to lack of Germinal Centers (Nielsen et al., 1996; Schubart et al., 1996; Kim et al., 1996). Syk is a central tyrosine kinase in the BCR signaling, and p35 was reported to modulate its stability, thus potentially regulating BCR signaling (Siegel et al., 2006). Furthermore the Ets factor *Spi-B* which modulates BCR signaling was identified as a direct target gene of OBF-1 (Bartholdy et al., 2006). These observations suggested that the lack of p35 might impair the immune response. However the Germinal Center formation and the immune response were absolutely normal in the *BAC p34* mice (Fig. 2a and b). These observations imply that the p34 isoform is the main player in the antigen dependent immune response.
The spleen composition was investigated in more detail to characterize the *BAC p34* mice. The lack of *OBF-1* was reported to decrease Transitional B cell seeding in the spleen (Schubart et al., 2000; Hess et al., 2001). In fact the Transitional B cell population was not impaired in the *BAC p34* mice. Furthermore the Syndecan1 expression profile was the same as *OBF-1*$^{+/-}$ mice. Surprisingly the number of Syndecan1 positive mature B cells from *BAC WT* mice was slightly reduced. The *BAC WT* mice expressed more OBF-1 than *OBF-1*$^{+/-}$ mice, so it seems that there is a direct negative correlation between OBF-1 and Syndecan1 expression (Fig. 3). OBF-1 expression peaks in cycling, low-density splenic B cells (including Germinal Center centroblasts) and can also be induced to high levels by stimulation that mimics T-cell help or bacterial Toll-like receptor 4 (TLR4) responses to lipopolysaccharide (LPS) (Qin *et al.*, 1998; Stevens *et al.*, 2000; Greiner *et al.*, 2000; Schubart *et al.*, 2001). On the contrary OBF-1 is not detected in resting, high-density splenic B cells (naïve B cells, memory B cells and GC centrocytes). In addition the mRNA levels of *OBF-1* are high and in similar amounts in all B cell populations suggesting that there is a posttranslational regulation of OBF-1 protein expression (Tiedt *et al.*, 2001; Boehm et al., 2001). Interestingly GFP expression increased significantly from the resting Transitional B cells to the more active mature B cells, indicating that the translational rate of the *OBF-1* transcript may also be regulated in splenic B cells (Fig. 3). BrdU incorporation in splenic B cells correlated with the Transitional B cell profile (data not shown). These results show that the splenic B cell populations of the *BAC p34* mice are normal, and that p35 is dispensable in the spleen.
The splenic B cell populations and the immune response were not impaired in the *BAC p34* mice. Therefore CFSE labelled mature B cells were stimulated with α-CD40 and IL-4 to mimic T-dependent B cell activation and Plasma cell differentiation *in vitro*, since it was reported that *OBF-1*$^{-/-}$ B cells have an altered response under these culture conditions (Corcoran et al., 2005). After 5 days culture the Syndecan1 profiles of proliferating cells were compared (Fig.4b). In fact the ability to differentiate into Syndecan1 high expressing cells was fully restored in the cells from *BAC p34* mice. Upon α-CD40 and IL-4 stimulation the B cells undergo reprogramming of their transcriptional machinery from a Germinal Center B cell profile till a Plasma cell profile. During this transition the cells upregulate *Irf4* and *Blimp1* and downregulate *Pax5*. In addition *Bcl6* is transiently expressed before the final stage of Plasma cell differentiation (Corcoran et al., 2006; Shapiro-Shelef et al., 2005). Corcoran et al.

showed that the Plasma cell differentiation defect of *OBF-1*$^{-/-}$ B cells *in vitro* was the consequence of an impaired transcriptional reprogramming, which is necessary for this cell type transition (Corcoran et al., 2005). Therefore it makes sense that reconstituting the B cells with a nuclear OBF-1 transcription factor leads to normal Plasma cell differentiation.

The number of bone marrow Transitional B cells is reduced in *OBF-1* knock out mice partly due to increased apoptosis (Hess et al., 2001). The bone marrow B cell populations of the *BAC WT* and *BAC p34* mice were normal and the transitional/mature B cell defect was also observed in the *BAC GFP* mice (Fig. 5a and b). The proportion of GFP positive cells is relatively high even at the pro and preB cell stage indicating that OBF-1 is translated early in B cell ontogeny (data not shown). CD22 is a negative regulator of BCR signaling and was reported to be upregulated in Transitional B cells of *OBF-1* knock-out mice (Hess et al., 2001). Indeed the *BAC p34* mice were able to downregulate CD22 indicating that the molecular link between OBF-1 and CD22 takes place in the nucleus (Fig.5c).

B cell commitment occurs at the ProB cell stage (Rolink et al., 2000). Indeed GFP started to be expressed after the EPLM cell stage indicating that OBF-1 expression correlates with B cell commitment (Fig. 6a). The ProB subpopulations (EPLM and preBI) can be cultivated with IL-7 on OP9 feeder cells and can proliferate for a long time under this culture system (Balciunaite et al., 2005). The EPLM and preBI cells were used to investigate Syk level and to monitor proliferation respectively (Fig. 6).

The EPLM cells in culture upregulate very rapidly CD19 (Fig. 6b), which promotes OBF-1 expression, as observed by Western blot (data not shown). Syk level was reported to be decreased in IL-7 dependent ProB cells from *OBF-1*$^{-/-}$ mice (Siegel et al., 2006). Indeed Syk was slightly downregulated in cells lacking p35 (Fig. 6c). This observation is consistent with previous results (Siegel *et al.*, 2006), although p34 was reported to also rescue Syk level.

In preBI cell culture from *BAC p34* mice the number of cells in S phase was higher than the cells expressing both nuclear and cytoplasmatic OBF-1 isoforms suggesting that p35 might play a role in the proliferation rate of early B cells (Fig. 6d). This observation is consistent with the decreased Syk level (Fig. 6c). Although the notion that OBF-1 could modulate the stability of Syk to slow down ProB cell proliferation *in vitro* (Siegel et al., 2006), it is not clear whether this mechanism plays a role *in vivo*, as the number of ProB cells is normal in *OBF-1*$^{-/-}$ mice.

It was evident that the nuclear OBF-1 isoform could rescue all the *in vivo* defects in the *OBF-1*$^{-/-}$ mice. However it is possible that the cytoplasmic OBF-1 isoform has a more subtle cellular role, which has not been identified yet in mice deficient for OBF-1. Therefore we performed microarray analysis in splenic B cell populations to investigate the contribution of each isoform in the regulation of OBF-1 dependent genes. The analysis started with the identification of the genes that were likely to be dependent on OBF-1. The comparison of two independent sets of microarray data between mice expressing or lacking OBF-1 allowed the characterization of 88 common misregulated genes (OBF-1 dependent genes) with a very stingent p value (Fig. 7a). As expected, *Pou2af1,* which is the name of the *OBF-1* gene, belonged to the OBF-1 dependent genes showing that the microarray analysis is relevant (Fig. 7c).

In fact most of these OBF-1 dependent genes were under the control of the p34 isoform, although about half of these genes were partially rescued by p34. The observation that an important part of the genes were not fully rescued by p34 can have two origins:

-The *BAC WT* mice express twice more OBF-1 than *BAC p34* mice (Fig. 1c) resulting in a stronger transcriptional impact on the p34 specific genes. This mechanism can be illustrated with *Syndecan1* (Fig. 3 and 7c), *Gad1* or *Emr1* (Fig. 7c).
-The p35 isoform can synergize with p34 to induce a normal OBF-1 dependent gene expression. This mechanism can be illustrated with *Pfn2*, *Rgs2* or *Smad1* (Fig. 7c).
Only 7 genes appeared to be independent of p34, and were therefore considered to be p35 specific (Fig. 7b and Table 1). Among these genes, four have an unknown function, and for the three other genes it is not clear what the molecular link with the *OBF-1* knock out phenotype could be. B cell novel protein isoform 1 (*BCNP1*) is a member of the genes with an unknown function and was upregulated in cells deficient for p34 (Fig. 7c). BCNP1 is a putative transmembrane protein and is only expressed in B cells. Interestingly BCNP1 was reported to be overexpressed on the plasma membrane in chronic lymphocytic leukemia (CLL), which is derived from mature B cells (Boyd et al., 2003). In fact OBF-1 was reported to be aberrantly expressed in some mature B cell derived human lymphomas. For example OBF-1 is absent in pre- and post-GC derived lymphomas (Greiner et al., 2000; Pileri et al., 2003) and silenced in the malignant Reed-Sternberg subtype of classical Hodgkin disease, which derives from GC B cells (Saez et al., 2002; Re et al., 2001; Jundt et al., 2002; Hertel et al., 2002; Theil et al., 2001; Stein et al., 2001). Therefore it is possible that some chronic lymphocytic leukemia overexpress BCNP1 due to the lack of cytoplasmic OBF-1 isoform expression in these cells, as *BCNP1* is a p35 specific gene (Fig. 3c and Table 1).
OBF-1 is known as a transcription coactivator (Babb et al., 1997; Luo et al., 1998). Interestingly 75% of the OBF-1 dependent genes were upregulated in cells deficient for OBF-1, suggesting that these genes were indirect OBF-1 target genes (Fig. 7c). In line with this observation, most of the p35 specific genes such as *BCNP1* had a higher expression in cells lacking OBF-1 (Fig. 7c). Furthermore the expression values in the bone marrow mature B cells correlated with the ones from the splenic B cell populations (Fig. 7c). This observation demonstrates that OBF-1 dependent genes have similar expression profiles in bone marrow and splenic B cell populations.

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3 Discussion

In this thesis the two main approaches to investigate the physiological functions of the different OBF-1 isoforms were to overexpress the nuclear OBF-1 isoform in B cells (BCS mice) and to engineer BAC transgenic mice that express only one isoform. In this section the results from the BCS and BAC transgenic mice are discussed in more detail. This discussion is mainly structured on the different microarray analyses that were performed in early and splenic B cells. Furthermore a DIGE assay from EPLM cultures of BAC transgenic mice (see Appendix) will also be described. This section gives no definite conclusions, but rather provides supportive notions that might be useful.

3.1 Overexpression of OBF-1 in B cells

OBF-1 is an essential transcription factor in B cells and allows proper T-dependent immune responses. So far only few direct target genes have been identified, and we do not have a clear understanding of the role of OBF-1 in the transcriptional machinery leading to antigen stimulated B cell response and germinal center formation. A transgenic mouse line overexpressing OBF-1 in T cells allowed to identify the Ets factor *Spi-B* as a direct target gene (Bartholdy et al., 2006), and this transcriptional network could explain partly the *OBF-1*$^{-/-}$ phenotype. We generated a transgenic mouse line overexpressing *OBF-1* under the control of the *Ig* μ enhancer and *VDJ* promoter, which had been previously characterized to confer B cell-specific expression (Banerji et al., 1983; Dreyfuss et al., 1987; Gillies et al., 1983; Grosschedl and Baltimore, 1985; Mason et al., 1985).

3.1.1 Phenotypic abnormalities

This transgenic mouse line overexpressing OBF-1 in B cells was expected to have an opposite phenotype than the *OBF-1*$^{-/-}$ mice such as increased B cell numbers and enhanced immune responses. New OBF-1 target genes were also expected to be identified. We were surprised to find that these mice had an impaired T-dependent immune response, a strongly reduced splenic B cell compartment and lack of transgene expression.

3.1.1.1 Spleen and immune response

First the splenic populations and the immune response were investigated. The transgenic mice had decreased number of immature and follicular B cells; however the number of marginal zone (MZ) B cells was normal. The T-dependent immune response was severely defective, but the T-independent and MZ B cells response were not affected. These observations suggested that the immunodeficiency was due to the reduction of follicular B cells. As expected the transgene was expressed in thymocytes, however transgene expression was undetectable in the spleen.

3.1.1.2 Bone marrow

The bone marrow B cell populations were investigated to identify the origin of the compromised splenic B cell compartment. Indeed the B cell ontogeny was strongly impaired at the large preBII cell stage when the cells downregulate CD43, which is a

marker for early B cell commitment and which is downregulated when the cells become highly proliferative. Furthermore the transgene was post-translationally downregulated as the HA signal by FACS analysis gradually disappeared in successive developmental stages while the mRNA level was not affected. This suggests that it is a condition for developmental progression. At the large preBII cell stage the *V* to DJ_H rearrangement occurs followed by $V\text{-}J_L$ rearrangement. OBF-1 was reported to be involved in the expression of a subset of *Ig* light chain genes, so possibly it could contribute to the development block. It was evident that overexpressing OBF-1 early in B cell ontogeny is deleterious. This notion is supported by the fact that overexpressing Spi-B, which is an OBF-1 target gene, in hematopoietic progenitor cells can block T, B, and NK cell development *in vitro* (Schotte et al., 2003).
An earlier developmental block was also observed at the EPLM-preBI transition, which defines the B cell commitment stage. In normal condition OBF-1 is not expressed in EPLM cells, however the transgene was effectively expressed in these cells suggesting that OBF-1 level has to be low prior to B cell lineage commitment. *In vitro* investigation of these EPLM cells showed that they have a strong proliferation and differentiation deficiency. Furthermore the proliferating cells have downregulated the transgene indicating that OBF-1 expression is detrimental at this stage. Interestingly our data and Siegel's data showed that *in vitro* proliferation of IL-7 dependent cells is inversely correlated to OBF-1 level (Siegel et al., 2006). Reconstitution experiments clearly showed that the impaired B cell development in the transgenic mice was intrinsic to the B cells and not due to the microenvironment.
Microarray analysis of the EPLM and large preBII cells provided a potential molecular explanation for the differentiation blocks in the BCS mice. qPCR confirmed that Id2 and Id3 were upregulated in EPLM and preBII cells of BCS mice. Furthermore *Id2* and *Id3* are OBF-1 direct target genes, as OBF-1 can bind octamer sequences in their respective promoters. Indeed Id proteins are known to be negative regulators of B cells, which explains why the B cells are impaired when these proteins are overexpressed. Additionnally *EBF1*, which induces OBF-1 expression, was found to be also an OBF-1 direct target gene.
These observations demonstrate that *OBF-1* expression has to be tightly regulated to allow normal B cell development. However it is possible that the block at the large preBII cell stage could also be the result of secondary cellular modifications due to the defective differentiation at the EPLM stage.

In the next section we will discuss in some detail the genes that were identified in our microarray experiments and mention what is known about their functions in B cells.

3.1.2 Microarray analysis

In order to understand the differentiation blocks in the transgenic mice, we performed microarray analysis comparing WT and transgenic EPLM and large preBII cells. 569 and 287 genes were misregulated (at least 2fold) in the EPLM and large preBII cells respectively (see Fig. 5 and Table 1 in chapter 2.1). Therefore the common 40 misregulated genes might have a molecular link with the aberrant OBF-1 expression. This section first describes some interesting genes from the common misregulated gene group, then *Id3* that belongs to the EPLM misregulated gene group and finally some genes misregulated in large preBII cells including *Id2*.

3.1.2.1 Common misregulated genes

3.1.2.1.1 *Syndecan-1* (downregulated in BCS mice):

Syndecan-1 is a heparin sulphate-bearing proteoglyclan, which functions as a matrix receptor by binding cells to interstitial collagens, fibronectin, and thrombospondin. In the B cell lineage Syndecan-1 is expressed on the surface of preB and plasma cells (Sanderson et al., 1989). The physiological function of Syndecan-1 is not clear; however it is a marker for plasma cell differentiation and tumorigenesis. The Syndecan-1 family proteins act as adhesion molecules, modulators of growth factor function, and coreceptors in processes such as morphogenesis, tissue repair, host defense, tumor development, and energy metabolism (Bernfield et al., 1999; Echtermeyer et al., 2001). The heparin sulphate chains of Syndecans, which are the main functional domains, have been implicated in regulating the activity of chemokines (Webb et al., 1993; Wang et al., 2003; Slimani et al., 2003). Data suggest that Syndecan-1 might be involved in generation of chemokine gradients (Li et al., 2002; Marshall et al., 2003). Furthermore the Syndecan ectodomains can be cleaved by protease to become soluble effectors (Fitzgerald et al., 2000). *Syndecan-1*$^{-/-}$ mice display increased leukocyte-endothelial interactions and angiogenesis, as well as increased inflammatory responses in experimentally induced delayed-type hypersensitivity (Gotte et al., 2002; Gotte et al., 2003). The ability of Syndecan-1 to generate chemokine gradients was demonstrated only in neutrophils (Gotte et al., 2003), however very little is known regarding its function in early B cells and plasma cells.

Syndecan-1 was downregulated in EPLM and large preBII cells of the BCS mice. In fact Syndecan-1 level is increased on the surface of mature B cells from *OBF-1*$^{-/-}$ mice indicating that there is an inverse correlation between OBF-1 and Syndecan-1 expression.

3.1.2.1.2 S100 calcium binding protein A10 (*calpactin*, downregulated in BCS mice):

S100A10 is a member of the EF-hand calcium-binding proteins and is important in intracellular calcium signalling, which regulates a variety of cellular processes, such as cell proliferation and gene transcription (Bhattacharya et al., 2004). The S100 proteins regulate intracellular processes such as cell growth and motility, cell cycle, transcription and differentiation (Heizmann et al., 2002). Indeed S100A10 was reported to be overexpressed in various cancers confirming its role in cellular growth (El-Rifai et al., 2002; Teratani et al., 2002; Cross et al., 2005). S100A10 is part of a bigger complex consisting of two S100A10 and two annexin-A2 subunits and is involved in Ca^{2+}-mediated lipid raft organization and vesicle movement (Gerke and Moss, 2002).

S100A10 was downregulated in EPLM and large preBII cells of BCS mice. Furthermore in a microarray study combined with Northern blotting, Kim et al. suggested that OBF-1 is required to downregulate S100A10 in mature B cells stimulated with anti-IgM, anti-CD40 and IL-4 (Kim et al., 2003). Although its function in early and mature B cell is not clear, it is interesting to notice that S100A10 is upregulated in cancer cells and its depletion might be associated with impaired cell proliferation.

3.1.2.1.3 Hairy and enhancer of split 1 (*Hes1*, upregulated in BCS mice):
Hes1 is a basic helix-loop-helix (bHLH) transcription factor and was reported to be a direct target gene of Notch signalling (Jarriault et al., 1998; Lu and Lux, 1996). The Notch-Hes1 axis is necessary for T cell differentiation from common lymphoid progenitor (CLP). Hes1 represses the transcription of target genes by interfering with other bHLH transcription factors like E2A via heterodimerization (Sasai et al., 1992), by direct binding to promoter regions of target genes like *ASH1* and *Hes1* itself (Chen et al., 1997; Takebayashi et al., 1994), and by collaborating with other transcription regulators like Myb (Allen et al., 2001). Overexpressing Hes1 suppresses the development of B cell lineage *in vivo* (Kawamata et al., 2002). Furthermore a constitutively active *Hes1* causes cell death in neuronal cells (Strom et al., 1997).
Hes1 was upregulated in EPLM and large preBII cells of the BCS mice suggesting that it might be involved in the differentiation blocks.

3.1.2.2 EPLM specific genes

3.1.2.2.1 *Id3* (upregulated in BCS mice):
The Id proteins are HLH proteins that lack a basic region. The Id proteins heterodimerize with E-proteins and inhibit their DNA binding ability since they lack the basic domain (Benezra et al., 1990). The resulting loss of E-protein activity can lead to impaired gene expression and disruption of the differentiation program (Chen et al., 1997; Desprez et al., 1995; Jen et al., 1992; Kreider et al., 1992; Moldes et al., 1997; Shoji et al., 1994). Id proteins can also interact with other transcription factors such as the ETS family, Pax family and retinoblastoma protein RB transcription factors (Iavarone et al., 1994; Yates et al., 1999; Roberts et al., 2001).
Id proteins are highly expressed in proB cells and are downregulated upon maturation (Sun et al., 1991; Wilson et al., 1991). In B cells, expression of these proteins was shown to repress transcriptional activity of the *IgH* and κ *intronic* as well as the κ*3' enhancer* (Pongubala and Atchison, 1991; Sun et al., 1991; Wilson et al., 1991). Id3 was also shown to interact directly with the E-protein E2A *in vivo* (Rivera et al., 2000; Yan et al., 1997) resulting in the promotion of T versus B lymphopoiesis. Constitutive expression of Id proteins in B cells impairs B cell development at an early stage (Sun et al., 1994). Furthermore Id3 was reported to inhibit B lymphocyte progenitor growth and survival (Kee et al., 2001).
In our microarray experiments Id3 was upregulated 3.8 fold in EPLM and 1.4 fold in large preBII cells of the BCS mice. The murine and human *Id3* gene promoters contain one and two octamer sites respectively. However these octamer sequences do not overlap between these species. Interestingly Id3 was expressed in preBI cells when endogenous OBF-1 expression begins, supporting the notion that *Id3* is a direct OBF-1 target gene.
OBF-1 protein level was reported to be high in IgM^- B cells and low in IgM^+ B cells in the bone marrow (Qin et al., 1998). As the mRNA level of *OBF-1* was not downregulated during B cell ontogeny, the decrease of OBF-1 protein level was probably post-translational. This decreased expression during B cell differentiation correlates with the downregulation of Id proteins and supports the idea that *Id3* is a direct OBF-1 target gene. Therefore the overexpression of OBF-1 in BCS mice might upregulate Id3 and inhibits EPLM differentiation

potentially in an E2A dependent manner. Furthermore Id3 was not downregulated downstream of preBI cells suggesting that it might be the cause for the differentiation block at the large preBII cell stage.
SIAH1 was reported to induce proteasome mediated downregulation of OBF-1 in mature B cells (Tiedt et al., 2001; Boehm et al., 2001). Therefore the downregulation of OBF-1 in the bone marrow might be due to SIAH1. However in our microarray analysis SIAH1 absolute expression was decreased about 2 fold from the EPLM to large preBII cells and there was no difference of expression between the cells from WT and BCS mice. This observation does not rule out the potential implication of SIAH1 in OBF-1 downregulation, as the expression level is not known in the preBI cells and the microarray measures only the mRNA level.

3.1.2.3 Large preBII specific genes

3.1.2.3.1 *Id2* (upregulated in BCS mice):
Id2 belongs also to the Id protein family and a knock out study showed that Id2 is a negative regulator of B cell development by regulating E2A activity (Ji et al., 2008). Indeed overexpressing Id2 blocks lymphocyte development (Morrow et al., 1999).
In our microarray Id2 was upregulated 3.1 fold in EPLM and 2 fold in large preBII cells of the BCS mice. Interestingly Id2 negatively controls differentiation into mature B2 cells while allowing the commitment to MZ B cells (Becker-Herman et al., 2002). This observation correlates perfectly with the impaired follicular B cell and the unaltered MZ B cell populations in the BCS mice. QPCR analysis of Id2 showed that it was expressed at the large preBII $CD43^+$ cells and then was downregulated. Indeed the BCS mice were not able to downregulate properly Id2 after the large preBII $CD43^+$ cell stage and Id2 was also upregulated in EPLM cells. Furthermore the *Id2* gene promoter has a conserved octamer site, which can be bound by OBF-1, supporting the notion that it is a direct OBF-1 target gene.
Therefore Id2 might be the molecular cause for both EPLM and large preBII developmental blocks. Both Id2 and Id3 proteins have a negative effect on B cell development; however knock out studies showed that Id1 has no intrinsic effect on B cell ontogeny (Ji et al., 2008). It is possible that Id2 and Id3 contribute both to the impaired B cell differentiation of the BCS mice. Interestingly in WT mice the expression peak of Id2 was in a more differentiated cell stage than Id3. Their respective octamer sites might have a different sensitivity to OBF-1, as OBF-1 is upregulated in large preBII cells.

3.1.2.3.2 *Gadd45β* (upregulated in BCS mice):
Gadd45β is a growth-arrest and DNA-damage-inducible gene that gets activated in response to various cellular stress signals, and also by inflammatory cytokines, such as IL-6, IL-18 and IL-12, TNF and TGF-β1. It can also be induced by treatment with lipopolysaccharide (LPS). Gadd45β is involved in cell cycle arrest and apoptosis and is required for the generation of T_{H1} cells *in vivo* (Lu et al., 2001). Gadd45 is transcriptionally upregulated by p53 via an intronic p53 consensus-binding site in the *Gadd45* gene (Kastan et al., 1992), but it can also be regulated in a p53-independent manner (Zhan et al., 1996). It was shown that the *Gadd45* promoter contains binding sites for

Oct-1 and NF-Y, which are required for the p53-independent activation of *Gadd45* following binding to BRCA1 (Fan et al., 2002) and following treatment with the HDAC inhibitor TSA (Hirose et al., 2003). Moreover, *Gadd45β* expression is regulated by NF-κB (Jin et al., 2002), resulting in the suppression of JNK signalling by targeting MKK7/JNKK2 and inhibiting its enzymatic activity. This inhibition is crucial for the suppression of TNF-α induced apoptosis and links the NF-κB signals to the MAPK pathway (De Smaele et al., 2001; Papa et al., 2004; Tang et al., 2001).
In our microarray analysis, Gadd45β was upregulated in the large preBII cells from transgenic mice. This could be explained by the direct binding of OBF-1 together with Oct-1 on the perfect octamer site present in the *Gadd45β* promoter. Chromatin Immunoprecipitation (ChIP) experiment on WT Abelson cells showed that OBF-1 does indeed bind the *Gadd45β* promoter (see Appendix). Furthermore Boris Bartholdy had also identified *Gadd45β* as a potential OBF-1 direct target gene in mice overexpressing OBF-1 in T cells. Hoffmann et al. reported in a microarray experiment that Gadd45β was upregulated in large preBII cells (Hoffmann et al., 2002). However the potential physiological implication of OBF-1 in inducing *Gadd45β* is not clear. It is possible that Gadd45β has to be upregulated at the preBII cell stage in response to stress or DNA-damage signals, such as single or double strands breaks upon V(D)J recombination.

3.1.2.3.3 *Cyclin D2* (upregulated in BCS mice):
Cyclin D2 is critical in cell cycle regulation for the G1-S progression (Resnitzky et al., 1994; Herrera et al., 1996) and was reported to be an essential mediator of BCR-induced proliferation (Chiles 2004).
In our microarray Cyclin D2 was upregulated in the large preBII cells of BCS mice where the preBCR is expressed. Chromatin immunoprecipitation was performed in wild type Ableson cells (see Appendix) showing that OBF-1 might bind to the *Cyclin D2* promoter. As overexpression of Cyclin D2 is correlated with tumorigenesis and prevention of apoptosis, it is unlikely that it played a role in the large preBII block. However investigation of *Cyclin D2*$^{-/-}$ mice shows that it plays a role for early B cell development and BCR-mediated mature B cell stimulation (Solvason et al., 2000). In fact OBF-1 is upregulated at the preBI stage and upon B cell stimulation which coincides with Cyclin D2 function. The figure 11 depicts the signalling pathways in B cells resulting in Cyclin D2 expression.

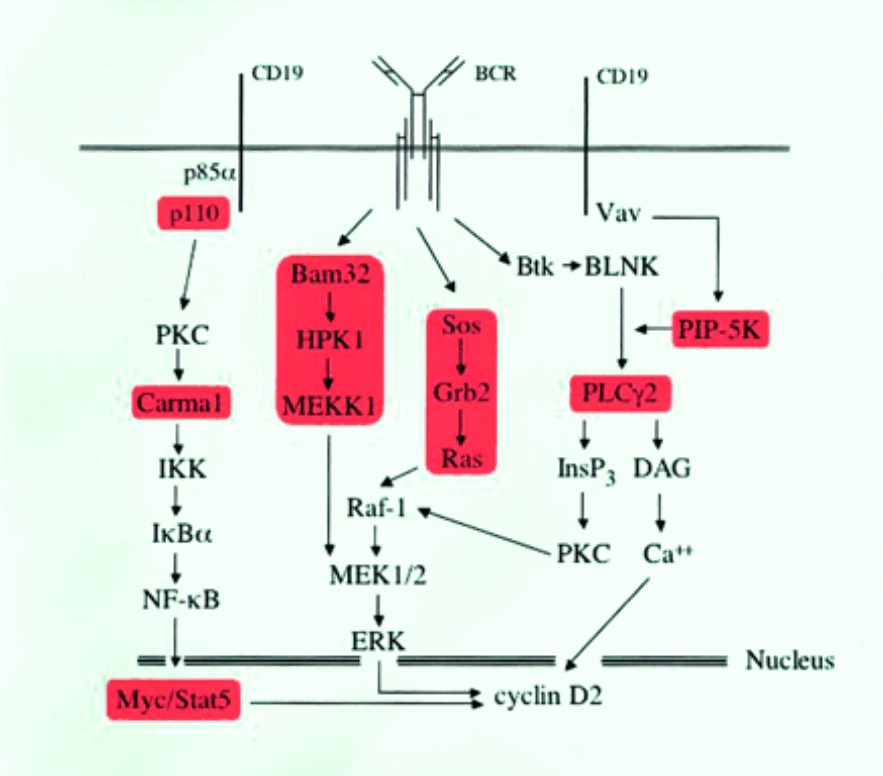


Figure 11: Cyclin D2 activation in B cells

BCR-induced signal transduction pathways that positively regulate cyclin D2 expression. In response to BCR cross-linking, the signalosome components, Btk, BLNK, and Vav, contribute to PLCγ2 activation, which in turn leads to the generation of diacylgycerol (DAG) and inositol 1,4,5-trisphosphate (InsP3) and subsequent increase in intracellular Ca^{2+} and activation of PKC, both of which are necessary for cyclin D2 induction. PKC and Sos-Grb2-Ras activate the Raf1-MEK1/2-ERK signaling module; MEK1/2 is also activated by the Bam32-HPK1-MEKK1 signaling module. The p85α subunit of PI3K acts to link the BCR to cyclin D2 induction by way of a PKC-Carma1-IKK-IκBα-NF-κB signaling module. In addition, the production of phosphatidylinositol 3,4,5-trisphosphates by PI3K represents an important target of pleckstrin homology (PH) domain-containing proteins, including Btk, Vav, and PLCγ2. CD19 is one of the main regulators of PI3K activity in B cells. The cytosolic tail of CD19 contains tandem YXXM motifs that are phosphorylated following BCR ligation and associate with the Src homology 2 domains of class I PI3K regulatory subunits. The individual signal transduction molecules, which have not yet been definitively linked to cyclin D2 induction in B cells, are highlighted in red (from Chiles 2004).

3.1.2.3.4 *Foxp1* (downregulated in BCS mice):

Foxp1 is an essential transcription factor in early B cell development as *Foxp1* defective mice have a differentiation block at the preBI to preBII transition and impaired *V(D)J* rearrangement (Hu et al., 2006). Foxp1 was shown to bind to the *Erag* enhancer and regulates *Rag1* and *2* expressions.

In our microarray Foxp1 was downreguled in large preBII cells of the BCS mice suggesting that it might be one cause for the differentiation block. However, Rag proteins were not misregulated indicating that the impaired differentiation, potentially mediated by the reduction of Foxp1 level, is due to other Foxp1 target genes.

3.2 Reconstitution of p34 isoform in *OBF-1*$^{-/-}$ mice

The *OBF-1* gene can generate different isoforms depending on which start codon is translated. However most of the investigations on OBF-1 were performed with *OBF-1*$^{-/-}$ mice which disrupts all OBF-1 isoforms. Therefore very little is known about the function of the membrane bound p35 isoform. Siegel et al. has reported that OBF-1 can interact with Syk and Galectin1 in the cytoplasm (Siegel et al., 2006; Yu et al., 2006), but no phenotypic investigation was performed on mice expressing only one isoform. BAC transgenic mice expressing the WT OBF-1, the p34 isoform and GFP in an *OBF-1*$^{-/-}$ background were generated to investigate the contribution of each OBF-1 isoform. This section describes some interesting genes identified from our microarray analysis that were p34 or p35 dependent. Some comments about proteins identified in EPLM cells using 2-D fluorescence difference gel electrophoresis (2D-DIGE) will follow. Finally we will discuss about a potential link between OBF-1 and the protein tyrosine kinase Syk.

3.2.1 Spleen

First the immune response and the splenic populations of the BAC transgenic mice were investigated as the main *OBF-1*$^{-/-}$ phenotypes occur in mature B cells and germinal center formation. The p34 isoform was able to fully rescue the number and the size of the germinal centers in immunised mice. In addition the IgM and IgG responses in immunised mice and the B cell populations associated with the Syndecan1 expression profile were not impaired in the *BAC p34* mice. These observations suggested that p35 might play a role either on a specific aspect in splenic B cells or in early B cells in the bone marrow. Plasma cell differentiation can be mimicked *in vitro* by stimulating naïve mature B cells with α-CD40 and IL-4. The differentiation and proliferation potential of mature B cells from the different BAC transgenic mouse lines were investigated with Syndecan1 and CFSE staining respectively. Indeed neither the differentiation nor the proliferation were impaired in the cells from *BAC p34* mice.

3.2.2 Bone marrow

The bone marrow populations were next investigated as *OBF-1*$^{-/-}$ mice have a decreased number of transitional B cells. The transitional B cells as well as their CD22 intensity were fully rescued in the *BAC p34* mice. Siegel et al. reported that OBF-1 could play a role in the very early B cell stage by stabilizing Syk (Siegel et al., 2006). In fact the proliferation of preBI cells *in vitro* were only partially rescued in the *BAC p34* mice and, in line with the results of Siegel et al., this hyperproliferation was associated with Syk downregulation. Therefore p35 might be involved in early B cell proliferation potentially by stabilizing Syk. However it is not clear yet what is the physiological function of p35 as its potential role was observed only *in vitro*.

3.2.3. Microarray analysis

Chapter 2.2 mainly focused on the microarray data in splenic B cells. Several potential OBF-1 target genes could be found in this microarray. The following genes could be interesting, although they were not discussed in chapter 2.2. Bcl2 modifying factor (Bmf) plays a critical role in apoptosis and can function as a tumor suppressor. Interleukin-1 receptor-associated kinase 3 (IRAK3) is a negative regulator of Toll-like receptor signaling. B3galt7 is involved in glycosylation; interestingly B4galt1 that have a similar function was found to be OBF-1 dependent (Teitell, 2003). Bmf, IRAK3 and B3galt7 were all rescued by p34 and downregulated in mice deficient for *OBF-1* (Fig. 7c in chapt. 2.2). This microarray analysis highlighted only a small number of p35 specific genes, and only one of these genes (*BCNP1*) could have a relevant physiological significance.
In this section we will discuss about potential interesting genes found in microarray analysis of bone marrow (large preBII and mature B cells) and splenic (total splenic B cells) populations.

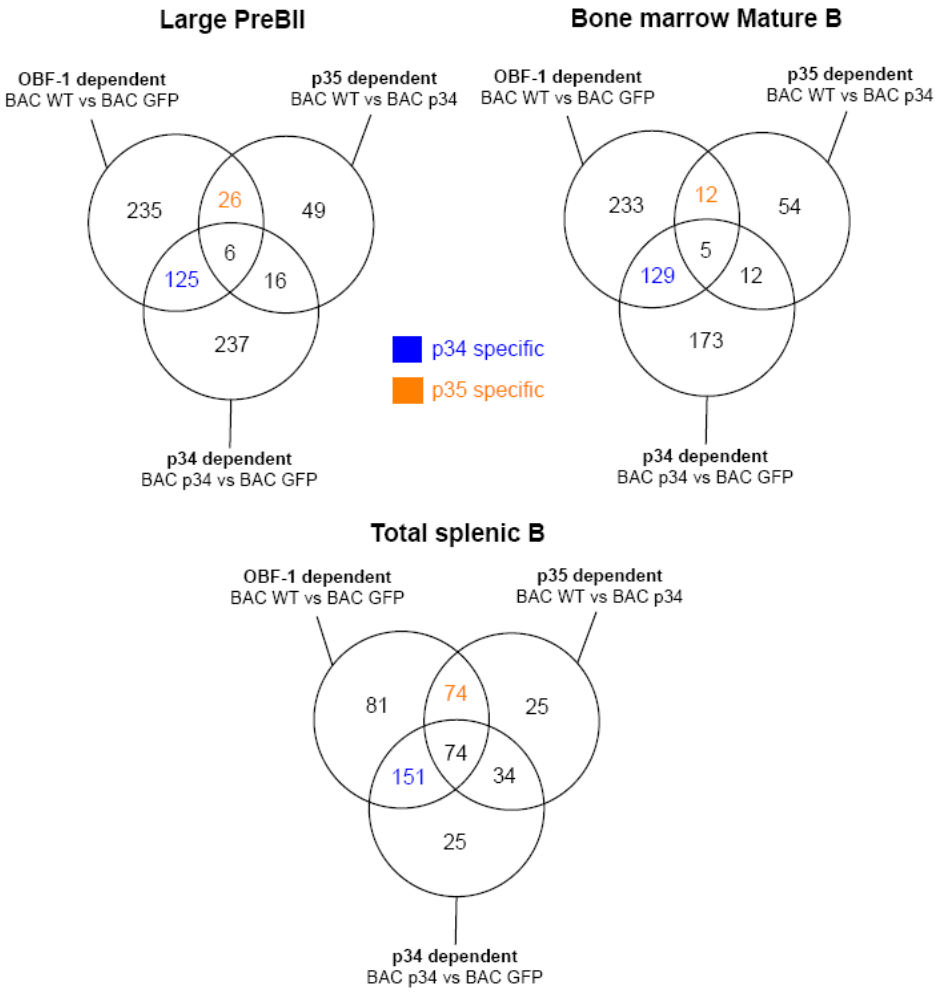


Figure 12 : Microarray analysis in different cell populations of BAC transgenic mice
The large preBII cells were FACS sorted with anti-B220-APC and anti-CD25-PE antibodies. The bone marrow mature B cells were FACS sorted with anti-B220-APC and anti-IgM-biotin antibodies, followed by Streptavidin-Cy5.5. The total splenic B cells were FACS sorted with B220-APC antibody. For every cell population three individual mice were used. The

microarray data were analyzed with the Expressionist program; genes with a fold median change of at least 1.5 and a p value of at least 0.01 were considered misregulated.

Microarray analysis of the *BAC WT*, *BAC p34* and *BAC GFP* mice were performed to identify the p34 and p35 dependent genes. The RNA from large preBII, bone marrow mature B cells and total splenic B cells were purified and processed for microarray analysis. The genes that were at least 1.5 fold misregulated with a p value of at least 0.01 were compared between the genotypes. The genes that were misregulated between the *BAC WT* and *BAC GFP* mice were called *OBF-1* dependent genes. The genes that were misregulated between the *BAC p34* and *BAC GFP* mice were called p34 dependent genes. The genes that were misregulated between the *BAC WT* and *BAC p34* mice were called p35 dependent genes. The intersections between the OBF-1 and p34 dependent groups were considered as p34 specific genes. The intersections between the OBF-1 and p35 dependent groups were considered as p35 specific genes. The microarray data scheme is depicted in Figure 12.

3.2.3.1 p34 specific genes

3.2.3.1.1 *FOG1* (upregulated by p34 in large preBII cells):

Friend of GATA-1 (FOG1) is encoded by the gene *Zfpm1* and is a binding partner of GATA-1 (Tsang et al., 1997; Boyes et al., 1998; Nichols et al., 2000). FOG1 contains nine putative zinc fingers and four of the fingers interact independently with GATA-1 (Fox et al., 1999). FOG1 interacts also with CtBP (Katz et al., 2002) and the NuRD corepressor complex (Hong et al., 2005) to promote normal erythropoiesis *in vivo*. FOG1 can activate or repress target genes by facilitating the binding of GATA factors to DNA, by recruiting chromatin remodelling complex, and by stabilizing tissue specific chromatin loops. FOG1 is highly expressed together with GATA-1 in multipotent progenitors and in erythroid and megakaryocytic cells. *FOG1*$^{-/-}$ mice die of anaemia by embryonic day 11.5 (Tsang et al., 1998). The megakaryocytes do not develop in *FOG1*$^{-/-}$ mice, however the erythroid lineage is partially blocked. FOG1 level is low in hematopoietic stem cells and in lymphoid cells and there is no FOG1 expression in the myeloid lineage. The role of FOG1 is not clear in the lymphoid lineage. FOG1 was reported to repress GATA-3-dependent induction of Th2 development. FOG1 is downregulated in mature B cells and in plasma cells suggesting that low level of FOG1 is required for late B cell differentiation or to maintain B cell identity.

In our microarray study FOG1 was downregulated in the large preBII cells from the *BAC GFP* mice. Furthermore *FOG1* was also identified as a potential OBF-1 target gene in mircroarray data performed by Alexander Karnowski. OBF-1 starts to be expressed at the preBI cell stage when FOG1 is expressed suggesting that their might be a direct link between these transcription factors.

3.2.3.1.2 *Cyclin D2* (upregulated by p34 in mature B cells):

Cyclin D2 and *Gadd45β* were identified as potential targets of p34 in the BCS transgenic mice. In fact in our microarray analysis *Cyclin D2* and *Gadd45β* were also identified as p34 specific genes and were downregulated in mature B cells and large preBII + mature B cells in *BAC GFP* mice respectively.

Therefore p34 might upregulate Cyclin D2 and Gadd45β in early B cells by binding to their respective promoters.

3.2.3.1.3 *Hes1* (upregulated by p34 in total splenic B cells):
Hes1 is hypothesized to play a role in the differentiation blocks of the BCS transgenic mice. Interestingly *Hes1* was identified as a p34 specific gene in our microarray data and was downregulated in total splenic B cells of *BAC GFP* mice. Therefore *Hes1* expression could be sensitive to p34 level in early and late B cell stages.

3.2.3.1.4 *Blimp1* (downregulated by p34 in total splenic B cells):
Blimp1 is a transcriptional repressor encoded by the *Prdm1* gene; it contains five zinc finger motifs that confer DNA binding ability (Keller et al., 1992). Blimp1 is expressed in plasma cells (Angelin-Duclos et al., 2000) and is a crucial transcription regulator for plasma cell differentiation. Blimp1 was reported to repress more than 225 genes and activate more than 30 (Shaffer et al., 2002). The genes regulated by Blimp1 are involved in proliferation, Ig secretion, germinal center (GC) function and B cell identity (Shaffer et al., 2002). At the GC B cell stage BCL-6 is downregulated, which allows Blimp1 expression (Reljic et al., 2000; Shaffer et al., 2000; Vasanwala et al., 2002). Then Blimp1 represses genes required for BCR signalling and GC reactions such as *Id3*, *c-Myc* (Lin et al., 1997; Lin et al., 2000), *Spi-B*, *CIITA* and *Pax5* (Shaffer et al., 2002). Once expressed, Blimp1 itself represses *BCL-6* to make sure the plasma cell differentiation is irreversible (Calame et al., 2003).
Blimp1 was identified as a p34 specific gene from our microarray analysis of total splenic B cells (Chapter 2.2 Fig. 7c). However Blimp1 was upregulated in mice lacking OBF-1, which contradicts the notion that it could be a direct OBF-1 target gene. We have to consider this observation carefully, because Corcoran et al. reported that Blimp1 was strongly downregulated in mature B cells from *OBF-1*$^{-/-}$ mice stimulated with α-CD40 and IL-4 (Corcoran et al., 2005).

3.2.3.1.5 *NFATc1* (downregulated by p34 in mature and total splenic B cells):
NFATc1 is a member of the Ca^{2+}-sensitive NFAT transcription factor family (Timmerman et al., 1997). NFAT are activated by BCR crosslinking or CD40 ligation and can transactivate target genes (Rao et al., 1997; Kiani et al., 2000; Crabtree and Olson, 2002; Verweij et al., 1990; Choi et al., 1994; Venkataraman et al., 1994). Calcium signaling activates the phosphatase calcineurin and induces movement of NFATc proteins into the nuleus, where they interact with other proteins such as AP-1 to regulate genes (Macian et al., 2001; Crabtree et al., 2002). The *NFATc1*$^{-/-}$ mice have reduced levels of serum IgG1 (Yoshida et al., 1998; Ranger et al., 1998) and IgE (Ranger et al., 1998), but probably due to impaired IL-4 production by T cells (Yoshida et al., 1998; Ranger et al., 1998). The physiological role of NFAT in B cells is not clear so far. Targeted disruption of *NFATc1* leads to overproduction of IgG1 and IgE and a polyclonal plasma cell infiltration of end organs (Peng et al., 2001). Furthermore this IgG1 and IgE overproduction was independent of IL-4 and was observed *in vitro* with isolated B cells. In addition NFATc1 was reported to play a role in B-1a cell development (Berland and Wortis, 2003).

NFATc1 was identified as a p34 specific gene and was upregulated in mature and total splenic B cells of *BAC GFP* mice (Fig. 12). The same observation was done in chapter 2.2 (Fig. 7c). Possibly the role of p34 might be to downregulate NFATc1, likely in an indirect manner, to promote class switched immunoglobulin production.

3.2.3.2 p35 specific genes

3.2.3.2.1 *Thioredoxin-like2* (upregulated by p35 in large preBII cells):

Thioredoxin-like protein 2 (Txnl2 / PICOT / Glutaredoxin3) consists of an N-terminal domain typical of thioredoxins and a C-terminal domain belonging to the nucleoside-diphosphate kinase family, separated by a small interface domain (Sadek et al., 2003). Txnl2 is ubiquitously expressed and was shown to bind microtubules (Sadek et al., 2003). Trxnl2 was also reported to interact with protein kinase C θ (PKC-θ) via its thioredoxin domain and acts as an important modulator of T cell receptor (TCR)-signalling (Witte et al., 2000). Overexpressing Txnl2 in Jurkat T cells suppressed the activation of JNK and the transcription factors AP-1 and NF-κB, by the active form of PKC-θ or by T cell-activating stimuli (Witte et al., 2000). PKC-θ depends on diacylglycerol (DAG), but not on Ca^{2+}. PKC-θ plays also a role in the activation of ERK, *IL-3* gene transcription and degranulation in response to FcεRI aggregation in mast cells (Liu et al., 2001). Txnl2 was shown to regulate also FcεRI-mediated mast cell activation via PKC-θ (Kato et al., 2008). In mast cells Txnl2 is a positive regulator of *IL-4* and *TNF-α* expression, NFAT and degranulation signal pathways and a negative regulator on a JNK signal pathway (Kato et al., 2008).
In our microarray data *Txnl2* was identified as a p35 specific gene and was downregulated in large preBII cells from *BAC GFP* mice. It is known that the redox status is important for early B cell development; therefore p35 might modulate it by upregulating Txnl2.

3.2.3.2.2 BMP signalling:
The ligand for the bone morphogenetic proteins type 2 receptor (Bmpr2), which is a serine/threonine kinase receptor, is BMP-6. BMPs belong to the TGF-β superfamily and are secreted proteins with pleiotropic roles, such as proliferation, differentiation, migration and apoptosis (Chen et al., 2004).
BMP-6 was reported to reduce the number of cobblestone-area-forming cells (CAFC) of normal human haematopoietic cells, which represent the functional primitive repopulating haematopoietic stem cells in long-term bone marrow culture (Ahmed et al., 2001). BMP-6 is also associated to poor prognosis in diffuse large B cell lymphomas (Rosenwald et al., 2002). Upon BMP-6 binding, the Bmpr2 signals by phosphorylating the receptor Smads (Smad-1, Smad-5, and Smal-8). The R- Smads then form complexes with the co-Smad (Smad4) and are translocated into the nucleus where they regulate gene expression (Chen et al., 2004; Waite et al., 2003). BMP-6 was reported to inhibit the proliferation of preBI cells by upregulating Id1 and Id3 (Kersten et al., 2006). In mature B cells the phosphorylation of Smad1/5/8 upon BMP-6 stimulation results in upregulation of Id1 mRNA. Id proteins inhibit E-proteins and Pax5 by forming heterodimers with them and preventing their DNA binding ability.

Furthermore BMP-6 inhibits growth of naïve and memory B cells stimulated with anti-IgM or in combination with CD40L. In addition BMP-6 induces apoptosis of activated memory B cells (Kersten et al., 2005).
Bmpr2 can also activate the MAP kinase-dependent pathways (Gilboa et al., 2000; Gallea et al., 2001; von Bubnoff and Cho, 2001; Wozney, 2002; Hassel et al., 2003). The MAP kinase pathway associated with Bmpr2 is less understood than the Smad pathway. It has been suggested that TAB1 and TAK1, which are MAPKKK, may form a complex with BMP receptors (Gallea et al., 2001; von Bubnoff and Cho, 2001; Nohe et al., 2002, 2004; Wozney, 2002; Hassel et al., 2003; Zwijsen et al., 2003). However the activation of Erk or p38 downstream of BMP receptors is not clear yet.
Hassel et al. reported a cross-talk between Bmpr2 and c-kit signalling involving Smad proteins, Erk and p38 kinases (Hassel et al., 2006). They showed that Bmpr2 and c-kit form a complex and that they can cooperate upon BMP2 and SCF stimulation in osteoblastic differentiation.
In our microarray experiments *Bmrp2* and *Smad1* were identified as p35 specific genes and were upregulated in the total splenic B cells of *BAC GFP* mice. It is possible that p35 modulates the response of mature B cells by downregulating the receptor for BMP-6. Bmpr2 plays also a role in early B cells and was shown to cooperate with c-kit suggesting that p35 might also regulate the Bmpr2/c-kit mediated preBI cell differentiation. Therefore p35 might modulate BMP signalling by downregulating Bmpr2 and Smad1.
Furthermore MEKK1 is involved in Bmpr2 signalling upon BMP-2 stimulation (Hassel et al., 2006). MEKK1 is encoded by the gene *Map3k1* and is part of the MAP kinase pathway. Gallagher et al. reported that MEKK1 is also required for CD40-dependent activation of the kinases Jnk and p38, germinal center formation, B cell proliferation and antibody production (Gallagher et al., 2007). In our microarray experiments *MEKK1* was identified as a p35 specific target gene and was upregulated in the splenic B cells of *BAC GFP* mice. This observation suggests that p35 might modulate the germinal center formation and the B cell response by downregulating MEKK1.
Altogether it seems that p35 may be involved in Smad1 related pathways as it downregulates Bmrp2, Smad1 and MEKK1. The Figure 13 depicts the different pathways regulating the stability of Smad1, which is a key transcription factor in BMP signalling.

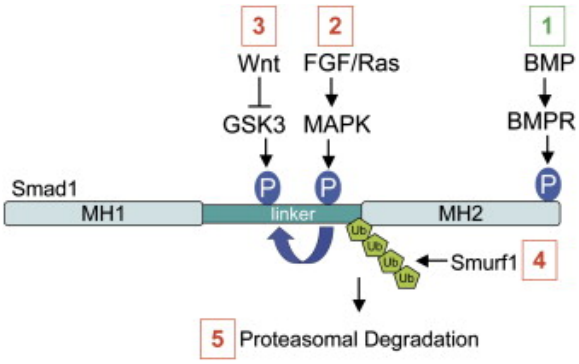


Figure 13: Sequential Events in Smad1 Degradation.
Activation of BMP receptor kinase activity leads to C-terminal phosphorylation of Smad1 (1). Activated MAPKs downstream of multiple inputs such as FGF or Ras can phosphorylate the linker domain (2), which provides a primed substrate for GSK3, which in turn is active in the absence of Wnt signaling (3). Smurf1 recognizes the doubly phosphorylated linker domain of Smad1 and polyubiquitinates (Ub) it (4), targeting Smad1 for degradation in centrosomes (5) (from Verheyen et al., 2007)

3.2.3.2.3 *Klf12* (upregulated by p35 in total splenic B cells):
The Krüppel-like zinc finger protein AP-2rep (KLF12) belongs to a family of transcriptional repressors with three Gli-Krüppel C_2H_2-type zinc fingers and was shown to inhibit *AP-2α* gene expression by binding to its promoter (Imhof et al., 1999). The AP-2 transcription factors (AP-2α, AP-2β, AP-2γ) play a role in embryonic development (Schorle et al., 1996; Zhang et al., 1996), regulation of programmed cell death (Moser et al., 1997), and cell growth and differentiation (Byrne et al., 1994). Decreased level of *AP-2* gene expression is associated with malignant transformation and overexpression of growth factor receptors in cancers (Kannan et al., 1994; Bosher et al., 1995; Turner et al., 1998). The AP-2 transcription factor can bind many proteins such as c-Myc (Gaubatz et al., 1995), p300/CBP (Braganca et al., 2003), p53 (McPherson et al., 2002), Rb (Wu et al., 1998; Batsche et al., 1998) or YY1 (Wu et al., 2001). Disruption of *AP-2α* leads to facial and limb development disturbances (Schorle et al., 1996; Zhang et al., 1996), however it does not seem to play a role in B cells.
KLF12 was identified as a p35 specific gene and was downregulated in total splenic B cells of *BAC GFP* mice. So far the functions of KLF12 are not clear yet, and there may be a link with AP-2 transcription factors in B cells or with unknown KLF12 targets.

3.2.3.2.4 *Sox4* (downregulated by p35 in total splenic B cells):
Sox4 is a member of the HMG box family of T cell transcription factors (Kuo et al., 1999) and is highly related to TCF1, LEF1 and Sry (van de Wetering et al., 1993). Sox4 was shown to bind to *LEF1*- and *TCF1*-binding motifs and can activate transcription via its C-terminal serine-rich domain (van de Wetering et al., 1993). Reconstitution experiments with *Sox4*$^{-/-}$ fetal liver cells demonstrated that Sox4 is required for the expansion and differentiation of proB cells (Schilham et al., 1996). Furthermore IL-7 dependent cultures of *Sox4*$^{-/-}$ fetal liver cells have an impaired proliferation (Schilham et al., 1996). So far Sox4 is an orphan transcription factor as no direct target gene was identified. Furthermore Sox4 was shown to interact with the IL-5Rα via Syntenin and IL-5 treatment of a preB cell line induced Sox4 activation (Geijsen et al., 2001). Interestingly Syntenin was reported to associate also with Syndecan1 (Grootjans et al., 1997), whose expression is modulated by OBF-1. *IL-5Rα*$^{-/-}$ mice have impaired development of B1 cells (Hiroi et al., 1999; Kopf et al., 1996; Yoshida et al., 1996). Furthermore the Syntenin binding domain of IL-5Rα was reported to be important for regulating B cell differentiation (Moon et al., 2001). Therefore IL-5Rα-induced *Sox4* activation could play a role in the regulation of early B cell development.
In our microarray data *Sox4* was identified as a p35 specific gene and was upregulated in the total splenic B cells of *BAC GFP* mice. It is thus possible that p35 downregulates Sox4 to modulate B cell development.

3.2.4 EPLM cultures

3.2.4.1 2-D fluorescence difference gel electrophoresis (2-D DIGE)

The 2-D DIGE assay is a method to compare the protein levels in 2D polyacrylamide gels between different cell samples. The cell extracts are

partially stained with a fluorescent dye in order to stain the most abundant proteins. Then the respective 2D gels are compared for spots which have different fluorescence levels. The cell extracts of EPLM cultures from *WT* and *OBF-1*$^{-/-}$ mice were analysed by 2D-DIGE assay to investigate the potential cause for the hyperproliferation of the cells from *OBF-1*$^{-/-}$ mice (see Appendix). The identified spots were then characterized by mass spectrometry. With this approach several proteins were identified and are depicted in Figure 25 in the Appendix. We describe here three proteins, which were all upregulated in EPLM cells deficient for OBF-1 and might have a physiological relevance.

3.2.4.1.1 PCNA:

PCNA is a cofactor of DNA polymerases that encircles DNA and is required for DNA replication (Moldovan et al., 2007). In cells from *OBF-1*$^{-/-}$ mice PCNA was upregulated suggesting that it might be one cause for hyperproliferation. OBF-1 might modulate preBI cell proliferation by maintaining PCNA at a low level. Interestingly the *OBF-1* promoter has a CREB binding site (Stevens et al., 2000). It was reported that cAMP, which activates CREB, has an antiproliferative effect on lymphocytes by inhibiting DNA replication via dissociation of PCNA from chromatin in S phase cells (Naderi et al., 2005).

3.2.4.1.2 Protein Disulfide-Isomerase A6:

Protein Disulfide-Isomerase A6 (PDIA6 / ERp5) catalyses the isomerization of disulfide bond formation in the oxidative environments of the endoplasmatic reticulum (Collet et al., 2002; Gruber et al., 2006). In cells from *OBF-1*$^{-/-}$ mice PDIA6 was upregulated and its overexpression was reported to promote *in vitro* migration and invasion and *in vivo* metastasis of breast cancer cells (Gumireddy et al., 2007). p35 is present in the endoplasmatic reticulum (Appendix), where PDIA6 is expressed. It is possible that p35 destabilizes PDIA6 to modulate cell proliferation.

3.2.4.1.3 S100 Calcium-binding protein A4 (S100A4):

Metastasis-promoting Mts1 (S100A4) protein belongs to the S100 family of Ca^{2+}-binding proteins. S100A4 is strongly associated with stimulation of metastasis, however the mechanism is poorly understood (Naaman et al., 2004). It was reported that S100A4 interacts with heavy chain of nonmuscle myosin (Kriajevska et al., 1994; Ford and Zain, 1995), liprinβ-1 (Kriajevska et al., 2002), p53 (Grigorian et al., 2001) and methionine aminopeptidase (Endo et al., 2002) suggesting a role in cell motility, adhesion and proliferation. In cells from *OBF-1*$^{-/-}$ mice S100A4 was upregulated suggesting a link with the hyperproliferation of these cells.

3.2.4.2 Putative involvement of the protein tyrosine kinase Syk

The existence of a form of OBF-1 at the membrane suggests that it may be playing a role there. The observation that BCR stimulation seems to be defective in *OBF-1* knock out mice (Kim *et al.*, 1996) suggests that OBF-1 might interact with components of signal transduction complexes. Siegel et al. identified Syk as an interacting partner of OBF-1 (Siegel et al., 2006).
Syk is a protein tyrosine kinase of 72 kD which is an important component of the B cell receptor signalling. Syk consists of two NH_2-terminal SH2 domains

and a COOH-terminal protein tyrosine kinase domain (PTK). Siegel et al. reported that Syk was hypophosphorylated in B cells from *OBF-1* knock out mice, suggesting that OBF-1 might interact with Syk and plays a role on Syk phosphorylation. The activation of B lymphocytes is initiated when the B cell receptor for antigen (BCR) is aggregated by interactions with polyclonal antigens. The cytoplasmic domains of the Ig-α and Ig-β components of the BCR complex link the receptor to cytoplasmic protein-tyrosine kinases, the activation of which elicits a cascade of biochemical responses. The protein-tyrosine kinases activated most proximal to the receptor are Syk and Lyn. In the resting state the SH2 domains of Syk inhibit its kinase activity presumably by blocking access of ATP to the kinase domain (autoinhibition). When bound to the BCR, Syk can assume an open conformation and phosphorylates the two ITAM tyrosines of Ig-α and Ig-β. The tandem SH2 domains of Syk then bind to the doubly phosphorylated ITAM tyrosines (ppITAM), thus fixing the kinase in an open and active conformation. It results a rapid phosphorylation of neighboring ITAM sequences, further Syk recruitment and the amplification of the BCR signal by a positive enzyme/product (Syk/ITAM) feedback. Fully activated Syk phosphorylates several cytosolic substrates, thereby leading to the activation of downstream signalling pathways.
Reconstitution of the BCR complex and several of its key signalling elements in the evolutionary distant environment of the Drosophila S2 Schneider cell line (Wossning *et al.*, 2004) was used to determine whether OBF-1 can modulate the function of kinases in the BCR pathway and modify the BCR response to antigen stimulation. However our experiments and Peter Nielsen's experiments were not able to confirm this interaction in splenic B cells (Appendix).

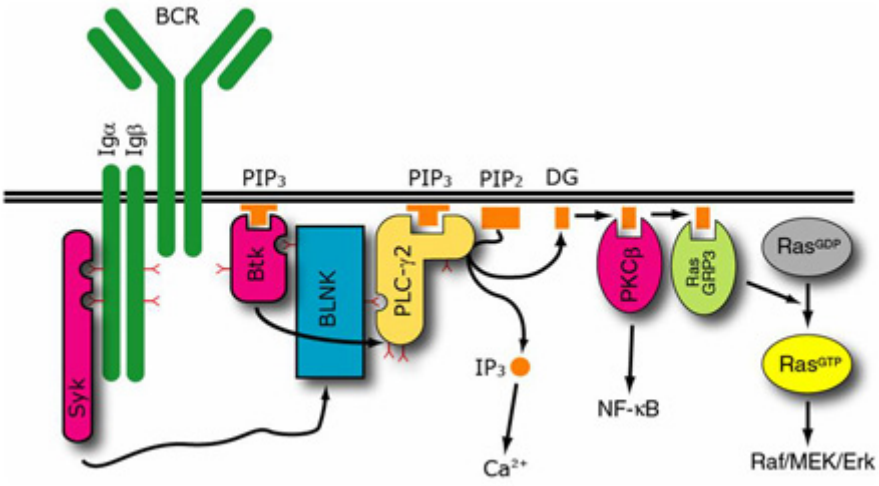


Figure 14: BCR-mediated Ras signaling pathway
Stimulation of BCR leads to activation of proximal protein tyrosine kinases including Syk and Btk. Btk phosphorylates several tyrosine residues on PLC-γ2, and subsequently activates PKCβ. PKCβ then phosphorylates RasGRP3, which results in the activation of Ras signaling cascade (**web.rcai.riken.jp/en/labo/lympho/research.html**).

Since no effect was detected in splenic B cells, we investigated the early B cells as Siegel et al. suggested that OBF-1 might stabilize Syk and modulate preBCR signalling in proB cells (Siegel et al., 2006). In this case Syk protein level was reduced in EPLM cultures from *BAC p34*, *BAC GFP* and *OBF-1*$^{-/-}$ mice suggesting that p35 might stabilize Syk under these culture conditions. Figure 14 illustrates BCR-mediated Syk activation, which drives the activation of the Ras signalling pathway. One should note that Syk induces also the activation of NF-κB and NFAT via PKCβ and Ca^{2+} respectively.

3.3 Summary

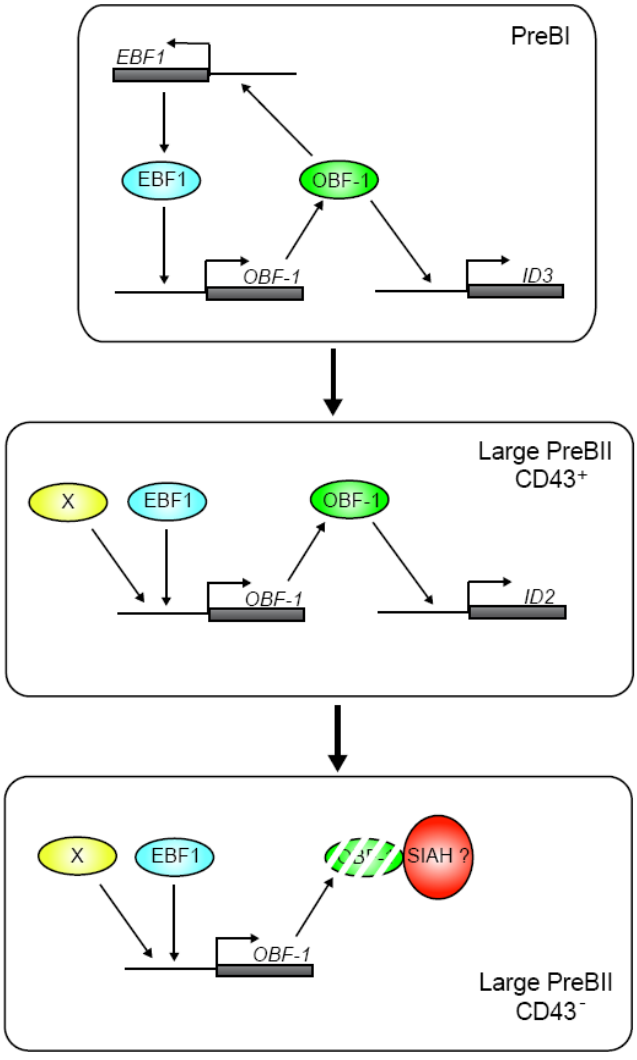
The main *OBF-1*$^{-/-}$ phenotype takes place in secondary lymphoid organs. However there is growing evidence that OBF-1 plays an important role in early B cells. In fact OBF-1 promotes the survival of transitional B cells (Hess et al., 2001; Schubart et al., 2000) and favours specific subsets of *IgVκ* genes during *V(D)J* recombination (Casellas et al., 2002; Jankovic et al., 2003). OBF-1 cooperates with Aiolos in the preBII to immature B cell transition (Sun et al., 2003), and Siegel et al. reported that OBF-1 might modulate the stability of Syk in preB cells, thus regulating preBCR signalling (Siegel et al., 2006). In the present thesis the experiments suggest that the nuclear OBF-1 isoform is the main player in mature B cells and that its expression level is crucial for early B cell development. Indeed mice expressing only the p34 isoform are essentially equivalent to mice expressing both isoforms. Furthermore expressing p34 too early in B cell ontogeny results in a severe differentiation block after the EPLM stage. This phenotype could seem irrelevant for normal B cell development as endogenous OBF-1 is expressed from the preBI cell stage onwards. However a second differentiation block occurs downstream at the large preBII cell stage in the BCS mice suggesting that OBF-1 overexpression in committed B cells is also deleterious. Furthermore *ID2* and *ID3* were identified as OBF-1 direct target genes, an observation which could explain both differentiation blocks. Interestingly ID2 and ID3 were both upregulated in preB cells, but with a different kinetic, and this is concomitant with OBF-1 expression level. Therefore *ID2/3* are the first genes which could be directly modulated by OBF-1 in early B cells (Figure 15). Interestingly OBF-1 can also bind to the *EBF1* promoter, this mechanism induces a positive feedback loop as *OBF-1* is induced by EBF1. Therefore OBF-1 expression contributes to the irreversibility of B cell commtiment.

Gadd45β and *CyclinD2* are involved in p53 signalling and were also identified as potential OBF-1 target genes by our microarray study and ChIP assays. Interestingly *CyclinD2* was also identified as a p34 specific gene in the BAC transgenic mice. It is not yet clear what the physiological relevance of this observation is.

The potential physiological role of the cytoplasmic OBF-1 isoform was observed only in early B cells as the IL-7 dependent proB cells from mice expressing only the p34 isoform were still hyperproliferative. The physiological roles of the bone morphogenetic proteins in the bone marrow concern mainly osteogenesis and stem cell differentiation. However it was reported that Bmrp2 could cooperate with c-Kit (Hassel et al., 2006). p35 seemed to have a negative effect on BMP signalling as Bmrp2, Smad1 and MEKK1 were upregulated in mice expressing only the p34 isoform. Therefore p35 could potentially modulate the preBI cell development when c-Kit is expressed. Furthermore microarray analysis showed that few genes were regulated by p35, and *BCNP1* might be one of these genes.

The observation that p34 level associated with ID proteins expression and the presence of the p35 isoform are crucial for early B cell differentiation and proliferation respectively argue for an important role of both isoforms in early B cells. The hyperproliferation origin of IL-7 dependent proB cells from *OBF-1*$^{-/-}$ mice is not fully understood, however the 2D-DIGE assay of EPLM culture cells showed that PCNA, ERp5 and S100A4 were upregulated in cells from *OBF-1*$^{-/-}$ mice, which correlates with their hyperproliferation and probably is associated with an increased stress. Furthermore S100A4 is a Ca^{2+}-binding protein which might potentially modulate the preBCR signalling. In fact S100A10 was downregulated in EPLM and large preB cells of BCS mice, which suggests that there is a negative correlation between OBF-1 and S100 calcium binding proteins expression. Western blot analysis of EPLM cells showed that Syk was downregulated in the cells lacking p35, and this defect could also modulate the preBCR response.
In brief, we have generated transgenic mice overexpressing OBF-1 in B cells as well as mice expressing specific OBF-1 isoforms. The former showed that proper OBF-1 expression level is crucial for early B cell development. The latter showed that the nuclear OBF-1 isoform plays a role during all B cell ontogeny with a main function in the spleen and that the cytoplasmatic isoform has a secondary function probably specifically in early B cells. These findings give a more detailed understanding of OBF-1 functions in all the B cell developmental stages.

Figure 15: Putative model for OBF-1 in preB cells
In preBI cells EBF1 activates the transcription of *OBF-1*, which might induce *Id3* expression. OBF-1 in turn induces *EBF1* expression in a positive feedback loop. In large preBII CD43^{+} cells *OBF-1* is upregulated potentially via a unknown factor (X). OBF-1 then might active *Id2* transcription. Finally in large preBII CD43^{-} cells *Id2* and *Id3* are downregulated potentially because of SIAH mediated proteasome degradation of OBF-1.



4 Materials and methods

This section describes the materials and methods that were used in the Appendix.

Mouse strains and cell line

BCS mice:
The *Eμ-V$_H$-OBF-1* construct used to generate the BCS mouse line contains an N-terminally HA epitope-tagged human *OBF-1* cDNA under the control of the murine VDJ enhancer (*Eμ*) coupled to the *V$_H$* promoter. The transgenic mouse line was obtained and bred in *B6CF1* x *C57BL/6* background and was bred to a wild type partner of the same genetic background.

BAC transgenic mice:
Modified BACs were used to generate transgenic founders in a *B6CF1* x *C57BL/6* background. The mice were then crossed with *OBF-1*$^{-/-}$ mice which were in a *129SV-C57BL/6* background.

Abelson cells:
The Abelson pro-B cell line was derived from a wild type mouse as described previously (Schubart et al., 1996b).

ET cloning

In a two-step approach a counter-selection cassette is first introduced at the location to be modified and in the second step replaced by non-selectable DNA (containing a mutation for instance). The counter-selection cassette contains the *rpsL* and the *neomycin* genes which confer Streptomycin sensitivity and Kanamycin resistance respectively. The first selection is performed with Kanamycin and the counter-selection for non-selectable DNA replacement is done with Streptomycin.

PAGE oligonucleotide purification

Polyacrylamide Gel Electrophoresis (PAGE) purification of long primers was performed to generate PCR fragments for ET cloning. 10% TBU was prepared by dissolving 96 g urea in 66.66 ml acrylamide/Bis-acrylamide 30% and by heating. ddH_2O was added till a 180 ml volume followed by the addition of 20 ml 10xTBE buffer (0.89M Tris, 0.89M boric acid, 20mM EDTA). Then the 10% polyacrylamide gel (8M urea) was prepared by mixing 80 ml 10% TBU, 500 μl APS 10% and 80 μl TEMED and the gel was polymerized between two glass plates. A pre-run was performed for 30 minutes at 500 V with 0.5xTBE in the upper container and 1xTBE in the lower container.
The oligonucleotides were EtOH precipitated and resuspended in 75 μl loading buffer (1.8ml formamide, 87.5μl ddH_2O, 87.5μl 10xTBE, 20μl EDTA 500mM) by heating at 75°C for 10 minutes.
After loading the oligonucleotides, the gel was run at 500-550 V for about 2h30. The primers were then cut out of the gel after UV shadowing visualization. The DNA was eluted overnight at 4°C by shaking with 600 μl TE buffer (10mM Tris, 1mM EDTA,

pH8.0). The primers were then EtOH precipitated and resuspended in 60 µl TE buffer. The oligonucleotides were finally recovered with a Biospin 6 column.

PCR

To determine the BAC transgenic genotype, genomic DNA was isolated from tail biopsies and used as a template for polymerase chain reaction (PCR).

The following primers were used to detect the plasmidic part of the BAC:
5'-TAC GGC GGC GGC ACG AAC TTC-3' and 5'-GGG GGA TCG CCA ACA AAT ACT ACC-3'

The following primers were used to detect the *Neo* cassette of the *OBF-1* KO locus:
5'-GGC TTA GAT AAC AAA GCG TGT GCT C-3' and 5'-GCG TGC CCA TCT TGT TCA ATG G-3'

Genomic QPCR was performed to identify which BAC transgenic mice is in an *OBF-1*$^{-/-}$ background. 1.5 µl genomic DNA (1:20 diluted) were used per total 25 µl reaction mix. The following primers were used to determine the number of *Neo* cassettes and amplify the reference gene *18S* respectively:
Neo: 5'-TTC CTT GCT CCT GCC GAG AAA GT-3' and 5'-TTC GCT TGG TGG TCG AAT G-3'
18S: 5'-TTG ACG GAA GGG CAC CAC CAG-3' and 5'-GCA CCA CCA CCC ACG GAA TCG-3'

The *BCS* transgene was detected using the following primer combinations:
5'-CTG TCC AGC CCC ACC AAA CCG-3' and 5'-CAC ACG GAC GCC CTG GTA TGG-3'

The following primers were used to perform qPCR analysis in BAC transgenic mice:
Smad1: 5'-TTT CAG ATG CCA GCT GAC ACA C-3' and 5'-GCA ACT GCC TGA ACA TCT CCT C-3'
Map3K1: 5'-AGA GAA GAC GCT GAG TGG CTG A-3' and 5'-TGC TCG GAG GAT GTG TTT CTG-3'
KLF12: 5'-AGT GCC GGT TGT CTA CAC AGC T-3' and 5'-ACA TTT GGC AGG TCA TCA TCG T-3'

Quantitative real-time PCR (QPCR) was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using a SybrGreen-based kit from Eurogenetics.

Cell culture

COS7 and 293T cells were maintained in DMEM plus 10% fetal calf serum. Abelson cells were grown in RPMI supplemented with 10%FCS, L-Glutamine and 50 mM β-mercaptoethanol.
The COS7 and 293T cells were transfected with the Ca-P method. Briefly for a 10cm dish, 125 µl 4x Ca^{2+}-Mix (0.5 M $CaCl_2$ / 0.1 M Hepes pH7) is added to 125 µl ddH_20 containing 10 µg total DNA (plasmid and Salmon Sperm DNA) and vortexed for few seconds. Then the mix is incubated 1 minute with 250 µl 2x P-Mix (0.75 mM

Na_2HPO_4 / 0.75 mM NaH_2PO_4 / 0.05 M Hepes pH7 / 0.28 M NaCl) and added dropwise on the cells. After 12-14 hours the cells are washed 2-3 times with PBS.

Protein extract, Western blotting and luciferase assay

For Western blotting, the cells were lysed by repeated cycles of freeze and thaw in 20 mM HEPES pH7.9, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 400 mM KCl and Complete Protease Inhibitor cocktail (Roche). Protein concentrations were determined with Bradford reagent (BioRad). The protein extracts were resolved by SDS-PAGE with subsequent transfer onto Protran Nitrocellulose membrane (Whatman). The proteins were detected with specific antibodies and with the ECL system (Amersham).
The Promega Dual Luciferase assay kit was used for the luciferase and mammalian two-hybrid assays. Briefly the 293T cells in a 5cm dish were washed with PBS and lysed in 400 µl 1xPLB by 2 cycles of freeze and thaw. 20 µl of supernatant (1:10 dilution) were measured in a Bertold Mikras Luminometer. The luciferase and Renilla signals were measured after 50 µl LARII and 25 µl STOP&GLO injection.

Immunofluorescent staining and flow cytometry (FACS) analysis

Single cell suspensions of bone marrow or spleen were prepared, stained and analyzed according to standard procedures (Rolink et al., 1994). FACS analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA), and fluorescence data were collected by gating on living cells and typical forward-side scatter appearance of lymphocyte. CellQuest and FlowJo softwares were used for collecting and analyzing the data respectively. Cell sorting was performed on a MoFlo (DakoCytomation).
Biotinylated peanut agglutinin (PNA-biotin, Vector Lab) was used for immunohistochemistry.

RNA preparation and hybridization to Affymetrix Microarrays

Cells were FACS sorted and RNA was purified with Rneasy Microkit from Qiagen. Total RNA (~50 ng) from each replicate was reverse transcribed and labeled using the Affymetrix 2-cycles labeling kit according to manufacturer's instructions. Biotinylated cRNA was fragmented by heating with magnesium (as per the Affymetrix instructions) and this fragmented cRNA was hybridized to Mouse 430v2 GeneChips (Affymetrix, Santa Clara, Calif.). Data were analyzed using Expressionist (Genedata AG). The normalized data were subjected to a Student *t*-test ($P < 0.01$) and were required to have a median fold change of 2 and 1.5 for BCS mice and BAC transgenic mice respectively.

Coimmunoprecipitation (CoIP)

The cells were washed with PBS and lysed in NP-40 lysis buffer (150 mM NaCl / 1% NP-40 / 50 mM Tris pH8.0 / complete protease inhibitor / 25 mM NaF) (1ml buffer for 10^7 cells) for 30 minutes on ice. After spinning at 10'000g for 10 minutes at 4°C, the α-HA antibody (1 µg for 500 µl sup) was added to the supernatant. After overnight incubation at 4°C, the immune complex was captured by adding 25 µl Protein A beads 50% slurry with 3 hours incubation at 4°C with rocking. The beads were then

washed three times with NP-40 lysis buffer. The beads were finally heated with 50 µl Laemmli buffer at 85°C for 10 minutes.

Chromatin Immunoprecipitation (ChIP)

5 x 10^7 Abelson B cells were harvested, suspended in 30 ml RPMI 1640 and crosslinked for 10 minutes at room temperature with 810µl 37% formaldehyde. Crosslinking was stopped by addition of 3 ml 1.25 M Glycine. Cells were washed twice with 15 ml PBS at room temperature, resuspended in 1 ml Cell Lysis Buffer (85 mM KCl / 0.5% NP40 / pH 8.0 / 1mM PMSF) + Complete Protease Inhibitor (Roche) and incubated on ice for 10 minutes. Nuclei were spun down for 5 minutes at 4°C at 5'000 rpm, then lysed for 10 minutes on ice in 2.2 ml Nuclei Lysis Buffer (50 mM Tris, pH 8.0 / 10 mM EDTA / 1% SDS / 1 mM PMSF) + Complete Protease Inhibitor (Roche). Chromatin was sonicated with a Branson 250 sonicator: 12 X 12'' pulses with 35% intensity and with 30'' pause interval, precleared by centrifugation at 13'200 rpm for 10 minutes at room temperature. Chromatin was transferred to fresh vials and stored at -80°C.
To immunoprecipitate chromatin with the anti-OBF-1 antibody, 500 µl chromatin were diluted with 1.5 ml ChIP Dilution Buffer (0.01% SDS / 1.1% Triton X-100 / 1.2 mM EDTA / 16.7 mM Tris pH8.1 / 167 mM NaCl / 1mM PMSF / Complete Protease Inhibitor). The diluted chromatin was precleaned with 40 µl Salmon Sperm DNA-ProteinA-Agarose 50% slurry and incubated at 4°C with rotation for 30 minutes. Immunoprecipitation of 2 ml supernatant was performed with 5 µg monoclonal OBF-1 antibody C-20 (SC-955X, Santa Cruz Biotechnology, CA). 5 µg rIgG were added to the control tube. Immune complexes were captured with rotation at 4°C for 2.5 hours with 60 µl Salmon Sperm DNA-ProteinA-Agarose 50% slurry. Beads were washed with 500 µl Low Salt Immuno Complex wash buffer (0.1% SDS / 1% TritonX-100 / 2 mM EDTA / 20 mM Tris pH8.1 / 150 mM NaCl), then with 500 µl High Salt Immuno Complex wash buffer (0.1% SDS / 1% TritonX-100 / 2 mM EDTA / 20 mM Tris pH8.1 / 500 mM NaCl), then with 500 µl LiCl Immuno Complex wash buffer (0.25 M LiCl / 1% NP40 / 1% deoxycholic acid / 1 mM EDTA / 10 mM Tris pH8.1) and finally twice with 500 µl 1xTE buffer. Immunoprecipitated chromatin fragments were eluted twice with 250 µl 0.1M $NaHCO_3$, 1%SDS for 15 min. at room temperature with rotation and crosslinking was reversed with 200 mM NaCl at 68°C overnight. The protein were digested by adding 10 µl 0.5 M EDTA, 20 µl 1 M Tris pH 6.8 and 1 µl 20 mg/ml Proteinase K and incubating 1 hour at 45°C. After extraction with phenol-chloroform and chloroform, DNA was precipitated with Ethanol and resuspended in 50 µl ddH_2O. 1-3µl immunoprecipitated DNA and 1/200 diluted input chromatin were analysed by PCR.
The following primers were used to amplify the promoter containing octamer site by PCR:
Gadd45β: 5'-CGA GGC CAG GAC CCA GG-3' and 5'-AGG TGA CTG AAA GGC AGC CG-3'
CyclinD2: 5'-CCA TTG ATA GGG TCC GAG GAG-3' and 5'-TAG CGG AGG CTG AGG CTG-3'

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Curriculum Vitae

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Education

2004 - 2008	**PhD in biochemistry, Friedrich Miescher Institute in Basel, Switzerland**
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Jan. - Feb. 2004 **Fribourg University, Switzerland**
Technician in biochemistry
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Oct. 2006 - Feb. 2007 **Venture Challenge Workshop, Basel University, Switzerland**
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List of Publications

Bartholdy B., Du Roure C., Bordon A., Emslie D., Corcoran L.M., Matthias P. (2006) The Ets factor Spi-B is a direct critical target of the coactivator OBF-1. *PNAS*, **103**, 11665-11670.

Albrecht U. and Bordon A. (2007) The Multiple Facets of Per2. *Cold Spring Harbour Meeting on Chronobiology*.

Langmesser S., Tallone T., Bordon A., Rusconi S., Albrecht U. (2008) Interaction of circadian clock proteins Per2 and Cry with Bmal1 and Clock. *Molecular Cell Biology*, **9**:41.

Bordon A., Bosco N., Du Roure C., Bartholdy B., Kohler H., Matthias G., Rolink A.G., Matthias P. (2008) Enforced expression of the transcriptional coactivator factor OBF1 impairs B cell differentiation at the earliest stage of development (submitted to PLoS ONE).

Bordon A., Bosco N., Kohler H., Rolink A.G., Matthias P. (2008) Dissecting the function(s) of the OBF-1 isoforms (manuscript in preparation).

Bordon A. and Matthias P. (2008) Intrinsic and extrinsic factors in early B cell development (manuscript in preparation).

6 Appendix

6.1 ET cloning detail

6.1.1 BAC structure

The BAC clone (*RP24 -173L14*) containing the mouse *OBF-1* gene was ordered at the BACPAC Resource Center (BPRC) at the Children's Hospital Oakland Research Institute in Oakland, California. The BAC was constructed by inserting the mouse genomic DNA containing the *OBF-1* gene into the *pTARBAC1* vector (Fig. 16) between the BamHI sites. The resulting BAC clone was then modified by ET cloning.

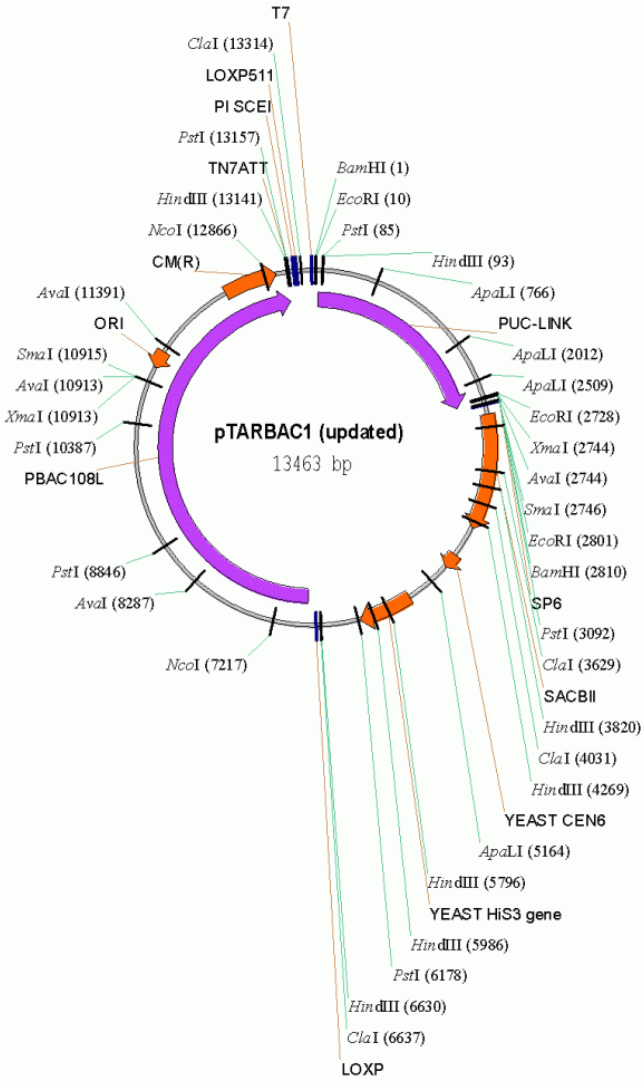


Figure 16: Scheme of the plasmid that was used to construct the BAC at the BPRC. The structure of the cloning vector with the different restriction sites is depicted. The cloning vector confers Chloramphenicol resistance (CM). The genomic DNA was inserted between the BamHI restriction sites.

6.1.2 ET cloning scheme

The following ET cloning procedure was adapted from the Counter-Selection BAC modification kit protocol from GeneBridges, Dresden, Germany. The name ET cloning comes from the RecE/RecT (or Redα/Redβ in our protocol) recombinases which are used to recombine homologous sequences. Briefly in a first step the *rpsL-neo* cassette is introduced in the location of interest. After selection against the selectable marker *neo* with Kanamycin the correct recombinants can be verified. In a second modification step the *rpsL-neo* cassette is replaced by a PCR generated non-selectable fragment. By selecting against the counter-selectable marker *rpsL* with Streptomycin, only bacteria which have undergone successful substitution by the non-selectable fragment will grow (Fig. 17).

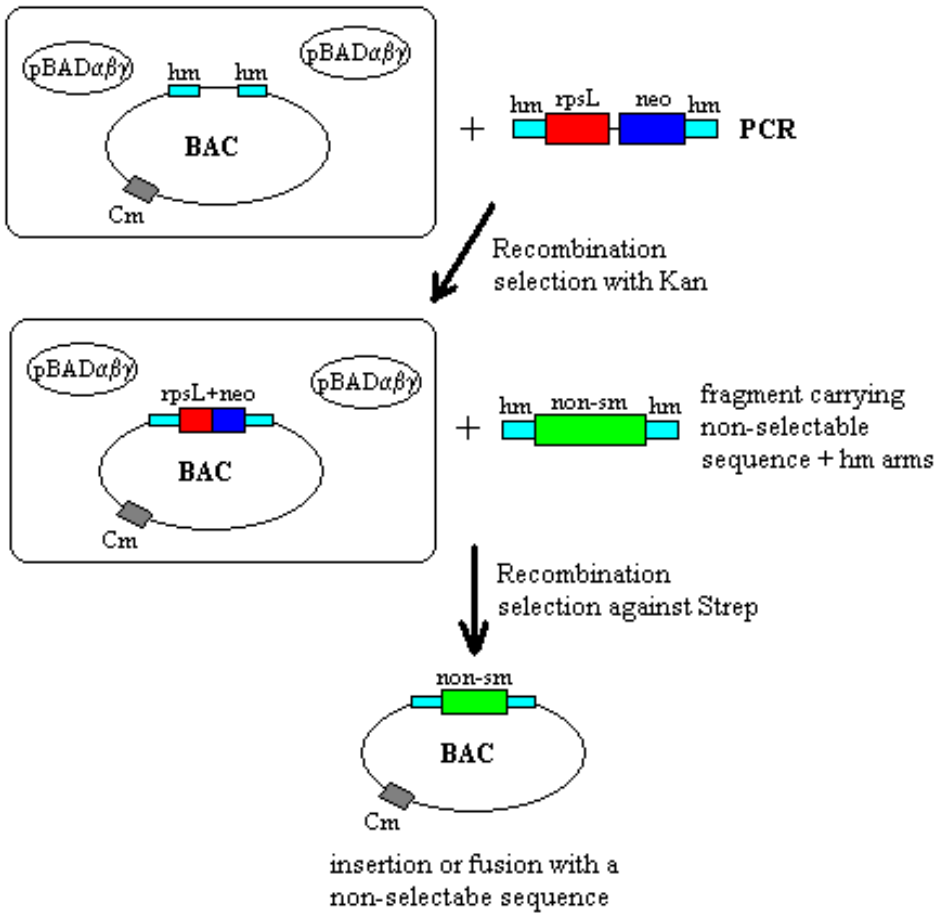


Figure 17 : ET cloning scheme

The procedure is described in the text. The plasmid pBADαβγ (Ampr) expresses the ET recombinase. hm = homology, *Cm* = Chloramphenicol resistance gene, non-sm = non-selectable marker

The plasmid *pBADαβγ(Amp^r)*, which encodes the recombinases and is L-arabinose inducible, was a gift from Prof. Busslinger and the plasmid *pRpsLneo* was ordered at GeneBridges to generate PCR fragments containing the *rpsL* and *neo* cassette.

6.1.3 ET cloning protocol

6.1.3.1 Electroporation of the plasmid *pBADαβγ(Amp^r)*

Electroporate the plasmid *pBADαβγ* as the GeneBridges protocol. The plasmid we have in the Matthias lab is a bit different from the ET cloning kit of GeneBridges. Our plasmid is not temperature sensitive, so you can grow the bacteria at 37°C without loosing it. The required ampicillin concentration is **100μg/ml** !

GeneBridges protocol for plasmid electroporation:

Before starting:

- Chill ddH_2O on ice for at least 2 hours.
- Chill electroporation cuvettes (1 mm).
- Chill the cuvette holder.

Set up an overnight culture. Pick at least ten colonies carrying the BAC and inoculate them together in an Eppendorf tube containing 1 ml LB medium with chloramphenicol (15 μg/ml) to select for the endogenous BAC. Puncture a hole in the lid for air. Incubate over night at 37°C with shaking. For testing of the streptomycin resistance, streak some colonies carrying the BAC on agar plates containing 50μg/ml streptomycin in addition to chloramphenicol (15 μg/ml). The colonies should grow on streptomycin plates (*rpsL-neo* counter-selection only works in E.coli strains carrying a mutated *rpsL* gene conferring to a streptomycin resistant phenotype).

The next day set up an Eppendorf tube containing fresh 1.4 ml LB medium conditioned with chloramphenicol and inoculate with 30 μl of fresh overnight culture. Culture for 2-3 hours at 37°C, shaking at 1000 rpm.

Prepare the cells for electroporation.
Move to the cold room and centrifuge for 30 seconds at 11,000 rpm in the benchtop centrifuge. Discard the supernatant by quickly tipping out the supernatant twice, and place the pellet on ice. Resuspend the pellet with 1 ml chilled ddH_2O, pipetting up and down three times to mix the suspension. Repeat the centrifugation and resuspend the cells again. Centrifuge and tip out the supernatant once more; 20 to 30 μl will be left in the tube with the pellet. Keep the tube on ice.

Wash several times a 1 mm electroporation cuvette (~ 4x) with chilled ddH_2O. Add 0.2-0.5 μg of the *pBADαβγ* plasmid to the pellet in the Eppendorf tube. Pipette several times a yellow tip in the chilled ddH_2O and pipette the mixture of bacteria and DNA into the chilled electroporation cuvette.

Electroporate at 1350 V, 10 μF, 600 Ohms. The pulse should be about 5 ms.

Add 1 ml SOC medium at RT without antibiotics to the cuvette. Mix the cells carefully by pipetting up and down and pipette back into the Eppendorf tube. Incubate the cultures at 37°C, shaking for 70 min.

Using a small loop, plate 100 μl bacteria on a LB agar plate containing ampicillin (100μg/ml) and chloramphenicol (15μg/ml). Incubate the plate at 37°C overnight.

6.1.3.2 Generation of a *rpsL-neo* PCR product flanked by homology arms

The oligonucleotides must be PAGE purified and are suspended in ddH_2O at a final concentration of 25 pmol/μl.

PCR reaction (in 50 μl)

28.5 μl	ddH_2O
5 μl	10xPCR buffer w/o $MgCl_2$
3 μl	$MgCl_2$
10 μl	dNTPs 1mM
1 μl	*pRpsL-neo* (10ng/μl)
1 μl	upper oligonucleotide
1 μl	lower oligonucleotide
0.5 μl	Taq polymerase

PROGRAM

94°C	3min.	
95°C	1min.	X 35
62°C	1min.	
72°C	1min/kb	
72°C	10min.	
4°C	∞	

Purify the PCR fragment by Qiagen kit (elution with ddH2O 1x with 50 μl and 1x with 40 μl). Add 10μl 10xNEB1 buffer and 2 μl DpnI. Digest O/N at 37°C.

Extract with 100 μl Phenol:CHCl3, add 12 μl NaAc 3M and 360 μl EtOH. Incubate 10 min. at –80°C and centrifuge 5 min. at 4°C. Wash the DNA pellet with 70%EtOH. Dry the pellet at 37°C using a heating block for 5-10 min. Resuspend in 5 μl Tris-HCl, pH 8.0 (0.2-0.5 μg/μl) or in ddH_2O.

The Taq polymerase is used to generate rpsL-neo fragments; however this enzyme can induce some mutations in the amplicon. Therefore the recombined colonies in the following step will have to be screened for Streptomycin sensitivity. Although the Taq polymerase works well, one can use the Pfu polymerase instead, which is not mutagenic and do not require colony screening.

6.1.3.3 Inserting the *rpsL-neo* cassette into a BAC (modified GeneBridges protocol)

The following GeneBridges protocol works well, but the Stewart method (described for non-selectable fragment insertion) for preparing bacteria for electroporation works probably even better.

Before starting:

- Chill ddH_2O on ice for at least 2 hours.
- Chill electroporation cuvettes (1 mm).
- Chill the cuvette holder.

To start O/N cultures, pick a colony from the plate with the bacteria containing the *pBADαβγAmp*^{r} plasmid and inoculate Eppendorf tubes containing 1 ml LB medium plus ampicillin (100 μg/ml) and chloramphenicol (15 μg/ml). Puncture a hole in the lid for air. Incubate the culture while shaking at 37°C O/N in the thermoshaker.

The next day, set up 4 lid-punctured Eppendorf tubes (2 for your own experiment and 2 for checking the OD_{600}) containing 1.4 ml fresh LB medium conditioned with Amp and Cm, and inoculate each with 30 μl fresh overnight culture. Incubate the tubes at 37°C shaking at 1100 rpm until $OD6_{00}$ ~ 0.14. To check $OD6_{00}$ take 100 μl from one of the 2 extra tubes and add into 400 μl LB (1:5 dilution). The best OD_{600} of the diluted sample is ~0.027. Do not let the culture overgrow beyond an OD_{600} of 0.2 (undiluted)!

Add 20 μl 10% L-arabinose to half of the tubes (1 for your own and 1 for the extra tubes), giving a final concentration of 0.1%-0.2%. This will induce the expression of the Red/ET recombination proteins. Don't use D-arabinose. Leave the other tubes without induction as negative controls. Incubate at 37°C, shaking for 1 hour until OD_{600} ~0.35 – 0.4 (OD_{600} of the diluted sample ~0.07 – 0.08). Do not let the culture overgrow beyond an OD_{600} of 0.4 (undiluted)! In the mean time let 2 Eppendorf tubes containing 1 ml SOC medium warm up at RT.

Prepare the cells for electroporation.
Move to the cold room and centrifuge for 30 seconds at 11,000 rpm in the benchtop centrifuge. Discard the supernatant by quickly tipping out the supernatant twice, and place the pellet on ice. Resuspend the pellet with 1 ml chilled ddH_2O, pipetting up and down three times to mix the suspension. Repeat the centrifugation and resuspend the cells again. Centrifuge and tip out the supernatant once more; 20 to 30 μl will be left in the tube with the pellet. Keep the tube on ice.

Wash several times the electroporation cuvettes (~ 4x) with chilled ddH_2O. Add 1 μl (0.2-0.3 μg) of your prepared linear *rpsL-neo* fragment with homology arms to the pellet in the Eppendorf tube. Pipette several times a yellow tip in the chilled ddH_2O and pipette the mixture of bacteria and DNA into the chilled electroporation cuvette.

Electroporate at 1350 V, 10 μF, 600 Ohms. The pulse should be about 5 ms.

Add 1 ml SOC medium at RT without antibiotics to the cuvette. Mix the cells carefully by pipetting up and down and pipette back into the Eppendorf tube. Incubate the cultures at 37°C, shaking for 70 min. Recombination will now occur.

Centrifuge the culture to pellet the bacteria, remove the supernatant in order to have about 100 μl left in the tube and streak the cultures with a loop onto LB agar plates containing chloramphenicol (15μg/ml), kanamycin (15μg/ml) and ampicillin (100μg/ml). Incubate the plates at 37°C O/N.

Pick 10 colonies from the plates (induced) and inoculate them in 100 μl of LB medium with Cm+Km+Amp (15+15+100 μg/ml).

Incubate at 37°C with shaking at 1100 rpm for 1-2 hours.

After 1-2 hours, use a loop to streak a small sample of the culture on Streptomycin (50 μg/ml), Kanamycin (15μg/ml) and Chloramphenicol (15 μg/ml).

Incubate the plate at 37°C O/N to test the function of the *rpsL-neo* cassette.

Transfer 30 μl of culture from the shaking at 37°C into 2 ml of fresh LB culture with the appropriate antibiotics (Cm+Km or Cm). Incubate at 37°C O/N with shaking at 1100 rpm. These cultures will be used for preparing BAC DNA and/or for PCR verification.

Add 300 μl of LB medium into the tubes from the shaking at 37°C with Cm+Km+Amp (15+15+100 μg/ml) and incubate at 37°C O/N. These cultures will be used for a second round of Red/ET to replacing rpsL-neo cassette by a non-selectable gene or an oligonucleotide.
One can make a glycerol stock of the bacteria, but before using the glycerol stock for the replacement of the rpsl-neo cassette one has to plate the bacteria first on a Cm/Km/Amp plate and then prepare the bacteria for electroporation otherwise the bacteria will dye after electroporation.

After successful confirmation that the clones carrying the *rpsL-neo* insertion in the BAC (restriction pattern or PCR product) and that the *rpsL* gene is not mutated (function test: streptomycin sensitivity), you can go on with the next steps. It is very important to make sure that the plasmid *pBAD*$\alpha\beta\gamma$ is still present in the bacteria (Amp resistance / miniprep as GeneBridges protocol). If you use 50 μg/ml Ampicillin instead of 100μg/ml, the bacteria will grow even if it has lost the *pBAD*$\alpha\beta\gamma$ plasmid, so make sure to use the right Amp concentration.

6.1.3.4 Generation of a non-selectable PCR product flanked by homology arms

So far the replacement of the *rpsL-neo* cassette by a non-selectable PCR fragment worked only when the PCR fragment has homology arms longer than 50 bp.
I suggest that the region to be modified in the BAC is subcloned into a working vector like *pBluescript*, and then the final desired modification is performed in this vector with conventional methods. Now a PCR fragment with longer homology arms can be easily generated using small primers (it is not absolutely necessary to do PAGE purification of small primers) and Pfu polymerase.

If you want to try anyway to generate a PCR fragment with 50 bp homology arms like the GeneBridges protocol, you can use the following procedure (the oligonucleotides

must be PAGE purified and are suspended in ddH_2O at a final concentration of 25 pmol/μl):

PCR reaction (in 50 μl)

29.5 μl	ddH_2O
5 μl	10xPCR buffer w/o $MgSO_4$
3 μl	$MgSO_4$
10 μl	dNTPs 1mM
1 μl	upper oligonucleotide
1 μl	lower oligonucleotide
0.5 μl	Pfu polymerase

Resuspend bacteria containing the wild type BAC in the PCR mix.

PROGRAM

94°C	4min.	
95°C	1min.	X 35
Tm°C	1min.	
72°C	2min/kb	
72°C	10min.	
4°C	∞	

Purify the PCR fragment by Qiagen kit (elution with ddH_2O 1x with 50 μl and 1x with 40 μl). Add 10μl 10xNEB1 buffer and 2 μl DpnI. Digest O/N at 37°C.

Extract with 100 μl Phenol:CHCl3, add 12 μl NaAc 3M and 360 μl EtOH. Incubate 10 min. at –80°C and centrifuge 5 min. at 4°C. Wash the DNA pellet with 70%EtOH. Dry the pellet at 37°C using a heating block for 5-10 min. Resuspend in 5 μl Tris-HCl, pH 8.0 (0.2-0.5 μg/μl) or ddH_2O.

6.1.3.5 Replacing the *rpsL-neo* cassette by non-selectable DNA (Stewart method for preparing bacteria for electroporation)

Preparation of the bacteria as the Stewart method:

Transfer 0.7 ml O/N culture of bacteria into 70 ml of $LB_{+antibiotics}$ and grow them at 37°C with shaking.

Prepare 10% glycerol with ddH_2O, and cool down on ice for at least 3 hours before using.

When the cells reach OD_{600} = 0.1-0.15, add 0.7 ml 10%L-arabinose to induce ET protein expression and incubate at 37°C.

After a further 1 hour, the bacteria should be at OD_{600} of 0.3-0.4.

Make sure the centrifuge and the rotor is very cold by centrifuging for 10 min., -5°C at 4000rpm.

Spin 35 ml bacteria in an Oak Ridge tube for 10 min. at 7000 rpm at –5°C. Put the other 35 mls on ice.

Pour away the supernatant, add the second 35 ml and respin.

Pour away supernatant, add the second 35 ml and respin.

Pour away supernatant, put tube on ice, resuspend bacteria in 5 ml ice cold 10%glycerol with an ice cold 5 ml pipette. Add a further 25 ml and centrifuge.

Repeat the above step twice.

Pour away supernatant and immediately dry the tube out with Kleenex tissue taking care not to touch the pellet.

Resuspend the bacteria in the remaining liquid (you should have a little more than 100 μl final resuspended volume).

Transfer 50 μl of bacteria into each pre-cooled eppendorf tube with a pre-cooled tip and freeze in liquid N_2 or use immediately.

It is **very** important that all the manipulations are performed as cold as possible !!!

Electroporation of the PCR fragments (2mm cuvettes)

(If cuvettes were reused) Wash cuvettes at least 10 times with cold ddH_2O, precool them on ice for at least 5 min.

Thaw competent bacteria on ice and add 1-2 μl PCR fragment (you should electroporate more than 1 μg PCR fragment).

Electroporate the bacteria at 2.3kV, 25 μF with Pulse controller set to 200Ω (~5ms).

Add 1 ml SOC medium at RT and transfer back into the eppendorf tube.

Incubate at 37°C for 70 min. with shaking (1100 rpm).

Plate 20μl on agar plate (15 μg/ml Cm; 50 μg/ml Streptomycin) and incubate O/N at 37°C.

For the replacement of the *rpsL-neo* cassette, you should obtain more than 500 colonies and about 50% of the colonies should have recombined.

The electroporation efficiency of the GeneBridges procedure should be a bit more than 10^8 colonies per μg DNA (high copy number plasmid).

The electroporation efficiency of the Stewart method should be a bit more than $2*10^8$ colonnies per μg DNA (high copy number plasmid).
The electroporation efficiency must be at least 10^8 colonies per μg DNA for a successful ET cloning, otherwise you will have a hard time !

6.1.3.6 Test of colonies for ET recombination

Perform colony PCR of several colonies to test ET recombination as standard protocols. It is very important to use one primer in the electroporated PCR fragment and the other primer outside of the PCR fragment. Then you can purify the PCR fragments of the recombined colonies with the Qiagen kit and send for sequencing. If you use both primers in the electroporated PCR fragment, you will have an amplified fragment in all the colonies (even if the colonies didn't recombine), but when you make a liquid culture of these colonies and perform a PCR, then no fragment will be amplified (for the unrecombined colonies).
Make minipreps of the colonies as the GeneBridges protocol and perform a digestion control of the BAC. Once the positive colonies have been identified, it is important that they would lose the *pBAD* plasmid. In order to select for colonies that do not contain it any more, proceed as follows: streak the O/N culture of the positive clone on Cm plate in order to obtain single colonies (the best is to dilute the culture 1:10000 and then plate 40 μl). The colonies will grow O/N at 37°C. Pick single colonies and spot them in parallel on a Cm plate and an Amp plate. Grow them O/N at 37°C. Select for those colonies that only grow on Cm but not Amp. If you have really problems to get rid off the *pBAD* plasmid, you can purifiy the BAC (*pBAD*$\alpha\beta\gamma$ will be copurified) and then electroporate DH10B bacteria. With this method for sure several colonies will not have integrated the *pBAD* plasmid.

To be 100% sure that the BAC has no internal rearrangements, digest the purified BAC with a restriction enzyme and perform a Pulse Field Electrophoresis (Biorad).

6.1.4 Preparation of BAC for microinjection into blastocystes

You shoul perform a CsCl purification of the BAC as follows in order to get rid off endotoxin.

Inoculate 2 ml O/N culture into 2 liters LB/Cm (15 μg/ml). Incubate O/N at 37°C.

Pour cells into 2 x 1l bottles (900ml/bottle) and one 250 ml bottle. Centrifuge 30 min. at 5000 RPM at 4°C.

Pour off supernatant and resuspend in 50 ml lysis solution I (25 ml per liter culture) (put in a 250ml bottle)

Lysis Solution I

50mM glucose
20mM Tris pH 8.0
10mM EDTA pH 8.0
Store 1 month at 4°C

Add 1.25 ml lysozyme (100 mg/ml) to 2.5 mg/ml final concentration. Mix by inversion.

Add 100 ml lysis solution II (50ml per liter culture) and mix well but gently by inversion. Let stand on ice for 90 sec. only.

Lysis Solution II

0.2M NaOH
1% SDS
(prepare fresh each day)

Add 75 ml lysis solution III (37.5 ml per liter of culture) and mix immediately by gentle swirling. Let stand on ice 10 min.

Lysis Solution III

60 ml 5M KOAc
11.5 ml glacial acetic acid
28.5 ml ddH_2O
Store 1 month at 4°C

Centrifuge the mixture 30 min at 8000 RPM at 4°C.

Pour supernatant twice through filter paper into a 1 liter bottle.

Precipitate the DNA by adding 135 ml isopropanol (0.6 vol) to the lysate and mix gently but thoroughly.

Centrifuge 30 min at 5000 RPM at 4°C. Pour off supernatant and resuspend DNA pellet in 2 ml TE buffer.
Place a 14 ml Falcon tube on a balance and set the balance to zero. Add the DNA dissolved in TE to a final weight of 2.6 g. Add 2.8 g CsCl and 200 µl EtBr (1%) to the DNA/CsCl and dissolve by inverting the tube carefully several times.

Transfer to a TLA 100.3 tube (maximum volume 3.5ml/tube), seal using Quickseal sealer, and centrifuge 18 hr at 80000 RPM at 20°C in a TLA 100 mini-ultracentrifuge.

Remove tubes and collect BAC DNA. Pierce the top of the tube with a 18-G needle as an air outlet and remove lower band (containing supercoiled plasmid DNA) by carefully withdrawing with a 18-G needle attached to a 1 ml syringe. Transfer the DNA to a new TLA 100.3 tube and fill the tube with 2.8g TE/2.8g CsCl mix (no EtBr).

Centrifuge 6 hrs at 80000 RPM at 20°C.

Withdraw the DNA by a syringe and extract the DNA in CsCl 3 to 4 times by adding an equal volume of water saturated isoamyl alcohol, inverting the tube several times, and microcentrifuging 1 min. at 13000xg at RT. Remove and discard the upper (deep pink) organic layer which contains the EtBr.

Finally, transfer the DNA to one or several 1.5ml microcentrifuge tubes and add 3 vol water to dilute the CsCl.

Add 2.5 vol of EtOH and mix carefully to precipitate the BAC DNA.

Microcentrifuge 10 min. at 13000xg at RT. Remove supernatant and wash the pellet 2 times in 70%EtOH. Air dry at RT and resuspend in 100µl TE buffer pH 8.5.

Measure the DNA concentration and check by pulse field electrophoresis.

Optional: You can linearise the BAC with the restriction enzyme PIscel if you want to make sure the BAC is properly inserted in the mouse genome. However the linearised BAC will be more fragile.

Perform a dialysis with a Millipore membrane floating on injection buffer.

Injection Buffer: 10mM Tris pH 7.5
0.1mM EDTA
100mM NaCl

Float the Millipore membrane filter disc on the surface of the injection buffer.

Deposit 20-30 µl of BAC on the center of the membrane (use a tip with a cut end so that the BAC is not sheared, never vortex the BAC).

Dialyse the BAC for about 2 hours.

Check the dialysed BAC DNA by pulse field electrophoresis.

Dilute the BAC DNA in injection buffer to 1.6 - 2 ng/µl.

Mix 50µl 2xPolyamine Buffer (60 µM Spermine, 140 µM Spermidine) with 50µl dialysed BAC (final conc. 0.8-1.0 ng/µl), incubate 48 hrs at 4°C and send for microinjection.

6.2 *In vitro* and *in vivo* studies

The section 6.2 gives an overview of some experiments that were performed during the PhD thesis to have a better understanding of the mechanism responsible for the different intracellular localizations of the OBF-1 isoforms (6.2.1 and 6.2.2), of some potentially interesting genes related to the BAC transgenic mice (6.2.3), of the 2D-DIGE assay from EPLM cells (6.2.4), of the Syk behaviour in *OBF-1*$^{-/-}$ mice (6.2.5) and of potential OBF-1 target genes in early B cells (6.2.6).

6.2.1 Distinct subcellular localizations of OBF-1 isoforms in transfected cells

Yu et al. was the first to report the existence of a cytoplasmatic OBF-1 isoform (Yu et al., 2001). Transfection experiments with GFP fusion constructs were performed to get a better understanding of the OBF-1 isoforms localizations. The p40 and p34 isoforms were found to be nuclear and the p35 isoform was cytoplasmatic. Furthermore p35 formed nuclear speckles after Leptomycin B (LMB) treatment, which is a specific inhibitor of CRM1 (nuclear exportin) (Fig. 18). This observation suggested that p35 had the potency to be exported out of the nucleus in a CRM1 dependent manner. The nuclear speckles are structures that contain high concentrations of splicing snRNPs and other splicing-related proteins (Handwerger and Gall, 2006). Nuclear speckles are thought to serve as a reservoir for factors that participate in the cotranscriptional splicing of mRNA at the chromosomes. Furthermore it is thought to be a way-station for components that accompany mRNA to the nuclear pore and/or cytoplasm (Handwerger and Gall, 2006).

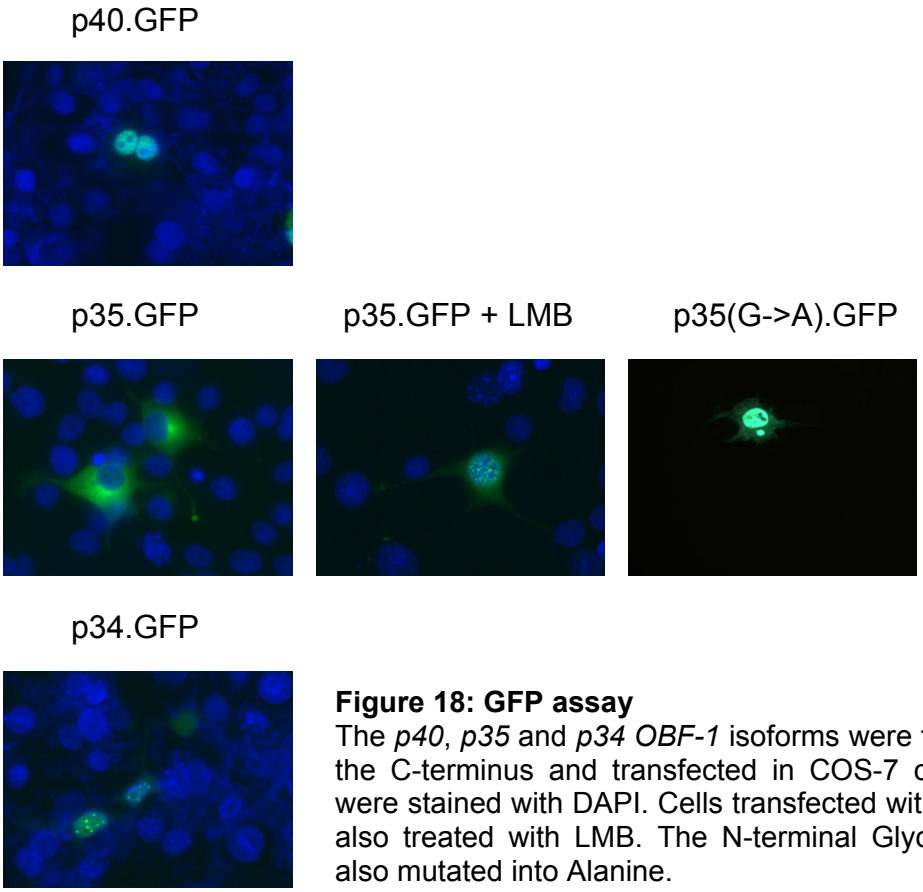


Figure 18: GFP assay
The *p40*, *p35* and *p34 OBF-1* isoforms were fused to *GFP* on the C-terminus and transfected in COS-7 cells. The nuclei were stained with DAPI. Cells transfected with *p35.GFP* were also treated with LMB. The N-terminal Glycine of *p35* was also mutated into Alanine.

The translocation of p35 in these speckles upon LMB treatment is not understood yet. Furthermore p35 had a nuclear localization when its N-terminal Glycine was mutated into Alanine (prevents myristoylation) indicating that only the myristoylation of OBF-1 prevents it from being nuclear.

Luciferase assays were performed to confirm the intracellular localizations of the OBF-1 isoforms. 293T cells were transfected with increasing amounts of expression vectors for p40, p35 or p34, together with a luciferase reporter plasmid containing the *IgK* promoter and the *pRL* control plasmid (Fig. 19). The p40 isoform was able to activate the *IgK* promoter, which confirmed its nuclear localization. Interestingly p35 induced luciferase transcription at a weaker level, indicating that this isoform was able to translocate into the nucleus transiently.

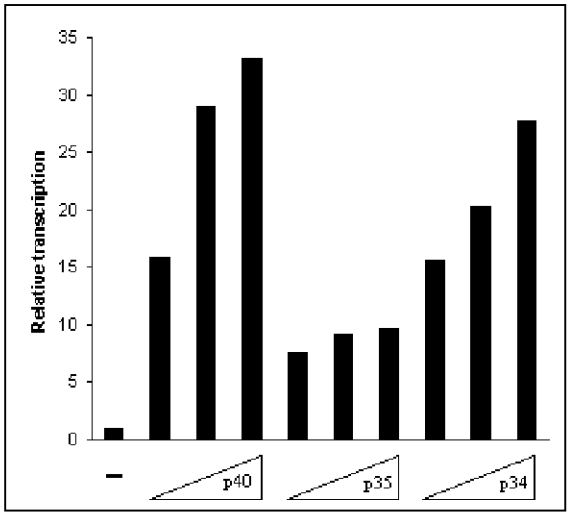


Figure 19: Luciferase assay
293T cells were transfected with a luciferase reporter plasmid and a *pRL* control plasmid. 2 fold increasing amounts of *OBF-1* plasmids were cotransfected.

6.2.2 p35 interacts with CRM1

GFP constructs transfected in COS7 cells showed that the p35 precursor p40 was restricted to the nucleus, and that p35, which is exclusively cytoplasmic, translocates into the nucleus after LMB treatement. Furthermore luciferase assays showed that p35 could translocate transiently in the nucleus. These observations led us to investigate the possibility that p40 could be cleaved into p35 in the nucleus and the latter would be exported into the cytoplasm to undergo myristoylation.
CRM1 is the best characterized nuclear exportin and is a specific target of the nuclear exportin inhibitor Leptomycin B (LMB) (Xu *et al.*, 2004). LMB induces the dissociation of the Nuclear Exporting Sequence (NES) from the nuclear export machinery by directly binding to CRM1 (Yashiroda *et al.*, 2003). CRM1 is dependent on Ran-GTP for Cargo binding. In the nucleus Ran is predominantly loaded with GTP by the guanine-nucleotide-exchange factor RCC1, whereas in the cytoplasm the Ran-bound-GTP is immediately hydrolysed into GDP by RanGAP1 thus releasing the Cargo (Fig. 20). This asymmetric distribution of RCC1 and RanGAP1 determines the direction of transport of the Cargo from the nucleus to the cytoplasm (Yashiroda *et al.*, 2003).

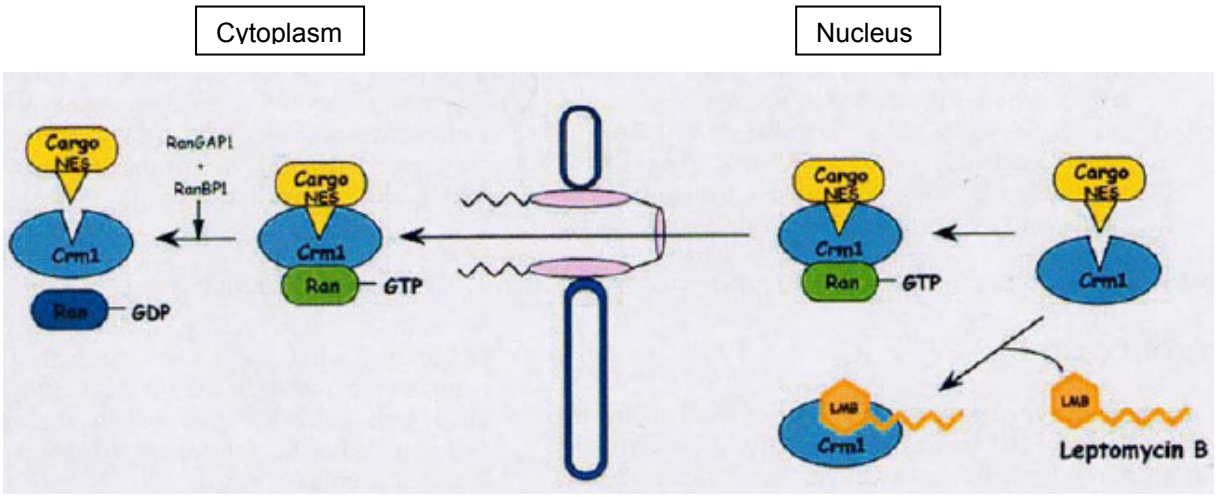


Figure 20: Nuclear exportin machinery
CRM1 is the main nuclear exportin protein and LMB inhibits its activity by antagonizing the binding of Cargo proteins and preventing Ran-GTP interaction (from Yashiroda et al., 2003).

Coimmunoprecipitation assays were performed to investigate whether OBF-1 would interact with CRM1. 293T cells were transfected with *GAL4.CRM1* and *OBF1.GFP* constructs. The HA tagged GAL4.CRM1 (GAL4 fused to CRM1) was immunoprecipitated by α-HA antibody and the OBF1-GFP fusion proteins were detected with α-GFP antibody by WB (Fig. 21). As a result p40.GFP, p35.GFP and p34.GFP to a lesser extend were coimmunoprecipitated with GAL4.CRM1 indicating that indeed the OBF-1 isoforms can bind to CRM1.

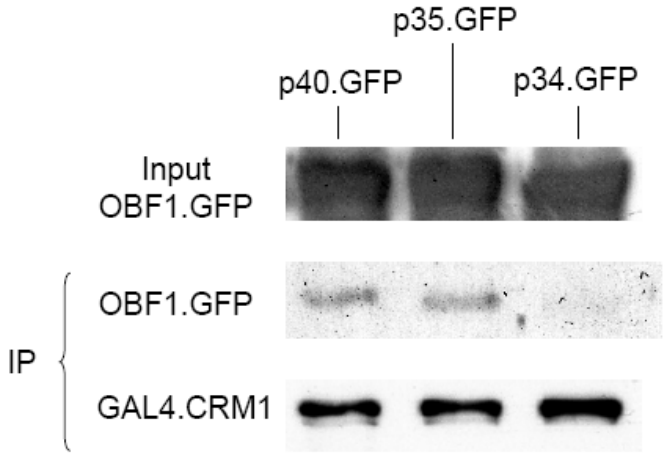


Figure 21: CoIP OBF1/CRM1

293T cells were transfected with *OBF1.GFP* and *GAL4.CRM1* constructs. GAL4.CRM1 was immunoprecipitated with α-HA Ab. The coimmunoprecipitated OBF1.GFP were detected with α-GFP Ab.

The Mammalian two-hybrid assay is a method to investigate interactions between two proteins. A reporter plasmid bearing the luciferase gene with a promoter

containing 5 *GAL4* binding sites is transfected with the *Renilla* reference plasmid. A schematic representation of the Mammalian two-hybrid assay is illustrated in Figure 22.

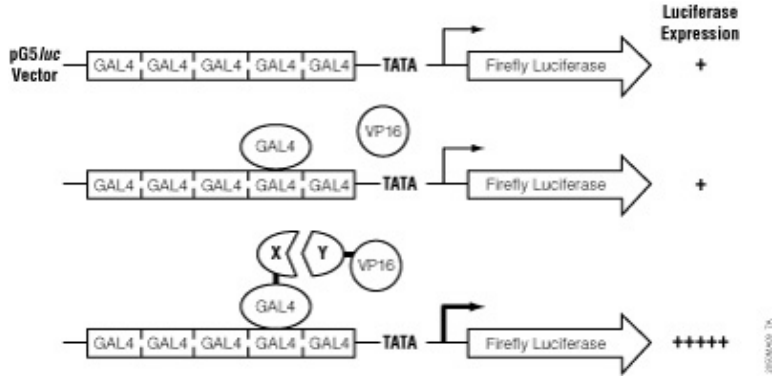


Figure 22: Schematic representation of the Mammalian Two-Hybrid System.
The pG5LucVector contains five GAL4 binding sites upstream of a minimal TATA box, which in turn, is upstream of the firefly luciferase gene. In negative controls, the background level of luciferase is measured in the presence of GAL4 (from the pBIND Vector) and VP16 (from the pACT Vector). Interaction between the two test proteins, as GAL4-X and VP16-Y fusion constructs, results in an increase in luciferase expression over the negative controls (www.promega.com/pagide/chap11.htm).

CRM1 and *OBF-1* were fused to *GAL4* and *VP16* respectively. VP16 induces the luciferase transcription when the protein partners interact (Fig. 23). Indeed CRM1 could bind to OBF-1 and the interaction was better with p35 than with p40.

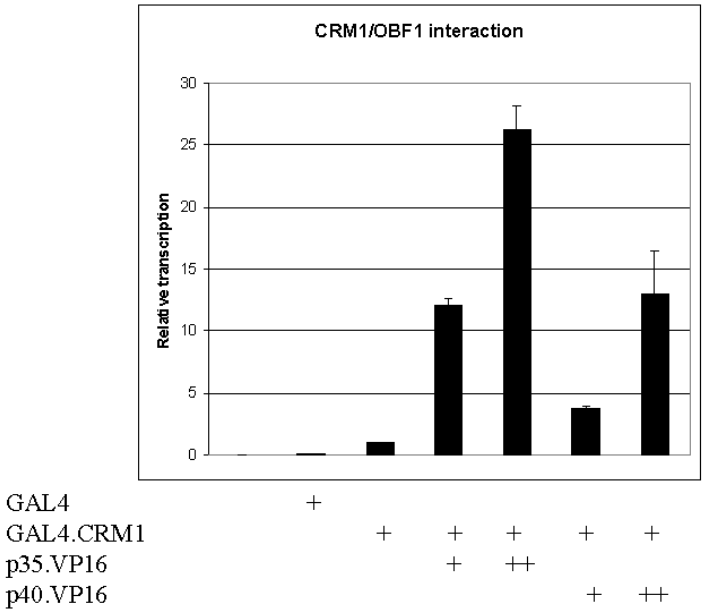


Figure 23: Mammalian two-hybrid assay
293T cells were transfected with luciferase reporter plasmid containg GAL4 binding sites, with a pRL control plasmid, with CRM1 fused to GAL4 and with OBF-1 fused to VP16. The values correspond of the mean ± SE of 2 independent experiments.

6.2.3 qPCR analysis of BAC transgenic mice

Smad1, *Map3k1* and *KLF12* were identified as p35 specific genes in the microarray experiment in total splenic B cells from BAC transgenic mice (Fig. 12). To investigate their expression pattern in stimulated B cells, mature B cells were CFSE labelled and stimulated with α-CD40 and IL-4. The cells expressing different level of Syndecan1 were sorted by FACS after 5 days. QPCR analysis revealed that the cells from *BAC p34* mice could not downregulate properly *Smad1* and *Map3k1*. On the other hand the overexpression of *KLF12* in the cells from *OBF-1*$^{-/-}$ mice was rescued by p34 (Fig. 24).

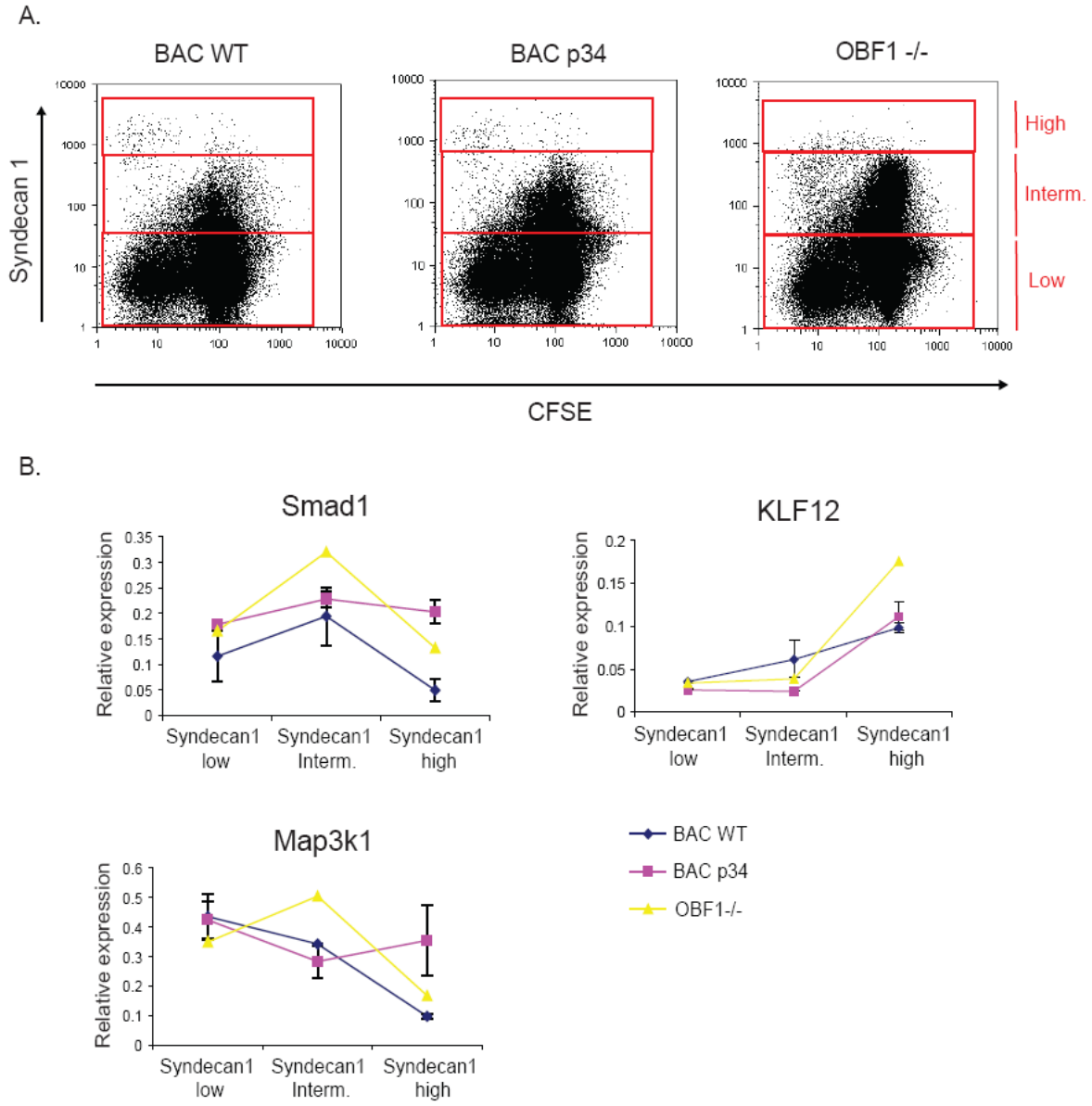
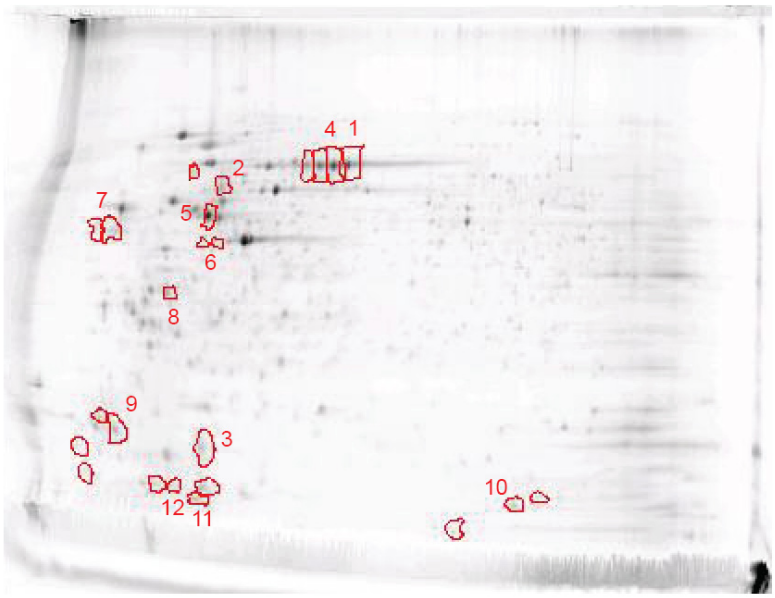


Figure 24: qPCR of *in vitro* stimulated mature B cells
(A) Mature B cells were CFSE stained and cultured for 5 days with α-CD40 and IL-4. The cells were then stained with α-Syndecan1-APC antibody. The red gates represent the populations that were sorted for RNA analysis. (B) QPCR analysis of the populations expressing different levels of Syndecan1. The values represent mean ± SE of two independent mice for *BAC WT* and *BAC p34* genotypes and one *OBF-1*$^{-/-}$ mouse.

6.2.4 2D-DIGE assay

The deletion of *OBF-1* results in hyperproliferation of ProB cells *in vitro* (see chapter 2.2). To have a better understanding of molecular origin of hyperproliferation, 2D-DIGE assay was performed with EPLM cell cutlures from *BAC WT* and *OBF-1*$^{-/-}$ mice (Fig. 25). In fact the cells from *OBF-1*$^{-/-}$ mice overexpress PCNA, PDIA6 and S100A4, which might explain the hyperproliferation of these cells. These results are discussed in the section 3.2.4.

Figure 25: DIGE assay DIGE assay was performed on EPLM cell cultures of *BAC WT* and *OBF-1*$^{-/-}$ mice. The proteins upregulated in cells from *OBF-1*$^{-/-}$ mice are in red.



1: BSA
2: Vimentin
3: not identified
4: BSA + HSP7C
5: PDIA6 + ATPB
6: αActin
7: not identified
8: AnnexinV + PCNA
9: not identified
10: CH10 (Heat Shock Protein (mitochondrial))
11/12: S100A4

increased in cells from *OBF1-/-* mice

decreased in cells from *OBF1-/-* mice

6.2.5 Collaboration with Peter Nielsen: putative involvement of the protein tyrosine kinase Syk

Roeder reported that OBF-1 might interact with Syk in splenic B cells. Therefore we started a collaboration with Dr. Peter Nielsen at the Max Planck Institute in Fribourg (Germany) to try confirming his results. However the experiments did not confirm an interaction between Syk and OBF-1 in mature B cells.

Drosophila S2 cells were transfected with *Syk* fused to *RFP* and *OBF-1* isoforms fused to *GFP* (Fig. 26). In the cells cotransfected with *Syk* and *p35*, blobs on the cell periphery where Syk and p35 colocalize appeared. The origin of these blobs was not understood.

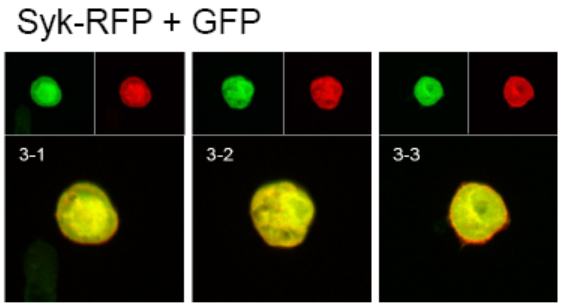
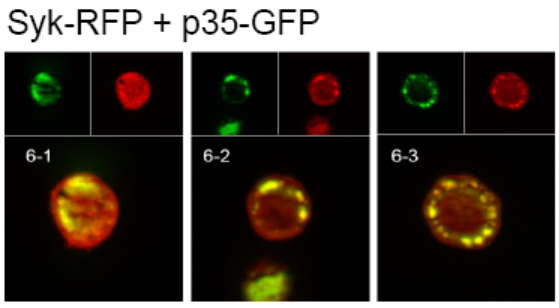
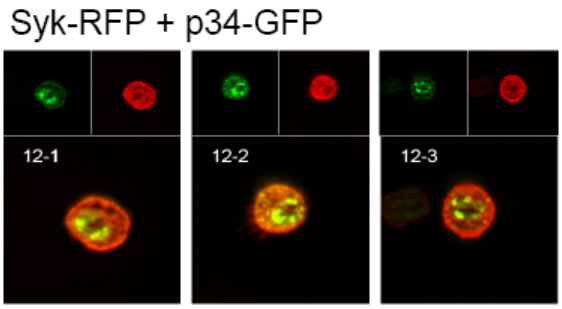


Figure 26 : Cotransfection experiments in S2 cells
S2 cells were transfected with *Syk-RFP* and *OBF1-GFP*.





An artificial BCR can be reconstituted in these S2 cells by transfecting its components (Wossning and Reth, 2004). Syk is the main BCR kinase and phosphorylates SLP-65 upon activation. When the BCR is reconstituted in the S2 cells, Syk is activated and phosphorylates SLP-65. Peter Nielsen performed transfection experiments in S2 cells to investigate Syk response and stability upon *OBF-1* cotransfection (Fig. 27). When *OBF-1* was cotransfected, Syk appeared to be hypophosphorylated. Furthermore Syk level might be decreased in presence of p35 isoform.

Plasmid:	1	2	3	4	5
hSyk	X	X	X	X	X
SLP 65	X	X	X	X	X
BCR		X	X	X	X
p35BOB			X		
p35BOB ΔATG				X	
p34BOB					X
pRmHa-3	15	3	--	--	--

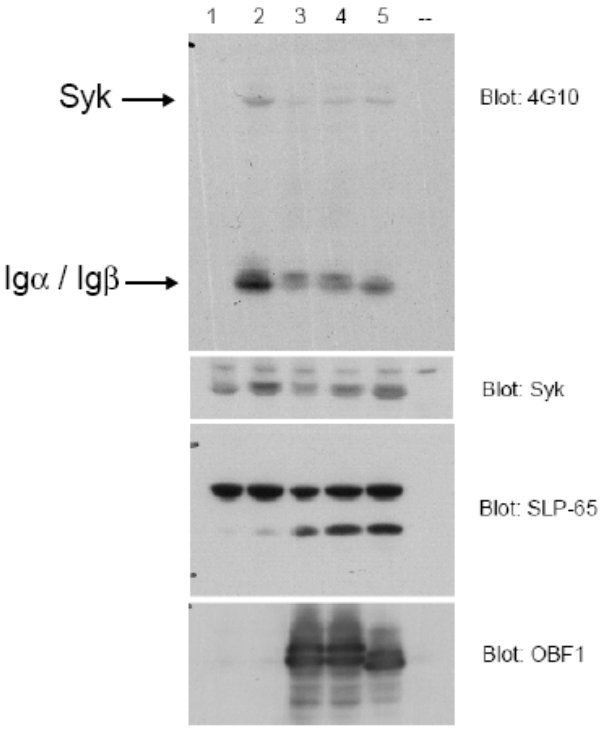


Figure 27: Syk response in transfected S2 cells

S2 cells were transfected as the scheme. Tyrosine phosphoryation (4G10), Syk, SLP-65 and OBF-1 level were detected by WB.

Although the S2 cell transfection allowed investigating individually the components of the BCR signalling, it is an artificial method. Therefore primary mature B cells from two independent *OBF-1*$^{-/-}$ mice strains (those from our lab and those from Nielsen et al.) were analysed. Figure 28 shows the result from a Western blot of stimulated and unstimulated splenic B cells. Stimulating the B cells with anti-IgM for 15 hours induced Syk expression both in *WT* and *KO* mice, to roughly the same extent. It also looked like Syk expression in B cells from *OBF-1*-deficient mice was at least as strong as in B cells from heterozygote mice. This does not correlate with the results published by Roeder's group in Cell where they showed that Syk expression seemed to decrease after stimulation and was significantly lower in B cells from *OBF-1*-deficient mice.

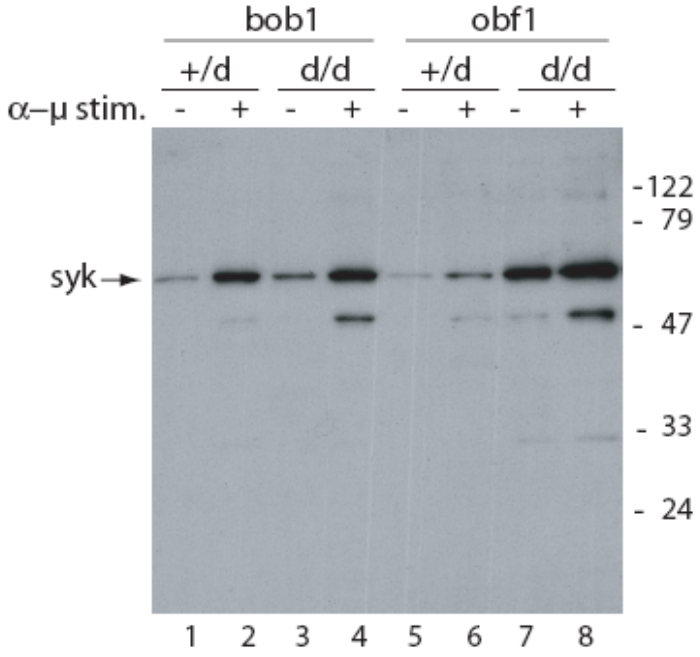


Figure 28: *In vitro* B cell stimulation
Splenic mature B cells were stimulated for 15 hours with anti-IgM. Bob1 and obf1 are mice from Fribourg and Basel respectively. WB of Syk was performed with the cell extracts.

Coimmunoprecipitation experiments were also performed in transfected 293T cells, however only the first experiment seemed to show an interaction between Syk and OBF-1. All the subsequent attempts failed to show this interaction (data not shown). Furthermore mammalian two-hybrid assays with *GAL4.Syk* and *OBF1.VP16* did not allow to evidence any interaction (data not shown). Therefore neither Peter Nielsen nor our lab could reproduce Roeder's results in mature B cells and confirm a direct interaction between Syk and OBF-1, although these two proteins colocalized in transfected S2 cells.

6.2.6 *Gadd45β* and *Cyclin D2* are potential OBF-1 direct target genes

Microarray data from EPLM and large preBII cells of BCS mice suggested that *Gadd45β* and *Cyclin D2* were direct target genes of p34 (2.1). Indeed octamer sites were discovered on their promoters. Chromatin immunoprecipitation (ChIP) of OBF-1 in *WT* Abelson cells was performed to investigate the OBF-1 binding potential on these octamer sites (Fig. 29). The ChIP procedure is described in Material and Methods. In fact OBF-1 binding was detected on the respective promoters suggesting that OBF-1 might be involved in *Gadd45β* and *Cyclin D2* expression in early B cells.

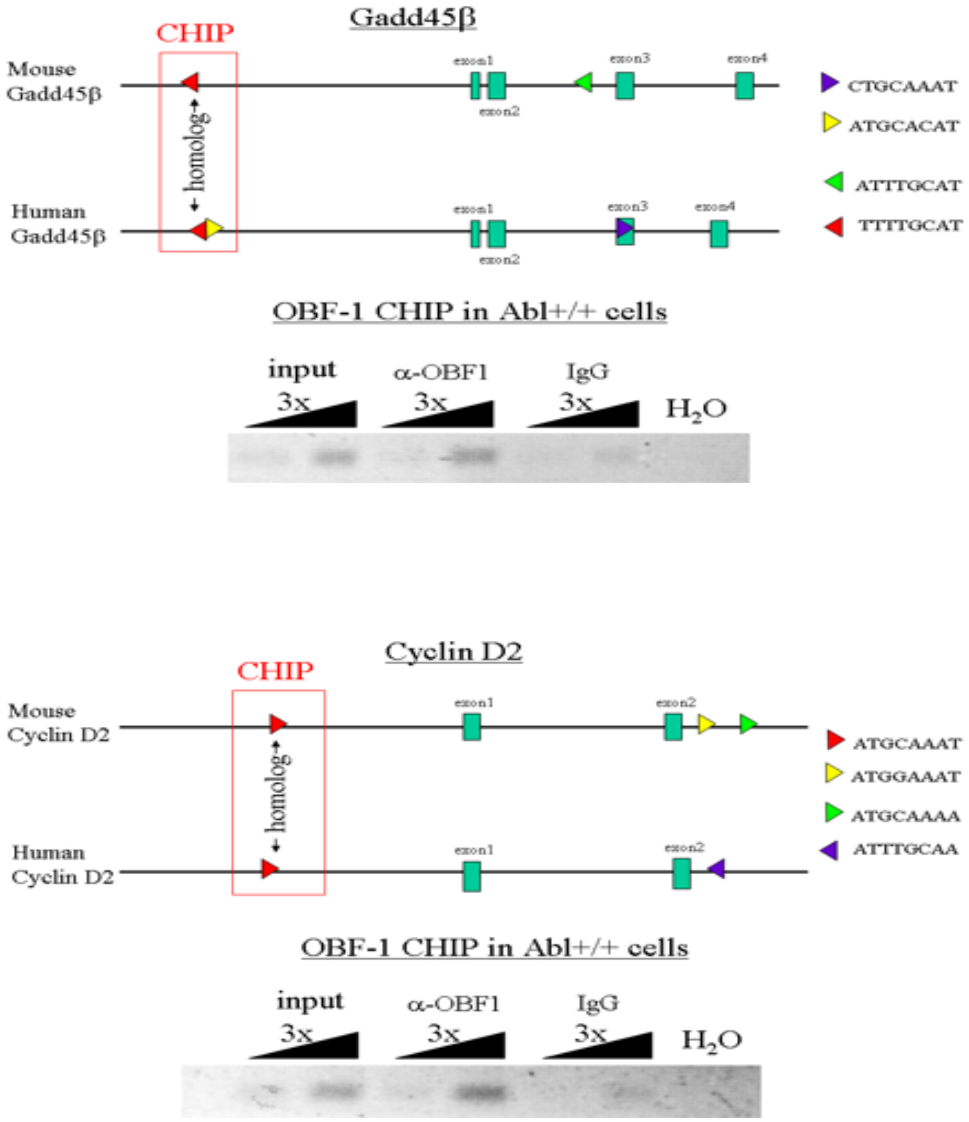


Figure 29: Chromatin Immunoprecipitation (ChIP) of *Gadd45β* and *Cyclin D2* octamer sites
ChIP was performed from *WT* Abl cells. α-OBF1 Ab and control rabbit IgG were used. The regions that were amplified by PCR are depicted by a red rectangle.

6.3 Abbreviations

Ab	antibody
BAC	bacterial artificial chromosome
BAFF	B-cell activating factor
BAFFR	BAFF receptor
β_2AR	β_2-adrenergic receptor
BCMA	B-cell maturation factor
BCR	B Cell Receptor
BCS	B Cell Specific
bHLH	basic helix-loop-helix
BLC	B-lymphocyte chemoattractant
BLR1	Burkitt lymphoma receptor 1
B-NHLs	non-Hodgkin B-cell lymphomas
Bp	base pairs
CAFC	cobblestone-area-forming cells
cAMP	adenosine 3',5'-cyclic monophosphate
cHD	classical Hodgkin disease
ChIP	chromatin immunoprecipitation
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CoIP	coimmunoprecipitation
CREB	cAMP response element binding protein
CSR	class switch recombination
DMEM	Dulbecco's modified Eagle medium
DN	double negative
DNP-KLH	dinitrophenyl-keyhole limpet hemocyanin
DP	double positive
ELISA	enzyme-linked immunosorbent serologic assay
EPLM	Early Progenitor with Lymphoid and Myeloid potential
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FDC	follicular dendritic cell
FITC	fluorescein isothiocyanate
GC	germinal center
GFP	green fluorescent protein
HA	hemagglutinin
HSC	hematopoietic stem cell
IDC	interdigitating dendritic cell
Ig	Immunoglobulin
ITAM	immunoreceptor tyrosine-based activation motif
Kb	1000 base pairs
KO	knock out
LB	Luria-Bertani
LCMV	lymphocytic choriomeningitis virus
LMB	Leptomycin B
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MPP	multipotent progenitor
MZ	marginal zone

NES	nuclear exporting sequence
NK	natural killer
O/N	overnight
PAGE	polyacrylamide gel electrophoresis
PALS	Periarteriolar Lymphoid Sheath
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PKA	protein kinase A
PNA	peanut agglutinin
QPCR	Quantitative Polymerase Chain Reaction
Rpm	revolutions per minute
RS	Reed-Sternberg
RSS	Recombination Signal Sequence
SD	standard deviation
SE	standard error
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
snRNPs	small nuclear ribonucleotide proteins
SOC	Super Optimal broth with Catabolite repression
SP	single positive
SS	single stranded
SSBs	single strand breaks
TCR	T cell receptor
TSA	Trichostatin A
UPR	unfolded protein response
UV	ultraviolet
VSV	Vesicular stomatitis virus
WB	Western blot
WT	wild type