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# **Regulation of B Cell Development by Posttranslational Modifications of Ebf1**

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## Abbreviation

AGM	Aorta–gonad–mesonephros
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BCR	B cell receptor
BD	Becton Dickinson
BP1	Beta protein 1
BSA	Bovine serum albumin
CIP	Calf intestinal alkaline phosphatase
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DBD	DNA-binding domain
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxid
DTT	Dithiothreitol
EMSA	Electrophoretic mobility shift assay
FCS	Fetal calf serum
GFP	Green fluorescent protein
HLH	Helix-loop-helix
HSC	Hematopoietic stem cell
H3K4me2	Di-methylation of histone 3 at lysine 4
Ig	Immunoglobulin
IP	Immunoprecipitation
IPT(=TIG)	Ig-like /plexins /transcription factors
IRES	Internal ribosomal entry site
IL7	Interleukin 7
LT-HSC	Long-term HSC
M-CSFR	Macrophage colony-stimulating factor receptor
Mg <sup>2+</sup>	Magnesium
MIG	MSCV-IRES-GFP
MPP	Multipotent progenitors
MSCV	Murine stem cell virus
MZ	Marginal zone

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NuRD	Nucleosome remodeling deacetylase
-OH	Hydroxyl
-PO <sub>4</sub> <sup>2-</sup>	Phosphate group
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
pS	Phosphoserine
pT	Phosphothreonine
PTMs	Post-translational modifications
pY	Phosphotyrosine
RAG	Recombination activating gene
RSSs	Recombination signal sequences
S <sub>13</sub> A	Serine <sub>13</sub> to alanine
S <sub>14</sub> A	Serine <sub>14</sub> to alanine
S <sub>13, 14</sub> A	Serine <sub>13</sub> and serine <sub>14</sub> to alanine
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
SL	Surrogate light
ST-HSC	Short-term HSC
SWI/SNF	SWItch/Sucrose non-fermentable
TAD	Transactivation domain
TdT	Terminal deoxynucleotidyl transferase
TEMED	Tetramethylethylenediamine
TIG (=IPT)	Transcriptional Immunoglobulin factor
VCAM1	Vascular cell adhesion molecule 1
VDJ <sub>H</sub>	Heavy chain variable, diversity and joining
VLA4	Very late antigen-4

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## Summary

Differentiation of B lymphocytes requires the function of multiple transcription factors that determine lineage specification and commitment. Ebf1 is a key regulator of the transcriptional network in B cell development. It is expressed from common lymphoid progenitors (CLPs) to mature B cells. It participates in the regulation of immunoglobulin heavy chain D and J rearrangement and activates, represses and poises approximate 3000 target genes, which are involved in B cell development.

To find an explanation for the diverse roles of Ebf1, post-translational modifications were studied. The phosphorylation of Ebf1 at serine13 and serine14 (S13 and S14) was discovered by mass spectrometry and we confirmed this finding in B cells.

Phosphorylation of Ebf1 at both of these sites is required for early B cell development as substitution by alanine results in a strong developmental impairment.

Phosphorylation of Ebf1-S13 and S14 does not influence DNA binding but is important for transactivation of target genes like *Pax5*. Interestingly, immunoglobulin heavy chain D and J rearrangement does not depend on phosphorylation of Ebf1-S13 and S14.

The expression of the *Ebf1* gene results in two protein isoforms,  $\alpha$ *Ebf1* and  $\beta$ *Ebf1*.  $\alpha$ *Ebf1* lacks 14 N-terminal amino acids of  $\beta$ *Ebf1*, however, no functional difference between the two Ebf1 isoforms has yet been described. I find that  $\alpha$ Ebf1 regulates target genes from CLPs to pre-pro-B cells, such as *Rag1*, which mediates DJ<sub>H</sub> rearrangement. The expression of  $\beta$ *Ebf1* starts from the pro-B stage and regulates *Pax5*,  *$\lambda$ 5*, *mb1*, *CD19* and *VpreB* in an Ebf1-S13 and S14 phosphorylation-dependent manner. This data indicates that  $\alpha$ Ebf1 and  $\beta$ Ebf1 regulate early B cell development at different stages by inducing different target genes.

Taken together, my studies contribute to a better understanding of the diverse functions of Ebf1 and I show that the two Ebf1 isoforms are functionally different during early B cell development.

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## Zusammenfassung

Die Differenzierung von B-Lymphozyten hängt von der Funktion mehrerer Transkriptionsfaktoren ab, welche diese Zellart spezifizieren und determinieren. Ebf1 ist ein Schlüsselfaktor des transkriptionellen Netzwerks, und ist exprimiert von gemeinsamen lymphoiden Vorläuferzellen (CLP) bis zu reifen B-Zellen. Es reguliert die Umordnung der Immunglobulingene D und J, und aktiviert, reprimiert und beeinflusst ungefähr 3000 Zielgene, welche in der B Zellentwicklung eine Rolle spielen.

Um eine Erklärung für die verschiedenen Rollen von Ebf1 zu finden, wurden post-transkriptionelle Modifikationen untersucht. Die Phosphorylierung von Ebf1 an den Serinen 13 und 14 (S13, S14) wurde durch Massenspektroskopie entdeckt und hier unabhängig davon in B Zellen bestätigt. Der Austausch dieser beiden Aminosäuren durch Alanin resultiert in einer starken Verminderung der B Zellentwicklung, was die Wichtigkeit dieser Phosphorylierungen zeigt. Diese beeinflussen nicht die DNA-Bindung, sind aber wichtig für die Transaktivierung von Zielgenen wie *Pax5*. Interessanterweise hängt die Umordnung der D und J Immunglobulingene jedoch nicht von dieser Phosphorylierung ab.

Die Expression des *Ebf1* Gens resultiert in zwei verschiedenen Proteinisoformen,  $\alpha$ Ebf1 und  $\beta$ Ebf1.  $\alpha$ Ebf1 fehlen die ersten 14 Aminosäuren von  $\beta$ Ebf1, bisher konnten jedoch keine funktionellen Unterschiede zwischen diesen zwei Isoformen gezeigt werden. Ich beschreibe hier die differentielle Regulierung verschiedener Zielgene, wie zum Beispiel von *Rag1* in CLPs und die Umordnung der DJ<sub>H</sub> Immunglobulingene durch  $\alpha$ Ebf1. Die Expression von  $\beta$ Ebf1 beginnt im pro B-Zell Stadium und führt zur Regulation anderer Zielgene, wie zum Beispiel *Pax5*,  *$\lambda$ 5*, *mb1*, *CD19*, und *VpreB* in Abhängigkeit der Phosphorylierung von S13 und S14. Diese Daten zeigen, daß  $\alpha$ Ebf1 und  $\beta$ Ebf1 die frühe B Zellentwicklung in unterschiedlichen Stadien durch die Induktion verschiedener Zielgene regulieren.

Zusammengenommen tragen diese Studien zu einem besseren Verständnis der Regulation der frühen B-Zellentwicklung durch differentielle Funktionen der beiden

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Isoformen von Ebf1 bei. Erstmals können hier funktionelle Unterschiede zwischen  $\alpha$ Ebf1 und  $\beta$ Ebf1 während dieser Entwicklung gezeigt werden.

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# 1 Introduction

## 1.1 The development of the hematopoietic system

Hematopoietic stem cells (HSCs) can differentiate into all functional blood cells (Lemischka 1991, Seita and Weissman 2010, Spangrude et al. 1991). They are adult stem cells located in the bone marrow containing the potential for self-renewal and differentiation in a homeostatic balance (Becker, Mc and Till 1963, Chotinantakul and Leeanansaksiri 2012, Dzierzak and Medvinsky 2008, Till and Mc 1961)

HSCs are divided into long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs). LT-HSCs possess self-renewing ability throughout life to generate sufficient numbers of ST-HSC. ST-HSCs maintain self-renewing ability for a limited time period and then develop into multipotent progenitors (MPPs) (Morrison and Weissman 1994, Seita and Weissman 2010, Weissman 2000). MPPs lose self-renewal ability and start a functionally irreversible maturation process (Morrison and Weissman 1994, Seita and Weissman 2010, Christensen and Weissman 2001). Further downstream, MPPs develop into common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs) (Akashi et al. 2000, Karsunky et al. 2008, Kondo, Weissman and Akashi 1997, Serwold, Ehrlich and Weissman 2009), losing their lineage potential step by step until the progenitor becomes a mature functional cell. CLPs can develop into B cells, T cells, NK cells and dendritic cells (Karsunky et al. 2008, Kondo et al. 1997). CMPs can develop into platelets, erythrocyte, granulocytes and macrophages (Akashi et al. 2000).

During mouse ontogeny the earliest HSC are found in the intra-embryonic aorta–gonad–mesonephros (AGM) region of the 10 days old embryo (E10) (Medvinsky and Dzierzak 1996, Muller et al. 1994). From E11.5 on, HSCs are found in the yolk sac and the fetal liver (Huang and Auerbach 1993, Medvinsky and Dzierzak 1996, Moore and Metcalf 1970, Muller et al. 1994), which is the last place before they migrate to the bone marrow at birth (Godin and Cumano 2005). With the expression of SOX17, fetal HSC switch into adult HSCs (He et al. 2011). Functionally, E14.5 fetal liver HSCs possess the same self-renewal and multi-potency capacity as bone marrow HSCs (Rebel et al. 1996b, Rebel et al. 1996a, Jordan, McKearn and Lemischka 1990, Harrison and Astle 1997).



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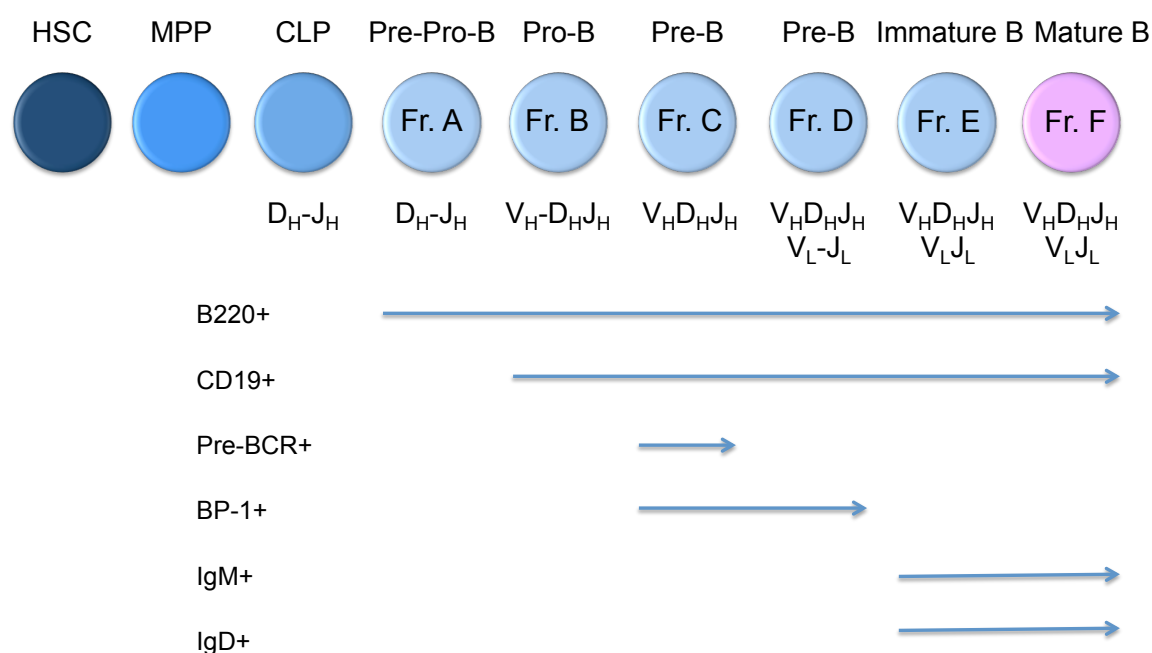
## 1.2 The development of B cells

### 1.2.1 The differentiation of B cells

B cell development starts in the fetal liver during embryonic development and continues in the bone marrow after birth (Whitlock and Witte 1982, Melchers 1977, Hardy and Hayakawa 2001, Cumano, Dieterlen-Lievre and Godin 1996, Ceredig et al. 1999).

Initially MPPs get in contact with bone marrow stromal cell via the binding of VCAM1 on the stromal cell to VLA-4 on the progenitor cell (Oostendorp and Dormer 1997). This facilitates the binding of stem cell factor (SCF) on the stromal cell and c-kit on the stem cell as a second step (Flanagan and Leder 1990, Williams et al. 1990, Zsebo et al. 1990). The VLA4 and VCAM1 coupling drives MPPs to develop into CLPs and causes the CLPs to express the IL7 receptor. IL7 is released from stromal cells (Hardy and Hayakawa 2001) and initiates differentiation of pre-pro-B cells. The development of B cells in the bone marrow is divided to pre-pro-B cells, pro-B cells, pre-B cells and immature-B cells stages depending on the expression of phenotypic markers as well as the status of immunoglobulin locus rearrangement (Figure 1) (Hardy and Hayakawa 2001, John 2007, Melchers et al. 2000, Osmond, Rolink and Melchers 1998, Rolink et al. 1993, Rolink et al. 1999b, ten Boekel, Melchers and Rolink 1995)

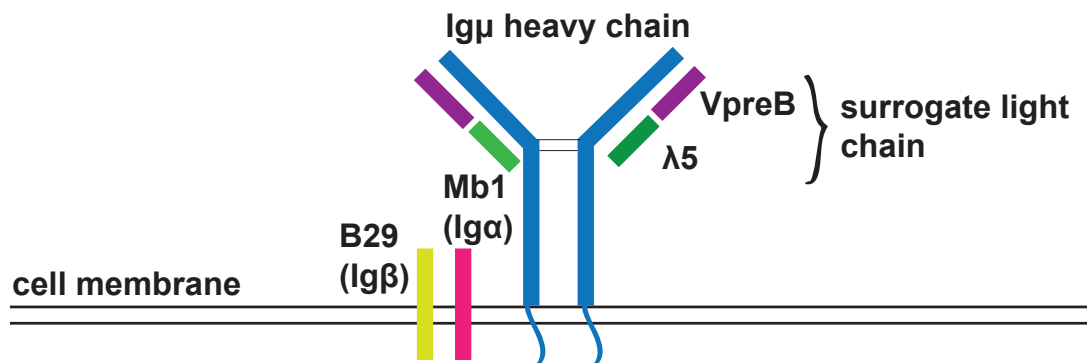
The first stage is the pre-pro-B cell (Rolink et al. 1993), which can also be called fraction A (Hardy and Hayakawa 2001). The cells start to express B220, which is the earliest B cell marker, but lack canonical B-cell markers including CD19 (Allman, Li and Hardy 1999, Hardy and Hayakawa 2001, Li et al. 1996, Ogawa, ten Boekel and Melchers 2000, ten Boekel et al. 1995) and synthesise terminal deoxynucleotidyl transferase (TdT) and recombination activating gene1 and 2 (Rag1 and Rag2) (Schatz, Oettinger and Baltimore 1989, Oettinger et al. 1990). The rearrangement of the immunoglobulin heavy chain locus is initiated by Rag1 and Rag2 at the diversity (D) and joining (J) segments (Schlissel 2003, Herzog, Reth and Jumaa 2009). Almost all pro-B cells have D<sub>H</sub> to J<sub>H</sub> rearranged at two alleles, suggesting that both alleles are accessible for variable (V) to D<sub>H</sub>J<sub>H</sub>-rearrangements (Ehlich et al. 1994, ten Boekel et al. 1995).



**Figure 1 Illustration of B cell development**

B cell development is divided into pre-pro-B, pro-B, pre-B and immature-B cell stages. Alternatively, this process can also be divided into fraction A-F. During development, B cells express different surface markers: B220 is expressed on B-biased progenitors and all subsequent stages of B cell development. CD19 is expressed on pro-B cells and later stages until terminal differentiation results in plasma cells. The pre-BCR, comprising Ig heavy chains and  $\lambda 5$  and VpreB surrogate light chains, is present on pre-B cells. BP-1 is expressed during the entire large and small pre-B cell stages. The BCR on immature B cells comprises  $\mu$  heavy and Ig light chains. Mature B cells display IgD in addition to IgM BCRs. The sequential immunoglobulin heavy chain and light chain rearrangement accompany the entire B cell development. HSC: hematopoietic stem cell, MPP: multipotent progenitor, CLP: common lymphoid progenitor, BCR: B cell receptor.

The joining of a heavy chain V region segment to the D-J locus happens in pro-B cells (fraction B) (Hardy and Hayakawa 2001). At this stage, pro-B cells start CD19 expression, a protein whose expression characterizes all later B lineage stages (ten Boekel et al. 1995, Hardy and Hayakawa 2001, Rolink et al. 1993). Cells in fraction B have up-regulated expression of heat stable antigen,  $\lambda 5$ , VpreB, Ig $\alpha$  and Ig $\beta$ , which are part of the pre-BCR, and grow in response to IL-7 and stromal cells (Herzog et al. 2009).



**Figure 2 Structure of the pre-B cell receptor (pre-BCR)**

Scheme of the pre-BCR complex, composed of the Ig  $\mu$ H chain, the surrogate light chains VpreB and  $\lambda$ 5 and the signal-transducing heterodimer Ig $\alpha$  (Mb1) / Ig $\beta$  (B29).

Following the completion of the heavy chain V to D-J rearrangement, pro-B cells become large pre-B cells (fraction C) (Hardy and Hayakawa 2001). These cells are responsive to IL-7 in the absence of stromal cells (Winkler, Melchers and Rolink 1995). As with all immunoglobulin gene rearrangements, VDJ<sub>H</sub> junctions are generated randomly (Tonegawa 1983). The success of VDJ<sub>H</sub> rearrangements is controlled via pairing of the resulting  $\mu$ H chain with the surrogate light (SL) chain (Zhang, Srivastava and Lu 2004, Martensson and Ceredig 2000, ten Boekel et al. 1995, Melchers et al. 1999). The surrogate light chain consists of two proteins: a V-like sequence called Vpre-B and a C-like sequence called  $\lambda$ 5 (Melchers et al. 1999, Sakaguchi and Melchers 1986, Kudo et al. 1987, Melchers et al. 2000). The complex of  $\mu$ H and SL chain associates with the Ig $\alpha$  (Mb1) / Ig $\beta$  (B29) heterodimer, which allows a pre-BCR to be deposited on the surface membrane of large pre-B cells (Figure 2) (Winkler et al. 1995, Brouns, de Vries and Borst 1995, Gauthier et al. 2002, Karasuyama, Kudo and Melchers 1990, Melchers et al. 2000, Tsubata and Reth 1990). Once finishing the assembly of pre-BCR on the cell surface, a downstream signaling cascade starts, resulting in a very strong proliferation of large pre-B cells (Melchers et al. 2000, Rolink et al. 2000, Parker et al. 2005, Hardy and Hayakawa 2001). The pre-BCR has two tasks: The first task is to shut down the activities and expression of the enzyme machinery catalyzing the rearrangements of the H-chain gene segments (ten Boekel, Melchers and Rolink 1998). The second task is to

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initiate the rearrangement of the L-chain genes (Schlissel and Morrow 1994, Constantinescu and Schlissel 1997).

After undergoing three to five rounds of cell division, large pre-B cells stop proliferation and differentiate into small non-dividing pre-B cells (fraction D) (Zhang et al. 2004, Hardy and Hayakawa 2001). At this stage, RAG proteins are expressed and light chain V-J joining takes place (Hardy and Hayakawa 2001, Rolink and Melchers 2000, ten Boekel et al. 1995, Ehlich et al. 1994). Successfully rearranged  $\kappa$  or  $\lambda$  L-chains replace VpreB /  $\lambda 5$  of the pre-BCR and form IgM with the H-chain (Schlissel and Morrow 1994, Constantinescu and Schlissel 1997, Iglesias 1991, Tsubata, Tsubata and Reth 1992).

After lineage commitment and differentiation through pro- and pre-B cell stages in the bone marrow, the expression of surface IgM starts the immature B cell stage (fraction E). 10-20% of these immature B leave their environment and migrate to secondary lymphoid organs as transitional B cells, where they complete their maturation by differentiating into naive, follicular, or marginal zone (MZ) B cells (fraction F) (Allman et al. 2001, Rolink et al. 1999b, Carsetti, Kohler and Lamers 1995, Loder et al. 1999). Each immature B cell exclusively expresses IgM on its surface that is tested for reactivity against autoantigens. The binding between antigen and BCR determines whether the immature B cell will be selected into the peripheral B-cell repertoire. High affinity to multivalent (cell-associated) self antigens in the marrow lead to B cell apoptosis and clonal deletion. Low affinity to soluble self antigen does not kill the B cell, but it cannot respond to antigen with IgD expression and has a short life span, a state called anergic. Cells which do not bind self antigens and express normal levels of IgM and IgD can enter the lymphoid follicles and survive for a few weeks until they either encounter their specific antigen or die (Nossal and Schrader 1975, Norvell, Mandik and Monroe 1995, Goodnow et al. 1988, Nemazee and Burki 1989)

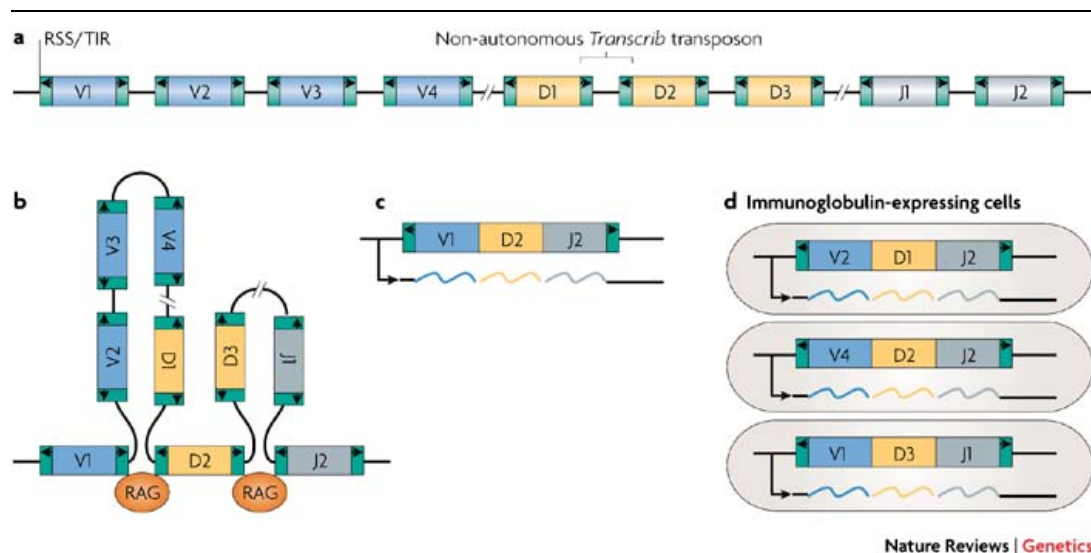
### **1.2.2 Immunoglobulin recombination**

B cell development is tightly associated with immunoglobulin recombination. The V(D)J recombination assembles immunoglobulin and T cell receptor genes during lymphocyte development through a series of DNA breakage and rejoining events

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(Schatz and Swanson 2011, Fugmann et al. 2000, Gellert 2002). The antigen-binding domain of the antibody consists of V (variable), D (diversity) and J (joining) segments (Hozumi and Tonegawa 1976). Totally, the immunoglobulin heavy chain region contains 44 V genes, 27 D genes and 6 J genes (Matsuda et al. 1998, Li et al. 2004). During the recombination process, each individual segment can be chosen randomly resulting in several possibilities of the junction, which is the fundamental reason for the diversity of antigen-receptor. This process minimizes the coding capacity of germline but tremendously amplify the possibility of the potential antigen-binding specificities (Roth 2014).

D-to-J joining starts with the binding of the recombinase. The recombinase consists of two lymphoid-specific proteins, Rag1 and Rag2, which recognize the flanking recombination signal sequences (RSSs). The RSSs consist of conserved heptamer and nonamer elements, separated by either 12 or 23 nucleotides of less conserved “spacer” sequence (Helmink and Sleckman 2012, Bassing et al. 2000). Once RAG binds to DNA, it cleaves and re-joins the DNA streams (van Gent et al. 1997). The RAG folds together the D and J segments to be recombined and cleaves the DNA exactly at the junction of the gene segment and the joining RSS motif. This cleavage creates a hairpin of DNA at the end of D and J segments and double strand breaks at the end of the RSS motif. The hairpins are opened by the activity of Artemis which is a crucial step of immunoglobulin rearrangement. The DNA ends are aligned via XRCC4, DNA ligase IV and Cernunnos, and non-templated nucleotides are randomly added via terminal deoxynucleotidyl transferase (TdT) to the coding end to make the two ends compatible for joining (Gauss and Lieber 1996). Finally, the processed coding ends are ligated by DNA ligase IV (van Gent and van der Burg 2007). V segment can join the rearranged D-J segment with the same process as D-to-J ( Figure 3) (Schatz and Swanson 2011, Helmink and Sleckman 2012).



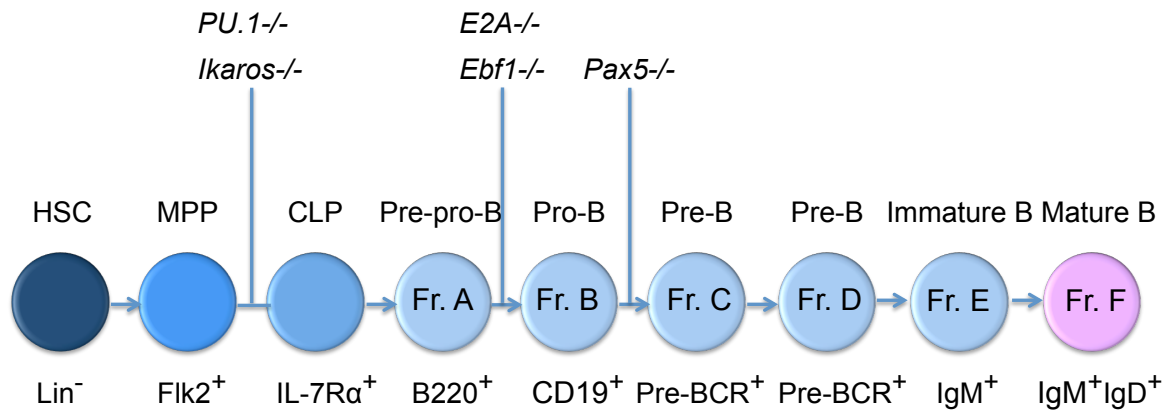
**Figure 3 Immunoglobulin rearrangement of V, D and J segments to produce a functional antigen-receptor**

(a) Ig gene contains several V, D and J segments. Each of them is flanked by a recombination signal sequence. (b) RAG proteins bind randomly to different combination of RSS sequences guiding the DNA folding and cleavage of the internal region (hairpin). (c) The V(D)J rearrangement is completed resulting in functional yet diverse immunoglobulin genes. (d) The Ig rearrangement exists in each cell on a clonal basis. (Figure is taken from Slotkin and Martienssen 2007).

### 1.2.3 Regulation of B cell development

The development of HSCs into B cells is characterized by modulating the activity or the expression of lineage-determining transcription factors and cytokines (Hagman and Lukin 2006, Santos et al. 2011, Northrup and Allman 2008, Dahl et al. 2003). Several transcription factors (*PU.1*, *Ikaros*, *E2A*, *Ebf1* and *Pax5*) and two cytokine receptors (*Flk2/Flt3* and *IL-7R $\alpha$* ) have been shown to be critical for the development of B cell precursors (Georgopoulos et al. 1994, Scott et al. 1994, Bain et al. 1994, Zhuang, Soriano and Weintraub 1994a, Nutt, Thevenin and Busslinger 1997a, Peschon et al. 1994, Lin and Grosschedl 1995). *PU.1* and *Ikaros* are expressed in HSCs and MPPs and mutation of these genes affects multiple lineages (Yoshida et al. 2006, Laslo et al. 2006, Zhuang et al. 2004). In contrast, *Ebf1* and *Pax5* expression is B-lineage specific (Urbanek et al. 1994, Lin and Grosschedl 1995, Thevenin, Nutt and Busslinger 1998, Hagman, Travis and Grosschedl 1991, Hagman et al. 1993) and their deletion interrupts B cell development without affecting other hematopoietic lineages. *Flk2* and *IL-7* regulate early B cell development and are

involved in the regulated expression of B lineage-specific transcription factors (Figure 4) (Satterthwaite and Witte 1996).



**Figure 4 Regulation of B development**

The approximate points at which development is arrested in *PU.1*, *Ikaros*, *E2A*, *Ebf1*, and *Pax5*-deficient mice are indicated above. Deletion of *PU.1* and *Ikaros* genes influences multiple lineages. The developmental arrest in *E2A* or *Ebf1*-deficient mice results in cells that are similar to B-biased progenitors, but which lack all Ig gene rearrangements. *Pax5*-deficient mice enter the early stage of B cell development and undergo D-J heavy chain rearrangement, but lack all stages beyond fraction C of B cell development and rearrangement of distal V<sub>H</sub> segments.

### 1.2.3.1 Transcription factors regulating early B cell development

The Ets family transcription factor PU.1 is required for the generation of both the innate and adaptive immune systems (Hromas et al. 1993, Scott et al. 1994, McKercher et al. 1996). *PU.1* is expressed in hematopoietic stem cells, multipotent progenitors, and all differentiating cells except erythroblasts, megakaryocytes, and T cells (Akashi et al. 2000, Hromas et al. 1993, Anderson et al. 1999). Deletion of the *PU.1* gene affects multiple lineages (Laslo et al. 2006). Many studies have confirmed the importance of PU.1 at early stages of hematopoietic development, modulating the chromatin structure of target genes (Iwasaki et al. 2005, Dakic et al. 2005, McDevit and Nikolajczyk 2006, Marecki, McCarthy and Nikolajczyk 2004, Nikolajczyk, Sanchez and Sen 1999, Stopka et al. 2005). For example, PU.1 has been discovered as an important regulator for *IL-7Rα*, *Ebf1* and *Pax5* gene

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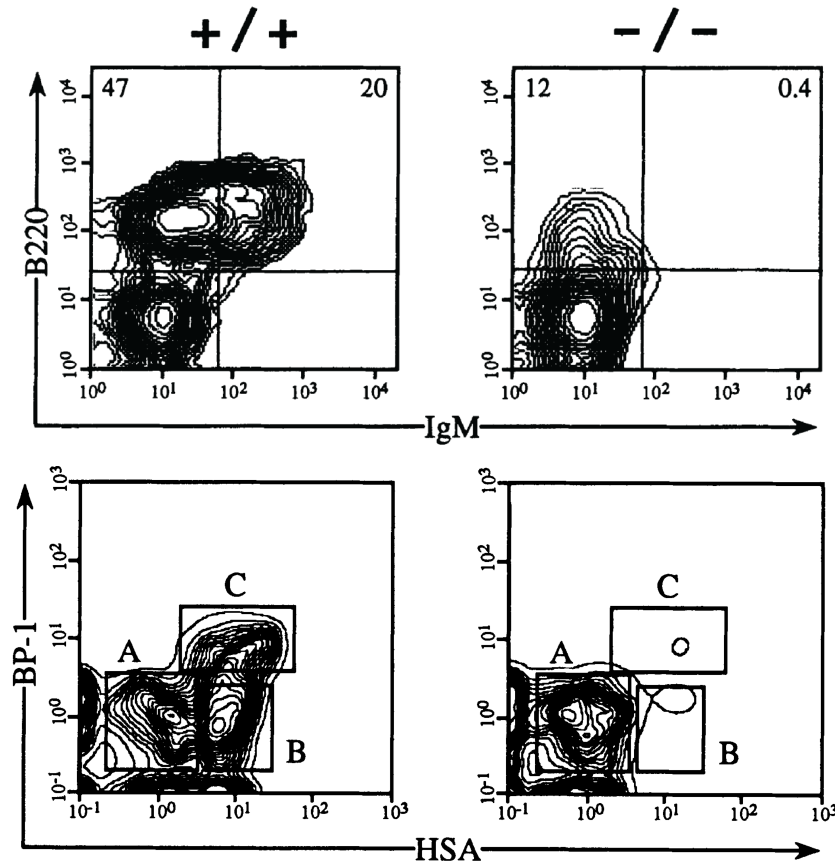
expression and immunoglobulin rearrangement (DeKoter, Lee and Singh 2002, McDevit et al. 2005, Marecki et al. 2004, Maitra and Atchison 2000, Nikolajczyk et al. 1999, Torlakovic et al. 2005). Graded expression of *PU.1* specifies either the myeloid or lymphoid fate of early hematopoietic progenitors. Low *PU.1* expression induces B cell development, whereas high *PU.1* levels suppress the B cell fate and promote instead macrophage differentiation (DeKoter and Singh 2000).

Ikaros is a central regulator of hematopoiesis by acting downstream of PU.1 to promote the differentiation of a MPP into a component of the lymphocyte lineage (Georgopoulos et al. 1994). Ikaros is required for the development of the earliest B cell progenitors and at later stages for VDJ recombination and B cell receptor expression (Sellars, Kastner and Chan 2011). Ikaros<sup>-/-</sup> mice lack pre-pro-B cells and exhibit a complete block in B lymphopoiesis due to a role for Ikaros in promoting *Flt3* and/or *IL-7R* expression in early hematopoietic progenitors (Wang et al. 1996, Sitnicka et al. 2003, Nichogiannopoulou et al. 1999, Yoshida et al. 2006). Ikaros likely contributes to *Ebf1* expression in part by activating *IL-7R* expression, as IL-7 signals are required for *Ebf1* transcription (Dias et al. 2005, Kikuchi et al. 2005, Reynaud et al. 2008). Ikaros regulates IgH rearrangement via activation of *Rag* genes (Reynaud et al. 2008). Additionally, Ikaros is thought to down-regulate preBCR signaling by repressing IgH1 ( $\lambda$ 5) transcription in preB cells (Thompson et al. 2007, Sabbattini et al. 2001). Ikaros<sup>-/-</sup> pro-B lines can be differentiated into macrophages when cultured with macrophage colony-stimulating factors (Reynaud et al. 2008).

The *E2A* gene encodes a pair of closely related proteins, E12 and E47, which are generated by alternative splicing of RNA transcripts (Murre, McCaw and Baltimore 1989). Targeted disruption of the *E2A* gene revealed that its products are required for the generation of functional B cells and for the expression of a B cell-specific gene program. In the absence of E2A, early B cell development is blocked at the pre-pro B stage (Fraction A) (Zhuang et al. 1994a, Bain et al. 1994) and immunoglobulin gene rearrangements are undetectable (Mombaerts et al. 1992, Shinkai et al. 1992). Analysis of mice expressing an *E2A-GFP* knock-in allele has shown that *E2A* expression in B lineage cells is up regulated concomitantly with the induction of *Ebf1* and initiation of D<sub>H</sub>-J<sub>H</sub> recombination (Zhuang et al. 2004). A functional binding site



for E2A has been found in the *Ebf1* promoter, suggesting that E2A is directly involved in the regulation of *Ebf1* expression (Smith, Gisler and Sigvardsson 2002).



**Figure 5 B cell differentiation in *Ebf1*-deficient mice is blocked at the pre-pro-B cell stage**

Flow cytometric analysis of bone marrow cells from wt (left) and *Ebf1*<sup>-/-</sup> mice using B220 and IgM expression (upper panel). In the lower panels, B220<sup>+</sup> and CD43<sup>+</sup> cells were gated and analysed for fraction A, B and C of pro-B cells using BP-1 and HSA expression. (Figure is taken from Lin and Grosschedl 1995).

*Ebf1* is a key factor in B lineage determination (Hagman and Lukin 2006). Loss of *Ebf1* results in a block in the transition of fraction A to fraction B (Figure 5) and the initiation of D-J rearrangements at the IgH locus. Enforced expression of *Ebf1* in *Ebf1*-deficient B220<sup>+</sup>IL-7R<sup>+</sup> B cell progenitors reversed the arrest of B cell development and activated  $\lambda 5$ , *VpreB*, *Rag1* and *Rag2*, germline *I $\mu$*  transcripts and *Pax5* (Medina et al. 2004). *Ebf1* activates chromatin remodelling of the *mb-1*

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promoter necessary for its transcriptional activation by Pax5 (Maier et al. 2003). E2A and Ebf1 function synergistically to activate transcription of B lineage genes, as *E2A/Ebf1* compound-mutant heterozygotes display a more severe defect during B lymphopoiesis than the single heterozygous animals (O’Riordan and Grosschedl 1999). Consistent with this possibility, ectopic expression of E2A and Ebf1 results in activation of the  $\lambda 5$  and *VpreB* genes and initiation of D-J<sub>H</sub> rearrangement (Romanow et al. 2000). The expression of Ebf1 correlates with the expression of 565 genes associated with pre-BCR and BCR signaling (Mansson et al. 2004, Treiber et al. 2010b). Ebf1 expression is compromised in *PU.1*<sup>-/-</sup> fetal liver hematopoietic progenitors, suggesting that PU.1 participates in regulation of the *Ebf1* gene (Maier et al. 2004).

In contrast to E2A and Ebf1, the transcription factor Pax5 is not required for specification of the B cell fate but is essential for commitment (Schebesta, Heavey and Busslinger 2002). Once committed to the B-lineage, B cells require Pax5 function to maintain their B-lymphoid identity throughout their development (Nutt et al. 2001). *Pax5*<sup>-/-</sup> pro-B cells express the early B-lineage genes and undergo D<sub>H</sub>-J<sub>H</sub>, and proximal V<sub>H</sub>-DJ<sub>H</sub> gene rearrangements (Nutt et al. 1997b). Interestingly, *Pax5*<sup>-/-</sup> pro-B cells exhibit extensive developmental plasticity allowing them to differentiate into all other hematopoietic lineages, which is suppressed upon restoration of *Pax5* expression (Nutt et al. 2001). Pax5 actively and continuously represses the expression of myeloid genes such as M-CSFR (macrophage colony-stimulating factor receptor) while inducing B-lineage genes like mb-1, CD19 and BLNK (Souabni et al. 2002). On this basis, E2A and Ebf1 can be regarded as primary B cell fate determinants and Pax5 as a B cell fate commitment factor (Singh, Medina and Pongubala 2005).

### **1.2.3.2 Cytokines regulating early B cell development**

The expression of Flk2/Flt3 is the earliest regulatory event that appears to trigger B cell development within MPPs (Adolfsson et al. 2001). Targeted inactivation of the Flk2/Flt3 gene results in a severe deficiency in the generation of B lineage progenitors (Mackarechtschian et al. 1995). Consistent with the requirement for Flk2/Flt3 signaling in the development of B-lineage progenitors is the significant

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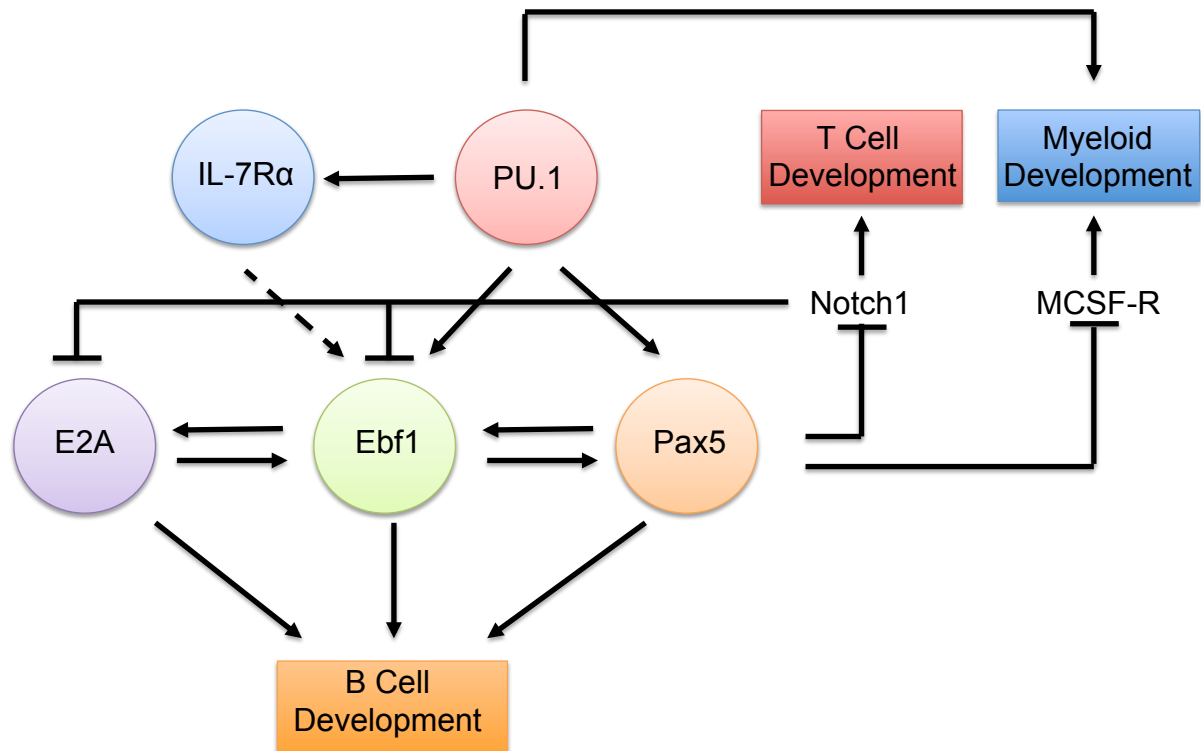
decrease in CLPs observed in mice deficient in Flk2/Flt3 ligand (Sitnicka et al. 2002). The signaling pathway through which Flk2/Flt3 selectively favors the generation of B lineage progenitors is unknown, but *in vitro* data suggest that activation of this receptor promotes expression of the *IL-7* receptor  $\alpha$  (*IL-7R $\alpha$* ) gene (Borge et al. 1999).

The development of pro-B cells in the bone marrow requires signaling through IL-7R $\alpha$  (Miller et al. 2002). A stromal-free culture system shows IL-7R signaling is sufficient to induce the differentiation of CLPs into B cells, suggesting that signaling through the IL-7R $\alpha$  may activate the expression or modulate the activity of key transcriptional regulators like E2A and/or Ebf1, which are required for specification of the B cell fate (Sitnicka et al. 2003, Vosshenrich et al. 2003, Dias et al. 2005, Kikuchi et al. 2005, Kikuchi et al. 2008). *IL-7<sup>-/-</sup>* mice have a profound block in B cell generation and a reduction of CLPs in the bone marrow (Dias et al. 2005). Compared to their wild-type counterparts, CLPs generated in the absence of IL-7 have normal T/NK differentiation potential, but severely impaired B potential. Accordingly, CLPs from *IL-7*-deficient mice express lower amounts of Ebf1 and Pax5 than wild-type CLPs, but similar amounts of GATA-3 (Dias et al. 2005, Carvalho et al. 2001, Rothenberg 2002). Overall, Flk2/Flt3 and IL-7R $\alpha$  induce many lineage-specific transcription factors in MPPs and CLPs.

### **1.2.3.3 Early B cell development is controlled by a complex network of transcription factors**

Early B cell development is a highly regulated process controlled by a complex network. Several transcription factors and signaling pathways have been implicated in the regulation of this process (Kee and Murre 1998, Medina et al. 2004, Reynaud et al. 2008, DeKoter et al. 2002, Greenbaum and Zhuang 2002, O'Riordan and Grosschedl 1999, Maier et al. 2004, Sigvardsson et al. 2002, Schebesta, Pfeffer and Busslinger 2002, Nutt et al. 2001). Because each factor regulates multiple target genes, and these targets often include genes encoding one or more of the aforementioned transcription factors, it is often said that these proteins comprise a transcriptional hierarchy that serves to orchestrate early B cell development and B

cell specification (Nutt et al. 2001, Medina et al. 2004). A provisional model for this hierarchy is illustrated in Figure 6.



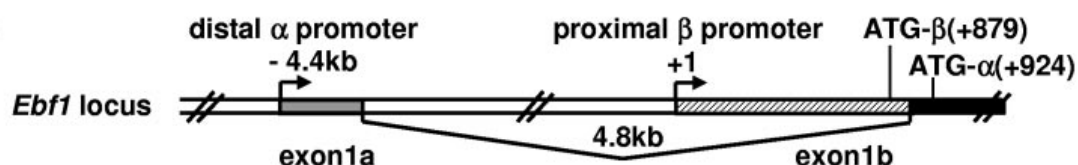
**Figure 6 Early B cell development is regulated by a complex network of transcription factors**

Representation of essential transcription factors responsible for the initiation of B-cell differentiation. An arrow represents a direct activation and a dashed arrow shows indirect activation; inhibition is shown using a blocked line (Mandel and Grosschedl 2010).

Regulation by PU.1 is dose-dependent, as low *PU.1* expression induces B cell development, whereas high PU.1 levels suppress the B cell fate and promotes gtmacrophage differentiation instead (DeKoter and Singh 2000). PU.1 regulates early B cell development by including the expression of the *IL-7* receptor  $\alpha$  chain (*IL-7R $\alpha$* ), and thus rendering lymphoid progenitors responsive to the pro-B cell differentiation and survival factor IL-7 (DeKoter et al. 2002). *IL-7*-deficient mice have no significant difference in the number of CLPs, but their ability to differentiate into B-lineage cells is greatly diminished (Dias et al. 2005). The *IL-7R $\alpha$*  gene was identified as a direct

target of PU.1, since it contains a functional PU.1 binding site in its 5' region and fails to be transcribed in *PU.1<sup>-/-</sup>* progenitors (DeKoter et al. 2002, Sitnicka et al. 2002). IL-7 signaling activates the transcription factor STAT5, which can rescue the lymphoid defect in mice with a targeted mutation of the *IL-7R $\alpha$*  gene (Goetz et al. 2004).

For the B-lineage transcriptional hierarchy, *Ebf1* plays a critical and dominant role (Medina et al. 2004). B cell development from *E2A<sup>-/-</sup>* progenitor cells is restored by introducing *Ebf1* (Seet, Brumbaugh and Kee 2004). *Ebf1* also bypasses the requirement for *Ikaros* as well as IL-7R $\alpha$  and PU.1 (Medina et al. 2004, Dias et al. 2005, Kikuchi et al. 2008, Reynaud et al. 2008). In each of these cases, ectopic expression of *Pax5* did not rescue B lymphopoiesis, indicating that *Ebf1* performs other functions in addition to activating *Pax5* expression (Dias et al. 2005, Kikuchi et al. 2008, Medina et al. 2004, Seet et al. 2004).



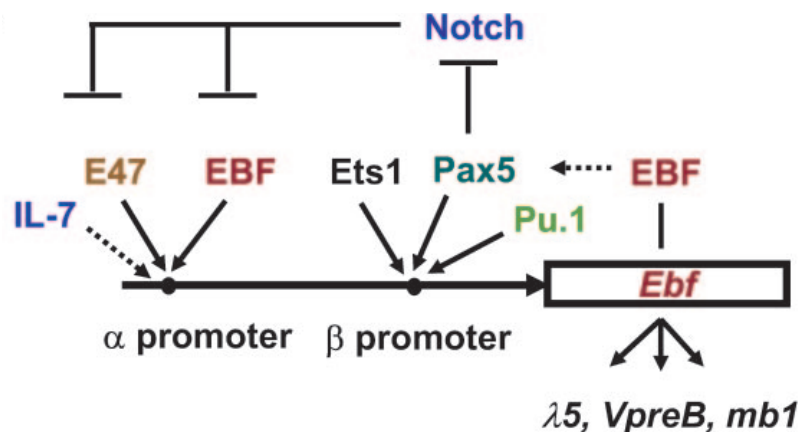
**Figure 7 Schematic overview of the distal *Ebf1 $\alpha$*  and proximal *Ebf1 $\beta$*  promoters.**

Two different first exons of *Ebf1*, exon1a and exon 1b, are indicated. The distal *Ebf1 $\alpha$*  promoter is located upstream of exon 1a and 4.4k nucleotides upstream of the proximal *Ebf1 $\beta$*  promoter, which is located within the first intron of *Ebf1 $\alpha$*  mRNA and 879 nucleotides upstream of the ATG- $\beta$ . (Figure is taken from Roessler et al. 2007).

*Ebf1* is controlled through two distinct promoters, the distal  $\alpha$ -promoter and the stronger proximal  $\beta$ -promoter (Smith et al. 2002, Roessler et al. 2007b).

Transcription from these promoters results in the expression of two *Ebf1* isoforms, *Ebf1 $\alpha$*  and  $\beta$ . Due to alternative splicing, transcripts from the distal  $\alpha$ -promoter lack the start codon that is used for translation of *Ebf1 $\beta$* , generating an isoform that lacks 14 N-terminal amino acids of the *Ebf1 $\beta$*  isoform. However, no functional difference between the two *Ebf1* isoforms has been described so far (Figure 7) (Roessler et al. 2007b).

The distinct proteins activate the Igll1 promoter *in vitro* equivalently, suggesting that the alternative proteins maybe a consequence of the need for differential promoter regulation rather than distinct biological functions. The Ebf1 $\alpha$  promoter is regulated by E2A and IL7 (Roessler et al. 2007b). Because STAT5 is activated by IL-7R signaling, this finding provides a possible explanation for the dependence of initial Ebf1 expression on both E2A and IL-7R (Dias et al. 2005, Kikuchi et al. 2005, Seet et al. 2004). The Ebf1 $\beta$  promoter is regulated via Ets1, PU.1 and Pax5 (Roessler et al. 2007b). Ebf1 regulates its own expression directly through induction of the Ebf1 $\alpha$  promoter and indirectly through up-regulation of Pax5, suggesting an auto-regulatory function for Ebf1. Compared to wild-type pro-B cells, Pax5<sup>-/-</sup> pro-B cells have reduced Ebf1 mRNA resulting predominantly from decreased transcription from the Ebf1 $\beta$  promoter (Roessler et al. 2007b). This feedback regulation by Pax5 on Ebf1 could function to amplify B cell-specific gene expression and solidify commitment to the B cell pathway (Figure 8).



**Figure 8 Representation of the complex regulatory network at the two Ebf1 promoters**

The two promoters of Ebf1 along with the known regulators are indicated. The gene product of the two promoters differs by 14 amino acids at the N terminus. An arrow represents a direct activation and a dashed arrow shows indirect activation, inhibition is depicted using a blocked line. (Figure is taken from Roessler et al. 2007).

The transcription factors E2A and Ebf1 are required at a similar step in early B cell differentiation (Schlissel, Voronova and Baltimore 1991, Choi et al. 1996, Kee and Murre 1998). B cell development is impaired at the pro-B cell stage in E2A<sup>+/-</sup> Ebf1<sup>+/-</sup> mice, because of reduced expression of multiple B-lymphoid genes including Rag1

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and Rag2 (O'Riordan and Grosschedl 1999). E2A induces *Ebf1* in a macrophage line and strongly synergizes with Ebf1 to regulate the expression of the surrogate light chain ( $\lambda 5$ , VpreB) and signaling components (Ig $\alpha$ , Ig $\beta$ ) of the pre-BCR (Kee and Murre 1998, Sigvardsson, O'Riordan and Grosschedl 1997, Sigvardsson 2000, Sigvardsson et al. 2002, Gisler and Sigvardsson 2002). The promoters of both the  $\lambda 5$  and VpreB genes contain functional binding sites for Ebf1 and E2A (Kudo and Melchers 1987, Sigvardsson et al. 1997).

Further evidence shows that *E2A* acts upstream of *Ebf1* in the genetic hierarchy of early B cell development (Smith et al. 2002). Pre-pro B cells express almost normal levels of *E2A* mRNA in *Ebf1*<sup>-/-</sup> mice, whereas *Ebf1* transcripts appear to be reduced in *E2A*<sup>-/-</sup> bone marrow cells (Bain et al. 1997, Lin and Grosschedl 1995). Ectopic expression of E2A could activate endogenous *Ebf1* gene in contrast to Ebf1, which is unable to induce E2A transcription (Kee and Murre 1998).

Pax5 represses the expression of myeloid genes such as M-CSFR and T-lineage genes such as Notch1, which actively inhibit B cell generation providing a molecular explanation for the lineage plasticity of the *Pax5*<sup>-/-</sup> pro-B cells (Souabni et al. 2002, Tagoh et al. 2006, Nutt et al. 1999). *Pax5* is a direct target of *Ebf1* and can regulate the expression of *Ebf1* via the positive feedback loop involving the  $\beta$ *Ebf1* promoter (Roessler et al. 2007b, Decker et al. 2009). The activation of IgH-VDJ rearrangement initiates a feed forward loop between Pax5 and Ebf1 and eliminates the potential for alternative cell fate (Decker et al. 2009). The target genes of Ebf1 are detectable in *Pax5*<sup>-/-</sup> pro-B cells, showing that Pax5 functions downstream of Ebf1 in the control of B cell development (Nutt et al. 1997b). In contrast, *Pax5* expression is reduced in pro-B cells of compound heterozygous *E2A*<sup>+/-</sup>*Ebf1*<sup>+/-</sup> mice and can be induced by ectopic expression of E2A or Ebf1 in a 70Z/3 macrophage cell line (Kee and Murre 1998, O'Riordan and Grosschedl 1999).

### 1.3 Early B cell factor (Ebf1)

Ebf1 is a helix-loop-helix (HLH) transcription factor belonging to a protein family consisting of 4 highly conserved members. It was discovered due to its binding to a functionally important palindromic site within the early B cell-specific CD79a promoter

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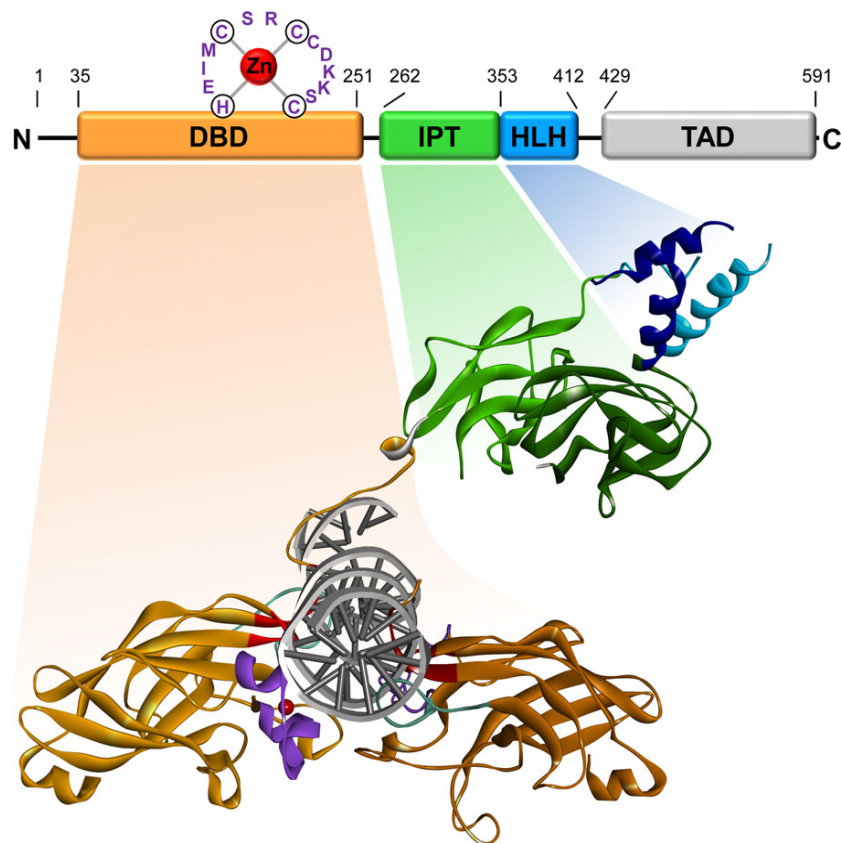
(Hagman et al. 1993, Feldhaus et al. 1992). The CD79a promoter drives expression of Ig- $\alpha$ , a transmembrane protein that is essential for assembly and signaling function of the pre-B cell receptor (pre-BCR) and the BCR on the B cell plasma membrane (Hombach et al. 1990, Campbell et al. 1991, Gold et al. 1991).

### **1.3.1 Protein structure of Ebf1**

The major isoform of Ebf1 consists of 591 amino acids, which includes an N-terminal DNA-binding domain (DBD) and an Ig-like/plexins/transcription factors (IPT) domain, a helix-loop-helix (HLH) dimerization domain, and a C-terminal transactivation domain (Hagman and Lukin 2005, Boller and Grosschedl 2014, Treiber et al. 2010a). Ebf1 binds its palindromic DNA-binding motif 5'-TCCCNNGGGA as a homo-dimer of two 65 kDa subunits with high affinity (Figure 9) (Travis et al. 1993).

The DBD of Ebf1 folds into a  $\beta$ -sandwich consisting of a four- and five-stranded anti-parallel  $\beta$ -sheet (Liberg, Sigvardsson and Akerblad 2002). Ebf1 keeps contacts with the major and minor grooves of the palindromic DNA site through three distinct motifs: a central module, the zinc knuckle and the GH loop. The major contribution to sequence specific DNA binding is provided by the central module comprising small  $\beta$ -sheets and loops which recognize bases within the major groove of one half-site of the palindromic binding site. The zinc knuckle establishes contacts with the minor groove of the other half- site (Treiber et al. 2010a). Biochemical analysis demonstrates a zinc-coordination motif, H-X3-C- X2-C-X5-C, located between amino acids 157 and 170 of Ebf1 (Treiber et al. 2010a, Fields et al. 2008). An expanded view of the zinc knuckle highlights the histidine and three cystine residues that coordinate the zinc ion required for DNA binding (Treiber et al. 2010a). Because of its difference to the canonical zinc finger structure, this atypical zinc finger motif was named 'zinc knuckle' or 'COE motif' (Hagman and Lukin 2005). In addition, the GH loop protrudes into the minor groove outside of the conserved recognition motif, which is important for DNA-binding affinity but does not appear to contribute to the specificity of sequence recognition (Treiber et al. 2010a).





**Figure 9 Structure of Ebf1**

The domains of Ebf1 are labeled and colored consistently throughout. A schematic representation of the domains in Ebf1<sub>1-591</sub> includes the DNA-binding domain (DBD) with a unique zinc knuckle involving a central motif (red) and its coordinating motif (purple). The DBD (orange), TIG/IPT and HLH domains all participate in Ebf1 dimerization. The carboxyl terminus includes the Ser/Thr/ Pro-rich transactivation domain (light grey). The amino acids demarcating each domain are numbered (upper part). The structure of an Ebf1<sub>35-412</sub> dimer interacts with DNA (grey) via the zinc ions shows as red spheres. The perspective is parallel to the helical axis of the DNA molecule. The visible portion of the HLH domain, the TIG/IPT domain, the DBD domains and the zinc knuckle motifs are indicated (red; lower part). (Figure is taken from Boller and Grosschedl 2014).

The Ebf1 dimer assembles a unique symmetric clamp extending across both half-sites of the palindrome. This configuration explains the strict requirement for a two-nucleotide spacer between the half-sites recognized by Ebf1, as has been noticed in mutation analyses (Travis et al. 1993). The mutagenesis of contact residues of Ebf1

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confirmed their importance for DNA binding (Fields et al. 2008, Siponen et al. 2010, Treiber et al. 2010a).

The C-terminus of the DBD is followed by an IPT domain folding in an immunoglobulin-like structure that extends from amino acid 262 to 353 (Bork et al. 1999). Although the IPT domain participates in the dimerization of Ebf1, the major part of the contact area is contributed by the HLH domains, which are necessary for dimerization in the absence of DNA (Hagman et al. 1995). A single HLH domain consists of two amphipathic helices (Hagman et al. 1993, Wang, Tsai and Reed 1997, Wang, Betz and Reed 2002), and four helices form a helix bundle of dimerized HLH domains (Ma et al. 1994, Hagman and Lukin 2005, Treiber et al. 2010a). The second helix is duplicated in vertebrates, resulting in a helix-loop-helix-loop-helix motif, for which no additional electron density has been detected arguing against a compact inclusion but raising the possibility of interaction with other proteins (Treiber et al. 2010a, Siponen et al. 2010).

### **1.3.2 Molecular mechanism of Ebf1 function**

Within the hematopoietic system, Ebf1 is expressed exclusively in B cells from the earliest step on, and is functionally required for early B cell development targeting around 3000 genes, which are strongly associated with B cell function and BCR signaling (Mandel and Grosschedl 2010, Nutt and Kee 2007, Zandi, Bryder and Sigvardsson 2010, Treiber et al. 2010b). The 3000 target genes can be divided into three groups: activated, repressed and poised genes, which are bound by Ebf1 in early B-cell stages but expression is detected only at later stages of the B lineage (Treiber et al. 2010b).

Independent of transcriptional activation or repression, ectopic expression of Ebf1 in a pre-T cell line results in the modification of histone 3 at lysine 4 (H3K4me2) of B cell specific Ebf1 targets (Treiber et al. 2010b). During B cell development, the CD79a promoter is activated by the collaboration of Ebf1, Runx1, E2A and Pax5 (Maier et al. 2004, Sigvardsson et al. 2002, Maier et al. 2003). In studies of the Ebf1 target gene *CD79a*, Ebf1 has been shown to contribute to epigenetic regulation of the promoter through CpG demethylation and nucleosomal remodelling. These

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modifications increase local chromatin accessibility, but they do not lead to transcription in the absence of other transcription factors (Maier et al. 2004). Ebf1 function is also associated with the chromatin-remodeling complexes SWI/SNF and Mi-2/NuRD. Ebf1-mediated induction of chromatin accessibility at the *Cd79a* promoter is dependent on the SWI/SNF complex, whereas the Mi-2/NuRD complex is involved in chromatin compaction and DNA hypermethylation at the *Cd79a* promoter (Gao et al. 2009). The Ebf1-activated targets *CD79a*, *Gfra2*, and *Pax5* gain H3K4 methylation and H3 acetylation marks upon the transition to the pro-B stage, correlating with their transcriptional activation (Treiber et al. 2010b). Although some targets bound by Ebf1 specifically in mature B cells show H3K4me2 modifications already in pro-B cells, H3K4 di-methylation with Ebf1 occupancy is mainly detected in mature B cells (Gyory et al. 2012).

## **1.4 Post-translational modification**

The human genome comprises 20,000 to 25,000 genes while the proteome is over 1 million, indicating that single genes encode multiple proteins (Jensen 2004). The complexity is further increased from the level of the genome to the proteome by post-translational modifications (PTMs), which are known to be essential mechanisms used by eukaryotic cells to diversify their protein functions and dynamically coordinate their signaling networks (Wang, Peterson and Loring 2014).

PTMs are chemical modifications that play a key role in functional proteomics, because they regulate activity, localization and interaction of proteins with other cellular molecules such as proteins, nucleic acids, lipids, and cofactors (Jensen 2004). Over 200 types of PTMs occur by enzymatic activity (Walsh, Garneau-Tsodikova and Howard-Jones 2006). These enzymes include kinases, phosphatases, transferases and ligases, which add or remove functional groups, proteins, lipids or sugars to or from amino acid side chains, and proteases, which cleave peptide bonds to remove specific sequences or regulatory subunits. Many proteins can also modify themselves using autocatalytic domains, such as autokinase and autoprotolytic domains (Krishna and Wold 1993).

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Post-translational modifications are changes made to a polypeptide or protein any time after translation. For example, many proteins are modified shortly after translation to mediate proper protein folding or stability or to direct the nascent protein to distinct cellular compartments (Grotenbreg and Ploegh 2007). Other modifications occur after folding and localization in order to activate or inactivate catalytic activity or to influence the biological activity of the protein (Geiss-Friedlander and Melchior 2007).

The most common types of PTMs studied in protein research today are focused on phosphorylation, glycosylation, ubiquitination, acetylation and methylation. PTMs can be experimentally detected by a variety of techniques, including mass spectrometry, eastern blot and western blot (Gianazza, Crawford and Miller 2007).

### **1.4.1 Phosphorylation**

Phosphorylation affects a large number of proteins in eukaryotic cells.

Phosphorylation is associated with protein activity and plays a key role in protein function regulation. The change of conformation can alter the catalytic activity of a protein, which means the protein can be activated or inactivated. Another effect of phosphorylation is to recruit other proteins, which contain a domain recognizing and binding to phosphomotifs. For example, MH2 and WW domains can recognize and bind to phosphoserine; SH2 and PTB domains can recognize and bind to phosphotyrosine; FHA domains can recognize and bind to phosphothreonine. Thus phosphorylation can regulate protein interactions resulting in signal transduction via downstream effector proteins (Yaffe 2002). This intracellular signal transduction is involved in the mediation of cell cycle progression, differentiation, development, peptide hormone response, adaptation and transformation (Hubbard and Cohen 1993, Pawson and Scott 1997, Hunter 2000, Toska et al. 2002).

Phosphorylation only occurs at the side chain of serine, threonine and tyrosine. The amino acids have a hydroxyl group that attacks the terminal phosphate group ( $\text{PO}_4^{2-}$ ) on a universal phosphor donor adenosine triphosphate (ATP), resulting in the transfer of the phosphate group to the amino acid side chain. This transfer is facilitated by magnesium ( $\text{Mg}^{2+}$ ). Protein phosphorylation is a reversible PTM that is

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mediated via kinases or phosphatases (Toska et al. 2002). The reversibility of protein phosphorylation also contributes to signal transduction allowing cells to respond to intracellular or extracellular stimuli (Johnson and Lewis 2001). In many cases, receptors activate downstream kinases, which then phosphorylate and activate their cognate downstream substrates, including additional kinases, until the specific response is achieved.

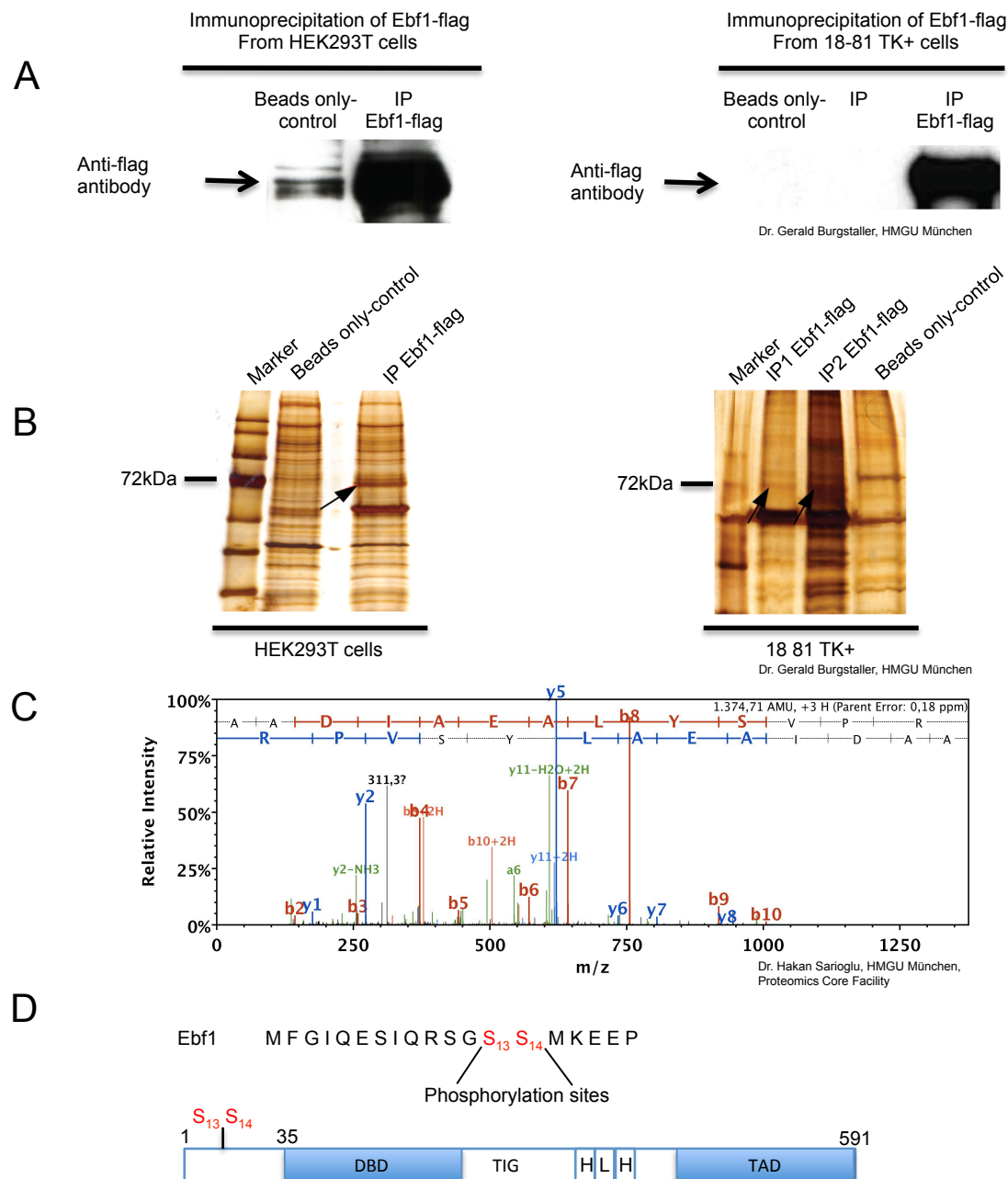
Substrates for kinase activity are diverse and include lipids, carbohydrates, nucleotides and proteins. An important co-substrate for almost all protein kinase is ATP, which is an ideal structure for the transfer of phosphate groups for nucleotidyl-, pyrophosphoryl- or phosphoryltransfer, respectively (Maltese 1990). Protein kinases are categorized into subfamilies that catalyse distinct domains. Approximately 80% of the mammalian kinome comprises serine and threonine kinases, and over 90% of the phosphoproteome consists of pS and pT. Indeed, the relative abundance ratio of pS: pT: pY in a cell is 1800:200:1 (Mann et al. 2002). Specificity is achieved via sequences flanking the consensus sites (Pawson and Nash 2003). Based on the consensus sequence, kinases not only phosphorylate a single protein but multiple substrates if the kinase-specific consensus sequences are available (Maltese 1990).

#### **1.4.2 Post translational modification of Ebf1**

Ebf1 was first described in 1991 since when the biological and biochemical function of Ebf1 have been studied (Hagman et al. 1991, Feldhaus et al. 1992). Conditional deletion of Ebf1 shows that it fulfills different roles during B cell development: activation, repression and poising of different target genes. Since these processes differ strongly, Ebf1 very likely needs to have different interaction partners and likely differs itself in these processes.

Post-translational modifications can significantly regulate activation, localization and interaction with other proteins, and all of these processes potentially affect the role of Ebf1 during B cell differentiation. Mass spectrometry is a classic method for the identification of PTM and amino acids affected thereby (Jensen 2004, Jiang, den Hertog and Hunter 2000). To isolated Ebf1 protein, *Ebf1-flag* was over expressed in HEK293T and 18-81 TK+ cell, and immunoprecipitated using anti-flag antibody

(Dr. Gerald Burgstaller, HMGU Munich). The purified Ebf1 protein was analysed via mass spectrometry under conditions allowing the identification of PTM (Dr. Hakan Sarioglu, HMGU Munich)



**Figure 10 Identification of post-translational modifications of Ebf1**

A: Western blot analysis of immunoprecipitated Ebf1-flag from HEK293T (left) and 18-81 TK+ cells, which ectopically express Ebf1-flag via transient transfection. Western blot and immunoprecipitation (IP) were performed using an anti-flag antibody. IP is a control to show the endogenous Ebf1 expression in 18-81 TK+ cells.

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Beads only is a negative control. B: Silver staining of immunoprecipitated proteins from HEK293T (left) and 18-81 TK+ cells. The precipitated proteins are isolated via a denaturing SDS-polyacrylamide gel. Ebf1 protein is indicated by an arrow, the protein size is approximately 72 kDa. Two independent IPs are shown (IP1, IP2). C: Phosphorylated Ebf1-S<sub>13</sub> and S<sub>14</sub> are identified by mass spectrometry. The phosphorylated Ebf1-S<sub>13</sub> and S<sub>14</sub> were spiked at equimolar level. D: The N-terminal amino acid sequence of βEbf1. S<sub>13</sub> and S<sub>14</sub> are marked in red to indicate the sites of phosphorylation. The methionine (M) located next to S<sub>14</sub> is the starting of αEbf1. DBD: DNA binding domain, TIG: immunoglobulin-like plexin. HLH: helix loop helix, TAD: transactivation domain.

Recording coverage of more than 60% of total amino acids of Ebf1, three sites for phosphorylation were identified, two of which are studied here: Serines located at position 13 and 14 of Ebf1 (Ebf1-S<sub>13</sub>, Ebf1-S<sub>14</sub>). The phosphorylation of Ebf1 at S<sub>13</sub> and -S<sub>14</sub> are only detectable in pre-B cell line cells (18-81 TK+), but not in human kidney cells (HEK293T) indicating a certain cell type specificity. As αEbf1 lacks the first 14 amino acids, phosphorylated Ebf1-S<sub>13</sub> and -S<sub>14</sub> are only part of in βEbf1. This phosphorylation of Ebf1-S<sub>13</sub> and -S<sub>14</sub> might regulate its interaction with other proteins, thereby allowing a differential influence on early B cell development.

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## 2 Aim of Study

Early B cell differentiation is regulated via a complex transcriptional network. Ebf1 is a central part of it, regulating around 3000 target genes associated with B cell function. Among these target genes, Ebf1 plays diverse roles to activate, repress or poise gene expression. Post-translational modification represents a potential explanation for these diverse roles. Specifically, phosphorylation of Ebf1 might contribute to the diverse functions of Ebf1 during B cell development.

The hypothesis of this study is that phosphorylation of Ebf1 at S<sub>13</sub> and S<sub>14</sub> plays a role in B cell development, and the aim of this study is to clarify the role of phosphorylation on Ebf1 S<sub>13</sub> and S<sub>14</sub> in early B cell development.

This study confirms the post-translational modification of endogenous Ebf1 via monoclonal antibodies, which only recognized the phosphorylated Ebf1-S<sub>13</sub> and S<sub>14</sub>. We investigate the role of phosphorylated Ebf1-S<sub>13</sub> and S<sub>14</sub> in primary B cells. As a transcription factor, the molecular mechanism of Ebf1 is to bind to DNA and regulate the expression of target genes. Therefore, we analyse the biological and biochemical function of phosphorylated Ebf1-S<sub>13</sub> and S<sub>14</sub> in B cell development.



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## 3 Material and Methods

### 3.1 Material

#### 3.1.1 Instruments

Manufacturers	Device
Peqlab Biotechnologie GmbH	Agarose Gel Chambers
CP2245; Sartorius AG	Accuracy Weighing Machine
B6120, Heraeus; Thermo Scientific	Bacterial Incubator
Trans-Blot Semi-Dry Transfer Cell; Bio-Rad	Blotting Chamber
CASY TTC, Innovatis	Cell Counter
Rotina 38R; Hettich Micro 200R; Hettich Rotanta 460R; Hettich Eppendorf centrifuge 5424; Eppendorf	Centrifuges
CB150; Binder	CO2-Incubator
TYPON Optimax; Raymed Imaging AG	Film Developer
Gene Pulser®; Bio-Rad	Electroporation System
FACSCalibur™ Flow Cytometer; BD Biosciences FACSCanto™ Flow Cytometer; BD Biosciences FACSAria™III Flow Cytometer; BD Biosciences	Flow Cytometry
Bio-Rad	Gel Dryer
Thermomixer Compact; Eppendorf	Heating Block
AF 100; Scotsman®	Ice Machine
Karl Hecht GmbH&Ko KG	Improved Counting Chamber

Herasafe KS12; Thermo Electron Corporation	Laminar Flow
LightCycle® 480 II; Roche	Light Cyclers
Microplate Luminometer Orion II; Berthold Detection Systems	Luminometer
RCT basic safety control (IKA)	Magnet Stirrer
Panasonic	Microwave
Axiovert25; Zeiss	Microscope
TV21; CTI GmbH	Native PAGE-Apparatus
DNA Engine®; Bio-Rad	PCR Machine
Mini Trans-Blot® Cell; Bio-Rad	SDS-PAGE Chamber
BioPhotometer; Eppendorf	Spectrophotometer
DNA-Engine®; Bio-Rad	PCR Machine
Photometer Bio 6131; Eppendorf	Photometer
Gilson	Pipettes
763 Multi Calimolic; Knick	pH-Meter
0.2 ml PCR-Reaction tube; Biozym Diagnostik 15 ml, 50 ml; Falcon 0.5 ml, 1.5 ml, 2 ml, Eppendorf	Reaction Vessels
Polymax 1040; Heidolph	Shakers
Light Cycler 480II; Roche	Thermo Cycler
Transilluminator TL33; HEROLAB GmbH	UV-Transilluminator
BVC 21; Vacuubrand	Vacuum Pump
Vortex Genius (IKA)	Vortexer
PowerPack300X; BioRad	Voltage Source
Sub6; Grant	Waterbath

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### 3.1.2 Consumables

Manufacturers	Products
ART® Molecular BioProducts	Pipette Tips
Becton Dickinson (BD) GmbH	Round-Bottom Tube: 5 ml Round-Bottom Tube with Strainer Cap: 5 ml Conical Tubes: 15 ml, 50 ml Luer-Lok™ Syringe: 20 ml Cell Culture Flasks: 75 cm <sup>2</sup> , 175cm <sup>2</sup> Cell Culture Plates: various Tissues Culture Dish: 10 cm <sup>2</sup> Cell Strainer: 70 µm Nylon, 100 µm Nylon
BioRad	Gene Pulser® Cuvettes
Biozym Diagnostik GmbH	PCR Tube: 0.2 ml
Brand	Disposable Cuvettes
Braun	Sterican® Cannulas 0.4 x 20 mm, 27G x 3/4", Size 20
CEA GmbH	Medical X-Ray Screen Film Blue Sensitive
Corning Incorporated	Sterile Disposable Pipettes
Eppendorf	Cuvette® 220-16 mm; Test Tube: 1.5 ml, 2 ml
Gilson	D10, D200, D1000 Diamond® Tower Pack™ tips
Millipore	Syringe Filter: 0.22 µm
Nunc GmbH	Cryo Tube™ Vials
Pharmacia	Sephadex® G-50 Column
Protran	Nitrocellulose Membrane
Roche Diagnostics	LightCycler® 480 Multiwell Plate 96 LightCycle® 480 Sealing Foil
Schott, Braun	Glass Ware
Sigma	Kodak BioMax MS Film
USA scientific	Lumitrac™ 2000 96W Microplate
Whatman GmbH	Gel-Blotting-Paper: 0.34 mm; 1.2mm

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### 3.1.3 Chemicals and Enzymes

Manufactures	Products
Applichem	Propidium Iodide
Applied Biosystems	Emerald™ Enhancer; Galacton-Plus® Chemiluminescent Substrate for β-Galactosidase
Bayer	Ciprobay® 400
Biochrom AG	Fetal calf serum (FCS)
Biolab Products	CRYSTAL agarose
Fermentas	dNTPs (100 mM) Glycogen
Gibco BRL	Dulbecco's Modified Eagle Medium (DMEM); Dulbecco's Phosphate Buffered Saline (PBS); L-Glutamine, 200 mM, 100 x; OptiMEM®; Penicillin/Strptomycin (10.000 U/ml & 10.000 µg/ml); Polyethylenimin (PEI); RPMI 1640; Sodium Pyruvate, 100 mM; Trypsin-EDTA, 0.05%
Hartmann Analytic	<sup>32</sup> P-Alpha-dCTP (3000 Ci/mol)
Invitrogen Life Technologies	5 x First Strand Buffer; Dithiothreitol (DTT); Oligo (dT) 12-18 Primer (0.5 µg/µl); SuperScript® II Reverse Transcriptase (200 U/µl)
New England Biolabs	1 x NEBuffer 2 & 3 Calf Intestinal Alkline Phophatase (10 U/µl)
PAA Laboratories	Bovine Serum Albumin (BSA)
PAN Biotech	Fetal calf serum (FCS)
Peqlab	peqGOLD TriFast™
Promega	RNasin® (40 U/µl)

R & D Systems	Recombinant Mouse IL-3; Recombinant Mouse IL-6; Recombinant Mouse SCF
Roche Diagnostics	Acetyl-Coenzyme A; Aprotinin; ATP disodium salt; Complete, Mini, EDTA-free Tablet; D(-)-Luciferin; Klenow Fragment, 2 U/μl; Leupeptin; LightCycler® 480 SYBR Green I Master; Phosphatase Inhibitor Cocktail Tablet; PMSF
Roth	Ammonium Persulfate ( Knapstein et al.); Ampicillin; Dimethylsulfoxid (DMSO); EDTA; Glycerin; Glycin; Ponceau S; Proteinase K; Rotiphorese® Gel 40 (Acrylamid/Bisacrylamid 29:1); N,N,N,N-Tetramethylethylenediamin (TEMED); Tris; Powdered milk; Tween® 20
Sigma	2-Mercaptoethanol; Chloroform; Luminol Sodium Salt; Nonidet™ P40 Substitute; p-Coumaric Acid; Polybren; Poly (deoxyinosinic-deoxycytidylic) Acid Sodium Salt (Poly (dl-dC))
Solis BioDyne	1 x FirePol Puffer B FIREPol® DNA Polymerase (5 U/μl)
Thermo Scientific	BamHI (10 U/μl); 10 x BamHI Buffer; BglII (10 U/μl); 10 x B Buffer; SpeI (10 U/μl); 10 x 0 Buffer; 10 mM dNTP; EcoRI (10 U/μl); 5 x Phusion HF Buffer; Phusion DNA Polymerase; pJET1.2/blunt Cloning Vector (50 ng/μl); 2x Reaction Buffer; StuI (10 U/μl); T4 DNA Ligase

	(5 U/μl); XhoI (10 U/μl);
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### 3.1.4 Kits

Manufactures	Kits
LightCycler® 480 SYBR Green I Master	Roche
Pierce® BCA Protein Assay Reagent	Thermo Scientific
QIAGEN® Plasmid Midi/Maxi Kit	QIAGEN GmbH
Quik Change®, Site-Directed Mutagenesis Kit	Stratagene
Wizard® SV Gel and PCR Clean-Up System	Promega

### 3.1.5 Size Markers

Manufactures	Products
GeneRuler™ 100 bp Plus DNA Ladder	Fermentas
PageRuler™ Prestained Protein Ladder	Fermentas

### 3.1.6 Antibodies

#### 3.1.6.1 Peptide anti-Ebf1-S13 (p), 14 (p) antibodies

The peptide sequence used to generate Anti-Ebf1-S13<sub>(p)</sub> and –Ebf1-S13<sub>(p)</sub> S14<sub>(p)</sub> Antibodies is: SIQRSGS<sub>(p)</sub>S<sub>(p)</sub>MKEEPC (Ebf1: amino acids 7-19). The C-terminal cysteine was added. Dr E.Kremmer and colleagues, HMGU Munich, generated the antibodies.

### 3.1.6.2 Antibodies for western blot and electrophoretic mobility shift assay (EMSA)

Antibody	Clone	Host Species	Manufactures	Dilution
Anti- $\beta$ -Actin	AC-74 Monoclonal	Mouse	Sigma	1:10000
Anti-Ebf1-S13 <sub>(p)</sub>	14H6, Isotype IgGG1, Monoclonal	Rat	E.Kremmer,	1:1
Anti-Ebf1-S13 <sub>(p)</sub> S14 <sub>(p)</sub>	1C4, Isotype IgGG1, Monoclonal	Rat	E.Kremmer HMGU	1:1
Anti-Ebf1-S13 <sub>(np)</sub> S14 <sub>(np)</sub>	1E12, Isotype IgG2c, Monoclonal	Rat	E.Kremmer HMGU	1:1
Anti-FLAG® M2	Monoclonal	Mouse	Sigma	1:10000
Anti-Pan-Ebf1	5E6, Isotype IgG2a, Monoclonal	Rat	E.Kremmer, HMGU	1:1
Anti-Mouse IgG	Peroxidase Polyclonal	Goat	Sigma	1:5000
Anti-Rat IgG-IgM	Peroxidase- Polyclonal	Goat	Jackson Immuno Research	1:5000

p: phosphor specific antibody; np: non-phosphor specific antibody

Undiluted antibodies are derived from hybridoma supernatant.

### 3.1.6.3 Antibodies for flow cytometry

Antibody	Clone	Host Species	Manufactures	Dilution
Anti-B220-FITC	RA3-6B2	Mouse	BD Diosciences	1:500
Anti-BP-1-Biotin	6C3	Mouse	eBioscience	1:500
Anti-CD19-Biotin	1D3	Mouse	eBioscience	1:500
Anti-CD24-PE	30F1	Mouse	eBioscience	1:2500
Anti-CD43-APC	S7	Mouse	eBioscience	1:500
Anti-CD45.2-APC	104	Mouse	eBioscience	1:500
Anti-IgD-Biotin	11-26	Mouse	eBioscience	1:500
Anti-IgM-APC	II/41	Mouse	eBioscience	1:250
Streptavidin-PE			eBioscience	1:500
Streptavidin-PerCP-Cy5.5			eBioscience	1:500

### 3.1.7 Oligonucleotides

All oligonucleotides listed are purchased from Metabion Company.

#### 3.1.7.1 Oligonucleotides for quantitative PCR

Oligonucleotide	Sequence 5' → 3'	Amplification Product (bp)
<i>β-Actin</i> FP	TGT GGT GGT GAA GCT GTA GC	380
<i>β-Actin</i> RP	GAC GAC ATG GAG AAG ATC TGG	
<i>B29</i> FP	AGC CGG AGC TCC ATG GTG AAG	216
<i>B29</i> RP	TGG CGC TGT CAC ATT TCT GC	
<i>CD19</i> FP	CGG GAC CCA GAA CCA GTA CG	247
<i>CD19</i> RP	CTG CCC AAG GTT GGA GTC GT	
<i>E2A</i> FP	GAC TCC GGG AGC GAG ATG AA	196
<i>E2A</i> RP	ATG AGG AAC CTG CGC TCC TG	
<i>EBF1</i> FP	CAT GTC CTG GCA GTC TCT GA	240
<i>EBF1</i> RP	CAA CTC ACT CCA GAC CAG CA	



<i>FOXO1</i> FP	GGA GAC AGC CCT GGG TAT CA	183
<i>FOXO1</i> RP	GGA ATG CAC GTC CCC ATC TC	
<i>Mb1</i> FP	TCA GAG CCC TGC CTC TCC TC	199
<i>Mb1</i> RP	ACT GGG GGC CCA TGT GAT GTT	
<i>Pax5</i> FP	TGG ACC GCG TGT TTG AGA GA	226
<i>Pax5</i> RP	AGG GTT GTG CTC GCC AAG TC	
<i>Rag1</i> FP	CCT TTG AAA AGG CAC CCG AGG	207
<i>Rag1</i> RP	GCC TGG CTT GGT GAA CTG CT	
<i>VpreB</i> FP	GCT CAT GCT GCT GGC CTA TC	224
<i>VpreB</i> RP	TCC AAG GGA AGA AGA TGC TAA	
$\lambda$ 5 FP	TGG AGC TTC AGT GGG AAG CA	152
$\lambda$ 5 RP	GGG TCC CAC CAC CAA AGA CA	

### 3.1.7.2 Oligonucleotides for mouse genotyping

Oligonucleotide	Sequence 5' → 3'	Amplification Product (bp)
<i>Ebf1</i> FP	GGA AAA GTT GCC TTC AAG TTG	150
<i>Ebf1</i> RP	TGT AGA GGA GCT GGA GCC G	
<i>Ebf1</i> Neo RP	GCG ATG CCT GCT TGC CGA A	400

### 3.1.7.3 Oligonucleotides for site-directed mutagenesis

Oligonucleotide	Sequence 5' → 3'
<i>Ebf1</i> -S13A FP	CAA CGG AGT GGA GCC AGT ATG AAG GAA GAG
<i>Ebf1</i> -S13A RP	CTC TTC CTT CAT ACT GGC TCC ACT CCG TTG
<i>Ebf1</i> -S14A FP	CAA CGG AGT GGA GGC GCT ATG AAG GAA GAG
<i>Ebf1</i> -S14A RP	CTC TTC CTT CAT AGC GCT TCC ACT CCG TTG
<i>Ebf1</i> -S13A, S14A FP	CAA CGG AGT GGA GCC GCT ATG AAG GAA GAG
<i>Ebf1</i> -S13A, S14A RP	CTC TTC CTT CAT AGC GGC TCC ACT CCG TTG

### 3.1.7.4 Oligonucleotides for D-J rearrangement of immunoglobulin heavy chain locus

Oligonucleotide	Sequence 5'→3'
<i>D<sub>H</sub>3-1</i> FP	AGG CTC TGA GAT CCC TAG ACA G
<i>GL5-1</i> RP	CCC GGA CAG AGC AGG CAG GTG G
<i>D<sub>H</sub>5-1</i> RP	ACA AGC TTC AAA GCA CAA TGC CTG GCT
<i>D<sub>H</sub>5-2</i> FP	ACG TCG ACT TTT GTC AAG GGA TCT ACT ACT GT
<i>J<sub>H</sub>4</i> RP	GGG TCT AGA CTC TCA GCC GGC TCC CTC AGG G
<i>GL3-2</i> FP	CGA AGT ACC AGT AGC AC
<i>GL5-2</i> RP	GAG TTG ACT GAG AGG ACA

### 3.1.7.5 Oligonucleotides for cloning

Oligonucleotide	Sequence 5'→3'
<i>αEbf1</i> FP	GAA TTC ATG AAG GAA GAG CCG CTG GG
<i>αEbf1</i> RP	CTC GAG TCA CAT GGG AGG GAC AAT CA
<i>Ebf1-ΔTAD</i> FP	GAA TTC ATG TTT GGG ATC CAG GAA AG
<i>Ebf1-ΔTAD</i> RP	CTC GAG CGA AGT GTT AGC AAG GGC TG
<i>αEbf1-ΔTAD</i> FP	GAA TTC ATG AAG GAA GAG CCG CTG GG
<i>αEbf1-ΔTAD</i> RP	CTC GAG CGA AGT GTT AGC AAG GGC TG

### 3.1.7.6 Oligonucleotides for EMSA

Oligonucleotide	Sequence 5'→3'
<i>Mb-1</i> FP	CCC CGA CCC CAC GCA CTA GAG AGA GAC TCA AGG GAA TTG TGG
<i>Mb-1</i> RP	CCC TGC ACC TGG GCT GGC CAC AAT TCC CTT GAG TCT CTC TC

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### 3.1.7.7 Oligonucleotides for sequencing

Oligonucleotide	Sequence 5'→ 3'
MSCV-EcoR1- <i>Ebf1</i> FP	CTG CAG CAT CGT TCT GTG TT
MSCV-Xho1- <i>Ebf1</i> RP	TAC GCT TGA GGA GAG CCA TT
pCMVcyto- <i>Ebf1</i> FP	TAC ATC AAT GGG CGT GGA TA
pCMVcyto- <i>Ebf1</i> RP	CCT ACT CAG ACA ATG CGA TGC
pcDNA3.1-flag- <i>Ebf1</i> FP	TCG GAA GGT ACG CCC TCT TA
pcDNA3.1-flag- <i>Ebf1</i> RP	TCC CAT ACA GGG CTT CAA CC

### 3.1.8 Plasmids

**MSCV-IRES-GFP** (MIG, Dr. Richard Moriggl, Ludwig Boltzmann Institute, Wien)

The murine stem cell virus (MSCV) vector carries the *Ebf1* gene (Dr. Silvia Hiechinger and Dr. Saihong Jin HMGU Munich) in front of an internal ribosomal entry site and the green fluorescent protein gene (IRES-GFP). The vector MIG-*Ebf1* was used for *Ebf1* site-directed mutagenesis (MIG-*S*<sub>13</sub>A), and  $\alpha$ *Ebf1*, *Ebf1*- $\Delta$ TAD and  $\alpha$ *Ebf1*- $\Delta$ TAD cloning. All of the modified MIG-*Ebf1* were finally used with the retroviral packaging cell line GP+E86 cells.

**Helper-Plasmid** (Dr. Richard Moriggl, Ludwig Boltzmann Institute, Wien)

This vector is necessary for the beginning of replication and transfer of other plasmids from a donor to a recipient. Helper-plasmid was used for transfer of MIG-*mutant Ebf1*,  $\alpha$ *Ebf1* and *-deleted Ebf1* into retrovirus packaged GP+E86 cells.

**pBL-Lambda5-Luc** (pBL-Luc empty vector: Dr. Ursula Zimbro Strobl, HMGU Munich)

This vector contains the lambda5 promoter including 3 *Ebf1* binding sites and the luciferase gene (Dr. Silvia Hiechinger, HMGU Munich). This vector was used in luciferase reporter assay.

**pcDNA3.1-Flag-*Ebf1*** (pcDNA3.1-Flag empty plasmid: Dr. Hermann Silje, MPI Munich)

pcDNA3.1-flag- *Ebf1*-S<sub>13</sub>A, -S<sub>14</sub>A and -S<sub>13, 14</sub>A using for electrophoretic mobility shift assay were generated from pcDNA3.1-Flag-*Ebf1*( Dr. Saihong Jin, HMGU Munich).

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**pCMVcyto** (Invitrogen Life Technologies)

pCMV-Ebf1-S<sub>13</sub>A, Ebf1-S<sub>14</sub>A, Ebf1-S<sub>13,14</sub>A, αEbf1, Ebf1-ΔTAD and αEbf1-ΔTAD were generated from pCMVcyto-Ebf1 (Dr. Silvia Hiechinger) and used for the overexpression in luciferase reporter assay.

**pCMVcyto-LacZ** (Dr. Ursula Zimmer Strobl, HMGU Munich)

This vector was used for over expression of the *LacZ* gene and normalization in luciferase reporter assay.

### 3.1.9 Bacteria

#### XL1-Blue

This is a E.coli stain. The genotype is F': Tu 10 proA + B lacIq D (lacZ) M15/recA1 end A1 gyr A96 (Nalr) thi hsd R17 (rk-mk +) glu V44 rel A1 lac. The original strain was purchased from Stratagene.

### 3.1.10 Cell lines

#### 18-81 TK+ (Siden et al., 1979)

Pre-B-cell line isolated from murine bone marrow provided by Dr. Hans-Martin-Jäck, Nikolaus-Fiebiger-Zentrum, Erlangen. The cell line was used for anti-Ebf1-S13<sub>(p)</sub>, and anti-Ebf1-S13<sub>(p)</sub> S14<sub>(p)</sub> phosphorylation antibodies test.

#### Ba/F3 (Palacios et al., 1984 & 1985)

IL-3-dependent pro-B cell line derived from murine bone marrow provided by Dr. Rudolph Grosschedl, MPI Freiburg. This cell line was used for electrophoretic mobility shift assay and luciferase reporter assay.

#### HEK293T (Graham et al., 1977)

Transformed fibroblast-like cell line derived from human embryonic kidney cells constitutively expressing the SV40 large T antigen. This cell line was used for the propagation of retroviruses with the murine stem cell virus and the protein expression test of mutant and truncated Ebf1. Dr. Rudolph Grosschedl, MPI Freiburg, provided this cell line.

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**GP+E86** (Markowitz et al., 1988)

Fibroblast-like retrovirus packaging cell line that was isolated from murine embryo. This cell line contains the gag, pol and env genes of the Moloney murine leukemia virus. This cell line was used for generation of helper-free recombinant retrovirus to transduce murine cells. Dr. Stefan Bohlander, University of Munich Großhadern, provided this cell line.

### **3.1.11 Mouse strain**

***Ebf1*<sup>-/-</sup>** (Lin & Grosschedl, 1995)

C57BL/6 transgenic mouse strain carries a targeted mutation in deletion of the Ebf1 gene residing on chromosome 11. In genomic sequence, functionally important portion of the DNA-binding domain of Ebf1 was replaced with a *neomycin-resistance* gene cassette. This mouse strain carries the CD45.2 allele of the tyrosine phosphatase CD45.

**C57BL/6** (Charles River, WIGA)

This mouse strain was used for generating the Ebf1-heterozygous mice to obtain a wild-type allele. In addition, he served the production of virgin wt bone marrow and spleen cells. This mouse strain carries the CD45.2 that is allogeneic the tyrosine phosphatase CD45.

**B6.2JL-Ptprc<sup>a</sup> Pepc<sup>b</sup> /BoyJ** (CD45.1; Charles River, WIGA)

C57BL/6 mouse strain carries the CD45.1 allele of the tyrosine phosphatase CD45 instead of CD45.2. These mice bone marrow cell was used as for Whitlock-Witte culture.

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### 3.1.12 Software

Software	Function
4Peaks 1.7.2	Analysis of DNA-Sequence
Adobe Illustrator CS5	Vector Drawing Program
BD FACSDiva Software	Analysis of Flow Cytometry and Cell Sorting
Cell Quest Becton Dickinson	Acquisition and Analysis of Flow Cytometry
FlowJo 9.3, Tree Star Inc.	Analysis of Flow Cytometry and Cell Sorting
Insilico Biotechnology	Ligation Calculation
LightCycler® SYBR Green Software	Recording and Evaluation of Quantitativ Real-Time PCR Reactions
MecVector with Assembler 12.7.5	Analysis of Plasmid Sequence
Primer3	Primer Design

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## **3.2 Methods**

### **3.2.1 Molecular biology methods**

#### **3.2.1.1 RNA**

##### **3.2.1.1.1 RNA isolation from mammalian cells**

The total RNA was isolated following peqGOLD TriFast<sup>TM</sup> manufacturer's protocol. FACS Aria<sup>TM</sup> III sorted mammalian cells were collected and centrifuged at 160 g, 4 °C for 6 min. After removal of the supernatant, cells were lysed in 1 ml peqGOLD TriFast<sup>TM</sup> and incubated at room temperature for 5 min. Then, 0.2 ml chloroform (Sigma) was added. The whole samples were mixed vigorously and incubated at room temperature for 3 min. The organic and aqueous phases were separated by centrifugation (12000 g, 5 min at 4 °C). The upper aqueous phase was transferred into a new 1.5 ml tube and mixed with 0.5 ml chilled isopropanol and 1 µl of glycogen (Ambion). After 30 minutes incubation on ice, the total RNA was precipitated by centrifugation (12000 g, 15 min at 4 °C). The supernatant was discarded and the RNA pellet was washed with 1 ml 70% (v/v) chilled ethanol (DEPC water). After centrifugation (12000 g, 15 min at 4 °C) the supernatant was removed and the RNA pellet was dried at room temperature. Finally, the RNA pellet was resuspended in 20 µl RNase free ddH<sub>2</sub>O. The concentration of isolated RNA was measured by photometric determination. The RNA were either directly used for cDNA synthesis or stored at -20 °C.

##### **3.2.1.1.2 cDNA synthesis**

cDNA was synthesized according to the manufacturer's protocol of First-Strand cDNA Synthesis, using Superscript<sup>TM</sup> II (Invitrogen). 1 ng – 5 µg of total RNA were gently mixed with 0.5 µg oligo (dT), 12-18 Primer (Invitrogen), 0.5 mM dNTP (Fermentas) and added dH<sub>2</sub>O up to 12 µl. The mixture was heated at 65 °C for 5 minutes and quickly chilled on ice. After a brief centrifugation to collect all contents of the tube, the 12 µl mixture was gently mixed with 1 x First-Strand Buffer, 5 mM DTT, 40 U RNaseOUT<sup>TM</sup> and 200 U SuperScript<sup>TM</sup> II (Invitrogen). The whole sample final amount was incubated at 42 °C for 50 min and inactivated at 70 °C for 15 min. The

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final volume of the mixture is 20 µl. The cDNA was either directly used for rtPCR or stored at -20 °C.

### **3.2.1.1.3 Quantitative real time polymerase chain reaction**

Gene expression analyses were performed according to the manufacturer's protocol of LightCycle® 480 SYBR Green I Master (Roche). 1 µl of cDNA was gently mixed with 5 µl of SYBR Green Master (Roche), 1 µl FP (10 pmol), 1 µl RP (10 pmol) and 2 µl ddH<sub>2</sub>O in 96-well microtiter plate. The oligonucleotides (see 3.1.7.1) were designed to be intron spanning using tools provided on the internet (Primer3). The 96-well-microtiter plate was subsequently covered by LightCycle® 480 Sealing Foil (Roche) centrifuged for 2 min at 12000 g and run in LightCycle® 480 II machine (Roche).

Real time PCR for Ebf1 target gene expressions were under this condition:

Cycle step	Temperature	Time	Cycle
Initial polymerase activation	95°C	10 min	1
Denaturation	95°C	10 sec	45
Annealing	65°C	10 sec	
Elongation	72°C	15 sec	
Creating the melting curve	65-97 °C	0.11 °C/sec	1

Actin was used as a housekeeping gene. All of the target genes cross point (CP) values were normalized to actin CP value. To verify the specificity of the amplified products, melting curve analysis were performed.

### **3.2.1.2 DNA**

#### **3.2.1.2.1 Genomic DNA isolation from mouse tail cells**

To isolate genomic DNA from mouse tail cells for genotyping, approximately 0.3 cm mouse tail tip was cut and incubated on Thermomixer compact (Eppendorf) with 100 µl lysis buffer (1x FirePol Polymerase Buffer (Solis BioDyne), 1.5 mM MgCl<sub>2</sub>, 40 µg/µl proteinase K (Ricaud Rothiot et al.)) at 55 °C, 400 rpm for at least 1 hour. After inactivating the proteinase K at 95 °C for 15 minutes, 1 µl of the solution from the genomic DNA was used in PCR reaction (3.2.1.2.3.1)



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### 3.2.1.2.2 Genomic DNA isolation from mouse fetal liver cells

To isolate genomic DNA of fetal liver cells from Ebf1-deficient mouse embryos (CD45.2), the cells were sorted (3.2.5.3) directly into lysis buffer (1x FirePol Polymerase Buffer (Solis BioDyne), 1.5 mM MgCl<sub>2</sub>, 40 µg/µl proteinase K (Ricaud Rothiot et al.)) and incubated on Thermomixer compact (Eppendorf) at 55 °C, 400 rpm for 1 hour. To inactivating the proteinase K, the mixture was heat at 95 °C for 15 minutes. 5 µl of the solution was used in the PCR of D-J Rearrangement of Immunoglobulin Heavy Chain Locus reaction.

### 3.2.1.2.3 Polymerase chain reaction (PCR)

#### 3.2.1.2.3.1 PCR for genotyping

DNA fragments were amplified in PCR reactions for Ebf1 mouse genotyping. Genomic DNA isolated from mouse tail cells were gently mixed with 1 U FirePol DNA polymerase (Solis BioDyne), 1 x FirePol buffer B (Solis BioDyne), 0.5 mM MgCl<sub>2</sub>, 1 mM dNTP (Fermentas), 1 pmol Ebf1 FP, 1 pmol Ebf1 RP, 1 pmol Ebf1 Neo RP (3.1.7.2.) and 17.9 µl ddH<sub>2</sub>O. Briefly spin down total 25 µl reagents, whole sample was run by PCR machine (DNA-Engine®; Bio-Rad).

The PCR program for Ebf1 mouse genotyping is:

Cycle step	Temperature	Time	Cycle
Initial denaturation	95 °C	3 min	1
Denaturation	95 °C	0.5 min	30
Annealing	56 °C	0.5 min	
Extension	72 °C	0.5 min	
Final extension	72 °C	5 min	1
	4 °C	hold	

The amplified PCR DNA was detected by 1.5% agarose gel.

#### 3.2.1.2.3.2 PCR for site-directed mutagenesis

Ebf1 S<sub>13</sub>, and S<sub>14</sub> were mutated following the manufacturer's protocol of Quik Change® Site-Directed Mutagenesis Kits (Stratagene). 55ng MSCV-IRES-GFP-*Ebf1* (MIG-*Ebf1*; Dr. Silvia Hiechinger and Dr. Saihong Jin; 3.1.8), pCMVcyto-*Ebf1* (Dr. Silvia Hiechinger; 3.1.8) or pcDNA3.1-Ebf1-Flag (Dr. Saihong Jin; 3.1.8) were

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separately mixed with 1x buffer, 2.5 U Pfu Polymerase (Stratagene), 10 pmol FP, 10 pmol RP (See 3.1.7.3), 1 mM dNTP (Fermentas) and 39  $\mu$ l ddH<sub>2</sub>O. After spinning down the total 50  $\mu$ l reagents, whole sample was run by PCR machine (DNA-Engine®; Bio-Rad).

The PCR program of Ebf1 S<sub>13</sub> and S<sub>14</sub> site-directly mutagenesis is:

Cycle step	Temperature	Time	Cycle
Initial denaturation	95 °C	0.5 min	1
Denaturation	95 °C	0.5 min	16
Annealing	55 °C	1 min	
Extension	68 °C	8 min	
	4 °C	hold	

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The mutant Ebf1 was digested and transformed into XL1 Blue bacterial following the mutagenesis manufacturer's protocol. After plasmid isolation (see 3.2.1.2.4.3), the sequence of mutant Ebf1 was control by sequencing.

### **3.2.1.2.3.3 PCR for D-J rearrangement of immunoglobulin heavy chain locus**

DNA fragments of sorted cells were amplified in nested PCR reactions for D-J IgH locus rearrangement. 5  $\mu$ l genomic DNA isolated from sorted cells was gently mixed with 1 U FirePol DNA polymerase (Solis BioDyne), 1 x FirePol buffer B (Solis BioDyne), 0.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP (Fermentas), 0.4  $\mu$ M *D<sub>H</sub>3-1* FP, 0.4  $\mu$ M *D<sub>H</sub>5-1* RP, 0.4  $\mu$ M *GL5-1* RP (3.1.7.4.) and 30  $\mu$ l ddH<sub>2</sub>O. After spinning down the total 50  $\mu$ l reagents, whole sample was run by PCR machine (DNA-Engine®; Bio-Rad) for the first round amplification.

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The PCR for D-J IgH rearrangement locus was performed under this condition:

Cycle step	Temperature	Time	Cycle
Initial denaturation	95 °C	1 min	1
Denaturation	95 °C	1 min	30
Annealing	63 °C	1 min	
Extension	72 °C	1.5 min	
Final extension	72 °C 4 °C	10 min hold	1

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2 µl of the first round amplified DNA was mixed with 1 U FirePol DNA polymerase (Solis BioDyne), 1 x FirePol buffer B (Solis BioDyne), 0.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP (Fermentas), 2 µM *D<sub>H</sub>5-2* FP, 2 µM *J<sub>H</sub>4* RP, 2 µM *GL3-2* FP, 2 µM *GL5-2* RP (3.1.7.4.) and 34 µl ddH<sub>2</sub>O. After spinning down the total 50 µl reagents, whole sample was run again by PCR machine (DNA-Engine®; Bio-Rad) in the same program.

The final PCR amplified DNA was loaded on 1.5% agarose gel for D-J IgH rearrangement locus determination.

### 3.2.1.2.3.4 PCR for cloning

For *αEbf1*, *Ebf1-ΔTAD* and *αEbf1- ΔTAD* cloning MIG-*Ebf1* was amplified following the protocol of Thermo Scientific Phusion High-Fidelity DNA Polymerase. 500 ng MIG-*Ebf1* plasmid (Dr. Silvia Hiechinger and Dr. Saihong Jin; 3.1.7) was gently mixed with 1 x Phusion HF buffer, 200 µM dNTPs, 0.5 µM primers, 0.02 U/µl Phusion DNA polymerase (Thermo Scientific) and dH<sub>2</sub>O up to 50 µl. *αEbf1*, *Ebf1-ΔTAD* and *αEbf1- ΔTAD* were amplified using primer pairs (3.1.7.5), which contain EcoRI recognition site at the 5' end of the forward primer and XhoI recognition site at the 5' end of the reverse primer. The total PCR mixture is run by PCR-machine (DNA-Engine®; Bio-Rad).

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PCR for Ebf1 deletions were performed under this condition:

Cycle step	Temperature	Time	Cycle
Initial denaturation	98 °C	30 sec	1
Denaturation	98 °C	10 sec	25-35
Annealing	69 °C	20 sec	
Extension	72 °C	15 sec/kb	
Final extension	72 °C	5 min	1
	4 °C	hold	

The amplified DNA was loaded on 1.5% agarose gel to control the correct size of *αEbf1*, *Ebf1-ΔTAD* and *αEbf1- ΔTAD*.

#### 3.2.1.2.4 Gel-DNA extraction

To purify PCR amplified DNA, *αEbf1*, *Ebf1-ΔTAD* and *αEbf1- ΔTAD* DNA was loaded on agarose gel. The size of wild type *αEbf1* is 1746 bp, *Ebf1-ΔTAD* is 1284 bp and *αEbf1-ΔTAD* is 1239 bp. The band containing correct size of amplified DNA was cut out and dissolved in the same amount of Membrane Binding Solution (Promega). The DNA was purified by PCR Clean-Up System kit (Promega) following the manufacturer's protocol and eluted in 50 μl nuclease-free water, store at 4 °C or -20 °C.

#### 3.2.1.2.5 DNA cloning

##### 3.2.1.2.5.1 Ligation

To insert *αEbf1*, *Ebf1-ΔTAD* or *αEbf1-ΔTAD* into MIG, pCMVcyto and pcDNA3.1-Flag vector ligation was performed. 50 ng of pJET1.2 vector was ligated with 150 ng of Wild type *αEbf1*, *Ebf1-ΔTAD* or *αEbf1-ΔTAD*. Following online ligation calculator (Insilico Biotechnology), the ratio of vector and insert DNA is 1 to 3, 5000 ng of MIG was ligated with 4127 ng *αEbf1*, 3035 ng *Ebf1-ΔTAD* or 2929 ng *αEbf1-ΔTAD*. 5000 ng of pCMVcyto was ligated with 5363 ng *αEbf1*, 3944 ng *Ebf1-ΔTAD* or 3806 ng *αEbf1-ΔTAD*.

The mixture of vector and insert DNA was gently mixed with 1 x reaction buffer and 5 U of T4 DNA ligase (Thermo Scientific) filling in ddH<sub>2</sub>O up to 20 μl. The total mixture

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was incubated at room temperature 5 min for pJET1.2 ligation and overnight for MIG and pCMVcyto ligation.

#### **3.2.1.2.5.2 Bacterial transformation**

To amplify the plasmid, 10 µl plasmid was mixed with 100 µl XL1 blue bacteria (3.1.9) and heated at 42 °C for 45 sec. The sample was cooled down on ice for 5 min. 200 µl LB media, without ampicillin, was added into XL1 blue bacteria and incubated at 37°C, 400 rpm for 30 min on Thermomixer compact (Eppendorf). The 310 µl mixture of XL1blue bacteria, LB media and plasmid was spread on ampicillin LB plate and incubated at 37 °C overnight.

#### **3.2.1.2.5.3 Bacterial culture and DNA isolation**

After overnight incubation, the colonies of transformed XL1 blue bacteria were carefully scraped from ampicillin LB plate and incubated in 100-200 ml ampicillin LB media at 37 °C overnight.

Next day, the bacterial DNA was isolated using QIAGEN Plasmid Mini/Maxi Kit (QIAGEN GmbH) according to the manufacturer's instructions.

#### **3.2.1.2.5.4 DNA test digestion**

To test the correct insertion of *αEbf1*, *Ebf1-ΔTAD* or *αEbf1-ΔTAD*, 1 µl plasmid was gently mixed with 10 U of restriction enzyme and 1 x buffer (Thermo Scientific) filling in dH<sub>2</sub>O up to 20 µl. The mixture was incubated at 37 °C 1 hour, and then loaded on 1.5% agarose gel.

All of the plasmids were digested as following:

Cloning	Restriction enzyme	Buffer	Correct size (bp)
pJET1.2- αEbf1	XhoI, EcoRI	0	1746
pJET1.2-Ebf1-ΔTAD	XhoI, EcoRI	0	1284
pJET1.2-αEbf1-ΔTAD	XhoI, EcoRI	0	1239
MIG-αEbf1	BamHI, SpeI	BamHI	1207 and 7102
MIG-Ebf1-ΔTAD	BglII, SpeI	B	1898 and 5691
MIG-αEbf1-ΔTAD	BglII, SpeI	B	1856 and 5691
pCMVcyto-αEbf1	StuI, BglII	B	1748 and 4859
pCMVcyto-Ebf1-ΔTAD	StuI, BglII	B	1256 and 4937
pCMVcyto-αEbf1-ΔTAD	StuI, BglII	B	1256 and 4895

### 3.2.1.2.5.5 DNA sequencing

50-100 ng/μl DNA and 100 pmol/μl sequencing primers (3.1.7.7) were send to Eurofins MWG Operon for sequencing. Sequencing data were read in MacVector software.

## 3.2.2 Biochemical methods

### 3.2.2.1 Protein preparation

#### 3.2.2.1.1 Protein isolation for western blot

For the extraction of protein from cell line cells and murine cells,  $1 \times 10^7$  cells were harvested and washed by PBS. Cell pellet was resuspended in 200 μl of lysis buffer (1% Nonidet<sup>TM</sup> P40 (Sigma), 50 mM Tris/HCl, pH 8, 150 mM NaCl) and incubated on ice for 40 min. To minimize degradation or dephosphorylating of proteins, the lysis buffer protease inhibitors (Complete Mini EDTA-Free Tablets, Roche) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail Tablets, Roche) were added according to the manufacturer's instructions. Finally, the lysates were centrifuged for 5 min at 18600 g, 4 °C. The supernatants were used for further analysis or stored at – 20 °C.

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### **3.2.2.1.2 Protein isolation from Ba/F3 cells for electrophoretic mobility shift assay (EMSA)**

$2 \times 10^7$  Ba/F3 cells were harvested and washed by pre-cold PBS for the extraction of protein from cells nuclear using in EMSA (3.2.2.2). The cell pellet was dissolved in 200  $\mu$ l sedimentation buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 EGTA) freshly adding 1 mM DTT (Invitrogen Life Technologies) and 1 mM proteinase inhibitor and incubated for 15 min on ice. Afterwards, 12.5  $\mu$ l of 10% Nonidet<sup>TM</sup> P 40 (Sigma) were added to specifically break cell membrane, without impairing the nuclear membrane. The mixture was strongly shaken for 5 min and subsequently centrifuged for 10 min at 18600 g, 4 °C. The pellet was washed in 1.5 ml sedimentation buffer and centrifuged again. The cell pellet was resuspended in 40  $\mu$ l of protein extraction buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1mM EGTA) freshly adding 1 mM DTT (Invitrogen Life Technologies) and 1 mM proteinase inhibitor (Complete Mini EDTA-Free Tablets, Roche) and then incubated for 30 min at 4 °C with strong shake. After 10 min centrifugation (18600 g, 4 °C), supernatant was used for further analysis or shock frozen in liquid nitrogen and stored at - 80 °C.

### **3.2.2.1.3 Protein isolation from Ba/F3 cells for luciferase-reporter-assay**

$1 \times 10^7$  Ba/F3 cells were harvested and washed with pre-cooled PBS for the extraction of protein used in luciferase reporter assays (3.2.2.3). The cell pellet was resuspended in 100  $\mu$ l luciferase protein extraction buffer (25 mM Tris/HCl, pH 7.8, and 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM DTT (Invitrogen Life Technologies)) and incubated on ice for 15 min. The mixture was centrifuged (18600 g) for 15 min at 4 °C. The supernatant was frozen in liquid nitrogen and stored at - 80 °C.

### **3.2.2.1.4 Phosphatase-treatment of protein extract**

For phosphatase treatment of protein extracts from 18-81 TK+ cells (3.1.10), 20  $\mu$ g of total protein were incubated with 15 U calf intestinal alkaline phosphatase (CIP, New England Biolabs, 10 U/ $\mu$ l) and 1 x NEBuffer3 (New England Biolabs) in a total

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volume of 40 µl. The mixture was incubated for 1 h at 37 °C and used for western blot (3.2.2.1.6).

### **3.2.2.1.5 Protein concentration measurement**

For the determination of extracted protein concentration, the BCA Protein Assay Reagent (Thermo Scientific) was used, following the manufacturer's instructions. Reagent A was mixed with reagent B in the ratio 50:1. To create a calibration curve BSA (PAA Laboratories) was added at the concentration of 0, 2, 4, 8, 10, 15 µg/µl separately into 400 µl total volume of reagent A/reagent B mixture. To determine the concentration of extracted protein, 1 µl of protein solution was added into 399 µl of reagent A/reagent B mixture. After 30 min incubation at 37°C, the absorbance at a wavelength of 562 nm was determined by photometry (BioPhotometer; Eppendorf).

### **3.2.2.1.6 SDS-polyacrylamide gel electrophoresis**

For protein separation, 20 µg protein was mixed with Laemmli buffer (20 % glycerol, 250 mM Tris, pH 6.8, 5 % 2-mercaptoethanol (Sigma), 4 % SDS, 0.01 % bromophenolblue) and shaken at 95 °C for 15 min. Subsequently, the proteins were separated by using a SDS-PAGE comprising a 5 % stacking gel (250 mM Tris/HCl pH 6.8, 0.1% SDS, 5% Rotiphorese® Gel 40 (Roth; Acrylamid/Bisacrylamid 29:1); 0.1% APS, 0.004% TEMED (Ricaud Rothiot et al.) and a 10-12% resolving gel (375 mM tris/HCl pH 8.8, 0.1% SDS, 10-12% Rotiphorese® Gel 40; 0.1% APS, 0.004% TEMED). PageRuler™ prestained protein ladder (Fermentas) was used as a protein size marker. The gel was run in a Mini Trans-Blot Cell® (Bio.Rad) electrophoresis chamber, using 1 x SDS running buffer (25 mM Tris, 1 % SDS, 192 mM glycine) at 160 V.

### **3.2.2.1.7 Transfer of electrophoretic separated protein to nitrocellulose membranes**

After SDS-PAGE, the separated proteins were transferred to a nitrocellulose membrane (Protran) using Semi-Dry Transfer Cell apparatus (BioRad). The nitrocellulose membrane and gel blotting paper (Whatman GmbH, 1.2 mm) were



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placed in 1x transfer buffer (20 % ethanol, 80 % 1 x SDS running buffer (25 mM Tris, 1 % SDS, 192 mM glycine)) before the transfer. The nitrocellulose membrane was placed on the top of SDS-PAGE and stacked with gel blotting paper on both side as a sandwich. The transfer was performed at 10 V, 300 mA for 1.5 hours. To check the transfer efficiency, the nitrocellulose membrane was stained in Ponceau S solution (2 % Ponceau S red, 30 % trichloroacetic acid and 30 % sulfosalicylic acid) for few seconds. After washing away the Ponceau S solution, the membrane was ready for immuno-detection.

### **3.2.2.1.8 Immuno-detection of protein**

The proteins transferred onto the nitrocellulose membrane were analysed by immuno-detection. The membrane was incubated with 5 % milk (5 % dry milk power in 0.4 % Tween® 20) or 5 % BSA (5 % dry BSA powder in 0.4 % Tween® 20) for 30 minutes at room temperature. The primary antibodies were incubated with membranes in 5 % milk or 5 % BSA solution in pre-tested dilution (3.1.6.2) at 4 °C with gentle shaking. Subsequently, the membrane was washed three times for 10 minutes each in 1x TBS + 0.4 % Tween® 20. According to the primary antibody, the secondary antibody was diluted appropriately (3.1.6.2) in the same solution as primary antibody at RT for 45 minutes with gentle shaking. After washing three times in 1x TBST for 10 min each, the membrane was incubated with ECL detection reagent (1 ml reagent A (0.1 M Tris/HCl pH 8.6, 0.25 mg/ml Luminol sodium salt (Sigma)), 100 µl reagent B (DMSO (Roth), 1.1 mg/ml p- Coumaric acid (Sigma)), 0.3 µl reagent C (30% H<sub>2</sub>O<sub>2</sub> )) for few minutes. The membrane was placed onto a light sensitive film (Medical X-Ray Film Blue Screen Sensitive (CEA GmbH) and developed in a X-ray film developing machine TYPON Optimax (Raymed Imaging).

### **3.2.2.2 Electrophoretic mobility shift assay (EMSA)**

#### **3.2.2.2.1 Preparation of oligonucleotides**

The complementary oligonucleotides (3.1.7.6) at the same concentration (250 ng/µl) were resuspended in annealing buffer (10mM Tris/HCl pH7.4, 10 mM MgCl<sub>2</sub>, 50 mM NaCl) and heat up to 95 °C for 10 minutes in a Thermomixer compact (Eppendorf). After switching the Thermomixer compact off, the samples were incubated in the

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Thermomixer compact until the temperature cooled down to room temperature. For the image of DNA-binding, the double-stranded oligonucleotides were radioactively labelled or stored at -20 °C for future work.

#### **3.2.2.2.2 Labelling and purification of oligonucleotides**

To label double-stranded oligonucleotides the providing Klenow fragment of *E. coli* DNA Pol I was used as a 3' to 5' end labelling. For this purpose, 2 µl oligonucleotides (25 ng/µl), 2 µl Klenow fragment (Roche Diagnostics, 2 U/µl), 2 µl NEB2 buffer (New England Biolabs, 10 x), 5 µl <sup>32</sup>P-alpha-dCTP (Hartmann Analytic, 3000 Ci/mol), 2 µl dNTPs (dATP, dGTP, dTTP, 500 µM; Fermentas) and 7 µl ddH<sub>2</sub>O were gently mixed and incubated at 37 °C for 1 hour. Subsequently to purify the labelled oligonucleotides, TE buffer (10 mM Tris, pH 8, 1 mM EDTA) and illustra NICK Columns (GE Healthcare Life Sciences) were used following the manufacturer's protocol. Afterward, scintillation counter (BioScan, Inc) were used following manufacturer's protocol to count the amount of labelled oligonucleotides. Labelled oligonucleotides were stored until use at -20 °C.

For preparing unlabelled oligonucleotides, 2 µl oligonucleotides (250 ng/µl), 2 µl Klenow fragment (2 U/µl), 2 µl NEB2 buffer (10 x), 2 µl of dNTPs (dATP, dCTP, dGTP, dTTP; 500 µM) and 12 µl ddH<sub>2</sub>O were incubated at 37 °C for 1 hour. The oligonucleotides were stored until use at -20 °C.

#### **3.2.2.2.3 Buffer preparation**

For the binding reaction of proteins and oligonucleotides, binding buffer was prepared. 5 µl 4 x binding buffer (40 mM HEPES pH 7.9, 280 mM KCl, 4 mM DTT (Invitrogen Life Technologies), 16 % Glycerin, 4 mM EDTA, 10 mM MgCl<sub>2</sub>), 2 µl poly dI-dC (1 mg/ml, (Sigma)) and 2 µl BSA (1 mg/ml, (PAA Laboratories)) were mixed with 3 - 4 µg protein from Ba/F3 cell lysates (3.2.2.1.2) and incubated on ice for 5 minutes. Subsequently, 2 µl radioactively labelled or unlabelled oligonucleotides and in some cases 2 µl Anti-FLAG® M2 antibody (3.1.6.2) was added to the binding buffer mixture, adding ddH<sub>2</sub>O to a final volume 20 µl. This sample was incubated on ice for 30 minutes and then loaded on a non-denaturing polyacrylamide gel.

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#### **3.2.2.2.4 Gel running and detection**

For the non-denaturing polyacrylamide gel (4 % Rotiphorese® Gel 40 (Roth), 0.5 x TBE pH8.3, 0.1 % APS (Ricaud Rothiot et al.) and 0.003 % TEMED (Ricaud Rothiot et al.)) running, 1 x TBS (pH 8.3) buffer was used. The protein, oligonucleotides and antibodies were separated at 130 V for 2 hours at 4 °C. Subsequently, the polyacrylamide gel was dried on a gel blot paper (Whatman Ltd., 0.34 mm) for 1 hour using a gel dryer (Bio-Rad) with vacuum and temperature at 80 °C. Finally, the dried gel was incubated with a light-sensitive film (Kodak Biomax MS Film, (Sigma)) at -80 °C for 4 hours. The film was developed in the developing machine (Raymed Imaging TYPON Optimax).

#### **3.2.2.3 Luciferase-reporter-assay**

##### **3.2.2.3.1 Luciferase-measurement**

To measure luciferase activity, 10 µl Ba/F3 cell protein extract (3.2.2.1.3) was added into a 96 well Microplate (Lumitrac™ 2000, (U.S. Scientific)). Additionally, 50µl luciferase assay buffer (20mM Tricin, 1 mM magnesium-carbonat pentahydrate, 3 mM Mg<sub>2</sub>SO<sub>4</sub>, 0.1 mM EDTA, 33.3 mM DTT (Invitrogen Life Technologies), 270 µM acetyl-Coenzyme A (Roche Diagnostics), 470 µM luciferin (Roche Diagnostics), 530 µM ATP disodium salt (Roche Diagnostics)) for each sample were prepared. The measurement of bioluminescence was performed using a luminometer (Berthold Detection Systems) at 560 nm wavelength and luciferase activity was calculated by normalizing to the β-galactosidase activity (3.2.2.3.2).

##### **3.2.2.3.2 β-Galactosidase measurement**

To normalise the transfection efficiency, a plasmid encoding gene lacZ was co-transfected in reporter assay. To measure β-galactosidase activity, 10 µl Ba/F3 cell protein extraction (3.2.2.1.3) were mixed with 100 µl β-galactosidase assay buffer (100 mM Na-P, pH8.0; 1 % Galacton Plus® Chemiluminescent Substrate for β-galactosidase (Applied Biosystems), 1 mM MgCl<sub>2</sub>) and incubated for 15 minutes at RT. Additionally, 50 µl β-galactosidase amplification buffer (0.2 M NaOH, 10% Emerald™ Enhancer (Applied Biosystems)) was used for the luminometer (Berthold

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Detection Systems). The  $\beta$ -galactosidase measurement was performed using a luminometer at 475 nm wavelength.

### 3.2.3 Cell culture

#### 3.2.3.1 General cell culture techniques

All cell culture procedures described below were carried out under a forced airflow hood (Thermo Scientific) and performed using sterile solutions and disposable pipettes (Corning Incorporated). Cells were cultured in the respective cells adapted media (3.2.3.2) and incubated in a CO<sub>2</sub> incubator (Binder) at 37 °C with 5% CO<sub>2</sub> and 95% humidity. Adherent cells and suspension cells were passaged every 3-4 days and washed in sterile PBS (Gibco BRL) using centrifugation at 160 x g for 6 minutes.

#### 3.2.3.2 Cell culture media

##### 18-81 TK+:

RPMI 1640 (Gibco BRL)	
Heat- inactivated FCS (PAA Laboratories)	10%
Sodium Pyruvate (100 mM; Gibco BRL)	1%
Penicillin/Streptomycin (10.000 U/ml & 10.000 µg/ml, Gibco BRL)	1%
$\beta$ -Mercaptoethanol (Sigma)	500 µM

##### Ba/F3:

RPMI 1640 (Gibco BRL)	
Heat- inactivated FCS (PAA Laboratories)	10%
Filtered IL-3-containing WEHI-3B supernatant	10%
L-Glutamine, 200 mM (100 x; Gibco BRL)	1%
Penicillin/Streptomycin (10.000 U/ml & 10.000 µg/ml, Gibco BRL)	1%

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**HEK293T and GP+E86:**

DMEM (Gibco BRL)	
Heat- inactivated FCS (PAA Laboratories)	10%
L-Glutamine, 200 mM (100 x; Gibco BRL)	1%
Penicillin/Streptomycin (10.000 U/ml & 10.000 µg/ml, Gibco BRL)	1%

**3.2.3.3 Cell counting**

The cell number was determined using CASY TTC (Innovatis) according to the manufacturer's instructions.

**3.2.3.4 Thawing and freezing of cells**

Adherent cells and suspension cells were fed with fresh medium one day before freezing.  $1 \times 10^6$  cells were washed with sterile PBS and centrifuged at  $160 \times g$  for 6 minutes. The cells were resuspended in freezing medium (10% DMSO (Roth), 90% heat inactivated FCS (PAA Laboratories)) and stored in a Cryo Tube™ Vials (Nunc GmbH). In order to continuously and slowly cool down the cells, the tubes were stored in a isopropanol freezing container (Nalgene, USA) at  $-80\text{ }^{\circ}\text{C}$  for 24 hours and then transferred into liquid nitrogen ( $-196\text{ }^{\circ}\text{C}$ ) for long time storage.

For thawing, the cells were rapidly warmed to  $37\text{ }^{\circ}\text{C}$  in a sterile water bath and gently added to 10 ml pre-warmed corresponding culture medium. To remove the DMSO, the cells were centrifuged and resuspended in fresh medium for further cultivation.

**3.2.3.5 Detachment of adherent cells**

Adherent cell lines (HEK293T and GP+E86) were detached by brief treatment with the proteolytic enzyme trypsin (trypsin-EDTA; 0.05%, Gibco BRL). For this purpose, the cells were gently washed with sterile PBS and then incubated with pre-warm 1 ml trypsin for 2-5 min at  $37\text{ }^{\circ}\text{C}$ . The enzyme reaction was stopped via adding 10 ml respective culture medium. After a centrifugation, the cells were either used for further cultivation or further analysis.

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### 3.2.3.6 Transient transfection of mammalian cells

#### 3.2.3.6.1 Transient transfection of HEK293T cells by PEI

For transient transfection,  $7.5 \times 10^5$  HEK293T cells were cultured in a 10 cm cell culture dish 24 hours before transfection. Afterwards, the cells were incubated with 3 ml DMEM (Gibco BRL) + 1% FCS (PAA Laboratories) for 20 minutes. Meanwhile, 6  $\mu$ g DNA were mixed with 200  $\mu$ l OptiMEM® (Gibco BRL) and 12  $\mu$ l Polyethylenimin (PEI, 1 mg/ml; Gibco BRL) and incubated at RT for 20 minutes. The 200  $\mu$ l DNA, OptiMEM® and PEI mixture were slowly added to 1% FCS DMEM cultured HEK293T cells and incubated for 4 hours in cell culture incubator. Subsequently, the transfection medium was replaced by normal corresponding culture medium (3.2.3.2) and cells were kept 24 to 48 hours in cell culture incubator.

#### 3.2.3.6.2 Transient transfection of Ba/F3 cells by electroporation

Transient transfections of Ba/F3 cells were performed for EMSA (3.2.2.2) and luciferase reporter assay (3.2.2.3) to investigate the biochemical function of Ebf1. The cells were fed with their appropriate medium (3.2.3.2) 24 hours before transfection.  $1 \times 10^7$  cells/transfection were washed three times with RPMI 1640. After the last centrifugation, the cell pellet was resuspended in a mixture of 300  $\mu$ l OptiMEM® and 20  $\mu$ g plasmid (see below) and transferred to an electroporation cuvette (Wuelfing and Brandau). Electroporation was performed using the Gene Pulser® II (BioRad) at 230 V and 975  $\mu$ F. Transfected cells were immediately transferred into 20 ml pre-warmed culture medium in a 6-well-plate (Becton Dickinson, GmbH) and incubated for 24 to 48 hours in cell culture incubator.

The plasmids using for EMSA transfection were: pcDNA3.1-*mock-Flag*, -*Ebf1-Flag*, -*Ebf1-S<sub>13</sub>A-Flag*, -*Ebf1-S<sub>14</sub>A-Flag* and -*Ebf1-S<sub>13, 14</sub>A-Flag*.

The plasmids using for luciferase reporter assay transfection were: pCMV-*mock*, -*Ebf1*, -*Ebf1-S<sub>13</sub>A*, -*Ebf1-S<sub>14</sub>A*, -*Ebf1-S<sub>13, 14</sub>A*, - $\alpha$ *Ebf1*, *Ebf1- $\Delta$ TAD* and - $\alpha$ *Ebf1- $\Delta$ TAD*. Each plasmid was mixed with pBL-*Lambda5-Luc* or pBL-empty vector and 4  $\mu$ g pCMVcyto-*LacZ* vector (3.1.8).

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### **3.2.3.7 Preparation of GP+E86 packaging cells and retroviral transduction of murine cells**

#### **3.2.3.7.1 Preparation of retrovirus packaging GP+E86 cells**

To produce retrovirus-packaging GP+E86 cells, HEK293T cells were first transfected by 6 µg MSCV-IRES-GFP (MIG)-*mock*, *-Ebf1*, *-Ebf1-S<sub>13</sub>A*, *-Ebf1-S<sub>14</sub>A*, *-Ebf1-S<sub>13, 14</sub>A* and 2 µg helper-plasmid (3.1.8) using PEI (3.2.3.6.1). 48 hours after transfection, the HEK293T cell culture supernatant containing viral particles was filtered through a sterile syringe filter (Millipore, 0.22 µm) and added onto GP+E86 cells growing in a 10 cm cell culture dish. To increase the efficiency of infection, 6 µg/ml polybrene (Sigma) were added to the GP+E86 cells supernatant. This process was repeated 4-6 times every 8-12 hours. In order to obtain maximum virus titers, infected GP+E86 cells, which should express GFP protein, were sorted by flow cytometry (3.2.5.2) two times. Sorted cells now had a genomic integration of the retrovirus constructs. The percentage of the *gag*, *pol* and *env* genes of the *Moloney murine leukemia virus* are released into the cell culture supernatant permanently by GP+E86 cells, which was used to transduce murine cells in the future.

#### **3.2.3.7.2 Retroviral transduction of murine cells**

For retroviral transduction of murine cells (CD45.2 *Ebf1*-deficient fetal liver cells), 2 x 10<sup>6</sup> cells were added to a 10cm cell culture dish retrovirus-producing GP+E86 culture medium (RPMI 1640, 10 % FCS, Penicillin/Streptomycin (10.000 U/ml & 10.000 µg/ml). 10 ng/ml IL-3, 5 ng/ml IL-6 and 10 ng/ml SCF (R & D Systems) were used for co-culture. After 48 hours incubation of the co-culture in a cell culture incubator, transduced fetal liver cells were washed with sterile PBS and used for Whitlock-Witte culture (3.2.3.8).

#### **3.2.3.8 Whitlock-Witte culture of murine bone marrow cells and fetal liver cells**

CA Whitlock and ON Witte established culture condition that support the expansion of B cells from primary murine bone marrow cells in 1982 (Whitlock C.A., 1982). This culture system is used for *in vitro* differentiation of *Ebf1*-deficient and retrovirally

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reconstituted hematopoietic cells.  $13.5 \times 10^6$  CD 45.1 wild type murine bone marrow cells were isolated (3.2.4.2.2) and cultured with 13.5 ml RPMI 1640 medium, which was mixed with 5 % heat-inactivated FCS (PAN Fetal Bovin Swe. Org. South America) and 1.7  $\mu$ l mercaptoethanol (Sigma) for 24 hours as a feeder cells. Then transduced CD45.2 fetal liver cells (3.2.3.7.2) were co-cultured with the feeder cells and incubated for ten to fourteen days. The co-culture cell medium was partially changed every three or four days until the cells were analysis by FACS Aria<sup>TM</sup> III flow cytometer (BD Biosciences).

### **3.2.4 Mouse analysis**

#### **3.2.4.1 Generation of *Ebf1*-deficient embryos**

To isolate *Ebf1*-deficient fetal liver cells (3.2.4.2), two heterozygous *Ebf1* mice (3.1.11) were crossed. The 14.5 to 16.5 days old *Ebf1*-deficient (E14.5 – E16.5) embryos were defined by genotyping of their tails (3.2.1.2.3.1).

#### **3.2.4.2 Isolation of organs and cells from mice**

##### **3.2.4.2.1 Isolation of fetal liver cells from mouse embryos**

To isolate fetal liver cells, the whole fetal liver from *Ebf1*-deficient mouse embryos (E14.5 – E16.5) was isolated carefully. The tissue was forced through a 70-micron nylon mesh and added into culture medium (RPMI 1640, 10 % FCS, Penicillin/Streptomycin (10.000 U/ml & 10.000  $\mu$ g/ml). Subsequently, the fetal liver cells were co-cultured with transduced retrovirus packaging GP+E86 cells (3.2.3.7.1) and used for Whitlock-Witte culture (3.2.3.8).

##### **3.2.4.2.2 Isolation of mouse bone marrow**

For the isolation of bone marrow, 3-24 week old femur and tibia were isolated. The bone marrow was flushed out with sterile PBS using a syringe (0.4 x 20 mm, G x 3/4, Size 20, Brown) and then flushed through a 100-micron nylon mesh (BD GmbH). The bone marrow cells were used in Whitlock-Witte cultures (3.2.3.8) or Hardy B cell fraction sorting (3.2.5.1).



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### **3.2.5 Flow cytometry**

#### **3.2.5.1 Purification of hardy B cell fraction sorting**

A method for the fractionation of B cells was established by Richard R. Hardy and Kyoko Hayakawa (1993). Murine B cell development was separated into fractions A, B and C in bone marrow and fractions D, E and F in the spleen using a combination of four different cell surface markers. For fractions A-C, cell surface marker B220+ and CD43+ bone marrow cells were further separated using Bp1 and CD24 (fraction A: BP1-, CD24-; fraction B: BP1-, CD24+; fraction C: BP1+, CD24+).

To isolate fraction A-C, wild type mouse bone marrow cells were isolated (3.2.4.2.2).  $2 \times 10^7$  bone marrow cells were resuspended in 500  $\mu$ l PBS and incubated with 1  $\mu$ l anti-B220, -CD43, -BP1 and -CD24 antibodies for 25 minutes at 4 °C. The stained cells were washed once with PBS and resuspended in 1 ml PBS. The bone marrow cells were sorted using a FACSaria<sup>TM</sup>III flow cytometer (BD Biosciences) directly into PBS. Purified fraction A-C cells were collected by centrifugation and cell pellets were sorted at -20 °C or used for protein isolation (3.2.2.1.1) for western blot.

#### **3.2.5.2 GP+E86 cells sorting of GFP positive retroviruses**

To increase the virus titer of MIG infected GP+E86 cells (3.2.3.7.1), cells were sorted with a FACSaria<sup>TM</sup>III flow cytometer. The infected GP+E86 cells (GFP +) were detached (3.2.3.5) and washed by PBS, and then directly sorted into corresponding culture medium and cultured with ciprobay (5 $\mu$ l/ml of medium, Bayer). This sorting process was repeated two or three times and subsequently the GFP positive GP+E86 cells were used for retroviral transduction of murine cells (3.2.3.7.2)

#### **3.2.5.3 Cells sorting of transduced fetal liver cells**

To investigate the influence of Ebf1-S<sub>13</sub>, S<sub>14</sub> phosphorylation,  $\alpha$ Ebf1 and truncated Ebf1 on target gene expression, GFP and CD45.2 positive transduced fetal liver cells were sorted. Ebf1-deficient CD45.2 fetal liver cells were transduced by MIG based plasmid (3.2.3.7), which contain different modified Ebf1 and GFP. The transduced fetal liver cells were co-cultured with Whitlock-Witte cells for ten to fourteen days (3.2.3.8) before sorting. The whole Whitlock-Witte co-culture cells were collected and

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washed by PBS. The cell pellets were resuspended by 500 µl PBS and incubated with 1 µl anti-CD45.2, 1 µl anti-CD19-Biotin and 1 µl Streptavidin-PE (3.1.6.3) antibodies for 25 minutes at 4 °C. After washing the stained co-culture cells with PBS, the GFP and CD45.2 double positive cells were sorted directly into PBS and used for RNA isolation (3.2.1.1.1).

### **3.2.6 Statistics**

The mean values, standard deviation and significance values (P) shown in the results were calculated by Microsoft Excel 2011. The P values were calculated from biological triplicate experiments. Real time PCR analyses were repeated as a technical triplicate and the data were analysed using  $\Delta C_p$  method.

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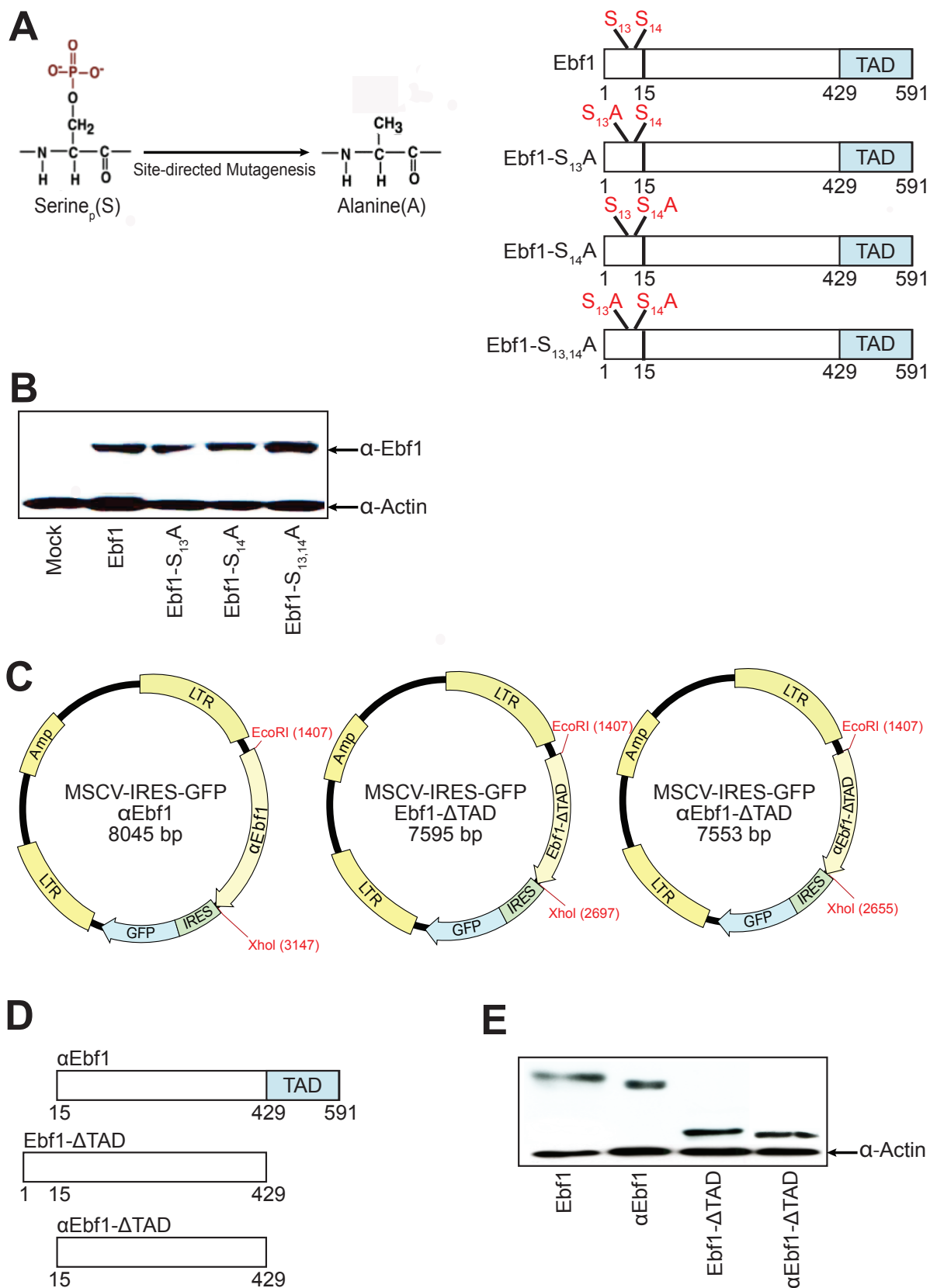
## 4 Results

### 4.1 Generation of truncated Ebf1 and mutant Ebf1

#### 4.1.1 Generation of mutant Ebf1

Ebf1 is a transcription factor (Hagman and Lukin 2005). Two promoters ( $\alpha$  and  $\beta$ ) generate two different protein isoforms and regulate their expression.  $\alpha$ Ebf1 is 15 amino acids shorter than  $\beta$ Ebf1 and the corresponding  $\alpha$  promoter locates 4.4 kb upstream of the  $\beta$  promoter. The  $\alpha$  promoter is indirectly regulated by IL7 and directly regulated by E2A and Ebf1, and the  $\beta$  promoter is associated with Pax5 and Pu.1 in B cells (Roessler et al. 2007b).

In the pre-B cell line 18-81 TK+, Ebf1 is phosphorylated at S<sub>13</sub> and S<sub>14</sub> (Dr. Gerald Burgstaller and Dr. Hakan Sarioglu, HMGU Munich), which are only present in the longer isoform  $\beta$ Ebf1 (Roessler et al. 2007b). To clarify the function of phosphorylated S<sub>13</sub> and S<sub>14</sub> in normal B cell development, we performed site-directed mutagenesis of *Ebf1* in the retroviral vector MSCV-IRES-GFP-*Ebf1* (MIG-*Ebf1*; Dr. Silvia Hiechinger and Dr. Saihong Jin; 3.1.8), resulting in the replacement of the serine residues with alanine. Serine has a hydroxyl group that can be phosphorylated by kinases, which is missing in the structurally similar alanine. We generated three constructs including mutations of S<sub>13</sub> to alanine (S<sub>13</sub>A), S<sub>14</sub> to alanine (S<sub>14</sub>A) and S<sub>13</sub> and S<sub>14</sub> to alanine (S<sub>13, 14</sub>A; Figure 11A). After the site-directed mutagenesis, the PCR amplified *Ebf1*-mutants were transformed into bacteria for plasmid isolation, and were controlled by sequencing. Expression of Ebf1-mutant proteins was investigated by western blot 24 hours after transfection of HEK293T cells (Figure 11B). The protein expression is comparable to wild type Ebf1 in HEK293T cells.



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## Figure 11 Generation of mutant and truncated Ebf1

(A) Left panel: schematic diagram of the molecular structure of serine and alanine. Right panel: schematic overview of the mutations introduced into Ebf1. Serine 13 and 14 of Ebf1 were mutated to alanine either alone or in combination.

(B) Western blot analysis of mutant Ebf1 protein expression 24 hours after transfection of HEK293T cells. Actin was used as a loading control. Mock is empty MIG vector transfection. Each transfection was performed 3 times.

(C) Plasmid maps show the short isoform of Ebf1 ( $\alpha$ ) and the truncated isoform lacking the transactivation domain ( $\Delta$ TAD).  $\alpha$ Ebf1, Ebf1- $\Delta$ TAD, and  $\alpha$ Ebf1- $\Delta$ TAD were amplified using forward primer containing EcoRI at the 5' end and reverse primer containing XhoI at the 5' end. The amplified PCR products were ligated into MSCV-IRES-GFP (MIG) vector via the restriction site EcoRI and XhoI.

(D) Schematic representation of the protein structure of  $\alpha$ Ebf1, Ebf1- $\Delta$ TAD and  $\alpha$ Ebf1- $\Delta$ TAD. The transactivation domain of Ebf1 starts at amino acid 429 and ends at amino acid 591; Ebf1- $\Delta$ TAD begins at amino acid 1 and ends at amino acid 429;  $\alpha$ Ebf1- $\Delta$ TAD begins at amino acid 15 and ends at amino acid 429 of wild type Ebf1.

(E) Western blot analysis of  $\alpha$ Ebf1, Ebf1- $\Delta$ TAD and  $\alpha$ Ebf1- $\Delta$ TAD expression 24 hours after transfection of HEK293T cells. Actin was used as a loading control. n = 3 for each transfection and western blot.

### 4.1.2 Generation of $\alpha$ Ebf1 and truncated Ebf1

To clarify the function of the first 15 amino acids, which are only present in  $\beta$ Ebf1 and of the transactivation domain in B cell development, we generated wild type  $\alpha$ Ebf1,  $\beta$ Ebf1 without the transactivation domain ( $\beta$ Ebf1- $\Delta$ TAD) and  $\alpha$ Ebf1- $\Delta$ TAD from the MIG- $\beta$ Ebf1 plasmid.  $\alpha$ Ebf1 and truncated isoforms of Ebf1 were amplified using a forward primer containing EcoRI at the 5' end and reverse primer containing XhoI at the 5' end (Figure 11C).  $\alpha$ Ebf1 and truncated Ebf1 were inserted separately into the MIG vector (Figure 11C), which is a retroviral system allowing the introduction and expression of target genes in primary murine cells. The ligations of restriction sites were performed as described in 3.2.1.2.5.1. The correct insertion was controlled by restriction digest and sequencing.

To confirm protein expression, the MIG- $\alpha$ Ebf1 and MIG-truncated Ebf1 plasmids were transfected into HEK293T cells via polyethylenimine (PEI). 24 hours after transfection, HEK293T cells were harvested for protein isolation. Western blot was performed to detect the protein expression (Figure 11E). The translational start site of wild type  $\alpha$ Ebf1 is 45bp downstream of  $\beta$ Ebf1 (Roessler et al. 2007b)

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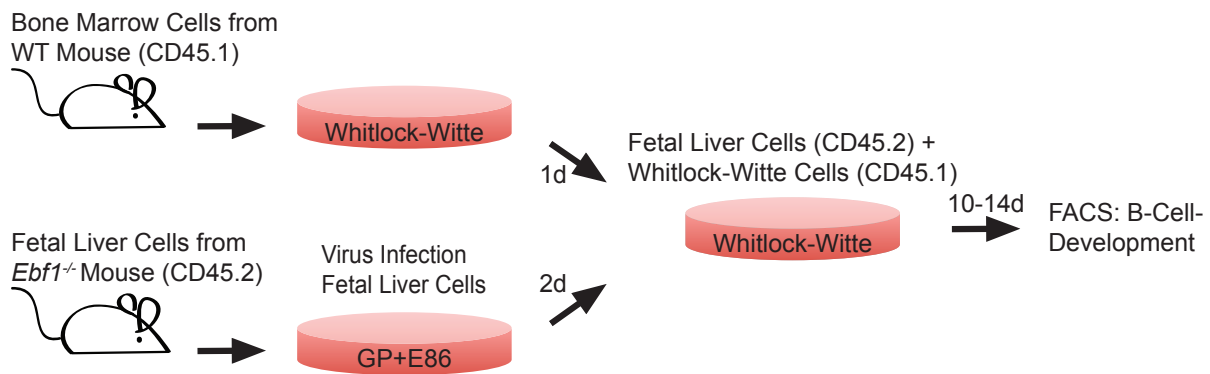
corresponding to 62.97kD of  $\alpha$ Ebf1 and 64.48kD of  $\beta$ Ebf1 protein size.  $\beta$ Ebf1- $\Delta$ TAD protein is 48.15kD and  $\alpha$ Ebf1- $\Delta$ TAD protein is 46.64kD (Figure 11D and 11E).  $\alpha$ Ebf1 and truncated isoforms of Ebf1 show comparable expression to  $\beta$ Ebf1 in transfected HEK293T cells.

## **4.2 Serine 13, 14 and TAD of *Ebf1* are required for B cell development**

In *Ebf1*-deficient mice, B cell differentiation is blocked at the pre-pro B cell stage (Lin and Grosschedl 1995). The ectopic expression of Ebf1 in hematopoietic progenitors from the fetal liver of *Ebf1*-deficient mice restores B cell development (Pongubala et al. 2008). CD19 is a surface antigen from the pro-B cell stage, whose expression is completely missing in absence of Ebf1 (Rolink et al. 1993). The role of Ebf1 in B cell development is well studied, however, post-translational modifications of Ebf1 and their function in B cell development are completely unclear.

The biological function of the phosphorylation of S<sub>13</sub> and S<sub>14</sub> of  $\beta$ Ebf1,  $\alpha$ Ebf1 and TAD in B cell differentiation was analysed by an established long-term cell culture system that supports the B cell differentiation *in vitro* (Whitlock-Witte culture) (Whitlock and Witte 1982). Fetal liver cells (CD45.2) from *Ebf1*-deficient mouse embryos (E14.5-E16.5) were isolated and co-cultured with retrovirus producing GP+E86 cells for Ebf1-S<sub>13</sub>A, -S<sub>14</sub>A, -S<sub>13, 14</sub>A,  $\alpha$ Ebf1, Ebf1- $\Delta$ TAD, or  $\alpha$ Ebf1- $\Delta$ TAD. Meanwhile, bone marrow cells were isolated from CD45.1 mice to generate a Whitlock-Witte culture (3.2.3.8). Following the two-day retroviral transduction, the fetal liver cells (CD45.2) were co-cultured with the feeder layer cells (CD45.1) for ten to fourteen days (Figure 12) to allow B cell development.

After ten to fourteen days WW culture, the transduced fetal liver cells were analysed by flow cytometry for expression of CD19. The CD45.1 and CD45.2 system allowed distinction of *Ebf1*-deficient fetal liver cells (CD45.2) from wild type CD45.1 mice bone marrow cells. The infected cells co-express GFP via a downstream IRES-GFP cassette, distinguishing the transduced from untransduced cells (Figure 13A).

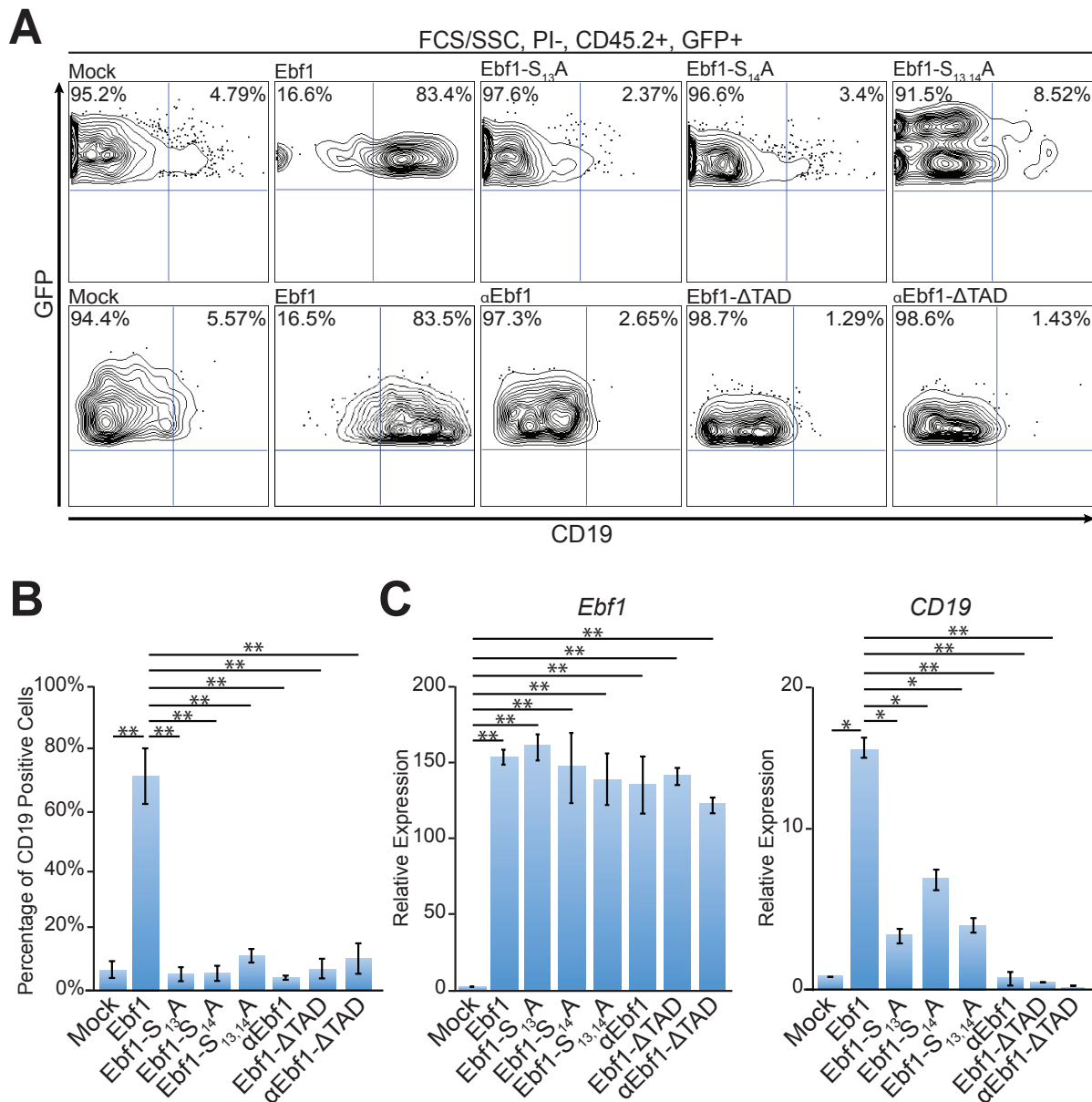


**Figure 12 Illustration of the experimental system to study B-cell development *in vitro***

Fetal liver cells (CD45.2) from *Ebf1*-deficient mouse embryos (E14.5-E16.5) were infected by co-culture with transduced GP+E86 cells over two days. Afterwards, the fetal liver cells were co-cultured for 10 to 14 days with Whitlock-Witte cells (CD45.1). This was followed by analysis of B-cell development via flow cytometry.

As Pongubala et al. (2008) described, the ectopic expression of  $\beta$ Ebf1 in *Ebf1*-deficient fetal liver cells restores B cell differentiation. In my study, the ectopic expression of  $\beta$ Ebf1 in *Ebf1*-deficient fetal liver cells could restore around 70% CD19-positive transduced cells compared to mock transduced cells. The ectopic expression of *Ebf1*-S<sub>13</sub>A, -S<sub>14</sub>A, -S<sub>13, 14</sub>A,  $\alpha$ Ebf1, *Ebf1*- $\Delta$ TAD, and  $\alpha$ Ebf1- $\Delta$ TAD cannot increase the percentage of CD19-positive cells (~ 5%) compared to mock transduced cells. This data shows an arrest in B cell differentiation upon ectopic expression of  $\alpha$ Ebf1 or without expression of serine 13, 14 or TAD of wild type *Ebf1* (Figure 13B).

To further control the retroviral transduction and WW co-culture, GFP and CD45.2 double positive cells were purified by flow cytometry and investigated for expression of *Ebf1* and *CD19* mRNA. No significant difference *Ebf1* mRNA expression was detected between all expressions of the different versions of  $\alpha$  and  $\beta$ Ebf1. The expression of CD19 was significantly decreased in fetal liver cells expressing  $\alpha$ Ebf1 or modified versions of *Ebf1* compared to  $\beta$ Ebf1 (Figure 13C)



**Figure 13 Serine 13, 14 and transactivation domain of Ebf1 are required for B-cell development**

(A) Flow cytometry analysis of B-cell differentiation of transduced *Ebf1*-deficient fetal liver cells (CD45.2). The percentage of CD19+ cells was analysed to monitor B-cell differentiation. Cells are gated for FCS/SSC, PI-, CD45.2+ and GFP+. CD45.2+ and GFP+ cells are transduced fetal liver cells from *Ebf1*-deficient mouse embryos (E14.5-E16.5). Mock is empty MIG vector. n = 3.

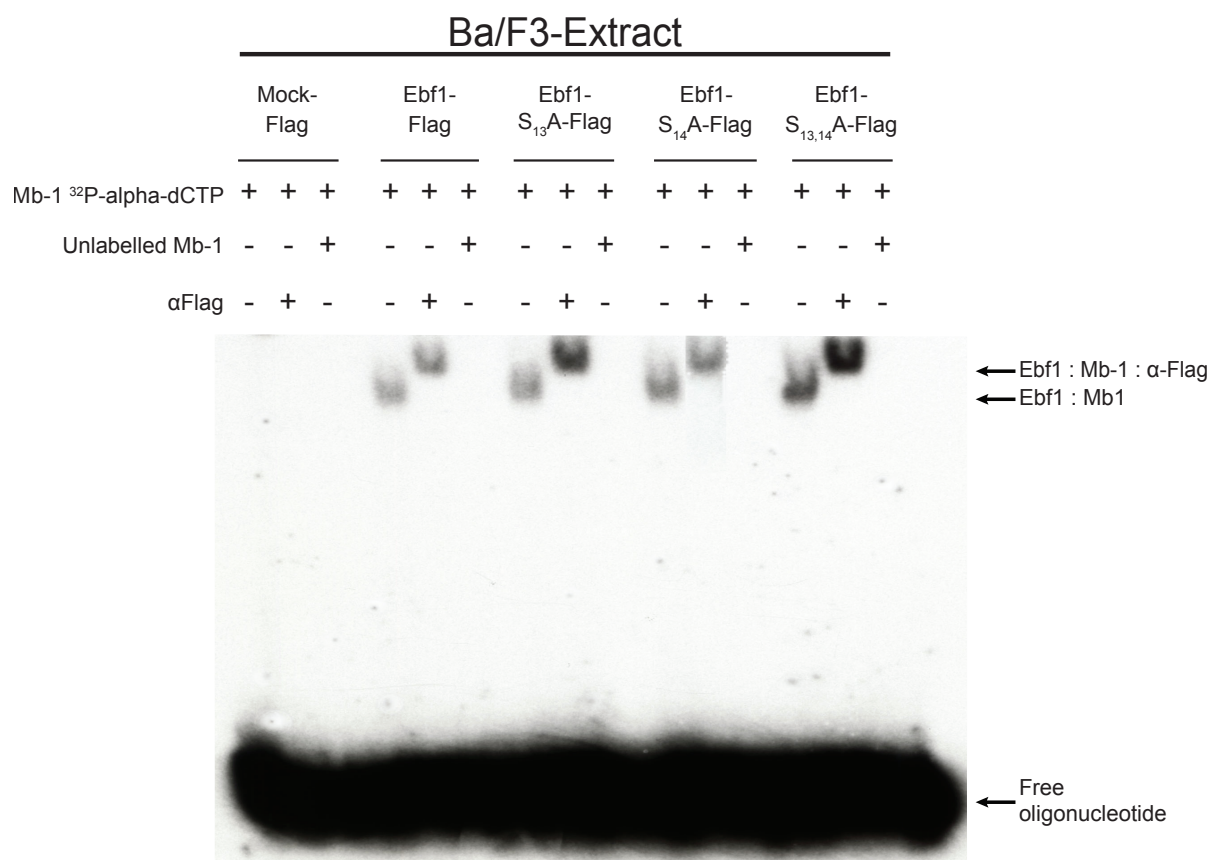
(B) Percentage of CD19+ cells of transduced fetal liver cells corresponding to panel (A). \*\* p < 0.005; n = 3.

(C) Determination of *Ebf1* and *CD19* transcripts of transduced fetal liver cells (CD45.2+, GFP+) corresponding to panel (A). After WW co-culture, GFP and CD45.2 double positive cells were purified by flow cytometry for analysis by qPCR. \* p < 0.05, \*\* p < 0.005; n = 3.



### 4.3 Ebf1 serine 13 and serine 14 are not required for DNA binding

Hagman et al. (1991) and Feldhaus et al. (1992) established that Ebf1 binds to the nucleotide sequence 5-CAAGGGAAT-3 found in the *mb-1* promoter. To study the DNA binding activity of Ebf1-S<sub>13</sub>A, -S<sub>14</sub>A and -S<sub>13,14</sub>A, electrophoretic mobility shift assay (EMSA) were performed. In the binding reaction, the cell protein lysates of transfected Ba/F3 cells were used. The Ba/F3 cells were transiently transfected with pcDNA3.1-flag-βEbf1, Ebf1-S<sub>13</sub>A, -S<sub>14</sub>A and -S<sub>13,14</sub>A. The empty vector as used as a negative control.



**Figure 14 Serine 13 and serine 14 of Ebf1 are not required for DNA binding**

Representative electrophoretic mobility shift assay of cell lysates from transiently transfected Ba/F3 cells with pcDNA3.1-mock-Flag, -Ebf1-Flag, Ebf1-S<sub>13</sub>A-Flag, Ebf1-S<sub>14</sub>A-Flag or Ebf1-S<sub>13,14</sub>A-Flag. Mb-1 <sup>32</sup>P-alpha-dCTP: radiolabeled oligonucleotide. Unlabelled Mb-1: non-radioactively labelled oligonucleotide. Lower band shows DNA and protein complex obtained. Higher band shows flag antibody binding to DNA and protein complex. At the bottom of the gel is free oligonucleotide. n = 3.

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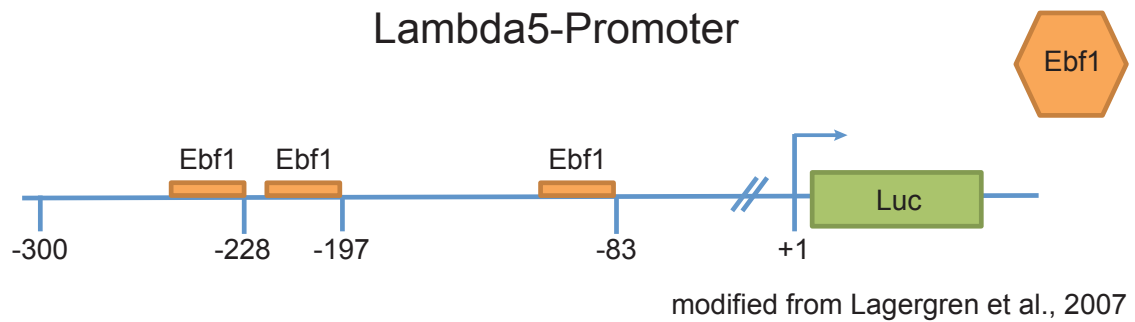
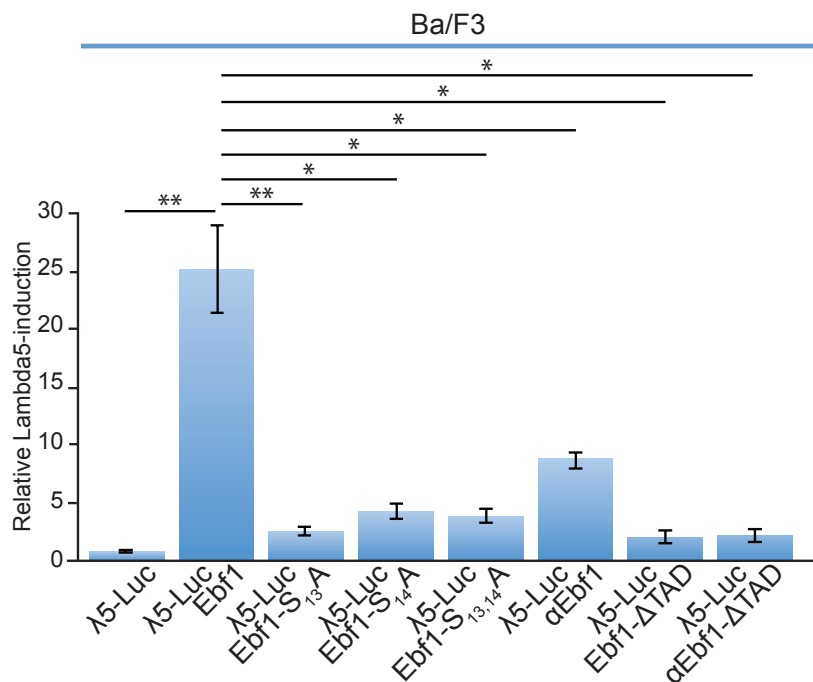
The EMSA data shows that the binding activity of mutant Ebf1 to the Mb-1 promoter-binding site is comparable to wild type Ebf1. The binding specificity was detected by competition with a non-radioactively labelled oligonucleotide, as well as by adding anti-flag antibody. The specific binding of this antibody to the Ebf1-flag: DNA complex slows down running behaviour of the complex in the gel (super shift, Figure 14).

#### **4.4 Transactivation of Ebf1 depends on serine 13 and serine 14**

The lambda5 promoter has been established as a model for the transactivation potential of Ebf1 in Ba/F3 cells (Sigvardsson et al. 2002). The murine pro-B cell line, Ba/F3 is particularly suitable for the luciferase reporter assay because the amount of Ebf1 in Ba/F3 cells is undetectable (Dr. Inga Ludenberg).

To study the transactivation potential of Ebf1 influenced by modifications of serine 13 and 14, wild type  $\alpha$ Ebf1 and the TAD, luciferase reporter assays were performed. pCMV- mock,  $\beta$ Ebf1, Ebf1-S<sub>13</sub>A, Ebf1-S<sub>14</sub>A, Ebf1-S<sub>13,14</sub>A,  $\alpha$ Ebf1,  $\beta$ Ebf1- $\Delta$ TAD and  $\alpha$ Ebf1- $\Delta$ TAD were separately transiently transfected with reporter construct pBL-Lambda5-Luc (Sigvardsson et al. 2002) into Ba/F3 cells. This reporter plasmid contains the lambda5 promoter including three Ebf1-binding sites in front of the luciferase reporter gene (Figure 15A). To normalize the luciferase activity, the Ba/F3 cells were additionally transfected with the pCMV- LacZ plasmid.

The relative induction of the lambda5 promoter was measured by luciferase activity 48 hours after transfection (Figure 15B). The transfection of Ba/F3 cells with  $\beta$ Ebf1 activates the lambda5 promoter ~ 25 folds, while the transactivation with modified Ebf1 was approximately ~ 5 folds reduced (Ebf1-S<sub>13</sub>A: 2.97 folds, Ebf1-S<sub>14</sub>A: 4.86 folds, Ebf1-S<sub>13,14</sub>A: 4.42 folds,  $\alpha$ Ebf1: 8 folds, Ebf1- $\Delta$ TAD: 4.97 folds and  $\alpha$ Ebf1- $\Delta$ TAD: 4.09 folds activation of lambda5 promoter). Thus, the inductions were significantly affected by mutation of serine 13, serine 14 and  $\alpha$ Ebf1, as well as Ebf1- $\Delta$ TAD and  $\alpha$ Ebf1- $\Delta$ TAD.

**A****B**

**Figure 15 Transactivation of Ebf1 depends on serine 13, serine 14 and the transactivation domain**

(A) Schematic diagram of the luciferase reporter assay. Ebf1 is expressed from pCMVcyto plasmids. The reporter plasmid contains the lambda5 promoter including three Ebf1 binding sites and the luciferase gene (pBL-Lambda5-Luc).

(B) Relative induction of Lambda5 promoter was measured by luciferase activity 24 hours after transfection in Ba/F3 cells transfected with pCMV-, mock, Ebf1, Ebf1-S<sub>13</sub>A, Ebf1-S<sub>14</sub>A, Ebf1-S<sub>13,14</sub>A, αEbf1, Ebf1-ΔTAD and αEbf1-ΔTAD. Luciferase values were first normalized to β-galactosidase activity and then to the luciferase activity in cells transfected with the empty pCMVcyto plasmid (pCMV-mock). \*  $p < 0.05$ , \*\*  $p < 0.005$ ;  $n = 4$ .

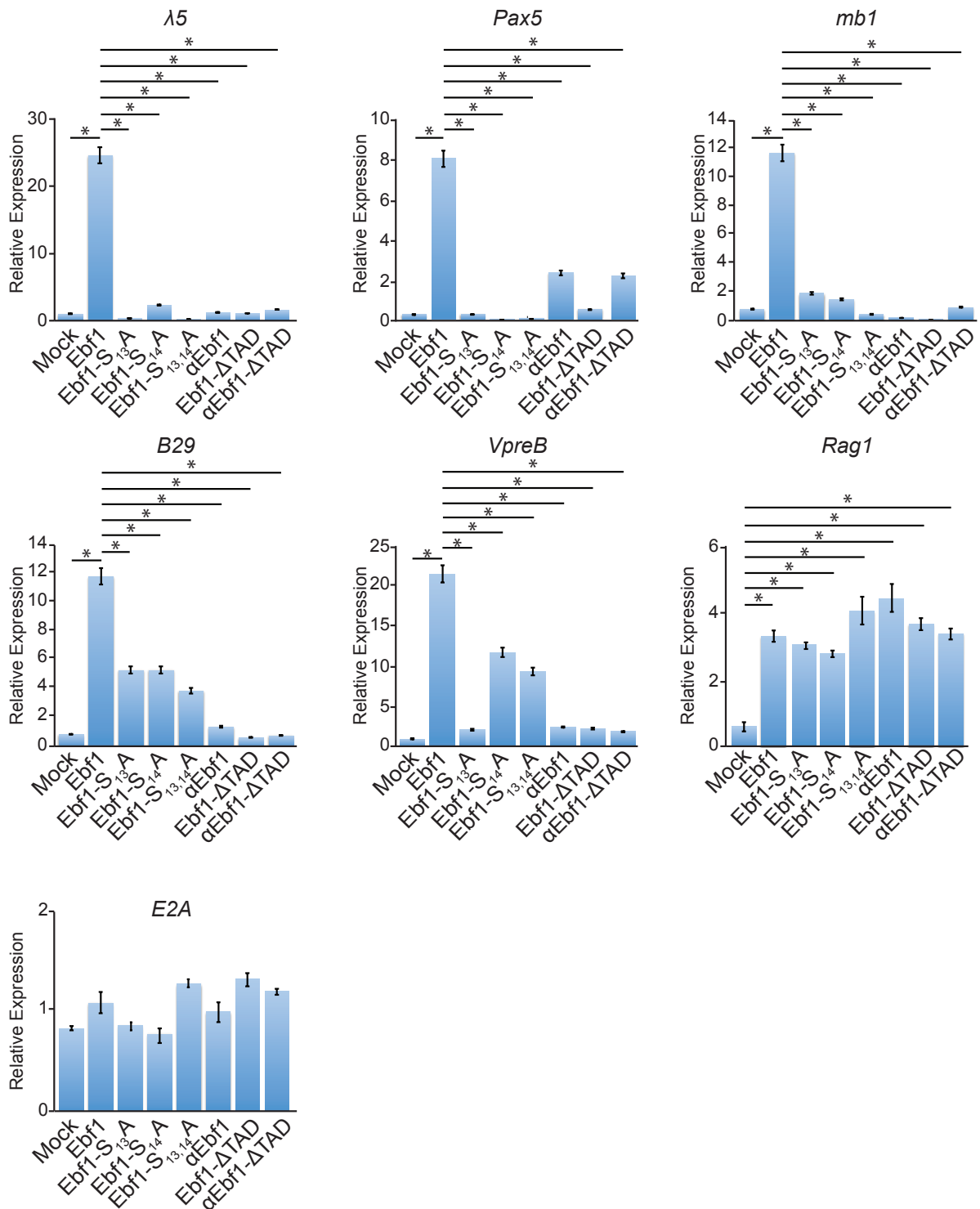
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## 4.5 Expression of Ebf1 target genes requires serine 13 and serine 14

Ebf1 is a transcription factor that influences the expression of many B cell genes. Loss of Ebf1 leads to the down-regulation or loss of mRNAs for  $\lambda 5$ , *Pax5*, *mb-1*, *B29*, *VpreB* and *Rag1* in common lymphoid progenitor cells (Zandi et al. 2010).

To investigate the influence of serine<sub>13</sub> and serine<sub>14</sub> phosphorylation and the importance of the TAD, the expression of B lineage genes were analysed by real-time PCR in transduced *Ebf1* deficient fetal liver cells. After 10 – 14 days WW co-culture (Figure 12) GFP and CD45.2 double positive cells were sorted by flow cytometry and the mRNA expression of  $\lambda 5$ , *Pax5*, *mb1*, *B29*, *VpreB*, *Rag1* and *E2A* were investigated by real time PCR.  $\lambda 5$  is expressed around 25-fold higher in wild type  $\beta$ Ebf1 transduced fetal liver cells, compared to only 1-2-fold in  $\alpha$ Ebf1 and all modified Ebf1 transduced fetal liver cells. *Pax5* is expressed 8-fold higher in  $\beta$ Ebf1 transduced cells compared to less than 1-fold in S<sub>13</sub>A, S<sub>14</sub>A, S<sub>13, 14</sub>A and Ebf1- $\Delta$ TAD, and 2-fold in  $\alpha$ Ebf1 and  $\alpha$ Ebf1- $\Delta$ TAD transduced fetal liver cells. *mb1* is expressed 11.5-fold higher in  $\beta$ Ebf1 transduced fetal liver cells, compared to less than 2-fold in all modified Ebf1 transduced fetal liver cells. The expression of *B29* in wild type Ebf1 transduced cells is 11.7-fold higher compared to only 4-5-fold in S<sub>13</sub>A, S<sub>14</sub>A and S<sub>13, 14</sub>A and less than 1 fold in  $\alpha$ Ebf1, Ebf1- $\Delta$ TAD and  $\alpha$ Ebf1- $\Delta$ TAD transduced fetal liver cells. *VpreB* shows 21-fold higher expression in  $\beta$ Ebf1 transduced fetal liver cells, compared to 1-fold in S<sub>13</sub>A,  $\alpha$ Ebf1, Ebf1- $\Delta$ TAD and  $\alpha$ Ebf1- $\Delta$ TAD and around 10-folds in S<sub>14</sub>A and S<sub>13, 14</sub>A.

The expression of *Rag1* is induced approximately 3-fold by  $\beta$ Ebf1 as well as by  $\alpha$ Ebf1 and all modified versions of Ebf1 in transduced fetal liver cells. Similarly, *E2A* is induced approximately 1-fold by  $\beta$ Ebf1 and  $\alpha$ Ebf1 and all modified Ebf1 versions. The mRNA expression of Ebf1 downstream B lineage genes are significantly reduced, but *Rag1* and *E2A* are not affected by  $\alpha$  or  $\beta$ Ebf1 or any of the modifications.



**Figure 16 Expression of Ebf1 target genes requires serine 13, serine 14 and the transactivation domain of Ebf1**

Quantitative RT-PCR analysis of indicated genes in sorted transduced fetal liver cells from *Ebf1*-deficient mouse (CD45.2+, GFP+) after 10 days of WW-culture corresponding to Figure 12 and Figure 13 panel (A). The fetal liver cells were transduced with Ebf1, Ebf1-S<sub>13</sub>A, Ebf1-S<sub>14</sub>A, Ebf1-S<sub>13,14</sub>A, αEbf1, Ebf1-ΔTAD and αEbf1-ΔTAD. Mock is empty MIG vector. \* p < 0,05; n = 3.

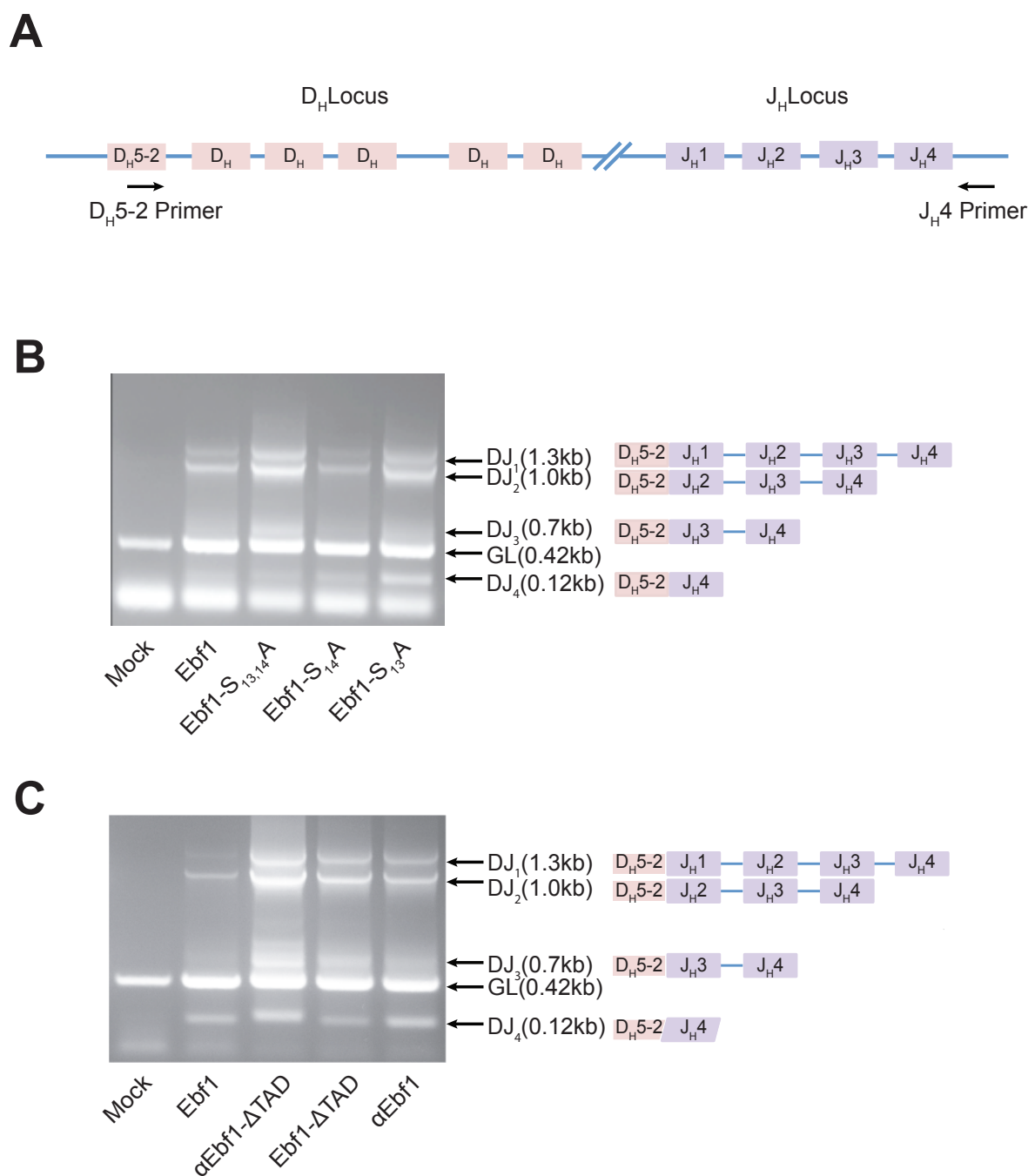
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## 4.6 D-J rearrangement requires *Ebf1* but is independent of serine 13, 14 and TAD

D-J rearrangement of immunoglobulin heavy chain locus is absent in *Ebf1*-deficient common lymphoid progenitor cells (Reynaud et al. 2008, Bassing et al. 2000, Lin and Grosschedl 1995).

To study whether the D-J IgH locus rearrangement is influenced by the modification of serine 13, serine 14,  $\alpha$ Ebf1,  $\beta$ Ebf1- $\Delta$ TAD and  $\alpha$ Ebf1- $\Delta$ TAD, PCR was performed with transduced *Ebf1*-deficient fetal liver cells. After 10 – 14 days WW co-culture (Figure 12) GFP and CD45.2 double positive cells were sorted by flow cytometry and the D-J rearrangement at the IgH locus was analysed. Using D<sub>H</sub>5-2 and J<sub>H</sub>4 primer flanking the D and J regions of the genomic DNA, the products result in a “ladder” of 4 bands corresponding to the joints DJ<sub>1</sub>, DJ<sub>2</sub>, DJ<sub>3</sub> and DJ<sub>4</sub> (Figure 17A, B and C).

$\beta$ Ebf1,  $\beta$ Ebf1-S<sub>13</sub>A,  $\beta$ Ebf1-S<sub>14</sub>A,  $\beta$ Ebf1-S<sub>13,14</sub>A (Figure 17B),  $\alpha$ Ebf1,  $\beta$ Ebf1- $\Delta$ TAD and  $\alpha$ Ebf1- $\Delta$ TAD (Figure 17C) transduced *Ebf1*-deficient fetal liver cells exhibited all bands corresponding to the DJ<sub>1</sub>, DJ<sub>2</sub>, DJ<sub>3</sub>, DJ<sub>4</sub> and germline which means that the modification of serine 13 and 14,  $\alpha$ Ebf1 and TAD do not influence D-J IgH rearrangements during B cell development.



**Figure 17 D-J Rearrangement of the immunoglobulin heavy chain locus requires *Ebf1*, but is independent of serine 13, serine 14 and the transactivation domain**

(A) Schematic diagram of the D-J IgH locus and the primers flanking the D<sub>H</sub>5-2 and J<sub>H</sub>4 regions used for the amplification of genomic DNA.

(B and C) Sorted transduced fetal liver cells from *Ebf1*-deficient mouse embryos (CD45.2<sup>+</sup>, GFP<sup>+</sup>) after 10-14d co-culture with Whitlock-Witte cells were examined for D-J rearrangement by PCR. The fetal liver cells were transduced with β*Ebf1*, *Ebf1*-S<sub>13</sub>A, *Ebf1*-S<sub>14</sub>A, *Ebf1*-S<sub>13,14</sub>A (B), α*Ebf1*, *Ebf1*-ΔTAD and α*Ebf1*-ΔTAD (C). Arrows indicate germline (GL) DNA and rearrangements of D to J<sub>1</sub>, J<sub>2</sub>, J<sub>3</sub> or J<sub>4</sub>. n = 3.

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## 4.7 Generation of antibodies specific for phosphorylated serine 13 and serine 14 of Ebf1

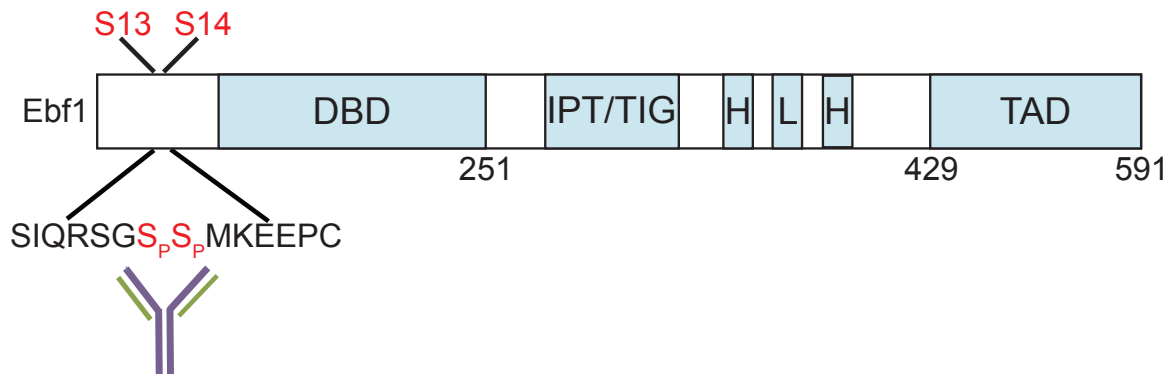
To confirm the phosphorylation of Ebf1 at serine<sub>13</sub> and serine<sub>14</sub> identified by mass spectrometry (Dr. Gerald Burgstaller, Dr. Hakan Sarioglu) and to be able to study the dynamics of this modification, we wanted to generate monoclonal antibodies recognizing the phosphorylated serine<sub>13</sub> and serine<sub>14</sub> of Ebf1. This task was conducted with the cooperation of Dr. Elisabeth Kremmer (Inst. for Molecular Immunology, HMGU Munich). We also included non-phospho specific peptide sequences. The peptide sequence is SIQRSGS<sub>(p)</sub>S<sub>(p)</sub>MKEEPC, which locates from amino acid 7 to 19 of Ebf1 (Figure 18A). The C-terminal cysteine was added to allow coupling of the peptide.

Totally, 60 monoclonal antibodies were generated. To analyse these clones by western blot, proteins were isolated from the Ebf1 expressing pre-B cell line 18-81 TK+, which also has been used to determine the post-translational modifications of Ebf1 by mass spectrometry. The protein extract was split and one half treated with calf intestinal alkaline phosphatase (CIP), to remove potential phosphorylation. CIP treated and non-treated proteins were blotted on nitrocellulose membrane and tested with the newly generated antibodies by immuno-detection.

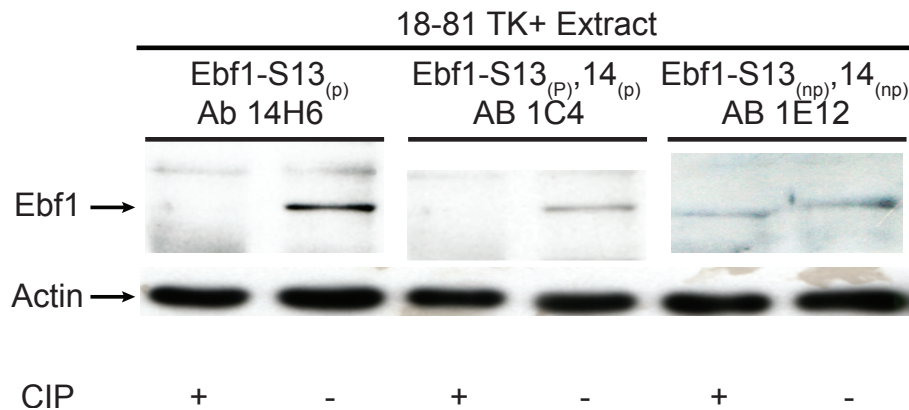
The supernatants of two monoclonal hybridoma clones, 14H6 and 1C4, could recognise CIP-untreated but not CIP-treated protein extracts (Figure 18B). 14H6 represents an Ebf1-S13<sub>(p)</sub> specific antibody and 1C4 represents an Ebf1-S13<sub>(p)</sub> S14<sub>(p)</sub> specific antibody. The supernatants of hybridoma clones 1E12 could recognize both CIP-treated and CIP-untreated protein extracts. Therefore, it is a non-phospho specific antibody used as a control in further experiment. As expected, the CIP-treated protein extracts in which phospho-groups were removed, show a lower molecular weight band compared to the untreated protein extracts (Figure 18B). Therefore, we conclude that endogenous Ebf1 of 18-81 TK+ cells is phosphorylated at serine<sub>13</sub> and serine<sub>14</sub> corresponding to the mass spectrometry data from Dr. Gerald Burgstaller and Dr. Hakan Sarioglu.



**A**



**B**



**Figure 18 Generation of antibodies specific for phosphorylated serine 13 and serine 14 of Ebf1**

(A) Schematic overview of the position and the sequence of the peptide used to generate Ebf1-S13<sub>(p)</sub>, and Ebf1-S13<sub>(p)</sub> S14<sub>(p)</sub> specific antibodies. The synthetic peptide locates from amino acids 7 to 19. p: phospho-specific antibody; np: non-phospho antibody.

(B) Representative western blot analysis for CIP-treated protein extract from 18-81 TK+ cells using Ebf1 phosphorylation specific or unspecific antibodies (clone 14H6, 1C4 and 1E12). +: CIP-treated protein extracts, -: CIP-untreated protein extracts. CIP-untreated protein extracts were used as a negative control. Actin was used as a loading control. n = 3

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## 5 Discussion

### 5.1 Verification of Ebf1-S<sub>13</sub> and S<sub>14</sub> phosphorylation

Ebf1 is a key regulator of early B-cell differentiation participating in a complex regulatory network. Ebf1 is expressed already in CLPs, and the functional activity of Ebf1 is required for the generation of pro-B cells (fraction B cells) that undergo the rearrangement of immunoglobulin heavy chain genes (Lin and Grosschedl 1995). Genome-wide ChIP-seq analysis in pro-B cells showed that Ebf1 occupies regulatory sequences of about 3000 genes, which are strongly associated with B cell identity and the (pre-)BCR signaling cascade (Treiber et al. 2010b). Ebf1 can activate, repress or poise those targets during B cell development raising the question how these biochemically very different activities are achieved. A possibility to explain these different behaviours is post-translational protein modification.

Therefore, transiently transfected Ebf1-flag was purified from the murine pre-B cell line 18-81 TK+ and HEK293T cells via immunoprecipitation and mass spectrometry was performed. The data shows that Ebf1-S<sub>13</sub> and S<sub>14</sub> are phosphorylated and this phosphorylation is present only in 18-81 TK+ cells, but not HEK293T cells, demonstrating cell-type specificity. Phosphorylation can regulate activation, localization and interaction with other proteins. For example the transcription factor E2A, which mediates the specification of the B cell lineage in cooperation with Ebf1, needs to be phosphorylated during B cell development (Sloan et al. 1996). Therefore, phosphorylation of Ebf1-S<sub>13</sub> and S<sub>14</sub> might be important biologically and biochemically for the various functions of Ebf1 during B cell development.

To confirm the finding from mass spectrometry independently on endogenous Ebf1 in cells, we tried to generate monoclonal antibodies. The peptide sequence used for antibody production is SIQRSGS<sub>(p)</sub>S<sub>(p)</sub>MKEEPK, located from amino acid 7 to 19 of Ebf1 (Figure 18). 14H6 is an Ebf1-S13<sub>P</sub> specific antibody and 1C4 is an Ebf1-S13<sub>P</sub>S14<sub>P</sub> specific antibody. In this study, endogenous protein was isolated from 18-81 TK+ cells, and the phosphorylation of Ebf1-S<sub>13</sub> and S<sub>14</sub> were detected via western blot using the newly generated monoclonal antibodies 14H6 and 1C4. In this western blot, the phospho-specific detection of S13 and S14 was controlled via treatment with calf intestinal phosphatase, which leads to the removal of phospho-groups. Our data

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shows that endogenous serine 13 and 14 of Ebf1 are phosphorylated, confirming the mass spectrometry data.

The mass spectrometry on transiently transfected Ebf1-flag also shows that approximately half of Ebf1 protein is phosphorylated at S<sub>13</sub> and S<sub>14</sub> in 18-81 TK+ cells showing flexibility in the biochemical properties of Ebf1 and thus offering potential explanation. Compared to other Ebf family members, which are expressed in tissues unrelated to hematopoietic cells, none of them present a serine at or close to this position. This could be another indirect evidence to support our conclusion that phosphorylation of Ebf1-S<sub>13</sub> and S<sub>14</sub> is cell-type specific.

The expression of Ebf1 is mediated via two distinct promoters, the distal  $\alpha$ -promoter and the proximal  $\beta$ -promoter. Transcription from these promoters results in the expression of two Ebf1 isoforms,  $\alpha$ Ebf1 and  $\beta$ Ebf1. Due to alternative splicing, transcripts from the distal promoter use a different start codon compared to  $\beta$ Ebf1, generating an isoform that lacks 14 N-terminal amino acids of the  $\beta$ Ebf1 isoform. Therefore, the phosphorylation of this two positions might also help to resolve potentially different functions between  $\alpha$  and  $\beta$ Ebf1 during B cell development.

## **5.2 The biological function of Ebf1-S<sub>13</sub>, S<sub>14</sub>-phosphorylation during B cell development**

As a key regulator, Ebf1 plays a 'pioneer' role for B cell development (Hagman and Lukin 2005). *Ebf1*-deficient mice display a complete block of B lymphopoiesis at the pre-pro-B-cell (fraction A) stage. No rearrangement at the Ig $\mu$  locus is detected *in vivo*, and *Ebf1*-deficient CLP show no D-to-J<sub>H</sub> rearrangement (Pongubala et al. 2008). The ectopic expression of *Ebf1* in hematopoietic progenitors from the fetal liver of *Ebf1*-deficient mice could restore B cell development (Pongubala et al. 2008). Since we found the phosphorylation of Ebf1-S<sub>13</sub> and S<sub>14</sub> specifically in B cells, we continued to study the potential biological functions of this two position. We generated *Ebf1*-S<sub>13</sub>A, S<sub>14</sub>A and S<sub>13,14</sub>A via site-directed mutagenesis at serine 13 and 14 replacing the serine residues with alanine. Serine has a hydroxyl group that can be phosphorylated by kinases, which is missing in the structurally similar alanine. However, this mutagenesis should not change the native structure. Our western blot

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shows that the expression of Ebf1-S<sub>13</sub>A, S<sub>14</sub>A and S<sub>13,14</sub>A is comparable to wild type Ebf1 in HEK293T cells suggesting that the mutagenesis does not change the stability of native protein (Figure 11b). As a transcription factor, Ebf1 protein forms a dimer and binds to DNA via its DBD. Our EMSA assays show that the binding activity of mutant Ebf1 to its binding site in the mb-1 promoter is comparable to wild type Ebf1 (Figure 14). Therefore, the phosphorylation of S13 and 14 is not associated with DNA binding activity of Ebf1. It also demonstrates that the mutant versions of Ebf1 are folded correctly, otherwise no DNA binding should be observed.

To investigate the biological function of the phosphorylation of Ebf1, *Ebf1*-S<sub>13</sub>A, -S<sub>14</sub>A and -S<sub>13,14</sub>A are analysed by an established long-term cell culture system that supports B cell differentiation of primary hematopoietic cells *in vitro* (WW culture). Mutant versions of Ebf1 are introduced into *Ebf1*<sup>-/-</sup> fetal liver cells via a co-culture with retrovirus producing GP+E cells. The transduced fetal liver cells undergo 10-14 days of co-culture with hematopoietic cells to allow B cell development. We use CD19 to identify B cell development as CD19 is a surface antigen from the pro-B cell stage on (fraction B). Our data shows that only 2%-8% cells express CD19 upon reintroduction of *Ebf1*-S<sub>13</sub>A, S<sub>14</sub>A and S<sub>13,14</sub>A, compared to wild type *Ebf1*. Therefore, the phosphorylation of serine 13 and 14 of Ebf1 are required for B cell development. This data is confirmed via analysis of *CD19* mRNA expression. Transduced *Ebf1*<sup>-/-</sup> fetal liver cells are sorted and analysed via real time-PCR, which shows that the expression of *CD19* mRNA is significantly decreased in cells expressing *Ebf1*-S<sub>13</sub>A, S<sub>14</sub>A and S<sub>13,14</sub>A.

D-J rearrangement of the immunoglobulin heavy chain locus is absent in *Ebf1*-deficient common lymphoid progenitor cells (Reynaud et al. 2008, Lin and Grosschedl 1995). This study shows that after 10-14 days WW culture, DJ<sub>H</sub> rearrangement is detectable not only in wild-type Ebf1 transduced fetal liver cells, but also in *Ebf1*-S<sub>13</sub>A, S<sub>14</sub>A and S<sub>13,14</sub>A expressing cells, suggesting that DJ<sub>H</sub> is independent of the phosphorylation of serine 13 and 14 (Figure 17). Furthermore, this confirms the functionality of the mutant versions of *Ebf1*, since they are able to rescue part of the defects of *Ebf1*-deficient cells. DJ<sub>H</sub> rearrangement is regulated by Ebf1 via the induction of RAG genes. Therefore the expression of RAG gene has also been studied. After 10-14 days WW culture, the transduced *Ebf1*<sup>-/-</sup> fetal liver

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cells were sorted and analysed via real time-PCR. Our data shows that the mRNA expression of *Rag1* in fetal liver cells expressing mutant versions of *Ebf1* is comparable to wild type *Ebf1*, which corresponds to the DJ<sub>H</sub> rearrangement. This could be one of the reasons for the diversity of Ebf1, which means not all of its functions are dependent on phosphorylation.

The double mutation, Ebf1-S<sub>13</sub>S<sub>14</sub>A, does not show a synergistic effect in enhancing the block of B cell development, as well as in the expression of target genes. In contrast, a few more percentage of CD19<sup>+</sup> cells is present upon double mutation suggesting an overlap between the biological functions of phosphorylated Ebf1-S<sub>13</sub> and Ebf1-S<sub>14</sub>. The data is confirmed by mRNA expression of *CD19* isolated from *Ebf1*-S<sub>13</sub>S<sub>14</sub>A transfected fetal liver cells (Figure 13C). The decrease of *CD19* mRNA is similar in *Ebf1*-S<sub>13</sub>S<sub>14</sub>A and *Ebf1*-S<sub>13</sub>A transfected cells where S14 seems to have a reduced effect, revealing a dominant effect of S13 in this case.

The fundamental phenotypes of *Ebf1*<sup>-/-</sup> hematopoietic cells are a complete block of B cell development in fraction A and a lack of DJ<sub>H</sub> rearrangement in CLP. During our *in vitro* experiment, we show that the early B cells need the phosphorylation of Ebf1 at S<sub>13</sub> and S<sub>14</sub> to develop into fraction B. The same phenotypes also occur upon deletion of the transactivation domain (Ebf1-ΔTAD and αEbf1-ΔTAD) suggesting the possibility that the block of B cell development caused by mutant *Ebf1* is related to the transactivation.

To study whether the transactivation potential of Ebf1 is influenced by modifications of serine 13 and 14, luciferase reporter assays were performed. We used a pre-B cell line, Ba/F3 cells, which does not express Ebf1 (Smith et al. 2002). βEbf1, Ebf1-S<sub>13</sub>A, Ebf1-S<sub>14</sub>A and Ebf1-S<sub>13,14</sub>A were transfected with a reporter construct containing the λ5 promoter including three Ebf1-binding sites in front of the luciferase reporter gene (Figure 15A; Sigvardsson et al. 1997). Our data shows that the inductions were significantly affected by the mutation of serine 13 and serine 14, suggesting that the transactivation of the λ5 promoter depends on the phosphorylation of Ebf1-S<sub>13</sub> and S<sub>14</sub>. Our study reveals important differences in Ebf1 transactivation. Phosphorylation of S<sub>13</sub> and S<sub>14</sub> in Ebf1 is required to activate the λ5 promoter in B cells involved in pre-BCR signaling cascades, but Ebf1 transactivation is only partially phospho-

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dependent. For example, the mRNA level of *Rag1*, which is directly participating in the Ig rearrangement, is comparable between wild type and mutant versions of Ebf1 in primary B cell culture. This might be because the *Rag1* promoter differs structurally from the  $\lambda 5$  promoter. Ebf1 can bind on the *Rag1* promoter and co-operate with other components to trans-activate Rag1, which does not require the phosphorylation of Ebf1-S<sub>13</sub> and S<sub>14</sub>. Another reason for the differences in Ebf1 transactivation might be the two Ebf1 isoforms. The first expressed isoform of Ebf1 is  $\alpha$ Ebf1 in CLPs, where the D-J<sub>H</sub> rearrange starts (Roessler et al. 2007b).  $\alpha$ Ebf1 lacks the first 14 amino acids, including S<sub>13</sub> and S<sub>14</sub>. Therefore a subset of Ebf1 target genes, initiated by  $\alpha$ Ebf1 in CLPs seems to be independent of S<sub>13</sub> and S<sub>14</sub> phosphorylation. In our  $\lambda 5$  promoter transactivation experiment, we also studied  $\alpha$ Ebf1, which showed that the induction by  $\alpha$ Ebf1 is significantly decreased compared to  $\beta$ Ebf1. This data suggests that the two isoforms of Ebf1 are functional different. However concerning co-transactivation together with E2A, similar activation of the endogenous  $\lambda 5$  gene was observed with  $\alpha$ Ebf1 and  $\beta$ Ebf1 suggesting that both isoforms can synergise with E2A (Roessler et al. 2007b).

### **5.3 The regulation of Ebf1 by phosphorylation of S<sub>13</sub> and S<sub>14</sub> during B cell development**

Early B-cell differentiation is a highly regulated process in which a multipotential progenitor cell is converted into a cell that expresses the B-cell antigen receptor. Several transcription factors and signaling pathways have been implicated in the regulation of this process. The targeted inactivations of the *Ebf1* and *E2A* genes result in similar blocks of B-cell differentiation, preceding the onset of rearrangement of the immunoglobulin heavy chain D<sub>H</sub> and J<sub>H</sub> segments (Bain et al. 1994, Zhuang et al. 1994b, Lin and Grosschedl 1995). Forced expression of *Ebf1* in hematopoietic progenitor cells skews the differentiation along the B-cell pathway, and genetic bypass experiments have shown that Ebf1 can promote B-cell differentiation in *E2A*<sup>-/-</sup> hematopoietic progenitors (Medina et al. 2004, Seet et al. 2004). Another important event in the differentiation of B-lineage cells, the commitment step, is regulated by the transcription factor Pax5. *Pax5*-deficient mice generate pro-B cells that express early B-cell markers and undergo D-to-J<sub>H</sub> and proximal V<sub>H</sub>-to-DJ<sub>H</sub> rearrangements (Nutt et al. 1999). *Pax5* is also required continuously for the maintenance of B-cell

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identity (Rolink et al. 1999a). The analysis of the regulatory hierarchy of transcription factors has suggested that E2A activates the expression of *Ebf1* and, together, both transcription factors induce the expression of several B-lineage genes, including *Rag1*, *Pax5*,  $\lambda 5$ , *VpreB*, *B29* and *mb1*, which encode components of the pre-B-cell receptor (O'Riordan and Grosschedl 1999).

Since we found that phosphorylation of Ebf1 at S13 and 14 influences B cell development via transactivation of the  $\lambda 5$  promoter, we further investigated downstream genes. After 10-14 days WW co-culture, the transduced *Ebf1*<sup>-/-</sup> fetal liver cells were sorted and analysed via real time-PCR. Our data shows that the expression of *Pax5*,  $\lambda 5$ , *VpreB*, *B29* and *mb1* is significantly decreased in cells expressing *Ebf1*-S<sub>13</sub>A, S<sub>14</sub>A, S<sub>13,14</sub>A as well as  $\alpha$ *Ebf1* or *Ebf1*- $\Delta$ TAD indicating that the expression of these B lineages genes is associated with the phosphorylation of S13 and 14. However the expression of *Rag1* is comparable between  $\alpha$ ,  $\beta$  and modified versions of *Ebf1*, as discussed before (5.2).

*Ebf1* and *E2A* appear to synergize in the activation of B-lineage gene expression because *Ebf1*/*E2A* double-heterozygous mutant mice have a more severe defect in B-cell differentiation than the single-heterozygous mice (O'Riordan and Grosschedl 1999). Therefore, we also studied the expression of *E2A*. The data shows no significant difference in the expression of *E2A* in cells expressing *Ebf1*-S<sub>13</sub>A, S<sub>14</sub>A, S<sub>13,14</sub>A,  $\alpha$ *Ebf1*, *Ebf1*- $\Delta$ TAD and  $\alpha$ *Ebf1*- $\Delta$ TAD. The expression of *E2A* is initiated upstream of *Ebf1* (Seet et al. 2004), however, the expression of *E2A* is impaired in *Ebf1*-deficient mice, suggesting that Ebf1 may also regulate the expression of *E2A* (Zhuang et al. 2004). Therefore, as with the *Rag1* gene, *E2A* may be regulated via  $\alpha$ Ebf1 in CLP.

Taken together, the expression of *E2A* is regulated via  $\alpha$ Ebf1, and *E2A* co-operates with  $\alpha$ Ebf1 to mediate gene expression in CLP. The expression of *Pax5*,  $\lambda 5$ , *VpreB*, *B29* and *mb1* is regulated by the co-activation of *E2A* and  $\beta$ Ebf1 depending on the phosphorylation at serine13 and 14, which means the two isoforms of Ebf1 are functionally different in the downstream expression and this difference might occur at different stages of B cell development. This data also shows that part of the target genes require the phosphorylation of serine 13 and 14 in Ebf1, giving a potential

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explanation for the precise timing in the expression of Ebf1 targets during B cell development.

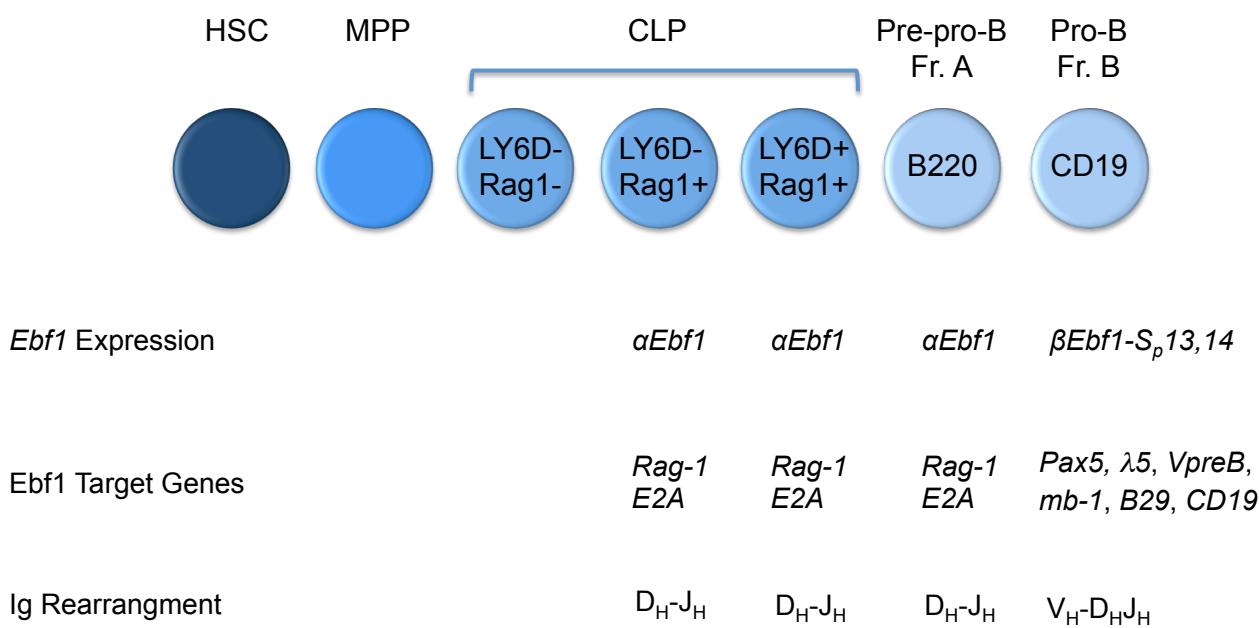
## 5.4 Two isoforms of Ebf1 are functionally different during B cell development

The expression of the *Ebf1* gene involves two promoters (distal promoter  $\alpha$  and proximal promoter  $\beta$ ) that are differentially regulated and generate distinct protein isoforms ( $\alpha$ Ebf1 and  $\beta$ Ebf1). Transcription from the  $\alpha$  promoter results in the expression of  $\alpha$ Ebf1, generating an isoform that lacks 14 N-terminal amino acids of  $\beta$ Ebf1 (Roessler, Budhu and Wang 2007a). However, no functional difference between the two Ebf1 isoforms has yet been described. The  $\alpha$  promoter is directly regulated by E2A and indirectly regulated by the IL-7 receptor. IL-7 signaling activates *Ebf1* expression indirectly by providing permissive conditions for *Ebf1* activation by other factors (Boller and Grosschedl 2014). *IL-7* deficient mice show a developmental arrest at the CLP stage and impaired *Ebf1* expression (Roessler et al. 2007b, Kikuchi et al. 2005). Forced expression of Ebf1 can partially rescue B-cell development in *IL-7R*-deficient mice (Kikuchi et al. 2005). The expression of  $\beta$ Ebf1 is regulated by Ets1, Runx1, Pax5 and PU.1 (Roessler et al. 2007b). Runx1 can change the epigenetic landscape at the Ebf1  $\beta$  promoter (Seo et al. 2012). Recent studies show that the SWI/SNF complex, which is associated with the epigenetic regulation of *Ebf1*, facilitates transcription from the Ebf1  $\beta$  promoter.

Since S13 and 14 only appear in  $\beta$ Ebf1, the modification of these two positions open a new view of the biological function of the two Ebf1 isoforms. In our WW co-culture, B cell development shows a strong impairment in  $\alpha$ Ebf1 expressing cells, while  $\beta$ Ebf1 can rescue the *Ebf1*<sup>-/-</sup> hematopoietic cells developing into B cells. This data suggests that  $\alpha$ Ebf1 and  $\beta$ Ebf1 are functionally different.  $\alpha$ Ebf1 starts to be expressed from the middle stage of CLP (Iy6d-, Rag1+; P, Joseph and Jacob 2012) synergising with E2A in inducing the expression of Rag1, which mediates the DJ<sub>H</sub> rearrangement.  $\alpha$ Ebf1 can support the development of CLPs to the pre-pro-B stage (fraction A), and then  $\beta$ Ebf1 is required for further B cell development. Our study of Ebf1 targets shows that B lineage genes (*Pax5*,  *$\lambda$ 5*, *mb1*, *B29* and *VpreB*) are regulated by  $\beta$ Ebf1 in a S<sub>13</sub> and S<sub>14</sub> phosphorylation-dependent manner. Pax5



maintains the B lineage from pro-B stage (fraction B) on (Nutt et al. 1997b). Therefore  $\beta$ Ebf1 starts to regulate B cell development from pro-B stage.  $\lambda 5$ , mb1, B29 and VpreB are the components of pre-BCR, which regulate the pre-B cell proliferation and development. Therefore  $\beta$ Ebf1 maintains B lineage in a phosphorylation of S<sub>13</sub> and S<sub>14</sub> dependent manner at least from the pro-B cell stage (Figure 19).



**Figure 19 Representation of the expression of Ebf1 isoforms and corresponding target genes from HSC to pro-B cells**

CLP are separated into three developmental stages. Expression of  $\alpha$ Ebf1 starts from the middle stage of CLP till pre-pro-B cells and regulates the expression of E2A and Rag1, allowing DJ<sub>H</sub> rearrangement. Expression of  $\beta$ Ebf1 starts from pro-B cells in a S<sub>13</sub> and S<sub>14</sub> phosphorylation dependent manner and regulates the expression of *Pax5*,  $\lambda 5$ , *VpreB*, *mb-1*, *B29* and *CD19*. HSC: hematopoietic stem cell; MPP: multi-potential progenitor; CLP: common lymphoid progenitor.

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## 5.5 Future work

The regulation of Ebf1 target genes requires collaboration of Ebf1 with other transcription factors. Many Ebf1-regulated genes, including *Vpreb1*, *Igll1*, and *CD79a* contain binding sites for E2A (Treiber et al. 2010b). However, during B cell development additional *Ebf1*-occupied sites are gained while others are lost resulting in a shift in the composition of neighboring binding sites between pro-B and mature B cells (Gyory et al. 2012). Our data shows that in the pre-B cell line Ba/F3 cells, the phosphorylation of Ebf1-S<sub>13</sub> and S<sub>14</sub> are required for the transactivation of  $\lambda 5$ . For the future work, to further prove the biochemical function of the phosphorylation of Ebf1-S<sub>13</sub> and S<sub>14</sub>, the co-transactivation between E2A and Ebf1-S<sub>13</sub>A and S<sub>14</sub>A should be studied.

Ebf1 plays different role during B cell development. It regulates the Ig rearrangement and the expression of B lineage genes (Lin and Grosschedl 1995). Our data shows that the development of CLPs to pre-pro-B cells is mediated via  $\alpha$ Ebf1, and then  $\beta$ Ebf1 starts to regulate pro-B development into pre-B cells in a S<sub>13</sub> and S<sub>14</sub> dependent manner. However, the regulation of the phosphorylation of Ebf1 at S<sub>13</sub> and S<sub>14</sub> in mature B is unclear. We generated Ebf1-S<sub>13</sub> and S<sub>14</sub> specific antibodies, 14H6 and 1C4, which can be used to detect at later stages of B cell development the phosphorylation states of S<sub>13</sub> and S<sub>14</sub>.

The expression of 3000 Ebf1 target genes is regulated by Ebf1 in three different ways: activation, repression and poising (Treiber et al. 2010b). We studied activation of the targets *Pax5*,  $\lambda 5$ , *mb1*, *CD19*, *VpreB*, *Rag1* and *E2A*, and found the expression of *Pax5*,  $\lambda 5$ , *mb1*, *CD19* and *VpreB* associated with the phosphorylation of Ebf1-S<sub>13</sub> and S<sub>14</sub>. For the future work, the repressed and poised genes should also be studied in Ebf1-S<sub>13</sub>A and S<sub>14</sub>A expressing cells to open a new view of the multi-function of Ebf1 during B cell development.

RSK and cdc2 are candidates to regulate the phosphorylation of Ebf-S<sub>13</sub>, and CKII and PKC potentially regulate the phosphorylation of Ebf1-S<sub>14</sub> (NerPhosK 1.0 Server, Technical University of Denmark). Since we found that the phosphorylation of Ebf1-

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S<sub>13</sub> and S<sub>14</sub> plays an important role during B cell development, the kinases responsible for this PTM and this regulation should be characterized.

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## **Declaration**

### **Eidesstattliche Erklärung**

Ich versichere hiermit an Eides statt, dass die von mir vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt ist.

München, den \_\_\_\_\_

\_\_\_\_\_  
Qiongman Wang

### **Erklärung**

Hiermit erkläre ich, dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist, und dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

München, den \_\_\_\_\_

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Qiongman Wang

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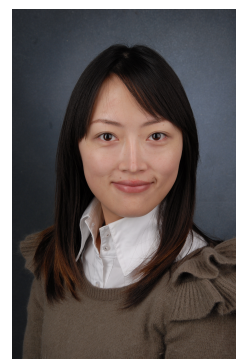
A specific thanks for my parents who encouraged me all the time, and my husband, Chuanxin Liu, who pay a lot of patient and endless love to support my study. Without their help and belief, I can't finish my thesis as quick as I expect.

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## Education:

<b>Aug. 2011</b>	<b>Oct.2015</b>	<b>Ludwig-Maximilian-University of München, Germany</b> Ph.D of Biology  <b>Institute of Clinical Molecular Biology and Tumor Genetics (KMOLBI) in Helmholtz Zentrum München</b> <i>Ph.D Thesis: Regulation of B cell Development by Posttranslational Modifications of Ebf1</i>
<b>Oct. 2008</b>	<b>Mar. 2011</b>	<b>Martin-Luther-University of Halle-Wittenberg, Germany</b> Master of Biomedical Engineering  <b>Institute of Lung Biology and Disease (iBLD) in Helmholtz Zentrum München</b> <i>Master Thesis: Expression profiling of Protein Arginine Methyltransferase (PRMT) Isoforms in Chronic Obstructive Pulmonary Disease (COPD)</i>
<b>Sep. 2002</b>	<b>Jul. 2007</b>	<b>Third Military Medical University, China</b> Medicine  Third Military Medical University Scholarship for distinguished Grade.

## Working Experience:

<b>Jan. 2011</b>	<b>Mar. 2011</b>	<b>Institute of lung biology and disease in Helmholtz Zentrum München, Germany</b> <i>Student work: PRMT regulate macrophage activation during COPD pathogenesis.</i>
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		<ul style="list-style-type: none"> <li>▪ Writing project proposal independently</li> <li>▪ Lavage mouse lung alveolar macrophage</li> <li>▪ Culture cell with cigarette smock extract</li> <li>▪ PRMT expression by real time PCR</li> </ul>
<b>Jul. 2007</b>	<b>Sep. 2008</b>	<b>ZunYi Medical College Hospital, China</b> Working in Ophthalmology as a resident <ul style="list-style-type: none"> <li>▪ Chief surgeon in the enucleation of the eyes</li> <li>▪ Assistant in the glaucoma and cataract surgery</li> </ul>

## **Skills:**

### **Languages:**

- |           |                 |
|-----------|-----------------|
| ▪ Chinese | Native          |
| ▪ English | Business fluent |
| ▪ German  | Basic           |

### **Knowledge of Major:**

- Biochemistry
- Pathology
- Neurology
- Immunology
- Physiology
- Medicine
- Chirurgery

### **Experiment Skill:**

- Mice Experiment: Mice lung lavage, Bone marrow isolation
- Primary cell Co-Culture
- Flow cytometry analysis
- Western Blot
- Immunohistochemistry
- Electrophoretic mobility shift assay<sup>n</sup>
- Reporter Assay
- Bacterial DNA transformation
- RNA isolation
- cDNA synthesis
- Real time PCR
- Plasmid DNA isolation
- Primer design

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## **Reference:**

### **Ph.D Supervisor:**

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