universität wien

MASTERARBEIT

Titel der Masterarbeit

„Generation of conditional alleles for the analysis of *Ikaros* and *Aiolos* function“

verfasst von

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angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 066 877
Studienrichtung lt. Studienblatt:	Masterstudium Genetik und Entwicklungsbiologie
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1. Zusammenfassung

Immunzellen entstehen aus hämatopoetischen Stammzellen (HSC). Für die korrekte Entwicklung der Blutzellen und Immunzellen spielen eine Reihe von Faktoren eine Rolle. Zwei Transkriptionsfaktoren, die vor allem für die Differenzierung von B Lymphozyten aus hämatopoetischen Stammzellen entscheidend sind, sind Ikaros (*Ikzf1*) und Aiolos (*Ikzf3*).

Bisherige Studien zeigten, dass beide Transkriptionsfaktoren für die Entwicklung von Lymphozyten, vor allem von B Zellen, essentiell sind. In Knockout-Experimenten wurde gezeigt, dass die Abwesenheit einer der beiden Transkriptionsfaktoren wesentliche Einflüsse auf die Produktion von B Zellen hat. *Ikzf1* Knockouts führten schon in einem frühen Entwicklungsstadium zu einer vollständigen Blockade der weiteren B Zell-Differenzierung. *Ikzf3*-defiziente Mäuse zeigten aberrante B Zell-Entwicklung, begleitet von einer Hyperaktivierung der reifen B Zellen.

Im Laufe meiner Masterarbeit habe ich zwei konditionelle Allele entwickelt. Das erste Allel enthielt die *Ikzf1* cDNA, reguliert von einem starken Promoter im *Rosa26* Genlocus von embryonalen Stammzellen (ES Zellen) der Maus. Für das Allel positive ES Zellklone wurden in Maus-Blastozysten injiziert um chimäre Mäuse zu erschaffen, die das konditionelle *Ikzf1* Allel tragen. *Ikzf1* Überexpression kann in den Mäusen erzielt werden, indem die dem *Ikzf1* Minigen vorangeschaltete gefloxte Stopkassette durch die Cre Rekombinase deletiert wird.

Weiters wurde ein Allel entworfen, bei dem das letzte Exon des *Ikzf3* Gens (Exon 8) von *LoxP* Stellen begrenzt wurde. Für das gefloxte *Ikzf3* Allel positive ES Zellen werden in Maus-Blastozysten injiziert. Die Expression der Cre Rekombinase führt dann zur Deletion des letzten *Ikzf3* Exons und zur Translation eines nichtfunktionellen Aiolos Proteins.

Zukünftige Pläne sind, die chimären Mäuse mit Mäusen zu kreuzen, die konditionell die Cre Rekombinase exprimieren, und dadurch zu einer konditionellen *Ikzf1* Überexpression bzw. *Ikzf3* Deletion führen. Die Analyse der Mäuse wird Aufschluss über die Funktionen von Ikaros und Aiolos in den verschiedenen Entwicklungsstadien von B Lymphozyten geben.

2. Summary

During hematopoiesis, blood and immune cells are generated from hematopoietic stem cells (HSC). A network of transcription factors regulates the differentiation pathway of HSCs towards the several blood cell lineages. Two important regulatory factors for B lymphocyte development are Ikaros (*Ikzf1*) and Aiolos (*Ikzf3*).

Previous studies showed that the transcription factors Ikaros and Aiolos are critical for the development of cells of the lymphoid lineage, especially for B cell lymphopoiesis. Studies have been conducted, knocking out *Ikzf1* or *Ikzf3*. These experiments led to the conclusion that both transcription factors were essential for the proper development of B cells, as *Ikzf1* null mutants showed a complete block of B cell development, while *Ikzf3* knockouts led to aberrant B cell development and hyperproliferation of mature B cells.

To analyze the role of the two transcription factors in different stages of B cell development, conditional alleles for the induction of *Ikzf1* overexpression on the one hand, and conditional *Ikzf3* deletion on the other hand, are of great essence.

In my master thesis, I have generated an allele containing the *Ikzf1* cDNA under the control of a strong promoter within the *Rosa26* locus of mouse embryonic stem (ES) cells. Successfully targeted ES cells were injected into mouse blastocysts to create chimeric mice carrying the conditional *Ikzf1* overexpression allele. Hence, *Ikzf1* overexpression can be achieved in mice upon Cre-mediated deletion of the upstream located floxed stop cassette.

Further, a conditional *Ikzf3* allele was designed, in which the last exon of the *Ikzf3* gene was flanked by *loxP* sites. Successfully targeted ES cells are to be injected into mouse blastocysts. The expression of Cre recombinase in mice carrying the conditional *Ikzf3* allele will result in the expression of a non-functional Aiolos protein.

The future aims of the projects will be the analysis of the impact of *Ikzf1* overexpression and *Ikzf3* deletion on murine B cell development. For this purpose, the chimeric mice will be bred to different C57BL/6J Cre lines to induce the expression of the conditional alleles. Resulting mice will express the Cre recombinase in different stages of lymphopoiesis, leading to the overexpression of *Ikzf1* or the deletion of *Ikzf3* in different stages of B cell development. It will be important to provoke *Ikzf1* overexpression and *Ikzf3* deletion in early lymphoid progenitors, in pro B cells as well as in mature B cells to reveal the impacts of the two hematopoietic transcription factors in the entire B cell lineage.

3. Introduction

3.1 Hematopoiesis generates blood and immune cells

During the process of hematopoiesis all types of blood cells are generated from hematopoietic stem cells (HSCs). HSCs have two main characteristics, to self-renew and to differentiate into all blood cell lineages. Self renewal ensures the maintenance of the hematopoietic stem cell pool while differentiation produces blood and immune cells which are essential for the organism (Mikkola et al. 2006; Mangel et al. 2013). In the fetus, hematopoietic stem cells are located in distinct anatomical sites like the yolk sac, the placenta, the fetal liver, and the aorta-gonad-mesonephros region (AGM), while postnatally hematopoiesis takes place in the bone marrow (Mikkola et al. 2006; 2011).

To produce blood cells, first, the c-kit and ScaI positive HSC generates multipotent progenitor (MPP) cells which then split into distinct developmental pathways. The MPP develops into a lymphoid-primed multipotent progenitor (LMPP), an erythroid/megakaryocytic (MEP) or a granulocyte/macrophage (GMP) progenitor. The LMPP further differentiates into common lymphoid progenitors (CLPs) or myeloid progenitors (GMPs) (Lin et al. 2010; Mercer et al. 2011). CLPs express high levels of Flt3 and IL7R but are low in c-kit expression (Mercer et al. 2011), while myeloid markers are amongst others CD11b and CD14 (Appleby et al. 2012). Erythrocytes, megakaryocytes, granulocytes and macrophages derive from the erythroid and myeloid lineages, while CLPs give rise to NK cells, B lymphocytes, and early thymocyte progenitors (ETPs), which will generate T lymphocytes after several transitional cell stages (Mandel et al. 2010; Kanji et al. 2011).

The dendritic cells (DCs) are another group of immune cells, which derive from HSCs. DCs are antigen-presenting cells and trigger T cell and B cell mediated immunity. They are further subdivided into conventional dendritic cells and plasmacytoid dendritic cells (Satpathy et al. 2012; Hanke et al. 2013).

The developmental decision between self renewal and differentiation and between the several differentiation pathways depends on internal signals like the expression of lineage-specific genes and external environmental signals from the stem cell niche (Georgopoulos 2002; Kanji et al. 2011). Hematopoiesis is dependent on multiple factors acting in a complex regulatory network. Regulators like hedgehog signaling are important for

primitive hematopoiesis in the fetus, while transcription factors like GATA-2, PU.1 and Runx1 are required for both, primitive and adult hematopoiesis. A lack of these factors impairs proper hematopoiesis (Kanji et al. 2011).

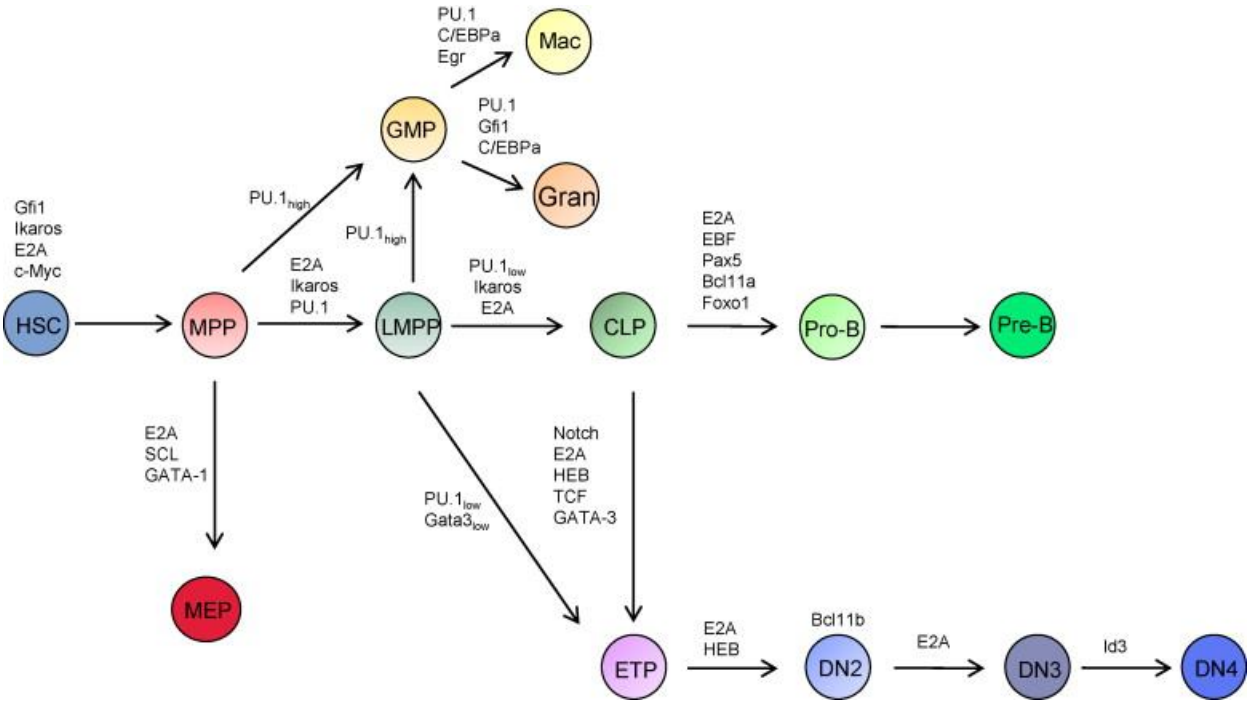


Figure 1. Hematopoiesis and its important regulatory factors (Mercer et al. 2011)

For the specification of the lymphoid lineage PU.1 and Ikaros are essential during early hematopoiesis. During lymphocyte development, transcription factors like E2A, EBF1, FOXO1 and Pax5 and other Ikaros family zinc-finger transcription factors including Helios and Aiolos are key regulators (Figure 1). (Zon 2001; Nutt et al. 2007; Mandel et al. 2010; Mercer et al. 2011)

3.2 Acquired Immunity and the relevance of B cells

The immune system applies two strategies to fight foreign pathogens, these are innate and acquired immunity. The main characteristic of these systems is to distinguish between self and non-self. An innate immune response occurs immediately after invasion and targets common but non-specific structures of pathogens. Acquired immunity, in contrast, is more specific. It uses B and T lymphocytes with unique antigen receptors to fight all kinds of antigens. Acquired immunity is furthermore equipped with a memory to be able to elicit fast immune responses against familiar antigens. (Abbas et al. 2007)

3.2.1 The significance of B cells and antibodies in an immune response

B cells produce antibodies to antagonize antigens. Antibodies can be found in a secreted or a membrane-bound form (the B cell receptor) and they are made of immunoglobulin (Ig) heavy and light chains consisting of constant and variable regions. The constant regions regulate the antibody isotype, while the variable regions are essential for antigen binding specificity (Almqvist et al. 2012). Upon antigen recognition, affinity binding leads to a process of maturation and enables a B cell to produce antibodies to fight foreign microbes (LeBien et al. 2008; Cerutti et al. 2012).

3.2.1.1 B cells originate from hematopoietic stem cells

B cells evolve from hematopoietic stem cells through sequential differentiation of progenitor cells in an ordered maturation and selection process. During early B cell development, the recombination of the immunoglobulin heavy chain (IgHC) and light chain (IgLC) loci is the principal task. The B cell receptor genes are assembled by random recombination of the Ig variable (V), diversity (D) and joining (J) gene segments. These recombination events are regulated by the recombination-activating genes (*RAG*). The HSC generates lymphoid progenitors which first differentiate to pro-B cells. In the pro-B cell, rearrangement of the immunoglobulin heavy chain (IgH) locus takes place. For this purpose, the IgH locus undergoes DJ recombination followed by V to DJ recombination resulting in the production of a µHC (Kirstetter et al. 2002; Almqvist et al. 2012). Pro-B cells express high levels of B220 and start to upregulate CD19 when they rearrange the IgH locus (Mercer et al. 2011). To form the pre-B cell receptor (pre-BCR) the µHC joins with the invariant surrogate light chain. This leads to the transition to the CD25 positive pre-B cell stage, where the recombination of

the κ and λ light chains occurs. A light chain assembles with the μHC leading to the expression of the B cell receptor (BCR) (Almqvist et al. 2012). Pre-B cells further develop into immature B cells after successful pairing of light and heavy chains and the expression of the BCR. This early B cell development takes place in the fetal liver and in the adult bone marrow. Immature B cells can be characterized by the surface expression of IgM and are capable to leave the bone marrow, circulate in the blood and enter secondary lymphoid organs as naïve B cells, which further upregulate IgD. Upon antigen triggering, naïve B cells mature to antibody producing plasma cells (Figure 2) (Kirstetter et al. 2002; Barneda-Zahonero et al. 2012).

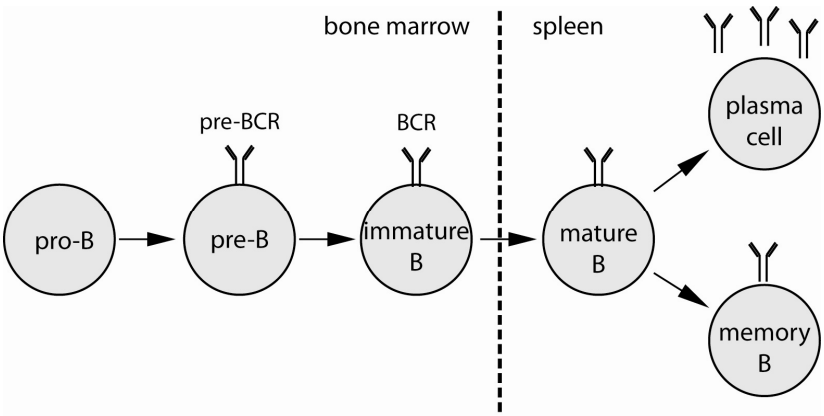


Figure 2. B cell development

3.2.1.2 T cell dependent antibody production in follicular B2 B cells

There are subsets of B lymphocytes, which react differently on antigen encounter. Conventional follicular B2 B cells are activated by protein-based antigen presentation through the help of T cells. Naïve recirculating B cells circulate through blood and lymph vessels, capture antigens, and present processed protein antigens via the major histocompatibility complex class II (MHC II) to helper T (T_H) cells at the boundary of the T cell zone and the B cell follicle in lymph nodes. T_H cells then trigger the clonal expansion and differentiation of the activated B cell (Reif et al. 2002; Ramiscal et al. 2013). As a consequence, the B2 B cells give rise to the germinal center (GC) in the follicle, mature to long lived plasma cells, and produce high affinity antibodies and memory B cells (Cerutti et al. 2012).

3.2.1.3 Affinity maturation of B2 B cells in germinal centers

GCs are a niche for affinity maturation of B cells where somatic hypermutation (SHM) and Ig class switching take place. Upon antigen recognition SHM occurs, leading to mutations in the variable regions of the Ig genes. The process of SHM selects for B cells expressing an Ig receptor with an increased affinity for the immunizing antigen. Low-affinity or self-reactive B cell clones are sorted out (Ramiscal et al. 2013). In contrast to SHM, the process of Ig isotype switching leads to mutations in the constant regions of Ig genes. The purpose of this process is to diversify effector functions by changing the B cell's antibody production from one antibody isotype to another. Maintaining the variable regions thereby ensures the original antigen binding specificity (Shanmugam et al. 2000). The GC consists of a dark and a light zone. In the dark zone, B cells undergo SHM and the antigen specific cells proliferate. When these cells migrate to the light zone they are selected. Antigen specific T cells send signals to the B cells. Only cells with highest BCR-affinity to the antigen are able to take up the antigen, present it on MHC complex and, therefore, receive signals from T cells to be released as high affinity plasma cells. Others are sent for recycling through the GC or undergo apoptosis (Cortes et al. 2004; Victora et al. 2012).

3.2.1.4 T cell independent immune response in B1 B cells and marginal zone B cells

Other subsets of B cells do not form germinal centers but rather grow extrafollicularly to short lived plasmablasts in case of antigen encounter (MacLennan et al. 2003). In contrast, these extrafollicular B cells, comprising B1 B cells and marginal zone (MZ) B cells are able to trigger an immune response in a T cell independent manner. They respond rapidly but polyspecifically to microbial conserved carbohydrates and glycolipids by the production of low affinity antibodies (Cerutti et al. 2012). These short-lived plasma cells have an immediate and quick effect on antigen clearance (Cortes et al. 2004).

3.2.1.5 Immune responses in the marginal zone of the spleen

The CD21 and CD1d expressing MZ B cells are located at the marginal sinus in the spleen. On the one hand, they have a role in T cell dependent immune response by transporting captured antigens from the vicinity into the splenic follicle to the follicular B cells. On the other hand, they can be activated T cell independently by pathogenic polysaccharides and

proteins to enter the splenic red pulp and produce oligospecific antibodies (Allman et al. 2008).

3.2.1.6 Low affinity antibody production by B1 B cells

B1 B cells inhabit peritoneal and pleural cavities. Upon antigen encounter, B1 B cells differentiate into IgM secreting short lived plasma cells and can also switch to IgA production. Without T cell triggering they provide a first defense against T cell independent antigens by the fast production of low affinity antibodies in the non-adaptive phase of the immune response. Their activation can be induced by cytokines like IL-5, IL-10 or through antigen presentation from dendritic cells (Allman et al. 2008; Brenner et al. 2011).

B1 B cells are further divided into B1a and B1b B cells although they originate from different precursors. While B1a B cells express high levels of CD5 and contribute to innate-like immunity, B1b B cells are CD5 negative and belong to adaptive immunity. B1b B cells are reported to derive from B2 B cells (Allman et al. 2008).

3.3 Regulation of B cell development by a transcriptional network

The developmental decisions towards the lymphoid lineage and the B cell developmental pathway are regulated on a transcriptional level. Different transcription factors affect B cell lymphopoiesis by epigenetic and chromatin regulations. In the decision towards lymphocyte development and during early B cell development, the transcription factors Ikaros and PU.1 play major roles. They mainly regulate the development of CLPs out of MPPs (Barneda-Zahonero et al. 2012). Other transcription factors, like E2A and EBF1, are further responsible for the commitment of the CLP towards the B cell lineage. The downstream regulated transcription factor Pax5 subsequently regulates the maintanence of B cell identity (Figure 3) (Nutt et al. 2007; McManus et al. 2011).

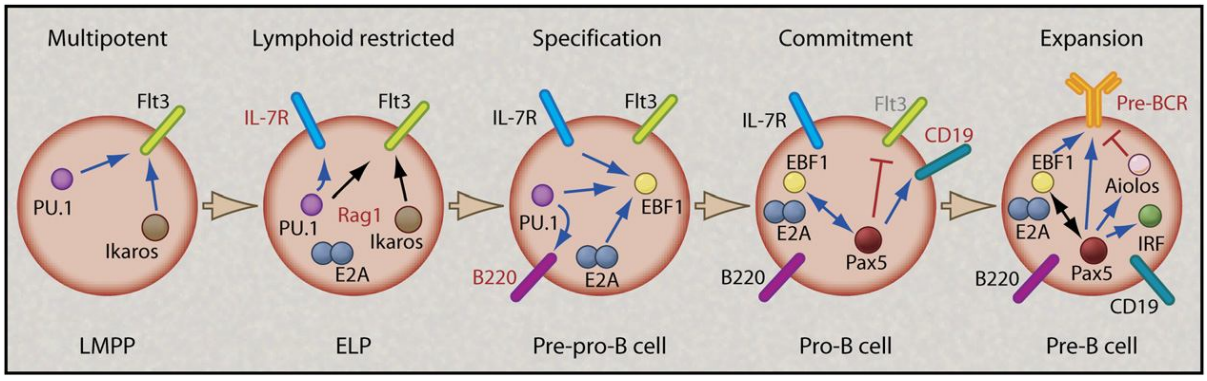


Figure 3. Key transcription factors and cell surface markers during early B cell development (Nutt et al. 2007)

3.3.1 The role of Ikaros family proteins in lymphocyte development

The Ikaros family members Ikaros (*Ikzf1*), Helios (*Ikzf2*) and Aiolos (*Ikzf3*) play pivotal roles in lymphocyte development and differentiation. They control the expression of target genes and the deregulation of these transcription factors is reported to have an impact on the development of leukaemias and lymphomas. The proteins share amino- and carboxyterminal zinc finger domains and can dimerize with each other (Rebollo et al. 2003).

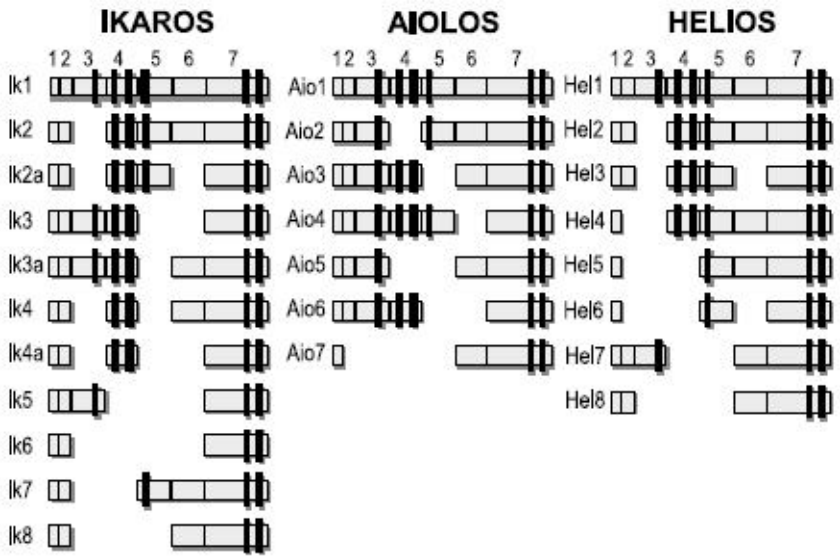


Figure 4. Ikaros, Aiolos and Helios isoforms. The black bars symbolize the zinc-finger motifs. (Rebollo et al. 2003)

3.3.1.1 The hematopoietic transcription factor Ikaros (*Ikzf1*)

The *Ikzf1* gene encodes the Ikaros protein containing several zinc finger motifs (Nichogiannopoulou et al. 1998). There are eight isoforms of the Ikaros protein generated by alternative splicing. All isoforms contain C-terminal zinc finger motifs responsible for dimerization with another Ikaros protein or with other Ikaros protein family members like Aiolos and Helios. The last C-terminal exon further contains a transcriptional activation domain (Wang et al. 1996). In addition to the dimerization and transcriptional activation domain at the C-terminus, the N-terminal zinc fingers are indispensable for protein function. These zinc finger motifs are essential for DNA binding but their number differs in various Ikaros isoforms. Ikaros isoforms with at least three zinc fingers at the N-terminus can bind DNA. Only protein dimers are able to interact with DNA as transcription factors.

The final role of the dimerized Ikaros protein is to bind to target genes and regulate their expression by regulatory effects like chromatin remodeling (Nichogiannopoulou et al. 1998; Zon 2001; Georgopoulos 2002).

Ikaros is reported to act as both, a transcriptional activator and repressor as Ikaros proteins were detected together with transcriptional activators, like the SWI/SNF complex, as well as with repressors, like the NURD complex (Kirstetter et al. 2002). On the one hand, it was published that Ikaros proteins associate with heterochromatin in nuclei of lymphocytes and recruit chromatin remodeling complexes and histone deacetylases to repress

transcription. (Brown et al. 1997; Kim et al. 1999). On the other hand, Kim J., Sif S. et al. (1999) reported an activating role of the Ikaros protein during transcription.

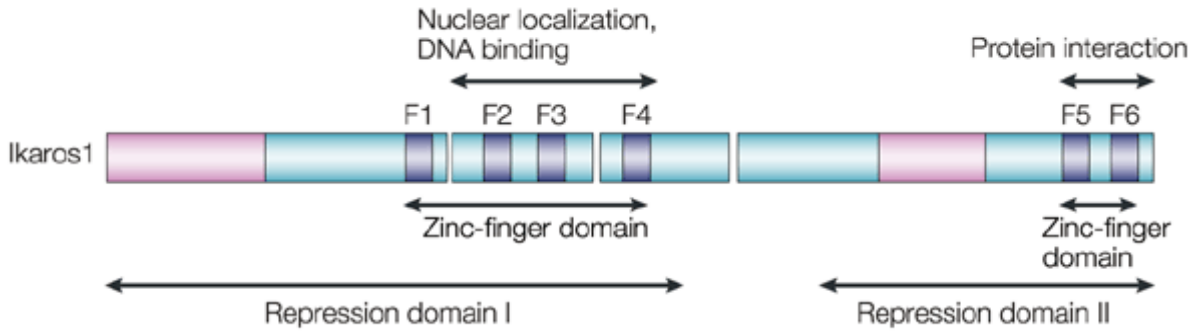


Figure 5. Ikaros protein - isoform 1 with the N-terminal DNA binding domain and the C-terminal dimerization domain (Georgopoulos 2002)

Research of the last twenty years revealed several developmental defects in Ikaros-mutant mice. Experiments comprising the deletion of the last exon of *Ikzf1* in the C-terminal dimerization and transcriptional activation domain resulted in an Ikaros null (*Ikzf1*$^{-/-}$) phenotype. No Ikaros activity was detected in *Ikzf1*$^{-/-}$ mice resulting in severe defects in lymphopoiesis like a very early block in B cell development (Wang et al. 1996; Nichogiannopoulou et al. 1998). Further experiments were carried out deleting the Ikaros DNA binding domain at the N-terminus. Ikaros proteins were stably produced and formed dimers with their dimerization partners. The abolishment of the DNA binding ability in one of the dimerization partners, however, led to an incapacity in DNA binding of the protein dimer, resulting in an Ikaros dominant negative (*Ikzf1*^{DN}) mutant phenotype (Nichogiannopoulou et al. 1998).

Both Ikaros mutations (*Ikzf1*$^{-/-}$ and *Ikzf1*^{DN}) displayed severe impacts on lymphocyte development. *Ikzf1*$^{-/-}$ mutant mice were able to produce lymphocytes, although B cell development was blocked quite early in differentiation and T cells showed a deregulated developmental pattern resulting in T cell malignancies (Nichogiannopoulou et al. 1998). Mice homozygous for the dominant negative Ikaros mutation (*Ikzf1*$^{DN/DN}$) showed a complete block in lymphopoiesis and the generation of all lymphoid lineages was disabled. Furthermore, lymph nodes and mature thymi were absent (Georgopoulos et al. 1994). In a heterozygous *Ikzf1*$^{DN/+}$ mutant, in contrast, a normal development of the lymphoid

compartment was observed at the beginning. In adult mice, a lymphoid hyperplasia was observed due to a lower TCR stimulation threshold, resulting in the appearance of leukaemias and lymphomas (Wang et al. 1996; Nichogiannopoulou et al. 1998; Rebollo et al. 2003).

While Ikaros deletions had negative effects on lymphocyte development, the differentiation of HSCs towards the myelo-erythroid lineage seemed to be even upregulated. Also, the spleen of Ikaros mutant mice was enlarged and populated by an increased number of myeloid and erythroid cells. (Georgopoulos et al. 1994).

Summarizing those findings, researchers ascribe a central role to the transcription factor Ikaros during the whole differentiation pathway of hematopoietic stem cells to mature lymphocytes. Ikaros is essential for the development of B and T cells. In contrast, Ikaros seems to have a repressing effect on the development of other hematopoietic lineages (Georgopoulos et al. 1994).

3.3.1.2 The B cell-restricted transcription factor Aiolos (*Ikzf3*)

Aiolos is an Ikaros-homologue. The four N-terminal zinc-finger domains are responsible for DNA binding while the two C-terminal motifs are necessary for dimerization. Although the two proteins share similarities in those domains, Aiolos is a stronger transcriptional activator than Ikaros (Rebollo et al. 2003). Aiolos seems to play a major role in late B cell development as gene inactivation has severe impacts on B cell differentiation. In contrast to Ikaros, which is essential from the HSC on, Aiolos cannot be detected in early hematopoietic progenitors, emphasizing its role in later B cell maturation. Aiolos expression can first be measured in pre-B cells and in double negative thymocytes but its expression finally peaks in mature B cells (Morgan et al. 1997; Wang et al. 1998).

To study the role of Aiolos during lymphocyte development, Aiolos null mice (*Ikzf3*$^{-/-}$) were investigated. It was found that *Ikzf3*$^{-/-}$ mutant B cells were activated faster and proliferated at lower BCR-signalling levels in comparison to Aiolos expressing B cells. Due to the reduced BCR stimulation threshold, the formation of germinal centers was facilitated and elevated serum IgE and IgG levels were detected. Hyperproliferation of follicular B cells resulted in the development of B cell lymphomas in adult mice. While conventional B2 B cells were increased, the numbers of B1 B cells and MZ B cells were drastically reduced in Aiolos knockout mice. Furthermore, a breakdown in B cell tolerance was observed leading to the production of autoantibodies (Wang et al. 1998; Zon 2001). While the numbers of both,

the pre B cells and germinal center B cells, are reported to be increased in *Ikzf3*$^{-/-}$ mice, the naïve B cell number is decreased. This indicates that the number of activated B cells must be upregulated due to the reduced BCR signaling activation threshold. T cell development seems to be hardly affected from Aiolos knockout while B cells display a hyperreactive phenotype in the absence of Aiolos. (Wang et al. 1998; Zon 2001; Rebollo et al. 2003)

3.3.1.3 The Ikaros-family protein Helios (*Ikzf2*)

Helios is another Ikaros-homologue encoding a zinc finger DNA binding protein. It is upregulated in early hematopoiesis, while its expression levels decrease towards differentiating lymphocytes, erythrocytes and macrophages. Helios expression is low in the bone marrow, high in thymi but not even detectable in further secondary lymphoid organs (Rebollo et al. 2003).

3.4 Aim of the project

In my master thesis, I wanted to investigate the roles of the zinc-finger transcription factors Ikaros and Aiolos during lymphocyte development. The aim of the project was the generation of mice, which conditionally overexpressed or deleted Ikaros and Aiolos, respectively, to analyze and compare their function and significance in murine B lymphocyte development.

3.4.1 Conditional *Ikzf1* overexpression

Ikaros is an important transcription factor during hematopoiesis. It is essential for the differentiation of hematopoietic stem cells to B and T lymphocytes as Ikaros null mutant mice display severe defects in lymphocyte development (Wang et al. 1996). Several papers have been published ascribing a central role to Ikaros in lymphocyte development, as very early blocks in B cell development and the development of T cell malignancies were reported in Ikaros knockout mice (Wang et al. 1996; Nichogiannopoulou et al. 1998).

Experiments, which conditionally deleted *Ikzf1* in different stages of lymphocyte development, revealed a pivotal role of the transcription factor in the proper development of B cells. Arrest of B cell development at the pro-B as well as at the mature B cell stage was detected in conditional *Ikzf1* deletion experiments (unpublished data). Even more intriguingly, conditional *Ikzf1* deletions at early B cell developmental stages disclosed distinct effects of the transcription factor on the development of different B cell subsets (unpublished data). While the development of B2 B cells was blocked upon *Ikzf1* deletion, the generation of B1 B cells was increased. First, I wanted to consolidate these results by the analysis of *Ikzf1* deletion in lymphoid progenitors.

The main project was the generation of an allele allowing the conditional overexpression of the *Ikzf1* protein just upon Cre-mediated deletion of a stop cassette in different stages of B cell development.

This project should give us deeper insight into the importance of Ikaros during the generation of B cells as well as into its diverging impacts on the development of different B cell subsets. Based on previous results, we would predict that conditional Ikaros overexpression should have contrary effects compared to the conditional Ikaros deletion experiments and should promote B2 B cell differentiation at the expense of B1 B cell development in early B lymphopoiesis.

3.4.2 Conditional *Ikzf3* deletion

Aiolos is a lymphocyte-restricted transcription factor having major effects on B cell development, like its protein family member Ikaros. Aiolos is an important regulatory factor during later B cell development. It is expressed from the pre-B cell stage on and its expression peaks in mature B cells. *Ikzf3* knockouts are reported to lead to uncontrolled hyperproliferation and differentiation of mature B cells resulting in the development of B cell lymphomas (Wang et al. 1998). Although Ikaros and Aiolos belong to the same protein family of zinc-finger transcription factors and are both essential for lymphocyte development, they do not seem to perform identical roles. While an *Ikzf1* knockout blocks B cell development and does not allow the B cell to differentiate into an antibody producing plasma cell at al (Wang, Nichogiannopoulou et al. 1996), *Ikzf3* knockouts are reported to promote the differentiation of mature B cells towards the effector state due to a reduced BCR signaling activation threshold (Wang et al. 1996; Wang et al. 1998).

The second project that I worked on, dealt with the generation of a floxed *Ikzf3* allele, allowing conditional *Ikzf3* deletion during lymphocyte development. The main purpose of this project was to produce a comparable model to the established conditional *Ikzf1* deletion model in the lab. This would allow the comparison of the effects of the hematopoietic transcription factors Ikaros and Aiolos on B cell development. Based on results published about *Ikzf3* knockout experiments, conditional *Ikzf3* deletions should result in a phenotype that varies significantly from our conditional *Ikzf1* deletion phenotype. Due to its role in the regulation of B cell activation (Wang et al. 1998), *Ikzf3* deletion in mature B cells is expected to result in B cell hyperactivation.

Taken together, the chosen methods using conditional alleles provide advantageous tools to make more significant statements about the effects and roles of the transcription factors Ikaros and Aiolos in B lymphocyte development, while T cell development stays unaffected. Although Ikaros and Aiolos are reported to have equivalent roles during lymphocyte development, their potential redundant roles can be verified just upon the analysis of the conditional *Ikzf1* and *Ikzf3* mice.

4. Results

4.1 *Ikzf1* deletion in early stages of lymphocyte development has diverging effects on B2 and B1 B cell development

To confirm the differential role of Ikaros on B1 and B2 B cell development, we bred conditional *Ikzf1*$^{fl/-}$ with *Rag1-Cre* mice, in which gene deletion is initiated in common lymphoid progenitors.

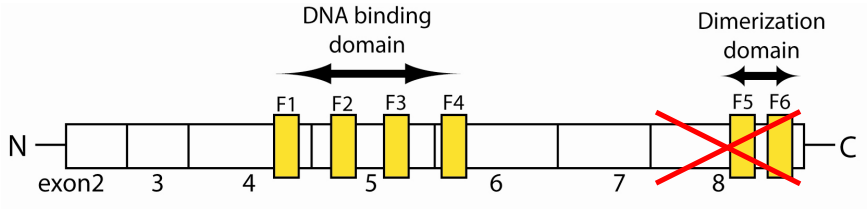


Figure 6. Conditional *Ikzf1* deletion. For the generation of the conditional *Ikzf1* allele, the last Exon of *Ikzf1* was flanked by *loxP* sites to be deleted upon the activity of the Cre recombinase. This allowed the conditional elimination of the dimerization domain leading to the translation of a nonfunctional Ikaros protein.

Bone marrow cells of experimental *Ikzf1*$^{fl/-}$ *Rag1-Cre* mice and control *Ikzf1*$^{+/+}$ *Rag1-Cre* mice were isolated and analyzed by flow cytometry.

While the development of myeloid cells was unaffected, *Ikzf1* gene ablation initiated before B cell commitment resulted in a drastic reduction of total B cell numbers with an almost complete loss of pre-B cells and all following developmental stages in the bone marrow of *Ikzf1*$^{fl/-}$ *Rag1-Cre* mice compared to *Ikzf1*$^{+/+}$ *Rag1-Cre* littermates. Conventional B2 B cell numbers were diminished, whereas an increase in B1 B cell numbers was identified in the bone marrow of experimental mice compared to control mice (Figures 7 and 8).

In summary, while B2 B cell development was impaired, the generation of B1 B cells was promoted when Ikaros was absent before B cell commitment. Therefore, I could confirm the differential effects of Ikaros on B1 versus B2 B cell development.

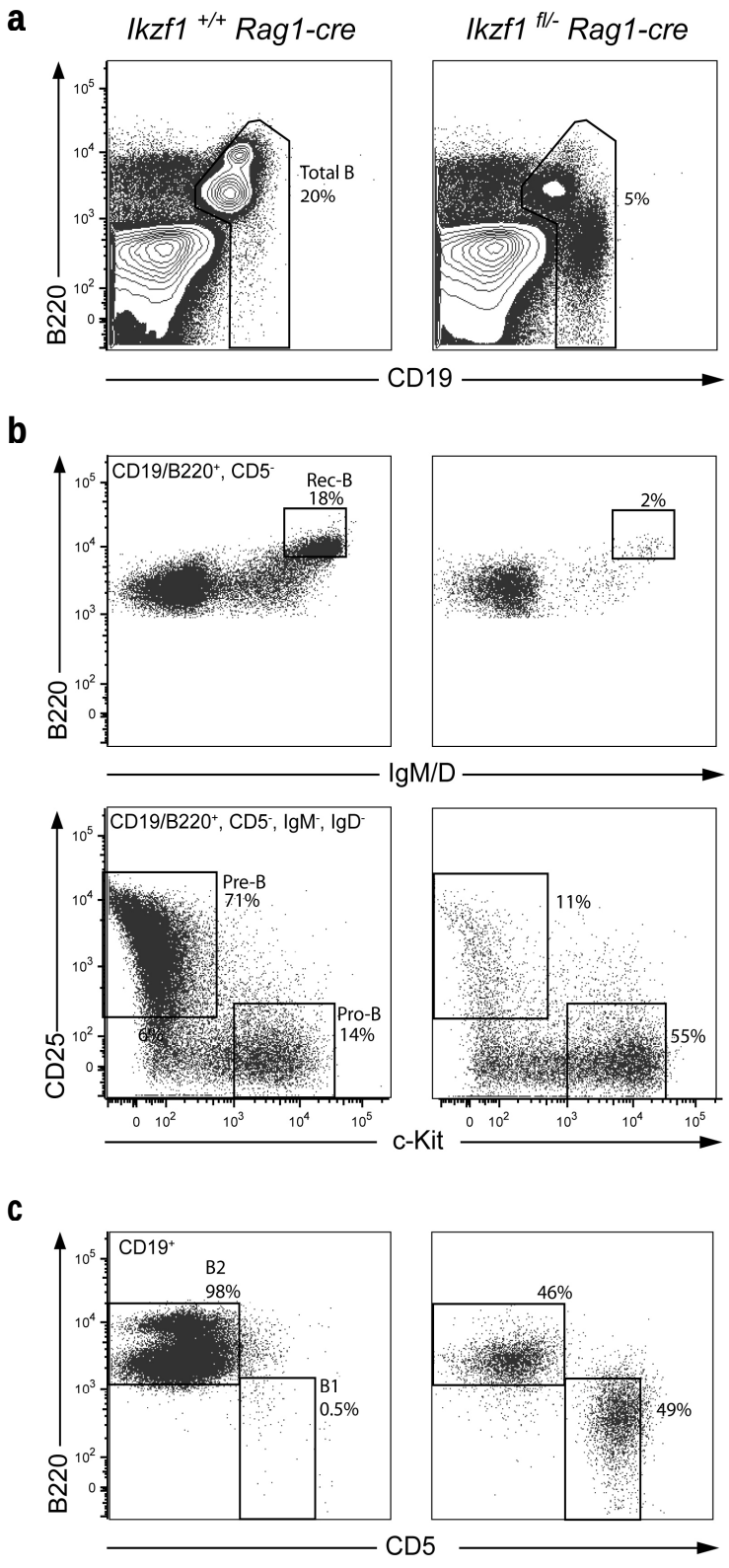


Figure 7. FACS analysis of bone marrow cells of a *Ikzf1*$^{fl/-}$ *Rag1-Cre* mouse and a control mouse (FACS plots). **(a)** In the experimental mouse (*Ikzf1*$^{fl/-}$ *Rag1-Cre*, right, a four-fold decrease in total B cells is observable compared to the control mouse (*Ikzf1*$^{+/+}$ *Rag1-Cre*, left). **(b)** The number of recirculating B cells as well as of pre-B cells is drastically diminished in the *Ikzf1*-deleted mouse, while pro-B cell numbers are higher than in the control mouse. **(c)** While B2 B cell numbers decline, B1 B cell numbers increase highly.

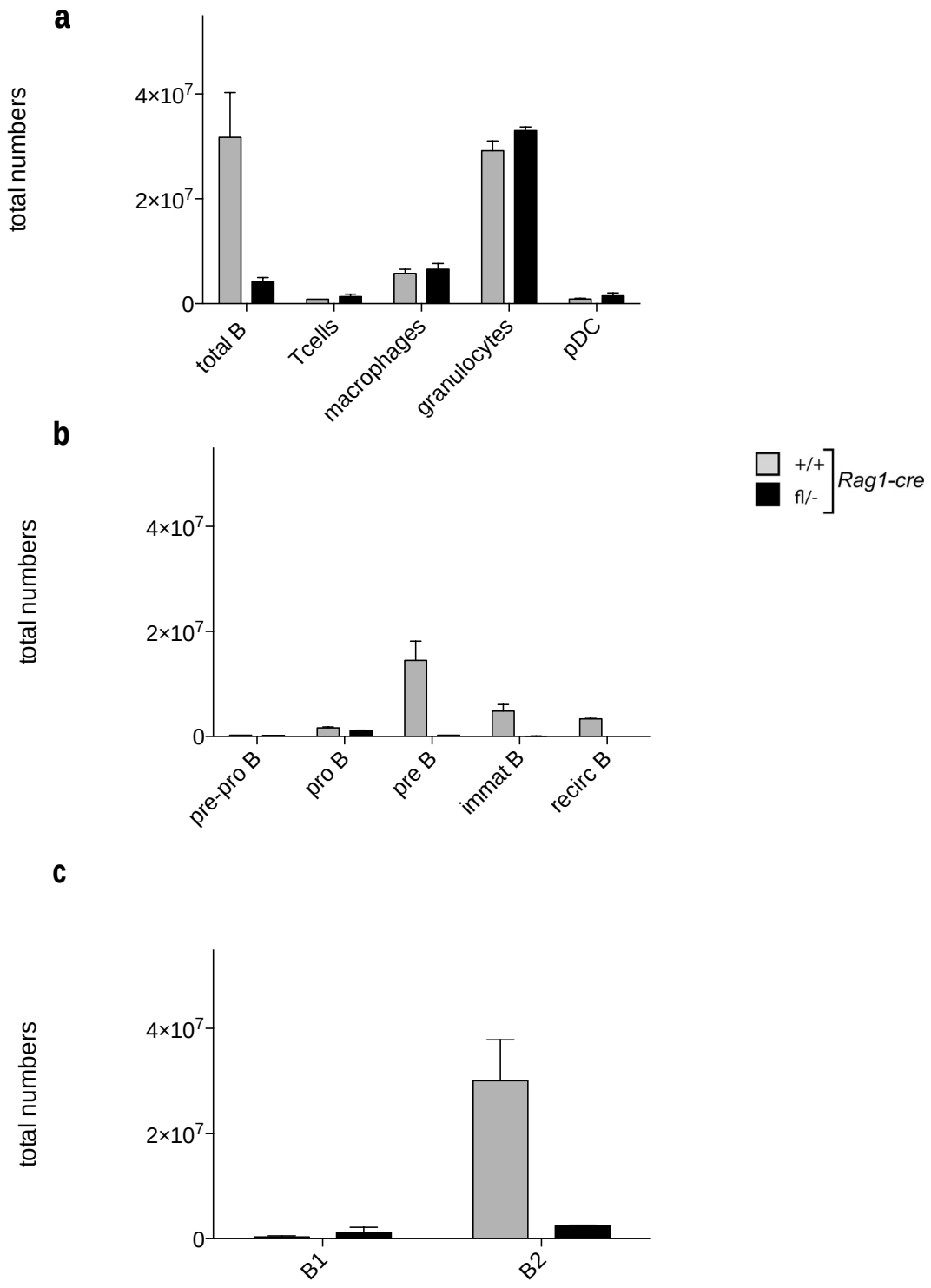


Figure 8. FACS analysis of bone marrow cells of two *Ikzf1*$^{fl/-}$ *Rag1-Cre* mice and two control mice (statistics). **(a)** While T cells, macrophages, granulocytes, and dendritic cells (pDC) do not change significantly upon early *Ikzf1* deletion, the total number of B cells is drastically reduced in experimental mice (fl/-) compared to control mice (+/+); **(b)** While pre-pro-B and pro-B cell numbers do not show significant changes in comparison to the control numbers, experimental mice display a drastic decrease in pre-B, immature B and mature B cell numbers (10-2 fold) upon early Ikaros deletion; **(c)** B2 B cells are reduced in *Ikzf1*$^{fl/-}$ *Rag1-Cre* mice, whereas B1 B cells are increased.

To further investigate the role of Ikaros and to gain new insights into the requirements for B1 versus B2 B cell development, I generated a model to study the conditional overexpression of *Ikzf1* before and during B cell commitment.

4.2 Generation of a conditional *Ikzf1* overexpression allele

4.2.1 Construction of the targeting vector for *Ikzf1* overexpression

For the generation of the conditional *Ikzf1* overexpression allele, the first step was the construction of a targeting vector containing the *Ikzf1* cDNA. For that purpose, the *Ikzf1* cDNA including a Gly-FLAG-V5-TEV-biotin tag (*Ikzf1-bio*) was cloned into the *CAG-stop-GFP* vector. The *Ikzf1-bio* sequence was cloned downstream of the cytomegalovirus early enhancer element and the chicken beta-actin promoter (CAG) followed by a stop cassette and downstream, the IRES-GFP reporter. The neomycin resistance gene (neoR), regulated by the thymidine kinase promoter (TK), served as stop cassette to prevent the ectopic expression of Ikaros protein in the mouse. The neoR cassette was flanked by *loxP* sites to allow conditional *Ikzf1* expression in mice only upon Cre-mediated deletion of the stop cassette (Figure 9).

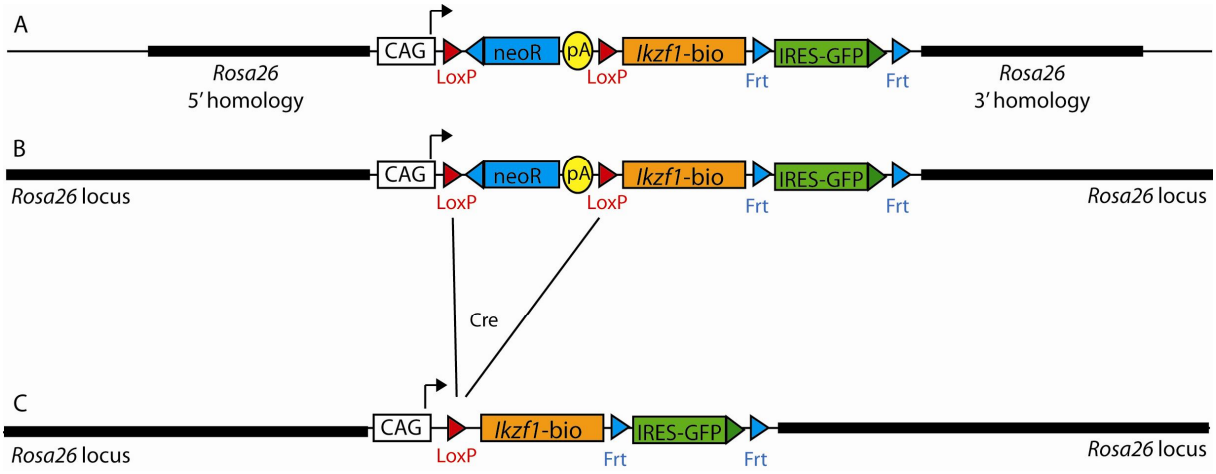


Figure 9. Strategy for conditional *Ikzf1* overexpression. (A) Targeting vector construct including *Rosa26* homology arms, CAG promoter, floxed neoR stop cassette, *Ikzf1-bio*, and an IRES-GFP reporter flanked by *frt* sites; (B) Targeting *CAG-stop-Ikzf1-bio-GFP* into the *Rosa26* locus of A9 ES cells through homologous recombination, (C) Cre-mediated deletion of the neoR-stop cassette to achieve *Ikzf1* overexpression.

First, the *Ikzf1-bio* sequence was isolated from a donor plasmid and cloned into the polylinker sequence of the pSP64 plasmid containing appropriate restriction sites. To introduce the sequence of interest into the *CAG-stop-GFP* vector, *AscI*-digested fragments of the *Ikzf1-bio* element as well as of the acceptor vector were gel-purified and ligated. *DH5α* competent *E. coli* cells were transformed with the targeting vector. For bacterial selection the *CAG-stop-Ikzf1-bio-GFP* targeting plasmid contained an ampicillin resistance gene.

Plasmids were isolated from the bacterial cells and an *AscI* restriction digest revealed that 10 out of 30 analyzed plasmids (33.3%) were the desired *CAG-stop-Ikzf1-bio-GFP* targeting plasmid (Figure 11 A).

The *AscI* restriction digest-positive plasmids were further reviewed for correct direction of the *Ikzf1-bio* fragment insertion. To this end, a forward primer in the *Ikzf1* cDNA (5'-AGTGGCGAGCAGCTGAAG-3' (12059)) was used for Sanger sequencing (Figure 10).

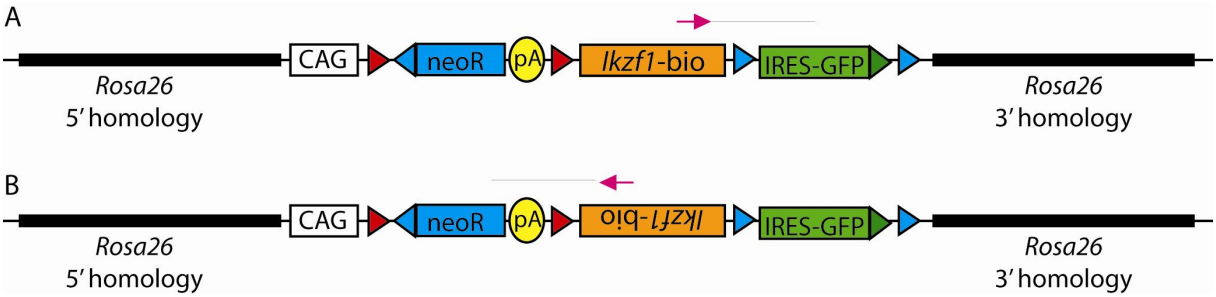


Figure 10. Checking for correct direction of insertion of the *Ikzf1-bio* sequence into the *CAG-stop-GFP* vector by Sanger sequencing. The correct direction of insertion (A) led to a sequencing result within the *Ikzf1-bio* and the IRES-GFP sequences, while an incorrect direction of insertion (B) resulted in the sequencing of the *Ikzf1-bio* and the neoR genes. The pink arrows indicate the primer used for sequencing.

Three of 10 sequenced plasmids showed a correctly inserted *Ikzf1-bio* fragment. Finally, a last control digest with the restriction enzymes *MluI* and *SmaI* disproved a double insertion of the *Ikzf1-bio* fragment into the targeting vector cassette in 2 out of 10 plasmids (Figure 11B).

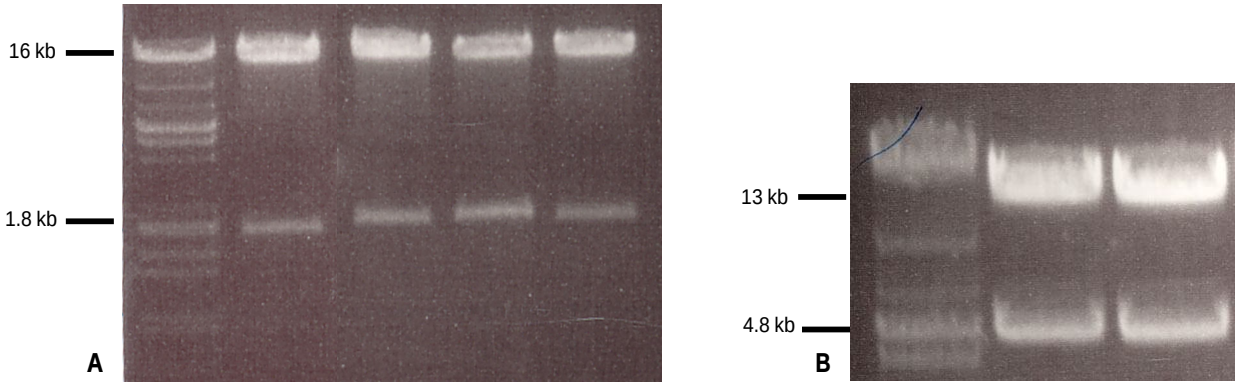


Figure 11. Control digests of the *CAG-stop-Ikzf1-bio-GFP* targeting vector. (A) Control digest with *AscI* yielded the 1.8 kb *Ikzf1-bio* and the 16 kb *CAG-stop-GFP* backbone fragment. (B) Control digests with *MluI* and *SmaI* yielded fragments of 4.8 and 13 kb size, rebutting a double insertion of *Ikzf1-bio* (λ marker used as DNA ladder)

4.2.2 Targeting *CAG-stop-Ikzf1-bio-GFP* into the *Rosa26* locus of A9 mouse embryonic stem cells

After the successful construction of the *CAG-stop-Ikzf1-bio-GFP* plasmid, A9 mouse embryonic stem cells (ES cells) were transfected with the *AgeI*-linearized and phenol-chloroform cleaned targeting vector. A9 ES cells are hybrid ES cells carrying a 129/Sv and a C57BL/6J allele. The aim of the transfection was the homologous integration of the *CAG-stop-Ikzf1-bio-GFP* targeting sequence into the *Rosa26* locus of the ES cells. The transfected ES cells were grown on γ-irradiated primary mouse embryonic fibroblasts (pMEFs) in selection medium containing *G418*. The antibiotic selected for cells, which had integrated the targeting sequence, including the neo gene conferring resistance to *G418*.

4.2.3 Screening of *Rosa26* targeted ES cells by nested PCR and Southern Blot analysis

Ninety-six ES cell clones were analyzed by nested PCR. For the nested PCR the Kapa2G robust DNA polymerase was used in the supplemented GC buffer.

The forward primers (12219/12221) annealed at an endogenous sequence of the *Rosa26* locus while the reverse primers (12220/12222) (Table 1) bound to a sequence specific for the introduced DNA sequence. This primer combination yielded in an 1196 bp PCR product only in ES cell clones, where homologous recombination of the targeting sequence into the *Rosa26* gene locus had taken place. Out of 96 picked ES cell clones, 11 showed to carry the *Rosa26*^{Ikzf1} allele (Figure 13).

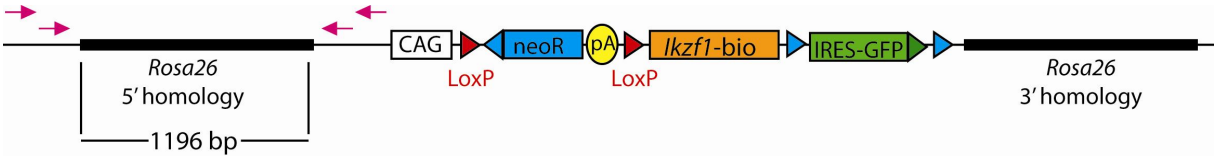


Figure 12. PCR screening strategy for *Rosa26* targeted ES cells. The pink arrows indicate the primers used for the nested PCR, generating an 1196 bp PCR product in positively targeted ES cells.

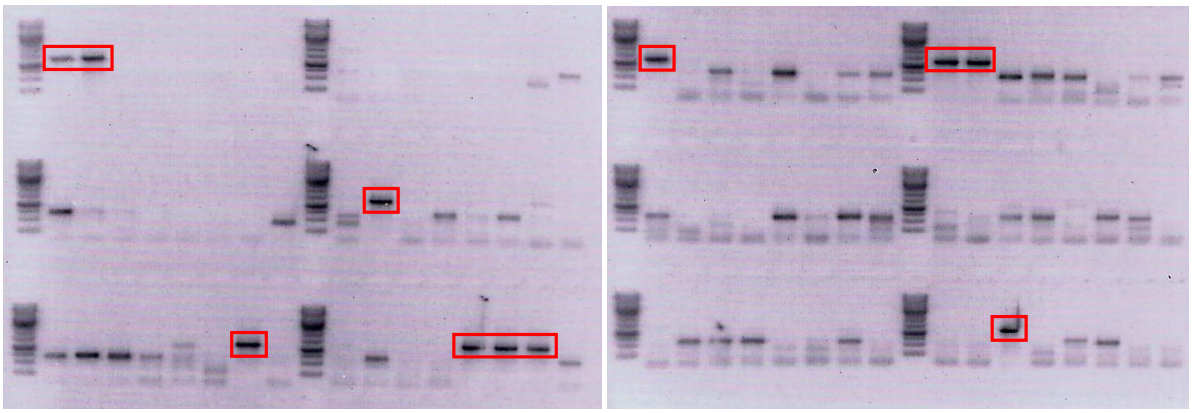


Figure 13. **Nested PCR of *Rosa26* targeted ES cell clones.** PCR screen of 96 ES cell clones revealed 11 positively targeted clones (marked by red boxes) carrying the *Rosa26*^{Ikzf1} allele, characterized by a PCR product size of 1196 bp

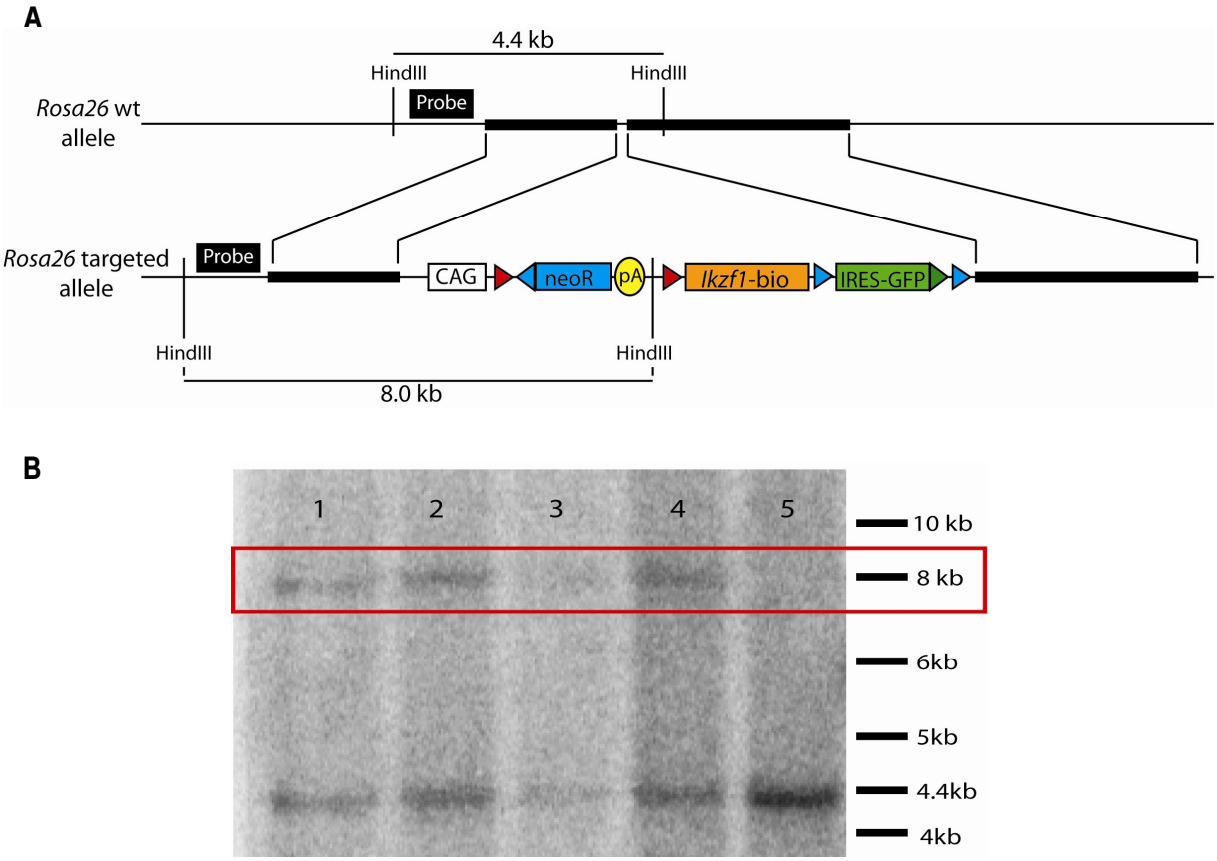


Figure 14. Southern blot strategy and results after *Rosa26* targeting. (**A**) The radioactively labeled probe marks a 4.4 kb *HindIII*-fragment in the wild-type *Rosa26* allele and an 8.0 kb *HindIII*-fragment in the targeted *Rosa26*^{Ikzf1} allele; (**B**) Southern blot analysis revealed 4 positively targeted ES cell clones (1,2,3,4) characterized by labeled DNA bands of 8.0 kb and 4.4 kb size.

Five of 11 PCR-positive clones were expanded for 3 passages, several aliquots were frozen and one was used for Southern blot analysis. ES cell DNA was digested with the restriction enzyme *HindIII* and loaded onto a 0.9% agarose gel to separate the genomic DNA fragments. After gel preparation, the DNA was blotted onto a nylon membrane. After overnight blotting, the membrane was hybridized with an α^{32}P-ATP radioactively labeled DNA probe. The probe corresponded to a DNA sequence of the endogenous *Rosa26* locus, which is located 650 bp upstream of the introduced *CAG-stop-Ikzf1-bio-GFP* sequence. The probe hybridized with a DNA fragment with a size of 4.4 kb in the wild-type *Rosa26* allele, while it hybridized with an 8.0 kb fragment in the targeted *Rosa26^{Ikzf1}* allele. After overnight incubation of the hybridized membrane with a phosphorimager plate, the labeled DNA bands were visualized by analysis with the phosphorimager (Personal Molecular Imager^{TM}, Biorad). The Southern blot analysis revealed 4 positively targeted ES cell clones, which had integrated the *CAG-stop-Ikzf1-bio-GFP* sequence into the *Rosa26* genomic locus (Figure 14).

4.2.4. Generation of chimeric mice carrying the *Rosa26^{Ikzf1}* allele

Clones 1, 2 and 4 (Figure 14 B) were chosen for blastocyste injection. All clones gave rise to chimeric mice. Judging from the fur color, one male with 70% and two males with 35% chimerism were obtained. The rest of the litter displayed lower chimerism (< 15%). The three highest chimeric mice were used for breeding with C57BL/6J females.

The litters of the breeding were genotyped for the presence of the targeted *Rosa26^{Ikzf1}* allele. For this purpose the primer pair 10629/8325 was used to yield a 210 bp fragment in the wild-type *Rosa26* allele and the primer pair 8642/8325 to generate a 520 bp PCR product in the targeted allele. If germline transmission of the *Rosa26^{Ikzf1}* allele had taken place this should lead to the generation of bands of both sizes (Table 1, Figure 15).

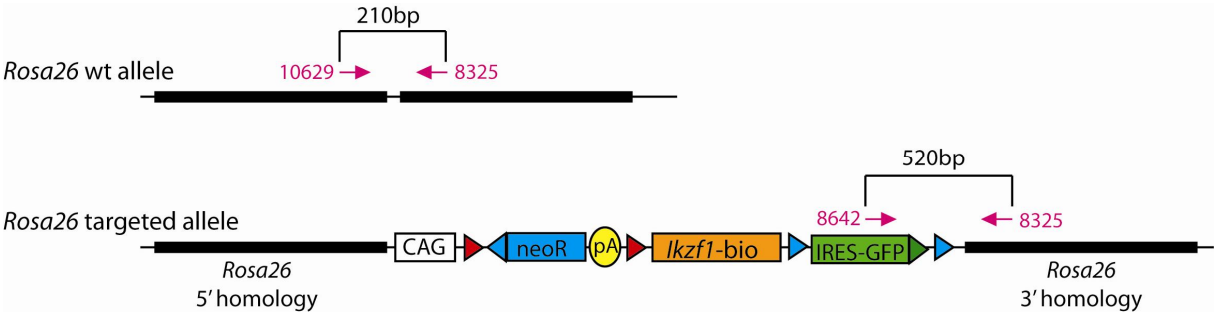


Figure 15. Genotyping the *Rosa26* gene locus. From the wild-type allele a 210bp product was PCR-amplified, while from the targeted *Rosa26*^{Ikzf1} allele a 520bp product was obtained.

Unfortunately, so far genotyping of 15 litters with approximately 7 pups each showed no germline transmission of the targeted *Rosa26*^{Ikzf1} allele (Figure 16).

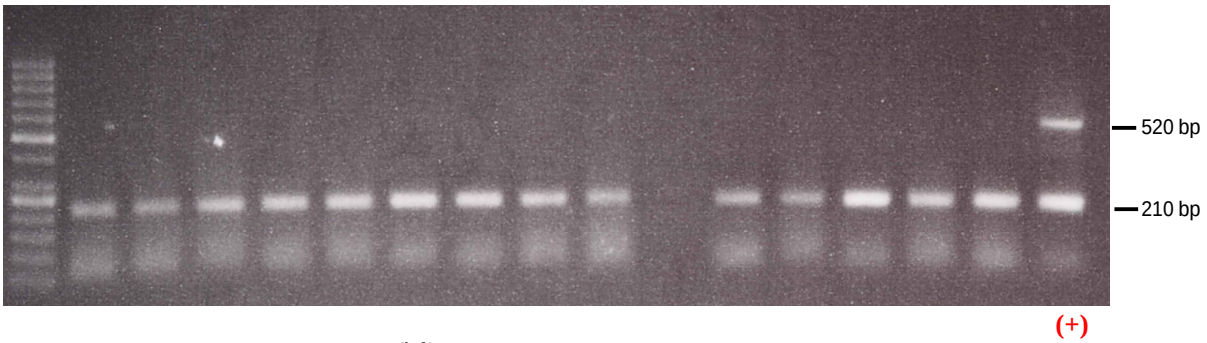


Figure 16. Genotyping of *Rosa26*^{Ikzf1} chimera offspring revealed that no germline transmission of the Rosa26^{Ikzf1} allele had taken place. The outer right band represents a positive control (+) yielding in a 520bp PCR product for the targeted allele as well as a 210bp product for the wild-type *Rosa26* allele. In contrast, all the genotyped pups only showed a band with 210bp of size, meaning that they do not carry the desired *Rosa26*^{Ikzf1} allele (50bp ladder used as reference).

To pursue our goal of generating mice for conditional *Ikzf1* overexpression, clones 1 and 2 were reinjected and clone 3 was newly injected into mouse blastocystes for the generation of *Rosa26*^{Ikzf1} chimeric mice.

4.3 Generation of a conditional *Ikzf3* allele

For the generation of the conditional *Ikzf3* allele, I first constructed a targeting vector containing the last *Ikzf3* exon (exon 8) flanked by two *loxP* sites, a GFP reporter element, and a neomycin resistance cassette (neoR). Homology arms of the *Ikzf3* locus flanked the whole sequence of interest. Subsequent ES cell targeting led to the integration of the modified *Ikzf3* exon into the endogenous locus of A9 ES cells. After the generation of chimeric mice carrying the floxed *Ikzf3* allele, the expression of the Cre recombinase can ablate *Ikzf3* function in knockin mice and therefore allow for the analysis of Aiolos action during B cell development (Figure 17).

4.3.1 Construction of the targeting vectors for the conditional deletion of *Ikzf3* function

Two targeting vectors for conditional *Ikzf3* deletion were generated, one of them expressing an IRES-GFP reporter (*Ikzf3^{fl}-IRES-GFP*), the second one resulting in the generation of a GFP-fusion protein (*Ikzf3^{fl}-fusion-GFP*) upon Cre-mediated deletion of *Ikzf3* exon 8.

Two vectors were constructed to test two different strategies of reporter gene expression. The *Ikzf3^{fl}-fusion-GFP* vector contained a 3' splice site-GFP exon which will be spliced to the 5' splice site of the *Ikzf3* exon 7 after the Cre-mediated deletion of exon 8, resulting in the expression of a GFP-fusion protein. In contrast, the *Ikzf3^{fl}-IRES-GFP* vector contained an internal ribosome entry site (IRES) upstream of the GFP sequence, resulting in the translation of two separate proteins from one mRNA, the non-functional Aiolos protein lacking exon 8 on the one hand, and the GFP reporter on the other hand.

The central part of the targeting constructs contained *Ikzf3* exon 8 including 6X poly-A sites flanked by *loxP* sites, the GFP reporter element and a neomycin resistance cassette (neoR) regulated by the phosphoglycerate kinase 1 (PGK) promoter for selection of positively targeted ES cells. The 6X poly-A sites will ensure the proper expression of the complete *Ikzf3* gene as long as *Ikzf3* exon 8 is not deleted. Besides, they prevent transcription and splicing of the reporter gene. The *Ikzf3^{fl}* targeting vectors contained furthermore an ampicillin resistance gene to select for transformed bacterial cells during cloning and the *diphtheria toxin A* gene (DT-A), whose product is toxic for ES cells. During ES cell targeting, random integration of the targeting sequence leads to the co-integration of the *diphtheria toxin A* gene and the subsequent gene expression and production of the toxin kills randomly

targeted ES cells. Only in ES cells, where homologous recombination of the floxed *Ikzf3* sequence into the *Ikzf3* locus takes place, no diphtheria toxin A is produced, selecting for positively targeted ES cells. (Figure 17).

Using recombineering technology, homology arms of the *Ikzf3* gene locus were cloned into a building vector to flank the sequence of interest. For this purpose, I first PCR-amplified homology boxes flanking the *Ikzf3* long arm, *Ikzf3* exon 8, *Ikzf3* short arm and *Ikzf3* control arm regions from the *Ikzf3* BAC RP23 441N6. I cloned the boxes into the pSP65 vector to be able to subsequently retrieve the homology arms from the BAC RP23 441N6 by homologous recombination (see section 6.2.5). From the pSP65 plasmid, I could then isolate the homology arms and exon 8 of the *Ikzf3* gene by restriction digests. The *Ikzf3* long arm sequence and *Ikzf3* exon 8 were then cloned into a transition vector (pSP65) containing a *loxP* site. Finally, *Ikzf3* long arm and exon 8, separated by the *loxP* site, were cloned upstream of the GFP reporter element and the neomycin resistance cassette. The *Ikzf3* short arm was introduced into the targeting plasmid downstream of the neomycin resistance gene to complete the targeting vector construction.

The *Ikzf3* control arm sequence overlapped with the short arm sequence but was about 800 bp longer. It was cloned into a targeting vector instead of the short arm sequence to generate a control vector. The purpose was to generate a positive control for ES cell PCR screens. In contrast to the short arm, the control arm region contained the sequences for primer annealing for nested PCR. As a consequence, nested PCR of ES cell clones transfected with the control vector should always lead to a positive PCR signal while transfection with the targeting vector only leads to a signal in ES cell clones where homologous recombination took place. In this way, the control vector served as positive control for PCR-screens of positively targeted ES cells.

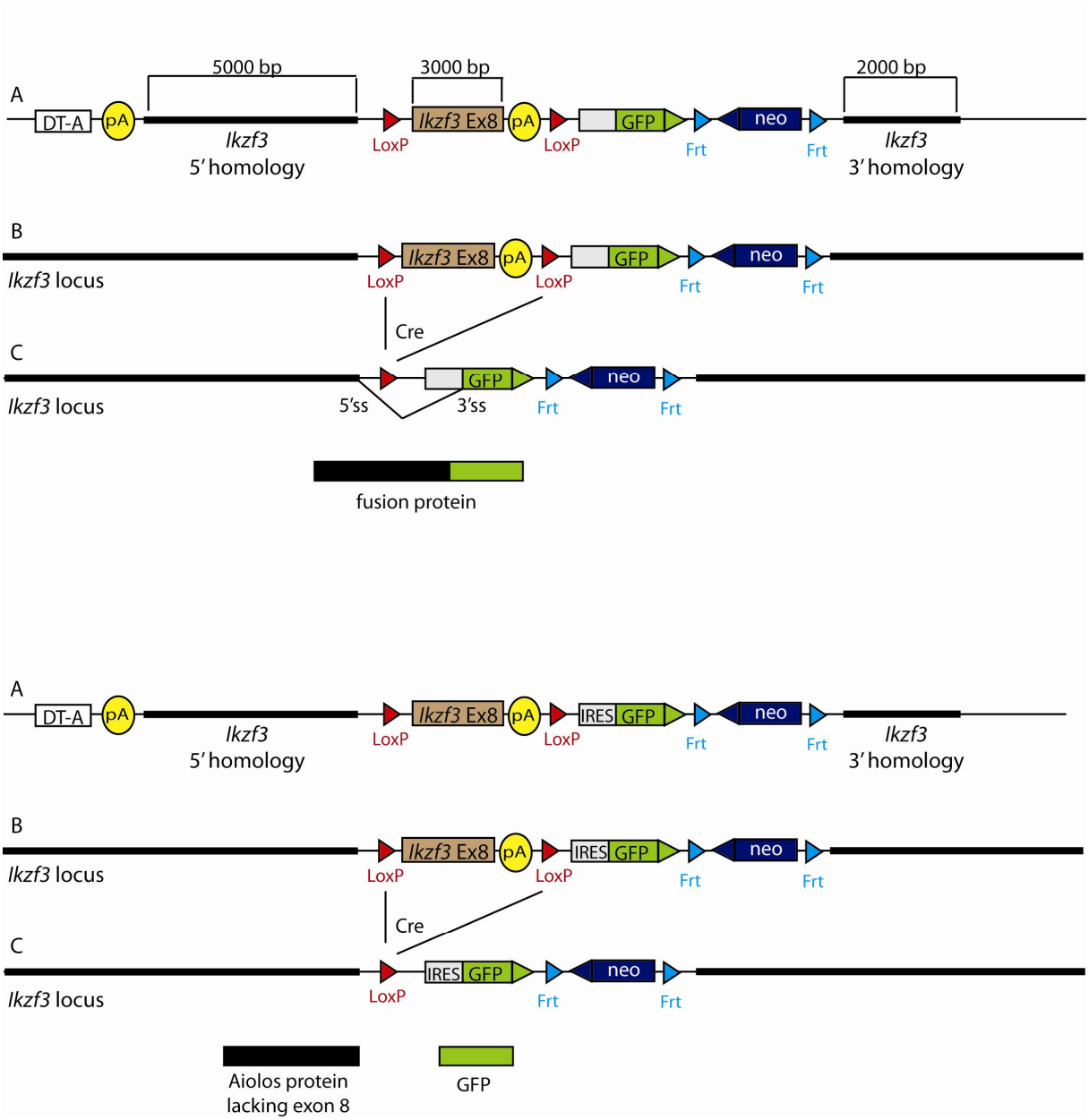


Figure 17. Strategy for conditional Aiolos deletion in knockin mice. (A) Targeting vector constructs containing *Ikzf3* homology arms, floxed *Ikzf3* exon 8, the *GFP* reporter element, a *neomycin* resistance cassette (*neoR*) and the *diphtheria toxin A* (*DT-A*) selection gene; (B) Targeting the *Ikzf3*^{fl}*-GFP-neo* sequence into the endogenous *Ikzf3* locus of A9 embryonic stem cells by homologous recombination, (C) Cre-mediated deletion of *Ikzf3* exon 8 leading to the expression of a non-functional Aiolos protein In knockin mice.

During cloning, I had to switch from competent *DH5α E. coli* cells to competent *Stbl3 E. coli* cells to be able to generate the 20 kb sized *Ikzf3*^{fl} targeting vector. The transformed *Stbl3* cells were grown at 25°C for 24h. Only from these cells, I was able to isolate the correct targeting vector. In contrast, plasmids isolated from transformed *DH5α* cells grown at 37°C or 30°C lacked different parts of the targeting plasmid in all samples.

Finally, restriction enzyme digests and Sanger Sequencing confirmed the correctness of the *Ikzf3*^{fl} targeting vectors (Figure 19).

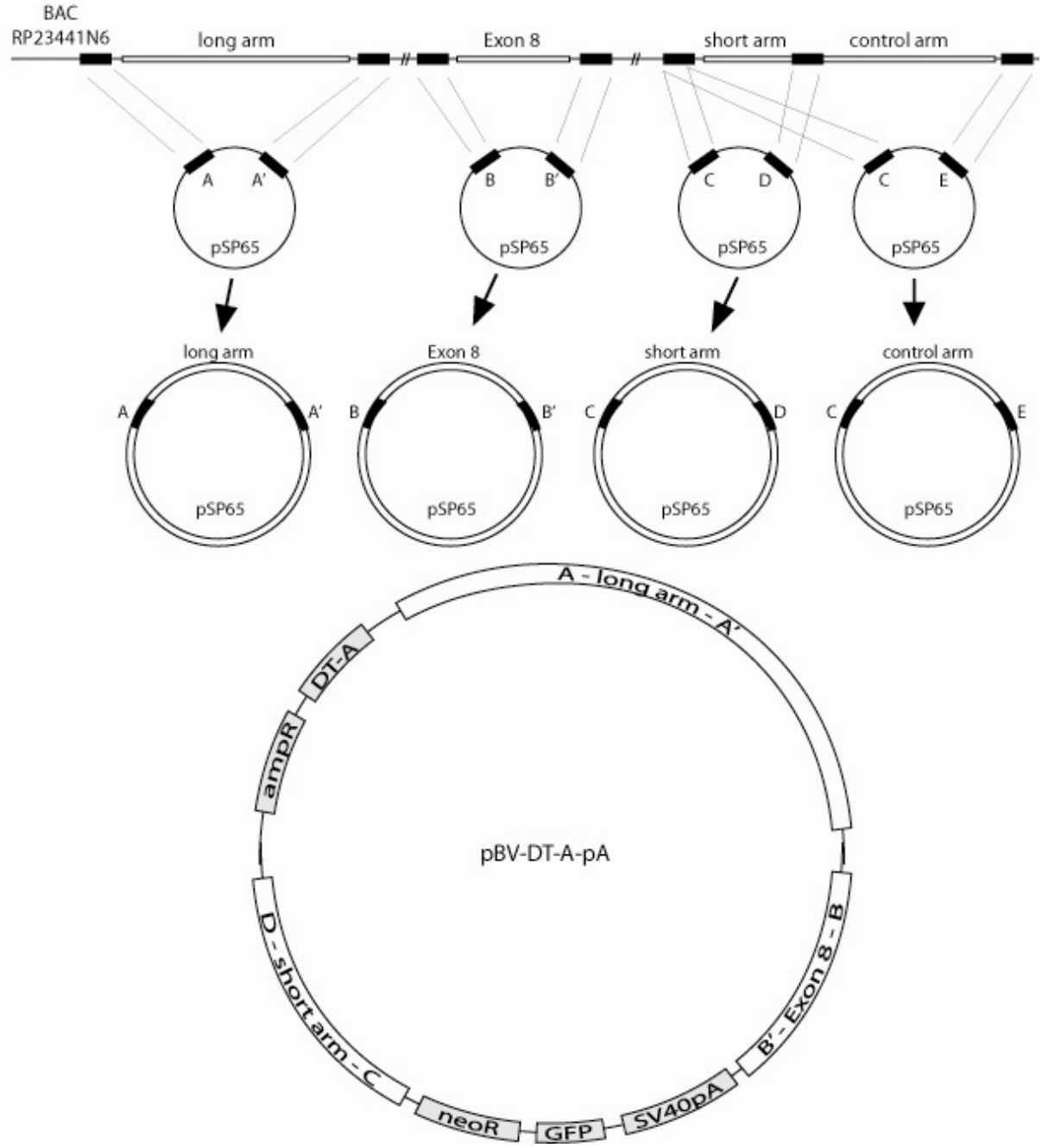
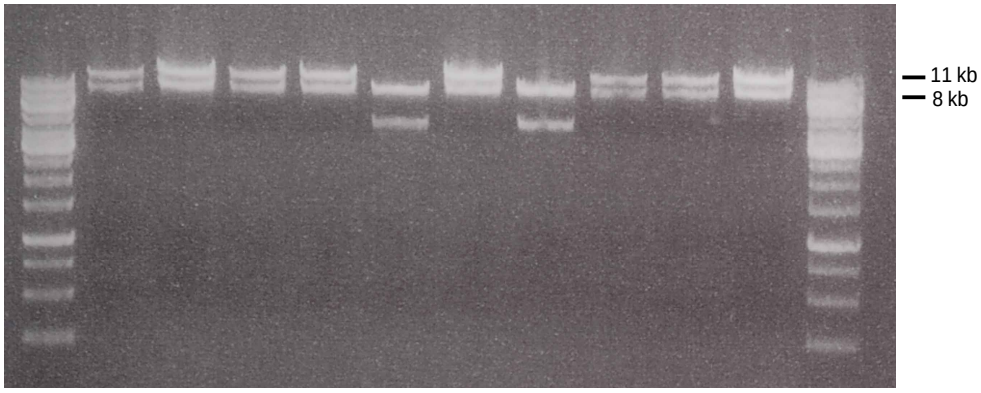
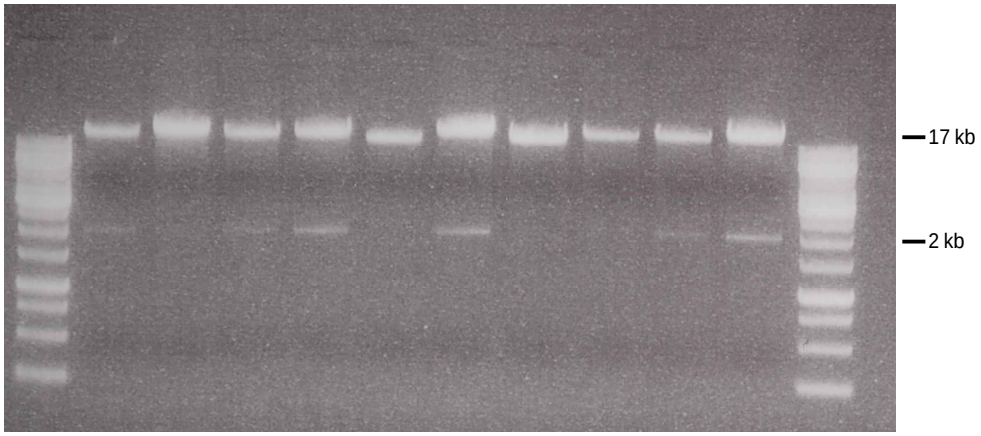


Figure 18. Isolation of *Ikzf3* homology arms and *Ikzf3* exon 8 by BAC recombineering. Homology boxes A, A', B, B', C, D and E were PCR amplified from the BAC RP23 441N6 and cloned into the pSP65 vector. Using recombineering technology, the *Ikzf3* long arm, exon 8, short arm and control arm were retrieved from the BAC into the pSP65 transition vector. Finally, the *Ikzf3* homology arms and *Ikzf3* exon 8 could be cloned into the *pBV-DT-A-pA* targeting vector, containing the *diphtheria toxin A* (*DT-A*) gene, a SV40 polyA stop element, the GFP reporter, a neomycin resistance cassette as well as an ampicillin resistance for bacterial selection.



(**A**) *AscI-NotI* digest



(**B**) *XmaI-BsiWI* digest

Figure 19. Control restriction digests of *Ikzf3^{fl}-IRES-GFP* targeting plasmids. (**A**) *AscI-NotI* digests revealed that 8 out 10 analyzed plasmids were the desired *Ikzf3^{fl}*-IRES-GFP targeting vectors, characterized by band sizes of 11kb and 8kb. (**B**) The *XmaI-BsiWI* restriction digests resulted in DNA fragments with sizes of 17 kb and 2 kb in positive *Ikzf3^{fl}-IRES-GFP* plasmids (1kb DNA ladder as reference).

4.3.2 Targeting the *Ikzf3^{fl}* vector into the endogenous *Ikzf3* gene locus of A9 embryonic stem cells

A9 mouse embryonic stem cells were transfected with 20 µg of the *SgrAI* - linearized and phenol-chloroform extracted targeting plasmid. Correct targeting should lead to the integration of the sequence of interest into the endogenous *Ikzf3* locus of the ES cells. The transfected ES cells were grown on γ-irradiated feeder cells in medium containing *G418* for the selection of positively targeted ES cells. The *DT-A* gene served as a second selection criterium. Nonhomologous integration of the targeting sequence including the *DT-A* gene led to the production of the diphtheria toxin and killed wrongly targeted ES cells.

4.3.3. Nested PCR screen of *Ikzf3* targeted ES cell clones

Around 700 ES cell clones were analyzed by nested PCR. Forward primers (10512/10513) and reverse primers (12570/12724) (Table 1) annealed in the *PGK* promoter and in an endogenous region of the *Ikzf3* gene locus, respectively (Figure 20).

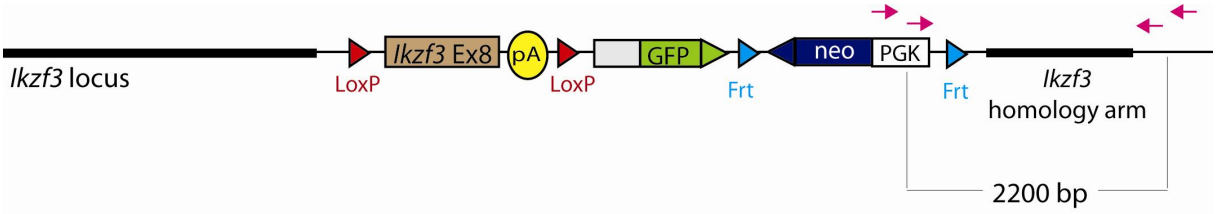


Figure 20. Nested PCR screening strategy of *Ikzf3* targeted ES cells. The nested PCR yielded a 2200 bp fragment, the pink arrows indicate the used primers.

The nested PCR yielded a PCR fragment of 2200 bp of size in correctly targeted ES cell clones. Out of 700 picked ES cell clones, 6 had integrated the *Ikzf3^{fl}-fusion-GFP-neo* and 17 the *Ikzf3^{fl}-IRES-GFP-neo* sequence correctly in the *Ikzf3* gene locus (Figures 21 and 22).

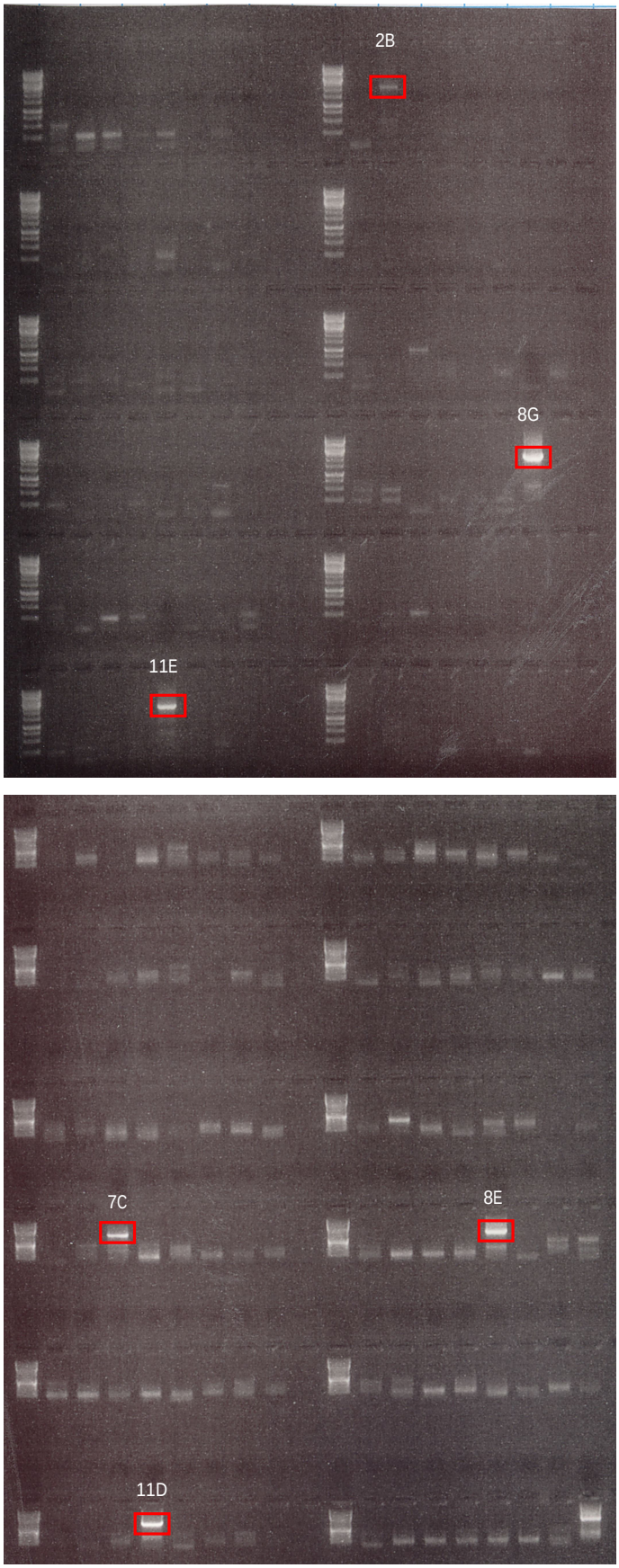


Figure 21. Nested PCR screen of targeted ES cell clones (fusion-GFP). The PCR screen revealed 6 positively targeted ES cell clones for the *Ikzf3*^{fl}*-fusion-GFP-neo* sequence characterized by a PCR product of 2200 bp; (+) indicates the positive control, (1 kb ladder as reference)

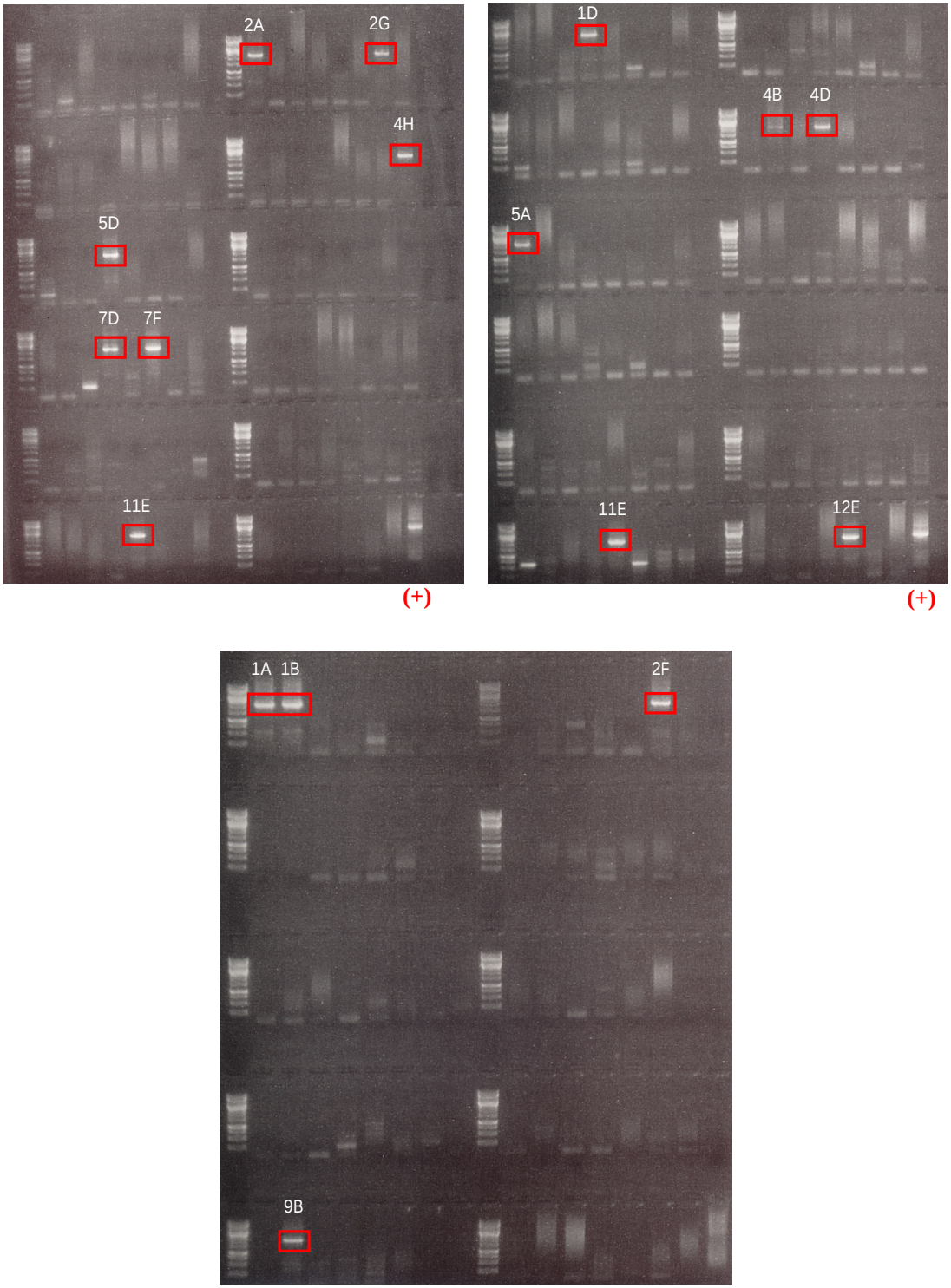


Figure 22. Nested PCR screen of targeted ES cell clones (IRES-GFP). The PCR screen revealed 17 positively targeted clones (indicated by the red boxes) for the *Ikzf3*^{fl}*-IRES-GFP-neo* sequence characterized by a PCR product of 2200 bp; the red (+) indicates positive controls, (1 kb ladder as reference)

Of 23 positively targeted ES cell clones, a screen for the presence of the upstream *loxP* site was conducted, carrying out a PCR with the primer pair 12768/12769 (Table 1). The forward primer annealed before the first *loxP* site within the *Ikzf3* long arm region, while the reverse primer annealed after the first *loxP* site in the last exon of *Ikzf3 (*Figure 23). The PCR yielded a PCR fragment of 270 bp of size in ES cell clones containing the *loxP* site, but only a 210 bp fragment, where the *loxP* site got lost due to homologous recombination within the *Ikzf3* exon 8 region (Figure 24).

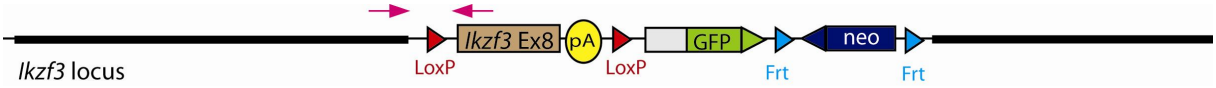


Figure 23. Screening for the presence of the upstream *loxP* site after ES cell targeting. The pink arrows indicate the primers used for the PCR screen.

Screening for the presence of the *loxP* site was essential as a lack of the upstream *loxP* site would not allow the Cre-mediated deletion of *Ikzf3* exon 8, invalidating the whole purpose of the experiment. Homologous recombination can take place in each homology sequence. As not only the *Ikzf3* long arm but also the exon 8 region shows sequence homology with the endogenous *Ikzf3* locus of the ES cells, homologous recombination can occur within both regions. While homologous recombination within the *Ikzf3* long arm region results in the integration of the upstream *loxP* site, recombination within the *Ikzf3* exon 8 region does not, but, in contrast, leads to the loss of this *loxP* site.

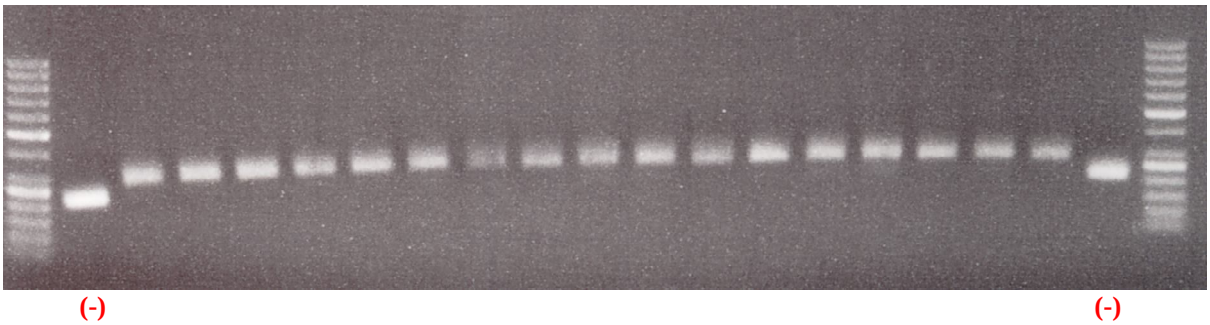


Figure 24. Screen for *loxP* site upstream of the *Ikzf3* Exon 8. Negative controls (-) yielded a fragment size of 210 bp while *loxP*–positive clones yielded a 270 bp fragment (50bp ladder as reference)

Surprisingly, all screened ES cell clones contained the *loxP* site upstream of *Ikzf3* exon 8. This means, that homologous recombination took place in all the positively targeted ES cell

clones within the long arm region and never within *Ikzf3* exon 8. A reasonable explanation for this phenomenon could be that the long arm is 2000 bp longer than the exon 8 region, providing a better opportunity for homologous recombination to take place.

4.3.4 Expansion of PCR-positive ES cell clones

After the PCR-check I expanded five nicely growing ES cell clones. These clones were expanded for 3 passages from the 96-well plates to 6cm plates. On plates without feeder cells, the ES cell DNA was enriched to be further isolated by phenol-chlorophorm extraction and ethanol precipitation.

The purified DNA of the ES cell clones was used to repeat a PCR screen using one of the primer pairs that I used for the nested PCR screen before (10512/12570 or 10513/12724) (Table 1).

The PCR screen revealed 3 positively targeted ES cell clones out of 5 (Figure 25). Experience of colleagues implies that these PCR-positive clones will also prove to be positive in Southern blot analysis.

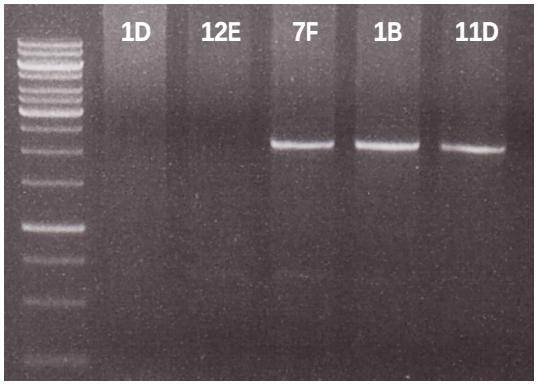


Figure 25. PCR screen after the isolation of ES cell DNA. Bands at 2.2kb prove correct *Ikzf3* targeting in the *Ikzf3^{fl}-IRES-GFP* clones 7F and 1B and the *Ikzf3^{fl}-GFP* clone 11D.

For time reasons, a more accurate analysis of the ES cell clones could not be conducted.

However, these 3 ES cell clones are further to be analyzed by Southern Blot analysis to confirm the correct targeting events. The DNA of the 5 clones is to be *AvrII*-digested, separated on a gel, blotted onto a nylon membrane and finally hybridized with a α^{32}P-ATP radioactively labeled probe DNA (see section 6.3.7).

To this end, I PCR-amplified and gel-eluted a DNA fragment of the 3' end of the *Ikzf3* gene locus from the BAC RP23 441N6, which will serve as probe DNA for Southern blot hybridization. The DNA probe will hybridize with a 9.2 kb DNA fragment in the wild-type

allele and with 13.8 kb (*Ikzf3*^{fl}*-fusion-GFP*) or 7.8 kb (*Ikzf3*^{fl}*-IRES-GFP*) fragments in the targeted alleles (Figure 26).

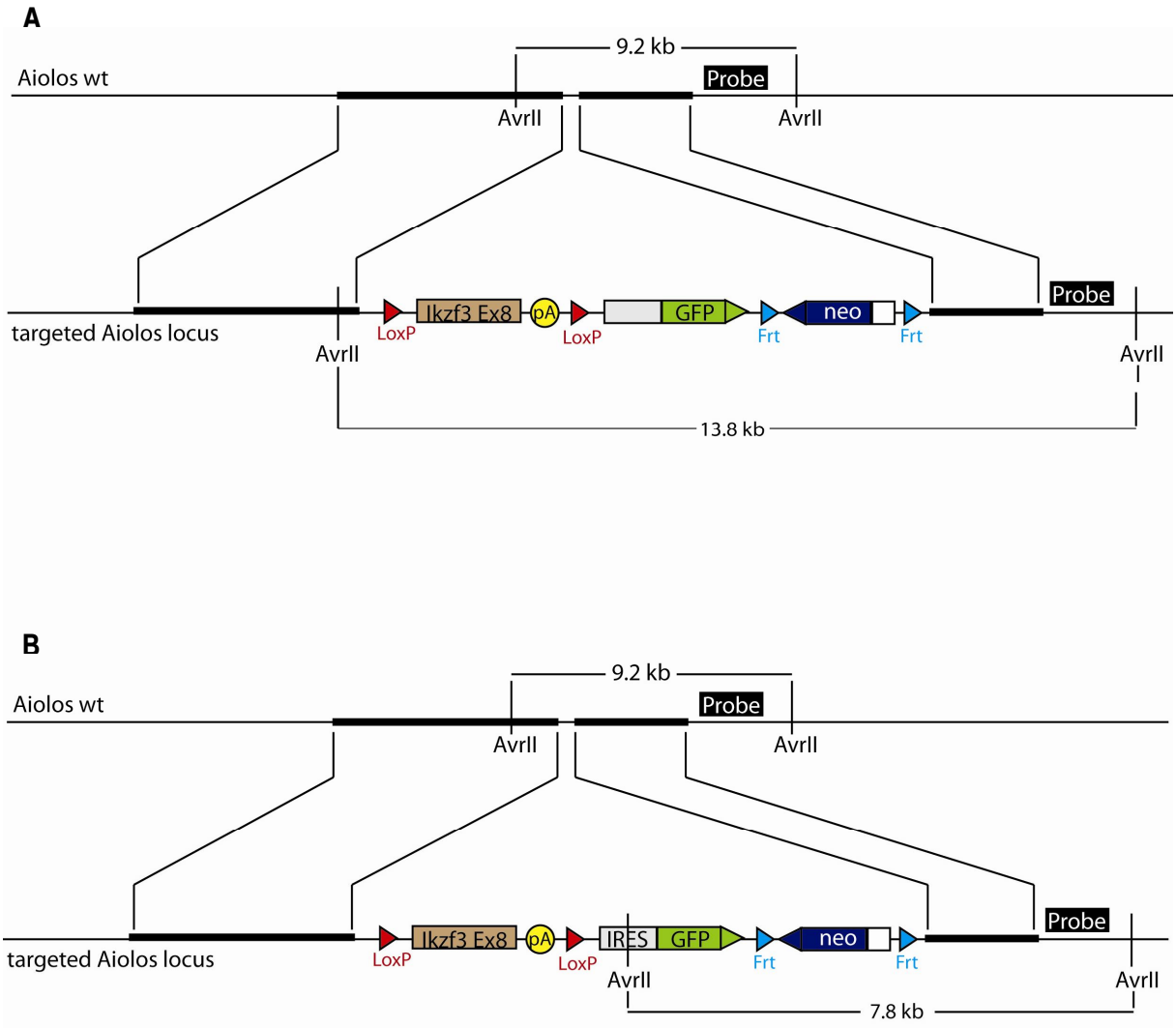


Figure 26. Southern blot Strategy for the detection of *Ikzf3* positively targeted ES cells. The α^{32}P-ATP radioactively labeled probe will hybridize with a 9.2 kb DNA fragment in the wild-type *Ikzf3* allele and with a 7.8kb (*Ikzf3*^{fl}*-IRES-GFP*) (B) or a 13.8kb (*Ikzf3*^{fl}*-fusion-GFP*) (A) fragment in the targeted alleles.

PCR- and Southern blot-positive ES cell clones will then be injected into blastocystes to generate chimeric mice carrying the *Ikzf3*^{fl} allele.

5. Discussion and Outlook

During my master thesis, I worked on two projects, both focusing on the roles and importance of Ikaros family zinc-finger transcription factors during B cell development. For this purpose, I constructed two conditional alleles based on the Cre-*lox* recombination system.

The first conditional allele should allow the overexpression of the Ikaros protein in mice by the introduction of the *Ikzf1* cDNA into the ubiquitously expressed *Rosa26* locus and its regulation by a strong promoter.

The second allele was constructed enabling the conditional deletion of the last exon of *Ikzf3*, thus, resulting in the expression of a non-functional Aiolos protein.

5.1 Conditional *Ikzf1* overexpression

Breeding *Rosa26*^{Ikzf1} chimeric mice with C57BL/6J mice did not generate pups carrying the targeted allele. Blastocyste injections of targeted A9 ES cells resulted in the generation of chimerice mice, displaying 70%, 35% or lower chimerism, judging from the fur colour. We expected to get germline transmission using the 70% male for breeding with C57BL/6 females. The 35% males were also used for breeding, although the chance to get germline transmission here was very low, judging from the experience of colleagues. Unfortunately, no germline-transmission took place in any of the breeding.

My last step, before I finished my master work, was to do another injection of *Rosa26*^{Ikzf1} targeted ES cells into blastocystes of C57BL/6J mice to increase the probability of germline transmission.

In the future, the chimeric mice could be bred with C57BL/6J mice carrying an allele which expresses the Cre-recombinase under the control of different gene promoters. If germline transmission takes place, resulting pups will overexpress the Ikaros protein. For this purpose *Rag-1*-Cre, *Cd79a*-Cre, as well as *Cd23*-Cre lines could be used for breeding to induce *Ikzf1*-overexpression in early lymphocyte development, in early B cell developmental stages and in mature B cells, respectively.

What is expected from these experiments is to get deeper insight into the essential role of the transcription factor Ikaros in different stages of B cell development. In contrast to conditional *Ikzf1* deletion experiments, where upon *Ikzf1* deletion no further B cell

development occurred, *Ikzf1*-overexpressing mice are expected to still produce B cells and even boost B cell production. The expected changes in B cell development will be the object of analysis.

Another focus lies on the comparison of the impact of *Ikzf1* expression on B1 and B2 B cell development. In *Ikzf1* deletion experiments, an increase in B1 and a loss of B2 B cell development was observed when *Ikzf1* was deleted already during early lymphocyte development (*Rag1-Cre*). In contrast, when deleted later in early B cells (*Cd79a-Cre*) the block in B2 B cell development was still observed but there was no significant change in B1 B cell numbers compared to wild-type mice (data not shown). These results suggest that B1 and B2 B cell developmental pathways separate in progenitor cell stages before B cell commitment. Based on this hypothesis, *Ikzf1* overexpression experiments could allow us to make statements about the diverging development of B1 and B2 B cells. If these two B cell subtypes developed early on separately from each other, *Ikzf1* overexpression in the progenitor state (*Rag1-Cre*) would be supposed to have repressing effects on B1 B cell development but promote B2 B cell generation. In contrast, overexpression in B cell committed stages (*Cd79a-Cre*) would not effect B1 B cell development because its developmental pathway already had split up earlier from the conventional B cell pathway that is affected by Ikaros.

Furthermore, one could do a rescue experiment by breeding *Ikzf1*$^{+/-}$ *Vav-Cre* mice with the *Rosa26*$^{Ikzf1/+}$ chimeras. In these mice the Cre recombinase will be expressed in the whole hematopoietic system. F2 breeding will lead to the generation of a small percentage of *Rosa26*$^{Ikzf1/+}$ *Ikzf1*$^{-/-}$ *Vav-Cre* mice. From previous experiments, we know that *Ikzf1*$^{-/-}$ mice are not viable due to the loss of *Ikzf1* function. Introducing the *Rosa26*^{Ikzf1} allele into these mice could give insight into the functionality of the generated overexpression allele. Viability of *Rosa26*$^{Ikzf/+1}$ *Ikzf1*$^{-/-}$ *Vav-Cre* mice will already be an important hint, if the ectopically overexpressed Ikaros protein is able to rescue the phenotype. The result of this experiment would not only make a statement about the functionality of the bio-tagged Ikaros protein, but it would also provide information about the protein level necessary to rescue the null phenotype.

To make more concrete statements about the protein amount produced from the ectopically expressed *Ikzf1* gene, it will be important to do a Western blot to contrast the Ikaros protein level generated in wild-type mice and in *Rosa26*$^{Ikzf1/+}$ *Ikzf1*$^{-/-}$ *Vav-Cre* mice.

5.2 Conditional *Ikzf3* deletion

Ikaros and Aiolos are often reported to have redundant function as transcription factors during lymphocyte development. To test this hypothesis, I designed an allele allowing the conditional deletion of *Ikzf3* at different stages of lymphocyte development. Thus, I generated a complementary model to the *Ikzf1* deletion model established in our lab.
Previous experiments targeting *Ikzf1* deletion in early B cells (*Cd79a-Cre*) led to a block of B cell development at the pro-B cell stage. More intriguingly, it was found that *Ikzf3*, which is usually expressed from the pre-B cell stage on, was already upregulated in pro-B cells when *Ikzf1* was deleted (manuscript under preparation).

This raises the question whether the upregulation of *Ikzf3* rescues pro-B cells because it is able to take over the functions of *Ikzf1* or if this earlier *Ikzf3* expression has no impact on early B cell development at all.

To answer this question, I generated a conditional *Ikzf3* allele (*Ikzf3*^{fl}). The *Ikzf1*^{fl} and the *Ikzf3*^{fl} mouse models will allow the simultaneous deletion of both transcription factors upon breeding with C57BL/6J Cre lines. Only this approach will allow us to make statements about potential redundant roles of Ikaros and Aiolos in lymphocyte development.

To that end, it will be essential to achieve a double deletion of *Ikzf1* and *Ikzf3* in early B cells generating *Ikzf1*^{fl} *Ikzf3*^{fl} *Cd79a-Cre* mice. Taken together with the results of conditional *Ikzf1* deletion, if the double deletion leads to the loss of pro-B cells, we can conclude that *Ikzf3* is able to partially rescue *Ikzf1* function during early B cell development and maintain the generation of pro-B cells. That would prove that Ikaros and Aiolos can perform redundant roles.

On the other hand, if the *Ikzf1*^{fl} *Ikzf3*^{fl} *Cd79a-Cre* mice show equal phenotypes to the *Ikzf1*^{fl} *Cd79-Cre* mice, we could conclude that Aiolos has no effect on the generation of the early B cells and that Ikaros and Aiolos do not have redundant function at this early B cell developmental stage.

Ikzf3 knockout mice were reported to develop B cell leukemias and lymphomas due to lower BCR-signaling thresholds resulting in the hyperactivation of B cells. Conventional B cell numbers were highly increased, whereas the numbers of B1 B cells and MZ B cells were reduced (Wang et al. 1998).

To check whether the *Ikzf3* knockout really had only effects on B cells, as reported from Wang et al. (1998), or if the observed phenotype was due to an impact on T cells, two different approaches can be applied. Deletion of *Ikzf3* only in T cells (*Ikzf3*$^{fl/-}$ *Cd4-Cre* mice) will allow testing a potential effect of *Ikzf3* deficiency on those cells. Furthermore, the generation of *Ikzf3*$^{fl/-}$ *Cd23-Cre* and *Ikzf3*$^{fl/-}$ *Cd79a-Cre* mice will enable the investigation of the effect of *Ikzf3* deficiency only in B cells. Therefrom, statements about the role of Aiolos on B cell development only can be made, excluding its effects on other lymphoid cells.

Finally, one could compare the phenotypes of *Ikzf3* deficiency and *Ikzf1* deficiency or overexpression in different stages of B cell development. The results will allow making statements about the roles of Ikaros and Aiolos during lymphocyte development, their potential redundant roles and their diverging effects on different B cell subsets. Furthermore, the divergent developmental pathways of different B cell subtypes can be studied, using the conditional *Ikzf1* and *Ikzf3* models.

6. Material and Methods

6.1 Material

6.1.1 Standard Buffers and Solutions

The following standard buffers, solutions and culture media were provided from the IMP/IMBA media kitchen:

0.5 M Ethylenediaminetetraacetate (EDTA), pH 8

3 M Sodium acetate (NaAc), pH 5.2

5 M Sodium chloride (NaCl)

1 M sodium phosphate buffer (NaPi), pH 7.2

1x Phosphate buffered saline (PBS)

20x saline-sodium citrate buffer (SSC)

10x Tris-acetate-EDTA (TAE)

1x Tris-EDTA (TE)

1 M Tris-HCl, pH 8

Sterile MonoQ water (H_2O)

LB growth medium

LB agar plates

SOB medium

Dulbecco's Modified Eagle's Medium (DMEM)

Other commonly used reagents were obtained from different companies:

Ampicillin (50mg/mL stock in H_2O; Amresco)

Chloramphenicol (12.5mg/mL stock in EtOH; Fluka)

Ethanol absolute (EtOH) (Merck)

Glycerol (AppliChem)

2-propanol (Isopropanol) (Sigma)

Chloroform (Fluka)

Phenol-chloroform-isoamylalcohol (Sigma)

Penicillin-Streptomycin solution (10000U/mL penicillin, 10mg/mL streptomycin; Sigma)

L-Glutamine Solution (200mM, Sigma)

Fetal calf serum (FCS) (PAA, Lot 10810-2125)

β-Mercapto-Ethanol (Merck)

MEM non essential aminoacid solution (Sigma)

Sodiumpyruvat solution (100mM; Sigma)

Leukemia inhibitory factor (ESGRO LIF) (106U/mL; Millipore)

Sodium Dodecyl Sulfate (SDS) (Serva)

Albumin-Fraction V (BSA) (Applichem)

Agarose (Sigma)

Ethidiumbromide solution (10mg/mL, Bio-rad)

Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich)

6.1.2 Media for embryonic stem cell culture

pMEF medium (culture medium for primary mouse embryonic fibroblasts):

DMEM supplemented with

10% FCS

1% Penicillin-Streptomycin solution

1% L-Glutamine Solution

0.1% β-Mercapto-Ethanol

ES cell medium (culture medium for A9 embryonic stem cells):

DMEM supplemented with

15% FCS

1% Penicillin-Streptomycin solution

1% L-Glutamine Solution

0.1% β-Mercapto-Ethanol

1% MEM non essential aminoacid solution

1% Sodiumpyruvat solution

0.1% ESGRO LIF

6.1.3 Solutions for DNA preparation

Tail Buffer: 100mM Tris pH 8

200mM NaCl

5mM EDTA

0.1% SDS

6.1.4 Solutions for Southern Blot Analysis

Denaturation solution:	20mM NaOH
	0.6M NaCl
Neutralization solution:	40% Tris pH 7.5
	1.5M NaCl
Church solution:	1% BSA
	1mM EDTA
	0.5M NaPi pH 7.2
	7% SDS
Washing Solution:	1% SDS
	40mM NaPi pH 7.2

6.1.5 Commonly used Kits

The following Kits were used according to the manufacturers' protocols.

Kit	*Purpose*
QIAquick Gel Extraction Kit (Qiagen)	purification of DNA fragments out of an agarose gel
QIAprep Spin Miniprep Kit (Qiagen)	plasmid purification out of competent bacterial cells
Qiagen Plasmid Maxi Kit (Qiagen)	purification of larger amounts of plasmid DNA out of bacterial cells
Prime It II – Random Primer Labeling Kit (Agilent Technologies)	probe labeling for Southern Blot hybridization
Illustra ProbeQuant G-50 Micro Columns (GE Healthcare)	Nucleotide removal kit for the probe purification for Southern Blot hybridization

6.2 Standard Molecular Techniques

6.2.1 Vector construction

The main purpose of cloning was the ligation of different DNA fragments to generate a target vector. A DNA fragment was isolated from one vector and introduced into another one. For this purpose, the donor as well as the acceptor vector was digested with the same restriction enzymes. The acceptor vector was afterwards dephosphorylated by the addition of 1µL of Alkaline Phosphatase (1U/µL, Roche) and incubation for 30 minutes at 37°C. The restriction digests were then loaded onto an agarose gel to separate the DNA fragments (see 6.2.7). The target bands of the vector and the insert were cut out of the gel and the DNA was extracted using the *QIAquick Gel Extraction Kit*. Gel eluted DNA fragments were then ligated using 1µL of *T4 DNA ligase* (Thermo Scientific) in the supplemented 10x buffer in a ligation mix of 10µL in total for 1h at room temperature. The ligation mix contained a 5-fold molar excess of insert DNA compared to the dephosphorylated vector DNA. The ligation mix was used to transform competent bacterial cells.

6.2.1.1. Construction of the *CAG-stop-Ikzf1-bio-GFP* targeting vector

To generate the conditional *Ikzf1* overexpression allele, the *Ikzf1-bio* DNA fragment was isolated from the *MSCV-bio-iPuro-Ikzf1* plasmid (plasmid database) using the restriction enzymes *XhoI* and *EcoRI* (Roche). Separation of the digested DNA on a 1% agarose gel enabled the subsequent gel-purification of the *Ikzf1-bio* fragment with a size of 1700bp. The *Ikzf1-bio* fragment was then inserted into the polylinker sequence of the pSP64 plasmid containing appropriate restriction sites. The last cloning step was the ligation of the *AscI* - *Ikzf1-bio* fragment into the *AscI* (NEB) digested *CAG-stop-GFP* targeting vector. Competent bacteria used for all transformation steps were the *DH5α E. coli* cells. Control digests with *AscI* (NEB) or *MluI* and *SmaI* (Roche) were conducted to reveal positive plasmids.

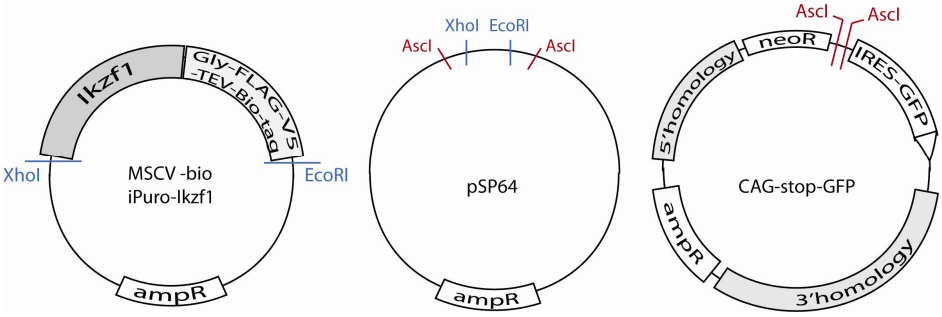


Figure 27. Plasmids used for the construction of the *CAG-stop-Ikzf1-GFP* targeting vector

6.2.1.2 Construction of the *Ikzf3^{fl}* targeting vector

For the construction of the *Ikzf3^{fl}* vector, the *pBV-DT-A-pA* plasmid served as backbone. First, the *IRES-GFP* sequence was isolated from the *pK11-IRES-GFP* plasmid (*ClaI*, *MluI* (Roche)) to be ligated into the *pBV-DT-A-pA* plasmid.

Homology boxes of the *Ikzf3* gene locus were amplified using PCR and cloned into the pSP65 vector (see figure 18). The *pSP65* vector was digested between the two homology boxes (*HindIII*, *XhoI* (Roche)) and homology arms were inserted between the homology boxes using recombineering technology (see 6.2.5). Finally, the *Ikzf3* short arm (*XmaI*, *BsiWI* (NEB)) and subsequently the long arm (*AscI* (NEB), *NotI* (Roche)) were cloned into the *pBV-DT-A-pA-GFP-neo* backbone to obtain the *Ikzf3^{fl}* targeting vector. Those final cloning steps were carried out in *Stbl3* competent *E.coli* cells. To test for the correctness of the plasmid, control digests (*XmaI* and *BsiWI*; *NotI* and *AscI*) as well as Sanger Sequencing were conducted.

6.2.2 Bacterial culture

Transformation steps were conducted using competent *DH5α* or *Stbl3 Escherichia coli* (*E.coli*) cells provided by IMP/IMBA service facilities. BAC DNA was propagated in *DH10B E.coli* cells and recombineering was conducted using competent *EL350 E.coli* cells. The cells were grown in LB medium and on LB agar plates with or without selection medium. For selection ampicillin (50µg/mL) or chloramphenicol (12.5µg/mL) were used.

Transformed *DH5α* cells were grown at 37°C overnight while *Stbl3* cells transformed with more unstable plasmids were grown at 25°C for 24 hours on selective LB agar plates. For the propagation of transformed bacterial colonies, I inoculated them into 2mL selective LB medium and let them grow in the same conditions as described above at 180rpm in a bacterial shaker for subsequent plasmid purification.

6.2.3. Transformation of competent bacteria

400µL of competent bacterial cells were thawed on ice. The ligation mix or the plasmid was added and the cells were incubated on ice for 30 minutes. After 1 minute heat-shock at 42°C, cells were incubated on ice again for 10 minutes. For the regeneration of the cells, 500mL SOB was added and the cells were incubated for 20 minutes in the bacterial shaker. Finally, the transformed cells were plated on selective LB agar plates and incubated at 37°C, 30°C, or 25°C until colonies were visible.

6.2.4 BAC DNA preparation

For the isolation of BAC DNA out of *DH10B E.coli* cells, I first inoculated an overnight culture in 2mL LB medium containing 25µg/mL chloramphenicol and let it grow at 37°C in a bacterial shaker. The next day, the bacterial cells were pelleted (3000rpm, 10min). The supernatant was discarded and the pellets were resuspended in 300µl P1 buffer (Qiagen) and mixed with 300µL P2 buffer (Qiagen) to lyse the cells. The cells were incubated for 5 minutes at room temperature before 300µL of buffer P3 (Qiagen) were added for neutralization. After 10 minutes incubation on ice, the lysate was centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was mixed with 800µL ice-cold isopropanol and incubated on ice for a few minutes. To precipitate the DNA the sample was centrifuged at 4000 rpm for 15 minutes at 4°C. The DNA pellet was then washed with 70% ethanol (4000 rpm, 5 min, 4°C). After the removal of ethanol, the DNA pellet was air-dried and finally dissolved in 40µL 1x TE buffer. The BAC DNA was then ready to be used.

6.2.5 Recombineering

For recombineering, the BAC DNA was first transferred into competent *EL 350 E.coli* cells. *EL 350 E.coli* cells were grown in 5mL LB medium at 30°C overnight. The next day, 1mL of the pre-culture was transferred into 20mL LB medium and the cell culture was grown to an OD_{600}=0.5 before the cells were pelleted. To make them competent they were washed 2x with 1mL ice-cold water followed by 3x washing with 1mL 15% ice-cold glycerol (4000rpm, 5min, cold room). Finally, the bacterial pellet was resuspended in 50µL ice-cold 15% glycerol and transferred to the 0.2cm Gene Pulser™ electroporation cuvette (Bio-Rad) to be electroporated with 100ng of BAC DNA (Gene Pulser™ II, Bio-Rad). The cells were incubated in 1mL LB medium at 30°C for 1h before they were plated on LB-chloramphenicol plates and grown at 30°C.

Subsequently, a single colony of *EL350* cells containing the BAC DNA was grown overnight in 5mL LB-chloramphenicol medium at 30°C. 1mL of overnight-culture was transferred to 20mL selection medium and grown to an OD_{600}=0.5. After the induction of recombination enzymes through the incubation of the bacterial culture for 15 minutes at 42°C, the cells were made competent (see before). Finally, the competent *EL350* cells containing BAC DNA were electroporated with 1µL of the linearized retrieval plasmid, recovered in LB at 30°C and plated onto selective LB plates. The plates were incubated at

30°C until colonies were visible and could be picked to isolate the plasmids using the *QIAprep Spin Miniprep Kit*.

6.2.6 Restriction enzyme digests

For restriction enzyme digests 5 units of enzyme were used for the digest of 1µg of DNA in the supplemented 10x buffers. The digests were conducted at 37°C for at least one hour unless recommended otherwise by the supplier. Restriction enzymes were supplied by Roche or New England Biolabs (NEB).

6.2.7 Gel electrophoresis

For gel electrophoresis 0.8-2% agarose gels were prepared in 1x TAE buffer depending on the sizes of the expected DNA fragments. The gels were supplied with Ethidiumbromide. The DNA was loaded onto the gel in 6x DNA loading dye (Thermo Scientific). As references, the GeneRuler 50bp, 100bp and 1kb DNA ladders (Thermo Scientific) were used. Gel electrophoresis was conducted in 1x TAE buffer for 30-50 minutes at 140-200V. The DNA bands could be visualized by the exposure to UV light (Alpha Imager, Alpha Innotech).

6.2.8 Polymerase chain reaction (PCR)

For PCRs, I used the following standard protocol applying *Taq* polymerase in the supplemented buffer (5prime), a desoxynucleotide triphosphate mix (dNTPs) (Thermo Scientific), a primer pair and the template DNA. Primers were designed using the Primer3 software (primer3.wi.mit.edu) and supplied by Invitrogen or Sigma. For ES cell screening I additionally added 3% DMSO and 1x $MgCl_2$ (25mM) to increase the PCR efficiency or I used a different DNA polymerase, the Kapa2G Robust DNA polymerase (5U/µL) in the supplemtented 5x GC buffer (peqlab). The Kapa2G Robust DNA polymerase was established for colony-PCRs with high reproducibility and has the advantage to be less error-prone and more tolerant against inhibitors like SDS, EtOH and EDTA in comparison to the *Taq* polymerase.

PCR reaction		*PCR program*		
template DNA	1µL	95°C	3min	
10x Taq buffer	2.5µL	95°C	30sec	38x
dNTPs (10mM)	0.5µL	T_a	30sec	
primer 1 (10mM)	0.5µL	72°C	t_E	
primer 2 (10mM)	0.5µL	72°C	10min	
Taq polymerase (5U/µL)	0.25 – 0.5µL	T_a...annealing temperature (T_m of primers – 5°C)		
H_2O	up to 25µL	t_E...extension time (1 min per 1000bp)		

6.2.9 DNA purification by phenol-chlorophorm extraction

Phenol-chloroform extractions were applied to purify linearized plasmid DNA and ES cell DNA. First, the DNA solution was mixed with 1 Volume of phenol-chloroform-isoamylalcohol and centrifuged at high speed (5 minutes, 15000xg). The aqueous phase containing the DNA was transferred to a new tube and the process was repeated. Then 1 volume of chloroform was added, thoroughly mixed with the DNA solution and centrifuged for 5 minutes. Also this step was repeated before the aqueous phase was again transferred to a new tube to precipitate the DNA. For DNA precipitation 1 volume of isopropanol and 1/10 volume of NaAc (3M, pH 5.2) was added to purify linearized plasmid DNA, while 3 volumes of absolute ethanol were added to precipitate ES cell DNA. I inverted the tube to precipitate the DNA. Then I took out the white fluffy DNA and washed it in 70% ethanol before I dissolved it in pre-warmed 1x TE.

6.3. Embryonic stem cell culture

6.3.1. General culture conditions

ES cells were plated on a layer of irradiated feeder cells and grown at 37°C in 5% CO_2 in a humidity comprising more than 95%. ES cell medium was changed daily and the ES cells were split every second day in a ratio of 1:7. Harvesting and passaging of cells included the removal of the medium, washing the cells with 1x PBS and trypsinization with 1x Trypsin (Gibco) for 3-4 minutes in the 37°C incubator. After trypsinization the cells were harvested in ES cell medium, pelleted (1200xg, 3min), resuspended and distributed in fresh ES cell medium on new feeder plates.

For freezing, ES cells were collected like for passaging and frozen in 50% ES cell medium, 40% FCS and 10% DMSO in 1mL aliquots in cryotubes (Thermo Scientific). They were wrapped into paper towels to be slowly frozen in the -80°C freezer before they were transferred for long-term storage to the liquid nitrogen tank.

6.3.2 Preparation of irradiated primary mouse embryonic fibroblasts

DR4 primary mouse embryonic fibroblasts (pMEFs) were supplied by the lab and used as feeder cells. pMEFs at passage 1 (P1) were thawed in the waterbath (37°C), resuspended in pMEF medium, centrifuged (1300xg, 3min) and plated in 15mL pMEF medium on Ø15cm tissue culture plates. I split the cells every other day in a ratio of 1:3 when they showed confluency. At P5, I harvested the cells in pMEF medium, irradiated them for 4 minutes (1300-1900 cGy, Gammacell 3000 Elan) and froze them in 1mL aliquots (50% cells in pMEF medium, 40% FCS, 10% DMSO).

6.3.3 ES cell targeting

A9 murine embryonic stem cells (ES cells) were supplied by the lab. I thawed ES cells on an Ø15cm tissue culture plate on a layer of pMEFs in ES cell medium. When the cells had grown to 70% confluency, I harvested them in 800µL 1xPBS. I transfected the ES cells with 20µg linearized and phenol-chloroform purified targeting vector by electroporation in a 4mm Gene Pulser cuvette (Bio-Rad). Electroporation was conducted at 0.24kV and 500F in the Gene Pulser™ (Biorad). After transfection, the cells regenerated for 10min in the laminar flow hood before they were distributed on four Ø10cm feeder plates in ES cell medium. The medium was renewed every day.

6.3.4 ES cell selection

30-36 hours after transfection selection for positively targeted ES cells was started by the addition of 360µg/mL *G418* to the ES cell medium. The selection medium was changed every day till the ES cell colonies were picked.

6.3.5 ES cell colony picking

When resistant ES cell clones became visible on the tissue culture plates (~ 7 days after transfection), the colonies were picked under the microscope in 20µL 1x PBS using a 20µL micropipette. The cells were transferred into a 96-well PCR plate and trypsinized (20µL trypsin). After 5 minutes incubation at 37°C, half of the cell suspensions were transferred to the corresponding wells on a 96-well tissue culture plate containing pMEFs in selection medium. The trypsinization in the rest of the cell suspensions was stopped by the addition of 90µL 1x PBS. The DNA of those remaining cells was used for PCR analysis.

6.3.6 PCR screening

To prepare the ES cell DNA for the PCR screen, I first centrifuged the cells in the 96-well PCR plate (5min, 1500xg) and discarded the supernatant. I added 5µL of a Proteinase K (Invitrogen) and Tail buffer mix (Proteinase K : Tail buffer = 1 : 20) to each well and incubated the PCR-plate at 55°C for 1 hour. Afterwards I added 50µL of H_2O and boiled the samples at 95°C for 10 minutes to make the ES cell DNA ready for the PCR screen.

I screened the ES cells for correct targeting using a nested PCR approach. For that purpose I designed two primer pairs specific for the targeted allele, the forward primers located in an endogenous sequence of the targeted gene locus, the reverse primers located in the introduced sequence of interest or vice versa. For the first PCR I used 1µL of the prepared ES cell DNA and the outer primer pair. Subsequently, I used 1µL of the first PCR reaction to conduct the second PCR using the inner primer pair. PCRs were conducted according to the protocols described in section 6.2.3. The PCR products were analyzed using gel electrophoresis (see section 6.2.2).

6.3.7. Southern Blot analysis

6.3.7.1 DNA preparation

PCR-screen positive ES cell clones were used for Southern Blot analysis. For this purpose, ES cell clones were expanded from the 96-well tissue culture plate to 24-well plates, 6-well

plates and finally to Ø6cm plates containing feeder cells. In each expansion step, I aspirated the medium, washed the cells with 1x PBS, trypsinized them and resuspended them in ES cell medium. I always froze aliquots to keep them for potential blastocyste injections and plated the rest of the cells. For Southern Blot analysis I needed to prepare large amounts of ES cell DNA. For that reason, I plated ES cells on Ø6cm plates in ES cell medium without feeder cells. I harvested the cells about 7 days after plating when they had grown highly confluent. I incubated the cells with 100µL Proteinase K and 5mL Tail Buffer at 55°C overnight. I purified the ES cell DNA by phenol-chloroform extraction (6.2.7) and subsequently digested 15µg of DNA with an appropriate restriction enzyme overnight to obtain distinguishable DNA fragments in the wild-type and the targeted allele.

6.3.7.2 Blotting

After the overnight digest I loaded the 40µL restriction digest as well as 15µL marker DNA on a 0.9% agarose gel (+EtBr) and did a gel electrophoresis for 5h at 140V. The gel could then be prepared for the blotting. Therefore I incubated the gel first with 500mL of 0.25M HCl solution for depurination for 7 minutes on a shaker. After depurination, the gel was incubated with denaturation solution for 30 minutes followed by neutralization for 30 minutes. Between each incubation step, the gel was rinsed with H_2O. Finally the gel was blotted onto a nylon membrane. For the blotting I put the gel onto 3 sheets of 3MM Whatman paper, the first sheet reaching into 20x SSC buffer to ensure the capillary transfer of the DNA onto the membrane. Onto the gel I put the nylon membrane, and again to sheets of Whatman paper and a stack of paper towels. I encumbered the whole construct and did the blotting overnight. The next day I disassembled the blot, rinsed the membrane in 2x SSC, wrapped the membrane into saran wrap and UV-crosslinked the DNA onto the membrane (UV Stratalinker 2400, Stratagene).

6.3.7.3 Hybridization and Detection

For pre-hybridization, I put the membrane into a hybridization flask in 15mL Church buffer and incubated it rotating for 1 hour at 65°C. In the meantime I prepared the radioactively labeled DNA probe using the *Prime-It II Random Primer Labeling Kit*. The DNA probe was generated by PCR amplification and gel-elution. For the hybridization, 25ng of probe DNA were mixed with 10µL random primers and H_2O to a final volume of 24µL. The DNA was denatured (95°C, 5min). After cooling the probe, I added 10µL 5x dATP buffer and 1µL *Exo (-)*

Klenow polymerase (5U/µL) before I labeled it with 5µL of [a^{32}-P] dATP. The probe was incubated at 37°C for 15 minutes before it was purified using a nucleotide removal kit according to the manufacturer's protocol. The purified probe was mixed with 100µL salmon sperm and denatured for 3 minutes at 95°C before it was added to the membrane in the church solution. Hybridization was performed overnight in the 65°C rotator.

After hybridization I discarded the church buffer and rinsed the membrane with washing solution. I added 100mL washing solution to the membrane in the flask and incubated it at 65°C for 30 minutes. I repeated this step until I only detected very low or no background levels of radioactivity on the membrane. The membrane was wrapped into saran wrap and exposed to a storage phosphor screen (Molecular Dynamics) for a few days. Thereafter the signal of radioactively labeled DNA bands could be detected using a phosophoimager (Personal Molecular Imager (PMI^{TM}), Bio-Rad).

6.3.8 Blastocyste Injection

Injection of ES cells into murine blastocysts was conducted by the IMP transgenic service facility. To prepare the ES cells for injection, I thawed Southern Blot – positive ES cells clones and plated them on Ø6cm feeder plates in ES cell medium. When they were confluent I passaged them to Ø10cm feeder plates. On the injection day, I harvested the confluent cells. After resuspension in ES cell medium, I plated the cells onto an empty tissue culture plate and left them there for 15-20 minutes to reduce the amount of pMEFs due to their adherence to the plate. Subsequently, I gently transferred the non-adherent ES cells into a 15mL falcon tube, centrifuged them (1200xg, 3min) and finally resuspended the cells in 1mL ES cell medium to hand them over to the transgenic service facility for blastocyste injection.

6.4 FACS analysis of *Ikzf1$^{fl/-}$ Rag1-cre* mice

6.4.1 Isolation of cells

For FACS analysis, *Ikzf1$^{fl/-}$ Rag1-cre* mice as well as control mice (*Ikzf1$^{+/+}$ Rag1-cre)* were sacrificed and their femora and tibiae were harvested to isolate bone marrow cells. Using a cell strainer, single cell suspensions of the organs were prepared in FACS buffer and the cell concentrations of the suspensions were measured using the CASY counter (Schärfe). For cell staining and subsequent FACS analysis, 5*10^6 cells were used.

6.4.2. Antibody – staining

For FACS analysis, bone marrow cells were stained with antibodies conjugated with fluorophores (Table 2). For staining I used 5*10^6 cells. After 10 minutes incubation with F_c block (1:500), I added biotinylated antibody to the cells and incubated for another 15-30 minutes. After washing with FACS buffer (1500xg, 5min, 4°C), I incubated the cells with directly fluorophor-conjugated primary antibodies and with streptavidin for detection of biotinylated antibodies for 20 minutes on ice. After 2x washing with FACS buffer the cells were resuspended in FACS buffer and analyzed by flow cytometry. Flow cytometric analyses were conducted on a FACS LSR Fortessa (405/488/561/640nm lasers) and the data was analyzed using FlowJo software (version 8.7, TreeStar).
The different hematopoietic cell types were identified as follows:

cell type	*markers*
pro-B cells	CD19$^+$ cKit$^+$ CD25$^-$ IgM$^-$ IgD$^-$
pre-B cells	CD19$^+$ cKit$^-$ CD25$^+$ IgM$^-$ IgD$^-$
immature B cells	CD19$^+$ IgM$^+$ IgD^{low} B220$^+$
mature B cells	CD19$^+$ IgM^{low} IgD$^+$ B220^{hi}
B2 B cells	CD19$^+$ B220$^+$ CD5$^-$
B1 B cells	CD19$^+$ B220^{low} CD5$^+$
T cells	TCRβ^+
Granulocytes	Gr-1^{hi} CD11b$^+$
Macrophages	CD115$^+$ CD11b$^+$

7. Supplementary

Primer	*Primer sequence*	*Purpose*
12219	CGCCTAAAGAAGAGGCTGTG	outer forward primer for nested PCR of *Rosa26* targeted ES cells
12220	GAAAGACCGCGAAGAGTTTG	outer reverse primer for nested PCR of *Rosa26* targeted ES cells
12221	CAGAGAGCCTCGGCTAGGTA	inner forward primer for nested PCR of *Rosa26* targeted ES cells
12222	TCATCAAGGAAACCCTGGAC	outer reverse primer for nested PCR of *Rosa26* targeted ES cells
10629	AAGGGAGCTGCAGTGGAGTA	forward primer for genotyping the *Rosa26* wild-type locus
8642	CGCCGCCGGGATCACTCTCG	forward primer for genotyping the *Rosa26* targeted locus
8325	TAAGCCTGCCCAGAAGACTC	reverse primer for genotyping the wild-type and the targeted *Rosa26* locus
10512	GGGGAACTTCCTGACTAGGG	outer forward primer for nested PCR of *Ikzf3* targeted ES cells
12570	AGACCAGCATGCCTTTCAGT	outer reverse primer for nested PCR of *Ikzf3* targeted ES cells
10513	GCTTGGCTGGACGTAAACTC	inner forward primer for nested PCR of *Ikzf3* targeted ES cells
12724	TGCTCTGGGGTATGTTTCAA	inner reverse primer for nested PCR of *Ikzf3* targeted ES cells
12768	TCATGACATGGCCTCACCTA	*loxP* screen forward primer annealing within the *Ikzf3* long arm region
12769	TGTTTCCCAGAGTCCCTCAG	*loxP* screen reverse primer annealing within *Ikzf3* exon 8

Table 1. List of primers

Antibody	*Fluorophor*	*Dilution*	*Supplier*
B220	Brilliant Violet 421	1/250	Biolegend
CD19	Brilliant Violet 421	1/300	BD Bioscience
TCRbeta	Brilliant Violet 421	1/300	Biolegend
Gr-1	eFluor 450	1/300	eBioscience
Ter119	eFluor 450	1/250	eBioscience
CD5	Fitc	1/250	eBioscience
CD117 (c-Kit)	Fitc	1/200	Pharmingen
CD19	PerCP-Cy5.5	1/500	Biolegend
Streptavidin	PerCP-Cy5.5	1/500	eBioscience
Ly6c	PE	1/10000	Home Made
CD115	PE	1/2000	eBioscience
NK1.1	PE	1/500	Biolegend
CD25	PE-CF594	1/1000	BD Bioscience
CD117 (c-Kit)	PE-Cy7	1/1000	eBioscience
CD11b	PE-Cy7	1/2000	eBioscience
Sca1	PE-Cy7	1/1000	eBioscience
IgD	APC	1/1000	eBioscience
IgM	APC	1/1000	eBioscience
Gr-1	APC	1/500	Biolegend
CD127 (IL-7R)	APC	1/200	eBioscience

Table 2. FACS antibodies and their conjugated fluorophores

Abbas, A. K., A. H. Lichtmann, et al. (2007). "Cellular and Molecular Immunology."

Allman, D. and S. Pillai (2008). "Peripheral B cell subsets." Curr Opin Immunol **20**(2): 149-57.

Almqvist, N. and I. L. Martensson (2012). "The pre-B cell receptor; selecting for or against autoreactivity." Scand J Immunol **76**(3): 256-62.

Appleby, S. L., M. P. Cockshell, et al. (2012). "Characterization of a distinct population of circulating human non-adherent endothelial forming cells and their recruitment via intercellular adhesion molecule-3." PLoS One **7**(11): e46996.

Barneda-Zahonero, B., L. Roman-Gonzalez, et al. (2012). "Epigenetic regulation of B lymphocyte differentiation, transdifferentiation, and reprogramming." Comp Funct Genomics **2012**: 564381.

Brenner, S., D. Drewel, et al. (2011). "A hypomorphic IgH-chain allele affects development of B-cell subsets and favours receptor editing." Embo J **30**(13): 2705-18.

Brown, K. E., S. S. Guest, et al. (1997). "Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin." Cell **91**(6): 845-54.

Cerutti, A., I. Puga, et al. (2012). "New helping friends for B cells." Eur J Immunol **42**(8): 1956-68.

Cortes, M. and K. Georgopoulos (2004). "Aiolos is required for the generation of high affinity bone marrow plasma cells responsible for long-term immunity." J Exp Med **199**(2): 209-19.
Aiolos.

Georgopoulos, K. (2002). "Haematopoietic cell-fate decisions, chromatin regulation and ikaros." Nat Rev Immunol **2**(3): 162-74.

Georgopoulos, K., M. Bigby, et al. (1994). "The Ikaros gene is required for the development of all lymphoid lineages." Cell **79**(1): 143-56.

Hanke, N., D. Alizadeh, et al. (2013). "Dendritic cell tumor killing activity and its potential applications in cancer immunotherapy." Crit Rev Immunol **33**(1): 1-21.

Kanji, S., V. J. Pompili, et al. (2011). "Plasticity and maintenance of hematopoietic stem cells during development." Recent Pat Biotechnol **5**(1): 40-53.

Kim, J., S. Sif, et al. (1999). "Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes." Immunity **10**(3): 345-55.

Kirstetter, P., M. Thomas, et al. (2002). "Ikaros is critical for B cell differentiation and function." Eur J Immunol **32**(3): 720-30.

LeBien, T. W. and T. F. Tedder (2008). "B lymphocytes: how they develop and function." Blood **112**(5): 1570-80.

Lin, Y. C., S. Jhunjhunwala, et al. (2010). "A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate." Nat Immunol **11**(7): 635-43.

MacLennan, I. C., K. M. Toellner, et al. (2003). "Extrafollicular antibody responses." Immunol Rev **194**: 8-18.

Mandel, E. M. and R. Grosschedl (2010). "Transcription control of early B cell differentiation." Curr Opin Immunol **22**(2): 161-7.

Mangel, M. and M. B. Bonsall (2013). "Stem cell biology is population biology: differentiation of hematopoietic multipotent progenitors to common lymphoid and myeloid progenitors." Theor Biol Med Model **10**(1): 5.

Martensson, I. L., N. Almqvist, et al. (2010). "The pre-B cell receptor checkpoint." FEBS Lett **584**(12): 2572-9.

McManus, S., A. Ebert, et al. (2011). "The transcription factor Pax5 regulates its target genes by recruiting chromatin-modifying proteins in committed B cells." Embo J **30**(12): 2388-404.

Mercer, E. M., Y. C. Lin, et al. (2011). "Multilineage priming of enhancer repertoires precedes commitment to the B and myeloid cell lineages in hematopoietic progenitors." Immunity **35**(3): 413-25.

Mercer, E. M., Y. C. Lin, et al. (2011). "Factors and networks that underpin early hematopoiesis." Semin Immunol **23**(5): 317-25.

Mikkola, H. K. and S. H. Orkin (2006). "The journey of developing hematopoietic stem cells." Development **133**(19): 3733-44.

Morgan, B., L. Sun, et al. (1997). "Aiolos, a lymphoid restricted transcription factor that interacts with Ikaros to regulate lymphocyte differentiation." Embo J **16**(8): 2004-13.

Nichogiannopoulou, A., M. Trevisan, et al. (1998). "Ikaros in hemopoietic lineage determination and homeostasis." Semin Immunol **10**(2): 119-25.

Nutt, S. L. and B. L. Kee (2007). "The transcriptional regulation of B cell lineage commitment." Immunity **26**(6): 715-25.

Ramiscal, R. R. and C. G. Vinuesa (2013). "T-cell subsets in the germinal center." Immunol Rev **252**(1): 146-55.

Rebollo, A. and C. Schmitt (2003). "Ikaros, Aiolos and Helios: transcription regulators and lymphoid malignancies." Immunol Cell Biol **81**(3): 171-5.

Reif, K., E. H. Ekland, et al. (2002). "Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position." Nature **416**(6876): 94-9.

Satpathy, A. T., X. Wu, et al. (2012). "Re(de)fining the dendritic cell lineage." Nat Immunol **13**(12): 1145-54.

Shanmugam, A., M. J. Shi, et al. (2000). "Evidence for class-specific factors in immunoglobulin isotype switching." J Exp Med **191**(8): 1365-80.

Victora, G. and M. Nussenzweig (2012). "Germinal centers." Annual Review of Immunology **30**: 429-457.

Wang, J. H., N. Avitahl, et al. (1998). "Aiolos regulates B cell activation and maturation to effector state." Immunity **9**(4): 543-53.

Wang, J. H., A. Nichogiannopoulou, et al. (1996). "Selective Defects in the Development of the Fetal and Adult Lymphoid System in Mice with an Ikaros Null Mutation." Immunity **5**: 537-549.

Zon, L. I. (2001). "Hematopoiesis - A developmental approach."

Deutsche Zusammenfassung

Immunzellen entstehen aus hämatopoetischen Stammzellen (HSC). Für die korrekte Entwicklung der Blutzellen und Immunzellen spielen eine Reihe von Faktoren eine Rolle. Zwei Transkriptionsfaktoren, die vor allem für die Differenzierung von B Lymphozyten aus hämatopoetischen Stammzellen entscheidend sind, sind Ikaros (*Ikzf1*) und Aiolos (*Ikzf3*).

Bisherige Studien zeigten, dass beide Transkriptionsfaktoren für die Entwicklung von Lymphozyten, vor allem von B Zellen, essentiell sind. In Knockout-Experimenten wurde gezeigt, dass die Abwesenheit einer der beiden Transkriptionsfaktoren wesentliche Einflüsse auf die Produktion von B Zellen hat. *Ikzf1* Knockouts führten schon in einem frühen Entwicklungsstadium zu einer vollständigen Blockade der weiteren B Zell-Differenzierung. *Ikzf3*-defiziente Mäuse zeigten aberrante B Zell-Entwicklung, begleitet von einer Hyperaktivierung der reifen B Zellen.

Im Laufe meiner Masterarbeit habe ich zwei konditionelle Allele entwickelt. Das erste Allel enthielt die *Ikzf1* cDNA, reguliert von einem starken Promoter im *Rosa26* Genlocus von embryonalen Stammzellen (ES Zellen) der Maus. Für das Allel positive ES Zellklone wurden in Maus-Blastozysten injiziert um chimäre Mäuse zu erschaffen, die das konditionelle *Ikzf1* Allel tragen. *Ikzf1* Überexpression kann in den Mäusen erzielt werden, indem die dem *Ikzf1* Minigen vorangeschaltete gefloxte Stopkassette durch die Cre Rekombinase deletiert wird.

Weiters wurde ein Allel entworfen, bei dem das letzte Exon des *Ikzf3* Gens (Exon 8) von *LoxP* Stellen begrenzt wurde. Für das gefloxte *Ikzf3* Allel positive ES Zellen werden in Maus-Blastozysten injiziert. Die Expression der Cre Rekombinase führt dann zur Deletion des letzten *Ikzf3* Exons und zur Translation eines nichtfunktionellen Aiolos Proteins.

Zukünftige Pläne sind, die chimären Mäuse mit Mäusen zu kreuzen, die konditionell die Cre Rekombinase exprimieren, und dadurch zu einer konditionellen *Ikzf1* Überexpression bzw. *Ikzf3* Deletion führen. Die Analyse der Mäuse wird Aufschluss über die Funktionen von Ikaros und Aiolos in den verschiedenen Entwicklungsstadien von B Lymphozyten geben.

Acknowledgements

First of all, I would like to thank Meinrad Busslinger for giving me the chance to do my master thesis in such a great scientific environment, working together with people dedicated to science.

Furthermore, I thank Tanja Schwickert, who was the most important contact person for me during my year at the IMP and always supported me with advice and ideas for my project.

I thank all the people of the Busslinger lab for the fantastic working atmosphere, their helpfulness and the nice breaks we had together. I have learned a lot during my year in your lab, thank you all.

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