Generation of a conditional *Pax5-Etv6* translocation allele for establishing an ALL mouse model

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Abstract

The transcription factor *PAX5* is an essential master regulator of B cell development. It is expressed exclusively in the B-lymphoid lineage of the hematopoietic system and is required for progression beyond the pro-B cell stage. Pax5 fulfills a dual role by repressing B-lineage-‘inappropriate’ genes and simultaneously activating B cell-specific genes (Cobaleda et al., 2007). Pax5 is known to play an oncogenic role in a subset of B-cell non-Hodgkin’s lymphoma where its intact sequence is brought under control of strong enhancers or promoters from the *IGH* locus. Recently, Pax5 was reported to be a frequent target of somatic mutations in pediatric acute lymphoblastic leukemia (ALL), being altered in more than 30% of the examined cases (Mullighan et al., 2007). Moreover, *Pax5* is a reported target of different chromosomal translocations in ALL resulting in fusion genes encoding chimeric proteins with novel functions. One of them is the dicentric translocation (9;12)(p13;p13) resulting in the fusion of *Pax5* to the transcriptional repressor gene *Etv6*. This was one of the first *Pax5* fusions in ALL to be reported and is a recurrent chromosome abnormality that accounts for close to 1% of childhood ALL. The resulting fusion protein is comprised of the entire DNA-binding paired domain of Pax5 fused to the nearly intact sequence of Etv6 with its DNA- and protein-interacting domains. The translocation affects only one allele of *Pax5* leaving the second one intact. However, there is no apparent explanation for the cause of leukemogenesis since it is known that heterozygous *Pax5*+/− mice have no apparent defects in B cell development (Urbanek et al., 1994; Nutt et al., 1999). This fact paves the way for the hypothesis that the fusion product resulting from the translocation must act as a negative regulator to interfere with the function of remaining wild type Pax5 protein. Recent *in vitro* transient transfection assays of PAX5-ETV6 in cultured pro-B cells have supported this hypothesis.

To investigate the functions of PAX5-ETV6 fusion protein leading to leukemogenesis I established *in vivo* mouse model by generating a conditional *Pax5-Etv6* translocation allele. The analysis of this mouse model is likely to provide novel important insight into the role of Pax5 in ALL development.
Zusammenfassung

Um die Rolle des PAX5-ETV6 Fusionsproteins bei der Entstehung der B-ALL zu untersuchen, habe ich im Zuge meiner Diplomarbeit ein in vivo Mausmodell erschaffen, welches ein konditionell aktivierbares Pax5-Etv6 Translokationsallel trägt. Die Analyse dieses Mausmodells soll zum besseren Verständnis der Rolle von Pax5 während der ALL Entwicklung beitragen.
1 Introduction

1.1 Hematopoiesis and the generation of B cell antigen receptors

The cells of the immune system originate in the bone marrow, where many of them also mature. They then migrate to guard the peripheral tissues, circulating in the blood and in a specialized system of vessels called the lymphatic system. All the cellular elements of blood, including the red blood cells that transport oxygen, the platelets that trigger blood clotting in damaged tissues, and the white blood cells of the immune system, derive ultimately from the same progenitor or precursor cells - the pluripotent hematopoietic stem cells which reside in the bone marrow (Figure 1). Initially they give rise to stem cells of more limited potential, the common myeloid progenitors (CMPs) and the common lymphoid progenitors (CLPs). The CMP is the immediate precursor of the oxygen carrying red blood cells and platelets but will also give rise to granulocytes, macrophages, dendritic cells, and mast cells of the immune system. The CLP gives rise to the lymphocytes and natural killer cells. There are two major types of lymphocyte: B lymphocyte or B cell, which mature in the bone marrow and T lymphocytes or T cells which mature in the thymus. Each individual lymphocyte matures bearing a unique variant of a prototype antigen receptor and is able to mount a specific immune response against virtually any foreign antigen, a molecule, which will bind the lymphocyte receptor. The B and T lymphocytes collectively bear a huge repertoire of receptor molecules on their surface that are highly diverse in their antigen binding sites. Adaptive immune responses are initiated in the peripheral lymphoid tissues such as spleen, lymph nodes or mucosal associated lymphoid tissues. T cells that encounter antigen proliferate and differentiate into antigen specific effector cells capable of killing cells infected with viruses, while B cells proliferate and differentiate into antibody secreting cells. Antibodies are the secreted form of the B-cell antigen receptor (BCR). The stepwise expression and assembly of components of the functional receptor for antigen is the hallmark of early B cell development.

An antibody molecule also known as immunoglobulin (Ig) molecule is composed of two distinct regions. One is the constant region that can take one of only four or five
biochemically distinguishable forms; the other is the variable region that can take an apparently infinite variety of subtly different forms that allow it to bind specifically to an equally vast variety of different antigens.

The genes for immunoglobulin variable regions are inherited as sets of gene segments, each encoding a part of the variable region of one of the immunoglobulin polypeptide chains.

During B cell development in the bone marrow, these gene segments are irreversibly joined by DNA recombination to form a stretch of DNA encoding a complete variable region. Because there are many different gene segments in each set, and different gene segments are joined together in different cells, each cell generates unique genes for the variable regions of the heavy and light chains of the immunoglobulin molecule. This process is called $V_{H}$-$DJ_{H}$ recombination.

Rearrangement of Ig heavy chain (IgH) genes proceeds in two steps, first by assembling diversity (D) and joining (J) segments (D-J rearrangements), followed by the joining of variable regions to D-J segments to create mature V(D)J joints. Productively rearranged genes encode H chains that participate in assembly of the pre-BCR on the plasma membrane. The pre-BCR consists of H chains, surrogate light ($\Psi L$) chain polypeptides encoded by the $\lambda 5$ and $VpreB$ genes, and Ig$\alpha$ and Ig$\beta$ (encoded by the $mb$-1 and $B29$ genes, respectively), a pair of membrane-spanning polypeptides required for display of Ig on the cell surface and for signal transduction (Maier and Hagman, 2002). Signaling through the pre-BCR is required for rearrangement and expression of Ig light chain genes (Ig$\kappa$ or Ig$\lambda$) that replace $\Psi L$ chains to form the mature BCR (Maier and Hagman, 2002).

Once these recombination events have succeeded in producing a functional receptor, further rearrangement is prohibited. Thus, this mechanism, enables the generation of a vast number of different proteins from limited number of gene segments, grants each lymphocyte a unique receptor specificity as well as ensures that all progeny of a single recombined B cell will carry the same genetic composition for receptor specificity. B cells displaying the mature BCR on the plasma membrane migrate to the periphery and respond to specific antigens by differentiating into antibody-secreting plasma cells and entering the memory pool.
Figure 1. All the cellular elements of blood, including the lymphocytes of the adaptive immune system, arise from hematopoietic stem cells in the bone marrow.
1.2 Transcription factor PAX5 and its role in early B cell development

Hematopoietic stem cells develop into B cells by first generating lymphoid primed multipotent progenitors, which subsequently differentiate via the earliest lymphocyte progenitor cell stage to common lymphoid progenitors (CLPs) with their characteristic B, T and natural killer cell potential (Busslinger, 2004). B cell lymphopoiesis is initiated by the entry of progenitors into the B-cell lineage transcription programme and the concomitant sequential rearrangement of the immunoglobulin genes through V_H-DJ_H recombination (Fuxa and Skok, 2007).

Entry of CLPs into the B cell lineage depends on three transcription factors: a basic helix-loop-helix protein E2A, the early B cell factor EBF1 and Pax5, also known as B-cell specific activator protein BSAP, one of nine paired box family transcription factors and the only one of them expressed within the mammalian hematopoietic system. B-cell development is blocked at its earliest stages in the absence of any one of these transcriptional regulators (Cobaleda et al., 2007). E2A and EBF1 activate B-lymphoid genes thus acting as lineage specification factors. Pax5 controls the commitment of lymphoid progenitors to the B-cell lineage. Pax5-deficient pro-B cells are uncommitted progenitors and have the potential to develop into various other hematopoietic cell types except for B-cells, which can only develop upon the reactivation of Pax5 expression, which restricts the developmental potential of progenitor cells to the B cell pathway (Nutt et al., 1999). At the molecular level Pax5 has a dual function. During B cell commitment it represses the transcription of B-lineage inappropriate genes (Delogu et al., 2006) and simultaneously activates the expression of B cell-specific genes (Schebesta et al., 2007) thus restricting the broad signaling capacity of B cell- progenitors to the B cell pathway (Figure 2), inducing V_H-DJ_H recombination, enabling (pre) B cell receptor signaling as well as regulating adhesion and migration that way promoting the development towards the mature B cell stage (Delogu et al., 2007; Schebesta et al., 2007).

Chromatin profiling by ChIP on-chip analysis has shown that Pax5 directly activates the chromatin at promoters or putative enhancers of its target genes (Schebesta et al., 2007).

Pax5 is continuously and exclusively expressed in the B lymphoid lineage beginning at the committed pro B cell stage to the mature B cell stage where its continuous expression is
required for the maintenance of the B cell identity and function (Horcher et al. 2001; Fuxa and Busslinger, 2007). Its expression is subsequently repressed during the terminal plasma cell differentiation (Fuxa and Busslinger, 2007).

Surprisingly, conditional Pax5 deletion allows mature B cells from peripheral lymphoid organs to dedifferentiate in vivo back to early uncommitted progenitors in the bone marrow, which are subsequently able to develop into T cells. Hence the loss of Pax5 can induce the conversion of mature B lymphocytes into functional T cells via dedifferentiation to uncommitted progenitor cells (Cobaleda et al., 2007). Together these studies have identified Pax5 as a crucial guardian of B lymphocyte identity and function (Cobaleda et al., 2007).

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**Figure 2.** The dual role of Pax5 during B lineage commitment.

Pax5 activates B cell specific genes (e.g. CD19, CD22 and mb-1) and simultaneously represses lineage-inappropriate genes at B cell commitment. The latter are essential for diverse cellular functions such as cell–cell communication, adhesion and migration in T lymphoid, myeloid and erythroid cells as well as in multipotent progenitors (Fuxa and Skok 2007).
1.3 Structure of PAX5

The central feature of paired box transcription factors is the highly conserved DNA-binding region of about 128 amino acids, the paired domain (Figure 3.), which was originally described in the Drosophila protein paired (Underhill, 2000). This domain consists of two homeodomain like helix-turn-helix subdomains, the N-terminal PAI and the C terminal RED domain (Breitling and Gerber, 2000), each with distinct sequence recognition properties (Czerny et al. 1993), which enable them to bind a distinct half-site of the Pax5 recognition sequence in adjacent major grooves of the DNA helix (Garvie et al. 2001). Both subdomains can bind DNA independently (Czerny et al. 1993, Epstein et al. 1994, Vogan et al. 1996, Kozmik et al. 1997). The paired domain has retained a high degree of homology between mouse and humans (Ghia et al., 1998). Pax5 also contains an amino terminal remnant of a homeodomain (Dahl et al. 1997), as well as a protein-protein interacting octapeptide motif (Eberhard et al. 2000), that is conserved in all Pax proteins with the exception of Pax4 and Pax6. The C terminal protein interaction motifs are responsible for the repression and activation functions of Pax5 (Cobaleda et al., 2007; Dörfler und Busslinger, 1996; Eberhard und Busslinger, 1999).

Figure 3. Schematic representation of Pax5 (Maier, Hagman 2002)

Oct: octapeptide motif, HD: partial homeodomain, Activ.: TAD/transcriptional activation domain, Repr.: inhibitory domain
1.4 Oncogenic role of Pax5 in B cell malignancies

Over the past years there has been a lot of evidence for the involvement of Pax5 in specific chromosomal translocations, which have lead to its oncogenic activation and consequently to malignancies of hematopoietic origin.

One example is the misguided class switch recombination responsible for the generation of the t(9;14)(p13;q32) translocation, which leads to the deregulation of Pax5 expression in non-Hodgkin lymphoma.

In this recurrent translocation t(9;14)(p13;q32), Pax5 gene sequence of chromosome 9p13 is inverted and juxtaposed to the constant gene region of the IGH gene locus (Busslinger et al., 1996; Iida et al., 1996; Morrison et al., 1998). The transcription of the structurally intact PAX5 gene is deregulated by insertion of the potent IGH Eµ enhancer or an antisense IGH Sµ promoter (Iida et al., 1996; Morrison et al., 1998; Sonoki et al., 2004) (Figure 4).

Another example for the Pax5 involvement in malignant chromosomal translocations are the recently reported cases of specific chromosomal translocations in pediatric B cell precursor acute lymphoblastic leukemia (BCP-ALL) patients, which lead to the fusion of
the N terminal part of Pax5 to the C terminal sequences of different fusion partner proteins this way generating novel transcription factors (Nebral et al., 2008). The first translocation to be described was Pax5 and Etv6 (Carroll et al., 1987; Cazzaniga et al., 2001; Strehl et al., 2003). Subsequently eleven additional translocations were identified; Pax5-foxp1, Pax5-znf521, Pax5-pml1, Pax5-eln, Pax5-dach1, Pax5-brd1, Pax5-pom121, Pax5-hipk1, Pax5-jak2, Pax5-auts2 and Pax5-c20orf112 (see Table 1, Figure 5). These translocations fused Pax5 to six different transcription factors (Etv6, Foxp1, Znf521, Pml, Dach1 and Brd1), two kinases (Hipk1 and Jak2), two structural proteins (Pom121 and Elastin) as well as two proteins of unknown function (Auts2 and C20orf112). In most BCP-ALL cases analyzed, these translocations were unbalanced as the reciprocal translocation could not be detected (Cazzaniga et al., 2001; Strehl et al., 2003; Nebral et al., 2008; Kawamata et al., 2008; An et al., 2008).
Table 1. PAX5 fusion partners in BCP-ALL

<table>
<thead>
<tr>
<th>Partner gene</th>
<th>Chr.</th>
<th>Localization/Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETV6</td>
<td>12p13</td>
<td>nuclear, transcriptional repressor</td>
<td>1, 2, 3</td>
</tr>
<tr>
<td>Ets variant gene 6 (TEL oncogene)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXP1</td>
<td>3p13</td>
<td>nuclear, transcription factor</td>
<td>3, 4</td>
</tr>
<tr>
<td>Forkhead box P1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZNF521</td>
<td>18q11</td>
<td>nuclear, transcription factor</td>
<td>4</td>
</tr>
<tr>
<td>Zinc finger protein 521</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PML</td>
<td>15q21</td>
<td>nuclear, PML bodies, transcription factor</td>
<td>5</td>
</tr>
<tr>
<td>Promyelocytic leukemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DACH1</td>
<td>13q24</td>
<td>nuclear, transcription factor</td>
<td>6</td>
</tr>
<tr>
<td>Dachshund homolog 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRD1</td>
<td>22q13</td>
<td>nuclear, putative transcription factor</td>
<td>6</td>
</tr>
<tr>
<td>Bromodomain containing 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POM121</td>
<td>7q11</td>
<td>nuclear pore membrane, assembly of nuclear envelope</td>
<td>6</td>
</tr>
<tr>
<td>POM121 membrane glycoprotein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIPK1</td>
<td>1p13</td>
<td>nuclear, speckles, transcriptional regulation</td>
<td>6</td>
</tr>
<tr>
<td>Homeodomain interacting protein kinase 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAK2</td>
<td>9p24</td>
<td>cytoplasmic, tyrosine kinase, receptor signaling</td>
<td>6</td>
</tr>
<tr>
<td>Janus kinase 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELN</td>
<td>7q11</td>
<td>extracellular matrix, elastic fibers, structural protein</td>
<td>7</td>
</tr>
<tr>
<td>Elastin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUTS2</td>
<td>7q11</td>
<td>intracellular, unknown</td>
<td>3</td>
</tr>
<tr>
<td>Autism susceptibility candidate 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20orf112</td>
<td>20q13</td>
<td>unknown, unknown</td>
<td>3, 6</td>
</tr>
<tr>
<td>Chromosome 20 open reading frame 112</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Table 1. A list of reported Pax5 fusion partners in BCP-ALL. Image courtesy of M. Busslinger
1.5 Transcription factor ETV6 (TEL)

The short arm of chromosome 12 has been long known as a hot spot for chromosomal rearrangements in diverse types of leukemias and myelodysplastic syndromes (Bohlander, 2005). These rearrangements include balanced translocations with a great number of different chromosome partner bands as well as unbalanced translocations and deletions, which have led to the loss of genetic material from 12p. Molecular cytogenetic studies showed that more than half the observed balanced translocations had breakpoints that were clustered in a single yeast artificial chromosome clone located at 12p13 (Kobayashi et al., 1994). More than 40 translocations with ETV6 involvement have been reported (Odero et al., 2001).

In 1994, Todd Golub in Gary Gillilands group succeeded in cloning the ETV6/PDGFRB fusion gene, which was the result of a balanced t(5;12)(q31:p13) translocation that had been found in malignant cells from a patient with chronic myelomonocytic leukemia (CMML) a myelodysplastic syndrome which is characterized by progression to acute myeloid leukemia (AML) (Golub et al., 1994). They found a fusion of the platelet derived growth factor receptor beta gene located at band 5q31 that was known already at the time to a previously unknown gene from chromosome 12 band p13, which they called TEL (Translocation Ets Leukemia gene). TEL was later renamed to ETV6 (ets variant gene 6) by the nomenclature commission in order to avoid confusion with the abbreviation for telomere.

ETV6 is a member of the ets (E-26 transforming specific) family of transcription factors. All ets family proteins share a very conserved protein domain of about 88 amino acids in length the so-called ets domain (Bohlander, 2005) (Figure 6).

The ets domain is a sequence-specific DNA-binding domain but it also mediates protein-protein interaction, it is evolutionarily highly conserved and found also in invertebrates such as Drosophila and C.elegans (Oikawa and Yamada, 2003; Wasylyk et al., 1993). Interestingly, the ets domain of ETV6 is more closely related to the ets domain of the Drosophila protein yan than to the ets domain of the human ETS1 or SPI1 (PU.1) genes (Golub et al., 1994).

The other evolutionarily conserved domain in the 652 amino acids of Etv6 is the N-terminally located pointed or sterile alpha motif (SAM) domain whose 3D structure has
recently been elucidated (Tran et al., 2002; Kim et al., 2001). This domain, also known as HLH domain is even more highly conserved in evolution and can be found in many different ets family members (Bohlander, 2005). It is found in yeast proteins and has been shown to be involved in homo- and heterodimerization of transcription factors and in signal transducing proteins (e.g. of the MAPK pathway) (Grimshaw et al., 2004). Etv6 contains two alternative translation start codons at positions 1 and 43, which lead to the expression of two isoforms of the transcription factor (Bohlander, 2005).

ETV6 has been found fused to many different partners, some of them prominent players in leukemogenesis such as RUNX1 and ABl1 (Golub et al., 1995; Golub et al., 1996; Papadopoulos et al., 1995) (Figure 7). The different types of fusion partners include protein tyrosine kinases as well as transcription factors. A number of fusions do not seem to lead to meaningful fusion proteins and are therefore regarded as “unproductive”.

![Diagram of ETV6 showing the location of the 56th amino acid](image)

*Figure 6. ETV6: a schematic representation. The PAX5 fusion breakpoint located in intron 2 of Etv6 at the 56th amino acid is indicated with the number 56. The remaining numbers indicate the length of the protein and corresponding domains in amino acids.*
Figure 7. ETV6 and its fusion partners in leukemia.
1.6 B-cell precursor Acute Lymphoblastic Leukemia (BCP - ALL)

B-cell precursor Acute Lymphoblastic Leukemia (BCP-ALL) belongs to the family of genetically heterogeneous lymphoid neoplasms derived from B-lymphoid progenitors and is the main type of childhood leukemia with a marked age incidence peak at 2-5 years (Greaves ans Wiemels, 2003). It has a B-cell precursor phenotype; 'Acute' refers to the undifferentiated, immature state of the circulating lymphocytes ("blasts"), and to the rapid progression of the disease, during which lymphoblasts uncontrollably multiply and are overproduced in the bone marrow, outcrowing other cells present and as a result of this can produce lymphocytosis, spread to other organs and form metastases which can be fatal in weeks to months if left untreated.

As with the other acute leukemias, the most common symptoms experienced by patients include fatigue, bleeding, and recurrent infections resulting from the suppression of normal hematopoiesis in the bone marrow by the accumulating blasts (Randolph, 2004). ALL exhibits the best response to standard chemotherapy as compared to acute myeloblastic leukemias (AML). Further, remission rates are highest among ALL patients, many of whom are experiencing sustained remissions suggesting cure.

Although advances in treatment have led to an overall favourable outcome with a cure rate of~80%, some subtypes of leukaemia still remain unresponsive to therapy (Greaves ans Wiemels, 2003). Approximately 25% of the patients suffer from relapse, which diminishes the chances of complete cure considerably (Pui et al., 2004).
1.7 Subtypes of BCP-ALL

In light of early treatment successes, researchers began to investigate modifications of standard treatment regimens to accommodate variability in weight, age, and response to therapy among children with ALL. Individualized treatment plans were implemented where some patients received a reduced intensity course of therapy to minimize drug toxicity while others received drug intensification to maximize response.

More recently, research efforts have been directed at the elucidation of leukemogenic mechanisms implicated in ALL to identify specific protein mutants that can be used to design drugs tailored to interfere with the activity of these mutant protein targets. Identification of chimeric proteins produced from chromosomal translocations and gene expression profiles from microarray analyses are the primary techniques used to identify the potential therapeutic targets.

The main subtypes of BCP-ALL involve a large variety of genetic alterations, including point mutations and cryptic deletions, but they are characterized by gross chromosome changes such as hyperdiploidy (in ~35% of BCP-ALL cases) leading to an increased gene dosage or translocations, the latter seem to arise mainly before birth at the level of hematopoietic stem cells during fetal hematopoiesis (Greaves and Wiemels, 2003).

Chromosome translocations, mostly caused by double-strand DNA breaks involve illegitimate recombination or juxtapositioning of normally separate genes to active regulatory elements as in case of MYC with the Immunoglobulin Heavy Chain (Rabbitts 1994, 1993).

However, more commonly in leukemia an in-frame chimeric or fusion gene is created, generating a hybrid protein with altered properties - usually resulting in activated kinase activity or novel transcriptional regulation (Look, 1997; Rowley, 1998) (Tables 1 and 2).
<table>
<thead>
<tr>
<th>Chromosome abnormality</th>
<th>Molecular lesion</th>
<th>Frequency (%)</th>
<th>Functional Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(12;21)(p13;q22)</td>
<td>TEL (ETV6)‐AML1 fusion</td>
<td>~ 20 in BCP- ALL</td>
<td>Chimeric Transcription Factor∗</td>
</tr>
<tr>
<td>t(1;19)(q23;p13)</td>
<td>E2A‐PBX1 fusion</td>
<td>~ 5 in BCP- ALL</td>
<td>Chimeric Transcription Factor</td>
</tr>
<tr>
<td>t(9;22)(q34;q11)</td>
<td>BCR‐ABL fusion</td>
<td>~ 5 in BCP- ALL</td>
<td>Activated Kinase</td>
</tr>
<tr>
<td>dic(9;12)(p13;p13)</td>
<td>PAX5‐TEL (ETV6) fusion</td>
<td>~ 1 in BCP- ALL</td>
<td>? *</td>
</tr>
</tbody>
</table>

Table 2. AML1, which is an important positive transcriptional regulator, might be switched to a transcriptional repressor in these fusions. The function of PAX5‐TEL (ETV6) fusion product (Ets variant gene 6 = TEL oncogene) resulting in a dicentric chromosome has yet to be elucidated.
1.8 PAX5-ETV6 fusion

PAX5 was recently shown to be the most frequent target of somatic mutation in BCP-ALL. Mono-allelic deletions, cryptic translocations or point mutations of Pax5 were detected in about 30% of BCP-ALL cases examined (Mullighan et al., 2007).

PAX5-ETV6 fusion defines most of the cases with dic(9;12)(p13;p13). It is a recurrent chromosome abnormality that accounts for ~1% of childhood ALL, almost exclusively BCP-ALL (Strehl et al., 2003; Fazio et al., 2008).

This fusion product was first described 8 years ago. Until today not much is known about the molecular or cellular functions of the resulting fusion product. It is known that this translocation results in a most frequently dicentric fusion of the N-terminal DNA binding paired box domain of Pax5 on chromosome 9 to the almost full sequence of ETV6/TEL gene on chromosome 12 (Cazzaniga et al., 2001). In this fusion, ETV6 retains all of its DNA or protein interacting domains, HLH (SAM/pointed) and Ets. The combination of the mentioned facts and the physiological roles of the genes involved, leads to the assumption that the PAX5-ETV6 fusion protein could act as an aberrant transcription factor, retaining the DNA binding domain of PAX5 and both the dimerization and DNA binding domains of ETV6. Ten years ago ETV6 was shown to act as a potent transcriptional repressor (Lopez et al. 1999). Recently (2008), the group of Giovanni Cazzaniga was the first to publish an in vitro transient transfection study investigating the oncogenic role of PAX5-ETV6 fusion protein. Indeed, they were able to show that PAX5-ETV6 is a nuclear protein thus supporting the possibility of it being a transcription factor. They were also able to show the specific interaction of PAX5-ETV6 and mSin3A corepressor complex. The regulatory domains of ETV6 are known to recruit transcriptional cofactors such as mSin3A, NCoR and HDAC3 leading to transcriptional repression of its target genes (Chakrabarti et al., 1999; Guidez et al., 2000; Wang et al., 2001). Since Pax5 inhibitory and activator domains are lost, it would make sense that PAX5-ETV6 is guided by the actions of the untouched regulatory domains of ETV6. The DNA binding paired domain of PAX5 which is left intact during the fusion might still be able to bind its targets, which would mean the deregulation of PAX5 target genes by ETV6.

At this point, all BCP-ALL cases analyzed have shown only a monoallelic loss of PAX5, indicating a haploinsufficiency or the generation of hypomorphic alleles. However, this
does not explain leukemogenesis, as B cell development is normal in heterozygous Pax5+/- mice (Urbanek et al., 1994; Nutt et al., 1999). Therefore, the PAX5 fusion proteins are thought to contribute to BCP-ALL formation by interfering with the function of the Pax5 wild-type allele (Cobaleda et al., 2007). The group of G. Cazzaniga was able to support this hypothesis in vitro by showing that PAX5-ETV6 was able to interfere with the transactivaton function of wild-type Pax5 in transient transfection assays (Fazio et al. 2008). Therefore, the possibility prevails, that the PAX5-ETV6 fusion protein could contribute to BCP-ALL formation by acting as constitutive repressor to antagonize PAX5 activity, which is provided by the second, wild-type Pax5 allele.

The Pax5 gene contains a potent B-cell specific enhancer in the 5’ part of the large intron 5 (Decker et al., 2008). Interestingly, breakpoints of all previously mentioned Pax5 fusion proteins (Table 1), except for PAX5-ETV6 lie downstream of this enhancer. The breakpoints of PAX5-ETV6 translocation lie in the intron 4 of Pax5 and intron 2 of Etv6. Importantly, recent chromatin profiling has revealed a putative enhancer in the 3’ sequences of the Etv6 intron 2, which likely directs transcription of the Pax5/Etv6 fusion gene in BCP-ALL in the absence of the Pax5 enhancer (McManus S., Busslinger M., unpublished data).
1.9 **Aim of the thesis project**

Most current treatments of childhood ALL are biologically crude and, although effective, are associated with considerable toxicity and morbidity (Greaves, Wiemels, 2003). Clearly, there is a need for further improvements in treatment, preferably based on the biological features of the disease. It is widely anticipated that the next major advance in the treatment of ALL will involve the use of designer therapies developed to specifically interfere with particular molecular abnormalities producing the leukemogenic aberration to the normal signal transduction pathways.

The dicentric translocation dic(9;12)(p13;p13) was first described more than two decades ago (Carroll et al., 1987), it was identified as a Pax5-Etv6 translocation 8 years ago (Cazzaniga et al., 2001). Still, the molecular and cellular effects of this particular translocation, responsible for 1% of all childhood BCP-ALL cases, stay largely unknown (Strehl et al., 2003; Fazio et al., 2008). The elucidation of these questions is often made difficult by the following facts. The Pax5-Etv6 translocation is not a very frequent event therefore little primary tumor tissue can be made available for analysis. There is usually enough material for the identification of the responsible translocation, but not for allowing a systemic investigation of its molecular effects. Furthermore, the malignant B-lymphocytes cannot be cultured in vitro and thus no established cell lines are available for analysis.

The aim of the project to be described was the reconstruction of the Pax5-Etv6 translocation by generating a conditional knock-in allele in the mouse (*Mus musculus*).

The conditional activation of Pax5-Etv6 translocation allele with a B-cell-specific Cre line will, for the first time, enable the investigation of the following questions:

1.) **Upon short activation, does the Pax5-Etv6 translocation allele interfere with the normal B-cell development?**

2.) **Which pathways and/or molecular functions of the wild-type Pax5 protein are affected by this fusion product?**
3.) Does this translocation perturb the B-cell regulatory network by interfering with the function of other relevant transcription factors?

4.) Upon long-term activation, will this translocation lead to the development of B cell-specific leukemia?

5.) Which oncogenes or tumor suppressor genes cooperate with PAX5-ETV6 fusion product to accelerate the formation of a neoplastic disease?

We hope that the generation of this transgenic animal will help to answer some questions about the cellular and the molecular nature of acute lymphoblastic leukemia and therefore to contribute on the long run to more precise and effective treatment of the affected children.
2 Approach and Results

Generation of a knock-in mouse requires several well-planned, sequential steps. First is the generation of the desired allele in the form of a targeting vector. This step is routinely accomplished by cloning and/or recombinENGINEering methods in the bacterial organism *E.coli*. Second, the isolated DNA of the targeting cassette has to be brought into the genome of the embryonic stem cells of the mouse (mESCs). The rare event of homologous recombination has to be successfully identified by PCR and Southern Blotting techniques before the mESCs can be prepared for injection into mouse blastocysts. Finally, pups born have to be inspected for the degree of their chimerism, which is estimated from their fur color. High degree of chimerism means higher probability for the following crossings to result in offspring with germline transmission of the targeted allele.

2.1 Generation of conditional *Pax5* knock-in alleles

2.1.1 Strategy

A targeting vector can have multiple functions, which allow the introduction of genetic alterations into the genes of interest by homologous recombination in the embryonic stem (ES) cells of the mouse. The following is a general strategy outline for the generation of the *Pax5-Etv6* targeting vector (Figure 8).

A *loxP* site was inserted into intron 3, and a stop codon at the end of the paired domain (Prd) in exon 4 of *Pax5*. A transcription stop cassette (six SV40 polyA sites) and a second *loxP* site follow. Downstream of the floxed exon 4 stop cassette, we inserted the same 3’ splice site and exon 4, which this time is fused to the human *Etv6* cDNA sequence and a C-terminal biotin tag (de Boer et al. 2003). The presence of the stop cassette makes sure that the *Pax5*<sup>Prd</sup> allele should only express the paired domain of *Pax5*. The entire biotinylated PAX5-ETV6 protein from the *Pax5<sup>Etv6</sup>* allele should be expressed only upon the Cre-mediated deletion of the floxed exon 4 stop cassette. A faithful, B-cell-specific expression of the *Pax5* knock-in allele is made sure by the presence of a powerful upstream promoter and a downstream enhancer in intron 5.
Figure 8. Generation of the Pax5Prd and Pax5ETV6 alleles by the insertion of the Etv6 gene into the Pax5 locus. Image courtesy of M. Busslinger.

The multiple tags, including the biotin tag at the end of the fusion cassette, will be used for the isolation, purification and localization of PAX5-ETV6 protein (Figure 9). The glycine linker, consisting of four glycines, functions as a flexible linker between the last amino acid of PAX5-ETV6 and the tag. The following V5 tag can be useful for further purification steps. Monoclonal antibodies with specificity for this epitope are available. V5 tag was recently reported to give almost as great results as the biotin tag when used for chromatin-immunoprecipitation (ChIP) (Kolodziej et al. 2009). Therefore, the V5 tag could substitute for an inefficient biotin tagging in case of reduced accessibility in a cross-linked chromatin. The following two TEV cleavage sites allow the elimination of the biotin tag after protein purification by a specific proteolytic cleavage. The last one is the biotin acceptor sequence, followed by a stop codon. The lysine on the biotin acceptor sequence can be specifically biotinylated by the E.coli biotin ligase BirA. BirA is ubiquitously expressed from the Rosa26BirA allele; endogenous proteins are not recognized by the enzyme and are not biotinylated. The biotinylation enables a strong biotin-streptavidin interaction, which in turn allows easy one-step protein-protein and protein-chromatin pull-downs with streptavidin beads.
Our targeting vector also carries genes for positive and negative selection. Positive selection was used to propagate only those mESCs in which homologous recombination had occurred. For this purpose we used the neomycin gene under the control of the eukaryotic phosphoglycerate kinase promoter (PGK-neo), which renders cells resistant to neomycin/G418. The selection gene was flanked by Frt sites, which allowed its subsequent deletion by Flp recombinase.

Negative selection was used to kill the cells, in which random integration had occurred instead of homologous recombination. Therefore the DT-A (diphtheria toxin A) gene was present outside the homology regions and could thus only be inserted into the genome by random integration, leading to the expression of the lethal toxin.

Another important feature of the described strategy is the future possibility to generate additional Pax5 translocation alleles by cDNA replacement. This would allow the elucidation of the functions of some other Pax5 fusion products such as Pax5-Foxp1,
Pax5-Znf521 and Pax5-ELN. All of them are thought to somehow interfere with the wild type Pax5 function and consequently with B cell development. The sequences of the mentioned fusion partner genes are similar to ETV6 in that they also provide a second DNA binding domain and therefore could bind their target genes not only through the paired domain of Pax5 but also through their very own binding domains. All of the above mentioned fusion proteins were shown to interfere with the transactivation function of the wild type Pax5 in transiently transfected cells (Bousquet et al., 2007; Mullighan et al., 2007; Kawamata et al., 2008), this is an indication for a similar function of these fusion proteins and is a good reason for further investigation of their function in vivo. The unique restriction sites BspEI and KpnI upstream of the biotin tag will allow the replacement of the Pax5-Etv6 sequence with the corresponding Pax5-Foxp1, Pax5-Znf521 and Pax5-ELN cDNA sequences (Figure 10).

![Figure 10](image.png)

*Figure 10. The presence of unique BspEI and KpnI restriction sites allows the generation of additional Pax5 translocation alleles by cDNA replacement. Image courtesy of M. Busslinger*

### 2.1.2 Stepwise cloning of Pax5-Etv6 targeting vector

The Pax5-Etv6 targeting vector was cloned from scratch. Therefore, a polylinker with the necessary restriction sites had to be constructed first. The multiple cloning site (MSC), carrying the following restriction sites: NotI, Sall, Ndel, BsiWI, BspEI, KpnI, Ascl, Pmel, SacI, PacI, was subsequently inserted into a DT-A containing retrieval plasmid. This plasmid was going to be used for all the following successive cloning steps (Figure 11).
The next step was the generation of two very similar constructs both of which would carry the long homology arm and the neomycin resistance cassette under control of a PGK promoter. Second homology arms differed in their lengths, the short one being used for the real targeting vector whereas the construct with the second long homology arm was used as a control vector for testing the nested PCR analysis.

The first gene to be inserted into the polylinker was the PGK-neo. During the cloning process a mutation in the DNA sequence of neomycin was discovered. The neomycin resistance gene, originally derived from transposon Tn5, encodes the enzyme neomycin.
phosphotransferase II, which confers resistance to various aminoglycoside antibiotics, including kanamycin and G418. The mutation involved a glutamic to aspartic acid conversion at residue 182 of the chimeric gene, which was previously reported to substantially reduce phosphotransferase activity, without affecting the stability of the neomycin phosphotransferase II mRNA or protein (Yenofsky et al. 1990). We had to repair the neomycin resistance cassette and then subclone it into the polylinker-carrying plasmid.

The homology arms were obtained by recombineneering also known as recombination mediated genetic engineering in E.coli and subsequently cloned into the previously mentioned DT-A plasmid.

In parallel to the cloning of the two different homology arm/neo resistance constructs, a fusion construct was being cloned (top of Figure 11). The fusion construct consists of six pieces, which had to be cloned in six subsequent steps in a separate plasmid, then cut out with the restriction endonucleases Sall and Ascl and inserted into the homology arm constructs creating the complete targeting and control vectors. This was the most efficient strategy due to the restrictions given by the enzyme restriction sites available in the polylinker sequence.

Many of the fragments used for cloning and recombineneering were obtained by PCR, some of the fragments were directly cut out of different plasmids already carrying the genes necessary for our construct.

### 2.2 ES cell targeting

The DNA used for ES cell targeting was obtained by maxi preparations and a subsequent phenol/chlorophorm extraction. After a round of ES cell work practice with feeder-free CCE ES cells (cell line derived from 129/Sv mouse strain), feeder-dependent A9 mES cells were targeted. A9 mES cells are dependent on mouse embryonic fibroblasts (MEFs) derived from the DR4 mouse strain (Tucker et al. 1997). The feeder layer is resistant to neomycin/G418, 6-thioguanine, puromycin and hygromycin, and survives the selection stress during the targeting process.
A9 ES cells were targeted twice with the vector Pax5-Etv6-BIO as well as the control vector. 352 Pax5-Etv6-BIO clones and 32 controls were picked.

### 2.2.1 Screening for positive clones by nested PCR

After the completion of the targeting and control vectors, several PCR primers were ordered for nested PCR. Nested PCR was used to screen for ES cell clones positive for homologous recombination. The primers were accordingly chosen to ensure a PCR product only when homologous recombination had occurred, or when the control vector was used. Three forward primers were located at the beginning of the neomycin resistance cassette, four reverse primers were located outside the short homology arm at the end of the long homology arm, which is present only in the control vector and in the genome. This implicated additional caution for the handling of the cells carrying the control vector during the ES cell work. Contamination with control vector would result in false positive clones. The primers were subsequently tested for the best combination during the practice round with the CCE mES cells to ensure optimal PCR amplification. The reaction was also optimized using different dilutions of the control vector mixed with unrelated genomic DNA in order to ensure the generation of a PCR product even at very low DNA concentrations. Forward primers 2 (MB 9559)+6 (MB 9560) and reverse primers 3 (MB 9561)+5 (MB 9562) performed best and were chosen for ES cell screening. Subsequently, nested PCR was run on the targeted clones. Both rounds together yielded four positive clones. Two clones from the first and two from the second round, resulting in a targeting frequency as low as 1%.
Figure 12. Nested PCR. A) seven nested primers were ordered, reverse primers were designed to bind outside of the short homology arm. B) After the first test round, primers 2 (MB 9559) and 6 (MB 9560) were chosen for the outer reaction, 3 (MB 9561) and 5 (MB 9562) for the inner reaction. This figure shows the nested PCR test-round with increasing dilutions of control vector plasmid DNA mixed with genomic DNA. The last three rows show the test run on genomic DNA targeted with the control vector. +cnt/-cnt/-mix : positive, negative and PCR-mix controls.
2.2.2  Verification of correct integration by Southern blot

2.2.2.1  Strategy

All four positive clones were expanded for genomic DNA preparation for the southern blot. Four ES cell clones obtained with control vector were chosen for expansion to test whether some of those had undergone homologous recombination as well. The second, longer homologous arm might have been an advantage in that case.

The Southern blot strategy was accordingly designed (Figure 14). The radioactive probe is complementary to the sequence of Pax5 intron 4 and hybridizes with both the long homology arm as well as an external, non-vector genomic sequence. Digestion with the restriction enzyme BglII yields a 7872 base pair fragment from the wild type Pax5 allele and a 4731 base pair fragment from the Pax5-Etv6-Bio fusion allele.
Figure 14. Southern blot strategy. The radioactive 1021 base pair 3’ probe anneals to the sequence at the boundary of the long homology arm and the genomic intron 4 sequence of Pax5. The digest with BglI results in a 7.8 kb fragment of the wild type allele and a 4.7 kb fragment of the knock in allele.

2.2.2.2 Results

The restriction enzyme BglI was used to digest the ES-cell DNA. Clone nr 10C from the first ES-cell targeting round proved positive for correct integration of the fusion knock-in allele (Figure 15). The positive clone was gently thawed and expanded for the upcoming blastocyst injection.
Figure 15. Southern blot analysis of the nested PCR-positive A9 clones. Knock-in allele is present in clone 10 C. No homologous recombination took place in the control vector clones, despite the second longer homology arm present in the targeting vector.

2.3 Generation of chimeric mice

The correctly targeted clone was injected into 3.5dpc C57BL/6 blastocysts in two injection rounds. The blastocysts were subsequently implanted into the uteri of pseudopregnant B6CBA females. In total, eight 100% chimeric male pups were born.

The degree of chimerism is determined by the contribution of the injected mES cells to the chimeric mouse. This is estimated from the fur color of the animal (Figure 16). Brown is dominant over black. A9 is a hybrid mES cell line (129Sv x C57BL/6) deriving from a hybrid mouse consisting of the brown 129SV and the “black” C57BL/6 background. Therefore, black fur derives from C57BL/6 blastocysts, whereas brown fur can only be derived from the injected A9 cells. A high degree of chimerism means high probability for the consequent crossings of chimeras with C57BL/6 mice to result in offspring with germline transmission of the targeted allele.
Figure 16. One of the eight 100% chimeras with the Pax5⁺/Pax5ETV6⁺ genotype. Photograph taken at the age of 6 weeks.

2.4 Germline transmission and neomycin cassette deletion

Germline transmission occurs when germ cells derived from the injected A9 cells are passed on from the chimera to the next generation. The chimeras were crossed with FLPe deleter mice. Southern blot analysis was performed to prove germline transmission as well as neomycin cassette deletion. Figure 17 portraits the Southern blot strategy used for the detection of neomycin cassette deletion. Figure 18 shows the proof for the successful germline transmission and neomycin cassette deletion.

Figure 17. Southern blot strategy for the detection of neomycin cassette deletion. After the deletion of the neomycin resistance cassette, digest with BglII results in a 7.8 kb fragment of the wild type allele and a 12.7 kb fragment of the knock-in allele.
Figure 18. A) From right to left Southern blot analysis of two of the eight chimeras confirming the correct integration of the knock-in allele. The remaining two lanes with a knock-in size band result from a crossing of a chimera with an FLPe mouse (FLP deleter with a C57Bl/6 background). The knock in band confirms the germline transmission. B) Southern blot analysis of pups from another cross of a chimera and an FLPe. Five of the analyzed pups show successful germline transmission of the knock in allele. One of them also carries a deletion of the neomycin cassette.

The mouse breedings and Southern blot analysis was performed by Barbara Werner.
3 Outlook and Discussion

Aim of thesis project was the generation of a Pax5-Etv6 knock-in mouse, which was successfully accomplished in a time span of one year. Germline transmission and neomycin cassette deletion were successful as well. A new and promising model organism was therefore made available for leukemia research.

From now on the new model organism will be monitored and thoroughly analyzed for short as well as long-term effects of the knock-in allele on B cell development.

Before the conditional activation of the knock-in allele, the function of the $\text{Pax}5^{\text{Prd}/+}$ allele alone can be examined. Flow cytometry analysis of the B cell compartment in the bone marrow and spleen of heterozygous $\text{Pax}5^{\text{Prd}/+}$ mice will determine whether the polypeptide resulting from the Pax5 paired domain alone can act as a dominant-negative protein interfering with the function of the wild type Pax5 protein.

The hematopoietic-specific Vav-cre line (de Boer et al., 2003) will subsequently be used to generate $\text{Pax}5^{\text{ETV6}/+}$ allele in the Vav-cre $\text{Pax}5^{\text{Prd}/+}$ mouse. The resulting $\text{Pax}5^{\text{ETV6}/+}$ mice can then be compared to the $\text{Pax}5^{\text{Prd}/+}$ mice. This should directly determine the additional B cell developmental defects contributed by the ETV6 sequence to the fusion protein.

In order to gain some insight of the $\text{Pax}5^{\text{Prd}/+}$ and $\text{Pax}5^{\text{ETV6}/+}$ functions at the molecular level, a cDNA microarray analysis can be performed to determine the gene expression pattern of the pro-B cells. This can be subsequently compared to the already available published data on the expression patterns of the wild-type as well as $\text{Pax}5^{-/-}$ pro-B cells (Delogu et al., 2006; Schebesta et al., 2007).

Pro-B cells can also be cultured in vitro. The biotinylation of Pax5-Etv6 allows streptavidin-mediated chromatin precipitation and subsequent Solexa sequencing of the precipitated DNA thus leading to the genome-wide identification of Pax5-Etv6 target genes. In vitro cultured pro-B cells of the $\text{Pax}5^{\text{ETV6}/+}$ mouse, which also express the $E.coli$ BirA ligase from the Rosa26$^{\text{BirA}}$ allele (Driegen et al., 2005), can be used for this purpose.
This kind of a genome-wide Pax5-Etv6 target map can then be generated and compared to the available genome-wide target map obtained from the pro-B cell analysis of Pax5\textsuperscript{bio/bio} Rosa26\textsuperscript{BirA/+} mice in M. Busslinger’s laboratory (IMP Vienna). The comparison of the wild-type Pax5 target genes with the target genes of Pax5-Etv6 will reveal the alternative targets of the fusion protein as well as the usual targets, which might now be deregulated by the Pax5 fusion protein.

In the long run, it will be interesting to see whether Pax5\textsuperscript{ETV6/+} mice develop leukemia. Tumorigenesis is a multistep process and usually, a single chromosomal translocation is not sufficient to induce leukemia. Cooperating oncogenic lesions might be necessary (Hanahan and Weinberg, 2000; Mullighan et al., 2007). Chromosomal aberrations thought to be exclusively associated with leukemias and lymphomas have been detected in normal individuals, indicating that such translocations can be generated without apparent oncogenic consequences (Brassesco et al., 2008). Besides, studies with ETV6-RUNX1 knock-in mice have shown that the expression of this hybrid gene is not sufficient for the induction of ALL (Andreasson et al., 2001). Human BCP-ALL cases characterized by PAX5 mutations frequently contain additional heterozygous Ikaros (Ikzf1) mutations or ETV6-RUNX1 translocations (Mullighan et al., 2007). Moreover, BCP-ALL is often characterized by the deletion of CDKN2A locus coding for the p16\textsuperscript{INK4A} inhibitor of cyclin D-dependent kinases (Sulong et al., 2008). Therefore, it would make sense to cross the Pax5\textsuperscript{ETV6/+} mice with Ikzf1\textsuperscript{+/-} (Wang et al., 1996), Cdkn2a\textsuperscript{+/-} (Kamijo et al., 1997) or conditional Etv6-Runx1 knock-in mice (in collaboration with Dr. H. Hock, Harvard Medical School, Boston) and see if tumors arise. If tumors arise, leukemic cells of these mice can be further characterized by analyzing their gene expression profile using cDNA microarray analysis. Direct targets of PAX5-ETV6 protein can then be identified by ChIP sequencing. Finally the expression of all newly identified and relevant genes can be compared to the available human BCP-ALL samples (in collaboration with Dr. S. Strehl, Children’s Cancer Research Institute, Vienna).

Additionally, as described earlier, the cloning strategy of the Pax5-ETV6 fusion envisions the quick generation of the fusion product Pax5-Foxp1, Pax5-Znf521 and Pax5-Eln knock-in alleles as well. As soon as these targeted mice become available, a comparison of
Pax5<sup>ETV6</sup>/+, Pax5<sup>Foxp1</sup>/+, Pax5<sup>Znf521</sup>/+ and Pax5<sup>Eln</sup>/+ mice could reveal whether all four fusion-proteins interfere with B cell development in similar or different ways.

To summarize, PAX5 has been shown to be the target of somatic mutation in about 30% of pediatric ALL cases. The identified mutations resulted in reduced levels of PAX5 protein or the generation of hypomorphic alleles (Mullighan et al., 2007). In childhood BCP-ALL PAX5 rearrangements occur at an incidence rate of about 2.5% (Nebral et al., 2008). The dicentric translocation resulting in PAX5-ETV6 fusion is present in about 1% of all childhood BCP-ALL cases (Strehl et al., 2003). The resulting product retains the DNA-binding paired domain of PAX5 and gains the DNA binding ETS as well as the transcription factor homo and heterodimerization SAM domains of the transcriptional repressor ETV6. The second wild-type allele of Pax5 is left intact. As Pax5<sup>+/−</sup> mice show no defects in B cell development (Cobaleda et al., 2007), a hypothesis can be put forward that the fusion protein negatively interferes with the wild-type PAX5 functions.

Of course it could as well be that cooperating somatic lesions of the second Pax5 allele such as deletions, copy number changes or internal amplifications are responsible for the complete inactivation of both alleles.

The fusion partner ETV6 is a member of the ETS family of transcription factors. It is known that Ets proteins and Pax-5 interact via multiple contacts between their respective DNA-binding domains (Garvie et al., 2001). For instance, binding of the mb-1 promoter by Pax-5 and Ets proteins is required for functional promoter activity (Fitzsimmons et al., 1996), but interestingly, only the paired domain of Pax-5 is required for activation (Nutt et al., 1998). PAX5-ETV6 fusion protein could bind the wild-type PAX5 and aversely regulate PAX5 functions via its Ets domain. Recent data (Mullighan et al., 2007) also showed that most of somatic mutations affecting Pax5 localize to the paired domain of the protein.
4 Materials and Methods

4.1 Standard techniques used for molecular biology

4.1.1 Deoxyribonucleic acid extraction and purification protocols

4.1.1.1 Plasmid DNA mini-preparation

Minipreparation of plasmid DNA is a rapid, small-scale isolation of plasmid DNA from bacteria. It is based on the alkaline lysis method invented by the researchers Birnboim and Doly in 1979.

When bacteria are lysed under alkaline conditions both DNA and proteins are precipitated. After the addition of acetate-containing neutralization buffer the large and less supercoiled chromosomal DNA and proteins precipitate, but the small bacterial DNA plasmids can stay in solution.

Minipreparation of plasmid DNA was used for the preparation of up to 20 µg of high-copy plasmid DNA from cultures of Escherichia coli using the QIAprep® miniprep kit from Qiagen. A single colony was picked from a freshly streaked selective plate and a culture of 1–5 ml LB (Luria-Bertani) medium containing the appropriate selective antibiotic was inoculated. After 12–16 h incubation at 37°C with vigorous shaking plasmid DNA was isolated according to the manufacturers protocol.

4.1.1.2 Mini-preparation from BAC clones

Qiagen QIAprep® buffers were used for the following procedure:

1. Inoculate a single, isolated bacterial colony in 3ml LB medium supplemented with 25µg/ml chloramphenicol. Use a 12-15ml snap-cap polypropylene tube. Incubate at 37°C for 12-16h with vigorous shaking.
2. Pellet cells by centrifugation at 3000 rpm for 10 min
3. Aspirate supernatant. Resuspend the pellet in 0.3ml of buffer P1 containing 100 µg/ml RNase A (stored at 4°C).
4. Add 0.3ml of buffer P2 and mix gently. Incubate at room temperature for 5 min.
   The appearance of the solution should change from turbid to almost translucent.
5. Slowly add buffer P3 to each tube and gently shake. A thick, white precipitate of
protein and \textit{E.coli} DNA will form. Incubate on ice for 5-10 minutes.

6. Centrifuge the suspension at 10000 rpm for 10 min at 4°C.

7. Keep on ice immediately after centrifugation. Remove supernatant and add a microcentrifuge tube containing 0.8ml ice-cold isopropanol. Mix well by inverting the tube few times and keep on ice for 5min. At this stage, if necessary samples can be frozen at -20°C o/n.

8. Spin at 4°C in a microcentrifuge for 15 min at 13000 rpm.

9. Carefully remove the supernatant and add 0.5ml of 70% ethanol to wash the pellet. Centrifuge at 4°C for 5 min at 13000 rpm. Repeat this step if necessary.

10. Carefully aspirate the supernatant and let the pellet air-dry at room temperature. Resuspend in 40µl TE buffer. Do not pipet to resuspend the pellet. Gently tap the tube at the bottom several times to resuspend the BAC DNA. For large PAC clones resuspension may take up to 1h.

\textbf{4.1.1.3 Plasmid DNA maxi-preparation}

DNA maxi preparations were done using the Qiagen Plasmid Maxi Kit. A single colony was picked from a freshly streaked selective plate and a culture of 1–5 ml LB (Luria-Bertani) medium containing the appropriate selective antibiotic was inoculated. After 6-8 h incubation at 37°C with vigorous shaking the starter culture was diluted 1/500 to 1/1000 into selective LB medium. It was subsequently grow at 37°C for 12–16 h with vigorous shaking (approx. 300 rpm). The bacterial cells were harvested by centrifugation at 6000 x g for 15 min at 4°C. The following DNA maxi preparation was done according to the manufacturers protocol. The protocol used was designed for preparation of up to 100µg of high- or low-copy plasmid DNA.

\textbf{4.1.1.4 DNA extraction from agarose gels}

Gel electrophoresis was used as a preparative technique to separate DNA according to its size prior to cloning. Low-melting Seaplaque Agarose gels in TAE buffer were used. Ethidium Bromide was added to the gel as intercalating agent for staining nucleic acids. DNA bands were visualized in UV-light (366nm) and excised with a scalpel. QIAquick Gel Extraction Kit from Qiagen was used fro the DNA extraction from the agarose gels. The procedure was performed according to the manufacturers protocol.
4.1.1.5 DNA precipitation using Ethanol

DNA had to be precipitated during cloning between double digest cloning steps with unmatching restriction endonuclease buffers.

Procedure

1. Add 0.03Vol of 3M NaAc pH 5.2 and 2.5Vol of pure Ethanol to the cloning reaction-mix and precipitate the DNA at -20°C for 20 minutes.
2. Spin for at 13000rpm for 10 min in a table-top microcentrifuge.
3. Wash the pellet with 70% Ethanol
4. Re-dissolve the pellet in sterile MonoQ water
5. Go on with cloning → add the second restriction endonuclease and the matching buffer

4.1.1.6 Plasmid DNA purification by phenol/chlorophorm extraction

The targeting vector plasmid DNA obtained from a QIAGEN Plasmid Maxi preparation had to be linearized and Phenol/Chloroform purified before electroporation into ES cells.

This method is effectively used for protein removal from nucleic acids. Proteins precipitate at the organic/aqueous interphase in form of a white cloudy substance. The aqueous phase is extracted several times using Phenol-Chloroform-Isoamylalcohol (25:24:1) mix until no more protein precipitates. The final chloroform extraction is used to remove phenol leftovers, which might still be dissolved in the aqueous phase.

Procedure

1. Linearize the vector by digesting 100µg of the maxiprep DNA for 4h with the unique cutter (PvuI or SgrAI) restriction endonuclease (0.5U/µg) in a total volume of 40µl.
2. Fill up to 500µl with sterile MonoQ water (use safe lock eppendorf microcentrifuge tubes )
3. Add 1Vol of Phenol, shake and spin at 13000 rpm for 5 min at RT
4. Transfer aqueous phase into a fresh safe lock tube
5. Purify again with phenol
6. Purify 2x with chloroform
7. Fill up to 500µl with sterile MonoQ water
8. Add 10% (50µl) 3M NaAC
9. Add 0.7 Vol (385µl) Isopropanol, shake gently, DNA precipitates.
10. Spin at 13000 rpm for 15 min at RT
11. Wash the pellet with 70% EtOH (500µl)
12. Spin at 13000 rpm for 5 min at RT
13. Air dry pellet and resuspend in 20µl TE Buffer pH 8.0
14. Run 1µl of the purified and linearized DNA on 0.9% Agarose gel.
15. Measure the DNA concentration, 10 - 25µg of targeting vector are required for the electroporation into the ES cells.

4.1.2 Measuring DNA concentrations

The concentration and quality of sample DNA is measured with a UV spectrophotometer. A solution containing 50 µg/ml of double strand DNA (dsDNA) has an absorbancy (optical density) of 1.0 at a wavelength of 260 nm.

\[
\frac{50}{1.0} = \frac{\text{DNA}}{\text{OD read}} \\
\text{DNA} = 50 \times \text{OD read}
\]

The essential amino acid tryptophan has a wavelength of maximum absorption of 280 nm and is used as an indicator for purity vs. protein contamination of the DNA sample. If the sample is contaminated, additional OD will be measured, which decreases the OD ratio between 260 and 280 nm. Clean DNA has an OD 260/280 ratio between 1.8 and 2.0. If the measured value is somewhat smaller, it indicates a contamination.

All the concentration measurements were done using Nano Drop™ 1000 spectrophotometer:
1. With the sample apparatus open, a droplet of sample (1µl) is pipetted onto the measurement pedestal.
2. When the sample apparatus is closed, the sample arm slightly compresses the droplet and a sample column is drawn. Surface tension alone holds the sample in place. The spectral measurement is then made and quantification is made based on the tightly controlled path length of 1mm.
3. When the measurement is complete, the sample apparatus is opened and the
sample is simply wiped from both the sample arm and sample pedestal using an ordinary dry laboratory wipe.

4.1.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis is the method used to separate DNA or RNA molecules according to their size. This is achieved by moving negatively charged nucleic acids through agarose gel matrix with an applied electric field. DNA and RNA molecules are negatively charged in aqueous solutions due to their phosphate groups and will travel to the positive electrode. Nucleic acids starting with the size of 50 base pairs to several thousand base pairs can be separated by this method. Smaller molecules will travel faster than the large molecules and therefore appear under the large DNA fragments on the gel. In general, gels with 0.7 (good separation or resolution of large 5–10kb DNA fragments) to 2% good resolution for small 0.2–1kb fragments) of agarose are used for this purpose. The higher the agarose concentration, the lower the speed at which molecules will move through the gel matrix.

The most common dye used to make DNA or RNA bands visible for agarose gel electrophoresis is ethidium bromide (EtBr). It fluoresces under UV light when intercalated into DNA or RNA. EtBr is a known carcinogen. Protective clothing should be used (e.g. nitrile gloves) at all times; however, safer alternatives are also available, such as SYBR green and SYBR safe stains.

After electrophoresis the gel is illuminated with an ultraviolet lamp (usually by placing it on a light box, while using protective gear to limit exposure to ultraviolet radiation) to view the DNA bands. The ethidium bromide fluoresces reddish-orange in the presence of DNA, any band containing more than ~20ng DNA becomes distinctly visible. The DNA band can also be cut out of the gel, and can then be dissolved to retrieve the purified DNA. The gel can then be photographed usually with a digital camera.

Agarose gel electrophoresis was used for:

- restriction mapping of cloned DNA
- separation of restricted genomic DNA prior to Southern analysis
- PCR analysis
The agarose was always dissolved in a 0.5 x TAE (Tris Acetate EDTA) buffer and melted in a microwave oven. The solution was cooled on ice by vigorous shaking to the temperature of ~60°C. Ethidium bromide was added (final concentration 1 µg/ml) and the liquid gel was poured into appropriate form to solidify.

### 4.1.4 Polymerase Chain Reaction (PCR)

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers containing sequences complementary to the target region along with a DNA are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

PCR technique was frequently used to amplify DNA necessary for the construction of the targeting vector. It was also used during the ES cell screening for homologous recombination of the targeting vector.

### 4.1.5 Genetic engineering

#### 4.1.5.1 Polylinker construction

Polylinker construction was the first step towards the generation of Pax5 fusion alleles. The following technique was used:

**STEP 1 for each oligo (4X)**

- Undiluted oligo (100ng/µl)  0,5 µl
- Polynucleotide kinase  1 µl
- 10x PNK Buffer  1 µl
- 10mM dATP  1 µl
- H2O  6,5 µl
Incubate for 1h at 37°C
Pool all reactions
Incubate at 95°C for 5 min
Cool down
(This is included in the PCR machine Program LINKER, but you have to change some conditions, I think Heat is on 70°C...)

STEP 2
Ligation into Vector DNA
Since the polylinker DNA concentration is pretty high, use very little insert DNA
I did 3 ligations with decreasing insert concentrations 1μl, 0.5μl and 0.3μl, -CNT

STEP 3
Transform into DH5α

4.1.5.2 Recombeneering

Recombeneering (recombination-mediated genetic engineering) is a technique based on homologous recombination systems mediated by bacteriophage \( \lambda \) proteins in \( E.Coli \) to modify DNA. These homologous recombination systems mediate the efficient recombination of a target fragment into the DNA construct. The method makes use of a defective \( \lambda \) prophage to supply functions to \( E.Coli \) that protect and recombine the electroporated linear DNA. This defective prophage carries a deletion between \( cro \) and \( bioA \). The PL operon encoding \( gam \) and the red recombination genes, \( exo \) and \( bet \), is under the tight control of the temperature sensitive repressor (allele \( cI857 \)). Recombination functions can thus be transiently supplied by shifting the cultures to 42°C for 15 min. This recombination system does not require RecA function and depends primarily on the expression of Exo, Beta, and Gam. Gam inhibits the \( E. coli \) RecBCD nuclease from attacking the electroporated linear DNA, while Exo and Beta generate recombination activity.
The sequence homologies (or arms) flanking the desired modifications are homologous to regions 5’ and 3’ to the region to be modified. Importantly, the recombination is proficient with DNA homologies as short as 30–50 bps, making it possible to use PCR-amplified fragments as the targeting cassette.
It is a powerful method for fast and efficient construction of vectors for subsequent
manipulation of the mouse genome or for use in cell culture experiments. It is also an efficient way of manipulating the bacterial genome directly.

*E.Coli* strain EL 350 was used in our experiments. These bacteria contain a defective λ prophage with recombination proteins *exo*, *bet*, and *gam* being controlled by the temperature-sensitive repressor *cl857*. EL350 also contains a tightly controlled arabinose-inducible *cre* gene. *cre* will mediate recombination between two identical loxP sites.

**Transfer of BAC DNA into EL 350 *E.coli* cells**

**Procedure**

1. Pick a single colony of EL 350 *E.coli* from a freshly streaked selective plate and inoculate a culture of 5ml LB (Luria-Bertani) medium. Incubate for 12–16 h at 30-32°C with vigorous shaking.
2. Transfer 1 ml of this culture to 20 ml of fresh LB medium.
3. Incubate for ~2h at 30-32°C with vigorous shaking until the OD = 0.5.
4. To make the cells competent work on ice and use pre-cooled reagents and instruments, such as tubes, pipettes, etc. Use 10ml of bacterial culture per preparation.
5. Incubate on ice for 5 min.
6. Pellet bacteria by centrifugation at 4000rpm for 5 min at 0°C.
7. Remove the supernatant and re-suspend the pellet in 1ml of ice-cold sterile water and transfer the cell suspension to pre-cooled 1.5ml microcentrifuge tube.
8. Centrifuge the cell suspension for at 4000rpm for 5 min at 0°C.
9. Wash the pellet with ice-cold sterile water, then with 15% ice-cold glycerol.
10. Resuspend the pellet in 50µl of 15% ice-cold glycerol and transfer to electroporation cuvette (BioRad Gene Pulser cuvette, 0.2 cm).
11. Electroporate 1µl (~100ng) of BAC DNA using Bio Rad Gene Pulser set at 2.5kV, 25µF with the pulse controller set at 200Ω.
12. Add 1ml of LB medium to cells, incubate at 32°C for 1.5h with shaking and spread on chloramphenicol selective agar media.

**Insertion of targeting cassette**

**Procedure**

1. Cut out the targeting cassette flanked by the homology boxes with the appropriate
restriction enzymes from vector DNA and purify.

2. Pick a single colony of EL 350 E.coli containing the BAC of interest from a freshly streaked selective plate and inoculate a culture of 5ml medium. Incubate for 12–16 h at 30-32°C with vigorous shaking.

3. Transfer 1 ml of this culture to 20 ml of fresh selective LB medium and incubate at 30-32°C for ~2h with vigorous shaking until OD=0.5.

4. Transfer 10ml of the bacterial culture to a separate flask and incubate at 42°C for 15min with shaking to induce recombination enzymes.

5. Place the flask on ice immediately and incubate for 5min. To make cells competent follow steps 4-10 from previous protocol (Transfer of BAC DNA into EL 350 E.coli cells).

6. Electroporate 1-2µl of purified, linear targeting cassette DNA (same settings as in step 11 from previous protocol (Transfer of BAC DNA into EL 350 E.coli cells)).

7. Add 1ml of LB medium to cells, incubate at 32°C for 1.5h with shaking and spread on appropriate selective agar media.

### 4.1.5.3 Molecular cloning of targeting vectors

Restriction endonuclease digestion, ligation and heat shock-transformation were the standard techniques used for the generation of the targeting cassettes. DH5α cells were used for all procedures. For the long-term preservation of cells carrying cloned constructs Hogness buffer was used.

Hogness buffer for long term preservation of bacterial cells:

- 36mM K₂HPO₄ · 3H₂O
- 13mM KH₂PO₄
- 20mM Na₃-Citrat · 2H₂O
- 10mM MgSO₄ · H₂O
- 44% Glycerol

### 4.1.5.4 Electroporation

Electroporation is a quick and stable transfection method, used to deliver DNA for homologous recombination into bacterial or animal cells. It is a dynamic phenomenon that depends on the local transmembrane voltage at each cell membrane point. The cell membrane permeability to ions and macromolecules is increased, by exposing the cell to
short (microsecond to millisecond), high voltage electric field pulses. It is generally accepted that for a given pulse duration and shape, a specific transmembrane voltage threshold exists for the manifestation of the electroporation phenomenon (from 0.5V to 1V). This leads to the definition of an electric field magnitude threshold for electroporation (E\text{th}). That is, only the cells within areas where E≥E\text{th} are electroporated. If a second threshold is reached or surpassed, electroporation will compromise the viability of the cells and result in irreversible electroporation.

High DNA concentrations will increase transfection efficiency. Highly compromised cell viability leads to high electroporation efficiencies.

Electroporation was used for recombineering in \textit{E.coli} cells as well as for targeting mouse (\textit{mus musculus}) Embryonic Stem (ES) cells.

\subsection*{4.2 mES cell Culture}

\subsubsection*{4.2.1 General principles}

The culture of mES cells, whilst not difficult, is a labour-intensive process that requires considerable care and attention to detail. Although mES cells may continue to proliferate under suboptimal conditions, they readily lose their ability to contribute to chimeras and be transmitted through the germline. Hence it is vitally important to maintain optimal culture conditions at all times.

All used reagents were tissue-culture grade, and solutions made up with Mono-Q® or Milli-Q® water. Disposable plastics were used wherever applicable. Rigorous aseptic technique was maintained.

All cell cultures were maintained in a humidified incubator, gassed with 5% CO\textsubscript{2} in air, at 37°C.

The quality of the fetal-calf serum (FCS) in the culture medium is a critical factor determining the success of mES-cell derivation. Sera vary considerably between batches in their ability to support the proliferation of pluripotent mES cells in the undifferentiated state, and it is therefore crucial to test a range of samples and select only the best for mES cell culture. An FCS previously successfully tested on ES cells in our laboratory was used. Established mES cell lines divide rapidly and are maintained at a relatively high density, and therefore require regular maintenance: the medium was changed daily to prevent its acidification, and the cells passaged approximately every 2-3 days.
More efficient germline transition is achieved when targeted cells in addition to being co-cultured with feeder cells, are maintained in a medium containing leukemia inhibitory factor (LIF).

### 4.2.2 Mycoplasma contamination

Contamination with mycoplasma can be highly problematical for mES cell culture, being readily apparent neither macroscopically nor microscopically, and having calamitous consequences for the pluripotency of mES cells. Crucially, blastocyst injection of mycoplasma-contaminated mES cell lines results in embryo lethality, and a dramatic reduction in the numbers of chimeras obtained as well as the level of mES cell contribution to their tissues. Elimination of mycoplasma contamination is impractical, treatment with antimycoplasma agents being detrimental to mES cells. Therefore once mycoplasma is detected in either mES cells or feeder cells, the best course of action is to discard the cultures. The most common sources of mycoplasma infection are other contaminated cultures, sera, or laboratory personnel using poor aseptic technique.

### 4.2.3 Tissue culture Equipment

1. Water bath set at 37°C
2. Differently sized sterile tissue-culture dishes and flasks (e.g. 96, 24, 12, 6 well dishes, Nunclon).
3. Polystyrene pipets (25ml, 10ml, 5ml, 2ml, 1ml)
4. Filtered pipet tips (e.g. from Molecular Bio Products)
5. Sterile Falcon tubes (15ml, 50ml, Falcon)
6. Cryovials (1ml, Nunc)
7. 70% Alcohol
8. Phosphate-buffered saline (PBS) without Ca\(^{2+}\) or Mg\(^{2+}\)
9. High Glucose Dulbecco’s modified Eagle’s medium (DMEM)
10. Fetal-calf serum (FCS): this must be tested for optimal ability to support mES cell culture, aliquot into 50ml and store at -20°C (Batch No. ... Sigma)
11. \(\beta\)-mercaptoethanol stock solution (100x): Add 35.7\(\mu\)l to 50ml of PBS. Filter sterilize, aliquot 5ml per tube and store at -20°C
12. Penicillin (Cat. No. P4687 Sigma)
13. Streptomycin (Cat. No. S1277 Sigma)
14. Glutamine, 200mM (Cat. No. G7513 Sigma)
15. Murine recombinant LIF (ESGRO®; Cat. No. ESG 1106, Chemicon International)
16. MEM non-essential amino acids, 100x solution (Cat. No. M7145 Sigma)
17. Na-Pyruvate
18. Trypsin solution: make 5ml aliquots from the 10x stock solution and freeze immediately to maintain maximum activity. Always thaw a fresh aliquot before use.
19. Geneticin /G418
20. ESGRO® LIF (Leukemia inhibitory factor)
21. DMSO (Dimethyl sulfoxide, Sigma)

4.2.4 Tissue culture reagents

4.2.4.1 MEF culture medium

Combine the following constituents:
1. 450ml High glucose DMEM
2. 50ml FCS
3. 10ml PSG (Penicillin Streptomycin Glutamine premade aliquot)

4.2.4.2 MEF freezing medium

Prepare fresh before use
Combine the following constituents:
1. 50% FCS
2. 40% MEF culture medium
3. 10% DMSO

4.2.4.3 mES cell culture medium

1. 450ml High glucose DMEM
2. 5ml β-mercaptoethanol aliquot
3. 5ml Na-Pyruvate aliquot
4. 5ml aliquoted Non-essential amino acids
5. 10ml PSG (Penicillin Streptomycin Glutamine premade aliquot)

4.2.4.4 FCS/LIF medium mES cell medium

For full FCS/LIF medium combine:

1. 85ml mES cell culture medium (premade)
2. 15ml FCS
3. 10µl ESGRO® LIF (1000U/ml)

mES cell medium containing FCS and LIF has to be made fresh every second day.

4.2.4.5 G418 selection medium

For G418 selection medium combine:

1. 99ml full mES cell medium with FCS /LIF
2. 1ml of a 100X G418 stock solution (e.g. to make a 100x stock solution take 300mg of active geneticin (418mg of 716µg/mg active geneticin) and dissolve it in 10ml full mES cell medium)

4.2.4.6 mES cell freezing medium

Prepare fresh before use

Combine the following constituents:

1. 50% FCS
2. 40% full mES cell culture medium
3. 10% DMSO

4.2.5 Preparation of MEFs

ES cells were always cultured on irradiated, mitotically inactivated feeder layer of mouse embryonic fibroblasts (DR4 pMEF).

MEFs proliferate for only a limited period in culture; new stocks have to be prepared regularly. Aliquots may be cryopreserved, and so fresh stocks need only be prepared every few months.

Feeder cells must be mitotically inactivated to prevent their proliferation and consequent overgrowth of the mES cell culture. This can be achieved using e.g. γ-irradiation.
4.2.5.1 Expansion and gamma (γ) - irradiation.

1. Rapidly thaw a frozen aliquot of MEFs by holding the vial momentarily in a water bath set at 37°C; thoroughly swab the outside of the vial with 70% alcohol, and transfer the contents to a centrifuge tube containing 10ml MEF medium.

2. Pellet the cells in a benchtop centrifuge at 1500rpm for 5 min. Aspirate the supernatant, and gently resuspend the cell pellet in 2ml MEF medium. Add the cell suspension to 23ml MEF medium in a 140cm² cell culture dish and incubate.

3. Change the medium the following day only; there is no need for further medium change between passages. The dish should reach confluence on the second day after incubation. MEFs can be subcultured three or four times from this stage (i.e. to passage 5 or 6) at a split ratio of 1:3, following which they usually cease to proliferate.

4. To γ-irradiate the MEFs add 2ml of 1 x trypsin solution to the confluent 140cm² cell culture dishes and incubate at 37°C for 4-5 min. Rock the plate until the cells detach, add 5ml MEF medium, pipet up and down to give a single cell suspension and collect the detached cells in 50ml sterile Falcon tubes with MEF medium.

5. Place the Falcon tubes inside a ⁶⁰Co source, and γ-irradiate the cell suspension with 3000rads.

6. Pellet the cells in a benchtop centrifuge at 1500rpm for 5 min. Aspirate the supernatant, and gently resuspend the cell pellet in 0.5ml MEF medium per 140cm² cell culture dish.

7. Transfer 0.5ml aliquots of cell suspension into cryovials containing 0.5ml freezing medium, mix gently and freeze slowly at -70°C, before storing under liquid nitrogen.

8. After thawing one 0.5 MEF aliquot is sufficient to seed a 140cm² cell culture dish. Place the vial in a 37°C water bath thaw quickly, swab with 70% alcohol and follow the protocol from steps 1 and 2.

4.2.6 Mouse embryonic stem cell targeting

A9 mES cells were used for targeting.

Feeder cells were always plated one day before or at least 3 hours prior to ES cells to allow attachment to the tissue culture dish.
4.2.6.1 Expansion

DAY 1 Take ES cells with a low passage number and rapidly thaw the frozen aliquot by holding the vial momentarily in a water bath set at 37°C; swab the outside of the vial with 70% alcohol and transfer the contents to a centrifuge tube containing 10ml full ES cell medium. Pellet the cells in a benchtop centrifuge at 1000rpm for 5 min. Aspirate the supernatant and gently resuspend the cell pellet in 1ml full ES cell medium. Aspirate and replace the MEF medium on the 140cm² dish with 20 ml full ES cell medium. Add the ES cell suspension to the full ES cell medium on the 140cm² tissue culture dish and incubate. DAY 2 Change mES cell culture medium by carefully aspirating the old culture medium from the plate and slowly adding fresh medium to one side of the plate without disturbing the cells.

DAY 3 Passage the ES cells in ratio of 1:8, keep two 140cm² tissue culture dishes (one plate per targeting vector/control vector electroporation)

Note: Wash cells twice with PBS. Trypsinize by adding 2ml trypsin solution to the plate and incubating for 5 min at 37°C. Halt trypsinization by adding 1ml of mES cell medium. It is essential at this point to dissociate colonies into single mES cells, which is achieved by pipeting the cell suspension up and down. Monitor the progress of dissociation under a microscope and once a single cell suspension is obtained, transfer this into a sterile tube with mES cell medium, pellet the cells by centrifugation, aspirate the supernatant, resuspend the pellet in 8ml of mES cell medium and add 1ml to a 140cm² dish (previously covered with MEFs) with 20 ml full ES cell medium.

DAY 4 Change mES cell culture medium (FCS/LIF)

4.2.6.2 Electroporation

DAY 5 Electroporation

Procedure

1. Detach the cells by trypsinization (make sure it is a single cell suspension) and pellet by centrifugation. Dissolve cells (10 × 10⁶) in 0.8ml PBS (Phosphate Buffered Saline)
2. Add 15-25µg of purified targeting vector/control vector DNA to the cell suspension and mix well
3. Transfer the suspension to electroporation cuvette (BioRad Gene Pulser cuvette, 0.4cm)
4. Electroporate using Bio Rad electroporator at 0.24kV, 500F. White foam of dead
cells will form on the top of the cell suspension indicating successful electroporation.

5. Leave the cells in the cuvette; let them rest for 10min at room temperature in the sterile hood.

6. Transfer the electroporated cells into full ES cell medium, mix well and split to cell culture plates. (Split 1:4, keep four 56cm² plates for ES cells electroporated with targeting vector and one plate for cells carrying the control vector)

DAY6 Change ES cell culture medium (FCS/LIF)
36 hours post electroporation Start selection with G418 containing mES cell selection medium. Change selection medium every day.

4.2.6.3 Picking

Pick mES cell clones 5-7 days post electroporation.
Whether the colonies are ready to be picked has to be assessed on individual basis and depends on the ongoing stage of selection with G418, as well as the size and density of the colonies.

Procedure:
Prepare a 96 well cell culture dish by plating feeder cells in MEF medium at least 3 hours prior to picking. Exchange the MEF feeder medium with 200µl full mES cell medium immediately before picking. Only ideal colonies should be picked. Colonies should be slightly smaller than the end of a 20µl pipette tip. Edges should be clearly defined and there should be no cells flattening or differentiating along the outside edges.

1. Wash the 140cm² cell culture dish containing targeted mES cell colonies twice with PBS, carefully aspirate and add 10ml PBS to the dish.
2. Pick clones in 20µl PBS by detaching from the plate with the pipette tip aspirating in one motion. Place the clones into a 96 well PCR plate. This should be done quickly; cells should not be without medium for much longer than 30 min.
3. Add 20µl of 2x trypsin solution to each well and incubate at 37°C for 5min.
4. Pipet up and down in order to obtain a single cell suspension, transfer 20µl to the 96-well plate with feeders and incubate.
5. Add 100µl PBS to the cell suspension left on the PCR plate and continue picking the next plate.
4.2.6.4 Nested PCR

Primers used: Forward: MB 9559/MB9560; Reverse: MB9561/MB9562

DNA was prepared for PCR:

1. Spin the 96 well PCR plates in a bench-top centrifuge for 5min at 1500rpm.
2. Remove supernatant.
3. Add 10µl Proteinase K solution (1mg/ml) to each well and incubate for 1h at 55°C in a heated PCR block.
4. Add 50µl of sterile Water to each well and inactivate Proteinase K by incubating for 10min at 95°C

2µl DNA of ES cell genomic DNA was used for the reaction, controls were included.

Optimized Program was used:
94°C: 5min
94°C: 30sec
55.2°C: 30sec
72°C: 3min 15sec
72°C: 5min
10°C: Forever

PCR product was run on agarose gel.

Positive clones were expanded and aliquots frozen to obtain enough DNA for a southern blot.

4.2.7 Southern Blot Analysis

Southern blotting is the transfer of DNA fragments from an electrophoresis gel to a membrane support, resulting in immobilization of the DNA fragments, so the membrane carries a semi permanent reproduction of the banding pattern of the gel. After immobilization, the DNA can be subjected to hybridization analysis, enabling bands with sequence similarity to a labeled probe to be identified.

4.2.7.1 Necessary reagents

Proteinase K buffer (w/o SDS):
50mM Tris-HCl pH 8.0, 20m EDTA, 100mM NaCl
Depurination solution: 12.3ml 10.18M 37% HCL in 487.7ml sterile MonoQ water
Denaturaturation buffer: 0.2M NaOH, 0.6M NaCl
Neutralization buffer: 0.5M Tris pH7.5, 1.5M NaCl
Church buffer: 1% BSA, 1mM EDTA, 0.5M NaPi pH 7.2, 7% SDS
Wash Buffer: 1% SDS, 40mM NaPi pH 7.2

4.2.7.2 Proteinase K digestion and DNA preparation for Southern Blot

1. Expand the PCR-positive mES cell clones by passaging the cells every 2-3 days from a 96-well plate to a 24, 12, 6-well and finally a 140cm² plate and let grow for approximately one week.
2. Wash the cells twice with PBS, add 2ml trypsin solution and incubate for 5 min at 37°C.
3. Collect the cells with a pipette and add to a 15ml Falcon tube containing 5ml mES cell medium.
4. Pellet the cells by centrifugation at 1500 rpm for 5 min.
5. Aspirate the supernatant and dissolve the pellet in 4.5ml 1x Proteinase K buffer (without SDS).
6. Add 300µl Proteinase K (stock solution: 20mg/ml, final concentration: 1mg/ml) and mix well.
7. To lyse the cells quickly add 250µl of 20% SDS (final concentration 1% SDS) solution.
8. Incubate at 55°C over night.
9. Next day add 200µl of 5M NaCl (0.3M NaCl final concentration).
10. Extract 2x with 1 Volume (5ml) of Phenol, spin for 5min at 4000rpm (4°C)
11. Extract 2x with 1 Volume (5ml) of Chloroform, spin for 5min at 4000rpm (4°C)
12. Remove the aqueous phase and fill it up to the volume of 5ml with sterile water
13. Add 3 Vol. (15ml) of Ethanol, shake gently and DNA will precipitate.
14. Fish the DNA with a Pasteur pipette tip, wash with 70% Ethanol and dissolve in 400µl preheated TE buffer (65°C).
15. Measure the DNA concentration.
4.2.7.3 DNA digestion and separation

DAY 1
30U of Restriction endonuclease BglII was used to digest 15-20 µg of genomic DNA prepared from ES cells in a volume of 50 µl.

DAY2
10U of restriction enzyme were added for 2 more hours.
The DNA fragments were then run on 0,9% agarose gel in 0,5 X TAE buffer for 6 hours at 130V and subsequently photographed with a fluorescent ruler.
Prior to blotting the gel was
- Depurinated for 15 min in 500ml of diluted HCl acid, breaking the large DNA pieces into smaller ones in order to allow more efficient transfer from the gel to the membrane.
- Washed for 30 min in 500ml of an alkaline buffer (0.2M NaOH, 0.6M NaCl) to denature the dsDNA into ssDNA for later hybridization to the probe, destroy any residual RNA that may still be present and improve binding of the negatively charged DNA to the positively charged membrane.
- Washed for 30 min in 500ml of neutralization buffer (0.5M Tris pH7.5, 1.5M NaCl)
- Rinsed with sterile MonoQ water and kept in 20 X SSC buffer until blotting

4.2.7.4 Blotting

Whatman® paper and the nylon membrane are soaked in 20 x SSC prior to blotting. A
sheet of nylon membrane is placed on top of the agarose gel. Pressure is applied evenly to the gel (by placing a stack of paper towels and a weight on top of the membrane and gel), to ensure good and even contact between gel and membrane. Buffer transfer by capillary action from a region of high water potential to a region of low water potential is then used to move the DNA from the gel on to the membrane. Ion exchange interactions bind the DNA to the membrane due to the negative charge of the DNA and positive charge of the membrane.

4.2.7.5 Radioactive labeling

DAY 3
The membrane is exposed to UV radiation to permanently and covalently crosslink the DNA to the membrane.
To ensure the binding specificity of the probe to the sample DNA, membrane is pre-hybridized with Church buffer for 1h at 65°C.
The membrane is then exposed to hybridization probe: a single DNA fragment (amplified by PCR from a template sequence) with a specific sequence whose presence in the target DNA is to be determined.
The probe is radioactively labeled so that it can be detected by autoradiography.
Preparation of the probe:
• Add TE Buffer to 25ng of gel-purified DNA to the volume of 24µl.
• Add 10µl of random primers (Stratagene Random primer labeling kit)
• Heat at 95°C for 5 min
• Cool down to RT
• Add 10µl of 5 x Buffer (dATP/ dCTP) and 1µl of Klenow polymerase

Hot Lab
• Add 5µl of 32P dATP/dCTP, mix well
• Incubate for 10 min at 37°C
• Vortex a G-25 Sephadex spin column (GE Healthcare), put it into a microcentrifuge tube
• Centrifuge for 1 min at 3000 rpm, discard flow
• Put column into a fresh tube, put probe onto the column
• Spin for 1 min at 3000 rpm
• Discard column, heat the probe at 95°C for 5 min
• Exchange the Church buffer with a preheated (65°C) fresh buffer (25ml)
• Carefully add the radioactive probe and incubate o/n at 65°C

DAY4
The probe is poured off, the membrane is washed twice with 25ml of pre-heated wash buffer at 65°C for 20-30 min and subsequently put into a developing box over night.

DAY5
Autoradiography and analysis of results
5 References


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6 Curriculum Vitae

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PERSONAL DATA
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EDUCATION
Studies of Biology at the GSAS, New York University, NYC, USA, 2009
Studies of Molecular Biology, University of Vienna 2002 - 2010, Austria
Primary and secondary school education in Yerevan, Armenia; Riga, Latvia; Vienna, Austria

EMPLOYMENT
12 / 2007 - 12 / 2008 Diploma Student, M.Busslinger laboratory, IMP Vienna, Austria
Generation of a conditional Pax5-Etv6 translocation allele for establishing an ALL mouse model.
09 / 2007 - 09 / 2008 Histology, Medical Laboratory Dr. J. Lorber, Vienna, Austria
07 / 2006 – 02 / 2007 Technical Assistant, Prof. Small Group, IMBA Vienna, Austria
01 - 06 / 2006 Technical Assistant, VDRC/ Dickson Group, IMP/IMBA Vienna, Austria

INTERNSHIPS
01 / 2009 - 05 / 2009 C. Desplan Laboratory, GSAS, New York University, NYC, USA.
Warts/Hippo pathway signaling mechanisms in a post-mitotic fate decision.
04 / 2007 - 07 / 2007 J.M.Penninger Laboratory, IMBA Vienna, Austria.
Genetics of common lung injury pathways in bird flu.
07 / 2006-12 / 2006 B.J.Dickson Laboratory, IMP Vienna, Austria.
Neuromuscular junctions and sarcomere structure.
01 / 2006 - 06 / 2006 RNAi Laboratory, IMBA Vienna, Austria.
Neuronal and Muscle RNAi screens in Drosophila.

PUBLICATIONS
Systematic genetic analysis of muscle morphogenesis and function in Drosophila
Frank Schnorrer, Cornelia Schönbauer, Christoph Langer, Georg Dietzl, Katharina Schernhuber, Michaela Fellner,
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MISCELLANEOUS
Fluent in English, German, Russian, Latvian and Armenian.
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