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Analysis of the Leukemogenic Potential of the *CALM/AF10* Fusion Gene in Patients, Transgenic Mice and Cell Culture Models

Thesis for the attainment of the title Doctor in Veterinary Medicine from the Faculty of Veterinary Medicine of the Ludwig-Maximilians-University, Munich

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Untersuchung des leukämogenen Potentials des *CALM-AF10* Fusionsgens im Patienten, im transgenen Mausmodell und in Zellkulturmodellen

Inaugural-Dissertation
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To my Family

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1. ABBREVIATIONS

μg	Microgram
μl	Microliter
μM	Micromolar
μm	Micrometer
AF10	ALL 1 fused gene from chromosome 10 (MLL10)
AGM	aorta/gonad/mesonephros region
ALL	Acute lymphoid leukemia
AML	Acute myeloid leukemia
ANTH	AP180 N-terminal homology
APC	Allophycocyanin
AUL	Acute Undifferentiated Leukemia
bp	base pair
BSA	Bovine serum albumin
BMI1	B lymphoma Mo-MLV insertion region
CALM	Clathrin Assembly Lymphoid Myeloid Leukemia Gene
CCR	Cysteine-rich region
CDH15	Cadherin 15, M-cadherin (myotubule)
CEBP	CAAT /enhancer binding protein
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta
CFU	Colony forming unit
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CTNNA1	Catenin (cadherin-associated protein), alpha 1, 102kDa
CTSZ	Cathepsin Z
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethilsulfoxid acid
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide Triphosphate
Dox	doxycycline
DPBS	Distilled phosphate-buffered saline
EBV	Epstein-Barr virus
ECFP	Enhanced Cyan Fluorescent Protein
EDTA	Ethilene Diamine Tetracetic Acid
EGFP	Enhanced Green Fluorescent Protein
ext-PHD	extended plant homeodomain
EYFP	Enhanced Yellow Fluorescent Protein
FBS	Fetal Bovine Serum

FCS	Fetal Calf Serum
g	gram
H2AFJ	H2A histone family, member J
HCK	Hemopoietic cell kinase
HEK	Human embryonic kidney cell line
HOX	Homeo box
HOXB4	Homeo box B 4
HRP	Horse Radish Peroxidase
HSC	Hematopoietic Stem Cell
IgH	Immunoglobulin heavy chain
JAG1	Jagged 1
kb	kilo base pair
kDa	kilo dalton
l	liter
LAP	Leukemia-associated protein
lck	Lymphocyte protein tyrosine kinase
LT-HSC	Long-Term Hematopoietic Stem Cell
LZ	Leucine ziper
M	Molar
m	meter
MEIS1	Myeloid Ecotropic viral Integration Site 1
MLL	Mixed Lineage Leukemia or Myeloid/Lymphoid Leukemia Gene
MLLT10	Mixed Lineage Leukemia or Myeloid/Lymphoid Leukemia Gene Translocated to 10
mm	millimeter
mM	mili Molar
MPP	Multipotent progenitor
ng	nanogram
NLS	Nuclear localization signal
OM	Octapeptide domain
ON	overnight
PAGE	Polyacrylamid Gel Electrophoresis
Pax 5	Paired box gene 5
PBS	Phosphate-buffered saline
PCR	Polimerase Chain Reaction
PE	Phycoerythrin
pg	picogram
PHD	Plant homeodomain
PMSF	Phenylmethylsulfonyl fluoride

RAB34	RAB34, member RAS oncogene family
RNA	Ribonucleic acid
RPMI	Rosewell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SET	Su (var) 3-9, Enhancer-of-zeste, Thithorax
SSC	Sodium Solution Concentrated
ST7	Suppression of Tumorigenicity 7
ST-HSC	Short-Term Hematopoietic Stem Cell
SV-40	Simian virus 40
TBS	Tris-buffered saline
TCR	T-cell receptor
TEM7R	Tumor endothelial marker 7-related precursor
tet	tetracycline
TKR2	Toll-like receptor 2
TRIO	Triple functional domain (PTPRF interacting)
USP3	Ubiquitin specific protease 3
UV	Ultra violet radiation
Zf	Zink finger
ZNF185	Zink finger protein 185 (LIM domain)

2. INTRODUCTION AND OBJECTIVES

The analysis of genetic alterations in leukemia has been essential in understanding the pathogenesis of this disease. Somatic acquired chromosomal translocations or inversions occur in about 65% of acute leukemia-patients (Rowley, 1990).

There are two major mechanisms by which translocations affect the genes near the translocation breakpoints: 1) fusion genes are generated and 2) the expression of one gene is altered by the juxtaposition of a different promoter/enhancer. The t(10;11)(p13;q14) translocation is a rare but recurring translocation and has been reported in patients with acute myeloid leukemia (AML), T-cell acute lymphoid leukemia (ALL), acute megakaryoblastic leukemia, acute byphenotypic leukemia and malignant lymphoma (Dreyling *et al.*, 1998; Bohlander *et al.*, 2000). It is associated with a poor prognosis. The exact role of the *CALM/AF10* fusion gene in leukemogenesis is not known. At the present time we do not know, whether the expression of the CALM/AF10 fusion protein affects normal haematopoietic development and if this fusion-protein alone can cause malignant transformation.

To answer the question whether this translocation occurs in stem cells or in committed progenitor cells (myeloid or lymphoid) leading to leukemia, the following approaches were persued:

Transgenic mice were generated which expressed the *CALM/AF10* fusion gene under the control of the immunoglobulin heavy chain enhancer promoter or under the control of the murine proximal *Lck* promoter.

Molecular characterization of the t(10;11)(p13;q14) as well as gene expression profiling was performed in a series of *CALM/AF10* positive patient samples.

An inducible cell line model was established in which the *CALM/AF10* expression can be induced by doxycycline.

3. REVIEW OF THE LITERATURE

3.1. Hematopoiesis and Stem Cells

Hematopoiesis is a tightly controlled process of blood cell production maintaining homeostasis (Smith, 2003). It is the differentiation of committed multipotent progenitors (MPP) giving rise to either lymphoid or erythro-myeloid lineages, resulting in the formation of common lymphoid (CLP) or common myeloid (CMP) progenitors (Busslinger, 2004). Some MPPs divide assymetricaly generating a non-committed MPP (self renew) and a committed MPP (differentiation).

3.1.1. Early Hematopoiesis

Hematopoiesis is a tissue-specific process which is developmentally regulated. The primary sites and patterns of hematopoiesis change throughout murine embryogenesis (Yoder, 2004).

During embryonic development, hematopoiesis occurs sequentially in distinct organs. There is a close association between angiogenesis and hematopoiesis. The co-existence of vascular and blood cells found in the same embryonic structures indicate the existence of a common precursor, the hemangioblast. These structures, known as blood islands, are part of the haemogenicum endothelium and are distributed in limited locations in the embryo, such as the floor of the aorta and the proximal umbilical and vitelline arteries (Orkin, 2000). In the mouse, blood and endothelial progenitors are first found in these structures at about embryonic day 7.5 (E7.5) (Haar and Acherman, 1971, cited by Kondo *et al*., 2003). The liver is the principal site of hematopoiesis from E12.0 through birth. At the same time, the spleen and the bone marrow become active sites of hematopoiesis prior to birth (E18.0) (Yoder, 2004). It is accepted that definitive hematopoiesis begins already in the embryo before the onset of circulation (at ~ E8.5). Cells giving rise to hematopoietic cells could be obtained in culture from the yolk sac and the aorta/gonad/mesonephros (AGM) region. The exact relationship between primitive yolk sac hematopoietic stem cells (HSCs) and definitive HSCs is still discussed (Yoder, 2001; Cumano and Godin, 2001 and Keller *et al*. 1999), but there is a consensus about the hepatic origin of the first stem cells which seed the bone marrow (Yoder, 2001).

Independently of their precise linear relationship, primitive and definitive hematopoiesis are regulated by different and independent microenvironments and transcription factors (Kondo *et al*., 2003).

3.1.2. Definitive Hematopoiesis

The ability of a population of progenitors to repopulate the hematopoietic system in adult recipients for a long period is defined as definitive hematopoiesis. These progenitors are defined as long term repopulation stem cells (LTR). LTR cells are found in the fetal liver and

bone marrow (Cumano and Godin, 2001). The hematopoietic activity of the spleen was experimentally demonstrated by Godin *et al.* (1999). It starts from E.16-E.17 until aproximately the first week after birth. They could detect the presence of long-term reconstituting progenitors and an increasing number of lymphoid and erythro-myeloid colony-forming-units (CFU) at this embryonic stage. Shortly after birth, the bone marrow becomes the principal hematopoietic organ (Jain, 1993; Keller *et al.* 1999).

3.1.3. Stem cells

Stem cell defines a cell with differentiation potential and self-renewal capacity, giving rise to different lineages and multiple cell types. The stem cells can be classified into pluripotent and multipotent stem cells:

Pluripotent stem cells, which are able to differentiate into cells of all three germ layers: ectoderm, mesoderm and endoderm. Embryonic stem cells are pluripotent stem cells which originate from the inner cell mass of the blastocyst. These cells have the ability to generate all cell types in the body and can be cultured *in vitro* almost infinitely. A common use for these cells is for the generation of gene-targeted mice (knock-in or knock-out) using homologous recombination (Kondo *et al.*, 2003).

Multipotent stem cells are cells with lineage specificity and can be isolated from various tissues of fetal and adult animals. Examples of multipotent stem cells are neuronal stem cells, hepatic stem cells and hematopoietic stem cells (HSC) (Kondo *et al.*, 2003). Orlic and Bondine (1994) defined a murine HSC as a cell with the ability to restore normal hematopoiesis for a prolonged period in mice transplanted after lethal irradiation. This is the generally accepted definition of HSCs (Cumano and Godin, 2001; Weissman, 2002; Passegué *et al.*, 2003).

Hematopoietic stem cells are functionally divided into a long-term subset (LT-HSC), capable of indefinite self-renewal, and a short-term subset (ST-HSC) that self-renew for a defined interval (Weissman *et al.* 2001; Passegué *et al.*, 2003; Coulombel, 2004). The lineage commitment in the blood cell development, progressing from pluripotent HSCs, through oligolineage progenitors, immature cells, and finally to functionally mature cells is accompanied by a loss of self-renewing capacity (Passegué *et al.*, 2003). An overview of hematopoiesis is shown in figure 1.

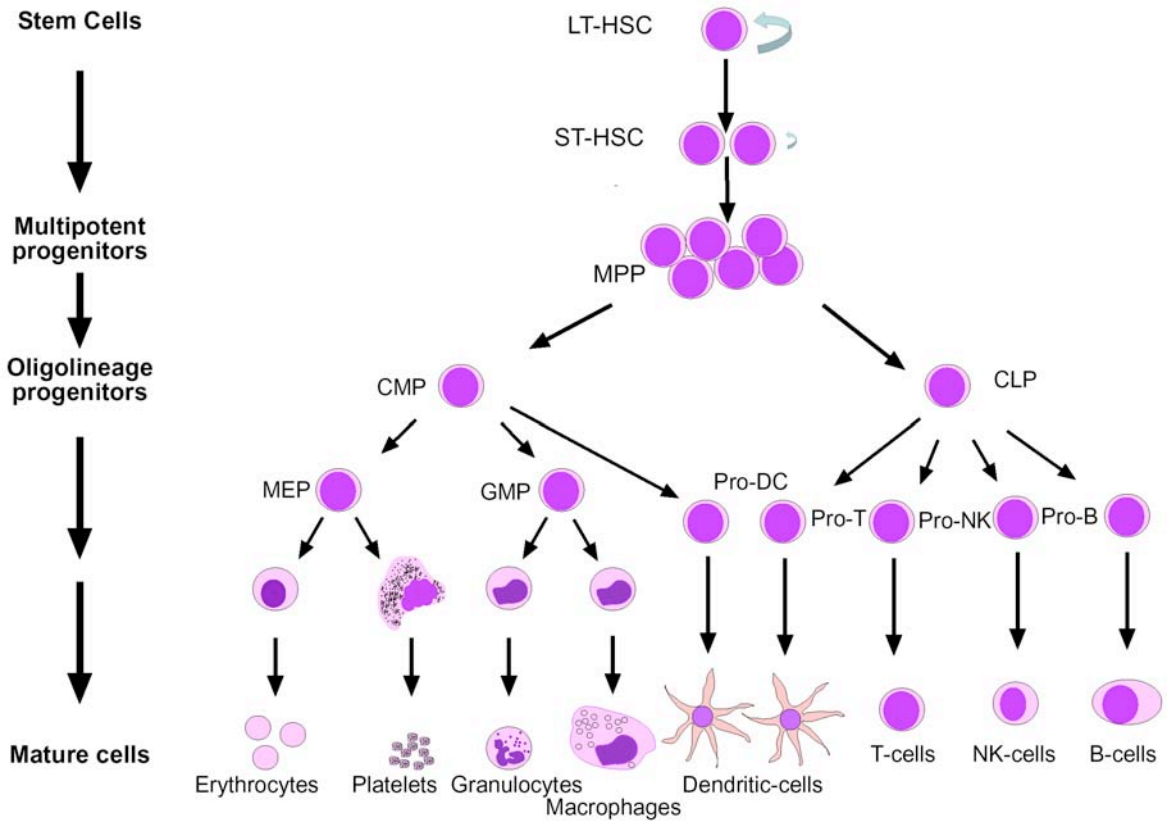

Figure 1: Hematopoietic and progenitor cell lineages. HSCs can be divided into LT-HSCs, highly self-renewing cells, ST-HSC, limited self-renewing cells. ST-HSCs differentiate to MPPs (Multipotent progenitors), which differentiate into oligolineage-restricted progenitors CMPs (common myeloid progenitors) and CLPs (common lymphoid progenitors). The CMPs give rise to the GMPs (myelomonocytic progenitor) and MEPs (megakaryotic/erythroid progenitors). These oligolineage-restricted progenitors give rise to the differentiated mature blood cells (adapted from Passegué *et al.*, 2003).

Hematopoietic differentiation is dependent on an elaborated interplay between the intrinsic genetic processes of blood cells and external factors (microenvironment) (Tenen *et al.,* 1997; Hackney *et al.*, 2002). All these genetic and environmental mechanisms that are responsible for blood production act affecting the relative equilibrium of critical cellular processes. The sum of all these processes and factors will finally determine whether HSCs, progenitors, and mature blood cells remain quiescent, proliferate, differentiate, self-renew, or undergo apoptosis. In a physiological situation, most of HSCs and many progenitor cells are quiescent in the G_0 phase of the cell cycle; however, many of the more mature progenitors are proliferating and giving rise to mature cells. In the absence of any stress factor, i.e. infection, poisoning or blood loss, the rate of apoptosis in progenitors and mature cells determines the rate of proliferation and differentiation (Smith, 2003). The possibility of examining gene expression of sorted cells by molecular biology has been essential to elucidate the function of

some genes involved in hematopoiesis. It remains a controversial question wether external induction instructs multipontent stem cell to go down a specific differentiation pathway or if the microenvironment only offers the conditions to the stem cell to carry out an intrinsically and pre-determined differentiation program (Schroeder *et al*., 2003). In this respect, two models for the developmental fate of stem cells are proposed: The instructive (extrinsic or deterministic) and the stochastic (intrinsic) model.

The instructive or deterministic model: this model postulates that the cell fate of HSCs and progenitors is determined by external factors driving the cells toward any of the different maturation stages or cell lineages. HSCs or multipotential progenitors would be induced to follow a particular developmental pathway due to genetic changes directed by the environment (intercellular interactions or diffusible signals) (Lemischka, 1992; Enver *et al*., 1998; Metcalf, 1998).

Cytokines are very important players among the environmental regulators of hematopoiesis (Zhu *et al*, 2002). Cytokines are a family of proteins that mediate positive and negative effects on cellular quiescence, differentiation, proliferation, and apoptosis. They are produced by hematopoietic cells and frequently by non-hematopoietic cells such as stromal cells form the bone marrow and endothelium. As a rule, cytokines function by stimulating a specific receptor and activating several different signalling pathways. One example is the activation of tyrosine kinases, such as focal adhesion kinase, pp60src, cABL, MAP (mitogen-activated protein) kinases, jun Kinase (JNK), and protein kinase C (PKC). Some cytokines induce cell proliferation, such as interleukin-3 and GM-CSF, while others protect cells from apoptosis making the cells able to respond to cytokines that promote cell growth. To this group belong the flt-3 ligand and kit ligand (Smith 2003). Cytokines may also work as a bridge between the stem cells and the elements in the microenvironment including extra cellular matrix (ECM) components (Smith, 2003). Regulators of HCSs including transforming growth factor-beta (TGF-β) and tumor necrosis factor-alpha (TNF-α) modulate cell cycle activity and engraftment. The *Wnt* pathway and the *notch* ligand family of transmembrane receptors have also important effects on the regulation of hematopoiesis (Hackney *et al*., 2002; Schroeder *et al*., 2003).

An important role as environmental regulators of hematopoiesis is also exerted by the chemokines. Chemokines are a large family of proteins which are involved in inflammation, leukocyte development and tissue recruitment, neovascularization, tumor development and metastasis. The name chemokines is a contraction of chemotatic cytokine, because they have the ability to stimulate leukocyte motility (Abbas *et al*., 1997). These molecules regulate HSC migration and homing to sites of need (Wright *et al*., 2002) and may also be negative and positive growth regulators. In hematopoiesis, they are able to inhibit progenitor growth, mediate thymic T-cell development and regulate the migration of hematopoietic progenitors (Smith, 2003).

Hematopoiesis is also regulated by a series of other essential molecules, and cells, such as: the extra-cellular matrix (ECM) components, hematopoietic and stromal cells, nutrients and vitamins, and a several different physiologic processes. ECM components bind to HSCs and progenitors providing the right conditions to these cells to interact with a large spectrum of stimulating and inhibiting cytokines and growth regulators (Hackney *et al*. 2002). Some of these ECM molecules are heparin sulfates, chemokines, collagens, thrombospondin-1, fibronectin and laminin. Additionally, adhesion molecules on progenitors and HSCs mediate the interaction with stromal cells which can induce growth, protect the cells from apoptosis and modulate regulatory factors. The binding of the HSCs and progenitors to ECM components is mediated by molecules localized on the surface of these cells. Examples for these molecules are integrins, selectins and mucins. The cells which may regulate hematopoiesis include NK cells, T-cells, macrophages, fibroblasts, osteoblasts, adipocytes, and perhaps neurons (Smith, 2003).

The second model proposed for the developmental fate of hematopoietic cells is the stochastic model (Ogawa, 1993; Enver *et al*., 1998; Bonnet, 2002). This model proposes that many genetic events are pre-programmed in a certain sequence and timing by intrinsic genetic processes as it is the case in embryogenesis. The *Rb* family, the *E2Fs*, cyclins, *SCL*, *Hox*, *Polycomb*, and other gene families appear to regulate proliferation and self-renewal of early hematopoietic cells (Pineault *et al.*, 2002; Park *et al*., 2003; Smith, 2003; Valk-Lingbeek *et al.*, 2004). The *bcl* family and others regulate apoptosis in hematopoietic cells.

The observation that the addition of exogenous growth factors to hematopoietic progenitor cells in culture allowed growth and development of cell lineages, but did not influence their commitment (Enver *et al*., 1998) supports the idea that the primary function of the extrinsic signals, including the growth factors, would be to give conditions for the survival and development of committed cells, whereas intrinsic mechanisms have already driven the lineage commitment (Bonnet, 2002).

Independently of whether the instructive or the intrinsic (stochastic) model is considered, the common pathway which will finally drive the progenitors to choose their differentiation to the one or the other cell lineage is directed by the alternative expression of a specific combination of transcription factors, inducing the expression of growth factors or their receptors (Tenen *et al*., 1997).

Specific nuclear (transcription) regulatory factors are expressed in particular lineages, establishing specific gene expression programs related to cell diversification (Orkin, 2000). The observation that many genes which were cloned at the site of leukemic translocations are transcription factors suggests that they play a crucial role in leukemia development and normal hematopoiesis (Look, 1997; Tenen *et al*., 1997; Orkin, 2000). Table 1 lists some of these hematopoietic transcription factors, their expression pattern, overexpression effects and knock-out phenotypes.

Table 1. Transcriptional regulators of hematopoiesis (Orkin, 2000)

Factor	Type	Expression pattern	Overexpression effects	Phenotype of mouse knockout
GATA-1	Zn finger	Prog, E, Meg, Eos, Mast	↑ E, Meg, Eos, □ myeloid	Blocked E, Meg
GATA-2	Zn finger	Prog, Meg, Mast	□ Mature E	□ Prog
GATA-3	Zn finger	Prog, T-cells, Th2	↑ Th2 □ Th1	No T-cells
PU.1	Ets	Prog, myeloid, B-cells	↑ Myeloid	No myeloid, T or B-cells
FOG-1	Multi-type-Zn fingers	Prog, E, Meg, Mast	□ Eos	Blocked E, no Meg
C/EBP□	B-zipper	Myeloid, Eos	↑ Eos	no Neutr, Eos
MafB	B-zipper	Monocyte	↑ Monocyte	
Runx1 (AML1)	Runt	Hematopoietic		No definitive hematopoiesis
T-bet	T-box	T_H1 cells	↑ T_H1 □ T_H2	
Pax5	Paired box	B-cells	□ Other lineages	No B-cells
Ikaros	Zn finger	Prog, T-cells		No lymphoid cells

Prog: progenitors, Eos: eosinophils, E: erythrocytes, Meg: megakariocytes, Neutr: neutrophils.

3.2. Leukemia and Leukemic Stem Cells

Leukemia can be defined as the generation of a new population of blood cells resulting from an aberrant and poorly regulated proliferation process of a few leukemic stem cells (Passegué, 2003). In the case of solid tumors as well as in leukemias it is difficult to identify the primary cell which suffered the transforming event leading to malignancy. Normal and leukemic stem cells (LSC) have in common the self-renew potential and share several developmental pathways. These similarities led to the conclusion that LSCs are HSCs that become leukemic as the result of accumulated mutations. HSCs have the machinery for self-renewal already activated and therefore may require fewer mutations to maintain it than more differentiated cells would require to re-activate self-renewal. HSCs also persist throughout life and therefore have much greater opportunities to accumulate mutations than more mature cells which persist only for a shorter period (Gilliland *et al.*, 2004). Leukemia can be the result of a mutation which conferred growth advantage and/or caused developmental arrest of progenitor blood cells (Guzman and Jordan, 2004).

Leukemic stem cells are defined as transformed hematopoietic stem cells or committed progenitor cells that have amplified or acquired the stem cell capacity for self-renewal (Lin and Sessa, 2004) but do not differentiate and have impaired functional properties (Guzman and Jordan, 2004). With the development of appropriate functional assays and methods for cell sorting, investigators have isolated and characterized malignant cells for both acute and chronic myelogenous leukemia (Gilliland *et al.*, 2004). The immunophenotypying of normal and leukemic stem cells can demonstrate that certain subtypes of human AML and CML are the consequence of mutations that accumulate in HSCs. There are experimental evidences suggesting that for most AML, independently of the lineage differentiation and phenotype of the leukemic cells HSCs and not committed progenitors are the target for leukemic transformation. This can be concluded analyzing the fact that, with exception of the M3 acute promyelocytic leukemia (APML), only the cells which are phenotypically similar to normal stem cells ($CD34^+$, $CD38^-$) were able to cause AML in transplanted nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, whereas leukemic $CD34^+$, $CD38^+$ cells were not able to cause disease (Passegué *et al.*, 2003).

Based on their clinical courses and on their lineage commitment, leukemias have been classified as acute or chronic or lymphoid versus myeloid, respectively. Based on the cytomorphology of the blast cells the FAB (French-American-British consortium) developed a widely used classification system for acute leukemias (Bennet *et al.*, 1976; Bennet *et al.*, 1985). This system identifies eight categories (M0-M7) of acute myeloid leukemia (AML) and three categories (L1-L3) of acute lymphoblastic leukemia (ALL). This classification established a standardization of diagnoses allowing comparisons of efficiency between different treatment regimens, especially in AML. The distinction between L1 and L2 subtypes in ALL is of no clinical importance. The cytomorphological observations, however, are still very important in diagnosis and treatment of leukemia. The informations obtained by immunologic techniques such as immunophenotyping or immunocytochemistry have proven to be extremely useful in the analysis of lineage commitment and leukemic cell differentiation (Burmeister and Thiel, 2001). Analyses of leukemia-specific changes on a molecular level using cytogenetic analysis, supplemented by fluorescence in situ hybridization (FISH) and molecular techniques, such as polymerase chain reaction (PCR) together with the cytomorphology and immunophenotyping are essential to provide a final diagnosis and to give an insight into the pathogenesis (Burmeister and Thiel, 2001; Haferlach *et al.*, 2005).

3.3. Chromosomal Translocations

A chromosomal translocation is defined by a rearrangement in which part of a chromosome is detached by breakage and then becomes attached to another chromosome (Lewin, 2000). Chromosomal abnormalities in tumors were already recognized since the beginning of the 20th

century, but their significance has only become clear in the last decade (Rabbitts, 1994). The analysis of clonal chromosomal abnormalities in blast cells from leukemia patients gained lot of importance for understanding of the molecular changes involved in leukemogenesis. The importance of chromosomal abnormalities in leukemias is demonstrated by the fact that more than 65% of acute leukemias cases are found to have non-random, somatically acquired chromosomal translocations or inversions (Rowley, 1990; Look, 1997).

On the molecular level, there are two major mechanisms by which chromosomal translocations lead to malignant cell transformation. They can either lead to the formation of fusion genes (e.g. the t(9;22)(q34;q11) results in the BCR/ABL fusion gene) or they cause gene deregulation (e.g. the t(8;14)(q24;q11) causes up-regulation of *CMYC*) (McKeithan *et al.*, 1986; Rabbitts, 1994).

3.4. The t(10;11)(p13;q14)

The t(10;11)(p13;q14) translocation was described in the U937 cell line which was derived from a diffuse hystiocytic lymphoma (Ralph *et al.*, 1976). It was noted that this translocation was distinct from a similar 10;11 translocation, the t(10;11)(p11-13;q23), found in AML, which results in the fusion of the *MLL* gene at 11q23 to the *AF10* gene at 10p12 (Chaplin et al., 1995). The t(10;11)(p13;q14) translocation was shown by Bohlander and coworkers to lead to the fusion of *CALM* (Clathrin assembly lymphoid myeloid leukemia), a member of the ap-3-like family of clathrin assembly proteins with the putative zinc finger transcription factor AF10 (ALL 1 fused gene from chromosome 10) located at 10p13 (Dreyling *et al.*, 1996). A schematic representation of the t(10;11)(p13;q14) translocation and the orientation of the resulting fusion genes is shown in figure 2.

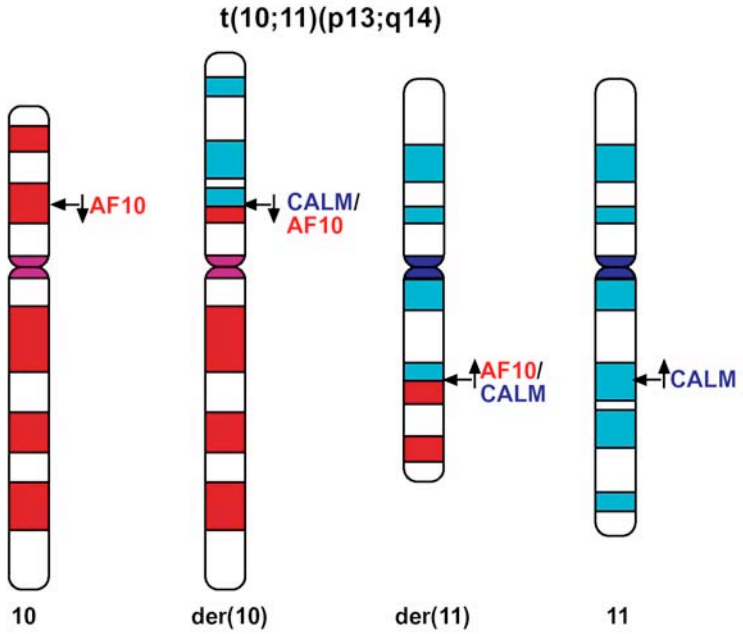

Figure 2: Diagram of the t(10;11)(p13;q14) translocation and the *CALM/AF10* fusion gene on the der(10) chromosome and *AF10/CALM* fusion gene on the der(11) chromosome.

3.5. *CALM, AF10* and *CALM/AF10*

3.5.1. *CALM*

The *CALM* (clathrin assembly lymphoid myeloid leukemia gene) or *PICALM* (phosphatidylinositol-binding clathrin assembly protein) gene encodes a 652 amino acid long ubiquitously expressed protein, which is a member of the AP-3 like family of clathrin assembly proteins (Dryeling *et al.*, 1996). Clathrin assembly proteins have a C-terminal domain which mediates the association between AP-3 and clathrin and play a critical role in the clathrin assembly in endocytosis (Dell'Angelica *et al.*, 1998; Marsh and McMahon, 1999).

Clathrin-mediated endocytosis is a process by which virtually all eukaryotic cells internalize nutrients, antigens, growth factors, pathogens and cell surface receptors (receptor recycling). Endocytosis of plasma membrane or synaptic vesicle component is performed by the progressive and sequential assembly of clathrin-coated vesicles that serve to concentrate cargo proteins and lipids into the emerging vesicle and provide a mechanical means to deform the membrane into a vesicular bud. The assembly of clathrin-coated buds is aided by an array of mostly cytosolic proteins that form a dynamic network of protein-protein interactions by associating with multiple partner proteins during the different stages of endocytosis (Takei and Haucke, 2001). Clathrin is the major component of the coat forming protein, a highly ordered

structure on the cytoplasmic surface of the vesicle. Soluble clathrin is composed of three identical 160 kDa heavy chains and three 22-28 kDa light chains. These three heavy and light chain complexes form three legged trimers, called clathrin triskelions. Triskelions assemble into a basketlike framework of hexagons and pentagons to form coated pits on the cytoplasmic surface of the membrane. The second major coat proteins are adaptor proteins (AP, assembly or adaptor proteins). CALM and AP180 are monomeric APs involved in the formation of the clathrin coats (Kim and Kim, 2001; Kalthoff *et al.*, 2002; Stahelin *et al.*, 2003).

CALM is the non neuronal homologue of the adaptor protein 180 (AP180) and interacts with phosphatidylinositol with its N-terminal domain which is homologous to AP180 (ANT180) (Ford *et al.*, 2001; Legendre-Guillemin *et al.*, 2004). Tebar *et al.* (1999) showed that the major binding partner of CALM is clathrin and that the over-expression of the clathrin-binding fragments of CALM dramatically affects clathrin-mediated endocytosis and the trafficking of receptors between the trans-Golgi network (TGN) and endosomes. CALM appears to assist the coat formation in clathrin-mediated endocytosis defining the size of the coated vesicle (Takei and Haucke, 2001). In subcellular localization studies, CALM was shown diffusely distributed within the cytoplasm when a GFP-CALM fusion was overexpressed in COS-7 cells, and also appeared to accumulate in the Golgi area (Tebar *et al.,* 1999). Vecchi and coworkers (2001) showed accumulation of CALM in the nucleus of COS-7 cells when nuclear export was blocked by leptomycin B. Two other genes (*AF-1p* and *EEN*) which encode endocytosis-related proteins are also involved in chromosomal translocations in leukemia, suggesting a potential link between endocytic proteins and cancer (Floyd and De Camilli, 1998). Both *AF-1p* and *EEN* are found fused to *MLL*, a gene involved in translocations with more than 50 different fusion partner genes, including AF10 (So *et al.*, 2003).

Our group performed yeast two hybrid interaction studies to identify CALM interacting proteins in order to learn more about the possible functions of CALM. CALM was found to interact with CATS, a protein with nuclear localization which is probably involved in cell proliferation. Overexpression of CATS in NIH3T3 cells was able to change the subcellular localization of both CALM and CALM/AF10 (Bohlander *et al.*, 2003). CALM was found also to interact with the four and a half LIM domain protein 2 (FHL2) (Pasalic *et al.*, 2005). FHL2 is an antagonist to the promyelocytic leukemia zink finger protein (PLZF), which is fused to the retinoic acid receptor-α as a result of the chromosomal translocation t(11,17)(q23;q21). Interestingly, FHL2 interacts with the proto-oncogene *SKI1* and with β-catenin, both having important roles in the *Wnt* signaling pathway (McLoughlin *et al.*, 2002), which is important in the process of T-cell development (Osborne and Kee, 2005).

CALM was recently found fused to another gene, *MLL*, in an infant AML case (Wechsler *et al.*, 2003). Curiously, as mentioned above, *AF10* was first identified as the fusion partner of *MLL* in the t(10;11)(p12;q23) which is found in AML. This observations lead to the

interesting question of whether the role played by the *CALM* portion in the *CALM/AF10* fusion gene is the same as the role of the *MLL* portion in the *MLL/AF10* fusion gene in leukemogenesis.

Potter and coworkers (1997) performed a phenotypical characterization of mice expressing a N-ethyl-N-nitrosourea (ENU) mutation in the mouse *Calm* (*Fitness 1* or *Picalm*) and observed that the mice suffered from a severe hypochromic anemia, characterized by anisocytosis and poikilocytosis. The hematopoiesis was shown to be defective in *fit1* mutants at an early progenitor stage, and cells representative of multiple hematopoietic lineages are significantly reduced. In addition to these hematopoietic defects, *fit 1* mutants were growth-retarded *in uterus* and remained dwarfed throughout their shortened life span. Detailed studies on an allele of intermediate severity, $fit1^{4R}$, showed that the anemia was mildly regenerative and demonstrated functional iron deficiency and abnormal iron distribution suggestive of impaired iron transport from the liver to other tissues. These mice also had increased myeloid/erythroid ratios in the bone marrow, scoliosis and lumbar vertebral abnormalities (Klebig *et al.*, 2003).

The structure of CALM is schematically represented in the figure 3.

3.5.2. *AF10*

AF10 (*ALL* 1 fused gene from chromosome 10), also called *MLLT10* (myeloid lymphoid leukemia gene, translocated to 10) was cloned as a fusion partner of *MLL* in the t(10;11)(p12;q23) in three AML patients. This rearrangement fuses the carboxy-terminal portion of AF10 to the amino-terminal third of MLL. *AF10* encodes a 109 kDa protein of 1,027 amino acids (Chaplin *et al.*, 1995). The predicted structure of AF10 contains also an N-terminal LAP/PHD-finger (PHD = plant homeodomain), followed by a cysteine-rich region or extended PHD finger region (CRR), a putative AT-hook, a nuclear localization signal (NLS) and a C-terminal leucine zipper (amino acids 766 to 794) (Linder *et al.*, 2000). The leukemia-associated-protein (LAP) motif is a cysteine-rich region (CRR) which binds DNA in a sequence-independent manner (Saha *et a.l*, 1995). LAP domains are zink fingers which are found in chromatin-associated proteins, e.g. CBP and MLL, suggesting that this domain could be involved in chromatin-mediated gene expression mechanisms (Perrin *et al,* 2003). The CRR or extended PHD finger domain, localized C-terminal to the LAP/PHD-finger, interacts with itself, and is probably responsible for the oligomerization of AF10. The AT-hook binds to cruciform DNA. The NLS identified by protein sequence analysis was demonstrated to direct AF10 to the nucleus (Linder *et al.*, 2000). The zinc fingers (ZF) take their name from its structure, in which a small group of conserved amino acids binds to a zinc ion and forms a relatively independent domain in the protein. ZF are a common motif in DNA binding and protein interaction and are found in transcription factors that assist both RNA polymerases II and III. Zn finger are one of the most abundant domains in the human genome (Lewin, 2000;

Petsko and Ringe, 2004). The leucine zipper (LZ) is a stretch of amino acids rich in leucine residues that provide a dimerization motif. Dimer formation itself has emerged as a common principle in the action of proteins (transcription factors) that recognize specific DNA sequences. An adjacent region of the LZ is responsible for binding to DNA (Lewin, 2000). The *Drosophila* AF10 (*dAF10*) contains a pentameric protein-protein interaction motiv and can associate *in vitro* with HP1, the *Drosophila* heterochromatin protein 1, which is encoded by the *Su(var)2-5* gene. HP1 takes part in a gene silencing phenomenon, termed position effect variegation (PEV), which refers to mosaic expression of euchromatic genes if they become relocated next to heterochromatin due to chromosomal rearrangements. This interaction suggests that *dAF10* participates in chromatin mediated gene silencing (Linder *et al*., 2001).

AF10 was shown to bind to GAS41 (glioma amplified sequence 41), a protein previously identified as the product of an amplified gene in glioblastoma. GAS41 shows significant homology to the *Sacharomyces cerevisae* protein ANC1 and to the human MLL fusion partners AF9 and ENL. In the same study, GAS41 was coimmunoprecipitated with INI1 (integrase interactor 1) and INI1 was present in the AF10 immunoprecipitate. INI1 is the human homologue of the yeast SNF5 protein, a component of the SWI/SNF complex, which acts to remodel chromatin and to modulate transcription (Debernardi *et al*., 2002).

DiMartino and colleagues (2002) observed that the N-terminal portion of *MLL*, which is present in the *MLL-AF10* fusion, alone is incapable of immortalizing myeloid progenitors and suggested that portions of *AF10* were required for the transforming activity of *MLL/AF10*. To investigate this requirement, they tested the ability of various deletion mutants of *MLL/AF10* to immortalize myeloid progenitors *in vitro*. They found one 82 amino acid region containing two sequences that are highly conserved in AF10-related proteins from *Drosophila* and *C elegans*. These homology regions include an almost perfectly conserved octapeptide (EQLLERQW) motiv (OM) separated by a nonconserved sequence from a leucine zipper (LZ). A minimal portion of AF10 encoding amino acids spanning the OM and LZ homology regions fused to the N terminal portion of *MLL* was sufficient for immortalization. These studies suggested that the ability to recruit parts of the transcriptional machinery may be the function of *AF10* in *MLL/AF10*-mediated leukemogenesis and that the OM/LZ motif is essential for this function (DiMartino *et al*., 2002). Perrin and colleagues (2003) showed that the LZ alone, without the PHD domain, of the *Drosophila* homolog of *AF10* (*dAF10*), activates the *Polycomb* group-responsive elements (PREs). The absence of the PHD domain was essential to confer this activity of the LZ. This deregulation is conserved in the human AF10 leucine zipper domain, which confers the same activity on an oncogenic MLL/AF10 fusion protein expressed in *Drosophila melanogaster*. MLL is a protein which shows homology to the *Drosophila trithorax* protein (TRX) in several domains. The *trx* gene and related *trx*- group genes are required to positively maintain the correct expression of a number

of *Drosophila* loci, including the homeotic genes. It is thought that TRX regulates homeotic gene expression at the level of chromatin organization, and it is likely that MLL plays the same role in vertebrates (Yu *et al.*, 1995). Another group of genes, the *Polycomb* group, has the opposite function, since these genes are required to maintain the repressed state of the same *Drosophila* loci (Perrin and Dura, 2004).

Covalent modifications of histone tails are very important features in regulating chromatin dynamics and gene expression (Strahl and Allis, 2000). Among the various modifications, histone methylation has attracted great attention due to its diverse functions, which include transcriptional regulation (Zhang and Reinberg, 2001; Lachner *et al.,* 2003). Many of the methylases have been linked to cancer, for example, the H3-lysine 4 (H3-K4) methyltransferase *MLL*, has been found to be involved in up to 50 leukemia-associated chromosomal translocations (Ayton and Cleary, 2001; So *et al.*, 2003). hDOT1L is a histone methyltransferase which methylates lysine 79 of histone H3 (H3-K79). hDOT1L was found to interact with AF10. This interaction is mediated by the octapeptide motiv and by the leucine zipper of AF10 (Okada *et al.*, 2005). The same authors showed that in the MLL/AF10 fusion, the OM/LZ domain is necessary to recruit hDOT1, and that the transformation of murine myeloid progenitor cells by a fusion of *MLL* and *hDOT1L (MLL/hDOT1L)* requires the histone methyltransferase activity of hDOT1L. The same OM/LZ domain of *AF10* was mentioned before as essential for the malignant transformation capability of the MLL/AF10 fusion (DiMartino *et al.*, 2002).

The important protein domains of CALM and AF10 and of the fusion proteins AF10/CALM and CALM/AF10 are shown in figure 3.

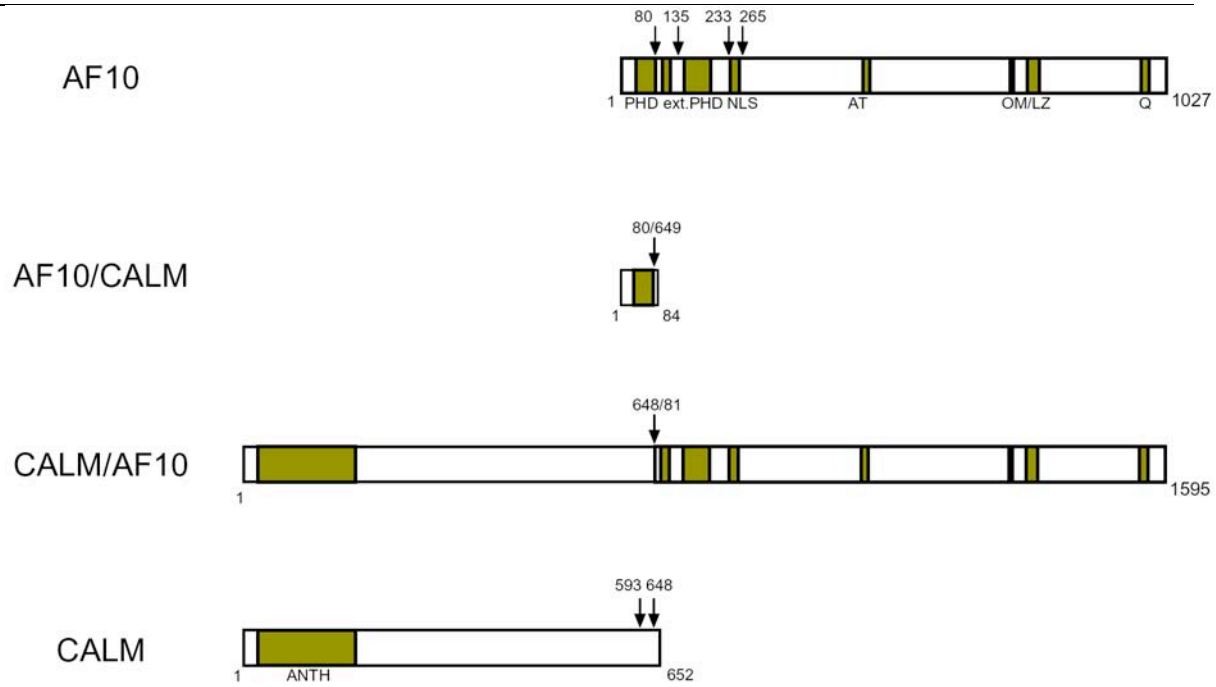

Figure 3: Diagram of AF10, CALM, CALM/AF10 and the AF10/CALM fusion protein. PHD: plant homeo domain; ext PHD: extended PHD; NLS: nuclear localization signal; AT: AT hook; OM/LZ: octapeptide/leucine zipper; Q: glutamic acid rich; ANTH: AP180 N-terminal homology. The arrows point to the alternative breakpoints in AF10 and CALM.

3.5.3. *CALM/AF10*

The *CALM/AF10* fusion gene found in the U937 cell line comprises almost the complete open reading frame of CALM and of AF10, whereas the *AF10/CALM* fusion gene would encode for only of the first 80 amino acids of AF10 and the last four amino acids of CALM (Dreyling *et al.*, 1996). Both fusion mRNA (*CALM/AF10* and *AF10/CALM*) can be detected in U937 (Dreyling *et al.* 1996). This translocation is a rare, but recurring translocation in AML associated with a poor prognosis (Dreyling *et al.*, 1998).

The *CALM/AF10* fusion transcript has been observed in the leukemic cells of patients with AML, ALL, malignant lymphoma (Bohlander *et al.*, 2000; Narita *et al.*, 1999; Ou *et al.*, 2004), acute megakaryoblastic leukemia (Jones *et al.*, 2001), monocytic leukemia with mediastinal infiltration (Nakamura *et al.* 2003), as well as in the leukemia-derived cell lines U937 and two other monocytic cell lines (P31/Fujioka, KP-Mo-TS) (Narita *et al.* 1999). Kumon *et al.* (1999) detected co-expression of both myeloid and lymphoid antigens on the leukemic cells of 7 out of 9 *CALM/AF10* positive leukemias, suggesting that *CALM/AF10* leukemias may be characterized by a mixed-lineage immunophenotype with coexpression of T-cell and myeloid antigens. Salmon-Nguyen *et al.* (2000) reported also the association of an inversion in the chromosome 11 and the t(10;11) translocation in a *CALM/AF10* patient. The singular feature in this T-cell ALL patient was a marked abnormal eosinophil proliferation.

Three different breakpoints of *CALM* (1926, 1987 and 2091), and four of *AF10* (423, 589, 883, 979) are described in *CALM/AF10* fusions (Dreyling et al., 1998; Kumon *et al*., 1999; Bohlander *et al*., 2000). Silliman *et al*. (1998) analyzed a T-ALL *CALM/AF10* – positive patient and detected multiple variants of the *AF10/CALM* and *CALM/AF10* fusion transcripts which arose from alternative splicing of *CALM* or *AF10*.

CALM/AF10 fusion transcripts were found in 9% of 131 unselected T-ALL patients and were described as the most common fusion gene in patients with T-ALL with T cell receptor □□ rearrangements (Asnafi *et al*., 2003). The reciprocal fusion transcript, *AF10/CALM,* is not found in all *CALM/AF10* patients (Dreyling et al., 1998; Narita *et al*., 1999; Bohlander *et al. 2000*; Carlson *et al*., 2000; Krause *et al*., 2004). It should be noted that cytogenetics alone is often insufficient to distinguish a t(10;11) leading to a *CALM/AF10* rearrangement from a t(10;11) which results in an *MLL/AF10* rearrangement (Kobayashi *et al*., 1997; Klaus *et al*., 2003).

Dik *et al*. (2005) performed expression profile analysis of + T-ALL patients with the *CALM/AF10* and observed that these leukemias are characterized by the overexpression of *HOXA5, HOXA9, HOXA10, MEIS1* and *BMI1*.

In a mouse bone marrow transplantation model, mouse bone marrow cells, retrovirally engineered to express the *CALM/AF10* fusion, were able to cause leukemia in all transplanted animals (n=13). The transplanted mice died of the leukemia after a mean survival of 125 days. The leukemia-initiating cells were identified as progenitor cells with lymphoid characteristics. These cells could give rise to leukemic cell co-expressing myeloid and lymphoid markers or myeloid markers alone. All cells were positive for the genomic DJ rearrangement at the *IgH* locus, demonstrating its lymphoid origin (Deshpande *et al*., 2005). These results clearly showed that the *CALM/AF10* fusion gene is a very potent inducer of leukemia.

3.6. *HOX* genes

The homeobox gene family encodes a large group of transcription factors which all contain a similar DNA-binding region of around 60 amino acids called the homeodomain. The homeodomain contains a helix-turn-helix DNA binding motif which is characteristic of many DNA-binding proteins. This domain is encoded by a DNA sequence of 180 base pairs termed the homeobox. Many homeobox genes are involved in development, and the homeobox was originally identified in genes that control patterning in *Drosophila* development (Acampora *et al*., 1989). The name ‘homeobox’ comes from the fact that mutations in some of these genes result in what is known as a homeotic transformation, in which one structure of the body plan is replaced by another. For example, in one homeotic mutation in *Drosophila*, a segment in

the fly's body that does not normally bear wings is transformed into an adjacent segment that does bear wings, resulting in a fly with four wings. Clusters of homeotic genes involved in specifying segment identity were first discovered in *Drosophila*. Similar complexes of homeotic genes have been identified in many animals. In vertebrates, the related clusters are known as the *Hox* complexes, and the homeoboxes of these genes are related to the Antennapedia homeobox of *Drosophila*.

In humans and mice there are 39 *Hox* genes organized in four clusters (A, B, C and D) on four different chromosomes (McGinnis and Krumlauf, 1992). In the hematopoietic system, the *Hox* gene expression was first analyzed by Adams and co-workers, who showed the presence of several *Hox* transcripts some of which appeared specific to subsets of hematopoietic cells (Kongsuwan *et al.*, 1988). Important clues to their potential roles have emerged from more detailed analysis of their expression pattern, which revealed that the members of the A, B and C, but none of the *Hox* D genes were most highly expressed in purified sub-populations of human BM cells enriched in stem cells and primitive progenitors, and that their expression was extinguished or sharply reduced in more differentiated cells (Pineault *et al.*, 2002). The function of *Hox* genes in hematopoiesis was initially investigated through overexpression studies showing that *Hoxb4* overexpression in mouse bone marrow cells conferred an enhanced proliferative and self-renewal potential to the bone marrow cells. The *Hoxb4*-overexpressing HSCs were 43-55 times more competitive than untransduced cells in reconstituting the HSC compartment *in vivo* (Sauvageau *et al.*, 2004). In addition, several members of the *HoxB* cluster were shown to be up-regulated following cytokine-induced differentiation of human BM progenitors in liquid culture (Gianpaolo *et al.*, 1994). The overexpression of *HOXB4* in mouse embryonic stem cells (ES) by retroviral transduction showed marked enhancement in the generation of definitive erythroid and mixed colony progenitors providing evidence implicating *Hox* genes in the control of very early stages of hematopoietic development (Helgason *et al.,* 1996).

Hox genes have also been associated with leukemic transformation. The first link between *Hox* gene overexpression and leukemia was obtained from genetic analyses of the WEHI-3B leukemic cell line, which was shown to contain proviral integrations resulting in the transcriptional activation of *Hoxb8* and interleukin-3 (IL-3) expression (Blatt *et al.,* 1988). Mice transplanted with bone marrow overexpressing *Hoxb8* and IL-3 died from an aggressive, polyclonal acute leukemia (Kroon *et al.,* 1998). A human *HOX* gene has also been implicated in leukemic transformation. *HOXA9* is overexpressed in a subset of human myeloid leukemias in the form of a fusion with a sub-domain of *NUP98,* as the result of a reciprocal translocation between chromosomes 7 and 11 (Borrow *et al.*, 1996).The importance of murine *Hoxa9* in the regulation of hematopoietic cell proliferation was revealed directly through gene targeting procedures, which showed that *Hoxa9*$^{-/-}$ mice have abnormal B lymphopoiesis and granulocyte-macrophage progenitors (CFU-GM) differentiating to cells from the lymphoid

lineage (Lawrence *et al*., 1997). In contrast, CFU-GM are hyperproliferative in *Hoxa10*$^{-/-}$ mice (Zhang *et al*., 1996), suggesting that proliferation of GM progenitors is at least in part regulated by *Hoxa9* and *Hoxa10*. The *trx* gene and related *trx*-group genes are required to positively maintain the correct expression of a number of *Drosophila* loci, including the homeotic genes.

As already mentioned, *MLL,* the human homologue of the *Drosophila Trx*, is involved in the regulation of the *HOX* genes. Ayton and Cleary (2003) showed that the myeloid transformation observed in murine primary myeloid progenitor cell lines immortalized by *MLL* fusion genes is associated with the expression of a specific subset of *HOX* genes from the A cluster. Cell lines immortalized by five different *MLL* fusion genes showed a characteristic *Hox A* gene expression profile. All lines expressed *Hoxa7, Hoxa9, Hoxa10* and *Hoxa11*. They demonstrated also that the *MLL* fusion proteins were required for the *Hox A* gene expression in the transduced cell lines. *Hoxa7* and *Hoxa9* were found to contribute independently to efficient *MLL*-associated myeloid immortalization *in vitro* and that *Hoxa9* was essential for induction of *MLL*-associated myeloid leukemias *in vivo*. Soulier *et al*. (2005) identified a new recurring translocation t(7;7)(p15;q34) in a T-ALL patient, targeting the major homeobox gene cluster *HOXA* and the *TCRB* locus and found that the expression of the whole *HOXA* gene cluster was dramatically dysregulated in the *HOXA*-rearranged cases. Upregulation of *HOXA* cluster genes was also demonstrated in cases with *MLL* and *CALM/AF10* rearrangements, suggesting that the *HOXA* genes are oncogenic in these leukemias. *CALM/AF10* positive T-ALL leukemias are characterized by overexpression of certain *HOXA* genes and also show overexpression of the *HOX* cofactor and TALE-homeobox gene (three amino acid loop extension) *MEIS1* (Myeloid Ecotropic viral Integration Site 1) (Bürglin, 1997). In the same study, the authors found overexpression of the polycomb group gene *BMI1* (B lymphoma Mo-MLV insertion region) (Dik *et al.,* 2005).

The polycomb group (PcG) gene family is highly conserved (Müller *et al*., 1995). PcG genes are implicated in *Homeobox* gene regulation and their biological activity lies in stable silencing of specific sets of genes through chromatin modifications (Valk-Lingbeek *et al*., 2004). The PcG antagonize the positive influence exerted by *MLL* on the regulation of the *HOX* genes. PcG genes are required to maintain the repressed state of the same *Drosophila* loci (Perrin and Dura, 2004). Some evidences indicate that the PcG are important for stem cell fate. *Bmi1*-deficient mice suffer from progressive loss of hematopoietic stem cells and cerebellar neurons. In addition, Mph1/Rae28, which directly interacts with Bmi1 in the PRC1 complex, is required for sustaining activity of hematopoietic stem cells as well as the proliferation of early cerebellar progenitors (Valk-Lingbeek *et al*., 2004). *Bmi1* is regulated by an extracellular-signaling molecule, the morphogen Sonic hedgehog (Shh), providing a connection between PcG genes and a major developmental signaling pathway (Leung *et al*., 2004). Park and co-workers (2003) showed that *Bmi1* is required for maintenance of adult

self-renewing HSCs in mice. The mechanism by which *Bmi1* modulates HSC self-renewal could be by the regulation of genes important for stem cell fate decisions as well as that of survival genes, anti-proliferative genes and stem-cell-associated genes.

3.7. The transgenic mouse model

3.7.1. Transgenic animals

Transgenic animals have a DNA sequence (the transgene) experimentally introduced and stably integrated in their genomes (Gassman *et al*, 1998).

The introduction of gene sequences into mammalian embryos has become a powerful tool to understand gene function. Transgenic animals have been instrumental in providing new insights into mechanisms of development, developmental gene regulation, into the action of oncogenes (Hanahan, 1984), and into the intricate cell interactions in the immune system. The transgenic technology offers exciting possibilities for generating precise animal models for human genetic diseases and for producing large quantities of economically important proteins by means of genetically engineered farm animals (Jaenisch, 1988).

The so called classic method for generation of transgenic mouse consists in the microinjection of cloned DNA directly into the male pronucleus of fertilized mouse oocytes. Viable embryos are subsequently implanted into pseudopregnant foster mothers (Gassmann and Hennet, 1998). This technique has been developed by Gordon and co-workers (1980) and has the principal advantage in the efficiency of generating transgenic lines that express most genes in a predictable manner (Jaenisch, 1988). Although the transgenic DNA is present in all cells, transgene expression is dependent on many factors such as the chosen promoter and enhancer elements, the number of incorporated copies, and the locus of integration. The main limitation of the classical transgenic approach is linked to the uncontrolled integration of the transgene into the genome. This random integration may influence the expression of genes situated close to the transgene, and the chromatin environment of the integration site may affect the expression of the transgene itself (Gassmann and Hennet, 1998).

Transgenic mice provide the means to assess directly the potency of oncogenes *in vivo*. Since a gene injected into a fertilized oocyte typically integrates into a host chromosome within a few cell divisions, all tissues of the transgenic mouse usually acquire the gene, but the regulatory elements of the gene may direct tissue-specific expression (Palmiter and Brinster, 1985).

3.7.2. Tissue-specific gene expression

The specificity of gene expression for cell types of developmental stages and the response of genes to physiological stimuli are mediated through a combitional interaction of promoter sequences, enhancers, and suppressors. These regulatory elements are composed of short stretches of DNA that are generally found in the promoter region of genes but also can be located in introns or dispersed over many kilobases upstream and downstream of genes (Brenner *et al.*, 2001). The design of the transgenic construct will determine the specificity and level of expression in the tissues.

The first cellular eukaryotic enhancer to be identified was the murine *Ig* heavy chain gene. This enhancer lies between the J_H gene segment cluster and μ constant region coding sequences. Upon V region assembly, a V region promoter comes to lie near the *IgH* introns enhancer (E*IgH*) and thereby becomes subject to its control. The discovery of the *IgH* intron enhancer proved of particular interest because of the tissue specificity of the enhancer (Eckhardt, 1992). The expression of microinjected Ig μ gene was shown to be tissue-specific in transgenic mice, and although the functionally rearranged heavy chain gene was present in all organs, it did not produced gene-specific transcripts in non-lymphoid tissues. This lead to the conclusion that cis-acting DNA sequences residing in the introduced rearranged μ gene must control its selective transcription (Grosschedl *et al.*, 1984). Adams and colleagues (1985) described the expression of the *c-myc* oncogene in B-cells driven by the Ig heavy chain enhancer causing lymphoid malignancy in transgenic mice. To mimic the translocated *myc* genes found in lymphoid tumors, they introduced a DNA sequence designated Eμ-*myc*, isolated from a mouse plasmacytoma in which a normal *myc* gene had become coupled to the Ig heavy chain enhancer. The resultant mice expressed the transgene exclusively in B-lymphoid cells and developed tumors within 6 to 14 weeks among 12 founders, another mouse died at the 34th week. The *IgH* enhancer was shown to be specifically active in splenic B-cells in these mice (Harris *et al.*, 1988). Gerlinger and colleagues (1986) targeted the expression of the rabbit β-globin structural gene to lymphocytes with the immunoglobulin heavy chain (*IgH*) enhancer and promoter sequences from either the chicken conalbumin or mouse κ light chain genes. They could demonstrate that a rabbit β-globin gene, in which the chicken conalbumin promoter replaced β-globin promoter elements, was not expressed in five transgenic mouse lines. The addition of the *IgH* enhancer allowed the specific expression in B lymphocytes. Nevertheless, replacing the conalbumin promoter by the Ig κ promoter resulted in higher specific expression. These results showed the specificity of the *IgH* enhancer in stimulating transcription in B-lymphocytes, and that an immunoglobulin promoter element can increase the level of specific expression.

The *Lck* gene encodes a lymphocyte-specific protein-tyrosine kinase ($p56^{Lck}$) that is a member of the *src* family. The *src* gene family includes seven closely related sequences (*fgr, fyn, hck,*

Lck, lyn, src and *yes*), all of which diverged from a common evolutionary precursor before the mammalian radiation. Considerable evidence suggests that the *src* family kinases can participate in the regulation of cell growth (Marth *et al.*, 1988). The expression of *Lck* is found in essentially all T cells, in most B cells, and in a few human cell lines derived from colon adenocarcinomas and small cell lung carcinomas (Adler *et al.*, 1998). The *Lck* gene is specifically implicated in the pathogenesis of lymphoid malignancy because of its rearrangements and overexpression in two independently obtained murine lymphoma cell lines, and by its localization at a site of frequent chromosomal abnormalities in human non Hodgkin lymphomas (Garvin *et al.* 1988). In mammals, the transcription of the *Lck* gene is regulated by two independent promoters, the proximal promoter, which is active in thymocytes, and the distal promoter, which predominates in mature T cells (Brenner *et al.*, 2001). Transcripts from the *Lck* locus are detectable as soon as hematopoietic progenitors first colonize the thymic anlage and are present in all thymocyte subpopulations defined by CD4 and CD8 expression. Since that the CD4 molecule appears on at least a fraction of immature lymphoid there is reason to believe that $p56^{Lck}$ contributes to signal transduction in these cells. Studies with transgenic animals using the mouse *Lck* gene, demonstrate that the proximal and the distal promoter elements can function independently (Allen *et al.*, 1992). T cells contain two kinds of *Lck* mRNA, which are called type I and type II. The proximal promoter is responsible for the transcription of the type I mRNA and the distal promoter, for the type II mRNA. Reynolds and colleagues (1990) analysed the relative abundance of type I and type II *Lck* mRNA transcripts and observed that the type I mRNA, product of transcription under the control of the proximal promoter, is expressed in all T-cells, but predominantly in immature thymocytes.

3.8. Inducible gene expression

The ability to individually manipulate the activity of a single gene or a few genes in a cell or a whole living organism represents a very powerful approach for study of complex biological processes.

Several inducible gene expression systems have been developed in an attempt to meet the need for regulated gene expression. Most of the commonly used methods for inducing gene expression, such as heat shock, steroids, or metallothionein, presents as disadvantages either high basal levels of gene expression under noninduced conditions (noise), pleiotropic effects on host cell genes, or both. Two systems have been developed that appear to overcome many of the problems associated with the first generation of inducible vectors in that the inducers are specific to the gene of interest and lead to low basal and high inducible levels of gene expression: the tetracycline (tet) and the progesterone antagonist (RU486) regulatable systems (Hoffmann *et al.*, 1996).

The tetracycline-regulatable gene expression system (Tet system) has, for a number of reasons, gained wide acceptance. By adapting prokaryotic transcription control elements to the eukaryotic transcriptional machinery, a highly selective regulatory circuit was established (Gossen and Bujard, 1992).

Tetracycline inhibits bacterial growth by binding to ribosomes and disrupting codon-anticodon interactions. Specifically, tetracycline prevents attachment of aminoacyl-tRNA to the acceptor site on the 30S ribosomal subunit. Tetracycline binds tightly, albeit reversibly, to a single site on the 30S subunit that is composed of residues from at least four proteins (S3, S7, S14 and S19) and residues from the 893-1054 regions of 16S rRNA. The principal mechanism by which *E.coli* becomes resistent to tetracycline involves multimeric antiporter proteins, known as Tet proteins, that are embedded in the bacterial inner membrane and, in exchange for a proton, catalyse the outward transport of tetracycline-Mg^{+2} complexes from the cytosol. Of the several known classes of Tet antiporters, the TetA proteins encoded by transposon Tn*10* and the plasmid pBR322 are the most important in molecular cloning; 399 amino acids in length, they consist of two domains, each containing six transmembrane segments. The two domains are connected by a cytoplasmic loop of 30-40 amino acids rich in positively charged residues. When TetA is present in high concentrations, cations are transported from the bacterial cell at such a rate that the membrane becomes depolarized and the viability of the cell is threatened. To prevent catastrophe, expression of TetA is tightly controlled by a helix-turn-helix repressor protein (TetR), the product of the *tetR* gene. In the absence of antibiotic, homodimers of the repressor bind tightly to the major grooves of two 15-bp palindromic operator sequences (*tetO* 1,2), thus preventing expression of the divergently transcribed genes *tetR* and *tetA*. TetR is therefore a powerful negative regulator of transcription both of its own gene and of *tetA* (Sambrook and Russel, 2001).

Two complementary systems have been developed in which the addition of the tetracycline derivative doxycycline (Dox) either switches transcription of a target gene off (tTA or 'Tet Off' system) (Gossen and Bujard, 1002) or on (rtTA or 'Tet On' system) (Gossen *et al.*, 1995). An additional advantage of the system is that it also enables control of the level of gene expression by titrating the dose of Dox. The Tet system, as initially designed, consists of two components: a tetracycline controlled transcriptional activator (rTA or rtTA) and a tTA/rtTA responsive promoter P_{tet} directing the transcription of the gene of interest. Bidiretional rTA/rtTA responsive promoters, P_{tet}bi-1 have been developed, allowing the coordinate expression of two genes, of which one may be used as a reporter for the expression of the gene of interest. The best results are obtained when the genes encoding the two components of the Tet system including the genes of interest, are introduced into cell lines or transgenic animals separately from each other in a two-step procedure, whereby in a first step, cell or mouse lines, for example, are generated to produce appropriately tTA or rtTA. These lines are suitable for introducing a variety of genes of interest controlled by P_{tet} or P_{tet}bi-1 (Bornkamm *et al.*, 2005).

In the case of cell lines, this second step is accomplished by a second transfection and selection of appropriate clones, whereas for transgenic animals double transgenic individuals are obtained by breeding. In practice, the establishment of well-regulated clones using the system with two plasmids is a technically difficult process and results in a low number of inducible clones (Hoffmann *et al*., 1996). The arguments exposed indicate a series of advantages in a system based on the one-step transfer of P_{tet} controlled genes along with the tTA/rtTA encoding transcription.

Bornkamm and colleagues (2005) reported the development of an EBV (Epstein-Barr virus) - based vector that contains all the elements for controlling the activity of a gene of interest via Dox. This vector, designated pRTS-1, accommodate all the elements required for Dox-regulated gene expression on one episomally replicating plasmid including the gene of interest. It is reported to show low background, high inducibility and a proportional rate of response to different Dox concentrations.

4. MATERIALS AND METHODS

4.1 Patients

Bone marrow cells or RNA samples from five patients, three with acute myeloid leukemia (AML) and two with T-cell acute lymphoid leukemia (T-ALL) with translocation t(10;11) were kindly provided by Dr. Cristina Mecuci, Perugia, Italy. cDNA from seven patients with AML, ALL or acute undifferentiated leukemia (AUL) with translocation t(10;11) were obtained from the Laboratory for Leukemia Diagnostics, Grosshadern. Bone marrow cell samples from twenty-nine T-ALL patients with TCR - □ rearrangement were obtained from Prof. Dr. Ludwig from the Immunologic Marker Laboratory, Charité University Clinic, Berlin.

4.2 Animals

For the production of transgenic mice, the inbred mouse strain FVB/N, established at the National Institute of Health (Bethesda, USA) in the late 70's, (Taketo *et al.*, 1991) was used. The transgenic mice were obtained by the classic transgenic method of microinjection in the male pronucleus (Gordon and Ruddle, 1982). The mice were maintained in the animal facilities of the Gene Center under non-barrier conditions. Acidified water (6 mM HCl) was provided ad libitum in bottles. Pregnant mice received breeding diet (Ssniff®M-Z extruded pellets) ad libitum. Maintenance diet (Ssniff®R/M-H pellets) was fed ad libitum to the others. Fodder pellets, as well as any other solid objects entering the mouse facility, were autoclaved. Mice were kept, separated by sex, in standard macrolon cages with grid lids on softwood fibre, paper and hay. Enrichment was provided in all cages of sufficient size with aspen wood pieces and activity wheels. The environmental conditions in the mouse facility were: Conventional facility (non-barrier), temperature 21°C (+/- 1°C), relative humidity 60% (+/- 5%), ventilation rate 15 air changes/hour, 2 pascal positive air-pressure and a lighting regimen of 12 hours light/12 hours dark.

Breeding strategy

Founders obtained from DNA-microinjection were bred from the age of 8 weeks to 6 months (females) or up to 12 months (males) with FVB/N inbred mice to generate transgenic lines. Female and male mice were caged together until the appearance of a vaginal plug. Males were thereafter caged singly.

4.3 Material and Reagents

Table 2a: Chemicals, commercial solutions and kits

Reagent	Company
T4 DNA Ligase	New England Biolabs, Frankfurt, Germany
100 MM dNTP Set, PCR Grade	Invitrogen life technologies, Karlsruhe, Germany
1Kb DNA Ladder	Invitrogen life Life Technologies, Karlsruhe, Germany
Acetic acid	Merck, Darmstadt, Germany
Agar-Agar	Carl Roth GmbH, Karlsruhe, Germany
Agarose	ICN Biomedicals, Inc.
BigDye® Terminator Cycle Sequencing	Applied Biosystems, Foster City, CA, USA
Bio-rad Protein Assay	Bio-rad Laboratories GmbH, Munich, Germany
Boric Acid	Carl Roth GmbH, Karlsruhe, Germany
Bromophenol blue	Carl Roth GmbH, Karlsruhe, Germany
Buffer Y/Tango®	MBI Fermentas, St. Leon-Roth, Germany
Chloroform	Sigma-Aldrich, Steinheim, Germany
Coomassie blue	Bio-Rad Laboratories, Hercules, California, US
Deoxyribonuclease I, Amplification Grade	Invitrogen life technologies, Karlsruhe, Germany
Dextransulfate	Carl Roth GmbH, Karlsruhe, Germany
Dietyl pyrocarbonate (DEPC)	Sigma-Aldrich Chemie GmbH
DMEM	PAN Biotech GmbH
DPBS	PAN Biotech GmbH
ECL® Western blotting Detection reagents	Amersham Biosciences, Buckinghamshire, England
Endofree® Plasmid Maxi Kit (10)	Qiagen GmbH, Hilden, Germany
Ethanol	Merck, Darmstadt, Germany
Ethidiumbromide	Carl Roth GmbH, Karlsruhe, Germany
Fetal Bovine Serum	Gibco BRL, Life Technologies. Paisley, Scotland
Giemsa's solution	Merck KgaA, Darmstadt, Germany
Glycerin	Carl Roth GmbH, Karlsruhe, Germany
Glycin	Carl Roth GmbH, Karlsruhe, Germany
Hydrochloridric acid 37%	Merck, Darmstadt, Germany
Isopropanol	Carl Roth GmbH, Karlsruhe, Germany
May-Grünwald's eosine-methylene blue solution modified	Merck KgaA, Darmstadt, Germany
Megaprime DNA Labelling System	Amersham Biosciences, Buckinghamshire, England
Methanol	Merck, Darmstadt, Germany
Milk powder	Carl Roth GmbH, Karlsruhe, Germany
Oligo $(dT)_{12-18}$ Primer	Invitrogen Life Technologies, Karlsruhe, Germany

Table 2b: Chemicals, commercial solutions and kits

Reagent	Company
Paraformaldehyd	Carl Roth GmbH, Karlsruhe, Germany
Penicilin/Streptomycin	PAN Biotech GmbH, Aidenbach, Germany
Peptone, meat pancreatic digested	Merck, Darmstadt, Germany
Phenol-chloroform	Carl Roth GmbH, Karlsruhe, Germany
PolyFect® Transfection Reagent	Qiagen GmbH, Hilden, Germany
Propidium iodide	Calbiochem, San Diego, USA
Proteinase inhibitor cocktail	Sigma-Aldrich Chemie –GmbH, Steinheim, Germany
Proteinase K	Sigma-Aldrich Chemie –GmbH, Steinheim, Germany
QIAshredder®	Qiagen GmbH, Saint Louis, USA
Restriction enzymes and buffers	MBI Fermentas, St. Leon-Roth, Germany and New England Biolabs, Frankfurt, Germany
RNeasy® Mini Kit	Qiagen GmbH, Hilden, Germany
RPMI 1640	PAN Biotech GmbH, Aidenbach, Germany
SDS ultra pure	Carl Roth GmbH, Karlsruhe, Germany
SeeBlue® Plus@	Invitrogen life technologies, Karlsruhe, Germany
Shrimp Alkaline Phosphatase	MBI Fermentas, St. Leon -Rot, Germany
Sodium acetate	Sigma-Aldrich, Steinheim, Germany
Sodium acetate	Merck, Darmstadt, Germany
Sodium chloride	Merck, Darmstadt, Germany
Sodium citrate	Carl Roth GbmH, Karlsruhe, Germany
Sodium hydroxide	Merck, Darmstadt, Germany
SuperScript® II Rnase H- Reverse Transcriptase	Invitrogen Life Technologies, Karlsruhe, Germany
Taq DNA Polymerase	New England Biolabs, Frankfurt, Germany
T4 DNA Ligase	New England Biolabs, Frankfurt, Germany
TEMED	Carl Roth GmbH, Karlsruhe, Germany
Testes salmon DNA	Sigma-Aldrich, Steinheim, Germany
TRIS – (hydroxymethl)-aminomethane	Carl Roth GmbH, Karlsruhe, Germany
Trypsin/EDTA	PAN Biotech GmbH, Aidenbach, Germany
Tween® 20	Carl Roth GmbH, Karlsruhe, Germany
Yeast extract	Carl Roth GmbH, Karlsruhe, Germany

Table 3: Labware and suppliers

Material	Company
1.5 ml microcentrifuge tube	Eppendorf, Hamburg, Germany
15 ml polypropylene conical tubes	Becton Dickinson Europe, Meylan Cedex, France
50 ml polypropylene conical tubes	Becton Dickinson Europe, Meylan Cedex, France
6 Well cell culture plate	Nunc Brand Products, Roskilde, Denmark
Cell culture flask 75cm^2	Sarstedt, Nümbrecht, Germany
Cell culture flask 75cm^2	Sarstedt, Nümbrecht, Germany
Cell culture plate 35 mm^2	Nunc Brand Products, Roskilde, Denmark
Cell Scraper 25cm	Sarstedt, Inc., Newton, USA
Cuvettes 10x4x45 mm	Sarstedt AG & Co., Nümbrecht, Germany
Nunc Cryotube™ vials	Nalge Nunc International, Denmark
FALCON® 14 ml Polystrene Round-bottom tube	Becton Dickinson Europe, Meylan Cedex, France
Gel-Blotting paper 0.8mm	Schleicher & Schleicher, Dassel, Germany
Hybond®-P PVDF Transfer membrane 0.45μm	Amersham Biosciences, Bucks, UK
Hypercassete 18x24 cm	Amersham Biosciences, Buckinghamshire, England
Hyperfilm® ECL Chemiluminescence reagent	Amersham Biosciences, Buckinghamshire, England
Hyperfilm® ECL High performance chemiluminescence film	Amersham Biosciences, Buckinghamshire, England
Lab-Tek II Chambered Coverglass w/cover	Nalge Nunc International, Naperville, IL, USA
MicroSpin® S-300 HR Columns	Amersham Biosciences, Piscataway, NJ, USA
Nescofilm sealing film	Carl Roth GmbH, Karlsruhe, Germany
Pipette tips 0.5-10 μl	Carl Roth GmbH, Karlsruhe, Germany
Pipette tips 100-1000 μl	Carl Roth GmbH, Karlsruhe, Germany
Pipette tips 1-200 μl	Carl Roth GmbH, Karlsruhe, Germany
Surgical blade	Feather Safety Razor Co. Med. Div., Japan
Syringe 10 ml	Braun, Melsungen, Germany
Syringe Driven Filter Unit Millex -GP 0.22 µm	Millipore, Carrigtwohill, Ireland
Syringe Driven Filter Unit Millex -HV 0.45 µm	Millipore, Carrigtwohill, Ireland
Syringe Driven Filter Unit Millex -LG 0.20 µm	Millipore, Japan
Tissue Culture Plate 6-Well Flat Bottom Cell+	Sarstedt, Inc. Newton, USA
Tissue Culture Plate 6-Well Flat Bottom Cell+	Sarstedt, Inc. Newton, USA

Table 4: Antibodies

Antibody	Isotype	Label	Company	Concentration
Anti-CALM C -18	Goat IgG	Primary	Santa Cruz Biotechnology	200μg/ml
Anti-CALM S -19	Goat IgG	Primary	Santa Cruz Biotechnology	200μg/ml
Anti-CALM G -17	Goat IgG	Primary	Santa Cruz Biotechnology	200μg/ml
Anti-GFP IgG	Rabbit IgG	Primary	Molecular Probes	2 mg/ml
Anti-goat IgG HRP – conjugated	Donkey IgG	HRP	Santa Cruz Biotechnology	400μg/l
Anti-rabbit IgG	Donkey IgG	HRP	Santa Cruz Biotechnology	400μg/ml
GR-1	Rat IgG2b	PE/APC	BD Pharmingen	200μg/ml
CD11b (Mac-1)	Rat IgG2b	PE/APC	BD Pharmingen	200μg/ml
Sca-1	Rat IgG2b	PE/APC	BD Pharmingen	200μg/ml
Ter119	Rat IgG2b	PE/APC	BD Pharmingen	200μg/ml
B220	Rat IgG2b	PE/APC	BD Pharmingen	200μg/ml
CD3	Rat IgG2b	PE/APC	BD Pharmingen	200μg/ml
CD4	Rat IgG2b	PE/APC	BD Pharmingen	200μg/ml
CD8	Rat IgG2a	PE/APC	BD Pharmingen	200μg/ml
CD19	Rat IgG2b	PE/APC	BD Pharmingen	200μg/ml
CD43	Rat IgG2b	PE/APC	BD Pharmingen	200μg/ml
CD117 (c-kit)	Rat IgG2b	PE/APC	BD Pharmingen	200μg/ml
Isotype control	Rat IgG2b	PE/APC	BD Pharmingen	200μg/ml

Table 5: Plasmids

Plasmid	Supplier
pEGFP-C1	BD Biosciences Clontech, Heidelberg, Germany
pEYFP-C1	BD Biosciences Clontech, Heidelberg, Germany
pECFP-C1	BD Biosciences Clontech, Heidelberg, Germany
pPepB-Splice	Werner Müller
pGEMT -easy™ vector	Promega Corporation, Madison, USA.
pRTS-1	Georg Bornkam, GSF, Grosshadern, Germany
pUC19Sfi1	Robert Chapmann, GSF, Grosshadern, Germany

Table 6: Primers used for CALM/AF10 amplification

Primer Name	Annealing (°C)	Nucleotide position in cDNA and genomic sequence*	Sequence
NG.T 45	57	CALM(1730-1753) Exon 15	5' – CCAAACTCCCACCTAGCAAGTTAG – 3'
NA.T 501	57	CALM(1998-2023) Exon 20	5' – GGAAGTGTTCCTGTAATGACGCAAC – 3'
ANB 497	57	CALM(2165-2144) Exon 22	5'– AAGGATTTTGCTGCTTGAGCAC – 3'
AF10 949B	57	AF10(949-929) Exon 14	5' – CCAATGCAGGTGATGGTTCTG – 3'
AF10 1086B	57	AF10(1086-1066) Exon 15	5' – GGTGTGTGCAGAGACTTCCTG – 3'
AF10 651B	57	AF10(651-632) Exon 11	5' – TGTCATGCAAGCACCAGTGG – 3'
AF10 T288	57	AF10(288-308) Exon 2	5'– CGAGAACCCGCTGGTTTATTG – 3'
CALM 1900T	56	CALM(1900-1920) Exon 17	5' – ACAACCGCTTGGAATGCTGC – 3'
AF10448B	56	AF10(448-424) Exon 8	5' – CCTTATGGGGACAAAGTTCACATC – 3'
CAJN fw	58	CALM(1929-1948) Exon 19	5' – ACCCCCTGTAATGGCCTATC – 3'
CAJN rev	58	AF10(636-617) Exon 11	5' – AGTGGCTGCTTTGCTTTCTC – 3'
CA2	60	CALM(448-471) Exon 3	5' – AACACGTTGTTTAACTTAAGCAA – 3'
CA3	60	CALM(682-704) Exon 7	5' – CTTGACATCTATAAGAAGTTCC – 3'
CA4	60	CALM(1195-1216) Exon 11	5' – CCTCATACCTCTTTAACAACTG – 3
CA5	60	CALM(1497-1519) Exon 13	5' – CATTTCTTCAGATGTATCTACTT – 3'
CA6	60	CALM(1808-1831) Exons 16 and 17	5'– GAAATGGAACCACTAAGAATGATG – 3'
CA7	60	AF10(443-460) Exon 8	5' – CCCATAAGGATGGAGCTTTAA – 3'
CA8	60	AF10(737-757) Exon 12	5' – CCGATAATGTCCAATACTGTG – 3'
CA9	60	AF10(1144-1162) Exon 15	5' – TCAGCTCACAGCTCAGGTC – 3'
CA10	60	AF10(1427-1446) Exon 16	5' – GTACCTTAATTGGCCTCCCT – 3'
CA11	60	AF10(1764-1783) Exon 16	5' – TTTACAGAGCCTCAGTGTTG – 3'
CA12	60	AF10(2132-2150) Exon 22	5' – CTCTCAGTCAGGCACCATC – 3'
CA13	60	AF10(2491-2509) Exon 25	5' – AACCGAAGATTAGAGGAAC – 3'
CA14	60	AF10(2839-2859) Exon 27	5' – GTCAATGGCGTGACAGTGGGG – 3'
CA15	60	CALM/AF10 (4782-4800)	5' – CTTCAGCAGCTGCAGATCC – 3'
AF10T182 XhoI	58	AF10(182-199) Exon 2	5' - CATCCTCGAGGAATGGTCTCTAGCGACC - 3'
AF10B728 SalI	58	AF10(728-705) Exon 12	5' – CCTTCTTCTTCACAAAGCAGTCCG – 3'

* Gene Bank acc. NM_007166 (CALM) and NM_08705.15 (AF10).

Table 7: PCR primers used to generate inserts for the various expression constructs or for house keeping genes.

Primer Name	Annealing (°C)	Nucleotide position	Sequence
IgHStartXhoI[a]	60	Mouse Chr 12	5' – CTTCTCGAGTAAATACATTTTAGAAGTCGATA - 3'
IgHProEndSac1[b]	60	Mouse Chr 12	5' – CTTGAGCTCGTGAGGTCCTGTGTGCTC – 3'
SV40splStartXba1	60		5' – CTTTCTAGATCTTTGTGAAGGAACCTTAC – 3'
SV40polAEndNot1	60		5' – CTTGCGGCCGCCTAATTTAAATGAGGAC – 3'
MLCKprom64-84topXhoI	59	64-84	5' – GTACTCGAGGACCAGTGCTCAGGAAGG – 3'
MLCKprom702-681bottomSac1	59	702-681	5' – GATGAGCTCAAGCTCCTGACTGGGTTCC – 3'
D10 Mit105F	56	Mouse STS Chr 10	5' - AATCTGGTCACACAGATTTGACC – 3'
D10 Mit105R	56	Mouse STS Chr 10	5' - GTCATCCCTGTGTAGTGTCAACA – 3'
mouseGAPDHf[c]	60	271 - 291	5' – CATCACCATCTTCCAGGAGC – 3'
mouseGAPDHr	60	714 - 695	5' – ATGACGTTGCCCACAGCCTT – 3'
pEGFP-C11240	59	1240 - 1258	5'- AAAGACCCCAACGAGAAGC -3'
hTBPf[d] exon 5 and 6	57	653 - 672	5' – GCACAGGAGCCAAGAGTGAA – 3'
hTBPr exon 6	57	779 - 760	5' – TCACAGCTCCCCACCATGTT – 3'
mHPRTf[e] exon 3	59	295 - 314	5' – GGGGGCTATAAGTTCTTTGC – 3'
mHPRTr exon 6	59	606 - 587	5' – TCCAACACTTCGAGAGGTCC – 3'

Access number: [a]NT_039551.4, [b]NC_000078.2, [c]NM_001001303, [d]NM_007583.11, [e]NM_013556

4.4 Media and Solutions

4.4.1Cell Culture

Media and phosphate-buffered-saline were purchased from Pan Biotech GmbH.

Complete cell culture medium
50 ml heat-inactivated FCS, sterile filtered
5 ml penicillin/streptomycin solution (10,000 U penicillin/ml, 10 mg/ml, respectively)
RPMI1640 or DMEM to complete 500 ml volume

Cell-freezing solution
10% DMSO sterile-filtered diluted in sterile filtered and heat-inactivated FCS

DMEM
With 4.5 g/l glucose, L-Glutamine, Sodium Pyruvate, 3.7g $NaHCO_3$

Sterile filtered

Doxycyclin Stock Solution
1 mg doxycyclin hyclate/ ml DPBS

Puromycin Stock Solution
1 mg puromycin/ml DPBS

4.4.2 Western blotting

RIPA buffer

1X PBS,
1% TritonX100.
0.5% sodium deoxycholate
0.1%SDS
Protease inhibitors (freshly added):
PMSF (stock 10 mg/ml diluted in isopropanol) 10 μl/ml RIPA
Sodium orthovanadate (100 mM stock diluted in distilled water) 10 μl/ml RIPA
Proteinase inhibitors cocktail (Sigma) 1:100 dilution

Tris buffered saline (TBS)

10 MM Tris, pH adjusted to 8.0 with HCl
150 MM NaCl

Phosphate buffered saline (PBS)

9.1 mM dibasic sodium phosphate
1.7 mM monobasic sodium phosphate
150 MM NaCl
pH 7.4

Blotto A

1 x TBS, 5% milk powder 0.05% Tween 20

Transfer Buffer for Western blotting

25 mM Tris HCl (pH 8.8)
200 MM glycin
20% methanol

Separating electrophoresis gel buffer

1.5 m Tris-HCl pH 8.8, SDS 2%

Stacking Gel buffer

1.5 m Tris-HCl, SDS 2% pH 6.8

Electrophoresis running buffer

250 mM Tris (HCl, pH 8.5)
2 M Glycin
1% SDS

Electrophoresis Sample buffer

125 mM Tris
4% SDS
20% Glycerol
10% β-Mercaptoethanol
0.01% bromophenol blue

Table 8: SDS Electrophoresis Separating Gel 6% for a 5 ml single mini gel (Sambrook and Russel, 2001)

H_2O	2.7 ml
1.5 m Tris (pH 8.8)	1.3 ml
30% Acrylamide mix	1 ml
10%SDS	50 μl
TEMED	4 μl
10% APS	50 μl

Table 9: SDS Electrophoresis Stacking Gel 5% for a single 2 ml mini gel (Sambrook and Russel, 2001)

H_2O	1.4 ml
1.5 m Tris (pH 6.8)	0.25 ml
30% Acrylamide mix	0.33 ml
10%SDS	20 μl
TEMED	4 μl
10% APS	20 μl

4.4.3 Southern Blotting

Lysis buffer for mouse tail genomic DNA extraction

100 mM EDTA pH 8.0
100 mM NaCl
50 mM Tris HCl pH 8.0
0.5 mg/ml proteinase K

Denaturation solution

1.5 M NaCl
0.5 N NaOH

Neutralization solution

1 M Tris HCl pH 7.4
1.5 M NaCl

20 x SSC pH7.0

3 M NaCl
0.3 M Sodium Citrate

Hybridization solution

0.2 g fat free milk
17 ml water
2 g dextran sulfate
6 ml 20 x SSC
2 ml Formamide
1 ml 20% SDS
80 μl 500 mM EDTA

DEPC water

0.1% diethylpyrocarbonate in bi-distilled water
mixed vigorously and autoclaved

4.4.4 Bacterial Culture, DNA preparation/manipulation/extraction

LB (Luria Bertani) Medium

10g Peptone
5g Yeast extract
10g NaCl
1l H_2O
For plates, add 15g Agar-Agar

Solution E1 (Cell Resuspending):

50 mM Tris HCl (pH 8.0)
10 mM EDTA
Adjust pH with HCl to 8.0
Add RNaseA to 100μg per ml final concentration, store at 4°C

Solution E2 (Cell Lysis)

200 mM NaOH
1% SDS

Solution E3 (Neutralization)

3.1 M potassium acetate
adjust pH to 5.0 with acetic acid

TE buffer

10 mM Tris-HCl, pH 8.0
1 mM EDTA, pH 8.0

TE buffer for DNA microinjection

5 mM Tris-HCl, pH 7.4
0.1 mM EDTA, pH 7.4

4.4.5 FACS Solutions

FACS Buffer

PBS
1% FCS
1 mg/l propidium iodide

4.5 Methods

4.5.1 Chemically Competent Bacteria

Competent bacterial cells of *E. coli* strain XL-1 blue (Stratagene) were prepared according to the calcium chloride method (Sambrook and Russel, 2001). A single colony was picked and transferred to a 14 ml falcon tube containing 5 ml of LB culture medium and incubated overnight in a shaking incubator at 37ºC and 200 rpm. This pre-culture was transferred to an Erlenmeyer flask containing 200 ml of LB and incubated in a shaker at 37ºC, 200 rpm for about 2 hours until the OD600 was between 0.5 and 0.7. After centrifuging for 10 minutes 6000 rpm, the bacterial pellet was resuspended in 60 ml TFB I and incubated 10 minutes on ice. Finally, the cells were centrifuged again and the pellet was resuspended in 4 ml TFB II. The bacteria were transferred to Eppendorf microcentrifuge tubes in 100 μl aliquots. The cells were used immediately for transformation or stored at –80ºC after shock freezing in liquid nitrogen. The competence of bacteria was tested using 10 pg of plasmid DNA to transform the bacteria. Typically 10^7-10^8 colonies per μg of plasmid DNA were obtained.

4.5.2 Electrocompetent Bacteria

Eletrocompetent bacterial cells of *E. coli* strain XL-1 blue (Stratagene) were prepared according to Sambrook and Russel, 2001. A bacterial pre-culture was prepared overnight as described for chemically competent bacteria. This pre-culture was transferred to a tube containing 400 ml of LB medium and incubated in a shaking incubator at 37ºC and 200 rpm as described until reaching the same OD600 range for chemically competent bacteria. The cells were pelleted and immediately washed (3000 rpm at 4ºC) with ice cold sterile distilled water. The pellet was washed again with ice cold sterile water containing 10% glycerol. The bacterial pellet was re-suspended in 4 ml water with 10% glycerol and 50 ml bacterial aliquots were transferred to Eppendorf microcentrifuge tubes in 50 μl aliquots and used or stored as described above. Typically 10^8 – 10^9 colonies per μg of plasmid DNA were obtained.

4.5.3 Bacterial Transformation

Chemical Transformation

For transformation experiments, 10 pg of DNA or a 5 μl aliquot from a 20 μl ligation reaction was used. 100 μl of freshly prepared or stored competent bacteria were put on ice and the DNA for transformation was pipetted into the tube. The DNA and the bacteria were carefully mixed by flicking the tube. After 50 minutes incubation on ice, the bacteria were heat shocked by transferring them to a 42ºC water bath for exactly 90 seconds. The tube was immediately chilled on ice. After 2 minutes, 120 μl of 37ºC pre-warmed LB medium was pipetted into the tube, which was incubated in a water bath or thermoblock at 37ºC. After 30 minutes

incubation, the bacteria were plated on LB agar plates containing the specific antibiotic (ampicilin, 50 μg/ml or kanamycyn 25 μg/ml) for selection of transformed bacteria. The plates were incubated overnight at 37ºC.

Electroporarion

For electroporation procedures, 10 pg of DNA or a 2 μl aliquot from a 20 μl ligation reaction was used. 50 μl of freshly prepared or stored competent bacteria were put on ice and the DNA for transformation was pipetted into the tube. The DNA and the bacteria were carefully mixed by flicking the tube. After one minute incubation on ice, the bacteria and DNA were transferred to the electroporation cuvette which was put in the electroporator (Easyjet Prima, Equibio) and submitted to a pulse of 1800 v. One ml of 37ºC pre-warmed LB medium was pipetted into the cuvette and the content was transferred to an 1,5 ml eppendorf tube, which was incubated in a thermomixer at 37ºC for one hour. After one hour incubation, the bacteria were plated on LB agar plates containing the specific antibiotic (ampicilin, 50 μg/ml or kanamycyn 25 μg/ml) for selection of transformed bacteria. The plates were incubated overnight at 37ºC.

4.5.4 Plasmid Preparation

Depending on the DNA amount and purity desired, plasmid DNA was obtained by alkaline lysis method from bacteria using mini, midi or maxi endonuclease-free preparation kit.

Miniprep

Miniprep plasmid preparations were performed using solutions prepared in our laboratory and a modification of the alkaline lysis protocol from the Genomed Company:

The bacterial culture was grown overnight in a polystyrene Round-bottom tube (FALCON, BD Labware, France) containing 4 ml LB medium at 37ºC in a shaking incubator with a rotation of 220 rpm. 1.5 ml of the culture was pelleted in an Eppendorf tube by micocentrifugation 10000 rpm for 5 minutes at room temperature. The pellet was resuspended in 300 μl of E1 solution. After resuspension of the bacterial pellet, 300 μl E2 lysis solution was added and 5 minutes room temperature incubation was carried out to allow complete lysis of bacteria. After 5 minutes, 300 μl of the neutralization buffer E3 was added and gently mixed by inverting the tube about ten times. The tubes were centrifuged 14000 rpm in a microcentrifuge (Eppendorf 1754c) at room temperature for 15 minutes.

After centrifugation, the supernatant was transferred to a new Eppendorf tube and 630 μl Isopropanol was added for DNA precipitation. The tube was put at -20ºC for 10 minutes and subsequently centrifuged at 20000x g at 4ºC for 20 minutes. After centrifugation, the supernatant was discarded and the DNA pellet was washed using 400 μl 70% ethanol. The

tube was centrifuged again at 4ºC for 15 minutes. The ethanol was carefully removed and the pellet air-dried. The DNA pellet was dissolved in 30 μl TE.

4.5.5 Determination of DNA concentration

The DNA concentration was determined either by spectrophotometry or by comparing different dilutions of DNA with a standard amount of DNA in an agarose Gel.

4.5.6 Agarose Gel Electrophoresis

To test restriction enzyme reactions, size of DNA fragments, estimate DNA concentration, DNA fragment extraction, analysis of PCR reactions products, separation of DNA or RNA for subsequent Southern or Northern blotting procedures, horizontal agarose gel electrophoresis was performed. The agarose concentration of the gel was between 0.7 and 1.5% in 1X TBE buffer, depending on the expected size of the DNA bands. The agarose was dissolved in boiling TBE in an Erlenmeyer bottle and cooled down to approximately 55ºC. Ethidium bromide was added at a concentration of 0.3μg/ml to allow the DNA visualization on the gel under UV light The agarose was poured in the gel cassette and allowed to solidify for about 40 minutes. Before applying the samples, the DNA to be applied was diluted 1:1 in 2X loading buffer. 300 ng DNA standard were also loaded to determine the size or to estimate the concentration of the fragments separated on the gel. The electrophoresis was carried out at room temperature and at constant voltage of (100V for a 13 cm minigel) (Bio-Rad Powerpac 200).

4.5.7 DNA Gel Extraction

For gel extraction of PCR products or DNA enzymatically digested for cloning or microinjection, the QIAquick Gel Extraction Kit was used (QIAGEN GmbH, Hilden) according to the manufacturater's instructions. The desired DNA band visualized on the gel under UV light was cut out using a sterile surgical blade, put into a sterile 1.5 ml Eppendorf tube and weighed. For each 100 mg of gel, 300 μl of buffer QG solution was added to the tube. The tube was incubated 10 minutes at 50°C. After complete gel dissolution, an amount of isopropanol equal to the gel volume was added and the gel was homogenized by vortexing. The content of the tube was transferred to the QIAquick column provided in the kit. The tube was centrifuged for 1 minute at top speed in a microcentrifuge. The flow-through was discarted, the column put back in the same collection tube and 500 μl of buffer QG were added to the column to remove all traces of agarose. To wash the column, 750 μl of buffer PE containing ethanol were added and the column was centrifuged as in the previous steps. To remove ethanol residues from the column, after centrifugation and discarding the flow-through, the column was centrifuged for one more minute. Then, the column was placed in a

new 1.5 ml Eppendorf tube. For elution, 50 µl of EB buffer was pipetted directly to the center of the membrane and centrifuged as before.

The eluted DNA was visualized on an agarose gel to verify the recovered amount from the gel and estimate the concentration.

4.5.8 Restriction enzyme digestion of plasmid and genomic DNA

The restriction digestion of DNA was carried out to determine the size of plasmid DNA fragments, for cloning procedures (in this case, vector or insert fragments were purified by gel extraction), prior to Southern transfer or to separate the DNA constructs from the vector backbone for microinjection.

Table 10: Conditions for restriction enzyme digestion

Plasmid DNA (testing)	Plasmid DNA (cloning)	Genomic DNA
1 µg DNA	5 µg DNA	10 µg DNA
10 U enzyme	30U enzyme	80 U enzyme
2 µl enzyme buffer	5 µl enzyme buffer	10 µl enzyme buffer
H_2O to 20 µl	H_2O to 50 µl	H_2O to 100 µl
1 to 2 hours incubation	3 to 4 hours incubation	12 to18 hours incubation

The cleavage reaction was pipetted in a 1.5 ml Eppendorf tube and incubated in a thermoblock at the optimal temperature as recommended for each enzyme.

4.5.9 DNA cloning

For plasmid construction standard procedures as described by Sambrook and Russel, 2001 were used. After plasmid preparation, the cDNA was digested with restriction enzymes to generate suitable 5´ and 3´ ends for directional cloning, separated by agarose gel electrophoresis and purified by gel extraction. The vector and insert concentration was estimated by comparing the bands in an ethidium bromide stained agarose gel with known amounts of DNA from a DNA ladder. The molar ratio between insert and vector was adjusted to about 3:1. The ligation reaction was performed as recommended by the suppliers of T4 DNA ligase:

x µl vector DNA
y µl insert DNA

1 μl T4 DNA ligase (400U/μl) (New England Biolabs, Frankfurt)
2 μl T4 DNA ligase buffer
z μl Water to 20 μl

The ligation was carried out at room temperature for 2 hours, followed by an overnight incubation at 4°C or 16°C.

4.5.10 Automated fluorescent sequencing of PCR products or plasmid DNA

For the sequencing reaction, we used a DNA sequencing Kit (ABI Prism Sequencing Kit) and the following reagents were pipetted into a 0.2 ml PCR tube as shown in table 11:

Table 11: Reagents for sequencing reaction

Reagent	Volume (μl)	Final Concentration
DNA	2	0.7 to 1 μg
Big Dye Sequence Terminator	4	
Sequencing primer	0.5	10 pmol
Distilled H_2O	3.5	

The table 12 shows the sequencing program for all the sequencing reactions performed:

Table 12: Program for Sequencing reaction

		25 cycles		
Denaturation	Denaturation	Annealing	Elongation	Cooling
96°C: 2 min	96°C: 30 sec	50°C: 15 sec	60°C: 4 min	4°C: •

4.5.11 Cell culture

The cell culture procedures were carried out in a vertical laminar flow hood (BDK Luft- und Reinraumtechnick GmbH) to ensure the sterile conditions required for mammalian cell culture. The cells were grown in commercially available cell culture flasks or plates (see table 3) and incubated at 37ºC in a humid atmosphere containing 5% CO_2. The cell culture medium used for adherent cells was commercial-available DMEM, supplemented with 10% FCS and antibiotics (100U penicillin and 100 ng streptomycin/ml medium). The cells were split according to the cell growth rate and density on the culture flasks or plates. In order to maintain the cell lines in a stock, 6 millions of healthy cells in the growing phase were suspended in 1.5 ml of FCS with 10% DMSO in 1.8 ml cryotubes and frozen in liquid nitrogen. The cells were frozen in three cooling steps: Primarily, the suspension of cells was chilled in ice for 5 minutes in pre-cold freezing medium. The tubes were transferred to a -80°C freezer for two hours and further stored in liquid nitrogen. The table 13 lists the cell lines cultivated in this project;

Table 13: Cell lines used for co-localization, protein expression and protein-targeting studies.

Cell Line	Origin	Morphology
HEK293T*	Established from a human primary embryonal kidney transformed by adenovirus type 5 (Ad 5)	adherent fibroblastoid cells growing as monolayer
NIH3T3*	Swiss mouse embryo	adherent fibroblast cells growing as monolayer

- reference in www.dsmz.de

4.5.12 Examination of CALM/AF10 transgenicity in FVB mice by PCR and Southern blotting

Mouse-tail genomic DNA extraction

To isolate genomic DNA from mouse tails we followed a protocol that was recommended by The Jackson's Laboratory (www.jax.org/imr/tail_phenol.html): A 0.5cm tail piece from a one month old FVB mouse was cut and digested overnight at 50°C on a rotator (900 rpm) in an Eppendorf tube containing 0.5 ml of proteinase K (0.5 mg/ml) diluted in lysis buffer. After complete digestion of the tail tissue, 0.7 ml phenol-chloroform-isoamyl alcohol was added to the tube and put on the rotator. After one hour, the Eppendorf tube was centrifuged at 14 000 rpm for 15 minutes at RT. In the tube three phases separated out: the upper phase, containing genomic DNA, the middle phase, containing RNA and protein debris and the bottom phase containing cell debris. The upper phase was carefully transferred to a new sterile 1.5 ml Eppendorf tube. To the supernatant, 1 ml of absolute ethanol was added and the tube was mixed for one minute by gentle inversion. The tube was centrifuged at 4°C (22,800 x g). The supernatant was carefully removed and the DNA pellet was washed with 70% ethanol. The tube was centrifuged again at top speed at 4°C. The ethanol was further removed and the pellet was air-dried for 10 minutes. The genomic DNA was resuspended in TE by pipette homogenization and to facilitate DNA solubilization, incubated 65°C for 15 minutes. The quality of genomic DNA was assessed by agarose electrophoresis and the concentration measured by spectrophotometry.

Polymerase Chain Reaction (PCR)

The PCR reaction was used as screening to identify the transgenic mice. The primer used for the reaction anneal in CALM and AF10 regions adjacent to the breakpoint of CALM/AF10 in the transgenic construct The table 14 illustrates the general condition for genomic DNA PCR

Table 14: General setting-up for PCR samples

Reagent	Volume (μl)	Final Concentration
Template DNA (200 ng/μl)	1	10 pg
Taq polymerase (5 U/μl)	0.125	0.75 U
Forward primer (10 μM)	0.5	0.25 μM
Reverse primer (10 μM)	0.5	0.25 μM
10 X Buffer	2	1 x
dNTP mix	2	0.2 mM/each
$MgCl_2$ (50 mM)	0.6	1.5 mM

PCR to verify the mouse DNA quality:

The PCR program for this primer pair is shown in Table 14:

Table 15: PCR program for STS primers

		25 cycles		
Denaturation	Denaturation	Annealing	Elongation	Cooling
95°C: 5 min	95°C: 45 sec	56°C: 45 sec	72°C: 45 sec	72°C 7 min; 4°C: •

Southern Blotting

To confirm the presence of the transgene, Southern hybridisation was performed. 15 μg of mouse genomic DNA was digested overnight at 37°C with *EcoR1* and size-fractionated on a 0.7% agarose gel. The gel was photographed with a ruler before Southern transfer. To improve the transfer of DNA fragments bigger than 15 kb, the gel was immersed for 10 minutes in 0.1 N HCl (acid depurination). The gel was then rinsed with distilled water and soaked for 45 minutes in several volumes of 1.5 m NaCl, 0.5 N NaOH with constant gentle agitation for DNA denaturation. After denaturation, the gel was briefly rinsed in deionized water, and then neutralized by soaking for 30 minutes in several volumes of a solution of 1 M Tris (pH 7.4), 1.5 m NaCl at room temperature with constant, gentle agitation. The neutralization solution was changed and the gel incubated for further 15 minutes. While the gel was in the neutralization solution, a piece of whatman 3 MM paper was cut in the same size of the agarose gel. Another paper was cut to have the same width, but was left long enough to reach the bottom of both sides of the electrophoresis chamber used for the blotting. The chamber was filled with transfer buffer (10XSSC) until the level of the liquid reached almost the top of the gel support. When the 3 MM paper on the top of the support was thoroughly wet, all air bubbles were removed using a 10 ml pipette. Using a paper cutter a piece of nitrocellulose filter about 1 Mm larger than the gel in both dimensions was cut and floated on the surface of a dish containing deionized water until it was completely wet, and then immersed in transfer buffer for at least 5 minutes. Using a clean scalpel blade a corner from the nitrocellulose filter

was cut to match the cut corner of the gel. The gel was removed from the neutralization solution and inverted so that its bottom side was on the top. The inverted gel was placed on the support so that it was centred on the wet 3 MM papers. Air bubbles between the 3 MM paper and the gel were removed. To prevent liquid flowing directly from the reservoir to the paper towels placed on top of the gel, which would impair DNA transfer, sealing film (Nescofilm, Carl Roth, Karlsruhe) was placed around the gel and membrane. The wet nitrocellulose was placed to the top of the gel, avoiding air bubbles. Two pieces of 3 MM paper (cut to exactly the same size as the gel) were wet in 2XSSC and placed top of the wet nitrocellulose filter. On top of the last two 3 MM papers, a stack of paper towels (5-8cm high) just smaller than the 3 MM papers was placed. A glass plate was put on top of the stack and weighed down with a 500-g weight. The objective was to set up a flow of liquid from the reservoir through the gel and the nitrocellulose filter, so that fragments of denatured DNA were eluted from the gel and were trapped in the nitrocellulose filter. The transfer was carried out for 14 to 18 hours. After the transfer, the presence of ethidium bromide stained DNA on the membrane could be observed under UV light.

DNA labelling

The labelling of the Southern probes with radiocative isotopes was performed using the Megaprime DNA Labelling System (Amersham Biosciences, England). In a 1.5 ml Eppendorf tube, 30 ng DNA, 5 µl Primer and 22 µl TE were pipetted to a final volume of 33 µl. The tube was put in a thermoblock at 98°C for 5 minutes. After denaturation, the tube was cooled down on ice for 5 minutes. 10 µl of 5x reaction buffer (dATP, dGTP, dTTP), 2 µl Klenow fragment and 5 µl (50µCi) [α-32P] dCTP were added. The tube was incubated for 1 hour at 37°C in a water bath. After vortexing and centrifugating a microspin column at 3000 rpm for one minute, 25 µl TE were added to the labeled DNA probe which was applied on the microspin column and centrifuged using the same spin and time. After collecting the probe in a new Eppendorf tube, 200 µl salmon sperm DNA (9.5 mg/ml) were added to the solution.

DNA hybridization on nitrocellulose membranes

After the DNA transfer, the DNA was cross-linked to the membrane using an UV irradiation. The membrane was transferred to a glass tube and pre-heated with 25 ml of pre-hybridization solution for 30 minutes at 62°C. 100 µl of salmon sperm were denatured at 100°C and added to the pre-hybridization solution with the membrane. The pre-hybridization was carried out for a further 2 hours at 62°C. After this time, the labeled probe was denatured at 98°C and added. The hybridization was performed overnight at 62°C in a hybridization oven.

After the hybridization, the membrane was washed four times at low stringency: twice for 10 minutes in 2 X SSC at 65°C and twice for ten minutes in 2 X SSC + 0.1% SDS at 65°C. Then a high stringency wash for ten minutes with 0.2 X SSC + 0.1% SDS at 65°C.

Film Exposure

The membrane was taken out of the tube, wrapped in Saran wrap and put into the film cassette (Hypercassete™, Amersham Biosciences, England). The film was put over the membrane and the exact location of the membrane on the film was marked with a pen. A first test-exposure was carried out for 15 minutes and the film was developed. If the signals on the film were weak, the exposure was extended for some hours or even days. In these cases, the film was kept at -80°C.

4.5.13 Transient transfection of mammalian cells

For co-localization and protein expression experiments, mouse (NIH3T3) or human (293T) cell lines were transiently transfected using an activated-dendrimer transfection reagent (Hudde *et al.*, 1999) from a commercial kit (PolyFect® Transfection Reagent, Qiagen).

Protein expression:

One day before the transfection, cells in an exponential stage of growth were washed once with PBS, detached from the 75 cm^2 cell culture flasks with 2 ml of trypsin-EDTA solution, counted using a Neubauer improved hemocytometer and put in 100 mm cell culture plates at a density of 10^6 cells/plate.

The plates were 80% confluent on the next day. For the transfection, 23 μg of plasmid DNA was suspended in 625 μl of DMEM without FCS and antibiotics. This solution was mixed with 135 μl of Polyfect and incubated at room temperature for 10 minutes. To the DNA/Polyfect complex, 6.7 ml of complete DMEM medium (with 10% FCS and antibiotics) was added and added to the cells. After 2 hours and 30 minutes, the cells were washed once with PBS and incubated with new complete DMEM. The transfection efficiency was controlled 20 hours later for the expression of the reporter gene using an inverted fluorescence microscope (Axiovert 200 Carl Zeiss, Germany).

Co-localization:

24 hours before transfection, exponentially growing mouse fibroblast cells (NIH3T3) in 75 cm^2 cell culture flasks were washed, detached and the cell number was determined as described. 8 x 10^4 cells were transferred to a 4-well (2 cm^2) special chamber (Lab-Tek II Chambered coverglass, Nalge Nunc) with a borosilicate bottom for visualization of cells transfected with GFP constructs by fluorescence microscopy. The transfection followed the protocol of the manufacturer: Initially, the DNA was suspended in 20 μl of DMEM without FCS and antibiotics. To the DNA/medium mix, 2 μl of PolyFect were pipetted and vortexed

for 10 sec. The DNA/transfection reagent complex was incubated at RT for 10 min. During the incubation time, the cells were washed once with PBS and 300 µl of complete DMEM medium was added to the cells. 120 µl DMEM containing 10% FCS and antibiotics were added to the DNA complex, the content of the tube was mixed by pipetting up and down and added to the cells. The cells were incubated at 37°C under 5% CO_2 atmosphere for 22 to 26 hours before the cells were examined with the inverted fluorescence microscope.

Cell fixation for microscopy

After the observation of the positively transfected cells on the inverted microscope, the cells were fixed with formaldehyde as described by Dernburg and Sedat (1998). Briefly, the cell culture medium was removed, the cells washed once with PBS and put in a 3% formaldehyde diluted in PBS for 10 min at RT. The cells were washed three times with PBS and analysed on the microscope or stored at 4°C protected from the light.

4.5.14 Stable transfection of mammalian cells

In order to establish stable cell lines for the inducible expression of CALM/AF10, expression constructs were trasfected into NIH3T3 cells as described for transient transfection. The efficiency of the transfection was evaluated 22 hours after transfection by observing the green GFP fluorescence in cells transfected with the pEGFP control plasmid. 48 hours after transfection, 1 µg/ml medium with puromycin (was added for the selection of the stably transfected cells). The addition of puromycin for the selection was carried out for two weeks.

4.5.15 Obtaining and processing of microscope images

The transfected cells were observed using an Axiovert 200 fluorescent microscope (Zeiss, Germany), the images captured using a digital camera (Hamamatsu C474295) and processed in a Power Mac G4 computer using the Open Lab software (Improvision, Heidelberg, Germany). The pictures were saved as Tiff files and the images processed using the program adobe photoshop.

4.5.16 Examination of CALM/AF10 expression at RNA Level by Reverse Transcriptase PCR and Microarrays

RNA extraction from frozen cells

The RNA extraction was performed using a denaturing guanidine isothiocyanate-containing buffer for cell lysis and a silica-gel-based membrane for RNA isolation. The reagents and the protocol from the Rneasy® MiniKit (Qiagen) were used as described: Initially, the cells were thawed in a water bath at 37oC and quickly centrifuged at 300 x g for 5 minutes at 4°C. The

cell pellet was immediately suspended in lysis solution RLT (350 µl or 600 µl RLT buffer with 1% freshly added beta mercaptoethanol depending on the cell number available). The sample was homogenized using a QIAshredder spin column and mixed with an equal volume of 70% ethanol. The sample was applied to an RNeasy mini column placed in a 2 ml collection tube and centrifuged for 15 seconds at 8000 x g. The flow-through was discarded, and 700 µl of RW1 buffer was added to the Rneasy column. The column placed in the same collection tube was centrifuged as before. The column was transferred into a new 2 ml collection tube and 500 µl of buffer RPE were pipetted into the column, which was centrifuged as before. This step was repeated and the column was put into a new RNase free 1.5 ml Eppendorf tube. 30 to 50 µl RNase free water was pipetted into the column for RNA elution. The column was centrifuged for one minute at 8000 x g. The RNA obtained was used for cDNA systhesis or analyzed with an Affymetrix gene chip array at the laboratory for leukemia diagnostics, Department of Medicine III at the Klinikum Grosshadern. The expression profiling with the Affymetrix chips was performed by Alexander Kohlmann.

For microarray analysis, the GeneChip System was used (Affymetrix, Santa Clara, CA). The targets for GeneChip analysis were prepared according to the current Expression Analysis Technical Manual. 5 to 10 µg total RNA was used as a started material in the subsequent cDNA synthesis with oligo [(dT)24T7promotor]65 primer (cDNA Synthesis System, Roche Diagnostics). The cDNA was purified by phenol/chlorophorm/isoamylalcohol extraction (Ambion, Austin, TX) and acetate/ethanol-precipitated overnight. For detection of the hybridized target nucleic acid biotin-labelled ribonucleotides were incorporated during the *in vitro* transcription (Enzo BioArray High Yield RNA Transcript Labelling Kit, Enzo Diagnostics). After quantification of the purified cRNA (RNeasy Mini Kit, Qiagen), 15 µg was fragmented by alkaline treatment (200 mM Tris-acetate, pH 8.2 / 500 mM potassium acetate / 150 mM magnesium acetate) and added to the hybridization cocktail sufficient for five hybridizations on standard GeneChip microarray. Before hybridization onto U95Av2, Test3 microarrays (AffymetricX) were chosen for monitoring the integrity of the cDNA. Washing and staining of the probe arrays were performed according to the current protocols (Micro_1v1, EukGE-WS2v2). The Affymetrix software (Microarrays Suite, version 4.0.1) extracted fluorescence intensities from each element on the microarrays as detected by confocal laser scanning according to the manufacturer's recommendations. The hybridization cocktails which demonstrated high-quality cRNA characteristics were selected for the analysis.

The expression analysis of the patients samples was carried out by Alexander Kohlmann, using the unsupervised analysis methods, principal component analysis (PCA) and hierarchical clustering. In the PCA, most of expression information is reduced dimensionally and represented in a linear transformation matrix. Principal components are the projections of the data in a three-dimensional vector. The hierarchical clustering organizes the expression data into groups with similar expression signatures (Schock *et al.,* 2004).

RNA from FACS-sorted cells

Cells from spleen, bone marrow or thymus were obtained from organs of mice immediately after euthanasia and passed through a cell strainer to obtain separate cells. The cells were suspended in DMEM or RPMI containing 15% FCS and the erythrocytes were lysed for 20 minutes RT using ammonium chloride 1:1 diluted in the cell suspension. The cells were count, ressuspended in PBS, transferred to FACS tubes and incubated 20 minutes at 4°C with the antibodies used for the cell sorting. After incubation, the cells were washed once (300 x g at 4°C for 10 minutes) and ressuspended in FACS buffer. Sorted cells were collected in eppendorf or FACS tubes containing cell culture medium with 50% FCS. The cells were centrifuged and lysed with RLT buffer according to the cell number and the RNA of the sorted cells was extracted as as described for frozen cells using the Rneasy® MiniKit.

RNA preparation from mouse tissue

RNA extraction of mouse tissue was carried out using the peqGOLD TriFast™ kit (Peqlab Biotechnologie GmbH, Germany). This kit is based on guanidine isothiocyanate lysis buffer and a phenol chloroform RNA extraction. The tissue RNA extraction was carried out as follow: Immediately after resection from euthanized mice, spleen, thymus and bone marrow were put into Eppendorf tubes and frozen in liquid nitrogen. The frozen organs were subsequently processed or stored at –80°C. For RNA extraction a steel bar was washed with autoclaved bidistilled DEPC-treated (0.1%) water. The frozen organ was transferred to a 15 ml falcon tube, which was kept in a vessel containing liquid nitrogen. The steel bar was used to grind the organ until it became as fine as sand. The organ fragments were lysed with 1 ml of peqGOLD Trifast solution (containing phenol and guanidin isothiocyanate) for each 100 mg of organ. After 5 minutes incubation at RT, 0.2 ml chloroform was added and the tube vortexed for 15 seconds. The tube was incubated for 3 to 5 minutes at RT and further centrifuged at 1400 rpm, RT for 5 minutes. Three phases were obtained after centrifugation: the upper phase, containing the RNA, the intermediate phase, containing DNA and protein, and the lower phase, containing phenol-chloroform. The upper phase was transferred to a new tube and precipitated using 0.5 ml of 100% ethanol for each 1 ml of peqGOLD Trifast solution initially used for the lysis. After mixing and centrifuging the tubes (14000 rpm for 15 minutes at 4°C), the RNA pellet was washed with 500 μl of 75% ethanol and centrifuged as before. The supernatant was carefully removed, the RNA air-dried and resuspended in RNAse free water. To assess the RNA quality after extraction, 2 μl of RNA were loaded on a 0.7% agarose gel containing ethidium bromide and observed with UV light.

cDNA synthesis (Reverse Transcriptase Reaction)

For cDNA synthesis, the reagents and protocols used were purchased from Invitrogen. To avoid DNA contamination, the RNA was treated with Deoxyribonuclease I before the cDNA

synthesis. For DNase treatment of RNA, 1 µg RNA was mixed with 1 µl 10X DNase I reaction buffer, 1 µl DNase I (1 U/µl) and in a total volume of 10 µl. The tube was incubated at RT for 40 minutes and the DNase was inactivated by adding 1 µl 25 mM EDTA to the Eppendorf tube and incubating at 65°C for ten minutes. After DNase treatment, an RNA aliquote was loaded on an agarose gel for quality control and to estimate the RNA concentration. For the Reverse Transcriptase reaction, 2 to 4 µl of RNA were pipetted into an 1.5 ml RNase-free Eppendorf tube together with 1 µl Oligo $(dT)_{12-18}$, 1 µl 10 MM dNTP mix (containing 10 MM each dATP, dGTP, dCTP and dTTP at neutral pH) and sterile water in a total volume of 12 µl. The mixture was heated to 65°C for five minutes and quickly chilled on ice. To the tube contents 4 µl 5 x First-Strand Buffer, 2 µl 0.1 M DTT and 1 µl RNaseOUT™ were added. The tube contents was mixed and incubated at 42°C. After 2 minutes, 1 µl of Superscript™ II Reverse Transcriptase was added, and the incubation at 42 °C was continued for 50 Min. The reverse transcriptase was then heat-inactivated at 70°C for 15 min.

4.5.17 Western blotting

Sample preparation

Cell lysis (total cell extract)

Between 5 to $10x10^7$ cells in a cell culture dish (e.g. transiently transfected 293 cells) were lysed using 150 µl RIPA buffer with freshly added protease inhibitors. The cells with the RIPA buffer were transferred to an Eppendorf microcentrifuge tube and mixed by inversion for 30 minutes at 4°C. After this step, the sample was centrifuged at 14000 rpm for 30 minutes and the supernatant was transferred to a new Eppendorf tube, the protein concentration was determined by the Bradford method (see below) and frozen at -80°C. To obtain protein extracts form the tissues of transgenic mice, 50 to 100 mg of organ tissue (e.g. thymus or spleen) were taken from euthanized mice and frozen in liquid nitrogen. The organs were smashed using a steel bar in a 15 ml falcon tube submersed in liquid nitrogen to avoid thawing of the sample. After complete rupture of the tissue, RIPA buffer was added to the cells and the lysis procedure was carried out as described above.

Determination of Protein Concentration

Protein concentrations were determined with the Bradford (Bio-Rad Protein Assay, Germany) method. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The extinction of a dye-albumin complex solution is

proportional to the protein amount over a 10-fold concentration range (5-25 μg/ml). The protein concentration of the sample was determined by comparing the extinction of the sample to values obtained from a range of protein standards. The protein standard used was Bovine Serum Albumin (BSA). Six different amounts of albumin (2.5 μg, 5 μg, 10 μg, 15 μg, 20 μg and 25 μg) were diluted in distilled water to a final volume of 800 μl. One μl of cell lysate was diluted in distilled water for the measurement. 200 μl of Protein Assay solution was added to the tubes. The tubes were incubated at RT for 15 minutes. For the measurements in spectrophotometer polystyrol cuvettes (10x4x45 mm Sarstedt, Germany) were used.

Protein gel electrophoresis

Total cell extract (TCE) proteins were separated with SDS-PAGE . A 5% polyacrylamid stacking gel and a 6 to 10% separation gel (tris-glycine buffer) were used (Sambrook and Russel, 2001). The protein lysates were diluted 1:1 with 2x loading buffer and heated in a boiling water bath for 10 minutes. 80 μg of protein in 15 to 30 μl volume was loaded in each gel lane. The electrophoresis was performed with constant voltage (100 V for a 15 cm gel) for 1 hour and 30 minutes in the cold room at 4°C.

Western blotting and Detection

After electrophoresis, the gel was taken from the cassette and washed once with TBS. For the blotting, the wet system was used (Bio-Rad Miniprotean® II system, Biorad Laboratories, Germany). To permit a better transfer of large molecular weight proteins such as CALM/AF10, which has a predicted size of about 170 kDa, a PVDF membrane was chosen after comparing the transfer efficiency between PVDF and nitrocellulose. The PVDF membrane was wetted in methanol for 30 seconds, rinsed in distilled water and equilibrated in transfer buffer for 10 minutes. The transfer system was assembled by putting a sponge on the bottom of the sandwich (in contact to the negative pole), a 0.8 mm filter paper in contact to the sponge, and the gel on the paper. Care was taken to eliminate all air bubbles in the stack. The transfer assembly was completed by another filter and a second sponge. The PVDF membrane was oriented to the positive pole to permit the protein (negatively charged) to migrate from the gel to the membrane. The transfer was performed at constant amperage of 250 mA for 4 hours at 4°C with buffer recirculation. The observation of the high molecular weight proteins of the pre-stained protein standard (SeeBlue Plus2, Invitrogen, Germany) on the membrane was used as an indicator for successful transfer. The proteins were fixed by air-drying to the membrane. The antibody-detection of protein was performed following the instructions of the antibody's supplier (Santa Cruz Biotechnology, USA). After the transfer, the membrane was blocked to prevent non-specific binding of antibodies to the membrane by incubating with Blotto A buffer for one hour at room temperature or overnight at 4°C with constant agitation. All incubation of the membrane were performed in a 50 ml Falcon tube on a roller mixer (CAT RM5 Digisystem Laboratory Instruments Inc). The membrane was washed once with TBS for

five minutes and incubated with the primary antibody at the appropriate dilution (e.g. 1:1000 in 4 ml volume) in Blotto A overnight. After incubation with the primary antibody, the membrane was washed three times with TBS 0.05% Tween-20 (TBST). The secondary antibody conjugated to Horse Radish Peroxidase (HRP) was diluted 1:2000 in Blotto A (4 ml) and put on the membrane for a 45 to 90 minutes-incubation at room temperature. The membrane was rinsed with distilled water, washed again tree times with TBST and once with TBS for 5 minutes. To detect the antibodies on the membrane, a commercial chemiluminescence kit (ECL Western blotting Detection Reagents Amersham Biosciences, Germany) was used according to the manufacturer's instructions. After washing, the ECL detection solution (0.125 ml/cm^2 membrane) was put on the membrane for 90 seconds. The exceeding amount of ECL was removed from the membrane using tissue paper. The membrane was covered with a plastic film and put in a film cassette and exposed to a chemiluminescence film4 for various exposure times (15 sec to 10 minutes).

4.5.18 Blood analysis of transgenic mice

Venous blood from transgenic mice was obtained from the orbital sinus using a microhematocrit tube under general anaesthesia with ethyl ether. The blood was collected in an EDTA microtube (Mikro-Probengefass, Sarstedt, Germany), mixed gently by inverting ten times and analyzed within two hours after blood collection (Jain, 1993). A blood smear was made on a glass slide at the collection time and air dried before staining. For the staining, the glass slide was put in a chamber with May-Grünwald solution for three minutes, washed with distilled water for two minutes, and immersed in a chamber with a 10% Giemsa solution for 30 minutes. After Giemsa staining, the slide was washed again with distilled water and air-dried. The cell morphology and relative blood cell count were analyzed with a standard optic microscope (Carl Zeiss, Germany). The complete blood cell count was performed using a Neubauer hemocytometer. For counting the erythrocytes, the blood was diluted 1:1000 in PBS, and for counting the leukocytes dilution was 1:50 in PBS with 3% glacial acetic acid for red blood cell lysis. The hematological values obtained were compared to reference values for the FVB/N mouse strain (www.jax.org).

[4] Hyperfilm ECL Amersham Biosciences

4.5.19 Flow cytometry

In order to analyse the development of the lymphoid lineages of the transgenic mice, spleen, thymus, lymph nodes and bone marrow cells were taken and analyzed for the expression of surface antigens. The mice were euthanized as described, the organs removed and the cells harvested and passed trough a cell strainer to obtain a cell suspension. The cells were suspended in RPMI1640 with 5% FCS and put on ice. The cell number was determined by counting using a Neubauer chamber as described above (chapter 4.5.16). 100,000 cells were resuspended in 100 μl PBS and incubated with different fluorescence–conjugated antibodies against cell surface markers for 20 min on ice avoiding light exposure. After incubation, the cells were washed once with PBS (300 x g for 10 minutes at 4ºC) and ressuspended in 100 μl of FACS buffer for immediate analysis or stored with PBS-4% paraformaldehyde solution at 4°C under light protection for subsequent analysis. Antibodies used for FACS were labelled with phycoerythrin for Gr-1, CD11b (Mac-1), Sca-1, Ter119, CD4, CD19, CD23, CD24, CD43, sIgM, F4/80 and allophycocyanin conjugated CD11b (Mac-1), CD117 (c-kit), B220, and CD8. Fluorescence was detected using a FACSCalibur flow cytometer and analyzed using the CellQuest software. Dead cells were gated out by high propidium iodide (PI) staining and forward light scatter.

4.5.20 *Post mortem* analysis of transgenic mice

Transgenic and control FVB mice were anesthetized with ethyl ether and euthanized by cervical dislocation. The mice were wetted with 70% ethanol, put on a clean paper towel and fixed to a corkboard with their limbs. The dissection instruments were autoclaved and soaked in ethanol before use. A vertical, ventral, midline incision was made from the neck to pubis. The skin was stretched laterally and pinced to the board. The peritoneum was opened and the diaphragm cut along the border of the ribs. The mouse was submersed in a vessel containing a 4% paraformaldehyde solution for at least 24 hours. The post mortem and histo-pathological examination were performed at the Institute of Pathology, GSF, Neuherberg by Dr. Quintanilla-Fend.

5. RESULTS

5.1. Cell Biology

5.1.1.Sequencing of CALM/AF10

The CALM/AF10 fusion gene used in all experiments was derived from the the U937 cell line (Dreyling *et al*., 1996) and had been cloned in Prof. Bohlander laboratory in Chicago and Göttingen into the pEGFP-C1 vector (Clontech). The CALM/AF10 cDNA was cloned in frame at the C-terminus of the EGFP coding sequence using *Xho I* and *SacII* sites at the amino and carboxy terminal ends, respectively. This construct was verified by sequencing before starting the cloning of the construct that was used in this thesis. The sequencing revealed almost the whole cDNA sequence of CALM (exons one to twenty, without exon 18) with the breakpoint at nucleotide 2091 of the CALM sequence (Dreyling et al., 1996) (gene bank accession number NM_001008660). The breakpoint in AF10 was located at nucleotide 424, corresponding to the start of exon 8. The cDNA of AF10 in this clone corresponds to the transcript variant 1 of AF10 (accession NT_004641) and lacks exons 18, 19 and 20. The AF10 sequence in this insert has a different 3' end from the published AF10 reference sequence (Chaplin *et al.,* 1995) (accession NT_004641). In our sequenced AF10 fragment, there are 123 extra nucleotides between exon 28 and 29. The sequence of the complete CALM/AF10 insert with the restriction enzyme sites *XhoI* at the 5' end and *SacII* at the 3' end is shown in the figure 4.

```
           10          20          30          40          50          60
CT CGA GGT CGA CGG TAT CGG GGG ATC GAT CCG CAT GCG AGC TCG GTA CCC CGG CTG CTG
 XhoI
           70          80          90         100         110
 AGC GGG TGG GGT GGT GGA GGA GCT GCA GAG ATG TCC GGC CAG AGC CTG ACG GAC CGA ATC
                                        M   S   G   Q   S   L   T   D   R   I>
120         130         140         150         160         170
 ACT GCC GCC CAG CAC AGT GTC ACC GGC TCT GCC GTA TCC AAG ACA GTA TGC AAG GCC ACG
  T   A   A   Q   H   S   V   T   G   S   A   V   S   K   T   V   C   K   A   T>
180         190         200         210         220         230
 ACC CAC GAG ATC ATG GGG CCC AAG AAA AAG CAC CTG GAC TAC TTA ATT CAG TGC ACA AAT
  T   H   E   I   M   G   P   K   K   K   H   L   D   Y   L   I   Q   C   T   N>
240         250         260         270         280         290
 GAG ATG AAT GTG AAC ATC CCA CAG TTG GCA GAC AGT TTA TTT GAA AGA ACT ACT AAT AGT
  E   M   N   V   N   I   P   Q   L   A   D   S   L   F   E   R   T   T   N   S>
300         310         320         330         340         350
 AGT TGG GTG GTG GTC TTC AAA TCT CTC ATT ACA ACT CAT CAT TTG ATG GTG TAT GGA AAT
  S   W   V   V   V   F   K   S   L   I   T   T   H   H   L   M   V   Y   G   N>
360         370         380         390         400         410
 GAG CGT TTT ATT CAG TAT TTG GCT TCA AGA AAC ACG TTG TTT AAC TTA AGC AAT TTT TTG
  E   R   F   I   Q   Y   L   A   S   R   N   T   L   F   N   L   S   N   F   L>
420         430         440         450         460         470
 GAT AAA AGT GGA TTG CAA GGA TAT GAC ATG TCT ACA TTT ATT AGG CGG TAT AGT AGA TAT
  D   K   S   G   L   Q   G   Y   D   M   S   T   F   I   R   R   Y   S   R   Y>
480         490         500         510         520         530
 TTA AAT GAG AAA GCA GTT TCA TAC AGA CAA GTT GCA TTT GAT TTC ACA AAA GTG AAG AGA
  L   N   E   K   A   V   S   Y   R   Q   V   A   F   D   F   T   K   V   K   R>
540         550         560         570         580         590
 GGG GCT GAT GGA GTT ATG AGA ACA ATG AAC ACA GAA AAA CTC CTA AAA ACT GTA CCA ATT
  G   A   D   G   V   M   R   T   M   N   T   E   K   L   L   K   T   V   P   I>
600         610         620         630         640         650
 ATT CAG AAT CAA ATG GAT GCA CTT CTT GAT TTT AAT GTT AAT AGC AAT GAA CTT ACA AAT
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   I   Q   N   Q   M   D   A   L   L   D   F   N   V   N   S   N   E   L   T   N>
660         670         680         690         700         710
  GGG GTA ATA AAT GCT GCC TTC ATG CTC CTG TTC AAA GAT GCC ATT AGA CTG TTT GCA GCA
   G   V   I   N   A   A   F   M   L   L   F   K   D   A   I   R   L   F   A   A>
720         730         740         750         760         770
  TAC AAT GAA GGA ATT ATT AAT TTG TTG GAA AAA TAT TTT GAT ATG AAA AAG AAC CAA TGC
   Y   N   E   G   I   I   N   L   L   E   K   Y   F   D   M   K   K   N   Q   C>
780         790         800         810         820         830
  AAA GAA GGT CTT GAC ATC TAT AAG AAG TTC CTA ACT AGG ATG ACA AGA ATC TCA GAG TTC
   K   E   G   L   D   I   Y   K   K   F   L   T   R   M   T   R   I   S   E   F>
840         850         860         870         880         890
  CTC AAA GTT GCA GAG CAA GTT GGA ATT GAC AGA GGT GAT ATA CCA GAC CTT TCA CAG GCC
   L   K   V   A   E   Q   V   G   I   D   R   G   D   I   P   D   L   S   Q   A>
900         910         920         930         940         950
  CCT AGC AGT CTT CTT GAT GCT TTG GAA CAA CAT TTA GCT TCC TTG GAA GGA AAG AAA ATC
   P   S   S   L   L   D   A   L   E   Q   H   L   A   S   L   E   G   K   K   I>
960         970         980         990        1000        1010
  AAA GAT TCT ACA GCT GCA AGC AGG GCA ACT ACA CTT TCC AAT GCA GTG TCT TCC CTG GCA
   K   D   S   T   A   A   S   R   A   T   T   L   S   N   A   V   S   S   L   A>
1020        1030        1040        1050        1060        1070
  AGC ACT GGT CTA TCT CTG ACC AAA GTG GAT GAA AGG GAA AAG CAG GCA GCA TTA GAG GAA
   S   T   G   L   S   L   T   K   V   D   E   R   E   K   Q   A   A   L   E   E>
1080        1090        1100        1110        1120        1130
  GAA CAG GCA CGT TTG AAA GCT TTA AAG GAA CAG CGC CTA AAA GAA CTT GCA AAG AAA CCT
   E   Q   A   R   L   K   A   L   K   E   Q   R   L   K   E   L   A   K   K   P>
1140        1150        1160        1170        1180        1190
  CAT ACC TCT TTA ACA ACT GCA GCC TCT CCT GTA TCC ACC TCA GCA GGA GGG ATA ATG ACT
   H   T   S   L   T   T   A   A   S   P   V   S   T   S   A   G   G   I   M   T>
1200        1210        1220        1230        1240        1250
  GCA CCA GCC ATT GAC ATA TTT TCT ACC CCT AGT TCT TCT AAC AGC ACA TCA AAG CTG CCC
   A   P   A   I   D   I   F   S   T   P   S   S   S   N   S   T   S   K   L   P>
1260        1270        1280        1290        1300        1310
  AAT GAT CTG CTT GAT TTG CAG CAG CCA ACT TTT CAC CCA TCT GTA CAT CCT ATG TCA ACT
   N   D   L   L   D   L   Q   Q   P   T   F   H   P   S   V   H   P   M   S   T>
1320        1330        1340        1350        1360        1370
  GCT TCT CAG GTA GCA AGT ACA TGG GGA GAT CCT TTC TCT GCT ACT GTA GAT GCT GTT GAT
   A   S   Q   V   A   S   T   W   G   D   P   F   S   A   T   V   D   A   V   D>
1380        1390        1400        1410        1420        1430
  GAT GCC ATT CCA AGC TTA AAT CCT TTC CTC ACA AAA AGT AGT GGT GAT GTT CAC CTT TCC
   D   A   I   P   S   L   N   P   F   L   T   K   S   S   G   D   V   H   L   S>
1440        1450        1460        1470        1480        1490
  ATT TCT TCA GAT GTA TCT ACT TTT ACT ACT AGG ACA CCT ACT CAT GAA ATG TTT GTT GGA
   I   S   S   D   V   S   T   F   T   T   R   T   P   T   H   E   M   F   V   G>
1500        1510        1520        1530        1540        1550
  TTC ACT CCT TCT CCA GTT GCA CAG CCA CAC CCT TCA GCT GGC CTT AAT GTT GAC TTT GAA
   F   T   P   S   P   V   A   Q   P   H   P   S   A   G   L   N   V   D   F   E>
1560        1570        1580        1590        1600        1610
  TCT GTG TTT GGA AAT AAA TCT ACA AAT GTT ATT GTA GAT TCT GGG GGC TTT GAT GAA CTA
   S   V   F   G   N   K   S   T   N   V   I   V   D   S   G   G   F   D   E   L>
1620        1630        1640        1650        1660        1670
  GGT GGA CTT CTC AAA CCA ACA GTG GCC TCT CAG AAC CAG AAC CTT CCT GTT GCC AAA CTC
   G   G   L   L   K   P   T   V   A   S   Q   N   Q   N   L   P   V   A   K   L>
1680        1690        1700        1710        1720        1730
  CCA CCT AGC AAG TTA GTA TCT GAT GAC TTG GAT TCA TCT TTA GCC AAC CTT GTG GGC AAT
   P   P   S   K   L   V   S   D   D   L   D   S   S   L   A   N   L   V   G   N>
1740        1750        1760        1770        1780        1790
  CTT GGC ATC GGA AAT GGA ACC ACT AAG AAT GAT GTA AAT TGG AGT CAA CCA GGT GAA AAG
   L   G   I   G   N   G   T   T   K   N   D   V   N   W   S   Q   P   G   E   K>
1800        1810        1820        1830        1840        1850
  AAG TTA ACT GGG GGA TCT AAC TGG CAA CCA AAG GTT GCA CCA ACA ACC GCT TGG AAT GCG
   K   L   T   G   G   S   N   W   Q   P   K   V   A   P   T   T   A   W   N   A>
1860        1870        1880        1890        1900        1910
  GCA ACA ATG GCA CCC CCT GTA ATG GCC TAT CCT GCT ACT ACA CCA ACA GGC ATG ATA GGA
   A   T   M   A   P   P   V   M   A   Y   P   A   T   T   P   T   G   M   I   G>
1920        1930        1940        1950        1960        1970
  TAT GGA ATT CCT CCA CAA ATG GGA AGT GTT CCT GTA ATG ACG CAA CCA ACC TTA ATA TAC
   Y   G   I   P   P   Q   M   G   S   V   P   V   M   T   Q   P   T   L   I   Y>
1980        1990        2000        2010        2020        2030
  AGC CAG CCT GTC ATG AGA CCT CCA AAC CCC TTT GGC CCT GTA TCA GGA GCA CAG AGA TGT
   S   Q   P   V   M   R   P   P   N   P   F   G   P   V   S   G   A   Q   R   C>
2040        2050        2060        2070        2080        2090
  GAA CTT TGT CCC CAT AAG GAT GGA GCT TTA AAA AGA ACA GAT AAT GGG GGT TGG GCC CAT
   E   L   C   P   H   K   D   G   A   L   K   R   T   D   N   G   G   W   A   H>
2100        2110        2120        2130        2140        2150
  GTG GTT TGT GCC CTG TAT ATT CCA GAG GTA CAA TTT GCC AAT GTT TCC ACA ATG GAA CCA
   V   V   C   A   L   Y   I   P   E   V   Q   F   A   N   V   S   T   M   E   P>
2160        2170        2180        2190        2200        2210
  ATT GTT TTA CAG TCT GTT CCG CAT GAT CGT TAT AAT AAG ACT TGC TAC ATT TGT GAT GAA
   I   V   L   Q   S   V   P   H   D   R   Y   N   K   T   C   Y   I   C   D   E>
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2220         2230         2240         2250         2260         2270
   CAA GGA AGA GAA AGC AAA GCA GCC ACT GGT GCT TGC ATG ACA TGT AAT AAA CAT GGA TGT
    Q   G   R   E   S   K   A   A   T   G   A   C   M   T   C   N   K   H   G   C>
2280         2290         2300         2310         2320         2330
   CGA CAG GCT TTC CAT GTA ACA TGC GCT CAG TTT GCC GGA CTG CTT TGT GAA GAA GAA GGT
    R   Q   A   F   H   V   T   C   A   Q   F   A   G   L   L   C   E   E   E   G>
2340         2350         2360         2370         2380         2390
   AAT GGT GCC GAT AAT GTC CAA TAC TGT GGC TAC TGT AAA TAC CAT TTT AGT AAG CTG AAA
    N   G   A   D   N   V   Q   Y   C   G   Y   C   K   Y   H   F   S   K   L   K>
2400         2410         2420         2430         2440         2450
   AAG AGC AAA CGG GGA TCT AAT AGG TCA TAT GAT CAA AGT TTA AGT GAT TCT TCC TCT CAC
    K   S   K   R   G   S   N   R   S   Y   D   Q   S   L   S   D   S   S   S   H>
2460         2470         2480         2490         2500         2510
   TCT CAG GAT AAA CAT CAT GAG AAA GAG AAA AAA AAA TAT AAA GAG AAG GAC AAA CAC AAA
    S   Q   D   K   H   H   E   K   E   K   K   K   Y   K   E   K   D   K   H   K>
2520         2530         2540         2550         2560         2570
   CAG AAA CAC AAG AAG CAG CCA GAA CCA TCA CCT GCA TTG GTT CCA TCC TTG ACT GTT ACT
    Q   K   H   K   K   Q   P   E   P   S   P   A   L   V   P   S   L   T   V   T>
2580         2590         2600         2610         2620         2630
   ACA GAA AAA ACT TAT ACA AGC ACT AGC AAC AAC TCT ATA TCT GGA TCA TTG AAG CGC TTG
    T   E   K   T   Y   T   S   T   S   N   N   S   I   S   G   S   L   K   R   L>
2640         2650         2660         2670         2680         2690
   GAA GAT ACT ACT GCA CGA TTT ACA AAT GCA AAT TTC CAG GAA GTC TCT GCA CAC ACC TCT
    E   D   T   T   A   R   F   T   N   A   N   F   Q   E   V   S   A   H   T   S>
2700         2710         2720         2730         2740         2750
   AGT GGA AAA GAT GTT TCA GAG ACT AGA GGG TCA GAG GGC AAA GGG AAG AAA TCT TCA GCT
    S   G   K   D   V   S   E   T   R   G   S   E   G   K   G   K   K   S   S   A>
2760         2770         2780         2790         2800         2810
   CAC AGC TCA GGT CAA AGG GGA AGA AAG CCT GGT GGT GGA AGA AAT CCA GGA ACA ACT GTG
    H   S   S   G   Q   R   G   R   K   P   G   G   G   R   N   P   G   T   T   V>
2820         2830         2840         2850         2860         2870
   TCA GCA GCT AGC CCT TTT CCT CAA GGC AGT TTT TCA GGA ACT CCA GGC AGT GTA AAG TCA
    S   A   A   S   P   F   P   Q   G   S   F   S   G   T   P   G   S   V   K   S>
2880         2890         2900         2910         2920         2930
   TCT TCT GGA AGT TCA GTG CAG TCT CCC CAG GAT TTC CTG AGC TTT ACA GAC TCA GAT CTG
    S   S   G   S   S   V   Q   S   P   Q   D   F   L   S   F   T   D   S   D   L>
2940         2950         2960         2970         2980         2990
   CGT AAT GAC AGT TAC TCT CAC TCC CAA CAG TCA TCA GCA ACC AAA GAT GTA CAT AAA GGA
    R   N   D   S   Y   S   H   S   Q   Q   S   S   A   T   K   D   V   H   K   G>
3000         3010         3020         3030         3040         3050
   GAG TCT GGA AGC CAG GAA GGG GGG GTA AAT AGT TTT AGT ACC TTA ATT GGC CTC CCT TCA
    E   S   G   S   Q   E   G   G   V   N   S   F   S   T   L   I   G   L   P   S>
3060         3070         3080         3090         3100         3110
   ACC TCA GCT GTT ACT TCA CAG CCT AAA AGC TTT GAA AAT TCA CCT GGA GAT TTG GGT AAT
    T   S   A   V   T   S   Q   P   K   S   F   E   N   S   P   G   D   L   G   N>
3120         3130         3140         3150         3160         3170
   TCC AGC CTT CCT ACA GCA GGA TAT AAG CGG GCT CAA ACT TCT GGC ATA GAA GAA GAA ACT
    S   S   L   P   T   A   G   Y   K   R   A   Q   T   S   G   I   E   E   E   T>
3180         3190         3200         3210         3220         3230
   GTA AAG GAA AAG AAA AGG AAA GGA AAT AAA CAA AGT AAG CAT GGG CCT GGC AGA CCC AAA
    V   K   E   K   K   R   K   G   N   K   Q   S   K   H   G   P   G   R   P   K>
3240         3250         3260         3270         3280         3290
   GGA AAC AAA AAT CAA GAG AAT GTT TCT CAT CTC TCA GTT TCT TCT GCT TCA CCA ACA TCA
    G   N   K   N   Q   E   N   V   S   H   L   S   V   S   S   A   S   P   T   S>
3300         3310         3320         3330         3340         3350
   TCT GTA GCA TCA GCT GCA GGA AGC ATA ACA AGC TCT AGT CTG CAG AAA TCT CCT ACA TTG
    S   V   A   S   A   A   G   S   I   T   S   S   S   L   Q   K   S   P   T   L>
3360         3370         3380         3390         3400         3410
   CTC AGG AAT GGA AGT TTA CAG AGC CTC AGT GTT GGC TCA TCT CCA GTT GGT TCA GAA ATT
    L   R   N   G   S   L   Q   S   L   S   V   G   S   S   P   V   G   S   E   I>
3420         3430         3440         3450         3460         3470
   TCC ATG CAG TAT CGG CAT GAT GGA GCT TGC CCA ACA ACT AGT ATT TAT AAC AGC AAT GAT
    S   M   Q   Y   R   H   D   G   A   C   P   T   T   S   I   Y   N   S   N   D>
3480         3490         3500         3510         3520         3530
   GTA GCA GTA TCG TTT CCA AAT GTA GTA TCT GGC TCG GGA TCT AGT ACT CCT GTC TCC AGC
    V   A   V   S   F   P   N   V   V   S   G   S   G   S   S   T   P   V   S   S>
3540         3550         3560         3570         3580         3590
   TCT CAC TTA CCT CAG CAG TCT TCT GGG CAT TTG CAA CAA GTA GGA GCG CTC TCT CCC TCA
    S   H   L   P   Q   Q   S   S   G   H   L   Q   Q   V   G   A   L   S   P   S>
3600         3610         3620         3630         3640         3650
   GCT GTG TCA TCT GCA GCC CCT GCT GTT GCT ACA ACT CAG GCA AAT ACT CTA TCT GGA TCT
    A   V   S   S   A   A   P   A   V   A   T   T   Q   A   N   T   L   S   G   S>
3660         3670         3680         3690         3700         3710
   TCT CTC AGT CAG GCA CCA TCT CAT ATG TAT GGC AAT AGA TCA AAT TCA TCA ATG GCA GCT
    S   L   S   Q   A   P   S   H   M   Y   G   N   R   S   N   S   S   M   A   A>
3720         3730         3740         3750         3760         3770
   CTT ATA GCT CAG TCT GAA AAC AAT CAA ACA GAT CAA GAT CTT GGA GAC AAT AGC CGC AAC
    L   I   A   Q   S   E   N   N   Q   T   D   Q   D   L   G   D   N   S   R   N>
3780         3790         3800         3810         3820         3830
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   CTA GTT GGC AGA GGA AGC TCA CCC CGA GGA AGT CTC TCG CCA CGA TCC CCT GTA AGC AGC
    L   V   G   R   G   S   S   P   R   G   S   L   S   P   R   S   P   V   S   S>
3840         3850         3860         3870         3880         3890
   TTA CAG ATT CGC TAT GAT CAA CCA GGC AAC AGC AGT TTG GAA AAT CTG CCT CCA GTA GCA
    L   Q   I   R   Y   D   Q   P   G   N   S   S   L   E   N   L   P   P   V   A>
3900         3910         3920         3930         3940         3950
   GCC AGC ATA GAA CAG CTT TTG GAG AGG CAG TGG AGT GAA GGA CAG CAA TTT TTA CTA GAA
    A   S   I   E   Q   L   L   E   R   Q   W   S   E   G   Q   Q   F   L   L   E>
3960         3970         3980         3990         4000         4010
   CAG GGT ACT CCT AGT GAC ATT TTA GGA ATG CTG AAG TCA TTA CAC CAA CTT CAA GTT GAA
    Q   G   T   P   S   D   I   L   G   M   L   K   S   L   H   Q   L   Q   V   E>
4020         4030         4040         4050         4060         4070
   AAC CGA AGA TTA GAG GAA CAA ATT AAA AAC TTG ACT GCC AAA AAG GAA CGG CTT CAG TTA
    N   R   R   L   E   E   Q   I   K   N   L   T   A   K   K   E   R   L   Q   L>
4080         4090         4100         4110         4120         4130
   TTG AAT GCA CAG CTT TCA GTG CCT TTT CCA ACA ATA ACA GCA AAT CCT AGT CCG TCT CAT
    L   N   A   Q   L   S   V   P   F   P   T   I   T   A   N   P   S   P   S   H>
4140         4150         4160         4170         4180         4190
   CAA ATA CAC ACA TTT TCA GCA CAG ACT GCT CCT ACT ACT GAT TCC TTG AAC AGC AGT AAG
    Q   I   H   T   F   S   A   Q   T   A   P   T   T   D   S   L   N   S   S   K>
4200         4210         4220         4230         4240         4250
   AGC CCT CAT ATA GGA AAC AGC TTT TTA CCT GAT AAT TCT CTT CCT GTA TTA AAT CAG GAC
    S   P   H   I   G   N   S   F   L   P   D   N   S   L   P   V   L   N   Q   D>
4260         4270         4280         4290         4300         4310
   TTA ACC TCC AGT GGA CAA AGT ACC AGC AGC TCA TCA GCT CTT TCT ACC CCA CCT CCT GCT
    L   T   S   S   G   Q   S   T   S   S   S   S   A   L   S   T   P   P   P   A>
4320         4330         4340         4350         4360         4370
   GGG CAG AGT CCG GCT CAA CAA GGC TCA GGA GTG AGT GGA GTT CAG CAG GTC AAT GGC GTG
    G   Q   S   P   A   Q   Q   G   S   G   V   S   G   V   Q   Q   V   N   G   V>
4380         4390         4400         4410         4420         4430
   ACA GTG GGG GCA CTA GCT AGT GGA ATG CAG CCT GTA ACT TCC ACC ATT CCT GCC GTG TCT
    T   V   G   A   L   A   S   G   M   Q   P   V   T   S   T   I   P   A   V   S>
4440         4450         4460         4470         4480         4490
   GCA GTG GGT GGA ATA ATT GGA GCT TTG CCA GGT AAC CAA CTG GCA ATT AAT GGC ATT GTA
    A   V   G   G   I   I   G   A   L   P   G   N   Q   L   A   I   N   G   I   V>
4500         4510         4520         4530         4540         4550
   GGA GCT TTA AAT GGG GTT ATG CAG ACT CCT GTC ACA ATG TCC CAG AAC CCT ACC CCT CTC
    G   A   L   N   G   V   M   Q   T   P   V   T   M   S   Q   N   P   T   P   L>
4560         4570         4580         4590         4600         4610
   ACC CAC ACA ACC GTA CCA CCT AAT GCA ACA CAT CCA ATG CCA GCT ACA CTG ACT AAC AGT
    T   H   T   T   V   P   P   N   A   T   H   P   M   P   A   T   L   T   N   S>
4620         4630         4640         4650         4660         4670
   GCC TCA GGA CTA GGA TTA CTT TCT GAC CAG CAA CGA CAA ATA CTT ATT CAT CAA CAG CAG
    A   S   G   L   G   L   L   S   D   Q   Q   R   Q   I   L   I   H   Q   Q   Q>
4680         4690         4700         4710         4720         4730
   TTT CAG CAG TTG TTA AAT TCT CAA CAG CTC ACA CCA GAA CAA CAT CAA GCC TTT TTG TAT
    F   Q   Q   L   L   N   S   Q   Q   L   T   P   E   Q   H   Q   A   F   L   Y>
4740         4750         4760         4770         4780         4790
   CAG TTA ATG CAA CAT CAC CAC CAG CAG CCG CAC CAA CCT GAA CTT CAG CAG CTG CAG ATC
    Q   L   M   Q   H   H   H   Q   Q   P   H   Q   P   E   L   Q   Q   L   Q   I>
4800         4810         4820         4830         4840         4850
   CCT GGA CCA ACA CAA ATA CCC ATA AAC AAC CTT CTT GCA GGT ACA CAG GCA CCC CCA CTT
    P   G   P   T   Q   I   P   I   N   N   L   L   A   G   T   Q   A   P   P   L>
4860         4870         4880         4890         4900         4910
   CAC ACA GCT ACC ACC AAC CCA TTT CTC ACC ATC CAT GGA GAT AAT GCA AGT CAG AAA GTA
    H   T   A   T   T   N   P   F   L   T   I   H   G   D   N   A   S   Q   K   V>
4920         4930         4940         4950         4960         4970
   GCA AGA CTT AGT GAT AAA ACT GGG CCT GTA GCT CAA GAG AAA AGT TGA CAC CTG AGA AAC
    A   R   L   S   D   K   T   G   P   V   A   Q   E   K   S   *   H   L   R   N>
4980         4990         5000
   ATC TAG AGC GGC CGC CAC GC GG
    I   *   S   G   R   H   R   G>
                          SacII
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Figure 4: Sequence of the insert CALM/AF10 from *XhoI* to *SacII*. The restriction sites for these enzymes are underlined. The colored nucleotides represent the last nt. of CALM (red, 2091 in the genomic cDNA sequence) and the first nt. of AF10 (blue, 424 in the genomic cDNA sequence) close to the breakpoint.

5.1.2.Sub-cellular localization and co-localization of CALM/AF10, CALM and AF10

Cloning

In order to analyse the sub-cellular localization and co-localization of CALM/AF10, CALM and AF10, the corresponding open-reading frames were cloned in frame with the genes coding for the Enhanced yellow Protein and Enhanced Cyan Protein (Clontech expression vectors pEYFP and pECFP). A co-localization using CFP and YFP fluorescent vectors fused to the gene of interest allowed us to determine the localization of the fusion proteins in the cell. Before these cloning steps, we modified the 3' region of the CALM/AF10 coding sequences in the pEGFP-CALM/AF10 plasmid to eliminate extra stop codons present in a region between two *Xba1* restriction sites at the positions 6404 and 6568, figure 71 (appendix). One microgram of this vector was cut with *Xba1*, religated overnight (16°C) and transformed into XL1-Blue bacteria as described in the methods section. Recombinant plasmids were sequenced and the modified pEGFP-CALM/AF10 vector lacking the region between nucleotides 6458 and 6622 in this vector was named pEGFP-CALM/AF10Δ*Xba1*. Two micrograms of pEGFP-CALM/AF10Δ*Xba1* was digested first with the restriction enzyme *SacII*. This first digestion was controlled on an agarose gel and the linearized DNA was cut with the enzymes *XhoI* (the enzyme chosen for the cloning in the correct reading frame of the pEYFP and pECFP) and *Nsi1*. The digestion with *NsiI* allowed us to cut the vector backbone and easily identify the CALM/AF10 insert on the agarose gel. After digestion and separation by eletrophoresis, the CALM/AF10 digested fragment was cut and extracted from the gel. Following the gel extraction, the expected size of the insert was verified on an agarose gel and the concentration was estimated by visually comparing the band to a known concentration of the DNA standard (1 Kb Ladder Invitrogen Life Technologies, Germany). The digestion, separation and gel extraction procedures are shown for CALM/AF10 in figure 5 (A, B and C).

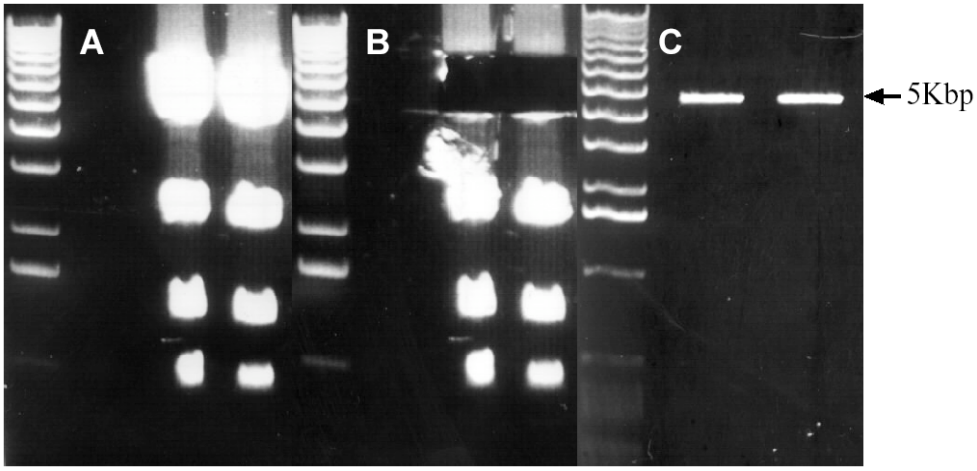


Figure 5: pEGFP-CALM/AF10 digested with XhoI, SacII and NsiI, separated on an 0.8% agarose gel in TBE, stained with ethidium bromide (0.3μg/ml) and visualized under ultra violet light Digested product (A), CALM/AF10 fragment cut from the gel (B), insert after gel extraction (C).

To clone the CALM/AF10 insert into the vectors pEYFC-C1 and pECFP-C1, the vectors were digested with *XhoI* and *SacII*. Insert and vectors were ligated (overnight at 16°C) to each other and transformed into XL1-Blue bacteria. Recombinant plasmids were sequenced across the 5' cloning site to confirm that the insert was in frame with the CFP or YFP open reading frame. For sequencing the 5' pEGFP primer EGFPC 1240-1258 was used. The CALM coding region clone used for the colocalization experiments had been cloned by Professor Stefan Bohlander into the vector pEGFP-C1 using the same enzymes as for CALM/AF10 coding region (*Xho1* and *SacII*). The CALM cDNA was cut out from this vector using *Xho1* and *SacII* and cloned into pEYFP-C1 and pECFP-C1 using the same restriction enzyme sites. The resulting constructs were named: pEYFP-CALM and pECFP-CALM. In order to obtain a CFP-AF10 (with the full length AF10 coding region) construct, we amplified the first 550 nucleotides of AF10 (encoding the first 80 amino acids of AF10 which are absent in the CALM/AF10 fusion). The 5' primer (AF10 T182) contained an *XhoI* site and the 3' primer (AF10 B728) contained a *SalI* restriction site. The 5' AF10 fragment amplified by these two primers contains a *Sal1* restriction enzyme site at position 668 of AF10. This 5' AF10 fragment was cut with *Xho1* and *Sal1* and inserted into pECFP-CALM/AF10□*Xba1* from which the CALM coding region and part of AF10 had been removed using these two enzymes. The product of this cloning procedure was the pECFP-AF10 construct. The proteins which are encoded by these constructs are schematically represented in the figures 6, 7, 8 and 9.

Figure 6: Schematic representation of the protein encoded by the pEYFP-CALM/AF10Δ*Xba1* construct.

Figure 7: Schematic representation of the protein encoded by the pECFP-CALM/AF10Δ*Xba1* construct.

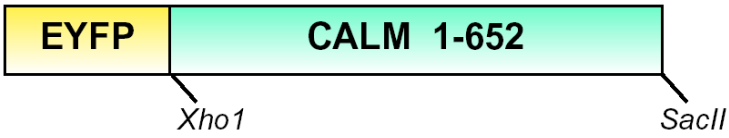


Figure 8: Schematic representation of the protein encoded by the pEYFP-CALM construct.

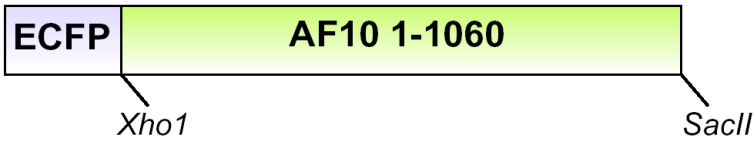


Figure 9: Schematic representation of the protein encoded by the pECFP-AF10 construct.

Transfection and sub-cellular localization experiments

We analysed the sub-cellular localization of fluorescent protein tagged CALM, AF10 and CALM/AF10 proteins by transiently expressing these proteins in the mouse fibroblast cell line NIH3T3. Cells were observed between 20 and 26 hours after transfection with an inverted epi-fluorescence microscope. In these experiments, CALM and CALM/AF10 localize in the cytoplasm of the cells and they co-localize in co-transfected cells (Figures 11 and 12). CFP-AF10 localizes to the nucleus in a speckled pattern and does not obviously colocalize with CALM or CALM/AF10. A fraction of YFP-CALM/AF10 can also be seen in the nucleus (figure 10 and 14, A and C). The nuclear fraction of YFP-CALM/AF10 seems to be enriched in big spots in the nucleus which might correspond to nucleoli. We cannot rule out that some of the nuclear YFP-CALM/AF10 co-localizes with CFP-AF10. However, most of the nuclear fraction of YFP-CALM/AF10 and CFP-AF10 seem to occupy distinct areas in the nucleus (fig. 14). Figures 10 to 14 illustrate the sub-cellular localization results

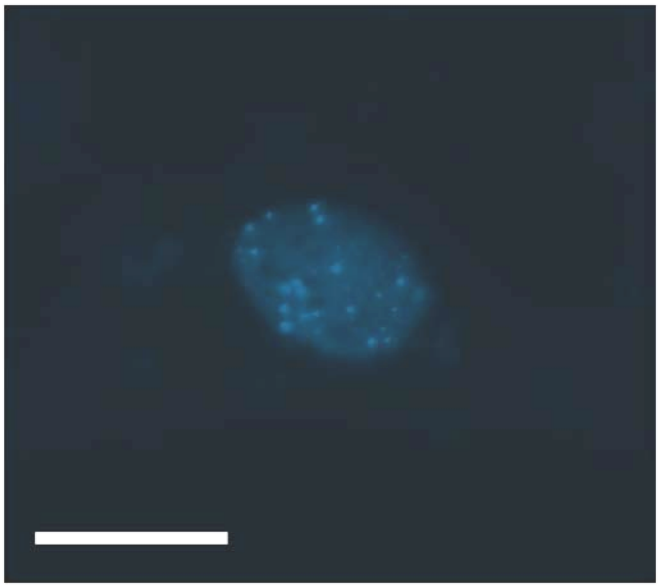


Figure 10: NIH3T3 cell transiently transfected with pECFP-AF10. Visualization with CFP filter. Scale = 10 µm

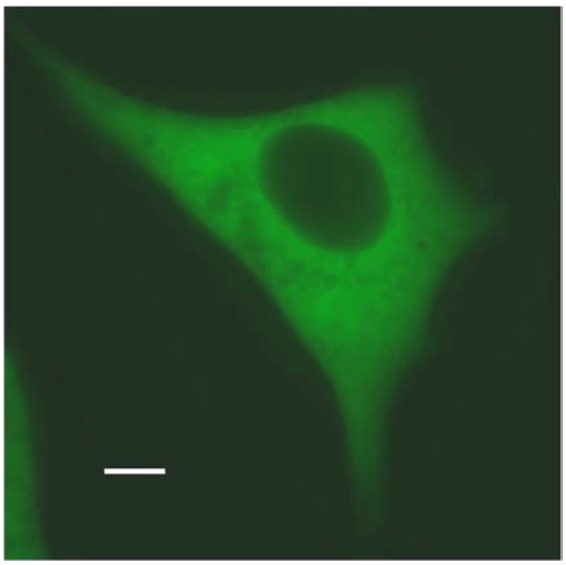


Figure 11: NIH3T3 cell transiently transfected with pEYFP-CALM. Visualization with YFP filter. Scale = 10 µm

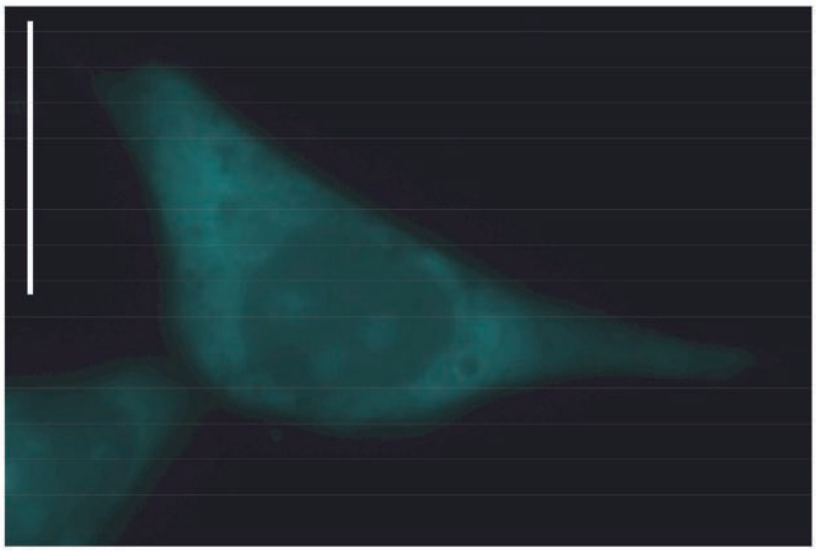


Figure 12: NIH3T3 cell transiently transfected with pECFP-CALM/AF10. Visualization with CFP filter. Scale = 10 µm

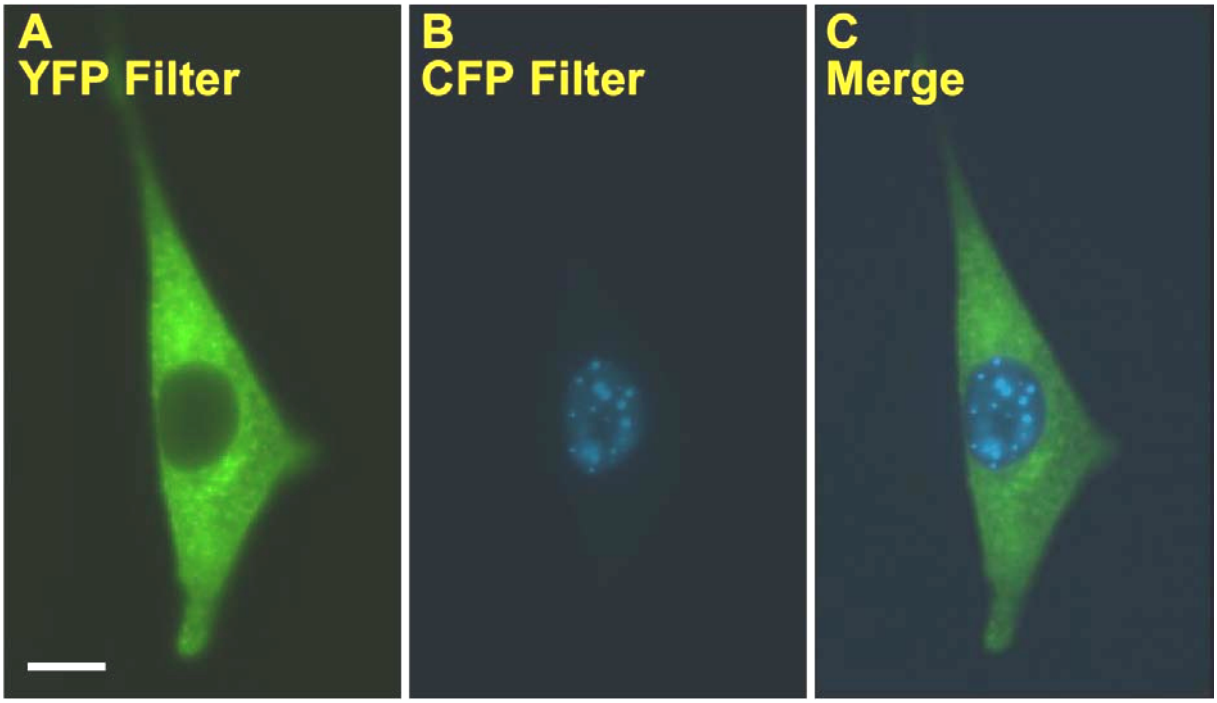


Figure 13: NIH3T3 cell transiently co-transfected with pECFP-AF10 and pEYFP-CALM. Visualization under YFP filter (A), CFP filter (B) and merged image (C). Scale = 10 µm

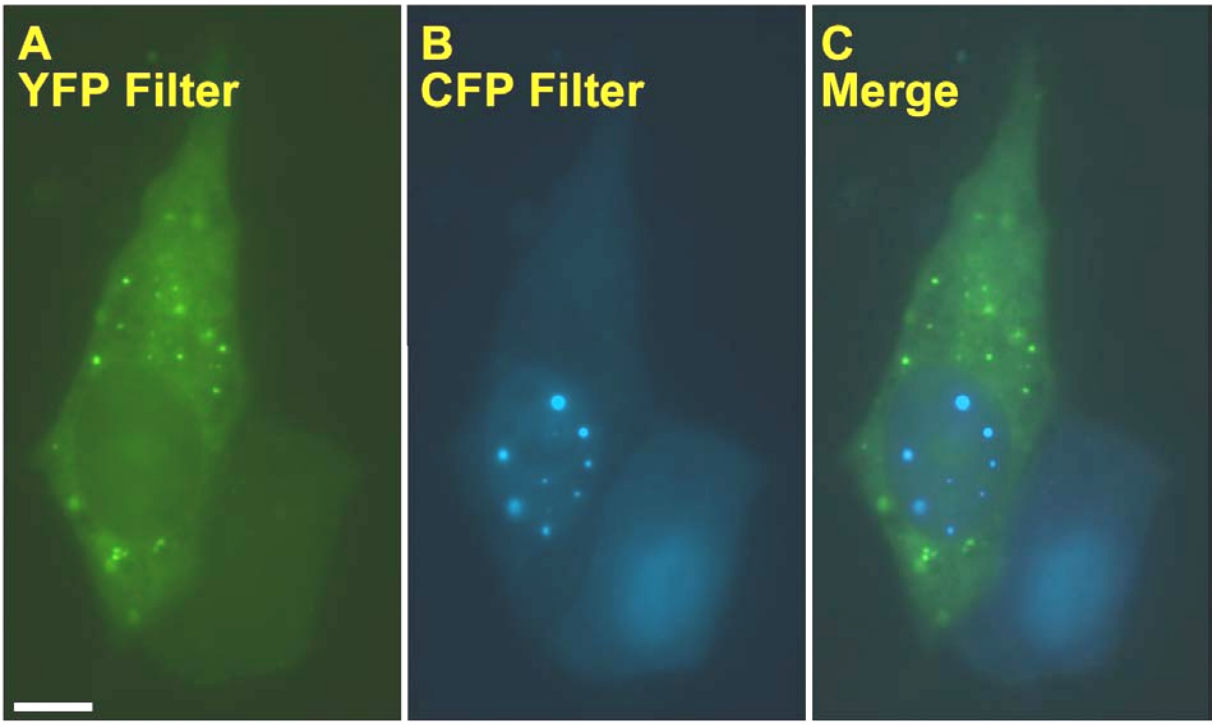


Figure 14: NIH3T3 cell transiently co-transfected with pECFP-AF10 and pEYFP-CALM/AF10. Visualization with YFP filter (A), CFP filter (B) and merged image (C). Scale = 10 µm

Protein expression

In order to confirm expression of the AF10, CALM and CALM/AF10 proteins in our fluorescent constructs, we over-expressed the proteins in the human embryonal kidney cell line (HEK293T) and performed Western analysis. We monitored the complete transfer of the proteins to the PVDF membrane by observing the transfer of the pre-stained molecular weight protein standard bands. The incubation time of the primary antibodies had to be optimized to 18 hours at 4°C. We could detect the proteins YFP-CALM and CALM/AF10 and the endogenous CALM using a pool of the commercial anti-CALM antibodies (S-19, G-17 and C-18) (figure 16). The YFP-CALM, GFP-CALM/AF10 and CFP-AF10 proteins were also detected using an anti-GFP antibody (Figures 15 to 17).

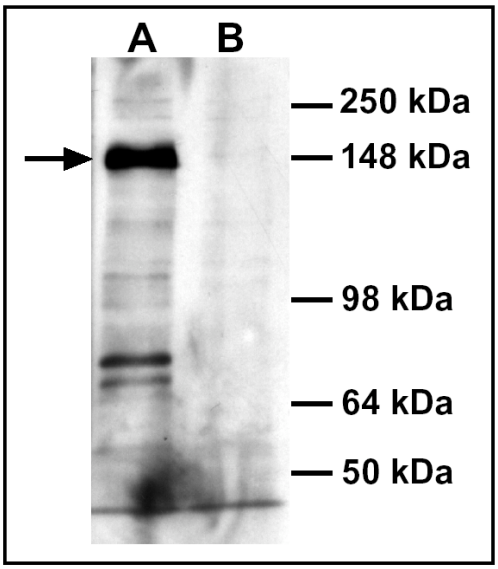


Figure 15: 6%SDS PAGE, Western blot of protein extract (80μg/lane) from 293 cells transiently-transfected with pEYFP-CALM/AF10 (A) and non-transfected (B), incubated with an rabbit anti-GFP antibody (1:3000 in blotto A) for 2 hours at RT. Secondary antibody, donkey anti-rabbit HRP-conjugated. Detection using ECL chemiluminescence's kit, exposure-time of 15 sec. The arrow shows the band corresponding to the YFP-CALM/AF10 protein (expected molecular weight 207 kDa).

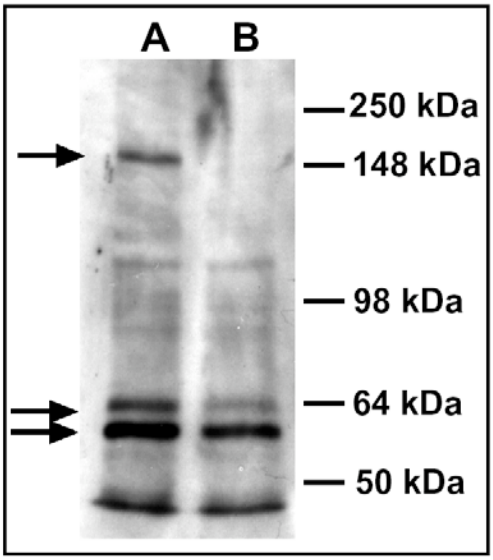


Figure 16: 6%SDS PAGE, Western blot of protein extract (80 μg/lane) from HEK293T cells transiently-transfected with pEYFP-CALM/AF10 (A) and non-transfected (B). The primary antibodies used were Anti- CALM C-18, S-19 and G-17, each diluted 1:1000 in blotto A, incubated over night at 4°C. Secondary antibody: donkey anti-goat HRP-conjugated. Detection using ECL chemiluminescence's kit with exposure time of 30 sec. The upper arrow shows the band corresponding to the YFP-CALM/AF10 protein (expected molecular weight 207kDa). The two lower arrows indicate two bands of the endogenous CALM protein (expected molecular weight about 70 kDa).

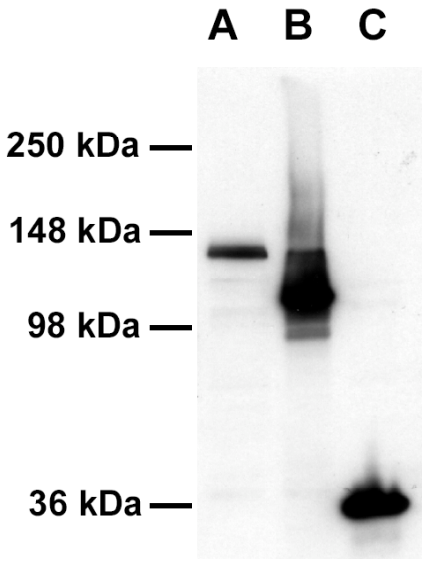


Figure 17: 10%SDS-PAGE, Western blot of protein extracts (50μg/lane) from HEK293T cells transiently-transfected with pECFP-AF10 (A), pEYFP-CALM (B), pEGFP-C1 (C) and non-transfected cells (D), incubated with rabbit anti-GPF antibody 1:3000. Secondary antibody: donkey anti-rabbit HRP-conjugated. Detection using ECL chemiluminescence's kit, exposure time of 20 sec.The bands observed in the lanes A, B, and C correspond to the fusion proteins CFP-AF10, YFP-CALM and the GFP protein, respectively.

5.2. Transgenic Mice

5.2.1. FVB mice expressing CALM/AF10 under control of a *IgH* enhancer/promoter

Cloning of the transgenic construct

In order to achieve transgenic expression of CALM/AF10 in B- cell precursors, we designed a construct in which the expression of CALM/AF10 was controlled by the *IgH* enhancer/promoter. To enhance post-transcriptional processing (splicing etc.) of the transgene, a small intron and a poly-adenylation signal (SV-40 splice/polyA fragment) were introduced at the 3' end of the CALM/AF10 encoding sequences. We designed two primer pairs to amplify the *IgH* enhancer/promoter (1 kb) and the SV-40 splice/polyA fragment (1.6 kb) from the plasmid pPepB-Splice (table 6). The amplified products were cloned into the PCR fragment cloning vector pGEMT -easy™. The primers (*IgH*StartXhoI and *IgH*ProEndSac1) for the *IgH* enhancer/promoter contained the enzymes *XhoI* (5') and *SacI* (3'). The primers for the amplification of the SV-40 splice/polyA fragment (SV40splStartXba1 and SV40polAEndNot1) contained the restriction enzyme sites for *XbaI* (5') and *NotI* (3'). First the SV-40 splice/polyA fragment was inserted 5' of the CALM/AF10 cDNA via the *Xho1* and *Sac1* sites. This order of cloning was chosen because an *Xba1* site is present in the *IgH* enhander/promoter fragment. The final construct was named pIGCASV-40 and is shown in figure 18.

Figure 18: Schematic representation of the pIGCASV-40 construct.

Generation of transgenic lines

After cloning the construct was linearized for the microinjection into the male pronucleus of zygotes from FVB mice. The transgenic construct was liberated from the vector backbone by digesting with the restriction enzymes *MluI* and *NsiI*. The linearized construct was eletrophoretically separated from the vector backbone and purified by gel extraction. To estimate the concentration of the linearized transgenic construct, the DNA was loaded in different amounts on an agarose gel together with a standard concentration of the DNA ladder. Figure 19 illustrates the DNA concentration estimation.

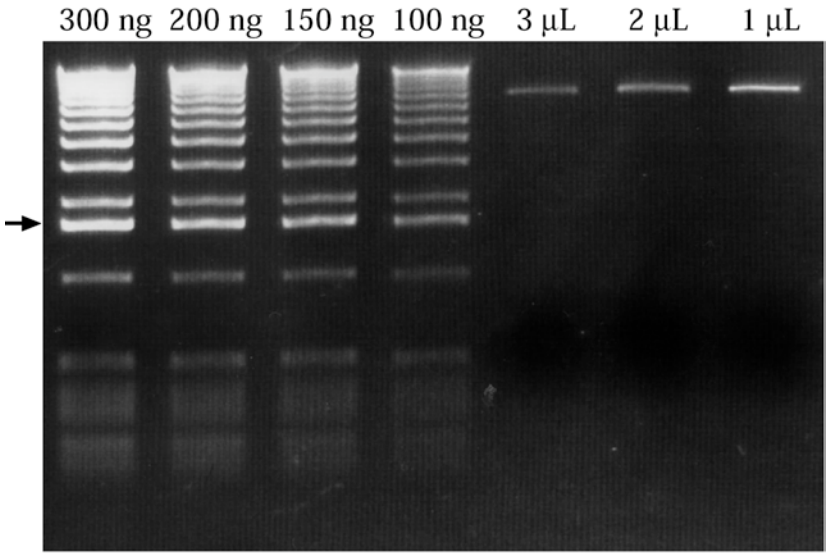


Figure 19: Agarose gel electrophoresis (0.7%, stained with Ethidium bromide (0.3µg/ml), visualized under ultra-violet light) of the linearized pIGCASV-40 for estimation of the concentration by comparison with the DNA 1Kb Ladder. The 1,6 kb (arrow) fragment of the ladder represents 10% of the total DNA contained in the ladder.

The linearized construct was filter sterilized with a syringe filter (0.2 m) and the DNA concentration was adjusted to 2 ng/l TE, pH 7.4, as recommended by Ms. Tamara Holy, who performed the microinjection and embryo-transfer procedures at the Gene Center. The mice generated by the microinjection and embryo transfer procedures were tested at 4 weeks of age, when the end of the tail (0.5cm) was taken for genomic DNA extraction and PCR testing. For the pIGCASV-40 transgenic construct, two lines were generated. The parameters of the transgenic mouse generation are summarized in the table16:

Table 16: Parameters from the generation of transgenic mice with the pIGCASV- 40 construct

Number of injected embryos	673
Viable embryos	365
Transferred embryos	356
Negative sections*	7
Number of transfers**	16
Embryonic reabssortion in uterus	14
Mice born	22
Number of dead new born mice	9
Number of transgenic mice	2
IgH transgenic lines generated	2

* Negative sections refers to euthanized non-pregnant receptor female

** Number of embryo transfer procedures to receptor female mice

Identification of transgenic mice

PCR

Transgenic mice were identified by PCR using genomic DNA as template. In order to verify the integrity and quality of the genomic DNA extracted from the mouse tails, a control PCR was performed using the primer pair D10Mit105, which amplifies a sequence tagged site (STS) on mouse chromosome 10. The PCR product has a length range between 145 and 189 bp depending on the mouse strain (www.informatics.jax.org/searches/probe.cgi?44349). Primers binding at the CALM and AF10 sequence close to the breakpoint of the fusion gene were used to identify CALM/AF10 transgenic mice (see figure 18 for primer location). Figure 20 illustrates the PCR screening of pIGCASV-40 mice.

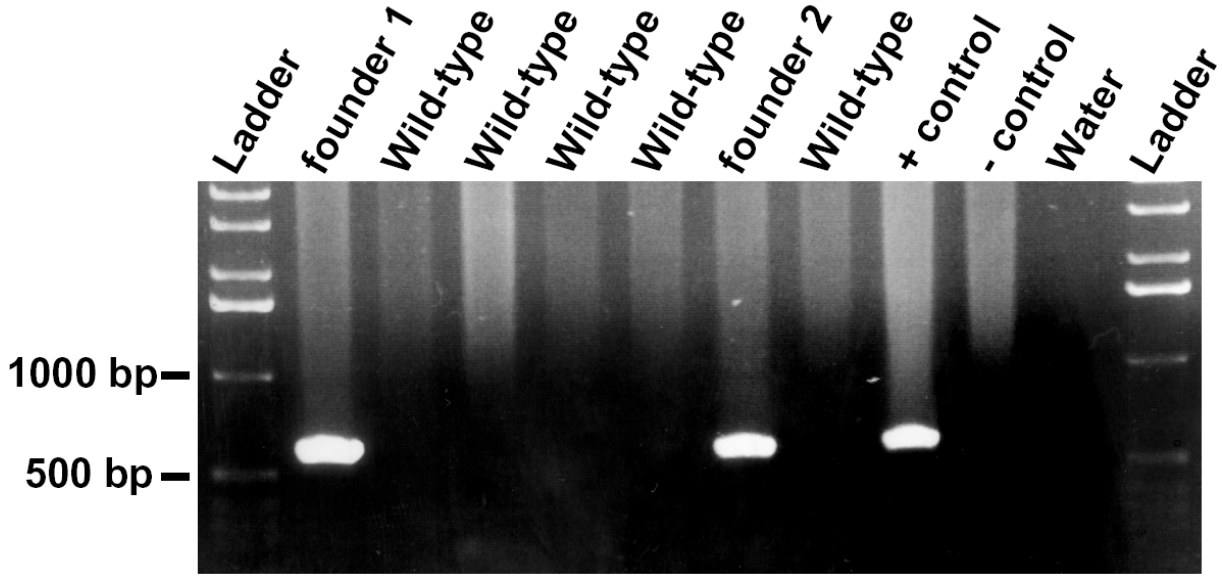


Figure 20: PCR screening of pIGCASV-40 transgenic mice. Primer pair used: NAT501 (CALM1998-2023) and AF10949B (AF10949-929). Size of the CALM/AF10 fragment: 593bp. PCR conditions: 35 cycles, annealing temperature 58°C.The positive control used was 30 pg of the transgenic construct mixed with wild type mouse genomic DNA. Approximately 100 ng of genomic DNA were used as template in each reaction.

Southern Blotting

Southern blotting was used to confirm the PCR results and to determine the number of transgene integration sites in the mouse lines. We could observe one integration site of the transgenic construct in the lines 1 and 2 of the pIGCASV-40 mice (figure 21a). Figure 21b illustrates the pIGCASV-40 construct which was linearized with the enzymes *Mlu1* and *Xho1* for the microinjection in FVB mouse zygote for the trangene mouse generation. The figure shows also the position of the fragment SV-40 used as radioactive probe for the Southern analysis and the position of the *Eco*R1 restriction site. The genomic DNA was digested with *Eco*R1

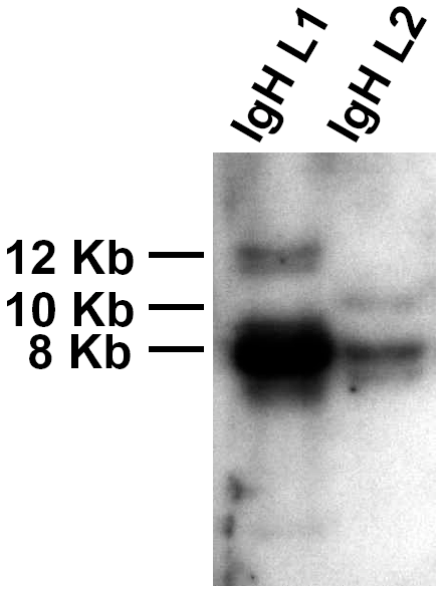


Figure 21a: Southern blot analysis of pIGCASV-40 transgenic mice. Lanes 1 and 2 contained 20 □g of genomic DNA digested overnight with EcoR1 and hybridized with 30ng of P^{32}-labelled SV-40 probe. 48 hours exposure. This southern was performed together with the pLCKCASV-40 mice, and the negative control is shown in the figure 41a.

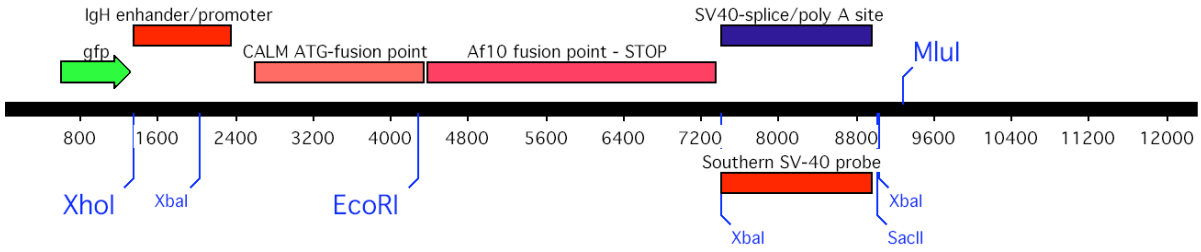


Figure 21b: Schematic representation of the pIGCASV-40 transgenic construct, with the restriction enzyme sites used for cloning or linearization for the transgenic mouse generation and the position of the sequence used as a radioactive probe for the Southern blot analysis. The transgenic construct was linearized with *Xho1* and *Mlu1* before microinjection.

Analysis of transgene expression by RT-PCR

In order to verify the expression of *CALM/AF10* in different organs and cell types, we extracted RNA from different haematological and non haematological tissues and from sorted cells representing specific haematological cell lineages and performed expression analysis by RT-PCR. Prior to the cDNA synthesis, the RNA was treated with DNase A (Invitrogen) to eliminate any contaminating genomic DNA containing the *CALM/AF10* transgene. This genomic DNA contamination would result in an amplification product of the same size as the product amplified from the *CALM/AF10* cDNA. Before cDNA synthesis, the RNA treated with DNase A was used as PCR template to verify that no genomic DNA was present. The

pictures 22 to 27 show the results obtained from the PCR of the different organs of the pIGCASV-40 mice, lines 1 and 2 for *Gapdh* and *CALM/AF10*. The organs analysed were blood, bone marrow, spleen, thymus, lungs, skeletal muscle and brain. Figures 24 and 27 illustrate the expression of the *CALM/AF10* transgene, showing stronger expression of *CALM/AF10* in haematological organs. In order to confirm and analyse the specific expression of *CALM/AF10* in the different blood cell types, we FACS sorted (Fluorescent Activated Cell Sorting) cells from spleen and bone marrow with lineage specific cell surface antibodies. The cell populations were: B220 positive and negative cells from spleen (B-lymphoid cells), Gr-1+ Mac-1+ double positive cells (myeloid lineage), CD117 (cKit) / Sca-1 double positive cells (progenitor cells) and the Sca-1 positive cells from the bone marrow. The house-keeping gene controls and the expression results of *CALM/AF10* in the sorted subpopulations are illustrated in the figures 28 to 31.

CALM/AF10 was expressed differently between the transgenic lines 1 and 2: In the line 1, *CALM/AF10* transcripts could be detected in splenocytes B220 positive and B220 negative cells, in the myeloid Gr-1+Mac-1+ double positive cells and CD117 (c-Kit) negative / Sca-1 positive cells cells from the bone marrow. In the line 2, only the B220 negative cells and in a lower intensity, the B220 positive cells were expressed *CALM/AF10.*

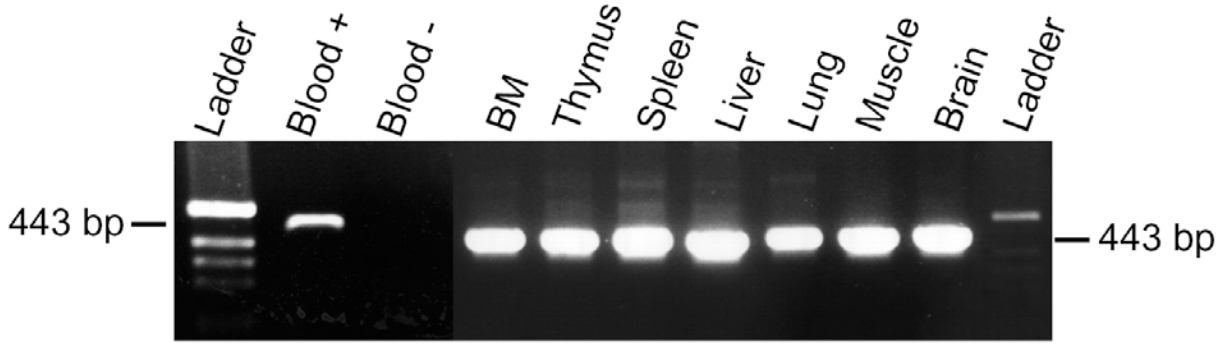


Figure 22: RT-PCR for cDNA quality control from different organs of pIGCASV-40 mouse Line 1. RNA digested with DNaseI without reverse transcriptase as negative control for blood cDNA (blood -). Primer pair used: mouse Gapdh. PCR conditions: 30 cycles, annealing temperature 60°C.

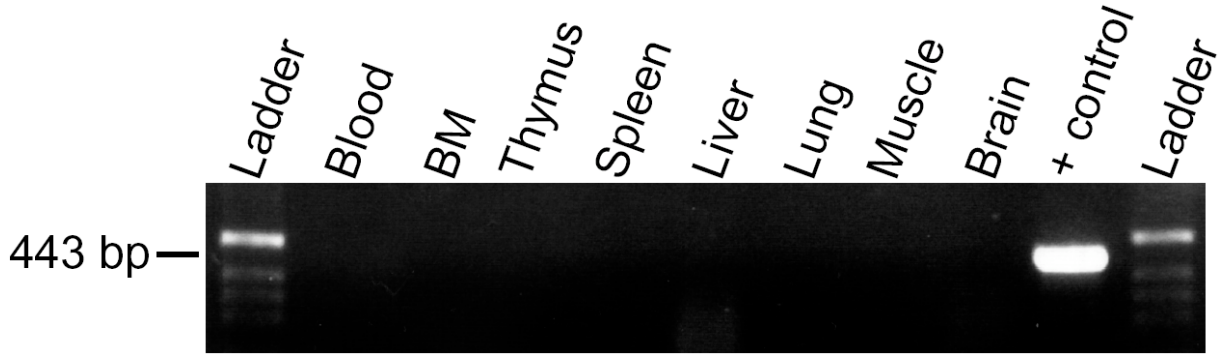


Figure 23: PCR of DNase-diggested mouse RNA form different organs of pIGCASV-40 mouse Line 1. Positive control: 2 ng of pIGCASV-40 plasmid. Primer pair used: mouse gapdh. PCR conditions: 30 cycles, annealing temperature 60°C.

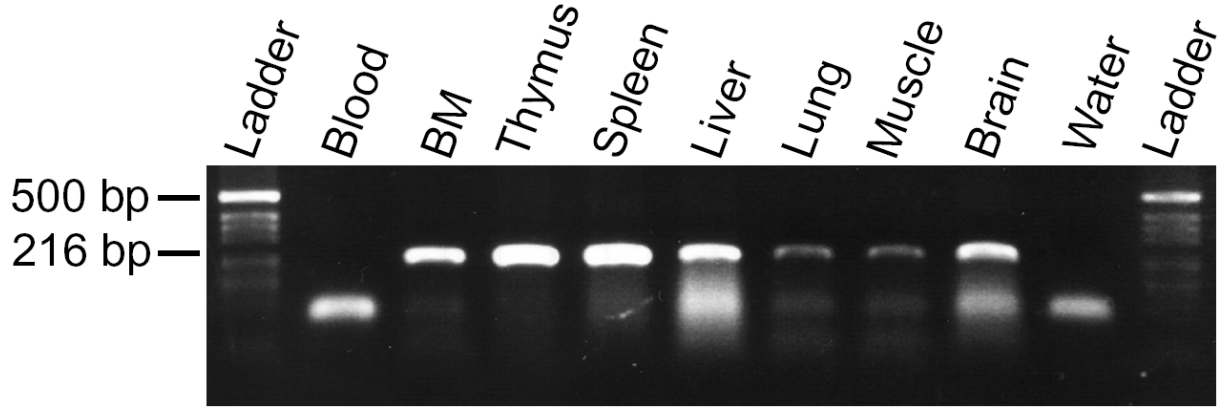


Figure 24: RT-PCR of different organs of pIGCASV-40 mouse Line 1. Primer pair used: CALM(1900-1920), AF10(448-424). PCR conditions: 30 cycles, annealing temperature 56°C.

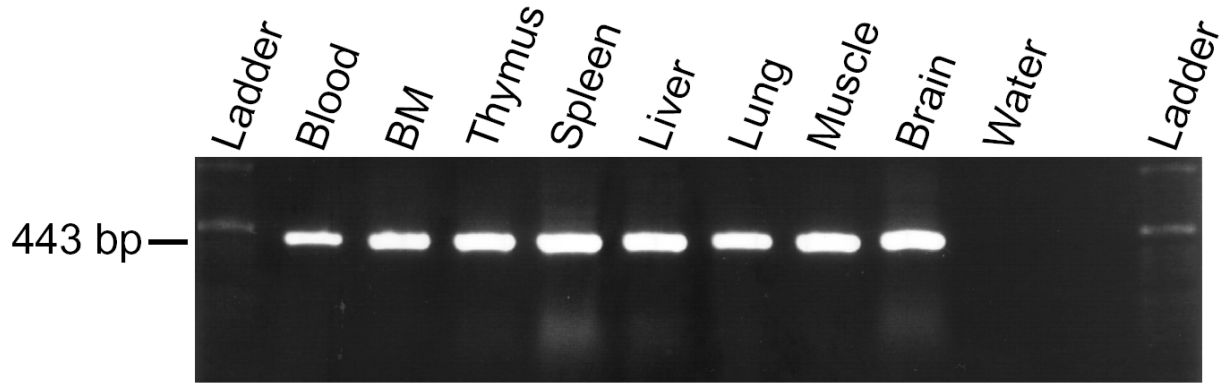


Figure 25: RT-PCR for cDNA quality control from different organs of pIGCASV-40 mouse Line 2. Primer pair used: mouse Gapdh. PCR conditions: 30 cycles, annealing temperature 60°C.

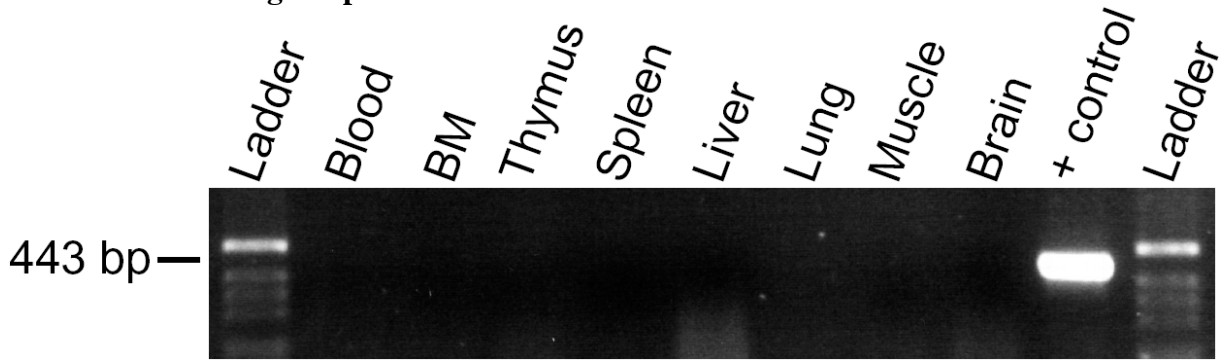


Figure 26: PCR of DNase-diggested mouse RNA for quality control from different organs of pIGCASV-40 mouse Line 2. Primer pair used: mouse Gapdh. PCR conditions: 35 cycles, annealing temperature 60°C.

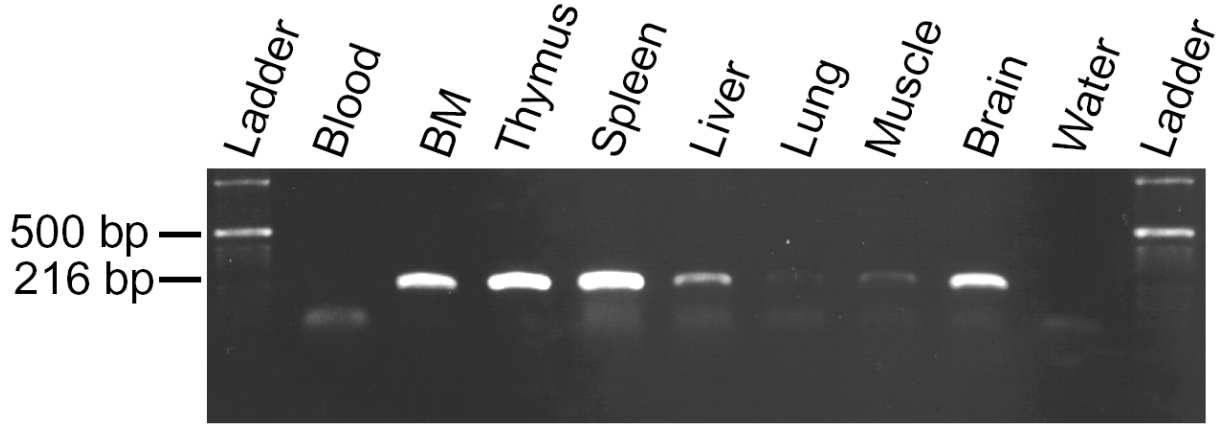


Figure 27: RT-PCR of different organs of pIGCASV-40 mouse Line 2 showing expression of the CALM/AF10 transgene. Primer pair used: CALM(1900-1920), AF10(448-424). PCR conditions: 30 cycles, annealing temperature 56°C.

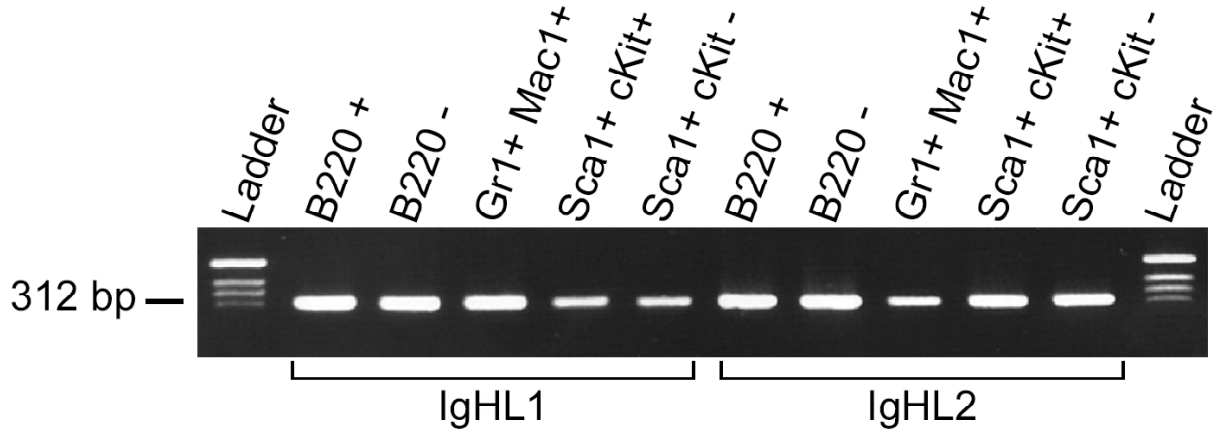


Figure 28:RT-PCR for cDNA quality control from sorted cells from spleen (B220 + and -) and bone marrow (Gr-1, Mac-1, Sca-1 and cKit) of pIGCASV-40 mouse Lines 1 and 2. Primer pair used: mouse Hprt. PCR conditions: 30 cycles, annealing temperature 59°C.

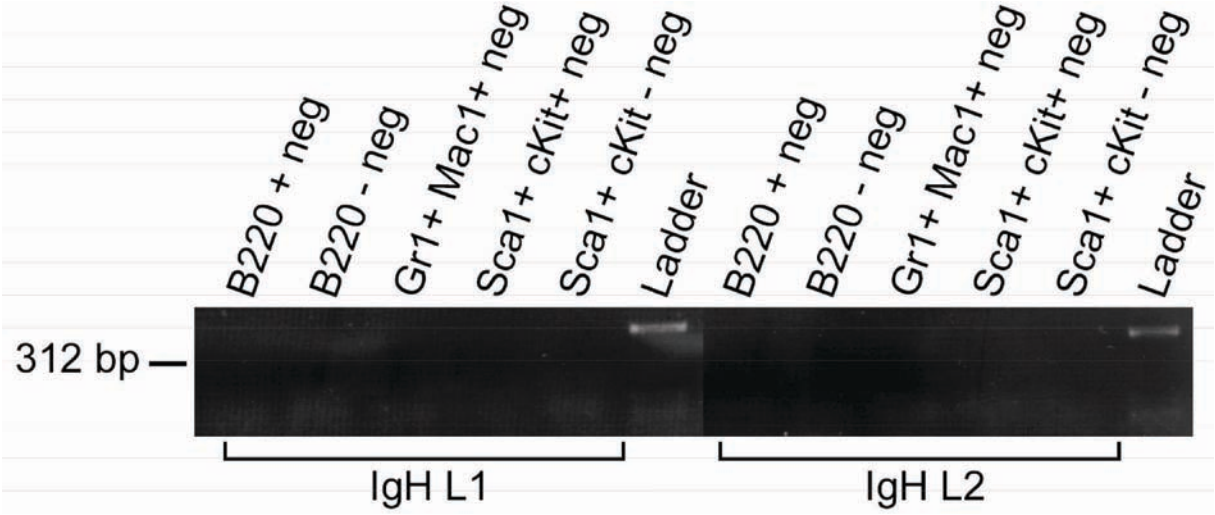


Figure 29: PCR for RNA quality control from sorted cells from spleen (B220 + and -) and bone marrow (Gr-1, Mac-1, Sca-1 and cKit) of pIGCASV-40 mouse Lines 1 and 2. RNA digested with DNaseI as negative control for cDNA of sorted cells. Primer pair used: mouse Hprt. PCR conditions: 30 cycles, annealing temperature 59°C.

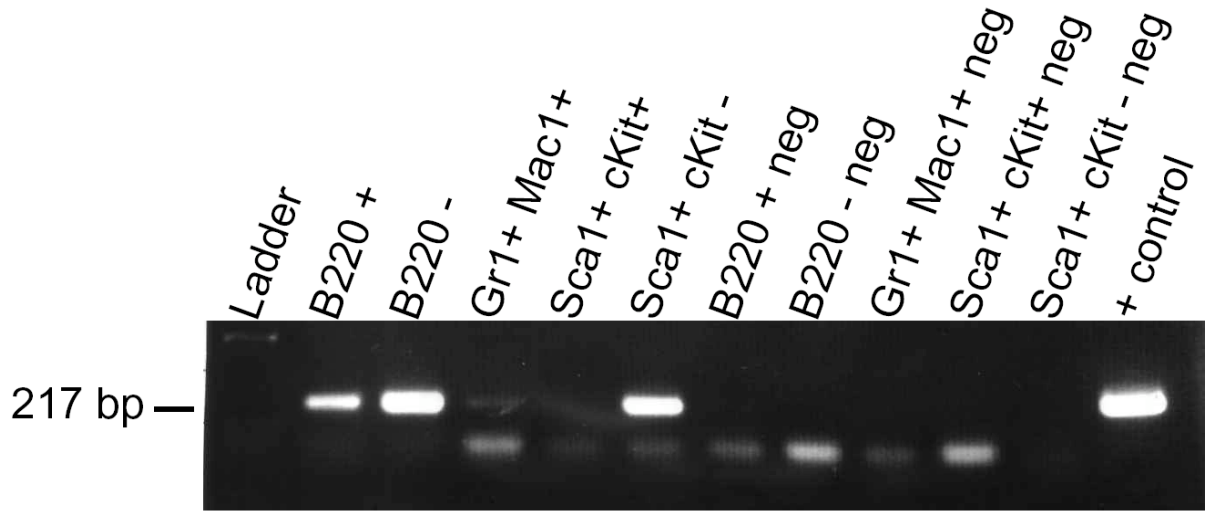


Figure 30: RT-PCR of sorted cells from spleen (B220 + and -) and bone marrow (Gr-1, Mac-1, Sca-1 and cKit) of pIGCASV-40 mouse Line 1 showing expression of the CALM/AF10 transgene. Primer pair used: CALM(1900-1920), AF10(448-424). PCR conditions: 30 cycles, annealing temperature 56°C.

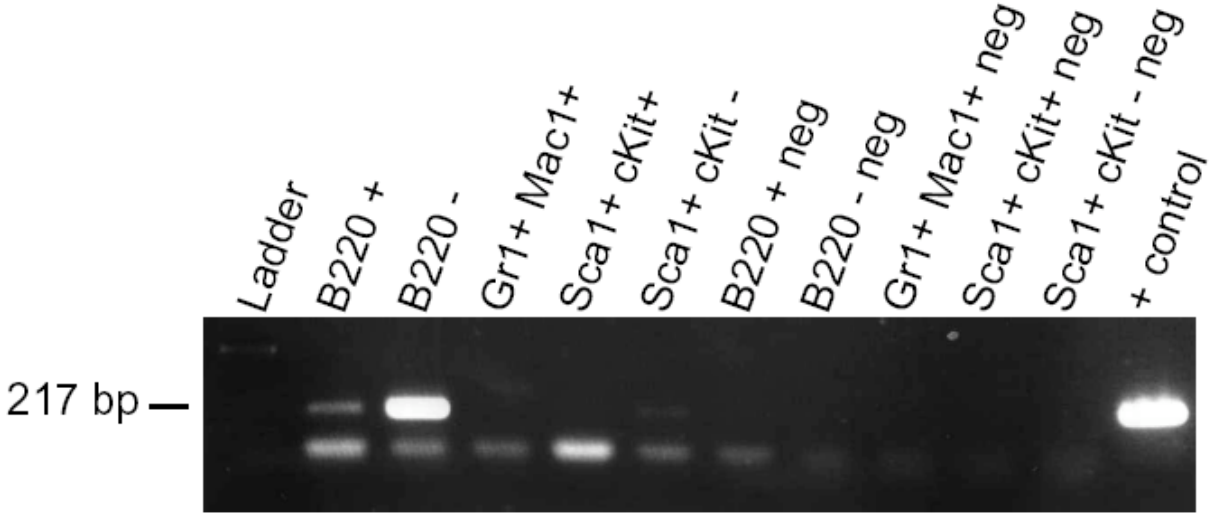


Figure 31: RT-PCR of sorted cells from spleen (B220 + and -) and bone marrow (Gr-1, Mac-1, Sca-1 and cKit) of pIGCASV-40 mouse Line 2 showing expression of the CALM/AF10 transgene. Primer pair used: CALM(1900-1920), AF10(448-424). PCR conditions: 30 cycles, annealing temperature 56°C.

Analysis of transgene expression by Western blot

Western blot analysis was performed with cell extracts of thymus, liver and spleen of pIGCASV-40 mice lines 1 and 2, and HEK293T cells transiently transfected with pEYFP-CALM/AF10 as positive control. The technical procedures and antibodies used were the same as described for the transiently transfected cells on page 64. We were not able to detect CALM/AF10 protein in any of the lines.

Analysis of the phenotype of the transgenic mice

The transgenic mice from the lines 1 and 2 were observed up to 15 months and no clinical alteration was observed when comparing to the wild type mice. The body weight of 29 transgenic was compared with 28 wild type adult mice (ages between 8 and 15 months) and there was no significative difference. Because the transgene was expected to be expressed specifically in B-cell progenitors (Eckhardt, 1992; Harris *et al.*, 1988), we analysed the hematopoietic system by hemogram, serum immunoglobulin concentration, flow cytometry of splenocytes, bone marrow cells and blood. Histopathologic and immunohistopatological analysis was also carried out to assess any alteration of the hematopoietic system in the transgenic mice.

Hemogram

Hemograms of 45 adult transgenic and 21 wild type FVB mice aged between 5 months and one year were done to observe hematological difference between transgenic and control mice. The white blood cell (WBC) count and red blood cell (RBC) count and the WBC differential count was performed. The RBC count of the transgenic mice was $6.36 \times 10^9 \pm 0.91 \times 10^9$ cells/ml and $6.22 \times 10^9 \pm 0.57 \times 10^9$ cells/ml for the control mice. The WBC count of the transgenic mice was $3.44 \times 10^6 \pm 1.22 \times 10^6$ cells/ml and $3.59 \times 10^6 \pm 1.09 \times 10^6$ cells/ml in the control mice. The total WBC and RBC count did not differ significantly between transgenic and control mice ($p>0.05$). On the microscopical examination, there was no morphological difference observed between transgenic and control mice blood cells. There was no statistical difference in the WBC differential between transgenic animals of pIGCASV-40 mice lines 1 and 2 and control animals. The hematological values and the U Mann-Withney statistic test for non paired samples results are shown in the table 17.

Table 17: Median, median standard-deviation and statistic results for WBC differential count values for pIGCASV-40 mice lines 1 and 2

pIGASV-40 mice	WBC	Median ± standard deviation		P value	Result
		Positive*	Negative**		
L1	Lymph	87.00 ± 10.95	83.00 ± 4.03	0.2212	POS = NEG
	Neutr	8.00 ± 8.28	9.00 ± 3.89	0.8721	POS = NEG
	Mono	4.00 ± 4.37	6.00 ± 3.35	0.0509	POS = NEG
	Eosin	0.00 ± 1.30	1.00 ± 0.98	0.5929	POS = NEG
	Baso	0.00 ± 0.53	0.00 ± 0.37	0.9604	POS = NEG
L2	Lymph	78.50 ± 12.41	80.50 ± 13.27	0.6719	POS = NEG
	Neutr	15.00 ± 8.00	11.00 ± 9.60	0.8157	POS = NEG
	Mono	6.00 ± 6.06	7.00 ± 5.24	0.7504	POS = NEG
	Eos	0.00 ± 1.37	0.00 ± 0.99	0.7470	POS = NEG
	Baso	0.00 ± 0.85	0.00 ± 0.36	0.4952	POS = NEG

*n = 27 (L1), n = 18 (L2) ** n = 10 (L1) n = 11 (L2)

Analysis of the phenotype at the immunological level

In order to detect any alterations in B-cell function in the pIGCASV-40 transgenic mice, the serum of five transgenic mice from the line 1, four transgenic mice from the line 2 and 11 wild type mice was analyzed. The samples were sent to the Institute of Pathology of the Technical University and the concentrations of the antibodies: IgA, IgG1, IgG2a, IgG2b, IgG3 and IgM were measured by ELISA. These tests were carried out by Olaf Gross at the Institute of Pathology of the Technical University of Munich. The values obtained for the optical density for the different serum antibodies were statistically analysed using the T- Students test for non paired samples considering the populations with similar variance and comparing the transgenic mice (Tg) and the wild type control mice (WT) from the two lines. The measurements were performed at different time points for the different lines and their respective controls. These results are graphically illustrated in the figure 32.

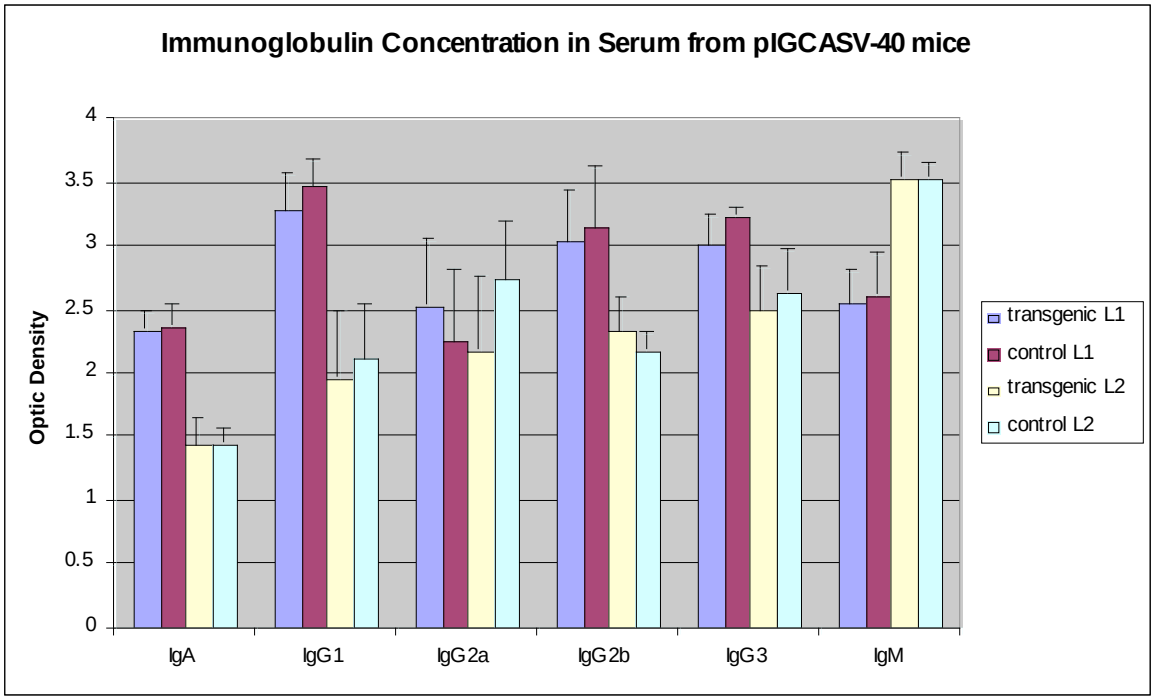


Figure 32: Graphic representation of serum immunoglobulin values for pIGCASV-40 mice lines 1 and 2 and controls.

Flow cytometry

Analysis of immunophenotype of WBCs in blood, bone marrow and spleen was performed by flow cytometry aiming to observe any difference between transgenic and wild type mice in the WBC cell distribution in these organs. The cells were obtained from organs of euthanized mice and incubated with the antibodies as described in the methods section. The surface markers analyzed were: for the stem cell compartment: c-Kit and Sca-1, for the myeloid progenitor compartment Gr-1, Mac-1, and for the lymphoid compartment: B220, CD19, CD43 and CD3. The distribution of the cell types was compared between transgenic and wild-type mice using the t-Test ($p>0.05$). The results are shown in the tables 22 to 25 (appendix) and graphically represented in the figures 33 to 35.

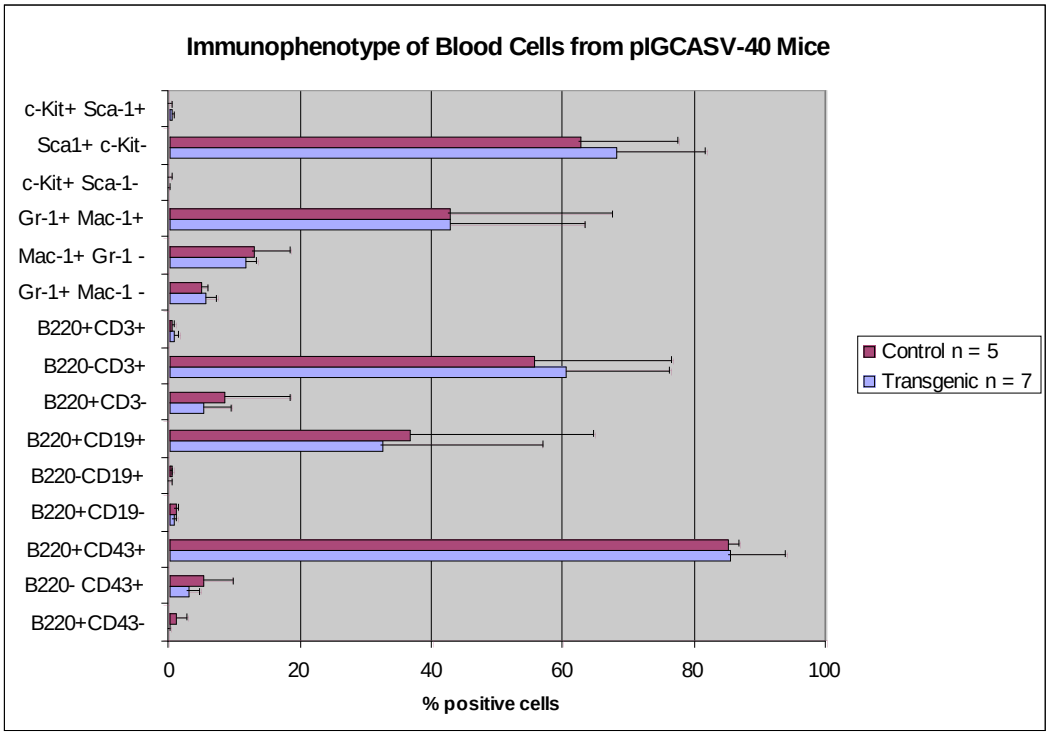


Figure 33: Immunophenotype of blood cells from pIGCASV-40 mice and FVB control, graphic representation showing the average values of c-Kit, B220, Mac-1, Gr-1, CD3, CD19 and CD43 positive WBCs.

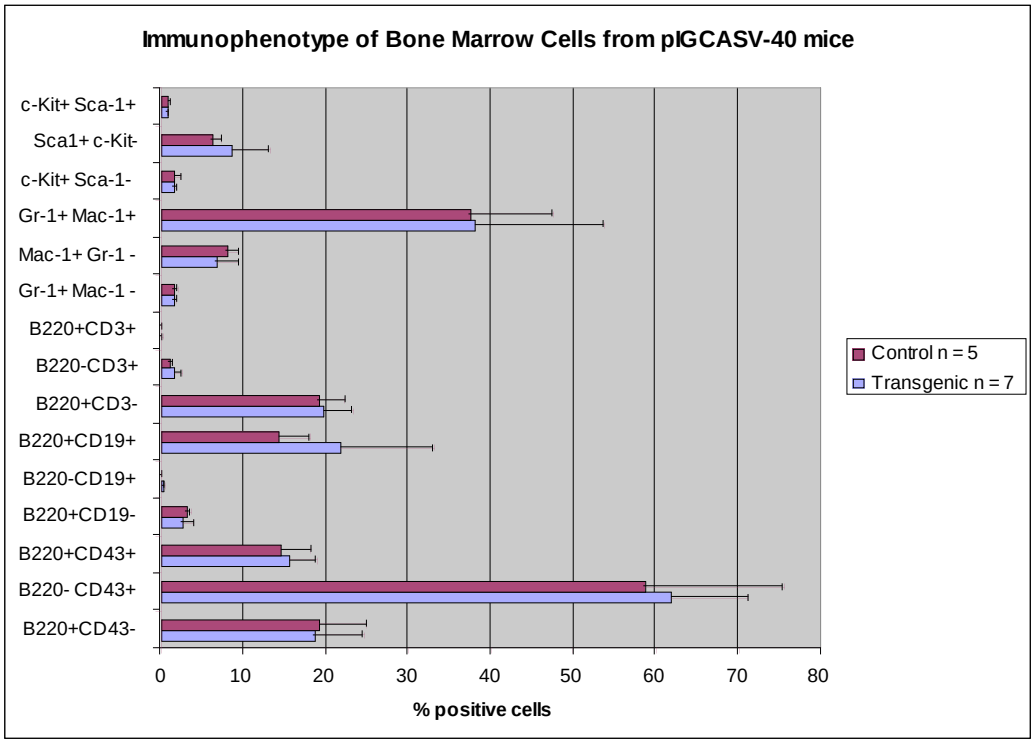


Figure 34: Immunophenotype of bone marrow cells from pIGCASV-40 mice and FVB control, graphic representation showing the average values of c-Kit, B220, Mac-1, Gr-1, CD3, CD19 and CD43 positive WBCs

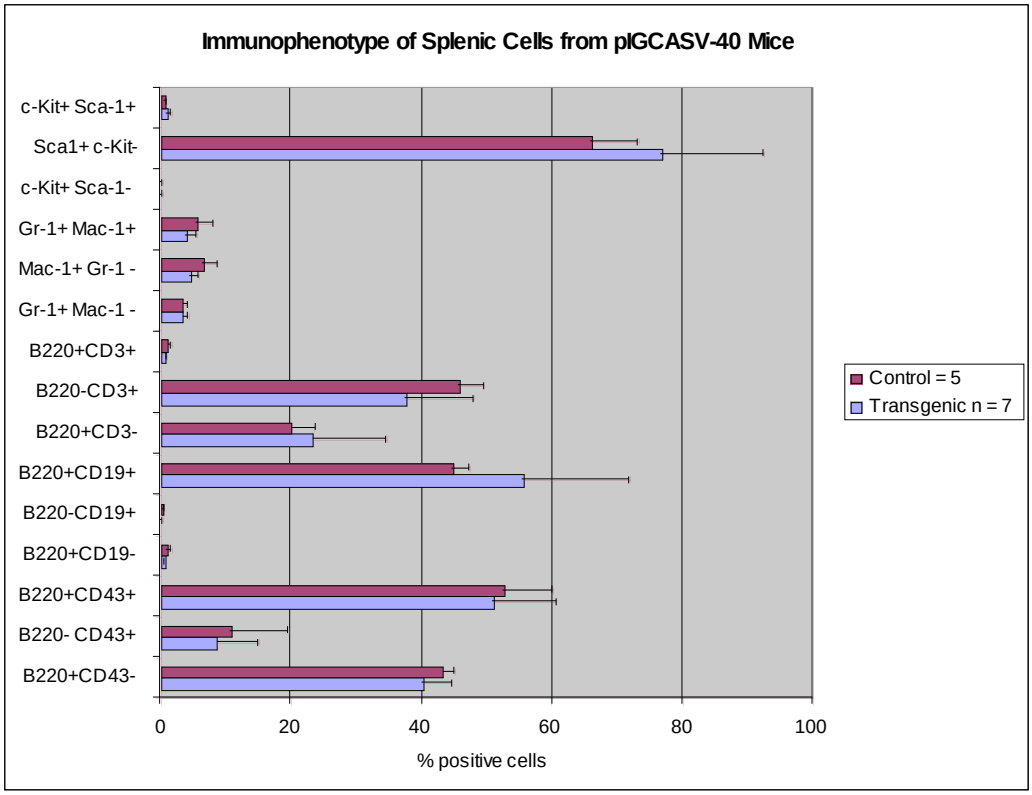


Figure 35: Immunophenotype of splenic cells from pIGCASV-40 mice and FVB control, graphic representation showing the average values of c-Kit, B220, Mac-1, Gr-1, CD3, CD19 and CD43 positive WBCs

Analysis of the phenotype of the pIGCASV-40 mice at the pathological level

Histopathology

In order to observe any pathological alterations in the transgenic mice related to the expression of CALM/AF10 in transgenic lines 1 and 2, we sent four mice to Dr. Leticia Quintanilla-Fend at the Institute of Pathology of the GSF Research Center for Environment and Health in Neuherberg. At the time point of this analysis, the mice were kept in a temporary facility without specific pathogen free (SPF) conditions and presented positive serology for the mouse hepatitis virus. The histologic examination of kidneys, liver, spleen, intestine, thymus and mesenteric lymph nodes from the control mouse revealed no alterations. For the mouse from the line 1, the same organs were analyzed as described above. The small intestine showed increased number of plasma cells in the lamina propria with mild atrophy of the villi. No evident Peyer´s patches could be observed. The liver showed conspicuous regenerative changes with nodules of hepatocytes with large nuclei. Another prominent feature was a marked hypertrophy and hyperplasia of Kupffer cells and sinusoidal lining cells, which were often laden with pigment. Focally, small inflammatory infiltrates with necrosis of hepatocytes

were found. The mesenterial lymph node showed germinal center hyperplasia accompanied by hyperplasia of the paracortical area. The thymus and the spleen had a normal appearance. The mouse from the pIGCASV-40 line 2 had the same organs analyzed as described above. The small intestine also showed increased number of plasma cell in the lamina propia. However, the plasma cell infiltration was less prominent than the one observed in the animal from the line 1. The mesenteric lymph nodes showed paracortical hyperplasia with the presence of multiple histiocytes. The kidneys showed focal infiltration with plasma cells. In some areas mitotic figures could be observed in the plasma cells. The liver, thymus and spleen appeared normal. Figures 36a to 36c show the alterations observed in these mice.

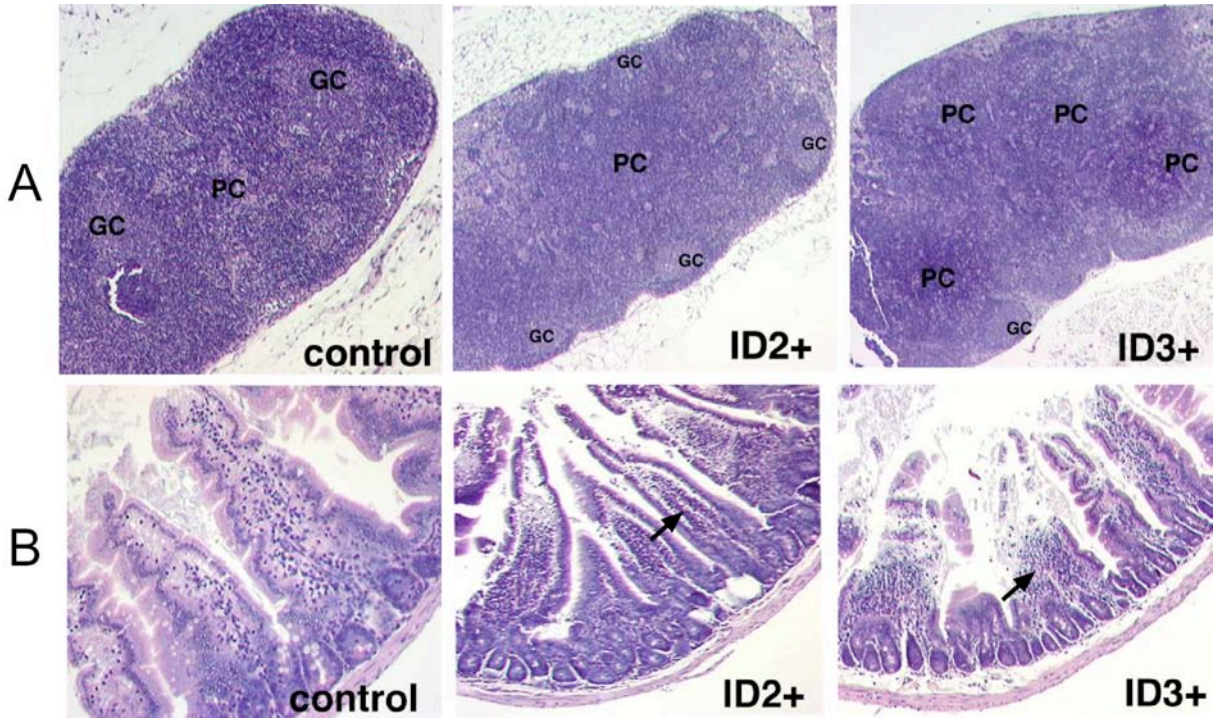


Figure 36a: Histopathology of pIGCASV-40 transgenic mice. Pictures in the line A show mesentheric lymph nodes exhibiting germinal center and paracortical area hyperplasia (ID2, Line 1 and ID3, line 2). The pictures in the line B show increased number of plasma cells (arrows) in the lamina propria of the small intestines. These alterations were not observed in the control animal.

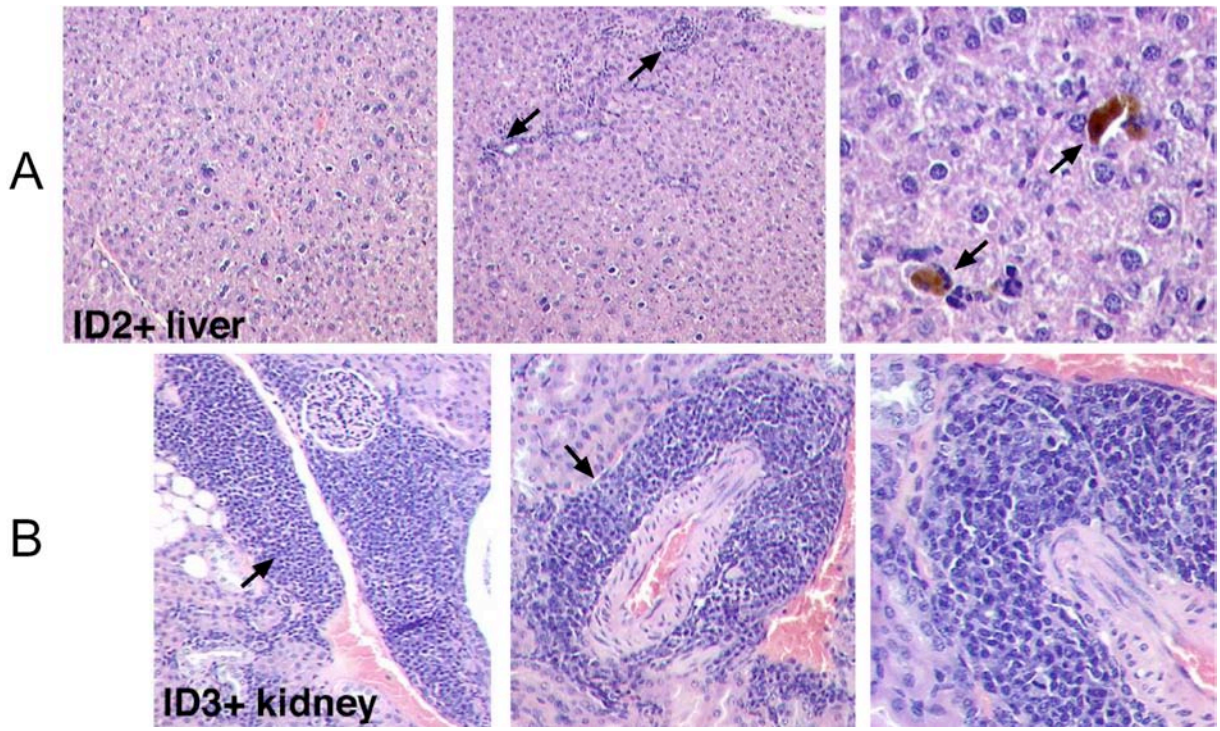


Figure 36b: Histopathology of pIGCASV-40 transgenic mice from the lines 1 and 2. Pictures in the line A show the liver from a mouse from the pIGCASV-40, line 2 showing regenerative changes with nodules of hepatocytes with large nuclei (arrows middle picture). Marked hypertrophy and hyperplasia of Kupfer cells and sinusoidal lining cells, often laden with pigment (arrows, last picture). The pictures at line B show focal plasma cell infiltrations in kidney (arrows).

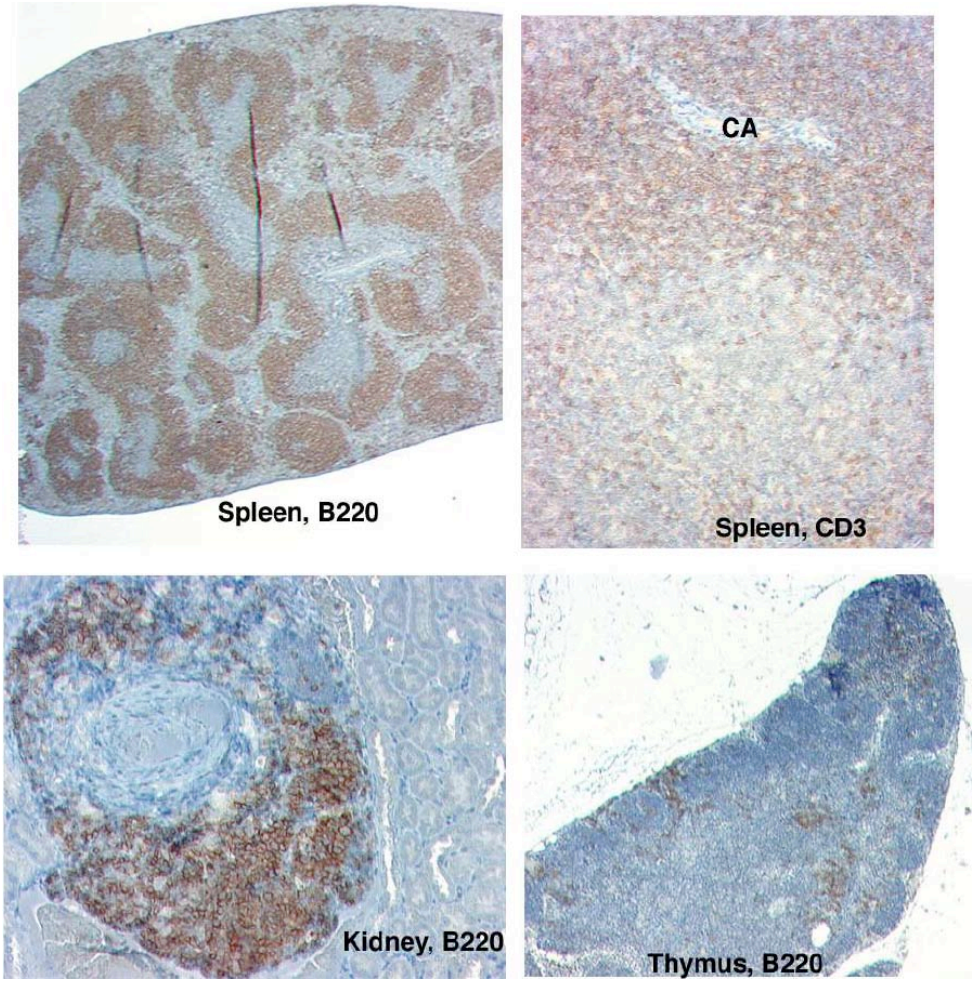


Figure 36c: Immunohistopathology of a pIGCASV-40 transgenic mouse from the line 2. The pictures show immunohystochemistry for B-cells (B220) confirming the plasma cell infiltration previously described in the kidney. Spleen and thymus showed normal distribution of T and B cells, as detected with B220 and CD3 antibodies. CA = central arteriole.

After this first analysis, all the transgenic mice were transferred to the SPF facilities at the Gene Center and, from these animals, we sent 2 clinically healthy adult mice of each line and 2 control-littermates with ages of 15 months for pathological analysis. All transgenic mice analyzed showed pathological alterations in the lungs, such as papillary adenocarcionomas or papillary adenomas. The term adenocarcionoma is used according to the recommendations of the mouse models of Human Cancers Consortium for the classification of proliferative pulmonary lesions of the mouse (Nikitin *et al.*, 2004). Four adult mice (3 from the line 1 and 1 from the line 2) showing severe dyspnea and weight loss were sacrificed and sent for pathological analysis and the same pulmonary lesions were found. Figure 37 shows a euthanized mouse that was sent to pathology and figure 38 illustrates the pathological findings found in these mice. The control mice had normal lungs and did not show any other

pathological findings. There were no plasma cell abnormalities in these animals as observed in the previous pathological analysis.

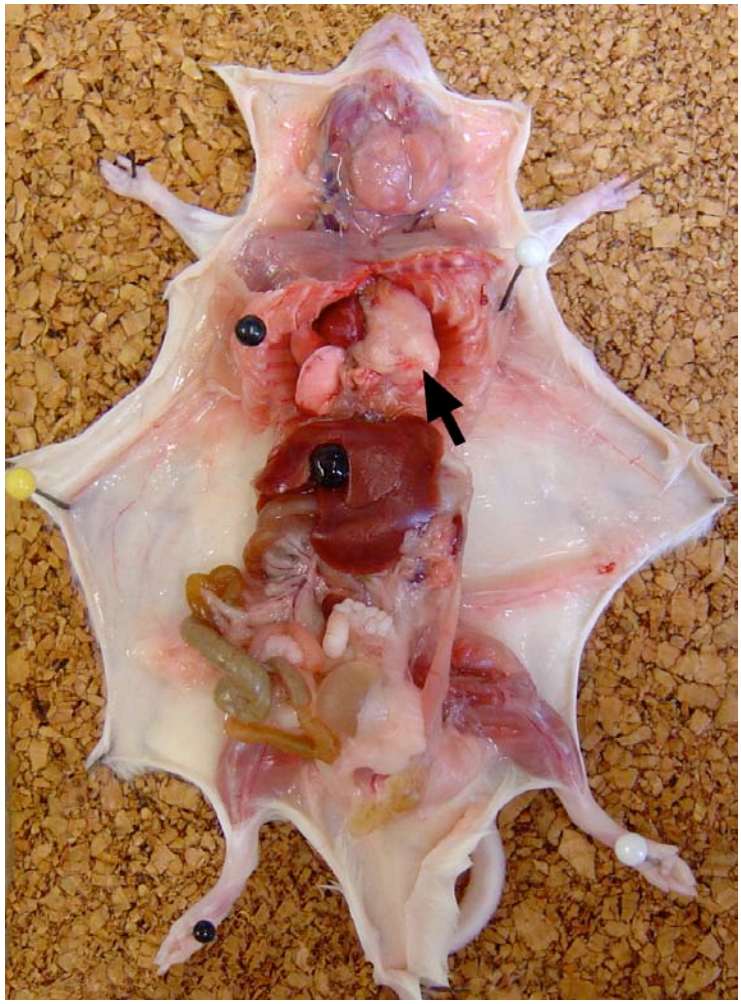


Figure 37: Macroscopic view of a pIGCASV-40 transgenic mouse from the line 1. The arrow indicates a lung tumor diagnosed as papillary adenocarcinoma on histopathology

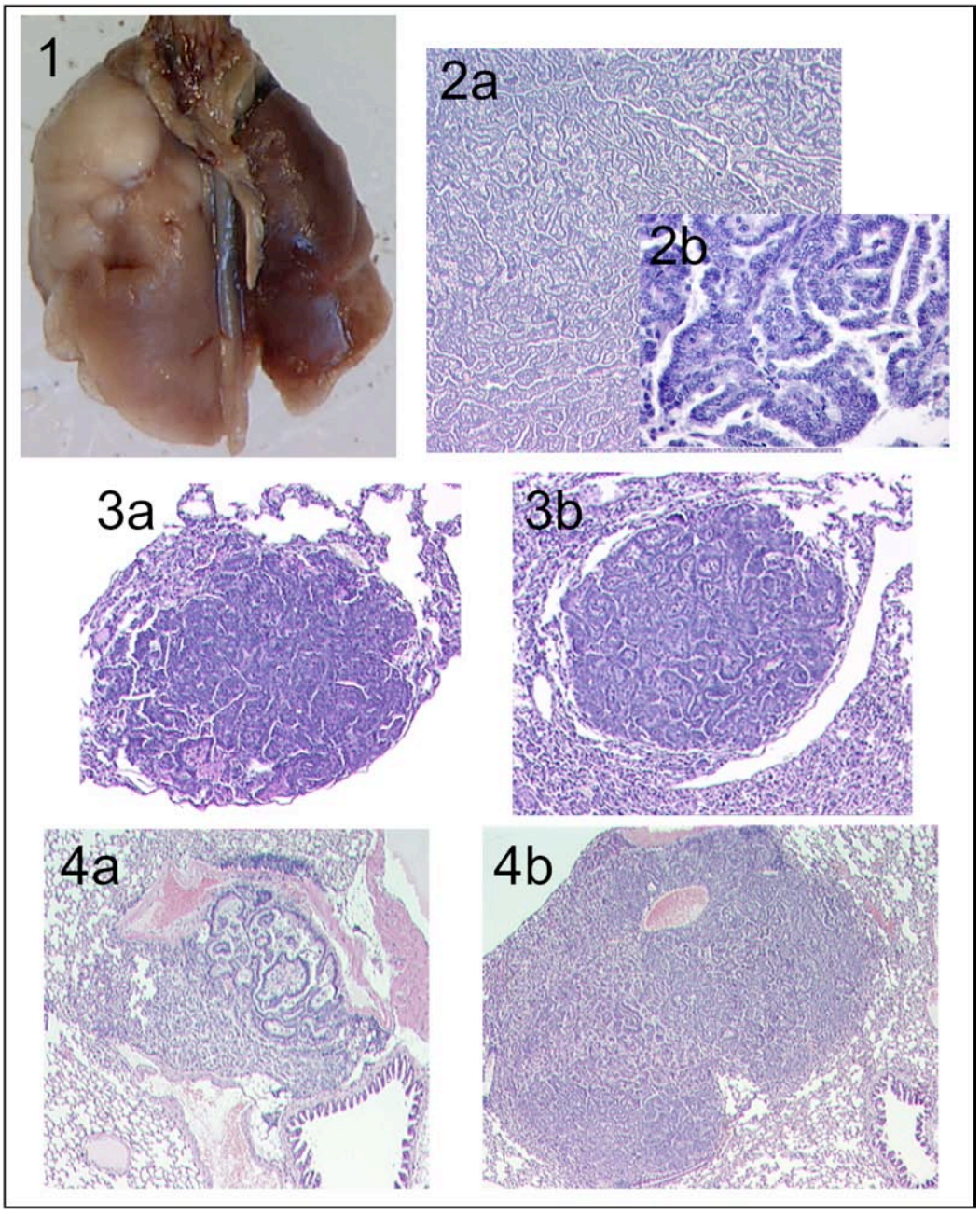


Figure 38: Lung tumors observed in pIGCASV-40 transgenic mice from lines 1 and 2. Picture 1 shows a macroscopic view of the lung of a mouse from the line 1 with an evident tumor. Picture 2a and 2b show the histopathology of the same organ with well differentiated papillary adenocarcinoma. Pictures 3a and 3b show two papillary adenomas from the same mouse. Pictures 4a and 4b show two papillary adenomas from a mouse from the line 2. Staining: hematoxilin-eosin.

5.2.2. FVB mice expressing CALM/AF10 under the control of the proximal *Lck* promoter

Cloning of the transgenic construct

CALM/AF10 transcripts have been detected in several patients with T-ALL (Silliman *et al.*, 1998; Narita *et al.*, 1999; Bohlander *et al.*, 2000; Asnafi *et al.*, 2003 and Ou *et al.*, 2004). With the objective of expressing *CALM/AF10* in T-cell precursors, we decide to use the *Lck* proximal promoter, reported as to induce gene expression specifically into immature T-cell populations (Allen *et al.*, 1992; Brenner *et al.*, 2001).

The proximal murine *Lck* promoter was amplified by PCR primers (M*LCK*prom64-84topXho1 and M*LCK*prom702-681bottomSac1) from mouse genomic DNA (Garvin *et al.*, 1988). The 5' primer contained an *Xho1* restriction site and the 3' primer a *Sac1* restriction site. Using an *XhoI* and *SacI* digest, the *IgH* promoter/enhancer fragment was released from the pIGCASV-40 plasmid and replaced by the 630 bp proximal *Lck* promoter fragment. The resulting p*LCK*-CASV-40 construct was used for transgenic mouse production and is schematically shown in figure 39.

Figure 39: Schematic representation of the pLCKCASV-40 construct.

Generation of transgenic lines

The transgenic construct was purified from the vector backbone and prepared for pro-nucleus injection as described above for the pIGCASV-40. The parameters for the generation of the transgene mice for this construct are summarized in the table 18.

Table 18: Parameters for the generation of transgenic mice with the pLCKCASV-40 construct

Number of injected embryos	377
Viable embryos	259
Transferred embryos	247
Negative sections*	2
Number of transfers**	11
Embryonic re-absorption in uterus	22
Number of mice born	21
Number of dead new born mice	0
Number of transgenic mice	3
LCK transgenic lines generated	3

* Negative sections refers to euthanized non-pregnant receptor female

** Number of embryo transfer procedures to receptor female mice

Identification of transgenic mice

PCR

Transgenic pLCKCASV-40 mice were identified by PCR using genomic DNA as template as described for the pIgCASV-40 transgenic mice.

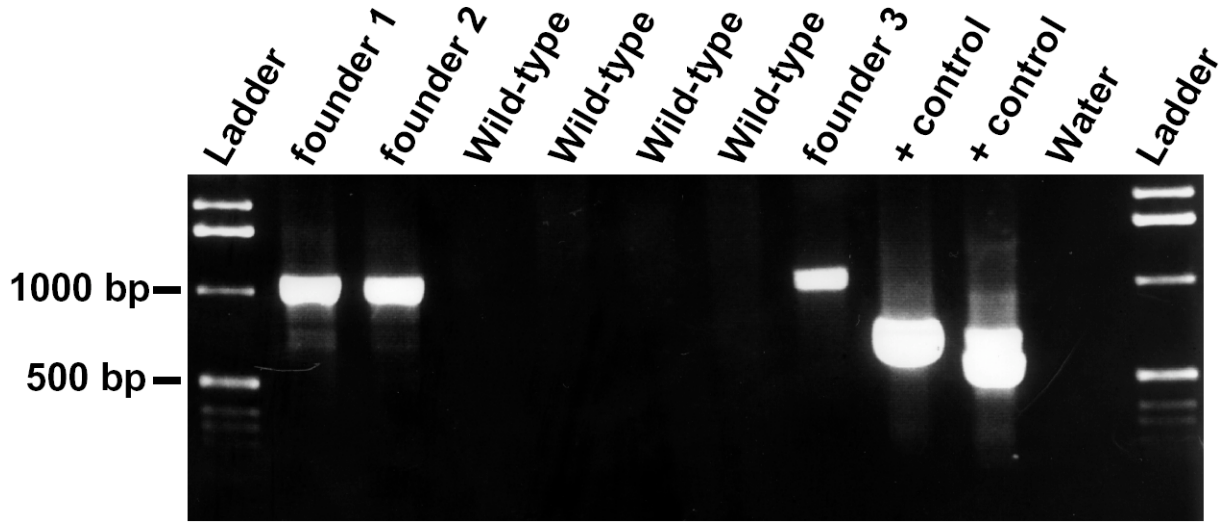


Figure 40: PCR screening of pLCKCASV-40 transgenic mice. Primer pair used: CALM(1730-1753) and AF10(1086-1066). Expected size of PCR product in the transgenic animals: 1000bp. conditions: 35 cycles, annealing temperature 58°C. cDNA from two CALM/AF10-positive patient samples were used as positive control. Note that the CALM/AF10 fusion transcripts in the patients have different breakpoints from the CALM/AF10 fusion used for the generation of the transgenic mice. These different fusion breakpoints result in smaller PCR products in the positive controls.

Southern Blot Analysis

Southern blot analysis was carried out to confirm the presence of the transgene in the founder mice and to determine the number of transgenic integrations in the mouse lines obtained. Although the mice from the line 3 tested positive by PCR for the *CALM/AF10* transgene, using the SV-40 probe we could not identify any positive band on Southern blot analysis in more than 10 blots with different enzymes for the genomic DNA digestion. Figure 41a shows the Southern blot results for the pLCKCASV-40 mice from the lines 1, 2 and 3. The figure 41b illustrates the pLCKCASV-40 construct linearized with the enzymes *Mlu1* and *Xho1* for the trangenic mice generation. The figure shows also the position of the SV-40 probe and the position of the *EcoR1* enzyme used for the genomic DNA digestion.

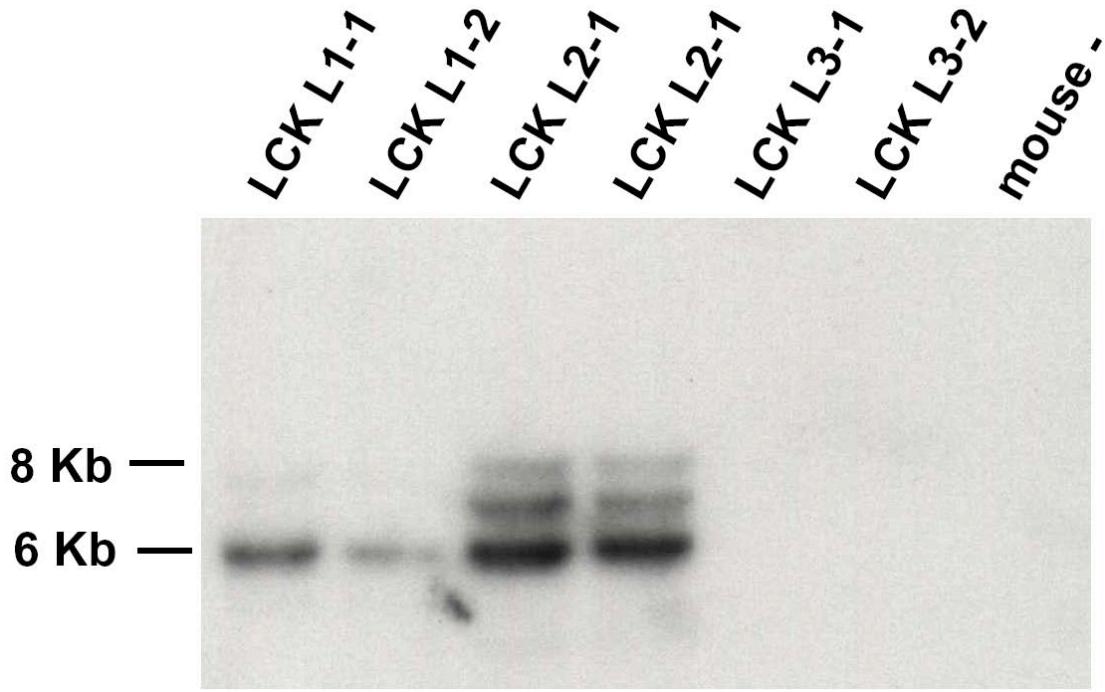


Figure 41a: Southern blot analysis of pLCKCASV-40 transgenic mice. Lanes 1 to 7 contained 20 □g of genomic DNA digested overnight with *EcoR1* and hybridized with 30ng of P^{32}-labelled SV-40 probe. 48 hours exposure.

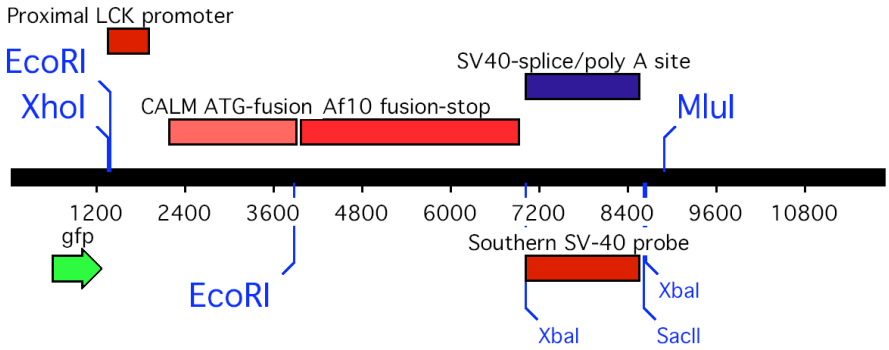


Figure 41b: Schematic representation of the pLCKCASV-40 transgenic construct, with the restriction enzyme sites used for cloning (*Xho*1 and *SacII*) and linearization (*Xho*1 and *Mlu*1) for the transgene and the position of the sequence used as a probe for Southern analysis.

Analysis of transgene expression by RT-PCR

In order to verify the expression of *CALM/AF10* in different organs and cell types from the pLCKCASV-40 transgenic mice, we extracted RNA from different haematological and non-haematological tissues and from sorted cells from specific haematological cell lineages and performed expression analysis by RT-PCR. The organs analysed were blood, bone marrow, spleen, thymus, lungs, skeletal muscle and adipose tissue. Pictures 42a to 47 show the cDNA controls and the *CALM/AF10* expression in these organs. In order to analyse the specific expression of *CALM/AF10* in different blood cell types, we sorted B220 positive B-cells from the spleen. From the bone marrow, the myeloid lineage double positive Gr-1+ Mac-1+ were sorted and from the thymus the T-cells CD4+CD8+ double positive, the T-helper CD4+ cells, the cytotoxic CD8 positive cells, and CD3 positive CD4 and CD8 negative cells early T-cells. Prior to the cDNA synthesis, the RNA obtained was treated with DNase A (Invitrogen) aiming to avoid contamination with genomic DNA and the *CALM/AF10* transgene. This genomic DNA contamination could generate an amplification product with the same size of the desired product amplified from CALM/AF10 cDNA. Before cDNA synthesis, the RNA treated with DNase A was tested by PCR to verify that no genomic DNA remained. The pictures 42a to 52 show the expression results obtained for the mouse Gapdh and Hprt genes (house-keeping genes) and for CALM/AF10 in different organs and sorted cells.

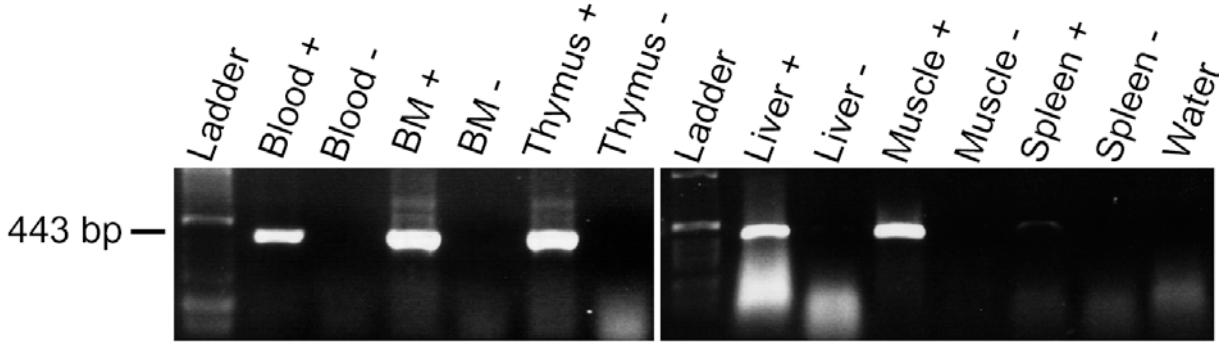


Figure 42a: RT-PCR for cDNA quality control from different organs of pLCKCASV-40 mouse Line 1. RNA digested with DNaseI without reverse transcriptase (-) and with reverse transcriptase (+). Primer pair used: mouse Gapdh. PCR conditions: 30 cycles, annealing temperature 60°C

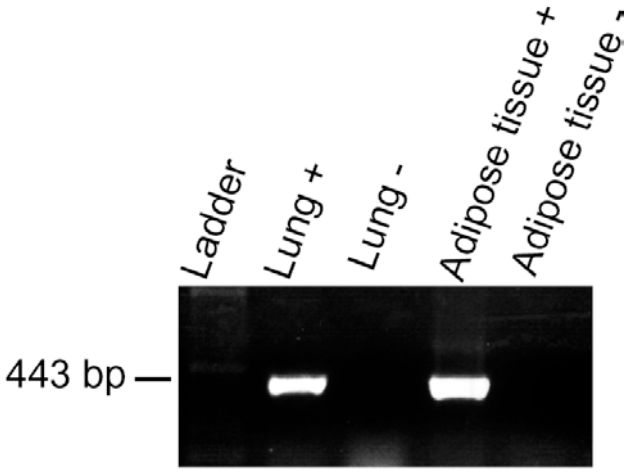


Figure 42b: RT-PCR for cDNA quality control from different organs of pLCKCASV-40 mouse Line 1. RNA treated with DNaseI without reverse transcriptase (-) and with reverse transcriptase (+). Primer pair used: mouse Gapdh. PCR conditions: 30 cycles, annealing temperature 60°C.

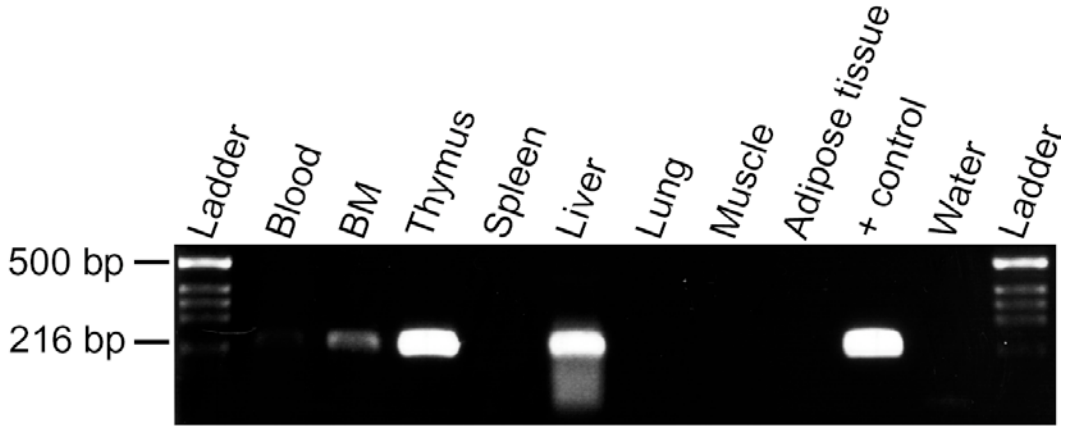


Figure 43: RT-PCR of different organs of pLCKCASV-40 mouse Line 1. Primer pair used: CALM(1900-1920), AF10(448-424). PCR conditions: 30 cycles, annealing temperature 56°C.

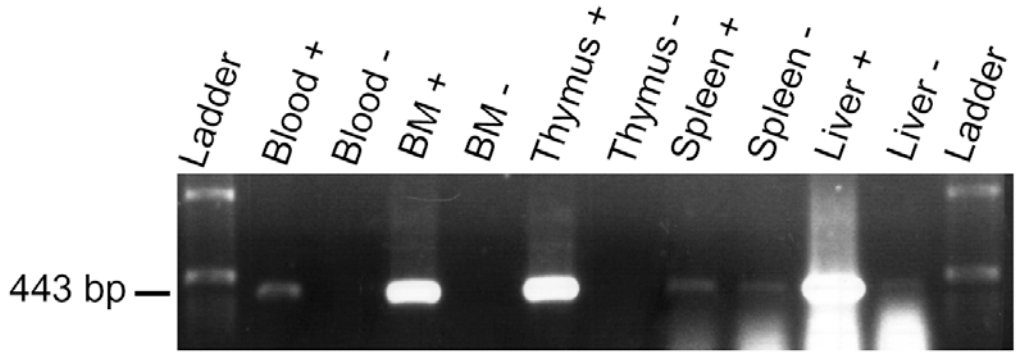


Figure 44a: RT-PCR for cDNA quality control from different organs of pLCKCASV-40 mouse Line 2. RNA treated with DNaseI without reverse transcriptase (-) and with reverse transcriptase (+). Primer pair used: mouse Gapdh. PCR conditions: 30 cycles, annealing temperature 60°C.

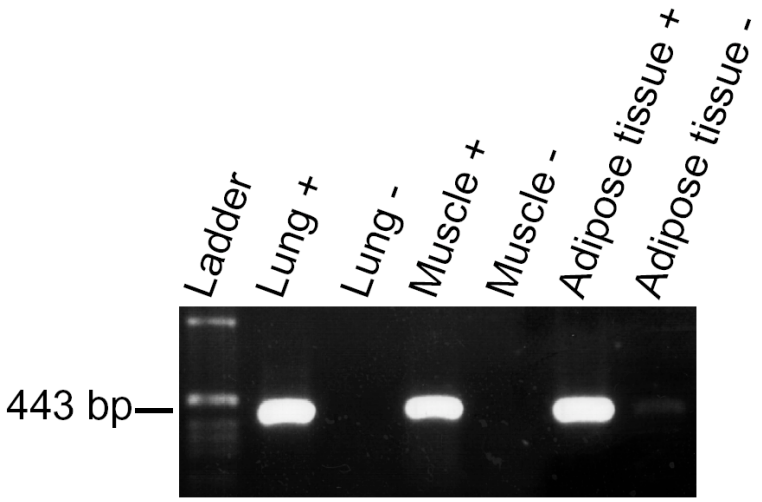


Figure 44b: RT-PCR for cDNA quality control from different organs of pLCKCASV-40 mouse Line 2. RNA treated with DNaseI without reverse transcriptase (-) and with reverse transcriptase (+). Primer pair used: mouse Gapdh. PCR conditions: 30 cycles, annealing temperature 60°C.

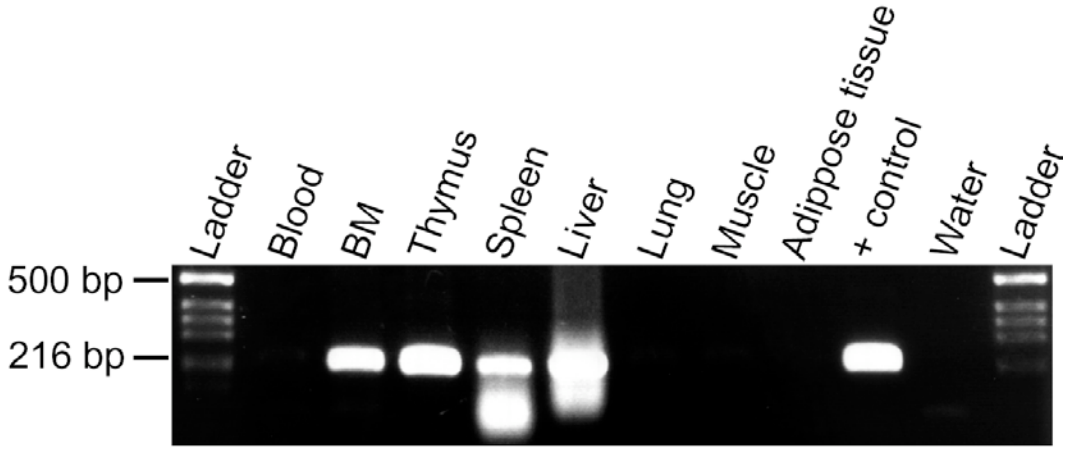


Figure 45 RT-PCR of different organs of pLCKCASV-40 mouse Line 2. Primer pair used: CALM(1900-1920), AF10(448-424). PCR conditions: 30 cycles, annealing temperature 56°C.

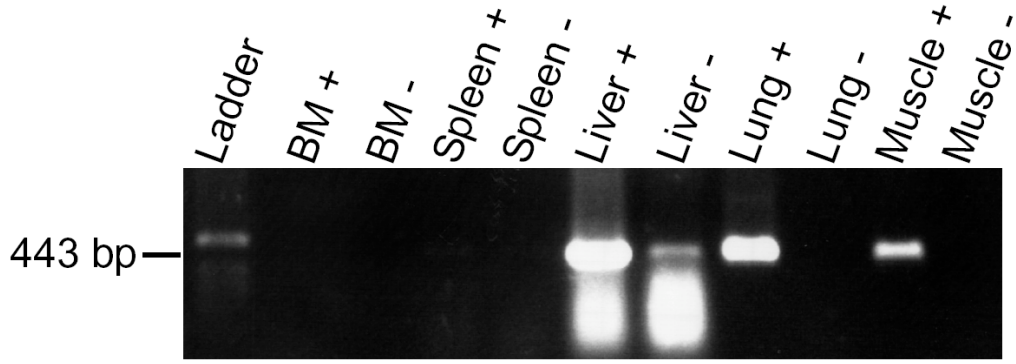


Figure 46a: RT-PCR for cDNA quality control from different organs of pLCKCASV-40 mouse Line 3. RNA treated with DNaseI without reverse transcriptase (-) and with reverse transcriptase (+). Primer pair used: mouse Gapdh. PCR conditions: 30 cycles, annealing temperature 60°C.

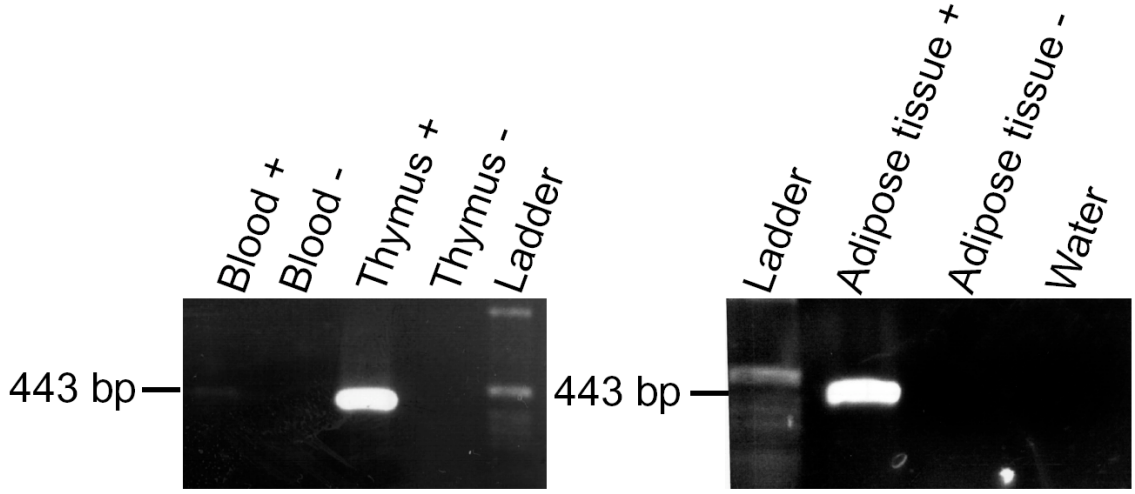


Figure 46b: RT-PCR for cDNA quality control from different organs of pLCKCASV-40 mouse Line 3. RNA treated with DNaseI without reverse transcriptase (-) and with reverse transcriptase (+). Primer pair used: mouse Gapdh. PCR conditions: 30 cycles, annealing temperature 60°C.

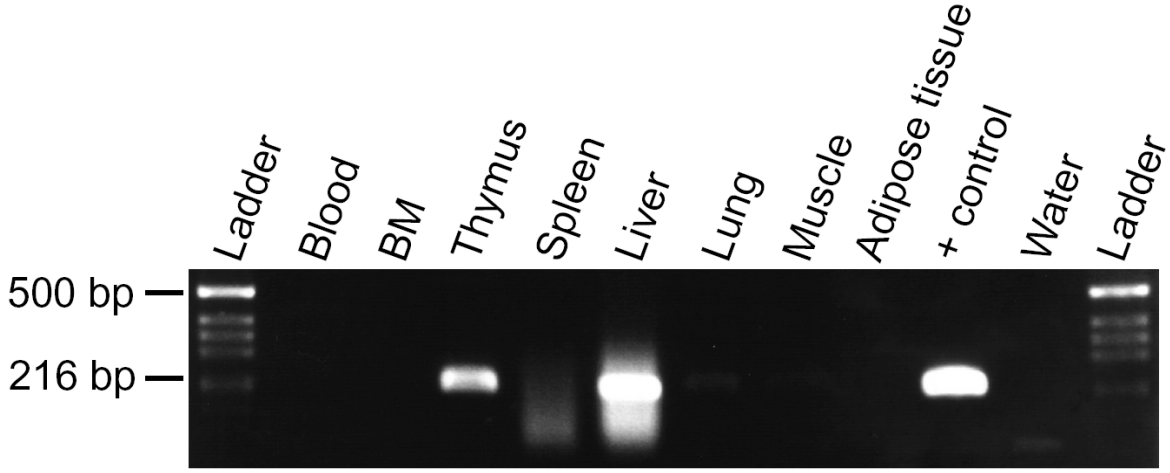


Figure 47: RT-PCR of different organs of pLCKCASV-40 mouse Line 3. Primer pair used: CALM(1900-1920), AF10(448-424). PCR conditions: 30 cycles, annealing temperature 56°C.

The amount of RNA obtained from FACS-sorted cells from organs of mice from the

pLCKCASV-40 line 1 was sufficient to obtain amplification of the house-keeping gene Hprt in the following subpopulations B220+, Gr-1+Mac-1+, CD4+CD8+, CD4+CD8-. We could detect less amplification of Hprt in CD4-CD8+ and no amplification in CD4-CD8-CD3+ cells, due to the very low number of cells obtained by sorting.
In the pLCKCASV-40 line 1, we detected the CALM/AF10 transcript in CD4+CD8+ cells and at lower expression levels in B220+ cells (figure 50).

In pLCKCASV-40 line 2, the RNA amount obtained was sufficient to observe Hprt amplification for all cell types, although the cDNA obtained from the CD4+CD8-, CD4-CD8+ and CD4-CD8-CD3+ cells showed only low amplification levels of the Hprt genes with 30 PCR cycles (figures 48 and 49). In this line, we could detect the *CALM/AF10* transcript only in the CD4+CD8+ and in the CD4+CD8- cells (figure 51). For the cytotoxic T cells (CD8+) the amount of RNA and cDNA was low for all three pLCKCASV-40 trangenic lines (figures 48 and 49).

Although the RT-PCR product using the Hprt primer pair was almost absent on the agarose gel for these cells, in the cells obtained from a mouse from pLCKCASV-40 line 3, the *CALM/AF10* transcript could be detected only in the cytotoxic T cells (figure 52).

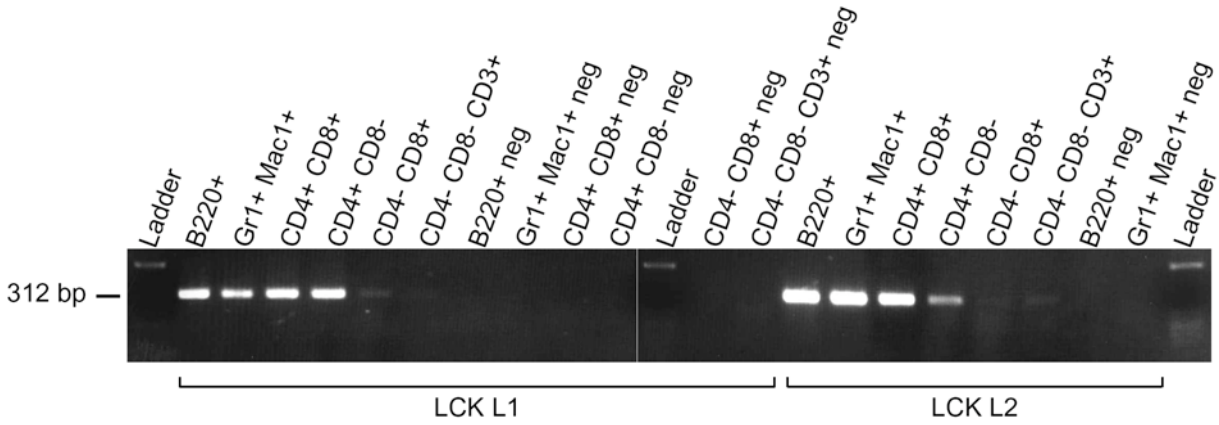


Figure 48: RT-PCR for cDNA quality control from sorted cells from spleen (B220 +), bone marrow (Gr1, Mac1) and thymus (CD4, CD8 and CD3) of pLCKCASV-40 mouse Lines 1 and 2. "neg" denotes RNA without reverse transcriptase. Primer pair used: mouse Hprt. PCR conditions: 30 cycles, annealing temperature 59°C.

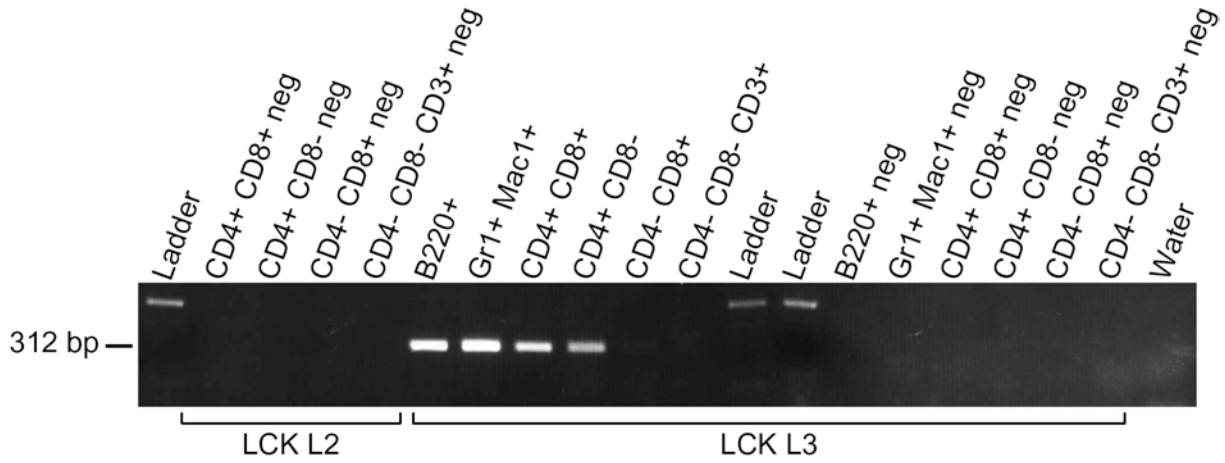


Figure 49: RT-PCR for cDNA quality control from sorted cells from spleen (B220 +), bone marrow (Gr-1, Mac-1) and thymus (CD4, CD8 and CD3) of pLCKCASV-40 mouse Lines 2 and 3. "neg" denotes RNA without reverse transcriptase. Primer pair used: mouse Hprt. PCR conditions: 30 cycles, annealing temperature 59°C.

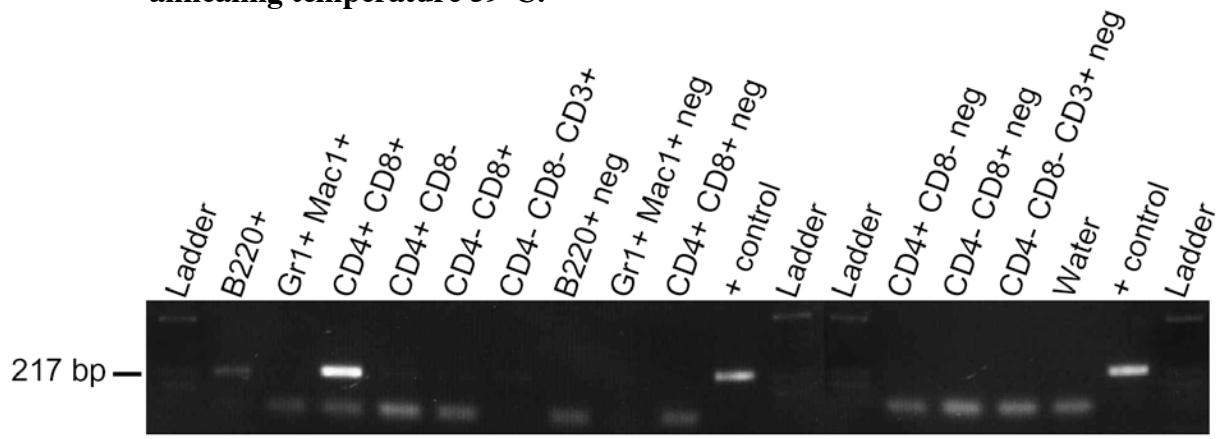


Figure 50: RT-PCR of sorted cells from spleen (B220 + and -) and bone marrow (Gr-1, Mac-1, Sca-1 and cKit) of pLCKCASV-40 mouse Line 1 showing expression of the CALM/AF10 transgene. "neg" denotes RNA without reverse transcriptase. Primer pair used: CALM(1900-1920), AF10(448-424). PCR conditions: 30 cycles, annealing temperature 56°C.

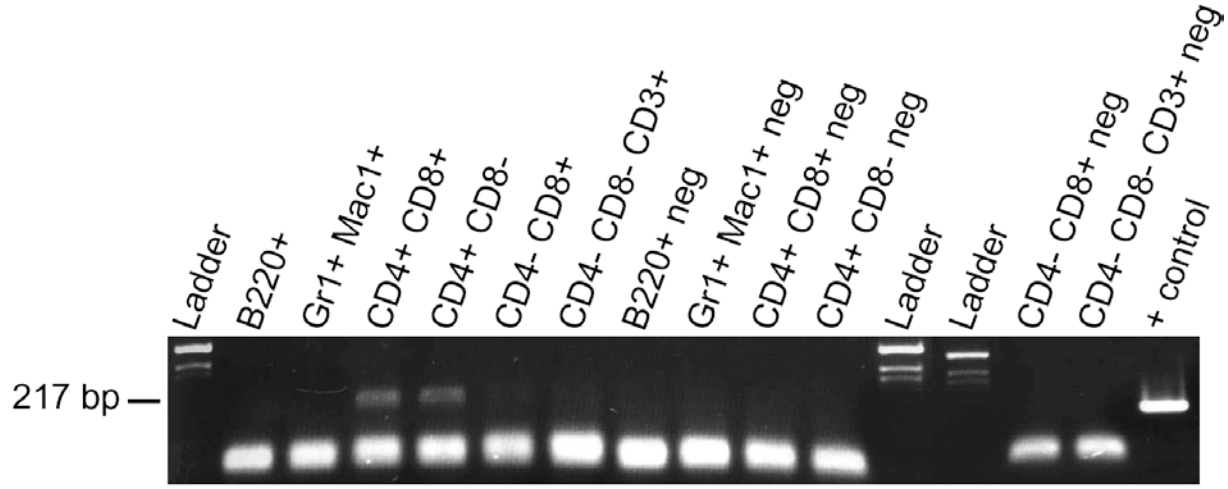


Figure 51: RT-PCR of sorted cells from spleen (B220 + and -) and bone marrow (Gr-1, Mac-1, Sca-1 and cKit) of pLCKCASV-40 mouse Line 2 showing expression of the CALM/AF10 transgene. "neg" denotes RNA without reverse transcriptase. Primer pair used: CALM(1900-1920), AF10(448-424). PCR conditions: 30 cycles, annealing temperature 56°C.

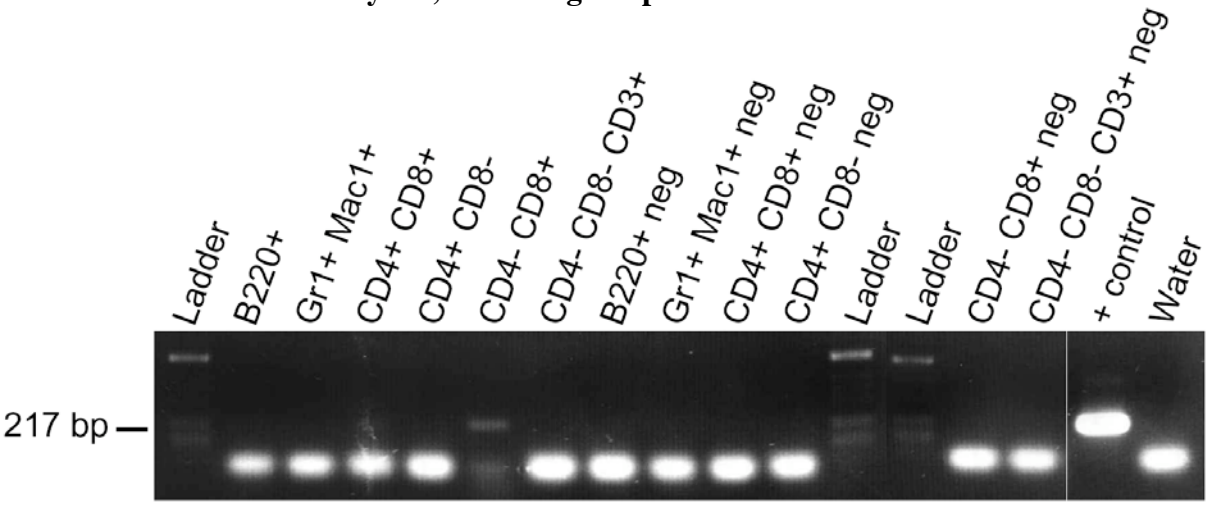


Figure 52: RT-PCR of sorted cells from spleen (B220 + and -) and bone marrow (Gr-1, Mac-1, Sca-1 and cKit) of pLCKCASV-40 mouse Line 3 showing expression of the CALM/AF10 transgene. "neg" denotes RNA without reverse transcriptase. Primer pair used: CALM(1900-1920), AF10(448-424). PCR conditions: 30 cycles, annealing temperature 56°C.

Analysis of transgene expression by Western blot

Western blot analysis was performed with cell extracts of thymus, liver and spleen of pLCKCASV-40 mice lines 1, 2 and 3 as described for the pIGCASV-40 mice. We were unable to detect the CALM/AF10 protein in the organs from the transgenic mice in these experiments.

Analysis of the phenotype at haematological level

Hemogram

Hemograms of 11 adult transgenic and 5 wild type FVB mice aged between 5 months and one year were performed to observe differences between transgenic and control mice. The white blood cell (WBC) count and red blood cell (RBC) count and the WBC differential count was performed. The RBC count of the transgenic mice was 7.23 x 10^9 $\pm$ 0.61 x 10^9 cells/ml and 6.57 x 10^9 $\pm$ 1.11 x 10^9 cells/ml for the control mice. The WBC count of the transgenic mice was 2.78 x 10^6 $\pm$ 1.21 x 10^6 cells/ml and 3.328 x 10^6 $\pm$ 1.25 x 10^6 cells/ml for the control mice. There was no statistically significant difference between transgenic and control mice in the total WBC and RBC count ($p>0.05$). We analyzed also the blood cell morphology and the relative WBC and RBC count in these animals. We did not observe any RBC or WBC morphological difference between transgenic and control mice blood cells. There was no statistical difference in the WBC differential count between transgenic animals of pLCKCASV-40 mice lines 1 and 2 and 3 and control animals. The hematological values evaluated and the U Mann-Withney statistic test for non paired samples results are shown in table 19.

Table 19: Median, median standard-deviation and statistic results for WBC differential count values for pLCKCASV-40 mice

WBC	Median ± standard deviation		P value	Result
	Positive %*	Negative %**		
Lymph	86.00 ± 5.79	90.00 ± 2.54	0.0571	POS = NEG
Neutr	9,00 ± 4.71	8,00 ± 1.87	0.2202	POS = NEG
Mono	2.5 ± 3.57	2.0 ± 3.35	0.1121	POS = NEG
Eosin	0.00 ± 1.50	0.00 ± 0,98	0.4472	POS = NEG
Baso	0.00	0.00	-	POS = NEG

*n = 11 , **n = 5

Flow cytometry

To analyze whether the pLCKCASV-40 mice showed alterations in the development and distribution of blood cells, we performed the analysis of different hematopoietic cell subpopulations: the bone marrow precursor markers CD117 (c-Kit) and Sca-1, the erythrocyte marker Ter119, the B-cell marker B220 and the myeloid markers Gr-1 and Mac-1 were used

to analyze the bone marrow samples from 3 transgenic and 3 control mice. The lymphoid antigens CD4, CD8 and CD3 were used to analyze the thymocytes of the same animals and cells from the spleen were analyzed with the surface antigens CD3 and B220. The values obtained were tested using the T-student test ($p>0.05$). Figure 53 shows the average values obtained for each antigen. There was no significant difference between transgenic and control mice. Table 26 (Appendix) shows the average, standard deviation and the t-test result for each surface antigen analyzed.

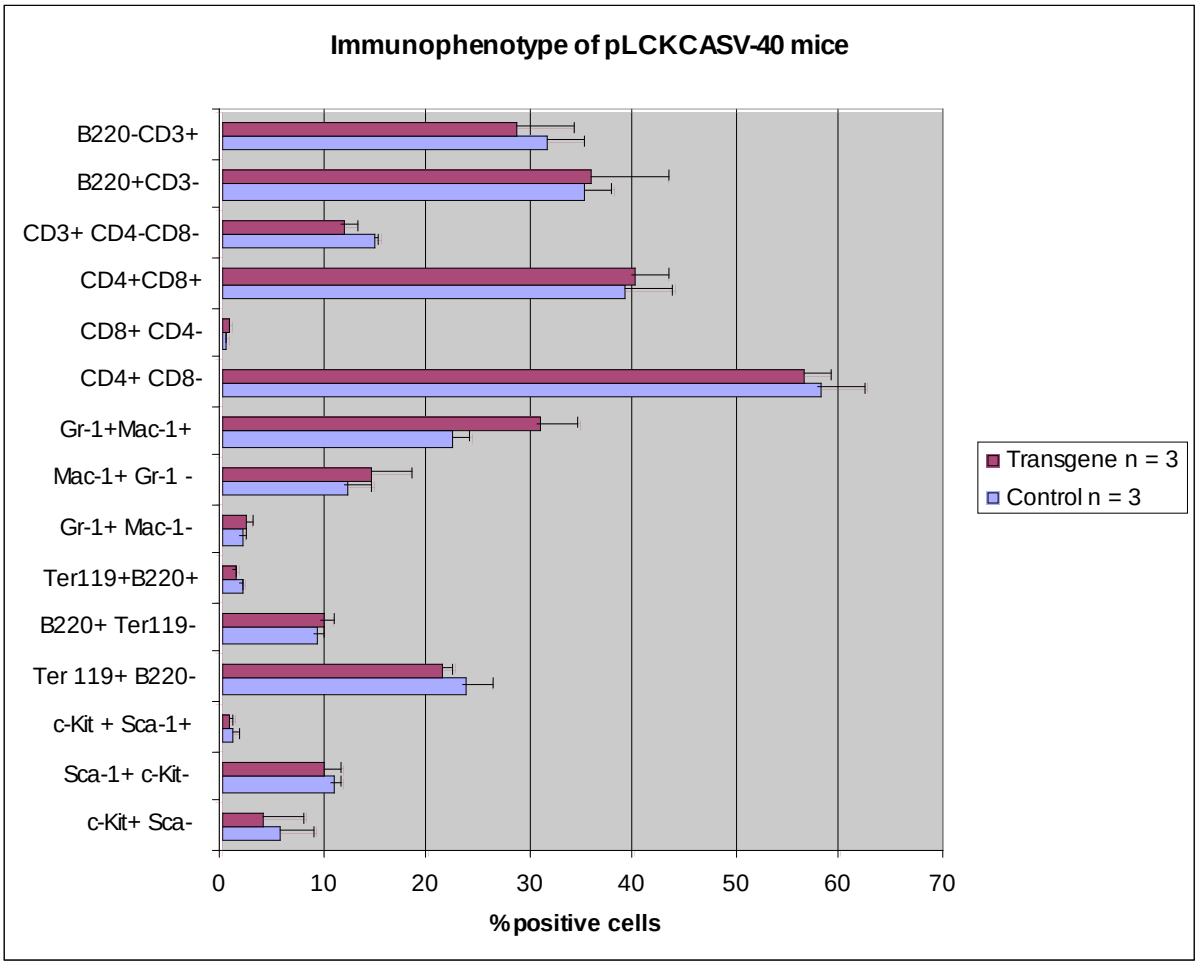


Figure 53: Immunophenotyping of cell surface markers from bone marrow (cKIT, Sca-1, GR1, Mac1), thymus (CD4, CD8 and CD3) and spleen (CD3 and B220) from pLCKCASV-40 transgenic mice and control ($p>0.05$).

Analysis of the phenotype of pLCKCASV-40 transgenic mice at the pathological level

Histopathology

For the hystopatological analysis we sent one mouse from each line ans two control mice. The main abnormal histological findings were in the liver, spleen, and thymus. The liver showed

hyperplasia of the hepatocytes surrounding the central veins. The spleen showed hyperplasia of the white pulp (Figure 54b). The immunohistochemistry revealed an increase of B-cells (B220), however, the relationship between B- and T-cells remained normal (Figure 54b). The thymus was normal in size and morphology. In the thymus medulla, a slight increase in B-cells could be observed (Figure 54b). The lung showed no alterations in any of the transgenic animals. The control animal also showed hyperplasia of the hepatocytes surrounding the central vein. In addition, in the spleen and thymus there was hyperplasia of lymphoid tissue, mainly from the B-cell compartment. The thymus was normal in size and morphology.
The pathological findings in all transgenic pLCKCASV-40 and in the control mouse were similar. Thus these findings are unlikely to be due to the action of the transgene. Figures 54a and 54b illustrate these findings.

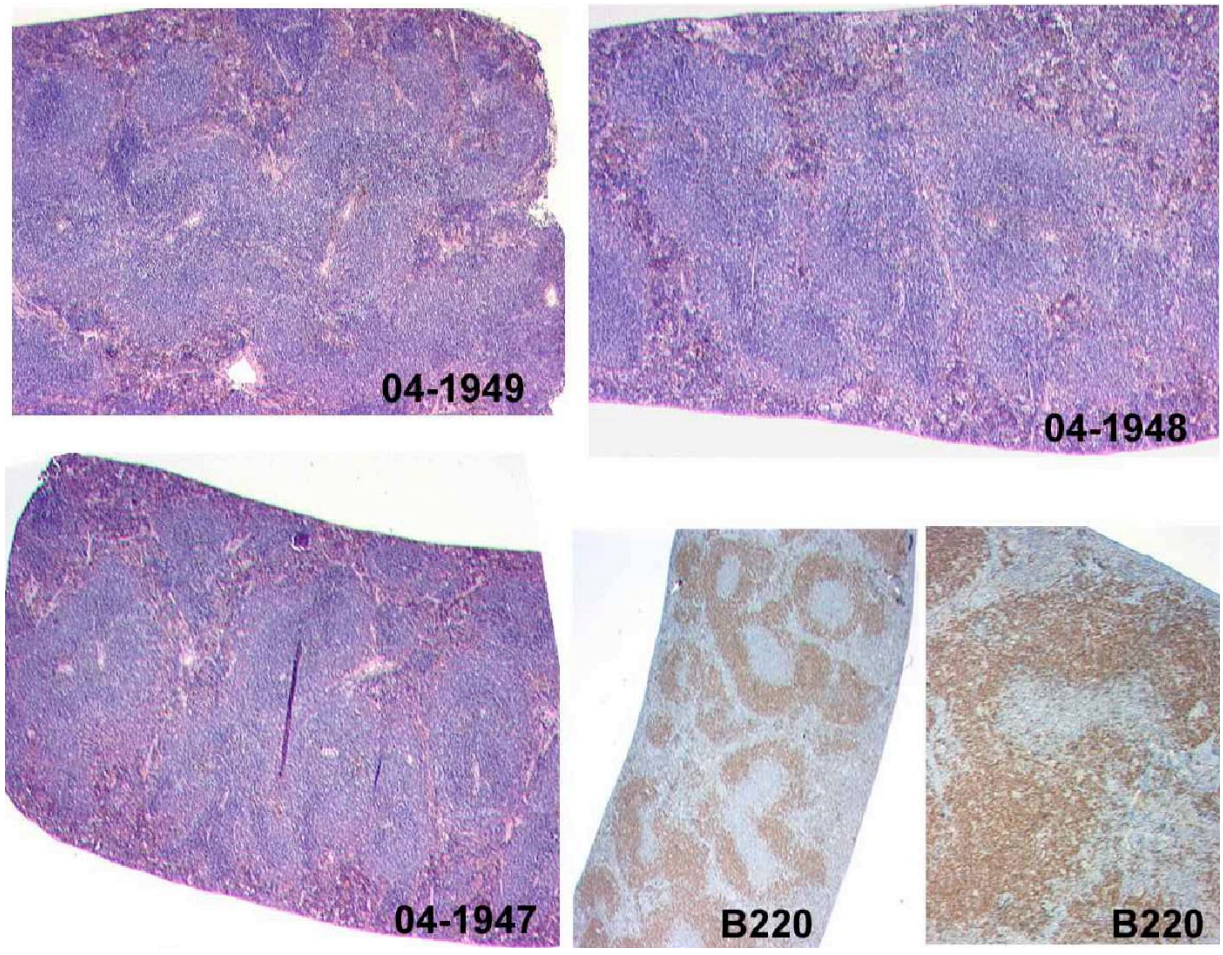


Figure 54a: Immunohistopathology of pLCKCASV-40 transgenic and control mice. The pictures show haematoxylin-eosin staining and immunohystochemistry for B-cells (B220) in the spleen. The staining shows hyperplasia of the white pulp (04-1947 to 1949) and an increase in B cell number (B220).

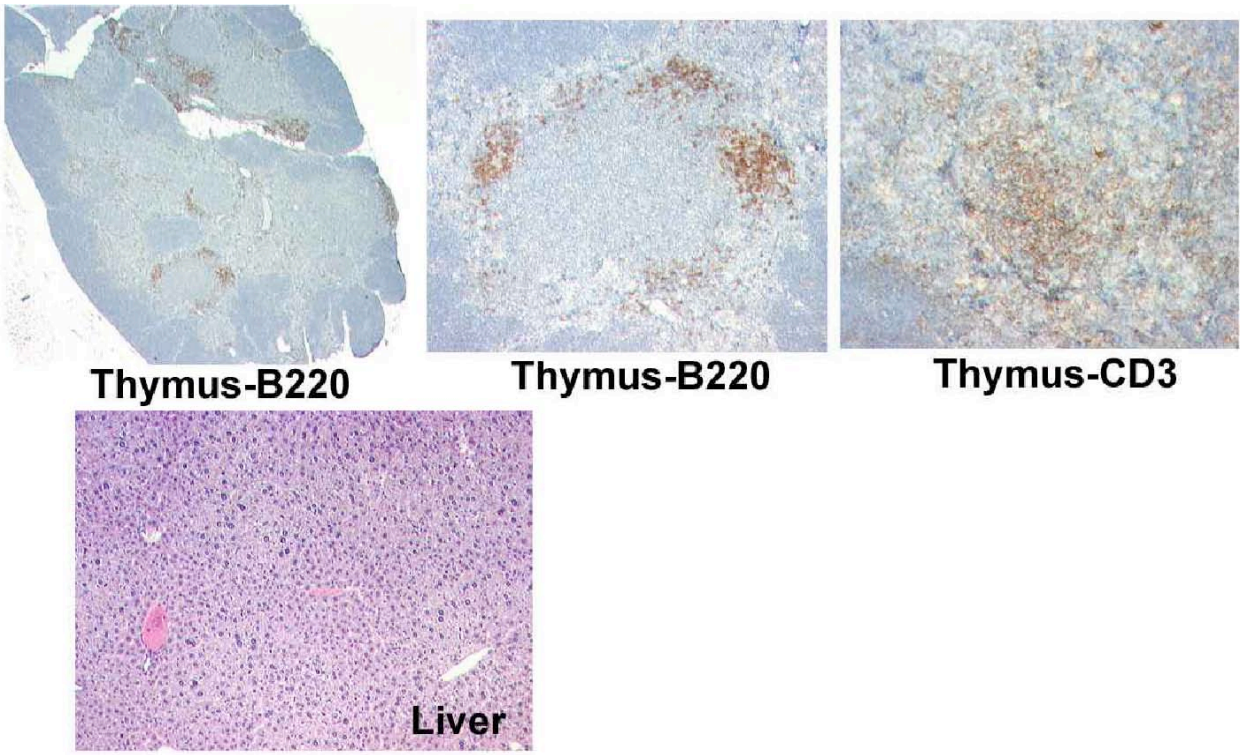


Figure 54b: Immunohistopathology of pLCKCASV-40 transgenic mice. The pictures show immunohystochemistry of thymus for B-cells (B220) and T-cells (CD3) demonstrating the relationship of T and B cells in this organ. The liver shows hyperplasia of hepatocytes around the central vein.

5.3. Analysis of the *CALM/AF10* fusion in patient samples

In order to better understand the importance of the t(10;11)(p13;q14) in leukemogenesis, we analyzed patient samples with a t(10;11) diagnosed by cytogenetics and molecular cytogenetics (fluorescence in situ hybridization, FISH) for the presence of CALM/AF10 transcripts by RT-PCR.

We obtained bone marrow cells or RNA samples from five patients, three with acute myeloid leukemia (AML) and two with T-cell acute lymphoid leukemia (T-ALL) from Dr. Cristina Mecucci, Perugia, Italy. cDNA from seven patients with AML, ALL or acute undifferentiated leukemia (AUL) with translocation t(10;11) were obtained from the Laboratory for Leukemia Diagnostics, Grosshadern. It was reported that the *CALM/AF10* fusion gene is the most common fusion gene among patients with T-ALL with T cell receptor □□ rearrangement (Asnafi *et al.,* 2003). To confirm these results, we tested bone marrow cell samples from twenty-nine T-ALL patients with TCR-□□ rearrangement kindly provided from Prof. Dr. Ludwig from the Immunologic Marker Laboratory, Charité University Clinic, Berlin. The samples from one patient were obtained from Prof. Dr. Borkhardt, Kinderklinik, LMU. A total of 17 CALM/AF10 positive patient samples were identified.

5.3.1. Analysis of *CALM/AF10* expression by RT-PCR and expression profiling of *CALM/AF10* positive patients using microarray technology

Identification of *CALM/AF10* transcripts in patient samples

The patients with a t(10;11), suggesting a *CALM/AF10* rearrangement, were tested by RT-PCR for the presence of *CALM/AF10* fusion transcripts (figures 56 to 60). FISH analysis had excluded an involvement of the MLL gene in these patients. All the patients with t(10;11) tested positive for the *CALM/AF10* transcript (figures 56 to 59 and tables 20a and b).

The CALM/AF10 fusion gene was reported to be the most common fusion gene among patients with T-ALL with T cell receptor □□ rearrangement (Asnafi *et al.,* 2003). To confirm these results, we tested a series of 29 patients with T-ALL with T-cell receptor □□ rearrangement for the presence of *CALM/AF10* transcripts and four of these (4/29; 13.79%) were positive for a *CALM/AF10* fusion transcript (figure 59; patients 10 to 13).

In seven patients it was also possible to amplify the reciprocal *AF10/CALM* fusion transcript (cases 1, 3, 4, 8, 9, 10 and 14; figure 60). The position of the primers used for the CALM/AF10 transcript identification is schematically shown in figure 55.

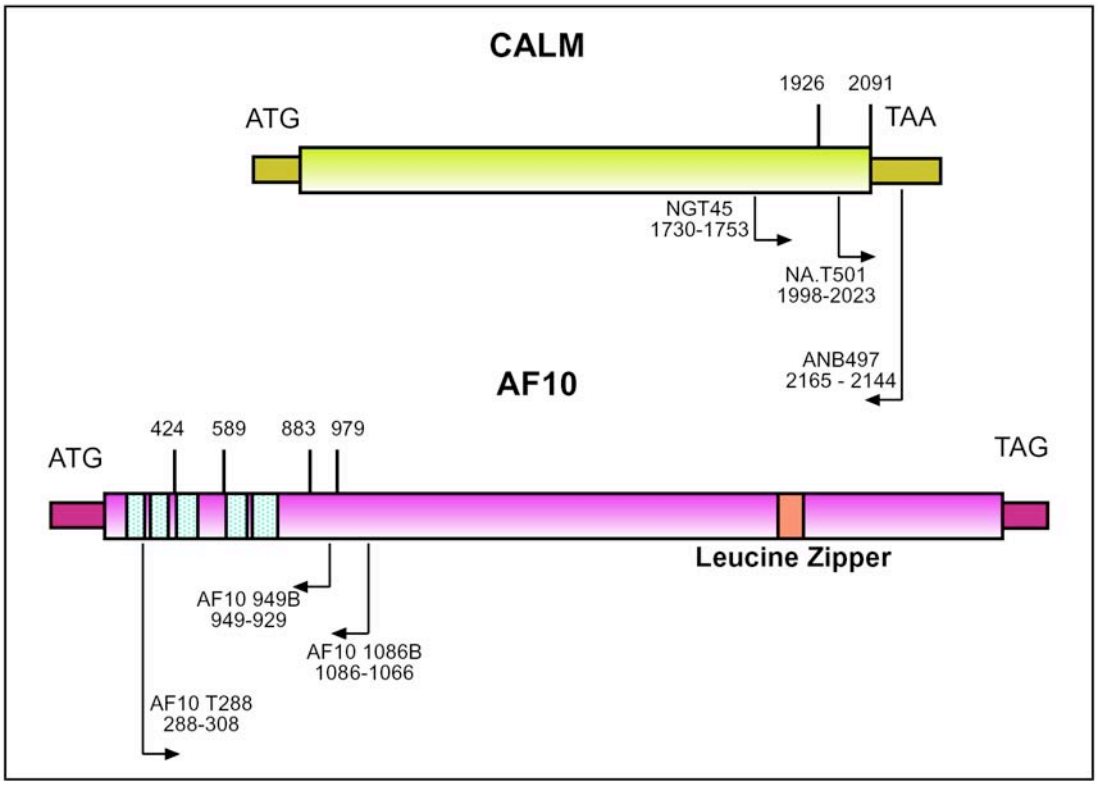


Figure 55: Diagrammatic representation of the primers used for the identification of fusion transcripts in patient cDNA in the *CALM* and *AF10* sequence.

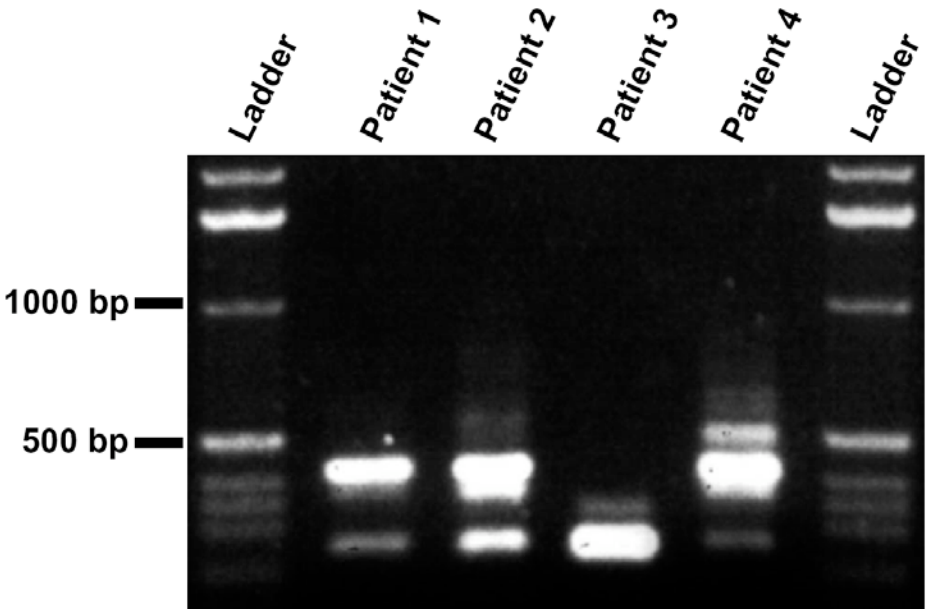


Figure 56: RT-PCR of patient samples. Primer pair used: CALM(1730-1753) and AF10(949-929). PCR conditions: 35 cycles, annealing temperature 58°C.

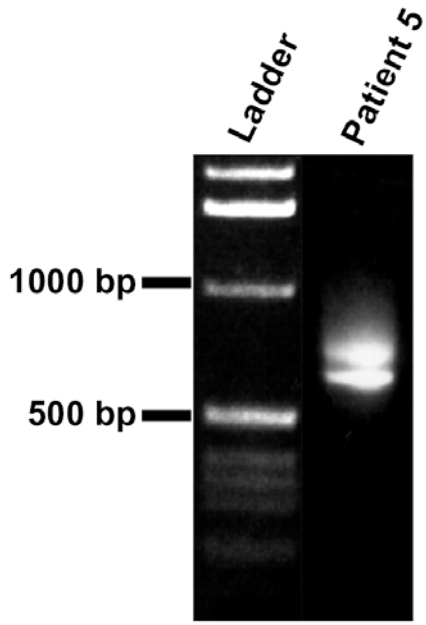


Figure 57: RT-PCR of patient sample. Primer-pair used: CALM(1998-2023) and AF10(1086-1066). PCR conditions: 35 cycles, annealing temperature 58°C.

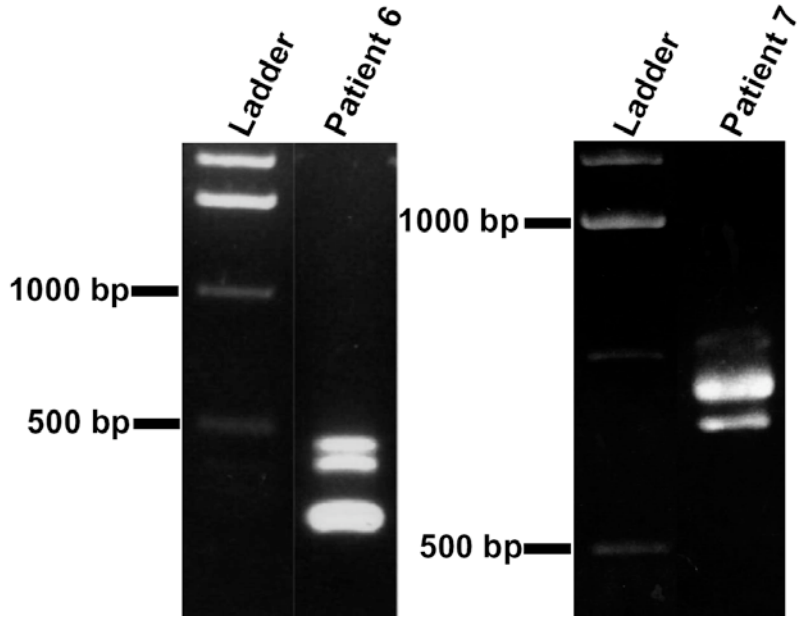


Figure 58: RT-PCR of patient samples. Primer pair used: CALM(1730-1753) and AF10(1086-1066). PCR conditions: 35 cycles, annealing temperature 58°C.

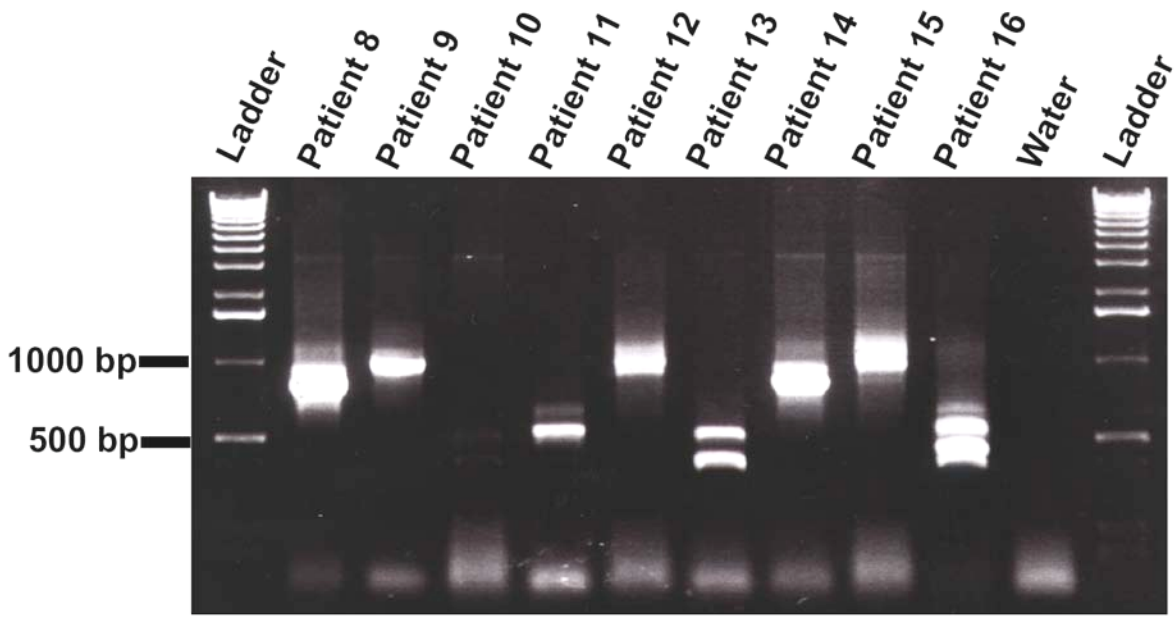


Figure 59: RT-PCR of patient samples. Primer pair used: CALM(1730-1753) and AF10(1086-1066). PCR conditions: 35 cycles, annealing temperature 58°C.

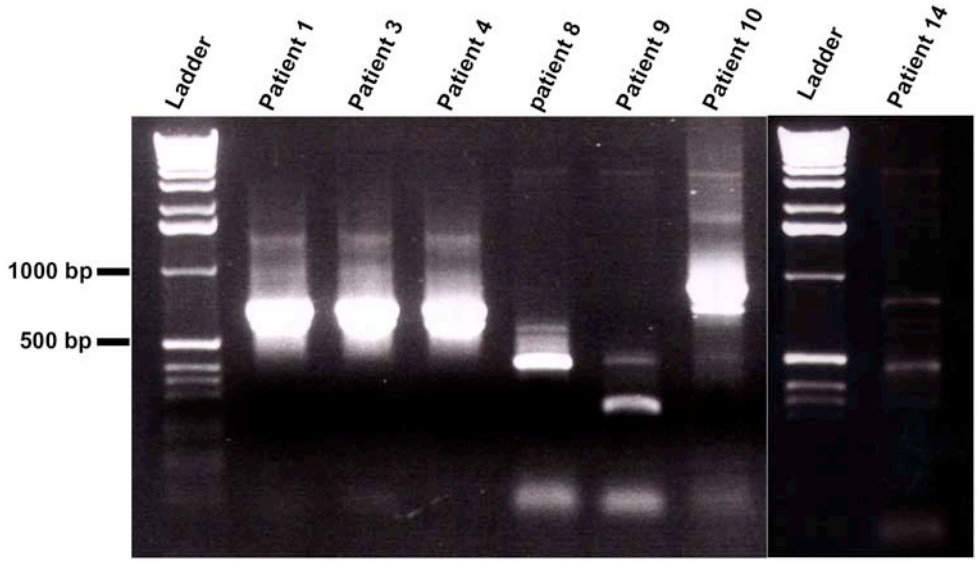


Figure 60: RT-PCR of patient samples. Primer pair used: AF10(288-308) and CALM(2165-2144). PCR conditions: 35 cycles, annealing temperature 57°C.

Sequencing of *CALM/AF10* fusion transcripts in patient samples

The RT-PCR products subcloned into the pGEMT-easy vector for sequencing. For sequencing, the same primers used for amplify of the *CALM/AF10* transcripts were used. Figures 61 and 62 show eletropherograms from the breakpoint regions in patient 5.

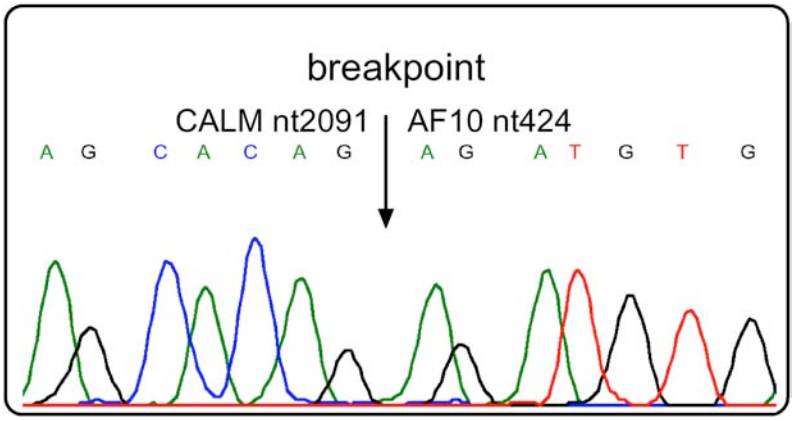


Figure 61: Eletropherogram of the sequence of the breakpoint segment of the CALM/AF10 fusion transcript amplified from the cDNA of patient 5. Sequencing primer used: CALM(1730-1750).

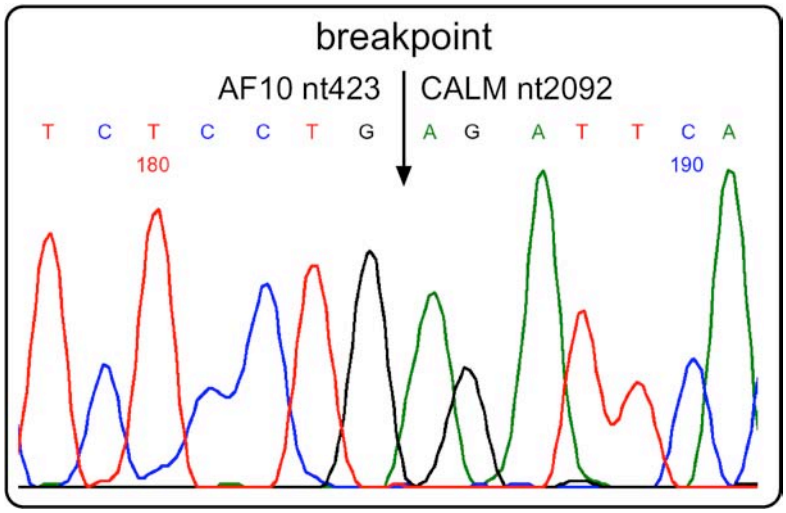


Figure 62: Eletropherogram from the sequence of the breakpoint of the AF10/CALM fusion transcript amplified from the cDNA of the patient 4. Primer used AF10(288-308).

The sequence analysis from all CALM/AF10 positive patient samples revealed three different breakpoints in CALM at nucleotide 1926, 2091 and 2064. The patient with the breakpoint at position 2064 in CALM showed a 106 base pair insertion which had not been described as a CALM exon before.

In AF10 four breakpoints were identified: at nucleotide position 424, 589, 883 and 979. There was one extra-exon in AF10 between exons 6 and 7, with 205 bases inserted 5' of nucleotide 424 of AF10. This exon contains a stop codon after 162 bases. The use of this exon would result in the translation of a truncated AF10 protein with 195 amino acids and a truncated CALM/AF10 protein with 751 amino acids. In this patient (case 16) another splice variant with the breakpoint 424 in AF10 was also seen. We did not observe any correlation between

disease phenotype and breakpoint location. Table 20a summarizes the patient data and the results observed.

Table 20a: Clinical data and sequencing results from CALM/AF10 patients

Patient No.	Sex	Type of leukemia	Age at diagnosis	Overall survival or longest follow-up (days)	Breakpoint in CALM (last nt in fusion)	Breakpoint in AF10 (first nt in fusion)
1	Female	AML M2	33	36 (dead)	1926/2091	883
2	Male	ABL	22	1067 (dead)	2091	883
3	Male	PreT-ALL	46	143 (dead)	1926	883
4	Male	AUL	25	886	2091/2064 +164bp	883
5	Male	PreT-ALL	35	650	2091	424
6	Male	ProT-ALL	33	641	1926	979
7	Female	ProT-ALL	28	275	1926	424
8	Female	AML	12	922 (at 22.06.05)	2091	424/589
9	Male	ALL	12	876 (at 22.06.05)	2091	424
10	Male	T-ALL□□	5	n.a.	1926	883
11	Male	T-ALL□□	32	n.a.	2091	883
12	Female	T-ALL□□	10	n.a.	2091	424
13	Female	T-ALL□□	5	n.a.	1926	883
14	Female	T-ALL	n.a.	n.a.	2091	589
15	Female	AML	n.a.	n.a.	1926	883
16	Female	AML	n.a.	n.a.	2091	205bp before424/424
17	Female	AML	12	n.a.	1926	883

n.a. = non acessed

From 9 of the patients we obtained the karyotype. Only three patients (1, 8 and 9) had the t(10,11)(p13;14) as the only chromosomal aberration. All the others presented additional karyotypic alterations and patients 2 and 4 had a complex aberrant karyotype (table 20b).

Table 20b: Cytogenetical data from CALM/AF10 patients

Patient	Karyotype
1	46,XX,t(10;11)(p13;q14) [20]
2	47,XY,dup(1)(q21q44),del(5)(q13q31),del(8)(p21),+del(8)(p21),der(10)t(10;11)(p12;q13),\nder(11)t(
	10;11)(p12;p13)del(11)(q13),del(12)(p11.2p13) [12]
	46,XY [3]
	nach CGH:\n47,XY,der(1)del(1)(p36.1)ins(1;1)(q23;p31.2p36.1),del(5)(q15),del(7)(q32),del(8)
3	47,XY,t(10;11)(p12;q21),+13 [2]
	46,XY [7]
4	46,XY,del(5)(q13),t(10;11)(p12;q13),der(10)t(5;10)(q23;p15)ins(10;5)(q24;q?q?) [5]
	6,XY [15]
5	46,XY,t(10;11)(p12;q13),del(12)(p11) [4]
	46,XY [5]
6	47,XY,t(10;11)(p13;q14),+21 [2]
	46,XY [18]
7	46,XX,del(5)(q22q33),t(10;11)(p13;q14) [14]
	46,XX [6]
8	46,XX,t(10;11)(p13;q14)
9	46,XY,t(10;11)(p13;q14)

The leucine zipper domain of AF10 was present in all putative CALM/AF10 fusion proteins found in the patients. We did not observe any correlation between breakpoint and diagnosis or diagnosis and the presence of the reciprocal fusion transcript *AF10/CALM*, which was found in seven patients. Figure 63 is a diagram of the fusion found in the patients and the resulting fusion proteins.

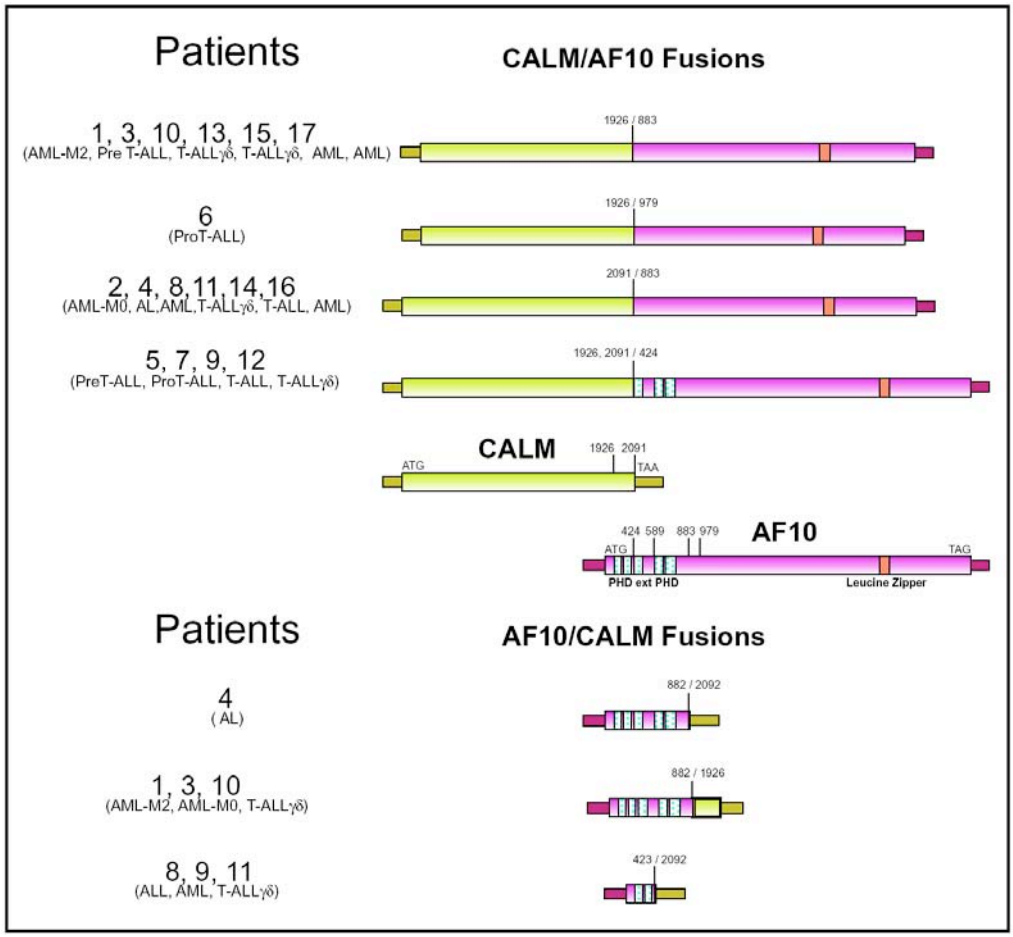


Figure 63: Putative CALM/AF10 and AF10/CALM fusion proteins derived from fusion mRNA in 17 CALM/AF10 patients. The numbers indicate breakpoint locations in nucleotides.

Gene expression analysis of CALM/AF10 patients

The gene expression profile of ten CALM/AF10 patient samples (patients 1, 2, 3, 5, 6, 7, 8, 9, 10 and 11) was analysed using oligonucleotide microarrays representing 33,000 different genes (U133 set, Affymetrix). These analyses revealed high expression levels of the polycomb group gene *BMI1*, the homeobox gene *MEIS1* and certain *HOXA* cluster genes: *HOXA4*, *HOXA5*, *HOXA7*, *HOXA9* and *HOXA10*. This expression pattern is similar to the pattern observed for patients with acute leukemia with *MLL* rearragements. Figure 64 shows a hierarchical cluster analysis of 50 adult ALL and AML samples including the 10 *CALM/AF10* positive patient samples using a subset of *HOX* genes and the TALE homeobox gene *MEIS1*. Figure 65 shows the same results plotted in three-dimensional space using the three components capturing most of the variance in the data set (principal component analysis). In this picture, the *CALM/AF10* patients cluster with patients with AML and *MLL* rearrangement. Only one of the *CALM/AF10* patients analyzed showed a slighty different expression pattern for these genes. Although the expression pattern of this patient was more similar as far as the

HOXA genes were concerned to the expression pattern found in AML-M2 patients with t(8;21), the expression of *MEIS1* was high as in the other *CALM/AF10* patients.

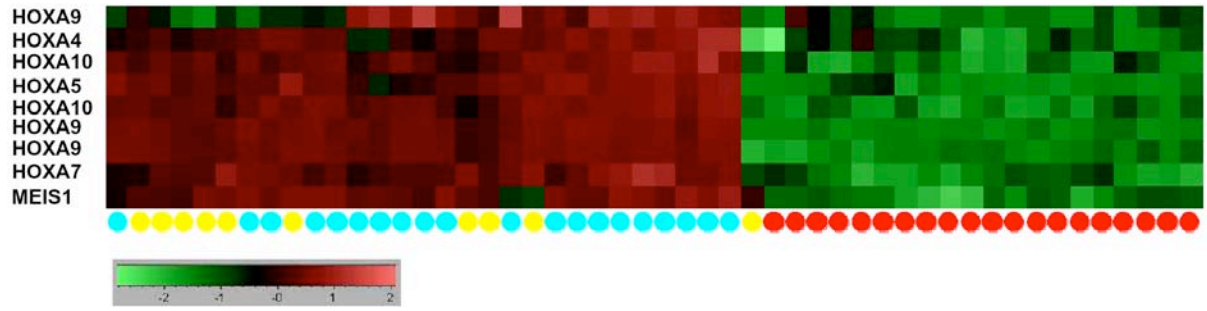


Figure 64: Hierarchical cluster analysis based on U133A microarray expression data of 50 adult ALL and AML (with MLL rearrangement, blue dots, and with AML M2 and t(8;21), red dots, columns) samples including ten *CALM/AF10* positive samples (yellow dots, columns) using a subset of genes (rows) (*Meis 1*, *HOXA 4*, 5, 7, 9 and 10). The normalized expression value for each gene is coded by color with the scale shown at the lower left (standard deviation from mean) Red cells indicate high expression and green cells indicate low expression. *MEIS1* (bottom) is highly expressed in all *CALM/AF10*-positive leukemias.

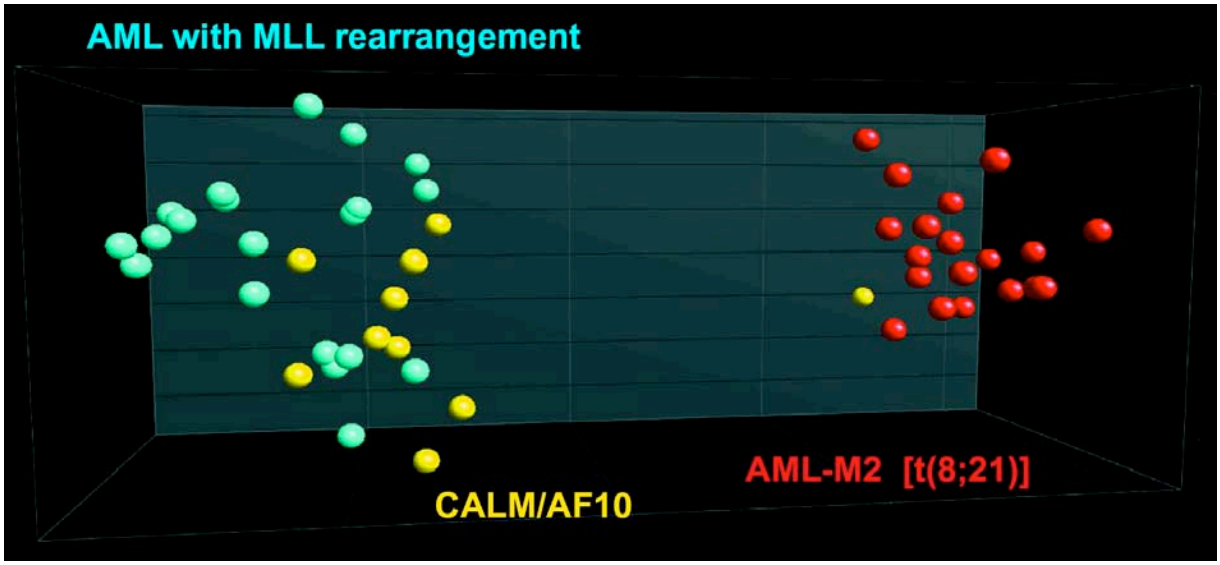


Figure 65: Principal Component Analysis (PCA) of the expression data from 50 leukemia patients including the 10 *CALM/AF10* positive patients. The data of the samples are plotted in a three-dimensional space using the three components capturing most of the variance in the data set (principal component analysis, PCA). Each patient sample is represented by a color-coded sphere. The PCA was calculated from the expression data of the following genes: *MEIS1, HOXA4, HOXA5, HOXA7, HOXA9* and *HOXA10*.

A second analysis was performed to compare the gene expression of *CALM/AF10* patients with groups patients, each of them consisting of 15 samples of patients with different leukemia diagnosis: T-ALL, ALL with *MLL* rearrangements, AML with *MLL* rearrangements, AML with inversion of the chromosome 16, AML showing normal karyotype, AML t(8;21), AML with acute promyelocytic leukemia, AML with complex abberant karyotype, and healthy bone marrow. This analysis showed that among the 100 statistically most significantly differentially expressed genes most genes (98 of 100) were expressed at a lower level in the CALM/AF10 patient samples when compared to the other groups. Table 21 shows a selection of genes which were down-regulated in *CALM/AF10* patients in comparison to the other groups.

The only consistently upregulated genes in the CALM/AF10 patient samples were *BMI1*, *DNAJC1* and *COMMD3*. Interestingly, all these genes lie immediately centromeric to the breakpoint of the t(10;11)(p21;q14) on 10p21.

Table 21: Downregulated genes in *CALM/AF10* patients compared to normal bone marrow and leukemic patients samples.

Gene	Affy id	Fold expression change	Map location	Description
RAB34	224710_at	-146.846	17q11.1	RAB34, member RAS oncogene family
TRIO	208178_x_at	-9.6969	5p15.1-p14	Triple functional domain (PTPRF interacting)
CEBPD	203973_s_at	-12.153	8p11.2-p11.1	CCAAT/enhancer binding protein (C/EBP), delta
ST7	207871_s_at	-14.214	7q31.1-q31.3	Suppression of Tumorigenicity 7
H2AFJ	225245_x_at	-5.19594	12p12	H2A histone family, member J
JAG1	216268_s_at	-23.7935	20p12.1-p11.23	jagged 1 (Alagille syndrome)
HCK	208018_s_at	-6.31031	20q11-q12	Hemopoietic cell kinase
TEM7R	227276_at	-4.18613	10p12.1	Tumor endothelial marker 7-related precursor
CDH15	206327_s_at	-3.01741	16q24.3	Cadherin 15, M-cadherin (myotubule)
CTSZ	210042_s_at	-6.5899	20q13	Cathepsin Z
CTNNA1	200765_x_at	-4.6203	5q31	Catenin (cadherin-associated protein), alpha 1, 102kDa
TKR2	224301_x_at	-6.689412	4q32	Toll-like receptor 2
HOXB4	204924_at	-9.855904	17q21-q22	Homeo box B 4
USP3	231767_at	-3.558808	15q22.3	Ubiquitin specific protease 3
ZNF185	203585_at	-3.74636	Xq28	Zink finger protein 185 (LIM domain)

5.4. Inducible expression of *CALM/AF10* in cell lines

In order to observe identifiy direct target genes of *CALM/AF10,* we conditionally expressed *CALM/AF10* using a tetracycline-regulatable gene expression system, which allows the controlled expression of *CALM/AF10* by adding docycycline to the cell culture medium. This system allows a kinetic study of the expression of *CALM/AF10* and its target genes in the cell. The objective was also to establish an *in vitro* system where we could experimentally confirm the results obtained from the RNA expression analysis of the patients.

Cloning

In order to achieve inducible expression of the CALM/AF10 fusion protein, we cloned the *CALM/AF10* coding sequence into the pRTS-1 vector designed by Bornkamm *et al* (2005). This is a doxycycline-regulatable vector with a unique combination of features. It carries all the elements for conditional gene expression including the gene of interest (*CALM/AF10*) on one EBV-derived episomally replicating plasmid. The vector is characterized by its low background activity in the absence of doxycycline, its high inducibility in the presence of doxycycline, and its graded response to increasing doxycycline. The chicken beta actin promoter and an element of the murine immunoglobulin heavy chain intron enhancer drive constitutive expression of a bicistronic expression cassette that encodes the optimized, highly doxycycline-sensitive reverse transactivator-VP16 fusion protein (rtTA2s-M2) (referred to as the reverse tet-transactivator) and a tetracycline repressor-KRAB fusion protein (referred ot as tet-silencer) placed behind an internal ribosomal entry site. *CALM/AF10* is expressed from a bidirectional doxycycline regulatable promoter that allows simultaneous expression of a second gene (GFP), used as a marker for the *CALM/AF10* expression. Tight regulation of the gene of interest is achieved by binding of the repressor to the doxycycline-regulated bi-directional promoter in the absence of doxycycline, and combined relief of repression and binding of the reverse transactivator in the presence of doxycycline. To facilitate the cloning of the 5.2 kbp *CALM/AF10* fusion cDNA into this vector, it was first inserted into a shuttle vector (pUC19Sfi1), a derivative of pUC19 modified by Rob Chapman (GSF). This vector has a multiple cloning site which is flanked by two *Sfi1* sites which are compatible with the *Sfi1* sites which flank the luciferase gene in the pRTS-1vector. The *CALM/AF10* coding sequence was inserted into this vector using the *Xho1* at the 5' and *Xba1* at the 3' side. The *CALM/AF10* coding fragment was then released by *Sfi1* digestion from pUC19Sfi1 *CALM/AF10* and used to replace the luciferase gene in pRTS-1 which had been removed by *Sfi1* digestion from this vector. The resulting plasmid was named pRTS-1-*CALM/AF10* and is schematically shown in figure 66.

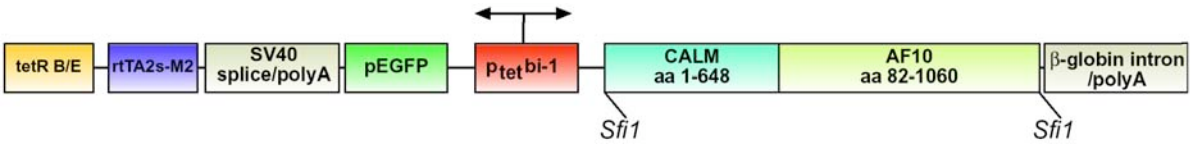
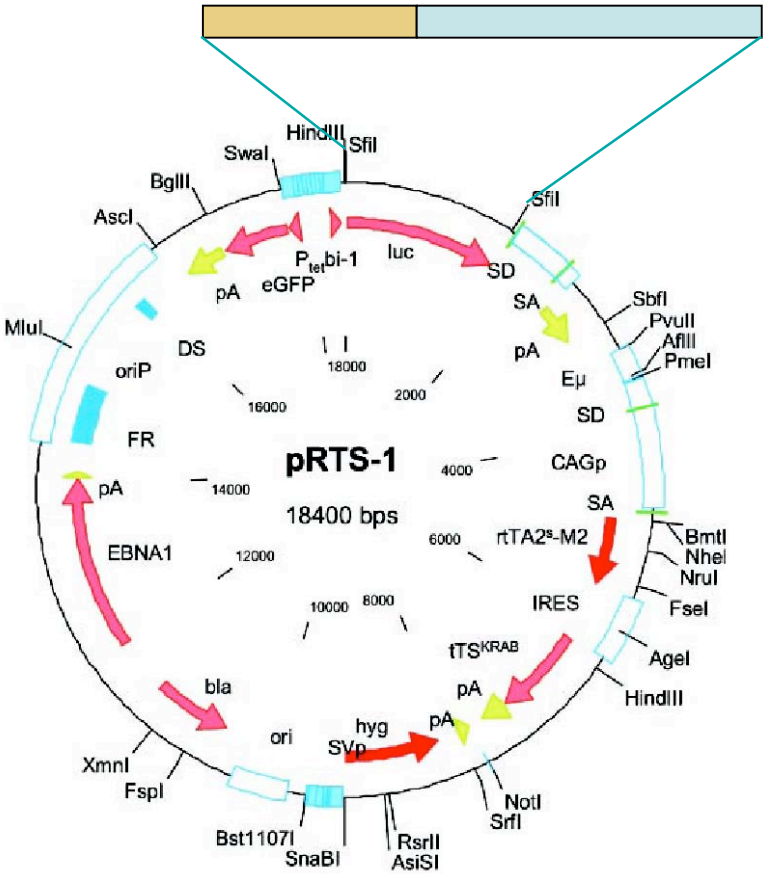


Figure 66: Schematically representation of the construct pRTS-1-CALM/AF10.



Stable transfection of mammalian cells

Endo-free plasmid DNA preparations of the pRTS-1-CALM/AF10 construct and of the empty vector pRTS-1 were used to transfect mouse NIH3T3 fibroblast cells. For 10^6 cells (80% confluent in a 100 mm cell culture dish) 3 μg of DNA was used. A control transfection using the pEGFP vector was carried out and showed about 70% GFP-positive cells 22 h after transfection. To select for stably transfected cell, 1 μg/ml of puromycin was added to the medium 48 h after transfection. 24 h after the addition of puromycin about 80% of the cells transfected with pEGFP had detached from the plate. In the plates containing pRTS-1-CALM/AF10-transfected cells and cells transfected with the pRTS-1, about 40% of the cells died. 48 h after the addition of puromycin to the medium, all pEGFP-transfected cells were dead. The plates containing the pRTS-1 and pRTS-1-CALM/AF10 transfected cells were 80%

confluent. After two weeks of puromycin selection, the expression of *CALM/AF10* and GFP (for pRTS-1-CALM/AF10 cells) or the expression of GFP and luciferase (for pRTS-1 cells) was induced using 1 μg of doxycycilin/ml of DMEM. 24 h after doxycyclin induction, FACS analysis of the pRTS-1-CALM/AF10- transfected cells showed 27.54% GFP positive cells. Figure 67 illustrates the cells transfected with pRTS-1-CALM/AF10 (without and with doxycyclin induction after 24 h).

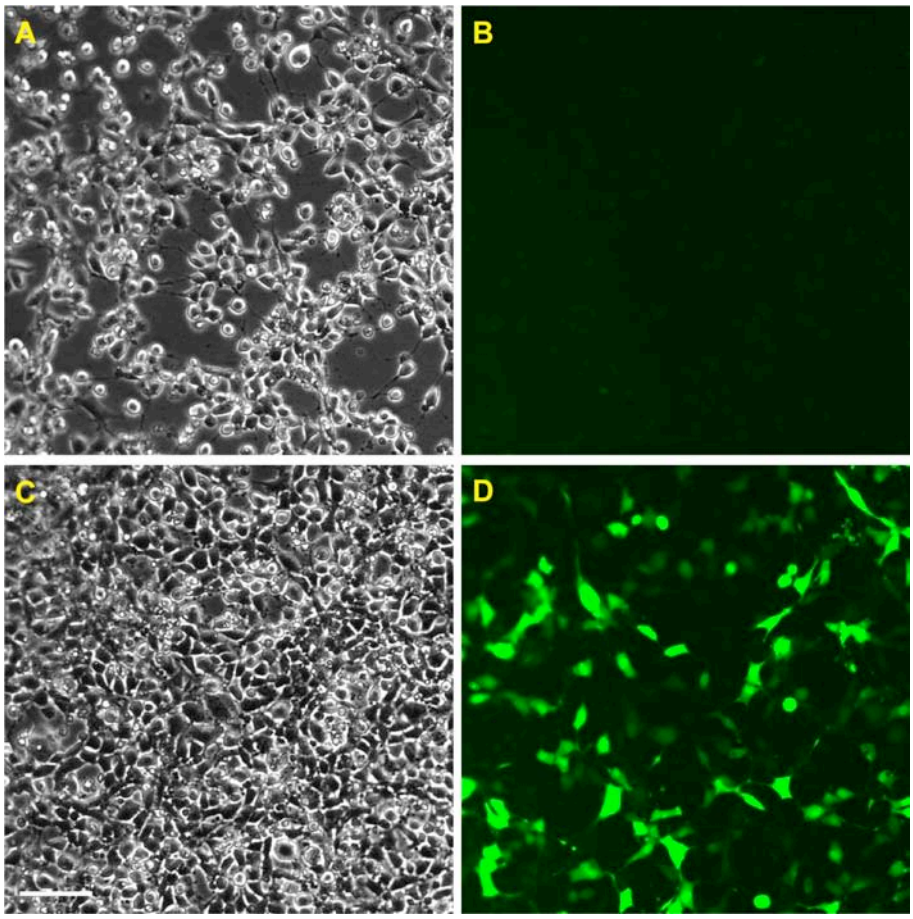


Figure 67: Inducible expression of CALM/AF10 in NIH3T3 cells stably transfected with pRTS-1-CALM/AF10: A, without doxycyclin (phase contrast), B without doxycyclin (GFP channel); C, with doxycyclin (phase contrast) and D, with doxycyclin (GFP channel) after 24 hours. Scale = 50 □m

CALM/AF10 Expression Analysis of Induced Cells

Expression Analysis at Transcriptional Level

The RNA of cells transfected with pRTS-1-CALM/AF10 cells was extracted and 200 ng were used for cDNA synthesis. The cDNA quality was assessed by RT-PRC using primers for mouse Gapdh. In order to exclude the possibility of genomic DNA contamination, we treated

the RNA preparation with desoxyribonuclease I before cDNA synthesis. Figure 68 illustrates the Gapdh PCR and the control for genomic DNA contamination. Figure 69 shows the expression of *CALM/AF10* in cells stably transfected with the pRTS-1-CALM/AF10 construct at different time points after doxycyclin induction. The DNase-treated RNA preparation without addition of reverse transcriptase was used as negative control for the RT-PCR reaction and is labeled "–". The "+" symbol indicates RNA with reverse transcriptase.

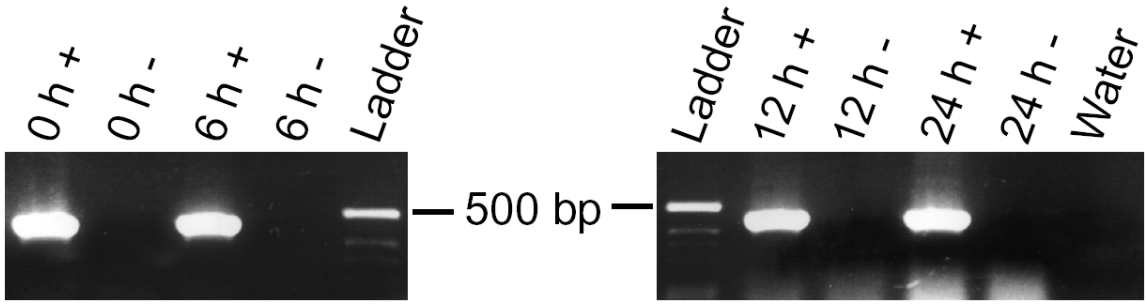


Figure 68: RNA quality control: RT-PCR of NIH3T3 cells stably transfected with pRTS-1-CALM/AF10 before (0 h+) and after (6 h+, 12 h+ and 24 h+) doxycyclin induction. Primer pair used: mouse Gapdh. PCR conditions: 30 cycles, annealing temperature 60°C. "+" with reverse transcriptase, "-" without reverse transcriptase.

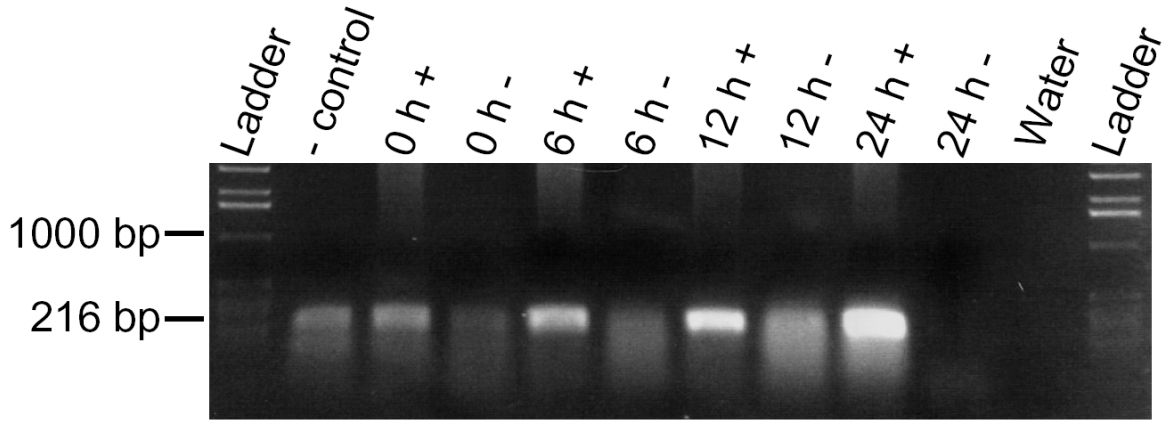


Figure 69: Time course of *CALM/AF10* fusion transcript induction. RT-PCR of NIH3T3 cells stably-transfected with pRTS-1-CALM/AF10 before (0 h+) and after (6 h+, 12 h+ and 24 h+) doxycyclin induction. Negative control: non-transfected NIH3T3 cells. Primer pair used: CALM(1900-1920), AF10(448-424). PCR conditions: 30 cycles, annealing temperature 56°C. "+" with reverse transcriptase, "-" without reverse transcriptase.

Expression Analysis at Protein Level

In order to verify the expression of the CALM/AF10 protein induced by addition of doxycyclin to the medium, we transiently transfected HEK293T cells with the pRTS-1-CALM/AF10 plasmid and performed Western analysis with the cell extracts 48 hours after transfection and 24 hours after dox addition to the cell culture medium. For that, 40 g of protein extracts were loaded on a 6% SDS PAGE gel and a Western analysis was performed to observe the presence of the CALM/AF10 protein in the induced cells. Figure 70 shows the presence of a low amount of CALM/AF10 only in the induced cells transiently transfected with pRTS-1-CALM/AF10.

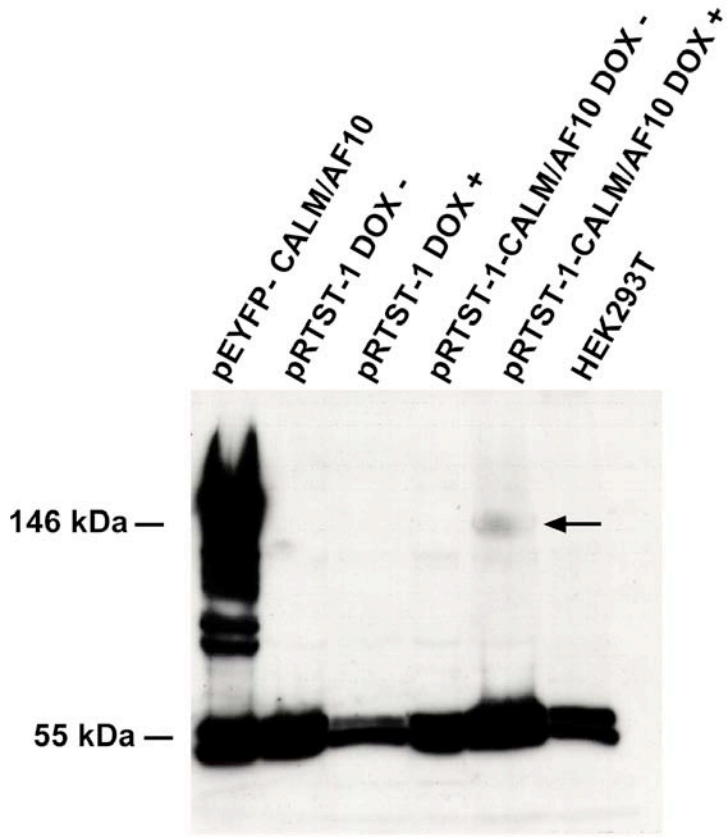


Figure 70: Western blot of specific CALM/AF10 protein expression 24h after addition of doxycyclin to the medium (arrow).

6. DISCUSSION

6.1. Cell biology

6.1.2. Sequencing of *CALM/AF10*

We completely sequenced the *CALM/AF10* expression cassette that was used in our experiments and observed that the *CALM* sequence was formed by exons 1 to 17 and 19 to 20, with an open reading frame region of 652 amino acids. This sequence and the breakpoint location correspond to the original sequence described for *CALM* in the U937 cell line (Dreyling *et al*., 1996). The breakpoint in *AF10* corresponded also to the published breakpoint in U937 and was located at nucleotide 424. AF10 was composed of exons 8, 10 to 17 and 21 to 30. In addition, we identified 123 extra nucleotides inserted between exons 28 and 29 of *AF10*, adding up to an open reading frame of 2932 nucleotides. These extra nucleotides were in frame with the *AF10* open reading frame and did not contain a stop codon. As a result our sequenced construct has a total open reading frame of 4875 nt coding for a 1625 amino acid CALM/AF10 protein. With the exception of the amino terminal PHD domain of AF10 (Linder *et al.,* 2000), all the important protein domains described for CALM (Bohlander, 2000; Ford *et al*., 2001; Legendre-Guillemin *et al*., 2004) and AF10 (Linder *et al*., 2000; DiMartino *et al*., 2002) were present in the construc used in this project.

6.1.2. Sub-cellular localization and co-localization of CALM/AF10, CALM and AF10

In the sub-cellular localization experiments, we were able to observe the CALM and CALM/AF10 proteins tagged with fluorescent protein mostly in the cytoplasm, and AF10 was found in the nucleus, in a speckled pattern. Some of the CALM/AF10 protein could also be seen in the nucleus. This relatively small fraction of CALM/AF10 protein could potentially interact with the native AF10. AF10-AF10 interaction can occur through the CCR or extended PHD finger domain, which is present in the wild type AF10 and is also retained in the AF10 part of the CALM/AF10 fusion. This motif, probably responsible for the oligomerization of AF10 (Linder *et al*., 2000) would allow the binding between CALM/AF10 and the wild type AF10 in the nucleus. Considering this nuclear interaction between CALM/AF10 and AF10, one could speculate that this binding could interfere in the normal function of AF10.

Interestingly, Vecchi and colleagues (2001) showed that CALM shuttles between cytoplasm and nucleus. They performed a GAL4-based transactivation assay and demonstrated that CALM modulated transcription and hypothesised that, as endocytic proteins contain multiple protein-protein interaction sites, they could act structurally as scaffold for the nuclear localization of transcription factors. Under normal conditions, CALM is very quickly exported to the cytoplasm, but the nuclear localization of CALM/AF10 could also interfere in possible nuclear functions of CALM.

Expression of the CALM/AF10 protein

Western analysis performed to detect the CALM/AF10 protein in transiently transfected HEK293T cells (with the constructs pEYFP-CALM/AF10, pEYFP-CALM and pECFP-AF10) was able to demonstrate the presence of CALM/AF10, CALM and AF10 proteins fused to the fluorescent proteins. pEYFP-CALM and pEYFP-CALM/AF10 were detected using anti-CALM antibodies and anti-GFP antibodies; pECFP-AF10 was detected using anti-GFP antibodies. These results are important to confirm the expression of the CALM/AF10 protein from our construct, which was used for transgenic mice production. The bands obtained on the Western analysis for YFP-CALM and CFP-AF10 were of the predicted sizes of 99 kDa and 127 kDa, respectively (Tebar *et al.*, 1999; Chaplin *et al.*, 1995). For YFP-CALM/AF10, the predicted size is about 200 kDa. The smaller size of approximately 160 kDa obtained for CALM/AF10 in our Western analysis (figures 15 and 16) could be due to protein degradation. When longer exposure times were used, we observed a smear pattern in the lane with the CALM/AF10 protein, probably due to degradation. Kim and Kim (2001) predicted five potential caspase 3 cleavage sites in CALM. This target sites for caspase 3 in CALM could explain the differences between predicted sizes and Western results for the CALM/AF10 protein and could also explain the 'smear' pattern observed on longer exposures.

6.2. Transgenic mice

6.2.1. FVB mice expressing *CALM/AF10* under control of a *IgH* enhancer/promoter

Generation of transgenic lines

We obtained two transgenic mouse lines which expressed *CALM/AF10* under the control of the *IgH* enhancer/promoter. Considering that the off-spring of transgenic crossed with wild type animals were approximately 50% transgenic, it was expected that the transgene had only one integration site in these lines (Gannon *et al.,* 1990). Southern analysis confirmed this assessment. As shown in the figure 21a we can observe two bands in line 1: one strong band with an approximate size of 8 kb and a second band of about 12 kb. In line 2, we can observe a fragment of 8 kb (as observed in line 1) and a second fragment of about 10 kb. It is very likely that this common 8 kb fragment is due to multiple tandem integrations of the transgene (transgene array) at the same genomic locus, because the 8 kb length corresponds to the length of the full construct. The occurrence of integration of several copies of the injected DNA, arrayed in a head-to-tail manner, at random sites in the mouse genome has been reported as a common phenomenon (Hamada *e al.,* 1993; Schneider and Wolf, 2005). Because the restriction enzyme used for genomic DNA digestion in this Southern analysis (*EcoR1*) has only one recognition site in the pIGCASV-40 construct, the product of digestion of various copies integrated in an array gives products with the length of the transgene construct (8 kb). This fragment is detected as a strong band corresponding to the number of integrated copies. The second band corresponds to the line specific flanking fragment obtained by the digestion

of the construct and the adjacent *Eco R1* restriction site in the chromosome. These line-specific bands have different sizes of approximately 12 kb for the line 1 and of approximately 10 kb for the line 2, indicating the occurrence of the integration of the transgene cassette in different locations of the genome.

Analysis of transgene expression by RT-PCR

The results obtained for the expression of *CALM/AF10* in the different organs have to be looked critically. The presence of blood cells in non hematopoietic organs could lead to false-positive *CALM/AF10* results in organ tissue. Sorting of blood cell subpopulations allowed us to assess *CALM/AF10* expression specifically in these cells. These results lead us to conclude that the expression of *CALM/AF10* in both transgenic lines was specific and restricted to the hematopoetic lineages.

In mice from line 1, the expression of CALM/AF10 was present in bone marrow immature c-Kit negative /Sca-1 positive cell fraction and in Gr-1/Mac-1 double positive cells. The former cell population can contain immature common myeloid progenitors (CMPs) (Akashi *et al.*, 2000), in which lymphocyte differentiation is inhibited by the expression of Sca-1 (Bradfute *et al.*, 2005). The Gr-1/Mac-1 cells are monocyte (macrophage) or granulocyte precursors, in which the *IgH* enhander is not active (Harris *et al.*, 1988; Eckhardt, 1992).

The *CALM/AF10* transcript was not detected in the very early stem cell population c-Kit/Sca-1 double positive cell fraction. This can be explained by the fact that the *IgH* enhancer/promoter is not active in these cells (Karasuyama *et al.*, 1990; Tsubata *et al.*, 1990).

The B220+ and B220- splenocytes also expressed *CALM/AF10.* In the B220- fraction, *CALM/AF10* could be present due to the very early cells, which do not present B220 in the cell surface or present at very low levels. These cells are the earliest lymphocyte progenitors (ELP) fraction in which the *IgH* enhancer is already active and these cells start to undergo D_H-J_H rearrangements at the *IgH* locus (Koyama *et al.,* 1997; Hardy *et al.*, 2000; Busslinger, 2004).

In mice from line 2 CALM/AF10 expression was observed only in B220 positive and in B220 negative splenocytes indicating a very specific expression of the *CALM/AF10* transgene. This is in line with the expression data reported for the transgene expression under the control of the IgH enhancer promoter in the literature (Harris *et al.*, 1988; Eckhardt, 1992).

Analysis of transgene expression by Western blot

The fact that we were unable to detect the CALM/AF10 fusion protein in the transgenic mice could be due to a low level of CALM/AF10 protein expression. This is in line with our attempts to detect the fusion protein in the U937 cell line, which has the CALM/AF10 fusion, and in the leukemic cell lines from CALM/AF10 transduced bone marrow cells, suggesting that a very low CALM/AF10 expression is sufficient to cause leukemia.

Another explanation for the failure to detecting the CALM/AF10 fusion protein in our model could be that the CALM/AF10 transcript is degraded by teh nonsense-mediated mRNA decay (NMD) pathway. NMD is a "quality control mechanism" which leads to the degradation of mRNAs that harbour premature translational stop codons. This mechanism prevents the synthesis of potentially harmful truncated proteins. NMD involves proteins described to bind to regions present in SV-40 mRNA which are part of our trangenes (Lejeune et al., 2002).

Hemogram

The transgenic animals were observed for a period of over 16 months without showing any alteration related to the hematopoietic system in comparison to the control animals. To analyse more closely the hematopoietic system, we performed total and relative WBC count and RBC count. The total WBC counts (3.44 $\pm$ 1.22 x 10^6/µl leukocytes for transgenic animals and 3.59 $\pm$ 1.09 x 10^6/µl for controls) were markedly lower than those reported for adult FV/B mice (5.6 $\pm$ 1.37 for males and 5.73 $\pm$ 0.79 for females) (www.jax.org). Since there were no differences between transgenic and control mice in the WBC count, we concluded that the differences of our values compared to the values reported in the literature are due to the presence of platelet aggregates observed in almost all blood slides analyzed. Jain (1993) mentions that the presence of blood clots are an important source for errors in the total WBC count. Because of these technical limitation, we also evaluated the WBC differential count. The values obtained did not differ significantly between transgenic and control animals and were similar to the values reported for the FVB/N strain (www.jax.org). The blood cell morphology was also assessed and no difference was observed between transgenic and control animals.

Mice with *N*-ethyl-*N*-nitrosourea (ENU)-induced mutations at the *Picalm* locus displayed a severe phenotype, with a shortened life span, growth retard *in utero*, iron metabolism deficiency, severe anemia and B-cell deficiency (Potter *et al.*, 1997; Klebig *et al.*, 2003). None of our transgenic lines showed such a phenotype.

Analysis of the phenotype at the immunological level

Serum immunoglobulin levels in transgenic mice expressing *CALM/AF10* under the control of the *IgH* enhancer/promoter were determined with the objective to assess the function of the B-cells in these animals (Yu *et al.*, 2004). As no difference was observed between transgenic and control mice in the immunoglobulin levels, we concluded that the *CALM/AF10* transgene expression in B-cells of the transgenic mice did not impair the immunoglobulin production in these animals. These results are in line with the observation that the transgenic animals remained clinically healthy for the whole observation period of over 15 months.

Flow cytometry

Flow cytometry of the WBCs from blood, bone marrow and spleen was performed to assess the hematopoietic development in the transgenic animals. As no significant differences were observed between progenitors and differentiated cells from the myeloid and lymphoid compartments between transgenic and wild type mice, we concluded that the expression of the *CALM/AF10* transgene in the cells of the B-lineage was not able to induce any obvious pathological or functional alteration.

Analysis of the phenotype at pathological level

Transgenic mice which expressed *CALM/AF10* under the control of the *IgH* enhancer/promoter were sent for pathological analysis at two different time points. At the time of the first analysis, the mice had been housed in a non-SPF animal facility and had positive serology for the mouse hepatitis virus. In these animals, the histopathology detected plasma cell infiltration in the lamina propria of the small intestines and focal infiltration of plasma cells in the kidney (line 2 only). The regenerative lesions seen in the liver tissue could be consequence of viral infection (Quintanilla-Fend, personal communication). The plasma cell infiltration in the intestine and the germinal center hyperplasia of mesenterial lymph node, accompanied by hyperplasia of the paracortical area could be also a consequence of local infectious processes. Curiously, both control animals did not show any related pathological alteration.

The transgenic mice from these two lines were transferred to animal facilities with SPF conditions via embryo transfer and, after 13 months were again analysed by histopathology. In these animals, there was no histopathological alteration related to the finding reported in the first mice sent to analysis. The histopathology findings did not diferred between transgenic and control animals, except for the lung altherations. Two transgenic animals, which were sent to analysis because the presented with respiratory distress, hyperpneia and cyanosis, had lung tumors. However, Mahler and colleagues (1996) described a high incidence of lung tumors (14% of males and 26% of females) in 14 month old FVB/N mice. 24 month old FVB/N mice had tumor rates ranging from 55% in females to 66% in males. The authors suggested that the high incidence of lung tumors in FVB/N mice should be considered when analyzing the phenotypes of transgenic FVB/N mice.

We tested *CALM/AF10* transgene expression in lung tissue by RT-PCR. The very low expression of CALM/AF10 found in the mice from the pIGCASV-40 line 1 could be the result of the presence of blood cells in the lung. The *CALM/AF10* transcript could not be detected in the lung tissue of pIGCASV-40 line 2. This findings support the argument that the occurrence of lung tumors in these mice is an event not related to the expression of the *CALM/AF10* transgene.

6.2.2. FVB mice expressing *CALM/AF10* under control of the *Lck* proximal promoter

Generation of transgenic lines

We obtained three lines of transgenic mice expressing *CALM/AF10* under the control of the *LCK* proximal promoter. The transgene transmission to the offspring was close to 50% in these three lines, leading us to conclude together with the results from the Southern analysis, that only one integration site was present in each line. The Southern analysis in LCK lines 1 and 2 showed that there was a single integration of multiple transgene copies. The enzyme *EcoR1* has two recognition sites in the transgene cassette, which are situated 5 prime relative to the hybridization probe. The product of the restriction enzyme digestion of the construct is a DNA fragment of 6 kb, the size of the stronger band detected on the Southern analysis. As discussed for the results of the pIGCASV-40 lines, the occurrence of higher molecular weight bands which were different between the lines (about 7 kb for line 1 and about 8 kb for line 2) corresponds to the line-specific bands. Although the *CALM*/AF10 transgene was detected by PCR from the tail DNA of mice from line 3, and the transgene transcript was also detected in different cells, the results of the Southern blot analysis for this line were negative. The probe used for the Southern analysis corresponds to the SV-40 region of the transgene construct. Our hypothesis is that the transgene is possibly truncated in this region.

Analysis of transgene expression by RT-PCR

We tested different organs and cells for the expression of *CALM/AF10* in the pLCKCASV-40 mice with emphasis on the expression in T-cells. The *Lck* proximal promoter is reported to be active in very early thymocytes (Brenner *et al.*, 2001). The expression of the Lck proximal promoter driven transgene was specific for hematopoietic organs. No *CALM/AF10* transcript was detected in lung or brain from any of the three lines. The thymus was the organ which showed expression in all transgenic lines. In order to confirm the specificity, we tested the following sub-populations: from the bone marrow Gr-1+/Mac-1+ cells, from the spleen B220+ cells, early CD4+/CD8+ T-cell progenitors, CD4+/CD8- T-helper cells, CD4-/CD8+ cytotoxic T-cells and very early CD4-/CD8-/CD3+ T cell progenitors. Unfortunately, the amount of cells obtained for this latter sub-population was not sufficient for RT-PCR analysis. Our expression results, showing *CALM/AF10* expression in CD4+/CD8+ double positive T cell progenitors confirmed the activity of the *Lck* proximal promoter in early T-cells. The expression of the transgene in B220 cells found in the line 1 agrees with results from Adler and colleagues (1998). The expression of *CALM/AF10* found only in CD8+ cells in line 3 shows a specific T-cell expression, however in mature cells. The *Lck* distal promoter is reported to be predominantly active in mature T-cells (Reynolds *et al.*, 1990).

Analysis of transgene expression by Western blot

The Western blot results for the pLCKCASV-40 transgenic mouse lines were similar as for the pIGCASV-40 mice. The reasons attributed for the non-detection of the fusion protein could be the same as for the other transgenic model.

Hemogram

The transgenic animals from the three pLCKCASV-40 lines were observed for a period of over 14 months and, like the IgH transgenic lines, did not show any clinical peculiarity. We carried out haematological analyis as described for the pIGCASV-40 mice. The values obtained for RBC count and WBC total and differential count did not differ significantly between transgenic and control mice. Considering these results, we decided to analyze the hematopoietic development by flow cytometry.

Flow cytometry

Our results for the transgenic mice expressing *CALM/AF10* under the control of the *Lck* proximal promoter were comparable to results obtained for the two pIGCASV-40 transgenic lines. There was no difference between transgenic animals and wild type control animals.

Analysis of the phenotype at pathological level

Like in the IgH-CALM/AF10 transgenic lines, there were no pathological findings. The apparent abnormal histological findings in the organs analysed could be related to the FV/B mouse strain and are probably not specifically related to the expression of the CALM/AF10 transgene.

6.2.2. General considerations about the *CALM/AF10* transgenic animals

As reported in several experiments with transgenic mice, the expression of a transgene early in embryonic development can lead to embryonic dead, making it difficult to study the fuction of the transgene in hematopoiesis (Okuda *et al*., 1996; Yergeau *et al*., 1997; Rhoades *et al*., 2000). To avoid this problem, we decide to restrict the expression of the CALM/AF10 fusion gene to immature B or T cells with the objective to generate transgenic lines which develop leukemia. There are *CALM/AF10* positive leukemias which show a more differentiated phenotype (Narita *et al.,* 1999; Carlson *et al.,* 2000; Jones *et al*., 2001; Nakamura *et al.,* 2003). On the other hand, *CALM/AF10*-associated leukemias are non-lineage specific and can be myeloid, lymphoid or biphenotypic. This observation supports the notion that the target cell for the leukemic transformation of *CALM/AF10* is a quite immature cell (Ralph *et al.,* 1983; Kumon *et al*., 1999). This would explain the inability of our constructs, in which the transgene expression is driven by either a B-cell or T-cell specific promoter, to cause leukemia.
Another relevant fact which could be associated with the absence of leukemia in mice expressing *CALM/AF10* under the control of an *IgH* enhancer/promoter might be due to a

functional interaction with the *Pax5* (Paired box gene 5) gene. In the leukemic blasts from a CALM/AF10 positive bone marrow transplant model, *Pax5*, also known as *BSAP* (B-cell-specific activator protein), is not expressed in B220 positive cells (Deshpande, personal communication). The expression of this gene is important for B-cell lineage commitment (Busslinger, 2004). In absence of *Pax5*, lymphopoiesis is blocked at the early pro-B stage, at which the cells still retain myeloid-lymphoid developmental potential (Rolink *et al*., 1999). Interestingly, Pax5 was shown to induce locus contraction of the *IgH* gene (Fuxa *et al*., 2004). In the absence of *Pax5*, a dramatic reduction of the V_H-DJ_H is reported. If the expression of *CALM/AF10* causes *Pax5* down-regulated, we would have a negative feed-back in our IgH-CALM/AF10 transgenic mice: the CALM/AF10 expression is directed by the *IgH* enhancer/promoter, which in turn is dependent on the Pax5 expression. However, we do not know whether the CALM/AF10 fusion protein is indeed able to directly or indirectly affect *Pax5* transcription.

In a mouse bone marrow transplantation model, Deshpande *et al*. (2005) transformed mouse bone marrow progenitor with a *CALM/AF10* retroviral construct and transplanted the transduced bone marrow cells into lethally irradiated mice. All transplanted animals (n=13) developed leukemia within a short period of three to four months. The leukemic cells identified in this model expressed myeloid and lymphoid markers, supporting the hypothesis of a very early target cell for the leukemic transformation by CALM/AF10. Stem cells, with their un-limited potential to self renew, have a much higher chance to accumulate enough mutations to develop leukemia (Gilliland, 2004). Curiously, some *CALM/AF10* leukemia patients cited in the literature and described in this work show the t(10;11) translocation as the sole cytogenetic chromosomal abnormality (Kumon *et al.,* 1999; Bohlander *et al*., 2000; Krause *et al.,* 2004) which indicates that the CALM/AF10 fusion might not require many other cooperating genetic events to cause leukemia. Dyrnan and colleagues (2005) generated *MLL* fusions-associated tumors in mice using Cre-*loxP* recombination *in vivo*. In this system, they used an *Lck-Cre* allele to express the *MLL/AF9* (reported in myeloid leukemia) and *MLL/ENL* (observed in myeloid and lymphoid leukemias) in T cells and their progenitors. After a period of 18 months, 90% of the *Lck-Cre –MLL/ENL* mice developed either myeloid or lymphoid tumors, whereas no *MLL/AF9* mouse developed leukemia. As the cells causing myeloid leukemia in the *Lck-Cre –MLL/ENL* mice were most likely derived from early T-cells, they assumed that the *MLL/ENL* gene targets the cells around the time of V-D-J recombinase gene expression. They confirmed this hypothesis demonstrating that the myeloid tumor cells showed recombinations at the T-cell receptor or the immunoglobulin H chain genes. They concluded that *MLL/ENL* is able to transform partially commited cells and drive it differentiation to other lineages.
Comparing these results with the results obtained by Deshpande (2005) in the *CALM/AF10* mouse transplantation model in which the tumor cells presented myeloid and lymphoid

markers, we can assume that a longer period of observation of the pLCKCASV-40 mice might be required. However, we can not rule out that the expression of the CALM/AF10 transgene is not strong enough in our transgenic lines or that the expression of the transgene occurs in cells that are already too committed to a certain cell lineage.

6.3. Analysis of CALM/AF10 expression by RT-PCR and expression profiling of CALM/AF10 positive patients using microarray technology

We were able to analyse samples from 17 *CALM*/AF10–positive leukemic patients. The patients were children or young adults, between 5 and 46 (average = 22) years old, a similar age distribution was reported in other studies for CALM/AF10 patients (Narita *et al.,* 1999; Salmon-Nguyen *et al*., 2000; Bohlander *et al.*, 2000). All leukemias were acute leukemias, undifferentiated or from the lymphoid or myeloid lineage. We did not see any obvious correlation between age, diagnosis and the location of the breakpoints in *CALM* or *AF10* in our patients.

Among a series of 29 patients with T-ALL with T-cell receptor □ rearrangement, four (14%) were found positive for *CALM/AF10* transcripts, confirming a previous report of the high incidence of *CALM/AF10* among this group of leukemias (Asnafi *et al*., 2003).

The reciprocal *AF10/CALM* transcript was identified in 7 of the 17 patients. The observation that the *AF10/CALM* transcripts can only be found in some patients has also been reported previously (Bohlander *et al.*, 2000; Carlson *et al.,* 2000), suporting the notion that CALM/AF10 is the relevant fusion protein in these leukemias.

Gene expression profiling is becoming an important tool in the analysis of leukemias (Andersson *et al.,* 2005; Haferlach *et al*., 2005). ALL with chromosomal rearrangements involving the *MLL* gene can be distinguished from ALL without *MLL* involvement. Three cytogenetically defined subtypes of AML: t(15;17), t(8;21) and inv(16) can also be recognized based on the gene expression profile (Schoch *et al*., 2002).

Among our 17 *CALM*/AF10 patients, we were able to analyze the gene expression profile for 10 of them. Hierarchical clustering analysis was performed using in addition the gene expression profile of 20 AML patients with MLL rearrangements and 20 patients with t(8;21) positive AML. The hierarchical clustering analysis was restricted to the *HOX A* cluster genes and *MEIS1*. Furthermore, the gene expression profile of the CALM/AF10 positive patients was compared to the profile of groups of 15 patients from each of 9 other leukemia subtypes: T-ALL, ALL with MLL rearrangements, AML with MLL rearrangements, AML with inversion of the chromosome 16, AML showing normal karyotype, AML t(8;21), AML with acute promyelocytic leukemia, AML with complex aberrant karyotype, as well as from 15 normal bone marrow control samples.

The hierarchical clustering revealed high expression levels of the homeobox gene *MEIS1*, and the *HOXA* cluster genes *HOXA1*, *HOXA4*, *HOXA5*, *HOXA7*, *HOXA9*, *HOXA10*. The CALM/AF10 patients had a very similar expression pattern to patients with MLL rearrangements as far as these genes were concerned. Curiously, from the ten CALM/AF10 patients analyzed, only two had a diagnosis of AML and one of an acute biphenotypic leukemia. The other seven patients had been diagnosed as ALL (1), T-ALL (2), Pro T-ALL (2) and Pre T-ALL (2). These results led to the conclusion of a common expression pattern between *CALM/AF10* patients independently of the type of leukemia (lymphoid or myeloid), considering the expression of the above-cited genes.

When looking for differentially expressed genes that were specific for CALM/AF10 leukemias, only three genes (DNAJC1, COMMD3 and BMI1) were found to be consistenly higher expressed in CALM/AF10 patients compared to all other leukemia groups analyzed. Since these three genes are located downstream (centromeric) to the 10p21 breakpoint, a position effect caused by the juxtapposition of the strong CALM promoter or enhancer in the 10;11 translocation can be assumed. This phenomenon has also been observed by Dik and colleagues, who showed overexpression of *HOXA* genes and *BMI1* and the two other genes (DNAJC1 and COMMD3) which lie in the vicinity of the breakpoint on 10p12 (Dik *et al*., 2005). They analyzed six *CALM/AF10* T-ALL patients and compared their expression profiles to 17 T-ALL *CALM/AF10* negative patients. However, they did not see higher expression levels of *HOXA7*.

The involvement of *HOX* genes in acute leukemias has been widely reported (Blatt *et al*., 1988; Kroon *et al*., 1998; Thorsteinsdottir *et al*., 2001; Ayton and Cleary, 2003; Soulier *et al*., 2005). Ayton and Cleary (2003) showed the dependence of *MLL* fusion proteins on *Hoxa9* and *Hoxa7* to transform myeloid progenitors in a mouse transplantation model. The mechanism by which *MLL* fusion genes can induce leukemia is probably related to chromatin structure. *MLL* has intrinsic histone H3, lysine 4 methyltransferase activity encoded by its SET (Su(var)3-9, Enhancer-of-zeste, Thithorax) domain and in addition recruits the SWI/SNF chromatin remodelling complex. These domains are lost in *MLL* fusions so that the chromatin-associated functions might come from the fusion partner (Zeisig *et al*., 2005). Yi Zhang's group (Okada *et al*. 2005) found an interaction between AF10 and MLL/AF10 and the human histone methyltransferase hDOT1L (disruptor of telomeric silencing 1). hDOT1L was shown to be able to transform myeloid progenitors when fused to MLL. It was also shown that the octapeptide-leucine zipper motif of AF10 is necessary and sufficient for the interaction with hDOT1L. MLL/AF10 associated myeloid transformation was accompanied by up-regulation of *Hoxa9* and *Hoxa7*. In addition, AF10 also interacts with GAS41, a protein which interacts with the SWI/SNF complex. Soulier *et al*. (2005) observed upregulation of all *HOXA* genes in T-ALL patients, suggesting that *HOXA* up-regulation as a common feature of these leukemias. The TALE (three amino acid loop extension) homeobox cofactor *Meis1* was shown to collaborate with *HOXB3* in leukemogenesis and accelerate the onset of *HOXA*-induced

leukemias as well as to reduce the latency of leukemias in mice transplanted with cells retrovirally-expressing the fusion gene *NUP98-HOXD13* (Thorsteinsdottir *et al.,* 2001; Pineault *et al.,* 2003).

Considering the similar overexpression pattern of *HOX* genes observed in *MLL*- and in CALM/AF10-induced leukemias, the leukemogenic mechanisms in both entities could have a common pathway.

When the gene expression profiles of the CALM/AF10 patients was compared to the expression profiles of other leukemia patients or normal bone marrow it became apparent that more than 90% of the top 100 differentially regulated genes were down-regulated in the CALM/AF10 patients. Some of the more interesting down-regulated genes (Table 21) are discussed in the following paragraphs.

RAB34: was the gene with the strongest repression in the CALM/AF10 samples. The Rab family is associated with vesicle membrane compartment functions and is important in vesicle trafficking (Sun *et al.,* 2003).

TRIO (Triple fuctional domain) protein gene, which is involved in phagocytosis of apoptotic cells was found expressed at a high frequence in oral carcinomas (de Bakker *et al.,* 2004; Baldwin *et al.,* 2005).

The *CEBPD* (CAAT/enhancer binding protein □) gene belongs to the C/EBP family of transcription factors and is important in proliferation, differentiation and apoptosis. *CEBPD* is a putative tumor suppressor gene whose deficiency in mouse fibroblasts cells caused impaired growth control and was associated with chromosomal instability (Huang *et al.,* 2004). Mutations in *C/EBPD* gene are common in human breast cancer (Tang and DeWille, 2002).

ST7 is a tumor suppressor gene which is downregulated by promoter methylation mediated by the PRMT5 (protein arginine methyltransferase), a protein associated with the human SWI/SNF complex (Pal *et al.*, 2004).

The expression of *H2AFJ* (histone family member J) was correlated with human melanocytic tumour (de Wit *et al.*, 2005).

JAG1 (jagged1) is a *NOTCH* ligand, whose expression is associated with bad prognosis in human breast cancer (Reedijk *et al,* 2005).

HCK (hematopoietic cell kinase) is a *src* kinase family member, expressed in hematopoietic cells including myeloid cells and to a minor extent in B-cells (Quintrel *et al.,* 1987). The inactivation of the *HCK* gene results in defects in phagocytosis in macrophages (Hauses *et al.*, 1998).

TEM7R (tumor endothelial marker 7) is abundantly expressed in tumor endothelial cells (Nanda *et al., 2004).*

CDH15 (Cadherin 15): Cadherins are a family of cell surface molecules involved in cellular structural and functional organization. The loss of heterozigosity of cadherin gene found in solid tumors makes these family of genes candidate tumor supressor genes (Kremmidiotis *et al.*, 1998; Cool and Jolicoer, 1999).

CTSZ (Cystein Protease Cathepsin Z) is expressed in pancreatic cell lines (Mahlamäki *et al*., 2002).
CTNNA1(□E-catenin) can suppress tumor invasion in human cancer (Vermeulen *et al*., 1999).
TKR2 (Human toll-like receptor 2) is a membrane receptor playing a role in innate immunity which activates NF-□B (Yang *et al*., 1999).
HOXB4 is a member of the *HOX B* gene cluster. *HOXB4* expression has been shown to increase the self-renewal of HSCs without disrupting differentiation (Abramovich *et al*., 2005).
USP3 (ubiquitin specific protease 3): functions in deubiquitination processes (Sloper-Mould *et al*., 1999).
ZNF185 (zink finger protein 185) was identified by expression profiling in prostate cancer. The methylation of the ZNF185 promoter transcriptional silencing of this gene is implicated in prostate tumorigenesis (Vanaja *et al*., 2003).
The transcriptional repression of these genes of which several have important functions in membrane trafficking, cellular growth, self-renewal, differentiation, angiogenesis and tumor suppression, could very well contribute to the transforming and leukemogenic potential of CALM/AF10. However, more detailed functional studies as well as the proof that these genes are direct targets of CALM/AF10-mediated gene regulation (see also next paragraph) are required to corroborate these assumptions.

6.3. Inducible expression of *CALM/AF10* in cell lines

Stable and transient transfection of mammalian cells

Stable transfection of mouse fibroblast with the pRTS-CALM/AF10 plasmid using puromycin selection resulted in a polyclonal cell line that was still expressing *CALM/AF10* at low basal levels. The inducibility of these cells, assessed flow cytometry of GFP-positive cells 24 h after induction, was about 30%. We asked for advise about the use of this system in the group which developed this vector (Michael Hölzer, personal communication) and received the suggestion to induce the cells a short period after transient transfection. It had been observed that the expression of the gene of interest from this vector became leaky after the cells were in culture for a period of longer than two months. The cells we were working with had been in culture for about 4 months and had been frozen and thawed.
Nevertheless, this tet-inducible system was shown to be useful to induce the gene expression and we could obtain doxycycline-inducible CALM/AF10 protein expression.
This system will be an important tool to define direct target genes of the CALM/AF10 fusion protein. Using this inducible system, we will also be able to answer the question of whether *CALM/AF10* induced transcriptional activation or repression can become epigenitically fixed or whether the continued presence of the CALM/AF10 fusion protein is required to maintain the CALM/AF10 specific gene expression pattern.

7. SUMMARY

The t(10;11)(p13;q14) is a recurring translocation resulting in the fusion of the *CALM* and *AF10* genes. The leukemogenic CALM/AF10 fusion genes codes for a 1595 amino acids protein. This translocation was first identified in a patient with hystiocytic lymphoma and was subsequently found in patients with AML, T-ALL and malignant lymphoma. This translocation is found in younger patients and is associated with a poor prognosis.

The *CALM*/AF10-associated leukemias can exhibit myeloid, lymphoid or mixed lymphoid-meyloid features, indicating a stem cell or an early commited progenitor as the target cell of leukemic transformation. At the present time the target cells in *CALM/AF10*-associated leukemogenesis are unknown. It is also not known which target genes are up or downregulated by the presence of the CALM/AF10 fusion protein.

To answer these questions, the following experiments were performed:

1) Five transgenic mouse lines, two expressing *CALM/AF10* under the control of the immunoglobulin heavy chain enhancer promoter and three under the control of the murine proximal Lck promoter were generated. Although the *CALM/AF10* expression was confirmed to be present and specific to the cells targeted by the promoters used (B- and T-cell progenitors for IgH and Lck promoters, respectively), the transgenic animals did not show a phenotype that could be detected after meticulous clinical, haematological, immunological, flow cytometrical and immunohistopatological analysis .

2) We performed molecular characterization of several *CALM/AF10* patient samples: A group of 13 patients with different types of leukemia: case 1 (AML M2), case 2 (Acute Biphetnotypic leukemia), case 3 (Pre T-ALL), case 4 (Acute Undifferentiated Leukemia), case 5 (PreT-ALL), cases 6 and 7 (ProT-ALL), case 8 (T-ALL), case 9 (AML), case 14 (T-ALL), case 15, 16 and 17 (AML) with a t(10;11) translocation detected by cytogenetic analysis suggesting a *CALM/AF10*-rearrangement. The samples were analyzed for the presence of the *CALM/AF10* and *AF10/CALM* fusion transcripts by RT-PCR and sequence analysis. All these patients were found to be positive for the CALM/AF10 fusion. In addition, we analyzed a series of twenty-nine patients with T-ALL with T-cell receptor □□ rearrangement. Among these patients, four (case 10 to 13) were positive for the *CALM/AF10* fusion transcript, indicating a high incidence of *CALM/AF10* fusions in this group of leukemia.

   Three different breakpoints in *CALM* at nucleotide 1926, 2091 and a new exon, with 106 bases inserted after nt 2064 of CALM in patient 4 were found. In AF10 four breakpoints were identified: at nucleotide position 424, 589, 883 and 979. In patient 16 we found an extra exon before nt 424 of AF10. In seven patients it was also possible to amplify the reciprocal *AF10/CALM* fusion transcript (case 1, 3, 4, 8, 9, 10 and 14). There was no

correlation between disease phenotype and breakpoint location. Ten *CALM/AF10* positive patients were analyzed using oligonucleotide microarrays representing 33,000 different genes (U133 set, Affymetrix). Analysis of microarray gene expression signatures of these patients revealed high expression levels of the polycomb group gene *BMI1*, the homeobox gene *MEIS1* and the *HOXA* cluster genes *HOXA1, HOXA4*, *HOXA5*, *HOXA7*, *HOXA9*, and *HOXA10*. The overexpression of HOX genes seen in these CALM/AF10 positive leukemias is reminiscent to the pattern seen in leukemias with rearrangements of the *MLL* gene, normal karyotypes and complex aberrant karyotypes suggesting a common effector pathway (i.e. *HOX* gene deregulation) for these diverse leukemias. In addition, the general pattern of gene expression of *CALM/AF10* patients when compared to other leukemia subtypes and to normal bone marrow was dominated by a global downregulation of genes some of them with function identified as related to important molecular mechanisms, such as membrane trafficking, cell growth regulation, proliferation, differentiation and tumor suppression.

3) We cloned *CALM/AF10* fusion gene into a vector that allowed us to induce the expression of CALM/AF10 using doxycycline in transiently and stably-transfected NIH3T3 and HEK293 cells. This system will be an important tool to identify direct CALM/AF10 target genes and to answer the question whether continued CALM/AF10 expression is necessary to maintain the CALM/AF10-associated expression pattern.

8. ZUSAMMENFASSUNG

Die t(10;11)(p13;q14) ist eine seltene aber wiederholt auftretende Translokation, die eine Fusion zwischen den Genen CALM and AF10 verursacht. Das leukämogene CALM/AF10 Fusionsgen kodiert für ein ca. 1600 Aminosäure langes Protein. Diese Translokation wurde in der U937 Zellinie, die von einem Patienten mit hystiozytischem Lymphom etabliert wurde, zuerst beschrieben und wurde in der Folge auch bei Patienten mit AML, T-ALL und malignem Lymphom gefunden. Diese Translokation kommt in jüngeren Patienten vor und geht mit einer eher schlechten Prognose einher.

Die CALM/AF10-positiven Leukämien haben einen myeloischen, lymphatischen oder gemischte lymphatisch-meyloischen Phenotyp, was darauf hindeutet, dass eine hämatopoetische Stammzelle oder eine sehr frühe Blutvorläuferzelle die Zielzelle bei der CALM/AF10-bedingten Transformation ist. Diese Zielzelle ist noch unbekannt. Weiterhin ist nicht bekannt, was die Zielegene des CALM/AF10 Fusionsproteins sind. Um diese Fragen zu beantworten, wurden die folgende Experimente durchgeführt:

1) Es wurden fünf transgenen Mauslinien, zwei die das CALM/AF10-Gen unter die Kontrolle des Immunoglobulin Heavy Chain Enhancer Promoters exprimieren und drei weitere, die das Transgen unter der Kontrolle proximalen murinen Lck Promoters exprimieren. Obwohl die zellspezifische Expression des Transgens auf RNA-Ebene nachgewiesen wurde, konnte nach sorgfältigen klinischen, hämatologischen, immunologischen, zytometrischen und immunohistopathologischen Untersuchungen keine durch das Transgen verursachte pathologische Veränderungen nachgewiesen werden.

2) Es wurden CALM/AF10-positive Patientenproben molekular charakterisiert: Eine Gruppe von 13 Patienten mit verschiedenen Leukämien und zytogenetisch nachgewiesener t(10;11)(p12;q14): Patient 1 (AML M2), Patient 2 (Akute biphänotypische Leukämie), Patient 3 (Pre T-ALL), Patient 4 (Akute undifferentierte Leukämia), Patient 5 (Pre T-ALL), Patienten 6 und 7 (ProT-ALL), Patient 8 (T-ALL), Patient 9 (AML), Patient 14 (T-ALL), Patienten 15, 16 und 17 (AML). Die Patientenproben wurden auf das Vorhandensein von CALM/AF10 und AF10/CALM Fusionstranskripten mittles RT-PCR untersucht. Bei allen Patienten wurden ein CALM/AF10 Fusionstranskript gefunden. Weiterhin wurde eine Gruppe von 29 Patienten, bei denen eine T-ALL mit T-cell Receptor g/d vorlag, auf das Vorhandensein des CALM/AF10 Fusionstranskriptes getestet. Bei vier dieser Patienten konnte das CALM/AF10 Fusionsgen nachgewiesen werden, was eine hohe Prävalenz der CALM/AF10 Fusion bei dieser Gruppe von Leukämien anzeigt.

Drei Bruchpunkte wurden in CALM gefunden: Bei Nukleotid 1926, 2091 und 2064. Bei Patient 4 wurde ein bisher nicht beschriebenes CALM Exon, mit 106 Basen gefunden. In AF10 wurden vier Bruchpunkte identifiziert: bei Nukleotid 424, 589, 883 und 979. Bei Patient 16 wurde einen zusätzliches Exon vor Nt 424 von AF10 gefunden. Bei sieben

Patienten (Nr 1, 3, 4, 8, 9, 10 and 14) konnte auch das reziproke AF10/CALM Fusiontranskript mittels RT-PCR amplifiziert werden. Es wurde keine Korrelation zwischen der Art der Leukämie und der Lokalisation der Bruchpunkte in CALM oder AF10 festgestellt. Bei Leukämieproben von zehn CALM/AF10 positive Patienten wurden mittels Oligonukleotide Microarrays, die 33,000 verschiedenen Genen entschprachen (U133 set, Affymetrix) ein Expressionsprofil erstellt. Die Analyse von Microarray Gene Expression Signaturen dieser Patienten zeigte hohe Expressionsraten des Polycombgruppe Gens BMI1, des Homeobox Gens MEIS1 und der HOXA Gen Cluster-Gene HOXA1, HOXA4, HOXA5, HOXA7, HOXA9 und HOXA10. Die Überexpression von HOX Genen bei CALM/AF10 positiven Leukämie wird in ähnlicher Weise bei Leukämien mit MLL Rearrangements und normalen so wie komplex aberranten Karyotypen beobachtet. Dies weist auf einen gemeisamen Pathway (i.e. HOX Gen-Deregulierung) bei diesen Leukämien hin. Insgesamt wurde bei den CALM/AF10 positiven Leukämien im Vergleich zu anderen Leukämieentitäten und zu normalem Knochenmark eine globale Repression der Genexpression beobachtet. Einige dieser signifikant reprimierten Gene sind an wichtigen zellulären Prozessen wie Membrantransport, Zellwachstum, Zellproliferation, Differenzierung und Tumorsupression beteiligt.

3) Das CALM/AF10 Fusionsgen wurde in einen Vector kloniert, der die induzierbares Expression von CALM/AF10 in transient und stabil transfizierten NIH3T3 und HEK293 ermöglicht. Mithilfe dieses Systems werden sich die unmittelbaren CALM/AF10 Zielgene identifizieren lassen. Weiterhin wird untersucht werden können, ob eine kontinuierliche Expression von CALM/AF10 notwendig ist, um das Expressionmuster der Zielgene aufrechtzuerhalten.

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10. APPENDIX

Table 22: Average, Standard Deviation and Statistical Analysis of Serum Immunoglobulins of pIGCASV-40 mice and FVB control mice.

Line	Immunoglobulin	Average ± Standard deviation		P value	Result
		POS	NEG		
L1	IgA	2,342 ± 0,142	2,374 ± 0,179	0,7782	POS = NEG
	IgG1	3,276 ± 0,295	3,473 ± 0,204	0,3133	POS = NEG
	IgG2a	2,537 ± 0,516	2,255 ± 0,563	0,4649	POS = NEG
	IgG2b	3,038 ± 0,409	3,142 ± 0,475	0,7384	POS = NEG
	IgG3	3,005 ± 0,242	3,135 ± 0,068	0,2842	POS = NEG
	IgM	2,547 ± 0,254	2,620 ± 0,327	0,7284	POS = NEG
L2	IgA	1,433 ± 0,218	1,452 ± 0,116	0,8603	POS = NEG
	IgG1	1,964 ± 0,528	2,120 ± 0,426	0,6017	POS = NEG
	IgG2a	2,182 ± 0,583	2,743 ± 0,457	0,1063	POS = NEG
	IgG2b	2,338 ± 0,264	2,179 ± 0,135	0,2284	POS = NEG
	IgG3	2,493 ± 0,344	2,649 ± 0,326	0,4609	POS = NEG
	IgM	3,542 ± 0,185	3,539 ± 0,103	0,9785	POS = NEG

Table 23: Average, Standard Deviation and Statistical Analysis of Immunophenotype from Blood Cells of pIGCASV-40 mice and FVB control mice.

Antigen	Average ± standard deviation		P value	Result
	Transgenic %*	Control %**		
c-Kit+ Sca-1+	0.44 ± 0.298	0.22 ± 0.269	0.178	Transgenic = Control
c-Kit- Sca-1+	68.06 ± 13.61	62.51 ± 15.05	0.321	Transgenic = Control
c-Kit+ Sca-1-	0.0525 ± 0.098	0.236 ± 0.304	0.203	Transgenic = Control
Gr-1+ Mac-1+	42.737 ± 20.64	42.6 ± 24.87	0.497	Transgenic = Control
Gr-1- Mac-1 +	11.68 ± 1.834	12.806 ± 5.789	0.386	Transgenic = Control
Gr-1+ Mac-1-	5.62 ± 1.821	4.9 ± 0.953	0.265	Transgenic = Control
B220+ CD3+	0.872 ± 0.752	0.526 ± 0.463	0.244	Transgenic = Control
B220- CD3+	60.467 ± 15.87	55.56 ± 21.001	0.376	Transgenic = Control
B220+CD3-	5.345 ± 4.297	8.57 ± 9.954	0.320	Transgenic = Control
B220+ CD19+	32.43 ± 24.38	36.67 ± 28.108	0.422	Transgenic = Control
B220- CD19+	0.175 ± 0.254	0.333 ± 0.3401	0.269	Transgenic = Control
B220+ CD19-	0.605 ± 0.737	0.93 ± 0.5678	0.269	Transgenic = Control
B220+ CD43+	85.225 ± 8.637	85.06 ± 1.82	0.486	Transgenic = Control
B220- CD43+	2.897 ± 1.934	5.316 ± 4.485	0.227	Transgenic = Control
B220+ CD43-	0.152 ± 0.199	1.1 ± 1.75	0.223	Transgenic = Control

*n = 7, **n = 5

Table 24: Average, Standard Deviation and Statistical Analysis of Immunophenotype from Bone Marrow Cells of pIGCASV-40 mice and FVB control mice.

Antigen	Average ± standard deviation		P value	Result
	Transgenic %*	Control %**		
c-Kit+ Sca-1+	0.775 ± 0.143	0.9 ± 0.221	0.225	Transgenic = Control
c-Kit- Sca-1+	8.71 ± 4.465	6.193 ± 1.145	0.174	Transgenic = Control
c-Kit+ Sca-1-	1.612 ± 0.294	1.676 ± 0.747	0.449	Transgenic = Control
Gr-1+ Mac-1+	38.112 ± 15.645	37.5 ± 10.019	0.476	Transgenic = Control
Gr-1- Mac-1 +	6.747 ± 2.631	8.046 ± 1.375	0.219	Transgenic = Control
Gr-1+ Mac-1-	1.55 ± 0.410	1.52 ± 0.5	0.468	Transgenic = Control
B220+ CD3+	0.117 ± 0.080	0.0733 ± 0.0635	0.227	Transgenic = Control
B220- CD3+	1.635 ± 0.964	1 ± 0.398	0.148	Transgenic = Control
B220+CD3-	19.757 ± 3.509	19.086 ± 3.245	0.402	Transgenic = Control
B220+ CD19+	21.84 ± 11.279	14.383 ± 3.738	0.143	Transgenic = Control
B220- CD19+	0.312 ± 0.267	0.04 ± 0.0346	0.068	Transgenic = Control
B220+ CD19-	2.632 ± 1.357	3.04 ± 0.582	0.308	Transgenic = Control
B220+ CD43+	15.655 ± 3.177	14.593 ± 3.683	0.354	Transgenic = Control
B220- CD43+	61.915 ± 9.250	58.646 ± 16.793	0.390	Transgenic = Control
B220+ CD43-	18.572 ± 6.019	19.286 ± 5.665	0.439	Transgenic = Control

*n = 7, **n = 5

Table 25: Average, Standard Deviation and Statistical Analysis of Immunophenotype from Splenocytes of pIGCASV-40 mice and FVB control mice.

Antigen	Average ± standard deviation		P value	Result
	Transgenic %*	Control %**		
c-Kit+ Sca-1+	0.982 ± 0.729	0.64 ± 0.164	0.212	Transgenic = Control
c-Kit- Sca-1+	76.75 ± 15.81	65.95 ± 7.208	0.144	Transgenic = Control
c-Kit+ Sca-1-	0.1925 ± 0.095	0.236 ± 0.021	0.214	Transgenic = Control
Gr-1+ Mac-1+	3.785 ± 1.726	5.48 ± 2.484	0.188	Transgenic = Control
Gr-1- Mac-1 +	4.625 ± 1.251	6.493 ± 2.220	0.141	Transgenic = Control
Gr-1+ Mac-1-	3.492 ± 0.783	3.393 ± 0.734	0.435	Transgenic = Control
B220+ CD3+	0.832 ± 0.170	1.116 ± 0.537	0.244	Transgenic = Control
B220- CD3+	37.587 ± 10.408	45.686 ± 3.863	0.112	Transgenic = Control
B220+CD3-	23.41 ± 11.15	20.126 ± 3.487	0.305	Transgenic = Control
B220+ CD19+	55.512 ± 16.310	44.79 ± 2.549	0.141	Transgenic = Control
B220- CD19+	0.225 ± 0.0858	0.323 ± 0.182	0.228	Transgenic = Control
B220+ CD19-	0.602 ± 0.106	1.016 ± 0.681	0.201	Transgenic = Control
B220+ CD43+	50.997 ± 9.678	52.663 ± 7.504	0.404	Transgenic = Control
B220- CD43+	8.74 ± 6.302	10.746 ± 8.942	0.379	Transgenic = Control
B220+ CD43-	40.192 ± 4.436	38.25 ± 1.775	0.235	Transgenic = Control

*n = 7, **n = 5

Table 26: Average, Standard Deviation and Statistical Analysis of Immunophenotype from WBCs of pLCKCASV-40 mice and FVB control mice.

WBC antigen	Average ± standard deviation		P value	Result
	Transgenic %*	Control %**		
B220- CD3+	28.703 ± 5.504	31.643 ± 3.668	0.484	Transgenic = Control
B220+ CD3-	35.843 ± 7.567	35.193 ± 2.810	0.895	Transgenic = Control
CD3+CD4-CD8-	11.783 ± 1.486	14.94 ± 0.521	0.053	Transgenic = Control
CD4+CD8+	40.073 ± 3.297	39.18 ± 4.702	0.800	Transgenic = Control
CD4-CD8+	0.8166 ± 0.174	0.516 ± 0.066	0.082	Transgenic = Control
CD4+CD8-	56.5 ± 2.748	57.96 ± 4.616	0.662	Transgenic = Control
Gr-1+ Mac-1-	30.863 ± 3.882	22.516 ± 1.744	0.0478	Transgenic = Control
Gr-1- Mac-1 +	14.633 ± 3.898	12.066 ± 2.698	0.401	Transgenic = Control
Gr-1+ Mac-1+	2.423 ± 0.669	1.926 ± 0,525	0.369	Transgenic = Control
Ter119+ B220+	1.38 ± 0.194	1.963 ± 0.359	0.068	Transgenic = Control
Ter119- B220+	9.903 ± 1.125	9.106 ± 0.815	0.381	Transgenic = Control
Ter119+ B220-	21.546 ± 1.082	23.526 ± 2.819	0.319	Transgenic = Control
c-Kit+ Sca-1+	0.843 ± 0.527	1.17 ± 0.615	0.524	Transgenic = Control
c-Kit- Sca-1+	10.063 ± 1.673	10.786 ± 1.054	0.561	Transgenic = Control
c-Kit+ Sca-1-	4.14 ± 4.028	5.74 ± 3.420	0.628	Transgenic = Control

*n = 3, **n = 3

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1    TAG TTA TTA ATA GTA ATC AAT TAC GGG GTC ATT AGT TCA TAG CCC ATA TAT GGA GTT CCG
61   CGT TAC ATA ACT TAC GGT AAA TGG CCC GCC TGG CTG ACC GCC CAA CGA CCC CCG CCC ATT
181  GAC GTC AAT AAT GAC GTA TGT TCC CAT AGT AAC GCC AAT AGG GAC TTT CCA TTG ACG TCA
241  ATG GGT GGA GTA TTT ACG GTA AAC TGC CCA CTT GGC AGT ACA TCA AGT GTA TCA TAT GCC
301  AAG TAC GCC CCC TAT TGA CGT CAA TGA CGG TAA ATG GCC CGC CTG GCA TTA TGC CCA GTA
361  CAT GAC CTT ATG GGA CTT TCC TAC TTG GCA GTA CAT CTA CGT ATT AGT CAT CGC TAT TAC
421  CAT GGT GAT GCG GTT TTG GCA GTA CAT CAA TGG GCG TGG ATA GCG GTT TGA CTC ACG GGG
481  ATT TCC AAG TCT CCA CCC CAT TGA CGT CAA TGG GAG TTT GTT TTG GCA CCA AAA TCA ACG
541  GGA CTT TCC AAA ATG TCG TAA CAA CTC CGC CCC ATT GAC GCA AAT GGG CGG TAG GCG TGT
601  ACG GTG GGA GGT CTA TAT AAG CAG AGC TGG TTT AGT GAA CCG TCA GAT CCG CTA GCG CTA
     CCG GTC GCC ACC ATG GTG AGC AAG GGC GAG GAG CTG TTC ACC GGG GTG GTG CCC ATC CTG
                       M   V   S   K   G   E   E   L   F   T   G   V   V   P   I   L>
721  GTC GAG CTG GAC GGC GAC GTA AAC GGC CAC AAG TTC AGC GTG TCC GGC GAG GGC GAG GGC
      V   E   L   D   G   D   V   N   G   H   K   F   S   V   S   G   E   G   E   G>
781  GAT GCC ACC TAC GGC AAG CTG ACC CTG AAG TTC ATC TGC ACC ACC GGC AAG CTG CCC GTG
      D   A   T   Y   G   K   L   T   L   K   F   I   C   T   T   G   K   L   P   V>
841  CCC TGG CCC ACC CTC GTG ACC ACC CTG ACC TAC GGC GTG CAG TGC TTC AGC CGC TAC CCC
      P   W   P   T   L   V   T   T   L   T   Y   G   V   Q   C   F   S   R   Y   P>
901  GAC CAC ATG AAG CAG CAC GAC TTC TTC AAG TCC GCC ATG CCC GAA GGC TAC GTC CAG GAG
      D   H   M   K   Q   H   D   F   F   K   S   A   M   P   E   G   Y   V   Q   E>
961  CGC ACC ATC TTC TTC AAG GAC GAC GGC AAC TAC AAG ACC CGC GCC GAG GTG AAG TTC GAG
      R   T   I   F   F   K   D   D   G   N   Y   K   T   R   A   E   V   K   F   E>
1021 GGC GAC ACC CTG GTG AAC CGC ATC GAG CTG AAG GGC ATC GAC TTC AAG GAG GAC GGC AAC
      G   D   T   L   V   N   R   I   E   L   K   G   I   D   F   K   E   D   G   N>
1081 ATC CTG GGG CAC AAG CTG GAG TAC AAC TAC AAC AGC CAC AAC GTC TAT ATC ATG GCC GAC
      I   L   G   H   K   L   E   Y   N   Y   N   S   H   N   V   Y   I   M   A   D>
1141 AAG CAG AAG AAC GGC ATC AAG GTG AAC TTC AAG ATC CGC CAC AAC ATC GAG GAC GGC AGC
      K   Q   K   N   G   I   K   V   N   F   K   I   R   H   N   I   E   D   G   S>
1201 GTG CAG CTC GCC GAC CAC TAC CAG CAG AAC ACC CCC ATC GGC GAC GGC CCC GTG CTG CTG
      V   Q   L   A   D   H   Y   Q   Q   N   T   P   I   G   D   G   P   V   L   L>
1261 CCC GAC AAC CAC TAC CTG AGC ACC CAG TCC GCC CTG AGC AAA GAC CCC AAC GAG AAG CGC
      P   D   N   H   Y   L   S   T   Q   S   A   L   S   K   D   P   N   E   K   R>
1321 GAT CAC ATG GTC CTG CTG GAG TTC GTG ACC GCC GCC GGG ATC ACT CTC GGC ATG GAC GAG
      D   H   M   V   L   L   E   F   V   T   A   A   G   I   T   L   G   M   D   E>
1381 CTG TAC AAG TCC GGA CTC AGA TCT CGA GGT CGA CGG TAT CGG GGG ATC GAT CCG CAT GCG
      L   Y   K   S   G   L   R   S   R   G   R   R   Y   R   G   I   D   P   H   A>
1441 AGC TCG GTA CCC CGG CTG CTG AGC GGG TGG GGT GGT GGA GGA GCT GCA GAG ATG TCC GGC
      S   S   V   P   R   L   L   S   G   W   G   G   G   G   A   A   E   M   S   G>
1501 CAG AGC CTG ACG GAC CGA ATC ACT GCC GCC CAG CAC AGT GTC ACC GGC TCT GCC GTA TCC
      Q   S   L   T   D   R   I   T   A   A   Q   H   S   V   T   G   S   A   V   S>
1561 AAG ACA GTA TGC AAG GCC ACG ACC CAC GAG ATC ATG GGG CCC AAG AAA AAG CAC CTG GAC
      K   T   V   C   K   A   T   T   H   E   I   M   G   P   K   K   K   H   L   D>
1621 TAC TTA ATT CAG TGC ACA AAT GAG ATG AAT GTG AAC ATC CCA CAG TTG GCA GAC AGT TTA
      Y   L   I   Q   C   T   N   E   M   N   V   N   I   P   Q   L   A   D   S   L>
1681 TTT GAA AGA ACT ACT AAT AGT AGT TGG GTG GTG GTC TTC AAA TCT CTC ATT ACA ACT CAT
      F   E   R   T   T   N   S   S   W   V   V   V   F   K   S   L   I   T   T   H>
1741 CAT TTG ATG GTG TAT GGA AAT GAG CGT TTT ATT CAG TAT TTG GCT TCA AGA AAC ACG TTG
      H   L   M   V   Y   G   N   E   R   F   I   Q   Y   L   A   S   R   N   T   L>
1801 TTT AAC TTA AGC AAT TTT TTG GAT AAA AGT GGA TTG CAA GGA TAT GAC ATG TCT ACA TTT
      F   N   L   S   N   F   L   D   K   S   G   L   Q   G   Y   D   M   S   T   F>
1861 ATT AGG CGG TAT AGT AGA TAT TTA AAT GAG AAA GCA GTT TCA TAC AGA CAA GTT GCA TTT
      I   R   R   Y   S   R   Y   L   N   E   K   A   V   S   Y   R   Q   V   A   F>
1921 GAT TTC ACA AAA GTG AAG AGA GGG GCT GAT GGA GTT ATG AGA ACA ATG AAC ACA GAA AAA
      D   F   T   K   V   K   R   G   A   D   G   V   M   R   T   M   N   T   E   K>
1981 CTC CTA AAA ACT GTA CCA ATT ATT CAG AAT CAA ATG GAT GCA CTT CTT GAT TTT AAT GTT
      L   L   K   T   V   P   I   I   Q   N   Q   M   D   A   L   L   D   F   N   V>
2041 AAT AGC AAT GAA CTT ACA AAT GGG GTA ATA AAT GCT GCC TTC ATG CTC CTG TTC AAA GAT
      N   S   N   E   L   T   N   G   V   I   N   A   A   F   M   L   L   F   K   D>
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2101 GCC ATT AGA CTG TTT GCA GCA TAC CAT GAA GGA ATT ATT AAT TTG TTG GAA AAA TAT TTT
      A   I   R   L   F   A   A   Y   H   E   G   I   I   N   L   L   E   K   Y   F>
2161 GAT ATG AAA AAG AAC CAA TGC AAA GAA GGT CTT GAC ATC TAT AAG AAG TTC CTA ACT AGG
      D   M   K   K   N   Q   C   K   E   G   L   D   I   Y   K   K   F   L   T   R>
2221 ATG ACA AGA ATC TCA GAG TTC CTC AAA GTT GCA GAG CAA GTT GGA ATT GAC AGA GGT GAT
      M   T   R   I   S   E   F   L   K   V   A   E   Q   V   G   I   D   R   G   D>
2281 ATA CCA GAC CTT TCA CAG GCC CCT AGC AGT CTT CTT GAT GCT TTG GAA CAA CAT TTA GCT
      I   P   D   L   S   Q   A   P   S   S   L   L   D   A   L   E   Q   H   L   A>
2341 TCC TTG GAA GGA AAG AAA ATC AAA GAT TCT ACA GCT GCA AGC AGG GCA ACT ACA CTT TCC
      S   L   E   G   K   K   I   K   D   S   T   A   A   S   R   A   T   T   L   S>
2401 AAT GCA GTG TCT TCC CTG GCA AGC ACT GGT CTA TCT CTG ACC AAA GTG GAT GAA AGG GAA
      N   A   V   S   S   L   A   S   T   G   L   S   L   T   K   V   D   E   R   E>
2461 AAG CAG GCA GCA TTA GAG GAA GAA CAG GCA CGT TTG AAA GCT TTA AAG GAA CAG CGC CTA
      K   Q   A   A   L   E   E   E   Q   A   R   L   K   A   L   K   E   Q   R   L>
2521 AAA GAA CTT GCA AAG AAA CCT CAT ACC TCT TTA ACA ACT GCA GCC TCT CCT GTA TCC ACC
      K   E   L   A   K   K   P   H   T   S   L   T   T   A   A   S   P   V   S   T>
2581 TCA GCA GGA GGG ATA ATG ACT GCA CCA GCC ATT GAC ATA TTT TCT ACC CCT AGT TCT TCT
      S   A   G   G   I   M   T   A   P   A   I   D   I   F   S   T   P   S   S   S>
2641 AAC AGC ACA TCA AAG CTG CCC AAT GAT CTG CTT GAT TTG CAG CAG CCA ACT TTT CAC CCA
      N   S   T   S   K   L   P   N   D   L   L   D   L   Q   Q   P   T   F   H   P>
2701 TCT GTA CAT CCT ATG TCA ACT GCT TCT CAG GTA GCA AGT ACA TGG GGA GAT CCT TTC TCT
      S   V   H   P   M   S   T   A   S   Q   V   A   S   T   W   G   D   P   F   S>
2761 GCT ACT GTA GAT GCT GTT GAT GAT GCC ATT CCA AGC TTA AAT CCT TTC CTC ACA AAA AGT
      A   T   V   D   A   V   D   D   A   I   P   S   L   N   P   F   L   T   K   S>
2721 AGT GGT GAT GTT CAC CTT TCC ATT TCT TCA GAT GTA TCT ACT TTT ACT ACT AGG ACA CCT
      S   G   D   V   H   L   S   I   S   S   D   V   S   T   F   T   T   R   T   P>
2881 ACT CAT GAA ATG TTT GTT GGA TTC ACT CCT TCT CCA GTT GCA CAG CCA CAC CCT TCA GCT
      T   H   E   M   F   V   G   F   T   P   S   P   V   A   Q   P   H   P   S   A>
2941 GGC CTT AAT GTT GAC TTT GAA TCT GTG TTT GGA AAT AAA TCT ACA AAT GTT ATT GTA GAT
      G   L   N   V   D   F   E   S   V   F   G   N   K   S   T   N   V   I   V   D>
3001 TCT GGG GGC TTT GAT GAA CTA GGT GGA CTT CTC AAA CCA ACA GTG GCC TCT CAG AAC CAG
      S   G   G   F   D   E   L   G   G   L   L   K   P   T   V   A   S   Q   N   Q>
3061 AAC CTT CCT GTT GCC AAA CTC CCA CCT AGC AAG TTA GTA TCT GAT GAC TTG GAT TCA TCT
      N   L   P   V   A   K   L   P   P   S   K   L   V   S   D   D   L   D   S   S>
3121 TTA GCC AAC CTT GTG GGC AAT CTT GGC ATC GGA AAT GGA ACC ACT AAG AAT GAT GTA AAT
      A   N   L   V   G   N   L   G   I   G   N   G   T   T   K   N   D   V   N>
3181 TGG AGT CAA CCA GGT GAA AAG AAG TTA ACT GGG GGA TCT AAC TGC GAA CCA AAG GTT GCA
      W   S   Q   P   G   E   K   K   L   T   G   G   S   N   C   E   P   K   V   A>
3241 CCA ACA ACC GCT TGG AAT GCT GCA ACA ATG GCA CCC CCT GTA ATG GCC TAT CCT GCT ACT
      P   T   T   A   W   N   A   A   T   M   A   P   P   V   M   A   Y   P   A   T>
3301 ACA CCA ACA GGC ATG ATA GGA TAT GGA ATT CCT CCA CAA ATG GGA AGT GTT CCT GTA ATG
      T   P   T   G   M   I   G   Y   G   I   P   P   Q   M   G   S   V   P   V   M>
3361 ACG CAA CCA ACC TTA ATA TAC AGC CAG CCT GTC ATG AGA CCT CCA AAC CCC TTT GGC CCT
      T   Q   P   T   L   I   Y   S   Q   P   V   M   R   P   P   N   P   F   G   P>
3421 GTA TCA GGA GCA CAG AGA TGT GAA CTT TGT CCC CAT AAG GAT GGA GCT TTA AAA AGA ACA
      V   S   G   A   Q   R   C   E   L   C   P   H   K   D   G   A   L   K   R   T>
3481 GAT AAT GGG GGT TGG GCC CAT GTG GTT TGT GCC CTG TAT ATT CCA GAG GTA CAA TTT GCC
      D   N   G   G   W   A   H   V   V   C   A   L   Y   I   P   E   V   Q   F   A>
3541 AAT GTT TCC ACA ATG GAA CCA ATT GTT TTA CAG TCT GTT CCG CAT GAT CGT TAT AAT AAG
      N   V   S   T   M   E   P   I   V   L   Q   S   V   P   H   D   R   Y   N   K>
3601 ACT TGC TAC ATT TGT GAT GAA CAA GGA AGA GAA AGC AAA GCA GCC ACT GGT GCT TGC ATG
      T   C   Y   I   C   D   E   Q   G   R   E   S   K   A   A   T   G   A   C   M>
3661 ACA TGT AAT AAA CAT GGA TGT CGA CAG GCT TTC CAT GTA ACA TGC GCT CAG TTT GCC GGA
      T   C   N   K   H   G   C   R   Q   A   F   H   V   T   C   A   Q   F   A   G>
3721 CTG CTT TGT GAA GAA GAA GGT AAT GGT GCC GAT AAT GTC CAA TAC TGT GGC TAC TGT AAA
      L   L   C   E   E   E   G   N   G   A   D   N   V   Q   Y   C   G   Y   C   K>
3781 TAC CAT TTT AGT AAG CTG AAA AAG AGC AAA CGG GGA TCT AAT AGG TCA TAT GAT CAA AGT
      Y   H   F   S   K   L   K   K   S   K   R   G   S   N   R   S   Y   D   Q   S>
3841 TTA AGT GAT TCT TCC TCT CAC TCT CAG GAT AAA CAT CAT GAG AAA GAG AAA AAA AAA TAT
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      L   S   D   S   S   S   H   S   Q   D   K   H   H   E   K   E   K   K   K   Y>
3901 AAA GAG AAG GAC AAA CAC AAA CAG AAA CAC AAG AAG CAG CCA GAA CCA TCA CCT GCA TTG
      K   E   K   D   K   H   K   Q   K   H   K   K   Q   P   E   P   S   P   A   L>
3961 GTT CCA TCC TTG ACT GTT ACT ACA GAA AAA ACT TAT ACA AGC ACT AGC AAC AAC TCT ATA
      V   P   S   L   T   V   T   T   E   K   T   Y   T   S   T   S   N   N   S   I>
4021 TCT GGA TCA TTG AAG CGC TTG GAA GAT ACT ACT GCA CGA TTT ACA AAT GCA AAT TTC CAG
      S   G   S   L   K   R   L   E   D   T   T   A   R   F   T   N   A   N   F   Q>
4081 GAA GTC TCT GCA CAC ACC TCT AGT GGA AAA GAT GTT TCA GAG ACT AGA GGG TCA GAG GGC
      E   V   S   A   H   T   S   S   G   K   D   V   S   E   T   R   G   S   E   G>
4141 AAA GGG AAG AAA TCT TCA GCT CAC AGC TCA GGT CAA AGG GGA AGA AAG CCT GGT GGT GGA
      K   G   K   K   S   S   A   H   S   S   G   Q   R   G   R   K   P   G   G   G>
4201 AGA AAT CCA GGA ACA ACT GTG TCA GCA GCT AGC CCT TTT CCT CAA GGC AGT TTT TCA GGA
      R   N   P   G   T   T   V   S   A   A   S   P   F   P   Q   G   S   F   S   G>
4261 ACT CCA GGC AGT GTA AAG TCA TCT TCT GGA AGT TCA GTG CAG TCT CCC CAG GAT TTC CTG
      T   P   G   S   V   K   S   S   S   G   S   S   V   Q   S   P   Q   D   F   L>
4321 AGC TTT ACA GAC TCA GAT CTG CGT AAT GAC AGT TAC TCT CAC TCC CAA CAG TCA TCA GCA
      S   F   T   D   S   D   L   R   N   D   S   Y   S   H   S   Q   Q   S   S   A>
4380 ACC AAA GAT GTA CAT AAA GGA GAG TCT GGA AGC CAG GAA GGG GGG GTA AAT AGT TTT AGT
      T   K   D   V   H   K   G   E   S   G   S   Q   E   G   G   V   N   S   F   S>
4441 ACC TTA ATT GGC CTC CCT TCA ACC TCA GCT GTT ACT TCA CAG CCT AAA AGC TTT GAA AAT
      T   L   I   G   L   P   S   T   S   A   V   T   S   Q   P   K   S   F   E   N>
4501 TCA CCT GGA GAT TTG GGT AAT TCC AGC CTT CCT ACA GCA GGA TAT AAG CGG GCT CAA ACT
      S   P   G   D   L   G   N   S   S   L   P   T   A   G   Y   K   R   A   Q   T>
4561 TCT GGC ATA GAA GAA GAA ACT GTA AAG GAA AAG AAA AGG AAA GGA AAT AAA CAA AGT AAG
      S   G   I   E   E   E   T   V   K   E   K   K   R   K   G   N   K   Q   S   K>
4621 CAT GGG CCT GGC AGA CCC AAA GGA AAC AAA AAT CAA GAG AAT GTT TCT CAT CTC TCA GTT
      H   G   P   G   R   P   K   G   N   K   N   Q   E   N   V   S   H   L   S   V>
4681 TCT TCT GCT TCA CCA ACA TCA TCT GTA GCA TCA GCT GCA GGA AGC ATA ACA AGC TCT AGT
      S   S   A   S   P   T   S   S   V   A   S   A   A   G   S   I   T   S   S   S>
4741 CTG CAG AAA TCT CCT ACA TTG CTC AGG AAT GGA AGT TTA CAG AGC CTC AGT GTT GGC TCA
      L   Q   K   S   P   T   L   L   R   N   G   S   L   Q   S   L   S   V   G   S>
4801 TCT CCA GTT GGT TCA GAA ATT TCC ATG CAG TAT CGG CAT GAT GGA GCT TGC CCA ACA ACT
      S   P   V   G   S   E   I   S   M   Q   Y   R   H   D   G   A   C   P   T   T>
4861 ACG TTC TCA GAG TTG CTG AAT GCA ATA CAC AAC GAC AGA GGT GAC AGT TCT ACA CTA ACA
      T   F   S   E   L   L   N   A   I   H   N   D   R   G   D   S   S   T   L   T>
4921 AAG CAA GAA CTT AAA TTC ATA GGT ATT TAT AAC AGC AAT GAT GTA GCA GTA TCG TTT CCA
      K   Q   E   L   K   F   I   G   I   Y   N   S   N   D   V   A   V   S   F   P>
4981 AAT GTA GTA TCT GGC TCG GGA TCT AGT ACT CCT GTC TCC AGC TCT CAC TTA CCT CAG CAG
      N   V   V   S   G   S   G   S   S   T   P   V   S   S   S   H   L   P   Q   Q>
5041 TCT TCT GGG CAT TTG CAA CAA GTA GGA GCG CTC TCT CCC TCA GCT GTG TCA TCT GCA GCC
      S   S   G   H   L   Q   Q   V   G   A   L   S   P   S   A   V   S   S   A   A>
5101 CCT GCT GTT GCT ACA ACT CAG GCA AAT ACT CTA TCT GGA TCT TCT CTC AGT CAG GCA CCA
      P   A   V   A   T   T   Q   A   N   T   L   S   G   S   S   L   S   Q   A   P>
5161 TCT CAT ATG TAT GGC AAT AGA TCA AAT TCA TCA ATG GCA GCT CTT ATA GCT CAG TCT GAA
      S   H   M   Y   G   N   R   S   N   S   S   M   A   A   L   I   A   Q   S   E>
5221 AAC AAT CAA ACA GAT CAA GAT CTT GGA GAC AAT AGC CGC AAC CTA GTT GGC AGA GGA AGC
      N   N   Q   T   D   Q   D   L   G   D   N   S   R   N   L   V   G   R   G   S>
5281 TCA CCC CGA GGA AGT CTC TCG CCA CGA TCC CCT GTA AGC AGC TTA CAG ATT CGC TAT GAT
      S   P   R   G   S   L   S   P   R   S   P   V   S   S   L   Q   I   R   Y   D>
5341 CAA CCA GGC AAC AGC AGT TTG GAA AAT CTG CCT CCA GTA GCA GCC AGC ATA GAA CAG CTT
      Q   P   G   N   S   S   L   E   N   L   P   P   V   A   A   S   I   E   Q   L>
5401 TTG GAG AGG CAG TGG AGT GAA GGA CAG CAA TTT TTA CTA GAA CAG GGT ACT CCT AGT GAC
      L   E   R   Q   W   S   E   G   Q   Q   F   L   L   E   Q   G   T   P   S   D>
5461 ATT TTA GGA ATG CTG AAG TCA TTA CAC CAA CTT CAA GTT GAA AAC CGA AGA TTA GAG GAA
      I   L   G   M   L   K   S   L   H   Q   L   Q   V   E   N   R   R   L   E   E>
5521 CAA ATT AAA AAC TTG ACT GCC AAA AAG GAA CGG CTT CAG TTA TTG AAT GCA CAG CTT TCA
      Q   I   K   N   L   T   A   K   K   E   R   L   Q   L   L   N   A   Q   L   S>
5581 GTG CCT TTT CCA ACA ATA ACA GCA AAT CCT AGT CCG TCT CAT CAA ATA CAC ACA TTT TCA
      V   P   F   P   T   I   T   A   N   P   S   P   S   H   Q   I   H   T   F   S>
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5640 GCA CAG ACT GCT CCT ACT ACT GAT TCC TTG AAC AGC AGT AAG AGC CCT CAT ATA GGA AAC
      A   Q   T   A   P   T   T   D   S   L   N   S   S   K   S   P   H   I   G   N>
5701 AGC TTT TTA CCT GAT AAT TCT CTT CCT GTA TTA AAT CAG GAC TTA ACC TCC AGT GGA CAA
      S   F   L   P   D   N   S   L   P   V   L   N   Q   D   L   T   S   S   G   Q>
5761 AGT ACC AGC AGC TCA TCA GCT CTT TCT ACC CCA CCT CCT GCT GGG CAG AGT CCG GCT CAA
      S   T   S   S   S   S   A   L   S   T   P   P   P   A   G   Q   S   P   A   Q>
5821 CAA GGC TCA GGA GTG AGT GGA GTT CAG CAG GTC AAT GGC GTG ACA GTG GGG GCA CTA GCT
      Q   G   S   G   V   S   G   V   Q   Q   V   N   G   V   T   V   G   A   L   A>
5881 AGT GGA ATG CAG CCT GTA ACT TCC ACC ATT CCT GCC GTG TCT GCA GTG GGT GGA ATA ATT
      S   G   M   Q   P   V   T   S   T   I   P   A   V   S   A   V   G   G   I   I>
5941 GGA GCT TTG CCA GGT AAC CAA CTG GCA ATT AAT GGC ATT GTA GGA GCT TTA AAT GGG GTT
      G   A   L   P   G   N   Q   L   A   I   N   G   I   V   G   A   L   N   G   V>
6001 ATG CAG ACT CCT GTC ACA ATG TCC CAG AAC CCT ACC CCT CTC ACC CAC ACA ACC GTA CCA
      M   Q   T   P   V   T   M   S   Q   N   P   T   P   L   T   H   T   T   V   P>
6061 CCT AAT GCA ACA CAT CCA ATG CCA GCT ACA CTG ACT AAC AGT GCC TCA GGA CTA GGA TTA
      P   N   A   T   H   P   M   P   A   T   L   T   N   S   A   S   G   L   G   L>
6121 CTT TCT GAC CAG CAA CGA CAA ATA CTT ATT CAT CAA CAG CAG TTT CAG CAG TTG TTA AAT
      L   S   D   Q   Q   R   Q   I   L   I   H   Q   Q   Q   F   Q   Q   L   L   N>
6181 TCT CAA CAG CTC ACA CCA GAA CAA CAT CAA GCC TTT TTG TAT CAG TTA ATG CAA CAT CAC
      S   Q   Q   L   T   P   E   Q   H   Q   A   F   L   Y   Q   L   M   Q   H   H>
6241 CAC CAG CAG CAG CAC CAA CCT GAA CTT CAG CAG CTG CAG ATC CCT GGA CCA ACA CAA ATA
      H   Q   Q   Q   H   Q   P   E   L   Q   Q   L   Q   I   P   G   P   T   Q   I>
6301 CCC ATA AAC AAC CTT CTT GCG AGG TCC ACA GCA CCC CCA CTT CAC ACA GCT ACC ACC AAC
      P   I   N   N   L   L   A   R   S   T   A   P   P   L   H   T   A   T   T   N>
6361 CCA TTT CTC ACC ATC CAT GGA GAT AAT GCA AGT CAG AAA GTA GCA AGA CTT AGT GAT AAA
      P   F   L   T   I   H   G   D   N   A   S   Q   K   V   A   R   L   S   D   K>
6421 ACT GGG CCT GTA GCT CAA GAG AAA AGT TGA CAC CTG AGA AAC ATC TAG AAA TTG CCT ATC
      T   G   P   V   A   Q   E   K   S   *>
6481 CTG CTG TTC TAG CAC TTC ATC TGG CTG CCT TTG CAG TCC TTT TAC TAC AGC TAT GAA GAA
6541 ACG CAA CAA GAA ACT CAA TGC ACA ACA AAG GAT TAA TTG CTG CAA GGA CAT TCT TGT AAG
6601 GCT TGA ATT CCC GGG GGA TCC ACT AGT TCT AGA GCG GCC GCC ACC GCG GGC CCG GGA TCC
6661 ACC GGA TCT AGA TAA CTG ATC ATA ATC AGC CAT ACC ACA TTT GTA GAG GTT TTA CTT GCT
6721 TTA AAA AAC CTC CCA CAC CTC CCC CTG AAC CTG AAA CAT AAA ATG AAT GCA ATT GTT GTT
6781 GTT AAC TTG TTT ATT GCA GCT TAT AAT GGT TAC AAA TAA AGC AAT AGC ATC ACA AAT TTC
6841 ACA AAT AAA GCA TTT TTT TCA CTG CAT TCT AGT TGT GGT TTG TCC AAA CTC ATC AAT GTA
6961 AAT CAG CTC ATT TTT TAA CCA ATA GGC CGA AAT CGG CAA AAT CCC TTA TAA ATC AAA AGA
7021 ATA GAC CGA GAT AGG GTT GAG TGT TGT TCC AGT TTG GAA CAA GAG TCC ACT ATT AAA GAA
7081 CGT GGA CTC CAA CGT CAA AGG GCG AAA AAC CGT CTA TCA GGG CGA TGG CCC ACT ACG TGA
7141 ACC ATC ACC CTA ATC AAG TTT TTT GGG GTC GAG GTG CCG TAA AGC ACT AAA TCG GAA CCC
7201 TAA AGG GAG CCC CCG ATT TAG AGC TTG ACG GGG AAA GCC GGC GAA CGT GGC GAG AAA GGA
7261 AGG GAA GAA AGC GAA AGG AGC GGG CGC TAG GGC GCT GGC AAG TGT AGC GGT CAC GCT GCG
7321 CGT AAC CAC CAC ACC CGC CGC GCT TAA TGC GCC GCT ACA GGG CGC GTC AGG TGG CAC TTT
7381 TCG GGG AAA TGT GCG CGG AAC CCC TAT TTG TTT ATT TTT CTA AAT ACA TTC AAA TAT GTA
7441 TCC GCT CAT GAG ACA ATA ACC CTG ATA AAT GCT TCA ATA ATA TTG AAA AAG GAA GAG TCC
7501 TGA GGC GGA AAG AAC CAG CTG TGG AAT GTG TGT CAG TTA GGG TGT GGA AAG TCC CCA GGC
7561 TCC CCA GCA GGC AGA AGT ATG CAA AGC ATG CAT CTC AAT TAG TCA GCA ACC AGG TGT GGA
7621 AAG TCC CCA GGC TCC CCA GCA GGC AGA AGT ATG CAA AGC ATG CAT CTC AAT TAG TCA GCA
7681 ACC ATA GTC CCG CCC CTA ACT CCG CCC ATC CCG CCC CTA ACT CCG CCC AGT TCC GCC CAT
7741 TCT CCG CCC CAT GGC TGA CTA ATT TTT TTT ATT TAT GCA GAG GCC GAG GCC GCC TCG GCC
7801 TCT GAG CTA TTC CAG AAG TAG TGA GGA GGC TTT TTT GGA GGC CTA GGC TTT TGC AAA GAT
7861 CGA TCA AGA GAC AGG ATG AGG ATC GTT TCG CAT GAT TGA ACA AGA TGG ATT GCA CGC AGG
7921 TTC TCC GGC CGC TTG GGT GGA GAG GCT ATT CGG CTA TGA CTG GGC ACA ACA GAC AAT CGG
7981 CTG CTC TGA TGC CGC CGT GTT CCG GCT GTC AGC GCA GGG GCG CCC GGT TCT TTT TGT CAA
8041 GAC CGA CCT GTC CGG TGC CCT GAA TGA ACT GCA AGA CGA GGC AGC GCG GCT ATC GTG GCT
8101 GGC CAC GAC GGG CGT TCC TTG CGC AGC TGT GCT CGA CGT TGT CAC TGA AGC GGG AAG GGA
8161 CTG GCT GCT ATT GGG CGA AGT GCC GGG GCA GGA TCT CCT GTC ATC TCA CCT TGC TCC TGC
8221 CGA GAA AGT ATC CAT CAT GGC TGA TGC AAT GCG GCG GCT GCA TAC GCT TGA TCC GGC TAC
8281 CTG CCC ATT CGA CCA CCA AGC GAA ACA TCG CAT CGA GCG AGC ACG TAC TCG GAT GGA AGC
8341 CGG TCT TGT CGA TCA GGA TGA TCT GGA CGA AGA GCA TCA GGG GCT CGC GCC AGC CGA ACT
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8401 GTT CGC CAG GCT CAA GGC GAG CAT GCC CGA CGG CGA GGA TCT CGT CGT GAC CCA TGG CGA
8461 TGC CTG CTT GCC GAA TAT CAT GGT GGA AAA TGG CCG CTT TTC TGG ATT CAT CGA CTG TGG
8521 CCG GCT GGG TGT GGC GGA CCG CTA TCA GGA CAT AGC GTT GGC TAC CCG TGA TAT TGC TGA
8581 AGA GCT TGG CGG CGA ATG GGC TGA CCG CTT CCT CGT GCT TTA CGG TAT CGC CGC TCC CGA
8641 TTC GCA GCG CAT CGC CTT CTA TCG CCT TCT TGA CGA GTT CTT CTG AGC GGG ACT CTG GGG
8701 TTC GAA ATG ACC GAC CAA GCG ACG CCC AAC CTG CCA TCA CGA GAT TTC GAT TCC ACC GCC
8761 GCC TTC TAT GAA AGG TTG GGC TTC GGA ATC GTT TTC CGG GAC GCC GGC TGG ATG ATC CTC
8821 CAG CGC GGG GAT CTC ATG CTG GAG TTC TTC GCC CAC CCT AGG GGG AGG CTA ACT GAA ACA
8881 CGG AAG GAG ACA ATA CCG GAA GGA ACC CGC GCT ATG ACG GCA ATA AAA AGA CAG AAT AAA
8941 ACG CAC GGT GTT GGG TCG TTT GTT CAT AAA CGC GGG GTT CGG TCC CAG GGC TGG CAC TCT
9001 GTC GAT ACC CCA CCG AGA CCC CAT TGG GGC CAA TAC GCC CGC GTT TCT TCC TTT TCC CCA
9061 CCC CAC CCC CCA AGT TCG GGT GAA GGC CCA GGG CTC GCA GCC AAC GTC GGG GCG GCA GGC
9121 CCT GCC ATA GCC TCA GGT TAC TCA TAT ATA CTT TAG ATT GAT TTA AAA CTT CAT TTT TAA
9181 TTT AAA AGG ATC TAG GTG AAG ATC CTT TTT GAT AAT CTC ATG ACC AAA ATC CCT TAA CGT
9241 GAG TTT TCG TTC CAC TGA GCG TCA GAC CCC GTA GAA AAG ATC AAA GGA TCT TCT TGA GAT
9301 CCT TTT TTT CTG CGC GTA ATC TGC TGC TTG CAA ACA AAA AAA CCA CCG CTA CCA GCG GTG
9361 GTT TGT TTG CCG GAT CAA GAG CTA CCA ACT CTT TTT CCG AAG GTA ACT GGC TTC AGC AGA
9421 GCG CAG ATA CCA AAT ACT GTC CTT CTA GTG TAG CCG TAG TTA GGC CAC CAC TTC AAG AAC
9481 TCT GTA GCA CCG CCT ACA TAC CTC GCT CTG CTA ATC CTG TTA CCA GTG GCT GCT GCC AGT
9541 GGC GAT AAG TCG TGT CTT ACC GGG TTG GAC TCA AGA CGA TAG TTA CCG GAT AAG GCG CAG
9601 CGG TCG GGC TGA ACG GGG GGT TCG TGC ACA CAG CCC AGC TTG GAG CGA ACG ACC TAC ACC
9661 GAA CTG AGA TAC CTA CAG CGT GAG CTA TGA GAA AGC GCC ACG CTT CCC GAA GGG AGA AAG
9721 GCG GAC AGG TAT CCG GTA AGC GGC AGG GTC GGA ACA GGA GAG CGC ACG AGG GAG CTT CCA
9781 GGG GGA AAC GCC TGG TAT CTT TAT AGT CCT GTC GGG TTT CGC CAC CTC TGA CTT GAG CGT
9841 CGA TTT TTG TGA TGC TCG TCA GGG GGG CGG AGC CTA TGG AAA AAC GCC AGC AAC GCG GCC
9901 TTT TTA CGG TTC CTG GCC TTT TGC TGG CCT TTT GCT CAC ATG TTC TTT CCT GCG TTA TCC
9961 CCT GAT TCT GTG GAT AAC CGT ATT ACC GCC ATG CAT
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Figure 71: pEGFP-CALM/AF10 construct used for the cloning procedures. The nucleotides in bold format in the sequence represent the excised fragment which contained 4 stop codons (in blue). The *Xba1* restriction sites are underlined.

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12. CURRICULUM VITAE

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1974 – 1981	Junior High School: Escola NS da Conceicão, Taquari, RS, Brazil
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