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Transformationspotential von *NPM-ALK*, *p21SNFT* und *Tax* in Reifen T Lymphozyten

Dissertation
zur Erlangung des Doktorgrades der Medizin
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vorgelegt von

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aus Kandiari/Pakistan

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From
Institute for Biomedical Research
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Abstract in German

Bislang ist nicht bekannt, in welchem Differenzierungsstadium reife T- Zell- Leukämien/ Lymphome initiiert werden. Frühere Studien in unserer Gruppe haben eine Resistenz reifer T-Zellen gegenüber der Transformation gezeigt. Nun sollte das transformierende Potential von *NPM-ALK, p21SNFT* und des viralen Onkoproteins *Tax* in reifen T- Zellen weiter untersucht werden. Zunächst wurden die Effekte der drei Proteine auf das Zellwachstum in vitro untersucht, indem humane T- Zelllinien mit gammaretroviralen Vektoren, die diese Gene kodierten, transduziert wurden. Für alle drei Gene konnte kein proliferationsfördernder Effekt beobachtet werden. Im zweiten Teil des Projekts wurden murine reife T- Zellen oder hämatopoetische Stammzellen (HPCs/ HSCs) mit diesen Vektoren transduziert und in Rag-1 knockout Mäuse transplantiert. Alle Mäuse, die mit *NPM-ALK* transduzierten monoklonalen reifen T- Zellen (OT-1) transplantiert wurden, entwickelten Leukämien/ Lymphome. Im Gegensatz dazu entwickelten nur wenige der mit *NPM-ALK* transduzierten polyklonalen T-Zellen oder HPCs/ HSCs transplantierten Tiere Leukämien/ Lymphome. In der *p21SNFT* Gruppe zeigten nur zwei der Mäuse, die mit transduzierten OT-1 T- Zellen transplantiert wurden, Leukämien/ Lymphome. Diese exprimierten eGFP in hohem Maße und interessanterweise auch CD19. Für alle *Tax* transplantierten Tiere konnten bislang keine Leukämien/ Lymphome beobachtet werden. Das weiteren zeigten diese Tiere keine eGFP Expression im peripheren Blut.

Zusammenfassend zeigen diese Resultate, dass monoklonale T- Zellen verglichen mit polyklonalen T- Zellen nach gammaretroviralem Transfer von *NPM-ALK* oder *p21SNFT* leichter transformierbar sind.

Abstract in English

To date it is not clear at which stage of differentiation mature T cell leukaemia/lymphoma is initiated. Previous studies in our group showed that mature T cells are relatively resistant to transformation. We wanted to further investigate the transformation potential of *NPM-ALK*, *p21SNFT* and the viral oncoprotein *Tax* on mature T cells. First, we analyzed the effects on T cell growth in vitro after transducing human T cell lines with gammaretroviral vectors encoding these genes. No growth or proliferation promoting effect of all three genes was observed. In the second part of the project, we transduced murine, mature T cells and/or haematopoietic stem cells (HPCs/HSCs) and transplanted these cells into Rag-1 deficient recipients. All mice transplanted with *NPM-ALK* transduced monoclonal mature T cells (OT-1) developed leukaemia/lymphoma. In contrast, only few *NPM-ALK* transduced polyclonal T cell and HPC/HSC transplanted mice developed leukaemia/lymphoma. From the *p21SNFT* group, only two mice transplanted with transduced OT-1 T cells developed leukaemia/lymphoma, which showed high eGFP and interestingly CD19 expression. No malignancies were observed in *Tax* transplanted animals so far. Furthermore, the recipients do not show any eGFP marking in the periphery.

In conclusion, our results show that compared to polyclonal T cells, monoclonal T cells are transformable after gammaretroviral transfer of *NPM-ALK* and *p21SNFT*.

1. Introduction

1.1 Haematopoiesis

1.1.1 Definition of haematopoiesis

Haematopoiesis is a Greek word that means production of blood (haimatos - blood, poiesis - production). All blood components are derived from the haematopoietic stem cell. Growth factors and cytokines play an important role in the maturation of different cells of blood and the immune system. During the embryonic period of life, the haematopoiesis begins in the extraembryonic mesoderm. During mid gestation, the main haematopoetic organs are liver and spleen. At the end of the gestation, it takes place in the bone marrow, where it remains throughout the adult life [1,2].

Blood contains different types of cells to fulfil different functions. The haematopoietic stem cell (HSC) has the ability of self-renewal and differentiation to all blood cell types [1,3,4]. In figure 1, the hierarchy of haematopoiesis is shown. The HSC can maintain haematopoiesis of an organism due to its pluripotent and lifelong self-renewal ability. It is accepted that the number of these HSCs is relatively conserved within the group of the mammals [5]. This was described for cat, mouse and humans and is about 10.000-20.000 HSCs per organism. The monoclonal regeneration from each HSC of the complete haematological system takes about 15 cell divisions. This points at the enormous stress, regeneration ability and transformation resistance of HSCs. From this cell two main lines are derived, the lymphoid (common lymphoid progenitor, CLP), which inturn differentiates to give rise to T cells, B cells, and natural killer cells, and the myeloid (common myeloid progenitor, CMP), which differentiates into neutrophils, eosinophils, monocytes/macrophages, basophils, erythrocytes and platelets.

For myeloid leukaemias there is growing evidence that they originate in haematopoietic stem cells/haematopoietic progenitor cells (HSCs/HPCs) or early myeloid progenitors [6,7]. However, compared with other mature cell lineages, fully differentiated lymphocytes claim a special position in haematopoiesis. They show long life-spans, sustained proliferation and the ability of self-renewal [8]. For B cells it was recently shown that fully mature B lymphocytes can still be transformed [9]. However, for mature T cells, several observations indicate that these cells are less susceptible to transformation than HSCs/HPCs. Here, we describe the development and maturation of T cells in more detail (see 1.1.2) as they are used in the available work to further investigate the transformation susceptibility of mature T cells.

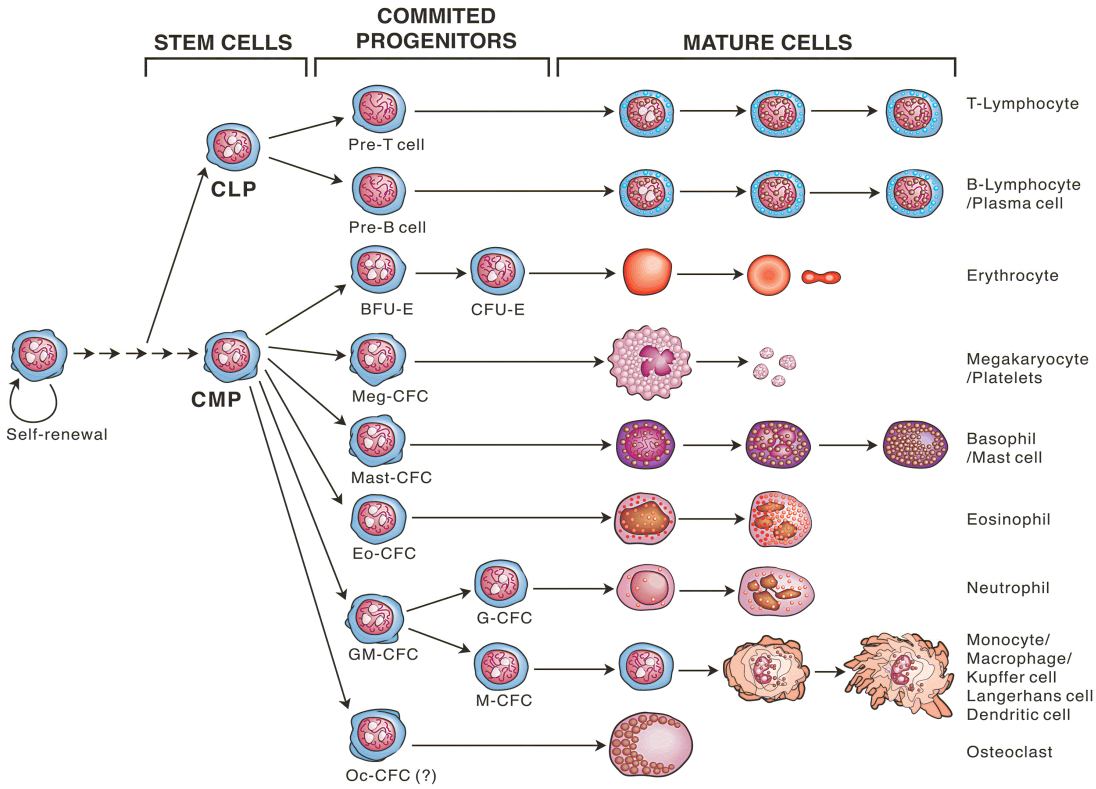


Figure 1. Hierarchy of haematopoiesis. The pluripotent haematopoietic stem cell has the capability of self-renewal, and it gives rise to all blood-cell types. The T lymphoid lineage in mature stage is highlighted (blue box), since experiments in the available thesis are mainly focused on these cells. Modified according to [10].

1.1.2 Development of mature T cells

T lymphocytes play an important role in the adaptive immune response. These CD3+ cells express a T cell receptor (TCR) on their cell surface which recognizes foreign antigens. T lymphocytes do not recognize free but cell bound antigens, which are presented to them by other cells (APC, antigen presenting cells) by means of MHC (major histocompatibility complex) molecules [11]. While these cells develop in the thymus, they rearrange the genes for the TCR. Either TCRα and TCRβ or TCRγ and TCRδ can be rearranged by the activity of the recombinase enzymes RAG-1 and RAG-2 [11,12]. The factors involved in the rearrangement of TCRα and TCRβ or TCRγ and TCRδ are not well-understood. This rearrangement leads to a very large diversity within the T cell population. Theoretically, a diversity of approximately 10^{18} different TCRs is possible, whereby for each antigen a specific T cell clone with a suitable TCR is conceivable. But the large diversity harbours the risk of self-reactivity. Therefore, during the selection process in T cell development, the essential self-tolerance and the broad spectrum against potential pathogens are maintained. During its development, the undifferentiated T cell progenitor migrates from the bone-marrow to the

thymus and goes through two fundamental selection stages: the positive and negative selection. The thymus stroma carries essential molecules like interleukins or MHC proteins for the T cell-development that act by direct cell cell contact. The developing lymphocytes whose receptors interact weakly with self antigens in connection with self MHC molecules, or bind in a particular way, receive a signal that enables them to survive; this type of selection is known as positive selection. This selection ensures that an individual will have a repertoire of T cells that can respond to peptides bound to his or her own MHC molecules.

In contrast, lymphocytes whose receptors bind strongly to self antigen-MHC complex receive signals that lead to their death via apoptosis; this type of selection is known as negative selection. Strongly self-reactive lymphocytes are therefore removed from the repertoire before they become fully mature and might initiate damaging autoimmune reactions. In this way immunological “self-tolerance” is established to ubiquitous self antigens [13]. In the thymus, the earliest cell population does not express CD4, CD8 and T cell receptor complex molecules (CD3, and the TCR α and β chains), they are called ‘double-negative’ (DN) thymocytes. These precursor cells give rise to two T cell lineages: the minority population of γ:δ T cells (which lack CD4 or CD8 even when mature), and the majority α:β T cell lineage. The development of prospective α:β T cells proceeds through stages in which both CD4 and CD8 are expressed by the same cell; these are known as ‘double-positive’ (DP) thymocytes. At first, double-positive cells express the pre-T cell receptor (p α:β T). These cells enlarge and divide. Later, they become small resting double-positive cells in which low level of T cell receptor (α:β) itself is expressed. Most of the thymocytes (about 98%) die within the thymus after becoming small double-positive cells. Those cells whose receptors can interact with self-peptide:self-MHC class I molecular complexes lose the expression of CD4 and become CD8 single positive, while the cells whose receptors can interact with self-peptide:self-MHC class II molecular complexes lose the expression of CD8 and become CD4 single positive. The outcome of this process is the ‘single positive’ (SP) thymocytes, which after maturation are exported from the thymus as mature single positive CD4 or CD8 T cells [14-16].

1.2 Malignant diseases of the blood

Malignant proliferative diseases constitute the most important disorders of the blood. They are organized in two common broad categories, which are described as below:

1.2.1 Myeloid neoplasms

These neoplasms arise from haematopoietic progenitor cells that give rise to cells of the myeloid (i.e., erythroid, granulocytic, and/or thrombocytic) lineage. There are further three categories of myeloid neoplasia: acute myelogenous leukaemia, in which immature progenitor cells accumulate in the bone marrow; myelodysplastic syndromes, which are associated with ineffective haematopoiesis and resultant peripheral blood cytopenias; and chronic myeloproliferative disorders, in which increased production of one or more terminally differentiated myeloid elements (e.g., granulocytes) usually leads to elevated peripheral blood counts [17].

1.2.2 Lymphoid neoplasms

These are the neoplasms of lymphoid cells, the T cells, B cells and NK cells. The vast majority of these neoplasms (80-85%) is of B cell origin, most of the remainder being T cell tumors; only rarely are tumors of NK origin.

Lymphoid neoplasms encompass a diverse group of entities. In 1994, a group of haematopathologists, oncologists, and molecular biologists came together to create the Revised European-American Classification of Lymphoid Neoplasms (REAL). Of importance, this classification scheme incorporated objective criteria, such as immunophenotype and genetic aberrations, together with morphologic and clinical features, to define distinct clinicopathologic entities. Most entities in the REAL classification can be diagnosed reproducibly by experienced pathologists and categorize patients into good or bad prognostic groups. More recently, an international group of haematopathologists and oncologists convened by the World Health Organisation (WHO) reviewed and updated the REAL classification, resulting in the inclusion of a number of additional rare entities [17]. The WHO classification sorts the lymphoid neoplasms into four broad categories, based on their cell of origin and immunophenotype:

1. Precursor B cell neoplasms (neoplasms of immature B cells)
2. Peripheral B cell neoplasms (neoplasms of mature B cells)
3. Precursor T cell neoplasms (neoplasms of immature T cells)
4. Peripheral T cell and NK cell neoplasms (neoplasms of mature T cells and natural killer cells). The sub types of this neoplasm are as under:

    - Adult T cell leukaemia/lymphoma
    - Anaplastic large cell lymphoma
    - Mycosis fungoides/Sezary syndrome

- Large granular lymphocytic leukaemia
- T cell prolymphocytic leukaemia
- Peripheral T cell lymphoma, unspecified
- Angioimmunoblastic T cell lymphoma
- Enteropathy-associated T cell lymphoma
- Panniculitis-like T cell lymphoma
- Hepatosplenic γδ T cell lymphoma
- NK/T cell lymphoma, nasal type
- NK cell leukaemia

The immature B cell and T cell leukaemia/lymphoma originate in the lymphoid progenitor cells in the bone marrow and the thymus respectively. For the mature B cell neoplasms, it has recently been shown that mature B cells are transformable [9]. But for the mature T cell neoplasms, it has not yet been known whether they originate from the mature T cells or the progenitor cells. To investigate this question, we have used the following potent T cell oncogenes in the available work:

1.3 Used potent T cell oncogenes

- *NPM-ALK*, the fusion oncogene
- *Tax*, the HTLV-1 viral oncoprotein
- *p21SNFT* (T cell potential oncogene)

1.3.1 *NPM-ALK* and anaplastic large cell lymphoma

NPM–ALK (nucleophosmin-anaplastic lymphoma kinase) is a fusion oncogene derived from the chromosomal translocation t(2;5)(p23;q35). This translocation fuses the gene encoding ALK receptor tyrosine kinase located on chromosome 2p23 with the housekeeping gene nucleophosmin (NPM) on chromosome 5q35. *NPM-ALK* chimeric gene encodes a constitutively activated tyrosine kinase that is oncogenic [18]. The ALK is the oncogene of most anaplastic large cell lymphomas (ALCL), deriving transformation through many molecular mechanisms. Nevertheless, ~15-40% of ALCL do not express ALK or other recurrent translocations. Approximately 70-80% of ALK-positive ALCLs express the NPM–ALK fusion protein, while the remaining 20-30% of ALK-positive ALCLs express other fusions like TPM3-ALK, TFG-ALK and CTLC1-ALK. ALCL has a peak incidence in childhood,

accounting for approximately 40% of NHL cases diagnosed in paediatric patients, whereas it accounts for <5% of NHL in adults and it is seen mostly in males.

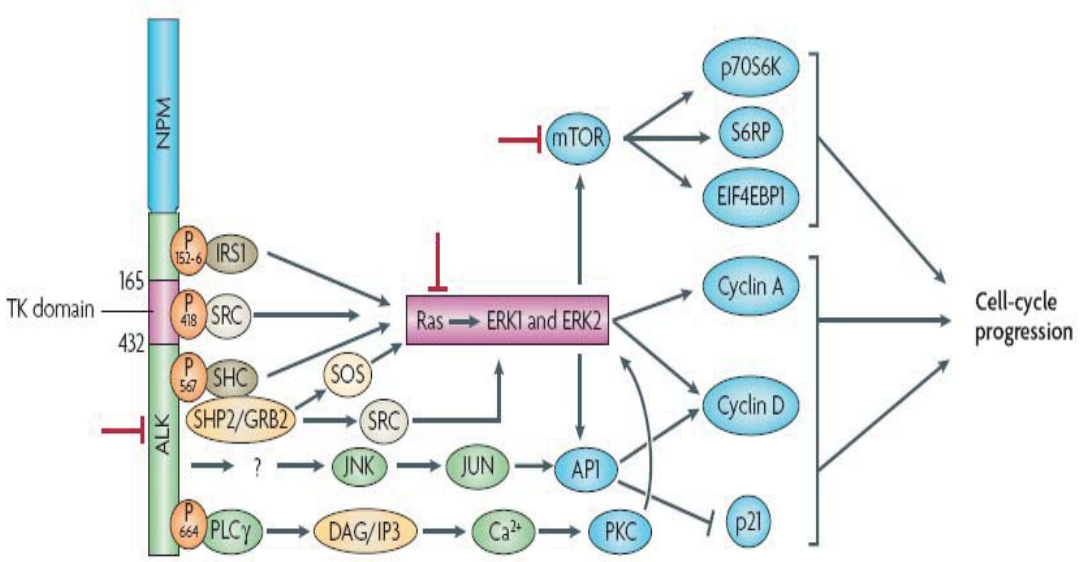


Figure 2. *NPM-ALK* and Ras–ERK Pathway. Mitogenic signalling by NPM–ALK is mainly due to the activation of the Ras–extracellular signal regulated kinase (ERK) pathway through the direct binding of insulin receptor substrate 1 (IRS1), SRC homology 2 domain-containing (SHC) and SRC to specific tyrosine residues of ALK. The SHP2–GRB2 (growth factor receptor-bound protein 2) complex interacts with ALK and SHC to enhance phosphorylation of ERK1 and ERK2 through SRC. Phospholipase C-γ (PLCγ) is directly bound and activated by ALK to trigger mitogenic stimuli by the generation of diacylglycerol (DAG) and inositol triphosphate (IP3), which, in turn, mobilize calcium stores from the endoplasmic reticulum and activate protein kinase C (PKC). ALK-induced mammalian target of rapamycin (mTOR) activation is transduced through the ERK signalling pathway and ends in the phosphorylation of the mTOR targets ribosomal protein S6 kinase (p70S6K) and S6 ribosomal protein (S6RP), which in turn stimulate protein translation and ribosome biogenesis. Activation of mTOR also leads to phosphorylation and inactivation of the eukaryotic initiation factor 4E-binding protein 1 (EIF4EBP1), dissociating EIF4EBP1 from the RNA cap-binding protein EIF4E, thus promoting cap-dependent translation of mRNA. Note that the NPM–ALK fusion protein is not drawn to scale. Potential therapeutic targets (indicated by red inhibitory arrows) are already being validated in clinical trials. JNK: JUN N-terminal kinase; SOS: son of sevenless. Modified according to [19].

NPM-ALK exerts its oncogenic effects via three main well characterized pathways [19]:

1. Ras–extracellular signal regulated kinase (ERK) pathway which leads to mitogenic signaling.
2. Phosphatidylinositol 3-kinase (PI3K) pathway which results in the antiapoptotic signals.
3. Janus kinase 3 (JAK3)–STAT3 pathway which leads to increased survival and proliferation.

Overall, the Ras–ERK pathway is essential mostly for ALCL proliferation which is described in more detail in the figure 2, whereas the JAK3–STAT3 pathway and the PI3K–Akt pathway have been shown to be vital primarily for cell survival and phenotypic changes.

1.3.2 *Tax* and adult T cell leukaemia/lymphoma

Tax is a viral oncoprotein that is encoded by the Human T cell leukaemia virus type 1 (HTLV-1). The *Tax* oncoprotein causes mature T cell malignancy, the adult T cell leukaemia/lymphoma (ATL). Only a small minority of HTLV-1-infected dividuals progress to ATL. Indeed, the cumulative risks of developing ATL among virus carriers are estimated to be approximately 6.6% for males and 2.1% for females. *Tax* exerts its oncogenic effects via different pathways to cause the increased cell proliferation, abnormal cell cycle, DNA damage and inhibition of apoptosis (see figure 3) [20,21].

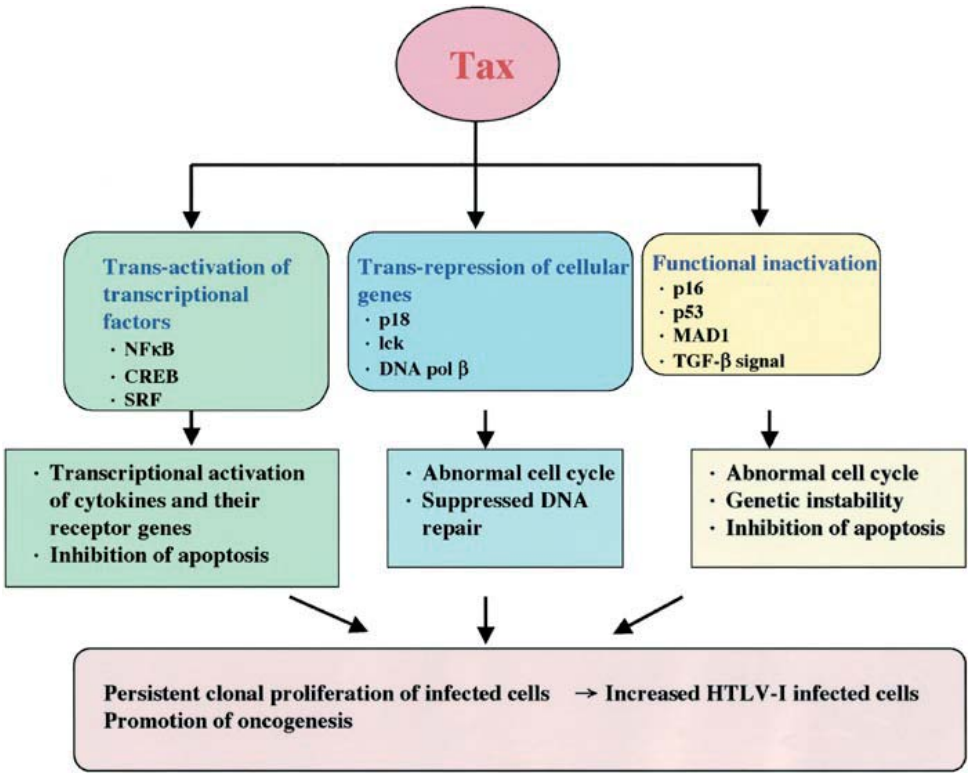


Figure 3. Pleiotropic activities of *Tax*. The oncogenic effects of *Tax* via different pathways are depicted which lead to increased cell proliferation, abnormal cell cycle, DNA damage and inhibition of apoptosis.

The HTLV-1 *Tax* protein is required for the virus to transform cells [22]; however, *Tax* transcripts are detected in only ~40% of all ATLs. Analyses of HTLV-1 proviruses and *Tax* transcripts in ATL cells revealed three ways in which cells can silence *Tax* expression, which are:

(i) Accumulation of nonsense mutations, insertions and deletions in *Tax* [23,24].

(ii) Silencing viral transcription by DNA methylation of the Provirus [24,25].

(iii) Deletion of the proviral 5' LTR [26].

The currently accepted view for the silencing of *Tax* expression by the cells is that *Tax* is needed early after infection with HTLV-1 to initiate transformation, but is not required later to maintain the transformed phenotype of ATL cells. As *Tax* is the main target of the host's CTLs (cytotoxic T lymphocytes), cells that down-regulate *Tax* expression (using one of the three genetic or epigenetic means described above) have an advantage in evading immunosurveillance and are preferentially selected *in vivo* during disease progression [24].

1.3.3 p21SNFT (T cell potential oncogene)

p21SNFT (21-kDa small nuclear factor isolated from T cells) is a human protein of the basic leucine zipper family. The over expression of *p21SNFT* causes specific repression of both human IL-2 promoter activity and the production of IL-2 by activated Jurkat cells. IL-2 (interleukin 2) is the major autocrine and paracrine growth factor for T cells and its production is indicative of T cell activation. IL-2 is highly regulated by multiple transcription factors, particularly AP-1, which coordinately activate the IL-2 promotor. The transcription factor AP-1 consists of two heterodimers, the Fos and the Jun. *p21SNFT* competes with the Fos protein for Jun dimerization. This structural change in the AP-1 by *p21SNFT* causes repressive effect on the IL-2 promotor and thereby IL-2 production [27]. But surprisingly, microarray analysis has shown that *p21SNFT* is up-regulated in T cell leukaemia/lymphoma [28]. To investigate its potential leukemogenic effects, we have transduced this gene into murine stem cells and mature T cells.

1.4 Gene Transfer

1.4.1 Definition of gene transfer

In broad term, gene transfer is the transfer of one DNA molecule to another DNA molecule. It is used for two main purposes, gene therapy and research. Here, we focus on gene transfer into cells, for which there are different methods which are described as below (see section 1.4.2).

1.4.2 Methods for gene transfer

Viral and non viral methods can be used for gene transfer (see figure 4). In viral methods, selected viral genomes are used to carry the gene of interest. Different viruses are used for this purpose (see section 1.4.3). The non viral methods consist of nacked DNA, DNA/Lipid:

Liposomal complex and bacterial vector methods. The genetic information is transferred by two different procedures, *ex vivo* or *in vivo*. In *ex vivo* gene transfer, the target cells are genetically modified (with gene of interest) out side the body and then transplanted back to the patient. In contrast to this, in *in vivo* gene transfer, the genetic information (gene of interest) is directly applied to the patient via different methods like local injection, systemic infusion, or gene gun.

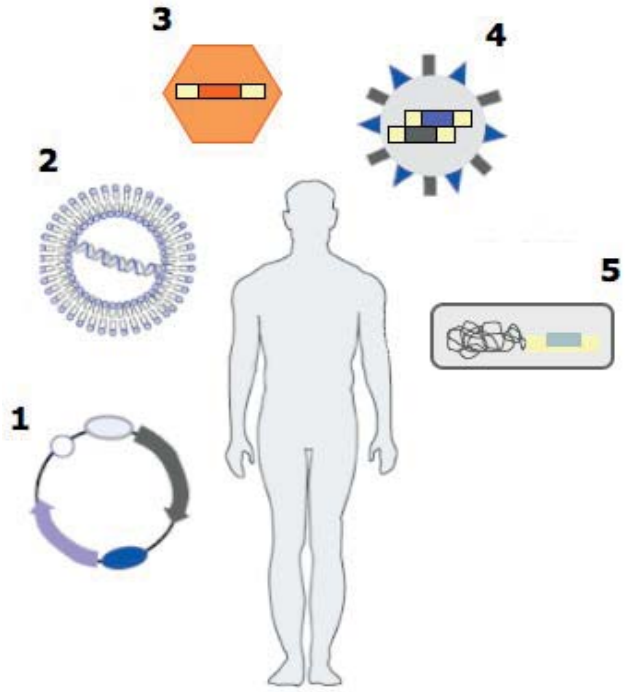


Figure 4. Gene Transfer-Vectors/-Methods. Non viral methods: Nacked DNA (1), DNA/Lipid: Liposomal complex (2), Bacterial vector (5). Viral methods: Adenoviral vector (3), Retroviral vector (4).

Here, we describe the viral methods as they are used in the available work (see 1.4.3).

1.4.3 Viral Gene-Transfer Vectors

Viral gene transfer vectors are derived from multiple viruses: adenoviral vectors, retroviral vectors derived from mouse retroviruses or lentiviruses, parvoviral vectors based on adeno-associated viruses, vectors derived from vaccinia virus, baculovirus, simian virus 40, epstein barr virus or herpes simplex virus.

Retroviral and adenoviral vectors are most widely used. However, only the retroviral vectors ensure a stable and persistent genome integration [29]. Retroviral vectors have some

disadvantages, like, their small transgene capacity of approximately 8-10kb. Further, they show a high recombination and mutation rate. They, with the exception of lentiviral vectors, can infect only dividing cells. During vector production, replication competent retrovirus can be generated [30]; but, the use of 'three plasmid system' enables production of replication-incompetent viruses. In table 1, some important characteristics of viral vectors are listed [31,32].

Table 1. Major groups of retroviral vectors.

Category	Retrovirus	AAV*	Adenovirus	Vaccinia-Virus	Herpes virus	Autonomous Parvovirus
Genome	ssRNA	ssDNA	dsDNA	dsDNA	dsDNA	ssDNA
Transgene capacity	8-10kb	<5kb	8-30kb	25kb	40-150kb	<4kb
Titer	10^8	10^{10}	10^{11}	10^8	10^7	10^8
Genome integration	yes	no, >10%	no	no	no	no
Transduction (quiescent cells)	no #	yes	yes	yes	yes	no
Immune reaction	light	strong	strong	strong	strong	unknown

* Adeno-associated Viruses. # except Lentiviral vectors (18 kb).

In this thesis, gammaretroviral vectors were used, derived from murine leukaemia viruses from the genus of the gammaretrovirus.

1.5 Retroviruses and their use in gene-transfer

1.5.1 Retroviruses and their genome

Retroviruses belong to the family of enveloped RNA viruses. They `reverse transcribe' (RT) their genome from RNA to DNA [33]. The virion measures 120 nm in diameter and contains two identical copies of positive strand RNA genome complexed with Nucleocapsid proteins. The Nucleocapsid, which is the inner portion of the virion, also contains Reverse

transcriptase (RT), Integrase (IN) and Protease (PR) proteins. A protein shell, formed by Capsid proteins, encloses the Nucleocapsid and delimits the viral core [34]. Matrix (MA) proteins form a layer outside the core and interact with an envelope consisting of a lipid bilayer, which surrounds the viral core particle. The envelope originates from the cellular membrane and incorporates viral Envelope glycoproteins (Env). The Envelope glycoprotein, responsible for the virus interaction with the specific receptor, is the only viral protein on the surface of the particle. It is formed by two subunits: TM (transmembrane), which anchors the protein into the envelope membrane, and SU (surface), which binds to the cellular receptor. Figure 5 shows the structure of a retroviral particle.

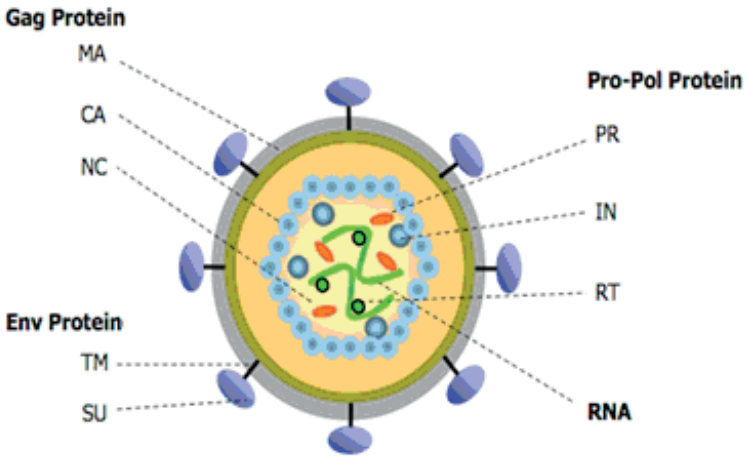


Figure 5: Structure of a retroviral particle. Gag proteins: Matrix-Protein (MA), Capsid (CA), Nucleocapsid (NC); Pro-Pol proteins: Protease Protein (PR), Integrase (IN), Reverse Transcriptase (RT); Env Protein: Transmembrane-Protein (TM), Surface-Proteine (SU).

In the genome of all retroviruses, the basic translated region is organised in four genes:

1. Gag, which encodes three main structural proteins: Matrix (MA), Capsid (CA) and Nucleocapsid (NC).
2. Pro, which encodes the Protease (PR), responsible for gag and gag-pol cleavage and the maturation of the viral particles during or after their budding.
3. Pol, which encodes the enzyme Reverse transcriptase (RT), responsible for reverse transcription of the viral RNA to DNA during the infection process, and Integrase (IN), which catalyses the integration of the proviral DNA into the host genome.
4. Env, which encodes two subunits of the Envelope glycoprotein: surface (SU) and transmembrane (TM).

In addition to gag, pro, pol, and env, 'complex' retroviruses, such as lentiviruses have accessory genes, whose concerted activities regulate viral gene expression, assembly of

infectious particles and modulate viral replication in infected cells. Together with the genes encoding the viral proteins, the retroviral genome is flanked by cis-acting sequences such as two LTRs (long terminal repeats), which contain elements required to drive gene expression, reverse transcription and integration into the host chromosome (Figure 6).

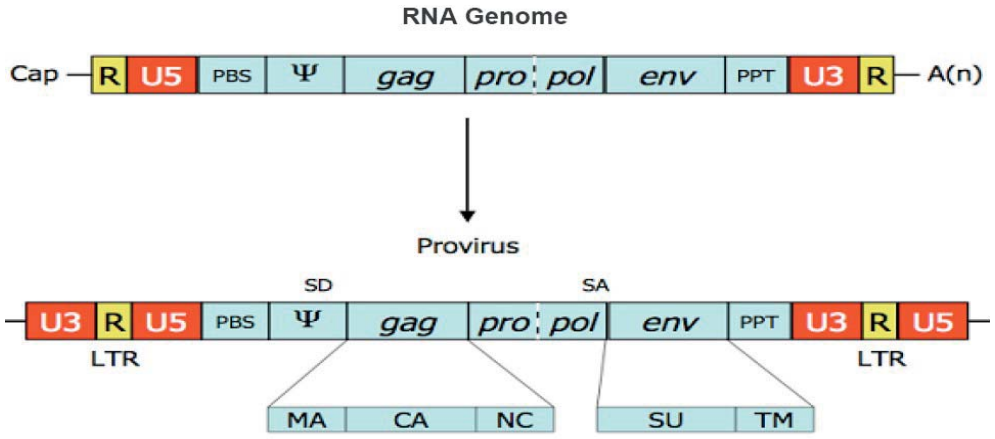


Figure 6. RNA genome and the integrated provirus of a gammaretrovirus. RNA viral genome is reverse transcribed into a DNA provirus by the activity of the viral Reverse transcriptase. During the RT process, U3 and U5 regions are duplicated and give rise to two identical LTRs (long terminal repeats). The dashed line between pro and pol indicates that the two genes share the open reading frame. Cap: 5′ capping. SD: splice donor site. SA: splice acceptor site. PPT: polypurine tract. PBS: primer binding site. A(n): poly(A) tail. Ψ: packaging signal.

The R region forms a direct repeat at both ends of the RNA genome and provides the homology sequence necessary for strand transfer during reverse transcription process. R at the 3' of the genome is polyadenylated with 50-200 noncoding adenylate residues. During the reverse transcription process, the U5 and U3 regions are duplicated. The U3 region contains promoter and enhancer elements that regulate gene expression. The untranslated region (leader region) at the 5' end of the genome contains a primer binding site (PBS), located next to the 3' end of the U5, complementary to the 3' terminus of the primer t-RNA which is used by the RT to initiate reverse transcription. A packaging signal Ψ sequence, required for the specific packaging of the RNA into newly formed virions, is located partly in leader region and partly in the first portion of the translated gag region. A polypurine tract (PPT), located upstream of U3, functions as the site of the initiation of the positive strand DNA synthesis, during reverse transcription [35].

1.5.2 Life cycle of retroviruses

The life cycle of a retrovirus begins with its interaction with the host cell surface (Figure 7). The binding of the viral glycoprotein to a specific receptor complex on the cell surface causes

virus and cell membranes to fuse and the viral particle to be internalised. After virus-cell membrane fusion, the virus core is released into the cytoplasm. The viral core is partially degraded to form a large nucleoprotein particle containing the viral genome (preintegration complex) and is transported into the nucleus.

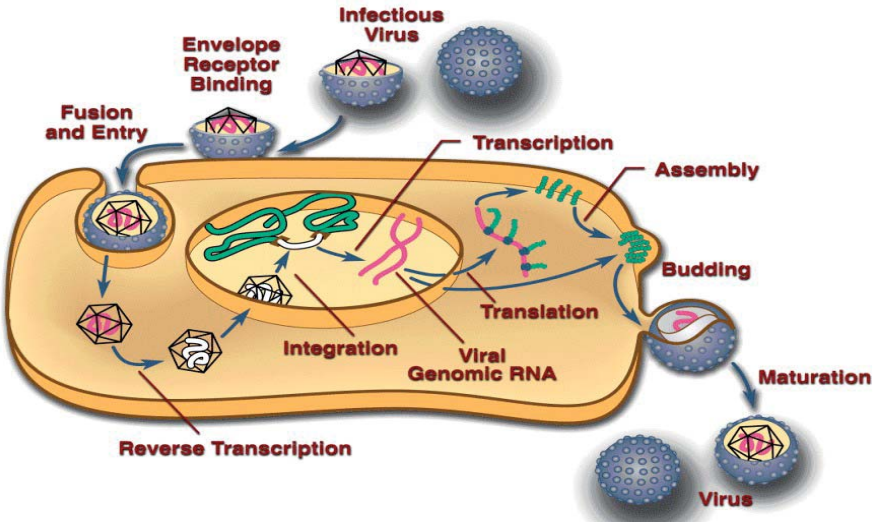


Figure 7. Retrovirus life cycle. The life cycle is completed with seven stages, which are: (1) Receptor binding, (2) Membrane fusion, (3) Reverse transcription, (4) Integration in the host genome, (5) Viral assembly, (6) Budding of viral particles, (7) Maturation of viral particles. Modified after [36]

The process of entry into the nucleus differs between oncoviruses (e.g. MLV) and lentiviruses (e.g. HIV). For HIV-1, it was established that efficient transport to the nucleus is active [37] and uses the cellular nuclear import machinery. MLV entry to the nucleus was reported to be mitosis-dependent [38, 39]. It was therefore assumed that MLV, and perhaps all oncoviruses, cannot transit through the pores of the nuclear membrane but gain the access to the nuclear area only during mitosis, when the nuclear membrane is disassembled. During this process, the viral RNA is reverse transcribed into a double stranded proviral DNA. The provirus is permanently integrated as part of the host genome, which is then transcribed to give rise to new viral genomic RNA and hence to all viral proteins required for the formation of new virions. Subsequently, viral RNA transcripts undergo splicing events in the cell nucleus, similarly to the host transcripts. Simple retroviruses can undergo single splicing, as in the case of MLV, which generates spliced mRNA expressing env. More complicated splicing occurs in complex retroviruses for the generation of the accessory proteins. The packaging signal (Ψ) in the viral RNA allows encapsidation of the unspliced RNA only, [40-42]. After assembly of the proteins around the viral RNA, the viral particle undergoes budding process from the cell membrane and takes the Env-protein. Within this particle, Protease catalyses

the polyproteins into the individual proteins like, MA, IN, RT, NC and CA. The virus particle is mature now to infect further cells with its Env receptor.

1.5.3 Production of replication incompetent retroviral particles

The 'three-plasmid system' allows the production of replication-incompetent viruses by a so-called packaging cell line (Figure 8) [43].

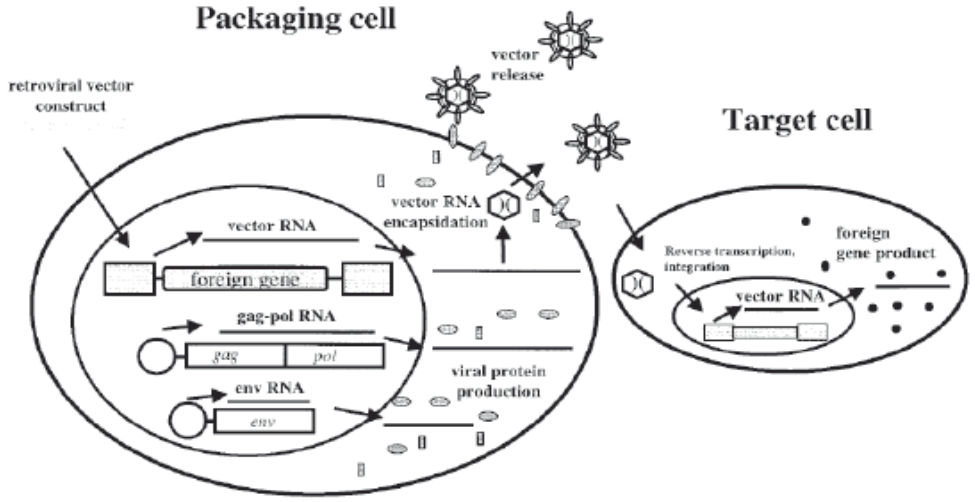


Figure 8. Three-plasmid packaging system for the production of replication-incompetent retroviral vectors. Viral particles are produced by transfection of two helper plasmids, which do not contain a packaging signal, and a transgene encoding plasmid that contains a packaging signal but does not code for any viral proteins. Transfected packaging cells produce the required gag/pol and env proteins for viral particle production. The transgenic vector is replicated and packaged into the virions. Thus, viral particles are produced that only contain the transgenic vector but no genetic information for viral replication or pathogenicity factors.

This cell line is transfected with a vector that only contains the transgene and the packaging signal (Ψ) but does not code for any viral proteins. In parallel, two further plasmids are transfected into the packaging cell line coding for env and gag/pol, respectively. These two plasmids contain the information for the production of gag/pol and env proteins for viral particle production but do not possess the packaging signal. Therefore, the transgenic vector is replicated and packaged into the virions. Thus, viral particles are produced that only contain the transgenic vector but no genetic information for viral replication or pathogenicity factors. The produced viral particles are used for transduction of target cells.

For the selective transduction of a certain cell type, replication-incompetent viruses can be targeted by pseudotyping. This process describes the production of viral vectors with a foreign envelope gene (VSV-G, gp160, HA). Pseudotyped viral particles can only infect cells expressing the respective receptor for the viral envelope protein.

Objective

The objective of my thesis was to investigate the stage of maturation at which the mature T cell leukaemia/lymphoma is initiated, because it is not yet clear whether these malignancies originate from the HSC/HPC, T cell precursors or the mature T cells themselves. As already described (see 1.1), for myeloid leukaemias there is a growing evidence that they originate in haematopoietic stem cells/haematopoietic progenitor cells (HSCs/HPSs) or early myeloid progenitors. However, compared with other mature cell lineages, fully differentiated lymphocytes claim a special position in haematopoiesis. They show long life-spans, sustained proliferation and the ability of self-renewal. For B cells, it was shown that fully mature B cells can be transformed. However, for mature T cells, several observations indicate that these cells are less susceptible to transformation than HSCs/HPCs. Among human haematologic malignancies, mature T cell leukaemias/lymphomas are less frequent, which occur predominantly in older patients and have a very poor prognosis. Therefore, to further investigate the transformation susceptibility of mature T lymphocytes in comparison with HSCs/HPCs, we transduced both of these cell types with the potent T cell oncogenes (*NPM-ALK* and *Tax)* and the potential oncogene (*p21SNFT*).

2. Materials

Chemicals used in the context of this thesis were obtained from Merck AG (Darmstadt), Roth GmbH (Karlsruhe), Santa Cruz Biotechnology Inc. (Heidelberg) and Sigma Aldrich GmbH (Hamburg).
Plastic material was obtained from Greiner Bio-One GmbH (Frickenhausen), Becton Dickinson GmbH (Heidelberg), Thermo Fischer Inc. (Bonn), Costar Inc. (Bodenheim), Corning Inc. (Schiphol-Rikjund, The Netherlands), Sarstedt AG & Co (Nürnbrecht) and Millipore GmbH (Schwalbach).
For tissue culture work, media and compounds were obtained from Lonza AG (Basel, Switzerland), Invitrogen GmbH (Karlsruhe), PAA Laboratories GmbH (Pasching, Austria) and PAN Biotech GmbH (Aidenbach, Austria).
Escherichia coli TOP10 bacteria and supplies were obtained from Invitrogen GmbH (Karlsruhe). Oligonucleotides were obtained from Sigma-Aldrich Genosys GmbH (Hamburg).

2.1 Buffers, compounds, media and plastic material

Media and buffers were prepared using deionized water from a Milli-pore filter system (Millipore GmbH, Schwalbach). Media were usually autoclaved for 20 minutes at 121°C and 2 bar pressure, while buffers were sterile filtered. Generally, buffers and compounds were stored at room temperature unless stated otherwise.

Table 2. Buffers, compounds and media for molecular biology

Buffer/Compound/Media	Ingredients/Supplier
Ampicillin stock solution	100mg/ml Ampicillin (Roth, Karlsruhe) dissolved in dH_2O
Annealing buffer (5x)	0,5M Tris (pH 7,4-7,5), 0,35M $MgCl_2$.
BW buffer (2x)	10mM Tris (pH 7,5), 1mM EDTA, 2,0M NaCl.
LB medium	20g LB (Lennox L Broth base) (Invitrogen, Karlsruhe), ad 1l dH_2O, autoclave
TAE (50 x)	1220g TRIS-HCl (Roth, Karlsruhe), 285.5g acetic acid (Roth, Karlsruhe), 500ml 0.5M EDTA (Roth, Karlsruhe), adjust to pH 8.0, ad 5l dH_2O
Agarose (1% / 2%)	1g / 2g agarose (Roth, Karlsruhe); ad 100ml dH_2O; heat in microwave until totally dissolved

Ethidiumbromide (10mg/ml)	BioRad Laboratories (München) Working solution: 0.25mg/ml Ehtidiumbromide (125µl ethidiumbromie, ad 5ml dH_2O)
DNA loading buffer (6 x)	0.05% bromophenol blue (Serva, Heidelberg), 0.05% xylene (Merck, Darmstadt), 15% Ficoll Type 400 (PAA, Pasching, Austria), ad 100% dH_2O
Dimethylsulfoxid (DMSO) (7%)	Roth (Karlsruhe) in dH_2O
DNA ladder (100bp / 1kbp)	100µl stock DNA ladder (New England Biolabs, Schwalbach), 500µl 6 x DNA loading buffer; ad 1.5ml dH_2O (store at -20°C)
LB-Agar ampicillin	32g LB-Agar (Invitrogen, Karlsruhe), ad 1l dH_2O, autoclave; add 40µg/ml ampicillin before pouring into the plates; (plates stored at 4°C)
Isopropanol	Roth (Karlsruhe)
Ethanol	Roth (Karlsruhe)
Bovine serum albumine (100 x)	New England Biolabs (Schwalbach)
NEB-buffers 1-4	New England Biolabs (Schwalbach)
dNTPs	New England Biolabs (Schwalbach)
Oligonucleotides	Sigma Genosys (Deisenhofen)
LB-ampicillin	20g LB (Lennox L Broth base) (Invitrogen, Karlsruhe), ad 1l dH_2O, autoclave, add 1ml of 100mg/ml ampicillin (Roth, Karlsruhe); (store at 4°C)

Table 3. Buffers, compounds and media for Western blotting

Buffer/Compound/Media	Ingredients/Supplier
25 x Protease Inhibitor Mix	1 tablet Protease Inhibitor Cocktail® (Roche, Basel, Switzerland); ad 2ml dH_2O; (store at 4°C)
50mM TRIS-HCl washing buffer	Roth (Karlsruhe), adjust pH to 8.0 with HCl
Acrylamid/Bisacrylamid solution (30%)	Bio-Rad Laboratories GmbH (München); (store at 4°C)
Ammonuim persulfate (APS) (10%)	10g APS (Merck, Darmstadt); ad 100ml dH_2O; (store at -20°C)
Glutaric dialdehyde (25%)	Merck (Darmstadt)
Glycerol	Merck (Darmstadt)
HCl	Merck (Darmstadt)

Isopropanol	Roth (Karlsruhe)
Milk in PBST (5%) (MBPST)	5g milk powder (Reformhaus), ad 100ml with PBST; (store at 4°C; use at day of preparation)
Neufeld washing buffer	10mM TRIS-HCl, pH 8.5; 0.6M NaCl, 0.1% SDS, 0.05% NP40
NP40 cell lysis buffer	150mM NaCl (Roth, Karlsruhe), 1% NP40 (Merck, Darmstadt), 50mM TRIS-HCl, pH 8.0 (Roth, Karlsruhe), 1mM PMSF (Sigma, Deisenhofen), 1x Protease Inhibitor Cocktail® (Roche, Basel, Switzerland); (store at 4°C)
PBS (10x)	Lonza (Basel, Switzerland)
PBS (1x)	100ml 10x DPBS (Dulbecco's phosphate buffered saline) (Lonza, Basel, Switzerland); ad 1l dH_2O
PBST (0.1%)	100ml 10x DPBS (Dulbecco's phosphate buffered saline) (Lonza, Basel, Switzerland), 1 ml Tween®-20 (Merck, Darmstadt); ad 1l dH_2O
Protein-A agarose (25%, 2 ml)	Santa Cruz (Heidelberg)
RIPA cell lysis buffer	150mM NaCl (Roth, Karlsruhe), 1% NP40 (Merck, Darmstadt), 50mM TRIS-HCl, pH 8.0 (Roth, Karlsruhe), 0.5% Sodium Deoxycholate (DOC) (Merck, Darmstadt), 0.1% SDS (Roth, Karlsruhe), 1mM PMSF (Sigma, Deisenhofen), 1x Protease Inhibitor Cocktail® (Roche, Basel, Switzerland); (store at 4°C)
SDS-PAGE running buffer (1 x)	100ml 10 x SDS-PAGE running buffer, 10ml 10% SDS solution, ad 1l dH_2O
SDS-PAGE running buffer (10 x)	30.2g TRIS-HCl (Roth, Karlsruhe), 144g glycine (Roth, Karlsruhe); adjust pH to 8.8; ad 1l dH_2O; (store at 4°C)
SDS-PAGE sample loading buffer (3x)	300mM TRIS-HCl, 9% SDS; 30% glycerol; 0.05% bromophenol blue
SDS-PAGE separation gel buffer, (1.5 M TRIS-HCl, pH 8.8)	91g TRIS-HCl (Roth, Karlsruhe), adjust pH to 8.8; ad 500ml dH_2O; (store at 4°C)

SDS-PAGE stacking gel buffer, (0.5 M TRIS-HCl, pH 6.8)	30.25g TRIS-HCl (Roth, Karlsruhe), adjust pH to 6.8; ad 500ml dH_2O; (store at 4°C)
Sodium dodecyl sulfate (SDS) (10%)	10g SDS (Roth, Karlsruhe); ad 100ml dH_2O
Tetramethylethylendiamine (TEMED)	Merck (Darmstadt); (store at 4°C)
Triton-X 100 lysis buffer	50mM HEPES (pH 7.5), 150mM NaCl, 1% Triton-X 100, 10% Glycerol, 2mM EDTA, 2% Aprotinin, 1mM PMSF, 1x Roche® Protease Inhibitor Cocktail, 2mM Pefabloc® SC
Triton-X 100, 10%	10ml Triton-X 100 (Roth, Karlsruhe); ad 100ml dH_2O
Western blot transfer buffer	200ml MeOH (Roth, Karlsruhe); 100ml 10x SDS-PAGE running buffer; ad 1l dH_2O
β-mercaptoethanol	Roth (Karlsruhe); store at 4°C

Table 4. Buffers, compounds and media for tissue culture

Buffer/Compound/Media	Ingredients (Supplier)
DMEM (Dulbecco's Modified Eagles Medium)	Containing 4.5g/l Glucose (Lonza, Basel. Switzerland); (store at 4°C)
DMEM-standard medium	500ml DMEM, 5% FCS, 1% penicillin-streptomycin (PAA, Pasching, Austria), 2% L-glutamine (PAA, Pasching, Austria)
2.5M Calcium chloride ($CaCl_2$)	Sigma (Deisenhofen); sterile filtered (0.22 μm filter); (store at 4°C)
HEPES (2x, 200mM)	23.83g HEPES (Roth, Karlsruhe); adjust pH 7.00; add 500ml dH_2O; sterile filtered (0.22μm filter); (store at 4°C)
Chloroquine (25mM)	Chloroquine (Sigma, Deisenhofen); sterile filtered (0.22μm filter); (store at 4°C)
PBS (Dulbecco's phosphate buffered saline) (1 x)	PAA Laboratories (Pasching, Austria)
Trypsin (0.25%) – EDTA (1mM)	Invitrogen (Karlsruhe); (store at -20°C)
RPMI 1640 (Roswell Park Memorial Institute 1640)	Lonza (Basel, Switzerland); (store at 4°C)

RPMI-standard medium	10% FCS (PAN Biotech, Aidenbach, Austria), 2% L-glutamine (Lonza, Basel, Switzerland), 1% Penicillin/Streptomycin (PAA, Pasching, Austria) in RPMI 1640 (Lonza, Basel, Switzerland); (store at 4°C)
Fetal calf serum (FCS)	PAN Biotech (Aidenbach, Austria); (store at -20°C)
Penicillin-Streptomycin (100 x, 10.000U, 10mg/ml)	PAA (Pasching, Austria); (store at -20°C)
2% BSA solution	2 % BSA (in PBS), Invitrogen (Karlsruhe); (store at -20°C)
Hanks balanced salt solution (HBSS)	Sigma, Deisenhofen
L-glutamine (100 x, 200mM)	PAA (Pasching, Austria); (store at -20°C, few weeks at 4°C)
Mouse interleukin 3 and 6 (IL-3, IL6)	Chiron (Ratingen)
Mouse stem cell factor	Chiron (Ratingen)
HEPES (25mM)	Roth (Karlsruhe), adjust with NaOH to pH 7.4; (store at -20°C)
X-Vivo-15 medium	Lonza (Basel, Switzerland); (store at -20°C)
Human interleukin-2 (IL-2) (10000U/ml)	Novatis (Nürnberg); (store at -20°C)
Human AB serum (hABS)	Sigma (Deisenhofen); (store at -20°C)
Pancoll	PAN Biotech (Aidenbach, Austria)
FACS-buffer	PBS (PAA, Pasching, Austria), 2% FCS and 0.05% NaN_3, (store at 4°C)
Retronectin	Invitrogen (Karlsruhe); (store at -20°C, few weeks at 4°C)
Anti-CD3-Anti-CD28 Dynabeads® (1x 10^8/ml)	Invitrogen (Karlsruhe)
Sodium pyruvate (100 x)	Invitrogen (Karlsruhe)

Table 5. Plastic material for tissue culture and molecular biology

Plastic material	Supplier
Tissue culture dishes (10cm)	Greiner Bio-One (Frickenhausen)
Conical polysterene tubes (15ml/ 50ml)	BD (Heidelberg)
Cryotubes	Sarstedt (Nürnbrecht)
Reaction tubes (1.5ml, 2ml)	Sarstedt (Nürnbrecht)
Cell culture flasks (175cm^2, 75cm^2)	BD (Heidelberg)

Pipettes (sterile) (2ml, 5ml, 10ml, 25ml)	BD (Heidelberg)
Tissue culture plates (6, 12, 24, 96 well)	Corning (Schiphol-Rikjund, The Netherlands)
Non-tissue culture plates (6, 24 well)	Corning (Schiphol-Rikjund, The Netherlands)
Sterile centrifugation tubes (FACS tubes)	BD (Heidelberg)
Sterile filter (0.22μm / 0.45μm)	Millipore (Schwalbach)
Syringe 10ml	Dahlhausen (Köln)
Non tissue culture plates	BD (Heidelberg)
Pipette tips (10μl, 200μl, 1000 μl)	Sarstedt (Nürnbrecht)

2.2 Kits

Table 6. Kits

Kit	Supplier
Annexin V-PE Apoptosis detection kit I for FACS	BD Falcon, Heidelberg
Calcium Phosphat Transfection Kit	Sigma, Deisenhofen
Caltag CAL-LYSE™	Caltag, Hamburg
DNeasy Blood & Tissue Kit	Qiagen, Hilden
EasySep Murine SCA1 Selection Kit	StemCell technologies, Kanada
ECL-Plus Western Blot Kit	Amersham Bioscience, England
E.N.Z.A. Cycle Pure Kit	peQLab (Erlangen)
JETquick Plasmid miniprep Spin Kit	Genomed, Löhne
JETquick Blood DNA Spin Mini Kit	Genomed, Löhne
Jetquick Gel Extraction Kit	Genomed, Löhne
Mouse Vβ TCR Screening Panel for FACS	BD Falcon, Heidelberg

Pfu Turbo PCR Amplification	Stratagene, Heidelberg
Qiagen Plasmid Maxi Kit	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden

2.3 Enzymes

Table 7. Enzymes and standard markers

Enzyme	Supplier
100bp-Standard marker	New England Biolabs, Frankfurt am Main
1kb-Standard marker	New England Biolabs, Frankfurt am Main
Calf Intestine Phosphatase (CIP)	New England Biolabs, Frankfurt am Main
Restriction enzymes	New England Biolabs, Frankfurt am Main
Klenow	New England Biolabs, Frankfurt am Main
Pfx-DNA-Polymerase	Invitrogen, Karlsruhe
Protein marker for Western-Blot	New England Biolabs, Frankfurt am Main
Proteinase K	Qiagen, Hilden
T4 DNA-Ligase	New England Biolabs, Frankfurt am Main

2.4 Oligonucleotides

Table 8. Oligonucleotides

Name	Primer-Sequencez 5´→3´	Application
*NPM-ALK*_For1	GTCTGAAAATTAGCTCGAC	Sequencing
*NPM-ALK*_For2	GCGGCTCTGGCCCAGTGCATATC	Sequencing
*NPM-ALK*_For3	GATCTGAAGAGCTTCCTGAG	Sequencing

*NPM-ALK*_For4	GAGGACAGGCCCAACTTCGC	Sequencing
*NPM-ALK*_For5	GAAGGCAGCTGCACCGTGC	Sequencing
*NPM-ALK*_Rev1	CACACCGGCCTTATTCCAAGC	Sequencing
*NPM-ALK*_Rev2	GTTCCTGCTGCCGTGCACCTTG	Sequencing
*NPM-ALK*_Rev3	CTTGCTGGGGTAGGGCATG	Sequencing
*NPM-ALK*_Rev4	GCTCATCCTGCTCGCTGC	Sequencing
*NPM-ALK*_Rev5	GCCTCCACGATGTGCAGCTCGTC	Sequencing
*Tax*_For1	CTCCAAGCTCACTTACAGGC	Sequencing
*Tax*_For2	CCACGTGATCTTTTGCCACC	Sequencing
*Tax*_Rev1	GCTTCGGCCAGTAACGTTAG	Sequencing
*Tax*_Rev2	CTGTGGTCAGGCTGATCTTG	Sequencing
Biotin A1	(biotin) CTG GGG ACC ATC TGT TCT TGG CCC T	LM-PCR
A2 RV	GCC CTT GAT CTG AAC TTC TC	LM-PCR
A3 RV	CCA TGC CTT GCA AAA TGG C	LM-PCR
OC1/FW	GAC CCG GGA GAT CTG AAT TC	LM-PCR
OC2/FW	AGT GGC ACA GCA GTT AGG	LM-PCR
Linker 1 FW	GAC CCG GGA GAT CTG AAT TCA GTG GCA CAG CAG TTA GG	LM-PCR
Linker 2 RV	(phosphate)CCT AAC TGC TGT GCC ACT GAA TTC AGA TCT CCC G	LM-PCR
RASEQ	CTT GCA AAA TGG CGT TAC	LM-PCR

2.5 Plasmids

Table 9. Plasmids

Internal Lab.number	Official name	Description	Reference/Source
M187	Eco-env	Expression plasmid for ecotropic envelope protein from MLV.	[44]
M387	MP91-eGFP	Retroviral vector with optimized MP91-leader (MPSV-LTR and MESV-vector), coding for IRES-eGFP, and a packaging signal.	C. Baum (MHH) and D. von Laer
M579	MLV gag-pol	Expression plasmid for MLV Gag/Pol with SV40-Promotor.	C. Baum (MHH)
M620	GALV-env	Expression plasmid for ecotropic envelope protein from GALV.	Plasmid factory, Bielefeld
M896	B-*Tax M*	Cloning plasmid cDNA of viral oncoprotein *Tax M*.	GENEART
M899	B-*NPM-ALK*	Cloning plasmid cDNA of human fusion oncogene *NPM-ALK*.	GENEART
M900	B- *P21SNFT*	Cloning plasmid cDNA of human *P21SNFT*.	GENEART
M905	MP91-*Tax M*	Retroviral vector with optimized MP91-leader (MPSV-LTR and MESV-vector), coding for *Tax M*-IRES-eGFP, carrying a packaging signal.	Cloned in this work.
M906	MP91-*NPM-ALK*	Retroviral vector with optimized MP91-leader (MPSV-LTR and MESV-vector), coding for *NPM-ALK*-IRES-GFP, carrying a packaging signal.	Cloned in this work.
M907	MP91- *P21SNFT*	Retroviral vector with optimized MP91-leader (MPSV-LTR and MESV-vector), coding for *P21SNFT*-IRES-eGFP, carrying a packaging signal.	Cloned in this work.
pCR 2.1-TOPO	pCR 2.1 TOPO	Cloning plasmid for sequencing.	Invitrogen

MHH: Medicine High School Hannover (Medizinische Hochschule Hannover).

2.6 Antibodies

Table 10. Antibodies

Primary antibodies (clone)	Marker	Supplier	Application
Hamster-α-Mouse CD3 (145-2C11)	-	BD	Stimulation
Hamster-α-Mouse CD3 (145-2C11)	R-Phycoerythrin-Cy5	Invitrogen	FACS
Rat-α-Mouse CD4 (RM4-5)	R-Phycoerythrin-Cy5.5	Invitrogen	FACS
Rat-α-Mouse CD8a (CT-CD8a)	R-Phycoerythrin	Invitrogen	FACS
Mouse-α-Mouse CD11b (M1/A70)	Allophycocyanin	BD	FACS
Rat-α-Mouse CD19 (6D5)	R-Phycoerythrin-Cy5.5	Invitrogen	FACS
Hamster-α-Mouse CD28 (37.51)	-	BD	Stimulation
Mouse-α-Mouse CD45.1 (A20)	R-Phycoerythrin	BD	FACS
Mouse-α-Mouse CD45.2 (104)	PerCP-Cy5.5	BD	FACS
Hamster-α-Mouse TCRβ (H57-597)	Allophycocyanin	BD	FACS
Mouse IgG1-α-Mouse Pre-alpha-TCR	-	BD	FACS
Mouse-α-Mouse NK1.1	Allophycocyanin	BD	FACS
Fc-Block-α-Mouse CD16/CD32 (2,4G2)	-	BD	FACS-block
Rabbit-α-ALK	-	abcam	Western-blot
Mouse-α-*P21SNFT*	-	Abnova	Western-blot
Mouse-α-HTLV-I *Tax*	-	abcam	Western-blot
Secondary antibodies/Reagents	**Marker**	**Supplier**	**Application**
Streptavidin	Allophycocyanin	BD	FACS
Rat-α-Mouse IgG1	Biotin	BD	FACS
Goat-α-Rat-IgG	PE	Invitrogen	FACS

Goat-α-Hamster-IgG	PE	Invitrogen	FACS
Goat-α-Mouse-IgG	Allophycocyanin	Invitrogen	FACS
Goat-α-Rabbit	HRPO	Santa Cruz Biot.	Western blot
Streptavidin	HRPO	Santa Cruz Biot.	Western blot

2.7 Bacteria

Molecular biology work was performed with E. coli TOP10 supplied by Invitrogen (Karlsruhe). The genotype of E. coli TOP_10 is: F^- mcrA Δ(mmr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(araleu)7697 galK rpsL (Str^R) endA1 nupG.

2.8 Cell lines and primary cells

Table 11. Cell lines and primary murine cells

Cell line/Primary cells	Description/Medium
293T	Human embryonic kidney epithelial cells transduced with T-antigen of SV40 [45]. For packaging of constructs. →DMEM-standard
SC-1	Murine Fibroblast cell line [46]. For Titration of produced retroviral (Eco env) supernatant. →DMEM-standard.
TE671	Human medulloblastoma cell line [47]. For Titration of produced retroviral (GALV env) supernatant. →DMEM-standard.
PM-1	Human T cell line originating from HUT 78 cell line, expressing CCR5 and CXCR4 [48] →RPMI standard.
Jurkat, clone E6-1	Human T cell line, a clone of the Jurkat-FHCRC cell line.

	→RPMI-standard.
Primary T lymphocytes	Isolated from spleen and lymph nodes of C57BL/6 Ly5.1 or OT-I donor mice. → Mouse medium special: RPMI 1640 with 10% FCS, 2% Glutamine, 1 % Pen/ Strep, Sodium Pyruvat (1x), NEAA (1x) and β-Mercaptoethanol (1x) + 100U/ml IL-2.
Primary lineage negative stem cells	Isolated from the bone marrow of six to eight weeks old C57BL/6 Ly5.1 donor mice. → RPMI with 10% FCS, 50ng/ml mSCF, 10ng/ml mIL-3, 50ng/ml mIL-6.

2.9 Instruments/Equipments

Table 12. Instruments/Equipments

Instrument	Supplier
Analytical scales	Kern & Sohn (Balingen-Frommern)
Autoclave HST 4-5-6 E	Zirbus Techonolgy (Bad Grund)
BIOBEAM 2000 Cs-137 (Radiation source + accessoriy)	Eckert & Ziegler, Berlin
Incubator	Heraeus, Hanau
Cell Counter Casy Tone TT	Schärfe Systems (Reutlingen)
Centrifuge (cooled) Avanti J20	Beckman (München)
Centrifuge (cooled) Rotina 48R	Hettich (Tuttlingen)
Clean bench HERAsafe HS12	Heraeus (Hanau)
DNA-electrophoresis-chamber	BioRad (München)
DNA-Sequencer	Applied Biosystems (Weiterstadt)
EasySep Magnet	StemCell technologies, Canada
FACScan	Beckton Dickinson (Heidelberg)

Film developing machine Optimax Type 1R	Schroeder und Henke (Wiesloch)
Fluorescent microscop Eclipse TE300	Nikon, Düsseldorf
Heating block DRI-BLOCK BD 2D	Techne (England)
Incubator for molecular biology	Heraeus (Hanau)
Incubator for tissue culture	Heraeus (Hanau)
Magnetic stirrer	IKA (Staufen)
Microscope	Leica (Bensheim)
Nano-Drop spectrometer	Peq-Lab (Erlangen)
PCR-Cycler	Biometra (Göttingen)
pH-meter Toledo MP 220	Mettler (Gießen)
Pipetboy acu	Intergra Biosciences (Fernwald)
Pipettes	Gilson (USA)
Scil Vet ABC (*Animal Blood Counter*)	Scil animal care company, Viernheim
Shaking incubator TH25	Edmund Bühler (Tübingen)
Swinging bucket centrifuge Megafuge 1.0R	Heraeus (Hanau)
Table top centrifuge Megafuge 1.0R	Heraeus (Hanau)
Typhoon Phosphor Imager	GE (Freiburg)
UV transluminator GelDoc 2000	BioRad (München)
Vortex Genie 2	Bender und Hobein (Switzerland)
Water bath	GFL (Burgwedel)

2.10 Materials for animal experiments

2.10.1 Mouse strains and husbandry conditions

Six to eight week old C57BL/6J.Ly5.1 (CD45.1+) and C57BL/6J.Ly5.2 (CD45.2+) RAG-1 deficient mice were obtained from Charles River laboratories (Sulzfeld, Germany) and Jackson laboratory (Bar Harbor, USA). Animals were bred and maintained under specific pathogen-free (SPF) like conditions in the animal facilities of the Georg-Speyer-Haus. Cages were individually ventilated (IVC). Symptomatic/leukaemic or healthy animals (donors) were sacrificed after anesthesia by cervical dislocation and examined for pathological abnormalities, including histology, morphology, white blood counts (WBC) and flow cytometry. The experiments were performed in compliance with the local animal experimentation guidelines. Animal experiments were approved by the regional council (Regierungspräsidium) Darmstadt, Hessen, Germany.

Tabel 13. Mouse strains

Name/Reference	Internal name	Supplier
B6.SJL- *Ptprc*^{a} *Pep3*^{b}/BoyJ [49]	Ly5.1	Charles River Laboratories Jaxmice, USA
B6.129S7-Rag-1^{tm1Mom}/J [50]	Rag-1	Charles River Laboratories Jaxmice, USA
C57BL/6Tg (TcraTcrb) 1100Mjb/j [51]	OT-I	Charles River Laboratories Jaxmice, USA

T lymphocytes or lineage negative stem cells were isolated from OT-I or Ly5.1 donor mice. Rag-1 deficient mice do not have any T and B lymphocytes and served as receiver animals. Ly5.1 haematopoietic cells could be differentiated by the antigen CD45.1 from the Rag-1 recipient (background C57BL/6 Ly5.2) cells having CD45.2 cells.

Transgenic OT-I mice possess only one type of exogenous TCR in the context of H2Kb (MHC-I) specifically directed against an epitope (amino acid residues 257-264) of Ovalbumin (OVA). Due to MHC-I context, almost exclusively cytotoxic T lymphocytes (CD8+) are generated in these animals. The donor cells were used as monoclonal T cell population.

2.10.2 Animal-experiment materials

Table 14. Animal-experiment materials and their applications

Materials	Application	Supplier
Ear hole puncher (Napox)	Marking of laboratory animals	Heiland, Hamburg
Histosette	Organ embedding	Simport, Canada
Microvette (EDTA-coated)	Blood taking	Sarstedt, Nümbrecht
Corktray (Hebu)	Dissection	Heiland, Hamburg
Insulin syringe	Intravenous transplantation	BD, Heidelberg
Cell strainer (100μm)	Homogenization of organs and filtration of cell suspension	BD, Heidelberg

3. Methods

3.1 Molecular Biology

3.1.1 DNA digestion with restriction enzymes

For the cloning or subcloning of plasmids and restriction analyses, plasmid DNA was digested with restriction endonucleases. Restriction enzymes were obtained from New England Biolabs and used in the recommended 10x buffers. For a preparative digestion 2-4μg of plasmid DNA was incubated for 1 to 12 hours with 5 to 20 units of restriction endonuclease in 35μl total sample volume. The digest was monitored by agarose gel electrophoresis (see section 3.1.3). For subcloning, bands were isolated from the gel and treated as described in section 3.1.4. For restriction analysis, 0.5 to 1μg plasmid DNA was digested in 20μl sample volume with 5 to 10 units restriction enzyme for 1 to 2 hours. Digestion was monitored by agarose gel electrophoresis.

3.1.2 Dephosphorylation of DNA fragments at the 5' end

Before ligation with insert, digested linearized vector plasmid was dephosphorilated to prevent religation. For this purpose, 1 μl (10 units) of calf intestinal phosphatase (CIP) was added to the restriction digest and incubated for 45 to 60 minutes at 37°C. Thereafter, the digested plasmids were run on the agarose gel to isolate the required fragments for ligation.

3.1.3 Agarose gel electrophoresis

The separation of double stranded DNA fragments was performed in a horizontal agarose gel in 1x TAE buffer. Generally, 1% agarose gel was used for separation. Smaller fragments (<500bp) were separated in 2% agarose gels. The agarose powder was dissolved in 1x TAE by heating in a microwave. Ethidiumbromide (1%) was added in a 1/1000 dilution before pouring the agarose into a gel-casting chamber. The polymerized agarose was placed in an electrophoresis chamber filled with 1x TAE. The DNA samples were mixed with 6x DNA loading buffer and pipetted into gel slots. As size markers, 10 to 12μl (~400ng) 100bp and 1kbp ladder (NEB) were loaded onto the gel. Electrophoresis was performed at 100V for 30 to 40 minutes. The gel was placed onto a UV transilluminator and scanned in a BioRad® Geldoc 2000®. The appropriate fragments were cut out.

3.1.4 Isolation of DNA fragments from agarose gels

For the isolation of DNA fragments the required fragments were cut out of the agarose gel with a scalpel and transferred into an Eppendorf reaction tube. New scalpel was used for each fragment. The extraction of DNA was performed with a JETquick gel extraction kit according to the manufacturer's instructions.

3.1.5 Ligation of DNA

The covalent ligation of DNA fragments was performed with the Quick-ligase®. The ligation was performed out in a total volume of 20μl with approximately 200ng linearized, dephosphorylated plasmid DNA and a three-fold excess of the insert for 15-20 minutes at room temperature. 10 units of Quick-ligase® (1μl) were added and the provided 2x Quick-ligase® buffer was used. The ligation mixture and a control sample without insert were used to transform competent E. coli TOP10® bacteria.

3.1.6 Transformation of Escherichia coli with plasmid DNA

Ligation products were transformed into E. coli TOP 10 bacteria. 50μl of competent bacteria were thawed and mixed with 20μl ligation samples. Cells were incubated on ice for 30 minutes and subsequently heat shocked for 2 minutes at 42°C. This heat shock leads to the uptake of DNA into competent cells. Subsequently, cells were cooled on ice for two minutes. 500μl LB medium was added and cells were incubated at 37°C for 45 minutes in a shaking incubator. Afterwards, cells were pelleted and supernatant was removed. Pellets were carefully resuspended in 50μl LB medium and cell suspensions were plated on an ampicillin (amp) supplemented LB-agar dish. After over night incubation at 37°C, the bacterial colonies were picked and used to inoculate mini DNA preparations (see section 3.1.8).

3.1.7 Preparing competent Escherichia coli for transformation

For transformation of ligation products, competent E. coli bacteria were prepared as follows: 2ml of E. coli TOP10 over night culture was mixed with 200ml of LB-medium and incubated until the culture reached an OD (optical density) of 0.5 to 0.7. The bacterial suspension was transferred into 50ml Falcon tubes and incubated for 10 minutes on ice. Afterwards, the Bacteria were harvested via centrifugation at 2890xg, 4°C for 10 minutes. Pellets were resuspended in 40ml of ice-cold 0.1M $MgCl_2$ solution. Afterwards, cells were pelleted, the supernatant was discarded and cells were resuspended in 20ml of 50mM $CaCl_2$ solution.

After 30 minutes incubation on ice, bacteria were pelleted again and resuspended in 2ml of 50mM $CaCl_2$-15% glycerol solution. Aliquots of 200µl were prepared and shock-frozen in liquid nitrogen and subsequently stored at -80°C. The competence was measured for 1x 10^6 colonies for 1µg plasmid DNA. The competent cells were prepared by Tefik Merovci, AG von Laer.

3.1.8 Preparations of DNA plasmid from Escherichia coli

Small-scale plasmid DNA preparations for analytic purposes were done using the peqGOLD Plasmid Miniprep Kit I. 3ml of an overnight culture of transformed E. coli TOP_10 bacteria was used according to manufacturers' instructions. The typical plasmid DNA yield was in-between 5-10µg in a total volume of 50-100µl elution buffer.
For preparative purposes. maxi preparations were performed using a total bacterial culture volume of 250ml. Maxi preparations were performed using the Nucleobond AX PC500 Maxiprep Kit, yielding plasmid DNA amounts in-between 0.5-1.5mg. Plasmid DNA concentrations were adjusted to 1mg/ml and stored in 200µl working aliquots at -20°C. All plasmid DNA concentrations were determined using a Nanodrop™ 1000 spectrophotometer.

3.1.9 Culture conditions and preservation of Escherichia coli

After transformation, plasmid-containing E. coli bacteria were cultured in ampicillin supplemented LB-medium (100µg/ml). Single clone colonies were produced by plating E. coli bacteria on LB-agar and incubating at 37°C for 12 to 16 hours. Agar plates were stored at 4°C and used for inoculation of mini and maxi preparations for several weeks. For cryo stock preparation, E. coli bacteria were cultured over night in a 37°C shaking incubator. 500µl of the culture was mixed with 500µl 7% DMSO in H_2O and stored at -80°C. Fresh cultures were inoculated by scratching a little amount of bacteria out of the cryo tube and transferring into LB-ampicillin medium.

3.1.10 Sequencing of DNA preparations

DNA sequencing was performed in the sequencing core facility of Georg-Speyer-Haus using a 3100 Avant Genetic Analyzer (Sanger procedure). For sequencing, 10µl mini DNA aliquot was mixed with 1µl of 10µM oligonucleotide solution and water was added to a total volume of 16µl. 0.5µg maxi preparation DNA was used in 16µl total sample volume, containing 1µl oligonucleotide solution.

3.1.11 LM-PCR and integration site analysis

In order to determine the integration site of the retroviral provirus in the host genome, the flanking sequences have to be identified. LM-PCR (ligation mediated PCR) is a very powerful method [52] for the amplification of DNA fragments whose sequences are partially known. For this purpose, the DNA was first isolated from the cell suspensions or tumors using DNeasy Blood & Tissue Kit and then digested with the restriction enzyme Tsp509I. This enzyme recognizes and digests palindromic sequences with AATT nucleotide sequence and produces sticky ends. Digestion occurs three times in the proviral sequence - once in the 5' and the 3' LTR sequence and once between them as well as several times in the host genome. Afterwards, the strand extension (primer extension) is performed in a one-step-PCR, creating single stranded DNA with a biotin molecule, which can be isolated by paramagnetic-Streptavidin coated particles (Dyna beads). Finally, for every integration site, two fragments are cleaned up, one fragment containing the 3´LTR and part of the proviral sequence (internal control band); and the other containing the 5´LTR and a part of the unknown host genome sequence (external band), (after trimming the external band, this sequence is aligned or compared to the whole genome). Thereafter, an adapter cassette (Linker) is ligated to the end of the unknown host DNA fragments as well as to the internal band (Adapter Ligation). The following steps are for a PCR and a 'nested' PCR.

For the PCR, two oligonucleotides (primers) are used, one complementary to the adapter (OC1/FW), and the other complementary to the known part of the LTR sequence (A2RV). In order to achieve a higher sensitivity and a stronger amplification of the target sequence, a 'nested' PCR with the oligonucleotides OC2/FW and A3RV is performed. Both primers bind inward compared to the PCR primers. With this method, the target sequence is amplified first in a standard PCR (external PCR) and subsequently, a part of this reaction is used as a template for the 'nested' PCR (internal PCR). The procedure in the available work was adapted according to Schmidt et al [53] and is schematically represented in figure 9. After 'nested' PCR, products are isolated from a 2% agarose gel. In each case, the individual fragment was cut out with a new scalpel to prevent contamination with other fragments. The DNA was then eluted by gel purification as described in 3.1.4. Afterwards, the eluted DNA was sequenced directly with the oligonucleotide A3RV. Since this did not work in every case, DNA fragments were subcloned into TOPO-vector pCR2.1 (Invitrogen). After following the manufacturers' instructions, the ligation proceeded for 30-45min. The transformation was done into chemically competent E.coli TOP10-cells (see section 3.1.6). On the next day, 8 positive white clones (per subcloned DNA fragment) were picked for a Colony PCR. The amplification of the fragments took place with the oligonucleotide pair OC2/FW + A3RV or M13for(-40) + M13rev. Part of the PCR product was analyzed on a 2% agarose gel and the correctly amplified PCR products were cleaned up by Sodium-Acetate/Ethanol precipitation

and then sequenced. The sequencing took place with the oligonucleotide A3RV and/or M13for(-20).

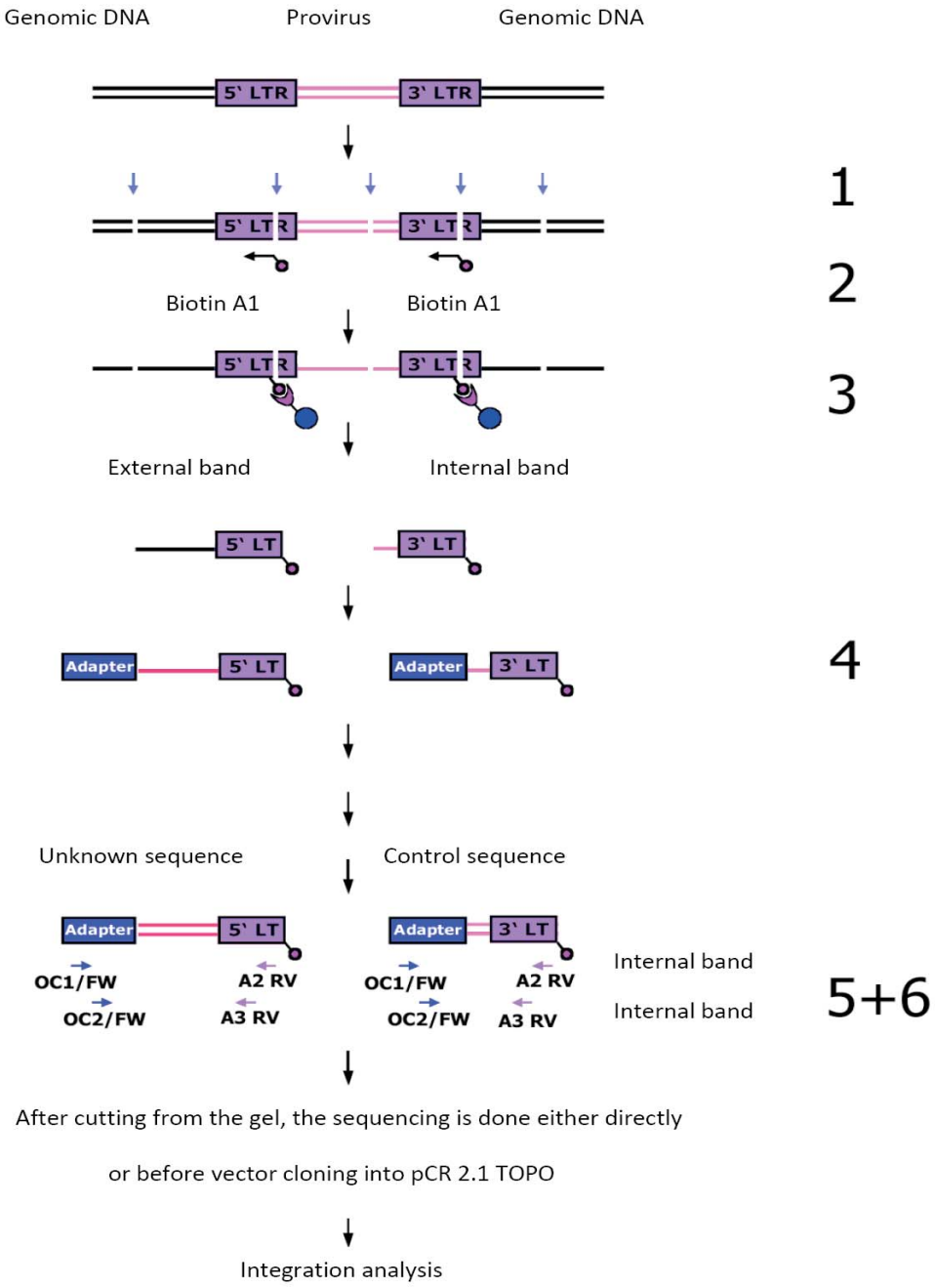


Figure 9. Individual steps of ligation mediated PCR.

The sequencing results were trimmed by the program 'LASERGENE® v8.0 SeqMan' to obtain the flanking genomic sequence, which could be compared to the mouse genome (NCBI 37). The individual steps of the LM-PCR are described more accurately in the following procedure:

1. Restriction of the genomic DNA

For this, 0.25-1μg of cleaned-up genomic DNA and the enzyme Tsp509I were used. According to standard, 500ng DNA was used in a total volume of 30μl. The buffer, indicated by the manufacturer was used and the incubation period was kept up to 2h at 65°C. After digestion, the DNA was purified by using the 'MinElute Reaction CleanUp Kit' (Qiagen). After following the manufacturers' instructions, the DNA was eluted with 10μl Elution buffer.

2. Strand extension (Primer extension)

This step was performed with the 'Phusion® High-Fidelity' DNA-polymerase enzyme and the oligonucleotide 'Biotin A1'.

Table 15. Composition of the primer extension reaction

15μl	DNA
5μl	5x Phusion buffer
1μl	5mM dNTP
1μl	Primer Biotin A1 (0.25 pmol/μl)
1μl	Phusion DNA Polymerase
2μl	H_2O

Table16. Incubation program for primer extension reaction

Step	Temperature (°C)	Duration (Min.)
1	95	5
2	65	30
3	72	15
4	4	∞

After this step, the DNA was cleaned up using the 'MinElute Reaction CleanUp Kit' (Qiagen). After following the manufacturers' instructions, the DNA was eluted twice with 20µl Elution buffer.

3. Biotin-Streptavidin interaction

20µl (corresponding to 200µg) of Streptavidin conjugated Dyna-Beads per sample were used. The beads were washed twice with 100µl 2xBW buffer and resuspended in 40µl of 2xBW buffer in a 2ml reaction tube. Now, 40µl DNA was added to the 40µl prepared beads. The mixture was incubated for 1h on the shaking-rotator with ~1000 rpm at room temperature (25°C). Subsequently after two times washing with H_2O, the DNA Bead-conjugate was resuspended in 5µl H_2O.

4. Ligation of Linker cassette

In each sample of resuspended 5µl DNA bead-conjugate, the following ligation preparations were added:

Table 17. Composition of ligation preparation

5µl	DNA-bead-conjugate
0,2µl	T4 Ligase
2µl	10x Ligation-buffer
1µl	Polylinker
11,8µl	H_2O

The ligation reaction was incubated over night at 16°C in the shaking-incubator with ~1100 rpm (2min. on / 5min. off). Subsequently, this reaction was washed twice with 100µl H_2O and resuspended in 10µl H_2O.

The Polylinker was assembled as follows: 40µl H_2O and each 20µl of Linker1FW (200pmol/µl) and Linker2RV (200pmol/µl) was incubated for 5 min at 70°C in the water bath and afterwards 20µl of 5x Annealing buffer was added. A further incubation for 5min at 70°C in the water bath was followed. Subsequently, the water bath was switched off and the

samples slowly cooled down over night. Afterwards, 10µl aliquots were made and kept at -20°C. The aliquots were used only once.

5+6. LM-PCR and nested PCR

Both, the actual LM-PCR and the following 'nested' PCR were performed with the 'Extensor Hi-Fidelity Kit' (ABgene). Both reactions have the same composition and the same PCR program except for the oligonucleotides. After the LM-PCR the reaction product was diluted to 1:50 with H_2O and used in the nested PCR.

Table 18. Composition of LM-PCR and nested PCR

1µl	DNA-bead-conjugate and/or LM-PCR-product (1:50)
12,5µl	Extensor Hi-Fidelity PCR Master Mix
1µl	OC1/FW (25pmol/µl) and/or OC2/FW (25pmol/µl)
1µl	A2RV (25pmol/µl) and/or A3RV (25pmol/µl)
9,5µl	H_2O

Table 19. PCR Program for LM-PCR and nested PCR

Step	Temperature(°C)	Duration (sec)	
1	94	120	
2	94	15	
3	60	30	
4	68	120	To step 2, 30 cycles
5	68	600	
6	4	∞	

After running on a 2% agarose gel, the remarkable fragments were cut out and either directly sequenced with A3RV-oligonucleotide or subcloned into the TOPO-vector pCR 2.1.

Subsequently after sequencing, the integration site analysis (already mentioned) was performed. In case of the subcloning (already mentioned), a Colony PCR was performed after picking the positive, white E.coli colonies. For this, a picked clone was directly used in a 50µl PCR reaction mix and incubated for 10min. The following PCR begins with a long step of 10min at 94°C, in which the bacteria are lysed and the plasmid DNA is set free. The TOPO vector with ligated 'Insert' from the mouse genome and the oligonucleotide of the PCR come into contact.

Table 20. Composition of Colony PCR

1 µl	10µM M13 rev bzw. A3RV
1 µl	10µM M13 for (-40) bzw. OC2/FW
1 µl	10 mM dNTP's
5 µl	15mM buffer (+$MgCl_2$)
0.3 µl	5 U/µl Taq-Polymerase
41,7 µl	H_2O

Table 21. PCR program for Colony PCR

Step	Temperature (°C)	Duration (sec.)	
1	94	600	
2	94	30	
3	53	30	
4	72	60	To step 2, 27 cycles
5	72	300	
6	4	∞	

Further procedure for cleaning up of the PCR products and the sequencing has already been described.

3.1.12 Western blotting

Western blotting was performed to monitor the transgene expression. For that reason, transfected 293T cells (see section 3.2.3.1) or transduced SC-1 cells (see section 3.2.3.2) were used two days after transfection/transduction. The cells were split via trypsination as follows:

The cells were washed once with ~3ml 1xPBS. After aspiration of the PBS, 1ml trypsin was added to the cells for ~30 seconds. Trypsin was inactivated by addition of 4ml DMEM-standard medium. Cells were resuspended using a 5ml-pipette and counted using the Casy cell counter.

$3x10^6$ cells were transferred to 2ml reaction tube and centrifuged for 2 minutes at 13000rpm. Afterwards, the cell pellets were washed with 1ml cold sterile 1xPBS. The cell pellets were resuspended in 80µl ice-cold lysis buffer and vortexed and incubated for 15 minutes on ice. The lysates were then spinnned down for 10 minutes at 13000rpm in a microcentrifuge (4°C) to settle the cell debris down. Proteins in the supernatant were harvested. Afterwards, the supernatant was transferred to a fresh 1.5ml reaction tube and 3x protein loading buffer was added to obtain a final loading buffer concentration of 1x. This was then heated for 5 minutes at 97°C in a heat block, and then stored at -20°C until further use.

3.1.12.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a denaturing separation method that separates the proteins according to their molecular size in an electrical field irrespective of their structure and net charge. Denaturing conditions are achieved by addition of the anionic detergent sodium dodecyl sulfate (SDS) to the protein samples. SDS mainly binds to the hydrophobic amino acids of proteins, thereby applies negative charge to each protein in proportion to its mass. Furthermore, the dodecyl sulfate anion compensates the proteins' own charges giving every protein a highly negative net charge. These two effects allow the separation of proteins based solely on protein size. SDS-PAGE is mostly a discontinuous electrophoretic method meaning that two types of polyacrylamide gels are casted. The lower separation gel has an alkaline pH value (8.8) and a polyacrylamide concentration ranging from 9 to 16%. The concentration of polyacrylamide directly determines the mesh size of the matrix, which should be adapted to the molecular weight of the protein of interest. The stacking gel is laid upon the separation gel and has a neutral pH value of 6.8 and a low polyacrylamide concentration leading to a wide matrix meshwork. In the discontinuous SDS-PAGE, the separation of proteins respective to their size takes place in the alkaline separation gel. The neutral stacking gel serves to focus the proteins in each lane to a small band at the interphase between stacking and separation gel.

This is necessary, because all proteins must enter the separation gel at the same time to obtain sharp protein bands. This is achieved by the high glycine concentration in the gel and the running buffer. Glycine is the smallest amino acid and has a pH of ~6.0. At the pH of the stacking gel, it is minimally negatively charged and mainly exists in its zwitterionic form. Therefore, the glycine ions are the speed-limiting factor of current flow in the stacking gel, which leads to a protein band focusing. In the separation gel, the glycine ions are anionic and proteins are separated by running at their respective speeds. The two gels were prepared according to the following table.

Table 22. Reagents for SDS-PAGE, stacking gels (4%) and resolving gels (10/16%)

Gel percentage	ddH_2O [ml]	30% degassed acrylamide-bisacrylamide solution [ml]	Gel buffer [ml]	10% SDS (w/v) [ml]
4%	6.1	1.3	2.5[1]	0.1
10%	4.1	3.3	2.5[2]	0.1
16%	2.1	5.3	2.5[2]	0.1

[1]Stacking gel: 0.5 M TRIS-HCl, pH 6.8

[2]Resolving gel: 1.5 M TRIS-HCl, pH 8.8

The polymerization was initiated by addition of 50μl 10% ammonium persulfate and 5μl TEMED to the 10ml gel solutions (for the stacking gel 10μl TEMED were added). Gels were generally prepared with a BioRad Mini Protean III system. The separation gel solution was pipetted between the two glass plates fixed onto the casting stand. Isopropanol was layered on top of the gel to obtain a smooth phase transition. The gel was allowed to polymerize for 30 to 40 minutes. The isopropanol was removed and the top of the separation gel was rinsed with deionized water. APS and TEMED were added to the prepared stacking gel solution and poured onto the separation gel. The comb was inserted and the gel was allowed to polymerize for 40 to 50 minutes. Polymerized gels were wrapped in wet tissue papers and stored at 4°C until use.

3.1.12.2 Immunoblotting

Immunoblotting allows the specific detection of proteins following their separation via SDS-PAGE. For this, the separated proteins are immobilized on a membrane and are detected by specific antibodies, which are directed against the protein of interest. Secondary antibodies that are chemically coupled to a reporter system, such as the horseradish peroxidase, in turn

bind to the primary antibodies. The signals produced by the reporter system can then be detected. The respective dilutions for the used antibodies are summarized in table 23:

Tabel 23. Dilutions for the Western blot antibodies

Primary antibody	**Marker**	**Dilution (5% milk in PBST)**
Rabbit-α-Alk	-	1:400
Mouse-α-p21SNFT	-	1:2000
Mouse-α-HTLV I *Tax*	-	1:400
Secondary antibody/Reagent		
Goat-α-rabbit	HRPO	1:10000
Goat-α-mouse	HRPO	1:10000

Molecular sizes of detected proteins were calculated by referring to a marked protein ladder (10-250 kDa) from New England Biolabs (Ipswich USA), run in parallel on each gel. Here, the protein transfer onto the nitrocellulose membrane was performed by the semi-dry approach. The blot was assembled as follows:

1. Whatman paper, soaked in transfer buffer
2. Membrane
3. Poly-acryl amide gel
4. Whatman paper, soaked in transfer buffer

The membrane and the Whatman papers were cut to the size of the gel. Air bubbles were removed from the blot and transfer was performed for 30 to 45 minutes at 15 volts. The blot was disassembled and the primary antibody was applied at appropriate concentrations over night at 4°C in 5% milk in PBST (MPBST). On the next day, primary antibody was discarded and the membrane was washed three times for 10 minutes with 20ml PBST. The secondary antibody was applied at appropriate concentrations in MPBST for 2 hours at room temperature. The antibody solution was discarded and the membrane was washed again three times for 10 minutes with PBST. For the development of the blot, ECL® or ECL plus®

kit from Amersham Bioscience® was used. For this, the two provided substrates were mixed 1:1 or 40:1, respectively. Blots were incubated 5 minutes with the mixed substrate in the dark. After removing excess substrate from the membrane, it was then placed into a plastic foil and then transferred into an Amersham Hypercassette®. Hyperfilm ECL® photo films were applied under safelight conditions to the blots for various exposure times and the films were developed in the film developing machine.

3.2 Tissue culture Culture conditions for eukaryotic cell lines

The cell lines used during this work were adherent (293T) and suspension (PM-1 and Jurkat). 293T cells were cultured in DMEM containing 10% fetal calf serum (DMEM-standard). $1.5x10^7$ 293T cells were seeded in a $175cm^2$ cell culture flask. Cells were split every 3 to 4 days via trypsination as follows: 293T cells were washed once with 20ml 1xPBS. After aspiration of the PBS, 5ml trypsin was added to the cells and left for half to 1 minute. Trypsin was then inactivated by addition of 25ml DMEM-standard medium. Cells were resuspended using a ten-milliliter-pipette. The separation of cells was monitored under the microscope. Cells were counted using the Casy cell counter.
PM-1 and Jurkat cells were cultured in RPMI supplemented with 2% glutamine, 5% FCS and 1% Pen/Strep (RPMI-standard). Cells were split after every 3 to 4 days at a 1:3 ratio and 12ml fresh RPMI-standard medium was added in a $75cm^2$ cell culture flask. For cell expansion, 6ml of PM-1/Jurkat cell suspension (containing $9x10^6$ cells) was added to a total volume of 50ml RPMI-standard in a $175cm^2$ cell culture flask. Cells were harvested at a density of $1.5x10^6$ cells per ml (3-4 days after seeding).

3.2.1 Freezing and thawing of cells

For long-term storage, cells were kept in liquid nitrogen. The cultured suspension cells were counted. Adherent cells were trypsinized, diluted in excess of complete culture medium and counted. The cell suspensions were then transferred to a 50ml Falcon tube and pelleted by centrifugation for 10 minutes at 1200rpm and 4°C. The supernatants were discarded and the cell pellets were resuspended in 90% FCS, 10% DMSO at a concentration of 10^7 cells/ml. 1ml aliquots of the cell suspensions were transferred to cryotubes in a pre-cooled (4°C) freezing container and were incubated at -80°C overnight before the cryotubes were stored in liquid nitrogen.
To restore cryo-stocks of cells, cells were thawed rapidly in a waterbath at 37°C. The cell suspension was diluted in 12ml pre-warmed medium in a 15ml Falcon tube and centrifuged for 5 minutes at 1200rpm. The DMSO-containing supernatant was discarded and the cell

pellet was resuspended using 12ml of complete medium. Finally, the cell suspension was transferred into a T-75 cell culture flask and cultured at 37°C.

3.2.2 Isolation and stimulation of primary murine MNCs/HSCs

3.2.2.1 Isolation of primary murine MNCs

Murine MNCs (mononuclear cells) were isolated from WT-mice (Ly5.1 or OT-I mice). After sacrificing the mice by breaking the nape, skin was disinfected with 70% EtOH. Spleen and lymph nodes (mesenteric and superficial inguinal) were removed and transferred into a 50ml Falcon tube containing 10-20ml ice-cold 1xPBS. Spleen and lymph nodes with the whole volume of PBS were homogenized with a syringe stamp through the screen cup in a 10cm tissue culture dish to get a single cell suspension. The whole volume of single cell suspension was filtrated through a cell strainer into a new 50ml tube. The dish was washed twice with 10ml PBS and the washing solutions were filtrated through the same cell strainer into the same 50ml tube. Cells were centrifuged for 10min. at 360RCF and room temperature. Supernatant was removed and cell pellet was resuspended with 10ml PBS. The removed supernatant was centrifuged again. This time supernatant was discarded. The second cell pellet was resuspended with 10ml PBS. Both cell suspensions were combined in one tube to get a total 20ml cell suspension. The cell suspension was carefully underlayed with 10ml Histopaque (room temperature) using a 10ml pipette and centrifuged for 30min. at 625RCF, room temperature and without break (for gradient stability). The interphase containing murine MNCs was carefully collected with a 5ml pipette in a new 50ml tube and diluted with 10ml PBS. Cells were centrifuged for 10min at 360RCF and room temperature. Supernatant was removed and cell pellet was resuspended with 10ml PBS. The washing step was repeated and cell pellet was resuspended with 10ml PBS. Cells were counted and stored on ice until further use.

3.2.2.2 Coating of Dynal-Epoxy beads with mAbs: α-CD3 and α-CD28

For the stimulation of murine T lymphocytes, magnetic particles (M-450 Epoxy Beads) were coated with antibodies α-CD3 and α-CD28. Dynabeads M-450 Epoxy were resuspended by vortexing the container before opening. After opening the container, beads were resuspended by pipetting and 1ml (4 x10^8 beads) was transferred to a sterile FACS tube. The tube was placed in a magnetic separator. All liquid was removed and discarded with a 1000µl pipette, while tube was still in the magnetic separator. After removing from the separator, 1ml 1xPBS was added to the tube and vortexed until beads were resuspended. The tube was again placed in the magnetic separator and the washing step was repeated. 60µg (60µl) of

each monoclonal antibody anti-CD3 (Clone: 145-2C11) and anti-CD28 (Clone: 37.51) were added to resuspended 1ml bead suspension and vortexed. The lid around the top of tube was tightly closed and wrapped with a parafilm. The tube was placed in a 50ml Falcon tube and incubated on a rotator for 16–24h at 37°C. Next day, the tube with beads was removed from rotator and placed in a magnetic separator. All liquid was removed and discarded with a 1000µl pipette, while tube was still in the magnetic separator. The tube was again placed in the magnetic separator and the washing step was repeated. 1ml PBS/0.1% MSA was added to the beads and the tube was placed on the rotator for washing/blocking for 5min. at room temperature. The tube was removed from the rotator and placed in a magnetic separator. All liquid was removed and discarded with a 1000µl pipette, while tube was still in the magnetic separator. Washing with 1ml PBS/0.1% MSA was repeated twice. The beads were resuspended in 1ml PBS/0.1% MSA and incubated on a rotator for 16–24h at 2–8°C. Next day, tube with beads was removed from the rotator and placed in a magnetic separator. All liquid was removed and discarded. The beads were resuspended with 1ml PBS/0.1% MSA and transferred to a new sterile FACS tube. The beads were stored (for at least 4 weeks) at 4°C for further use.

3.2.2.3 Stimulation of primary murine T lymphocytes

For stimulation, murine T lymphocytes were prepared in mouse special medium. After washing with 2ml PBS, anti-CD3/-CD28 coupled beads were added in 3:1 beads to cell ratio after resuspending in 1ml of prepared cell suspension. After mixing of cell suspension and beads by pipetting carefully up and down, the cell/bead suspension was seeded in 6–well culture plates with a density of 4.2-4.9 x 10^6 cells/7ml per well. Cells were kept under standard cell culture conditions for 4 days. 100U/ml IL-2 was added on each day for better stimulation. On fourth day, cells were collected in a 50ml Falcon tube. After rigorous pipetting 8 to 10 times up and down with a 10ml pipette, cells were debeaded by placing in a magnetic separator. Afterwards, the medium containing debeaded cells was transferred to a new 50ml Facon tube. The cells were counted and used for transduction (see sections 3.2.6/3.2.5).

3.2.2.4 Isolation and stimulation of murine lineage negative HSCs/HPCs

Murine lineage negative haematopoietic stem cells/haematopoietic progenitor cells (lin-HSCs/HPCs) were isolated from the bone marrow of WT mice (Ly5.1). After killing the mice by a nape-break, skin was disinfected with 70% EtOH. Tibia and Femur bones were removed from the animals (without touching the fur) and raw parts of the muscles were cut away. After isolation, Tibia and Femur were stored on ice in a 50ml Falcon tube containing isolation-buffer. For further preparation, three 10cm tissue culture dishes were prepared:

1. EtOH, 2. PBS, 3. Isolation-buffer. Tibia and Femur, as well as surgical instruments were washed with 70% EtOH for sterility and rinsed in 1xPBS. The bones were stored in the dish plate containing isolation-buffer. Muscle tissues and tendons were carefully removed from the bones to obtain a clear view on the red shimmering bone marrow. The ends of the bones were cut away and bone marrow was flushed with isolation-buffer filled syringe. Clumps were dispersed by gently resuspending the bone marrow. The cells were centrifuged for 8min. at 320RCF and RT. Cell pellet was resuspended in 6ml isolation-buffer. Remaining clumps of cells and debris were removed by passing cell suspension through a 100µm mesh nylon strainer. Cells were counted and centrifuged for 8min. at 320RCF and RT. Supernatant was discarded and cells were resuspended in isolation-buffer at 1 x 10^8 cells/ml. Isolation of lineage negative stem cells (lin-HSCs/HPCs) was carried out according to the manufacturers' instructions by using 'Lineage Cell Depletion Kit' (Miltinye Biotec). 5x10^5 to 1x10^6 lin-HSCs/animal were yielded. This procedure depletes the mature haematopoietic cells, such as T cells, B cells, monocytes/macrophages, granulocytes, erythrocytes, and their committed precursors from the bone marrow, thus leaving the lineage negative cells untouched that express CD117 or Sca-1 marker. The cells were cultured for 3 days in RPMI (Bio Whittaker, Rockville,USA), supplemented with the following cytokines: 10ng/ml murine IL-3, 50ng/ml murine IL-6, and 50ng/ml murine stem cell factor (SCF) (Tebu-bio, Offenbach, Germany). The cells were counted and used for the transduction (see sections 3.2.6/3.2.5).

3.2.3 Production of Eco/GALV viral particles

3.2.3.1 Transient transfection of 293T cells

This technique is based on the unspecific uptake of plasmid-DNA-$CaCl_2$ crystals by 293T cells. Since the plasmids do not integrate into the genome but exist as episomes in the cells, the transgene is only transiently expressed. For this purpose, 5x10^6 293T cells were seeded in 8ml DMEM-standard per 10cm dish. On the next day, the medium was exchanged to 0.25μM chloroquine in DMEM-standard (8ml). Master mixes were prepared as follows (for one sample):

	7.5	μg	plasmid of interest
	1	μg	M187/ M620 (coding for Eco/GALV env, respectively)
	12.5	μg	M579 (coding for gag/pol)
	50	μl	$CaCl_2$ (2.5 M)
Ad	500	μl	dH_2O

This mix was added drop-wise to 500µl (per sample) of 2x HEPES (pH: 7.0/7.05/7.1) under vigorous vortexing. Mixture was incubated for 20min. at room temperature. After incubation, 1ml from the transfection mix was pipetted to each plate under continuous shaking to yield an evenly distribution of transfection crystals. Cells were incubated for 8h and then the medium was exchanged by 6ml DMEM-standard. Next day, supernatant was harvested and new 6ml DMEM standard was added. Supernatant was collected 24, 36 and 48 hours after transfection. Collected supernatant was sterile filtrated through 0.45 µm membrane and stored at 4°C. All supernatants were pooled and titrated on the embryonic murine fibroblast SC-1 cell line (for Eco env-supernatant) or TE671 cell line (for GALV env-supernatant).

3.2.3.2 Titration of the produced vector particles

In order to determine the quantity of infectious particles in the produced gammaretroviral supernatant, the murine adherent cell line SC-1 and human adherent cell line TE671 were transduced with eco-supernatant and GALV-supernatant, respectively. For this purpose, one day before titration, 5×10^4 cells (SC-1/TE671)/well in 1ml DMEM standard medium were seeded in a 24-well tissue culture plate. On the next day, different quantities (between 0 and 1000µl) of produced viral supernatant were added on to the cells and centrifuged for 1 hour with 2380rpm at 31°C. The total volume was kept to 1ml per well. After 2-3 days, the medium was removed and the cells were washed once with 1xPBS and then were trypsinized. The cells were resuspended with 1ml DMEM standard medium and transferred to FACS tubes. After washing with 2ml FACS buffer, cells were resuspended into 300µl FACS buffer and analyzed for eGFP fluorescence in the FACS. The titres of the viral particles were calculated by the following formula:

Titre [infectious particles/ml] = (transduced cells [%]/100) x seeded cells x dilution factor.

All the dilutions were set in duplicates and the average value was determined. The average titre of the produced supernatants was achieved from $1x10^5$ to $4x10^6$ particles per ml.

3.2.4 Transduction of human T cell lines (PM-1 and Jurkat)

In a 6-well plate, $1x10^6$ cells (PM-1/Jurkat)/well/3ml (RPMI standard medium) were seeded in a 6-well tissue culture plate. Different quantities (between 0 and 1000µl) of produced viral supernatant were added on to the cells to achieve ~15% eGFP positive cells (to get single copy of transgene/cell) and centrifuged for 1 hour with 2380rpm at 31°C. The total volume

was kept to 3ml per well. Afterwards, the cells were cultured for three days and analyzed by FACS (see 3.2.8).

The MOI (multiplicity of infectious particle) was calculated by the following formula:

MOI [infectious particles/cell] = (Titre [transfer units/ml] x volume of supernatant [ml]) / cell number.

3.2.5 Transduction of stimulated T cells or HSCs/HPCs

Stimulated T cells or HSCs/HPCs were transduced by culturing on virus-preloaded six-well plates. For this purpose, non-tissue culture treated six well plates were coated with 1ml retronectin solution (fibroncetin protein) over night at 4°C. The plates were blocked with 3ml of 2% BSA solution and subsequently washed with 3ml HBSS. 3ml virus containing supernatant was added to each well and six-well plates were centrifuged for 30 minutes at 962xg at 4°C. This step was repeated twice. Virus in the supernatant adheres unspecifically to the retronectin-coated surface, thus allowing concentration of retroviral particles at the bottom of the plate. $1x10^6$ stimulated lin-HSCs/HPCs in stem cell medium and 3 to $4x10^6$ stimulated T cells in mouse special medium were added per well of virus-preloaded plate and incubated overnight. Next day, the cells were transferred onto a second virus-preloaded plate (procedure repeated as above) and cultured for two days.

3.2.6 Cell counting by means of 'counting chamber'

The cell number was determined by using 'Neubauer cell counting chamber'. 10µl of well-resuspended cell suspension was mixed (diluted) with 1x trypan blue solution. Trypan blue is able to stain only dead cells. Thus, viable cells (unstained) were counted and dead cells (blue cells) were not. The mixture (approx. 10µl) was laid on to the counting chamber and cells were counted.

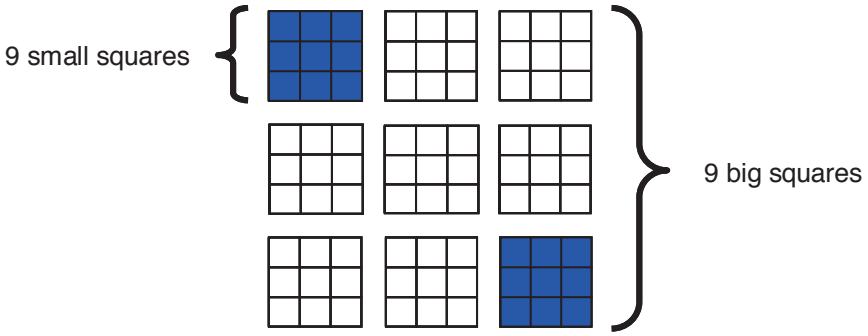


Figure 10. Cell counting by using Neubaur counting chamber.

Figure 10 shows the schematic representation of counting chamber. The cell number in two blue big squares was determined and then divided by 2 to get the average count in one big square. The exact cell count was calculated by the following formula:

Cells/ml = average count in one big square x dilution ratio (cell suspension:trypan blue) x 10^4

3.2.7 Animal experimental methods

3.2.7.1 Animal husbandry conditions

The mice were maintained and bred according to the guidelines of FELASA (Federation of European Laboratory Animal Science Associations) in the animal husbandry facility of George-Speyer-Haus. Cages were individually ventilated (IVC). The area was entered only after wearing the protective clothings, face mask, gloves and overshoes. The temperature was constantly maintained to 22°C with the air humidity from 50 to 60%. The light rhythm (light/dark) followed at the interval of 12 hours. The animals were cared and checked for general state of health by animal care-takers, maintenance staff as well as by a veterinary control supervisor.

3.2.7.2 Blood withdrawal for FACS and haemogram

Up to 10% of the blood volume from the mice can be withdrawn usually without any recognizable side effects. Blood was taken from the mice either from the tail vein or by heart puncture (if the mouse was sacrificed). For withdrawal from the tail vein, the mice were warmed up with red light lamp, then the tail vein was cut with a scalpel and the blood was collected with EDTA coated capillary (Microvette). 50-100µl blood was taken and used for FACS analysis and haemogram. ~50µl blood was used for FACS analysis (see 3.2.8). ~5µl blood was used for haemogram and the blood cell count was determined by using Scil Vet ABC (animal blood counter) in order to get a reference over the leukaemic status of the experimental animals. For the heart puncture, the animals were first anesthetised with Enfluran and then sacrificed by a neck break. The animal was fixed on a cork tray. After disinfecting with 70% ethanol, the chest wall was opened and blood was withdrawn with a 1ml syringe by puncturing the heart with a scissors. Blood could be taken up to 1ml. Heparin was used as an anticoagulant. For the determination of haemogram, EDTA could only be used as an anticoagulant. Therefore, also while scarificing the animal, some blood was taken with EDTA-coated Microvette.

3.2.7.3 Sacrificing and sectioning of the animals

Obviously sick recipients and the donor animals were anesthetized with Enfluran and afterwards sacrificed by a neck break. The dead animals were fixed in upside-down position on a cork-tray and disinfected with 70% ethanol. The skin was removed from the abdominal wall without damaging the internal organs. After opening the abdominal wall, all macroscopically visible changes of the internal organs were documented and the following organs were removed for further investigations: spleen, lymph nodes, thymus (if available), kidneys, liver, heart, lungs, brain and bones (Tibia and Femur) and/or the appropriate tumors (lymphomas). Before further preparation, the organs were transferred into 1xPBS and stored on ice.

3.2.7.4 Processing of the organs for FACS and histology

After isolation of the lymphatic organs, the single cell suspensions were made for FACS analysis and the part of the organs was fixed for histological analysis. From the sick animals, lymphatic organs (spleen, lymph nodes, thymus and bone marrow) were analyzed by FACS and histological examination. The individual sample was homogenized through a cell strainer (filter mesh) with the help of a syringe stamp in a 6-well plate and collected in a 50ml Falcon tube. The cell suspensions were washed once with 1xPBS and the cell number was determined. An appropriate cell quantity was then used for the FACS analysis and the remaining part of the cells was preserved at -80°C. Further, the appropriate organs were fixed in the 10% ZINC FORMAL FIXX solution. After 24-36 hours, the Fixx solution was removed and 70% ethanol was added to the stored organs. Histological sections of the fixed organs were made according to the regulation of the pathological data base RITA [54] and stained with Haematoxylin and Eosin (HE). This was done by a commercial service of the company MFD DIAGNOSTICS GmbH (Wendelsheim). The histological investigation was done in co-operation with Dr. Silvia Hartmann and Professor Dr. Martin-Leo Hansmann from Pathology Institute of the JWG University of Frankfurt.

3.2.8 FACS analysis

Via Flow cytometry, different physical and chemical characteristics of individual cells or particles are measured at the same time. The cells are arranged one behind the other in a flow direction and examined separately by means of a laser beam. Due to its scattered light characteristics, cell size (forward scatter, FSC) and granularity (sideward scatter, SSC) can be determined for several thousand individual cells. After staining with a fluorochrome conjugated-antibody or by the expression of fluorescent protein (e.g. green fluorescent

protein, GFP), the cells are sensed by the laser beam. Surface proteins can be recognized with a primary fluorochrome-coupled antibody. For FACS analysis, CellQuest pro (BD) was used in the available work. For this purpose, ~5 x 10^5 cells were transferred into 5ml round bottom FACS tubes. The cells were washed twice with 2ml FACS-buffer by centrifugation. Supernatant was discarded and cells were resuspended in 300μl FACS-buffer. For blood or homogenized organ cell suspensions, erythrocytes were lysed by using cal-lyse solution. After staining with antibodies, the cells (WBCs) were washed twice with PBS and resuspended in 300μl FACS-buffer. Afterwards, the cells were analyzed by FACS.

4. Results

It is not clear yet whether the mature T cell leukaemias/lymphomas originate from the haematopoietic stem cells/haematopoietic progenitor cells (HSC/HPC), later T cell progenitors, or the mature T cells themselves. As already described (see 1.1) for myeloid leukaemias, there is a growing evidence that they originate from HSCs/HPCs or early myeloid progenitors. For B cells it was recently shown that mature B lymphocytes can still be transformed; but for mature T cells, several observations indicate that these cells are less susceptible to transformation than HSCs/HPCs. To further investigate the transformation susceptibility of mature T cells in comparison with HSCs/HPCs, we introduced gammaretroviral vectors encoding the potent T cell oncogenes *NPM-ALK* (a fusion oncogene) and *Tax* (a viral oncogene), and the T cell potential oncogene *p21SNFT* into both cell types and then transplanted them into mouse models.

4.1 Cloning of gammaretroviral vectors

For the transduction of HSCs/HPCs and mature T cells, gammaretroviral vectors were cloned that coded for the potent T cell oncogenes *NPM-ALK* and *Tax,* and the T cell potential oncogene *p21SNFT*. Figure 9 shows a schematic representation of the cloned vectors.

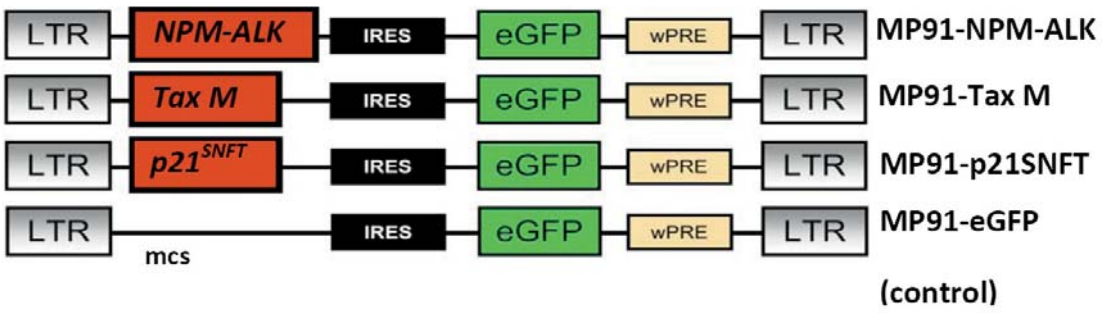


Figure 11. Structure of the gammaretroviral vectors. For cloning, MP91-eGFP gammaretroviral vector (control) was used, which encodes mcs, an IRES-element and the expanded green fluorescent protein (eGFP). To enhance the expression of the respective transgene, a wPRE element (Woodchuck hepatitis virus post transcriptional regulatory element) was used.

The gammaretroviral vector MP91-eGFP was used as the basic construct for the cloning. This vector encodes for a mcs (multiple cloning site), an IRES- element (internal ribosome entry site) and the gene for the expanded green fluorescent protein (eGFP), which serves as

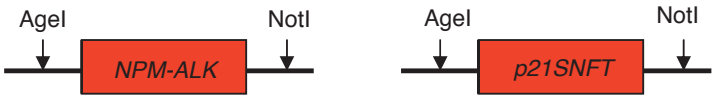
a marker and a control gene. The cDNAs of *NPM-ALK*, *Tax* and *p21SNFT* were cloned individually into the MP91-eGFP vector in the available work.

4.1.1 Cloning of *NPM-ALK* and *p21SNFT*

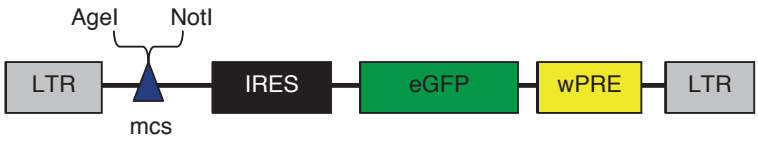
The plasmid B-*NPM-ALK* (containing *NPM-ALK* insert) and the gammaretroviral vector MP91-eGFP were both digested with the restriction enzymes AgeI and NotI (for maps see appendix). The digestion cuts the *NPM-ALK* insert out of the B-*NPM-ALK* plasmid and the MP91-eGFP retroviral vector in the mcs to produce corresponding sticky ends. The isolated fragments were then ligated to get the resultant vector MP91-*NPM-ALK* (see figure 12).

p21SNFT was also cloned with the same strategy, resulting in the vector MP91-*p21SNFT* (see figure 12).

(1) Digestion of plasmids containing *NPM-ALK* and *p21SNFT* with AgeI and NotI:



(2) Digestion of MP91-eGFP gammaretroviral vector at mcs with AgeI and NotI:



(3) Resultant MP91-*NPM-ALK* vector after ligation of the *NPM-ALK* insert (from step 1) and the MP91-eGFP gammaretroviral vector (from step 2):

(4) Resultant MP91-*p21SNFT* vector after ligation of the *p21SNFT* insert (from step1) and the MP91-eGFP gammaretroviral vector (from step 2):

Figure 12. Cloning strategy for *NPM-ALK* and *p21SNFT*. (1) Digestion of B-*NPM-ALK* plasmid (containing *NPM-ALK* insert) and B-*p21SNFT* plasmid (containing *p21SNFT* insert) with restriction enzymes AgeI and NotI. (2) Digestion of gammaretroviral vector MP91-eGFP with restriction enzymes AgeI and NotI. (3) Resultant vector MP91-*NPM-ALK* after ligation of *NPM-ALK* insert and digested MP91-eGFP retroviral vector. (4) Resultant vector MP91-*p21SNFT* after ligation of *p21SNFT* insert and digested MP91-eGFP retroviral vector.

4.1.2 Cloning of *Tax M*

For *Tax*, its murine codon optimized form '*Tax M*' was used for expression in murine cells. As the restriction sites in the plasmid 'B-*Tax M*' and gammaretroviral vector MP91-eGFP did not match for cloning, the gene for *Tax M* was first cloned into the Bluescript plasmid M23 (which had resembling restriction sites), which was then transferred into gammaretroviral vector MP91-eGFP (for maps see appendix). The whole cloning strategy for *Tax M* is depicted in figure 13 A and B.

(1) Digestion of the Bluescript M23 with XmaI, and the plasmid B-*Tax M* (containing *Tax M* insert) with NotI

(2) Linearized Bluescript M23 and *Tax M* plasmids with sicky ends:

(3) Treatment of the above products (step 2) with Klenow to generate blunt ends:

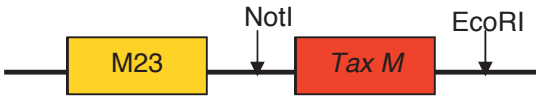
(4) Digestion of linearized blunt ended M23 and *Tax M* plasmids (step 3) with BamHI:

(5) Intermediate vector 'M23-*Tax M*' after ligation of the above products (step 4):

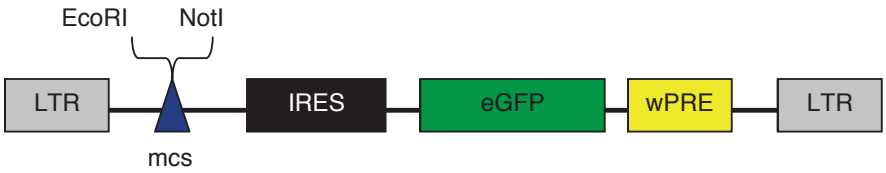
Figure 13 A. Cloning of *Tax M* into Bluescript M23. (1) Digestion of the Bluescript M23 with XmaI, and the plasmid B-*Tax M* (containing *Tax M* insert) with NotI to linearize (open) both the plasmids. (2) Linearized Bluescript M23 and B-*Tax M* plasmids with sicky ends, resulting from the step 1 digestion. (3) Treatment of both sticky ended M23 and *Tax M* plasmids with Klenow to generate blunt ends. (4) Digestion of both linearized blunt ended M23 and *Tax M* plasmids with BamHI to produce the corresponding sites. (5) Ligation of step 4 products, resulting in the intermediate vector M23-*Tax M*'.

The plasmid B-*Tax M* (containing *Tax M*) was digested with NotI, and the Bluescript plasmid M23 with XmaI to open (linearize) both the plasmids. As this digestion resulted in mismatched sticky ends due to the use of different sticky cutters (restriction enzymes), both vectors (linearized B-*Tax M* and M23) were treated with Klenow to generate blunt ends. Afterwards, both vectors were digested with BamHI. The digestion cuts the *Tax M* insert out of linearized B-*Tax M* plasmid and the M23 linearized vector to produce corresponding sticky ends. The fragments were ligated. Thus, an intermediate vector 'M23-*Tax M*' was cloned (see figure 13 A), which carried matching restriction sites for cloning into the gammaretroviral vector. The intermediate cloned plasmid 'M23-*Tax M*' and the gammaretroviral vector MP91-eGFP both were digested with EcoRI and NotI enzymes. The digestion cuts the *Tax M* insert out of 'M23-*Tax M*' and the MP91-eGFP gammaretroviral vector in the mcs. The isolated fragments were then ligated to get the resultant vector MP91-*Tax M* (see figure 13 B).

(1) Digestion of intermediate vector M23-*Tax M* with EcoRI and NotI:



(2) Digestion of gammaretroviral vector MP91-eGFP with EcoRI and NotI:



(3) Resultant MP91-*Tax M* vector after ligation of the *Tax M* insert (from step 1) and the MP91-eGFP retroviral vector (from step 2):

Figure 13 B. Cloning of *Tax M* (from intermediate vector M23-*Tax M*) into gammaretroviral vector M91-eGFP. (1) Digestion of intermediate vector M23-*Tax M* with EcoRI and NotI. (2) Digestion of gammaretroviral vector MP91-eGFP with EcoRI and NotI. (3) Resultant MP91-*Tax M* vector after ligation of the *Tax M* insert (from step 1) and the MP91-eGFP retroviral vector (from step 2).

Thus, the isolated fragments of all the three genes (*NPM-ALK*, *p21SNFT* and *Tax M*) were individually inserted into MP91-eGFP vector directly before the IRES element in order to enable bicistronic expression of oncogene and marker gene (eGFP). All cloned constructs were verified by sequencing. Possible mutations were excluded.

4.2 Packaging of cloned constructs

Packaging was performed in 293T cells as described in section 3.2.3.1. For this purpose, the envelopes derived from the Gibbon ape leukaemia virus (GALV) and the ecotropic murine leukaemia virus (Eco) were used to produce supernatants for the transduction of human T cell lines and murine cells, respectively. One day after packaging, green cells under the fluorescent microscope confirmed successful transfection (Figure 14).

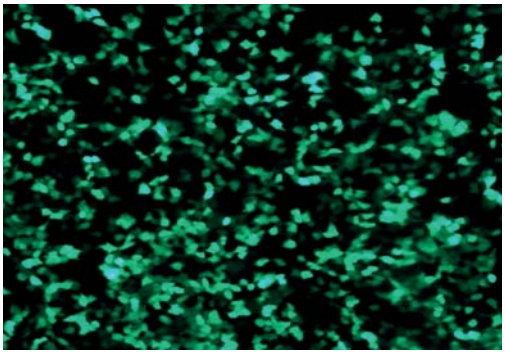


Figure 14. Green cells observed in the fluorescent microscope 24 hours after transfection.

The 'GALV' supernatant was used for *in vitro* experiments with human T cell lines (PM-1 and Jurkat), while the 'Eco' supernatant was used for *in vivo* experiments with murine models. All supernatants were pooled at 4^0C for at least 10 days until further use.

4.3 Western blot analysis for protein expression

Western blotting was performed to check for protein expression of cloned constructs. As *Tax* is a viral oncoprotein, transfected human '293T' cells were used for its expression. But, ALK and *p21SNFT* are human genes and they could be expressed naturally in human cells, as antibodies used for the detection of *NPM-ALK* expression bind to its ALK portion. Therefore, to exclude a positive signal from the corresponding endogenous proteins in human cells, different species cells (murine cells 'SC-1') were transduced with both of these constructs individually and analyzed for respective protein expression. For this purpose, *Tax* transfected 293T cells (see section 3.2.3.1) and *NPM-ALK* or *p21SNFT* transduced SC-1 cells (see section 3.2.3.2) were used. Two days after transfection/transduction, cells were trypsinized and washed with PBS to be used for Western blotting. Western blotting was performed as described in the section 3.1.12. Expression was confirmed for all three construts by comparing their sizes with the standard marker 'kDa'. As negative controls, untransfected 293T cells for *Tax* and untransduced SC-1 cells for *NPM-ALK* and *p21SNFT* were used. Thus, the expression of all these oncogenes on protein level was confirmed (see figure 15).

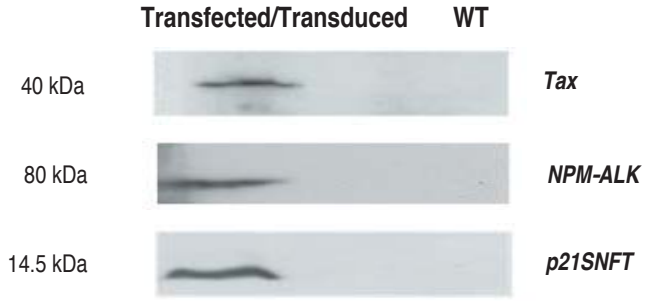


Figure 15. Protein expression of transfected/transduced oncogenes. *Tax* expression in 293T cells (upper band, 40 kDa). *NPM-ALK* expression in SC-1 cells (middle band, 80 kDa). *p21SNFT* expression in SC-1 cells (lower band, 14.5 kDa). As negative controls, corresponding untransfected/ untransduced cells (wild type) were used (right empty portion of the corresponding gel). WT: wild type.

4.4 In vitro Experiments

4.4.1 Effects of *p21SNFT*, *Tax M* and *NPM-ALK* on human T cell lines

To observe the in vitro effects on cell growth of T cells, two T cell lines (Jurkat and PM-1) were transduced with *p21SNFT*, *Tax M* and NPM-ALK supernatants (GALV env) and followedup for eGFP marking and cell growth. eGFP transduced cells were used as controls. Both cell lines showed comparative transduction efficacies for transferred genes (see figure 16).

Different MOIs of vector supernatants were used to achieve ~15% eGFP positive cells to confer single copy of gene in the transduced cells (see section 3.2.4). Gene marking and the growth (counting) were monitored every second day. For *NPM-ALK* and *Tax M*, eGFP marking declined from ~15% to <1% in both T cell lines over 12 to 14 days after transduction, while the eGFP marking for the control cells remained stable (Figure 17 A/B). For both, *NPM-ALK* and *Tax M*, cells also grew slower as compared to the control eGFP transduced cells (Figures 17 E/F).

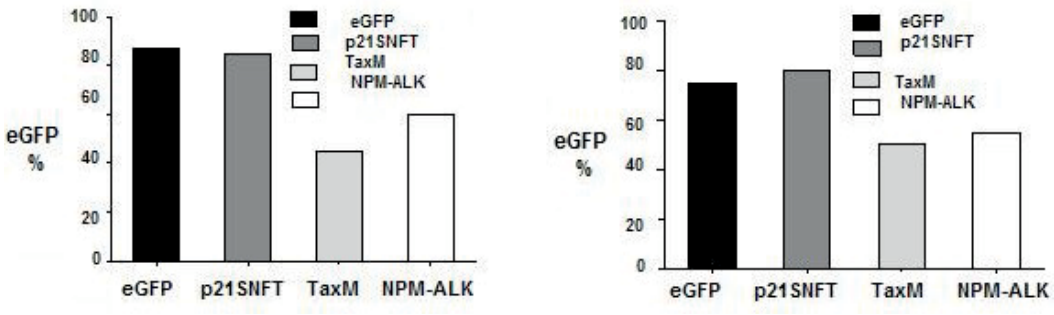


Figure 16. (A) Transduction efficacies of constructs in PM-1 cell line. eGFP control: 87%; *p21SNFT*: 85%; *Tax M*: 45%; *NPM-ALK*: 60%. **(B) Transduction efficacies of constructs in Jurkat cell line.** eGFP control: 75%; *p21SNFT*: 80%; *Tax M*: 50%; *NPM-ALK*: 55%. Black bars: eGFP control; dark grey bars: *p21SNFT*; light grey bars: *Tax M*; white bars: *NPM-ALK*.

For *p21SNFT*, the eGFP marking declined very slowly from 13% to 5% in both T cell lines over 42 days after transduction, while the eGFP marking of the control cells remained stable (Figures 17 A/B). The cell counts for both, *p21SNFT* and the control eGFP were comparable in both T cell lines and no big difference could be observed (Figures 17 C/D).

Thus, no growth or proliferation promoting effect of all these genes could be observed. As for *NPM-ALK* and *Tax M*, eGFP marking declined from ~15% to <1%, LM-PCR was performed

to further investigate if the transgene was still present in the transduced cells, (see section 4.4.2).

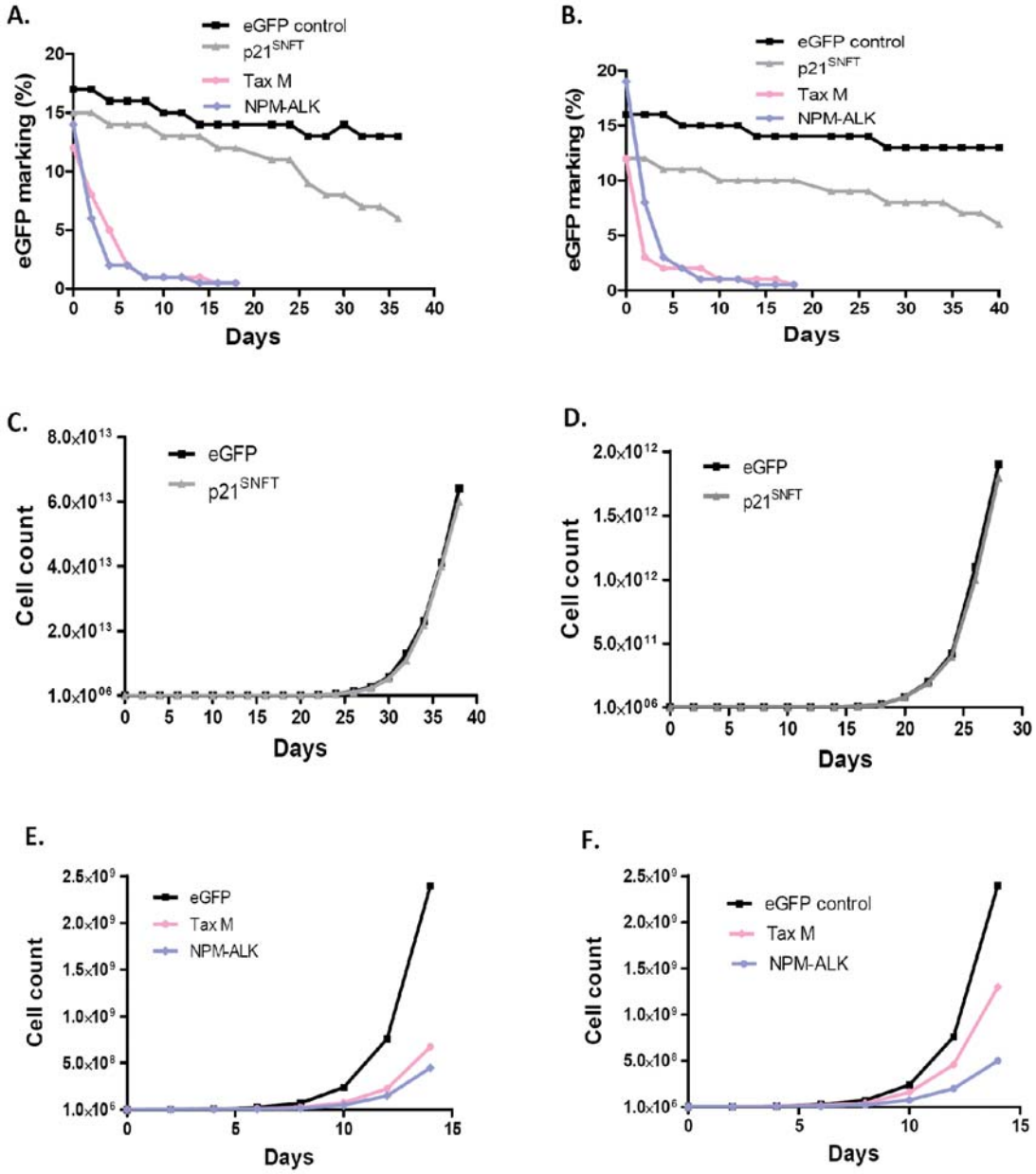


Figure 17. Effects of *p21SNFT*, *Tax M* and *NPM-ALK* on human T cell lines.
eGFP marking for all three oncogenes declined over time, while the control cells stably expressed eGFP in both T cell lines, (A) PM-1, (B) Jurkat. Cell growth for *p21SNFT* was comparable to eGFP control in both T cell lines, (C) PM-1, (D) Jurkat. Cell growth for *NPM-ALK* and *Tax M* was less exponential in comparison to eGFP control in both T cell lines, (E) PM-1, (F) Jurkat. Black line: eGFP control; grey line: *p21SNFT*; red line: *Tax M*; blue line: *NPM-ALK*. Note: *NPM-ALK* and *Tax M* transduced cells were observed for 14 days due to loss of eGFP marking.

4.4.2 LM-PCR for *NPM-ALK* and *Tax M* transduced T cell line

NPM-ALK and *Tax M* transduced both T cell lines (PM-1 and Jurkat) showed decline in eGFP marking from ~15% to <1%. LM-PCR was performed to check if the transgene was still present in the transduced cells. For this purpose, DNA was isolated from *NPM-ALK* and *Tax M* transduced Jurkat cells before and after the decline of eGFP expression (~15% and <1%). LM-PCR was performed as described in the section 3.1.11. Figure 18 shows the result of the LM-PCR, in which both (*NPM-ALK* and *Tax M*) transduced cells with ~15% eGFP expression show oligoclonal integration pattern (≤ 8 bands), while cells with <1% eGFP expression show only one band which refers to the internal control (figure 18, band depicted in blue box). An explanation for that fact is described in the discussion part (see 5.1).

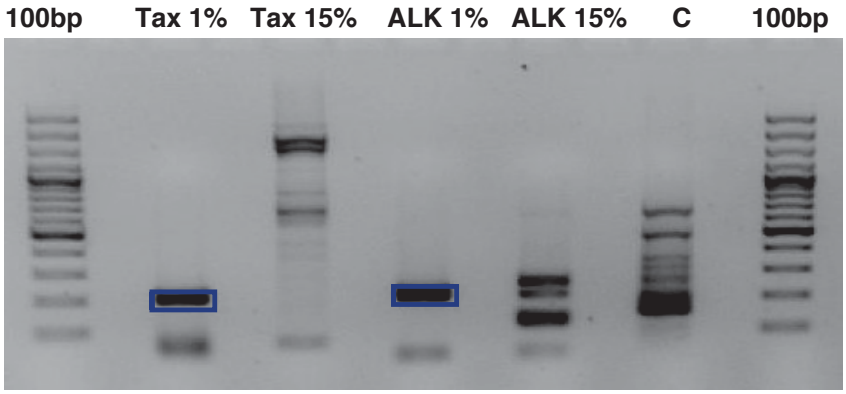


Figure 18. LM-PCR of *Tax M* and *NPM-ALK* in Jurkat T cell line. For both (*Tax M* and *NPM-ALK*), oligoclonal integration pattern (≤ 8 bands) can be seen in the cells expessing 15% eGFP, while no integration in the cells expressing <1% eGFP could be observed except for the internal control band (blue box). C: eGFP control cells expressing ~15% eGFP.

4.5 *In vivo* experiments

4.5.1 Mouse models: Transplantation protocol and follow up

After transduction (see section 3.2.5), 1-2x10^7 polyclonal T cells (from C57BL/6J.Ly5.1 mice) or monoclonal T cells (from C57BL/6Tg.Ly5.2 mice) and 3-5x10^5 HSCs/HPCs (from C57BL/6J.Ly5.1 mice) were transplanted into RAG-1 deficient recipient mice (C57BL/6J.Ly5.2 background). The cells were injected into the tail vein of the recipients. One day before transplantation with murine stem cells, recipient mice were sublethally irradiated with 5Gy. After 16-24 weeks, animals that received T cell transplants developed a massive colitis and were sacrificed. T cells were isolated from spleen and lymph nodes and serially transplanted into the secondary recipients. The basic transplantion protocol is shown in figure 19.

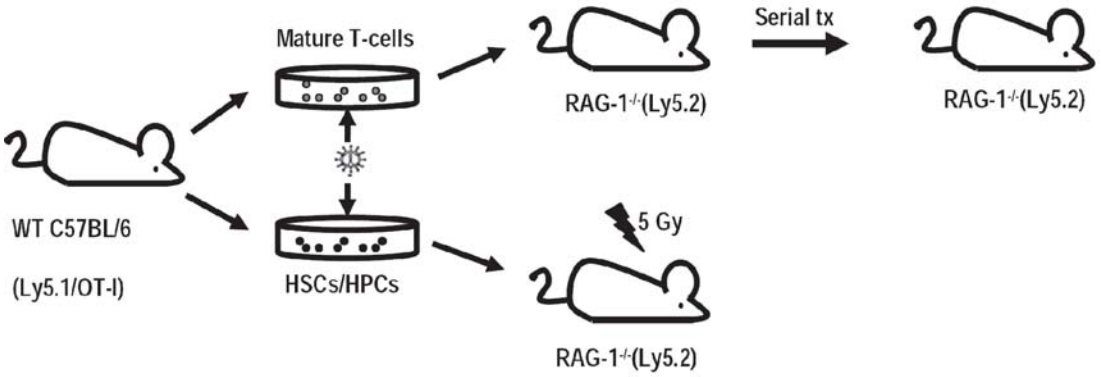


Figure 19. Transplantion principle and serial transplantation. C57BL/6 (Ly5.1 or OT-I) WT mice were used as donors for murine T cells (TC) and lineage negative HSCs/HPCs. Isolated lineage negative HSCs/HPCs were stimulated for 3 days and mature T cells for 4 days. Subsequently after retroviral transduction, the cells were transplanted in to Rag-1 deficient mice. After 16-24 weeks, polyclonal T cell transplanted animals developed massive colitis and were sacrificed. After isolating T cells from spleen and lymph nodes, those cells were serially transplanted into secondary recipients (always one donor for one recipient).

4.5.2 Comparable levels of retroviral transduction and expression of the oncogenes in HSCs/HPCs, mature T cells and their respective progeny

After isolation, mature T cells were stimulated with anti-CD3/-CD28 coupled paramagnetic beads for 4 days, and HSCs/HPCs were stimulated with mIL-3, mIL-6 and mSCF for 3 days (see section 3.2.2). After two rounds of transductions with ecotropic vector supernatants, the

transgene expression was measured by FACS. For mature T cells, 20 to 67% and for HSCs/HPCs, 25 to 48% transduction efficacies were achieved (see figure 20).

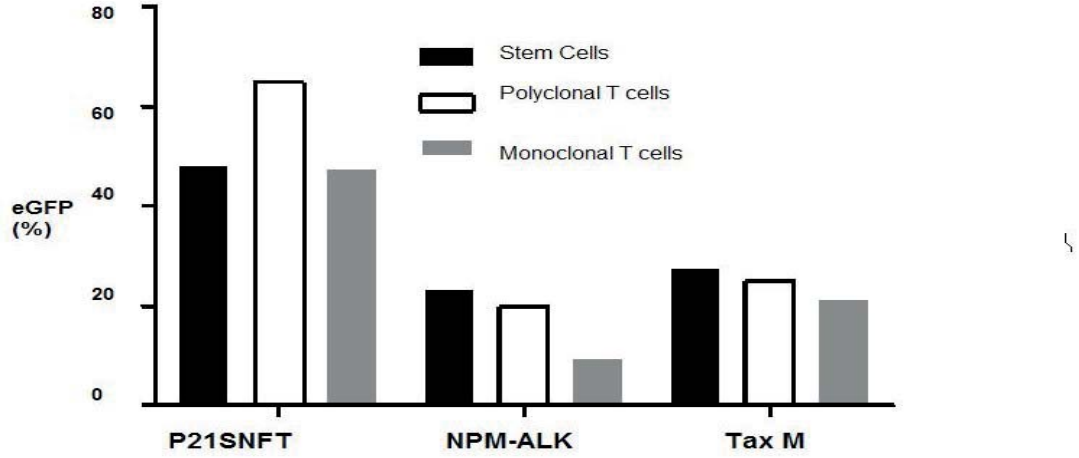


Figure 20. Transduction efficacies of oncogenes in stimulated murine HSCs/HPCs and mature T cells. The overall transduction efficacies achieved for each construct were: *p21SNFT*: 47 to 65%; *NPM-ALK*: 9 to 25%; *Tax M*: 21 to 27%. Black bars: haematopoietic stem cells; white bars: polyclonal T cells; gray bars monoclonal T cells (OT-1).

After transduction, the cells were transplanted into Rag-1 deficient mice. Table 24 shows the number of transplanted mice with each cell type (transduced with each construct).

Table 24. Number of transplanted mice with each cell type/construct.

Construct	HSCs transplanted mice	Polyclonal T cell transplanted mice	Monoclonal T cell transplanted mice
p21SNFT	5	6	7
NPM-ALK	3	5	5
Tax M	10	6	12

The animals were analyzed for donor cell engraftment and transgene (eGFP) expression 6 weeks after transplantation by collecting blood from the tail vein. Expression of donor marker (CD45.1), T cell marker (CD3) and the transgene (eGFP) was determined by FACS analysis. This procedure was performed every 6 to 8 weeks to check for the transgene expression and any indication for the development of leukaemia/lymphoma. Figure 21 shows eGFP expression in the T cell compartment of all transplanted groups 6 weeks after transplantation. The overall eGFP expression in T cell compartment was 20 to 73% for all the *p21SNFT*

transplanted groups (haematopoietic stem cell, polyclonal T cell and monoclonal T cell). For *NPM-ALK* animals, eGFP expression could not be observed 6 weeks after transplantation. Subsequently, the *NPM-ALK*_OT-I transplanted group expressed eGFP 7 to 9 weeks after transplantation. From *Tax M* group, the eGFP expression could not be observed for the whole observation period of ~8 months, although enfragted cells were positive for the donor marker (CD45.1) and T cell marker (CD3). MP91-eGFP control vector transplanted mice were already used and showed a stable expression of eGFP [55].

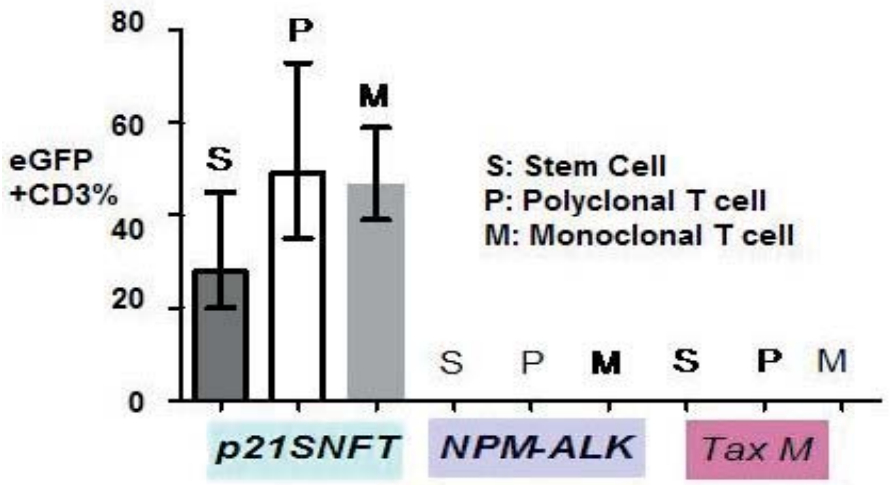


Figure 21. eGFP expression in T cell compartment of all transplanted groups 6 weeks after transplantation. For *p21SNFT*: stem cell transplanted group: 20 to 45% (black bar), polyclonal T cell transplanted group 35 to 73% (white bar), monoclonal T cell (OT-1) transplanted group: 39 to 59% (gray bar). For *NPM-ALK* and *Tax M* transplanted groups, no eGFP marking could be observed. S: stem cell; P: polyclonal T cell; M: monoclonal T cell.

4.5.3 Oncogenes transform primary murine mature T cells and HSCs/HPCs after retroviral transduction

After transplantation of oncogene-transduced HSCs/HPCs and mature T cells, some of the animals from the *NPM-ALK* and *p21SNFT* groups developed haematological malignancies with characteristic latencies. The tumors showed massive enlargements of spleen (splenomegaly) and, mesenteric and paraspinal lymph nodes (lymphoma). In some cases, white blood cell counts (WBC) were elevated (upto 74 x 10^3/µl, control 15 x 10^3/µl) and showed leukaemic characteristics. The details of these tumors are described as below:

4.5.3.1 *NPM-ALK* induced tumors

4.5.3.1.1 Tumor development after transplation of *NPM-ALK* transduced OT-1 cells

All 5 mice transplanted with *NPM-ALK* transduced monoclonal mature T cells (OT-I) developed leukaemia/lymphoma with massive enlargements of liver (hepatomegaly), spleen (splenomegaly) and mesenteric lymph nodes 45 to 64 days after transplantation. Figure 22A shows the survival curve of this group (red line). Only 3 out of 5 mice could be analyzed because, unfortunately 2 of them died overnight and were autolytic. Nevertheless, autopsy of these two mice showed massive enlargements of spleen and lymph nodes. In FACS analysis, the tumors from analyzed mice were positive for 6 to 45% eGFP marking (figure 22B), but very low percentages of different cell populations like, TCRα/β (common TCR), Vα2/Vβ5 (OT-I specific TCR), CD8, CD4, CD19, NK1 (natural killer), and a high percentage of CD11b (myeloid marker) population in the eGFP negative population. The eGFP expressing cells did not express any lineage marker.

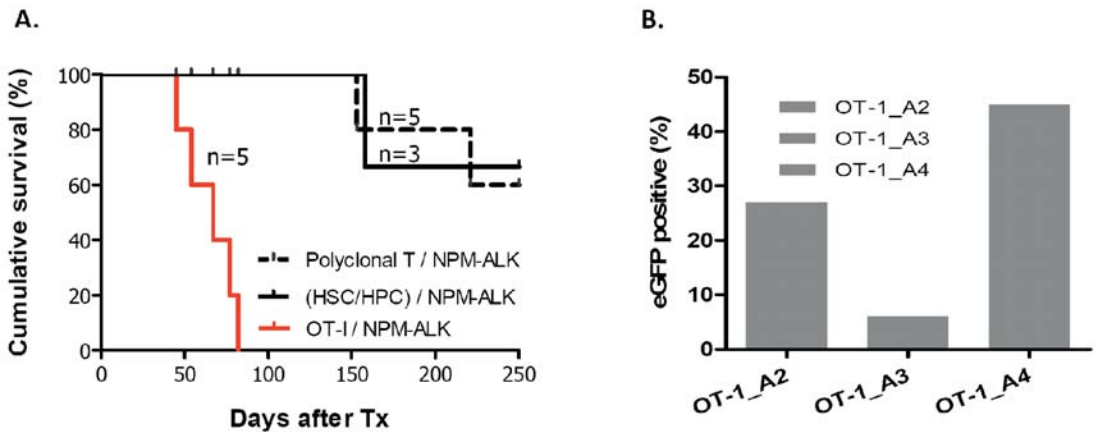


Figure 22. (A) Survival curve of *NPM-ALK* transplanted mice. All 5 mice transplanted with transduced monoclonal T cells (OT-1) developed leukaemia/lymphoma (red line), while only 2 out of 5 mice transplanted with transduced polyclonal T cells (black dotted line) and 1 out of 3 mice transplanted with transduced haematopoietic stem cells (black line) developed leukaemia/lymphoma. **(B) eGFP expressions in *NPM-ALK*_OT-I tumors.** Mouse # 2 (A2): 27%, Mouse # 3 (A3): 6%, Mouse # 4 (A4): 45%.

To exclude contaminating (non malignant) cells from the tumors, the tumor cells (supplemented with IL-2) were kept in culture for two weeks and again analyzed by the FACS. CD11b marker as well as the other markers remained undetected. Furthermore, the tumor cells from three mice of this group were serially transplanted into Rag-1 deficient mice. After 3 to 4 weeks of transplantation, two of them developed the same tumors with the same phenotype corresponding to the donor mice tumors 3 to 4 weeks after serial transplantation.

Thus, again, the tumors could not be analyzed to a clear haematopoietic lineage by FACS analysis. The FCAS findings of these tumors are summed up in Table 25.

Table 25. FACS findings of tumors from *NPM-ALK*_OT-I cell transplanted mice.

Muose	eGFP	TCR α/β	Vα2	Vβ5	CD8	CD4	CD19	CD11b	NK1	WBC > 15000/µl
2	27	2	7	0	0	0	1	30	1	No
3	6	2	11	9	11	2	<1	76	6	Yes
3 (sr.tx)	2	<1	<1	0	0	0	0	76	1	n.m
4	45	1	0	0	0	0	2	25	<1	n.m
4 (sr.tx)	17	<1	0	0	0	0	0	54	0	Yes

The tumors were positive for 6 to 45% eGFP marking (figure 22B), but very low percentages of different cell populations like, TCRα/β (common TCR), Vα2/Vβ5 (OT-I specific TCR), CD8, CD4, CD19, NK1 (natural killer), and a high percentage of CD11b (myeloid marker) population in the eGFP negative compartment. The eGFP expressing cells did not express any lineage marker. All the values are in percentages (%). tx: transplantation; sr.tx: serially transplanted; n.m: not measured.

Histopathological analysis for these tumors revealed ‘anaplastic lymphoma’ phenotype (Figure 23 A/B, data not shown – M. L. Hansmann).

4.5.3.1.2 Tumor development after transplantation of *NPM-ALK* transduced polyclonal T cells

Two out of five mice transplanted with *NPM-ALK* transduced polyclonal T cells developed leukaemia/lymphoma with massive enlargements of liver (hepatomegaly) and spleen (splenomegaly) 153 to 250 days after transplantation (figure 22 A). In FACS analysis, no phenotype of the tumors could be identified.

Table 26. FACS findings of tumors from *NPM-ALK*_polyclonal T cell transplanted mice.

Mouse	eGFP	CD3	CD45.1	CD8	CD4	CD19	CD11b	NK1	WBC >15000/µl
3	2	17	18	10	8	1	10	1	No
5	0	0	12	0	0	0	0	0	No

Tumors showed different cell populations which were positive for donor cell marker (CD45.1) and either T cell (CD3), B cell (CD19), natural killer (NK), or myeloid cell (CD11b) marker but did not show any eGFP marking except one tumor (mouse 3) which showed 2% eGFP as well. The eGFP expressing cells did not show any linage marker.

Although the tumors showed different cell populations which were positive for donor cell (CD45.1) and either T cell, B cell or myeloid cell marker, but they did not express any eGFP

marking except for one tumor which expressed 2% eGFP as well. These 2% eGFP positive cells did not show any lineage specific marker. Table 26 shows the FACS findings of these tumors.

Histopathological analysis for these tumors revealed anaplastic lymphoma phenotype (Figure 23 C/D, data not shown – M. L. Hansmann).

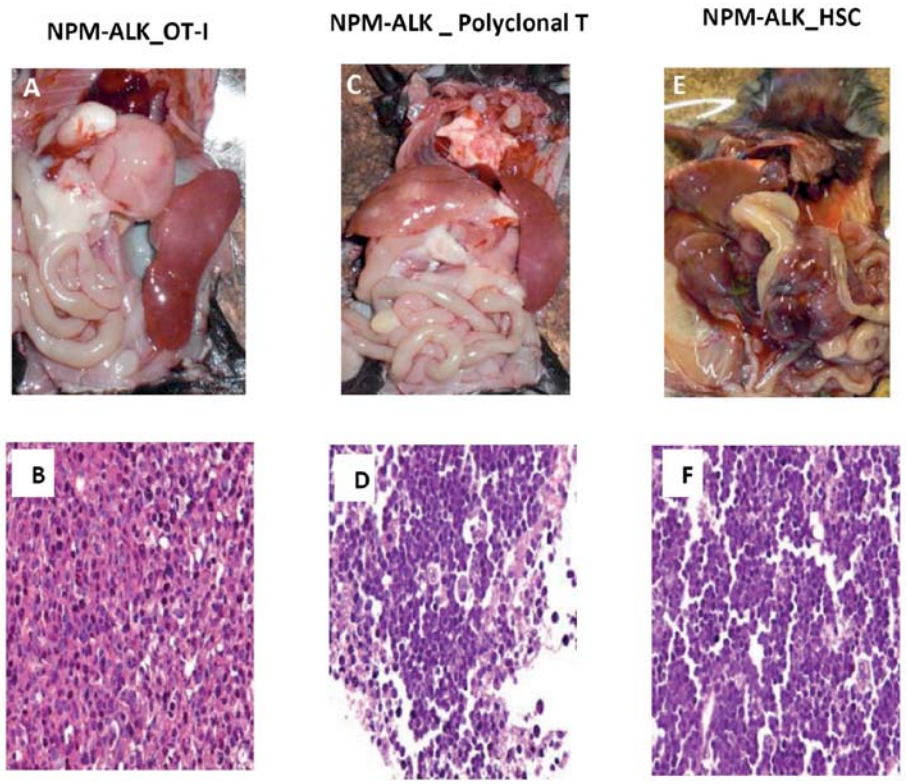


Figure 23. (A) Histopathology of *NPM-ALK* induced leukaemia/lymphoma.
(A) Autopsy of animal 3_*NPM-ALK*_OT-I. Gorss enlargement of mesenteric lymph nodes and spleen (splenomegaly) can be seen. **(B)** Mesenteric lymph node of animal 3_ *NPM-ALK*_OT-I. Tumor section showing undifferentiated lymphocytes (anaplasia), infiltrated with granulocytes. **(C)** Autopsy of animal 3_*NPM-ALK*_Polyclonal T cell. Gorss enlargements of liver (hepatomegaly) and spleen (splenomegaly) with tumor masses (white patches) can be seen. **(D)** Spleen of animal 3_ *NPM-ALK*_Polyclonal T cell. Undifferentiated lymphocytic infiltration (anaplasia) can be seen. **(E)** Autopsy of animal 1_*NPM-ALK*_HSC. A grossly enlarged haemorrhagic mesenteric mass infiltrated into intestines. **(F)** Mesenteric tumor mass of animal 1_*NPM-ALK*_HSC. Tumor section showing undifferentiated lymphocytic infiltration (anaplastic).

4.5.3.1.3 Tumor development after transplation of *NPM-ALK* transduced HSCs/HPCs

One out of the three mice transplanted with *NPM-ALK* transduced HSCs/HPCs developed a massively enlarged mesenteric tumor mass (lymphoma) 158 days after transplantation. In the

FACS analysis, the tumor showed different cell populations which were positive for donor cell (CD45.1) and either T cell, B cell or myeloid cell marker, but did not express any eGFP marking (see table 27).

Table 27. FACS findings of the tumor after transplation of *NPM-ALK* transduced HSCs/HPCs.

Mouse	eGFP	CD3	CD45.1	CD8	CD4	CD19	CD11b	NK1	WBC >15000/µl
1	0	33	60	18	20	14	4	2	n.m

The tumor showed different cell populations which were positive for donor cell marker (CD45.1) and either T cell (CD3), B cell (CD19), natural killer (NK), or myeloid cell (CD11b) marker but did not show any eGFP marking. n.m: not measured.

Histopathological analysis for the tumor revealed anaplastic lymphoma type (Figure 23 E/F, data not shown – M. L. Hansmann).

4.5.3.2 *P21SNFT* induced tumors

From *p21SNFT* group, only 2 out of 7 mice transplanted with *p21SNFT* transduced monoclonal mature T cells (OT-I) developed leukaemia/lymphoma (see section 4.5.3.2.1). None of the mice transplanted with *p21SNFT* transduced HSCs/HPCs or polyclonal T cells developed leukaemia/lymphoma upto 240 days post-transplantation (figure 24 A).

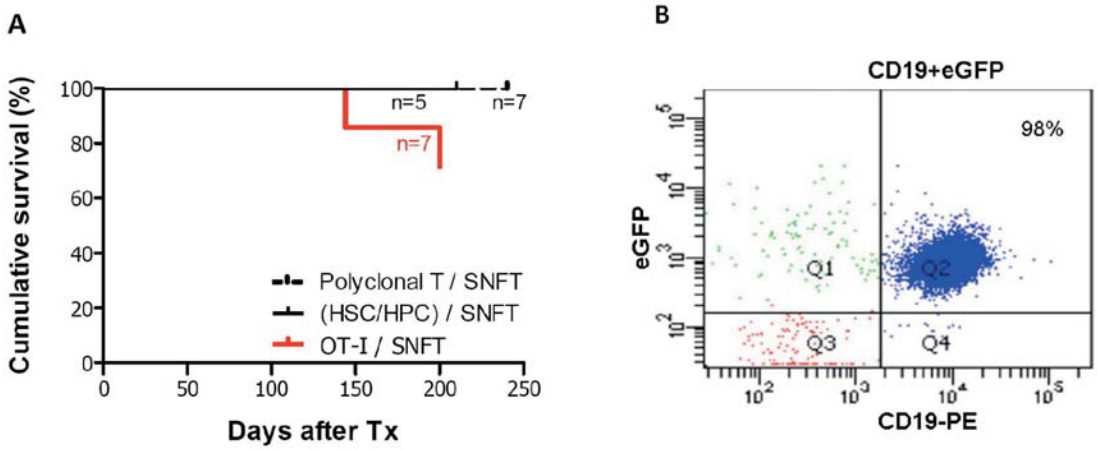


Figure 24 A. Survival curve of *p21SNFT* transplanted mice. Only 2 out of 7 mice transplanted with p21SNFT transduced monoclonal T cells (OT-1) developed leukaemia/lymphoma (red line). None of the mice transplanted with p21SNFT transduced polyclonal T cells (black dotted line) and haematopoietic stem cells (black line) developed leukaemia/lymphoma up to followup of 240 days. **B. FACS analysis of *p21SNFT*_OT1 transplanted mice tumors.** Enlarged spleens/lymph nodes (lymphomas) were analyzed by FACS. Almost all cells (98%) were double positive for eGFP and CD19 in both mice tumors.

4.5.3.2.1 Tumor development after transplantation of *p21SNFT* transduced OT-I cells

2 out of 7 mice transplanted with *p21SNFT* transduced monoclonal mature T cells (OT-I) developed leukaemia/lymphoma 144 to 200 days after transplantation (figure 24 A). The tumors showed massive enlargements of mesenteric, paraspinal and superficial inguinal lymph nodes (lymphomas) infiltrating the surrounding organs. In FACS analysis, the tumors showed high eGFP and CD19 markings (figure 24 B).

Histopathological analysis for these tumors revealed 'anaplastic lymphoma' type (Figure 25, data not shown – M. L. Hansmann).

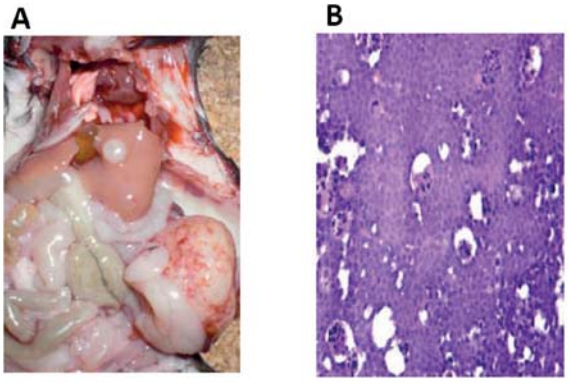


Figure 25. Histopathology of *p21SNFT* induced leukaemia/lymphoma. (A) Autopsy of animal 3_21SNFT_OT-I. Gorssly enlarged mesenteric lymph node mass, and liver with a tumor mass (round white). **(B)** Mesenteric lymph node (lymphoma) of animal 3_ *p21SNFT*_OT-I. Tumor section showing undifferentiated lymphocytes (anaplasia).

4.5.4 LM-PCR analysis for the *Tax* transplanted group

As already mentioned (see section 4.5.2), form *Tax* transplanted group, none of the mice expressed any eGFP marking, although they expressed the donor cell marker CD45.1/OT-I and T cell (CD3) marker. Furthermore, the recipients did not show any signs for leukaemia/lymphoma. To further investigate if the gene modified cells were present in the recipients, LM-PCR was performed by isolating DNA from one mouse of each *Tax*-transplanted group (HSC, polyclonal T cell and OT-I T cell). LM-PCR was performed as described in the section 3.1.11. For this purpose, animals were sacrificed and DNA was isolated from the splenocytes. LM-PCR revealed oligoclonal pattern (≤10 bands) of the transduced cells in all three groups of animals (figure 26), which indicates that *Tax* transduced cells were still repopulating the recipients, but did not express the transgene anymore.

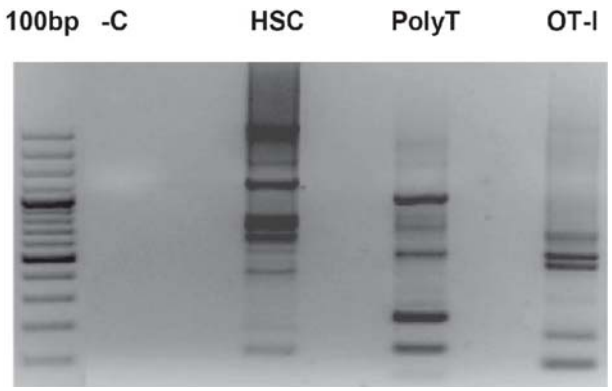


Figure 26. LM-PCR of *Tax* transplanted group. Oligoclonal pattern (≤10 bands) of the transduced cells can be seen in all three groups. HSC: Haematopoietic stem cell. PolyT: Polyclonal T cell. OT-I: Monoclonal T cell (OT-I). –C: Negative control.

4.5.5 Analyses of clonal pattern and gammaretroviral integration sites for the tumors via LM-PCR

With the help of LM-PCR, clonal patterns and gammaretroviral integration sites for all the *NPM-ALK* and *p21SNFT* induced tumors were analyzed, which are as follows:

4.5.5.1 Clonal pattern of *NPM-ALK* induced tumors

From the *NPM-ALK* transplanted group, all induced tumors showed oligo- to monoclonal pattern. They are individually described as below:

4.5.5.1.1 Clonal pattern of *NPM-ALK*_OT-1 tumors

Three tumors were analyzed from this group. Mouse 2 and 3 showed oligoclonal, while mouse 4 showed monoclonal pattern (figure 27). The cells from all these tumors were serially transplanted into secondary recipients as well as cultured in the cell culture. Two of these mice (mouse 3 and 4) developed leukaemia/lymphoma 3 to 4 weeks after serial transplantation. Also in the cell culture, tumor cells from these mice (3 and 4) survived. In LM-PCR analysis, mouse 3 tumor cells (both, serially transplanted mouse-tumor and cultured cells) showed monoclonal pattern of the same fragment size (Figure 27, lane 3, 4, 5), while for serially transplanted mouse 4 tumor, no DNA fragment could be observed but the cultured cells from mouse 4 tumor showed the same monoclonal integration pattern of the same fragment size (Figure 27, lane 6,7,8).

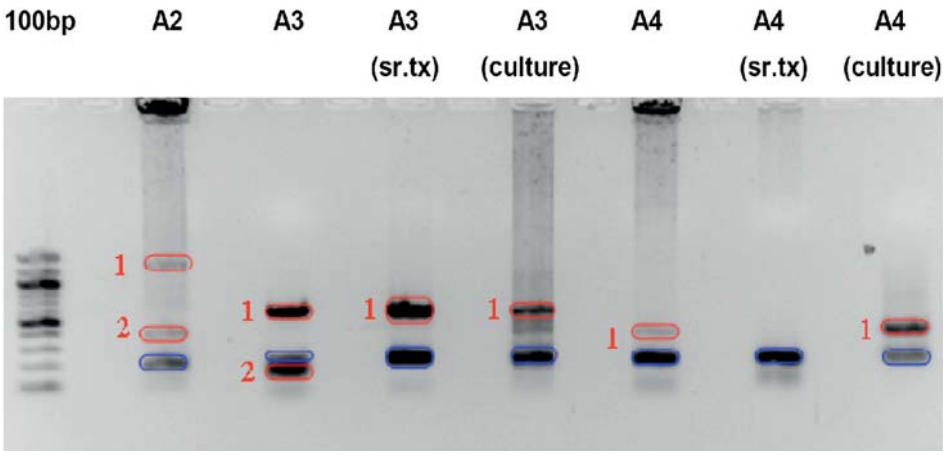


Figure 27. LM-PCR of *NPM-ALK*_OT-1 tumors. Mono- to oligoclonal pattern in all tumors (red box), Internal control (blue box). A2 shows 2 fragments. A3 shows 2 fragments, but after serial transplantation (A3 sr.tx) and culturing (A3 culture), the cells show single fragment. A4 shows single fragment, but after serial transplantation (A4 sr.tx), required fragment could not be detected, while the cultured cells (A4 culture) show the single fragment of same size. A: animal number, sr.tx: serially transplanted.

4.5.5.1.2 Clonal pattern of *NPM-ALK*_polyclonal T cell tumors

From this group, 2 out of 5 mice (mouse 3 and 5) developed tumors. In LM-PCR analysis, mouse 3 showed monoclonal, while mouse 5 showed oligoclonal integration pattern (Figure 28, lane 2 and 3.)

4.5.5.1.3 Clonal pattern of *NPM-ALK*_HSC tumors

From this group, only 1 out of 3 mice developed tumor. In LM-PCR analysis, the tumor showed oligoclonal pattern (Figure 28, lane 1).

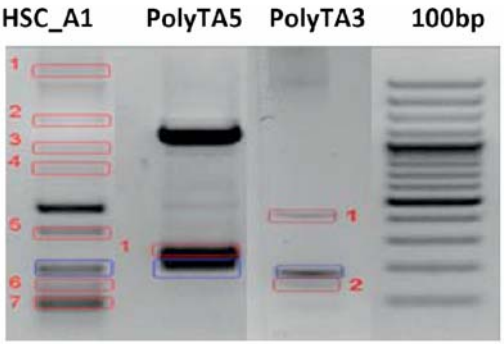


Figure 28. LM-PCR of *NPM-ALK* (HSC/Polyclonal T cell group) tumors. Mono- to oligoclonal pattern in all tumors (red box), Internal control (blue box). Tumor from *NPM-ALK*_HSC transplanted mouse 1 (lane 1) shows oligoclonal pattern. Tumors from *NPM-ALK*_polyclonal T cell transplanted mouse 5 (lane 2) shows monoclonal, while mouse 3 (lane 3) shows oligoclonal pattern. A: animal number; HSC: haematopoietic stem cell; PolyT: Polyclonal T cell.

4.5.5.2 Clonal pattern of *p21SNFT* induced tumors

From this group, only 2 mice (mouse 3 and 5) transplanted with *p21SNFT* transduced monoclonal T cells (OT-I) developed leukaemia/lymphoma. Only one of these tumors (mouse 3) was analyzed in LM-PCR, which showed monoclonal pattern (Figure 29).

Figure 29. LM-PCR of *p21SNFT*_OT-I tumor. Tumor from *p21SNFT*_OT-1 transplanted mouse 3 shows monoclonal pattern (red box). Internal control (blue box). A: animal number.

4.5.5.3 Gammaretroviral integration site analysis for *NPM-ALK* and *p21SNFT* induced tumors

Gammaretroviral integration sites were analyzed via LM-PCR for all induced tumors to see the transgene-surrounding oncogenes that might have contributed in the development of tumor. For *NPM-ALK* and *p21SNFT* oncogene vectors, genomic regions flanking the retroviral integrations in tumor cells were obtained by using LM-PCR. They were sequenced and aligned to the mouse genome. Hit loci were examined for the nearest genes to the vector integrations as well as the genes within a 200kb upstream and downstream window. Genes in this window were analyzed for appearance in the RTCGD (retroviral tagged cancer gene database) [56] and NCBI database, which describe the preferential integration of gammaretroviruses and a list of mouse genome, respectively. When comparing genes within +/-200kb window of the integrations with RTCGD database, from *NPM-ALK*_polyclonal T cell A3 tumor, only 1 out of 29 integration site flanking genes (~3%) was found in the RTCGD, which was Irak2, while from *p21SNFT*_OT-I A3 tumor, 2 out of 9 integration site flanking genes (22%) were found in the RTCGD, which were Hck and Plagl2. In general, when compared to the mouse genome, there were some interesting genes near the transgene integrations which are known to play an important role in cell cycle, cell survival and proliferation, and apoptosis. Some of those genes are as follows:

- In the *NPM-ALK*_OT-I group: Map4k, Bfkbib, Jak3, Pak4, IL-17, Eif3s12.

- In the *NPM-ALK*_polyclonal T cell group: Irak2, IL-6, IL-17rc, IL-17rE, Cidec.
- In the *NPM-ALK*_stem cell group: Trafs, Macc1.
- In the *p21SNFT* group: Hck, Plagl2.

A detailed list of all integration sites and their surrounding genes in *NPM-ALK* and *p21SNFT* induced tumors can be found in the supplementary data (see appendix, 7.3).

5. Discussion

To investigate at which stage of differentiation mature T cell leukaemia/lymphoma is initiated, we introduced gammaretroviral vectors encoding the potent T cell oncogenes *NPM-ALK* and *Tax,* and the T cell potential oncogene *p21SNFT* into different cell types, i.e, haematopoietic stem cells, polyclonal T cells and monoclonal T cells, and then transplanted into Rag-1 deficient mice. First, we analyzed the in vitro effects of these genes on two different human T cell lines (Jurkat and PM-1). No growth or proliferation promoting effect for the analyzed genes was observed. In the second part of the project, we investigated these genes *in vivo*. After transducing these genes into three different cell types (haematopoietic stem cells, polyclonal T cells and monoclonal T cells) individaully, these cells were transplanted into Rag-1 deficient mice. A total of 18 HSC/HPC, 17 mature polyclonal T cell and 24 mature monoclonal T cell (OT-I) transplanted mice were evaluated (see table 24). From the *NPM-ALK* group, all five mice transplanted with transduced mature monoclonal T cells (OT-1) developed leukaemia/lymphoma, while 1 out of 3 mice transplanted with transduced HSCs/HPCs and 2 out of 6 mice transplanted with transduced mature polyclonal T cells developed leukaemia/lymphoma. From *p21SNFT* group, only two mice transplanted with transduced mature monoclonal T cells (OT-1) developed leukaemia/lymphoma.

5.1 Effects of *NPM-ALK*, *Tax* and *p21SNFT* on human T cell lines

Gammaretroviral vectors encoding control vector, *p21SNFT, Tax* or *NPM-ALK* were transduced into two different human T cell lines (PM-1 and Jurkat). Only single copy transduced cells (~15% eGFP positive) were used to achieve the same copy number of each used transgene and to decrease the risk of insertional mutagenesis. The risk of insertional mutagenesis has already been described [57]. After transduction, the cells were followed up for gene marking and cell growth. For *NPM-ALK* and *Tax* transduced T cell lines, the eGFP markings declined from ~15 to <1% 8 to 10 days after transduction, while the control cells transduced with the control gene (eGFP) showed stable expression (see figure 17 A/B). Oncogene transduced cells also showed less exponential growth as compared to control cells (see figure 17 E/F). For *p21SNFT*, the eGFP marking declined very slowly from ~15 to ~6% in both transduced T cell lines (PM-1 and Jurkat) over a period of several weeks, while the control cells transduced with the control gene (eGFP) showed stable expression (see figure 17 A/B). The cell growth for *p21SNFT* transduced cells was comparable with the control cells in both T cell lines (see figure 17 C/D). The decline in eGFP for *p21SNFT* transduced cells might have been due to survival disadvantage from this gene. As it has

already been shown by a group that *p21SNFT* causes decreased production of IL-2 (interleukin 2) in Jurkat T cells [27]. IL-2 is a very important growth factor for T cells. Lack of IL-2 might have resulted in the survival disadvantage of *p21SNFT* expressing T cells.

As the eGFP marking for *NPM-ALK* and *Tax* transduced T cell lines (PM-1 and Jurkat) declined from ~15 to <1%, LM-PCR was performed before and after decline of eGFP marking in Jurkat cells to further investigate if the cells were still containing the proviral sequence coding for *NPM-ALK* and *Tax*. For both oncogenes (*NPM-ALK* and *Tax*), LM-PCR revealed oligoclonal pattern in the cells expressing ~15% eGFP, while the cells expressing <1% eGFP showed only one band, which has been the internal control (see figure 18). There are two possibilities for this fact. On the one hand, it might be that an error during the LM-PCR performance occured, resulting in the amplification of only the internal control band, which is extremely unlikely. On the other hand, it is more likely that the signal for the internal control band was caused by a contamination with a plasmid during DNA extraction from the cells. A contamination during the LM-PCR procedure can be excluded because the negative control revealed no signal (data not shown). The band appearing at ~50bp length was subcloned, but it did not reveal any usable sequence.

Another way to test if there has still been proviral sequence in the transduced cell lines, would have been the usage of a second restriction enzyme in the beginning of the LM-PCR procedure (step 1) in a separate approach. In this approach, the restriction enzyme Tsp509I would have been used. In future experiments, one could perform LM-PCR with the MspI, which digests the palindromic nucleotide sequence CCGG instead of AATT in the case of the usage of Tsp509I. Via this approach, using 2 or 3 different restriction enzymes, probability of finding all gammaretroviral insertions in the cells' genome is increased.

Thus, no any growth or proliferation promoting effect of all these oncogenes on both human T cell lines was observed, rather those cells probably died, indicated by decreased exponential growth, decline in eGFP marking and the absence of the proviral sequence in the LM-PCR (in NPM-ALK and Tax transduced cells). In contrast to that, control vector transduced cells grew better with stable expression of eGFP marking. Unfortunately, only one LM-PCR was performed with DNA, extracted from control vector transduced cells 5 days after transduction, but not from later time points. These time points might have shown some interesting integration sites that might cause the survival advantage of these cells due to possible transactivation of surrounding genes (proto-oncogenes). In this case, one could have investigated the altered gene expression of these surrounding genes via qPCR or microarray analysis.

5.2 Transformation potential of *NPM-ALK* in primary murine cells

5.2.1 Transformtion of murine HSCs/HPCs after gammaretroviral mediated *NPM-ALK* transduction

Haematopoietic stem cells/haematopoietic progenitor cells (HSCs/HPCs) were transduced with the potent T cell oncogene '*NPM-ALK*' and transplanted into Rag-1 deficient mice. 1 out of 3 mice transplanted with transduced HSCs/HPCs developed leukaemia/lymphoma 158 days after transplantation. In FACS analysis, no phenotype of the tumor could be identified. Although the tumor showed different cell populations which were positive for the donor cell marker (CD45.1) and either T cell, B cell, NK cell or myeloid cell marker, but it did not express any eGFP marking (see section 4.5.3.1.3). The LM-PCR revealed an oligoclonal pattern of the tumor (figure 28). The histopathologic analysis revealed this tumor as 'anaplastic lymphoma' phenotype (see figure 23). As the tumor cells lost eGFP marking, RTqPCR or microarray analysis should be performed to look for the expression of the *NPM-ALK* and surrounding genes. The tumorigenic role of *NPM-ALK* transduced bone marrow precursors in a mouse model has already been described by others. Here, 4 out of 7 mice developed tumors, which were 'large B cell lymphomas' [61]. By using T cell lineage-restricted promoters, transgenic mice have been established in which *NPM-ALK* expression leads to T cell transformation [62,63]. Similarly, in these *NPM-ALK* transgenic models, B cell lymphomas were also observed [62,64,65], suggesting that the inappropriate activation of ALK in B cells can elicit signals promoting their transformation and supporting the pathogenetic role of this kinase in the rare ALK-DLBCL. Therefore, in further steps, this tumor should be analyzed for BCR/TCR rearrangement on genomic level and further markers of early B and T cell development to identify if it is B cell or T cell lymphoma.

5.2.2 Transformation of murine polyclonal T cells after gammaretroviral mediated *NPM-ALK* transduction

Only 2 out of 5 mice transplanted with *NPM-ALK* transduced polyclonal T cells developed leukaemia/lymphoma 153-250 days after transplantation. In FACS analysis, no phenotype of the tumors could be identified, as the tumors showed different cell populations which were positive for donor cell marker (CD45.1) and either T cell, B cell, NK cell or myeloid cell marker but did not express any eGFP marking except one tumor which expressed 2% eGFP as well (see section 4.5.3.1.2). These 2% eGFP positive cells did not show any lineage specific marker. The LM-PCR revealed monoclonal to oligoclonal pattern of these tumors (see figure 28). The histopathologic analysis revealed these tumors as 'anaplastic lymphoma'

(see figure 23). As the tumor cells lost eGFP marking, RTqPCR or microarray analysis should be performed to look for the expression of *NPM-ALK* and surrounding genes. In further steps, these tumors should be analyzed for BCR/TCR rearrangement on genomic level and for additional markers of early lymphoid development to know if they are B cell or T cell lymphomas. Although before transplantation we transduced T cells, but it might have been possible that some B cells survived in the culture and were transduced with NPM-LK before transplantation. In our group the experiments are ongoing in which some of B cells survived in the cell culture and were transduced with the oncogene (ΔTrkA). It has already been shown that mature B cells can be transformed [9]. Although the relative resistance to transformation of polyclonal T cells by potent T cell oncogenes LMO2, ΔTrkA and TCL-1 has already been investigated in our group [55], but in comparison to the oncogenes used previously, *NPM-ALK* is known to cause mature form of T cell and also B cell leukaemia/lymphoma [61,62,63]. Additionally, it might be possible that *NPM-ALK* is a more potent oncogene and therefore could transform mature polyclonal T cells. Furthermore, as only 2 out of 6 mice transplanted with *NPM-ALK* transduced polyclonal T cells developed leukaemia/lymphoma after longer latencies (153-250 days after transplantation) in comparison to all 5 mice transplanted with *NPM-ALK* transduced monoclonal mature T cells (OT-I), which developed leukaemia/lymphoma within shorter latencies (45-64 days after transplantation), an interesting potential control mechanism in the leukaemogenesis by mature T cells could be the clonal competition of polyclonal mature T cells for stimulatory niches, which is well described in the following section 5.2.3.

5.2.3 Transformtion of murine monoclonal T cells after gammaretroviral mediated *NPM-ALK* transduction

In our experimental setup, we used monoclonal mature T cells (OT-I) to analyze the hypothesis of ‘clonal competition’ by polyclonal T cells for stimulatory niches. The peripheral T lymphocytes get their survival signal by interacting with specific major histocompatibility complex (MHC)/self peptide complex, presented by APC (antigen presenting cells). Homeostatic mechanisms conserve the polyclonality of the T cell population [66] and regulates the proliferation of peripheral T lymphocytes [67]. This mechanism could possibly hinder the outgrowth of premalignant T cell clones. In a polyclonal situation, potentially preleukaemic clones would not get sufficient survival signals because of the competition within the stimulatory niches and thus their out growth would be controlled (see figure 30 A). Therefore, OT-I TCR transgenic mice were chosen as donors to use monoclonal mature T cells to exclude polyclonal situation. These monoclonal T cells are mainly CD8+ in these

mice models. These mice express only one transgenic TCR [68] and thus the control mechanism of the MHC-TCR niches is abolished.

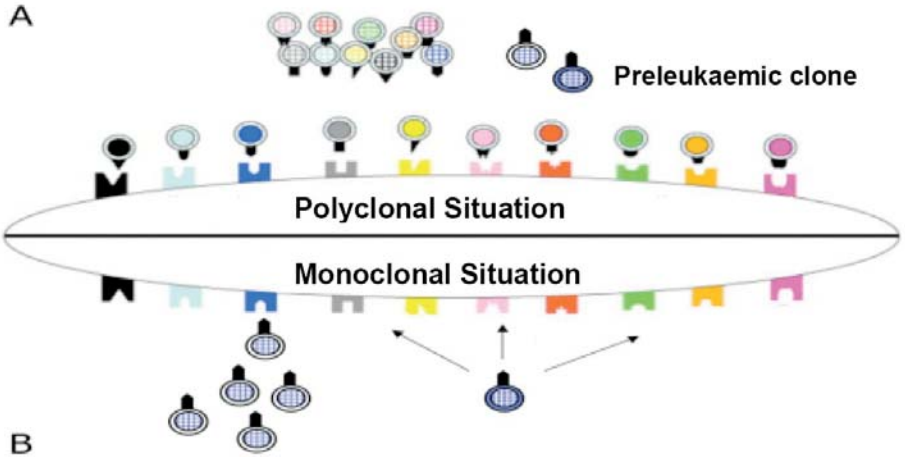


Figure 30. Suggested niche model in controlling preleukaemic clones. (A) Polyclonal situation. All stimulatory niches are occupied by competing T cells. In this situation the preleukaemic clones could either be lost or controlled through the homeostatic mechanisms. **(B) Monoclonal situation (OT-I transplant).** All niches are free to be occupied by preleukaemic clones, as there is no competition due to absence of polyclonal T cells. Potential leukaemic clones would find sufficient stimulatory niches to attain full growth and possibly develop leukaemia/lymphoma. Modified after [66].

The hypothesis is that, by the restriction of the T cell repertoires, free MHC niches could possibly be occupied by potentially transformable T cells. The preleukaemic clone could attain a full growth signal within the open niches without any competition of polyclonal T cells (see figure 30 B).

For this purpose, after transducing the monoclonal T cells (OT-I) with *NPM-ALK*, Rag-1 deficient mice were transplanted with these transduced cells. All 5 mice from this group developed leukaemia/lymphoma after short latencies (45-64 days post transplantation). In FACS analysis, these tumors were positive for 6 to 45% eGFP marking, but very weak percentages of different cell populations like, TCRα/β (common TCR), Vα2/Vβ5 (OT-I specific TCR), CD8, CD4, CD19, NK1 (natural killer), and a high percentage of CD11b (myeloid marker) population. The eGFP expressing cells did not express any lineage marker (see section 4.5.3.1.1). The histopathologic analysis revealed ‘anaplastic lymphoma’ phenotype (see figure 23). LM-PCR revealed an oligoclonal to monoclonal pattern of these tumors (see figure 27).

As all 5 mice from this group (*NPM-ALK* transduced OT-I) developed leukaemia/lymphoma in comparison to *NPM-ALK* transduced HSCs transplanted mice, from which 1 out of 3 mice and polyclonal T cell transplanted mice from which 2 out of 6 mice developed

leukaemia/lymphoma, these results might support our hypothesis of 'stimulatory niches' (mentioned above). Higher portions of polyclonal T cells might have occupied an increased number of stimulatory niches and controlled the full growth of premalignant clones. However in contrast to that, it was described that activated/memory T cells do not dependent upon MHC-TCR interaction for survival signals and their proliferation is essentially controlled by cytokines [69,70,71]. In the presented work, only memory T cells were used since the *ex vivo* stimulation with anti CD3/CD28 antibodies converted the naive mature T cells to memory T cells [72]. On the other hand, it could be shown that after the ablation of TCR, naive and memory T cells disappeared due to deprivation of MHC-TCR signal [73,74]. It would also be possible that OT-I lymphocytes have an increased, so far undiscovered, intrinsic transformation potential. Moreover, OT-I TCR could be particularly activated by high MHC affinity and thus support a malignant transformation. The high affinity of OT-I TCR was described previously [75,76]. In order to examine this hypothesis more closely for clonal compitition, experiments are ongoing in our group in which oncogene transduced monoclonal OT-I T cell population is cotransplanted with different populations of polyclonal T cells.

5.3 Transformation potential of *p21SNFT*

5.3.1 Transformtion of murine monoclonal T cells after gammaretroviral mediated *p21SNFT* transduction

From *p21SNFT* group, 2 out of 7 mice transplanted with transduced monoclonal mature T cells (OT-I) developed leukaemia/lymphoma 144-200 days after transplantation, while none of the mice transplanted with *p21SNFT* transduced polyclonal T cells or haematopoietic stem cells developed leukaemia/lymphoma, although they highly expressed eGFP (see section 4.5.3.2). An interesting potential mechanism in the leukaemogenesis by monoclonal mature T cells (OT-I) could be the free stimulatory niches (MHC/self peptide), occupied by the transformed clones, which has already been described in the section 5.2.3. Histopathologic analysis of these tumors revealed 'anaplastic lymphoma' (see figure 25). LM-PCR was performed for one of these tumors which revealed monoclonal pattern (see figure 29). In FACS analysis, these tumors showed high eGFP and interestingly CD19 expression (see 4.5.3.2.1). Although CD19 is a specific marker for B cells, it can aberrantly be expressed by T cell leukaemia/lymphoma [77,78]. Therefore, this tumor should be investigated for BCR/TCR rearrangement on genomic level to indentify if they are B cell or T cell lymphomas. As already mentioned that, before transplantation we transduced T cells with these oncogenes, but it might have been possible that some B cells survived in the culture and were transduced with *p21SNFT* before transplantation. In our group the experiments are ongoing in which the

B cells survived in the T cell culture and were transduced with the oncogene (ΔTrkA). It has already been shown that mature B cells can be transformed [9].

5.3.2 Mice transplanted with *p21SNFT* transduced HSCs/HPCs and polyclonal T cells

None of the mice transplanted with *p21SNFT* transduced HSCs/HPCs and polyclonal T cells developed leukaemia/lymphoma over the followup of ~240 days after transplantation, although they highly expressed eGFP for the transgene (see 4.5.3.2). In our experimental setup, from the whole *p21SNFT* group, only 2 out of 7 mice transplanted with *p21SNFT* transduced monoclonal T cells (OT-I) developed leukaemia/lymphoma 144-200 days after transplantation. The observation period and the number of transplanted mice are too small and need to be increased for further studies. Therefore, it might be possible that after a longer observation period, these mice develop leukaemia/lymphoma.

5.4 *Tax* transplanted group

None of the *Tax* transplanted mice develop leukaemia/lymphoma. Furthermore, the recipients did not show any eGFP marking in the periphery and lymphatic organs after a followup of ~250 days post-transplantation, although they expressed donor cell (CD45.1 or OT-I) and T cell (CD3) markers. To further investigate if *Tax*-transduced cells were present in the recipients, LM-PCR was performed by isolating DNA from one mouse of each *Tax*-transplanted group (HSC, polyclonal T cell and OT-I T cell group). For this purpose, animals were sacrificed and DNA was isolated from the splenocytes. LM-PCR revealed oligoclonal pattern of the transduced cells in all three group animals (see section 4.5.4), which indicates that *Tax* transduced cells were still repopulating the recipients, but did not express the transgene anymore. It might be possible that the gene modified cells silenced or mutated the transgene. It has already been shown that the HTLV-I *Tax* protein is required for the virus to transform cells [22]; however, *Tax* transcripts are detected in only ~40% of all ATLs. Analyses of HTLV-I proviruses and *Tax* transcripts in ATL cells revealed three ways in which cells can silence *Tax* expression, which are:

- Accumulation of nonsense mutations, insertions and deletions in *Tax* [23,24].
- Silencing viral transcription by DNA methylation of the Provirus [24,25].
- Deletion of the proviral 5' LTR [26].

The currently accepted view for silencing of *Tax* is that, it is required to initiate transformation, but is not required later to maintain the transformed phenotype of ATL cells. As *Tax* is the main target of the host's CTLs (cytotoxic T lymphocytes), cells that down-regulate *Tax*

expression (using one of the three genetic or epigenetic means described above) have an advantage in evading immunosurveillance and are preferentially selected *in vivo* during disease progression [24]. In our used mouse models, *Tax* transduced cells might have down-regulated the *Tax* gene by the mechanisms mentioned before. Furthermore, another group has shown that only few *Tax*-transgenic mice develop leukaemia/lymphoma 18 months after birth [79]. In our *Tax*-transplanted mice, the observation period is still too short (<260 days). It might be possible that these mice develop leukaemia/lymphoma after a longer observation period.

5.5 Integration site analysis of gammaretroviral vectors in the tumors

Gammaretroviral integration sites were analyzed via LM-PCR for all induced tumors. For *NPM-ALK* and *p21SNFT* oncogene vectors, genomic regions flanking the retroviral integrations in tumor cells were obtained by using LM-PCR. They were sequenced and aligned to the mouse genome to identify adjacent genes that might have a cooperative effect with the oncogenes. Hit loci were examined for the nearest genes to the vector integrations as well as the genes within a 200kb upstream and downstream window. Genes in this window were analyzed for appearance in the RTCGD [56] and NCBI database, which describe the preferential integration of gammaretroviruses and a list of mouse genome, respectively. When comparing genes within +/-200kb window of the integrations with RTCGD database, from *NPM-ALK*_polyclonal T cell A3, only 1 out of 29 integration site flanking genes (~3%) was found in the RTCGD, which was Irak2, while from *p21SNFT*_OT-I A3, 2 out of 9 integration site flanking genes (22%) were found in the RTCGD, which were Hck and Plagl2. In the other tumors of *NPM-ALK* group, the integration site flanking genes were not found in the RTCGD database. But in general, when aligned to the mouse genome, there were some interesting genes near the transgene integrations (see section 4.5.5.3) which are known to play an important role in cell cycling, survival, proliferation and apoptosis. Some of those are described as follows:

In *NPM-ALK_OT-I* group tumors, Jak3 and Pak4 genes were present near the integration of the transgene. Jak3 is known to causes increased cell survival and proliferation. It is involved in one of the important pathways (Jak3/Stat3) by which *NPM-ALK* exerts its oncogenic effects to induce leukaemia/lymphoma [19]. For Pak4, one group has shown that its overexpression or activation plays a key role in oncogenic transformation due to its ability to

promote cell survival and subsequent uncontrolled proliferation. It is overexpressed in many types of cancers, like, colon, esophageal and mammary tumors [80].

In *NPM-ALK_HSC/HPC* group tumor, TRAFS was present near the transgene integration, which prevents the lymphoma cells from spontaneous and induced apoptosis [81].

In *p21SNFT_OT-I* group tumor, PLAG1 was present near the transgene integration. Oncogenic activation of PLAG1 is a crucial event in human tumours like, pleomorphic adenomas of the salivary glands, lipoblastoma, hepatoblastoma, and acute myeloid leukaemia (AML) [82].

These genes might have contributed to the development of leukaemia/lymphoma in both groups (*NPM-ALK* and *p21SNFT* induced tumors) described above. The risk of insertional mutagenesis due to retroviral mediated gene transfer was previously described [58],[59],[60]. A detailed list of all integration-sites in our induced tumor groups can be found in the supplementary data (see appendix 7.3). Infact, these results do not prove that the transgene (*NPM-ALK*/*p21SNFT*) was up-regulated by the surrounding genes via LTR transactivation, or its insertion caused transactivation of surrounding genes. This should be checked by further experiments like microarray, or RT or qPCR to look for the expression of these genes.

5.6 Conclusion

All mice transplanted with *NPM-ALK* transduced mature monoclonal T cells (OT-I) developed leukaemia/lymphoma as compared to *NPM-ALK* transduced HSCs/HPCs and mature polyclonal T cells transplanted mice, from which only few recipients developed leukaemia/lymphoma. Furthermore, from the *p21SNFT* group, 2 out of 7 mice transplanted with mature monoclonal T cells (OT-I) developed leukaemia/lymphoma. Thus, in comparison to polyclonal T cells, monoclonal T cells (OT-I) are more readily transformable with T cell oncogenes *NPM-ALK* and *p21SNFT*. Competition of T cell clones might control the outgrowth of pre-malignant T cells and which may be the mechanism for the relative resistance of polyclonal mature T cells to transformation. The mechanisms underlying the control of malignant outgrowth in polyclonal T cell populations will be further analyzed in our group on the molecular level and on the level of clonal dynamics.

6. References

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7. Appendix

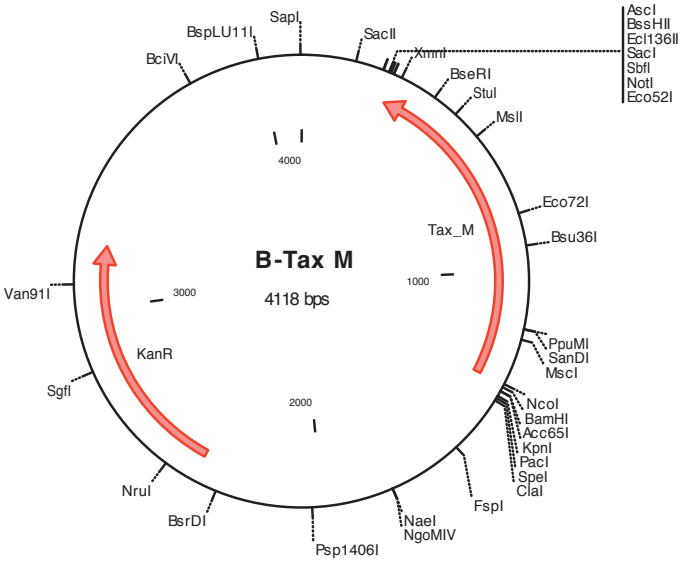
7.1 Abbreviations

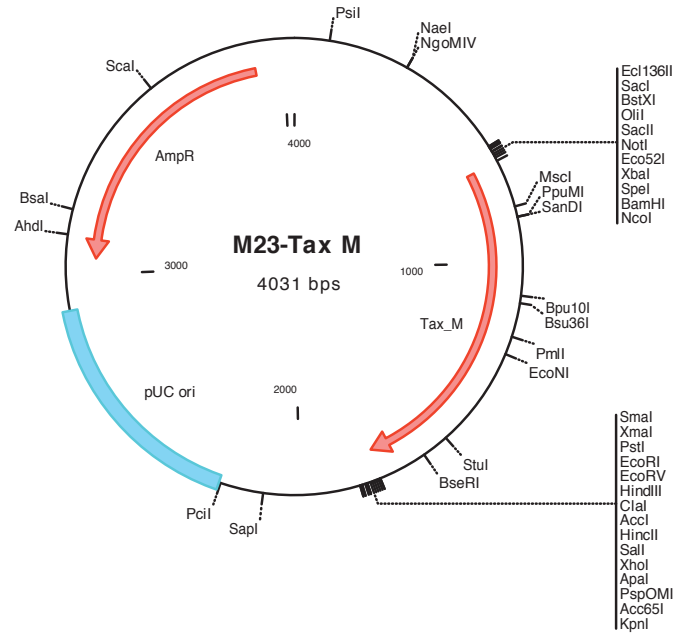
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
AAV	Adeno-associated virus
bp	Basepair
BSA	Bovine serum albumin
cDNA	Complimentary DNA
CIS	Common integration sites
ddH_2O	Deionized distilled water
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides
DP	Double positive
E.coli	Escherichia coli
EDTA	Ethylendiamintetraessigsäure
eGFP	Enhanced green fluorescent protein
Env	Envelope
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FCS	Fetal Calf Serum
Gag	Group-specific antigen
GALV	Gibbon Ape leukaemia virus
HBSS	Hanks buffered salt solution
HE	Haematoxilin eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HRPO	Horse-radish peroxidase
HSC/HPC	Haematopoietic stem cell/ Haematopoietic progenitor cell
HTLV-I	Human T-lymphotropic Vírus type 1
Ig	Immunoglobulin
IL	Interleukin
IN	Integrase
IRES	Internal ribosome entry site
IVC	Individual ventilated cage
kb	Kilobase
kDa	Kilodalton

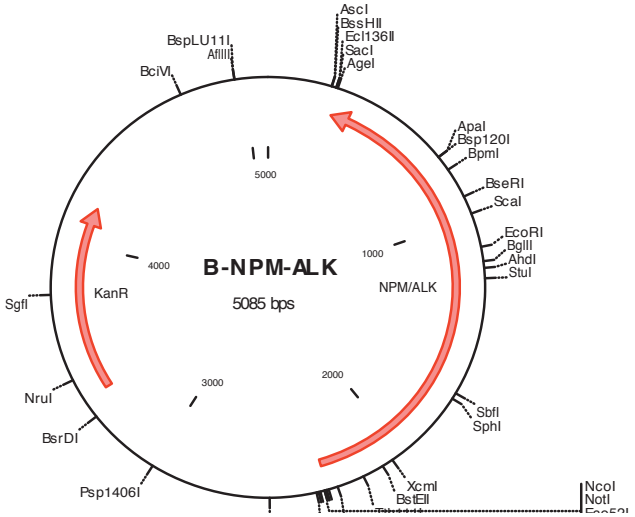
LB	Lennox L Broth base
LIC	Leikemia initiating cell
LM-PCR	Ligation mediated Polymerase chain reaction
LN	Lymph node
LTR	Long terminal repeats
M	Molar
MA	Matrix protein
mcs	Multiple cloning site
MHC	major histocompatibility complex
min.	Minute
MLV	Murine leukaemia virus
MNC	Mononuclear cells
MPBST	5% milk in PBST
mRNA	Messenger RNA
MSA	Murines Serum Albumin
NC	Nucleocapsid protein
NEB	New England Biolabs
NHL	Non Hodgkin Lymphoma
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PBST	Phosphate-buffered saline – Tween 20
PE	Phycoerythrine
PCR	Polymerase chain reaction
Pol	Polymerase
PR	Protease
RCV	Replication competent virus
RCR	Replication competent Retrovirus
RNA	Ribonucleic acid
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute
RRE	Rev responsive element
RT	Reverse transcriptase
RT	Room temperature
SCID	Severe Combined Immunodeficiency
SDS	Sodium dodecyl sulfate
Sec.	Second
SIN	Self inactivating
SV40	Simian virus 40
TAE	Tris-Acetat-EDTA
TCR	T cell receptor
TM	Transmembrane

TRIS	Tris(hydroxymethyl)amino-methane
TFG	TRK-fused gene
UTR	Untranslated region
UV	Ultra-violet light
V	Volt
v/v	volume per volume
w/v	weight per volume
wPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
WT	Wild type
µg	Micrograms
µl	Microliter
µm	Micrometer

7.2 Plasmid maps



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StuI
SbfI
SphI
XcmI
BstEII
NcoI
NotI
BspLU11I
AflIII
BciVI
B-NPM-ALK
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NPM/ALK
KanR
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NruI
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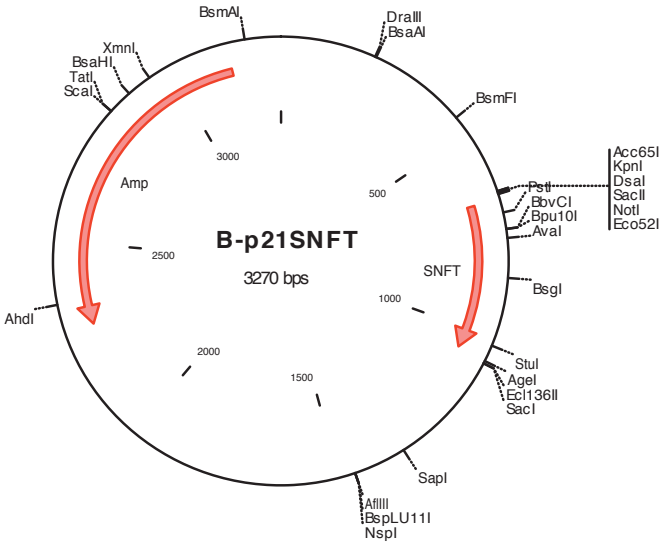
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AvaI
BsgI
StuI
AgeI
Ecl136II
SacI
SapI
AflIII
BspLU11I
NspI
AhdI
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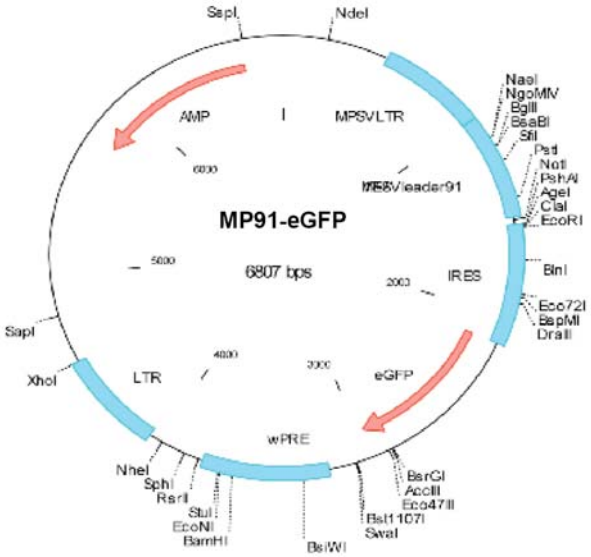
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BlnI
Eco72I
BspMI
DraII
SapI
XhoI
NheI
SphI
RsrII
StuI
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BamHI
BsiWI
BsrGI
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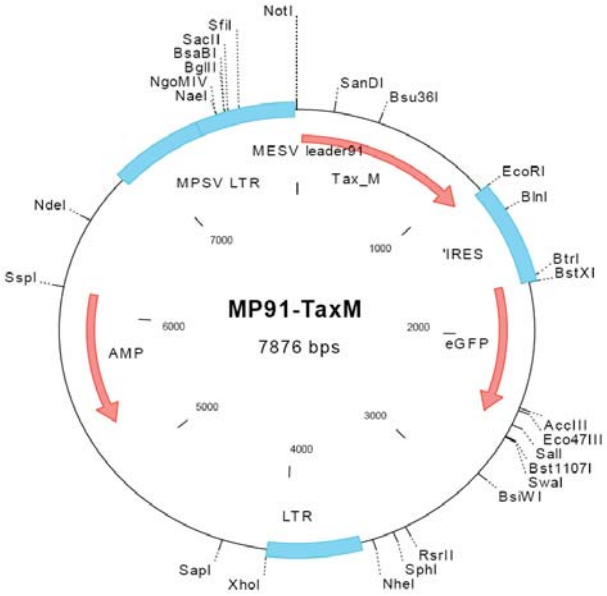
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MPSV LTR
Tax_M
EcoRI
BlnI
NdeI
IRES
BtrI
BstXI
SspI
MP91-TaxM
7876 bps
AMP
eGFP
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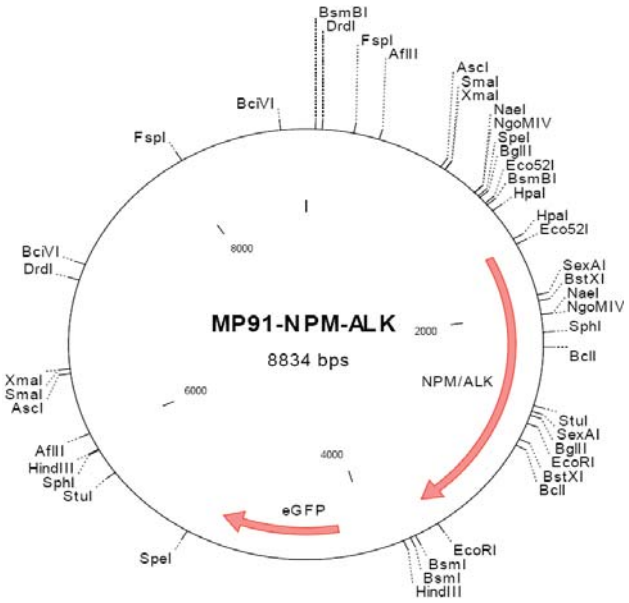
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NgoMIV
SpeI
BglII
Eco52I
BsmBI
HpaI
HpaI
Eco52I
SexAI
BstXI
NaeI
NgoMIV
SphI
BclI
StuI
SexAI
BglII
EcoRI
BstXI
BclI
EcoRI
BsmI
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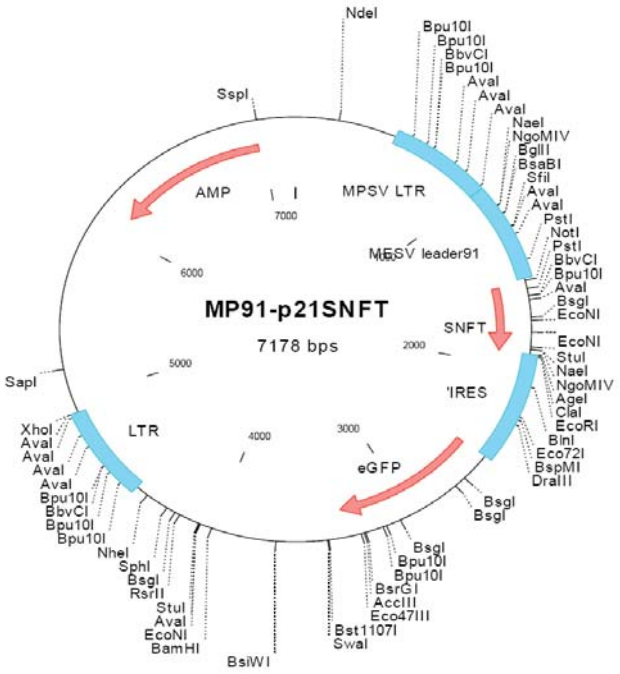
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Bpu10I
Bpu10I
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AvaI
AvaI
AvaI
AvaI
XhoI
SapI
SspI

7.3 Integration site analysis for the tumors

Table. Integration site analysis for the tumors. Ticket: ' 6009008b-7850-41c2-a9a9-5ba96f3ac6ed' .

Bandwidth: 200000 **Query Type: surrounding genes**

ID	Chromo-some	Location	Type	Gene ID	Gene Symbol	Distance	Start	End	Ori	Rel. Orient.	Band	Synonyms	Description	RTCGD
1.1	6	113420397	surrounding	14311	Cidec	-34648	113385749	113374630	-	F	6 E3	CIDE-3lFsp27	cell death-inducing DFFA-like effector c	no
	6	113420397	surrounding	18294	Ogg1	143392	113277005	113284178	+	R	6 E-F1l6 48.0 cM	Mmh	8-oxoguanine DNA-glycosylase 1	no
	6	113420397	surrounding	22346	Vhlh	-153618	113574015	113581627	+	R	6 E3l6 49.45 cM	Vhlh	von Hippel-Lindau syndrome	no
	6	113420397	surrounding	52163	Camk1	-126481	113293916	113284118	-	F	6 E3l6 48.7 cM	Al505105lCaMKIalphaID6Ertd263e	calcium/calmodulin-dependent protein kinase I	no
	6	113420397	surrounding	57890	Il17re	11919	113408478	113420745	+	R	6 E3	AA589509IIL-17REIII25r	interleukin 17 receptor E	no
	6	113420397	surrounding	66087	Tmem111	61235	113481632	113464881	-	F	6 E3	0610039A15RiklAI225901IAW260416IPob	transmembrane protein 111	no
	6	113420397	surrounding	67767	Jagn1	27768	113392629	113398221	+	R	6 E3	5830427H10RiklAW146438	jagunal homolog 1 (Drosophila)	no
	6	1134203	surround	68089	Arpc4	92290	113328	113339	+	R	6 E3	20kDal5330419I20RiklAI327076Ip20-Arc	actin related protein 2/3 complex, subunit 4	no

		97	ing				107	643						
	6	113420397	surrounding	70979	4931417G12Rik	130250	113550647	113546756	-	F	6 E3	-	RIKEN cDNA 4931417G12 gene	no
	6	113420397	surrounding	78783	Brpf1	163206	113257191	113274703	+	R	6 E3	4833438B11Rikl4930540D11RiklBrpf2	bromodomain and PHD finger containing, 1	no
	6	113420397	surrounding	101100	Ttll3	72645	113347752	113364558	+	R	6 E3	4833441J24RiklAI450050	tubulin tyrosine ligase-like family, member 3	no
	6	113420397	surrounding	101122	Rpusd3	-51063	113369334	113365313	-	F	6 E3	AI527266	RNA pseudouridylate synthase domain containing 3	no
	6	113420397	surrounding	101206	Tada3l	-92883	113327514	113316649	-	F	6 E3	1110004B19RiklADA3lAI987856	transcriptional adaptor 3 (NGG1 homolog, yeast)-like	no
	6	113420397	surrounding	101314	6720456B07Rik	-134369	113554766	113566945	+	R	6 E3	AW011779	RIKEN cDNA 6720456B07 gene	no
	6	113420397	surrounding	108960	Irak2	-168100	113588497	113644745	+	R	6 E3l6 49.5 cM	6330415L08RiklAI649099IIRAK-2lMGC102586	interleukin-1 receptor-associated kinase 2	yes (2)
	6	113420397	closest	171095	Il17rc	-1171	113421568	113433132	+	R	6 E3	1110025H02RiklIL-17RCIIL17-RCIIL17-RLIIl17rl	interleukin 17 receptor C	no
	6	113420397	surrounding	171508	Creld1	-13166	113433563	113443332	+	R	6 E3l6 49.5 cM	AI843811	cysteine-rich with EGF-like domains 1	no
	6	113420397	surrounding	210673	Prrt3	31415	113451812	113444089	-	F	6 E3	6330505P20lB230206N24Rik	proline-rich transmembrane protein 3	no
	6	113420397	surrounding	211232	Cpne9	188096	113232301	113255565	+	R	6 E3	A730016F12RiklKIAA4217lmKIAA4217	copine family member IX	no

	6	113420397	surrounding	211651	Fancd2	-61282	113481679	113546279	+	R	6 E3	2410150O07RiklAU015151IBB137857IFA-D2IFA4IFACDIFADIFANCD	Fanconi anemia, complementation group D2	no
1.2.1	9	9113294	closest	71544	9030420J04Rik	125807	9239101	8994328	-	R	9 A1	BE136619	RIKEN cDNA 9030420J04 gene	no
1.2.2	5	30276422	surrounding	16193	Il6	-63279	30339701	30346508	+	R	5 B1I5 17.0 cM	Il-6	interleukin 6	no
	5	30276422	surrounding	22171	Tyms	123699	30400121	30385367	-	F	5 B1I5 18.2 cM	TS	thymidylate synthase	no
	5	30276422	surrounding	242914	EG242914	149860	30426282	30422546	-	F	5 B1I5	Kif2c	predicted gene, EG242914	no
	5	30276422	surrounding	242915	Gm444	-155320	30431742	30445522	+	R	5 B1	-	gene model 444, (NCBI)	no
	5	30276422	surrounding	433865	EG433865	-154663	30121759	30120688	-	F	5 B1	-	predicted gene, EG433865	no
	5	30276422	surrounding	100034662	LOC100034662	-116467	30159955	30158270	-	F	5 B1I5	-	predicted gene, 100034662	no
	5	30276422	closest	100039369	LOC100039369	59070	30335492	30322681	-	F	5 B1I5	-	predicted gene, 100039369	no
	5	30276422	surrounding	100039405	LOC100039405	-141554	30134868	30132993	-	F	5 B1I5	-	predicted gene, 100039405	no

2.1	8	74302896	surrounding	16336	Insl3	89745	74213151	74214474	+	R	8 B3.3I8 33.0 cM	RIfIRInI	insulin-like 3	no
	8	74302896	surrounding	16453	Jak3	102423	74200473	74214473	+	R	8 B3.3I8 33.0 cM	faelwil	Janus kinase 3	no
	8	74302896	surrounding	66171	Pgls	186813	74116083	74120166	+	R	8 C1I8	1110030K05RiklAI447866IPlgs	6-phosphogluconolactonase	no
	8	74302896	surrounding	72297	B3gnt3	-77205	74225691	74215618	-	F	8 C1	2210008L19Rik	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 3	no
	8	74302896	surrounding	74015	Fcho1	-53351	74249545	74232545	-	F	8 B3.3	3322402E17RiklKIAA0290IN28152	FCH domain only 1	no
	8	74302896	surrounding	170938	Zfp617	-143839	74446735	74458528	+	R	8 B3.3	Zfps11-6	zinc finger protein 617	no
	8	74302896	surrounding	234407	Glt25d1	168001	74134895	74148810	+	R	8 B3.3	2810024B22RiklMGC38524	glycosyltransferase 25 domain containing 1	no
	8	74302896	surrounding	234413	BC049349	-172047	74474943	74494232	+	R	8 B3.3	mszf37lmszf55-2	cDNA sequence BC049349	no
	8	74302896	surrounding	236193	Zfp709	-103071	74405967	74416463	+	R	8 B3.3	BC021921IHIT-40IMGC28841	zinc finger protein 709	no
	8	74302896	surrounding	382018	Unc13a	-107350	74195546	74153322	-	F	8 B3.3	2410078G03RiklC630025L14IMunc13-1	unc-13 homolog A (C. elegans)	no
	8	7430289	surround	385024	LOC385024	78104	743810	743690	-	F	8 B3.3I8	-	predicted gene, 385024	no

		6	ing				00	43						
	8	74302896	surrounding	665991	EG665991	26935	74275961	74284493	+	R	8 B3.3	-	predicted gene, EG665991	no
	8	74302896	closest	665996	LOC665996	13914	74288982	74303673	+	R	8 B3.3	-	similar to Gene model 1082, (NCBI)	no
	8	74302896	surrounding	670453	LOC670453	140127	74443023	74440390	-	F	8 B3.3I8	-	predicted gene, 670453	no
	8	74302896	surrounding	675190	LOC675190	-98750	74401646	74402559	+	R	8 B3.3	-	vomeronasal 2, receptor, pseudogene 91	no
	8	74302896	surrounding	100037278	Bcnp1	181351	74121545	74132048	+	R	8 B3.3	Bcnp1	family with sequence similarity 129, member C	no
	8	74302896	surrounding	100042289	LOC100042289	83470	74219426	74220368	+	R	8 B3.3I8	-	predicted gene, 100042289	no
2.2	14	46559339	surrounding	218963	LOC218963	-144363	46703702	46704660	+	F	14 C1	-	ubiquitin pseudogene	no
	14	46559339	surrounding	625377	LOC625377	-84076	46643415	46648459	+	F	14 C1	-	predicted gene, EG625377	no
	14	46559339	surrounding	666834	LOC666834	-81118	46478221	46477700	-	R	14 C1	-	predicted gene, EG666834	no
	14	46559339	closest	100041685	LOC100041685	-16462	46542877	46538762	-	R	14 C1	-	similar to H3 histone, family 3A	no

3.1	7	29574836	surrounding	15388	Hnrpl	-21096	29595932	29607263	+	F	7 A3	C79783ID830027H13RiklHnrpl	heterogeneous nuclear ribonucleoprotein L	no
	7	29574836	surrounding	16855	Lgals4	-44173	29619009	29626722	+	F	7 A3l7 4.0 cM	galectin-4	lectin, galactose binding, soluble 4	no
	7	29574836	surrounding	16858	Lgals7	-74569	29649405	29651303	+	F	7 A3	Galectin-7IMGC151215IMGC151217	lectin, galactose binding, soluble 7	no
	7	29574836	surrounding	18036	Nfkbib	-23293	29551543	29543270	-	R	7 A3-B3	IKB-betaIIkBIIkBbIMGC36057	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, beta	no
	7	29574836	surrounding	24030	Mrps12	-48036	29526800	29524660	-	R	7 A3	AI327385IRpms12Irps12	mitochondrial ribosomal protein S12	no
	7	29574836	surrounding	26411	Map4k1	-193037	29767873	29788298	+	F	7 B1	Hpk1ImHPK1	mitogen-activated protein kinase kinase kinase kinase 1	no
	7	29574836	surrounding	50760	Fbxo17	72944	29501892	29523163	+	F	7 A3	Fbg4IFbx17IFbxo26	F-box protein 17	no
	7	29574836	surrounding	51798	Ech1	-35521	29610357	29617258	+	F	7 A3	AA617331IMGC107274	enoyl coenzyme A hydratase 1, peroxisomal	no
	7	29574836	surrounding	60594	Capn12	-91840	29666676	29678604	+	F	7 A3	-	calpain 12	no
	7	29574836	surrounding	60595	Actn4	172463	29747299	29678273	-	R	7 A3	C77391IMGC102374	actinin alpha 4	no
	7	29574836	surrounding	64383	Sirt2	23034	29551802	29573679	+	F	7 B1-2	5730427M03RiklSIR2L2ISir2l	sirtuin 2 (silent mating type information regulation 2, homolog) 2 (S. cerevisiae)	no
	7	29574836	surrounding	70584	Pak4	-191633	29383203	29343838	-	R	7 A3	5730488L07RiklAW555722ImKIAA1142	p21 (CDKN1A)-activated kinase 4	no

	7	29574836	surrounding	71984	Sars2	47821	29527015	29538874	+	F	7 A3|7 9.5 cM	2410015F05Rik|D7Ertd353e|SerRSmt	seryl-aminoacyl-tRNA synthetase 2	no
	7	29574836	surrounding	73830	Eif3s12	191997	29766833	29756392	-	R	7 A3-B1	1200009C21Rik|Eif3s12	eukaryotic translation initiation factor 3, subunit K	no
	7	29574836	surrounding	101744	C330005M16Rik	-158802	29416034	29392656	-	R	7 A3	AU017076|Papl	RIKEN cDNA C330005M16 gene	no
	7	29574836	surrounding	233040	Fbxo27	96968	29477868	29484353	+	F	7 A3	E130008B10Rik|FBG5|Gm161	F-box protein 27	no
	7	29574836	closest	320435	5830482F20Rik	840	29573996	29583982	+	F	7 A3	5830482F20Rik|9930116N10Rik|MGC51433	Ras and Rab interactor-like	no
	7	29574836	surrounding	624918	EG624918	33638	29541198	29543763	+	F	7 A3	-	predicted gene, EG624918	no
	7	29574836	surrounding	100093674	Trnak-uuu	4243	29579079	29579007	-	R	-	-	transfer RNA lysine (anticodon UUU)	no
3.2	8	84391865	closest	234515	Inpp4b	152766	84239099	84646460	+	F	8 C2|8 38.0 cM	E130107I17Rik	inositol polyphosphate-4-phosphatase, type II	no
4.1	7	4625921	surrounding	16156	Il11	103712	4729633	4724657	-	R	7 A1|7 2.0 cM	IL-11	interleukin 11	no
	7	4625921	surrounding	19943	Rpl28	-118646	4744567	4746149	+	F	7 A1|7 4.0 cM	D7Wsu21e|MGC107666|MGC115078	ribosomal protein L28	no
	7	4625921	surround	21954	Tnni3	-15187	447404	446991	-	R	7 A1|7	Tn1|cTnI	troponin I, cardiac 3	no

			ing			6	5	0			9.0 cM			
	7	4625921	surrounding	21955	Tnnt1	-158344	4467577	4456265	-	R	7 A1|7 9.0 cM	AW146156|Tntl|sTnT	troponin T1, skeletal, slow	no
	7	4625921	surrounding	53420	Syt5	-127752	4498169	4491367	-	R	7 A1	SytV	synaptotagmin V	no
	7	4625921	closest	66245	1500019G21Rik	10644	4636565	4612123	-	R	7 A1	Hspbp1	RIKEN cDNA 1500019G21 gene	no
	7	4625921	surrounding	67441	Isoc2b	191860	4817781	4796562	-	R	7 A1	0610042E07Rik|AI553388|Isoc2	isochorismatase domain containing 2b	no
	7	4625921	surrounding	68255	Tmem86b	-43837	4582084	4579640	-	R	7 A1	C330014O21Rik|MGC118389	transmembrane protein 86B	no
	7	4625921	surrounding	77891	Ube2s	138021	4763942	4759616	-	R	7 A1	0910001J09Rik|6720465F12Rik|AA409170|E2-EPF|MGC101982	ubiquitin-conjugating enzyme E2S	no
	7	4625921	surrounding	78052	4930572D21Rik	-108629	4734550	4735943	+	F	7 A1	4930572D21Rik	transmembrane protein 190	no
	7	4625921	surrounding	232807	Ppp1r12c	-172639	4453282	4433123	-	R	7 A1	2410197A17Rik|AI839747|B430101M08|Mbs85	protein phosphatase 1, regulatory (inhibitor) subunit 12C	no
	7	4625921	surrounding	232811	Suv420h2	-65869	4691790	4699089	+	F	7 A1	BC024816|KMT5C|MGC36471|Suv4-20h2	suppressor of variegation 4-20 homolog 2 (Drosophila)	no
	7	4625921	surrounding	232813	D430041B17Rik	164885	4790806	4780076	-	R	7 A1	-	RIKEN cDNA D430041B17 gene	no
	7	4625921	surrounding	243819	Saps1	-15369	4610552	4583097	-	R	7 A1	2010309P17Rik|AI836219|B430201G11Rik|D030074N20Rik|MGC62441|Pp6r1|mKIAA1115	SAPS domain family, member 1	no

	7	4625921	surrounding	243822	4930401F20Rik	96983	4722904	4704831	-	R	7 A1	-	RIKEN cDNA 4930401F20 gene	no
	7	4625921	surrounding	330460	BC022651	50932	4676853	4658434	-	R	7 A1	A630041N19IMGC31268	cDNA sequence BC022651	no
	7	4625921	surrounding	333182	Cox6b2	78775	4704696	4703396	-	R	7 A1	BC048670ICOXVIB2IMGC130249IMGC130250	cytochrome c oxidase subunit VIb polypeptide 2	no
	7	4625921	surrounding	381979	Brsk1	-16609	4642530	4667599	+	F	7 A1	Gm1100IMGC99905ISAD-BISADB	BR serine/threonine kinase 1	no
	7	4625921	surrounding	436022	6030429G01Rik	-141877	4484044	4474559	-	R	7 A1	-	RIKEN cDNA 6030429G01 gene	no
	7	4625921	surrounding	545902	Ptprh	-70278	4555643	4500216	-	R	7 A1	-	protein tyrosine phosphatase, receptor type, H	no
	7	4625921	surrounding	628211	EG628211	35783	4590138	4590992	+	F	7 A1I7	-	predicted gene, EG628211	no
	7	4625921	surrounding	664968	2210411K11Rik	115241	4741162	4736387	-	R	7 A1	-	RIKEN cDNA 2210411K11 gene	no
	7	4625921	surrounding	669557	LOC669557	62684	4563237	4563908	+	F	7 A1	-	hypothetical LOC669557	no
6.1	7	29574836	surrounding	15388	Hnrpl	-21096	29595932	29607263	+	F	7 A3	C79783ID830027H13RiklHnrpl	heterogeneous nuclear ribonucleoprotein L	no
	7	29574836	surrounding	16855	Lgals4	-44173	29619009	29626722	+	F	7 A3I7 4.0 cM	galectin-4	lectin, galactose binding, soluble 4	no
	7	2957483	surround	16858	Lgals7	-74569	296494	296513	+	F	7 A3	Galectin-7IMGC151215IMGC151217	lectin, galactose binding, soluble 7	no

		6	ing				05	03						
	7	2957483 6	surround ing	18036	Nfkbib	-23293	295515 43	295432 70	-	R	7 A3-B3	IKB-betaIIkBIIkBbIMGC36057	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, beta	no
	7	2957483 6	surround ing	24030	Mrps12	-48036	295268 00	295246 60	-	R	7 A3	AI327385IRpms12Irps12	mitochondrial ribosomal protein S12	no
	7	2957483 6	surround ing	26411	Map4k1	- 19303 7	297678 73	297882 98	+	F	7 B1	Hpk1ImHPK1	mitogen-activated protein kinase kinase kinase kinase 1	no
	7	2957483 6	surround ing	50760	Fbxo17	72944	295018 92	295231 63	+	F	7 A3	Fbg4IFbx17IFbxo26	F-box protein 17	no
	7	2957483 6	surround ing	51798	Ech1	-35521	296103 57	296172 58	+	F	7 A3	AA617331IMGC107274	enoyl coenzyme A hydratase 1, peroxisomal	no
	7	2957483 6	surround ing	60594	Capn12	-91840	296666 76	296786 04	+	F	7 A3	-	calpain 12	no
	7	2957483 6	surround ing	60595	Actn4	17246 3	297472 99	296782 73	-	R	7 A3	C77391IMGC102374	actinin alpha 4	no
	7	2957483 6	surround ing	64383	Sirt2	23034	295518 02	295736 79	+	F	7 B1-2	5730427M03RiklSIR2L2lSir2l	sirtuin 2 (silent mating type information regulation 2, homolog) 2 (S. cerevisiae)	no
	7	2957483 6	surround ing	70584	Pak4	- 19163 3	293832 03	293438 38	-	R	7 A3	5730488L07RiklAW555722lmKIAA1142	p21 (CDKN1A)-activated kinase 4	no
	7	2957483 6	surround ing	71984	Sars2	47821	295270 15	295388 74	+	F	7 A3l7 9.5 cM	2410015F05RiklD7Ertd353elSerRSmt	seryl-aminoacyl-tRNA synthetase 2	no
	7	2957483 6	surround ing	73830	Eif3s12	19199 7	297668 33	297563 92	-	R	7 A3-B1	1200009C21RiklEif3s12	eukaryotic translation initiation factor 3, subunit K	no
	7	2957483	surround	101744	C330005M1	- 15880	294160	293926	-	R	7 A3	AU017076IPapl	RIKEN cDNA C330005M16 gene	no

		6	ing		6Rik	2	34	56						
	7	29574836	surrounding	233040	Fbxo27	96968	29477868	29484353	+	F	7 A3	E130008B10RikIFBG5IGm161	F-box protein 27	no
	7	29574836	closest	320435	5830482F20Rik	840	29573996	29583982	+	F	7 A3	5830482F20RikI9930116N10RikIMGC51433	Ras and Rab interactor-like	no
	7	29574836	surrounding	624918	EG624918	33638	29541198	29543763	+	F	7 A3	-	predicted gene, EG624918	no
	7	29574836	surrounding	100093674	Trnak-uuu	4243	29579079	29579007	-	R	-	-	transfer RNA lysine (anticodon UUU)	no
7.1	1	193768409	surrounding	18005	Nek2	122968	193645441	193656921	+	F	1 H6I1 103.0 cM	AA617254IC77054	NIMA (never in mitosis gene a)-related expressed kinase 2	no
	1	193768409	surrounding	22033	Traf5	57247	193825656	193821088	-	R	1 H6I1 105.0 cM	-	Tnf receptor-associated factor 5	no
	1	193768409	surrounding	22033	Traf5	148040	193916449	193878231	-	R	1 H6I1 105.0 cM	-	Tnf receptor-associated factor 5	no
	1	193768409	surrounding	22782	Slc30a1	37749	193730660	193737126	+	F	1 H6I1 106.0 cM	AI839647IC130040I11RikIZnt1	solute carrier family 30 (zinc transporter), member 1	no
	1	193768409	closest	74023	3322402L07Rik	-32861	193801270	193809795	+	F	1 H6	3322402L07RikIMGC117971IMGC151312IMGC151314Ird-3Ird3	retinal degeneration 3	no
	1	193768409	surrounding	98736	4930557M11Rik	-45401	193723008	193714109	-	R	1 H6	BB016121	RIKEN cDNA 4930557M11 gene	no

	1	193768409	surrounding	214742	Rcor3	193260	193961669	193924340	-	R	1 H6	4921514E24RiklC730034D20RiklE130101E15RiklMGC28186	REST corepressor 3	no
	1	193768409	surrounding	100042611	LOC100042611	-48296	193816705	193817417	+	F	1 H6l1	-	predicted gene, ENSMUSG00000063025	no
	1	193768409	surrounding	100042625	LOC100042625	129996	193898405	193897612	-	R	1 H6l1	-	predicted gene, 100042625	no
7/2	6	144695379	surrounding	66760	4933425H06Rik	73991	144621388	144642354	+	R	6 G3	-	RIKEN cDNA 4933425H06 gene	no
	6	144695379	closest	665149	EG665149	23290	144672089	144673427	+	R	6 G3	-	predicted gene, EG665149	no
7/3	12	120,491,065	closest	320910	Itgb8	-14316	120401267	120476749	-		12q F2	-	Integrin beta 8	no
	12	120,491,065	surrounding	238445	Macc1	190818	120681883	120705403	+		12q F1	-	Metastasis associated in colon cancer 1	no
7/4	9	3374604	surrounding	67667	Alkbh8	39373	3335231	3385847	+	F	9 A1	4930562C03Rikl8030431D03Rikl9430088N01RiklAbh8lMGC10235	alkB, alkylation repair homolog 8 (E. coli)	no
	9	3374604	surrounding	114673	4930433N12Rik	-174802	3199802	3133847	-	R	9 A1	-	RIKEN cDNA 4930433N12 gene	no
	9	3374604	surrounding	234889	Gucy1a2	-15775	3532354	3897342	+	F	9 A1	9430053E15	guanylate cyclase 1, soluble, alpha 2	no

						0								
	9	3374604	closest	244672	Cwf19l2	-29481	3404085	3479236	+	F	9 A1	3230401L03Riklg9030209E10	CWF19-like 2, cell cycle control (S. pombe)	no
	9	3374604	surrounding	384911	LOC384911	122867	3497471	3494531	-	R	9 A1	-	similar to DNA topoisomerase	no
	9	3374604	surrounding	404381	Olfr369-ps1	141261	3233343	3236366	+	F	9 A1	-	olfactory receptor 369, pseudogene 1	no
	9	3374604	surrounding	664797	LOC664797	187720	3186884	3188334	+	F	9 A1	-	similar to bM573K1.5 (novel Ulp1 protease family member)	no
7/5	13	67332494	surrounding	72807	2810487A22Rik	168209	67500703	67490263	-	R	13 B3	2810487A22RiklAI929863lRsl2	zinc finger protein 429	no
	13	67332494	surrounding	78251	Zfp712	-170396	67162098	67139530	-	R	13 B3	4921504N20Riklmszf31lmszf89	zinc finger protein 712	no
	13	67332494	closest	218311	Zfp455	37052	67295442	67310234	+	F	13 B3l13 40.0 cM	3732412P20RiklRslcan-10	zinc finger protein 455	no
	13	67332494	surrounding	218314	A230042K10Rik	101002	67433496	67413934	-	R	13 B3	A230042K10RiklRslcan23	zin finger protein 595	no
	13	67332494	surrounding	238690	Zfp458	37510	67370004	67355854	-	R	13 B3l13 40.3 cM	BC062958lRslcan-7lmszf59-2	zinc finger protein 458	no
	13	67332494	surrounding	268670	Zfp759	103330	67229164	67242723	+	F	13 B3l13 39.9 cM	BC028265lRslcan-8lRslcan8	zinc finger protein 759	no
	13	67332494	surrounding	328274	Zfp459	189860	67522354	67506658	-	R	13 B3l13 41.1 cM	9930025G17RiklRslcan-14	zinc finger protein 459	no

	13	67332494	surrounding	380855	Rsl1	58376	67274118	67284435	+	F	13 B3|13 40.0 cM	MGC118400|rslcan-9	regulator of sex limited protein 1	no
	13	67332494	surrounding	408065	Zfp456	144205	67476699	67464520	-	R	13 B3|13 40.9 cM	BC065418|MGC73892|Rslcan-13	zinc finger protein 456	no
	13	67332494	surrounding	431706	Zfp457	74854	67407348	67393386	-	R	13 B3|13 40.5 cM	Rslcan-6	zinc finger protein 457	no
	13	67332494	surrounding	432769	Zfp708	-133582	67198912	67170334	-	R	13 B3	AU021768|BC038328|MGC102251|Rslcan11	zinc finger protein 708	no
	13	67332494	surrounding	629016	E130120F12Rik	129014	67461508	67440245	-	R	13 B3	-	RIKEN cDNA E130120F12 gene	no
	13	67332494	surrounding	674699	LOC674699	44173	67288321	67289116	+	F	13 B3	-	similar to STAT3-interacting protein as a repressor	no
7/6	5	43607499	closest	231207	Cpeb2	-17699	43625198	43677429	+	F	5 B	A630055H10Rik	cytoplasmic polyadenylation element binding protein 2	no
	5	43607499	surrounding	665934	EG665934	-47944	43559555	43542925	-	R	5 B3	-	predicted gene, EG665934	no
														no
7/7	5	91314134	surrounding	13874	Ereg	-189509	91503643	91522675	+	F	5 E1	EPR|MGC36144	epiregulin	no
	5	91314134	closest	14825	Cxcl1	-6202	91320336	91322141	+	F	5 E-F|5 51.0 cM	Fspl|Gro1|KC|MgsaIN51|Scyb1|gro	chemokine (C-X-C motif) ligand 1	no
	5	9131413	surround	20309	Cxcl15	90574	912235	912320	+	F	5 E1|5	Scyb15|lungkine|weche	chemokine (C-X-C motif) ligand 15	no

		4	ing				60	93			51.5 cM			
	5	9131413 4	surround ing	20309	Cxcl15	80281	912338 53	912253 39	+	F	5 E1|5 51.5 cM	Scyb15|lungkine|weche	chemokine (C-X-C motif) ligand 15	no
	5	9131413 4	surround ing	20310	Cxcl2	-18791	913329 25	913349 64	+	F	5 E1|5 51.0 cM	CINC-2a|GROb|Gro2|MIP-2|MIP-2a|Mgsa-b|Mip2|Scyb|Scyb2	chemokine (C-X-C motif) ligand 2	no
	5	9131413 4	surround ing	20311	Cxcl5	12580 9	911883 25	911906 51	+	F	5 E1|5 53.0 cM	AMCF-II|ENA-78|GCP-2|LIX|Scyb5|Scyb6	chemokine (C-X-C motif) ligand 5	no
	5	9131413 4	surround ing	20311	Cxcl5	12168 7	911924 47	911901 09	+	F	5 E1|5 53.0 cM	AMCF-II|ENA-78|GCP-2|LIX|Scyb5|Scyb6	chemokine (C-X-C motif) ligand 5	no
	5	9131413 4	surround ing	56744	Cxcl4	11259 5	912015 39	912024 09	+	F	5 E1	Cxcl4|Scyb4	platelet factor 4	no
	5	9131413 4	surround ing	56744	Cxcl4	10993 5	912041 99	912033 29	+	F	5 E1	Cxcl4|Scyb4	platelet factor 4	no
	5	9131413 4	surround ing	57349	Ppbp	11659 0	911975 44	911990 86	+	F	5 E1	2400003M24Rik|AI854500|CTAP3|CTAPIII|Cxcl7|LA-PF4|LDGF|MDGF|NAP-2|NAP-2-L1|Scyb7|TGB|TGB1|THBGB1|b-TG1|beta-TG	pro-platelet basic protein	no
	5	9131413 4	surround ing	57349	Ppbp	11325 2	912008 82	911993 40	+	F	5 E1	2400003M24Rik|AI854500|CTAP3|CTAPIII|Cxcl7|LA-PF4|LDGF|MDGF|NAP-2|NAP-2-L1|Scyb7|TGB|TGB1|THBGB1|b-TG1|beta-TG	pro-platelet basic protein	no
	5	9131413 4	surround ing	71920	Epgn	-14240 9	914565 43	914642 38	+	F	5 E2	2310069M11Rik|epigen	epithelial mitogen	no
	5	9131413 4	surround ing	330122	Gm1960	99005	912151 29	912171 16	+	F	5 E1	Dcip1|Gm1960	chemokine (C-X-C motif) ligand 3	no
	5	9131413	surround	330122	Gm1960	95241	912188	912169	+	F	5 E1	Dcip1|Gm1960	chemokine (C-X-C motif) ligand 3	no

		4	ing				93	03						
	5	91314134	surrounding	665563	1110019K23Rik	-46088	91360222	91450394	+	F	5 E1	1110019K23RikIC630010D07Rik	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2-like	no
8.1	2	153068063	surrounding	15162	Hck	133819	152934244	152977172	+	F	2 H1|2 86.0 cM	AI849071IBmkIHck-1IMGC18625	hemopoietic cell kinase, Evi47	yes(6)
	2	153068063	surrounding	16569	Kif3b	-49102	153117165	153157950	+	F	2 86.0 cM	AI854312IAW549267ImKIAA0359	kinesin family member 3B	no
	2	153068063	surrounding	54711	Plagl2	-969	153067094	153053505	-	R	2 H2	AU018672IAW552839ImKIAA0198	pleiomorphic adenoma gene-like 2	yes(3)
	2	153068063	surrounding	72326	2500004C02Rik	103746	153171809	153168231	-	R	2 H1	-	RIKEN cDNA 2500004C02 gene	no
	2	153068063	surrounding	99237	Tm9sf4	81026	152987037	153036199	+	F	2 H1	AA986553IAU045326IB930079E06IKIAA0255 IMGC117930ImKIAA0255	transmembrane 9 superfamily protein member 4	no
	2	153068063	closest	140484	Pofut1	795	153067268	153095989	+	F	2 H2	KIAA0180IO-FucT-1ImKIAA0180	protein O-fucosyltransferase 1	no
	2	153068063	surrounding	228788	BC020535	176372	152891691	152907462	+	F	2 H1	MGC19360	cDNA sequence BC020535	no
	2	153068063	surrounding	228790	Asxl1	-103812	153171875	153229743	+	F	2 H1	DKFZp434N0535ImKIAA0978	additional sex combs like 1 (Drosophila)	no
	2	153068063	surrounding	241732	Tspyl3	-16944	153051119	153048106	-	R	2 H1	AW212607IGm36IMGC58351	TSPY-like 3	no

9.1, 9.2, 9.3	7	29574438	surrounding	15388	Hnrpl	-21494	29595932	29607263	+	R	7 A3	C79783ID830027H13RiklHnrpl	heterogeneous nuclear ribonucleoprotein L	no
	7	29574438	surrounding	16855	Lgals4	-44571	29619009	29626722	+	R	7 A3I7 4.0 cM	galectin-4	lectin, galactose binding, soluble 4	no
	7	29574438	surrounding	16858	Lgals7	-74967	29649405	29651303	+	R	7 A3	Galectin-7IMGC151215IMGC151217	lectin, galactose binding, soluble 7	no
	7	29574438	surrounding	18036	Nfkbib	-22895	29551543	29543270	-	F	7 A3-B3	IKB-betaIIkBIIkBbIMGC36057	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, beta	no
	7	29574438	surrounding	24030	Mrps12	-47638	29526800	29524660	-	F	7 A3	AI327385IRpms12Irps12	mitochondrial ribosomal protein S12	no
	7	29574438	surrounding	26411	Map4k1	-193435	29767873	29788298	+	R	7 B1	Hpk1ImHPK1	mitogen-activated protein kinase kinase kinase kinase 1	no
	7	29574438	surrounding	50760	Fbxo17	72546	29501892	29523163	+	R	7 A3	Fbg4IFbx17IFbxo26	F-box protein 17	no
	7	29574438	surrounding	51798	Ech1	-35919	29610357	29617258	+	R	7 A3	AA617331IMGC107274	enoyl coenzyme A hydratase 1, peroxisomal	no
	7	29574438	surrounding	60594	Capn12	-92238	29666676	29678604	+	R	7 A3	-	calpain 12	no
	7	29574438	surrounding	60595	Actn4	172861	29747299	29678273	-	F	7 A3	C77391IMGC102374	actinin alpha 4	no
	7	29574438	surrounding	64383	Sirt2	22636	29551802	29573679	+	R	7 B1-2	5730427M03RiklSIR2L2ISir2l	sirtuin 2 (silent mating type information regulation 2, homolog) 2 (S. cerevisiae)	no
	7	29574438	surrounding	70584	Pak4	-191235	29383203	29343838	-	F	7 A3	5730488L07RiklAW555722ImKIAA1142	p21 (CDKN1A)-activated kinase 4	no

	7	29574438	surrounding	71984	Sars2	47423	29527015	29538874	+	R	7 A3|7 9.5 cM	2410015F05Rik|D7Ertd353e|SerRSmt	seryl-aminoacyl-tRNA synthetase 2	no
	7	29574438	surrounding	73830	Eif3s12	192395	29766833	29756392	-	F	7 A3-B1	1200009C21Rik|Eif3s12	eukaryotic translation initiation factor 3, subunit K	no
	7	29574438	surrounding	101744	C330005M16Rik	-158404	29416034	29392656	-	F	7 A3	AU017076|Papl	RIKEN cDNA C330005M16 gene	no
	7	29574438	surrounding	233040	Fbxo27	96570	29477868	29484353	+	R	7 A3	E130008B10Rik|FBG5|Gm161	F-box protein 27	no
	7	29574438	closest	320435	5830482F20Rik	442	29573996	29583982	+	R	7 A3	5830482F20Rik|9930116N10Rik|MGC51433	Ras and Rab interactor-like	no
	7	29574438	surrounding	624918	EG624918	33240	29541198	29543763	+	R	7 A3	-	predicted gene, EG624918	no
	7	29574438	surrounding	100093674	Trnak-uuu	4641	29579079	29579007	-	F	-	-	transfer RNA lysine (anticodon UUU)	no
10.1	7	4625921	surrounding	16156	Il11	103712	4729633	4724657	-	R	7 A1|7 2.0 cM	IL-11	interleukin 11	no
	7	4625921	surrounding	19943	Rpl28	-118646	4744567	4746149	+	F	7 A1|7 4.0 cM	D7Wsu21e|MGC107666|MGC115078	ribosomal protein L28	no
	7	4625921	surrounding	21954	Tnni3	-151876	4474045	4469910	-	R	7 A1|7 9.0 cM	Tn1|cTnI	troponin I, cardiac 3	no
	7	4625921	surrounding	21955	Tnnt1	-15834	4467577	4456265	-	R	7 A1|7 9.0 cM	AW146156|TntIs|TnT	troponin T1, skeletal, slow	no

						4								
	7	4625921	surrounding	53420	Syt5	-127752	4498169	4491367	-	R	7 A1	SytV	synaptotagmin V	no
	7	4625921	closest	66245	1500019G21Rik	10644	4636565	4612123	-	R	7 A1	Hspbp1	RIKEN cDNA 1500019G21 gene	no
	7	4625921	surrounding	67441	Isoc2b	191860	4817781	4796562	-	R	7 A1	0610042E07RiklAI553388Ilsoc2	isochorismatase domain containing 2b	no
	7	4625921	surrounding	68255	Tmem86b	-43837	4582084	4579640	-	R	7 A1	C330014O21RiklMGC118389	transmembrane protein 86B	no
	7	4625921	surrounding	77891	Ube2s	138021	4763942	4759616	-	R	7 A1	0910001J09Rikl6720465F12RiklAA409170IE2-EPFIMGC101982	ubiquitin-conjugating enzyme E2S	no
	7	4625921	surrounding	78052	4930572D21Rik	-108629	4734550	4735943	+	F	7 A1	4930572D21Rik	transmembrane protein 190	no
	7	4625921	surrounding	232807	Ppp1r12c	-172639	4453282	4433123	-	R	7 A1	2410197A17RiklAI839747IB430101M08IMbs85	protein phosphatase 1, regulatory (inhibitor) subunit 12C	no
	7	4625921	surrounding	232811	Suv420h2	-65869	4691790	4699089	+	F	7 A1	BC024816IKMT5CIMGC36471ISuv4-20h2	suppressor of variegation 4-20 homolog 2 (Drosophila)	no
	7	4625921	surrounding	232813	D430041B17Rik	164885	4790806	4780076	-	R	7 A1	-	RIKEN cDNA D430041B17 gene	no
	7	4625921	surrounding	243819	Saps1	-15369	4610552	4583097	-	R	7 A1	2010309P17RiklAI836219IB430201G11RiklD030074N20RiklMGC62441IPp6r1ImKIAA1115	SAPS domain family, member 1	no
	7	4625921	surrounding	243822	4930401F20Rik	96983	4722904	4704831	-	R	7 A1	-	RIKEN cDNA 4930401F20 gene	no

	7	4625921	surrounding	330460	BC022651	50932	4676853	4658434	-	R	7 A1	A630041N19IMGC31268	cDNA sequence BC022651	no
	7	4625921	surrounding	333182	Cox6b2	78775	4704696	4703396	-	R	7 A1	BC048670ICOXVIB2IMGC130249IMGC130250	cytochrome c oxidase subunit VIb polypeptide 2	no
	7	4625921	surrounding	381979	Brsk1	-16609	4642530	4667599	+	F	7 A1	Gm1100IMGC99905ISAD-BISADB	BR serine/threonine kinase 1	no
	7	4625921	surrounding	436022	6030429G01Rik	-141877	4484044	4474559	-	R	7 A1	-	RIKEN cDNA 6030429G01 gene	no
	7	4625921	surrounding	545902	Ptprh	-70278	4555643	4500216	-	R	7 A1	-	protein tyrosine phosphatase, receptor type, H	no
	7	4625921	surrounding	628211	EG628211	35783	4590138	4590992	+	F	7 A1I7	-	predicted gene, EG628211	no
	7	4625921	surrounding	664968	2210411K11Rik	115241	4741162	4736387	-	R	7 A1	-	RIKEN cDNA 2210411K11 gene	no
	7	4625921	surrounding	669557	LOC669557	62684	4563237	4563908	+	F	7 A1	-	hypothetical LOC669557	no
11/1	11	86436031	surrounding	56427	Tubd1	77521	86358510	86380862	+	F	11 C	4930550G19RiklTubd	tubulin, delta 1	no
	11	86436031	surrounding	67300	Cltc	134963	86570994	86508155	-	R	11 C	3110065L21RiklCHCIMGC92975IR74732	clathrin, heavy polypeptide (Hc)	no
	11	86436031	surrounding	67487	Dhx40	185131	86621162	86582351	-	R	11 C	2410016C14RiklARG147IDDX40IPAD	DEAH (Asp-Glu-Ala-His) box polypeptide 40	no
	11	8643603	surround	72508	Rps6kb1	-77755	863582	863274	-	R	11 C	2610318I15RiklI4732464A07RiklI70kDalAA959758IAI256796IAI314060IS6K1Ip70/85s6kIp70	ribosomal protein S6 kinase, polypeptide 1	no

		1	ing				76	85				s6k		
	11	86436031	surrounding	75909	Tmem49	61293	86497324	86397367	-	R	11 C	3110098I04RikI4930579A11RikIAI787464Ini-2	transmembrane protein 49	no
	11	86436031	surrounding	76892	0610013E23Rik	137872	86298159	86312509	+	F	11 C	0610013E23RikIAV007605	ring finger protein, transmembrane 1	no
	11	86436031	surrounding	217057	Ptrh2	-61454	86497485	86505825	+	F	11 C	A230072I16RikIBit1ICGI-147	peptidyl-tRNA hydrolase 2	no
	11	86436031	closest	387140	Mirn21	-38372	86397659	86397568	-	R	-	mmu-mir-21	microRNA 21	no
	11	86436031	surrounding	100043687	LOC100043687	44033	86391998	86394271	+	F	11 CI11	-	predicted gene, 100043687	no

Lebenslauf

ASHOK KUMAR

Geboren am 25.05.1977 Nationalität: Pakistani

Akademische Ausbildung

Abschluss	Universität	Jahr des Abschlusses	Note	Schwerpunkte
Doktor der Medizin	Johann Wolfgang Goethe-Universität, Frankfurt am Main	2010	Magna Cum Laude	Molekulare Biology, Tumor Biologie
D.C.P (Diploma in Clinical Pathology)	LUMHS, Jamshoro, Pakistan	2008	A (sehr gut)	Hämatologie, Mikrobiologie, Biochemie, Histopathologie
Bachelor in Medizin, Bachelor in Chirurgie (M.B;B.S)	LUMHS, Jamshoro, Pakistan	2003	A (sehr gut)	Medizin, Chirurgie
Higher Secondary College (Abitur)	Sindh Model College, Tandoallahyar	1996	A (sehr gut)	Englisch, Biologie, Physik, Chemie
SSC (Realschul-abschluss)	Government High School, Kandiari	1993	A1 (mit Auszeichnung)	Englisch, Biologie, Physik, Chemie

Unterschrift _____

Schriftliche Erklärung

Ich erkläre, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel:

Transformationspotential von NPM-ALK, p21SNFT und Tax in reifen T Lymphozyten

in dem Georg-Speyer-Haus, Frankfurt am Main
unter Betreuung und Anleitung von Prof. Dr. Dorothee von Laer
mit Unterstützung durch.....
ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.
Ich habe bisher an keiner in - oder ausländischen Universiät ein Gesuch um Zulassung zur Promotion eingereicht. *)
Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Frankfurt am Main .....

(Unterschrift)

*) Im Falle des Nicht zutreffnes streichen.