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GENETIC ALTERATIONS IN LYMPHOMA

- WITH FOCUS ON THE *IKAROS*, *NOTCH1* AND *BCL11B* GENES

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True knowledge exists in knowing you know nothing.
(Socrates, 470-399 B.C.)

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ABSTRACT

Cell proliferation is a process that is strictly regulated by a large number of proteins. An alteration in one of the encoding genes inserts an error into the regulative protein, which may result in uncontrolled cell growth and eventually tumor formation. Lymphoma is a cancer type originating in the lymphocytes, which are part of the body's immune defence. In the present thesis, *Znfn1a1*, *Notch1* and *Bcl11b* were studied; all involved in the differentiation of T lymphocytes. The three genes are located in chromosomal regions that have previously shown frequent loss of heterozygosity in tumor DNA.

Ikaros is a protein involved in the early differentiation of T lymphocytes. In this thesis, mutation analysis of the *Znfn1a1* gene in chemically induced murine lymphomas revealed point mutations and homozygous deletions in 13 % of the tumors. All of the detected deletions lead to amino acid substitutions or abrogation of the functional domains in the Ikaros protein. Our results support the role of Ikaros as a potential tumor suppressor in a subset of tumors.

Notch1 is a protein involved in many differentiation processes in the body. In lymphocytes, Notch1 drives the differentiation towards a T-cell fate and activating alterations in the *Notch1* gene have been suggested to be involved in T-cell lymphoma. We identified activating mutations in *Notch1* in 39 % of the chemically induced murine lymphomas, supporting the involvement of activating *Notch1* mutations in the development of T-cell lymphoma.

Bcl11b has been suggested to be involved in the early T-cell specification, and mutations in the *Bcl11b* gene has been identified in T-cell lymphoma. In this

thesis, point mutations and deletions were detected in the DNA-binding zinc finger regions of Bcl11b in 15 % of the chemically induced lymphomas in C57Bl/6×C3H/HeJ F_1 mice. A mutational hotspot was identified, where four of the tumors carried the same mutation. Three of the identified alterations, including the hotspot mutation in Bcl11b, increased cell proliferation when introduced in a cell without endogenous Bcl11b, whereas cell proliferation was suppressed by wild-type Bcl11b in the same cell line. Mutations in Bcl11b may therefore be an important contributing factor to lymphomagenesis in a subset of tumors.

A germ line point mutation was identified in *BCL11B* in one of 33 human B-cell lymphoma patients. Expression of BCL11B in infiltrating T cells was significantly lower in aggressive compared to indolent lymphomas, suggesting that the infiltrating T cells may affect the B-cell lymphomas.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I **Anneli Karlsson**, Peter Söderkvist and Shi-Mei Zhuang.
Point mutations and deletions in the *Znfn1a1/Ikaros* gene in chemically induced murine lymphomas.
Cancer Research (2002) 62: 2650-2653.

II **Anneli Karlsson**, Anna Rasmussen and Peter Söderkvist.
Notch1 is a frequent mutational target in chemically generated lymphoma in mouse.
Submitted for publication

III **Anneli Karlsson**, Amanda Nordigården, Jan-Ingvar Jönsson and Peter Söderkvist.
Bcl11b mutations identified in murine lymphomas increase the proliferation rate in hematopoietic progenitor cells.
BMC Cancer (2007) 7:195

IV **Anneli Karlsson**, Charlotte Witell, Mats Karlsson, Christina Karlsson, Peter Söderkvist.
Mutation in the *BCL11B* gene in human B-cell lymphoma.
Manuscript

LIST OF ABBREVIATIONS

CDK	cyclin dependent kinase
CIP	CDK inhibitor protein
DN	double negative thymocyte
DP	double positive thymocyte
DSL	Delta, Serrate, LAG-2
ICN	intracellular Notch1
INK	inhibitor of CDK4
KIP	kinase inhibitor protein
PEST	rich in amino acids P, E, S and T
SP	single positive thymocyte
T-ALL	T-cell acute lymphoblastic leukemia
TCR	T-cell receptor

INTRODUCTION

Cancer is one of the most common diseases in the Western world. In Sweden, every third person will develop cancer at some point during their life time. A tumor may develop from any tissue in the body, causing various types of cancer with different underlying mechanisms and symptoms. Common for all cancers is the uncontrolled cell growth, eventually intruding on the normal tissue functions. Development of target specific treatment against cancer is of highest importance for present and future research, and more knowledge must therefore be achieved about the genes and the signalling mechanisms underlying tumor formation. In this thesis, three genes have been studied; *Ikaros*, *Notch1* and *Bcl11b*; that are involved in the development of lymphoma. Hopefully, the results obtained in this thesis will add a small piece to the great puzzle of cancer.

Cancer development

Cancer has long been known as a genetic disease. As early as 1914, Boveri reported about gross chromosomal abnormalities in malignant tumors, and suggested these alterations to be responsible for carcinogenesis (Boveri, 1914). In 1951, Muller suggested that a cell must contain more than one mutation in order to transform into a neoplasm, based on the long latency time between exposure of mutagenic radiation and the appearance of a tumor (Muller, 1951). A few years later, Foulds described tumor progression as a step-wise process where the cell acquires different qualities at each step, e.g. growth stimulatory, anti-apoptotic or invasive qualities (Foulds, 1957). Hereditary mutations can therefore be seen as predisposing factors for cancer development, as these cells already carry a genetic defect (reviewed in Hemminki et al., 2006). However,

somatic mutations are required to trigger the neoplasm and cancer is one of the few diseases where a somatic mutation actually is pathogenic to its bearer (reviewed in Vogelstein & Kinzler, 2004). In the seventies, Nowell presented the theory of clonal expansion in tumors: a cell which acquires a somatic mutation will achieve a growth advantage over adjacent normal cells which eventually leads to clonal expansion (Nowell, 1976), a theory which nowadays is more or less the definition of a neoplasm (Vogelstein & Kinzler, 2004).

Three categories of genes are known to influence cell growth and therefore being targets for mutation in tumor cells; cell growth regulating genes, genes controlling genetic stability and genes involved in invasion and metastasis. The first group of genes is the type regulating cell growth, positively or negatively. To this group belongs oncogenes and tumor suppressor genes (which will be described in detail below), but also genes involved in programmed cell death, apoptosis and regulating telomere repeat length, which may prolong the survival of neoplastic cells (reviewed in Hanahan & Weinberg, 2000). The second group consists of genes which control the genetic stability. Here we find genes that control the DNA repair system of the cell and genes controlling the chromosomal stability. When mutated, the overall genetic control is out of order and more mutational events in the cell are likely to follow (Hanahan & Weinberg, 2000). The third group of genes regulates the ability for cells to invade tissues; the ability of metastasis (Hanahan & Weinberg, 2000). This ability only needs to be acquired by solid tumors, as lymphoma and leukemia both originate in cell types which already possesses this quality (Nowell, 2002).

The described genes encode proteins, which are all taking part in signalling pathways in the cell. Each cell type has its own specific pattern of signals, and one particular mutation may therefore have different effects depending on the cell type it occurs in. Some proteins have different functions in different pathways, and may thus have both oncogenic and tumor suppressive effects depending on cell type (reviewed in Vogelstein & Kinzler, 2004). Previous mutations may also influence whether the new mutation has any impact on the cell growth or not. Genes within the same pathway are rarely mutated in the same tumor, since one mutation is often enough to interfere with the regulation of the pathway. For example, mutations in *PTEN* and *PIK3CA* in

the *Akt* pathway have been described as mutually exclusive in lymphoma (Abubaker et al., 2007).

The cell cycle

The phases of cell growth are the same, whether in a tumor or in normal cells, as for example in fetal development. Certain signals command the cell to start doubling its DNA content by replication during the S phase. When this is accomplished, the cell has also doubled its size and will be divided into two equal daughter cells during the M phase (mitosis).

The cell cycle (figure 1) is normally under strict regulation and before replication there is a quality control of the DNA (the G_1 checkpoint), where it is decided whether or not the cell may enter the next phase of the cell cycle (reviewed in Sandal, 2002). At the G_2 checkpoint, preceding mitosis, there is also a quality control of the cell material that makes sure that the newly synthesized DNA is identical to the original DNA and that division occurs equally between the daughter cells (Hickman et al., 2002). There is also a resting phase (G_0), where the cell exits the cell cycle temporarily in response to contact inhibition or permanently due to telomere shortening (reviewed in Kiyokawa, 2006).

The cell cycle is mainly regulated by cyclin-dependent kinases (CDK) and corresponding cyclins (reviewed in Sandal, 2002). In the G_1 phase, CDK4/cyclin D and CDK6/cyclin D are required for progression to the G_1/S restriction point, where CDK2/cyclin E takes over and irreversibly pushes the cell into the S phase (Sandal, 2002). In the same way, activation of the CDK1/cyclin B complex is required before the cell can enter M phase (Le Breton et al., 2005).

The CDKs and cyclins are regulated by many proteins. CDK inhibitors, such as $p21^{CIP1}$, $p27^{KIP1}$, $p57^{KIP2}$, $p15^{INK4b}$, $p16^{INK4a}$ and $p14^{ARF}$ can inactivate the CDKs by phosphorylation, arresting the cell cycle in response to antiproliferative signals (reviewed in Sandal, 2002). Tumor suppressor genes may also arrest the cell cycle when needed. In a resting cell, the retinoblastoma (RB) protein is bound to E2F, preventing entry into S phase. However, the nuclear transcription factor E2F, which is involved in replication, is released by phosphorylation of RB by CDK4/cyclin D or CDK2/cyclin E (Sandal, 2002).

The tumor suppressor p53 is known as the gate-keeper of the genome, as it may prevent DNA replication if the DNA is damaged. After DNA repair is performed the cell cycle may continue, but if the damage is too large to be repaired the cell may instead undergo apoptosis.

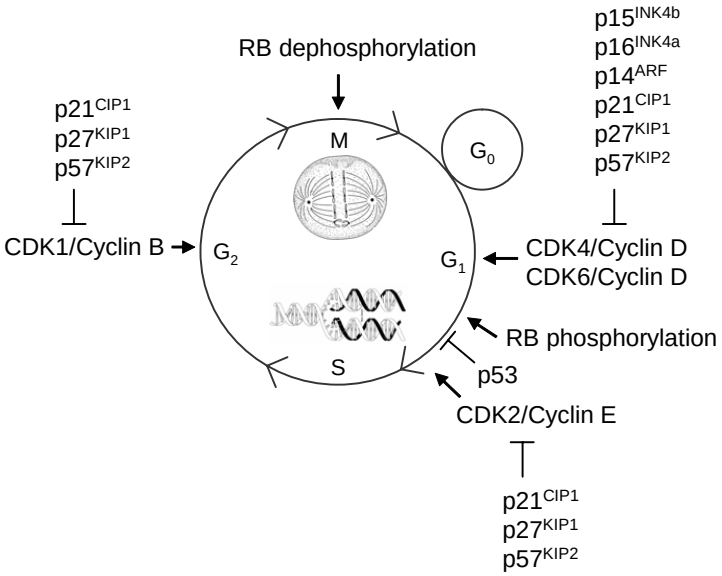

Figure 1. The cell cycle progression is favoured by cyclin-dependent kinases (CDK) and the corresponding cyclins. These are regulated by the CDK/kinase inhibitor protein (CIP/KIP) family and inhibitor of CDK4 (INK4) family of proteins. S - DNA synthesis, M - mitosis, G_1, G_2 and G_0 - gaps. Arrows indicate progression and T indicate inhibition of the cell cycle. (Modified from Donovan & Slingerland, 2000)

Oncogenes and tumor suppressor genes

Proto-oncogenes and tumor suppressor genes are normally expressed in the cell in purpose of regulating cell growth. Proto-oncogenes encode proteins that induce cell growth; however, these genes are strongly regulated in the cell in order to control the cell growth. Mutations in proto-oncogenes may render them consecutively active. They are then called oncogenes, and may drastically accelerate the proliferation rate.

Tumor suppressor genes normally inhibit cell growth, but may be inactivated for example by a point mutation, deletion or methylation of the promoter region. The cellular consequences of tumor suppressor gene inactivation is the same as for activated oncogenes; uncontrolled cell growth. Tumor suppressor genes are generally believed to affect tumor formation only when both alleles are inactivated (Knudson, 1971), as one allele of the protein may cover for the loss of the second allele. Inactivation of one allele is referred to as allelic loss or loss of heterozygosity, and predisposes the cell for cancer as only one more inactivating mutation is needed for complete inactivation (Knudson, 1971). However, some tumor suppressor genes seem to affect tumorigenesis even when only one allele is inactivated (reviewed in Santarosa & Ashworth, 2004). This phenomenon is referred to as haploinsufficiency, and has been observed for e.g. *p53*, *p27*^{Kip1} and *PTEN* in some tumors (Di Cristofano et al., 1998; Fero et al., 1998; Venkatachalam et al., 1998). The mechanism behind haploinsufficiency is so far unknown, but may be the result of imprinting or dominant mutations blocking the function of the normal allele. The reduction in gene expression yields a phenotype which contributes to carcinogenesis (Santarosa & Ashworth, 2004).

Lymphoma

Lymphoma is one of the top ten cancers in Sweden with approximately 1600 new cases per year, and occurs with similar frequencies in men and women (Swedish National Board of Health and Welfare, 2007). These tumors originate in the lymphocytes, which are part of the immune defence, and are grossly divided into Hodgkin's Lymphoma and Non-Hodgkin's Lymphoma. Non-Hodgkin's Lymphoma may be further classified into a number of different lymphoma types based on their differential stage, with varying grade of aggressiveness. The symptoms are similar to a common infection; 70 % of the cases have enlarged lymph nodes, fever and fatigue is common and some patients may also experience loss of appetite and weight. Surgical removal of the lymphomas, followed by chemotherapy and/or radiotherapy is the main treatment of patients with lymphoma. In severe cases a stem cell or bone marrow transplantation is performed. Over the years, the prognosis for lymphoma patients has been improved, with a 5 years survival of 54 % for Non-Hodgkin's Lymphoma and 85 % for Hodgkin's Lymphoma (Swedish Cancer Foundation and Swedish National Board of Health and Welfare, 2005).

Lymphocyte development

The lymphocytes originate in the bone marrow from a pluripotent hematopoietic stem cell that give rise to all types of blood cells. The differentiation into mature lymphocytes is a highly restricted step-wise process that via a lymphoid progenitor may create B- and T-lymphocytes as well as natural killer cells (reviewed in Orkin, 1995). The differentiation from the lymphoid progenitor into mature B- and T-lymphocytes is regulated by a number of proteins, for example Ikaros and PU.1 in early lymphocyte development, GATA-3 and Notch1 in the T-cell lineage, and Pax5, E2A and EBF in the B-cell lineage (Orkin, 1995; Rothenberg, 2007; Singh & Pongubala, 2006). When the lymphocytes have committed to the T-cell lineage, the cells undergo further maturation steps by expressing certain surface receptors (figure 2). At the first, double negative (DN) stage, the cells lack expression of the T-cell receptor (TCR) and other important surface markers. At the pre-T cell stage, the cells express the TCR-β, CD3 and pre-Tα, which comprises the

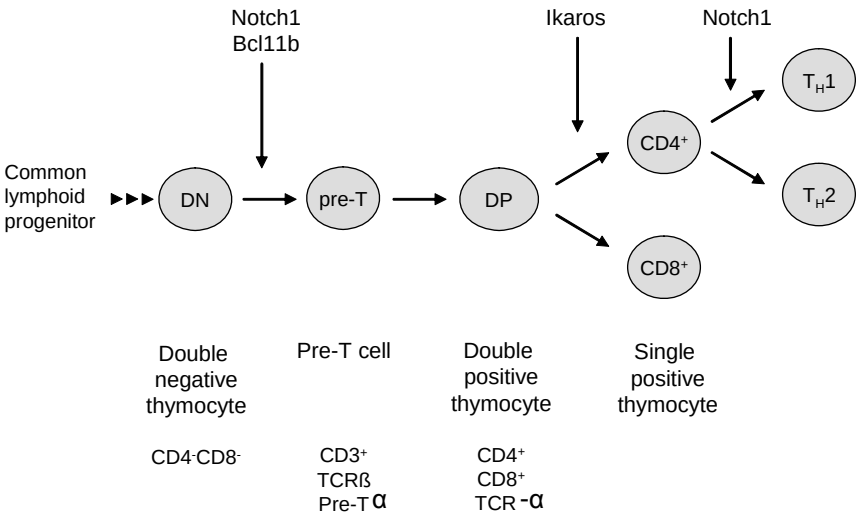

Figure 2. Schematic figure of the differentiation of T cells, including some of the transcription factors required for the different differential stages. (Modified from Osborne & Kee, 2005)

pre-TCR. If any of these markers are absent, the cell will be arrested at this stage of maturation. At the double positive (DP) stage of maturation, the CD4 and CD8 surface receptors are expressed. These receptors are able to recognize antigens presented to the circulating T cells. Also, the TCR-α is expressed at the DP stage, which completes the T-cell receptor. Finally, the cells undergo positive and negative selection, which allows maturation into single positive T cells (SP; $CD4^+$ or $CD8^+$). (reviewed in Fischer & Malissen, 1998)

Genetic rearrangements and alterations in lymphoma

Many types of lymphoma exhibit translocations in the genome and this has given much information about the genes involved in lymphomagenesis (reviewed in Rowley, 1998). For example, Burkitt's lymphoma has been associated with a translocation between regions on chromosome 8 and 14, containing the proto-oncogene *MYC* and the immunoglobulin heavy chain (*IGH*) gene, respectively (Dalla-Favera et al., 1982; Taub et al., 1982). MYC promotes cell proliferation by activating genes involved in cell cycle control, such as CDK4, Cyclin D1, D2 and E, and by suppressing genes involved in growth arrest, such as $p27^{KIP1}$, $p21^{CIP1}$ and $p15^{INK4b}$ (Rui & Goodnow, 2006). The 8;14 translocation does not alter the structure of the *MYC* protein, but instead switches the regulatory sequences, resulting in constitutive expression of *MYC* as the *IGH* gene is actively transcribed in B cells (Janz, 2006). However, in most other chromosomal translocations associated with lymphoid malignancies, the two genes located at the breakpoints are rearranged to form a fusion protein, which contains parts of both of the original proteins and a deregulated activity (Rowley, 1998).

Genes studied in this thesis

Much of what is known about cancer today is received from studies on mouse models. Many genes in the human and the murine genomes are homologous and the cancer forms are similar. Previous studies on chemically induced murine lymphoma have shown frequent allelic loss in chromosomes 4, 11 and 12 (Zhuang et al., 1996), indicating that these chromosomes contain candidate tumor suppressor genes. In this thesis, we have focused on three genes that are located in these chromosomal regions; *Ikaros*, *Notch1* and *Bcl11b*, all involved in the differentiation of T lymphocytes (Grabher et al., 2006; Ng et al., 2007; Tydell et al., 2007).

Ikaros

Ikaros is an lymphoid-specific transcription factor that belong to the Ikaros family of proteins, which play an important role in the development and differentiation of lymphocytes (Georgopoulos et al., 1992; Morgan et al., 1997; Sridharan & Smale, 2007). Ikaros is encoded by the *Znfn1a1* gene, which is highly conserved between the murine and human genomes, and located on mouse chromosome 11. The protein comprises seven exons, which may be alternatively spliced into 8 different isoforms (Hahm et al., 1998). Ikaros contains six zinc finger structures, whereof the four N-terminal zinc fingers are critical to Ikaros function as they enable binding to the core motifs in the promoter region of target genes (Koipally et al., 2002). The two C-terminal zinc fingers mediate dimerization with other Ikaros isoforms or with other Ikaros family members, in order to enhance Ikaros activity and allow transcription (Hahm et al., 1998; Morgan et al., 1997; Sun et al., 1996). Some of the isoforms lack one or more of the N-terminal zinc fingers, resulting in varying ability to bind DNA (Sun et al., 1996). Only two of the Ikaros isoforms are able to bind

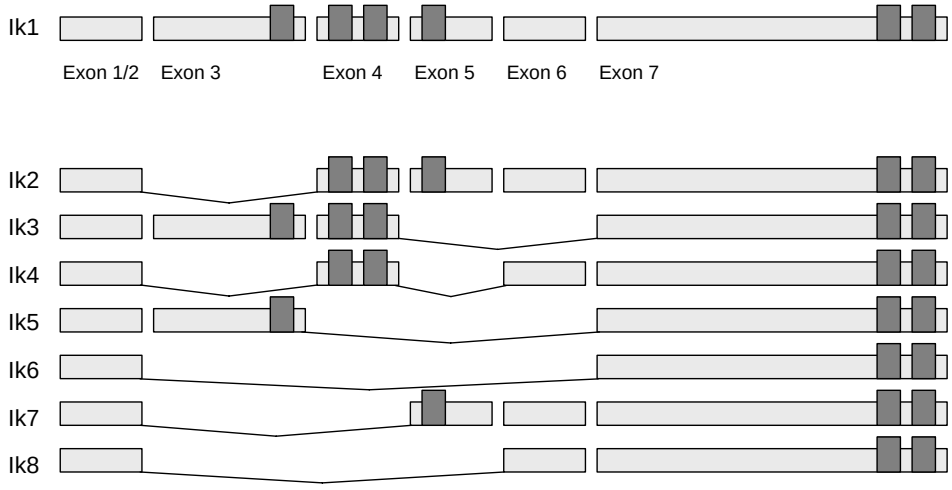

Figure 3. The eight isoforms of Ikaros. Grey squares indicate the four N-terminal and the two C-terminal zinc finger domains. (Modified from Sun et al., 1996)

DNA efficiently enough to activate transcription of target genes. The C-terminal zinc fingers enabling dimerization are present in all isoforms, and transcriptionally inactive isoforms will therefore exert a dominant negative effect on the dimer, thereby inhibiting transcription (Hahm et al., 1994; Molnar & Georgopoulos, 1994; Sun et al., 1996). The strongest inhibitory isoform, Ik6, is deficient of all four DNA-binding zinc fingers and has been implicated in lymphoid malignancies (Nakase et al., 2000; Sun et al., 1999; Yagi et al., 2002). The expression of isoforms varies during differentiation, and it is therefore possible that there exists some kind of fine-tuning system of Ikaros activity (Klug et al., 1998)

In the nucleus, Ikaros binds to the ATPase Mi-2, which is associated with the chromosome remodeling and deacetylase (NuRD) complex (Kim et al., 1999; Sridharan & Smale, 2007). The combination of histone deacetylation and chromatin remodeling ATPase activities in the same complex may, together with Ikaros, repress transcription of the pre-B cell receptor component λ5 (Denslow & Wade, 2007; Sabbattini et al., 2001). However, Ikaros/Mi-2/NuRD is also reported to activate transcription in vitro (Koipally et al., 2002).

Studies in mouse models have shown that homozygous deletion of *Znfn1a1* results in deficiency in T, B, and natural killer cells, as well as their early progenitors (Georgopoulos et al., 1994). Mice with heterozygous deletions, on the other hand, rapidly develop T-cell lymphoma or leukemia. Genetic analysis of the tumors from these mice revealed loss of the wild-type *Ikaros* allele (Winandy et al., 1995), suggesting that Ikaros may function as a potential tumor suppressor in lymphomagenesis.

Notch

The Notch family of receptor proteins has four members named Notch1-4, which act as key regulators of several critical cell functions, such as proliferation, survival and differentiation (Grabher et al., 2006). Of the four Notch family members, Notch1 plays a crucial role for maintenance of hematopoietic stem cells (Radtke et al., 2004) and is especially important for T-cell commitment, at the expense of B cells (Wilson et al., 2001). Notch1 is highly expressed in double negative T cells ($CD4^-CD8^-$), downregulated in double positive T cells ($CD4^+CD8^+$), and returning to an intermediate level in mature single positive T cells ($CD4^+$ or $CD8^+$) (Hasserjian et al., 1996).

Notch1 is translated as one polypeptide, but is cleaved during maturation and the two parts are associated by non-covalent bonds before localisation to the membrane as a heterodimer (Logeat et al., 1998). The Delta, Serrate and LAG-2 (DSL) family have been identified as ligands to extracellular Notch1 (Kojika & Griffin, 2001). The ligand-binding domain of Notch1 contains EGF-repeats that may be modified during maturation. Glycosylation of the EGF-repeats by the glycosyltransferase Fringe is important for the specificity of the ligand binding and may regulate the affinity to the DSL ligands, predicted to have different biological outcomes (Kojika & Griffin, 2001; Yang et al., 2005). Upon ligand binding to the Notch1 receptor, proteolytic cleavage will release the intracellular domain of Notch (ICN), which can then enter the nucleus and bind to the coactivators Mastermind and CSL (also known as CBF-1 and RBP-Jκ) thereby initiating transcription of target genes such as Hes1, Hey1 and pTα, all important for efficient T-cell development (Brou et al., 2000; De Strooper et al., 1999; Fischer & Gessler, 2007; Zweidler-McKay & Pear, 2004). The latter, pTα, is a critical component of the pre-T cell receptor, mediating proliferation and activation of T cells (Zweidler-McKay & Pear, 2004). Without Notch signal, CSL is bound to the mentioned target genes, acting as a transcriptional repressor.

The turnover of Notch1 is handled by the E3-ligase CDC4 (also called SEL-10 or FBXW7), which ubiquitylates the C-terminal PEST region (rich in amino acids P, E, S and T) of Notch1, thereby marking the ICN domain for degradation via the ubiquitin-proteasome pathway (Gupta-Rossi et al., 2001).

Notch1 is a typical proto-oncogene in T cells, and may be constantly activated by genetic alterations. Mutations resulting in activation of Notch has been detected both in human and murine T-cell neoplasms, mainly in the heterodimerization and PEST regions (Breit et al., 2006; Lee et al., 2005; Lin et al., 2006; Mansour et al., 2006; O'Neil et al., 2006; Shimizu et al., 2007; Weng et al., 2004; Zhu et al., 2006). V(D)J recombination of the *Notch1* gene that deletes part of the ligand binding region of Notch1 has also been detected in thymic mouse lymphoma (Tsuji et al., 2004). A deletion following upon recombination will create a cryptic transcription start site halfway through the *Notch1* gene, which produces a protein lacking most of the extracellular domain that will be constantly active in the cell (Tsuji et al., 2003). Stabilization of Notch1 could also be due to mutations in the *CDC4* gene, where Arg residues in the target

binding region is frequently altered in T- cell acute lymphoblastic leukemia (T-ALL) (Malyukova et al., 2007; Maser et al., 2007). The frequent occurrence of activating *Notch1* mutations in T-cell lymphoma suggests that the *Notch1* gene is a critical target for the development of lymphoma.

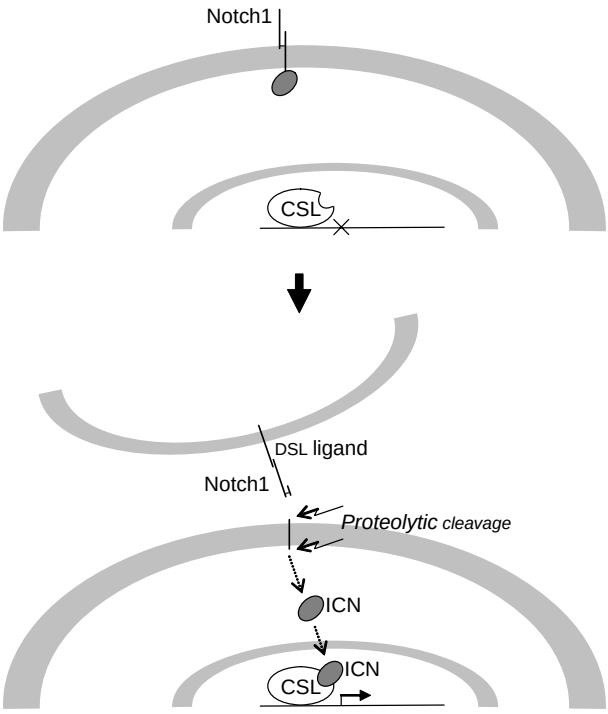

Figure 4. Without Notch signal, CSL is bound to target genes, acting as a transcriptional repressor. Upon ligand binding to the Notch1 receptor, two proteolytic cleavages will release the intracellular domain of Notch (ICN), which can then enter the nucleus and bind to CSL and other cofactors, initiating transcription of target genes such as Hes1, Hey1 and pTα, all important for efficient T-cell development. (Modified from Bray, 2006)

BCL11B/Bcl11b

BCL11B is a zinc finger protein expressed exclusively in the T cells during the double negative stage of differentiation (Tydell et al., 2007; Wakabayashi et al., 2003b). BCL11B has been implicated in the development of lymphoma by its frequent involvement in translocations in human T-ALL (Bernard et al., 2001; Bezrookove et al., 2004; MacLeod et al., 2003; Nagel et al., 2003; Przybylski et al., 2005). Murine studies on chemically induced T-cell lymphoma have previously shown frequent allelic loss in the telomeric region of chromosome 12 where mouse homologue *Bcl11b* is located (Zhuang et al., 1996). Additionally, point mutations and deletions in the *Bcl11b* gene have been detected in radiation-induced lymphoma (Sakata et al., 2004; Wakabayashi et al., 2003a), supporting a role of Bcl11b in regulation of cell growth.

BCL11B encodes a protein which contains six DNA-binding zinc-finger structures, as well as a proline-rich domain and an acidic domain that may possibly transactivate target genes (Satterwhite et al., 2001). In vitro studies have pointed out Bcl11b as a strong transcriptional repressor (Avram et al., 2002), and other studies have shown that Bcl11b interacts with the histone deacetylase SIRT1 within a larger protein complex in mammalian cells to repress transcription of target genes (Senawong et al., 2003). BCL11B also interacts with the nucleosome remodelling and deacetylase (NuRD) protein complex in T lymphocytes (Cismasiu et al., 2005). Both SIRT1 and NuRD bind

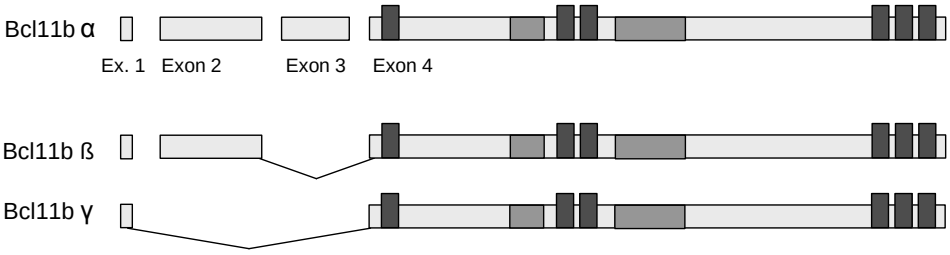

Figure 5. The three isoforms of Bcl11b. Dark grey squares indicate the zinc finger domains, light grey squares the proline-rich and acidic domains. (Modified from Wakabayashi et al., 2003a)

to and deacetylate p53, thereby repressing p53-mediated transactivation (Langley et al., 2002; Luo et al., 2000). Contradictory, Bcl11b has also been reported to act as a transcriptional activator, initiating IL-2 transcription in CD4+ T cells (Cismasiu et al., 2006). In addition, Bcl11b has been assigned anti-apoptotic properties, since knock-down of Bcl11b expression with RNA interference induced apoptosis in T cell lines, via simultaneous activation of the death receptor-mediated and mitochondrial pathways (Grabarczyk et al., 2006; Kamimura et al., 2007a).

The *BCL11B/Rit1/CTIP2* gene was first identified in human chromosome 14q32.2 (Satterwhite et al., 2001), as a homologue to *BCL11A/CTIP1*, which is known to be involved in translocations in human leukemia (Martin-Subero et al., 2002; Satterwhite et al., 2001). Bcl11b shares many of its properties with its homologue Bcl11a. Bcl11a also regulates transcription by binding to the same target sequence as Bcl11b (Avram et al., 2002). However, BCL11A is expressed in CD34+ myeloid precursors, B cells, monocytes, megakaryocytes and very weakly in T cells (Saiki et al., 2000). BCL11A is frequently translocated in B-cell malignancies (Martin-Subero et al., 2002; Satterwhite et al., 2001). Full-length BCL11A can dimerize with BCL6, which is known to be oncogenic in human B cells (Liu et al., 2006; Nakamura et al., 2000) and physically interacts with the NuRD complex to repress transcription in vitro (Fujita et al., 2004). BCL11A also interacts with SIRT1 in the same manner as BCL11B, repressing transcription in vitro (Senawong et al., 2005). The similarities between BCL11A and BCL11B concerning structure, target sequence and binding partners, suggest that the two proteins may act with similar mechanisms although in different cell types.

AIMS OF THE THESIS

The overall aim of this thesis was to study genes that may affect the initiation of lymphoma formation. As a model, we used chemically induced T-cell lymphoma from mouse. These tumors have previously been analyzed for genome-wide allelic loss, a study that showed frequent loss of heterozygosity in chromosomes 2, 4, 11 and 12 (Zhuang et al., 1996). From these chromosomal regions, we chose to analyze two candidate tumor suppressor genes (*Ikaros*, chromosome 11; *Bcl11b*, chromosome 12) in this thesis, to establish their involvement in lymphoma development. For the same purpose, we studied the oncogene *Notch1*, located on mouse chromosome 2. Specific aims were to study:

- the involvement of *Ikaros* inactivation due to point mutations, deletions or allelic loss, in the development of chemically induced murine T-cell lymphoma.
- genetic alterations of *Notch1* and *CDC4*, a regulator of *Notch1* located on mouse chromosome 3, and establish their role in development of T-cell lymphoma.
- mutations in the *Bcl11b* gene in chemically induced murine T-cell lymphoma, and investigate what cellular effect these mutations may entail.
- genetic alterations in *BCL11B* and *BCL11A*, as well as *BCL11B* expression, in human B-cell lymphoma, in order to determine whether alterations in these genes may contribute to B-cell lymphoma.

MATERIALS AND METHODS

Tissue specimens

Murine T-cell lymphoma

In papers I-III, we studied chemically induced T-cell lymphomas from three different mouse strains. Tumor induction was performed at the National Institute of Environmental Health Sciences, Triangle Park, North Carolina, USA, by using the chemicals phenolphthalein, 1,3-butadiene or 2',3'-dideoxycytidine. These chemicals are all known to induce high incidence of lymphoma in mice (Dunnick & Hailey, 1996; Melnick et al., 1990; Rao et al., 1996). Phenolphtalein has been used as an ingredient in laxatives, whereas butadiene is a gas extensively used in the plastic industry and dideoxycytidine is a drug that has been approved for treatment of HIV-positive patients. The mouse strains that were used for carcinogen exposure were C57Bl/6×C3H/HeJ F_1 mice, NIH Swiss mice and heterozygous *p53*-deficient C57Bl/6 (TSG-p53™) mice. A total of 104 lymphomas were received from the carcinogen exposure groups; 31 butadiene-induced lymphomas (BLF) and 16 dideoxycytidine-induced lymphomas in C57Bl/6×C3H/HeJ F_1 mice (DLF); 47 dideoxycytidine -induced lymphomas in NIH Swiss mice (DLS); and 10 phenolphtalein-induced lymphomas in TSG-p53™ mice (PL). All lymphomas were of T-cell origin, and collected mainly from thymus and spleen.

Human B-cell lymphoma

In paper IV, we studied human B-cell lymphomas that were collected at Örebro University Hospital, Sweden, from 1999 through 2002. In total, 32 tumors were studied from 15 female and 17 male patients between 19-82 years,

(average 63 years). The tumors were classified as 12 large B-cell lymphomas, 2 mantle cell lymphomas, 11 follicular centre lymphomas and 7 small lymphocytic lymphomas. Large B-cell lymphomas, follicular centre lymphomas grade 3B and mantle cell lymphomas are classified as aggressive lymphomas (n=16), whereas indolent lymphomas are chronic lymphocytic leukemias and the remaining grades of follicular centre lymphomas (n=16).

The study was approved by the Ethical Committee at the Faculty of Health Sciences, Linköping, Sweden (no. 109-06).

Laboratory methods

The methods have been described in detail in each individual paper, and therefore only a brief discussion on the methods will follow.

Mutation analysis

All four papers are based on mutation analysis of DNA material. Rapid screening for point mutations and small insertions/deletions was performed by means of **Single Strand Conformation Analysis** (SSCA). With this method it is possible to detect any mutation with as little as one nucleotide difference in a fragment of up to approximately 300 bp. The technique is based on the unique single strand conformation of each fragment, and consequently the individual mobility in non-denaturing polyacryl-amide gel electrophoresis. In paper IV, we used **denaturating HPLC** (WAVE) for mutational screening of *BCL11B* in normal population. This technique separates hetero- and homoduplex DNA fragments by their differences in solubility in different solvents and binding capacity to a solid hydrophobic matrix. This method is very quick and efficient to detect a specific mutation, but may also be used for mutation screening of longer fragments.

For detection of larger deletions, we used **Southern blot analysis** in paper I (*Ikaros*) and **PCR/DNA sequencing** in paper II (*Notch1*). Southern blot is a reliable and common method for detecting larger deletions, however, it is very time-consuming and requires large quantities of DNA. In paper II, we were looking for deletions caused by the V(D)J recombination machinery, hence we knew the approximate location of the expected deletions and analyzed for the exact break point. PCR with Phusion™ high fidelity DNA polymerase,

followed by DNA sequencing was a quick and efficient method for this purpose. Tumors with deletions yielded a ~300 bp PCR product, and tumors without deletions a ~16 kb product. Allelic loss was detected in the chromosomal region close to *Ikaros* (I), using **^{32}P-labelling of microsatellite markers** by PCR and subsequent separation with PAGE and the visual inspection of allelic imbalance compared to normal tissue.

Expression analysis

In paper IV, we measured gene expression of *BCL11B* by two different methods. **Real-time PCR** is a quantitative method where a relative value of the expression in relation to the expression of a house-keeping gene is obtained, whereas **immunohistochemistry staining** is a qualitative method, where antibody labelling of a protein can reveal in which cell type the protein is expressed, and the staining intensity may give an estimate of the amounts of protein expressed in the cell.

Proliferation assays

In paper III, we decided to study whether the mutations detected in *Bcl11b* provided any effect on cell growth. We therefore inserted the wild-type *Bcl11b* gene into the pCI-neo plasmid and introduced the three different point mutations by site-directed **mutagenesis**. Because Bcl11b has been described as a tumor suppressor in previous studies (Wakabayashi et al., 2003a), we wanted to imitate homozygous mutants when evaluating the effects of the mutations. For this purpose, we chose the myeloid cell line FDC-P1 as a host cell line, as FDC-P1 does not express any endogenous Bcl11b.

Proliferation rate was first assessed by **[^{3}H]-thymidine incorporation,** which is a quantitative method for studying cell proliferation. [^{3}H]-labeled thymidine is incorporated into the DNA of proliferating cells during DNA-synthesis. The amount of incorporated [^{3}H]-thymidine is assessed in a β-counter, yielding the rate of proliferation.

F**low cytometry** is used for separation of cells of different size and inner complexity. Labeling the cells with different substances such as **propidium iodide** or **5-carboxyfluorescein diacetate succimidylester** (CFSE), may give

additional information. Living cells are impermeable to propidium iodide, whereas the damaged cell membrane of late apoptotic and necrotic cells allows propidium iodide to enter the cell and to intercalate with the DNA. Flow cytometry separates cells by propidium iodide content, yielding the percentage of viable cells in a sample.
CFSE is a non-fluorescent cell permeant molecule which is cleaved after entering the cell, rendering the molecule impermeant and fluorescent. The CFSE molecules are then divided equally between daughter cells during mitosis, which makes the cells less fluorescent for each cell division. Flow cytometry detects the amount of CFSE in each cell, and the fluorescence decay gives the rate of cell proliferation.

RESULTS AND DISCUSSION

Paper I

The Ikaros protein, encoded by the *Znfn1a1* gene, is a critical transcription factor involved in the early development and differentiation of the T-cell lineage (Ng et al., 2007). The normal function of Ikaros depends on three domains, including sequence-specific DNA binding, transactivation, and dimerization domains (Sun et al., 1996). These three domains were analyzed for mutations, deletions and allelic loss in the chemically induced murine lymphomas.
SSCA revealed missense or nonsense mutations, small deletions or insertions in eleven of 104 tumors (11 %). Eight of the alterations occurred in the DNA binding domain, which contains four zinc fingers shown to be the most critical part of Ikaros repression (Koipally et al., 2002). The DNA-binding domain consists of four zinc fingers encoded by exons 3, 4, and 5, which is a candidate mutation cluster and supports the critical role for the zinc finger domains in the normal function of the Ikaros protein. The three remaining alterations identified were all insertions in exon 7, which encode the transactivation and dimerization domains. The insertions caused altered reading frame in the three tumors, resulting in abrogation of the transactivation domain as well as the dimerization domain.

In addition, exons 3-5 and 7 were studied by Southern blot analysis. Southern blot needs large quantities of DNA and unfortunately only 68 tumors had enough material for this analysis. Two of these tumors revealed homozygous deletions of exons 3-5 and one sample disclosed homozygous deletion of exons 5 and 7. The homozygous deletions resulted in complete loss of the

DNA binding domain or transactivation domain, which will abrogate the function of Ikaros. Ik6, one of the isoforms of Ikaros, lacks the whole DNA-binding region, making it the strongest repressor of the Ikaros isoforms (Koipally et al., 1999). It has also been implicated in the development of leukemia (Winandy et al., 1995), indicating that the deletions detected in this study also are contributing to lymphomagenesis, acting in a dominant-negative fashion.

Allelotyping of the chromosomal region where the *Znfn1a1* gene is located revealed LOH in 26% of the BLF and 29% of the DLF samples, implying that allelic loss of *Znfn1a1* may be an important event in lymphomagenesis. However, only two of the tumors with allelic loss showed simultaneous point mutations in the examined regions of the *Znfn1a1* gene.

As described above, it is also possible that other mechanisms may be involved in the inactivation of Ikaros, such as overexpression of the dominant negative isoforms. In contrast to normal lymphocytes that predominantly express the active isoforms of Ikaros, it has been shown that leukemic T cells express an increased level of inactive isoforms, which interfere with the active isoforms and exert dominant-negative functions (Nakase et al., 2000; Nakayama et al., 1999; Sun et al., 1999).

Similar frequencies of point mutations and deletions in *Znfn1a1* have been identified in radiation-induced mouse thymic lymphomas (Okano et al., 1999). This indicates the involvement of Ikaros inactivation in the development of lymphomas induced both chemically (by dideoxycytidine, butadiene or phenolphtalein) and by radiation.

Paper II

Notch1 is a membrane-bound receptor involved in many cellular processes, for example in differentiation of T cells (Grabher et al., 2006). Accumulation of intracellular Notch1 in the nucleus leads to consistent transcriptional activation of target genes like *e.g. Hes1* and *Hey1* (Fischer & Gessler, 2007). The accumulation has previously been described as a consequence of different types of mutations in *Notch1*. Mutations in the heterodimerization domain result in ligand-independent signalling (Breit et al., 2006; Lee et al., 2005; Lin et

al., 2006; Mansour et al., 2006; O'Neil et al., 2006; Shimizu et al., 2007; Weng et al., 2004; Zhu et al., 2006), whereas mutations in the PEST domain lead to a stabilization and a constant activation of ICN (Breit et al., 2006; Lee et al., 2005; Lin et al., 2006; Mansour et al., 2006; O'Neil et al., 2006; Shimizu et al., 2007; Weng et al., 2004; Zhu et al., 2006). Deletions have also been described in the N-terminal part of the protein, creating a cryptic splice site and a protein lacking most of the extracellular domain (Tsuji et al., 2004; Tsuji et al., 2003). Moreover, the *CDC4* gene is involved in the ubiquitylation and degradation of Notch1, and mutations in the region of *CDC4* that bind to ICN also result in an accumulation of ICN (Malyukova et al., 2007; Maser et al., 2007).

In paper II, we analyzed chemically induced mouse lymphomas for mutations in the *Notch1* and *CDC4* genes. Exons 1b-2 of *Notch1* encodes the ligand-binding domain of Notch1 and were analyzed by PCR and MegaBACE™ sequencing. Furthermore, the HD region, encoded by exons 26-27, and the PEST domain, encoded by exon 34, were analyzed by SSCA and MegaBACE™ sequencing, as well as exons 8 and 9 of *CDC4*, encoding the ICN-binding region. In total, 40 of 103 lymphomas (39 %) displayed *Notch1* mutations, making *Notch1* an important target gene for mutations in chemically induced lymphomas.

The PEST domain of *Notch1* disclosed genetic alterations in 29 of 103 tumors. All of the insertions, deletions and duplications in exon 34 resulted in an altered reading frame of *Notch1*, in part or totally deleting the PEST region. The short amino acid sequence WSSSSP, located at residues 2495-2500 within the PEST domain, has been suggested to be critical for the degradation of Notch1, and protein termination and abrogation of this sequence consequently results in accumulation of a truncated Notch1 which cannot be degraded (Chiang et al., 2006). However, the truncated Notch1 still possesses binding and transactivation capacities. The mutations identified in exon 34, all truncated the protein between residues 2326-2494, upstreams of the WSSSSP sequence, indicating a stabilization of ICN in the tumor cells.

Degradation may also be lost by mutations in *CDC4*, where mutational hot-spots have been identified in Arg residues in the region that normally binds the Notch1 PEST domain, most frequently in Arg^{465}, Arg^{479} and Arg^{505} (Malyukova et al., 2007; Maser et al., 2007). The mutations in *CDC4* Arg

residues preclude ubiquitylation and proteosomal degradation of ICN. However, in our material no such mutations in *CDC4* could be detected.

The HD domain of *Notch1* showed point mutations in eight of 103 tumors. Five of these tumors displayed the same point mutation altering Leu to Pro in codon 1668, which thus represents a mutational hotspot. The corresponding mutation has also been reported both in human (Breit et al., 2006; Zhu et al., 2006) and in murine lymphoma (Lin et al., 2006). Proline is known to cause α-helix disruption in protein structures, indicating that Leu1668Pro mutation may enable ligand-independent activation of Notch1. Another potential hotspot for mutations may be the Ala residue in codon 1690, which was mutated in two tumors. One of these mutations, Ala1690Asp, has also been described previously (Breit et al., 2006), whereas Ala1690Pro is novel.

N-terminal deletions of exons 1b and 2 and large intronic sequences surrounding these exons were detected in 16 of 103 tumors. Fifteen of these tumors also displayed small insertions of 1-8 nucleotides accompanying the deletions. Similar deletions have earlier been described and suggested to contribute to a higher malignancy in radiation induced mouse lymphomas (Tsuji et al., 2004).
The deletion breakpoints were closely located to sequences similar to the recombination signalling sequences (RSS) that are normally found in the gene encoding the T-cell receptor. A high variability is crucial for the T-cell receptor in order to recognize the wide variety of antigen that the T cells may be exposed to. The variability is generated from rearrangement of certain gene segments, an event referred to as V(D)J recombination, which is dependent upon the RSS being recognized and cleaved by Rag enzymes, followed by end-processing and ligation by the V(D)J recombination machinery (Gellert, 1997). The RSS are DNA stretches consisting of a highly conserved heptamer (7 bp), a 12 or 23 bp spacer and a highly conserved nonamer (9 bp) that specifically signal to the V(D)J recombinase about rearrangement (Gellert, 1997). The V(D)J recombinase is a cluster of enzymes that rearrange the gene segments, and contains some enzymes that are unique for the lymphocytes, e.g. Recombination-activating gene 1 and 2 (RAG1, RAG2), while some are ubiquitous DNA repair enzymes (Gellert, 1997). The V(D)J recombinase introduces double strand breaks adjacent to the RSS, whereas RAG1 and -2 creates hairpin structures at the ends before they are rejoined at the new site.

At the splice sites, nucleotides may be inserted before the rejoining, adding further variability to the receptor (Gellert, 1997). The deletions detected in this study may be due to illegitimate recombination of the *Notch1* gene by the V(D)J recombination machinery, which would also explain the small insertions accompanying the deletions. A deletion caused by recombination creates a cryptic transcription start site in the *Notch1* gene, producing a protein that lacks most of the extracellular domain (Tsuji et al., 2003). As a consequence, the Notch1 receptor will be more exposed to S3-cleavage and hence over-activated in the cell.

A reporter-gene assay previously showed that mutations in both the HD and PEST regions on the same allele synergistically amplified the luciferase activity 20-40-fold, whereas the mutations acting alone caused 1.5 to 9-fold increase compared to normal Notch1 activity (Weng et al., 2004). In our material, five samples had point mutations in the HD region as well as a protein truncation in exon 34. In addition, four tumors displayed *Notch1* truncations in both alleles of exon 34, while eight tumors contained both deletions in the target binding region and truncations in the PEST domain. It is plausible to believe that these mutations also may have a synergistic effect on transcriptional activity.

The chemically induced lymphomas have earlier also been analyzed for mutations in p53 (Zhuang et al., 1997). Two other research groups have suggested that wild-type p53 may directly induce Notch1 expression in epithelial cells, via sequence-specific p53 binding sites in the *Notch1* promoter (Lefort et al., 2007; Yugawa et al., 2007). Contradictory, in thymocytes low expression or absence of p53 is correlated with an increased expression of Notch1. Down-regulation of p53 was shown to increase presenilin expression, resulting in increased levels of ICN and subsequent Hes1 expression (Laws & Osborne, 2004). However, yet another study suggested an alternative mechanism, where p53 levels are regulated by Notch1, possibly by a Mdm2-dependent mechanism (Beverly et al., 2005). In addition, p53 has been reported to induce CDC4 expression (Perez-Losada et al., 2005), which in turn ubiquitylates Notch1 for degradation. Together, previous studies indicate a cell type specific and complex regulation of Notch1 and p53 activities, which remains to be fully elucidated. Our data do not show mutual exclusivity between *Notch1* and *p53* mutations but indicate a reverse relationship ($p=0.14$;

χ^2-test). The relationship is supported by the low *Notch1* mutation frequency in phenolphtalein-induced tumors on $p53^{+/-}$ mice (PL 10%), compared to tumors induced in Swiss or C57Bl/6×C3H/HeJ F_1 mice by dideoxycytidine (DLS 52 %, DLF 44 %) or butadiene (BLF 26 %). Both mutational inactivation of p53 and activation of Notch1 result in cell growth, why mutations in both genes may be unnecessary for lymphomagenesis. The difference between the two subgroups of mice is therefore most likely due to heterozygous inactivation of p53 in mice treated with phenolphtalein.

Paper III-IV

BCL11B is mainly expressed in thymocytes, where down-regulation of the gene results in a differentiation block at the $CD4^-CD8^-$ stage, indicating a role for BCL11B in regulation of T-cell differentiation (Wakabayashi et al., 2003b). *BCL11B* have previously been implicated in lymphomagenesis, as alterations of *BCL11B* are detected in human and murine lymphoblastic malignancies of T-cell origin (Bernard et al., 2001; Bezrookove et al., 2004; MacLeod et al., 2003; Nagel et al., 2003; Przybylski et al., 2005; Sakata et al., 2004; Wakabayashi et al., 2003a). This was further supported in paper III, where seven out of 47 (15 %) chemically induced lymphomas in C57Bl/6×C3H/HeJ F_1 mice displayed somatic frameshift or point mutations in *Bcl11b*. No differences could be seen between tumors induced by butadiene compared to dideoxycytidine. Interestingly, all point mutations were located between residues 778-844, which is within the region encoding the three C-terminal zinc fingers of Bcl11b. A hotspot for mutations was suggested at codon 828, where four samples displayed the same mutation. The frameshift mutation was detected in the same region, deleting the two C-terminal zinc fingers. Studies on other zinc-finger containing transcription factors have shown that the zinc finger structures are likely to be important for the DNA-binding function of the protein (Koipally et al., 2002).

To elucidate if any of these mutations influence cell function, FDC-P1 cells were transfected with either wild-type *Bcl11b* or any of the four mutants of *Bcl11b*, S778N, K828T, Y844C or FS823. Loss of function in *Bcl11b* has previously been shown to contribute to lymphomagenesis (Wakabayashi et al., 2003a), suggesting a role as a tumor suppressor, and we therefore wanted to resemble a homozygous Bcl11b mutant state to evaluate the effects of the mutations. FDC-P1 cells do not express endogenous Bcl11b and is widely used

to study proliferation and apoptosis in hematopoietic cells, and was therefore chosen for these experiments. FDC-P1 cells are dependent on IL-3 for continuous growth but respond to other cytokines such as stem cell factor (SCF; also called c-kit ligand) by slower cell cycle kinetics and inhibition of apoptosis (Mohle & Kanz, 2007). In addition, SCF may synergize with other stimuli to enhance proliferation (Karlsson et al., 2003). The clones were grown in the absence of cytokines to analyze the ability to sustain survival. This was measured by propidium iodide staining followed by flow cytometry. However, none of the clones were able to support survival without IL-3. In addition, Bcl11b was analyzed for synergistic effects in combination with SCF, as measured by ^{3}H-thymidine incorporation and CFSE staining followed by flow cytometry. Interestingly, FDC-P1 cells expressing the wild-type Bcl11b showed reduced proliferation rate with more than 50% compared to non-transfected cells. In contrast, expression of the K828T, Y844C and FS823 mutated variants of Bcl11b resulted in increased proliferation both by [^{3}H]-thymidine incorporation and by CFSE staining. Interestingly, the K828T mutation that was detected in four tumor samples seemed to increase proliferation rate most efficiently among the three different missense mutants. In addition, the frameshift mutant also increased cell growth to the same extent as K828T. However, the S778N mutant did not differ compared to untransfected cells. These results from the proliferation assays demonstrate that the mutations identified in paper III are functional and affect cell proliferation.

BCL11A, a homologue of BCL11B, is expressed in CD34+ myeloid precursors, B cells, monocytes, megakaryocytes and very weakly in T cells (Saiki et al., 2000) and translocations of BCL11A is often involved in B-cell malignancies (Martin-Subero et al., 2002; Satterwhite et al., 2001). BCL11A binds to the same target sequence as BCL11B (Avram et al., 2002) and interacts with the histone deacetylase SIRT1 to regulate transcription, in the same manner as BCL11B (Senawong et al., 2005). Also, BCL11A can dimerize with BCL6, which alike BCL11B can interact with the NuRD complex to repress transcription (Fujita et al., 2004).

The high conservation and the many shared properties between BCL11A and BCL11B suggest that both genes may act with similar mechanisms although in different cell types. We therefore analyzed human B-cell lymphomas for

mutations in the *BCL11A* and *BCL11B* genes, revealing a novel germ-line *BCL11B* point mutation (1610C>G) in one of 32 tumors (3 %), that created a P537R shift close to one of the DNA-binding zinc fingers. Normal DNA from 700 healthy individuals was also screened for this point mutation with denaturing HPLC analysis, but no mutation was found, and we could therefore not exclude the possibility that mutations in *BCL11B* may be a predisposing factor for B-cell lymphoma. To evaluate the importance of the mutation in the B-cell lymphoma, BCL11B expression levels were assessed with real-time PCR and IHC. Increased BCL11B expression was detected with real-time PCR in 26 samples compared to normal peripheral B cells. However, IHC showed that all of the expression was solely produced by infiltrating T cells within the B cell lymphomas. Unexpectedly, the aggressive tumors displayed a significantly lower BCL11B expression compared to the indolent tumors ($p=0.01$; Mann-Whitney U-test).

Normal development contains many events that are also associated with tumor formation, e.g. proliferation, apoptotic resistance and invasion (Tlsty, 2001; Wiseman & Werb, 2002). These events are often induced by extracellular signals from adjacent or stromal cells. Stromal cells have even been shown to play an active role in development of mammary tumors (Barcellos-Hoff & Ravani, 2000), where mammary cells grown together with irradiated stroma cells became more malignant than mammary cells grown with non-irradiated stroma cells. It may therefore be possible that the infiltrating T cells somehow could affect the malignant B cells. For example, BCL11B directly binds to the IL-2 promoter to activate its transcription (Cismasiu et al., 2006). IL-2, acting in concert with CD40-CD40L interaction, has been shown to be critical for the immune response in B lymphocytes (Grabstein et al., 1993; Johnson-Leger et al., 1998), and we therefore speculate that the BCL11B expression in the T cells infiltrating the B-cell lymphomas in this study may influence the formation of these lymphomas, perhaps via IL-2 signalling, although the expression does not occur in the malignant cells. However, it is still possible that the variation in BCL11B expression is normal fluctuation in different T-cell differentiation stages and the missense mutation detected in one sample is just a passive mutation. Further evaluation of the effects of cells surrounding the tumor must therefore be done before any certain conclusions can be drawn.

Table 1. Summary of mutations detected in the chemically induced murine lymphomas.
FS, Frameshift; ■, Retention of C57Bl/6 allele; ¤, Retention of C3H/HeJ allele; N.A., not analyzed

	Notch1	*Bcl11b (#12)*	*LOH #12*	*Ikaros (#11)*	*LOH #11*[a]	*p53*[b] *(#11)*
	n=103	n=47	Bcl11b, bas 729, 1386	Mut n=104, Del n=68	n=45	n=78
BLF 1					■	
2	FS					
3		K828T				
4	Del ex 1b-2	K828T				
5	Del ex 1b-2		■	FS		
6			¤			
7	Del ex 1b-2		¤		■	N236S
8			¤		¤	C173F
9	L1668P, 2×FS			C60W	■	FS
10					¤	H176L
11			■			
12				R126X	■	FS
14					¤	
15	Del ex 1b-2	K828T	¤			
17	FS Del ex 1b-2	FS				
20			■			
21					■	
24		S778N				
25						R153G
30	FS					
DLF 1	Del ex 1b-2	Y844C		Del ex 3-5	¤	N.A.
2	A1690D		■			N.A.
3	FS, Del ex 1b-2		¤		■	N.A.
5	FS			D99A		N.A.
6			■	Del ex 3-5		N.A.
8	Del ex 1b-2		¤			N.A.
9	Del ex 1b-2				■	N.A.
11			¤			N.A.
12			¤			N.A.

	Notch1	*Bcl11b (#12)*	*LOH #12*	*Ikaros (#11)*	*LOH #11*[a]	*p53*[b] *(#11)*
	n=103	n=47	Bcl11b, bas 729, 1386	Mut n=104, Del n=68	n=45	n=78
DLF13			■			N.A.
14	FS Del ex 1b-2		■	Del ex 5, 7	■	N.A.
15			■	FS		N.A.
16		K828T				N.A.
DLS 1	ins ex27, FS		N.A.		N.A.	
2	A1690P, FS		N.A.		N.A.	
5	2×FS, Del ex 1b-2		N.A.		N.A.	
6			N.A.	R75G	N.A.	
7	FS		N.A.		N.A.	
9	Del ex 1b-2		N.A.		N.A.	
10			N.A.	L90P, Del p.404-429	N.A.	
11			N.A.		N.A.	Del ex 5-9
12			N.A.	D99A	N.A.	
13	FS		N.A.		N.A.	
15	FS		N.A.		N.A.	
16	S2398X, Del ex 1b-2		N.A.		N.A.	
19	FS		N.A.		N.A.	
21	FS		N.A.		N.A.	
22			N.A.	FS	N.A.	
25	L1668P, FS		N.A.		N.A.	
27	L1668P		N.A.		N.A.	
28	FS		N.A.		N.A.	
29			N.A.	G64A	N.A.	
30	L1668P		N.A.		N.A.	
31	FS		N.A.		N.A.	
32	FS		N.A.		N.A.	
34	A2399X		N.A.		N.A.	
35	E2387X		N.A.		N.A.	
37	L1668P, FS		N.A.		N.A.	
38	FS		N.A.		N.A.	
39			N.A.		N.A.	L262Q
43	FS, Del ex 1b-2		N.A.		N.A.	

	Notch1	*Bcl11b (#12)*	*LOH #12*	*Ikaros (#11)*	*LOH #11*[a]	*p53*[b] *(#11)*
	n=103	n=47	Bcl11b, bas 729, 1386	Mut n=104, Del n=68	n=45	n=78
DLS44	FS, Del ex 1b-2		N.A.		N.A.	
45	FS		N.A.		N.A.	C272R
46			N.A.		N.A.	A158S, H176Q
PL 2			N.A.	FS	N.A.	N.A.
7	FS, Del ex 1b-2		N.A.		N.A.	N.A.

[a](Zhuang et al., 1996)
[b](Zhuang et al., 1997)

General Discussion

This thesis has focused on two tumor suppressors located in mouse chromosome 11 (*Ikaros*) and 12 (*Bcl11b*). Also, the oncogene *Notch1*, located on chromosome 2, was studied. The genes in focus are all involved in lymphocyte development and differentiation, and it has been shown that there are connections and similarities between them.

Ikaros has recently been reported to down-regulate the Notch pathway, by binding to the same consensus site as CSL in the Hes1 promoter (Beverly & Capobianco, 2003; Dumortier et al., 2006). Hes1 transcription is normally repressed by CSL, but is activated when Notch1 binds to CSL. Competitive binding of Ikaros to the DNA target sequence of CSL, TGGGAA, represses Hes1 expression in a dose-dependent manner. Also, dominant-negative isoforms of Ikaros may antagonize the Hes1 repression, by blocking the full-length functional Ikaros (Beverly & Capobianco, 2003). Ikaros is mainly involved in directing development of hematopoietic stem cells into lymphoid progenitors, and may also be involved in T-cell differentiation (Clevers & Grosschedl, 1996). Notch1, on the other hand, is important for the specification into T cells among lymphoid progenitors and perhaps also for commitment into the T-cell lineage, possibly by up-regulation of Bcl11b (Tydell et al., 2007). The Notch pathway may therefore be blocked by Ikaros during early differentiation, and the different Ikaros isoforms may later on offer a fine-tuning system for Notch1 regulation during T-cell differentiation. In paper I, 13 % of the chemically induced lymphomas displayed *Ikaros* mutations, mainly in the DNA binding region. Mutations in *Notch1* and *Ikaros* were not mutually exclusive ($p=0.82$; χ^2-test) since five of the tumors had mutations in both *Notch1* and in *Ikaros*, but together the genes displayed a total mutational frequency of 47 % in these tumors.

Ikaros has also been shown to repress transcription by binding to Mi-2β within the NuRD complex (Sridharan & Smale, 2007). However, in vitro studies have shown that the complex also may activate transcription (Koipally et al., 2002). Forthermore, Mi-2β has been shown to be critical for transcription of CD4 (Williams et al., 2004). It is possible that the positive regulation Ikaros has on T-cell differentiation may depend on CD4 expression, since the expression of the CD4 surface coreceptor is one of the key events of T-cell maturation. The NuRD complex has also been reported to bind to BCL11B via the metastasis-

associated proteins MTA1 and MTA2, repressing transcription in vitro (Cismasiu et al., 2005). However, no target genes of BCL11B-NuRD are known at present. As the NuRD complex interact with Ikaros and Bcl11b, as well as with Bcl6 (Fujita et al., 2004), it may be important to further study this protein complex.

The previously detected allelic loss in chromosome 12 could be confirmed in paper III, by two intragenic single nucleotide polymorphisms in *Bcl11b* (729A>G and 1386G>A). Another research group has previously detected point mutations and allelic loss in *Bcl11b* in radiation-induced lymphoma in agreement with the two-hit hypothesis (Wakabayashi et al., 2003a). However, none of the samples with mutations in *Bcl11b* in our study showed simultaneous allelic loss. Bcl11b has been suggested to be haploinsufficient for suppression of tumors, since $Bcl11b^{+/-}p53^{+/-}$ mice generates more spontaneous tumors than do $Bcl11b^{+/+}p53^{+/-}$ mice (Kamimura et al., 2007b). The phenomenon of haploinsufficiency, where one single copy of the gene may be incapable of providing sufficient protein in order to maintain normal cell function, may explain the findings in paper III. Haploinsufficiency has previously been demonstrated for other tumor suppressors as well, *e.g.* p53, $p27^{Kip1}$ and PTEN (Santarosa & Ashworth, 2004). Of the 14 *Ikaros* mutant tumors in paper I, only two showed simultaneous allelic loss, hence haploinsufficiency may be considered to contribute to tumor development in *Ikaros*-heterozygous tumors as well.

In the study on radiation-induced mouse lymphomas, mutations in *Bcl11b* were found to be mutually exclusive with *p53* mutations, suggesting a common pathway for tumor formation (Wakabayashi et al., 2003a). Of the 47 tumors analyzed in paper III, 13 revealed mutations in either *Bcl11b* or *p53* (Zhuang et al., 1997), but no tumor carried mutations in both genes, supporting mutational complementarity. Furthermore, Bcl11b has been shown to interact with the histone deacetylase protein complex designated NuRD (Cismasiu et al., 2005), which reduces the levels of acetylated p53, repressing p53 dependent transcription and thereby modulating p53-mediated cell growth arrest and apoptosis (Luo et al., 2000). Likewise, the interaction between Bcl11b and another histone deacetylase, SIRT1 (Senawong et al., 2003), also leads to transcriptional repression of p53 (Langley et al., 2002).

However, due to the effects on proliferation detected here, it is possible that Bcl11b may act by an alternative mechanism.

The mechanisms by which Bcl11b execute growth suppression is still largely unknown. However, a previous report has demonstrated that Bcl11b is able to repress transcription from a consensus response element (Avram et al., 2002),

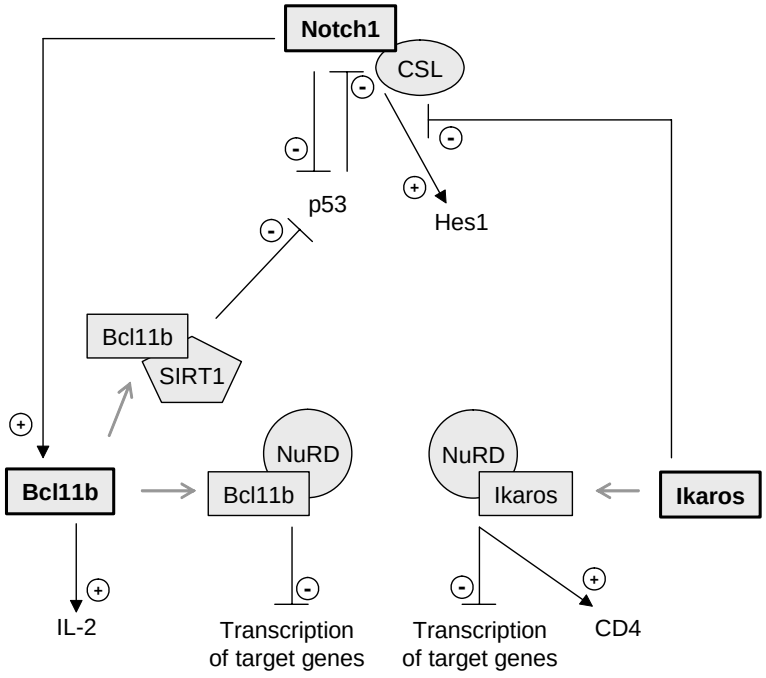

Figure 6. Summary of the connections between the three genes studied in this thesis. Ikaros and Bcl11b both bind to the NuRD complex, regulating transcription of target genes (Cismasiu et al., 2005; Williams et al., 2004). Notch1 and Bcl11b (by interaction with the SIRT1 complex) both inhibit p53 expression (Beverly et al., 2005; Senawong et al., 2003; Langley et al., 2002). Also, direct effects between the genes have been described; Notch1 may upregulate Bcl11b expression (Tydell et al., 2007), whereas Ikaros directly inhibits Hes transcription by competitive binding to the ICN/CSL regulatory element (Beverly & Capobianco, 2003; Dumortier et al., 2006).

supporting our notion of a suppressive role for Bcl11b on cell cycle. The same group has recently proved that Bcl11b directly activates IL-2 expression in $CD4^+$ T lymphocytes (Cismasiu et al., 2006). However, it is possible that the protein may have both activating and repressing functions depending on which other proteins it associates with and in which cell type it is expressed. Interestingly, the consensus sequence of Ikaros, TGGGAA, is found also in the IL-2 receptor β subunit gene (Beverly & Capobianco, 2003), suggesting that there may be a functional connection between Ikaros and Bcl11b.

CONCLUSIONS

In paper I, we identified point mutations and homozygous deletions in the *Znfn1a1* gene in 13 % of the chemically induced murine lymphomas, all of which lead to amino acid substitutions or abrogation of the functional domains in the Ikaros protein. Our results additionally support the role of Ikaros as a potential tumor suppressor.

In paper II, we identified activating mutations in *Notch1* in 39 % of the chemically induced murine lymphomas. *Notch1* mutations is the single most frequent gene in this set of chemically induced murine lymphomas, and may be one of the most important mutational targets in the development of T-cell lymphoma.

In paper III, point mutations and deletions in the three C-terminal zinc fingers of Bcl11b were revealed in 15 % of the chemically induced lymphomas in C57Bl/6×C3H/HeJ F_1 mice. A mutational hotspot was detected in codon 828, where four of the tumors displayed the same mutation. Three of the identified alterations, including the hotspot mutation in Bcl11b, increased cell proliferation when introduced in a cell without endogenous Bcl11b expression, wheras cell proliferation was suppressed by wild-type Bcl11b in the same cell line. Mutations in Bcl11b may therefore be an important contributing factor to lymphomagenesis in a subset of tumors.

In paper IV, a BCL11B germ-line mutation was identified in one of 33 human B-cell lymphomas. Expression of BCL11B in infiltrating T cells was

significantly lower in aggressive compared to indolent lymphomas, suggesting that the infiltrating T cells may affect the B-cell lymphomas.

Collectively, this suggests that *Ikaros*, *Notch1* and *Bcl11b* are important targets for development of subsets of murine T-cell lymphoma. The role of *BCL11B* in human B-cell lymphoma is still not fully elucidated.

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